Determinants of Atmospheric Trace Gas Oxidation in Bacteria

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This thesis includes **three** original papers published in peer reviewed journals. The core theme of the thesis is discovering **determinants of atmospheric trace gas oxidation in bacteria.** The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the **School of Biological Sciences, Monash University** under the supervision of **A/Prof Chris Greening**.
The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of **Chapters 2, 3, and 4,** my contribution to the work involved the following:

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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

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ABSTRACT

Recent research has shown that two atmospheric trace gases, molecular hydrogen (H_2) and carbon monoxide (CO), are key energy sources for aerobic bacteria. Several actinobacterial species, including *Mycobacterium smegmatis*, have been shown to use these gases as alternative energy sources for persistence when they are starved of organic carbon sources required for growth. This process is hypothesised to support the productivity and resilience of oligotrophic ecosystems. Moreover, microbial consumption of trace gases controls the chemical composition of the atmosphere and in turn regulates greenhouse gas levels. Despite this recent progress, there are many gaps in our understanding of the microbial and enzymatic determinants of bacterial trace gas oxidation.

In this work, we expand on our understanding of the diversity of trace gas scavenging in bacteria by addressing four main questions: (i) 'how widespread across bacterial phyla is the process of atmospheric gas oxidation?', (ii) 'is the energy derived used primarily for persistence or growth?', (iii) 'what are the molecular determinants of atmospheric gas oxidation?', and (iv) 'does atmospheric CO oxidation also enhance bacterial survival?'. We address these knowledge gaps using a multidisciplinary approach, combining bacterial physiology, genetic dissection, biochemical assays, and phylogenetic analyses.

Most environmental bacteria exist in a dormant state, though questions still remain as to how they are able to persist when deprived of their preferred energy substrates. We investigated the energy sources that supported persistence in the widespread yet understudied bacterial phylum Chloroflexota. To do this, we combined cutting-edge transcriptomics analysis with complementary biochemical and phylogenetic assays, using the distantly related thermophilic strains, *Thermomicrobium roseum* (class Chloroflexia) and *Thermogemmatispora* sp. T81 (class Ktedonobacteria). We infer that these bacteria shift from growing on organic compounds to persisting on inorganic compounds, including atmospheric H_2 and CO. In turn, we confirmed that both strains oxidise atmospheric H_2 and CO during persistence, and that this process supports aerobic respiration. Phylogenetic analysis suggests that the genetic determinants of trace gas oxidation, the group 1h [NiFe]-hydrogenases and carbon monoxide dehydrogenases, were independently acquired by these bacteria through separate horizontal acquisition events. In turn, this study extends the phenomenon of atmospheric H_2 oxidation to a third phylum and suggests atmospheric CO is also a critical energy source for persistence.

We subsequently wanted to determine whether an additional hydrogenase family, the group 2a [NiFe]-hydrogenases, also mediated atmospheric H_2 oxidation. To do so, we investigated the expression, activity, and role of this hydrogenase in three environmentally and phylogenetically distinct species, *Gemmatimonas aurantiaca* (phylum Gemmatimonadota), *Acidithiobacillus ferrooxidans* (phylum Proteobacteria) and *Chloroflexus aggregans* (phylum Chloroflexota). We show that all strains are able oxidise atmospheric H_2, thus revealing new phyla and enzymes that contribute to the biological sink of the global H_2 cycle. Moreover, in contrast to the currently held paradigm that atmospheric trace gases are primarily used for persistence, we show that expression and activity of these strains is upregulated during mixotrophic growth. Phylogenetic analysis suggests the group 2a [NiFe]-hydrogenase is encoded by 13 bacterial phyla, suggesting atmospheric H_2 is a major energy source supporting microbial growth.

Finally, we aimed to elucidate the molecular mechanisms that underpin atmospheric H_2 scavenging within bacteria. We show that most bacteria encoding group 1h and 2a [NiFe]-hydrogenases harbour putative iron-sulfur cluster proteins, herein named HhyE and HucE. We show through a combination of genetic manipulation and biochemical assays using *M. smegmatis* that these proteins are essential for H_2 oxidation; H_2 consumption was completely eliminated in a double deletion mutant, and single mutants phenocopied their respective hydrogenase catalytic subunit mutants. Loss of the genes encoding these proteins also compromised mycobacterial growth and survival. We propose that these putative iron-sulfur cluster proteins serve as electron conduits between the catalytic subunits of their respective hydrogenases and the menaquinone pool of the respiratory chain.

This work provides a broad overview of the determinants of atmospheric gas oxidation in bacteria, spanning from whole organism level down to molecular modulators. We provide axenic culture-based evidence for atmospheric H_2 oxidation in three additional bacterial phyla, Chloroflexota, Gemmatimonadota, and Proteobacteria, as well as the first demonstrations that atmospheric CO oxidation fuels persistence. Concomitantly, these findings validate genomic surveys of potential trace gas oxidisers, provide pure culture evidence to complement ecosystem-based analyses, and highlight that trace gases not only support persistence but also mixotrophic growth. On this basis, we propose that trace gas oxidation is an extremely widespread strategy that supports productivity and resilience of aerobic bacteria in diverse ecosystems.

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ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CFU	Colony forming unit
CH_4	Methane
CN^-	Cyanide ion
CO	Carbon monoxide
CODH	Carbon monoxide dehydrogenase
C_T	Threshold cycle
E_o'	Electric potential
EPR	Electron paramagnetic resonance
ETF	Electron transfer flavoprotein complex
FAD	Flavin adenine dinucleotide
FeS	Iron-sulfur cluster
g_{dw}	Grams of dry weight
H_2	Hydrogen
HdB	Hartmans de Bont
Hhy	Group 1h [NiFe]-hydrogenase
HMP	Hydrogenase maturation protease
HQNO	2-Heptyl-4-hydroxyquinoline n-oxide
Huc	Group 2a [NiFe]-hydrogenase
HydDB	Hydrogenase database
kDa	(kilo) Daltons
LB(T)	Lysogeny broth (with Tween80)
mA	Milliamps
MAG	Metagenome assembled genome
mL^{-1}; L^{-1}	Per millilitre; per litre
mM; µM; nM	Millimolar; micromolar; nanomolar
mV; V	Millivolts; volts
N_2	Nitrogen
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium chloride
NCBI	National Center for Biotechnology Information
NHL	ncl-1, HT2A and lin-41
NMHC	Non-methane hydrocarbon
$OD_{(max)}$	Optical density (maximum)
OH^-	Hydroxyl radical
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonyl fluoride
ppbv	Parts per billion by volume
ppmv	Parts per million by volume
qRT-PCR	(Quantitative reverse transcription) polymerase chain reaction
rpm	Revolutions per minute
Tg yr^{-1}	Teragrams per year
v/v; w/v	Volume per volume; weight per volume
WT	Wild-type

1 INTRODUCTION

1.1 Atmospheric trace gas oxidation

1.1.1 Atmospheric trace gases

Bacteria possess hidden metabolic flexibility. They often use multiple energy sources to sustain themselves in ecosystems with low or variable nutrient levels. For example, some bacteria simultaneously use multiple energy sources to grow, whereas others switch from growing on preferred energy sources to persisting on alternate sources (Carbonero et al., 2014, Freilich et al., 2009). In this regard, recent studies have shown that atmospheric trace gases, such as hydrogen (H_2) and carbon monoxide (CO), support microbial communities in ecosystems with low resource availability or stability. While these two gases are found at relatively low atmospheric mixing ratios of 0.53 and 0.1 parts per million (ppmv) respectively (Conrad, 1996), they are nevertheless ubiquitously available. They are also ideal energy sources given they readily diffuse through bacterial cell membranes (Greening et al., 2016), have low activation energies, and yield much free energy when aerobically respired (Cammack et al., 2001, Hanson and Hanson, 1996, Meyer and Schlegel, 1983, Greening et al., 2016). The ability of bacteria to oxidise these gases, either alternatively or simultaneously with other substrates (e.g. organic carbon), confers a selectively advantage in resource-poor or resource-variable ecosystems. The capacity of soils to consume atmospheric H_2 has been recognised for decades (Conrad, 1996), yet only recently has it been confirmed that bacteria mediate this process, and the specific phyla and enzymes responsible for this uptake have been identified. Likewise, there remains many gaps in the understanding of the microbial mediators and physiological role of atmospheric CO oxidation.

1.1.1.1 Hydrogen

Hydrogen is an important trace gas in the atmosphere that is produced by a combination of anthropogenic, geochemical and biological processes **(Figure 1-1)** (Conrad, 1996, Constant et al., 2009, Ehhalt and Rohrer, 2009, Schwartz et al., 2013). This gas is maintained at a stable concentration of 0.53 ppmv as a result of several H_2-producing (sources) and H_2-oxidising (sinks) processes (Novelli et al., 1999). The primary sources of H_2 in the atmosphere are due to photochemical reactions of methane and other hydrocarbons (~54%; 41 Tg yr^{-1}), biomass burning (~20%; 15 Tg yr^{-1}), fossil fuels and industrial usage (~14%; 11 Tg yr^{-1}), and nitrogen fixation on land and in the oceans (~12%; 9 Tg yr^{-1}) (Ehhalt and Rohrer, 2009, Greening et al., 2015b). Contrastingly, there are only two main sinks of atmospheric H_2, via hydroxyl radical oxidation in the troposphere (~24%; 19 Tg yr^{-1}) and biological soil uptake.

Soil microorganisms constitute the largest sink of hydrogen, accounting for approximately 70 million tonnes of hydrogen uptake from the atmosphere per year (Ehhalt and Rohrer, 2009), which is the equivalent of 75-80% of the net H_2 lost from the atmosphere (Constant et al., 2009, Greening et al., 2015b, Schwartz et al., 2013). Despite hydrogen being rapidly turned over, increasing fossil fuel usage as well as higher rates of methane oxidation have the potential to increase the amount of hydrogen in the troposphere (Constant et al., 2009, Greening et al., 2015b). A potential increase in tropospheric H_2 levels may be in turn counteracted by an increase in microbial uptake, offsetting any excess H_2 in the atmosphere to maintain a H_2 concentration equilibrium. Further characterisation of bacteria able to oxidise atmospheric H_2 (H_2 scavengers) is required to gain a better understanding of the nature and responsiveness of this sink.

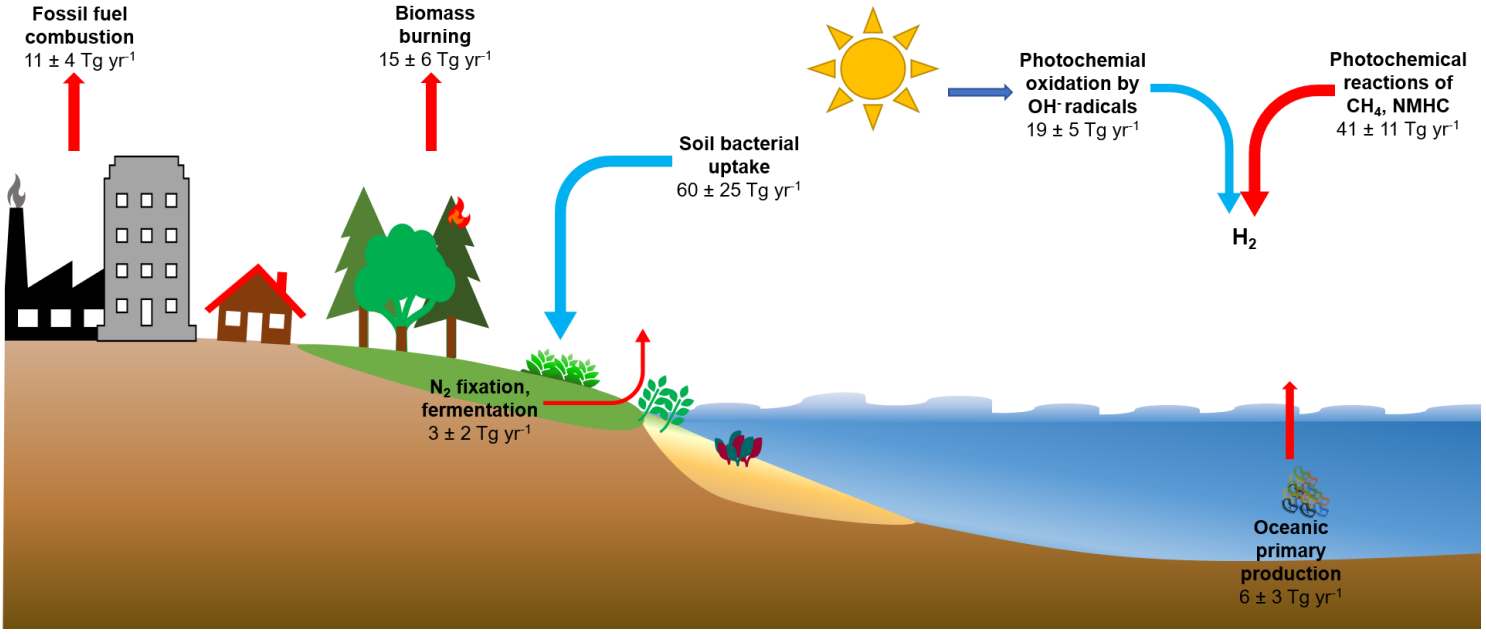

Figure 1-1: **The biogeochemical H_2 cycle**. Hydrogen is maintained in the atmosphere at a stable concentration of 0.53 ppmv, with a number of sources (red arrows) and sinks (blue arrows). NMHC = non-methane hydrocarbon. Figure adapted from (Constant et al., 2009).

Within the environment, there are numerous ways in which microorganisms can utilise hydrogen as an energy source. The major processes resulting in the evolution of hydrogen in microbial metabolism are fermentation, hydrogenogenic respiration processes, N_2 fixation and phosphite oxidation (Schwartz et al., 2013, Constant et al., 2009, Richardson, 2000). Conversely, there are a number of metabolic processes which use hydrogen as an energy source, by coupling it to the reduction of other metabolites, including in aerobic respiration, nitrate respiration, sulfate respiration, methanogenesis, acetogenesis, dehalorespiration and fumarate respiration (Schwartz et al., 2013, Constant et al., 2009). Hydrogenotrophic bacteria can be found in essentially all ecosystems including in soils (Conrad, 1999a), oceans (Adam and Perner, 2018), geothermal hot springs (Pumphrey et al., 2011), wetlands (Simankova et al., 2000, Hanson and Hanson, 1996, Kotsyurbenko et al., 2001), and as part of the human microbiome (Vianna et al., 2008, Gaci et al., 2014, Benoit et al., 2020). Within anoxic environments, a substantial amount of H_2 is produced, largely due to fermentation. However, as there is a high level of inter- and intra-species H_2 recycling, there is little to no net evolution of H_2 in these ecosystems (Conrad, 1996, Constant et al., 2009). Conversely, in primarily oxic systems, atmospheric sources of H_2, rather than biotic, are thought to be the primary energy source for H_2-oxidising microorganisms (Greening et al., 2016).

The primary enzymatic mediator of hydrogen uptake from the atmosphere are hydrogenases. Hydrogenases are biological metalloenzymes that catalyse the reversible heterolytic cleavage of elemental hydrogen (H_2) into two protons and electrons (Equation 1) (Lubitz et al., 2014).

$$H_2 \leftrightharpoons [H^+ + H^-]^+_+ \leftrightharpoons 2H^+ + 2e^-$$

Equation 1: **Heterolytic cleavage of molecular hydrogen by hydrogenases**

The oxidation of hydrogen by hydrogenases is a highly thermodynamically favourable reaction in the presence of respiratory electron acceptors. The electrons generated can be shuttled to electron acceptors to support aerobic or anaerobic respiration, or can be used as a reductant for chemosynthetic carbon fixation (Schwartz et al., 2013). There are three major types of hydrogenase that differ in metal centre composition, [NiFe], [FeFe] and [Fe], which are each ligated by inorganic CO and CN^- ligands (Lubitz et al., 2014, Vignais et al., 2001). Hydrogenases are further classified into different subgroups and subtypes based on their amino acid sequence phylogeny; their phylogenetic grouping also correlates with differences in physiological role, biochemical characteristics, metal-binding motifs, and predicted genetic organisation (Greening et al., 2016). To date, genomic and metagenomic studies have determined that at least 51 bacterial and archaeal phyla possess hydrogenases that are capable of using hydrogen, indicating that utilisation of this gas is more important than previously thought (Carere et al., 2017, Greening et al., 2016, Ji et al., 2017, Piché-Choquette et al., 2017).While most characterised hydrogenases are irreversibly inhibited when O_2 binds at their H_2-activating active sites (Stripp et al., 2009, Vincent et al., 2005), there are a number of subgroups that have been determined to be oxygen-tolerant, including the group 1d, 1f, 1l, 1h, and 2a [NiFe]-hydrogenases (Greening et al., 2016, Pandelia et al., 2012, Søndergaard et al., 2016, Ortiz et al., 2020). These subgroups of hydrogenases are often present in aerobic bacteria, with group 1h hydrogenases thought to be the primary enzymatic mediator of the biogeochemical hydrogen cycle. Despite numerous metagenomic and genomic surveys highlighting the prevalence of oxygen-tolerant hydrogenase genes in a range of ecosystems (Constant et al., 2011b, Greening et al., 2016, Ji et al., 2017, Kessler et al., 2019, Leung et al., 2020), culture-based assays have only proven the aerobic hydrogen scavenging capacity of three bacterial phyla, the Acidobacteriota, the Actinobacteriota and the Verrucomicrobiota (Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Greening et al., 2015a,

Meredith et al., 2014, Myers and King, 2016, Schmitz et al., 2020). The amount of H_2 uptake mediated by microorganisms suggests that more organisms than currently characterised scavenge at an atmospheric level. Thus, understanding both the bacterial players and the molecular mechanisms that enable this rapid and constant uptake will provide a clearer indication of how microorganisms can control trace gas cycling.

1.1.1.2 Carbon monoxide

Carbon monoxide (CO) is another important trace gas within the atmosphere. It is maintained globally at an average mixing ratio of ~0.1 ppmv, though there are daily, seasonal and spatial variations in CO levels (Khalil and Rasmussen, 1990, Novelli et al., 1998). Moreover, there are a number of areas that act as CO hotspots, for example volcanic zones, where geothermal activity can raise local CO concentrations up to ~6000 ppmv (Shock et al., 2010). Natural sources of atmospheric CO, in addition to geothermal activity, include oceanic production through photochemical and thermal processes, production by phytoplankton and photoproduction by leaves of plants, together accounting for ~40% of CO emissions (Conte et al., 2019, Seiler and Giehl, 1977, Tarr et al., 1995). However, most emissions of CO into the atmosphere are due to anthropogenic activities such as the combustion of fossil fuels and oxidation of hydrocarbons (~60%) (Khalil and Rasmussen, 1990). Analogous to the hydrogen cycle, the major mechanisms of CO removal from the atmosphere are through oxidation by free hydroxyl radicals (~85%) and uptake by soil microorganisms (~15%) (Bartholomew and Alexander, 1981, Khalil and Rasmussen, 1990). While these processes are currently maintained at a relatively stable equilibrium, it is hypothesised that increasing anthropogenic CO emissions as a result of greater fossil fuel usage and land clearing practices may shift this balance (Khalil and Rasmussen, 1990, Dey and Dhal, 2019, Novelli et al., 1998). The interaction of hydroxyl radicals with increasing CO concentrations in the atmosphere has climatological effects, as it lowers the amount of hydroxyl radicals freely available to react with the highly climate active

gas methane (Conrad, 2009, Novelli et al., 1998). As such, it is becoming increasingly important to understand the key processes controlling the biogeochemical CO cycle, including the contribution of the only biological CO sink: soil microorganisms.

CO oxidation by soil microorganisms accounts for the net removal of ~250 Tg yr^{-1} of CO from the troposphere per year (Bartholomew and Alexander, 1981, Inman et al., 1971). Broadly, these CO-oxidising soil microorganisms fall into two classes: carboxydotrophs, which are able to grow chemolithoautotrophically on CO at elevated levels (King and Weber, 2007, Conrad, 1996), and carboxydovores, which are adapted to oxidise CO at lower concentrations (King, 2003b, King and Weber, 2007). Both aerobic carboxydovory and carboxydotrophy are mediated by a form I carbon monoxide dehydrogenase (CODH); this enzyme shuttles electrons yielded from CO to the respiratory chain to support ATP generation or to the Calvin-Benson cycle to facilitate CO_2 fixation. The CO utilised by carboxydovores have been hypothesised to support aerobic respiration but not carbon fixation. To date, the activity of these enzymes has been experimentally confirmed in 11 bacterial and seven archaeal genera to date, though the enzyme has been identified in a much larger number of genera via genomic surveys (King and Weber, 2007, Cordero et al., 2019a, Meyer and Schlegel, 1983). Additionally, there are a number of anaerobic carboxydotrophic bacteria and archaea that grow on CO using a distinct nickel-containing CODH (Dobbek et al., 2001, Robb and Techtmann, 2018). As this thesis focuses primarily on ambient gases, only aerobic carboxydovores will be discussed in detail.

While the purpose of CO oxidation in carboxytrophs is well established, the physiological role of CO oxidation in carboxydovores was not resolved until recently. The form I CODH of these bacteria are typically thought to have higher affinity but lower activity than to those of carboxydotrophs (Cordero et al., 2019a, King, 2003b). A study by Cunliffe (2013) determined that the marine Alphaproteobacteria *Ruegeria pomeroyi* showed no growth enhancement in the presence of excess CO, nor was the profile of cellular metabolites

influenced (Cunliffe, 2013). A persistence-centric model of the physiological role of CO oxidation was more recently established based on a study of *Mycobacterium smegmatis* CODH; through a combination of proteomic, genetic and biochemical analysis, it was confirmed that aerobic oxidation of atmospheric CO enhances microbial survival in nutrient-limited cells (Cordero et al., 2019a). This finding is supported by additional studies showing CODH is upregulated in oxic nutrient-limited cultures of *Phaeobacter sp.* strain MED193 (Muthusamy et al., 2017), *Rhodococcus jostii* RHA1 (Patrauchan et al., 2012) and *M. smegmatis* (Berney and Cook, 2010). Moreover, an in-depth survey of genomic, metagenomic, and metatranscriptomic datasets highlighted the presence of a form I CODH in 196 bacterial and archaeal genera (Cordero et al., 2019a), including within seven of the nine most dominant soil phyla, the Proteobacteria, Actinobacteriota, Acidobacteriota, Chloroflexota, Firmicutes, Gemmatimonadota and Bacteroidota (Janssen, 2006). The presence of a CODH in such a large subset of aerobic bacteria suggests that the microbial mediators of the CO cycle are much more diverse than previously thought. However, axenic culture studies would be necessary to resolve the specific capacity of additional bacterial and archaeal phyla to oxidise atmospheric CO. Furthermore, this also supports the notion that CO may serve as a dependable source of energy for bacteria in nutrient limited environments. An additional role for atmospheric CO oxidation may be to aid in mixotrophic growth of some strains, though this requires further investigation.

1.1.1.3 Methane

Methane (CH_4) is one of the most climatologically important gases within the atmosphere, accounting for approximately 20% of greenhouse-gas induced global warming over the past ~150 years (Kirschke et al., 2013). Methane emissions worldwide have been increasing, largely due to anthropogenic activities increasing biotic and abiotic production, such as ruminant and rice agriculture, waste treatment and fossil fuel usage. As a result of these activities, global mixing ratios of the gas have risen to 1.77 ppmv (Kirschke et al., 2013).

Similar to the hydrogen and carbon monoxide cycles, the concentration of CH_4 in the atmosphere is controlled by a number of sources (e.g. methanogenic archaea, fossil fuels) and sinks (e.g. photochemical oxidation by OH^- radicals, microbial oxidation in soil) (Conrad, 2009).

Broadly, aerobic methane-oxidising bacteria can be classified into four main groups, Alphaproteobacteria, Gammaproteobacteria, Verrucomicrobiota and Methylomirabiolta methanotrophs (Carere et al., 2017, Dumont and Murrell, 2005, Dunfield et al., 2007, Ettwig et al., 2010, Hanson and Hanson, 1996, Mohammadi et al., 2017, Mohammadi et al., 2019, Murrell and Jetten, 2009, Tveit et al., 2019). All four groups have been shown to aerobically oxidise methane primarily using the particulate membrane-bound methane monooxygenase complex (pMMO) (Bender and Conrad, 1992, McDonald et al., 2008). While the predominant theory is that most methanotrophs are obligate methane oxidisers, growing evidence suggests that a number of aerobic methanotrophs are facultative, and can mixotrophically oxidise methane in conjunction and alternatively with other electron sources such as short chain organic compounds (e.g. acetate, propane, ethanol) and other atmospheric trace gases (e.g. H_2) (Carere et al., 2017, Crombie and Murrell, 2014, Im et al., 2011). For example, verrucomicrobial methanotrophs within the genus *Methylacidiphilum* are able to grow autotrophically on H_2 and some can even oxidise this gas at atmospheric concentrations (Carere et al., 2017, Carere et al., 2019, Mohammadi et al., 2017, Mohammadi et al., 2019, Schmitz et al., 2020).

1.1.2 Biogeochemical implications

The role of microorganisms in controlling biogeochemical cycling of atmospheric gases has been well defined over the course of the last 40 years, with strong evidence of microbial influences on atmospheric composition extending as far back as the Early Precambrian era (Conrad, 1996, Holland et al., 1986, Schidlowski, 1983). Despite this, the majority of the

microbial players for the H_2 and CO cycles remain elusive. Notably, the predominant theory of how H_2 was removed from the atmosphere in the past was that cell-free soil hydrogenases mediated the process (Conrad, 1996). This paradigm was only recently overturned with the discovery of the high-affinity H_2 oxidising strain, *Streptomyces* sp. PCB7, by Constant et al. (2008), and was furthered by the genetic confirmation of atmospheric scavenging hydrogenases in *M. smegmatis* by Greening et al. (2014) (Greening et al., 2014a, Constant et al., 2008). Similarly, while bacterial-mediated uptake of atmospheric CO has been known for some time, with an initial demonstration of atmospheric CO oxidation by *Oligotropha carboxidovorans* by Conrad and Seiler (1980) (Conrad and Seiler, 1980), the genetic basis for aerobic CO scavenging was only unambiguously confirmed by Cordero et al. (2019) in *M. smegmatis* (Cordero et al., 2019a). With only a limited number of cultivated aerobic high-affinity H_2- and CO-oxidisers, questions still remain as to whether the currently characterised physiological roles for each trace gas hold true for the majority of microbial phyla determined to encode these high-affinity enzymes.

It is well known that microorganisms have diverse effects on biogeochemical cycles, with methanogenic archaea and denitrifying bacteria in agricultural settings, for instance, greatly increasing greenhouse gas emissions (Conrad, 2009, Hu et al., 2015). Conversely, atmospheric H_2, CO and CH_4 scavengers play a key role in the mitigation of anthropogenic emissions and thus control atmospheric composition. As such, understanding the precise microbial influencers on the biogeochemical cycles of atmospheric, particularly climate-active, gases is imperative for any future strategic mitigation of anthropogenic climate change (Singh et al., 2010). While H_2 and CO are not traditionally classified as a climate-active gases, their non-biological sinks are reliant on photochemical oxidation by hydroxyl radicals (Constant et al., 2009, Ehhalt et al., 1990, Ehhalt and Rohrer, 2009, Khalil and Rasmussen, 1990, Monson and Holland, 2001). As a result of hydroxyl radical chemistry, increasing H_2 and CO emissions

in turn increase CH_4 concentrations in the atmosphere (Kirschke et al., 2013, Monson and Holland, 2001, Greening et al., 2015b, Constant et al., 2009, Novelli et al., 1998). As such, having a greater understanding of the microbial sinks of H_2, CO and even CH_4 will potentially assist with the development of land management strategies to help preserve these natural sinks.

While the predominant biological sinks of atmospheric H_2 and CO are due to microbial activity primarily in soils, the microbial sink CH_4 appears to be less active, for currently unknown reasons. The comparatively smaller number of phyla encoding the genetic determinants for aerobic CH_4 oxidation (three phyla) than for CO (seven phyla) or H_2 oxidation (~13 phyla) may indicate lower capacity for atmospheric uptake (Kirschke et al., 2013). Interestingly, despite any potential increases in anthropogenic H_2 emissions over the past 40 years, the bacterial sink of H_2 appears to adapt efficiently, maintaining a relatively stable global H_2 equilibrium (Greening et al., 2015b, Schultz et al., 2003). This suggests that the bacterial sink of H_2 is well poised to mitigate excessive H_2 in the atmosphere, potentially liberating hydroxyl radicals to interact with other climate active gases. As the ability to oxidise hydrogen at atmospheric concentrations is encoded by at least 13 bacterial phyla (Greening et al., 2016, Ji et al., 2017, Piché-Choquette et al., 2017, Barz et al., 2010, Tamagnini et al., 2002), there is likely to be a sufficiently large sink for microbes to be able to efficiently consume anthropogenic hydrogen emissions and thus maintain atmospheric H_2 at a stable equilibrium. Similarly, it is believed that the levels of CO in the atmosphere are stably maintained globally, in part due to the strength of the bacterial CO sink (King and Weber, 2007). Additionally, a small subset of bacterial phyla encode genes for both aerobic H_2 and CO oxidation, suggesting that some microorganisms have an effect on both gas cycles. Understanding the key players in all three trace gas cycles through axenic culture characterisation studies will allow for a greater understanding of how resilient the microbial sinks are to increasing anthropogenic emissions.

1.1.3 Ecological implications

As previously mentioned, atmospheric trace gases are dependable alternate energy sources in dynamic ecosystems due to their diffusibility, ubiquity, low activation energy and relatively high energy yield (Conrad, 1996, Greening et al., 2016, Thauer, 2011). Utilisation of these trace gases as part of a broader flexible metabolic strategy may influence the spatial distribution of microorganisms as well as community structure (Carbonero et al., 2014, Greening et al., 2019). Indeed, whereas using atmospheric trace gases is a highly dependable and minimalistic strategy for bacterial maintenance in environments with low bioavailable organic carbon, alternative strategies such as mobilising recalcitrant organic polymers would likely confer major energetic costs, given the genes, enzymes, and ATP required to activate them (Martínez-Cano et al., 2015).

Bacteria often inhabit ecosystems where the availability of their preferred energy sources are low or variable. They depend on metabolic flexibility to adapt to such low resource availability or stability. As a result, most bacteria in soil ecosystems and other natural environments exist in a dormant or persisting state, associated with reduced metabolic expenditure. Among the common phenotypic forms of bacterial dormancy include: differentiation into 'resting' structures (e.g. spores), reduction in cell size (miniaturisation), thickening of cell walls and the production of extracellular polymeric substances (Kjelleberg et al., 1987, Lennon and Jones, 2011, Novitsky and Morita, 1976, Madigan and Martinko, 1997). The predominant dormancy strategies can greatly vary between environments, with marine bacteria for instance more likely to adopt a miniaturised form (Kjelleberg et al., 1987). While the energy requirements for dormant cells are typically 1000 times lower than that of vegetative cells, dormant bacteria still require minimal energy inputs for maintenance, including but not limited to macromolecular repair, cell wall integrity, membrane potential maintenance and sensing functions (Price and Sowers, 2004, Morita, 1982). There are a number

of ways in which bacteria can potentially obtain this energy, including through necromass consumption, reliance on endogenous storage compounds (e.g. glycogen, polyhydroxyalkanoates) (González-Pastor et al., 2003, Kadouri et al., 2005, Lennon and Jones, 2011, Wiebe and Bancroft, 1975, Carere et al., 2019, Strong et al., 2016) or through the utilisation of atmospheric trace gases (discussed in Section 1.2.2). Thus, bacteria within ecosystems with high degrees of organic carbon variability may utilise one or more of the aforementioned strategies to generate maintenance energy.

There are a wide range of ecosystems where atmospheric trace gases may support bacterial growth or persistence. Low levels of organic carbon and other nutrient levels appear to be a selective pressure favouring trace gas oxidisers in different environments (Ji et al., 2017, Leung et al., 2020, Lynch et al., 2014, Constant et al., 2009, Ehhalt and Rohrer, 2009, Greening et al., 2015b, Greening and Cook, 2014, King, 2003a, Moran et al., 2004). A well-studied example of natural ecosystems with extreme physicochemical conditions are geothermal springs; these ecosystems are traditionally limited by organic carbon, experience variability in chemical properties, and can experience extreme temperatures (> 50°C) and pH (Colman et al., 2016, Loiacono et al., 2012, Power et al., 2018, Spear et al., 2005). Geothermal springs are also known to harbour higher concentrations of H_2 and CO which have been shown to be actively consumed by microorganisms (King et al., 2008, Lindsay et al., 2019, Otaki et al., 2012, Brady et al., 2015), suggesting these gases serve as dependable energy sources for both growth and persistence in these ecosystems (Shock et al., 2010, Spear et al., 2005). Conversely, within oligotrophic ecosystems, the ability to utilise atmospheric trace gases may confer a selective advantage for those bacteria as well as potentially shape the community structure in these ecosystems. Additionally, human-engineered environments such as acidic mine drainage and wastewater treatment plants also experience high degrees of variation in organic carbon content and can undergo oxic-anoxic cycles, potentially driving community composition to select for

bacteria that can use atmospheric trace gases either aerobically or anaerobically (Ferrera and Sánchez, 2016, Mielke et al., 2003).

Significantly, microbial oxidation of H_2 and CO in conjunction with CO_2 fixation appears to be key drivers of ecosystem resilience in both hot and cold deserts, particularly in the absence of typical phototrophs (Leung et al., 2020, Lynch et al., 2014). Specifically, a study by Ji & Greening et al. (2017) analysed the metabolic potential of organisms found in the surface soils of the Robinson Ridge, Adams Flat and the McMurdo Dry Valley regions of Antarctica and found that the genetic determinants for H_2 and CO oxidation were encoded by multiple lineages, including the candidate phyla Dormibacterota (formerly AD3) and Eremiobacterota (formerly WPS-2). Moreover, gas chromatograph analysis of sampled soils from Robinson Ridge and Adams Flat were shown to aerobically oxidise both H_2 and CO at rates modelled to be sufficient to sustain the energy needs of ~5.5 to 8.0 x 10^7 bacteria per gram of soil (Ji et al., 2017). However, determining whether the energy derived from trace gas oxidation in these ecosystems is sufficient to promote bacterial growth, rather than just mediating bacterial persistence, still requires more research. In particular, while this study provides both metagenomic and microcosm-based evidence of trace gas oxidation, pure culture studies would be required to resolve the precise microbial mediators of trace gas oxidation.

1.2 The physiology of trace gas oxidation

1.2.1 Integration in respiratory chain

The main role of atmospheric trace gas oxidation is to provide electrons to the aerobic respiratory chain, either during bacterial persistence (see Section 1.2.2) or as an additional substrate during mixotrophic growth (see Section 1.2.3). As such, the enzymes involved are likely to be intimately associated with the respiratory chain, and as a result, are typically membrane-bound or membrane-associated (Melo and Teixeira, 2016, Poole and Cook, 2000, Richardson, 2000, Spero et al., 2016). The aerobic respiratory chain contains a number of

conserved elements, though the specific subtypes of each enzyme can differ greatly between bacteria (Poole and Cook, 2000, Berney and Cook, 2010). Typical components of the aerobic respiratory chain of bacteria are: primary dehydrogenases (e.g. NADH dehydrogenases, succinate dehydrogenases), quinone electron carriers (e.g. menaquinone), terminal oxidases (e.g. cytochrome *bd* oxidase, haem-copper oxidases) and an ATP synthase (Poole and Cook, 2000, Greening et al., 2015b, Cordero et al., 2019b), with the presence of multiple types of each component common (Poole and Cook, 2000). The first three components are used to sequentially transfer electrons derived from the oxidation of substrates through the respiratory chain to reduce oxygen, yielding water; this energises the translocation of protons (through vectorial and scalar mechanisms) across the cell membrane, generating a proton-motive force that drives ATP synthesis (Poole and Cook, 2000). In periods of nutrient limitation, metabolically flexible bacteria have been shown to downregulate primary dehydrogenases associated with organic carbon oxidation in favour of enzymes involved in the metabolism of alternate energy sources to fuel the aerobic respiratory chain (Berney et al., 2014b, Constant et al., 2011b, Cordero et al., 2019a, Cordero et al., 2019b, Greening et al., 2014a, Liot and Constant, 2016, Poole and Cook, 2000, Carbonero et al., 2014, Freilich et al., 2009). Some bacteria also use alternate electron acceptors during periods of prolonged hypoxia or anoxia (Berney and Cook, 2010, Poole and Cook, 2000, Berney et al., 2014a).

As previously stated, the ubiquity and high-energy yield of H_2 and CO make them dependable alternate energy sources. Additionally, H_2 and CO oxidation yields high-potential electrons that can be relayed into the menaquinone pool and to terminal oxidases (Berney and Cook, 2010, Berney et al., 2014b, Greening et al., 2014a, Cordero et al., 2019a, Conrad, 1996). Case studies using the metabolically flexible soil bacterium *M. smegmatis* have determined that hydrogen oxidation is tightly coupled to the aerobic respiratory chain (Berney and Cook, 2010, Cordero et al., 2019b, Greening et al., 2014a). Cordero et al. (2019b) determined that the

two uptake hydrogenases of *M. smegmatis* differentially interact with the respiratory chain, with the persistence-linked group 1h hydrogenase (Hhy) shuttling electrons to the cytochrome *bd* oxidase complex, whereas the growth-associated group 2a hydrogenase (Huc) was shown to donate electrons to cytochrome *bcc-aa3* oxidase supercomplex (Cordero et al., 2019b). Moreover, addition of the respiratory uncoupler nigericin (Nicholls and Ferguson, 2013) and the menaquinone analogue HQNO resulted in reduction of H_2 oxidation activity for both hydrogenases to different levels; this suggests they both obligately associate with the aerobic respiratory chain, and input electrons that are transferred to the quinone pool and terminal oxidases (Cordero et al., 2019b). However, whether this intimate association with the aerobic respiratory chain is specific to actinomycetes or is a more general trend across aerobic trace gas scavenging bacteria requires more experimental evidence. A schematic of aerobic respiration during nutrient replete (**Figure 1-2a**) and nutrient limited conditions (**Figure 1-2b**), including trace gas oxidation, in an organotrophic microorganism is depicted in **Figure 1-2**.

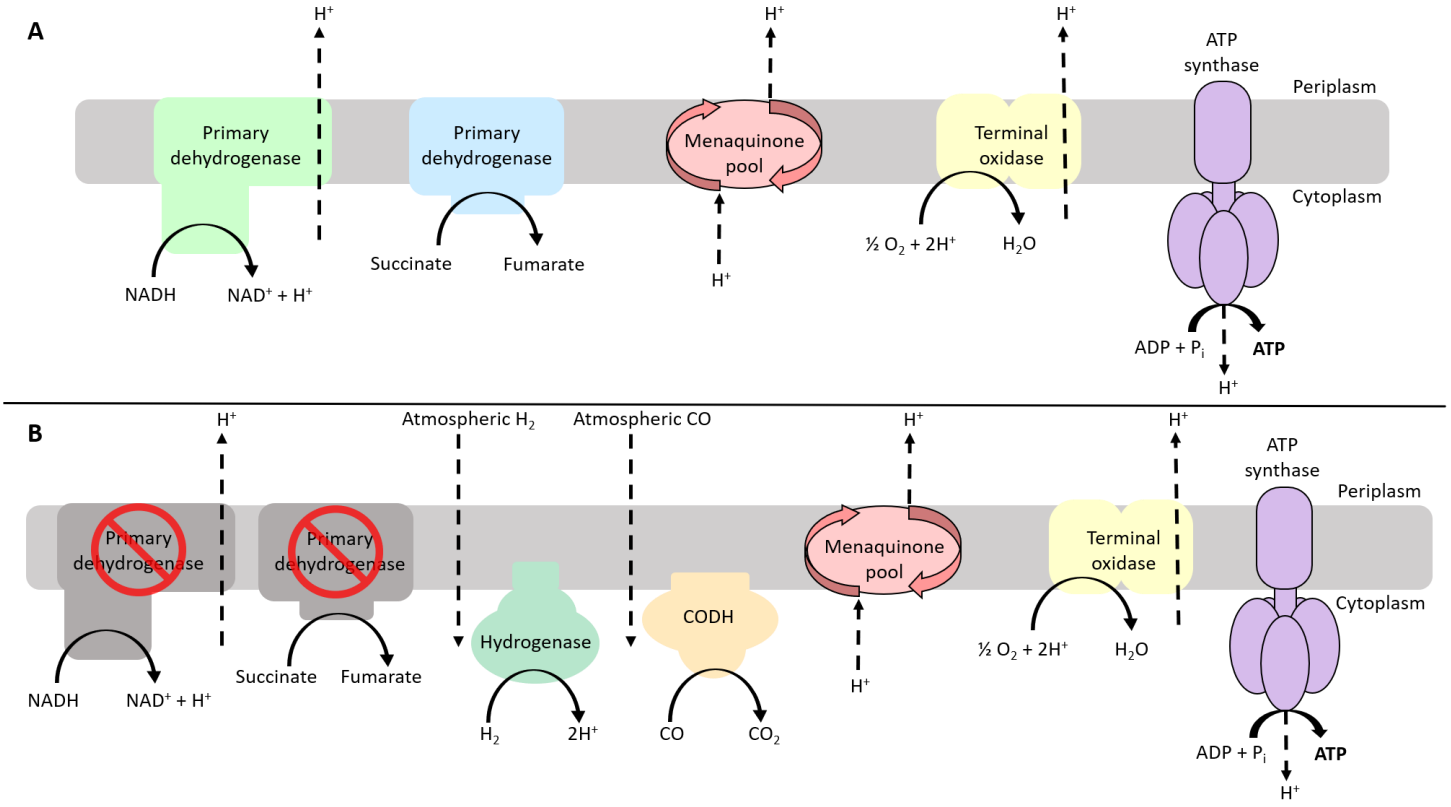

Figure 1-2: **Schematic of an organotrophic bacterial aerobic respiratory chain under (a) nutrient replete and (b) nutrient limited conditions**. In nutrient replete conditions, the aerobic respiratory chain progresses as normal, with protons generated by the breakdown of organic carbon used to facilitate ATP synthesis. In nutrient limited conditions, primary dehydrogenases are downregulated in favour of alternative metabolic enzymes, such as hydrogenases and carbon monoxide dehydrogenases. These enzymes input electrons into the menaquinone pool, thus driving the proton motive force and ATP synthesis, though less than that of organic carbon breakdown. Note: not all terminal oxidases are capable of pumping protons. Figure adapted from (Greening et al., 2015b).

1.2.2 Role in survival

The two physiological roles of atmospheric trace gas scavenging are for bacterial persistence during nutrient limitation and during mixotrophic growth (see Section 1.2.3). As the energy requirements for survival are much lower than that for growth (Price and Sowers, 2004, Morita, 1982), trace gases are an ideal energy source for bacterial maintenance (Constant et al., 2011b, Greening et al., 2015b, Cordero et al., 2019a), particularly in ecosystems devoid of more energy-dense compounds. As stated earlier, trace gases have been inferred to support ecosystem productivity and resilience in environments devoid of traditional primary producers, supporting the energetic requirements of 10^7 to 10^8 bacteria per gram of soil (Ji et al., 2017, Constant et al., 2011b, Constant et al., 2010). However, the role of atmospheric H_2 utilisation supporting survival for specific bacterial phyla has only been determined in two phyla to date, the Actinobacteriota and the Acidobacteriota. Additionally, persistence on atmospheric CO has been only been genetically proven in one phylum, the Actinobacteriota, though it has also been inferred for some marine proteobacterial species and other bacterial phyla shown to encode a CODH (Berney and Cook, 2010, Christie-Oleza et al., 2012, Cordero et al., 2019a, Cunliffe, 2013, Muthusamy et al., 2017, Patrauchan et al., 2012).

Across both the Actinobacteriota and Acidobacteriota, there have been seven pure culture studies that have demonstrated the use of atmospheric H_2 via a group 1h [NiFe]-hydrogenase during persistence (Berney et al., 2014b, Myers and King, 2016, Meredith et al., 2014, Greening et al., 2015a, Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Cordero et al., 2019b). Upon transitioning to stationary phase, *M. smegmatis* broadens its catabolic repertoire during persistence, shifting from organic compounds only to a broad range of trace organic and inorganic compounds (Berney and Cook, 2010, Cordero et al., 2019b, Berney et al., 2014b, Greening et al., 2014a). This is contrary to the classical view that, to persist, bacteria simply restrict metabolic activity. Similarly, the acidobacterial thermophile

Pyrinomonas methlyaliphatogenes upregulates expression of its hydrogenase four-fold and rapidly consumes atmospheric H_2 upon transition to stationary phase (Greening et al., 2015a). Moreover, hydrogen scavenging was also determined to be a viable strategy for maintaining exospores of numerous streptomyces species (Constant et al., 2010, Constant et al., 2008, Liot and Constant, 2016, Meredith et al., 2014). The results of these experiments provide clear evidence that hydrogen is a dependable source of energy during bacterial persistence, particularly in environments where nutrients are scarce.

Genetic knockout studies have confirmed trace gas oxidation enhances long-term survival of both *M. smegmatis* and *Streptomyces avermitilis*. Exospores of *S. avermitilis* were shown by Liot et al. (2015) to be ~75% less viable than wild-type strains when their hydrogenase was knocked out (Liot and Constant, 2016). Previously, studies by Berney et al. (2010) and Greening et al. (2014) highlighted that energy-limited hydrogenase mutant strains experienced a ~40% reduction in viability compared to wild-type counterparts, suggesting that atmospheric hydrogen is a critical alternative electron donor for *M. smegmatis* (Berney and Cook, 2010, Greening et al., 2014b). It is important to note here that *M. smegmatis* uses additional metabolic strategies to persist, including CO oxidation, so the degree of viability reduction is to be expected (Berney and Cook, 2010, Cordero et al., 2019a). Consistently, a CODH-mutant of *M. smegmatis* also demonstrated a reduction in long-term survival, with a similar decrease observed as that of the hydrogenase mutants (~45% reduction after four weeks) (Cordero et al., 2019a). Together these genetic studies highlight the overall importance of trace gas oxidation for bacterial persistence, though knockout or inhibitor studies would be required to determine whether trace gas scavenging fulfils a similar purpose in other phyla hypothesised to survive nutrient limitation in this manner. However, as genetic manipulation tools are not readily available for the majority of bacterial phyla, whole cell physiological assays would also be required.

1.2.3 Role in growth

The role of atmospheric trace gases in mixotrophic bacterial growth is less studied than that of its role in energy generation during persistence. While all three gases have been experimentally determined to be incapable of supporting autotrophic growth (Conrad, 1984, Conrad, 1996, Conrad, 1999b), recent studies on the methanotrophs *Methylacidiphilum fumariolicum* SolV (Schmitz et al., 2020) and *Methylocapsa gorgona* MG08 (Tveit et al., 2019) have inferred the possibility of autotrophic growth using all three trace gases. However, little research has been carried out to determine their role in mixotrophic growth, with co-utilisation of atmospheric trace gases with organic carbon sources the likely mixotrophic strategy of heterotrophic bacteria.

Hydrogen oxidation during mixotrophic growth has been suggested to play a key role in generating energy in the absence of their preferred energy sources for the nitrite oxidiser *Nitrospira moscoviensis* and the methane oxidisers *Methylacidiphilum* sp. RTK17.1 and *Methylocystis* sp. SC2 (Carere et al., 2017, Koch et al., 2014, Hakobyan et al., 2020). Research by Koch et al. (2014) has suggested that despite formerly being classified as an obligate chemolithoautotroph that solely used nitrite, *N. moscoviensis* is in fact more metabolically flexible; this bacterium can couple H_2 oxidation to CO_2 fixation in oxygenic conditions and in the absence of nitrite (Koch et al., 2014). Similarly, the verrucomicrobial chemoorganoautotrophic methanotroph, *Methylacidiphilum* sp. RTK17.1, was shown by Carere et al. (2017) to grow optimally by co-oxidising CH_4 and H_2 as opposed to autotrophically on either gas (Carere et al., 2017). More recently, hydrogen oxidation was shown by Hakobyan et al. (2020) to increase the biomass yield of the alphaproteobacterial chemoorganoheterotrophic methanotroph *Methylocystis* sp. SC2 during co-oxidation of CH_4 in methane and oxygen-limited conditions (Hakobyan et al., 2020). These three studies provide a broader framework in which the co-metabolism of atmospheric gases may represent a common

growth strategy across bacterial phyla found in dynamic ecosystems and suggest that previously characterised obligate specialist bacteria are the exception rather than the norm. However, for all three organisms, it is yet unclear whether atmospheric concentrations of H_2 would elicit the same growth augmentation response.

To date, there have yet to be any studies focused specifically on the potential for atmospheric CO to fuel mixotrophic bacterial growth, despite evidence of both autotrophy on excess CO (Conrad, 1996, King and Weber, 2007, Meyer and Schlegel, 1983) and persistence on atmospheric CO (Berney and Cook, 2010, Christie-Oleza et al., 2012, Cordero et al., 2019a, Cunliffe, 2013, Muthusamy et al., 2017, Patrauchan et al., 2012). However, as the energy derived from atmospheric CO oxidation is likely shuttled through the aerobic respiratory chain, it is theoretically possible that atmospheric CO could also be used during mixotrophic growth.

1.3 The biochemistry of trace gas oxidation

1.3.1 Group 1h [NiFe]-hydrogenase

Research has shown that high-affinity [NiFe]-hydrogenases are responsible for the biogeochemically and ecologically significant process of atmospheric hydrogen oxidation (Berney et al., 2014b, Greening et al., 2014a). To date, there are only four known classes that have been determined to be oxygen tolerant, the 1d, 1f, 1h, and 2a subgroups (Greening et al., 2016, Pandelia et al., 2012, Søndergaard et al., 2016). Of these four classes, only three have been experimentally determined to oxidise atmospheric H_2, the group 1f, 1h and 2a hydrogenases. The prevalence of the group 1h hydrogenase across bacterial phyla has been highlighted through a number of metagenomic and genomic surveys, whereby at least 13 bacterial phyla encode the genetic determinants (Greening et al., 2016, Ji et al., 2017, Piché-Choquette et al., 2017). However, to date, only isolates from three phyla have been axenically determined to scavenge H_2, suggesting further work is required to determine whether the remaining 10 phyla also oxidise atmospheric H_2 via a group 1h hydrogenase.

Crystallisation studies performed on the low-affinity group 1h hydrogenase from *Ralstonia eutropha*, have revealed that the enzyme is comprised of a closely associated large and small subunit that exists in homodimeric form (Schäfer et al., 2016, Schäfer et al., 2013). The enzyme has been experimentally determined to be transcribed in two distinct operons, a structural operon which contains the catalytic subunits, and an accessory operon which contains maturation factors (Berney et al., 2014b, Greening et al., 2015b). Based on RT-PCR in *M. smegmatis*, the structural operon encodes: a large subunit (HhyL) containing the Ni-based active centre ligated by CN^- and CO that binds H_2; a small subunit (HhyS) containing three FeS clusters; an uncharacterised protein with a possible role in electron transfer (HhyE); and two chaperones supporting nickel insertion (HypA and HypB). The accessory operon encodes four maturation factors (HypC-F) and three hypothetical proteins (HhaA-C) (Berney et al., 2014b, Greening et al., 2015b). Currently, the exact mechanism behind the high-affinity nature of the *M. smegmatis* group 1h hydrogenase is unknown; resolving the crystal structure of it and comparing it to that of the low-affinity enzyme in *R. eutropha* may provide deeper insights.

The group 1h [NiFe]-hydrogenase has been determined to mediate atmospheric hydrogen oxidation in three distinct bacterial phyla to date: Actinobacteriota, Acidobacteriota, and Verrucomicrobiota (Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Greening et al., 2015a, Meredith et al., 2014, Myers and King, 2016, Schmitz et al., 2020). The affinity for hydrogen of this enzyme is up to 20-fold greater than other characterised hydrogenases, though the apparent Michaelis constants ($K_{m\ app}$) can vary greatly between species (Greening et al., 2015b). In general, for a hydrogenase to be classified as high affinity for H_2, their $K_{m\ app}$ should be in the range of 30-130 nM (Greening et al., 2014a), which has been experimentally determined in two bacterial phyla, the Actinobacteriota and Acidobacteriota (Constant et al., 2011b, Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Greening et al., 2015a). However, more recent whole-cell kinetics measurements

have determined that other species harbouring a group 1h hydrogenase have the capacity to oxidise atmospheric concentrations of hydrogen with a higher $K_{m\ app}$ (range of 100 – 1000 nM), namely the verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* (Schmitz et al., 2020). Conversely, the Knallgas betaproteobacteria *R. eutropha* has been shown to encode a low affinity group 1h hydrogenase ($K_{m\ app}$ of 1-5 µM) and, to date, has not been experimentally verified to be able to oxidise atmospheric concentrations of H_2 (Schäfer et al., 2013). The large kinetic range suggests that the interplay between the catalytic subunits, the accessory proteins and the rest of the respiratory chain may play a key role in determining whether a microorganism that encodes a group 1h hydrogenase is capable of atmospheric hydrogen oxidation. More recently, studies have theorised that proximal electron acceptors may play a role in facilitating electron transfer between the catalytic subunits and the respiratory chain (Berney et al., 2014b, Greening et al., 2015b, Schäfer et al., 2013), potentially contributing to the high-affinity nature of the hydrogenase. However, as to date no high-affinity group 1h hydrogenase has been purified, inferences on what enables the high affinity nature of the group 1h hydrogenase cannot be definitively stated.

1.3.2 Group 2a [NiFe]-hydrogenase

The group 2a [NiFe]-hydrogenase is another oxygen-tolerant uptake hydrogenase that has also been determined to be high-affinity in the aerobic soil microorganism, *M. smegmatis* (Berney et al., 2014b, Greening et al., 2014a, Søndergaard et al., 2016). Currently, the only experimentally determined phylum capable of atmospheric H_2 scavenging via this enzyme is the Actinobacteriota, with a *M. smegmatis* mutant containing only a group 2a hydrogenase shown to still have the capacity to oxidise atmospheric hydrogen, albeit at a slower rate than that of its wild-type counterpart (Greening et al., 2014a, Cordero et al., 2019b). Furthermore, genomic and metagenomic surveys have routinely determined that at least eight other bacterial phyla encode the genetic determinants for the group 2a hydrogenase (Greening et al., 2016,

Søndergaard et al., 2016), including Cyanobacteria, suggesting that this hydrogenase may play a wider role than previously proposed. Additionally, these studies have identified the presence of this hydrogenase in a number of low nutrient ecosystems including oceans, permafrost soils, hot springs and bog soils, albeit in low abundance (Greening et al., 2016, Barz et al., 2010, Tamagnini et al., 2002).

Within the Cyanobacteria, there have been extensive studies focused on the purported role of the group 2a uptake hydrogenase in the recycling of by-products of nitrogenase reactions, primarily within heterocysts (Bothe et al., 2010, Papen et al., 1986, Tamagnini et al., 2007). It is hypothesised that, for the majority of the nitrogen-fixing Cyanobacteria discovered so far, an uptake hydrogenase is also present in its genome, though the presence of an uptake hydrogenase may not be contingent on N_2-fixing capacity (Tamagnini et al., 2002). As such, three distinct roles for the hydrogenase were proposed: 1) as an additional source of ATP to be used for a range of metabolic processes, 2) to ensure availability of reducing agents for nitrogen fixation and 3) to remove excess oxygen from the active site of the nitrogenase (Eisbrenner and Bothe, 1979, Houchins, 1984, Tamagnini et al., 2007, Zhang et al., 2014). A study by Troshina et. al. (1996) on *Anabaena variabilis* ATCC 29413 demonstrated that hydrogenase activity occurred in the absence of nitrogenase activity, though higher levels of activity were not seen upon the addition of exogenous hydrogen (Troshina et al., 1996). Moreover, a number of other studies have suggested that the amount of hydrogen produced by nitrogenase reactions within heterocysts may be sufficient for the induction of the group 2a hydrogenase and results in the offsetting of hydrogen emissions by Cyanobacteria (Bothe et al., 2010, Houchins, 1984, Lindblad and Tamagnini, 2001, Tamagnini et al., 2002, Tamagnini et al., 2007). Taken together, these findings may indicate that the uptake hydrogenases of Cyanobacteria have similar affinities to the group 2a hydrogenase characterised in *M. smegmatis*, suggesting that its ability to utilise low concentrations of hydrogen may be more generalisable across bacterial

phyla, though to date this has not been tested. Moreover, there is still some debate around the oxygen-tolerance of Cyanobacteria-associated group 2a hydrogenases, given they are primarily localised to the thylakoid membranes of heterocysts or have been shown to be preferentially expressed during dark phase nitrogenase reactions in nonheterocystous Cyanobacteria (Tamagnini et al., 2007, Camsund et al., 2011, Seabra et al., 2009).

Similar to the group 1h hydrogenase, the group 2a hydrogenase is transcribed in two main operons, one encoding structural proteins and the other accessory/maturation factors. The structural subunits are arranged into a large catalytic subunit and a small subunit consisting of three iron-sulfur clusters, namely two [4Fe-4S] clusters and one [3Fe-4S] cluster, based on EPR spectroscopic studies on the cyanobacterium *Nostoc punctiforme* ATCC 29133 and the proteobacterium *Acidithiobacillus ferrooxidans* (Raleiras et al., 2013, Fischer et al., 1996, Schröder et al., 2007). Within the structural operon of *M. smegmatis*, a putative iron-sulfur protein has also been shown to be co-transcribed with the catalytic subunits, suggesting that this may be the means by which electron transfer to the respiratory chain occurs (Berney et al., 2014b, Greening et al., 2015b). However, alternative electron transfer proteins have been suggested for the cyanobacterial strains due to its localisation, primarily in the form of a *b*-type cytochrome (Tamagnini et al., 2002), though these may not serve as the immediate electron acceptor for all cyanobacterial species. Indeed, this potential structural difference between cyanobacterial species and non-cyanobacterial species may play a key role in the efficiency of the hydrogenase to utilise low concentrations of H_2 or may be a determinant of the physiological role of the hydrogenase in different species.

Additionally, genetic determinants for the group 2a hydrogenase have been identified in isolates from human-engineered ecosystems such as the acidic mine drainage acidophile *A. ferrooxidans*, the wastewater treatment plant nitrifier *N. moscoviensis* and the solfataric thermoacidophile *Hydrogenobacter acidophilus* (Koch et al., 2014, Shima and Suzuki, 1993,

Drobner et al., 1990, Schröder et al., 2007). As the aforementioned species have been shown to be capable of hydrogenotrophic growth, it would be interesting to investigate whether the group 2a hydrogenases in these species, as well as others, can mediate atmospheric H_2 oxidation. Altogether the presence of the group 2a hydrogenase in diverse organisms, spanning obligate photolithoautotrophs, chemoorganoheterotrophs and chemolithoautotrophs within different ecosystems suggests that this hydrogenase may play a key role in enabling bacterial survival in the face of nutrient limitation.

1.3.3 Accessory hydrogenase proteins

Whereas the structural genes for the [NiFe] hydrogenase are encoded on one operon, other maturation and additional factors are generally encoded on another operon, the accessory operon. Previous RT-PCR experiments have suggested that the accessory operon encodes a number of genes necessary for hydrogenase maturation including factors to synthesis the active site and specific endopeptidases (Greening et al., 2015b). Moreover, conserved with the structural operons of both the group 1h and 2a hydrogenases are putative iron-sulfur cluster proteins and conserved hypothetical proteins, which may act as electron transfer proteins and/or could be involved in tethering the hydrogenase to the cell membrane, though more research is required to definitively determine their physiological roles.

1.3.3.1 Putative iron-sulfur cluster proteins

Iron-sulfur (FeS) cluster proteins are involved in a multitude of reactions including catalysis, oxygen sensing, gene regulation and electron transfer (Beinert et al., 1997, Johnson et al., 2005, Lill, 2009). One of the most common roles for FeS cluster proteins is mediating electron transfer through the ability of Fe to switch from a +2 to a +3 redox state (Johnson et al., 2005, Lill, 2009). Structurally, FeS cluster proteins are typically found in three main arrangements, Fe_2S_2, Fe_3S_4 and Fe_4S_4, though other structural arrangements as well as novel coordination ligands are possible (Beinert et al., 1997, Goris et al., 2011). These proteins are typically

characterised by the presence of highly conserved CxxC (NifU-like) or CxH(x_{15-18})CxxC (Rieske-like) motifs in their sequences, which have been experimentally determined to be involved in the coordination of FeS clusters (Beinert et al., 1997, Schmidt and Shaw, 2001, Py et al., 2012). Present across all domains of life, FeS clusters are able to mediate a range of functions depending on the number and type of ligands coordinated to the centre as well as the associated proteins that make up the cluster (Beinert et al., 1997, Fontecave, 2006). For example, three FeS clusters are present within the small subunit of all uptake [NiFe]-hydrogenases. They serve to transfer electrons derived from H_2 oxidation to the respiratory chain, and in the case of oxygen-tolerant enzymes, are thought to aid in enzyme reactivation following oxygen poisoning of the active site (Ogata et al., 2016).

Specific proteins mediate the transfer of electrons from the small subunits of uptake [NiFe]-hydrogenases to the menaquinone pool. The immediate electron acceptor of many classes of uptake hydrogenases, including the oxygen-tolerant group 1d [NiFe] hydrogenase, is a membrane-bound *b*-type cytochrome subunit (Frielingsdorf et al., 2011, Volbeda et al., 2013, Greening et al., 2016). However, the equivalent proteins were not detected in the structural operons of group 1h or 2a hydrogenases (Berney et al., 2014b). Instead, two conserved putative iron-sulfur cluster proteins, denoted as HhyE (MSMEG_2718) and HucE (MSMEG_2268), have been suggested as the potential electron transfer proteins associated with group 1h and 2a hydrogenases respectively. It is predicted that these proteins have a higher standard redox potential (-0.2 V) than that of menaquinone (-0.07 V), but lower than that of H_2 (-0.42 V), resulting in a highly thermodynamically favourable reaction (Berney et al., 2014b, Brown et al., 2008, Greening et al., 2014a). Resolving their precise role in high affinity hydrogen oxidation requires the generation of knockout mutants, the purification of the proteins in order to perform redox chemical analysis, and interaction studies with their yet-to-be purified hydrogenases.

1.3.3.2 Maturation proteins

The biosynthesis and maturation of the multi-subunit metalloenzyme, the [NiFe]-hydrogenase, is typically tightly regulated and is controlled by a number of proteins collectively termed maturation and accessory factors (Böck et al., 2006, Lacasse and Zamble, 2016, Senger et al., 2017). For hydrogenases, this accessory operon usually consists of six main genes, termed *hypA-F*, which encode proteins primarily involved in nickel insertion, CN^- and CO synthesis, and proteolytic cleavage to generate the functional hydrogenase. These six genes have been extensively studied, primarily in relation to *E. coli* (Lacasse and Zamble, 2016), though their functionality is largely conserved across [NiFe]-hydrogenase expressing species including *M. smegmatis* (Berney et al., 2014b, Greening et al., 2015b).

In brief, the determined roles of these six proteins are as follows. The metallochaperone HypA has primarily been characterised as a nickel insertase that works in conjunction with the nickel chelator and NTPase, HypB, to bind and selectively deliver nickel to the structural subunits during hydrogenase maturation (Hube et al., 2002, Lacasse and Zamble, 2016, Olson et al., 1997). Conversely, HypC, HypD, HypE and HypF are predominantly associated with the assembly of the iron-centre of the large catalytic subunit, including the biosynthesis and coordination of the CN^- ligand (Böck et al., 2006, Lacasse and Zamble, 2016). HypF has been characterised to be a carbomoyl transferase that is involved in the conversion of carbamoyl phosphate into a carbamoyl moiety which is transferred to HypE, which then catalyses the dehydration of the S-carboxamide moiety to yield HypE-thiocyanate (Lacasse and Zamble, 2016, Rangarajan et al., 2008, Magalon and Böck, 2000, Paschos et al., 2002, Senger et al., 2017, Watanabe and Miki, 2011). Further downstream processing by the HypC-D complex then catalyses the transfer of the CN^- ligand from HypE to the iron atom in the large subunit precursor molecule (Lacasse and Zamble, 2016, Watanabe and Miki, 2011). Additional analysis of HypC and HypD has suggested that, when complexed, these proteins serve as both

a source for and a scaffold for iron, which can then be ligated by the CN^- and CO ligands prior to final large subunit processing (Lacasse and Zamble, 2016, Soboh et al., 2012). Final processing of the primitive large subunit is typically carried out by an endopeptidase, a hydrogenase maturation protease (HMP), whereby the C terminus of the large subunit is cleaved, causing a conformational change via the bridging of Fe and Ni atoms to close the active centre (Lacasse and Zamble, 2016, Senger et al., 2017, Theodoratou et al., 2005). This complex then associates with the small subunit containing FeS clusters to form a functional enzyme capable of transferring the electrons derived from hydrogen oxidation to the menaquinone pool via an electron transfer protein intermediate such as a *b*-type cytochrome in group 1d [NiFe]-hydrogenases (Pinske et al., 2011, Vignais and Billoud, 2007, Volbeda et al., 2013).

1.3.3.3 Conserved hypothetical proteins

There are several hypothetical proteins within the *M. smegmatis* Hhy accessory protein operon, including MSMEG_2715 (HhaA), MSMEG_2716 (HhaB) and MSMEG_2717 (HhaC), which are highly conserved across organisms encoding group 1h [NiFe]-hydrogenases, yet are of completely unknown function (Greening et al., 2015b). Protein family searches using the functional analytics software packages InterPro (Hunter et al., 2009, Mitchell et al., 2018) and HMMER (Potter et al., 2018) for all three proteins result in no known homology to other characterised proteins nor the presence of conserved domains. Despite the current lack of characterisation, a study by Schäfer et al. (2013) suggested that a homolog to MSMEG_2717, PHG067, in the betaproteobacterium *R. eutropha*, may mediate the transfer of electrons from the catalytic subunit of the hydrogenase to the respiratory chain (Schäfer et al., 2013). As such, it is possible that the three conserved hypothetical proteins, HhaA-C, may serve as the electron acceptors for a number of high affinity hydrogenases, or may play another role altogether such as in membrane tethering.

1.3.4 Carbon monoxide dehydrogenase

The oxidation of CO is primarily mediated by a form I carbon monoxide dehydrogenase (CODH) in both carboxydovores and carboxydotrophs (Cordero et al., 2019a, King, 2003b, King and Weber, 2007), though there are distinct physiological differences between the two. However, despite differences in physiological role, the underlying enzymatic determinant of aerobic CO oxidation is the same enzyme, a form I CODH. Conversely, an alternative CODH, containing a Ni-[4Fe-5S] centre, is primarily utilised by anaerobic carboxydotrophs to fix CO via the Wood-Ljungdahl pathway (Dobbek et al., 2001, Xavier et al., 2018, Robb and Techtmann, 2018). As the subject of this thesis focuses on aerobic atmospheric trace gas oxidation, the anaerobic CODH will not be discussed further.

In aerobic microorganisms, the form I CODH is an oxygen-tolerant dimer of heterotrimers consisting of three structural subunits, large (CoxL), medium (CoxM) and small (CoxS) (Dobbek et al., 2002, Dobbek et al., 1999, Schübel et al., 1995, Lorite et al., 2000, Meyer et al., 1986). The large subunit, which contains a molybdenum-copper centre that binds cytosine dinucleotides, catalyses the conversion of CO to CO_2 using water as an oxidant (Schübel et al., 1995, King and Weber, 2007, Meyer et al., 2000, Dobbek et al., 2002). An EPR study on the carboxydotroph *Oligotropha carboxidivorans* showed that the small subunit consists of two distinct sets of [2Fe-2S] clusters can bind cysteine and histidine ligands (Bray et al., 1983, Schübel et al., 1995). The medium subunit has been determined to be involved in binding a flavin adenine dinucleotide (Fuhrmann et al., 2003, Kang and Kim, 1999). The liberated electrons can then be shuttled to terminal electron acceptors, such as oxygen and nitrate via the respiratory chain. Carboxydotrophs also support CO_2 fixation using this enzyme (King and Weber, 2007, Meyer et al., 1986, Cordero et al., 2019a, Frunzke and Meyer, 1990). Additionally, similar to hydrogenases, a variable number of accessory proteins are also associated with optimal maturation and functioning of the CODH, including CoxG which has

been postulated to be involved in anchoring the CODH to the cytoplasmic membrane (Fuhrmann et al., 2003, Santiago et al., 1999). It is possible that this variation in number and function of accessory proteins influences the thresholds of CO able to be utilised by bacteria, though more research is required to characterise these accessory proteins and their degree of conservation across atmospheric CO oxidising bacteria.

The genetic determinants for CO oxidation have been identified in seven dominant soil phyla, the Proteobacteria, Actinobacteriota, Acidobacteriota, Chloroflexota, Firmicutes, Gemmatimonadota and Bacteroidota (Cordero et al., 2019a, Janssen, 2006), with atmospheric scavenging theorised for Proteobacteria, Actinobacteriota and Chloroflexota (Gadkari et al., 1990, King and King, 2014a, King, 2003b, King, 2003c, Weber and King, 2017). Atmospheric CO oxidation has been experimentally observed in 18 bacterial and archaeal genera (Cordero et al., 2019a, Gadkari et al., 1990, King, 2003b, Weber and King, 2017, King and King, 2014a), including the marine strains *Ruegeria pomolori* and a Phaeobacter isolate (Christie-Oleza et al., 2012, Muthusamy et al., 2017). However, to date, only one species of Actinobacteriota, *M. smegmatis*, has been genetically validated to oxidise atmospheric concentrations of CO via a type I CODH for bacterial persistence (Cordero et al., 2019a). As such, further pure culture-based studies are required to definitively determine whether other phyla are also capable of this ability, and to determine when atmospheric CO is being utilised.

1.4 Knowledge gaps and thesis aims

1.4.1 Knowledge gaps

Genomic and metagenomic surveys suggest representatives from several dominant soil bacterial phyla from a range of ecological niches have enzymes to oxidise atmospheric trace gases. These include isolates within the phyla Chloroflexota, Proteobacteria and Gemmatimonadota (Greening et al., 2016, Ji et al., 2017, Leung et al., 2020). However, culture-dependent experimental validation is needed to test if these observations are valid (Overmann

et al., 2017, Pham and Kim, 2012). To address this knowledge gap, this thesis will use representative cultured strains of each of the aforementioned phyla to determine their capacity to utilise trace gases.

In addition to characterising additional microbial players in the biogeochemical cycle of hydrogen, expanding the enzyme families capable of mediating this process is of great interest. To date, all species that have been experimentally validated to oxidise atmospheric hydrogen harbour a group 1h [NiFe]-hydrogenase (Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Greening et al., 2015a, Meredith et al., 2014, Myers and King, 2016, Schmitz et al., 2020), and use this enzyme to support survival in response to nutrient starvation (Berney and Cook, 2010, Berney et al., 2014a, Greening et al., 2014b, Liot and Constant, 2016). While the group 1h [NiFe]-hydrogenase is likely the primary determinant of atmospheric hydrogen oxidation, another potentially high-affinity, oxygen-tolerant subgroup of [NiFe]-hydrogenase has been identified and characterised, the group 2a [NiFe]-hydrogenase (Cordero et al., 2019b, Greening et al., 2014a, Greening et al., 2016, Søndergaard et al., 2016). While most research on the group 2a [NiFe]-hydrogenase has primarily focused on its role in recycling the by-products of the nitrogenase reaction in Cyanobacteria (Bothe et al., 2010, Tamagnini et al., 2007), its presence in the genomes of eight additional phyla (Søndergaard et al., 2016) and studies in *M. smegmatis* have indicated that it may play a broader role in enabling mixotrophic growth on hydrogen (Berney et al., 2014b, Cordero et al., 2019b). Culture-dependent studies of other organisms encoding a group 2a [NiFe]-hydrogenase are required to determine whether this ability is generalisable across other bacterial phyla or is a specific mycobacterial adaptation.

Also of interest are whether proteins co-expressed with group 1h and 2a [NiFe]-hydrogenases support atmospheric H_2 oxidation. Previous molecular studies using the model soil actinobacterium *M. smegmatis* and proteobacterium *R. eutropha* have identified conserved

structural and accessory operons that encode a number of hypothetical proteins with little to no homology to any currently characterized proteins which may confer the high affinity nature of these enzymes (Greening et al., 2014a, Schäfer et al., 2013). Of these proteins that co-transcribe with the structural hydrogenase operons are proteins that encode putative FeS clusters, which are predicted to transfer the electrons liberated from the oxidation of hydrogen from the small subunits to the menaquinone pool of the mycobacterial respiratory chain (Berney et al., 2014b, Greening et al., 2014a, Greening et al., 2015b). As such, understanding the molecular determinants that confer high-affinity hydrogen oxidation will allow for a greater understanding of how these enzymes function, potentially contributing to a biotechnological application for these hydrogenases within a hydrogen fuel-based economy.

Finally, it is also of growing importance to understand the biogeochemical cycling process of other atmospheric trace gases, such as carbon monoxide. As microorganisms represent the largest biological sink of this gas (Bartholomew and Alexander, 1981, Khalil and Rasmussen, 1990), it is critical to resolve which microorganisms mediate this process and why. While there have been a number of organisms determined to be able to grow on excess amounts of CO (King and Weber, 2007), little research has been conducted to investigate whether it could also serve as a dependable energy source during bacterial persistence or mixotrophic growth, similar to the proposed role of hydrogen.

1.4.2 Thesis aims

The overall aim of this thesis was to expand our understanding of the determinants of atmospheric gas oxidation in bacteria, spanning from whole organism level down to molecular modulators. This aim was subdivided into four main objectives, which are reflected in the three data chapters and general discussion:

1. How widespread is atmospheric H_2 oxidation across bacterial phyla?
2. Can atmospheric H_2 support mixotrophic growth?

3. What accessory genes enable atmospheric H_2 oxidation?
4. Does atmospheric CO oxidation support bacterial persistence?

Chapter 2 demonstrates the phylum Chloroflexota is capable of mediating the processes of atmospheric H_2 and CO oxidation, while addressing the importance of both gases for persistence. Chapter 3 investigates the role of the group 2a [NiFe]-hydrogenase in mixotrophic growth for three bacterial phyla and simultaneously provides axenic evidence for H_2 scavenging in two additional bacterial phyla, Gemmatimonadota and Proteobacteria. Chapter 4 highlights the integral role of putative iron-sulfur cluster proteins for high affinity, aerobic hydrogen oxidation in two subgroups of [NiFe]-hydrogenases. The role of CO oxidation for aerobic bacteria is discussed in Chapter 2 and additionally expanded on in the general discussion section, with specific reference to a co-authored publication on the physiological role of the *M. smegmatis* carbon monoxide dehydrogenase that is not included in this thesis.

2 TWO CHLOROFLEXI LINEAGES INDEPENDENTLY EVOLVED THE ABILITY TO PERSIST ON ATMOSPHERIC HYDROGEN AND CARBON MONOXIDE

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2.1 Abstract

Most aerobic bacteria exist in dormant states within natural environments. In these states, they endure adverse environmental conditions such as nutrient starvation by decreasing metabolic expenditure and using alternative energy sources. In this study, we investigated the energy sources that support persistence of two aerobic thermophilic strains of the environmentally widespread but understudied phylum Chloroflexi. A transcriptome study revealed that *Thermomicrobium roseum* (class Chloroflexia) extensively remodels its respiratory chain upon entry into stationary phase due to nutrient limitation. Whereas primary dehydrogenases associated with heterotrophic respiration were downregulated, putative operons encoding enzymes involved in molecular hydrogen (H_2), carbon monoxide (CO), and sulfur compound oxidation were significantly upregulated. Gas chromatography and microsensor experiments showed that *T. roseum* aerobically respires H_2 and CO at a range of environmentally relevant concentrations to sub-atmospheric levels. Phylogenetic analysis suggests that the hydrogenases and carbon monoxide dehydrogenases mediating these processes are widely distributed in Chloroflexi genomes and have probably been horizontally acquired on more than one occasion. Consistently, we confirmed that the sporulating isolate *Thermogemmatispora* sp. T81 (class Ktedonobacteria) also oxidises atmospheric H_2 and CO during persistence, though further studies are required to determine if these findings extend to mesophilic strains. This study provides axenic culture evidence that atmospheric CO supports bacterial persistence and reports the third phylum, following Actinobacteria and Acidobacteria, to be experimentally shown to mediate the biogeochemically and ecologically important process of atmospheric H_2 oxidation. This adds to the growing body of evidence that atmospheric trace gases are dependable energy sources for bacterial persistence.

2.2 Introduction

Bacteria from the phylum Chloroflexi are widespread and abundant in free-living microbial communities (Delgado-Baquerizo et al., 2018, Mehrshad et al., 2018b, Sunagawa et al., 2015, Thompson et al., 2017). One reason for their success is their metabolic diversity; cultured strains from the phylum include heterotrophs, lithotrophs, and phototrophs adapted to both oxic and anoxic environments (Whitman, 2015). Cultured representatives of the phylum are classified into four classes by the genome taxonomy database (Parks et al., 2018), the primarily aerobic Chloroflexia and Ktedonobacteria and the anaerobic Anaerolineae and Dehalococcoidia (Whitman, 2015). Studies have provided insight into the metabolic strategies that anaerobic classes within the phylum use to adapt to oligotrophic niches (Dong et al., 2019, Hug et al., 2013). However, surprisingly little is known about how aerobic heterotrophic Chloroflexi colonise oxic environments. Global surveys have reported that Chloroflexi comprise 4.3% of soil bacteria (Delgado-Baquerizo et al., 2018) and 3.2% of marine bacteria (Sunagawa et al., 2015). However, the most dominant lineages within these ecosystems (notably Ellin6529 and SAR202) have not been cultivated (Delgado-Baquerizo et al., 2018, Mehrshad et al., 2018a, Parks et al., 2018). Instead, most of our knowledge about the ecophysiological strategies of aerobic heterotrophic Chloroflexi is derived from studies on thermophilic isolates. Various strains from the classes Chloroflexia and Ktedonobacteria have been isolated and characterised from hot springs and geothermal soils (Jackson et al., 1973, King and King, 2014a, King and King, 2014b, Stott et al., 2008, Houghton et al., 2015).

Within geothermal environments, Chloroflexi strains are likely to encounter temporal and spatial variations in the availability of organic carbon compounds and other nutrients (Power et al., 2018). It is currently unknown how members of this phylum stay energised in response to these environmental perturbations. Carbon monoxide (CO) and molecular hydrogen (H_2) of both geothermal and atmospheric origin are available in such environments

and may be particularly important energy sources for sustaining growth and persistence (King, 2003a, King and Weber, 2008, Shock et al., 2010, Spear et al., 2005, Yang et al., 2015). Consistently, genomic, and metagenomic studies have revealed that Chloroflexi encode carbon monoxide dehydrogenases (King and Weber, 2007, Wu et al., 2009, Quiza et al., 2014) and hydrogenases (Greening et al., 2016, Ji et al., 2017, Søndergaard et al., 2016, Wu et al., 2009) known to mediate aerobic respiration of these gases. Chloroflexi isolates have been shown to aerobically oxidise CO at a range of environmentally significant concentrations: *Thermomicrobium roseum* consumes high concentrations of CO when available during growth (Wu et al., 2009) and multiple *Thermogemmatispora* isolates have been shown to oxidise CO, including *T. carboxidovorans* to atmospheric concentrations (0.10 ppmv) (King and King, 2014a). While H_2 oxidation has yet to be reported in aerobic heterotrophic Chloroflexi, strains of the phylum are known to encode the high-affinity group 1h [NiFe]-hydrogenase (Greening et al., 2016, Greening et al., 2015b). This enzyme class has been shown to support bacterial persistence by mediating oxidation of atmospheric H_2 (0.53 ppmv) (Berney et al., 2014a, Constant et al., 2010, Greening et al., 2014a, Greening et al., 2015a, Greening et al., 2014b, Ji et al., 2017, Kanno et al., 2016, Khdhiri et al., 2015, Liot and Constant, 2016, Meredith et al., 2014). To date, atmospheric H_2 oxidation has only been experimentally confirmed in Actinobacteria (Berney et al., 2014a, Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Liot and Constant, 2016, Meredith et al., 2014) and two acidobacterial isolates (Greening et al., 2015a, Myers and King, 2016).

In this study, we investigated the persistence strategies of thermophilic isolates from two classes of Chloroflexi. We focused primarily on *Thermomicrobium roseum* (class Chloroflexia, formerly class Thermomicrobia (Parks et al., 2018), a strain originally isolated from Toadstool Spring of Yellowstone National Park, USA (Jackson et al., 1973). This obligately aerobic bacterium is known to grow heterotrophically on a variety of carbohydrates,

organic acids, and proteinaceous substrates (Houghton et al., 2015, Jackson et al., 1973, Wu et al., 2009). Previous analyses have shown *T. roseum* encodes a type I carbon monoxide dehydrogenase and a group 1h [NiFe]-hydrogenase (Greening et al., 2016, Wu et al., 2009), and can oxidise high concentrations of CO. However, the bacterium appears to be incapable of supporting chemolithoautotrophic growth and lacks key enzymes for the Calvin-Benson cycle (King and King, 2014b, Wu et al., 2009). A combination of transcriptome sequencing and targeted activity assays were used to holistically determine the metabolic basis of persistence in this organism, including demonstrating that CO and H_2 are oxidised by this strain during nutrient limitation. To help generalise these findings, we also investigated *Thermogemmatispora* sp. T81 (class Ktedonobacteria), a cellulolytic thermophilic strain which we previously isolated from geothermal soils in Tikitere, New Zealand (Stott et al., 2008, Tomazini et al., 2018, Vyssotski et al., 2012). Collectively, our results demonstrate that atmospheric H_2 and CO serve as important energy sources that support the persistence of members of this phylum.

2.3 Materials and Methods

2.3.1 Bacterial strains

Thermomicrobium roseum DSM 5159 (Houghton et al., 2015, Jackson et al., 1973) and *Thermogemmatispora* sp. T81 (Stott et al., 2008, Vyssotski et al., 2012) were imported from the Extremophiles Research Group (GNS Science, Wairakei, New Zealand) culture collection in February 2017. Cultures of both bacterial isolates were routinely maintained in 120 mL serum vials sealed with treated lab-grade butyl rubber stoppers. Cultures of *T. roseum* contained 30 mL Castenholz media (Ramaley and Hixson, 1970) supplemented with 1 g L^{-1} yeast extract and 1 g L^{-1} tryptone, whereas *Thermogemmatispora* sp. T81 cultures were maintained in 30 mL 10% R2A media (Reasoner and Geldreich, 1985). Unless otherwise stated, both strains were incubated at 60 °C at an agitation speed of 150 rpm in an Eppendorf

40 Incubator. For *T. roseum*, cultures were inoculated to an OD_{600} of 0.03 and cells entered stationary phase within 48 h (OD_{max} = 0.75 to 1.0); growth curves confirmed that the strain entered stationary phase as a result of nutrient limitation, likely because of exhausting organic carbon supplies **(Figure 2-3)**. For *Thermogemmatispora* sp. T81, 1 mL of stationary-phase cells were inoculated into 29 mL medium and cultures were incubated for 294 h before gas consumption assays were performed. Sporulation of *Thermogemmatispora* sp. T81 at this timepoint was verified by light microscopy of Gram-stained cultures. Gram staining and 16S rRNA gene amplicon sequencing also confirmed that both cultures were axenic.

2.3.2 Transcriptomics

Full transcriptome sequencing (RNA-Seq) was used to compare gene expression in *T. roseum* cultures under nutrient-rich (exponential phase; 10 mL, OD_{600} of 0.3) and nutrient-exhausted (stationary phase; 10 mL, OD_{600} of 0.75, 48 h post OD_{max}) conditions. Biological triplicate samples for each condition were harvested by centrifugation (21,000 × *g*, 15 min, 4 °C), the supernatants were removed, and the cell pellets were resuspended in 1 mL of RNAlater Stabilisation Solution (ThermoFisher Scientific) prior to freezing at −20 °C. Extraction and sequencing of RNA was performed by Macrogen Inc., Seoul, Korea. Briefly, RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), libraries were constructed using a TruSeq RNA v2 Sample Prep Kit (Illumina), and rRNA was removed using the Ribo-Zero rRNA Removal Kit (Illumina). The resultant complementary DNA was sequenced on an Illumina HiSeq4000 platform using a paired-end, 100 bp high-throughput protocol. Sequence analysis was performed using the automated cloud-based transcriptomics pipeline, AIR (Sequentia Biotech). Briefly, the steps performed were read trimming, read quality analysis using FastQC (Andrews, 2010), and read mapping against the *T. roseum* reference genome (NCBI ID: NC_011959.1 (Wu et al., 2009)) using an intrinsic platform read aligner with default parameters. Aligned reads were checked for quality prior to data normalisation using the

trimmed mean of M-values method (Robinson and Oshlack, 2010) and the 'normalizaData' command within the R package HTSFilter (Rau et al., 2013). A principle component analysis was then performed on the normalised data prior to statistical analysis using edgeR (Robinson et al., 2010) to obtain differential gene expression counts.

2.3.3 Gas chromatography

Gas chromatography measurements were performed to determine whether the two Chloroflexi strains could use atmospheric levels of CO and H_2. Briefly, sealed serum vials containing stationary-phase cultures of *T. roseum* (72 hr post OD_{max} / 120 h post-inoculation) and sporulating cultures of *Thermogemmatispora* sp. T81 (294 h post-inoculation) were opened, equilibrated with ambient air (1 h), and resealed. These vials were then amended with H_2 (via 1% v/v H_2 in N_2 gas cylinder, 99.999% pure) or CO (via 1% v/v CO in N_2 gas cylinder, 99.999% pure) to achieve headspace concentrations of ~14 ppmv for each gas. The first headspace samples were collected within minutes after closure in order to measure the initial gas concentrations. The vials were maintained at the growth temperature (60 °C) and agitated (150 rpm) for the entire incubation period (75 h) to enhance H_2, CO, and O_2 transfer to the cultures. Six to nine headspace samples (1 mL) were collected at different time intervals using a gas-tight syringe to measure H_2 and CO. Concomitantly, headspace gas concentrations in heat-killed negative controls (autoclaved; 30 mL) were measured to confirm that observed rates of gas consumption occurred due to a biotic process. Headspace gas concentrations were determined by gas chromatography using a pulsed discharge helium ionization detector (Wentworth et al., 1992, Novelli et al., 2009). This customized trace gas analyser (model TGA-6791-W-4U-2, Valco Instruments Company Inc.) is designed to analyse a suite of atmospheric gases across six orders of magnitude of concentrations. Briefly, the system is configured to use two valves as injectors/backflushers, and two valves to front flush or heart cut from the precolumns (Mole Sieve 5A, set at 140 °C). Gases are then separated on the main columns (5′

X 1/8" HayseSep Db, set at 55 °C). The fifth valve is used as a sample loop selector to accommodate a larger range of gas concentrations. Concentrations of H_2 and CO in each sample were regularly calibrated against ultra-pure H_2 and CO gas standards of known concentrations. With the standards used, the limit of detection was 42 ppbv H_2 and 9 ppbv CO.

2.3.4 Kinetic measurements

The whole-cell kinetic parameters of H_2 and CO oxidation in *T. roseum* were measured by comparing rates of gas consumption at different substrate concentrations. Briefly, the headspace of stationary-phase cultures (72 h post OD_{max}) were amended with 100, 1000, or 4000 ppmv H_2 or CO; whereas 100 ppmv mixing ratios were attained as described above, 100% H_2 and 100% CO cylinders (99.999% pure) were used to attain mixing ratios of 1000 and 4000 ppmv. Cultures were incubated at 60 °C at an agitation speed of 300 rpm in an Eppendorf 40 Incubator. Headspace gas samples were measured at various time intervals (0, 0.5, 1, 2, 3, 4, and 5 h after substrate addition) by gas chromatography as described above. Reaction velocity relative to the gas concentration was measured at each timepoint and plotted on a Michaelis–Menten graph. Curves of best fit, $V_{max\ app}$ values, and $K_{m\ app}$ values were initially calculated in GraphPad Prism (version 7.01) using non-linear regression models (enzyme kinetics – substrate vs. velocity, Michaelis–Menten, least squares fit). Michaelis–Menten parameters were also derived using linear regressions based on Lineweaver-Burk, Eadie-Hofstee, and Hanes-Woolf plots (Price, 2002).

2.3.5 Activity staining

Hydrogenase and carbon monoxide dehydrogenase activity was stained using whole-cell lysates of stationary-phase cultures of *T. roseum*. Five hundred mL of culture was harvested by centrifugation (10,000 × *g*, 10 min, 4 °C), washed in phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4), and resuspended in 16 mL lysis buffer (50 mM Tris-Cl, pH 8.0, 1 mM PMSF, 2 mM $MgCl_2$,

5 mg ml^{-1} lysozyme, 40 µg ml^{-1} DNase, 10% glycerol). The resultant suspension was then lysed by passage through a Constant Systems cell disruptor (40,000 psi, four times), with unbroken cells removed by centrifugation (10,000 × *g*, 20 min, 4 °C). Protein concentration was calculated using the bicinchoninic acid assay (Smith et al., 1985) against bovine serum albumin standards. Next, 20 µg protein was loaded onto a native 7.5% (w/v) Bis-Tris polyacrylamide gel prepared as described elsewhere (Walker, 2002) and run alongside a protein standard (NativeMark Unstained Protein Standard, Thermo Fisher Scientific) at 25 mA for 1.5 h. The gel was cut into three sections that were stained either for total protein, hydrogenase activity, or carbon monoxide dehydrogenase activity. For total protein staining, the gel section was incubated in AcquaStain Protein Gel Stain (Bulldog Bio) at room temperature for 3 h. For hydrogenase staining (Greening et al., 2014a), the gel section was incubated in 50 mM potassium phosphate buffer (pH 7.0) supplemented with 500 µM nitroblue tetrazolium chloride (NBT) in an anaerobic jar (5% H_2, 10% CO_2, 85% N_2 v/v) at 60 °C for 1 h. For carbon monoxide dehydrogenase staining (Lorite et al., 2000), the gel section was incubated in 50 mM Tris-HCl buffer (pH 7.5) containing 50 µM NBT and 100 µM phenazine methosulfate in an anaerobic jar (100% CO v/v atmosphere) maintained at 60 °C for 1 h.

2.3.6 Electrode measurements

For *T. roseum* cultures, rates of H_2 oxidation with and without treatment of respiratory chain uncouplers were measured amperometrically, following previously established protocols (Berney et al., 2014b, Carere et al., 2017). Prior to the start of measurement, a Unisense H_2 microsensor electrode was polarised at +800 mV for 1 h using a Unisense multimeter and calibrated with standards of known H_2 concentration. Gas-saturated PBS was prepared by bubbling the solution with 100% (v/v) of either H_2 or O_2 for 5 min. For untreated cells, 1.1 mL microrespiration assay chambers were sequentially amended with stationary-phase *T. roseum* cell suspensions (OD_{600} = 1; 0.9 mL), H_2-saturated PBS (0.1 mL), and O_2-saturated

PBS (0.1 mL) stirred at 250 rpm, 37 °C. Following measurements of untreated cells, the assay mixtures were treated with 100 μM carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), 10 μM nigericin, or 10 μM valinomycin. Changes in H_2 concentrations were recorded using Unisense Logger Software. Upon observing a linear change in H_2 concentration, initial rates of consumption were calculated over a period of 20 s and normalised against total protein concentration.

2.3.7 Phylogenetics analyses

Phylogenetic trees were constructed to investigate the evolutionary history and distribution of uptake hydrogenases and carbon monoxide dehydrogenases within the Chloroflexi phylum. Specifically, the catalytic subunits of [NiFe]-hydrogenases (HhyL and homologues) and type I carbon monoxide dehydrogenases (CoxL) were retrieved from Chloroflexi genomes and metagenome-assembled genomes (MAGs) in the NCBI RefSeq database via protein BLAST (Altschul et al., 1990) in October 2018. Using MEGA7 (Kumar et al., 2016), the amino acid sequences were aligned with reference sequences (Greening et al., 2016, Quiza et al., 2014) with ClustalW and evolutionary relationships were visualised by constructing a maximum-likelihood phylogenetic tree; specifically, initial trees for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. Gaps were treated with partial deletion and trees were bootstrapped with 100 replicates.

2.4 Results and Discussion

2.4.1 Thermomicrobium roseum *upregulates hydrogenase and carbon monoxide dehydrogenase expression during a coordinated response to nutrient starvation*

We compared the transcriptomes of triplicate *T. roseum* cultures under nutrient-rich (exponential phase) and nutrient-limited (stationary phase) conditions. A total of 401 genes were significantly upregulated and 539 genes were significantly downregulated by at least two-fold ($p < 10^{-6}$) in response to nutrient limitation **(Figure 2-1a; Table 7-1)**. Three major trends were observed with respect to energy acquisition and utilisation. Firstly, genes associated with energetically expensive processes were downregulated, including those encoding ribosomal proteins, cytochrome *c* and menaquinone biosynthesis enzymes, and the megaplasmid-encoded chemotactic and flagellar apparatus **(Table 7-1)**. Secondly, there was evidence of mobilisation of internal carbon stores, including an acetoin dehydrogenase complex and an electron transfer flavoprotein complex (ETF). Thirdly, the expression profiles indicate there is extensive remodelling of the respiratory chain. Two primary respiratory dehydrogenases involved in heterotrophic growth (type I and II NADH dehydrogenases) were downregulated, whereas complexes involved in lithotrophic energy generation and a succinate dehydrogenase were upregulated **(Figure 2-1a; Table 7-1)**. In both conditions, the terminal oxidases that mediate aerobic respiration were highly expressed and there was no evidence of the use of other electron acceptors; the cytochrome aa_3 oxidase was expressed in both phases and the alternative cytochrome bo_3 oxidase was upregulated during stationary phase. In contrast, the F_1F_o-ATPase (ATP synthase) was downregulated, a finding consistent with an expected decrease in the availability of respiratory electron donors during nutrient limitation (**Table 7-1)**.

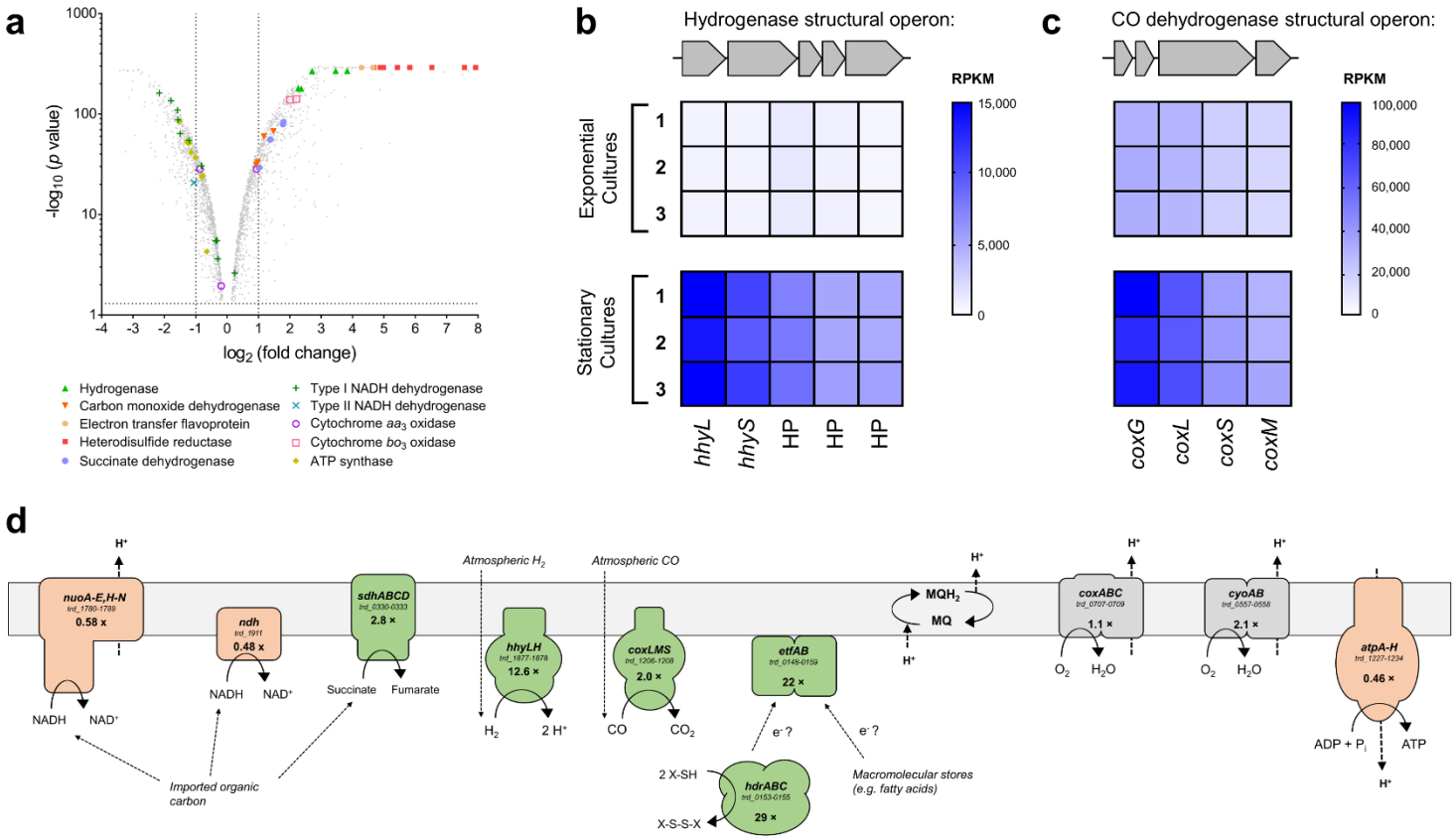

Figure 2-1: **Differential gene expression of nutrient-rich (exponential phase) and nutrient-limited (stationary phase) cultures of *Thermomicrobium roseum*. a)** Volcano plot showing relative expression change of genes following nutrient limitation. The fold-change shows the ratio of normalised transcript abundance of three stationary phase cultures divided by three exponential phase cultures (biological replicates). Each gene is represented by a grey dot and respiratory genes are highlighted as per the legend. **b, c** Heat maps of normalised abundance of the putative operons encoding the structural subunits of the group 1h [NiFe]-hydrogenase (hhyLS; **b**) and type I carbon monoxide dehydrogenase (coxLSM; **c**) The read counts per kilobase million (RPKM) are shown for three exponentially growing and three stationary phase biological replicates. HP = hypothetical protein. **d)** Differential regulation of the respiratory complexes mediating aerobic respiration of organic and inorganic compounds. Complexes are differentially shaded depending on whether they are significantly upregulated (green), downregulated (orange), or unchanged (grey) in nutrient-limited compared to nutrient-rich cultures. Gene names, loci numbers, and average fold changes in transcriptome abundance are shown for each complex. Shown are the structural subunits of type I NADH dehydrogenase (nuoA-E,H-N), type II NADH dehydrogenase (ndh), succinate dehydrogenase (sdhA-D), group 1h [NiFe]-hydrogenase (hhyLS), type I carbon monoxide dehydrogenase (coxLMS), heterodisulfide reductase (hdrABC), electron transfer flavoprotein (etfAB), sulfur-carrier protein (tusA), cytochrome aa_3 oxidase (coxABC), cytochrome bo_3 oxidase (cyoAB), and ATP synthase (atpA-H). Note that the physiological role of the highly upregulated hdrABC, etfAB, and tusA genes is yet to be experimentally validated in *T. roseum*

Thermomicrobium roseum upregulates genes associated with H_2 and CO metabolism under nutrient-limiting conditions. The genes encoding the structural subunits of a group 1h [NiFe]-hydrogenase (*hhyLS*; trd_1878–1877) (Constant et al., 2011b, Greening et al., 2016, Søndergaard et al., 2016), which are a class of oxygen-tolerant enzymes known to mediate atmospheric H_2 oxidation (Constant et al., 2010, Greening et al., 2014a, Greening et al., 2015a, Schäfer et al., 2013, Schäfer et al., 2016), were upregulated by an average of 12.6-fold **(Figure 2-1b)**. Also upregulated were the conserved hypothetical proteins *hhaABC* (trd_1876–1874; 5.5-fold) (Greening et al., 2015b), encoded on the same putative operon as the structural subunits, as well as a separate putative operon of maturation factors (trd_1873–1863; 3.1-fold) **(Figure 2-2; Table 7-1)**. The structural (trd_1206–1208) and maturation (trd_1209–1215) subunits encoding a type I carbon monoxide dehydrogenase were upregulated by an average of two-fold **(Figure 2-1c & Figure 2-2)** in response to nutrient limitation. Consistent with previous reports of CO utilisation during growth in this organism (Wu et al., 2009), carbon monoxide dehydrogenase genes were highly expressed in both exponential and stationary-phase cultures **(Figure 2-1c; Table 2-1).** This suggests that *T. roseum* uses CO to supplement available organic carbon during growth (mixotrophy) and persistence. These findings are broadly similar to observations made in other phyla, notably Actinobacteria and Proteobacteria, that hydrogenase and carbon monoxide dehydrogenase expression are induced by organic carbon limitation (Berney and Cook, 2010, Christie-Oleza et al., 2012, Constant et al., 2010, Greening et al., 2014a, Muthusamy et al., 2017, Patrauchan et al., 2012, Santiago et al., 1999).

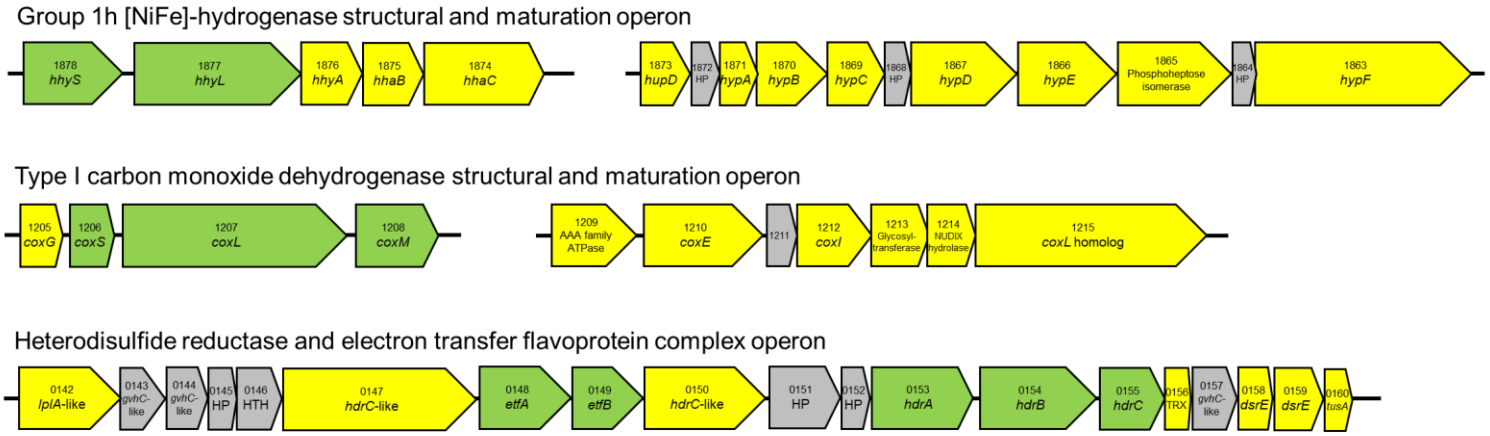

Figure 2-2 (Supplement): **Structure of the putative operons encoding the group 1h [NiFe]-hydrogenase, type I carbon monoxide dehydrogenase and heterodisulfide reductase/electron transfer flavoprotein complex in *Thermomicrobium roseum*.** Genes are differentially coloured depending on whether they encode structural subunits (green), accessory and maturation factors (yellow), or hypothetical proteins (grey).

Overall, the greatest differential in gene expression involved a 19-gene cluster (trd_0160–0142) putatively involved with the oxidation of sulfur compounds. The cluster contains gene encoding a putative soluble heterodisulfide reductase (*hdrABC*), an electron transfer flavoprotein complex (*etfAB*), three sulfur-carrier proteins (*tusA*, *dsrE1*, *dsrE2*), three lipoate-binding proteins (*lbpA*), and various hypothetical proteins, which are upregulated by an average of 45-fold during persistence. Most of these components have homologues in a system recently shown to mediate the oxidation of diverse organic and inorganic sulfur compounds in *Hyphomicrobium denitrificans* (Koch and Dahl, 2018, Cao et al., 2018). One role of this cluster may be to mediate the activation and oxidation of endogenous or exogenous thiol-containing compounds. To achieve this, we predict that the Hdr complex catalyses disulfide bond formation between the thiol compound and a sulfur-carrier protein (e.g., TusA); the Hdr complex then transfers the liberated electrons into the respiratory chain, possibly via the ETF complex. Supporting this notion, thiol oxidation to disulfide is exergonic with oxygen as the terminal electron acceptor. While Hdr complexes are best-characterized for their roles in heterodisulfide reduction in methanogenic archaea (Wagner et al., 2017), they have also been studied in sulfur-oxidizing and sulfate-reducing bacteria, where they have been predicted to be physiologically reversible (Ramos et al., 2014, Boughanemi et al., 2016). Consistently, the Hdr complex of *T. roseum* is most closely related to those of sulfur-oxidising *Sulfobacillus*, *Hyphomicrobium*, and *Acidithiobacillus* strains (Justice et al., 2014, Koch and Dahl, 2018, Quatrini et al., 2009). It seems plausible that *T. roseum* would benefit from a survival advantage if it can harness reduced sulfur compounds available in geothermal springs. However, further work is needed to verify the activity, substrates, and physiological role of this system.

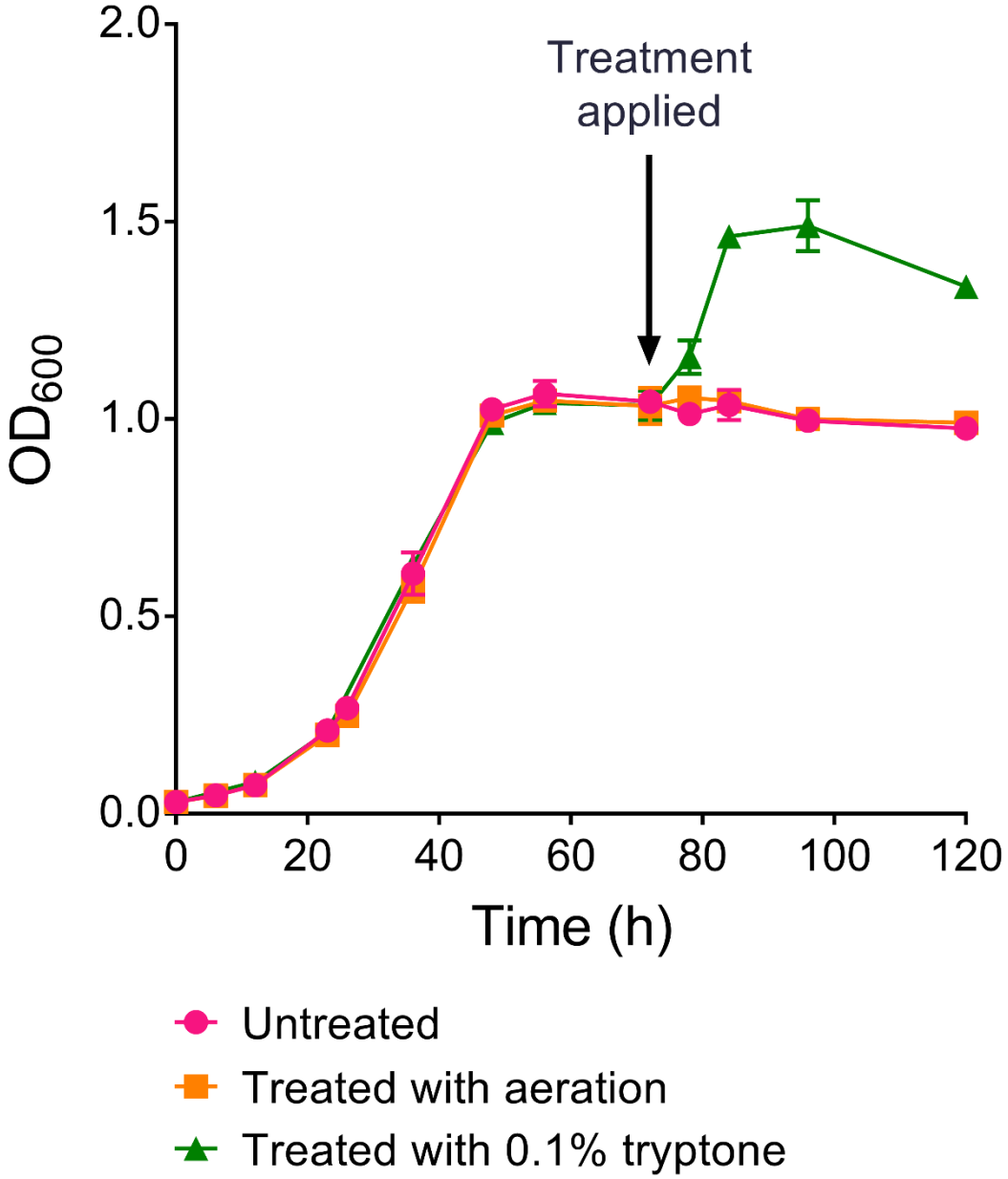

Figure 2-3 (Supplement): **Growth curve of *Thermomicrobium roseum* DSM 5159**. Cultures were grown in 120 mL serum vials containing 30 mL Castenholz media supplemented with 1 g L^{-1} yeast extract and 1 g L^{-1} tryptone. They were inoculated at a starting OD_{600} of 0.03 and incubated in an orbtial shaker at 150 rpm, 60°C. After cells entered stationary-phase, at 72 h the cultures were either supplemented with 1 g L^{-1} tryptone, reaerated for 1 h, or left untreated. This confirmed that cultures entered stationary-phase due to nutrient-limitation rather than oxygen-limitation.

Collectively, these findings show that *T. roseum* is more metabolically flexible than previously thought. **Figure 2-1d** illustrates the predicted remodelling of the respiratory chain that occurs during the transition from nutrient-rich to nutrient-limited conditions. The upregulation of enzymes involved in harnessing inorganic compounds, in conjunction with the downregulation of gene clusters involved in NADH oxidation, suggests that *T. roseum* has evolved mechanisms to maintain aerobic respiration despite nutrient fluctuations and deprivation within its environment.

2.4.2 T. roseum *aerobically oxidises H_2 and CO at a wide range of concentrations, including sub-atmospheric levels, during persistence*

The high expression levels for genes encoding the group 1h [NiFe]-hydrogenase and type I carbon monoxide dehydrogenase suggested that *T. roseum* may support persistence by oxidising atmospheric H_2 and CO. To test this, we incubated nutrient-limited cultures of *T. roseum* in an ambient air headspace supplemented with ~14 ppmv of either H_2 or CO and monitored their consumption using gas chromatography. In agreement with our hypothesis, cultures aerobically oxidised both gases in a first-order kinetic process; within 71 h, mixing ratios of these gases (103 ppbv H_2, 22 ppbv CO) were five times below atmospheric levels **(Figure 2-4a, b)**. This constitutes the first observation of both aerobic H_2 respiration and atmospheric H_2 oxidation within the phylum Chloroflexi.

Whole-cell kinetic measurements revealed that *T. roseum* efficiently oxidises H_2 and CO across a wide range of concentrations through hydrogenase and carbon monoxide dehydrogenase activity. In cultures, the enzymes display a moderate apparent velocity ($V_{max\ app}$ of 376 nmol H_2 and 149 nmol CO g^{-1} of protein min^{-1}) and moderate apparent affinity ($K_{m\ app}$ of 569 nM H_2 and 285 nM CO) for these substrates **(Figure 2-4c, d; Table 2-1)**. With respect to carbon monoxide dehydrogenase, these observations are consistent with the organism being able to utilise CO at elevated concentrations for growth (Wu et al., 2009) and

atmospheric concentrations for persistence. The apparent kinetic parameters of the group 1h [NiFe]-hydrogenase are more similar to those recently described for the verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* (K_m = 600 nM) (Mohammadi et al., 2017) than to the high-affinity, low-activity hydrogenases of previously described atmospheric H_2 scavengers (K_m < 50 nM) (Constant et al., 2010, Greening et al., 2014a, Greening et al., 2015a). Altogether, these findings suggest that *T. roseum* can take advantage of the elevated H_2 and CO concentrations when available through geothermal activity and subsist on atmospheric concentrations of these gases otherwise.

Consistent with the observed whole-cell activities, cell-lysates run on native polyacrylamide gels strongly stained for hydrogenase and carbon monoxide dehydrogenase activity **(Figure 2-4e)**. The molecular weight of the major bands were, respectively, at the expected molecular weight for a carbon monoxide dehydrogenase dimer [266 kDa, $(CoxLMS)_2$] and slightly below the expected molecular weight of a hydrogenase dimer [210 kDa, $(HhyLS)_2$]. This is compatible with biochemical studies in other organisms that have shown type I carbon monoxide dehydrogenases and group 1h [NiFe]-hydrogenases form homodimers (Dobbek et al., 1999, Schäfer et al., 2016, Schäfer et al., 2013). We next verified that the hydrogenase was coupled to the respiratory chain by measuring H_2 oxidation using a H_2 electrode under aerobic conditions. Untreated cells oxidised H_2 at a rapid rate. This activity decreased by 2.5-fold upon addition of the respiratory uncoupler CCCP and ceased upon addition of the ionophore valinomycin, whereas no significant change in H_2 oxidation rate was observable with the protonophore nigericin **(Figure 2-4f)**. The combination of these results suggests that the oxidation of hydrogen is tightly coupled to the respiratory chain and this interaction may be linked to the electrical gradient ($\Delta\psi$), but not pH gradient (ΔpH), of the membrane.

Findings from the transcriptome analysis and activity studies therefore suggest that *T. roseum* persists through oxidation of atmospheric H_2 and CO. We propose that the group 1h [NiFe]-hydrogenase and type I carbon monoxide dehydrogenase directly use electrons derived from atmospheric H_2 and CO to support aerobic respiration **(Figure 2-1d**). It is probable that these electrons are relayed via electron carriers into the menaquinone pool and are subsequently transferred to the terminal oxidases. However, further studies are needed to confirm how these proteins functionally and physically interact with the respiratory chain, including their localisation and which electron carriers they interact with.

Due to the genetic intractability of Chloroflexi and the lack of specific hydrogenase or carbon monoxide dehydrogenase inhibitors, we were also unable to determine the necessity of either H_2 or CO oxidation for prolonged survival for this organism. However, previous studies have demonstrated that genetic deletion of the group 1h [NiFe]-hydrogenase reduces longevity of *M. smegmatis* cells (Berney and Cook, 2010, Berney et al., 2014a, Greening et al., 2014b) and *Streptomyces avermitilis* exospores (Constant et al., 2010, Liot and Constant, 2016).

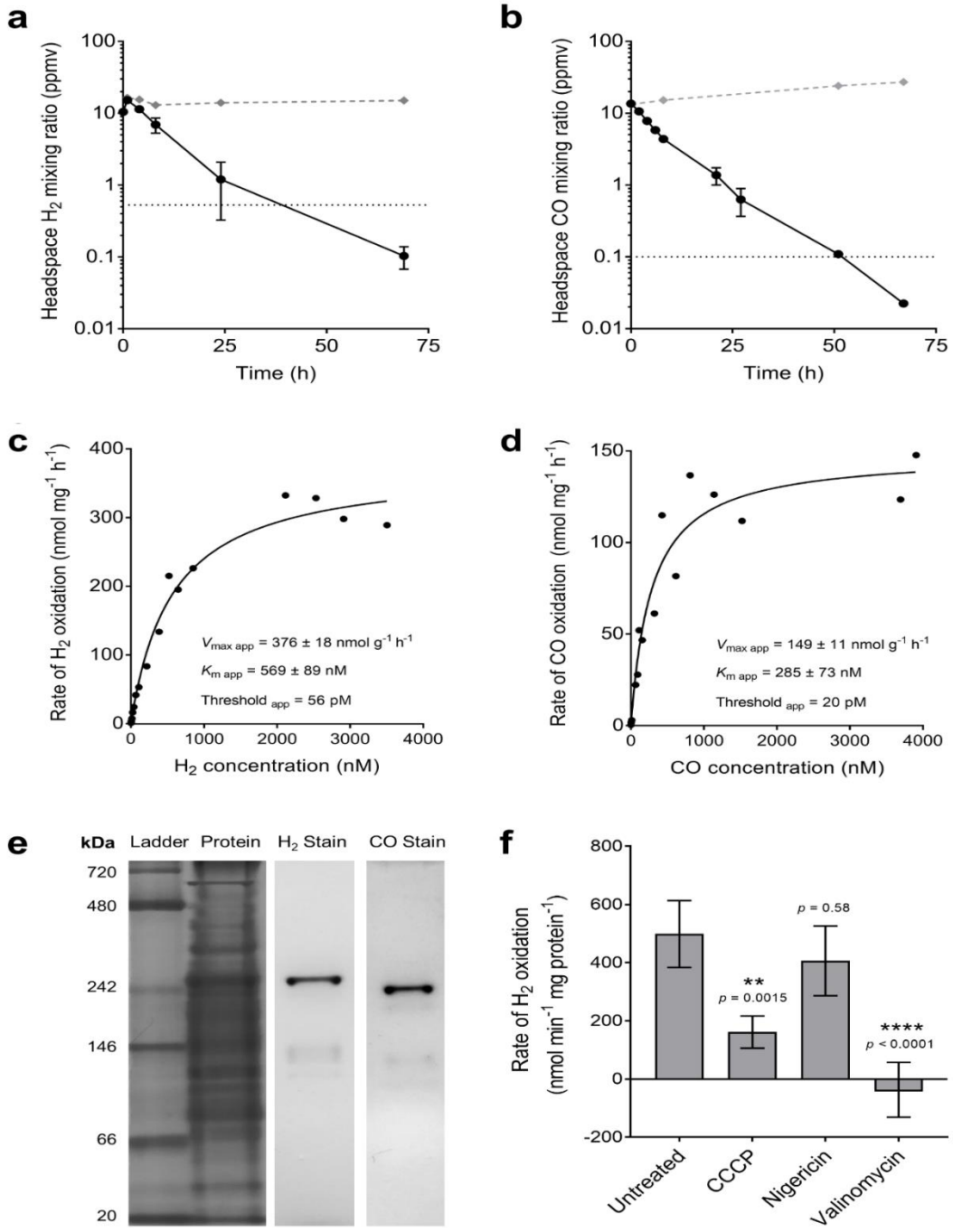

Figure 2-4: **Hydrogenase and carbon monoxide dehydrogenase activity of *Thermomicrobium roseum* cultures during nutrient limitation**. **a, b** Oxidation of molecular hydrogen (H_2; **a**) and carbon monoxide (CO; **b**) to sub-atmospheric levels by *T. roseum cultures*. Error bars show standard deviations of three biological replicates, with heat-killed cells monitored as a negative control (grey dashed lines). Mixing ratios of H_2 and CO are displayed on a logarithmic scale and dotted lines show the average atmospheric mixing ratios of H_2 (0.53 ppmv) and CO (0.10 ppmv). **c, d** Apparent kinetic parameters of H_2 (**c**) and CO (**d**) oxidation by *T. roseum* whole cells. Curves of best fit and kinetic parameters were calculated based on a Michaelis–Menten non-linear regression model. Values calculated based on Lineweaver-Burk, Hanes-Woolf, and Eadie-Hofstee plots are shown in **Table 2-1**. **e** Zymographic observation of hydrogenase and carbon monoxide dehydrogenase activity in *T. roseum* whole-cell lysates. The first two lanes show protein ladder and whole protein stained with Coomassie Blue. The third and fourth lanes show hydrogenase and carbon monoxide dehydrogenase activity stained with the artificial electron acceptor nitroblue tetrazolium in a H_2-rich and CO-rich atmosphere respectively. **f** Amperometric measurements of hydrogenase activity in *T. roseum* whole cells. The rate of H_2 oxidation was measured with a hydrogen electrode before and after treatment with the respiratory uncouplers and ionophores carbonyl cyanide m-chlorophenyl hydrazine (CCCP), nigericin, and valinomycin.

Table 2-1 (Supplement): **Comparison of four methods to determine apparent kinetic parameters for H_2 and CO oxidation for whole cells of *Thermomicrobium roseum*.**

Method	$V_{max\ app}$ **H_2**	$K_{m\ app}$ **H_2**	$V_{max\ app}$ **CO**	$K_{m\ app}$ **CO**
Nonlinear regression	376	569	149	285
Lineweaver-Burk plot	319	688	175	427
Hanes-Woolf plot	372	624	133	220
Eadie-Hofstee plot	377	639	137	244
Average	361	630	149	294

2.4.3 Scavenging of atmospheric gases is potentially a common persistence strategy within the aerobic heterotrophic Chloroflexi

Having demonstrated that *T. roseum* oxidises atmospheric trace gases during persistence, we subsequently investigated whether this is a common strategy employed by the Chloroflexi. We first analysed the respiratory capabilities of *Thermogemmatispora* sp. T81, a heterotrophic cellulolytic and sporulating thermophile, which we previously isolated from geothermal soils from Tikitere, New Zealand (Stott et al., 2008, Tomazini et al., 2018). Analysis of the organism's genome (Assembly ID: GCA_003268475.1) indicated that it encodes core respiratory chain components similar to *T. roseum*, including primary dehydrogenases (*nuo*, *ndh*, *sdh*), terminal oxidases (*cox*, *cyo*), and ATP synthase (*atp*). The genome also encodes putative operons for the structural subunits of a group 1h [NiFe]-hydrogenase, the maturation factors of this hydrogenase, and structural subunits of a type I carbon monoxide dehydrogenase **(Figure 2-5)**. However, homologues of the putative heterodisulfide reductase and ETF complexes encoded by *T. roseum* are absent from the *Thermogemmatispora* sp. T81 genome.

We verified that sporulating cultures of *Thermogemmatispora* sp. T81 actively consume H_2 and CO. The organism slowly oxidised available H_2 and CO in the headspace to sub-atmospheric levels (120 ppbv H_2, 70 ppbv CO) over ~320 h **(Figure 2-6a, b)**. Although this strain has previously been shown to oxidise carbon monoxide (King and King, 2014b), this is the first observation that it can do so to sub-atmospheric concentrations and during persistence. These results suggest that, despite their distinct evolutionary histories and ecological niches, *Thermogemmatispora* sp. T81 and *T. roseum* have both evolved similar metabolic strategies to survive nutrient limitation.

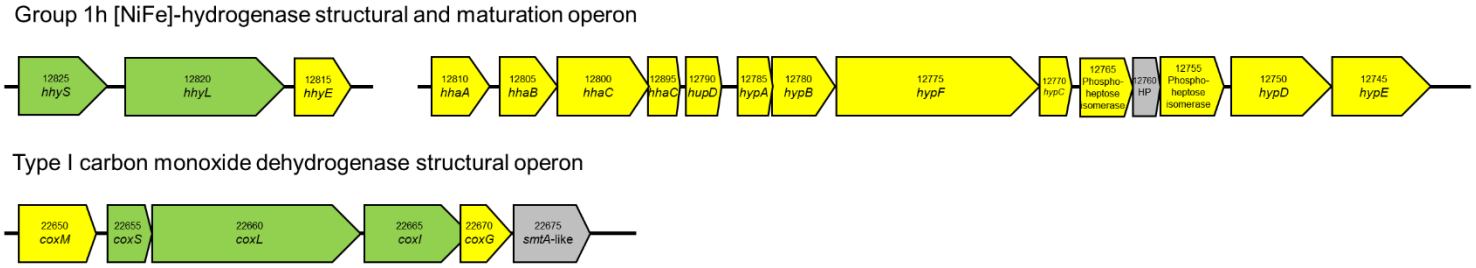

Figure 2-5 (Supplement): **Structure of the putative operons encoding the group 1h [NiFe]-hydrogenase and type I carbon monoxide dehydrogenase in *Thermogemmatispora sp.* T81**. Genes are differentially coloured depending on whether they encode structural subunits (green), accessory and maturation factors (yellow), or hypothetical proteins (grey).

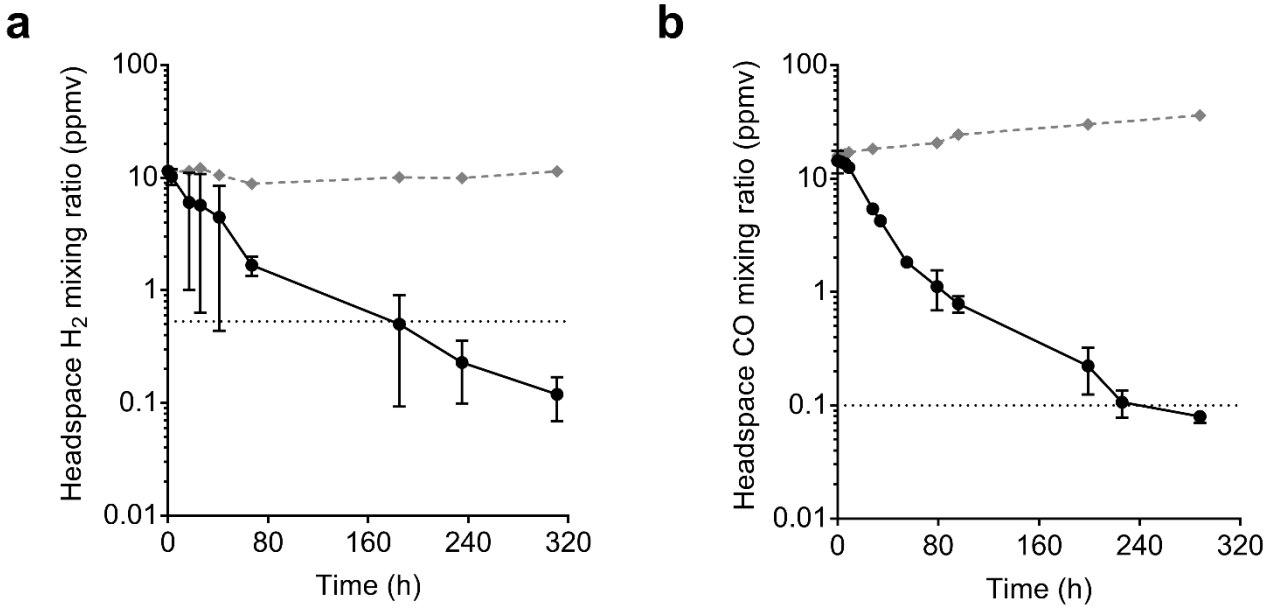

Figure 2-6: **Hydrogenase and carbon monoxide dehydrogenase activity of *Thermogemmatispora sp.* T81 during sporulation**. Oxidation of molecular hydrogen (H_2; **a**) and carbon monoxide (CO; **b**) to sub-atmospheric levels by *Thermogemmatispora sp*. T81 cultures. Error bars show standard deviations of three biological replicates, with heat-killed cells monitored as a negative control (grey dashed lines). Mixing ratios of H_2 and CO are displayed on a logarithmic scale and dotted lines show the average atmospheric mixing ratios of H_2 (0.53 ppmv) and CO (0.10 ppmv).

Analysis of the distribution of hydrogenases and carbon monoxide dehydrogenases within publicly available reference genomes showed that genetic capacity for trace gas scavenging is a common trait among aerobic Chloroflexi. Specifically, group 1h [NiFe]-hydrogenases and type I carbon monoxide dehydrogenases were encoded in three of the four reference genomes within the Thermomicrobiales (class Chloroflexia) and four of the five reference genomes within the Ktedonobacteriales (class Ktedonobacteria) **(Figure 2-7a, b)**. The latter includes the genomes of the heterotrophic soil bacterium *Ktedonobacter racemifer* (Cavaletti et al., 2006) and the nitrite-oxidising bioreactor isolate *Nitrolancea hollandica* (Sokolova et al., 2004). In addition, seven strains within the photosynthetic order Chloroflexales encoded group 1f and/or group 2a [NiFe]-hydrogenases **(Figure 2-8)**. These hydrogenase classes have been shown to mediate aerobic H_2 oxidation in a range of bacteria, including to sub-atmospheric concentrations in *Acidobacterium ailaaui* and *M. smegmatis* respectively (Greening et al., 2014a, Myers and King, 2016). Moreover, a metatranscriptome study revealed that homologs of the group 1f [NiFe]-hydrogenase of *Roseiflexus* species are highly expressed in geothermal microbial mats at night (Klatt et al., 2007). Hence, it is likely that the traits of aerobic H_2 respiration and possibly atmospheric H_2 oxidation extends to the photosynthetic strains of this phylum. A range of metagenome-assembled genomes, including from the abundant candidate class Ellin6529 (Delgado-Baquerizo et al., 2018, Ji et al., 2017), also encoded genes for aerobic H_2 and CO oxidation **(Figure 2-8 & Figure 2-9)**. Consistent with previous reports, Dehalococcoidia encode group 1a [NiFe]-hydrogenases known to facilitate dehalorespiration (Hartwig et al., 2017, Jayachandran et al., 2004, Nijenhuis and Zinder, 2005).

Our analyses suggest that the capacity for atmospheric H_2 and CO oxidation may have evolved on two or more occasions within the Chloroflexi. Phylogenetic trees show that the group 1h [NiFe]-hydrogenases from Chloroflexia and Ktedonobacteria are divergent and fall

into two distinct, robustly supported branches **(Figure 2-7a)**. It is therefore more likely that Chloroflexia and Ktedonobacteria independently acquired these enzymes, for example as a result of horizontal gene transfer events from other Terrabacteria, rather than vertically inheriting them from a common ancestor. Phylogenetic analysis also suggests that the type I carbon monoxide dehydrogenase may have also been acquired on two or three occasions in this phylum **(Figure 2-7b)**. In line with their probable independent acquisition, the putative operons encoding the hydrogenase and carbon monoxide dehydrogenase in *T. roseum* **(Figure 2-2**) and *Thermogemmatispora* sp. T81 **(Figure 2-5)** are distinctly organized. For example, the structural and accessory factors of carbon monoxide dehydrogenase are encoded in a single putative operon in *Thermogemmatispora* sp. T81 (*coxMSLIG*), but are separated into a structural operon (*coxGSLM*) and accessory operon (including *coxG* and *coxE*) in *T. roseum*. These findings agree with previous inferences of horizontal dissemination of *hhyL* and *coxL* genes (Greening et al., 2016, Greening et al., 2015b, Quiza et al., 2014) and suggest there is strong selective pressure for the acquisition of metabolic enzymes that support persistence. However, other explanations for their observations cannot be ruled out and further analysis is required to unravel the complex evolutionary histories of hydrogenases and carbon monoxide dehydrogenases.

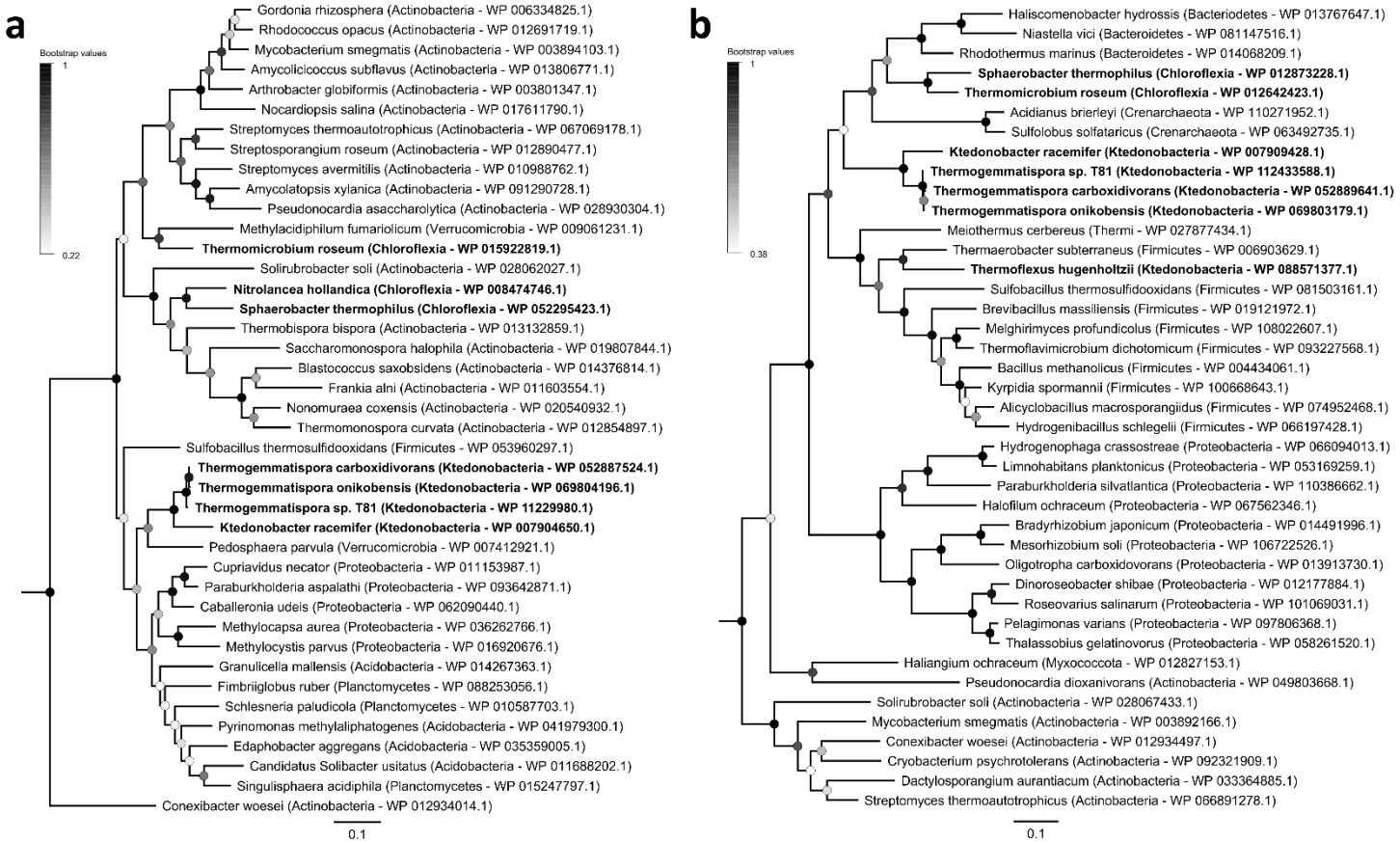

Figure 2-7: **Evolutionary history of the group 1h [NiFe]-hydrogenase and type I carbon monoxide dehydrogenase**. Phylogenetic trees showing the distribution and evolutionary history of the catalytic (large) subunits of the group 1h [NiFe]-hydrogenase (hhyL; **a**) and type I carbon monoxide dehydrogenase (coxL; **b**) in the phylum Chloroflexi. Chloroflexi sequences (labelled by class) are shown in bold against reference sequences (labelled by phylum). Trees were constructed using amino acid sequences through the maximum-likelihood method (gaps treated with partial deletion) and were bootstrapped with 100 replicates. The trees were respectively rooted with group 1g [NiFe]-hydrogenase sequences (WP_011761956.1, WP_048100713.1) and type II carbon monoxide dehydrogenase sequences (WP_011388721.1, WP_012893108.1). The distribution of other respiratory uptake hydrogenases within genomes and metagenome-assembled genomes (MAGs) in the phylum Chloroflexi is shown in **Figure 2-8**. The distribution of type I carbon monoxide dehydrogenases within metagenome-assembled genomes (MAGs) in the phylum Chloroflexi is shown in **Figure 2-9.**

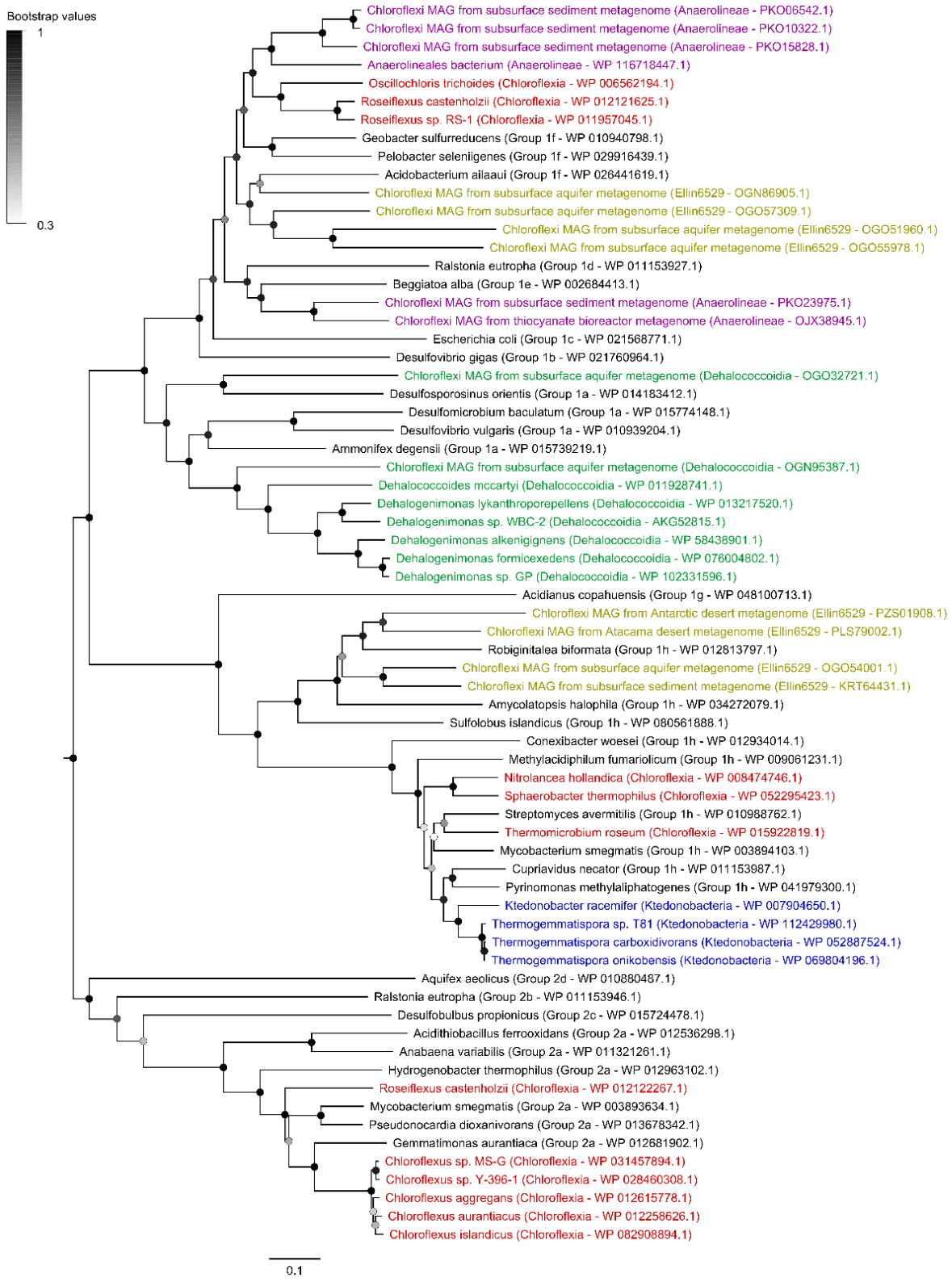

Figure 2-8 (Supplement): **Phylogenetic tree showing the distribution of uptake respiratory hydrogenases in Chloroflexi genomes and metagenome-assembled genomes (MAGs)**. The genomes encode catalytic subunits of group 1a, group 1e, group 1f, group 1h, and group 2a [NiFe]-hydrogenases. Sequences are coloured by class, where blue = Ktenodobacteria, red = Chloroflexia, green = Dehalococcoidia, purple = Anaerolineae, and yellow = candidate class Ellin6529, with reference sequences in black. The tree was constructed using amino acid sequences of the hydrogenase large subunit using the neighbour-joining method and was bootstrapped with 100 replicates.

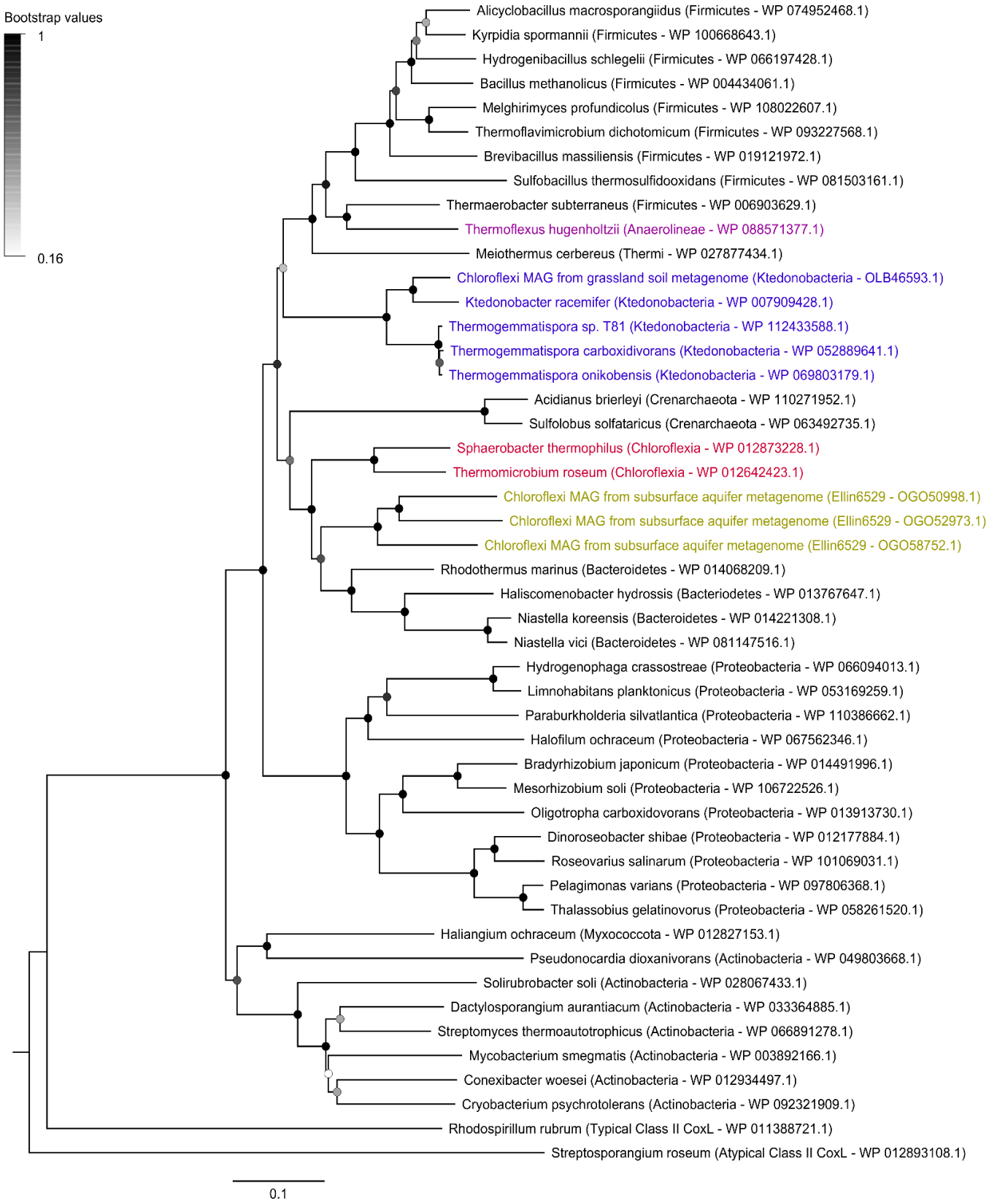

Figure 2-9 (Supplement): **Phylogenetic tree showing the distribution of type I carbon monoxide dehydrogenases in Chloroflexi genomes and metagenome-assembled genomes (MAGs)**. Sequences are coloured by class, where blue = Ktenodobacteria, red = Chloroflexia, purple = Anaerolineae, and yellow = candidate class Ellin6529, with reference sequences in black. The tree was constructed using amino acid sequences of the carbon monoxide dehydrogenase large subunit using the neighbour-joining method and was bootstrapped with 100 replicates.

2.4.4 Ecological and biogeochemical significance of metabolic flexibility and trace gas oxidation in Chloroflexi

Aerobic heterotrophic bacteria from the phylum Chloroflexi are more metabolically versatile than previously thought. The transcriptome analyses clearly show that *T. roseum* regulates its metabolism in response to nutrient limitation, enabling persistence on a combination of exogenous inorganic compounds and likely endogenous carbon reserves. In support of this, gas chromatography measurements showed that the bacterium efficiently oxidises H_2 and CO down to sub-atmospheric concentrations during persistence through an aerobic respiratory process. We made similar findings for the ktedonobacterial isolate *Thermogemmatispora* sp. T81, suggesting that trace gas scavenging might be a common persistence strategy employed by aerobic Chloroflexi. Analyses of primary sequence phylogeny and operon structure indicate that the group 1h [NiFe]-hydrogenases and carbon monoxide dehydrogenases within these organisms fall into different clades and are relatively divergent. Hence, it is probable that these organisms have horizontally acquired the capacity to oxidise atmospheric H_2 and CO via separate events, though other explanations are possible. The apparent convergence in persistence strategies is notable given the distinct evolutionary histories, persistence morphologies (i.e., sporulation in T81), and ecological niches of these bacteria. Resource generalism is therefore likely to be a common ecological strategy for the survival of Chloroflexi in environments where organic carbon and other nutrients may be periodically scarce.

More broadly, these findings provides pure culture support for the hypothesis that atmospheric carbon monoxide serves as an energy source for persistence (Ji et al., 2017). Our findings suggest that the expression and activity of carbon monoxide dehydrogenase is linked to persistence, and provide evidence that atmospheric CO may serve as an electron donor for the aerobic respiratory chain in this condition. Indeed, as with atmospheric H_2, atmospheric

CO is likely to be a dependable energy source for microbial survival given its ubiquity, diffusibility, and energy density. Integrating these findings with the wider literature, it is probable that atmospheric CO oxidation is a general strategy supporting long-term survival of aerobic heterotrophic bacteria. Indeed, various heterotrophic bacteria have previously been inferred to be capable of oxidising atmospheric CO, including Proteobacteria (King, 2003b, King, 2015, Weber and King, 2012, Weber and King, 2017), Actinobacteria (Gadkari et al., 1990, King, 2003c), and a *Thermogemmatispora* strain (King and King, 2014a). Moreover, other datasets have shown that carbon monoxide dehydrogenase expression is activated during nutrient limitation in other aerobic organisms (Berney and Cook, 2010, Christie-Oleza et al., 2012, Muthusamy et al., 2017, Patrauchan et al., 2012, Santiago et al., 1999). However, in contrast to atmospheric H_2 (Greening et al., 2014b, Liot and Constant, 2016), it remains to be validated through genetic and biochemical studies that atmospheric CO oxidation can enhance survival of bacteria during persistence. In line with previous activity-based measurements (Wu et al., 2009), the transcriptome analysis shows that *T. roseum* expresses carbon monoxide dehydrogenase at high levels during growth. Unlike carboxydotrophs such as *Oligotropha carboxidovorans* (Dobbek et al., 1999, Santiago et al., 1999, Meyer and Schlegel, 1978), *T. roseum* as a carboxydovore cannot grow chemolithoautotrophically (King and King, 2014b, Wu et al., 2009) and instead appears to use CO as an additional energy source during heterotrophic growth. The wide kinetic range of the *T. roseum* carbon monoxide dehydrogenase in whole cells likely enables this isolate to both persist on ubiquitously available atmospheric CO (Khalil and Rasmussen, 1990) and grow mixotrophically in microenvironments where CO is available at elevated concentrations (up to 6000 ppmv) through geothermal activity (Shock et al., 2010).

Finally, this study establishes Chloroflexi as the third phylum experimentally shown to scavenge atmospheric H_2, following the Actinobacteria (Constant et al., 2011b, Constant et al.,

2010, Constant et al., 2008, Greening et al., 2014a, Meredith et al., 2014) and Acidobacteria (Greening et al., 2015a, Myers and King, 2016). The findings made here are similar to those previously reported for the actinobacterium *Mycobacterium smegmatis* (Berney and Cook, 2010, Greening et al., 2014a) and acidobacterium *Pyrinomonas methylaliphatogenes* (Greening et al., 2015a), both of which also shift from heterotrophic respiration to atmospheric H_2 oxidation in response to energy limitation, including through expressing group 1h [NiFe]-hydrogenases. Given at least four other cultured phyla **(Figure 2-7a)** and two candidate phyla (Ji et al., 2017) also encode group 1h [NiFe]-hydrogenases, it seems increasingly likely that atmospheric H_2 serves as a general energy source for aerobic heterotrophic bacteria. This observation is also potentially biogeochemically significant, given aerobic soil bacteria are known to be the main sink in the global hydrogen cycle (Ehhalt and Rohrer, 2009). Further work, however, is needed to test these whether these principles extend to the still enigmatic Chloroflexi species inhabiting mesophilic soil environments.

3 A WIDELY DISTRIBUTED HYDROGENASE OXIDISES ATMOSPHERIC H_2 DURING BACTERIAL GROWTH

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3.1 Abstract

Diverse aerobic bacteria persist by consuming atmospheric hydrogen (H_2) using group 1h [NiFe]-hydrogenases. However, other hydrogenase classes are also distributed in aerobes, including the group 2a [NiFe]-hydrogenase. Based on studies focused on Cyanobacteria, the reported physiological role of the group 2a [NiFe]-hydrogenase is to recycle H_2 produced by nitrogenase. However, given this hydrogenase is also present in various heterotrophs and lithoautotrophs lacking nitrogenases, it may play a wider role in bacterial metabolism. Here we investigated the role of this enzyme in three species from different phylogenetic lineages and ecological niches: *Acidithiobacillus ferrooxidans* (phylum Proteobacteria), *Chloroflexus aggregans* (phylum Chloroflexota), and *Gemmatimonas aurantiaca* (phylum Gemmatimonadota). qRT-PCR analysis revealed that the group 2a [NiFe]-hydrogenase of all three species is significantly upregulated during exponential growth compared to stationary phase, in contrast to the profile of the persistence-linked group 1h [NiFe]-hydrogenase. Whole-cell biochemical assays confirmed that all three strains aerobically respire H_2 to sub-atmospheric levels, and oxidation rates were much higher during growth. Moreover, the oxidation of H_2 supported mixotrophic growth of the carbon-fixing strains *C. aggregans* and *A. ferrooxidans.* Finally, we used phylogenomic analyses to show that this hydrogenase is widely distributed and is encoded by 13 bacterial phyla. These findings challenge the current persistence-centric model of the physiological role of atmospheric H_2 oxidation and extend this process to two more phyla, Proteobacteria and Gemmatimonadota. In turn, these findings have broader relevance for understanding how bacteria conserve energy in different environments and control the biogeochemical cycling of atmospheric trace gases.

3.2 Introduction

Aerobic bacteria mediate the biogeochemically and ecologically important process of atmospheric hydrogen (H_2) oxidation (Greening et al., 2015b). Terrestrial bacteria constitute the largest sink of this gas and mediate the net consumption of approximately 70 million tonnes of atmospheric H_2 per year (Constant et al., 2009, Ehhalt and Rohrer, 2009). The energy derived from this process appears to be critical for sustaining the productivity and biodiversity of ecosystems with low organic carbon inputs (Greening et al., 2016, Ji et al., 2017, Kanno et al., 2016, Kessler et al., 2019, Khdhiri et al., 2015, Lynch et al., 2014). Atmospheric H_2 oxidation is thought to be primarily mediated by group 1h [NiFe]-hydrogenases, a specialised oxygen-tolerant, high-affinity class of hydrogenases (Constant et al., 2011b, Constant et al., 2010, Greening et al., 2014a, Greening et al., 2016, Schäfer et al., 2013). To date, aerobic heterotrophic bacteria from four distinct bacterial phyla, the Actinobacteriota (formerly Actinobacteria; (Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Meredith et al., 2014)), Acidobacteriota (formerly Acidobacteria; (Greening et al., 2015a, Myers and King, 2016)), Chloroflexota (formerly Chloroflexi; see **Chapter 2**), and Verrucomicrobiota (formerly Verrucomicrobia; (Schmitz et al., 2020)), have been experimentally shown to consume atmospheric H_2 using this enzyme (Chaumeil et al., 2019). This process has been primarily linked to energy conservation during persistence. Reflecting this, the expression and activity of the group 1h hydrogenase is induced by carbon starvation across a wide range of species (see **Chapter 2** and (Berney and Cook, 2010, Berney et al., 2014a, Constant et al., 2010, Cordero et al., 2019b, Greening et al., 2014a, Greening et al., 2015a)). Moreover, genetic deletion of hydrogenase structural genes results in impaired long-term survival of *Mycobacterium smegmatis* cells and *Streptomyces avermitilis* spores (Berney and Cook, 2010, Berney et al., 2014a, Greening et al., 2014b, Liot and Constant, 2016).

Genomic and metagenomic surveys have suggested that other uptake hydrogenases are widely distributed among aerobic bacteria and potentially have a role in atmospheric H_2 uptake (Greening et al., 2016, Søndergaard et al., 2016). These include the widely distributed group 2a [NiFe]-hydrogenases. This hydrogenase class has primarily been investigated in Cyanobacteria, where it is encoded by most diazotrophic strains; the enzyme recycles H_2 released as a by-product of the nitrogenase reaction and inputs the derived electrons into the respiratory chain (Bothe et al., 2010, Houchins, 1984, Papen et al., 1986, Tamagnini et al., 2002). However, according to HydDB, group 2a hydrogenases are also encoded by isolates from at least eight other phyla (Søndergaard et al., 2016), spanning both obligate organoheterotrophs (e.g., *Mycobacterium*, *Runella*, *Gemmatimonas*) and obligate lithoautotrophs (e.g., *Acidithiobacillus*, *Nitrospira*, *Hydrogenobacter*) (Drobner et al., 1990, Greening et al., 2014a, Koch et al., 2014). In *M. smegmatis*, this enzyme has a sufficiently high apparent affinity to oxidise H_2 even at sub-atmospheric levels (Cordero et al., 2019b, Greening et al., 2014a) and is maximally expressed during transitions between growth and persistence (Berney et al., 2014b, Cordero et al., 2019b). In common with the group 1h hydrogenase also encoded by this bacterium, the group 2a hydrogenase requires potential electron-relaying iron-sulfur proteins for activity (see **Chapter 4**) and is obligately linked to the aerobic respiratory chain (Cordero et al., 2019b). However, it remains unclear if atmospheric H_2 oxidation by the group 2a hydrogenase reflects a general feature of the enzyme or instead is a specific adaptation of the mycobacterial respiratory chain.

In this study, we investigated whether group 2a [NiFe]-hydrogenases play a general role in atmospheric H_2 consumption. To do so, we studied this enzyme in three species, *Gemmatimonas aurantiaca*, *Acidithiobacillus ferrooxidans*, and *Chloroflexus aggregans*, that differ in their phylogenetic affiliation, ecological niches, and metabolic strategies. The obligate chemoorganoheterotroph *G. aurantiaca* (phylum Gemmatimonadota;

formerly Gemmatimonadetes) was originally isolated from a wastewater treatment plant and to date has not been shown to utilise H_2 (Zhang et al., 2003). The obligate chemolithoautotroph *A. ferrooxidans* (phylum Proteobacteria) was originally isolated from acidic coal mine effluent, and has been extensively studied for its energetic flexibility, including the ability to grow exclusively on H_2 (Drobner et al., 1990, Razzell and Trussell, 1963, Valdés et al., 2008). The metabolically flexible *C. aggregans* (phylum Chloroflexota), a facultative chemolithoautotroph and anoxygenic photoheterotroph, was originally isolated from a Japanese hot spring and is capable of hydrogenotrophic growth (Hanada et al., 1995, Kawai et al., 2019, Otaki et al., 2012). The organisms differ in their carbon dioxide fixation pathways, with *A. ferrooxidans* mediating the Calvin-Benson cycle *via* two RuBisCO enzymes, *C. aggregans* encoding the 3-hydroxypropionate cycle (Heinhorst et al., 2002, Klatt et al., 2007, Valdés et al., 2008), and *G. aurantiaca* unable to fix carbon dioxide (Zhang et al., 2003). While all three species have previously been shown to encode group 2a [NiFe]-hydrogenases (Greening et al., 2016, Valdés et al., 2008), it is unknown whether they can oxidise atmospheric H_2. To resolve this, we investigated the expression, activity, and role of this enzyme in axenic cultures of the three species.

3.3 Materials and Methods

3.3.1 Bacterial growth conditions

Gemmatimonas aurantiaca (DSM 14586), *Acidithiobacillus ferrooxidans* (DSM 14882), and *Chloroflexus aggregans* (DSM 9486) were imported from DSMZ. All cultures were maintained in 120 mL glass serum vials containing a headspace of ambient air (H_2 mixing ratio ~0.5 ppmv) sealed with lab-grade butyl rubber stoppers. Prior to use, stoppers were treated to prevent H_2 release by boiling twice in 0.1 M sodium hydroxide for 2 h, and twice in deionised water for 2 h, prior to baking in a 70°C overnight. Broth cultures of *G. aurantiaca* were grown in 30 mL of NM1 media as previously described (Zhang et al., 2003) and incubated at 30°C at

an agitation speed of 180 rpm in a New Brunswick Scientific Excella E24 incubator. Cultures of *C. aggregans* were maintained chemoheterotrophically in 30 mL of 1/5 PE media, as previously described (Hanada et al., 1995), and incubated at 55°C at an agitation speed of 150 rpm in an Eppendorf 40 Incubator in the dark. Cultures of *A. ferrooxidans* were maintained in 30 mL DSMZ medium 882 supplemented with an additional 13 g L^{-1} of $FeSO_4$.7H2O (pH 1.2) and incubated at 30°C at an agitation speed of 180 rpm in a New Brunswick Scientific Excella E24 incubator. To assess whether bacterial growth was enhanced by the presence of H_2 for each species, ambient air headspaces were amended with either 1% or 10% H_2 (via 99.999% pure H_2 gas cylinder). Growth was monitored by determining the optical density (OD_{600}) of periodically sampled 1 mL extracts using an Eppendorf BioSpectrophotometer.

3.3.2 RNA extraction

Triplicate 30 mL cultures of *G. aurantiaca*, *A. ferrooxidans* and *C. aggregans* were grown synchronously in 120 mL sealed serum vials. Whereas one set of triplicate cultures were grown in an ambient air headspace, another set was grown in an ambient air headspace supplemented with H_2 to a final concentration of 10% v/v (*via* a 99.999% pure H_2 cylinder). Cultures were grown to either exponential phase (OD_{600} 0.05 for *G. aurantiaca*; OD_{600} 0.1 for *C. aggregans*; OD_{600} 0.05 for *A. ferrooxidans*) or stationary phase (Day 10 for *G. aurantiaca*; Day 4 for *C. aggregans*; Day 14 for *A. ferrooxidans*). For *G. aurantiaca and C. aggregans,* cells were then quenched using a glycerol-saline solution (−20 °C, 3:2 v/v), harvested by centrifugation (20,000 × *g*, 30 min, −9 °C), resuspended in 1 mL cold 1:1 glycerol:saline solution (−20 °C), and further centrifuged (20,000 × *g*, 30 min, −9 °C). Briefly, resultant cell pellets were resuspended in 1 mL TRIzol Reagent (Thermo Fisher Scientific), mixed with 0.1 mm zircon beads (0.3 g), and subject to beat-beating (five cycles, 4000 rpm, 30 s) in a Mini-Beadbeater 96 (Biospec) prior to centrifugation (12,000 × *g*, 10 min, 4 °C). Total RNA was extracted using the phenol-chloroform method as per manufacturer's

instructions (TRIzol Reagent User Guide, Thermo Fisher Scientific) and resuspended in diethylpyrocarbonate-treated water. RNA was treated using the TURBO DNA-free kit (Thermo Fisher Scientific) as per manufacturer's instructions. RNA from *A. ferrooxidans* was extracted using a previously described extraction method optimised for acid mine drainage microorganisms (Zammit et al., 2011). RNA concentration and purity were confirmed using a NanoDrop ND-1000 spectrophotometer.

3.3.3 Quantitative RT-PCR

Quantitative reverse transcription PCR (qRT-PCR) was used to determine the expression profile of all hydrogenase genes present in each species during different growth phases with and without supplemental H_2. cDNA was synthesised using a SuperScript III First-Strand Synthesis System kit for qRT-PCR (Thermo Fisher Scientific) with random hexamer primers, as per manufacturer's instructions. For all three species, the catalytic subunit gene of the group 2a [NiFe]-hydrogenase (*hucL*) was targeted. In addition, transcript levels of the catalytic subunit of all additional [NiFe]-hydrogenases present in these strains were analysed, i.e., group 3d (*hoxH*) for *C. aggregans* and both group 1e (*hyiB*) and group 3b (*hyhL*) for *A. ferrooxidans*. Quantitative RT-PCR was performed using a LightCycler 480 SYBR Green I Master Mix (Roche) as per manufacturer's instructions in 96-well plates and conducted in a LightCycler 480 Instrument II (Roche). Primers used in the study (**Table 3-1**) were designed using Primer3 (Untergasser et al., 2012). Copy numbers of each gene were interpolated from standard curves of each gene created from threshold cycle (C_T) values of amplicons that were serially diluted from 10^8 to 10 copies ($R^2 > 0.95$). Hydrogenase expression data were then normalised to housekeeping genes in exponential phase under ambient air conditions for each species (16S rRNA gene for *G. aurantiaca* and *C. aggregans*; DNA-directed RNA polymerase subunit beta gene *rpoC* for *A. ferrooxidans*). All biological triplicate samples, standards, and negative controls were run in technical duplicate.

Table 3-1 (Supplement): **Oligonucleotide primers used in this study.**

Primer	**Sequence (5' to 3')**	**Tm (°C)**
G. aurantiaca 16S_fwd (515F)	GTG YCA GCM GCC GCG GTA A	54
G. aurantiaca 16S_rvs (806rB)	GGA CTA CNV GGG TWT CTA AT	54
G. aurantiaca HucL_fwd	TGC ATG GAC CGA AGC AAG	62
G. aurantiaca HucL_rvs	AAT GAG CGT GGC GTT GTG	62
C. aggregans 16S_fwd	CGA AAG AAC CTT ACC CGG GC	61
C. aggregans 16S_rvs	CGA TCT GCA CTG AGA CCA CG	61
C. aggregans HucL_fwd	CAT CGA GGG GAG AAA TGC GG	61
C. aggregans HucL_rvs	GGA AGA GCG GGT CGT AGT CT	61
C. aggregans HoxH_fwd	CGA TCC GAT TGA CTA CCG CG	61
C. aggregans HoxH_rvs	GGC GCG AAG GTT AGG TGA AA	61
A. ferrooxidans rpoC_fwd	GGT GCA GCA GGA TTC GTT CA	61
A. ferrooxidans rpoC_rvs	ACG AGG GAG GTC ATG GGA AG	61
A. ferrooxidans HucL_fwd	GGT TGG GCA AGT ACG TCT CG	61
A. ferrooxidans HucL_rvs	GCG ATC AGT TGC CGG GAT AG	61
A. ferrooxidans HyiB_fwd	GGA GAG CAA GAT CAT CGC CG	61
A. ferrooxidans HyiB_rvs	GGG AGT TCG GTG CCT TTG AG	61
A. ferrooxidans HyhL_fwd	CCA AAG CCG TGA GAT CAG CC	61
A. ferrooxidans HyhL_rvs	TGT ATC CAC TCG CCG CAG TA	61

3.3.4 Gas chromatography

Gas chromatography measurements were used to determine the capacity of the three species to use sub-atmospheric concentrations of H_2. For initial experiments, H_2 consumption by triplicate cultures in vials containing an ambient air headspace was monitored during growth; H_2 mixing ratios were measured immediately following inoculation (mixing ratio = 440 ± 34 ppbv), then at mid-exponential and late stationary phase. In subsequent experiments, to determine H_2 oxidation rate constants, biological triplicate cultures of each species were opened, equilibrated with ambient air (1 h), and resealed. These re-aerated vials were then amended with H_2 (*via* 1% v/v H_2 in N_2 gas cylinder, 99.999% pure) to achieve final headspace concentrations of ~10 ppmv. Headspace mixing ratios were measured immediately after closure and at regular intervals thereafter for 200 h or until the limit of quantification of the gas chromatograph was reached (42 ppbv H_2). This analysis was performed for both exponential phase and stationary phase cultures. For H_2 quantification, 2 mL headspace samples were measured using a pulsed discharge helium ionisation detector (model TGA-6791-W-4U-2, Valco Instruments Company Inc.) calibrated against standard H_2 gas mixtures of known concentrations (0.1, 0.5, 1, 5, 20, 50, 100, 150, 500, 1000, 2500, 5000, and 7000 ppmv), prepared by diluting either 99.999% pure H_2 gas cylinder in synthetic air (20.5% v/v O_2 in N_2) or 1% v/v H_2 in N_2 gas into He (99.999% pure) as described previously (see **Chapter 2**). The vials for each species were maintained at their respective growth temperatures and agitation speeds for the entire incubation period to facilitate H_2 and O_2 transfer between the headspace and the culture. Concurrently, headspace mixing ratios from media-only negative controls (30 mL of media for each species) were measured to confirm that observed decreases in gas concentrations were biological in nature. First order rate constants (k values) for exponential and stationary phase H_2 consumption were determined using the exponential function in GraphPad Prism (version 8.0.2).

3.3.5 Phylogenetic analysis

A phylogenetic tree was constructed to investigate the distribution and evolutionary history of group 2a [NiFe]-hydrogenases across bacterial phyla. Amino acid sequences of the catalytic subunit of the group 2a [NiFe]-hydrogenase (HucL) and related enzymes were retrieved from the National Center for Biotechnology Information (NCBI) Reference Sequence database by protein BLAST in February 2020. The resultant sequences were then classified using HydDB (Søndergaard et al., 2016), with sequences matching group 2a [NiFe]-hydrogenases retained and any duplicate and multispecies sequences removed. The 207 amino acid sequences representative of genus-level diversity were aligned with reference sequences using Clustal W in MEGA X (Kumar et al., 2008). Evolutionary relationships were visualised by constructing a maximum-likelihood phylogeny etic tree, with Neighbour-Joining and BioNJ algorithms applied to a matrix of pairwise distances that were estimated using a JTT model and topology selected by superior log-likelihood value. Gaps were treated with partial deletion, the tree was bootstrapped with 500 replicates, and the tree was midpoint rooted. Sequences used in this analysis are listed in **Table 7-2**. In addition, 20 annotated reference genomes (representative of order-level diversity) were retrieved from the NCBI GenBank database and manually analysed for putative group 2a [NiFe]-hydrogenase gene clusters. The web-based software Properon (doi.org/10.5281/zenodo.3519494) was used to generate to-scale gene organisation diagrams of these group 2a [NiFe]-hydrogenases. All species names and taxonomic assignments follow the Genome Taxonomy Database (Chaumeil et al., 2019, Parks et al., 2018).

3.4 Results

3.4.1 The expression profile of group 2a [NiFe]-hydrogenases is antithetical to group 1h [NiFe]-hydrogenases

We used qRT-PCR to quantify the expression of the large subunit of the group 2a [NiFe]-hydrogenase (*hucL*). The gene was expressed at moderate to high levels in all three strains during aerobic growth on preferred energy sources (organic carbon for *G. aurantiaca* and *C. aggregans*, ferrous iron for *A. ferrooxidans*) (**Figure 3-1**). Expression levels did not significantly differ between strains grown in an ambient air headspace containing atmospheric H_2 or supplemented with 10% H_2 (**Figure 3-1**). This suggests hydrogenase expression is constitutive and occurs even when atmospheric concentrations of the substrate are available.

Across all three strains, hydrogenase expression significantly decreased during the transition from growth to persistence. For *G. aurantiaca*, high expression was observed during exponential phase under both H_2-supplemented and H_2-unamended conditions (av. 8.4×10^6 copies per g_{dw}) and decreased 51-fold during stationary phase (av. 1.6×10^5 copies g_{dw}^{-1}; $p = 0.012$) (**Figure 3-1a**). Hydrogenase expression of *A. ferrooxidans* was moderate during growth (av. 1.8×10^6 copies per g_{dw}) and dropped 3.9-fold in stationary phase cultures (av. 4.5×10^5 copies per g_{dw}; $p = 0.013$) (**Figure 3-1b**), whereas expression in *C. aggregans* was very high during exponential growth (av. 2.9×10^9 copies g_{dw}^{-1}) and fell 15,000-fold during persistence (av. 1.9×10^5 copies g_{dw}^{-1}; $p = 0.003$) (**Figure 3-1c**). Overall, while expression levels greatly vary between species, these results clearly show the group 2a [NiFe]-hydrogenase is expressed primarily in growing cells. These expression profiles contrast with the group 1h [NiFe]-hydrogenase, which is induced during long-term persistence in a range of species (see **Chapter 2** and (Berney and Cook, 2010, Berney et al., 2014a, Constant et al., 2010, Cordero et al., 2019b, Greening et al., 2015a)).

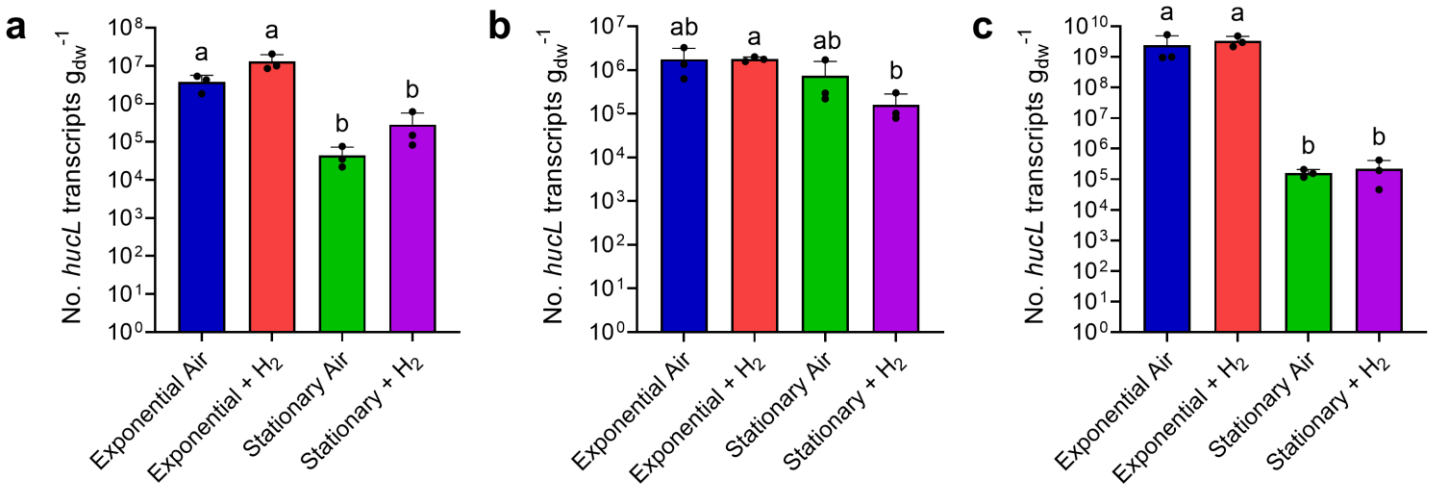

Figure 3-1: **Expression of the group 2a [NiFe]-hydrogenase in three bacterial strains during growth and survival.** The normalised transcript copy number of the large subunit gene (*hucL*) are plotted for (**a**) *Gemmatimonas aurantiaca* (locus GAU_0412), (**b**) *Acidithiobacillus ferrooxidans* (locus AFE_0702), and (**c**) *Chloroflexus aggregans* (locus CAGG_0471). Copy number was analysed by qRT-PCR in cultures harvested during exponential phase and stationary phase, in the presence of either ambient H_2 or 10% H_2. Error bars show standard deviations of three biological replicates (averaged from two technical duplicates) per condition. Values denoted by different letters were determined to be statistically significant based on a one-way ANOVA with post-hoc Tukey's multiple comparison ($p < 0.05$).

3.4.2 Group 2a [NiFe]-hydrogenases oxidise H_2 to sub-atmospheric levels

Hydrogenase activity of the three strains was inferred from monitoring changes in headspace H_2 mixing ratios over time by gas chromatography. In line with the expression profiles (**Figure 3-1**), we observed that all three strains oxidised atmospheric H_2 during growth in an ambient air headspace (**Figure 3-2**). These observations extend the trait of trace gas scavenging to three more species and suggest that group 2a [NiFe]-hydrogenases broadly have the capacity to oxidise H_2 at atmospheric levels. We subsequently monitored the consumption of H_2 by exponential and stationary phase cultures in ambient air supplemented with 10 ppmv H_2. For *G. aurantiaca* and *A. ferrooxidans*, H_2 was oxidised to sub-atmospheric levels under both conditions in an apparent first-order kinetic process (**Figure 3-3a, b**). However, biomass-normalised first-order rate constants were higher in exponential than stationary phase cells by 23-fold ($p = 0.0029$) and 120-fold ($p < 0.0001$), respectively (**Figure 3-3d**). For *C. aggregans*, H_2 was oxidised at rapid rates in exponentially growing cells, but occurred at extremely slow rates in stationary cells (**Figure 3-3c, d**). These observations support the qRT-PCR results by showing hydrogenase activity predominantly occurs during growth.

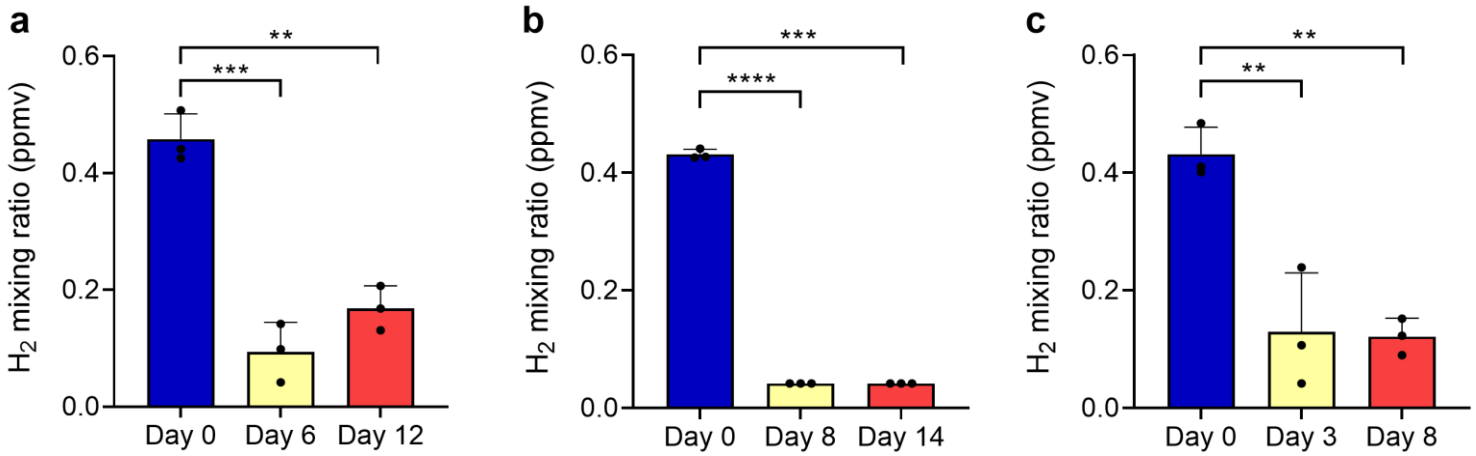

Figure 3-2 (Supplement): **Consumption of atmospheric H_2 by three bacteria encoding group 2a [NiFe]-hydrogenases.** H_2 mixing ratios of cultures of **(a)** *Gemmatimonas aurantiaca*, **(b)** *Acidithiobacillus ferrooxidans*, and **(c)** *Chloroflexus aggregans* are shown. Ratios were measured upon inoculation (blue bars), during mid-exponential growth (yellow bars), and in late stationary phase (red bars); the different sampling times between the cultures reflect their distinct growth parameters. Error bars show standard deviations of three biological replicates and statistical significance was tested using a two-way ANOVA with post-hoc Tukey's multiple comparison (** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

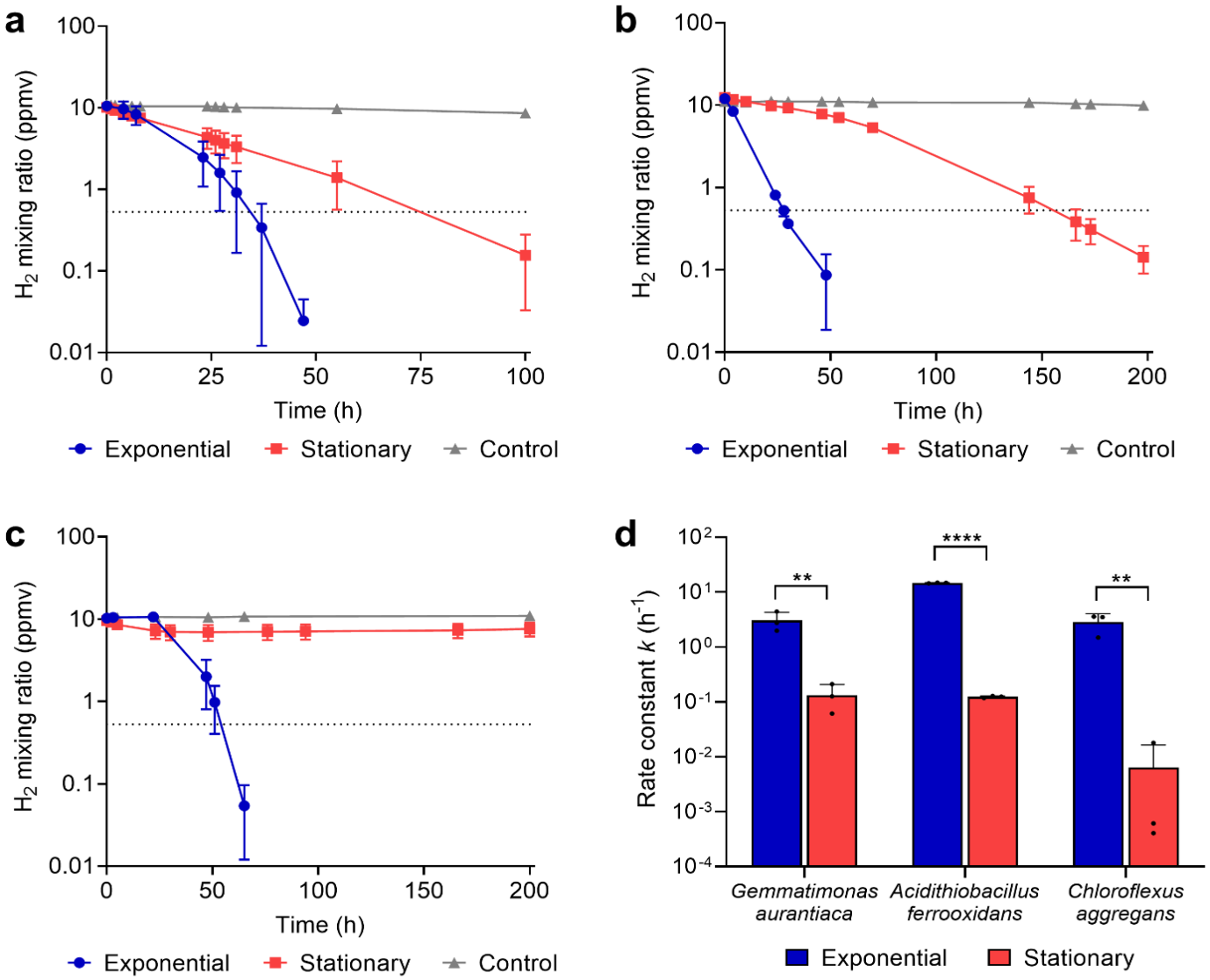

Figure 3-3: **Hydrogenase activity in three bacterial strains during growth and survival**. H_2 oxidation by cultures of (**a**) *Gemmatimonas aurantiaca*, (**b**) *Acidithiobacillus ferrooxidans*, and (**c**) *Chloroflexus aggregans*. Error bars show the standard deviation of three biological replicates, with media-only vials monitored as negative controls. Dotted lines show the atmospheric concentration of hydrogen (0.53 ppmv). **d** Biomass-normalised first-order rate constants based on H_2 oxidation observed in exponential and stationary phase cultures. Error bars show standard deviations of three biological replicates and statistical significance was tested using a two-way ANOVA with post-hoc Tukey's multiple comparison (** = $p < 0.01$; **** = $p < 0.0001$).

It should be noted that additional [NiFe]-hydrogenases are encoded by both *C. aggregans* (group 3d) and *A. ferrooxidans* (group 1e and 3b), but not for *G. aurantiaca*. These additional hydrogenases are expressed at tenfold lower levels for *C. aggregans*, but at similar levels for *A. ferrooxidans*, and hence may contribute to H_2 uptake (**Figure 3-4**). It is nevertheless likely that the group 2a [NiFe]-hydrogenases mediate atmospheric H_2 uptake given (i) the H_2 uptake activities of *C. aggregans* and *A. ferrooxidans* mimic that of *G. aurantiaca*, which lacks additional hydrogenases; (ii) previous genetic studies show group 2a enzymes mediate high-affinity aerobic H_2 uptake in mycobacteria (Cordero et al., 2019b, Greening et al., 2014a); and (iii) group 1e and 3b/3d enzymes are likely incapable of atmospheric H_2 oxidation given their respective characterised roles in anaerobic respiration and fermentation (Søndergaard et al., 2016).

3.4.3 H_2 consumption enhances mixotrophic growth in carbon-fixing strains

The observation that expression and activity of the group 2a [NiFe]-hydrogenase is optimal during growth suggests this enzyme supports mixotrophic growth. To test this, we monitored growth by optical density of the three strains in headspaces containing H_2 at either ambient, 1%, or 10% mixing ratios. No growth differences in the obligate heterotroph *G. aurantiaca* were observed between the conditions ($p = 0.30$) (**Figure 3-5a**). In contrast, H_2-dependent growth stimulation was observed for the obligate autotroph *A. ferrooxidans* (1.4-fold increase; $p = 0.0003$) (**Figure 3-5b**) and facultative autotroph *C. aurantiaca* (1.2-fold increase; $p = 0.029$) (**Figure 3-5c**). This suggests that reductant derived from H_2 oxidation can be used by these bacteria to fix CO_2 through the Calvin-Benson and 3-hydroxypropionate cycles, respectively.

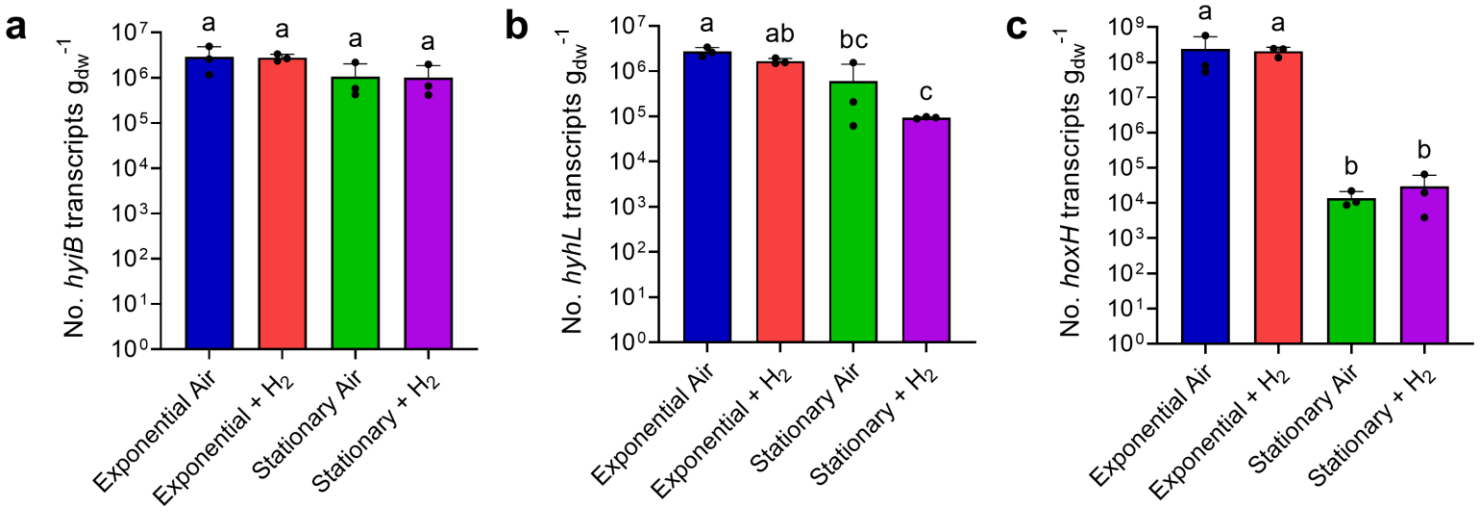

Figure 3-4 (Supplement): **Expression of other hydrogenases during growth and survival.** The normalised number of transcriptions of the large subunit genes of the **(a)** group 1e [NiFe]-hydrogenase of *Acidithiobacillus ferrooxidans* (*hyiB*, locus AFE_3286), **(b)** group 3b [NiFe]-hydrogenase of *Acidithiobacillus ferrooxidans* (*hyhL*, locus AFE_0937), and **(c)** group 3d [NiFe]-hydrogenase of *Chloroflexus aggregans* (*hoxH*, locus CAGG_2476). *Gemmatimonas aurantiaca* only encodes the group 2a hydrogenase. Results are shown for cultures harvested during exponential phase and stationary phase, in the presence of either ambient H_2 or 10% H_2. Error bars show standard deviations of three biological replicates (averaged from two technical duplicates) per condition. Values denoted by different letters were determined to be statistically significant based on a one-way ANOVA with post-hoc Tukey's multiple comparison ($p < 0.05$).

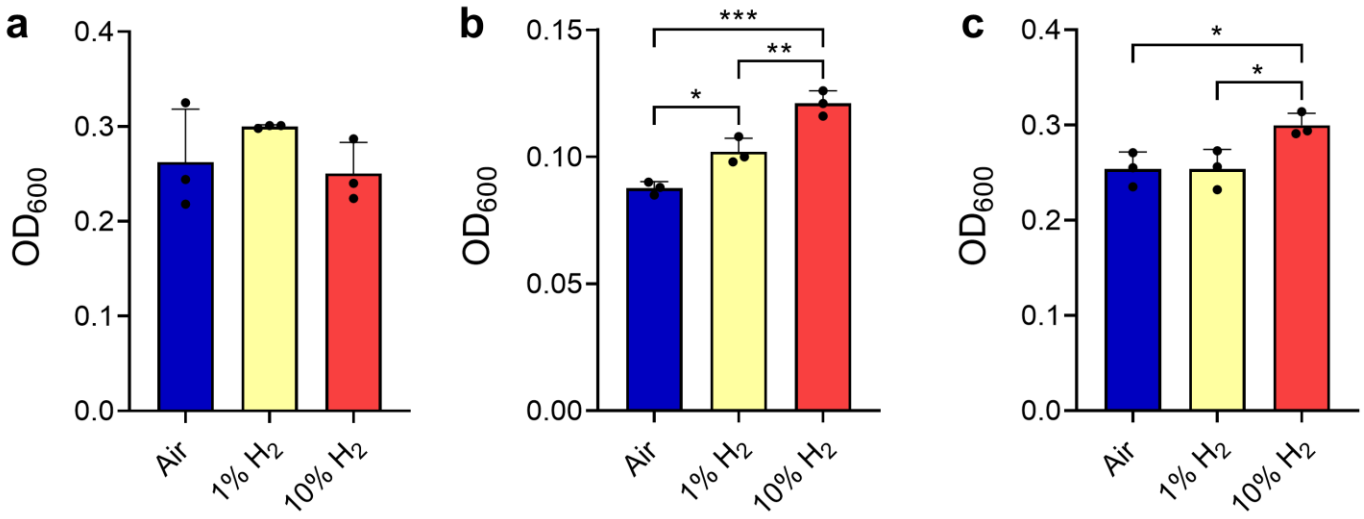

Figure 3-5: **Effects of H_2 supplementation on growth of three bacterial strains**. The final growth yield (OD_{600}) of (**a**) *Gemmatimonas aurantiaca,* (**b**) *Acidithiobacillus ferrooxidans*, and (**c**) *Chloroflexus aggregans* is shown in ambient air vials containing H_2 at either ambient, 1%, or 10% concentrations. Error bars show the standard deviation of three biological replicates and statistical significance was tested using a one-way ANOVA with post-hoc Tukey's multiple comparison (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

3.4.4 Hydrogenases with common phylogeny and genetic organisation are widely distributed across 13 bacterial phyla

Finally, we surveyed the distribution of group 2a [NiFe]-hydrogenases to infer which other bacteria may oxidise atmospheric H_2. We detected the large subunit of this hydrogenase (HucL) across 171 genera and 13 phyla (**Table 7-2; Figure 3-6**); this constitutes a 3.2-fold increase in the number of genera and 1.4-fold increase in the number of phyla previously reported to encode this enzyme (Greening et al., 2016, Søndergaard et al., 2016). This increase in HucL distribution is due to the increase in available genome sequences since previous analyses were performed. The HucL-encoding bacteria include various known hydrogenotrophic aerobes, such as *Nitrospira moscoviensis* (Nitrospirota) (Koch et al., 2014), *Hydrogenobacter thermophilus* (Aquificota) (Kawasumi et al., 1984), *Kyrpidia tusciae* (Firmicutes) (Hogendoorn et al., 2020, Klenk et al., 2011), *Sulfobacillus acidophilus* (Firmicutes) (Hedrich and Johnson, 2013), and *Pseudonocardia dioxanivorans* (Actinobacteriota) (Grostern and Alvarez-Cohen, 2013), suggesting these strains may also consume atmospheric H_2. The hydrogenase was also distributed in various lineages of Bacteroidota, Alphaproteobacteria, Gammaproteobacteria, and Deinococcota for which H_2 oxidation has not, to our knowledge, been reported.

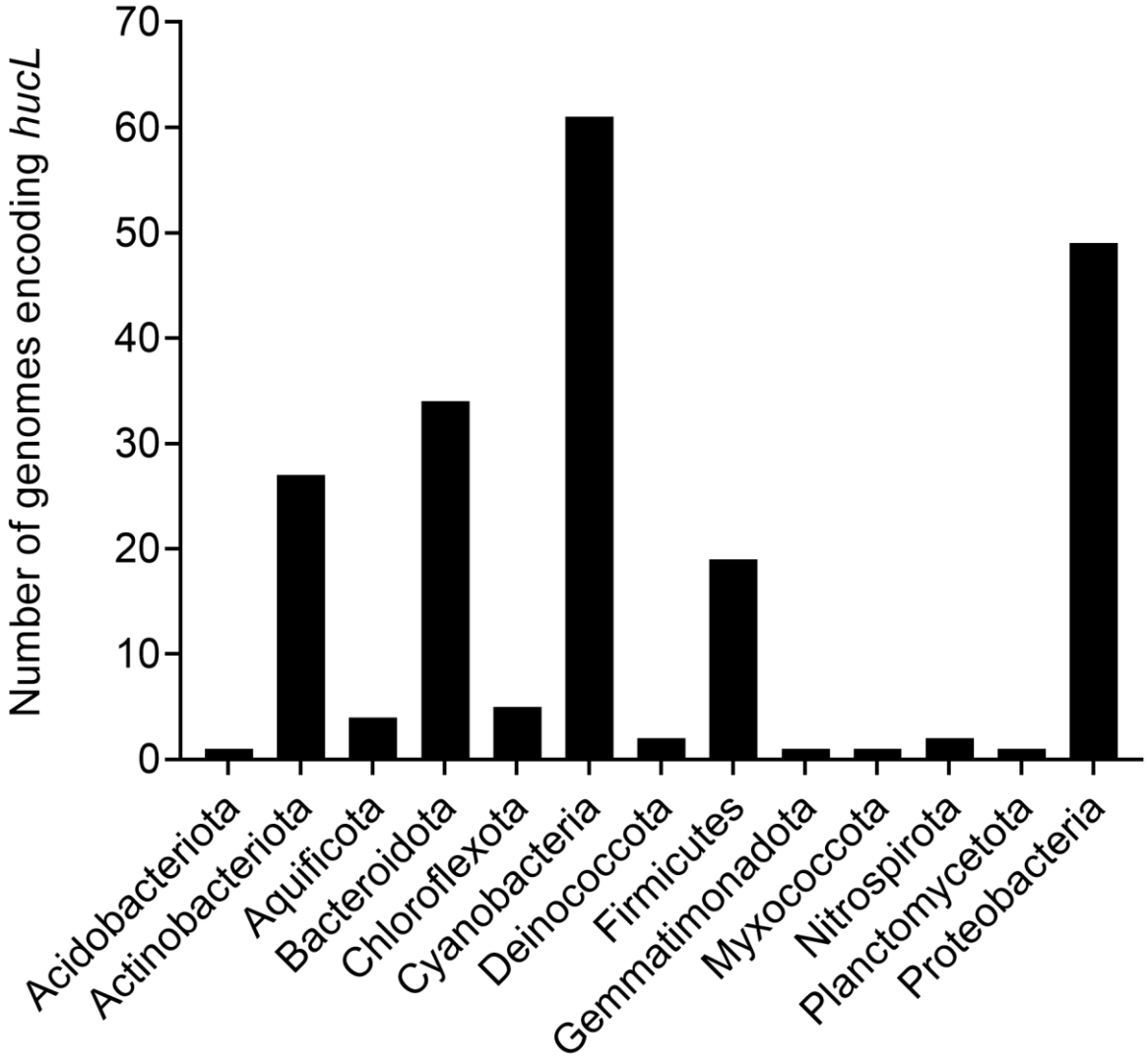

Figure 3-6 (Supplement): **Distribution of group 2a [NiFe]-hydrogenases across microbial genomes.** Results are based on the number of genomes in which the large subunit gene was detected (*hucL*) and are shown by phylum.

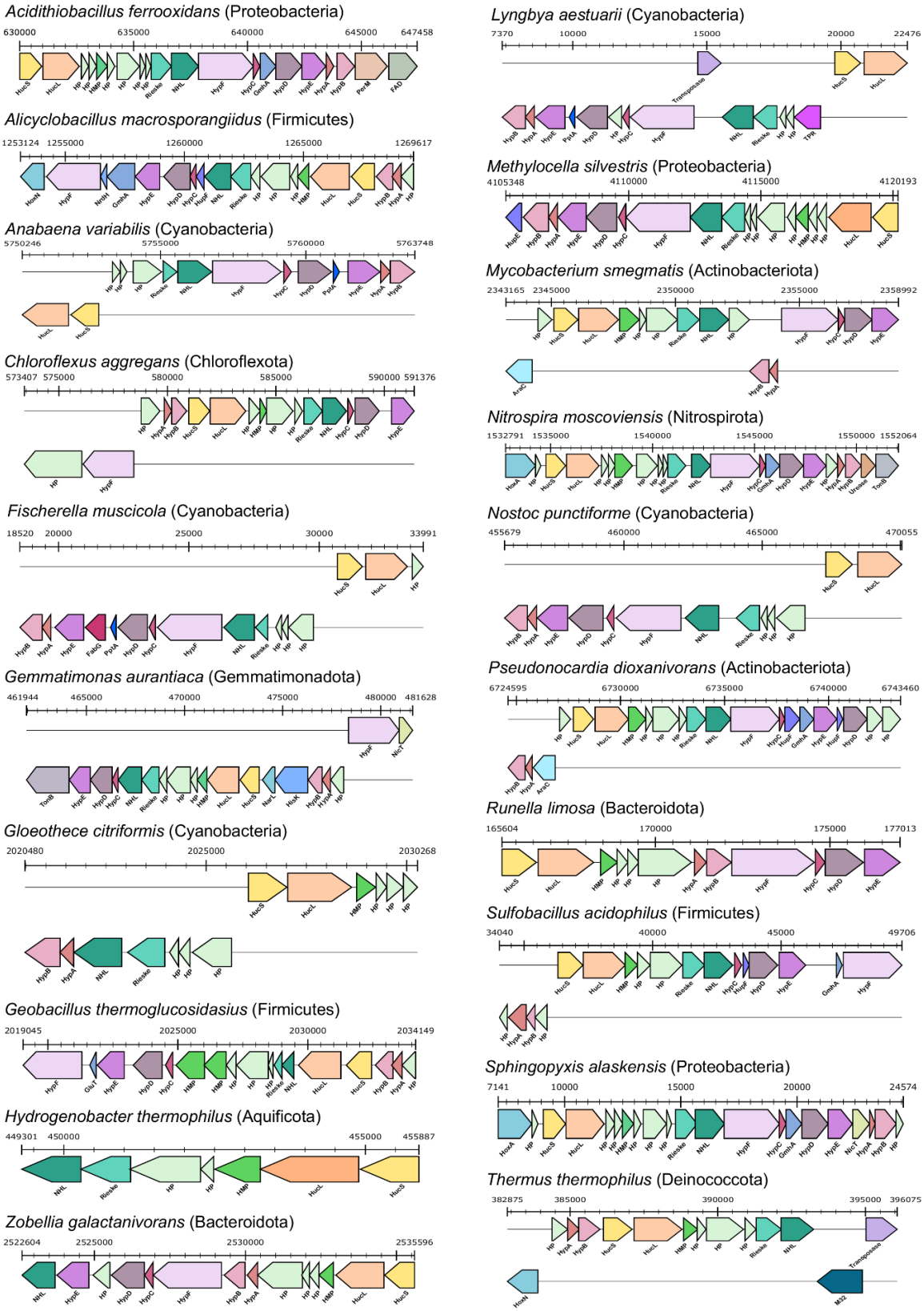

Figure 3-7 (Supplement): **Genetic organisation of group 2a [NiFe]-hydrogenases across ten phyla.** Abbreviations: HucL = hydrogenase large subunit; HucS = hydrogenase small subunit; HypABCDEF = hydrogenase maturation factors; HMP = hydrogenase maturation peptidase; NHL = NHL repeat protein; HP = conserved hypothetical protein; GmhA = putative phosphoheptose isomerase; Rieske = Rieske-like iron-sulfur protein (HucE); FAD = putative FAD-dependent oxidoreductase. Gene length is shown to scale and gene identifiers are as per the nomenclature of HydDB.

A maximum-likelihood phylogenetic tree showed the retrieved HucL sequences form a well-supported monophyletic clade. Most sequences clustered into four major radiations, Bacteroidota-associated, Cyanobacteria-associated, Proteobacteria-associated (including *A. ferrooxidans*), and a mixed clade containing sequences from seven phyla (including *G. aurantiaca* and *C. aggregans*) (**Figure 3-8**). Several genes were commonly genomically associated with *hucL* genes in putative operons, including the hydrogenase small subunit (*hucS*), a Rieske-type iron-sulfur protein (*hucE*) (see **Chapter 4**), hypothetical proteins (including NHL-repeat proteins) (Berney et al., 2014b), and various maturation factors (**Figure 3-7**). The group 2a [NiFe]-hydrogenases are distinct in both phylogeny and genetic organisation to the two most closely related hydrogenase subgroups, the previously described group 2e [NiFe]-hydrogenases of aerobic hydrogenotrophic Crenarchaeota (Auernik and Kelly, 2010, Søndergaard et al., 2016) and the novel group 2f [NiFe]-hydrogenases that are distributed sporadically in bacteria and archaea (**Figure 3-8**).

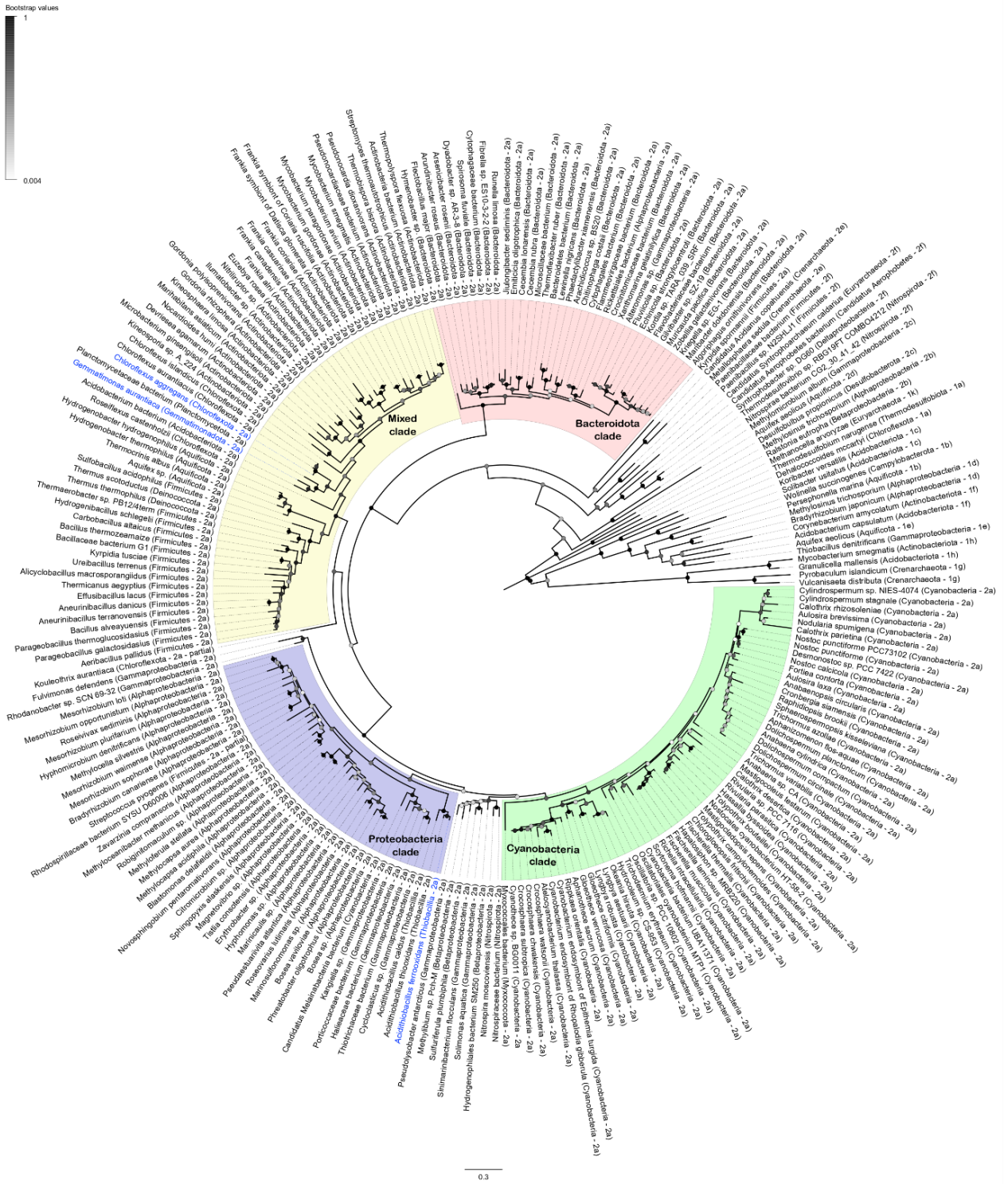

Figure 3-8: **Radial phylogenetic tree showing the distribution and evolutionary history of the group 2a [NiFe]-hydrogenase**. Amino acid sequences of the catalytic subunit of the group 2a [NiFe]-hydrogenase (hucL) are shown for 171 bacterial genera. The taxon names of the three study species, *G. aurantiaca, A. ferrooxidans*, and *C. aggregans*, are coloured in blue. The tree was constructed using the maximum-likelihood method (gaps treated with partial deletion), bootstrapped with 500 replicates, and rooted at the mid-point. Accession numbers and amino acid sequences used to construct the tree are listed in **Table 7-2**. The total number of genomes identified per phylum are as follows: Acidobacteriota (1), Actinobacteriota (27), Aquificota (4), Bacteroidota (34), Chloroflexota (5), Cyanobacteria (61), Deinococcota (2), Firmicutes (19), Gemmatimonadota (1), Myxococcota (1), Nitrospirota (2), Planctomycetota (1), Proteobacteria (49).

3.5 Discussion

Overall, these findings demonstrate that atmospheric H_2 oxidation is not solely a persistence-linked trait. We infer that group 2a [NiFe]-hydrogenases are optimally expressed and active during exponential phase, consume H_2 at sub-atmospheric concentrations, and support mixotrophic growth. Largely concordant findings were made in three phylogenetically, physiologically, and ecologically distinct bacterial species. These findings contrast with multiple pure culture studies that have linked expression, activity, and phenotypes associated with group 1h [NiFe]-hydrogenases to survival rather than growth (see **Chapter 2** and (Berney and Cook, 2010, Constant et al., 2010, Greening et al., 2014a, Greening et al., 2015a, Greening et al., 2014b, Liot and Constant, 2016)). However, a growth-supporting role of atmospheric H_2 oxidation is nevertheless consistent with several surprising recent reports: the measurement of atmospheric H_2 oxidation during growth of several strains (Greening et al., 2014a, Greening et al., 2014b, Mohammadi et al., 2017, Schmitz et al., 2020); the discovery of an Antarctic desert community driven by trace gas oxidation (Ji et al., 2017); and the isolation of a proteobacterial methanotroph thought to grow on air alone (Tveit et al., 2019). Together, these findings suggest that the current persistence-centric model of atmospheric H_2 utilisation is overly generalised and that this process also supports mixotrophic growth.

Atmospheric H_2 oxidation during growth is likely to primarily benefit bacteria that adopt a mixotrophic lifestyle. While atmospheric H_2 alone can sustain bacterial maintenance, theoretical modelling suggests this energy source is insufficiently concentrated to permit growth as the sole energy source (Conrad, 1996, Greening et al., 2015b). Instead, bacteria that co-oxidise this dependable gas with other organic or inorganic energy sources may have significant selective advantages, especially in environments where resource availability is very low or variable. Likewise, it is probable that many bacteria in natural environments supplement growth by taking advantage of transient increases in H_2 availability. For example, the metabolic

generalist *C. aggregans* may facilitate its expansion in geothermal mats by simultaneously utilising geothermal and atmospheric sources of H_2, in addition to sunlight and organic compounds (Hanada et al., 1995, Otaki et al., 2012, Spear et al., 2005). Similarly, in the dynamic environment of wastewater treatment plants, *G. aurantiaca* may be well-suited to take advantage of fermentatively-produced H_2 released during transitions between oxic and anoxic states (Park et al., 2017, Ferrera and Sánchez, 2016).

The ability to consume atmospheric H_2 may also be particularly advantageous during early stages of ecological succession. Indeed, *A. ferrooxidans* may initially rely on this atmospheric energy source as it colonises barren tailings and establishes an acidic microenvironment conducive for iron oxidation (Mielke et al., 2003). Hydrogen synthesis in tailings can further benefit *A. ferrooxidans* as acid conditions and more complex bacterial consortia develop. Specifically, acetate-dependent growth of dissimilatory sulfate reducing bacteria in tailings (Spear et al., 2005) will initiate endogenous geochemical production of trace hydrogen ($FeS + H_2S \rightarrow FeS_2 + H_2$). As tailings cycle between aerobic (vadose) and anaerobic (water-saturating) conditions, the H_2 available from atmospheric and geochemical sources respectively may provide a continuous energy source for *A. ferrooxidans*. In addition, any environments possessing sulfate and iron, i.e., "downstream" from acid-generating ecosystems (including marine sediments), can generate hydrogen through bacterial organotrophic sulfate reduction.

This study also identifies key microbial and enzymatic players in the global hydrogen cycle. The group 2a [NiFe]-hydrogenase is the second hydrogenase lineage shown to have a role in atmospheric H_2 oxidation across multiple bacterial phyla. The group 1h enzyme is probably the main sink of the H_2 cycle given it is the predominant hydrogenase in most soils (Constant et al., 2011a, Constant et al., 2011b, Greening et al., 2016). However, the group 2a enzyme is moderately to highly abundant in many soil, marine, and geothermal environments

(Cordero et al., 2019a), among others, and hence is also likely to be a key regulator of H_2 fluxes. This study also reports atmospheric H_2 oxidation for the first time in two globally dominant phyla, Proteobacteria and Gemmatimonadota, and uncovers *A. ferrooxidans* as the first H_2-scavenging autotroph. Until recently, atmospheric H_2 oxidation was thought to be primarily mediated by heterotrophic Actinobacteriota (Constant et al., 2011b, Constant et al., 2010, Greening et al., 2014a, Greening et al., 2015b), but it is increasingly apparent that multiple aerobic lineages are responsible (see **Chapter 4** and (Greening et al., 2016, Greening et al., 2015a, Myers and King, 2016, Schmitz et al., 2020)). Some six phyla have now been described that are capable of atmospheric H_2 oxidation and, given the group 2a [NiFe]-hydrogenase is encoded by at least eight other phyla, others will likely soon be described. It is possible that atmospheric H_2 oxidation extends to other important groups, such as nitrite-oxidising Nitrospirota (Koch et al., 2014), methane-oxidising Proteobacteria (Grostern and Alvarez-Cohen, 2013), and potentially even oxygenic phototrophs; while Cyanobacteria are known to recycle endogenously-produced H_2 (Houchins, 1984, Papen et al., 1986, Eichner et al., 2019), it should be tested whether they can also scavenge exogenous H_2. Indeed, while atmospheric H_2 oxidisers were only recently discovered (Constant et al., 2010, Constant et al., 2008, Greening et al., 2019), it is now plausible that these bacteria may represent the rule rather than the exception among aerobic H_2 oxidisers.

4 PUTATIVE IRON-SULFUR PROTEINS ARE REQUIRED FOR HYDROGEN CONSUMPTION AND ENHANCE SURVIVAL OF MYCOBACTERIA

Article published

Frontiers in Microbiology

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4.1 Disclaimer

The contents of this chapter are from the revised manuscript draft submitted to *Frontiers in Microbiology* in November 2019. During the review process, we carried out extra experiments including gene complementation and performing activity staining via the protocol described by Cordero et al. (2019b) to better target Huc expression (Cordero et al., 2019b). The new results led us to amend the manuscript text to incorporate the largely concordant findings, address reviewer comments and to strengthen the manuscript overall. These complementation experiments (generation of complemented strains, activity staining and respirometry measurements of the complemented strains) were primarily carried out by Paul Cordero, who was promoted to co-first author in the revised manuscript. As such, these additional experiments performed during the revision process are not included in this thesis.

4.2 Abstract

Recent work has shown that aerobic soil bacteria persist by scavenging molecular hydrogen (H_2) from the atmosphere. This key process serves as the primary sink in the biogeochemical hydrogen cycle and supports the productivity of oligotrophic ecosystems. In *Mycobacterium smegmatis*, atmospheric H_2 oxidation is catalysed by two phylogenetically distinct [NiFe]-hydrogenases, Huc (group 2a) and Hhy (group 1h). However, it is currently unresolved how these enzymes transfer electrons derived from H_2 oxidation into the aerobic respiratory chain. In this work, we used genetic approaches to confirm that two novel putative iron-sulfur cluster proteins encoded on the hydrogenase structural operons, HucE and HhyE, are required for H_2 consumption in *M. smegmatis*. Sequence analysis show that these proteins, while homologous, fall into distinct phylogenetic clades and have distinct metal-binding motifs. H_2 oxidation was reduced when the genes encoding these proteins were deleted individually and was eliminated when they were deleted in combination. In turn, the growth

yield and long-term survival of these deletion strains was significantly lower than the parent strain. In both biochemical and phenotypic assays, the mutant strains lacking the putative iron-sulfur proteins phenocopied those of hydrogenase structural subunit mutants. We therefore hypothesize that these proteins mediate electron transfer between the catalytic subunits of the hydrogenases and the menaquinone pool of the *M. smegmatis* respiratory chain. However, further studies on the purified proteins are required to confirm this. These proteins may also play a role in hydrogenase maturation. The conserved nature of these proteins within most other Hhy- or Huc-encoding organisms suggest that these proteins are important determinants of atmospheric H_2 oxidation.

4.3 Introduction

Over the last decade, various studies have revealed that aerobic bacteria conserve energy during persistence through aerobic respiration of atmospheric hydrogen (H_2) (Constant et al., 2010, Greening et al., 2014a, Greening et al., 2015b, Islam et al., 2019a, Liot and Constant, 2016, Meredith et al., 2014). This process is now recognized to be important for biogeochemical and ecological reasons. Gas-scavenging soil bacteria serve as the primary sink in the global hydrogen cycle and are responsible for the net consumption of approximately 70 million tonnes of H_2 each year (Constant et al., 2009, Ehhalt and Rohrer, 2009, Greening et al., 2014a, Piché-Choquette et al., 2018). More recently, it has been inferred that this process supports the productivity and biodiversity of various ecosystems, especially low-carbon soils (Bay et al., 2018, Greening et al., 2016, Ji et al., 2017, Kanno et al., 2016, Kessler et al., 2019, Khdhiri et al., 2015, Lynch et al., 2014, Piché-Choquette and Constant, 2019). Atmospheric H_2 oxidation appears to be a widespread trait among soil bacteria. To date, bacteria from three phyla have been experimentally shown to oxidize atmospheric H_2, Actinobacteriota (Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Meredith et al., 2014), Acidobacteriota (Greening et al., 2015a, Myers and King, 2016), and Chloroflexota (**Chapter**

2). However, genomic surveys indicate at least 13 other phyla can mediate this process (Carere et al., 2017, Greening et al., 2016, Ji et al., 2017, Piché-Choquette et al., 2017).

The genetic basis and physiological role of atmospheric H_2 oxidation is now largely understood. This process has been most comprehensively studied in the genetically tractable soil actinobacterium *Mycobacterium smegmatis* (Greening and Cook, 2014). In this organism, atmospheric H_2 oxidation is mediated by two membrane-bound, oxygen-tolerant hydrogenases: Huc (group 2a [NiFe]-hydrogenase, also known as Hyd1 or cyanobacterial-type uptake hydrogenase) and Hhy (group 1h [NiFe]-hydrogenase, also known as Hyd2 or actinobacterial-type uptake hydrogenase). Additionally, *M. smegmatis* has a third [NiFe]-hydrogenase, Hyh (Hyd3), which is responsible for hydrogen production and primarily plays a role during hypoxia (Berney et al., 2014a). Both H_2 oxidizing hydrogenases are each composed of a large and small subunit (Huc with HucS and HucL; Hhy with HhyS and HhyL) (Berney et al., 2014b). These two hydrogenases are upregulated in stationary-phase cells, including in response to organic carbon limitation (Berney and Cook, 2010, Berney et al., 2014b). Consistently, when the structural subunits of these hydrogenases are deleted, strains show reduced growth yield and impaired long-term survival during starvation (Berney and Cook, 2010, Berney et al., 2014a, Greening et al., 2014b). Similar findings have been made in *Streptomyces avermitilis*; the sole hydrogenase of this organism, Hhy, is exclusively expressed in exospores and strains lacking this enzyme exhibit severe survival defects (Constant et al., 2010, Liot and Constant, 2016). Given these findings, it is proposed that bacteria shift from growing on organic compounds to persisting on atmospheric trace gases. Indeed, theoretical calculations indicate that the energy derived from atmospheric H_2 oxidation can sustain the maintenance of 10^7 to 10^8 cells (Conrad, 1999b).

Despite this progress, little is currently known about the biochemical basis of atmospheric H_2 oxidation. One outstanding question is how electrons derived from H_2

oxidation are transferred to the respiratory chain. Most classes of respiratory uptake hydrogenases are predicted to be co-transcribed with a cytochrome *b* subunit (Greening et al., 2016, Søndergaard et al., 2016). For example, such subunits interact with the prototypical oxygen-tolerant hydrogenases (group 1d [NiFe]-hydrogenases) of *Escherichia coli* and *Ralstonia eutropha*; they anchor the hydrogenase to the membrane and transfer electrons from the hydrogenase small subunit to the quinone pool (Frielingsdorf et al., 2011, Volbeda et al., 2013). However, we did not detect equivalent proteins in the operons encoding the structural subunits of Huc (MSMEG_2261 – 2270) or Hhy (MSMEG_2722 – 2718) in *M. smegmatis* (Berney et al., 2014b). Putative iron-sulfur proteins, tentatively annotated as HucE (MSMEG_2268) and HhyE (MSMEG_2718), were encoded downstream of the hydrogenase structural subunits and may potentially fulfil this role instead (Berney et al., 2014b, Greening et al., 2015b). In this work, we characterized the effects of deleting these genes on hydrogenase activity, growth, and survival in *M. smegmatis*. We also investigated their broader conservation in hydrogenase-encoding bacteria.

4.4 Materials and Methods

4.4.1 Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in **Table 4-1**. *Escherichia coli* TOP10 was maintained on lysogeny broth (LB; 10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract, 15 g/L agar) agar plates, while *Mycobacterium smegmatis* mc^2155 (Snapper et al., 1990) and derived mutants were maintained on LB agar plates supplemented with 0.05% (w/v) Tween80 (LBT). For broth culture, *E. coli* was grown in LB. *M. smegmatis* was grown in either LBT or in Hartmans de Bont (HdB) minimal medium (Hartmans and De Bont, 1992) supplemented with 0.2% (w/v) glycerol. In all cases, liquid cultures were grown in rotary incubators at 37°C with agitation (200 rpm).

Table 4-1 (Supplement): **Bacterial strains and plasmids used in this study**

Strain	**Description**	**Reference**
mc^{2}155	Electrocompetent, wild-type strain of *Mycobacterium smegmatis*	(Snapper et al., 1990)
Δ*hupE*	mc^{2}155 with markerless deletion of MSMEG_2268	This study
Δ*hhyE*	mc^{2}155 with markerless deletion of MSMEG_2718	This study
Δ*hupE*Δ*hhyE*	mc^{2}155 with markerless deletions of MSMEG_2268 and MSMEG_2718	This study
Δ*hupS*	mc^{2}155 with markerless deletion of MSMEG_2262	(Berney et al., 2014b)
Δ*hhyL*	mc^{2}155 with markerless deletion of MSMEG_2719	(Berney and Cook, 2010)
Δ*hupS*Δ*hhyL*	mc^{2}155 with markerless deletions of MSMEG_2262 and MSMEG_2719	(Berney et al., 2014b)
TOP10	Chemically competent, wild-type strain of *Escherichia coli* F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ$^-$ *rps*L(Str^R) *end*A1 *nup*G	Thermo Fisher
Plasmid	**Description**	**Reference**
pX33	Gm^r, *sacB*, mycobacterial Ts ori, p15A ori, *xylE*	(Gebhard *et al.*, 2006)
pX33-*hupE*	pX33 harbouring the MSMEG_2268 deletion construct	This study
pX33-*hhyE*	pX33 harbouring the MSMEG_2718 deletion construct	This study

4.4.2 Mutant strain construction

Allelic exchange mutagenesis was used to produce markerless deletions of the genes encoding two putative iron-sulfur proteins, *hucE* (MSMEG_2268) and *hhyE* (MSMEG_2718). Briefly, a fragment containing fused left and right flanks of the MSMEG_2268 (1800 bp) and MSMEG_2718 (3098 bp) genes were synthesized by GenScript. These fragments were cloned into the SpeI site of the mycobacterial shuttle plasmid pX33 (Gebhard et al., 2006) to yield the constructs pX33-*hucE* and pX33-*hhyE* (**Table 4-1**). These constructs were propagated in *E. coli* TOP10 and transformed into wild-type *M. smegmatis* mc^2155 cells by electroporation. Gentamycin (5 µg mL^{-1} for *M. smegmatis* or 20 µg mL^{-1} for *E. coli*) was used in selective solid and liquid medium to propagate pX33. Creation of the double iron-sulfur cluster mutant (Δ*hucE*Δ*hhyE*) was achieved by transformation of Δ*hhyE* electrocompetent *M. smegmatis* mc^2155 with the pX33-*hucE* construct. Briefly, to allow for permissive temperature-sensitive vector replication, transformants were incubated on LBT gentamycin plates at 28°C until colonies were visible (5-7 days). Resultant catechol-positive colonies were subcultured onto fresh LBT gentamycin plates and incubated at 40°C for 3-5 days to facilitate integration of the recombinant plasmid flanks into the chromosome. The second recombination event was facilitated by subculturing catechol-reactive and gentamycin-resistant colonies onto LBT agar plates supplemented with 10% sucrose (w/v) and incubating at 40°C for 3-5 days. Catechol-unreactive colonies were subsequently screened by PCR to discern wild-type revertants from Δ*hucE*, Δ*hhyE* and Δ*hucE*Δ*hhyE* mutants. Primers used for the generation of mutants and for screening are listed in **Table 4-2**.

Table 4-2 (Supplement): **Primers used in this study**

Primer	**Sequence (5' to 3')**	**Enzyme**
del2268_fwd	aaaACTAGTGTGTCAGGTTCGCATCGAC	SpeI
del2268_rvs	aaaACTAGTTCACAACCGATCACGACCG	SpeI
2267_chrom_fwd	AGACCCTGTGCACGCGATC	
2269_chrom_rvs	CGGCGACCGCGTCGGAC	
del2718_fwd	aaaACTAGTATGACCACCACAGCTCCCAA	SpeI
del2718_rvs	aaaACTAGTTCATGGGATGTCCTCCCGC	SpeI
2717_chrom_fwd	GCAGCGTCCTTCACGGA	
2719_chrom_rvs	GGTAACCCTCGTAGAGCA	

4.4.3 Respirometry measurements

Cultures of wild-type and derived mutant strains of *M. smegmatis* were grown in 125 mL aerated conical flasks containing 30 mL HdB medium supplemented with 0.2% glycerol. Respirometry measurements were performed with mid-stationary phase cells, i.e. 72 h post OD_{max} (~0.9). A Unisense H_2 microsensor electrode was polarised at +800 mV for 1 h using a Unisense multimeter and calibrated against standards of known H_2 concentration. Gas-saturated PBS was prepared by bubbling the solution with 100% (v/v) of either H_2 or O_2 for 5 min. The 1.1 mL microrespiration assay chambers were sequentially amended with stationary-phase cultures (0.9 mL, OD_{600} = 0.9), H_2-saturated PBS (0.1 mL), and O_2-saturated PBS (0.1 mL). Chambers were stirred at 250 rpm, 37°C. Changes in H_2 concentration were recorded using Unisense Logger Software, and upon observing a linear change in H_2 concentration, rates of consumption were calculated over a period of 20 s corresponding to the most linear uptake of the hydrogen by the cells. Oxidation rates were normalised against total protein concentration, which was determined by the bicinchoninic acid method (Smith et al., 1985) with bovine serum albumin standards.

4.4.4 Activity staining

Cultures of wild-type and derived mutant strains of *M. smegmatis* were grown in 2.5 L aerated conical flasks containing 500 mL HdB medium supplemented with 0.2% glycerol. Mid-stationary phase cultures (72 h post OD_{max}, ~3.8) were harvested by centrifugation (10,000 x *g*, 10 min, 4°C), washed in phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and resuspended in 16 mL lysis buffer (50 mM Tris-Cl, pH 8.0, 1 mM PMSF, 2 mM $MgCl_2$, 5 mg mL^{-1} lysozyme, 40 µg mL^{-1} DNase, 10% glycerol). Resultant cell suspensions were passed through a Constant Systems cell disruptor (40,000 psi, four times), with unbroken cells removed by centrifugation (10,000 x *g*, 20 min, 4°C) to yield whole-cell lysates. Protein concentration was determined using a bicinchoninic

acid assay (Smith et al., 1985) with bovine serum albumin standards. Next, 20 µg of each whole-cell lysate was loaded onto two native 7.5% (w/v) Bis-Tris polyacrylamide gels prepared as described elsewhere (Walker, 2002) and run alongside a protein standard (NativeMark Unstained Protein Standard, ThermoFisher Scientific) for 1.5 h at 25 mA. One gel was stained overnight at 4°C with gentle agitation using AcquaStain Protein Gel Stain (Bulldog Bio) for total protein determination. The other gel was incubated in 50 mM potassium phosphate buffer (pH 7.0) supplemented with 500 µM nitroblue tetrazolium chloride (NBT) in an anaerobic jar amended with an anaerobic gas mixture (5% H_2, 10% CO_2 85% N_2 v/v) overnight at room temperature.

4.4.5 Growth and survival assays

Cultures of wild-type and derived mutant strains of *M. smegmatis* were inoculated into 125 mL conical flasks containing 30 mL LBT medium (initial OD_{600} of 0.001). Growth was monitored by measuring optical density at 600 nm (1 cm cuvettes; Eppendorf BioSpectrometer Basic); when OD_{600} was above 0.5, cultures were diluted ten-fold in LBT before measurement. Specific growth rate during mid-exponential growth was calculated for each replicate using GraphPad Prism (nonlinear regression, exponential growth equation, least squares fit). The long-term survival of the cultures was determined by counting colony forming units (CFU mL^{-1}) of cultures 21 days post-OD_{max}. Cultures were serially diluted in HdB containing no carbon source and spotted on to agar plates in technical quadruplicates. After incubation at 37°C for three days, the resultant colonies were counted.

4.4.6 Sequence and phylogenetic analysis

Sequences homologous to *M. smegmatis* HucE (MSMEG_2268) and HhyE (MSMEG_2718) were retrieved by protein BLAST (Altschul et al., 1990) using the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) (Pruitt et al., 2007). The retrieved hits were cross-referenced with the hydrogenase database (HydDB)

(Søndergaard et al., 2016) in order to determine which organisms co-encode HucE with HucL and HhyE with HhyL. For downstream phylogenetic and motif analysis, sequences were filtered to remove truncated HupE/HhyE proteins and retain one protein sequence per genus. This resulted in a representative subset of 52 full-length HucE and 26 full-length HhyE sequences. The retrieved sequences were aligned using ClustalW in MEGA7 (Kumar et al., 2016). The phylogenetic relationships of these sequences were visualized on a maximum-likelihood tree based on the Poisson correction method and bootstrapped with 100 replicates. In addition, WebLogo (Crooks et al., 2004) was used to analyze the conserved motifs containing cysteine and histidine residues predicted to bind iron-sulfur clusters.

4.5 Results

4.5.1 HucE and HhyE are predicted to be novel iron-sulfur proteins associated with group 2a and group 1h [NiFe]-hydrogenases

We investigated the diversity of putative iron-sulfur cluster proteins associated with [NiFe]-hydrogenases by conducting a homology-based search using the amino acid sequences of HucE (MSMEG_2268) and HhyE (MSMEG_2718) from *M. smegmatis*. Homologous sequences were retrieved from 14 phyla and 104 genera of bacteria (**Figure 4-1 & Table 7-3**). The evolutionary relationship of these proteins were visualised on a maximum-likelihood phylogenetic tree (**Figure 4-1**).

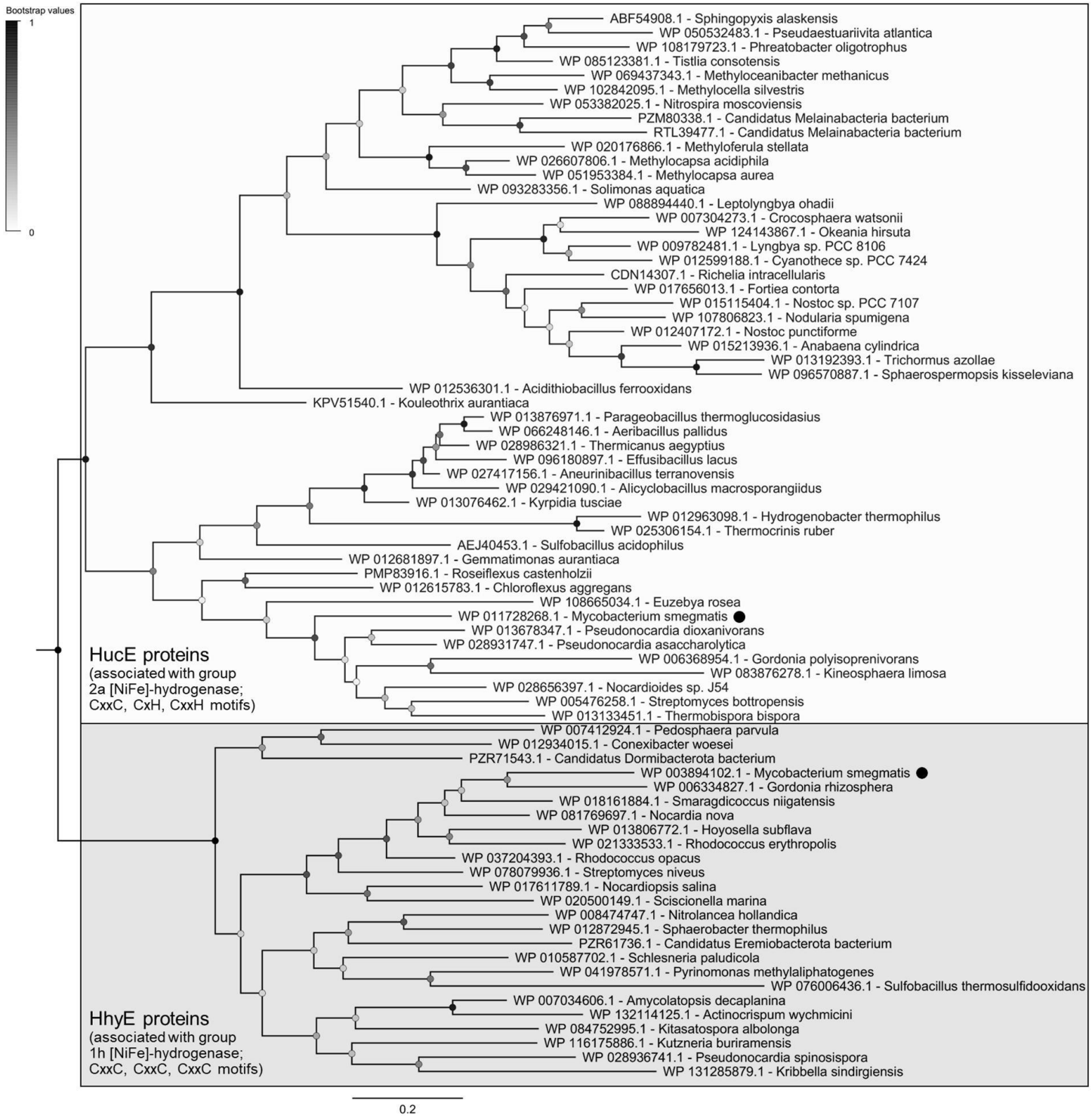


Figure 4-1: **Phylogenetic tree of HucE and HhyE proteins associated with group 2a and 1h [NiFe]-hydrogenases.** The tree visualizes the evolutionary relationships between a representative subset of 52 full-length HucE and 26 full-length HhyE sequences. The proteins encoded by *Mycobacterium smegmatis* are emphasised. The tree was constructed using the maximum-likelihood method (gaps treated with partial deletion), bootstrapped with 100 replicates, and rooted at the mid-point. The sequences used to create this tree are provided in **Table 7-3**.

All retrieved sequences fall into two robustly supported clades, the HucE proteins associated with group 2a [NiFe]-hydrogenases (Huc) and the HhyE proteins associated with group 1h [NiFe]-hydrogenases (Hhy), that share approximately 27% amino acid identity. HhyE proteins were encoded by various atmospheric H_2 oxidisers, including *Streptomyces* (Berney and Cook, 2010), *Rhodococcus* (Meredith et al., 2014), *Pyrinomonas* (Greening et al., 2015a), and *Thermogemmatispora* (see **Chapter 2**). HucE proteins were encoded by various Cyanobacteria, which are known to recycle H_2 produced during the nitrogenase reaction via group 2a [NiFe]-hydrogenases (Houchins and Burris, 1981, Tamagnini et al., 2002), as well as genera capable of aerobic hydrogenotrophic growth such as *Nitrospira* (Koch et al., 2014), *Pseudonocardia* (Grostern and Alvarez-Cohen, 2013), and *Acidithiobacillus* (Schröder et al., 2007). Of the hydrogenase-positive species surveyed, 9.5% lacked HucE and HhyE, including *Thermomicrobium* (see **Chapter 2**), and *Methylacidiphilum* (Mohammadi et al., 2017) species known to synthesise mid-affinity group 1h [NiFe]-hydrogenases. In contrast, no HucE or HhyE sequences were retrieved from organisms that lack hydrogenases.

Multiple sequence alignments show that HucE and HhyE proteins contain highly conserved motifs potentially involved in binding iron-sulfur clusters (**Figure 4-3 & Figure 4-5**). Both HucE and HhyE contain a CxxC motif within a domain homologous to NifU proteins (Yuvaniyama et al., 2000). The C-terminus of HhyE proteins contains two CxxC motifs typical of iron-sulfur proteins (e.g. rubredoxins). In contrast, the HucE proteins contain an N-terminal motif CxH(x15-18)CxxC that matches the signature motif of Rieske iron-sulfur clusters (Schmidt and Shaw, 2001) (**Figure 4-3**). A subset of the species surveyed contain truncated HucE and HhyE proteins that contain the NifU-like domain but lack the C-terminal domains (**Figure 4-2 & Figure 4-5**).

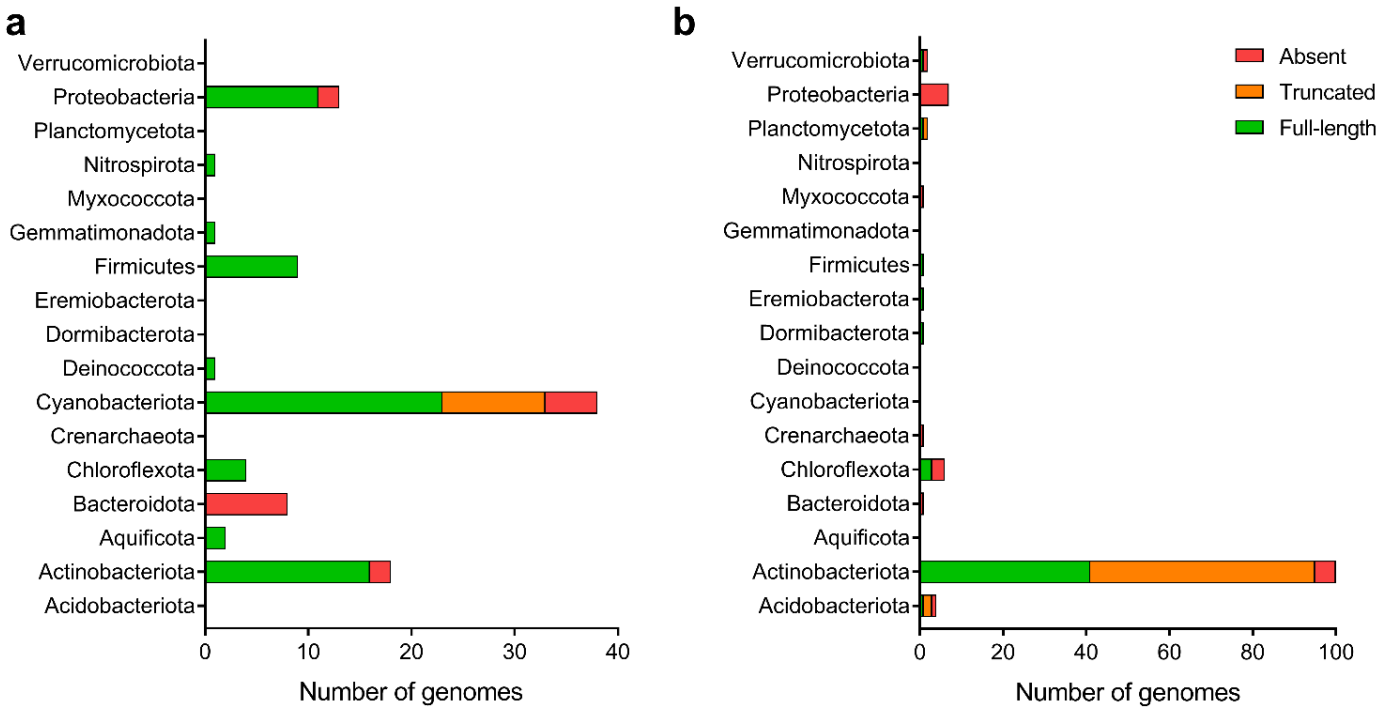


Figure 4-2 (Supplement): **Distribution of HucE and HhyE proteins in hydrogenase-encoding microorganisms**. **(a)** Distribution of HucE proteins in genomes of bacteria that encode group 2a [NiFe]-hydrogenases. **(b)** Distribution of HhyE proteins in genomes of bacteria and archaea that encode group 1h [NiFe]-hydrogenases. Genomes are coloured depending on whether a full-length protein is present, a truncated protein is present, or the protein is absent.

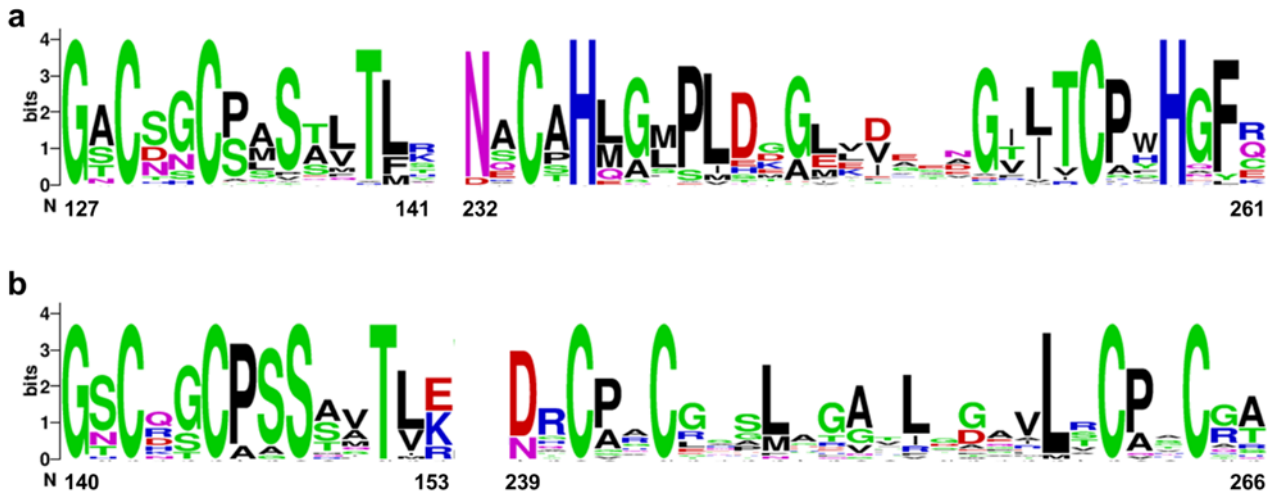


Figure 4-3 (Supplement): **Conservation of cysteine and histidine residues predicted to bind iron-sulfur clusters in HucE and HhyE proteins. (a)** WebLogo of the 52 full-length HucE sequences shown in **Figure 4-1(b)** WebLogo of the 25 full-length HhyL sequences shown in **Figure 4-1**. The numbering refers to the residues of *M. smegmatis* HucE and HhyL respectively as per **Figure 4-2**.

In *M. smegmatis*, the putative localization of these proteins with their greater hydrogenase operons has been shown to be downstream of the catalytic subunits (Berney et al., 2014b), HucL and HhyL (**Figure 4-4**). Whilst there are similarities in the accessory and maturation proteins encoded within the greater gene clusters, there are some unique accessory proteins present for the Group 2a hydrogenase (e.g. AraC and a NHL repeat domain protein), possibly pertaining to the observed differences in H_2 oxidation activity of these enzymes (Cordero et al., 2019b).

4.5.2 HucE and HhyE are essential for H_2 oxidation in Mycobacterium smegmatis

We used allelic exchange mutagenesis to generate markerless single and double mutants of the *hucE* and *hhyE* genes in *M. smegmatis*, i.e. Δ*hucE*, Δ*hhyE*, and Δ*hucE*Δ*hhyE*. Gene deletion was confirmed by PCR targeting chromosomal sequences adjacent to the flanking regions used for homologous recombination **(Figure 4-6)**. Assays were used to compare H_2 oxidation of these strains with the wild-type strain and strains containing previously generated deletions of the hydrogenase structural subunits, i.e. Δ*hucS*, Δ*hhyL*, and Δ*hucS*Δ*hhyL*, that lack hydrogenase activity (Berney and Cook, 2010, Berney et al., 2014b, Greening et al., 2014a).

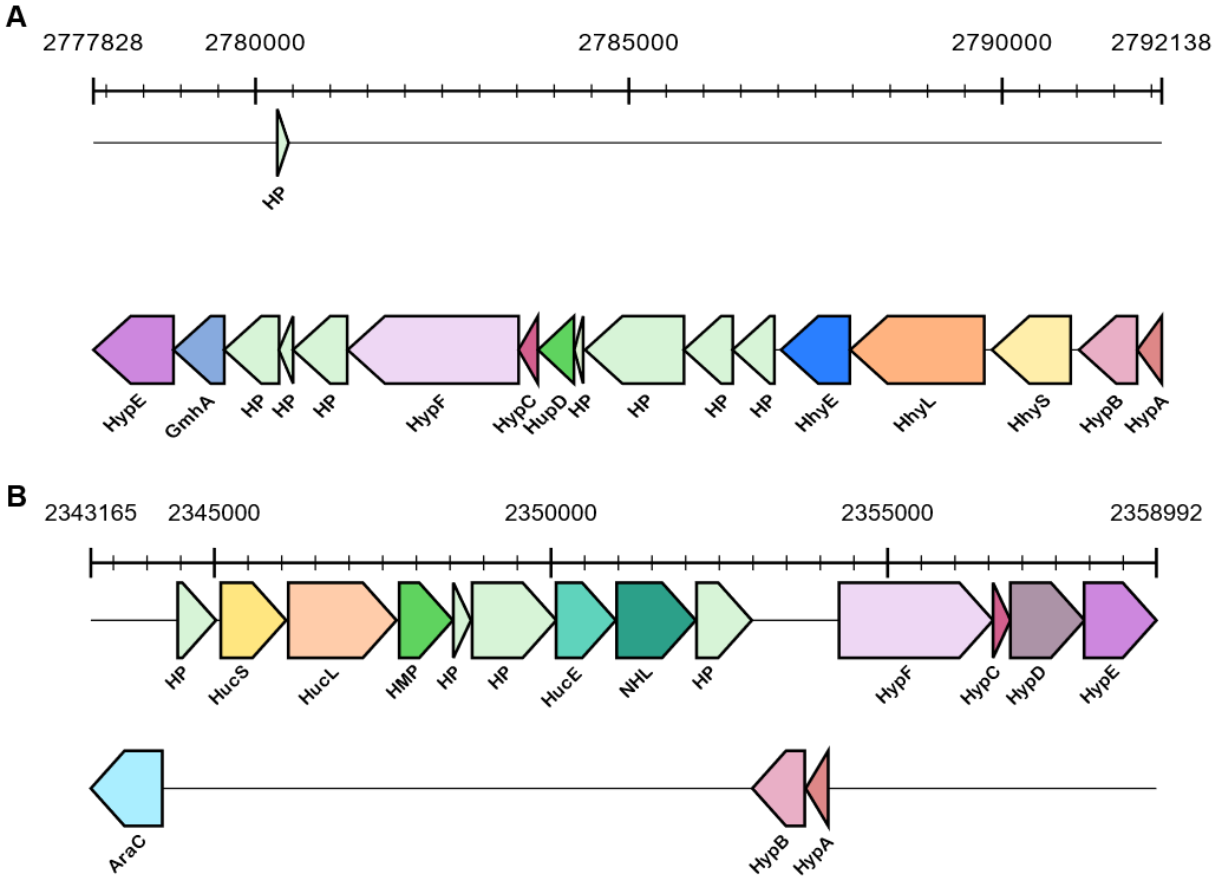
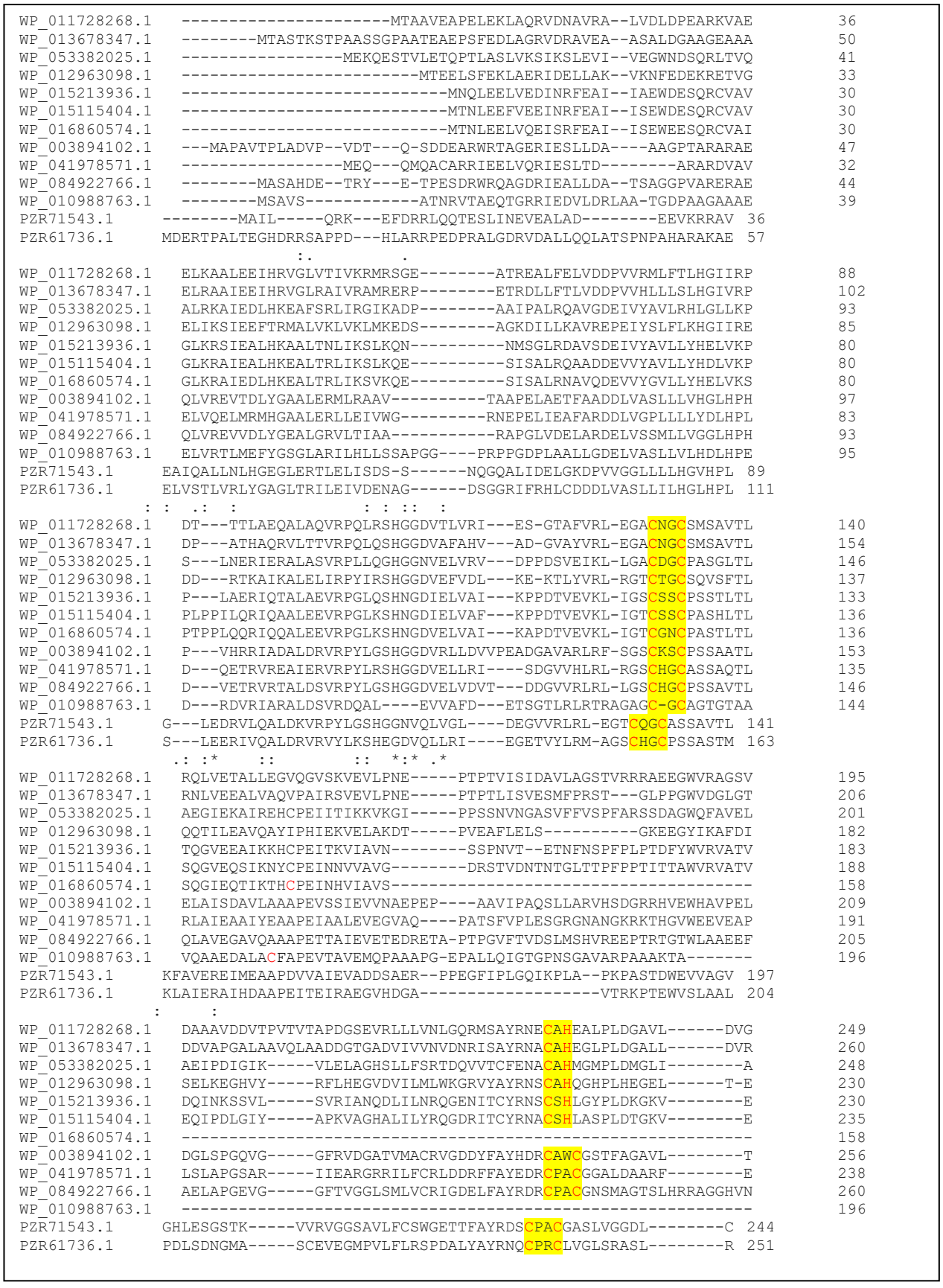


Figure 4-4 (Supplement): **Predicted genetic organisation of the Group 1h and 2a hydrogenase operons of *M. smegmatis*.** (**A**) Gene cluster organisation of the Group 1h [NiFe]-hydrogenase, Hhy. (**B**) Gene cluster organisation of the Group 2a [NiFe]-hydrogenase, Huc. The genes surrounding the catalytic subunits of each hydrogenase are represented to scale. Genes have been colour coded as follows: yellow = small subunit; orange = catalytic subunit; light green = conserved hypothetical protein; red = HypA; rose = HypB; crimson = HypC; mauve = HypD; purple = HypE; lilac = HypF; bright green = hydrogenase maturation protease; forest green = NHL repeat protein; teal = HucE; blue = HhyE; indigo = GmhA; light blue = AraC. Genes have been labelled according to previously defined nomenclature (Berney et al., 2014b).


WP_011728268.1 ----------MTAAVEAPELEKLAQRVDNAVRA--LVDLDPEARKVAE 36
WP_013678347.1 --------MTASTKSTPAASSGPAATEAEPSFEDLAGRVDRAVEA--ASALDGAAGEAAA 50
WP_053382025.1 ----------MEKQESTVLETQPTLASLVKSIKSLEVI--VEGWNDSQRLTVQ 41
WP_012963098.1 ----------MTEELSFEKLAERIDELLAK--VKNFEDEKRETVG 33
WP_015213936.1 ----------MNQLEELVEDINRFEAI--IAEWDESQRCVAV 30
WP_015115404.1 ----------MTNLEEFVEEINRFEAI--ISEWDESQRCVAV 30
WP_016860574.1 ----------MTNLEELVQEISRFEAI--ISEWEESQRCVAI 30
WP_003894102.1 ---MAPAVTPLADVP--VDT---Q-SDDEARWRTAGERIESLLDA----AAGPTARARAE 47
WP_041978571.1 ----------MEQ---QMQACARRIEELVQRIESLTD--------ARARDVAV 32
WP_084922766.1 --------MASAHDE--TRY---E-TPESDRWRQAGDRIEALLDA--TSAGGPVARERAE 44
WP_010988763.1 --------MSAVS----------ATNRVTAEQTGRRIEDVLDRLAA-TGDPAAGAAAE 39
PZR71543.1 --------MAIL-----QRK---EFDRRLQQTESLINEVEALAD--------EEVKRRAV 36
PZR61736.1 MDERTPALTEGHDRRSAPPD---HLARRPEDPRALGDRVDALLQQLATSPNPAHARAKAE 57
WP_011728268.1 ELKAALEEIHRVGLVTIVKRMRSGE--------ATREALFELVDDPVVRMLFTLHGIIRP 88
WP_013678347.1 ELRAAIEEIHRVGLRAIVRAMRERP--------ETRDLLFTLVDDPVVHLLLSLHGIVRP 102
WP_053382025.1 ALRKAIEDLHKEAFSRLIRGIKADP--------AAIPALRQAVGDEIVYAVLRHLGLLKP 93
WP_012963098.1 ELIKSIEEFTRMALVKLVKLMKEDS--------AGKDILLKAVREPEIYSLFLKHGIIRE 85
WP_015213936.1 GLKRSIEALHKAALTNLIKSLKQN----------NMSGLRDAVSDEIVYAVLLYHELVKP 80
WP_015115404.1 GLKRAIEALHKEALTRLIKSLKQE----------SISALRQAADDEVVYAVLLYHDLVKP 80
WP_016860574.1 GLKRAIEDLHKEALTRLIKSVKQE----------SISALRNAVQDEVVYGVLLYHELVKS 80
WP_003894102.1 QLVREVTDLYGAALERMLRAAV----------TAAPELAETFAADDLVASLLLVHGLHPH 97
WP_041978571.1 ELVQELMRMHGAALERLLEIVWG---------RNEPELIEAFARDDLVGPLLLLYDLHPL 83
WP_084922766.1 QLVREVVDLYGEALGRVLTIAA----------RAPGLVDELARDELVSSMLLVGGLHPH 93
WP_010988763.1 ELVRTLMEFYGSGLARILHLLSSAPGG----PRPPGDPLAALLGDELVASLLVLHDLHPE 95
PZR71543.1 EAIQALLNLHGEGLERTLELISDS-S------NQGQALIDELGKDPVVGGLLLLHGVHPL 89
PZR61736.1 ELVSTLVRLYGAGLTRILEIVDENAG------DSGGRIFRHLCDDDLVASLLILHGLHPL 111
WP_011728268.1 DT---TTLAEQALAQVRPQLRSHGGDVTLVRI---ES-GTAFVRL-EGACNGCSMSAVTL 140
WP_013678347.1 DP---ATHAQRVLTTVRPQLQSHGGDVAFAHV---AD-GVAYVRL-EGACNGCSMSAVTL 154
WP_053382025.1 S---LNERIERALASVRPLLQGHGGNVELVRV---DPPDSVEIKL-LGACDGCPASGLTL 146
WP_012963098.1 DD---RTKAIKALELIRPYIRSHGGDVEFVDL---KE-KTLYVRL-RGTCTGCSQVSFTL 137
WP_015213936.1 P---LAERIQTALAEVRPGLQSHNGDIELVAI---KPPDTVEVKL-IGSCSSCPSSTLTL 133
WP_015115404.1 PLPPILQRIQAALEEVRPGLKSHNGDIELVAF---KPPDTVEVKL-IGTCSSCPASHLTL 136
WP_016860574.1 PTPPLQQRIQQALEEVRPGLKSHNGDVELVAI---KAPDTVEVKL-IGTCGNCPASTLTL 136
WP_003894102.1 P---VHRRIADALDRVRPYLGSHGGDVRLLDVVPEADGAVARLRF-SGSCKSCPSSAATL 153
WP_041978571.1 D---QETRVREAIERVRPYLRSHGGDVELLRI----SDGVVHLRL-RGSCHGCASSAQTL 135
WP_084922766.1 D---VETRVRTALDSVRPYLGSHGGDVELVDVT---DDGVVRLRL-LGSCHGCPSSAVTL 146
WP_010988763.1 D---RDVRIARALDSVRDQAL----EVVAFD---ETSGTLRLRTRAGAGC-GCAGTGTAA 144
PZR71543.1 G---LEDRVLQALDKVRPYLGSHGGNVQLVGL----DEGVVRLRL-EGTCQGCASSAVTL 141
PZR61736.1 S---LEERIVQALDRVRVYLKSHEGDVQLLRI----EGETVYLRM-AGSCHGCPSSASTM 163
WP_011728268.1 RQLVETALLEGVQGVSKVEVLPNE-----PTPTVISIDAVLAGSTVRRRAEEGWVRAGSV 195
WP_013678347.1 RNLVEEALVAQVPAIRSVEVLPNE-----PTPTLISVESMFPRST---GLPPGWVDGLGT 206
WP_053382025.1 AEGIEKAIREHCPEIITIKKVKGI-----PPSSNVNGASVFFVSPFARSSDAGWQFAVEL 201
WP_012963098.1 QQTILEAVQAYIPHIEKVELAKDT-----PVEAFLELS----------GKEEGYIKAFDI 182
WP_015213936.1 TQGVEEAIKKHCPEITKVIAVN--------SSPNVT--ETNFNSPFPLPTDFYWVRVATV 183
WP_015115404.1 SQGVEQSIKNYCPEINNVVAVG--------DRSTVDNTNTGLTTPFPPTITTAWVRVATV 188
WP_016860574.1 SQGIEQTIKTHCPEINHVIAVS---------- 158
WP_003894102.1 ELAISDAVLAAAPEVSSIEVVNAEPEP----AAVIPAQSLLARVHSDGRRHVEWHAVPEL 209
WP_041978571.1 RLAIEAAIYEAAPEIAALEVEGVAQ----PATSFVPLESGRGNANGKRKTHGVWEEVEAP 191
WP_084922766.1 QLAVEGAVQAAAPETTAIEVETEDRETA-PTPGVFTVDSLMSHVREEPTRTGTWLAAEEF 205
WP_010988763.1 VQAAEDALACFAPEVTAVEMQPAAAPG-EPALLQIGTGPNSGAVARPAAAKTA------- 196
PZR71543.1 KFAVEREIMEAAPDVVAIEVADDSAER--PPEGFIPLGQIKPLA--PKPASTDWEVVAGV 197
PZR61736.1 KLAIERAIHDAAPEITEIRAEGVHDGA--------VTRKPTEWVSLAAL 204
WP_011728268.1 DAAAVDDVTPVTVTAPDGSEVRLLLVNLGQRMSAYRNECAHEALPLDGAVL------DVG 249
WP_013678347.1 DDVAPGALAAVQLAADDGTGADVIVVNVDNRISAYRNACAHEGLPLDGALL------DVR 260
WP_053382025.1 AEIPDIGIK-----VLELAGHSLLFSRTDQVVTCFENACAHMGMPLDMGLI--------A 248
WP_012963098.1 SELKEGHVY-----RFLHEGVDVILMLWKGRVYAYRNSCAHQGHPLHEGEL------T-E 230
WP_015213936.1 DQINKSSVL-----SVRIANQDLILNRQGENITCYRNSCSHLGYPLDKGKV--------E 230
WP_015115404.1 EQIPDLGIY-----APKVAGHALILYRQGDRITCYRNACSHLASPLDTGKV--------E 235
WP_016860574.1 ---------- 158
WP_003894102.1 DGLSPGQVG-----GFRVDGATVMACRVGDDYFAYHDRCAWCGSTFAGAVL--------T 256
WP_041978571.1 LSLAPGSAR-----IIEARGRRILFCRLDDRFFAYEDRCPACGGALDAARF--------E 238
WP_084922766.1 AELAPGEVG-----GFTVGGLSMLVCRIGDELFAYRDRCPACGNSMAGTSLHRRAGGHVN 260
WP_010988763.1 ---------- 196
PZR71543.1 GHLESGSTK-----VVRVGGSAVLFCSWGETTFAYRDSCPACGASLVGGDL--------C 244
PZR61736.1 PDLSDNGMA-----SCEVEGMPVLFLRSPDALYAYRNQCPRCLVGLSRASL--------R 251

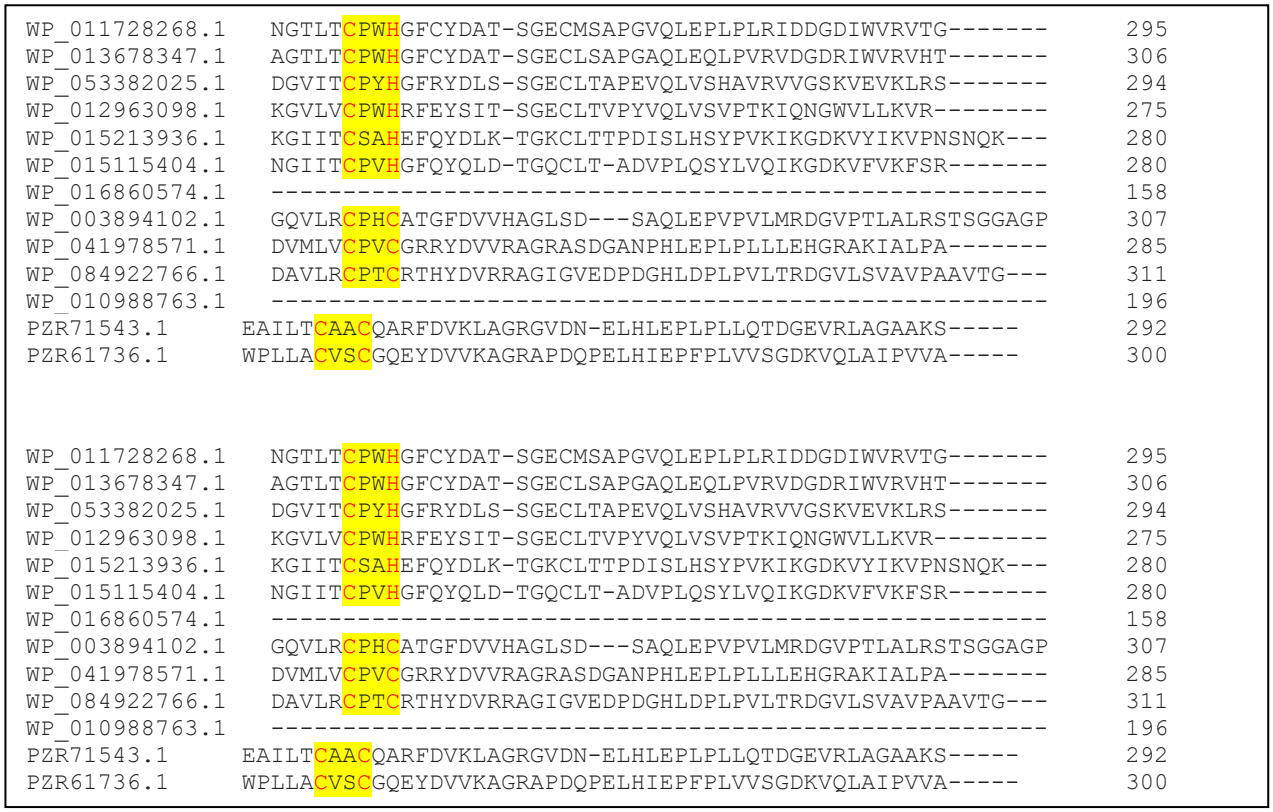


Figure 4-5 (Supplement): **Multiple sequence alignment of the HucE and HhyE family proteins from bacteria that have been shown or predicted to mediate aerobic H_2 respiration.** The proteins shown are: WP_011728268.1 *(Mycobacterium smegmatis* HucE*),* WP_013678347.1 *(Pseudonocardia dioxanivorans* HucE*),* WP_053382025.1 *(Nitrospira moscoviensis* HucE), WP_012963098.1 *(Hydrogenobacter thermophilus* HucE), WP_015213936.1 *(Anabaena cylindrica* HucE), WP_015115404.1 *(Nostoc sp. PCC 7107* HucE), WP_016860574.1 (*Fischerella muscicola* HucE truncated), WP_003894102.1 *(Mycobacterium smegmatis* HhyE), WP_041978571.1 *(Pyrinomonas methylaliphatogenes* HhyE), WP_084922766.1 *(Rhodococcus equi* HhyE), WP_010988763.1 *(Streptomyces avermitilis* HhyE truncated), PZR71543.1 *(Candidatus Dormibacterota* bacterium HhyE), PZR61736.1 *(Candidatus Eremiobacterota* bacterium HhyE). The conserved cysteine and histidine residues predicted to bind iron-sulfur clusters are coloured in red and the motifs are highlighted in yellow.

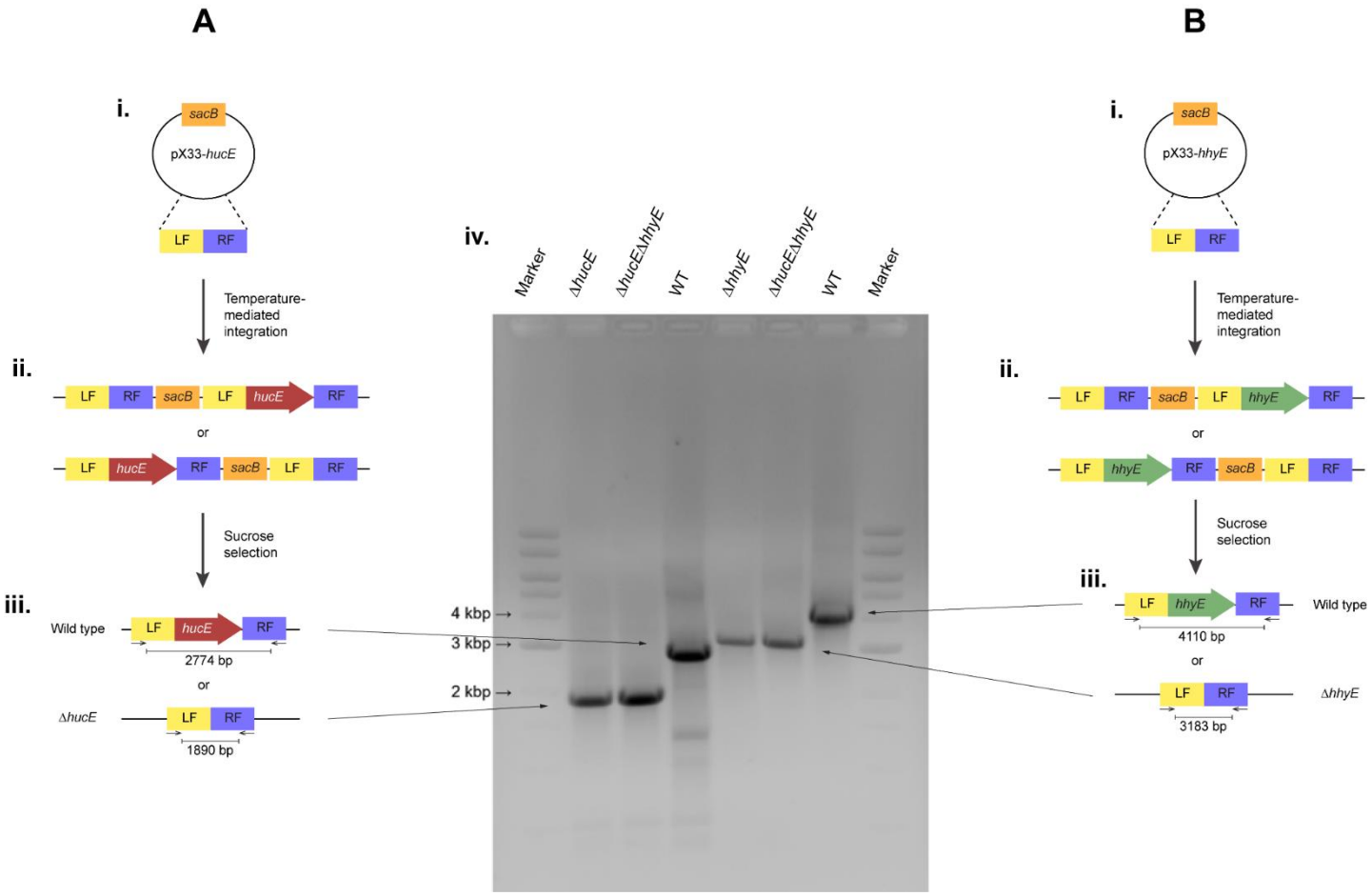


Figure 4-6 (Supplement): **Deletion of the *hucE* and *hhyE* genes in *Mycobacterium smegmatis.*** The schematic shows the four main steps that led to the production of knockouts of (A) *hucE* (MSMEG_2268) and (B) *hhyE* (MSMEG_2718). (i) Construction of the pX33-*hucE* / pX33-*hhyE* vector containing a fused left flank (LF) and right flank (RF) of the gene. (ii) Temperature-mediated integration of the vector into the *M. smegmatis* chromosome via either the left flank or right flank of the chromosomal *hucE* / *hhyE* gene. (iii) Chromosomal excision of the vector due to sacB-mediated sucrose toxicity to either wild-type revertants or Δ*hucE* / Δ*hhyE* mutants. (iv) PCR-based screening through primers (2267_chrom_fwd, 2269_chrom_rvs, 2717_chrom_fwd, and 2719_chrom_rvs) targeting the flanks to confirm whether colonies are (A) wild-type revertant (2774 bp product) or Δ*hucE* mutant (1890 bp product) and (B) wild-type revertant (4110 bp product) or Δ*hhyE* mutant (3183 bp product). Both primer sets were used to confirm the double mutant.

We first used a H_2 electrode to measure rates of aerobic H_2 respiration mediated by whole cells of each strain. There were significant differences in the rate of H_2 oxidation for all deletion strains compared to the wild-type **(Figure 4-7)**. Loss of *hucE* and *hhyE* resulted in reductions of 1.8-fold and 8.4-fold respectively; such reductions were statistically indistinguishable from those observed in the mutants of the hydrogenase structural subunits *hucS* and *hhyL*. Deletion of both iron-sulfur proteins (Δ*hucE*Δ*hhyE*) or both hydrogenase structural subunits (Δ*hucS*Δ*hhyL*) caused complete cessation of H_2 oxidation, highlighting that these two hydrogenases are solely responsible for H_2 oxidation and that the putative iron-sulfur proteins are indispensable for this process. The negative rates of H_2 oxidation in Δ*hucE*Δ*hhyE* and Δ*hucS*Δ*hhyL* strains is most likely background noise due to the detection limit of the electrode rather than actual H_2 production by Hyh (Hyd3), since this hydrogenase is only upregulated during hypoxia (Berney et al., 2014a). The lack of significant differences between the strains containing deletions of the catalytic subunits, compared to the putative iron-sulfur proteins, are consistent with the model that HucE and HhyE are functionally linked with the Huc and Hhy hydrogenases.

In an interrelated assay, we performed activity staining of the Huc and Hhy hydrogenases using whole-cell lysates of wild-type and deletion mutant strains in the presence of the artificial electron acceptor nitroblue tetrazolium. Bands corresponding to functional hydrogenase activity could be observed for wild-type, Δ*hucE*, and Δ*hucS* strains, but not the other strains (**Figure 4-8**). The similarity in the staining bands observed between Δ*hucS* and Δ*hucE* strains or between Δ*hhyL* and Δ*hhyE* indicate that the putative iron-sulfur proteins HucE and HhyE, like their respective catalytic subunits HucS and HhyL, are important for hydrogenase activity. The artificial electron acceptor cannot compensate for the loss of HucE/HhyE and neither can HucE for HhyE nor HhyE for HucE. This further supports the model that HucE and HhyE form a functional association with Huc and Hhy, respectively.

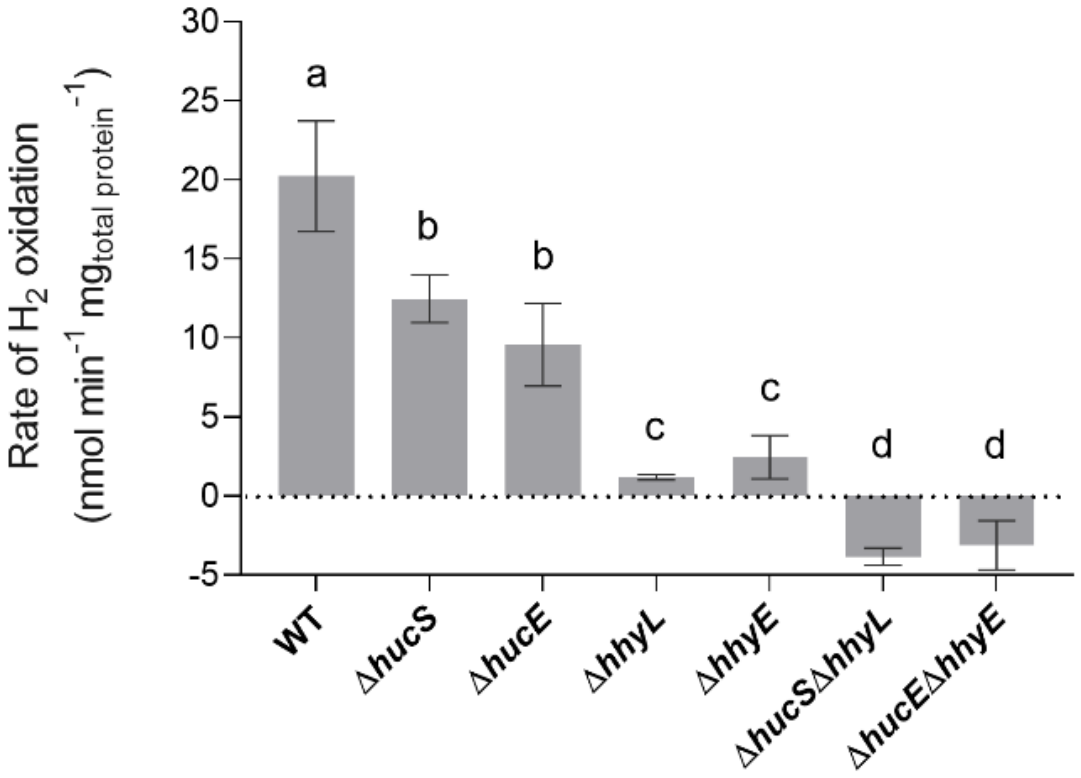


Figure 4-7: **Hydrogen oxidation by wild-type and derived mutant strains of *M. smegmatis.*** H_2 uptake by whole cells in mid-stationary phase (72 h post OD_{max}) was measured amperometrically using a Unisense H_2 electrode. (A) Comparison of the rates of H_2 oxidation between wild type, single and double mutants of the iron-sulfur proteins (Δ*hucE*, Δ*hhyE*, Δ*hucE*Δ*hhyE*), and single and double mutants of hydrogenase structural subunits (Δ*hucS*, Δ*hhyL*, Δ*hucS*Δ*hhyL*). Error bars show standard deviations of three biological replicates and values labelled with different letters are significantly different ($p < 0.05$) based on a one-way ANOVA. The respirometry measurements in this figure were performed by Paul Cordero.

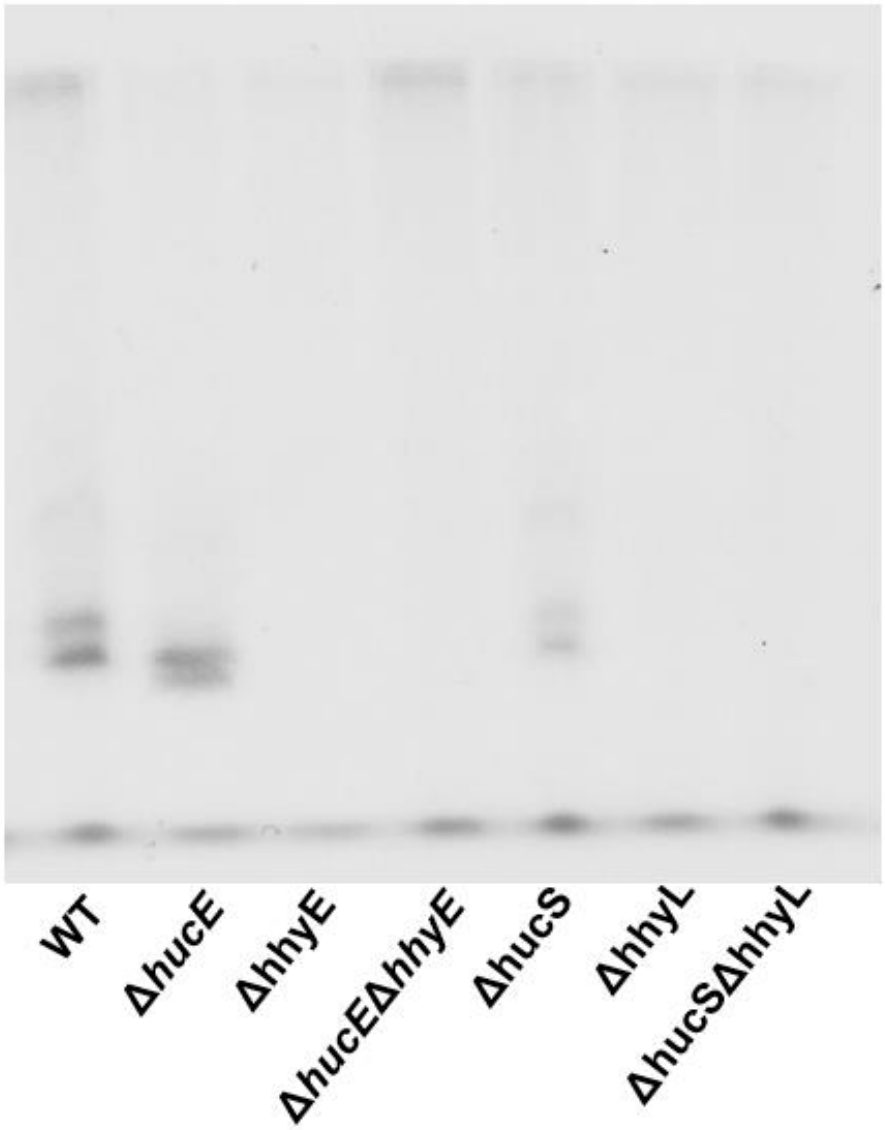


Figure 4-8: **Comparison of hydrogenase activity of wild-type and derived mutant strains of *Mycobacterium smegmatis.*** Seven strains were tested in mid-stationary phase (three days post OD_{max}): wild-type (WT), single and double mutants of the iron-sulfur proteins (Δ*hucE*, Δ*hhyE*, Δ*hucE*Δ*hhyE*), and single and double mutants of hydrogenase structural subunits (Δ*hucS*, Δ*hhyL*, Δ*hucS*Δ*hhyL*). Zymographic staining of H_2 uptake of whole-cell lysates using the artificial electron acceptor nitroblue tetrazolium in a H_2-rich atmosphere. The original gel and Coomassie stain are shown in **Figure 4-9**.

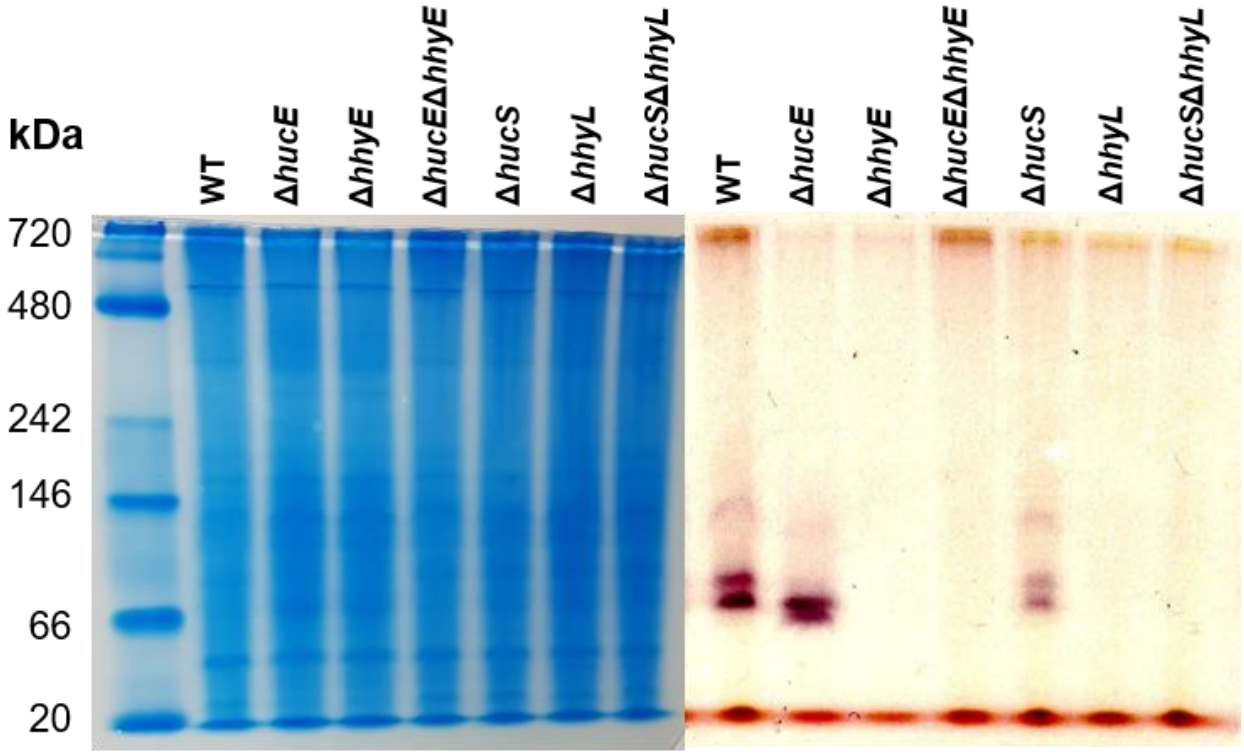


Figure 4-9 (Supplement**): Original native polyacrylamide gels showing staining of hydrogenase activity**. The right gel shows hydrogenase activity stained with the artificial electron acceptor nitroblue tetrazolium in a H_2-rich atmosphere. The left gel shows protein marker and total protein stained with Coomassie Blue.

4.5.3 HucE and HhyE mutant strains have significant growth and survival defects

Previous genetic studies have shown that the hydrogenases modestly increase growth yield and long-term survival of *M. smegmatis* (Berney and Cook, 2010, Greening et al., 2014b). We therefore tested whether these findings extended to the putative iron-sulfur proteins by analysing the growth rate, growth yield, and long-term survival of the seven aforementioned strains when cultured aerobically on rich media (LBT). In line with previous findings (Berney et al., 2014b, Greening et al., 2014b), no significant differences in specific growth rate were observed between the strains **(Figure 4-10a)**. However, there was a 10% reduction in the specific growth yield of the HhyE mutant compared to the wild-type strain ($OD_{max\ wt}$ = 4.19 ± 0.21; $OD_{max\ \Delta hhyE}$ = 3.81 ± 0.09; p = 0.008) **(Figure 4-10b)**. This phenotype extended to the double mutant strain (Δ*hucE*Δ*hhyL*) and again phenocopied single and double mutants lacking the *hhyL* gene.

We also tested whether the strains were defective in long-term survival by counting colonies 21 days following OD_{max}. There were significant reductions in the survival of all strains, apart from the Δ*hucE*, compared to the wild-type **(Figure 4-10c)**. Cell counts were approximately two-fold lower for the Δ*hhyE* and Δ*hhyL* strains ($p < 0.02$), and four-fold lower for the double mutant strains ($p < 0.002$), relative to the wild-type. These findings agree with previous reports that atmospheric H_2 oxidation by the hydrogenases enables *M. smegmatis* to survive energy starvation (Greening et al., 2014b) and further supports that the putative iron-sulfur proteins contribute to this function.

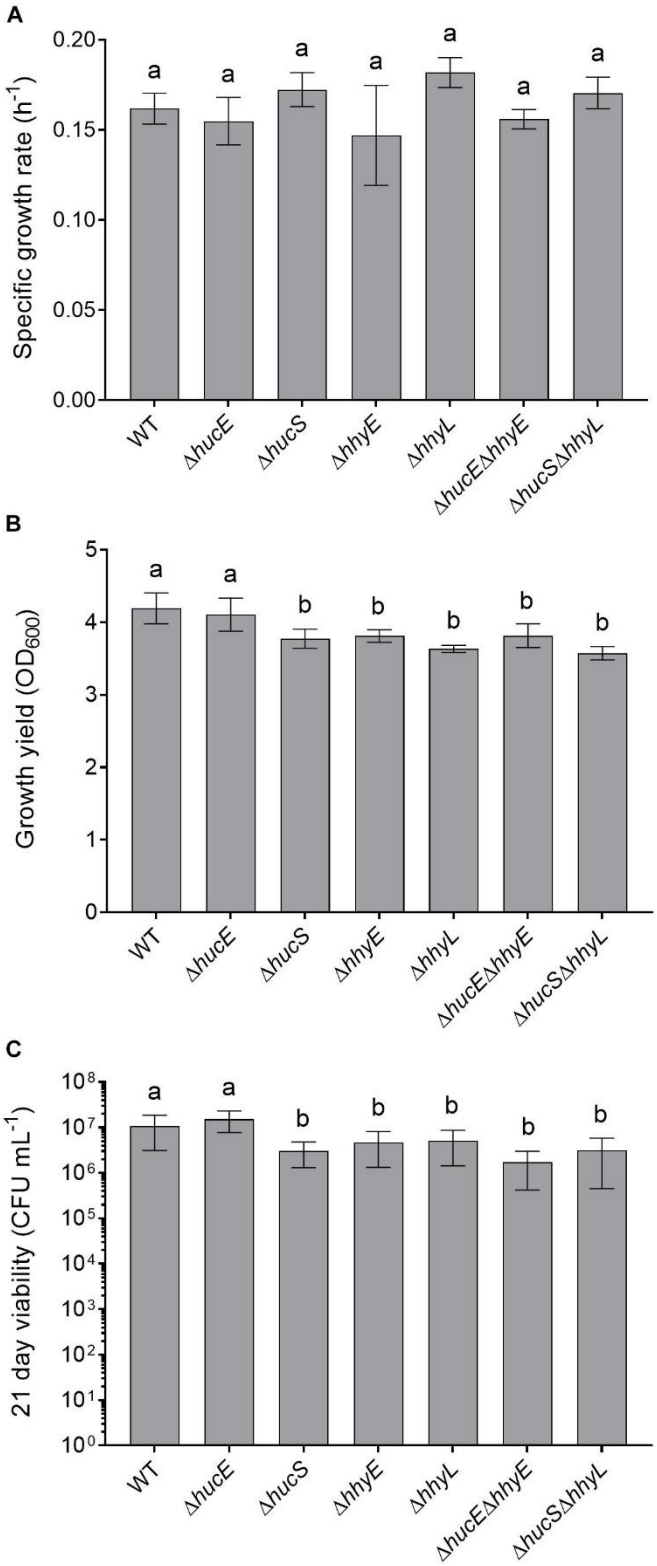


Figure 4-10: **Comparison of growth and survival between wild-type and mutant strains of *M. smegmatis*.** Seven strains were grown on lysogeny broth supplemented with Tween80 (LBT): wild-type, single and double mutants of the iron-sulfur proteins (Δ*hucE*, Δ*hhyE*, Δ*hucE*Δ*hhyE*), and single and double mutants of hydrogenase structural subunits (Δ*hucS,* Δ*hhyL*, Δ*hucS*Δ*hhyL*). (**a**) Specific growth rate (μ) during exponential phase. (**b**) Final growth yield (OD_{max}) at 24 hours post-stationary phase. (**c**) Long-term survival (CFU mL^{-1}) at 21 days post-stationary phase. Error bars show standard deviations of six biological replicates. Values labelled with different letters are significantly different ($p < 0.05$) based on a one-way ANOVA.

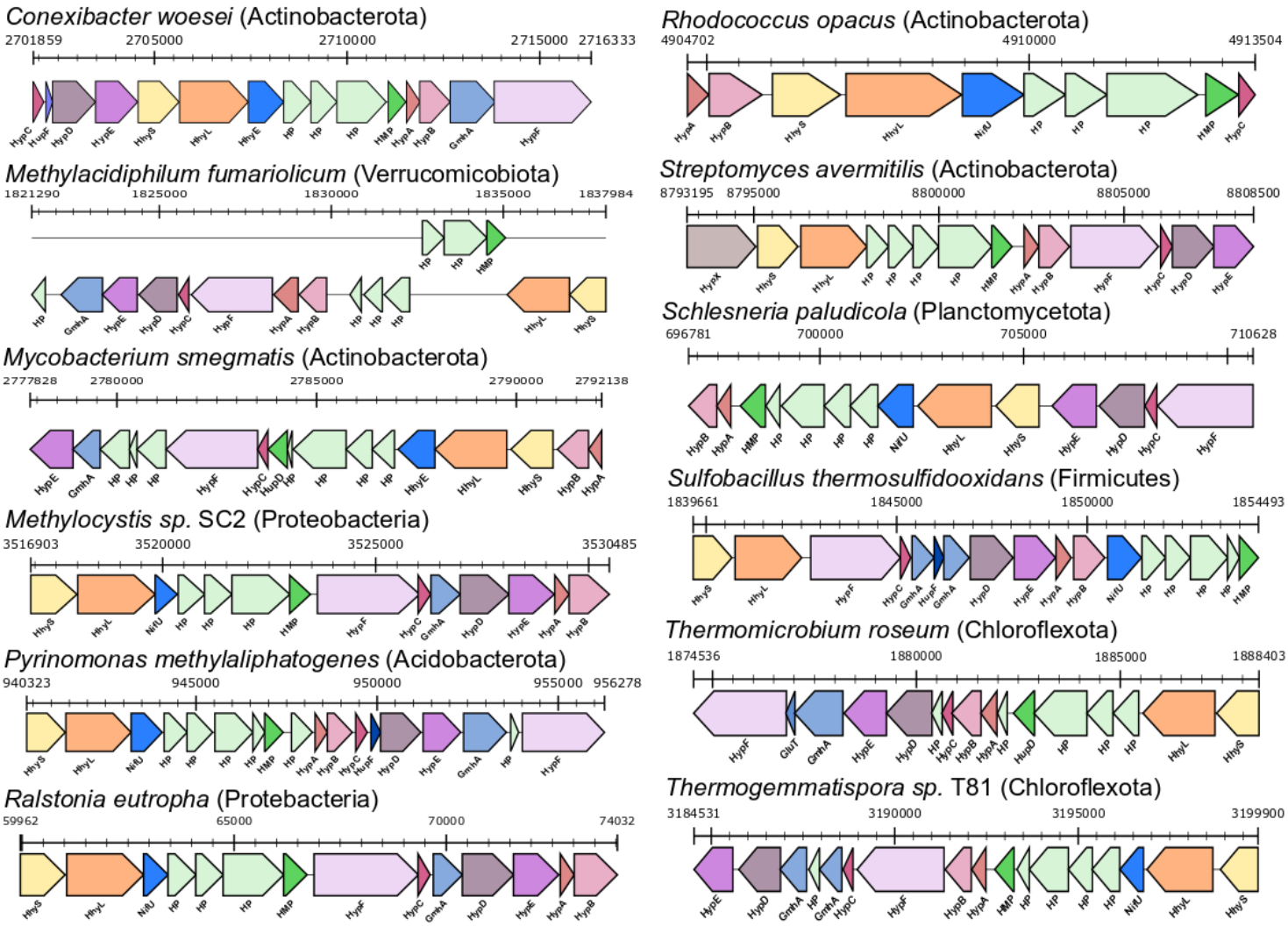


Figure 4-11 (Supplement): **Genetic organisation of group 1h [NiFe]-hydrogenases in 12 distinct bacterial species.** Abbreviations: HhyL = hydrogenase large subunit; HhyS = hydrogenase small subunit; HypABCDEF = hydrogenase maturation factors; HMP = hydrogenase maturation peptidase; HP = conserved hypothetical protein; GmhA = putative phosphoheptose isomerase; NifU = NifU-like iron-sulfur protein (HhyE); GluT = gluteredoxin. Gene length is shown to scale and gene identifiers are as per the nomenclature of HydDB.

4.6 Discussion

In summary, this study shows that HucE and HhyE are required for the enzymatic activity and physiological function of the mycobacterial uptake hydrogenases. Strains lacking these proteins showed no hydrogenase activity in either amperometric or zymographic assays. Furthermore, they exhibited growth and survival phenotypes similar to those of knockouts of hydrogenase structural subunits (Greening and Cook, 2014), in part due to the numerous survival mechanisms present in *M. smegmatis* such as persistence on carbon monoxide (Cordero et al., 2019a). Despite some sequence similarity between the two proteins, they are non-redundant, as there was no compensation in hydrogenase activity in the single mutant strains. The genomic survey and phylogenetic analysis indicate that *hucE* and *hhyE* genes co-evolved with the genes encoding the structural subunits of the group 2a and group 1h [NiFe]-hydrogenases. Their detection in the genomes of most but not all characterized high-affinity H_2 oxidizers encoding group 1h [NiFe]-hydrogenases indicate they are an important but overlooked mediators of atmospheric H_2 oxidation (**Figure 4-11**). They are also associated with the group 2a [NiFe]-hydrogenases of H_2-recycling Cyanobacteriota and various aerobic hydrogenotrophic bacteria that are not currently known to oxidize atmospheric H_2.

This study lends some support to the hypothesis that these proteins serve as the immediate electron acceptors for the group 2a and group 1h [NiFe]-hydrogenases. There are broadly four lines of evidence that support this hypothesis: (i) the presence of highly conserved motifs for binding iron-sulfur clusters, (ii) the essentiality of these proteins for the function of these hydrogenases, (ii) their association with the structural rather than maturation operons of the hydrogenases (Berney et al., 2014b), and (iv) their genomic association with hydrogenases that lack known electron transfer subunits (e.g. cytochrome *b* subunits). With the respect to the latter point, it is interesting that these proteins are conserved in Cyanobacteria, given the immediate electron acceptors of their uptake hydrogenases have long remained enigmatic

(Tamagnini et al., 2002). It is also notable that HucE proteins encode the signature motifs of a Rieske iron-sulfur cluster. Given their unusual ligands, these clusters have a higher standard redox potential (E_o' > -150 mV) than most iron-sulfur clusters (e.g. ferredoxins) (Brown et al., 2008). They would therefore be well-poised to accept the relatively high-potential electrons derived from atmospheric H_2 and transfer them to menaquinone. Consistently, zymographic studies suggest that the high-affinity hydrogenases operate at higher redox potential than prototypical hydrogenases, given they are reactive with the nitroblue tetrazolium (E_o' = -80 mV) but not viologen compounds (E_o' = -360 mV) (Greening et al., 2014a, Pinske et al., 2012). Comparatively, the HhyE proteins are more similar to traditional bacterial ferredoxins, suggesting that the differences between the two putative iron-sulfur cluster proteins are specific for the functions of group 2a and 1h hydrogenases, rather than interchangeable.

While this study demonstrates HucE and HhyE are important for mycobacterial growth as well as hydrogenase function, further work is ultimately needed to resolve their respective functions. While a role in electron transfer is most plausible, other roles are also possible and compatible with the available evidence, for example as specific assembly factors and/or structural scaffolds for the hydrogenases. Another potential role could be in metal cofactor oxygen tolerance, as previously determined for the group 1d [NiFe]-hydrogenase in *R. eutropha* (Fritsch et al., 2014). Furthermore, the presence of conserved hypothetical proteins of yet unknown function downstream of HucE and HhyE may also be potential electron acceptor candidates, in particular MSMEG2717 which shares homology to PHG067, the proposed electron acceptor of *R. eutropha* (Schäfer et al., 2013). Biochemical studies, including studying the redox chemistry of these proteins and their interactions with the as-yet-unpurified hydrogenases, are now required to distinguish these possibilities and develop a sophisticated understanding of their function.

5 DISCUSSION AND OUTLOOK

5.1 Discussion

It is critical to determine the genetics, biochemistry, physiology, and distribution of atmospheric trace gas oxidation to understand how microorganisms influence biogeochemical cycling. By using an interdisciplinary approach, this thesis has provided evidence that numerous bacterial phyla mediate atmospheric H_2 and CO oxidation, validating previous genomic and metagenomic surveys on the distribution of aerobic trace gas oxidisers (Constant et al., 2011b, Greening et al., 2016, Ji et al., 2017, King and Weber, 2007, Leung et al., 2020). The thesis has also shed light on the molecular mechanisms through which high-affinity H_2 oxidation occurs. Moreover, these results have challenged prevailing paradigms on the role of energy derived from oxidising trace atmospheric gases, demonstrating that H_2 can be utilised during both mixotrophic growth and persistence, and that CO can be used during persistence. Despite the advances generated in this thesis, there remain a number of yet unanswered questions concerning the molecular determinants that enable atmospheric H_2 oxidation, the metabolic flexibility of other previously characterised microorganisms and the resilience of the microbial sinks of trace atmospheric gases in a changing global environment.

5.1.1 Evolutionary, ecological, and biogeochemical ramifications of the diversity of trace gas oxidisers

Using a combination of culture-based physiological, biochemical and phylogenetic analysis, this thesis has highlighted that bacteria previously shown by genomic surveys to harbour potential high affinity hydrogenase and carbon monoxide dehydrogenases are indeed capable of scavenging H_2 and CO, and thus many more bacterial phyla than previously reported are able to oxidise at atmospheric concentrations. In spite of the critical role of microorganisms in constituting the main sinks for both atmospheric H_2 and CO (Ehhalt and Rohrer, 2009, Khalil

and Rasmussen, 1990), only a tiny fraction of the microorganisms capable of atmospheric oxidation have been experimentally validated. Bacteria capable of using atmospheric concentrations of H_2 were only discovered in 2008, with the isolation of the first-high affinity H_2 oxidiser *Streptomyces sp.* PCB7 (Constant et al., 2008). This discovery sparked new culture-based and culture-independent research to identify additional soil bacteria capable of oxidising atmospheric H_2. Multiple species from the phylum Actinobacteriota have been shown to harbour genes encoding a high-affinity, oxygen tolerant hydrogenase (Constant et al., 2011b, Greening et al., 2016), while genes encoding high-affinity hydrogenases have also been observed in at least 13 bacterial phyla (Barz et al., 2010, Greening et al., 2016, Ji et al., 2017, Piché-Choquette et al., 2017, Tamagnini et al., 2002). To date, atmospheric H_2 oxidation has now been experimentally verified in six bacterial phyla, Actinobacteriota (Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Meredith et al., 2014), Acidobacteriota (Greening et al., 2015a, Myers and King, 2016), Verrucomicrobiota (Schmitz et al., 2020), and through this work, Chloroflexota (**Chapters 2** and **3**; (Islam et al., 2019a, Islam et al., 2020)), Proteobacteria (**Chapter 3**; (Islam et al., 2020)) and Gemmatimonadota (**Chapter 3**; (Islam et al., 2020)). Similarly, whilst aerobic carbon monoxide dehydrogenase genes are encoded by at least 16 microbial phyla, only four phyla to date have been shown to utilise atmospheric levels as an alternate energy source during microbial persistence: Actinobacteriota (*Mycobacterium smegmatis*; (Cordero et al., 2019a)), Proteobacteria (*Ruegeria pomeroyi*; (Christie-Oleza et al., 2012, Moran et al., 2004)), Halobacteriota (*Haloferax namakaokahaiae*; (McDuff et al., 2016)) and Chloroflexota (*Thermomicrobium roseum, Thermogemmatispora* sp. T81; (**Chapter 3;** (Islam et al., 2019a)). In effect, the works contained within this thesis have doubled the number of phyla experimentally verified to aerobically oxidise atmospheric concentrations of H_2 and provide evidence of atmospheric CO oxidation in two additional species.

The widespread distribution of the determinants of atmospheric H_2 and CO oxidation suggests this process confers a selective advantage in many ecosystems. This work supports the possibility of multiple horizontal acquisitions of hydrogenase- and CODH-encoding genes in different bacteria (Greening et al., 2016, Greening et al., 2015b, Quiza et al., 2014). For example, in Chloroflexota, group 1h hydrogenases fall into two distinct highly-supported clades within the Chloroflexia and Ktedonobacteria (**Chapter 2**), suggesting independent acquisition from other bacteria rather than vertical inheritance from a common ancestor. This pattern of horizontal acquisition suggests atmospheric H_2 oxidation confers a significant survival advantage to these bacteria. In turn, we theorise that the ability of numerous lineages of bacteria to utilise atmospheric concentrations of H_2 and CO influences the diversity of microorganisms in different ecosystems. The genetic determinants of aerobic H_2 and CO oxidation have previously been identified in the majority of oxygenated ecosystems on Earth, including soils, oceans, sediments and plants (Constant et al., 2011b, Cordero et al., 2019a, Greening et al., 2016, Ji et al., 2017, King and Weber, 2007, Piché-Choquette et al., 2017, Tolli et al., 2006, Kessler et al., 2019, Greening and Maier, 2016, Kanno et al., 2016). Through this work, we have expanded the diversity of ecosystems with high-affinity H_2 and CO oxidisers to include bacteria isolated from three additional ecosystems: geothermal springs (**Chapters 2** and **3**), acidic mine drainage (**Chapter 3**) and wastewater treatment plants (**Chapter 3**). The characterisation of bacteria from these ecosystems suggests an advantage for trace gas scavenging in ecosystems that experience extreme variability in H_2 and CO concentrations (i.e. geothermal springs; (King et al., 2008, Shock et al., 2010, Spear et al., 2005)), but also those which are human-engineered (i.e. mine drainage, wastewater; (Ferrera and Sánchez, 2016, Mielke et al., 2003)). Moreover, the characterisation of aerobic H_2 oxidation in two understudied yet dominant soil phyla, Chloroflexota and Gemmatimonadota (Janssen, 2006), extends this process to two additional phyla that have the potential to be the major oxidisers in

other non-extreme environments. As such, this work indicates that atmospheric trace gas oxidation supports growth and persistence of bacteria across a much wider range of ecosystems that previously thought.

The widespread distribution of enzymes for atmospheric trace gas oxidation across different bacteria and ecosystems may contribute to the overall stability of biogeochemical cycles. In contrast to atmospheric methane oxidation that is restricted to just three phyla, this thesis and previous studies suggests that most dominant soil phyla can oxidise atmospheric H_2 and/or CO. In turn, this suggests that the bacterial sinks for these gases are likely to be fairly resilient to increasing emissions driven by anthropogenic activities. In spite of increasing anthropogenic emissions of H_2 and CO since the Industrial Revolution, the global concentrations of H_2 and CO have largely remained stable due to the strength of the microbial soil sinks (Greening et al., 2015b, King and Weber, 2007, Schultz et al., 2003, Khalil and Rasmussen, 1988). Thus, by identifying new microbial sinks for these gases, this study provides further insights into how resilient these are in a changing global environment. Our results suggest that the atmospheric H_2 sink may not be constricted to organisms encoding group 1h [NiFe]-hydrogenases, with three bacterial phyla capable of atmospheric H_2 oxidation via group 2a [NiFe]-hydrogenases (**Chapter 3**). Moreover, recent studies have suggested that the group 1f hydrogenases (Myers and King, 2016) and group 1l hydrogenases (Ortiz et al., 2020) are novel lineages of oxygen-tolerant high-affinity hydrogenases, indicating that the H_2 sink may be even broader than previously considered.

5.1.2 Dual role of atmospheric H_2 oxidation in mixotrophic growth and persistence

The discovery of atmospheric H_2 oxidising microorganisms has rapidly expanded over the course of the past decade, with an additional three bacterial phyla being experimentally validated to oxidise atmospheric H_2 in this work (**Chapters 2** and **3).** Of the six bacterial phyla that have been experimentally validated to oxidise atmospheric H_2, this study reveals there is a

clear disjuncture between the classes of [NiFe]-hydrogenase that enable aerobic atmospheric H_2 scavenging and which growth phase H_2 is used in. Broadly, group 1h [NiFe]-hydrogenases appear to be primarily used during bacterial persistence, whereas group 2a [NiFe]-hydrogenases are used during mixotrophic growth. To date, the phyla Actinobacteriota (Constant et al., 2010, Constant et al., 2008, Greening et al., 2014a, Meredith et al., 2014), Acidobacteriota (Greening et al., 2015a, King and King, 2014a) and Chloroflexota (**Chapter 2**) have been experimentally shown to primarily use atmospheric H_2 during bacterial persistence. This persistence-linked uptake of atmospheric H_2 is primarily mediated by a group 1h [NiFe]-hydrogenase, though the aforementioned 1f and 1l hydrogenases have also been hypothesised to mediate H_2-dependent persistence (Ortiz et al., 2020). Significantly, these three bacterial phyla have been determined to be the predominant bacterial phyla in highly oligotrophic ecosystems, for example desert ecosystems (Ortiz et al., 2020, Ji et al., 2017), where the oxidation of atmospheric H_2 enables continuous energy generation despite a scarcity of organic substrates (Leung et al., 2020).

Conversely, the phyla that have been characterised to use atmospheric H_2 during mixotrophic growth include Actinobacteriota (*M. smegmatis* specifically; (Berney et al., 2014b, Cordero et al., 2019b, Greening et al., 2014a)), Chloroflexota (**Chapter 3**), Verrucomicrobiota (Schmitz et al., 2020), Gemmatimonadota (**Chapter 3**) and Proteobacteria (**Chapter 3**). To date, the ability to oxidise atmospheric H_2 during growth largely seems to be linked to the possession of a group 2a [NiFe]-hydrogenase, with the exception of the verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* SolV which uses a group 1h [NiFe]-hydrogenase. This distinction suggests more competitive environments might select for the retention of the group 2a hydrogenase that supports mixotrophic growth, whereas energy-limited ecosystems select for the retention of the group 1h hydrogenase that supports bacterial persistence. The presence of group 2a hydrogenases in 13 bacterial phyla, including lineages

not currently known to oxidise H_2 (Islam et al., 2020), suggests that mixotrophic utilisation of atmospheric H_2 may represent a wider energy supplementation strategy for bacteria in environments with variable nutrient availability. As this hydrogenase has also been demonstrated to rapidly oxidise higher concentrations of H_2 (Kawai et al., 2019, Koch et al., 2014, Islam et al., 2020), it may also be able to differentially respond to changes in H_2 concentrations, as potentially mediated by two-component regulatory systems, an AraC or a σ_{54} transcription factor encoded upstream of the structural operon (Lenz et al., 1997, Skibinski et al., 2002, Cordero et al., 2019b).

Despite the apparent duality in roles for atmospheric H_2, there are several actinobacterial species that encode both group 1h and 2a [NiFe]-hydrogenases. These include isolates within the actinobacterial genera *Mycobacterium*, *Frankia*, *Gordonia*, *Pseudonocardia*, *Streptomyces*, and *Thermobispora* (Søndergaard et al., 2016). The presence of both hydrogenases is likely to influence the speed at which H_2 can be taken up from the atmosphere and oxidised, as observed for *M. smegmatis*, which oxidised tropospheric H_2 at significantly faster rates than cultured streptomycetes harbouring only a group 1h hydrogenase (Greening et al., 2014a). Moreover, it is becomingly increasingly clear that group 1h and 2a hydrogenases are differentially expressed and integrated into the aerobic respiratory chain, suggesting atmospheric H_2 can be used in different ways to support the transition between growth and persistence (Cordero et al., 2019b). As such, the prevalence of high-affinity hydrogenases in Actinobacteriota and their ability to utilise H_2 during mixotrophic growth and/or persistence may contribute to their dominance in oxic soil environments, as is also supported by genetic studies showing impaired growth and survival in mutant strains lacking these hydrogenases (Berney and Cook, 2010, Berney et al., 2014a, Greening et al., 2014b, Islam et al., 2019b, Liot and Constant, 2016).

5.1.3 Persistence centric model of CO oxidation

Aerobic carboxydovores harbouring high-affinity CODHs (Cordero et al., 2019a, King and Weber, 2007) represent the largest biological sink of atmospheric CO, accounting for 250 million tonnes of CO removal per year (Bartholomew and Alexander, 1981, Inman et al., 1971). However, the purpose of this process remained unclear: is CO used for growth or for persistence? Previous studies have demonstrated CODHs being upregulated during bacterial persistence in cultures of *Phaeobacter sp.* strain MED193, *Rhodococcus jostii* RHA1 and *Mycobacterium smegmatis* (Berney and Cook, 2010, Muthusamy et al., 2017, Patrauchan et al., 2012). However, these studies did not consider atmospheric CO as a fuel source and were devoid of genetic and biochemical studies that validated the use of CO to enhance bacterial persistence.

In **Chapter 2**, we provided clear genetic and biochemical evidence of bacterial persistence in two Chloroflexota species on atmospheric CO. Previously, both species, *Thermomicrobium roseum* and *Thermogemmatispora sp.* T81, were experimentally shown to use excess concentrations of CO (King and King, 2014a, Wu et al., 2009). This study represents the first indication of atmospheric CO-mediated bacterial persistence and lends credence to the theory of atmospheric CO driving bacterial survival in the same manner as atmospheric H_2. As both species were isolated from geothermal environments that are known to experience large fluctuations in CO concentration (Shock et al., 2010), the ability to utilise sub-atmospheric concentrations would be an evolutionarily advantageous persistence strategy. We extended the findings using the genetically tractable soil bacteria, *M. smegmatis*, whereby we demonstrated lower long-term survival rates in CODH knockout mutants (Cordero et al., 2019a). Concordant with our findings in Chloroflexota, the CODH of *M. smegmatis* was also highly upregulated during carbon starvation and supported aerobic respiration (Cordero et al., 2019a). Taken in

conjunction, these two studies clearly shift the paradigm on CO oxidation in bacteria, suggesting that atmospheric CO may be primarily used during microbial persistence.

5.1.4 Two putative iron-sulfur proteins are critical determinants of atmospheric H_2 oxidation

Within the operons of all [NiFe]-hydrogenases, including those associated with the group 1h and 2a [NiFe]-hydrogenases, there are a number of conserved genes associated with hydrogenase maturation and function, some with unknown functions. The *hypA-F* genes and the conserved maturation endopeptidase have clearly defined roles in hydrogenase maturation (Böck et al., 2006, Greening et al., 2015b, Lacasse and Zamble, 2016, Senger et al., 2017). In this study, we also show that two conserved putative iron-sulfur cluster proteins, HucE and HhyE, are generally clustered in the operons encoding most group 1h and 2a [NiFe]-hydrogenases. We show these are necessary for aerobic H_2 oxidation in *M. smegmatis*, and hypothesise they serve as the electron conduits between the catalytic subunit and the menaquinone pool of the aerobic respiratory chain (**Chapter 4**; (Islam et al., 2019b)). However, further work involving purified HucE and HhyE would be required to validate that they are FeS proteins and that they interact with their respective hydrogenases.

This potential mechanism of electron transfer is not fully conserved across aerobic high-affinity H_2-oxidising organisms. For example, it is hypothesised that the betaproteobacterium *R. eutropha* instead relays electrons into the respiratory chain via a conserved hypothetical protein PHG067 (a HhaA homolog), rather than a HhyE homologue (**Chapter 4;** (Islam et al., 2019b, Schäfer et al., 2013)). This discrepancy may correlate with the difference in thresholds of H_2 able to be oxidised between *R. eutropha* and other organisms harbouring group 1h [NiFe]-hydrogenases, such as *M. smegmatis*¸ *Thermogemmatispora* T81, *R. opacus,* and *P. methylaliphatogenes* (Greening et al., 2014a, Greening et al., 2015a, Islam et al., 2019a, Meredith et al., 2014, Islam et al., 2019b). These aforementioned species have all been

experimentally verified to scavenge H_2 whereas *R. eutropha* is unable to oxidise atmospheric concentrations of H_2 (Schäfer et al., 2013). Thus, it is possible that atmospheric H_2 oxidation is dependent on the association of the catalytic hydrogenase subunits with a putative iron-sulfur cluster protein and potentially also the influences of other conserved hypothetical proteins. However, as there are a number of species that have been verified to utilise atmospheric H_2 but do not encode putative HhyE homologues (**Figure 4-11**), such as *Streptomyces sp.* PCB7, *S. avermitilis*, *T. roseum* and *M. fumariolicum* SolV (Constant et al., 2008, Islam et al., 2019a, Islam et al., 2019b, Liot and Constant, 2016, Schmitz et al., 2020), this suggests there may be other proteins that enable high-affinity H_2 oxidation.

5.2 Outlooks

5.2.1 Molecular determinants of atmospheric H_2 oxidation require characterisation

Understanding the enzymology of atmospheric H_2 oxidation is critical for understanding how bacteria control the biogeochemical H_2 cycle. This will be advanced through ongoing work to purify and characterise the group 1h and 2a [NiFe]-hydrogenases, together with hypothetical proteins that they are co-encoded with. For example, there are three conserved hypothetical proteins of unknown function that are encoded within the *M. smegmatis* group 1h hydrogenase accessory operon, HhaA, HhaB and HhaC (Greening et al., 2015b). To date, my attempts to over-express these proteins have been unsuccessful, though some progress was made with regards to creating knockouts using allelic exchange mutagenesis for analysis of their physiological role. In conjunction with HucE and HhyE, characterisation of the functions of these proteins via protein purification, and associated biochemical and biophysical assays are important for determining what role these proteins play in mediating high affinity H_2 oxidation. Moreover, understanding how these proteins interact with purified high-affinity catalytic subunits using approaches such as co-immunoprecipitation or cross-linking mass spectrometry assays (Rao et al., 2014, Berggård et al., 2007) would provide a more holistic

understanding of the molecular determinants of high-affinity aerobic H_2 oxidation. These approaches would also enable better in-silico modelling approaches, which would then provide information on how these proteins interact with each other on a sub-atomic level (Ritchie, 2008).

Supporting our previous inferences (Cordero et al., 2019b), a study by Schmitz et al. (2020) on *M. fumariolicum* SolV's group 1h hydrogenase has also provided evidence that high-affinity H_2 scavenging is dependent on the association of hydrogenases with the cytoplasmic membrane (Schmitz et al., 2020). This highlights another area prime for resolution: how are aerobic high affinity hydrogenases tethered to the membrane in the absence of traditional localisation signals and proteins? Though challenging, purification of the majority of components, i.e. a high-affinity hydrogenase, the putative iron-sulfur cluster protein and the conserved hypothetical proteins that enable atmospheric H_2 oxidation will allow for a deeper understanding of the physiology and biochemistry of these abundant yet understudied hydrogenases. This characterisation would then potentially enable biotechnological applications, such as forming parts of oxygen-tolerant hydrogen fuel cells (Cracknell et al., 2008).

The majority of environmental bacteria exist in an uncultured state, with even fewer cultivated representatives having tools available for genetic manipulation and expression studies. This represents a significant problem for the characterisation of the diversity of hydrogenases associated with a range of ecologically important species, as gene knockout/knockdown studies and recombinant protein expression is currently limited to bacteria that have genetic tools readily available (e.g. *E. coli* and *M. smegmatis*; (Guilhot et al., 1994, Pontrelli et al., 2018)). While some structural characteristics of group 1h and 2a hydrogenases are likely to be generalisable across species, there have been instances of physiological differences between species encoding the same type of hydrogenase. For

example, the group 1h hydrogenase from genetically tractable betaproteobacterium *R. eutropha* has been demonstrated to be low affinity (Schäfer et al., 2013) compared to those from *M. smegmatis* (Greening et al., 2014a), *Streptomyces sp.* PCB7 (Constant et al., 2008), *T. roseum* (**Chapter 2**), *Thermogemmatispora sp.* T81 (**Chapter 2**) and *P. methylaliphatogenes* (Greening et al., 2015a). As such, going forward it would be ideal to develop molecular tools targeting understudied phyla such as Chloroflexota and Gemmatimonadota to gain a holistic understanding of the variations within hydrogenases of the same class.

5.2.2 Characterisation of the hidden metabolic flexibility of traditionally 'obligate' substrate utilising bacteria

A prevailing question is how microorganisms can survive in the absence of their preferred energy sources. In general, microorganisms within environments that are subject to variations in available nutrients must employ strategies that will guarantee their prolonged survival until conditions improve (Lennon and Jones, 2011, van Vliet, 2015). In particular, it is becoming increasingly apparent that microorganisms previously characterised as metabolically restricted are in fact able to use a larger variety of substrates than previously thought.

Numerous genomic and metagenomics surveys have identified the genetic determinants of both H_2 and CO oxidation in obligate organoheterotrophs as well as obligate lithoautotrophs (Cordero et al., 2019a, Greening et al., 2016, King and Weber, 2007, Søndergaard et al., 2016). Our observation that the well-known hydrogen-oxidising chemolithoautotroph *Acidithiobacillus ferrooxidans* (Drobner et al., 1990) is capable of atmospheric H_2 oxidation (**Chapter 3**; (Islam et al., 2020)) warrants further testing of the capacity of atmospheric H_2 oxidation in other species. Of the obligate chemolithoautotrophic phyla that have been identified to encode high-affinity hydrogenases, the nitrite-oxidising phylum Nitrospirota has been shown to be able to utilise grow on excess concentrations of H_2 (Koch et al., 2014, Boone

et al., 2001); however, whether they can do so on atmospheric H_2 has not been tested yet. Likewise, the observation that proteobacterial and verrucomicrobial methanotrophs can support autotrophic or mixotrophic growth using H_2 shifts paradigms regarding the substrate specialism of these key greenhouse gas regulators (Carere et al., 2017, Hakobyan et al., 2020, Mohammadi et al., 2017, Schmitz et al., 2020). Better characterising hydrogen metabolism in taxa such as these would enable a better understanding of not only the metabolic flexibility of so-called 'obligate' chemolithoautotrophs and chemoorganoheterotrophs, but also provide greater clarity on the species involved in the biogeochemical H_2 cycle. Furthermore, the discovery of potentially high-affinity hydrogenases (**Chapter 3;** (Islam et al., 2020, Ortiz et al., 2020, Auernik and Kelly, 2010, Søndergaard et al., 2016)) in numerous archaeal lineages, namely the group 2e [NiFe]-hydrogenases in aerobic thermophilic Crenarchaeota (Laska et al., 2003, Urbieta et al., 2017) and the group 1l [NiFe]-hydrogenases in halophilic Halobacterota (Greening et al., 2016, Ortiz et al., 2020), justifies testing whether some archaea can also oxidise atmospheric H_2. Their capacity to oxidise atmospheric CO has already been proven (King, 2015).

In addition to bacteria that have not yet been characterised as being able to oxidise atmospheric H_2, the use of H_2 by Cyanobacteria remains similarly understudied. Many Cyanobacteria harbour two types of hydrogenases, an aerobic uptake hydrogenase (group 2a [NiFe] hydrogenases) and a bidirectional hydrogenase (usually group 3d [NiFe] hydrogenases), though there are some strains that only possess one type (Søndergaard et al., 2016, Tamagnini et al., 2007). Based on studies in nitrogen-fixing marine Cyanobacteria, the current paradigm is that the function of the group 2a hydrogenase is primarily to recycle the by-products of the nitrogenase reaction, thus potentially maintaining the levels of intracellular reducing equivalents, providing ATP via aerobic hydrogenotrophic respiration and aiding in the exclusion of oxygen from nitrogenase (Tamagnini et al., 2002). Although all Cyanobacteria

that encode an uptake hydrogenase are nitrogen-fixing (Tamagnini et al., 2007), it should be explored whether this hydrogenase exclusively recycles endogenously produced H_2 or can also oxidise exogenous atmospheric H_2. The oxygen-tolerance of the cyanobacterial group 2a hydrogenase, the immediate and terminal electron acceptors of the hydrogenase, and the threshold of H_2 able to be oxidised are also research avenues that need to be explored.

5.2.3 A changing global environment may affect sinks of atmospheric H_2 and CO

The ecological range and phylogenetic diversity of microorganisms able to scavenge atmospheric trace gases reflects their critical role in maintaining the biogeochemical cycles of these gases. However, questions still remain about whether or not these microbial sinks are able to cope with continual global increases in H_2 and CO, together with other local and global pressures. It is expected that, like all other living organisms on Earth, microorganisms will be similarly negatively affected by a changing global climate (Cavicchioli et al., 2019). Climate models have predicted that increasing global temperatures will affect both terrestrial and marine ecosystems, with wide reaching consequences including ocean acidification, changes to seasonal blooming cycles, stability of symbiotic relationships, increasing rates of microbial decomposition of organic carbon and increased production of toxins (Hoffmann and Sgrò, 2011, Huisman et al., 2018, Stevnbak et al., 2012, Torda et al., 2017, Ziegler et al., 2017). Thus, it is possible that the combination of these climatological effects would influence the diversity, distribution and abundance of different groups of microorganisms (Cavicchioli et al., 2019), potentially affecting their ability to uptake atmospheric trace gases such as H_2 and CO. Understanding the diversity of the microbial players active in these sinks will allow for greater predictive models focused on determining how affected the biogeochemical cycles will be in a changing global environment, and whether or not the plasticity of the microbial sinks is sufficient to counteract any changes in phylum abundance. This would ideally be combined with manipulative soil microcosm-based and culture-based studies investigating how the

expression and activities of hydrogenases change in response to temperature and moisture changes.

5.2.4 Towards an ecosystem-wide understanding of trace gas oxidation in other ecosystems

Whereas studies on trace gas oxidation have mostly focused on soils to date (Constant et al., 2011b, Cordero et al., 2019a, Greening et al., 2016, King and Weber, 2007), this study highlights that this process is likely to be important in a wider range of ecosystems. Comprehensive metagenomic, metatranscriptomic, and biogeochemical studies are required to resolve the mediators and significance of trace gas oxidation in these ecosystems, ideally paired with culture-based characterisation. While soils have been fairly thoroughly studied in terms of the genetic determinants of aerobic high-affinity H_2 oxidation and CO oxidation, other ecosystems have been comparatively less surveyed. For instance, there have been some attempts to characterise the capacity of oceans to aerobically oxidise atmospheric CO (Moran et al., 2004, Tolli et al., 2006, Zafiriou et al., 2003). A range of bacteria from the marine *Roseobacter* clade (e.g. *Ruegeria pomeroyi*) can oxidise atmospheric CO (Christie-Oleza et al., 2012, Muthusamy et al., 2017), but it is not yet established whether this process also supports persistence in the oligotrophic open ocean. Concordant with genomic surveys highlighting the prevalence of CODH genes within marine ecosystems (Moran et al., 2004, Tolli et al., 2006, Zafiriou et al., 2003), it is possible that atmospheric CO oxidation is also a common strategy supporting the persistence of marine microorganisms. Indeed, there have been numerous reports of marine bacteria that have hypothesised their capacity for aerobically oxidising CO during bacterial persistence, yet to date these have not been experimentally validated. The concentration of CO in marine surface waters is subject to photoproduction as well as phytoplankton-driven fluctuations (Conte et al., 2019, Gros et al., 2009) and can differ both diurnally and latitudinally (Khalil and Rasmussen, 1990, Xie and Gosselin, 2005). Owing to this variability and the scarcity of organic carbon sources, the presence of a high-affinity

aerobic CODH would potentially be evolutionarily advantageous, allowing for persisting marine species to maintain their basal energetic requirements. As the favoured persistence strategy of marine bacteria is cell miniaturisation (Kjelleberg et al., 1987, Novitsky and Morita, 1976), in common with *T. roseum* (**Chapter 2**), atmospheric CO oxidation may also be sufficient to support persistence. Atmospheric CO may be particularly desirable in mesopelagic and bathypelagic zones of oceans, where organic carbon and light availability are both highly limiting.

To date, even less is known about the total abundance of high-affinity hydrogenases within oceanic environments, whether these hydrogenases are active, and whether there are distribution differences (e.g. between coastal and open waters). To this effect, we wanted to investigate the ability of the abundant marine bacterium *Sphingopyxis alaskensis*, which encodes a group 2a hydrogenase, to oxidise atmospheric H_2. My experiments show that stationary phase *S. alaskensis* cultures were able to oxidise 10 ppmv of H_2 to sub-atmospheric concentrations in ~48 hours (**Figure 5-1**). This bacterium is thus the first experimentally validated marine microorganism capable of consuming atmospheric H_2. Additional physiological experiments will be carried out in a similar manner to that of **Chapter 3**, to further investigate whether H_2 is oxidised during bacterial growth, whether H_2 has a growth augmentation effect, and to quantify the expression of the 2a hydrogenase across growth phases. This work will then form part of a greater research project investigating marine trace gas scavenging, paired with metagenomic and biogeochemical data across marine transects.

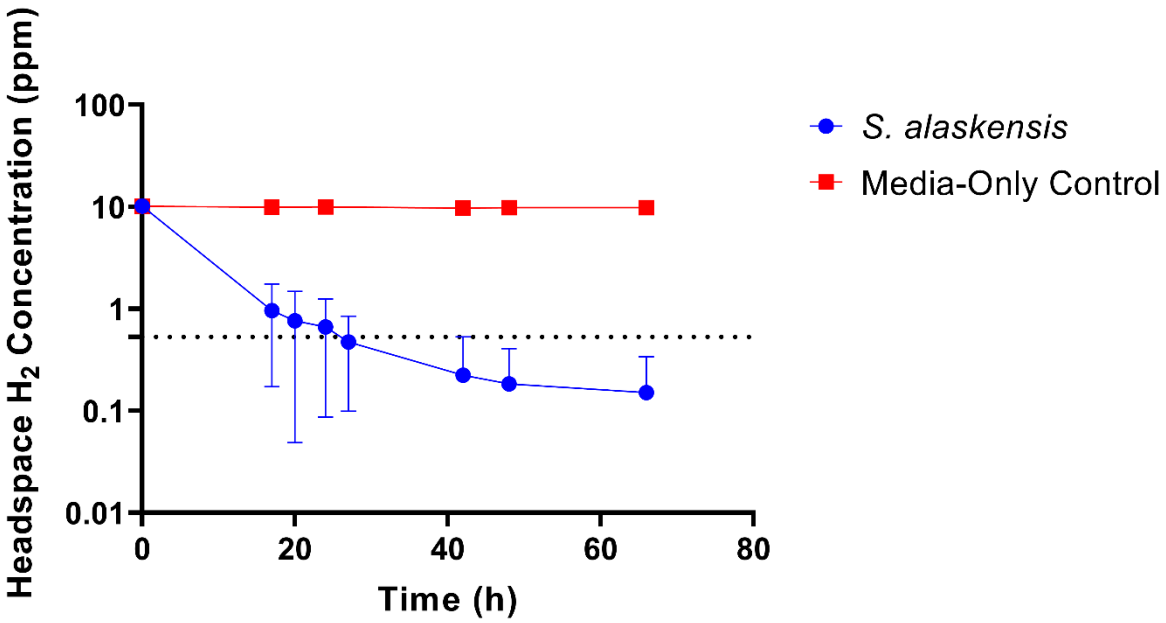


Figure 5-1: **H_2 oxidation by stationary phase cultures of *Sphingopyxis alaskensis*.** Error bars show the standard deviation of three biological replicates, with media only vials monitored as negative controls. Dotted line represents the atmospheric concentration of hydrogen (0.53 ppmv).

Human-engineered ecosystems such as mine tailings, wastewater treatment plants, landfill, urban areas and nuclear waste facilities have also been understudied with respect to trace gas metabolism (Baker and Banfield, 2003, Fernandes et al., 2018, Fields et al., 2005, Meyer-Dombard et al., 2020, Santini et al., 2015, Ferrera and Sánchez, 2016, Clerbaux et al., 2008). These ecosystems represent novel areas to be investigated with reference to bacterial succession and resilience in response to variations in nutrient availability and other physicochemical stressors. Due to their ubiquity and diffusibility, atmospheric trace gases are a favourable alternate energy source for microorganisms in these ecosystems, suggesting that targeted meta-omics surveys and cultivation studies would potentially uncover a greater diversity of atmospheric H_2 and CO oxidisers than currently characterised.

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7 SUPPLEMENTARY DATA

Chapter 2:

Table 7-1 (Supplement): **Transcript read count for RNA-seq experiment comparing gene expression of three exponential phase (nutrient-rich) and three stationary phase (nutrient-limited) cultures of *Thermomicrobium roseum* (xlsx).**

Chapter 3:

Table 7-2 (Supplement): **Microbial distribution and amino acid sequences of the group 2a [NiFe]-hydrogenase large subunit HucL (xlsx).**

Chapter 4:

Table 7-3 (Supplement): **Distribution and sequences of HucE and HhyE proteins in microorganisms encoding the group 2a and 1h [NiFe]-hydrogenases (xlsx).**