MONASH UNIVERSITY
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REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
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Sec. Research Graduate School Committee

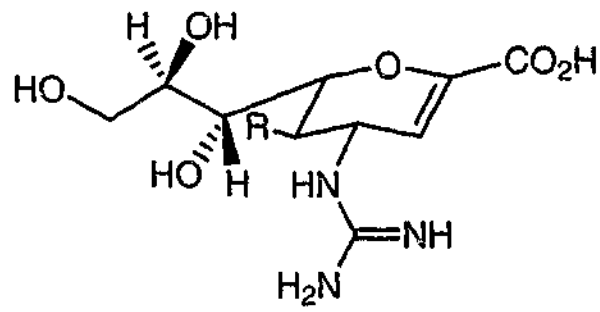
Addendum and Corrigendum

p3 line 21-22, the references to be cited at the end of this sentence are: Jawetz *et al.* 1997 and Forbes *et al.* 1998

p23 line 24 The subtype of the Influenza A virus neuraminidase was N2

p23 paragraph 2, the references to be cited at the end of this paragraph are: Taylor 1996 and Roggentin *et al.* 1993a

p33 Figure 1-11 should be presented as:



R = NHAc

(Zanamivir)

p38 line 28-29 replace "mucin" with "bovine submaxillary mucin"

p38 line 28-29 Comment: bovine submaxillary mucin contains the α(2→6) linkage

p52 line 4, Table 2-1 and Table 4-5 Correction: Fetuin contains both α(2→3) and α(2→6) linkages

p91 Table 4-5 Mixed bovine gangliosides - α(2→8) should read Mixed bovine gangliosides - Mixed linkages

p91 Table 4-5 Colominic acid - Mixed linkages should be Colominic acid - α(2→8)

p91 line 12 "In these experiments, the α(2→8)-linked glycoprotein, colominic acid..." should read "In these experiments, the α(2→8)-linked polysaccharide, colominic acid..."

p91 line 3-4 Following the sentence "The results of the relative hydrolysis rates of the substrates tested are summarised in Table 4-5" insert "The relative hydrolysis rate was determined from the calculated released molar amount of sialic acid."

p91 Table 4-5 Caption, include in the caption, "The relative hydrolysis rate was determined from the calculated molar amount of sialic acid."

p127 line 19 replace "Pshezetsky" with "Pshezhetsky"

A preliminary investigation of a sialidase activity associated with *M. smegmatis*

A thesis submitted for the degree of Doctor of Philosophy

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Statement of Originality

I declare that this thesis is less than one hundred thousand words in length, and has not been submitted in any form for another degree or diploma at any university. The material discussed in this thesis is my own work, and information derived from the literature or unpublished work of others has been acknowledged in the text and a list of references provided.

Carolyn Joy Trower

Abstract

Mycobacteria are a genus of bacteria that are responsible for a wide spectrum of disease in humans and animals worldwide. The most commonly known infection is tuberculosis (TB) caused by *Mycobacterium tuberculosis*. The genus is characterised by the presence of thick waxy cell walls containing many unusual glycoconjugates including glycolipids. Intrinsic resistance to antibiotics and resistance to available agents characterise the species. It is hence important that new agents are created to treat the infections that these organisms cause.

The genome sequence of *M. tuberculosis* H37Rv was recently solved (Cole *et al.* 1998). A sequence known as Rv3463 was determined to have sialidase-like structural characteristics, indicating the existence of a sialidase in *M. tuberculosis*. Sialidases are glycohydrolases that cleave sialic acid residues from glycoproteins, glycolipids and polysaccharides. In bacteria they are utilised in both nutritional and pathogenic roles. Sialidases are also of great interest in therapeutic discovery, demonstrated by the development of the drugs zanamivir and oseltamivir. These drugs are both sialidase inhibitors and are the first commercially available antiviral agents active against both influenza A and B infection. Thus the existence of a sialidase in mycobacteria could prove to be very interesting.

Sequence similarity searches of Rv3463 against a range of known bacterial and viral sialidases revealed no significant match, however it was decided to evaluate *M. smegmatis* mc^2155 and *M. bovis* BCG cell lysates for the presence of a sialidase activity. The cell lysates were found to hydrolyse 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (MUN), indicating the possible presence of an enzyme with sialidase-like activity. The enzyme was then partially purified using an affinity matrix protocol comprising of *N*-acetylneuraminic acid linked to epoxy-activated Sepharose 6B by a thioglycosidic linkage.

The partially purified enzyme was observed to cause hydrolysis of a wide variety of sialoglycoconjugates. It was also shown to hydrolyse both α(2,3)- and α(2,6)-sialyllactose using ^{1}H NMR spectroscopy. The enzyme was found to follow Michaelis-Menten kinetics, determined to have an optimal pH of 5.0 and act optimally at 37 °C. It was activated by bovine serum albumin (BSA) and divalent cations (Ca^{2+}

and Mn^{2+}) and inhibited by 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en) and EDTA. Substrate specificity of the partially pure enzyme against a range of sialoglycoconjugates showed that it preferentially hydrolyses α(2,3)-linkages more rapidly than α(2,6)-linkages.

Interestingly, while *M. smegmatis* lysates exhibited sialidase-like activity, evaluation of *M. tuberculosis* cell lysates showed that this organism did not possess a sialidase-like enzymatic activity. This confirmed that the gene Rv3463 was not the enzyme in question.

The characteristics of the partially purified enzyme from *M. smegmatis* appear to be those of a sialidase. It is hoped that once the sequence is identified its similarity to other known sialidases can be examined and similar sequences found in other mycobacterial species may lead to a new target in the treatment of these resistant pathogens.

Acknowledgments

I would like to extend my sincerest thanks to my supervisors Professor Mark von Itzstein and Professor Ross Coppel for all of their patience, assistance and advice during the course of my PhD. Without their experience and kindness this thesis would cease to exist. I would also like to thank my associate supervisors Dr Gaik Kok, Dr Ossama El Kabbani and Professor Peter Scammells for their presence in the absence of Mark.

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Dr Milton Kiefel, Dr Joe Tiralongo, Mr Pas Florio, Mr Tho van Phan, Dr Periera and Dr Julian Rood must also be thanked for their synthesis of substrates, inhibitors, matrices and supply of enzymes and clones. I must also thank my proofreaders, Dr Samia Abo, Dr Jenny Wilson, Dr Thomas Haselhorst and Ms Connie Darmanin for reading my thesis and correcting my erroneous grammar before I sent it to my supervisors! Thank you to the members of the Department of Medicinal Chemistry, and Department of Pharmacology, VCP and the Centre for Biomolecular Science and Drug Discovery, Griffith University for their friendship and support.

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Publications

Part of this work has been presented at a conference and submitted for journal publication:

i. Trower C.J., Abo S., Billman-Jacobe, H., Wilson, J.C., Coppel, R.L. & von Itzstein M.

A preliminary study of a putative sialic acid-recognising enzyme from Mycobacterium smegmatis.

The International Glyconjugate Organisation Meeting (GLYCO XVI).

The Hague, Netherlands. 2001

ii. Trower C.J., Abo S., Billman-Jacobe, H., Wilson, J.C., Coppel, R.L. & von Itzstein M.

Identification and Characterisation of a sialic acid-recognising enzyme from Mycobacterium Smegmatis mc^2155

Submitted for publication

Abbreviations

A. viscosus	*Actinomyces viscosus*
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
βGal-D-1,3- D-GalNAc	2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactopyranose
BCG	Bacille Calmette Guerin
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
C. perfringens	*Clostridium perfringens*
C. diptheriae	*Clostridium diptheriae*
C. sordelli	*Clostridium sordelli*
Ca	Calcium
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine 5'-monophosphate
CDC	Centre for Disease Control
cm	Centimetre
CMP	Cytidine monophosphate
CNS	Central nervous system
CPM	Counts per minute
Cu	Copper
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
DOT	Directly observed therapy
DTT	Dithiothreitol
E. coli	*Eschericia coli*
EB	Elution buffer
EDTA	Ethylene diaminetetraacetic acid
$FeCl_3$	Ferric chloride
H. parasuis	*Haemophilus parasuis*
h	Hour
HCl	Hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid

His	Histidine
HIV	Human immunodeficiency virus
Hg	Mercury
HRP	Horseradish peroxidase
H_2SO_4	Sulfuric acid
IEF	Isoelectric focusing
INH	Isonicotinic acid hydrazine (isoniazid)
IPTG	Isopropylthio β-D-galactoside
kb	kilobase pairs
KCl	Potassium chloride
kDa	kilo Dalton
KDN	2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; 3-deoxy-D-*glycero*-D-*galacto*-nonulsonic acid; Deaminoneuraminic acid
K_i	Dissociation constant of inhibitor
K_m	Michaelis-Menten constant
kV	kilo Volt
L	Litre
LB	Luria Bertaini
M	Molar
M. smegmatis	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
M. viridifaciens	*Micromonospora viridifaciens*
MAC	*Mycobacterium avium complex*
Ma	Milliamp
MCS	Multiple cloning site
MDR	Multi-drug resistant
MES	2-(*N*-Morpholino) ethane sulfonic acid
mg	Milligram
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MHz	Mega hertz

min	Minute
mL	Millilitre
mM	Millimolar
Mn	Manganese
$MnCl_2$	Manganese chloride
MU	4-Methylumbelliferone
MUN	4-methylumbelliferyl α-D-*N*-acetylneuraminic acid
Mw	Molecular weight
$NaAsO_3$	Sodium arsenite
NaCl	Sodium chloride
$NaIO_4$	Sodium periodate
NAL	*N*-acetyllactosamine
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
Neu5Ac	*N*-acetylneuraminic acid
Neu5Ac2en	2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid
ng	nanogram
nm	nanometre
NTM	Nontuberculous mycobacteria
NMR	Nuclear magnetic resonance
OD	Optical density
P. multocida	*Pasteurella multocida*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PMSF	Phenylmethyl sulfonyl fluoride
PMN	Polymorphonuclear cells
pmol	picomole
PTA	Phosphotungstic acid
RNA	Ribonucleic acid
rpm	Revolutions per minute
S. oralis	*Streptococcus oralis*
S. typhimurium	*Salmonella typhimurium*

s	second
SAR	Structure activity relationship
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	Tuberculosis
TBS	Tris buffered saline
TE	Tris-EDTA buffer
TIGR	The Institute for Genomic Research
U	Units
UK	United Kingdom
USA	United States of America
UV	Ultra violet
μl	microlitre
μm	micrometre
V	Volt
V. cholerae	*Vibrio cholerae*
VIDRL	Victorian Infectious Diseases Reference Laboratory
V_{max}	Maximum velocity (or rate)
v/v	Volume/volume
W	Watts
WEHI	Walter & Eliza Hall Institute
WHO	World Health Organisation
WW	World war
3D	Three dimensional
Ω	Ohms
λ	Wavelength

1 Introduction

1.1 General

Mycobacteria are a genus of bacteria characterised by the presence of thick waxy cell walls containing many unusual glycolipids. Intrinsic resistance to antibiotics and resistance to available agents characterise this species (Jawetz *et al.* 1987; Forbes *et al.* 1998). Mycobacteria are ubiquitous in nature and are responsible for a wide spectrum of disease worldwide (Table 1-1) (Benenson 1990; Griffith *et al.* 1993; Tomashefski *et al.* 1996; 1997; Raju and Schluger 2000; Khoor *et al.* 2001). The most well known organism of this genus is *Mycobacterium tuberculosis*, which is responsible for the pulmonary disease, tuberculosis (TB).

TB is the major cause of death worldwide, associated with a single infectious disease in adults. The majority of deaths due to TB occur in developing countries (Suffys *et al.* 1997). The increasing incidence of TB in industrialised countries such as the United States of America (USA) and the United Kingdom (UK) has been associated with an increase in immigration from developing countries such as Asia (Efferen and Hyman 1996; De Cock and Low 1997; Cowie *et al.* 1998). The significant increase in the incidence of TB over the last few decades has resulted in the World Health Organisation (WHO) declaring in 1993 the TB epidemic a global emergency (Efferen and Hyman 1996; Suffys *et al.* 1997).

M. tuberculosis is not the only infectious mycobacterium. Apart from pulmonary disease, mycobacteria are able to cause systemic blood-borne disease, lymphadenitis, skin ulcers, abscesses, and wound infections in both healthy and immunocompromised individuals (Benenson 1990; Griffith *et al.* 1993; Tomashefski *et al.* 1996; 1997; Raju and Schluger 2000; Khoor *et al.* 2001). Most mycobacterial infections are difficult to treat requiring costly long-term multi-drug therapy or invasive surgical debridement and excision. Full chemotherapeutic compliance is often difficult to achieve, with many patients failing to complete therapies. Non-compliance has exacerbated the difficulty of treating many of these diseases due to the emergence of multi-drug resistant (MDR) organisms, which further complicate treatment.

Prevention of infection is currently the most effective way of dealing with mycobacteria. With respect to TB, the Centre for Disease Control (CDC) guidelines recommend that institutional and health care facilities prevent the spread of nosocomial infection by rapid identification of the mycobacteria, isolation of those infected, and immediate treatment of patients with known or suspected infection (Efferen and Hyman 1996). The CDC also recommends that appropriate environmental and engineering requirements of isolation facilities are used and all staff and visitors use personal protective devices. These preventative measures are costly and only available in the developed world where the epidemic is not as severe. It is thus important to determine more cost effective and accessible options.

The challenge, as Barry III (Barry 1997) states, is to improve the efficacy of current therapies and develop truly short course chemotherapeutic regimes which will encourage patient compliance and decrease the development of resistance. New chemotherapeutics with novel targets need to be developed. A successful example of this is the development of zanamivir to treat the influenza virus.

Zanamivir is a sialidase inhibitor which has become the first commercially available antiviral active against both influenza A and B (MIST Group 1998). Previously available therapies for the treatment of influenza; amantadine and rimantadine were only active against influenza A (MIST Group 1998), which later acquired resistance to them (Van Voris and Newell 1992). Resistance to zanamivir is thought to be less likely because key amino acid residues in the active site of the sialidase are highly conserved (von Itzstein *et al.* 1993).

Examination of the *M. tuberculosis* H37Rv genome revealed the presence of a possible sialidase (Cole *et al.* 1998). The highly conserved active site of the sialidases, and the fact that sialidase inhibitors are already being used as chemotherapeutics made this enzyme an attractive candidate for examination. If a sialidase exists in mycobacteria and it is determined to be essential for pathogenesis in the species it may provide a valuable chemotherapeutic target.

1.2 Mycobacteria

1.2.1 History of Tuberculosis

Mycobacteria are ancient pathogens. It is believed that TB was recorded as far back as 3,000 years ago as the cause of death in Egyptians (1996). *M. tuberculosis* was first isolated and identified in 1882 by Koch (Koch, 1882). Serious epidemics occurred during the 18th and 19th Centuries in Europe and the UK due to changes in the demographic patterns caused by the industrial revolution. Large populations of malnourished factory workers living in crowded, unsanitary conditions concentrated in most major industrial cities. These "slums" caused TB to spread rapidly with transmission to the upper classes and rural areas. The disease was so feared it was named the "white plague". Eventually a decrease in over-crowding and poverty caused a decrease in incidence (Salyers and Whitt 1994).

Notably, a resurgence of the disease occurred during World War II (WWII), again due to malnourishment and over-crowding. It was particularly prevalent in concentration camps but disappeared rapidly once the war ended.

Recently, an increase of TB infection rates has occurred, especially in the USA amongst those populations occupying the inner city slums, homeless centres and prisons. The increasing incidence of TB is again due to over-crowding, malnourishment (prevalently caused by alcoholism, smoking and drug abuse) and immune suppression (caused by HIV and AIDS) (Salyers and Whitt 1994). Unfortunately, the incidence of other pathogenic mycobacterial infection is also increasing.

1.2.2 The Morphology and Epidemiology of Mycobacteria

TB is caused by *M. tuberculosis*, a member of the genus *Mycobacterium*. This genus is the only one classed in the *Mycobacteriaceae* family, which belong to the *Actinomycetes* class. Mycobacteria are thin, rod-shaped (0.2-0.4 x 2-10 μm), non-motile bacteria. They are obligate aerobes and non-sporeforming (Figure 1-1) (Jawetz *et al.* 1987; Heifets 1997; Forbes *et al.* 1998).

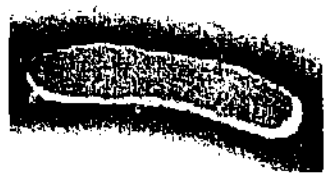


Figure 1-1: Electron micrograph of *M. tuberculosis*

Mycobacteria are known as "acid-fast" bacilli due to their unusual cell wall structure that contains *N*-glycolylmuramic acid and many lipids (Figure 1-2) (Jawetz *et al.* 1987; Forbes *et al.* 1998). The cell wall prevents the bacteria from being easily stained but once they are stained they resist decolourisation by acidified alcohol, thus the name "acid-fast". This important property helps to distinguish mycobacteria from other genera. Due to high lipid content, the cell surface is highly hydrophobic which causes the cells to clump during growth. Clumping results in the slow growth of the cells as nutrients are prevented from easily entering the cells.

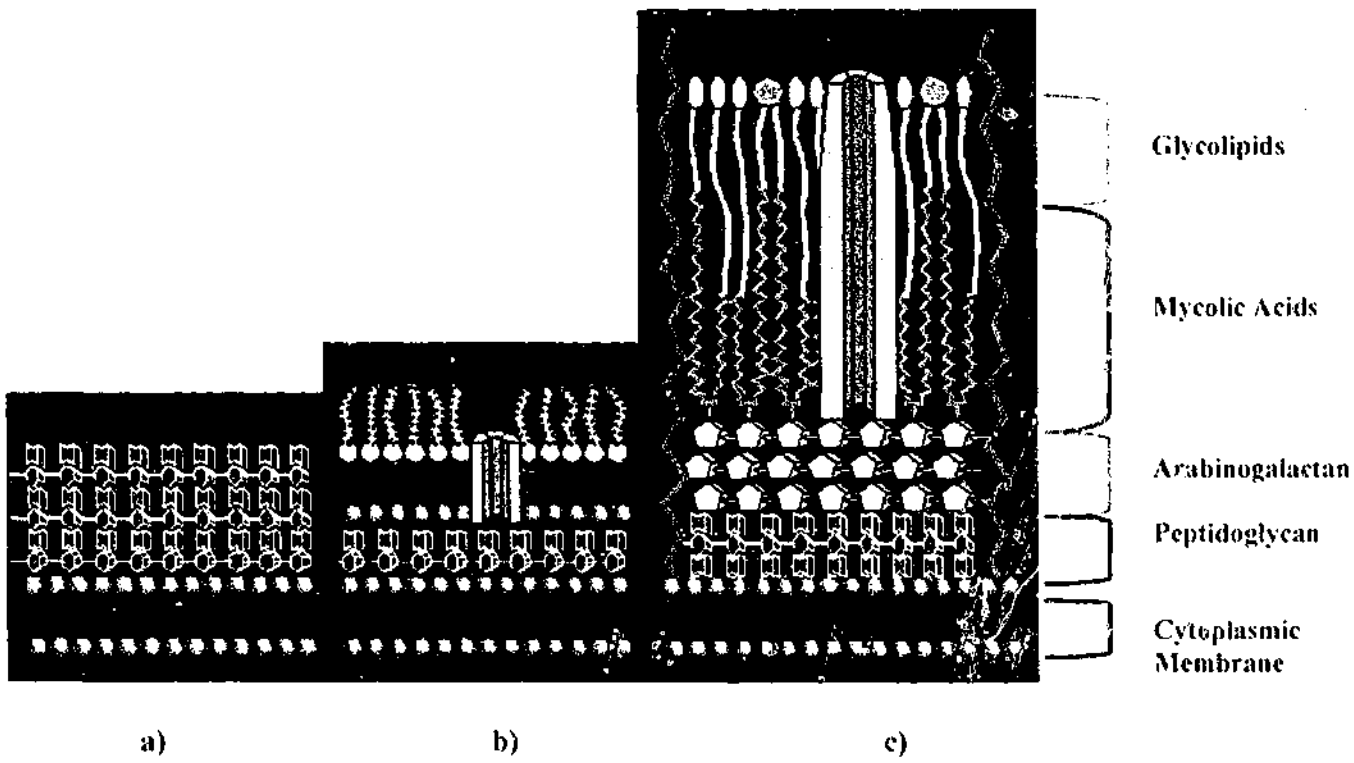


Figure 1-2: a) the gram positive cell wall, b) the gram negative cell wall and c) the mycobacterial cell wall.

There are approximately 71 recognised species of mycobacteria that cause a range of infections in humans and animals (Forbes *et al.* 1998). They are divided into two broad groups known as the *M. tuberculosis* complex and the non-tuberculous mycobacteria group (NTM, atypical). Ten percent of mycobacterial infections seen

clinically are caused by the atypical mycobacteria. They are the most common opportunistic infections in patients with advanced HIV (1997). Atypical mycobacteria are not communicable from person to person like their tuberculous counterparts.

Table 1-1 summarises some of the common mycobacterial groups. In general, members of the *M. tuberculosis* complex group cause TB infections, are slow growers (they take greater than seven days for colonies to appear on solid media) and have non-pigmented colonies. NTM comprise all other species of mycobacteria and are further subclassed into groups based upon growth rate and pigmentation. Photochromogens produce pigment in light but not in darkness, scotochromogens develop pigment when grown in the dark or light and nonphotochromogens remain non-pigmented. All three of these subclasses take greater than seven days for colonies to appear on solid media. Rapid-growers are the final class of NTM defined by the appearance of colonies on solid media, in less than seven days. *M. leprae* is closely related to *M. tuberculosis* but is classed as an NTM. It belongs in its own subcategory, as it is non-cultivatable *in vitro* and can only be grown in the armadillo and footpads of mice. It causes leprosy, a chronic skin, mucous membrane and nerve tissue disease. It is more common in tropical, developing countries and is spread by infected humans via inhalation or contact with infected skin.

Mycobacterium tuberculosis complex			
Name	**Epidemiology**	**Pathogen**	**Type of infection**
M. tuberculosis	Humans (worldwide)	Yes	Tuberculous, pulmonary, disseminated
M. bovis	Humans and animals (worldwide)	Yes	Tuberculous, pulmonary
M. bovis BCG	Humans (also vaccine)	Potential	Tuberculous, pulmonary
M. africanum	Humans (East and West Africa)	Yes	Tuberculous, pulmonary
Nontuberculous Mycobacteria			
Photochromogens			
M. kansasii	Humans	Potential	Pulmonary and extrapulmonary (lymphadenitis and cutaneous)
M. asiaticum	Uncommon, primarily found in Australia	Potential	Pulmonary
M. marinum	Fresh and salt water, fish	Potential	Cutaneous
Scotochromogens			
M. szulgai	Water and soil	Potential	Pulmonary, cervical adenitis, bursitis
M. scrofulaceum	Raw milk and other dairy products, soil, water	Potential	Cervical adenitis in children
M. gordonae	Tap water, water, soil	Nonpathogenic	N/A
M. cookii	Sphagnum, surface water in New Zealand	Nonpathogenic	N/A
Nonphotochromogens			
Name	**Epidemiology**	**Pathogen**	**Type of infection**
M. avium complex	Natural water, humans, birds, water and soil	Yes	Pulmonary, lymphadenitis, disseminated disease in the immune suppressed
M. xenopi	Water, aerosols	Yes	Pulmonary
M. ulcerans	Tropical and temperate climates	Yes	Buruli ulcers and other cutaneous infections
Rapid-growers			
M. absessus	Environment worldwide	Potential	Skin and soft tissue, pulmonary, post operative infection
M. fortuitum	Soil and water	Potential	Skin and soft tissue, lymphadenitis
M. chelonae	Soil and water	Potential	Skin and soft tissue, post operative, keratitis
M. smegmatis	Environment worldwide	Potential (rare)	Skin and soft tissue

Table 1-1 Mycobacteria and associated diseases

1.2.3 *M. tuberculosis*

M. tuberculosis is spread from person to person via prolonged aerosol exposure from a patient with active disease to other susceptible individuals (Salyers and Whitt 1994; 1997). Droplets dispersed by coughing are sufficiently small enough (1-5 μm) to evade the upper respiratory tract defence mechanisms. As aerosols they remain suspended longer than larger droplets (Jawetz *et al.* 1987; Salyers and Whitt 1994).

Clinical symptoms of TB include fever, a productive cough (often with bloody sputum), weight loss and fatigue (Salyers and Whitt 1994; 1997). TB causes progressive lung destruction and may eventually progress to systemic disease which is often fatal. TB can infect any region of the body including joints, spleen, liver, bone, gastrointestinal tract and the brain. It is a slow, progressive disease, but can kill more rapidly in AIDS patients where the fatality rate is nearly 80% (Salyers and Whitt 1994).

TB is difficult to diagnose as it can infect many people. A person is considered infected with TB when a positive conversion to the TB skin test occurs. A positive result indicates *M. tuberculosis* has been inhaled and survived long enough in the lung to stimulate the host's immune system. Only 3-5% of persons with a positive skin test actually develop active disease.

The main defence of the lung against TB infection is the alveolar macrophage. Unfortunately, *M. tuberculosis* has the ability to survive and grow in inactivated alveolar macrophages. The mechanism of entry into the macrophage is still not fully known.

Activated macrophages can kill the bacterium. If the immune system responds rapidly to mount a T cell response, macrophage activation may occur leading to the prevention of symptomatic disease, however, a positive skin test may still result. When a person (elderly, young, and immunocompromised) cannot mount a rapid enough T cell response the bacterium continues to grow in lung macrophages. New T cells, PMN's and macrophages are attracted to the area and accumulate around the sites where the bacteria are reproducing. Tissue in these areas becomes damaged and the immune cells form a tubercule. These can calcify and form hard edged lesions visible in chest X-rays. In these lesions some active TB may remain to cause

reactivation TB later in life when a person's immune defences become weak (ie AIDS, cancer) (Salyers and Whitt 1994; 1997).

Treatment of TB requires long-term therapy with a combination of antibiotics that include isoniazid, ethionamide, ethambutol, pyrazinamide, rifampicin and streptomycin (Jawetz *et al.* 1987; Melmon *et al.* 1992; Salyers and Whitt 1994). Resistance to these agents occurs at a relatively high frequency when they are used alone. For this reason, TB therapy uses a combination of these drugs. Isoniazid and rifampicin has been one of the most effective combinations and is commonly used. Effective treatment for pulmonary TB includes both six and nine month regimens (Melmon *et al.* 1992). Table 1-2 explains the treatment regimens recommended by the Advisory council for the elimination of tuberculosis Morbid Mortal Wkly Rep (MMWR) 1993; 42(RR-7): 1 (1997)

The emergence of resistance to such therapy has occurred because prolonged treatment is required and patient compliance is generally low. The ugly side effect profile also encourages patients to treat themselves less often leading to treatment failures and resistance (Salyers and Whitt 1994; 1997). To increase compliance, many health care institutions have implemented directly observed therapy which requires the patient to ingest the medications in front of a health care worker (1997). The CDC also recommends directly observed therapy for those patients with drug-resistant TB and all patients receiving intermittent therapy (two-three times weekly) (1997).

TB without HIV			TB with HIV
Option 1	**Option 2**	**Option 3**	
Daily doses of isoniazid, rifampin and pyrazinamide for 8 weeks. Follow with 16 weeks of isoniazid and rifampicin used daily 2-3 times weekly. In areas where the isoniazid resistance is not <4 % ethambutol or streptomycin should be added to the initial regimen until susceptibility to isoniazid and rifampin is demonstrated. Treatment should continue for at least 6 months and 3 months beyond culture conversion.	Daily doses of isoniazid, rifampin, pyrazinamide and streptomycin or ethambutol for 2 weeks followed by twice weekly directly observed of the same drugs for 6 weeks. Followed by directly observed twice-weekly doses of isoniazid and rifampin for 16 weeks.	Treat with directly observed therapy three times a week with isoniazid, rifampin, pyrazinamide and ethambutol or streptomycin for 6 months	Options 1, 2 or 3 can be used but treatment regimens should continue for 9 months and at least 6 months beyond culture conversion.

Table 1-2 Treatment regimens recommended by the Advisory council for the elimination of tuberculosis MMWR Morbid Mortal Wkly Rep 1993; 42(RR-7): 1

In cases of TB outbreaks resistant to isoniazid and rifampicin, the CDC recommends that initial five-drug or six-drug regimens should be used. Drug susceptibility tests should be completed to guide the subsequent therapy. If resistance to isoniazid and rifampicin is confirmed, a regimen that includes three or more drugs active against the organism should be continued for 12-24 months after sputum cultures become negative (1997).

Preventative therapy is also recommended for HIV patients with a positive skin test, and HIV patients who are at a high risk of TB but have negative skin tests. People who have been in close contact with patients recently infected with TB (especially children) should receive preventative therapy.

1.2.4 *M. Avium complex* (MAC)

M. avium complex consists of both *M. avium* and *M. intracellulare*. They can cause a wide spectrum of disease, which include asymptomatic infection, coin lesions, bronchitis and invasive pulmonary disease. The *M. avium complex* is the most common cause of non-tuberculous mycobacterial disease in many countries including the USA, Australia, Switzerland, Hong Kong and Japan (Benson 1993; Hosker *et al.* 1995; Raska *et al.* 1995; Horsburgh Jr 1996; O'Brien *et al.* 2000; Raju and Schluger 2000; Khoor *et al.* 2001). It is also the most common disseminated disease in patients with late stage HIV infection, with persistent fever and weight loss the common features (Raska *et al.* 1995; Raju and Schluger 2000). Therapies include a wide range of agents used in combination to prevent secondary resistance developing. The most common are cycloserine, ethionamide, azithromycin, clarithromycin, rifabutin, clofazimine, ethambutol, amikacin and ciprofloxacin.

1.2.5 *M. leprae*

M. leprae is the causative agent of leprosy. Leprosy is a bacterial skin disease that can also affect the peripheral nerves and upper airways. Like *M. tuberculosis*, it is also of socioeconomic importance being endemic in poor Third world countries such as South East Asia, tropical Africa and Latin America. It can also be found in the USA in small numbers as it is endemic in Hawaii, Texas, California and Louisiana (Benenson 1990). However, the majority of persons in the USA infected by *M. leprae* are mostly immigrants and refugees who contracted the bacilli before arriving in the country.

Humans are the only significantly proven reservoir of leprosy infection with the mode of transmission requiring prolonged close contact. Millions of bacilli are liberated daily from the nasal discharge of untreated patients and these have been shown to be viable for up to one week in dried nasal secretions. Cutaneous ulcers also shed high amounts of the bacilli.

Therapy is long-term, with infectiousness being lost in patients within three months of treatment with dapsone or clofazimine or within three days with rifampicin therapy. None of these agents are used alone as resistance has already developed to dapsone and the number of resistant cases is rising with rifampicin. Therapy usually includes

rifampicin once a month with a daily dose of dapsone or a high dose of clofazimine once a month with a daily maintenance dose of the same agent. Therapy is long, with treatment continuing for at least two years after skin smears have become negative.

1.2.6 Pulmonary diseases caused by other non-tuberculous mycobacteria

After MAC, *M. kansasii* appears to be the most common nontuberculous mycobacteria (NTM) to cause pulmonary disease similar to that of TB (Horsburgh Jr 1996; Khoor *et al.* 2001). Unlike TB its progression is slower. Patients who present with pulmonary disease from NTM generally present with a persistent cough or a history of recurrent bronchitis. Most NTM infections occur in patients with pre-existing lung disease or those over the age of 50 (Griffith *et al.* 1993; Raska *et al.* 1995; Tomashefski *et al.* 1996; O'Brien *et al.* 2000; Khoor *et al.* 2001). They are treated after antibiotic sensitivity is determined with both intravenous and oral antibiotics for a duration of at least six months.

Other NTM that cause pulmonary disease include *M. abscessus, M. xenopi, M. gordonae, M. fortuitum* and *M. chelonae* (Griffith *et al.* 1993; Khoor *et al.* 2001). The latter two also cause pneumonia-like symptoms. Treatment of these organisms is usually based upon initial sensitivity tests, as many of them are uniformly resistant to antituberculosis drugs. *M. abscessus* can be extremely difficult to eradicate. It has, however been successfully treated by amikacin and cefoxitin or imipenem for a duration of up to three months followed by surgical resection of localised disease (Griffith *et al.* 1993).

1.2.7 Lymphadenitis

Lymphadenitis is an inflammation of the lymph nodes. It is mostly caused by *M. tuberculosis* in disseminated disease, however when it is caused by other mycobacteria it almost always appears limited to the cervical area. It is the most common manifestation of infection from NTM in children (Hazra *et al.* 1999). Most cases in paediatrics were caused by NTM such as *M. scrofulaceum* and rarely by *M. kansasii, M. bovis, M. fortuitum* and *M. chelonae* (Horsburgh Jr 1996; 1997). MAC is also emerging as a prominent cause (Evans *et al.* 1998; O'Brien *et al.* 2000). When lymphadenitis is caused by nontuberculous mycobacteria it almost always infects children (Horsburgh Jr 1996; Evans *et al.* 1998; O'Brien *et al.* 2000). Therapy for

lymphadenitis caused by *M. tuberculosis* consists of the previously explained TB medications in combination with one another and should be continued for at least nine months. Treatment for the other mycobacteria is usually by surgical excision (Hazra *et al.* 1999; O'Brien *et al.* 2000).

1.2.8 Skin and soft tissue infection

This group of infections include abscesses, septic arthritis and osteomyelitis. Most symptoms are usually localised and include pain, swelling and mild serous drainage. Immunosuppressed persons appear to be at a greater risk of contracting these infections than healthy individuals. Infection usually results from a direct inoculation of the mycobacteria, haematogenous dissemination or a surgical complication such as catheterisation, dialysis, cardiac surgery and recently cosmetic surgery (Wallace Jr *et al.* 1998; Wallace Jr 1989; Midani and Rathore 1999; Murillo *et al.* 2000). The most common organisms for this type of infection are *M. chelonae* and *M. fortuitum*. Most cases occur in the extremities and initially present as nodules or ulceration with abscess formation.

The above mycobacteria are traditionally resistant to the usual antimicrobials used to treat TB. Instead, antibiotics such as erythromycin, doxycycline, amikacin, cefoxitin, sulfonamides, imipenem and ciprofloxacin are used. Initial therapy is once again difficult with parenteral antibiotics given over several weeks followed by oral agents to which the organism has been tested for sensitivity (Schulz *et al.* 2001). Surgical debridement of the tissue is also utilised (Wallace Jr 1989; 1997; Midani and Rathore 1999). The duration of therapy, as is characteristic with other mycobacterial infections, continues for several months after the lesions have healed.

M. marinum is another mycobacteria which has been commonly associated with nodular skin lesions (Horsburgh Jr 1996). Its source has been traditionally located to persons swimming in non-chlorinated water. The lesion is treated with doxycycline, minocycline or co-trimoxazole (sulfamethoxazole and trimethoprim).

M. ulcerans causes severe large ulcerative lesions and is a prevalent disease in Africa (Horsburgh Jr 1996). Therapy is usually restricted to severe measures such as surgical excision and skin grafting. In severe cases amputation of the affected limb is the only treatment option.

Rarely *M. smegmatis* and *M. haemophilum* have also been attributed to causing skin infection (Horsburgh Jr 1996; O'Brien *et al.* 2000).

1.2.9 Mycobacterial infection therapies: methods and complications

As briefly mentioned in the preceding sections, a wide spectrum of antimicrobials are used to treat mycobacterial infections. Unfortunately, in almost all situations therapy is complicated due to the problem of increasing resistance achieved by the highly adaptable mycobacterial cell system. Accordingly, antimicrobial agents must be given in combination of two or more for lengthy periods of time. For compliance in patient care, this is less than desirable. Multi-drug therapy for the patient can be confusing with hard to remember non-daily dosing. Many patients also cease therapy before completion of the course is scheduled because they "feel" better, or they stop due to the side effects that they perceive to be worse than the illness. The high costs of many medications over many months may also tempt a patient to cease therapy early. Unfortunately, these medication problems are not the only complicating factors in the treatment of mycobacterial infections. Many of the antimicrobials necessary for treatment may only be administered intravenously, and other infections, namely the skin and soft tissue infections can only be treated by surgical excision, which is painful, invasive and can be disfiguring. Investigation of the groups of antimicrobials used to treat mycobacterial infections allows examination in more detail of the problems outlined.

Isoniazid (isonicotinic acid hydrazine, INH) (Figure 1-3 a)) was introduced in 1952 as a water soluble, synthetic, orally active antibiotic. It is the most active drug for the treatment of TB in those who can tolerate it (Jawetz 1992) however, it is only active against actively growing mycobacteria. INH passes freely into mammalian cells so is thus effective against intracellular organisms being actively taken up by tubercule bacilli. Its mechanism of action is not fully known but it is believed to inhibit the synthesis of mycolic acids, which interferes with the formation of mycobacterial cell walls. INH may also combine with an enzyme that is found uniquely in isoniazid sensitive strains of mycobacteria resulting in disorganisation of cell metabolism. Resistance by mycobacteria to INH occurs by the organism preventing the penetration of the drug by a decrease in catalase activity. Structurally similar to pyridoxine, it can bind with pyridoxal to cause a pyridoxine deficiency, which can cause neuritis and

other symptoms such as insomnia, muscle twitching and convulsions. These effects are prevented by pyridoxine supplementation thus INH is never administered as a monotherapy. Other side effects are usually dependent on dose and duration of therapy and occur in about 5% of those treated. They include allergic skin reactions, fever, liver toxicity, blood disorders, arthritis, vasculitis, CNS and peripheral nervous system disorders. Patients with glucose-6-phosphate deficiency are at risk of haemolytic anaemia. Isoniazid can also cause phenytoin toxicity as it increases the serum levels of the drug.

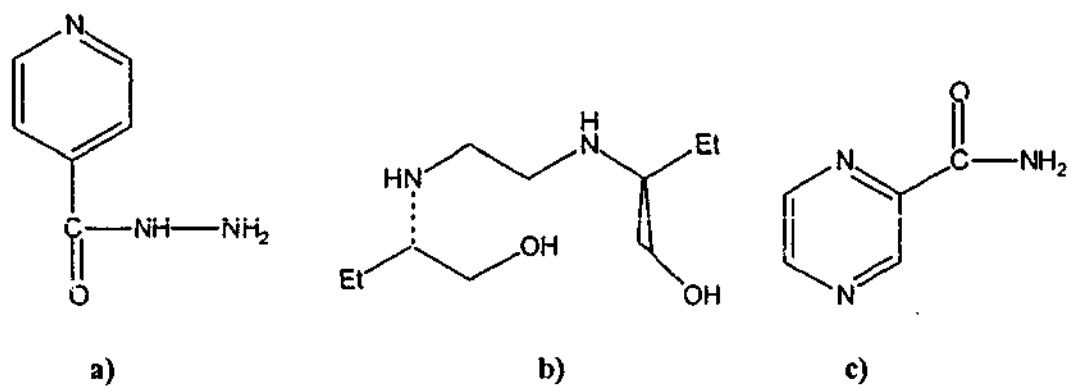


Figure 1-3 Structures of a) isoniazid, b) ethambutol and c) pyrazinamide

Ethambutol (Figure 1-3 b)), active as the dextro-rotary isomer is only effective against mycobacteria (Jawetz 1992). Its mechanism of action is unknown however, it may interfere with carbohydrate metabolism. Resistance can develop quickly if it is used alone. Side effects include optic neuritis, which will occur if renal function is low, red/green colour blindness and a decrease in visual acuity, which are dose related. Other side effects include gastrointestinal disturbances, arthralgia, headaches, giddiness, mental disturbances and a decrease in uric acid excretion, which increases blood urate.

Pyrazinamide (Figure 1-3 c)) is a tuberculostatic drug at acidic pH, but is inactive at neutral pH. It is only effective against intracellular organisms in macrophages. Side effects usually occur in 1-5 % of patients and range from arthralgia, hyperuricaemia, gastrointestinal, malaise and fever. With high doses, serious hepatic damage can occur.

Rifampicin (rifampin) (Figure 1-4) is a semi-synthetic derivative of the macrolide antibiotic rifamycin from *Streptomyces*. It acts by binding and inhibiting DNA-

dependent RNA polymerase to prevent RNA synthesis in prokaryotic cells. It does this by inhibition of mRNA synthesis caused by binding to subunit β-RNA polymerase encoded by the *rpoβ* gene (Ortona and Cingolani 1998). It has no activity against eukaryotic cells. Used alone, resistance builds quickly due to chromosomal mutation (mutation of the *rpoβ* gene) of mycobacteria. Rifampicin causes body fluids to turn pink (ie tears, sweat). Side effects occur in less than 4% of patients, these include rash, fever, gastrointestinal upsets, liver problems with jaundice (associated commonly with patients who have had prior liver disease), influenza-like syndrome and CNS disturbances. Rifampicin can induce liver enzymes thus it increases the metabolism of warfarin, glucocorticoids, narcotic analgesics, antidiabetic agents, dapsone and oestrogens (oral contraceptives).

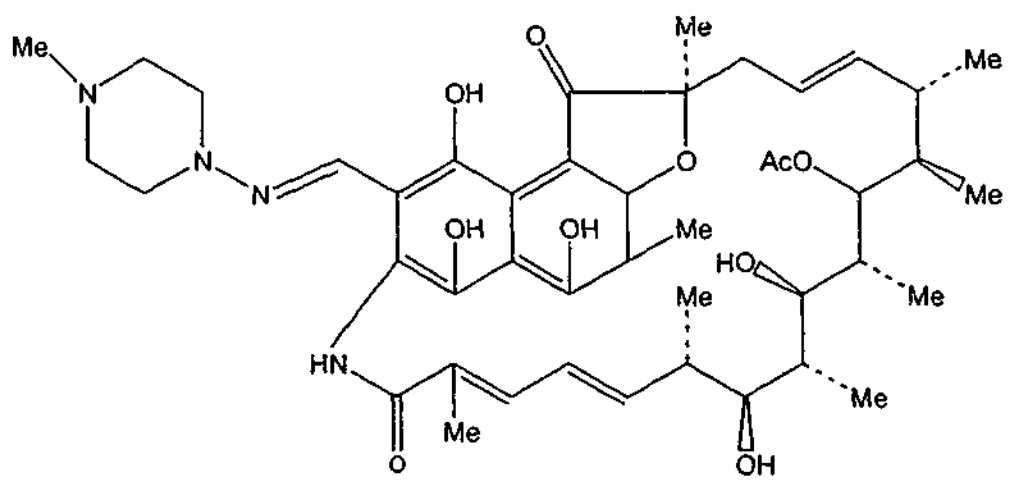
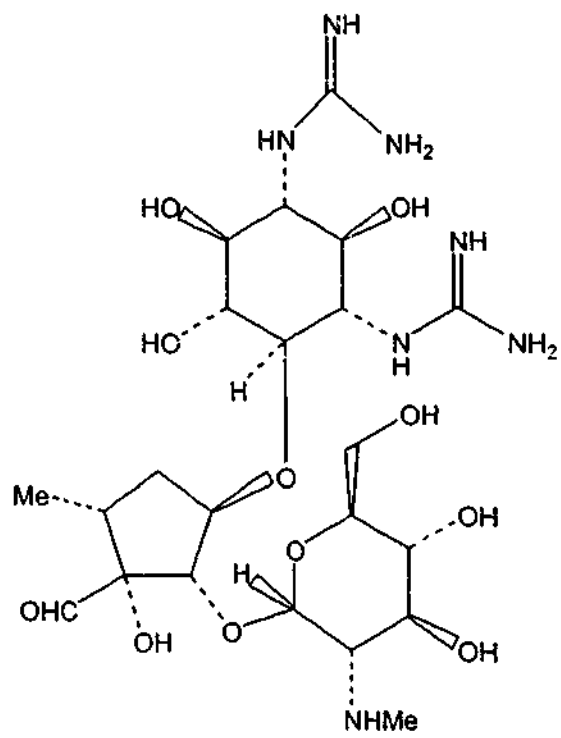


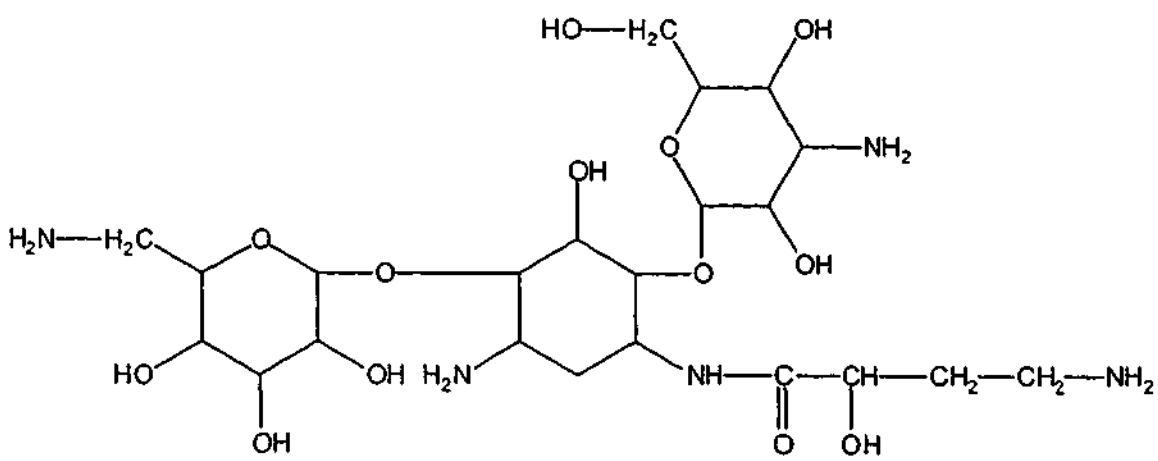
Figure 1-4 Structure of rifampicin

The aminoglycoside antibiotics (which include streptomycin, amikacin, kanamycin and tobramycin) (Figure 1-5) are agents that act by inhibiting bacterial protein synthesis. They bind to the 30S subunit of the bacterial ribosome, which leads to the misreading of mRNA and thus defective proteins. To get to the site of action they need to penetrate the cell membrane using oxygen dependent transport systems so are more active when given with an agent that disturbs the cell wall such as isoniazid. They are poorly absorbed from the gastrointestinal tract and are therefore only administered by intravenous, subcutaneous, intramuscular or intrathecal injection. Resistance is a major problem with this group of antibiotics and can develop by the bacteria acquiring the ability to produce enzymes that inactivate the drugs by

adenylation, acetylation or phosophorylation. Alteration of the cell wall surface to prevent drug penetration or the receptor on the 30S subunit being deleted or altered due to chromosomal mutation are other methods of resistance. Side effects are severe and include both ototoxicity and nephrotoxicity. Their use is restricted in patients with severe renal failure and all patients being treated with this group must have their renal function monitored.



a)



b)

Figure 1-5 Examples of the aminoglycoside antibiotics; a) streptomycin and b) amikacin

Capreomycin, cycloserine and ethionamide are second line drugs used when the previously explained ones have become too toxic, or the mycobacteria have acquired

resistance to them. Capreomycin is a peptide antibiotic administered by intramuscular injection. It can cause severe kidney damage, eighth nerve injury, deafness and ataxia. It is contraindicated with streptomycin due to the nephro- and ototoxicity. Toxicity can be reduced if the therapeutic dose is given only two to three times weekly. Cycloserine (Figure 1-6 a)) prevents cell wall synthesis by inhibition of alanine racemase thus stopping the formation of D-alanine and of the D-alanine peptide that forms peptidoglycan. CNS side effects are the most common experienced and these are dose related. Unfortunately, doses needed to treat mycobacterial infections are high thus these side effects are common. Ethionamide (Figure 1-6 b)) may act similarly to isoniazid by inhibiting the synthesis of mycolic acids, a major component of cell wall. It is however rarely used, as resistance develops rapidly and the doses required to treat infection are so high that they cause gastric irritation and neurologic symptoms.

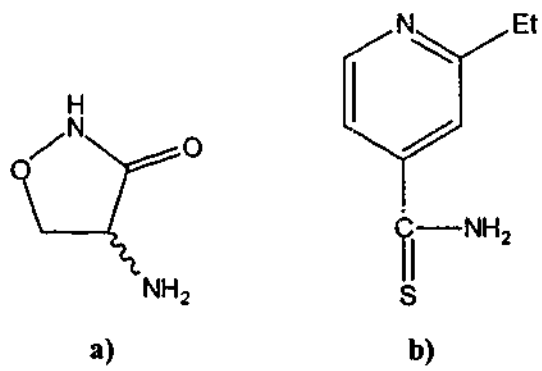
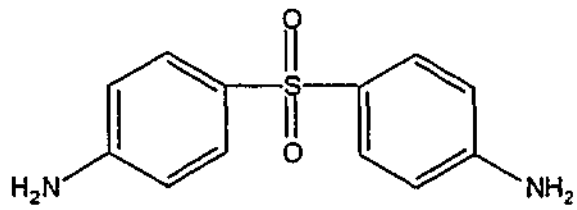
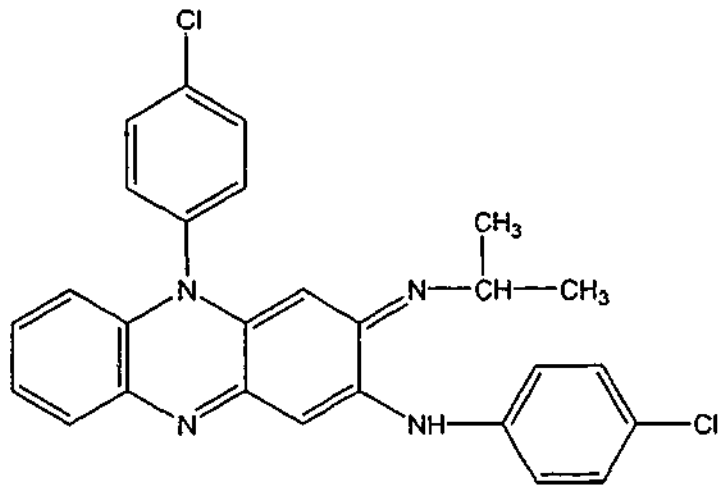


Figure 1-6 Structures of a) cycloserine and b) ethionamide

Apart from rifampicin, other agents used to treat leprosy are dapsone and clofazimine (Figure 1-7). Dapsone is closely related to the sulfonamides and it is believed it acts orally to inhibit folate synthesis. Resistance develops when low doses are used therefore it is used in combination with rifampicin. Side effects are common and include haemolysis of red blood cells, methemoglobinaemia, anorexia, nausea, vomiting, skin rashes and allergy. Clofazimine is a dye used as an alternative to dapsone. Its mechanism of action is unknown but may involve DNA binding. It is given orally and its main adverse effect is skin discolouration ranging from red-brown to black. It also has some gastrointestinal side effects.



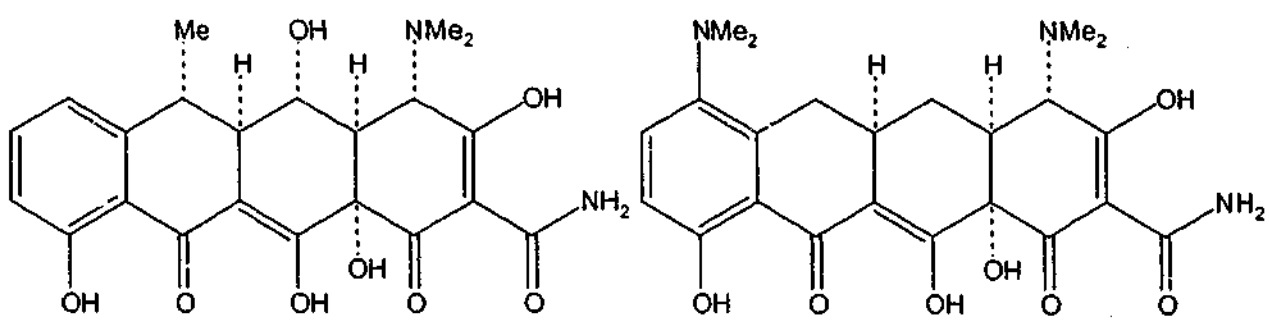
a)



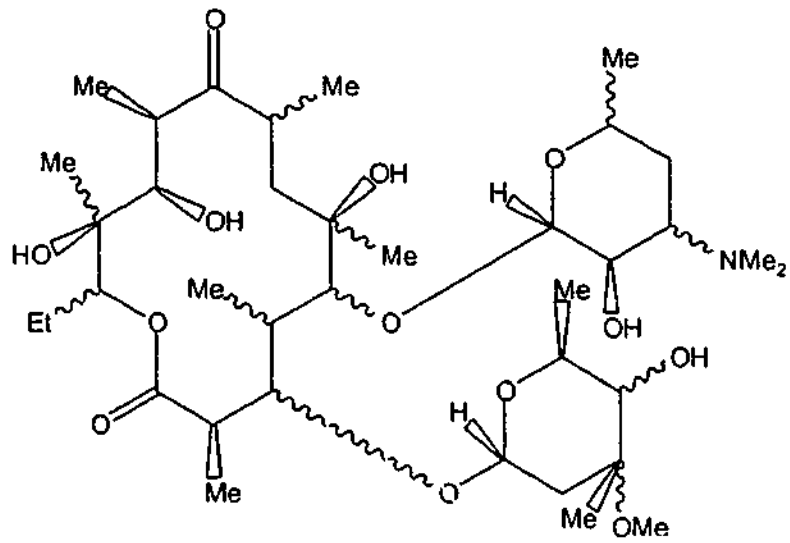
b)

Figure 1-7 : Structures of a) dapsone and b) clofazimine

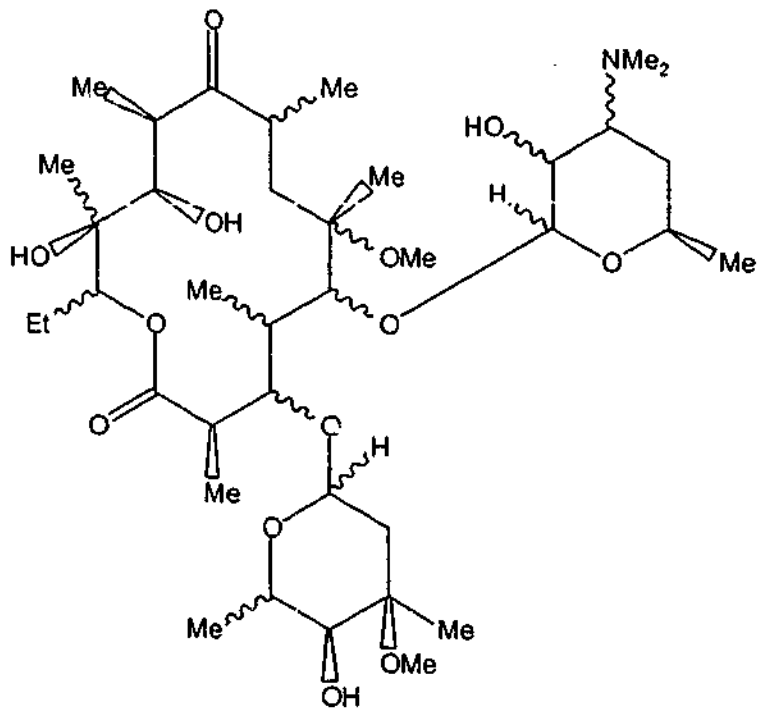
Other agents used are common antimicrobials that include the tetracyclines (doxycycline and minocycline), erythromycins (erythromycin, clarithromycin and azithromycin), ciprofloxacin and imipenem (Figure 1-8). All are used after susceptibility tests to them are complete; the results of which can take weeks. They cannot be used alone due to resistance and all have limitations. Imipenem for example can only be given intravenously. The tetracyclines can upset the stomach and are only bacteriostatic; the erythromycins cause liver problems if used in high doses for longer than 14 days and ciprofloxacin can cause blood dyscrasias.



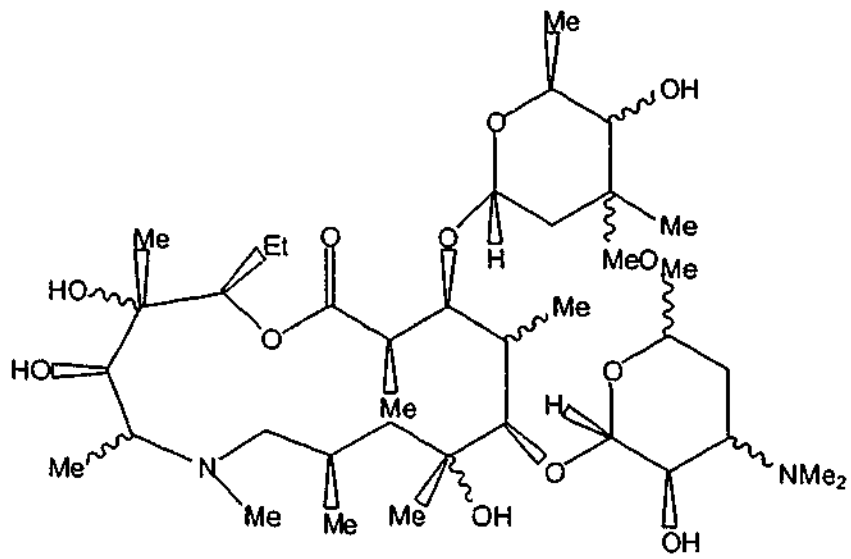
a) b)



c)



d)



e)

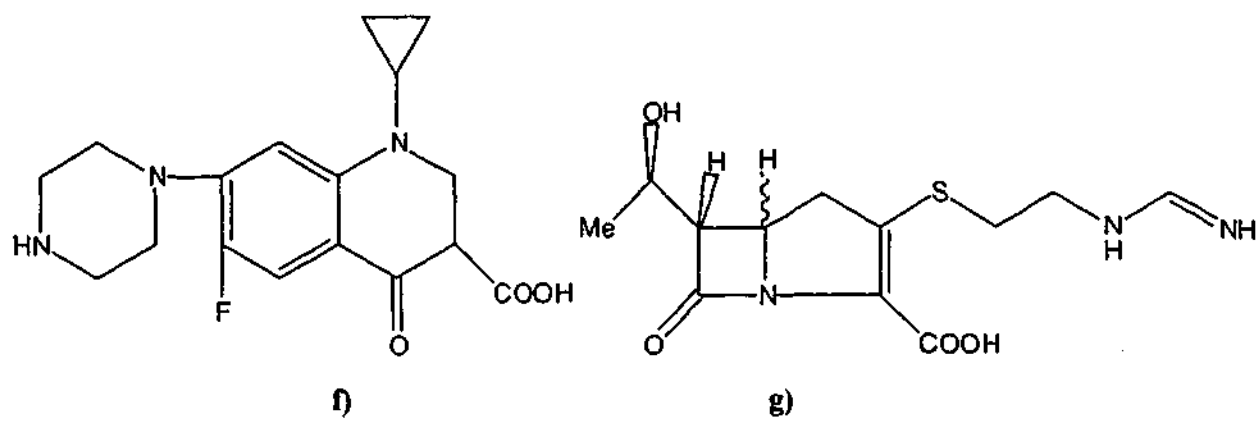


Figure 1-8 Chemical structures of a) doxycycline, b) minocycline, c) erythromycin, d) clarithromycin, e) azithromycin, f) ciprofloxacin and g) imipenem

1.2.10 Mycobacterial infections and treatment: what should we aim for?

As has been previously discussed, the genus mycobacterium contains many species of organism that are able to cause a wide spectrum of clinical disease. The diseases they cause have been shown to be very difficult to treat requiring long term, multi-drug therapy or invasive surgical procedures. All current chemotherapeutics available need to be given in high dose combinations with others. High doses increase the incidence of side effects with these agents, many which are already severe and damaging to major organs of the body. None of these factors help patient compliance and when a patient stops taking their medication the organism can build more resistance making it harder to treat. These factors alone show that it is necessary to develop new agents to treat this family of microorganisms. We need to discover novel targets within the bacteria and develop newer agents that will be less susceptible to resistance, yet powerful enough to treat the infections alone at doses that will minimise the advent of side effects.

The TB genome was thus examined to determine if it contained any genes that code for enzymes that were already novel drug targets in other disease-causing organisms. One gene was annotated to say that it contained sequence similarity to other genes that code for the sialidase enzyme. Inhibitors of sialidases were the first successful treatments towards the influenza virus making this discovery worthy of further investigation.

Subsequent testing for this enzymatic activity in other mycobacteria, namely *M. smegmatis* and *M. bovis* BCG showed that they appeared to possess a sialidase-like activity. The reason why mycobacteria would contain such an enzyme is unclear. It

may be to facilitate cell entry and adhesion as it is for the influenza virus as both replicate within host cells. It may however, be present as a nutritional source to unmask carbohydrates for energy. This would not be an unlikely hypothesis as sialidase activity is present in *M. smegmatis* which is typically non-pathogenic.

The following sections examine and explain the functions, properties and uses of the sialidase family. Understanding the characteristics of the putative mycobacterial sialidase and comparing it with the properties of those from other organisms may prove it to be a worthy target for drug development as it has been for treating influenza virus infection.

1.3 Sialidases

1.3.1 History

Sialidases are enzymes grouped under the heading of *N*-acetylneuraminosyl glycohydrolase. They are also known as neuraminidases. They are responsible for the cleavage and release of sialic acids from a wide range of sialic acid-containing compounds (sialoglycoconjugates).

Initial work implicating the existence of sialidases as an enzyme were based in virology (Hirst 1941). An enzyme activity responsible for the release of influenza virus from the surface of chicken erythrocytes agglutinated by the virus particles was proposed. The enzyme was later termed 'receptor destroying enzyme', as extracts of *Vibrio cholerae* and *Clostridium perfringens* were also able to destroy the receptor sites for the influenza virus on the surface of the human red blood cell (Burnet *et al.* 1946). The demonstration of sialic acid release from a salivary mucin by a *Pneumococcal* extract led to the proposal that this enzyme should be called a sialidase (Heimer and Meyer 1956). A year later the name neuraminidase was put forward (Gottschalk 1957). Both names are now used interchangeably. Purified sialidase was first obtained from *V. cholerae* and shown to be identical to the receptor destroying enzyme (Ada *et al.* 1961).

1.3.2 Sialidase distribution and function

Sialidases are widely distributed though-out nature, being found in a range of organisms, which include viruses, bacteria, fungi, protozoan and vertebrates (Ada *et al.* 1961; Miyagi and Tsuiki 1985; Hiraiwa *et al.* 1988; Roggentin *et al.* 1988; Kitajama *et al.* 1994; Taylor 1996; Amino *et al.* 1998; Saldana *et al.* 1998; Byers *et al.* 2000; Kobayashi *et al.* 2000). The substrate, sialic acid is glycosidically linked to saccharide moieties and can be synthesised in higher organisms such as echinoderms. It is not, however synthesised by plants, lower metazoans or most microbes (Warren 1963; Traving and Schauer 1998; Nadanaka *et al.* 2001).

Sialidases have been found to act in both the pathogenicity and nutrition of many organisms and a deficiency in human cells has also been linked to a host of disease states such as sialidosis (Lukong *et al.* 2001; Pshezetsky and Ashmarina 2001).

Mammalian sialidases have been located in several tissues, where the enzymes perform many roles that have been thought to include the regulation of cell proliferation, clearance of plasma proteins and the catabolism of gangliosides and glycoproteins (Pshezetsky and Ashmarina 2001; Potier 1981).

In viruses such as influenza, sialidases are a major virulence factor. Along with haemagglutinin, which binds the virus to sialic acid on the host cell membrane, the viral sialidases process progeny virus particles when they bud from an infected cell and remove sialic acids to halt self-agglutination.

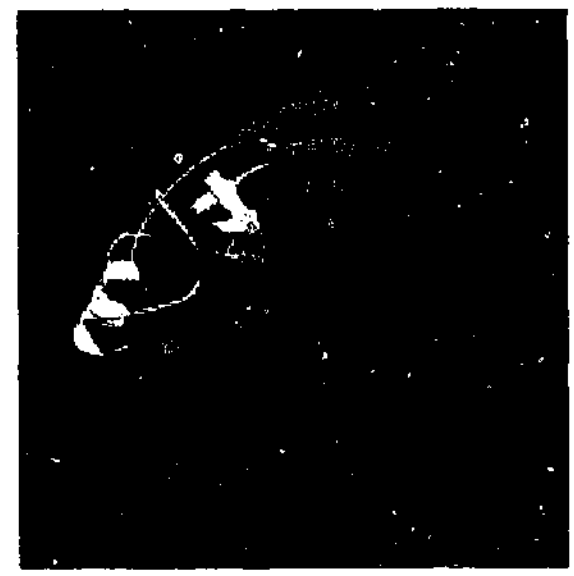
In bacteria, sialidases also have important roles. Although most bacteria do not produce sialic acid themselves they produce a sialidase that allows them to use environmental sialoglycoconjugates as carbon and energy sources (Vimr and Troy 1985). After cleavage of the sialic acid from sialoglycoconjugates, bacteria use transport systems to transport sialic acid into the cell for catabolism. It has even been shown that bacteria such as *Eschericia coli* which do not produce a sialidase still contain the genes for sialic acid uptake and degradation (Vimr and Troy 1985). This suggests that the scavenging of this sugar by bacteria may make it a common nutritional source. Sialidases in pathogenic organisms have also had defined roles in disease aetiology. *V. cholerae* sialidase for example, removes sialic acid from gangliosides to unmask GM_1, the binding site for cholera toxin (Galen *et al.* 1992). Parasites such as the *Trypanosoma* species also utilise sialidases as a virulence factor in host cell adhesion and invasion (Buschiazzo *et al.* 1997; Magdesian *et al.* 2001).

1.3.3 Structure

The structure of sialidase from the influenza virus was elucidated in 1983 for two strains of the virus (Tokyo/3/67 and A/RI/5^+/57) (Varghese *et al.* 1983). The structures were resolved to 2.9Å and at this resolution and refinement the two strains did not appear to have any significant differences. The enzyme was determined to have a tetrameric association of identical monomers (Figure 1-9 a)). The polypeptide folded into six identical four-stranded β-sheets that were arranged as the blades of a propeller. Further research using a number of sialidases from a variety of influenza strains determined that the influenza sialidase is a box-shaped globular head attached to a thin stalk which was embedded into the viral membrane (Colman *et al.* 1983).

The four catalytic sites were on the top face of the globular heads and sialic acid bound here in large pockets lined with charged residues. It was revealed that the residues are highly conserved among influenza sialidases despite low sequence identities (Colman *et al.* 1983).

a)



b)

Figure 1-9 a) The tetrameric structure of viral Influenza N9 sialidase, b) The monomeric structure of bacterial *S. typhimurium* sialidase

Further analysis of other sialidases showed that the sequence identity between influenza sialidase and bacterial sialidases was low (approximately 15%). However, comparison of non-viral sialidases with one another also showed low sequence homology at around 30%. Despite low sequence identity, all sialidases appear to fold as β-sheets in the shape of a propeller. The topology of the catalytic domain also remains conserved and the active sites of the enzymes share many features (Hoyer *et al.* 1992; Taylor *et al.* 1992; Crennell *et al.* 1993; Roggentin *et al.* 1993a; Fronda *et al.* 1999; Mizan *et al.* 2000).

Today there are many complete sialidase gene sequences available for bacterial and parasitic sialidases, and although the sialidases differ in length they all exhibit significant similarities after alignment of their primary sequences (Roggentin *et al.* 1993a). In 1989 Roggentin *et al.* observed that in bacterial sialidases the most characteristic feature was that all contained a motif of 8-12 residues that were highly conserved and repeated up to five times within the primary sequence (Roggentin *et al.*

1989; Roggentin *et al.* 1993a). This conserved sequence is known as the Asp box motif (S-X-D-X-G-X-T-W) and it appears in topologically identical positions within the β-propeller. Another significant motif is the RIP/RLP motif. It is usually located near the *N*-terminus of bacterial and parasitic sialidase sequences and contains one conserved proline and an arginine that interacts with a sialic carboxylate group. It has been found that if the arginine residue is changed to a lysine by site directed mutagenesis it decreases the affinity of the enzyme for sialic acid (Roggentin *et al.* 1993a).

Recently it has been found that mammalian sialidases also contain two Asp box motifs and a RIP motif near their *N*-terminal (Fronda *et al.* 1999; Kotani *et al.* 2001). Interestingly, the influenza virus sequences do not contain Asp boxes but do have a REP motif, which is comparable with the RIP motif. Although the lack of Asp boxes is a significant difference between viral and bacterial sialidases, the 3-dimensional structure (3D) of bacterial sialidases still appear to fold in the same way as that of the influenza virus. An example of this is the crystal structure of *Salmonella typhimurium* LT2 which is a monomeric sialidase with a sequence identity to the influenza sialidase of only 15% (Figure 1-9 b)) (Crennell *et al.* 1993). Although the influenza sialidase is a tetramer, the *S. typhimurium* monomer is of a similar size to each of the influenza monomers that make up the tetramer. The two structures were similar in general fold and spatial arrangement of the catalytic residues. Both also contain an arginine triad in the active site, a hydrophobic pocket and a key tyrosine and glutamic acid (Crennell *et al.* 1993).

It thus appears that although sequence homology is low for sialidases of all species they share common structural similarities. Key factors for all appear to be the folding into β-propellers, the existence of hydrophobic pockets, and a RIP/RLP/REP motif near the *N*-terminus. Amongst non-viral sialidases, the Asp-box motif that occurs at topologically equivalent positions on the structure is also a key element to this enzyme.

1.3.4 Biochemical properties

As mentioned in Section 1.3.3, sialidases may occur naturally as monomers and oligomers, thus the molecular mass distribution of this group of enzymes can differ

significantly. Bacterial sialidases for example are generally monomers with the exception of *C. chauvoei* (dimer) and *C. septicum* (trimer) (Roggentin *et al.* 1993a). Influenza virus sialidase however exists as a tetramer (Varghese *et al.* 1983) as does the large (360 kDa) bacteriophage-associated sialidase (Machida *et al.* 2000). The size difference of the sialidases also influences their different enzymatic and biochemical properties. Sialidases in general can range in size from as large as 360 kDa to as small as 20 kDa, with the average size of the bacterial sialidases in the range of 40-150 kDa.

Bacterial sialidases are generally classed into two groups based on their size. In general, enzymes of around 40 kDa are considered small and those of 60 kDa or more (eg *V. cholerae* sialidase) are considered large (Hoyer *et al.* 1992). The larger enzymes tend to have multiple protein domains in addition to the sialidase domain, which may account for some of their different biochemical properties (Vimr 1994).

Bacterial sialidases may be secreted and others are localised on the cell surface or found intracellularly. In general, it appears that the small sialidases are localised in the cell, for example the small isoform of *C. perfringens* and the sialidase of *S. typhimurium* (Roggentin *et al.* 1988; Hoyer *et al.* 1992). *A. viscosus* is also an example of a cell surface associated sialidase (Teufel *et al.* 1989).

Common properties of bacterial sialidases include their optimal pH which is usually acidic and their preference for cleaving α-(2,3)-linked, terminally-bound sialic acids over other linkages (Moriyama and Barksdale 1967; Potier *et al.* 1979; Berry *et al.* 1996; Lichtensteiger and Vimr 1997; Byers *et al.* 2000; Mizan *et al.* 2000). Although the preference is towards α(2,3)-linked sialic acids, larger sialidases can cleave sialic acid attached via other linkages at a high rate. This is not true for the smaller sialidases of *C. perfringens, C. sordelli* and *S. typhimurium* that hydrolyse only a small number of substrates of differing linkages.

Like their optimal pH, most bacterial sialidases also have an isoelectric point (pI) that is acidic, with the exception of *S. typhimurium* which focuses at a basic pH greater than 9 (Roggentin *et al.* 1993a; Teufel *et al.* 1989). Most sialidases appear to have optimal hydrolytic activity at a temperature of 37 °C. The exception is the large isoform of *C. perfringens* which hydrolyses with most activity at 55 °C (Roggentin *et al.* 1993a).

Further properties of the sialidase can differ greatly between the enzymes but can include activation by divalent cations and, as expected inhibition by chelators. Well studied examples of these properties include *V. cholerae* and *C. diptheriae* sialidases which both have enhanced enzymatic activity from the addition of Ca^{2+} ions and are inhibited by EDTA (Ada *et al.* 1961; Moriyama and Barksdale 1967). Both Mg^{2+} and Mn^{2+} also enhance sialidase activity to varying degrees as Ca^{2+} does. Enhancement of sialidase activity by Ca^{2+} is not unique to bacterial sialidases. It may also enhance the activity of some mammalian sialidases such as the human exo-α-sialidase (Achyuthan and Achyuthan 2001).

There are also many sialidases for which divalent cations have no effect or act as an inhibitor. Neither *S. oralis*, *A. viscosus* or *H. parasuis* sialidase activity is affected by the addition of divalent cations, however the divalent cations including Cu^{2+} inhibit the cytosolic sialidase from rat liver (Miyagi and Tsuiki 1985; Teufel *et al.* 1989; Lichtensteiger and Vimr 1997; Byers *et al.* 2000). Metal ions such as Cu^{2+} have inhibitory effects on other sialidases. Cu^{2+} is inhibitory towards another mammalian sialidase from pig liver and Hg^{2+} can inhibit *A. viscosus* activity (Teufel *et al.* 1989; Kobayashi *et al.* 2000).

The sialidase family of enzymes have evolved into many subgroups of wide size distribution. Some exist as monomers, others as oligomers, and all with the ability to cleave sialic acid from many substrates to varying degrees. Although the bacterial sialidases share properties in common, such as an optimal activity at an acidic pH and an optimal temperature of 37 °C they still have individual requirements that make them a unique and interesting group of enzymes to study.

1.3.5 Sialidase in disease

The family of sialidases has been linked to many infections and disease states. In the human body a lack of this endogenous enzyme can cause defects in bodily growth and function. In viruses, bacteria and parasites, sialidases can act as virulence factors aiding in cell attachment and invasion of the host. In this way, it is almost a case of can't live with them, can't live without them! In this section, the absence of this enzyme and its detrimental effects to human health, and the roles that sialidases have in virulence and pathogenicity will be discussed.

Human lysosomal sialidase is involved in the degradation of sialoglycoproteins in the body. Sialidosis and galactosialidosis are associated with a defect and deficiency of this enzyme (Cantz *et al.* 1981; Lowden *et al.* 1981; O'Brien 1981; Suzuki 1995). Galactosialidosis also involves a deficiency of β-galactosidase. Both disorders are autosomal recessive, sialidosis being a disorder of lysosomal acid sialidase deficiency and galactosialidosis a lysosomal storage disease. Patients suffering sialidosis excrete high amounts of sialyloligosaccharides and sialylglycopeptides in their urine. Clinically, there are two types of sialidosis termed early or late onset of the disease. Late onset occurs in patients over eight years of age and usually manifests as cherry-red spots, myoclonus, seizures, neuropathy and difficulties in walking. Early onset is often fatal with severe symptoms including those previously mentioned, as well as mental and psychomotor retardation, bone abnormalities, progressive neurological disorders and hepatosplenomegaly. Similarly, galactosialidosis patients excrete large amounts of complex carbohydrate mixtures rich in sialic acids. Symptoms depend on the age of onset and can include those mentioned for sialidosis. The earlier the onset of galactosialidosis the more severe the symptomatology. At present there is no known cure for either disease but with the genes involved in the disease now known, a cure should not be far away (Achyuthan and Achyuthan 2001; Lukong *et al.* 2001).

As discussed above, a deficiency of endogenous sialidase in humans causes disease. Conversely, existence of sialidases in other organisms such as viruses, bacteria and parasites facilitates their invasion and consequent infection of humans. As briefly mentioned in Section 1.3.2, sialidases assist the viral spread of influenza by cleaving sialyl residues during the budding of the infectious particles to remove sialic acids that would otherwise be trapped by haemagglutinin-host sialyl interactions. They also aid influenza infection by reducing the aggregating properties of the virus particles and reducing the viscosity of mucin for travel of the virus through mucous membranes. Human parainfluenza also contains a haemagglutinin sialidase that appears to operate in the same way as influenza. Paramyxoviruses too utilise a haemagglutinin sialidase, which functions to recognise sialic acid for cell attachment to allow viral attack. It also acts to cleave sialic acid for viral budding and release.

The virulence of bacterial sialidases has been recognised for some time now. In 1967, although no mechanism was determined, the sialidase of *C. diptheriae* was found to

be a virulence factor when it was shown that antiserum to the sialidase protected against infection (Moriyama and Barksdale 1967). This phenomenon has also been shown in mice that were treated with antiserum to the sialidase from *P. multocida* (Straus *et al.* 1996). The bacterial and parasitic sialidases are thought to be used by the organisms to scavenge carbohydrates from host cells for nutrition and survival. They may also be used to remove sialic acids from host glycoconjugates to facilitate the unmasking of receptors needed to colonise or invade host cells (Corfield 1992). Interfering with the hosts defence system, by decreasing the viscosity of mucin or altering the functions of immune and inflammatory mediators and cells is also a potential function the sialidase may undertake to be labelled a virulence factor (Corfield 1992).

C. perfringens is the cause of gas gangrene. In this infection wounds become less oxygenated due to the damage of surrounding blood vessels which leads to a contamination of the anaerobic bacteria (Cato *et al.* 1986). In the absence of oxygen the bacteria divide rapidly and release a cocktail of enzymes, including a sialidase to degrade the host structures. Sialidases are released in high amounts and cleave the terminal sialic acids on host cells to help cell invasion of the bacteria (Nees *et al.* 1975). Clinically, the high levels of sialidase in the wounds are used as an indicator for *C. perfringens* infection and as a marker for diagnosis (Roggentin *et al.* 1993b).

V. cholerae, an intestinal pathogen, colonises the gastrointestinal tract and excretes a potential enterotoxin to cause the disease cholera (Lockman *et al.* 1984). The toxin must bind to the ganglioside receptors (GM_1) to be internalised into the cells. The binding event causes an increase in cAMP which leads to inhibition of cell salt transport, causing an eflux of water into the colon and severe diarrhoea. Sialidases aid the pathogenesis of *V. cholerae* by removing sialic acid from higher order gangliosides, to unmask GM_1 the receptor site for cholera toxin to enable it to bind (Galen *et al.* 1992).

The sialidase from the *Actinomyces* species, responsible for periodontal disease is thought to unmask galactose residues on the host cell surface to permit binding of the bacteria (Teufel *et al.* 1989). Recently, *S. oralis* sialidase has been examined to determine if it may be a contributing virulence factor in endocarditis (Byers *et al.* 2000). Byers *et al.* determined that the sialidase is a component of a protein complex

and hypothesised that if this complex contains other glycosidic and proteolytic activities like *V. cholerae* sialidase, it may act to break down host glycoproteins. This activity may facilitate adhesion of the organism to epithelial surfaces and circulatory glycoproteins. It was also found that the enzyme has a high specificity for human sialic acids.

Trypanosome species such as *Trypanosoma cruzi* which causes Chagas disease possess sialidases and *trans*-sialidases on their cell surface for cell attachment. Since the parasite is unable to synthesise sialic acid it transfers it from the host to the parasite's own surface glycoproteins. The sialylation enables the parasite to invade host cells and evade the immune system (Ming *et al.* 1993; Buschiazzo *et al.* 1997; Traving and Schauer 1998).

In summary, sialidases have been determined to be virulence factors for a host of bacterial, viral and parasitic organisms. Information learned about the 3D structure of the influenza sialidase enzyme after its virulence had been determined led to the development of more effective treatments of the "flu" (MIST Group 1998; Nicholson *et al.* 2000; Dreitlein *et al.* 2001). As more knowledge is gained about the mechanisms of actions of the bacterial and parasitic sialidases they too may become useful targets for the design of novel chemotherapeutics.

1.3.6 Therapeutic potential and a history of rational drug design of sialidase inhibitors

Most research into the therapeutic potential of sialidases began with the influenza virus. Before any structural information was known, early research work focussed on finding an inhibitor that was based around the natural substrate sialic acid. Key functional groups were modified on sialic acid and other functional groups were added in a "hit and miss" approach. The most significant inhibitor to evolve from this work was 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en) (Figure 1-10) (Meindl *et al.* 1974). It contains a double bond in the ring which induces planarity that is purported to resemble the transition state of the hydrolysis reaction between it and the enzyme. The transition-state is a conformation where binding of a substrate is changed so that it fits with the highest energy into the binding site of the enzyme (Chong *et al.* 1991; Chong *et al.* 1992).

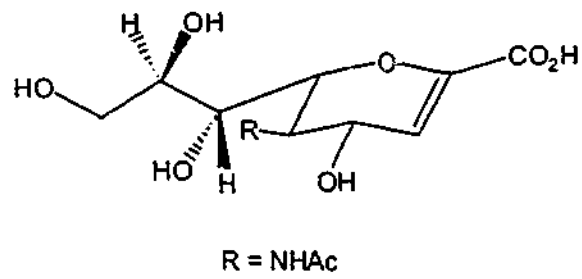


Figure 1-10 Structure of 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en)

The affinity of Neu5Ac2en to the influenza sialidase was measured at 4 x 10^{-6} M (Holzer *et al.* 1992). It was also found to inhibit other sialidases from bacteria and mammals in concentrations of about 10^{-6} M, which made it a non-specific inhibitor and not therapeutically effective. The challenge was to design an inhibitor with high specificity for the influenza virus sialidase that would also be active *in vivo*.

Elucidation of the influenza sialidase 3D structure facilitated the rational drug design of a sialidase inhibitor (Varghese *et al.* 1983). Influenza virus sialidase was an excellent target for the design of therapeutic agents against influenza as the active site of this enzyme contains a number of amino acid residues that are highly conserved and it was thought that resistance would be less likely to develop (von Itzstein *et al.* 1993). The crystallographically determined 3D structure of influenza virus sialidase was examined using the program GRID. The active site was analysed to explore whether any of the functional groups around sialic acid could be replaced with other functional groups that interacted more strongly with the sialidase (von Itzstein *et al.* 1993). The analysis revealed a pocket close to the hydroxyl group at the four position of sialic acid was lined by two glutamic acid residues which were conserved among all known strains of the influenza A and B viruses. Replacement of the hydroxyl group at the four position of Neu5Ac2en with an amino group was calculated by computer analysis to increase overall binding activity due to salt bridge formation with a carboxylic acid group of Glu119 (von Itzstein *et al.* 1993). It was also determined that replacement of that four position hydroxyl by the more basic guanidinyl group should further enhance binding affinity for the active site, due to lateral binding through the terminal nitrogens of the guanidino group with the two glutamic acid residues (Glu119 and Glu 227). Further studies of the active site of the influenza sialidase using GRID once again with various probes (amino, carboxy,

hydroxy, methyl, etc) also suggested that the addition of an amino functionality at the C4 position should increase overall binding (von Itzstein *et al.* 1996).

The 4-amino and 4-guanidino inhibitors were prepared though selective reduction and further functional analysis of 4-azido-4-deoxy-Neu5Ac2en (von Itzstein *et al.* 1993; von Itzstein *et al.* 1994). Crystallographic studies of the two compounds complexed to influenza sialidase were then completed. These analyses confirmed that for the 4-amino a salt bridge formed between the Glu119 and the 4-amino group. For the complex with the guanidino group it was confirmed that the guanidino group bound to Glu227 and the carboxylate of Glu119 was within 3.5Å of the other terminal nitrogen of the guanidinium moiety.

The direct measurement of the two compounds (ie the 4-amino and 4-guanidino derivatives) using the MUN assay (Chapter 2, Section 2.2.4), showed that they were high-affinity inhibitors for the influenza sialidase (inhibition constants of 5×10^{-8} and 2×10^{-10} M respectively) (von Itzstein *et al.* 1993). The 4-guanidino-4-deoxy-Neu5Ac2en is a slow binding inhibitor of influenza A and B, possibly due to conformational changes in either the protein or ligand to accommodate it. The slow-binding step of influenza sialidase and the 4-guanidino-Neu5Ac2en was found to involve the expulsion of a water molecule located in the active site to allow room for the large guanidino moiety (Pegg and von Itzstein 1994).

Cell culture tests performed to investigate inhibition showed that both the 4-amino and 4-guanidino derivatives were effective against both influenza A and B sialidases. They were also found to have very low IC_{50} values (concentration required to reduce plaque formation in MDCK cells by 50%) compared with amantadine, ribavirin and Neu5Ac2en (von Itzstein *et al.* 1993). Evaluation of the two inhibitors for anti-viral activity in animal models also proved promising. Intranasal administration of both inhibitors found that each eliminated viral shedding and decreased pyrexia. The effective dose of the 4-amino derivative was around 6 mg kg^{-1} twice a day and the dose of the 4-guanidino derivative was 50 μg kg^{-1} twice daily (around 1,000 times more effective than amantadine) (von Itzstein *et al.* 1993). Specificity tests also showed that unlike their parent compound, both were highly specific for influenza sialidase and were poor inhibitors of both nonviral and paramyxoviral haemagluttinin sialidases (Holzer *et al.* 1992).

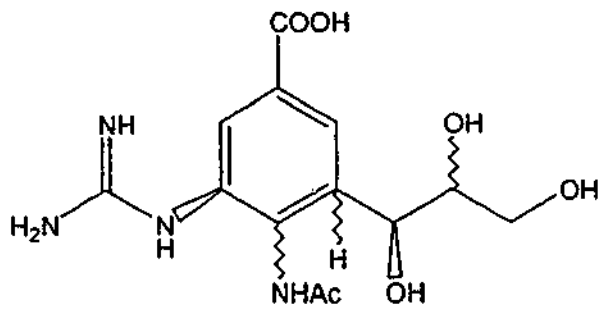


Figure 1-11 Structure of zanamivir

4-deoxy-4-guanidino-Neu5Ac2en (zanamivir) (Figure 1-11) was not orally active due to a short serum half-life and poor tissue distribution, however, it was selected for clinical trials as an orally inhaled antiviral. Zanamivir performed well in clinical trials when it was administered via inhalation or intranasally (MIST Group 1998; Dreitlein *et al.* 2001). It was shown to decrease viral shedding and influenza symptoms by up to two days in patients with clinical disease with minimal side effects. Although it was renally excreted, patients with some renal failure were able to use it without dose changes (Dreitlein *et al.* 2001). It is now commercially available as a powder for inhalation under the name of Relenza® and is given at a dose of 5 mg twice daily for five days. The advantages of inhalation are that the medication is working locally at the site of influenza infection and thus it minimises systemic side effects that often occur from oral administration. However, it is not always the most convenient dosage form, especially for young children and the elderly where respiratory function and co-ordination is not always at its optimum, thus research has continued to find an inhibitor that is orally bio-available.

In another approach, researchers moved away from the design of inhibitors based on a carbohydrate framework and instead designed inhibitors using a cyclohexene ring (Kim *et al.* 1997). This strategy was adopted to increase the lipophilicity of the inhibitors and therefore increase their bioavailability. It was proposed that the chemically simple cyclohexene ring could be readily functionalised so that suitable pharmacological properties could be easily optimised. Furthermore, the cyclohexene ring was determined to be chemically more stable and easier to modify for optimal antiviral and pharmacological properties. These workers synthesised many derivatives of cyclohexene and analysed them for those that most resembled the

planar transition state of the sialidase hydrolysis reactions. These compounds were then manipulated to find which side chains gave optimal inhibition at the active site of the sialidase using structure activity relationships (SAR) and X-ray crystallography. Analysis of the new cyclohexene family proved that the closer the structure is to the transition state, the more potent it is as an inhibitor (Kim *et al.* 1997).

Crystallographic examination of a number of cyclohexene derivatives with the influenza virus sialidase revealed the existence of a hydrophobic pocket in the region of the active site in which the glycerol side-chain of sialic acid usually resides. This hydrophobic pocket was exploited in the design of cyclohexene derivatives with the glycerol side-chain replaced by hydrophobic groups. Increased inhibition occurred (the IC_{50} of the enzyme being 3,700 nM for a CH_3 side chain, improving to 1 nM for the $(CH_3CH_2)_2CH$ side chain) (Kim *et al.* 1997). It was found that the 3-pentyl analogue was the optimal hydrophobic group for activity, and the addition of an ethyl ester to the acid group gave a compound that exhibited good oral bio-availability in test animals (mice, rats and dogs) and had good oral efficacy in the mouse and ferret influenza model (Kim *et al.* 1997). The addition of the ethyl ester to the 3-pentyl analogue gave a prodrug, which was rapidly converted to its active form after gastrointestinal absorption.

The prodrug, GS4104 (oseltamivir) (Figure 1-12), underwent clinical trials. Like its carbohydrate-like predecessor, zanamivir, treatment resulted in less viral shedding and alleviation of influenza symptoms faster than when untreated (Nicholson *et al.* 2000). Oral administration of oseltamivir led to the side effects of mild nausea and vomiting but this appears to resolve itself in one to two days (Nicholson *et al.* 2000). If gastrointestinal side effects were severe then the orally inhaled zanamivir may be the preferred treatment option. Like zanamivir, oseltamivir too is now available on prescription. It is sold under the trade name Tamiflu™ and is available as 75 mg capsules given twice daily for five days to treat persons with influenza over 13 years of age. It is renally excreted and being orally absorbed, the dose for patients with renal failure must be reduced to 75 mg once daily. This dose also has a prophylactic effect and can be given to persons at risk of influenza infection (Dreitlein *et al.* 2001). It is also available as a syrup for children.

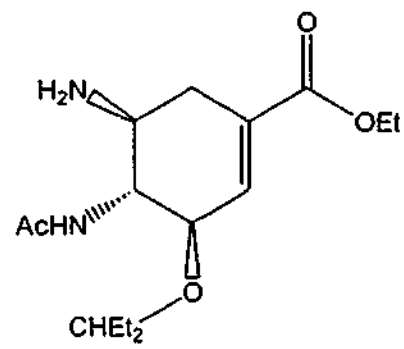


Figure 1-12 Structure of oseltamivir

Currently zanamivir and oseltamivir are the only sialidase inhibitors available for the treatment of influenza infection. Much focus still evolves around inhibitors for the treatment of influenza. New cyclopentane inhibitors have been designed with features of the parent compounds zanamivir and oseltamivir (Sidwell *et al.* 2001; Smee *et al.* 2001). These inhibitors have shown higher potency than either of zanamivir and oseltamivir and were effective and well tolerated in animal models at high doses (Sidwell *et al.* 2001; Smee *et al.* 2001). Other influenza sialidase inhibitors involving the glycerol side chain of zanamivir being replaced with a lipophilic carboxamide moiety have shown potent inhibition and selectivity for the influenza A virus (Wyatt *et al.* 2001).

Some work has looked into the design of sialidase inhibitors for bacterial infection. Mimetics of Neu5Ac2en based on Δ^4-β-D-glucopyranosiduronic acids were synthesised and found to inhibit both bacterial and viral sialidase although in this case the compounds were more potent towards viral sialidase (Florio *et al.* 1999). More recently, sialic acid based α-*C*-glycosides have been synthesised and those compounds that contained hydrophobic substituents (a common feature of sialidase inhibitors) at the 2-position of sialic acid gave high inhibition of *C. perfringens* sialidase (Wang *et al.* 2000). As more structural and functional knowledge is gained about the bacterial sialidases it is hoped that chemists will be able to design inhibitors that, like zanamivir and oseltamivir will be effective at treating the myriad of infections that organisms such as *V. cholerae*, *clostridium* species, and others can cause. The brief overview given here of current ongoing work illustrates that more effective inhibitors synthesised using SAR and rational drug design should not be too far away.

1.4 Definition of the Investigation

In the preceding sections it has been shown that the species of mycobacteria are able to cause a wide spectrum of disease worldwide and treatment of these organisms is difficult, lengthy and costly. TB for example is able to evade the immune system by surviving and replicating in alveolar macrophages, the very cells that are the main defence of the lung against this infection.

The discovery of a sialidase in some species of the organism could lead to a new approach for the treatment of the wide spectrum of diseases that mycobacteria are able to cause. Sialidases have already proven useful targets for treating the influenza virus that uses this enzyme to facilitate cell adhesion and infiltration. Perhaps mycobacteria are able to do the same by cleaving sialic acids that exist on the surface of the macrophage (Chmiela *et al.* 1997).

Research undertaken to produce inhibitors against some infectious microbial sialidases has the potential to lead to new treatments against the diseases caused by these micro-organisms. The following investigation aims to identify and biochemically characterise a potential enzyme containing sialidase-like activity from mycobacteria. It is hoped that the results obtained about the characteristics of this mycobacterial enzyme may lead to its identification as a novel chemotherapeutic target that in the future will make the treatment and severity of some mycobacterial infections a bleak memory of the past.

2 Preliminary Investigation of Putative Sialidases in Mycobacteria

2.1 General

The genome of *M. tuberculosis* had recently been solved (Cole *et al.* 1998). It was of interest to examine the genomic sequence for enzymes that may be amenable to inhibition. During examination of the genome, Rv3463 a gene with its function annotated as "a putative sialidase" was discovered. This was of interest as the laboratory was experienced with sialidases and it was chosen as a possible enzyme to screen for. *M. smegmatis* mc^2155 was chosen as the initial strain to examine as its growth rate is faster compared with other mycobacterial strains and it is rarely a pathogenic strain (Table 1-1). Another mycobacterial species, *M. bovis* BCG was also available to test. Although *M. bovis* BCG has a slower growth rate than. *M. smegmatis* mc^2155 it would be screened if any positive results were obtained with *M. smegmatis* mc^2155 to determine if it also contained a putative sialidase.

2.2 Materials and Methods

2.2.1 Bacterial strains and media

M. smegmatis mc^2155 and *M. bovis* BCG were kindly donated by Professor Ross Coppel from the Department of Microbiology, Monash University, Clayton.

Cells were propagated in two different media. All media was sterilised by autoclaving for 15 min at 121 °C unless otherwise stated. The Luria Bertaini broth (LB) was supplied by the Sigma Chemical Co. and was prepared according to manufacturers instructions. LB agar was prepared by the addition of 6 g of bacteriological agar (Sigma Chemical Co.) to 400 mL of the LB broth. Difco Laboratories supplied Middlebrook 7H9 media, which is a defined media, used specifically for the growth of mycobacteria (Appendix 1). It was also prepared according to the manufacturers instructions. After sterilisation the Middlebrook 7H9 media was supplemented with 0.05% v/v Tween 80 and a solution of glucose and sodium chloride (NaCl) (Jacobs *et al.* 1991) (Appendix 1). Both supplemented solutions were filter sterilised through a 0.2 μm acetate filter membrane (Millipore Australia). All cultures were incubated in a Ratek orbital mixer incubator at 37 °C.

2.2.2 Culturing

Single colonies of *M. smegmatis* mc^2155 were streaked onto LB agar plates and incubated at 37 °C under aerobic conditions for three days. After this time 10 mL liquid broths of Middlebrook 7H9 media containing 0.05% v/v of Tween 80 and enriched with the solution of glucose and NaCl were inoculated with fresh single colonies (Jacobs *et al.* 1991). Broths were incubated with shaking for three days at 37 °C to give late log to stationary phase cultures. Large Middlebrook 7H9 broths were then grown with the starter culture in a dilution of 1/100. They were incubated with shaking for 24 h at 37 °C.

After this time cells were harvested by centrifugation at 7,000 rpm for 15 min. The cell pellet was resuspended in phosphate buffered saline (PBS), pH 7.0 and sonicated on ice 15 times for 30 s intervals. To separate the cell debris and cell wall, the cells were centrifuged at 7,000 rpm for 30 min at 4 °C. The pellet consisting of cell debris and cell wall was stored at –20 °C. The supernatant (cell lysate) was either stored at -20 °C and subsequently assayed for sialidase activity or underwent further fractionation.

Fractionation separated the cell membrane from the cell cytosol. To achieve fractionation the cell lysate was centrifuged at 37,000 rpm for 2 h at 4 °C (Sorensen *et al.* 1995; Amara *et al.* 1998). The pelleted material consisted of the cell membrane and was solubilised with 50 mM sodium acetate buffer pH 6.0.

The protein content of all samples was measured using the Bio-Rad protein estimation reagents with bovine serum albumin (BSA) (CSL) as the standard (Bradford 1976).

2.2.3 Chemical reagents and equipment

For the fluorescence assay 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (MUN) was kindly synthesised and supplied by Dr Milton Kiefel from the Centre for Biomolecular Science and Drug Discovery Griffith University and Mr Pas Florio from the Department of Medicinal Chemistry, Monash University. The α(2,6)-sialyllactose was also synthesised and supplied by Dr Milton Kiefel for use in the thiobarbituric acid assay along with α(2,3)-sialyllactose, fetuin, mucin and colominic acid which were supplied by the Sigma Chemical Co. ICN chemicals supplied the

mixed bovine gangliosides for this assay. The periodate solution, arsenite solution, thiobarbituric acid reagent and butanol solution were prepared according to Aymard-Henry *et al.* (Aymard-Henry *et al.* 1973). Recipes for these reagents are given in Appendix 2. One unit of Type II *Vibrio cholerae* sialidase was purchased from Sigma Chemical Co. One unit is defined as liberating one μmol of sialic acid per minute at 37 °C.

N-acetyllactosamine (NAL) and ^{14}C *N*-acetyllactosamine used in the *trans*-sialidase and sialyltransferase assays were supplied by the Sigma Chemical Co. and Cytidine 5' monophosphate sialic acid (CMP-[^{14}C]-Neu5Ac) was purchased from NEN chemicals, USA. *Trans*-sialidase from *T. cruzi* was kindly provided by Dr M.E.A. Pereira from the New England Medical Centre Hospital, Department of Medicine, Boston, MA. 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactopyranose (βGal-D-1,3-D-GalNAc) was supplied by Sigma Chemical Co. and rat liver sialyltransferases were supplied by Calbiochem. ICN chemicals supplied both the Ecolite and Cytoscint scintillation fluids.

The affinity matrix for purification was kindly synthesised and made by Dr Milton Kiefel from the Centre for Biomolecular Science and Drug Discovery Griffith University, according to the methods published in Ciccotosto *et al* (Ciccotosto *et al.* 1998). The DEAE ion exchange matrix was from Pharmacia and the Fetuin agarose from Sigma Chemical Co. A Bio-Rad Econocolumn 1.0 x 120 cm was used for size exclusion chromatography. All other purification matrices were packed into Poly-Prep (0.8 x 4 cm) and Econo-Pac (1.5 x 12 cm) columns purchased from Bio-Rad. Protein purification columns were attached to an automated protein fraction system (Pharmacia) during all purifications and fractions concentrated with 50 and 250 mL Amicon stirred cell concentrators from Grace Company. The Miniprotean II gel electrophoresis unit from Bio-Rad was used for SDS-PAGE and also Western Blot transfers.

Fluorescence was measured in a Hitachi F-1200 fluorescence spectrophotometer. A Pharmacia Biotech Ultraspec 2000 spectrophotometer was used for protein estimation, culture optical densities and colorimetric assays. ^{14}C Counts for the *trans*-sialidase and sialyltransferase assays were measured in a United Technologies Packard 2000CA Tri-Carb liquid scintillation analyzer.

Unless otherwise stated all volumes of less than 1 mL were centrifuged in a benchtop Heraeus Biofuge 13. Samples greater than 1 mL were centrifuged in a Beckman Avanti (J-25 and 14) centrifuge. Cell fractionation at high speed was achieved in a Beckman Optima XL-100K ultra-centrifuge.

All other primary chemicals were supplied commercially and were of analytical grade.

2.2.4 Fluorescence assay

Primary activity was determined by a fluorometric assay that observes the hydrolysis of 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (MUN) (Potier *et al.* 1979). As previously discussed in Chapter 1, sialidases cleave terminal sialic acids that are α-ketosidically linked to glycoproteins, glycolipids and oligosaccharides. In this way sialidases will cleave the MUN to liberate the fluorescent metabolite 4-methyl umbelliferone (MU) and free sialic acid (Figure 2-1) (Potier *et al.* 1979).

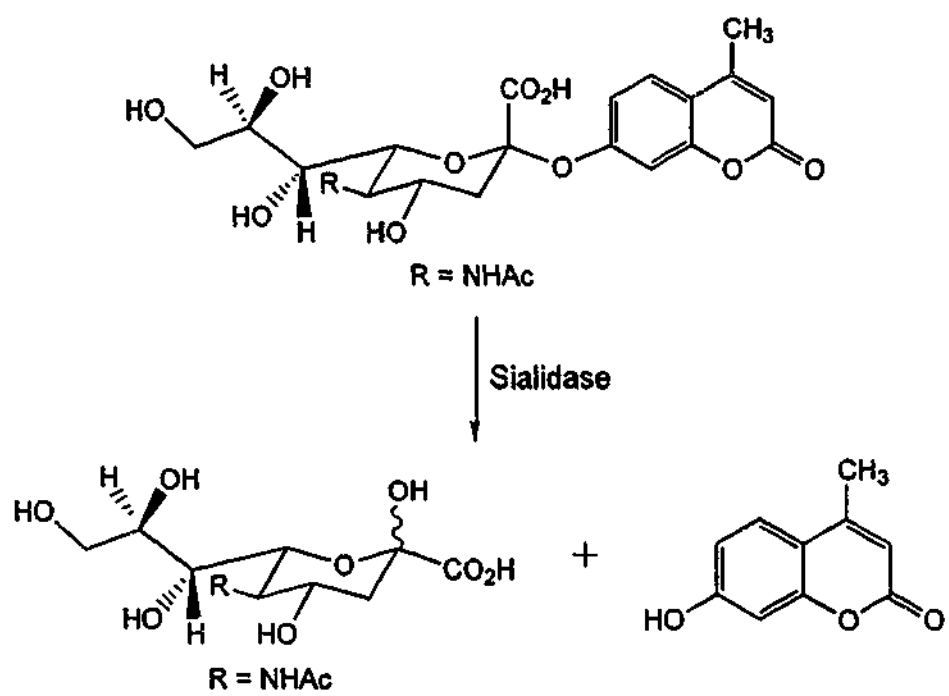


Figure 2-1: Chemical equation of MUN being cleaved by a sialidase to produce 4-methylumbelliferone (MU) and free sialic acid

A typical assay to test the mycobacterial sialidase consisted of up to 50 μl of enzyme solution, 35 μl of 50 mM sodium acetate buffer, pH 3.8 for crude cell lysate or pH 5.0 for partially pure enzyme, 15 μl of 1 mM MUN and water to a final volume of 100 μl. The reaction was incubated at 37 °C with shaking for up to 1 h. After this time the

reaction was stopped by adding 2.4 mL of 0.25 M glycine pH 10. Fluorescence emission was read at 400 nm after excitation at 365 nm in a Hitachi F-1200 fluorescence spectrophotometer. A negative control containing no enzyme and a positive control (*V. cholerae* sialidase in 50 mM 2-(*N*-morpholino)-ethane sulfonic acid (MES) containing 6 mM calcium chloride ($CaCl_2$) pH 6.5) were also assayed (Potier *et al.* 1979).

2.2.5 Colorimetric assay

The thiobarbituric acid assay or 'Warren method' was the original assay for testing for sialidase activity (Warren 1959; Moriyama and Barksdale 1967; Arden *et al.* 1972; Aymard-Henry *et al.* 1973). It is a colorimetric assay. This type of assay is not as sensitive as the MUN assay, thus weak enzyme activity may produce false negative readings (Potier *et al.* 1979). Although solutions must be highly active for this assay to work effectively, it has the advantage of being able to test the substrate specificity of the sialidase to sialic acid-linked compounds, including oligosaccharides, gangliosides (glycolipids), and glycoproteins. This is an advantage as sialidases may have a higher specificity for different linkages or sialyloglycoconjugates which cannot be determined using the fluorometric assay.

Substrate	Linkage	Stock concentration	Final concentration
α(2,3)-sialyllactose	α(2→3)	3.8 mM	0.75 mM
α(2,6)-sialyllactose	α(2→6)	3.8 mM	0.75 mM
Fetuin	α(2→3)	22.5 mg/mL	4.5 mg/mL
Mucin	α(2→6)	22.5 mg/mL	4.5 mg/mL
Colominic acid	α(2→8)	2.8 mg/mL	0.56 mg/mL
Mixed bovine gangliosides	Mixed linkages	7 mg/mL	1.4 mg/mL

Table 2-1: Substrate concentrations used in the thiobarbituric acid assay (Yamamoto and Nishimura 1987). When gangliosides were used a final concentration of 0.1% (w/v) Triton X was also added to the reaction mixture. The concentration of sialic acid in each of these substrates has been determined experimentally (see 2.2.6), and the results are found in Appendix 4.

As the enzyme activity in *M. smegmatis* was low, the thiobarbituric acid assay was slightly modified. A reaction consisted of up to 100 µl of enzyme, 100 µl of test substrate (Table 2-1), 50 µl of buffer (50 mM sodium acetate pH 3.8 (crude) or pH 5.0 (partially pure)) and distilled water to 250 µl. Reactions were incubated overnight (up to 22 h) at 37 °C with shaking. After this time the reaction was stopped by the addition of 250 µl of 5% phosphotungstic acid (PTA) in 2 M hydrochloric acid (HCl). Reactions were allowed to stand at room temperature for 20 min and any resultant precipitate removed by centrifugation (13,000 rpm for 5 min). A 200 µl aliquot of clear supernatant was taken and placed in a fresh tube. One hundred microlitres (100 µl) of periodate solution (Appendix 2) was added and the reaction placed at room temperature for another 20 min. After this time, 1 mL of arsenite solution (Appendix 2) was added and the reaction shaken to remove any colour. Three mL of thiobarbituric acid reagent (Appendix 2) was then added and the reaction shaken and placed in a boiling water bath for 20 min. Solutions were cooled in iced water for 5 min and then 4.2 mL of butanol solution (Appendix 2) was added and mixed well. The solutions were centrifuged for 5 min at 2,000 rpm and then the absorbance of the butanol layer was read on a spectrophotometer at 549 nm. *V. cholerae* sialidase was used (50 mM MES containing 6 mM $CaCl_2$) as a positive control, and a reaction without enzyme was used as a negative control. The negative control was used in all assays.

2.2.6 Quantitation of sialic acid from substrates

The thiobarbituric acid assay can also be used to quantitatively determine the amount of free sialic acid that results from the cleavage of the substrates by the enzyme. The final amount of free sialic acid was determined from a standard curve with known concentrations of sialic acid that was assayed using the thiobarbituric acid assay. The standard curve is found in Appendix 3.

In order to determine the proportion of sialic acid cleaved in the thiobarbituric acid assay the amount of bound sialic acid that the substrates contained before cleavage was determined using the Bial assay (Schauer 1978; Schauer and Corfield 1982; Manzi and Varki 1993). The Bial assay (ferric/orcinol) determines the amount of sialic acid that the substrate contains from its ability to form a blue-purple

chromophore. The chromophore is soluble in organic solvents when heated with orcinol/Fe^{2+} in concentrated HCl (Schauer 1978; Schauer and Corfield 1982; Manzi and Varki 1993).

Substrates (50 µl) at the concentrations in Table 2-1 were mixed with 50 µl of water and 100 µl of orcinol reagent (81.4 mL concentrated HCl, 0.2 g orcinol, 2 mL of 1% ferric chloride ($FeCl_3$) solution and water to 100 mL). The reactions were incubated at 95 °C for 15 min and then cooled on ice for 10 min. After this time 700 µl of amyl alcohol was added and the reactions vortexed. They were then centrifuged at 3,000 rpm for 5 min and the absorbance determined in a spectrophotometer at 572 nm. A standard curve for this experiment with known concentrations of sialic acid was determined (Appendix 4).

2.2.7 *Trans*-sialidase and sialyltransferase assays

The *trans*-sialidase and sialyltransferase assays were performed on *M. smegmatis* to determine if the mycobacterium contained an enzyme capable of transferring sialic acids to acceptors. *Trans*-sialidase enzymes transfer sialic acid molecules directly from one glycosidic linkage to another carbohydrate residue. The sialyltransferase however, adds sialic acids to terminal positions of the oligosaccharide chains of glycoproteins and glycolipids from the nucleotide sugar CMP-Neu5Ac, which acts as the donor molecule.

The *trans*-sialidase assay followed the method by Scudder *et al.* (Scudder *et al.* 1993). The reaction mixture consisted of 3.3 µl of 15.2 mM α(2,3)-sialyllactose (the donor substrate), 9.7 µl of *N*-acetyllactosamine (NAL) (the acceptor substrate) (containing 0.1 µl, approximately 40,000 CPM of ^{14}C NAL), 10-20 µl of *M. smegmatis* cell lysate or partially purified enzyme and 50 mM cacodylic acid buffer, pH 6.9, containing 0.02% sodium azide and 1 mg/mL of bovine serum albumin (BSA) to 50 µl. Reactions were incubated at 37 °C for 30 min and then stopped by the addition of 1 mL of water. The reaction solutions were then passed down glass pasteur pipette columns containing 500 µl of Q sepharose fast flow (Pharmacia) that had been equilibrated with 1 M sodium acetate solution and washed with water. The reactions were also washed three times with water and the flowthrough was discarded. The bound product was eluted from the column with 2.5 mL of 0.8 M ammonium acetate

and collected into scintillation vials. A volume of 10 mL of Ecolite scintillant was added to the reactions which were then counted in a scintillation counter. Purified *T. cruzi trans*-sialidase was used as a positive control and a reaction containing no enzyme was used as a negative control.

The sialyltransferase assay measures the enzymatic transfer of labelled sialic acid to a synthetic acceptor, βGal-D-1,3-D-GalNAc or NAL. βGal-D-1,3-D-GalNAc will accept sialic acid at a α(2,3) position to produce βGal-D-1,3-D-GalNAc-2,3-Neu5Ac and NAL at a α(2,6) position to produce NAL-2,6-Neu5Ac. The modified assay was based on that developed by Wen *et al.* and Williams *et al.* (Wen *et al.* 1992; Williams *et al.* 1995). Briefly, each reaction contained 10 μl of 20 mM βGal-D-1,3-D-GalNAc or NAL, 0.5 μl of CMP-[^{14}C]-Neu5Ac (0.74 MBq/mL), 5 μl 50 μM CMP-Neu5Ac, 0.2 μl of 100 mM adenosine triphosphate (ATP), 4.7 μl of 400 mM HEPES buffer containing 32 mM $MnCl_2$ pH 7.5, *M. smegmatis* fractions and water to 50 μl. The reactions were incubated at 37 °C for 2 h for the α(2,3) reaction (βGal-D-1,3-D-GalNAc) and for 1 h for the α(2,6) (NAL). Reactions were terminated by the addition of 1 mL Dowex suspension (100g in 500 mL water) (Bio-Rad), thoroughly vortexed and centrifuged at 13,000 rpm for 5 min. Six hundred μl (600 μl) of the supernatant was then transferred to scintillation vials and mixed with 3 mL of Cytoscint scintillation fluid. Counts were determined using a scintillation counter.

Rat liver sialyltransferases were used as the positive control and a negative control containing all substrates and buffers without the addition of a sialyltransferase was used.

2.2.8 Protein purification methods

To obtain pure and active protein from the *M. smegmatis* cell lysates, various purification strategies were attempted. All procedures were completed at 4 °C unless otherwise stated. Before any purification procedure was used, ammonium sulfate precipitation was completed to decrease the amount of contaminants. Ammonium sulfate was added to the *M. smegmatis* cell lysate at 80% saturation with stirring for 5 h. After this time the mixture was centrifuged at 7,000 rpm for 30 min. The resulting pellet was redissolved in PBS and the activity tested using MUN. Unfortunately, a large amount of enzymatic activity was lost at this step and dialysis of this sample

against 50 mM sodium acetate pH 6.2 to remove the salts resulted in complete loss of activity. The loss of sialidase activity from dialysis has been reported during the purification of influenza virus sialidase and was thus not considered an unusual result (Chong *et al.* 1991). Ammonium sulfate precipitation and the dialysis approach were therefore abandoned and a new culture was grown for purification.

The first method of choice for the purification of the enzyme was size exclusion chromatography; a method that separates proteins on the basis of their size. A Sephacryl S-100 (Pharmacia) gel filtration column (1.0 x 1.2 cm Bio-Rad Econocolumn) equilibrated with 50 mM sodium acetate pH 6.0 was used. To maximise activity, the *M. smegmatis* cell lysate was concentrated using an Amicon 50 mL stirred cell concentrator. One millilitre of cell lysate containing approximately 25 mg of protein was loaded onto the column and 5 mL fractions were collected overnight in the same buffer. Protein estimation of the collected fractions was achieved by measuring the absorbance at 280 nm on a spectrophotometer. Those fractions with similar apparent protein content and of similar size were pooled and concentrated using the Amicon 50 mL stirred cell concentrator. The concentrated fractions were then tested for sialidase activity using the MUN assay. A 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to observe the purity of the concentrated fractions (200 V for 45 min).

As explained in Section 2.3.2, the activity and purity of the enzyme was not high from this purification approach and therefore an affinity chromatography method was attempted. The rationale behind this approach was to obtain a pure protein from one single step and to eliminate the multi-step purification processes that were utilised previously (Ada *et al.* 1961; Miyagi and Tsuiki 1985; Kobayashi *et al.* 2000). Affinity chromatography has long been considered the most specific method for protein purification as it is based upon the unique specificity inherent in an enzyme-substrate interaction (Narayanan 1994).

A volume of 0.5 to 1 mL of crude *M. smegmatis* cell lysate or active fractionated lysate, containing approximately 5 mg of protein was loaded onto an affinity column equilibrated with 50 mM sodium acetate buffer, pH 6.0 containing 5 mL bed volume of *N*-acetylneuraminic acid attached to epoxy-activated Sepharose 6B (Pharmacia) by a metabolically stable thioglycosidic linkage (Ciccotosto *et al.* 1998). The

thiosialosides are thought to be resistant to hydrolysis by sialidases, thus attaching Neu5Ac to the Sepharose 6B matrix with such a linkage should ensure that the matrix was stable to the enzyme being purified (Suzuki *et al.* 1990; Ciccotosto *et al.* 1998). After loading the enzyme onto the column, the column was washed with 50 mM sodium acetate buffer, pH 6.0 until all unbound protein was eluted. The enzyme itself was eluted with a linear gradient of NaCl in the same buffer (ranging from 50 mM increasing to 500 mM NaCl). Twenty mL fractions were collected and concentrated on ice to 1-2 mL using Amicon 250 mL and 50 mL stirred cell concentrators. Concentrated fractions were subsequently tested for sialidase activity using MUN and examined for purity using 12% SDS-PAGE.

Other methods to purify the enzyme were also attempted. Namely, DEAE (Pharmacia) ion exchange chromatography and affinity chromatography using the α(2,3)-linked glycoprotein, fetuin (Sigma Chemical Co.). The method used for both of these matrices was the same as the affinity chromatography experiment explained previously. The rationale and results for both of these columns is discussed in Section 2.3.2.

2.3 Results and Discussion

2.3.1 Screening cell lysates for enzymatic activity: primary results

Originally, *M. smegmatis* was grown in LB broth. The crude cell lysate, supernatant and a sample of sterile LB broth incubated under the same conditions as the bacteria were assayed using the MUN assay. In this assay, the buffer used was 50 mM 2-(*N*-morpholino)-ethane sulfonic acid (MES) containing 6 mM calcium chloride ($CaCl_2$), identical to the procedure for *V. cholerae* sialidase (Section 2.2.4) (Potier *et al.* 1979). The samples were incubated for 30 min at 37 °C. *V. cholerae* sialidase was used as a positive control. Results for this experiment showed low sialidase activity in all test samples. The cell lysate had the lowest activity and the supernatant the highest. The media alone also gave a positive result which was thought to have been due to the yeast content in the broth. This result made it difficult to determine whether or not *M. smegmatis* had sialidase activity. Accordingly, the growth media was changed to Middlebrook 7H9 (Difco) which is a defined media containing no components that could mimic or contain a sialidase activity (Appendix 1).

The MUN assay was repeated on the cell lysate, supernatant, a sample of the sterile Middlebrook 7H9 media incubated in the same manner as the bacterial culture and a sample of non-sterile Middlebrook 7H9 media. In this assay only the cell lysate recorded a positive result indicating that *M. smegmatis* possessed some ability to cleave MUN. As the activity was weak the incubation time was increased to one hour. Different buffers and pH's were investigated to enhance the sialidase activity. The optimum pH for the crude *M. smegmatis* was determined to be pH 3.8 (Table 2-2), which is much more acidic than the optimal pH for *V. cholerae* sialidase. This pH was used for all subsequent experiments associated with the crude enzyme. After determining the optimum pH *M. bovis* BCG was also grown in Middlebrook 7H9 and its crude cell lysate assayed for the ability to cleave *N*-acetylneuraminic acid from MUN. Like *M. smegmatis*, *M. bovis* BCG also gave a positive result for sialidase activity however the observed activity was not as high as that of *M. smegmatis*.

pH	% Activity
3	85
3.4	90
3.6	91
3.8	100
4	78
7.1	68

Table 2-2: The % activity of crude *M. smegmatis* cell lysate at different pH.

It is interesting to note that previous research in this field had stated that no sialidase activity was apparent for mycobacteria (Arden *et al.* 1972; Raynaud *et al.* 1998). A report of sialidase activity in an organism called *Actinomyces bovis* ATCC 19009 along with many other *Actinomyces* species had however been published (Moncla and Braham 1989; Teufel *et al.* 1989; Yeung and Fernandez 1991). This is significant since, as mentioned in Chapter 1, mycobacteria belong in the *Actinomycetes* class.

The finding of the sialidase activity in *M. smegmatis* and *M. bovis* BCG was therefore not unusual for this genus.

All crude fractionated sections of *M. smegmatis* were assayed for sialidase activity using an acidic buffer and MUN. It was found that the cell wall did not contain a sialidase activity. The cytosol and membrane however did contain sialidase activity. Further centrifugation and fractionation was unable to separate the sialidase activity into one fraction. The specific activity was also similar for both fractions however, the membrane activity was always more stable and was retained longer than that of the cytosol. It was thus concluded that the activity was cell bound and not secreted, but its location within the cell is still unknown. It is believed that it may be membrane bound but point into the cytosol in which case it would be useful in a nutritional role. This would be a logical assumption, as *M. smegmatis* is rarely pathogenic.

The disadvantage of using MUN is its susceptibility to degradation in the presence of light and extremes of pH or temperature. Therefore, the crude cell lysate of *M. smegmatis* was also assayed using the thiobarbituric acid assay with α(2,3)-sialyllactose, as the substrate. *M. bovis* BCG was not assayed using this method as its activity was too low. This assay which utilises any sialylglycoconjugate was performed to support the findings for MUN to detect sialidase activity. Sialylglycoconjugates are generally not susceptible to degradation by light, and are less likely to degrade from extremes of pH and temperature.

The assay was modified from the previous assays used to evaluate sialidase activity in mycobacteria (Arden *et al.* 1972; Uchida *et al.* 1974; Raynaud *et al.* 1998). Before adding the cell lysate to the reaction, it was concentrated to a small volume to increase its protein concentration and subsequent activity. The lysate was also added into the assay in the maximal volume of 100 μl. Other modifications to the assay included incubating the reaction overnight (previously it had only been incubated for 4 h (Arden *et al.* 1972), or 30 min (Uchida *et al.* 1974), and using an acidic buffer at pH 3.8, compared with less acidic buffers (Arden *et al.* 1972; Uchida *et al.* 1974). Positive results were obtained for the *M. smegmatis* cell lysate, although the activity was low.

As the activity for both the *M. smegmatis* and *M. bovis* BCG cell lysates were low, it was thought that the enzymatic activity may be due to *trans*-sialidase or sialyltransferase activities which often have cross-over activity with sialidases. Both enzymatic activities were evaluated for using their respective assays with *M. smegmatis* cell lysate (Section 2.2.7). Neither *trans*-sialidase nor sialyltransferase activity was found. Both experiments were repeated with longer incubation times and more acidic buffers but this did not improve the activity which was significantly lower than the negative controls. It was therefore concluded that the enzymatic activity determined in *M. smegmatis* was due to the cleavage of *N*-acetylneuraminic acid glycosides and not transfer. The enzyme was most likely a sialidase or an enzyme that exhibits sialidase-like activity.

2.3.2 Purification of the *M. smegmatis* enzyme

M. smegmatis was selected as the organism to purify as it had shown higher activity than *M. bovis* BCG and was a faster grower, with cells available for harvest and purification within one week compared with one month. As briefly mentioned in Section 2.2.8, the first purification method of the *M. smegmatis* was size exclusion chromatography. Pooled and concentrated fractions were analysed and it was found that sialidase activity albeit weak, was located in only some fractions. Some separation of the proteins was shown to occur, as sialidase activity was not found in all fractions. Unfortunately, analysis of the degree of purification by 12% SDS-PAGE (Figure 2-2) showed that no single fraction was clean. It was therefore necessary to use another purification method.

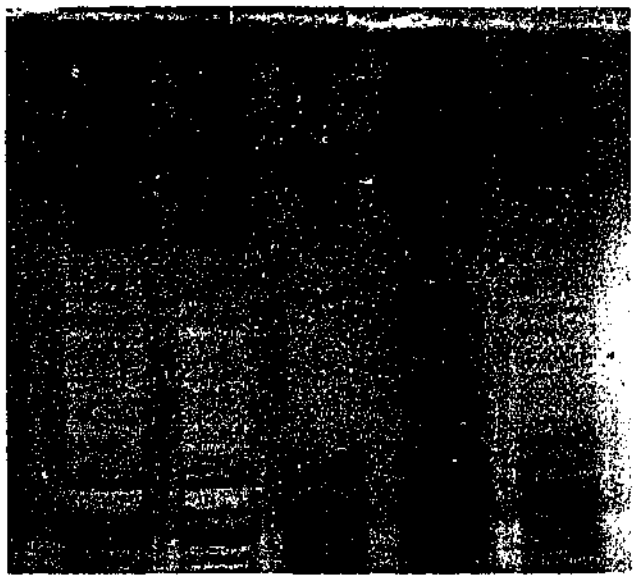


Figure 2-2: 12% SDS-PAGE showing the impure fractions from Sephacryl S-100 gel filtration.

There had been some success within the von Itzstein group with the purification of sialidases using affinity matrix columns (Ciccotosto *et al.* 1998). This approach was adopted in an attempt to purify the *M. smegmatis* sialidase-like activity. Purified fractions assayed with MUN were shown to have a large increase in activity compared with the crude fractions. Specific activity had risen by over 1,000 times in some cases. Repeat purifications using the column also verified this trend (Table 2-3). Affinity chromatography using the same affinity matrix to purify rat cytosolic sialidase was also shown to increase enzymatic activity (unpublished results: von Itzstein, Abo & Alafaci).

Analysis of the purity of the enzyme posed another problem. Although there was high activity from the purified samples, the amount of protein in most samples was so low that visualisation on a SDS-PAGE gel after silver staining was not possible (Figure 2-3). The protein purity or its approximate molecular weight was therefore not determined.

Date	Sample Type	Fraction (salt elution)	Specific Activity (U/mg)	Increase in activity from load
12/8/99	Crude cell lysate	Load	0.228	n/a
		100 mM salt	42.920	188 x
		100 mM salt	32.340	142 x
		300 mM salt	48.100	210 x
13/9/99	Crude cell lysate	Load	0.177	n/a
		100 mM salt	23.000	130 x
		300 mM salt	40.961	232 x
		500 mM salt	57.507	325 x
05/10/99	Crude cell lysate	Load	0.247	n/a
		100 mM salt	97.918	396 x
		300 mM salt	70.859	287 x
		500 mM salt	41.939	170 x
10/03/00	Fractionated membrane	Load	0.225	n/a
		*100 mM salt	≥395.380	≥1757 x
		*300 mM salt	≥105.440	≥468 x
		*500 mM salt	≥105.440	≥468 x
23/11/00	Fractionated membrane	Load	0.100	n/a
		50 mM salt	1040.00	1.04×10^4 x
		100 mM salt	550.000	5500 x
		300 mM salt	620.000	6179 x

Table 2-3: Selected fractions from a range of purifications show the large increase in specific activity in the MUN assay after purification on the affinity matrix. *Samples were so active that they were off-scale therefore the activity was calculated using the highest value on the fluorimeter with the protein content. Protein content was calculated using the Bio-Rad protein estimation system (Section 2.2.2)

Other purification methods were then attempted to obtain an active band on an SDS-PAGE that could be visualised. The first alternative method attempted was DEAE ion exchange chromatography. Crude *M. smegmatis* cell lysate was loaded onto this column in the same manner as the affinity column and eluted with a gradient of NaCl (50-500 mM NaCl). Fractions were analysed on a 12% SDS-PAGE and were found to contain clean single bands. Unfortunately, none of the bands contained sialidase activity. The purification was repeated with the addition of BSA and $CaCl_2$ to the

buffer to see if like *V. cholerae* sialidase (Potier *et al.* 1979), they stabilised the enzyme, but no activity was recovered.

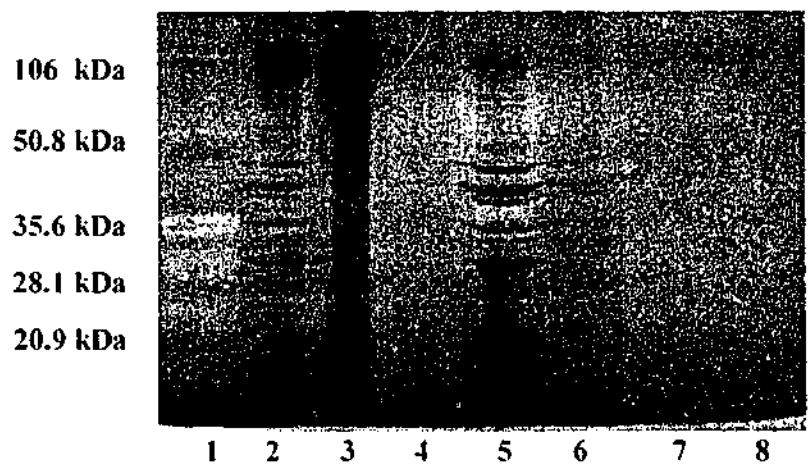


Figure 2-3: 12% SDS-PAGE of purified *M. smegmatis* cell lysate from the affinity column. Lane 1 is the molecular weight markers, 2 is the crude cell lysate before purification, 3 and 4 are two buffer washes to remove unbound protein, 5 is a 50 mM salt elution, 6 is a 100 mM salt elution (where high activity is usually found), 7 is a 300 mM salt elution and 8 is a 500 mM salt elution.

The final method of purification utilised another affinity matrix, fetuin agarose. Fetuin is a α(2,3)-linked glycoprotein. It was already known that the putative sialidase was able to cleave the α(2,3)-linkage of sialyllactose in the thiobarbituric acid assay thus this protein may have been able to bind to fetuin. The cell lysate was loaded and eluted with a NaCl gradient as before. Analysis by protein estimation, the MUN assay and SDS-PAGE showed that none of the lysate had bound and all protein had been washed through the column in the flowthrough. This may have been due to the enzyme having no affinity for the fetuin and thus not binding. Alternatively, the enzyme may have had high affinity for the fetuin and cleaved the sialic acid from the column completely so that there was no substrate left to which it could successfully bind to the column. As the traditional biochemical methods had failed to varying degrees it was decided that taking a molecular approach and cloning the sialidase gene may improve further characterisation of the activity. In this way the protein could be overexpressed and easily purified using a hexa-his tag attached to the protein, as had been previously successful for the purification of *C. perfringens* sialidase (Kruse *et al.* 1996).

3 Genetic Manipulation and Analysis of Mycobacteria and Sialidases

3.1 General

Due to the difficulties of purifying the *M. smegmatis* putative sialidase using the methods described in the previous chapter, it was decided that cloning the putative gene may improve the expression and purification of the enzyme. At this time the sequence of both the *M. smegmatis* and *M. bovis* BCG genomes were in the early stages of determination so it was decided that the cloning should proceed with genes from *M. tuberculosis* for which the genome sequence was complete (Cole *et al.* 1998).

The cloning method chosen had been previously published by Kruse *et al.* (Kruse *et al.* 1996) for the "small" 1.4 kb (42 kDa) sialidase of *C. perfringens*. In summary, the method involves cloning the sialidase gene into a vector containing a hexa–histidine affinity tag (His_6) which is translationally fused to the *N*-terminus of the protein. Expression of the recombinant gene is controlled by a vector-based promoter which can be induced with isopropylthio β-D-galactoside (IPTG). To detect expression of the protein after induction, a Western Blot using anti-hexa-His antibodies can be performed. If the protein has expressed it should be readily purified using metal chelate chromatography. To determine if the expressed protein was a sialidase it would be assayed using MUN as was described in Chapter 2 (Section 2.2.4).

The difficulty of this experiment for the *M. tuberculosis* sialidase was that there was no confirmed sialidase gene identified on the genome. To overcome this problem a group of well known bacterial, viral and mammalian sialidase sequences were used to identify a likely "panel" of potential *M. tuberculosis* sialidases. The closest matches between the bacterial sialidases and the *M. tuberculosis* genome were cloned. The *C. perfringens* sialidase was also cloned as a positive control. Clones were expressed, assayed for sialidase activity and tested for expression using Western Blots. If clones contained a sialidase activity they were purified using metal affinity columns.

3.2 *Materials and Methods*

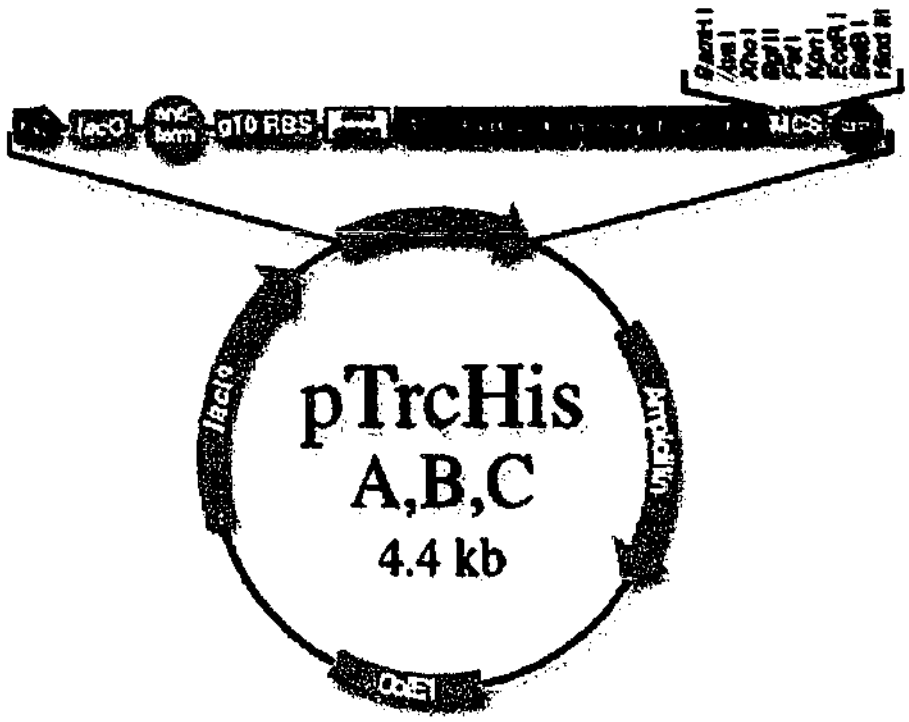
3.2.1 Strains and Plasmids

E. coli BL21 (DE3) pLysS and *E. coli* XL1-Blue MRF' were obtained from Stratagene. Both bacteria were propagated in SOB media (Appendix 5) and prepared as competent cells for electrotransformation of plasmid DNA (Appendix 6).

The *C. perfringens* clone (*E. coli* DH5α pUC19) was kindly donated by Dr. Julian Rood, Department of Microbiology at Monash University (Roggentin *et al.* 1988; Kruse *et al.* 1996). The vector, pTrcHisA from Invitrogen Pty Ltd (Figure 3-1) was transformed into *E. coli* XL1-Blue MRF'.

M. tuberculosis H37Rv, *M. tuberculosis* 01117727, *M. tuberculosis* 01118961 and *M. smegmatis* ATCC 607 were propagated and supplied by Dr Janet Fyfe at the Victorian Infectious Diseases Reference Laboratory (VIDRL). The *M. tuberculosis* cells obtained from VIDRL were harvested for 15 min at 4,000 rpm and the pellets were resuspended in PBS (Section 2.2.2). Cells were sonicated on ice once for 10 min and twice for 5 min in a GenProbe Bransonic ultrasonic cleaner. They were then pelleted at 13,000 rpm in a benchtop centrifuge (Heraeus) and filtered through 0.2 μm acetate filter membranes (Millipore) to remove any unlysed cells. Dr Helen Billman-Jacobe from the Department of Microbiology and Immunology, University of Melbourne, extracted the *M. tuberculosis* H37Rv genomic DNA by the method detailed in Appendix 7.

E. coli was propagated at 37 °C in Luria Bertaini (LB) broth (Sambrook *et al.* 1989), or on LB agar (Section 2.2.1). After electrotransformation which was performed in a Gene Pulser II from Bio-Rad, cells were grown in SOC medium (Appendix 5) (Sambrook *et al.* 1989). Media was supplemented with filter sterilised ampicillin (100 mg/L) (Sigma Chemical Co.) for selection of pTrcHis vectors. One hundred mM isopropylthio-β-D-galactoside (IPTG) was used to induce gene transcription from the pTrcHisA vector.



pTrcHis A

pTrcHis forward primer

RBS

Mini Cistron

360 AAAATTAAAG AGGTATATAT TA ATG TAT CGA TTA AAT AAG GAG GAA TAA
ACC

Met Tyr Arg Leu Asn Lys Glu Glu ***

ProBond binding domain

412 ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr

Gene 10 leader

Anti-Xpress™ Antibody Epitope

EK recognition sequence

EK cleavage site

460 GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG ▼GAT
Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp

Bam HI *Xho* I *Sac* I *Bgl* II *Pst* I *Kpn* I *Eco* RI *Bst* BI

508 CGA TGG GGA TCC GAG CTC GAG ATC TGC AGC TGG TAC CAT ATG GGA ATT
Arg Trp Gly Ser Glu Leu Glu Ile Cys Ser Trp Tyr His Met Gly Ile

Hind III

556 CGA AGC TTG GCT GTT TTG GCG GAT GAG AGA AGA TTT TCA GCC TGA
Arg Ser Leu Ala Val Leu Ala Asp Glu Arg Arg Phe Ser Ala ***

Figure 3-1 The pTrcHis vector map and cut sites in the multiple cloning site (MCS)

3.2.2 Chemical Reagents and equipment

Restriction enzymes *Hind*III, *Bam*HI, *Pst*I and *Eco*RI were obtained from Amersham Pharmacia Biotech. *Taq* polymerase and its corresponding buffer were both purchased from Amersham Pharmacia Biotech. *Pfx*, platinum buffer, 50 mM $MgSO_4$ and enhancer solution were obtained from Gibco BRL. Dinucleotide tri-phosphate (dNTP's) (containing dATP, dCTP, dGTP, dTTP) (10 mM) were supplied by Amersham Pharmacia Biotech and T4 ligase 3 U/µl and its ligase buffer were from Promega.

The Talon® resin was obtained from Clontech Laboratories and the antibodies used in the Western blot were Tetra-His™ antibodies from Qiagen and anti-mouse affinity isolated HRP conjugated antibodies from Silenus laboratories. The immunofluorescence kit used was the BM Chemiluminescence Blotting substrate (POD) from Roche. The PCR machine used was Perkin Elmer Gene amplification PCR system. DNA purification systems used included the Geneclean II® kit from Bio 101 Inc. and the High Pure Plasmid Isolation Kit® from Roche. The former was used to purify DNA from agarose gels and the latter kit was used to isolate highly pure DNA for nucleotide sequencing reactions. For general plasmid DNA isolation, minipreparations were used. These are described in Appendix 8 (Birnboim and Doly 1979; Sambrook *et al.* 1989).

All other equipment and chemicals are listed in Chapter 2 and were of analytical grade.

3.2.3 Bioinformatics

A group of bacterial sialidases had previously been analysed with regard to structure and motifs (Roggentin *et al.* 1993a). Roggentin *et al.* determined that sequence homology between bacterial sialidases was quite poor (commonly around 20%), but all contain Asp boxes (SXDXGXTW) and significant structural amino acids such as the RIP motif (as detailed in Chapter 1, Section 1.3.3). Using this information, each of these bacterial sialidases was aligned with the *M. tuberculosis* H37Rv genome using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1997), specifically tblastn and tblastp from the National Centre for Biotechnology

Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Sialidase structures were obtained from Entrez and Genbank in the NCBI website. Known viral and mammalian sialidase sequences were also compared to the *M. tuberculosis* H37Rv genome.

Resulting alignments were analysed for Asp boxes and significant structural amino acids such as the RIP motif. If significant amino acid content was high (> 20%), the corresponding *M. tuberculosis* gene sequence was identified. Both TubercuList from the Institut Pasteur, Paris and the *M. tuberculosis* database, from the Sanger Centre were accessed to obtain the full gene sequence:

http://genolist.pasteur.fr/TubercuList

http://www.sanger.ac.uk/Projects/M_tuberculosis

If the identified genes were not already assigned a function they were selected to be cloned

3.2.4 Amplification Methods

The following amplification methods were completed for the selected genes. The rationale for the use of these selected genes is explained in Section 3.2.3 and 3.3.1.

3.2.4.1 *C. perfringens*

Purified *C. perfringens* clone (*E. coli* DH5α pUC19) DNA was used as the template. The template DNA was linearised by digestion for 1 h at 37 °C with *Hind*III to reduce supercoiling. A reaction mix containing 1 μg of template DNA, 20 pmol of both a forward and reverse primer (Figure 3-4), 1 μl of 10 mM dNTP's, 5 μl of 10-fold concentrated polymerase buffer containing 15 mM $MgCl_2$, 2.5 U of *Taq* polymerase DNA and water to a final volume of 50 μl was made. The reaction underwent 35 cycles of the PCR. The cycles consisted of denaturation (95 °C for 30 s), annealing (46 °C for 30 s) and extension (72 °C for 1 min).

3.2.4.2 Rv3463

The template DNA was obtained from *M. tuberculosis* H37Rv. A reaction mix containing 100 ng of template DNA, 20 pmol of both a forward and reverse primer (Figure 3-5, 1a) and 1b)), 1 μl of 10 mM dNTP's, 5 μl of 10-fold platinum PCR

amplification buffer, 1.5 µl of 50mM $MgSO_4$, 2.5 U *Taq* polymerase DNA, 2.5 µl of 10 times *Pfx* enhancer solution and water to a final volume of 50 µl was used. Thirty five cycles of PCR were carried out that consisted of denaturation (96 °C for 30 s), annealing (56 °C for 30 s) and extension (72 °C for 30 s).

3.2.4.3 Rv1733c

The template DNA was from *M. tuberculosis* H37Rv. A reaction mix containing 100 ng of template DNA, 20 pmol of each primer (Figure 3-5 2a) and 2b)), 1 µl of 10 mM dNTP's, 5 µl of 10-fold concentrated buffer containing 1.5 mM $MgCl_2$, 2.5 U *Taq* polymerase DNA and water to a final volume of 50 µl was used. Thirty five cycles of denaturation (96 °C for 30 s), annealing (56 °C for 30 s) and extension (72 °C for 30 s) were applied.

3.2.4.4 Rv1968

The template DNA was from *M. tuberculosis* H37Rv. A reaction mix containing 100 ng of template DNA, 20 pmol of each primer (Figure 3-5 3a) and 3b)), 1 µl of 10 mM dNTP's, 5 µl of 10-fold platinum PCR amplification buffer, 1.5 µl of 50mM $MgSO_4$, 0.25 µl *Pfx*, 10 µl of 10 times *Pfx* enhancer solution and water to a final volume of 50 µl was used. This reaction underwent touchdown PCR which consisted of an initial denaturing cycle (95 °C for 2 min), denaturation (96 °C for 30 s), annealing for 30 s starting at 60 °C and decreasing by 0.5 °C each cycle until it reached a temperature of 50 °C, and extension (68 °C for 1 min). The reaction then underwent a further 20 cycles of denaturation (95 °C for 30 s), annealing (50 °C for 30 s) and extension (60 °C for 1 min).

3.2.5 Cloning

3.2.5.1 Purification of the DNA fragments

The *M. tuberculosis* H37Rv genomic DNA used in Section 3.2.4 for amplification of the genes was extracted by Dr Helen Billman-Jacobe using the method detailed in Appendix 7.

The *C. perfringens* clone (*E. coli* DH5α pUC19) was used as the DNA template for amplification of the *C. perfringens* sialidase gene (Section 3.2.4.2). It was grown overnight at 37 °C in 10 mL LB broth containing ampicillin. The template DNA was then purified from the clone via a DNA minipreparation based on an alkaline lysis procedure explained in Appendix 8 (Birnboim and Doly 1979; Sambrook *et al.* 1989).

Similarly, the pTrcHisA plasmid vector DNA was also purified from an overnight 10 mL LB and ampicillin culture of *E. coli* XL1-Blue MRF' using the DNA minipreparation procedure (Appendix 8) (Birnboim and Doly 1979; Sambrook *et al.* 1989). Following minipreparation, the pTrcHisA DNA was digested for 2 h at 37 °C with either *Bam*HI and *Pst*I or *Bam*H1 and *Eco*R1. It was then electrophoresed on a 1% agarose gel and stained with ethidium bromide. After electrophoresis the pTrcHisA DNA was purified from the agarose using the Geneclean II® kit from Bio 101 Inc.

3.2.5.2 Purification of the PCR products

To purify the PCR products they were initially precipitated at –20 °C for 20 min after the addition of 0.1 volumes of 3 M sodium acetate and 2 volumes of 100% ethanol. After this time the mixes were centrifuged at 13,000 g for 30 min at room temperature. The precipitated DNA was washed twice with 70% ethanol and resuspended in sterile distilled water.

The suspended PCR DNA then underwent digestion for 2 h at 37 °C with *Bam*HI and *Pst*I (*C. perfringens* and Rv3463 PCR product) or *Bam*H1 and *Eco*R1 (Rv1733c and Rv1968 PCR product). Each of these were then electrophoresed on a 1% agarose gel and stained with ethidium bromide. After electrophoresis the PCR DNA was purified from the agarose using the Geneclean II® kit from Bio 101 Inc.

3.2.5.3 Ligation

The purified PCR amplified DNA of all genes was individually ligated to *Bam*HI/*Eco*RI or *Bam*HI/*Pst*I digested pTrcHisA vector in a 15 µl reaction containing 1.5 U of T4 ligase, 10 fold concentrated ligase buffer, and water to volume. The reaction was incubated overnight at 15 °C and was stopped the following day by heat inactivation at 65 °C for 10 min.

3.2.5.4 Transformation

The ligated PCR DNA and vector of the *C. perfringens* sialidase and Rv3463 were transformed into electro-competent *E. coli* BL21 (DE3) pLysS cells (Appendix 6) by electrotransformation at 2.5 kV, 200 Ω resistance and 25 μF capacitance. Ligated Rv1733c and Rv1968 PCR DNA and vector were introduced into electro-competent *E. coli* XL1-blue MRF' cells in the same manner. Cells were resuspended in 1 mL of SOC broth and allowed to recover for 1 h at 37 °C before being plated onto LB ampicillin plates. The plates were incubated overnight at 37 °C.

3.2.5.5 Analysis of the clones

Overnight colonies were individually re-plated onto LB ampicillin plates and incubated overnight at 37 °C. Colonies were then grown overnight in 10 ml LB and ampicillin liquid cultures. Potential clones were analysed by extraction of plasmid DNA using DNA minipreparation as described previously (Appendix 8) (Birnboim and Doly 1979; Sambrook, Fritsch *et al.* 1989), with the addition of a phenol chloroform extraction step for the *C. perfringens* and Rv3463 clones (Appendix 9) (Sambrook *et al.* 1989). The DNA was once again digested for 2 h at 37 °C with *Bam*HI and *Pst*I or *Bam*HI and *Eco*R1 and analysed on a 1% agarose gel. If the inserted band of the correct size appeared in the stained gel it confirmed insertion of the gene.

Rv3463, Rv1733c and Rv1968 recombinant plasmids were also sequenced. *C. perfringens* was not sequenced due to its high amount of sialidase activity after protein expression (refer to Section 3.3.3). High purity DNA was needed for sequencing reactions, so Rv3463 DNA was extracted from an overnight 10 mL LB and ampicillin broth of *E. coli* BL21 (DE3) pLysS by minipreparation and phenol chloroform extraction. The DNA was transformed into electro-competent *E. coli* XL1-Blue MRF' cells (at 1.8 kV, 200 Ω resistance and 25 μF capacitance). The transformed cells were allowed to recover in 1 mL of SOC broth at 37 °C for 1 h and were then plated onto LB ampicillin plates and grown overnight at 37 °C.

Single colonies of Rv3463 were plated to purity on LB ampicillin plates. Rv1733c and Rv1968 clones were already cloned into *E. coli* XL1-Blue MRF' so they too were

streaked to purity as described for Rv3463. All three were then inoculated into 10 mL LB and ampicillin broths and grown overnight at 37 °C.

The High Pure Plasmid Isolation Kit® from Roche was used to purify the plasmid DNA. Three mL of each overnight culture was pelleted by centrifugation at 13,000 rpm for 5 min and the supernatant discarded. The cell pellets were resuspended in 250 µl of resuspension buffer containing RNAse. 250 µl of lysis buffer was then added and the tubes mixed gently and incubated at room temperature for 5 min. After this time 350 µl of chilled binding buffer was added and gently mixed. The tubes were incubated for 5 min on ice and then centrifuged for 10 min. The resulting pellet was discarded. The remaining supernatant was transferred to a Roche filter tube and centrifuged again for 1 min. The filtrate was discarded and 500 µl of wash buffer was added to the filter tube and it was once again centrifuged for 1 min. This step was repeated with 700 µl of wash buffer. The clean DNA was eluted from the filter tube by the addition of 100 µl of elution buffer accompanied by centrifugation for 1 min.

A sample of the resulting DNA was run on a 1% agarose gel to determine the quantities of DNA recovered, as a typical sequencing reaction needs up to 500 ng of plasmid DNA.

For each gene, two reactions were made, the first containing the pTrcHis forward primer and the second containing its reverse primer (Figure 3-6). Each reaction was made according to the Walter and Eliza Hall Institute (WEHI) "Big dye Terminator reaction protocol" and contained up to 500 ng of plasmid DNA, 3.2 pmol of pTrcHis primer, 8 µl of Terminator Mix and water to 20 µl:

(http://www.wehi.edu.au/dsl/BDTprotocol.html#Precipitate).

The thermocycler sequencing reaction consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min.

The reaction products were purified and precipitated for analysis. Each reaction was mixed with 20 µl of distilled deionised water and 60 µl of 100% isopropanol and incubated at room temperature for 15 min to precipitate the extension products. After this time the extension products were pelleted by centrifugation at 13,000 rpm for 20 min. The supernatant was discarded. The pellet was rinsed with 250 µl of 75% isopropanol, vortexed and centrifuged for 5 min at 13,000 rpm. The supernatant was

then aspirated and the resulting pellet allowed to dry. The pelleted extension products were then taken to WEHI who performed gel sequencing. The results were analysed from sequence chromatograms obtained from WEHI using BLAST searches and Sequencher™.

3.2.6 Expression

E.coli transformants containing the correct gene insertion from previous analysis of the clones (Section 3.2.5.5), were inoculated into 10 mL LB and ampicillin broths and grown at 37 °C overnight. One hundred µl (100 µl) of the overnight culture was inoculated into another 10 mL LB and ampicillin broth. This was grown for 2 to 4 h at 37 °C until an OD of 0.6 had been reached. After this time gene expression was induced by the addition of IPTG to a final concentration of 1 mM and further incubated for another 3 to 4 h. Cells were harvested by centrifugation at 3,000 rpm for 10 min at 4 °C.

A Western Blot was performed using anti-His antibodies to test for protein expression. A 12% SDS-PAGE gel was run with samples of the crude lysates from the clones loaded into the gel at a concentration of approximately 15 ng of protein per well. The pTrcHisA vector was used as a negative control and the *C. perfringens* clone as a positive control. After the gel had run, the proteins were transferred to a PVDF membrane. The membrane was washed twice with TBS buffer (4.38 g NaCl, 3.03 g Tris and water to 500 mL pH 7.4) for 10 min each. The membrane was then blocked with TBS-T (TBS containing 0.5 mL Tween 20 per litre) and 1% BSA for 1 h at room temperature. After this time the membrane was probed for 1 h at room temperature with a 1 in 1,500 dilution of Tetra-His™ antibodies (Qiagen) (3 µg of antibody in total) in TBS-T with 1% BSA. It was then washed 3 times for 10 min each with TBS-T and probed with secondary antibodies (anti-mouse HRP antibodies, 1 in 3,000) in TBS-T with 1% BSA for 1 h at room temperature. The bands on the membrane were then detected using the BM Chemiluminescence Blotting Substrate kit (Boehringer Mannheim) according to the manufacturer's instructions.

3.2.7 Cell lysis

The *C. perfringens* sialidase was extracted using lysozyme buffer (25 mM Tris, 2 mM EDTA 3 mg/mL lysozyme) (Kruse *et al.* 1996). Four mL of buffer was added for

every gram of wet weight pelleted cells. After addition of the buffer the cells were allowed to incubate with shaking for 1 h at room temperature. The sialidase was then harvested by centrifugation at 11,000 g for 10 min at 4 °C and was located in the supernatant (Kruse *et al.* 1996). To improve sialidase collection the addition of lysozyme buffer to the pellet was repeated. The sialidase was stabilised by the addition of 1 mM dithiothreitol (DTT) and stored at 4 °C until needed.

The protein from the cloned TB genes (Rv3463, Rv1733c and Rv1968) was extracted by five 30 s cycles of sonication on ice.

3.2.8 Purification using Talon chromatography

If the Western Blot (Section 3.2.6) showed reactivity to the His antibody with the approximately correct size, the proteins were then purified.

Before purification, proteins were dialysed overnight in elution buffer (EB; 300mM NaCl and 50 mM sodium phosphate pH 7.0) to remove the EDTA. The clones were then purified using a metal affinity resin called Talon®, which binds polyhistidine–tagged proteins. The pTrcHisA vector contained a hexa-his sequence, which produces a hex-his tag attached to the *N*-terminal of the protein after expression.

The Talon® resin was equilibrated with the EB buffer. Proteins were mixed with the resin and gently agitated at room temperature for 1 h to allow protein binding. After this time the protein and resin complex was placed in a 10 mL column connected to a fraction collector (Pharmacia). Once the mix had settled EB buffer was washed through the column at a flow rate of 0.5 mL/min until all unbound proteins had been eluted. Elution of the bound protein was achieved by the use of an increasing imidazole gradient in EB buffer. Concentrations ranged from 100 to 500 mM of imidazole and 5 mL fractions were collected. Once again all similar fractions were pooled and concentrated together using a 50 mL Amicon stirred cell concentrator. The fractions were then analysed for sialidase activity and purity using the MUN assay and a 12% SDS-PAGE.

3.2.9 Sialidase assays

The extracted *C. perfringens* sialidase was assayed for activity using MUN, as previously described (Potier *et al.* 1979; Kruse *et al.* 1996). Briefly, the reaction mix

contained 10 µl of enzyme, 80 µl of 50 mM sodium acetate buffer pH 6.1 and 10 µl of 1 mM MUN. The reaction was incubated for 10 min at 37 °C with shaking.

The cloned TB genes were assayed with MUN in the same manner as the *M. smegmatis* crude cell lysate in Chapter 2 (Section 2.2.4). After the appropriate times the reactions were stopped by the addition of 2.4 mL of 0.25 M glycine pH 10.2 and read in a fluorimeter at 400 nm after excitation at 365 nm. In all assays, strains harbouring the pTrcHisA alone were used as negative controls.

3.2.10 TB isolate testing

After the gene cloning had been completed for *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv, *M. tuberculosis* 01117727, *M. tuberculosis* 01118961 and *M. smegmatis* ATCC607 from Dr Janet Fyfe (VIDRL) were obtained. H37Rv was a laboratory strain and the other *M. tuberculosis* strains had recently been isolated from patients with active TB infection. *M. smegmatis* ATCC 607 was used as a control to test if it also possessed sialidase activity like *M. smegmatis* mc^2155. All of the above bacteria were assayed with MUN in the same way as *M. smegmatis* mc^2155 and *M. bovis* BCG (Chapter 2, Section 2.2.4). Incubation times used were 1 and 2 h. The addition of 1% final concentration of BSA or 1 mM Ca^{2+} was tested to see if they were stabilising agents for the enzyme as they are for *V. cholerae*. The buffer was used at pH 3.8, 5.0 and 6.0.

3.2.11 *M. smegmatis* mutant library screen

Ms Dharshini Jeevarajah and Dr. Helen Billman-Jacobe from the Department of Microbiology and Immunology at the University of Melbourne had created a *M. smegmatis* mutant gene library. Screening the mutant bacterial colonies for lack of sialidase activity may determine the gene that coded for the enzyme in *M. smegmatis*. The library contained thousands of mutants, thus it was impractical to screen colonies using the traditional MUN assay (Chapter 2, Section 2.2.4). It was therefore necessary to devise more rapid methods to screen the library.

The first method was to spray colonies grown on agar plates with MUN and incubate them in the dark at 37 °C for 1 h or more to detect fluorescence (Roggentin *et al.* 1988). As a positive control, the *C. perfringens* clone from Dr. Julian Rood was

tested as this method had previously been successful for this enzyme (Roggentin *et al.* 1988). The clone was grown, harvested and extracted in the same method as the re-cloned *C. perfringens* sialidase gene (Section 3.2.6 and 3.2.7). The cell lysate was tested for sialidase activity using MUN (Section 3.2.9). The cloned *C. perfringens* sialidase was found to have extremely high activity. It was then grown overnight at 37 °C on LB ampicillin agar plates and sprayed the following day with MUN as described. After 1 h incubation, the plates were sprayed with 0.25 M glycine, pH 10 and examined for fluorescing colonies under UV light at 350 nm. If fluorescing colonies could be visualised the method would be repeated using *M. smegmatis* mutant library in the same manner.

The second method was a scaled-down MUN assay in a microplate as a higher amount of colonies could be screened. This method had also been used previously (O'Brien and Mitsuoka 1991). The difficulty was lysing mycobacterial cells in a microplate. Mycobacterial cells usually need sonication to be successfully lysed which cannot be done in a microplate thus, alternative cell lysis procedures were attempted.

To test different lysis methods, Middlebrook 7H9 broths were inoculated with *M. smegmatis* and grown for 3 days at 37 °C. They were subcultured into the same media and grown overnight at 37 °C. The cells were then aliquotted into 1 mL microcentrifuge tubes and pelleted by centrifugation at 13,000 rpm.

Table 3-1 summarises the cell lysis procedures used.

	Lysis Method	Reference
1.	Whole cells washed with buffer *.	This study
2.	Cells were resuspended in 1 mL of buffer and sonicated on ice (15 x 30 s). Cells were centrifuged# and the supernatant assayed.	This study
3.	Cells resuspended in 1 mL of buffer and snap frozen with dry ice and ethanol. Cells were thawed at 65 °C for 5 min. Freezing and thawing was repeated 5 times. Cells were centrifuged and the supernatant assayed.	(Miller, Bryant *et al.* 1999)
4.	0.5 mL 40% sucrose and 1.5 mM EDTA added to cells. Cells were incubated at room temp. for 15 min and centrifuged. 0.5 mL of ice cold water was added and cells were incubated on ice for 10 min, centrifuged and the supernatant assayed	Osmotic Shock protocol from Qiagen
5.	Cells were resuspended in 0.5 mL of buffer and 0.5 mL of 10% SDS. They were incubated at room temp or 37 °C for 15 min, centrifuged and the supernatant assayed.	(Miller, Bryant *et al.* 1999)
6.	Cells were resuspended in 1 mL 0.2 M NaOH and 2.5% SDS in buffer and incubated at 100 °C for 5 min or 37 °C for 15 min. They were centrifuged and the supernatant assayed	(Gueriava, Izac *et al.* 1998)
7.	Cells were resuspended in 1 mL buffer with 1 drop of 10% SDS and 2 drops of chloroform added, incubated at room temp for 15 min, centrifuged and the supernatant assayed	(Sambrook, Fritsch *et al.* 1989)
8.	Cells were resuspended in 0.5 mL of buffer and 0.5 mL of acetonitrile, incubated at 65 °C for 10 min, centrifuged and the supernatant assayed	(Unson, Newton *et al.* 1998)

Table 3-1 Methods used to lyse M. smegmatis cells and release enzyme to cleave MUN. *The buffer used at all times was 50 mM Sodium Acetate buffer pH 6.0. # Cells were centrifuged at 7,000 rpm at 4 °C.

3.3 Results

3.3.1 Bioinformatics

It was necessary to identify a panel of putative sialidase genes from *M. tuberculosis* H37Rv to clone. The *C. perfringens* "small" 1.4 kb (42 kDa) sialidase had been previously cloned (Kruse *et al.* 1996) and was selected as the positive control.

As briefly mentioned in Chapter 2 (Section 2.1), Rv3463 (Appendix 11) had been given the annotation "function: probable neuraminidase" (Sanger Centre UK and TubercuList, the Institut Pasteur, Paris). Rv3463 sequence was aligned with a large selection of bacterial, viral and mammalian sialidases using BLAST. The Asp box

alone was also aligned with Rv3463. The sequence was not found to contain any similarity with the sialidase enzymes or motifs. Due to its annotation, Rv3463 was still chosen to be cloned so that its potential function could be investigated.

To determine if any other gene sequence belonging to the *M. tuberculosis* genome was a putative sialidase, the range of bacterial sialidases that were aligned and compared by Roggentin *et al.* (Roggentin *et al.* 1993a), and some selected viral and mammalian sialidases were aligned against all genes in the *M. tuberculosis* H37Rv genome. Atypical sialidases such as sialidase L (Chou *et al.* 1994) and the motif from KDN sialidase (SGDSAGIWVLSA) (Kitajama *et al.* 1994) were also aligned against the genome. The Asp box alone was used as a search sequence but no significant matches were obtained. Matches were analysed and compared for sequence similarity with particular interest in the binding domain, Asp boxes and other significant amino acids.

Significant matches included the KDN sialidase motif and *Micromonospora viridifaciens* to Rv1733c, *V. cholerae* sialidase to Rv1968, *M.viridifaciens* sialidase to Rv2264c and *M.viridifaciens* sialidase to Rv3512.

The KDN sialidase motif matched to a sequence in Rv1733c, a sequence that also had some similarity to *M. viridifaciens* sialidase (Figure 3-2). Rv1733c was also thought to be a membrane protein, which agreed with the preliminary activity results from *M. smegmatis* mc^2155 (Chapter 2, Section 2.3.1).

a) emb|AL123456|MTBH37RV Mycobacterium tuberculosis H37Rv complete genome
Length = 4411529

```
Score = 21.6 bits (44), Expect = 22
Identities = 9/12 (75%), Positives = 9/12 (75%)
Frame = -3

Query: 1          SGDSAGIWVLSA      12
                  SGD  GIWV SA
Sbjct: 1960080    SGDRVGIWVDSA      1960045
```

b) Query Mycobacterium tuberculosis H37Rv complete genome,
Subject sialidase GI585539, *M.viridifaciens*

```
Score = 25.1 bits (53), Expect = 299
Identities = 17/51 (33%), Positives = 22/51 (42%), Gaps = 4/51 (7%)

Query: 32         NPLVRGTDRLEAVVMLLAVTVSLLTI----PFAAAAGTAVQDSRSHVYAHQ
                  NP +R   R AV   LLA  ++  T+    P  A AG  V      +Y  Q
Sbjct: 4          NPYLRRLPRRRAVSFLLAPALAAATVAGASPAQAIAGAPVPPGGEPLYTEQ
```

Figure 3-2: a) the sequence alignment and identity of Rv1733c (subject) with the KDN sialidase motif (query) b) the sequence alignment and identity of Rv1733c with *M. viridifaciens* sialidase

Rv1968 contained a sequence match the *V. cholerae* sialidase (Figure 3-3). It was determined to be a gene that was part of the *mce*3 operon. The *mce* operons are a group of four operons within the *M. tuberculosis* genome that contain mammalian cell entry genes (*mce*). These genes as the name suggests were previously characterised as coding for proteins involved in the invasion of host cells (Cole *et al.* 1998; Brosch *et al.* 2000). As sialidases too have been used to unmask sialic acids from host cells to facilitate cell invasion and infection by other organisms, this made Rv1968 an interesting gene to examine.

emb|AL123456|MTBH37RV Mycobacterium tuberculosis H37Rv complete genome
Length = 4411529

```
Score = 33.6 bits (75), Expect = 0.17
Identities = 21/65 (32%), Positives = 31/65 (47%), Gaps = 2/65 (3%)
Frame = -1

Query: 248      GGDPGALSNTND--IITRTSRDGGITWDTELNLTEQINVSDEFDFSDPRP 295
                GGDPGAL       ++ R  R+GG   D +    + +  +  F F   RP
Sbjct: 2211755  GGDPGALVEKRQPLVLQRGQRNGGDDDDADCADGQTVAFGEGFHFGAHRP

Query: 296      IYDPSSNTVLVSYARWP
                +  P+S    + Y  WP
Sbjct: 2211581  LS*PAS----LMYTGWP
```

Figure 3-3: The sequence alignment and identity of Rv1968 (subject) with the *V. cholerae* sialidase enzyme (query).

Both Rv2264c and Rv3512 were excluded from cloning. Rv2264c is a proline rich protein which would make it too difficult to clone, and Rv3512 was already identified as a PE PGRS protein. PE PGRS (Rv3512) belongs to a group of unusual proteins that are unique to mycobacteria which would make it unlikely to be a sialidase.

Table 3-2 summarises the genes that were selected to be cloned. Protein and amino acid sequences of the chosen genes and their expressed proteins can be found in Appendix 10.

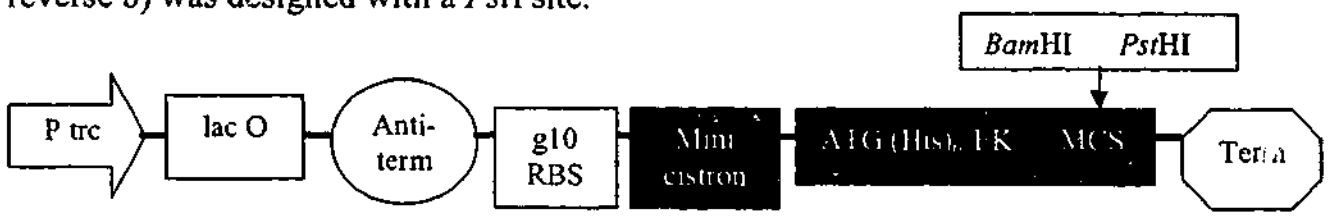
Name	Description	Organism of sialidase match
Rv3463 (Mw: 30.652 kDa)	Function: probable neuraminidase	None determined Sanger suggest a match with Influenza A
Rv1733c (Mw: 22.459 kDa)	Probable membrane protein	KDN sialidase, *M. viridifaciens*
Rv1968 (Mw: 43.673 kDa)	Part of *mce*3 operon	*V. cholerae*

Table 3-2: The *M. tuberculosis* genes selected to clone

3.3.2 Cloning of sialidase candidates

3.3.2.1 The PCR primers

In order to amplify the gene for insertion into the pTrcHisA vector the primers shown in Figure 3-4 for the *C. perfringens* sialidase were synthesised. The forward primer a) was based on the *Bam*HI cleavage site from Kruse *et al.* (Kruse *et al.* 1996) and the reverse b) was designed with a *Pst*I site.



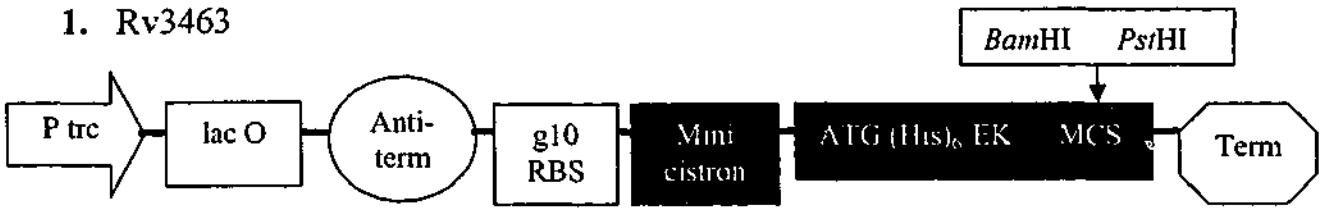
a) 5′ GGG G↓GA TCC ATG TAA CAA AAA CAA TAC C 3′
*Bam*HI

b) 5′ GGG CTG CA↓G TTA TTG TTT ATT AAT TAG TGA 3′
*Pst*I

Figure 3-4: Primers synthesised for the amplification of the *C. perfringens* sialidase. a) The forward primer containing a *Bam*HI cleavage site and b) The reverse primer containing a *Pst*I restriction site.

The primers for Rv3463, Rv1733c and Rv1968 were designed in a similar fashion to the *C. perfringens* sialidase. Each was designed with the forward primer containing a *Bam*HI site. The reverse primer for Rv3463 contained a *Pst*I site and the reverse primer for both Rv1733c and Rv1968 contained an *Eco*R1 site. The computer program "Sequencher™" was utilised to map each genes cut sites. The resulting primers are shown below in Figure 3-5.

1. Rv3463



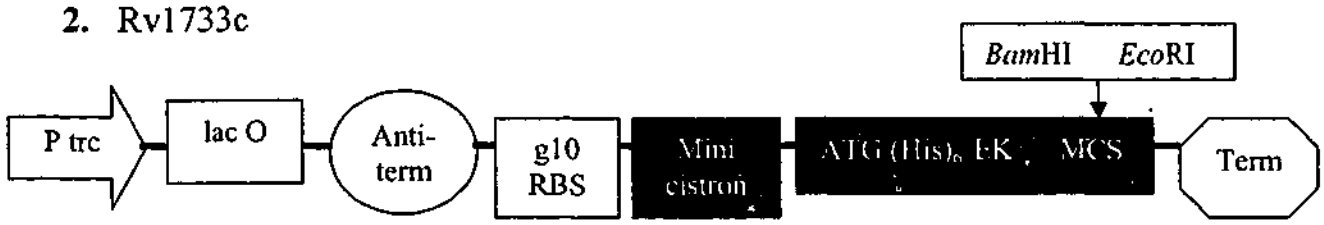
a) 5′ AAA G↓GA TCC ACC AAT TGT GCC GCC GGC 3′

*Bam*HI

b) 5′ AAA CTG CA↓G TCA GTC GGA GCG GCT TCG 3′

*Pst*I

2. Rv1733c



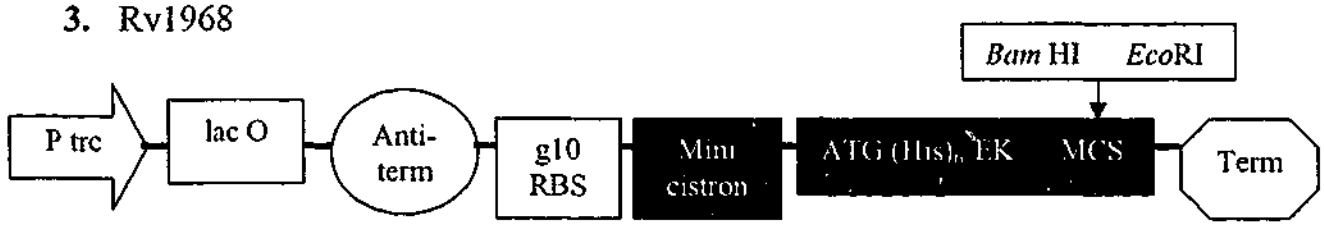
a) 5′ AAA G↓GA TCC ATC GCC ACA ACC CGC GAT C 3′

*Bam*HI

b) 5′ AAA G↓AA TTC TCA CCG CTG CGT GCA GAA C 3′

*Eco*RI

3. Rv1968



a) 5′ AAA G↓GA TCC CTG CAA TAC CAG CGG CTG 3′

*Bam*HI

b) 5′ AAA G↓AA TTC TCA CGG TTG GTC TCC CG 3′

*Eco*RI

Figure 3-5: Forward and reverse primers designed for PCR of the chosen genes. In all cases a) is the forward and b) is the reverse.

Rv1968 contained a large hydrophobic signal peptide sequence of 30 residues at the *N*-terminal of the protein. This was taken into consideration during primer design and the sequence was not included. The start codon for the primer was located where the sequence for the predicted mature protein was determined to begin.

The pTrcHisA primers were also designed for the sequencing reaction detailed in Section 3.2.5.5 (Figure 3-6).

a) 5′ GGC TAG CAT GAC TGG TGG 3′

b) 5′ GAC CGC TTC TCG GTT CTG 3′

Figure 3-6: pTrcHisA primers for sequencing a) the forward primer and b) the reverse primer

3.3.2.2 PCR amplification results

From the amplification methods detailed in Section 3.2.4, a PCR product of 1.16 kb was produced for *C. perfringens*, a 1 kb PCR product was obtained for both Rv3463 and Rv1968 and a PCR product of 600 base pairs was obtained for Rv1733c. These products are shown in (Figure 3-7a)-d)). Figure 3-8 represents the standard base pair markers used in the agarose gels to determine the size of the PCR products.

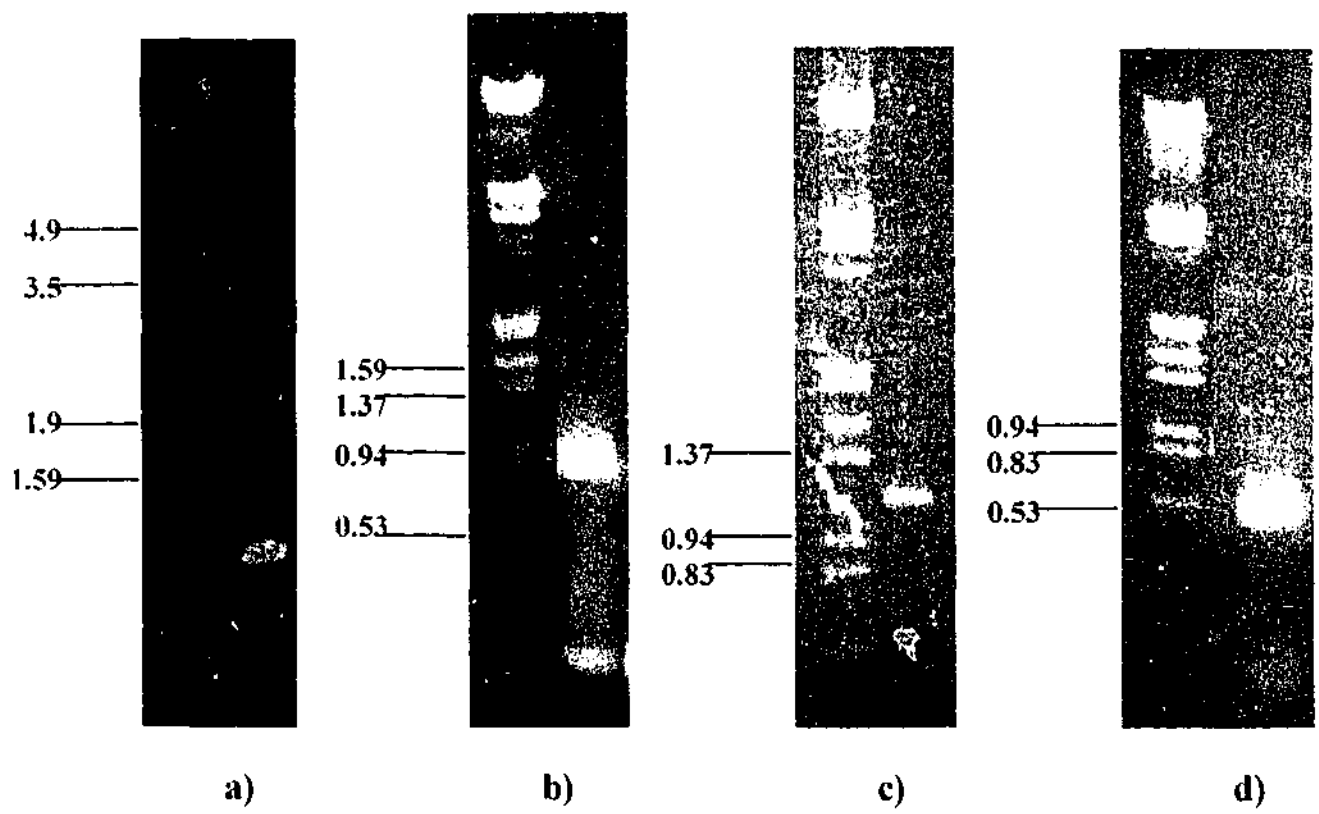


Figure 3-7 PCR amplification products of a) *C. perfringens*, b) Rv3463, c) Rv1968 and d) Rv1733c

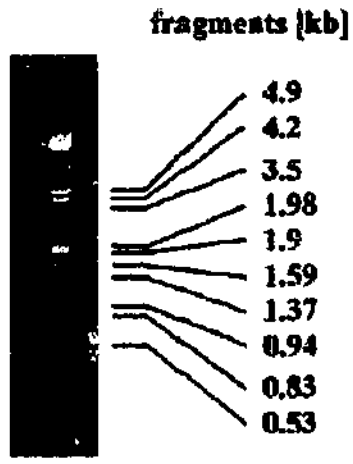


Figure 3-8 Base pair markers

3.3.2.3 Gene sequencing

The genes were sequenced to ensure that they did code for the correct sequence. The sequencing chromatograms and cut site results can be found in Appendix 11. In summary, Rv3463, Rv1733c and Rv1968 were found to be in frame with the pTricHisA hexa-his sequence, no other sequence errors were observed. Sequencher™ was used to determine the enzymatic cut sites and they were all found to be correct and in the predicted positions. These results indicated that the inserted gene sequences of the DNA were correct.

3.3.3 Expression and purification of putative sialidases

Eleven colonies from the *C. perfringens* cloning (positive control) were expressed. Following induction with IPTG their cell lysates were extracted and assayed for sialidase activity (Sections 3.2.7 and 3.2.9). All expressed protein from the chosen colonies were shown to produce sialidase activity (Table 3-3).

.Sample	Activity (mU)	Result
Blank 1 (water)	49	negative
Blank 2 (lysozyme buffer)	45	negative
Negative control (pTricHisA)	86.5	negative
Colony number 1	1235	positive
Colony number 2	Off scale	positive
Colony number 3	1180	positive
Colony number 4	785	positive
Colony number 5	1050	positive
Colony number 6	966	positive
Colony number 7	1042	positive
Colony number 8	1048	positive
Colony number 9	990	positive
Colony number 10	1016	positive
Colony number 11	Off scale	positive

Table 3-3 *C. perfringens* sialidase assay results

The pTrcHisA vector was transformed into *E. coli* and was used as a negative control. When it was grown and harvested in the same way as the *C. perfringens* clone it was shown to have no sialidase activity (Table 3-3).

C. perfringens sialidase was then purified using the Talon® resin as explained in Section 3.2.8 and it was shown that a high degree of purification could be achieved (Figure 3-9).

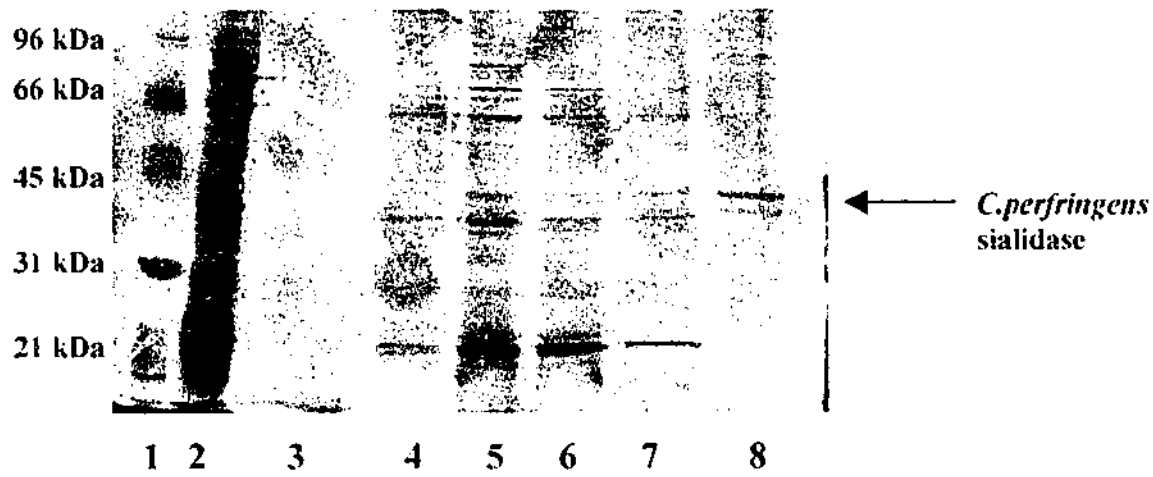


Figure 3-9: 12% SDS-PAGE of the Talon® purification of the *C. perfringens* sialidase. Lane 1 are the molecular weight markers, 2 is the crude cell lysate before purification, lanes 3-5 are washes with EB, Lanes 6 and 7 are 100 mM imidazole elutions and 8 is a 200 mM imidazole solution. Lane 8 contains a relatively pure band in the 45 kDa range which is the sialidase that can only be seen faintly in other lanes.

Rv3463, Rv1733c and Rv1968 clones were induced with IPTG as previously explained. Cells were harvested and lysed with sonication. The crude cell lysates were assayed for sialidase activity in the same assay explained in Chapter 2 (Section 2.2.4). None of these clones were found to possess the ability to cleave MUN.

An SDS-PAGE was run of the crude protein extracts to detect expression of the proteins from Rv3463, Rv1733c and Rv1968. *C. perfringens* was run as a positive control as it had already been shown to express a sialidase with high activity (Table 3-3). Unfortunately, in the crude protein extracts, detection of the expressed proteins was not possible, even for *C. perfringens* (Figure 3-9 and Figure 3-10).

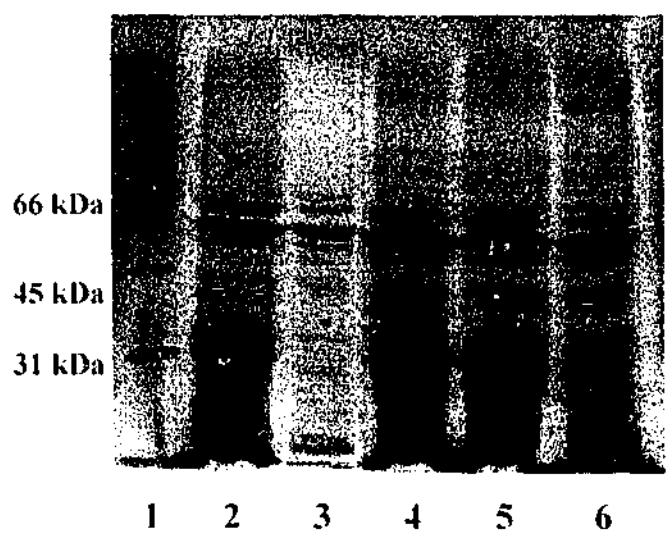


Figure 3-10: Shows that the expression of the genes is poor compared with the negative control pTrcHisA which appears to contain all of the same bands. All samples are the crude cell lysates. Lane 1 are the molecular weight markers, 2 is pTrcHisA, 3 is *C. perfringens*, 4 is Rv3463, 5 is Rv1733c and 6 is Rv1968.

The expressed protein from the clones were then probed with anti-His antibodies in a Western Blot to determine if expression had occurred. The Western Blot would also enable a determination of the approximate size of the expressed protein for comparison with the predicted size. *C. perfringens* sialidase was used as the positive control and the pTrcHisA vector as the negative control.

Figure 3-11 shows the results of the Western Blot. From this Figure it can be seen that all proteins except Rv1733c were expressed and of approximately the correct size. All should be 3 kDa larger than their usual molecular weights (Table 3-2), due to the additional His tag. In this instance it appeared that proteins of the correct size were being expressed for all but Rv1733c, which did not express well. As there was no sialidase activity in Rv3463 and Rv1968 the proteins were not purified.

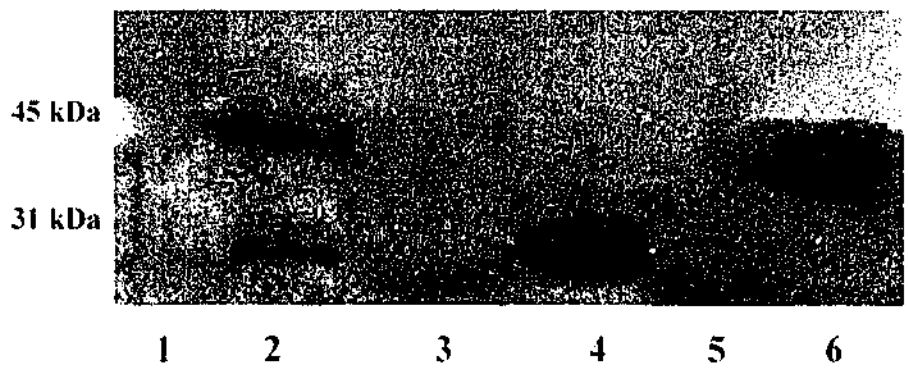


Figure 3-11: A Western Blot of the clones. Lane 1 contains the molecular weight markers which did not react with the antibodies (they were visible on the original gel), Lane 2 shows the *C. perfringens* sialidase (the positive control) (42 kDa), Lane 3 shows pTrcHis A (the negative control), Lane 4 shows Rv3463 (31 kDa), Lane 5 is Rv1733c (22 kDa) and Lane 6 shows Rv1968 (44 kDa).

3.3.4 TB isolate testing

All *M. tuberculosis* cell lysates assayed in Section 3.4.2 did not contain sialidase activity indicating that there did not appear to be a gene coding for this enzyme in an active form in this organism (Table 3-4).

M. smegmatis ATCC 607 was found to contain sialidase activity and supported the findings for *M. smegmatis* mc^2155 (Table 3-4).

Strain	Buffer	Incubation time	Activity (mU)
M. smegmatis mc^{2}155	50 mM Sodium acetate pH 3.8	1	21
M. smegmatis mc^{2}155	50 mM Sodium acetate 1% BSA, pH 3.8	1	50
M. smegmatis ATCC607	50 mM Sodium acetate pH 3.8	1	16.5
M. smegmatis ATCC607	50 mM Sodium acetate 1% BSA, pH 3.8	1	48
M. tuberculosis H37Rv	50 mM Sodium acetate pH 3.8	1	0
M. tuberculosis H37Rv	50 mM Sodium acetate 1% BSA, pH 3.8	2	7.5
M. tuberculosis H37Rv	50 mM Sodium acetate pH 3.8	1	0
M. tuberculosis H37Rv	50 mM Sodium acetate 1% BSA, pH 3.8	2	0
M. tuberculosis H37Rv	50 mM Sodium acetate pH 6.0	2	0
M. tuberculosis 01117727	50 mM Sodium acetate pH 3.8	1	0
M. tuberculosis 01117727	50 mM Sodium acetate 1 mM Ca^{2+}, pH 5.0	1	1
M. tuberculosis 01118961	50 mM Sodium acetate pH 3.8	1	4
M. tuberculosis 01118961	50 mM Sodium acetate 1 mM Ca^{2+}, pH 5.0	1	0

Table 3-4 Assay results of *M. tuberculosis* and *M. smegmatis* isolates

The lack of sialidase activity of the *M. tuberculosis* cell lysates answered the question of the inactivity of the expressed proteins from the cloned genes. The genes did not code for sialidases. After these results were determined it was decided that recloning Rv1733c to obtain expressed protein was not useful to this research as it was unlikely to be a sialidase.

3.3.5 *M. smegmatis* mutant library screen

To screen the *M. smegmatis* mutant library the first method employed was to spray colonies grown on agar plates with MUN (Roggentin *et al.* 1988). As a positive control, the *C. perfringens* clone from Dr. Julian Rood was tested first as this method had previously been successful for this enzyme (Roggentin *et al.* 1988). After incubation and spraying with glycine no fluorescence was detected from the *C. perfringens* colonies. This experiment was therefore not used upon *M. smegmatis* colonies, as wildtype *M. smegmatis* mc^2155 was previously determined to contain less enzymatic activity than the *C. perfringens* sialidase (Chapter 2, Section 2.3.1).

The second method selected to screen the mutant library was a scaled-down MUN assay in a microplate that had also been successfully utilised previously (O'Brien and Mitsuoka 1991). Before this method could proceed it was necessary to determine an adequate method for lysing mycobacterial cells in a small volume, whilst retaining sufficient enzymatic activity. As Table 3-5 shows, no cell lysis procedure was adequate to produce sufficient lysis and sialidase activity which could be applicable to a microplate experiment. Hence, the microplate screening of the *M. smegmatis* mutants would not be feasible.

	Lysis Method	Cell lysis	Sialidase activity
1.	Whole cells washed with buffer *.	No (negative control)	No
2.	Cells were resuspended in 1 mL of buffer and sonicated on ice (15 x 30 s). Cells were centrifuged# and the supernatant assayed.	Yes (positive control)	Yes
3.	Cells resuspended in 1 mL of buffer and snap frozen with dry ice and ethanol. Cells were thawed at 65 °C for 5 min. Freezing and thawing was repeated 5 times. Cells were centrifuged and the supernatant assayed.	No	No
4.	0.5 mL 40% sucrose and 1.5 mM EDTA added to cells. Cells were incubated at room temp. for 15 min and centrifuged. 0.5 mL of ice cold water was added and cells were incubated on ice for 10 min, centrifuged and the supernatant assayed	A small amount of lysis occurred	No
5.	Cells were resuspended in 0.5 mL of buffer and 0.5 mL of 10 % SDS. They were incubated at room temp or 37 °C for 15 min, centrifuged and the supernatant assayed.	Unclear	Some activity was detected but it was due to the SDS degrading MUN
6.	Cells were resuspended in 1 mL 0.2 M NaOH and 2.5% SDS in buffer and incubated at 100 °C for 5 min or 37 °C for 15 min. They were centrifuged and the supernatant assayed	Solution too cloudy to determine if cells were lysed	No
7.	Cells were resuspended in 1 mL buffer with 1 drop of 10% SDS and 2 drops of chloroform added, incubated at room temp for 15 min, centrifuged and the supernatant assayed	A small amount of lysis occurred	No
8.	Cells were resuspended in 0.5 mL of buffer and 0.5 mL of acetonitrile, incubated at 65 °C for 10 min, centrifuged and the supernatant assayed	No	No

Table 3-5: The results of the lysis methods for *M. smegmatis* cells *The buffer used at all times was 50 mM sodium acetate buffer pH 6.0. # Cells were centrifuged at 7,000 rpm at 4 °C. The assay contained 1 mM MUN and 50 µl enzyme and the above buffer (pH 3.8) to 100 µl. They were incubated for 1 h at 37 °C

3.4 Discussion

Using bioinformatics, genes from *M. tuberculosis* H37Rv which contained sequence similarities to bacterial sialidases (Rv1968 and Rv1733c) and a gene which was labelled a putative sialidase (Rv3463; Sanger Centre) were cloned. *C. perfringens* sialidase was also cloned as a positive control.

After cloning, all genes were sequenced and shown to code for the correct sequence. The protein from these clones was then expressed successfully for all except Rv1733c. After protein expression each clone was tested for sialidase activity. The cloned *C. perfringens* gene was the only one to provide an expressed protein containing sialidase activity.

The possible reasons for lack of activity in Rv3463 and Rv1968 included; the cloned gene did not express a sialidase enzyme, the clone did express a sialidase but it was not expressed in an active form, or the expressed protein produced did not contain the correct sequence.

The later availability of the *M. tuberculosis* isolates from VIDRL and subsequent testing indicated that *M. tuberculosis* did not contain an active sialidase. From these results it was concluded that the lack of activity shown in the expressed protein from Rv1968 and Rv3463 was due to the fact that they were not sialidase enzymes. At this time it was thus decided that Rv1733c would not be recloned to obtain expressed protein as it was unlikely that it would express a sialidase.

As Rv3463 was labelled a putative sialidase on the Sanger Centre genome site, and it was then shown to contain no sequence similarity to any sialidases or activity after expression, the Sanger Centre in the UK were contacted and asked why Rv3463 had been annotated as a putative sialidase. The original BLAST search had apparently found a small match between the gene and a sialidase, but the researchers of the Sanger Centre agreed with the findings in this thesis and in the next update of the genome this annotation would be revised (Appendix 12).

Alternative methods used to determine the gene that codes for the enzyme producing the sialidase like activity in *M. smegmatis* were also unsuccessful due to the characteristics of the species (ie the thick cell wall).

Screening the *M. smegmatis* mutant library may enable detection of a mutant lacking sialidase activity leading to determination of the missing gene which would be responsible for activity, however the large number of mutants made screening using the usual MUN assay time consuming so development of a larger screen was necessary.

Spraying plated colonies with MUN to differentiate between colonies that could or could not fluoresce was unsuccessful with *C. perfringens* which expresses a highly active sialidase and would thus not be successful with an organism less active. Similarly a microplate MUN assay would be useful in a large screening experiment to detect active and inactive mutants, however, due to the characteristics of the mycobacterial cell wall a successful small volume cell lysis method could not be determined and the mutant library screen was postponed.

In summary, the time taken to clone the genes from *M. tuberculosis* H37Rv was of no benefit in creating a better purification of a mycobacterial sialidase. The only outcome was that Rv3463 was shown not to be a sialidase. This finding supported the BLAST searches that had found no similarities with Rv3463 and other sialidases (Section 3.2.3). Work is still ongoing by The Institute for Genomic Research (TIGR) in the USA to solve the genome of *M. smegmatis* but it is already known that its genome is larger than that of *M. tuberculosis* H37Rv (TIGR, Coppel *et al.* unpublished results) thus it may contain a larger variety of genes coding for other enzymes. The genome *M. bovis* BCG is also being sequenced and at this time it is known that it contains at least one region that is not in *M. tuberculosis*. As a weak sialidase activity was found in this organism the gene coding for this enzyme may be located within that region. When both genomes are solved the task of finding the putative sialidase will be easier as they can be compared directly to *M. tuberculosis* and known sialidases and a putative gene determined. Due to the time constraints for this research, the genomic work was postponed so that the partially pure enzyme from the Neu5Ac-linked affinity column purification (Chapter 2, Section 2.2.8) could be characterised to elucidate more biochemical information about the enzyme.

4 Biochemical Characterisation of the *M. smegmatis* Sialidase

4.1 General

As determined in the previous chapter, a selection of *M. tuberculosis* H37Rv genes, including Rv3463, a gene assigned as a putative sialidase were cloned. None of the resulting protein from the expression of the genes contained sialidase activity. The sialidase assay of a selection of *M. tuberculosis* strains also proved that *M. tuberculosis* did not appear to contain a sialidase enzyme.

Due to the length of time that would be involved in screening the *M. smegmatis* mutant gene library for mutants without sialidase activity, the biochemical analysis of the enzyme using the partially purified *M. smegmatis* fractions obtained from affinity chromatography in Chapter 2 (Section 2.2.8) was continued. Most experiments in this Chapter, unless otherwise stated, used the fluorometric assay explained in Chapter 2 (Section 2.2.4) that observes the hydrolysis of MUN (Potier *et al.* 1979).

All experiments completed in this chapter were repeated at least twice unless otherwise stated.

4.2 Materials

Pronase was purchased from Boehringer Mannheim. The protease inhibitors used were EDTA from BDH and phenylmethyl sulfonyl fluoride (PMSF) from Sigma Chemical Co.

The sialidase inhibitors were kindly donated and synthesised by Dr Milton Kiefel from the Centre of Biomolecular Science and Drug Discovery, Griffith University, and Mr Pas Florio and Mr Tho Van Phan from the Department of Medicinal Chemistry, Monash University (Appendix 14). The fluorescence overlay experiment used 10% native tris/tricene gels purchased from Bio-Rad.

All H^1 NMR spectroscopic experiments were performed on a 600 MHz Bruker spectrometer from the Centre of Biomolecular Science and Drug Discovery, Griffith University.

All kinetic data was analysed using the Enzyme Kinetics Program (version 2.0; Hearne Scientific Software). This program fits the initial velocity measurements as a

function of substrate concentration using an iterative least-square function. The program was adapted from that described by Cleland (Cleland 1979). The kinetics data were graphed using proFit 5.5.3 from QuantumSoft, Switzerland (www.quansoft.com).

Other chemicals and reagents are either listed in Chapter 2, Section 2.2.3 or were of analytical grade.

4.3 Biochemical Investigations

4.3.1 Degradation of Sialidase Activity by the addition of Pronase

Pronase is a mixture of several proteolytic enzymes, including endo- and exo-proteinases, thus it can cleave almost any peptide bond and can be useful to inactivate enzymes (Narahashi 1970). In order to conclude that the apparent sialidase activity detected in crude cell lysates of *M. smegmatis* mc^2155 was due to an enzyme and not degradation of the substrate, pronase was used as a test.

Pronase was added to the crude cell membrane lysate, crude cytosolic lysate and some partially pure fractions obtained from the purification of the putative sialidase on the thiosialoside-linked affinity column (Chapter 2, Section 2.2.8) to determine if any loss of activity could be obtained.

Pronase was added to samples at a final concentration of 1 mg/mL and the mixtures were incubated overnight at 37 °C with shaking (Rudbach and Johnson 1964; D'Aniello *et al.* 1993; Ueno *et al.* 1999). Samples without pronase were also incubated overnight at 37 °C as negative controls.

After overnight incubation the above samples, along with fresh samples stored overnight at –20 °C were assayed for sialidase activity with MUN at the appropriate pH as previously explained.

It was shown that all samples without pronase added had retained their enzymatic activity and were not significantly different in activity to the samples stored at –20 °C. Therefore, any activity loss in the test samples could be attributed to pronase. After activity determination using MUN as the substrate it was found that the crude membrane fraction appeared unaffected by the pronase treatment and the crude cytosol lost a small amount of activity. All partially pure fractions lost complete

activity. The differences in activity loss was explained by the amounts of protein in each sample, the level of purity of the putative sialidase and the pH of the samples. The optimal pH for pronase activity is pH 7-8 (Garner *et al.* 1974). All tested samples were suspended in 50 mM sodium acetate buffer pH 6.0 which would slightly decrease the pronase's ability to cleave peptide bonds. The crude membrane fraction contained mg amounts of protein whereas the purified samples contained μg amounts. It is recommended that pronase be added to protein solutions in a ratio of 100:1 for complete digestion and even then this may not be achieved without the further addition of aminopeptidase (Garner *et al.* 1974). The high protein content of the crude samples would have required such a large amount of pronase that this ratio could not be practically achieved without dilution of the crude samples. Dilution was not feasible due to the low sialidase-like activity in the crude samples. Thus in crude samples the pronase became saturated faster and was unable to completely digest all peptide bonds as it was not used at its optimal concentration.

In summary, the results indicated that the sialidase-like activity was affected by pronase addition indicating that the degradation of MUN was most likely due to an enzyme.

4.3.2 Protease inhibition

Possible loss of enzymatic activity during some purification processes may have been due to attack of the enzyme by proteases existing in the cell lysate. As had been determined by the addition of pronase, cruder samples were less affected by attack due to the protective functions of other proteins. In order to test this hypothesis EDTA and PMSF were added to various fractions and crude samples to determine the effects. EDTA and PMSF are protease inhibitors and in theory if the loss of activity was due to proteases these substrates should provide some protection to the enzyme.

EDTA and PMSF were added to crude and partially pure enzyme samples at final concentrations of 1.3 mM and 1 mM respectively (Roche Molecular Biochemicals guide for protease inhibition 2000). Samples were evaluated with MUN as the substrate immediately after the addition of EDTA and PMSF and after storage at –20 °C for 24 h. Identical samples containing no protease inhibitors were assayed at the same time. The results are summarised in Table 4-1.

As Table 4-1 shows, the protease inhibitors had the opposite effect to that desired. All samples lost significant activity after addition of the inhibitors thus they were not deemed a useful agent for stabilising the enzyme and retaining its activity during purification.

Possible reasons for the loss of activity could be that like *V. cholerae* sialidase the *M. smegmatis* sialidase-like enzyme may require the use of cations for activity (Ada *et al.* 1961). If this was the case then EDTA, in its action as a metal chelator would have mopped up any free cations in the solution to inhibit the activity. Mixed protease inhibitors containing PMSF had also been reported to decrease rat liver lysosomal sialidase activity, although no logical explanation could be given (Miyagi and Tsuiki 1985). Other stabilising agents including cations were therefore investigated for their effect upon the sialidase-like enzyme.

Sample	% Original Activity	% Activity with protease inhibitors	% Activity with protease inhibitors after 24 h
Crude Membrane	100	62	71
Crude Cytosol	100	89	92
100 mM salt elution (23/11/00)	100	23.8	13.2
200 mM salt elution (23/11/00)	100	5.9	0.8
50 mM salt elution (27/11/00)	100	0	0
100 mM salt elution (27/11/00)	100	1.3	0.6
200 mM salt elution (27/11/00)	100	14.3	7.1
300 mM salt elution (27/11/00)	100	12.8	11.1

Table 4-1: The effect of protease inhibitors upon the activity of the crude and partially pure sialidase enzyme from *M. smegmatis* when MUN is used as a substrate

4.3.3 The effect of sugars (stabilising agents), cations, chelators and detergents upon enzymatic activity

A wide range of compounds were tested to see if they had any effect upon the enzymatic activity of the putative *M. smegmatis* sialidase. In the first instance stabilising agents were assessed to determine if they could increase the activity and

stability of the enzyme. Table 4-2 summarises the results of the assays where MUN was used as the substrate. The stabilisers were added to the partially purified *M. smegmatis* membrane fractions in the appropriate concentrations before incubation (Saito and Komamine 1976; Holmes *et al.* 1997; Ward *et al.* 1999).

Additive	Final concentr-ation	Reference	% Activity Crude	% Activity 100 mM	% Activity 200 mM	% Activity 300 mM
None			100	100	100	100
BSA	2%		97	2035	680	906
BSA	0.1%		n/a	n/a	n/a	525
Sorbitol	1 mg/mL	(Holmes, Scopes *et al.* 1997)	78	193	108	147
Glutathione	1 mM	(Saito and Komamine 1976)	72	76	60	145
DTT	1 mM	(Saito and Komamine 1976)	70	n/a	91	118
Mercapto-ethanol	1 mM	(Saito and Komamine 1976)	70	n/a	106	141
Sucrose	1 mg/mL	(Ward, Adams *et al.* 1999)	81	n/a	n/a	115
Glucose	1 mg/mL	(Ward, Adams *et al.* 1999)	144	n/a	n/a	149
Lactose	1 mg/mL	(Ward, Adams *et al.* 1999)	56	n/a	n/a	135
Glycerol	5 %	(Saito and Komamine 1976)	105	n/a	n/a	569

Table 4-2: The effect of stabilising agents upon the activity of the sialidase enzyme from *M. smegmatis* where MUN is used as the substrate. Where n/a is written samples were not assayed.

Table 4-2 demonstrates that glycerol and BSA were the only two compounds that had significant protecting abilities. Even at a lower concentration BSA was able to

increase the enzymatic activity to a high degree. The increase in activity seen with the addition of BSA indicates that the enzyme needs extra non-enzymatic protein as a stabilising agent. Glycerol was used at a high concentration thus the large increase in activity may not have been as significant in the lower concentrations used by other sugars.

Once again, the crude cell lysate was generally unaffected by the addition of stabilising agents as it contains numerous proteins that may offer stabilisation. For this reason it was no longer useful in further assays to determine the affect of cations and detergents upon enzymatic activity as it predicably would not give definitive results. BSA and glycerol had shown positive results and were assayed again as controls. The results of the effect of the addition of cations and detergents are given in Table 4-3. Once again the samples were assayed with MUN at the appropriate pH. In this case a single purified fraction of *M. smegmatis* membrane fraction was used to compare the effects directly.

Additive	Final concentration	Activity (mU)	Change in Activity
None	-	28.5	1 x
BSA	1 %	650	22 x
Triton X	1 %	249	8.7 x
Glycerol	1 %	148	5.2 x
EDTA	10 mM	8	0.28 x
Cholic acid	10 mM	0.5	0.02 x
$CuCl_2$	10 mM	16.5	0.58 x
$MgCl_2$	10 mM	164	5.8 x
NaCl	10 mM	139.5	4.9 x
$CaCl_2$	10 mM	772.5	27 x
$MnCl_2$	10 mM	486	17 x

Table 4-3: The effect of cations, detergents and non-enzymatic protein upon the activity of the partially pure sialidase enzyme from *M. smegmatis*

Table 4-3 shows that in general bivalent cations (except for Cu^{2+}) appear to significantly increase the activity of the sialidase. This result corresponds well with the fact that EDTA inhibited activity. EDTA is a metal ion chelator and will mop up and bind cations so that they are unavailable for use by the enzyme. This is not an unusual result as many other sialidases require cations for activity (Ada *et al.* 1961; Moriyama and Barksdale 1967), and may be inhibited by Cu^{2+} (Miyagi and Tsuiki 1985; Teufel *et al.* 1989; Trabelsi *et al.* 1991; Lichtensteiger and Vimr 1997; Byers *et al.* 2000).

Once again both BSA and glycerol were able to stabilise and increase the activity of the enzyme. Glycerol was added at 1/5 the concentration that it was used in Table 4-2 and at this concentration it was still able to increase activity by five times. Glycerol was thus determined to be a better stabiliser than originally thought.

Triton X also increased the activity of the enzyme which supported the primary results and ideas in Chapter 2, Section 2.3.1 that this enzyme may be membrane bound. Triton X is known to be a detergent that may remove enzymes from the membrane surface. The addition of Triton X to crude cell lysates before fractionation however, did not help activity or increase the amount of enzymatic activity found in the membrane compared with the cytosolic fraction.

The above assay was repeated with Ca^{2+}, Mn^{2+} and Mg^{2+} at final concentrations of 1 mM and 5 mM and BSA, Triton X and Glycerol at 0.1 and 0.5%. At both 5 mM and 1 mM final concentrations, Ca^{2+} and Mn^{2+} were still able to cause a significant increase in activity of up to 8 times. Mg^{2+} also increased activity by 3 times at a 1mM final concentration. BSA, Glycerol and Triton X were also able to increase activity by 3-4 times at both the 0.1 and 0.5% concentrations.

As Ca^{2+} consistently appeared to have the greatest results it was then added to the assay buffer for all further assays unless otherwise stated. This result compared well with other bacterial sialidases such as *V. cholerae* and *C. diptheriae* sialidases which have also been shown to be activated by cations with Ca^{2+} being able to improve activity to the greatest degree (Ada *et al.* 1961; Moriyama and Barksdale 1967). The new assay buffer therefore consisted of 50 mM sodium acetate containing 1 mM Ca^{2+} pH 6.5. In cases of low activity, assays were also supplemented with BSA to a final concentration of 1%.

4.3.4 Optimisation of assay conditions: temperature and pH

Using MUN as a substrate, the optimal pH of the partially pure enzyme was determined by assaying the enzyme at different buffer pH's ranging between pH 3 and pH 7. The results are shown in Figure 4-1, where it can be seen that the highest activity was obtained at pH 5. Once the pH became more basic there was a sharp decrease in activity. This was shown to be true for all partially pure fractions compared with the acidic pH optimum of the crude cell lysates. From the results of this experiment all partially pure fractions were thus assayed at pH 5.0 unless otherwise stated. A result such as this is not unique as crude preparations of cytosolic sialidase from rat liver also exhibit a lower optimal pH than their pure counterparts (Miyagi and Tsuiki 1985).

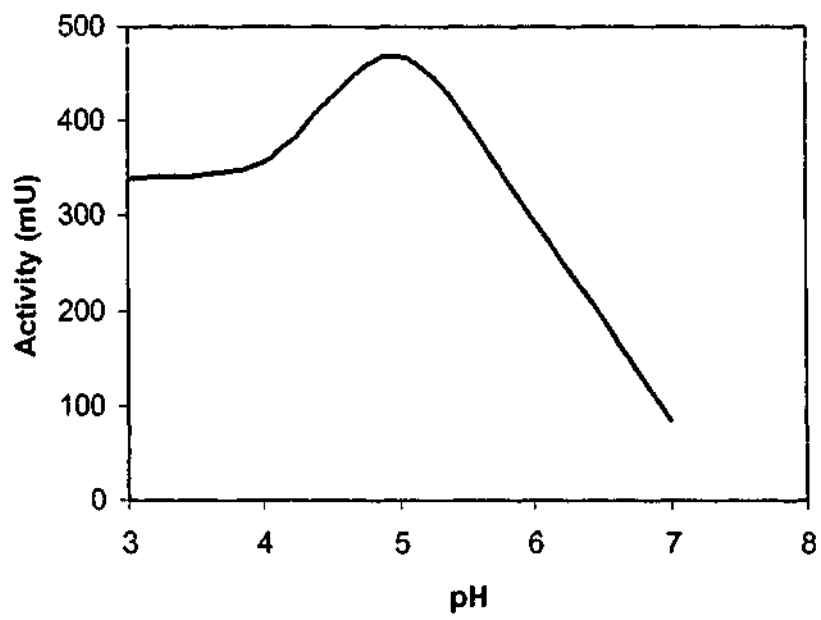


Figure 4-1: Optimal pH of the partially pure sialidase enzyme from *M. smegmatis*, measured in mU.

The optimal temperature was determined using the substrate MUN with a partially pure enzyme and Ca^{2+} added to the buffer at pH 5.0 as previously determined. Enzyme mixtures were incubated at 6, 19, 25, 30, 37, 40, 45, 50 and 60 °C for 1 h. Figure 4-2 shows that that the optimal temperature for the enzyme was 37 °C, which is the same as most sialidases. *A. viscosus* DSM 43798, a close relative of mycobacteria for example has a sialidase with an optimal activity at a temperature of 37 °C (Teufel *et al.* 1989).

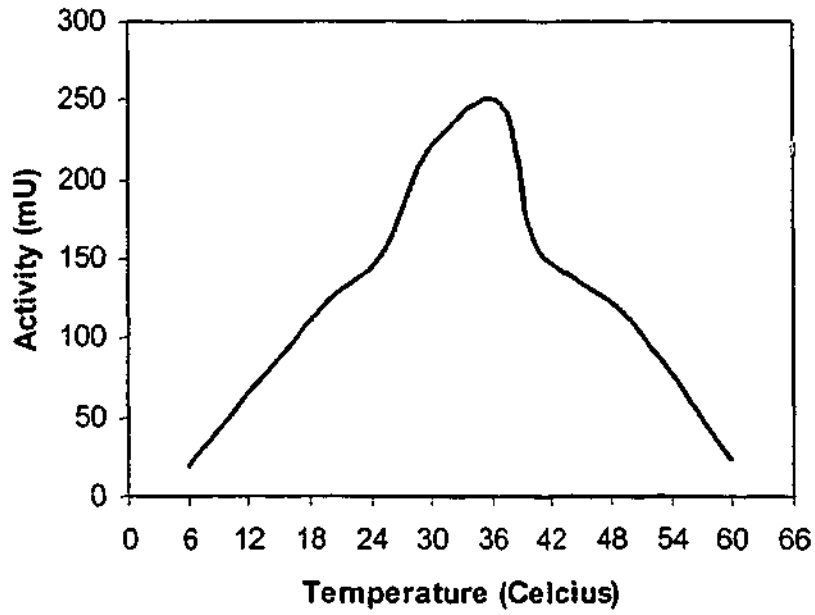


Figure 4-2: Optimum temperature of the partially pure sialidase enzyme from *M. smegmatis* measured in mU.

Although *M. smegmatis* grows well at 37 °C it is reported to grow best at 30 °C, thus one might expect that its enzymatic activity should be high at 30 °C also. This was shown to be the case. Incubation at 30 °C was determined to give the second highest activity and it was not significantly lower than the optimum activity at 37 °C when compared with the other incubation temperatures (Table 4-4).

Temperature (°C)	% Activity
6	7.9
19	47
25	61
30	89
37	100
40	65
45	54
50	45
60	9.3

Table 4-4: Percentage activity of the sialidase enzyme from *M. smegmatis* after incubation at different temperatures

4.3.5 Substrate and linkage specificity

Bacterial sialidases cleave sialoglycoconjugates to use sialic acids as carbon and energy sources for the bacterial cell (Vimr and Troy 1985). The organism may also use the sialidases to cleave sialic acids that mask binding sites on host cells so that they can adhere and invade such cells (Nees *et al.* 1975; Corfield 1992; Galen *et al.* 1992). In this way the sialidase enzymes may be required to cleave different types of sialoglycoconjugates some of which have different linkages. The sialidase must therefore have the ability to cleave different linkages to perform their role adequately.

To investigate the linkage/substrate specificity of the *M. smegmatis* putative sialidase the thiobarbituric acid assay explained in Chapter 2 (Section 2.2.5) was employed. In this assay free sialic acid reacts to form a coloured chromophore (pink-red) which absorbs light at 549 nm (Warren 1959; Moriyama and Barksdale 1967; Arden *et al.* 1972; Aymard-Henry *et al.* 1973). As this assay only detects free sialic acid it may be used to test for sialic acid cleaved from any sialoglycoconjugate. The approach was therefore used to determine the specificity of the substrates listed in Table 4-5. The

amount of bound sialic acid of each substrate before cleavage was determined by the Bial ferric/orcinol assay explained in Chapter 2 (Section 2.2.6).

The results of the relative hydrolysis rates of the substrates tested are summarised in Table 4-5. Hydrolysis of the α(2→3)-linked glycoprotein fetuin occurred at the highest rate indicating an α(2→3) specificity which is common in many sialidases (Moriyama and Barksdale 1967; Potier *et al.* 1979; Berry *et al.* 1996; Lichtensteiger and Vimr 1997; Byers *et al.* 2000; Mizan *et al.* 2000). The rate of hydrolysis of mixed gangliosides was the next highest but this reaction would only proceed in the presence of detergents (0.1% Triton X). After the mixed gangliosides, the rate of hydrolysis for α(2,3)-sialyllactose was the next most rapid. α(2→6) linked substrates were also hydrolysed with no significant difference between sialyllactose and mucin. In these experiments, the α(2→8)-linked glycoprotein, colominic acid, was not hydrolysed, indicating that there was no α(2→8)-linkage specificity under these conditions. The results of this experiment were similar to those of small sialidases as this enzyme did not hydrolyse a wide range of substrates to a high degree and showed specificity towards the more common linkages. Larger sialidases tend to hydrolyse all linkages to some degree as explained in Chapter 1 (Section 1.3.4).

Substance	Linkage	Relative hydrolysis rate %
α(2,3)-sialyllactose	α(2→3)	100
α(2,6)-sialyllactose	α(2→6)	57
Fetuin	α(2→3)	350
Mucin	α(2→6)	51
Mixed bovine gangliosides	α(2→8)	180
Colominic acid	Mixed linkages	No hydrolysis detected

Table 4-5: The relative hydrolysis of synthetic and natural substances compared to α(2,3)-sialyllactose, determined by the thiobarbituric acid assay.

4.3.6 Further linkage studies: ^{1}H NMR spectroscopy

^{1}H NMR spectroscopy is a useful tool for studying the kinetics of enzymatic reactions catalysed by sialidases and *trans*-sialidases because the level of both the substrate and products can be measured simultaneously. Indeed ^{1}H NMR spectroscopy has been successfully used to investigate the mechanism of hydrolysis of MUN, sialyllactose and other α-ketosides of Neu5Ac by various bacterial, viral and mammalian sialidases and protozoal *trans*-sialidases (Friebolin *et al.* 1981a; Friebolin *et al.* 1981b; Chong *et al.* 1992; Wilson *et al.* 1995; Wilson *et al.* 1996; Kao *et al.* 1997; Terada *et al.* 1997; Todeschini *et* al 2000; Wilson *et al.* 2000). Specifically, the stereoselectivity of the action of sialidases can be monitored determined using this technique since the anomeric configuration of the released sialic acid is readily monitored (Friebolin *et al.* 1981a).

Studies have shown that the chemical shift of the H3 axial and equatorial protons are diagnostic of the anomer of sialic acid (Friebolin *et al.* 1981a; Friebolin *et al.* 1981b; Chong *et al.* 1992; Wilson *et al.* 1995; Wilson *et al.* 1996; Kao *et al.* 1997; Terada *et al.* 1997; Todeschini *et* al 2000; Wilson *et al.* 2000).

Crystallographic studies of sialidases and *trans*-sialidases from viral, bacterial and protozoal sources have shown that in the sialidase or *trans*-sialidase/Neu5Ac complex, sialic acid, the natural substrate for these enzymes, binds exclusively in the α-configuration even though the α-anomer is observed to be the minor solution conformer (von Itzstein *et al.* 1993; Pegg and von Itzstein 1994).

Previous ^{1}H NMR studies have shown that the hydrolysis of a series of α-ketosides of Neu5Ac proceeds, as catalysed by sialidases or *trans*-sialidases from many sources, with retention of anomeric configuration. In this reaction, it has been observed that the sialic acid portion of the α-ketoside binds to the catalytic site in the α-configuration and that the first product of release in the catalytic reaction is the α-anomer of sialic acid. In regard to monitoring this reaction, the chemical shift of the H3 axial and equatorial protons of sialic acid have been extremely useful and diagnostic for revealing the anomeric configuration of sialic acid in solution (Friebolin *et al.* 1981a; Friebolin *et al.* 1981b; Chong *et al.* 1992; Wilson *et al.* 1995; Wilson *et al.* 1996; Kao *et al.* 1997; Terada *et al.* 1997; Todeschini *et* al 2000; Wilson *et al.* 2000).

When the catalysis reaction is monitored by ^{1}H NMR it appears that sialidases and *trans*-sialidases follow a similar pattern reaction profile (Friebolin *et al.* 1981a; Wilson *et al.* 1995; Kao *et al.* 1997; Terada *et al.* 1997; Todeschini *et* al 2000; Wilson *et al.* 2000). At time zero, before the addition of sialidase or *trans*-sialidase only the $H3_{axial}$ and $H3_{equatorial}$ protons of the substrate appear in the ^{1}H NMR spectrum. After addition of the enzyme the hydrolysis reaction begins, and the $H3_{axial}$ and $H3_{equatorial}$ signals appear at chemical shift values that corresponding to those observed for the α-anomer of sialic acid start to appear. These signals increase in intensity with time as the reaction proceeds, with a concomitant decrease in the H3 signals of the substrate. After a short period of time, it is also observed that an additional set of signals corresponding to the formation of the β-anomer of Neu5Ac appear in the ^{1}H spectrum. The β-anomer of Neu5Ac is formed due to the mutatrotation of the free α-anomer of Neu5Ac in solution. Due to this process the time the H3 signals of the α-anomer of sialic acid begin to progressively decrease in intensity in the ^{1}H NMR spectrum. The reaction continues until all substrate is hydrolysed and a final solution equilibrium mixture of the two sialic acid anomers is established.

An example of a bacterial sialidase that catalyses this reaction is *S. typhimurium* sialidase. An ^{1}H NMR study of the hydrolysis of the synthetic sialidase substrate MUN catalysed by *S. typhimurium* sialidase has previously been reported (Wilson *et al.* 1995). In this study the $H3_{equatorial}$ and $H3_{axial}$ resonances of the substrate (MUN) were visible in the ^{1}H NMR spectrum at 2.78 ppm and 1.91 ppm respectively. During the time-course of the experiment the α-Neu5Ac resonances became visible at 2.64 and 1.53 ppm and increased in intensity with a concomitant decrease of the substrate protons (Wilson *et al.* 1995). This demonstrated that the product retained the same anomeric configuration as that of the substrate. Mutarotation of the reaction product also occurred with the intensity of the resonances of the α-anomer decreasing after 37 min until it reached a final equilibrium of 95% β- and 5% α-Neu5Ac (Wilson *et al.* 1995).

As shown for *S. typhimurium*, in solution free sialic exists as an equilibrium of the β-anomer and α-anomer. The free sialic acid prefers the more thermodynamically stable β-anomer and will usually be found in equilibrium concentrations of 90-95% β- and

5-10% α-Neu5Ac (Wilson *et al.* 1995; Kao *et al.* 1997; Terada *et al.* 1997; Todeschini *et* al 2000).

Previous studies to date describe all sialidases as retaining enzymes and not inverting enzymes (Friebolin *et al.* 1981c; Wilson *et al.* 1995; Kao *et al.* 1997; Terada *et al.* 1997; Todeschini *et* al 2000). This means that all enzymes are shown to proceed with the initial release of the α-anomer of sialic acid. Inverting enzymes release the β-anomer only without the need for formation of the α-anomer and subsequent mutarotation.

^{1}H NMR spectroscopy was used in this current investigation to observe the hydrolysis of sialyllactose and further confirm the linkage specificity determined in Section 4.3.5.

Partially purified *M. smegmatis* mc^{2}155 samples were freeze dried and reconstituted with 50 mM deuterated sodium acetate buffer pH 5.0, containing 1 mM Ca^{2+} and 0.1% BSA. An amount of 1 mg of either α(2,3)- or α(2,6)-sialyllactose was added to the samples. Control solutions were also prepared to include the above buffer with 1 mg of α(2,3)-or α(2,6)-sialyllactose. Spectra were acquired on a 600 MHz Bruker spectrometer at 298 K.

Figure 4-3 and Figure 4-4 show the ^{1}H NMR spectrum (600 MHz, 298 K) of α(2,3)-, and α(2,6)-sialyllactose respectively, between 2.0 to 2.8 ppm in the presence of the partially purified sialidase from *M. smegmatis*. The catalytic reaction proceeded in a similar fashion to that described previously for other sialidases (Friebolin *et al.* 1981a; Friebolin *et al.* 1981b; Chong *et al.* 1992; Wilson *et al.* 1995; Wilson *et al.* 1996; Kao *et al.* 1997; Terada *et al.* 1997; Todeschini *et* al 2000; Wilson *et al.* 2000).

The $H3_{equatorial}$ protons of the sialic acid of α(2,3)-sialyllactose (2.7 ppm) and α(2,6)-sialyllactose (2.65 ppm) were used to monitor the hydrolysis reaction over time. As the reaction proceeded the $H3_{equatorial}$ protons of free sialic acid (β-anomer) became visible at 2.15 ppm in both spectra, with a concomitant decrease in the $H3_{equatorial}$ protons of sialyllactose. The putative sialidase continued to show a preference for hydrolysis of the α(2,3)-sialyllactose, as this reaction appeared to proceed at a faster rate and thus supports the previous substrate specificity results determined form the thiobarbituric acid assay in Section 4.3.5.

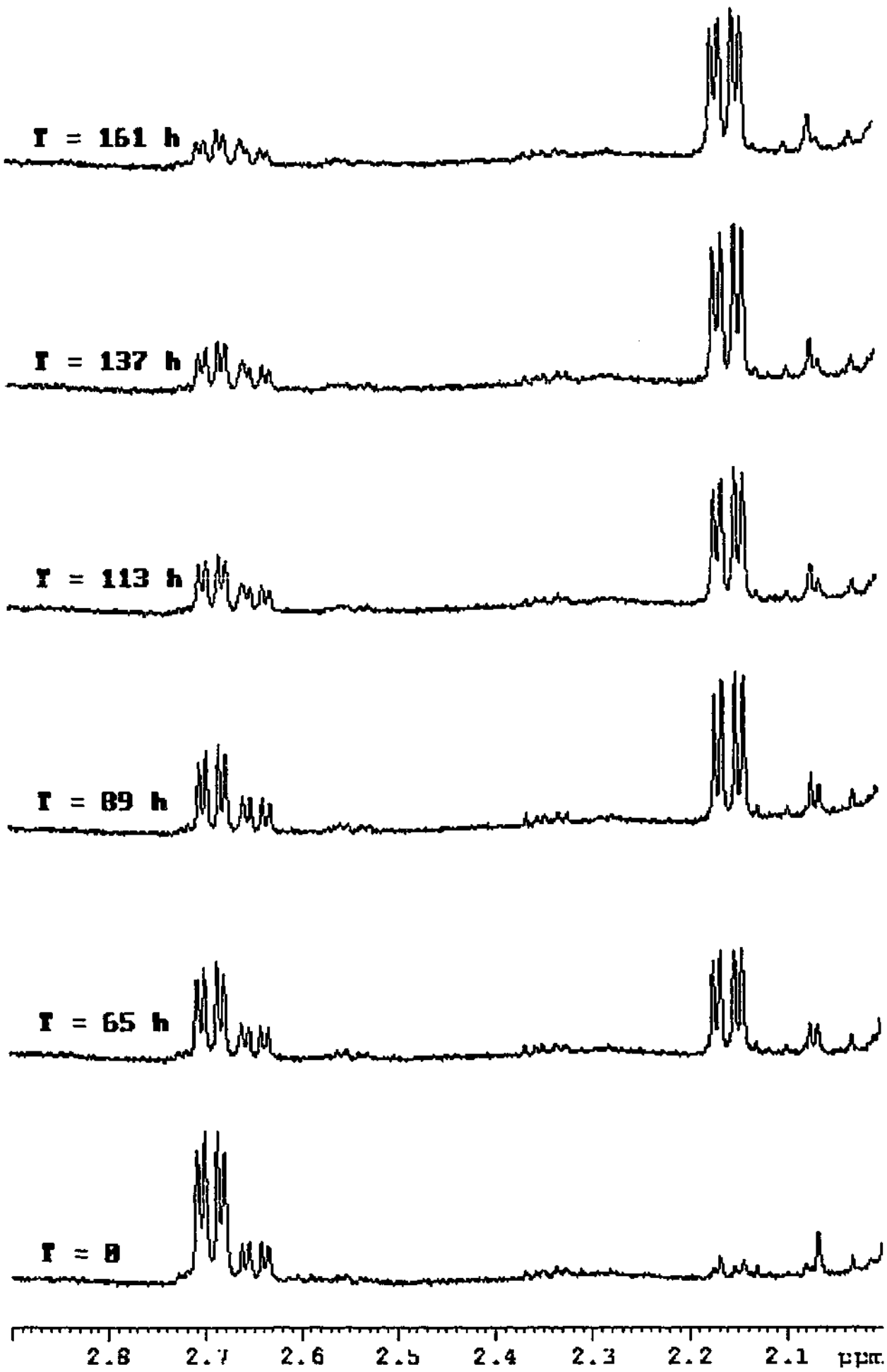


Figure 4-3: ^{1}H NMR time course reaction for the hydrolysis of α(2,3)-sialyllactose in the presence of partially purified *M. smegmatis* sialidase (600 MHz, 298 K).

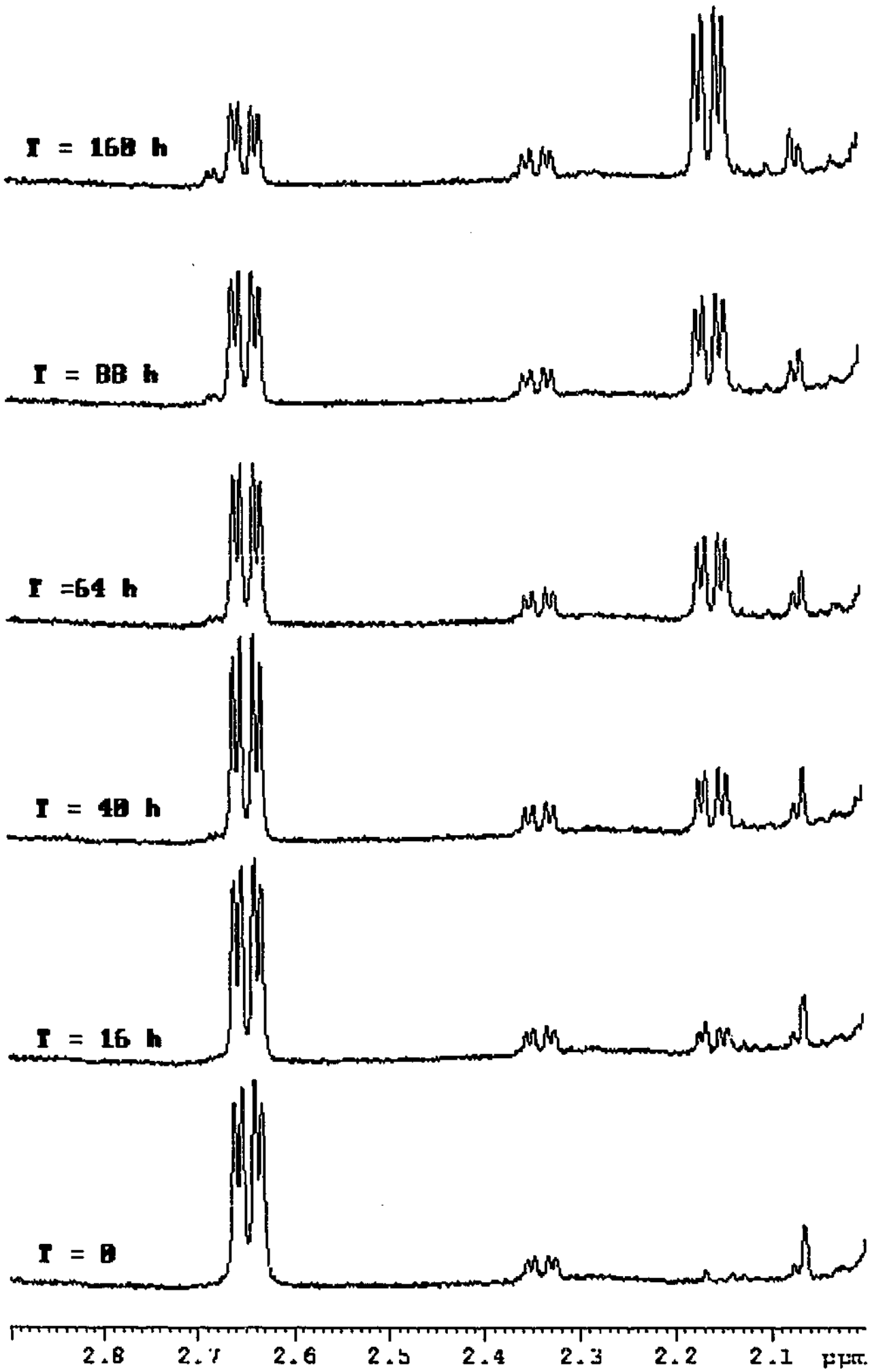


Figure 4-4: ^{1}H NMR time course reaction for the hydrolysis of α(2,6)-sialyllactose in the presence of partially purified *M. smegmatis* sialidase (600 MHz, 298 K).

4.3.7 SDS-PAGE, Fluorescence overlay and Isoelectric Focussing

Protein purification thus far had been unable to produce sufficient amounts of active protein to enable protein visualisation on SDS-PAGE (Chapter 2, Section 2.3.2) (Figure 2-3). To solve this problem it was decided that an overlay of the gel with MUN and its subsequent hydrolysis by the enzyme might provide localisation and visualisation of the protein. The sensitivity of this type of staining had been previously reported to be 10,000 fold greater than staining with coomassie brilliant blue (Berg *et al.* 1985). If a clear fluorescent band was obtained it may also be possible to extract it and obtain an *N*-terminal sequence using mass spectrometry. This method had previously been successful (Berg *et al.* 1985; Samollow *et al.* 1985; Bischoff *et al.* 1998).

Other advantages apart from sensitivity that this method provided included that fact that other options such as Western blotting with antibodies would be more costly and technically demanding (Bischoff *et al.* 1998), and the available antibodies for sialidases are only for the mammalian enzyme and would therefore not be very specific, whereas MUN was already specific for the *M. smegmatis* enzyme.

A 12% SDS-PAGE was loaded with partially pure enzyme in a buffer containing 1 mM Ca^{2+} and 1% BSA. Pure commercial *V. cholerae* sialidase was run as a positive control. After electrophoresis in the cold for 1 h at 200 V the gel was rinsed with 50 mM sodium acetate buffer pH 5.0 and overlaid with Whatman #1 filter paper soaked in 1 mM MUN. This was incubated in the dark for 1 h at 37 °C (Berg *et al.* 1985; Samollow *et al.* 1985). After this time the reaction was stopped with 0.25 M glycine pH 10. The gel was observed under an UV light at 330 nm but no bands were present, not even those of *V. cholerae* sialidase which was of extremely high activity.

As neither the positive control nor the sample had fluoresced, it was determined that denaturation of the enzymes by the SDS occurred to such an extent as to render the enzymes inactive, thus a native gel may be more successful. The only drawback to using a native gel was that the approximate molecular weight of the protein could not be determined.

Partially purified enzyme containing Ca^{2+} and BSA as before were run on a 10% native tricine gel (Bio-Rad). Pure *V. cholerae* sialidase was run on the gel as a positive control, heat denatured *V. cholerae* sialidase and heat denatured partially pure

M. smegmatis enzyme were run as negative controls. The gel was run at 150 V on ice for 1-2 h with a two-buffer system (Samollow *et al.* 1985; Schagger and von Jagow 1991; Schagger *et al.* 1994). The inner buffer consisted of 4.844 g of tris and 7.168 g of tricene in 1 L of water. The outer buffer contained 9.688 g of tris and 1.2 mL of concentrated HCl in 1 L of water (pH 8.9). After the gel had run it was rinsed with 50 mM sodium acetate buffer pH 5.0 containing 1 mM Ca^{2+} and was then overlaid with filter paper soaked in a solution of 1 mM MUN in the same buffer. The overlaid gel was incubated in darkness for 1.5 h at 37 °C (Samollow *et al.* 1985). After this time the bands were observed under an UV light at 330 nm.

Figure 4-5 shows the sialidase bands from both *V. cholerae* and *M. smegmatis* on a native gel that was overlaid with MUN. Denatured enzyme did not hydrolyse the MUN and thus did not show any bands, indicating that there was no background reaction. Due to the impressive fluorescence it was hoped that the band from the gel could be extracted to obtain an *N*-terminal sequence. Unfortunately, after staining the fluorescent band was located in exactly the same position as the BSA band making it impossible to extract a pure protein. As there was little enzyme, sequencing the band would probably only result in determining a BSA sequence.

1. 2. 3. 4.

Figure 4-5: Native gel overlaid with MUN. Lane 1 contains pure *V. cholerae* sialidase (1 in 1,000 dilution); Lane 2 contains heat killed *V. cholerae* sialidase; Lane 3 containes the partially pure *M. smegmatis* enzyme; Lane 4 contains the heat killed *M. smegmatis* enzyme.

Isoelectric focusing (IEF) is a method used to determine the isoelectric point (pI) of a protein by carrying out electrophoresis in a gel containing a pH gradient. A protein applied to the gel will be either positively or negatively charged depending on the local pH at that point in the gel. Upon application of a current, the protein will move until it encounters that part of the gel which corresponds to its pI at which point it will have no net charge and will stop migration.

IEF is run on a native gel and was examined for two reasons. Firstly to determine the pI of the *M. smegmatis* putative sialidase for further characterisation and comparison with other sialidases, and secondly to determine if a different gel method could also provide a fluorescent band to provide further support to the results of the native gel overlaid with MUN (Figure 4-5). This experiment had also been previously successful to stain both *C. sordelli* and *V. cholerae* sialidases (Berg *et al.* 1984).

IEF was performed according to the manufacturer's instructions with partially pure *M. smegmatis* putative sialidase (Bio-Rad). The ampholyte solution used (Bio-Rad) was in a pH range of 3-10. The gel was run at 100 V, 5 mA and 0.5 W for 15 min, 200 V 5 mA and 1 W for 15 min and then 450 V, 4 mA and 2 W for 1h. After this time the gel was rinsed with buffer, overlaid with filter paper soaked in MUN and incubated in darkness for 1.5 h as had been performed for the native gel experiment (Samollow *et al.* 1985). The gel was then observed under an UV light at 330 nM and the isoelectric point was thus determined to lay between pH 5.0 and 6.0. Between this pH a broad fluorescent band had appeared which was consistent with its optimal pH of 5.0 and the IEF point of other sialidases (Chapter 1, Section 1.3.4). No photograph of this gel is shown as it was an extremely thin gel that could not be separated from the filter paper to obtain a clear picture.

4.3.8 Further optimisation of conditions

In Section 4.3.3 it was shown that BSA, Ca^{2+} and Mn^{2+} were capable of stabilising and increasing the activity of the partially pure *M. smegmatis* putative sialidase. This Section studied the optimal concentrations and combinations of these compounds to determine the optimal activity of the enzyme.

All compounds were initially assayed alone in a range of concentrations to determine the lowest concentration of the additive to provide optimal activity. The assay used was the MUN assay as previously described (Potier *et al.* 1979). The results are shown in Table 4-6.

Additive	Final concentration	Activity (mU)	% Activity
None	-	83.5	100
BSA	0.1 %	130	155
BSA	0.5 %	183.5	220
BSA	1.0 %	212.5	254
BSA	1.5 %	239.5	286
BSA	2.0 %	281	336
Ca^{2+}	0.5 mM	124.5	149
Ca^{2+}	1.0 mM	124.5	149
Ca^{2+}	5 mM	122	146
Ca^{2+}	10 mM	122	146
Mn^{2+}	0.5 mM	99	118.5
Mn^{2+}	1.0 mM	103	123
Mn^{2+}	5 mM	110	132
Mn^{2+}	10 mM	117	140

Table 4-6: Activity of the partially purified enzyme from *M. smegmatis* after the addition of cations and BSA at different concentrations

As the concentration of BSA increased so did the activity but using a 2% final concentration of BSA was too high in many assay systems so a 1% final concentration was chosen as a suitable concentration to use in combination with the cations. The activity of Ca^{2+} and Mn^{2+} were not significantly different at any of the concentrations and had previously been found to increase the activity of the enzyme to a higher degree (Table 4-3). The reasons for this may have been that the enzyme may have degraded since the previous experiments so that there was less protein content for the cations to activate and thus a lower increase in activity was observed compared to the previous experiments (Table 4-3) and that saturation of the enzyme by the cations

occurs at less than 0.5 mM. Using the above results and those from Section 4.3.3 it was decided that a 5 mM final concentration would be a workable concentration to use in combination with each other and BSA, as this should ensure saturation of the enzyme. The results of the combinations of the three additives showed that BSA and Ca^{2+} induced the most enzymatic activity from the sialidase (Table 4-7). The combination of Ca^{2+} and Mn^{2+} did not produce any synergistic effects with the activity being comparable to each cation being used alone (Table 4-6). Once again this would be due to saturation of the cationic sites. The combination of all three compounds gave the second highest activity after the BSA and Ca^{2+} also indicating that two different cations were not useful in optimising activity.

Additive	Activity (mU)	% Activity
None	73.5	100
BSA & Ca^{2+}	240.5	327
BSA & Mn^{2+}	176	239
Ca^{2+} & Mn^{2+}	100.5	136
BSA, Ca^{2+} & Mn^{2+}	222.5	302

Table 4-7: The effect of 1% BSA, 5 mM Ca^{2+} and 5 mM Mn^{2+} on the activity of the partially purified enzyme from *M. smegmatis* when used in combination.

As BSA and Ca^{2+} gave the highest activity in combination with each other they were then assayed at differing concentrations to determine if a lower concentration of the two together was still able to improve the sialidase activity. Table 4-8 shows this to be so with a high activity found when the concentrations of BSA and Ca^{2+} were 1% and 1 mM respectively. It appeared that lower concentrations of Ca^{2+} with a higher concentration of BSA were the most useful. The reason for this could be that there is only a limited amount of enzymatic sites to which Ca^{2+} can bind hence high concentrations will lead to saturation so that Ca^{2+} can no longer bind to enhance enzymatic activity. Extremely high concentrations of the two were shown to enhance activity and would most likely be due to the BSA's stabilising effect. These

concentrations were deemed too high to be practical in the usual assays thus it was determined that for optimal activity a final concentration of 1% BSA and 1 mM Ca^{2+} should be used.

Concentration of BSA	Concentration of Ca^{2+}	% Activity
0.0 %	0 mM	100
0.5 %	1 mM	680
1.0 %	1 mM	807
1.0 %	5 mM	700
1.0 %	10 mM	523
2.0 %	5 mM	877

Table 4-8: The change in activity of the partially purified enzyme from *M. smegmatis* after the addition of differing concentrations of BSA and Ca^{2+}.

4.3.9 Kinetics

Kinetic studies for the partially purified *M. smegmatis* enzyme were performed with MUN and fetuin under normal assay conditions and under optimal conditions. Normal conditions mean that the buffer used was 50 mM sodium acetate buffer pH 5.0 containing 1 mM Ca^{2+}. Optimal conditions utilised the same buffer with the addition of 1% BSA. MUN was analysed using the fluorescence assay previously described and fetuin using the thiobarbituric acid assay (Warren 1959; Potier 1981). The concentration of Neu5Ac in the fetuin was determined by the orcinol assay (Chapter 2, Section 2.2.6 and Appendix 3). The Enzyme Kinetics Program (version 2.0; Hearne Scientific Software) analysed all kinetics data. This program fits the initial velocity measurements as a function of substrate concentration using an iterative least-square function and Michaelis-Menten kinetics. Table 4-9 summarises the K_m and V_{max} for the experiments and their respective Michaelis-Menten graphs and Lineweaver-Burk plots are also shown in Figure 4-6 and Figure 4-7. The Michaelis-Menten and Lineweaver-Burk calculations are described in Appendix 13.

Substrate	Conditions	K_m of sialic acid	V_{max} (U)
MUN	Normal	23.25 mM	28.22
MUN	Optimal	0.897 mM	2.521
Fetuin	Normal	5.60 μM	4.070
Fetuin	Optimal	0.247 μM	0.349

Table 4-9: The K_m and V_{max} of MUN and the Neu5Ac in fetuin. At optimal conditions both substrates have an increased rate of reaction as would be expected.

Table 4-9 shows that the K_m and V_{max} for both MUN and the Neu5Ac in the fetuin decreases at optimal conditions. The decrease in K_m for both substrates is about 23 fold and the drop in V_{max} is 11 fold. It is interesting to note that both substrates increase the rate of reaction at optimal conditions by the same proportion.

Compared with other sialidases the values determined for the above substrates are not unusual. Sialidases from different organisms and species can produce a wide range of K_m and V_{max} values. A few examples are given in Table 4-10 for comparison.

Sialidase Type	Reference	Substrate	K_m	V_{max}
Rat Liver	(Miyagi and Tsuiki 1985)	Fetuin	2.10 mM	41 μmol/h/mg
Rat Liver	(Miyagi and Tsuiki 1985)	MUN	0.67 mM	100 μmol/h/mg
Pig Liver	(Kobayashi, Ito *et al.* 2000)	MUN	0.07 mM	150 nmol/min/mg
Human Placental	(Hıraiwa, Nishizawa *et al.* 1988)	Fetuin	2.53 mM	n/d
Rabbit sperm	(Prakash, Srivastava *et al.* 1977)	Fetuin	0.01 mM	0.112 μmol/min/mg
A. viscous	(Teufel, Roggentin *et al.* 1989)	MUN	0.1 mM	406 U/mg
T. brucei	(Engstler, Reuter *et al.* 1992)	MUN	0.16 mM	3.5 mU
Triatoma infestans	(Amino, Marques Porto *et al.* 1998)	MUN	0.19 mM	73 x 10^{-6} units
V. cholerae	(Potier, Mameli *et al.* 1979)	MUN	0.22 mM	n/d
V. cholerae	(Ciccotosto, Kiefel *et al.* 1998)	MUN	0.08 mM	n/d
Human plasma	(Monti, Bassi *et al.* 2000)	MUN	0.14 mM	7.6 mU/mg
Human erythrocyte	(Venerando, Fiorilli *et al.* 1997)	Fetuin	n/d	24.8
Human erythrocyte	(Venerando, Fiorilli *et al.* 1997)	MUN	n/d	66.6 μU/mg

Table 4-10: Sialidases and their K_m and V_{max} values with MUN and fetuin as substrates.

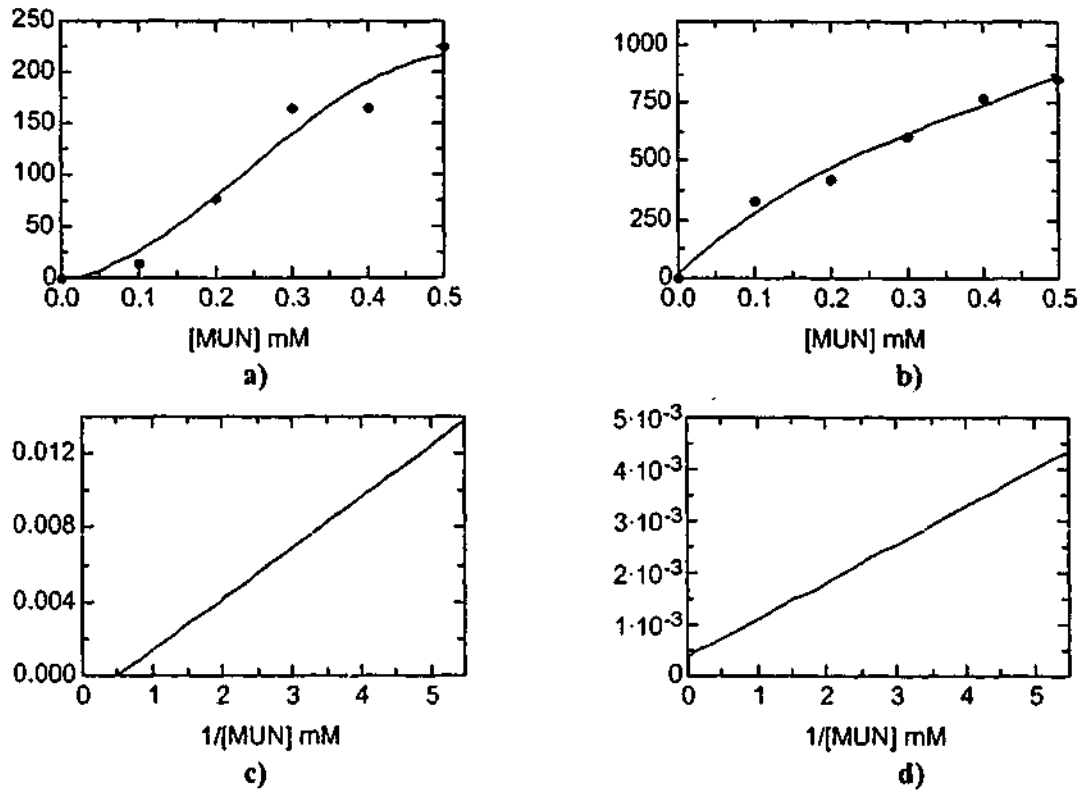


Figure 4-6: Comparison of the kinetics of the *M. smegmatis* sialidase at normal and optimal buffer conditions where MUN is the substrate. a) The change in velocity versus the concentration of MUN (mM) at normal conditions, b) The change in velocity versus the concentration of MUN (mM) at optimal conditions, c) The Lineweaver Burk plot of a) and d) The Lineweaver Burk plot of b).

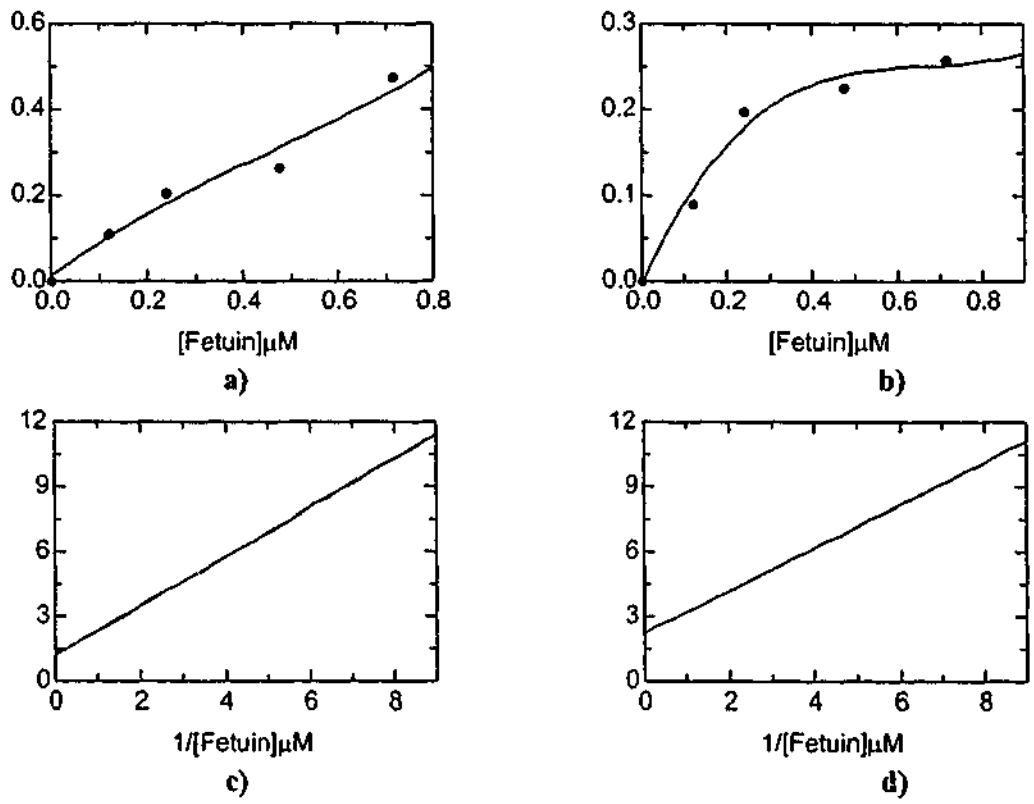


Figure 4-7: Comparison of the kinetics of the *M. smegmatis* sialidase at normal and optimal buffer conditions where fetuin is the substrate. a) The change in velocity versus the concentration of fetuin (μM) at normal conditions, b) The change in velocity versus the concentration fetuin (μM) at optimal conditions, c) The Lineweaver Burk plot of a) and d) The Lineweaver Burk plot of b).

4.3.10 Inhibition

To provide evidence to the hypothesis that the partially pure enzyme from *M. smegmatis* was a sialidase, inhibition studies were completed using the fluorescence assay with the normal buffer (Potier 1981). The enzyme was tested against Neu5Ac, Neu5Ac2en and a range of inhibitors that had been synthesised in the laboratory and were based on Neu5Ac. The structures of all inhibitors are found in Appendix 14. Neu5Ac2en, the PFV and TP inhibitors activity against other viral and bacterial sialidases is also given in Appendix 15. Of these synthesised inhibitors, none gave a significant inhibition against any of the bacterial sialidases except 1 mM PFV 110 against *S. typhimurium* sialidase and 1 mM PFV 112 against *C. perfringens* sialidase (Appendix 16). Most were able to significantly inhibit viral sialidases at 0.1 mM concentrations.

Table 4-11 gives a summary of the preliminary results of inhibition against the partially pure *M. smegmatis* enzyme. As discussed in the introduction, Neu5Ac can cause some inhibition of sialidases at a high concentration. At 8 mM final concentration this was found to be true for *M. smegmatis* enzyme with almost complete inhibition of enzymatic activity. MJK 1.120, DRG A22 and PFV 108 were found to give a high inhibition in this initial screening and were thus chosen for further investigation. MJK 2.5 did not give consistent results between duplicates so it was also re-assayed.

Inhibitor	Final concentration	Activity (mU)	% inhibition
None	-	357.5	-
Neu5Ac	8 mM	22.5	93 %
6TP.41.DD	1 mM	346.5	3 %
MJK 1.122	1 mM	313.5	12 %
MJK 1.126	1 mM	211.5	41 %
MJK 1.120	1 mM	24	93 %
DRG A22	1 mM	46	87 %
MJK 2.10	1 mM	380.5	0
MJK 2.5	1 mM	362	0*
PFV 112	1 mM	152.5	57 %
PFV 140	1 mM	142.5	60 %
PFV 102	1 mM	161	55 %
PFV 106	1 mM	194.5	46
PFV 108	1 mM	80	78 %
PFV 110	1 mM	254.5	29 %

Table 4-11: The activity of inhibitors to the *M. smegmatis* enzyme. * MJK 2.5 was retested, as the duplicates were not accurate. Neu5Ac2en was not tested in this screen as at this time new stock was being synthesised.

Table 4-12 shows that the inhibitors that gave a high decrease in enzymatic activity in Table 4-11 were still able to do so. In this experiment a lower concentration of Neu5Ac was used and still showed some inhibitory activity. Neu5Ac2en was also tested for its inhibitory activity and was shown to give a characteristically high inhibition that is seen for most other sialidases (Appendix 15) (Meindl *et al.* 1974; Holzer *et al.* 1992). MJK 2.5 was retested and did not give any enzymatic inhibition.

Inhibitor	Final concentration	Activity (mU)	% inhibition
None	-	109.5	-
Neu5Ac	1 mM	62.5	43 %
Neu5Ac2en	1 mM	5	95 %
MJK 1.120	1 mM	30.5	72 %
DRG A22	1 mM	16.5	85 %
MJK 2.5	1 mM	191.5	0
PFV 108	1 mM	4.5	95 %

Table 4-12: Repeat test of inhibitors to the *M. smegmatis* enzyme that gave good inhibition in Table 4-11. Neu5Ac2en was tested and shown to give high inhibition. A lower concentration of Neu5Ac continued to give a small amount of inhibition

Inhibition constants were determined for all inhibitors in Table 4-12 except DRG A22 and Neu5Ac. DRG A22 was not analysed, as there was not enough inhibitor available to do so. Neu5Ac was not analysed, as it was a poor inhibitor. All other inhibitors were determined to be linear competitive and their K_i was calculated using Michaelis-Menten kinetics and the Enzyme Kinetics Program (version 2.0; Hearne Scientific Software).

All were assayed using MUN and the normal buffer except Neu5Ac2en, which was assayed twice, once with the normal buffer and again with the optimal buffer for comparison (see Section 4.3.9). All assays were completed at two concentrations of substrate (0.15 and 0.30 mM MUN). Inhibitors were assayed in a range of concentrations; Neu5Ac2en between 0-0.1 mM; PFV 108 between 0-0.5 mM; and MJK 1.120 between 0-1 mM. The results are shown in Table 4-13.

Inhibitor	Condition	K_i
Neu5Ac2en	Normal	2.2583×10^{-5} M
Neu5Ac2en	Optimal	1.5714×10^{-5} M
PFV 108	Normal	1.5571×10^{-4} M
MJK 1.120	Normal	6.3860×10^{-5} M

Table 4-13: K_i values for inhibitors of the putative *M. smegmatis* sialidase enzyme.

As Table 4-13 shows, all inhibitors were determined to have high a K_i. The value for Neu5Ac2en did not appear to differ significantly between normal and optimal conditions indicating that the conditions may alter the activity of the enzyme but do not appear to change the binding ability of the inhibitor. The K_i value for Neu5Ac2en was also similar to the estimated K_i that was determined for the other bacterial sialidases shown in Appendix 15.

Structurally, all synthesised inhibitors are related to the parent, Neu5Ac. At this time it is difficult to determine why structures so similar to one another differ so greatly in activity. For example in the MJK series MJK 1.122 showed 12% inhibition compared with MJK 1.120 implying that the acetyl group in the 9 position was necessary for recognition. This was not the case for MJK 2.10 (Appendix 14). MJK 2.10 however, is also acetylated at the C-4 hydroxyl of galactose which may change its specificity. To understand this phenomenon clearly it is necessary to solve the structure of the enzyme which will then answer the questions of binding specificity and structural requirements.

4.4 Discussion

The results from this chapter have shown that the enzymatic activity that was found in the crude cell lysates of *M. smegmatis* is most likely that of a sialidase. Initial assays showed that the enzymatic activity could be lost through proteolysis with pronase and chelation of cations by EDTA.

The enzyme shares common properties of other bacterial sialidases such as an optimal pH that is slightly acidic, an optimal temperature of 37 °C and stabilisation by BSA and Ca^{2+}. Like most other sialidases it prefers to hydrolyse $\alpha(2\rightarrow3)$ linkages to other glycosidic linkages. Although no size could be determined for the enzyme it contained the ability to fluoresce on a native gel in the same position as *V. cholerae* sialidase. Kinetically, it was also shown to be an enzyme by being saturated at high substrate concentrations. Most importantly Neu5Ac2en, the original sialidase inhibitor that is capable of inhibiting almost all sialidases to a high degree, inhibited the *M. smegmatis* enzyme to a degree that is comparable with other bacterial sialidases.

The information gathered in this chapter should enable the further study of this enzyme to be easier as the optimal conditions and inhibitory substances are now known and can be used or avoided as necessary.

5 Conclusions

Mycobacteria as a species have the ability to cause a wide spectrum of disease in both humans and animals. TB is the most commonly known infection from these organisms and is responsible for a great deal of morbidity and mortality worldwide. The primary objective of the work described in this thesis was to identify a suitable enzyme from this genus that had the potential of being a target for chemotherapeutic agents.

Initial analysis of the *M. tuberculosis* H37Rv genome revealed Rv3463 as a gene annotated to be a putative sialidase. Sialidases which cleave sialic acids are implicated in the nutrition and pathogenicity of many organisms (Moriyama and Barksdale 1967; Nees *et al.* 1975; Corfield *et al.* 1981; Vimr and Troy 1985; Teufel *et al.* 1989; Galen *et al.* 1992; Straus *et al.* 1996; Buschiazzo *et al.* 1997; Byers *et al.* 2000; Magdesian *et al.* 2001). The strategy of using a sialidase as a chemotherapeutic target has been successfully demonstrated in the treatment of influenza.

Examination of the Rv3463 gene for structural similarities such as the Asp or RIP/RLP motifs, common in all bacterial sialidases, did not support its annotation (Roggentin *et al.* 1989; Roggentin *et al.* 1993a). Neither did further sequence analysis of the gene with other sialidase enzymes. Rv3463 appeared to have no common features with the sialidase enzymes.

To investigate whether a sialidase existed in mycobacteria, *M. smegmatis* and *M. bovis* BCG cell cultures were tested for sialidase activity and interestingly an enzyme resembling a sialidase was found to exist in the cell lysate. No activity was found in the cell culture media indicating that this enzyme was not secreted and was more likely cell bound, similar to sialidases from *C. perfringens*, *S. typhimurium* and *A. viscosus* (Roggentin *et al.* 1988; Teufel *et al.* 1989; Hoyer *et al.* 1992). Further fractionation of the cell lysate revealed activity in both the membrane and cytosol but not the cell wall confirming the initial results. The enzyme displayed weak sialidase activity and was thus tested for *trans*-sialidase and sialyltransferase activity but was found to possess neither.

As further examination of the enzymatic activity was necessary, purification methods were utilised. Cultures of *M. smegmatis* grew faster than those of *M. bovis* BCG and

M. smegmatis also displayed more sialic acid cleaving ability. Based on these characteristics, *M. smegmatis* was selected as the organism to purify and characterise the putative enzyme from. Preliminary attempts to purify the enzyme were difficult. Most column matrices employed failed to give pure, active enzyme. The most successful purification method utilised an affinity column that contained sialic acid thioglycosidically-linked to Sepharose 6B (Ciccotosto *et al.* 1998). This purification method gave extremely low yields of the protein which precluded determination of the purity and approximate size of the enzyme however, the enzymatic activity of the material produced was extremely high and useful for analysis. The partially purified protein was also extremely stable with enzymatic activity being retained for at least six months when the enzyme was stored at –20 °C. Nevertheless, the amount of protein was so low that it was not useful for analysis, therefore a bio-molecular approach was adopted to solve this problem.

Currently the genome for *M. smegmatis* is being determined so it was not available at the time of cloning for examination of its genes to determine a putative sialidase. Accordingly, it was necessary to use *M. tuberculosis* H37Rv DNA for cloning of the gene. Rv3463 and genes considered to have similarity to sialidases (Rv1733c and Rv1968) from sequence similarity matching were cloned in an attempt to identify a sialidase enzyme. This method did not detect any sialidase activity from the expressed protein obtained cloned genes. Sialidase activity testing of *M. tuberculosis* cell lysates also showed that the organism did not independently produce any sialidase activity indicating that the annotation for Rv3463 was incorrect and the lack of activity found in the clones was true. Correspondence with the authors of the genome has ensured that Rv3463 will have its annotation rectified when the genome is next updated (Appendix 12).

Further examination of the *M. smegmatis* mutant gene library also proved difficult. Screening agar plates for colonies without sialidase activity could not be undertaken as the activity in the cells was already low so that most colonies containing a sialidase would probably not fluoresce when sprayed with MUN and would give false negative results. Moreover, mycobacteria have a thick cell wall making it difficult for the substrate to permeate this barrier preventing the sialidase reaction from occurring. The mycobacterial cell wall also thwarted attempts to screen the library with MUN

using a microplate assay. No successful method could be devised to lyse the cells within the plates. The genetic approach was thus abandoned/postponed until the *M. smegmatis* genome is completed.

Biochemical analysis was undertaken on the active partially purified fractions of *M. smegmatis* to further characterise the enzyme. As no sialidase activity was detected in *M. tuberculosis*, it was initially decided that it was important to confirm that the activity found in *M. smegmatis* was due to enzymatic activity and not degradation of MUN from an alternate source. Digestion of the enzyme with pronase confirmed that the activity was enzymatic with a corresponding decrease in the hydrolysis of MUN. Protease inhibitors were also added to the cell lysate to investigate whether they would provide any stabilising effects. It was rationalised that the observed loss of activity in some purifications could be due to the possibility that the semi-pure enzyme was more susceptible to attack by natural proteases that exist within the cell lysate. Unfortunately, the protease inhibitors appeared to inhibit the sialidase-like enzyme activity and were therefore not deemed to be useful. The rationale for the inhibition of sialidase activity was the fact that later results showed that cations are able to increase enzymatic activity for the putative sialidase, and EDTA, one of the protease inhibitors functions as a cationic chelator inhibiting this effect. PMSF had also been reported to decrease the sialidase activity of rat liver lysosomal sialidase (Miyagi and Tsuiki 1985).

Other stabilising agents were tested for their effect on the enzyme. Both BSA and glycerol increased the enzymatic activity to a higher degree than other known stabilisers (Table 4-2). The increase in enzymatic activity after the addition BSA confirmed that the enzyme required non-enzymatic protein for stabilisation. This observation explained why some pure fractions with little protein content would rapidly lose activity. Addition of BSA restored activity to fractions where enzymatic activity had decreased. In all cases the protein appeared to behave as an enzyme, but was it actually a sialidase? More evidence was needed to convince the sceptics.

The effect of cations and soaps on enzymatic activity was examined. The addition of bivalent cations to enzyme fractions as previously mentioned, significantly increased the degree of enzymatic activity, with Ca^{2+} and Mn^{2+} increasing activity to the greatest degree (Section 4.3.3, Table 4-3). Inhibition of the enzyme from the addition

of EDTA, a cationic chelator and protease inhibitor was thus a correct observation. Similar inhibition by EDTA, and enzyme activity enhancement by Ca^{2+} and Mn^{2+} was observed with *V.cholerae*, *C. diptheriae*, and human exo-α-sialidase (Ada *et al.* 1961; Moriyama and Barksdale 1967; Achyuthan and Achyuthan 2001). Cu^{2+} was shown to inhibit the enzymatic activity, as observed for the cytosolic sialidase from rat liver (Miyagi and Tsuiki 1985), the guinea pig pulmonary parenchyma sialidase (Trabelsi *et al.* 1991), and the pig liver sialidase (Kobayashi *et al.* 2000). The enzymatic properties of the *M. smegmatis* enzyme correlated well with the characteristics of other sialidases.

Determination of the optimal pH, pI and temperature of the *M. smegmatis* enzyme was also observed to be comparable to other well characterised sialidases. Originally, the optimal pH of the crude enzyme in the cell lysate was determined to be pH 3.8. Once the protein had been partially purified the optimal pH was pH 5.0. This observation was also described for the cytosolic sialidase from rat liver (Miyagi and Tsuiki 1985). Most bacterial sialidases have optimal pH values in the acidic range, such as *A. viscosus* (pH 5.0) (Teufel *et al.* 1989) and *V. cholerae* sialidase that has a maximal activity at pH 4.6 (Potier 1981). The pI was determined to lie between pH 5.0 and 6.0, in accordance with that observed for other bacterial sialidases (Teufel *et al.* 1989; Roggentin *et al.* 1993a). The temperature at which the *M. smegmatis* enzyme possessed maximal activity was determined to be 37 °C, and again consistent with the majority of sialidases, including *A. viscosus* sialidase (Teufel *et al.* 1989).

Further proof that the enzyme from *M. smegmatis* was a sialidase was determined by substrate and linkage specificity studies. The enzyme demonstrated a high specificity to α(2,3)-linked sialic acids over other linkages. Sialidases from *S. pneumoniae, P. multocida, C. diphtheriae, V. cholerae, S. oralis* also show the same preference to α(2,3)-linked sialic acids (Moriyama and Barksdale 1967; Potier *et al.* 1979; Berry *et al.* 1996; Byers *et al.* 2000; Mizan *et al.* 2000). Further examination of the relative hydrolysis rates of the substrates revealed that the *M. smegmatis* enzyme (Table 4-5) followed a similar pattern of specificity as that of *V. cholerae* and *C. perfringens* (Corfield 1992). The only exceptions to this was mucin and colominic acid. The *M. smegmatis* enzyme could not hydrolyse mucin with high activity and could not

hydrolyse colominic acid at all. *V. cholerae* and *C. perfringens* sialidases hydrolyse mucin to a high degree and weakly hydrolyse colominic acid (Corfield 1992).

Kinetic and inhibition studies confirmed that the enzyme behaved as a sialidase. At the determined optimal conditions with MUN the enzyme had a K_m of 0.897 mM, comparable to the K_m observed for *A. viscosus, T. brucei, Triatoma infestans* and *V. cholerae* sialidases (Table 4-10).

The K_m for fetuin was lower than the other sialidases shown in Table 4-10, however these values were obtained from mammalian, not bacterial sialidases which could possibly account for the difference. Inhibition studies showed that the enzyme is strongly inhibited by Neu5Ac2en (K_i 1.57 x 10^{-5} M) and other inhibitors based on sialic acid such as PFV 108 and MJK 1.120 (Appendix 14). The K_i of Neu5Ac2en was comparable to that of other bacterial sialidases (Appendix 16), further supporting the sialidase-like characteristics of the *M. smegmatis* enzyme.

The data and evidence gathered and discussed in this thesis, strongly suggest the existence of a sialidase enzyme in *M. smegmatis*. Unfortunately, the difficulties experienced in the purification of the enzyme prevented determination of an approximate size or *N*-terminal sequence of the enzyme which will be addressed in future studies. Until such time as the genome is completed, further purification to obtain active protein may be undertaken using an approach based upon affinity matrix techniques with a pH gradient. Elution of the protein should occur in the pH 5.0 range. This may solve the problem of protein being eluted in more than one fraction leading to a more enzyme-enriched fraction. The completion of the *M. smegmatis* genome will make the search and identification of the sialidase a much easier task. From here, successful cloning may be able to increase the expression to produce more protein that will be easier to purify and further characterise.

These studies have confirmed that there does not appear to be a sialidase associated with *M. tuberculosis*, therefore new, potent chemotherapeutics for TB will have to be sought by other approaches. This is not to say that the determined lack of existence of a sialidase in this mycobacterial species precludes the existence of sialidases in other mycobacterial species. There are many other mycobacterial species that cause serious disease such as *M. ulcerans* and *M. avium* that have different and larger genomes than *M. tuberculosis* and may contain the sialidase detected in *M. smegmatis* and *M. bovis*

BCG. Therefore the information that has been gained for *M. smegmatis* and *M. bovis* BCG may have important implications for the treatment of diseases caused by other mycobacterial species. Determination of the gene from *M. smegmatis* will potentially enable searching the other organisms faster, as its sequence can be used to align with sequences identified in other complete and incomplete mycobacterial genomes. The process is faster and easier than screening all organisms in the laboratory with conventional assays, some which take months to culture or are too pathogenic to handle in a normal laboratory setting.

It is really only a matter of time before more information can be gathered about the *M. smegmatis* sialidase and the genome is nearing completion to make this work faster and easier. All results obtained in this thesis indicate a sialidase enzyme that may be cell bound, is most active at acidic pH and at 37 °C, is specific for α(2,3)-linkages and is inhibited by Neu5Ac2en. It is the first report and characterisation of a sialidase from mycobacteria. Understanding the sialidase's role in the organism and determining its existence in the pathogenic species of this family may determine it to be a useful target in the design and treatment of a new generation of antimicrobials for mycobacteria. Its existence has opened up a new area into the research of the mycobacterial species, a genus that was never considered to produce a sialidase enzyme; proof that a sialidase exists in *M. smegmatis* is, in itself a very interesting discovery.

Appendix 1

Composition of Middlebrook 7H9 (Difco)

Ammonium Sulfate	0.5000 g
Monopotassium Phosphate	1.0000 g
Disodium Phosphate	2.5000 g
Sodium citrate	0.1000 g
Magnesium sulfate	0.0500 g
Calcium Chloride	0.0005 g
Zinc Sulfate	0.0010 g
Copper Sulfate	0.0010 g
L-Glutamic Acid	0.5000 g
Ferric Ammonium Citrate	0.0400 g
Pyridoxine	0.0010 g
Biotin	0.0005 g

Solution g/litre (final solution) 4.7 g/l. Final pH 6.6 at 25 °C.

To prepare one litre of media, suspend 4.7 g of dehydrated powder in 900 mL of distilled and deionised water containing 2 mL of glycerol. Sterilise in the autoclave for 15 mins at 121 °C. Cool, and aseptically add 2.5 mL of sterile 20 % Tween 80 and 100 mL of filter sterilised enrichment solution containing 2 g glucose and 0.85 g NaCl per 100 mL.

Appendix 2

Reagents used in the thiobarbituric acid assay

All reagents were prepared according to Aymard-Henry *et al* (Aymard-Henry *et al.* 1973).

Periodate reagent

Dissolve 4.28 g of sodium periodate ($NaIO_4$) in 38 mL of distilled water.

Add 62 mL of syrupy orthophosphoric acid and mix well.

Store in a glass-stoppered brown bottle.

Arsenite reagent

Dissolve 10 g of sodium arsenite ($NaAsO_3$) and 7.1 g of sodium sulfate in 100 mL of distilled water.

Add 0.3 mL of concentrated sulfuric acid (H_2SO_4)

Thiobarbituric acid reagent

Dissolve 1.2 g of thiobarbituric acid (4,8-Dihydroxy-2-mercaptopyridine) and 14.2 g of anhydrous sodium sulfate in 200 mL of distilled water by heating in a boiling water bath.

Make up fresh each week.

Butanol

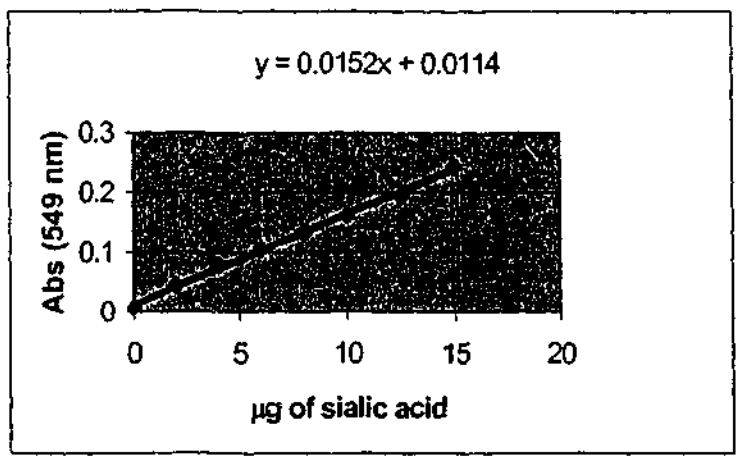
Add 5 mL of concentrated HCl to 100 mL of butanol.

Appendix 3

Standard curve of the concentration of Neu5Ac using the thiobarbituric acid assay

A standard curve for the concentration of Neu5Ac was set up according to the following table. As there was no need to cleave sialic acid, no incubation was necessary and the thiobarbituric acid assay was completed by the addition of PTA as outlined in Chapter 2 (Section 2.2.5). Results were read at 549 nm in the spectrophotometer and were graphed to give the standard curve. The resulting curve was used to determine the amount of cleaved sialic acid resulting after incubation with enzyme and substrates (Section 2.2.5).

Sample	Amount of Neu5Ac in 50 µl (µg)	Volume of 1 mg/mL Neu5Ac (µl)	Volume of Distilled Water (µl)	Volume of 50 mM Na Acetate pH 3.8 (µl)	Average absorbance of samples at 549 nm
1	0	0	150	100	0.006
2	2	2	148	100	0.044
3	4	4	146	100	0.078
4	6	6	144	100	0.105
5	8	8	142	100	0.132
6	10	10	140	100	0.162
7	12.5	12.5	137.5	100	0.1945
8	15	15	135	100	0.244



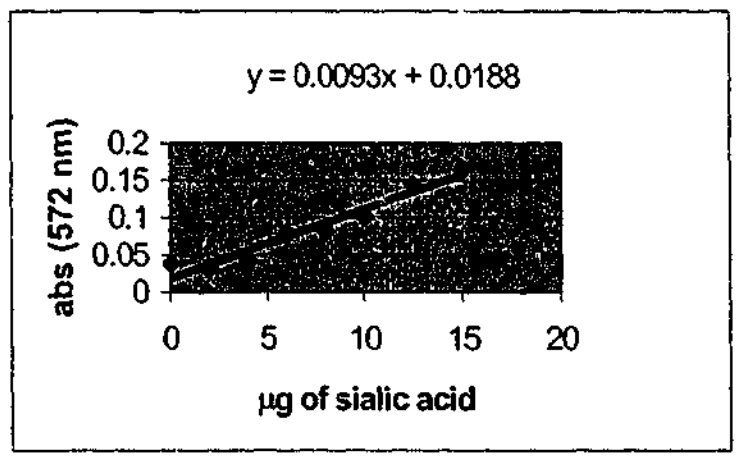
Appendix 4

Standard curve of the concentration of Neu5Ac using the Bial ferric/orcinol assay

A standard curve to determine the starting concentration of Neu5Ac in the thiobarbituric acid assay was completed using the first table. Results were read at 572 nm in the spectrophotometer and graphed to give the standard curve. From the standard curve, the amount of sialic acid in each substrate was determined and is shown in the second table on the following page. The experimental method for these results can be found in Chapter 2 (Section 2.2.6).

Sample	[Neu5Ac] µg/mL	Volume of 1 mg/mL Neu5Ac (µl)	Volume of Distilled Water (µl)	Volume of Orcinol reagent*	Average absorbance of samples at 572 nm
1	0	0	100	100	0.038
2	20	2	98	100	0.033
3	40	4	96	100	0.0405
4	60	6	94	100	0.078
5	80	8	92	100	0.0825
6	100	10	90	100	0.1025
7	125	12.5	87.5	100	0.143
8	150	15	85	100	0.1655

* Orcinol reagent contains 81.4 mL of concentrated HCl, 0.2 g of orcinol, 2 mL of 1 % $FeCl_3$ solution and distilled water to 100 mL.



Substrate	Stock concentration	Average absorbance of samples at 572 nm	Amount of Neu5Ac µg/mL
2,3-sialyllactose	3.8 mM	0.462	953.12
2,6-sialyllactose	3.8 mM	0.446	918.71
Fetuin	22.5 mg/mL	0.361	735.91
Mucin	22.5 mg/mL	0.6445	1345.59
Colominic acid	2.8 mg/mL	0.5245	1087.53
Mixed bovine gangliosides	7 mg/mL	0.527	1092.90

Appendix 5

Media recipes for SOB and SOC broths

SOB

Tryptone	20.00 g
Yeast extract	5.000 g
NaCl	0.584 g
KCl	0.186 g

Make up to 1 litre with distilled water and sterilise by autoclaving at 121 °C for 15 mins.

SOC

Tryptone	20.00 g
Yeast extract	5.000 g
NaCl	0.584 g
KCl	0.186 g
$MgCl_2.6H_20$	2.030 g
$MgSO_4.7H_20$	2.460 g
Glucose	3.600 g

Make up to 1 litre with distilled water and sterilise by autoclaving at 121 °C for 15 mins.

Appendix 6

Preparation of competent cells for electrotransformation of plasmids

Refer to Sambrook *et al.* 1989

1. Grow an overnight culture of *E. coli* BL 21 (DE3) pLys S or *E. coli* XL1-Blue MRF' cells in 10 mL of SOB media at 37 °C with shaking.
2. Inoculate 250 mL of SOB media in a pre-warmed 2 litre flask with 2.5 mL of the overnight culture.
3. Grow for 2-4 h until the OD_{600} is 0.7 (no more than 0.8).
4. Put the culture on ice for 10 min and keep all following steps on ice or at 4 °C.
5. Centrifuge cells for 10 min at 5,000 rpm.
6. Pour off the supernatant and the resuspend pellet in 10 mL of ice cold sterile 10% glycerol.
7. Add more glycerol to fill centrifuge tube and centrifuge for 10 min at 5,000 rpm as before.
8. Pour off supernatant and repeat steps 6 and 7.
9. Pour off as much supernatant as quickly as possible.
10. Resuspend the pellet in any remaining solution using a sterile 1 mL pipette.
11. Dispense into chilled, sterile microfuge tubes into 40-60 µl aliquots and snap freeze on a dry ice/ethanol bath.
12. Store at –70 °C until needed for electrotransformation of cells.

Appendix 7

Method of extraction of M. tuberculosis H37Rv DNA

1. Grow *M. tuberculosis* H37Rv in 20 mL broths of Middlebrook 7H9 media with Tween 80 and ADC enrichment (Jacobs *et al.* 1991) at 37 °C with shaking to mid-log phase.
2. Add cycloserine to a final concentration of 1 mg/mL.
3. Incubate at 37 °C with shaking for 48-72 h.
4. Centrifuge at 4,000 rpm for 15 min at 4 °C
5. Discard the supernatand and wash the cell pellet once with 10 mL of STE (0.1 M NaCl, 0.01 M Tris pH 7.5 and 0.1 mM EDTA)
6. Centrifuge at 4,000 rpm for 15 min at 4 °C
7. Resuspend the pellet in 4 mL of lysis solution (15% sucrose, 0.05 M Tris pH 8.5, 0.05 M EDTA, 1 mg/mL lysozyme and 10 µl Rnase (10mg/mL)
8. Incubate at 37 °C for 30 min
9. Add SDS to 4% (4 mL of 10% SDS)
10. Add proteinase K to 100 µg/mL (50 µl of 20 mg/mL suspension)
11. Increase the volume to 10 mL with TE (Appendix 8).
12. Incubate at 37 °C for 30 min
13. Incubate at 70 °C for 5 min
14. Extract with phenol/chloroform (1:1). Use 10 mL shake and centrifuge at 20 °C for 15 min at 4,000 rpm
15. Precipitate with 0.2 M NaCl and absolute ethanol. Decant the top layer into a 50 mL tube containing 400 µl of 5 M NaCl, then add 20 mL of ethanol.
16. Store at –20 °C for more than one hour (preferably overnight)
17. Centrifuge at 4,000 rpm for 30 min at 4 °C
18. Decant and dry pellet
19. Resuspend in 400 µl TE and transfer to a microfuge tube.
20. Extract with an equal volume of phenol/chloroform
21. Take the top layer to a new microfuge tube
22. Extract with an equal volume of chloroform
23. Take the top layer to a new microfuge tube

24. Precipitate with NaCl/ethanol- 4 μl NaCl/100 μl sample, then 2 times volume of ethanol. Centrifuge at high speed for 15 min at 4 °C.
25. Remove ethanol and air-dry the tube until all of the ethanol has evaporated.
26. Resuspend the pellet in TE (200 μl or so).
27. Check the concentration of DNA.

Appendix 8

Minipreparation method

Refer to Birnboim and Doly 1979; Sambrook *et al.* 1989.

Solutions

Cell resuspension solution: 50 mM Tris-HCl, pH 7.5
10 mM EDTA
10 μg/mL DNase free RNase A

Lysis solution: 0.2 M NaOH
1 % SDS

Neutralisation solution: 1.32 M potassium acetate, pH 4.8
1 mM EDTA

TE buffer: 10 mM Tris-HCl pH 7.5
1 mM EDTA

Procedure

1. Centrifuge cells from 1.5 mL of an overnight culture and discard the supernatant.
2. Resuspend cell pellet in 200 μl of resuspension solution.
3. Add 200 μl of lysis solution and mix by inversion until suspension clears.
4. Add 200 μl of neutralisation solution and mix by inversion.
5. Centrifuge at 13,000 rpm for 10 min.
6. Carefully decant the supernatant into a new tube and discard the pellet.
7. Add two volumes of 100% isopropanol to precipitate the DNA. Mix by inversion and leave at room temperature for 10 min.
8. Centrifuge for 15 min at 13,000 rpm and discard the supernatant.
9. Wash pellet twice with cold 70 % ethanol and repeat step 8 in between each wash.
10. Dry the pellet and then resuspend the DNA in 20 μl of TE or distilled water.

Appendix 9

Phenol chloroform extraction method

Refer to Birnboim and Doly 1979; Sambrook *et al.* 1989.

Procedure

1. Add an equal volume of phenol: chloroform to the nucleic acid sample.
2. Mix until an emulsion forms.
3. Centrifuge at 12,000 g for 15 s at room temperature.
4. Transfer the aqueous phase to a clean tube (upper phase).
5. Repeat steps 1 to 4 until no protein is visible at the interface.
6. Add an equal volume of chloroform and repeat steps 1 to 4.
7. Recover the nucleic acid by precipitation with ethanol (refer to step 7-10 Appendix 8)

Appendix 10

Sequence and BLAST matches of genes selected for cloning

Exo-Alpha Sialidase (cloning positive control) from *C.perfringens*

DEFINITION exo-alpha-sialidase (EC 3.2.1.18) - *Clostridium perfringens.*
ACCESSION S01339
PID g80529
SOURCE *Clostridium perfringens.*
AUTHORS Roggentin, P., Rothe,B., Lottspeich,F. and Schauer,R.
TITLE Cloning and sequencing of a *Clostridium perfringens* sialidase gene
JOURNAL *FEBS Lett.* 238 (1), 31-34 (1988)

AMINO ACID SEQUENCE:

```
mcnknntfek nldishkpep lilfnkdnni wnskyfripn iqllndgtil tfsdiryngp
ddhayidias arstdfqktw syniamknnr idstysrvmd sttvitntgr iiliagswnt
ngnwamttst rrsdwsvqmi ysddngltws nkidltkdss kvknqpsnti gwlggvgsgi
vmddgtivmp aqislrenne nnyysliiys kdngetwtmg nkvpnsntse nmvieldgal
imstrydysg yraayishdl gttweiyepl ngkiltgkgs gcqgsfikat tsnghrigli
sapkntkgey irdniavymi dfddlskgvq eicipypedg nklgggyscl sfknnhlgiv
yeangnieyq dltpyyslin kq
```

ORIGIN:

```
   1 - cttatacctt cttaaagtta taaaagctaa aattttatag aaaactccaa aaacaattat
  61 - atttgcctta ttccatagta aagaatttca aatactgtta taatttattt gaaaaacttc
 121 - tataattttc aatatagaag tttttaatag ttaggtttct aaagctattt ataagaaaaa
 181 - taacttttaa attaatgggt aaatatgaat ttatggagga gattatattt atgtgtaaca
 241 - aaaacaatac ctttgaaaag aatctagata taagccataa accagaacca ctaatactat
 301 - ttaacaagga taataacata tggaattcaa agtattttag aattcccaat atacaattat
 361 - taaatgatgg tacaatttta acctttcag atattcgtta taatggtcct gatgaccatg
 421 - cttatataga catagcttct gcacgtagta ctgattttgg aaagacatgg agctataaca
 481 - tagcaatgaa aaataatcgt attgactcta cttattctcg tgtaatggac tccacaacag
 541 - ttattacaaa tacaggtaga ataatattaa ttgcaggctc atggaataca aatggaaact
 601 - gggcaatgac tacttctaca agaagaagtg attggtctgt ccaaatgatt tattctgatg
 661 - acaatggatt aacttggtct aataaaatag atttaactaa ggactcttca aaagtaaaaa
 721 - atcaaccaag taatacaatt ggatggctag gaggagttgg ctcaggtatt gtaatggatg
 781 - atggaacaat agttatgcca gcacaaattt ccttaagaga aaataatgaa aataactatt
 841 - attcattaat tatctattca aaggataatg gtgaaacatg gacaatggga aacaaggttc
 901 - ctaattcaaa cacctccgaa aatatggtaa tagaattaga tggcgcttta attatgagta
 961 - caagatatga ttactctggc tatagggcag catacatctc tcatgattta ggaaccactt
1021 - gggaaatata tgaaccttta aacggtaaaa ttttaactgg taagggctct ggatgccaag
1081 - gttcctttat taaggctact acttcaaatg gacatagaat aggattaatt tcagcaccta
1141 - aaaacactaa aggtgaatat ataagagaca atattgccgt ttatatgatt gactttgatg
1201 - atttatctaa aggagttcaa gaaatatgca ttccttatcc tgaagacggt aacaaattag
1261 - gtggtggcta ttcttgtcta tcatttaaaa ataaccatct aggcattgtt tatgaagcca
1321 - atggaaatat agaatatcaa gacttaacac cttattactc actaattaat aaacaataat
1381 - aaaat
```

Rv3463 (similarity to sialidases) (3880903-3881757)

Starts at: 3880903
Ends at: 3881757
Molecular weight: 30652 Da
Gene name: Rv3463
Function: probable neuraminidase.
FASTA results: NRAM_IAQIT Q07584 neuraminidase (ec 3.2.1.18) opt: 116; E(): 0.97; (24.8 % identity in 214 aa overlap)

AMINO ACID SEQUENCE:

```
MTNCAAGKPSSGPNLGRFGSFGRGVTPQQATEIEALGYGAVWVGGSPPAA
LSWVEPILQATTTLCVATGIVNIWSAPAQRVAESFHRIEAAYPGRFLLGI
GVGHAEMISEYRKPYNALVEYLDRLDDYGVPANRRVVAALGPRVLGLSAR
RSAGAHPYLTTPEHTARARELIGPSAFLAPEHKVVLTTDSARARTVGRQA
LDMYFNLANYRNNWKRLGFTDDEVSRPGSDRLVDAVVAYGTPDAIAARLN
EHLLAGADHVPIQVLTEDDNLVSALTELAKPLRLT
```

ORIGIN:

>*M. tuberculosis* genome: 1055 bp - region from 3880803 bp to 3881857 bp

```
   1 - caccgccaac ccacattacc cgcatcgccg tgctctgcgc aaaacgccgt aggccacgcg
  61 - ctcaccggaa tagcaccggt gagccgagcg gttagagcaa ccatgaccaa ttgtgccgcc
 121 - ggcaaaccca gctcaggccc taacctcggc cgattcggat cgttcggacg cggcgtcacc
 181 - ccccagcagg ccacagaaat cgaggcgctg ggctacgggg cggtctgggt gggaggctca
 241 - ccacccgccg cactgtcctg ggtggacccg attctgcaag cgaccaccac attgtgtgtg
 301 - gccaccggca ttgtcaatat ctggtcggca ccggcccagc gagtcgccga atcgttccac
 361 - cgcatcgagg cggcctaccc gggccgcttt ctgctgggta tcggagtcgg gcatgccgag
 421 - atgatcagtg agtaccgcaa gccctacaac gcgctggtgg aatacctaga ccggctcgac
 481 - gactatgggg tgcccgccaa ccgccgggtg gtggccgcac tgggcccccg ggtcctgggc
 541 - ctgtccgcac gccgcagcgc cggggcgcac ccgtacctga ccacacccga acacacggca
 601 - cgggcccgtg agctgattgg tccgtcggcg ttcctggcgc ccgaacacaa ggtggtgctg
 661 - accaccgact cggcaagggc ccgtacggtg ggacgccagg cgctcgatat gtacttcaac
 721 - ctggctaact accgcaacaa ctggaaacgg ctgggcttca ccgacgacga agtctcccgg
 781 - ccgggcagcg accgcctggt tgacgccgtg gtcgcctacg gcactccaga cgcgatcgcg
 841 - gcacggctga acgaacacct gcttgcaggc gccgaccatg tccctattca ggtcctcacc
 901 - gaagatgaca acctggtgtc ggcgctgacc gaactcgcga agccgctccg actgacttga
 961 - tcccgaaacg gagggttgcg aacccaactg gtcgcggctc cactcggtta aggctcggtt
1021- agggtttgat ccatgcggtt gctagtcacc ggtgg
```

Genes in region from 3880.8 kb to 3881.9 kb (1054 bp)

Gene name	Length	A. number	Coordinates	Orientation	Description
InfA	219	Rv3462c	3880433..3880651	-	initiation factor IF-1
Rv3463	855	Rv3463	3880905..3881759	+	probable neuraminidase
RmlB	993	Rv3464	3881835..3882827	+	dTDP-glucose 4,6-dehydratase

BLAST Results:

No significant matches found

Rv1733c (KDN sialidase *N*-terminal sequence match)(1959856-1960485)

Starts at:	1959856
Ends at:	1960485
Molecular weight:	22459 Da
Gene name:	Rv1733c
Function:	probable membrane protein

AMINO ACID SEQUENCE:

MIATTRDREGATMITFRLRLPCRTILRVFSRNPLVRGTDRLEAVVMLLAV
TVSLLTIPFAAAAGTAVQDSRSHVYAHQAQTRHPATATVIDHEGVIDSNT
TATSAPPRTKITVPARWVVNGIERSGEVNAKPGTK**SGDRVGIWVDSA**GQL
VDEPAPPARAIADAALAALGLWLSVAAVAGALLALTRAILIRVRNASWQH
DIDSLFCTQR

Blasted with *N*-terminal sequence of KDN sialidase from Pavlova *et al.* (Pavlova, Yuziuk *et al.* 1999)

(Match in sequence with *N*-terminal sequence of KDN sialidase (SGDSAGIWVLSAR) is in bold and the underlined section was a match with *M. viridifaciens* sialidase (GI:585539))

ORIGIN:

>*M. tuberculosis* genome: 830 bp - region from 1959756 bp to 1960585 bp

```
  1 - ggtgccgagg gcgagcatgc tggattcaac ggccatgccg tccagagtac ggtcgcggtc
 61 - cagcttggcg gagccctggt tgccgctacc ggacggttgt caccgctgcg tgcagaacag
121 - gctgtcgatg tcgtgttgcc aactggcgtt gcgaacgcgg atcagaatcg cccgagtgag
181 - cgccagcagg gcgcccgcaa ccgcggcgac gctcaaccag agtcccaagg cggccagggc
241 - cgcatccgca atggcacggg ccggcggagc tggttcatcg accagctgac cggcactgtc
301 - gacccaaatg ccgacgcggt caccggattt ggttcccggc ttcgcgttga cctcaccgct
361 - gcgttctatt ccgttcacga cccatcgggc aggcacggtg atcttcgtgc gcggcggcgc
421 - tgacgtggcg gtcgtgttgc tgtcgatcac cccctcgtga tcgatcacgg tcgcggttgc
481 - gggatggcgg gtctgggcct ggtgggcata gacgtggctg cgggaatcct ggactgcggt
541 - gccggccgcg gcggcgaacg ggatagtcag cagcgagacc gtgacggcca gcagcatgac
601 - gaccgcctcg agtcgatccg tcccacgcac cagcggattg cggctgaaca cccgcagtat
661 - cgtccggcac ggcaagcgca gcctaaacgt gatcatggtg gctccttcac gatcgcgggt
721 - tgtggcgatc atcgctgtga attgctcgtg gctcctaggg tcgttcggcc ttggggctgg
781 - ggacgtcggt cacgaatggc tgggcgccgt gcatatcggg tgaaccgggc
```

Genes in region from 1959.8 kb to 1960.6 kb (828 bp)

Gene name	length	A. number	Coordinates	Orientation	Description
Rv1732c	546	Rv1732c	1959246..1959791	-	Unknown
Rv1733c	630	Rv1733c	1959858..1960487	-	possible membrane protein

BLAST Results:

BLAST with KDN sialidase *N*-terminal sequence (Pavlova, Yuziuk *et al.* 1999).

Hypothetical protein Rv1733c [*Mycobacterium tuberculosis* H37Rv]

Length = 210, Score = 21.6 bits (44), Expect = 10, Identities = 9/12 (75 %), Positives = 9/12 (75 %)

```
Query:     1     SGDSAGIWVLSA 12
                 SGD  GIWV SA
Subject:   136   SGDRVGIWVDSA 147
```

Rv1968 (*V. cholerae* match) (2211624-2212853)

Starts at: 2211624
Ends at: 2212853
Molecular weight: 43673 Da
Gene name: Rv1968

Function: Unknown but similar to several *M. tuberculosis* proteins eg MTC28.11

FASTA results: Z97050|MTCI28_11 (515 aa) opt: 751 z-score: 555.6 E(): 2e-23; 32.1 % identity in 448 aa overlap. Contains hydrophobic signal-sequence like region in *N*-terminal 30 residues. Also similar to MTV023 and MTV051, TBparse score is 0.875

AMINO ACID SEQUENCE:

```
MKSFAERNRLAIGTVGIVVVAAVALAALQYQRLPFFNQGTRVSAYFADAG
GLRTGNTVEVSGYPVGKVSSISLDGPGVLVEFKVDTDVRLGNRTEVAIKT
KGLLGSKFLDVTPRGDGRLDSPIPIERTTSPYQLPDALGDLAATISGLHT
ERLSESLATLAQTFADTPAHFRNAIHGVARLAQTLDERDNQLRSLLANAA
KATGVLANRTDQIVGLVRDTNVVLAQLRTQSAALDRIWANISAVAEQLRG
FIAENRQQLRPALDKLNGVLAIVENRKERVRQAIPLINTYVMSLGESLSS
GPFFKAYVVNLLPGQFVQPFISAAFSDLGLDPATLLPSQLTDPPTGQPGT
PPLPMPYPRTGQGGEPRLTLPDAITGNPGDPRYPYRPEPPAPPPGGPPPG
PPAQQPGDQP
```

ORIGIN:

>*M. tuberculosis* genome: 1432 bp - region from 2211524 bp to 2212955 bp

```
   1 - cgccttctac ctgtgcgacg tcgtgctcaa ggtcaacggc aagggcggcc agccggtgta
  61 - catcaagctg gccggtcagg acagcgggcg gtgcgcgccg aaatgaaatc cttcgccgaa
 121 - cgcaaccgtc tggccatcgg cacagtcggc atcgtcgtcg tcgccgccgt tgcgctggcc
 181 - gcgctgcaat accagcggct gccgtttttc aaccagggca ccagggtctc cgcctatttc
 241 - gccgacgccg gcgggctgcg caccggcaac accgtcgagg tctccggcta tccggtggga
 301 - aaagtgtcca gcatctcgct cgacggaccg ggcgtgctgg tggagttcaa ggtcgacacc
 361 - gacgtccgac tcggaaaccg caccgaagtg gcaatcaaaa ccaagggctt gttgggcagc
 421 - aagttcctcg acgtcacccc ccgcggggac ggccgactcg attctccgat cccgatcgag
 481 - cggaccacgt cgccctacca actgcccgac gcccttggcg atttggccgc cacgatcagc
 541 - gggttgcaca ccgagcggct gtccgaatcg ctggccaccc tggcgcagac ctttgccgat
 601 - acgccggcgc acttccgcaa cgccatacac ggggtggccc ggctcgccca aaccctcgat
 661 - gagcgcgaca accaactgcg cagcctgctg gccaacgcgg ccaaagccac cggggtgctg
 721 - gccaaccgca ccgaccagat cgtcggcctg gtgcgcgaca cgaatgtggt cttggcgcag
 781 - ctgcgcaccc aaagcgccgc cctggaccgg atctgggcga acatctcggc ggtggccgaa
 841 - caactgcggg gcttcatcgc tgagaaccgc cagcagctgc gcccggcgct ggacaagctc
 901 - aacggggtgc tggctatcgt cgaaaaccgc aaagagcgtg tgcggcaggc catcccgctg
 961 - atcaacacct atgtcatgtc gctgggtgag tcgctgtcgt cgggcccgtt cttcaaggca
1021 - tacgtggtga acctgctgcc gggtcagttc gtgcaaccgt tcatcagcgc cgcgttctcc
1081 - gacctggggc tcgacccggc cacgttgctg ccgtcgcagc tgaccgaccc accgaccggt
1141 - caacccggaa ccccgccgtt gccgatgccc tacccgcgca cgggccaggg cggtgagccg
1201 - cggctgacgc tgcccgacgc gatcaccggc aatcccggcg atccgcgcta tccgtaccgg
1261 - ccggagccgc ccgcgccgcc gcccggcggg ccgccgcccg gcccgcccgc gcagcagccg
1321 - ggagaccaac cgtgacaacg aaactcagac gtgcccgctc ggtgttggcg accgccctgg
1381 - tgctggtcgc gggcgtgatc ctggccatgc gcaccgccga cgccgccgcc cg
```

BLAST Results:

BLAST with *V. cholerae* sialidase, GI 547993 (Galen, Ketley *et al.* 1992)

Part of mce3 operon [*Mycobacterium tuberculosis* H37Rv]

Length = 4411529, Score = 33.5 bits (75), Expect = 0.57, Identities = 21/67 (31 %), Positives = 31/67 (45 %), Gaps = 2/67 (2 %)

```
Query: 248     GGDPGALSNTND--IITRTSRDGGITWDTELNLTEQINVSDEFDFSDPRPIYDPSSNTVL
305            GGDPGAL       ++ R  R GG   D +    + +   + F F   RP+  P+S
Sbjct:2211755  GGDPGALVEKRQPLVLQRGQRNGGDDDDADCADGQTVAFGEGFHFGAHRPLS*PAS----
2211588

Query: 306    VSYARWP 312
               + Y  WP
Sbjct:2211587  LMYTGWP 2211567
```

Appendix 11

Sequencing results of Rv3463, Rv1733c and Rv1968

Rv3463 – Forward Primer

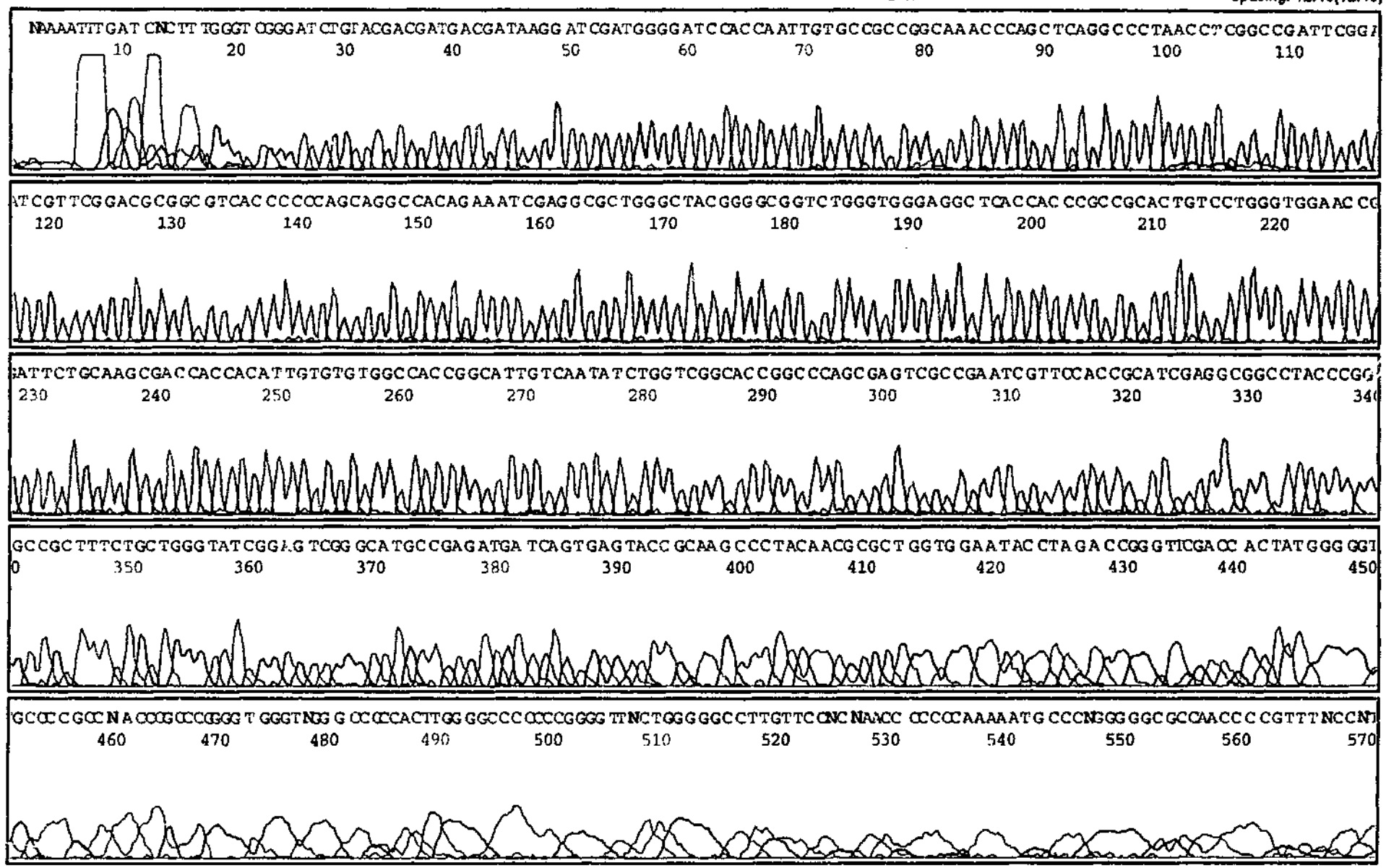
ABI PRISM

Model 373
Version 3.4.1
SeqAdaptive
Version 3.3.1

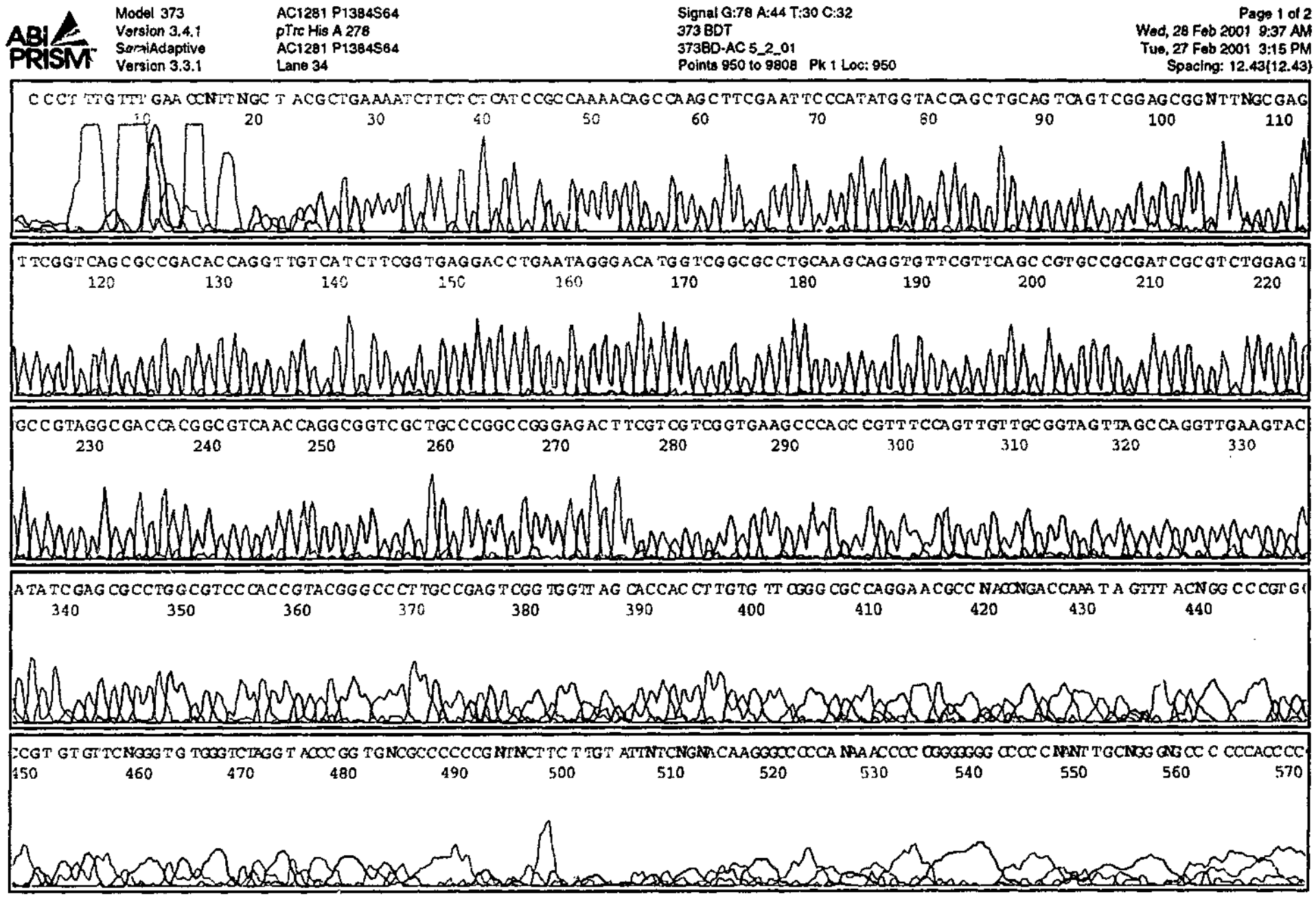
AC1281 P1384S65
pTrc His A 279
AC1281 P1384S65
Lane 35

Signal G:138 A:72 T:38 C:51
373 BDT
373BD-AC 5_2_01
Points 950 to 9808 Pk 1 Loc: 950

Page 1 of 2
Wed, 28 Feb 2001 9:37 AM
Tue, 27 Feb 2001 3:15 PM
Spacing: 12.40(12.40)



Rv3463 – Reverse Primer



Rv1733c – Forward Primer

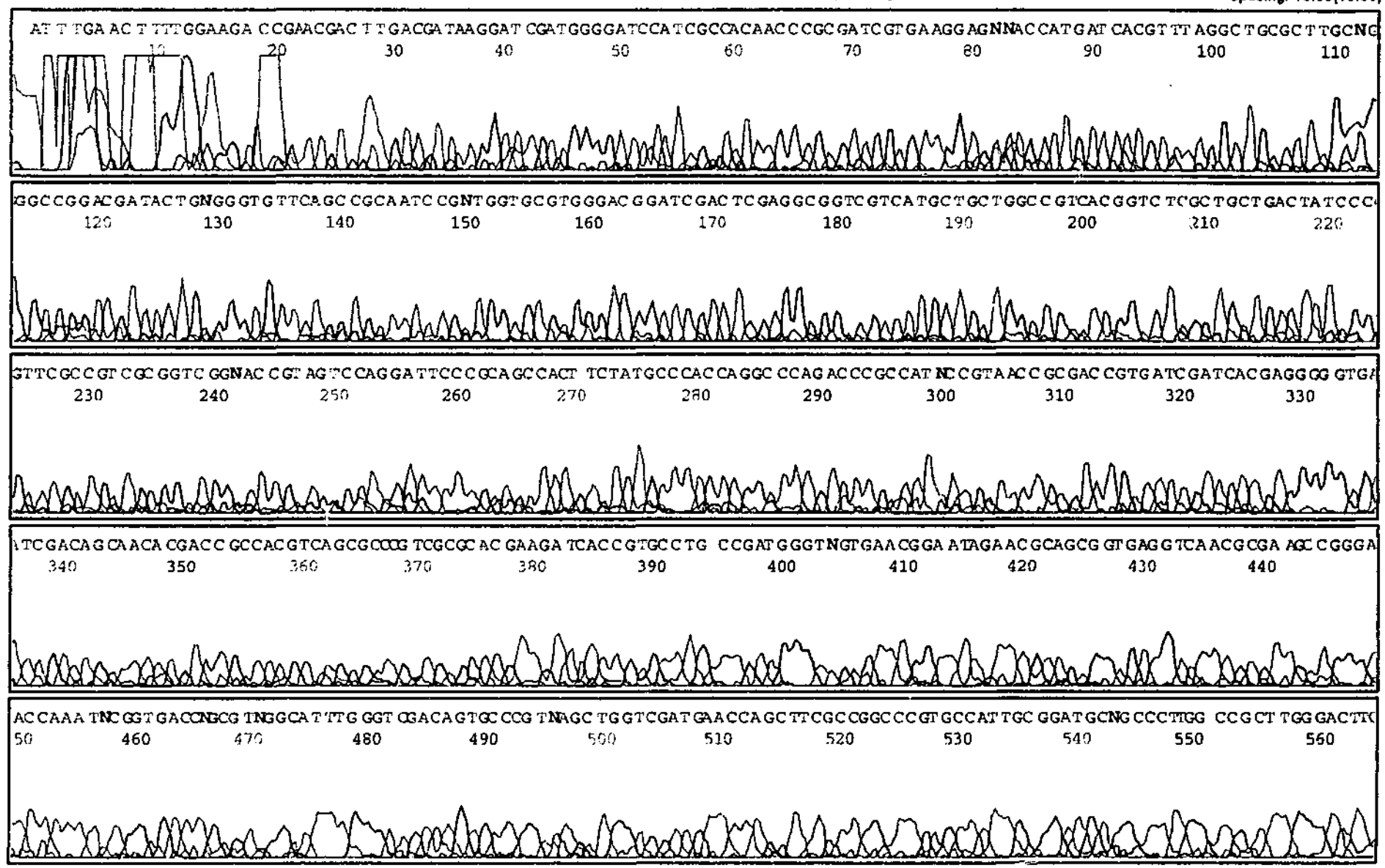
ABI PRISM

Model 377
Version 3.4.1
ABI100
Version 3.3.1

AD1343 P1384S69
pTrcHis A 279 Rv1733c
AD1343 P1384S69
Lane 47

Signal G:59 A:41 T:60 C:57
DT (BD Set Any-Primer)
373BD Simon 24/8/00
Points 950 to 6852 Pk 1 Loc: 950

Thu, 29 Mar 2001 11:40 AM
Wed, 28 Mar 2001 4:48 PM
Spacing: 10.60(10.60)



Rv1733c – Reverse Primer

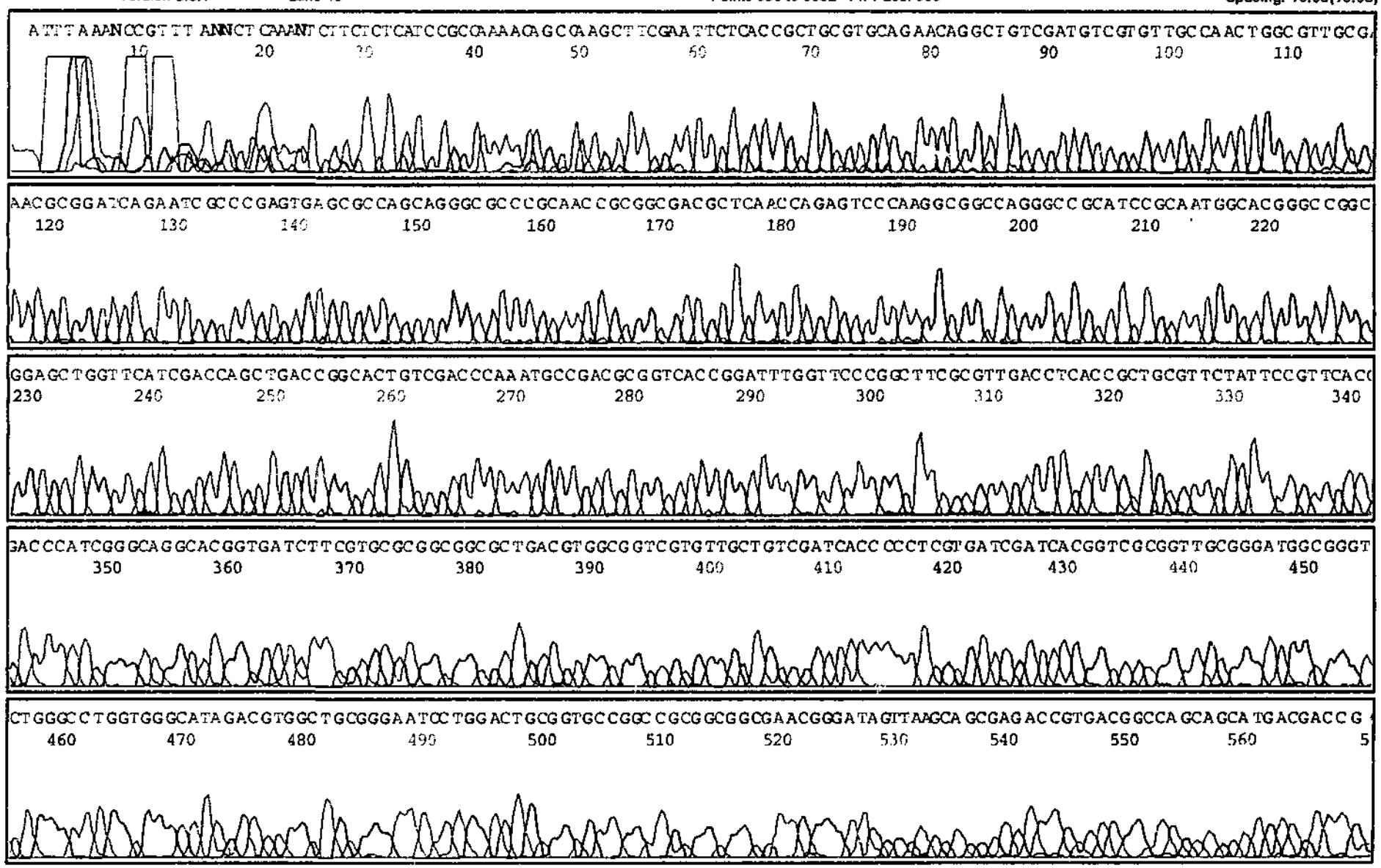
ABI PRISM

Model 377
Version 3.4.1
ABI100
Version 3.3.1

AD1343 P1384S68
pTrcHis A 278 Rv1733c
AD1343 P1384S68
Lane 46

Signal G:146 A:88 T:71 C:123
DT (BD Set Any-Primer)
373BD Simon 24/8/00
Points 950 to 8852 Pk 1 Loc: 950

Thu, 29 Mar 2001 11:39 AM
Wed, 28 Mar 2001 4:48 PM
Spacing: 10.68(10.68)



Rv1968 – Forward Primer

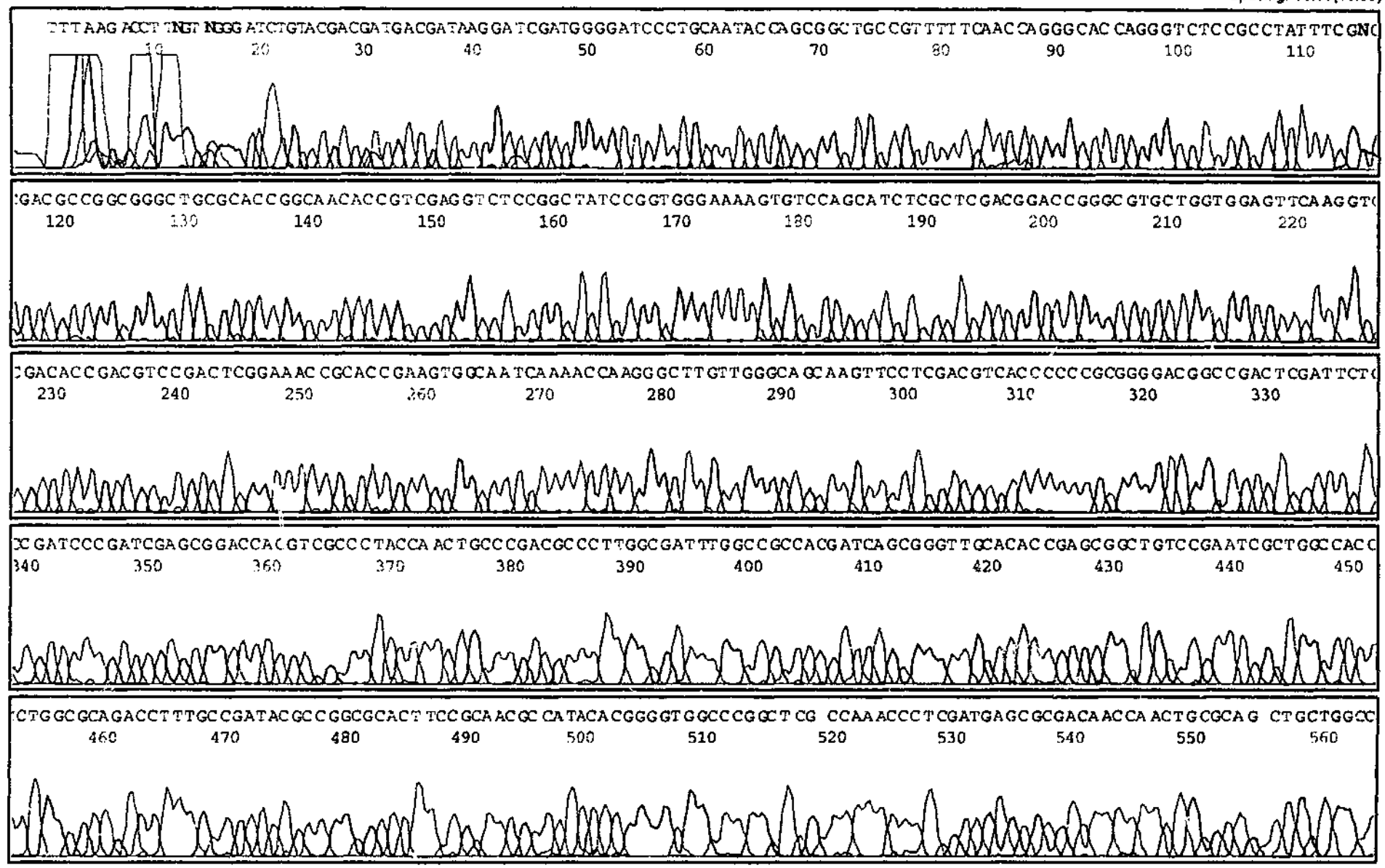
ABI PRISM

Model 377
Version 3.4.1
ABI100
Version 3.3.1

AD1343 P1384S71
pTrcHis A 279 Rv1968
AD1343 P1384S71
Lane 50

Signal G:280 A:164 T:120 C:257
DT (BD Set Any-Primer)
373BD Simon 24/8/00
Points 950 to 8852 Pk 1 Loc: 950

Thu, 29 Mar 2001 11:40 AM
Wed, 28 Mar 2001 4:48 PM
Spacing: 10.59(10.59)



Rv1968 – Forward Primer

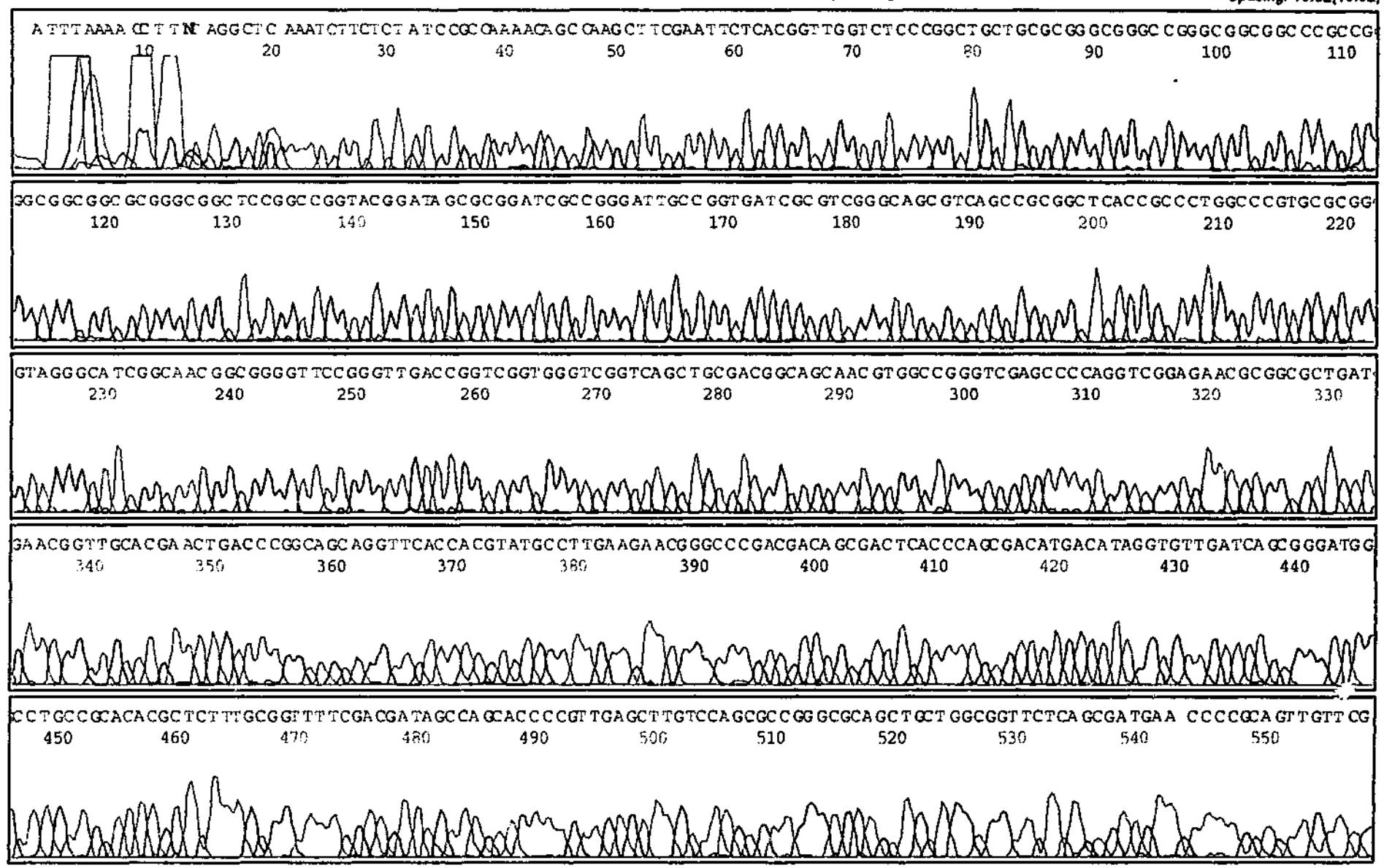
ABI PRISM

Model 377
Version 3.4.1
ABI100
Version 3.3.1

AD1343 P1384S70
pTrcHis A 278 Rv1968
AD1343 P1384S70
Lane 49

Signal G:262 A:124 T:104 C:197
DT (BD Set Any-Primer)
373BD Simon 24/8/00
Points 950 to 8852 Pk 1 Loc: 950

Thu, 29 Mar 2001 11:40 AM
Wed, 28 Mar 2001 4:48 PM
Spacing: 10.52(10.52)



Appendix 12

The correspondence and reply to Rv3463 from The Sanger Centre UK

Subject: Rv3463 a probable sialidase?

Date: Wed, 28 Mar 2001 14:14:53 +0100

From: Julian Parkhill <parkhill@sanger.ac.uk>

Organisation: The Sanger Centre

To: carolyn.trower@vcp.monash.edu.au

CC: Bart Barrell <barrell@sanger.ac.uk>, stcole@pasteur.fr

> **-----Original Message-----**

From: Carolyn Trower [mailto: carolyn.trower@vcp.monash.edu.au]

Sent: Monday, March 26, 2001 03:50

To: barrell@sanger.ac.uk

Subject: Rv3463 a probable sialidase?

Dear Dr. Barrell,

I am a postgraduate student that has been studying sialidases (neuraminidases) for the past few years. I am curious to know why the gene Rv3463 has been given the annotation of "probable sialidase". My colleagues and I have been BLAST searching this sequence and attempting to align it with many other sialidases, including the one used on your database, and have been unable to match it with any other known sialidases. We have also searched using the common sialidase motifs, still to no avail. If you or your colleagues are able to help us understand why this gene has been given this annotation it would be greatly appreciated.

Sincerely,

Carolyn Trower

> **-----Reply-----**

The annotation apparently came from a weak match that was present in the original database searches. Subsequent searches have shown that this annotation is probably not correct, and we will remove it next time we update the databases. Thanks for pointing this out.

Yours,

Julian Parkhill

Appendix 13

Kinetics calculations and theory

The theory for this Appendix was taken from Harper's Biochemistry (Rodwell and Kennelly 2000).

The Michaelis-Menten equation

The substrate concentration that produces half of the maximal velocity (K_m) may be determined by graphing the velocity as a function of [S].

When [S] is approximately equal to the K_m the velocity is highly responsive to changes in [S] and the enzyme is working at approximately half of the maximal velocity. This is expressed mathematically as:

$$v_i = \frac{V_{max}[S]}{K_m + [S]}$$

It is shown schematically as:

$$\text{Enz} + \text{S} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \text{EnzS} \xrightarrow{k_2} \text{E} + \text{Product}$$

A linear form of the equation is used to determine the K_m and V_{max}. A direct measurement of the numeric value for V_{max} and thus the calculation of K_{eq} may require impractically high substrate concentrations to achieve saturating conditions in the laboratory. In this way a straight line permits extrapolation of the K_m and V_{max} from less than saturating conditions. The Michaelis-Menten equation is inverted to give a straight-line equation, which is plotted to give a double reciprocal or Lineweaver-Burk plot:

$$v_i = \frac{V_{max}[S]}{K_m + [S]}$$

Inverted to:

$$\frac{1}{v_i} = \frac{K_m + [S]}{V_{max}[S]}$$

Factor:

$$\frac{1}{v_i} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

This is the equation for a straight line ($y = mx + c$) where:

$$y = \frac{1}{v_i} \text{ and } x = \frac{1}{[S]}$$

Competitive inhibition

Competitive inhibition is defined as competition of the inhibitor with the substrate for the binding position in the active sites of the enzyme. Most cases of this inhibition involve an inhibitor which resembles the substrate. In our example the substrate for a sialidase is sialic acid and all inhibitors used in this study contain a sialic acid moiety (Appendix 15). Competitive inhibition is usually a reversible reaction and can be shown by the following scheme:

$$\text{Enz} - \text{I} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \text{Enz} + \text{I}$$

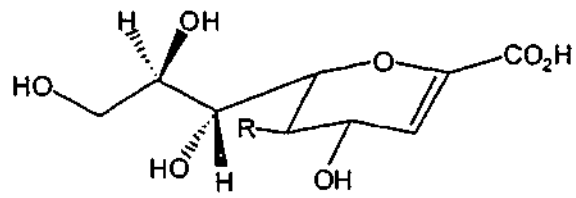
From this scheme the equilibrium constant K_i can be calculated:

$$K_i = \frac{[\text{Enz}]\,[\text{I}]}{[\text{Enz-I}]} = \frac{k_1}{k_{-1}}$$

A competitive inhibitor raises the apparent K_m of the substrate and the lower the K_i of an inhibitor the more powerful it is.

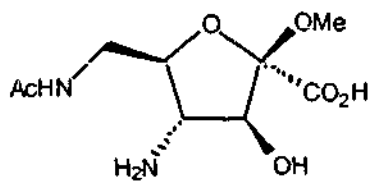
Appendix 14

The chemical structures of the sialidase inhibitors

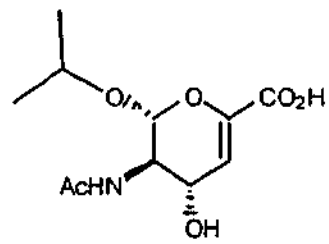


R = NHAc

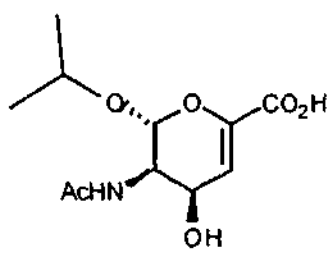
2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en)



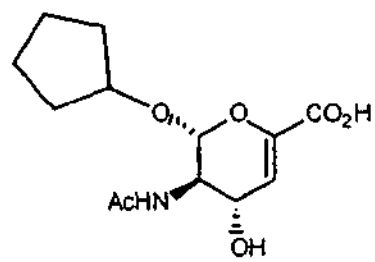
6.TP.41.DD



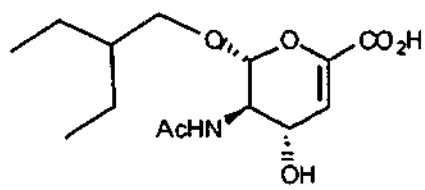
PF-IV-140



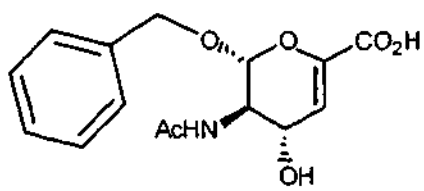
PF-V-102



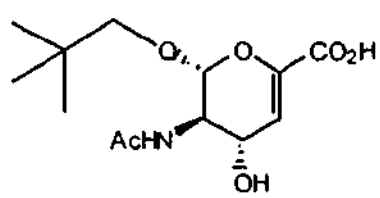
PF-V-106



PF-V-108

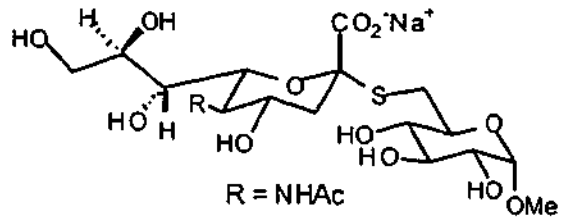


PF-V-110

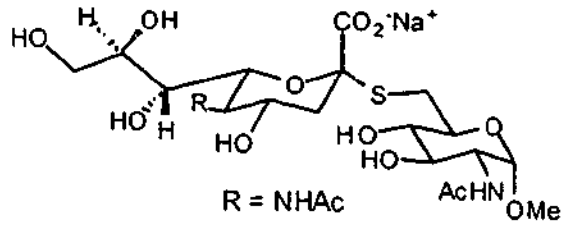


PF-V-112

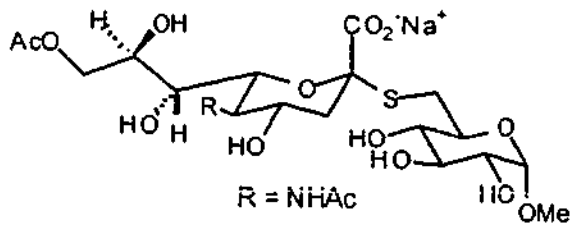
MJK 1.122(2)
$C_{18}H_{30}NO_{13}SNa$
MW = 523.49



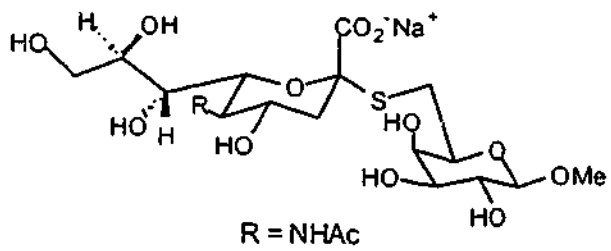
MJK 1.126(1)
$C_{20}H_{33}N_2O_{13}SNa$
MW = 564.54



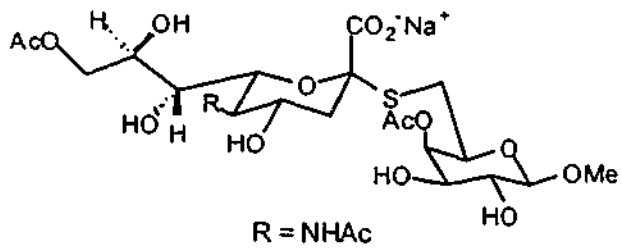
MJK 1.120(2)
$C_{20}H_{32}NO_{14}SNa$
MW = 565.52



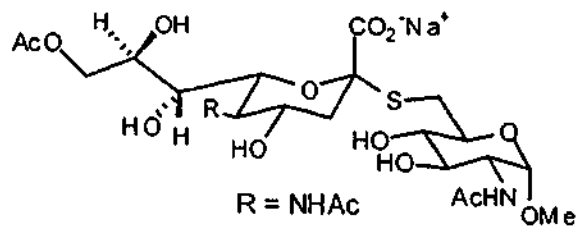
DRG A-22(1)
$C_{18}H_{30}NO_{13}SNa$
MW = 523.49



MJK 2.10(2)
$C_{22}H_{34}NO_{15}SNa$
MW = 607.56



MJK 2.5
$C_{22}H_{35}N_2O_{14}SNa$
MW = 606.57



Appendix 15

% Inhibition of the TP and PFV inhibitors to bacterial and viral sialidases

Organism	Neu5Ac2en 0.1 mM	Neu5Ac2en 1 mM
N9	87	100
N2	78	98
ST	43	92
CP	42	75
VC	98	97

Organism	6TP.41.DD 1 mM
N9	11
N2	20
VC	0

Organism	PFV-112 0.1 mM	PFV-112 0.5 mM	PFV-112 1 mM
N9	93	-	97
N2	95	-	96
ST	0	-	52
CP	12	14	89
VC	11	-	47

Organism	PFV-140 0.1 mM	PFV-140 0.5 mM	PFV-140 1 mM
N9	97	-	99
N2	92	-	99
ST	0	-	20
CP	0	6	0
VC	32	67	58

Organism	PFV-102 0.1 mM	PFV-102 0.5 mM	PFV-102 1 mM
N9	96	-	95
N2	89	-	98
ST	0	-	31
CP	1	6	0
VC	31	-	19

Organism	PFV-106 0.1 mM	PFV-106 0.5 mM	PFV-106 1 mM
N9	97		98
N2	95		98
ST	0		66.5
CP	6	12	0
VC	37		75

Organism	PFV-108 0.1 mM	PFV-108 0.5 mM	PFV-108 1 mM
N9	96	-	97
N2	95	-	99
ST	25	-	69
CP	3	6	0
VC	16	-	48

Organism	PFV-110 0.1 mM	PFV-110 0.5 mM	PFV-110 1 mM
N9	36	42	30
N2	11	-	65
ST	51	63	90
CP	50	76	73
VC	12	-	12

Approximate K_i (M) of sialidases at 1 mM final inhibition concentration.

Inhibitor	Neu5Ac2en	6TP.41.DD	PFV-112	PFIV-140	PFV-102	PFV-106	PFV-108	PFV-110
Organism								
N9	1×10^{-7}	2.5×10^{-3}	1×10^{-5}	5×10^{-6}	1×10^{-5}	5×10^{-6}	1×10^{-5}	5×10^{-4}
N2	5×10^{-6}	1×10^{-3}	1×10^{-5}	5×10^{-6}	5×10^{-6}	5×10^{-6}	1×10^{-6}	3×10^{-4}
ST	2.5×10^{-5}	nd	2.5×10^{-4}	1×10^{-3}	5×10^{-4}	1×10^{-4}	2.5×10^{-4}	5×10^{-5}
CP	1×10^{-4}	nd	5×10^{-5}	0	0	0	0	1×10^{-4}
VC	1×10^{-5}	0	2.5×10^{-4}	2.5×10^{-4}	1×10^{-3}	1×10^{-4}	2.5×10^{-4}	2.5×10^{-3}

Approximate Ki (M) of sialidases at 0.1 mM final inhibition concentration.

Inhibitor	Neu5Ac2en	PFV-112	PFIV-140	PFV-102	PFV-106	PFV-108	PFV-110
Organism							
N9	5×10^{-6}	1×10^{-6}	1×10^{-6}	1×10^{-6}	1×10^{-6}	1×10^{-6}	5×10^{-5}
N2	1×10^{-5}	1×10^{-6}	1×10^{-6}	5×10^{-6}	1×10^{-6}	1×10^{-6}	2.5×10^{-4}
ST	5×10^{-5}	0	0	0	0	1×10^{-4}	2.5×10^{-5}
CP	5×10^{-5}	2.5×10^{-4}	0	5×10^{-3}	5×10^{-4}	1×10^{-3}	2.5×10^{-5}
VC	5×10^{-7}	2.5×10^{-4}	5×10^{-5}	5×10^{-5}	5×10^{-5}	1×10^{-4}	2.5×10^{-4}

nd = not determined

Approximate K_i's were determined according to the table given in Appendix 16.

N9 and N2 are influenza sialidases, ST is the *S. typhimurium* sialidase, CP is the *C. perfringens* sialidase and VC is the *V. cholerae* sialidase

All reactions consisted of enzyme, 10 µl of 1 mM MUN and buffer to 100 µl. They were incubated for 15 min at 37 °C and stopped with 2.4 ml of 0.25 M glycine, pH 10. Fluorescence emission was read at 400 nm after excitation at 365 nm in a Hitachi F-1200 fluorescence spectrophotometer.

The buffer used for N9 and ST was 50 mM Sodium phosphate buffer with 100 mM NaCl pH 6.8. For CP the buffer used was 50 mM Sodium acetate pH 6.1 and for N2 and VC the buffer, was 50 mM MES containing 6 mM $CaCl_2$, pH 6.5

Appendix 16

K_i estimates for bacterial and viral sialidases

Conditions of the assay

[MUN] = 100 μM

[I] = 0.1, 1.0 or 10 mM

$K_m = 4 \times 10^{-5}$ M

Assumed competitive linear inhibition

$$v = \frac{V_{max}}{1 + K_m / [MUN] (1 + [I] / K_i)}$$

	% inhibition	
K_i (M)	**0.1 mM**	**1.0 mM**
1×10^{-7}	100	100
5×10^{-7}	98	100
1×10^{-6}	97	100
5×10^{-6}	85	98
1×10^{-5}	74	97
5×10^{-5}	36	85
1×10^{-4}	22	74
5×10^{-4}	5	36
1×10^{-3}	3	22
5×10^{-3}	0.6	5
1×10^{-2}	0.3	3

This data was kindly provided by Dr. Andrew Chong (Victorian College of Pharmacy, Monash University).

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