DISSIMILATORY IRON-REDUCING AND ENDOSPORULATING BACTERIA

by

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(Under the Direction of Juergen Wiegel)

ABSTRACT

This dissertation represents a diversified study of the biochemical, physiological, and genetic traits of members of the low G+C subdivision of the Gram-type positive bacteria, also known as the '*Firmicutes*'. The phylum '*Firmicutes*' contains a diverse array of taxa that are not easily separated into coherent phylogenetic groups by any one physiological trait, such as endospore-formation or dissimilatory iron reduction. This dissertation considers numerous contemporary, and highly convergent in providing breadth and scope of the subject matter, methods of study. The principle aim was to examine the lineage '*Firmicutes*' by a) a genomic study of the occurrence or absence of endosporulation genes in numerous members of the lineage, b) classic biochemical studies of the enzymes responsible for biotic iron reduction, and c) culture-dependent studies and isolations of various '*Firmicutes*' to both identify new iron-reducers and better resolve the taxonomy of the lineage. The work with endosporulation genes has shown there might not be a distinct set of "endosporulation-specific" genes. This raises several new questions about this exceptionally complex process. The work described here on "ferric reductases" suggests there are enzymes capable of iron reduction that also have additional activities. The isolation of novel bacteria presented here have added to the diversity of the '*Firmicutes*' but have also added to the phylogenetic and taxonomic complexity of this group.

Traditional boundaries for families and genera have been weakened or shown to be in need of further studies.

INDEX WORDS: Gram-type positive bacteria, Firmicutes, Thermophiles, Endospores, Dissimilatory iron reduction, Quinones, Oxidative stress, The University of Georgia

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BACTERIA, OXIDOREDUCTASE ENZYMES, AND THE ENDOSPORULATING

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B.S., The University of Georgia, 2000

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial

Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2006

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December 2006

ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to all of my family, friends, and members of the University of Georgia/Athens community who have made my matriculation such a rich and fulfilling period of my life. I thank my parents for their continued support of me as I have progressed forward in my life. I thank Juergen Wiegel, my advisor and friend, for his guidance and support, and my committee members for all of their thoughtful suggestions and willingness to foster my academic development. Finally, I would like to say that I have been privileged to have had the opportunity to work with so many wonderful people in the Department of Microbiology.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Thermophiles

A thermophile is generally defined as a microorganism with an optimum growth temperature between 50ºC and 75ºC (Wiegel 1998b). Extreme thermophiles and hyperthermophilic bacteria and archaea have temperature optima above 75ºC and up to 105ºC (Wiegel 1998b).

Even though the study of thermophiles dates back nearly a century, laboratory studies focused on a relatively small subset of organisms (e.g., *Geobacillus stearothermophilis* and the actinomycetes) up until the last four decades (Miehe 1907; Morrison and Tanner 1922; Emoto 1933; Brock 1967; Campbell and Pace 1968; Cross 1968; Brock and Freeze 1969). Knowledge of thermophiles has increased tremendously over the last two decades (Stetter 1986; Wiegel 1992). Two well-studied examples of the multitude of problems which play a part in determining the upper temperature limit for microbial growth are: the maintenance of a stable and fluid membrane and the stability of protein components within the cell at elevated tempertures.

A general thermophilic modification to elevated temperatures is the increased saturation of fatty acids to maintain membrane fluidity (Brock 1978). The increase in saturated fatty acid composition in the membrane corresponds to a more rigid (stable) structure of lipids (Brock 1978). In addition, the archaea have a unique membrane structure that may contribute to thermostability. Archaeal lipid composition consists of phytanyl chains which are linked via ether bonds rather than ester bonds as in the bacteria (Brock 1978; Konings et al. 2002). The acyl

chains of archaeal lipids are usually fully saturated isoprenoids (Brock 1978; Konings et al. 2002). Most archaea growing under moderate conditions contain a lipid bilayer membrane just as their bacterial and eukaryal counterparts (Brock 1978; Konings et al. 2002). However, in extreme thermophilic archaea, a monolayer in which the lipids span the whole membrane is formed (Brock 1978; Konings et al. 2002).

Studies have shown that thermophilic enzymes are not only heat tolerant but also function optimally under elevated temperature conditions (Hibino et al. 1974; Wedler and Hofman 1974; Brock 1978). However, thermophilic enzymes are only marginally different from their mesophilic counterparts when only primary sequence is considered (Jaenicke 2000). It is clear that a number of factors (e.g., localized packing of the polypeptide chain, secondary and supersecondary structural elements, domains and subunits, etc.) contribute to protein stability at elevated temperatures (Jaenicke 2000).

As is evident from the above-described thermophilic traits, very few generalizations can be made about thermophiles. Thermophiles have been isolated and described from a diverse array of environments and are represented by a multitude of distant taxa within the archaea and bacteria (Brock 1978; Stetter 1986; Wiegel and Adams 1998; Wiegel 1992, 1998b; Wiegel et al. 2004).

The '*Firmicutes*'

The low G+C subdivision of the Gram-type (Wiegel 1981) positive bacteria, or phylum BXIII '*Firmicutes*', are divided into three classes: the '*Clostridia*', *Mollicutes*, and '*Bacilli*'; whereas other Gram-type positive bacteria, such as *Corynebacterium*, are in the phylum '*Actinobacteria',* the second phylum containing Gram-type positive bacteria (Gibbons and Murray 1978; Garrity et

al. 2002; Table 1.2.; Figs. 1.1., 1.2., and A.5.). However, 16S rRNA cataloging has demonstrated there is considerable heterogeneity among the aerobic and anaerobic, endospore-forming and non-endospore-forming bacilli of the '*Firmicutes*' (Ash et al. 1991; Farrow et al. 1992; Wisotzkey et al. 1992; Collins et al 1994; Nazina et al. 2001). The '*Bacilli*' alone contain at least six highly divergent, taxonomic lineages (Ash et al. 1991; Farrow et al. 1992; Wisotzkey et al. 1992; Nazina et al. 2001). Accordingly, it was no surprise when Sokolova et al. (2006) recently described the genus *Thermolithobacter* as a member of a novel distinct lineage of the '*Firmicutes*'. The levels of 16S rRNA gene sequence similarity were less than 85% between the lineage containing the *Thermolithobacter* and the well-established members of the three classes of the '*Firmicutes*' and warranted the creation of a new class within the '*Firmicutes*', *Thermolithobacteria* (Sokolova et al. 2006).

Exemplary of the diversity found within the phylum '*Firmicutes*' are the genera *Thermoanaerobacterium* and *Thermoanaerobacter* (Onyenwoke and Wiegel, in press; see Chapters 2 and 3, this dissertation). Both genera are members of the '*Thermoanaerobacteriales*', (order II of the class '*Clostridia*') with the *Thermoanaerobacterium* belonging to the family '*Thermoanaerobacteriaceae*' and the *Thermoanaerobacter* belonging to the family '*Thermoanaerobacteriaceae*' (Garrity et al. 2002). However, differentiating between members of the *Thermoanaerobacterium* and the *Thermoanaerobacter* is quite difficult if only using physiological traits, such as endospore formation, growth temperature and pH ranges, and thiosulfate reduction, are considered.

Although all members of both genera have a Gram-type positive cell wall, the Gram-staining reaction is highly variable among the member species. Endospore formation has been observed for some species of both genera, and the presence or absence of endospores had, in the

past, typically been used as a defining characteristic among the member species. However, the formation of endospores is no longer regarded as a strong taxonomic property as several species of bacteria never shown to produce endospores have been demonstrated to contain characteristic spore specific genes (Brill and Wiegel 1997; Onyenwoke et al. 2004). Thus, species of *Thermoanaerobacterium* and *Thermoanaerobacter* for which no spores have been observed may now be regarded as asporogenic, i.e., containing several characteristic spore specific genes but never shown to produce endospores, as well as endospore-forming and non-endospore-forming (Onyenwoke and Wiegel, in press; this dissertation Chapter 5). Growth temperature and pH ranges are extremely broad, and many species exhibit a temperature span for growth over 35°C. However, temperature versus growth rate plots, which exhibit a biphasic curve, indicate changes in the rate limiting steps. Interestingly, Wiegel (1990) suggested the members of these genera contain, for some critical metabolic steps, two enzymes: one for the lower temperature of the growth range and one for the higher. This could have an evolutionary relevance (Wiegel 1990; Wiegel 1998a). Reduction of thiosulfate to elemental sulfur (S^0) by all members of the *Thermoanaerobacterium* had previously been used as a differentiating characteristic between the *Thermoanaerobacterium* and the *Thermoanaerobacter*, which reduce thiosulfate to H_2S (Lee et al. 1993d), until the isolation of: *Thermoanaerobacterium* species capable of reducing thiosulfate to sulfide (Collins et al. 1994), *Thermoanaerobacterium* species incapable of thiosulfate reduction (Cann et al. 2001), and *Thermoanaerobacter* species cabable of reduceing thiosulfate to either both H_2S and S^0, or only S^0 (Kozianowski et al. 1997; Mona Dashti, M. S. thesis, The University of Georgia; Lee et al. in preparation).

Taxonomy

Because of taxa such as the *Thermoanaerobacterium* and the *Thermoanaerobacter*, a natural system of taxonomy/classification, e.g., 16S rRNA based, is typically the method employed for systematic nomenclature (Woese et al. 1975; Stackebrandt and Woese 1984; Woese 1987). Because of the use of 16S rRNA gene sequence data for classification, phylogenetic relationships can be systematically inferred using sequence analysis tools instead of simply relying only upon physiological similarities. Such tools include: BLAST (BLASTN), CLUSTAL_X (Thompson et al. 1997), the phylogeny inference package (PHYLIP) software (Felsenstein 1989), the software suite ARB (Ludwig et al. 2004), and the neighbour-joining algorithms (Saitou and Nei 1987), to name a few. In addition, the use of natural classification, e.g., 16S rRNA based, can often clear up incorrect taxonomy, as was the case with *Thermoanaerobium acetigenum* (Nielsen et al. 1993).

Thermoanaerobium acetigenum* strain X6B^{T} is, based upon many characteristics, i.e., a Gram-type positive (Wiegel 1981), low-G+C content rod, a typical member of the '*Firmicutes*'. Based on its physiological properties alone, it was placed in the genus *Thermoanaerobium*, the type species of which was *Thermoanaerobium brockii* (Zeikus et al. 1979). However, the 16S rRNA gene sequence for *Thermoanaerobium acetigenum* was not determined when originally described by Nielsen et al. (1993). Therefore, the classification of *Thermoanaerobium acetigenum* was based only on some physiological similarities, i.e., not natural classification. Later the type species of *Thermoanaerobium*, *Thermoanaerobium brockii*, would be reclassified as *Thermoanaerobacter brockii* by Lee et al. (1993d) and, subsequently, as *Thermoanaerobacter brockii* subsp. *brockii* by Cayol et al. (1995). *Thermoanaerobium acetigenum* X6B^{T} was not transferred to the genus *Thermoanaerobacter*, or reclassified at all, because of the lack of 16S

rRNA gene sequence analysis (Wiegel and Ljungdahl 1981). Onyenwoke et al. (2006; this dissertation Chapter 4) would later reassign *Thermoanaerobium acetigenum* to the genus *Caldicellulosiruptor*; a member of the order *Clostridiales*, and not to the genus *Thermoanaerobacter*; as *Caldicellulosiruptor acetigenus*, based on 16S rRNA gene sequence, DNA–DNA hybridization analysis and retesting of its properties (Garrity et al. 2002).

Endosporualtion: taxonomic and phylogenetic importance

Endosporulation is known to occur only among the bacteria belonging to the '*Firmicutes*' (Errington 1993; Brill and Wiegel 1997; Nicholson et al. 2000; Byrer et al. 2000; Stragier 2002) and until recently was used as a mandatory characteristic for inclusion within, and differentiation among, genera of the '*Firmicutes*', such as: *Bacillus*, *Desulfotomaculum*, *Clostridium*, *Thermoanaerobacterium*, and *Thermoanaerobacter* (Sneath 1984; Hippe et al. 1992; Slepecky and Hemphill 1992; see also above in **The '*Firmicutes*'**). Based on 16S rRNA gene analysis, species forming endospores do not truly form easily-defined, coherent groups, e.g., the *Bacillus* and *Clostridium* are not coherent genera and are interspersed with genera partly or exclusively consisting of species for which endospore formation has not been observed (Collins et al. 1994). The juxtaposition of closely related, non-endospore-forming species among endospore-forming species suggests endosporulation is an unsuitable taxonomoic trait/marker. However, the phylogenetic intermingling of non-endospore-forming and endospore-forming species does raise interesting issues as to how the process of endosporulation evolved.

Most of the processes of endosporulation investigated to date appear to be highly similar among all endospore-forming species, and thus it is usually assumed that all endospore-forming species most likely arose from the same sporulating ancestor (Errington 1993; Gerhardt and

Marquis 1989; Nakamura et al. 1995; Sauer et al. 1994, 1995). However, the process is complex;

requiring intricate networks of temporal and compartmental regulation as well as possibly more

than 150 different gene products, of which about 75 must act sequentially (Errington 1993;

Gould 1984; Grossman 1995; Ireton and Grossman 1994; Nicholson et al. 2000; Paidhungat et

al. 2001; Setlow 1995, 2001). The complexity makes endosporulation vulnerable to disruption,

i.e., if a single, required component functions incorrectly, endospore formation will not be

observed. Subsequently, a non-endospore-forming phenotype would easily evolve, even though

many functional sporulation genes would still be present.

Based on this hypothesis, Brill and Wiegel (1997) attempted to develop a fast method to

separate novel isolates that do not form endospores into asporogenic, i.e., bacteria with an

impaired sporulation process but containing the majority of sporulation genes, or non-spore-

forming, i.e., absence of sporulation specific genes such as in Gram-type-negative *Escherichia

coli* or *Pseudomonas* spp. Brill and Wiegel (1997) went on to describe a PCR and Southern-

hybridization-based assay to distinguish between asporogenic and non-spore-forming species by

employing probes directed against specific sporulation genes. This PCR and Southern-

hybridization-based assay system was used, and expanded on to include genome analyses, by

Onyenwoke et al. (2004; Chapter 5 this dissertation). By searching for sequences with similarity

to endosporulation-related genes identified from the genome of the endosporulation model

microorganism *Bacillus subtilis*, they were able to show 1) several endosporulating species

lacked sequences with significant similarity to those of *B. subtilis*, and 2) gene sequences with

weak similarity to genes thought to be endosporulation-specific could be identified in non-spore-

forming bacteria outside of the low G+C Gram-type-positive phylogenetic branch and in the

archaea (Stragier 2002). The obtained results raised interesting questions regarding the evolution

of sporulation among the '*Firmicutes*'. It might be that drastic changes in the genes could have rendered them so different from the original genes that they are no longer recognizable when compared to the *B. subtilis* genes. The other possibility is that the *B. subtilis* genes themselves might have been changed from their original state.

Isolation and characterization of '*Firmicutes*'

Descriptions of '*Firmicutes*' isolations are extremely diverse. Members of this taxa have been found distributed in a wide array of environments; too wide to adequately describe in the context and scope of this literature review (refer to Ljungdahl and Wiegel 1986; Wiegel 1992, 1998b; Wiegel and Adams 1998; Garrity et al. 2002; and material cited therein for thorough reviews). Even the known habitats and points of isolation for the thermophilic *Thermoanaerobacterium* and *Thermoanaerobacter* are very diverse and include: alkaline and neutral hot springs and pools, organic waste piles, sediments of acid springs, various soils, tartrate infusion of grape residue, fruit juice waste products, pond sediment, thermal volcanic algal-bacterial mats, high temperature petroleum reservoirs and other deep subsurface environments, etc. (Onyenwoke and Wiegel, in press; this disseratation Chapters 2 and 3).

Thermoanaerobacterium* and *Thermoanaerobacter* have commonly been isolated by the direct supplementation of glucose and either yeast extract, peptone, tryptone or casamino acids to media, i.e., chemoorganoheterotrophic type metabolism (Grassia et al. 1996). However, there have been other reports of *Thermoanaerobacter* strains capable of coupling H_2 oxidation directly to growth, i.e., chemolithotrophic metabolism (Fardeau et al. 1993, 1994). Slobodkin et al. (1999a, 1999b), Zhou et al. (2001) and Roh et al. (2002) have further reported on the isolation and characterization of chemolithotrophic metal-reducing (specifically iron-reducing)

Thermoanaerobacter strains from deep subsurface environments. This type of metabolism, referred to as chemolithotrophic, dissimilatory metal reduction, has become a topic of interest due to its possible role in biogeochemical cycling and potential importance in the evolution of microbial life (Lovley 1991; Liu et al. 1997). Of particular interest are the thermophilic, iron-reducing, chemolithoautotrophs (i.e., able to grow with CO_2, H_2, and amorphous iron oxides alone), which include several '*Firmicutes*' (Slobodkin et al. 1997, 1999b; Sokolova et al., in press; Figure 1.3.; this dissertation Appendix A and Appendix B). Despite observations dating back nearly 80 years that bacteria were capable of biotic iron reduction (Harder 1919; Pringsheim 1949), it had simply been assumed that the reduction of iron, Fe(III) to Fe(II), was an abiotic reaction. However, mesophilic iron-reducing bacteria have been well-documented and studied in recent years (Lovley and Longergan 1990; Longergan et al. 1996); whereas thermophilic iron-reducers have been less frequently described (Boone et al. 1995; Slobodkin et al. 1997, 1999b; Greene et al. 1997; Kashefi et al. 2003; Sokolova et al., in press; this dissertation Appendix A and Appendix B). A thermophilic iron-reducer was not even described until *Bacillus infernus* (Boone et al. 1995). Slobodkin and Wiegel (1997) showed that several different Fe(III)-reducing microorganisms able to grow at temperatures up to 90°C must exist through their work with Fe(III)-reducing enrichments at elevated temperatures.

Of the first described iron-reducers, all required organic carbon sources, i.e., were heterotrophs. Slobodkin et al. (1997) first demonstrated chemolithoautotrophic growth with *Carboxydothermus ferrireducens* and later with *Thermoanaerobacter siderophilus* (Figure 1.3.; Slobodkin et al. 1999b, 2006). Thermophilic, autotrophic, iron reduction has gained interest because it 1) is under-represented by current culture collections (not a routine characterization step for novel microorganisms and therefore assumed to be much more prevalent than currently

reported), 2) may exist in biosphere pockets deep within the Earth (and possibly other planets) (Gold 1992), and 3) may have been involved in low temperature, banded iron formation. In addition, iron reduction can impact environmental systems in a number of ways: organic matter oxidation, aromatic degradation, and inhibition/stimulation of other microbial populations (Lovley 1995a).

Biotic metal reduction

An appreciation for the impact of biotic metal reduction has begun to flourish in recent years with the discovery of microbes capable of the reduction of numerous transition metals, e.g., iron, manganese, uranium, and arsenic (Boone et al. 1995; Caccavo et al. 1996b; Greene et al. 1997; Kieft et al. 1999; Slobodkin et al. 1997; Myers and Nealson 1988a; Lovley and Phillips 1988b). But the microbially-mediated reduction of metals is a phenomenon that was first explored decades ago (Adeney 1894; King and Davis 1914; Harder 1919; Allison and Scarseth 1942; Wachstein 1949; Terai et al. 1958; Tucker et al. 1962; Johnson and Stokes 1966).

Fe(III) and Mn(IV) have become the central players in the study of metal reduction because of 1) their relative abundance (though iron is usually 5-10 times more abundant than manganese) in anaerobic sediments, and 2) the number of microbes discovered that can reduce them for the generation of energy, i.e., dissimilatory metal-ion-reducing microbes (DMRM) (Myers and Nealson 1988a, 1988b; Myers and Nealson 1990; Nealson and Saffarini 1994; Guerinot 1994). As might be inferred from their similar redox potentials, Fe(III) and Mn(IV) reduction often occur in close spatial proximity with areas of Mn reduction activity always occurring above areas of Fe reduction activity in stratified environments (Nealson and Myers 1992). The fact of the matter is any organism capable of Fe reduction is a potential indirect Mn

reducer via indirect chemical reduction (Nealson and Myers 1992). The activity of biotic iron reduction plays an important role in geochemical iron cycling that is not, altogether, fully understood.

Iron is one of the most abundant elements in the earth's crust (2nd most abundant metal after aluminum and 3rd most abundant element overall [Howard 1999; McGeary and Plummer 1997]) but was probably even more abundant in prebiotic times (Egami 1975; Cox 1994). Its flexible redox potential (+300 mV in *a*-type cytochromes to -490 mV in certain iron-sulfur proteins) has been exploited by incorporation into numerous proteins, i.e., assimilatory iron reduction [the incorporation of Fe(II), the biologically active form of iron, into enzymes] (Guerinot 1994; Andrews et al. 1999). Such proteins bind oxygen and/or are involved in electron transfer (Egami 1975; Payne 1993; Cox 1994; Andrews et al. 1999). The redox potential of the ferrous/ferric couple is pH related (Straub et al. 1996) and is most positive at extremely acidic pH [+770 mV at pH 2, the oxygen/water couple is estimated to be between +820 and +830 mV at circum-neutral pH (Thauer et al. 1977; see also Table 1.1; Johnson and Bridge 2002)]. Iron exists predominately in its ferric form in an oxic environment and is highly insoluble, and therefore biologically unavailable, at a neutral pH (approximately 10^{-18}-10^{-17} M, far below the optimal required for microbial growth [estimated to be 10^{-8} to 10^{-6} M]), as compared to a value of 100 mM for free ferrous iron (Guerinot 1994; Andrews et al. 1999). Fe(III) and Mn(IV) form a variety of oxide and oxyhydroxide phases leading to varying mineral formations, which complicate this energetic picture as various oxides can have different redox potentials (Nealson and Myers 1992). Adding to the complexity of iron cycling, excess iron is toxic, possibly by the interaction of reduced iron with oxygen creating hydroxyl radicals (Dancis 1998).

Manganese

Additionally, as stated above, manganese reduction is also an important process in nature because of its abundance and intertwined nature with iron. Manganese reduction is thought to be especially important in the deep sea and deep sea sediments. The microbially-mediated reduction of manganese (IV) oxide and the enzymatic machinery of the reaction have been extensively studied (Ehrlich 1963, 1966, 1970, 1971, 1974; Ghiorse and Ehrlich 1976; Myers and Nealson 1988a, 1988b, 1990). For example, Mn is substituted for Fe in many cases in *Lactobacillus plantarum*. *L. plantarum* contains a Mn cofactored catalase instead of heme groups and millimolar concentrations of non-enzymatic Mn(II) take on the role of the superoxide dismutase (SOD) for this microorganism (Archibald 1986). Myers and Nealson (1988a, 1988b, 1990) have demonstrated that respiratory proton translocation can be coupled to the anaerobic respiration of manganese. Mn oxidation activity, i.e., Mn(II) to insoluble Mn(IV) oxide, has even been shown for supposedly inactive bacterial endospores (van Waasbergen et al. 1993, 1996; Francis and Tebo 2002). A *Bacillus* sp. strain SG-1 is capable of Mn(II) oxidation over a wide range of: temperatures (3-70ºC), Mn(II) concentrations (less than nM to more than mM), and ionic strengths (van Waasbergen et al. 1996; Francis and Tebo 2002).

Iron transport, binding, and acquisition

Before progressing any further on the subject of biotic iron reduction, it is worthwhile to briefly review what is currently known about iron transport and acquisition in some of the best-studied, microbial models where many proteins, and the genes encoding for those proteins, have already been identified and well-characterized.

Iron acquisition, transport, and storage have been well-studied in many genera of fungi. The use of these eukaryotic microorganisms to elucidate many of the mechanisms of iron utilization in a biological setting has produced many physiologically relevant results. Perhaps the yeast *Saccharomyces cerevisiae* is the best-studied. *S. cerevisiae* makes use of an NADPH-dependent, plasma membrane-localized ferric reductase (Fre1) for both reduction of ferric iron and for its assimilation (Anderson et al. 1992; Dancis et al. 1992; Kaplan and O'Halloran 1996; Eide and Guerinot 1997). The budding yeast in general has both a low (K_m= 40 µM) and high (K_m= 0.15 µM) affinity transport system for Fe (Kaplan and O'Halloran 1996). The low affinity transporter, *fet4* gene product, can also transport other metals, such as manganese and cadmium (Dix et al. 1994; Kaplan and O'Halloran 1996) while the high affinity transporter, *fet3* gene product, is specific for and regulated by Fe (Askwith et al. 1994; Kaplan and O'Halloran 1996). Fet3 is a multicopper oxidase that couples the oxidation of ferrous iron back to ferric iron with the reduction of molecular oxygen to water (Askwith et al. 1994; De Silva et al. 1995; Kaplan and O'Halloran 1996). Together with a permease component (Ftr1), Fet3 makes up the high affinity Fe transport system (Ftr1-Fet3) (Stearman et al. 1996; Kaplan and O'Halloran 1996; Severance et al. 2004). Ferrous iron uptake is independent of *fre1* (Dancis et al. 1992).

It is also likely the production of siderophores plays some role in accessing insoluble, extracellular stores of Fe in aerobic and facultative organisms (Guerinot 1994; Luu and Ramsay 2003). Strict anaerobes and the lactic acid bacteria have never been shown to produce siderophores. Wide structural variation exists among siderophores. However, they may be generally classified as either hydroxamates or phenolates/catecholates. Typically, the iron is bound to the siderophore by the O_2 atoms of these functional groups. Then, the iron-siderophore

complex binds a specific membrane-localized receptor. Finally, the siderophore releases the iron into the cell (Luu and Ramsay 2003).

Work on siderophores has focused on the Gram-type negative bacteria, but work on *Bacillus subtilis*, and in particular its catechol siderophore (Grossman et al. 1993) which has similarity to enterobactin, suggests similarities also exist between the Gram-type positive and negative bacteria (Guerinot 1994). These compounds bind with high affinities (K_m) for Fe, e.g., 10^{-52} M in *E. coli*, and are well-known for their stability (Brickman and McIntosh 1992). The Fe-bound siderophore is then transported across the membrane through an interaction with a protein thought to be able to couple the membrane electrochemical potential to active transport, the TonB protein in *E. coli* (Hancock and Braun 1976; Bradbeer 1993; Postle1993; Larsen et al. 2003). The release of the Fe might then be mediated by a decrease in affinity of the siderophore for the Fe. In the case of enterochelin, Fe release is mediated by an esterase that cleaves the ester backbone of the siderophore, which in turn leads to a decrease in affinity of the siderophore for Fe, 10^{-52} M to 10^{-8} M (Brickman and McIntosh 1992).

It is plausible some of the above-mentioned transport molecules/siderophores and ferric reductases, or analogs of either type, are utilized for the transport and reduction of highly insoluble forms of Fe(III) by the '*Firmicutes*'.

Dissimilatory Fe(III) reduction

Dissimilatory iron (Fe) reduction, the use of Fe(III) as the terminal electron acceptor in electron transport, may be the most important chemical change that takes place in anaerobic soils and sediments (Ponnamperuma 1972; Lovley 1991; Longergan et al. 1996; Das and Caccavo 2000). Until recently, the reduction of Fe(III) to Fe(II) had been regarded as a primarily abiotic,

chemical, process (Fenchel and Blackburn 1979; Ghiorse 1988; Zehnder and Strumm 1988). The isolation of microorganisms capable of metal reduction may have remained elusive because of 1) culturing techniques using highly crystalline oxides that are difficult for microorganisms to reduce, and 2) the use of glucose as the carbon source during isolations, which led to acidic fermentation end-products and low pH conditions that reduced the metals abiotically (Nealson and Saffarini 1994). However, the discovery of a diverse number of microorganisms capable of Fe(III) reduction has proven the preconceived notion of Fe(III) reduction being primarily abiotic to be a falsehood (Balashova and Zavarzin 1980; Obuekwe et al. 1981; Semple and Westlake 1987; Lovley and Phillips 1988; Lovley et al. 1993; Boone et al. 1995; Caccavo et al. 1996b; Bowman et al. 1997; Slobodkin et al. 1997, 1999a, 1999b; Greene et al. 1997; Kieft et al. 1999; Coates et al. 2001; Roh et al. 2002) and has led to the suggestion that the last common ancestor of all extant life on Earth may have been an Fe(III)-reducing microorganism (Walker 1987; Wiegel and Adams 1998; Kieft et al. 1999; Lovley and Coates 2000).

The ability to reduce Fe(III) is a highly conserved characteristic among many microorganisms, both archaea and bacteria (Huber et al. 1987; Lovley 1991; Boone et al. 1995; Slobodkin et al. 1997, 1999b; Vargas et al. 1998; Kieft et al. 1999; Kashefi and Lovley 2000; Kashefi et al. 2002a, 2002b, 2003). The first Fe(III)-reducer discovered in the modern era shown to link growth to the reduction of Fe(III) oxides was a *Pseudomonas* species [probably a member of the group *Shewanella putrefaciens*] (Balashova and Zavarzin 1980; Obuekwe et al. 1981; Semple and Westlake 1987). Among the classes of bacteria that include Fe(III)-reducers are: the *Proteobacteria*, overwhelmingly the gamma and delta subclasses (Balashova and Zavarzin 1980; Obuekwe et al. 1981; Semple and Westlake 1987; Lovley and Phillips 1988a, 1988b; Lovley et al. 1989; Gorby and Lovley 1991; Myers and Nealson 1991; Caccavo et al. 1992, 1994; Roden

and Lovley 1993; Lovley et al. 1993, 1995; Coates et al. 1996, 2001), and the '*Firmicutes*' (Boone et al. 1995; Slobodkin et al. 1997, 1999b; Kieft et al. 1999; Roh et al. 2002; Sokolova et al., in press; this dissertation Chapter 6 and Appendix A). Fe(III)-reducers are also both mesophiles, typically the Gram–type negative bacteria (Lovley and Phillips 1988b; Lovley et al. 1989, 1993, 1995; Gorby and Lovley 1991; Myers and Nealson 1991; Caccavo et al. 1992, 1994; Roden and Lovley 1993; Coates et al. 1996, 2001), and (hyper)thermophiles, typically the '*Firmicutes*' and archaea (Figure 1.3.; Boone et al. 1995; Slobodkin et al. 1997, 1999b; Roh et al. 2002; Kashefi et al. 2002a; Kashefi and Lovley 2000). Though thermophilic Fe(III)-reducers are known outside of these taxa, e.g., the Gram-type negative *Flexistipes*, *Deferribacter thermophilus* (Greene et al. 1997); the *Proteobacteria*, '*Geothermobacterium ferrireducens*' (Kashefi et al. 2002b) and *Geothermobacter ehrlichii* (Kashefi et al. 2003); the *Thermatogales*, *Thermotoga maritima* (Huber et al. 1986; Vargas et al. 1998); and the *Deinococcus-Thermus* clade, *Thermus scotoductus* (Balkwill et al. 2004).

However, the ability of Gram-type positive bacteria to reduce Fe(III) at high temperatures (above 60°C in some instances) has not been well-described or studied (Boone et al. 1995; Slobodkin and Wiegel 1997; Slobodkin et al. 1997, 1999b; Table 1.2.). General differences that exist between the '*Firmicutes*' and the well-studied Gram-type negative microorganisms raise questions regarding the differences which must exist between their respective mechanisms of Fe(III) reduction. For example, owing to the fact that the '*Firmicutes*' have a cell wall with a significantly different structural architecture than that of Gram-type negative microorganisms, it would be expected that the mechanisms for Fe(III) reduction would differ between the lineages. Also, many '*Firmicutes*' have elevated temperature optima for Fe(III) reduction in comparison to Gram-type negative microorganisms.

In dealing with diverse thermal environments, psychrophilic, mesophilic, and thermophilic (hyperthermophilic) are the categories of microorganisms immediately identified (Wiegel 1990). In general, however, thermophiles and psychrophiles have been less studied. Thermophiles, for instance, are typically more useful than their mesophilic counterparts for developing strategies for the bioremediation efforts of thermally-heated waters contaminated with uranium, technetium, chromium, and other toxic metals (Lovley 1995; Lovley and Coates 1997; Kashefi and Lovley 2000; Roh et al. 2002). To summarize, studies focusing on the diversity of Fe(III)-reducers using different sources of Fe(III), including various minerals and minerals of varying crystallinity, would provide useful data for comparisions of differing methodologies used for microbial Fe(III) reduction at the very least.

Possible mechanisms for Fe(III) reduction

Direct cell contact required for the reduction of insoluble Fe(III) (hydr)oxides

Fe(III) (hydr)oxide minerals, as previously stated above, are highly insoluble but are the most abundant form of available iron in terrestrial, aerobic habitats. They exist in the form of minerals such as: ferrihydrite, lepidocrocite, maghemite, magnetite ($Fe(II)Fe(III)_2O_4$), hematite ($Fe(III)_2O_3$), and goethite ($\alpha \cdot Fe(III)OOH$) (Lovley 1991; Phillips et al. 1993; Frederickson and Gorby 1996; Hernandez and Newman 2001). The presence of high abundances of such highly insoluble Fe(III) oxides would suggest that direct cell contact to these minerals is the most feasible way for the electron transfer to occur. Work performed by Munch and Ottow (1977, 1980, 1983), Arnold et al. (1988), Myers and Nealson (1988a, 1988b), and Myers and Myers (1992) is also suggestive of this case, i.e., direct cell contact to insoluble Mn(IV) and Fe(III)

oxides is required for metal reduction. In particular, *Shewanella putrefaciens* has been shown to have Fe (hydr)oxides (ferrihydrite, geothite, and hematite) tightly attached to and penetrating its outer membrane and peptidoglycan layer (Glauser et al. 2001). Furthermore, cells of many dissimilatory Fe(III)-reducers, in general, have been frequently observed attached to particles of Fe(III) oxides, as it has been observed with *Shewanella putrefaciens*, *Carboxydothermus ferrireducens*, *Shewanella alga* BrY, and *Thermolithobacter ferrireducens* (Arnold et al. 1988; Lovley and Phillips 1988b; Slobodkin et al. 1997, 2006; Das and Caccavo 2000, 2001; Glasauer et al. 2001, Sokolova et al., in press; this dissertation Appendix A). Interestingly, the adhesion appears to be dictated by the surface chemistry of the cells and the oxides and not due to the crystallinity (available surface area) of the particular Fe(III) oxide (Das and Caccavo 2001).

The cellular machinery, or at least parts of the machinary, required to allow for direct cell contact and adhesion to insoluble Fe(III) oxides has recently begun to be elucidated. *Shewanella putrefaciens* might make use of a type II protein secretion system, encoded by *ferE*, for the transport of a 91 kDa heme-containing protein to its outer membrane (DiChristina et al. 2002). This heme-containing protein could potentially have a role in the direct reduction of Fe(III) and Mn(IV) (DiChristina et al. 2002). *Geobacter metallireducens* accesses insoluble Mn(IV) and Fe(III) oxides by producing flagella and pili, which are not observed when the bacterium is grown on soluble Fe(III) citrate (Childers et al. 2002).

Other evidence has been generated using cell-free filtrates and semipermiable barriers, i.e., dialysis membranes and alginate beads, to support the direct cell contact model for Fe(III) reduction. This data argue against the electron shuttle theory for Fe(III) reduction, i.e., the major opposing theory of direct cell contact that proposes a soluble, iron-reducing intermediate/shuttle is being produced to carry reducing equivalents/electrons from inside cells out to the insoluble

Fe(III) oxides. Using the cell-free filtrates from cultures of *G. metallireducens*, Nevin and Lovley (2000) showed no compound was produced capable of reducing Fe(III) oxides, i.e., no production of an extracellular, electron-shuttling compound. These filtrates did not stimulate the reduction of the Fe(III) minerals (Nevin and Lovley 2000). However, the addition of the commonly tested quinone analog 9,10-anthraquinone 2,6-disulfonic acid (AQDS), a proposed electron shuttle, did stimulate the accumulation of Fe(II). In addition, if a semipermiable barrier (300 kDa dialysis membrane; allowing movement of low molecular weight compounds such as quinones) was placed between *G. metallireducens* or *Shewenella* cells and insoluble Fe(III) oxides, no iron reduction was observed (Munch and Ottow 1977, 1980, 1983; Arnold et al. 1988; Lovley and Phillips 1988b; Caccavo et al. 1992). Nevin and Lovley (2000) showed a similar lack of Fe(III) reduction by *G. metallireducens* when the Fe(III) oxides were entrapped within alginate beads. This experiment was repeated with a soluble source of Fe(III) by solubilizing the Fe(III) oxides with nitrilotriacetic acid (Nevin and Lovley 2000). Again, no Fe(III) reduction was observed. This evidence would support the other side of the story. This data supports the idea direct cell contact is required for Fe(III) oxide reduction and argues against the electron shuttle theory of Fe(III) reduction (Nevin and Lovley 2000), specifically for *G. metallireducens*. Other bacteria, such as *Shewanella alga* (Caccavo et al. 1992) and *Geothrix fermentens* (Coates et al. 1999), might be a different story.

The growth of *Shewanella alga* and *Geothrix fermentens* on Fe(III) oxides was shown to require solubilzation of the Fe(III) with nitrilotriacetic acid (Nevin and Lovley 2000). *Shewanella oneidensis* (Newman and Kolter 2000) and *Geothrix fermentens* (Nevin and Lovley 2002) both apparently produce an electron-shuttling compounds, as well as possible Fe(III) solubilization compounds (Nevin and Lovley 2002), thereby eliminating their need for direct cell

contact to insoluble Fe(III) oxides. Apparently attachment may not even be essential for Fe(III) reduction by *Shewanella alga* as attachment deficient *S. alga* cells still reduce Fe(III) oxides and the rate of Fe(III) oxide reduction in culture is not even lowered (Caccavo et al. 1997).

The shuttle mechanism, i.e., extracellular compounds may be produced to carry reducing equivalents to insoluble Fe(III) oxides

It has been proposed some microorganisms can produce secreted, extracellular compounds that reduce insoluble Fe(III) oxides (Lovley et al. 1996; Greene et al. 1997; Lovley and Blunt-Harris 1999; Hernandez and Newman 2001). This theory is backed by evidence linking the ability of all Fe(III)-reducers to make use of quinones, or quinone analogs such as anthraquinone-2,6-disulfonate (AQDS), as terminal electron acceptors (Lovley et al. 1998, 2000; Coates et al. 1998; Lovley and Coates 2000; Newman and Kolter 2000; Hernandez and Newman 2001; Straub et al. 2001). Quinones typically act as intermediates in electron transport in many electron transport chains in natural habitats (Lovley et al. 1996). The quinone moieties of humic substances function as the electron shuttle to Fe(III) oxides (Lovley and Blunt-Harris 1999; Hernandez and Newman 2001). A similar phenomenon has been demonstrated by McKinlay and Zeikus (2004) with neutral red mediating iron reduction in fermentative, not anaerobically respiring, *Escherichia coli*. This enzymology would also seem to be coupled to hydrogenase activity, i.e., hydrogen oxidation, as hydrogen was consumed during iron reduction (McKinlay and Zeikus 2004).

Work with Fe(III)-reducers, such as *Thermus* isolate SA-01, has demonstrated the importance of electron shuttles, such as quinones. *Thermus* isolate SA-01 is able to reduce (soluble) Fe(III) complexed to citrate or NTA, but the amount of hydrous ferric oxide reduced in

the absence of AQDS is relatively small, i.e., 0.025 mmol reduced without AQDS and 0.5 mmol reduced with AQDS (Kieft et al. 1999). Another interesting study involving AQDS was performed by Fredrickson et al. (2001), who showed nickel substitution in hydrous ferric oxides inhibited dissimilatory iron reduction by *Shewanella putrefaciens* strain CN32 only in the absence of AQDS. This result might suggest an additional role for AQDS, i.e., facilitating the immobilization of Ni within the crystal structure of biogenic magnetite (Fredrickson et al. 2001).

The work with semipermeable barriers described above in "**Direct cell contact required for the reduction of insoluble Fe(III) (hydr)oxides**" may not be applicable to all Fe(III)-reducers as well. Luu et al. (2003) was able to achieve the reduction of Fe(III) oxides placed in either dialysis membranes or alginate beads when soil and NTA were present in the medium. They theorized the NTA solubilized some component of the soil, not the Fe(III) but possibly humic material, thereby facilitating Fe(III) reduction (Luu et al. 2003). Unfortunately, this piece of work was performed using an enrichment, and not a pure, culture.

Iron reductases

One definition for a ferric reductase would be any protein with the ability to transfer electrons directly to ferric iron and reduce it to ferrous iron. The most frequently reported proteins involved in dissimilatory and assimilatory iron reduction are: cytochromes or protein complexes containing cytochromes (Roden and Lovley 1993; Magnuson et al. 2000; Assfalg et al. 2002; Barton et al. 2003; see also **"Cytochromes"** in this literature review), and flavin-containing proteins, also known as flavoproteins (Fontecave et al. 1987; Halle and Meyer 1992; Mazoy and Lemos 1996; Vadas et al. 1999; Mazoy et al. 1999; Mazoch et al. 2004; see also **"Flavin reductases"** this literature review), respectively. However, this fact has greatly complicated the

study of ferric iron reduction as both classes of proteins are highly promiscuous enzymes, i.e., they can reduce other compounds besides iron. In particular, cytochromes have been shown to play a functional role in the reduction of several electron acceptors, e.g., elemental sulfur, iron, and manganese, in addition to ferric iron (Roden and Lovley 1993; Assfalg et al. 2002). Therefore, it has been quite difficult in many instances to prove iron is the true substrate for the presumed ferric reductase beyond demonstrations of kinetic parameters that would indicate this is the case, i.e., high specific activities for iron reduction and high substrate affinities for iron (Vadas et al. 1999; Mazoy et al. 1999; Magnuson et al. 2000; Kaufmann and Lovley 2001; Mazoch et al. 2004). Onyenwoke et al. (in preparation; this dissertation Chapter 6) report partly on this issue in their characterization of a presumed ferric reductase (better described as a highly promiscuous oxidoreductase) from the '*Firmicutes*' *Carboxydothermus ferrireducens*. This oxidoreductase is capable of the reduction of chromium, AQDS, and numerous other quinones and metals, in addition to ferric iron.

Cellular localization of iron reductases

Membrane-bound iron reductases

It has been hypothesized, and it is tempting to think, dissimilatory iron reductases should be membrane-localized for direct contact to occur. The knowledge that direct cell contact with insoluble Fe(III) oxides may be necessary for the Fe(III) reduction in some microorganisms, e.g., *Geobacter* species but possibly not in *Shewanella* species*,* (see above in **"Possible mechanisms for Fe(III) reduction"**; Arnold et al. 1988; Lovley and Phillips 1988b; Caccavo et al. 1996a; Slobodkin et al. 1997; 1999b; Das and Caccavo 2000, 2001; Chiu et al. 2001; Glasauer et al.

2001). Indeed, the initial studies with the Fe(III)-reducers *Geobacter metallireducens*, *Shewanella putrifaciens*, and *Geobacter sulfurreducens* have confirmed iron reductases are found within the content of membrane fractions (Gaspard et al.1998; Gorby and Lovley 1991; Myers and Myers 1993; Magnuson et al. 2000, 2001; Childers et al. 2002). These findings fit very well into the Mitchell theory of chemi-osmotic electron transport phosphorylation, i.e., the coupling of an ATPase to electron and hydrogen flow which requires a charge impermeable membrane for the tight coupling of the electrochemical gradient of H^+ ions across the membrane (Mitchell 1961). This theory points to the essential need for a (charge impermeable) membrane to generate a proton motive force which would essentially fuel the energy production of the cell. This line of reasoning is suggestive of the hypothesis that an Fe(III) reductase would be membrane-bound to localize the energy generating machinery of the cell to a common location.

Soluble iron reductases

It should be noted the majority of reported Fe(III) reductases reside in the cytoplasm (Mazoch et al. 2004). The vast majority of studied Fe(III) reductases probably play a role in assimilatory processes (Moody and Dailey 1985; Fontecave et al. 1987; Halle and Meyer 1992; Mazoy and Lemos 1996; Vadas et al. 1999; Mazoy et al. 1999; Mazoch et al. 2004). In addition, the fact remains some environments contain chelated (soluble) ferric iron as the dominant species, and there have been reportings of Fe(III) reductases isolated from dissimilatory iron-reducers that were localized to cytoplasmic cell fractions as well (Luther et al. 1996; Fortin et al. 2000).

An Fe(III) reductase was isolated from the cytoplasmic fraction of the dissimilatory iron-reducer *Geobacter sulfurreducens* (Kaufmann and Lovley 2001). This reductase was unusual in its strict preference for NADPH as electron donor over NADH, which was not utilized at all

(Kaufmann and Lovley 2001). This preference might be viewed as surprising as NADPH has been dogmatically viewed as an electron donor only for assimilatory processes, such as photosynthesis, even though recent studies, such as acetate oxidation in *Geobacter* species being dependent on NADPH as an intermediate of the tricarboxcylic acid cycle, have demonstrated otherwise (Champine et al. 2000; Galushko and Schink 2000). These traits were found to be echoed by *Pyrobaculum islandicum* when an iron reductase was characterized from this iron-reducing member of the archaea, i.e., the activity of the iron reductase was localized primarily to the cytoplasm and NADPH was the preferred electron donor over NADH (Childers and Lovley 2001). The hyperthermophilic archaeon *Archaeoglobus fulgidus* also contains a ferric reductase (FeR) localized to the cytoplasmic frasction, but either NADPH or NADH can serve as the electron donor (Vadas et al. 1999; Chiu et al. 2001). Even though dissimilatory iron reduction has never been shown for *A. fulgidus*, 1) the reduction of Fe(III) by H_2 and FeR, and 2) the relative abundance of the enzyme in the soluble cell fraction (~0.75%) suggests a catabolic role for the enzyme (Vadas et al. 1999; Chiu et al. 2001).

However, the role of these soluble enzymes *in vivo* has been difficult to assess as they could function in either assimilatory or dissimilatory processes. The *G. sulfurreducens* soluble iron reductase was found to be constitutively expressed, i.e., found with or without iron in the growth media as the electron acceptor, as is also true for *P. islandicum* (Kaufmann and Lovley 2001; Childers and Lovley 2001). Currently, no work has been performed to establish the effect of growth conditions (an abundant or low iron concentration in growth media) on the abundance, or activity, of FeR in *A. fulgidus*.

Cytochromes

The most frequently reported proteins involved in metal reduction are the cytochromes (Barton et al. 2003). Cytochromes, especially *c*-type cytochromes, play some role in Fe(III) reduction as either membrane-associated electron carriers or terminal Fe(III) reductases (Lojou et al. 1998a, 1998b; Gaspard et al. 1998; Magnuson et al. 2000, 2001; Leang et al. 2003; Lloyd et al. 2003; Butler et al. 2004; DiDonato et al. 2004; Mehta et al. 2005). Lovley (2000) has proposed a model for Fe(III) reduction for *Geobacter sulfurreducens* involving cytochromes in which a 41 kDa cytochrome, a 9 kDa cytochrome, and an 89 kDa cytochrome are positioned in the outer membrane, periplasm, and cytoplasmic membrane, respectively. The oxidation of cytoplasmic compounds leads to a cascade/chain of events in which electrons are passed through the cell wall and membranes along this cytochrome-linked "bridge" to the awaiting Fe(III).

In summarizing what is currently known about the correlation between Fe(III) reduction and cytochromes, it should be noted cytochromes tend to localize to either the periplasmic space or membrane of cells, as might be expected when growth is contingent upon the reduction of an insoluble Fe(III) oxide (Myers and Myers 1992, 1993 1997, 2000). The cytochrome content of the well-described Fe(III)-reducer *Shewenella putrefaciens* is markedly localized to the outer membrane when *S. putrefaciens* is grown anoxically (Myers and Myers 1992, 1997). Secondly, the production of *c*-type cytochromes tends to be induced during growth on ferric compounds (Dobbin et al. 1999). As example, a 63.9 kDa periplasmic tetrahaem flavocytochrome c_3 (Ifc$_3$) is expressed when *Shewenella frigidimarina* NCIMB400 is grown anaerobically with ferric citrate or ferric pyrophosphate (Dobbin et al. 1999). Disruption of the *ifcA* chromosomal gene leads to no significant rate of Fe(III) reduction but does lead to an increased production of other *c*-type cytochromes. Also, the Fe(III)-reducer *Geobacter sulfurreducens* produces a *c*-type cytochrome

involved in Fe(III) reduction (Seeliger et al. 1998). This cytochrome was found in the membrane fraction, the periplasmic space, and the surrounding medium in equal amounts (an interesting combination of the proposed theories for Fe(III) reduction have been discussed here in "**Possible mechanisms for Fe(III) reduction**") (Seeliger et al. 1998). The described cytochrome can be oxidized by both soluble Fe(III) citrate and Fe(III)-NTA and insoluble Fe(III) oxides (Seeliger et al. 1998). This scenario offers the alternatives that this cytochrome may have activity as either a membrane-localized protein or as a secreted, extracellular carrier protein. However, the work of Seeliger et al. (1998) has been called into question by Lloyd et al. (1999), who concluded the cytochrome described by Seeliger et al. (1998) was not involved in Fe(III) reduction. Additionally, it has been shown that *Geobacter metallireducens* GS-15 mediates electron transfer during growth on insoluble Fe(III) via a type *b* cytochrome and membrane-bound Fe(III) reductase (Gorby et al. 1988; Gorby and Lovley 1991). The evidence linking cytochromes to Fe(III) reduction is both convincing and plentiful; however, it is known, and comes as no surprise, that cytochromes are highly promiscuous enzymes and serve multiple cellular functions in respect to bioenergetics (see "**Iron reductases**").

It is apparent that low-potential multiheme cytochromes, e.g., cyt c_7 and cyt c_3, interface with numerous electron acceptors as nonspecific metal dehydrogenases (Barton et al. 2003). Multiheme cytochromes may play a role in the reduction of elemental sulfur, iron, and manganese (Roden and Lovley 1993; Assfalg et al. 2002) in *Desulfuromonas acetoxidans*. Since *G. metallireducens* and *G. sulfurreducens* both have triheme cyt c_7, it is appropriate to consider these electron carriers also function in metal reduction in a manner similar to that reported for the cyt c_7 of *Desulfuromonas acetoxidans* (Seeliger et al. 1998; Afkar and Fukumori 1999; Champine et al. 2000; Barton et al. 2003). But different methodologies for Fe(III) reduction must

exist. For instance, *Pyrobaculum islandicum* does not contain *c*-type cytochromes (Childers and Lovley 2001), but it can conserve energy via dissimilatory Fe(III) oxide reduction (Kashefi and Lovley 2000; see also "**Possible mechanisms for Fe(III) reduction"**).

Other possible mechanisms for Fe(III) reductases

Quinones and quinone reduction

In many (an)oxic soils and sediments, it is the quinone moieties of humic substances that function as the electron shuttles to the highly prevalent Fe(III) oxides (Lovley et al. 1996; Hernandez and Newman 2001). In addition, the importance of quinones to bacterial respiration has been described. For instance, it has been shown *Shewanella oneidensis* requires menaquinone (MK) during growth on several electron acceptors, including Mn(IV), Fe(III), fumarate, nitrate, nitrite, thiosulfate, dimethyl sulfoxide (DMSO), and AQDS (Myers and Myers 1993b, 1994, 2004, 2000; Newman and Kolter 2000; Schwalb et al. 2003). In addition, mutants of *Shewanella putrefaciens* strain MR-1 deficient in fumarate, iron, and nitrate respiration also lack menaquinone. The addition of menaquinone restored respiration on the aforementioned electron acceptors (Myers and Myers 1994). Based on the above information, it is obvious quinones play some role in dissimilatory iron reduction. Therefore, it is not inconceivable to argue that the role of "ferric iron reductases" may be intertwined with quinone reduction schemes as well.

It is well-known from work done with *E. coli* and other *Proteobacteria* that quinones are involved in respiration; specifically it has even been generalized that ubiquinones (UQs), i.e., benzoquinone isoprenologues, are essential components of oxygen and nitrate respiration,

whereas menaquinones (MKs), i.e., naphthquinone isoprenologues, are more functional in other anaerobic respirations (Polglase et al. 1966; Gennis and Stewart 1996; Sohn et al. 2004; White et al. 2005). Indeed, differences in midpoint potentials between UQ/reduced UQs (UQH$_2$) [E_m +113 mV] and MK/MKH$_2$ [E_m -74 mV] would dictate MKs are more suitable for a respiratory chain utilizing lower-potential electron acceptors, e.g., fumarate, whereas UQs are better suited for oxygen and nitrate respiration (Gennis and Stewart 1996). However, this scheme may be oversimplified. UQ's also function as the electron carriers between *b*-type cytochromes and various terminal oxidases (Sùballe and Poole 1998) and have been found as the dominate quinone species (and in unusually large amounts that equate to 5- to 20-fold greater than aerobically grown *E. coli*) in strains of the strictly anaerobic bacteria *Dehalococcoides* (White et al. 2005) and *Carboxydothermus ferrireducens* (Onyenwoke et al., in preparation; this dissertation Chapter 7). Still, MKs, and not UQs, might still be the actual electron acceptors for anaerobic respirers, such as *Dehalococcoides* sp. and *C. ferrireducens*, with the highly abundant UQs [60-85 and 70 mol% in *Dehalococcoides* sp. (White et al. 2005) and *C. ferrireducens*, respectively (Onyenwoke et al., in preparation; this dissertation Chapter 6)] playing some other role in these strains.

Apart from their role in respiration, reduced UQs (UQH$_2$) have been shown to scavenge lipid peroxyl radicals and thereby prevent a chain reaction of oxidative damage to the polyunsaturated fatty acids of biological membranes, a process known as lipid peroxidation (Forsmark-Andrée et al. 1995). Therefore, the amount of UQH$_2$ and other antioxidants, such as vitamin E, present in low-density lipoprotein is of vital importance for the prevention of oxidative, cellular damage (cytotoxic effects) and even diseases such as atherosclerosis (Forsmark-Andrée et al. 1995). Pools of UQH$_2$, and other reduced quinones are presumably

maintained by soluble quinone oxidoreductases, e.g., DT-diaphorase, also known as NAD(P)H:
quinone oxidoreductase, (EC 1.6.99.2); lipoamide dehydrogenase; the quinone oxidoreductases
MdaB and ChrR of *Helicobacter pylori* and *Pseudomonas putida*, respectively; and possibly the
Fe(III)-reducing enzyme described by Onyenwoke et al. (in preparation; this dissertation Chapter
6; see also **"Iron reductases"** in this literature review) (Beyer et al. 1996; Siegel et al. 2004;
Olsson et al. 1999; Wang and Maier 2004; Gonzalez et al. 2005). Thus, pools of UQH_2 protect
against cytotoxic and carcinogenic effects. Therefore, any inhibition in the quinone reductase
activity would result in an increase in free radical damage (Beyer et al. 1996). The role of
protection from oxidative stress has already been theorized by White et al. (2005) by their work
on *Dehalococcoides* strains. These authors speculated UQs, specifically UQ-8, play some role in
oxidative stress management. Further evidence from Abhilashkumar et al. (2001) also suggests
UQ-8 is an antioxidant for the cattle filarial parasite *Setaria digitata*.

An iron and a quinone reductase?

As described above, there is a relationship between iron and quinone reduction. This duality
makes sense when it is realized most proteins termed "iron reductases" might more aptly be
referred to as "flavin reductases" (see "**Iron reductases"** and **"Flavin reductases"**) and are
generally constitutively expressed, i.e., high or low iron concentrations do not affect ferric
reductase activity. One notable exception is the ferric reductase of *Magnetospirillum
magnetotacticum* (Noguchi et al. 1999; Schroder et al. 2003). This terminology is validated by
the work of Magnuson et al. (2000) and Onyenwoke et al. (in preparation; this dissertation
Chapter 6) in the isolations of ferric reductases from *Geobacter sulfurreducens* and
Carbxoydothermus ferrireducens, respectively. The quinone reductase activity was even

significantly higher with a quinone rather than iron as substrate for the *G. sulfurreducens* enzyme. This line of reasoning would ultimately lead Onyenwoke and Wiegel (submitted; this dissertation Chapter 7) to examine the inverse proposal: If ferric reductases are highly promiscuous enzymes that reduce several substrates, including quinones, are quionone reductases similarly promiscuous, i.e., reducing several different electron acceptors, and possibly iron?

For this particular inquiry, the well described quinone reductase NAD(P)H:quinone oxidoreductase (NQO1), also known as DT- diaphorase (Ernster 1958, 1967, 1987), was chosen for study (this dissertation Chapter 7). NQO1 is a widely-occurring dimeric flavoprotein, contains one (1) catalytically essential, non-covalently bound FAD prosthetic group (Ernster et al. 1962; Prochaska and Talalay 1986; Prochaska 1988; Smith et al. 1988; Faig et al. 2000; Cavelier and Amzel 2001), and typically catalyzes a two electron transfer (Iyanagi and Yamazaki 1969, 1970; Powis and Appel 1980). This two electron transfer presumably plays a role in protecting cells from reactive oxygen species that can be formed when one electron transfer products, e.g., semiquinones, are produced in the presence of oxygen (Ross 2004). It has already been shown NQO1 is active upon numerous substrates including various quinones and quinone analogs, azo dyes, superoxide, chromium, and various other electron acceptors (Ernster and Navazio 1958; Cui et al. 1995; Petrilli and de Flora 1988; Aiyar et al. 1992; Siegel et al. 2004). Therefore, NQO1 is, by definition, a highly promiscuous and functionally diverse protein.

NQO1 was found to be capable of the enzymatic reduction of Fe(III) to Fe(II) via the oxidation of the cosubstrate NADH and studied to obtain kinetic data by Onyenwoke and Wiegel (this dissertation Chapter 7). However, the exact implications of this iron reduction activity by NQO1 are still unclear. As decribed above, the highly promiscuous nature of NQO1 makes it

difficult to assess whether Fe(III) is a true substrate for the enzyme. This observed Fe(III) reduction activity, and the broad substrate range of NQO1, may be further allusion to the true activity of NQO1: a flavin reductase.

Flavin reductases

An Fe(III) reductase could actually function as an FAD reductase (Fontecave et al. 1994). As previously mentioned, many presumed ferric reductases are flavin-containing proteins, or flavoproteins (see also **"Iron reductases" in this literature review**). Estimates of total cellular FAD and free FAD in microorganisms are estimated to be 51 µM and 13 µM, respectively (Bochner and Ames 1982; Ohnishi et al. 1994). Flavin reductases are chromophore-less enzymes which catalyze the reduction of these free flavins, i.e., FAD, FMN, or riboflavin, with the concomitant oxidation of NADPH or NADH (Fontecave et al. 1994; Pierre et al. 2002). Here, the product of the reaction is actually the reduced flavin which is released into the cytoplasm. In terms of iron reduction, the reduced flavin might then chemically reduce the Fe(III) adventitiously, a possible rationale for the observed extremely broad substrate specificity of Fe(III) reductases.

Table 1.1. Energetics of various compounds used as electron acceptors. Abbreviations: DMSO, dimethyl sulfoxide; DMS, dimethyl sulfide; TMAO, trimethylamine N-oxide; TMA, trimethylamine. (Adapted from Thauer et al. 1977).

Electron acceptor	Reduction half reaction	Number of electrons transferred	E_0' (mV)
Oxygen	O_2/H_2O	2	+818
Soluble Fe(III)	Fe^{3+}/Fe^{2+}	1	+772
Nitrate	NO_3^-/NO_2^-	2	+433
Mn(IV)	MnO_2/Mn^{2+}	2	+380
Nitrite	NO_2^-/NO	1	+350
DMSO	DMSO/DMS	2	+160
TMAO	TMAO/TMA	2	+130
Fumarate	Fumarate/Succinate	2	+33
Tetrathionate	$S_4O_6^{2-}/2S_2O_3^{2-}$	2	+24
Sulfite	HSO_3^-/HS^-	6	-116
Carbon dioxide	CO_2/CH_4	8	-244
Sulfate	SO_4^{2-}/SO_3^{2-}	2	-480
Sulfate	SO_4^{2-}/HSO_3^-	2	-516

Table 1.2. Examples of the taxa found within the three classes (i.e. the '*Clostridia*', the '*Bacilli*', and the *Mollicutes*) of the phylum '*Firmicutes*'. [1]Examples of species within the indicated class and orders. [2]Orders *Bacillales* and '*Lactobacillales*' are equivalent to '*Bacilli*' I and '*Bacilli*' II, respectively, in Figure 1.2.

Class	Order	Examples[1]
'Clostridia'	Clostridiales	Clostridium butyricum, Peptostreptococcus anaerobius, Peptococcus niger
	'Thermoanaerobacteriales'	Thermoanaerobacterium thermosulfurigenes, Thermoanaerobacter ethanolicus, Thermoanaerobacter tengcongensis
	Halanaerobiales	Halanaerobium praevalens, Halobacteroides halobius
'Bacilli'	Bacillales[2]	Bacillus subtilis subsp. subtilis, Alicyclobacillus acidocaldarius, Bacillus anthracis
	'Lactobacillales'	Lactobacillus delbrueckii, Enterococcus faecium, Leuconostoc mesenteroides, Streptococcus pyogenes
Mollicutes	Mycoplasmatales	Mycoplasma pneumoniae, Mycoplasma genitalium
	Entomoplasmatales	Entomoplasma ellychniae, Spiroplasma citri
	Acholeplasmatales	Acholeplasma laidlawii
	Anaeroplasmatales	Anaeroplasma abactoclasticum

Table 1.3. Fe(III)-reducing, thermophilic bacteria. Abbreviations: ND, not determined.

Properties	*Thermolithobacter ferrireducens* Sokolova et al. 2006	*Bacillus infernus* Boone et al. 1995	*Thermoterrabacterium ferrireducens* Slobodkin et al. 1997; Khijniak et al. 2005	*Deferribacter thermophilus* Greene et al. 1997
Chemolithoautotrophic	+	-	+	-
Organic electron donors	none	sugars	sugars	sugars
Metabolism	obligate anaerobe	obligate anaerobe	obligate anaerobe	microaerophilic
Spore formation	not observed	yes	not observed	not observed
Flagella	2-3, peritrichous	none	none	monotrichous (polar)
Motility	tumbling	none	tumbling	none
Cell size (avg)	0.5 x 1.8 μm	0.7 x 6.0 μm	0.3 x 2.0 μm	0.4 x 3.0 μm
Temp range (°C)	50-75, opt. 72	39-65, opt. 61	50-74, opt. 65	50-65, opt. 60
pH range	6.5-8.5, opt. 7.3	7.3-7.8, opt. 7.3	5.5-7.6, opt. 6.1	5.0-8.0, opt. 6.5
Gram stain	+	+	+	-
Reduction of Mn(IV)	-	+	-	+
G + C content (mol%)	53	ND	41	34
Other metals reduced	**none**	**none**	**U(VI)**	**none**

Thermoanaerobacter siderophilus Slobodkin et al. 1999	*Thermotoga maritima* Huber et al. 1986; Vargas et al. 1998	*'Geothermobacterium ferrireducens'* Kashefi et al. 2002b	*Geothermobacter ehrlichii* Kashefi et al. 2003	*Thermus scotoductus* Balkwill et al. 2004
+	-	+	-	-
sugars	sugars	none	sugars, amino acids, peptides	sugars and amino acids
obligate anaerobe	obligate anaerobe	obligate anaerobe	obligate anaerobe	facultative anaerobe
not observed	not observed	not observed	not observed	not observed
2-3, peritrichous	monotrichous (subpolar)	monotrichous	monotrichous (subpolar)	none
tumbling	+	+	+	none
0.5 x 6.5 μm	0.6 x 5.0 μm	0.5 x 1.0 μm	0.5 x 1.2 μm	0.5 x 1.5 μm
39-78, opt. 70	55 -90, opt. 80	65 -100, opt. 85 -90	35 -65, opt. 55	42-73, opt. 65
4.8-8.2, opt. 6.4	5.5-9.0, opt. 6.5	ND (grew 6.8 -7.0)	5.0-7.75, opt. 6.0	6-8, opt. 7.5
+	-	-	-	-
+	**ND**	-	-	**ND**
32	46	ND	62.6	64.5
none	**none**	**none**	**none**	**Cr(VI), U(VI), Co(III)**

Fig. 1.1. The simplified universal phylogenetic tree of life. The numbers on the branch tips correspond to the following groups. The bacteria: 1, the Thermotogales; 2, the flavobacteria and relatives; 3, the cyanobacteria; 4, the purple bacteria; **5, the Gram-type positive bacteria=** ***'Firmicutes'***; and 6, the green nonsulfur bacteria. The archaeon: kingdom Crenarchaeota:7, the genus *Pyrodictium*; and 8, the genus *Thermoproteus*; and the archaeon kingdom Euryarchaeota: 9, the Thermococcales; 10, the Methanococcales;11, the Methanobacteriales; 12, the Methanomicrobiales; and 13, the extreme halophiles. The Eucarya: 14, the animals; 15, theciliates; 16, the green plants; 17, the fungi; 18, the flagellates; and 19, the microsporidia. Taken from Woese et al. 1990.
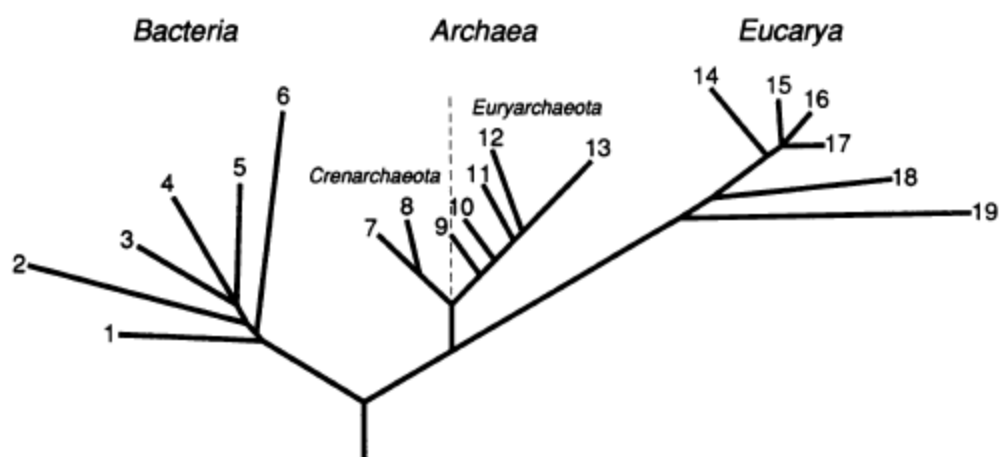
Fig. 1.2. Schematic (unrooted) representation of relationships within the '*Firmicutes*' and other taxa. '*Firmicutes*' are indicated in bold. The orders of the '*Clostridia*' and the *Mollicutes* have been omitted for clarity. Modified from Wolf et al. 2004.
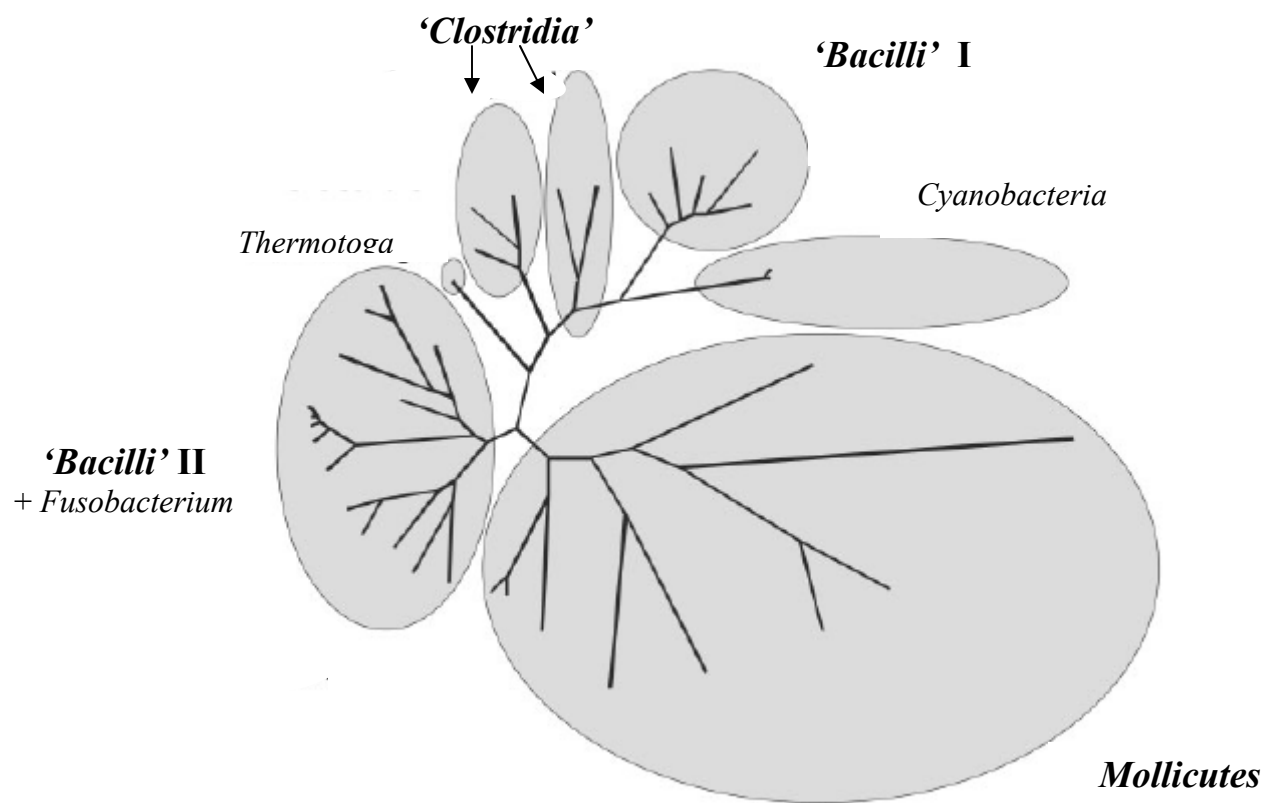
Fig. 1.3. Phylogenetic tree of the thermophilic, iron-reducing bacteria. Fitch tree showing the estimated phylogenetic relationships of thermophilic, iron-reducing bacteria based on 16S rRNA gene sequence data with Jukes-Cantor correction for synonymous changes. Numbers at nodes indicate bootstrap support percentages for 100 replicates. Bar, 0.02 nucleotide substitutions per site. GenBank accession numbers are indicated after the strain identifier. The superscript "T" denotes the strain is the type strain for the species. The thermophilic, iron-reducing bacteria are indicated in bold.

JW/JH-Fiji-1 (AF286862)

Clostridium thermopalmarium DSM 5974[T] (X72869)

Clostridium thermobutyricum DSM 4928[T] (X72868)

Clostridium butyricum DSM 552[T] (M59085)

Bacillus infernus DSM 10277[T] (U20384)

Caloramator fervidus DSM 5463[T] (L09187)

JW/JH-1 DSM 13655 (AF286863)

Caloramator indicus ACM 3982[T] (X75788)

Thermobrachium celere DSM 8682[T] (X99238)

Geothermobacter ehrlichii DSM 15274[T] (AY155599)

Thermolithobacter ferrireducens Fiji-2 (AF282252)

Thermolithobacter ferrireducens JW/KA-2[T] seq. B (AF282254)

Thermolithobacter ferrireducens JW/KA-2[T] seq. A (AF282253)

Thermoterrabacterium ferrireducens DSM 11255[T] (U76363)

Thermoanaerobacter siderophilus DSM 12299[T] (AF120479)

Thermoanaerobacter ethanolicus JW 200[T] (L09162)

Deferribacter thermophilus ACM 5093[T] (U75602)

Thermus scotoductus DSM 8553[T] (AF032127)

'*Geothermobacterium ferrireducens*' ATCC BAA-426 (AF411013)

Thermotoga maritima DSM 3109[T] (M21774)

0.02

CHAPTER 2

THE *THERMOANAEROBACTERIUM* [1]

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[1]Onyenwoke, R. U., and J. Wiegel. Genus I. *Thermoanaerobacterium*. Submitted to *Bergey's Manual of Systematic Bacteriology*.

Abstract

Ther.mo.an.ae.ro.bac'te.ri.um. Gr. n. *thermos*, hot; Gr. pref. *an*, not; Gr.n. *aer*, air; Gr.n. bacterion, a small rod; M.L. neut. n. *Thermoanaerobacterium,* rod which grows in the absence of air at high temperatures.

Cells are Gram-type positive (Wiegel 1981), but stain Gram-negative (besides *T. polysaccharolyticum* which is Gram-stain variable). Cells are rod-shaped and motile by peritrichous flagella. All are obligate anaerobes, have thermophilic growth temperature optima (between 60 to 70°C) and catalase negative. However, the temperature growth range for the members of the genus is extremely broad and ranges from 35 to 75°C (for discussion see Wiegel 1990, 1998). The pH range for growth is equally broad (3.8 to 8.5). The pH optima vary from a low of 5.2 for *T. aotearoense* (the lowest pH optimum for anaerobic, thermophilic bacteria) to an alkaline high of 7.8-8.0 for *T. thermosaccharolyticum*. All members are chemoorganotrophic with yeast extract stimulating growth for most species. Endospores have been observed for some species, and their presence or absence has, in the past, typically been used as a defining characteristic of the member species from one and other. However, the observation of sporulation or not sporulating is no longer regarded as a strong taxonomic property (Onyenwoke et al. 2004).The configuration of quinone systems, cellular fatty acids and diaminopimelic acids in *Thermoanaerobacterium* species have been described by Yamamoto et al. (1998). Members of the genus include some well- characterized, such as: *T. thermosaccharolyticum* (McClung 1935; Prévot 1938; Collins et al. 1994), and some recently characterized "canned food spoilers" (McClung 1935; Dotzauer et al. 2002).

G+C content: 29 to 46 mol%

Type species: *Thermoanaerobacterium thermosulfurigenes* (Basonym: *Clostridium thermosulfurogenes,* Corrig. Schink and Zeikus, 1983, 896[VP]) Lee Y.E., M.K. Jain, C. Lee, S.E. Lowe and J.G. Zeikus. 1993, 48[VP]

Further descriptive information

Reduction of thiosulfate to elemental sulfur was used as the differentiating characteristic of the genus *Thermoanaerobacterium* from *Thermoanaerobacter* (Lee et al. 1993d) until: the isolation and characterization of *T. polysaccharolyticum* (thiosulfate is reduced to sulfide) and *T. zeae* (thiosulfate is not reduced) (Cann et al. 2001), *T. thermosaccharolyticum* (thiosulfate is reduced to sulfide) was added to the genus *Thermoanaerobacterium* (Collins et al. 1994) and '*Thermoanaerobacter sulfurigignens*' was described to reduce thiosulfate to elemental sulfur. (Dashti, M., M.S. thesis, The University of Georgia).

Thermoanaerobacterium species produce thermostable enzymes that have, and will have, important industrial uses: alpha-galactosidase (King et al. 2002), pullulanases, glucoamylases, alpha-glucosidases (Ganghofner et al. 1998; Hyun and Zeikus 1985a; Madi et al. 1987; Haeckel and Bahl 1989; Burchhardt et al. 1991; Matuschek et al. 1994), endoxylanase (Shao et al. 1995a; Liu et al. 1996b), beta-xylosidase, a novel acetyl xylan esterase (Shao and Wiegel 1995; Lorenz and Wiegel 1997) and D-xylose ketol-isomerase (Kim et al. 2001; Liu et al. 1996c; Meng et al. 1993a). Glucose-isomerases in relation to saccharidase synthesis and development of single-step processes for sweetener production have been studied by Lee et al. (1990) and Lee and Zeikus (1991). Further work with *Thermoanaerobacterium* species has included: metabolic engineering to eliminate the production of organic acids by thermophilic bacteria and to increase yields of

desired fermentation end-products (e.g. ethanol) (Klapatch et al. 1994; Simpson and Cowan 1997; Cameron et al. 1998; Altaras et al. 2001; Desai et al. 2004), characterization of β-D-xylosidases (Lee and Zeikus 1993; Vocadlo et al. 2002a; Vocadlo et al. 2002b; Yang et al. 2004) and glucuronidases (Shao et al. 1995b; Bronnenmeier et al. 1995) and the development of these bacteria as possible alternate expression hosts, especially for genes with a thermophilic origin (Mai and Wiegel 1999; Mai and Wiegel 2001).

Enrichment and isolation procedures

Known habitats include: various hot springs and pools, organic waste piles, sediments of acid springs, various soils, tartrate infusion of grape residue, pond sediment, thermal volcanic algal-bacterial mats and fruit juice waste products.

Thermoanaerobacterium have been isolated from high temperature petroleum reservoirs by the direct supplementation of production waters with glucose and either yeast extract, peptone, tryptone or casamino acids (Grassia et al. 1996).

Taxonomic comments

The genus *Thermoanaerobacterium* is a member of the '*Thermoanaerobacteriales',* (order II of the class '*Clostridia*') and belong to the family '*Thermoanaerobacteriaceae'* (Garrity et al. 2002). Cellular polyamine distribution profiles have been investigated for this genus and show some differences from the phylogenetically related thermophilic anaerobes *Moorella*, *Dictyoglomus*, and *Thermoanaerobacter* (Hamana et al. 2001).

List of species in the genus *Thermoanaerobacterium*

Thermoanaerobacterium aotearoense Liu S.Y., F.A. Rainey, H.W. Morgan, F. Mayer and J. Wiegel. 1996, 395[VP]

ao.te.a'ro.en.se. Maori n. *ao* cloud; Maori adl. *tea*, white; Maori adj. *roa,* long white cloud, referring to the native Maori name for New Zealand, Aotearoa, Land of the Long White Cloud.

This description is based on that of Liu et al. (1996a), and on study of the type strain JW/SL-NZ613.

Rods (0.7 to 1.0 by 2.1 to 14.3 μm) that are typically peritrichous exhibiting a tumbling motility. Cells stain Gram-negative but have a Gram-type positive cell wall covered with hexagonal S-layer lattices. Oval - terminal endospores (1.4 to 2.1 by 2.8 to 2.9 μm) in slightly swollen sporangia are produced by 5-15% of cells.

Growth characteristics

An obligate anaerobe with a temperature range for growth at pH 5.2 from 35 to 66°C (optimum temperature 60-63°C). The $pH^{25°C}$ range for growth at 60°C was about pH 3.8 to 6.8 (pH optimum 5.2). Substrates that are utilized in the presence of 0.055% yeast extract included: L-arabinose, galactose, cellobiose, glucose, mannose, fructose, lactose, pectin, sucrose, xylose, maltose, starch, rhamnose, pectin, xylan, N-acetylglucosamine and salacin (some strains utilized ribose and raffinose). Thiosulfate is reduced to elemental sulfur with sulfur deposition within the cell and also in the medium. No dissimilatory sulfate reduction with glucose, acetate, or lactate as electron donor.

Fermentation products: glucose $\rightarrow$ ethanol + acetic acid + 0.5 lactic acid 2 CO_2 + 2 H_2

Enrichment and habitat

T. aotearoense has only been isolated from various hot springs in New Zealand (North Island). The type strain was isolated from a small pool in the Weimangu thermal valley at the Warbick terrace. Strain was cultured under anaerobic conditions at 60°C (pH 4.5) on 0.1% yeast extract and 0.5% xylose (Liu et al. 1996a).

G+C content: 34.5 to 35 mol%

Available strains: JW/SL-NZ 604, JW/SL-NZ 606 = DSM 8692, JW/SL-NZ 611, JW/SL-NZ 613, JW/SL-NZ 614, JW/SL-NZ 625 (Liu et al., 1996a)

Type strain: JW/SL-NZ613 = DSM 10170

Gene Bank accession number (16S rRNA): X93359

Thermoanaerobacterium polysaccharolyticum Cann I.K.O., P.G. Stroot, K.R. Mackie, B.A. White and R.I. Mackie. 2001, 299[VP]

poly. sac.cha.ro.ly'ti.cum. Gr. n. *polysacchar*, many sugars; Gr. adj. *lyticus*, dissolving; N.L. neut. adj. *polysaccharolyticum*, many sugars dissolving.

This description is based on that of Cann et al. (2001), and on study of the type strain KMTHCJ.

Cells are straight rods occurring singly or sometimes in pairs with tumbling motility by flagella. Gram stain is variable but the ultrastructure determined by electron microscopy is Gram-type positive. Spores have not been observed. A surface layer-like protein has been

observed. Cells are catalase negative. Major fatty acids were determined to be iso 15:0 (70.6%), iso 17:0 (19.2%) and straight chain 16:0 (10.1%).

Growth characteristics

An obligate anaerobe that exhibits tolerance to air exposure, but the bacterium will grow in sealed tubes only after the reduction of an oxidized medium. Growth is only seen in the anaerobic region of a stab culture. Yeast extract is not required for growth. A wide variety of complex and simple carbohydrates, which include: melibiose, raffinose, arabinose, galactose, lactose, maltose, mannose, rhamnose, sucrose, trehalose, xylose, cellobiose and melezitose, are fermented but not cellulose, starch, xylan, cracked corn, pectin, sorbose, mannitol, malate, fumarate, citrate, glycerol or H_2/CO_2. Common end products of glucose fermentation are: ethanol and carbon dioxide with hydrogen, acetate, formate (more acetate than formate), and lactate being produced in lesser amounts. Glucose and xylose are used simultaneously when present in media in equal proportions. Doubling times at 68°C with glucose, raffinose, and melibiose as the carbon source are 2.1, 3.4 and 5.5 h, respectively. Thiosulfate is reduced to sulfide. Nitrate, sulfate and sulfur are not reduced. Indole is not produced. The optimum temperature for growth is between 65-68°C at pH 6.8 on glucose (max. is 70°C and growth does not occur below 45°C). The pH range for growth was between 5.0 and 8.0 (optimum pH was between 6.8-7.0 at 65°C).

Enrichment and habitat

Isolated from an organic waste pile from a canning factory in Hoopeston, IL, USA by the use of raffinose as the sole added carbon source at an incubation temperature of 60°C.

Industrial applications

T. polysaccharolyticum produces a highly active enzyme exhibiting both mannanase and endoglucanase activities (Cann et al. 1999). The thermostable alpha-galactosidase produced by the bacterium is also useful for pretreating food ingredients that can elict gastrointestinal disturbances if not modified by this enzyme (King et al. 2002). The utility of the CAK1-derived phagemid for use in the development of a gene transfer system for the clostridia was evaluated by using it to examine heterologous expression of *manA* derived from *T. polysaccharolyticum* in *E. coli* (Li et al. 2002). This is the second system available for thermophilic, anaerobic *Firmicutes* (Mai and Wiegel 1999).

G+C content: 46 mol%

Type strain: strain KMTHCJ = ATCC BAA-17 = DSM 13641 (Cann et al. 2001)

Gene Bank accession number (16S rRNA): U40229

Thermoanaerobacterium saccharolyticum Lee Y.E., M.K. Jain, C. Lee, S.E. Lowe and J.G. Zeikus. 1993, 49[VP]

sac.cha.ro.ly'ti.cum. Gr. n. *sacchar*, sugar; Gr. adj. *lyticus* dissolving; N.L. neut. adj. *saccharolyticum*, sugar dissolving.

This description is based on that of Lee et al. (1993d), and on study of the type strain B6A-RI.

Cells appear as rods (~ 0.8 to 1.0 by 3.0 to 15 μm; some cells as long as 30 μm) occurring in chains of varying lengths. Morphology is elongated during nutrient limitation or stationary phase. Cells are motile with peritrichous flagella, Gram-type positive but Gram-stain negative

and catalase negative. Cell wall contains three electron dense layers that are 5 nm thick and alternate with electron-light layers of similar thickness. Colonies on agar plates are soft, tan, circular, and convex with hollow centers ("donut"- shaped). Colony diameters range from 0.5-4.0 mm after 4 days at 55°C. Spores have not been observed. Cells contain a 1.5 MDa plasmid.

Growth characteristics

Bacterium is an obligate anaerobe existing chemoorganotrophically with growth stimulated by the presence of yeast extract. Growth is observed with xylan and starch but not cellulose, pectin, ribose, melibiose, melezitose, xylitol, or sorbitol. Hespell (1992) has further described the fermentation of xylans by strain B6A while Weimer (1985) described the fermentation of hemicellulose and hemicellulose-derived aldose sugars. Other complex and simple carbohydrates fermented include: maltose, lactose sucrose, cellobiose, glucose, xylose, galactose, mannose, fructose, trehalose, rhamnose, raffinose and mannitol. Landuyt et al. (1995) have also demonstrated growth on paraffin oil. Fermentation products from either glucose or xylan include: acetic acid and ethanol in approximately equal amounts, as well as H_2, lactic acid and CO_2. L-rhamnose fermentation yields equimolar amounts of 1,2-propandiol and a mixture of ethanol, acetic acid, lactic acid, H_2, and CO_2. No growth occurs in the absence of a fermentable carbohydrate. Thiosulfate is reduced to elemental sulfur which is deposited on the cell and in the media. The pH range for growth is 5.0 to less than 7.5 (the optimum pH is about 6.0). The temperature range for growth is a min. of 45°C to a max. of 68 to 70°C (60°C is the optimum). Cells can survive heating at 85°C for 15 min. but not 90°C for 5 min.

Growth inhibition

Growth is inhibited by penicillin G (200 µg/ml), 100 µg/ml of either chloramphenicol or neomycin, or O_2 (0.2 atm [ca. 20.26 kPa]). Growth occurs in the presence of up to 2% NaCl.

Enrichment and habitat

Isolation from geothermal sites in Yellowstone National Park and the Thermopolis areas, WY, USA and the Steamboat area, NV, USA. The type strain was isolated from sediments of the Frying Pan thermal acid spring in Yellowstone National Park. Isolation and enrichment of *T. saccharolyticum* was achieved using xylan as the carbon source (Lee et al. 1993d).

Industrial applications

T. saccharolyticum strain B6A-RI is currently being actively studied due to its production of a β-D-xylosidase (Lee and Zeikus 1993; Armand et al. 1996; Vocadlo et al. 2002a; Vocadlo et al. 2002b; Yang et al. 2004) and glucuronidases (Bronnenmeier et al. 1995). Lee et al. (1993a, 1993b) have cloned, sequenced, and biochemically characterized the gene for the endoxylanase of B6A-RI and are working on the regulation schemes of the xylanolytic enzymes. Saha et al. (1990) characterized an endo-acting amylopullulanase while Ramesh et al. (1994) cloned and sequenced the apu gene from B6A-RI and purified and characterized the amylopullulanase from *E. coli* The abundance of xylan in nature (the 2nd most abundant polymer in nature), cost and environmental considerations have caught the attention of the pulp and paper industry toward the application of this enzyme.

    T. saccharolyticum was also used for the cloning of the L-lactate dehydrogenase and the subsequent elimination of lactic acid as a fermentation product (Desai et al. 2004). The results

demonstrated progress toward metabolic engineering to eliminate the production of organic acids by thermophilic bacteria and to increase yields of desired fermentation end-products (e.g. ethanol). The xylose isomerase gene from *T. saccharolyticum* was cloned by complementation into an *E. coli* mutant (Lee et al. 1993c). New vectors constructed in order to express heterologous hydrolytic enzymes in *T. saccharolyticum* indicate this bacterium may function as an alternate expression host, especially for genes with a thermophilic origin (Mai and Wiegel 2001).

G+C content: 36 mol%

Type strain: strain B6A-RI = ATCC 49915 = DSM 7060 (Lee et al. 1993d)

Gene Bank accession number (16S rRNA): L09169

Thermoanaerobacterium thermosaccharolyticum (Basonym: *Clostridium thermosaccharo-lyticum,* McClung, 1935, 200[AL] Other synonyms: ("*Therminosporus thermosaccharolyticus"* (McClung, 1935) Prévot, 1938; *Clostridium tartarivorum*, (Hollaus and Klaushofer 1973; Matteuzzi et al. 1978)) Collins M.D., P.A. Lawson, A. Willems, J.J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe and J.A.E. Farrow. 1994, 824[VP]

ther.mo.sac.cha.ro.ly'ti.cum. Gr. adj. *thermos*, hot. Gr.n. *sacchar*, sugar; Gr. adj. *lytikos*, dissolving; N.L. neut. adj. *thermosaccharolyticum*, referring to thermophilic and sugar-dissolving.

This description is based on that of McClung (1935) and Hollaus and Sletyr (1972), and on study of the type strain LMG 2811.

Cells in PYG broth culture are Gram-stain negative (though Gram-type positive), typically motile, peritrichous and catalase negative. The *T. thermosaccharolyticum* S-layer represents the first observation of a bacterial S-layer glycan without a core region connecting the carbohydrate moiety with the polypeptide portion (Cejka and Baumeister 1987; Sara et al. 1988; Altman et al. 1990; Altman et al. 1995; Altman et al. 1996; Schaffer et al. 2000). The molecular mass of the monomeric subunit of the major S-layer protein from strain E207-71 was determined to be ~ 75 kDa (Allmaier et al. 1995). Cells appear to have an intracellular network of fibrils which could function as a cytoskeleton-like structure preserving cell shape (Mayer et al. 1998). Cells are rods (0.4 to 0.7 by 2.4 to 16 μm) occurring singly or in pairs but never in chains. Spores are observed as round or oval (1.3-1.5 μm) and located terminally distending the cell (Pheil and Ordal 1967; Campbell and Ordal 1968; Hsu and Ordal 1970). Sporulation is enhanced by several carbon sources that slow the growth rate of the cells (α- or β-methylglucoside, cellobiose, galactose, salicin and starch) (Hsu and Ordal 1969a, 1969b, 1970). Pheil and Ordal (1967) showed sporulation is enhanced by the addition of L-xylose or L-arabinose to a basal media (optimum pH for sporulation was 5.0-5.5). Cells become shorter and thicker in media containing glucose and do not sporulate when cultured on glucose. van Rijssel et al. (1992) isolated a lithotrophic *Clostridium* strain with extremely thermoresistant spores from a pectin-limited continuous culture of *T. thermosaccharolyticum*.

Colonies on pea-infusion agar are 2-4 mm, granular with indistinct feathered edges and are greyish-white with an often slightly raised center.

Growth characteristics

Obligate anaerobes with growth not occurring in the absence of a fermentable carbohydrate. Cell growth optimization experiments have been performed by Baskaran et al. (1995a). The optimum temperature for growth is between 55-62°C (some growth at 37°C, poor if any at 30°C, and no growth at 70°C). Tanaka et al. (1973) and Wilder et al (1963) have reported on the heat stable ferredoxins of *T. thermosaccharolyticum*. The pH for growth was between 6.5-8.5 (optimum pH 7.8) (Liu et al. 1996; Mosolova et al. 1991). Mosolova et al. (1991) has studied the effects of: pH, temperature and antibiotics on the growth and metabolism of *T. thermosaccharolyticum.*

Dextrin, pectin (Hollaus and Sleytr 1972), arabinose, fructose, galactose, glucose, mannose, xylose, cellobiose, lactose, maltose, sucrose, trehalose, glycogen, starch, xylan and salicin are fermented but not rhamnose, pyruvate, inulin or mannitol. However, it should be noted that cells that were originally cultured on pyruvate will ferment it. It is cells that were originally cultured with glucose that are unable to ferment pyruvate (Lee and Ordal 1967). Diauxic growth is observed when cells are cultured on glucose and xylose (Aduseopoku and Mitchell 1988). van Rijssel et al. (1993) showed the involvement of an intracellular oligogalacturonate hydrolase in the metabolism of pectin.

Common fermentation end-products from growth on PYG (peptone-yeast extract-glucose) broth include: acetic, butyric, lactic and succinic acids, ethanol (ethanol tolerance (Baskaran et al. 1995b) and production (Saddler and Chan 1984; Vancanneyt et al. 1987; Mistry and Cooney 1989) have been investigated in detail) and H_2 (Nikitina et al. 1993). Lynd et al. (2001) showed that ethanol, rather salt accumulation resulting from base added for pH control, was not the limiting factor for the growth of strain HG-8 at elevated feed xylose concentrations. Hill et al. (1993) have investigated end-product regulation during xylose fermentation under

nutrient limitations using continuous and batch cultures. Butanol formation at a neutral pH has been demonstrated by Freier-Schroder et al. (1989).

Nitrate is reduced and, sulfite and thiosulfate are reduced to H_2S. Meyer et al. (1990) have studied a rubredoxin from *T. thermosaccharolyticum* that might have a role in cellular sulfur metabolism. Nitrogen fixation and ammonia assimilation by cells has been studied by Clarke (1949) and Bogdahn and Kleiner (1986).

Enrichment and habitat

Cultured from: strains obtained from the National Canners' Research Laboratories in Washington, DC, USA and from original soil isolates by McClung (1935), tartrate infusion of grape residue (Mercer and Vaughn 1951) and pond sediment from the Netherlands (van Rijssel and Hansen 1989).

Industrial applications

The application of metabolic engineering for the development of new fermentation processes is an important technology for the conversion of renewable resources to chemicals (Saddler and Chan 1984).

T. thermosaccharolyticum has been used in microbial fermentations as an important organism for the production of 1,2-propanediol (1,2-PD) (Sanchezriera et al. 1987; Altaras et al. 2001; Cameron et al. 1998). *T. thermosaccharolyticum* is also of considerable interest as a producer of thermostable amylolytic enzymes. A thermoactive glucoamylase was purified and characterized by Specka et al. (1991). The bacterium has a soluble amylolytic enzyme system which was fractionated into a pullulanase, a glucoamylase and an alpha-glucosidase by

Ganghofner et al. (1998) and Feng et al. (2002). These enzymes are currently being further investigated (Aleshin et al., 2003).

Plasmids from strain DSM 571 have been mapped and further characterized by Belogurova et al. (1991, 1992, 2002) and Delver et al. (1996). Klapatch et al. (1996a, 1996b) have done some further molecular characterizations with strain HG-8 looking at: the specific endonuclease activity of the cell extract and electrotransformation of the cells.

G+C content: 29-32 mol% (Liu et al. 1996)

Available strains: DSM 571, ATCC 31925 = HG-6, ATCC 31960 = HG-8   (McClung 1935; Hollaus and Klaushofer 1973; Cameron and Cooney 1986), DSM 572 = ATCC 27384 = T9-1 (*Clostridium tartarivorum*), DSM 573 (Mercer and Vaughn 1951; Matteuzzi et al. 1978), DSM 869 = ATCC 25773 (Prévot 1938), DSM 7416 (van Rijssel and Hansen 1989), FH1 (Hoster et al. 2001)

Type strain: ATCC 7956 = DSM 571 = HAMBI 2225 = LMG 2811

Gene Bank accession number (16S rRNA): M59119

Thermoanaerobacterium thermosulfurigenes (Basonym: *Clostridium thermosulfurogenes*, Corrig. Schink and Zeikus, 1983b, 896[VP]) Lee Y.E., M.K. Jain, C. Lee, S.E. Lowe and J.G. Zeikus. 1993, 48[VP]

ther.mo.sul.fur.i'ge.nes. Gr. adj. *thermos*, hot. L.n. *sulfur*, brimstone; Gr. suff. *genes*, born from; N.L. neut. adj. *thermosulfurigenes*, releasing sulfur in heat.

Type species of *Thermoanaerobacterium*.

This description is based on that of Schink and Zeikus (1983a), and on study of the type strain 4B.

Cells are straight rods (0.5 by >2 μm), vary in length (from single cells 2 μm to filamentous chains greater than 20 μm, motile and peritrichous. Stains Gram-negative but electron micrographs reveal a double-layered wall without the presence of an outer membranous layer (Gram-type positive). Distinctive features include: double-layered wall architecture, numerous internal membranes that appeared vesicular, a thin cell wall, no outer cell wall and large and electron-dense cytoplasmic granules inside cells. The components of the cell envelope of strain EM1 were isolated, and the S-layer protein was purified and characterized by Brechtel et al. (1999). In strain EM1, the S-layer homology domains do not attach to the peptidoglycan (Brechtel and Bahl 1999). Significant differences in the cell envelope structure could be observed when the cells were grown in continuous culture under glucose limitation as compared to cells grown under starch limitation (Antranikian et al. 1987; Mayer et al. 1998). More than likely the phenomenon is due to the increase in production of α-amylase and pullulanase during starch limitation (Antranikian et al. 1987; Mayer and Gottschalk 2004). Agar embedded colonies are fluffy (0.5-1.5 mm in diameter with no pigment). Terminal, spherical, refractile, white spores (free spores rare) with distinctly swollen sporangia are observed with xylose or pectin as the energy source (but never with glucose). Catalase is not present, and cytochromes are not detected.

Growth characteristics

An obligate thermophile and anaerobe that grows chemoorganotrophically with yeast extract and tryptone enhancing growth. The optimum temperature was near 60°C (max. below 75°C and min. above 35°C). Narberhaus et al. (1994) has studied the synthesis of heat shock proteins in

strain EM1. The pH optimum for growth on glucose was 5.5-6.5 (with growth not observed below 4.0 or above 7.6).

Fermentable carbohydrates include: pectin, starch, polygalacturonic acid, amygdalin, aesculin, salicin, D-xylose, galactose, glucose, inositol, mannitol, melibiose, rhamnose, trehalose, mannose, cellobiose, maltose, L-arabinose and sucrose but no growth on H_2/CO_2, cellulose, arabinogalactan, galacturonate, citrate, pyruvate, lactate, tartrate, lactose, melezitose, raffinose, D-ribose, sorbitol, methanol, or glycerol. β-amylase is expressed with growth on maltose but not glucose or sucrose (Hyun and Zeikus 1985b). Doubling time on glucose or pectin was approximately 2 h. Glucose fermentation yields H_2/CO_2, ethanol, acetate, and lactate (whereas methanol and isopropanol were also produced during pectin fermentation). Ethanol is produced as the major, soluble, reduced end-product of growth and not butyrate or acetate. Fermentation products: glucose (0.5 %) → 207 ethanol + 152 acetic acid + 113 lactic acid + 317 CO_2 + 231 H_2 (μmol)

Thiosulfate is reduced to elemental sulfur with no sulfide or sulfite formed. Sulfate and nitrate are not reduced. Sulfur droplets were found localized in the cytoplasm (Liu et al. 1996). Addition of sulfite inhibited growth with no production of elemental sulfur. Without the presence of thiosulfate, growth occurred without the formation of inorganic sulfur metabolites. Gelatin is liquefied but does not produce indole, acetylmethylcarbinol or hydrogen sulfide.

Growth inhibition

Cell growth is inhibited by: 100 μg/ml of penicillin, streptomycin, cycloserine, tetracycline or chloramphenicol; 500 μg/mL sodium azide; 2% sodium chloride; O_2 (0.203×10^5 Pa); 2% NaCl or sulfite. No growth inhibition was observed due to the presence of hydrogen.

Enrichment and habitat

Isolated from a thermal (55-65°C), volcanic, algal-bacterial community (Octopus Spring) in Yellowstone National Park, WY, USA via enrichment with pectin as the energy source (Schink and Zeikus 1983a) and fruit juice waste products in Germany (Madi et al. 1987).

Industrial applications

T. thermosulfurigenes was found to have very active pectinases, a high ethanol/ lactate ratio during growth on pentoses and a pectin methylesterase and polygalacturonase hydrolase activity. *T. thermosulfurigenes* has also been studied because of the activity of its starch degrading enzymes (pullulanases and amylases), which are desirable due to their elevated temperature optima (temperature being an exploited property to improve the solubility of starch and to prevent bacterial contamination) (Hyun and Zeikus 1985a; Madi et al. 1987; Haeckel and Bahl 1989; Burchhardt et al. 1991; Matuschek et al. 1994; Sahm et al. 1996). Matuschek et al. have characterized the genes from strain EM1 that encode: two glycosyl hydrolases (1996) and a novel ABC transporter (1997). Huber et al. (1996) have investigated the structure and some of the kinetic properties of the β-galactosidase from strain EM1.

Lloyd et al. (1994) have performed the crystallization and preliminary X-ray diffraction studies of the xylose isomerase from strain 4B while Sriprapundh et al. (2000) reported on the thermal stability and activity of the isomerase. The D-xylose ketol-isomerase of *T. thermosulfurigenes* has also been studied for applications: in the food industry for the production of sweeteners (can use D-glucose as a substrate to yield D-fructose) (Kim et al. 2001; Meng et al. 1993a; Meng et al. 1993b) and as an effective selection agent of transgenic plant cells (using D-xylose as the selection agent) (Haldrup et al. 1998).

Knegtel et al. (1996) resolved the crystal structure of the cyclodextrin glycosyltransferase of strain EM1 at 2.3 A, and Leemhuis et al. (2003) and Wind et al. (1995, 1998) have done further work and characterization of this enzyme. Strain EM1 was used for studies of the three-dimensional structure of polypeptides and proteins, in particular beta-galactosidases (Zolotarev et al. 2003).

G+C content: 32.6 mol%

Available strains: 4B (Schink and Zeikus 1983a), DSM 3896 = EM1 (Madi et al. 1987)

Type strain: 4B = ATCC 33743 = DSM 2229

Gene Bank accession number (16S rRNA): L09171

Thermoanaerobacterium xylanolyticum Lee Y.E., M.K. Jain, C. Lee, S.E. Lowe and J.G. Zeikus. 1993, 48[VP]

xy.lan.o.ly'ti.cum. Gr. n. *xylanosum,* xylan; Gr. adj. *lyticus*, dissolving; N.L. adj. *xylanolyticum*, xylan dissolving.

This description is based on that of Lee et al. (1993d), and on study of the type strain LX-11.

Cells are Gram-stain negative but Gram-type positive, motile, short rods (0.8 to 1.0 by 2.0 to 7.0 μm) that occur singly or in pairs. An elongated morphology does not develop during nutrient limitation or stationary phase (unlike *T. saccharolyticum*), and the cytoplasm is much less granular. Terminal, spherical spores are formed with free spores seen rarely. Surface colonies on agar are circular, cloudy to white in color, 2 to 5 mm in diameter (rough surface texture) with smooth edges.

Growth characteristics

Bacterium is an obligate anaerobe that grows on xylan and starch but not cellulose, ribose, melibiose, melezitose, xylitol, pectin or lactate. Yeast extract stimulates growth. Other fermented carbohydrates include: maltose, lactose sucrose, cellobiose, glucose, xylose, galactose, mannose, fructose, rhamnose and mannitol. Fermentation products from either glucose or xylan include: acetic acid and ethanol in approximately equal amounts, as well as H_2 and CO_2 but no lactic acid. The pH range for growth is 5.0 to 7.5 (optimum pH is about 6.0) with a temperature range for growth from 45 to 70°C (60°C is the optimum). Thiosulfate is reduced to elemental sulfur, which is deposited on the cell and in the medium.

Growth inhibition

Cell growth is inhibited by penicillin G (200 µg/ml); 100 µg/ml of either neomycin sulfate, ampicillin, streptomycin sulfate, rifampin, polymyxin B, erythromycin, tetracycline, or acridine orange; or by 1% NaCl.

Enrichment and habitat

Isolated from geothermal sites (sediments of the Frying Pan thermal acid spring) in Yellowstone National Park, WY, USA using xylan as the carbon source (Lee et al. 1993d).

G+C content: 36.1 mol%

Type strain: LX-11 = ATCC 49914 = DSM 7097 (Lee et al. 1993d)

Gene Bank accession number (16S rRNA): L09172

Thermoanaerobacterium zeae Cann I.K.O., P.G. Stroot, K.R. Mackie, B.A. White and R.I. Mackie. 2001, 300[VP]

za.ae. Gr. n. *zeae* of corn, describing the use of corn as a substrate for growth.

This description is based on that of Cann et al. (2001), and on study of the type strain mel2.

Cells are motile by peritrichous flagella, straight rods, Gram-type positive, even though cells stain Gram variable, and occur singly or sometimes in pairs. Spore formation has never been observed. Growth will occur on solid media with the production of catalase negative cells.

Growth characteristics

Cells are obligately anaerobic but neither nitrate, thiosulfate, sulfate nor sulfur is reduced. Indole is not produced. A wide variety of complex and simple carbohydrates are fermented, which include: cracked corn, starch, xylan, glucose, melibiose, raffinose, arabinose, galactose, lactose, maltose, mannose, rhamnose, salicin, sucrose, trehalose, xylose, cellobiose, melizitose and pyruvate but not cellulose, pectin, sorbose, mannitol, malate, fumarate, citrate, glycerol or H_2/CO_2. The end products of glucose fermentation are ethanol and carbon dioxide with hydrogen, acetate, formate also been produced in lesser quantities (with more formate than acetate produced. The optimum temperature for growth (when glucose is used as the substrate) is between 65-70°C at pH 6.8 (max. is 72°C and growth does not occur below 37°C). The pH range for growth is 3.9-7.9. The fastest doubling time on glucose observed was 1.8 h at the temperature optimum.

Growth inhibition

Growth is completely inhibited by tetracycline, rifampicin, kanamycin, and erythromycin (50 µg/ml for all). However, growth does occur in the presence of concentrations of 300 mM NaCl, 450 mM KCl and 150 mM NH_4Cl.

Enrichment and habitat

Isolated from an organic waste pile from a canning factory in Hoopeston, IL, USA by the use of melibiose as the sole added carbon source at an incubation temperature of 60°C.

G+C content: 42 mol%

Type strain: mel2 = ATCC BAA-16 = DSM 13642 (Cann et al. 2001)

Gene Bank accession number (16S rRNA): U75993

Table 2.1. Comparison of physiological traits of the *Thermoanaerobacterium* species. All species are: motile and produce acetic acid, ethanol, H_2 and CO_2 when fermenting glucose.

Characteristics	*T. aotearoense*	*T. polysaccharolyticum*	*T. saccharolyticum*	*T. thermosaccharolyticum*	*T. thermosulfurigenes*	*T. xylanolyticum*	*T. zeae*
Spores	+	-	-	+	+	+	-
Gram Stain	neg	var	neg	neg	neg	neg	var
Opt. temp. (°C)	60-63	65-68	60	55-62	60	60	65-70
pH optimum	5.2	6.8-7.0	6.0	7.8	5.5-6.5	6.0	ND
G+C content (mol%)	34.5-35	46	36	29-32	32.6	36.1	42
Products of glucose fermentation							
Butyric acid	-	-	-	+	-	-	-
Formic acid	-	+	-	-	-	-	+
Lactic acid	+	+	+	+	+	-	-
Succinic acid	-	-	-	+	-	-	-
Pectin fermented	+	-	-	+	+	-	-
Lactose fermented	+	+	+	+	-	+	+
Thiosulfate reduction							
To H_2S	-	+	-	+	-	-	-
To S^0	+	-	+	-	+	+	-

Figure 2.1. Phylogenetic tree of the *Thermoanaerobacterium* species. Fitch tree showing the estimated phylogenetic relationships of *Thermoanaerobacterium* species based on 16S rRNA gene sequence data with Jukes-Cantor correction for synonymous changes. The 16S rRNA gene data used represent *Escherichia coli* DSM30083[T] nucleotide positions 105–1450. Numbers at nodes indicate bootstrap support percentages for 100 replicates. Bar, 0.05 nucleotide substitutions per site. GenBank accession numbers are indicated after the strain identifier. The superscript "T" denotes the strain is the type strain for the species.

Thermoanaerobacterium thermosulfurigenes DSM 2229[T] L09171
Thermoanaerobacterium xylanolyticum DSM 7097[T] L09172
Thermoanaerobacterium aotearoense JW/SL-NZ613[T] X93359
Thermoanaerobacterium saccharolyticum DSM 7060[T] L09169
Thermoanaerobacterium thermosaccharolyticum DSM 571[T] M59119
Thermoanaerobacterium polysaccharolyticum DSM 13641[T] U40229
Thermoanaerobacterium zeae DSM 13642[T] U75993
Thermoanaerobacter ethanolicus JW 200[T] L09162
Caldanaerobacter subterraneus subsp. subterraneus DSM 13054[T] AF195797
Thermacetogenium phaeum DSM 12270[T] AB020336
Thermosyntropha lipolytica JW/VS-265[T] X99980
Syntrophomonas wolfei subsp. wolfei DSM 2245[T] AF022248
Ammonifex degensii DSM 10501[T] U34975
Coprothermobacter proteolyticus DSM 5265[T] X69335
Clostridium stercorarium subsp. stercorarium NCIMB 11754[T] L09174
Moorella thermoacetica LJD M59121
Sporotomaculum hydroxybenzoicum DSM 5475[T] Y14845
Desulfotomaculum thermobenzoicum subsp. thermobenzoicum DSM 6193[T] AJ294429
Desulfotomaculum kuznetsovii DSM 6115[T] AJ294427
Desulfotomaculum thermocisternum ST90[T] U33455
Desulfotomaculum australicum DSM 11792[T] M96665
Bacillus subtilis subsp. subtilis DSM 10[T] AJ276351

0.05

CHAPTER 3

THE *THERMOANAEROBACTER* [1]

Abstract

Ther.mo.an.ae.ro.bac'ter Gr. adj. *thermus*, hot; Gr. pref. *an* not; Gr. n. *aer*, air; M. L. *bacter* masc. equivalent of Gr. neut. n. *bacterion* rod staff; M. L. muse. n. thermoanaerobacter, rod which grows in the absence of air at higher temperatures.

All species have a Gram-type positive cell wall (Wiegel 1981), but Gram-stain reaction is variable among the member species. The Gram-stain reaction is even variable for some of the individual species of the genus. Most species are motile by a peritrichous arrangement of flagella. Cells are rod-shaped and occur in various arrangements that vary by species. Many of the *Thermoanaerobacter* species, as first described for the type species (Wiegel and Ljungdahl 1981), form pleomorphic cells at the late exponential and stationary growth phases, assembling through non-symmetric cell division (forming coccoid cells frequently in chains alternating rod-shaped + coccoid shaped-cells). Other pleomorphic rods are formed, assumingly through weakening of the cell wall, through the possible mode of action of lysozyme-like enzymes that are the remnants of the sporulation process.

Further descriptive information

Endospore formation has been observed for all species except: *T. acetoethylicus***,** *T. ethanolicus,* and *T. sulfurophilus*. However, for several species the presence of the characteristic spore specific genes have been demonstrated, thus the species for which no spores have been observed are regarded as asporogenic (Onyenwoke et al. 2004). Spore formation in some species is rarely observed (e.g. *T. brockii* subsp. *brockii* (Cook et al. 1991)), whereas strains of *T. thermohydrosulfuricus* and *T. kivui* easily form spores with above 50% of vegetative cells sporulating. Growth temperature optima range from 55- 75°C with growth ranges of 35- 78°C.

Many species exhibit a wide temperature span for growth over 35°C, however, the temperature versus growth rate plots, which exhibit a biphasic curve, indicate changes in the rate limiting steps. Wiegel (1990) suggested that these bacteria contain, for some critical metabolic steps, two enzymes: one for the lower temperature of the growth range and one for the higher. This could also have an evolutionary relevance (Wiegel 1990; Wiegel 1998). Several species also exhibit a wide pH optimum over 3 pH units without a specific peak (e.g. *T. ethanolicus* JW 200). The pH growth range is also quite wide among species ranging from 4.0- 9.9 with pH optima between 4.8- 8.5.

One of the previous characteristics of the genus was the reduction of thiosulfate to H_2S by all of its member species (Lee et al. 1993d) until *T. italicus* (Kozianowski et al. 1997) and *'T. sulfurigignens'* (Dashti, M., S.Y. Liu, F. Rainey, F. Mayer, and J. Wiegel, thesis, The University of Georgia) were characterized and shown to produce: both H_2S and S^0, and only S^0, respectively. All species are capable of chemoorganotrophic growth with a requirement of yeast extract and a fermentable carbohydrate for many species. An exception is *T. wiegelii* which has no requirement for yeast extract (Cook et al. 1996). In addition, *T. kivui* (Leigh et al. 1981) and *T. siderophilus* (Slobodkin et al. 1999) are also capable of autotrophic growth. There are also reports of other *Thermoanaerobacter* strains that are capable of coupling H_2 oxidation to growth (Fardeau et al. 1993; Fardeau et al. 1994). Common fermentation end-products are: acetic acid, ethanol (Kannan and Mutharasan 1985), lactic acid, H_2 and CO_2.

Information about peptide and amino-acid oxidation in the presence of thiosulfate by members of the genus has been reported by Faudon et al. (1995). Cellular polyamine distribution profiles have been investigated for this genus and show some differences from the phylogenetically related thermophilic anaerobes *Moorella*, *Dictyoglomus*, and

Thermoanaerobacterium (Hamana et al. 2001). The configuration of quinone systems, cellular fatty acids and diaminopimelic acids in *Thermoanaerobacter* species have been described by Yamamoto et al. (1998). Chaperonins from *Thermoanaerobacter* species have been discussed by Scopes and Truscott (1998) and Truscott and Scopes (1998). Members of the genus include "canned food spoilers" (McClung 1935; Dotzauer et al. 2002). Subbotina et al. (2003a, 2003b) have developed specific oligonucleotide probes for the detection of *Thermoanaerobacter* species.

Isolation and enrichment

Thermoanaerobacter have been isolated from high temperature petroleum reservoirs by the direct supplementation of production waters with glucose and either yeast extract, peptone, tryptone or casamino acids (Grassia et al. 1996) and identified by 16S rDNA gene cloning and sequencing (Leu et al. 1998; Orphan et al. 2000). Szewzyk et al. (1994) isolated glucose- and starch-degrading strains related to *Thermoanaerobacter* from a deep borehole in granite. Slobodkin et al. (1999a, 1999b), Zhou et al. (2001) and Roh et al. (2002) have reported on the isolation and characterization of metal-reducing *Thermoanaerobacter* strains from deep subsurface environments.

G+C content: 30-38 mol%

Type species: *Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl 1981) Wiegel and Ljungdahl 1982, 384[VP]

Phylogenetic information

The genus *Thermoanaerobacter* is a member of the '*Thermoanaerobacteriales',* (order II of the class '*Clostridia*') and belong to the family '*Thermoanaerobacteriaceae'* (Garrity et al. 2002). *Thermoanaerobacter subterraneus* (Fardeau et al. 2000), *Thermoanaerobacter tengcongensis* (Xue et al. 2001) and *Thermoanaerobacter yonseiensis* (Kim et al. 2001) have been reassigned to the genus *Caldanaerobacter* by Fardeau et al. (2004) and renamed *Caldanaerobacter subterraneus*, *Caldanaerobacter subterraneus* subsp. *tengcongensis* and *Caldanaerobacter subterraneus* subsp. *yonseiensis*, respectively. This reassignment was based on phylogenetic and metabolic differences (e.g. production of L-alanine as a major fermentation product from growth on glucose and growth at higher temperatures (up to 80°C)) between these 3 species and the other *Thermoanaerobacter* (Fardeau et al. 2004).

Industrial applications

Thermoanaerobacter species have been used in studies to understand and exploit the huge array of enzymes that these bacteria produce to breakdown carbohydrates (Alister et al. 1990; Zeikus et al. 1991; Svensson 1994; Fardeau et al. 1996; Kuriki and Imanaka 1999) and because of the typical thermostable nature of the enzymes themselves (Zamost et al. 1991; Vieille and Zeikus 2001). Lind et al. (1989) identified a beta-galactosidase from a strain of *Thermoanaerobacter*, and Mitchell et al. (1982) characterized a beta-1, 4- glucosidase activity. The general mechanism for regulation of glucoamylase and pullulanase synthesis has been studied (Hyun and Zeikus 1985a; Mathupala et al. 1993; Lin and Leu 2002). Xylose and pectin metabolism have also been further elucidated by the studies of Erbeznik et al. (1998a, 1998b) and Kozianowski et al. (1997), respectively. Wynter et al. (1996, 1997) have described a novel thermostable dextranase from a

Thermoanaerobacter species. Keratinoltytic activity has been shown for a species of *Thermoanaerobacter*, '*Thermoanaerobacter keratinophilus*', by the demonstration of hydrolysis of feather meal (Riessen and Antranikian 2001). An industrial application was shown with production of ethanol in pretreated hemicellulosic hydrolysates from wheat straw (Ahring et al. 1996; Ahring et al. 1999; Sommer et al. 2004). High ethanol tolerances also make these bacteria useful for industrial production schemes (Wiegel and Ljungdahl 1981; Lovitt et al. 1984; Wiegel 1992; Larsen et al. 1997). The effects of temperature on the stereochemistry of enzymatic-reactions, with particular detail to alcohol dehydrogenases, has been studied by Phillips (1996, 2002b) and Phillips et al. (1994). Efficient and thermostable isomerases have also been characterized and studied (Jorgensen et al. 2004). Jorgensen et al. (1997) also identified and cloned the nucleotide sequence of a thermostable cyclodextrin glycosyltransferase from a *Thermoanaerobacter* species. Kim et al. (1997) also report on the production of cyclodextrin using raw corn starch and a cyclodextrin glycosyltransferase from *Thermoanaerobacter*. Martin et al. (2001, 2003, 2004) have used *Thermoanaerobacter* cyclodextrin glycosyltransferases as immobilized biocatalysts for the synthesis of oligosaccharides. Nakano et al. (2003) have reported on the synthesis of glycosyl glycerol by a cyclodextrin glucanotransferases. The effects of temperature on the kinetics of mesophillic and thermophilic (a *Thermoanaerobacter* species) 3-phosphoglycerate kinases has been described by Thomas and Scopes (1998).

List of species in the genus *Thermoanaerobacter*

Thermoanaerobacter acetoethylicus (Basonym: *Thermobacteroides acetoethylicus*, Ben-Bassat and Zeikus, 1981) Rainey and Stackebrandt 1993, 857[VP]

a.ce.to.e.thy.licus. L. n. *acetum* vinegar. M. L. n. *ethylicus* ethyl alcohol.

This description is based on that of Ben-Bassat and Zeikus (1981), and on study of the type strain HTB2.

Cells are motile rods (0.6 by 1.5-2.5 μm) that occur singly or pairs. Flagella are arranged peritrichously, and the cells have never been observed to form spores. The cells have a multi-layered Gram-type positive (though Gram-stain negative) cell wall with no outer wall membrane. Colonies are uniformly round, flat, mucoid and nonpigmented with a diameter of 3 mm after 48 h. Cytochrome pigment and catalase are both absent.

Growth characteristics

The optimum temperature for growth is 65°C (max. <80°C and min. >40°C). The pH range for growth is 5.5 to 8.5 (at 65°C). Cells are obligate anaerobes and chemoorganotrophic with a requirement for yeast extract. The glycolytic Embden-Meyerhof -Parnas pathway is used. Fermented carbohydrates include: glucose, mannose, cellobiose, maltose, sucrose, lactose and starch. The major end products of glucose fermentation were: acetic acid and ethanol (in equal amounts), as well as isobutyric acid, butyric acid, H_2 and CO_2 but not lactic acid. Thiosulfate (but not sulfate) is reduced to hydrogen sulfide.

Fermentation products (μmol): 130 glucose $\rightarrow$ 102 ethanol + 126 acetic acid + 157 CO_2 + 165 H_2

Growth inhibition

Growth is inhibited by: air; 2% NaCl; and 10 μg/ml of tetracycline, streptomycin, penicillin G, vancomycin and neomycin. H_2 at 2 atm. does not inhibit growth.

Enrichment and habitat

Isolated from an Octopus Spring algal-bacterial mat at Yellowstone National Park, WY, USA by the use of trypticase peptone-yeast extract glucose (TYEG) medium (pH 7.2).

G+C content: 31 mol%

Strains available: HTD1 (Zeikus et al. 1979; Zeikus et al. 1980).

Type strain: HTB2 = HTB2/W = ATCC 33265 = DSM 2359 (Zeikus et al. 1980).

Gene Bank accession number (16S rRNA): L09163

Thermoanaerobacter brockii* subsp. *brockii (Basonym: *Thermoanaerobium brockii*, Zeikus, Hegge and Anderson 1983, 673[VP]) (*Thermoanaerobacter brockii*, Lee, Jain, Lee, Lowe and Zeikus 1993, 49[AL]), emend. Cayol, Ollivier, Patel, Ravot, Magot, Ageron, Grimont and Garcia 1995, 787[VP]

brock'i.i. M.. L. gen. n. *brockii*, of Brock, named for Thomas Dale Brock, who performed pioneering studies on the physiological ecology of extreme thermophiles.

This description is based on that of Zeikus et al. (1979), and on study of the type strain HTD4.

Cells are: Gram-type positive (Gram-stain positive), short rods (1.0 by 2 to 20 μm). Cell size varies frequently by length (minicells common), and cells arrangement occurs in pairs, chains, and filaments. Round, heat-resistant terminal spores are observed. Cytochrome pigment and catalase are absent. Colonies on agar are circular (0.2 to 0.3 mm in diameter), flat, mucoid and nonpigmented. AGram-type positive cell wall architecture without an outer wall membrane is present.

Growth characteristics

Cells are chemoorganotrophic with yeast extract and a fermentable carbohydrate required for growth. The optimum temperature for growth is thermophilic (65 to 70°C with a temperature range of >35°C to <85°C). The doubling time at the optimum temperature is ~ 1 h. The pH range is 5.5 to 9.5 (with the optimum at 7.5). Cells are obligate anaerobes and ferment a wide variety of substrates including: glucose, maltose, sucrose, lactose, cellobiose, starch and pyruvate but not xylose, cellulose, arabinose, mannose, lactate, tartrate, ethanol, tryptone, casamino acids or pectin. Common end products of glucose fermentation include: acetic acid, lactic acid, ethanol, H_2 and CO_2 but not butyric acid. Exogenous H_2 addition (0.4 to 1.0 atm) to a cellobiose fermentation increased the ethanol/acetate ratio (Lamed and Zeikus 1980).

Fardeau et al. (1997) has reported on the utilization of serine, leucine, isoleucine, and valine by the bacterium. Maruta et al. (2002) identified the gene encoding a trehalose

phosphorylase. Thiosulfate is reduced to hydrogen sulfide. No reduction of oxygen, sulfate, nitrate or fumarate is observed. Also no protein hydrolysis occurs.

Growth inhibition

Cell growth is inhibited by: penicillin, cycloserine, streptomycin, tetracycline and chloramphenicol (100 µg/ml); exposure to air (21% O_2); and 2% NaCl .

Enrichment and habitat

Isolation of type strain originally from Washburn pool B spring sediment at Yellowstone National Park, WY, USA using TYEG media (pH 7.2-7.4).

Industrial applications

The alcohol dehydrogenases of this bacterium have been characterized and studied by numerous groups which include: Peretz and Burstein 1989; Bogin et al. 1997; Yang et al. 1997; Bogin et al. 1998a, 1998b; Korkhin et al. 1998; Peretz et al. 1997a, 1997b; Korkhin et al. 1999; Li et al. 1999; Kleifield et al. 2000; McMahon and Mulcahy 2002; Miroliaei and Nemat-Gorgani 2002; Kleifield et al. 2003a, 2003b, 2004. Rabinkov et al. (2000) investigated the disulfide modification and antioxidant properties of S-allylmercaptoglutathione using the alcohol dehydrogenase of *T. brockii* subsp. *brockii* as one of disulfide-containing test enzymes.

 T. brockii subsp. *brockii* is of interest because of its thermostable chaperone proteins (chaperonin 60 and chaperonin 10 in particular) (Truscott et al. 1994; Todd et al. 1995). The bacterium has also been used for studies on the reduction of β-oxoesters (Seebach et al. 1984). Breves et al. (1997) identified the genes encoding two different beta-glucosidases. Oligosaccharides synthesis and structural analysis has been performed by Okada et al. (2003).

G+C content: 30-31 mol%

 Available strains: HTA1, HTD4, HTD6, HTR1 = DSM 2599, ATCC 35047 (Zeikus et al. 1979)

Type strain: HTD4 =ATCC 33075 = ATCC 53556 = DSM 1457

Gene Bank accession number (16S rRNA): L09165

Thermoanaerobacter brockii subsp. *finnii* (Basonym: *Thermoanaerobacter finnii*, Schmid et al. 1986) Cayol, Ollivier, Patel, Ravot, Magot, Ageron, Grimont and Garcia 1995, 788[VP]

fin'ni.i. M. L. gen. n. *finnii*, of Finn, named for Robert K. Finn, who made important contributions to the development of the ethanol vacuum fermentation process.

This description is based on that of Schmid et al. (1986), and on study of the type strain AKO-1.

Short rods (0.4 to 0.6 by 1 to 4 μm), occurring singly, in pairs and in short chains (occasionally coccoid cells), which are motile. Cells are Gram-type positive **(Gram-stain variable)** and form heat-resistant terminal spores. Colonies are circular (1 to 3 mm in diameter) smooth, white and round. The cell wall contains peptidoglycan of the *meso*-diaminopimelic acid type.

Growth characteristics

Cells are thermophilic (optimum temperature of 65°C) with a temperature range from 40°C to 75°C. The optimum pH is between 6.5-6.8. An obligate anaerobe with a chemoorganotrophic

growth requirement describes *T.brockii* subsp. *finnii*. Growth requires the presence of yeast extract and a fermentable carbohydrate. Fermented sugars are numerous and include: glucose, fructose, galactose, mannose, cellobiose, maltose, melibiose, sucrose, lactose, xylose, ribose, mannitol and pyruvate. The end products of glucose and xylose fermentation are ethanol and CO_2, as well as minor amounts of acetic acid and L-lactic acid. Thiosulfate is reduced to hydrogen sulfide.

Fermentation products (µmol/mL): 12.1 glucose $\rightarrow$ 20.1 ethanol + 3.2 acetic acid + 4.6 lactic acid + 20.1 CO_2 + 3.0 H_2

Growth inhibition

Growth inhibition was by penicillin G and tetracycline (6 µg/ml).

Enrichment and habitat

Isolated from sediment sludge from Lake Kivu in East Africa using M-1 media (which includes lactic acid, glucose and yeast extract) at 60°C.

Industrial applications

This bacterium was used to develop an assay to determine pyridine nucleotide levels in cell extracts as low as 1 pmol (Schmid et al. 1989). *T. brockii* subsp. *finnii* has also been used in studies aimed at understanding the breakdown of carbohydrates found in sulfide-, elemental sulfur- or sulfate-rich thermal hot springs and oil fields (Fardeau et al. 1996). Antranikian (1989)

reported on the formation of an extracellular, thermoactive amylase and pullulanase in batch culture by *T. brockii* subsp. *finni*.

G+C content: 32 mol%

 Type strain: AKO-1 = ATCC 43586 = DSM 3389 (Schmid et al. 1986)

Gene Bank accession number (16S rRNA): L09166

Thermoanaerobacter brockii* subsp. *lactiethylicus Cayol, Ollivier, Patel, Ravot, Magot, Ageron, Grimont and Garcia 1995, 788[VP]

lac.ti.e.thy'li.cus. L. n. *lacticum*, lactic acid; M. L. n. *ethylicus*, ethyl alcohol; *lactiethylicus*, referring to the production of both lactic acid and ethanol.

This description is based on that of Cayol et al. (1995), and on study of the type strain SEBR 5268.

Cells are straight rods (0.5 by 2 to 3 μm), motile by means of peritrichous flagella and occur singly or in pairs in young cultures. Pleomorphic filamentous cells (length of 15 μm) appear in older cultures. Cells are Gram-type positive (Gram-stain positive) and form spores in a medium containing D-xylose as an electron donor and thiosulfate as an electron acceptor (but not if D-xylose is replaced with glucose). Colonies in a roll tube are: 4 mm in diameter after 2 days of incubation at 60°C, smooth, uniformly round, mucoid, nonpigmented and flat.

Growth characteristics

This obligate anaerobe is thermophilic (optimum temperature 55-60°C) with a temperature range between 40°C and 75°C. The pH range is 5.6 to 8.8. Yeast extract is required for fermentation of carbohydrates. The doubling time with glucose as the carbon source is 2h at 60°C. Fermentable substrates are: glucose, fructose, galactose, mannose, D-ribose, D-xylose, cellobiose, lactose, maltose, sucrose, mannitol, starch, yeast extract and pyruvate but not L-arabinose, cellulose, L-rhamnose, glycerol, ribitol, galactitol, sorbose or melibiose. End products of glucose fermentation are acetic acid, lactic acid, ethanol, H_2 and CO_2. Thiosulfate, sulfite and elemental sulfur are reduced to hydrogen sulfide. No reduction of sulfate, nitrate or fumarate occurs.

Growth inhibition

A concentration of 4.5% NaCl is tolerated (the optimum NaCl concentration is 1%).

Enrichment and habitat

Type strain isolated from geothermally heated (92°C) French, oil samples through the incubation of samples in an anaerobic, glucose-based medium at 60°C.

Industrial applications

T. brockii subsp. *lactiethylicus* has been studied, along with *T. brockii* subsp. *finnii*, in an attempt to understand the breakdown of carbohydrates found in sulfide-, elemental sulfur- or sulfate-rich thermal hot springs and oil fields (Fardeau et al. 1996).

G+C content: 35 mol%

Available strains: SEBR 7311, SEBR 7312 (Cayol et al. 1995)

Type strain: SEBR 5268 = DSM 9801 (Cayol et al. 1995)

Gene Bank accession number (16S rRNA): U14330

Thermoanaerobacter brockii subsp. '*pseudoethanolicus*' (Basonym: *Clostridium thermohydrosulfuricum* 39E (Zeikus et al. 1980); *Thermoanaerobacter ethanolicus* 39E (Lee, Jain, Lee, Lowe and Zeikus 1993, 47[VP]))

This description is based on that of Zeikus et al. (1980), and study of strain 39E. Based on the 16S rRNA sequence analysis (Fig. 1), it became obvious that strain 39E does not belong to the species *ethanolicus*. Thus, it is described here as a new subspecies *pseudoethanolicus* strain 39 E of *T. brockii*, naming is based on the 16S rRNA and its *T. ethanolicus* JW 200- like properties. The genome sequence of 39E will be available in the near future.

Cells are rod-shaped and sporulate when grown with xylose as substrate. Gram-stain reaction is variable, but the cell wall is Gram-type positive. The cells are motile and reduce thiosulfate.

Growth characteristics

Bacterium utilizes the Embden-Meyerhof-Parnas Pathway and the pentose-phosphate pathway, respectively, for the catabolism of hexoses and pentoses to pyruvate (Zeikus et al. 1981). Fermented carbohydrates include: xylose, cellobiose, starch, glucose, maltose and sucrose. Glucose is consumed in preference to cellobiose as an energy source for growth (Ng and Zeikus 1982.). A coculture of *T. brockii* subsp. *pseudoethanolicus* and *Clostridium thermocellum* is stable and leads to an increased yield of ethanol during cellulose fermentations (Ng et al. 1981). Jones et al. have studied high-affinity maltose (2000, 2002b) and xylose (2002a) binding and transport in strain 39E. No growth was observed using CO_2 / H_2. The temperature optimum is 65°C. The synthesis of very long bifunctional fatty acid species, making up about 40% of fatty acyl components of the membrane, is part of the microorganism's adaptation for optimum growth at extremely high temperatures (Jung et al. 1994). The generation time at 65°C is 75 min.

Fermentation products (μmol): glucose $\rightarrow$ 549 ethanol + 31 acetic acid + 50 lactic acid + 31 H_2

Growth inhibition

Ethanol tolerance is not temperature dependent in the wild-type strain (39E) (Burdette et al. 2002) but was shown to be temperature dependent in mutants. Glucose is not fermented at an ethanol concentration of 2.0% by wild-type 39E, but mutants have been shown to ferment glucose at 8.0% ethanol at 45°C (3.3% at 68°C) (Lovitt et al. 1984).

Enrichment and habitat

Isolated from the Octopus Spring algal-bacterial mat in Yellowstone National Park, WY, USA with TYEG media, or modified TYEG media that contained 5% xylose instead of glucose, at 65°C.

Industrial applications

The general mechanism for regulation of glucoamylase and pullulanase synthesis has been studied in strain 39E (Hyun and Zeikus 1985a, 1985b), as well as some studies on the active site, and characterization, of the amylopullulanase (Mathupala et al. 1993, 1994; Mathupala and Zeikus 1993; Lin and Leu 2002). Podkovyrov et al. (1993) provided analysis of the catalytic center of cyclomaltodextrinase. Xylose metabolism has also been further elucidated by the cloning and characterization of genes encoding: a xylose isomerase, a xylulose kinase (Erbeznik et al. 1998a, 1998b), a xylose ABC (ATP-binding cassette) transport operon (Erbeznik et al. 2004) and xylose binding protein, XylF (Erbeznik et al. 1998c).

The histidine biosynthesis pathway was identified in this bacterium making it the first thermophilic, anaerobic bacterium with identified *his* anabolic genes (Erbeznik et al. 2000). Other studied and characterized enzymes include: a lactate dehydrogenase (LDH), studied because of a link between an increase in the initial concentration of glucose and an increase in the activity of the LDH (Germain et al. 1986); a secondary-alcohol dehydrogenase; a primary-alcohol dehydrogenase and an acetaldehyde dehydrogenase (Pham et al. 1989; Pham and Phillips 1990; Phillips 1992, 2002a; Zheng and Phillips 1992; Zheng et al. 1992., 1994; Burdette and Zeikus 1994a, 1994b; Burdette et al. 1995, 1997, 2000, 2002; Arni et al. 1996; Secundo and

Phillips 1996; Tripp et al. 1998; Heiss and Phillips 2000; Heiss et al. 2001a, 2001b). The secondary-alcohol dehyrogenase (Burdette et al. 1996) and a thermostable beta-galactosidase (Fokina and Velikodvorskaia 1997) have been cloned and expressed in *E. coli*.

G+C content: mol%: not determined

Available strains: 39E = DSM 2355 = ATCC 33223 (Zeikus et al. 1980)

Gene Bank accession number (16S rRNA): L09164

***Thermoanaerobacter ethanolicus*[T]** (Wiegel and Ljungdahl 1981) Wiegel and Ljungdahl 1982, 384[VP]

e.tha.no.li.cus. IVS. n. ethanol. corresponding alcohol of ethane (ethane+ol) M. L. masc. adj. *ethanolicus*, indicating the production of ethanol. Type species of *Thermoanaerobacter*.

This description is based on that of Wiegel and Ljungdahl (1981), and on study of the type strain JW 200.

Cells in early logarithmic growth are 0.3-0.8 by 4 -8 µm (rods) often with pointed ends. In late logarithmic growth, cells are long filamentous cells (up to 100 µm). Cells have 1-12 peritrichously situated flagella (up to 50µm in length) and 4-8 pili. Cell division may lead to long chains of bacteria. Coccoid cells (0.8-1.5 µm) are frequently observed, but spores have never been observed. The Gram-stain is variable; however, the cell wall is Gram-type positive (*meso*-diaminopimelic acid type), as observed by electron microscopy. Peteranderl et al. (1993) demonstrated that strain JW200 is able to regenerate to a walled form after autolysis was induced to form stable, cell wall-free cells. Deep agar (2%) colonies at 60°C are 0.5-1.0 mm in diameter,

lenticular and white. After 40 h at 60°C, colonies are 2-4 mm in diameter, white, smooth, round to irregular and flat. In old cultures, the colonies turn brown, but no pigment has ever been identified.

Growth characteristics

Cells are obligate anaerobes that grow from pH 4.4-9.9 (optimum pH 5.8-8.5). The growth temperature is from 37-78°C with an optimum temperature at 69°C. Yeast extract and a fermentable carbohydrate are required for growth. Lacis and Lawford (1985) have studied the growth efficiencies of thermophilic and mesophilic anaerobes with an emphasis on strain JW 200. Glucose, fructose, galactose, mannose, ribose, xylose, cellobiose, lactose, maltose, sucrose, starch and pyruvate are fermented but not cellulose, raffinose, rhamnose, fucose, m-erythritol, m-inositol, xylitol, glycerol, mannitol, sorbitol, trehalose, melezitose, melibiose, niacinamide or amygdalin. The major end products of glucose fermentation are: ethanol (1.8 mol/mol of glucose) and CO_2, with minor amounts of acetic acid, lactic acid, and H_2 also produced. Variations in end-products are observed. For instance, a decrease in the yeast extract to fermented carbohydrate ratio leads to a decrease in ethanol production and an increase in acetic acid and lactic acid concentrations (Hild et al. 2003). Lacis and Lawford (1988a, 1988b, 1989, 1991, 1992) have reported on ethanol yields from fermentations involving both glucose and xylose. Wiegel et al. (1983) has described the production of ethanol from bio-polymers.

Esculin and gelatin are hydrolyzed, and cells are negative for: catalase, indole production and lipase (or cellulase activity).

Fermentation products (mol): 1.0 glucose + 0.1 H_2O → 1.8 ethanol + 0.1 acetic acid + 0.1 lactic acid + 1.9 CO_2 + 0.2 H_2

Growth inhibition

Cells from late logarithmic growth lyse on exposure to 100 µg/ml of polymyxin B for 30 min at 37°C or 50°C (pH 7.2). (This was not true of early logarithmic growth cells.) Cells are inhibited by chloramphenicol but are resistant to erythromycin, tetracycline and penicillin-G. H_2 is inhibitory at 10% (growth does not occur at 75% or higher concentrations). Lactic acid, acetic acid and ethanol also become inhibitory above 100 mM, 200 mM and 500 mM (65°C), respectively. Type strain can be adapted to growth on 8% ethanol.

Enrichment and habitat

Type strain isolated from water and mud samples (pH ~8.8, temp. 45-50°C) that were collected from an alkaline hot spring in White Creek (opposite the Great Fountain Geyser located at Fire Hole Lake Drive) in Yellowstone National Park, WY, USA.

Industrial applications

T. ethanolicus strain JW 200 produces an extensive number of enzymes that have been studied and well-characterized: the beta-xylosidase (Shao and Wiegel 1992) and alcohol dehydrogenases (Bryant and Ljungdahl 1981; Bryant et al. 1988, 1992; Holt et al. 2000) are examples. Also, the

gene for the bifunctional xylosidase-arabinosidase (xarB) from *T. ethanolicus* has been cloned, sequenced, and expressed in *Escherichia. coli* (Mai et al. 2000).

G+C content: 32 mol%

Available strains: JW 200, JW 201 (Wiegel and Ljungdahl 1981)

Type strain: JW 200 = ATCC 31550 = DSM 2246

Cell wall type: m-DAP

Gene Bank accession number (16S rRNA): L09162

Thermoanaerobacter italicus (Kozianowski et al. 1997) Kozianowski, Canganella, Rainey, Hippe and Antranikian 1998, 1084[VP]

i.ta.li.cus. L. n. *italia* Italy; M. L. masc. adj. *italicus* pertaining to Italy, where the organism was isolated.

This description is based on that of Kozianowski et al. (1997), and on study of the type strain Ab9.

Cells are rod-shaped (0.4-0.75 by 2 -6 µm) and chains up to 50 µm when grown on glucose. These cells are non-motile, Gram-stain negative and Gram-type positive (meso-diaminopimelic acid type in cell wall). Spherical, terminal spores are produced with xylose as substrate. Colonies on a glucose-containing agar are 2-3 mm in diameter, round with entire margins, greyish-white and opaque with a glassy surface.

Growth characteristics

Bacterium is thermophilic (optimum growth temperature around 70°C with growth occurring from 45 to 78°C), anaerobic, and a chemoorganotroph. The pH optimum is around 7.0. Doubling time at 70°C with pectin is 3 h (compared to 2.1 h with glucose). Fermentable carbohydrates include: amygdalin, arabinose, cellobiose, esculin, fructose, glucose, galactose, lactose, maltose, mannose, melezitose, melibiose, mannitol, raffinose, sucrose, trehalose, starch, xylan, glycogen, D-glucosamine, saccharose, inulin, pectin and xylose but not cellulose. End products of glucose fermentation (0.5%) are: ethanol (26 mM), lactic acid (12.4 mM), acetic acid (2.2 mM), succinate (0.3 mM), CO_2 and H_2 (end products of pectin or pectate fermentation are ethanol, lactic acid, acetic acid, CO_2 and H_2). Thiosulfate is reduced to both elemental sulfur and sulfide. Accumulation of sulfur in the media and in the cells can be observed.

Growth inhibition

1% NaCl did not inhibit growth; however, 10 μg/ml of cephalosporin, erythromycin, kanamycin or rifampicin totally inhibits growth.

Enrichment and habitat

Isolated from water and mud samples (40-70°C) in thermal spas collected from the north of Italy (Abano, Terme, Calzignano Terme, Montegrotto Terme, Battaglia Terme, Sirmione and Agano Terme) with the use of pectin and pectate (polygalacturonic acid) as substrates. The isolation of type strain occurred from medicinal mud (fango) of Abano Terme, Italy.

Industrial applications

Thermoactive xylanolytic (temperature optimum 70-75°C) enzymes and amylolytic and pullulolytic (temperature optimum 80-85°C) enzymes are produced. Two thermoactive pectate lyases were isolated after growth on pectin (pectate was the preferred substrate but higher enzyme yields were obtained with pectin).

G+C content: 34.4 mol%

Type strain: Ab9 = DSM 9252 (Kozianowski et al. 1997)

 Cell wall type: m-DAP

Gene Bank accession number (16S rRNA): no sequence

Thermoanaerobacter '***keratinophilus***' Riessen and Antranikian 2001

ke.ra.ti.no'phil.us. Gr. n. *keras* keratin; Gr. adj. *philos* loving; M. L. adj. *keratinophilus* keratin-loving.

This description is based on that of Riessen and Antranikian (2001), and on study of the type strain 2KXI.

The cells are rod-shaped, 0.2-0.3 by 1-3.0 µm in the exponential growth phase and occur singly, in pairs or in short chains. Cells are Gram-stain negative regardless of growth phase but have a Gram-type positive cell wall. Stationary growth phase cells are pleomorphic, including a

filamentous form up to 10 or 15 μm in length. Spores were never observed. The rods are slightly curved and commonly appear as coccoid-shaped.

Growth characteristics

The temperature optimum is 70°C, with growth occurring from 50°C to just below 80°C. The pH optimum for growth is 7.0 with a pH range of 5.0-9.0. The optimal NaCl concentration is 5-10 g/l with a range of 0-30 g/l. Growth is fermentative and strictly anaerobic.

Utilized substrates include: casein, bactopeptone, yeast extract, tryptone, collagen, gelatin, starch, pectin, glucose, fructose, galactose, mannose, pyruvate, maltose and cellobiose but not xylan, cellulose, pullulan, xylose, arabinose, lactose or olive oil. Yeast extract and tryptone (0.05% (wt/vol) of both) are required for growth on saccharolytic substances. The generation time of the type strain when grown with 5 g/l yeast extract and 5 g/l tryptone was 67 min. Growth can be achieved with merino wool and chicken feathers solely. Thiosulfate and sulfate can both serve as electron acceptors. Thiosulfate stimulates growth to approximately threefold that of sulfate but is not required for growth. Ampicillin, kanamycin and streptomycin inhibit growth at 10 μg/ml.

Enrichment and habitat

Isolation of the type strain was from hydrothermal vents, 74°C and pH 6.0, in the area of Furnas on the Azorean island São Miguel using merino wool and chicken feathers as sustrates at an incubation temperature of 70°C.

Industrial applications

Strain 2KXI possesses an intracellular protease and an extracellular keratinolytic enzyme with different properties. The ability to degrade keratin is a unique characteristic among the known Bacteria and Archaea (Friedrich and Antranikian 1996).

G+C content: 37.6 mol%

Type strain: 2KXI = DSM 14007 (Riessen and Antranikian 2001)

Gene Bank accession number (16S rRNA): AY278483

Thermoanaerobacter kivui (Basonym: *Acetogenium kivui*, Leigh and Wolfe 1983, 886[AL]) Collins, Lawson, Willems, Cordoba, Fernandez-Garayzabal, Garcia, Cai, Hippe and Farrow 1994, 824[VP]

ki'vui. M. L. adj. *kivuus* pertaining to Kivu, named for its source, Lake Kivu.

This description is based on that of Leigh et al. (1981), and on study of the type strain LKT-1.

Cells grown on H_2 and CO_2 are rod-shaped (0.7 by 2 -3.5 µm) but are longer, 0.7-0.8 by 5.5 -7.5 µm, when grown on glucose. Cells occur in pairs or chains and are non-flagellated. The Gram-stain is negative, but Gram-type positive cell wall is present. Cell wall is covered by a hexagonal S-layer that is composed of a single 80 kDa subunit (Lupas et al. 1994). Endospores are not observed. Colonies on agar plates are: convex, circular, entire, translucent, tan in color, and have a smooth, shiny surface (2 mm diameter after 1 week). Cells are catalase negative.

Growth characteristics

The temperature optimum is 66°C, with growth occurring from 50 to 72°C. Physiology is obligate anaerobic growth not occurring without a reducing agent such as cysteine-sulfide. However, small amounts of O_2 in semisolid and liquid media caused a lag phase but did not alter the ability of the bacterium to synthesize acetate via the acetyl coenzyme A pathway (Karnholz et al. 2002). The pH optimum is 6.4 with a pH range of 5.3-7.3. Fermentable carbohydrates include: glucose, mannose, fructose, and pyruvate, which yield acetic acid. However, galactose, maltose, raffinose, ribose, sucrose, lactose, trehalose, cellobiose, cellulose, pectin, starch, mannitol and inositol cannot serve as substrates. The bacterium oxidizes H_2 and reduces CO_2 to produce acetic acid as the sole product of metabolism (Ryabokon et al. 1995). Acetic acid formation has been studied by Kevbrina and Pusheva (1996) and Kevbrina et al. (1996). The doubling time on H_2 and CO_2 is 1.75-2.5 h at 60°C. Poor growth is observed on formate while yeast extract and trypticase increase cell yields.

$2 CO_2 + 4 H_2 \rightarrow$ acetic acid $+ 2 H_2O$
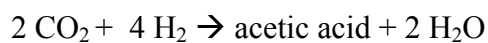
Enrichment and habitat

Isolated, so far, only from sediments of Lake Kivu, Africa, enrichment was done at 60°C with H_2 and CO_2 (67 and 33%, respectively).

G+C content: 38 mol%

Type strain: LKT-1= ATCC 33488 = DSM 2030 (Leigh et al. 1981)

Gene Bank accession number (16S rRNA): L09160

Thermoanaerobacter mathranii (Larsen et al. 1997) Larsen, Nielsen and Ahring 1998, 327[VP]

ma.thra.ni.i. M. L. gen. n., of Mathrani, in honor of the late Indra M. Mathrani, who contributed greatly to our understanding of thermophilic anaerobes from hot springs during his short career.

This description is based on that of Larsen et al. (1997) and Sonne-Hansen et al. (1993), and on study of the type strain A3.

Cells are Gram-stain variable (Gram-type positive), straight and rod-shaped occurring singly and when under under suboptimal conditions in long chains. The vegetative cells in exponential growth phase are 0.7 by 1.8 -3.9 µm, motile and spore-forming (terminal, spherical spores that swell cells). Cells are typically longer when either sporulated (6.4-8.2 µm) or grown under suboptimal conditions (e.g. a temp. of 75°C). Colonies are white and irregular with a 1 mm diameter after growth on beechwood xylan agar for 7 days. The surface of the colonies is granulated with top formation.

Growth characteristics

The optimum growth temperature of *T. mathranii* is between 70-75°C (no growth at 47 or 78°C). The pH optimum is 6.8-7.8 (4.7 and 8.8 are the extreme pH values for growth). Cells are catalase negative. The doubling time on xylose was 74 min. at 69°C (pH 7.0). Sulfide is produced from casein-peptone, sulfate or thiosulfate. Yeast extract is required for growth along with a fermentable carbohydrate but does not serve as a sole carbon/energy source. Carbohydrates used for growth include: amygdalin, L-arabinose, cellobiose, D-fructose, D-glucose, glycogen, lactose, maltose, D-mannitol, mannose, melezitose, melibiose, raffinose, D-ribose, sucrose, trehalose, xylan and D- xylose but not avicel, casein-peptone, cellulose, D-galactose, glycerol, inulin, pectin, L-rhamnose, salicin, sorbitol or yeast extract. The end-products of D-xylose fermentation are: ethanol (Ahring et al. 1999), lactic acid, acetic acid, CO_2 and H_2.

Fermentation products: xylose + H_2O → 1.1 ethanol + 0.4 acetic acid + 0.06 lactic acid + 1.81 CO_2 + 0.9 H_2

Growth inhibition

Growth is inhibited by 100 mg/l of tetracycline, chlorampenicol, penicillin G, neomycin or vancomycin. Growth is still seen in the presence of 10mg/l chlorampenicol and neomycin. *T. mathranii* is also insensitive to: 50.66 kPa overpressure of H_2, 2% NaCl and 5% ethanol. When *T. mathranii* was grown with a wet-oxidized wheat straw hydrolysate, inhibitory effects were also seen due to aromatic monomers (e.g. phenol aldehydes and to a lesser extent by phenol ketones) (Klinke et al. 2001).

Enrichment and habitat

Isolated from a biomat and sediment from a slightly alkaline (pH 8.5) hot spring (70°C) in Hverðagerdi-Hengil, Iceland (Sonne-Hansen et al. 1993). Beechwood xylan at 68°C (pH 8.4) was the substrate used for isolation and the subsequent enrichment of the bacterium.

Industrial applications

An industrial application was shown with production of ethanol in pretreated hemicellulosic hydrolysates from wheat straw (Ahring et al. 1996). Also the high tolerance to ethanol (up to 5%) makes this bacterium useful. One of the most efficient enzymes for converting D-galactose into D-tagatose was found in *T. mathranii* and subsequently produced heterologously in *E. coli* and characterized (Jorgensen et al. 2004). *T. mathranii* has also been used in the implementation of an Upflow Anaerobic Sludge Blanket (UASB) purification reactor step for the detoxification process of water derived from bioethanol production (Torry-Smith et al. 2003).

G+C content: 37 mol%

Type strain: A3 = DSM 11426 (Sonne-Hansen et al. 1993; Larsen et al. 1997)

Gene Bank accession number (16S rRNA): Y11279

Thermoanaerobacter siderophilus Slobodkin, Tourova, Kuznetsov, Kostrikina, Chernyh and Bonch-Osmolovskaya 1999, 1477[VP]

si.de.ro'phil.us. Gr. n. *sideros* iron; Gr. adj. *philos* loving; M. L. adj. *siderophilus* iron-loving.

This description is based on that of Slobodkin et al. (1999a), and on study of the type strain SR4.

Cells are straight to curved rods (0.4-0.6 by 3.5-9.0 l µm), Gram-type positive (Gram-stain positive) and occur singly or in short chains. Round, refractile, heat-resistant spores are formed in terminally swollen sporangia. Maximum sporulation was observed when growth was with 9,10-anthraquione 2,6-disulfonic acid (AQDS). Colonies in agar-shake cultures were: uniformly round, 0.5-1.0 mm in diameter and white. Cells exhibit slight tumbling motility due to peritrichous flagellation.

Growth characteristics

Bacterium is an anaerobe with a thermophilic growth optimum (69-71°C) and temperature growth range from 39-78°C. The pH range for growth is from 4.8 to 8.2 (with an optimum at 6.3-6.5). Substrates utilized in the presence, as well in the absence, of Fe(III) as an electron acceptor include: peptone, yeast extract, beef extract, casein, starch, glycerol, pyruvate, glucose, sucrose, fructose, maltose, xylose, cellobiose and sorbitol but not formate, acetate, lactate, methanol, ethanol, propanol, isopropanol, butanol, propionate, *n*-butyrate, succinate, malate, maleate, glycine, alanine, arginine, L-arabinose, olive oil, xylan or cellulose. Molecular hydrogen and CO_2 can be utilized in the presence of Fe(III) for growth. Fermentation products from glucose are: ethanol, lactate, H_2 and CO_2. Electron acceptors reduced include: amorphous iron(III) oxide, AQDS, sulfite, thiosulfate, elemental sulfur and MnO_2. The products of

amorphous iron(III) oxide reduction are magnetite and siderite. Sulfite, thiosulfate and elemental sulfur are reduced to hydrogen sulfide. Nitrate, sulfate and O_2 cannot be used for growth.

Growth inhibition

Growth is inhibited by chloramphenicol, neomycin, polymyxinB and kanamycin (100 µg/ml) but not by penicillin, ampicillin, streptomycin or novobiocin (100 µg/ml). Growth occurs with NaCl concentrations from 0-3.5%. It was established that Fe(III) reduction in *T. siderophilus* is carried out to relieve the inhibitory effect of hydrogen (Gavrilov et al. 2003).

Enrichment and habitat

Isolated from hydrothermal (70-94°C) vents in the area of Karymsky volcano on the Kamchatka peninsula, Russia using amorphous iron(III) oxide and peptone in an anaerobic media at pH 6.8-6.9 (70°C).

G+C content: 32 mol%

Type strain: SR4 = DSM 12299 (Slobodkin et al. 1999)

Gene Bank accession number (16S rRNA): AF120479

Thermoanaerobacter* '*sulfurigignens'** (Dashti, M., S.Y. Liu, F. Rainey, F. Mayer, Y.J. Lee and J. Wiegel, thesis, The University of Georgia)

sul.fu.ri.gig'nens. L. n. *sulfur*, sulfur, brimstone; L. v. *gignere*, to produce, *sulfurigignens* relating to the formation of sulfur droplets from thiosulfate.

This description is based on that of Dashti, thesis, The University of Georgia, and on study of the type strain JW/SL- NZ826.

Cells are motile by peritrichous flagella, rods (0.3 to 0.8 by 1.2 to 4.0 μm during exponential growth), non-pigmented and form spores (round, terminal spores 0.46 to 0.83 µm in diameter usually seen during the late exponential or early stationary growth phase). Cells tend to be longer during the stationary phase with lengths up to 35 μm observed. Elemental sulfur is deposited on the cell surface and in the medium during growth on thiosulfate. The Gram-stain reaction is negative but the cell wall is Gram-type positive. Colonies are creamy white, circular (1 to 2 mm in diameter).

Growth characteristics

The temperature range for the growth at pH 6.5 is 34-74°C (with an optimum from 63-65°C). The pH range for growth at 60°C is from 4.0 to 8.0 (with an optimum of 4.8-6.5). Xylose, glucose, starch, lactose, galactose, maltose, fructose, sucrose, mannose, cellibiose, raffinose, pyruvate, methanol and mannitol are fermented in the presence of 0.3% yeast extract. Ribose, arabinose, dextran, xylan, cellulose, glycerol, xyliotol, formate and gluconate are not used as

substrates. The addition of 0.5% peptone, tryptone, casein hydrolysate or casamino acids in the presence of 0.3% yeast extract does not increase cell growth. Weak growth is observed with fumarate and succinate. Sulfate and sulfite are not reduced. Thiosulfate is reduced to elemental sulfur inside the cells. The sulfur inclusion droplets are released into the media due to lysis of the cells. '*T. sulfurigignens*' can tolerate up to 800mM thiosulfate. Cells are catalase and indole negative.

Fermentation products (in the presence of 0.3% yeast extract): glucose (or xylose) $\rightarrow$ 0.7 mM ethanol + 0.4 mM acetic acid + 0.9 mM lactic acid + 1.1 mM CO_2 + 0.8 mM H_2

Growth inhibition

Growth is inhibited by neomycin and chloramphenical at concentrations of 100 µg/ml and gramicidin at 10µg/ml. Cells are resistant to vancomycin, bacitracin, tetracycline and ampicillin at 10µg/ml and kanamycin, streptomycin, cycloheximide and cycloserine at 100 µg/ml.

Enrichment and habitat

Isolated and enriched from an acidic volcanic stream outlet on White Island, New Zealand at 60°C (pH 5.0) using anaerobic media containing: 20 mM thiosulfate, 0.5% yeast extract and 0.5% xylose.

G+C content: 34.5 mol%

Available strains: JW/SL- NZ824 and JW/SL- NZ826

Type strain: JW/SL- NZ826 = DSM 13515 = ATCC 700320

Gene Bank accession number (16S rRNA): AF234164

Thermoanaerobacter sulfurophilus (Bonch-Osmolovskaya et al. 1997) Bonch-Osmolovskaya, E. A., M. L. Miroshnichenko, N. A. Chernykh, N. A. Kostrikina, E. V. Pikuta, and F. A. Rainey 1998, 631[VP]

sul.fu.ro.phi'lus. L. masc. adj., *sulfurophilus*, of L. n. *sulfur*- sulfur and GK. masc. adj. *philos*-liking: *sulfurophilus*-liking elemental sulfur.

This description is based on that of Bonch-Osmolovskaya et al. (1997), and on study of the type strain L-64.

Cells are curved, Gram-type positive rods (0.5 by 3-7 μm) with mini-cells common. Cells occur in long wound chains in older, or nutrient deficient, cultures. Spores have never been observed. Motility is through the use of peritrichous flagella.

Growth characteristics

The temperature range for the growth is 44-75°C with an optimum from 55-60°C. The pH range for growth is from 4.5 to 8.0 with an optimum at 6.8-7.2. Bacterium has a obligately anaerobic metabolism. Fermentable carbohydrates include: glucose, fructose, lactose, rhamnose, arabinose, xylose, sorbitol, inositol, mannitol, sucrose, cellobiose, maltose, starch, pectin, pyruvate and lactate, but no growth is observed on cellulose, succinate, citrate, formate, acetate, propionate, butyrate, methanol, ethanol or H_2 (in the presence or absence of elemental sulfur as the electron acceptor). Growth with glucose yields: H_2, CO_2, and acetate, minor amounts of lactate and ethanol (production of H_2S, no lactate and higher amounts of ethanol when elemental sulfur was also present). Elemental sulfur and thiosulfate are reduced to sulfide (addition of elemental sulfur also stimulates growth). Nitrate, sulfate and sulfite are not reduced.

Fermentation products: lactate → (μmol/mL of product) 3.0 H_2S + 0.14 acetic acid + 0.20 H_2

Enrichment and habitat

 Isolated and enriched from a sulfur-containing cyanobacterial mat occurring along the rim of a hot (53-58°C) pond from the Uzon caldera, Kamchatka by the use of a peptone, glucose, lactate medium (incubation at 55°C with a media pH of 7.0) (Bonch-Osmolovskaya et al. 1997).

G+C content: 30.3 mol%

Available strains: P-82, G-1, and L-64 (Bonch-Osmolovskaya et al. 1997)

Type strain: L-64 = DSM 11584

Gene Bank accession number (16S rRNA): Y16940

Thermoanaerobacter thermocopriae (Basonym: *Clostridium thermocopriae,* Jin, Yamasato, and Toda 1988, 280[AL]) Collins, Lawson, Willems, Cordoba, Fernandez-Garayzabal, Garcia, Cai, Hippe and Farrow 1994, 824[VP]

ther.mo.co'pri.ae. Gr. n. *thermos*, heat; Gr. n. *kopria*, compost; M. L. gen. n. *thermocopriae* of heat compost.

This description is based on that of Jin et al. (1988), and on study of the type strain JT3-3.

Cells are straight rods (0.5-0.7 by 2.2-6.0 μm) that produce terminal, spherical spores with a diameter of 1.2-1.6 μm which swell the cell. The Gram-type positive cells are motile and stain Gram negative.

Growth characteristics

The temperature range for growth of *T. thermocopriae* is 47-74°C with an optimum at 60°C. The pH range for growth is from 6.0 to 8.0 with an optimum at 6.5-7.3. The bacterium is an obligate anaerobe fermenting numerous carbohydrates: cellulose, hemicellulose, cellobiose, glucose, fructose, maltose, arabinose, lactose, trehalose, glycogen, starch, amygaldin, xylan and mannose but not melibiose, pectin, inulin or mannitol. The end products of carbohydrate fermentation are:

acetic acid, butyric acid, lactic acid, ethanol, H_2 and CO_2 but not propionic or valeric acid. Indole is not produced, esculin is hydrolyzed and gelatin is not digested. No nitrate reduction occurs.

Fermentation products: 1% cellibiose (100 mL culture) → (meq) 0.8 to 4.1 butyric acid + 0.3 to 1.7 acetatic acid + less than 0.8 lactic acid + 2 to 5 ethanol

Enrichment and habitat

Isolated from compost of camel feces (type strain), soil and a hot spring in Japan (The University of Tokyo and Kinugawa) using cellobiose or cellulose in anaerobic culture at 60°C (pH 7.1) (Jin and Toda, Abstr. Annu. Meet. Soc. Ferment. Technol. Jpn. 1987, p.134; Jin et al. 1988).

Industrial applications

An extracellular cellulose is produced by this bacterium.

G+C content: 37.2 mol%

Available strains: JT1, JT2, JT3-1, JT3-2, JT3-3, JT5 (Jin and Toda, Abstr. Annu. Meet. Soc. Ferment. Technol. Jpn. 1987, p.134; Jin et al. 1988)

Type strain: JT3-3 = ATCC 51646 = IAM 13577 = JCM 7501

Gene Bank accession number (16S rRNA): L09167

Thermoanaerobacter thermohydrosulfuricus (Basonym: *Clostridium thermohydrosulfuricum*, Klaushofer and Parkkinen 1965, 448[AL] (Skerman et al. 1980)) Lee, Jain, Lee, Lowe and Zeikus 1993, 49[VP]

ther.mo.hy.dro.sul.fur'i.cus. M. L. masc. n. *thermos*, hot; M. L. masc. adj. *hydrosulfuricum*, pertaining to hydrogen sulfide; M. L. masc. adj. *thermohydrosulfuricum*, indicating that the organism grows at high temperatures and reduces sulfite to H_2S.

This description is based on that of Hollaus and Sleytr (1972) and Wiegel et al. (1979), and on study of the type strain E100-69.

Cells occur singly, in short chains or (in some strains) in long filamentous groups. Cells are 0.3 to 0.6 by 2.0 to 13.0 μm and motile by peritrichous flagella. As shown by fracture planes, flagella appear to have empty cores that could possibly have a use for transport of flagellin molecules during flagellar assembly (Sleytr and Glauert 1973). Spores are spherical and terminal, and the sporangia swell the cells. Sporulating cultures tend to contain thinner, more elongated cells. The Gram-stain is variable but a Gram-type positive cell wall composed of two layers is present. The cell wall contains meso-diaminopimelic acid and is covered by an S-layer (Sára and Sleytr 1996a, 1996b). The S-layer shows hexagonal symmetry, a center-to-center spacing of the morphological units of 14.2 nm (Crowther and Sleytr 1977; Bock et al. 1994; Messner et al. 1995) and is composed of glycoprotein subunits with a molecular weight of ~20,000 (Christian et al. 1988; Sára et al. 1989). Peteranderl et al. (1993) demonstrated that strain JW102 is able to regenerate to a walled form after autolysis was induced to form stable, cell wall-free cells. The cells are catalase negative.

Growth characteristics

Anaerobic conditions are required for cell growth. Growth occurs at pH 5.5 to 9.2, and the optimum pH for growth is 6.9 to 7.5. The optimum growth temperature is between 67 and 69°C with no growth occurring at 76 to 78°C. Growth at 37°C is poor and no growth is observed at 25°C. H_2 in the gas phase inhibits growth as does lactate.

Fructose, galactose, glucose, mannose, xylose, cellobiose, maltose, sucrose, trehalose, pectin, esculin and salicin are fermented. Cook et al. has studied the phenomena of glucose (1993) and xylose (1994) uptake by this bacterium in detail. Fermentation of dextrin, potato starch, mannitol, dulcitol and sorbitol and coagulation of litmus milk are variable. Inositol, erythritol, glycerol, lactate, tartrate and cellulose are not fermented. When both glucose and xylose (Patel et al. 1988) or starch and glucose (Parkkinen 1986) are present in media, they are used simultaneously. Extracellular enzymatic starch degradation in relation to the bacterium's constant specific growth rate has been further investigated by Heitmann et al. (1996). Mori (1995) showed that strain YM3 required yeast extract to grow unless it was grown in co-culture with *Clostridium thermocellum* strain YM4, or the cell free broth of YM4. Growth with PYG media yields: H_2, CO_2, acetic acid, lactic acid and ethanol (formic, butyric, isovaleric and isocaproic acids, propanol and isopropanol may be detected). Cook and Morgan (1994) have studied ethanol production under hyperbolic growth conditios. Mayer et al. (1995) isolated mutants that were defective in acetate kinase and/or phosphotransacetylase in order to block acetate production by cells. (L-lactate was the main fermentation product.) H_2 and CO_2 are produced in media containing liver infusion. Methanol is the major metabolic end-product of pectin fermentation.

glucose + 0.5 H_2O → ethanol + 0.5 acetic acid + 0.5 lactic acid + 1.5 CO_2 + H_2
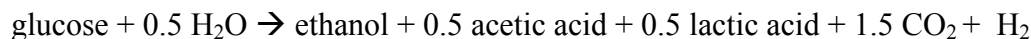
Sulfite and thiosulfate are reduced to hydrogen sulfide, but sulfate is not reduced. H_2S is produced from tryptophan, peptone and yeast extract in the growth media. Nitrate, but not nitrite, is reduced. Acetyl methyl carbinol and indole are not produced.

Enrichment and habitat

Isolated from: extraction juices from beet sugar factories (type strain) (Klaushofer and Parkkinen 1965; Hollaus and Klaushofer 1973), mud and soil (Wiegel et al. 1979; Klingeberg et al. 1990), hot springs in Utah and Wyoming, USA and a sewage plant in Georgia, USA (Wiegel et al. 1979), and a sugar refinery in Germany (Klingeberg et al. 1990).

Industrial applications

The S-layer glycoprotein of *T. thermohydrosulfuricus* strain L111-69 has been investigated for its possible use for carrying cell wall fragments (Sára and Sleytr 1996a, 1996b; Sleytr et al. 1999) (also macromolecules such as ferritin and invertase (Sára and Sletyr 1989)) as 'microparticles' for immunoassays: human IgG immobilization (Kupcu et al. 1995, 1996; Weber et al. 2001) and recombinant major birch pollen allergen Bet v 1 immobilization (Jahn-Schmid et al. 1996). Protein phosphorylation, as a regulatory mechanism, was studied using *T. thermohydrosulfuricus* to extend the limited range of knowledge on this subject to include a thermophile (Londesborough 1986). Sha et al. (1997) purified and characterized a thermostable

DNA polymerase from *T. thermohydrosulfuricus*. Thermostable lactate dehydrogenases (Turunen et al. 1987) and α-amylases and pullulanases (Antranikian et al. 1987; Melasniemi 1987; Melasniemi and Paloheimo 1989) have also been purified to homogeneity and characterized from the bacterium. An alpha-glucosidase exhibiting maltase, glucohydrolase and 'maltodextrinohydrolase' activity was isolated and purified from culture supernatants of *T. thermohydrosulfuricus* (Wimmer et al. 1997).

G+C content: 35-37 mol%

Available strains: E100-69, L 110-69 = DSM 568 (Klaushofer and Parkkinen 1965; Hollaus and Klaushofer 1973), L 77-66 = DSM 569, S 100-69 = DSM 570 (Hollaus and Klaushofer 1973), JW102 = DSM 2247 (Wiegel et al. 1979), DSM 7021, and DSM 7022 (Klingeberg et al. 1990), YM3 (Mori 1990)

Type strain: E100-69 = ATCC 35045 = DSM 567 = LMG 6659 = NCIB (now NCIMB) 10956

Cell wall type: m-DAP

Gene Bank accession number (16S rRNA): L09161

Thermoanaerobacter wiegelii Cook, Rainey, Patel and Morgan 1996, 126[VP]

wie.gel'i.i. M.L. gen. n. *wiegelii*, of Wiegel, in recognition of Juergen Wiegel's contributions to the study of thermophilic anaerobes.

This description is based on that of Cook et al. (1996), and on study of the type strain Rt8.B1.

Cells grown on solid trypticase peptone-yeast extract glucose (TYEG) medium produced nonpigmented colonies (0.5 to 2.0 mm) that were smooth and uniformly round. Cells obtained from isolated colonies were Gram-stain negative rods (Gram-type positive cell wall). Cells occurred singly, in pairs, or (less frequently) in chains and were 0.4 to 0.6 μm wide by 4 to 10 μm long. Electron micrographs of the cell wall revealed a two-layer structure. The inner layer, which was adjacent to the cytoplasmic membrane, stained intensely, whereas the outer layer was less dense. Cells were sluggishly motile by peritrichous flagella. Cells that were grown on TYEG medium did not sporulate. However, when the organisms were grown in a minimal medium at 65°C, the cells were long and filamentous and spores were produced. Spores are round and terminal, distend the cells and are brightly refractile. Spores survived more than 80 min of exposure at 115°C, which confirmed their heat resistance.

Growth characteristics

Anaerobic conditions are required for cell growth. Growth occurs at pH 5.5 to 7.2, but not at pH 5.0 or 7.25, and the optimum pH for growth is 6.8. The temperatures for growth range from 38 to 78°C with an optimum growth temperature between 65 and 68°C; no growth at 34 or 80°C. Yeast extract or trypticase is not required for growth, and both of these growth supplements can be replaced by vitamin-free casamino acids and vitamins. However, no growth occurs in trypticase peptone-yeast extract medium in the absence of a fermentable carbon source.

The utilized carbohydrates include: glucose, xylose, maltose, lactose, cellobiose, raffinose, glucosamine, galactose, fructose, mannose, sucrose, glycerol, soluble starch, pectin and chitin. Sorbitol, mannitol and trehalose are also fermented, but ethanol, DL-lactate, sodium citrate, sodium succinate, transaconitate, malonate, glutamate, glutamine, sodium pyruvate, 2-deoxyglucose, α -methyl-glucoside, L-arabinose, α -L-rhamnose, dulcitol, m-inositol, ribose, α-L-fucose and L-sorbose are not. Growth with glucose yields: H_2, CO_2, acetic acid, lactic acid and ethanol (up to 1.1 mol per mol of sustrate). Propionate is formed during growth on xylose or cellibiose. The doubling time on glucose is 72 min., and growth studies demonstrated that glucose and xylose were used simultaneously when they were supplied together at nonlimiting concentrations (similar to *T. ethanolicus*) (Carreira et al. 1983).

Sulfite and thiosulfate are reduced to hydrogen sulfide. Nitrate, oxygen, sulfate and sulfur are not reduced. Indole is not produced and esculin and gelatin are not hydrolyzed. Cells do not accumulate anthrone-reactive material when they are grown with glucose.

Growth inhibition

Cephalosporin C, erythromycin, bacitracin, tetracycline or polymyxin B completely inhibited growth at 10 mg/ml. Trimethoprim, rifampin, amphotericin B, D-cycloserine, penicillin G, streptomycin sulfate, chloramphenicol and ampicillin did not inhibit growth at concentrations up to 100 mg/mL. The metabolic inhibitors monensin (100 mM), 2,4-dinitrophenol (500 mM), tetrachlorosalicylanilide (10 mM), N,N-dicyclohexylcarboiimide (500 mM) and iodoacetate (500 mM) all inhibited growth when they were added to cultures that were growing exponentially on glucose. Sodium azide, sodium fluoride, potassium cyanide, and sodium arsenate completely

stopped growth at 65°C when they were added to a final concentration of 5 mM. Oxygen completely inhibits growth.

Enrichment and habitat

Isolated from neutral to alkaline freshwater in a geothermally (56 to 69°C) heated water source in Government Gardens, Rotorua, New Zealand. The primary enrichment cultures were prepared by adding 0.2 ml of pool water to 10 ml of prereduced TYEG medium under an N_2 atmosphere (incubation at 70°). Purification involved the use of TYEG agar deeps.

Industrial applications

T. wiegelii was used to study the effect of the external media pH on ATP synthesis rates by monitoring the proton motive force and membrane potential of the bacterium over its pH growth range (Cook 2000).

G+C content: 35.6 mol%

Type strain: Rt8.B1 = DSM 10319

Gene Bank accession number (16S rRNA): X92513

Table 3.1. Comparison of physiological traits of the *Thermoanaerobacter* species.

Characteristics	T. acetoethylicus	T. brockii subsp. brockii	T.brockii subsp. finnii	T. brockii subsp. lactiethylicus	T. brockii subsp. pseudoethanolicus	T. ethanolicus	T. italicus	T. kivui	T. mathranii	T. siderophilus	'T. sulfurigignens'	T. sulfurophilus	T. thermocopriae	T. thermohydrosulfuricus	T. wiegelii
Spores	-	+	+	+	+	-	+	-	+	+	+	-	+	+	+
Motility	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+
Inhibition by H_2	-	ND	ND	ND	ND	-	ND	ND	-	+	ND	ND	ND	+	ND
Inhibition by 2 % NaCl	+	+	ND	-	ND	ND	ND	ND	-	-	ND	ND	ND	ND	ND
Gram stain	neg	pos	var	pos	var	var	neg	neg	var	pos	neg	ND	neg	var	neg
Opt. temp. (°C)	65	65-70	65	55-60	65	69	70	66	70-75	69-71	63-67	55-60	60	67-69	65-68
G+C content (mol%)	31	30-31	32	35	ND	32	34.4	38	37	32	34.5	30.3	37.2	35-37	35.6
Products of fermentation															
Acetic acid	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Butyric acid	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Ethanol	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Isobutyric acid	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactic acid	-	+	+	+	+	+	+	-	+	-	+	+	+	+	+
Succinate	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
H_2	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
CO_2	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Pectin fermented	-	-	ND	ND	ND	+	+	-	-	ND	ND	+	-	+	+
Mannose fermented	+	-	+	+	ND	+	+	+	+	ND	+	ND	+	+	+
Xylose fermented	-	-	+	+	+	+	+	ND	+	+	+	+	ND	+	+
Pentoses fermented	-	ND	+	+	+	+	+	ND	+	+	+	+	ND	+	+
Thiosulfate reduction															
To H_2S	+	+	+	+	ND	+	+	ND	+	+	-	+	+	+	+
To S^0	ND	-	-	-	ND	-	+	ND	-	-	+	-	-	-	-

Figure 3.1. Phylogenetic tree of the *Thermoanaerobacter* species. Fitch tree showing the estimated phylogenetic relationships of *Thermoanaerobacter* species based on 16S rRNA gene sequence data with Jukes-Cantor correction for synonymous changes. The 16S rRNA gene data used represent *Escherichia coli* DSM30083[T] nucleotide positions 107–1450. Numbers at nodes indicate bootstrap support percentages for 100 replicates. Bar, 0.05 nucleotide substitutions per site. GenBank accession numbers are indicated after the strain identifier. The superscript "T" denotes the strain is the type strain for the species.

Phylogenetic tree showing relationships among *Thermoanaerobacter* and related taxa:

- 75 — *Thermoanaerobacter wiegelii* DSM 10319^T (X92513)
- *Thermoanaerobacter sulfurophilus* DSM 11584^T (Y16940)
- *Thermoanaerobacter acetoethylicus* DSM 2359^T (L09163)
- 99, 100 — *Thermoanaerobacter ethanolicus* JW 200^T (L09162)
- *Thermoanaerobacter siderophilus* DSM 12299^T (AF120479)
- 94 — *Thermoanaerobacter thermohydrosulfuricus* DSM 567^T (X92513)
- 93 — *Thermoanaerobacter brockii* subsp. *finnii* DSM 3389^T (L09166)
- 99 — *Thermoanaerobacter brockii* subsp. *brockii* DSM 1457^T (L09165)
- 84 — *Thermoanaerobacter pseudoethanolicus* 39E (L09164)
- 100 — *Thermoanaerobacter brockii* subsp. *lactiethylicus* DSM 9801^T (U14330)
- 93 — *Thermoanaerobacter kivui* DSM 2030^T (L09160)
- 100 — *Thermoanaerobacter mathranii* DSM 11426^T (Y11279)
- *Thermoanaerobacter thermocopriae* IAM 13577^T (L09167)
- 100 — 'Thermoanaerobacter sulfurigignens' (AF234164)
- *Caldanaerobacter subterraneus* subsp. *subterraneus* DSM 13054^T (AF195797)
- 92 — *Ammonifex degensii* DSM 10501^T (U34975)
- 83 — *Coprothermobacter proteolyticus* DSM 5265^T (X69335)
- *Thermoanaerobacterium thermosulfurigenes* DSM 2229^T (L09171)
- *Moorella thermoacetica* LJD (M59121)
- *Thermacetogenium phaeum* DSM 12270^T (AB020336)
- 100 — *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (AF022248)
- *Thermosyntropha lipolytica* JW/VS-265^T (X99980)
- 100 — *Desulfotomaculum thermocisternum* ST90^T (U33455)
- 100 — *Desulfotomaculum australicum* DSM 11792^T (M96665)
- 100 — *Desulfotomaculum kuznetsovii* DSM 6115^T (AJ294427)
- 94 — *Desulfotomaculum thermobenzoicum* subsp. *thermobenzoicum* DSM 6193^T (AJ294429)
- *Sporotomaculum hydroxybenzoicum* DSM 5475^T (Y14845)
- *Bacillus subtilis* subsp. *subtilis* DSM 10^T (AJ276351)

0.05

CHAPTER 4

RECLASSIFICATION OF *THERMOANAEROBIUM ACETIGENUM* AS

CALDICELLULOSIRUPTOR ACETIGENUS COMB. NOV. AND EMENDATION OF THE

GENUS DESCRIPTION[1]

Abstract

Although the type species of the genus *Thermoanaerobium*, *Thermoanaerobium brockii*, was transferred to *Thermoanaerobacter*, *Thermoanaerobium acetigenum* was not transferred. Therefore, *Thermoanaerobium acetigenum* should be reclassified. Based on 16S rRNA gene sequence analysis and re-examination of physiological properties of the type strain, X6B[T] (=DSM 7040[T]=ATCC BAA-1149[T]), we propose that *Thermoanaerobium acetigenum* should be reclassified as *Caldicellulosiruptor acetigenus* comb. nov. Strain X6B[T] contains two separate 16S rRNA genes bracketing another species in the phylogenetic 16S rRNA gene-based tree.

Results and discussion

Thermoanaerobium acetigenum strain X6B[T], an anaerobic, thermophilic bacterium, was isolated by Nielsen et al. (1993) using xylan as the substrate. This bacterium, a Gram-type positive (Wiegel 1981), low-G+C content rod, has many characteristics of a typical member of the *Firmicutes* (Gibbons and Murray 1978). Based on its physiological properties alone, it was placed in the genus *Thermoanaerobium*, the type species of which is *Thermoanaerobium brockii* (Zeikus et al. 1979).

Because the 16S rRNA gene sequence for *Thermoanaerobium acetigenum* X6B[T] had not been determined previously, the classification of *Thermoanaerobium acetigenum* X6B[T] was therefore based only on some physiological similarities. Although the type species of *Thermoanaerobium*, *Thermoanaerobium brockii*, was reclassified as *Thermoanaerobacter brockii* by Lee et al. (1993d) and, subsequently, as *Thermoanaerobacter brockii* subsp. *brockii*

(type strain HTD4^T) by Cayol et al. (1995), *Thermoanaerobium acetigenum* X6B^T was not transferred to the genus *Thermoanaerobacter* (Wiegel and Ljungdahl 1981) because of the lack of 16S rRNA gene sequence analysis. Here we report on the assignment of the type strain of *Thermoanaerobium acetigenum* to the genus *Caldicellulosiruptor* as *Caldicellulosiruptor acetigenus* comb. nov., based on 16S rRNA gene sequence, DNA–DNA hybridization analysis and retesting of its properties. Special attention was given to cellulose degradation, as all other presently known *Caldicellulosiruptor* species are cellulolytic, whereas strain X6B^T has been described as being non-cellulolytic.

Strain X6B^T was obtained as a freeze-dried culture of strain DSM 7040^T from the DSMZ (Braunschweig, Germany). To determine the 16S rRNA gene sequence, *Thermoanaerobium acetigenum* DSM 7040^T was grown under anaerobic conditions (Ljungdahl and Wiegel 1986; Angelidaki et al. 1990). A basal salts medium (final pH 7.3–7.4) was prepared as described by Nielsen et al. (1993). Strain DSM 7040^T was grown in basal salts medium supplemented with yeast extract (0.3%), tryptone (1.0%) and glucose (0.5%), and subjected to two rounds of isolation of single colonies using yeast extract, tryptone, glucose salts medium solidified with 2.2% Gelrite (colonies became visible after incubation at 65°C for 48–72 h). Because the initial 16S rRNA gene sequence analysis yielded two different 16S rRNA species, which bracketed another *Caldicellulosiruptor* species, it became necessary to confirm the purity of the culture further. Therefore, strain DSM 7040^T was grown using three different media (substrate conditions as described below), and each culture was then subjected to three subsequent rounds of single-cell colony isolation. To establish three lines of cultures, strain DSM 7040^T was grown in the above-described basal salts medium, supplemented with yeast extract, tryptone, glucose and brain heart infusion (0.2%) (termed BYTG medium). From this culture, three parallel

cultures were inoculated (0.1% inoculum) using the following media: (i) basal salts plus 0.2% arabinose medium, (ii) basal salts plus 0.2 % raffinose medium and (iii) BYTG medium. Arabinose- and raffinose-supplemented basal salts media were used because the closest *Caldicellulosiruptor* species to strain X6B[T] on the phylogenetic tree (Fig. 4.1.) are unable to use these substrates (Table 4.1.). After checking microscopically that the cultures were suspensions of individual cells and did not contain any clumps or associations of cells, each of the above cultures was used to inoculate dilution series of Gelrite shake-roll tubes (Ljungdahl and Wiegel 1986), with 2.2% (w/v) Gelrite, to obtain single-cell colonies. The Gelrite shake-roll tubes were incubated at 65°C for 48–72 h before colonies became visible. Colonies were picked in an anaerobic chamber (Coy Products) and resuspended in a tube containing 0.3–0.4 ml of the corresponding medium, which was then used to inoculate the next round of Gelrite shake-roll tubes. This process of colony picking was repeated for three rounds of colony isolation with three colonies being picked from each of the arabinose, raffinose and BYTG media after the third and final round. Each of the picked final colonies was reinoculated into a fresh tube of the medium from which it was isolated, resulting in nine cultures: three with the arabinose medium, three with the raffinose medium and three with BYTG medium.

Subsequent extraction of DNA from the nine cultures was performed using a DNeasy Tissue kit (Qiagen). The DNA was then amplified using a bacterial domain-specific primer set for 16S rRNA, 27 forward and 1492 reverse (Lane 1991). PCR was carried out as described previously (Lee et al. 2005). The PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and sequenced by Macrogen (Seoul, Korea). PCR products from the colonies were cloned using a TOPO TA Cloning kit (Invitrogen). Clones were randomly chosen, from which plasmid DNA was extracted by using an Eppendorf FastPlasmid Mini kit (Brinkman). The DNA

was subsequently amplified, purified and sequenced. The sequence similarities were determined using Sequencher v4.1.4 (Gene Codes). Ten clones were sequenced, resulting in two similar sets of sequences (Fig. 4.1.; termed T6 and T4, GenBank accession nos AY772477 and AY772476, respectively). Three clones with the same 16S rRNA gene sequence were never obtained from a single culture, suggesting that an even distribution of clones with the different sequences existed. Analysis of the 16S rRNA gene sequence using nucleotide to nucleotide BLAST (BLASTN) at NCBI (http://www.ncbi.nlm.nih.gov/blast/) to retrieve the most significant homologues of the query 16S rRNA gene sequence revealed that the most significant sequence alignments of *Thermoanaerobium acetigenum* X6B[T] were with *Caldicellulosiruptor* species (Sissons et al. 1987; Rainey et al. 1994), with *Caldicellulosiruptor lactoaceticus* 6A[T] (Mladenovska et al. 1995) and *Caldicellulosiruptor kristjanssonii* I77R1B[T] (Bredholt et al. 1999) as the closest relatives (Fig. 1). Subsequently, 16S rRNA gene sequence-based phylogenetic trees were generated using CLUSTAL_X (Thompson et al. 1997) for sequence alignments, and phylogeny inference package (PHYLIP) software (Felsenstein 1989) and neighbour-joining algorithms (Saitou and Nei 1987) to look at differing tree constructions and to generate distance matrices. TreeExplorer (Kumar et al. 1994), a supplemental program of MEGA, was used to view the tree. The phylogenetic trees generated (the neighbor-joining tree is shown in Fig. 4.1.) showed clearly that *Thermoanaerobium acetigenum* X6B[T] belongs to the clade of *Caldicellulosiruptor* and not to the genus *Thermoanaerobacter* or *Thermoanaerobacterium*. Each of the repurified strains exhibited the two different 16S rRNA sequences. In contrast to other reported cases, e.g., *Clostridium paradoxum* (Rainey et al. 1996), the two sequences were not juxtaposed, but rather separated by a sequence of another species, *Caldicellulosiruptor kristjanssonii*. A comparable situation has been reported by Amann et al. (2000), i.e., single-cell-derived pure cultures contained two

different 16S rRNA genes with about 5% inferred difference in substitutions and bracketing a different species. The relative distance between the two identified *Thermoanaerobium acetigenum* X6B[T] 16S rRNA gene sequences was about 2% and the distance to the closest neighbours *Caldicellulosiruptor lactoaceticus* 6A[T] and *Caldicellulosiruptor kristjanssonii* I77R1B[T] was around 1%. Therefore, DNA–DNA hybridization experiments were performed using the method described by De Ley et al. (1970) and modified by Huß et al. (1983). Chromosomal DNA for DNA–DNA hybridization was extracted and purified using a Maxi Genomic [0]DNA Prep kit (A&A Biotechnology).

DNA–DNA hybridization values between *Thermoanaerobium acetigenum* DSM 7040[T] and *Caldicellulosiruptor owensensis* OL[T], *Caldicellulosiruptor lactoaceticus* 6A[T] and *Caldicellulosiruptor kristjanssonii* I77R1B[T] were 34.3, 50.9 and 53.1%, respectively. These values are all significantly below the 70% relatedness mark that would indicate a relationship at the species level (Wayne et al. 1987) and clearly distinguish *Thermoanaerobium acetigenum* X6B[T] from these three *Caldicellulosiruptor* species that are its closest neighbours in the 16S rRNA gene-based phylogenetic tree (Fig. 4.1.). The repeated isolation of single-cell colonies after growth in different media, the observed homogeneity of the colony and cell morphologies with and among cultures (using microscopy) and the fact that all isolated colonies gave rise to the two different 16S rRNA sequences indicate that it is unlikely that the two sequences are due to a mixed culture having been analysed. These results indicate that *Thermoanaerobium acetigenum* X6B[T] belongs in the genus *Caldicellulosiruptor*, a member of the order *Clostridiales*, and not in the genus *Thermoanaerobacter*, order '*Thermoanaerobacteriales*' (Garrity et al. 2002).

Members of the genus *Caldicellulosiruptor* have the characteristic trait of coupling cellulose degradation to growth (Rainey et al. 1994). However, *Thermoanaerobium acetigenum* X6B[T] was characterized previously (Nielsen et al. 1993) as being incapable of cellulose degradation.

Thermoanaerobium acetigenum DSM 7040[T] was retested for the ability to degrade cellulose using Whatman no. 1 filter paper and carboxymethylcellulose (1.0% w/v, CMC 7LT or 7M; Hercules). In addition, cellulase activity was determined by the use of the reducing sugar assay employing *p*-hydroxybenzoic acid hydrazide and glucose as a standard (Lever 1973). *Thermoanaerobium acetigenum* DSM 7040[T] was incapable of degrading Whatman no. 1 filter cellulose with or without 0.05% (w/v) yeast extract, but utilized CMC, exhibiting moderate growth with 1.0% of the low substitution (substitution level 0.7 of 3) form Hercules 7LT or 7M. Eleven and 10 $\mu mol \cdot ml^{-1}$, respectively, of reduced sugar residues was released from the cultures after 4 days of incubation, with a requirement for yeast extract (0.05%, w/v) for growth. Growth was not observed with only CMC 7LT/7M present. More highly substituted (e.g. 1.2 out of 3) CMCs (Hercules 12M or 12L) did not serve as substrates.

The substrate utilization spectrum of *Thermoanaerobium acetigenum* DSM 7040[T], as performed by Nielsen et al. (1993), was re-examined by adding various carbohydrates (to a final concentration of 2 g $\cdot l^{-1}$) from autoclaved stock solutions (pyruvate was filter-sterilized) to the basal media. Cultures were incubated at 73°C for 48–72 h. Growth of cultures with insoluble substrates was determined by cell counts (Olympus model Vanox microscope with a Petroff-Hausser counting chamber). The results confirmed the previously published data.

The 16S rRNA gene sequence analysis, CMC-cellulase activity and growth observed on low-substituted CMC indicate that *Thermoanaerobium acetigenum* belongs to the genus *Caldicellulosiruptor*, and we propose the name *Caldicellulosiruptor acetigenus* comb. nov.

Emended description of the genus Caldicellulosiruptor Rainey et al. 1995.

The description is the same as that given by Rainey et al. (1994) with the addition that some members do not possess the capacity to degrade crystalline cellulose or filter paper and cannot use cellulose as a carbon and energy source, but can hydrolyse CMC.

Description of Caldicellulosiruptor acetigenus comb. nov.

Caldicellulosiruptor acetigenus (a.ce.ti.ge'nus. L. n. *acetum* vinegar; L. v. *genere*, *gignere* to produce; N.L. masc. adj. *acetigenus* vinegar- or acetic acid-producing).

Basonym: *Thermoanaerobium acetigenum* Nielsen et al. 1994.

The description is based mainly on that given by Nielsen et al. (1993). Cells stain Gram-negative but have a Gram-type positive cell wall structure, occur singly or in pairs, and are about 3.6–5.9 by 0.7–1.0 μm in size. Sometimes occur as chains of up to eight cells. On solidified xylan-containing medium, off-white, milky-coloured colonies are observed. Strictly anaerobic chemoorganoheterotroph. At pH 7.0, growth occurs between 50 and 78°C (optimum 65–68°C). Growth occurs at pH 5.2–8.6 (optimum 7.0). Doubling time under optimal conditions is

approximately 4 h. Arabinose, cellobiose, fructose, D-galactose, D-glucose, lactose, maltose, mannose, raffinose, soluble starch, sucrose, trehalose, D-xylose and xylan support growth. Growth and CMC-cellulase activity is observed when grown on carboxymethylcellulose (Hercules CMC, 7LT or 7M) in the presence of traces of yeast extract, but not with filter paper or crystalline (Avicel) cellulose. Acetate, CO_2, H_2, ethanol and traces of isobutyric acid (but not lactate) are formed during growth with glucose or D-xylose. The DNA G+C content of the type strain is 35.7±0.8 mol% (chromatographic method).

The type strain is X6B[T] (=DSM 7040[T]=ATCC BAA-1149[T]), which was isolated from a combined biomat and sediment sample taken from a slightly alkaline hot spring at Hverðagerdi, Iceland.

Table 4.1. Differential characteristics of *Caldicellulosiruptor acetigenus* X6B[T], *Caldicell-ulosiruptor kristjanssonii* I77R1B[T] and *Caldicellulosiruptor lactoaceticus* 6A[T]. Strains: 1, *Caldicellulosiruptor acetigenus* X6B[T]; 2, *Caldicellulosiruptor kristjanssonii* I77R1B[T]; 3, *Caldicellulosiruptor lactoaceticus* 6A[T]. *Determined at: a, pH 7.0 with xylose as substrate; b, pH 7.0 with cellobiose as substrate. †Determined at: *a*, 68°C with xylose; *b*, 70°C with cellobiose. ‡Substrate concentrations were 2.0 g l^{−1}. §NaCl concentrations tested were 0.2% for strains X6B[T] and I77R1B[T] and 0.5 % for strain 6A[T].

Characteristic	1	2	3
Temperature range for growth (optimum) (°C)*	50–78 (65–68)^a	50–82 (78)^b	50–78 (68)^b
pH range for growth (optimum)†	5.2–8.5 (7.0)^a	5.8–8.0 (7.0)^b	5.8–8.2 (7.0)^b
Substrate utilization‡:			
Arabinose	+	–	–
Fructose	+	+	–
D-Galactose	+	+	–
D-Glucose	+	+	–
Mannose	+	+	–
Raffinose	+	–	–
Sucrose	+	+	–
Trehalose	+	+	–
Avicel	–	+	+
Growth inhibition:			
H$_2$ (0.5 atm)	+	–	–
NaCl§	+	–	–
Lactate as a major fermentation product	–	+	+

Fig. 4.1. Neighbour-joining tree showing the estimated phylogenetic relationships of *Caldicellulosiruptor acetigenus* X6B^T based on 16S rRNA gene sequence data with maximum-likelihood correction for synonymous changes. The 16S rRNA gene data used represent *Escherichia coli* DSM30083^T nucleotide positions 42–1424. Numbers at nodes indicate bootstrap support percentages for 1000 replicates. Bar, 0.02 nucleotide substitutions per site. GenBank accession numbers are given in parentheses.

Caldicellulosiruptor acetigenus **DSM 7040^T T6 (AY772477)** — 96
Caldicellulosiruptor lactoaceticus DSM 9545^T (X82842) — 100
Caldicellulosiruptor acetigenus DSM 7040^T T4 (AY772476) — 97
Caldicellulosiruptor kristjanssonii DSM 12137^T (AJ004811) — 100
Caldicellulosiruptor owensensis DSM 13100^T (U80596) — 74
'*Anaerocellum thermophilum*' Z-1203 (L09180) — 100
Caldicellulosiruptor saccharolyticus DSM 8903^T (L09178)
'*Thermoanaerobacter cellulolyticus*' NA 10B (L09183) — 63
Thermoanaerobacterium thermosulfurigenes DSM 2229^T (L09171)
Thermoanaerobacter ethanolicus JW 200^T (L09162)

0·02

CHAPTER 5

SPORULATION GENES IN MEMBERS OF THE LOW G+C GRAM-TYPE POSITIVE

BRANCH ('*FIRMICUTES*')[1]

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[1]Onyenwoke, R. U., J. A. Brill, K. Farahi, and J. Wiegel. Submitted to the *Archives of Microbiology*.

Abstract

Endospore formation is a specific property found within bacteria belonging to the Gram-type-positive low G+C mol% branch ('*Firmicutes'*) of a phylogenetic tree based on 16S rRNA genes. Within the Gram-type-positive bacteria, endospore-formers and species without observed spore formation are widely intermingled. In the present study, a previously reported experimental method (PCR and Southern hybridization assays) and analysis of genome sequences from 52 bacteria and archaea representing sporulating, non-spore-forming, and asporogenic species were used to distinguish non-spore-forming (void of the majority of sporulation-specific genes) from asporogenic (contain the majority of sporulation-specific genes) bacteria. Several sporulating species lacked sequences similar to those of *Bacillus subtilis* sporulation genes. For some of the genes thought to be sporulation specific, sequences with weak similarity were identified in non-spore-forming bacteria outside of the Gram-type-positive phylogenetic branch and in archaea, rendering these genes unsuitable for the intended classification into sporulating, asporogenic, and non-spore-forming species. The obtained results raise questions regarding the evolution of sporulation among the '*Firmicutes'*.

Introduction

Endosporulation is known to occur only among the bacteria belonging to the phylogenetic branch of Gram-type-positive bacteria (Wiegel 1981; Wiegel and Quandt 1982), also called '*Firmicutes'*, within the prokaryotic domain Bacteria (Gibbons and Murray 1978; Woese 1987). Garrity et al. (2003) narrowed the term '*Firmicutes*' to the status of a phylum, containing only the classes '*Clostridia'*, *Mollicutes*, and '*Bacilli',* whereas other Gram-type-positive bacteria, such as *Corynebacterium*, are in the phylum '*Actinobacteria'*, the second phylum containing

Gram-type-positive bacteria. Being one of the most complex developmental processes in prokaryotes, endosporulation has been shown to require intricate networks of temporal and compartmental regulation in *Bacillus subtilis* and involves more than 150 different gene products, of which about 75 must act sequentially (Errington 1993; Gould 1984; Grossman 1995; Ireton and Grossman 1994; Nicholson et al. 2000; Paidhungat et al. 2001; Setlow 1995, 2001). This complexity makes the process of endosporulation vulnerable to disruptions. For instance, if not all the components that are required in the sequential process function correctly, spore formation will not be observed. Subsequently, an asporogenic phenotype can easily evolve, even though many functional sporulation genes still will be present. Most of the processes of endosporulation investigated to date appear to be highly similar among all endospore-forming species, and thus it is usually assumed that all endospore-forming species most likely arose from the same sporulating ancestor (Errington 1993; Gerhardt and Marquis 1989; Nakamura et al. 1995; Sauer et al. 1994, 1995).

Until recently, the ability to form endospores was used as a mandatory characteristic to include novel isolates into the genera *Bacillus*, *Desulfotomaculum*, and *Clostridium* (Hippe et al. 1992; Slepecky and Hemphill 1992; Sneath 1984). However, according to phylogenetic analyses based on 16S rRNA genes, *Bacillus* and *Clostridium* are not coherent genera and are interspersed with genera partly or exclusively consisting of species for which endospore formation has not been observed (Fig. 5.1.; Ash et al. 1991; Collins et al. 1994). Consequently, the genera *Bacillus* and *Clostridium* have been redefined and subdivided into novel families and genera (P. deVos, Universiteit Gent, in preparation; Garrity and Holt 2001; Wiegel et al. 2004). Several of the newly formed genera contain both endospore-forming species and species for which no endosporulation has been observed. This has led to the speculation that in a large number of

species that do not form endospores, the ability to sporulate has been lost due to an interruption of the sequential sporulation process.

Brill and Wiegel (1997) attempted to develop a fast method to separate novel isolates into asporogenic and non-spore-forming species on the basis of the presence and absence of sporulation genes, respectively. [The term asporogenic has lately been used, including by Brill and Wiegel (1997), in contrast to the use in the older literature and in several recent sporulation-related publications. To avoid further confusion, we have decided to use the term in agreement with the traditional use: "asporogenic" = bacteria with an impaired sporulation process but containing the majority of sporulation genes and "non-spore-forming" = absence of sporulation specific genes such as in Gram-type-negative *Escherichia coli* or *Pseudomonas* spp.] Another term for asporogenic bacteria would be cryptic spore-formers since the restoration of one or two gene products would presumably lead to spore formation. Brill and Wiegel (1997) described a PCR and Southern-hybridization-based assay to distinguish between asporogenic and non-spore-forming by employing probes directed against three representative and at the time assumed specific sporulation genes: *spo0A*, *ssp α/β* -type, and *dpaA/B*. We applied this assay but also extended our study to include analysis of available (up to February 2004) genome sequences. By searching for sequences with similarity to 66 sporulation related genes from the sporulation model microorganism *B. subtilis* (Stragier 2002), we sought to validate our results that some of the asporogenic members of the low G+C group of the '*Firmicutes*' do not contain "sporulation specific" genes. In the present study, we analyzed genes that are similar to *B. subtilis* sporulation-related genes using complete genome sequences from a multitude of prokaryotes.

Materials and methods

Organisms and growth conditions

The tested bacteria and their source are given in Table 5.1. and were cultivated as previously described (Brill and Wiegel 1997).

Genomic DNA isolation, PCR, Southern hybridizations, and sequencing

Genomic DNA isolation, PCR, Southern hybridizations, and Sequencing were carried out as previously described (Brill and Wiegel 1997).

Sequence retrieval and phylogenetic analysis

The following *B. subtilis* sequences served as the query sequences when searching against the databases of complete genome sequences.

− Preseptation: *minC*, *spo0A*, *spo0B*, *spo0H* (sigma factor), *rapA*, *spoVG*, *spoIIAA* (anti-sigma factor antagonist), *spoIIAB* (anti-sigma factor), *spoIIAC* (sigma factor), *spoIIB*, *spoIID*, *spoIIE*, *spoIIGA*, *spoIIGB* (sigma factor precursor), *spoIIM*, *spoIIP*, *spoIIQ*, *spoIIR*;

− Postseptation: *gerM*, *spoIIIA (A, B, C, D, E, F, G, H)*, *spoIVA*, *spoIIID*, *spoVB*, *spoVK*, *cotE*;

− Postengulfment: *spoVID*, *gerPA*, *spoIIIG* (sigma factor), *spoIVB*, *spoIVCB* (sigma factor precursor), *spoVA (A, B, C, D, E, F)*, *spoVM*, *dpaA*, *dpaB*, *hep1*, *sspA*, *sspE*, *gerA (A, B, C)*, *gerD*, *gpr*, *splB*, *sleB*, *cwlD*, *cwlJ*, *cotD*, and *yqfC*, *yqfD*, *yabP*, *yabQ*, *spmA*, *spmB*.

These sequences were obtained by screening of the amino acid sequences of the SwissProt/TrEMBL databases (http://us.expasy.org/sprot/). Non-redundant GenBank

(http://www.ncbi.nlm.nih.gov/, reference sequences are indicated for GenBank), SwissProt, and ERGO (ERGO-derived sequences are indicated by superscript 1) databases were used to obtain the gene assignments for the comparisons of the amino acid sequences. Basic Local Alignment Search Tool (BLAST) [(http://www.ncbi.nlm.nih.gov/BLAST)] searches were done on each open reading frame (ORF) using a basic protein-to-protein blast search of amino acid similarities to sequences in the GenBank, SwissProt, and ERGO non-redundant databases in February 2004. The results of all of these searches were used to provide a putative identication of probable ORF sequences with similarity to the *B. subtilis* query sequences. Probable similarity was based on the combination of identity and probability (*p*) scores, and *e*-values from BLAST analyses. Final assignments of similarity, as illustrated in Tables 2, 3, 4, 5, were based on comparisons of the *e*-value of the query *B. subtilis* sequence to the similar sequence that was delivered by the BLAST search. A (++) designation in the previously mentioned tables indicated a difference of less than 20-fold of the similar sequence *e*-value to the *B. subtilis* sequence. The (+) designation was a difference greater than 20-fold but less than or equal to 40-fold. The (+/–) designation indicated a similar sequence existed but with an *e*-value greater than 40-fold different from that of the *B. subtilis* query. The (–) designation indicated no similar sequence was present or that the similar sequence had an *e*-value greater than or equal to 0.001, which was used as the cut-off value for this work. The genomes of the following bacteria and archaea were used in the comparison, superscript T indicates it is the type strain: *Staphylococcus aureus* MSSA strain 476[1], *Enterococcus faecalis*[1], *Enterococcus faecium* DO(JGI)[1], *Lactococcus lactis* subsp. *lactis*[1] and reference sequence: NC_002662[T], *Listeria innocua*[T], *Listeria monocytogenes* EGD-e[1] and NC_003210, *Streptococcus agalactiae* 2603V/R NC_004116, *Streptococcus equi*[1] and NC_002955, *Streptococcus mutans* UA159[1] and NC_004350 *Streptococcus pneumoniae*

TIGR4[1], *Streptococcus pyogenes* SF 370-M1[1], *Streptococcus salvarius* subsp. *thermophilus*[1], *Mycoplasma pneumoniae* M129[1], *Streptomyces coelicolor* A3(2)[1], *Haemophilus actinomycetemcomitans* NC_002924 and HK 1651[1], *Caulobacter crescentus* CB15, NC_002696, *Escherichia coli* K12, NC_000913 *Escherichia coli* O157:H7, NC_002695 *Helicobacter pylori* J99[1] and NC_000921, *Myxococcus xanthus*[1] and NC_004802, *Salmonella enterica* subsp. *enterica* serovar *typhi NC_003198*, *Salmonella typhimurium* LT2[1] and NC_003197, *Borrelia burgdorferi* B31[1 T] and NC_004971, *Synechococcus* spp. PCC7002[1] and WH8102 NC_005070, *Prochlorococcus marinus* subsp. *pastoris* str. CCMP 1378 NZ_AAAW00000000, *Anabaena* spp. PCC7120[1], *Chloroflexus aurantiacus*[T], *Fusobacterium nucleatum* spp. *nucleatum* ATCC 25586[T], *Deinococcus radiodurans* R1[T], *Cytophaga hutchinsonii* DSM 4304[T], *Archaeoglobus fulgidus* DSM 4304[1 T] and NC_000917, *Halobacterium* spp. NRC-1 NC_002607, *Methanocaldococcus jannaschii* DSM 2661[T], *Pyrococcus furiosus* DSM 3638[T], *Aeropyrum pernix*[1] and K1[T], *Bacillus anthracis* strain Ames NC_003997, *Bacillus cereus* ATCC 14579[T], *Bacillus firmus*[1], *Bacillus halodurans* C-125[1] and NC_002570, *Bacillus licheniformis*[1], *Bacillus megaterium*[1], *Bacillus sphaericus*[1], *Geobacillus stearothermophilus* 10[1] and NC_002926, *Bacillus subtilis* subsp. *subtilis* strain 168[1] and NC_000964, *Bacillus thuringiensis*[1], *Bacillus thuringiensis* subsp. *israelensis* ATCC 35646 IG-59[1], *Clostridium acetobutylicum* ATCC824[T], *Clostridium botulinum* A NC_003223, *Clostridium difficile* 630[1] and NC_002933, *Clostridium perfringens* strain 13124[T], *Desulfitobacterium hafniense* NZ_AAAW00000000.

Tree Building

The Phylogeny Inference Package (PHYLIP) and Molecular Evolutionary Genetics Analysis (MEGA2) software contained all of the programs used for inferring phylogenies. The 16S rRNA

gene trees were generated using different algorithms [Fitch (Fig. 5.1.), neighbor-joining (data not shown), and UPGMA (data not shown)] to examine differing tree constructions as a factor in determining the interrelatedness of the representative microorganisms and to generate the distance matrixes. The ClustalX alignment tool Tree Explorer was used for tree viewing.

Results and discussion

Experimentally analyzed species

Based on the assumption that sporulation evolved only once, and thus all or the majority of sporulation-specific genes exhibit sequence similarity, we applied the method of Brill and Wiegel (1997) to analyze 28 '*Firmicutes'* species from 22 genera and to 16 strains of *Thermobrachium celere* (Engle et al. 1996; Table 5.1.). These asporogenic species included pairs of closely related (98% 16S rRNA sequence similarity) species representing isolates from a great variety of mesobiotic and thermobiotic environments. The selected and representative species covered eight different '*Firmicutes'* clusters as defined by Collins et al. (1994) as well as lactic acid bacteria and genera related to *Bacillus* (Table 5.1.). Many other species or genera from the same or other clusters, such as clusters 16–20 (Engle et al. 1996), could have been chosen. For most of the tested strains, the method of Brill and Wiegel (1997) yielded positive results, i.e., at least three of the five assays indicated the presence of the representative sporulation genes (Table 5.1.). The results suggested that this method provides a relatively fast experimental means to differentiate between non-spore-forming and asporogenic bacteria (Wiegel 1992), especially in species and strains for which no genome sequences are available. The data also support the notion that differentiation between some of the "clostridial" species and species of the genera

described as lacking sporulation should be reevaluated. As an example, the separation of *Clostridium hastiforme* from the *Tissierella* species based on sporulation appears to be no longer justified on the basis of the high 16S rRNA sequence similarity of these bacteria (Fig. 5.1.) combined with the presented data that the type species *Tissierella praeacuta* is asporogenic, i.e., contains sequences with similarity to sporulation genes from *B. subtilis* and other clostridia (Brill and Wiegel 1997). Thus, the genus *Tissierella* should include sporulating species such as *C. hastiforme*.

However, a few species did not yield indications for the presence of sporulation genes (Table 5.1.). These species would now be regarded as non-spore-forming. As evident from the phylogenetic tree (Fig. 5.1.), the non-spore-forming, Gram-type-positive bacteria identified here do not cluster all together, but belong to different sub-branches, e.g., the *Peptostreptococcus* species, or the *Enterococcus/Carnobacterium/Gemella/Staphylo-coccus* group, possibly constituting a non-spore-forming sub-branch of the otherwise spore-forming *Bacillales/Lactobacillales*.

Genomic analysis among Bacillus spp.

Due to some indications that the experimental test did not give unequivocal results (E. Stackebrandt, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH-Braunschweig, Germany, personal communication) and the comparison of sequences of sporulation genes done by Stragier (2002), we used available (February 2004) genome sequences to challenge our findings and to test for the assumed similarities of sporulation genes. Fifty-two bacteria and archaea, representing diverse phyla, were selected in order to search for the occurrence of 65 assumed "spore-specific" genes that were chosen because they are a

representative sampling of the genes required for each stage of sporulation: preseptation, postseptation, and postengulfment (Stragier 2002). The sequence identity matches were based on identity to the *B. subtilis* sequences (as explained in the "Materials and methods"), which were used as reference sequences. Among the chosen microorganisms were: 17 sporogenic bacteria ('*Firmicutes*'), 29 bacteria that do not form spores but belong to '*Firmicutes*', and as negative controls Proteobacteria (representing 4 of the 5 classes), spirochetes, cyanobacteria, green non-sulfur bacteria, *Fusobacteria*, *Deinococcus*, *Bacteroides*, and *Actinobacteria*, and five archaea (see "Materials and methods"). The choice of microorganisms was somewhat random but did depend on the availability of at least greater than 95% complete genome sequence as a prerequisite. Unfortunately, no genome sequences were available (February 2004) for most of the above-described experimentally tested bacterial species (Table 5.1.).

Occurrence and absence of sporulation specific genes in completed genome sequences

Based on the similarity of the predicted protein sequence, the analysis of the complete genome data indicated that, with a high probability, some "spore-specific" genes are in the genomes of additional '*Firmicutes*' that have never been shown to produce endospores (Table 5.4.). This includes several *Streptococcus* species, two *Enterococcus* species, *Lactococcus lactis*, and most numerously in the studied *Listeria* species. *Listeria monocytogenes* and *Listeria innocua* contain 17 supposedly "spore-specific" genes (Table 5.4.), which include many sigma-factor-related genes. In contrast to the microorganisms listed in Table 5.4., the analysis of several *Bacillus* spp. clearly demonstrates that the sporulating species *B. licheniformis*, *B. firmus*, *B. sphaericus*, and *B. thuringiensis* apparently have quite different or modified sporulation genes, showing no or low levels of similarity with the genes from *B. subtilis* (Table 5.2.). This finding is in agreement

with unpublished data from E. Stackebrandt (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH-Braunschweig, Germany, personal communication) using the experimental assay of Brill and Wiegel (1997). One strain of *Geobacillus stearothermophilus* revealed fewer genes with similarity to the *B. subtilis* genes than to the second analyzed strain that contained many genes with similar sequences [It needs to be pointed out that for many strains the assignment to *G. stearothermophilus* prior to the use of 16S rRNA sequence analysis is questionable (Ash et al. 1991)]. Stragier (2002), based on the comparison of the few *Bacillus* spp. and *Clostridium* spp. genome sequences available at that time of analysis, suggested already to some extent that the inferred diversity in the sequences of sporulation genes needs to be further analyzed and quantified in the future to further elucidate the phylogeny.

Phylogenetic ramifications

We failed to detect sequences similar to those of many sporulation genes of *B. subtilis* in genomes of sporulating species, e.g., members of the order *Clostridales* tested were shown to lack sequences similar to *sspE*. In additon, all of the *Clostridales* tested, except *Clostridium difficile* and *Desulfitobacterium hafniense*, lacked sequences similar to *dpaA/B* of *B. subtilis*. Even members of the order *Bacillales* were found to lack sequences similar to sporulation genes of type species *B. subtilis*: All of the *Bacillales* tested, besides *B. halodurans*, *B. megaterium*, *B. subtilis*, and *G. stearothermophilus*, lacked at least one *ssp* and all, besides *B. anthracis*, *B. cereus*, *B. halodurans*, *G. stearothermophilus* 10, *B. subtilis*, and *B. thuringiensis* subsp. *israelensis*, lacked *dpaA/B* (Table 5.2.). The lack of many identifiable similar sequences in *B. firmus* and *B. licheniformis* (besides those found also in the Gram-type-negative species) was surprising since these two species are closely related to *B. subtilis* (Fig. 5.1.). In contrast, *G.*

stearothermophilus is relatively distantly related. These findings lead us to several broader issues related to phylogeny and the presence or absence of genes, as discussed below.

1) The phylogenetic differences are relatively small (e.g., depicted by short branches), so the placement of the proposed non-spore-forming genera among the sporogenic taxa could be a product of the tree-building algorithm and the selection of included species, which also led to low boot strap values. It is possible that these taxa branched off from other ancestors of the *Bacillus*/*Clostridium* branches before sporulation evolved in the orders *Bacillales*, *Clostridales*, and *Thermoanaerobacteriales*, respectively (Garrity et al. 2003). However, the different tree-building methods [e.g., Fitch (Fig. 5.1.) and neighbor-joining (data not shown)] yielded similar tree morphology making this less likely. The placement of the non-spore-forming genera such as *Streptococcus*, *Listeria*, and *Enterococcus* in the phylogenetic branch of the '*Firmicutes*' has recently been confirmed by using DNA-dependent RNA polymerase phylogeny (Morse et al. 2002);

2) Heavy horizontal gene transfer or loss of the sporulation genes may have occurred among the early '*Firmicutes*'. However, these scenarios are doubtful as an explanation owing to the fact that genes involved in complex developmental processes are typically widely dispersed over the entire chromosome, as based on the arrangement of sporulation genes on the *B. subtilis* chromosome or the large amount and distribution of genes involved with multicellular developmental processes and regulation in the *Streptomyces coelicolor* genome (Bentley et al. 2002; Stragier 2002; Stragier and Losick 1996);

3) Our sequence similarity searches were mainly limited to using the *B. subtilis* gene sequences as reference because it is the model organism for studying endospore formation. Even the sequences from *C. acetobutylicum*, which were used partly as a reference set, were originally identified by similarities to those from *B. subtilis*. Despite this limitation, the data imply that either sporulation developed more than once or early sporulation genes underwent major changes during the evolution to the genes of present-day species such as *B. subtilis* and *C. acetobutylicum*.

Selection of genes involved solely in sporulation processes

In seeking to tabulate those genes that have one singular involvement in cells, namely sporulation, we arrived at a significantly modified table (Table 5.6.) than the one presented by Stragier (2002). Due to the much larger database of available genome sequences, our table is both drastically more restricted and smaller. It is unclear at this stage whether the observed division into genes present in *Bacillus* and genes present in *Clostridum* is artificial or due to phylogenetic differences. The presented analysis demonstrates clearly that there is a need to obtain a detailed analysis of sets of "sporulation-specific" genes from aerobic and anaerobic species lacking sequences with unequivocal similarities to the *B. subtilis* genes. It has been speculated that the anaerobic clostridial lineage is more ancient than the one of the *Bacillales*. Thus, the *Bacillus* and *Clostridium* species might have quite differently modified sporulation genes. Currently, there is not enough knowledge about the sporulation processes in clostridia to attempt such analysis, which should also include unusual sporulation systems such as the ones from *Epulopiscium* and *Metabacterium* (Angert et al. 1996; Siunov et al. 1999).

Acknowledgments

Table 5.1. Bacterial species experimentally tested for the presence of sporulation-specific genes *spo0A*, *ssp*, and *dpa* (*A/B*). Clusters are as assigned by Collins et al. (1994) and Farrow et al. (1995). Correct lengths of PCR-products are 100 bp for *ssp* and 300 bp for *spo0A*. For sequence of the selected strains see Brill and Wiegel (1997). *ND* Not determined. [a]Some strains did not yield unequivocal PCR-products for *ssp1*. Others did not yield positive hybridization bands with the *dpa* probe but four out of the five tests were always positive. [b]No unequivocal PCR product for *ssp1* obtained.

Bacteria	Clusters	PCR products for *ssp* and *spo0A* and positive Southern hybridizations for all *ssp*, *spo0A*, and *dpa* probes	PCR-control Universal eubacterial 16S rRNA gene probe
Thermobrachium celere DSM 8682	Between II and III	+	+
16 isolates of *Thermobrachium celere*		+[a]	ND
Thermoanaerobacter ethanolicus DSM 2246	V	+	ND
Thermobrachium acetoethylicus ATCC 2359	V	+	ND
Thermosyntropha lipolytica DSM 11003	VIII	+[b]	+
Megasphaera cerevisiae ATCC 43254	IX	+	+
Veillonella parvula ATCC 10790	IX	+	ND
Selenomonas ruminantium ATCC 12561	IX	+	+
Selenomonas sputigena ATCC 35185	IX	+	+
Anaerobranca horikoshii DSM 11003	Closest to IX	+	ND
Desulfitobacterium dehalogenans DSM 9161	Closest to IX	+	ND
Peptostreptococcus anaerobius ATCC 27337	XI	−	+
Clostridium thermoalkaliphilum DSM 7309	XI	+	+
Eubacterium tenue ATCC 25553	XI	+	+
Tissierella praeacuta ATCC 25539	XII	+	+
Peptostreptococcus magnus ATCC 15	XIII	−	+
Eubacterium limosum ATCC 8486	XV	+[b]	+
Leuconstoc mesenteroides ssp. *mesenteroides* ATCC 8293	Lactobacillales	−	+

Bacteria	Clusters	PCR products for *ssp* and *spo0A* and positive Southern hybridizations for all *ssp*, *spo0A*, and *dpa* probes	PCR-control Universal eubacterial 16S rRNA gene probe
Lactobacillus delbrueckii ssp. *delbrueckii* ATCC 9649	Lactobacillales	–	+
Lactococcus lactis ssp. *lactis* ATCC 19435 794	Lactobacillales	–	+
Streptococcus agalactiae ATCC 13813	Lactobacillales	–	+
Streptococcus pyogenes ATCC 12344	Lactobacillales	–	+
Carnobacterium divergens ATCC 35677	Lactobacillales	–	+
Gemella haemolysans ATCC 10379	Lactobacillales	–	+
Enterococcus faecalis ATCC 19433	Bacillales	–	+
Staphylococcus saprophyticus ATCC 15305	Bacillales	–	+
Staphylococcus aureus ATCC 12600	Bacillales	–	+
Listeria monocytogenes ATCC 15313	Bacillales	–	+
Brochothrix thermosphacta ATCC 11509	Bacillales	–	+

Table 5.2. Presence and absence of sporulation genes (with sequence similarity to *Bacillus subtilis* genes) in genomes of *Bacillus* and *Geobacillus* species. Designations are based on *p*-values and *e*-scores obtained from BLAST searches to identify relative similarity of gene sequence to the *B. subtilis* query gene sequences (see " Materials and methods " section " Sequence retrieval and phylogenetic analysis " for specific details of the analysis. (++) Scores identify sequence as similar to the *B. subtilis* query sequence. (+) Some similarity over parts of the sequence exists to the *B. subtilis* query sequence. (+/–) Score is too low to allow for a definitive classification of the sequence relative to the *B. subtilis* query sequence. (–) Score indicates that little to no similarity exists relative to the *B. subtilis* query sequence. The genes that are in *bold* represent similar sequences found in species outside the *Firmicutes* (Table 5). *Asterisk* Indicates the number of genes not found were too numerous to list and include those not otherwise indicated in the other columns. [a]Difference between the two *Geobacillus stearothermophilus* strains refers to the source of the genome data, see "Materials and methods".

Bacillales	(++)	(+)	(+/–)	(–)
Bacillus subtilis	All genes present (reference set)			
Bacillus anthracis	***spoVG***, ***spoIIA(A, B)***, ***spoIID***, ***spoIIE***, ***spoIIGB***, *spoIIP*, *gerM*, *spoIIIAE*, *spoIVA*, *spoIIID*, ***spoVB***, *spoVID*, ***spoIIIG***, *spoIVB*, *spoVA(D, F)*, ***sspA***, *gerA(A, C)*, *gpr*, ***splB***, *yqfC*, *yqfD*, *yabP*	***spo0A***, ***spo0H***, ***spoIIAC***, *spoIIIA(B, D)*, *cotE*, *gerPA*, *spoVA(B, C)*, ***dpaB***, *cwlJ*, ***spmA***, ***spmB***	***minC***, *rapA*, *spoIIGA*, *spo0B*, *spoIIB*, *spoIIM*, ***spoIIQ***, *spoIIR*, *spoIIIA(A, C, F, G, H)*, ***spoVK***, ***spoIVCB***, *spoVA(A, E)*, ***dpaA***, *hep1*, *gerAB*, *gerD*, ***sleB***, ***cwlD***, *cotD*, *yabQ*	*spoVM*, *sspE*
Bacillus cereus	***spoVG***, ***spoIIA(A, B)***, ***spoIID***, ***spoIIE***, ***spoIIGB***, *spoIIP*, *gerM*, *spoIIIAE*, *spoIVA*, *spoIIID*, ***spoVB***, *spoVID*, *spoIIIG*, *spoIVB*, *spoVA(D, F)*, *sspA*, *gerA(A, C)*, *gpr*, ***splB***, *yqfC*, *yqfD*, *yabP*	***spo0A***, ***spo0H***, ***spoIIAC***, *spoIIIA(B, D)*, *cotE*, ***gerPA***, ***spoVA(B, C)***, *dpaB*, ***cwlJ***, *spmA*, *spmB*	*minC*, *spo0B*, *rapA*, *spoIIB*, *spoIIGA*, *spoIIM*, ***spoIIQ***, *spoIIR*, *spoIIIA(A, C, F, G, H)*, ***spoVK***, ***spoIVCB***, *spoVA(A, E)*, ***dpaA***, *hep1*, *gerAB*, *gerD*, ***sleB***, ***cwlD***, *cotD*, *yabQ*	*spoVM*, *sspE*
Bacillus firmus		*sspA*	***spoVK***	*
Bacillus halodurans	***spoVG***, ***spoIIAA***, ***spoIID***, ***spoIIE***, ***spoIIGB***, *spoIIP*, *gerM*, *spoIIIAE*, *spoIVA*, *spoIIID*, ***spoVB***, *spoVID*, *spoIVB*, *spoVAF*, *spoVM*, *sspA*, *gerAA*, *gpr*, ***splB***, *yqfD*, *yabP*	***spo0A***, ***spo0H***, ***spoIIA(B, C)***, *spoIIIA(B, D)*, *cotE*, ***spoIIIG***, *spoVA(C, D)*, ***dpaB***, *sspE*, *gerAC*, *cwlJ*, *yqfC*, ***spmA***, ***spmB***	***minC***, *spo0B*, *rapA*, *spoIIB*, *spoIIGA*, *spoIIM*, ***spoIIQ***, ***spoIIR***, *spoIIIA(A, C, F, G, H)*, ***spoVK***, ***spoIVCB***, *spoVAE*, ***dpaA***, *hep1*, *gerAB*, *gerD*, ***sleB***, ***cwlD***, *cotD*, *yabQ*	*gerPA*, *spoVA(A, B)*
Bacillus licheniformis	***spo0H***, ***spoIIA(A, B, C)***	*rapA*, *yqfC*, *yqfD*	*spoIIB*, ***spoIIE***, ***spoIIGB***, ***spoIIIG***, ***spoIVCB***, ***cwlD***	*
Bacillus megaterium	***spoVG***, ***spoIIAA***, ***spoIIE***, *spoIIP*, *spoVID*, *sspA*, *gerAA*, *gpr*, *yqfC*, *yqfD*	***spo0H***, ***spoIIA(B, C)***, *sspE*, *gerAC*	***spo0A***, *spoIIG(A, B)*, ***spoIIIG***, ***spoIVCB***, *spoVAF*, *gerAB*, ***splB***, *cwlJ*	*

Bacillales	(++)	(+)	(+/–)	(–)
Bacillus sphaericus		**spoIIAB**	**spo0A**, **spoIIA(A, C)**, **spoIIGB**, **spoIIIG**, **spoIVCB**, *dpaA*	*
Bacillus thuringiensis	**spoIIGB**, *spoIIID*	*spo0A*	**spo0H**, *rapA*, **spoIIAC**, *spoIIGA*, **spoIIIG**, **spoIVCB**, *spoVAF*	*
Bacillus thuringiensis subsp. *israelensis*	**spoVG**, **spoIIA(A, B)**, **spoIIE**, **spoIIGB**, *spoIIP*, *gerM*, *spoIIIAC*, *spoIIID*, **spoVB**, *spoVID*, *spoIVB*, *spoVAC*, *sspA*, *gpr*, **splB**, *yqfC*, *yqfD*, *yabP*	*spo0A*, *gerA(A, C)*, **spoIIAC**, *spoIIIA(B, D, E)*, *cotE*, *gerPA*, **spoIIIG**, *spoVAB*, *cwlJ*, **spmB**	**minC**, *spo0B*, **spo0H**, *rapA*, *spoIIB*, **spoIID**, *spoIIGA*, *spoIIM*, **spoIIQ**, *spoIIR*, *spoIIIA(A,F, G, H)*, **spoIVCB**, *spoVA(A, D, E, F)*, *spoVK*, **dpaA**, **dpaB**, *hep1*, **sleB**, **cwlD**, *cotD*, *yabQ*, *spmA*, *gerAB*	*spoIVA*, **spoVB**, *spoVM*, *sspE*, *gerA(A, C)*
Geobacillus stearothermophilus[a]	**spoIIAA**, *sspA*, *sspE*	*spo0A*, **spoIIA(B, C)**	*rapA*, **spoIIGB**, **spoIIIG**, *hep1*, **spoIVCB**, **dpaA**	*
Geobacillus stearothermophilus 10	**spoVG**, **spoIIA(A, B)**, **spoIID**, **spoIIE**, **spoIIGB**, *spoIIP*, *gerM*, *spoIIIA(C, E)*, *spoIIID*, *spoIVA*, **spoVB**, *spoVID*, *gerPA*, **spoIIIG**, *spoIVB*, *spoVAF*, *gerAA*, *gpr*, **splB**, *yqfC*, *yqfD*, *yabP*, **spmA**	**spo0A**, **spoIIAC**, *spoIIIA(B, D)*, *cotE*, *spoVA(B, C, D)*, *cwlJ*, **spmB**	**minC**, *spo0B*, **spo0H**, *rapA*, *spoIIGA*, *spoIIM*, **spoIIQ**, *spoIIR*, *spoIIIA(A,F, G)*, **spoVK**, **spoIVCB**, *spoVA(A, E)*, **dpaA**, **dpaB**, *hep1*, *gerA(B, C)*, *gerD*, **sleB**, **cwlD**, *yabQ*	*spoIIB*, *spoIIIAH*, *spoVM*, *sspA*, *sspE*, *cotD*

Table 5.3. Presence and absence of sporulation genes (with sequence similarity to *B. subtilis* genes) in genomes of *Clostridium* and *Desulfitobacterium* species. Designations are based on *p*-values and *e*-scores obtained from BLAST searches to identify relative similarity of gene sequence to the *B. subtilis* query gene sequences. (++) Scores identify sequence as similar to the *B. subtilis* query sequence. (+) Some similarity exists over parts of the sequence to the *B. subtilis* query sequence. (+/–) Score is too low to allow for a definitive classification of the sequence relative to the *B. subtilis* query sequence. (–) Score indicates that little to no similarity exists relative to the *B. subtilis* query sequence. The genes that are in *bold* represent similar sequences found in species outside the *Firmicutes* (Table 5). *Asterisk* indicates the number of genes not found were too numerous to list and include all those not otherwise indicated in the other columns

Clostridiales	(++)	(+)	(+/–)	(–)
Clostridium acetobutylicum	spoIVA	**spoVG**, **spoIIE**, **spoIIGB**, spoIIID, **spoIIIG**, sspA, gerA(A, C), yqfC	**minC**, **spo0A**, **spo0H**, rapA, **spoIIA(A, B, C)**, **spoIID**, spoIIM, spoIIP, **spoIIQ**, spoIIR, spoIIIA(A, B, C, D, E, F, G, H), **spoVB**, **spoVK**, spoIVB, **spoIVCB**, spoVA(C, D, E, F), gerAB, gpr, **splB**, **sleB**, **cwlD**, cwlJ, yqfD, yabP, yabQ, **spmA**, **spmB**	spo0B, spoIIB, **spoIIGB**, gerM, cotE, spoVID, gerPA, spoVA(A, B), spoVM, **dpaA**, **dpaB**, hep1, sspE, gerD, cotD
Clostridium botulinum		gerAC	spoVAF, gerA(A, B)	*
Clostridium difficile	spoIVA	**spoVG**, **spoIIA(A, B)**, **spoIIE**, spoIIIA(C, D), spoIIID, **spoIIIG**	**minC**, **spo0A**, **spo0H**, rapA, **spoIIAC**, **spoIID**, spoIIGA, **spoIIGB**, spoIIM, spoIIP, **spoIIQ**, spoIIR, spoIVB, **spoIVCB**, spoIIIA(E, G, H), **spoVB**, **spoVK**, spoVA(C, D, E), **dpaA**, **dpaB**, gpr, **splB**, **sleB**, **cwlD**, yqfC, yqfD, yabP, yabQ, **spmA**, **spmB**	spo0B, spoIIB, gerM, spoIIIA(**A, B, F**), spoIVA, cotE, spoVID, gerPA, spoVA(A, B, F), spoVM, hep1, sspA, sspE, gerA(A, B, C), gerD, cwlJ, cotD
Clostridium perfringens	spoIVA	**spoVG**, **spoIIA(A, B)**, **spoIID**, **spoIIE**, **spoIIGB**, spoIIID, **spoIIIG**, sspA, yqfC	**minC**, **spo0A**, **spo0H**, **spoIIAC**, spoIIGA, spoIIM, spoIIP, **spoIIQ**, spoIIR, spoIIIA(**A, B, C, D, E, G, H**), **spoVB**, **spoVK**, spoIVB, **spoIVCB**, spoVA(C, D, E, F), gerAA, gpr, **splB**, **sleB**, **cwlD**, yqfD, yabP, yabQ, **spmA**, **SpmB**	spo0B, rapA, gerM, spoIIB, spoIIIAF, cotE, spoVID, gerPA, spoVA(A, B), spoVM, **dpaA**, **dpaB**, hep1, sspE, gerA(B, C), gerD, cwlJ, cotD
Desulfitobacterium hafniense		**spoVG**, spoIVA, spoIIID, **spoIIIG**, sspA, gerA(A, C), yqfC	**minC**, **spo0A**, **spo0H**, **spoIIA(A, B, C)**, **spoIID**, spoIIG(A, **B**), spoIIP, **spoIIQ**, spoIIR, spoIIIA(**A, B, D, E, G**), **spoVB**, **spoVK**, spoIVB, **spoIVCB**, spoVA(C, D, E, F), **dpaA**, **dpaB**, gerAB, gpr, **splB**, **sleB**, **cwlD**, cwlJ, yqfD, yabP, **spmA**, **spmB**	spo0B, rapA, spoIIB, **spoIIE**, spoIIM, gerM, spoIIIA(C, F, H), cotE, spoVID, gerPA, spoVA(A, B), spoVM, hep1, sspA, sspE, gerD, cotD, yabQ

Table 5.4. Gene sequences with similarity to sporulation genes observed in genomes of Gram-type-positive microorganisms that do not form endospores. (++) Scores identify a sequence as being similar to the *B. subtilis* query sequence. (+) Some similarity over parts of the sequence to the *B. subtilis* query sequence. (+/−) Score is too low to allow for a definitive classification of the sequence relative to the *B. subtilis* query sequence. The genes that are in *bold* represent similar sequences found in species outside the *Firmicutes* (Table 5.5.). The genes *underlined* represent similar sequences not found in species outside the *Firmicutes* (Tables 5.2.-5.4.).

Gram-type positive	(++)	(+)	(+/–)
Staphylococcaceae			
Staphylococcus aureus		*spoVG*, <u>*spoVID*</u>	*spo0A*, *spo0H*, *spoIIA(A, B, C)*, *spoIIGB*, *spoIIE*, *spoIIQ*, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*, *dpaA*, *cwlD*
Mycoplasmatales			
Mycoplasma pneumoniae			*spo0H*, *spoIIGB*, *spoVB*, *spoIIIG*
Lactobacillales			
Enterococcus faecalis			*spo0A*, *spo0H*, *spoIIA(B, C)*, *spoIIGB*, *spoIIQ*, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*
Enterococcus faecium			*spo0A*, *spo0H*, *spoIIGB*, *spoIIQ*, *spoVB*, *cwlD*
Lactococcus lactis			*spo0A*, *spoIIAC*, *spoIIGB*, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*
Listeria innocua	*spoVG*		*minC*, *spo0A*, *spo0H*, *spoIIA(A, B, C)*, *spoIIE*, *spoIIQ*, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*, *dpaA*, <u>*hep1*</u>, *cwlD*
Listeria monocytogenes	*spoVG*		*minC*, *spo0A*, *spo0H*, *spoIIA(A, B, C)*, *spoIIE*, *spoIIGB*, *spoIIQ*, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*, *dpaA*, <u>*hep1*</u>, *cwlD*
Streptococcus agalactiae			*spoIIAC*, *spoIIGB*, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*
Streptococcus equi			*spo0A*, *spo0H*, *spoIIAC*, *spoIIGB*, *spoIIQ*, *spoVB*, *spoVK*, *spoIIIG*
Streptococcus mutans			*spo0H*, *spoIIAC*, *spoIIGB*, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*
Streptococcus pneumoniae			*spo0A*, *spo0H*, *spoIIA(B, C)*, *spoIIGB*, <u>*spoIVA*</u>, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*
Streptococcus pyogenes		<u>*sspA*</u>	*spo0A*, *spo0H*, *spoIIAC*, *spoIIGB*, *spoVB*, *spoVB*, *spoVK*, *spoIIIG*, *spoIVCB*
Streptococcus salvarius subsp. *thermophilus*			*spoVB*
Actinomycetales			
Streptomyces coelicolor			*spo0A*, *spo0H*, *spoIIA (A, B, C)*, *spoIIGB*, *spoIVCB*, *spl*, *spoIIE*, *spoIIQ*, *spoVK*, *spoIIIG*, *spoIVCB*, *splB*

Table 5.5. Gene sequences with similarity to sporulation genes observed in genomes of Gram-type-negative microorganisms that do not form endospores. (++) Scores identify sequence as similar to the *B. subtilis* query sequence. (+) Some similarity over parts of the sequence to the *B. subtilis* query sequence. (+/–) Score is too low to allow for a definitive classification of the sequence relative to to the *B. subtilis* query sequence
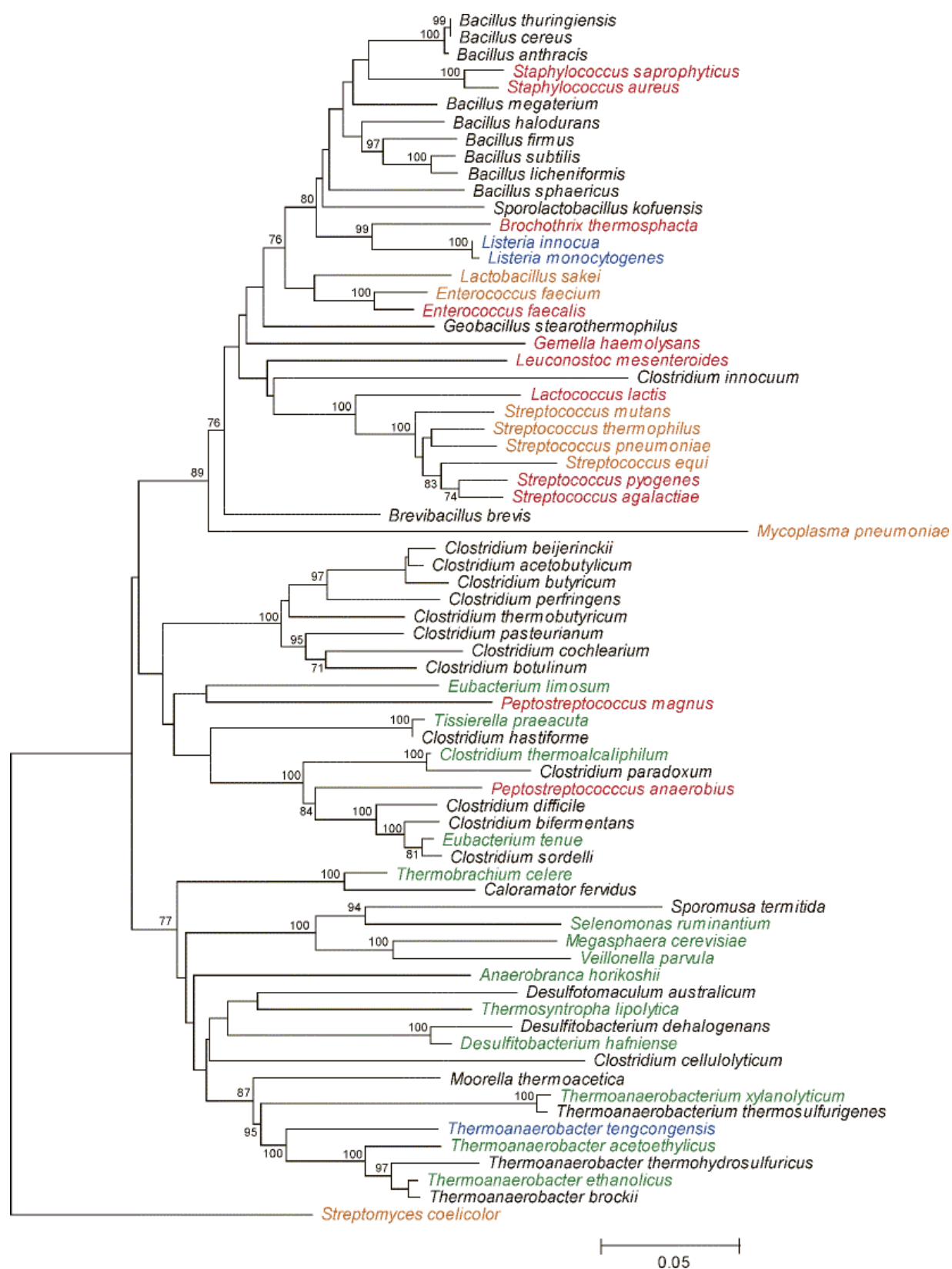
Gram-type negative	(++)	(+)	(+/–)
Proteobacteria			
Haemophilus actinomycetemcomitans			*spo0A, spoIIAC, spoIIQ, spoVK, spoIIIG, cwlD*
Caulobacter crescentus			*spoIIQ, spoIVCB, dpaA, sleB, cwlD*
Escherichia coli			*spo0A, spo0H, spoIIA(B, C), spoIIGB, spoIIQ, spoIIIAA, spoVK, spoIIIG, spoIVCB, cwID*
Escherichia coli O157:H7			*spoIVCB, cwlD, spo0A, spo0H, spoIIAB, spoIIGB, spoIIQ, cwID*
Helicobacter pylori J99			*spo0A, spoIIAC, spoIIGB, spoIIQ, spoVK, spoIIIG, spoIVCB, dpaA, cwlD*
Myxococcus xanthus			*spo0A, spo0H, spoIIA(B,C), spoIIGB, spoIIIG, spoIVCB, sleB, dpaA, cwlD*
Salmonella typhi			*spo0A, spo0H, spoIIAC, spoIIGB, spoIIQ, spoVK, spoIIIG, spoIVCB, cwlD*
Salmonella typhimurium			*spo0A, spo0H, spoIIAC, spoIIGB, spoIIQ, spoVK, spoIIIG, spoIVCB, cwlD*
Spirochaetes			
Borrelia burgdorferi			*spo0A, spo0H, spoIIAC, spoIIGB, spoVG, spoIIQ, spoVK, spoIIIG, spoIVCB*
Cyanobacteria			
Synechococcus spp.			*minC, spoIID, spoIIQ, spoIIIAA, spoVK, spoIVCB, cwID*
Prochlorococcus marinus			*spoIVCB, cwlD, minC, spoIID, spoIIQ, spoIIIAA, spoVK, spoIVCB, cwlD*
Anabaena spp.			*minC, spo0A, spoIIA(A, C), spoIID, spoIIQ, spoIIIAA, spoVK, sleB, cwlD*
Chloroflexi			
Chloroflexus aurantiacus			*minC, spoIIAB, spoIIE, spoIIIAA, spoIVCB*
Fusobacteria			
Fusobacterium nucleatum		*spoVG*	*minC, spo0A, spo0H, spoIIAA, spoIID, spoIIE, spoIIQ, spoVK, spoIVCB, splB, cwlD*
Deinococcus-Thermus			
Deinococcus radiodurans			*minC, spoIIAB, spoIID, spoIIQ, spoVK, cwlD*
Bacteroidetes			
Cytophaga hutchinsonii			*spoIIE, spmA, spmB*
Archaea			
Euryarchaeota			
Archaeoglobus fulgidus	*spoVG*		*spo0A, spoVB, spoVK, dpaB*
Halobacterium spp. NRC-1			*spo0A, spoVK, dpaB*

Gram-type negative	(++)	(+)	(+/–)
Methanocaldococcus jannaschii			*spoVB, spoVK*
Pyrococcus furiosus			*spoVB*
Crenarchaeota			
Aeropyrum pernix			*spoVK*

Table 5.6. Spore-specific genes observed in *Bacillus* and *Clostridium* and related species.

Classification	Genes
Present in some *Bacillus* spp. and some *Clostridium* spp., but no similar sequences observed in Gram-type-negative Proteobacteria or Cyanobacteria species	*rapA*, *spoIIGA*, *spoIIM*, *spoIIP*, *spoIIR*, *spoIIIA (B, C, D, E, F, G, H)*, *spoIVA*, *spoIIID*, *spoIVB*, *spoVA (C, D, E, F)*, *sspA*, *gerA (A, B, C)*, *gpr*, *cwlJ*, *yqfC*, *yqfD*, *yabP*, *yabQ*, *spo0B*
Present in some *Bacillus* spp. only and absent from *Clostridium* spp.	*spoIIB*, *gerM*, *cotE*, *spoVID*, *gerPA*, *spoVA (A, B)*, *spoVM*, *dpaA*, *hep1*, *sspE*, *gerD*, *cotD*

Fig. 5.1. Phylogenetic tree constructed from the 16S rRNA gene with maximum likelihood correction for synonymous changes using the Fitch algorithm. Strain designations were omitted for simplicity of the tree but are included in "Materials and methods". Sporogenic species are indicated in *black*. Asporogenic species, as determined by PCR and Southern-hybridization-based assay are indicated in *green*. Assignments as asporogenic species based on genome sequence analysis are indicated in *blue*. Non-spore-forming species are indicated in *red* for PCR and Southern-hybridization-based assay and in *orange* for genome analysis. *Numbers at nodes* indicate bootstrap support values for 100 replicates. *Scale bar* denotes number of nucleotide substitutions per site. Using the neighbor-joining algorithm (figure not shown), a very similar tree was obtained, except the relative position of *Geobacillus stearothermophilus* and *Bacillus brevis* were closer to the position of *Bacillus subtilis*, whereas *Clostridium innocuum* was closer to *Mycoplasma pneumoniae*.

99 | *Bacillus thuringiensis*
100 | *Bacillus cereus*
Bacillus anthracis
100 | *Staphylococcus saprophyticus*
Staphylococcus aureus
Bacillus megaterium
Bacillus halodurans
97 | *Bacillus firmus*
100 | *Bacillus subtilis*
Bacillus licheniformis
Bacillus sphaericus
80 | *Sporolactobacillus kofuensis*
99 | *Brochothrix thermosphacta*
76 | 100 | *Listeria innocua*
Listeria monocytogenes
Lactobacillus sakei
100 | *Enterococcus faecium*
Enterococcus faecalis
Geobacillus stearothermophilus
Gemella haemolysans
Leuconostoc mesenteroides
Clostridium innocuum
100 | *Lactococcus lactis*
Streptococcus mutans
100 | *Streptococcus thermophilus*
Streptococcus pneumoniae
Streptococcus equi
83 | *Streptococcus pyogenes*
74 | *Streptococcus agalactiae*
89 | *Brevibacillus brevis*
Mycoplasma pneumoniae
Clostridium beijerinckii
97 | *Clostridium acetobutylicum*
Clostridium butyricum
Clostridium perfringens
100 | *Clostridium thermobutyricum*
Clostridium pasteurianum
95 | *Clostridium cochlearium*
71 | *Clostridium botulinum*
Eubacterium limosum
Peptostreptococcus magnus
100 | *Tissierella praeacuta*
Clostridium hastiforme
100 | *Clostridium thermoalcaliphilum*
100 | *Clostridium paradoxum*
Peptostreptococccus anaerobius
84 | 100 | *Clostridium difficile*
100 | *Clostridium bifermentans*
Eubacterium tenue
81 | *Clostridium sordelli*
100 | *Thermobrachium celere*
Caloramator fervidus
Sporomusa termitida
94 | *Selenomonas ruminantium*
100 | 100 | *Megasphaera cerevisiae*
Veillonella parvula
Anaerobranca horikoshii
Desulfotomaculum australicum
Thermosyntropha lipolytica
Desulfitobacterium dehalogenans
100 | *Desulfitobacterium hafniense*
Clostridium cellulolyticum
Moorella thermoacetica
87 | 100 | *Thermoanaerobacterium xylanolyticum*
Thermoanaerobacterium thermosulfurigenes
95 | *Thermoanaerobacter tengcongensis*
100 | *Thermoanaerobacter acetoethylicus*
100 | *Thermoanaerobacter thermohydrosulfuricus*
97 | *Thermoanaerobacter ethanolicus*
Thermoanaerobacter brockii
77 | *Streptomyces coelicolor*

0.05

CHAPTER 6

CHARACTERIZATION OF A SOLUBLE OXIDOREDUCTASE WITH AN FE(III)

REDUCTION ACTIVITY FROM *CARBOXYDOTHERMUS FERRIREDUCENS* [1]

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[1]Onyenwoke, R. U., R. Geyer, and J. Wiegel. To be submitted to *Applied and Environmental Microbiology*.

Abstract

An NAD(P)H-dependent oxidoreductase has been purified approximately 40-fold from the soluble protein fraction of the dissimilatory iron-reducing, anaerobic, thermophilic bacterium *Carboxydothermus ferrireducens*. The enzyme has a broad substrate range and reduces Fe^{3+}, Cr^{6+}, and the quinone analog AQDS with rates of 0.31, 3.3, and 3.3 $U \cdot mg^{-1}$ protein, calculated turnover numbers for NADH oxidation of 0.25, 2.5, and 2.5 s^{-1}, respectively.

Introduction

Dissimilatory iron reduction is the use of Fe^{3+} as the terminal electron acceptor in anaerobic respiration (Myers and Myers 1992). This phenomenon has important environmental implications, particularly in anaerobic soils and may be related to the evolution of microbial life (Slobodkin et al. 1997). Dissimilatory iron-reducing bacteria, as a group, are phylogenetically diverse with members among many distinct taxa, e.g., the Gram-type negative bacteria (especially the Δ-Proteobacteria) and the Gram-type positive bacteria (Myers and Myers 1992; Coates et al. 2001; Slobodkin et al. 1997).

Fe^{3+} most commonly exists in the environment as a general class of insoluble compounds known collectively as 'Fe^{3+} oxides'. For this reason, as well as due to work with *Shewanella* and *Geobacter* (Lovley 1991; Myers and Myers 1992; Magnuson et al. 2000)*, it has been suggested that direct cell contact (and therefore a membrane-localized system of proteins) is required for the reduction of 'Fe^{3+} oxides'. However, solublized Fe^{3+} is important and in relatively high abundances in environments rich in organic chelating agents (Ratering and Schnell 2000), and soluble 'Fe^{3+} reductases' have been characterized (Kaufmann and Lovley 2001; Chiu et al. 2001). The role of these soluble enzymes is presently unknown but may be linked to Fe^{3+}

reduction via a soluble electron carrier, such as a quinone (Lovley et al. 1996; Fig. 6.1.). This hypothesis is strengthened by the reported ability of all described Fe^{3+}-reducers to make use of the quinone analog 9,10-anthraquinone-2,6-disulfonate (AQDS) as a terminal electron acceptor of the electron transport chain. Quinones typically act as intermediates in cellular, electron transport chains. In many (an)aerobic soils and sediments, it is the quinone moieties of humic substances that function as the electron shuttles to 'Fe^{3+} oxides' (Lovley et al. 1996; Hernandez and Newman 2001). In addition, the importance of quinones to bacterial respiration has been described. *Shewanella oneidensis* requires menaquinone (MK) during growth on several electron acceptors, including Mn^{4+}, Fe^{3+}, fumarate, nitrate, nitrite, thiosulfate, DMSO, and AQDS (Myers and Myers 1993b; Myers and Myers 1994; Myers et al. 2004; Myers and Myers 2000; Newman and Kolter 2000; Schwalb et al. 2003).

Here we report on an NAD(P)H-dependent oxidoreductase purified from the cytoplasmic (soluble) fraction of the Gram-type positive, anaerobic, thermophilic, dissimilatory iron-reducing bacterium *Carboxydothermus ferrireducens* (basonym: *Thermoterrabacterium ferrireducens*) (Slobodkin et al. 1997, 2006) capable of Fe^{3+} and quinone reduction. Insoluble 'Fe^{3+} oxides', chelated forms of Fe^{3+} (e.g., Fe^{3+} citrate and Fe^{3+}-EDTA), AQDS, and fumarate serve as electron acceptors for this bacterium (Slobodkin et al. 1997). Thus, the *C. ferrireducens* oxidoreductase (CFOR) may play some role in reduction-oxidation type reactions in this microorganism; possibly bridging the energetic gap between the quinone electron intermediates (shuttles) and the Fe^{3+} terminal electron acceptors.

Materials and methods

Materials

Unless otherwise noted, all chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA. A 1 mM stock of $FADH_2$ was prepared by adding solid sodium dithionite to FAD and following the loss of absorbance at 450 nm, i.e., the reduction of FAD to $FADH_2$ (Louie et al. 2003). Recombinant human (rh)NQO1, purified from *Escherichia coli* (Beall et al. 1994), was the generous gift of Dr. David Siegel, University of Colorado Health Sciences Center, Denver, Colorado, USA. The purified NQO1 was at a final protein concentration of 3.3 mg/ml in 25 mM Tris-HCL (pH 7.4) containing 250 mM sucrose and 5 μM FAD. Lyophilized guinea pig zeta-crystallin protein was the generous gift of Dr. J. Samuel Zigler, National Eye Institute (NIH), Bethesda, Maryland, USA.

Cell growth

 Carboxydothermus ferrireducens (DSM 11255) was obtained from the authors' laboratory culture collection and cultivated under anoxic conditions [N_2 headspace (Ljungdahl and Wiegel 1986)] in a minimal medium, described by Slobodkin et al. (1997), containing 40 mM glycerol and 10 mM fumarate in a 100 l fermentor at the University of Georgia's Fermentation Facility (Athens, GA). Cells (50 to 60 g wet weight) were: harvested by continuous centrifugation (13,000 *g*), washed and resuspended in 20 mM Tris buffer containing 10% (v/v) glycerol (pH 8.5) to a final concentration of 1.0 g cell mass/ml, and stored at -80ºC for further use.

Enzyme assays

Unless otherwise noted, all enzyme assays were performed in duplicate under anoxic conditions (N_2-sparged Hungate tubes) at 50°C for 15 min. The assay was linear for at least 30 min., and the data indicated ~15% of the substrate (Fe^{3+}) had been consumed in 15 min. (Fig. 6.2.). The reaction mixture (final volume 1.0 ml) contained: buffer [20 mM MES, 20 mM Tris, 20 mM MOPS, 20 mM TAPS, and 40 mM $MgCl_2$ (pH 6.5)], 0.01-10 mM NADH (or NADPH), 1.0-10 µM FAD, (FMN or riboflavin), 0.2 mM ferrozine, 0.01-500 µM Fe^{3+} citrate, and 0.5-50 µg of protein. The reaction was initiated by the addition of protein. Activity was monitored on a Beckman DU-64 spectrophotometer by either 1) following the reduction of Fe^{3+} to Fe^{2+} by the use of the Fe^{2+} capture reagent ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine) at 562 nm [molar extinction coefficient (ε_{562}) of 27.9 $mM^{-1}cm^{-1}$ (Dailey and Lascelles 1977)], or 2) observing the rate of NADH oxidation at 340 nm [ε_{340} = 6.22 $mM^{-1}cm^{-1}$ (Ernster et al. 1962)]. One milliunit (mU) is the amount of enzyme catalyzing the release of 1 nmol of product per min. The maximal pH for Fe^{3+} reduction activity was determined by adjusting the pH of the buffer (see above) using either 5.0 M HCl or 5.0 M NaOH.

Product inhibition studies were performed with the purified CFOR employing a reaction mixture containing 1) 0-50 µM NAD^+ with 100 nM Fe^{3+} citrate, 5.0 µM FAD, and 0-50 µM NADH; 2) 0-50 µM $FADH_2$ with 100 nM Fe^{3+} citrate, 50 µM NADH, and 0-50 µM FAD; or 3) 0-10 µM ferrous sulfate heptahydrate with 50 µM NADH and 5 µM FAD, and 0-10 µM Fe^{3+} citrate. Reaction was initiated by the addition of protein. Inhibition studies employing EDTA, NTA, or metals ($CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, Na_2CrO_4, MnO_2, Na_2SeO_3, or Co-EDTA) were conducted using 100 nM ferric citrate, 5 µM FAD, 50 µM NADH, and 2.5 µg protein.

For testing the reduction of other metals by the purified CFOR (added to the assay mixture to give a final concentration of 50 µM), iron and ferrozine were omitted from the reaction mixture. Manganese (Mn^{4+}) reduction to Mn^{2+} was determined using a formaldoxime-ammonia reagent (Brewer and Spencer 1971; Greene et al. 1997). The increase in absorbance at 450 nm (A_{450}) due to the production of Mn^{2+} was followed and compared to Mn^{2+} standards. Mn^{2+} standards were prepared by reducing a potassium permanganate solution with concentrated sulfuric acid. Cobalt (Co^{3+}) reduction was measured at 535 nm by observing the loss of Co^{3+} over time compared to sodium dithionite reduced standards (Caccavo et al. 1996b). Chromium (Cr^{6+}) reduction was determined by observing the increase in A_{540} using *s*-diphenylcarbazide and sodium dithionite reduced chromium (Cr^{6+}) standards (Urone 1955). Arsenic (As^{5+}) reduction to As^{3+} was determined using a molybdate color reagent (Johnson 1971). The decrease in A_{885} due to the loss of As^{5+} was monitored and compared to chemically reduced As^{3+} standards. Alternatively, the rate of NADH oxidation was also followed to determine all rates of metal reduction. NADH oxidation was used as the exclusive method to follow selenate ($Na_2Se^{6+}O_4$) reduction. The reduction of quinones (all at 50 µM) involved the same assay system in which metals were omitted for the appropriate quinone, and the rate of NADH oxidation was observed. The reduction of 9,10-anthraquinone-2,6-disulfonate (AQDS) was monitored by following the increase in A_{450} [$\varepsilon_{450} = 3.5$ $mM^{-1}cm^{-1}$] (Lovley et al. 1996).

The mechanism of quinone reduction was determined using cytochrome *c* as a trap for semiquinone generation (Gonzalez et al. 2005). The purified CFOR was tested along with two controls: guinea pig zeta-crystallin protein (Rao et al. 1992.) and human NAD(P)H:quinone oxidoreductase, also known as DT-diaphorase (Ernster 1987). The increase in A_{550} due to

reduction of cytochrome c [$\varepsilon_{550} = 31.2$ mM^{-1}cm^{-1}] by the semiquinone was monitored in an assay containing 50 µM 1,2-naphthoquinone.

Quinone analysis (Lipid extraction and fractionation)

Whole cell quinone analysis was performed as described by Sokolova et al. (in press). Briefly, duplicate samples of *C. ferrireducens* (0.5 g cell mass/ml) were extracted using a modified Bligh and Dyer (1959) extraction method (White et al. 2005). Lipids were separated on a silicic acid column with chloroform, acetone, and methanol into neutral lipid, glycolipid, and polar lipid fractions (Guckert et al. 1985). Solvents were removed under a gentle stream of nitrogen, and the fractions were stored at -20°C. The neutral lipid fraction was resolved in methanol and analyzed for respiratory quinones by HPLC-tandem mass spectrometry (Geyer et al. 2004).

Purification of a soluble oxidoreductase from C. ferrireducens with an Fe^{3+} reduction activity

All purification steps were performed aerobically at room temperature (~25ºC) unless otherwise noted. A cell lysate was prepared from approximately 20 ml of cell suspension (~20 g cell mass) by passage twice through a French pressure cell at 5500 kPa. The cell lysate was then centrifuged at 10,000 g for 30 min. (4ºC) to pellet cell debris and ultra-centrifuged [105,000 g for 1h (4ºC)] to pellet the membrane fraction from the soluble (cytoplasmic) fraction. The membrane fraction was discarded, and the soluble fraction was retained for the subsequent purification protocol.

For the following procedures, all buffers contained 10 µM FAD. In addition, the precaution of wrapping all columns, buffer-containing bottles, and containers used for dialysis in aluminum foil was taken to prevent the degradation of the FAD due to exposure to light. The

soluble fraction (~20 ml), prepared as described above, was applied to a Q-Sepharose Fast Flow column [1.0 x 30 cm (20 ml bed volume); Amersham Biosciences, Piscataway, NJ] that had been equilibrated with 20 mM Tris buffer (pH 8.5) containing 10% (v/v) glycerol (buffer A). The protein was eluted using buffer A + 1.0 M NaCl along a linear gradient of 0 to 0.6 M NaCl (total volume 100 ml) with a flow rate of 1.5 ml/min (4.0 ml fractions collected). Fractions containing Fe^{3+} reduction activity were eluted between 0.3 and 0.6 M NaCl and were pooled. The pooled fractions (~45 ml) were dialyzed using Spectra/Por 12-14 kDa cut-off dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA) overnight (2 exchanges for fresh buffer) against 400 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 10% (v/v) glycerol (buffer B). The pooled fractions were then loaded onto a HiTrap Blue HP column [1.6 x 2.5 cm (5 ml bed volume); Amersham Biosciences, Piscataway, NJ] that had been equilibrated using buffer B. After loading of the protein onto the HiTrap column, at least 5 column volumes of buffer B were used to remove any unbound protein before a step-wise elution (2.0 ml fractions collected) with buffer B + 1.0 mM NADH (30 ml used for elution) . The flow rate was maintained at 2.0 ml/min. The fractions containing the protein eluted by NADH were pooled (~30 ml) and dialyzed against 400 ml of buffer B (2 exchanges for fresh buffer) overnight. The pooled fractions were concentrated to ~5 ml using an Amicon 8400 ultra-filtration stirred cell (400 ml capacity; Millipore, Bedford, MA) equipped with a 10 kDa cut-off ultra-filtration membrane (Amicon, Danvers, MA) and applied to a Sephacryl S-300 HR (Sigma Chemical Co., St. Louis, MO) column [1.6 x 50 cm (100 ml bed volume); Amersham Biosciences, Piscataway, NJ] that had been equilibrated with buffer B + 0.15 M NaCl at 0.4 ml/min. After application of the sample, the protein was eluted using 120 ml of the equilibration buffer, and fractions (2.0 ml) were collected.

Determination of native molecular weight

The native molecular weight (M_r) of the enzyme was determined using gel filtration on the Sephacryl S-300 HR column. Apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa) were the molecular weight standards.

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmeli (1970). A Bio-Rad broad range molecular marker kit [(Hercules, CA); myosin (M_r 200, 000), β-galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,000), lysozyme (M_r 14,000), and aprotinin (M_r 6500)] was used as standards. Proteins were separated on 12% polyacrylamide gels and stained using the Pierce (Rockford, IL) GelCode stain reagent.

Determination of flavin and metal content

The purified CFOR was analyzed for flavin content, after perchloric acid extraction, by high-pressure liquid chromatography (HPLC) using a C-18 reversed phase column (25 x 4.6 cm) with a 20 mM ammonium acetate mobile phase (pH 6.0) containing 21% acetonitrile (v/v) at a flow rate of 0.6 ml/min (Hausinger et al. 1986). Various concentrations of FAD (retention time = 4.88 min) and FMN (retention time = 5.71 min) were prepared as standards to identify, and to determine the amount of, any possible bound flavin.

Metal content was determined using both the iron quantitation method of Fish (1988) with Fe^{3+} citrate standardsand inductively coupled plasma mass spectrometry (ICP-MS) analysis

(Chemical Analysis Laboratory, UGA, Athens, GA). Total protein was determined using a Bio-Rad protein assay kit (Richmond, CA) with a bovine serum albumin standard. The cofactor content of the CFOR was calculated based upon a molecular mass of 45 kDa.

Determination of N-terminal amino acid sequence

For N-terminal amino acid sequencing, the purified CFOR was separated by SDS-PAGE (12%) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was stained using 1% Amido black, and the selected bands were excised using a clean razor blade. The amino acid sequence was determined by automated Edman degradation (J. Pohl, Microchemical and Proteomics Facility, Emory University, Atlanta, GA).

Kinetic data analysis

Data were expressed as the double-reciprocal plots. Nonlinear regression analysis was performed with the kinetic software package Aca-Stat 5.1.25.

Results

Localization of the CFOR

Lysates prepared from cells grown on fumarate exhibited similar Fe^{3+} reduction rates, using the ferrozine assay, to cells grown on Fe^{3+} citrate, as has been previously described (Kaufmann and Lovley 2001). When the cell lysate was partitioned into soluble and membrane fractions, Fe^{3+} reduction activity was found to be equally localized to both fractions (Gavrilov et al. 2003, in press). The activity in the soluble fraction was chosen for further study.

Purification of the CFOR

Oxygen did not serve as a substrate (i.e., no NAD(P)H-O_2 activity), and NAD(P)H-dependent Fe^{3+} reduction activity was not inactivated by exposure to oxygen, i.e., the activity was equivalent whether purification occurred under aerobic or anaerobic conditions, as has also been previously observed with the strict anaerobe *Shewanella putrefaciens* strain MR-1 (Myers and Myers 1993a). Therefore, purification was performed under aerobic conditions. In addition, theactivity was stimulated by the addition of FAD. Therefore, purification of the CFOR was performed in the presence of 10 μM FAD (see Materials and methods). A three-step, chromatographic procedure (Table 6.1.) was then used to purify the CFOR to apparent, gel-electrophoretic purity with an enzymatic yield of ~25% and a specific activity of 560 mU $\cdot$ mg $^{-1}$ protein.

Physical and biochemical characteristics

The native molecular mass of the CFOR was determined to be ~200 kDa by gel filtration and native, gradient (4-16%) PAGE. SDS-PAGE analysis showed a single subunit with a relative molecular mass of ~45 kDa. Taken together, these data suggest an enzyme oligomer, possibly a tetramer or (less likely) a pentamer.

ICP-MS of the purified CFOR, which was dialyzed against distilled water to remove any cofactors not tightly associated, yielded an Fe content of 0.96±0.04 Fe per 45 kDa subunit of enzyme. Furthermore, an Fe content of 0.98±0.05 Fe per 45 kDa subunit was determined by the use of ferrozine to calculate the iron content spectrophotometrically (Fish 1988). HPLC analysis of the purified CFOR after perchloric acid extraction indicated each 45 kDa subunit contained 0.9±0.09 FAD.

The CFOR exhibited maximal activity around a pH of 6.5 and a temperature of 50ºC (Fig. 6.3.A. and 6.3.B., respectively). EDTA inhibited enzymatic activity by 25, 60, and 75% at 50, 100, and 1000 μM, respectively. NTA inhibited enzymatic activity by 25 and 50% at 10 and 100 μM, respectively. When added to the ferrozine assay (measuring rate of Fe(III) reduction), nickel ($NiCl_2 \cdot 6H_2O$) inhibited enzymatic iron reduction activity by 60% at 100 μM. The addition of 100 and 1000 μM manganese (MnO_2) increased enzymatic activity by 60 and 100%, respectively. The addition of chromium (Na_2CrO_4), selenium (Na_2SeO_3), cobalt (Co-EDTA), copper ($CuSO_4 \cdot 5H_2O$), and zinc ($ZnSO_4 \cdot 7H_2O$) [up to 1 mM of each tested] had no effect on enzymatic activity.

Enzymatic activities and kinetic studies

The CFOR reduced Cr^{6+}, 1,2-naphthoquinone and AQDS at rates comparable to or greater than the rate of Fe^{3+} reduction. Exogenous FAD resulted in higher activities, e.g., the Fe^{3+} reduction rate was 560 mU $\cdot$ mg^{-1} protein with 5 μM FAD, and 34 mU $\cdot$ mg^{-1} protein when exogenous FAD was absent. The CFOR reduced FAD when other substrates were absent but only at a rate of 14 mU $\cdot$ mg^{-1} protein. As a control, the abiotic Fe^{3+} reduction rate with chemically reduced FAD, i.e., no CFOR present, was determined. The Fe^{3+} reduction rate with 0.5 μM of reduced FAD, and no CFOR, in the assay was calculated as 0.28 mU, as compared to 2.8 mU with no reduced FAD and 5.0 μg NQO1, i.e., the ferrozine assay as described above. Other quinones and metals were also reduced but at lower rates than the above described activities (Table 6.2.).

Kinetic studies were performed using a three substrate, steady-state analysis according to the procedure of Segel (1975). Briefly, initial velocity studies were carried out by varying the concentrations of the electron acceptor, (i.e., Fe^{3+}, Cr^{6+}, or AQDS), FAD, and NADH.

(Onyenwoke and Wiegel, submitted; Chapter 7, this dissertation). As illustrated in Figs. 6.4.-6.6., the double-reciprocal plots with the patterns of lines intersecting the x-axis to the left of the y-axis were used for calculations of apparent Michaelis-Menten constants (K_m^{app}) and apparent maximum velocities (V_{max}^{app}). The determined parameters [K_m^{app}, V_{max}^{app}, and turnover number (k_{cat})] for the enzyme during the reduction of Fe^{3+}, as well as Cr^{6+} and AQDS, are shown in Table 6.3.

The best fit plot for NADH saturation in the presence of various concentrations of Fe^{3+} citrate yields an intersection at a point close to the x-axis (data not shown). The inverse plot, i.e., Fe^{3+} citrate saturation in the presence of a series of NADH concentrations, (Fig. 6.4.) also, roughly, intersects the x-axis at a single point. This data suggests Fe^{3+} citrate and NADH are binding independently [random sequential in the nomenclature of Cleland (1977)]. A similar result was obtained on examination of the effect of FAD with a series of [Fe^{3+} citrate], i.e., based on interpretation of these kinetic data FAD and Fe^{3+} citrate also bind independently of each other (data not shown). Figs. 6.5. and 6.6. show the best fit plots for AQDS and Cr^{6+} saturation, respectively, in the presence of various concentrations of NADH. Again the plots are indicative of a sequential reaction mechanism, though the plot for Cr^{6+} saturation indicates an ordered sequential mechanism (Cleland 1977). In order to obtain some further data on the proposed reaction mechanism (and to possibly determine if substrate binding and product release are random or ordered if the mechanism is indeed sequential), the product (ferrous sulfate, $FADH_2$, and NAD^+) inhibition patterns of the CFOR were analyzed with respect to various concentrations of the substrates (Fe^{3+} citrate and NADH) and FAD.

NAD^+ was found to be a mixed type inhibitor [properties of both competitive and uncompetitive inhibition] versus NADH (Fig. 6.7.A.) with an apparent $K_I' = K_I$ when Fe^{3+} citrate

was at a sub-saturating level (100 nM). The plot of the effect of Fe^{2+} sulfate versus Fe^{3+} citrate indicated uncompetitive inhibition, based on the best-fit plot/line from the data (Fig. 6.7.C.), when NADH was present at a sub-saturating level (50 μM). The plots of NAD^+ versus NADH and Fe^{2+} sulfate versus Fe^{3+} citrate indicate the reaction mechanism is specifically ordered sequential (Segel 1975), with further data necessary to determine the specific reaction mechanism according to the nomenclature of Cleland. Increasing $[FADH_2]$ versus FAD suggested the occurrence of activation instead of inhibition (Fig. 6.7.B.).

Quinone analysis and mechanism of quinone reduction

The neutral lipid fraction of *C. ferrireducens* contained the following respiratory quinones (mol%): ubiquinone (UQ)-8 (58) as the major compound, UQ-9 (4.6), UQ-7 (3.5), menaquinone (MK)-8 (15), demethylmenaquinone (DMK)-9 (12), MK-5 (3.4), and MK-4 (1.7) as minor compounds. The minor quinones, except UQ-9, were also found in the control (culture media) and could derive from passive accumulation in the membrane (Geyer, R., unpublished results).

When compared to the controls, i.e., guinea pig zeta-crystallin protein (single electron mechanism) and human NAD(P)H:quinone oxidoreductase (two electron mechanism), the CFOR exhibited a two electron mechanism for quinone reduction.

Amino acid sequence analysis of the enzyme

The N-terminal sequence of the CFOR as determined by automated Edman degradation was Met-Asn-Lys-Tyr-Val-His-Ala-Val-Pro-Asn-Phe.

This sequence was used to search databases [SwissProt/TrEMBL (http://us.expasy.org/sprot/) and GenBank (http://www.ncbi.nlm.nih.gov/)] for similar proteins

by using the BLAST algorithm (Altschul et al. 1990). The N-terminal sequence showed 100 and 72% identity to the electronically annotated: glutamate formiminotransferase of *Carboxydothermus hydrogenoformans* [305 amino acids with a predicted mol. mass of ~34 kDa] (Wu et al. 2005) and phosphotransferase EII fragment of *Mycoplasma capricolum* [118 amino acids with a predicted mol. mass of ~13 kDa] (Bork et al. 1995), respectively.

Discussion

At least two *C. ferrireducens* enzymes are capable of NAD(P)H-dependent Fe^{3+} reduction, one localized to the soluble protein fraction (the CFOR) and the second to the membrane fraction. The relationship between the two enzymes is currently not understood, but the two enzymes might not act independently of each other. An assimilatory (possibly soluble) Fe^{3+} reductase could attain a secondary (dissimilatory) function simply by adding the ability to couple Fe^{3+} reduction to the respiratory chain through a second (possibly membrane-bound) enzyme (Nealson and Saffarini 1994). The NAD(P)H-dependent Fe^{3+} reduction activity localized to the soluble protein fraction was chosen for further study, and an enzyme (the CFOR) was purified and characterized. The CFOR contains FAD and Fe (both at a ratio of ~1 per 45 kDa subunit of enzyme), and its NAD(P)H-dependent Fe^{3+} reduction activity is stimulated by FAD. From the kinetic data a sequential reaction mechanism is indicated and a simple mechanistic model can be proposed (Fig. 6.8.A.1. and 6.8.A.2.). However, due to a broad substrate range, e.g., reduces Fe^{3+}, Cr^{6+}, and AQDS, several possibilities exist for the physiological function of the CFOR.

The CFOR can function as an FAD reductase (Fontecave et al. 1994; Fig. 6.8.B.), as is supported by the $FADH_2$-dependent activation of Fe^{3+} reduction (Fig. 6.7.B.). The $FADH_2$ could then chemically reduce the substrate, e.g., Fe^{3+}, AQDS or Cr^{6+}. However, the determined FAD

reduction rate, i.e., no additional electron acceptor added to the assay, of 14 mU · mg^{-1} protein is significantly below other observed activities (Table 6.2.), and the low observed K_m's for Fe^{3+}, AQDS, and Cr^{6+} suggest a more direct role for the CFOR in their reduction.

The data also suggest that CFOR is a quinone oxidoreductase, as the highest observed activities were with quinones, i.e., AQDS and 1,2-naphthquinone. Thus, the quinone content of *C. ferrireducens* was determined. Interestingly, the major quinone present was a UQ (UQ-8) and not a MK, as would be expected. This is unusual as 1) data from *Escherichia coli* and other *Proteobacteria* and 2) differences in midpoint potentials indicate UQs are typically involved in oxygen and nitrate respiration, whereas MKs are more functional in anaerobic respiration (Polglase et al. 1966; Gennis and Stewart 1996; Sohn et al. 2004; White et al. 2005). However, this scheme may be oversimplified as UQs also function as the electron carriers between *b*-type cytochromes and various terminal oxidases (Sùballe and Poole 1998). UQs have been found as the dominate quinone species in other anaerobic bacteria (White et al. 2005). Still, MKs could be the species involved in anaerobic respiration with the UQs playing some other role.

Apart from their role in respiration, reduced UQs (UQH_2) scavenge lipid peroxyl radicals and thereby prevent a chain reaction of oxidative damage to the polyunsaturated fatty acids of biological membranes, a process known as lipid peroxidation (Forsmark-Andrée et al. 1995). Therefore, the [UQH_2] and other antioxidants, such as vitamin E, present in low-density lipoprotein is of vital importance for the prevention of oxidative damage (cytotoxic effects) and even diseases such as atherosclerosis (Forsmark-Andrée et al. 1995). UQH_2, and other reduced quinone, pools are maintained by soluble quinone oxidoreductases [e.g., DT-diaphorase, also known as NAD(P)H:quinone oxidoreductase, (EC 1.6.99.2); lipoamide dehydrogenase; the quinone oxidoreductases MdaB and ChrR of *Helicobacter pylori* and *Pseudomonas putida*,

respectively; and possibly the CFOR]. These enzymes protect against cytotoxic and carcinogenic effects (Beyer et al. 1996; Siegel et al. 2004; Olsson et al. 1999; Wang and Maier 2004; Gonzalez et al. 2005). Any inhibition of quinone reductase activity results in an increase in free radical damage (Beyer et al. 1996).

Quinone compounds that serve as pro-oxidants also arise from exogenous sources, e.g., plant secreted plumbagin and juglone (Soballe and Poole 1999; Gonzalez et al. 2005). These quinones are highly soluble, and their redox cycling imposes a severe oxidative stress (Soballe and Poole 1999). Semiquinones, which are the result of single electron reductions, are the damaging species (Gonzalez et al. 2005). Enzymes such as DT-diaphorase and the CFOR avert the production of the semiquinone by performing a two electron reduction to produce a less active hydroquinone. This activity protects cells from the reactive semiquinone species (Siegel et al. 2004). Thus, quinone metabolism has a direct effect upon how a cell manages oxidative stress (Soballe and Poole 1999; Wang and Maier 2004).

Possible roles for the CFOR in redox type reactions besides Fe^{3+} reduction have been suggested. In particular, the high activities reported for quinone reduction suggest a role in protection from oxidative stress by maintaining pools of UQH_2. Current evidence does suggest UQ's are antioxidants (Abhilashkumar et al. 2001; White et al. 2005). However, the CFOR is also an FAD reductase. The production of reduced FAD, which does reduce Fe^{3+}, could be the explanation for the broad substrate specificity exhibited by the enzyme (Table 6.2.). Some role in respiration is also plausible, but less likely as membrane-localized enzymes, such as the membrane-bound Fe^{3+}-reducing enzyme mentioned here, would be expected to fulfill this function.

Acknowledgements

Table 6.1. Purification of the CFOR. [a]Assays contained 50 µM NADH, 5 µM FAD, and 100 nM Fe^{3+} citrate.

Purification step	Amt of protein (mg)	Total activity[a] (mU)	Sp act (mU · mg^{-1})	Purification factor (fold)	Yield (%)
Soluble fraction	680	10,200	15	1	100
Q-Sepharose	99	7920	80	5	78
Blue Sepharose	13.5	7020	520	35	69
Sephacryl S-300 HR	4.5	2520	560	37	25

Table 6.2. Enzymatic activities associated with the CFOR. [a]Assays contained 50 µM NADH and 5 µM FAD and are the representative values of duplicate samples. [b]Represents the ratio of activity to the Fe^{3+} reductase activity.

NAD(P)H-dependent enzymatic activity (substrate is listed)	Avg sp act[a] (mU · mg^{-1})	Relative activity[b]
AQDS reduction	2100±50	3.8
1,2-naphthquinone reduction	870±80	1.6
Cr^{6+} reduction	650±30	1.2
Fe^{3+} reduction	560±55	1.0
2-hydroxy-1,4-naphthquinone reduction	300±32	0.54
duroquinone reduction	250±70	0.45
As^{5+} reduction	210±65	0.38
Fumarate reduction	210±25	0.38
Cu^{2+} reduction	180±20	0.32
1,4-naphthquinone reduction	180±14	0.32
Co^{3+} reduction	150±16	0.27
Mn^{4+} reduction	130±55	0.23
benzoquinone reduction	130±30	0.23
menadione reduction	110±28	0.20
Se^{6+} reduction	none detected	0

Table 6.3. Kinetic parameters of the CFOR. [a] k_{cat}'s (turnover numbers) were calculated by determining the amount of CFOR, based upon the molecular weight of the monomer (45 kDa) and assuming one catalytic site per CFOR monomer, present in the reaction mixture. The k_{cat}'s were then determined from the V_{max}^{app} values.

		K_m^{app} (µM)	V_{max}^{app} (mU · mg^{-1} protein)	k_{cat}^a (s^{-1})
Fe^{3+} reduction	Fe^{3+}	0.014	480	0.36
	FAD	1.2	230	0.18
	NADH	62	310	0.25
Cr^{6+} reduction	Cr^{6+}	0.6	10,000	7.5
	FAD	2.0	3300	2.5
	NADH	76	3300	2.5
AQDS reduction	AQDS	0.025	2500	1.9
	FAD	5.8	2500	1.9
	NADH	26	3300	2.5

Fig. 6.1. Proposed model in which an electron shuttle serves to reduce insoluble Fe^{3+} oxides. "Q" represents any electron shuttle, e.g., the quinone analog AQDS. "Q" is the oxidized form of the shuttle while "QH_2" is the reduced form. The shuttle passes into the cytoplasm of the dissimilatory iron-reducer and is reduced. The reduced shuttle carries the electrons outside to the insoluble Fe^{3+} source where the Fe^{3+} is reduced to Fe^{2+}.
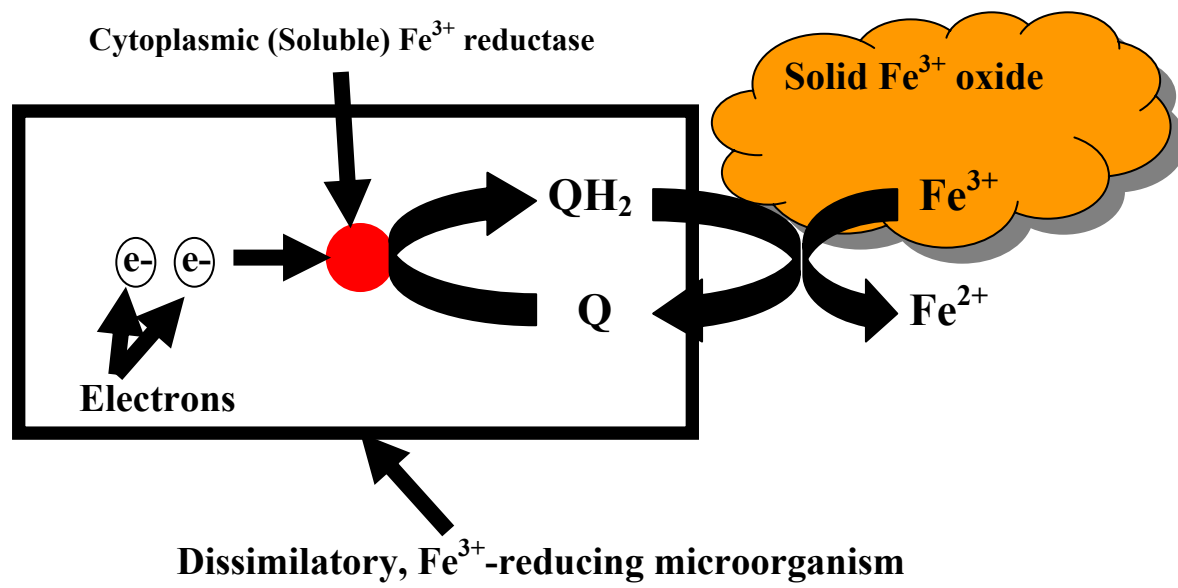
Cytoplasmic (Soluble) Fe^{3+} reductase

Solid Fe^{3+} oxide

QH$_2$

Fe^{3+}

e- e-

Q

Fe^{2+}

Electrons

Dissimilatory, Fe^{3+}-reducing microorganism

Fig. 6.2. Time course showing the linearity of the NAD(P)H-dependent Fe^{3+} reduction activity of the crude *C. ferrireducens* soluble (cytoplasmic) protein fraction. The assay was performed using the ferrozine method and contained: 50 µg of protein, 5 µM FAD, 0.05 mM NADH or NADPH, and 100 µM Fe^{3+} citrate.
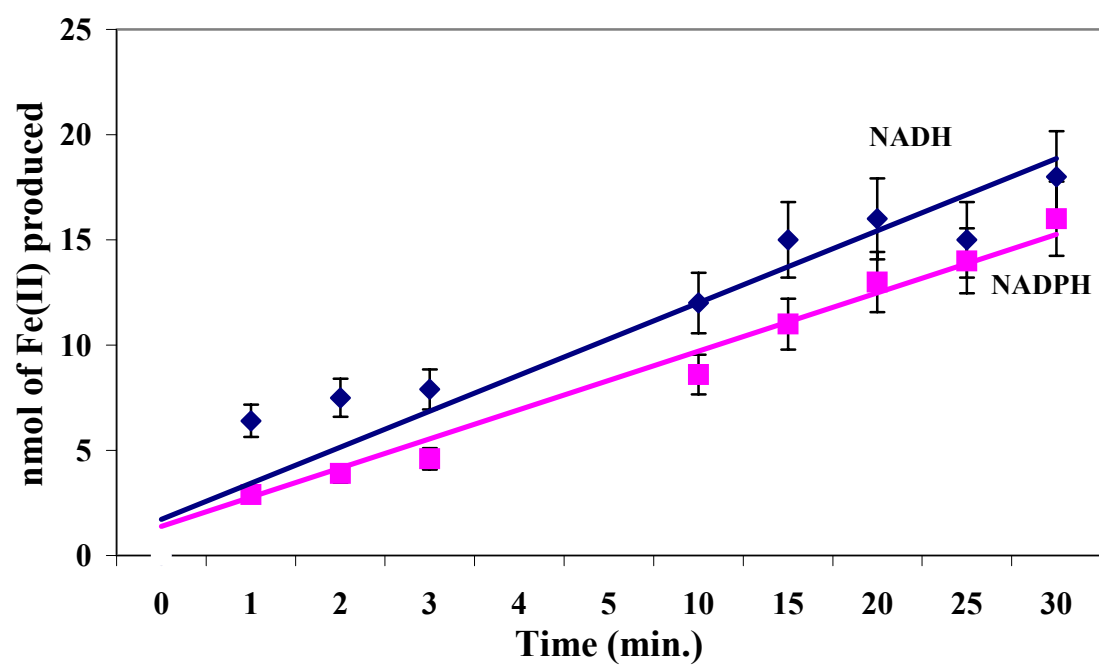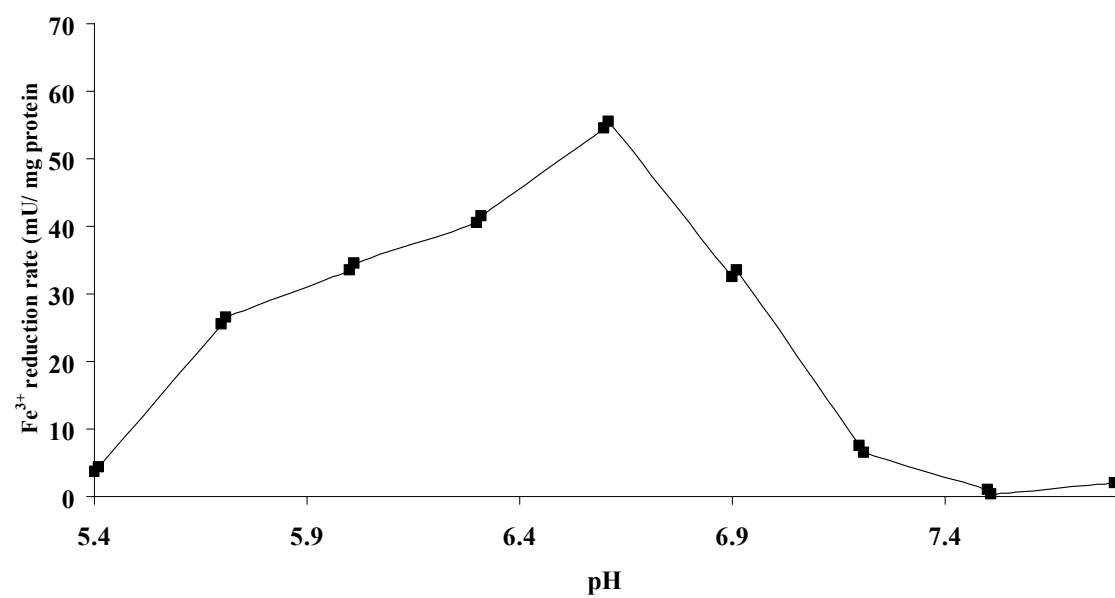
Fig. 6.3. The effects of pH **(A)** and temperature **(B)** on the NAD(P)H-dependent Fe^{3+} reduction activity of the CFOR. Assay mixtures contained: 2.5 μg of protein, 5 μM FAD, 0.05 mM NADH, and 100 nM Fe^{3+} citrate.
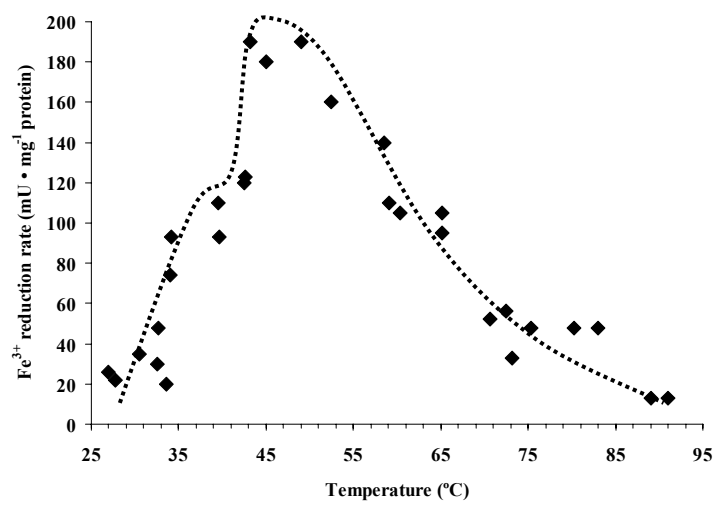
A

B

Fig. 6.4. Initial velocity kinetics of the Fe^{3+} reduction activity of the CFOR. All data are represented as the double- reciprocal plots. The effect of [Fe^{3+} citrate] on activity. Measurements represent a combined replot for three, independent series with NADH as the constant substrate. Assays followed the oxidation of NADH and contained: 2.5 μg of protein and 5 μM FAD.
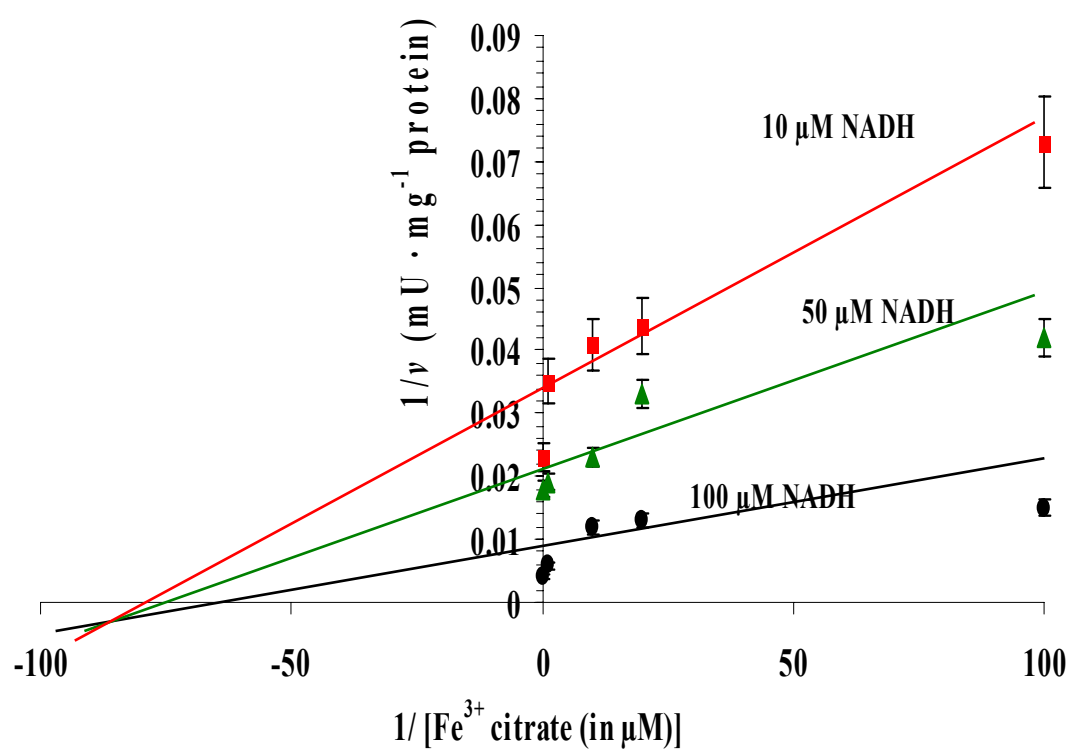
Fig. 6.5. The effect of [AQDS] on the AQDS reduction activity of the CFOR. Measurements represent a combined replot for three, independent series with NADH as the constant substrate. Assays followed the oxidation of NADH and contained: 2.5 µg of protein and 5 µM FAD.
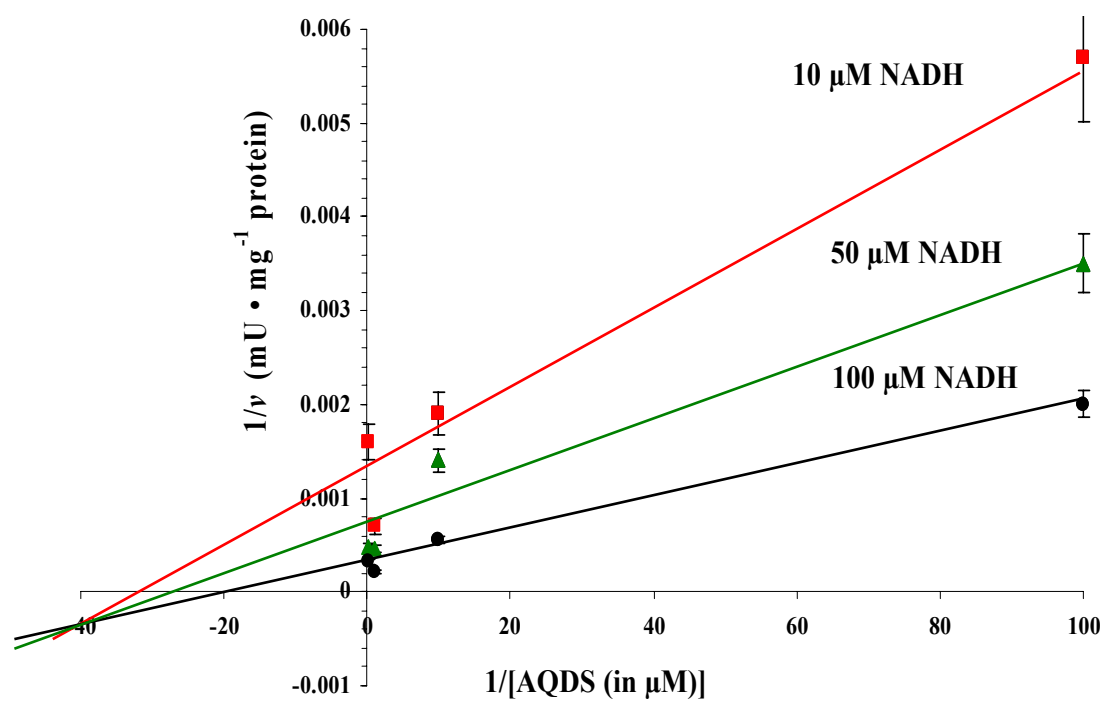
Fig. 6.6. The effect of $[Cr^{6+}]$ on the Cr^{6+} reduction activity of the CFOR. Measurements represent a combined replot for three, independent series with NADH as the constant substrate. Assays followed the oxidation of NADH and contained: 2.5 μg of protein and 5 μM FAD.
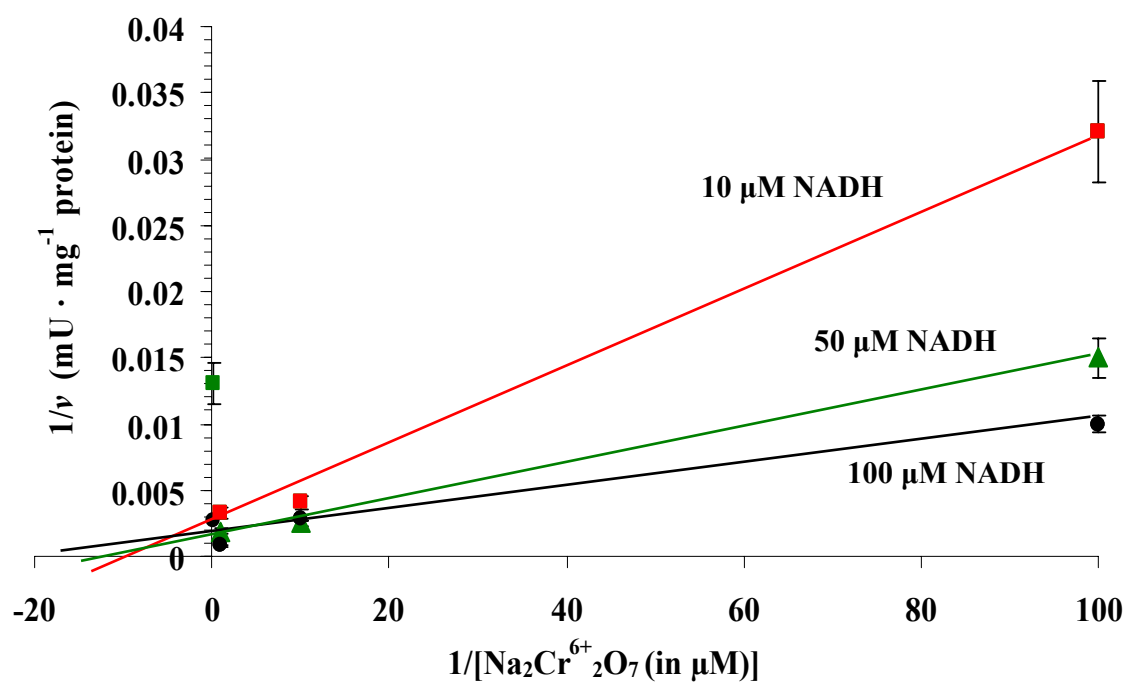
Fig. 6.7. Product inhibition patterns for Fe^{3+} reduction by the CFOR. **(A)** NAD^+ concentration kept constant with 100 nM Fe^{3+} citrate, 5.0 μM FAD, and 0-50 μM NADH. **(B)** $FADH_2$ concentration kept constant with 100 nM Fe^{3+} citrate, 50 μM NADH, and 0-50 μM FAD. **(C)** Fe^{2+} sulfate heptahydrate concentration kept constant with 50 μM NADH, 5 μM FAD, and 0-10 μM Fe^{3+} citrate. Assays were performed by observing NADH oxidation.
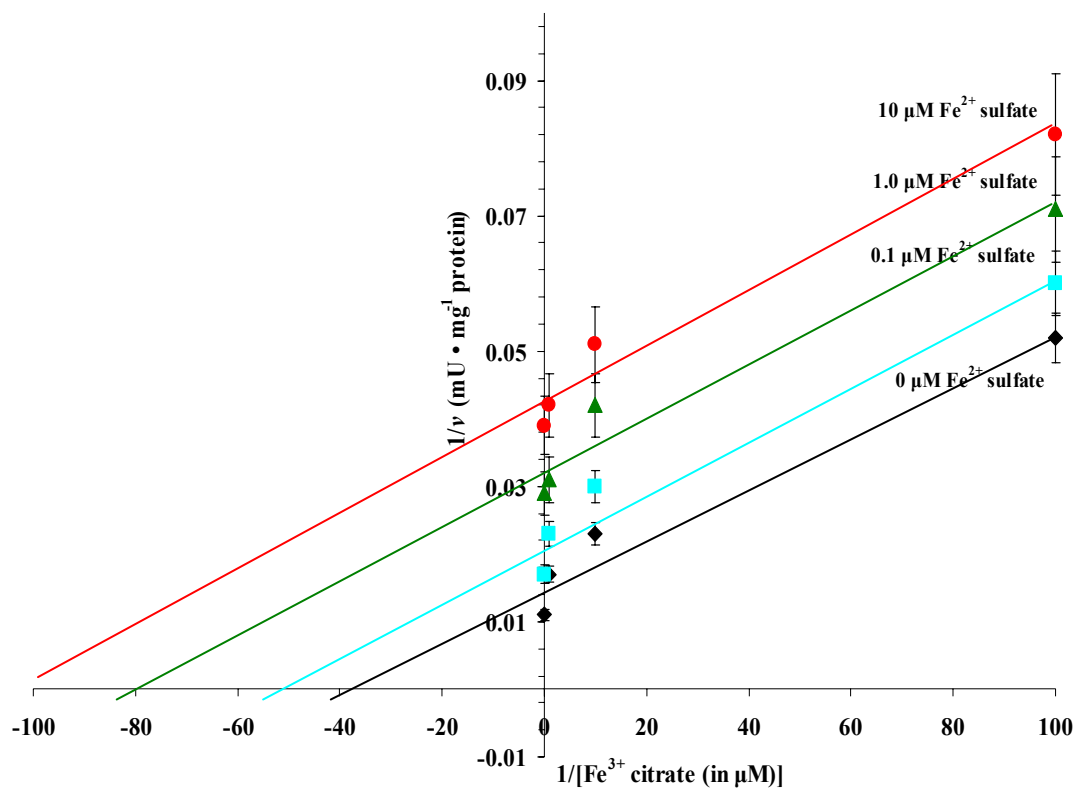
A
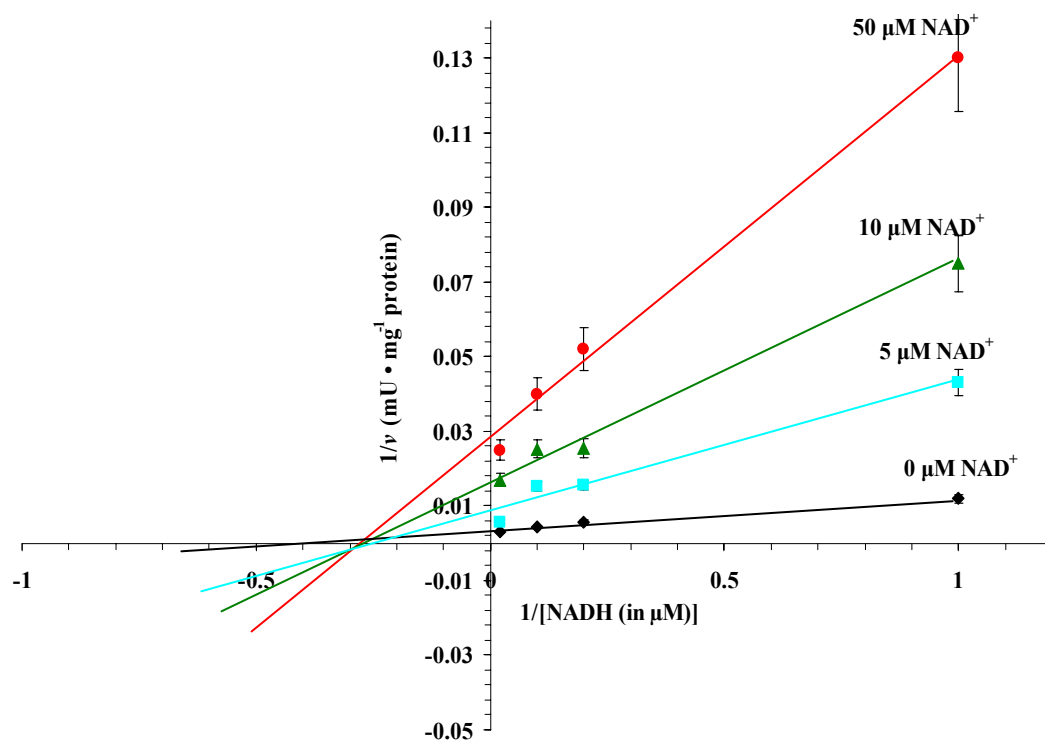
B
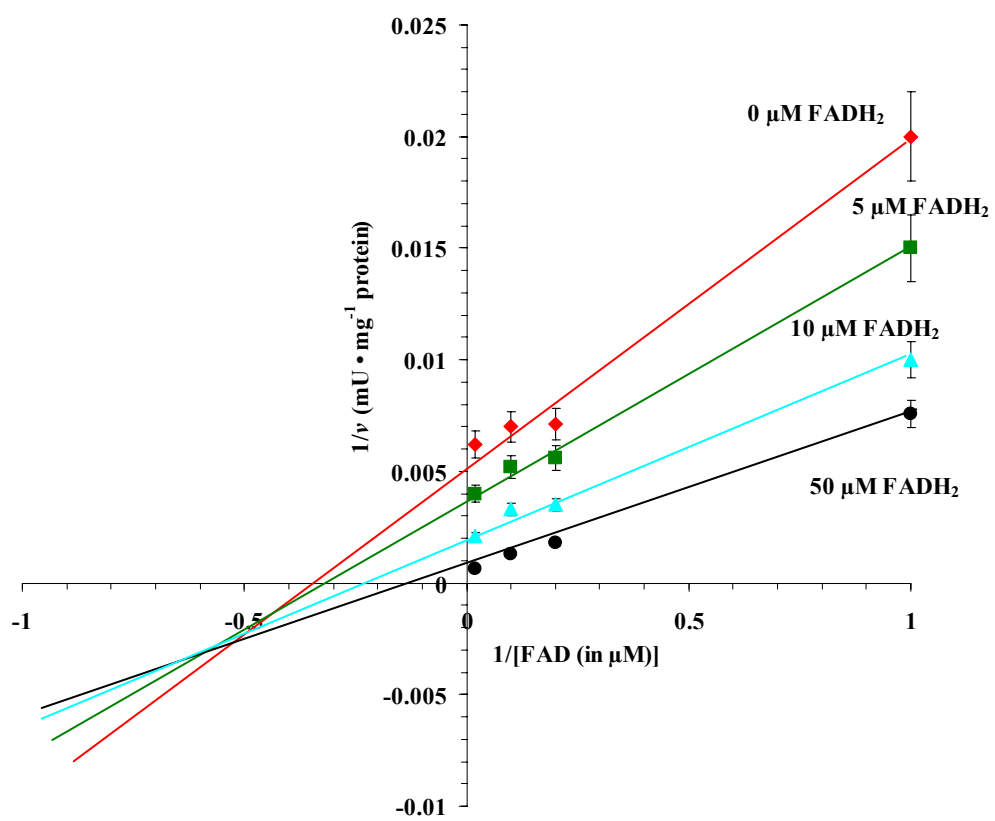
C

Fig. 6.8. Proposed mechanisms of substrate reduction by the CFOR. "X" refers to the substrate, e.g., Fe^{3+}, Cr^{6+}, or AQDS, reduced (X^{red}) or oxidized (X^{ox}). **(A)** 1. Substrate is reduced via the oxidation of NADH with the enzyme bound FAD (yellow) serving as a cofactor, i.e., electron mediator. Bound Fe (pink) is also present in the enzyme. 2. Exogenous FAD increases enzymatic activity (indicated by thicker arrows) and possibly occupies a secondary site on the CFOR. **(B)** Alternatively, exogenous FAD increases activity because the CFOR additionally functions as a flavin reductase. The exogenous FAD is reduced by the CFOR. The reduced FAD in turn reduces the substrate "X".

A1
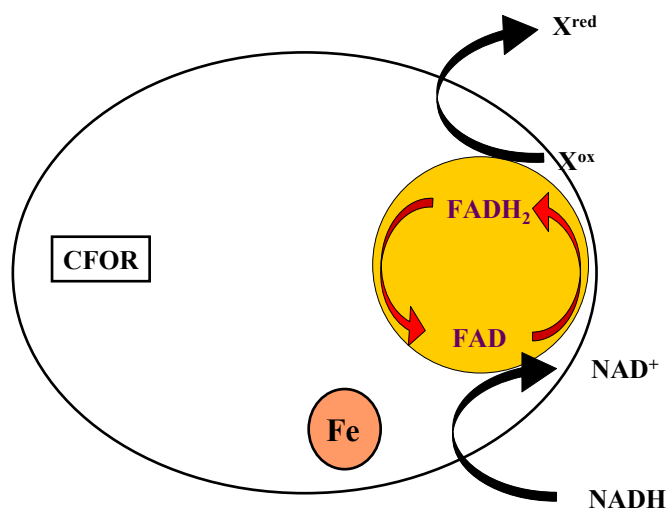
A2
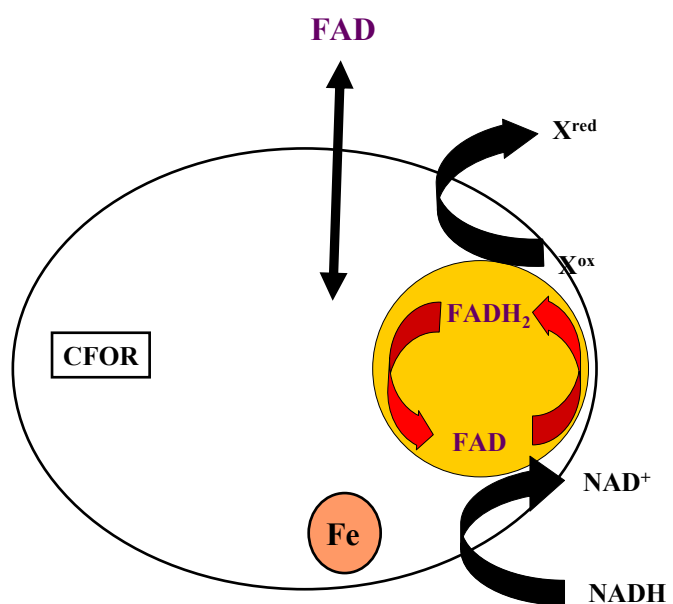
B
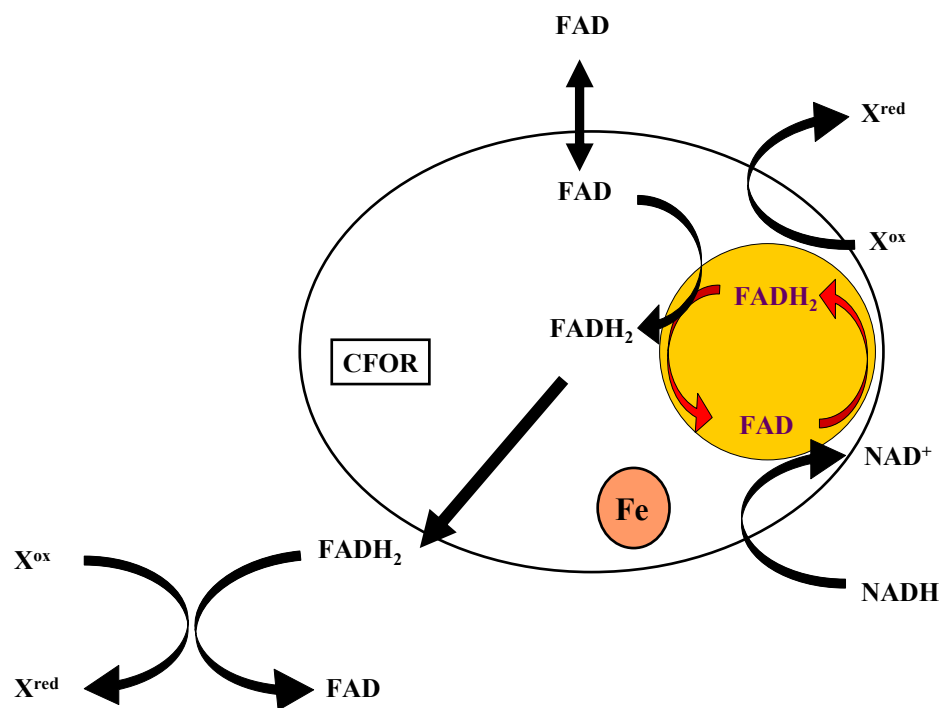
CHAPTER 7

IRON (III) REDUCTION: A NOVEL ACTIVITY OF THE HUMAN NAD(P)H

OXIDOREDUCTASE[1]

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[1]Onyenwoke, R. U., and J. Wiegel. To be submitted to *FEBS Journal*.

Abstract

NAD(P)H:quinone oxidoreductase (NQO1; EC 1.6.99.2) catalyzes a two electron transfer involved in the protection of cells from reactive oxygen species. These reactive oxygen species are often generated by the one electron reduction of quinones or quinone analogs. We report here on the previously unreported Fe(III) reduction activity of human NQO1. Under steady state conditions with Fe(III) citrate, the apparent Michaelis-Menten constant (K_m^{app}) was ~ 0.3 nM and the apparent maximum velocity (V_{max}^{app}) was 16 U · mg^{-1}. Substrate inhibition was observed above 5 nM. NADH was the electron donor, K_m^{app} = 340 μM and V_{max}^{app} = 46 U · mg^{-1}. FAD was also a cofactor with a K_m^{app} of 3.1 μM and V_{max}^{app} of 89 U · mg^{-1}. The turnover number for NADH oxidation was 25 s^{-1}. Possible physiological roles of the Fe(III) reduction by this enzyme are discussed.

Introduction

The enzymatic reduction of quinones has been documented in the literature as early as 1954 (Martius 1954, 1960, 1961; Martius and Nitz –Litzow 1954; Maerkif and Martius 1960, 1961; Ernster and Navazio 1958; Ernster et al. 1962; Hosoda et al. 1974; Ross 2004), with the majority of this work focusing on NAD(P)H:quinone oxidoreductase 1 (NQO1), known historically as DT-diaphorase for its unspecific reactivity with both DPNH and TPNH, i.e., NADH and NADPH (Ernster 1958, 1967, 1987). NQO1 is described as a tightly associated physiological dimer of identical subunits, each comprising 273 amino acids (Faig et al. 2000; Figure 7.1.). NQO1 is a flavoprotein with each monomer containing one (1) catalytically essential, non-covalently bound FAD prosthetic group (Smith et al. 1988, Faig et al. 2000, Cavelier et al. 2001). It is also known that the addition of 0.2-0.5 μM FAD stimulates menadione reduction 1.5-1.7

fold by a mechanism which is presently not understood (Smith et al. 1988). NQO1 occurs widely (Ernster et al. 1962; Prochaska and Talalay 1986; Smith et al. 1988; Prochaska 1988). Typically, it catalyzes a two electron transfer (Iyanagi and Yamazaki 1969, 1970; Powis and Appel 1980). This two electron transfer protects cells from reactive oxygen species formed when the products of one electron transfers, e.g., semiquinones, are produced in the presence of oxygen (Ross 2004). NQO1 has a broad substrate specificity, various quinones and quinone analogs, azo dyes, superoxide, and other electron acceptors are reduced (Ernster and Navazio 1958; Cui et al. 1995; Siegel et al. 2004).

Based on work with rat liver mitochondrial fractions, the reduction of hexavalent chromium [Cr(VI)] is one of the activities ascribed to NQO1 (Petrilli and de Flora 1988). Presumably, the enzyme a) carries out multiple one electron reductions, or b) performs a simultaneous reduction of two Cr(VI) to Cr(V), i.e., the typical two electron transfer model, to alleviate Cr(VI) toxicity (Aiyar et al. 1992). Cr(VI) compounds are noted for 1) their mobility in the environment (solubility), and 2) their DNA-damaging and cell-transforming effects (Levis and Bianchi 1982; Petrilli and de Flora 1982; Viamajala et al. 2004). They are human carcinogens (Yassi and Nieboer 1988; Hayes 1997). Reduced Cr is less mobile (less soluble) and, thus, less toxic. Presumably it is unable to cross membranes (Levis and Bianchi 1982; Langard 1982). Reduced Cr is even reported to be an essential nutrient (Cary 1982; Stearns et al. 1995).

NQO1 is one of the key enzymes in cellular chemoprotection. It is a highly promiscuous enzyme that reduces a large number of substrates. The importance and versatility of NQO1 makes the study of its substrate specificity important. We have recently investigated the involvement of NQO1 with the reduction of the key transition metal iron [Fe(III)]. To our

knowledge, Fe(III) has not been reported as a substrate for NQO1. Iron is one of the most abundant elements on earth and is incorporated into numerous proteins that bind oxygen or are involved in electron transfer reactions (Andrews et al. 1999). Iron reduction and oxidation is an important mediator of oxidative damage via Fenton chemistry [reaction **A**] (Zigler et al. 1985; Halliwell and Gutteridge 1984a, 1984b, 1992; Gutteridge and Halliwell 2000).

$$O_2^{\bullet-} + Fe(III) \quad \rightarrow \quad Fe(II) + O_2$$

$$2O_2^{\bullet-} + 2H^+ \quad \rightarrow \quad H_2O_2 + O_2 \qquad\qquad \textbf{(A)}$$

$$H_2O_2 + Fe(II) \quad \rightarrow \quad OH^- + {}^{\bullet}OH + Fe(III)$$
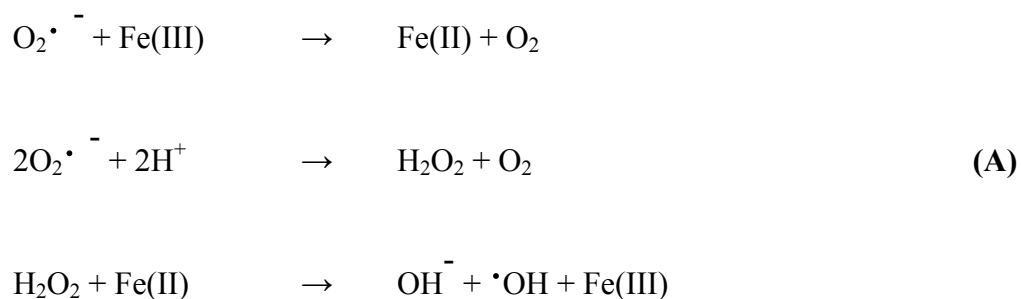
Here we report on some of the steady state kinetic properties of the human NQO1 and demonstrate the enzymatic reduction of Fe(III). The exact implications of an iron reduction activity by NQO1 are currently unclear.

Experimental procedures

Materials

β-NADH, NAD$^+$, FAD, ferric citrate, ferrous sulfate heptahydrate, and ferrozine (3- (2-pyridyl)-5,6-bis (4- phenyl-sulfonic acid)- 1,2,4-triazine) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Tris-HCl was purchased from J.T. Baker, Phillipsburg, NJ, USA. A 1 mM stock of FADH$_2$ was prepared by adding solid sodium dithionite to FAD and following the loss

of absorbance at 450 nm, i.e., the reduction of FAD to $FADH_2$ (Louie et al. 2003). Recombinant human (rh)NQO1, purified from *Escherichia coli* (Beall et al. 1994), was the generous gift of Dr. David Siegel, University of Colorado Health Sciences Center, Denver, Colorado, USA. The purified NQO1 was at a final protein concentration of 3.3 mg/ml in 25 mM Tris-HCL (pH 7.4) containing 250 mM sucrose and 5 µM FAD.

Enzyme Activity Assays

Unless otherwise noted, all enzyme assays were performed in triplicate under anaerobic conditions using N_2 sparged Hungate tubes (Ljungdahl and Wiegel 1986) at 37°C for 15 min. Data indicated ~15% of the substrate [Fe(III)] had been consumed in 15 min. Reduction of Fe(III) to Fe(II) was monitored by the use of the Fe(II) capture reagent ferrozine at 562 nm [molar extinction coefficient (ε_{562}) is 27.9 $mM^{-1}cm^{-1}$ (Dailey and Lascelles 1977)] and by measuring the rate of NADH oxidation at 340 nm [$\varepsilon_{340} = 6.22$ $mM^{-1}cm^{-1}$ (Ernster et al. 1962)] both on a Beckman DU-64 spectrophotometer. However, due to its higher sensitivity, the ferrozine assay was the only method employed when the concentrations of Fe(III) in the assay were below 0.1 nM. The reaction mixture (final volume 1.0 ml (pH 7.4) contained: 0.1 M Tris-HCl, 0.05-0.5 mM NADH, 0.5-10 µM FAD, 0.5 mM ferrozine (when required), 0.025 nM-100 µM Fe(III) citrate, and 0.5-2.5 µg of rhNQO1. Product inhibition studies were performed in the reaction mixture using 1) 0-50 µM NAD^+ with 0.5 nM Fe(III) citrate, 5.0 µM FAD, and 0-50 µM NADH; 2) 0-50 µM $FADH_2$ with 0.5 nM Fe(III) citrate, 50 µM NADH, and 0-50 µM FAD; or 3) 0-1 nM Fe(II) sulfate heptahydrate with 50 µM NADH and 5 µM FAD, and 0-1 nM Fe(III) citrate. Reactions were initiated by the addition of rhNQO1.

Kinetic data analysis

Data were expressed as the double-reciprocal plots. Nonlinear regression analysis was performed with the kinetic software package Aca-Stat 5.1.25.

Results

The enzyme NQO1 oxidizes NADH (34 ± 3 U · mg^{-1} protein) and reduces Fe(III) to Fe(II) by either a one electron reduction of one Fe(III) or by reducing 2 Fe(III) simultaneously, i.e., a two electron reaction mechanism. As already stated, FAD has been previously shown to stimulate menadione reduction (Smith et al. 1988). Therefore, it was not surprising that exogenous FAD resulted in higher activities, i.e., the Fe(III) reduction rate was 2.0-2.5 fold lower than the above reported velocity without 0.5 µM FAD (Fig. 7.2. and additional data not shown). As controls, the Fe(III) reduction rate with chemically reduced FAD and no NQO1 was determined as well as the rate of FAD reduction by NQO1 [no Fe(III) present]. The Fe(III) reduction rate with 0.5 µM of reduced FAD, and no NQO1, in the assay was 0.28 ± 0.03 mU total activity, as compared to 85 ± 7 mU with no reduced FAD and 2.5 µg NQO1 (i.e., a typical assay as described above). The rate of FAD (0.5 µM) reduction, i.e., no Fe(III) present, by NQO1 was 1.4 ± 0.2 mU total activity with 2.5 µg NQO1. Thus, the kinetic analysis of Fe(III) reduction by NQO1 requires consideration of three compounds: NADH, the complexed Fe(III), and FAD. Steady state kinetic data collected included initial velocity studies with concentrations of Fe(III) citrate, FAD, and NADH. The apparent Michaelis-Menten constant (K_m^{app}) and apparent maximum velocity (V_{max}^{app}) were: NADH ($K_m^{app} = 340$ µM and $V_{max}^{app} = 46$ U · mg^{-1}), FAD ($K_m^{app} = 3.1$ µM and $V_{max}^{app} = 89$ U · mg^{-1}), and Fe(III) citrate ($K_m^{app} = 0.3$ nM, $V_{max}^{app} = 16$ U · mg^{-1}). Furthermore, based on the

molecular weight of monomeric NQO1, a turnover number of 25 s^{-1} was calculated for NADH oxidation.

The steady state kinetic data were then used to investigate the reaction mechanism for the enzyme. The best fit plot (Fig. 7.3.) for Fe(III) citrate saturation in the presence of various NADH concentrations yields an intersection at a point close to the x-axis. This data suggests that Fe(III) citrate and NADH bind independently [random sequential in the nomenclature of Cleland (1977)]. A similar result was obtained on examination of the effect of FAD with a series of [Fe(III) citrate], i.e., based on interpretation of these kinetic data FAD and Fe(III) citrate also bind independently of each other (data not shown). A sequential reaction mechanism is again indicated by the effect of increasing [NADH] when FAD is treated as the variable substrate and increasing [FAD] when NADH is the variable substrate (data not shown).

In order to obtain some further data on this proposed reaction mechanism, and to possibly determine if substrate binding and product release are random or ordered, the product inhibition patterns were analyzed. As expected for a sequential type reaction mechanism, NAD$^+$ was a competitive inhibitor versus NADH (Fig. 7.4.A.) when Fe(III) citrate was at a sub-saturating level (0.5 nM). In contrast Fe(II) sulfate yielded mixed inhibition [properties of both competitive and uncompetitive inhibition] (Fig. 7.4.C.) with an apparent $K_I' < K_I$ (see Fig. 7.5.) when NADH was present at a sub-saturating level (50 μM). These results indicate that the reaction mechanism is specifically random sequential (Segel 1975), but due to the presently unknown role of FAD/FADH$_2$ (i.e., bound as cofactor vs. reversible binding as substrate forming an enzyme-substrate complex) further data is necessary to determine the specific reaction mechanism, e.g., random bi bi, random uni bi, etc. Increasing [FADH$_2$] stimulated rather than inhibited the reaction (Fig. 7.4.B.). Indeed the affinity for FAD is not even significantly altered by differing

concentrations of FADH$_2$ as the double-reciprocal plots converge at a point close to the x-axis (Fig. 7.4.B.).

Discussion

The calculated V_{max}^{app} values listed above for NQO1 are comparable to values obtained for soluble, bacterial metal reductases, e.g., the two Fe(III) reductases of *Paracoccus denitrificans* [FerA; Fe(III)-NTA (K_m = 17 μM) and NADH (K_m = 5.5 μM) with a velocity, or V_{max}, of 20 U · mg^{-1} and FerB; Fe(III)-NTA (K_m = 800 μM) and NADH (K_m = 2.6 μM) with a velocity of 5.6 U · mg^{-1} (Mazoch et al. 2004)], the Fe(III) reductase of *Geobacter sulfurreducens* [Fe(III)-NTA (K_m^{app} = 1 mM) and NADH (K_m^{app} = 25 μM) with a velocity of 65 U · mg^{-1} (Kaufmann and Lovley 2001)], and the Cr(VI) reductase of *Escherichia coli* [NADH; K_m = 17.2 μM and V_{max} = 130.7 U · mg^{-1} (Bae et al. 2005)]. However, the calculated values are significantly lower than the reported V_{max}'s of human NQO1 for menadione reduction [estimated K_m and V_{max} values for NADH and menadione are: 200 μM and 762 U · mg^{-1}, and 3.3 μM and 294 U · mg^{-1}, respectively (Smith et al. 1988)]. However, the human NQO1 exhibited a far higher affinity for Fe(III).

The random sequential reaction mechanism for NQO1 reported here is contrary to 1) work that has been done with human, rat, and mouse NQO1 to study the reaction mechanism of the enzyme through steady state and stopped flow kinetic methods (Hosoda et al. 1974), and 2) the mechanistic model predicted by crystallographic studies (Faig et al. 2000; Cavelier and Amzel 2001; Li et al. 1995; Figs. 7.6.-7.8.). These studies indicated a ping-pong type of reaction mechanism for the binding of NADPH, quinone, and/or the common NQO1 inhibitors dicumarol (Hosoda et al. 1974) or Cibacron Blue (Li et al. 1995; Fig. 7.6.). However, it should be noted that these authors were observing quinone reduction and not Fe(III) reduction. Crystal structures have

indicated the quinone and nicotinamide [NAD(P)$^+$] must occupy the same site (Faig et al. 2000; Cavelier and Amzel 2001; Li et al. 1995; Figs. 7.6.-7.7.). Therefore, the rationale for the ping-pong mechanism has been hydride transfer from NAD(P)H to the enzyme-bound FAD followed by the release of NAD(P)$^+$ and hydride transfer from reduced FAD to the quinone followed by the release of the hydroquinone (Cavelier and Amzel 2001). Interestingly, Sparla et al. (1999) have observed a similar disparity in mechanism for an enzymatic reaction when they reported their work with a NADH-specific, FAD-containing, soluble reductase from maize seedlings capable of Fe(III) citrate reduction. These authors report a sequential mechanism (whether random or ordered was not commented upon by the authors) when Fe(III) citrate was the electron acceptor but a ping-pong reaction mechanism when a quinone was the electron acceptor.

Another mechanistic issue is the activation of NQO1 by reduced FAD. This apparent activation raises yet another possibility that the enzyme could function as an FAD reductase (Fontecave et al. 1994; Fig. 7.9.B.). The FADH$_2$ would then chemically reduce Fe(III), or any of the other numerous substrates NQO1 has been reported to reduce, e.g., quinones and quinone analogs, azo dyes, superoxide, and Cr(VI). It is conceivable FAD occasionally dissociates from the enzyme, as it is non-covalently bound (Smith et al. 1988; Faig et al. 2000; Cavelier and Amzel 2001), and then reduces Fe(III) adventitiously. Even though NQO1 has been well-described as a flavoprotein, it could also have this dual activity as an FAD reductase, i.e., its activity is a combination of flavoprotein oxidoreductase and FAD reductase. When supplied as substrate, the FAD (0.5 μM) reduction rate was calculated as 560±50 mU · mg^{-1} protein. This concentration of FAD and concentrations used in this work (0.5-8.0 μM) are physiologically relevant as estimates of total cellular FAD in human tissue are 0.5-10 μM (Ortega et al. 1999).

Thus, NQO1 is an FAD reductase though this activity is significantly below the value of 34±3 U · mg^{-1} protein calculated for Fe(III) reduction.

Another issue of physiological relevance is substrate inhibition by 5 nM Fe(III) citrate. The fact that Fe(III) citrate becomes a potent inhibitor of Fe(III) reductase activity at elevated concentrations can not presently be explained beyond simple substrate inhibition. However, this inhibition is assumed to be physiologically irrelevant as the human body's pool of free, unbound iron is estimated to be well below 1 nM Fe(III) [complexed Fe^{3+} exists in equilibrium with free Fe^{3+} at $\sim10^{-17}$-10^{-18} M free Fe^{3+} (Dale et al. 2004)], a reason for the use of a multitude of relatively low Fe(III) citrate concentrations in the present study (Figs. 7.2., 7.3.; see Materials and Methods).

Finally, to address the physiological role of this enzymatic reduction of Fe(III), a role of this enzyme in the prevention of iron-mediated oxidative damage by superoxide (O$_2\cdot$) (Gutteridge and Halliwell 2000) seem possible despite the subsequent formation of the hydroxyl radical ($\cdot$OH). Dicker and Cederbaum (1993) showed the hydroxyl radical ($\cdot$OH) was formed during the reoxidation of the menadione semi(hydro)quinone, which is produced by rat liver cytosol (presumably the rat liver quinone oxidoreductase), only in the presence of a chelated Fe(III) [e.g. Fe(III) EDTA, Fe(III) ATP, or Fe(III) citrate] chemical catalyst, i.e., Fenton chemistry. However, no oxidation of NAD(P)H or reduction of Fe(III) in the absence of the menadione, which is reduced by the rat liver cytosol to produce the menadione semi-(hydro)quinone, was reported (Dicker and Cederbaum 1993).

The conclusion that Fe(III) is not an appropriate direct substrate for an NAD(P)H:quinone oxidoreductase, but rather a chemical catalyst, is in contrast to what has been seen in this present work; namely chelated Fe(III), and also FAD, can be directly enzymatically

reduced by the human NQO1 via the oxidiation of NADH (Fig. 7.9.). Differences in the assay conditions might explain these results, i.e., an anaerobic assay and purified, human oxidoreductase were used in this work while Dicker and Cederbaum (1993) used aerobic conditions and a crude rat liver soluble fraction. Clearly, more work is required. What has been shown here is that the human NAD(P)H:quinone oxidoreductase can function as a) an Fe(III) reductase at a low substrate concentration, i.e., in the 10^{-11} M Fe(III) citrate range, via a predicted sequential reaction mechanism, or b) an FAD reductase, a more likely role based on the available, intracellular concentration of Fe^{3+} ($\sim 10^{-17}$-10^{-18} M). Thus, novel activities have been demonstrated for this enzyme.

Acknowledgements

Fig. 7.1. Schematic representation (ribbon diagram) of the human NQO1 homodimer. The monomers are represented in light green and dark green. Secondary structure numbering is indicated. The bound FAD is shown in one of the two sites. Figure from Faig et al. 2000.
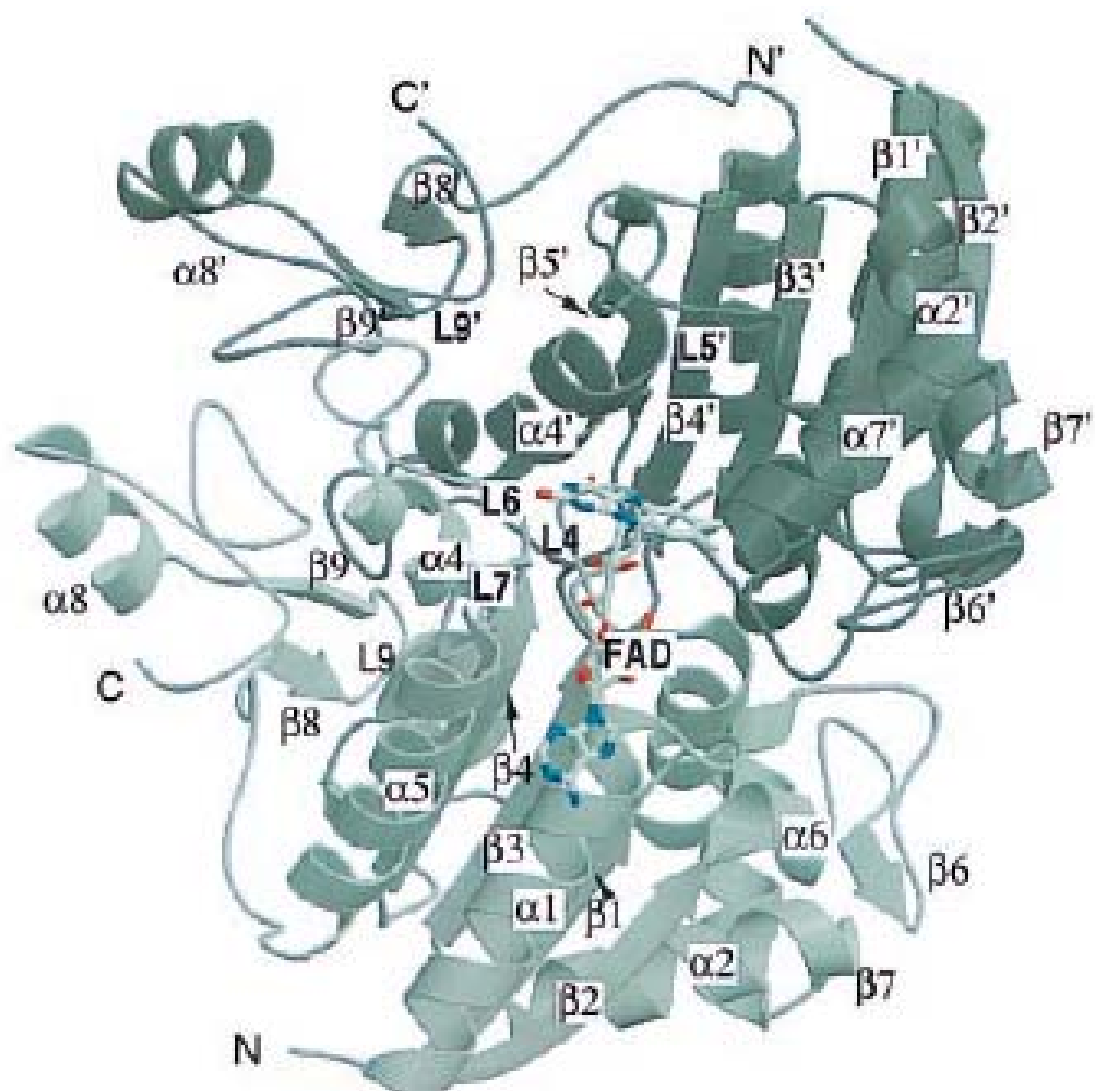
Fig. 7.2. Initial velocity kinetics of the iron reduction activity of human NQO1. All data are represented as the double- reciprocal plots. The effect of [Fe(III) citrate] on the iron reduction activity of human NQO1. Assays included 0.1 mM NADH. Ferrozine assays were employed.
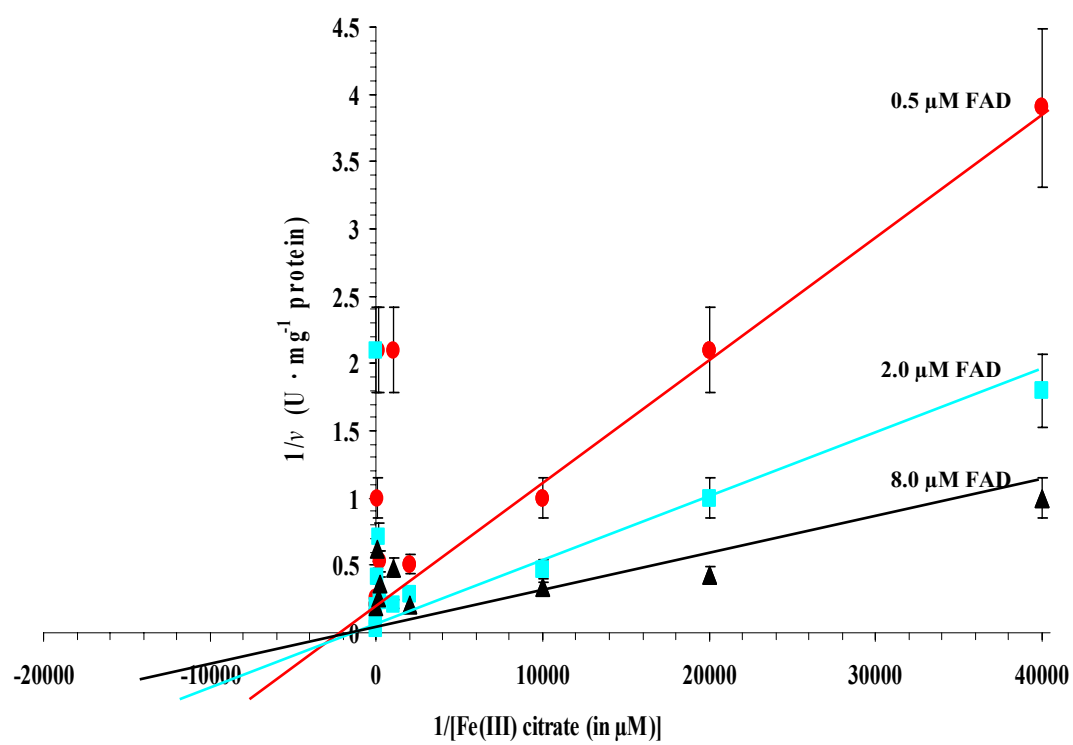
Fig. 7.3. The combined, double reciprocal replot of the effect of [Fe(III) citrate] on the iron reduction activity of human NQO1. Measurements represent a plot for three, independent series with NADH as the constant substrate. Ferrozine assays were employed.
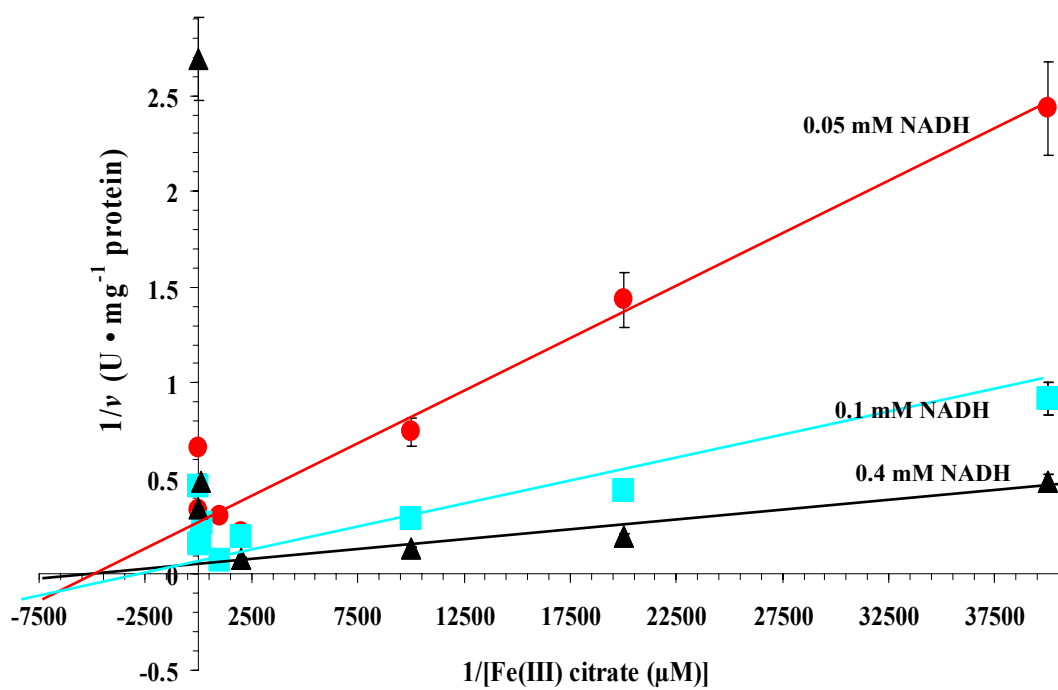
Fig. 7.4. Product inhibition patterns for the reaction catalyzed by human NQO1. **(A)** NAD^+ concentration kept constant with 0.5 nM Fe(III) citrate, 5.0 µM FAD, and 0-50 µM NADH. **(B)** $FADH_2$ concentration kept constant with 0.5 nM Fe(III) citrate, 50 µM NADH, and 0-50 µM FAD. **(C)** Fe(II) sulfate heptahydrate concentration kept constant with 50 µM NADH, 5 µM FAD, and 0-1 nM Fe(III) citrate. Assays followed NADH oxidation.

A
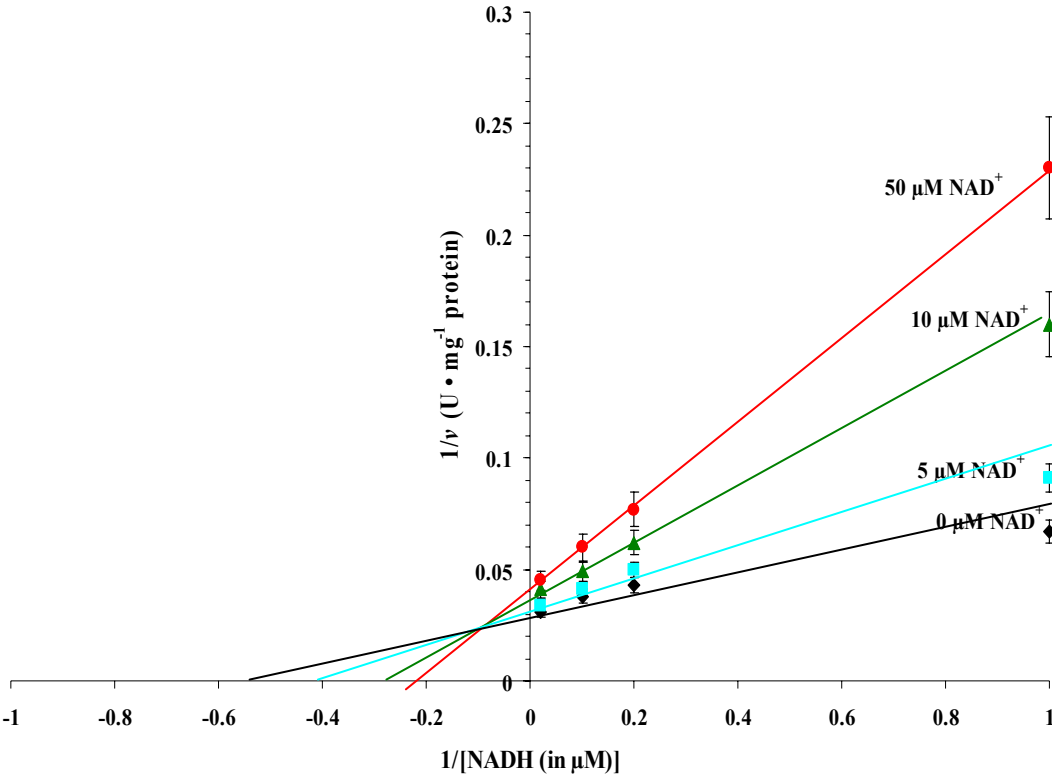
B
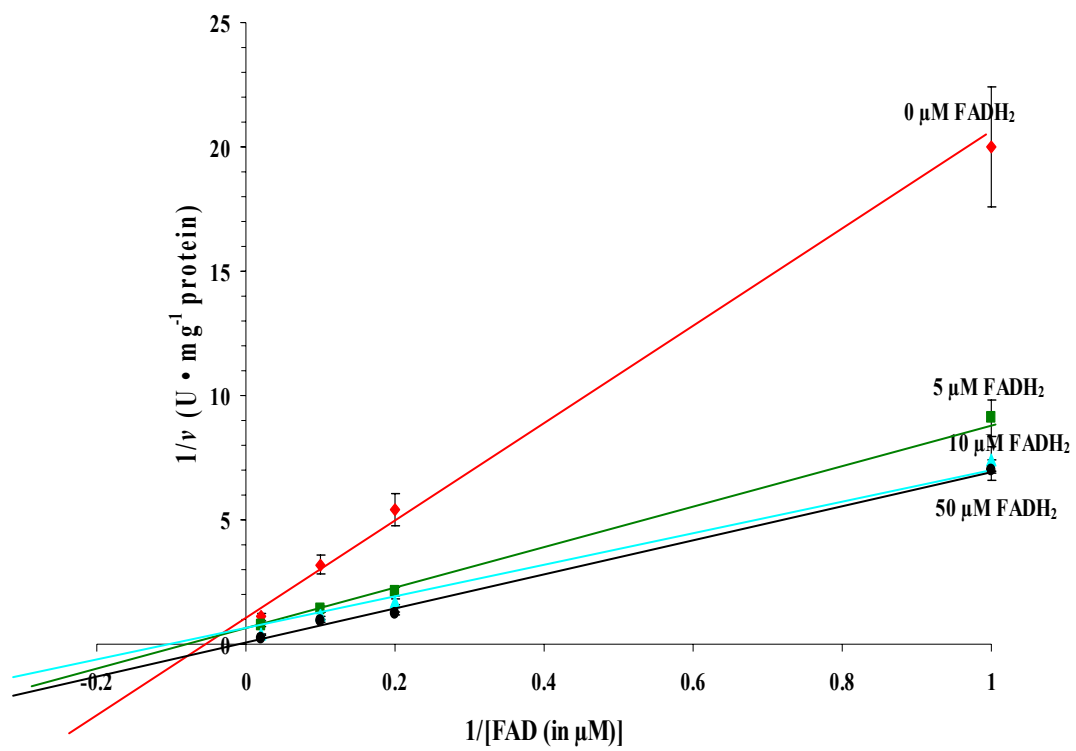
C
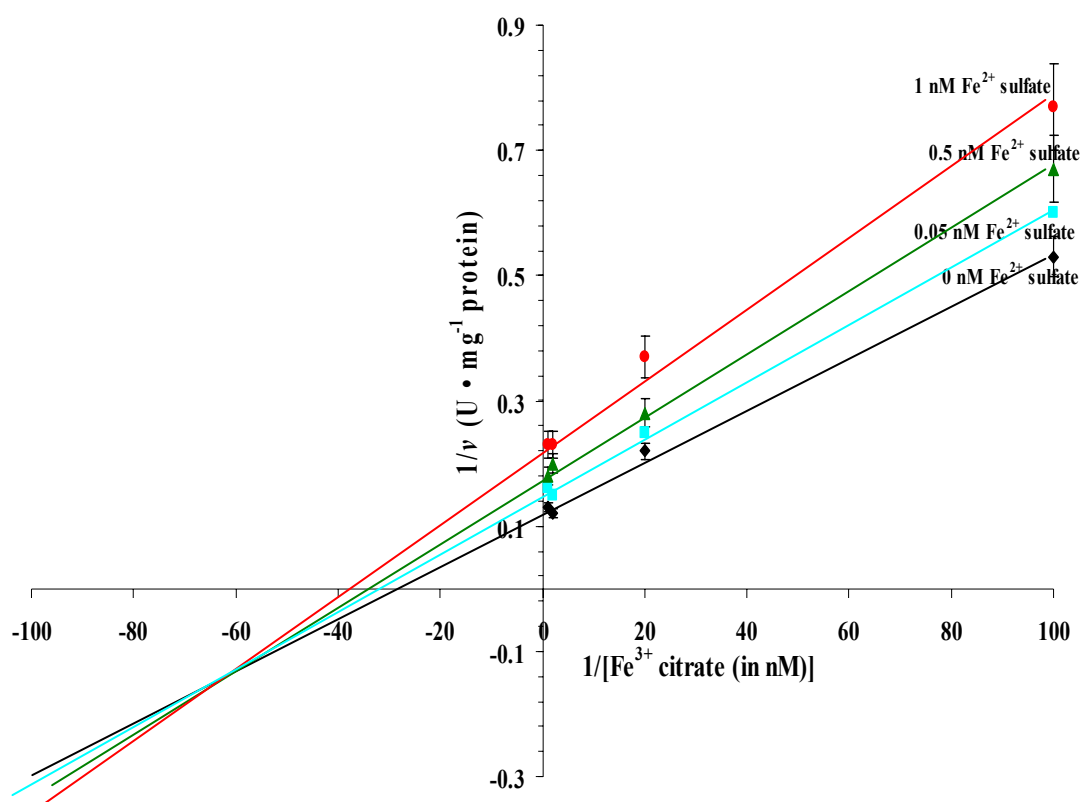
Fig. 7.5. The kinetic scheme for a reversible enzyme inhibitor (Segel 1975). Modified to show the possible binding scheme for substrate [Fe(III)] and product [Fe(II)] to NQO1.

$$NQO1 + Fe^{3+} \;\rightleftharpoons\; NQO1\text{-}Fe^{3+} \;\longrightarrow\; NQO1 + Fe^{2+}$$

$$+ \qquad\qquad\qquad +$$

$$Fe^{2+} \qquad\qquad\qquad Fe^{2+}$$

$$\updownarrow\, K_{\mathrm{I}} \qquad\qquad \updownarrow\, K_{\mathrm{I}}'$$

$$NQO1\text{-}Fe^{2+} + Fe^{3+} \;\longrightarrow\; NQO1\text{-}Fe^{2+}\text{-}Fe^{3+}$$
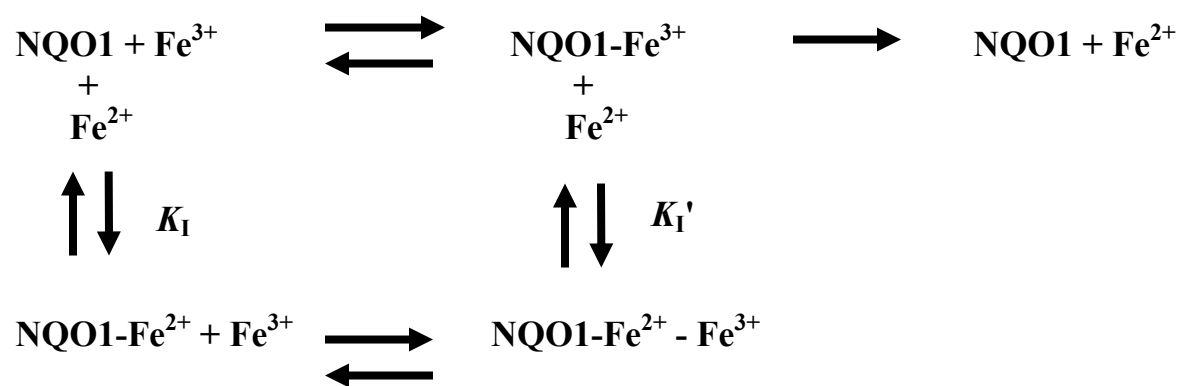
Fig. 7.6. The combined data from two (2) NQO1 complexes showing the superposition of cofactor (FAD), inhibitor (Cibacron blue), and substrate (duroquinone). Complex I contained NQO1 with bound FAD, Cibacron blue, and duroquinone. Complex II contained NQO1 with bound FAD and $NADP^+$. The information from complexes I and II was then combined. FAD is bound in the same position in both complexes. $NADP^+$ (carbon, gray; oxygen, red; nitrogen, blue; phosphorous, yellow) is in the position found in complex II. Duroquinone (green) is in the position found in complex I; it fully overlaps the nicotinamide ring of $NADP^+$ in complex II. Cibacron blue (blue) is in the position found in complex I. Three of its four rings overlap the position of the ADP of $NADP^+$ found in complex II. Figure from Li et al. 1995.
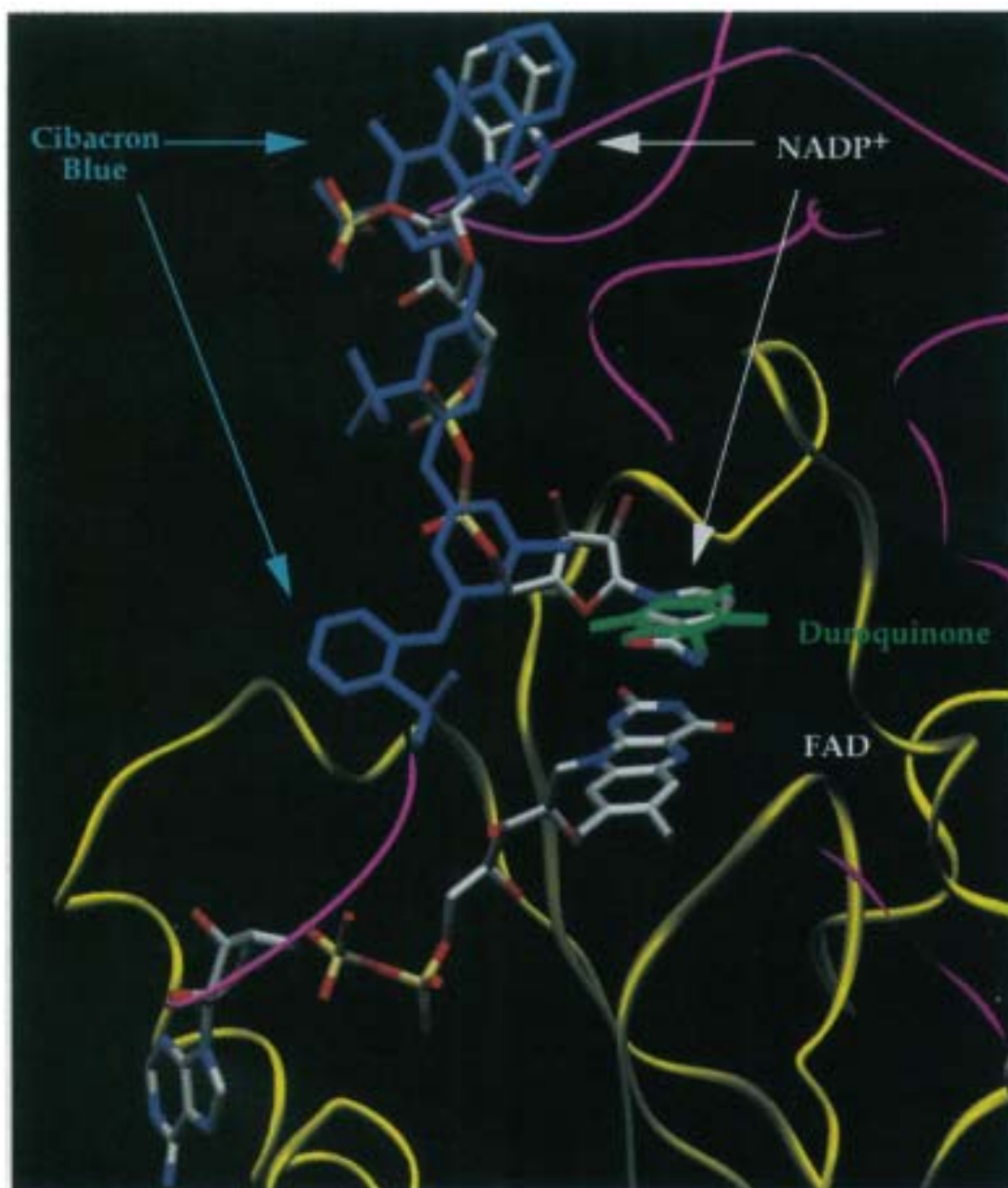
Fig. 7.7. The proposed mechanism of quinone reduction by NQO1. **(A)** The binding of NADP$^+$ to NQO1. **(B)** The binding of substrate (duroquinone) to NQO1. Both NADP$^+$ and duroquinone cannot simultaneously occupy the site. The quinone is in an optimal position to receive a hydride from the FADH$_2$ (FAD represented in schematic). Figure from Li et al. 1995.

A

B

Fig. 7.8. The proposed mechanism for the obligatory two-electron reduction of a quinone (benzoquinone = Q) by NQO1. The overall reaction is: $NADH + Q + H^+ \rightarrow NAD^+ + QH_2$. Figure from Li et al. 1995.
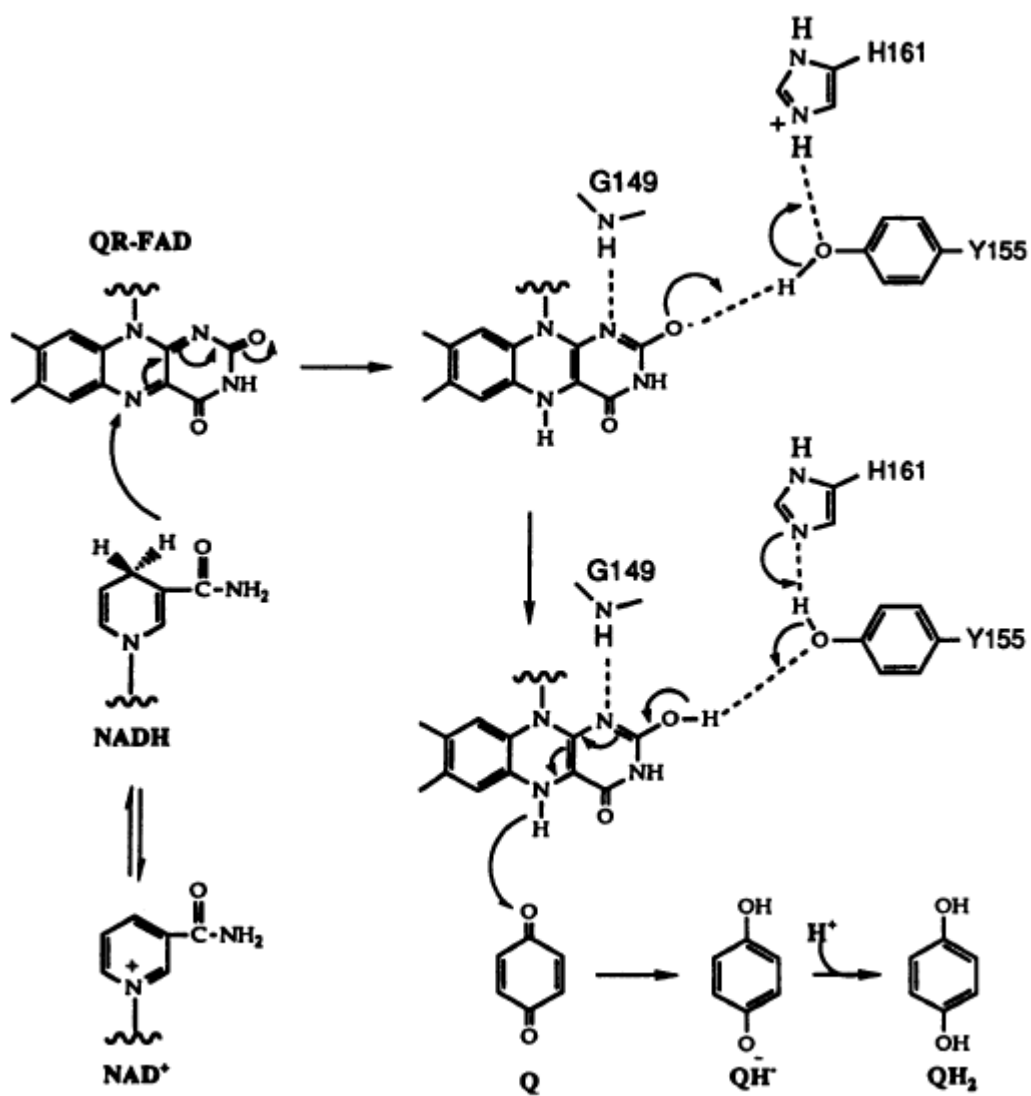
Fig. 7.9. Proposed mechanism of Fe^{3+} reduction by the NQO1. **(A)** 1. Fe^{3+} is reduced via the oxidation of NADH with the enzyme bound FAD (yellow) serving as cofactor, i.e., electron mediator. 2. Exogenous FAD increases enzymatic activity (indicated by thicker arrows) and possibly occupies a secondary site on the NQO1. **(B)** Alternatively, exogenous FAD increases activity because the NQO1 additionally functions as a flavin reductase. The exogenous FAD is reduced by the NQO1. The reduced FAD in turn reduces the Fe^{3+}.

A1
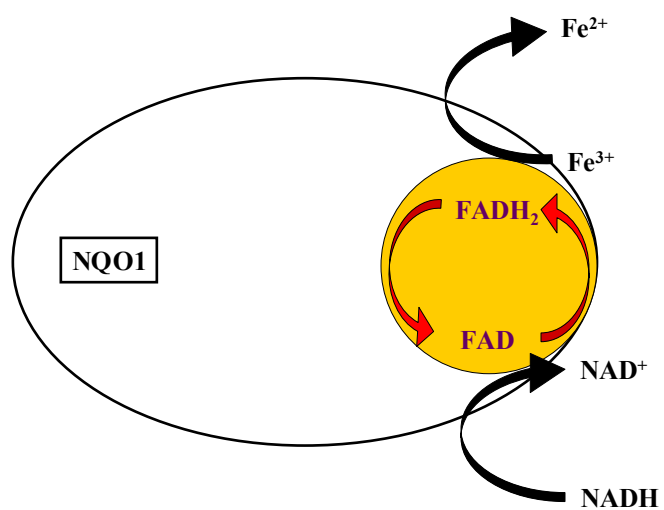
A2
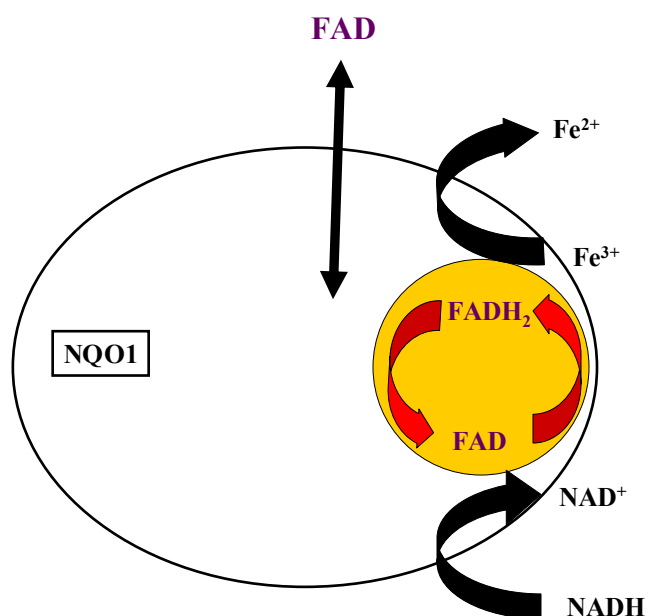
B
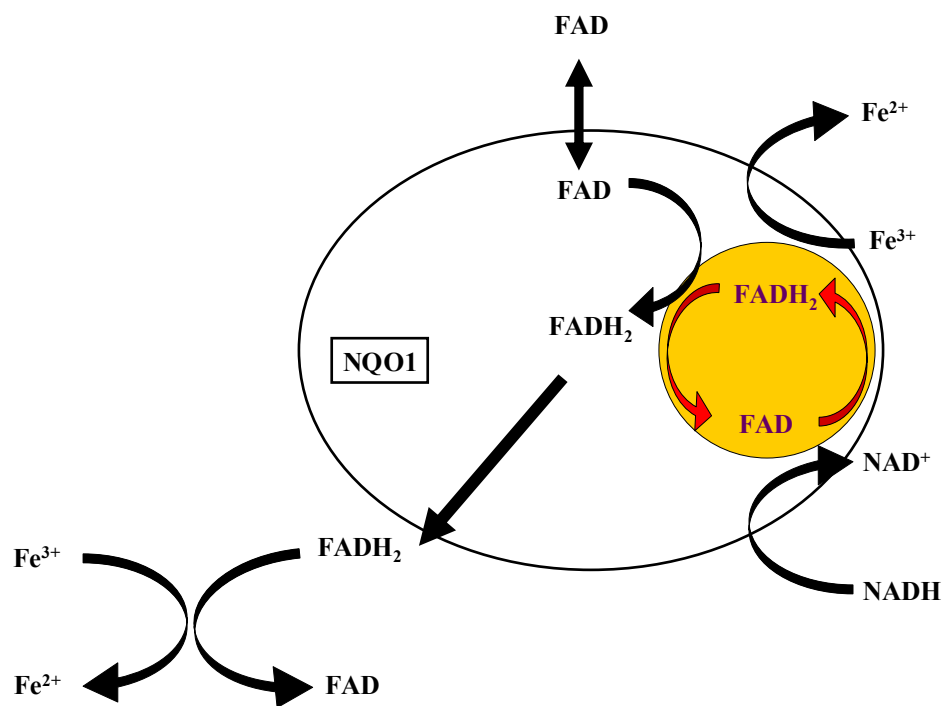
CONCLUSION

This dissertation has 1) provided a detailed summary of the '*Firmicutes*' in terms of a broad genomic overview of endosporulation in the lineage and a more focused exploration of the phylogeny using a few specific taxa (i.e., iron-reducing species, the *Thermoanaerobacterium,* the *Thermoanaerobacter*, and *Caldicellulosiruptor acetigenus*) as examples, and 2) described and provided novel biochemical characterizations of enzymes that are possibly applicable throughout the '*Firmicutes*' and beyond.

Endosporulation is a unique characteristic of the '*Firmicutes*' but is not restricted to, or a shared trait of, particular lineages within the '*Firmicutes*'. The study described here even indicates the genes for endosporulation may not be conserved among distinct phylogenetic lineages. This fact is suggestive of convergent evolution for the development of the endospore-forming phenotype, i.e., the ability to produce endospores arose independently multiple times. However, the complexity of the process, i.e., over 150 genes and gene products are involved, allows for other possible conclusions. It is likely the ability to identify all the genes responsible for endosporulation from whole genome sequences correctly is currently lacking, or the current system of phylogeny in use may not be making the correct assumptions about true lineage determinations for the non-sporulating genera, also lacking *B. subtilis*-related sporulation genes. Based on the work presented in this dissertation, it is apparent that many questions about the phylogenetic status of the '*Firmicutes*' have yet to be answered.

Apart from this global overview of the current status of the '*Firmicutes*' lineage, work has also been presented on the purification and characterization of a soluble oxidoreductase from

Carboxydothermus ferrireducens (the CFOR). The CFOR is likely involved in redox reactions; possibly having a functional role in quinone reduction due to its high activities with quinones, and due to the fact that the enzyme averts the production of the semiquinone radical by performing a two electron reduction step to produce an unreactive hydroquinone species. This activity is similar to the here reported novel iron reduction activity of the human NAD(P)H: quinone oxidoreductase, a well-described and characterized enzyme known to be highly promiscuous in terms of its substrate specificity.

Collectively, this dissertation serves as glimpse into the diversity of a lineage containing members with numerous distinctive physiologies and properties. Presently it is unknown how many, as of yet, undescribed taxa belong to the *'Firmicutes'*. With the notion that only about 1% of the estimated microorganisms have been isolated and described, the elucidation of possible novel lineages within the *'Firmicutes'* may help to clarify the systematics of this group.

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APPENDIX A

NOVEL CHEMOLITHOTROPHIC, THERMOPHILIC, ANAEROBIC BACTERIA

THERMOLITHOBACTER FERRIREDUCENS GEN. NOV., SP. NOV. AND

THERMOLITHOBACTER CARBOXYDIVORANS SP. NOV.[1]

Abstract

Three thermophilic strains of chemolithoautotrophic Fe(III)-reducers were isolated from mixed sediment and water samples (JW/KA-1 and JW/KA-2[T]: Calcite Spring, Yellowstone N.P., WY, USA; JW/JH-Fiji-2: Savusavu, Vanu Levu, Fiji). All were Gram stain positive rods (~0.5 x 1.8 µm). Cells occurred singly or in V-shaped pairs, and they formed long chains in complex media. All utilized H_2 to reduce amorphous iron (III) oxide/hydroxide to magnetite at temperatures from 50 to 75°C (opt. ~73°C). Growth occurred within the pH^{60C} range of 6.5 to 8.5 (opt. pH^{60C} 7.1-7.3). Magnetite production by resting cells occurred at pH^{60C} 5.5 to 10.3 (opt. 7.3). The iron (III) reduction rate was 1.3 µmol Fe(II) produced $\times$ h^{-1} $\times$ ml^{-1} in a culture with 3×10^7 cells, one of the highest rates reported. In the presence or absence of H_2, JW/KA-2[T] did not utilize CO. The G+C content of the genomic DNA of the type strain is 52.7±0.3 mol %. Strains JW/KA-1 and JW/KA-2[T] each contain two different 16S rRNA gene sequences. The 16S rRNA gene sequences from JW/KA-1, JW/KA-2[T], or JW/JH-Fiji-2 possessed >99% similarity to each other but also 99% similarity to the 16S rRNA gene sequence from the anaerobic, thermophilic, hydrogenogenic CO-oxidizing bacterium '*Carboxydothermus restrictus*' R1. DNA-DNA hybridization between strain JW/KA-2[T] and strain R1[T] yielded 35% similarity. Physiological characteristics and the 16S rRNA gene sequence analysis indicated that the strains represent two novel species and are placed into the novel genus *Thermolithobacter* within the phylum '*Firmicutes*'. In addition, the levels of 16S rRNA gene sequence similarity between the lineage containing the *Thermolithobacter* and well-established members of the three existing classes of the '*Firmicutes*' is less than 85%. Therefore, *Thermolithobacter* is proposed to constitute the first genus within a novel class of the '*Firmicutes*', *Thermolithobacteria*. The Fe(III)-reducing *Thermolithobacter ferrireducens* gen. nov., sp. nov. is designated as the type species with strain

JW/KA-2[T] (ATCC 700985 [T], DSM 13639 [T]) as its type strain. Strain R1[T] is the type strain for the hydrogenogenic, CO-oxidizing *Thermolithobacter carboxydivorans* sp. nov. (DSM 7242 [T], VKM 2359 [T]).

Introduction

Anaerobic, microbially-mediated processes, such as carbon monoxide oxidation and autotrophic iron (III) reduction, may have been important on primitive Earth, which contained a reduced atmosphere and very low levels of oxygen (Lovley 1991; Gold 1992). Increasingly biotic dissimilatory iron reduction has become a topic of interest due to its possible role in biogeochemical cycling and potential importance in the evolution of microbial life (Lovley 1991).

Iron is the third most abundant element in the Earth's crust (McGeary and Plummer 1997), and it is generally believed that iron-reducing bacteria are more abundant in nature than their representation in culture collections. Of particular interest are thermophilic, iron-reducing, chemolithoautotrophs, which could have been involved in the precipitation of ancient Banded Iron Formations. Bacteria from a variety of phylogenetic groups are capable of iron reduction (Boone et al. 1995; Lonergan et al. 1996; Slobodkin et al. 1997, 1999). Three out of eight published thermophilic, iron- reducing bacteria (Table A.1.) belong to the Gram-type positive '*Firmicutes*' (Gibbons and Murray 1978; Garrity et al. 2002). Others belong to the Gram-type negative *Flexistipes*, *Deferribacter thermophilus* (Greene et al. 1997); *Proteobacteria*, '*Geothermobacterium ferrireducens*' (Kashefi et al. 2002b) and *Geothermobacter ehrlichii* (Kashefi et al. 2003); *Thermatogales*, *Thermotoga maritima* (Huber et al. 1986; Vargas et al. 1998); and the *Deinococcus-Thermus* clade, *Thermus scotoductus* (Balkwill et al. 2004). In

addition, two archaeal, thermophilic iron-reducers are known: *Geoglobus ahangari* (Kashefi et al. 2002a), and *Pyrobaculum islandicum* (Kashefi and Lovley 2000).

Autotrophic, dissimilatory iron reduction was first demonstrated for *Thermoterrabacterium ferrireducens* and *Thermoanaerobacter siderophilus* (Slobodkin et al. 1997, 1999). Moreover, Slobodkin and Wiegel (1997) showed that several different Fe(III)-reducing microorganisms must exist growing at temperatures up to 90°C. Since that time, the autotrophic, dissimilatory iron-reducers *Geoglobus ahangari* (Kashefi et al. 2002a) and *'Geothermobacterium ferrireducens'* (Kashefi et al. 2002b) have been isolated, and *Pyrobaculum islandicum* has been shown to grow autotrophically (Kashefi and Lovley 2000).

Another type of chemolithotrophic growth that often presumably exists in tandem with dissimilatory, chemolithotrophic iron-reduction is hydrogenogenic, CO-oxidation. Members of both groups have been found to inhabit unique microbial communities and both often rely on the energy of reduced inorganic compounds (Slobodkin and Wiegel 1997; Slobodkin et al. 1999). Hydrogenogenic, CO-oxidizing anaerobic prokaryotes base their metabolism on the anaerobic oxidation of carbon monoxide to equimolar hydrogen gas and carbon dioxide production, according to the reaction $CO + H_2O \rightarrow CO_2 + H_2$ (ΔG^0 = -20 kJ). The name "hydrogenogens" has been proposed for this physiological group because hydrogen is the only reduced product of their metabolism, and physiological groups of anaerobic prokaryotes are frequently named after the major reduced product of their metabolism. CO-oxidizing hydrogenogenic prokaryotes belong to the bacterial genera *Carboxydothermus* (Svetlichnyi et al. 1991, 1994), *Caldanaerobacter* (Sokolova et al. 2001), *Carboxydocella* (Sokolova et al. 2002), *Thermosinus* (Sokolova et al. 2004a) and *Thermincola* (Sokolova et al. 2005) and to the hyperthermophilic archael genus *Thermococcus* (Sokolova et al. 2004b). Hydrogenogenic CO-oxidizing

prokaryotes have been shown to grow obligately dependent on CO (Sokolova et al. 2002), fermentatively (Sokolova et al. 2001; Sokolova et al. 2004a), and employing the reduction of Fe(III) and various other electron acceptors (Sokolova et al. 2004a, 2004b; Henstra and Stams 2004).

Three strains of thermophilic iron-reducers were isolated from hot springs in Yellowstone National Park and Fiji and have been partly described (Hanel, J., 2000, thesis, The University of Georgia; Wiegel et al. 2003). Based on their physiological features and 16S rRNA gene sequence analysis they were proposed as '*Ferribacter thermautotrophicus*' gen. nov., sp. nov. (Wiegel et al. 2003). However, their 16S rRNA gene sequences were similar to the sequence of the hydrogenogenic CO-oxidizing strain R1[T], which was described previously as '*Carboxydothermus restrictus*' sp. nov. (Svetlichnyi et al. 1994). Currently neither name has been validated. The similarity of the above-mentioned strains 16S rRNA gene sequences, and their lack of similarity with other described '*Firmicutes*' indicate that they should be 1) assigned as two novel species within a novel genus and 2) proposed as a novel lineage (class) within the '*Firmicutes*'. Because the previously suggested names would exclude the specific properties of the other species, we propose to assign them to the novel genus *Thermolithobacter*, a name which includes the key properties of both species.

Materials and methods

Environmental samples

A combined sample of water, organic filamentous material, and sediment containing 10-15 ppm Fe from a runoff of a hot spring close to the Yellowstone river at the Calcite Spring area from

Yellowstone National Park (44°54.291' N, 110°24.242' W) contained white and black bacterial filaments. The thermal spring sampling point had a temperature gradient from 60-85°C with a pH of 7.6. The sample from Fiji contained water and sediment [Na/K ratio was 25.7, 1158 ppm Na, 1606 ppm Ca, 5250 ppm Cl, 228 ppm SO_4, and 0.06 ppm Fe] (Cox 1981; Patel, B.K.C., 1985, thesis, Waikato University, Hamilton, New Zealand) from Nakama springs (main spring; used by local residents for cooking) south of the soccer field in Savusavu on Vanu Levu. The estimated temperature for the spring is around 170°C (boiling; partly superheated) with a pH ~7.5. Samples were collected in sterile, N_2-flushed 100 ml Pyrex jars, capped with butyl rubber stoppers and brought to the laboratory in Athens, GA, where they were stored at 4-7°C for several weeks before the enrichments were started.

Strain R1[T] was isolated from a terrestrial hot spring at the Raoul Island, Archipelago Kermadeck (New Zealand).

Media and cultivation

Mineral medium was prepared anaerobically by the Hungate technique (Ljungdahl and Wiegel 1986) under an atmosphere of hydrogen and carbon dioxide gases (80:20 v/v), or under 100% CO. It contained (per liter of de-ionized water): 0.33 g KH_2PO_4, 0.33 g NH_4Cl, 0.33 g KCl, 0.33 g $MgCl_2 \cdot 2H_2O$, 0.33 g $CaCl_2 \cdot 2H_2O$, 2.0 g $NaHCO_3$, 1 ml vitamin solution (Wolin et al. 1963), and 1.2 ml trace element solution (Slobodkin et al. 1997). The pH^{25C} was adjusted to 7.0 with 10% (w/v) NaOH under an atmosphere of either hydrogen and carbon dioxide gases (80:20 v/v) or 100% CO. To this mineral medium, 90 mM amorphous Fe(III) oxide/hydroxide [mineral Fe medium; prepared as described by Slobodkin et al. (1997)] or 20 mM 9,10-anthraquinone 2,6-disulfonic acid (AQDS) were typically added as electron acceptors. Medium was routinely

sterilized by autoclaving at $121^{\circ}C$ for 1 hr due to the possible presence of heat stable spores (Byrer et al. 2000).

JW/KA-1, JW/KA-2^T, and JW/JH-Fiji-2 were isolated from mineral Fe-medium enrichments positive for Fe reduction. Briefly, positive enrichments were used to inoculate (2% v/v) mineral AQDS medium. Agar shake roll tubes using AQDS medium supplemented with 2.5% Bacto agar were inoculated and incubated at $60^{\circ}C$. After 24 h, isolated colonies were picked in an anaerobic chamber (Coy Products) under a N_2/H_2 atmosphere (95:5 v/v) and used to inoculate AQDS medium. Cultures were incubated for 48 h at $60^{\circ}C$. Those tubes displaying AQDS reduction and cell growth were used to inoculate (2% v/v) mineral Fe medium. After 48 h, tubes displaying black magnetic precipitation and cell growth were repeatedly used for subsequent (nine times for JW/KA-2^T) picking of colonies from agar shake- roll-tube-dilution rows to ensure obtaining axenic cultures. The picked colonies were re-suspended in 0.5 ml of pre-reduced media and used to inoculate the subsequent round of agar shake-roll-tube-dilution rows. The cell suspensions were examined microscopically to ensure that they contained mainly single cells and no clumps of cells. Stock cultures were stored in 50% v/v glycerol in AQDS and mineral Fe media at $-75^{\circ}C$. For routine use, cultures were maintained in both iron oxyhydroxide and AQDS media.

The enrichments and pure cultures of JW/KA-1, JW/KA-2^T, and JW/JH-Fiji-2 were typically grown in Hungate or Balch tubes at $60^{\circ}C$, and all transfers were carried out with disposable syringes and hypodermic needles. No reducing agents were added because the reducing agents dithionite, cysteine, Ti(III) citrate (Gaspard et al. 1998) and HCl-cysteine reduce Fe(III). Strain R1^T was grown at 70-72$^{\circ}C$ in 50 ml serum flasks containing 10 ml of the liquid

mineral medium described above, except that AQDS and Fe(III) oxide/hydroxide were omitted (Svetlichnyi et al. 1994). The medium was reduced with 0.5 g/l Na_2S 9 H_2O under 100% of CO.

Determination of growth

Growth was determined by direct cell counts and visually by determinations of reduced iron (black magnetic precipitation). Besides cell counting and measuring the change in OD_{600nm}, the growth of the CO-utilizing strain was additionally measured by following CO utilization and H_2 production employing gas chromatography (Sokolova et al. 2004a).

Microscopy

Light microscopic observations were performed using slides coated by 2% (w/v) ultra pure agar and an Olympus VANOX equipped with phase-contrast objectives. Electron microscopy of negatively stained cells was performed with a JEOL CX-100 transmission electron microscope (Valentine et al. 1968). Light and electron microscopy of strain R1[T] were performed as described by Svetlichnyi et al. (1994).

Quantification of Fe(II)

Fe(II) was determined employing the 2,2-dipyridyl iron assay of Balashova and Zavarzin (1980). In this assay, all species of iron are dissolved in 0.5 ml of 0.6 M HCl, but only Fe(II) reacts with the added 2,2-dipyridyl. Iron was quantified using an Fe(II) ($FeCl_2$) standard curve.

Temperature and pH ranges for growth

The temperature range for the iron-reducers was determined in both mineral Fe- and AQDS-media at pH^{25C} 7.3 using a temperature gradient incubator (Scientific Industries, Inc., Bohemia, N.Y.) with shaking (~20 strokes/min.). To compensate for the effect of temperature on pH measurements, pH^{60C} was determined with a pH meter calibrated with standards and the electrode equilibrated at $60^{o}C$ (Wiegel 1998). Sterile anaerobic 1 M NaOH and 3 M HCl were used to adjust the pH of the medium prior to inoculation. The temperature and pH^{25C} ranges for the growth of strain R1[T] were determined as described by Svetlichnyi et al. (1994) without shaking.

Carbon sources (substrate utilization)

Growth on potential carbon sources was assessed using Hungate tubes containing mineral medium and Fe(III) in which the autotrophic carbon source, CO_2, was omitted and various other substrates were supplied. In the case of strain R1[T] potential carbon sources which could be utilized during growth on H_2 were assessed using 50 ml serum flasks containing 10 ml of the same mineral medium, except 20 mM AQDS was supplied instead of Fe(III). Cell numbers and iron or AQDS reduction were used to determine growth. Negative controls contained identical media and carbon supplements but were inoculated with autoclaved suspensions of cells. This control tested the possibility of abiotic Fe(III) or AQDS reduction, which is common with carbohydrates at high temperatures (Slobodkin et al. 1997). To avoid false positive results, cultures were transferred into the same media using 1% (v/v) inocula before being scored as positive.

Electron acceptors

The ability to utilize various electron acceptors was tested in mineral medium without the addition of Fe(III). Utilization was assumed when cell counts increased from 1.0×10^6 to 2.0×10^7 in the second and third subsequent subcultures, and when the reduction of ferric citrate and AQDS was accompanied by changes in color from black to clear (reduced form) and yellow to brown or black (reduced form), respectively. When nitrate, thiosulfate, sulfate or elemental sulfur was added as the potential electron acceptor, the medium was reduced with sodium sulfide. The inoculum was 1% (v/v). A culture without addition of an electron acceptor was used as a control.

Lipid extraction and fractionation

Duplicate samples of strain JW/JH-Fiji-2 (0.5 g/ml) were extracted using a modified Bligh and Dyer (1959) extraction method (White et al. 2005). Lipids were separated on a silicic acid column with chloroform, acetone, and methanol into neutral lipid, glycolipid, and polar lipid fractions (Guckert et al. 1985). Solvents were removed under a gentle stream of nitrogen, and the fractions were stored at -20°C. The neutral lipid fraction was resolved in methanol and analyzed for respiratory quinones by HPLC-tandem mass spectrometry (Geyer et al. 2004). The phospholipid fatty acids (PLFAs) in the polar lipid fraction were derivatized with trimethylchlorosilane in methanol to produce fatty acid methylesters (FAMEs) and analyzed on a GC-MS system after the addition of FAME 19:0 as an internal standard (Geyer et al. 2005).

G+C content

The G+C mol% was determined by the HPLC method of Mesbah et al. (1989). DNA for the analysis was extracted using the mini-BeadBeater DNA extraction system (Biospec Products). DNA from strain R1[T] was isolated as described by Marmur (1961).

DNA extraction, sequencing, and 16S rRNA gene sequence phylogenetic analysis

Genomic DNA was extracted from cell pellets obtained from cultures of JW/KA-2[T], JW/KA-1 and JW/JH-Fiji-2 (after multiple rounds of isolating single cell colonies) using the DNeasy Tissue kit (Qiagen). Purifed DNA was used to amplify the small-subunit rRNA gene by PCR as previously described (Götz et al. 2002). PCR products (approximately 1500 base pairs) were purified using the PCRpure Spin Kit (Intermountain Scientific, Kaysville, Utah) and cloned into pCR2.1 vector using the TOPO-TA cloning kit (Invitrogen). Plasmid DNA was prepared using an alkaline-lysis method (Sambrook et al. 1989). Sequencing reactions were performed using the ABI PRISM BigDyeTerminator Cycle Sequencing Kit and an ABI 310 Genetic Analyzer (PE Biosystems) according to the manufacturer's protocol. DNA was extracted, amplified, cloned, and sequenced three different times to note any discrepancies that might arise during these procedures. Three clones were sequenced of each strain to ensure the cultures were pure, and to again note any discrepancies that might exist within the sequences. Additionally, PCR products were sequenced directly to determine whether or not a single product was present. Both strands of clones JW/KA-1, JW/KA-2[T], and JW/JH-Fiji- 2 were completely sequenced using a suite of 16S rRNA-specific primers (Vetriani et al. 1999) to generate an overlapping set of sequences which were assembled into one contiguous sequence. Sequence alignments were performed using the software suite ARB (Ludwig et al. 2004). Phylogenetic analysis was performed using

1307 homologous nucleotides. Using a subset of sequences, and based on manual sequence alignments and secondary structure comparisons, all nucleotides were used to construct distance matrices by pairwise analysis with the Jukes and Cantor correction (Jukes and Cantor 1969). Maximum-likelihood, maximum-parsimony and neighbor-joining analyses were done as previously described (Götz et al. 2002). The FastDNAml (Olsen et al. 1994) tree is presented, and the T value was optimized to T=1.2. Bootstrap values were based on 1,000 trials.

DNA-DNA hybridization

DNA-DNA hybridization was performed by the German culture collection (DSMZ). DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications described by Huss et al. (1983), using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 x 6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

Nucleotide sequence accession numbers of novel isolates

The 16S rRNA gene sequences of strain JW/KA-2[T] sequences A and B and JW/JH-Fiji-2 were deposited into GenBank under the accession numbers AF282253, AF282254, and AF282252, respectively. The sequence of strain R1[T] was deposited as DQ095862.

Results

Morphology

As examined by phase contrast microscopy, the iron-reducing strains JW/KA-1, JW/JH-Fiji-2 and JW/KA-2[T] cells were slightly curved rods, approximately 1.8 by 0.5 μm. They were usually found in V-shaped pairs, although chains were not uncommon. In mineral Fe-media, tumbling motility was observed during light microscopic imaging of cultures incubated under optimal growth conditions. Electron microscopy revealed the presence of 2-3 peritrichously located flagella (Fig. A.1.). Spores were never observed by phase contrast or electron microscopy. Cultures were unable to withstand 5 min. of heat treatment at 100°C, thus, heat resistant spores appeared to be absent. Cells stained Gram positive. Colonies formed on the agar surface in roll tubes (mineral medium with AQDS as electron acceptor) and were typically small (1-2 mm in diameter), flat, round, and white in color after 24 hours.

The CO-oxidizing strain R1[T] cells were short, straight rods, motile with peritrichious flagella. They divided by binary fusion, and ultra-thin sections revealed a Gram-type positive cell wall (Wiegel 1981; Svetlichnyi et al. 1994).

Chemolithoautotrophic growth

The iron-reducing strain JW/KA-2[T] and the CO-oxidizing strain R1[T] were obligately anaerobic as O_2 concentrations of 0.2% in the gas phase were bactericidal. Although the relationship was not strictly proportional, an increase in cell number of strain JW/KA-2[T] was observed with a concomitant increase in magnetite formation (Fig. A.2.A.) during growth on CO_2/H_2 with Fe(III) as the electron acceptor. The amount of Fe(II) produced per cell in a growing culture of strain

JW/KA-2[T] was initially high, but steadily decreased as the cells entered the later phases of growth. Both resting cells and growing cells in the culture may have been responsible for Fe(III) reduction. Strain JW/KA-2[T] utilized CO_2, yeast extract, casamino acids, fumarate, and crotonate in the presence of H_2. In the absence of H_2, JW/KA-2[T] used formate as an electron donor and Fe(III) oxide/hydroxide, 9,10-anthraquinone 2,6-disulfonic acid, fumarate, and thiosulfate as terminal electron acceptors. None of the iron-reducing strains were able to grow on CO or a CO/H_2 mixture. In contrast, strain R1[T] grew chemolithoautotrophically on CO (Fig. A.2.B.) and produced equimolar quantities of H_2 and CO_2. The minimal generation time was 1.3 h. However, strain R1[T] did not grow with either Fe(III) citrate or Fe(III) oxide/hydroxide in mineral media supplied with HCO_3^-, acetate, lactate, yeast extract, formate, or citrate under 100% H_2 or 100% CO atmosphere. Strain R1[T] did not grow in media supplemented with AQDS, HCO_3^-, acetate, lactate, yeast extract, formate, or citrate under 100% H_2, and did not reduce AQDS during growth on CO. Strain R1[T] did not grow on yeast extract, formate, citrate, lactate or a H_2:CO_2 mixture in media supplemented with SO_4^{2-}, $S_2O_3^{2-}$, or elemental sulfur.

The temperature range for growth of the iron-reducing strain JW/KA-2[T] at pH^{60C} 7.0 was 50-75°C (opt. 73-74°C) [Fig. A.3.A.]; no growth occurred below 47°C or above 77°C. The pH^{60C} range for growth of JW/KA-2[T] was between 6.5 and 8.5 (opt. ~7.1-7.3) [Fig. A.3.B.]; no growth occurred below pH^{60C} 5.5 or above 9.0. Using a temperature of 72°C and a pH^{60C} of 7.3, the shortest doubling time observed was 46 minutes.

The temperature and pH ranges for magnetite production by resting cells of JW/KA-2[T] were broader than those for growth. The temperature range (Fig. A.3.C.) was from 50 to 90°C (opt. 74°C) and pH^{60C} range from 5.5 to 10.3 (opt. 7.3) [Fig. A.3.D.].

Experiments to determine an optimal headspace to liquid medium ratio revealed a requirement for ~1.5 volumes of headspace for each volume of liquid. Large headspace volumes (100:1 and 200:1) resulted in no growth or iron reduction (data not shown). Experiments with varying levels of atmospheric H_2 indicated that 100% H_2 was optimal for both cell yields and Fe(III) reduction. However, growth was observed at H_2 concentrations as low as 0.1% (vol/vol) in the headspace.

The observed stoichiometry of iron reduction to H_2 uptake was 1.3 mol of Fe(II) produced per mol of H_2 utilized. The theoretical ratio is 2 mol of Fe(II) produced per 1 mol of hydrogen utilized, but some of the hydrogen was used in reduction of CO_2 to biomass (0.35 mol CO_2 reduced/mol Fe(III) reduced).

By refeeding the JW/KA-2^T cultures at 24 h intervals with H_2/CO_2 gas mixtures, a maximal cell yield of 10^8 cells $\cdot$ ml^{-1} was obtained. The bacteria exhibited a high rate of Fe(III) reduction to Fe(II). For example, *Thermoterrabacterium ferrireducens* (Slobodkin et al. 1997), reduces Fe(III) to Fe(II) at a maximal rate of 0.6 μmol $\times$ h^{-1} $\times$ ml^{-1} in cultures containing 1.2 $\times$ 10^9 cells. Under optimal growth conditions, strain JW/KA-2^T reduced approximately 1.3 μmol of Fe(III) to Fe(II) $\times$ h^{-1} $\times$ ml^{-1} in cultures containing 3.0 $\times$ 10^7 cells.

Substrate utilization

In the presence of Fe(III) as an electron acceptor and H_2 as an electron donor, the iron-reducing strain JW/KA-2^T utilized CO_2, fumarate (20 mM), yeast extract (1%), casamino acids (1%), and crotonate (20 mM), and (without the addition of H_2) formate (20 mM) as carbon sources, but no growth (with or without Fe(III)) on tryptone (1.0%), glucose (20 mM), galactose (20 mM), xylose (20 mM), sucrose (20 mM), propionate (20 mM), starch (5 g/l), sodium acetate (30 mM),

succinate (20 mM), lactic acid (20 mM), glycerol (130 mM), cellobiose (20 mM), ethanol (20 mM), 1-butanol (20 mM), 2-propanol (20 mM), acetone (20 mM), phenol (10 mM), ethylene glycol (20 mM), 1,3 propanediol (20 mM), catechol (20 mM) or olive oil (10 ml/l) as carbon and energy sources. In the controls, glucose, sucrose, xylose, cellobiose, galactose, and starch reduced Fe(III) to Fe(II) abiotically at rates similar to those of a growing culture. However, no increase in cell numbers was observed with these substrates; i.e. growth was not observed with these particular substrates.

Electron acceptors

The oxidation of hydrogen gas by the iron-reducing strain JW/KA-2[T] was coupled to the reduction of Fe(III) to magnetite and siderite, as determined by x-ray diffraction analysis (data not shown). Strain JW/KA-2[T] also used AQDS (20 mM), thiosulfate (20 mM) and fumarate (20 mM) as electron acceptors but not ferric citrate, nitrate, sulfate (all at 20 mM), elemental sulfur (sublimated, 0.1% w/v), elemental sulfur (precipitated, 0.1% w/v), crystalline iron (III) oxide, 15 mM manganese (IV) added as MnO_2 (Kostka and Nealson 1998), 100 μM selenium (VI) added as selenium acetate, 10 μM uranium (VI) added as uranyl acetate, or 100 μM arsenic (V) added as potassium arsenate.

The CO-oxidizing strain R1[T] did not use either Fe(III) or AQDS as electron acceptors under 100% H_2, and it did not reduce AQDS during growth on CO. Strain R1[T] did not grow on CO in the presence of NO_3^-, Fe(III) oxide/hydroxide, Fe(III) citrate, or SO_3^{2-}. Strain R1[T] grew on CO in the presence of SO_4^{2-}, $S_2O_3^{2-}$, or fumarate, though the presence of the previously mentioned electron acceptors did not stimulate the growth and no indication of the reduction of either SO_4^{2-}, $S_2O_3^{2-}$, or elemental sulfur in media supplemented with yeast extract, formate,

acetate, pyruvate, citrate, succinate, lactate or $H_2:CO_2$, respectively, was observed. Strain R1[T] also did not use either NO_3^-, SO_3^{2-}, or fumarate as electron acceptor while growing on $H_2:CO_2$ (66:34).

Antibiotic susceptibility

During growth at 60°C in mineral iron medium, strain JW/KA-2[T] was resistant to tetracycline and ampicillin at 100 $\mu g \cdot ml^{-1}$ (higher concentrations not tested). It was resistant to 10 $\mu g \cdot ml^{-1}$ rifampicin, erythromycin, streptomycin and chloramphenicol but not at 100 $\mu g \cdot ml^{-1}$. The latter four were bactericidal at 100 $\mu g \cdot ml^{-1}$. It should be noted that tetracycline and ampicillin lose their effectiveness rapidly at this incubation temperature (Peteranderl et al. 1990), so the exact concentration of these antibiotics JW/KA-2[T] is resistant to is unclear. However, due to their bactericidal action they could still be used to grant some selective advantage to strain JW/KA-2[T] in enrichment cultures. Strain R1[T] grows with antibiotics as previously described (Svetlichnyi et al. 1994).

Lipid extraction and fractionation

The phospholipid fatty acid (PLFA) profile of strain JW/JH-Fiji-2, which showed 99% 16S rRNA gene sequence similarity to JW/KA-1 and JW/KA-2[T], contained the following fatty acids (mol%): i15:0 (32.2 ± 6.4), a15:0 (4.1 ± 0.9), i16:0 (0.8 ± 0.1), 16:0 (15.9 ± 1.0), i17:0 (35.0 ± 4.5), a17:0 (11.2 ± 1.5), and 18:0 (0.8 ± 0.2). The neutral lipid fraction of strain JW/JH-Fiji-2 contained the following respiratory quinones (mol%): demethylmenaquinone-9 (82.7) as the major compound, menaquinone (MK)-4 (2.2), MK-5 (0.7), MK-6 (2.2) and ubiquinone (UQ)-6 (9.0), UQ-7 (0.6), UQ-9 (1.4), UQ-10 (1.2) as minor compounds. The minor quinones, except

UQ-9, were also found in the control (culture media) and could derive from passive accumulation in the membrane (Geyer, R., unpublished results).

G+C content

The mean (± standard deviation from three measurements) guanosine (G) plus cytosine (C) content of the genomic DNA of strain JW/KA-2^T was 52.7 ± 0.3 mol% G+C as determined by HPLC (Mesbah et al. 1989). Strain R1^T DNA G+C content was 52 ± 1 mol% and was determined by melting-point analysis (Marmur and Doty 1962), using *Escherichia coli* K12 as a reference.

Phylogeny

DNA was extracted, amplified, cloned, and sequenced three different times from strains JW/KA-1, JW/KA-2^T, and JW/JH-Fiji- 2. Three clones were sequenced for each of the above three listed strains to ensure that the cultures were pure, and to note any discrepancies that may exist within the sequences. Additionally, PCR products were sequenced directly to determine whether or not a single product was present. Sequencing of the PCR products indicated that there were two 16S rRNA gene sequences, designated A and B, present in both JW/KA-1 and JW/KA-2^T, i.e. each strain has two 16S rRNA gene sequences. The two strains differed slightly in the cell and colony morphology at the time of isolation. When comparing the two 16S rRNA gene sequences from one strain, a similarity of 98.9% was observed with the changes distributed in the variable regions throughout the 16S rRNA gene sequence. The sequences were checked from three different colony isolations and three different DNA isolations of each strain after the strains were isolated nine times as a single colony in subsequent agar roll tube cultures. It should be noted

here that comparable situations have been reported before, i.e., single-cell-derived pure cultures contained two different 16S rRNA gene sequences with up to 5% inferred differences in substitutions and bracketing different species (Amann et al. 2000; Onyenwoke et al. 2006). For strain JW/JH-Fiji-2, only one 16S rRNA gene sequence was observed. Further isolates need to be obtained and analyzed to test whether the occurrence of two 16S rRNA gene sequences is common among strains of this novel species.

The 16S rRNA gene sequences of strain R1^T and strains JW/KA-2^T, JW/KA-1 and JW/JH-Fiji-2 possessed 99% sequence similarity. The 16S rRNA gene sequences of JW/KA-2^T, JW/KA-1 and JW/JH-Fiji-2 possessed only low similarity to any other previously described thermophilic or mesophilic iron-reducers. The closest neighbors on the 16S rRNA gene sequence-based phylogenetic tree is *Thermanaeromonas toyohensis* (Fig. A.4.), as well as the *Desulfotomaculum* species, several of which are also capable of chemolithoautotrophic growth, and the acetogenic *Moorella* species, of which two are also able to grow chemolithoautotrophically with CO (Menon and Ragsdale 1996; Wiegel unpublished results). The chemolithoautotrophic, iron-reducing strains do not produce acetate although they share with *Moorella* species a high (52 to 56) mol% G+C content.

DNA-DNA hybridization

DNA-DNA hybridization between strains R1^T and JW/KA-2^T was 35%.

Discussion

Iron-reducing strains

Physiologically, the three iron-reducing strains JW/KA-1, JW/KA-2^T, and JW/JH-Fiji-2 were different from other iron-reducing, thermophilic bacteria. They required H_2 or formate as their electron donor. They were not able to grow chemoorganotrophically. One of the distinguishing features of *Thermolithobacter ferrireducens* was that it only grew to low cell densities. However, the specific rates of Fe(III) reduction per biomass unit were approximately 10 times higher than those which have been reported for other iron-reducers (Table A.2.). The 16S rRNA gene sequence analysis has distinguished these strains from other thermophilic iron-reducers, such as *Bacillus infernus* (which is only capable of iron reduction under heterotrophic conditions) (Boone et al. 1995), *Thermoterrabacterium ferrireducens* (which utilizes glycerol, and grows to higher densities in similar media) (Slobodkin et al. 1997), and *Thermoanaerobacter siderophilus* (which also utilizes sulfite and elemental sulfur as an electron acceptor) (Slobodkin et al. 1999). In agreement with the 16S rRNA gene sequence analyses, we propose that the strains JW/KA-1, JW/JH-Fiji-2 and JW/KA-2^T represent a novel genus and species, *Thermolithobacter ferrireducens*.

These microorganisms may also be interesting models for early life on Earth, and possibly extra-terrestrial life forms. Of special note in this regard are their ability to grow autotrophically in mineral medium, the use of Fe(III) as a terminal electron acceptor, and a high Fe(III) reduction rate. Presuming that a reservoir of Fe(III) could be provided, JW/KA-2^T would require little more than H_2, CO_2, trace minerals, and elevated temperatures from geothermal activity to thrive; i.e., as might be present in deep subsurface environments. Properties of

Thermolithobacter ferrireducens, i.e., reducing Fe(III) in the range of pH^{60C} 5.5 to 10.3 and temperature ranges of 50 to 90°C, indicate that this or closely related organisms could have contributed to geologically important iron reduction, such as forming the Precambrian Banded Iron Formations, especially since the resting cells showed an extended pH and temperature range and high Fe(III) reduction rates. Studies have indicated that, on Earth, ultra-fine grain magnetite is frequently the product of microbial Fe(III)-reduction (Gold 1992). Similar ultra-fine grains of magnetite have been observed in the Martian meteorite ALH84001 (McKay et al. 1996). It is of note to mention *Thermoterrabacterium ferrireducens* (Slobodkin et al. 1997) was isolated from a hot spring runoff at Calcite Spring a few meters away from the sample site from which strain JW/KA-2^{T} was isolated. However, these sampling sites are far apart, which suggests that this bacterium could also be widespread in various geothermal environments (Slobodkin and Wiegel 1997).

CO-oxidizing strain

 The CO-oxidizing strain R1^{T} was distinguished by 16S rRNA gene sequence analysis from any other known hydrogenogenic, CO-trophic bacteria. Strain R1^{T} differed from *Carboxydothermus hydrogenoformans*, the type species of the genus that strain R1^{T} was originally assigned, by the inability of strain R1^{T} to grow employing AQDS reduction (Henstra and Stams 2004) and in the ability of strain R1^{T} to grow fermentatively on several carbohydrates while *C. hydrogenoformans* is unable to grow fermentatively.

Novel lineage Thermolithobacteria

In spite of their differences, strain R1[T] and strains JW/KA-1, JW/KA-2[T], and JW-Fiji-2 possessed sufficient 16S rRNA gene sequence similarity to assign them to the same genus. Taking into account that strains JW/KA-1, JW/KA-2[T], and JW-Fiji-2 only grow chemolithotrophically and strain R1[T] prefers chemolithoautotrophic growth (cell yield during the growth on CO is 10 times higher than during the growth on glucose, data not shown), we propose *Thermolithobacter* as a novel genus within the phylum '*Firmicutes*'.

The phylum '*Firmicutes*' is divided into three classes: the '*Clostridia*', *Mollicutes*, and '*Bacilli*' (Garrity et al. 2002). Levels of 16S rRNA gene sequence similarity between the lineage containing the *Thermolithobacter* and well-established members of the three classes of the '*Firmicutes*', i.e., represented by the type species of the type genus of the '*Bacilli*' and '*Clostridia*', *Bacillus subtilis* and *Clostridium butyricum*, respectively, are less than 85%, and the *Thermolithobacter* appear to form their own distinct lineage apart from the members of the three existing classes (Fig. A.5.). Even though *Heliobacterium chlorum* (a member of class 1) and *Alicyclobacillus acidocaldarius* (class 3) are the closest neighbors to the *Thermolithobacter* on the phylogenetic tree (Fig. A.5.), it should be noted that the classifications for these taxa are still tentative (Garrity et al. 2002). The 16S rRNA gene sequences of representative members of closely related (Schloss and Handelsman 2004) phylogenetic taxa outside the '*Firmicutes*' were included in the tree (Fig. A.5.). The tree also shows the phylogenetic position of *Thermolithobacter* is indicative of a distinct lineage within the '*Firmicutes*'. It is therefore proposed that the *Thermolithobacter* represent a new class within phylum BXIII '*Firmicutes*', *Thermolithobacteria* (Gibbons and Murray 1978; Garrity et al. 2002).

Description of *Thermolithobacteria* classis nov.

Thermolithobacteria (Ther.mo.li.tho.bac.te'ri.a. Gr. adj. *thermos*, hot; Gr. masc. n. *lithos*, stone; N. L. *bacter* masc. equivalent of Gr. neut. n. *baktron*, rod staff ; suff. –*ia*, ending proposed by Gibbons and Murray (1978) to denote a class; N. L. neut. pl. n. *Thermolithobacteria*, thermophilic, lithotrophically growing rods).

Class of the '*Firmicutes*'. Segregation of these organisms is warranted by: 1) their distinct phylogenetic lineage within the '*Firmicutes*', and 2) their obligate, or preferred, chemolithotrophic growth. Description is the same as that of the genus. Type order: *Thermolithobacterales*.

Description of *Thermolithobacterales* ord. nov.

Thermolithobacterales (Ther.mo.li.tho.bac.ter'ales. N.L. masc. n. *Thermolithobacter*, the type genus of the order; suff. –*ales*, ending to denote order; N.L. fem. pl. n. *Thermolithobacterales*, the order of the genus *Thermolithobacter*).

Description is the same as that for the genus. Type family: *Thermolithobacteraceae*.

Description of *Thermolithobacteraceae* fam. nov.

Thermolithobacteraceae (Ther.mo.li.tho.bac.te.ra'ce.ae. N.L. masc. n. *Thermolithobacter*, the type genus of the family; N.L. suff. –*aceae*, ending denoting a family; N.L. fem. pl. n. *Thermolithobacteraceae*, the family of *Thermolithobacter*).

Description is the same as that for the genus. Type genus: *Thermolithobacter*.

Description of *Thermolithobacter* gen. nov.

Thermolithobacter (Ther.mo.li. tho.bac'ter. Gr. adj. *thermos*, hot; Gr. masc. n. *lithos*, stone; N. L. masc. n. *bacter* equivalent of Gr. neut. n. *baktron*, rod staff; N. L. masc. n. *Thermolithobacter*, thermophilic lithotrophically growing rods). Cells are short rods. Gram-type positive cell wall. Bacterium. Obligate anaerobe. Thermophile. Neutrophile. Chemolithotroph. DNA G+C content is 52-53 $\pm$1 mol%. Type species: *Thermolithobacter ferrireducens*.

Description of *Thermolithobacter ferrireducens* sp. nov.

Thermolithobacter ferrireducens [fer.ri.re.du'cens. L.n. *ferrum*, iron; N.L. part. adj. *reducens*, leading back, bringing back, and in the chemistry converting to a different state; N. L. part. adj. *ferrireducens*, reducing iron (III)]. Has the characteristics of the genus. Rod-shaped cells, approximately 1.8 by 0.5 μm, occurring in singles, V-shaped pairs or chains. Exhibit tumbling motility via 2-3 peritrichously located flagella; spores not observed. Grows under anaerobic conditions in the presence of H_2/CO_2 in a temperature range of 50-75°C (opt. ~73°C). The pH^{60C} range for growth is 6.5-8.5 (opt. 7.1-7.3). Uses as electron acceptor, Fe(III), 9,10-anthraquinone-2,6-disulfonic acid, thiosulfate, and fumarate. No growth occurs with sulfur (precipitated or sublimated), nitrate, sulfate, ferric citrate, crystalline ferric hydroxide, Mn(IV), U(VI), Se(VI) or As(V). Besides growing with H_2/CO_2, the species is able to grow in the presence of H_2 with casamino acids, yeast extract, fumarate, and crotonate, and also without the addition of H_2 with formate. Resistant to tetracycline and ampicillin at 100 μg/ml (higher concentrations not tested), and resistant to 10 μg/ml rifampicin, erythromycin, streptomycin and chloramphenicol but not at 100 μg/ml (all tests performed at 60°C in mineral iron medium). The latter four were bactericidal at 100 g/ml. The phospholipid derived fatty acids consist mainly (78%) of terminal branched

species i15:0, i17:0, and a17:0 which is consistent with the affiliation to the Gram-type positive bacteria. The most abundant respiratory quinone species is demethylmenaquinone-9 (84%) if grown with yeast and H_2. The G+C content of the DNA of the type strain is 52.7 ± 0.3 mol %. Type strain is JW/KA-2[T] (=ATCC 700985[T] =DSM 13639[T]), isolated from a mixed sample of geothermally heated sediment and water collected from a hot spring outflow at Calcite Spring in the Yellowstone National Park (Wyoming, USA).

Description of *Thermolithobacter carboxidivorans* sp. nov.

Thermolithobacter carboxidivorans [car.bo.xy.di.vo'rans. N.L. neut. n. *carboxydum* carbon monoxide; L. part. adj. *vorans* devouring, digesting; N.L. part. adj. *carboxidivorans*, digesting carbon monoxide]. Has the characteristics of the genus. Cells are short, straight rods, about 0.5 by 1-2 μm, arranged singly or in short chains. Cells are motile due to peritrichous flagella. On solid medium produces round, white, semi- translucent colonies, one (1) mm in diameter. Grows within the temperature range from 40 to 78°C (opt. 70°C). The pH for growth ranges from 6.6 to 7.6 (opt. 6.8-7.0). Grows on CO autotrophically, producing hydrogen as the sole reduced product. Growth and CO consumption are inhibited by penicillin, erythromycin, and chloramphenicol but not streptomycin, rifampicin, or tetracycline (all 100 μg/ml). Does not reduce SO_4^{2-}, $S_2O_3^{2-}$, or fumarate during growth with CO. Does not reduce SO_4^{2-}, $S_2O_3^{2-}$, or elemental sulfur with yeast extract, formate, acetate, pyruvate, citrate, succinate, lactate or H_2:CO_2. Does not grow on a H_2:CO_2 mixture in the presence or absence of either NO_3^-, SO_3^{2-}, or fumarate. Does not grow on CO in the presence of Fe(III) citrate, Fe(III) oxide/hydroxide, or NO_3^-. The G+C content of the DNA of the type strain is 52 ± 1 mol%.The type strain is strain

R1^T (=DSM 7242^T, = VKM 2359^T), isolated from a terrestrial hot spring at Raoul Island, Archipelago Kermadeck.

Acknowledgements

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Table A.1. Differentiation of JW/KA-2$^{\text{T}}$ from other Fe(III)-reducing thermophilic micro-organisms.

Properties	Strain JW/KA-2^T	*Bacillus infernus* Boone et al. 1995	*Thermoterrabacterium ferrireducens* Slobodkin et al. 1997	*Deferribacter thermophilus* Greene et al. 1997
Chemolithoautotrophic	+	-	+	-
Organic electron donors	**none**	sugars	sugars	sugars
Metabolism	**obligate anaerobe**	obligate anaerobe	obligate anaerobe	microaerophilic
Spore formation	**not observed**	yes	not observed	not observed
Flagella	**2-3, peritrichous**	none	none	monotrichous (polar)
Motility	**tumbling**	none	tumbling	none
Cell size (avg)	**0.5 x 1.8 μm**	0.7 x 6.0 μm	0.3 x 2.0 μm	0.4 x 3.0 μm
Temp range (°C)	**50-75, opt. 72**	39-65, opt. 61	50-74, opt. 65	50-65, opt. 60
pH range	**6.5-8.5, opt. 7.3**	7.3-7.8, opt. 7.3	5.5-7.6, opt. 6.1	5.0-8.0, opt. 6.5
Gram stain	+	+	+	-
Reduction of Mn(IV)	-	+	-	+
G + C content (mol%)	**53**	ND	41	34

Thermoanaerobacter siderophilus Slobodkin et al. 1999	*Thermotoga maritima* Huber et al. 1986	*'Geothermobacterium ferrireducens'* Kashefi et al. 2002b	*Geothermobacter ehrlichii* Kashefi et al. 2003	*Thermus scotoductus* Balkwill et al. 2004
+	-	+	-	-
sugars	sugars	none	sugars, amino acids, peptides	sugars and amino acids
obligate anaerobe	obligate anaerobe	obligate anaerobe	obligate anaerobe	facultative anaerobe
not observed	not observed	not observed	not observed	not observed
2-3, peritrichous	monotrichous (subpolar)	monotrichous	monotrichous (subpolar)	none
tumbling	+	+	+	none
0.5 x 6.5 µm	0.6 x 5.0 µm	0.5 x 1.0 µm	0.5 x 1.2 µm	0.5 x 1.5 µm
39-78, opt. 70	55 -90, opt. 80	65 -100, opt. 85 -90	35 -65, opt. 55	42-73, opt. 65
4.8-8.2, opt. 6.4	5.5-9.0, opt. 6.5	ND (grew 6.8 -7.0)	5.0-7.75, opt. 6.0	6-8, opt. 7.5
+	-	-	-	-
+	ND	-	-	ND
32	46	ND	62.6	64.5

Table A.2. A comparison of the rates of Fe(III) reduction by *Thermolithobacter ferrireducens* strain JW/KA-2^T to other iron-reducing bacteria.

Microorganism $(T_{opt}/T_{max}, ^oC)$	Rate of Fe(III) reduction (μmol Fe(II) formed ml^{-1} h^{-1}) at:	
	(exponential cell density)	extrapolated for 10^8 cells ml^{-1}
Thermophilic		
Thermolithobacter ferrireducens (73/75)	**1.3 (3×10^7 cells/ml)**	**4.3**
Thermoterrabacterium ferrireducens (65/74)	0.6 (6×10^8 cells/ml)	0.1
Deferribacter thermophilus (60/65)	0.1 (5×10^7 cells/ml)	0.2
Thermoanaerobacter siderophilus (70/78)	0.3 (8×10^7 cells/ml)	0.4
Mesophilic		
Geobacter metallireducens (33/40)	0.4 (2×10^8 cells/ml)	0.2

Fig. A.1. Electron micrograph of JW/KA-2^T. Negative staining preparation using uranyl acetate. (↑) indicates flagella, (⬆) indicates Fe precipitation.

0.5 µm
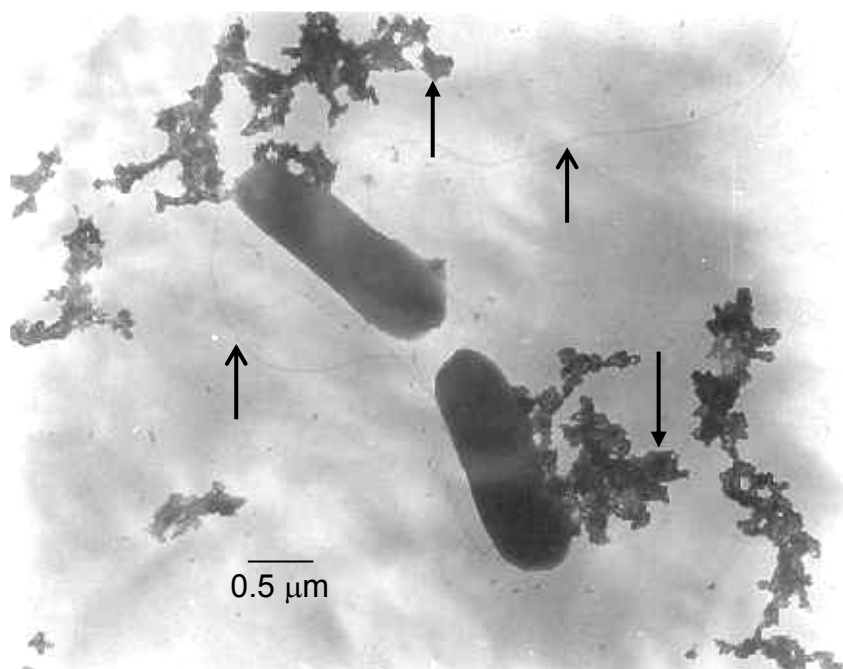
Fig. A.2. Growth and **(A)** Fe(II) formation by JW/KA-2[T] and **(B)** CO utilization/ H_2 production by strain R1[T]. **A.** Cell growth of strain JW/KA-2[T] (-♦-) paralleled Fe(II) accumulation (-•-) when cells were grown in a mineral Fe medium at pH^{25C} 7.2 and 60°C as compared to a sterile control (-∈-). Gas atmosphere was 80:20 H_2-CO_2 (vol/vol). **B.** Cell growth of strain R1[T] (-▲-) was accompanied by CO utilization (-♦-) and H_2 production (-■-) when cells were grown in a mineral medium at pH^{25C} 7.2 and 70-72°C. Gas atmosphere was 100% CO.

A
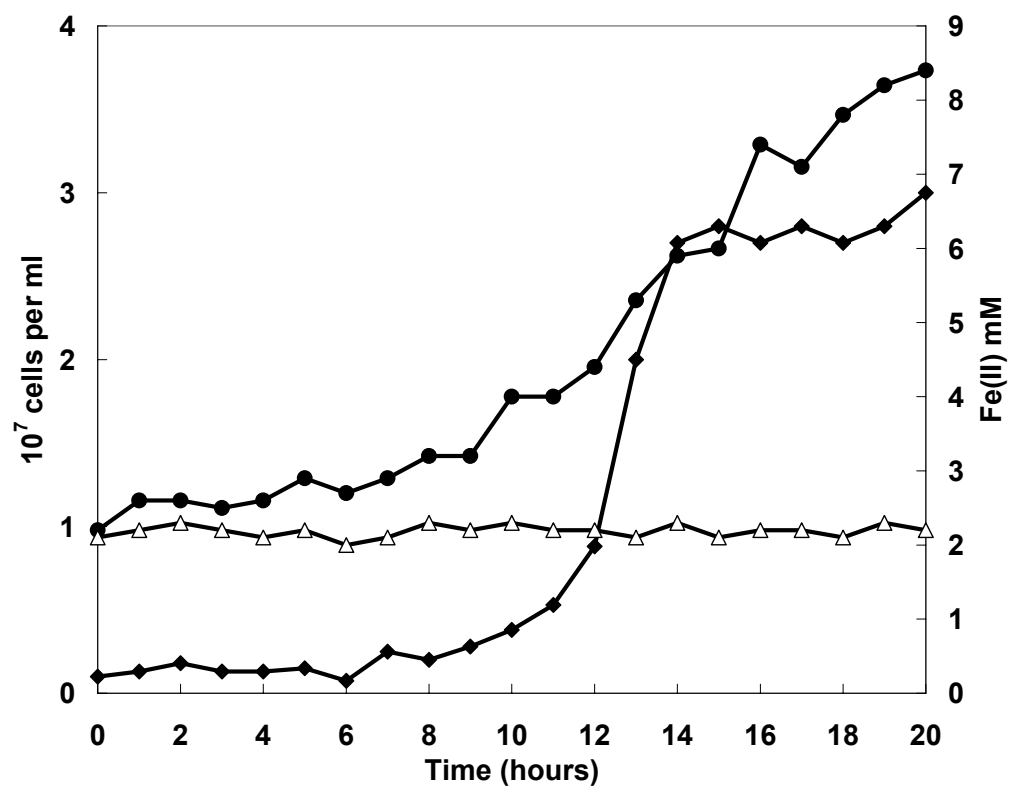
B
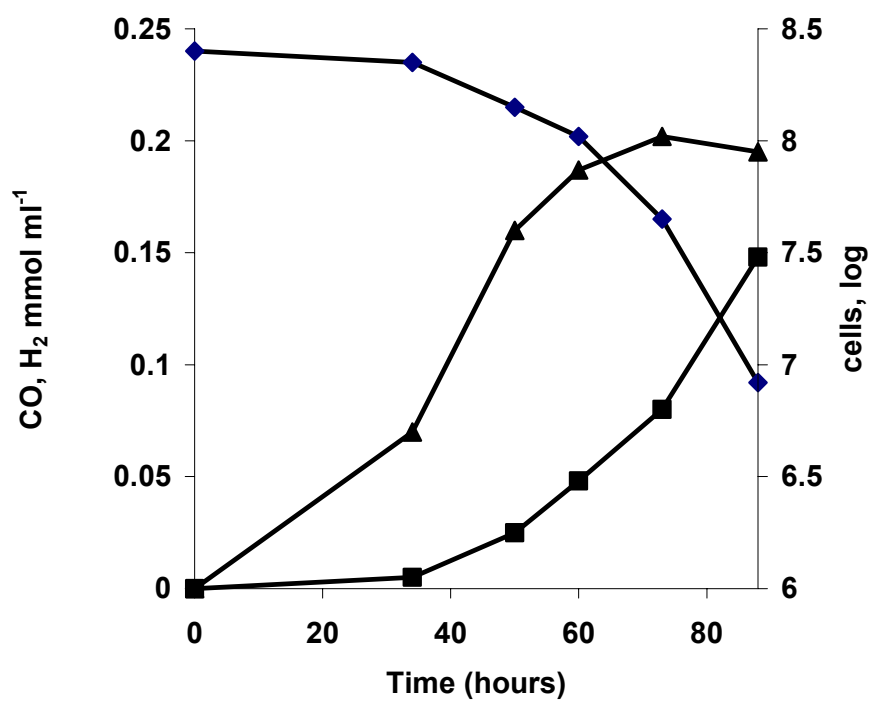
Fig. A.3. Influence of incubation **(A)** temperature and **(B)** pH on growth of JW/KA-2[T], and the influence of incubation **(C)** temperature and **(D)** pH on Fe(III) reduction by resting cells of JW/KA-2[T]. **A.** Fe-medium with a pH[60C] 7.3 was inoculated with 1×10^6 cells/ml. After 24 hour incubation with shaking (20 rpm), cells were counted using a Petroff-Hausser counting chamber. **B.** Fe medium was inoculated with 1×10^6 cells/ml and incubated at 72°C. After 24 hour incubation with shaking (20 rpm), cells were counted using a Petroff-Hausser counting chamber. **C.** Resting cells were inoculated to a concentration of 5×10^7 cells/ml, and incubated for 24 hours in mineral media with a pH[60C] of 7.2 under an 80:20 H_2-CO_2 (vol/vol) atmosphere. Fe(II) accumulation was monitored (-□-) as compared to a sterile control (-▲-). A pH meter calibrated at 60°C was used to determine pH of the media. **D**. Resting cells (-□-) were inoculated to a concentration of 5×10^7 cells/ml, incubated at 60°C with shaking (20 rpm). Fe(II) accumulation was monitored (-□-) as compared to a sterile control (-▲-).

A
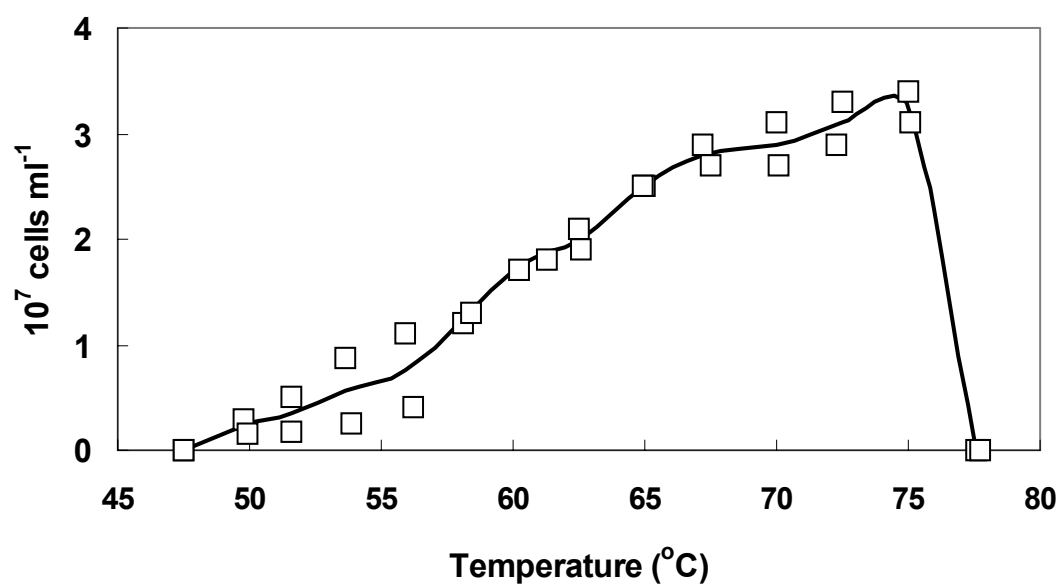
B
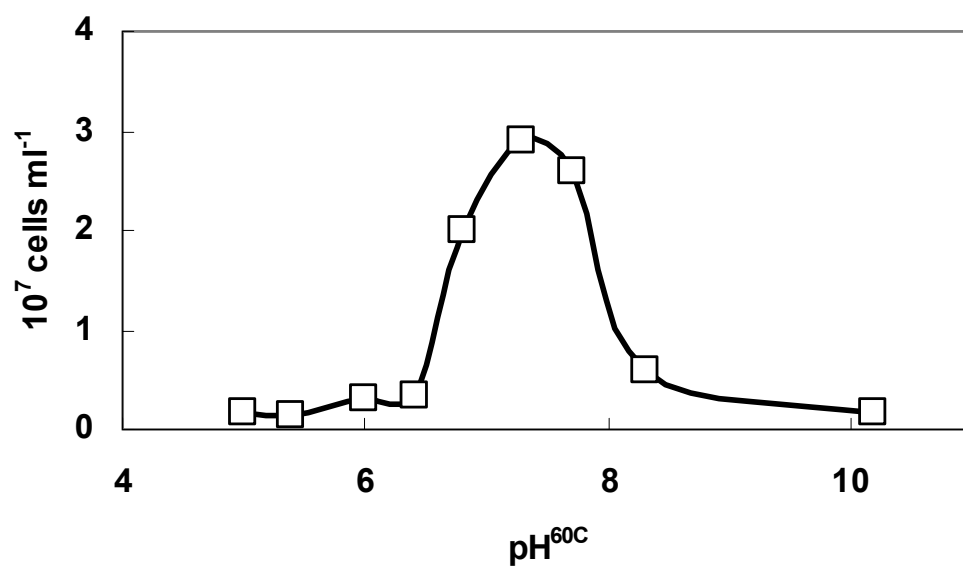
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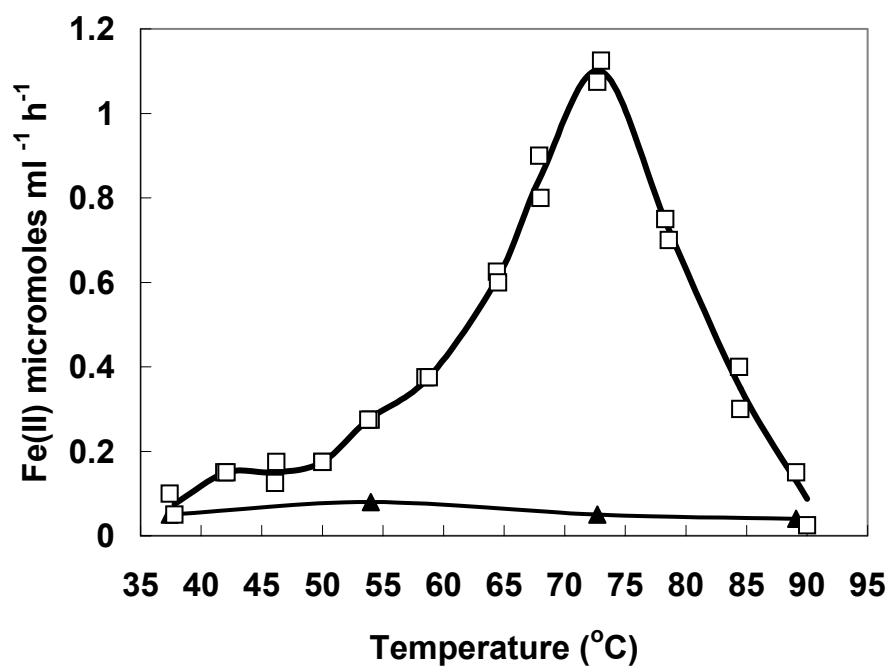
D
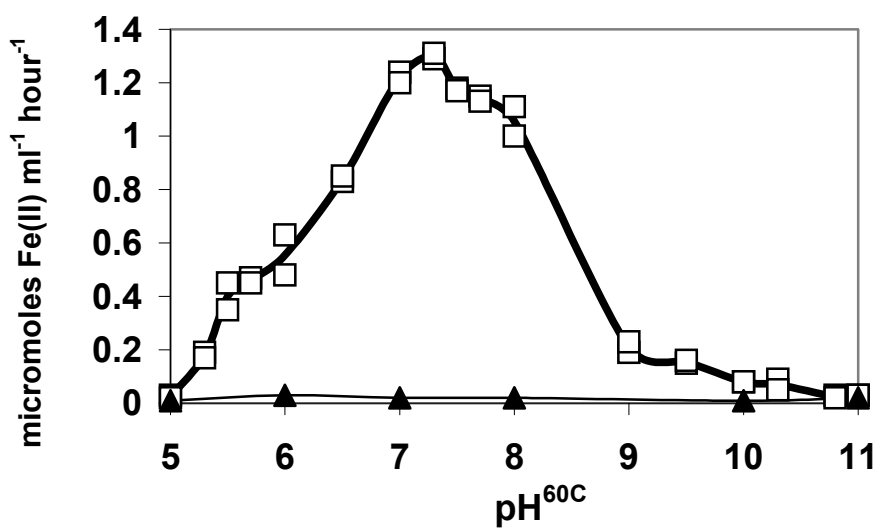
Fig. A.4. Phylogenetic tree**.** Unrooted phylogenetic tree showing the position of *Thermolitho-bacter ferrireducens* strains JW/KA-2^T, with sequences A and B, and JW/JH-Fiji-2, and *T. carboxydivorans* strain R1^T. Two 16S rRNA gene sequences were also observed for strain JW/KA-1. The tree was generated by maximum-likelihood. Reliability values at each internal branch are percentages obtained from 1000 trials. The scale bar represents the expected number of changes per sequence position. *Thermus aquaticus* was used as outgroup. The superscript "T" denotes that the strain is the type strain of the species.

Bacillus subtilis subsp. *subtilis* DSM 10^{T} (D26185)

Halanaerobium acetethylicum DSM 3532^{T} (X89071)

Peptostreptococcus anaerobius DSM 2949^{T} (L04168)

Clostridium butyricum DSM 552^{T} (M59085)

Desulfotomaculum halophilum DSM 11559^{T} (U88891)

Peptococcus niger DSM 20475^{T} (X55797)

Moorella thermoautotrophica DSM 1974^{T} (X77849)

Thermoanaerobacter ethanolicus JW 200^{T} (L09162)

Thermoanaerobacterium thermosulfurigenes DSM 2229^{T} (L09171)

Thermolithobacter ferrireducens str. KA-2^{T} seq.b (AF282254)

Thermolithobacter ferrireducens str. Fiji-2 (AF282252)

Thermolithobacter carboxydivorans str. R1^{T} (DQ095862)

Thermolithobacter ferrireducens str. KA-2^{T} seq. a (AF282253)

Thermanaeromonas toyohensis DSM 14490^{T} (AB062280)

Thermoterrabacterium ferrireducens DSM 11255^{T} (U76363)

Thermus aquaticus DSM 625^{T} (L09663)

0.10

Fig. A.5. Phylogenetic tree of higher taxa**.** Phylogenetic tree constructed from the 16S rRNA gene with maximum likelihood correction for synonymous changes using the neighbor-joining algorithm. GenBank accession numbers are indicated in parentheses. The three classes of the phylum '*Firmicutes*' are indicated. For the '*Clostridia*' (class 1), the order 1 *Clostridiales* are indicated by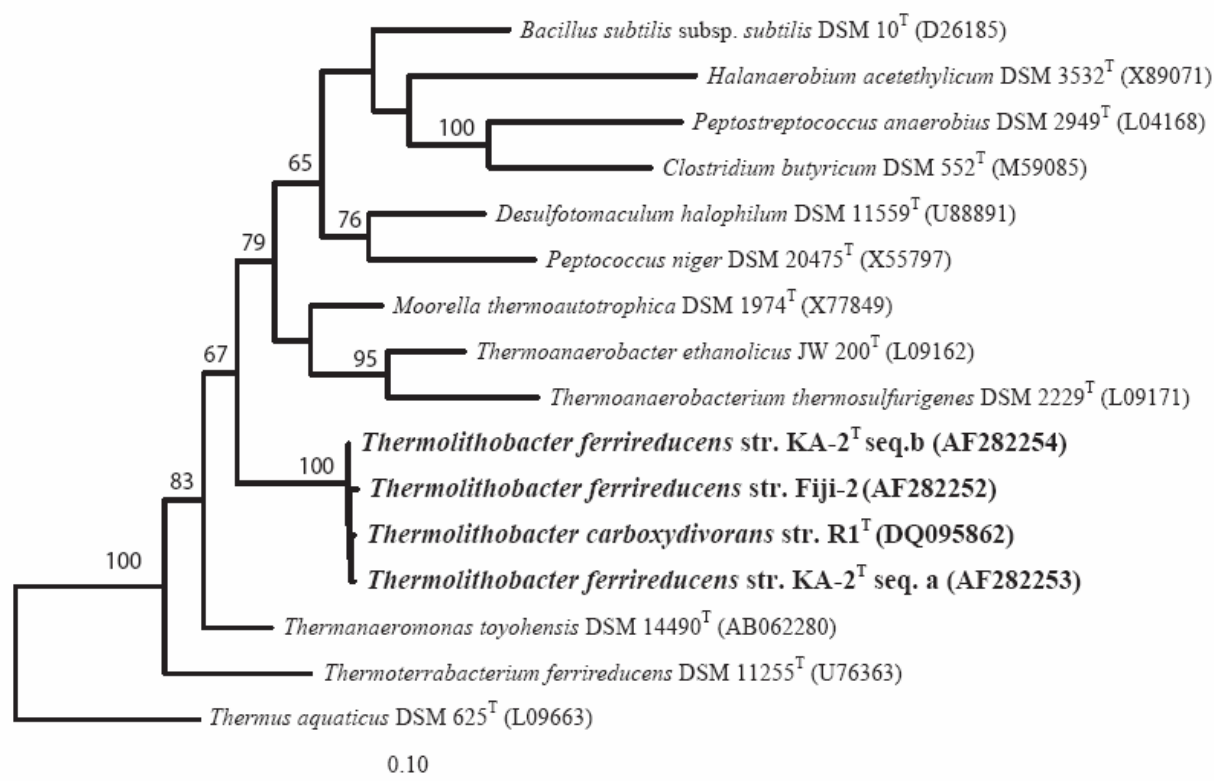 (■) while the order 2 '*Thermoanaerobacteriales*' and order 3 *Halanaerobiales* are indicated by (○) and (□), respectively. The class '*Bacilli*' is divided into the order 1 *Bacillales* (◆) and order 2 '*Lactobacillales*' (◇) while the members of the class *Mollicutes* are indicated by (●) with the order number indicated, i.e., [1]order 1 *Mycoplasmatales*, [2]order 2 *Entomoplasmatales*, [3]order 3 *Acholeplasmatales*, and [4]order 4 *Anaeroplasmatales* (Garrity et al. 2002). To have a meaningful tree, each order is only represented by the type species of the type genus for the class and for the first family for each of the different orders. Numbers at nodes indicate bootstrap support values for 100 replicates with only values above 75 displayed on the tree. Scale bar denotes number of nucleotide substitutions per site. *Escherichia coli* was used as the outgroup.

100 ● *Mycoplasma pneumoniae* [1] (M29061)
● *Mycoplasma genitalium* [1] (X77334)
96
79
100 ● *Acholeplasma laidlawii* [3] (U14905)
● *Anaeroplasma abactoclasticum* [4] (M25050)
90
● *Entomoplasma ellychniae* [2] (M24292)
98 ◆ *Bacillus subtilis* subsp. *subtilis* (AJ276351)
◇ *Lactobacillus delbrueckii* (X52654)
◇ *Aerococcus viridans* (M58797)
■ *Acidaminococcus fermentans* (X65935)
■ *Peptococcus niger* (X55797)
100 ■ *Syntrophomonas wolfei* subsp. *wolfei* (AF022248)
○ *Thermoanaerobacterium thermosulfurigenes* (L09171)
100 □ *Halanaerobium praevalens* (AB022034)
□ *Halobacteroides halobius* (X89074)
94 ■ *Eubacterium limosum* (M59120)
78
96 ■ *Clostridium butyricum* (M59085)
■ *Peptostreptococcus anaerobius* (D14150)
■ *Heliobacterium chlorum* (M11212)
◆ *Alicyclobacillus acidocaldarius* (AB060166)
Thermolithobacter ferrireducens JW/KA-2(A) (AF282253)
100 *Thermolithobacter ferrireducens* JW/KA-2(B) (AF282254)
93 *Thermolithobacter carboxydivorans* R1 (DQ095862)
Chloroflexus aurantiacus (D38365)
Acidimicrobium ferrooxidans (U75647)
97 *Bifidobacterium bifidum* (M38018)
98 *Mycobacterium tuberculosis* (X55588)
96 *Streptomyces albus* (X53163)
Spirochaeta stenostrepta (M88724)
Deinococcus radiodurans (Y11332)
Deferribacter thermophilus (U75602)
Escherichia coli (X80725)

0.05

APPENDIX B

FE(III) REDUCTION BY NOVEL CHEMOLITHOTROPHIC STRAINS OF GLYCOLYTIC

THERMOPHILES[1]

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[1]Onyenwoke, R. U., J. Hanel, R. C. Davis, and J. Wiegel. To be submitted to the *International Journal of Systematic and Evolutionary Microbiology*.

Abstract

Based on 16S rRNA analysis, two novel strains, JW/JH-1 from Yellowstone National Park, WY, USA and JW/JH-Fiji-1 from Fiji: Vanua Levu Island, were isolated as thermophilic, chemolithoautotrophic iron-reducers. They belong to the clade of the glycolytic '*Caloramator celere*' (basonym *Thermobrachium celere*) and *Clostridium thermobutyricum*, respectively. However, the glycolytic type strains of both species were unable to couple growth to the reduction of amorphous Fe(III) oxides, or Fe(III) citrate in the absence of H_2/CO_2.

Introduction

In recent years, evidence has indicated that a great deal more of the world's Fe(II) might be the product of microbial activity than was previously estimated. Despite observations dating back nearly 80 years that bacteria were capable of biotic iron reduction (Harder 1919; Pringsheim 1949), it had simply been assumed that the reduction of Fe(III) to Fe(II) was an abiotic reaction. Mesophilic iron-reducing bacteria have been well-documented and studied in recent years (Lovley and Longergan 1990; Longergan et al. 1996), however, thermophilic iron-reducers have been rarely described (Boone et al. 1995; Slobodkin et al. 1997, 1999; Greene et al. 1997; Kashefi et al. 2003; Sokolova et al., in press). A thermophilic iron-reducer was not even described until *Bacillus infernus* (Boone et al. 1995). Of the first described iron reducers, all required organic carbon sources, i.e., were heterotrophs. Slobodkin et al. (1997) demonstrated chemolithoautotrophic growth for *Thermoterrabacterium ferrireducens* (i.e. able to grow with CO_2, H_2, and amorphous Fe(III) oxides alone) and later also for *Thermoanaerobacter siderophilus* (Slobodkin et al. 1999). Thermophilic, autotrophic, iron reduction has gained interest because it: 1) is under-represented by current culture collections, and 2) may exist in

biosphere pockets deep within the Earth (and possibly other planets) (Gold 1992). This growing interest has been demonstrated by the more recent isolations of the autotrophic iron reducers *Geoglobus ahangari* (Kashefi et al. 2002a), *'Geothermobacterium ferrireducens'* (Kashefi et al. 2002b), and Thermolithobacter ferrireducens (Sokolova et al., in press; this dissertation Appendix A) and by the observation that *Pyrobaculum islandicum* is capable of autotrophic growth on iron (Kashefi and Lovley 2000).

Iron reduction can impact environmental systems in a number of ways: organic matter oxidation, aromatic degradation, banded iron formation, and inhibition/stimulation of other microbial populations (Lovley 1995). As mentioned earlier, biotic Fe(III) reduction has been an underestimated phenomenon and is, therefore, not a routine characterization step for novel microorganisms. We report here on Fe(III) reduction by anaerobic glycolytic and chemolithoautotrophic thermophiles carrying out Fe(III) reduction.

Materials and methods

Environmental samples

A combined water, organic filamentous material, and sediment sample from a runoff of a hot spring close to the river at the Calcite Spring area from Yellowstone National Park contained white and black bacterial filaments and about 10-15 ppm iron in the sediment. The sample from Fiji contained water and sediment from a hot spring runoff channel at the soccer field on Vanua Levu Island. Samples were collected in sterile, N_2-flushed 100 ml Pyrex jars, capped with butyl rubber stoppers, and brought to the laboratory in Athens, GA, where they were stored at 4-7°C for several weeks before the enrichments were started.

Enrichment and isolation of organisms

'Caloramator celere' strain JW/JH-1 and *Clostridium thermobutyricum* JW/JH-Fiji-1 were isolated from the Yellowstone and Fiji samples, respectively, using an anaerobic media prepared using the Hungate technique (Ljungdahl and Wiegel 1986; Hanel, J., M.S. thesis, The University of Georgia) under a headspace of H_2/CO_2 (80:20 v/v) containing (per liter deionized water): 0.33 g of KH_2PO_4, 0.33 g of NH_4Cl, 0.33 g of KCl, 0.33 g of $MgCl_2 \cdot 2H_2O$, 0.33 g of $CaCl_2 \cdot 2H_2O$, 2.0 g of $NaHCO_3$, 1 ml of vitamin solution (Wolin et al. 1963) and 1.2 ml of trace element solution (Slobodkin et al. 1997) with a pH^{25C} adjusted to 7.0 with 10% (w/v) NaOH. To this mineral medium, 90 mM amorphous Fe(III) oxide/hydroxide (mineral Fe medium; prepared as described by Slobodkin et al. 1997) or 20 mM 9,10-anthraquinone 2,6-disulfonic acid (AQDS medium) were typically added as electron acceptors. Medium was routinely sterilized by autoclaving at 121^oC for 1 hour due to the possible presence of heat stable spores with possible D_{10} times of nearly 2 hr (Byrer et al. 2000). The enrichment and pure cultures of JW/JH-1 and JW/JH-Fiji-1 were typically grown in Hungate or Balch tubes at 60^oC, and all transfers were carried out with disposable syringes and hypodermic needles. Since the reducing agents dithionite, cysteine, Ti(III) citrate (Gaspard et al. 1998) and HCl-cysteine (unpublished results) reduce Fe(III), no reducing agents were added.

For isolations, approximately 1 g of sediment and 2 ml of liquid from the mixed sample were transferred to 50 ml serum bottles containing 10 ml of the anaerobic mineral medium supplemented with 90 mM Fe(III) oxide/hydroxide and 20 mM formate, and the enrichments were incubated at 60^oC without shaking. After 72 hours, the presence of iron reducers was indicated by the occurrence of magnetic black precipitate (magnetite). Positive enrichments were transferred three times to the same medium to ensure viability. After the final transfer, the

enrichment displaying the most magnetic precipitate was selected and used to inoculate (2% v/v) several Hungate tubes containing mineral medium supplemented with AQDS as the soluble electron acceptor. All tubes were reduced (yellow to dark brown) in 24 h and displayed growth. The cultures were then inoculated into agar roll tubes containing AQDS media supplemented with 2.5% Bacto agar and incubated at 60°C. After 24 h, isolated colonies were picked in an anaerobic chamber (Coy Products) under N_2:H_2 (95:5 v/v) and inoculated into AQDS medium, which was then incubated for 48 h at 60°C. Tubes displaying positive AQDS reduction and cell growth were used to inoculate (2% v/v) mineral Fe media and were incubated for 48 h. Tubes displaying formation of black magnetic precipitation production and cell growth were then repeatedly transferred into agar roll tubes until pure cultures were obtained. Stock cultures were stored in glycerol (50% v/v) in both AQDS and mineral Fe media at -80°C. For routine use, cultures were maintained in both media.

Microscopy

Light microscopic observations were performed using an Olympus VANOX equipped with phase-contrast objectives and a Petroff-Hausser counting chamber.

Temperature and pH ranges for growth

The temperature range for the iron reducers was determined in both mineral Fe-medium and AQDS-media at pH^{25C} 7.3 (Wiegel 1998) using a temperature gradient incubator (Scientific Industries, Inc., Bohemia, N.Y.) with shaking (~20 strokes/min.). The pH^{60C} [pH determined with a pH meter calibrated with standards and electrode equilibrated at 60°C (Wiegel 1998)] was

determined in both media at 60°C under shaking at ~20 rpm. Sterile anaerobic 1 M NaOH and 3 M HCl were used to adjust the pH of the medium prior to inoculation.

Carbon sources (substrate utilization)

Growth on potential carbon sources was assessed using Hungate tubes containing mineral medium and Fe(III) in which the autotrophic carbon source, CO_2, was omitted and substrates were supplied. Cell numbers and iron or AQDS reduction were used to determine growth. Negative controls included the use of identical media and carbon supplements, inoculated with autoclaved, sterile suspensions in order to test the possibility of abiotic iron or AQDS reduction such as is common with carbohydrates (Slobodkin et al. 1997). All potential carbon sources were sterilized by autoclavation with the exception of pyruvate, which was filter-sterilized. The inocula were 1% (v/v).

Electron acceptors

The ability to utilize various compounds as electron acceptors was tested for growth in mineral medium without the addition of Fe(III). Utilization was assumed when optical densities (600 nm) increased by 0.2, and the reduction of ferric citrate and AQDS was accompanied by changes in color from black to clear (reduced form) and yellow to brown or black (reduced form), respectively. When nitrate, thiosulfate, sulfate or elemental sulfur was added as potential electron acceptors, the medium was reduced with sodium sulfide. The inoculum was 1% (v/v). A culture without addition of an electron acceptor was used as a control.

Iron reduction and Fe(II) analysis

Fe(II) was determined employing the 2,2-dipyridyl iron assay in which all species of iron are dissolved in 0.5 ml of 0.6 N HCl. Only Fe(II) will react with the added 2,2-dipyridyl in glacial acetic acid buffer (Balashova and Zavarzin 1980). Iron was quantified using an Fe(II) standard curve of $FeCl_2$ ranging from 0.5 mM to 50 mM.

Antibiotic susceptibility

Antibiotic susceptibility testing was carried out in mineral media supplemented with tryptone (1%) and yeast extract (0.4%) at 60^oC without shaking. The antibiotics were added to the media from filter sterilized stock solutions to the appropriate, final concentration (10 or 100 μg/ml). An inoculated control was made for each strain by the omission of an antibiotic to the media. Growth was observed at 12, 24, and 48 hrs.

G+C content

The G+C mol% was determined by the HPLC method of Mesbah et al. (1989). DNA for the analysis was extracted using the mini-BeadBeater DNA extraction system (Biospec Products).

DNA extraction, sequencing, and 16S rRNA phylogenetic analysis

Genomic DNA was extracted from cell pellets obtained from cultures of JW/JH-1 and JW/JH-Fiji-1 using the DNeasy Tissue kit (Qiagen). The 16S rRNA genes were amplified by PCR from genomic DNA in reactions containing 50 mM KCl, 30 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.05% Igepal (Sigma), 1 U *Taq* Polymerase, and 20 pmol each primer (8F, AGAGTTTGATCCTGGCTCAG and 1492R, GGTTACCTTGTTAC-

GACTT) with thermal cycling conditions as follows: 94°C for 2 min; 94°C for 30 s, 50°C for 30 s, 72°C for 1.5 min for 30 amplification cycles; and an extension cycle at 72°C for 10 min. PCR products (approximately 1500 base pairs) were purified using the PCRpure Spin Kit (Intermountain Scientific, Kaysville, Utah) and cloned into a pCR2.1 vector using the TOPO-TA cloning kit (Invitrogen). Plasmid DNA was prepared using an alkaline-lysis method (Sambrook et al. 1989). Sequencing reactions were performed using the ABI PRISM BigDyeTerminator Cycle Sequencing Kit and an ABI 310 Genetic Analyzer (PE Biosystems) according to the manufacturer's protocol. DNA was extracted, cloned, amplified and sequenced three different times. Three clones were sequenced of each strain to ensure that the cultures were pure, and to note any discrepancies that may exist within the sequences. Additionally, PCR products were sequenced directly to determine whether a single product was present. Both strands of JW/JH-1 and JW/JH-Fiji-1 were completely sequenced using a suite of 16S rRNA-specific primers (Vetriani et al. 1999) to generate an overlapping set of sequences which were assembled into one contiguous sequence. Sequence alignments were performed using the software suite ARB (Ludwig et al. 2004). The alignment was manually edited considering the expected sequence secondary structure. An unrooted phylogenetic tree was constructed by maximum-likelihood using the program FastDNAml (Olsen et al. 1994) embeded in ARB. Bootstrap values were based on 100 trials. The reliability value (bootstraps) of each internal branch indicates, as a percentage, how often the corresponding cluster was found. The organisms used in the phylogenetic analysis and the GenBank accession numbers for their 16S rRNA gene sequences were: *Clostridium butyricum* (X77834; outgroup), *Thermoanaerobacter ethanolicus* (L09162), *Moorella thermoautotrophica* (X77849), *Moorella thermoacetica* (M59121), *Moorella glycerini* (U82327), *Desulfotomaculum nigrificans* (AY742958), *Desulfotomaculum thermobenzoicum*

(AJ294429), *Desulfotomaculum thermocisternum* (U33455), *Desulfotomaculum geothermicum* (AJ294428).

DNA-DNA hybridization

DNA-DNA hybridizations were performed using…..

Nucleotide sequence accession numbers of novel isolates

The 16S rRNA sequences of strain JW/JH-1 and JW/JH-Fiji-1 were deposited into GenBank under the accession numbers AF286863 and AF286862, respectively.

Results and Discussion

Morphology

JW/JH-1 cells were rods ~0.5 by 10-12 μm, usually appearing as single cells, with some pairs, during early growth (24 hr) while older cultures (72 hr) contained long chains of cells, up to 10, in addition to paired and single cells (Fig. B.1.B.). Retarded motility was observed. Young cultures (6 hr) stained Gram positive while older cultures tended to stain Gram negative. Colonies formed on the agar (2.2%) surface in roll tubes (mineral medium supplemented with yeast extract (0.4%) and tryptone (1.0%)) and are ~1 mm in diameter, cream-colored, with filamentous edges.

As examined by phase contrast microscopy, cells of strain JW/JH-Fiji-1 were rods ~0.5 by 6-8 μm, usually arranged in pairs or appearing as single cells during early growth (24 hr) while older cultures (72 hr) often contain long chains, ≥12 cells (Fig. B.1.A.). Retarded motility

was observed. Young (6 hr) and older (72 hr) cultures always stained Gram negative. Colonies formed on the agar (2.2%) surface in roll tubes (mineral medium supplemented with yeast extract (0.4%) and tryptone (1.0%)) and are ~0.5 mm in diameter, cream-colored, with smooth edges.

Spores were never observed by phase contrast microscopy for either strain. Both strains were unable to withstand 5 min of heat treatment at 100°C. Thus, heat resistant spores appeared to be absent in these strains under the employed conditions.

Temperature and pH ranges for growth

The temperature range for growth of strain JW/JH-1 was 45-68°C (pH^{25C} 7.3) with an opt. 63°C (Fig. B.2.A.); no growth occurred below 40°C or above 70°C. The pH^{60C} range for growth of JW/JH-1 was 5.5-9.5, with an opt. ~8.0 (Fig. B.2.B.); no growth occurred below pH^{60C} 5.0 or above 10.

The temperature range for growth of strain JW/JH-Fiji-1 was 47-72°C (pH^{25C} 7.3), opt. 65°C (Fig. B.3.A.); no growth occurred below 40°C or above 75°C. The pH^{60C} range for growth of JW/JH-Fiji-1 was 5.5-8.5, opt. ~7.2 (Fig. B.3.B.); no growth occurred below pH^{60C} 5.0 or above 9.0.

Substrate utilization

Substrates utilized by strain JW/JH-1 include: yeast extract (0.3% and as low as 0.05%), sucrose (0.2%), raffinose (0.2%), inositol (0.2%), crotonate (0.1%), and benzoate (0.1%) but not fructose (0.2%), glucose (0.2%), or ribose (0.2%) (with either Fe(III) oxyhydroxide (90 mM) or AQDS (20 mM) as electron acceptor) (Table B.1.). In the presence of atmospheric H_2, but not in its

absence, as an electron donor, strain JW/JH-1 utilized: CO_2, formate (10 mM), glycerol (3 ml/l), and acetate (20 mM) but not casamino acids (1%), glucose (20 mM), cellobiose (20 mM), or pyruvate (20 mM).

Strain JW/JH-Fiji-1 utilized: yeast extract 0.3% (and as low as 0.05%), sucrose (0.2%), corn starch (0.1%), ribose (0.2%), fructose (0.2%), glucose (0.2%), succinate (10 mM), toluene (1 mM), acetone (40 mM), phenol (5 mM), benzoate (10 mM), formate (10 mM) but not galactose (0.2%), xylose (0.2%), or maltose (0.2%) (with either Fe(III) oxyhydroxide (90 mM) or AQDS (20 mM) as electron acceptor) (Table B.1.). JW/JH-Fiji-1 utilized CO_2 and formate (10 mM) in the presence of a H_2 headspace. Formate was also utilized in the absence of H_2. Neither casamino acids (1%), cellobiose (20 mM), glycerol (3 ml/l), acetate (20 mM) nor pyruvate (20 mM) was utilized. In the controls, glucose, sucrose, xylose, cellobiose, galactose, and starch reduced Fe(III) to Fe(II) abiotically at rates similar to that of a growing culture, however, no increase in cell numbers was observed.

Electron acceptors

The oxidation of hydrogen gas was coupled to the reduction of Fe(III) to magnetite and siderite (determined by x-ray diffraction analysis). Besides amorphous Fe(III) oxyhydroxide (90 mM), JW/JH-1 and JW/JH-Fiji-1 utilized AQDS (20 mM) and thiosulafate (20 mM) as electron acceptors with atmospheric H_2/CO_2 (80:20 vol/vol) but not Fe(III) citrate (25 mM), nitrate, sulfate, elemental sulfur (sublimated, 0.1%), elemental sulfur (precipitated, 0.1%), crystalline Fe(III) oxide, manganese (IV) (15 mM) added as MnO_2 (Kostka and Nealson 1998), or selenium (VI) (100 μM) added as selenium acetate. However, JW/JH-1 and JW/JH-Fiji-1 were unable to

utilize amorphous Fe(III) oxyhydroxide (90 mM), AQDS (20 mM) or thiosulafate (20 mM) as electron acceptors when the H_2/CO_2 headspace (80:20vol/vol) was replaced with CO (100%).

Both strains grew to higher cell densities (2.0×10^8 cells/ml) on an Fe(III) mineral medium supplemented with 1% yeast extract and 1% tryptone than in the absence of yeast extract and tryptone (3.0×10^7 cells/ml).

Iron reduction and Fe(II) analysis

In the process of dissimilatory Fe(III) reduction, 2 mol of Fe(II) were formed for every 1 mol of H_2 oxidized. Strains JW/JH-1 and JW/JH-Fiji-1 produced Fe(II) and oxidized H_2 at ratios of: 0.9-1.1 $\pm$ 0.2 and 0.8-0.9 $\pm$ 0.1, respectively. Thus, per mole of H_2 consumed for the reduction of Fe(III), 1 mole of H_2 was used for anabolic reactions.

Antibiotic susceptibility

Growing at 60°C on rich media, strain JW/JH-Fiji-1 was susceptible to 10 μg/ml: rifampicin, erythromycin, streptomycin, chloramphenicol, tetracycline, and ampicillin. Strain JW/JH-1 showed an identical susceptibility profile to JW/JH-Fiji-1, except JW/JH-1 was resistant to streptomycin at 10 μg/ml (but not at 100 μg/ml).

G+C content

The mean ($\pm$ standard deviation from three measurements) guanosine (G) plus cytosine (C) content of the genomic DNA of strains JW/JH-1 and JW/JH-1 Fiji-1 were ?? $\pm$?? and 62.5 $\pm$ 0.05 mol% G+C, respectively, as determined by HPLC (Mesbah et al. 1989).

Description of '*Caloramator celere*' strain JW/JH-1

Cells of strain JW/JH-1 are rods ~0.5 by 10-12 μm, usually appearing as single cells, with some pairs, during early growth (24 hr) while older cultures (72 hr) contain long chains of cells, up to 10, in addition to paired and single cells. Retarded flagellation and sluggish motility is observed. Young cultures (6 hr) stain Gram positive while older cultures tend to stain Gram negative. Colonies formed on the agar (2.2%) surface in roll tubes (mineral medium supplemented with yeast extract (0.4%) and tryptone (1.0%)) are ~1 mm in diameter, cream-colored, with filamentous edges.

The temperature range for growth of strain JW/JH-1 is 45-68°C [pH^{25C} 7.3] (opt. 63°C); with no growth at or below 40°C, or at or above 70°C. The pH^{60C} range for growth of JW/JH-1 is 5.5-9.5 (opt. ~8.0); no growth at or below pH^{60C} 5.0, or at or above 10. Strain JW/JH-1 utilizes yeast extract, sucrose, raffinose, inositol, crotonate, and benzoate. In the presence of atmospheric H$_2$ as an electron donor, strain JW/JH-1 utilizes: CO$_2$, formate, glycerol, and acetate. Amorphous Fe(III) oxyhydroxide, AQDS, and thiosulafate are utilized as electron acceptors with H$_2$/CO$_2$ (gas phase 80:20) but not Fe(III) citrate, nitrate, sulfate, elemental sulfur (sublimated), elemental sulfur (precipitated), crystalline Fe(III) oxide, manganese (IV) added as MnO$_2$, or selenium (VI).

Strain JW/JH-1 was isolated from a combined water, organic filamentous material, and sediment sample from a runoff of a hot spring close to the river at the Calcite Spring area from Yellowstone National Park containing white and black bacterial filaments and about 10-15 ppm iron in the sediment.

Strain JW/JH-1 was deposited in the American Type Culture Collection ATCC 700984 and the German Collection of Microorganisms DSM 13655.

Description of *Clostridium thermobutyricum* strain JW/JH-Fiji-1

Cells of strain JW/JH-Fiji-1 are rods ~0.5 by 6-8 μm, usually arranged in pairs or appearing as single cells during early growth (24 hr) while older cultures (72 hr) often contain long chains, ≥12 cells. Retarded flagellation and sluggish motility is observed. Young (6 hr) and older (72 hr) cultures stain Gram negative. Colonies formed on the agar (2.2%) surface in roll tubes (mineral medium supplemented with yeast extract (0.4%) and tryptone (1.0%) and are ~0.5 mm in diameter, cream-colored, with smooth edges. The temperature range for growth of strain JW/JH-Fiji-1 is 47-72°C [pH^{25C} 7.3] (opt. 65°C); no growth at or below 40°C, or at or above 75°C. The pH^{60C} range for growth of JW/JH-Fiji-1 is 5.5-8.5 (opt. ~7.2); no growth at or below pH^{60C} 5.0, or at or above 9.0. Strain JW/JH-Fiji-1 utilizes: yeast extract, sucrose, corn starch, ribose, fructose, glucose, succinate, toluene, acetone, phenol, benzoate, and formate. JW/JH-Fiji-1 utilizes CO_2 and formate (10 mM) in the presence of H_2 as headspace gas. Amorphous Fe(III) oxyhydroxide, AQDS, and thiosulfate can serve as electron acceptors with H_2/CO_2 as headspace gas but not Fe(III) citrate, nitrate, sulfate, elemental sulfur (sublimated), elemental sulfur (precipitated), crystalline Fe(III) oxide, manganese (IV) added as MnO_2, or selenium (VI).

Strain JW/JH-Fiji-1 was isolated from a sample from Fiji containing water and sediment from a hot spring runoff channel at the soccer field in Savusavu on Vanua Levu Island.

The G+C content of the DNA of the strain is 62.5 ± 0.05 mol %. Strain JW/JH-Fiji-1 was deposited in the American Type Culture Collection ATCC 700983 and the German Collection of Microorganisms DSM 13654.

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Table B.1. Substrates utilized by strains JW/JH-Fiji-1, *Clostrium thermobutyricum* JW171K[T] (Wiegel et al. 1989), JW/JH-1, and *Thermobrachium celere* JW/YL-NZ35[T] (Engle et al. 1996). The designation of: (+) indicates growth (a visibly turbid culture), (-) indicates no growth, and (±) indicates weak growth (not a visibly turbid culture) at OD 600 nm. OD reading for (±) designation was ≥0.03 as compared to both: an uninoculated control (media containing the substrate but uninoculated) and an inoculated control, i.e., this control was inoculated with the bacterium but lacks the substrate. [a]Reduction of Fe(III) oxide, formation of black precipitates from amorphous brown Fe(III) oxide, indicated growth. [b]Reduction of AQDS, yellow to black, indicated growth. NT=not tested. Neither strain JW/JH-Fiji-1 nor strain JW/JH-1 utilized: rhamnose or propionate (both at 0.2%), trehalose (0.1%), ethanol (0.3%), lactic acid (20 mM), 2-propanol (40 mM), CO + Fe(III) oxide (90 mM), or CO + AQDS (20 mM). A superscript "T" indicates the type strain of a species.

Substrate	JW/JH-Fiji-1	JW171K[T]	JW/JH-1	JW/YL-NZ35[T]
Yeast extract (0.3%)	+	+	+	+
Sucrose (0.2%)	+	-	+	+
Cellobiose (0.2%)	-	+	-	-
Corn starch (0.1%)	+	-	-	NT
Raffinose (0.2%)	-	-	$\underline{+}$	NT
Inositol (0.2%)	-	NT	$\underline{+}$	NT
Crotonate (0.1%)	-	NT	$\underline{+}$	NT
Xylose (0.2%)	-	+	-	-
Ribose (0.2%)	$\underline{+}$	+	-	-
Fructose (0.2%)	$\underline{+}$	+	-	+
Glucose (0.2%)	$\underline{+}$	+	-	+
Galactose (0.2%)	-	-	-	+
Maltose (0.2%)	-	+	-	+
Succinate (10 mM)	$\underline{+}$	-	-	NT
Fumarate (10 mM)	-	-	-	NT
Ethanol (0.3%)	-	-	-	NT
Toluene (1 mM)	$\underline{+}$	NT	-	NT
Acetone (40 mM)	+	NT	-	NT
Phenol (5 mM)	+	NT	-	NT
Benzoate (10 mM)	$\underline{+}$	NT	$\underline{+}$	NT
Formic acid (10 mM)	+	-	-	NT
H$_2$/CO$_2$ + Fe(III) oxide (90 mM)	[a]+	-	+	NT
H$_2$/CO$_2$ + AQDS (20 mM)	[b]+	-	+	NT

Fig. B.1. Phase-contrast images of: **(A)** JW/JH-Fiji-1 and **(B)** JW/JH-1. Bars are 10 μm in length.
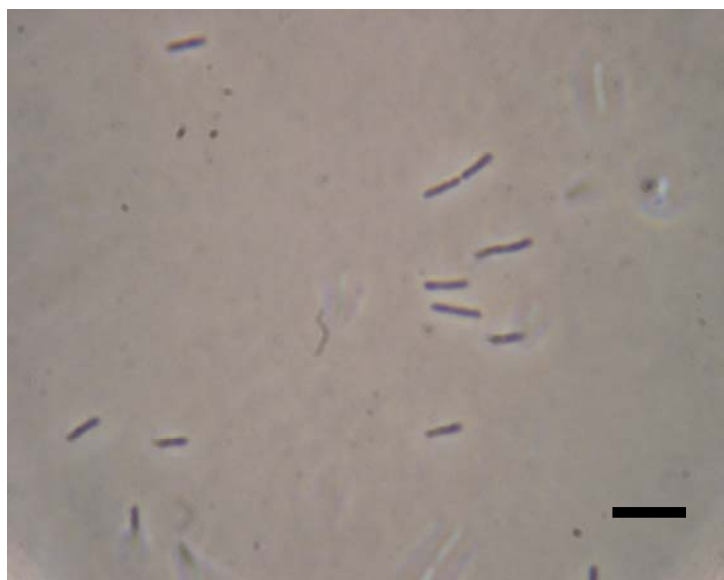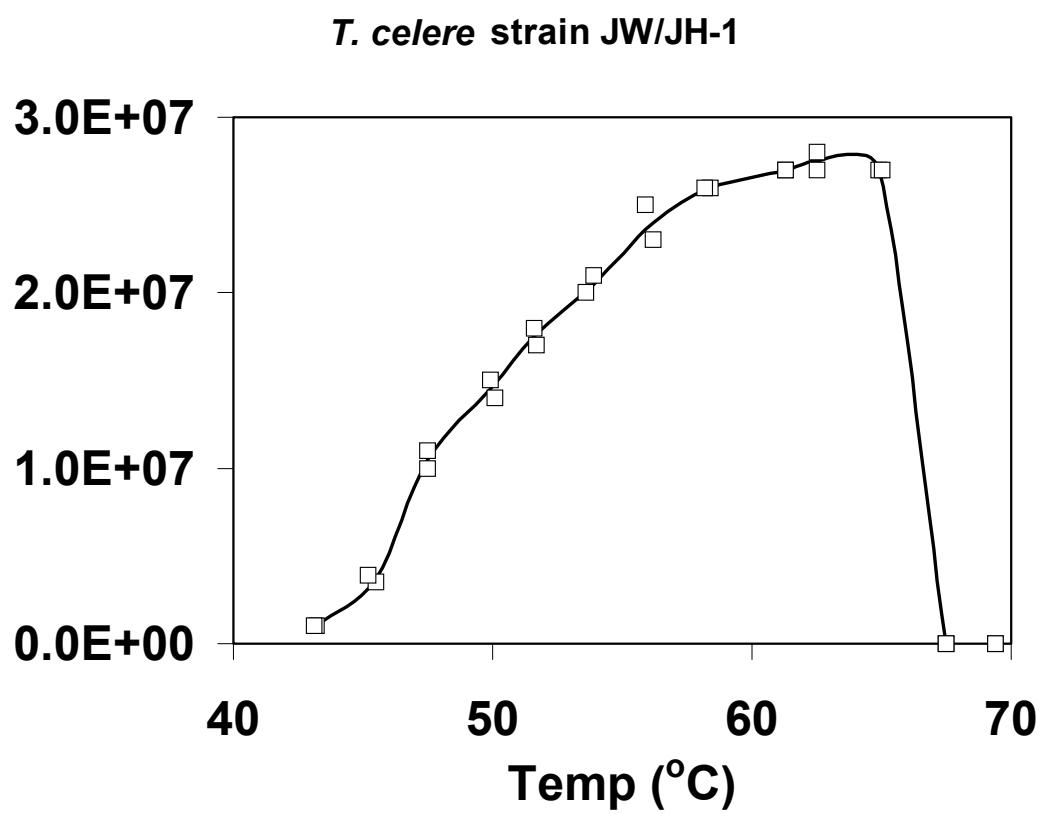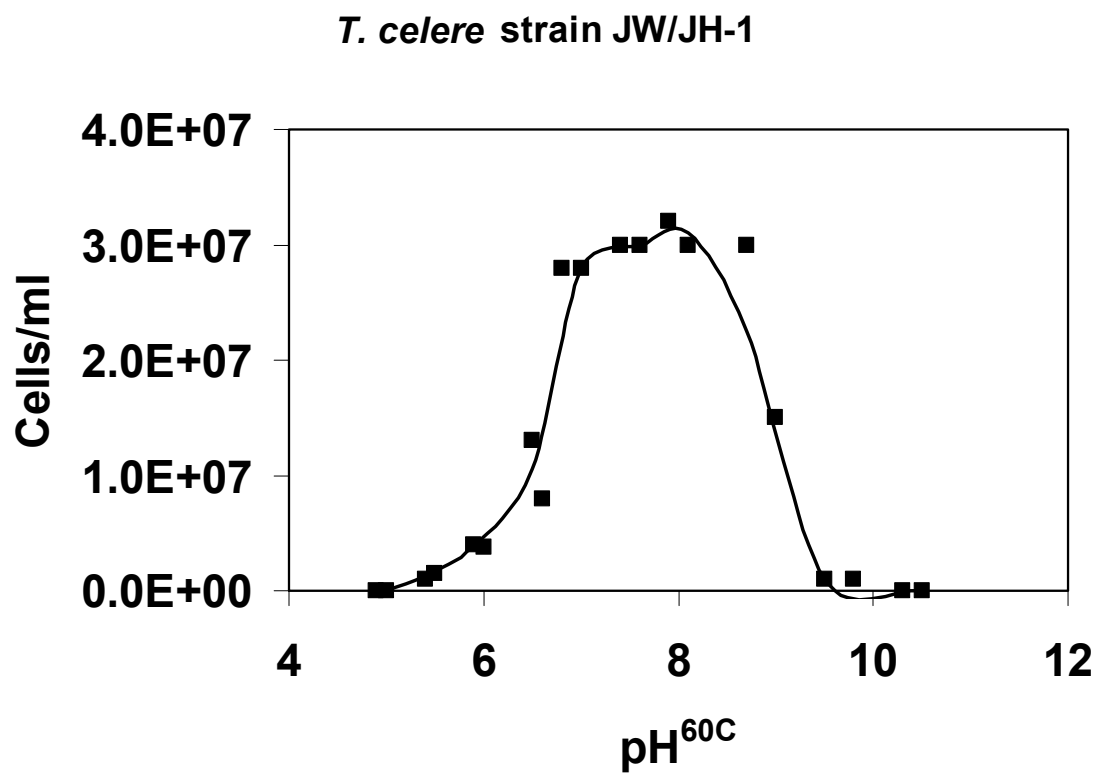
A

B

Fig. B.2. Influence of incubation **(A)** temperature and **(B)** pH on growth of JW/JH-1. **A.** Media with a pH^{60C} 7.3 was inoculated with 1×10^6 cells/ml. After 24 hours of incubation with shaking (20 rpm), cells were counted using a Petroff-Hausser counting chamber. **B.** Mineral media was inoculated with 1×10^6 cells/ml and incubated for 24 hours at 60°C with shaking (20 rpm).

A

T. celere strain JW/JH-1

B

T. celere strain JW/JH-1

Fig. B.3. Influence of incubation **(A)** temperature and **(B)** pH on growth of JW/JH-Fiji-1. **A.** Media with a pH^{60C} 7.3 was inoculated with 1×10^6 cells/ml. After 24 hours of incubation with shaking (20 rpm), cells were counted using a Petroff-Hausser counting chamber. **B**. Mineral media was inoculated with 1×10^6 cells/ml and incubated for 24 hours at 60°C with shaking (20 rpm).

A

C. thermobutryricum strain JW/JH-Fiji-1

B

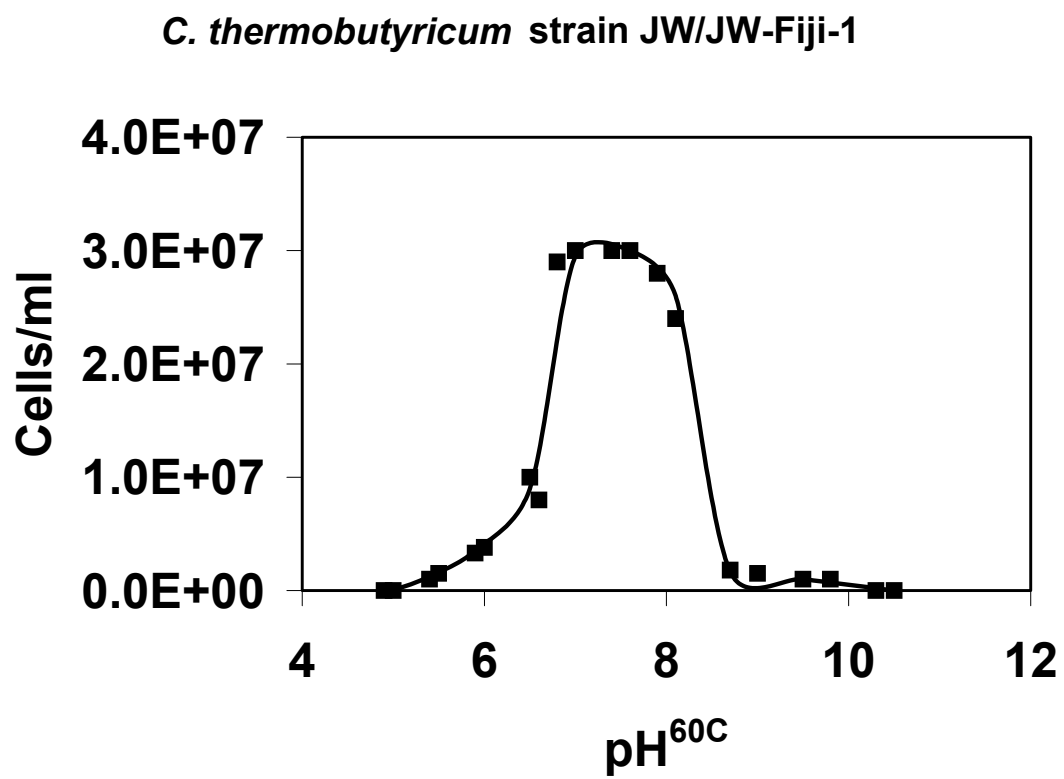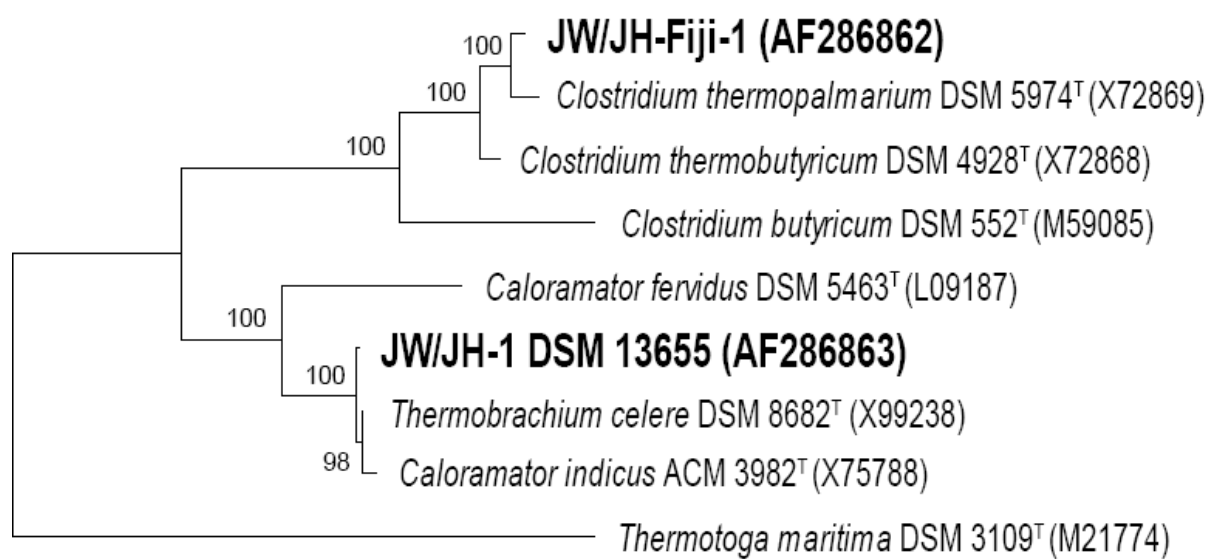*C. thermobutyricum* strain JW/JW-Fiji-1
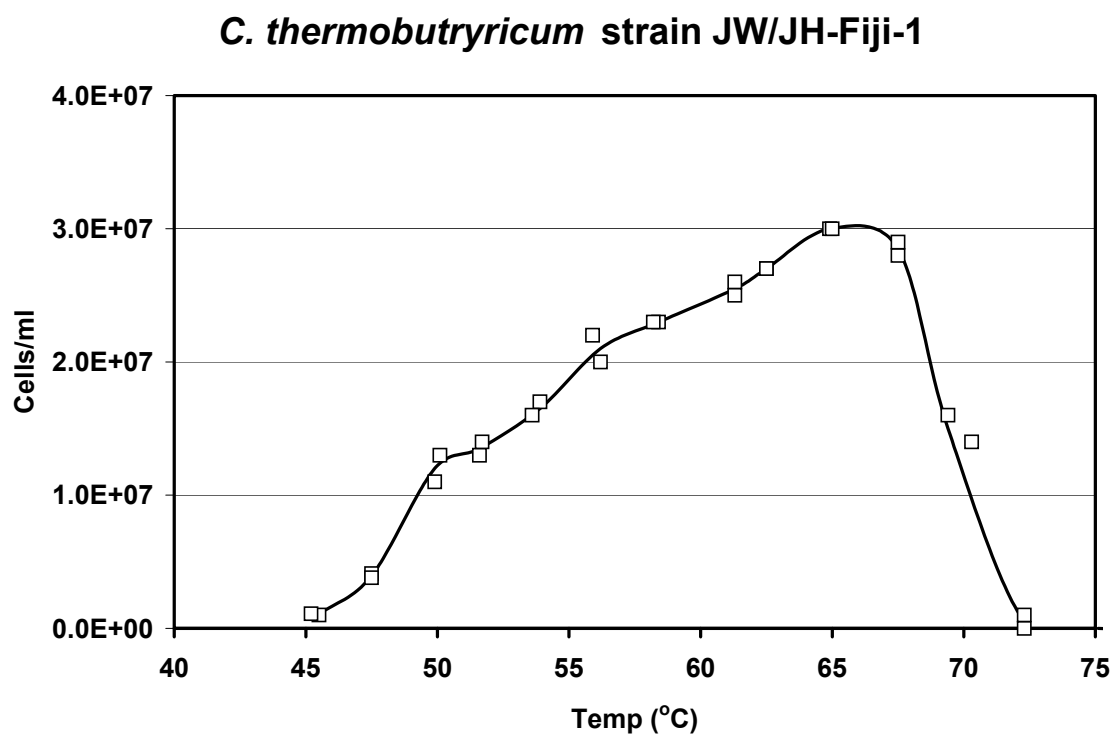
Fig. B.4. Fitch tree showing the estimated phylogenetic relationships of strains JW/JH-1 and JW/JH-Fiji-1 based on 16S rRNA gene sequence data with Jukes-Cantor correction for synonymous changes. The 16S rRNA gene data used represent *Escherichia coli* DSM30083[T] nucleotide positions 105–1450. Numbers at nodes indicate bootstrap support percentages for 100 replicates. Bar, 0.02 nucleotide substitutions per site. GenBank accession numbers are indicated after the strain identifier. The superscript "T" denotes the strain is the type strain for the species. The thermophilic, iron-reducing bacteria are indicated in bold.