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DOCTOR OF PHILOSOPHY
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.....[redacted].....
Sec. Research Graduate School Committee

## AMENDMENTS

**Table of Contents, page v, section 5.4 should read:** Failure to obtain an *rpoH* mutant of *N. gonorrhoeae*.

**Chapter Two, Table 2.2 should include:** Plasmid - p*tet*M25.2, Description - Conjugative plasmid of *N. gonorrhoeae*, Reference or Source - Kupsch *et al.* (1996).

**Chapter Three, Figure 3.7, Comment:** No negative control is shown in this figure as direct sequencing of the PCR products verified that they encoded the *RLS* sequence.

**Chapter Four, Figure 4.9, Comment:** A shorter exposure time for the membrane probed with 16S rRNA produced a less intense signal as expected, but there was no difference in the mRNA levels at the different time points.

**Chapter Five, page 93, subheading 5.4 should read:** Failure to obtain an *rpoH* mutant of *N. gonorrhoeae*.

**Chapter Five, page 93, line 11 should read:** Despite several attempts at this, no chloramphenicol resistant colonies were obtained for cultures incubated at 30°C and very few were recovered from those incubated at 37°C.

**Chapter Five, page 98, paragraph 1, line 9 should read:** The delayed increase in *rpoH* mRNA suggests that neither an increase in transcription nor translation of *rpoH* is responsible for this induction.

**Chapter Six, page 108, paragraph 3, line 6 should read:** *M. genitalium* and *M. pneumoniae* only contain $\sigma^{70}$ (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996) and *C. trachomatis* contains three sigma factors, $\sigma^{66}$, $\sigma^{28}$ and $\sigma^{54}$ (Kalman *et al.*, 1999).

# CHARACTERISATION OF ALTERNATIVE SIGMA FACTORS AND THE HEAT SHOCK RESPONSE IN *NEISSERIA GONORRHOEAE*

A thesis submitted for the degree of
Doctor of Philosophy at Monash University

by

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August 2003

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# ABSTRACT

The regulation of the gene encoding the gonococcal type 4 pilin subunit, *pilE*, is complex and has been under investigation for a long period of time. Analysis of the upstream region identified elements involved in $\sigma^{54}$ regulation including a $\sigma^{54}$ dependent promoter, P3, an upstream activator site and binding sites for integration host factor (IHF). In heterologous backgrounds this promoter was active in the presence of the appropriate activators. This study concentrated on identifying the gene encoding $\sigma^{54}$, *rpoN*, from *Neisseria gonorrhoeae*, and to determine its role, if any, in *pilE* regulation.

A region was identified in *N. gonorrhoeae* which, when translated, displayed significant similarity to $\sigma^{54}$ of *Escherichia coli*. This sequence was designated *RLS* for *rpoN*-like sequence. However, a deletion event appeared to have rendered *RLS* incapable of encoding a functional $\sigma^{54}$ protein as the regions essential for binding to promoter DNA were missing. This deletion appears to be widespread as it is also evident in the genomes of the pathogenic *Neisseria meningitidis* and the commensal *Neisseria subflava*. The exception to this was the commensal *Neisseria lactamica* which contained an intact *rpoN* homologue. This homologue was unable to complement a *Pseudomonas aeruginosa rpoN* mutant and did not seem to be expressed in *N. lactamica* under the conditions tested, suggesting that it may be redundant in this organism.

Molecular chaperones have been shown to have an important role in the pathogenesis of intracellular pathogens but have not been studied to a great extent in *N. gonorrhoeae*. Therefore, another aspect of this study was to characterise the heat shock response of *N. gonorrhoeae*. The genes encoding the molecular chaperones DnaK, DnaJ and GrpE were identified and transcriptional analyses demonstrated that they were upregulated from a $\sigma^{32}$dependent promoter upon stress. This was confirmed by mutational analysis of the $\sigma^{32}$ promoter of *dnaK*.

This subsequently led to the identification and characterisation of the $\sigma^{32}$ encoding gene, *rpoH*, from *N. gonorrhoeae*. This gene could complement the temperature sensitive phenotype of an *E. coli rpoH* mutant and was found to be essential, indicating that it is imperative for the homeostasis of *N. gonorrhoeae*. Transcriptional analyses and

protein studies determined that regulation of expression of *rpoH* was different to that identified in most other organisms, which occurs predominantly at the translational level. The studies suggested that an increase in the activity of pre-formed $\sigma^{32}$ was primarily responsible for induction of the heat shock response in *N. gonorrhoeae*. The mechanism via which this occurs has yet to be elucidated. Deletion of the *rpoH* leader sequence and subsequent transcriptional analysis suggested the binding of a putative repressor to this region during normal growth. Despite potential IHF binding sites identified within this sequence, gel shift assays revealed that this regulatory protein does not in fact bind to the region upstream of *rpoH* under the conditions tested.

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# LIST OF PUBLICATIONS

The following publication arose from the research presented in this thesis:

Laskos, L., Dillard, J. P., Seifert, H. S., Fyfe, J. A. and Davies, J. K. (1998). The pathogenic neisseriae contain an inactive *rpoN* gene and do not utilize the *pilE* $\sigma^{54}$ promoter. Gene 208, 95-102.

# DECLARATION OF AUTHENTICITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other institution, and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

Lina Laskos

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# ABBREVIATIONS

| | |
|---|---|
| $A_{260}$ | absorbance at 260nm |
| ADP | adenosine diphosphate |
| AMV | avian myelobastosis virus |
| Ap | ampicillin |
| ATP | adenosine triphosphate |
| α | alpha |
| β | beta |
| bp | base pair(s) |
| BSA | bovine serum albumin |
| cAMP | cyclic adenosine monophosphate |
| CAT | chloramphenicol acetyltransferase |
| cDNA | copy deoxyribonucleic acid |
| CIRCE | controlling inverted repeat of chaperone expression |
| cm | centimetre |
| Cm | chloramphenicol |
| CRP | cAMP receptor protein |
| δ | delta |
| dATP | deoxyadenosine triphosphate |
| ddUTP | dideoxyuridine triphosphate |
| DEPC | diethylpyrocarbonate |
| DIG | digoxigenin |
| DMIV | Deakin Modified Iso-Vitalex |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| DTT | dethiothreitol |
| dUTP | deoxyuridine triphosphate |
| ε | epsilon |
| ECF | extracytoplasmic function |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme linked immunosorbent assay |
| Erm | erythromycin |
| fmol | femptomole |
| *g* | gravitational constant |
| g | gram(s) |
| γ | gamma |
| Hsp | heat shock protein(s) |
| HTH | helix-turn-helix motif |
| HPD | histidine, proline, aspartic acid motif |
| IHF | integration host factor |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| kb | kilobase pair(s) |
| kcal/mol | kilocalorie per mole(s) |
| kDa | kilodalton(s) |
| L | litre(s) |
| LB | Luria-Bertani |
| μFarad | microFarad |
| μg | microgram(s) |
| μl | microlitre(s) |

| | |
|---|---|
| µM | micromolar(s) |
| M | molar |
| mg | milligram(s) |
| ml | millilitre(s) |
| mM | millimolar |
| mm | millimetre(s) |
| mRNA | messenger ribonucleic acid |
| Nal | nalidixic acid |
| ng | nanogram(s) |
| nm | nanometre(s) |
| NY | nutrient yeast |
| OD | optical density |
| $OD_{600}$ | optical density at 600nm |
| ORF | open reading frame(s) |
| P | promoter |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pg | picogram(s) |
| Ph | phosphorus |
| r | resistant |
| Rif | rifampicin |
| RBS | ribosome binding site |
| *RLS* | *rpoN*-like sequence |
| RNA | ribonucleic acid |
| RNaseA | ribonuclease A |
| RNaseH | ribonuclease H |
| RP | reverse primer |
| RT | reverse transcription/transcriptase |
| S | sulphur |
| σ | sigma |
| SDS | sodium dodecyl sulphate |
| SOE | splicing by overlap extension |
| SSC | salt, sodium, citrate buffer |
| TAE | Tris, acetate, EDTA buffer |
| TBE | Tris, borate, EDTA buffer |
| Tc | tetracycline |
| TE | 10mM Tris-HCl, 1mM EDTA, pH8.0 |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TEN | Tris, EDTA, NaCl buffer |
| Tsp | transcription start point(s) |
| UAS | upstream activator site |
| UP | universal primer |
| V | volts |
| v/v | volume per volume |
| W | watts |
| w/v | weight per volume |
| X-gal | 5-bromo-4chloro-3-indol-β-D-galactopyranoside |
| °C | degrees Celsius |
| ΔG | net change in free energy |
| Ω | ohms |
| % | percentage |

# CHAPTER ONE

# INTRODUCTION

## 1.1 Gonorrhoea

The sexually transmitted disease gonorrhoea is caused by the obligate human pathogen, *Neisseria gonorrhoeae*. This organism is a Gram negative diplococcus and the primary sites of colonisation are the mucous membranes of the urethra, endocervix, pharynx, rectum and conjunctiva (Britigan *et al.*, 1985; Stephens, 1989). The resulting disease is characterised by a purulent discharge consisting mainly of phagocytic cells (Handsfield, 1990). Complications can occur if the infection spreads to the upper genital tract to cause epididymitis in men or pelvic inflammatory disease in women, or it can disseminate and cause bacteraemia (Ross, 1996).

The diagnosis of gonorrhoea is of great importance as gonococcal infection remains one of the most common sexually transmitted infections among individuals in developing countries, despite the decline in developed countries in the past decade (De Schryver and Meheus, 1990). If left untreated it can lead to sterility in males and females (Hook and Handsfield, 1990). This is compounded by the asymptomatic nature of up to 50% of women and 10% of men infected (Bignell, 1999; Hook and Holmes, 1985). Thus gonorrhoea presents a global health problem.

Gram stains of exudate specimens are used to confirm gonococcal urethritis in men, but culture is necessary for the diagnosis of cervicitis in women. However, asymptomatic patients are more difficult to diagnose with microscopy (Jephcott, 1997; Sherrard and Barlow, 1996). This problem, in addition to the increasing incidence of gonococcal resistance to antimicrobial agents used for treatment (Fox and Knapp, 1999), has necessitated the surveillance of resistance patterns to facilitate treatment. Vaccine development has proved difficult as *N. gonorrhoeae* has acquired several strategies to evade the host immune response. Gonorrhoea, when left untreated, can also significantly facilitate the transmission of human immunodeficiency virus, which further requires the implementation of appropriate control measures (Grosskurth *et al.*, 1995).

It has been difficult to study the pathogenesis of *N. gonorrhoeae* as there is no accepted animal model. As an alternative *in vitro* cell and organ culture assays have been employed, utilising human cells and tissues from sites routinely infected with *N. gonorrhoeae* (Stephens, 1989). Recent *in vivo* studies with human male volunteers have confirmed and expanded the results obtained *in vitro* (Cohen *et al.*, 1994; Cornelissen *et al.*, 1998; Seifert *et al.*, 1994).

The first step of the gonococcal infection process is the attachment and colonisation of host non-ciliated, columnar epithelial cells located on mucosal surfaces (Stephens, 1989). This initial interaction is mediated by the adherence of gonococcal surface organelles, pili (Section 1.6), to the mucosal epithelium (Stephens, 1989; Swanson, 1973). Pili are fine, hairlike structures that protrude from the surface of the pathogen. Attachment to human cells is thought to occur via the PilC protein which appears to be located at the tip of the pilus fibre and act as an adhesin (Rudel *et al.*, 1995).

Following attachment of the pathogen the colony opacity associated (Opa) proteins, a family of phase-variable outer membrane proteins, mediate close interaction to the target epithelial cells (Blake *et al.*, 1995; Makino *et al.*, 1991). This event is essential for invasion of the host cells. Gonococci can express any of the eleven variant *opa* genes present in the gonococcal genome (Bhat *et al.*, 1991), generating heterogeneous bacterial populations. There are two classes of host cell receptors for Opa proteins and binding depends on the variant Opa protein in question. One class is the heparan sulfate proteoglycan receptor expressed by epithelial cells (van Putten and Paul, 1995). The other class is the carcinoembryonic antigens (CD66) which are members of the immunoglobulin superfamily (Gray-Owen *et al.*, 1997) and expressed differentially by multiple cell types throughout the human host. The cell tropism of *N. gonorrhoeae* is thus determined by the expression of variable Opa proteins (Kupsch *et al.*, 1993).

Additional putative virulence determinants have also been identified which affect gonococcal adherence and invasion of host cells. These include lipooligosaccharide (LOS; Minor *et al.*, 2000) and the porin PorB, a typical porin located in the outer membrane of the gonococcus (Bauer *et al.*, 1999).

Subsequent to the invasion and internalisation of host epithelial cells by a process termed parasite-directed endocytosis (McGee *et al.*, 1988), the gonococcus can reside in vacuoles or free in the cytoplasm (Shaw and Falkow, 1988). At this stage the bacteria can replicate intracellularly, a process dependent on host cell pyruvate and mediated by the Opa proteins (Williams *et al.*, 1998). This is followed by activation of the host immune response and a massive infiltration of phagocytic cells to the site of infection. Apoptosis of the infected cells permits the gonococci to infect adjacent epithelial cells or the recruited phagocytic cells, or they may disseminate via the bloodstream (McGee *et al.*, 1983). *N. gonorrhoeae* has evolved several mechanisms to avoid destruction by phagocytes once internalised (Haines *et al.*, 1991; Lorenzen *et al.*, 2000). Hence, the infected phagocytes can serve as a route of transmission of *N. gonorrhoeae* from one host to the next (Shafer and Rest, 1989).

## 1.2 Meningitis

*Neisseria meningitidis*, the other obligate human pathogenic neisserial species in addition to *N. gonorrhoeae*, is one of the causative agents of septicaemia and meningitis (Schwartz *et al.*, 1989). Meningococcal disease occurs world wide on an endemic scale and is a significant cause of morbidity and mortality with disease rates of industrialised countries significantly lower than those of third-world countries (van Deuren *et al.*, 2000).

The organism colonises the nasopharynx and 5-10% of adults are asymptomatic carriers of meningococcal strains that are predominantly non-pathogenic (Caugant *et al.*, 1994). Virulent strains can traverse the mucosal epithelium (Dehio *et al.*, 1998), gain access to the bloodstream and cause systemic disease. From here *N. meningitidis* can cross the blood-brain barrier to reach the cerebrospinal fluid and cause meningitis.

*N. meningitidis* shares a number of pathogenicity factors with *N. gonorrhoeae* inlcuding the outer membrane Opc protein (de Vries *et al.*, 1998), pili (Stephens, 1989), capsular polysaccharide (Stephens *et al.*, 1993) and IgA protease (Vitovski *et al.*, 1999). Additional pathogenicity factors include the lactoferrin binding receptor, LbpA, and the siderophore receptor, FrpB (Pettersson *et al.*, 1997).

Despite the differences in colonisation sites and the infectious process, *N. gonorrhoeae* and *N. meningitidis* are genetically very closely related. When housekeeping genes of the two species are compared the nucleotide sequence similarity exceeds 98% (Zhou and Spratt, 1992). The recent sequencing of the genomes has allowed a precise calculation with over 90% similarity at the amino acid sequence level (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000; Tinsley and Nassif, 2001).

## 1.3 Genetic systems of *N. gonorrhoeae*

The genome of *N. gonorrhoeae* is 2200 kilobase (kb) pairs in size (Dempsey *et al.*, 1991) with the potential to encode approximately 2000 gene products. During this project, sequencing of the *N. gonorrhoeae* strain FA1090 genome was completed (Genbank accession number AE004969) with annotation of the sequence still underway. This has allowed the identification of putative genetic loci involved in pathogenesis that may have the potential to be used as therapeutic targets or vaccine candidates.

Virtually all strains of *N. gonorrhoeae* possess a 4.2kb cryptic plasmid (Davies, 1990) and some also contain a 39kb conjugative plasmid (Davies, 1985). The *tetM* gene, conferring tetracycline resistance, has been found in a portion of these conjugative plasmids (Burdett *et al.*, 1982). Numerous repetitive DNA elements are found throughout the gonococcal genome (Correia *et al.*, 1986, 1988; Hamilton, 1999) and are postulated to be involved in recombination events, suggesting genome fluidity.

The two mechanisms of genetic exchange that operate in *N. gonorrhoeae* are transformation and conjugation. Transformation of chromosomal genes is extremely efficient with a frequency of 1% of cells serving as recipients in any given experiment (Seifert and So, 1991). Only piliated gonococcal cells are competent for transformation (Sparling, 1966) and preferentially take up DNA that contains a 10 base pair (bp) DNA uptake sequence (Goodman and Scocca, 1988). Double stranded DNA penetrates the cell and recombines into the chromosome by homologous recombination (Biswas and Sparling, 1981). The autolysis of gonococcal cells *in vivo* under poor growth conditions provides free DNA required for the transformation process (Hebeler and Young, 1975). Conjugation in *N. gonorrhoeae* occurs at a lower frequency and results in the mobilisation of plasmid DNA that often encodes antibiotic resistance genes (Norlander

*et al.*, 1979).

It has been difficult to study the genetic manipulation and mutagenesis of *N. gonorrhoeae* due to the lack of appropriate genetic systems. However, the development of the Hermes shuttle vectors has allowed the genetic complementation of *Neisseria* mutants (Kupsch *et al.*, 1996). In addition gonococcal chromosomal gene function can be determined using transposon shuttle mutagenesis systems (Kahrs *et al.*, 1994).

## 1.4 Genetics of gonococcal surface protein variation

Several of the virulence associated proteins produced by *N. gonorrhoeae* are subject to phenotypic variation. This produces a repertoire of variants from which the fittest bacteria can evade the host immune response (Meyer and van Putten, 1989), function at different stages of pathogenesis (Rudel *et al.*, 1992) and determine tissue tropism (Kupsch *et al.*, 1993; Seifert and So, 1988). The genetic processes that mediate this are phase and antigenic variation (Cannon and Sparling, 1984). Phase variation refers to the 'switch on' or 'switch off' of expression, and antigenic variation describes the expression of antigenically distinct forms of the same proteins (Meyer and van Putten, 1989; Seifert and So, 1988). A number of additional genes with varying functions and hypothetical proteins with the potential to be phase-variable have been identified in *N. gonorrhoeae* (Snyder *et al.*, 2001). Thus such processes provide the gonococcus with relatively simple mechanisms of environmental adaptation.

The surface components subject to these processes include pilin, the Opa proteins and LOS. Variations in pilin expression result from non-reciprocal recombination events between silent gene copies and the expression locus (Meyer and van Putten, 1989). Opa protein phase variation depends on slipped-strand mispairing of DNA during DNA replication (Belland *et al.*, 1989; Murphy *et al.*, 1989) and antigenic variation relies on reciprocal recombination between different *opa* genes (Connell *et al.*, 1988; Stern *et al.*, 1986). LOS phase variation results in a combination of intrinsic changes in its synthesis and differential sialylation of external moieties (McGee *et al.*, 1996; van Putten and Robertson, 1995).

## 1.5 Culture and colony morphology of *N. gonorrhoeae*

*N. gonorrhoeae* grows optimally *in vitro* at 37°C in an atmosphere of 5% (v/v) $CO_2$. Within 20 hours of inoculation piliated cultures appear as small, shiny, domed colonies with defined edges on solid media. With time pili are lost and colonies appear large and dull with an undefined edge (Swanson *et al.*, 1971). Cells expressing Opa proteins appear as opaque colonies (Swanson *et al.*, 1971) and those without them are described as transparent (Davies, 1990).

## 1.6 Type 4 pili

### 1.6.1 Introduction

Type 4 pili (or fimbriae) are hair-like surface appendages widely distributed in Gram negative bacteria. They are located at the pole of the cell, mediate attachment to host epithelial cells (Faranchych and Frost, 1988) and are therefore correlated with virulence. They also mediate twitching motility, the mechanism responsible for surface translocation, and thus movement of cells across mucosal surfaces (Bradley, 1980; Henrichsen, 1983). Bacteria that express type 4 pili include *N. gonorrhoeae*, (Hermodson *et al.*, 1978), *N. meningitidis* (Hermodson *et al.*, 1978), *Pseudomonas aeruginosa* (Johnson *et al.*, 1986), *Moraxella bovis* (Marrs *et al.*, 1985), *Moraxella lacunata* (Marrs *et al.*, 1990), *Dichelobacter nodosus* (McKern *et al.*, 1988), *Eikenella corrodens* (Hood and Hirschberg, 1995), *Aeromonas hydrophila* (Pepe *et al.*, 1996) and *Pasteurella multocida* (Ruffolo *et al.*, 1997).

The structure of type 4 pili from different species is very similar suggesting they may be processed, assembled and exported by a common mechanism (Strom and Lory, 1993). This has been supported by the expression of type 4 pili in heterologous species (Elleman *et al.*, 1986; Hoyne *et al.*, 1992; Mattick *et al.*, 1987). They are comprised of approximately 500-1000 identical subunits, denoted pilin (Strom and Lory, 1993) and are assembled in a helical manner to form a pilus (Strom and Lory, 1993). Pilin is synthesised as a precursor with a short, basic, amino terminal leader peptide which is cleaved by a prepilin peptidase prior to assemble of the pilin monomers (Strom and Lory, 1993). Each mature pilin subunit has the distinctive feature of a highly conserved

hydrophobic and methylated amino terminus (Strom and Lory, 1993).

### 1.6.2 Regulation of type 4 pili

The regulation of pilin production has been extensively studied in *P. aeruginosa*. In this organism pilin is encoded by the *pilA* gene and is transcribed using the alternative sigma ($\sigma$) factor, $\sigma^{54}$ or RpoN (Section 1.7), from a conserved $\sigma^{54}$ promoter (Ishimoto and Lory, 1989). Transcription initiation from $\sigma^{54}$ promoters is dependent on an activator protein which binds to a sequence termed the upstream activator site (UAS), situated approximately 100bp upstream of the promoter (Reitzer and Magasanik, 1986). Such an UAS was identified upstream of *pilA* (Pasloske *et al.*, 1989) to which the PilR activator protein binds and activates transcription (Ishimoto and Lory, 1992). PilR, in conjunction with the histidine kinase sensor protein, PilS, forms a two-component regulatory system essential for *pilA* expression (Hobbs *et al.*, 1993).

The regulatory mechanisms controlling pilin production have not been well characterised in other bacteria. However, consensus sequences for $\sigma^{54}$ promoters have been identified upstream of the genes encoding the pilin subunit in *D. nodosus* (Elleman and Hoyne, 1984), *M. lacunata* and *M. bovis* (Heinrich and Glasgow, 1997; Marrs *et al.*, 1985), *A. hydrophila* (Pepe *et al.*, 1996), *Myxococcus xanthus* (Wu and Kaiser, 1997) and *E. corrodens* (Villar *et al.*, 1999). This suggests that many type 4 pilin producing bacteria have systems controlling pilin expression analogous to that in *P. aeruginosa*.

### 1.6.3 Regulation of type 4 pilin expression in *N. gonorrhoeae*

The gene encoding the gonococcal pilin subunit, *pilE* (Meyer *et al.*, 1984), is regulated at the transcriptional level. Sequence analysis in conjunction with transcriptional experiments have identified three consensus sequences for promoters upstream of *pilE*. They include two $\sigma^{70}$ promoters (P1 and P2; Fyfe *et al.*, 1995; Hawley and McClure, 1983) and a $\sigma^{54}$ promoter (P3) that overlaps P1 (Figure 3.1; Fyfe *et al.*, 1995; Thony and Hennecke, 1989). Based on these observations it was postulated that pilin expression in *N. gonorrhoeae* required $\sigma^{54}$ and the cognate regulatory factors. However, *in vitro* it appears that only P1 is required for transcription of *pilE* in *N. gonorrhoeae* (Fyfe *et al.*, 1995). Transcription from P2 is evident only in *Escherichia coli* (Fyfe *et al.*, 1995). It

has previously been suggested that P3 is functional in *N. gonorrhoeae* (Taha and Giorgini, 1995) but site-directed mutagenesis experiments have proved that this is not the case *in vitro* (Fyfe *et al.*, 1995; Laskos *et al.*, 1998). It seemed possible that P3 may function *in vivo* due to the presence of an UAS located upstream of *pilE* (Carrick *et al.*, 1997; Fyfe *et al.*, 1993) that resembles the PilR binding site found upstream of *pilA* in *P. aeruginosa* (Pasloske *et al.*, 1989). This would rely on the presence of the appropriate activator.

Until recently, what was suggested to be a two-component regulatory system, PilA and PilB, was thought to regulate *pilE* transcription in *N. gonorrhoeae* (Taha *et al.*, 1988; Taha *et al.*, 1991). Subsequent studies revealed that PilA is in fact an FtsY homologue and does not affect *pilE* expression in the gonococcus (Arvidson *et al.*, 1999; Fyfe and Davies, 1998). The FtsY protein is involved in the proper localisation of inner membrane proteins (Luirink *et al.*, 1994). Additionally, PilB has been shown to function as a peptide methionine sulfoxide reductase (Wizemann *et al.*, 1996).

Identification of a chimeric gene, *rsp*, which, when translated, displays similarity to the functional sequences of PilR and PilS from *P. aeruginosa*, led to the hypothesis that it encoded the two-component regulatory system responsible for $\sigma^{54}$ dependent transcription of *pilE* (Carrick *et al.*, 2000). The same study revealed that this protein was not involved in pilin regulation *in vitro*.

Subsequent studies have demonstrated that the DNA binding protein, Integration Host Factor (IHF), and an AT rich sequence upstream of *pilE* are required for optimal expression (Fyfe and Davies, 1998; Hill *et al.*, 1997).

Collectively these results do not exclude the possibility that $\sigma^{54}$ is required for pilin expression. Alternative growth conditions may be required or $\sigma^{54}$ may not have been functional under the conditions tested.

## 1.7 The alternative sigma factor, $\sigma^{54}$

### 1.7.1 Introduction

$\sigma^{54}$ was initially described as an alternative σ factor based on its alternative mechanism of transcription initiation (Merrick, 1993) despite a lack of similarity at the amino acid level to any known sigma factor (Merrick and Gibbins, 1985). Genes encoding $\sigma^{54}$ have been identified from a number of bacteria using classical genetic approaches. These include *E. coli* (Jones *et al.*, 1994), *P. aeruginosa* (Ishimoto and Lory, 1989), *Bacillus subtilis* (Debarbouille *et al.*, 1991) and *Bradyrhizobium japonicum* (Kullik *et al.*, 1991). Recent genome sequencing projects have also identified open reading frames (ORFs) potentially encoding $\sigma^{54}$ from a diverse range of species within the phylogenetic domain *Bacteria* (Studholme and Buck, 2000). $\sigma^{54}$ is implicated in the expression of a wide variety of genes involved in assorted cellular functions such as the utilisation of nitrogen and carbon sources, amino acid transport, toluene and xylene catabolism, RNA metabolism and pilin synthesis (Merrick, 1993; Studholme and Buck, 2000).

### 1.7.2 $\sigma^{54}$ structure and promoter recognition

$\sigma^{54}$ promoters have the consensus sequence, 5' -TGGCACGNNNNTTGCa/t- 3', with the conserved GG and GC motifs (underlined) located at positions -24 and -12 relative to the transcription start point (tsp; Morett and Buck, 1989). Historically they were classified as -24/-12 promoters (Thony and Hennecke, 1989). Recent evidence has shown that the spacing of the -12 element relative to the mapped tsp is not absolute and the distance between them may vary between 8 and 21 nucleotides, with a spacing of 11-13 nucleotides most common (Barrios *et al.*, 1999). The appropriate spacing between the strictly conserved elements is essential as deletions in this region abolish promoter function (Buck, 1986; Keseler and Kaiser, 1995; Mullin and Newton, 1993). In order for $\sigma^{54}$ to bind, the two promoter motifs must be positioned on the same face of the DNA helix (Merrick, 1993). The degree of sequence conservation among $\sigma^{54}$ promoters is very high between distantly related bacteria allowing potential promoters to be readily identified (Barrios *et al.*, 1999; Studholme and Buck, 2000). In fact, potential $\sigma^{54}$ promoters have been identified upstream of several gonococcal genes in addition to *pilE*

(Section 3.1).

Alignment of the predicted amino acid sequence of various $\sigma^{54}$ proteins revealed three conserved regions (Merrick *et al.*, 1987; Merrick, 1993). Mutation analysis and protein fragmentation studies led to assigned functions for each region (Cannon *et al.*, 1995; Gallegos and Buck, 1999; Sasse-Dwight and Gralla, 1990; Tintut and Gralla, 1995; Wong *et al.*, 1994). The N-terminal 50 amino acids comprises Region I and is primarily responsible for $\sigma^{54}$ activation. It is rich in glutamine and leucine residues and has both positive and inhibitory functions during the transcription initiation process. Region I binds to the early melted -12 DNA element (Gallegos and Buck, 2000; Hsieh and Gralla, 1994; Wigneshweraraj *et al.*, 2000) and inhibits initiation of transcription prior to binding of the activator upstream of the promoter (Hsieh *et al.*, 1994; Syed and Gralla, 1997). This is achieved by the concomitant binding of Region I to core RNA polymerase which keeps the holoenzyme in such a conformational state that it can not isomerise (Casaz and Buck, 1999; Gallegos and Buck, 1999). Subsequent recruitment of the activator allows Region I to overcome this repression which results in isomerisation and conversion from a closed promoter complex to a stabilised open complex (Syed and Gralla, 1997, 1998; Wang *et al.*, 1995).

Region I is followed by a central region, Region II, that is variable in both length and sequence (Merrick, 1993). It can range in length from 26 residues in *Rhodobacter capsulatus* (Cullen *et al.*, 1994) to 110 residues in *B. japonicum* (Kullik *et al.*, 1991) and is characterised by a predominance of acidic residues. The acidic residues occur every third amino acid in the $\sigma^{54}$ protein of enteric bacteria and thus have been termed acidic trimer repeats (Wong and Gralla, 1992). This region is thought to influence the binding of $\sigma^{54}$ to core RNA polymerase, stabilise the holoenzyme and suppress non-specific DNA binding by the holoenzyme (Cannon *et al.*, 1999; Southern and Merrick, 2000). In addition it has been implicated in the melting of promoter DNA during transcription initiation (Wong and Gralla, 1992) and open complex formation prior to holoenzyme isomerisation (Southern and Merrick, 2000). However, the lack of Region II conservation suggests that none of its proposed activities are essential (Cannon *et al.*, 1999).

The carboxy-terminal Region III is approximately 400 residues in length and is primarily responsible for binding to promoter DNA sequences, modulation of DNA binding and core RNA polymerase binding. The conserved carboxy-terminal one-third of the protein has been shown to cross-link DNA and recognises the –12 and –24 promoter elements (Cannon *et al.*, 1994; Guo and Gralla, 1997). The conserved helix-turn-helix (HTH) motif, in collaboration with additional regions of the protein, is also involved in binding to the -12 element (Chaney and Buck, 1999; Merrick and Chambers, 1992). Region III contains a highly conserved stretch of 10 amino acids designated the RpoN box which is diagnostic of all $\sigma^{54}$ proteins (Merrick, 1993). It is required for DNA binding and implicated in recognition of the -24 element (Taylor *et al.*, 1996; Wang and Gralla, 2001). Adjacent sequences enhance this binding activity most likely through intramolecular interaction with the primary binding domain (Cannon *et al.*, 1997). The start of Region III contains a hydrophobic heptad repeat interdigitated with a high number of strongly acidic residues (Sasse-Dwight and Gralla, 1990). This section constitutes the main core RNA polymerase binding determinant (Gallegos and Buck, 1999; Hsieh *et al.*, 1999; Tintut *et al.*, 1994; Wong *et al.*, 1994). Parts of Region III are also involved to a lesser degree in polymerase isomerisation (Oguiza and Buck, 1997; Oguiza *et al.*, 1999), the early events of transcription initiation (Kelly and Hoover, 1999; Oguiza *et al.*, 1999) and activator responsiveness (Chaney and Buck, 1999; Wang and Gralla, 2001). Therefore, it seems that there is considerable overlap in the complex functions between the distinct domains of $\sigma^{54}$. This is further complicated by the appearance of interdomain communication between Region I and the DNA binding domain of Region III during transcription (Casaz and Buck, 1999; Wigneshweraraj *et al.*, 2000). This interaction forms an organising centre over the -12 region which acts as a molecular switch to prevent DNA melting prior to activation (Guo *et al.*, 1999; Wang *et al.*, 1999; Wigneshweraraj *et al.*, 2001).

### 1.7.3 Activation of $\sigma^{54}$ promoters

As stated previously, activation of expression from $\sigma^{54}$ promoters is absolutely dependent on a regulatory protein that binds to an UAS (Section 1.6.2). Bacterial UASs resemble eukaryotic transcriptional enhancers (Reitzer and Magasanik, 1986) defined as *cis*-acting DNA sequences that increase transcription rates of RNA polymerase II (Blackwood and Kadonaga, 1998). In order for the distally situated activator to interact

with the $\sigma^{54}$ core RNA polymerase complex, looping of the intervening DNA is required (Rippe *et al.*, 1997). This looping mechanism is often mediated and stabilised by the bending protein, IHF, which binds to a specific site located between the promoter and UAS (Claverie-Martin and Magasanik, 1991). IHF can also have the additional role of stimulating transcription initiation by recruiting $\sigma^{54}$-holoenzyme to the promoter (Bertoni *et al.*, 1998). For some promoter regions that lack the IHF binding site the intervening DNA has an intrinsic single bend that allows interaction between the activator and $\sigma^{54}$-holoenzyme promoter complex (Carmona *et al.*, 1997).

The vegetative sigma factor, $\sigma^{70}$, and related sigma factors function by binding to promoter specific sequences only when complexed with core RNA polymerase (Dombroski *et al.*, 1992). $\sigma^{54}$ differs as it is capable of promoter specific binding in the absence of core RNA polymerase, although binding is greatly increased in its presence (Cannon *et al.*, 1993), such that holoenzyme assembly occurs directly on the promoter (Merrick, 1993). The $\sigma^{54}$-holoenzyme in a closed promoter complex is controlled at the level of DNA melting by a proposed 'molecular switch' that involves Region I and the -12 element (Cannon *et al.*, 2000; Guo *et al.*, 1999; Guo *et al.*, 2000; Wang *et al.*, 1999), and is unable to form an open complex in the absence of activator (Popham *et al.*, 1989). Binding of the activator catalyses the isomerisation of the closed promoter complex to an open one via the energy generated from adenosine triphosphate (ATP) hydrolysis (Popham *et al.*, 1989). This occurs by turning the switch 'on' which leads to modification of both $\sigma^{54}$ interaction with DNA and holoenzyme conformation to suppress the transcriptional silencing (Guo *et al.*, 2000). Simultaneous conformational changes in $\sigma^{54}$ occur which also lead to this event (Cannon *et al.*, 1999; Gallegos *et al.*, 1999; Guo *et al.*, 1999). Thus holoenzyme isomerisation and DNA promoter melting lead to transcription initiation.

There is evidence to suggest that $\sigma^{54}$ and $\sigma^{70}$ bind to the same region of core RNA polymerase (Gallegos and Buck, 1999) and that their core-binding domains have similar shapes (Svergun *et al.*, 2000; Wigneshweraraj *et al.*, 2000). Therefore it appears the specialised properties of $\sigma^{54}$ holoenzyme are conferred to some extent by Region I and its interaction with core RNA polymerase.

### 1.7.4 $\sigma^{54}$ dependent transcriptional activators

Comprehensive studies of transcriptional activators that function with $\sigma^{54}$ suggest they operate via a common mechanism (Xu and Hoover, 2001). Activators contain three functionally distinct domains with varying degrees of homology (Shingler, 1996) and can be divided into subgroups that reflect the mechanisms of activation in response to biological input (Shingler, 1996). One subgroup, which includes PilR (Section 1.6.2; Ishimoto and Lory, 1992), is a member of two-component regulatory systems that respond to signals sensed by the cognate histidine kinase sensor protein (Gross *et al.*, 1989). Activation occurs by phosphorylation of the activator.

Several activators are capable of initiating transcription of functionally unrelated genes with $\sigma^{54}$ promoters in heterologous backgrounds. This includes activation of the gonococcal *pilE* $\sigma^{54}$ promoter (P3) by NifA from *E. coli* (Boyle-Vavra *et al.*, 1993) and PilR (Carrick *et al.*, 1997; Fyfe *et al.*, 1995) suggesting that a functional UAS is located in the region upstream of *pilE*. However, *N. gonorrhoeae* does not contain any intact activators that could direct transcription from $\sigma^{54}$ promoters (Genbank accession number AE004969; Carrick, 1998).

## 1.8 The bacterial heat shock response

The bacterial heat shock response is characterised by the rapid induction of a set of proteins in response to environmental stresses (Lindquist, 1986; Neidhardt *et al.*, 1984). These proteins and the genes that encode them have been historically referred to as heat shock proteins (Hsps) and heat shock genes, respectively, as a temperature upshift was the first stress to be tested and could be done so uniformly by most investigators (Macario *et al.*, 1999). Their biological role is to protect cells against the toxic effects generated by exposure to stress and they also have an essential role in protein function during normal growth conditions. The extensive structural homology between some of the Hsps from all phylogenetic kingdoms indicates that this response is universal and has been conserved during evolution (Bardwell and Craig, 1984; Hunt and Morimoto, 1985). It is found in nearly every cell type examined (Lindquist, 1986). The response was initially discovered in the fruit fly species *Drosophila* when a new set of puffs were

noticed on the salivary gland chromosomes when subjected to heat, dinitrophenol and sodium salicylate (Tissieres *et al.*, 1974).

Upon heat shock at least 20 Hsps can be identified in *E. coli* by 2-dimensional gel analysis (Neidhardt *et al.*, 1984) and with more recent recently developed techniques at least 100 induced proteins have been identified in *B. subtilis* (Bernhardt *et al.*, 1999).

Many Hsps act as molecular chaperones, and function in the folding and assembly of proteins. These include DnaK, DnaJ, GrpE, GroEL, GroES and HtpG (Section 1.9; Ellis, 1987). Hsps are also involved in all major growth-related cellular processes (Mager and De Kruijff, 1995).

There is strong evidence to suggest that the signal that induces the heat shock response is the accumulation of misfolded and denatured proteins upon stress (Goff and Goldberg, 1985; Kanemori *et al.*, 1994; Voellmy, 1996). Many proteins lose their native, functional configuration and tend to aggregate. Hsps can reverse this process allowing the proper intra- and inter-molecular folding and assembly of proteins (Lindquist, 1986).

Hsps are induced immediately in response to elevated temperature but the maximum induction temperature varies depending on the normal range of environmental exposure. In *E. coli*, Hsp synthesis is initiated when cells are shifted from 37°C to 42°C but the maximum response is observed at 45-50°C (Neidhardt *et al.*, 1984; Yamamori *et al.*, 1978). The response is transient but the length of time it is sustained differs and depends in part on the experimental parameters examined and undoubtedly the environmental conditions encountered *in vivo*. A modest 5°C increase from the optimal growth temperature of 37°C in *E. coli* results in rapid induction of Hsp synthesis which reaches its maximum at 5 minutes and declines to a new steady-state level at 20-30 minutes (Neidhardt *et al.*, 1984; Yamamori *et al.*, 1978). This decline is termed the adaptation phase of the heat shock response. Exposure to a more lethal temperature such as 50°C ensures that an elevated level of Hsp synthesis is maintained (Craig and Gross, 1991; Neidhardt *et al.*, 1984). Alternatively, Hsp synthesis in *Caulobacter crescentus* continues to escalate for at least 90 minutes following a temperature upshift (Baldini *et al.*, 1998). Exposure to stress induces activation of the heat shock genes but also results

in the down-regulation of many housekeeping genes.

Induction of bacterial heat shock genes upon stress is regulated at the transcriptional level (Yura *et al.*, 1993). However, the mechanisms controlling this induction vary greatly depending on the organism. Some bacteria positively regulate the heat shock response with the alternative sigma factor, $\sigma^{32}$ or RpoH (Section 1.12). Other bacteria negatively regulate heat shock genes by the Controlling Inverted Repeat of Chaperone Expression (CIRCE)/HrcA repressor system (Section 1.13). To further complicate matters the response in several bacteria is regulated via both positive and negative mechanisms (Section 1.13). In addition some heat shock genes are subject to post-transcriptional regulation which maintains the appropriate level of the protein in the cell at all times (Avedissian *et al.*, 1995; Parsons *et al.*, 1999).

In addition to elevated temperature, the heat shock response is elicited by a number of other physical or metabolic stresses. However, the importance of these stresses depends on the ecosystem in which the bacteria inhabit. They include an acid or alkaline shift, oxidative or osmotic stress, ethanol, DNA-damaging agents such as nalidixic acid and starvation for glucose, carbon, oxygen or phosphate (Bianchi and Baneyx, 1999; Hecker and Volker, 2001; Krueger and Walker, 1984; Meury and Kohiyama, 1991; Neidhardt *et al.*, 1984; Taglicht *et al.*, 1987). Under these conditions Hsps are produced in addition to proteins that can be specific for each inducing stress. Thus there appears to be considerable overlap between the various stress regulons.

## 1.9 Molecular chaperones

### 1.9.1 Introduction

Molecular chaperones are a conserved set of unrelated, specialised proteins found in all members of the phylogenetic domains *Eucarya*, *Archaea* and *Bacteria* (Hartl, 1996). The term was initially used by Laskey *et al.* (1978) to describe the role of nucleoplasmin in the assembly of DNA and histones into nucleosomes, without itself forming part of the overall structure. The major classes of chaperones identified, based on the apparent molecular weight, are the 40kDa heat shock proteins (Hsps; Hsp40/DnaJ family), the 70kDa Hsps (Hsp70/DnaK family), 60kDa Hsps (Hsp60/GroEL family), 90kDa Hsps

(Hsp90/HtpG), 100kDa Hsps (Hsp100/ClpABX:HslU) and the small Hsps such as IbpAB (the chaperone classifications in parentheses denote the eukaryotic and bacterial homologues, respectively; Ben-Zvi and Goloubinoff, 2001; Fink, 1999).

Despite the fact that the amino acid sequence of a polypeptide chain contains all the information that determines the three-dimensional structure of the functional protein, many proteins require additional assistance to fold correctly (Creighton, 1990). Molecular chaperones play an essential role in cell physiology and are defined as proteins that bind to and stabilise not only unstable, but apparently stable proteins, and by controlling the binding and release of the substrate protein promote its appropriate fate *in vivo*, without themselves becoming a component of the final structure (Hendrick and Hartl, 1993).

The Hsp60 and Hsp70 chaperones and their associated co-chaperones have been extensively studied in the eukaryotic organisms *Drosophila melanogaster* (Craig *et al.*, 1983; Martin *et al.*, 1995), *Saccharomyces cerevisiae* (Boorstein *et al.*, 1994), plants (Boston *et al.*, 1996), humans (Jindal *et al.*, 1989; Tavaria *et al.*, 1996) and the bacterial species *E. coli* (Kusukawa and Yura, 1988; Lund, 2001) and *B. subtilis* (Hecker *et al.*, 1996; Hecker and Volker, 2001; Volker *et al.*, 1994). The primary structure of these proteins is so similar they are thought to be the most conserved proteins found in all organisms and thus true evolutionary homologues (Ellis and van der Vies, 1991; Feder and Hofmann, 1999; Gupta and Golding, 1993; Zeilstra-Ryalls *et al.*, 1991). They can be found in all the major cellular compartments of eukaryotic organisms and are also present in multiple copies in some bacteria therefore comprising 'superfamilies' (Lindquist and Craig, 1988; Nimura *et al.*, 1994; Seaton and Vickery, 1994; Vickery *et al.*, 1997). Such homologues often differ in inducibility and function.

Specific peptide binding sites have been identified for some chaperones, as determined by their interaction with particular protein targets (Hardy and Randall, 1993), but they generally have relatively promiscuous specificity (Melnick and Argon, 1995). They bind non-covalently to hydrophobic residues on interactive nascent protein surfaces that are accessible transiently during cellular processes (Ellis and van der Vies, 1991). Release often involves hydrolysis of ATP and additional co-chaperone proteins (Skowyra *et al.*, 1990; Viitanen *et al.*, 1990).

In addition some chaperones can function as oligomers or complexes of several different chaperones, acting sequentially in protein folding pathways (Johnson and Craig, 1997). Each chaperone binds to protein intermediates at different stages of folding eventually releasing a competent native protein (Ruddon and Bedows, 1997; Zolkiewski, 1999). Protein folding by molecular chaperones has been studied in a few cases *in vivo* and *in vitro* and the pathways appear to be similar in each case (Mitraki *et al.*, 1991; Ruddon and Bedows, 1997). However some aspects of the folding process seem to be significantly different when comparing prokaryotic and eukaryotic systems (Feldman and Frydman, 2000; Netzer and Hartl, 1997).

### 1.9.2 Functions of molecular chaperones

Molecular chaperones are required for a multitude of cellular biochemical processes under all environmental conditions. Chaperones assist polypeptides to reach a biologically active conformation by preventing alternative pathways of assembly that can produce non-functional proteins and aggregates (Martin and Hartl, 1997). Protein aggregates can arise from the hydrophobic association of partially folded protein intermediates which collapse into a compact state and form a metastable secondary structure (Fink, 1995, 1998). Chaperones can also unfold proteins that have folded incorrectly and promote proper folding. They are involved in protein transport where proteins are required to enter eukaryotic organelles or the bacterial periplasm and therefore are required to traverse the membrane (Fink, 1999). In this process chaperones keep the newly synthesised protein precursors in a translocation-competent state until they have reached their final destination and subsequently aid in the refolding of the protein to its functional conformation (Hartl and Neupert, 1990; Lecker *et al.*, 1989). Cellular processes such as DNA replication, ribosome biogenesis, cell division, bacteriophage replication and those involving assembly of oligomeric complexes require chaperones at some stage for proper functioning (Alix and Guerin, 1993; Georgopoulos *et al.*, 1973; Gupta, 1990; Sakakibara, 1988). Chaperones are also involved in the degradation of unfolded proteins by targeting them for proteolysis by cytoplasmic proteases or ubiquitinating enzymes (Herman and D'Ari, 1998; Mathew and Morimoto, 1998).

Exposure to stress induces the expression of many molecular chaperones and therefore they are also classified as Hsps (Section 1.8). In some bacteria the chaperones DnaK, GroEL and their associated co-chaperones have the additional role of regulating the heat shock response by controlling the biological activity of specific transcription factors or regulatory elements (Sections 1.12.4 and 1.13; Mogk *et al.*, 1997; Straus *et al.*, 1990; Tilly *et al.*, 1983; Tomoyasu *et al.*, 1998). Chaperones can also function as pathogenicity factors and immunogens resulting in autoimmune disease in the host (Section 1.9.3).

### 1.9.3 Molecular chaperones as immunogens and pathogenicity factors

Several bacterial molecular chaperones have been identified as immunodominant antigens during infection with a number of pathogenic bacteria including *Chlamydia trachomatis* (Horner *et al.*, 1997), *Mycobacterium leprae* (McKenzie *et al.*, 1991), *Mycobacterium tuberculosis* (Shinnick *et al.*, 1988), *Treponema pallidum* (Hindersson *et al.*, 1987), *Listeria monocytogenes* (Kimura *et al.*, 1998) and a number of others (Lemos *et al.*, 2000; Qoronfleh *et al.*, 1993; Zugel and Kaufmann, 1999). Chaperones from the GroE, DnaK and HtpG families have been shown to induce very strong humoral and cellular immune responses by the host (Kaufmann, 1990; Shinnick, 1991; Zugel and Kaufmann, 1999). The extracellular location of some of these chaperones, especially GroEL, makes them readily accessible as targets for the immune response (Lund, 2001; Zhong and Brunham, 1992). This is of major significance as chaperones have been implicated in the pathogenesis of several autoimmune disorders (Kaufmann, 1990; Zugel and Kaufmann, 1999). The high degree of sequence homology between the bacterial and host chaperones can induce a cross-reactive immune response to conserved epitopes that leaves the host unable to distinguish between foreign and self antigens (Kaufmann, 1990; Theofilopoulos, 1995; Zugel and Kaufmann, 1999). The repertoire of autoimmune disorders is diverse and includes rheumatoid arthritis, diabetes and psoriasis (Elias *et al.*, 1990; McLean *et al.*, 1990; Rambukkana *et al.*, 1993; Zugel and Kaufmann, 1999).

Despite their role in autoimmunity, induction of a host immune response to bacterial chaperones can confer protective immunity against infection. Immunisation of mice with GroEL and GroES from *Helicobacter pylori* has been shown to protect against

subsequent challenge and gastroduodenal disease (Ferrero *et al.*, 1995). Such protective immunity is also displayed by mice when vaccinated with GroEL and DnaK from *M. tuberculosis* and several other intracellular pathogens (Blander and Horwitz, 1993; Lowrie *et al.*, 1997; McKenzie *et al.*, 1991; Noll *et al.*, 1994; Noll and Autenrieth, 1996; Silva and Lowrie, 1994; Zugel and Kaufmann, 1997).

Recent evidence suggests that chaperones have an important role in the pathogenesis of intracellular pathogens. DnaK and Hsp70-related proteins from some *Mycoplasma* species, *C. trachomatis*, *Haemophilus influenzae* and GroEL from *Haemophilus ducreyi* and *Legionella pneumophila* have been implicated in attachment to host cells, highlighting their function as potential virulence factors (Boulanger *et al.*, 1995; Frisk *et al.*, 1998; Garduno *et al.*, 1998; Hartmann and Lingwood, 1997; Hartmann *et al.*, 2001; Raulston *et al.*, 1993). DnaK from *Brucella suis* and *L. monocytogenes* is required for phagocytosis by macrophages and intracellular multiplication (Hanawa *et al.*, 1999; Kohler *et al.*, 1996). The Clp ATPase of *L. monocytogenes* is essential for *in vivo* survival and virulence in mice macrophages (Rouquette *et al.*, 1998).

Thus the involvement of chaperones in pathogenesis, either by directly mediating adhesion or by interaction with protein substrates required for intracellular survival, warrants investigation into their suitability as vaccine candidates (Rouquette *et al.*, 1998; Srivastava and Amato, 2001).

### 1.10 The GroE chaperone system

The GroE proteins of *E. coli*, encoded by the *groES* and *groEL* genes, were first identified because of their requirement for the assembly of several bacteriophages (Georgopoulos and Ang, 1990; Georgopoulos *et al.*, 1972). They are required for growth at all temperatures (Fayet *et al.*, 1989; Georgopoulos and Ang, 1990). The bacterial *groE* operon is composed of the genes, *groES* and *groEL*, respectively (Segal and Ron, 1996b). Some bacteria also contain an additional copy of the *groEL* gene arranged as a monocistronic operon (Segal and Ron, 1996b). At normal temperatures GroEL constitutes approximately 1% of total cellular protein but this increases to 10% under heat shock conditions (Herendeen *et al.*, 1979). Its abundance indicates that it is a major chaperone under stress conditions.

GroES comprises a single heptameric ring structure of 10 kilodaltons (kDa) and GroEL consists of 14 identical 60kDa subunits that form 2 heptameric ring structures with a central cavity (Figure 1.1; Braig *et al.*, 1994; Ellis and Hartl, 1996). Partially folded protein intermediates of up to 60kDa (Xu *et al.*, 1997) can fit into the GroEL cavity and then GroES together with ATP bind to the structure to form a lid over the cavity. ATP-dependent folding of the polypeptide follows and it is subsequently released into solution. If the protein is incompletely folded re-binding occurs and the process is repeated until it has reached the native state (Fenton and Horwich, 1997; Hartl, 1996). The GroE system can also unfold misfolded protein conformations such as those that occur in response to stress (Ranson *et al.*, 1995; Zahn *et al.*, 1996).

Under normal conditions GroEL appears to bind to about 10-15% of a defined set of newly synthesised cytoplasmic proteins and this increases to 30% following heat shock (Ellis and Hartl, 1996; Ewalt *et al.*, 1997). This is presumably due to the increased level of GroEL substrates that are misfolded upon stress (Lund, 2001). In some cases GroEL can associate with substrates following their release from the DnaK chaperone system (Section 1.11.2).

The majority of studies on GroE have been in *E. coli* and it is assumed that the chaperone system functions via a similar mechanism in all other cell types (Lund, 2001; Ruddon and Bedows, 1997). This has been suggested because of the high degree of amino acid sequence conservation amongst GroE homologues and the finding that expression of heterologous GroE proteins can complement *E. coli groE* mutants (Dionisi and Viale, 1998; Schon and Schumann, 1995).

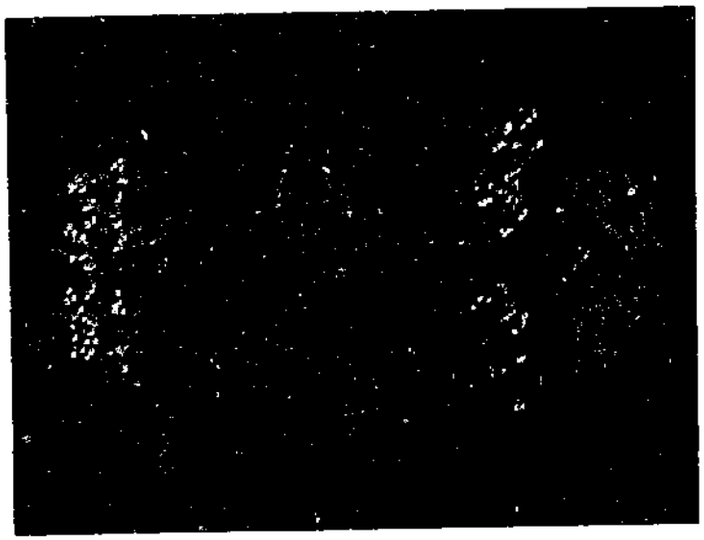
## 1.11 The DnaK chaperone system

### 1.11.1 Introduction

The DnaK chaperone system consists of the DnaK protein and associated co-chaperones DnaJ and GrpE and are encoded by the *dnaK*, *dnaJ* and *grpE* genes, respectively. The proteins were initially identified as host proteins of *E. coli* required for bacteriophage lambda DNA replication (Georgopoulos *et al.*, 1977; Saito and Uchida, 1977, 1978). In *E. coli* DnaK is essential for growth at high temperatures but its absence at

**Figure 1.1 Crystal structure of the GroEL/GroES/ADP complex (Xu *et al.*, 1997).**

The two GroEL heptameric rings are coloured in purple and the GroES heptameric ring is shown in green. The substrate binding sites are shown in space filling representation (yellow and blue). This figure has been adapted from Ranson *et al.* (1998).



physiological temperature results in slowed growth and cellular defects in chromosome segregation, cell division and motility (Bukau and Walker, 1989a, b; Paek and Walker, 1987). This is also true for DnaJ but loss of this protein has a less severe phenotype (Sell *et al.*, 1990) due to a complementing homologue (Ueguchi *et al.*, 1995). Alternatively, GrpE is essential for growth at all temperatures (Ang and Georgopoulos, 1989).

The DnaK chaperone system has been extensively studied at the genetic, biochemical and structural level in *E. coli* and the studies described below refer to this organism unless otherwise stated. As DnaK is the most abundant cytosolic chaperone and the function of the system can not be replaced *in vivo* (Mogk *et al.*, 1999) it qualifies as the central chaperone in the *E. coli* cytosol.

### 1.11.2 Function of the DnaK chaperone system

One of the major functions of the DnaK chaperone system at physiological temperature is to transiently bind nascent polypeptide chains and facilitate their folding at the same time as preventing premature aggregation and misfolding (Bukau and Horwich, 1998). This is done in co-operation with the ribosome-associated molecular chaperone, trigger factor (Deuerling *et al.*, 1999; Teter *et al.*, 1999; Valent *et al.*, 1997). Nascent polypeptides immediately interact with trigger factor following their biogenesis and either fold directly if they are short, or are transferred co- or post-translationally to DnaK and DnaJ if they are larger. It has been estimated that the folding of approximately 9-18% of newly synthesised polypeptides ranging in size from 16-167kDa is conducted via the DnaK chaperone system (Deuerling *et al.*, 1999; Teter *et al.*, 1999). However, the true figure is likely to be much higher as the instability of the DnaK/substrate complex during isolation renders it difficult to accurately measure the extent of these interactions (Teter *et al.*, 1999). DnaK has a preference for binding to longer polypeptides in the 30-75kDa range (Teter *et al.*, 1999).

Upon heat shock the DnaK chaperone system of *E. coli* is the most efficient at preventing aggregation of thermolabile proteins and actively resolubilises small aggregates of denatured proteins in the cell (Diamant *et al.*, 2000; Mogk *et al.*, 1999; Skowyra *et al.*, 1990; Ziemienowicz *et al.*, 1993). In conjunction with the protease ClpB (Woo *et al.*, 1992), the DnaK chaperone system can also solubilise large protein

aggregates and reactivate them (Goloubinoff *et al.*, 1999; Mogk *et al.*, 1999). In this instance ClpB is first thought to recognise and bind heat-induced aggregates and partially unfold them. New hydrophobic patches are transiently exposed on the surface of the aggregates that can subsequently bind DnaK and the chaperone system proceeds to disaggregate them. Progressive refolding of the entangled polypeptides may subsequently occur via the DnaK or GroE chaperone systems or they may be targeted to the protease network for degradation (Goloubinoff *et al.*, 1999; Mogk *et al.*, 1999; Tomoyasu *et al.*, 2001; Zolkiewski, 1999). This bi-chaperone system appears to be conserved as the DnaK and ClpB homologues of *Thermus thermophilus* and *S. cerevisiae* (Hsp70 and Hsp104, respectively) function in a similar manner (Glover and Lindquist, 1998; Motohashi *et al.*, 1999).

Under normal growth conditions the DnaK chaperone system is also involved in the processing of peptide intermediates from several sources including small heat shock proteins (Veinger *et al.*, 1998) and in translocation and secretion of some proteins (Diamant *et al.*, 2000). DnaK is involved in proteolysis by presenting some proteins to proteases for degradation. In addition DnaK has an integral role in regulation of the heat shock response in *E. coli* (Section 1.12.4) and maintains the negative supercoiling of DNA upon thermal stress (Ogata *et al.*, 1996).

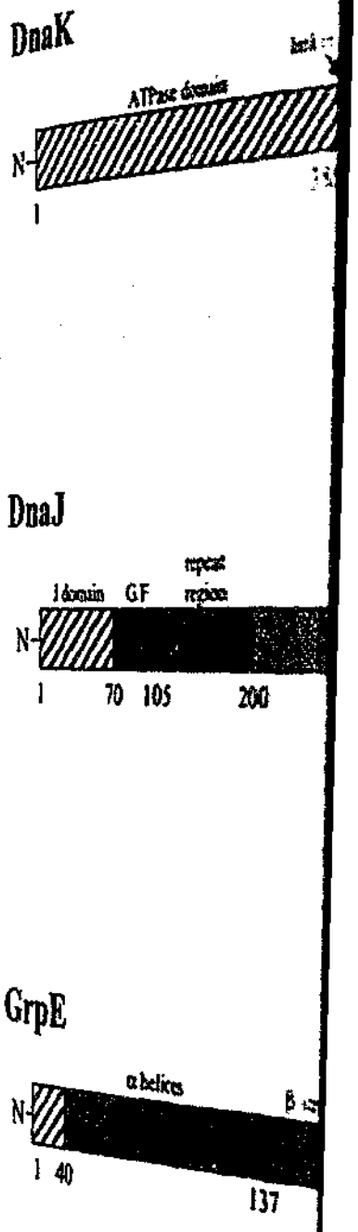
### 1.11.3 Structure and properties of DnaK

DnaK proteins and related Hsp70 homologues are composed of two major functional domains (Figure 1.2). The highly conserved N-terminal domain of 44kDa has ATPase activity and is followed by a more variable 15kDa peptide binding domain (Buchberger *et al.*, 1995). The function of the least conserved C-terminal 10kDa region is unclear but it is thought to contribute to a structural interaction with cofactors (Bertelsen *et al.*, 1999).

The crystal structures for the individual domains of DnaK and Hsp70 homologues have been determined and their architectures are nearly identical (Figure 1.3A). The ATPase domain constitutes two subdomains separated by a deep central cleft (Figure 1.3; Flaherty *et al.*, 1990; Harrison *et al.*, 1997). The subdomains are joined by two crossed alpha ($\alpha$) helices that divide the cleft so that nucleotide is bound to the upper region

**Figure 1.2 Schematic diagram of the general structures of DnaK, DnaJ and GrpE from *E. coli*.**

The black, vertical bars represent the four cysteine rich repeats found in DnaJ. The amino and carboxy termini are represented by N and C, respectively. The diagram is not drawn to scale.



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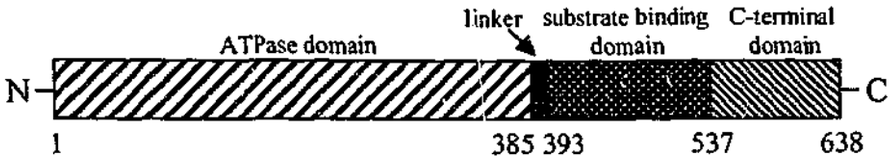
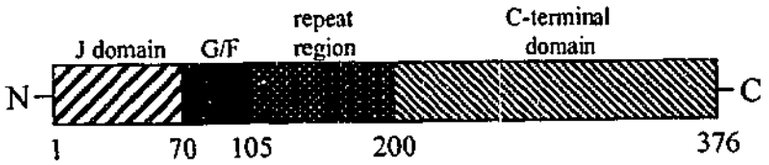
## DnaK




## DnaJ




## GrpE

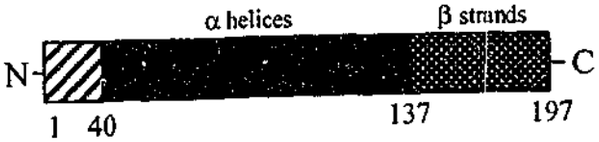


**Figure 1.3 Structures of the ATPase domains of DnaK and Hsc70 (Harrison *et al.*, 1997) and the substrate binding domain of DnaK (Zhu *et al.*, 1996).**

The structure of the ATPase domains of DnaK (red; residues 1-388) and Hsc70 (green), the bovine Hsp70 homologue, are overlapped and the two domains indicated (Panel A). The structure of the substrate binding domain (Panel B; residues 389-607) encompasses a portion of the C-terminal domain. The blue arrows denote β strands and the α helices are shown as blue ribbons. The amino acids that interact with the peptide substrate (green) are shown in space filling representation (grey and red). The hinge that connects the substrate binding domain to the C-terminal domain is indicated as is the helical lid. The amino and carboxy termini are represented by N and C, respectively. These figures have been modified from Harrison *et al.* (1997) and Mayer *et al.* (2000).

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(Flaherty *et al.*, 1994) and DnaJ binds to the lower portion of the cleft (Gassler *et al.*, 1998; Suh *et al.*, 1998). GrpE binds to an exposed, highly conserved loop in the ATPase domain adjacent to the nucleotide binding area and forms a stable interaction (Buchberger *et al.*, 1994).

The substrate binding domain consists of a beta (β) subdomain and a terminal α helical subdomain (Figure 1.3; Zhu *et al.*, 1996). Anti-parallel β strands and four upward-protruding connecting loops form a hydrophobic central pocket which binds substrate and an arch that encloses the substrate backbone. α-helices stacked on the β-sandwich form a helical lid that traps bound substrate.

The ATPase domain and substrate binding domain are linked by a highly conserved region of 13 amino acids which mediates the interdomain communication essential for DnaK function (Han and Christen, 2001).

The beginning of the C-terminal domain forms a helical structure (Figure 1.3) that is stably folded and rigid and is part of the lid that closes over the substrate binding site (Bertelsen *et al.*, 1999). The extreme terminal region is much more flexible in solution and thought to play a role in structural interaction (Bertelsen *et al.*, 1999).

Consistent with the hydrophobic nature of the substrate binding pocket, DnaK recognises and binds to small segments of unfolded or partially folded proteins containing 4-5 consecutive amino acids enriched in hydrophobic and aromatic residues flanked by regions enriched in basic residues (Rudiger *et al.*, 1997a; Rudiger *et al.*, 1997b). These high affinity binding sites occur on average every 36 amino acids but tend to be buried in the mature protein (Rudiger *et al.*, 1997b).

### 1.11.4 Structure and properties of DnaJ

The 44kDa DnaJ protein of *E. coli* (Bardwell *et al.*, 1986) consists of four distinct domains (Figure 1.2). The highly conserved J domain of approximately 70 amino acids located at the amino terminus defines the DnaJ family (Hennessy *et al.*, 2000). This is followed by a stretch of 35 amino acids rich in glycine and phenylalanine residues (G/F region; Cyr *et al.*, 1994) and a subsequent 50 residue region containing four repeats of

the sequence CXXCXGXG (C-cysteine, X- any amino acid, G-glycine; Lu and Cyr, 1998; Szabo *et al.*, 1996). The carboxy-terminus is the least conserved segment.

DnaJ-like proteins can be classified into three groups depending on their similarity to the domains mentioned above (Cheetham and Caplan, 1998). Type I proteins display similarity to DnaJ over all domains including the J domain, G/F region and repeat motif. Type II proteins have the J domain and G/F region in common and Type III proteins only encode the J domain which may be positioned at any site in the polypeptide.

The structure of the J domains from DnaJ and the human homologue, HDJ1, have been determined using nuclear magnetic resonance and are very similar (Pellecchia *et al.*, 1996; Qian *et al.*, 1996). This domain contains four α helices packed together in a compact structure with a highly conserved tripeptide of histidine, proline and aspartic acid residues (HPD) situated in a loop region between helices II and III (Figure 1.4; Cheetham and Caplan, 1998). It is the HPD motif and adjacent helix II region that interact and bind to the lower cleft of the ATPase domain of DnaK and stimulate its ATPase activity (Gassler *et al.*, 1998; Suh *et al.*, 1998; Suh *et al.*, 1999; Tsai and Douglas, 1996). DnaJ has also been shown to bind to the substrate binding pocket of DnaK (Suh *et al.*, 1998).

The G/F region is thought to have accessory functions to those of the J domain and may be involved in the formation of a ternary DnaJ-DnaK-substrate complex (Pellecchia *et al.*, 1996; Wall *et al.*, 1995). The structure for the repeat region has been determined and shown to resemble a zinc finger (Banecki *et al.*, 1996; Szabo *et al.*, 1996). This domain has a V-shaped structure with a zinc binding site in each side formed by the sulfur atoms of the four cysteine residues (Figure 1.4; Martinez-Yamout *et al.*, 2000)). It is responsible for binding to some unfolded protein substrates and the zinc finger structurally stabilises such interactions (Szabo *et al.*, 1996). The carboxy-terminal has been implicated in the binding of some substrates and conferring chaperone activity (Goffin and Georgopoulos, 1998; Lu and Cyr, 1998).

The DnaJ binding motif consists of a hydrophobic stretch of approximately eight consecutive residues enriched in aromatic and aliphatic hydrophobic residues, similar to the binding site for DnaK (Rudiger *et al.*, 2001). This is the reason, in addition to co-

**Figure 1.4 Ribbon structures of the DnaJ domain (Pellecchia *et al.*, 1996) and repeat region (Martinez-Yamout *et al.*, 2000).**

The structure of the DnaJ domain (residues 2-76) comprises four α helices, coloured in red and yellow and numbered, and the highly conserved tripeptide, the HPD motif (Panel A). The structure of the repeat region (residues 131-209) contains blue arrows that represent β strands and red spheres that depict the zinc molecules (Panel B). The yellow sticks denote the sulphur side chains of the cysteine residues within each repeat. The amino and carboxy termini are represented by N and C, respectively.

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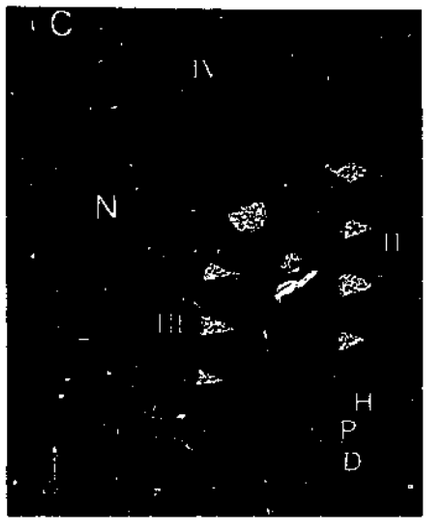


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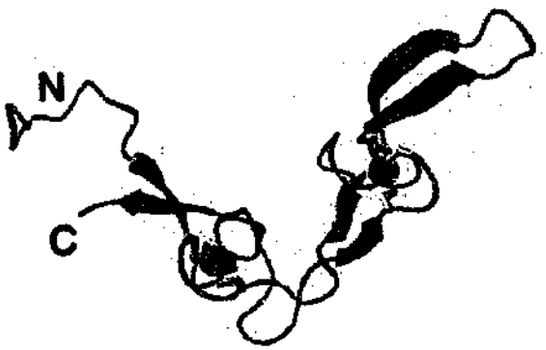

chaperone activity, DnaJ exhibits chaperone activity on its own and can prevent aggregation of misfolded proteins (Schroder *et al.*, 1993; Szabo *et al.*, 1994). However, unlike DnaK, binding of DnaJ to substrates is usually unstable and with low affinity (Rudiger *et al.*, 2001). This process allows DnaJ to target substrate to DnaK in its co-chaperone capacity by binding to DnaK via the J domain. Whether both chaperones bind the same substrate site is still unknown.

### 1.11.5 Structure and properties of GrpE

The 22kDa GrpE protein exists as an asymmetric homodimer (Groemping and Reinstein, 2001) and consists of two distinct domains (Figure 1.2). An N-terminal, long α-helical tail, to which each monomer contributes a helix, is followed by a compact C-terminal domain mainly composed of β sheets and loop structures (Figure 1.5; Harrison *et al.*, 1997). There are five discreet, conserved regions amongst GrpE sequences despite the considerable difference in length and low identity (Harrison *et al.*, 1997; Roberts *et al.*, 1996; Wu *et al.*, 1994). However, these regions contribute to an overall structure that appears to be conserved.

The α-helical domain is required for dimerisation of GrpE and thought to stabilise the structure of the two GrpE molecules (Wu *et al.*, 1996). The C-terminal domain is involved in binding to DnaK and through this action exerts its function as a nucleotide exchange factor (Harrison *et al.*, 1997). This is accomplished by recognising and binding to the ADP-bound state of DnaK and stimulating nucleotide dissociation and therefore peptide release from DnaK (Section 1.11.6; Liberek *et al.*, 1991; McCarty *et al.*, 1995; Szabo *et al.*, 1994). Interestingly, when bound to DnaK, the GrpE dimer is asymmetric in structure. It is curved towards DnaK so that the monomer proximal to DnaK contributes the majority of the residues for interaction (Figure 1.5; Harrison *et al.*, 1997).

**Figure 1.5 Ribbon diagram of the structure of GrpE complexed to the ATPase domain of DnaK (Harrison *et al.*, 1997).**

The GrpE monomers are indicated in orange and green and the DnaK ATPase domain in pink. α helices are depicted as ribbons and β strands as arrows. The first 33 amino acids of the GrpE molecules are not shown. The amino and carboxy termini are represented by N and C, respectively. This figure has been modified from Harrison *et al.* (1997).

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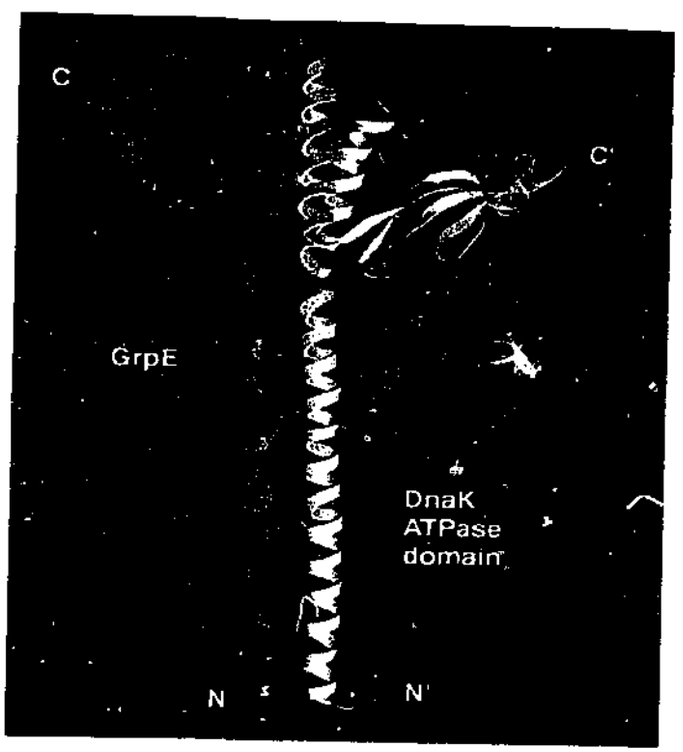


### 1.11.6 Mechanism of action

The reaction cycle of the DnaK chaperone system has been determined, however there is still some speculation over the finer details of the process. There are believed to be two starting points to the cycle. DnaK in its ATP-bound state (DnaK/ATP) may bind directly to exposed hydrophobic patches of unfolded or partially folded polypeptide substrates via its substrate binding cavity. Alternatively, DnaJ can first interact with the substrate and present it to DnaK for binding (Gamer *et al.*, 1996; Szabo *et al.*, 1994). Subsequent interaction of the J domain of DnaJ with the ATPase domain of DnaK stimulates cleavage of gamma (γ) phosphate resulting in a DnaJ/DnaK/ADP/substrate complex (Gassler *et al.*, 1998; Liberek *et al.*, 1991; McCarty *et al.*, 1995; Suh *et al.*, 1998). The ATPase activity of DnaK, which is intrinsically quite low, is increased by up to 15000 fold by DnaJ favouring the DnaK/ADP state (Buchberger *et al.*, 1995; Laufen *et al.*, 1999; McCarty *et al.*, 1995; Russell *et al.*, 1999). This activity causes the cleft of the substrate binding domain to close and results in a stable complex due to the increased affinity of DnaK in this state for peptide substrate (Mayer *et al.*, 1999). This process of ATP hydrolysis and substrate binding requires interdomain communication between the ATPase domain and substrate binding domain of DnaK and is coupled by DnaJ (Buchberger *et al.*, 1995; Mayer *et al.*, 1999). The energy generated by hydrolysis of ATP drives the proper folding of the substrate (Hartl, 1996; Pierpaoli *et al.*, 1997).

GrpE subsequently binds the complex and acts as a nucleotide exchange factor. It accelerates the dissociation of ADP from DnaK, by a factor of 5000, by inducing a conformational change in DnaK that destabilises the binding of purine and ribose rings of ADP (Packschies *et al.*, 1997). The nucleotide free, substrate bound DnaK rapidly binds ATP, now in its low affinity state for substrates, which induces a structural change in the substrate binding domain causing the lid on the cleft of DnaK to open and release the substrate (Buchberger *et al.*, 1995; Liberek *et al.*, 1991; Zhu *et al.*, 1996). The released substrates may either fold spontaneously and rapidly to their native state or require additional folding and re-enter the chaperone cycle (Hartl, 1996; Hendrick and Hartl, 1993). This is especially true for multidomain proteins due to their larger size (Tomoyasu *et al.*, 2001). Alternatively some of these proteins, stabilised by DnaK interaction, may be transferred to the GroE system for additional isomerisation (Fink, 1999; Hartl, 1996; Teter *et al.*, 1999).

GrpE is thought to be the rate-limiting factor of the DnaK chaperone cycle and responsible for its regulation under heat shock conditions (Groemping and Reinstein, 2001). This occurs as GrpE undergoes a thermal transition upon heat shock and the β sheet domain (binding site for DnaK) partially unfolds so that it can no longer function as a nucleotide exchange factor (Groemping and Reinstein, 2001). Thus the DnaK/ATP/substrate complex is favoured and prevents aggregation of substrates by 'holding' them and preventing their interaction with each other at elevated temperatures (Beissinger and Buchner, 1998). The temporary denaturation of GrpE is reversible and upon permissible conditions the protein refolds and catalyses nucleotide exchange and substrate release from DnaK conditions (Groemping and Reinstein, 2001). GrpE *of T. thermophilus* also functions in a similar fashion and undergoes a thermal transition (Groemping and Reinstein, 2001). Therefore it appears the general role of GrpE as a thermosensor is conserved among different species (Groemping and Reinstein, 2001).

## 1.12 The alternative sigma factor, $\sigma^{32}$

### 1.12.1 Introduction

The alternative sigma factor, $\sigma^{32}$, encoded by the *rpoH* gene is only found in Gram negative bacteria and has been identified from at least one member of each subdivision of the proteobacteria, excluding the epsilon (ε) subclass (Ono *et al.*, 2001). It has been identified and characterised from several bacteria of the α subdivision of proteobacteria including *C. crescentus* (Reisenauer *et al.*, 1996; Wu and Newton, 1996), *B. japonicum* (Narberhaus *et al.*, 1996; Narberhaus *et al.*, 1997), *R. capsulatus* (Emetz and Klug, 1998), *Rhodobacter sphaeroides* (Karls *et al.*, 1998), *Sinorhizobium meliloti* (Ono *et al.*, 2001), *Zymomonas mobilis* and *Agrobacterium tumefaciens* (Nakahigashi *et al.*, 1995). *rpoH* has been identified in a number of γ subdivision proteobacteria, the most extensively studied being that from *E. coli* (Nakahigashi *et al.*, 1995) and to a lesser extent from *P. aeruginosa* (Benvenisti *et al.*, 1995; Naczynski *et al.*, 1995; Nakahigashi *et al.*, 1998), *Pseudomonas putida* (Manzanera *et al.*, 2001), *Xanthomonas campestris* (Huang *et al.*, 1998), *Vibrio cholerae* (Sahu *et al.*, 1997), *Buchnera aphidicola* (Sato and Ishikawa, 1997) and several enteric bacteria (Nakahigashi *et al.*, 1995, 1998; Ramirez-Santos and Gomez-Eichelmann, 1998; Ramirez-Santos *et al.*, 2001). *rpoH* has been identified from several members of the β subdivision of proteobacteria but has yet

to be characterised from these organisms (Ono *et al.*, 2001; Yura and Nakahigashi, 1999). A variety of *sig* genes have been isolated from *Myxococcus xanthus*, a member of the delta (δ) subdivision of proteobacteria, that share a significant degree of homology with *rpoH* (Ueki and Inouye, 2001). However, inactivation of these genes does not affect heat shock protein induction but rather has an effect on multicellular differentiation (Ueki and Inouye, 2001).

### 1.12.2 $\sigma^{32}$ structure and promoter recognition

$\sigma^{32}$ promoters of *E. coli* have the consensus sequence, 5' -TNTCNCCCTTGAA (13-14bp) CCCCATTTA- 3', with the conserved motifs (underlined) located at positions -35 and -10 relative to the tsp (Cowing *et al.*, 1985). The $\sigma^{32}$ promoter sequences of α proteobacteria are slightly different to that of *E. coli*, and the consensus $\sigma^{32}$ promoter sequence for this group of bacteria has been modified to 5' -CTTG (17-18bp) C(C/T)TATNTNNG- 3' (Segal and Ron, 1995).

Analysis of the amino acid sequence of $\sigma^{32}$ reveals that it contains the four typical functional regions of sigma factors when compared to the sequence of those belonging to the $\sigma^{70}$ class (Nakahigashi *et al.*, 1995). $\sigma^{32}$ also contains a highly conserved segment of nine amino acids unique to this family of sigma factors, termed the RpoH box, with the sequence Q(R/K)(K/R)LFFNLR (Nakahigashi *et al.*, 1995). The RpoH box overlaps another conserved region, Region C, involved in high efficiency binding to RNA polymerase (Arsene *et al.*, 2000). This binding both stabilises $\sigma^{32}$ and provides a competitive advantage over other sigma factors during the heat shock response (Yura and Nakahigashi, 1999).

### 1.12.3 Regulation of $\sigma^{32}$ expression

The regulatory mechanisms controlling $\sigma^{32}$ levels vary considerably but can generally be characterised into two broad groups. One group includes the mechanisms operative in γ proteobacteria and the other includes those utilised by the α proteobacteria.

### 1.12.4 Post-transcriptional regulation of $\sigma^{32}$ synthesis in *E. coli*

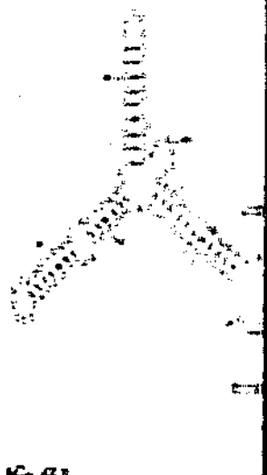
The heat shock response is elicited by a transient increase in the synthesis rate and subsequent cellular level of $\sigma^{32}$ in most of the proteobacteria examined (Huang *et al.*, 1998; Nakahigashi *et al.*, 1998; Ramirez-Santos and Gomez-Eichelmann, 1998). Further extensive analysis concerning the regulation of $\sigma^{32}$ levels from *E. coli* has been performed and is thus used as the paradigm for related bacteria.

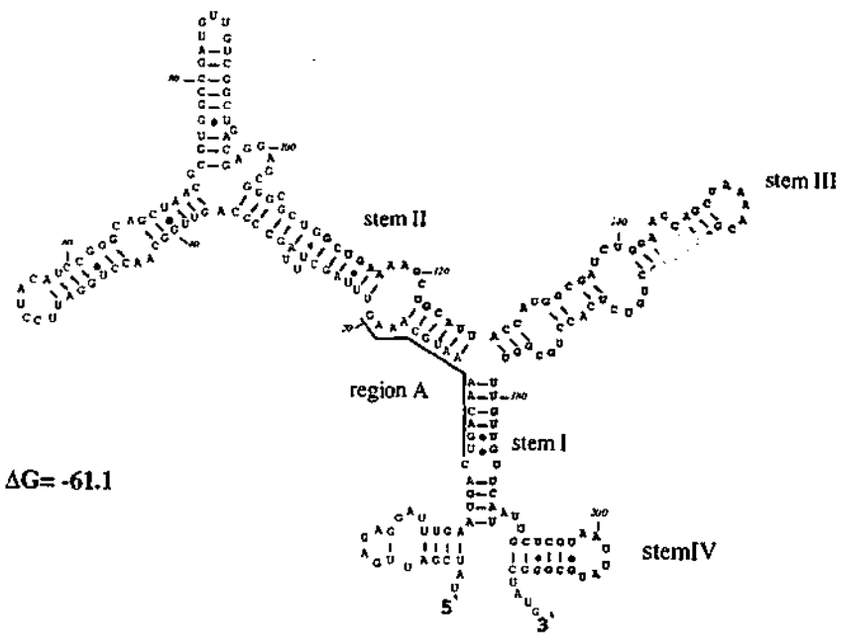
Upon a temperature upshift from 30°C to 42°C the cellular level of $\sigma^{32}$ increases dramatically within the first few minutes (induction phase) and declines at around 10 minutes post heat shock to a new steady-state level (adaptation phase; Straus *et al.*, 1987). The kinetics of this response is analogous to that of the induction of heat shock gene expression (Section 1.8). The observed increase in $\sigma^{32}$ is obtained predominantly by enhanced synthesis, stability and activity (Yura *et al.*, 1993). The increase in synthesis occurs at the translational level and is mediated via *cis*-acting elements located within the *rpoH* coding sequence (Nagai *et al.*, 1991a, b). Extensive deletion and mutation analyses of an *rpoH-lacZ* gene fusion identified positive (Region A) and negative (Region B) elements essential for induction (Figure 1.6; Morita *et al.*, 1999a; Morita *et al.*, 1999b; Nagai *et al.*, 1991a, b; Yuzawa *et al.*, 1993). Region A, represented by nucleotides 6-20 relative to the translation initiation codon, represents a 'downstream box' that is complementary to the 3' region of 16S rRNA and acts as a translational enhancer. Region B is represented by nucleotides 110-247 relative to the initiation codon. The secondary structure formed by base pairing between Region A and Region B at physiological temperature obstructs the translation initiation region, including the ribosome binding site (RBS), and therefore represses translation (Morita *et al.*, 1999a; Nagai *et al.*, 1991b; Nakahigashi *et al.*, 1995; Yuzawa *et al.*, 1993). An increase in temperature partially melts the structure which enhances ribosome entry and subsequent translation (Morita *et al.*, 1999a). It appears that the thermostability of the *rpoH* mRNA secondary structure at the translation initiation region is important for the induction of $\sigma^{32}$ synthesis at elevated temperatures and the transcript has intrinsic thermosensing capacity (Morita *et al.*, 1999a; Morita *et al.*, 1999b).

The enhanced stability and activity of $\sigma^{32}$ following heat shock contribute significantly to the enhanced levels observed and is achieved by the DnaK chaperone system (Section

**Figure 1.6 Predicted secondary structure of the 5' region of *rpoH* mRNA (nucleotides -20 to 210 relative to the initiation codon) from *E. coli* (Morita *et al.*, 1999a; Nagai *et al.*, 1991a).**

The structure was created using the MULFOLD program (Jaeger *et al.*, 1990). The calculated minimum free energy (ΔG, kcal/mol) is indicated and the ribosome binding site (RBS) and start codon are represented by blue and red text, respectively. The downstream box (Region A) is shown and text in bold type represents Region B that forms critical base-pairings with Region A (Morita *et al.*, 1999a). The segments of mRNA that correspond to each of the stem structures (I-IV) are shown (Morita *et al.*, 1999a). •, G-U pairs.






ΔG= -61.1

1.11). This system is involved in the negative feedback regulation of the heat shock regulon by direct interaction with $\sigma^{32}$ (Bukau, 1993; Gamer *et al.*, 1992; Liberek *et al.*, 1992; Tilly *et al.*, 1989; Yura *et al.*, 1993). During steady-state growth $\sigma^{32}$ is unstable and has a short half-life of approximately 1 minute (Straus *et al.*, 1987). At this time a modest 10-30 molecules of $\sigma^{32}$ are present per cell (Craig and Gross, 1991). The extreme instability of $\sigma^{32}$ *in vivo* results from degradation by the ATP-dependent metalloprotease FtsH (Herman *et al.*, 1995; Tatsuta *et al.*, 1998; Tomoyasu *et al.*, 1995). FtsH belongs to the conserved family of AAA proteins (ATPases associated with a variety of cellular activities) whose members are widely distributed among eukaryotic and prokaryotic organisms (Tomoyasu *et al.*, 1993b). It is a cytoplasmic membrane protein with its active domain located in the cytoplasm (Tomoyasu *et al.*, 1993a) and degrades a subset of cytoplasmic regulatory proteins and unassembled membrane proteins (Herman *et al.*, 1995; Ogura *et al.*, 1999; Tomoyasu *et al.*, 1995). In addition to FtsH several other ATP-dependent proteases including ClpAP, Lon and HslVU are involved in the degradation of $\sigma^{32}$ *in vivo* (Kanemori *et al.*, 1997; Kanemori *et al.*, 1999). Upon heat shock an 8-fold increase in $\sigma^{32}$ stability occurs for the first few minutes (Straus *et al.*, 1990; Straus *et al.*, 1987; Tilly *et al.*, 1989). This is followed by rapid destabilisation of $\sigma^{32}$ (half-life of 15-20 seconds) and a final return to steady-state stability levels at approximately 10 minutes post heat shock (Kanemori *et al.*, 1999; Morita *et al.*, 2000).

This transient stabilisation of $\sigma^{32}$ was thought to occur by a model where the misfolded proteins that accumulate in stressed cells titrate the DnaK chaperone system so that it no longer preferentially binds to $\sigma^{32}$ and target it for degradation by cellular proteases. $\sigma^{32}$ is then free to bind RNA polymerase, a complex which is resistant to degradation by FtsH, and initiate transcription of heat shock genes (Blaszczak *et al.*, 1999; Bukau, 1993; Craig and Gross, 1991; Tomoyasu *et al.*, 1998). During the adaptation phase the DnaK chaperone system would once again be free to bind $\sigma^{32}$, inactivating it and resuming its role in the rapid turnover of the sigma factor. Thus the role of the DnaK chaperone system would be to compete with RNA polymerase for binding to $\sigma^{32}$ and keeping it in a state sensitive to proteolysis (Blaszczak *et al.*, 1999). However Tatsuta *et al.* (2000) recently demonstrated, with *rpoH* mutants deficient in RNA polymerase binding, that the role of the chaperones was not simply to sequester $\sigma^{32}$ from RNA polymerase binding but to somehow actively modulate $\sigma^{32}$ stability. The precise

mechanism by which this occurs has yet to be determined but is thought to occur by maintaining $\sigma^{32}$ in an accessible and competent state for degradation by either keeping it soluble or in a particular structural form (Blaszczak *et al.*, 1999; Tatsuta *et al.*, 2000). The DnaK chaperone system constitutes a highly sensitive stress-sensing and transducing system of the *E. coli* heat shock response.

Therefore control of $\sigma^{32}$ during heat shock relies on a delicate balance between the level of *rpoH* mRNA translation by direct temperature sensing and the rate of $\sigma^{32}$ turnover determined by the DnaK chaperone system/protease mediated negative control pathway.

### 1.12.5 Transcriptional regulation of *rpoH* in *E. coli*

Upon heat shock, *rpoH* transcription is induced only moderately (2-fold) and does not contribute significantly to the overall increase in $\sigma^{32}$ levels during the induction phase or the decline during the adaptation phase (Erickson *et al.*, 1987; Fujita and Ishihama, 1987; Straus *et al.*, 1987). However transcription of *rpoH* is complex and appears to be responsible for maintaining appropriate levels of $\sigma^{32}$ during various metabolic processes (Bukau, 1993).

*rpoH* has four promoters, P1, P3, P4 and P5 (Figure 1.7; Erickson *et al.*, 1987; Nagai *et al.*, 1990). Promoters P1, P4 and P5 are $\sigma^{70}$ dependent and P3 is $\sigma^{24}$ dependent. $\sigma^{24}$ is a member of the extracytoplasmic function (ECF) subfamily of sigma factors that responds to extracytoplasmic stimuli and is especially active at elevated temperatures (Missiakas and Raina, 1998). P1 and P4 are the major promoters responsible for *rpoH* transcription under most growth conditions but at extreme heat shock of 50°C, P3 is the only active promoter (Erickson and Gross, 1989; Wang and Kaguni, 1989a; Yura *et al.*, 1993; Yura, 1996).

Binding sites for the regulatory proteins cyclic adenosine monophosphate (cAMP) receptor protein (CRP), CytR and DnaA are located in the region upstream of *rpoH* (Figure 1.7). These proteins modulate *rpoH* transcription linking its expression to global regulatory networks. Binding of DnaA, required for the initiation of DNA replication (Bramhill and Kornberg, 1988; Zyskind *et al.*, 1977), represses transcription from P3 and P4 *in vitro* and *in vivo* (Wang and Kaguni, 1989b). Two binding sites for CRP exist

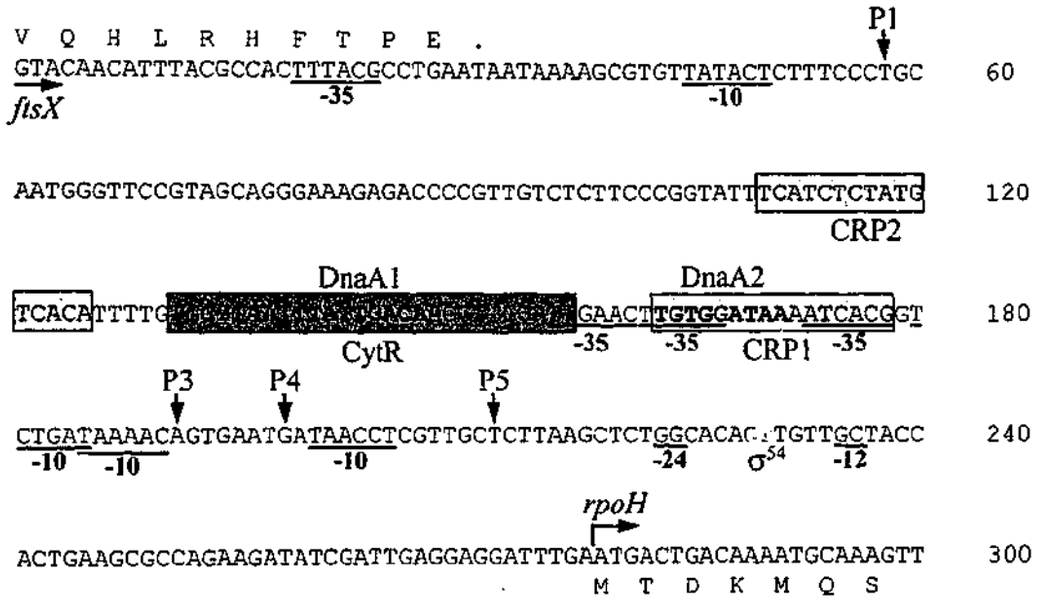
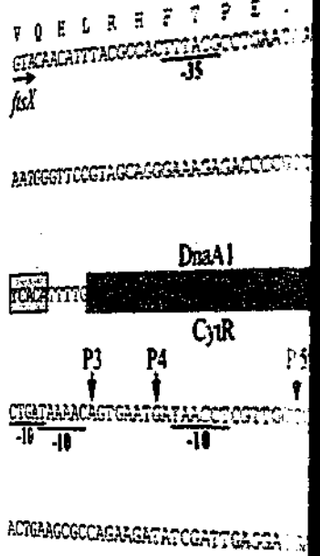
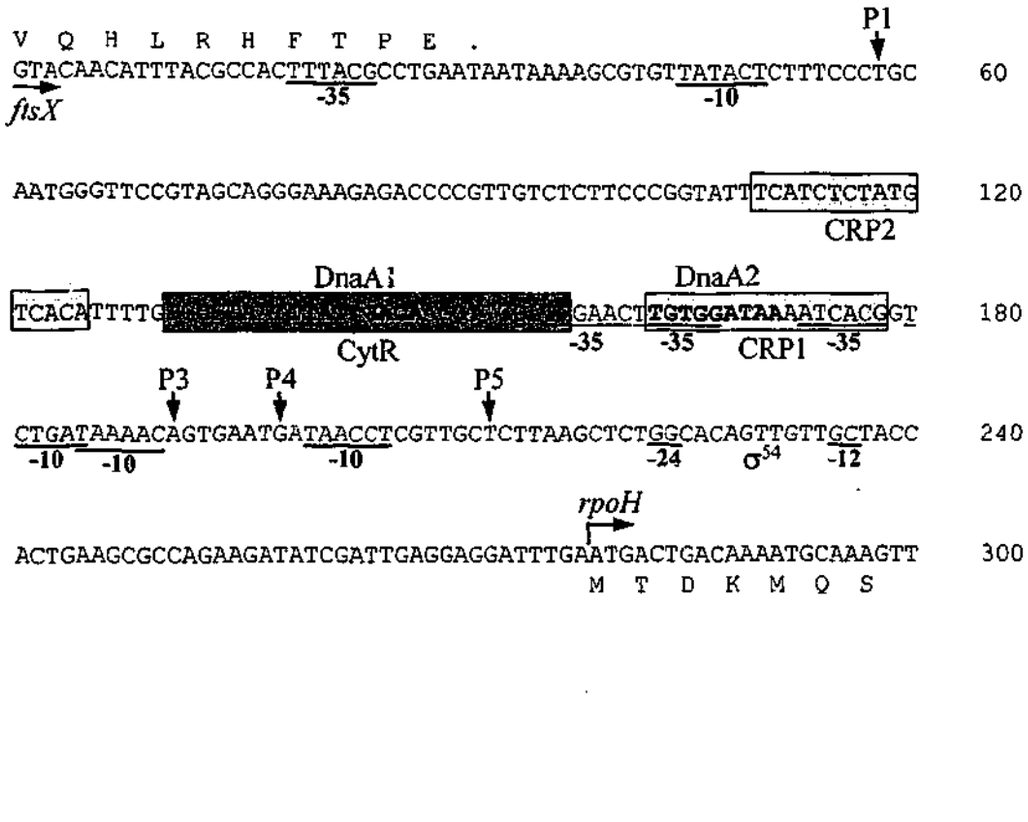

V Q H L R H F T P E .
P1
GTACAACATTTACGCCACTTTACGCCTGAATAATAAAAGCGTGTTATACTCTTTCCCTGC 60
fisX
-35
-10
AATGGGTTCCGTAGCAGGGAAAGAGACCCCGTTGTCTCTTCCCGGTATTTCATCTCTATG 120
CRP2
DnaA1
DnaA2
TCACATTTTG GAACTTGTGGATAAAATCACGGT 180
CytR
-35
-35
CRP1
-35
P3
P4
P5
CTGATAAAACAGTGAATGATAACCTCGTTGCTCTTAAGCTCTGGCACAC TGTTGCTACC 240
-10
-10
-10
-24
σ54
-12
rpoH
ACTGAAGCGCCAGAAGATATCGATTGAGGAGGATTTGAATGACTGACAAAATGCAAAGTT 300
M T D K M Q S

**Figure 1.7 Nucleotide sequence of the region upstream of the *rpoH* gene from *E. coli* (Erickson *et al.*, 1987).**

The amino acid sequences of *ftsX* and *rpoH* are shown above and below the corresponding nucleotide sequences, respectively (Erickson *et al.*, 1987). The *ftsX* stop codon is depicted by a dot (.). The $\sigma^{70}$ promoters, P1, P4 and P5 are shown (Erickson *et al.*, 1987; Kallipolitis and Valentin-Hansen, 1998; Nagai *et al.*, 1990; Ramirez-Santos *et al.*, 2001) as is the $\sigma^{24}$ promoter, P3 (Wang and Kaguni, 1989a) and the corresponding tsp of each promoter is denoted by a labelled arrowhead. The CRP binding sites are boxed in pink and the boxed region in green represents the CytR binding site (Kallipolitis and Valentin-Hansen, 1998; Nagai *et al.*, 1990). The nucleotides in bold indicate the two DnaA binding sites (Wang and Kaguni, 1989b). Nucleotides are numbered at the right of the figure.




V Q H L R H F T P E .
P1
GTACAACATTTACGCCACTTTACGCCTGAATAATAAAAGCGTGTTATACTCTTTCCCTGC 60
ftsX
-35
-10
AATGGGTTCCGTAGCAGGGAAAGAGACCCCGTTGTCTCTTCCCGGTATTTCATCTCTATG 120
CRP2
DnaA1
DnaA2
TCACATTTTG GAACTTGTGGATAAAATCACGGT 180
CytR
-35
-35
CRP1
-35
P3
P4
P5
CTGATAAAACAGTGAATGATAACCTCGTTGCTCTTAAGCTCTGGCACAGTTGTTGCTACC 240
-10
-10
-10
-24
σ54
-12
rpoH
ACTGAAGCGCCAGAAGATATCGATTGAGGAGGATTTGAATGACTGACAAAATGCAAAGTT 300
M T D K M Q S

and binding of the protein to the distal site (CRP1) increases transcription from P5 and decreases it from P4 (Nagai *et al.*, 1990). P5 is a weak catabolite-sensitive promoter requiring cAMP and CRP for its function (Nagai *et al.*, 1990). CRP can also function co-operatively with CytR and the complex formed, when bound to the appropriate binding regions in a protein stoichiometry of 1:2, respectively, can repress transcription from P3, P4 and P5 (Kallipolitis and Valentin-Hansen, 1998). This complex is proposed to respond to nucleoside catabolism (Valentin-Hansen *et al.*, 1996).

The physiological significance of coupling between *rpoH* regulation and the various cellular processes described remains unclear. The regulation of *rpoH* transcription is further complicated by the recent identification of a putative $\sigma^{54}$ dependent promoter upstream of the *rpoH* gene (Figure 1.7; Pallen, 1999). However its role, if any, has yet to be determined. Interestingly induction of $\sigma^{32}$ synthesis by the addition of ethanol or DNA gyrase inhibitors occurs primarily at the transcriptional level (Lopez-Sanchez *et al.*, 1997; Morita *et al.*, 1999b) in contrast to the translational induction witnessed with heat shock and hyperosmotic shock (Bianchi and Baneyx, 1999).

### 1.12.6 Regulation of $\sigma^{32}$ expression in other γ proteobacteria

The $\sigma^{32}$ proteins of *Serratia marcescens* and *P. aeruginosa* are unstable during normal conditions and show a marked stabilisation and transient induction upon heat shock, as in *E. coli* (Nakahigashi *et al.*, 1998). This increase in synthesis takes place at the translational level. The 5' *rpoH* mRNA sequences again have the potential to form the appropriate secondary structures for translational repression and also contain downstream boxes (Nakahigashi *et al.*, 1995). The *rpoH* mRNA of *Proteus mirabilis* also displays these features with the exception of the protein being very stable under steady-state conditions and the level increasing only modestly upon a thermal upshift (Nakahigashi *et al.*, 1998). In contrast, the $\sigma^{32}$ level of *Klebsiella pneumoniae* does not increase at all under heat shock (Ramirez-Santos and Gomez-Eichelmann, 1998). In addition to the above bacteria, *Citrobacter freundii*, *Enterobacter cloacae* and *P. aeruginosa* have the potential to form an *rpoH* mRNA secondary structure similar to that of *E. coli* (Nakahigashi *et al.*, 1995). The proposed *rpoH* mRNA secondary structures of *V. cholerae* and *P. putida* are different to that of *E. coli*, but as they contain a downstream box can still fit the model proposed for translational control (Manzanera

*et al.*, 2001; Sahu *et al.*, 1997). It appears that *rpoH* from *B. aphidicola* and *H. influenzae* lacks the characteristic mRNA secondary structure and therefore the potential for translational regulation (Fleischmann *et al.*, 1995; Nakahigashi *et al.*, 1998; Sato and Ishikawa, 1997).

Most of the bacteria mentioned, including the enteric bacteria *Salmonella enterica*, *Shigella flexneri*, *K. pneumoniae*, *Yersinia frederiksenii* and *Erwinia amylovora* have a consensus $\sigma^{24}$ dependent promoter and at least one consensus $\sigma^{70}$ dependent promoter in the region upstream of *rpoH* (Huang *et al.*, 1998; Manzanera *et al.*, 2001; Nakahigashi *et al.*, 1995; Ramirez-Santos *et al.*, 2001; Sahu *et al.*, 1997). However, regulation of *rpoH* transcription by additional cellular regulatory proteins such as DnaA, CytR and CRP seems to be confined to enteric bacteria (Ramirez-Santos *et al.*, 2001).

In summary it appears that the *rpoH* mRNA secondary structure involved in translational control and the $\sigma^{24}$ dependent promoter are found in most of the γ proteobacteria studied. For those that do not contain these regulatory elements it may be a reflection of the different ecological niches and hence the need to modulate $\sigma^{32}$ levels differently.

### 1.12.7 Regulation of $\sigma^{32}$ expression in α proteobacteria

In contrast to the translational regulation of $\sigma^{32}$ predominant in γ proteobacteria, $\sigma^{32}$ expression from the α proteobacteria seems to be regulated at the transcriptional and post-translational levels.

*B. japonicum* has three *rpoH* genes that differ in their primary sequence, their significance to survival and regulation (Narberhaus *et al.*, 1997). $rpoH_1$ is arranged in an operon with the heat shock gene, *hspA*, and transcription is induced upon heat shock from a $\sigma^{70}$ promoter located upstream of *hspA* (Narberhaus *et al.*, 1996). During normal growth conditions transcription is repressed by binding of a repressor to a negative regulatory element situated in the upstream region (Narberhaus *et al.*, 1998a). $rpoH_2$ is transcribed constitutively from a $\sigma^{70}$ promoter at physiological conditions and, as in *E. coli*, from a $\sigma^{24}$ promoter upon heat shock (Narberhaus *et al.*, 1997). $rpoH_3$ is arranged in an operon with the *ragA* and *ragB* genes and transcription is induced moderately from

a $\sigma^{32}$ promoter located upstream of the operon (Narberhaus *et al.*, 1997). Narberhaus *et al.* (1998b) showed that the $\sigma^{32}$ proteins have distinct promoter specificities *in vivo* and *in vitro* and that during normal growth conditions $RpoH_2$ is responsible for transcription of the heat shock genes, and that this function is assumed by $RpoH_1$ upon heat shock. The precise function of $RpoH_3$ remains unclear.

*S. meliloti* has two *rpoH* genes that differ in their primary sequence and function (Oke *et al.*, 2001; Ono *et al.*, 2001). $RpoH_1$ is required for growth at high temperatures and successful symbiosis whereas $RpoH_2$ appears to be redundant. The significance of this multigene family in these soil bacteria is unclear but highlights the complex strategies used to cope with various environmental conditions.

The *rpoH* genes of *C. crescentus*, *R. capsulatus* and *A. tumefaciens* are positively auto-regulated from a $\sigma^{32}$ promoter upon heat shock and, for the former two bacteria, from a $\sigma^{70}$ promoter under steady-state conditions and during various stages of the cell cycle (Emetz and Klug, 1998; Nakahigashi *et al.*, 1999; Nakahigashi *et al.*, 2001; Reisenauer *et al.*, 1996; Wu and Newton, 1996, 1997; Yura and Nakahigashi, 1999). The immediate increase in $\sigma^{32}$ levels following heat shock in these bacteria suggests that post-translational mechanisms of regulation are involved (Emetz and Klug, 1998; Nakahigashi *et al.*, 1999; Wu and Newton, 1997). The first example of $\sigma^{32}$ regulation at the level of activity, besides that described for *E. coli*, was recently demonstrated by Nakahigashi *et al.* (2001) for *A. tumefaciens*. $\sigma^{32}$ activity is negatively modulated by the free DnaK chaperone machinery such that upon stress it is activated and responsible for the induction phase of the heat shock response. However the precise mechanism of DnaK modulation has yet to be elucidated and may occur at any stage of $\sigma^{32}$ function. The transient increase in $\sigma^{32}$ upon heat shock was due to an increase in transcription followed by destabilisation of the usually stable $\sigma^{32}$ protein during the adaptation phase.

### 1.13 Negative regulation of heat shock genes in bacteria

In contrast to the positive regulation of the heat shock response exhibited in some Gram negative bacteria, the response in the majority of bacteria is controlled by negative regulation at the transcriptional level.

The most wide-spread, well characterised negative cis-acting element is CIRCE, a conserved 9bp inverted repeat separated by a 9bp spacer (Zuber and Schumann, 1994). It has the consensus sequence, TTAGCACTC - N9- GAGTGCTAA (Narberhaus, 1999). This element is found upstream of the *dnaK* and/or *groE* operons of a number of phylogenetically distant bacteria including Gram positive bacteria, cyanobacteria and proteobacteria (Segal and Ron, 1993; Segal and Ron, 1996b; Wetzstein *et al.*, 1992). It acts as a binding site for the repressor protein, HrcA, under normal growth conditions and repression is relieved upon stress (Roberts *et al.*, 1996; Yuan and Wong, 1995).

In *B. subtilis* the GroE chaperones modulate the heat shock response by binding to HrcA, preventing its aggregation and facilitating its proper folding (Mogk *et al.*, 1997). Titration of GroE by misfolded proteins upon stress produces an inactive HrcA protein and induces expression of the *dnaK* and *groE* operons. This is in contrast to the DnaK mediated modulation of the heat shock response in *E. coli* and *A. tumefaciens* (Sections 1.12.4 and 1.12.7).

Recently an additional function of CIRCE from *B. subtilis* was discovered. It seems that CIRCE can act as an mRNA destabiliser to modulate expression of individual genes of the *dnaK* operon at a post-transcriptional level (Homuth *et al.*, 1999; Yuan and Wong, 1995).

Interestingly, several different regulatory strategies can be used simultaneously to control particular sets of heat shock genes in some bacteria. In addition to CIRCE, *B. subtilis* employs three strategies to regulate different classes of heat-inducible genes (Hecker *et al.*, 1996; Hecker and Volker, 2001). This is also the case for *Streptomyces* species and *B. japonicum* which, including CIRCE, utilise three distinct mechanisms of negative regulation to modulate the stress response (Minder *et al.*, 2000; Servant and Mazodier, 2001). In the latter organism, which has multiple *groE* operons, one is regulated by $\sigma^{32}$ and two are negatively controlled by CIRCE/HrcA at normal temperature (Babst *et al.*, 1996; Minder *et al.*, 2000). The *groE* operons of *C. crescentus* and *A. tumefaciens* are under the dual control of $\sigma^{32}$ and CIRCE/HrcA. Upon heat shock, $\sigma^{32}$ increases transcription of *groE* while the repressor functions at physiological conditions (Baldini *et al.*, 1998; Nakahigashi *et al.*, 1999). This coordinate control presumably enables bacteria to cope with expression of different sets of heat shock

genes in response to specific environmental conditions and their requirement for differing induction kinetics (Narberhaus, 1999).

## 1.14 The gonococcal heat shock response

The gonococcus is subject to various environmental conditions throughout the infection cycle. In order to survive this process the organism must easily adapt to the sometimes hostile conditions it is exposed to. Such stresses include nutrient starvation, anaerobiosis, acidic pH and toxic oxygen species.

Preliminary characterisation of the gonococcal heat shock response was performed by Woods *et al.* (1990) and Klimpel and Clark (1989). They showed that synthesis of at least 12, and up to 37, proteins is increased upon a thermal upshift depending on the strain. This increase occurs as early as 5 minutes following a temperature upshift and remains stable for up to 60 minutes. Two of these proteins were identified as GroEL and DnaK by immunological analysis and putative GroES and GrpE proteins were identified based only on their apparent size with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Several of these proteins, including GroEL, are induced irrespective of the nature of the stress indicating that a general stress response is functional in *N. gonorrhoeae* (Chomvarin, 1993; Pannekoek *et al.*, 1992b). A subset of additional stress-specific proteins are induced to enhance survival and possibly virulence when subjected to iron limitation, anaerobiosis, acidic pH and glucose depletion (Clark *et al.*, 1987; Mietzner *et al.*, 1984; Pannekoek *et al.*, 1992a; Pettit *et al.*, 1996; West and Sparling, 1985; Woods *et al.*, 1990). Interestingly, growth in iron limited conditions and heat shock treatment of 44°C did not affect the level of GroEL in *N. meningitidis* suggesting differences in regulation and perhaps function when compared to *N. gonorrhoeae* (Arakere *et al.*, 1993).

Interestingly, recent work on the heat shock response of *N. meningitidis* using DNA microarray technology demonstrated that only two ORFs were deregulated significantly upon heat shock at 42°C and 43.5°C (Guckenberger *et al.*, 2002). The vast array of the ORFs, 55 in total, are deregulated when cultures are exposed to the higher temperature of 45°C (Guckenberger *et al.*, 2002). This is in contrast to the results obtained with *N. gonorrhoeae*, where induction of the heat shock genes is apparent at 42°C and 43°C

(Klimpel and Clark, 1989; Tauschek *et al.*, 1997; Woods *et al.*, 1990). Most of the meningococcal genes whose transcription was significantly upregulated represent typical heat shock genes such as *groES*, *groEL*, *dnaK*, *dnaJ* and *grpE* (Guckenberger *et al.*, 2002).

Several stress-specific proteins elicit an antibody response during natural infection (Clark *et al.*, 1988; Mietzner *et al.*, 1984). GroEL is a major target of the immune response for both *N. gonorrhoeae* and *N. meningitidis* (Pannekoek *et al.*, 1993; Pannekoek *et al.*, 1995). GroEL proteins have been implicated in the development of auto-immune responses resulting in some forms of trachoma, arthritis, diabetes and salpingitis (Elias *et al.*, 1990; Kiessling *et al.*, 1991; Morrison *et al.*, 1989; Taylor *et al.*, 1990). As some of these diseases can arise from gonococcal infection, it is tempting to speculate that GroEL, in addition to its integral function as a chaperone, is an important pathogenicity factor of this organism.

The DnaK chaperone system has not been characterised in *N. gonorrhoeae* but the *groE* operon was recently identified and characterised from strain MS11-A (Hamilton, 1999; Tauschek *et al.*, 1997). Transcriptional analyses performed allowed preliminary insight into regulation of the heat shock response of *N. gonorrhoeae*. The *groES* and *groEL* genes are organised in an operon and analysis of sequence upstream of the operon revealed overlapping consensus sequences for $\sigma^{70}$ and $\sigma^{32}$ dependent promoters. Upon heat shock, transcription emanating from either or both of these promoters increased significantly. The mechanism controlling regulation appears to be reminiscent of that in *E. coli* where transcription from the $\sigma^{70}$ promoter provides the organism with a basal level of GroES and GroEL under physiological conditions and the elevated levels required upon stress (Zhou *et al.*, 1988). However this has yet to be confirmed in *N. gonorrhoeae*.

In addition to a putative $\sigma^{32}$ promoter located upstream of *groE*, further evidence for a $\sigma^{32}$-like sigma factor from *N. gonorrhoeae* comes from the identification of a 40kDa protein which co-purified with RNA polymerase and reacted with polyclonal antisera to $\sigma^{32}$ of *E. coli* (Klimpel *et al.*, 1989). This suggests that the heat shock regulon in this organism is controlled by $\sigma^{32}$.

## 1.15 Aims and scope of this investigation

Type 4 pili are a major virulence factor of *N. gonorrhoeae* (Swanson, 1973). The existence of a multiple promoter system and several regulatory elements upstream of *pilE* (Carrick *et al.*, 1997; Fyfe *et al.*, 1993; Fyfe *et al.*, 1995; Fyfe and Davies, 1998) suggest that this gene is under complex regulatory control. Given the fact that *N. gonorrhoeae* possesses a $\sigma^{54}$ promoter and UAS, it seemed highly likely that a gonococcal $\sigma^{54}$ homologue existed. This study succeeded in identifying an *rpoN*-like sequence which, due to an internal deletion resulting in a frameshift mutation, was not capable of encoding a functional $\sigma^{54}$ protein. This redundant sequence was also apparent in several other *Neisseria* spp. examined, with the exception of *Neisseria lactamica*. This organism had an intact copy of *rpoN* that under the conditions tested did not appear to be expressed.

Molecular chaperones such as the GroE and DnaK chaperone systems are essential for the quality control of cellular proteins and can also play an important role in the immunogenicity and pathogenesis of bacterial disease (Hartl, 1996; Kaufmann, 1990). This study concentrated on the identification and characterisation of the genes encoding members of the gonococcal DnaK chaperone system, *dnaK*, *dnaJ* and *grpE*. Analysis of the deduced amino acid sequences of these genes revealed that they contained all the relevant features and characteristics required for proper function. Transcriptional analysis revealed that they were all induced upon stress and regulated by a $\sigma^{32}$ dependent promoter.

Identification of a $\sigma^{32}$ promoter upstream of the gonococcal *dnaK*, *dnaJ* and *grpE* genes and the *groE* operon (Tauschek *et al.*, 1997) indicated that these chaperones were positively regulated by the $\sigma$ factor $\sigma^{32}$. Identification of the *rpoH* gene from *N. gonorrhoeae* in this study and subsequent regulatory analysis revealed that it is predominantly regulated at the level of its activity, which provides the organism with a rapid response mechanism to stress.

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2, respectively.

## 2.2 Chemicals and reagents

The chemicals and reagents utilised in this study were of analytical grade and acquired from either Amersham Pharmacia Biotech, Ajax Chemicals, BDH Chemicals, Bio-Rad Laboratories, Difco, Life Technologies, Mallinckrodt, New England Biolabs, Oxoid, Promega, Sigma Chemical Company or Roche.

## 2.3 Media and culture conditions

*E. coli* strains were cultured in Luria-Bertani (LB) broth or on LB agar (Sambrook *et al.*, 1989). Plasmids were maintained by the addition of ampicillin (Ap; 50μg/ml), chloramphenicol (Cm; 30μg/ml) or erythromycin (Erm; 150μg/ml) as required.

*P. aeruginosa* strains were cultured in Nutrient Yeast (NY) broth or on NY agar (Appendix). Plasmids were maintained using carbenicillin (250μg/ml).
Broth cultures of *E. coli* and *P. aeruginosa* were incubated aerobically at 37°C overnight on a rotary shaker. Inoculated plates were incubated aerobically at 37°C overnight.

*Neisseria* strains were cultured on GC agar base (Oxoid) or in GC broth (Appendix) supplemented with 1% (w/v) Deakin Modified Iso-Vitalex (DMIV) (Appendix; Sarandopoulos and Davies, 1993). GC agar plates were supplemented with Erm (7μg/ml), Cm (8μg/ml), tetracycline (Tc; 12.5μg/ml), nalidixic acid (Nal; 10μg/ml) or rifampicin (Rif; 10μg/ml) as required. GC agar plates were incubated at 37°C overnight

## Table 2.1 Bacterial strains used in this study.

| Strain | Relevant Genotype or Phenotype | Reference or Source |
|---|---|---|
| ***Escherichia coli*** | | |
| DH5α | F′*supE*44 Δ*lacU*169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 | Bethesda Research Laboratories |
| DH12S | ϕ80d*lacZ*ΔM15 *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *araD*139 Δ(*ara,leu*)7697 Δ*lacX*74 *galU galK rpsL deoR nupG recA*1/F′*proAB*$^+$ *lacI*$^q$ZΔM15 | Gibco, BRL |
| JKD1595 | DH5α(pJKD1595) | Black *et al.* (1995) |
| JKD2282 | DH5α(pJKD2282) | This study |
| JKD2283 | DH5α(pJKD2283) | This study |
| JKD2284 | DH5α(pJKD2284) | This study |
| JKD2325 | DH5α(pJKD2325) | This study |
| JKD2326 | DH5α(pJKD2326) | This study |
| KY1608 | MC4100 Δ*rpoH30*::*kan zhf50*::Tn*10* | Nakahigashi *et al.* (1995) |
| YMC10 | Wildtype isolate | Reitzer and Magasanik (1986) |
| YMC18 | *rpoN*::Tn*10* derivative of YMC10 | Ueno-Nishio *et al.* (1983) |
| ***Neisseria gonorrhoeae*** | | |
| MS11-A | Derivative of MS11 | Segal *et al.* (1985) |
| JKD259 | Tc$^r$; harbours *ptet*M25.2 | This laboratory |
| JKD328 | Wildtype nasopharyngeal isolate | Microbial Diagnostic Unit, University of Melbourne |
| JKD331 | Wildtype nasopharyngeal isolate | Microbial Diagnostic Unit, University of Melbourne |
| JKD334 | Wildtype nasopharyngeal isolate | Microbial Diagnostic Unit, University of Melbourne |
| JKD359* | Nal$^r$ mutant of MS11-A | J. Fyfe |
| JKD457 | Cm$^r$; *RLS* mutant of MS11-A | This study |
| JKD466 | Cm$^r$; *rpoH* recombinant of MS11-A | This study |
| JKD467 | Cm$^r$; *rpoH* recombinant of MS11-A | This study |
| JKD468 | Cm$^r$; *rpoH* recombinant of MS11-A | This study |
| JKD469 | Cm$^r$; *rpoH* recombinant of MS11-A | This study |
| JKD483* | Rif$^r$ mutant of MS11-A | This study |
| JKD484 | JKD483 harbouring p*tet*M25.2 from JKD259 | This study |
| JKD487 | JKD483(p*tet*M25.2ΩpJKD1595) | This study |
| JKD488 | JKD484(p*tet*M25.2 ΩpJKD2325) | This study |
| JKD489 | JKD484(p*tet*M25.2 ΩpJKD2326) | This study |
| JKD491 | JKD484(p*tet*M25.2 ΩpJKD2282) | This study |
| JKD492 | JKD484(p*tet*M25.2 ΩpJKD2283) | This study |
| JKD493 | JKD484(p*tet*M25.2 ΩpJKD2284) | This study |
| JKD494 | JKD484(p*tet*M25.2 ΩpJKD2346) | This study |
| JKD5018 | JKD484(p*tet*M25.2 ΩpJKD2358) | This study |
| ***Neisseria meningitidis*** | | |
| MC58¢2 | | Mark Achtman |
| Z5498 | Derivative of C751 clinical isolate | Sarkari *et al.* (1994) |
| ***Neisseria subflava*** | | |
| JKD385 | Human isolate | Sexual Health Centre, Melbourne |
| ***Neisseria lactamica*** | | |
| JKD386 | Human isolate | Sexual Health Centre, Melbourne |
| ***Pseudomonas aeruginosa*** | | |
| PAK | Wildtype clinical isolate | David Bradley, Memorial University of Newfoundland |

| Strain | Relevant Genotype or Phenotype | Reference or Source |
|---|---|---|
| PAK-N1 | Tn*5G* PAK *rpoN* mutant | Ishimoto and Lory (1989) |

* spontaneous mutants selected by plating MS11-A onto nalidixic acid (10μg/ml) or rifampicin (10μg/ml)

## Table 2.2 Plasmids used in this study.

| Plasmid | Description | Reference or Source |
|---|---|---|
| Hermes-2 | $Erm^r$ *E. coli*/*N. gonorrhoeae* shuttle vector | Kupsch *et al.* (1996) |
| pKV3 | pBR322 containing the *rpoH* gene from *E. coli* | Tobe *et al.* (1984) |
| pKK232-8 | $Ap^r$ promoter selection vector | Brosius (1984) |
| pSU2718 | $Cm^r$ cloning vector | Martinez *et al.* (1988) |
| pUC18 | $Ap^r$ cloning vector | Yanisch-Perron *et al.* (1985) |
| pUCP18 | $Ap^r$ *E. coli*/*P. aeruginosa* shuttle vector | Schweizer (1991) |
| pJKD800 | pGEM-3Z containing the *cat* gene | J. Fyfe |
| pJKD861 | P*recA*::*cat* fusion inserted into pGEM-3Z | J. Fyfe |
| pJKD862 | P*pilE*::*cat* fusion inserted into pGEM-3Z | Fyfe *et al.* (1995) |
| pJKD1595 | Hermes-2 containing a promoterless *cat* gene | Black *et al.* (1995) |
| pJKD1870 | pUCP18 containing the *rpoN* gene from *P. aeruginosa* | J. Fyfe |
| pJKD1926 | Clone from an *N. gonorrhoeae* MS11-A *Sau*3A library in pUC18 containing *dnaK* | Hamilton (1999) |
| pJKD2026 | 5.5kb *Hin*cII genomic DNA fragment from *N. gonorrhoeae* containing *RLS* inserted into *Hin*cII digested pSU2718 | This study |
| pJKD2035 | 1.1kb *Sma*I-*Hin*cII fragment of pJKD861 containing P*recA*::*cat* inserted into pJKD2026 digested with *Ava*II-*Cla*I and filled in | This study |
| pJKD2044 | 0.45kb *Xba*I fragment of pJKD2026, containing part of *RLS*, inserted into *Xba*I digested pUC18 | This study |
| pJKD2045 | 0.8kb *Eco*RI-*Xba*I fragment of pJKD2026, containing part of *RLS*, inserted into *Eco*RI-*Xba*I digested pUC18 | This study |
| pJKD2101 | 2.37kb PCR product amplified from *N. gonorrhoeae* genomic DNA with oligonucleotide primers 5490 and 5492, filled in and inserted into *Hin*cII digested pSU2718 | This study |
| pJKD2107 | 1.5kb PCR product amplified from *N. gonorrhoeae* genomic DNA with primers 5608 and 5609, filled in and inserted into *Hin*cII digested pUC18 | This study |
| pJKD2108 | 0.95kb PCR product amplified from *N. gonorrhoeae* genomic DNA with primers 5610 and 5611, filled in and inserted into *Hin*cII digested pSU2718 | This study |
| pJKD2122 | 1.1kb *Sma*I-*Hin*cII fragment of pJKD861 containing P*recA*::*cat* inserted into pJKD2101 digested with *Hin*cII, in the same orientation as *rpoH* | This study |
| pJKD2124 | 1.1kb *Sma*I-*Hin*cII fragment of pJKD861 containing P*recA*::*cat* inserted into pJKD2101 digested with *Hin*cII, in the opposite orientation to *rpoH* | This study |
| pJKD2238 | 0.33kb PCR product amplified from *N. gonorrhoeae* genomic DNA with oligonucleotide primers 6034 and 6035, filled in and inserted into *Hin*cII digested pUC18 | This study |
| pJKD2239 | PCR fusion product amplified with oligonucleotide primers 6034 and 6035 using the PCR products amplified from pJKD2238 with oligonucleotide primer combinations 6034/7080 and 6035/7081 (Figure 4.2) as templates. The 0.33kb product was filled in and inserted into *Hin*cII digested pUC18 | This study |

**Table 2.2 continued:**

| Plasmid | Description | Reference or Source |
|---|---|---|
| pJKD2240 | PCR fusion product amplified with oligonucleotide primers 6034 and 6035 using the PCR products amplified from pJKD2238 with oligonucleotide primer combinations 6034/7078 and 6035/7079 (Figure 4.2) as templates. The 0.33kb product was filled in and inserted into *Hin*cII digested pUC18 | This study |
| pJKD2266 | 0.33kb *Bam*HI-*Hin*dII fragment of pJKD2239 inserted into pKK232-8 | This study |
| pJKD2267 | 0.33kb *Bam*HI-*Hin*dII fragment of pJKD2240 inserted into pKK232-8 | This study |
| pJKD2268 | 0.48kb PCR product amplified from *N. gonorrhoeae* genomic DNA with oligonucleotide primers 4527 and 7070, filled in and inserted into *Hin*cII digested pUC18 | This study |
| pJKD2270 | PCR fusion product amplified with oligonucleotide primers 4527 and 7070 using the PCR products amplified from pJKD2101 with oligonucleotide primer combinations 4527/7082 and 7070/7071 (Figure 5.3) as templates. The 0.36kb product was filled in and inserted into *Hin*cII digested pSU2718 | This study |
| pJKD2272 | 1.8kb PCR product amplified from *N. lactamica* genomic DNA with oligonucleotide primers 4878 and 4941, filled in and inserted into *Hin*cII digested pSU2718 | This study |
| pJKD2273 | 0.33kb *Bam*HI-*Hin*dII fragment of pJKD2238 inserted into pKK232-8 | This study |
| pJKD2274 | 0.6kb *Eco*RI fragment of pJKD2266 inserted into pJKD862 to replace gonococcal P*pilE::cat* | This study |
| pJKD2275 | 0.6kb *Eco*RI fragment of pJKD2267 inserted into pJKD862 to replace gonococcal P*pilE::cat* | This study |
| pJKD2281 | 0.6kb EcoRI fragment of pJKD2273 inserted into pJKD862 to replace gonococcal P*pilE::cat* | This study |
| pJKD2282 | 1.1kb *Bam*HI fragment of pJKD2281 inserted into Hermes-2 | This study |
| pJKD2283 | 1.1kb *Bam*HI fragment of pJKD2274 inserted into Hermes-2 | This study |
| pJKD2284 | 1.1kb *Bam*HI fragment of pJKD2275 inserted into Hermes-2 | This study |
| pJKD2319 | 0.48kb *Bam*HI-*Hin*dII fragment of pJKD2268 inserted into pKK232-8 | This study |
| pJKD2320 | 0.36kb *Bam*HI-*Hin*dII fragment of pJKD2270 inserted into pKK232-8 | This study |
| pJKD2321 | 1.8kb *Bam*HI-*Hin*dII fragment of pJKD2272 inserted into pUCP18 | This study |
| pJKD2322 | 0.75kb *Eco*RI fragment of pJKD2319 inserted into pJKD862 to replace gonococcal P*pilE::cat* | This study |
| pJKD2323 | 0.63kb *Eco*RI fragment of pJKD2320 inserted into pJKD862 to replace gonococcal P*pilE::cat* | This study |
| pJKD2325 | 1.25kb *Bam*HI fragment of pJKD2322 inserted into Hermes-2 | This study |
| pJKD2326 | 1.13kb *Bam*HI fragment of pJKD2323 inserted into Hermes-2 | This study |

**Table 2.2 continued:**

| Plasmid | Description | Reference or Source |
|---|---|---|
| pJKD2345 | PCR fusion product amplified with oligonucleotide primers 4526 and 4527 using the PCR products amplified from *N. gonorrhoeae* genomic DNA with oligonucleotide primer combinations 4527/9349 and 4526/9350 (Figure 5.3) as templates. The 1.37kb product was filled in and inserted into *Hinc*II digested pSU2718 | This study |
| pJKD2346 | 1.37kb *Bam*HI-*Hin*dIII fragment of pJKD2345 inserted into Hermes-2 | This study |
| pJKD2352 | 1.34kb PCR product amplified from *N. gonorrhoeae* genomic DNA with oligonucleotide primers 4526 and 4527, filled in and inserted into *Hinc*II digested pSU2718 | This study |
| pJKD2358 | 1.34kb *Bam*HI-*Hin*dIII fragment of pJKD2352 inserted into Hermes-2 | This study |

in a 5% $CO_2$ atmosphere. Inoculated GC broths were supplemented with 1% (v/v) $NaHCO_3$ and incubated aerobically at 37°C on a rotary shaker.

*E. coli* and *P. aeruginosa* strains were stored at -70°C in LB and NY broth, respectively, containing 20% sterile glycerol. *Neisseria* strains were stored at -70°C in GC broth supplemented with 20% sterile glycerol.

## 2.4 Preparation of DNA

### 2.4.1 Preparation of genomic DNA from *E. coli* and *P. aeruginosa*

Genomic DNA was extracted from *E.coli* and *P. aeruginosa* strains according to the method of Wilson (1993), finally resuspending the pellet in 100µl of sterile distilled water.

### 2.4.2 Preparation of genomic DNA from *Neisseria*

Bacterial growth was scraped from one quarter of a cultured GC agar plate, resuspended in 1ml of TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) and centrifuged at 13000*g* for 5 minutes. This wash step was repeated and the cell pellet finally resuspended in 300µl of TE buffer. Following the addition of 600µl of lysis buffer (1.6% (w/v) SDS, 10mM Tris, pH8.0) and 10µl of RNaseA (10mg/ml), the suspension was mixed by inversion and incubated at 56°C for 30 minutes (mixing every 5 minutes).

A phenol extraction was performed by adding 500µl of TE-saturated phenol to the lysed cells, mixing and centrifuging at 13000*g* for 5 minutes. The supernatant was transferred to a new tube and the extraction repeated twice, followed by 2 extractions with 500µl of chloroform. The DNA was precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH5.2) and 2.5 volumes of absolute ethanol and spooled. The DNA was washed twice with 70% ethanol, dried under vacuum using the Speed Vac SVC100 (Savant) and resuspended in 100µl of sterile distilled water.

### 2.4.3 Small scale preparation of plasmid DNA

Small scale plasmid extraction from *E. coli* and *P. aeruginosa* strains was performed by the alkaline lysis method described by Morelle (1989) with DNA finally suspended in 50µl of sterile distilled water.

Purification of Hermes-2 plasmid DNA (Table 2.2) and plasmid DNA used as template for sequencing reactions (Section 2.10) was achieved using the High Pure Plasmid Isolation Kit (Roche) according to the manufacturer's instructions.

## 2.5 DNA manipulation procedures

### 2.5.1 Restriction endonuclease digestion

DNA was digested with restriction endonucleases according to the instructions supplied by the manufacturer. Digests were performed in 20µl volumes and terminated by the addition of 0.2 volumes of loading dye (50% sucrose, 0.5% bromophenol blue, 5mM EDTA), followed by agarose gel electrophoresis (Section 2.5.3).

### 2.5.2 Creation of non-cohesive ends

If necessary following restriction endonuclease digestion or polymerase chain reaction (PCR) amplification, cohesive termini were filled in using the Double Geneclean II® Method, an addendum to the Geneclean II ® Kit (BIO101 Inc., Integrated Sciences; Section 2.5.4). Briefly, digested DNA was subjected to agarose gel electrophoresis (Section 2.5.3) and purified by the Geneclean II® method. Eluted DNA was added to a mixture consisting of 10µl of 10 x polymerase buffer, 1mM ATP (Promega), 200µM dNTPs (Promega), 10 units of T4 polynucleotide kinase (Promega) and 10 units of DNA polymerase I (Promega). Following an incubation at 37°C for 1 hour, DNA was purified once again using the Geneclean II® method and finally eluted in 10µl of distilled water.

### 2.5.3 Agarose gel electrophoresis

DNA samples containing 0.2 volumes of loading dye were electrophoresed in 0.8 - 1% (w/v) agarose gels containing 0.2µg/ml ethidium bromide. Gels were prepared and buffered in 1 x TAE buffer (Sambrook *et al.*, 1989) and electrophoresed at 100V for 30-60 minutes in a Bio-Rad gel apparatus. Bacteriophage lambda DNA (Roche) digested with *Hin*dIII or the 100bp ladder (Roche) served as molecular size standards. DNA was visualised using a TFL-20M Hybaid "Crosslinker" Transilluminator (Integrated Science Pty. Ltd.). Gels were photographed using a Mitsubishi Video Copy Processor, or alternatively a Polaroid MP4 Land Camera with Polaroid Type 667 Black and White Coaterless Land Film.

### 2.5.4 Purification of DNA from agarose gels

DNA fragments were purified from agarose gels using the Geneclean II Kit® (BIO101, Inc., Integrated Sciences) obtained from Bresatec, according to the instructions provided by the manufacturer.

### 2.5.5 Alkaline phosphatase treatment of vector DNA

Restriction endonuclease digested vector DNA was dephosphorylated by treatment with alkaline phosphatase (Roche). The 60µl reaction consisted of linearised vector, 6µl of 10 x dephosphorylation buffer (Roche), and 20 units of calf intestinal alkaline phosphatase. Following an incubation at 37°C for 1 hour, the reaction was terminated by the addition of 6.7µl of 10 x TEN buffer (Appendix) and incubation at 65°C for 15 minutes. The vector DNA was purified using the Geneclean II® method (Section 2.5.4) and eluted in 15µl of sterile distilled water.

### 2.5.6 Ligation of DNA

Ligation reactions were performed in a 10µl volume with a vector to insert ratio of approximately 1:5. Reactions included 1µl of T4 DNA ligase buffer and 1 unit of T4 DNA ligase (Promega) and were incubated overnight at 16°C.

### 2.5.7 Construction of *N. gonorrhoeae* genomic libraries

Approximately 1µg of genomic DNA from *N. gonorrhoeae* MS11-A (Table 2.1) was digested with the appropriate restriction endonuclease overnight (Section 2.5.1) and electrophoresed on an agarose gel (Section 2.5.3). Partial digests were performed with *Sau*3A by dilution in the corresponding buffer and incubating at 37°C for 30 seconds followed by electrophoresis. Fragments of the desired size range were purified using the Geneclean II® method (Section 2.5.4) and ligated to vector DNA (Section 2.5.6) digested with a compatible restriction endonuclease and treated with alkaline phosphatase (Section 2.5.5). The ligation mix was precipitated with 0.1 volumes of 3M sodium acetate (pH5.2) and 2.5 volumes of absolute ethanol and placed on ice for 30 minutes. The ligated DNA was pelleted by centrifugation at 13000*g* for 5 minutes, washed with 70% ethanol and dried under vacuum. Following resuspension in 10µl of sterile distilled water, half of the solution was used to transform the relevant competent species (Section 2.6).

## 2.6 Preparation of competent cells

### 2.6.1 *E. coli* competent cells

*E.coli* DH5α competent cells were prepared using the method described by Hanahan (1985). Cells were ultimately snap frozen in a dry ice-ethanol and stored at -70°C.

When competent cells of a different *E. coli* strain were required, a rapid small scale procedure was applied. 50µl of an overnight culture was used to inoculate a 10ml LB broth and incubated for 2.5 hours at 37°C. The culture was chilled on ice for 15 minutes and centrifuged at 1000*g* for 20 minutes at 4°C. The cell pellet was resuspended gently in 1ml of chilled 50mM $CaCl_2$ and an additional 4ml added. Following an incubation on ice for 30 minutes the suspension was centrifuged as described previously. The cell pellet was resuspended in 100µl of 50mM $CaCl_2$ and used for a single transformation experiment.

### 2.6.2 *E. coli* electrocompetent cells

*E. coli* DH12S electrocompetent cells were prepared using the method outlined by Smith *et al.* (1990). The cells were snap frozen in a dry-ice ethanol bath and stored at -70°C. Electrocompetent cells of the *E. coli rpoH* mutant, KY1608, were prepared by the same method but the culture was grown at 20°C instead of 37°C prior to the washing steps.

### 2.6.3 *P. aeruginosa* competent cells

Competent *P. aeruginosa* cells were prepared using a method modified from Elleman *et al.* (1986). An inoculated 5ml NY broth was incubated overnight without shaking and added to 100ml of NY broth. The culture was incubated at 37°C until an optical density at 600nm ($CD_{600}$) of 0.3 was reached as determined using the Spectronic 20 spectrophotometer (Bausch and Lomb). Cells were pelleted by centrifugation at 4300*g* for 5 minutes at 4°C and resuspended in 10ml of 0.1M $MgCl_2$. This washing step was repeated and the pelleted cells finally resuspended in 10ml of 0.15M $MgCl_2$ and incubated on ice for 1 hour. Following centrifugation at 4°C cells were resuspended in 1.5ml of 0.15M $MgCl_2$. 150µl of sterile glycerol was added to the mixture and cells were frozen in 100µl aliquots in a dry ice-ethanol bath and stored at -70°C.

## 2.7 Transformation procedures

### 2.7.1 Chemical transformation of *E. coli* and *P. aeruginosa* competent cells

*E. coli* competent cells were transformed with plasmid DNA as described by Sambrook *et al.* (1989). *P. aeruginosa* competent cells were transformed using the same method with the exceptions of substituting SOC broth (Sambrook *et al.*, 1989) with NY broth and plating cells onto NY agar. For blue-white selection of recombinant clones, 50µl of 2% 5-bromo-4-chloro-3-indol-β-D-galactopyranoside (X-Gal) dissolved in dimethylformamide was spread on the agar plate prior to plating of the transformation mix.

### 2.7.2 Transformation of electrocompetent *E. coli* cells

A 5µl volume of ligation mix (Section 2.5.6) was added to 25µl of thawed electrocompetent cells (Section 2.6.2) in a chilled 0.1cm electroporation cuvette (Bio-Rad). Electrotransformation was performed using a Bio-Rad Gene Pulser set at 25µFarad, 1.8V and 200Ω. Following pulsing, 1ml of SOC (Sambrook *et al.*, 1989) was added and the suspension incubated at 37°C for 1 hour with shaking. 100µl aliquots were plated onto LB agar containing the appropriate antibiotic (Section 2.3), X-Gal (Section 2.7.1) and 5µl of 840mM isopropyl-β-D-thiogalactopyranoside (IPTG). Plates were incubated overnight at 37°C.

### 2.7.3 Transformation of *N. gonorrhoeae*

*N. gonorrhoeae* transformations were performed by resuspending piliated cells from an overnight culture plate in 1ml of GC broth (Section 2.3). Approximately 1µg of linearised plasmid DNA was added to the culture and incubated at 37°C with shaking for 2 hours. 200µl aliquots of the mixture were plated onto GC agar containing the appropriate antibiotic (Section 2.3).

Recombination of DNA into the p*tet*M25.2 conjugative plasmid was achieved using the Hermes-2 plasmid (Kupsch *et al.*, 1996). Approximately 1µg of DNA was linearised with *Cla*I and added to a 1ml suspension of piliated JKD484 (Table 2.1) cells transferred from an overnight culture plate into GC broth. Following an incubation at 37°C for 2 hours with shaking cultures were plated onto GC agar containing Erm for selection of recombinants.

### 2.7.4 Conjugation of *N. gonorrhoeae*

A spontaneous rifampicin resistant mutant of *N. gonorrhoeae* MS11-A (Table 2.1) was selected by plating cells onto GC agar containing Rif (10µg/ml) and incubating at 37°C for several days. One resistant colony was selected and denoted JKD483 (Table 2.1). The p*tet*M25.2 conjugative plasmid was then transferred from JKD259 (Table 2.1) to JKD483 by conjugation. Gonococcal conjugation experiments with the p*tet*M25.2 conjugative plasmid or derivatives were performed by mixing donor and recipient

strains. A suspension of 5 x $10^8$ donor cells was mixed with a suspension of 5 x $10^9$ recipient cells on a small section of a GC agar plate and incubated overnight. The growth was transferred into 500µl of GC broth and 100µl aliquots were spread onto GC agar plates containing appropriate antibiotics (Section 2.3) for selection of transconjugants.

## 2.8 Preparation of oligonucleotide primers

Oligonucleotide primers were synthesised on an Applied Biosystems 381A DNA Synthesiser, and deprotected in a solution of 35% $NH_4OH$ at 56°C for 16 hours. They were then aliquoted, dried under vacuum and resuspended in 100µl of sterile distilled water. The concentration of oligonucleotide primers was determined by measuring the absorbance at 260nm ($A_{260}$), as an $A_{260}$ of 1.0 correlates to 33µg/ml of single stranded DNA. Oligonucleotide primers were stored at -20°C as anhydrous pellets or in solution. The sequences of oligonucleotide primers used in this study are shown in Table 2.3.

## 2.9 Polymerase chain reaction (PCR)

### 2.9.1 Amplification by PCR

PCR was performed using the GeneAmp® PCR System 2400 (Perkin Elmer) thermal cycler. Reaction volumes of 50µl contained approximately 100ng of genomic DNA or 1ng of plasmid DNA, 2µM of each oligonucleotide primer, 200µM dNTPs (Promega), 5µl of 10 x reaction buffer (Roche) and 2.5 units of *Taq* DNA polymerase (Roche). The program routinely used consisted of 30 cycles each of 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, followed by 1 cycle of 30 seconds at 94°C, 30 seconds at 55°C and 5 minutes at 72°C. Large PCR products required programs with longer extension times.

### 2.9.2 Construction of PCR fusions

PCR fusions were generated using the splicing by overlap extension (SOE) method described by Horton *et al.* (1989). Products to be fused were amplified by PCR (Section 2.9.1) and electrophoresed on an agarose gel (Section 2.5.3). Following extraction by

## Table 2.3 Oligonucleotide primers used in this study.

| Oligonucleotide Primer | Sequence | Reference or Source |
|---|---|---|
| RP | 5'-AGCGGATAACAATTTCACACAGGA-3' | New England Biolabs® Inc. |
| UP | 5'-GTTTTCCCAGTCACGAC-3' | New England Biolabs® Inc. |
| 102 | 5'-TTAACGCGTGAATTCAAAAATC-3' | Fyfe *et al.* (1993) |
| 1768 | 5'-ATTTTAGCTTCCTTAGCTCC-3' | Fyfe *et al.* (1995) |
| 2377 | 5'-GCGGATCC(G/A)TA(C/T)TTNGCNACNGT-3' | This laboratory |
| 3260 | 5'-CACACTGGGACTGAGACACG-3' | Black *et al.* (1998) |
| 3261 | 5'-CGGCAGTCTCATTAGAGTGC-3' | Black *et al.* (1998) |
| 4525 | 5'-GACGAGCAGGGCTACCTG-3' | This study |
| 4526 | 5'-TGTGGCAGTGTCTGGTGC-3' | This study |
| 4527 | 5'-CATATTGACCCTAGCCGC-3' | This study |
| 4857 | 5'-CGCATAAAATTTCACCTC-3' | Fyfe and Davies (1998) |
| 4877 | 5'-TCGTCCACGCCCAAGTCC-3' | This study |
| 4878 | 5'-TTATTCTGCGGTTTTGCG-3' | This study |
| 4940 | 5'-AACGCCTGCCCCAAACCG-3' | This study |
| 4941 | 5'-TTGTTCATGGTCAGATGG-3' | This study |
| 4942 | 5'-CCGGCGATGTTGGACAGC-3' | This study |
| 4943 | 5'-TTGGCTGCGGTTGCCGTC-3' | This study |
| 5490 | 5'-CGCCATTCCCATCATGCG-3' | This study |
| 5492 | 5'-CAAGTGCCGATTTATGCG-3' | This study |
| 5493 | 5'-TTGGATGGCGGGTAATGC-3' | This study |
| 5494 | 5'-TCGGTAGCTGCTCTTGCC-3' | This study |
| 5495 | 5'-TTTCCTACTGTCTCGACG-3' | This study |
| 5608 | 5'-GTACCCTATTTCCAAACG-3' | This study |
| 5609 | 5'-CGGCTTTGAACATGGACG-3' | This study |
| 5610 | 5'-TGCCAGAGGTCGAAACCG-3' | This study |
| 5611 | 5'-CGGCATACGGGTTGACCG-3' | This study |
| 6034 | 5'-TCATCGAGTCTTCACACG-3' | This study |
| 6035 | 5'-ACAAGAGTTGGTTGTACC-3' | This study |
| 7070 | 5'-CAGGATGAGTTGTTTGGC-3' | This study |
| 7071 | 5'-ACCGCCGATACGCAGTTTCAGCC-3' | This study |
| 7078 | 5'-CGGCGGGCTGTTTCCCGCTACAGCATGGC-3' | This study |
| 7079 | 5'-GCCATGCTGTAGCGGGAAACAGCCCGCCG-3' | This study |
| 7080 | 5'-CGATGGGCTGTAAATCTGGCGGGCGGCGGG-3' | This study |
| 7081 | 5'-CCCGCCGCCCGCCAGATTTACAGCCCATCG-3' | This study |
| 7082 | 5'-GGCTGAAACTGCGTATCGGCGGTGTAAACCTGATA GCTCAATTCG -3' | This study |
| 8102 | 5'-TTTCCTTCCATTCGGGCG-3' | This study |
| 8182 | 5'-TGCTCGTCAAGGAAATCG-3' | This study |
| 9349 | 5'-CGTCTTGAAGCCAGCAGCTTTCCACAATGC-3' | This study |
| 9350 | 5'-GCATTGTGGAAAGCTGCTGGCTTCAAGACG-3' | This study |
| 11076 | 5'-GACACCCAAATCGTCGGC-3' | This study |
| 11077 | 5'-CGGCTATGACGGCTACGGG-3' | This study |
| 11794 | 5'-CCATCGACTGGCTTGCCGACC-3' | This study |
| 13060 | 5'-TATCTGACCGACAGCATCG-3' | This study |
| 13061 | 5'-CCCAGCAGCTCAACCAGCG-3' | This study |

the Geneclean II Kit® (Section 2.5.4), 100ng of each DNA fragment was denatured, annealed and used as template in the PCR fusion reaction. The program used consisted of 30 cycles each of 2 minutes at 92°C, 2 minutes at 37°C and 7 minutes at 72°C.

### 2.9.3 Reverse transcriptase (RT) PCR

RT PCR was performed with the SUPERSCRIPT™ II RNaseH Reverse Transcriptase Kit (Life Technologies) using the method supplied by the manufacturer with some modifications. Copy DNA (cDNA) was created in a 20µl reaction containing 15µg of RNA (Section 2.14.2) and 0.5µM of the downstream priming oligonucleotide primer. The reaction was incubated at 70°C for 10 minutes then immediately placed on ice. Following the addition of 4.0µl of 5 x First Strand Buffer (Life Technologies), 2.0µl of 0.1M DTT (Life Technologies) and 200µM of dNTPs (Promega), the solution was prewarmed at 42°C for 2 minutes. 200 units of SUPERSCRIPT™ II RNaseH RT was added followed by an incubation at 42°C for 50 minutes. The enzyme was inactivated by placing the reaction at 70°C for 15 minutes and a 5µl aliquot was used directly as template in PCR. 4µM of each oligonucleotide primer and 2.5 units of *Taq* DNA polymerase (Roche) was added to the 25µl volume reaction and the program used was outlined in Section 2.9.1.

## 2.10 Automated sequencing

DNA sequencing was achieved using the PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the protocol supplied by the manufacturer. Following thermal cycling, extension products were precipitated from reactions by the addition of 2µl 3M sodium acetate pH5.2 and 50µl of 95% ethanol. The mixture was incubated on ice for 10 minutes and centrifuged for 30 minutes at 13000*g* at room temperature. The pellet was washed twice with 70% ethanol, dried under vacuum and analysed on an Applied Biosystems Model 373A DNA Sequencing System.

## 2.11 Computer analysis of nucleotide and protein sequences

Nucleotide sequence data and deduced amino acid sequences were analysed with the Sequencher™ 3.0 (Gene Codes Corporation) program. The BLAST program (Altschul

*et al.*, 1990) was used to compare all sequences to those submitted in the Genbank, SWISS-PROT and EMBL databases. Further analysis was performed using the Sequence Interpretation Tools database, which accessed CLUSTALW (Thompson *et al.*, 1994), a sequence alignment program, and MOTIF/SMART (Ogiwara *et al.*, 1996), for sequence motif analysis and retrieval. All of the mentioned databases were accessed on the World Wide Web via GenomeNet or NCBI using Netscape Communicator 4.6.

## 2.12 Non-radioactive labelling of probe DNA

DNA probes were labelled with digoxigenin (DIG) -11-dUTP using the Non-radioactive Labelling and Detection Kit (Roche) according to the manufacturer's instructions. Alternatively, PCR (Section 2.9.1) was used to label DNA probes by the addition of 1µl of DIG-11-dUTP to the reaction. Labelled PCR products were electrophoresed on a 1% low melting point agarose gel, excised and stored at -20°C. The gel slice was boiled for 10 minutes to denature the DNA and added to the hybridisation solution (Sections 2.13.3 and 2.14.3).

## 2.13 Transfer of DNA to nylon membrane

### 2.13.1 Southern blotting

Southern blotting was performed by a modification of the method described by Sambrook *et al.* (1989). Digested DNA was electrophoresed on a 1% agarose gel and subsequently photographed adjacent to a ruler to permit the size determination of hybridising fragments relative to the non-labelled molecular standards. The gel was then immersed in 0.25M HCl for 15 minutes, rinsed with distilled water, and soaked in denaturation solution (Appendix) for 15 minutes. Following a rinse with distilled water, the gel was soaked in neutralisation solution (Appendix) for 30 minutes. All incubations were performed at room temperature with shaking. Transfer of DNA to a Hybond $^{TM}$-N nylon membrane (Amersham) was performed by capillary transfer as described by Sambrook *et al.* (1989) with 20 x SSC (Appendix) as the transfer buffer. The gel was surrounded by parafilm and transfer allowed to proceed overnight. The DNA was crosslinked to the membrane using a TFL-20M Hybaid "Crosslinker" Transilluminator (Integrated Science Pty. Ltd.) for 5 minutes.

### 2.13.2 Colony hybridisation

Colony hybridisation was performed using a modification of the method described by Grunstein and Hogness (1975). Agar plates containing patched colonies from the fractionated *N. gonorrhoeae* genomic library (Section 2.5.7) were overlaid with a nylon membrane to permit the transfer of colonies. The filters were placed in a chamber of chloroform vapour for 20 minutes then incubated in 10ml of colony hybridisation lysis buffer (Appendix) for 45 minutes at 37°C with shaking. Filters were washed for 5 minutes in 2 x SSC (Appendix) then incubated in denaturation solution (Appendix) for 5 minutes. Filters were again washed in 2 x SSC and incubated in neutralisation solution (Appendix) for 5 minutes. All steps were carried out at room temperature with shaking. Following a final rinse with 2 x SSC, filters were dried and the DNA crosslinked to the membrane as for Southern blots (Section 2.13.1).

### 2.13.3 DNA-DNA hybridisation

Hybridisation for Southern blots and colony hybridisations was performed using the same procedure. Membranes were prehybridised at 65°C for 1-3 hours in 20ml of prehybridisation solution (Appendix) then drained. The labelled DNA probe (Section 2.12) was added to 3ml of prehybridisation solution and boiled for 10 minutes. The denatured probe was added to the membrane and incubated overnight at 65°C. Following hybridisation, high stringency washes were performed by washing the membrane twice with 2 x SSC, 0.1% (w/v) SDS for 5 minutes at room temperature with shaking followed by two washes in 0.1 x SSC, 0.1% (w/v) SDS at 65°C for 15 minutes with shaking.

### 2.13.4 Detection of hybridised probe DNA

Hybridised labelled probe DNA was detected using the CDP-*Star*™ Detection Kit (Roche) according to the supplied protocol.

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### 2.13.4 Detection of hybridised probe DNA

Hybridised labelled probe DNA was detected using the CDP-*Star*™ Detection Kit (Roche) according to the supplied protocol.

## 2.14 RNA analysis

### 2.14.1 Preparation of cultures

GC plates inoculated with neisserial strains were incubated overnight and the cells scraped off and resuspended in GC broth. This culture was used to inoculate a 50ml GC broth with a starting $OD_{600}$ of 0.05 and incubated at 37°C with shaking for 4 hours. Cells from 10ml samples were pelleted by centrifugation at 1680*g* for 5 minutes and total RNA extracted as described in Section 2.14.2.

Cultures subjected to heat shock treatment were incubated for 4 hours to mid-exponential phase and 10ml samples were transferred to 42°C for the required time. Samples were chilled on ice and centrifuged at 1680*g* for 5 minutes at 4°C followed by RNA extraction (Section 2.14.2).

### 2.14.2 Isolation of RNA

RNA was isolated from neisserial strains by the single step method (Chomczynski and Sacchi, 1987) using TRIZOL® (Life Technologies). The protocol supplied was followed with the exception of incubating the homogenate at 65°C for 10 minutes prior to the addition of chloroform to increase the efficiency of cell lysis and improve the yield of high molecular weight messenger RNA (mRNA) species. The RNA pellet was resuspended in 90µl of nuclease free water (Promega) and treated for contaminating DNA by the addition of 10µl of 10 x DNase I buffer (Appendix), 1 unit of RQ1 RNase free DNase (Promega) and 33 units of RNasin RNase Inhibitor (Promega). An incubation at 37°C for 1 hour was followed by a second round of RNA extraction using TRIZOL® (Life Technologies) and the RNA was finally resuspended in 100µl nuclease free water (Promega). The RNA concentration was determined by measuring the absorbance at 260nm, as an $A_{260}$ reading of 1.0 equates to 40µg/ml of RNA. Samples were precipitated with 0.1 volumes 2M NaCl (prepared with diethylpyrocarbonate (DEPC) treated water) and 2.5 volumes of absolute ethanol and stored at -70°C. When required the appropriate volume of RNA was centrifuged for 20 minutes at 4°C

(12000*g*) and the pellet washed with 75% ethanol by centrifuging for 5 minutes at 7500*g*. The pellet was air dried and finally resuspended in nuclease free water.

### 2.14.3 RNA dot blotting, DNA-RNA hybridisation and detection of hybridised probe DNA

RNA dot blotting was performed using a Schleicher & Schuell SRC96D Minifold I Dot Blotter (Bartelt Instruments Pty. Ltd.) after washing wells with 20 x SSC (Appendix). RNA samples were initially resuspended in dilution buffer containing DEPC treated water, 20 x SSC, and formaldehyde at a ratio of 5:3:2. Following an incubation at 65°C for 10 minutes, the RNA was transferred to a nylon membrane using the dot blotter. The membrane was then dried and crosslinked as outlined in Section 2.13.1.

Membranes were prehybridised in high SDS concentration hybridisation buffer (The DIG System User's Guide for Filter Hybridisation, Roche) for 2-5 hours at 65°C. Following addition of the denatured probe (Section 2.12) in the same buffer, membranes were incubated overnight at 65°C. High stringency washes were performed as described in Section 2.13.3 followed by detection of hybridised probe DNA (Section 2.13.4).

### 2.14.4 Primer extensions

Primer extension reactions were performed using the Primer Extension System (Promega) according to the instructions provided. Briefly, oligonucleotide primers were labelled at the 5' end with [$\gamma^{32}$P]-dATP (Amersham Pharmacia Biotech) and annealed to 50µg of total RNA. Following extension with avian myeloblastosis virus (AMV) reverse transcriptase, cDNA products were precipitated overnight at -20°C with 0.1 volumes of 3M sodium acetate pH 5.2 and 2.5 volumes of absolute ethanol. Samples were centrifuged at 12000*g* for 15 minutes, washed with 70% ethanol and resuspended in a 6µl solution consisting of half Loading Dye (Promega) and half sterile distilled water. Electrophoresis of the samples followed as described in Section 2.14.5. Sequencing reactions on the corresponding DNA were performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) to permit mapping of the tsps using the T7 Sequencing Kit (Amersham Pharmacia Biotech). The supplied protocol was followed using [$\alpha^{35}$S]-dATP (Amersham Pharmacia Biotech) and "Read Short" conditions.

### 2.14.5 Electrophoresis of primer extension and sequencing products

Primer extension and sequencing reactions were electrophoresed through an 8% (w/v) polyacrylamide gel (Appendix) in 1 x TBE buffer (Appendix). On ... dissolved by heating at 37°C the gel mixture was filtered through a Whatman No.1 filter and adjusted to 100ml with distilled water. Gel polymerisation was initiated by the addition of 400µl of 10% (w/v) ammonium persulfate and 40µl of N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and the solution poured between two glass plates and allowed to set overnight.

Samples were denatured by boiling prior to loading onto the gel. The gel was electrophoresed at 100W in 1 x TBE buffer using a Poker Face SE 1500 Sequencer (Hoefer Scientific Instruments). The gel was subsequently dried onto Whatman® 3MM chromatography paper using a Dual-Temperature Slab Gel Drier Model SE 1150 (Hoefer Scientific Instruments). The dried gel was exposure to Fuji X-ray film and developed using a Fuji RGII X-ray film processor.

## 2.15 Determination of chloramphenicol acetyltransferase (CAT) levels

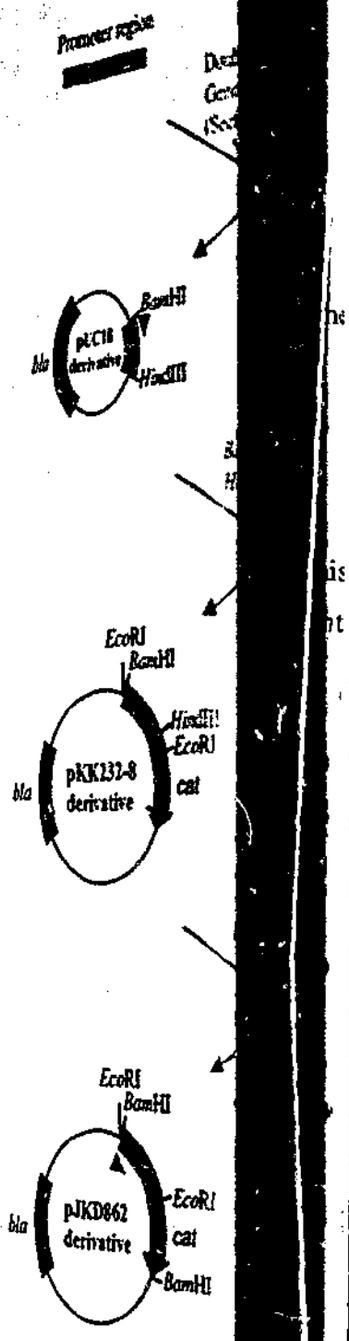
### 2.15.1 Construction of transcriptional reporters

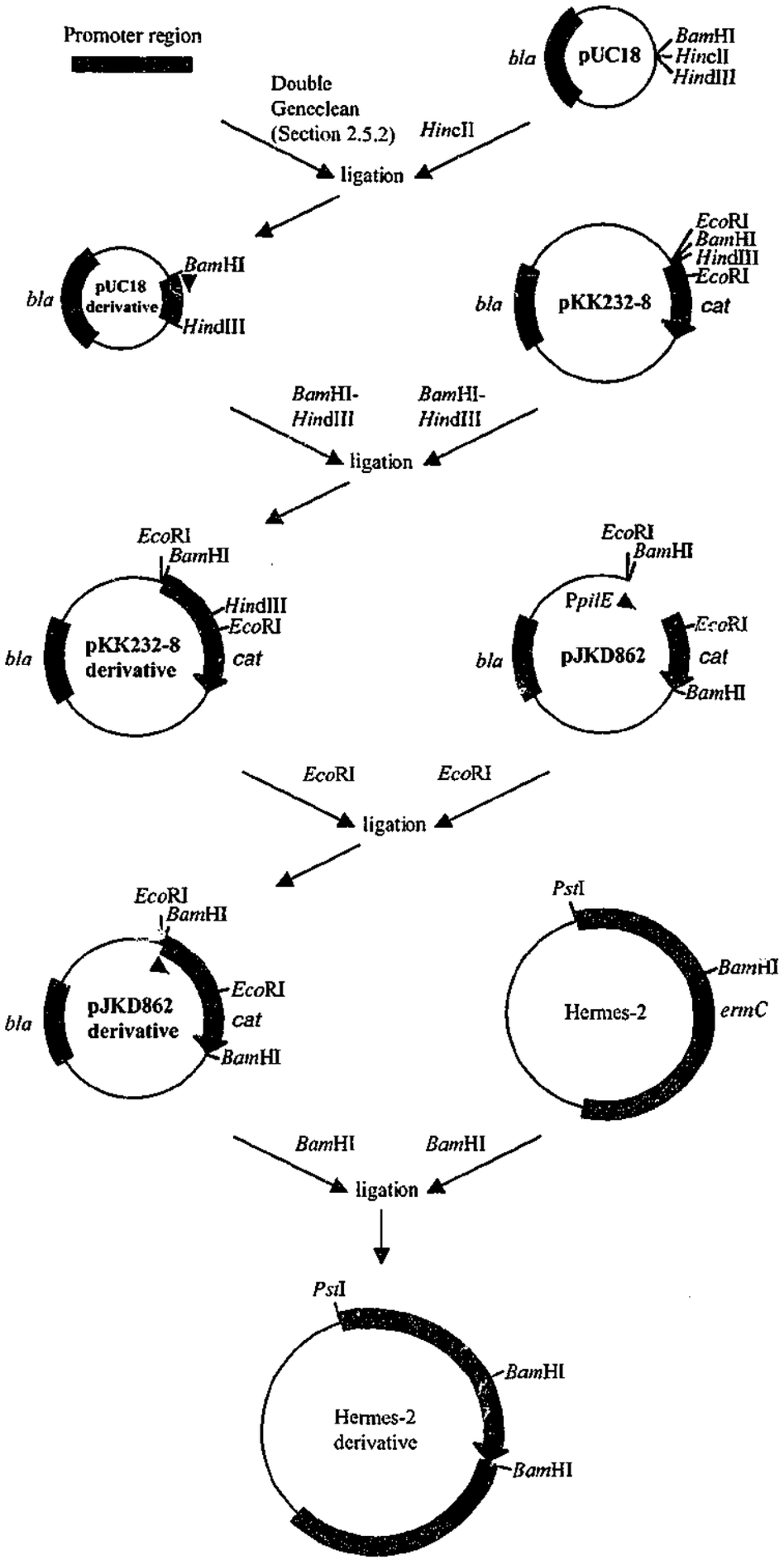
Transcriptional reporters were constructed by fusions between promoter regions and the promoterless *cat* gene, encoding chloramphenicol acetyltransferase (CAT), by PCR or ligation of restriction fragments. The cloning strategy used to create the fusions in plasmid vectors pKK232-8 and Hermes-2 is shown in Figure 2.1 and the resulting recombinant plasmids are listed in Table 2.2.

### 2.15.2 Preparation of *E. coli* cell extracts

A 10ml LB broth culture of the appropriate strain was incubated overnight at 37°C. 500µl of this culture was used to inoculate a 50ml LB broth and incubated at 37°C for 3 hours with shaking until the cells reached mid-exponential phase. 10ml samples were centrifuged at 3500*g* for 5 minutes at 4°C and washed with 5ml of cold phosphate buffered saline (PBS; Appendix). The cell pellet was resuspended in 2ml of cold PBS

**Figure 2.1 Strategy for the construction of transcriptional reporter plasmids.** Promoter fragments were amplified by PCR (red box), rendered blunt ended by the Double Geneclean II® Method (Section 2.5.2) and cloned into the *Hinc*II site of the plasmid vector pUC18. The orientation of the inserts were checked by restriction endonuclease digestion to ensure that the promoter fragments would be orientated in the same direction as the *cat* gene of the reporter plasmid. The inserts were then directionally subcloned into the *Bam*HI-*Hin*dIII sites located upstream of the promoterless *cat* gene (green box) in the promoter selection vector pKK232-8. The pKK232-8 derivates were digested with *Eco*RI and the promoter fragments subcloned into pJKD862 to replace the gonococcal *pilE* promoter region (P*pilE*; yellow box). This permitted the promoter::*cat* transcriptional fusions to be subcloned on *Bam*HI fragments into the *Bam*HI site of the *E. coli*/*N. gonorrhoeae* shuttle vector Hermes-2.




Promoter region
bla
pUC18
BamHI
HincII
HindIII
Double
Geneclean
(Section 2.5.2)
HincII
ligation
BamHI
pUC18
derivative
bla
HindIII
EcoRI
BamHI
HindIII
EcoRI
bla
pKK232-8
cat
BamHI-
HindIII
BamHI-
HindIII
ligation
EcoRI
BamHI
HindIII
EcoRI
bla
pKK232-8
derivative
cat
EcoRI
BamHI
PpilE
EcoRI
bla
pJKD862
cat
BamHI
EcoRI
EcoRI
ligation
EcoRI
BamHI
EcoRI
bla
pJKD862
derivative
cat
BamHI
PstI
BamHI
Hermes-2
ermC
BamHI
BamHI
ligation
PstI
BamHI
Hermes-2
derivative
BamHI

and passaged through a French Pressure Cell (Aminco, Silver Springs Md., USA). The lysed suspension was immediately centrifuged at 13000*g* for 15 minutes at 4°C. The supernatant was snap frozen in 250µl aliquots using a dry ice-ethanol bath and stored at -70°C. Different aliquots were used to measure total protein concentrations (Section 2.15.4) and CAT levels (Section 2.15.5).

Culture samples subjected to heat shock treatment were transferred to 42°C for various time intervals prior to centrifugation and passage through the French Pressure Cell.

### 2.15.3 Preparation of *N. gonorrhoeae* cell extracts

Cell extracts were prepared following the method supplied with the CAT Enzyme Linked Immunosorbent Assay (ELISA) kit (Roche) with some minor alterations. Cultures were grown to mid exponential phase as described in Section 2.14.1 and cells pelleted by centrifuging at 3500*g* for 5 minutes at 4°C. The cell pellet was washed twice with cold PBS, once with 1ml of TEN buffer (Appendix) and finally resuspended in 150µl of cold 250mM Tris-HCl pH7.8.

Cells were lysed by repeated freezing in dry ice-ethanol and thawing at 37°C for 5 minutes each. The suspension was centrifuged immediately at 12000*g* for 10 minutes at 4°C. The supernatant was snap frozen in 50µl aliquots using a dry ice-ethanol bath and stored at -70°C. Different aliquots were used to measure total protein concentrations (Section 2.15.4) and CAT levels (Section 2.15.5).

Culture samples subjected to heat shock treatment were transferred to 42°C for various time intervals prior to centrifugation and lysis.

### 2.15.4 Determination of the total protein concentration in cell extracts

The protein concentration (µg/ml) of cell extracts was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard. The OD was measured at 750nm on a Perkin Elmer 552 double-beam spectrophotometer.

### 2.15.5 Quantitative determination of CAT protein in cell extracts

The level of total CAT (pg/µg protein) in cell extracts was determined using the CAT ELISA kit (Roche). The protocol was adhered to with the exception of using various concentrations of total protein, ranging from 10ng to 1µg. All samples were measured in duplicate and substrate reactions were performed using the substrate ABTS® (Roche) without substrate enhancer. The absorbance of the samples was measured at 405nm on a Microplate Reader, Model 450 (Bio-Rad).

## 2.16 Protein analysis

### 2.16.1 Preparation of *E. coli* and *P. aeruginosa* cell extracts

Overnight cultures of *E. coli* and *P. aeruginosa* were used to prepare whole cell extracts. 200µl of each culture was centrifuged at 13000*g* for 3 minutes and the cell pellet resuspended in 80µl of 2 x SDS sample buffer (Appendix). Following a 5 minute boiling step, samples were centrifuged at 13000*g* for 1 minute and electrophoresed as described below.

### 2.16.2 Preparation of *N. gonorrhoeae* cell extracts

Whole cell gonococcal extracts used in the Western blot with the anti-RpoN primary antibody (Figure 3.11; Klimpel *et al.*, 1989) were obtained as follows. A quarter of the growth of an inoculated plate was resuspended in 80µl of 2 x SDS sample buffer (Appendix) and boiled for 5 minutes. Following a 1 minute centrifugation samples were electrophoresed as described below.

Whole cell gonococcal extracts used in the Western blot with the anti-RpoH primary antibody (Figure 5.12; donated by Bernd Bukau) were prepared as described in Section 2.15.3 with the following modification. Following centrifugation the cell pellet was immediately resuspended in 150µl of PBS and the cells lysed by repeated freezing and thawing.

### 2.16.3 SDS-polyacrylamide gel electrophoresis (PAGE)

Protein samples were electrophoresed in Laemmli (1970) SDS polyacrylamide gels consisting of 12% (w/v) resolving gels and 4% (w/v) stacking gels. Gels were poured between 7cm x 12cm and 9cm x 12cm glass plates with 1.5mm spacers.

The 12% resolving gel consisted of 2ml of resolving gel buffer (Appendix), 2.8ml of distilled water, 3.2ml of 30% acrylamide (Appendix), 30µl of 10% (w/v) ammonium persulfate and 8.75µl of TEMED. The gel was poured between the plates, secured in a casting stand (Bio-Rad) and overlayed with distilled water. Once the gel had polymerised, the water was drained and the 4% stacking gel poured. The stacking gel consisted of 625µl of stacking gel buffer (Appendix), 1.48ml of distilled water, 400µl of 30% acrylamide (Appendix), 15µl of 10% (w/v) ammonium persulfate and 2.5µl of TEMED. The comb was inserted and the gel allowed to polymerise.

The gel was assembled in a Mini Protean II™ cell (Bio-Rad). Protein samples and SeeBlue™ PreStained Standards (NOVEX™) used as molecular size markers were boiled, centrifuged (Section 2.16.1) and loaded. Electrophoresis was performed in freshly prepared Tris-glycine buffer (Appendix) at 200V for 45 minutes. The gel was then used for Western blotting (Section 2.16.4).

### 2.16.4 Western Blotting

Western blotting was performed as described by Sambrook *et al.* (1989). Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) using a Bio-Rad transblotter. Electrophoresis was performed in transblotting buffer (Appendix) at 100V for 1 hour. The membrane was then soaked overnight at 4°C in TBS-Tween (Appendix) containing 5% (w/v) skim milk powder. Western blots were detected using the ECL™ Detection Kit (Amersham Pharmacia Biotech) following the protocol supplied. The monoclonal anti-RpoN primary antibody (Klimpel *et al.*, 1989) was used in conjunction with the Peroxidase Conjugated Anti-Mouse Immunoglobulin (Silenus) secondary antibody. The polyclonal anti-RpoH primary antibody (donated by Bernd Bukau) was used in combination with the Peroxidase Conjugated Sheep Anti-Rabbit Immunoglobulin (Silenus) secondary antibody.

## 2.17 Gel shift assays

Gel shift assays were performed using the DIG Gel Shift Kit (Roche) according to the manufacturer's instructions. Target DNA fragments were amplified by PCR (Section 2.9.1), purified with the Geneclean II Kit® (Section 2.5.4) and labelled with DIG-11-ddUTP at the 3' termini. 15fmol of labelled DNA was used per 20µl reaction.

# CHAPTER THREE

## THE PATHOGENIC NEISSERIA, *NEISSERIA GONORRHOEAE* AND *NEISSERIA MENINGITIDIS*, CONTAIN AN INACTIVE *rpoN* GENE AND DO NOT UTILISE THE *pilE* $\sigma^{54}$ PROMOTER

### 3.1 Introduction

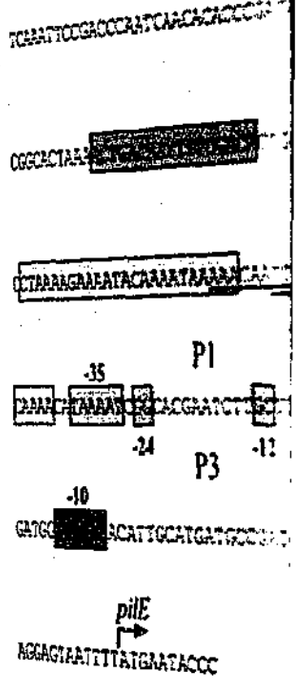
The $\sigma^{54}$ sigma factor, or RpoN, is required for transcription from $\sigma^{54}$ dependent promoters and has been identified in a number of phylogenetically distant bacteria ranging from proteobacteria (Merrick, 1993) to Gram positive bacteria (Gardan *et al.*, 1997; Studholme and Buck, 2000), and also in specialised pathogens (Studholme and Buck, 2000). In addition to $\sigma^{54}$, transcription initiation from these promoters requires an activator protein which usually binds to an UAS situated about 100bp upstream of the promoter (Reitzer and Magasanik, 1986).

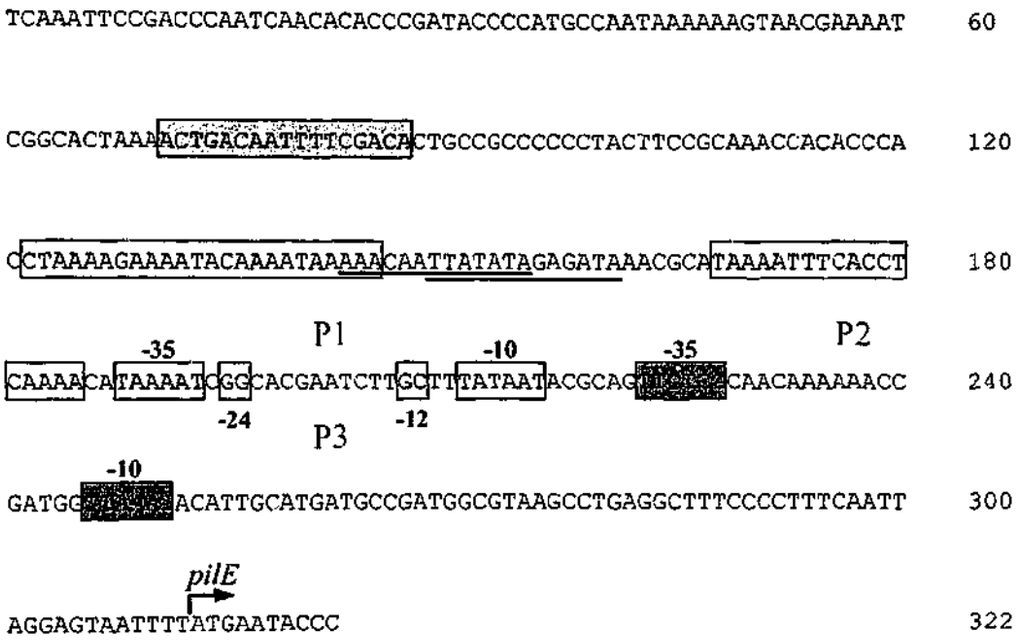
Based on comparisons with the deduced amino acid sequences from 17 *rpoN* genes, three distinct regions of the protein have been identified (Merrick, 1993). Region III contains the primary DNA binding regions including a DNA cross-linking region (Cannon *et al.*, 1994), the HTH motif (Merrick and Chambers, 1992) and the RpoN box (Taylor *et al.*, 1996). The latter two motifs have been implicated in promoter recognition (Merrick and Chambers, 1992; Taylor *et al.*, 1996).

Sequence analysis of the region upstream of the *pilE* gene from *N. gonorrhoeae* MS11-A (Meyer *et al.*, 1984) together with transcriptional analysis have identified several potential promoters (Figure 3.1). These include two overlapping consensus promoter sequences, one for a $\sigma^{70}$ promoter (P1), and the other for a $\sigma^{54}$ promoter (P3; Fyfe *et al.*, 1995; Meyer *et al.*, 1984; Thony and Hennecke, 1989). An additional $\sigma^{70}$ promoter (P2) is located downstream of these two promoters (Fyfe *et al.*, 1995). Fyfe *et al.* (1993) and Carrick *et al.* (1997) have also described an UAS located about 100bp upstream of P3 that shares similarity to the binding site of the PilR activator protein of *P. aeruginosa* (Pasloske *et al.*, 1989). This region is essential for maximal transcription from P3 in a *P. aeruginosa* background (see below) indicating it functions as a binding site for PilR (Carrick *et al.*, 1997). Sequence analysis of the corresponding region from the *N.*

**Figure 3.1 Nucleotide sequence of the region upstream of the *pilE* gene from *N. gonorrhoeae* MS11-A (Fyfe *et al.*, 1995).**

The $\sigma^{70}$ promoters, P1 and P2, are indicated (Fyfe *et al.*, 1995; Meyer *et al.*, 1984) as is the $\sigma^{54}$ promoter, P3 (Meyer *et al.*, 1984). The putative UP elements are boxed in yellow (Fyfe and Davies, 1998) and the boxed region in orange represents the UAS (Fyfe *et al.*, 1995). The overlapping, underlined sequences represent the IHF binding sites (Hill *et al.*, 1997). Nucleotides are numbered at the right of the figure.




TCAAATTCCGACCCAATCAACACACCCGATACCCCATGCCAATAAAAAAGTAACGAAAAT 60
CGGCACTAAAACTGACAATTTTCGACACTGCCGCCCCCCCTACTTCCGCAAACCACACCCA 120
CCTAAAAGAAAATACAAAATAAAAACAATTATATAGAGATAAACGCATAAAATTTCACCT 180
P1
P2
-35
-10
-35
CAAAACATAAAATCGGCACGAATCTTGCTTTATAATACGCAGCAACAAAAAACC 240
-24
-12
P3
-10
GATGCACATTGCATGATGCCGATGGCGTAAGCCTGAGGCTTTCCCCTTTCAATT 300
pilE
AGGAGTAATTTTATGAATACCC 322

*meningitidis* MC58¢2 *pil*E gene revealed that the P1, P3 and UAS sequences were conserved (Carrick *et al.*, 1997).

P1 is required for transcription of *pilE* in *N. gonorrhoeae* under *in vitro* conditions (Fyfe *et al.*, 1995). Transcription from P3 can only be detected in a background that contains the relevant activator, such as in *E. coli* in the presence of the activator protein NifA (Boyle-Vavra *et al.*, 1993) and in *P. aeruginosa* with a functional PilR protein (Carrick *et al.*, 1997; Fyfe *et al.*, 1995). Site-directed mutagenesis of the -24 box had no effect on the expression of a P*pilE*::*cat* transcriptional fusion, when compared with a similar construct with all the promoters intact (Fyfe *et al.*, 1995). Similarly, a P*pilE*::*lacZ* transcriptional fusion with both the $\sigma^{70}$ promoters (P1 and P2) mutated and only the $\sigma^{54}$ promoter intact, had no detectable transcriptional activity in *N. gonorrhoeae* (Laskos *et al.*, 1998). These observations suggested that under the *in vitro* growth conditions tested, the putative gonococcal RpoN was either unable to bind to the promoter or was not produced. Alternatively, different environmental conditions or *in vivo* growth may be required for RpoN expression.

In addition to the identification of a putative $\sigma^{54}$ binding site upstream of *pil*E, further evidence suggested that *N. gonorrhoeae* contained an *rpoN* gene. Consensus sequences for $\sigma^{54}$ dependent promoters have been identified upstream of a number of gonococcal genes including *pilC* (Taha *et al.*, 1996), *comA* (Facius and Meyer, 1993) and *pip* (Albertson and Koomey, 1993). Klimpel *et al.* (1989) also identified a 90 kDa gonococcal protein which co-purified with RNA polymerase and reacted with a monoclonal antibody raised against the *Salmonella typhimurium* $\sigma^{54}$. Therefore it was postulated that *N. gonorrhoeae* contained an *rpoN* gene and the focus of this chapter was to identify such a gene. Several other *Neisseria* spp. were investigated to determine whether this gene was widespread within the species.

## 3.2 Failure to identify an *rpoN* homologue by hybridisation or complementation

Initially, attempts were made to identify a *N. gonorrhoeae rpoN* homologue from strain MS11-A using a range of standard techniques. MS11-A genomic DNA was digested with a variety of restriction endonucleases and probed with the *rpoN* genes from *E. coli*, *P. aeruginosa* and *Acinetobacter calcoaceticus*, in Southern hybridisations at low

stringency. These bacteria are classified as proteobacteria and subsequently belong to the same evolutionary division as *N. gonorrhoeae*. Therefore it was assumed they would display the highest similarity to the putative gonococcal *rpoN* gene. No hybridising fragments were identified using any of these probes (data not shown). In addition, a gonococcal partial *Sau*3A genomic library was constructed using the *E. coli-P. aeruginosa* shuttle vector pUCP18 and screened in a *P. aeruginosa rpoN* mutant (PAK-N1). The ability to grow in the absence of glutamine was selected for, as glutamine auxotrophy is associated with the *rpoN* mutation (Totten *et al.*, 1990). This approach was also unsuccessful, as no clones capable of complementing this aspect of the *P. aeruginosa rpoN* mutation were obtained. A separate library of *N. gonorrhoeae* chromosomal DNA, partially digested with *Sau*3A and ligated into the vector pUC18, was used to transform an *E. coli rpoN* mutant (YMC18). Transformants were selected for growth in the absence of glutamine. Several colonies were obtained and preliminary sequencing with oligonucleotide primers, universal primer (UP) and reverse primer (RP; data not shown) led to the identification of the gonococcal glutamine synthetase gene, *glnA* (GenBank Accession Number M84113), required for glutamine biosynthesis, but did not identify an *rpoN* homologue.

### 3.3 Identification of *RLS* from *N. gonorrhoeae* strain FA1090

Following the unsuccessful attempts to isolate the putative gonococcal *rpoN* homologue from *N. gonorrhoeae* MS11-A, genomic sequence data from *N. gonorrhoeae* strain FA1090 was released on the University of Oklahoma *N. gonorrhoeae* Genome Database (Genbank accession number AE004969). Despite the preliminary and incomplete sequence data initially provided, BLAST analysis of this sequence revealed a region, which, when translated, displayed significant similarity (32% identity, excluding deleted regions) to the *E. coli* $\sigma^{54}$ amino acid sequence (Jones *et al.*, 1994). In particular, a nucleotide sequence potentially encoding the 10 amino acid RpoN box, close to the C-terminal end of the protein was identified (Figure 3.2). This motif plays a role in recognition of the -24 promoter element (Taylor *et al.*, 1996; Wang and Gralla, 2001) and is believed to be diagnostic of RpoN proteins, as database searches have failed to identify it in any other proteins (Merrick, 1993). Translation of the sequence further upstream revealed similarity to the N-terminal region of *E. coli* $\sigma^{54}$ (data not shown). However, this sequence was in a different reading frame, suggesting that the ORF might

**Figure 3.2 A translated region of the *N. gonorrhoeae* (Ng) strain FA1090 genomic sequence (Genbank accession number AE004969) that displayed significant similarity to the carboxy terminus of the RpoN protein from *E. coli* (Ec; Jones *et al.*, 1994) as observed by BLAST analysis.**

Amino acids in red text and the asterisk (*) represent identical residues. Conserved amino acids are depicted by green text and the colon (:). Amino acids with less similarity are indicated by blue text and the dot (.). The highly conserved RpoN box (Merrick, 1993) is boxed.

```
Ng      PKSYSDEALANLLAFRGIEVSRRTVAKYRESLEIPAAHKRKTAE
Ec      AKPLSDSKLTSLLSEQGIMVARRTVAKYRESLSIPPSNQRKQLV
        .*. **. *:.**: :** *.**********.**.:::**
```

not be capable of encoding an intact *rpoN* homologue. Consequently, this 914bp region was designated the *rpoN*-like sequence (*RLS*). To determine whether the apparent reading frameshift was potentially due to an error in the FA1090 sequence deposited in the database, it was decided to clone and characterise the corresponding region from the genome of strain MS11-A.

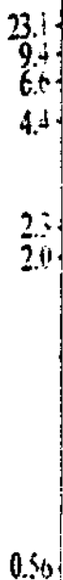
### 3.4 Cloning and nucleotide sequence analysis of *RLS* from *N. gonorrhoeae* strain MS11-A

*RLS* was isolated from strain MS11-A by screening of a genomic library. To obtain an *RLS*-specific probe, oligonucleotide primers 4525 and 4878 were designed, based on the *RLS* sequence of strain FA1090. These were used to amplify a 506bp fragment within *RLS*, representing the more highly conserved 3' end, from genomic DNA of strain MS11-A by PCR. The nucleotide sequence of this fragment was verified by direct sequencing of the product. The fragment was then used to probe genomic DNA from MS11-A completely digested with a variety of restriction endonucleases in a Southern hybridisation. The *RLS*-specific probe hybridised strongly to a single 5.5kb *Hin*cII fragment under high stringency (Figure 3.3). Hybridising fragments resulting from digests with other restriction enzymes were of a much larger size and would have proved more difficult to clone (data not shown). Genomic DNA from *E. coli* strain DH5α digested with *Cla*I-*Sal*I was used as a control and no hybridising fragments were obtained (Figure 3.3).

The 5.5kb hybridising fragment from MS11-A was subsequently cloned and identified from a size-fractionated library by digesting genomic DNA with *Hin*cII and ligating fragments of 4.0-7.0kb into the vector pSU2718 digested with *Hin*cII. The *RLS*-specific probe was used to screen the library by colony hybridisation using high stringency conditions. The clone retained for further study was designated pJKD2026. Restriction endonuclease analysis, combined with preliminary sequencing of the 5.5kb insert, succeeded in locating the *RLS* as shown in Figure 3.4. The nucleotide sequence was obtained for both strands of a 1.8kb region within the insert of pJKD2026 using a series of oligonucleotide primers (Figure 3.5). The MS11-A-derived *RLS* sequence was 914bp in length and highly similar to the sequence from FA1090, with only one base substitution noted (Figure 3.5). The first ATG codon of the ORF was assumed to be the

**Figure 3.3 Southern hybridisation analysis to confirm the location of *RLS* in *N. gonorrhoeae* strain MS11-A.**

Total genomic DNA from *N. gonorrhoeae* strain MS11-A and *E. coli* strain DH5α was digested with *Hinc*II (lane 2) and *ClaI*/*Sal*I (lane 3), respectively, and probed with an internal region of *RLS* amplified with oligonucleotide primers 4525 and 4878 (Figure 3.5). The hybridising fragment is indicated by the arrow. Lambda DNA digested with *Hind*III and labelled with DIG served as the size standards (kb; lane 1).



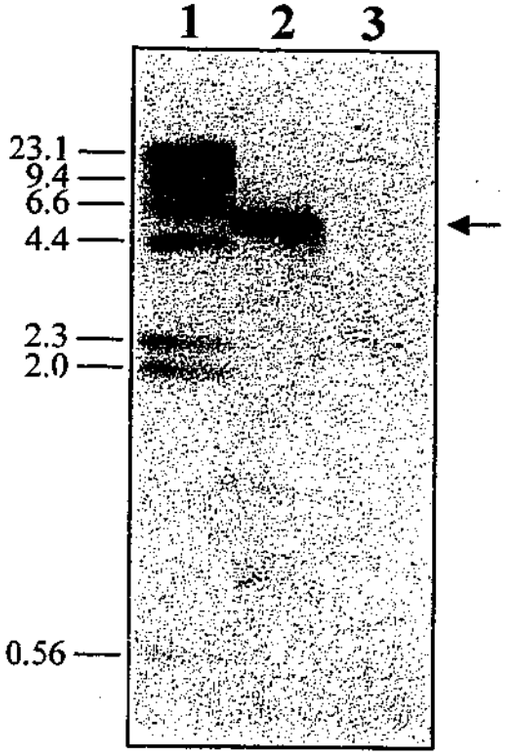

1
2
3
23.1
9.4
6.6
4.4
2.3
2.0
0.56

**Figure 3.4 Strategy for the sequencing of *RLS* from *N. gonorrhoeae* MS11-A.**

Physical map of the 5.5kb insert of pJKD2026 showing the position and orientation of *RLS* and the flanking *kat* (Johnson *et al.*, 1996) and *pglA* (Jennings *et al.*, 1998) genes. Subclones created to determine the complete sequence of *RLS* and adjacent regions are indicated on the right and the extent of cloned sequence is represented by pink horizontal lines. Arrows indicate the direction and extent of sequence determined from each clone using oligonucleotide primers. The dashed horizontal line represents vector sequence. The position of relevant restriction endonuclease sites is also shown. The location of the oligonucleotide primer 4941 is depicted by the yellow arrow. Abbreviations: HII, *Hin*cII; E, *Eco*RI; X, *Xba*I.

1 kb

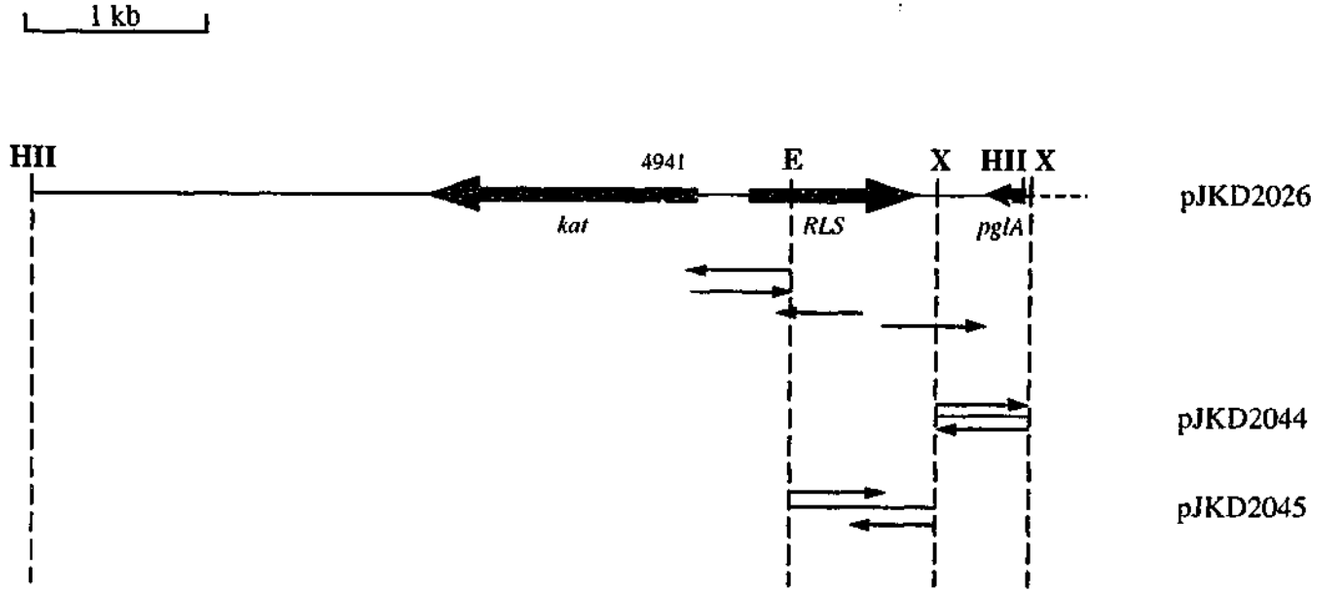
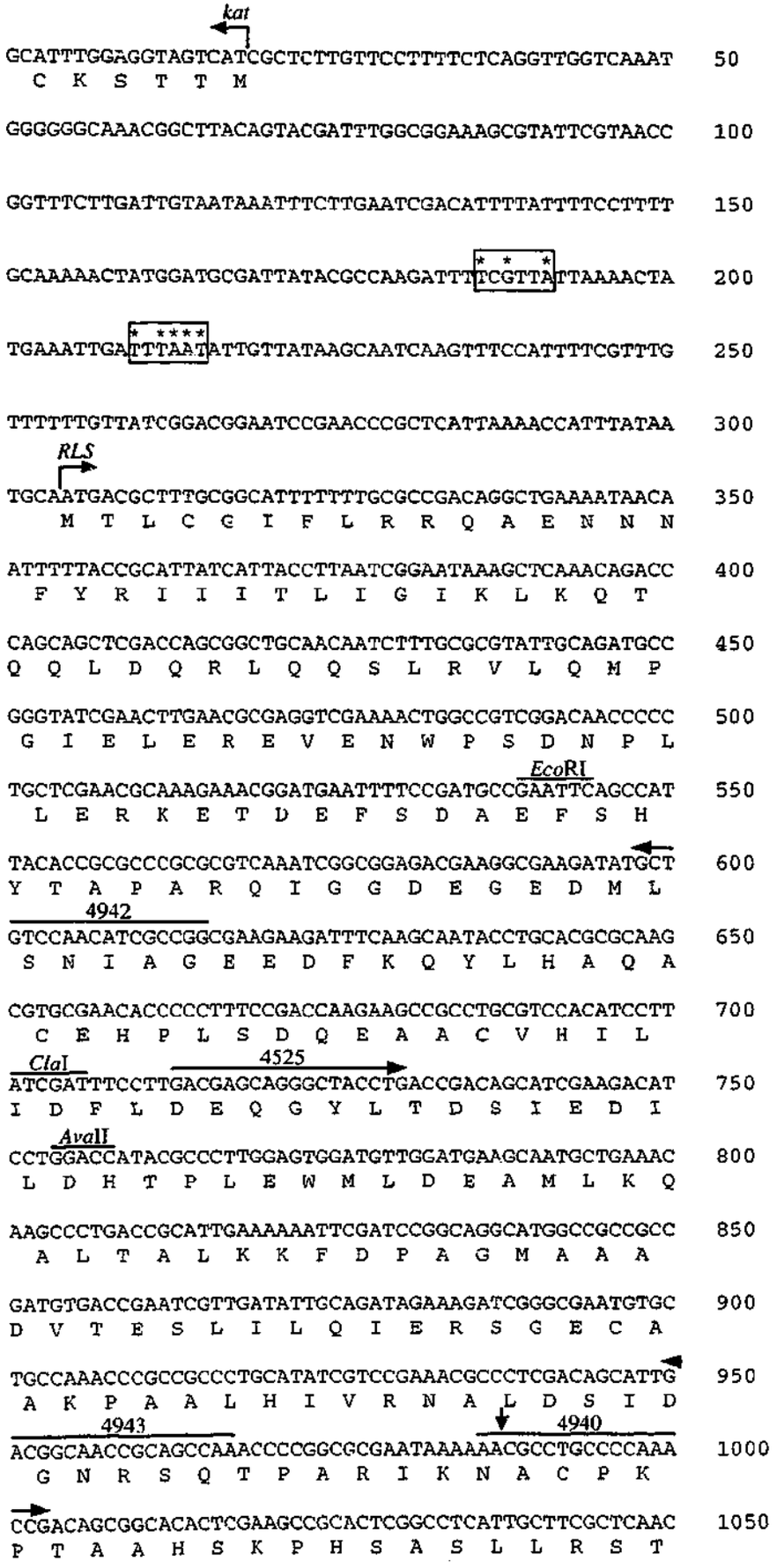
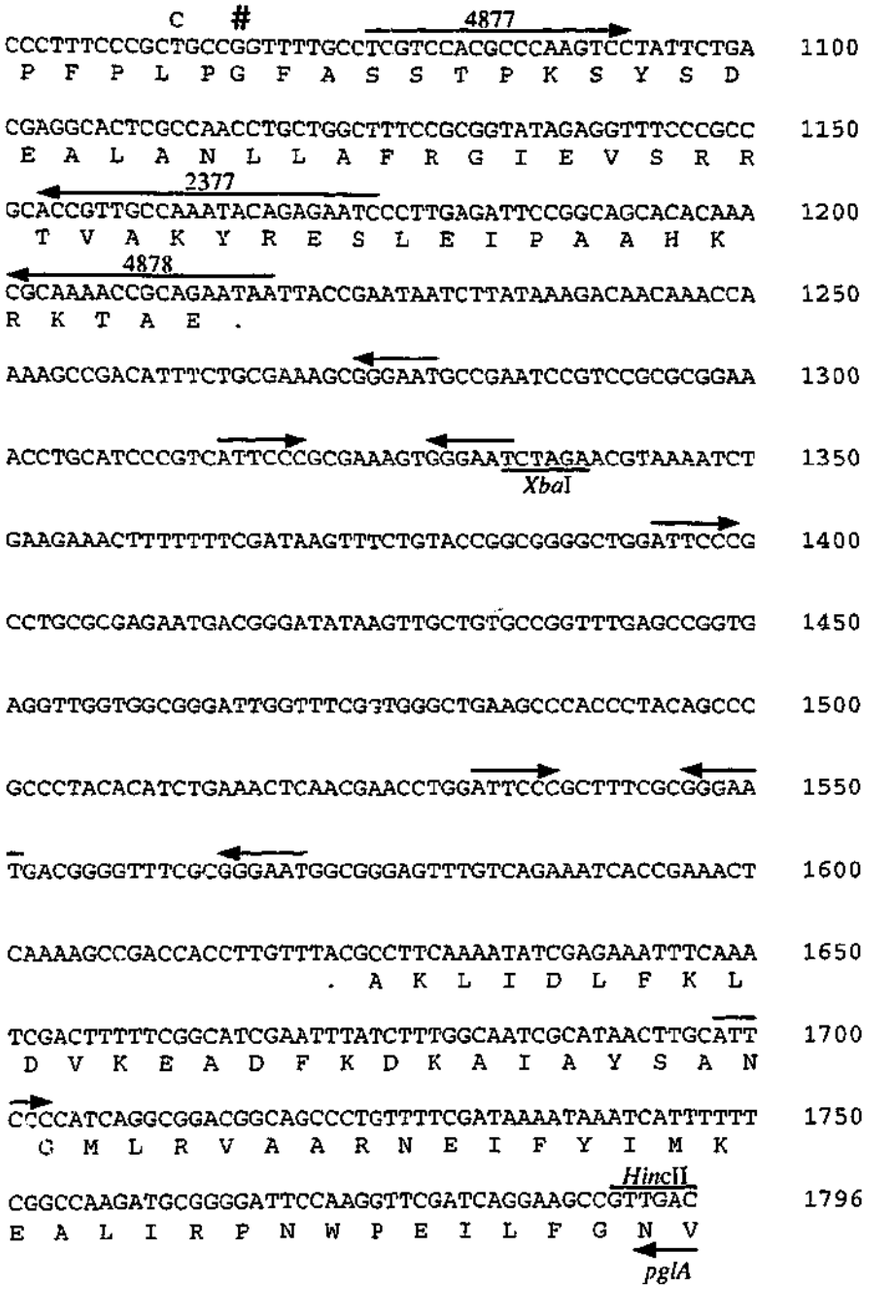

1 kb
HII
4941
E
X
HII X
kat
RLS
pglA
pJKD2026
pJKD2044
pJKD2045

**Figure 3.5 Nucleotide sequence of a 1.8kb region within the insert of pJKD2026 containing *RLS*.**

The deduced amino acid sequences of *RLS*, amino-terminal region of Kat (Johnson *et al.*, 1996) and carboxy-terminal region of PglA (Jennings *et al.*, 1998) are shown below the corresponding nucleotide sequence. The blue hash (#) denotes the position of the translational frame change of the *RLS* coding sequence and the deduced amino acid sequence shown from this point represents that in frame 1 (Section 3.4). Stop codons are indicated by a dot (.). The red coloured nucleotide located above the *RLS* sequence represents the bp substitution found in the corresponding region of strain FA1090. Arrows located in the *RLS-pglA* intergenic region denote core RS3 repeats (Haas and Meyer, 1986). Oligonucleotide primers used are depicted by numbered arrows above the nucleotide sequence. Restriction endonuclease sites are shown above or below the nucleotide sequence. The putative $\sigma^{70}$ promoter comprising the -10 and -35 regions is boxed and asterisks (*) indicate the nucleotides identical to the consensus sequence (Hawley and McClure, 1983). The downward pointing arrow represents the location of the additional adenine nucleotide in the corresponding region from *N. lactamica* (Section 3.8). Nucleotides are numbered at the right of the figure.


kat
GCATTTGGAGGTAGTCATCGCTCTTGTTCCTTTTCTCAGGTTGGTCAAAT 50
C K S T T M
GGGGGGCAAACGGCTTACAGTACGATTTGGCGGAAAGCGTATTCGTAACC 100
GGTTTCTTGATTGTAATAAATTTCTTGAATCGACATTTTATTTTCCTTTT 150
GCAAAAACTATGGATGCGATTATACGCCAAGATTTTCGTTATTAAAACTA 200
TGAAATTGATTTAATATTGTTATAAGCAATCAAGTTTCCATTTTCGTTTG 250
TTTTTTGTTATCGGACGGAATCCGAACCCGCTCATTAAAACCATTTATAA 300
RLS
TGCAATGACGCTTTGCGGCATTTTTTTGCGCCGACAGGCTGAAAATAACA 350
M T L C G I F L R R Q A E N N N
ATTTTTACCGCATTATCATTACCTTAATCGGAATAAAGCTCAAACAGACC 400
F Y R I I I T L I G I K L K Q T
CAGCAGCTCGACCAGCGGCTGCAACAATCTTTGCGCGTATTGCAGATGCC 450
Q Q L D Q R L Q Q S L R V L Q M P
GGGTATCGAACTTGAACGCGAGGTCGAAAACTGGCCGTCGGACAACCCCC 500
G I E L E R E V E N W P S D N P L
EcoRI
TGCTCGAACGCAAAGAAACGGATGAATTTTCCGATGCCGAATTCAGCCAT 550
L E R K E T D E F S D A E F S H
TACACCGCGCCCGCGCGTCAAATCGGCGGAGACGAAGGCGAAGATATGCT 600
Y T A P A R Q I G G D E G E D M L
4942
GTCCAACATCGCCGGCGAAGAAGATTTCAAGCAATACCTGCACGCGCAAG 650
S N I A G E E D F K Q Y L H A Q A
CGTGCGAACACCCCCTTTCCGACCAAGAAGCCGCCTGCGTCCACATCCTT 700
C E H P L S D Q E A A C V H I L
ClaI
4525
ATCGATTTCCTTGACGAGCAGGGCTACCTGACCGACAGCATCGAAGACAT 750
I D F L D E Q G Y L T D S I E D I
AvaII
CCTGGACCATACGCCCTTGGAGTGGATGTTGGATGAAGCAATGCTGAAAC 800
L D H T P L E W M L D E A M L K Q
AAGCCCTGACCGCATTGAAAAAATTCGATCCGGCAGGCATGGCCGCCGCC 850
A L T A L K K F D P A G M A A A
GATGTGACCGAATCGTTGATATTGCAGATAGAAAGATCGGGCGAATGTGC 900
D V T E S L I L Q I E R S G E C A
TGCCAAACCCGCCGCCCTGCATATCGTCCGAAACGCCCTCGACAGCATTG 950
A K P A A L H I V R N A L D S I D
4943
4940
ACGGCAACCGCAGCCAAACCCCGGCGCGAATAAAAAACGCCTGCCCCAAA 1000
G N R S Q T P A R I K N A C P K
CCGACAGCGGCACACTCGAAGCCGCACTCGGCCTCATTGCTTCGCTCAAC 1050
P T A A H S K P H S A S L L R S T

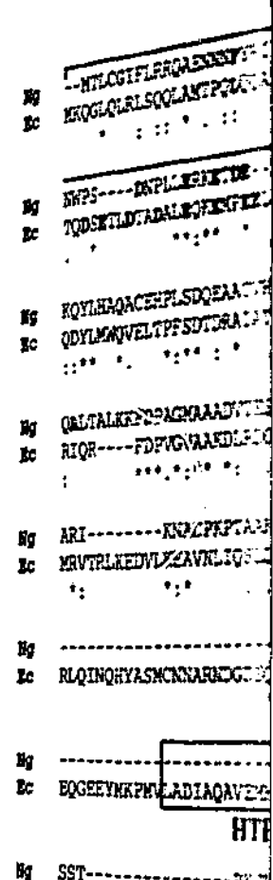

C #
4877
CCCTTTCCCGCTGCCGGTTTTGCCTCGTCCACGCCCAAGTCCTATTCTGA 1100
P F P L P G F A S S T P K S Y S D
CGAGGCACTCGCCAACCTGCTGGCTTTCCGCGGTATAGAGGTTTCCCGCC 1150
E A L A N L L A F R G I E V S R R
2377
GCACCGTTGCCAAATACAGAGAATCCCTTGAGATTCCGGCAGCACACAAA 1200
T V A K Y R E S L E I P A A H K
4878
CGCAAAACCGCAGAATAATTACCGAATAATCTTATAAAGACAACAAACCA 1250
R K T A E .
AAAGCCGACATTTCTGCGAAAGCGGGAATGCCGAATCCGTCCGCGCGGAA 1300
ACCTGCATCCCGTCATTCCCGCGAAAGTGGGAATCTAGAACGTAAAATCT 1350
XbaI
GAAGAAACTTTTTTTCGATAAGTTTCTGTACCGGCGGGGCTGGATTCCCG 1400
CCTGCGCGAGAATGACGGGATATAAGTTGCTGTGCCGGTTTGAGCCGGTG 1450
AGGTTGGTGGCGGGATTGGTTTCGTTGGGCTGAAGCCCACCCTACAGCCC 1500
GCCCTACACATCTGAAACTCAACGAACCTGGATTCCCGCTTTCGCGGGAA 1550
TGACGGGGTTTCGCGGGAATGGCGGGAGTTTGTCAGAAATCACCGAAACT 1600
CAAAAGCCGACCACCTTGTTTACGCCTTCAAAATATCGAGAAATTTCAAA 1650
. A K L I D L F K L
TCGACTTTTTCGGCATCGAATTTATCTTTGGCAATCGCATAACTTGCATT 1700
D V K E A D F K D K A I A Y S A N
CCCCATCAGGCGGACGGCAGCCCTGTTTTCGATAAAATAAATCATTTTTT 1750
G M L R V A A R N E I F Y I M K
HincII
CGGCCAAGATGCGGGGATTCCAAGGTTCGATCAGGAAGCCGTTGAC 1796
E A L I R P N W P E I L F G N V
pglA

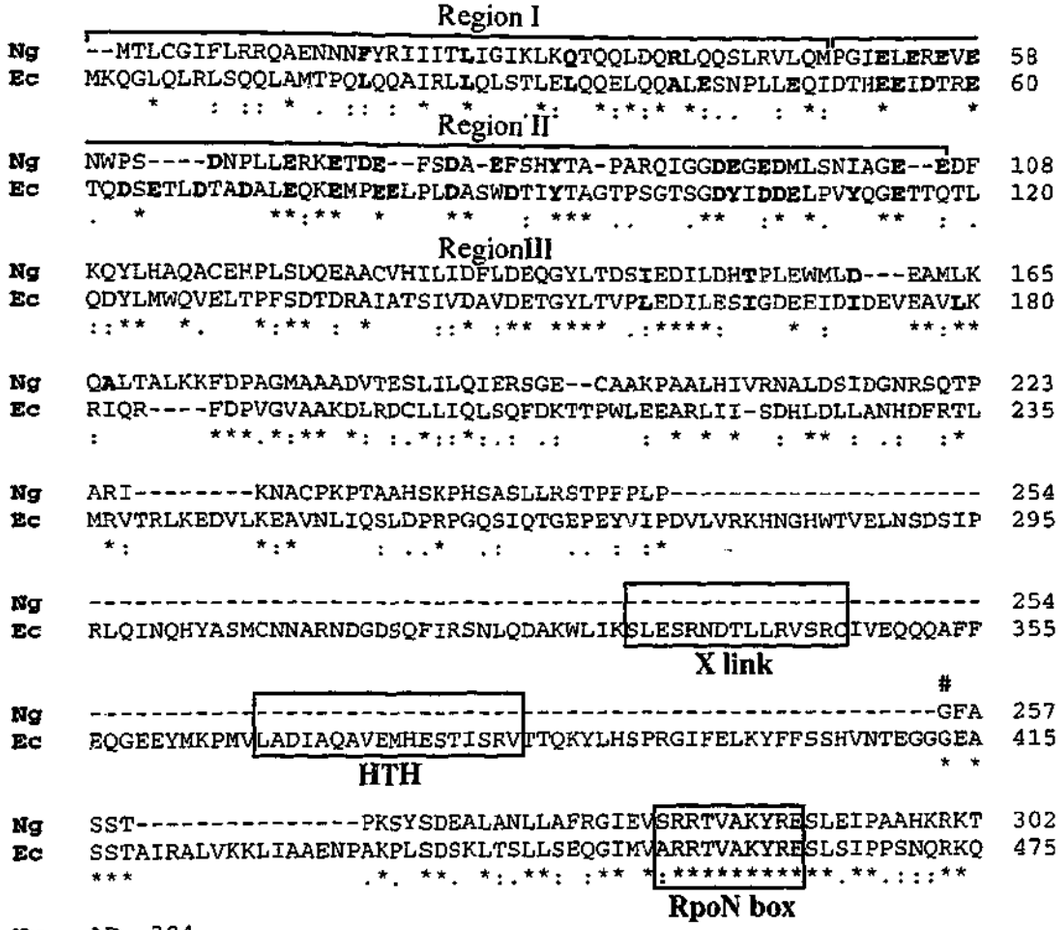
start codon. No nucleotide sequence corresponding to a RBS (Shine and Dalgarno, 1975) was found upstream of the putative ATG start codon. In addition, no consensus sequence depicting a Rho-independent transcriptional terminator (d'Aubenton Carafa *et al.*, 1990) was found in the region immediately downstream of the *RLS* stop codon. A putative $\sigma^{70}$ promoter (Hawley and McClure, 1983) was identified 89bp upstream of the putative start codon of *RLS* (Figure 3.5). The suggested -10 sequence, TTTAAT, shares five out of six bases with the consensus sequence TATAAT (Hawley and McClure, 1983). It is preceded at a distance of 18bp by a potential -35 region, TCGTTA, with three of six bases identical to the consensus sequence TTGACA (Hawley and McClure, 1983).

The 1.8kb sequence from MS11-A contained no single ORF, in any of the three reading frames, capable of encoding an intact *rpoN* gene. A similarity was observed between the deduced amino acid sequence of *RLS* translated in frame 2, and the *E. coli* RpoN protein, within Region I, Region II, and the N-terminal portion of Region III (Figure 3.6). It seems to have retained the glutamine rich amino terminus but not the hydrophobic heptad repeats found in Region I and Region III. The former two features are responsible for inhibition of polymerase isomerisation in the absence of activator (Cannon *et al.*, 1999; Wang *et al.*, 1995), enhancer responsiveness (Syed and Gralla, 1998) and DNA binding (Gallegos and Buck, 2000). The repeat in Region III is required for binding and recruiting of the core RNA polymerase to the promoter (Hsieh *et al.*, 1999; Tintut *et al.*, 1994). In addition, the acidic nature of Region II has been conserved (31% acidic residues compared to 32% in *E. coli*) and is thought to be involved in formation of the open complex prior to isomerisation of the holoenzyme (Southern and Merrick, 2000; Wong and Gralla, 1992). A region encoding the RpoN box (Merrick, 1993; Taylor *et al.*, 1996) was identified, as was observed for the FA1090 sequence, but this region was present in a different reading frame (frame 1). No sequence encoding the essential HTH motif (Merrick and Chambers, 1992) or DNA binding region (Cannon *et al.*, 1994; Cannon *et al.*, 1995) was identified within *RLS*. Overall, the translated amino acid sequence of *RLS* displayed 33% identity to the *E. coli* $\sigma^{54}$ sequence (excluding the deleted region). Based on this comparison it appears that an approximately 414bp deletion encompassing the region of the gene that encodes essential DNA binding motifs occurred in the ancestral *rpoN* homologue. This resulted in a frameshift mutation rendering the gene incapable of encoding a functional RpoN protein.

**Figure 3.6 Comparison between the derived amino acid sequences of *RLS* from *N. gonorrhoeae* (Ng) and *rpoN* from *E. coli* (Ec; Jones *et al.*, 1994).**

The amino acids are numbered at the right of the figure according to their position in the individual protein sequences. The alignment was performed with the aid of the CLUSTAL W program (Thompson *et al.*, 1994) and dashes (-) indicate gaps that have been introduced to maximise alignment. Identical amino acids are indicated by an asterisk (*), conserved amino acids are depicted by a colon (:) and residues with less similarity are represented by a dot (.). The hash (#) denotes the position of the translational frame change of *RLS*. The three regions of RpoN identified by Merrick (1993) are indicated. Regions I and II are overlined and the remainder of the sequence constitutes Region III. Amino acids in red text represent residues that comprise the hydrophobic heptad repeats located in Regions I and III (Tintut *et al.*, 1994; Wang *et al.*, 1995). Amino acids in green text indicate acidic residues found predominantly in Region II. The DNA cross-linking region (Cannon *et al.*, 1994), HTH motif (Merrick and Chambers, 1992) and RpoN box (Merrick, 1993) are indicated.




Region I
Region II
RegionIII
X link
HTH
RpoN box
Ng --MTLCGIFLRRQAENNNFYRIIITLIGIKLKQTQQLDQRLQQSLRVLQMPGIELEREVE 58
Ec MKQGLQLRLSQQLAMTPQLQQAIRLLQLSTLELQQELQQALESNPLLEQIDTHEEIDTRE 60
Ng NWPS----DNPLLERKETDE--FSDA-EFSHYTA-PARQIGGDEGEDMLSNIAGE--EDF 108
Ec TQDSETLDTADALEQKEMPEELPLDASWDTIYTAGTPSGTSGDYIDDELPVYQGETTQTL 120
Ng KQYLHAQACEHPLSDQEAACVHILIDFLDEQGYLTDSIEDILDHTPLEWMLD---EAMLK 165
Ec QDYLMWQVELTPFSDTDRAIATSIVDAVDETGYLTVPLEDILESIGDEEIDIDEVEAVLK 180
Ng QALTALKKFDPAGMAAADVTESLILQIERSGE--CAAKPAALHIVRNALDSIDGNRSQTP 223
Ec RIQR----FDPVGVAAKDLRDCLLIQLSQFDKTTPWLEEARLII-SDHLDLLANHDFRTL 235
Ng ARI--------KNACPKPTAAHSKPHSASLLRSTPFPLP---------- 254
Ec MRVTRLKEDVLKEAVNLIQSLDPRPGQSIQTGEPEYVIPDVLVRKHNGHWTVELNSDSIP 295
Ng ---------- 254
Ec RLQINQHYASMCNNARNDGDSQFIRSNLQDAKWLIKSLESRNDTLLRVSRQIVEQQQAFF 355
Ng ----------GFA 257
Ec EQGEEYMKPMVLADIAQAVEMHESTISRVTTQKYLHSPRGIFELKYFFSSHVNTEGGGEA 415
Ng SST----------PKSYSDEALANLLAFRGIEVSRRTVAKYRESLEIPAAHKRKT 302
Ec SSTAIRALVKKLIAAENPAKPLSDSKLTSLLSEQGIMVARRTVAKYRESLSIPPSNQRKQ 475
Ng AE 304
Ec LV 477

The putative *RLS* start codon was located 286 nucleotides from the start of the divergently transcribed *kat* gene (Johnson *et al.*, 1996) that encodes the catalase protein. This enzyme is required for protection against damage by hydrogen peroxide (Hassett and Cohen, 1989) and some protection against phagocytosis (Bishai *et al.*, 1994; Zheng *et al.*, 1992). Downstream of *RLS* is a 270bp region containing seven copies of the direct and inverted repeats, previously designated RS3 (Haas and Meyer, 1986). These are a family of repeats that consist of a common 6bp core sequence of 5'-ATTCCC-3' or 5'-GGGAAT-3' respectively, and are denoted in the sequenced region (Figure 3.5). The core sequences may be part of either direct or inverted repeats. Directly downstream of the RS3 elements, the 3' end of an ORF was identified that shares a significant homology with the corresponding region of the *pglA* gene of *N. meningitidis* (Jennings *et al.*, 1998). This gene encodes a glycosyl transferase involved in the biosynthesis of the pilin-linked trisaccharide structure.

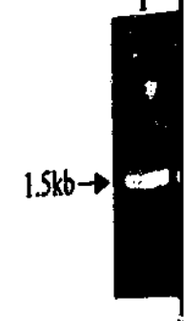
## 3.5 A comparison of *RLS* between pathogenic and non-pathogenic neisserial species

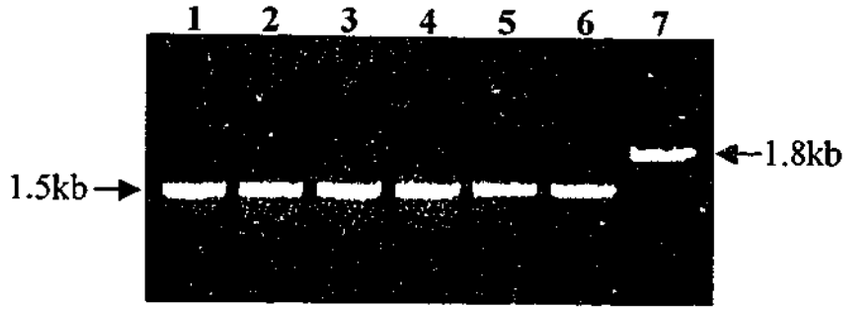
The genomes of *N. gonorrhoeae* strains FA1090 and MS11-A have been shown to have *RLS* containing a deletion of a section encoding an essential DNA cross-linking region and HTH motif. To determine whether this characteristic was shared by other gonococcal strains and neisserial species, PCR was performed. Genomic DNA was prepared from three isolates of *N. gonorrhoeae*, strains JKD328, JKD331 and JKD334, two previously described strains of *N. meningitidis*, strains MC58¢2 and Z5498, and the non-pathogenic *Neisseria subflava* JKD385 and *Neisseria lactamica* JKD386 (Table 2.1). DNA preparations were used as templates in PCR reactions using the oligonucleotide primers 4878 and 4941 (Table 2.3), the latter located within the gonococcal *kat* gene (Figure 3.4). The expected 1.5kb fragment was amplified from all the DNA samples tested, with the exception of DNA extracted from *N. lactamica* JKD386, which produced a larger fragment of approximately 1.8kb (Figure 3.7). This discrepancy in size will be further analysed in Section 3.8.

Direct sequencing of the 3' end of each fragment using oligonucleotide primer 4878 verified that the same apparent deletion was present in all the pathogenic strains tested and the non-pathogenic *N. subflava* (data not shown). In addition, this result indicates

**Figure 3.7 PCR analysis to determine the presence of *RLS* in various *Neisseria* spp.**

Total genomic DNA was isolated from *N. gonorrhoeae* strains JKD328 (lane 1), JKD331 (lane 2) and JKD334 (lane 3), *N. meningitidis* strains MC58¢2 (lane 4) and Z5498 (lane 5), *N. subflava* strain JKD385 (lane 6) and *N. lactamica* strain JKD386 (lane 7) and used as template in PCR with oligonucleotide primers 4878 and 4941 (Figures 3.4 and 3.5). The sizes of PCR products are indicated by arrows.




1
2
3
4
5
6
7
1.5kb
1.8kb

that the location of *RLS*, adjacent to *kat*, is conserved in *N. gonorrhoeae*, *N. meningitidis*, *N. subflava* and *N. lactamica*.

### 3.6 There is only a single copy of *RLS* in *N. gonorrhoeae* and *N. meningitidis*

To determine whether an intact copy of *rpoN* was present elsewhere on the chromosome Southern hybridisation using high stringency conditions was performed on genomic DNA from strain MS11-A and *N. meningitidis* strain MC58ϕ2 digested with a range of restriction endonucleases. A 150pb fragment containing the 3' end of *RLS* was used as the probe and obtained by amplification of this sequence using oligonucleotide primers 4877 and 4878 (Figure 3.5) from genomic DNA of strain MS11-A. Direct sequencing of this product confirmed the nucleotide sequence. As shown in Figure 3.8 single hybridising fragments were obtained for all the digests, suggesting that it is unlikely that a second, intact copy of *rpoN* is present in the genomes of either *N. gonorrhoeae* or *N. meningitidis*.

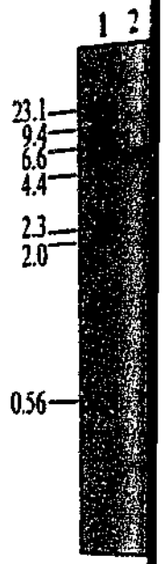
### 3.7 *RLS* is not associated with the 90kDa gonococcal protein that reacts with an anti-RpoN monoclonal antibody

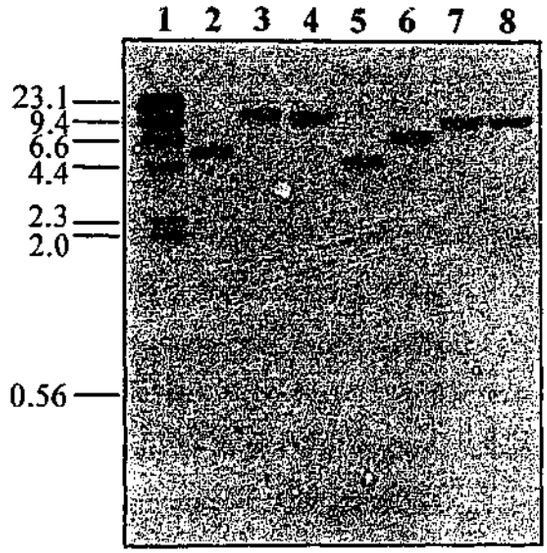
*N. gonorrhoeae* was originally thought to contain a $\sigma^{54}$ homologue because a monoclonal antibody raised against purified $\sigma^{54}$ protein from *S. typhimurium* appeared to react with a 90kDa gonococcal protein (Klimpel and Clark, 1989). This protein co-purified with RNA polymerase indicating that it was associated with transcription and was most likely $\sigma^{54}$. However, the largest ORF within *RLS*, potentially encoding the N-terminal portion of an RpoN-like protein, is only 834bp and would be expected to encode a polypeptide of approximately 30kDa (Figure 3.6). It was remotely possible that trimer formation of this 30kDa protein could be responsible for reacting with the antibody. To determine whether this ORF encodes the protein that reacts with the anti-RpoN monoclonal antibody, a gonococcal mutant derivative was constructed.

The 53bp *AvaII*/*ClaI* fragment internal to *RLS* from pJKD2026 (Figure 3.5) was replaced by a 1.1kb cassette containing the gonococcal *recA* promoter fused to a promoterless *cat* gene (Black *et al.*, 1995). The resulting construct, pJKD2035, was linearised by digestion with *PstI* and transformed into *N. gonorrhoeae* MS11-A (Figure

**Figure 3.8 Southern hybridisation analysis to verify only a single copy of *RLS* existed on the genomes of *N. gonorrhoeae* and *N. meningitidis*.**

Total genomic DNA from *N. gonorrhoeae* strain MS11-A was digested with *Hin*cII (lane 2), *Ava*I (lane 3), *Cla*I (lane 4) and *Dra*I (lane 5) and *N. meningitidis* strain MC58¢2 with *Ava*I (lane 6), *Cla*I (lane 7) and *Dra*I (lane 8). The membrane was probed with the 3' region of *RLS* amplified with oligonucleotide primers 4877 and 4878 (Figure 3.5). DIG labelled lambda DNA digested with *Hin*dIII served as the size standards (kb; lane 1).




1 2 3 4 5 6 7 8
23.1
9.4
6.6
4.4
2.3
2.0
0.56

3.9). Following homologous recombination, one chloramphenicol resistant clone was retained for further study and designated JKD457. Southern hybridisation analysis using high stringency conditions with two different probes was performed on total genomic DNA from strains MS11-A and JKD457 digested with *Hin*cII to confirm integration of the cassette. Probes included an internal 463bp fragment of *RLS*, amplified from pJKD2026 using oligonucleotide primers 4525 and 2377 (Figure 3.5), and the *cat* gene amplified from pJKD800 using oligonucleotide primers UP and RP. The expected 5.5kb and 6.6kb hybridising fragments for MS11-A and JKD457, respectively, were obtained when probing with the internal region of *RLS* (Figure 3.10). The same 6.6kb fragment from strain JKD457 hybridised to the *cat* gene (Figure 3.10) and no hybridising fragments were obtained for MS11-A, verifying the mutant derivative.

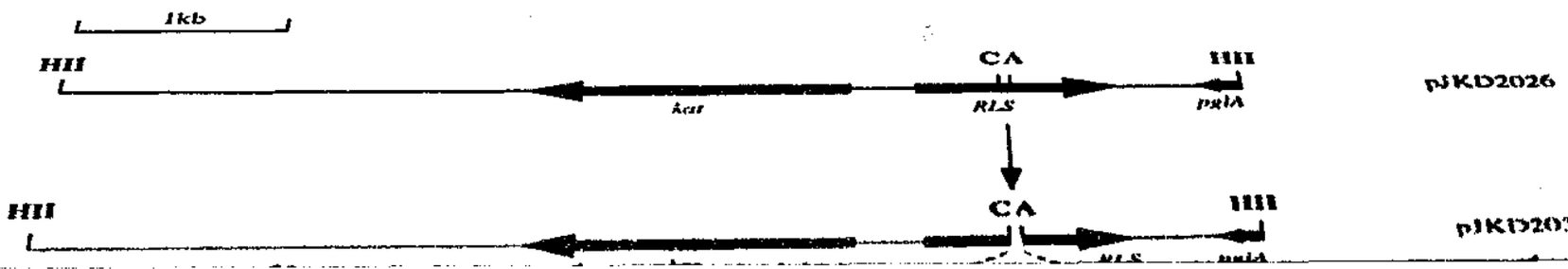
Western blot analysis was performed on whole-cell extracts prepared from *N. gonorrhoeae* strains MS11-A and JKD457, using the *S. typhimurium* anti-RpoN monoclonal antibody (Klimpel *et al.*, 1989). Cell extracts prepared from *E. coli* strain YMC10 and *P. aeruginosa* strain PAK were included as positive controls. As shown in Figure 3.11 both gonococcal strains produced a 90kDa protein that reacts with the antibody. This indicates that the largest ORF within *RLS* does not encode this protein. The expected 54kDa reacting protein was observed in *P. aeruginosa*, however a larger protein of approximately 75kDa was evident in *E. coli*.

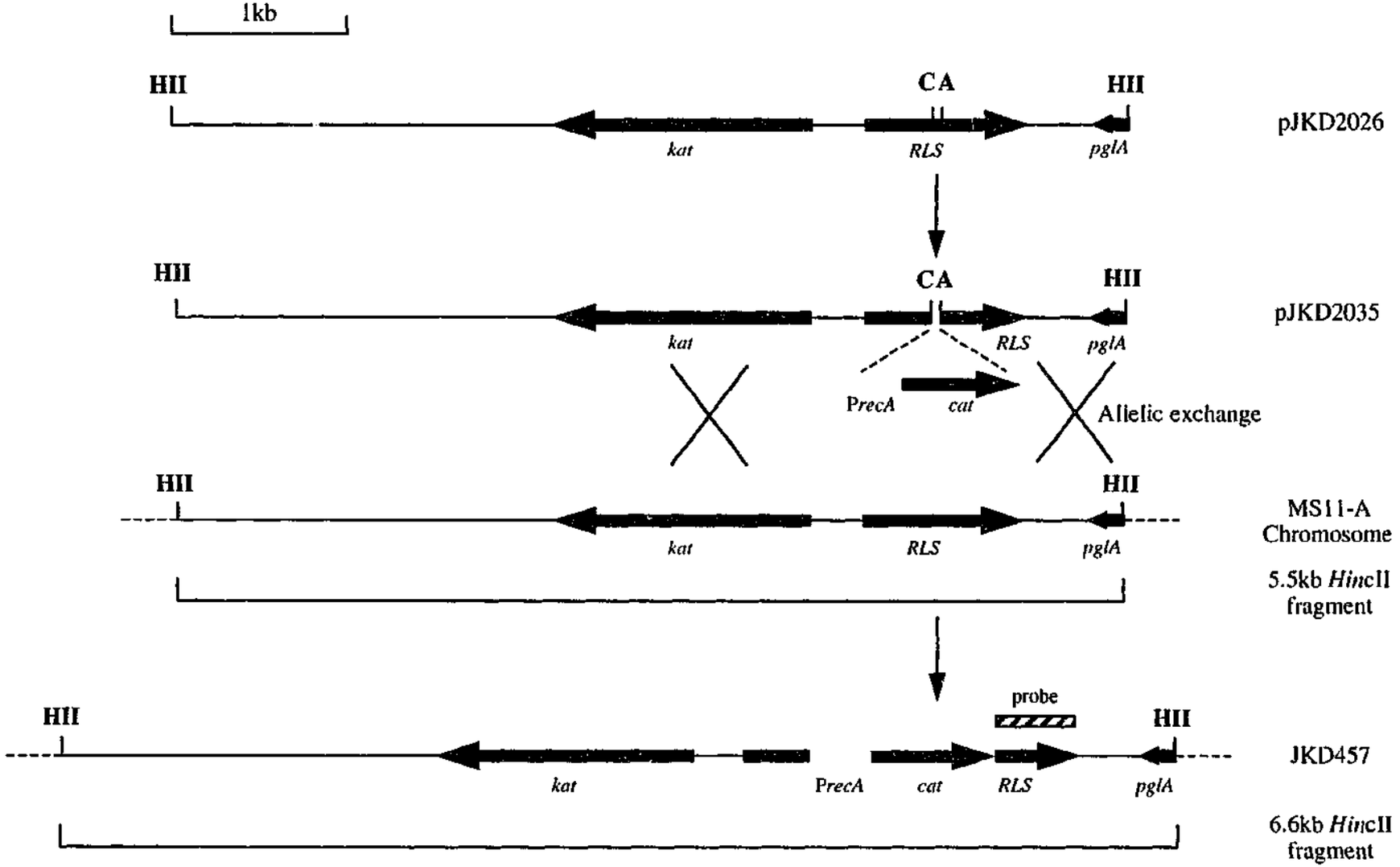
### 3.8 The non-pathogenic *N. lactamica* contains an intact copy of *rpoN*

As mentioned in Section 3.5, a DNA preparation from *N. lactamica* JKD386 used as template in PCR with the oligonucleotide primers 4878 and 4941 (Figures 3.4 and 3.5) yielded a 1.8kb fragment. This fragment was approximately 300bp larger than that obtained for the other neisserial strains. To establish if this product represented an intact copy of *rpoN*, it was cloned into the vector pSU2718 digested with *Hin*cII, and one clone, pJKD2272, was retained for further study. The nucleotide sequence was obtained for the entire insert of pJKD2272 using a series of oligonucleotide primers (Figure 3.12) and was found to be 1736bp in length. The *N. lactamica RLS* was 1344bp in length with the potential to encode a protein of 447 amino acids with a predicted molecular weight of 49.2kDa (Figure 3.13). No nucleotide sequence corresponding to a consensus RBS (Shine and Dalgarno, 1975) was found immediately upstream of the putative ATG start

**Figure 3.9 Strategy for construction of the gonococcal *RLS* mutant.**

The 53bp *AvaII*/*Cla*I fragment internal to *RLS* (green arrow) from pJKD2026 was replaced with the P*recA*::*cat* fusion (yellow-pink arrow) from pJKD861. The resulting plasmid pJKD2035 was linearised, transformed into *N. gonorrhoeae* MS11-A and integrated into the gonococcal chromosome by allelic exchange at the *RLS* locus to produce JKD457. The position of the probe used for subsequent Southern hybridisation analysis to confirm the mutant and the expected hybridising *Hinc*II fragments are shown. Dashed lines represent the gonococcal chromosome. Relevant restriction endonuclease sites are indicated. Abbreviations: A, *Ava*II; C, *Cla*I; HII, *Hinc*II.




1kb
HII
CA
HII
kat
RLS
pglA
pJKD2026
HII
CA
HII
kat
RLS
pglA
pJKD2035
PrecA
cat
Allelic exchange
HII
HII
kat
RLS
pglA
MS11-A
Chromosome
5.5kb HincII
fragment
probe
HII
HII
kat
PrecA
cat
RLS
pglA
JKD457
6.6kb HincII
fragment

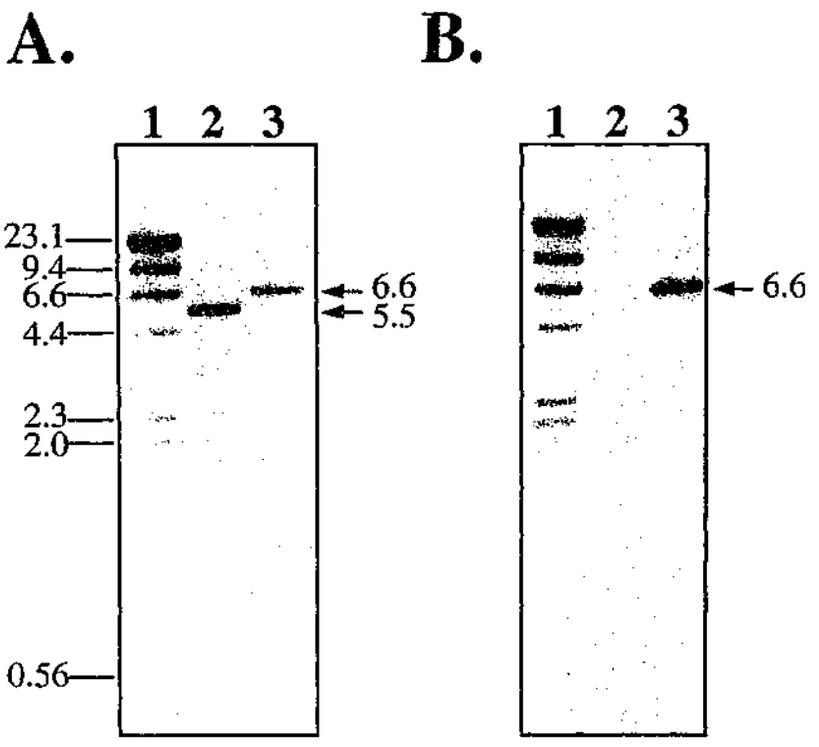

A.
B.
1 2 3
23.1
9.4
6.6
4.4
2.3
2.0
0.56
6.6
5.5
1 2 3
6.6

**Figure 3.11 Western blot analysis of the gonococcal *RLS* mutant.**

Whole cell extracts of *N. gonorrhoeae* strains MS11-A (lane 1) and JKD457 (lane 2), *E. coli* strain YMC10 (lane 3) and *P. aeruginosa* strain PAK (lane 4) were fractionated on a 12% PAGE gel, transferred to a nitrocellulose membrane and incubated with the monoclonal antibody 6RN3 (Klimpel *et al.*, 1989). Hybridising proteins (kDa) are indicated by the arrows.

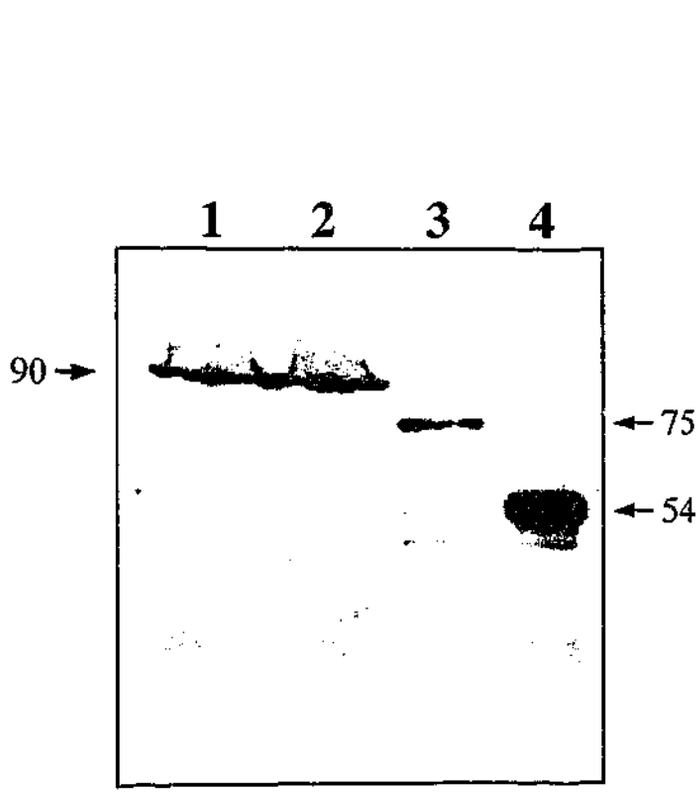

1
2
3
4
90
75
54

**Figure 3.12 Physical map of the 1736bp insert of pJKD2272 showing the position and orientation of *RLS* and the flanking *kat* gene (Johnson *et al.*, 1996).**

Arrows below the map indicate the direction and extent of sequence determined using the oligonucleotide primers printed above them. Universal primer (UP) and reverse primer (RP) bound to vector sequences.

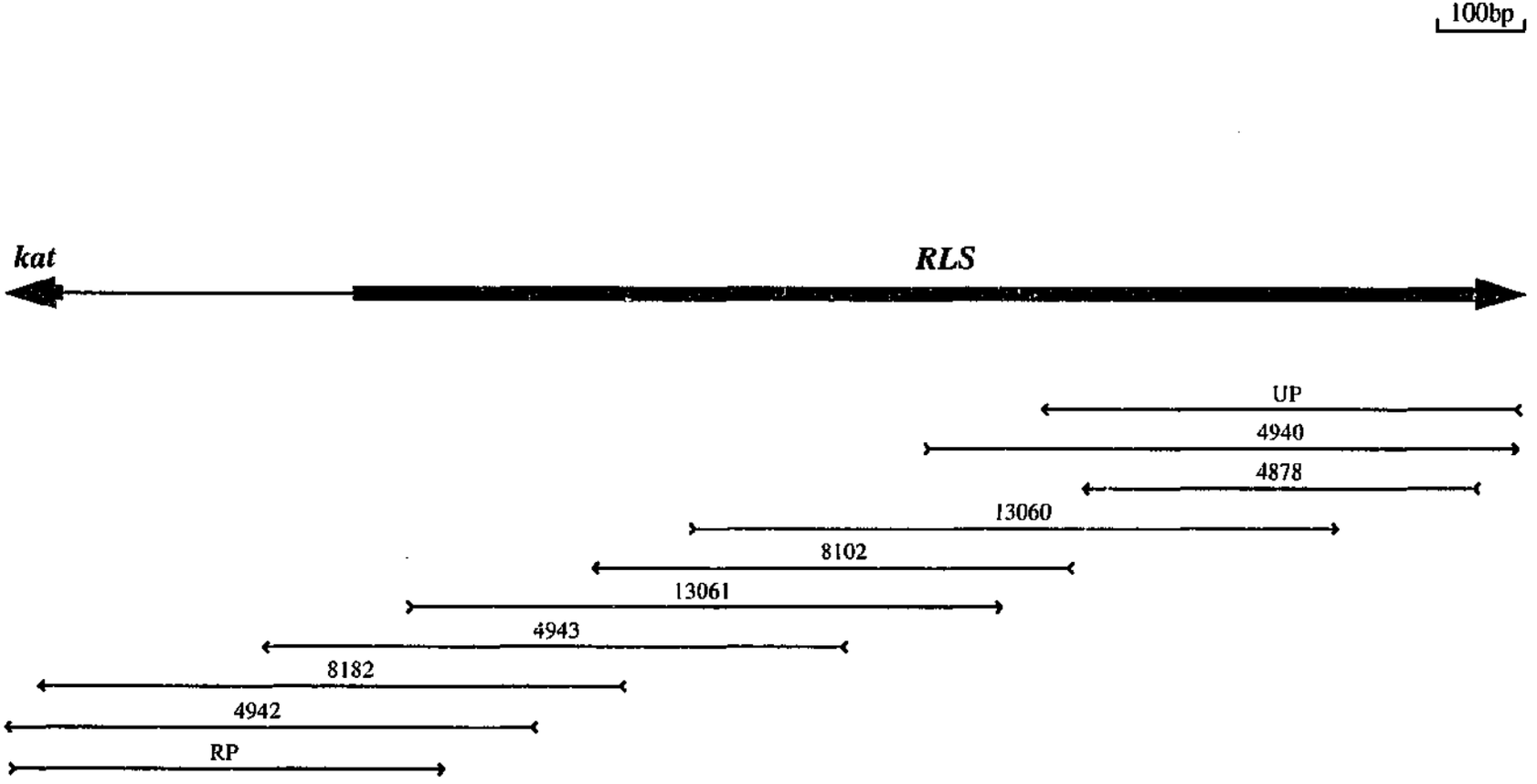
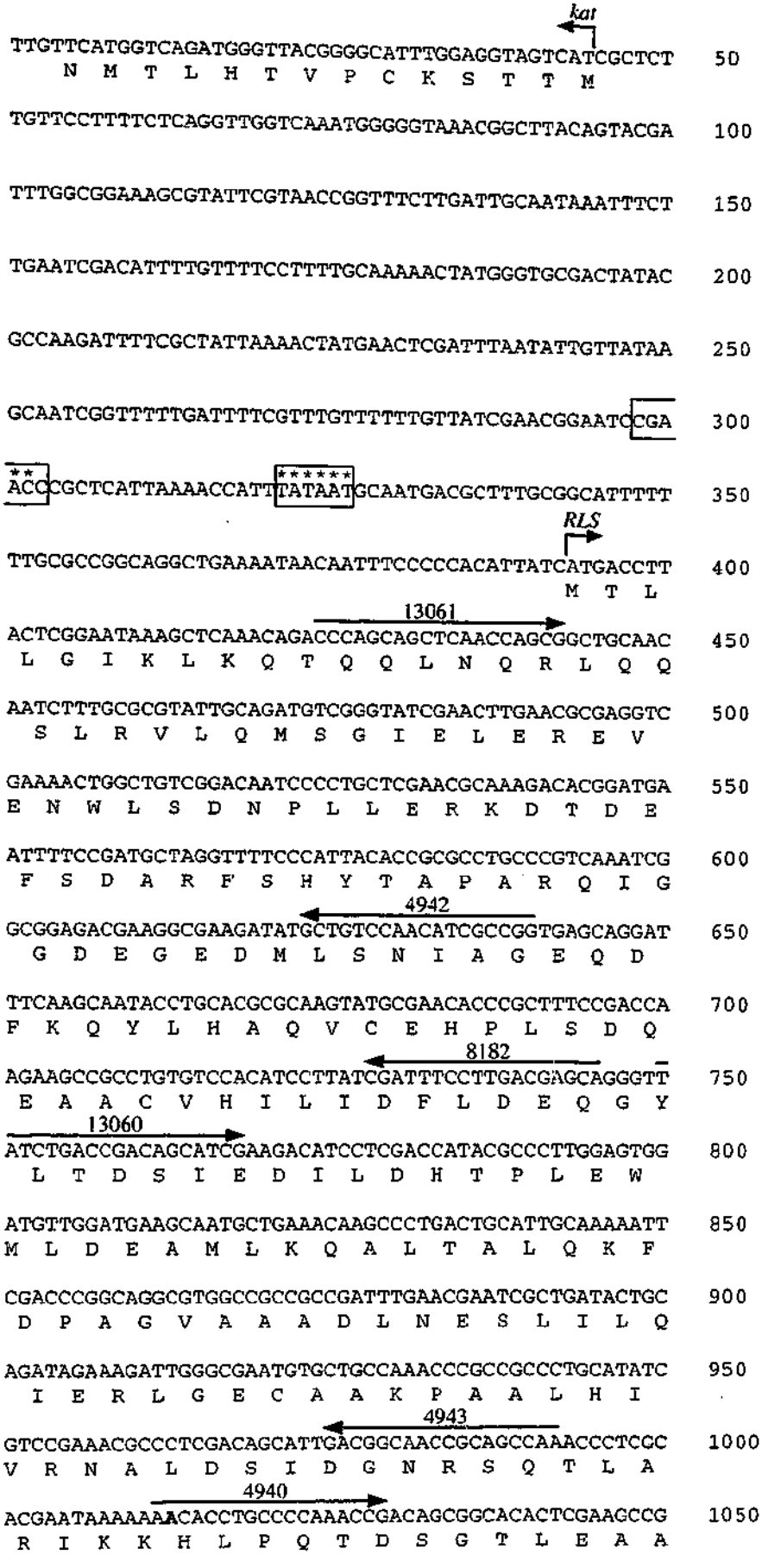
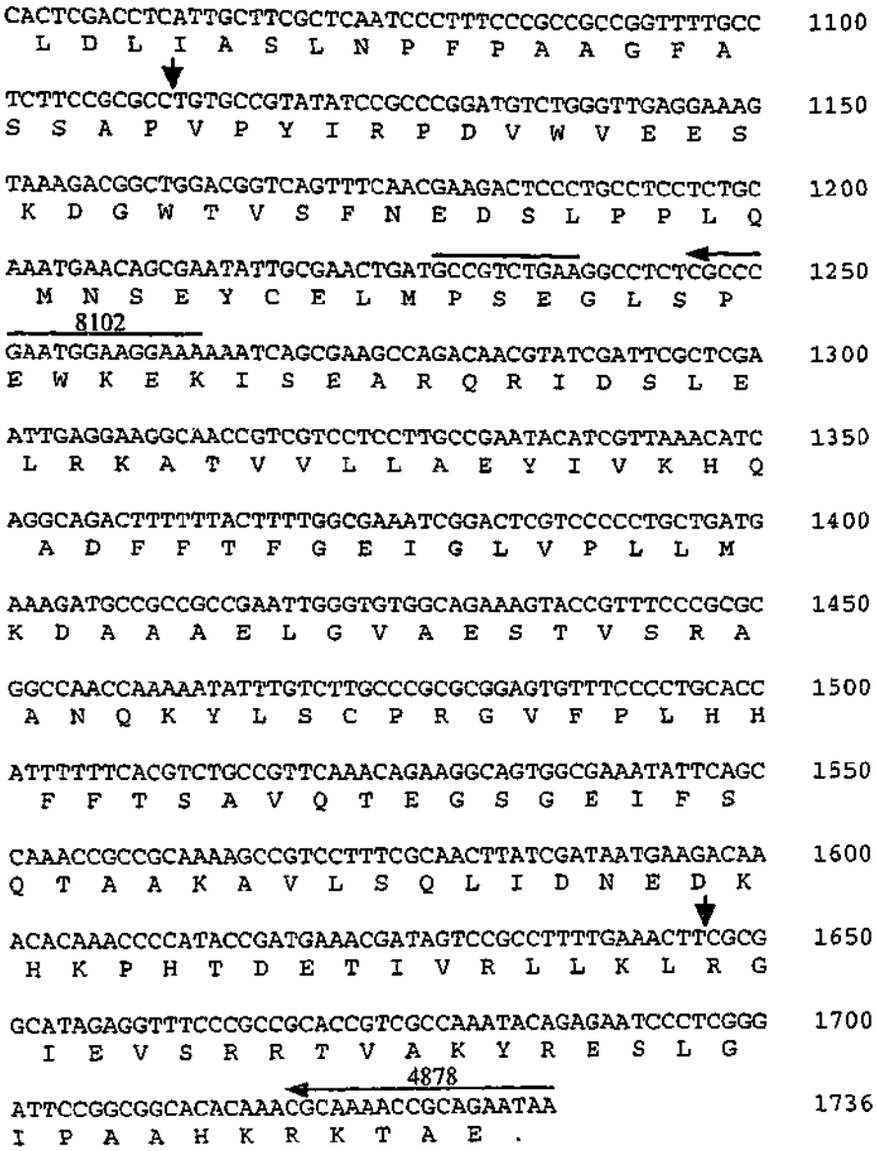

100bp
kat
RLS
UP
4940
4878
13060
8102
13061
4943
8182
4942
RP

**Figure 3.13 Nucleotide sequence of the 1736bp insert of pJKD2272 containing *RLS*.**

The deduced amino acid sequence of *RLS* and the amino terminal region of Kat (Johnson *et al.*, 1996) are shown below the corresponding nucleotide sequence. The stop codon is indicated by a dot (.). Oligonucleotide primers used are depicted by numbered arrows above the nucleotide sequence. The putative $\sigma^{70}$ promoter comprising the -10 and -35 regions is boxed and asterisks (*) represent the nucleotides identical to the consensus sequence (Hawley and McClure, 1983). The Neisserial DNA uptake sequence is overlined. The downward pointing arrows border the region that is deleted in gonococcal and meningococcal strains. The red coloured nucleotide depicts the additional adenine located at this position. Nucleotides are numbered at the right of the figure.


kat
TTGTTCATGGTCAGATGGGTTACGGGGCATTTGGAGGTAGTCATCGCTCT 50
N M T L H T V P C K S T T M
TGTTCCTTTTCTCAGGTTGGTCAAATGGGGGTAAACGGCTTACAGTACGA 100
TTTGGCGGAAAGCGTATTCGTAACCGGTTTCTTGATTGCAATAAATTTCT 150
TGAATCGACATTTTGTTTTCCTTTTGCAAAAACTATGGGTGCGACTATAC 200
GCCAAGATTTTCGCTATTAAAACTATGAACTCGATTTAATATTGTTATAA 250
GCAATCGGTTTTTGATTTTCGTTTGTTTTTTGTTATCGAACGGAATCCGA 300
ACCCGCTCATTAAAACCATTTATAATGCAATGACGCTTTGCGGCATTTTT 350
RLS
TTGCGCCGGCAGGCTGAAAATAACAATTTCCCCCACATTATCATGACCTT 400
M T L
13061
ACTCGGAATAAAGCTCAAACAGACCCAGCAGCTCAACCAGCGGCTGCAAC 450
L G I K L K Q T Q Q L N Q R L Q Q
AATCTTTGCGCGTATTGCAGATGTCGGGTATCGAACTTGAACGCGAGGTC 500
S L R V L Q M S G I E L E R E V
GAAAACTGGCTGTCGGACAATCCCCTGCTCGAACGCAAAGACACGGATGA 550
E N W L S D N P L L E R K D T D E
ATTTTCCGATGCTAGGTTTTCCCATTACACCGCGCCTGCCCGTCAAATCG 600
F S D A R F S H Y T A P A R Q I G
4942
GCGGAGACGAAGGCGAAGATATGCTGTCCAACATCGCCGGTGAGCAGGAT 650
G D E G E D M L S N I A G E Q D
TTCAAGCAATACCTGCACGCGCAAGTATGCGAACACCCGCTTTCCGACCA 700
F K Q Y L H A Q V C E H P L S D Q
8182
AGAAGCCGCCTGTGTCCACATCCTTATCGATTTCCTTGACGAGCAGGGTT 750
E A A C V H I L I D F L D E Q G Y
13060
ATCTGACCGACAGCATCGAAGACATCCTCGACCATACGCCCTTGGAGTGG 800
L T D S I E D I L D H T P L E W
ATGTTGGATGAAGCAATGCTGAAACAAGCCCTGACTGCATTGCAAAAATT 850
M L D E A M L K Q A L T A L Q K F
CGACCCGGCAGGCGTGGCCGCCGCCGATTTGAACGAATCGCTGATACTGC 900
D P A G V A A A D L N E S L I L Q
AGATAGAAAGATTGGGCGAATGTGCTGCCAAACCCGCCGCCCTGCATATC 950
I E R L G E C A A K P A A L H I
4943
GTCCGAAACGCCCTCGACAGCATTGACGGCAACCGCAGCCAAACCCTCCC 1000
V R N A L D S I D G N R S Q T L A
4940
ACGAATAAAAAAACACCTGCCCCAAACCGACAGCGGCACACTCGAAGCCG 1050
R I K K H L P Q T D S G T L E A A


CACTCGACCTCATTGCTTCGCTCAATCCCTTTCCCGCCGCCGGTTTTGCC 1100
L D L I A S L N P F P A A G F A
TCTTCCGCGCCTGTGCCGTATATCCGCCCGGATGTCTGGGTTGAGGAAAG 1150
S S A P V P Y I R P D V W V E E S
TAAAGACGGCTGGACGGTCAGTTTCAACGAAGACTCCCTGCCTCCTCTGC 1200
K D G W T V S F N E D S L P P L Q
AAATGAACAGCGAATATTGCGAACTGATGCCGTCTGAAGGCCTCTCGCCC 1250
M N S E Y C E L M P S E G L S P
8102
GAATGGAAGGAAAAAATCAGCGAAGCCAGACAACGTATCGATTCGCTCGA 1300
E W K E K I S E A R Q R I D S L E
ATTGAGGAAGGCAACCGTCGTCCTCCTTGCCGAATACATCGTTAAACATC 1350
L R K A T V V L L A E Y I V K H Q
AGGCAGACTTTTTTACTTTTGGCGAAATCGGACTCGTCCCCCTGCTGATG 1400
A D F F T F G E I G L V P L L M
AAAGATGCCGCCGCCGAATTGGGTGTGGCAGAAAGTACCGTTTCCCGCGC 1450
K D A A A E L G V A E S T V S R A
GGCCAACCAAAAATATTTGTCTTGCCCGCGCGGAGTGTTTCCCCTGCACC 1500
A N Q K Y L S C P R G V F P L H H
ATTTTTTCACGTCTGCCGTTCAAACAGAAGGCAGTGGCGAAATATTCAGC 1550
F F T S A V Q T E G S G E I F S
CAAACCGCCGCAAAAGCCGTCCTTTCGCAACTTATCGATAATGAAGACAA 1600
Q T A A K A V L S Q L I D N E D K
ACACAAACCCCATACCGATGAAACGATAGTCCGCCTTTTGAAACTTCGCG 1650
H K P H T D E T I V R L L K L R G
GCATAGAGGTTTCCCGCCGCACCGTCGCCAAATACAGAGAATCCCTCGGG 1700
I E V S R R T V A K Y R E S L G
4878
ATTCCGGCGGCACACAAACGCAAAACCGCAGAATAA 1736
I P A A H K R K T A E .

codon. A potential $\sigma^{70}$ promoter (Hawley and McClure, 1983) was identified 66bp upstream of the putative start codon. The probable -10 sequence, TATAAT, was identical to the consensus (Hawley and McClure, 1983) and is preceded at a distance of 17bp by a less conserved -35 region, CGAACC, with two out of six bases identical to the consensus sequence, TTGACA (Hawley and McClure, 1983). One copy of the 10bp neisserial DNA uptake sequence, 5' -GCCGTCTGAA- 3' (Goodman and Scocca, 1988), was identified within the *RLS* ORF.

Comparison of the *RLS* ORF from *N. lactamica* with the corresponding sequence from *N. gonorrhoeae* strain MS11-A (Figure 3.14) revealed that *N. lactamica* does not contain a deleted version of the *rpoN* homologue. Indeed, it seems to be the only neisserial species examined that has retained an intact *rpoN* homologue. An additional adenine nucleotide within *RLS* from *N. lactamica* at position 1013bp (Figure 3.13) shifts the frame compared to *N. gonorrhoeae* such that a small section of the alignment does not remain conserved prior to the deletion point (Figure 3.14). Comparison of the *RLS* ORF from *N. lactamica* with several other RpoN proteins revealed a 35.5% identity in a 484 amino acid overlap with RpoN from *E. coli*, 33.9% identity in a 484 amino acid overlap with RpoN from *K. pneumoniae*, 30.8% identity in a 490 amino acid overlap with RpoN from *A. calcoaceticus* and 30.2% identity in a 503 amino acid overlap with RpoN from *P. aeruginosa* (Figure 3.15). RLS seems to have retained all the features and motifs essential for $\sigma^{54}$ function. They include the glutamine rich amino terminus and hydrophobic heptad repeats found in Region I and Region III. Region II is quite short in length but this region is variable in size among the $\sigma^{54}$ proteins (Cullen *et al.*, 1994). However, it has conserved the acidic nature of this region (Wong and Gralla, 1992) with 26% of residues carrying a negative charge. In addition, the DNA cross-linking region, HTH motif and RpoN box have all been maintained.

## 3.9 The $\sigma^{54}$ homologue of *N. lactamica* can not complement a *P. aeruginosa rpoN* mutant

Sequence analysis of plasmid pJKD2272 revealed that *N. lactamica* contains an *rpoN* homologue. To establish whether this homologue could complement an *rpoN* mutant, the *P. aeruginosa rpoN* mutant, PAK-NI, was used as the host. On solid media, this mutant is non-piliated, as $\sigma^{54}$ is the sigma factor responsible for transcription of the *pilA*

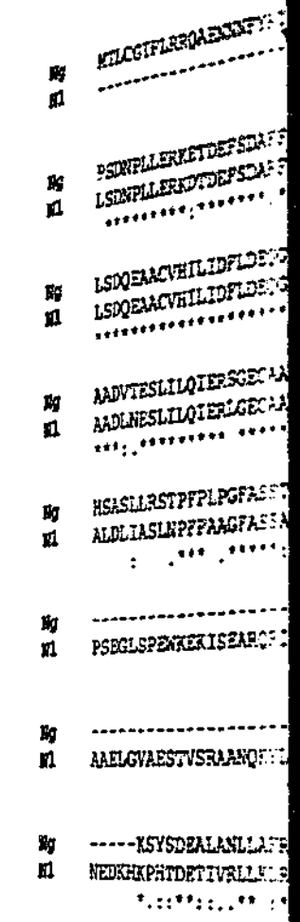


**Figure 3.14 Comparison between the derived amino acid sequences of *RLS* from *N. gonorrhoeae* and *N. lactamica*.**

The alignment was performed using the CLUSTAL W program (Thompson *et al.*, 1994) and dashes (-) indicate gaps that have been introduced to maximise alignment. Amino acids in red text and the asterisk (*) represent identical residues. Conserved amino acids are depicted by green text and the colon (:) and residues with less similarity are indicated by blue text and the dot (.). The downward pointing arrow represents the region that contains an additional adenine nucleotide. The amino acids are numbered at the right of the figure according to their position in the individual protein sequence.

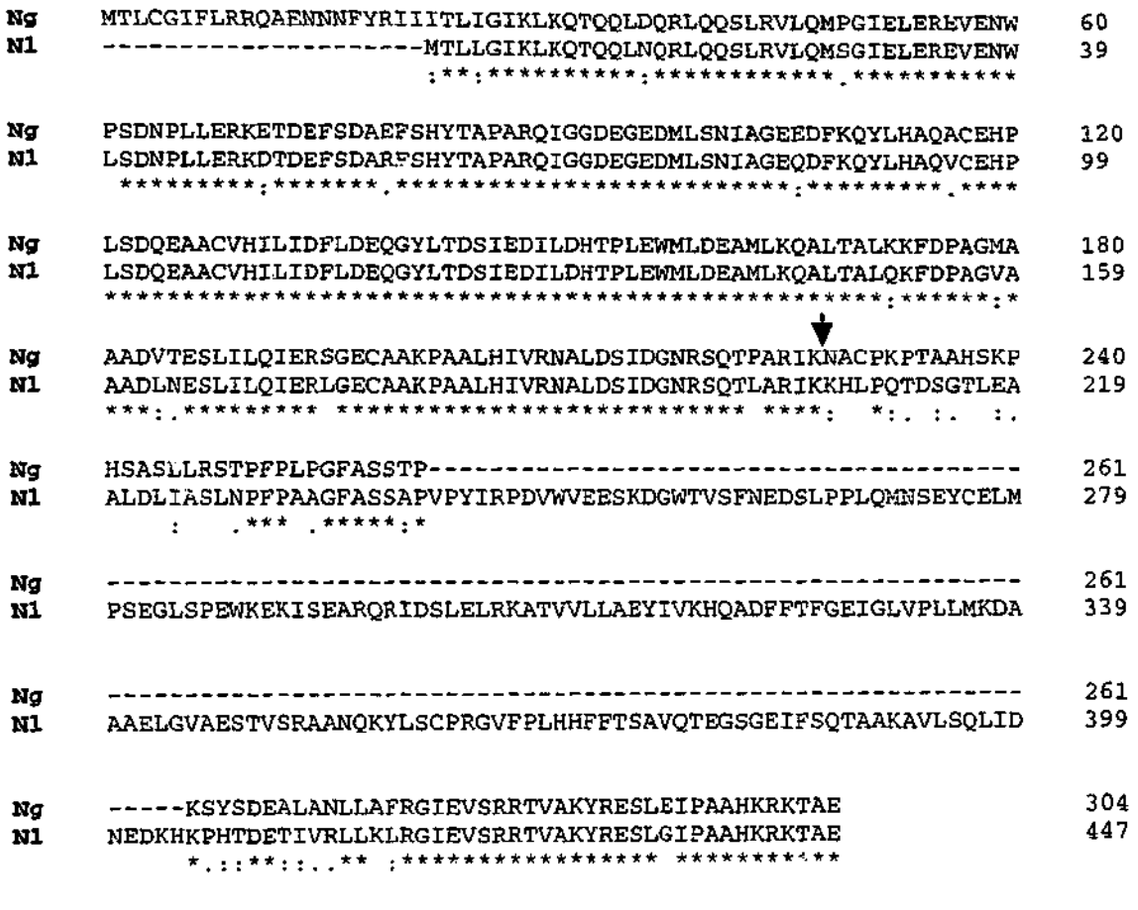
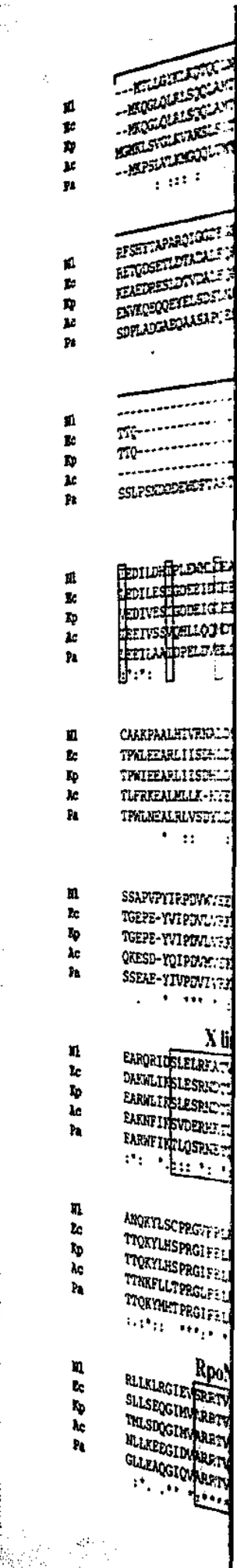
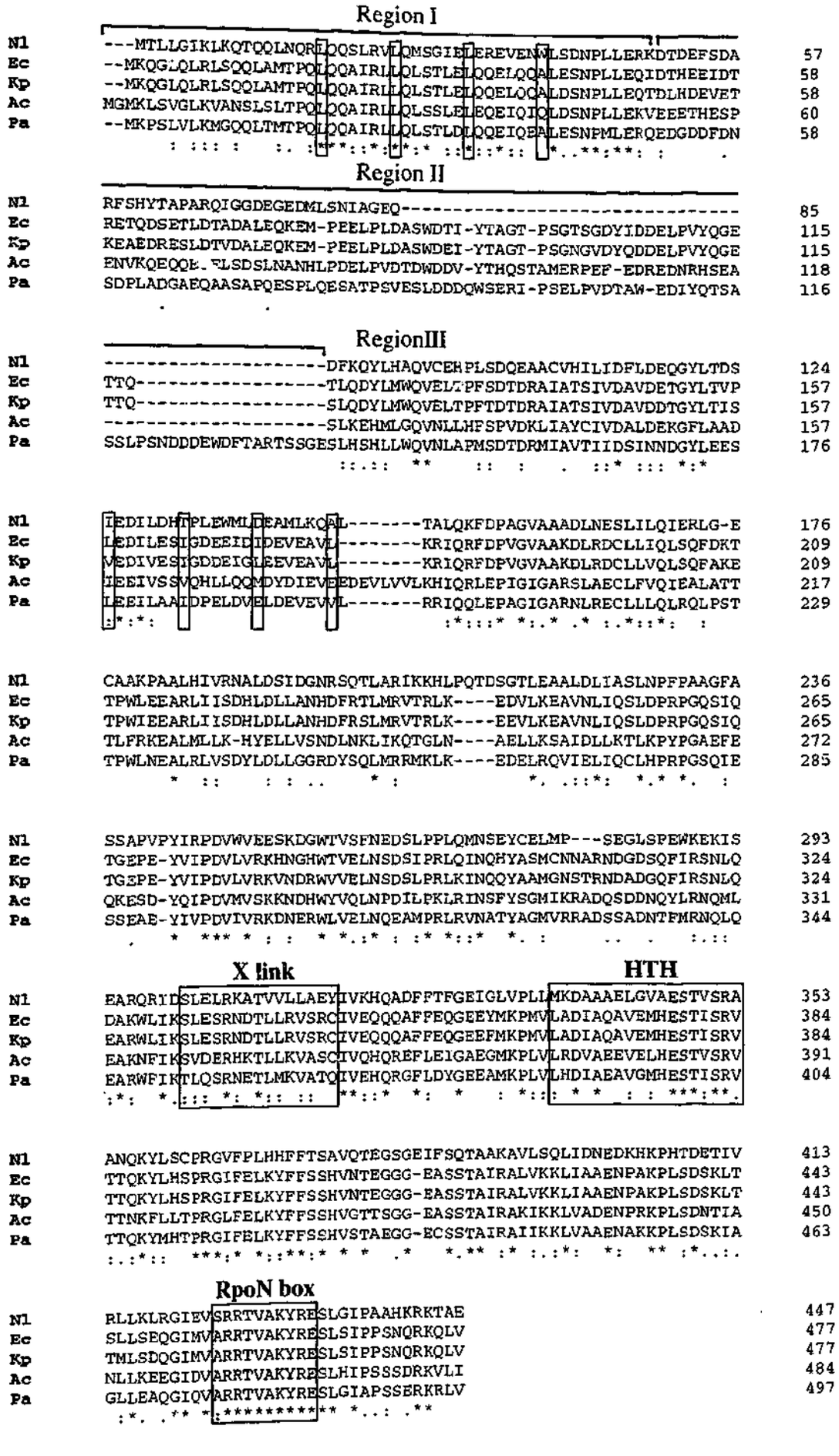

Ng MTLCGIFLRRQAENNNFYRIIITLIGIKLKQTQQLDQRLQQSLRVLQMPGIELEREVENW 60
N1 ----------MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENW 39
Ng PSDNPLLERKETDEFSDAEFSHYTAPARQIGGDEGEDMLSNIAGEEDFKQYLHAQACEHP 120
N1 LSDNPLLERKDTDEFSDARFSHYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHP 99
Ng LSDQEAACVHILIDFLDEQGYLTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGMA 180
N1 LSDQEAACVHILIDFLDEQGYLTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVA 159
Ng AADVTESLILQIERSGECAAKPAALHIVRNALDSIDGNRSQTPARIKNACPKPTAAHSKP 240
N1 AADLNESLILQIERLGECAAKPAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEA 219
Ng HSASLLRSTPFPLPGFASSTP---------- 261
N1 ALDLIASLNPFPAAGFASSAPVPYIRPDVWVEESKDGWTVSFNEDSLPPLQMNSEYCELM 279
Ng ---------- 261
N1 PSEGLSPEWKEKISEARQRIDSLELRKATVVLLAEYIVKHQADFFTFGEIGLVPLLMKDA 339
Ng ---------- 261
N1 AAELGVAESTVSRAANQKYLSCPRGVFPLHHFFTSAVQTEGSGEIFSQTAAKAVLSQLID 399
Ng -----KSYSDEALANLLAFRGIEVSRRTVAKYRESLEIPAAHKRKTAE 304
N1 NEDKHKPHTDETIVRLLKLRGIEVSRRTVAKYRESLGIPAAHKRKTAE 447

**Figure 3.15 Amino acid sequence comparison of the RLS ORF from *N. lactamica* and the RpoN proteins of *E. coli* (Ec; Jones *et al.*, 1994), *K. pneumoniae* (Kp; Merrick and Gibbins, 1985), *A. calcoaceticus* (Ac; Ehrt *et al.*, 1994) and *P. aeruginosa* (Pa; Jin *et al.*, 1994).**

The alignment was performed with the CLUSTAL W program (Thompson *et al.*, 1994) and dashes (-) indicate gaps that have been introduced to maximise alignment. Residues in red text and the asterisk (*) represent identical residues. Conserved amino acids are indicated by green text and the colon (:) and residues with less similarity are depicted by blue text and the dot (.). The three regions of RpoN identified by Merrick (1993) are indicated. Regions I and II are overlined and the remainder of the sequence constitutes Region III. The boxed residues represent amino acids that form the hydrophobic heptad repeats found in Regions I and III (Tintut *et al.*, 1994; Wang *et al.*, 1995). The DNA cross-linking region (Cannon *et al.*, 1994), HTH motif (Merrick and Chambers, 1992) and RpoN box (Merrick, 1993) are indicated. Amino acids are numbered at the right of the figure according to their position in each individual protein sequence.




Region I
Region II
RegionIII
X link
HTH
RpoN box
Nl
Ec
Kp
Ac
Pa

gene, encoding the type IV pilin subunit. Thus colonies appear small, shiny and domed. Conversely, piliated cells form large, dull flat colonies (Bradley, 1980).

The *Bam*HI/*Hin*dIII fragment of pJKD2272 containing the entire *rpoN* homologue was subcloned into similarly digested pUCP18, an *E. coli*/*P. aeruginosa* shuttle vector, generating pJKD2321. The insert contains sequence upstream of *RLS* so that transcription is expected to occur from its own promoter. pJKD1870 was used as a positive control as it contains the *P. aeruginosa rpoN* gene cloned into pUCP18 and vector alone was used as a negative control. Subsequently, pJKD2321 and pJKD1870 and pUCP18 were transformed into *P. aeruginosa* PAK-N1, plated onto NY agar and incubated at 37°C overnight. As expected, large colonies typical of piliated cells were produced from the strain harbouring pJKD1870 (Figure 3.16). However, both PAK-N1 (pJKD2321) and PAK-N1 (pUCP18) appeared to be non-piliated, suggesting that the *N. lactamica rpoN* homologue cannot complement the *P. aeruginosa rpoN* mutation. Alternatively, it may be that RLS is not expressed because the neisserial promoter is not recognised or functional in *P. aeruginosa*.

### 3.10 Transcriptional analysis of the *rpoN* homologue of *N. lactamica*

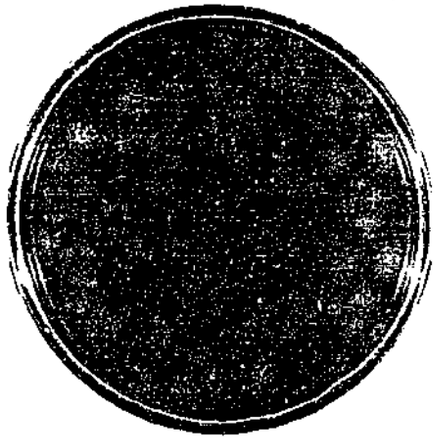
Results from the previous section demonstrate that the *rpoN* homologue of *N. lactamica* does not seem capable of complementing a *P. aeruginosa rpoN* mutant. RT PCR was performed to establish whether *RLS* was actually expressed in *N. lactamica*.

Oligonucleotide primers 4940 and 4878 were used as they primed internally to the coding sequence (Figure 3.12). Oligonucleotide primers 3260 and 3261, designed to an internal region of the gonococcal 16S rRNA gene (Rossau *et al.*, 1988), were used as a positive control due to the constitutive expression of this gene (Rossau *et al.*, 1988). Following reverse transcription with the downstream primer, cDNA products were used as template in PCR (Figure 3.17). The negative control reactions contained all the components of the RT mix except the reverse transcriptase. As expected no PCR products were observed for these reactions (lanes 3 and 6). The expected size products of 999bp for *RLS* and 835bp for the 16S rRNA gene were amplified from DNA templates used as positive controls for the PCR reaction (lanes 4 and 7). Lane 5 illustrates transcription of the 16S rRNA gene. However, lane 2 did not contain a PCR

**Figure 3.16 Complementation analysis of a *P. aeruginosa rpoN* mutant with *RLS* from *N. lactamica.***

Constructs pJKD1870 and pJKD2321, containing *rpoN* and *N. lactamica RLS*, respectively, were used to transform *P. aeruginosa rpoN* mutant PAK-NI. Colonies were obtained following an overnight incubation at 37°C and the phenotype is indicated to the right.

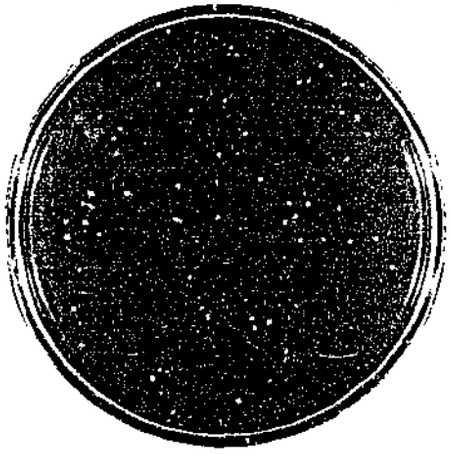
PAK-NI (pJKD1870)



Phenotype

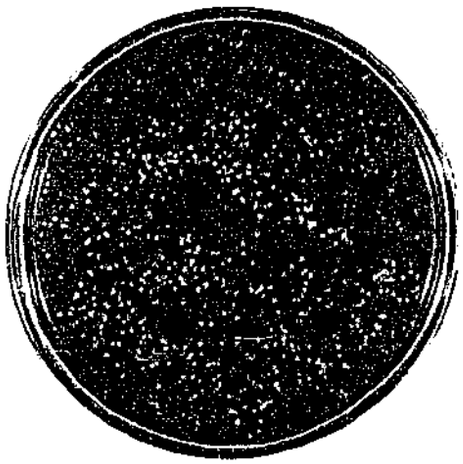
Pil+

PAK- N1 (pUCP18)



Pil-

PAK-NI (pJKD2321)

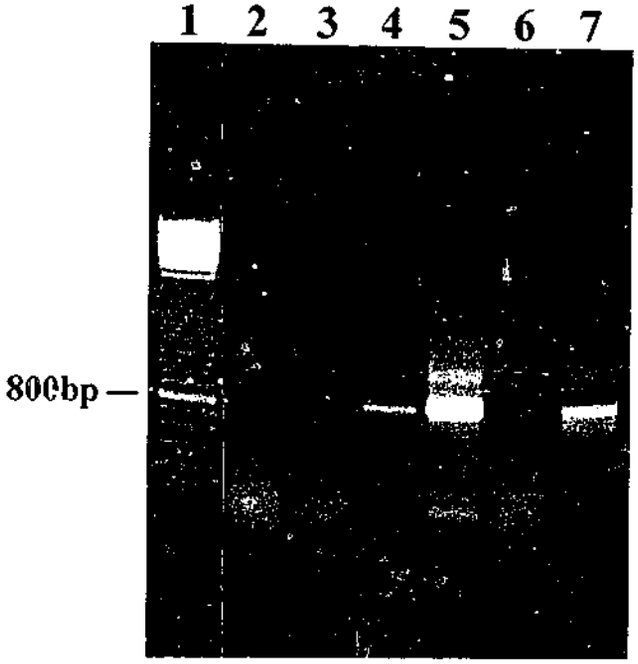


Pil-

**Figure 3.17 RT-PCR analysis of *RLS* from *N. lactamica*.**

The negative control reactions for the RT PCR did not contain any template for PCR amplification as there was no RT. The experimental reactions contain RNA template. DNA template was used as the positive control for the PCR reactions. Lane 2: *RLS* experimental reaction; Lane 3: *RLS* negative RT control; Lane 4: *RLS* DNA positive control for PCR; Lane 5: 16S rRNA experimental reaction; Lane 6: 16S rRNA negative RT control; Lane 7: 16S rRNA DNA positive control for PCR. Reactions in lanes 2-4 used oligonucleotide primers 4878 and 4940 and those in lanes 5-7 used oligonucleotide primers 3260 and 3261. Lane 1 contained a 100bp ladder that served as the size standards.

800bp


1 2 3 4 5 6 7
800bp

product suggesting that *RLS* is not transcribed. It may be that expression of this gene was not compatible with the environmental conditions used to grow *N. lactamica*, or *in vivo* growth conditions are necessary.

### 3.11 Discussion

It had long been thought that *N. gonorrhoeae* contained an *rpoN* gene and that the encoded sigma factor was responsible for *pilE* transcription from a putative $\sigma^{54}$ promoter (Thony and Hennecke, 1989). Subsequent analysis by Fyfe *et al.* (1995) and Laskos *et al.* (1998) revealed that *pilE* transcription in *N. gonorrhoeae* was in fact dependent on a $\sigma^{70}$ promoter *in vitro*, leading to the conclusion that the gonococcal $\sigma^{54}$ was either unable to bind to the promoter due to the presence of an anti-sigma factor (Brown and Hughes, 1995) or was not produced. Different environmental conditions to those used in the experiments, or *in vivo* growth, may have been required for RpoN expression.

This chapter aimed to identify the gonococcal *rpoN* gene and attempts were made using conventional methods. Southern hybridisations were performed probing gonococcal DNA at low stringency with the *rpoN* genes from *E. coli*, *P. aeruginosa* and *A. calcoaceticus*. As these bacteria belong to the proteobacteria subdivision, the same evolutionary group as *Neisseria* spp., it was postulated they would share the highest similarity to the proposed gonococcal *rpoN* homologue. No hybridising fragments were obtained (data not shown) indicating that the putative gonococcal homologue does not share a high degree of nucleotide sequence similarity with other *rpoN* genes. Complementation assays have previously been used to identify *rpoN* homologues but were not successful at identifying a gonococcal homologue (Ehrt *et al.*, 1994; Toukdarian and Kennedy, 1986). Collectively, the results indicate that perhaps the putative gonococcal *rpoN* homologue was non-functional in the species tested.

Fortuitously, at this time genomic sequence data from *N. gonorrhoeae* strain FA1090 was released on the University of Oklahoma *N. gonorrhoeae* Genome Database (Genbank accession number AE004969). BLAST analysis of this sequence revealed a region, which, when translated, displayed significant similarity to the *E. coli* $\sigma^{54}$ amino acid sequence (Jones *et al.*, 1994). In particular a region encoding the highly conserved

and characteristic RpoN box (Merrick, 1993) was identified. This sequence was consequently designated *RLS* (*rpoN*-like sequence) and a genomic library identified the corresponding region from *N. gonorrhoeae* strain MS11-A. Sequence analysis (Figure 3.5) revealed that translation of the sequence upstream of that encoding the RpoN box displayed similarity to the N-terminal region of *E. coli* $\sigma^{54}$, but was in a different reading frame, indicating that *RLS* might be incapable of encoding an intact *rpoN* homologue. In addition some of the motifs necessary for function were not conserved, including the hydrophobic heptad repeats located in Regions I and III, required for enhancer responsiveness (Syed and Gralla, 1998), DNA binding (Gallegos and Buck, 2000) and binding to core RNA polymerase (Hsieh *et al.*, 1999; Tintut *et al.*, 1994). Most surprising was the absence of an approximately 420bp region that encodes the essential DNA binding motifs (Cannon *et al.*, 1994; Cannon *et al.*, 1995; Merrick and Chambers, 1992). This would clarify the absence of hybridising fragments for *N. gonorrhoeae* when probed with the *rpoN* genes of various bacteria (Section 3.2) and for *E. coli* when probed with an internal region of *RLS* (Figure 3.3). It appears that a deletion containing this region occurred in the ancestral *rpoN* homologue and rendered the associated mutant sigma factor unable to bind DNA (Guo and Gralla, 1997) and hence function. This deletion appears to be widespread as it is also evident in the genomes of the pathogenic *N. meningitidis* and the commensal *N. subflava* as determined by PCR and sequence analysis (Figure 3.7).

The possibility that the *N. gonorrhoeae* and *N. meningitidis* genomes contained an additional, intact copy of *rpoN* was unlikely based on the Southern hybridisation results presented in Figure 3.8. A probe generated to hybridise to the most highly conserved RpoN box bound to single fragments when genomic DNA of the two species was digested with several restriction endonucleases, indicating that *RLS* is the only copy of an *rpoN*-like sequence.

The mystery remains as to the identity and function of the 90kDa gonococcal protein that binds the anti-RpoN monoclonal antibody (Figure 3.11). The presence of this protein, originally observed by Klimpel *et al.* (1989), was also observed in this study. However, the largest ORF within *RLS* would only be capable of encoding a 30kDa protein (Figure 3.5). It was possible that the ORF formed a trimer although interruption of this ORF had no effect on the amount or size of the protein reacting with the antibody

in a Western blot (Figure 3.11), confirming that this protein is not encoded by this ORF. The extract used in the original experiment (Klimpel *et al.*, 1989) was a crude RNA polymerase preparation so it is possible that this cross-reacting protein was a contaminant and is not involved in the transcriptional process. Further experimental procedures such as protein purification and amino terminal sequencing would establish the identity of this protein.

The fact that the *rpoN* deletion is present in the genomes of several *Neisseria* spp. indicates that a functional gene was once present in an ancestor of the neisseria. Perhaps the loss of *rpoN* is associated with the evolution of these species as obligate human colonisers. Interestingly, complete sequencing of the genomes of *H. influenzae* (Fleischmann *et al.*, 1995), *M. tuberculosis* (Cole *et al.*, 1998), *Mycoplasma genitalium* (Fraser *et al.*, 1995) and *Mycoplasma pneumoniae* (Himmelreich *et al.*, 1996), all obligate human pathogens, failed to reveal any *rpoN* homologues. $\sigma^{54}$ associated transcriptional regulation is very responsive to environmental conditions, and it may be that this type of regulation is no longer essential in this particular ecological niche. In fact RpoN is generally not essential for growth under optimal conditions, except in *M. xanthus* (Keseler and Kaiser, 1997). However, this does not explain the presence of an *rpoN* homologue in the genomes of the obligate human pathogens *C. trachomatis* (Kalman *et al.*, 1999; Stephens *et al.*, 1998), *T. pallidum* (Fraser *et al.*, 1998) and several others (Studholme and Buck, 2000).

The argument that the absence of *rpoN* in the former bacteria is a consequence of evolutionary 'streamlining' (Studholme and Buck, 2000) does not clarify the presence of *rpoN* in genomes smaller than that of *N. gonorrhoeae*, such as *T. pallidum* (Fraser *et al.*, 1998) and *C. trachomatis* (Stephens *et al.*, 1998). This also contradicts the theory that a larger chromosome is necessary for the $\sigma^{54}$ mode of transcription as it requires significant stretches of intergenic DNA for the DNA-looping mechanism of activator-RNA polymerase contact (Reitzer and Magasanik, 1986). The presence or absence of *rpoN* in particular species obviously involves additional factors and each case has to be examined individually. The consensus seems to be that $\sigma^{54}$ has a role in pathogenesis as it is expressed in the majority of bacterial pathogens investigated so far (Studholme and Buck, 2000). $\sigma^{54}$ is required for the expression of virulence factors in *V. cholerae* responsible for colonisation of the host (Klose and Mekalanos, 1998) and is possibly

involved in the expression of the *T. pallidum* bacterioferritin iron-storage protein that has neutrophil-activating and antigenic properties (Evans *et al.*, 1995; Noordhoek *et al.*, 1989). It is also required for expression of the *pspABCDE* operon in enteric bacteria (Model *et al.*, 1997) thought to be involved in efficient translocation of virulent proteins (Studholme and Buck, 2000).

A chimeric gene, *rsp*, was recently identified in *N. gonorrhoeae* (Carrick *et al.*, 2000). The *rsp* gene has sequence similarity to both the *pilS* and *pilR* genes of *P. aeruginosa* encoding a two-component regulatory system that controls piliation. This gene, like *RLS*, seems to have evolved either through a deletion and subsequent fusion event or a rearrangement event, and as such is no longer involved in gonococcal pilin regulation (Carrick *et al.*, 2000). Evidently, these species have evolved so that RpoN and the associated two-component regulatory system are no longer required for regulation of type 4 piliation. Transcriptional regulation of *pilE* occurs via a $\sigma^{70}$ dependent promoter (Fyfe *et al.*, 1995) and requires binding of the cofactor, IHF, to upstream sequences for optimal expression (Hill *et al.*, 1997). In addition, UP-like elements proximal to the promoter enhance transcription (Fyfe and Davies, 1998) although binding of the RNA polymerase $\alpha$ subunit to this region has yet to be demonstrated. Interestingly, IHF plays a role in the activation of $\sigma^{54}$ promoters in *E. coli* and other bacterial species (Goosen and van de Putte, 1995) and originally may have had this function in the neisserial progenitor.

Current *pilE* regulation provides the biological advantage of transcription without the need for additional energy expenditure by production of the alternative sigma factor and activator/sensor. In addition competition for core RNA polymerase between sigma factors is alleviated so that variations in the concentrations of sigma factors are not required (Studholme and Buck, 2000).

Downstream of *RLS* was a region containing copies of the direct and inverted repeats, designated RS3 ( Figure 3.4; Haas and Meyer, 1986). These are a family of repeats found in the flanking regions of a number of genes (Seiler *et al.*, 1996; van der Ende *et al.*, 1999) that consist of a common 6bp core sequence and thought to be involved in site-specific recombination (Seiler *et al.*, 1996). van der Ende *et al.* (1999) demonstrated that the RS3 repeats located upstream and downstream of the *porA* gene in *N.*

*meningitidis* are responsible for deletion of this gene by recombination resulting in a *porA*-negative variant.

In addition to the apparent deletion within *RLS*, subsequent mutation may have been responsible for the deletion of the adenine nucleotide in *N. gonorrhoeae* which renders it out of frame (Figures 3.5 and 3.14). Sequencing of the *N. meningitidis* genome (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000) revealed that the corresponding region of *RLS* has an extra adenine nucleotide so that it is in frame.

The discovery of an intact *rpoN* homologue in *N. lactamica* was surprising considering the deleted variants in the pathogenic neisseria (Figure 3.7). Sequence analysis revealed that the features characteristic of each of the three regions within RpoN (Merrick, 1993) were conserved in the *N. lactamica* homologue (Figure 3.15). The failure of this homologue to complement a *P. aeruginosa rpoN* mutant (Figure 3.16) indicates that it may not function as a sigma factor in this background. Alternatively, the *rpoN* homologue may have been poorly expressed or not expressed at all if the neisserial promoter was not recognised by the *P. aeruginosa* transcription machinery. Expression using a *P. aeruginosa* promoter would clarify this point. The absence of an RT PCR product for the *N. lactamica rpoN* gene (Figure 3.17) revealed that under the *in vitro* conditions tested it was not transcribed. Alternative environmental conditions or *in vivo* growth may be required for $\sigma^{54}$ expression and further experimental analysis is necessary.

# CHAPTER FOUR

# IDENTIFICATION AND REGULATORY ANALYSIS OF THE DnaK CHAPERONE SYSTEM IN *NEISSERIA GONORRHOEAE*

## 4.1 Introduction

Molecular chaperones are required for a variety of cellular processes ensuring the proper conformation and cellular location of a number of proteins (Thomas *et al.*, 1997). In particular, the reactivation and degradation of proteins impaired by heat stress or other types of stress is facilitated by chaperones (Ben-Zvi and Goloubinoff, 2001). The predominant cytoplasmic bacterial chaperone systems are the essential DnaK and GroEL groups that are highly conserved and have been extensively studied in *E. coli* (Georgopoulos and Welch, 1993; Hartl and Martin, 1995).

The stress response of *N. gonorrhoeae* had not been investigated until recently despite evidence that molecular chaperones induce an immune response (Kaufmann, 1990) and have a role in disease pathogenesis (Hanawa *et al.*, 1999; Kohler *et al.*, 1996; Pannekoek *et al.*, 1992a). Woods *et al.* (1990) initially identified the gonococcal DnaK and GroEL proteins by their immunological cross-reactivity to the *E. coli* counterparts. Further analysis found the GroEL homologue to be associated with the cell surface (Chomvarin, 1993). Such a location would render the protein capable of interaction with host surfaces and exposed to the immune response (Chomvarin, 1993). In agreement with this, it was demonstrated that GroEL is a major immunogen during natural infection of both *N. gonorrhoeae* (Pannekoek *et al.*, 1993) and *N. meningitidis* (Pannekoek *et al.*, 1995). Interestingly, GroEL from *N. meningitidis* can only be isolated from the cytosol and cannot be found in either the outer membrane or the periplasmic space (Arakere *et al.*, 1993).

Subsequent cloning and sequence analysis of the gonococcal *groES* and *groEL* homologues revealed that they were organised in a bicistronic operon (Tauschek *et al.*, 1997), an arrangement similar to that found in most bacterial species (Segal and Ron, 1996b). Overlapping $\sigma^{70}$ and $\sigma^{32}$ promoters have been identified upstream of the operon and transcription is induced upon thermal stress (Tauschek *et al.*, 1997). Transcription

from the $\sigma^{70}$ promoter probably occurs under normal conditions and an elevated level of transcription originates from the $\sigma^{32}$ promoter following exposure to stress, as occurs for the *E. coli groE* operon (Zhou *et al.*, 1988). However, contrary to a number of other species where GroEL is produced at a higher rate than GroES, as the chaperonins function in a 2:1 ratio, respectively (Segal and Ron, 1996b; Zeilstra-Ryalls *et al.*, 1991), an intergenic transcriptional terminator potentially results in a higher level of expression of GroES relative to GroEL in *N. gonorrhoeae* (Tauschek *et al.*, 1997). Therefore it seems that control of expression of the gonococcal *groE* operon is similar to that in *E. coli* but the regulatory mechanisms affecting mRNA levels of *groES* and *groEL* appear to differ.

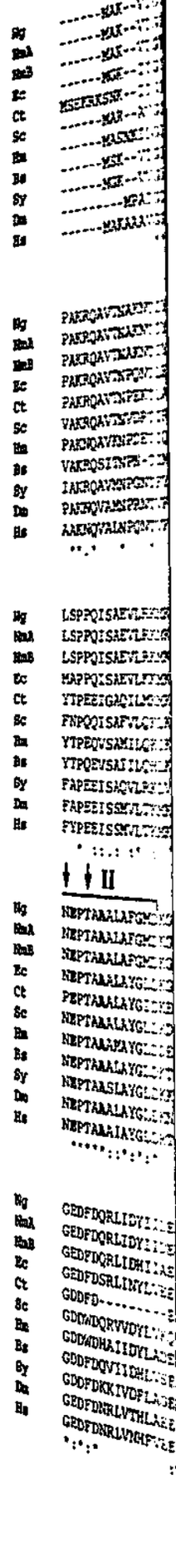
This chapter investigates the occurrence of the DnaK chaperone system, encoded by *dnaK*, *dnaJ* and *grpE* in the genomes of the neisserial strains that have been completely sequenced to date including *N. gonorrhoeae* strain FA1090 (Genbank accession number AE004969), *N. meningitidis* strain MC58 (Tettelin *et al.*, 2000) and *N. meningitidis* strain Z2491 (Parkhill *et al.*, 2000). In addition the transcriptional regulation of these genes was analysed from *N. gonorrhoeae* strain MS11-A and promoter-like elements identified.

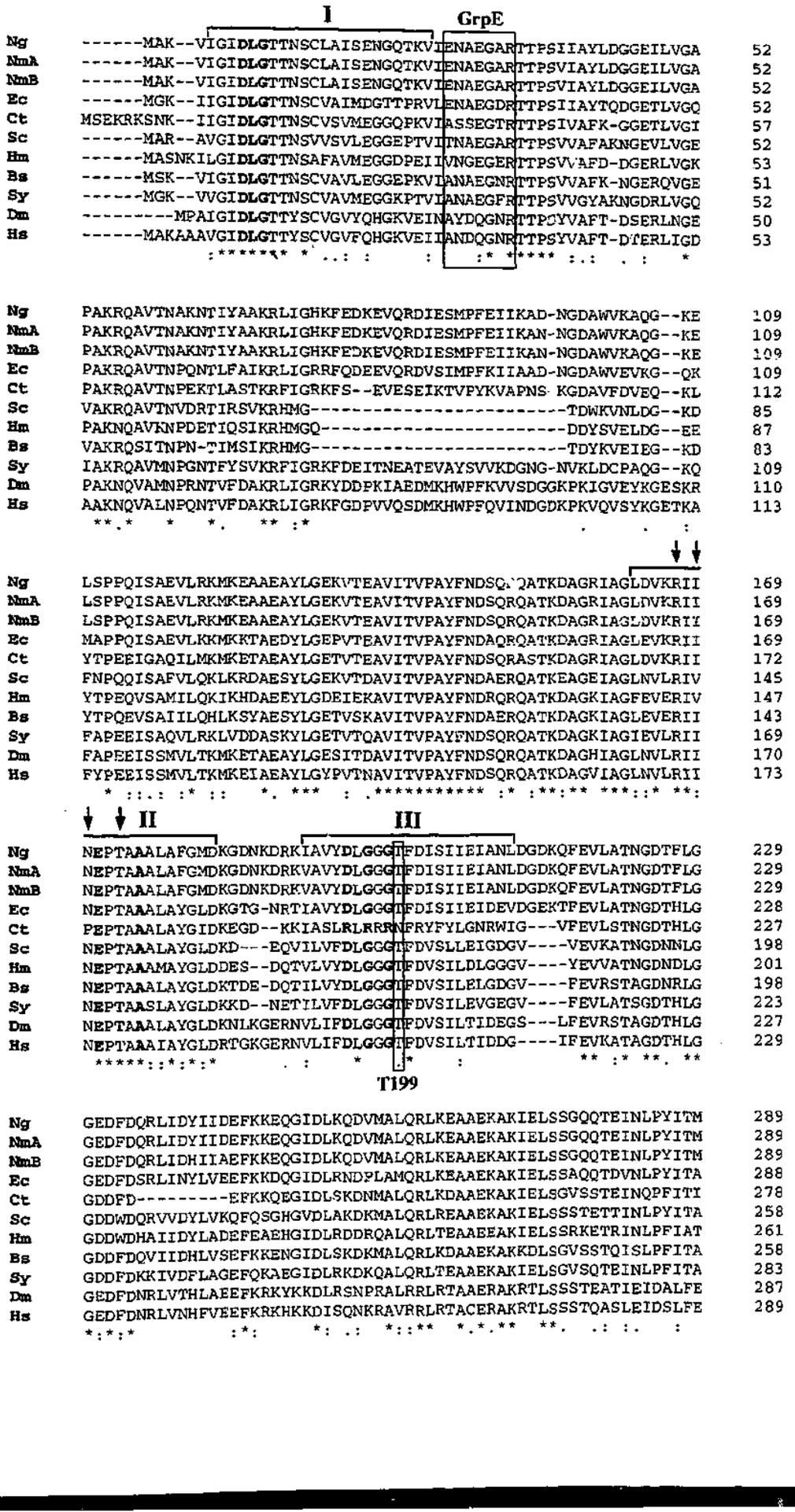
## 4.2 Identification of *dnaK* from *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strains MC58 and Z2491

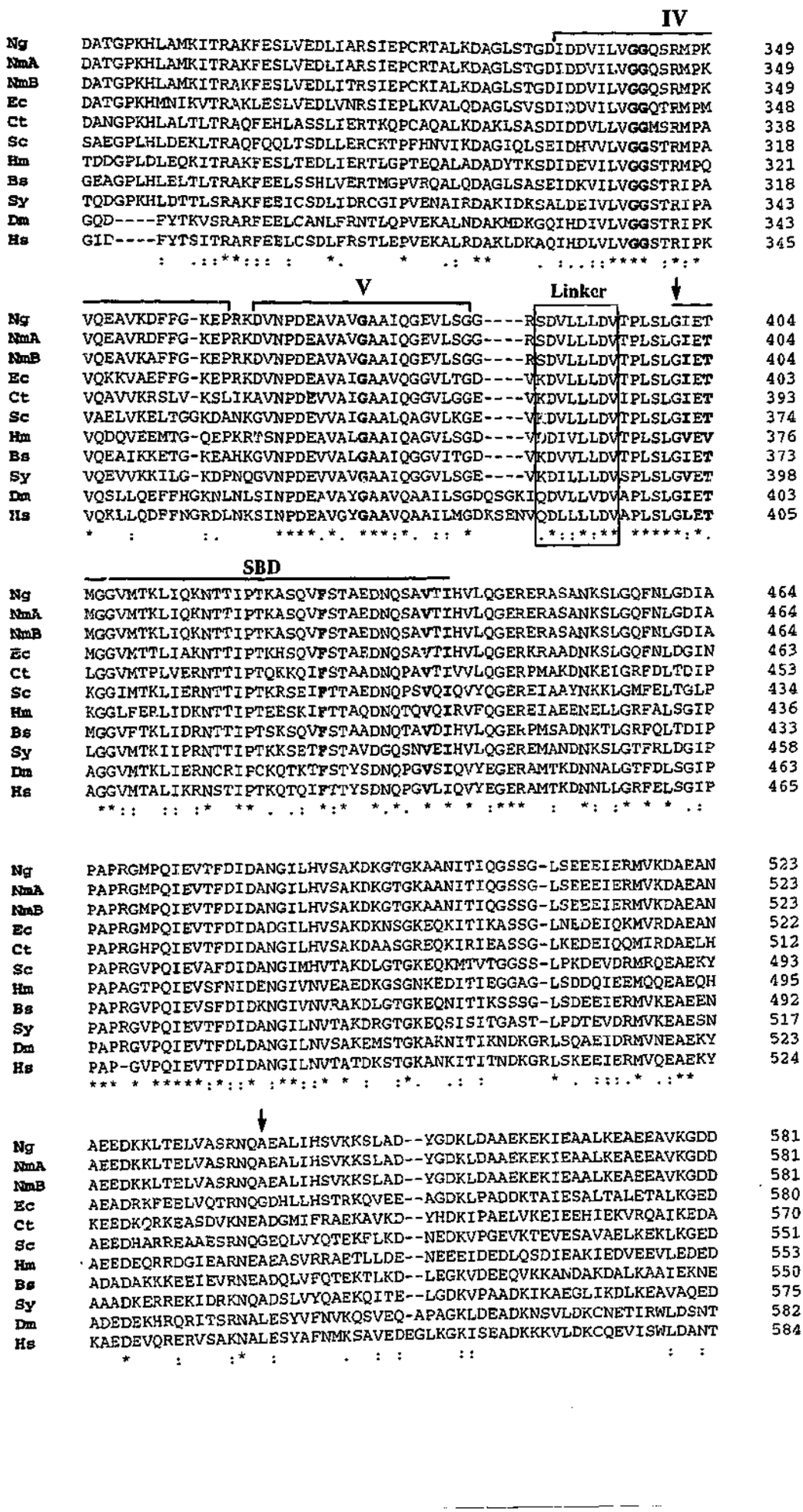
BLAST analysis of the *N. gonorrhoeae* strain FA1090 genomic database (Genbank accession number AE004969) revealed a region which, when translated, displayed significant similarity to the *E. coli* DnaK amino acid sequence (Bardwell and Craig, 1984) with a 72.4% identity in a 642 amino acid overlap (Figure 4.1). The gene was designated *dnaK* due to the high level of similarity. The gonococcal *dnaK* gene was 1926bp in length with the potential to encode a polypeptide of 643 amino acids with a predicted molecular mass of 70.7kDa (Figure 4.2). A potential RBS, AGGAG (Shine and Dalgarno, 1975), was located immediately upstream of the putative ATG start codon (Figure 4.2). A Rho-independent transcriptional terminator (d'Aubenton Carafa *et al.*, 1990) consisting of an 11bp inverted repeat was identified 17bp downstream of the stop codon. This could form a stable stem and loop structure with a $\Delta G$ value of -16 kcal/mol. A single copy of the 10bp neisserial DNA uptake sequence,

**Figure 4.1 Amino acid sequence comparison of the DnaK (Hsp70) proteins from a diverse spectrum of organisms.**

The DnaK (Hsp70) proteins of *N. gonorrhoeae* (Ng; Genbank accession number AE004969), *N. meningitidis* strains Z2491 (NmA; Parkhill *et al.*, 2000) and MC58 (NmB; Tettelin *et al.*, 2000), *E. coli* (Ec; Bardwell and Craig, 1984), *Chlamydia trachomatis* (Ct; Danilition *et al.*, 1990), *Streptomyces coelicolor* (Sc; Bucca *et al.*, 1993), *Halobacterium marismortui* (Hm; Gupta and Singh, 1992), *B. subtilis* (Bs; Wetzstein *et al.*, 1992), *Synechocystis* sp. (Sy; Chitnis and Nelson, 1991), *Drosophila melanogaster* (Dm; Ingolia *et al.*, 1980) and *Homo sapiens* (Hs; Hunt and Morimoto, 1985) were aligned with the aid of the CLUSTAL W program (Thompson *et al.*, 1994). Dashes (-) indicate gaps that have been introduced to maximise alignment. Residues in red text and the asterisk (*) represent identical residues. Conserved amino acids are indicated by green text and the colon (:) and residues with less similarity are depicted by blue text and the dot (.). Domains I-V denote the areas of structural similarity that confer ATPase activity to the N-terminal region of the protein (Bork *et al.*, 1992; Buchberger *et al.*, 1994). The most highly conserved residues located within these domains are indicated by bold type. The region that binds GrpE is boxed (Buchberger *et al.*, 1994) as is the linker region (Han and Christen, 2001) and the downward pointing arrows represent sites of DnaJ interaction (Gassler *et al.*, 1998; Suh *et al.*, 1998). The site of autophosphorylation (T199; McCarty and Walker, 1991) is boxed and the central substrate binding domain (SBD) is overlined (Zhu *et al.*, 1996). Amino acids are numbered at the right of the figure according to their position in each individual protein sequence.




I
GrpE
II
III
T199
Ng
NmA
NmB
Ec
Ct
Sc
Hm
Bs
Sy
Dm
Hs

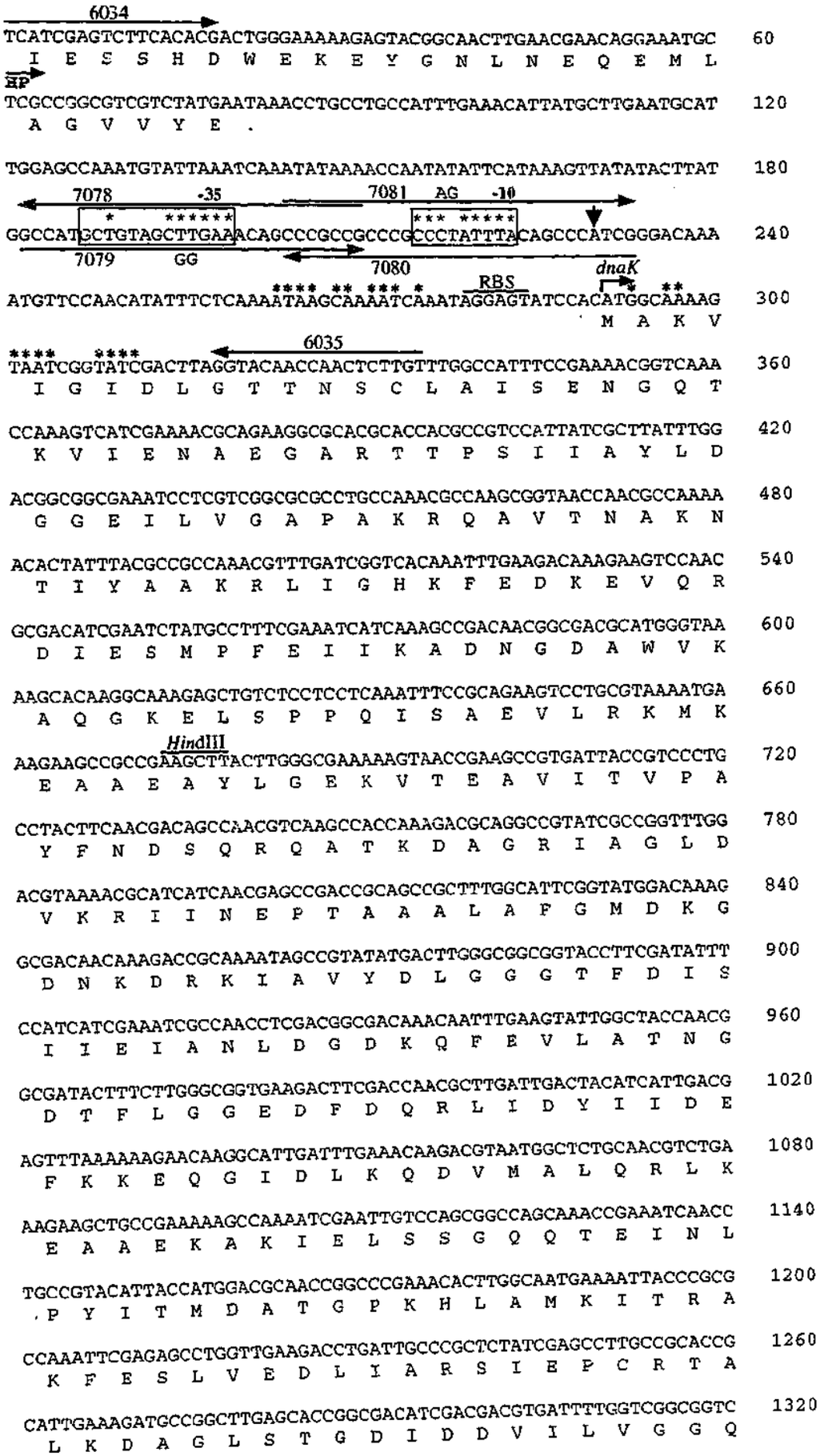

IV
V
Linker
SBD
Ng
NmA
NmB
Ec
Ct
Sc
Hm
Bs
Sy
Dm
Hs

```
Ng      ---KTAIDAKAEALGTASQKLGEMVYAQAQAEAQAGEG----------------------A   617
NmA     ---KTAIDAKAEALGTASQKLGEMVYAQAQAEAQAGEG----------------------A   617
NmB     ---KAAIDAKTEALGAASQKLGEMVYAQAQAEAQAGES----------------------E   617
Ec      ---KAAIEAKMQELAQVSQKL--MEIAQQQHAQQQTAG----------------------A   614
Ct      S--TTAIKAASDELSTHMQKIGEAMQAQSASAAASSAANAQGGPNINSEDLKKHSFSTRP   628
Sc      T---AEIRTATEKVAAVSQKLGQAMYAD-AQAAQAAG-------GEAPGADAG-------   593
Hm      AT-KEDYEAVTETLSEELQEIGKQMYQDQAQQAQAVPRALVRVARPAPGALPDRA----A   608
Bs      F---EEIKAKKDELQTIVQELSMKLYEEAAKAQQAQG------------GAN--------   587
Sy      ---DAKIQTVMPELQQVLYSIGSNMYQQAGAEAGVGAPG---AG---------------P   614
Dm      TAEKEEFDHKMEELTRHCSPIMTKMHQQGAGAAGGPGAN----------------------   621
Hs      LAEKDEFEHKRKELEQVCNPIISGLYQG-AGGPGPGGFG----------------------   622
                        :       :

Ng      QANASAKKDDDVVDADFEEVKDDKK--                                     642
NmA     QAESSAKKDDDIVDADFEEVKDDKK--                                     642
NmB     QANASAKKDDDVVDADFEEVKDDKK--                                     642
Ec      DASANNAKDDDVVDAEFEEVKDKK---                                     638
Ct      PAGGSASSTDNIEDADVEIVDKPE---                                     652
Sc      --AEGKGADDDVVDAEIVDDERKDGAA                                     618
Hm      QQAAAEQGAEEYVDADFEDVEESDEDE                                     635
Bs      ---AEGKADDNVVDAEYEEVNDDQNKK                                     611
Sy      EAGTSSGGGDDVIDAEFSEPEK-----                                     636
Dm      -CGQQAGGFGGYSGPTVEEVD------                                     641
Hs      -AQGPKGGSG--SGPTIEEVD------                                     640
                          ..      .
```

**Figure 4.2 Nucleotide sequence of the *dnaK* gene and adjacent sequences from *N. gonorrhoeae* FA1090 (Genbank accession number AE004969).**

The deduced amino acid sequence of *dnaK* is shown below the corresponding nucleotide sequence and the stop codon is depicted by a dot (.). HP refers to an ORF that displayed the highest similarity to a hypothetical protein in the Genbank database. Oligonucleotide primers are indicated by numbered arrows above or below the sequence. The coloured oligonucleotide primers indicate those used in site-directed mutagenesis and the purple coloured nucleotides represent the altered bases. The putative RBS is overlined as is the Neisserial DNA uptake sequence. The putative transcriptional terminator is denoted by dashed arrows. Restriction endonuclease sites are shown above the nucleotide sequence. The tsp (Figure 4.10) is indicated by an arrowhead and the associated $\sigma^{32}$ promoter sequences are boxed with asterisks (*) portraying the nucleotides identical to the consensus sequence (Cowing *et al.*, 1985). The red coloured nucleotides represent potential Fur binding regions and the asterisks (*) above them indicate the bases identical to the consensus sequence (Genco and Desai, 1996). Nucleotides are numbered at the right of the figure.


6034
TCATCGAGTCTTCACACGACTGGGAAAAAGAGTACGGCAACTTGAACGAACAGGAAATGC 60
I E S S H D W E K E Y G N L N E Q E M L
HP
TCGCCGGCGTCGTCTATGAATAAACCTGCCTGCCATTTGAAACATTATGCTTGAATGCAT 120
A G V V Y E .
TGGAGCCAAATGTATTAAATCAAATATAAAACCAATATATTCATAAAGTTATATACTTAT 180
7078 -35 7081 AG -10
GGCCATGCTGTAGCTTGAAACAGCCCGCCGCCCGCCCTATTTACAGCCCATCGGGACAAA 240
7079 GG 7080
dnaK
RBS
ATGTTCCAACATATTTCTCAAAATAAGCAAAATCAAATAGGAGTATCCACATGGCAAAAG 300
M A K V
6035
TAATCGGTATCGACTTAGGTACAACCAACTCTTGTTTGGCCATTTCCGAAAACGGTCAAA 360
I G I D L G T T N S C L A I S E N G Q T
CCAAAGTCATCGAAAACGCAGAAGGCGCACGCACCACGCCGTCCATTATCGCTTATTTGG 420
K V I E N A E G A R T T P S I I A Y L D
ACGGCGGCGAAATCCTCGTCGGCGCGCCTGCCAAACGCCAAGCGGTAACCAACGCCAAAA 480
G G E I L V G A P A K R Q A V T N A K N
ACACTATTTACGCCGCCAAACGTTTGATCGGTCACAAATTTGAAGACAAAGAAGTCCAAC 540
T I Y A A K R L I G H K F E D K E V Q R
GCGACATCGAATCTATGCCTTTCGAAATCATCAAAGCCGACAACGGCGACGCATGGGTAA 600
D I E S M P F E I I K A D N G D A W V K
AAGCACAAGGCAAAGAGCTGTCTCCTCCTCAAATTTCCGCAGAAGTCCTGCGTAAAATGA 660
A Q G K E L S P P Q I S A E V L R K M K
HindIII
AAGAAGCCGCCGAAGCTTACTTGGGCGAAAAAGTAACCGAAGCCGTGATTACCGTCCCTG 720
E A A E A Y L G E K V T E A V I T V P A
CCTACTTCAACGACAGCCAACGTCAAGCCACCAAAGACGCAGGCCGTATCGCCGGTTTGG 780
Y F N D S Q R Q A T K D A G R I A G L D
ACGTAAAACGCATCATCAACGAGCCGACCGCAGCCGCTTTGGCATTCGGTATGGACAAAG 840
V K R I I N E P T A A A L A F G M D K G
GCGACAACAAAGACCGCAAAATAGCCGTATATGACTTGGGCGGCGGTACCTTCGATATTT 900
D N K D R K I A V Y D L G G G T F D I S
CCATCATCGAAATCGCCAACCTCGACGGCGACAAACAATTTGAAGTATTGGCTACCAACG 960
I I E I A N L D G D K Q F E V L A T N G
GCGATACTTTCTTGGGCGGTGAAGACTTCGACCAACGCTTGATTGACTACATCATTGACG 1020
D T F L G G E D F D Q R L I D Y I I D E
AGTTTAAAAAAGAACAAGGCATTGATTTGAAACAAGACGTAATGGCTCTGCAACGTCTGA 1080
F K K E Q G I D L K Q D V M A L Q R L K
AAGAAGCTGCCGAAAAAGCCAAAATCGAATTGTCCAGCGGCCAGCAAACCGAAATCAACC 1140
E A A E K A K I E L S S G Q Q T E I N L
TGCCGTACATTACCATGGACGCAACCGGCCCGAAACACTTGGCAATGAAAATTACCCGCG 1200
P Y I T M D A T G P K H L A M K I T R A
CCAAATTCGAGAGCCTGGTTGAAGACCTGATTGCCCGCTCTATCGAGCCTTGCCGCACCG 1260
K F E S L V E D L I A R S I E P C R T A
CATTGAAAGATGCCGGCTTGAGCACCGGCGACATCGACGACGTGATTTTGGTCGGCGGTC 1320
L K D A G L S T G D I D D V I L V G G Q

```
AGTCCCGTATGCCGAAAGTACAAGAAGCCGTTAAAGACTTCTTCGGCAAAGAACCGCGCA   1380
  S  R  M  P  K  V  Q  E  A  V  K  D  F  F  G  K  E  P  R  K

AAGACGTGAACCCTGACGAAGCCGTTGCCGTAGGTGCAGCGATTCAAGGCGAAGTATTGA   1440
  D  V  N  P  D  E  A  V  A  V  G  A  A  I  Q  G  E  V  L  S

GCGGCGGCCGCAGCGACGTATTGCTGCTGGACGTAACCCCTCTGTCCTTGGGTATCGAAA   1500
  G  G  R  S  D  V  L  L  L  D  V  T  P  L  S  L  G  I  E  T

CCATGGGTGGTGTGATGACCAAGCTGATTCAAAAGAACACTACTATTCCGACTAAAGCGT   1560
  M  G  G  V  M  T  K  L  I  Q  K  N  T  T  I  P  T  K  A  S

CTCAAGTGTTCTCTACTGCCGAAGACAACCAAAGCGCAGTAACCATCCACGTACTGCAAG   1620
  Q  V  F  S  T  A  E  D  N  Q  S  A  V  T  I  H  V  L  Q  G

GCGAACGCGAACGCGCTTCTGCCAACAAATCTTTAGGTCAATTCAACTTGGGCGACATCG   1680
  E  R  E  R  A  S  A  N  K  S  L  G  Q  F  N  L  G  D  I  A

CACCCGCACCACGCGGTATGCCGCAAATTGAAGTTACTTTCGACATCGACGCCAATGGTA   1740
  P  A  P  R  G  M  P  Q  I  E  V  T  F  D  I  D  A  N  G  I

TCTTGCACGTTTCCGCCAAAGACAAAGGCACTGGCAAAGCGGCCAACATTACCATCCAAG   1800
  L  H  V  S  A  K  D  K  G  T  G  K  A  A  N  I  T  I  Q  G

GTTCTTCAGGTTTGAGCGAAGAAGAAATCGAACGCATGGTGAAAGATGCCGAAGCCAATG   1860
  S  S  G  L  S  E  E  E  I  E  R  M  V  K  D  A  E  A  N  A

CCGAGGAAGATAAAAAGCTGACTGAATTGGTCGCTTCCCGCAACCAAGCCGAAGCCCTGA   1920
  E  E  D  K  K  L  T  E  L  V  A  S  R  N  Q  A  E  A  L  I

TTCACTCCGTGAAAAAATCTTTGGCGGACTACGGCGACAAACTCGACGCTGCCGAGAAAG   1980
  H  S  V  K  K  S  L  A  D  Y  G  D  K  L  D  A  A  E  K  E

AAAAAATCGAAGCCGCGCTGAAAGAAGCCGAAGAAGCCGTGAAAGGCGACGACAAAACCG   2040
  K  I  E  A  A  L  K  E  A  E  E  A  V  K  G  D  D  K  T  A

       ClaI
CCATCGATGCCAAAGCCGAAGCACTGGGTACAGCCAGCCAAAAACTGGGCGAAATGGTTT   2100
  I  D  A  K  A  E  A  L  G  T  A  S  Q  K  L  G  E  M  V  Y

ACGCGCAAGCGCAAGCCGAAGCCCAAGCCGGCGAGGGCGCACAAGCCAATGCTTCTGCAA   2160
  A  Q  A  Q  A  E  A  Q  A  G  E  G  A  Q  A  N  A  S  A  K

AGAAAGACGATGATGTCGTAGATGCCGACTTTGAAGAAGTAAAAGACGACAAAAAATAAT   2220
  K  D  D  D  V  V  D  A  D  F  E  E  V  K  D  D  K  K  .

TGATGCCGTCTGAAAAAAACGCGAACCATTCGGTTCGCTTTTTTTTCAATTGAGATAAAA   2280

GACAATAGCA                                                     2290
```

5' -GCCGTCTGAA- 3' (Goodman and Scocca, 1988), was located immediately downstream of the stop codon.

The gonoccocal DnaK homologue exhibited a very high degree of sequence identity with the DnaK (Hsp70) proteins from members of the phylogenetic domains *Bacteria*, *Eucarya* and *Archaea* ranging from 72.4% with *E. coli* to 46.6% with *Homo sapiens* (Figure 4.1; Table 4.1), in accordance with the highly conserved nature of this protein. DnaK homologues were also identified from the two meningococcal strains sequenced to date, including strains Z2491 (Parkhill *et al.*, 2000) and MC58 (Tettelin *et al.*, 2000) and comparison with the gonococcal DnaK protein revealed identities of 97.8% and 98.9%, respectively (Table 4.1).

Analysis of the neisserial DnaK homologues reveals that the functional signature sequences characteristic of this protein have been conserved. Residues essential for activity within the N-terminal ATPase domain that constitute the ATP binding site, active sites for ATP hydrolysis, structural residues and coupling of the DnaK ATPase activity with substrate release (Figure 4.1; Bork *et al.*, 1992; Buchberger *et al.*, 1994) have been conserved. This includes the site of autophosphorylation, the threonine residue at position 199 in the *E. coli* sequence (Figure 4.1; McCarty and Walker, 1991). In addition the amino acids that form the hydrophobic pocket that binds substrate within the central substrate binding domain (Zhu *et al.*, 1996) have been retained in the neisserial DnaK proteins, as have the regions involved in DnaJ (Gassler *et al.*, 1998; Suh *et al.*, 1998) and GrpE (Buchberger *et al.*, 1994) interaction (Figure 4.1). The highly conserved region that links the ATPase domain and substrate binding domain has been conserved (Figure 4.1; Han and Christen, 2001). The fact that the C-terminal 100 amino acids are less conserved is not uncommon as this region is not essential for the activity of the protein (Buchberger *et al.*, 1995) but provides structural support (Han and Christen, 2001).

Homology searches of the flanking regions of the gonococcal *dnaK* homologue identified ORFs where the derived amino acid sequence displayed significant similarity to those of known or putative gene products deposited in the Genbank database (Figure 4.3). The arrangement upstream was identical to that identified in *N. meningitidis* strains MC58 (Tettelin *et al.*, 2000) and Z2491 (Parkhill *et al.*, 2000), however sequence

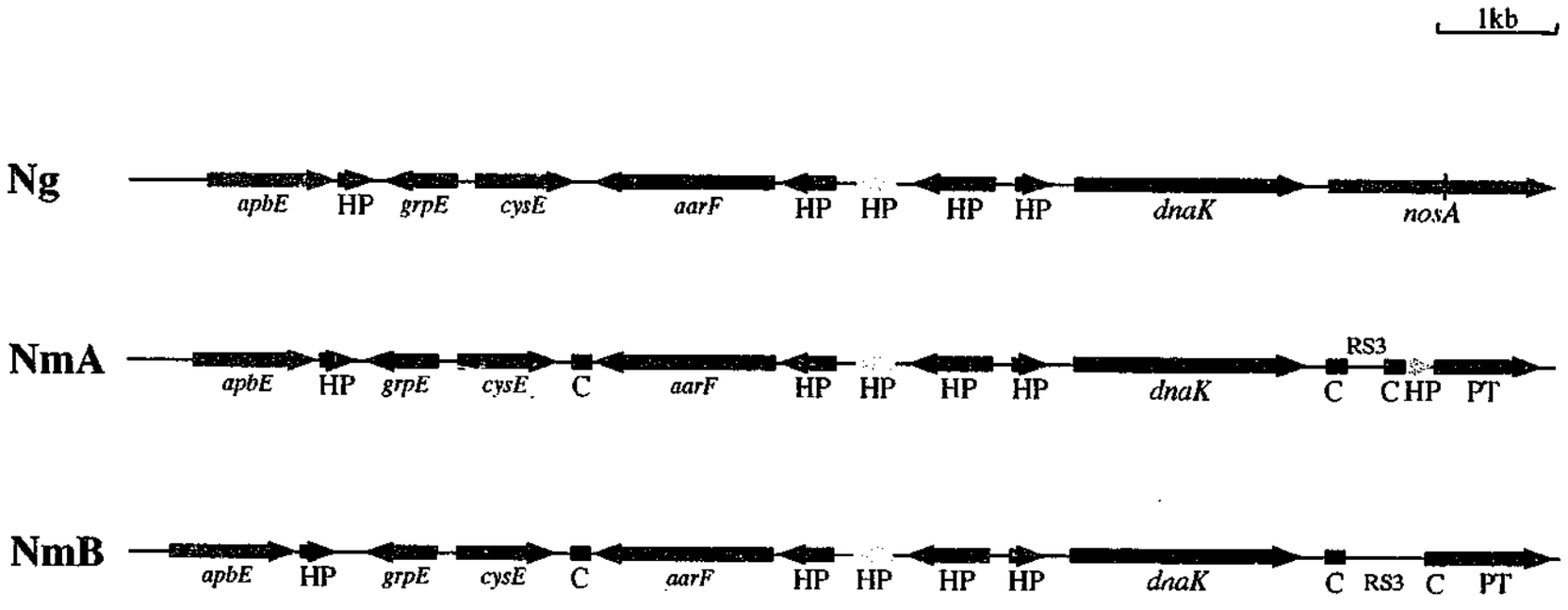
**Table 4.1 Amino acid comparison of the DnaK, DnaJ and GrpE proteins from *N. gonorrhoeae* FA1090 (Genbank accession number AE004969) with their homologues in other organisms.**

Alignments were performed with the CLUSTAL W program (Thomson *et al.*, 1994) and are expressed as percentage identity. Sequences were obtained through the Genbank database using the accession numbers listed. Sequences not available are referred to as N.A.

| Organism | Identity (%) | | | Accession Number |
|---|---|---|---|---|
| | DnaK | DnaJ | GrpE | |
| *N. meningitidis* (A) | 98.9 | 98.7 | 99.5 | AL162754, AL162752 |
| *N. meningitidis* (B) | 97.8 | 98.7 | 99.5 | AE002411, AE002365, AE002412 |
| *E. coli* | 72.4 | 59.1 | 34.3 | K01298, M12565, X07863 |
| *C. trachomatis* | 54.5 | 44.3 | 24.2 | M27580, AE001307, AE001312 |
| *S. coelicolor* | 54.9 | 38.3 | 19.5 | L46700 |
| *H. marismortui* | 49.2 | NA | NA | M84006 |
| *B. subtilis* | 56.7 | 49.2 | 29.7 | M84964 |
| *Synechocystis* sp. | 56.1 | 41.7 | 21.6 | M57518, D64006, D64001 |
| *D. melanogaster* | 46.7 | 26.3 | 28.7 | J01104, X95247, U34903 |
| *Homo sapiens* | 46.6 | 31.2 | 28.9 | M11717, L08069, AAG31605 |

**Figure 4.3 ORFs found flanking the *dnaK* and *grpE* genes of *N. gonorrhoeae* FA1090 (Ng; Genbank accession number AE004969) and *N. meningitidis* strains Z2491 (NmA; Parkhill *et al.*, 2000) and MC58 (NmB; Tettelin *et al.*, 2000).**

Schematic diagram indicating the orientation of putative ORFs named after the most similar known or putative genes in the Genbank database. HP indicates ORFs that displayed the highest similarity to hypothetical proteins in the database. PT refers to the ORFs that encode a putative transposase which in *N. meningitidis* MC58 contains a point mutation (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). C denotes the Correia element (Correia *et al.*, 1986) found in the meningococcal strains, and RS3 denotes a region containing RS3 repeats (Haas and Meyer, 1986). The black vertical line represents a translational frame shift in the gonococcal *nosA* sequence. ORFs with the same colour represent highly related sequences.


1kb
Ng
apbE
HP
grpE
cysE
aarF
HP
HP
HP
HP
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nosA
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RS3
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C
HP
PT
NmB
apbE
HP
grpE
cysE
C
aarF
HP
HP
HP
HP
dnaK
C
RS3
C
PT

downstream of the gonococcal *dnaK* gene diverged. Surprisingly, *dnaK* was not organised in an operon with either *dnaJ* or *grpE*, an arrangement detected in most bacteria (Segal and Ron, 1996b).

## 4.3 Identification of *dnaJ* from *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strains MC58 and Z2491

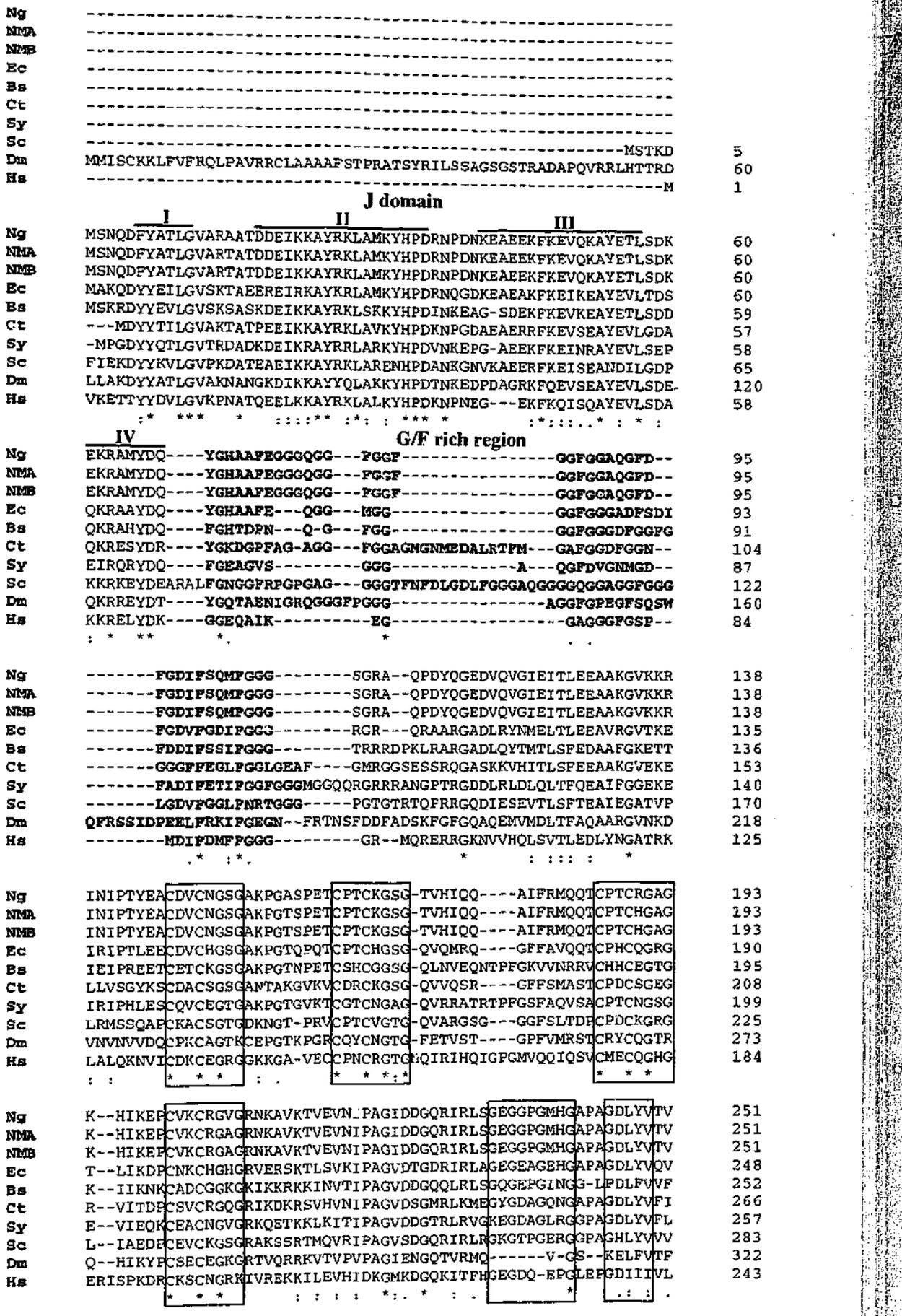
The gonococcal *dnaJ* homologue was identified by BLAST analysis of the *N. gonorrhoeae* strain FA1090 genomic database (Genbank accession number AE004969) with the *E. coli dnaJ* sequence (Bardwell *et al.*, 1986). The region identified, when translated, displayed 59.1% identity in a 381 amino acid overlap (Figure 4.4), and hence was designated *dnaJ*. The gonococcal *dnaJ* gene was 1119bp in length with the potential to encode a polypeptide of 373 amino acids with a predicted molecular mass of 41kDa (Figure 4.5). A potential RBS, AGATGA (Shine and Dalgarno, 1975) was located 4bp upstream of the putative ATG start codon (Figure 4.5). A potential Rho-independent transcriptional terminator (d'Aubenton Carafa *et al.*, 1990) was located 20bp downstream of the stop codon. The 13bp imperfect inverted repeat could form a stable stem and loop structure with a $\Delta G$ value of -24.8 kcal/mol.

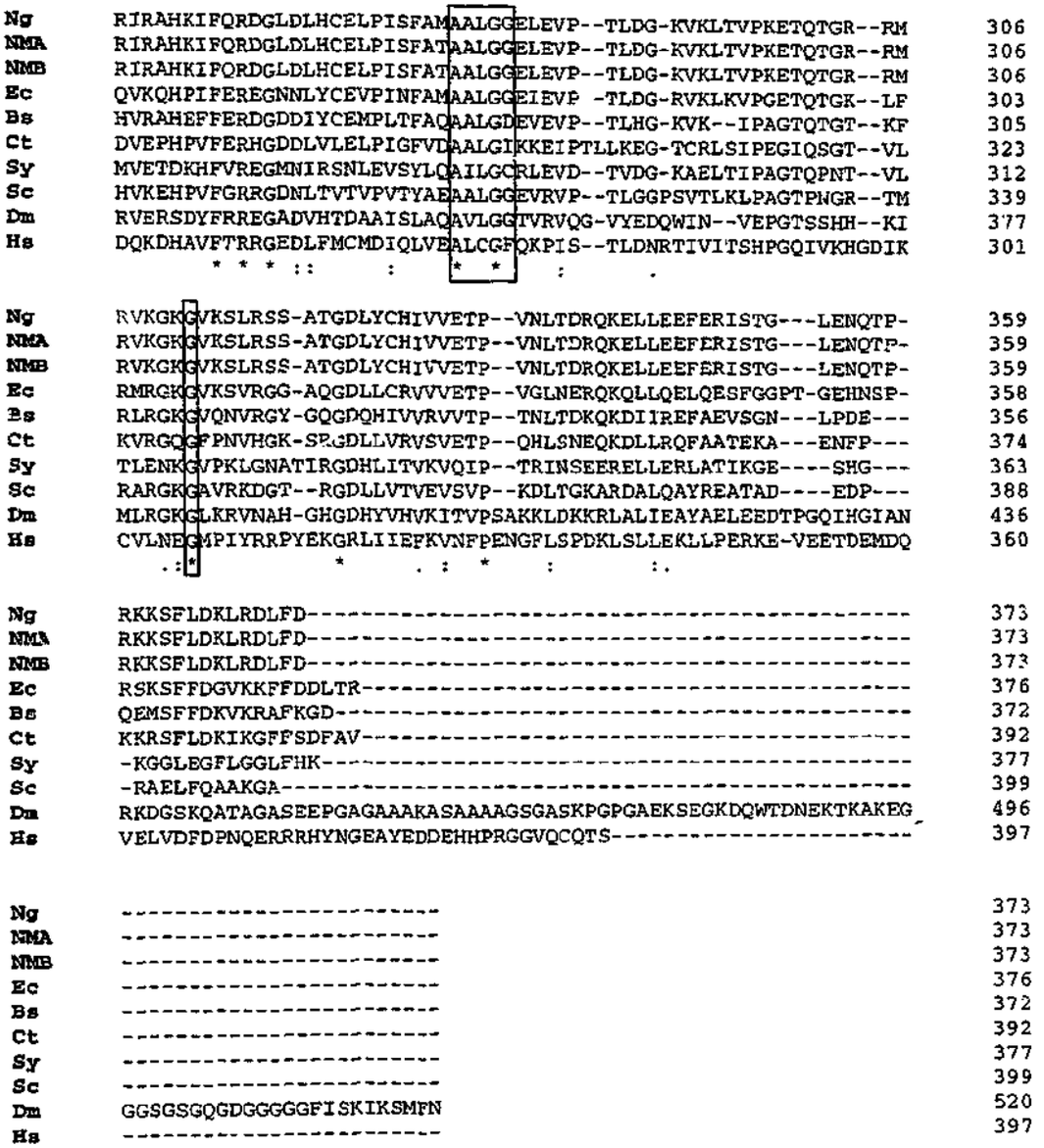
The gonoccocal DnaJ homologue exhibited a very high degree of sequence identity with the DnaJ (Hsp40) proteins from members of the domains *Bacteria*, *Eucarya* and *Archaea* ranging from 59.1% with *E. coli* to 26.3% with *D. melanogaster* (Figure 4.4; Table 4.1). In addition, DnaJ homologues were identified from the two meningococcal strains, Z2491 (Parkhill *et al.*, 2000) and MC58 (Tettelin *et al.*, 2000), both displaying 98.7% identity to the gonococcal DnaJ homologue (Figure 4.4; Table 4.1).

The structural organisation of the neisserial DnaJ proteins is characteristic of typical DnaJ (Hsp40) proteins with the following conserved features (Figure 4.4). The amino terminal consists of a 70 amino acid J domain, involved in binding to and regulation of the ATP hydrolytic cycle of DnaK (Cheetham and Caplan, 1998; Suh *et al.*, 1998; Tsai and Douglas, 1996), that forms four α-helices and a loop region (Pellecchia *et al.*, 1996; Szyperski *et al.*, 1994) and contains the highly conserved HPD motif (Figure 4.4). This is followed by a 35 amino acid region comprising approximately 50% glycine and phenylalanine residues that functions as a spacer (Cyr *et al.*, 1994) and is required in

**Figure 4.4 Amino acid sequence comparison of the DnaJ (Hsp40) proteins from a variety of organisms.**

The DnaJ (Hsp40) proteins of *N. gonorrhoeae* (Genbank accession number AE004969), *N. meningitidis* strains Z2491 (NmA; Parkhill *et al.*, 2000) and MC58 (NmB; Tettelin *et al.*, 2000), *E. coli* (Ec; Bardwell *et al.*, 1986), *B. subtilis* (Bs; Wetzstein *et al.*, 1992), *C. trachomatis* (Ct; Stephens *et al.*, 1998), *Synechocystis* sp. (Sy; Kaneko *et al.*, 1996), *S. coelicolor* (Sc; Bucca *et al.*, 1993), *D. melanogaster* (Dm; Kurzik-Dumke *et al.*, 1998) and *Homo sapiens* (Hs; Chellaiah *et al.*, 1993) were aligned using the CLUSTAL W program (Thompson *et al.*, 1994). Dashes (-) indicate gaps that have been introduced to maximise alignment. Amino acids coloured in red text and the asterisk (*) represent identical residues and conserved residues are depicted by green text and the colon (:). Amino acids with less similarity are indicated by blue text and the dot (.). Domains I-IV of the J domain form four α-helical structures (Pellecchia *et al.*, 1996; Szyperski *et al.*, 1994). The G/F rich region in bold type consists of approximately 50% glycine and phenylalanine residues (Cyr *et al.*, 1994). The boxed regions identify the zinc finger with four repeats of the sequence, CXXCXGXG, where X denotes any amino acid (Lu and Cyr, 1998; Szabo *et al.*, 1996). The boxed regions coloured in yellow are thought to be involved in substrate binding and chaperone activity (Goffin and Georgopoulos, 1998; Lu and Cyr, 1998). Amino acids are numbered at the right of the figure according to their position in each individual protein sequence.


J domain
I
II
III
IV
G/F rich region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NMA RIRAHKIFQRDGLDLHCELPISFATAALGGELEVP--TLDG-KVKLTVPKETQTGR--RM 306
NMB RIRAHKIFQRDGLDLHCELPISFATAALGGELEVP--TLDG-KVKLTVPKETQTGR--RM 306
Ec QVKQHPIFEREGNNLYCEVPINFAMAALGGEIEVP -TLDG-RVKLKVPGETQTGK--LF 303
Bs HVRAHEFFERDGDDIYCEMPLTFAQAALGDEVEVP--TLHG-KVK--IPAGTQTGT--KF 305
Ct DVEPHPVFERHGDDLVLELPIGFVLAALGIKKEIPTLLKEG-TCRLSIPEGIQSGT--VL 323
Sy MVETDKHFVREGMNIRSNLEVSYLQAILGCRLEVD--TVDG-KAELTIPAGTQPNT--VL 312
Sc HVKEHPVFGRRGDNLTVTVPVTYAEAALGGEVRVP--TLGGPSVTLKLPAGTPNGR--TM 339
Dm RVERSDYFRREGADVHTDAAISLAQAVLGGTVRVQG-VYEDQWIN--VEPGTSSHH--KI 377
Hs DQKDHAVFTRRGEDLFMCMDIQLVEALCGFQKPIS--TLDNRTIVITSHPGQIVKHGDIK 301
RVKGKGVKSLRSS-ATGDLYCHIVVETP--VNLTDRQKELLEEFERISTG---LENQTP- 359
RVKGKGVKSLRSS-ATGDLYCHIVVETP--VNLTDRQKELLEEFERISTG---LENQTP- 359
RVKGKGVKSLRSS-ATGDLYCHIVVETP--VNLTDRQKELLEEFERISTG---LENQTP- 359
RMRGKGVKSVRGG-AQGDLLCRVVVETP--VGLNERQKQLLQELQESFGGPT-GEHNSP- 358
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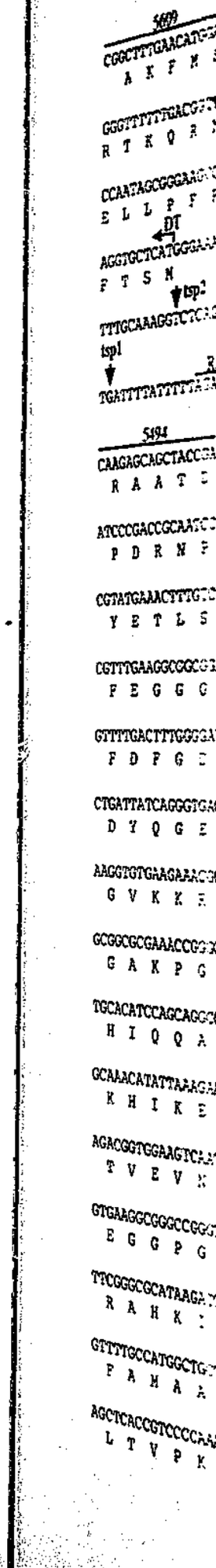
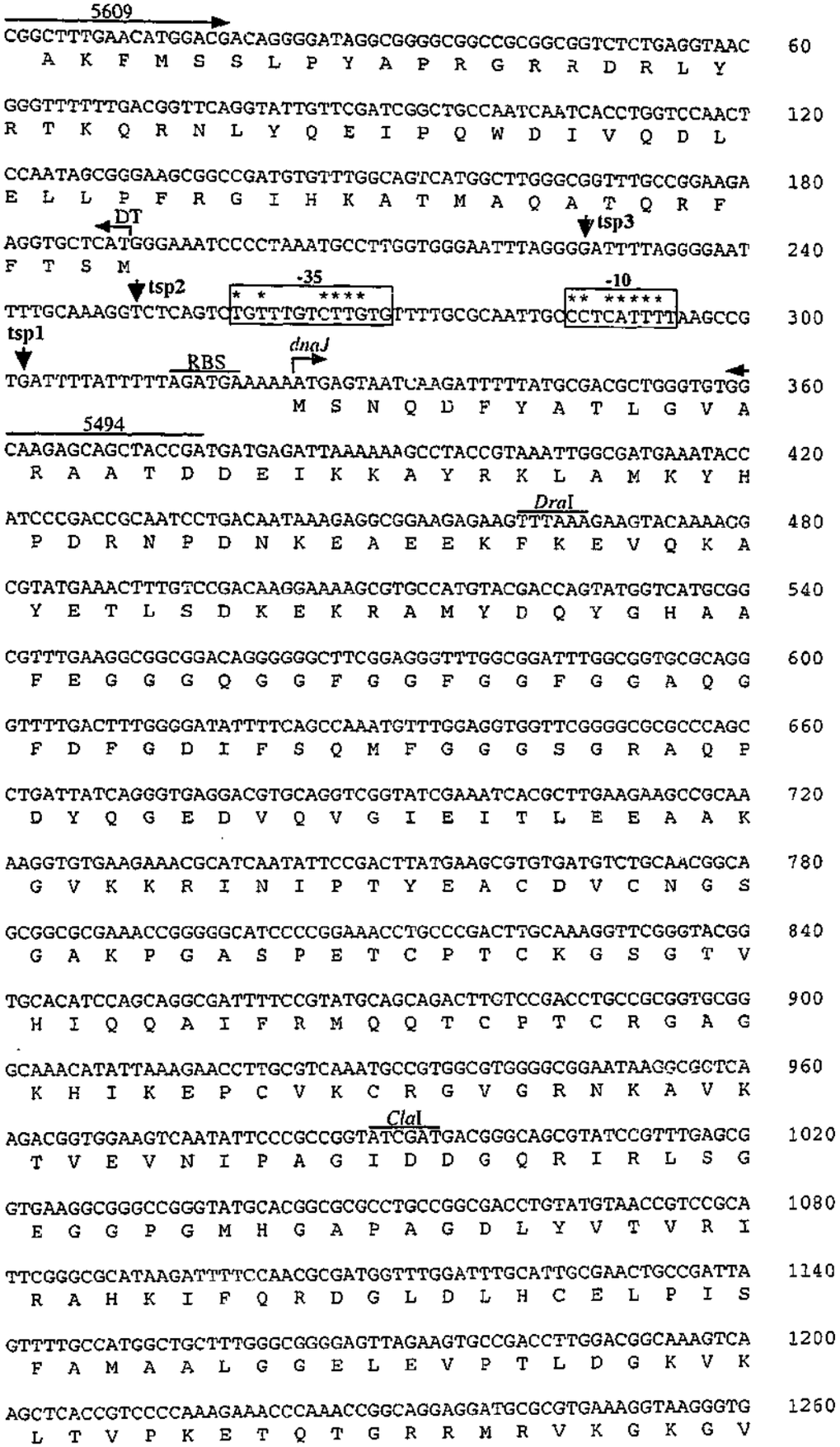
KVRGQGFPNVHGK-SRGDLLVRVSVETP--QHLSNEQKDLLRQFAATEKA---ENFP--- 374
TLENKGVPKLGNATIRGDHLITVKVQIP--TRINSEERELLERLATIKGE----SHG--- 363
RARGKGAVRKDGT--RGDLLVTVEVSVP--KDLTGKARDALQAYREATAD----EDP--- 388
MLRGKGLKRVNAH-GHGDHYVHVKITVPSAKKLDKKRLALIEAYAELEEDTPGQIHGIAN 436
CVLNEGMPIYRRPYEKGRLIIEFKVNFPENGFLSPDKLSLLEKLLPERKE-VEETDEMDQ 360
RKKSFLDKLRDLFD---------- 373
RKKSFLDKLRDLFD---------- 373
RKKSFLDKLRDLFD---------- 373
RSKSFFDGVKKFFDDLTR---------- 376
QEMSFFDKVKRAFKGD---------- 372
KKRSFLDKIKGFFSDFAV---------- 392
-KGGLEGFLGGLFHK---------- 377
-RAELFQAAKGA---------- 399
RKDGSKQATAGASEEPGAGAAAKASAAAAGSGASKPGPGAEKSEGKDQWTDNEKTKAKEG 496
VELVDFDPNQERRRHYNGEAYEDDEHHPRGGVQCQTS---------- 397
---------- 373
---------- 373
---------- 373
---------- 376
---------- 372
---------- 392
---------- 377
---------- 399
GGSGSGQGDGGGGGFISKIKSMFN 520
---------- 397

**Figure 4.5 Nucleotide sequence of the *dnaJ* gene and flanking regions from *N. gonorrhoeae* FA1090 (Genbank accession number AE004969).**

The deduced amino acid sequences of *dnaJ* and the hypothetical IS1106A2 transposase remnant (TR; Parkhill *et al.*, 2000) are shown below the corresponding nucleotide sequences. The stop codon is indicated by a dot (.). The putative ribosome binding site (RBS) is overlined and the putative transcriptional terminator is represented by dashed arrows. Oligonucleotide primers used are depicted by numbered arrows above or below the nucleotide sequence. Restriction endonuclease sites are shown above the nucleotide sequence. The tsps (Figure 4.11) are denoted by arrowheads and the corresponding $\sigma^{32}$ promoter sequence for tsp1 is boxed with asterisks (*) indicating the nucleotides identical to the consensus sequence (Cowing *et al.*, 1985). Nucleotides are numbered to the right of the figure.




5609
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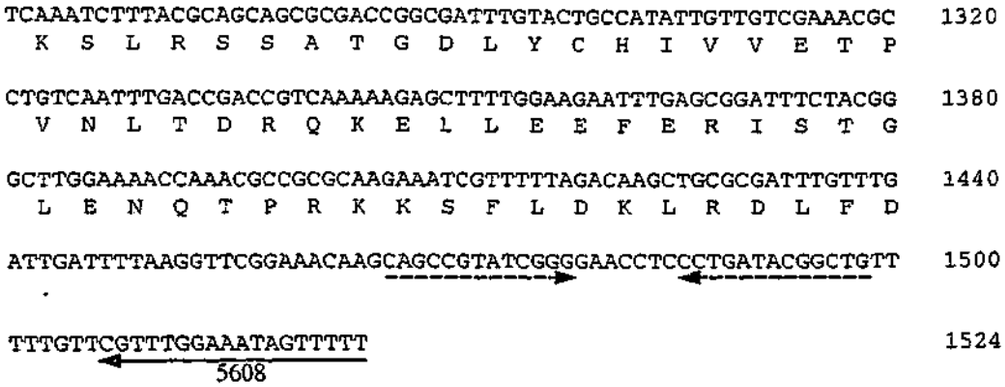
A K F M S S L P Y A P R G R R D R L Y
GGGTTTTTTGACGGTTCAGGTATTGTTCGATCGGCTGCCAATCAATCACCTGGTCCAACT 120
R T K Q R N L Y Q E I P Q W D I V Q D L
CCAATAGCGGGAAGCGGCCGATGTGTTTGGCAGTCATGGCTTGGGCGGTTTGCCGGAAGA 180
E L L P F R G I H K A T M A Q A T Q R F
DT
tsp3
AGGTGCTCATGGGAAATCCCCTAAATGCCTTGGTGGGAATTTAGGGGATTTTAGGGGAAT 240
F T S M
tsp2
-35
-10
TTTGCAAAGGTCTCAGTCTGTTTGTCTTGTGTTTTGCGCAATTGCCCTCATTTTAAGCCG 300
tsp1
dnaJ
RBS
TGATTTTATTTTTAGATGAAAAAATGAGTAATCAAGATTTTTATGCGACGCTGGGTGTGG 360
M S N Q D F Y A T L G V A
5494
CAAGAGCAGCTACCGATGATGAGATTAAAAAAGCCTACCGTAAATTGGCGATGAAATACC 420
R A A T D D E I K K A Y R K L A M K Y H
DraI
ATCCCGACCGCAATCCTGACAATAAAGAGGCGGAAGAGAAGTTTAAAGAAGTACAAAACG 480
P D R N P D N K E A E E K F K E V Q K A
CGTATGAAACTTTGTCCGACAAGGAAAAGCGTGCCATGTACGACCAGTATGGTCATGCGG 540
Y E T L S D K E K R A M Y D Q Y G H A A
CGTTTGAAGGCGGCGGACAGGGGGGCTTCGGAGGGTTTGGCGGATTTGGCGGTGCGCAGG 600
F E G G G Q G G F G G F G G F G G A Q G
GTTTTGACTTTGGGGATATTTTCAGCCAAATGTTTGGAGGTGGTTCGGGGCGCGCCCAGC 660
F D F G D I F S Q M F G G G S G R A Q P
CTGATTATCAGGGTGAGGACGTGCAGGTCGGTATCGAAATCACGCTTGAAGAAGCCGCAA 720
D Y Q G E D V Q V G I E I T L E E A A K
AAGGTGTGAAGAAACGCATCAATATTCCGACTTATGAAGCGTGTGATGTCTGCAACGGCA 780
G V K K R I N I P T Y E A C D V C N G S
GCGGCGCGAAACCGGGGGCATCCCCGGAAACCTGCCCGACTTGCAAAGGTTCGGGTACGG 840
G A K P G A S P E T C P T C K G S G T V
TGCACATCCAGCAGGCGATTTTCCGTATGCAGCAGACTTGTCCGACCTGCCGCGGTGCGG 900
H I Q Q A I F R M Q Q T C P T C R G A G
GCAAACATATTAAAGAACCTTGCGTCAAATGCCGTGGCGTGGGGCGGAATAAGGCGCTCA 960
K H I K E P C V K C R G V G R N K A V K
ClaI
AGACGGTGGAAGTCAATATTCCCGCCGGTATCGATGACGGGCAGCGTATCCGTTTGAGCG 1020
T V E V N I P A G I D D G Q R I R L S G
GTGAAGGCGGGCCGGGTATGCACGGCGCGCCTGCCGGCGACCTGTATGTAACCGTCCGCA 1080
E G G P G M H G A P A G D L Y V T V R I
TTCGGGCGCATAAGATTTTCCAACGCGATGGTTTGGATTTGCATTGCGAACTGCCGATTA 1140
R A H K I F Q R D G L D L H C E L P I S
GTTTTGCCATGGCTGCTTTGGGCGGGGAGTTAGAAGTGCCGACCTTGGACGGCAAAGTCA 1200
F A M A A L G G E L E V P T L D G K V K
AGCTCACCGTCCCCAAAGAAACCCAAACCGGCAGGAGGATGCGCGTGAAAGGTAAGGGTG 1260
L T V P K E T Q T G R R M R V K G K G V


TCAAATCTTTACGCAGCAGCGCGACCGGCGATTTGTACTGCCATATTGTTGTCGAAACGC 1320
K S L R S S A T G D L Y C H I V V E T P
CTGTCAATTTGACCGACCGTCAAAAAGAGCTTTTGGAAGAATTTGAGCGGATTTCTACGG 1380
V N L T D R Q K E L L E E F E R I S T G
GCTTGGAAAACCAAACGCCGCGCAAGAAATCGTTTTTAGACAAGCTGCGCGATTTGTTTG 1440
L E N Q T P R K K S F L D K L R D L F D
ATTGATTTTAAGGTTCGGAAACAAGCAGCCGTATCGGGGAACCTCCCTGATACGGCTGTT 1500
TTTGTTCGTTTGGAAATAGTTTTT 1524
5608

conjunction with the J domain for optimal interaction with DnaK (Figure 4.4; Karzai and McMacken, 1996; Wall *et al.*, 1995). A 50 residue region then follows containing a zinc finger with four repeats of the sequence, CXXCXGXG, responsible for binding to some denatured protein substrates and suppressing protein aggregation (Figure 4.4; Lu and Cyr, 1998; Szabo *et al.*, 1996). The carboxy-terminus is less conserved and also thought to be involved in binding to some substrates and conferring chaperone activity (Goffin and Georgopoulos, 1998; Lu and Cyr, 1998).

BLAST analysis of sequence adjacent to the gonococcal *dnaJ* gene revealed putative ORFs where the derived amino acid sequence displayed significant similarity to those of known or putative gene products deposited in the Genbank database (Figure 4.6). This configuration was almost identical to that in *N. meningitidis* strains Z2491 (Parkhill *et al.*, 2000) and MC58 (Tettelin *et al.*, 2000) with the exception of a truncated copy of the IS1106A2 transposase (Parkhill *et al.*, 2000) located immediately upstream of the gonococcal *dnaJ* gene . Interestingly, the neisserial *dnaJ* gene is not arranged in an operon with any other stress related genes, as occurs in the majority of bacteria investigated (Segal and Ron, 1996b).

## 4.4 Identification of *grpE* from *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strains MC58 and Z2491

BLAST analysis of the *N. gonorrhoeae* strain FA1090 genomic database (Genbank accession number AE004969) with the *E. coli* GrpE amino acid sequence (Lipinska *et al.*, 1988) revealed a region that displayed significant similarity when translated (Figure 4.7). The ORF was therefore designated *grpE*. The gonococcal *grpE* gene was 576bp in length, and could encode a deduced polypeptide of 192 amino acids with a molecular mass of 21.1kDa (Figure 4.8). A potential RBS, AGGGA (Shine and Dalgarno, 1975), was found 6bp upstream of the start codon. A putative Rho-independent transcriptional terminator (d'Aubenton Carafa *et al.*, 1990) consisting of an imperfect 15bp inverted repeat with a $\Delta G$ value of -22.3 kcal/mol was identified 74bp downstream of the stop codon. Two copies of the 10bp neisserial DNA uptake sequence, 5'-GCCGTCTGAA-3' (Goodman and Scocca, 1988), were located upstream and downstream of the ORF.

**Figure 4.6 ORFs found flanking the *dnaJ* gene of *N. gonorrhoeae* FA1090 (Ng; Genbank accession number AE004969) and *N. meningitidis* strains Z2491 (NmA; Parkhill *et al.*, 2000) and MC58 (NmB; Tettelin *et al.*, 2000).**

Schematic diagram indicating the orientation of putative ORFs named after the most similar known or putative genes in the Genbank database. HP indicates ORFs that displayed the highest similarity to hypothetical proteins in the database. DT refers to the truncated copy of the IS1106A2 transposase (Parkhill *et al.*, 2000) that is partially deleted. ORFs with the same colour represent highly related sequences.

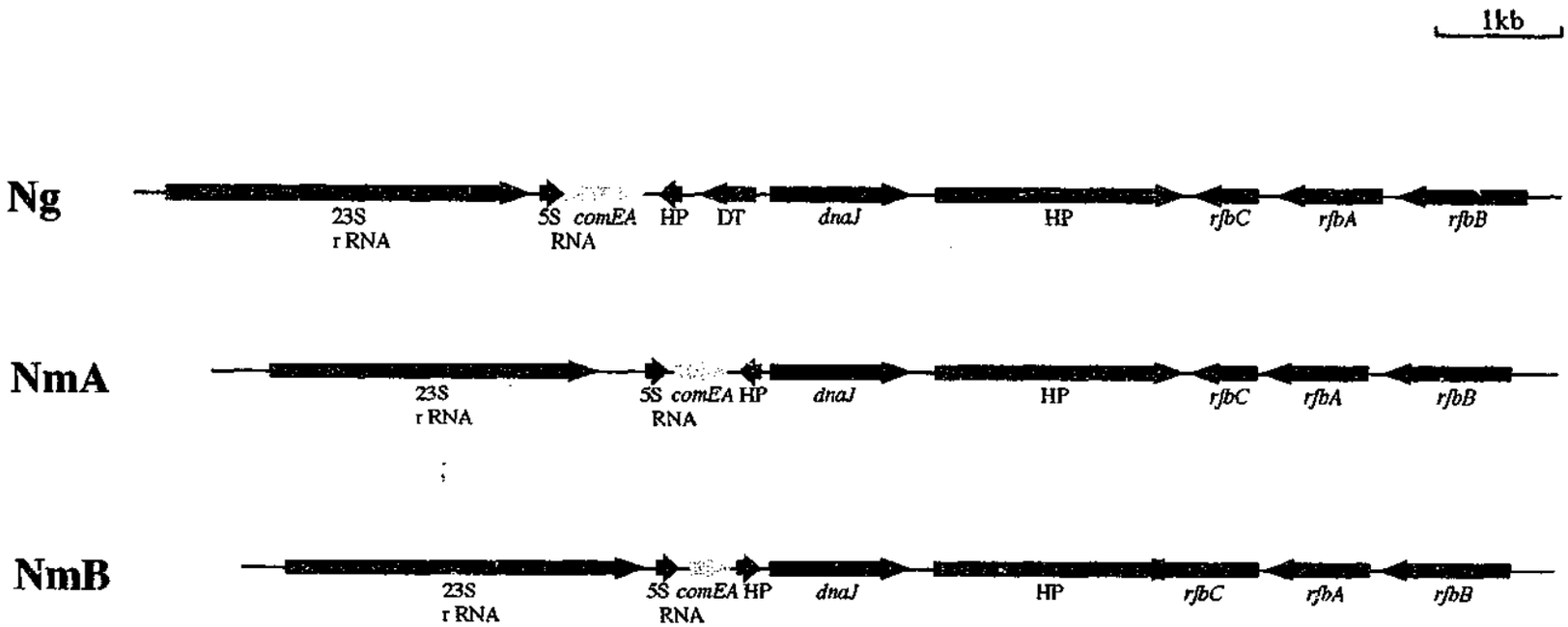

1kb
Ng
23S r RNA
5S RNA
comEA
HP
DT
dnaJ
HP
rfbC
rfbA
rfbB
NmA
23S r RNA
5S RNA
comEA
HP
dnaJ
HP
rfbC
rfbA
rfbB
NmB
23S r RNA
5S RNA
comEA
HP
dnaJ
HP
rfbC
rfbA
rfbB

**Figure 4.7 Amino acid sequence comparison of the GrpE proteins from various prokaryotes and eukaryotes.**

The GrpE proteins of *N. gonorrhoeae* (Ng; Genbank accession number AE004969), *N. meningitidis* strains Z2491 (NmA; Parkhill *et al.*, 2000) and MC58 (NmB; Tettelin *et al.*, 2000), *E. coli* (Ec; Lipinska *et al.*, 1988), *D. melanogaster* (Dm; Genbank accession number U34903), *Homo sapiens* (Hs; Genbank accession number AF298592), *B. subtilis* (Bs; Wetzstein *et al.*, 1992), *Synechocystis* sp. (Sy; Kaneko *et al.*, 1996), *C. trachomatis* (Ct; Stephens *et al.*, 1998) and *S. coelicolor* (Sc; Bucca *et al.*, 1993) were aligned using the CLUSTAL W program (Thompson *et al.*, 1994). Dashes (-) indicate gaps that have been introduced to maximise alignment. Residues in red text and the asterisk (*) represent identical residues. Conserved amino acids are indicated by green text and the colon (:) and residues with less similarity are depicted by blue text and the dot (.). Conserved domains (I-V) are overlined (Conway de Macario *et al.*, 1994; Wetzstein *et al.*, 1992; Wu *et al.*, 1994). Residues in bold type represent the coiled-coil region (Harrison *et al.*, 1997). Amino acids involved in DnaK binding and activation (Harrison *et al.*, 1997; Wu *et al.*, 1996) are boxed. Amino acids are numbered at the right of the figure according to their position in each individual protein sequence.

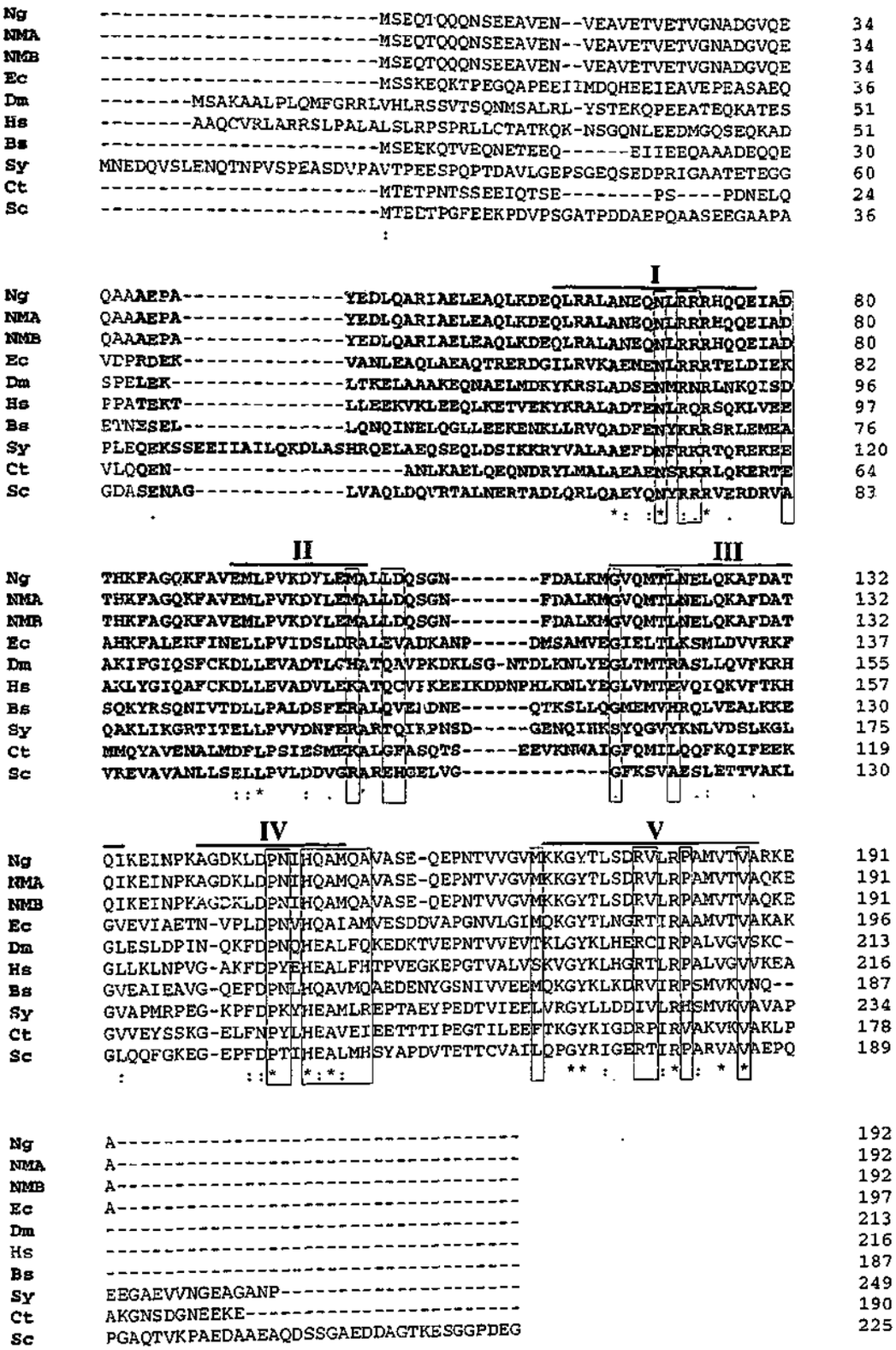
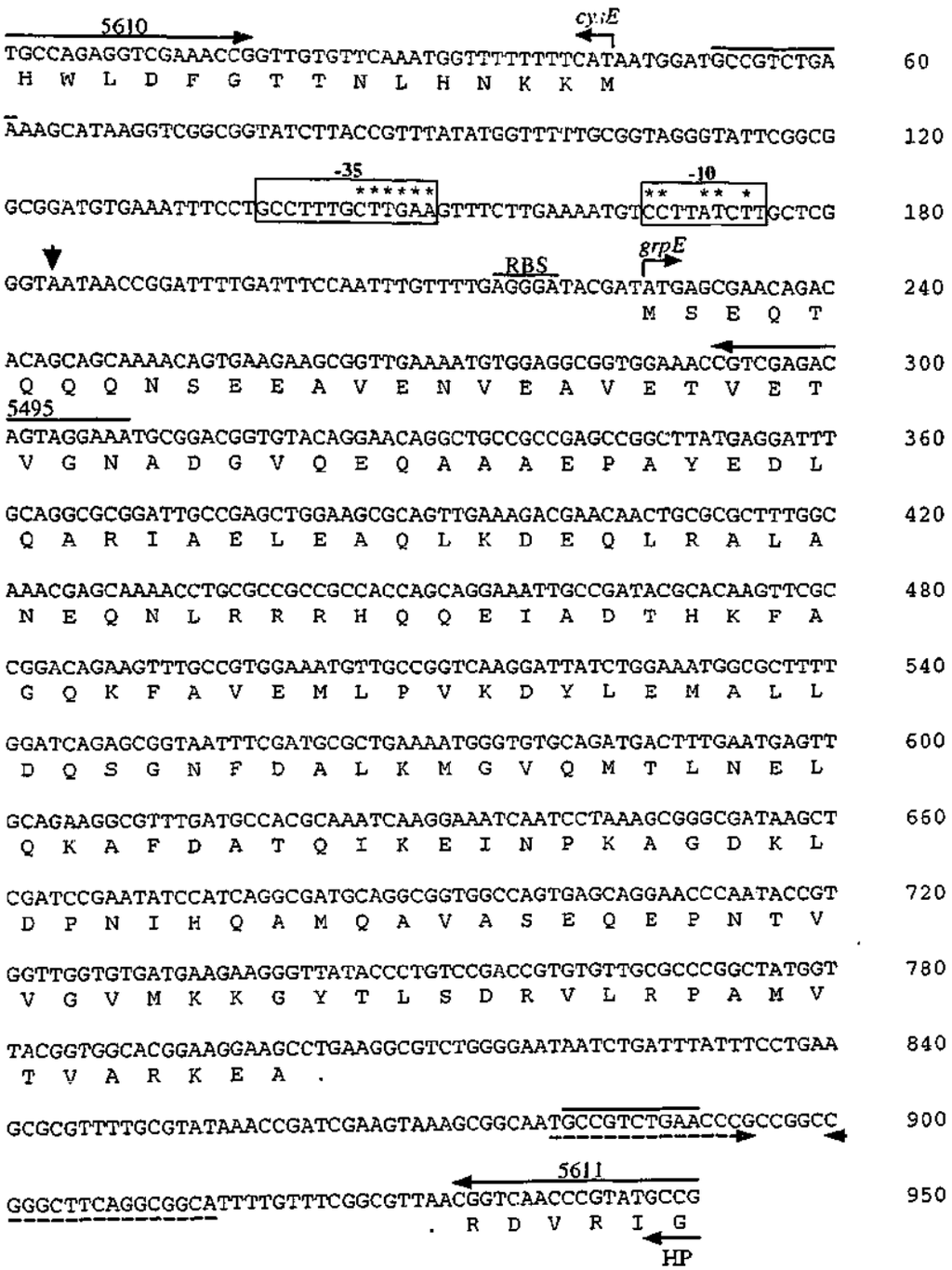

I
II
III
IV
V

**Figure 4.8 Nucleotide sequence of the *grpE* gene and flanking regions from *N. gonorrhoeae* FA1090 (Genbank accession number AE004969).**

The deduced amino acid sequence of *grpE*, *cysE* and the hypothetical protein (HP) are shown below the corresponding nucleotide sequences. Stop codons are indicated by a dot (.). The putative RBS is overlined as is the Neisserial DNA uptake sequence. The putative transcriptional terminator is denoted by dashed arrows. Oligonucleotide primers are depicted by numbered arrows above or below the nucleotide sequence. The tsp is indicated by an arrowhead and the associated $\sigma^{32}$ promoter sequences are boxed with asterisks (*) portraying the nucleotides identical to the consensus sequence (Cowing *et al.*, 1985). Nucleotides are numbered to the right of the figure.


5610
cysE
TGCCAGAGGTCGAAACCGGTTGTGTTCAAATGGTTTTTTTTCATAATGGATGCCGTCTGA 60
H W L D F G T T N L H N K K M
AAAGCATAAGGTCGGCGGTATCTTACCGTTTATATGGTTTTTGCGGTAGGGTATTCGGCG 120
-35
-10
GCGGATGTGAAATTTCCTGCCTTTGCTTGAAGTTTCTTGAAAATGTCCTTATCTTGCTCG 180
grpE
RBS
GGTAATAACCGGATTTTGATTTCCAATTTGTTTTGAGGGATACGATATGAGCGAACAGAC 240
M S E Q T
ACAGCAGCAAAACAGTGAAGAAGCGGTTGAAAATGTGGAGGCGGTGGAAACCGTCGAGAC 300
Q Q Q N S E E A V E N V E A V E T V E T
5495
AGTAGGAAATGCGGACGGTGTACAGGAACAGGCTGCCGCCGAGCCGGCTTATGAGGATTT 360
V G N A D G V Q E Q A A A E P A Y E D L
GCAGGCGCGGATTGCCGAGCTGGAAGCGCAGTTGAAAGACGAACAACTGCGCGCTTTGGC 420
Q A R I A E L E A Q L K D E Q L R A L A
AAACGAGCAAAACCTGCGCCGCCGCCACCAGCAGGAAATTGCCGATACGCACAAGTTCGC 480
N E Q N L R R R H Q Q E I A D T H K F A
CGGACAGAAGTTTGCCGTGGAAATGTTGCCGGTCAAGGATTATCTGGAAATGGCGCTTTT 540
G Q K F A V E M L P V K D Y L E M A L L
GGATCAGAGCGGTAATTTCGATGCGCTGAAAATGGGTGTGCAGATGACTTTGAATGAGTT 600
D Q S G N F D A L K M G V Q M T L N E L
GCAGAAGGCGTTTGATGCCACGCAAATCAAGGAAATCAATCCTAAAGCGGGCGATAAGCT 660
Q K A F D A T Q I K E I N P K A G D K L
CGATCCGAATATCCATCAGGCGATGCAGGCGGTGGCCAGTGAGCAGGAACCCAATACCGT 720
D P N I H Q A M Q A V A S E Q E P N T V
GGTTGGTGTGATGAAGAAGGGTTATACCCTGTCCGACCGTGTGTTGCGCCCGGCTATGGT 780
V G V M K K G Y T L S D R V L R P A M V
TACGGTGGCACGGAAGGAAGCCTGAAGGCGTCTGGGGAATAATCTGATTTATTTCCTGAA 840
T V A R K E A .
GCGCGTTTTGCGTATAAACCGATCGAAGTAAAGCGGCAATGCCGTCTGAACCCGCCGGCC 900
5611
GGGCTTCAGGCGGCATTTTGTTTCGGCGTTAACGGTCAACCCGTATGCCG 950
. R D V R I G
HP

The degree of homology among GrpE homologues is not as high as it is for DnaK and DnaJ proteins (Macario *et al.*, 1999). Comparisons with the gonococcal GrpE homologue reveals identities ranging from 34.3% with *E. coli* to 19.5% with *S. cerevisiae* (Figure 4.7; Table 4.1). GrpE homologues were also identified from *N. meningitidis* strains Z2491 (Parkhill *et al.*, 2000) and MC58 (Tettelin *et al.*, 2000), both displaying 99.5% identity to the gonococcal equivalent (Figure 4.7; Table 4.1).

Analysis of GrpE proteins reveals five highly conserved segments (Figure 4.7; Conway de Macario *et al.*, 1994; Wetzstein *et al.*, 1992; Wu *et al.*, 1994). The amino terminal region of the *E. coli* GrpE protein is predicted to form a coiled-coil region (amino acids 40-85; Figure 4.7; Wu *et al.*, 1994) involved in GrpE dimerisation and stabilisation. Determination of the crystal structure of GrpE has extended this α-helical region to amino acid 137 (Harrison *et al.*, 1997). Analysis of the corresponding regions of the neisserial homologues with the COILS program (Berger *et al.*, 1995) reveals that they too have the potential to form such a structure (data not shown). Residues involved in the stable binding of DnaK (Harrison *et al.*, 1997; Wu *et al.*, 1996) display a high degree of conservation among the neisserial GrpE homologues (Figure 4.7).

Putative ORFs where the derived amino acid sequence displayed significant similarity to those of known or putative gene products deposited in the Genbank database were identified proximal to the gonococcal *grpE* gene by BLAST analysis (Figure 4.3). This organisation was identical to that described in *N. meningitidis* strains MC58 (Tettelin *et al.*, 2000) and Z2491 (Parkhill *et al.*, 2000).

## 4.5 Transcriptional analysis of the *dnaK*, *dnaJ* and *grpE* genes of *N. gonorrhoeae* by RNA dot blot hybridisation

RNA dot blot hybridisation was employed to determine whether transcription of the gonococcal *dnaK*, *dnaJ* and *grpE* genes was induced when exposed to heat shock. The *dnaK* gene probe was prepared by purification of the 1370bp *Hin*dIII/*Cla*I fragment from pJKD1926 (Figure 4.2; Hamilton, 1999). Probes for *dnaJ* and *grpE* were prepared by first amplifying the entire ORFs from chromosomal DNA of *N. gonorrhoeae* MS11-A and cloning into pUC18 digested with *Hin*cII. Oligonucleotide primers 5608 and 5609 were used to amplify *dnaJ* (Figure 4.5) and 5610 and 5611 used to amplify *grpE* (Figure

4.8) and the subsequent products cloned to yield plasmids pJKD2107 and pJKD2108, respectively. The *dnaJ* gene probe was then created by purification of the 528bp *DraI/Cla*I fragment (Figure 4.5) of pJKD2107 and the *grpE* gene probe by purification of the entire insert of pJKD2108 PCR amplified with the oligonucleotide primers used to clone it.

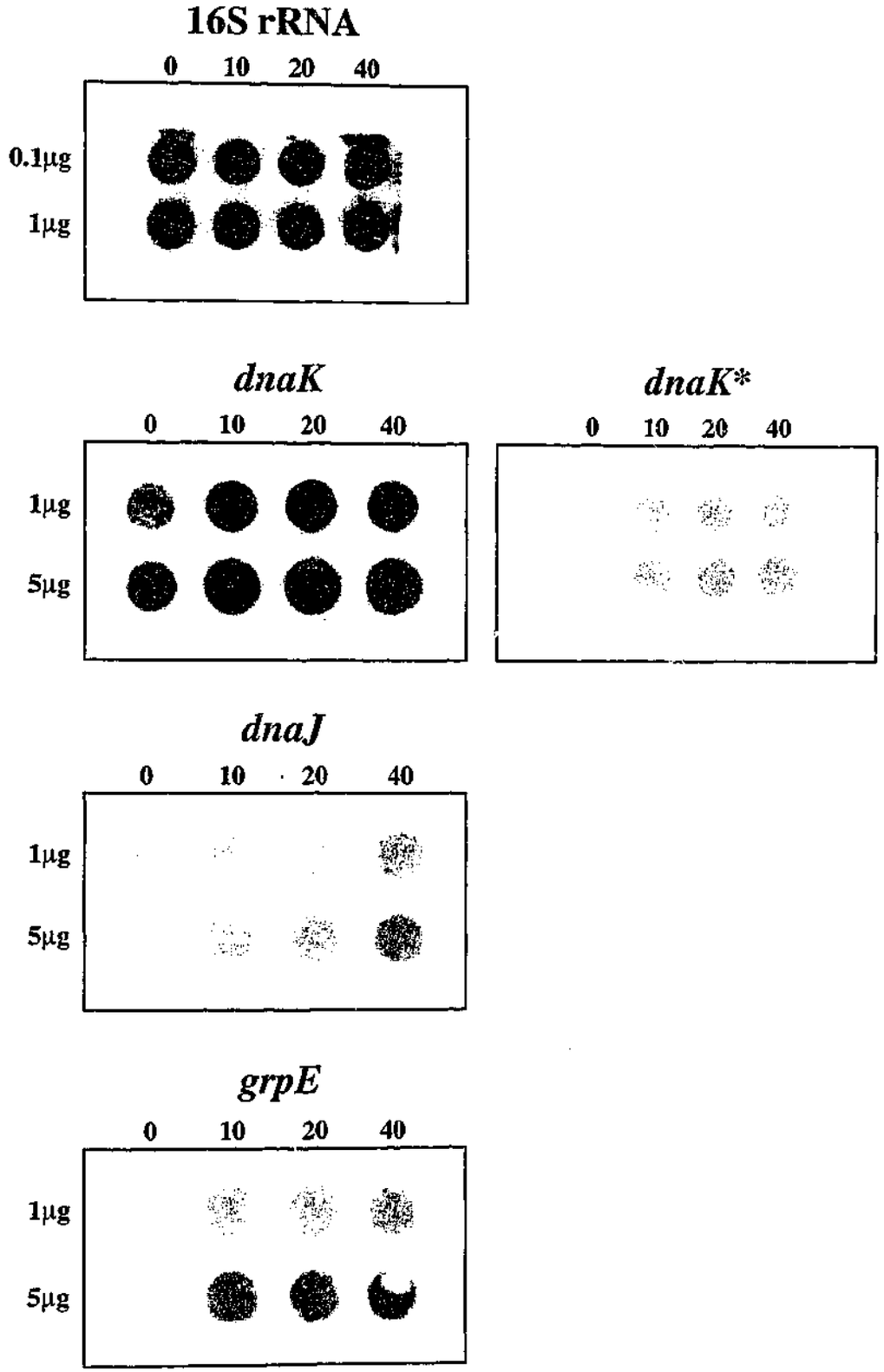
An internal region of the gonococcal 16S rRNA gene (Rossau *et al.*, 1988) was used as the control to confirm equivalent amounts of RNA were transferred to the nylon membranes. This probe was constructed by amplification of an 835bp fragment from *N. gonorrhoeae* MS11-A genomic DNA with oligonucleotide primers 3260 and 3261 (Black *et al.*, 1998).

Total RNA was extracted from exponentially growing cultures of *N. gonorrhoeae* MS11-A following exposure to heat shock at 42°C for 0, 10, 20 and 40 minutes. RNA samples were diluted to either 0.1, 1 or 5µg, transferred to nylon membranes and incubated with the generated probes. The hybridisation results are shown in Figure 4.9.

Probing with the 16S rRNA probe verified the transfer of equivalent amounts of RNA from each sample. A substantial increase in the level of *dnaK*, *dnaJ* and *grpE* specific transcript was observed following exposure to heat shock at 42°C for 10 minutes. This indicates that transcription of these genes is induced upon stress. Transcription of *dnaK* reached a maximum at 20 minutes exposure to heat shock and then appeared to decline slightly at 40 minutes. In contrast, *dnaJ* specific message seemed to intensify with prolonged exposure to heat shock, while the level of *grpE* transcription appeared consistently elevated. Interestingly, transcription of the *groE* operon reached a maximum at 20 minutes exposure to heat shock and then declined despite ongoing exposure (Hamilton, 1999). The level of *dnaK* specific transcript produced under physiological and heat shock conditions was more abundant than *grpE* specific transcript produced, while the expression of *dnaJ* appears to be the weakest.

**Figure 4.9 Northern dot blot analysis of RNA isolated from *N. gonorrhoeae* MS11-A following a temperature upshift.**

RNA was extracted from cells exposed to heat shock at 42°C for the number of minutes indicated above each well. The amount of RNA transferred to the membranes is indicated. Filters were probed with the gonococcal 16S rRNA, *dnaK*, *dnaJ* and *grpE* genes as indicated. The asterisk represents a shorter time exposure of the membrane probed with *dnaK*.


16S rRNA
0 10 20 40
0.1µg
1µg
dnaK
0 10 20 40
1µg
5µg
dnaK*
0 10 20 40
dnaJ
0 10 20 40
1µg
5µg
grpE
0 10 20 40
1µg
5µg

## 4.6 Mapping of the transcriptional start points of *dnaK*, *dnaJ* and *grpE* from *N. gonorrhoeae* by primer extension

To locate the functional promoters responsible for the transcription of the gonococcal *dnaK*, *dnaJ* and *grpE* genes, the tsps were mapped by primer extension. This was performed on the same RNA preparations used for the RNA dot blot hybridisations (Section 4.5), extracted before and after heat shock.

Oligonucleotide primers 6035 (Figure 4.2), 5494 (Figure 4.5), 5495 (Figure 4.8) were used to identify the tsps of *dnaK*, *dnaJ* and *grpE*, respectively. Sequencing reactions on the corresponding plasmid DNA, pJKD2238 (Section 4.7), pJKD2107 and pJKD2108 were performed with the same oligonucleotide primers.

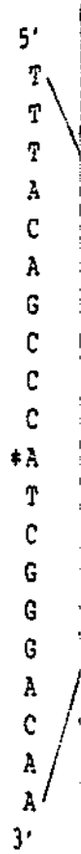
Primer extension products were obtained for all the genes at physiological temperature, however the signals were greatly intensified upon the temperature upshift (Figures 4.10, 4.11 and 4.12). This concurs with the RNA dot blot hybridisation results and confirms that transcription of *dnaK*, *dnaJ* and *grpE* is induced under stress conditions.

In contrast to the Northern dot blot hybridisation result, the primer extension product obtained for the *dnaK* specific message increased in intensity with continued exposure to heat shock. The tsp for *dnaK* under physiological and stress conditions was mapped to an A residue situated 61bp upstream of the putative start codon (Figure 4.2). Sequence upstream of the tsp displayed similarity to the -10 and -35 regions of $\sigma^{32}$ dependent promoters (Cowing *et al.*, 1985). The potential -10 box, CCCTATTTA, shared 8 out of 9 bases with the consensus sequence, CCCCATTTA. This was preceded at a distance of 15bp by a probable -35 box, GCTGTAGCTTGAA, with 7 out of 13 bases identical to the consensus sequence, TNTCNCCCTTGAA.

Three primer extension products were obtained for *dnaJ* under stress and non-stress conditions and were designated tsp1, tsp2 and tsp3. All three tsps were located at a G residue situated 22bp, 74bp and 97bp, respectively, upstream of the putative translational start site (Figure 4.5). Only the level of transcript from tsp1 increased with a thermal upshift. As for *dnaK*, the corresponding promoter for tsp1 shared similarity to the consensus sequence for $\sigma^{32}$ dependent promoters (Cowing *et al.*, 1985). The

**Figure 4.10 Primer extension analysis of the *dnaK* upstream region of *N. gonorrhoeae* MS11-A.**

Total RNA (50μg per lane) isolated from cells grown at 37°C and heat shocked at 42°C for 0 (lane 1), 10 (lane 2), 20 (lane 3) and 40 (lane 4) minutes was subjected to primer extension using oligonucleotide primer 6035 (Figure 4.2). The primer extension products obtained are indicated by the arrow. The sequencing ladder adjacent to the reactions and marked T, G, C, A was generated with the same oligonucleotide primer. The sequence obtained is listed to the left of the panel and the asterisk represents the tsp.



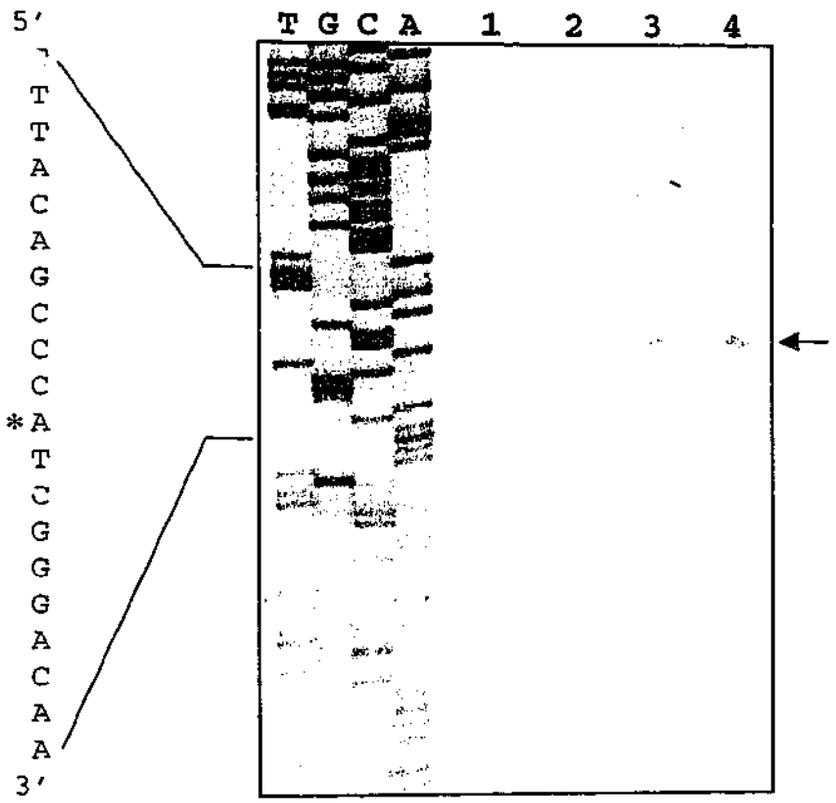
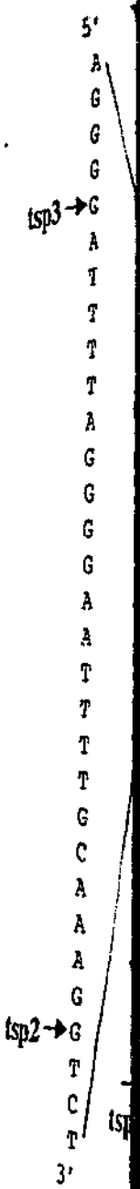

5'
T
T
A
C
A
G
C
C
C
C
*A
T
C
G
G
G
A
C
A
A
3'
T G C A
1
2
3
4

**Figure 4.11 Primer extension analysis of the *dnaJ* upstream region of *N. gonorrhoeae* MS11-A.**

Total RNA (50µg per lane) isolated from cells grown at 37°C and heat shocked at 42°C for 0 (lane 1), 10 (lane 2), 20 (lane 3) and 40 (lane 4) minutes was subjected to primer extension using oligonucleotide primer 5494 (Figure 4.5). The primer extension products obtained are indicated by the arrows. The sequencing ladder adjacent to the reactions and marked T, G, C, A was generated with the same oligonucleotide primer. The sequence obtained is listed to the left of the panel and the tsps are shown.



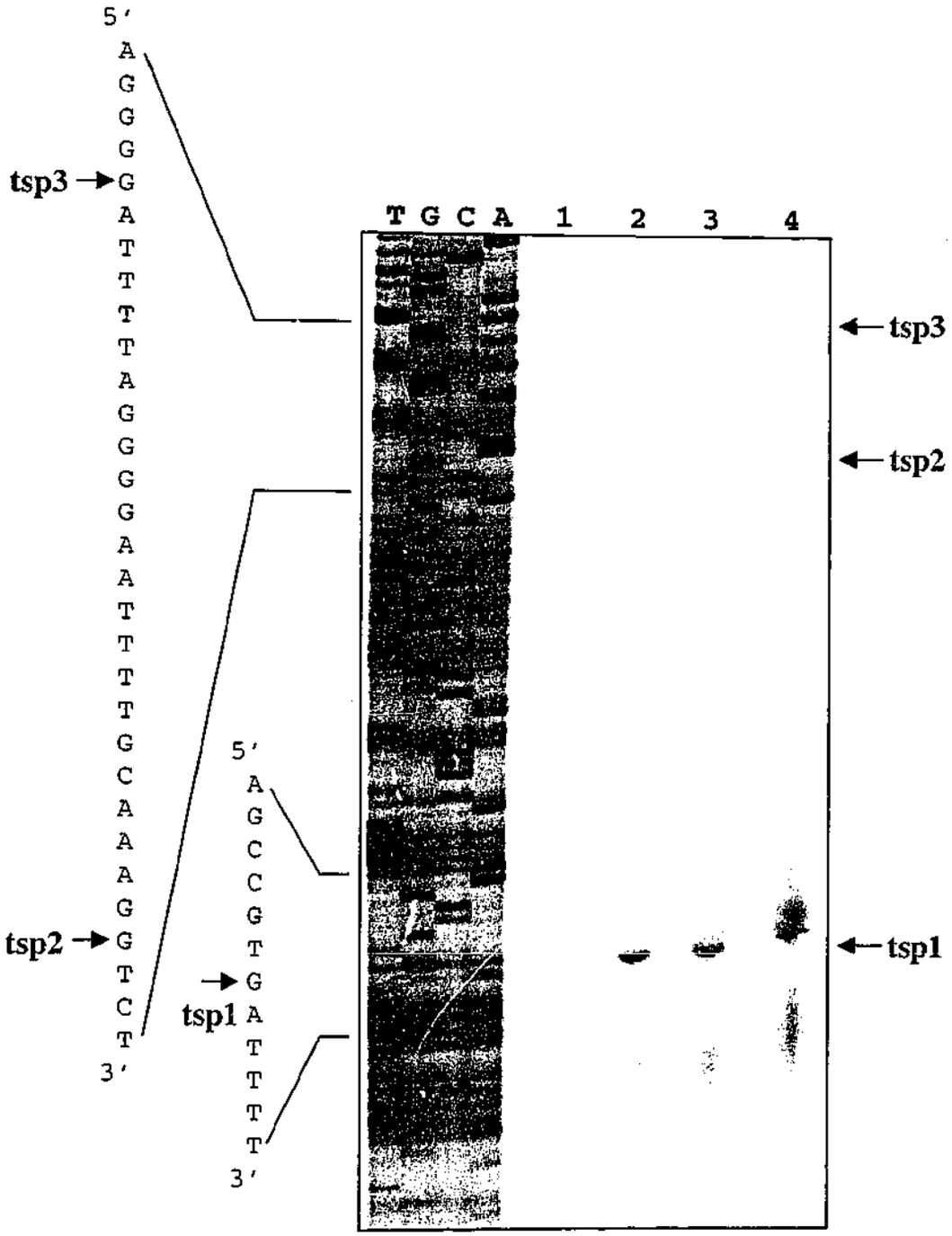
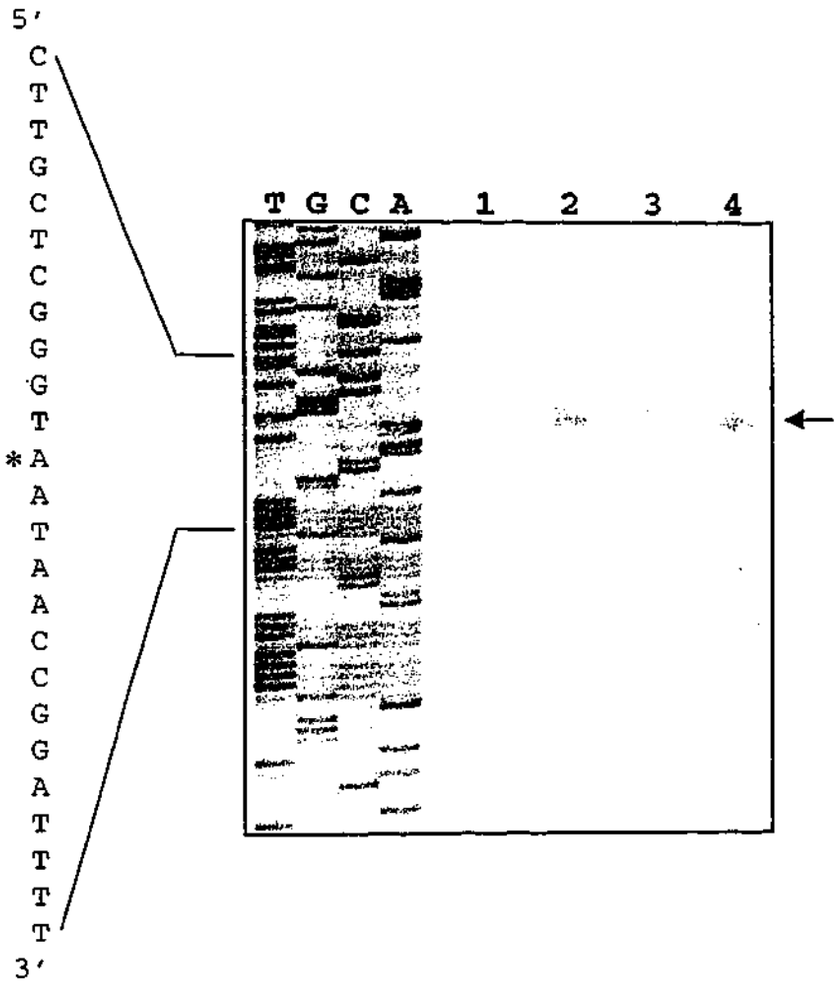

5′
A
G
G
G
G
tsp3
A
T
T
T
T
T
A
G
G
G
G
A
A
T
T
T
T
G
C
A
A
A
G
tsp2
T
C
T
3′
5′
A
G
C
C
G
T
G
tsp1
A
T
T
T
T
3′
T G C A 1 2 3 4
tsp3
tsp2
tsp1

**Figure 4.12 Primer extension analysis of the *grpE* upstream region of *N. gonorrhoeae* MS11-A.**

Total RNA (50μg per lane) isolated from cells grown at 37°C and heat shocked at 42°C for 0 (lane 1), 10 (lane 2), 20 (lane 3) and 40 (lane 4) minutes was subjected to primer extension using oligonucleotide primer 5495 (Figure 4.8). The primer extension products obtained are indicated by the arrow. The sequencing ladder adjacent to the reactions and marked T, G, C, A was generated with the same oligonucleotide primer. The sequence obtained is listed to the left of the panel and the asterisk represents the transcription start point.


5'
C
T
T
G
C
T
C
G
G
G
T
*A
A
T
A
A
C
C
G
G
A
T
T
T
3'
T G C A
1
2
3
4

probable -10 region, CCTCATTTT, shared 7 out of 9 bases with the consensus sequence, and 14bp upstream the -35 region, TGTTTGTCTTGTG, shared 6 out of 13 bases with the consensus sequence. Analysis of sequence the appropriate distance upstream of tsp2 and tsp3 revealed only weak matches to the consensus sequence for $\sigma^{32}$ promoters.

The intensity of the *grpE* specific signal was consistent for the duration of the temperature upshift, confirming the Northern dot blot results. Transcription of *grpE* at both temperatures emanated from an A residue 42bp upstream of the putative start codon (Figure 4.8), and the associated potential promoter displayed similarity to the consensus sequence for $\sigma^{32}$ promoters (Cowing *et al.*, 1985). The probable -10 box, CCTTATCTT, shared 5 out of 9bp with the consensus sequence and 15bp upstream the probable -35 box, GCCTTTGCTTGAA, had 6 out of 13bp identical to the consensus sequence.

The primer extension results reveal that the proposed $\sigma^{32}$ dependent promoters for *dnaK*, *dnaJ* and *grpE* are functional under stress conditions.

## 4.7 Mutational analysis of the *dnaK* promoter

To verify the activity of the putative $\sigma^{32}$ promoter identified upstream of *dnaK* under non-stress and stress conditions, site-directed mutagenesis of the promoter was performed. The nucleotide changes made to bases within the -10 and -35 regions were selected based on alignment of the gonococcal *dnaK* and *groE* $\sigma^{32}$ promoters, as they were the only promoters identified when mutational analysis was performed. This would have rendered the appropriate sigma factor incapable of recognising the promoter (Cowing *et al.*, 1985), and this was assayed in *E. coli* and *N. gonorrhoeae*.

The *dnaK* upstream region was amplified using oligonucleotide primers 6034 and 6035 (Figure 4.2) and genomic DNA from *N. gonorrhoeae* MS11-A as template. The PCR product was cloned into pUC18 digested with *Hin*cII and the resulting construct, pJKD2238, used as template for the site-directed mutagenesis reactions. The oligonucleotide primer 7080 and the complement 7081 were used to create the -10 box mutation, and 7078 and the complement 7079 were used to manufacture the -35 box

mutation (Figure 4.2). The mutated PCR fusions were cloned into *Hinc*II digested pUC18 to yield pJKD2239 and pJKD2240, respectively. The inserts of pJKD2238, pJKD2239 and pJKD2240 were cloned into the promoter selection vector pKK232-8 and the resulting promoter::*cat* transcriptional fusions were subcloned into the unique *Bam*HI site of the *E. coli*/*N. gonorrhoeae* shuttle vector Hermes-2 to yield pJKD 2282, pJKD2283 and pKD2284, respectively (Figure 2.1).

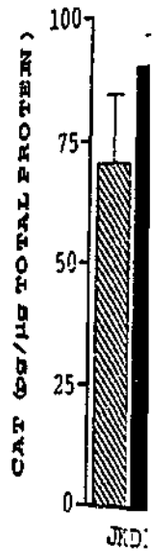
Exponential phase cultures of the corresponding strains were exposed to heat shock at 42°C for 0, 3 and 10 minutes. Cell extracts of the cultures were then prepared and the level of CAT enzyme determined (Figure 4.13). The 3 and 10 minute intervals were chosen as the heat shock response in *E. coli* is transient such that transcription of *dnaK* peaks at 4-5 minutes following exposure and then declines to a new steady-state level (Yamamori *et al.*, 1978; Yamamori and Yura, 1980).

Basal levels of CAT enzyme were detected for strain JKD1595 which contains a promoterless *cat* gene (Figure 4.13). Surprisingly, CAT levels similar to JKD1595 were observed for strain JKD2282 which contains the wild type *dnaK* promoter region. This indicates that the gonococcal $\sigma^{32}$ dependent promoter identified by primer extension (Section 4.6) is not functional in an *E. coli* background. This also explains the extremely low levels of CAT activity obtained for the mutated promoter constructs represented by strains JKD2283 and JKD2284 (Figure 4.13).

The Hermes-2 promoter::*cat* transcriptional fusions were then transferred to *N. gonorrhoeae* strain JKD484 by transformation and the level of CAT activity assessed. To confirm integration of the fusions into p*tet*M25.2 by homologous recombination, the plasmids were transferred by conjugation into strain JKD359. Exponential phase cultures of the resulting recombinant gonococcal strains were subjected to heat shock at 42°C for 0, 10 and 20 minutes. Cell extracts of the cultures were then prepared and the CAT levels determined (Figure 4.14). Basal levels of CAT were obtained for strain JKD487 which contains the promoterless *cat* gene. As expected much higher levels of CAT were obtained for strain JKD491 which contains the wild type *dnaK* promoter region and establishes the activity of the $\sigma^{32}$ dependent promoter. Comparison of the CAT levels from strain JKD491 prior and following heat shock did not reveal a significant difference. This may be a result of the cells proceeding into stress mode

**Figure 4.13 Transcriptional analysis of the gonococcal *dnaK* upstream region in *E. coli*.**

CAT levels (pg/μg total protein) were determined for *E. coli* strains JKD2238, JKD2239 and JKD2240 which contain the plasmid encoded *cat* reporter constructs carrying the wild type *dnaK* promoter region, the mutated –10 region and the mutated –35 region, respectively. Strain JKD1595 was the negative control as it contained the promoterless *cat* gene. Cell extracts were prepared from strains exposed to heat shock at 42°C for 0 minutes (hatched bars), 3 minutes (black bars) and 10 minutes (spotted bars). The CAT levels shown are the means of four separate assays. Error bars represent standard deviation.



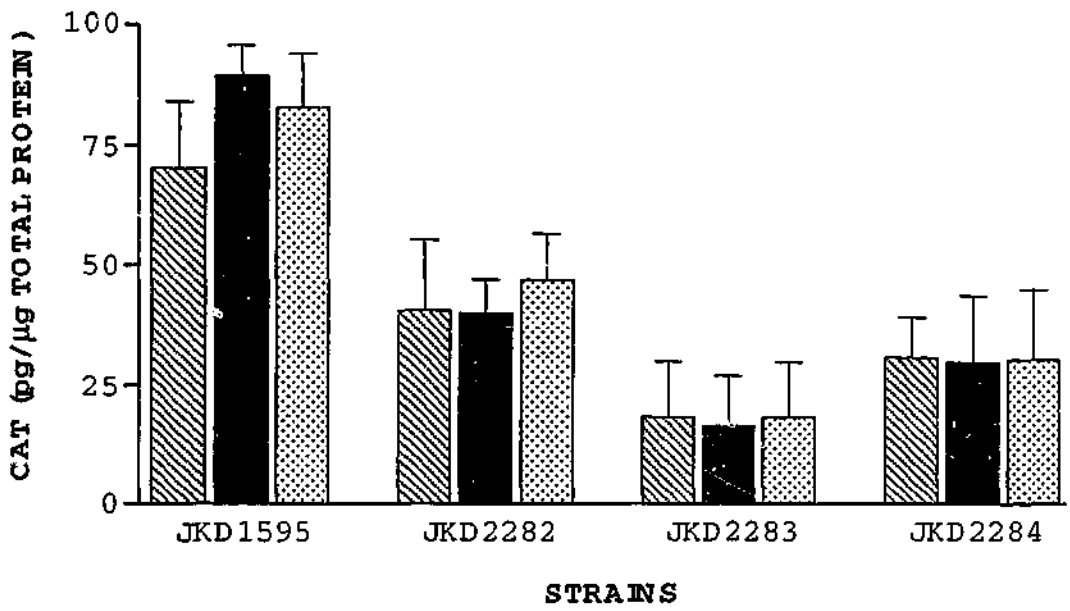
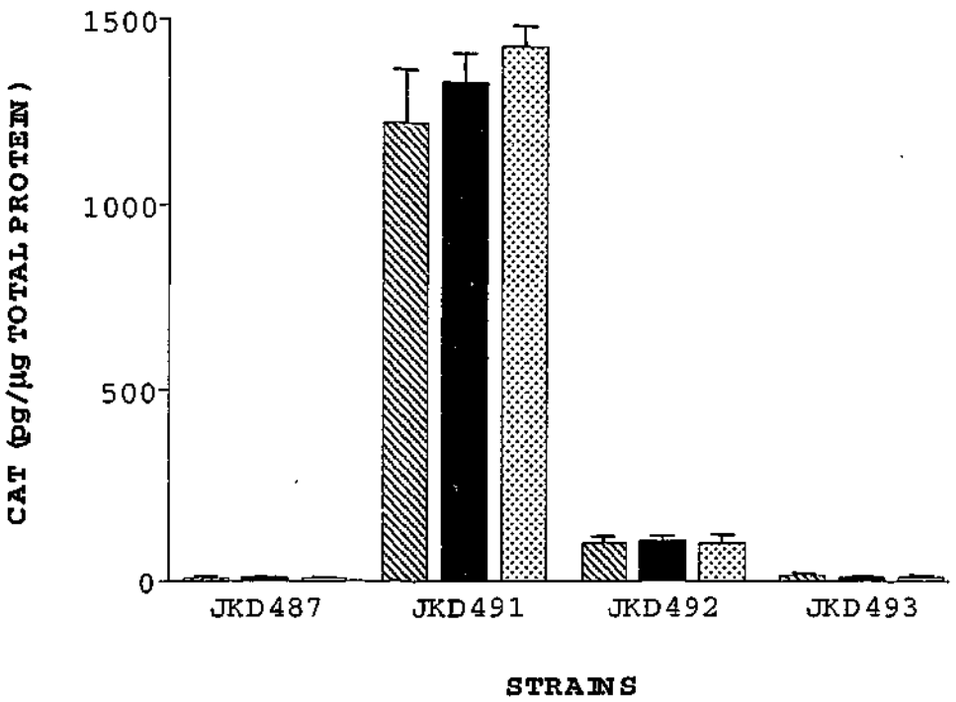

CAT (pg/µg TOTAL PROTEIN)
100
75
50
25
0
JKD1595
JKD2282
JKD2283
JKD2284
STRAINS

**Figure 4.14 Transcriptional analysis of the gonococcal *dnaK* upstream region in *N. gonorrhoeae*.**

CAT levels (pg/μg total protein) were determined for gonococcal strains JKD491, JKD492 and JKD493 which contain *cat* reporter constructs integrated into the conjugative plasmid p*tet*M25.2 carrying the wild type *dnaK* promoter region, the mutated –10 region and the mutated –35 region, respectively. The control strain was JKD487 as it encoded the promoterless *cat* gene. Cell extracts were prepared from strains exposed to heat shock at 42°C for 0 minutes (hatched bars), 10 minutes (black bars) and 20 minutes (spotted bars). The CAT levels shown are the means of four separate assays. Error bars represent standard deviation.


1500
1000
500
0
CAT (pg/µg TOTAL PROTEIN)
JKD487
JKD491
JKD492
JKD493
STRAINS

during the initial phase of the cell extract preparation procedure. The CAT levels obtained for strains JKD492 and JKD493, which contain the mutated -10 and -35 boxes, respectively, were greatly reduced. The CAT level for JKD492 was reduced almost 17-fold compared to the wild type, while that for JKD493 was similar to the control strain JKD487. These results indicate that the putative $\sigma^{32}$ dependent promoter is responsible for transcription of the *dnaK* gene and the bases mutated in the -35 region have a more pertinent effect on sigma binding than those mutated in the -10 region.

## 4.8 Discussion

This chapter identified and characterised the genes encoding the molecular chaperones DnaK, DnaJ and GrpE from *N. gonorrhoeae* strains FA1090 (Genbank accession number AE004969) and MS11-A. Comparison of the deduced amino acid sequences of these proteins with their homologues from other organisms revealed extensive conservation (Table 4.1). As DnaK (Hsp70) is the most conserved protein found in any species (Falah and Gupta, 1997) it was not unexpected that the gonococcal (Genbank accession number AE004969) and meningococcal (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000) homologues displayed the highest degree of conservation, followed by DnaJ and the less conserved GrpE.

Analysis of the neisserial DnaK, DnaJ and GrpE homologues revealed the retention of the proposed functional sequences and structural organisation of analogous proteins. The regions of DnaK involved in conferring ATPase activity and coupling (Bork *et al.*, 1992; Buchberger *et al.*, 1994), substrate binding (Zhu *et al.*, 1996) and interaction with DnaJ (Gassler *et al.*, 1998; Suh *et al.*, 1998) and GrpE (Buchberger *et al.*, 1994) have been conserved (Figure 4.1). The domains of DnaJ and GrpE responsible for binding and regulation of DnaK (Cheetham and Caplan, 1998; Harrison *et al.*, 1997; Suh *et al.*, 1998; Tsai and Douglas, 1996; Wu *et al.*, 1996) have also been retained in the neisserial homologues (Figures 4.4 and 4.7). This leads to the conclusion that the functional activity of the chaperones is similar to that observed in *E. coli*, where they have been extensively studied (Bukau, 1993; Fink, 1999) and also thought to be conserved within the Hsp70 family (Laufen *et al.*, 1999). Briefly, DnaK with the assistance of DnaJ and GrpE, refolds denatured proteins via an ATPase-driven cycle of substrate binding and release (Bukau and Horwich, 1998). DnaJ stimulates ATP hydrolysis of DnaK such that

protein substrates can be locked into the substrate binding domain of DnaK (Liberek *et al.*, 1991; McCarty *et al.*, 1995; Szabo *et al.*, 1996). Substrate release is then mediated by a process of ADP/ATP exchange that is catalysed by the nucleotide exchange factor GrpE (Liberek *et al.*, 1991; Packschies *et al.*, 1997; Szabo *et al.*, 1996).

Examination of sequence adjacent to *dnaK* and *grpE* from *N. gonorrhoeae* FA1090 revealed several copies of the neisserial DNA uptake sequence (Figures 4.2 and 4.8). This sequence occurs on average every kb in the gonococcal chromosome and is essential for species-specific DNA uptake (Elkins *et al.*, 1991; Goodman and Scocca, 1988). It often forms part of transcriptional terminators (Goodman and Scocca, 1988) and this is apparent in the case of *grpE*.

Further analysis of flanking regions indicated that the gonococcal and meningococcal *dnaK* and *dnaJ* genes were not arranged in an operon (Figures 4.3 and 4.6). In fact the ORFs identified adjacent to *dnaK*, *dnaJ* and *grpE* did not appear to be stress related. This finding was unexpected as *dnaK* and *dnaJ* from the majority of bacteria are arranged in an operon which occasionally includes *grpE* (Parsons *et al.*, 1999; Segal and Ron, 1996b). It is possible that different levels of *dnaK* and *dnaJ* expression are required, a situation that is observed in *E. coli* (Bardwell *et al.*, 1986) where it is due to infrequent translation initiation of *dnaJ* mRNA (Sell *et al.*, 1990). This suggested mode of transcriptional regulation may be more energy efficient and thus advantageous to *Neisseria* spp. than post-transcriptional mechanisms with the genes arranged in an operon. The gonococcal *grpE* gene is located approximately 5kb upstream of the *dnaK* gene and the intergenic sequence is highly conserved in *N. meningitidis* (Figure 4.3), most likely due to the importance of both of these genes (Ang and Georgopoulos, 1989; Paek and Walker, 1987). The exception is a copy of the Correia element found only in the meningococcal strains. This element is thought to be involved in homologous recombination (Dempsey *et al.*, 1995; Hamilton, 1999). This repetitive sequence is also located downstream of the *dnaK* gene in the meningococcal strains and may have arisen from a genomic rearrangement which would explain the divergent sequence compared to *N. gonorrhoeae* in this region. The sequence adjacent to *dnaJ* (Figure 4.6) is highly conserved within the species, once again probably due to the essential requirement of this gene in response to stress (Saito and Uchida, 1978). The exception is a non-

functional deleted copy of the transposase of IS1106A2 (Parkhill *et al.*, 2000) immediately upstream of *dnaJ* in *N. gonorrhoeae*.

RNA dot blot hybridisation was employed to investigate the regulation of transcription of the *dnaK*, *dnaJ* and *grpE* genes from *N. gonorrhoeae* MS11-A following exposure to heat shock. Figure 4.9 showed that expression of all three genes was significantly induced upon a thermal upshift but seemed to differ with prolonged exposure. Transcription of *dnaK* seemed to decline slightly at 40 minutes exposure, in contrast to the increased message obtained for the primer extension result at the same time (Figure 4.10; see below). This discrepancy is probably due to the non-specific binding of the probe, which contains the majority of the *dnaK* gene, to the gonococcal *hscA* homologue which shares 55% similarity with *dnaK* at the nucleotide level (data not shown). This proposed gene was identified in *N. gonorrhoeae* by BLAST analysis of the *N. gonorrhoeae* strain FA1090 genome database (Genbank accession number AE004969) with the *E. coli* homologue (Seaton and Vickery, 1994). A region was identified, which, when translated, displayed significant similarity to Hsc66 (data not shown). *hscA* encodes the molecular chaperone Hsc66 in *E. coli* (Seaton and Vickery, 1994) and is thought to be involved in the cold shock response (Lelivelt and Kawula, 1995) and other cellular functions (Hesterkamp and Bukau, 1998). The transcription of this gene remains constant upon heat shock (Lelivelt and Kawula, 1995) however the level of protein decreases (Hesterkamp and Bukau, 1998) suggesting that perhaps the mRNA has a short half-life. Therefore instability of the transcript at the elevated temperature may account for the RNA dot blot results.

Transcription of *dnaJ* appeared to increase and *grpE* expression remained constant upon ongoing exposure to heat shock (Figure 4.9), in agreement with the primer extension results (see below). In contrast, transcription of the *groE* operon is transient in that it peaks at 20 minutes exposure to heat shock and then declines (Hamilton, 1999). Such a transient response is observed in most bacteria studied, the only difference being the time of acclimatisation. This reason for this discrepancy is not clear, but may have to do with differing stabilities of the *groE* (Hamilton, 1999) and *dnaK* chaperone system transcripts or post-transcriptional regulatory mechanisms. Different levels of transcript for *dnaK*, *dnaJ* and *grpE* were observed and likely a result of variable protein concentration requirements for optimal function in both the stress response and other

cellular processes (Sakakibara, 1988). The results obtained for *N. gonorrhoeae* are in accordance with those obtained for *N. meningitidis* upon heat shock at 45°C where transcription of *dnaK*, *dnaJ* and *grpE* increased significantly (Guckenberger *et al.*, 2002).

The stress response of a number of bacteria has been studied and transcription of the chaperones *dnaK*, *dnaJ* and *grpE* has generally been found to be induced at the transcriptional level upon exposure to heat shock (Segal and Ron, 1996b; Wetzstein *et al.*, 1992; Yamamori *et al.*, 1978; Yamamori and Yura, 1980; Yura and Nakahigashi, 1999). Recently additional mechanisms of heat shock regulation at a post-transcriptional levels have been proposed. The substantial secondary structure predicted in the 5' leader sequence of *dnaK* mRNA from *C. crescentus* and *H. ducreyi* suggests that it attenuates expression by sequestering the RBS from interaction with 16S rRNA at physiological conditions (Avedissian *et al.*, 1995; Parsons *et al.*, 1999). mRNA processing and stability have also been proposed to contribute to *dnaK* mRNA regulation (Homuth *et al.*, 1997; Jayaraman *et al.*, 1997).

Analysis of the sequence surrounding the putative *dnaK* start codon revealed two potential Fur boxes (Figure 4.1; Genco and Desai, 1996). The 19bp DNA sequence that constitutes the Fur box is a binding site for the ferric uptake regulator protein Fur that results in transcriptional repression in the absence of extracellular iron (Hennecke, 1990). Potential Fur boxes have been identified proximal to the *dnaK* translational start site in a variety of bacteria indicating that iron may be involved in the negative regulation of DnaK (Parsons *et al.*, 1999). The importance of iron availability in gonococcal pathogenesis (Keevil *et al.*, 1989) suggests that DnaK has as essential role in this process. Thus it seems that regulation of the gonococcal stress response occurs primarily at the transcriptional level but further analysis is required to determine whether post-transcriptional regulatory mechanisms are also involved.

There appear to be two primary groups of transcriptional regulatory strategies employed by bacteria to regulate the *dnaK* chaperone system. The majority of Gram positive bacteria and several Gram negative bacteria utilise a negative control mechanism where stress exposure displaces a repressor protein bound to a conserved *cis*-acting inverted repeat located near the promoter and permits transcription via a vegetative sigma factor

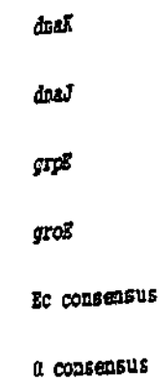
(Bahl *et al.*, 1995; Narberhaus, 1999; Roberts *et al.*, 1996; Segal and Ron, 1996a; Spohn and Scarlato, 1999; van Asseldonk *et al.*, 1993; Yuan and Wong, 1995; Zuber and Schumann, 1994). A BLAST search of the gonococcal database (Genbank accession number AE004969) with the conserved *cis*-acting inverted repeat, CIRCE (Zuber and Schumann, 1994), did not reveal any homologous sequences (data not shown). As this element is highly conserved and absent from *N. gonorrhoeae* then the alternative regulatory strategy was considered. Several species of Gram negative bacteria have adopted a positive mode of regulation of the *dnaK* operon where the alternative sigma factor, $\sigma^{32}$, directs transcription under stress (Fleischmann *et al.*, 1995; Karls *et al.*, 1998; Nakahigashi *et al.*, 1995; Narberhaus *et al.*, 1997; Ramirez-Santos and Gomez-Eichelmann, 1998; Ramirez-Santos *et al.*, 2001; Sahu *et al.*, 1997; Wu and Newton, 1996). Tauschek *et al.* (1997) demonstrated that transcription of the gonococcal *groE* operon during stress is initiated from a $\sigma^{32}$ promoter. The following chapter describes the identification of this alternative sigma factor in *N. gonorrhoeae*.

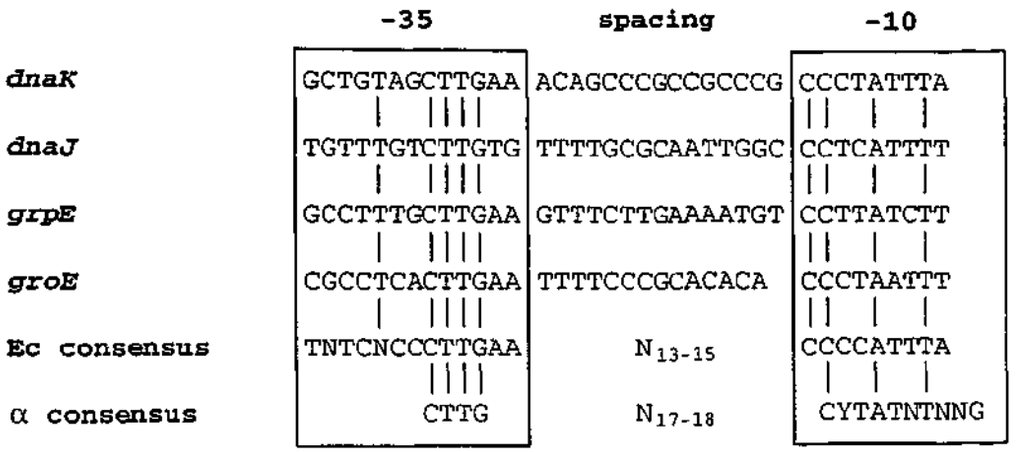
Primer extension analysis of *dnaK* and *grpE* revealed that transcription at steady-state and heat shock conditions emanated from promoters with similarity to the -10 and -35 regions of $\sigma^{32}$ promoters (Figures 4.10 and 4.12). Similarly, transcription of *dnaJ* was initiated from such a promoter upon thermal upshift and physiological conditions, however two additional tsps were also identified (Figure 4.11). Only poor matches to the consensus sequence for $\sigma^{32}$ promoters were identified upstream and the signals did not intensify upon subjection to high temperature. Alignment of the consensus sequences for heat shock promoters of *E. coli* (Cowing *et al.*, 1985), $\alpha$ purple proteobacteria (Segal and Ron, 1995), the gonococcal *groE* operon (Tauschek *et al.*, 1997) and those identified in this study revealed that the gonococcal $\sigma^{32}$ promoters shared extensive similarity to both of the consensus sequences (Figure 4.15). There does however appear to be some variation among the stretch of C residues in the -10 box of the gonococcal genes and the 5' end of the -35 box seems to be the least conserved when compared to the *E. coli* consensus sequence.

Based upon the above alignment, site-directed mutagenesis was performed on the *dnaK* promoter to confirm its activity and analysis was conducted in an *E. coli* and *N. gonorrhoeae* background. Highly conserved bases in the -10 and -35 regions (Figure 4.15) were chosen for mutagenesis. Surprisingly a low background level of CAT

**Figure 4.15 Nucleotide sequence alignment of $\sigma^{32}$ dependent promoters.**

The consensus sequences for heat shock promoters of *E. coli* (Ec consensus; Cowing *et al.*, 1985) and the alpha division of protcobacteria (α consensus; Segal and Ron, 1995) were compared with the $\sigma^{32}$ dependent promoters of the gonococcal *dnaK*, *dnaJ*, *grpE* genes identified in this study and that identified upstream of the gonococcal *groE* operon (Tauschek *et al.*, 1997). The consensus –10 and –35 regions are boxed.




-35
spacing
-10
dnaK
GCTGTAGCTTGAA
ACAGCCCGCCGCCCG
CCCTATTTA
dnaJ
TGTTTGTCTTGTG
TTTTGCGCAATTGGC
CCTCATTTT
grpE
GCCTTTGCTTGAA
GTTTCTTGAAAATGT
CCTTATCTT
groE
CGCCTCACTTGAA
TTTTCCCGCACACA
CCCTAATTT
Ec consensus
TNTCNCCCTTGAA
N13-15
CCCCATTTA
α consensus
CTTG
N17-18
CYTATNTNNG

enzyme was obtained for the wild type construct in *E. coli* ( Figure 4.13). This indicates that the gonococcal $\sigma^{32}$ promoter is not recognised by the *E. coli* RpoH sigma factor. This chapter provides results of the first gonococcal $\sigma^{32}$ promoter cloned in *E. coli* that is non-functional in this background. Interestingly the $\sigma^{32}$ regulated *groE* operon of *A. tumefaciens* and *dnaKJ* operon of *B. japonicum* are expressed poorly and not at all, respectively, in an *E. coli* background (Minder *et al.*, 1997; Segal and Ron, 1996a). This has been attributed to the differences in the heat shock promoter consensus sequences between $\alpha$ and $\gamma$ purple proteobacteria, where the -10 and -35 consensus sequences of the latter bacteria contain a larger number of C residues (Segal and Ron, 1995). The lack of recognition of the gonococcal *dnaK* promoter by the *E. coli* $\sigma^{32}$ protein may also be due to slight variations in sequence of the -10 and -35 regions as stated previously. Alternatively additional regulatory factors specific to *N. gonorrhoeae* may be required for *dnaK* expression. Transfer of the mutated promoter constructs to *N. gonorrhoeae* revealed CAT levels comparable to the negative control (Figure 4.14), confirming that the $\sigma^{32}$ promoter identified by primer extension analysis (Figure 4.10) is in fact used for *dnaK* transcription in *N. gonorrhoeae*.

# CHAPTER FIVE

# IDENTIFICATION AND CHARACTERISATION OF THE ALTERNATIVE SIGMA FACTOR, $\sigma^{32}$, FROM *NEISSERIA GONORRHOEAE*

## 5.1 Introduction

The alternative sigma factor, $\sigma^{32}$, responsible for the positive control of stress related genes has been identified in a variety of Gram negative bacteria from the $\alpha$ and $\gamma$ subdivision of proteobacteria (Benvenisti *et al.*, 1995; Emetz and Klug, 1998; Fleischmann *et al.*, 1995; Huang *et al.*, 1998; Karls *et al.*, 1998; Manzanera *et al.*, 2001; Naczynski *et al.*, 1995; Nakahigashi *et al.*, 1995; Narberhaus *et al.*, 1997; Ono *et al.*, 2001; Ramirez-Santos and Gomez-Eichelmann, 1998; Ramirez-Santos *et al.*, 2001; Reisenauer *et al.*, 1996; Sahu *et al.*, 1997; Sato and Ishikawa, 1997; Wu and Newton, 1996; Yura, 1996). However, it has only been identified, but not studied, in four members of the beta subdivision of proteobacteria including *Alcaligenes xylosoxydans* (Yura and Nakahigashi, 1999), *Methylovorus* sp., *Ralstonia* sp. and *Hydrogenophilus thermoluteolus* (Ono *et al.*, 2001).

The majority of bacteria respond to stress by enhancing the level of RpoH produced, and despite the similar structure and function of the protein, this is achieved via considerably different regulatory mechanisms among bacteria (Nakahigashi *et al.*, 1998). The regulation of RpoH in the majority of $\gamma$ subdivision proteobacteria occurs primarily at the translational level involving the secondary structure of the *rpoH* mRNA (Nagai *et al.*, 1991b; Nakahigashi *et al.*, 1998; Yuzawa *et al.*, 1993). Regulation of *rpoH* in most of these bacteria also occurs to a minor extent at the transcriptional level via $\sigma^{70}$ dependent promoters and in extreme temperature conditions from a $\sigma^{24}$ dependent promoter (Erickson *et al.*, 1987; Nakahigashi *et al.*, 1995; Ramirez-Santos *et al.*, 2001; Yura *et al.*, 1993). Regulatory elements in the promoter region linked to the carbon source and DNA replication are also found in some of the bacteria (Kallipolitis and Valentin-Hansen, 1998; Naczynski *et al.*, 1995; Nagai *et al.*, 1990; Ramirez-Santos *et al.*, 2001; Wang and Kaguni, 1989b). At present, two members of this group of bacteria, *H. influenzae* (Fleischmann *et al.*, 1995) and *B. aphidicola* (Sato and Ishikawa, 1997), appear to lack the *rpoH* mRNA secondary structure responsible for translational

regulation and this is thought to be a reflection of the ecological niche of these bacteria (Nakahigashi *et al.*, 1998).

The regulation of $\sigma^{32}$ in the $\alpha$ subdivision of proteobacteria occurs primarily at the transcriptional level. *rpoH* from *C. crescentus* (Reisenauer *et al.*, 1996; Wu and Newton, 1996, 1997), *R. capsulatus* (Emetz and Klug, 1998) and *A. tumefaciens* (Nakahigashi *et al.*, 1998; Yura and Nakahigashi, 1999) is positively autoregulated from a $\sigma^{32}$ dependent promoter upon heat shock. However, recent evidence indicates that $\sigma^{32}$ from *A. tumefaciens* is predominantly regulated at the post-translational level (Nakahigashi *et al.*, 2001). *B. japonicum* has three *rpoH* genes which are regulated by different mechanisms (Narberhaus *et al.*, 1997). Upon heat shock $rpoH_3$ is transcriptionally induced via a $\sigma^{32}$ dependent promoter located upstream of the adjacent *rag* genes, and $rpoH_1$ transcription is induced from a $\sigma^{70}$ dependent promoter (Narberhaus *et al.*, 1996). $rpoH_2$ transcription originates from a $\sigma^{70}$ promoter at normal temperature and is induced at extreme temperatures from a $\sigma^{24}$ dependent promoter (Narberhaus *et al.*, 1997), a situation reminiscent of that in *E. coli* (Erickson *et al.*, 1987; Yura *et al.*, 1993).

An additional regulatory mechanism employed by *E. coli* is the negative regulation of $\sigma^{32}$ activity by the DnaK chaperone system. During physiological conditions $\sigma^{32}$ interacts with DnaK (Gamer *et al.*, 1996; McCarty *et al.*, 1996; Nagai *et al.*, 1994) and together with DnaJ and GrpE this complex targets $\sigma^{32}$ to proteases for degradation (Herman *et al.*, 1995; Kanemori *et al.*, 1997; Tomoyasu *et al.*, 1995). Upon exposure to heat shock the chaperones are sequestered away from the sigma factor by misfolded proteins such that $\sigma^{32}$ function is restored (Gamer *et al.*, 1996). This strategy of regulating the activity of $\sigma^{32}$ was thought to be conserved in all species (Nakahigashi *et al.*, 1995). This suggestion was made because the region termed Region C, which was originally thought to bind DnaK and encompasses the RpoH box (McCarty *et al.*, 1996; Rudiger *et al.*, 1997b), is uniquely conserved (Nakahigashi *et al.*, 1995; Yura and Nakahigashi, 1999). However, subsequent *in vivo* studies revealed that this region does not in fact bind DnaK but instead is involved in the high affinity binding of RNA polymerase (Arsene *et al.*, 1999; Joo *et al.*, 1998).

In the previous chapter identification of $\sigma^{32}$ dependent promoters upstream of the gonococcal molecular chaperones encoded by *dnaK*, *dnaJ*, *grpE* and the *groE* operon (Tauschek *et al.*, 1997), together with the absence of the regulatory *cis*-acting inverted repeats (CIRCE) suggested that $\sigma^{32}$ was responsible for regulation of the heat shock response in *N. gonorrhoeae*. This chapter describes the cloning, nucleotide sequence and transcriptional analysis of the *rpoH* gene from *N. gonorrhoeae*.

## 5.2 Identification and nucleotide sequence analysis of the *rpoH* gene from *N. gonorrhoeae*

The *rpoH* gene of *N. gonorrhoeae* MS11-A was cloned following the initial identification of this gene from the genome database of *N. gonorrhoeae* FA1090 (Genbank accession number AE004969). BLAST analysis of the genome database with the *E. coli* RpoH amino acid sequence (Landick *et al.*, 1984) revealed a region, which, when translated, displayed 50 % similarity (data not shown), indicating the presence of an *rpoH* homologue in *N. gonorrhoeae*. Homology searches of the flanking regions of the gonococcal *rpoH* homologue identified ORFs where the derived amino acid sequence displayed significant similarity to those of known or putative gene products deposited in the Genbank database (Figure 5.1). This arrangement was very similar to that in *N. meningitidis* strains Z2491 (Parkhill *et al.*, 2000) and MC58 (Tettelin *et al.*, 2000) with the exceptions that in *N. gonorrhoeae* there is an apparent frameshift mutation in a gene encoding a hypothetical transmembrane protein with the highest similarity to the *H. influenzae* homologue HI0507 (Fleischmann *et al.*, 1995), and the absence of a Correia element (Dempsey *et al.*, 1995; Hamilton, 1999) immediately downstream of *rpoH*.

Based on flanking sequences, oligonucleotide primers 5490 and 5492 (Figure 5.1) were designed to amplify the corresponding region from genomic DNA of *N. gonorrhoeae* MS11-A by PCR. A large amount of sequence was included adjacent to *rpoH* to allow the subsequent construction of a mutant (Section 5.4). The expected size fragment of 2.37kb was obtained indicating the conservation of sequences adjacent to *rpoH* in gonococcal strains FA1090 and MS11-A (Figure 5.1). Double-stranded sequence was obtained for the portion of the PCR product that encoded the *rpoH* gene using a series of oligonucleotide primers that spanned this region (Figure 5.2). The nucleotide sequence

**Figure 5.1 ORFs found flanking the *rpoH* gene of *N. gonorrhoeae* FA1090 (Ng; Genbank accession number AE004969) and *N. meningitidis* strains Z2491 (NmA; Parkhill *et al.*, 2000) and MC58 (NmB; Tettelin *et al.*, 2000).**

Schematic diagram indicating the orientation of putative ORFs named after the most similar known or putative genes in the Genbank database. HP indicates ORFs that displayed the highest similarity to hypothetical proteins in the database. C denotes the Correia element (Correia *et al.*, 1986) found in the meningococcal strains (Haas and Meyer, 1986) and the black vertical line represents a translational frame shift in the sequence. Oligonucleotide primers are indicated by numbered arrows above the sequence. ORFs with the same colour represent highly related sequences.

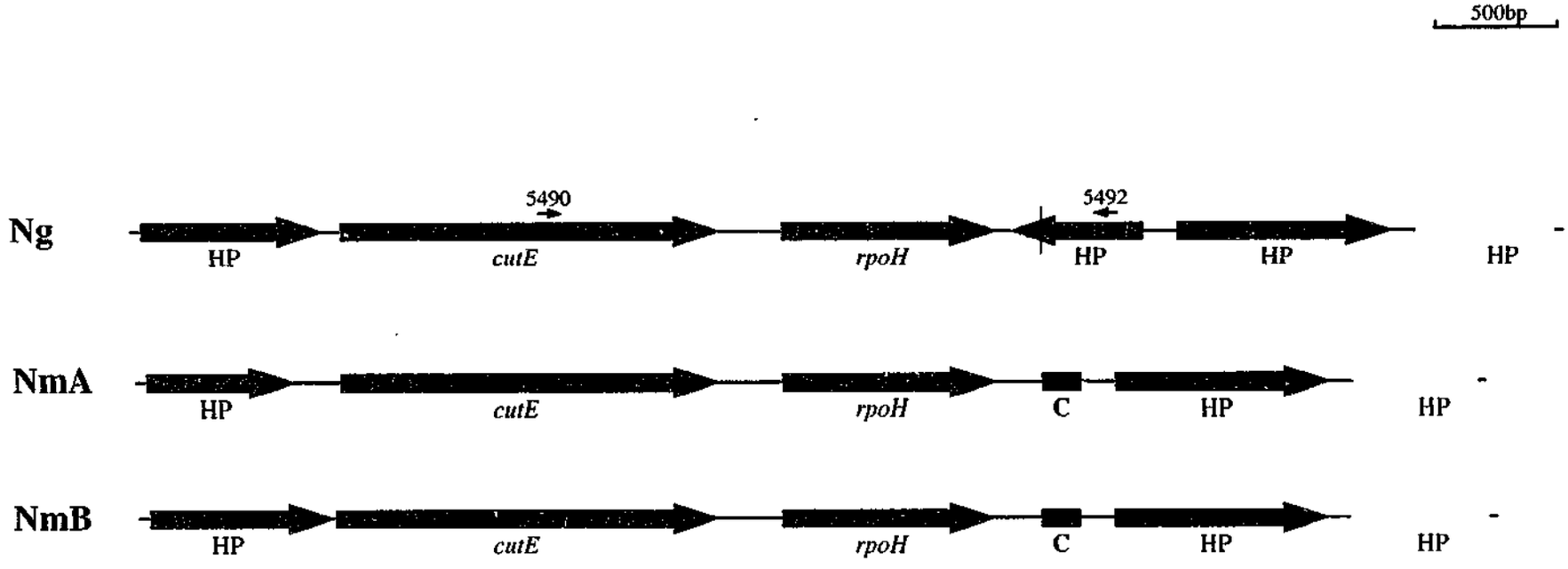
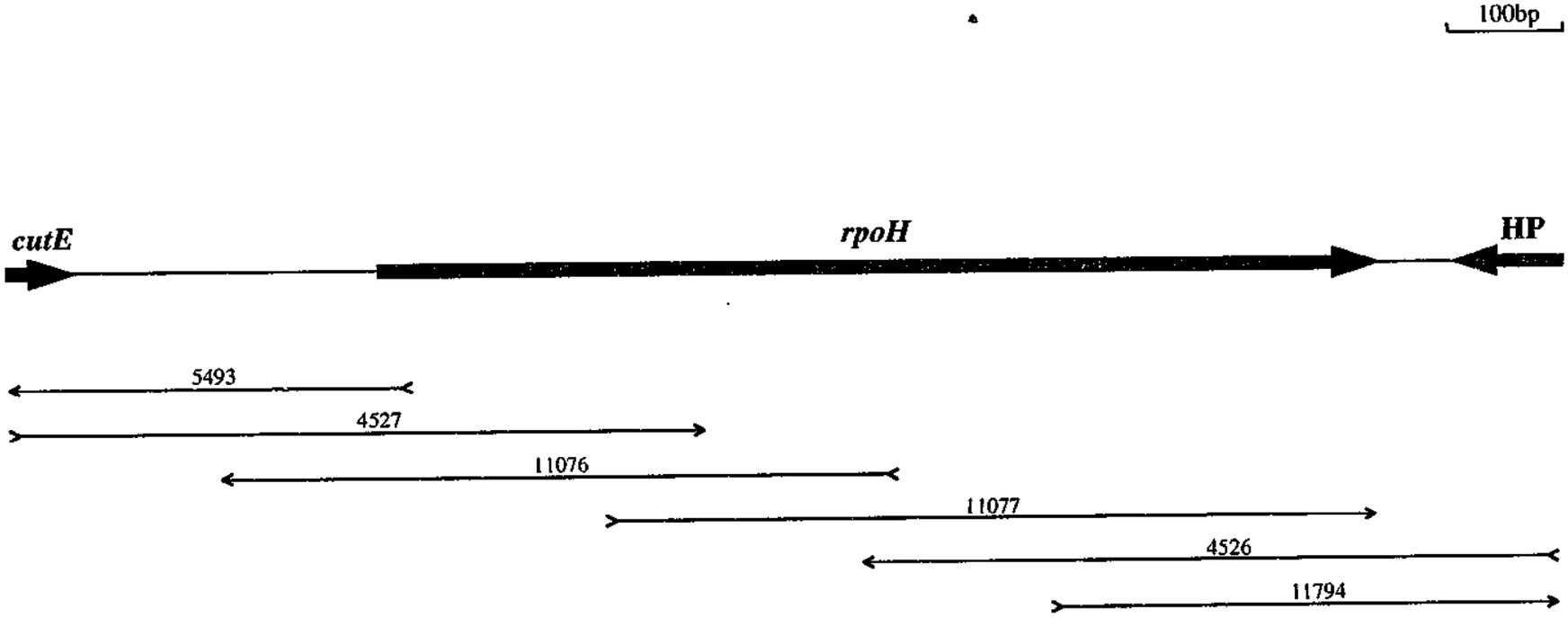

500bp
Ng
5490
5492
HP
cutE
rpoH
HP
HP
HP
NmA
HP
cutE
rpoH
C
HP
HP
NmB
HP
cutE
rpoH
C
HP
HP

**Figure 5.2 Physical map of the 1338bp fragment sequenced encompassing *rpoH* and flanking regions.**

Arrows below the map indicate the direction and extent of sequence determined using the oligonucleotide primers printed above them.


100bp
cutE
rpoH
HP
5493
4527
11076
11077
4526
11794

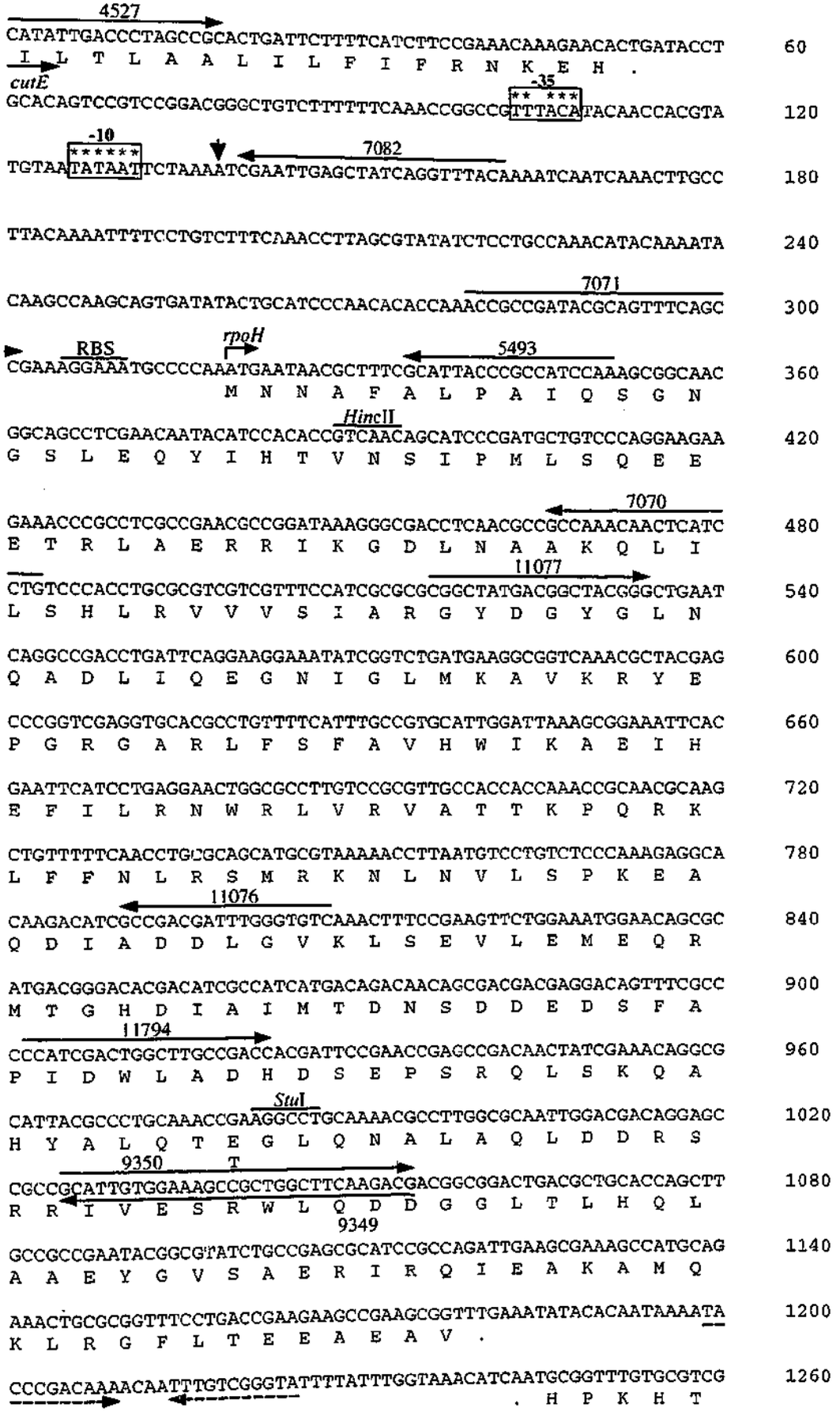
of a 1338bp fragment was determined (Figure 5.3). The PCR product was subsequently cloned into the vector pSU2718 digested with *Hin*cII and one clone, pJKD2101, was retained for further analysis. The gonococcal *rpoH* gene was 861bp in length with the potential to encode a protein of 287 amino acids with a predicted molecular weight of 31.6kDa (Figure 5.3). A potential RBS, AGGAAA (Shine and Dalgarno, 1975) was located 8bp upstream of the putative ATG start codon (Figure 5.3). A potential Rho-independent transcriptional terminator (d'Aubenton Carafa *et al.*, 1990) consisting of an 11bp inverted repeat was identified 16bp downstream of the stop codon. This could potentially form a stable stem and loop structure with a $\Delta G$ value of -14.3kcal/mol.

Alignment of the deduced amino acid sequence of the gonococcal *rpoH* gene with those from other organisms (Figure 5.4) revealed extensive identity to the $\sigma^{32}$ proteins of *N. meningitidis* Z2491 (98.3% in a 290 amino acid overlap), *N. meningitidis* MC58 (96.9% in a 290 amino acid overlap), *Methylovorus* sp. SS1 (60.9% in a 289 amino acid overlap), *A. xylosoxydans* (55.1% in a 312 amino acid overlap), *E. coli* (50.7% in a 290 amino acid overlap), *B. aphidicola* (49% in a 290 amino acid overlap), *H. thermoluteolus* (47.9% in a 311 amino acid overlap), *C. crescentus* (40.7% in a 300 amino acid overlap), *M. xanthus* (38.5% in a 300 amino acid overlap), *A. tumefaciens* (37.1% in a 300 amino acid overlap) and *Ralstonia* sp. CH34 (35.8% in a 330 amino acid overlap).

Analysis of the neisserial $\sigma^{32}$ homologues reveals that the proposed functional sequences characteristic of this protein have been conserved (Figure 5.4). The sequence similarity was very high for the conserved regions 2.4 and 4.2, responsible for promoter recognition, and the regions 2.1 and 2.2, which are involved in binding to core RNA polymerase (Lonetto *et al.*, 1992; Severinova *et al.*, 1996). Additional regions and specific residues proposed to be involved in the interaction of the sigma factor with core RNA polymerase have also been retained in the neisserial $\sigma^{32}$ proteins (Arsene *et al.*, 1999; Arsene *et al.*, 2000; Blaszczak *et al.*, 1999; Joo *et al.*, 1997; Joo *et al.*, 1998; Tomoyasu *et al.*, 2001). Also conserved is the RpoH box characteristic of $\sigma^{32}$ proteins (Nakahigashi *et al.*, 1995) and the overlapping Region C essential for high affinity binding to core RNA polymerase (Arsene *et al.*, 1999; Joo *et al.*, 1998). In addition, residues thought to be involved in abortive transcription (Joo *et al.*, 1998) and indirect interaction of the sigma factor with the -35 region of the promoter (Joo *et al.*, 1998)

**Figure 5.3 Nucleotide sequence of the *rpoH* gene and adjacent sequences from *N. gonorrhoeae* MS11-A.**

The deduced amino acid sequences of RpoH and carboxy-terminal regions of CutE (Rogers *et al.*, 1991) and HI0507 (HP; Fleischmann *et al.*, 1995) are shown below the corresponding nucleotide sequence. Stop codons are indicated by a dot (.). Oligonucleotide primers are indicated by numbered arrows above or below the sequence. The blue coloured oligonucleotide primers indicate those used in site-directed mutagenesis and the pink coloured nucleotide represents the altered base. The green coloured oligonucleotide primers represent those used in the SOE procedure. The putative RBS is overlined and the putative transcriptional terminator is denoted by dashed arrows. Restriction endonuclease sites are shown above the nucleotide sequence. The transcriptional start point is indicated by an arrowhead and the associated $\sigma^{70}$ promoter sequences are boxed with asterisks (*) portraying the nucleotides identical to the consensus sequence (Hawley and McClure, 1983). The red coloured nucleotides represent potential IHF binding regions. Nucleotides are numbered at the right of the figure.


4527
CATATTGACCCTAGCCGCACTGATTCTTTTCATCTTCCGAAACAAAGAACACTGATACCT 60
I L T L A A L I L F I F R N K E H .
cutE
-35
GCACAGTCCGTCCGGACGGGCTGTCTTTTTTCAAACCGGCCGTTTACATACAACCACGTA 120
-10
7082
TGTAATATAATTCTAAAATCGAATTGAGCTATCAGGTTTACAAAATCAATCAAACTTGCC 180
TTACAAAATTTTCCTGTCTTTCAAACCTTAGCGTATATCTCCTGCCAAACATACAAAATA 240
7071
CAAGCCAAGCAGTGATATACTGCATCCCAACACACCAAACCGCCGATACGCAGTTTCAGC 300
RBS
rpoH
5493
CGAAAGGAAATGCCCCAAATGAATAACGCTTTCGCATTACCCGCCATCCAAAGCGGCAAC 360
M N N A F A L P A I Q S G N
HincII
GGCAGCCTCGAACAATACATCCACACCGTCAACAGCATCCCGATGCTGTCCCAGGAAGAA 420
G S L E Q Y I H T V N S I P M L S Q E E
7070
GAAACCCGCCTCGCCGAACGCCGGATAAAGGGCGACCTCAACGCCGCCAAACAACTCATC 480
E T R L A E R R I K G D L N A A K Q L I
11077
CTGTCCCACCTGCGCGTCGTCGTTTCCATCGCGCGCGGCTATGACGGCTACGGGCTGAAT 540
L S H L R V V V S I A R G Y D G Y G L N
CAGGCCGACCTGATTCAGGAAGGAAATATCGGTCTGATGAAGGCGGTCAAACGCTACGAG 600
Q A D L I Q E G N I G L M K A V K R Y E
CCCGGTCGAGGTGCACGCCTGTTTTCATTTGCCGTGCATTGGATTAAAGCGGAAATTCAC 660
P G R G A R L F S F A V H W I K A E I H
GAATTCATCCTGAGGAACTGGCGCCTTGTCCGCGTTGCCACCACCAAACCGCAACGCAAG 720
E F I L R N W R L V R V A T T K P Q R K
CTGTTTTTCAACCTGCGCAGCATGCGTAAAAACCTTAATGTCCTGTCTCCCAAAGAGGCA 780
L F F N L R S M R K N L N V L S P K E A
11076
CAAGACATCGCCGACGATTTGGGTGTCAAACTTTCCGAAGTTCTGGAAATGGAACAGCGC 840
Q D I A D D L G V K L S E V L E M E Q R
ATGACGGGACACGACATCGCCATCATGACAGACAACAGCGACGACGAGGACAGTTTCGCC 900
M T G H D I A I M T D N S D D E D S F A
11794
CCCATCGACTGGCTTGCCGACCACGATTCCGAACCGAGCCGACAACTATCGAAACAGGCG 960
P I D W L A D H D S E P S R Q L S K Q A
StuI
CATTACGCCCTGCAAACCGAAGGCCTGCAAAACGCCTTGGCGCAATTGGACGACAGGAGC 1020
H Y A L Q T E G L Q N A L A Q L D D R S
9350
T
CGCCGCATTGTGGAAAGCCGCTGGCTTCAAGACGACGGCGGACTGACGCTGCACCAGCTT 1080
R R I V E S R W L Q D D G G L T L H Q L
9349
GCCGCCGAATACGGCGTATCTGCCGAGCGCATCCGCCAGATTGAAGCGAAAGCCATGCAG 1140
A A E Y G V S A E R I R Q I E A K A M Q
AAACTGCGCGGTTTCCTGACCGAAGAAGCCGAAGCGGTTTGAAATATACACAATAAAATA 1200
K L R G F L T E E A E A V .
CCCGACAAAACAATTTGTCGGGTATTTTATTTGGTAAACATCAATGCGGTTTGTGCGTCG 1260
. H P K H T

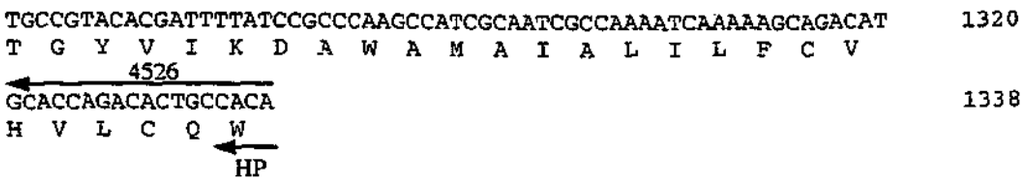
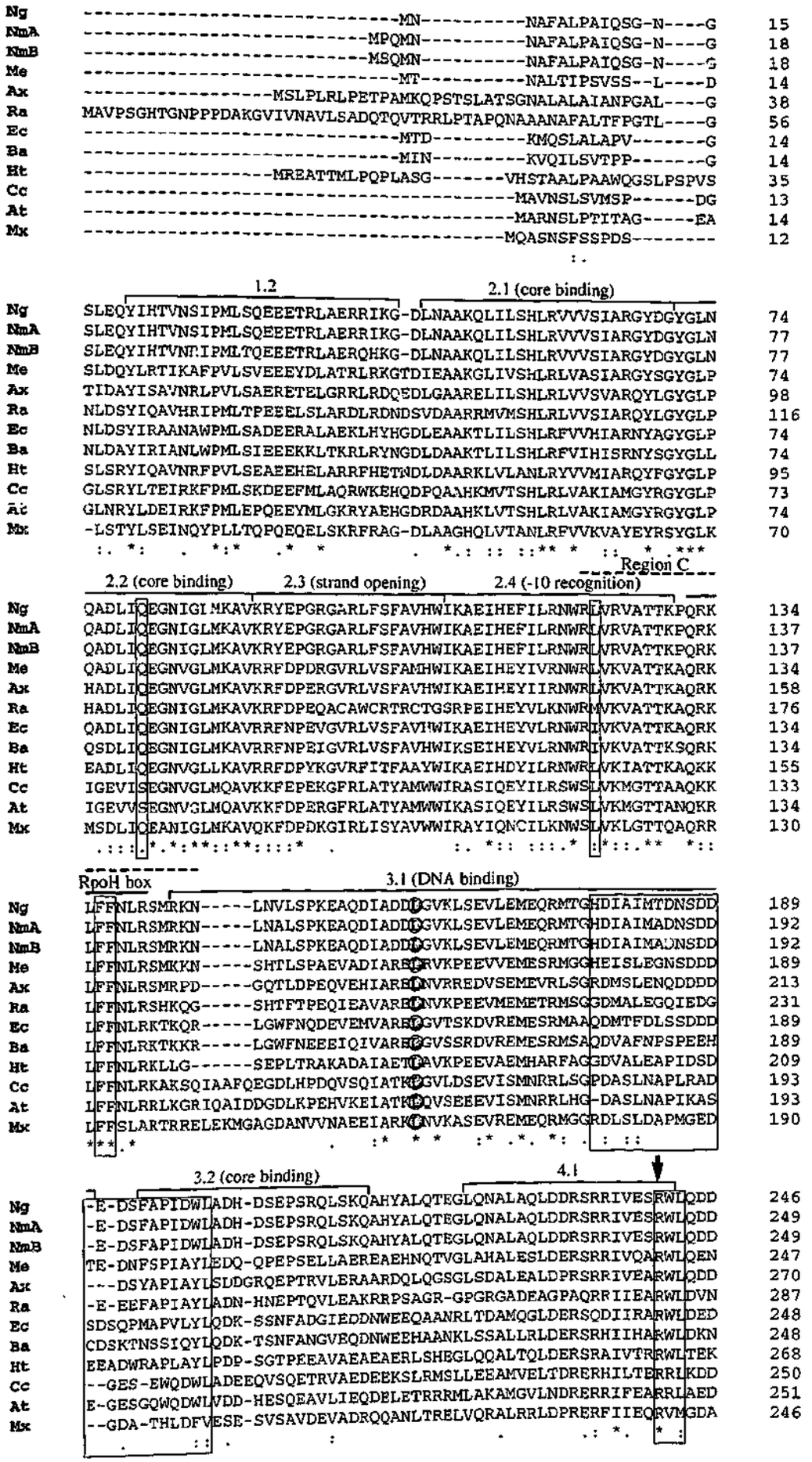
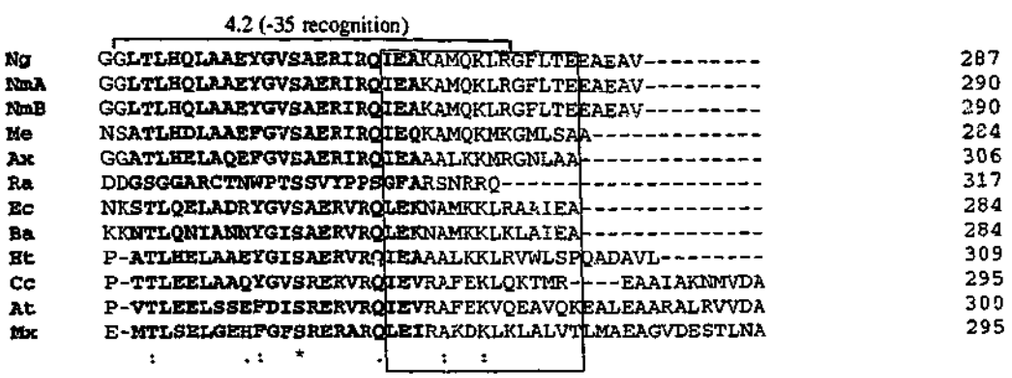

TGCCGTACACGATTTTATCCGCCCAAGCCATCGCAATCGCCAAAATCAAAAAGCAGACAT 1320
T G Y V I K D A W A M A I A L I L F C V
4526
GCACCAGACACTGCCACA 1338
H V L C Q W
HP

**Figure 5.4 Amino acid sequence comparison of the $\sigma^{32}$ proteins from a variety of bacteria.**

The $\sigma^{32}$ proteins of *N. gonorrhoeae* (Ng), *N. meningitidis* strains Z2491 (NmA; Parkhill *et al.*, 2000) and MC58 (NmB; Tettelin *et al.*, 2000), *Methylovorus* sp. SS1 (Me; Genbank accession number AF177466), *A. xylosoxydans* (Ax; Genbank accession number AB009990), *Ralstonia* sp. CH34 (Ra; Genbank accession number J05278), *E. coli* (Ec; Calendar *et al.*, 1988), *B. aphidicola* (Ba; Sato and Ishikawa, 1997), *H. thermoluteolus* (Ht; Genbank accession number AB009991), *C. crescentus* (Cc; Wu and Newton, 1996), *A. tumefaciens* (At; Nakahigashi *et al.*, 1995) and *M. xanthus* (Mx; Apelian and Inouye, 1993) were aligned with the aid of the CLUSTAL W program (Thompson *et al.*, 1994). Dashes (-) indicate gaps that have been introduced to maximise alignment. Residues in red text and the asterisk (*) represent identical residues. Conserved amino acids are indicated by green text and the colon (:) and residues with less similarity are depicted by blue text and the dot (.). The most highly conserved regions identified among sigma factors are overlined and numbered and their proposed functions stated (Helmann and Chamberlin, 1988; Lonetto *et al.*, 1992). Boxed residues are involved in high affinity binding to core RNA polymerase (Arsene *et al.*, 1999; Arsene *et al.*, 2000; Blaszczak *et al.*, 1999; Joo *et al.*, 1997; Joo *et al.*, 1998; Nagai *et al.*, 1994; Tomoyasu *et al.*, 2001; Zhou and Spratt, 1992). The highly conserved RpoH box is overlined and the overlapping Region C is denoted by a dashed line (Nakahigashi *et al.*, 1995). The boxed region coloured in yellow is thought to be involved in the indirect interaction of $\sigma^{32}$ with the -35 region of the promoter (Joo *et al.*, 1998). Residues in bold type represent the putative HTH motif (Helmann and Chamberlin, 1988) and the residue circled in blue is implicated in abortive transcription (Joo *et al.*, 1998). The downward pointing arrow represents the conserved amino acid modified in site-directed mutagenesis experiments.


1.2
2.1 (core binding)
Region C
2.2 (core binding)
2.3 (strand opening)
2.4 (-10 recognition)
RpoH box
3.1 (DNA binding)
3.2 (core binding)
4.1


4.2 (-35 recognition)
Ng GGLTLHQLAAEYGVSAERIRQIEAKAMQKLRGFLTEEAEAV--------- 287
NmA GGLTLHQLAAEYGVSAERIRQIEAKAMQKLRGFLTEEAEAV--------- 290
NmB GGLTLHQLAAEYGVSAERIRQIEAKAMQKLRGFLTEEAEAV--------- 290
Me NSATLHDLAAEPGVSAERIRQIEQKAMQKMKGMLSAA---------- 284
Ax GGATLHELAQEPGVSAERIRQIEAAALKKMRGNLAA---------- 306
Ra DDGSGGARCTNWPTSSVYPPSGFARSNRRQ---------- 317
Ec NKSTLQELADRYGVSAERVRQLEKNAMKKLRAAIEA---------- 284
Ba KKNTLQNIANNYGISAERVRQLEKNAMKKLKLAIEA---------- 284
Ht P-ATLHELAAEYGISAERVRQIEAAALKKLRVWLSPQADAVL--------- 309
Cc P-TTLEELAAQYGVSRERVRQIEVRAFEKLQKTMR----EAAIAKNMVDA 295
At P-VTLEELSSEFDISRERVRQIEVRAFEKVQEAVQKEALEAARALRVVDA 300
Mx E-MTLSELGEHFGFSRERARQLEIRAKDKLKLALVTLMAEAGVDESTLNA 295

have been conserved. The C-terminal portion of $\sigma^{32}$ forms a HTH motif thought to be involved in binding to the -35 element of the promoter (Helmann and Chamberlin, 1988) and analysis of the corresponding regions of the neisserial homologues with the HTH motif prediction program (Dodd and Egan, 1990) reveals that they too have the potential to form such a configuration (data not shown).

The putative *rpoH* start codon was located 263 nucleotides from the end of an ORF with significant homology to the *cutE* gene of *E. coli* (Figure 5.3; Rogers *et al.*, 1991). This gene encodes an apolipoprotein N-acetyltransferase which is an essential protein involved in copper transport and protein modification (Rogers *et al.*, 1991). Downstream of *rpoH* the 3' end of a divergently transcribed ORF was identified that shared the highest similarity to the hypothetical transmembrane protein, HI0507, from *H. influenzae* (Figure 5.3; Fleischmann *et al.*, 1995). However, a frameshift mutation in the middle of this gene would render it incapable of encoding a functional protein.

## 5.3 Complementation of an *E. coli rpoH* mutant with the gonococcal *rpoH* gene

To determine whether the gonococcal *rpoH* gene could complement the temperature sensitive phenotype of an *E. coli rpoH* mutant and thus confirm its identity, complementation studies were performed. The *rpoH* null mutant of *E coli*, KY1608 (Nakahigashi *et al.*, 1995), can only grow at or below 20°C. Electrocompetent cells of KY1608 were prepared and transformed with the plasmid pJKD2101. The plasmid pKV3, containing the *E. coli rpoH* gene (Tobe *et al.*, 1984) and the plasmid vector pSU2718 were also introduced into KY1608 as controls. The transformants were tested for growth by incubation at 20°C and 37°C on LB plates supplemented with and without chloramphenicol (Table 5.1). As expected at 20°C KY1608 cells carrying any of the plasmids grew equally well. At the higher temperature of 37°C growth of KY1608 was only supported by the positive control pKV3 and pJKD2101. These results indicate that the gonococcal *rpoH* gene is transcribed and translated in *E. coli* and the protein product is similar in function to the *E. coli* homologue.

**Table 5.1 Complementation of *E. coli rpoH* mutant KY1608.**

The *E. coli rpoH* mutant KY1608 was transformed with the plasmids pSU2718, pKV3 and pJKD2101 and incubated on LB agar supplemented with chloramphenicol (Cm) or without (LB). The colony-forming ability of each transformant was examined following overnight incubation at 20°C and 37°C. +, colony-forming ability; -, no colony-forming ability.

| **Strain/Plasmid** | **Colony formation** | |
|---|---|---|
| | 20°C | 37°C |
| KY1608 (LB) | + | - |
| KY1608 (Cm) | - | - |
| KY1608 (pSU2718) (LB) | + | - |
| KY1608 (pSU2718) (Cm) | + | - |
| KY1608 (pKV3) (LB) | + | + |
| KY1608 (pJKD2101) (LB) | + | + |
| KY1608 (pJKD2101) (Cm) | + | + |

## 5.4 The *rpoH* gene from *N. gonorrhoeae* is essential

An attempt was made to determine the importance of $\sigma^{32}$ in *N. gonorrhoeae* by insertional activation of the *rpoH* gene with an antibiotic resistance cassette. A 1.1kb cassette from pJKD861 containing the gonococcal *recA* promoter fused to a promoterless *cat* gene (Black *et al.*, 1995) was cloned into the *Hin*cII site located 70bp downstream of the putative translation start site of the *rpoH* gene in pJKD2101 (Figure 5.3 ). The insert was cloned in the same orientation as *rpoH* to yield plasmid pJKD2122 (Figure 5.5) and in the opposite orientation to yield pJKD2124. The plasmids were linearised with *Pst*I and transformed into *N. gonorrhoeae* MS11-A (Figure 5.5). Inoculated plates were incubated at 30°C and 37°C as the gonococcal *rpoH* mutant may have been temperature sensitive like its *E. coli* counterpart (Zhou *et al.*, 1988). Interestingly, no chloramphenicol resistant colonies were obtained for cultures incubated at 30°C and very few were recovered from those incubated at 37°C. They included three transformants resulting from recombination with pJKD2122 and one from recombination with pJKD2124 and were designated JKD466, JKD468, JKD469 and JKD467, respectively. Several additional small colonies were also obtained following incubation but did not grow upon subculture. Southern hybridisation analysis using high stringency conditions was performed on genomic DNA extracted from these strains and digested with *Sau*3A to confirm integration of the cassette (Figure 5.6). Probes included an internal 593bp fragment of *rpoH* obtained by digestion of pJKD2101 with *Hin*cII and *Stu*I (Figure 5.3) and the *cat* gene amplified from pJKD800 using oligonucleotide primers UP and RP. The expected 3.7kb hybridising *Sau*3A fragment was observed for strain MS11-A when probed with the internal region of *rpoH* (Figures 5.5 and 5.6). However, this fragment was also evident in all the transformants indicating that the *rpoH* gene was still intact (Figure 5.6B). Hybridising fragments of various size were obtained for the recombinant derivatives when probed with the *cat* gene (Figure 5.6B). This indicates that during the transformation procedure cointegrates formed that resulted from either random integration into sites other than the *rpoH* gene (lanes 3 and 4) or a single cross-over event (lanes 5 and 6). These result strongly suggest that the *rpoH* gene of *N. gonorrhoeae* is essential for growth at 30°C and 37°C.

**Figure 5.5 Strategy for construction of the gonococcal *rpoH* mutant.**

The P*recA*::*cat* fusion (yellow-blue arrow) from pJKD861 was inserted into the *Hinc*II site of *rpoH* (red arrow) in pJKD2101. The resulting plasmid pJKD2122 was linearised, transformed into *N. gonorrhoeae* MS11-A and if successful would have integrated into the gonococcal chromosome by allelic exchange at the *rpoH* locus. The position of the probe used for subsequent Southern hybridisation analysis to confirm potential mutants and the expected hybridising *Sau*3A fragments are shown. Dashed lines represent the gonococcal chromosome. Relevant restriction endonuclease sites are indicated. Abbreviations: HII, *Hinc*II; S, *Sau*3A.

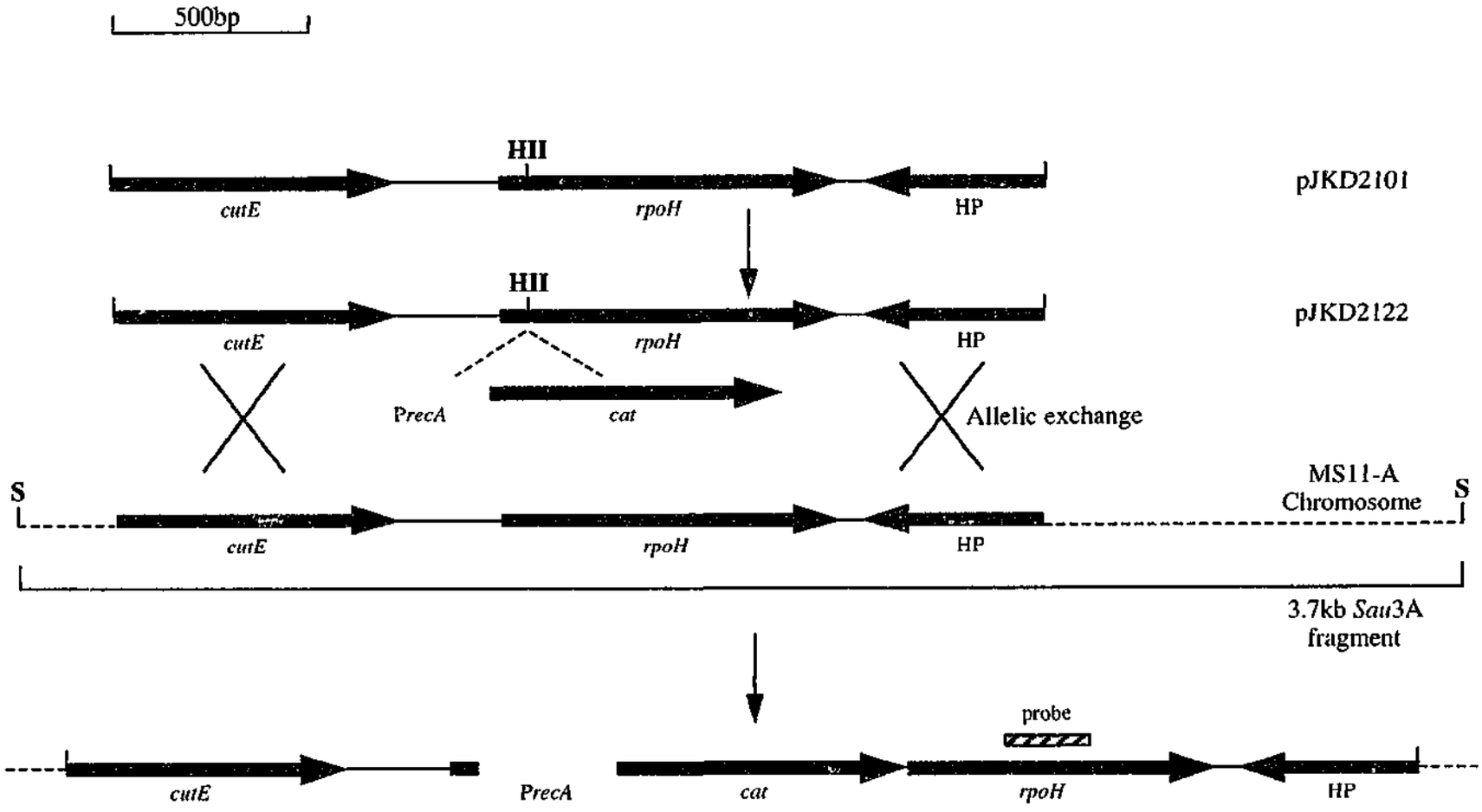
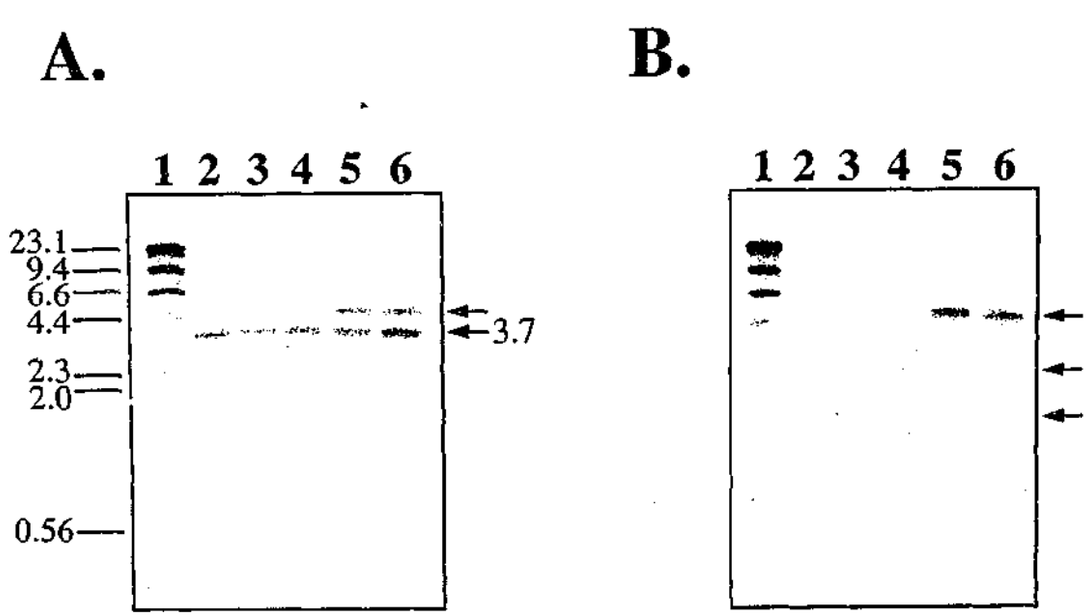

500bp
HII
cutE
rpoH
HP
pJKD2101
HII
cutE
rpoH
HP
pJKD2122
PrecA
cat
Allelic exchange
S
cutE
rpoH
HP
MS11-A
Chromosome
S
3.7kb Sau3A
fragment
probe
cutE
PrecA
cat
rpoH
HP

**Figure 5.6 Southern hybridisation analysis of the gonococcal *rpoH* recombinants.**

Total genomic DNA from *N. gonorrhoeae* MS11-A (lane2) and the gonococcal *rpoH* recombinants JKD466 (lane 3), JKD467 (lane 4), JKD468 (lane 5) and JKD469 (lane 6) was digested with *Sau*3A and probed with an internal region of *rpoH* (Panel A) as shown in Figure 5.5 and the *cat* gene (Panel B). The size of the hybridising band (kb) containing *rpoH* is indicated by an arrow and the additional arrows depict transformants that resulted from either random integration or single cross-over events. Lambda DNA digested with *Hin*dIII and labelled with DIG served as the size standards (kb; lane 1).


A.
B.
1 2 3 4 5 6
23.1
9.4
6.6
4.4
2.3
2.0
0.56
3.7
1 2 3 4 5 6

## 5.5 *In vivo* analysis of a point mutation on $\sigma^{32}$ function

As it was impossible to disrupt the gonococcal *rpoH* gene by insertional inactivation mutational analysis was performed. A number of mutations and deletions have been identified in the *E. coli rpoH* gene that affect function by disrupting core RNA polymerase binding, DNA binding, promoter recognition, transcriptional activation (Arsene *et al.*, 1999; Joo *et al.*, 1997; Joo *et al.*, 1998; Zhou *et al.*, 1992) or possibly degradation by FtsH (Blaszczak *et al.*, 1999). In order to construct a gonococcal *rpoH* mutant it was necessary to select a mutation that would provide substantial core RNA polymerase binding but reduced transcriptional activity. This would ensure that the mutant sigma factor could compete with the wild type for binding to core RNA polymerase but would be defective in initiating transcription of the heat shock genes upon stress. The level of specific message produced by these genes could subsequently be measured.

The mutation R243C occurs in a conserved region of *E. coli* $\sigma^{32}$ where an arginine residue is changed to a cysteine residue at amino acid position 243 (Figure 5.4; Joo *et al.*, 1998). It seemed to be the most appropriate mutation as it demonstrated a substantial decrease in the maximum yield of transcripts produced from the $\sigma^{32}$ dependent promoter of *dnaK* despite a moderate reduction in core RNA polymerase binding, indicating that its effect was at some stage of the transcription process (Joo *et al.*, 1998). This amino acid was conserved in the gonococcal homologue (Figure 5.4) and site-directed mutagenesis (Ho *et al.*, 1989) was performed to construct the mutant. The oligonucleotide primer 9350 and its complement 9349 (Figure 5.3) were designed to alter the C nucleotide at position 721 (Figure 5.3) to a T nucleotide. PCR was performed with genomic DNA from *N. gonorrhoeae* MS11-A as template and the oligonucleotide primer combinations 4527/9349 and 4526/9350. The expected size fragments of 1054 and 314bp were obtained and the PCR products were subsequently used as template in a PCR fusion reaction with oligonucleotide primers 4526 and 4527. As a control the wild type *rpoH* gene was amplified using the same oligonucleotide primers and genomic DNA of MS11-A as template. The products were cloned into the plasmid vector pSU2718 digested with *Hin*cII to yield the plasmids pJKD2352 and pJKD2345, which contain the wild type *rpoH* gene and mutated *rpoH* gene, respectively. The inserts were subcloned into the *Bam*HI and *Hin*dIII sites of the *E. coli*/*N. gonorrhoeae* shuttle vector

Hermes-2 to yield pJKD2358 and pJKD2346, respectively. The Hermes-2 derivatives were then linearised by digestion with *Cla*I and used to transform *N. gonorrhoeae* strain JKD484. To confirm integration of the constructs into p*tet*M25.2 by homologous recombination, the plasmids were transferred by conjugation into strain JKD359.

The biochemical activity of the sigma factors was determined by assaying the level of transcription emanating from the $\sigma^{32}$ dependent promoter of *dnaK* by primer extension. Total RNA was extracted from exponentially growing cultures of *N. gonorrhoeae* MS11-A, JKD494 (p*tet*M25.2 encoding mutant *rpoH*) and JKD5018 (p*tet*M25.2 encoding wild type *rpoH*) following exposure to heat shock at 42°C for 0, 10 and 20 minutes. The oligonucleotide primer 6035 was used to identify the *dnaK* tsp and for the corresponding sequencing reaction with pJKD2238 (Section 4.7). The expected profile was obtained for MS11-A (Figure 5.7) with an increase in the level of primer extension product following prolonged exposure to heat shock. This tsp originated from a $\sigma^{32}$ dependent promoter, as described in Section 4.6. When compared with MS11-A, an approximate two-fold increase in the level of primer extension product was obtained for strain JKD5018 at both 37°C and 42°C, indicative of the extra copy of *rpoH* in this strain. The increase confirms that this sigma factor is in fact responsible for *dnaK* transcription upon stress. The level of *dnaK* specific message from strain JKD494 was comparable to that of MS11-A at the different time points (Figure 5.7). This indicates that the mutated *rpoH* gene product was defective in function, and could not compete with the wild type protein. The level of heat shock gene expression appeared equivalent to a strain with one copy of *rpoH* per cell.

## 5.6 Regulatory analysis of the gonococcal *rpoH* gene

To elucidate the mechanisms responsible for regulation of the gonococcal *rpoH* gene a search was performed for the elements identified in *rpoH* regulation of other bacteria. In the majority of γ proteobacteria *rpoH* regulation seems to occur primarily at the translational level. Briefly, the 5' coding region of the mRNA contains a sequence that forms an internal secondary structure involving the initiation codon and downstream 20 nucleotides, termed the downstream box, that represses *rpoH* translation at physiological temperatures due to the inaccesibility of the ribosome binding site (Figure 5.8; Morita *et al.*, 1999a; Nagai *et al.*, 1991a; Nagai *et al.*, 1994; Yuzawa *et al.*, 1993).

**Figure 5.7 Primer extension analysis of the *dnaK* upstream region of the gonococcal strains MS11-A, JKD494 and JKD5018.**

Total RNA (50μg per lane) isolated from cells grown at 37°C (lanes 1, 4 and 7) and heat shocked at 42°C for 10 (lanes 2, 5 and 8) and 20 (lanes 3, 6 and 9) minutes was subjected to primer extension using oligonucleotide primer 6035 (Figure 4.2). RNA was extracted from strains JKD5018 (lanes 1-3), JKD494 (lanes 4-6) and MS11-A (lanes 7-9). The primer extension products obtained are indicated by the arrow. The sequencing ladder adjacent to the reactions and marked T, G, C, A was generated with the same oligonucleotide primer. The sequence obtained is listed to the left of the panel and the asterisk represents the transcription start point.

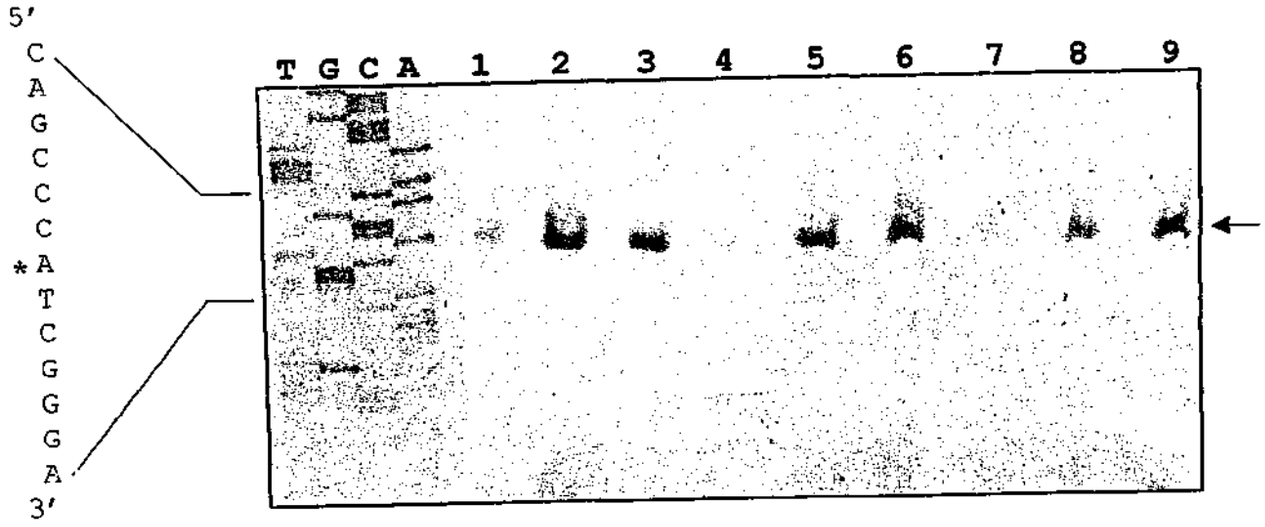

5′
C
A
G
C
C
C
*A
T
C
G
G
G
A
3′
T G C A 1 2 3 4 5 6 7 8 9

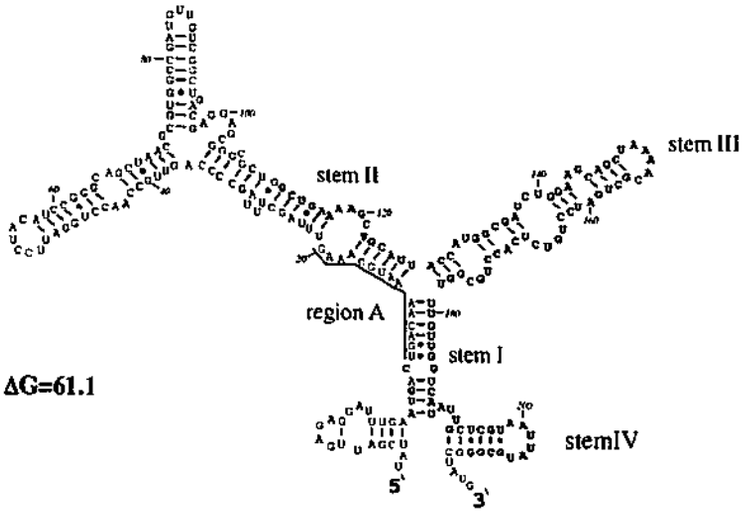
**Figure 5.8 Predicted secondary structures of the 5' region of *rpoH* mRNA (nucleotides –20 to 210 relative to the initiation codon) from *E. coli* (Panel A; Morita *et al.*, 1999a; Nagai *et al.*, 1991a) and *N. gonorrhoeae* (Panels B and C).** The structures in Panels A and B were the most stable predicted by the MULFOLD program (Jaeger *et al.*, 1990) and that in Panel C was predicted using the RNAFOLD program (Zuker, 1989). The calculated minimum free energy (ΔG, kcal/mol) for each structure is indicated and the RBS and start codon is represented by blue and red text, respectively. The downstream box (Region A) is shown and text in bold type represents Region B that forms critical base-pairings with Region A (Morita *et al.*, 1999a). Segments of *E. coli* mRNA that correspond to each of the stem structures (I-IV) are shown (Morita *et al.*, 1999a). •, G-U pairs.

A.

B.

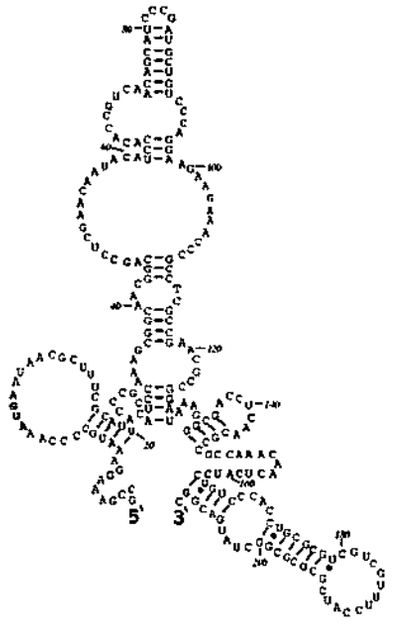
C.

A.



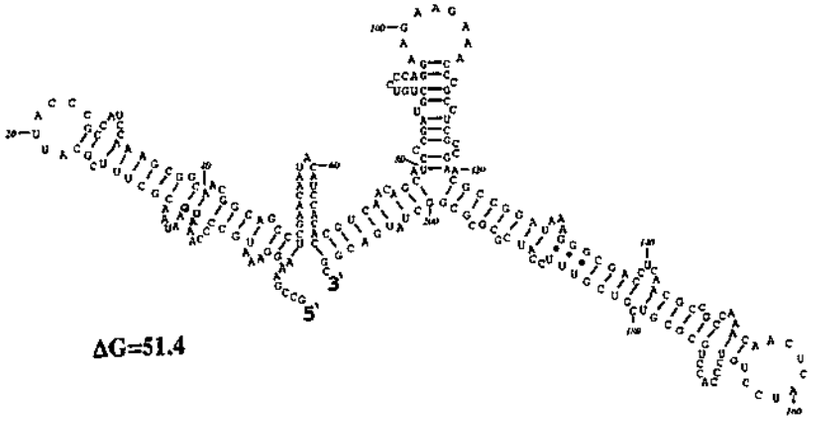

ΔG=61.1

B.




ΔG=43.1

C.




ΔG=51.4

Upon a temperature upshift the structure partially melts permitting an enhanced level of translation due to complementarity of the downstream box with 16S rRNA (Morita *et al.*, 1999b). The anti-downstream box of the gonococcal 16S rRNA gene was identified by BLAST analysis of the *N. gonorrhoeae* FA1090 genomic database with the 16S rRNA gene of *E. coli* (Noller and Woese, 1981). Alignment of this sequence with the region of the gonococcal *rpoH* gene that corresponds to the downstream box of *E. coli rpoH* revealed 5 matches, including the G:U pairs, giving a 33% identity (Figure 5.9). The complementarity found in *E. coli* is usually around 65-80% (Nakahigashi *et al.*, 1995) indicating that the gonococcal *rpoH* transcript lacks the positive regulatory element, the downstream box.

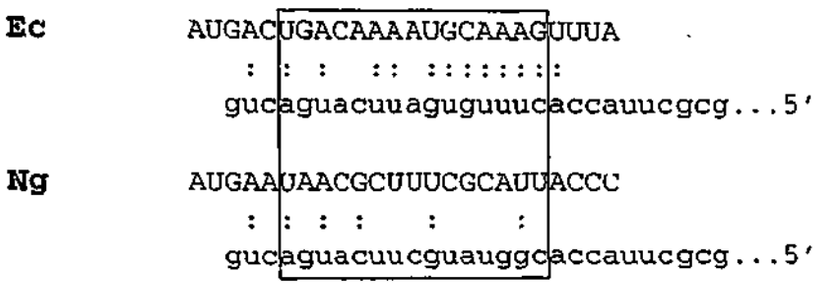
To predict the secondary structure of the *rpoH* message the computer program MULFOLD (Jaeger *et al.*, 1990) was used and visualised by LOOPDLOOP (Gilbert, 1996). These same programs have been utilised to predict the *rpoH* mRNA secondary structure for other organisms encompassing sequence immediately upstream of the initiation codon and 210 bases of downstream coding sequence (Nakahigashi *et al.*, 1995). The structure for the corresponding segment of the *N. gonorrhoeae rpoH* mRNA was predicted and is apparently different from that reported for other γ proteobacteria (Figure 5.8B). The general structure lacked the four characteristic stems and more notable was the absence of base pairing between the ATG start codon and surrounding region with part of the internal coding sequence. This suggests that this region is not sequestered from ribosome entry, which is crucial for translational repression at steady-state temperatures (Morita *et al.*, 1999a). However, when the same sequence was put through another RNA folding program, RNAFOLD (Zuker, 1989), a different structure was obtained with a higher ΔG value (Figure 5.8C). Despite the base pairing formed by part of the ATG start codon it is located close to an exposed loop that may be subject to ribosome entry. In addition part of the RBS forms base pairings which could hyperstabilise the secondary structure and abolish thermoregulation (Morita *et al.*, 1999a). These results do not exclude the possibility that the gonococcal *rpoH* gene is thermally regulated at the translational level by an alternative mechanism that does not involve positive regulation via a downstream box. However, further studies demonstrate that this does not appear to be the case (Section 5.7).

**Figure 5.9 Complementarity between the downstream box of *rpoH* and the anti-downstream box of 16S rRNA.**

Nucleotides 1-24 of *E. coli rpoH* (Ec) containing the downstream box (boxed residues) were aligned with the complementary anti-downstream box of 16S rRNA (lower sequence; Nakahigashi *et al.*, 1995). Base-pairings, including G-U pairs, are indicated by the colon (:). Alignment of the corresponding regions of *rpoH* and 16S rRNA from *N. gonorrhoeae* (Ng) is also shown.

Ec

Ng


Ec
AUGACUGACAAAAUGCAAAGUUUA
gucaguacuuaguguuucaccauucgcg...5′
Ng
AUGAAUAACGCUUUCGCAUUACCC
gucaguacuucguauggcaccauucgcg...5′

The regulation of *E. coli rpoH* also occurs to a minor extent at the transcriptional level (Erickson *et al.*, 1987; Straus *et al.*, 1987; Tilly *et al.*, 1986). Three promoters are $\sigma^{70}$ dependent (Erickson *et al.*, 1987; Fujita and Ishihama, 1987) and one is $\sigma^{24}$ dependent (Erickson and Gross, 1989; Wang and Kaguni, 1989a). The latter is predominantly responsible for *rpoH* transcription at very high temperatures. Transcription of *rpoH* is also modulated by DnaA, coupling it to DNA replication (Wang and Kaguni, 1989b) and the cAMP-CRP/CytR nucleoprotein complex indicating a role in various metabolic processes (Kallipolitis and Valentin-Hansen, 1998; Nagai *et al.*, 1990). Analysis of the gonococcal upstream region did not reveal a $\sigma^{24}$ dependent consensus sequence or potential binding sites for DnaA or CRP (Figure 5.3). The lack of any CytR binding sites was indicative of the absence of the encoding gene from the gonococcal genome as determined by BLAST analysis of the FA1090 genome database (data not shown).

The regulation of *rpoH* in the $\alpha$ proteobacteria occurs primarily at the level of transcription rather than translation. This occurs predominantly from a $\sigma^{32}$ dependent promoter in *C. crescentus*, *A. tumefaciens* and *R. capsulatus*, with post-transcriptional regulation also evident in the latter two bacteria (Emetz and Klug, 1998; Nakahigashi *et al.*, 1998; Nakahigashi *et al.*, 1999; Nakahigashi *et al.*, 2001; Reisenauer *et al.*, 1996; Wu and Newton, 1996). *B. japonicum* possesses three *rpoH* genes which are regulated at the transcriptional level and one of these is $\sigma^{32}$ dependent upon heat shock (Narberhaus *et al.*, 1996; Narberhaus *et al.*, 1997). Analysis of the gonococcal *rpoH* upstream region did not reveal any consensus sequences for $\sigma^{32}$ dependent promoters.

## 5.7 Transcriptional analysis of the *rpoH* gene from *N. gonorrhoeae*

RNA dot blot hybridisation was used to determine the transcriptional activity of *rpoH* at physiological temperature and upon heat shock. The *rpoH* gene probe was prepared by purification of the 593bp *Hin*cII/*Stu*I fragment of pJKD2101 (Figure 5.3). A gonococcal 16S rRNA gene probe was constructed by amplification of the 835bp fragment from *N. gonorrhoeae* MS11-A genomic DNA using oligonucleotide primers 3260 and 3261 to confirm the equivalent transfer of RNA for all samples to the nylon membranes.

Total RNA was extracted from an exponentially growing culture of *N. gonorrhoeae* MS11-A following exposure to heat shock at 42°C for 0, 10, 20 and 40 minutes. RNA samples were diluted to either 0.1, 1 or 5µg, transferred to nylon membranes and incubated with the generated probes. The hybridisation results are shown in Figure 5.10. Probing with the 16S rRNA gene revealed the equivalent transfer of RNA to the membranes. The *rpoH* specific message increased upon heat shock but only after 20 minutes and become much more intense at 40 minutes exposure. Interestingly, transcription of the heat shock genes is induced as early as 10 minutes following a thermal upshift (Section 4.5). The delayed increase in *rpoH* mRNA suggests that neither an increase in transcription or translation of *rpoH* is responsible for this induction. Rather, it would appear that the activity and/or stability of pre-formed $\sigma^{32}$ could play a major role in the initial induction of the heat shock proteins. This form of post-translational regulation has only been documented in one other bacterium, *A. tumefaciens*, where activation of pre-existing $\sigma^{32}$ primarily accounts for induction of the heat shock proteins immediately upon stress (Nakahigashi *et al.*, 2001).

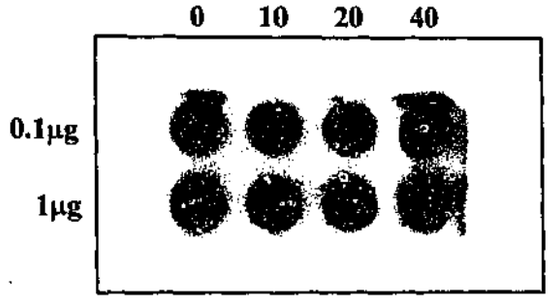
To elucidate whether the increase in *rpoH* specific message is due to an increase in transcription, primer extension analysis was performed. Total RNA was extracted from an exponentially growing culture of *N. gonorrhoeae* MS11-A, incubated at 37°C and subjected to heat shock at 42°C for 0, 10, 20, 30 and 40 minutes. The oligonucleotide primer 5493 was used for the primer extension reactions and for the sequencing reaction with plasmid pJKD2101 (Figure 5.3). A product was obtained for *rpoH* at physiological temperature and this signal intensified following the temperature upshift (Figure 5.11). The increase in transcript was slight at 10 minutes and much more prominent from 20 minutes onwards, consistent with the RNA dot blot hybridisation results. The tsp mapped to an A residue located 181bp upstream of the putative start codon (Figure 5.3). Sequence upstream of the tsp displayed similarity to the -10 and -35 regions of $\sigma^{70}$ dependent promoters (Hawley and McClure, 1983). The potential -10 box, TATAAT, was identical to the consensus and was preceded at a distance of 17bp by a putative -35 element, TTTACA, sharing 5 out of 6bp with the consensus, TTGACA.

Several additional primer extension products were obtained downstream of that mentioned above (Figure 5.11) but they seem to be artifacts of the process and most likely represent stalling of the RNA polymerase. Analysis of sequence the appropriate

**Figure 5.10 Northern dot blot analysis of RNA isolated from *N. gonorrhoeae* MS11-A following heat shock.**

RNA was extracted from cells exposed to heat shock at 42°C for the number of minutes indicated above each well. The amount of RNA transferred to the membranes is indicated. Filters were probed with the 16S rRNA and *rpoH* gene as indicated.

**16S rRNA**




***rpoH***

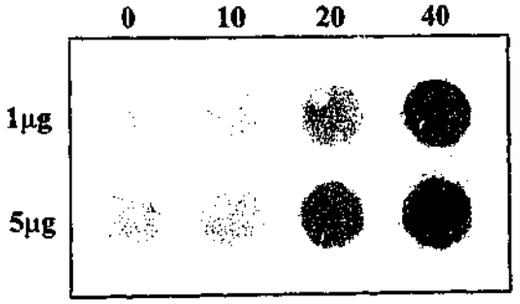
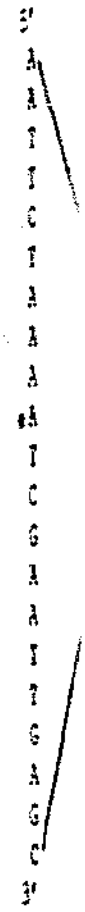
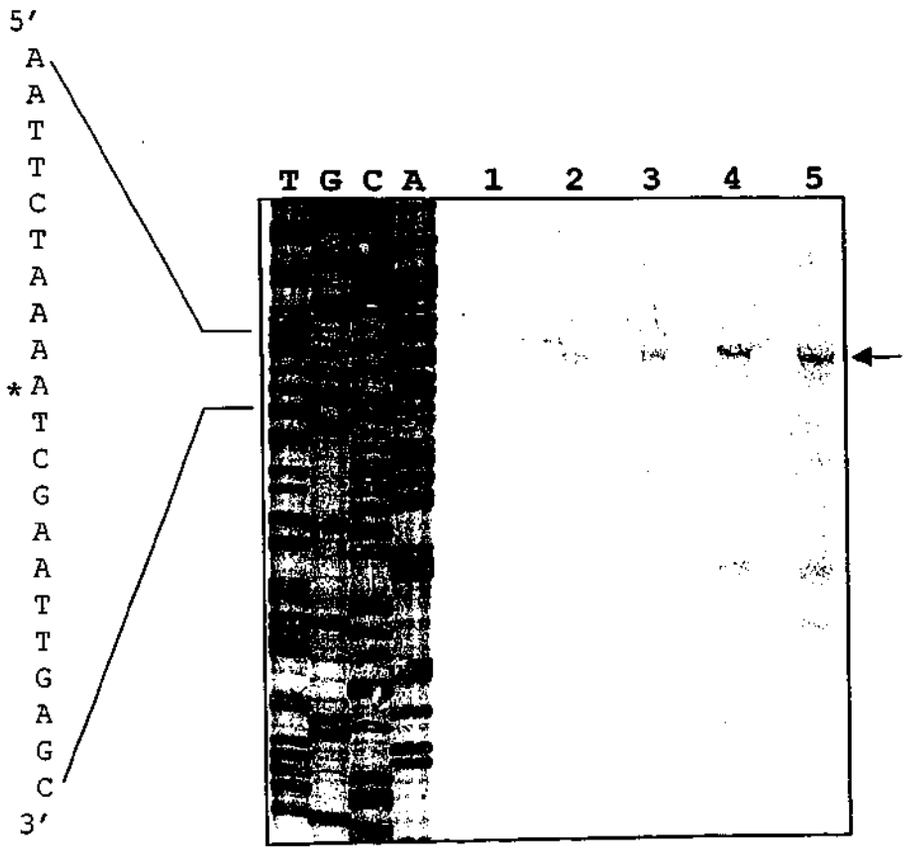


**Figure 5.11 Primer extension analysis of the *rpoH* upstream region of *N. gonorrhoeae* MS11-A.**

Total RNA (50µg per lane) isolated from cells grown at 37°C and heat shocked at 42°C for 0 (lane 1), 10 (lane 2), 20 (lane 3), 30 (lane 4) and 40 (lane 5) minutes was subjected to primer extension using oligonucleotide primer 5493 (Figure 5.3). The primer extension products obtained are indicated by the arrow. The sequencing ladder adjacent to the reactions and marked T, G, C, A was generated with the same oligonucleotide primer. The sequence obtained is listed to the left of the panel and the asterisk represents the tsp.




5′
A
A
T
T
C
T
A
A
A
*A
T
C
G
A
A
T
T
G
A
G
C
3′
T G C A
1
2
3
4
5

distance upstream revealed no matches to the consensus sequences for any known promoters.

The primer extension results reveal that only one $\sigma^{70}$ dependent promoter was identified for the *rpoH* gene of *N. gonorrhoeae* under steady-state and stress conditions unlike the multiple promoters of various specificity identified in the *rpoH* upstream region of other organisms (Section 5.6).

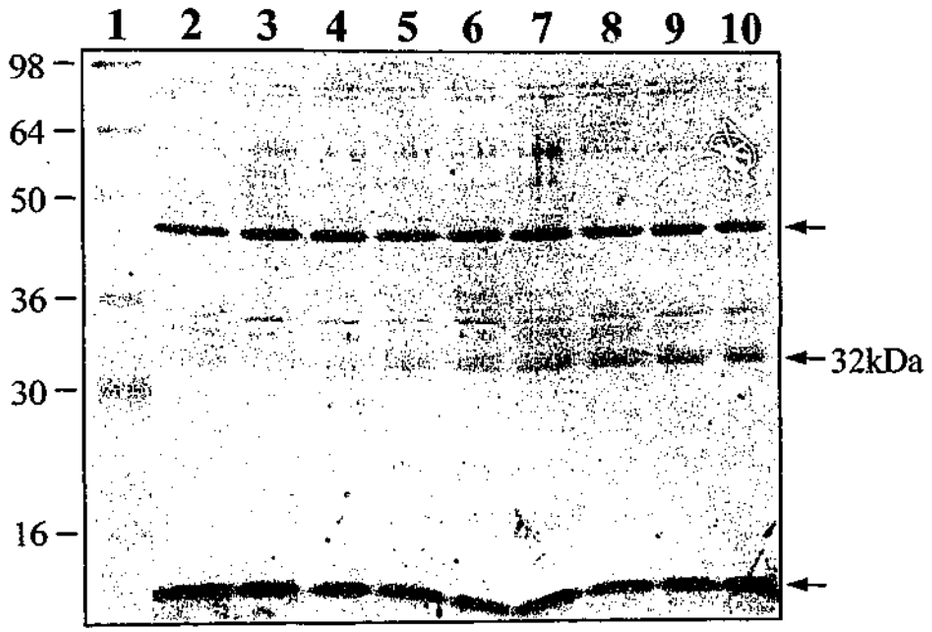
## 5.8 Heat induced synthesis of $\sigma^{32}$ in *N. gonorrhoeae*

Expression of the gonococcal $\sigma^{32}$ protein was investigated to establish if the rate of synthesis concomitantly increases with the observed increase in transcription (Section 5.7). Exponentially growing cells of *N. gonorrhoeae* MS11-A were heat shocked at 42°C for 0, 5, 10, 15, 20, 30, 40, 50 and 60 minutes and subsequently harvested. Cell extracts were prepared and equivalent amounts of protein separated on an SDS-polyacrylamide gel. Western blot analysis was performed with a polyclonal antibody raised against *E. coli* $\sigma^{32}$ (Figure 5.12). Fortuitously the antibody cross reacted with several proteins in the gonococcal extracts demonstrating the equivalent amount of protein in each sample. A protein of approximately 32kDa was recognised by the antibody and was induced substantially following incubation at 42°C. This induction seemed to occur at around 15-20 minutes and increased with prolonged exposure to heat shock. It coincides with the gradual increase in *rpoH* transcription as determined by primer extension (Figure 5.11). However, it should be noted that direct proof is lacking that the gonococcal *rpoH* gene does in fact encode the 32kDa protein.

## 5.9 Transcriptional regulation of the gonococcal *rpoH* gene

The discovery in Section 5.7 that the *rpoH* gene was regulated in part at the transcriptional level led to closer examination of the upstream region and the search for potential regulatory elements. An interesting finding was the presence of the relatively long leader sequence of 181bp (Figure 5.3). Most other promoters responsible for *rpoH* transcription do not have such a long leader sequence upstream of the initiation codon. The exceptions are the $\sigma^{32}$ promoter located 156bp upstream of the *rag* operon responsible for transcription of the adjacent $rpoH_3$ gene of *B. japonicum* (Narberhaus *et*

**Figure 5.12 Western blot analysis of *N. gonorrhoeae* exposed to heat shock.**
*N. gonorrhoeae* strain MS11-A was subjected to heat shock at 42°C for 0, 5, 10, 15, 20, 30, 40, 50 and 60 minutes and whole cell extracts subsequently prepared. Equal volumes of extract from each sample were fractionated on a 12% PAGE gel and lanes 2, 3, 4, 5, 6, 7, 8, 9 and 10 represent the heat shock time intervals in ascending order. The membrane was incubated with the polyclonal antibody raised against *E. coli* $\sigma^{32}$ (Bernd Bukau). Pre-stained standards served as the markers (kDa; lane 1). Proteins recognised by the antiserum are indicated by the arrows.


1 2 3 4 5 6 7 8 9 10
98
64
50
36
30
16
32kDa

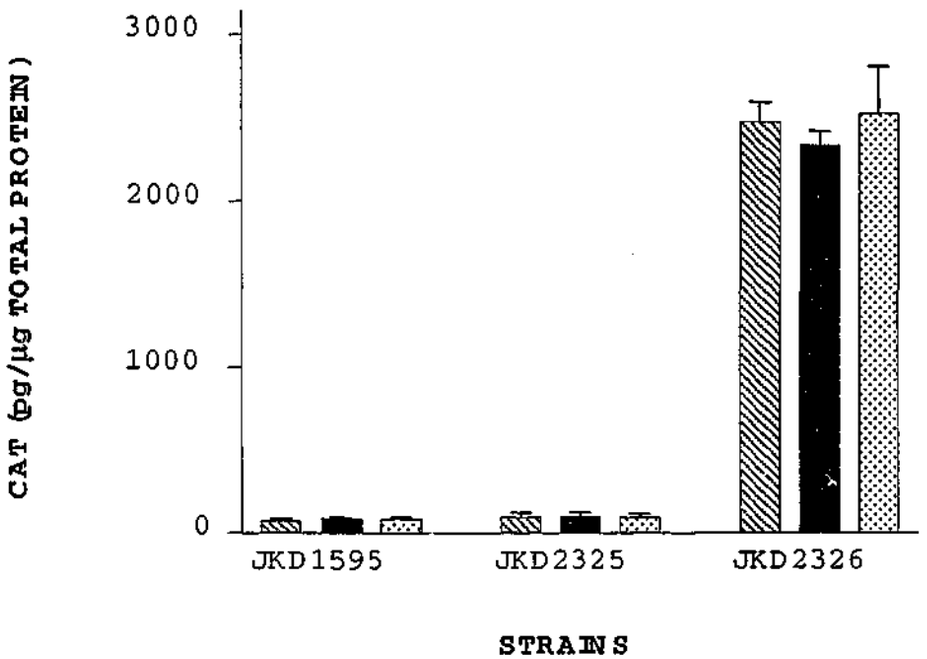
*al.*, 1997) and the $\sigma^{70}$ promoters, termed P1, of the *E. coli* and *P. putida rpoH* genes (Erickson *et al.*, 1987; Manzanera *et al.*, 2001). Despite the 220bp distance between P1 and the ATG start codon of *E. coli rpoH*, there are three other promoters and binding sites for DnaA, CRP and CytR in the intervening sequence (Figure 1.7). An additional promoter is also found in this region of the *rpoH* gene of *P. putida* (Manzanera *et al.*, 2001). To determine the transcriptional significance, if any, of the leader sequence deletion analysis using the SOE method was performed (Horton *et al.*, 1989) and transcriptional activity evaluated in *E. coli* and *N. gonorrhoeae*.

In order to delete the region between the tsp and the RBS, PCR was employed to obtain products upstream and downstream of this area. Oligonucleotide primer combinations 4527/7082 and 7071/7070 (Figure 5.3) were used to accomplish this with pJKD2021 as template. The expected size fragments of 161bp and 205bp were obtained and used as template in a PCR fusion reaction with the external oligonucleotide primers 4527 and 7070. The intact upstream region was also amplified with oligonucleotide primers 4527 and 7070 as a control. The expected size fragments were recovered and the wild type upstream region was cloned into *Hinc*II digested pUC18 to yield pJKD2268. The deleted upstream region was cloned into pSU2718 digested with *Hinc*II to yield pJKD2270. pJKD2268 and pJKD2270 were subsequently digested with *Bam*HI and *Hin*dIII and the inserts ligated to the similarly digested promoter selection vector pKK232-8. The subsequent promoter::*cat* transcriptional fusions were subcloned into the unique *Bam*HI site of the *E. coli*/*N. gonorrhoeae* shuttle vector Hermes-2 to yield the wild type upstream region, pJKD2325, and the deleted upstream region, pJKD2326 (Figure 2.1). Exponential phase cultures of the corresponding strains were heat shocked at 42°C for 0, 5 and 10 minutes. These time intervals were selected as transcription of *rpoH* in *E. coli* peaks at approximately 5 minutes following heat shock (Erickson *et al.*, 1987).

Basal levels of CAT enzyme were detected for strain JKD1595 which contains the promoterless *cat* gene (Figure 5.13). The levels of CAT obtained for JKD2325, containing the wild type *rpoH* promoter region, were equivalent to those observed for JKD1595. Similar levels of CAT were obtained upon the thermal upshift indicating that the gonococcal $\sigma^{70}$ promoter does not respond in an analogous manner to the $\sigma^{70}$ promoters responsible for the increase in transcription in *E. coli* (Erickson *et al.*, 1987).

**Figure 5.13 Transcriptional analysis of the gonococcal *rpoH* upstream region in *E. coli*.**

CAT levels (pg/μg total protein) were determined for *E. coli* strains JKD2325 and JKD2326 which contain the plasmid encoded *cat* reporter constructs carrying the wild type *rpoH* promoter region and the deleted leader sequence, respectively. Strain JKD1595 was the negative control as it contained the promoterless *cat* gene. Cell extracts were prepared from strains exposed to heat shock at 42°C for 0 minutes (hatched bars), 5 minutes (black bars) and 10 minutes (spotted bars). The CAT levels shown are the means of four separate assays. Error bars represent standard deviation.


3000
2000
1000
0
CAT (pg/µg TOTAL PROTEIN)
JKD1595
JKD2325
JKD2326
STRAINS

These results also suggest that the gonococcal *rpoH* gene is poorly expressed in *E. coli*. Primer extension analysis of RNA isolated from the same cultures revealed that transcription of the *rpoH* gene originated from the same promoter as that identified in *N. gonorrhoeae* (Figures 5.14 and 5.3). Deletion of the leader sequence in strain JKD2326 revealed 25-fold higher CAT levels compared to JKD2325 and a lack of heat induction (Figure 5.13). Primer extension analysis revealed that the *rpoH* specific message emanated from the $\sigma^{70}$ promoter identified previously (Figures 5.14 and 5.3) and the signals observed upon heat shock were more intense than those observed for JKD2325. The significant increase in promoter activity for JKD2326 suggests that regulation occurs at the level of transcription in *E. coli*. Deletion of the leader sequence could affect the stability of the mRNA. Alternatively, the deleted region may contain a binding site recognised by an *E. coli* transcriptional repressor, deletion of which results in the enhanced level of transcription observed. The reason as to the discrepancy between the low level of transcript and the high level of CAT observed for JKD2326 prior to heat shock remains unclear (Figures 5.13 and 5.14).

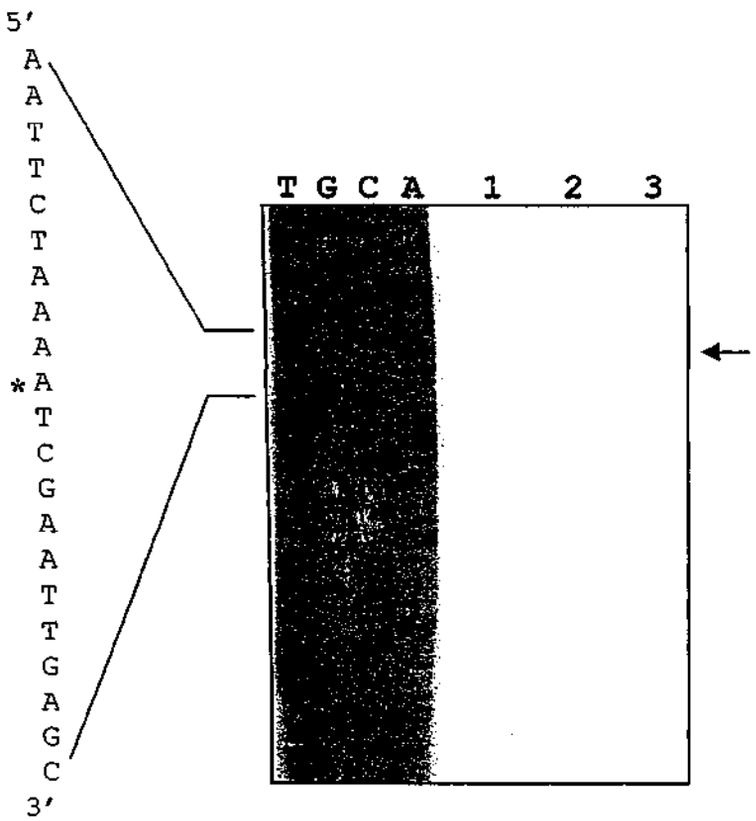
The Hermes-2 promoter::*cat* transcriptional fusions were then linearised with *Cla*I, transferred to *N. gonorrhoeae* strain JKD484 by transformation and the level of CAT activity assessed. Integration of the fusions into p*tet*M25.2 by homologous recombination was confirmed by transferring the plasmids into strain JKD359 by conjugation. Exponential phase cultures of the resulting recombinant gonococcal strains were subjected to heat shock at 42°C for 0, 20 and 40 minutes as *rpoH* transcription increases substantially at 20 minutes (Section 5.7). Cell extracts of the cultures were then prepared and the CAT levels determined (Figure 5.15).

Basal levels of CAT were obtained for strain JKD487 which contains the promoterless *cat* gene. Modest levels of CAT were produced for JKD488, containing the intact promoter region, prior to heat shock and remained constant following the temperature increase. This indicates that transcription was not heat induced and may depend on *rpoH* coding sequence not included in the constructs. Alternatively, the superhelical structure of the *rpoH* upstream region may have an effect on transcription upon heat shock and if it is altered in the reporter construct may render it constitutive. Deletion of the leader sequence in strain JKD489 resulted in an approximate 7-fold increase in CAT compared to JKD488 and was not induced significantly upon heat shock. These results reflect

**Figure 5.14 Primer extension analysis of the gonococcal *rpoH* upstream region in *E. coli*.**

Total RNA (50μg per lane) isolated from strains JKD2325 (Panel A) and JKD2326 (Panel B) grown at 37°C and heat shocked at 42°C. Strains JKD2325 and JKD2326 were heat shocked for 0 (lane 1), 5 (lane 2) and 10 (lane 3) minutes. Samples were then subjected to primer extension using oligonucleotide primer 5493 (Figure 5.3). The primer extension products obtained are indicated by the arrow. The sequencing ladder adjacent to the reactions and marked T, G, C, A was generated with the same oligonucleotide primer. The sequence obtained is listed to the left of each panel and the asterisk represents the tsp.

**A.**


5′
A
A
T
T
C
T
A
A
A
*A
T
C
G
A
A
T
T
G
A
G
C
3′
T G C A 1 2 3

**B.**

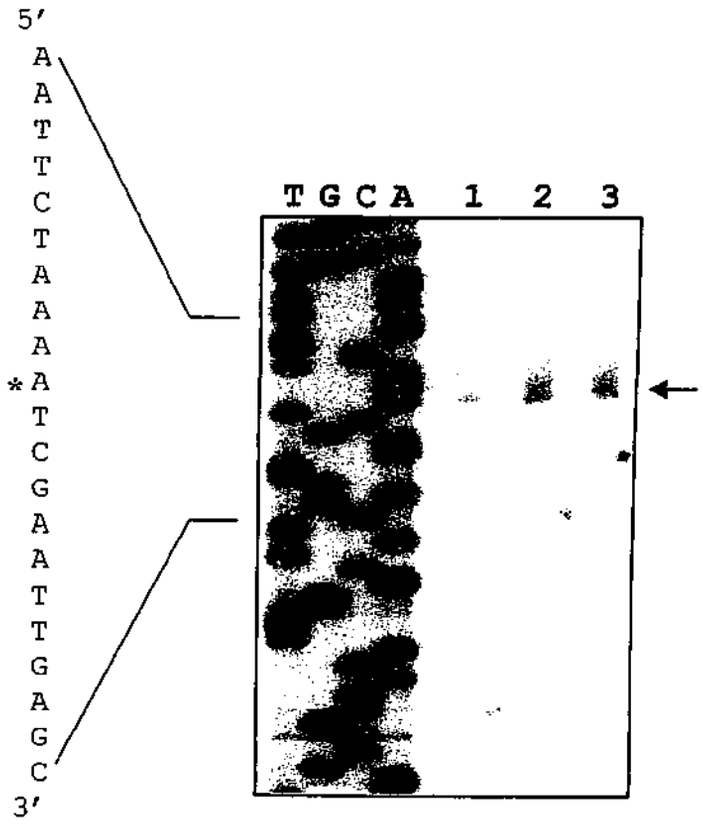
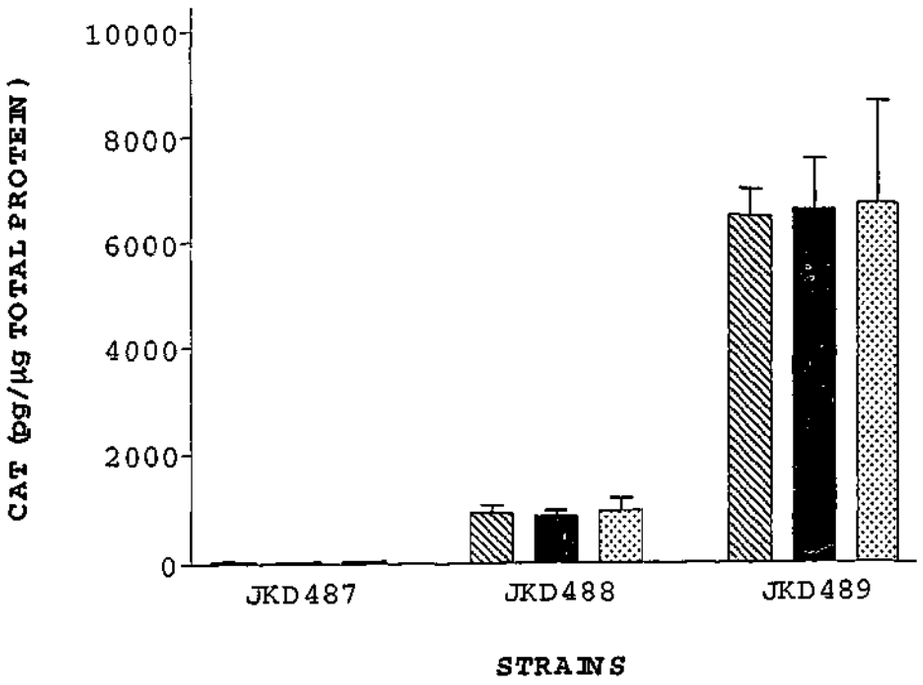

5′
A
A
T
T
C
T
A
A
A
*A
T
C
G
A
A
T
T
G
A
G
C
3′
T G C A 1 2 3

**Figure 5.15 Transcriptional analysis of the gonococcal *rpoH* upstream region in *N. gonorrhoeae*.**

CAT levels (pg/μg total protein) were determined for gonococcal strains JKD488 and JKD489 which contain the *cat* reporter constructs integrated into the conjugative plasmid p*tet*M25.2, carrying the wild type *rpoH* promoter region and that with the deleted leader sequence, respectively. The control strain JKD487 contained the promoterless *cat* gene. Cell extracts were prepared from strains exposed to heat shock at 42°C for 0 minutes (hatched bars), 20 minutes (black bars) and 40 minutes (spotted bars). The CAT levels shown are the means of four separate assays. Error bars represent standard deviation.


10000
8000
6000
4000
2000
0
CAT (pg/µg TOTAL PROTEIN)
JKD487
JKD488
JKD489
STRAINS

those obtained for *E. coli* and thus may be indicative of the difference in secondary structure between the wild type and deleted constructs which affects the level of transcription or mRNA stability. Alternatively, the leader sequence may encode a potential binding site for a gonococcal repressor.

## 5.10 Binding of IHF to the gonococcal *rpoH* upstream region

The possibility of a repressor binding to the *rpoH* upstream region led to further analysis of this sequence. Potential binding sites in the leader sequence were found for the DNA-binding, heterodimeric protein, IHF (Figure 5.3; Friedman, 1988). IHF is a multi-functional protein involved in a number of biological functions including site-specific recombination, DNA replication and the positive and negative regulation of gene expression (Freundlich *et al.*, 1992; Friedman, 1988). IHF was recently purified from *N. gonorrhoeae* and shown to bind to the *pilE* upstream region (Hill *et al.*, 1997) and enhance the level of transcription (Fyfe and Davies, 1998). Comparison of the *pilE* IHF binding sequence (Hill *et al.*, 1997) and *E. coli* core IHF binding consensus sequence with the putative *rpoH* IHF binding sequences revealed a high degree of similarity (Figure 5.16). To determine whether this protein bound to the *rpoH* upstream region and influenced transcription gel shift analysis was performed.

The *rpoH* upstream region was amplified by PCR with oligonucleotide primers 4527 and 5493 (Figure 5.3) using pJKD2101 as template. The insert of pJKD2270, containing the deleted leader sequence of *rpoH*, was also amplified as this fragment would lack the putative IHF binding sites and could serve as the negative control. The gonococcal *pilE* promoter region was used as a positive control and was amplified by PCR with oligonucleotide primers 102 (Fyfe *et al.*, 1993) and 1768 (Fyfe *et al.*, 1995) using pJKD862 as template (Fyfe *et al.*, 1995). A portion of the gonococcal *pilE* upstream region that does not contain the IHF binding site was used as a negative control by amplifying this region from pJKD862 with oligonucleotide primers 1768 and 4857 (Fyfe and Davies, 1998). The expected PCR products were obtained for all reactions, purified and subjected to gel shift analysis with the gonococcal IHF protein (Hill *et al.*, 1997). The results are shown in Figure 5.17. As demonstrated previously IHF bound to the *pilE* upstream region. However, when the binding site was omitted no shifting of the DNA fragment occurred. The IHF protein did not bind to either the wild

**Figure 5.16 Nucleotide sequence alignment of putative IHF binding sites.**
The putative *rpoH* IHF binding sequences (designated in the order that they appear upstream of the start codon; Figure 5.3) was compared to the degenerate IHF core binding consensus sequence of *E. coli* (Ec; Craig and Nash, 1984) and *pilE* IHF binding region (Hill *et al.*, 1997). Y, C/T nucleotides; R, A/G nucleotides. The underlined region of *pilE* represents the nucleotides that are protected by IHF.

| | |
|---|---|
| ***Ec*** | TNYAANNNRTTGAT |
| ***pilE*** | TACAAAATAAAACAATTATATAGA |
| ***rpoH$_1$*** | TACAAAATCAATCAAACTTGCCTT |
| ***rpoH$_2$*** | TACAAAATTTTCCTGTCTTTCAAA |
| ***rpoH$_3$*** | TACAAAATACAAGCCAAGCAGTGA |

**Figure 5.17 Gel shift analysis of IHF binding to the *pilE* and *rpoH* promoter regions.**

Gel shift assays with the wild type *pilE* promoter region (lanes 1-3), partial *pilE* promoter region lacking the IHF binding domain (lanes 4-6), wild type *rpoH* promoter region (lanes 7-9) and deleted *rpoH* promoter region (lanes 10-12) were performed with purified IHF from *N. gonorrhoeae* (Hill *et al.*, 1997). Lanes 1, 4, 7 and 10, no protein added; lanes 2, 5, 8 and 11, 0.1μg IHF; lanes 3, 6, 9 and 12, 0.001μg IHF. The arrow indicates the IHF bound DNA fragments.

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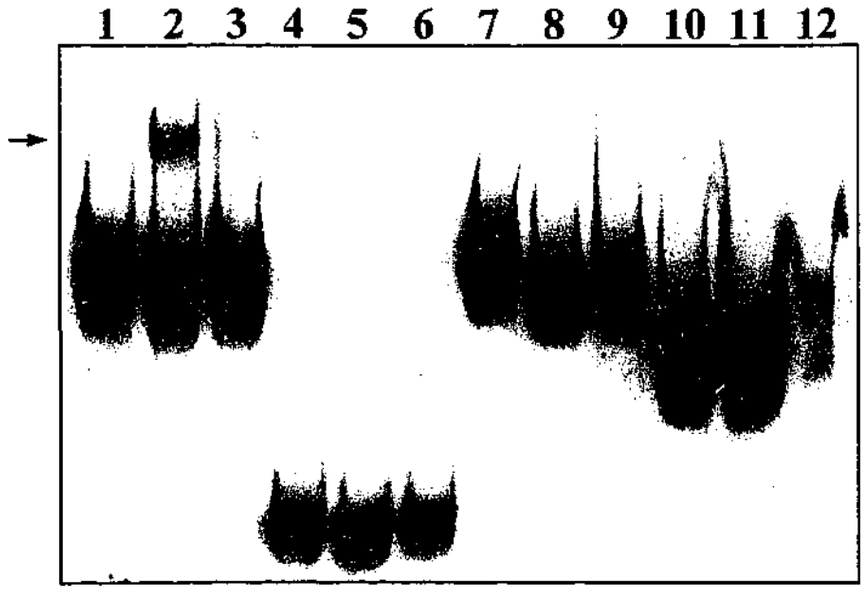

type *rpoH* upstream region or the DNA fragment with the deleted leader sequence. These results indicate that IHF does not bind to the *rpoH* promoter region and subsequently does not have an effect on the regulation of this gene in *N. gonorrhoeae* under the conditions tested.

## 5.11 Discussion

The heat shock response of a number of Gram negative bacteria is regulated positively at the transcriptional level by the alternative sigma factor, $\sigma^{32}$ (Yura, 1996). The identification of $\sigma^{32}$ promoters upstream of several heat shock genes in *N. gonorrhoeae* (Chapter 4) led to the conclusion that this sigma factor was responsible for induction of the stress response. This chapter identified and characterised the alternative sigma factor, $\sigma^{32}$, encoded by *rpoH* from *N. gonorrhoeae* strain MS11-A. A considerable degree of conservation is apparent when the deduced amino acid sequence of the gonococcal *rpoH* gene is compared to that from other organisms (Figure 5.4). As expected the gonococcal $\sigma^{32}$ protein was most similar to those from *N. meningitidis* followed by *Methylovorus* sp. SS1 and *A. xylosoxydans*. This reflects the phylogenetic relationship of the bacteria, with all these species belonging to the beta subdivision of proteobacteria.

The functional sequences and structural organisation of $\sigma^{32}$ have been conserved among the neisserial homologues (Figure 5.4). These include the regions involved in promoter recognition, core RNA polymerase binding and the RpoH box characteristic of $\sigma^{32}$ proteins (Arsene *et al.*, 1999; Arsene *et al.*, 2000; Blaszczak *et al.*, 1999; Helmann and Chamberlin, 1988; Joo *et al.*, 1997; Joo *et al.*, 1998; Lonetto *et al.*, 1992; Nakahigashi *et al.*, 1995; Severinova *et al.*, 1996). This leads to the conclusion that the structure and function of $\sigma^{32}$ is similar to that observed in *E. coli*, where it has been extensively studied (Arsene *et al.*, 2000). This is confirmed by restoration of the temperature sensitivity of an *E. coli rpoH* null mutant when complemented with the gonococcal homologue (Table 5.1). In addition, this suggests that $\sigma^{32}$ recognises promoters related to the *E. coli* $\sigma^{32}$ consensus sequence.

Examination of sequence flanking the *rpoH* gene from *N. gonorrhoeae* strains MS11-A and FA1090 (Figures 5.1 and 5.3) revealed similarity to the *cutE* gene (Rogers *et al.*,

1991) and hypothetical protein HI0507 (Fleischmann *et al.*, 1995) located upstream and downstream of *rpoH*, respectively. Interestingly the sequence encoding the putative hypothetical protein is displaced in *N. meningitidis* by the insertion of a Correia element (Correia *et al.*, 1986). The fact that this hypothetical protein is non-functional in *N. gonorrhoeae* due to a frameshift mutation demonstrates its redundance in the neisserial species. The neisserial *rpoH* genes are not located downstream of the essential cell division operon *ftsYEX*, an arrangement that is conserved in enteric bacteria (Crickmore and Salmond, 1986; Ramirez-Santos and Gomez-Eichelmann, 1998), *P. aeruginosa* (Stover *et al.*, 2000), *P. putida* (Aramaki *et al.*, 1999), *Buchnera* sp. APS (Shigenobu *et al.*, 2000) and *Aeromonas hydrophila* (Merino *et al.*, 2001). This finding is not surprising as *Neisseria* sp. belong to a different subdivision of proteobacteria.

It was not possible to create a gonococcal *rpoH* mutant by insertional inactivation indicating that this gene is essential for viability (Figure 5.6) as is the case in *E. coli* (Zhou *et al.*, 1988). Introduction of an additional copy of *rpoH* in *N. gonorrhoeae* with a mutation that has been shown to render $\sigma^{32}$ defective in transcription of *dnaK* in *E. coli* (Joo *et al.*, 1998) did not yield the same result (Figure 5.7). The level of *dnaK* transcription did not decline to the same extent in the gonococcal mutant as it did in *E. coli*. These results may be explained by the decreased ability of the gonococcal mutant $\sigma^{32}$ to compete with the wild type and influence transcription. The level of *dnaK* transcription approximately doubled upon introduction of a second wild type copy of *rpoH* into the cell, regardless of the temperature, confirming that the encoded $\sigma^{32}$ protein is indeed responsible for transcription of this gene (Figure 5.7).

To determine the mechanisms responsible for regulation of the gonococcal *rpoH* gene a search was performed for the regulatory elements operative in other organisms. The positive regulatory element, the downstream box, was lacking from the gonococcal transcript (Figure 5.9) and the 5' region did not form the characteristic secondary structure responsible for thermal regulation in *E. coli* and several other $\gamma$ subdivision proteobacteria (Figure 5.8; Morita *et al.*, 1999a; Nagai *et al.*, 1991a; Nagai *et al.*, 1994; Nakahigashi *et al.*, 1995; Yura, 1996; Yuzawa *et al.*, 1993).

Analysis of the *rpoH* upstream region of *N. gonorrhoeae* revealed no DnaA (Wang and Kaguni, 1989b) or CRP (Kallipolitis and Valentin-Hansen, 1998) consensus binding

sites (Figure 5.3). The lack of a $\sigma^{32}$ consensus sequence (Section 4.8; Figure 5.3) in the *rpoH* upstream region suggested that this gene is probably not auto-regulated. A $\sigma^{24}$ consensus sequence (Missiakas and Raina, 1998) was also not found.

To establish the mechanism of *rpoH* regulation from *N. gonorrhoeae* MS11-A transcriptional analysis by RNA dot blot hybridisation and primer extension was performed. Transcription of *rpoH* increased substantially following a temperature upshift to 42°C for 20 minutes (Figures 5.10 and 5.11). According to the primer extension results this increase began modestly at 10 minutes and increased with prolonged exposure to heat shock. Transcription emanated from a $\sigma^{70}$ promoter (Figure 5.3) and no other promoters were identified under the conditions tested. This is in contrast to the transcriptional regulation reported for the majority of *rpoH* genes which occurs from multiple promoter systems. The level of $\sigma^{32}$ protein was investigated following heat shock and appeared to increase slightly at 10 minutes followed by a more dramatic increase at 20 minutes (Figure 5.12). This elevated level remained constant for at least 60 minutes following the temperature upshift. The concomitant increase in transcription and translation suggests that *rpoH* from *N. gonorrhoeae* is controlled, at least partially, at the level of transcription following heat shock. This situation is similar to that in the $\alpha$ subdivision of proteobacteria where regulation occurs at the transcriptional level (Emetz and Klug, 1998; Nakahigashi *et al.*, 1998; Narberhaus *et al.*, 1997; Reisenauer *et al.*, 1996; Wu and Newton, 1996; Yura and Nakahigashi, 1999).

The increase in $\sigma^{32}$ synthesis following heat shock lagged well behind the observed increase in heat shock gene expression (Sections 4.4 and 4.5) indicating that additional non-transcriptional regulatory mechanisms must be involved. This immediate increase in heat shock protein synthesis prior to an increase in the $\sigma^{32}$ level was recently observed for *A. tumefaciens* (Nakahigashi *et al.*, 2001). Induction of the heat shock response in this bacterium is caused predominantly by an increase in the activity of pre-existing $\sigma^{32}$ and to a minor extent by the increase in $\sigma^{32}$ synthesis due to *rpoH* transcription. It appears that the DnaK chaperone system controls the activity of $\sigma^{32}$ and upon stress mediates its activation. However, whether the control is exerted by direct contact between the chaperones and $\sigma^{32}$, and thus regulated via a similar mechanism to that in *E. coli*, or by means of additional regulatory factors has yet to be determined (Nakahigashi *et al.*, 2001). Briefly, in *E. coli*, the DnaK chaperone system modulates the

cellular level of $\sigma^{32}$ by regulating its synthesis, stability and activity (Gamer *et al.*, 1996; Herman *et al.*, 1995; Nagai *et al.*, 1994; Straus *et al.*, 1990; Tomoyasu *et al.*, 1995). The control of activity and stability is exerted by preferential binding of the DnaK system to $\sigma^{32}$ under steady-state conditions rendering it sensitive to proteolysis and sequestering it away from core RNA polymerase (Herman *et al.*, 1995; Tatsuta *et al.*, 1998; Tomoyasu *et al.*, 1995; Tomoyasu *et al.*, 1998). It seems likely that this form of activity control of $\sigma^{32}$ is responsible for the immediate increase in heat shock gene expression upon heat shock in *N. gonorrhoeae*. Upon stress, the DnaK chaperone system would preferentially bind to misfolded or denatured proteins so that $\sigma^{32}$ was free to bind to core RNA polymerase and induce transcription of the heat shock genes. This form of activity regulation is also apparent in *E. coli* during a temperature downshift where there is a decline in the level of heat shock protein synthesis without a decrease in the level of $\sigma^{32}$ (Taura *et al.*, 1989). Negative modulation of the heat shock response by the DnaK chaperone system appears to be well conserved as Hsp70 in mammalian cells negatively regulates HSF1, a human heat shock transcription factor (Shi *et al.*, 1998).

Interestingly, $\sigma^{32}$ of *A. tumefaciens* is very stable at normal temperatures and gradually destabilises during the adaptation phase of the heat shock response until the level of $\sigma^{32}$ has declined to reach a new steady-state level (Nakahigashi *et al.*, 2001). In contrast, *E. coli* $\sigma^{32}$ is highly unstable at physiological temperature and stabilises upon induction of the heat shock response contributing to an increase in the level of $\sigma^{32}$. The stability of the gonococcal $\sigma^{32}$ protein was not determined in this study but would provide an insight into its role during the different phases of the heat shock response.

To further elucidate the regulatory mechanisms responsible for the transcriptional regulation of *rpoH* from *N. gonorrhoeae* the long leader sequence was deleted and transcriptional activity assessed in *E. coli* and *N. gonorrhoeae*. Transcription from the *rpoH* $\sigma^{70}$ promoter in *E. coli* was poor (Figure 5.13) but deletion of the leader sequence led to a significantly higher level of CAT in both *E. coli* and *N. gonorrhoeae* (Figures 5.13 and 5.15). This may be a result of the different secondary structures adopted by each of the individual transcripts which may affect transcription, mRNA stability or translation (Avedissian *et al.*, 1995; Parsons *et al.*, 1999; Reisenauer *et al.*, 1996). Transcription seems the more likely candidate as primer extension revealed an increase in the level of *rpoH* transcript from the deleted construct in *E. coli* (Figure 5.14). The

secondary structure of the intact *rpoH* promoter region would be more extensive and complex than the deleted one and may therefore decrease mRNA stability or increase the frequency of premature transcription termination resulting in a lower level of CAT (Avedissian *et al.*, 1995; Marolda and Valvano, 1998). The lack of heat induction of the *rpoH* wild type transcriptional fusion in *N. gonorrhoeae* (Figure 5.15) was unexpected but may be due to the altered topological configuration of the DNA by being located on the conjugative plasmid. Temperature changes have been shown to have conformational effects on the DNA supercoiling of some bacterial promoters and subsequently influence gene expression (Hurme and Rhen, 1998; Tse-Dinh *et al.*, 1997). If the increase in *rpoH* transcription upon thermal upshift is due to altered DNA topology this may account for the results observed.

An additional possibility for the elevated levels of CAT obtained for the deleted constructs in *E. coli* and *N. gonorrhoeae* was the binding of a putative repressor to the *rpoH* leader sequence. Closer inspection of the *rpoH* upstream sequence led to the identification of potential IHF binding sites (Figures 5.3 and 5.16). IHF has been implicated in the negative regulation of a number of genes in *E. coli* including the C (Mu phage) gene (Krause and Higgins, 1986), *sodB* (Dubrac and Touati, 2000), a number of *omp* genes (Goosen and van de Putte, 1995), the *ilv* operon (Pagel *et al.*, 1992) and *himA* (Ditto *et al.*, 1994). Gel shift analysis was used to determine if IHF bound to the *rpoH* upstream region. The results (Figure 5.17) demonstrated IHF did not bind to this region indicating that it does not have a role in the regulation of *rpoH* from *N. gonorrhoeae* under the conditions tested.

Overall, the data presented in this chapter suggest that the gonococcal *rpoH* gene is regulated predominantly at the level of activity immediately following a thermal upshift. An increase in *rpoH* transcription is then observed following ongoing exposure to heat shock. This mechanism of regulation is similar to that observed for *A. tumefaciens* (Nakahigashi *et al.*, 2001).

# CHAPTER SIX

## GENERAL DISCUSSION

Type 4 pili play a major role in the pathogenesis of gonococcal disease and therefore constitute a major virulence factor (Paranchych and Frost, 1988). Thus the regulation of the *pilE* gene has been, and still continues to be, under great scrutiny. Of the three identified *pilE* promoters, only the $\sigma^{70}$ promoter P1, is active *in vitro* (Fyfe *et al.*, 1995). However, the presence of a $\sigma^{54}$ promoter, P3, and UAS and IHF binding sites upstream of *pilE* led to the conclusion that this gene could be regulated by $\sigma^{54}$ under different environmental conditions (Carrick *et al.*, 1997; Fyfe *et al.*, 1993; Fyfe *et al.*, 1995; Fyfe and Davies, 1998). This hypothesis was supported by the fact that P3 was functional in heterologous backgrounds when the appropriate regulatory factors were present (Boyle-Vavra *et al.*, 1993; Carrick *et al.*, 1997; Fyfe *et al.*, 1995).

The results of this study show that *N. gonorrhoeae* contains the remnants of an *rpoN* homologue designated *RLS*, for *rpoN*-like sequence (Chapter 3). This sequence was missing the DNA binding motifs essential for function (Cannon *et al.*, 1994; Cannon *et al.*, 1995; Merrick and Chambers, 1992) due to an apparent deletion and so the associated mutant protein would have been unable to function as a sigma factor. It seems that an intact *rpoN* gene was once functional in an ancestor of *N. gonorrhoeae*, but was made redundant during the course of evolution. Such a deletion event also occurred for the gonococcal chimeric gene *rsp* so that it can no longer function as a two-component regulatory system controlling piliation (Carrick *et al.*, 2000).

Regulation of the *pilE* gene appears to have been streamlined so that energy expenditure is kept to a minimum. Mechanisms of transcriptional regulation other than those enforced by alternative sigma factors seem to be a growing trend. *H. influenzae* only contains three sigma factors including the housekeeping sigma factor $\sigma^{70}$, and the alternative sigma factors $\sigma^{32}$ (Chapter 5) and $\sigma^{24}$ (Section 1.12.5; Fleischmann *et al.*, 1995). *M. genitalium* and *M. pneumoniae* only contain $\sigma^{70}$ (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996) and *C. trachomatis* contains two $\sigma^{70}$ factors and $\sigma^{24}$ (Kalman *et al.*, 1999). *N. gonorrhoeae* is similar to *H. influenzae* in that it encodes a $\sigma^{70}$ factor,

$\sigma^{32}$ (Chapter 5) and an alternative sigma factor that is a member of the ECF family (Genbank accession number AE004969).

Analysis of *N. meningitidis* and the non-pathogenic *N. subflava* revealed that the same *rpoN* deletion was present in these species, although this was not the case with the commensal species *N. lactamica* (Section 3.8). *N. lactamica* contains an intact *rpoN* homologue and the deduced amino acid sequence shows that it has retained all the features necessary for $\sigma^{54}$ function. This homologue was not capable of complementing a *P. aeruginosa rpoN* mutant (Section 3.9) and under the conditions tested was not transcribed in *N. lactamica* (Section 3.10). Additional experiments using alternative expression systems compatible with the background host and functional assays would aid in the characterisation of this gene. Alternatively, it may be that *RLS* in *N. lactamica* is not expressed and as yet has not undergone an evolutionary recombination event to streamline the genome, as has occurred for the pathogenic neisseria. This seems likely as Carrick (1998) discovered that *rsp* was partially deleted in *N. lactamica* as well as *N. gonorrhoeae* (Carrick *et al.*, 2000), *N. meningitidis* and *N. subflava*.

Molecular chaperones play a significant role in the quality control of proteins and a variety of cellular processes under all environmental conditions (Lund, 2001). Their contribution to the pathogenesis and virulence of intracellular pathogens (Section 1.9.3) warrants a comprehensive analysis of these proteins. This study succeeded in identifying and characterising the DnaK chaperone system from *N. gonorrhoeae* (Chapter 4). The gonococcal DnaK, DnaJ and GrpE proteins have retained all of the necessary signature motifs identified in other bacteria suggesting that they function in a similar manner. This was also the case for *N. meningitidis* (Sections 4.1, 4.2, 4.3).

Analysis of the annotated meningococcal genomic sequences (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000) reveals that the two strains contain a putative DnaJ homologue, HscB, and a putative DnaK homologue, HscA, with *N. meningitidis* MC58 containing two copies of the HscA homologue. An ORF potentially encoding the *hscA* gene was also identified from *N. gonorrhoeae* FA1090 (Section 4.8). In *E. coli* HscB acts as the co-chaperone for HscA but function of this chaperone system does not require the nucleotide exchange activity of GrpE (Silberg *et al.*, 1998). This chaperone system can not restore the function of the DnaK chaperone system upon stress and is proposed to

have a different function (Silberg *et al.*, 1998). Therefore it appears that the DnaK chaperone system in *N. gonorrhoeae* and *N. meningitidis* constitutes the major network for the recovery of proteins upon stress.

Transcriptional analysis of the gonococcal *dnaK*, *dnaJ* and *grpE* genes, determined that all three are induced upon a thermal upshift and transcription emanated from a $\sigma^{32}$ promoter (Sections 4.5 and 4.6). This was confirmed for *dnaK* by mutational analysis of the $\sigma^{32}$ promoter (Section 4.7). Interestingly, the *E. coli* $\sigma^{32}$ protein was unable to recognise the gonococcal *dnaK* promoter under normal growth conditions (Section 4.7). This may have been due to the slight differences between the $\sigma^{32}$ promoter sequences. The gonococcal *dnaK* $\sigma^{32}$ promoter has a lower number of C residues found in the –10 and –35 boxes compared to the *E. coli* $\sigma^{32}$ consensus sequence (Figure 4.15).

Identification of $\sigma^{32}$ promoter sequences upstream of the genes encoding the major molecular chaperone system of *N. gonorrhoeae*, in addition to that identified upstream of *groE*(Tauschek *et al.*, 1997), suggested that this bacterium contained the alternative sigma factor $\sigma^{32}$. *rpoH* was identified and subsequently cloned and characterised from this bacterium (Chapter 5). This gene could complement an *E. coli rpoH* mutant indicating a similar function to the *E. coli* homologue (Section 5.3). Despite repeated attempts, a gonococcal *rpoH* mutant could not be obtained strongly suggesting it is essential (Section 5.4). This finding was not surprising as a basal level of the proteins encoded by the heat shock genes it transcribes would be required for normal growth and the maintenance of cellular functions (Arsene *et al.*, 2000). As stated earlier, *N. gonorrhoeae* has only three sigma factors indicating that $\sigma^{32}$ could play a very important role in the homeostasis of this organism.

Analysis of the regulation of the gonococcal *rpoH* gene did not appear to be consistent with that of *E. coli*, where the increase in the level of $\sigma^{32}$ responsible for induction of the heat shock response is predominantly due to translational derepression and an increase in stability and activity (Yura and Nakahigashi, 1999). In fact, it did not appear to contain any of the regulatory elements responsible for translational regulation (Section 5.6). Transcriptional and Western blotting analyses determined that the level of gonococcal $\sigma^{32}$ does not increase significantly until well after an increase in expression of the heat shock genes was observed (Sections 5.7 and 5.8). This suggests that

activation of pre-formed $\sigma^{32}$ was sufficient to induce the heat shock response. Such an energy efficient mode of regulation would be particularly advantageous to *N. gonorrhoeae* as it could respond rapidly to stress stimuli *in vivo*. Therefore, the increased amount of $\sigma^{32}$ obtained by transcription late in the heat shock response is probably a subsidiary mechanism for sustaining the elevated expression of Hsps.

The mechanism of $\sigma^{32}$ activation upon heat shock has yet to be determined. In *A. tumefaciens* and *E. coli* the DnaK chaperone system negatively modulates $\sigma^{32}$ by inhibiting its activity and/or stability under normal conditions (Nakahigashi *et al.*, 2001; Tatsuta *et al.*, 2000). This is achieved by direct interaction of DnaK with $\sigma^{32}$ in *E. coli* (Gamer *et al.*, 1996). To determine whether this apparently evolutionary conserved mechanism of regulation is functional in *N. gonorrhoeae*, strains that could differentially express the *dnaK* and *dnaJ* genes would need to be constructed and the levels of heat shock genes during steady-state growth monitored.

The stability of $\sigma^{32}$ at different phases of the heat shock response for *E. coli* and *A. tumefaciens* plays an important role in the transient nature of this response. $\sigma^{32}$ of *E. coli* is extremely unstable during normal growth, transiently stabilises during the induction phase of the heat shock response before finally returning to its unstable state during the adaptation phase (Tilly *et al.*, 1989). Alternatively, the *A. tumefaciens* $\sigma^{32}$ protein is stable during normal growth and its destabilisation during the adaptation phase returns the $\sigma^{32}$ level to normal (Nakahigashi *et al.*, 2001). The heat shock response of *N. gonorrhoeae* did not appear to be transient under the conditions tested (Sections 4.5, 4.6, 5.7, 5.8) but determining the stability of $\sigma^{32}$ would indicate whether it has a function in the regulation of this protein.

# APPENDIX

**NY Broth**

25g/L Nutrient broth No. 2 (Oxoid)

5g/L Yeast extract

Autoclaved at 121°C for 20 minutes.

**NY Agar**

As for NY broth with the addition of 10g Agar No. 1 (Oxoid) per litre of broth.

**GC Broth**

15g/L Special peptone (Oxoid)

1g/L Corn starch (Difco)

5g/L NaCl

4g/L $K_2HPO_4$

1g/L $KH_2PO_4$

Autoclaved at 121°C for 20 minutes. When cooled, 1% DMIV (see below) and 1% (v/v) of 1M $NaHCO_3$ was added.

**Deakin Modified Iso-Vitalex (DMIV)**

2.59g L-cysteine-HCl

1.1g L-cysteine

1.0g Adenine

0.03g Guanine-HCl

1.0g L-glutamine

0.013 p-aminobenzoic acid

0.25g di-phosphopyridine nucleotide

0.1g Cocarboxylase

0.02g Ferric nitrate

0.003g Thiamine-HCl

100g Glucose

0.01g Cyanocobalamin

The first four ingredients were dissolved separately in concentrated HCl prior to the addition of the remaining ingredients. The pH was adjusted to 9.0 and the volume made up to 1L with distilled water. The solution was filtered through a 0.45µm pore size filter and stored at -20°C.

**20 x SSC**

3.0M NaCl

0.3M Sodium citrate

pH7.4

**Denaturation Solution**

0.5M NaOH

1M NaCl

**Neutralisation Solution**

0.5M Tris-HCl

1.0M NaCl

pH7.4

**Prehybridisation Solution**

5 x SSC

0.1% (w/v) N-laurylsarcosine

0.02% (w/v) SDS

1% (w/v) Skim milk powder

**10 x Colony Hybridisation Lysis Buffer**

50mM Tris-HCl, pH8.0

150mM NaCl

5mM EDTA

3% Bovine Serum Albumin (BSA)

40µg/ml Lysozyme

A 10 x stock solution containing the first three ingredients was stored at room temperature. BSA and lysozyme were added to the required volume of diluted stock solution when needed.

**10 x DNase I Buffer**

20mM Sodium acetate

10mM $MgCl_2$

10mM NaCl

pH4.5

**10 x TBE Buffer**

0.9M Tris-borate

0.02M EDTA

**8% Polyacrylamide Gel**

7.6g Acrylamide

0.4g N, N'-methylenebisacrylamide

42g Urea

10ml of 10 x TBE

40ml Distilled water

All ingredients were dissolved by heating at 37°C, filtered through a Whatman No.1 filter and adjusted to 100ml with distilled water. 400µl of 10% (w/v) ammonium persulfate and 40µl of N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were added prior to pouring of the gel.

**1 x PBS**

137mM NaCl

2.7mM KCl

4.3mM $Na_2HPO_4$

1.4mM $KH_2PO_4$

pH7.2

**TEN Buffer**

40mM Tris-HCl

1mM EDTA

150mM NaCl

pH7.8

**2 x SDS Sample Buffer**

200mM Tris-HCl

2% (w/v) SDS

20% (v/v) Glycerol

10% (v/v) β-mercaptoethanol

0.2% (w/v) Bromophenol blue

pH6.8

**Resolving Gel Buffer**

1.5M Tris-HCl

0.4% (w/v) SDS

pH8.8

**Stacking Gel Buffer**

0.5M Tris-HCl

0.4% (w/v) SDS

pH6.8

**30% Acrylamide**

29.41% (w/v) Acrylamide

0.51% (w/v) N,N'-methylenebisacrylamide

Ingredients were dissolved in 80ml of distilled water at 37°C and filtered through a Whatman No.1 filter. The volume was adjusted to 100ml with distilled water and stored at 4°C in the dark.

**Tris-glycine Electrophoresis Buffer**

12.5mM Tris-HCl

100mM Glycine

0.05% (w/v) SDS

pH8.3

Made fresh.

**Transblotting Buffer**

12.5mM Tris-HCl

100mM Glycine

15% (v/v) Methanol

pH8.3

**TBS-Tween**

0.15M NaCl

0.05M Tris-HCl

0.05% Tween 20

pH7.4

# BIBLIOGRAPHY


Albertson, N.H., and Koomey, M. (1993) Molecular cloning and characterization of a proline iminopeptidase gene from *Neisseria gonorrhoeae*. *Mol Microbiol* **9**: 1203-1211.

Alix, J.H., and Guerin, M.F. (1993) Mutant DnaK chaperones cause ribosome assembly defects in *Escherichia coli*. *Proc Natl Acad Sci U S A* **90**: 9725-9729.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403-410.

Ang, D., and Georgopoulos, C. (1989) The heat-shock-regulated *grpE* gene of *Escherichia coli* is required for bacterial growth at all temperatures but is dispensable in certain mutant backgrounds. *J Bacteriol* **171**: 2748-2755.

Apelian, D., and Inouye, S. (1993) A new putative sigma factor of *Myxococcus xanthus*. *J Bacteriol* **175**: 3335-3342.

Arakere, G., Kessel, M., Nguyen, N., and Frasch, C.E. (1993) Characterization of a stress protein from group B *Neisseria meningitidis*. *J Bacteriol* **175**: 3664-3668.

Aramaki, H., Sagara, Y., and Fujita, M. (1999) Cloning and sequencing of *rpoH* and identification of *ftsE-ftsX* in *Pseudomonas putida* PpG1. *DNA Res* **6**: 241-245.

Arsene, F., Tomoyasu, T., Mogk, A., Schirra, C., Schulze-Specking, A., and Bukau, B. (1999) Role of region C in regulation of the heat shock gene-specific sigma factor of *Escherichia coli*, σ32. *J Bacteriol* **181**: 3552-3561.

Arsene, F., Tomoyasu, T., and Bukau, B. (2000) The heat shock response of *Escherichia coli*. *Int J Food Microbiol* **55**: 3-9.

Arvidson, C.G., Powers, T., Walter, P., and So, M. (1999) *Neisseria gonorrhoeae* PilA is an FtsY homolog. *J Bacteriol* **181**: 731-739.

Avedissian, M., Lessing, D., Gober, J.W., Shapiro, L., and Gomes, S.L. (1995) Regulation of the *Caulobacter crescentus dnaKJ* operon. *J Bacteriol* **177**: 3479-3484.

Babst, M., Hennecke, H., and Fischer, H.M. (1996) Two different mechanisms are involved in the heat-shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum*. *Mol Microbiol* **19**: 827-839.

Bahl, H., Muller, H., Behrens, S., Joseph, H., and Narberhaus, F. (1995) Expression of heat shock genes in *Clostridium acetobutylicum*. *FEMS Microbiol Rev* **17**: 341-348.

Baldini, R.L., Avedissian, M., and Gomes, S.L. (1998) The CIRCE element and its putative repressor control cell cycle expression of the *Caulobacter crescentus groESL* operon. *J Bacteriol* **180**: 1632-1641.

Banecki, B., Liberek, K., Wall, D., Wawrzynow, A., Georgopoulos, C., Bertoli, E., Tanfani, F., and Zylicz, M. (1996) Structure-function analysis of the zinc finger region of the DnaJ molecular chaperone. *J Biol Chem* **271**: 14840-14848.

Bardwell, J.C., and Craig, E.A. (1984) Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible *dnaK* gene are homologous. *Proc Natl Acad Sci U S A* **81**: 848-852.

Bardwell, J.C., Tilly, K., Craig, E., King, J., Zylicz, M., and Georgopoulos, C. (1986) The nucleotide sequence of the *Escherichia coli* K12 *dnaJ*+ gene. A gene that encodes a heat shock protein. *J Biol Chem* **261**: 1782-1785.

Barrios, H., Valderrama, B., and Morett, E. (1999) Compilation and analysis of $\sigma^{54}$-dependent promoter sequences. *Nucleic Acids Res* **27**: 4305-4313.

Bauer, F.J., Rudel, T., Stein, M., and Meyer, T.F. (1999) Mutagenesis of the *Neisseria gonorrhoeae* porin reduces invasion in epithelial cells and enhances phagocyte responsiveness. *Mol Microbiol* **31**: 903-913.

Beissinger, M., and Buchner, J. (1998) How chaperones fold proteins. *Biol Chem* **379**: 245-259.

Belland, R.J., Morrison, S.G., van der Ley, P., and Swanson, J. (1989) Expression and phase variation of gonococcal P. II genes in *Escherichia coli* involves ribosomal frameshifting and slipped-strand mispairing. *Mol Microbiol* **3**: 777-786.

Benvenisti, L., Koby, S., Rutman, A., Giladi, H., Yura, T., and Oppenheim, A.B. (1995) Cloning and primary sequence of the *rpoH* gene from *Pseudomonas aeruginosa*. *Gene* **155**: 73-76.

Ben-Zvi, A.P., and Goloubinoff, P. (2001) Review: mechanisms of disaggregation and refolding of stable protein aggregates by molecular chaperones. *J Struct Biol* **135**: 84-93.

Berger, B., Wilson, D.B., Wolf, E., Tonchev, T., Milla, M., and Kim, P.S. (1995) Predicting coiled coils by use of pairwise residue correlations. *Proc Natl Acad Sci U S A* **92**: 8259-8263.

Bernhardt, J., Buttner, K., Scharf, C., and Hecker, M. (1999) Dual channel imaging of two-dimensional electropherograms in *Bacillus subtilis*. *Electrophoresis* 20: 2225-2240.

Bertelsen, E.B., Zhou, H., Lowry, D.F., Flynn, G.C., and Dahlquist, F.W. (1999) Topology and dynamics of the 10 kDa C-terminal domain of DnaK in solution. *Protein Sci* **8**: 343-354.

Bertoni, G., Fujita, N., Ishihama, A., and de Lorenzo, V. (1998) Active recruitment of $\sigma^{54}$-RNA polymerase to the Pu promoter of *Pseudomonas putida*: role of IHF and αCTD. *Embo J* **17**: 5120-5128.

Bhat, K.S., Gibbs, C.P., Barrera, O., Morrison, S.G., Jahnig, F., Stern, A., Kupsch, E.M., Meyer, T.F., and Swanson, J. (1991) The opacity proteins of *Neisseria gonorrhoeae* strain MS11 are encoded by a family of 11 complete genes. *Mol Microbiol* **5**: 1889-1901.

Bianchi, A.A., and Baneyx, F. (1999) Hyperosmotic shock induces the $\sigma^{32}$ and $\sigma^{E}$ stress regulons of *Escherichia coli*. *Mol Microbiol* **34**: 1029-1038.

Bignell, C. (1999) National guideline for the management of gonorrhoea in adults. *Sexually Transmitted Infections* **75**: S13-S15.

Bishai, W.R., Howard, N.S., Winkelstein, J.A., and Smith, H.O. (1994) Characterization and virulence analysis of catalase mutants of *Haemophilus influenzae*. *Infect Immun* **62**: 4855-4860.

Biswas, G.D., and Sparling, P.F. (1981) Entry of double-stranded deoxyribonucleic acid during transformation of *Neisseria gonorrhoeae*. *J Bacteriol* **145**: 638-640.

Black, C.G., Fyfe, J.A., and Davies, J.K. (1995) A promoter associated with the neisserial repeat can be used to transcribe the *uvrB* gene from *Neisseria gonorrhoeae*. *J Bacteriol* **177**: 1952-1958.

Black, C.G., Fyfe, J.A., and Davies, J.K. (1998) Absence of an SOS-like system in *Neisseria gonorrhoeae*. *Gene* **208**: 61-66.

Blackwood, E.M., and Kadonaga, J.T. (1998) Going the distance: a current view of enhancer action. *Science* **281**: 61-63.

Blake, M.S., Blake, C.M., Apicella, M.A., and Mandrell, R.E. (1995) Gonococcal opacity: lectin-like interactions between Opa proteins and lipooligosaccharide. *Infect Immun* **63**: 1434-1439.

Blander, S.J., and Horwitz, M.A. (1993) Major cytoplasmic membrane protein of *Legionella pneumophila*, a genus common antigen and member of the hsp60 family of heat shock proteins, induces protective immunity in a guinea pig model of Legionnaires' disease. *J Clin Invest* **91**: 717-723.

Blaszczak, A., Georgopoulos, C., and Liberek, K. (1999) On the mechanism of FtsH-dependent degradation of the $\sigma^{32}$ transcriptional regulator of *Escherichia coli* and the role of the DnaK chaperone machine. *Mol Microbiol* **31**: 157-166.

Boorstein, W.R., Ziegelhoffer, T., and Craig, E.A. (1994) Molecular evolution of the HSP70 multigene family. *J Mol Evol* **38**: 1-17.

Bork, P., Sander, C., and Valencia, A. (1992) An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proc Natl Acad Sci U S A* **89**: 7290-7294.

Boston, R.S., Viitanen, P.V., and Vierling, E. (1996) Molecular chaperones and protein folding in plants. *Plant Mol Biol* **32**: 191-222.

Boulanger, J., Faulds, D., Eddy, E.M., and Lingwood, C.A. (1995) Members of the 70kDa heat shock protein family specifically recognize sulfoglycolipids: role in gamete recognition and mycoplasma-related infertility. *J Cell Physiol* **165**: 7-17.

Boyle-Vavra, S., So, M., and Seifert, H.S. (1993) Transcriptional control of gonococcal *pilE* expression: involvement of an alternate sigma factor. *Gene* **137**: 233-236.

Bradley, D.E. (1980) A function of Pseudomonas aeruginosa PAO polar pili: twitching motility. *Can J Microbiol* **26**: 146-154.

Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D.C., Joachimiak, A., Horwich, A.L., and Sigler, P.B. (1994) The crystal structure of the bacterial chaperonin GroEL at 2.8 A. *Nature* **371**: 578-586.

Bramhill, D., and Kornberg, A. (1988) Duplex opening by DnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. *Cell* **52**: 743-755.

Britigan, B.E., Cohen, M.S., and Sparling, P.F. (1985) Gonococcal infection: a model of molecular pathogenesis. *N Engl J Med* **312**: 1683-1694.

Brosius, J. (1984) Plasmid vectors for the selection of promoters. *Gene* **27**: 151-160.

Brown, K.L., and Hughes, K.T. (1995) The role of anti-sigma factors in gene regulation. *Mol Microbiol* **16**: 397-404.

Bucca, G., Smith, C.P., Alberti, M., Seidita, G., Passantino, R., and Puglia, A.M. (1993) Cloning and sequencing of the *dnaK* region of *Streptomyces coelicolor* A3(2). *Gene* **130**: 141-144.

Buchberger, A., Schroder, H., Buttner, M., Valencia, A., and Bukau, B. (1994) A conserved loop in the ATPase domain of the DnaK chaperone is essential for stable binding of GrpE. *Nat Struct Biol* **1**: 95-101.

Buchberger, A., Theyssen, H., Schroder, H., McCarty, J.S., Virgallita, G., Milkereit, P., Reinstein, J., and Bukau, B. (1995) Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. *J Biol Chem* **270**: 16903-16910.

Buck, M. (1986) Deletion analysis of the *Klebsiella pneumoniae* nitrogenase promoter: importance of spacing between conserved sequences around positions -12 and -24 for activation by the NifA and NtrC (GlnG) products. *J Bacteriol* **166**: 545-551.

Bukau, B., and Walker, G.C. (1989a) Δ*dnaK52* mutants of *Escherichia coli* have defects in chromosome segregation and plasmid maintenance at normal growth temperatures. *J Bacteriol* **171**: 6030-6038.

Bukau, B., and Walker, G.C. (1989b) Cellular defects caused by deletion of the *Escherichia coli dnaK* gene indicate roles for heat shock protein in normal metabolism. *J Bacteriol* **171**: 2337-2346.

Bukau, B. (1993) Regulation of the *Escherichia coli* heat-shock response. *Mol Microbiol* **9**: 671-680.

Bukau, B., and Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines. *Cell* **92**: 351-366.

Burdett, V., Inamine, J., and Rajagopalan, S. (1982) Heterogeneity of tetracycline resistance determinants in Streptococcus. *J Bacteriol* **149**: 995-1004.

Calendar, R., Erickson, J.W., Halling, C., and Nolte, A. (1988) Deletion and insertion mutations in the *rpoH* gene of *Escherichia coli* that produce functional $\sigma^{32}$. *J Bacteriol* **170**: 3479-3484.

Cannon, J.G., and Sparling, P.F. (1984) The genetics of the gonococcus. *Annu Rev Microbiol* **38**: 111-133.

Cannon, W., Claverie-Martin, F., Austin, S., and Buck, M. (1993) Core RNA polymerase assists binding of the transcription factor sigma-54 to promoter DNA. *Mol Microbiol* **8**: 287-298.

Cannon, W., Claverie-Martin, F., Austin, S., and Buck, M. (1994) Identification of a DNA-contacting surface in the transcription factor sigma-54. *Mol Microbiol* **11**: 227-236.

Cannon, W., Missailidis, S., Smith, C., Cottier, A., Austin, S., Moore, M., and Buck, M. (1995) Core RNA polymerase and promoter DNA interactions of purified domains of $\sigma^{N}$: bipartite functions. *J Mol Biol* **248**: 781-803.

Cannon, W., Chaney, M., and Buck, M. (1999) Characterisation of holoenzyme lacking $\sigma^{N}$ regions I and II. *Nucleic Acids Res* **27**: 2478-2486.

Cannon, W.V., Chaney, M.K., Wang, X., and Buck, M. (1997) Two domains within $\sigma^{N}$ ($\sigma^{54}$) cooperate for DNA binding. *Proc Natl Acad Sci U S A* **94**: 5006-5011.

Cannon, W.V., Gallegos, M.T., and Buck, M. (2000) Isomerization of a binary sigma-promoter DNA complex by transcription activators. *Nat Struct Biol* **7**: 594-601.

Carmona, M., Claverie-Martin, F., and Magasanik, B. (1997) DNA bending and the initiation of transcription at $\sigma^{54}$-dependent bacterial promoters. *Proc Natl Acad Sci U S A* **94**: 9568-9572.

Carrick, C.S., Fyfe, J.A., and Davies, J.K. (1997) The normally silent $\sigma^{54}$ promoters upstream of the *pilE* genes of both *Neisseria gonorrhoeae* and *Neisseria meningitidis* are functional when transferred to *Pseudomonas aeruginosa*. *Gene* **198**: 89-97.

Carrick, C.S. (1998) Regulation of piliation in pathogenic *Neisseria*. PhD Thesis. Monash Univeristy, Australia.

Carrick, C.S., Fyfe, J.A., and Davies, J.K. (2000) The genome of *Neisseria gonorrhoeae* retains the remnants of a two-component regulatory system that once controlled piliation. *FEMS Microbiol Lett* **186**: 197-201.

Casaz, P., and Buck, M. (1999) Region I modifies DNA-binding domain conformation of sigma 54 within the holoenzyme. *J Mol Biol* **285**: 507-514.

Caugant, D.A., Hoiby, E.A., Magnus, P., Scheel, O., Hoel, T., Bjune, G., Wedege, E., Eng, J., and Froholm, L.O. (1994) Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. *J Clin Microbiol* **32**: 323-330.

Chaney, M., and Buck, M. (1999) The sigma 54 DNA-binding domain includes a determinant of enhancer responsiveness. *Mol Microbiol* **33**: 1200-1209.

Cheetham, M.E., and Caplan, A.J. (1998) Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. *Cell Stress Chaperones* **3**: 28-36.

Chellaiah, A., Davis, A., and Mohanakumar, T. (1993) Cloning of a unique human homologue of the *Escherichia coli* DnaJ heat shock protein. *Biochim Biophys Acta* **1174**: 111-113.

Chitnis, P.R., and Nelson, N. (1991) Molecular cloning of the genes encoding two chaperone proteins of the cyanobacterium *Synechocystis* sp. PCC 6803. *J Biol Chem* **266**: 58-65.

Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156-159.

Chomvarin, C. (1993) Virulence associated proteins of *Neisseria gonorrhoeae*. PhD Thesis. Monash University, Australia.

Clark, V.L., Campbell, L.A., Palermo, D.A., Evans, T.M., and Klimpel, K.W. (1987) Induction and repression of outer membrane proteins by anaerobic growth of *Neisseria gonorrhoeae*. *Infect Immun* **55**: 1359-1364.

Clark, V.L., Knapp, J.S., Thompson, S., and Klimpel, K.W. (1988) Presence of antibodies to the major anaerobically induced gonococcal outer membrane protein in sera from patients with gonococcal infections. *Microb Pathog* **5**: 381-390.

Claverie-Martin, F., and Magasanik, B. (1991) Role of integration host factor in the regulation of the *glnHp2* promoter of *Escherichia coli*. *Proc Natl Acad Sci U S A* **88**: 1631-1635.

Cohen, M.S., Cannon, J.G., Jerse, A.E., Charniga, L.M., Isbey, S.F., and Whicker, L.G. (1994) Human experimentation with *Neisseria gonorrhoeae*: rationale, methods, and implications for the biology of infection and vaccine development. *J Infect Dis* **169**: 532-537.

Cole, S.T., and *et al.* (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537-544.

Connell, T.D., Black, W.J., Kawula, T.H., Barritt, D.S., Dempsey, J.A., Kverneland, K., Jr., Stephenson, A., Schepart, B.S., Murphy, G.L., and Cannon, J.G. (1988) Recombination among protein II genes of *Neisseria gonorrhoeae* generates new coding sequences and increases structural variability in the protein II family. *Mol Microbiol* **2**: 227-236.

Conway de Macario, E., Dugan, C.B., and Macario, A.J. (1994) Identification of a *grpE* heat-shock gene homolog in the archaeon *Methanosarcina mazei*. *J Mol Biol* **240**: 95-101.

Cornelissen, C.N., Kelley, M., Hobbs, M.M., Anderson, J.E., Cannon, J.G., Cohen, M.S., and Sparling, P.F. (1998) The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. *Mol Microbiol* **27**: 611-616.

Correia, F.F., Inouye, S., and Inouye, M. (1986) A 26-base-pair repetitive sequence specific for *Neisseria gonorrhoeae* and *Neisseria meningitidis* genomic DNA. *J Bacteriol* **167**: 1009-1015.

Correia, F.F., Inouye, S., and Inouye, M. (1988) A family of small repeated elements with some transposon-like properties in the genome of *Neisseria gonorrhoeae*. *J Biol Chem* **263**: 12194-12198.

Cowing, D.W., Bardwell, J.C., Craig, E.A., Woolford, C., Hendrix, R.W., and Gross, C.A. (1985) Consensus sequence for *Escherichia coli* heat shock gene promoters. *Proc Natl Acad Sci U S A* **82**: 2679-2683.

Craig, E.A., Ingolia, T.D., and Manseau, L.J. (1983) Expression of Drosophila heat-shock cognate genes during heat shock and development. *Dev Biol* **99**: 418-426.

Craig, E.A., and Gross, C.A. (1991) Is Hsp70 the cellular thermometer? *Trends Biochem Sci* **16**: 135-140.

Craig, N.L., and Nash, H.A. (1984) *E. coli* integration host factor binds to specific sites in DNA. *Cell* **39**: 707-716.

Creighton, T.E. (1990) Protein folding. *Biochem J* **270**: 1-16.

Crickmore, N., and Salmond, G.P. (1986) The *Escherichia coli* heat shock regulatory gene is immediately downstream of a cell division operon: the *fam* mutation is allelic with *rpoH*. *Mol Gen Genet* **205**: 535-539.

Cullen, P.J., Foster-Hartnett, D., Gabbert, K.K., and Kranz, R.G. (1994) Structure and expression of the alternative sigma factor, RpoN, in *Rhodobacter capsulatus*; physiological relevance of an autoactivated *nifU2-rpoN* superoperon. *Mol Microbiol* **11**: 51-65.

Cyr, D.M., Langer, T., and Douglas, M.G. (1994) DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. *Trends Biochem Sci* **19**: 176-181.

Danilition, S.L., Maclean, I.W., Peeling, R., Winston, S., and Brunham, R.C. (1990) The 75-kilodalton protein of *Chlamydia trachomatis*: a member of the heat shock protein 70 family? *Infect Immun* **58**: 189-196.

d'Aubenton Carafa, Y., Brody, E., and Thermes, C. (1990) Prediction of Rho-independent *Escherichia coli* transcription terminators. A statistical analysis of their RNA stem-loop structures. *J Mol Biol* **216**: 835-858.

Davies, J. (1985) Plasmids in local isolates of *Neisseria gonorrhoeae*. *Australian Microbiology* **6**: 289.

Davies, J. (1990) The genetics of gonococcal variation and virulence. *Today's Life Science* **2**: 12-20.

De Schryver, A., and Meheus, A. (1990) Epidemiology of sexually transmitted diseases: the global picture. *Bull World Health Organ* **68**: 639-654.

de Vries, F.P., Cole, R., Dankert, J., Frosch, M., and van Putten, J.P. (1998) *Neisseria meningitidis* producing the Opc adhesin binds epithelial cell proteoglycan receptors. *Mol Microbiol* **27**: 1203-1212.

Debarbouille, M., Martin-Verstraete, I., Kunst, F., and Rapoport, G. (1991) The *Bacillus subtilis sigL* gene encodes an equivalent of $\sigma^{54}$ from gram-negative bacteria. *Proc Natl Acad Sci U S A* **88**: 9092-9096.

Dehio, C., Gray-Owen, S.D., and Meyer, T.F. (1998) The role of neisserial Opa proteins in interactions with host cells. *Trends Microbiol* **6**: 489-495.

Dempsey, J.A., Litaker, W., Madhure, A., Snodgrass, T.L., and Cannon, J.G. (1991) Physical map of the chromosome of *Neisseria gonorrhoeae* FA1090 with locations of genetic markers, including *opa* and *pil* genes. *J Bacteriol* **173**: 5476-5486.

Dempsey, J.A., Wallace, A.B., and Cannon, J.G. (1995) The physical map of the chromosome of a serogroup A strain of *Neisseria meningitidis* shows complex rearrangements relative to the chromosomes of the two mapped strains of the closely related species *N. gonorrhoeae*. *J Bacteriol* **177**: 6390-6400.

Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., and Bukau, B. (1999) Trigger factor and DnaK cooperate in folding of newly synthesized proteins. *Nature* **400**: 693-696.

Diamant, S., Ben-Zvi, A.P., Bukau, B., and Goloubinoff, P. (2000) Size-dependent disaggregation of stable protein aggregates by the DnaK chaperone machinery. *J Biol Chem* **275**: 21107-21113.

Dionisi, H.M., and Viale, A.M. (1998) Purification and characterization of *Chromatium vinosum* GroEL and GroES proteins overexpressed in *Escherichia coli* cells lacking the endogenous *groESL* operon. *Protein Expr Purif* **14**: 275-282.

Ditto, M.D., Roberts, D., and Weisberg, R.A. (1994) Growth phase variation of integration host factor level in *Escherichia coli*. *J Bacteriol* **176**: 3738-3748.

Dodd, I.B., and Egan, J.B. (1990) Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. *Nucleic Acids Res* **18**: 5019-5026.

Dombroski, A.J., Walter, W.A., Record, M.T., Jr., Siegele, D.A., and Gross, C.A. (1992) Polypeptides containing highly conserved regions of transcription initiation factor $\sigma^{70}$ exhibit specificity of binding to promoter DNA. *Cell* **70**: 501-512.

Dubrac, S., and Touati, D. (2000) Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. *J Bacteriol* **182**: 3802-3808.

Ehrt, S., Ornston, L.N., and Hillen, W. (1994) RpoN ($\sigma^{54}$) is required for conversion of phenol to catechol in *Acinetobacter calcoaceticus*. *J Bacteriol* **176**: 3493-3499.

Elias, D., Markovits, D., Reshef, T., van der Zee, R., and Cohen, I.R. (1990) Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein. *Proc Natl Acad Sci U S A* **87**: 1576-1580.

Elkins, C., Thomas, C.E., Seifert, H.S., and Sparling, P.F. (1991) Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. *J Bacteriol* **173**: 3911-3913.

Elleman, T.C., and Hoyne, P.A. (1984) Nucleotide sequence of the gene encoding pilin of *Bacteroides nodosus*, the causal organism of ovine footrot. *J Bacteriol* **160**: 1184-1187.

Elleman, T.C., Hoyne, P.A., Stewart, D.J., McKern, N.M., and Peterson, J.E. (1986) Expression of pili from *Bacteroides nodosus* in *Pseudomonas aeruginosa*. *J Bacteriol* **168**: 574-580.

Ellis, J. (1987) Proteins as molecular chaperones. *Nature* **328**: 378-379.

Ellis, R.J., and van der Vies, S.M. (1991) Molecular chaperones. *Annu Rev Biochem* **60**: 321-347.

Ellis, R.J., and Hartl, F.U. (1996) Protein folding in the cell: competing models of chaperonin function. *Faseb J* **10**: 20-26.

Emetz, D., and Klug, G. (1998) Cloning and characterization of the *rpoH* gene of *Rhodobacter capsulatus*. *Mol Gen Genet* **260**: 212-217.

Erickson, J.W., Vaughn, V., Walter, W.A., Neidhardt, F.C., and Gross, C.A. (1987) Regulation of the promoters and transcripts of *rpoH*, the *Escherichia coli* heat shock regulatory gene. *Genes Dev* **1**: 419-432.

Erickson, J.W., and Gross, C.A. (1989) Identification of the $\sigma^{E}$ subunit of *Escherichia coli* RNA polymerase: a second alternate sigma factor involved in high-temperature gene expression. *Genes Dev* **3**: 1462-1471.

Evans, D.J., Jr., Evans, D.G., Lampert, H.C., and Nakano, H. (1995) Identification of four new prokaryotic bacterioferritins, from *Helicobacter pylori*, *Anabaena variabilis*, *Bacillus subtilis* and *Treponema pallidum*, by analysis of gene sequences. *Gene* **153**: 123-127.

Ewalt, K.L., Hendrick, J.P., Houry, W.A., and Hartl, F.U. (1997) In vivo observation of polypeptide flux through the bacterial chaperonin system. *Cell* **90**: 491-500.

Facius, D., and Meyer, T.F. (1993) A novel determinant (*comA*) essential for natural transformation competence in *Neisseria gonorrhoeae* and the effect of a *comA* defect on pilin variation. *Mol Microbiol* **10**: 699-712.

Falah, M., and Gupta, R.S. (1997) Phylogenetic analysis of mycoplasmas based on Hsp70 sequences: cloning of the *dnaK* (hsp70) gene region of *Mycoplasma capricolum*. *Int J Syst Bacteriol* **47**: 38-45.

Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989) The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J Bacteriol* **171**: 1379-1385.

Feder, M.E., and Hofmann, G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* **61**: 243-282.

Feldman, D.E., and Frydman, J. (2000) Protein folding *in vivo*: the importance of molecular chaperones. *Curr Opin Struct Biol* **10**: 26-33.

Fenton, W.A., and Horwich, A.L. (1997) GroEL-mediated protein folding. *Protein Sci* **6**: 743-760.

Ferrero, R.L., Thiberge, J.M., Kansau, I., Wuscher, N., Huerre, M., and Labigne, A. (1995) The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. *Proc Natl Acad Sci U S A* **92**: 6499-6503.

Fink, A.L. (1995) Compact intermediate states in protein folding. *Annu Rev Biophys Biomol Struct* **24**: 495-522.

Fink, A.L. (1998) Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Fold Des* **3**: R9-23.

Fink, A.L. (1999) Chaperone-mediated protein folding. *Physiol Rev* **79**: 425-449.

Flaherty, K.M., DeLuca-Flaherty, C., and McKay, D.B. (1990) Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature* **346**: 623-628.

Flaherty, K.M., Wilbanks, S.M., DeLuca-Flaherty, C., and McKay, D.B. (1994) Structural basis of the 70-kilodalton heat shock cognate protein ATP hydrolytic activity. II. Structure of the active site with ADP or ATP bound to wild type and mutant ATPase fragment. *J Biol Chem* **269**: 12899-12907.

Fleischmann, R.D., and *et al.* (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**: 496-512.

Fox, K.K., and Knapp, J.S. (1999) Antimicrobial resistance in *Neisseria gonorrhoeae*. *Curr Opin Urol* **9**: 65-70.

Fraser, C.M., and *et al.* (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**: 397-403.

Fraser, C.M., and *et al.* (1998) Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science* **281**: 375-388.

Freundlich, M., Ramani, N., Mathew, E., Sirko, A., and Tsui, P. (1992) The role of integration host factor in gene expression in *Escherichia coli*. *Mol Microbiol* **6**: 2557-2563.

Friedman, D.I. (1988) Integration host factor: a protein for all reasons. *Cell* **55**: 545-554.

Frisk, A., Ison, C.A., and Lagergard, T. (1998) GroEL heat shock protein of *Haemophilus ducreyi*: association with cell surface and capacity to bind to eukaryotic cells. *Infect Immun* **66**: 1252-1257.

Fujita, N., and Ishihama, A. (1987) Heat-shock induction of RNA polymerase sigma-32 synthesis in *Escherichia coli*: transcriptional control and a multiple promoter system. *Mol Gen Genet* **210**: 10-15.

Fyfe, J.A., Strugnell, R.A., and Davies, J.K. (1993) Control of gonococcal pilin-encoding gene expression in *Escherichia coli*. *Gene* **123**: 45-50.

Fyfe, J.A., Carrick, C.S., and Davies, J.K. (1995) The *pilE* gene of *Neisseria gonorrhoeae* MS11 is transcribed from a $\sigma^{70}$ promoter during growth in vitro. *J Bacteriol* **177**: 3781-3787.

Fyfe, J.A., and Davies, J.K. (1998) An AT-rich tract containing an integration host factor-binding domain and two UP-like elements enhances transcription from the *pilEp*1 promoter of *Neisseria gonorrhoeae*. *J Bacteriol* **180**: 2152-2159.

Gallegos, M.T., and Buck, M. (1999) Sequences in $\sigma^{N}$ determining holoenzyme formation and properties. *J Mol Biol* **288**: 539-553.

Gallegos, M.T., Cannon, W.V., and Buck, M. (1999) Functions of the $\sigma^{54}$ region I *in trans* and implications for transcription activation. *J Biol Chem* **274**: 25285-25290.

Gallegos, M.T., and Buck, M. (2000) Sequences in $\sigma^{54}$ region I required for binding to early melted DNA and their involvement in sigma-DNA isomerisation. *J Mol Biol* **297**: 849-859.

Gamer, J., Bujard, H., and Bukau, B. (1992) Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor $\sigma^{32}$. *Cell* **69**: 833-842.

Gamer, J., Multhaup, G., Tomoyasu, T., McCarty, J.S., Rudiger, S., Schonfeld, H.J., Schirra, C., Bujard, H., and Bukau, B. (1996) A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the *Escherichia coli* heat shock transcription factor $\sigma^{32}$. *Embo J* **15**: 607-617.

Gardan, R., Rapoport, G., and Debarbouille, M. (1997) Role of the transcriptional activator RocR in the arginine-degradation pathway of *Bacillus subtilis*. *Mol Microbiol* **24**: 825-837.

Garduno, R.A., Garduno, E., and Hoffman, P.S. (1998) Surface-associated Hsp60 chaperonin of *Legionella pneumophila* mediates invasion in a HeLa cell model. *Infect Immun* **66**: 4602-4610.

Gassler, C.S., Buchberger, A., Laufen, T., Mayer, M.P., Schroder, H., Valencia, A., and Bukau, B. (1998) Mutations in the DnaK chaperone affecting interaction with the DnaJ cochaperone. *Proc Natl Acad Sci U S A* **95**: 15229-15234.

Genco, C.A., and Desai, P.J. (1996) Iron acquisition in the pathogenic *Neisseria*. *Trends Microbiol* **4**: 179-184.

Georgopoulos, C., Georgiou, M., Selzer, G., and Eisen, H. (1977) Bacteriophage T4 mutants which propagate on *E. coli* K12 but not on *E. coli* B. *Experientia* **33**: 1157-1158.

Georgopoulos, C., and Ang, D. (1990) The *Escherichia coli* GroE chaperonins. *Semin Cell Biol* **1**: 19-25.

Georgopoulos, C., and Welch, W.J. (1993) Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol* **9**: 601-634.

Georgopoulos, C.P., Hendrix, R.W., Kaiser, A.D., and Wood, W.B. (1972) Role of the host cell in bacteriophage morphogenesis: effects of a bacterial mutation on T4 head assembly. *Nat New Biol* **239**: 38-41.

Georgopoulos, C.P., Hendrix, R.W., Casjens, S.R., and Kaiser, A.D. (1973) Host participation in bacteriophage lambda head assembly. *J Mol Biol* **76**: 45-60.

Gilbert, D.G. (1996) loopDloop in Java, a Java application for visualising RNA secondary structure. ftp://iubio.bio.indiana.edu/molbio/loopdloop/java/.

Glover, J.R., and Lindquist, S. (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* **94**: 73-82.

Goff, S.A., and Goldberg, A.L. (1985) Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. *Cell* **41**: 587-595.

Goffin, L., and Georgopoulos, C. (1998) Genetic and biochemical characterization of mutations affecting the carboxy-terminal domain of the *Escherichia coli* molecular chaperone DnaJ. *Mol Microbiol* **30**: 329-340.

Goloubinoff, P., Mogk, A., Zvi, A.P., Tomoyasu, T., and Bukau, B. (1999) Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proc Natl Acad Sci U S A* **96**: 13732-13737.

Goodman, S.D., and Scocca, J.J. (1988) Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. *Proc Natl Acad Sci U S A* **85**: 6982-6986.

Goosen, N., and van de Putte, P. (1995) The regulation of transcription initiation by integration host factor. *Mol Microbiol* **16**: 1-7.

Gray-Owen, S.D., Dehio, C., Haude, A., Grunert, F., and Meyer, T.F. (1997) CD66 carcinoembryonic antigens mediate interactions between Opa-expressing *Neisseria gonorrhoeae* and human polymorphonuclear phagocytes. *Embo J* **16**: 3435-3445.

Groemping, Y., and Reinstein, J. (2001) Folding properties of the nucleotide exchange factor GrpE from *Thermus thermophilus*: GrpE is a thermosensor that mediates heat shock response. *J Mol Biol* **314**: 167-178.

Gross, R., Arico, B., and Rappuoli, R. (1989) Families of bacterial signal-transducing proteins. *Mol Microbiol* **3**: 1661-1667.

Grosskurth, H., and *et al.* (1995) Impact of improved treatment of sexually transmitted diseases on HIV infection in rural Tanzania: randomised controlled trial. *Lancet* **346**: 530-536.

Grunstein, M., and Hogness, D.S. (1975) Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proc Natl Acad Sci U S A* **72**: 3961-3965.

Guckenberger, M., Kurz, S., Aepinus, C., Theiss, S., Haller, S., Leimbach, T., Panzner, U., Weber, J., Paul, H., Unkmeir, A., Frosch, M., and Dietrich, G. (2002) Analysis of the heat shock response of *Neisseria meningitidis* with cDNA- and oligonucleotide-based DNA microarrays. *J Bacteriol* **184**: 2546-2551.

Guo, Y., and Gralla, J.D. (1997) DNA-binding determinants of sigma 54 as deduced from libraries of mutations. *J Bacteriol* **179**: 1239-1245.

Guo, Y., Wang, L., and Gralla, J.D. (1999) A fork junction DNA-protein switch that controls promoter melting by the bacterial enhancer-dependent sigma factor. *Embo J* **18**: 3736-3745.

Guo, Y., Lew, C.M., and Gralla, J.D. (2000) Promoter opening by $\sigma^{54}$ and $\sigma^{70}$ RNA polymerases: $\sigma$ factor-directed alterations in the mechanism and tightness of control. *Genes Dev* **14**: 2242-2255.

Gupta, R.S. (1990) Mitochondria, molecular chaperone proteins and the in vivo assembly of microtubules. *Trends Biochem Sci* **15**: 415-418.

Gupta, R.S., and Singh, B. (1992) Cloning of the HSP70 gene from *Halobacterium marismortui*: relatedness of archaebacterial HSP70 to its eubacterial homologs and a model for the evolution of the HSP70 gene. *J Bacteriol* **174**: 4594-4605.

Gupta, R.S., and Golding, G.B. (1993) Evolution of HSP70 gene and its implications regarding relationships between archaebacteria, eubacteria, and eukaryotes. *J Mol Evol* **37**: 573-582.

Haas, R., and Meyer, T.F. (1986) The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. *Cell* **44**: 107-115.

Haines, K.A., Reibman, J., Tang, X.Y., Blake, M., and Weissmann, G. (1991) Effects of protein I of *Neisseria gonorrhoeae* on neutrophil activation: generation of diacylglycerol from phosphatidylcholine via a specific phospholipase C is associated with exocytosis. *J Cell Biol* **114**: 433-442.

Hamilton, C.W. (1999) Analysis of repeated sequence elements in pathogenic *Neisseria*. PhD Thesis. Monash Univeristy, Australia.

Han, W., and Christen, P. (2001) Mutations in the interdomain linker region of DnaK abolish the chaperone action of the DnaK/DnaJ/GrpE system. *FEBS Lett* **497**: 55-58.

Hanahan, D. (1985) Techniques for transformation of *E. coli*. In DNA cloning: a practical approach. Vol. 1. Glover D.M. (ed). Oxford: IRL Press, pp. 109-135.

Hanawa, T., Fukuda, M., Kawakami, H., Hirano, H., Kamiya, S., and Yamamoto, T. (1999) The *Listeria monocytogenes* DnaK chaperone is required for stress tolerance and efficient phagocytosis with macrophages. *Cell Stress Chaperones* **4**: 118-128.

Handsfield, H.H. (1990) *Neisseria gonorrhoeae*. In Principles and practice of infectious diseases. Mandell, G. L., Douglas, R.G., and Bennett, J.E. (eds). New York: Churchill Livingstone, pp. 1613-1631.

Hardy, S.J., and Randall, L.L. (1993) Recognition of ligands by SecB, a molecular chaperone involved in bacterial protein export. *Philos Trans R Soc Lond B Biol Sci* **339**: 343-352; discussion 352-344.

Harrison, C.J., Hayer-Hartl, M., Di Liberto, M., Hartl, F., and Kuriyan, J. (1997) Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. *Science* **276**: 431-435.

Hartl, F.U., and Neupert, W. (1990) Protein sorting to mitochondria: evolutionary conservations of folding and assembly. *Science* **247**: 930-938.

Hartl, F.U., and Martin, J. (1995) Molecular chaperones in cellular protein folding. *Curr Opin Struct Biol* **5**: 92-102.

Hartl, F.U. (1996) Molecular chaperones in cellular protein folding. *Nature* **381**: 571-579.

Hartmann, E., and Lingwood, C. (1997) Brief heat shock treatment induces a long-lasting alteration in the glycolipid receptor binding specificity and growth rate of *Haemophilus influenzae*. *Infect Immun* **65**: 1729-1733.

Hartmann, E., Lingwood, C.A., and Reidl, J. (2001) Heat-inducible surface stress protein (Hsp70) mediates sulfatide recognition of the respiratory pathogen *Haemophilus influenzae*. *Infect Immun* **69**: 3438-3441.

Hassett, D.J., and Cohen, M.S. (1989) Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. *Faseb J* **3**: 2574-2582.

Hawley, D.K., and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res* **11**: 2237-2255.

Hebeler, B.H., and Young, F.E. (1975) Autolysis of *Neisseria gonorrhoeae*. *J Bacteriol* **122**: 385-392.

Hecker, M., Schumann, W., and Volker, U. (1996) Heat-shock and general stress response in *Bacillus subtilis*. *Mol Microbiol* **19**: 417-428.

Hecker, M., and Volker, U. (2001) General stress response of *Bacillus subtilis* and other bacteria. *Adv Microb Physiol* **44**: 35-91.

Heinrich, D.W., and Glasgow, A.C. (1997) Transcriptional regulation of type 4 pilin genes and the site-specific recombinase gene, *piv*, in *Moraxella lacunata* and *Moraxella bovis*. *J Bacteriol* **179**: 7298-7305.

Helmann, J.D., and Chamberlin, M.J. (1988) Structure and function of bacterial sigma factors. *Annu Rev Biochem* **57**: 839-872.

Hendrick, J.P., and Hartl, F.U. (1993) Molecular chaperone functions of heat-shock proteins. *Annu Rev Biochem* **62**: 349-384.

Hennecke, H. (1990) Regulation of bacterial gene expression by metal-protein complexes. *Mol Microbiol* **4**: 1621-1628.

Hennessy, F., Cheetham, M.E., Dirr, H.W., and Blatch, G.L. (2000) Analysis of the levels of conservation of the J domain among the various types of DnaJ-like proteins. *Cell Stress Chaperones* **5**: 347-358.

Henrichsen, J. (1983) Twitching motility. *Annu Rev Microbiol* **37**: 81-93.

Herendeen, S.L., VanBogelen, R.A., and Neidhardt, F.C. (1979) Levels of major proteins of *Escherichia coli* during growth at different temperatures. *J Bacteriol* **139**: 185-194.

Herman, C., Thevenet, D., D'Ari, R., and Bouloc, P. (1995) Degradation of $\sigma^{32}$, the heat shock regulator in *Escherichia coli*, is governed by HflB. *Proc Natl Acad Sci U S A* **92**: 3516-3520.

Herman, C., and D'Ari, R. (1998) Proteolysis and chaperones: the destruction/reconstruction dilemma. *Curr Opin Microbiol* **1**: 204-209.

Hermodson, M.A., Chen, K.C., and Buchanan, T.M. (1978) *Neisseria* pili proteins: amino-terminal amino acid sequences and identification of an unusual amino acid. *Biochemistry* **17**: 442-445.

Hesterkamp, T., and Bukau, B. (1998) Role of the DnaK and HscA homologs of Hsp70 chaperones in protein folding in *E.coli*. *Embo J* **17**: 4818-4828.

Hill, S.A., Samuels, D.S., Carlson, J.H., Wilson, J., Hogan, D., Lubke, L., and Belland, R.J. (1997) Integration host factor is a transcriptional cofactor of *pilE* in *Neisseria gonorrhoeae*. *Mol Microbiol* **23**: 649-656.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B.C., and Herrmann, R. (1996) Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Res* **24**: 4420-4449.

Hindersson, P., Knudsen, J.D., and Axelsen, N.H. (1987) Cloning and expression of *Treponema pallidum* common antigen (Tp-4) in *Escherichia coli* K12. *J Gen Microbiol* **133 ( Pt 3)**: 587-596.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**: 51-59.

Hobbs, M., Collie, E.S., Free, P.D., Livingston, S.P., and Mattick, J.S. (1993) PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in *Pseudomonas aeruginosa*. *Mol Microbiol* **7**: 669-682.

Homuth, G., Masuda, S., Mogk, A., Kobayashi, Y., and Schumann, W. (1997) The *dnaK* operon of *Bacillus subtilis* is heptacistronic. *J Bacteriol* **179**: 1153-1164.

Homuth, G., Mogk, A., and Schumann, W. (1999) Post-transcriptional regulation of the *Bacillus subtilis dnaK* operon. *Mol Microbiol* **32**: 1183-1197.

Hood, B.L., and Hirschberg, R. (1995) Purification and characterization of *Eikenella corrodens* type IV pilin. *Infect Immun* **63**: 3693-3696.

Hook, E.W., 3rd, and Holmes, K.K. (1985) Gonococcal infections. *Ann Intern Med* **102**: 229-243.

Hook, E.W., 3rd, and Handsfield, H.H. (1990) Sexually Transmitted Diseases. Holmes, K.K. (ed). New York: McGraw-Hill Publishers, pp. 149-160.

Horner, P.J., Cain, D., McClure, M., Thomas, B.J., Gilroy, C., Ali, M., Weber, J.N., and Taylor-Robinson, D. (1997) Association of antibodies to *Chlamydia trachomatis* heat-shock protein 60 kD with chronic nongonococcal urethritis. *Clin Infect Dis* **24**: 653-660.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**: 61-68.

Hoyne, P.A., Haas, R., Meyer, T.F., Davies, J.K., and Elleman, T.C. (1992) Production of *Neisseria gonorrhoeae* pili (fimbriae) in *Pseudomonas aeruginosa*. *J Bacteriol* **174**: 7321-7327.

Hsieh, M., and Gralla, J.D. (1994) Analysis of the N-terminal leucine heptad and hexad repeats of sigma 54. *J Mol Biol* **239**: 15-24.

Hsieh, M., Tintut, Y., and Gralla, J.D. (1994) Functional roles for the glutamines within the glutamine-rich region of the transcription factor sigma 54. *J Biol Chem* **269**: 373-378.

Hsieh, M., Hsu, H.M., Hwang, S.F., Wen, F.C., Yu, J.S., Wen, C.C., and Li, C. (1999) The hydrophobic heptad repeat in Region III of *Escherichia coli* transcription factor sigma 54 is essential for core RNA polymerase binding. *Microbiology* **145 ( Pt 11)**: 3081-3088.

Huang, L.H., Tseng, Y.H., and Yang, M.T. (1998) Isolation and characterization of the *Xanthomonas campestris rpoH* gene coding for a 32-kDa heat shock sigma factor. *Biochem Biophys Res Commun* **244**: 854-860.

Hunt, C., and Morimoto, R.I. (1985) Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proc Natl Acad Sci U S A* **82**: 6455-6459.

Hurme, R., and Rhen, M. (1998) Temperature sensing in bacterial gene regulation-what it all boils down to. *Mol Microbiol* **30**: 1-6.

Ingolia, T.D., Craig, E.A., and McCarthy, B.J. (1980) Sequence of three copies of the gene for the major *Drosophila* heat shock induced protein and their flanking regions. *Cell* **21**: 669-679.

Ishimoto, K.S., and Lory, S. (1989) Formation of pilin in *Pseudomonas aeruginosa* requires the alternative σ factor (RpoN) of RNA polymerase. *Proc Natl Acad Sci U S A* **86**: 1954-1957.

Ishimoto, K.S., and Lory, S. (1992) Identification of PilR, which encodes a transcriptional activator of the *Pseudomonas aeruginosa* pilin gene. *J Bacteriol* **174**: 3514-3521.

Jaeger, J.A., Turner, D.H., and Zuker, M. (1990) Predicting optimal and suboptimal secondary structure for RNA. *Methods Enzymol* **183**: 281-306.

Jayaraman, G.C., Penders, J.E., and Burne, R.A. (1997) Transcriptional analysis of the *Streptococcus mutans hrcA*, *grpE* and *dnaK* genes and regulation of expression in response to heat shock and environmental acidification. *Mol Microbiol* **25**: 329-341.

Jennings, M.P., Virji, M., Evans, D., Foster, V., Srikhanta, Y.N., Steeghs, L., van der Ley, P., and Moxon, E.R. (1998) Identification of a novel gene involved in pilin glycosylation in *Neisseria meningitidis*. *Mol Microbiol* **29**: 975-984.

Jephcott, A.E. (1997) Microbiological diagnosis of gonorrhoea. *Genitourin Med* **73**: 245-252.

Jin, S., Ishimoto, K., and Lory, S. (1994) Nucleotide sequence of the *rpoN* gene and characterization of two downstream open reading frames in *Pseudomonas aeruginosa*. *J Bacteriol* **176**: 1316-1322.

Jindal, S., Dudani, A.K., Singh, B., Harley, C.B., and Gupta, R.S. (1989) Primary structure of a human mitochondrial protein homologous to the bacterial and plant chaperonins and to the 65-kilodalton mycobacterial antigen. *Mol Cell Biol* **9**: 2279-2283.

Johnson, J.L., and Craig, E.A. (1997) Protein folding in vivo: unraveling complex pathways. *Cell* **90**: 201-204.

Johnson, K., Parker, M.L., and Lory, S. (1986) Nucleotide sequence and transcriptional initiation site of two *Pseudomonas aeruginosa* pilin genes. *J Biol Chem* **261**: 15703-15708.

Johnson, S.R., Steiner, B.M., and Perkins, G.H. (1996) Cloning and characterization of the catalase gene of *Neisseria gonorrhoeae*: use of the gonococcus as a host organism for recombinant DNA. *Infect Immun* **64**: 2627-2634.

Jones, D.H., Franklin, F.C., and Thomas, C.M. (1994) Molecular analysis of the operon which encodes the RNA polymerase sigma factor $\sigma^{54}$ of *Escherichia coli*. *Microbiology* **140 ( Pt 5)**: 1035-1043.

Joo, D.M., Ng, N., and Calendar, R. (1997) A $\sigma^{32}$ mutant with a single amino acid change in the highly conserved region 2.2 exhibits reduced core RNA polymerase affinity. *Proc Natl Acad Sci U S A* **94**: 4907-4912.

Joo, D.M., Nolte, A., Calendar, R., Zhou, Y.N., and Jin, D.J. (1998) Multiple regions on the *Escherichia coli* heat shock transcription factor $\sigma^{32}$ determine core RNA polymerase binding specificity. *J Bacteriol* **180**: 1095-1102.

Kahrs, A.F., Bihlmaier, A., Facius, D., and Meyer, T.F. (1994) Generalized transposon shuttle mutagenesis in *Neisseria gonorrhoeae*: a method for isolating epithelial cell invasion-defective mutants. *Mol Microbiol* **12**: 819-831.

Kallipolitis, B.H., and Valentin-Hansen, P. (1998) Transcription of *rpoH*, encoding the *Escherichia coli* heat-shock regulator $\sigma^{32}$, is negatively controlled by the cAMP-CRP/CytR nucleoprotein complex. *Mol Microbiol* **29**: 1091-1099.

Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R.W., Olinger, L., Grimwood, J., Davis, R.W., and Stephens, R.S. (1999) Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* **21**: 385-389.

Kaneko, T., and *et al.* (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res* **3**: 109-136.

Kanemori, M., Mori, H., and Yura, T. (1994) Effects of reduced levels of GroE chaperones on protein metabolism: enhanced synthesis of heat shock proteins during steady-state growth of *Escherichia coli*. *J Bacteriol* **176**: 4235-4242.

Kanemori, M., Nishihara, K., Yanagi, H., and Yura, T. (1997) Synergistic roles of HslVU and other ATP-dependent proteases in controlling in vivo turnover of $\sigma^{32}$ and abnormal proteins in *Escherichia coli*. *J Bacteriol* **179**: 7219-7225.

Kanemori, M., Yanagi, H., and Yura, T. (1999) Marked instability of the $\sigma^{32}$ heat shock transcription factor at high temperature. Implications for heat shock regulation. *J Biol Chem* **274**: 22002-22007.

Karls, R.K., Brooks, J., Rossmeissl, P., Luedke, J., and Donohue, T.J. (1998) Metabolic roles of a *Rhodobacter sphaeroides* member of the $\sigma^{32}$ family. *J Bacteriol* **180**: 10-19.

Karzai, A.W., and McMacken, R. (1996) A bipartite signaling mechanism involved in DnaJ-mediated activation of the *Escherichia coli* DnaK protein. *J Biol Chem* **271**: 11236-11246.

Kaufmann, S.H. (1990) Heat shock proteins and the immune response. *Immunol Today* **11**: 129-136.

Keevil, C.W., Davies, D.B., Spillane, B.J., and Mahenthiralingam, E. (1989) Influence of iron-limited and replete continuous culture on the physiology and virulence of *Neisseria gonorrhoeae*. *J Gen Microbiol* **135 ( Pt 4)**: 851-863.

Kelly, M.T., and Hoover, T.R. (1999) Mutant forms of *Salmonella typhimurium* $\sigma^{54}$ defective in transcription initiation but not promoter binding activity. *J Bacteriol* **181**: 3351-3357.

Keseler, I.M., and Kaiser, D. (1995) An early A-signal-dependent gene in *Myxococcus xanthus* has a $\sigma^{54}$-like promoter. *J Bacteriol* **177**: 4638-4644.

Keseler, I.M., and Kaiser, D. (1997) $\sigma^{54}$, a vital protein for *Myxococcus xanthus*. *Proc Natl Acad Sci U S A* **94**: 1979-1984.

Kiessling, R., Gronberg, A., Ivanyi, J., Soderstrom, K., Ferm, M., Kleinau, S., Nilsson, E., and Klareskog, L. (1991) Role of hsp60 during autoimmune and bacterial inflammation. *Immunol Rev* **121**: 91-111.

Kimura, Y., Yamada, K., Sakai, T., Mishima, K., Nishimura, H., Matsumoto, Y., Singh, M., and Yoshikai, Y. (1998) The regulatory role of heat shock protein 70-reactive CD4+ T cells during rat listeriosis. *Int Immunol* **10**: 117-130.

Klimpel, K.W., and Clark, V.L. (1989) The heat shock response of type 1 and type 4 gonococci. *Sex Transm Dis* **16**: 141-147.

Klimpel, K.W., Lesley, S.A., and Clark, V.L. (1989) Identification of subunits of gonococcal RNA polymerase by immunoblot analysis: evidence for multiple sigma factors. *J Bacteriol* **171**: 3713-3718.

Klose, K.E., and Mekalanos, J.J. (1998) Distinct roles of an alternative sigma factor during both free-swimming and colonizing phases of the *Vibrio cholerae* pathogenic cycle. *Mol Microbiol* **28**: 501-520.

Kohler, S., Teyssier, J., Cloeckaert, A., Rouot, B., and Liautard, J.P. (1996) Participation of the molecular chaperone DnaK in intracellular growth of *Brucella suis* within U937-derived phagocytes. *Mol Microbiol* **20**: 701-712.

Krause, H.M., and Higgins, N.P. (1986) Positive and negative regulation of the Mu operator by Mu repressor and *Escherichia coli* integration host factor. *J Biol Chem* **261**: 3744-3752.

Krueger, J.H., and Walker, G.C. (1984) *groEL* and *dnaK* genes of *Escherichia coli* are induced by UV irradiation and nalidixic acid in an *htpR*$^{+}$-dependent fashion. *Proc Natl Acad Sci U S A* **81**: 1499-1503.

Kullik, I., Fritsche, S., Knobel, H., Sanjuan, J., Hennecke, H., and Fischer, H.M. (1991) *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the $\sigma^{54}$ gene (*rpoN*). *J Bacteriol* **173**: 1125-1138.

Kupsch, E.M., Knepper, B., Kuroki, T., Heuer, I., and Meyer, T.F. (1993) Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. *Embo J* **12**: 641-650.

Kupsch, E.M., Aubel, D., Gibbs, C.P., Kahrs, A.F., Rudel, T., and Meyer, T.F. (1996) Construction of Hermes shuttle vectors: a versatile system useful for genetic complementation of transformable and non-transformable *Neisseria* mutants. *Mol Gen Genet* **250**: 558-569.

Kurzik-Dumke, U., Debes, A., Kaymer, M., and Dienes, P. (1998) Mitochondrial localization and temporal expression of the *Drosophila melanogaster* DnaJ homologous tumor suppressor Tid50. *Cell Stress Chaperones* **3**: 12-27.

Kusukawa, N., and Yura, T. (1988) Heat shock protein GroE of *Escherichia coli*: key protective roles against thermal stress. *Genes Dev* **2**: 874-882.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Landick, R., Vaughn, V., Lau, E.T., VanBogelen, R.A., Erickson, J.W., and Neidhardt, F.C. (1984) Nucleotide sequence of the heat shock regulatory gene of *E. coli* suggests its protein product may be a transcription factor. *Cell* **38**: 175-182.

Laskey, R.A., Honda, B.M., Mills, A.D., and Finch, J.T. (1978) Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature* **275**: 416-420.

Laskos, L., Dillard, J.P., Seifert, H.S., Fyfe, J.A., and Davies, J.K. (1998) The pathogenic neisseriae contain an inactive *rpoN* gene and do not utilize the *pilE* $\sigma^{54}$ promoter. *Gene* **208**: 95-102.

Laufen, T., Mayer, M.P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J., and Bukau, B. (1999) Mechanism of regulation of Hsp70 chaperones by DnaJ cochaperones. *Proc Natl Acad Sci U S A* **96**: 5452-5457.

Lecker, S., Lill, R., Ziegelhoffer, T., Georgopoulos, C., Bassford, P.J., Jr., Kumamoto, C.A., and Wickner, W. (1989) Three pure chaperone proteins of *Escherichia coli* - SecB, trigger factor and GroEL - form soluble complexes with precursor proteins *in vitro*. *Embo J* **8**: 2703-2709.

Lelivelt, M.J., and Kawula, T.H. (1995) Hsc66, an Hsp70 homolog in *Escherichia coli*, is induced by cold shock but not by heat shock. *J Bacteriol* **177**: 4900-4907.

Lemos, J.A., Burne, R.A., and Castro, A.C. (2000) Molecular cloning, purification and immunological responses of recombinants GroEL and DnaK from *Streptococcus pyogenes*. *FEMS Immunol Med Microbiol* **28**: 121-128.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. (1991) *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc Natl Acad Sci U S A* **88**: 2874-2878.

Liberek, K., Galitski, T.P., Zylicz, M., and Georgopoulos, C. (1992) The DnaK chaperone modulates the heat shock response of *Escherichia coli* by binding to the $\sigma^{32}$ transcription factor. *Proc Natl Acad Sci U S A* **89**: 3516-3520.

Lindquist, S. (1986) The heat-shock response. *Annu Rev Biochem* **55**: 1151-1191.

Lindquist, S., and Craig, E.A. (1988) The heat-shock proteins. *Annu Rev Genet* **22**: 631-677.

Lipinska, B., King, J., Ang, D., and Georgopoulos, C. (1988) Sequence analysis and transcriptional regulation of the *Escherichia coli grpE* gene, encoding a heat shock protein. *Nucleic Acids Res* **16**: 7545-7562.

Lonetto, M., Gribskov, M., and Gross, C.A. (1992) The $\sigma^{70}$ family: sequence conservation and evolutionary relationships. *J Bacteriol* **174**: 3843-3849.

Lopez-Sanchez, F., Ramirez-Santos, J., and Gomez-Eichelmann, M.C. (1997) *In vivo* effect of DNA relaxation on the transcription of gene *rpoH* in *Escherichia coli*. *Biochim Biophys Acta* **1353**: 79-83.

Lorenzen, D.R., Gunther, D., Pandit, J., Rudel, T., Brandt, E., and Meyer, T.F. (2000) *Neisseria gonorrhoeae* porin modifies the oxidative burst of human professional phagocytes. *Infect Immun* **68**: 6215-6222.

Lowrie, D.B., Silva, C.L., Colston, M.J., Ragno, S., and Tascon, R.E. (1997) Protection against tuberculosis by a plasmid DNA vaccine. *Vaccine* **15**: 834-838.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *Journal of biological chemistry* **193**: 265-275.

Lu, Z., and Cyr, D.M. (1998) The conserved carboxyl terminus and zinc finger-like domain of the co-chaperone Ydj1 assist Hsp70 in protein folding. *J Biol Chem* **273**: 5970-5978.

Luirink, J., ten Hagen-Jongman, C.M., van der Weijden, C.C., Oudega, B., High, S., Dobberstein, B., and Kusters, R. (1994) An alternative protein targeting pathway in *Escherichia coli*: studies on the role of FtsY. *Embo J* **13**: 2289-2296.

Lund, P.A. (2001) Microbial molecular chaperones. *Adv Microb Physiol* **44**: 93-140.

Macario, A.J., Lange, M., Ahring, B.K., and De Macario, E.C. (1999) Stress genes and proteins in the archaea. *Microbiol Mol Biol Rev* **63**: 923-967, table of contents.

Mager, W.H., and De Kruijff, A.J. (1995) Stress-induced transcriptional activation. *Microbiol Rev* **59**: 506-531.

Makino, S., van Putten, J.P., and Meyer, T.F. (1991) Phase variation of the opacity outer membrane protein controls invasion by *Neisseria gonorrhoeae* into human epithelial cells. *Embo J* **10**: 1307-1315.

Manzanera, M., Aranda-Olmedo, I., Ramos, J.L., and Marques, S. (2001) Molecular characterization of *Pseudomonas putida* KT2440 *rpoH* gene regulation. *Microbiology* **147**: 1323-1330.

Marolda, C.L., and Valvano, M.A. (1998) Promoter region of the *Escherichia coli* O7-specific lipopolysaccharide gene cluster: structural and functional characterization of an upstream untranslated mRNA sequence. *J Bacteriol* **180**: 3070-3079.

Marrs, C.F., Schoolnik, G., Koomey, J.M., Hardy, J., Rothbard, J., and Falkow, S. (1985) Cloning and sequencing of a *Moraxella bovis* pilin gene. *J Bacteriol* **163**: 132-139.

Marrs, C.F., Rozsa, F.W., Hackel, M., Stevens, S.P., and Glasgow, A.C. (1990) Identification, cloning, and sequencing of *piv*, a new gene involved in inverting the pilin genes of *Moraxella lacunata*. *J Bacteriol* **172**: 4370-4377.

Martin, C.S., Flores, A.I., and Cuezva, J.M. (1995) Cpn60 is exclusively localized into mitochondria of rat liver and embryonic *Drosophila* cells. *J Cell Biochem* **59**: 235-245.

Martin, J., and Hartl, F.U. (1997) Chaperone-assisted protein folding. *Curr Opin Struct Biol* **7**: 41-52.

Martinez, E., Bartolome, B., and de la Cruz, F. (1988) pACYC184-derived cloning vectors containing the multiple cloning site and *lacZα* reporter gene of pUC8/9 and pUC18/19 plasmids. *Gene* **68**: 159-162.

Martinez-Yamout, M., Legge, G.B., Zhang, O., Wright, P.E., and Dyson, H.J. (2000) Solution structure of the cysteine-rich domain of the *Escherichia coli* chaperone protein DnaJ. *J Mol Biol* **300**: 805-818.

Mathew, A., and Morimoto, R.I. (1998) Role of the heat-shock response in the life and death of proteins. *Ann N Y Acad Sci* **851**: 99-111.

Mattick, J.S., Bills, M.M., Anderson, B.J., Dalrymple, B., Mott, M.R., and Egerton, J.R. (1987) Morphogenetic expression of *Bacteroides nodosus* fimbriae in *Pseudomonas aeruginosa*. *J Bacteriol* **169**: 33-41.

Mayer, M.P., Laufen, T., Paal, K., McCarty, J.S., and Bukau, B. (1999) Investigation of the interaction between DnaK and DnaJ by surface plasmon resonance spectroscopy. *J Mol Biol* **289**: 1131-1144.

Mayer, M.P., Schroder, H., Rudiger, S., Paal, K., Laufen, T., and Bukau, B. (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nat Struct Biol* **7**: 586-593.

McCarty, J.S., and Walker, G.C. (1991) DnaK as a thermometer: threonine-199 is site of autophosphorylation and is critical for ATPase activity. *Proc Natl Acad Sci U S A* **88**: 9513-9517.

McCarty, J.S., Buchberger, A., Reinstein, J., and Bukau, B. (1995) The role of ATP in the functional cycle of the DnaK chaperone system. *J Mol Biol* **249**: 126-137.

McCarty, J.S., Rudiger, S., Schonfeld, H.J., Schneider-Mergener, J., Nakahigashi, K., Yura, T., and Bukau, B. (1996) Regulatory region C of the *E. coli* heat shock transcription factor, $\sigma^{32}$, constitutes a DnaK binding site and is conserved among eubacteria. *J Mol Biol* **256**: 829-837.

McGee, D.J., Chen, G.C., and Rest, R.F. (1996) Expression of sialyltransferase is not required for interaction of *Neisseria gonorrhoeae* with human epithelial cells and human neutrophils. *Infect Immun* **64**: 4129-4136.

McGee, Z.A., Stephens, D.S., Hoffman, L.H., Schlech, W.F., 3rd, and Horn, R.G. (1983) Mechanisms of mucosal invasion by pathogenic *Neisseria*. *Rev Infect Dis* **5 Suppl 4**: S708-714.

McGee, Z.A., Gorby, G.L., Wyrick, P.B., Hodinka, R., and Hoffman, L.H. (1988) Parasite-directed endocytosis. *Rev Infect Dis* **10 Suppl 2**: S311-316.

McKenzie, K.R., Adams, E., Britton, W.J., Garsia, R.J., and Basten, A. (1991) Sequence and immunogenicity of the 70-kDa heat shock protein of *Mycobacterium leprae*. *J Immunol* **147**: 312-319.

McKern, N.M., Stewart, D.J., and Strike, P.M. (1988) Amino acid sequences of pilins from serologically distinct strains of *Bacteroides nodosus*. *J Protein Chem* **7**: 157-164.

McLean, I.L., Archer, J.R., Cawley, M.I., Pegley, F.S., Kidd, B.L., and Thompson, P.W. (1990) Specific antibody response to the mycobacterial 65 kDa stress protein in ankylosing spondylitis and rheumatoid arthritis. *Br J Rheumatol* **29**: 426-429.

Melnick, J., and Argon, Y. (1995) Molecular chaperones and the biosynthesis of antigen receptors. *Immunol Today* **16**: 243-250.

Merino, S., Altarriba, M., Gavin, R., Izquierdo, L., and Tomas, J.M. (2001) The cell division genes (*ftsE* and *X*) of *Aeromonas hydrophila* and their relationship with opsonophagocytosis. *FEMS Microbiol Lett* **198**: 183-188.

Merrick, M., Gibbins, J., and Toukdarian, A. (1987) The nucleotide sequence of the sigma factor gene *ntrA* (*rpoN*) of *Azotobacter vinelandii*: analysis of conserved sequences in NtrA proteins. *Mol Gen Genet* **210**: 323-330.

Merrick, M., and Chambers, S. (1992) The helix-turn-helix motif of $\sigma^{54}$ is involved in recognition of the -13 promoter region. *J Bacteriol* **174**: 7221-7226.

Merrick, M.J., and Gibbins, J.R. (1985) The nucleotide sequence of the nitrogen-regulation gene *ntrA* of *Klebsiella pneumoniae* and comparison with conserved features in bacterial RNA polymerase sigma factors. *Nucleic Acids Res* **13**: 7607-7620.

Merrick, M.J. (1993) In a class of its own - the RNA polymerase sigma factor $\sigma^{54}$ ($\sigma^{N}$). *Mol Microbiol* **10**: 903-909.

Meury, J., and Kohiyama, M. (1991) Role of heat shock protein DnaK in osmotic adaptation of *Escherichia coli*. *J Bacteriol* **173**: 4404-4410.

Meyer, T.F., Billyard, E., Haas, R., Storzbach, S., and So, M. (1984) Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. *Proc Natl Acad Sci U S A* **81**: 6110-6114.

Meyer, T.F., and van Putten, J.P. (1989) Genetic mechanisms and biological implications of phase variation in pathogenic Neisseriae. *Clin Microbiol Rev* **2 Suppl**: S139-145.

Mietzner, T.A., Luginbuhl, G.H., Sandstrom, E., and Morse, S.A. (1984) Identification of an iron-regulated 37,000-dalton protein in the cell envelope of *Neisseria gonorrhoeae*. *Infect Immun* **45**: 410-416.

Minder, A.C., Narberhaus, F., Babst, M., Hennecke, H., and Fischer, H.M. (1997) The *dnaKJ* operon belongs to the $\sigma^{32}$-dependent class of heat shock genes in *Bradyrhizobium japonicum*. *Mol Gen Genet* **254**: 195-206.

Minder, A.C., Fischer, H.M., Hennecke, H., and Narberhaus, F. (2000) Role of HrcA and CIRCE in the heat shock regulatory network of *Bradyrhizobium japonicum*. *J Bacteriol* **182**: 14-22.

Minor, S.Y., Banerjee, A., and Gotschlich, E.C. (2000) Effect of α-oligosaccharide phenotype of *Neisseria gonorrhoeae* strain MS11 on invasion of Chang conjunctival, HEC-1-B endometrial, and ME-180 cervical cells. *Infect Immun* **68**: 6526-6534.

Missiakas, D., and Raina, S. (1998) The extracytoplasmic function sigma factors: role and regulation. *Mol Microbiol* **28**: 1059-1066.

Mitraki, A., Fane, B., Haase-Pettingell, C., Sturtevant, J., and King, J. (1991) Global suppression of protein folding defects and inclusion body formation. *Science* **253**: 54-58.

Model, P., Jovanovic, G., and Dworkin, J. (1997) The *Escherichia coli* phage-shock-protein (*psp*) operon. *Mol Microbiol* **24**: 255-261.

Mogk, A., Homuth, G., Scholz, C., Kim, L., Schmid, F.X., and Schumann, W. (1997) The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*. *Embo J* **16**: 4579-4590.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rudiger, S., Roder, D., Langen, H., and Bukau, B. (1999) Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *Embo J* **18**: 6934-6949.

Morelle, G. (1989) A plasmid extraction procedure on a miniprep scale. *Focus* **11**: 7-8.

Morett, E., and Buck, M. (1989) *In vivo* studies on the interaction of RNA polymerase-$\sigma^{54}$ with the *Klebsiella pneumoniae* and *Rhizobium meliloti nifH* promoters. The role of NifA in the formation of an open promoter complex. *J Mol Biol* **210**: 65-77.

Morita, M., Kanemori, M., Yanagi, H., and Yura, T. (1999a) Heat-induced synthesis of $\sigma^{32}$ in *Escherichia coli*: structural and functional dissection of *rpoH* mRNA secondary structure. *J Bacteriol* **181**: 401-410.

Morita, M.T., Tanaka, Y., Kodama, T.S., Kyogoku, Y., Yanagi, H., and Yura, T. (1999b) Translational induction of heat shock transcription factor $\sigma^{32}$: evidence for a built-in RNA thermosensor. *Genes Dev* **13**: 655-665.

Morita, M.T., Kanemori, M., Yanagi, H., and Yura, T. (2000) Dynamic interplay between antagonistic pathways controlling the $\sigma^{32}$ level in *Escherichia coli*. *Proc Natl Acad Sci U S A* **97**: 5860-5865.

Morrison, R.P., Belland, R.J., Lyng, K., and Caldwell, H.D. (1989) Chlamydial disease pathogenesis. The 57-kD chlamydial hypersensitivity antigen is a stress response protein. *J Exp Med* **170**: 1271-1283.

Motohashi, K., Watanabe, Y., Yohda, M., and Yoshida, M. (1999) Heat-inactivated proteins are rescued by the DnaK.J-GrpE set and ClpB chaperones. *Proc Natl Acad Sci U S A* **96**: 7184-7189.

Mullin, D.A., and Newton, A. (1993) A $\sigma^{54}$ promoter and downstream sequence elements ftr2 and ftr3 are required for regulated expression of divergent transcription units *flaN* and *flbG* in *Caulobacter crescentus*. *J Bacteriol* **175**: 2067-2076.

Murphy, G.L., Connell, T.D., Barritt, D.S., Koomey, M., and Cannon, J.G. (1989) Phase variation of gonococcal protein II: regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence. *Cell* **56**: 539-547.

Naczynski, Z.M., Mueller, C., and Kropinski, A.M. (1995) Cloning the gene for the heat shock response positive regulator (sigma 32 homolog) from *Pseudomonas aeruginosa*. *Can J Microbiol* **41**: 75-87.

Nagai, H., Yano, R., Erickson, J.W., and Yura, T. (1990) Transcriptional regulation of the heat shock regulatory gene *rpoH* in *Escherichia coli*: involvement of a novel catabolite-sensitive promoter. *J Bacteriol* **172**: 2710-2715.

Nagai, H., Yuzawa, H., and Yura, T. (1991a) Interplay of two cis-acting mRNA regions in translational control of $\sigma^{32}$ synthesis during the heat shock response of *Escherichia coli*. *Proc Natl Acad Sci U S A* **88**: 10515-10519.

Nagai, H., Yuzawa, H., and Yura, T. (1991b) Regulation of the heat shock response in *E coli*: involvement of positive and negative cis-acting elements in translation control of $\sigma^{32}$ synthesis. *Biochimie* **73**: 1473-1479.

Nagai, H., Yuzawa, H., Kanemori, M., and Yura, T. (1994) A distinct segment of the $\sigma^{32}$ polypeptide is involved in DnaK-mediated negative control of the heat shock response in *Escherichia coli*. *Proc Natl Acad Sci U S A* **91**: 10280-10284.

Nakahigashi, K., Yanagi, H., and Yura, T. (1995) Isolation and sequence analysis of *rpoH* genes encoding $\sigma^{32}$ homologs from gram negative bacteria: conserved mRNA and protein segments for heat shock regulation. *Nucleic Acids Res* **23**: 4383-4390.

Nakahigashi, K., Yanagi, H., and Yura, T. (1998) Regulatory conservation and divergence of $\sigma^{32}$ homologs from gram-negative bacteria: *Serratia marcescens*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Agrobacterium tumefaciens*. *J Bacteriol* **180**: 2402-2408.

Nakahigashi, K., Ron, E.Z., Yanagi, H., and Yura, T. (1999) Differential and independent roles of a $\sigma^{32}$ homolog (RpoH) and an HrcA repressor in the heat shock response of *Agrobacterium tumefaciens*. *J Bacteriol* **181**: 7509-7515.

Nakahigashi, K., Yanagi, H., and Yura, T. (2001) DnaK chaperone-mediated control of activity of a $\sigma^{32}$ homolog (RpoH) plays a major role in the heat shock response of *Agrobacterium tumefaciens*. *J Bacteriol* **183**: 5302-5310.

Narberhaus, F., Weiglhofer, W., Fischer, H.M., and Hennecke, H. (1996) The *Bradyrhizobium japonicum rpoH*$_1$ gene encoding a $\sigma^{32}$-like protein is part of a unique heat shock gene cluster together with *groESL*$_1$ and three small heat shock genes. *J Bacteriol* **178**: 5337-5346.

Narberhaus, F., Krummenacher, P., Fischer, H.M., and Hennecke, H. (1997) Three disparately regulated genes for $\sigma^{32}$-like transcription factors in *Bradyrhizobium japonicum*. *Mol Microbiol* **24**: 93-104.

Narberhaus, F., Kaser, R., Nocker, A., and Hennecke, H. (1998a) A novel DNA element that controls bacterial heat shock gene expression. *Mol Microbiol* **28**: 315-323.

Narberhaus, F., Kowarik, M., Beck, C., and Hennecke, H. (1998b) Promoter selectivity of the *Bradyrhizobium japonicum* RpoH transcription factors in vivo and in vitro. *J Bacteriol* **180**: 2395-2401.

Narberhaus, F. (1999) Negative regulation of bacterial heat shock genes. *Mol Microbiol* **31**: 1-8.

Neidhardt, F.C., VanBogelen, R.A., and Vaughn, V. (1984) The genetics and regulation of heat-shock proteins. *Annu Rev Genet* **18**: 295-329.

Netzer, W.J., and Hartl, F.U. (1997) Recombination of protein domains facilitated by co-translational folding in eukaryotes. *Nature* **388**: 343-349.

Nimura, K., Yoshikawa, H., and Takahashi, H. (1994) Identification of *dnaK* multigene family in *Synechococcus* sp. PCC7942. *Biochem Biophys Res Commun* **201**: 466-471.

Noll, A., Roggenkamp, A., Heesemann, J., and Autenrieth, I.B. (1994) Protective role for heat shock protein-reactive alpha/beta T cells in murine yersiniosis. *Infect Immun* **62**: 2784-2791.

Noll, A., and Autenrieth, I.B. (1996) Immunity against *Yersinia enterocolitica* by vaccination with *Yersinia* HSP60 immunostimulating complexes or *Yersinia* HSP60 plus interleukin-12. *Infect Immun* **64**: 2955-2961.

Noller, H.F., and Woese, C.R. (1981) Secondary structure of 16S ribosomal RNA. *Science* **212**: 403-411.

Noordhoek, G.T., Hermans, P.W., Paul, A.N., Schouls, L.M., van der Sluis, J.J., and van Embden, J.D. (1989) *Treponema pallidum* subspecies *pallidum* (Nichols) and *Treponema pallidum* subspecies *pertenue* (CDC 2575) differ in at least one nucleotide: comparison of two homologous antigens. *Microb Pathog* **6**: 29-42.

Norlander, L., Davies, J., and Normark, S. (1979) Genetic exchange mechanisms in *Neisseria gonorrhoeae*. *J Bacteriol* **138**: 756-761.

Ogata, Y., Mizushima, T., Kataoka, K., Kita, K., Miki, T., and Sekimizu, K. (1996) DnaK heat shock protein of *Escherichia coli* maintains the negative supercoiling of DNA against thermal stress. *J Biol Chem* **271**: 29407-29414.

Ogiwara, A., Uchiyama, I., Takagi, T., and Kanehisa, M. (1996) Construction and analysis of a profile library characterizing groups of structurally known proteins. *Protein Sci* **5**: 1991-1999.

Oguiza, J.A., and Buck, M. (1997) DNA-binding domain mutants of sigma-N ($\sigma^N$, $\sigma^{54}$) defective between closed and stable open promoter complex formation. *Mol Microbiol* **26**: 655-664.

Oguiza, J.A., Gallegos, M.T., Chaney, M.K., Cannon, W.V., and Buck, M. (1999) Involvement of the $\sigma^N$ DNA-binding domain in open complex formation. *Mol Microbiol* **33**: 873-885.

Ogura, T., Inoue, K., Tatsuta, T., Suzaki, T., Karata, K., Young, K., Su, L.H., Fierke, C.A., Jackman, J.E., Raetz, C.R., Coleman, J., Tomoyasu, T., and Matsuzawa, H. (1999) Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in *Escherichia coli*. *Mol Microbiol* **31**: 833-844.

Oke, V., Rushing, B.G., Fisher, E.J., Moghadam-Tabrizi, M., and Long, S.R. (2001) Identification of the heat-shock sigma factor RpoH and a second RpoH-like protein in *Sinorhizobium meliloti*. *Microbiology* **147**: 2399-2408.

Ono, Y., Mitsui, H., Sato, T., and Minamisawa, K. (2001) Two RpoH homologs responsible for the expression of heat shock protein genes in *Sinorhizobium meliloti*. *Mol Gen Genet* **264**: 902-912.

Packschies, L., Theyssen, H., Buchberger, A., Bukau, B., Goody, R.S., and Reinstein, J. (1997) GrpE accelerates nucleotide exchange of the molecular chaperone DnaK with an associative displacement mechanism. *Biochemistry* **36**: 3417-3422.

Paek, K.H., and Walker, G.C. (1987) *Escherichia coli dnaK* null mutants are inviable at high temperature. *J Bacteriol* **169**: 283-290.

Pagel, J.M., Winkelman, J.W., Adams, C.W., and Hatfield, G.W. (1992) DNA topology-mediated regulation of transcription initiation from the tandem promoters of the *ilvGMEDA* operon of *Escherichia coli*. *J Mol Biol* **224**: 919-935.

Pallen, M. (1999) RpoN-dependent transcription of *rpoH*? *Mol Microbiol* **31**: 393.

Pannekoek, Y., Dankert, J., and van Putten, J.P. (1992a) Identification and characterization of a cross-reactive and a unique B-cell epitope on the hsp60 homologue from *Neisseria gonorrhoeae*. *FEMS Microbiol Lett* **78**: 23-29.

Pannekoek, Y., van Putten, J.P., and Dankert, J. (1992b) Identification and molecular analysis of a 63-kilodalton stress protein from *Neisseria gonorrhoeae*. *J Bacteriol* **174**: 6928-6937.

Pannekoek, Y., Schuurman, I.G., Dankert, J., and van Putten, J.P. (1993) Immunogenicity of the meningococcal stress protein MSP63 during natural infection. *Clin Exp Immunol* **93**: 377-381.

Pannekoek, Y., Dankert, J., and van Putten, J.P. (1995) Construction of recombinant neisserial Hsp60 proteins and mapping of antigenic domains. *Mol Microbiol* **15**: 277-285.

Paranchych, W., and Frost, L.S. (1988) The physiology and biochemistry of pili. *Adv Microb Physiol* **29**: 53-114.

Parkhill, J., and *et al.* (2000) Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature* **404**: 502-506.

Parsons, L.M., Waring, A.L., Limberger, R.J., and Shayegani, M. (1999) The *dnaK/dnaJ* operon of *Haemophilus ducreyi* contains a unique combination of regulatory elements. *Gene* **233**: 109-119.

Pasloske, B.L., Drummond, D.S., Frost, L.S., and Paranchych, W. (1989) The activity of the *Pseudomonas aeruginosa* pilin promoter is enhanced by an upstream regulatory site. *Gene* **81**: 25-34.

Pellecchia, M., Szyperski, T., Wall, D., Georgopoulos, C., and Wuthrich, K. (1996) NMR structure of the J-domain and the Gly/Phe-rich region of the *Escherichia coli* DnaJ chaperone. *J Mol Biol* **260**: 236-250.

Pepe, C.M., Eklund, M.W., and Strom, M.S. (1996) Cloning of an *Aeromonas hydrophila* type IV pilus biogenesis gene cluster: complementation of pilus assembly functions and characterization of a type IV leader peptidase/N-methyltransferase required for extracellular protein secretion. *Mol Microbiol* **19**: 857-869.

Pettersson, A., Poolman, J.T., van der Ley, P., and Tommassen, J. (1997) Response of *Neisseria meningitidis* to iron limitation. *Antonie Van Leeuwenhoek* **71**: 129-136.

Pettit, R.K., Filiatrault, M.J., and Martin, E.S. (1996) Alteration of gonococcal protein expression in acidic culture. *Infect Immun* **64**: 1039-1042.

Pierpaoli, E.V., Sandmeier, E., Baici, A., Schonfeld, H.J., Gisler, S., and Christen, P. (1997) The power stroke of the DnaK/DnaJ/GrpE molecular chaperone system. *J Mol Biol* **269**: 757-768.

Popham, D.L., Szeto, D., Keener, J., and Kustu, S. (1989) Function of a bacterial activator protein that binds to transcriptional enhancers. *Science* **243**: 629-635.

Qian, Y.Q., Patel, D., Hartl, F.U., and McColl, D.J. (1996) Nuclear magnetic resonance solution structure of the human Hsp40 (HDJ-1) J-domain. *J Mol Biol* **260**: 224-235.

Qoronfleh, M.W., Weraarchakul, W., and Wilkinson, B.J. (1993) Antibodies to a range of *Staphylococcus aureus* and *Escherichia coli* heat shock proteins in sera from patients with *S. aureus* endocarditis. *Infect Immun* **61**: 1567-1570.

Rambukkana, A., Das, P.K., Witkamp, L., Yong, S., Meinardi, M.M., and Bos, J.D. (1993) Antibodies to mycobacterial 65-kDa heat shock protein and other immunodominant antigens in patients with psoriasis. *J Invest Dermatol* **100**: 87-92.

Ramirez-Santos, J., and Gomez-Eichelmann, M.C. (1998) Identification of $\sigma^{32}$-like factors and *ftsX-rpoH* gene arrangements in enteric bacteria. *Can J Microbiol* **44**: 565-568.

Ramirez-Santos, J., Collado-Vides, J., Garcia-Vareia, M., and Gomez-Eichelmann, M.C. (2001) Conserved regulatory elements of the promoter sequence of the gene *rpoH* of enteric bacteria. *Nucleic Acids Res* **29**: 380-386.

Ranson, N.A., Dunster, N.J., Burston, S.G., and Clarke, A.R. (1995) Chaperonins can catalyse the reversal of early aggregation steps when a protein misfolds. *J Mol Biol* **250**: 581-586.

Ranson, N.A., White, H.E., and Saibil, H.R. (1998) Chaperonins. *Biochem J* **333 ( Pt 2)**: 233-242.

Raulston, J.E., Davis, C.H., Schmiel, D.H., Morgan, M.W., and Wyrick, P.B. (1993) Molecular characterization and outer membrane association of a *Chlamydia trachomatis* protein related to the hsp70 family of proteins. *J Biol Chem* **268**: 23139-23147.

Reisenauer, A., Mohr, C.D., and Shapiro, L. (1996) Regulation of a heat shock $\sigma^{32}$ homolog in *Caulobacter crescentus*. *J Bacteriol* **178**: 1919-1927.

Reitzer, L.J., and Magasanik, B. (1986) Transcription of *glnA* in *E. coli* is stimulated by activator bound to sites far from the promoter. *Cell* **45**: 785-792.

Rippe, K., Guthold, M., von Hippel, P.H., and Bustamante, C. (1997) Transcriptional activation via DNA-looping: visualization of intermediates in the activation pathway of *E. coli* RNA polymerase-$\sigma^{54}$ holoenzyme by scanning force microscopy. *J Mol Biol* **270**: 125-138.

Roberts, R.C., Toochinda, C., Avedissian, M., Baldini, R.L., Gomes, S.L., and Shapiro, L. (1996) Identification of a *Caulobacter crescentus* operon encoding *hrcA*, involved in negatively regulating heat-inducible transcription, and the chaperone gene *grpE*. *J Bacteriol* **178**: 1829-1841.

Rogers, S.D., Bhave, M.R., Mercer, J.F., Camakaris, J., and Lee, B.T. (1991) Cloning and characterization of *cutE*, a gene involved in copper transport in *Escherichia coli*. *J Bacteriol* **173**: 6742-6748.

Ross, J.D. (1996) Systemic gonococcal infection. *Genitourin Med* **72**: 404-407.

Rossau, R., Heyndrickx, L., and Van Heuverswyn, H. (1988) Nucleotide sequence of a 16S ribosomal RNA gene from *Neisseria gonorrhoeae*. *Nucleic Acids Res* **16**: 6227.

Rouquette, C., de Chastellier, C., Nair, S., and Berche, P. (1998) The ClpC ATPase of *Listeria monocytogenes* is a general stress protein required for virulence and promoting early bacterial escape from the phagosome of macrophages. *Mol Microbiol* **27**: 1235-1245.

Ruddon, R.W., and Bedows, E. (1997) Assisted protein folding. *J Biol Chem* **272**: 3125-3128.

Rudel, T., van Putten, J.P., Gibbs, C.P., Haas, R., and Meyer, T.F. (1992) Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. *Mol Microbiol* **6**: 3439-3450.

Rudel, T., Scheurerpflug, I., and Meyer, T.F. (1995) *Neisseria* PilC protein identified as type-4 pilus tip-located adhesin. *Nature* **373**: 357-359.

Rudiger, S., Buchberger, A., and Bukau, B. (1997a) Interaction of Hsp70 chaperones with substrates. *Nat Struct Biol* **4**: 342-349.

Rudiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997b) Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *Embo J* **16**: 1501-1507.

Rudiger, S., Schneider-Mergener, J., and Bukau, B. (2001) Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone. *Embo J* **20**: 1042-1050.

Ruffolo, C.G., Tennent, J.M., Michalski, W.P., and Adler, B. (1997) Identification, purification, and characterization of the type 4 fimbriae of *Pasteurella multocida*. *Infect Immun* **65**: 339-343.

Russell, R., Wali Karzai, A., Mehl, A.F., and McMacken, R. (1999) DnaJ dramatically stimulates ATP hydrolysis by DnaK: insight into targeting of Hsp70 proteins to polypeptide substrates. *Biochemistry* **38**: 4165-4176.

Sahu, G.K., Chowdhury, R., and Das, J. (1997) The *rpoH* gene encoding $\sigma^{32}$ homolog of *Vibrio cholerae*. *Gene* **189**: 203-207.

Saito, H., and Uchida, H. (1977) Initiation of the DNA replication of bacteriophage lambda in *Escherichia coli* K12. *J Mol Biol* **113**: 1-25.

Saito, H., and Uchida, H. (1978) Organization and expression of the *dnaJ* and *dnaK* genes of *Escherichia coli* K12. *Mol Gen Genet* **164**: 1-8.

Sakakibara, Y. (1988) The *dnaK* gene of *Escherichia coli* functions in initiation of chromosome replication. *J Bacteriol* **170**: 972-979.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning. A laboratory manual. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory Press.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**: 5463-5467.

Sarandopoulos, S., and Davies, J.K. (1993) Genetic organization and evolution of the cryptic plasmid of *Neisseria gonorrhoeae*. *Plasmid* **29**: 206-221.

Sarkari, J., Pandit, N., Moxon, E. R., and Achtman, M. (1994) Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. *Mol Microbiol* **13**: 207-217.

Sasse-Dwight, S., and Gralla, J.D. (1990) Role of eukaryotic-type functional domains found in the prokaryotic enhancer receptor factor $\sigma^{54}$. *Cell* **62**: 945-954.

Sato, S., and Ishikawa, H. (1997) Expression and control of an operon from an intracellular symbiont which is homologous to the *groE* operon. *J Bacteriol* **179**: 2300-2304.

Schon, U., and Schumann, W. (1995) Overproduction, purification and characterization of GroES and GroEL from thermophilic *Bacillus stearothermophilus*. *FEMS Microbiol Lett* **134**: 183-188.

Schroder, H., Langer, T., Hartl, F.U., and Bukau, B. (1993) DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. *Embo J* **12**: 4137-4144.

Schweizer, H. P. (1991) *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97**: 109-121.

Schwartz, B., Moore, P.S., and Broome, C.V. (1989) Global epidemiology of meningococcal disease. *Clin Microbiol Rev* **2 Suppl**: S118-124.

Seaton, B.L., and Vickery, L.E. (1994) A gene encoding a DnaK/hsp70 homolog in *Escherichia coli*. *Proc Natl Acad Sci U S A* **91**: 2066-2070.

Segal, E., Billyard, E., So, M., Storzbach, S., and Meyer, T. F. (1985) Role of chromosomal rearrangement in *N. gonorrhoeae* pilus phase variation. *Cell* **40**: 293-300.

Segal, G., and Ron, E.Z. (1993) Heat shock transcription of the *groESL* operon of *Agrobacterium tumefaciens* may involve a hairpin-loop structure. *J Bacteriol* **175**: 3083-3088.

Segal, G., and Ron, E.Z. (1995) The *dnaKJ* operon of *Agrobacterium tumefaciens*: transcriptional analysis and evidence for a new heat shock promoter. *J Bacteriol* **177**: 5952-5958.

Segal, G., and Ron, E.Z. (1996a) Heat shock activation of the *groESL* operon of *Agrobacterium tumefaciens* and the regulatory roles of the inverted repeat. *J Bacteriol* **178**: 3634-3640.

Segal, R., and Ron, E.Z. (1996b) Regulation and organization of the *groE* and *dnaK* operons in Eubacteria. *FEMS Microbiol Lett* **138**: 1-10.

Seifert, H.S., and So, M. (1988) Genetic mechanisms of bacterial antigenic variation. *Microbiol Rev* **52**: 327-336.

Seifert, H.S., and So, M. (1991) Genetic systems in pathogenic Neisseriae. *Methods Enzymol* **204**: 342-357.

Seifert, H.S., Wright, C.J., Jerse, A.E., Cohen, M.S., and Cannon, J.G. (1994) Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *J Clin Invest* **93**: 2744-2749.

Seiler, A., Reinhardt, R., Sarkari, J., Caugant, D.A., and Achtman, M. (1996) Allelic polymorphism and site-specific recombination in the *opc* locus of *Neisseria meningitidis*. *Mol Microbiol* **19**: 841-856.

Sell, S.M., Eisen, C., Ang, D., Zylicz, M., and Georgopoulos, C. (1990) Isolation and characterization of *dnaJ* null mutants of *Escherichia coli*. *J Bacteriol* **172**: 4827-4835.

Servant, P., and Mazodier, P. (2001) Negative regulation of the heat shock response in *Streptomyces*. *Arch Microbiol* **176**: 237-242.

Severinova, E., Severinov, K., Fenyo, D., Marr, M., Brody, E.N., Roberts, J.W., Chait, B.T., and Darst, S.A. (1996) Domain organization of the *Escherichia coli* RNA polymerase $\sigma^{70}$ subunit. *J Mol Biol* **263**: 637-647.

Shafer, W.M., and Rest, R.F. (1989) Interactions of gonococci with phagocytic cells. *Annu Rev Microbiol* **43**: 121-145.

Shaw, J.H., and Falkow, S. (1988) Model for invasion of human tissue culture cells by *Neisseria gonorrhoeae*. *Infect Immun* **56**: 1625-1632.

Sherrard, J., and Barlow, D. (1996) Gonorrhoea in men: clinical and diagnostic aspects. *Genitourin Med* **72**: 422-426.

Shi, Y., Mosser, D.D., and Morimoto, R.I. (1998) Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev* **12**: 654-666.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407**: 81-86.

Shine, J., and Dalgarno, L. (1975) Determinant of cistron specificity in bacterial ribosomes. *Nature* **254**: 34-38.

Shingler, V. (1996) Signal sensing by $\sigma^{54}$-dependent regulators: derepression as a control mechanism. *Mol Microbiol* **19**: 409-416.

Shinnick, T.M., Vodkin, M.H., and Williams, J.C. (1988) The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. *Infect Immun* **56**: 446-451.

Shinnick, T.M. (1991) Heat shock proteins as antigens of bacterial and parasitic pathogens. *Curr Top Microbiol Immunol* **167**: 145-160.

Silberg, J.J., Hoff, K.G., and Vickery, L.E. (1998) The Hsc66-Hsc20 chaperone system in *Escherichia coli*: chaperone activity and interactions with the DnaK-DnaJ-GrpE system. *J Bacteriol* **180**: 6617-6624.

Silva, C.L., and Lowrie, D.B. (1994) A single mycobacterial protein (hsp65) expressed by a transgenic antigen-presenting cell vaccinates mice against tuberculosis. *Immunology* **82**: 244-248.

Skowyra, D., Georgopoulos, C., and Zylicz, M. (1990) The *E. coli dnaK* gene product, the hsp70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. *Cell* **62**: 939-944.

Smith, M., Jessee, J., Landers, T., and Jordan, J. (1990) High efficiency bacterial electroporation: 1 x $10^{10}$ *E. coli* transformants/μg. *Focus* **12**: 38-40.

Snyder, L.A., Butcher, S.A., and Saunders, N.J. (2001) Comparative whole-genome analyses reveal over 100 putative phase-variable genes in the pathogenic *Neisseria* spp. *Microbiology* **147**: 2321-2332.

Southern, E., and Merrick, M. (2000) The role of region II in the RNA polymerase σ factor $\sigma^N$ ($\sigma^{54}$). *Nucleic Acids Res* **28**: 2563-2570.

Sparling, P.F. (1966) Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. *J Bacteriol* **92**: 1364-1371.

Spohn, G., and Scarlato, V. (1999) The autoregulatory HspR repressor protein governs chaperone gene transcription in *Helicobacter pylori*. *Mol Microbiol* **34**: 663-674.

Srivastava, P.K., and Amato, R.J. (2001) Heat shock proteins: the 'Swiss Army Knife' vaccines against cancers and infectious agents. *Vaccine* **19**: 2590-2597.

Stephens, D.S. (1989) Gonococcal and meningococcal pathogenesis as defined by human cell, cell culture, and organ culture assays. *Clin Microbiol Rev* **2 Suppl**: S104-111.

Stephens, D.S., Spellman, P.A., and Swartley, J.S. (1993) Effect of the (α 2→8)-linked polysialic acid capsule on adherence of *Neisseria meningitidis* to human mucosal cells. *J Infect Dis* **167**: 475-479.

Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R.L., Zhao, Q., Koonin, E.V., and Davis, R.W. (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **282**: 754-759.

Stern, A., Brown, M., Nickel, P., and Meyer, T.F. (1986) Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. *Cell* **47**: 61-71.

Stover, C.K., and *et al.* (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**: 959-964.

Straus, D., Walter, W., and Gross, C.A. (1990) DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of $\sigma^{32}$. *Genes Dev* **4**: 2202-2209.

Straus, D.B., Walter, W.A., and Gross, C.A. (1987) The heat shock response of *E. coli* is regulated by changes in the concentration of $\sigma^{32}$. *Nature* **329**: 348-351.

Strom, M.S., and Lory, S. (1993) Structure-function and biogenesis of the type IV pili. *Annu Rev Microbiol* **47**: 565-596.

Studholme, D.J., and Buck, M. (2000) The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. *FEMS Microbiol Lett* **186**: 1-9.

Suh, W.C., Burkholder, W.F., Lu, C.Z., Zhao, X., Gottesman, M.E., and Gross, C.A. (1998) Interaction of the Hsp70 molecular chaperone, DnaK, with its cochaperone DnaJ. *Proc Natl Acad Sci U S A* **95**: 15223-15228.

Suh, W.C., Lu, C.Z., and Gross, C.A. (1999) Structural features required for the interaction of the Hsp70 molecular chaperone DnaK with its cochaperone DnaJ. *J Biol Chem* **274**: 30534-30539.

Svergun, D.I., Malfois, M., Koch, M.H., Wigneshweraraj, S.R., and Buck, M. (2000) Low resolution structure of the $\sigma^{54}$ transcription factor revealed by X-ray solution scattering. *J Biol Chem* **275**: 4210-4214.

Swanson, J., Kraus, S.J., and Gotschlich, E.C. (1971) Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. *J Exp Med* **134**: 886-906.

Swanson, J. (1973) Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. *J Exp Med* **137**: 571-589.

Syed, A., and Gralla, J.D. (1997) Isolation and properties of enhancer-bypass mutants of sigma 54. *Mol Microbiol* **23**: 987-995.

Syed, A., and Gralla, J.D. (1998) Identification of an N-terminal region of sigma 54 required for enhancer responsiveness. *J Bacteriol* **180**: 5619-5625.

Szabo, A., Langer, T., Schroder, H., Flanagan, J., Bukau, B., and Hartl, F.U. (1994) The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system DnaK, DnaJ, and GrpE. *Proc Natl Acad Sci U S A* **91**: 10345-10349.

Szabo, A., Korszun, R., Hartl, F.U., and Flanagan, J. (1996) A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates. *Embo J* **15**: 408-417.

Szyperski, T., Pellecchia, M., Wall, D., Georgopoulos, C., and Wuthrich, K. (1994) NMR structure determination of the *Escherichia coli* DnaJ molecular chaperone: secondary structure and backbone fold of the N-terminal region (residues 2-108) containing the highly conserved J domain. *Proc Natl Acad Sci U S A* **91**: 11343-11347.

Taglicht, D., Padan, E., Oppenheim, A.B., and Schuldiner, S. (1987) An alkaline shift induces the heat shock response in *Escherichia coli*. *J Bacteriol* **169**: 885-887.

Taha, M.K., So, M., Seifert, H.S., Billyard, E., and Marchal, C. (1988) Pilin expression in *Neisseria gonorrhoeae* is under both positive and negative transcriptional control. *Embo J* **7**: 4367-4378.

Taha, M.K., Dupuy, B., Saurin, W., So, M., and Marchal, C. (1991) Control of pilus expression in *Neisseria gonorrhoeae* as an original system in the family of two-component regulators. *Mol Microbiol* **5**: 137-148.

Taha, M.K., and Giorgini, D. (1995) Phosphorylation and functional analysis of PilA, a protein involved in the transcriptional regulation of the pilin gene in *Neisseria gonorrhoeae*. *Mol Microbiol* **15**: 667-677.

Taha, M.K., Giorgini, D., and Nassif, X. (1996) The *pilA* regulatory gene modulates the pilus-mediated adhesion of *Neisseria meningitidis* by controlling the transcription of *pilC1*. *Mol Microbiol* **19**: 1073-1084.

Tatsuta, T., Tomoyasu, T., Bukau, B., Kitagawa, M., Mori, H., Karata, K., and Ogura, T. (1998) Heat shock regulation in the *ftsH* null mutant of *Escherichia coli*: dissection of stability and activity control mechanisms of $\sigma^{32}$ *in vivo*. *Mol Microbiol* **30**: 583-593.

Tatsuta, T., Joob, D.M., Calendar, R., Akiyama, Y., and Ogura, T. (2000) Evidence for an active role of the DnaK chaperone system in the degradation of $\sigma^{32}$. *FEBS Lett* **478**: 271-275.

Taura, T., Kusukawa, N., Yura, T., and Ito, K. (1989) Transient shut off of *Escherichia coli* heat shock protein synthesis upon temperature shift down. *Biochem Biophys Res Commun* **163**: 438-443.

Tauschek, M., Hamilton, C.W., Hall, L.A., Chomvarin, C., Fyfe, J.A., and Davies, J.K. (1997) Transcriptional analysis of the *groESL* operon of *Neisseria gonorrhoeae*. *Gene* **189**: 107-112.

Tavaria, M., Gabriele, T., Kola, I., and Anderson, R.L. (1996) A hitchhiker's guide to the human Hsp70 family. *Cell Stress Chaperones* **1**: 23-28.

Taylor, H.R., Maclean, I.W., Brunham, R.C., Pal, S., and Wittum-Hudson, J. (1990) Chlamydial heat shock proteins and trachoma. *Infect Immun* **58**: 3061-3063.

Taylor, M., Butler, R., Chambers, S., Casimiro, M., Badii, F., and Merrick, M. (1996) The RpoN-box motif of the RNA polymerase sigma factor $\sigma^{N}$ plays a role in promoter recognition. *Mol Microbiol* **22**: 1045-1054.

Teter, S.A., Houry, W.A., Ang, D., Tradler, T., Rockabrand, D., Fischer, G., Blum, P., Georgopoulos, C., and Hartl, F.U. (1999) Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. *Cell* **97**: 755-765.

Tettelin, H., and *et al.* (2000) Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. *Science* **287**: 1809-1815.

Theofilopoulos, A.N. (1995) The basis of autoimmunity: Part I. Mechanisms of aberrant self-recognition. *Immunol Today* **16**: 90-98.

Thomas, J.G., Ayling, A., and Baneyx, F. (1997) Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *E. coli*. To fold or to refold. *Appl Biochem Biotechnol* **66**: 197-238.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673-4680.

Thony, B., and Hennecke, H. (1989) The -24/-12 promoter comes of age. *FEMS Microbiol Rev* **5**: 341-357.

Tilly, K., McKittrick, N., Zylicz, M., and Georgopoulos, C. (1983) The DnaK protein modulates the heat-shock response of *Escherichia coli*. *Cell* **34**: 641-646.

Tilly, K., Erickson, J., Sharma, S., and Georgopoulos, C. (1986) Heat shock regulatory gene *rpoH* mRNA level increases after heat shock in *Escherichia coli*. *J Bacteriol* **168**: 1155-1158.

Tilly, K., Spence, J., and Georgopoulos, C. (1989) Modulation of stability of the *Escherichia coli* heat shock regulatory factor $\sigma^{32}$. *J Bacteriol* **171**: 1585-1589.

Tinsley, C., and Nassif, X. (2001) Meningococcal pathogenesis: at the boundary between the pre- and post-genomic eras. *Curr Opin Microbiol* **4**: 47-52.

Tintut, Y., Wong, C., Jiang, Y., Hsieh, M., and Gralla, J.D. (1994) RNA polymerase binding using a strongly acidic hydrophobic-repeat region of $\sigma^{54}$. *Proc Natl Acad Sci U S A* **91**: 2120-2124.

Tintut, Y., and Gralla, J.D. (1995) PCR mutagenesis identifies a polymerase-binding sequence of sigma 54 that includes a sigma 70 homology region. *J Bacteriol* **177**: 5818-5825.

Tissieres, A., Mitchell, H.K., and Tracy, U.M. (1974) Protein synthesis in salivary glands of *Drosophila melanogaster*: relation to chromosome puffs. *J Mol Biol* **84**: 389-398.

Tobe, T., Ito, K., and Yura, T. (1984) Isolation and physical mapping of temperature-sensitive mutants defective in heat-shock induction of proteins in *Escherichia coli*. *Mol Gen Genet* **195**: 10-16.

Tomoyasu, T., Yamanaka, K., Murata, K., Suzaki, T., Bouloc, P., Kato, A., Niki, H., Hiraga, S., and Ogura, T. (1993a) Topology and subcellular localization of FtsH protein in *Escherichia coli*. *J Bacteriol* **175**: 1352-1357.

Tomoyasu, T., Yuki, T., Morimura, S., Mori, H., Yamanaka, K., Niki, H., Hiraga, S., and Ogura, T. (1993b) The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J Bacteriol* **175**: 1344-1351.

Tomoyasu, T., and *et al.* (1995) *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor $\sigma^{32}$. *Embo J* **14**: 2551-2560.

Tomoyasu, T., Ogura, T., Tatsuta, T., and Bukau, B. (1998) Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in *Escherichia coli*. *Mol Microbiol* **30**: 567-581.

Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., and Bukau, B. (2001) Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the *Escherichia coli* cytosol. *Mol Microbiol* **40**: 397-413.

Totten, P.A., Lara, J.C., and Lory, S. (1990) The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. *J Bacteriol* **172**: 389-396.

Toukdarian, A., and Kennedy, C. (1986) Regulation of nitrogen metabolism in *Azotobacter vinelandii*: isolation of *ntr* and *glnA* genes and construction of *ntr* mutants. *Embo J* **5**: 399-407.

Tsai, J., and Douglas, M.G. (1996) A conserved HPD sequence of the J-domain is necessary for YDJ1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. *J Biol Chem* **271**: 9347-9354.

Tse-Dinh, Y.C., Qi, H., and Menzel, R. (1997) DNA supercoiling and bacterial adaptation: thermotolerance and thermoresistance. *Trends Microbiol* **5**: 323-326.

Ueguchi, C., Shiozawa, T., Kakeda, M., Yamada, H., and Mizuno, T. (1995) A study of the double mutation of *dnaJ* and *cbpA*, whose gene products function as molecular chaperones in *Escherichia coli*. *J Bacteriol* **177**: 3894-3896.

Ueki, T., and Inouye, S. (2001) SigB, SigC, and SigE from *Myxococcus xanthus* homologous to $\sigma^{32}$ are not required for heat shock response but for multicellular differentiation. *J Mol Microbiol Biotechnol* **3**: 287-293.

Ueno-Nishio, S., Backman, K. C., and Magasanik, B. (1983) Regulation at the *glnL*-operator-promoter of the complex *glnALG* operon of *Escherichia coli*. *J Bacteriol* **153**: 1247-1251.

Valent, Q.A., de Gier, J.W., von Heijne, G., Kendall, D.A., ten Hagen-Jongman, C.M., Oudega, B., and Luirink, J. (1997) Nascent membrane and presecretory proteins synthesized in *Escherichia coli* associate with signal recognition particle and trigger factor. *Mol Microbiol* **25**: 53-64.

Valentin-Hansen, P., Sogaard-Andersen, L., and Pedersen, H. (1996) A flexible partnership: the CytR anti-activator and the cAMP-CRP activator protein, comrades in transcription control. *Mol Microbiol* **20**: 461-466.

van Asseldonk, M., Simons, A., Visser, H., de Vos, W.M., and Simons, G. (1993) Cloning, nucleotide sequence, and regulatory analysis of the *Lactococcus lactis dnaJ* gene. *J Bacteriol* **175**: 1637-1644.

van der Ende, A., Hopman, C.T., and Dankert, J. (1999) Deletion of *porA* by recombination between clusters of repetitive extragenic palindromic sequences in *Neisseria meningitidis*. *Infect Immun* **67**: 2928-2934.

van Deuren, M., Brandtzaeg, P., and van der Meer, J.W. (2000) Update on meningococcal disease with emphasis on pathogenesis and clinical management. *Clin Microbiol Rev* **13**: 144-166, table of contents.

van Putten, J.P., and Paul, S.M. (1995) Binding of syndecan-like cell surface proteoglycan receptors is required for *Neisseria gonorrhoeae* entry into human mucosal cells. *Embo J* **14**: 2144-2154.

van Putten, J.P., and Robertson, B.D. (1995) Molecular mechanisms and implications for infection of lipopolysaccharide variation in *Neisseria*. *Mol Microbiol* **16**: 847-853.

Veinger, L., Diamant, S., Buchner, J., and Goloubinoff, P. (1998) The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J Biol Chem* **273**: 11032-11037.

Vickery, L.E., Silberg, J.J., and Ta, D.T. (1997) Hsc66 and Hsc20, a new heat shock cognate molecular chaperone system from *Escherichia coli*. *Protein Sci* **6**: 1047-1056.

Viitanen, P.V., Lubben, T.H., Reed, J., Goloubinoff, P., O'Keefe, D.P., and Lorimer, G.H. (1990) Chaperonin-facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (GroEL) are $K^+$ dependent. *Biochemistry* **29**: 5665-5671.

Villar, M.T., Helber, J.T., Hood, B., Schaefer, M.R., and Hirschberg, R.L. (1999) *Eikenella corrodens* phase variation involves a posttranslational event in pilus formation. *J Bacteriol* **181**: 4154-4160.

Vitovski, S., Read, R.C., and Sayers, J.R. (1999) Invasive isolates of *Neisseria meningitidis* possess enhanced immunoglobulin A1 protease activity compared to colonizing strains. *Faseb J* **13**: 331-337.

Voellmy, R. (1996) Sensing stress and responding to stress. *Exs* **77**: 121-137.

Voiker, U., Engelmann, S., Maul, B., Riethdorf, S., Volker, A., Schmid, R., Mach, H., and Hecker, M. (1994) Analysis of the induction of general stress proteins of *Bacillus subtilis*. *Microbiology* **140 ( Pt 4)**: 741-752.

Wall, D., Zylicz, M., and Georgopoulos, C. (1995) The conserved G/F motif of the DnaJ chaperone is necessary for the activation of the substrate binding properties of the DnaK chaperone. *J Biol Chem* **270**: 2139-2144.

Wang, J.T., Syed, A., Hsieh, M., and Gralla, J.D. (1995) Converting *Escherichia coli* RNA polymerase into an enhancer-responsive enzyme: role of an $NH_2$-terminal leucine patch in $\sigma^{54}$. *Science* **270**: 992-994.

Wang, L., Guo, Y., and Gralla, J.D. (1999) Regulation of sigma 54-dependent transcription by core promoter sequences: role of -12 region nucleotides. *J Bacteriol* **181**: 7558-7565.

Wang, L., and Gralla, J.D. (2001) Roles for the C-terminal region of sigma 54 in transcriptional silencing and DNA binding. *J Biol Chem* **276**: 8979-8986.

Wang, Q.P., and Kaguni, J.M. (1989a) A novel sigma factor is involved in expression of the *rpoH* gene of *Escherichia coli*. *J Bacteriol* **171**: 4248-4253.

Wang, Q.P., and Kaguni, J.M. (1989b) DnaA protein regulates transcription of the *rpoH* gene of *Escherichia coli*. *J Biol Chem* **264**: 7338-7344.

West, S.E., and Sparling, P.F. (1985) Response of *Neisseria gonorrhoeae* to iron limitation: alterations in expression of membrane proteins without apparent siderophore production. *Infect Immun* **47**: 388-394.

Wetzstein, M., Volker, U., Dedio, J., Lobau, S., Zuber, U., Schiesswohl, M., Herget, C., Hecker, M., and Schumann, W. (1992) Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. *J Bacteriol* **174**: 3300-3310.

Wigneshweraraj, S.R., Fujita, N., Ishihama, A., and Buck, M. (2000) Conservation of sigma-core RNA polymerase proximity relationships between the enhancer-independent and enhancer-dependent sigma classes. *Embo J* **19**: 3038-3048.

Wigneshweraraj, S.R., Chaney, M.K., Ishihama, A., and Buck, M. (2001) Regulatory sequences in sigma 54 localise near the start of DNA melting. *J Mol Biol* **306**: 681-701.

Williams, J.M., Chen, G.C., Zhu, L., and Rest, R.F. (1998) Using the yeast two-hybrid system to identify human epithelial cell proteins that bind gonococcal Opa proteins: intracellular gonococci bind pyruvate kinase via their Opa proteins and require host pyruvate for growth. *Mol Microbiol* **27**: 171-186.

Wilson, K. (1993) Preparation of genomic DNA from bacteria. In Current Protocols in Molecular Biology. Vol. 1. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds). United States of America: Green Publishing Associates, Inc. and John Wiley and Sons Inc., pp. 2.4.1-2.4.2.

Wizemann, T.M., Moskovitz, J., Pearce, B.J., Cundell, D., Arvidson, C.G., So, M., Weissbach, H., Brot, N., and Masure, H.R. (1996) Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens. *Proc Natl Acad Sci U S A* **93**: 7985-7990.

Wong, C., and Gralla, J.D. (1992) A role for the acidic trimer repeat region of transcription factor $\sigma^{54}$ in setting the rate and temperature dependence of promoter melting *in vivo*. *J Biol Chem* **267**: 24762-24768.

Wong, C., Tintut, Y., and Gralla, J.D. (1994) The domain structure of sigma 54 as determined by analysis of a set of deletion mutants. *J Mol Biol* **236**: 81-90.

Woo, K.M., Kim, K.I., Goldberg, A.L., Ha, D.B., and Chung, C.H. (1992) The heat-shock protein ClpB in *Escherichia coli* is a protein-activated ATPase. *J Biol Chem* **267**: 20429-20434.

Woods, M.L., 2nd, Bonfiglioli, R., McGee, Z.A., and Georgopoulos, C. (1990) Synthesis of a select group of proteins by *Neisseria gonorrhoeae* in response to thermal stress. *Infect Immun* **58**: 719-725.

Wu, B., Ang, D., Snavely, M., and Georgopoulos, C. (1994) Isolation and characterization of point mutations in the *Escherichia coli grpE* heat shock gene. *J Bacteriol* **176**: 6965-6973.

Wu, B., Wawrzynow, A., Zylicz, M., and Georgopoulos, C. (1996) Structure-function analysis of the *Escherichia coli* GrpE heat shock protein. *Embo J* **15**: 4806-4816.

Wu, J., and Newton, A. (1996) Isolation, identification, and transcriptional specificity of the heat shock sigma factor $\sigma^{32}$ from *Caulobacter crescentus*. *J Bacteriol* **178**: 2094-2101.

Wu, J., and Newton, A. (1997) The *Caulobacter* heat shock sigma factor gene *rpoH* is positively autoregulated from a $\sigma^{32}$-dependent promoter. *J Bacteriol* **179**: 514-521.

Wu, S.S., and Kaiser, D. (1997) Regulation of expression of the *pilA* gene in *Myxococcus xanthus*. *J Bacteriol* **179**: 7748-7758.

Xu, H., and Hoover, T.R. (2001) Transcriptional regulation at a distance in bacteria. *Curr Opin Microbiol* **4**: 138-144.

Xu, Z., Horwich, A.L., and Sigler, P.B. (1997) The crystal structure of the asymmetric GroEL-GroES-$(ADP)_7$ chaperonin complex. *Nature* **388**: 741-750.

Yamamori, T., Ito, K., Nakamura, Y., and Yura, T. (1978) Transient regulation of protein synthesis in *Escherichia coli* upon shift-up of growth temperature. *J Bacteriol* **134**: 1133-1140.

Yamamori, T., and Yura, T. (1980) Temperature-induced synthesis of specific proteins in *Escherichia coli*: evidence for transcriptional control. *J Bacteriol* **142**: 843-851.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**: 103-119.

Yuan, G., and Wong, S.L. (1995) Isolation and characterization of *Bacillus subtilis groE* regulatory mutants: evidence for *orf39* in the *dnaK* operon as a repressor gene in regulating the expression of both *groE* and *dnaK*. *J Bacteriol* **177**: 6462-6468.

Yura, T., Nagai, H., and Mori, H. (1993) Regulation of the heat-shock response in bacteria. *Annu Rev Microbiol* **47**: 321-350.

Yura, T. (1996) Regulation and conservation of the heat-shock transcription factor $\sigma^{32}$. *Genes Cells* **1**: 277-284.

Yura, T., and Nakahigashi, K. (1999) Regulation of the heat-shock response. *Curr Opin Microbiol* **2**: 153-158

Yuzawa, H., Nagai, H., Mori, H., and Yura, T. (1993) Heat induction of $\sigma^{32}$ synthesis mediated by mRNA secondary structure: a primary step of the heat shock response in *Escherichia coli*. *Nucleic Acids Res* **21**: 5449-5455.

Zahn, R., Perrett, S., and Fersht, A.R. (1996) Conformational states bound by the molecular chaperones GroEL and SecB: a hidden unfolding (annealing) activity. *J Mol Biol* **261**: 43-61.

Zeilstra-Ryalls, J., Fayet, O., and Georgopoulos, C. (1991) The universally conserved GroE (Hsp60) chaperonins. *Annu Rev Microbiol* **45**: 301-325.

Zheng, H.Y., Hassett, D.J., Bean, K., and Cohen, M.S. (1992) Regulation of catalase in *Neisseria gonorrhoeae*. Effects of oxidant stress and exposure to human neutrophils. *J Clin Invest* **90**: 1000-1006.

Zhong, G., and Brunham, R.C. (1992) Antibody responses to the chlamydial heat shock proteins hsp60 and hsp70 are H-2 linked. *Infect Immun* **60**: 3143-3149.

Zhou, J., and Spratt, B.G. (1992) Sequence diversity within the *argF*, *fbp* and *recA* genes of natural isolates of *Neisseria meningitidis*: interspecies recombination within the *argF* gene. *Mol Microbiol* **6**: 2135-2146.

Zhou, Y.N., Walter, W.A., and Gross, C.A. (1992) A mutant $\sigma^{32}$ with a small deletion in conserved region 3 of $\sigma$ has reduced affinity for core RNA polymerase. *J Bacteriol* **174**: 5005-5012.

Zhou, Y.N., Kusukawa, N., Erickson, J.W., Gross, C.A., and Yura, T. (1988) Isolation and characterization of *Escherichia coli* mutants that lack the heat shock sigma factor $\sigma^{32}$. *J Bacteriol* **170**: 3640-3649.

Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E., and Hendrickson, W.A. (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**: 1606-1614.

Ziemienowicz, A., Skowyra, D., Zeilstra-Ryalls, J., Fayet, O., Georgopoulos, C., and Zylicz, M. (1993) Both the *Escherichia coli* chaperone systems, GroEL/GroES and DnaK/DnaJ/GrpE, can reactivate heat-treated RNA polymerase. Different mechanisms for the same activity. *J Biol Chem* **268**: 25425-25431.

Zolkiewski, M. (1999) ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. A novel multi-chaperone system from *Escherichia coli*. *J Biol Chem* **274**: 28083-28086.

Zuber, U., and Schumann, W. (1994) CIRCE, a novel heat shock element involved in regulation of heat shock operon *dnaK* of *Bacillus subtilis*. *J Bacteriol* **176**: 1359-1363.

Zugel, U., and Kaufmann, S.H. (1997) Activation of CD8 T cells with specificity for mycobacterial heat shock protein 60 in *Mycobacterium bovis* bacillus Calmette-Guerin-vaccinated mice. *Infect Immun* **65**: 3947-3950.

Zugel, U., and Kaufmann, S.H. (1999) Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clin Microbiol Rev* **12**: 19-39.

Zuker, M. (1989) Computer prediction of RNA structure. *Methods Enzymol* **180**: 262-288.

Zyskind, J.W., Deen, L.T., and Smith, D.W. (1977) Temporal sequence of events during the initiation process in *Escherichia coli* deoxyribonucleic acid replication: roles of the *dnaA* and *dnaC* gene products and ribonucleic acid polymerase. *J Bacteriol* **129**: 1466-1475.

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# The pathogenic neisseriae contain an inactive *rpoN* gene and do not utilize the *pilE* $\sigma^{54}$ promoter

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# The pathogenic neisseriae contain an inactive *rpoN* gene and do not utilize the *pilE* $\sigma^{54}$ promoter

Lina Laskos [a], Joseph P. Dillard [b], H. Steven Seifert [b], Janet A.M. Fyfe [a], John K. Davies [a,*]

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**Abstract**

The $\sigma^{54}$ promoter (*P3*) upstream of the *pilE* gene in *Neisseria gonorrhoeae* was shown to be non-functional by transcriptional analysis of a *PpilE::lacZ* fusion containing only *P3*. A region on the chromosome of *N. gonorrhoeae* strain MS11-A was identified that potentially encodes a protein with a significant similarity to the *Escherichia coli* RpoN protein. However, this region (designated *RLS* for *rpoN*-like sequence) does not contain a single open reading frame (ORF) capable of encoding a functional RpoN protein. It appears that *RLS* may have arisen from an ancestral *rpoN* homologue that underwent a deletion removing the sequence encoding the essential helix–turn–helix (HTH) motif, and changing the subsequent reading frame. An *RLS* has been identified in several strains of *N. gonorrhoeae* and *N. meningitidis*. A 90-kDa gonococcal protein has previously been shown to react with a monoclonal antibody raised against the RpoN from *Salmonella typhimurium*. However, mutagenesis and Western blot analysis confirmed that the gene encoding this protein is not contained within *RLS*. © 1998 Elsevier Science B.V.

*Keywords:* RpoN; *RLS*; Pili; Gonococcus

## 1. Introduction

Type 4 pili synthesized by the pathogenic neisserial species, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, have been shown to be essential for the initial attachment to human epithelial and endothelial cells (McGee et al., 1981; Virji et al., 1992). Since the original cloning and sequencing of the gene encoding the *N. gonorrhoeae* pilin subunit, *pilE*, there has been considerable interest in the transcriptional regulation of this gene. Upstream of the major *pilE* transcription start site are two overlapping consensus promoter sequences, one for a $\sigma^{70}$ promoter (*P1*), and the other for a $\sigma^{54}$-dependent promoter (*P3*) (Fyfe et al., 1995). An additional $\sigma^{70}$ promoter (*P2*) is located downstream of these two promoters. Genes transcribed from $\sigma^{70}$ promoters may or may not be subject to regulation, whereas transcription from $\sigma^{54}$-dependent promoters absolutely requires both the alternative $\sigma$ factor (RpoN) and an activator protein, which generally binds to an upstream activator site (*UAS*) (Merrick, 1993). On the basis of results obtained from the transcriptional analysis of the *Pseudomonas aeruginosa pilA* gene (Ishimoto and Lory, 1989), it was reported that transcription of *pilE* in *N. gonorrhoeae* was $\sigma^{54}$-dependent (Thony and Hennecke, 1989). When expressed in an *Escherichia coli* strain containing an intact *rpoN* gene, but lacking an appropriate activator protein, transcription from the $\sigma^{70}$ promoter *P1* is repressed by the competitive binding of RpoN to the overlapping promoter sequence (Boyle-Vavra et al., 1993; Fyfe et al., 1995). Transcription from the $\sigma^{54}$ promoter can take place in *E. coli* in the presence of the cloned *nifA* gene (Boyle-Vavra et al., 1993), and also in *P. aeruginosa* where PilR (the activator protein required for the $\sigma^{54}$-dependent transcription of the *pilA* gene), is able to bind to a sequence approximately 100 nt upstream of the *pilE* promoter (Fyfe et al., 1995; Carrick et al., 1997). Klimpel et al. (1989) identified a 90-kDa gonococcal protein which co-purified with RNA polymerase, and reacted with a monoclonal antibody raised

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Abbreviations: aa, amino acid(s); Ab, antibody(ies): $\beta$Gal, $\beta$-galactosidase; HTH, helix–turn–helix; nt, nucleotide (s): oligo, oligodeoxyribonucleotide; ORF, open reading frame; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; $^R$, resistant; *RLS*, *rpoN*-like sequence; u, units (s); *UAS*, upstream activator site (s).


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against the *Salmonella typhimurium* RpoN. However, it was later shown that *pilE* transcription in *N. gonorrhoeae* strain MS11-A, at least when grown in vitro, was independent of the $\sigma^{54}$ promoter. Site-directed mutagenesis of the −24 box had no effect on the expression of a *PpilE::cat* transcriptional fusion, when compared with a similar construct with all the promoters intact (Fyfe et al., 1995). This observation suggested that under these growth conditions, the putative gonococcal RpoN was either not produced, or was unable to bind to the promoter.

In this study, we report evidence that the pathogenic neisseria do not produce a functional RpoN. A transcriptional fusion of the *pilE* promoter to *lacZ*, with both the $\sigma^{70}$ promoters (*P1* and *P2*) mutated and only the $\sigma^{54}$ promoter (*P3*) intact, has no detectable transcriptional activity in *N. gonorrhoeae*. We identified a nt sequence in several isolates of *N. gonorrhoeae* and *N. meningitidis* that shares a sequence similarity with the *rpoN* gene from *E. coli*, but it encodes a deleted version. This *rpoN*-like sequence (*RLS*) contains a large deletion incorporating the region that would encode the helix–turn–helix (HTH) motif, a region essential for promoter recognition, and subsequently alters the reading frame.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The media and culture conditions used have been described previously (Fyfe et al., 1995).

### 2.2. DNA techniques

Methods for DNA manipulation, PCR and nt sequencing have been described previously (Fyfe et al., 1995). The oligo primers used in this study were as follows: 4525 (5′ GACGAGCAGGGCTACCTG), 4877 (5′ TCGTCCACGCCCAAGTCC), 4878 (5′ TTATTCTGCGGTTTTGCG), 4941 (5′ TTGTTCATGGTCAGATGG), 102 (5′ TTAACGCGTGAATTCAAAAAT), and TN3RU (5′ GGATTCCCCCTTAACG). To create a size-fractionated plasmid library, total genomic DNA was digested with *Hin*cII and electrophoresed on an agarose gel. Fragments from the 4.5–6.5-kb fraction were purified using the BIO101 Geneclean Kit (Bresatec, Australia) and ligated into the plasmid pSU2718. After transformation into *E. coli* DH12S, the library was screened by colony hybridization to identify clones containing the desired DNA fragment. *N. gonorrhoeae* strains containing mutated *pilE* promoter constructs fused to *lacZ* were obtained by initially replacing the *P3* −24 box, the *P1* −10 box and the *P2* −10 box with restriction sites as previously described (Fyfe et al., 1995). Despite overlapping of the *P1* and *P3* promoters, the *P3* −24 box mutation did not alter the *P1* −35 box. The fragments containing the mutated promoters were then subcloned into a vector containing a gonococcal uptake sequence, and the resulting plasmids were transformed into strain MS11CmLac2, which contains a *PpilE*::mTn*CmlacZ* fusion (Boyle-Vavra and Seifert, 1993), and plated on to media containing XGal. White colonies were obtained at a frequency of 0.3–1%, and mutations were confirmed by amplifying the promoter region by PCR using primers 102 and TN3RU. The resulting fragments were digested with restriction enzymes specific for the mutated sequences. Southern blotting and nt sequencing were used to confirm the constructions.

### 2.3. Hybridization analysis

Southern hybridizations and colony hybridizations were performed using Hybond N nylon membranes (Amersham Corp.). Labeling of probes and detection of DNA were achieved using the Digoxigenin Nonradioactive DNA Labeling and Detection System (Boehringer Mannheim) according to the protocol supplied by the manufacturer.

### 2.4. Western blot analysis

*E. coli* and *P. aeruginosa* cells were grown in liquid media and *N. gonorrhoeae* cells were scraped from a plate. Proteins from these samples were solubilized in sample buffer (0.2 M Tris, 20% glycerol, 2.5% SDS, 10% β-mercaptoethanol, 0.1% Bromophenol Blue; pH 6.8) and electrophoresed at 200 V by SDS–PAGE. Transference of proteins to nitrocellulose membranes (Schleicher and Scheull) was done electrophoretically. Following transfer, membranes were blocked with 5% skim milk in Tris-buffered saline (0.05 M Tris, 0.15 M NaCl; pH 7.4) overnight and probed with a mouse monoclonal Ab, 6RN3 (diluted 1:4000), raised against RpoN from *S. typhimurium* (Klimpel et al., 1989). Following incubation with horse-radish peroxidase-conjugated anti-mouse secondary Ab (Silenus), the membranes were detected using the ECL detection kit (Amersham) as described in the manufacturer's instructions.

### 2.5. β-galactosidase assays

Cultures were grown in GCB liquid medium to the early stationary phase. Cultures (1 ml) were centrifuged (18 000 × *g* for 30 s) and resuspended in 1 ml Z-buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$). The cells were lysed by sonication (3 × 10 s, 0°C), and cell debris was removed by centrifugation (18 000 × *g* for 15 min). β-

Table 1
Bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristic | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH12S | f80d*lacZ*ΔM15 *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) *araD*139 Δ(*ara, leu*)7697 Δ*lacX*74 *galU galK rpsL deoR nupG recA*1/F′ *proAB*$^+$ *lacI*$^q$ ZΔM15 | *Gibco, BRL* |
| *YMC10* | *thi endA hsr* Δ*lacU*169 *hutC*$_K$ | *Reitzer and Magasanik (1986)* |
| *TH1* | Δ*lacU*169 *thi-1 endA supE*44 *hsdR*17 Δ*glnF* | de Bruijn and Ausubel (1983) |
| *P. aeruginosa* | | |
| PAK | wt | Bradley, Memorial University of Newfoundland |
| PAK-N1 | PAK mutant with Tn*5G* insertion in *rpoN* locus | Ishimoto and Lory (1989) |
| *N. gonorrhoeae* | | |
| MS11-A | Derivative of MS11 | Segal et al. (1985) |
| MS11CmLac2 | *pilE2*::mTn*CmLac* | Boyle-Vavra and Seifert (1993) |
| JD1504 | pJD1124 X MS11CmLac2 (*P1*$^-$*P2*$^-$*P3*$^+$) | This study |
| JD1516 | pJD1147 X MS11CmLac2 (*P1*$^-$*P2*$^-$*P3*$^-$) | This study |
| JKD328 | Clinical isolate | Microbiological Diagnostic Unit (MDU), University of Melbourne |
| JKD331 | Clinical isolate | MDU, University of Melbourne |
| JKD334 | Clinical isolate | MDU, University of Melbourne |
| JKD457 | Cm$^R$; *RLS* mutant of MS11-A | This study |
| *N. meningitidis* | | |
| 58¢2 | Clinical isolate | Carrick et al. (1997) |
| Z5498 | Derivative of C75 | Sarkari et al. (1994) |
| Plasmids | | |
| pSU2718 | Cm$^R$ cloning vector | Martinez et al. (1988) |
| pUP1 | Km$^R$ cloning vector containing the gonococcal uptake sequence | Elkins et al. (1991) |
| pJD1124 | 0.6-kb *Eco*RI fragment of pJKD1305 inserted into *Eco*RI site of pUP1, (*P1*$^-$*P2*$^-$*P3*$^+$) | This study |
| pJD1147 | 1.2-kb *Bam*HI of fragment of pJKD1836 cloned in *Bam*HI site pUP1, (*P1*$^-$*P2*$^-$*P3*$^-$) | This study |
| pJKD1305 | Mutated *PpilE::cat* (*P1*$^-$*P2*$^-$*P3*$^+$) in pGEM3z | This study |
| pJKD1836 | Mutated *PpilE::cat* (*P1*$^-$*P2*$^-$*P3*$^-$) in pGEM3z | This study |
| pJKD2026 | 5.5-kb *Hin*cII genomic DNA fragment from *N. gonorrhoeae*, containing *RLS*, inserted into *Hin*cII site of pSU2718 | This study |

Gal activity was measured spectrophotometrically using *o*-nitrophenyl β-D-galactoside and was standardized to total protein as determined using the BCA assay (Pierce).

## 3. Results

### 3.1. Construction and transcriptional analysis of PpilE::lacZ fusions containing mutated promoters

Previous studies have used transcriptional fusions of the *pilE* promoter to *cat* to examine *pilE* transcription. However, constructs lacking the major $\sigma^{70}$ promoter *P1* gave no Cm$^R$ transformants of *N. gonorrhoeae*. This result suggested that *P1* was necessary for *pilE* transcription, but provided no way to measure *pilE* transcription directly from the potential $\sigma^{54}$ promoter in *N. gonorrhoeae*. To circumvent this difficulty, promoter constructs containing an intact $\sigma^{54}$ promoter (*P3*) or no intact promoter sequences were subcloned into a vector containing a gonococcal uptake sequence and were transformed into a *N. gonorrhoeae* strain containing a *PpilE::lacZ* fusion. The *lacZ* fusion allowed us to screen for transformants that incorporated the mutated promoters, and to quantitate transcription from the *pilE* promoters by assaying β-Gal activity. The β-Gal activity in the strain with wt promoters was more than 750 times greater than that in the strain carrying only the $\sigma^{54}$ dependent promoter (Table 2). The strain containing no promoters gave a similar level of β-Gal activity to the strain with the $\sigma^{54}$ promoter, indicating that no transcription was emanating from the $\sigma^{54}$ promoter.

Table 2
Specific activities of β-Gal synthesized from various *PpilE::lacZ* fusions

| Strain | Promoter status | β-Gal activity[a] |
|---|---|---|
| MS11CmLac2 | wt | 2160±130 |
| JD1504 | $\sigma^{54}$ only | 2.6±0.1 |
| JD1516 | Promoterless | 3.1±0.2 |

[a]Means of triplicates ± standard deviations in u/mg protein.

### 3.2. Failure to identify an rpoN homologue by hybridization or complementation

Initially, attempts were made to identify a *N. gonorrhoeae rpoN* homologue from strain MS11-A using a range of standard techniques. For example, MS11-A genomic DNA was digested with a variety of restriction enzymes and probed with the *rpoN* genes from *E. coli*, *P. aeruginosa* and *Acinetobacter calcoaceticus*, in Southern hybridizations at low stringency. No hybridizing fragments were identified using any of these probes (data not shown). In addition, a gonococcal gene library was constructed using the *E. coli–P. aeruginosa* shuttle vector pUCP18, and screened in a *P. aeruginosa rpoN* mutant (PAK-N1), selecting for the ability to grow in the absence of glutamine, as glutamine auxotrophy is associated with the *rpoN* mutation (Totten et al., 1990). This approach was also unsuccessful, as no clones capable of complementing this aspect of the *P. aeruginosa rpoN* mutation were obtained. A separate library of *N. gonorrhoeae* chromosomal DNA was used to transform an *E. coli rpoN* mutant (TH1), and transformants were selected for growth in the absence of glutamine. These experiments led to the identification of the *N. gonorrhoeae* glutamine synthetase gene, *glnA* (GenBank Accession Number M4113), but did not identify an *rpoN* homologue.

### 3.3. Identification of an RpoN-like sequence (RLS) from N. gonorrhoeae strain FA1090

Subsequently, BLAST analysis of genomic sequence data from *N. gonorrhoeae* strain FA1090 released on the University of Oklahoma *N. gonorrhoeae* Genome Database (B.A. Roe, S. Clifton, D.W. Dyer; http://www.genome.ou.edu), revealed a region on contig 274 (26 April 1997 update), which, when translated, displayed significant similarity (32% identity, excluding deleted regions) to the *E. coli* RpoN aa sequence (Jones et al., 1994). In particular, a nt sequence potentially encoding the 10-aa RpoN box, close to the C-terminal end of the protein was identified. This motif plays a role in recognition of the −24/−12 promoter consensus and is believed to be diagnostic of RpoN proteins, as database searches have failed to identify it in any other proteins (Merrick, 1993). Translation of the sequence further upstream revealed a similarity to the N-terminal region of the *E. coli* RpoN. However, this sequence was in a different reading frame, suggesting that the ORF might not be capable of encoding an intact *rpoN* homologue. Consequently, this 914-bp region within contig 274 was designated the *rpoN*-like sequence (*RLS*). To determine whether the apparent reading frame shift was potentially due to an error in the FA1090 sequence deposited in the database, it was decided to clone and characterize the corresponding region from the genome of strain MS11-A.

### 3.4. Cloning and nt sequence of RLS from N. gonorrhoeae strain MS11-A

To obtain an *RLS*-specific probe, oligo primers 4525 and 4878 (Fig. 1) were designed, based on the *RLS* sequence of strain FA1090. These were used to amplify a 506-bp fragment within *RLS* from genomic DNA of strain MS11-A. The nt sequence of this PCR fragment was verified by direct sequencing of the product. The fragment was then used to probe genomic DNA from MS11-A, completely digested with *Hin*cII. The *RLS*-specific probe hybridized strongly to a single 5.5-kb fragment under conditions of high stringency. This hybridizing fragment was subsequently cloned and identified from a size-fractionated, pSU2718 derived, gene library. The clone retained for further study was designated pJKD2026. Restriction analysis, combined with preliminary sequencing of the 5.5-kb insert, succeeded in locating the *RLS* as shown in Fig. 1. The nt sequence was obtained for both strands of a 1.8-kb region within the insert of pJKD2026 (Fig. 1). When compared with the corresponding sequence from FA1090, the MS11-A-derived sequence was highly similar, in particular within the *RLS*, where only three base substitutions were noted (data not shown). In addition, as observed for FA1090, the putative *RLS* start codon from MS11-A was located 286 nt from the start of the divergently transcribed *kat* gene. Downstream of *RLS* is a 270-bp region containing seven copies of the direct and inverted repeats, previously designated *RS3* (Haas and Meyer, 1986). Directly downstream of the *RS3* elements, the 3′ end of an ORF was identified that shares a significant homology with the corresponding region of the *rfpB* determinant of *Shigella dysenteriae*.

The RpoN protein from *E.coli* contains 477 aa. Based on comparisons with the deduced aa sequences of 16 other *rpoN* genes, three distinct regions of the protein have been identified (Merrick, 1993). Region III contains two important motifs, a potential HTH motif, and the RpoN box (Merrick, 1993). Both of these motifs have been implicated in promoter recognition and DNA binding (Merrick and Chambers, 1992).

The 1.8-kb sequence from MS11-A contained no single ORF, in any of the three reading frames, capable of encoding an intact *rpoN* gene. A similarity was observed between the deduced aa sequence of *RLS* translated in frame 2, and the *E. coli* RpoN protein, within Region I, Region II, and the N-terminal portion of Region III (Fig. 2). A region encoding the RpoN box was identified, as was observed for the FA1090 sequence, but this region was present in a different reading frame (frame 1). No sequence encoding the essential HTH motif was identified within *RLS*. These

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f strain MS11-A.

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*11-A*

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and the N-terminal portion
region encoding the RpoN
observed for the FA1090
was present in a different
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dentified within *RLS*. These

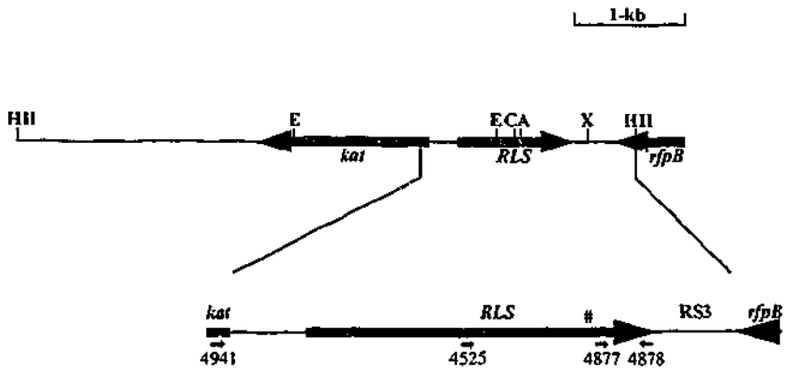


Fig. 1. Physical map of the 5.5-kb insert of pJKD2026 showing the position and orientation of *RLS* and the flanking *kat* (Genbank Accession Number U35457) and *rfpB* (Genbank Accession Number S73325) genes (thick arrows). The enlarged section depicts the 1.8-kb region sequenced in this study. Oligo primers are indicated by thin arrows. The hash (#) denotes the region of *RLS* where the frameshift occurs. RS3 denotes the location of the RS3 repeats. The position of relevant restriction enzyme sites are shown. Abbreviations: HII, *Hin*cII; E, *Eco*RI; C, *Cla*I; A, *Ava*II; X, *Xba*I.

```
Ng:  --MTLCGIFLRRQAENNNFYRIIITLIGIKLKQTQQLDQRLQQSLRVLQMPGIELEREVE  58
Ec:  MKQGLQLRLSQQLAMTPQLQQAIRLLQLSTLELQQELQQALESNPLLEQIDTHEEIDTRE  60

Ng:  NWPS----DNPLLERKETDE--FSDA-EFSHYTA-PARQIGGDEGEDMLSNIAGE--EDF  108
Ec:  TQDSETLDTADALEQKEMPEELPLDASWDTIYTAGTPSGTSGDYIDDELPVYQGETTQTL  120

Ng:  KQYLHAQACEHPLSDQEAACVHILIDFLDEQGYLTDSIEDILDHTPLEWMLD---EAMLK  165
Ec:  QDYLMWQVELTPFSDTDRAIATSIVDAVDETGYLTVPLEDILESIGDEEIDIDEVEAVLK  180

Ng:  QALTALKKFDPAGMAAADVTESLILQIERSGE--CAAKPAALHIVRNALDSIDGNRSQTP  223
Ec:  RIQR----FDPVGVAAKDLRDCLLIQLSQFDKTTPWLEEARLII-SDHLDLLANHDFRTL  235

Ng:  ARI--------KNACPKPTAAHSKPHSASLLRSTPFPLP----------------------  254
Ec:  MRVTRLKEDVLKEAVNLIQSLDPRPGQSIQTGEPEYVIPDVLVRKHNGHWTVELNSDSIP  295

Ng:  ------------------------------------------------------------  254
Ec:  RLQINQHYASHCNNARNDGDSQFIRSNLQDAKWLIKSLESRNDTLLRVSRCIVEQQQAFF  355

                                                                  #
Ng:  ------------------------------------------------------GFA  257
Ec:  EQGEEYMKPMVLADIAQAVEMHESTISRVTTQKYLHSPRGIFELKYFFSSHVNTEGGGEA  415
                HTH                                             * *

Ng:  SST----------------PKSYSDEALANLLAFRGIEVSRRTVAKYRESLEIPAAHKRKT  302
Ec:  SSTAIRALVKKLIAAENPAKPLSDSKLTSLLSEQGIMVARRTVAKYREELSIPPSNQRKQ  475
     ***                 * ** *. **. .** *.********** ** ...**
                                      RpoN box

Ng:  AE  304
Ec:  LV  477
```

Fig. 2. Comparison between the derived aa sequences of *RLS* from *N. gonorrhoeae* (Ng) and RpoN from *E. coli* (Ec) (Jones et al., 1994) performed with the aid of CLUSTALW. Identical aa are indicated by an asterisk, and conserved aa are indicated by dots. Dashes indicate gaps that have been introduced to maximize alignment. The hash (#) denotes the position of the change of frame of translation of *RLS*. The HTH motif (Merrick and Chambers, 1992) and RpoN box (Merrick, 1993) are boxed.

results suggest that a deletion occurred in the ancestral *rpoN* homologue, resulting in a frame-shift mutation, and rendering the gene incapable of encoding a functional RpoN protein.

### 3.5. *A comparison of* RLS *between different* N. gonorrhoeae *and* N. meningitidis *strains*

The genomes of *N. gonorrhoeae* strains FA1090 and MS11-A have been shown to have *RLS* containing an apparent deletion of a region thought to encode a HTH motif. To determine whether this characteristic was shared by other gonococcal strains, and *N. meningitidis*, genomic DNA was prepared from three clinical isolates of *N. gonorrhoeae*, strains JKD328, JKD331 and JKD334, and two previously described strains of *N. meningitidis*, strains 58∉2 and Z5498 (Table 1). These DNA preparations were used as templates in PCR reactions using the oligo primers 4878 and 4941 (Fig. 1). In each case, the expected 1.5-kb fragment was amplified,

and direct sequencing of the 3′ end of each fragment using primer 4878 verified that the same apparent deletion was present in all these gonococcal and meningococcal strains (data not shown). In addition, this result indicates that the location of RLS, adjacent to *kat*, is conserved in both *N. gonorrhoeae* and *N. meningitidis*.

### 3.6. *There is only a single copy of* RLS *in* N. gonorrhoeae *and* N. meningitidis

It remained possible that in addition to *RLS*, an intact copy of an *rpoN* homologue was present on a 5.5-kb *Hin*cII fragment in the gonococcal MS11-A chromosome, and that the sequence was not yet in the database for FA1090. If an intact copy of *rpoN* did exist, then it should contain the sequence encoding the RpoN box. To resolve this question, genomic DNA from strain MS11-A and *N. meningitidis* strain 58¢2, was digested with a range of restriction enzymes, and probed with a 150-bp fragment containing the 3′ end of *RLS* (amplified using primers 4877 and 4878), under high stringency conditions. As shown in Fig. 3, single hybridizing fragments were obtained for all digests, suggesting that it is unlikely that a second, intact copy of *rpoN* is present in the genomes of either *N. gonorrhoeae* or *N. meningitidis*.

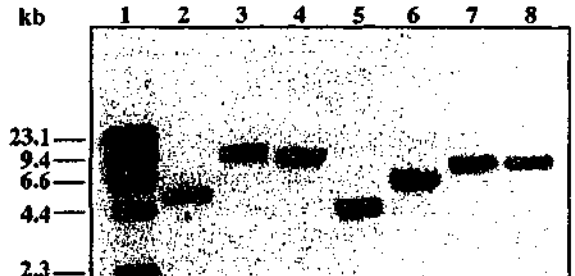


Fig. 3. Southern hybridization analysis of total genomic DNA from *N. gonorrhoeae* strain MS11-A digested with *Hin*cII (lane 2), *Ava*I (lane 3), *Cla*I (lane 4), *Dra*I (5), and *N. meningitidis* strain 58¢2 digested with *Ava*I (lane 6), *Cla*I (lane 7) and *Dra*I (lane 8). The membrane was probed with the 3′ region of *RLS* amplified with oligo primers 4877 and 4878. Molecular weight markers, a phage λ *Hin*dIII digest, are indicated in lane 1.

### 3.7. RLS *is not associated with the 90-kDa gonococcal protein that reacts with an anti-RpoN monoclonal Ab*

It has been shown previously that a monoclonal Ab raised against purified RpoN protein from *S. typhimurium* appears to react with a 90-kDa gonococcal protein, and that this protein co-purifies with RNA polymerase (Klimpel et al., 1989). However, the largest ORF within *RLS*, potentially encoding the N-terminal portion of an RpoN-like protein, is only 834 bp and would be expected to encode a polypeptide of approximately 30 kDa. To determine whether this ORF encodes the protein that reacts with the anti-RpoN monoclonal, a gonococcal mutant derivative (JKD457) was constructed, in which the 53-bp *Ava*II–*Cla*I fragment within this ORF (see Fig. 1) was replaced by a 1.1-kb cassette containing the *N. gonorrhoeae recA* promoter fused to a promoterless *cat* gene (Black et al., 1995), and confirmed by Southern hybridization (data not shown). Western blot analysis was performed on whole-cell extracts prepared from *N. gonorrhoeae* strains MS11-A and JKD457, using the anti-RpoN monoclonal Ab (Klimpel et al., 1989). Cell extracts prepared from *E. coli* strain YMC10 and *P. aeruginosa* strain PAK were included as positive controls. As shown in Fig. 4, both gonococcal strains produce a 90-kDa protein that reacts with the Ab, suggesting that this protein is not encoded by the ORF within *RLS*.

## 4. Discussion

We have reported previously that the *N. gonorrhoeae pilE* gene has a potentially functional $\sigma^{54}$ promoter (*P3*)

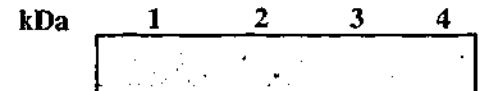


Fig. 4. Western blot analysis of whole-cell extracts from *N. gonorrhoeae* strains MS11-A (lane 1) and JKD457 (lane 2), *E. coli* strain YMC10 (lane 3) and *P. aeruginosa* strain PAK (lane 4), fractionated on a SDS 12% PAGE gel. The membrane was incubated with the monoclonal Ab 6RN3 (Klimpel et al., 1989).

-kDa gonococcal
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t al., 1989). Cell
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l strains produce a
Ab, suggesting that
RF within *RLS*.

the *N. gonorrhoeae*
$\sigma^{54}$ promoter (*P3*)

3 4

ts from *N. gonorrhoeae*
*E. coli* strain YMC10
fractionated on a SDS
d with the monoclonal

that binds RpoN in *E. coli*, and can direct transcription in *E. coli* or *P. aeruginosa* when the appropriate activator is present (Boyle-Vavra et al., 1993; Fyfe et al., 1995; Carrick et al., 1997). However, mutagenesis of this promoter had no effect on *pilE* transcription in *N. gonorrhoeae*. Now we have shown that no transcription is initiated from the region upstream of *pilE* if the $\sigma^{54}$ promoter is the only promoter present. These results suggest that a gonococcal RpoN protein is either not present or is non-functional under in vitro growth conditions. Putative $\sigma^{54}$ promoters have been reported upstream of other *N. gonorrhoeae* genes, including *pilC* (Taha et al., 1996), *comA* (Facius and Meyer, 1993) and *pip* (Albertson and Koomey, 1993), but we have not been able to detect transcription from these promoters in a gonococcal background (unpublished observations).

This study confirms that the *pilE P3* promoter is inactive in *N. gonorrhoeae* and suggests that the reason for this is the lack of a functional *rpoN* gene. Identification of *RLS* on the chromosome of gonococcal strain MS11-A, followed by a comparison of the deduced aa sequence with the *E. coli* RpoN protein, suggests that a major deletion involving the sequence encoding the essential HTH motif has occurred in an ancestral *rpoN* gene. This deletion would have rendered the associated mutant σ factor unable to bind DNA (Guo and Gralla, 1997).

The possibility that *N. gonorrhoeae* and *N. meningitidis* genomes contain an additional, intact copy of *rpoN* is unlikely based on the Southern hybridization results presented in Fig. 3. Assuming that an intact *rpoN* gene would at least contain a sequence encoding the highly conserved RpoN box, a probe was generated to hybridize to such a sequence, based on the 3′ end of *RLS*. Only single DNA fragments, generated using several restriction enzymes, hybridized to the probe, indicating that *RLS* is the only copy of an *rpoN*-like sequence in *N. gonorrhoeae* strain MS11-A and *N. meningitidis* strain 58¢2.

The mystery remains as to the identity and function of the 90-kDa gonococcal protein that binds the anti-RpoN monoclonal Ab. The presence of this protein, originally observed by Klimpel et al. (1989), was also observed in this study. However, the largest ORF within *RLS* would only be capable of encoding a 30-kDa protein. Interruption of this ORF had no effect on the amount or size of the protein reacting with the anti-RpoN Ab in a Western blot (Fig. 3), confirming that this protein is not encoded by this ORF.

The fact that the *rpoN* deletion is present in the genomes of several *N. gonorrhoeae* and *N. meningitidis* strains indicates that a functional gene may have been present in an ancestor of the pathogenic neisseria. Perhaps the loss of *rpoN* is associated with the evolution of these species as obligate human colonizers. Interestingly, complete sequencing of the genome of *Haemophilus influenzae* (Fleischman et al., 1995), another obligate human pathogen, failed to reveal an *rpoN* homologue. RpoN-associated transcriptional regulation is very responsive to environmental conditions, and it may be that this type of regulation is no longer essential in this particular ecological niche.

## References


Albertson, N.H., Koomey, M., 1993. Molecular cloning and characterization of a proline iminopeptidase gene from *Neisseria gonorrhoeae*. Mol. Microbiol. 9, 1203–1211.

Black, C.G., Fyfe, J.A.M., Davies, J.K., 1995. A promoter associated with the neisserial repeat (NR) can be used to transcribe the *uvrB* gene from *Neisseria gonorrhoeae*. J. Bacteriol. 177, 1952–1958.

Boyle-Vavra, S., Seifert, H.S., 1993. Shuttle mutagenesis: two mini-transposons for gene mapping and for *lacZ* transcriptional fusions in *Neisseria gonorrhoeae*. Gene 129, 51–57.

Boyle-Vavra, S., So, M., Seifert, H.S., 1993. Transcriptional control of gonococcal *pilE* expression: involvement of an alternative sigma factor. Gene 137, 233–236.

Carrick, C.S., Fyfe, J.A.M., Davies, J.K., 1997. The normally silent $\sigma^{54}$ promoters upstream of the *pilE* genes of both *Neisseria gonorrhoeae* and *Neisseria meningitidis* are functional when transferred to *Pseudomonas aeruginosa*. Gene 198, 89–97.

de Bruijn, F.J., Ausubel, F.M., 1983. The cloning and characterization of the *glnF* (*ntrA*) gene of *Klebsiella pneumoniae*: role of *glnF* (*ntrA*) in the regulation of nitrogen fixation (*nif*) and other nitrogen assimilation genes. Mol. Gen. Genet. 192, 342–353.

Elkins, C., Thomas, C.E., Seifert, H.S., Sparling, P.F., 1991. Species-specific uptake of DNA is mediated by a 10-base-pair sequence. J. Bacteriol. 173, 3911–3913.

Facius, D., Meyer, T.F., 1993. A novel determinant (*comA*) essential for natural transformation competence in *Neisseria gonorrhoeae* and the effect of a *comA* defect on pilin variation. Mol. Microbiol. 10, 699–712.

Fleischman, R.D. et al., 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269, 449–604.

Fyfe, J.A.M., Carrick, C.S., Davies, J.K., 1995. The *pilE* gene of *Neisseria gonorrhoeae* MS11 is transcribed from a $\sigma^{70}$ promoter during growth in vitro. J. Bacteriol. 177, 3781–3787.

Guo, Y., Gralla, J.D., 1997. DNA-binding determinants of sigma 54 as deduced from libraries of mutations. J. Bacteriol. 179, 1239–1245.

Haas, R., Meyer, T.F., 1986. The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. Cell 44, 107–115.

Ishimoto, K.S., Lory, S., 1989. Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (RpoN) of RNA polymerase. Proc. Natl. Acad. Sci. USA 86, 1954–1957.

Jones, D.H., Franklin, F.C., Thomas, C.M., 1994. Molecular analysis of the operon which encodes the RNA polymerase sigma factor sigma 54 of *Escherichia coli*. Microbiology 140, 1035–1043.

Klimpel, K.W., Lesley, S.A., Clarke, V.L., 1989. Identification of subunits of gonococcal RNA polymerase by immunoblot analysis: evidence for multiple sigma factors. J. Bacteriol. 171, 3713–3718.

McGee, Z.A., Johnson, A.P., Taylor-Robinson, D., 1981. Pathogenic mechanisms of *Neisseria gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or type 4. J. Infect. Dis. 143, 413–422.

Martinez, E., Bartolome, B., De La Cruz, F., 1988. pACYC184-derived cloning vectors containing the multiple cloning

site and *lacZa* reporter gene of pUC8/9 and pUC18/19 plasmids. Gene 68, 159–162.

Merrick, M.J., Chambers, S., 1992. The helix-turn-helix motif of $\sigma^{54}$ is involved in recognition of the −13 promoter region. J. Bacteriol. 174, 7221–7226.

Merrick, M.J., 1993. In a class of its own—the RNA polymerase sigma factor $\sigma^{54}$ ($\sigma^{N}$). Mol. Microbiol. 10, 903–909.

Reitzer, L.J., Magasanik, B., 1986. Transcription of *glnA* in *E. coli* is stimulated by activator bound to sites far from the promoter. Cell 45, 785–792.

Sarkari, J., Pandit, N., Moxon, E.R., Achtman, M., 1994. Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. Mol. Microbiol. 13, 207–217.

Segal, E., Billyard, E., So, M., Storzbach, S., Meyer, T.F., 1985. Role of chromosomal rearrangement in *N. gonorrhoeae* pilus phase variation. Cell 40, 293–300.

Taha, M.K., Giorgini, D., Nassif, X., 1996. The *pilA* regulatory gene modulates the pilus-mediated adhesion of *Neisseria meningitidis* by controlling the transcription of *pilC1*. Mol. Microbiol. 19, 1073–1084.

Thony, B., Hennecke, H., 1989. The −24/−12 promoter comes of age. FEMS Microbiol. Rev. 63, 341–358.

Totten, P.A., Lara, J.C., Lory, S., 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. 172, 389–396.

Virji, M., Alexandrescu, C., Fergusson, D.J.P., Saunders, J.R., Moxon, E.R., 1992. Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial cells. Mol. Microbiol. 6, 1271–1279.

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