Chapter 9

Members of the Order Thermotogales: From Microbiology to Hydrogen Production

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Summary

Members of the deep-branching order *Thermotogales* are widespread in various terrestrial, submarine and subterrestrial extreme environments. This bacterial order included both thermophilic and hyperthermophilic anaerobic microorganisms so far pertaining to ten genera. It is only recently (2011) that cultivation of a mesophilic member of this order belonging to a novel genus, *Mesotoga*, has been successful. All members, with the exception of Mesotoga spp., are recognized as high hydrogen producers having possible applications in biotechnology with a peculiar emphasis for members of the genus *Thermotoga* (e.g. *T. maritima* and *T. neapolitana*). The ecology, phylogeny and metabolism linked to hydrogen production of these bacteria, are reviewed.

I. Introduction

Members of the order *Thermotogales* (Fig. 9.1) were represented as a deep-branching lineage within the phylogenetic tree (Huber et al. 1986; Reysenbach et al. 2001; Huber and Hannig 2006) thus suggesting that representatives of this order might have arisen early during the first steps of bacterial evolution. However the phylogenetic position of *Thermotogales*, as the nature (hyperthermophile or mesophile) of the ancestor of *Bacteria*, still remains a matter of debate (Brochier and Philippe 2002; Zhaxybayeva et al. 2009). Most of these Gram-negative anaerobic bacteria possess an outer sheath like structure called a "toga" ballooning over the ends of the cell (e.g. *Thermotoga* and *Thermosipho* spp.) (Huber et al. 1986; Antoine et al. 1997). Terminal protuberances on one end of the cells and single sphere containing several cells have also been observed in particular in *Fervidobacterium* spp. (Patel et al. 1985). *Thermotogales* range from thermophiles to hyperthermophiles having optimum temperature for growth above 80 °C with possible growth up to 90 °C (e.g. *Thermotoga maritima*, *T. neapolitana*, and *T. hypogea*). They are recognized as non-sporing rods occurring singly, in pairs or in chains with the absence of meso-diaminopimelic acid in the peptidoglycan (Reysenbach et al. 2001; Huber and Hannig 2006). This order comprises ten genera: *Thermotoga*, *Thermosipho*, *Fervidobacterium*, *Geotoga*, *Petrotoga*, *Marinitoga*, *Thermococcoides* and the recently described genera *Kosmotoga*, *Oceanotoga* and *Defluviitoga* (Di Pippo et al. 2009; Jayasinghearachchi and Lal 2011; Ben Hania et al. 2012). Because of the almost identical 16S rRNA gene sequences of *K. olearia* and *Thermoccoides shengliensis*, and their many shared phenotypic features, *T. shengliensis* has been proposed to be reclassified within the genus *Kosmotoga* and named *K. shengliensis* (Feng et al. 2010; Nunoura et al. 2010). Recently, a mesophilic lineage (*Mesotoga*) within the *Thermotogales* has been evidenced by the detection of *Thermotogales* 16S rRNA gene sequences in many mesothermic environments (Nesbø et al. 2006, 2010). The mesophilic nature of such microorganisms has been established by their isolation and cultivation in 2011 ("*Mesotoga sulfurireducens*" strain PhosAc3, Ben Hania et al. 2011), in 2012 (*Mesotoga prima* strain MesG1.Ag.4.2, Nesbø et al. 2012) and in 2013 (*Mesotoga infera* strain VNs100T, Ben Hania et al. 2013) with *Mesotoga prima* being the first described representative and type species of genus *Mesotoga* (Nesbø et al. 2012). All isolated *Mesotoga* species were confirmed to grow optimally at mesothermic conditions (40 °C for "*Mesotoga sulfurireducens*", 45 °C for *M. infera* and 37 °C for *M. prima*) (Ben Hania et al. 2011, 2013; Nesbø et al. 2012) making them microorganisms of notable interest to understand bacterial evolution from mesophily to thermophily or *vice versa* (Nesbø et al. 2006; Ben Hania et al. 2011).

Abbreviations: CMC – Carboxy methyl cellulose; GAP deh – Glyceraldehyde-3-phosphate dehydrogenase; GghA – 1,4-β-D-glucan glucohydrolase; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MBS – Metabisulfite; NRO – NADH oxidoreductase; ORF – Open reading frames; RET – Reversed electron transport; ROS – Reactive oxygen species

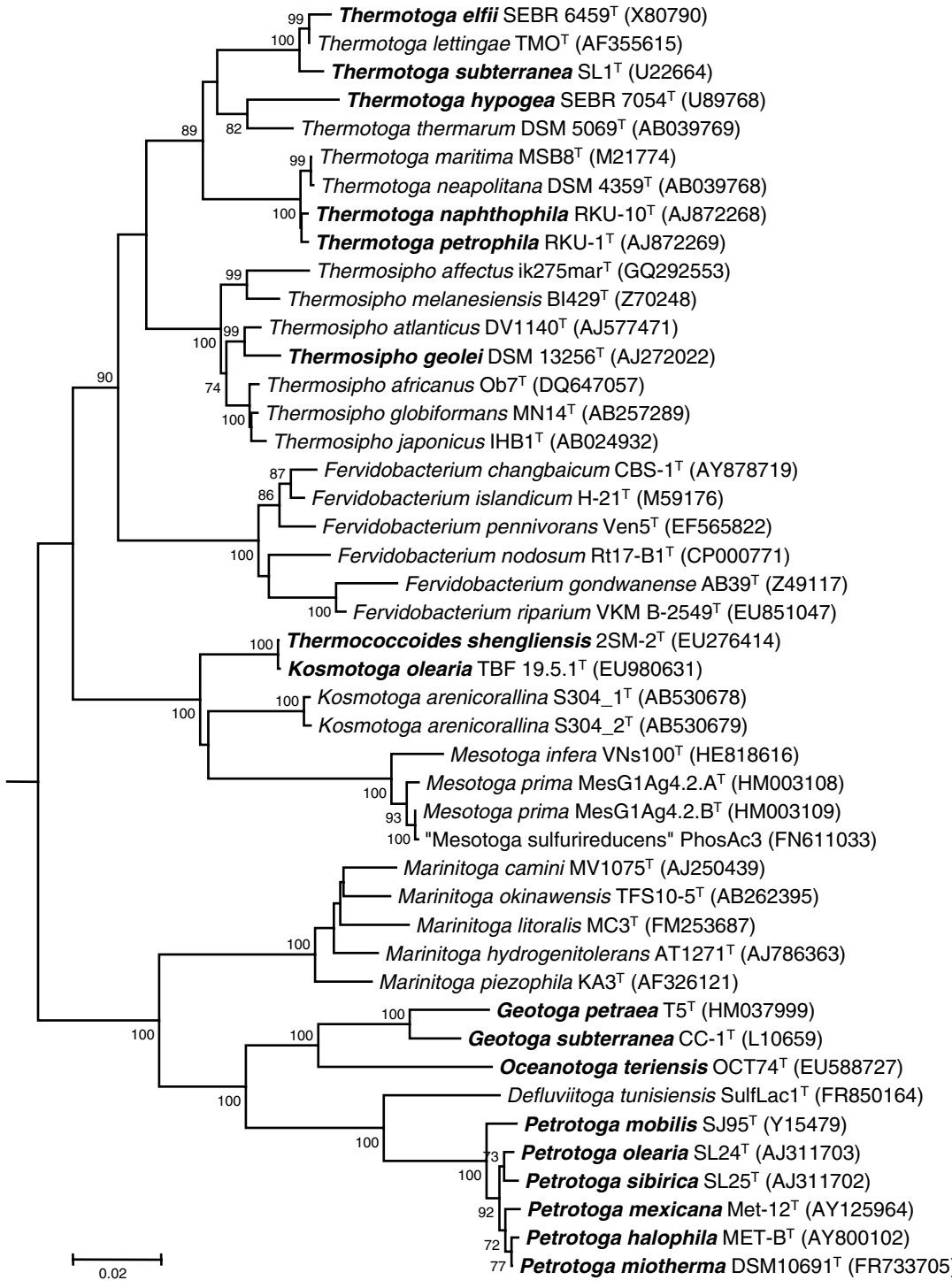

Fig. 9.1. Phylogenetic tree based on 16 rRNA gene sequences representing the position of oilfield microorganisms (*bold characters*) within the order *Thermotogales*. Neighbor joining method was used, calculation from 1,190 aligned pb, bootstrap from 1,000 replicates. *Aquifex aeolicus* (AJ309733) and *Bacillus subtilis* (K00637) were used as outgroup (not shown). *Bar scale*, 0.02 substitution per nucleotide.

II. Habitat

Most of the thermophilic *Thermotogales* have been detected by molecular approaches and/or cultural approaches from geothermally heated environments (Table 9.1) (Huber and Hannig 2006). Most *Fervidobacterium* spp. have been isolated from low saline terrestrial hot springs (Patel et al. 1985; Huber et al. 1990; Andrews and Patel 1996; Friedrich and Antranikian 1996), which is also the case of *Thermotoga thermarum* (Windberger et al. 1989). Numerous slightly halophilic bacteria pertaining to the genera *Marinitoga* (Wery et al. 2001; Alain et al. 2002; Postec et al. 2005, 2010; Nunoura et al. 2007) and *Thermosipho* originated from marine ecosystems (e.g. deep-sea hydrothermal vents) (Huber et al. 1989; Antoine et al. 1997; Takai and Horikoshi 2000; Urios et al. 2004). Many thermophilic *Thermotogales* have been recovered from oilfield waters and facilities (Ollivier and Cayol 2005). They include *Thermotoga* spp. (*T. elfii*, *T. petrophila*, *T. hypogea*) (Magot et al. 2000; Ollivier and Cayol 2005), and *Kosmotoga olearia* (Di Pippo et al. 2009); species of both these genera were also isolated from submarine thermal vents (e.g. *T. neapolitana* and *K. arenicorallina*) (Windberger et al. 1989; Nunoura et al. 2010). Interestingly *Petrotoga*, *Geotoga*, and *Oceanotoga* spp. have representatives which originated only from oil reservoirs thus suggesting that such bacteria might be indigenous to these extreme environments and might possibly be relevant for enhancing oil recovery in the petroleum industry (Magot et al. 2000; Ollivier and Cayol 2005; Jayasinghearachchi and Lal 2011). However the indigenous character of *Thermotogales* to the oil field ecosystems should be considered with caution as their presence may result from anthropogenic activities after drilling operations or water injections during oil exploration (Magot et al. 2000). Besides geothermally heated sediments, only a few thermophilic *Thermotogales* have been isolated from anaerobic digesters. These include *Thermotoga lettingae* (Balk et al. 2002) and the recently described *Defluviitoga tunisiensis* (Ben Hania et al. 2012). *Thermotoga lettingae* was isolated from a thermophilic sulfate-reducing bioreactor operating at 65 °C with methanol as the sole substrate. *D. tunisiensis* was isolated from a mesothermic bioreactor (37 °C) treating lactoserum and phosphogypsum (Balk et al. 2002; Ben Hania et al. 2012). As mentioned above, there is now evidence for the presence of *Thermotogales* (*Mesotoga*) in mesothermic environments such as enrichment cultures degrading chlorinated compounds, temperate hydrocarbon–impacted sites, oil reservoirs, oil gas storage, anaerobic bioreactors treating solvent-containing pharmaceutical wastewater, and a gas-fed bioreactor treating sulfate- and zinc-rich wastewater (Ben Hania et al. 2011, 2013; Nesbø et al. 2006, 2012). In this respect, we may expect *Mesotoga* spp. to be of ecological relevance in the bioremediation of polluted sites (Ben Hania et al. 2011).

III. Metabolic Features

A. Electron Donors

Members of the *Thermotogales* are considered as heterotrophic fermentative microorganisms able to ferment sugars (Table 9.1) (Reysenbach et al. 2001; Huber and Hannig 2006). All thermophilic *Thermotogales* have been demonstrated to grow on complex organic substrates such as peptone and yeast extract, the latter being required to ferment sugars (Huber and Hannig 2006). Besides monosaccharides (e.g. glucose, fructose, xylose), di- and trisaccharides (e.g. sucrose, lactose, cellobiose, raffinose), *Thermotogales* can also ferment polysaccharides (Huber and Hannig 2006). Their use of cellulose has been reported, in particular for *Thermotoga maritima*, *T. neapolitana*, *Fervidobacterium islandicum*, and *Marinitoga camini* (Huber et al. 1986, 1990; Windberger et al. 1989; Wery et al. 2001; Huber and Hannig 2006). *Thermotoga maritima*, *T. neapolitana*, and *M. camini* were also reported to ferment glycogen (Huber et al. 1986; Windberger et al. 1989; Wery et al. 2001; Huber and Hannig 2006). The use of starch has been reported many times within the *Thermotogales* (e.g. *Thermotoga*, *Geotoga*,

Table 9.1. Characteristics of ten genera of Thermotogales order.

Characteristic	"*Mesotoga*"	*Defluviitoga*	*Oceanotoga*	*Kosmotoga*	*Marinitoga*	*Geotoga*	*Petrotoga*	*Thermosipho*	*Thermotoga*	*Fervido-bacterium*
Isolation source (s)	Sediments, Mesothermic, Bioreactor	Mesothermic, Bioreactor	Oil reservoir	Oil reservoir, Shallow hydrothermal vent	Hydrothermal vents	Oil reservoirs	Oil reservoirs	Hydrothermal vents and oil reservoir	Hydrothermal vents, oil reservoirs and bioreactor	Terrestrial hot springs
Temperature optimum (°C)	37–40 °C	55	55–58	65	55–65	45–50	55–60	65–75	65–80	65–70
Oxygen tolerance (v/v)	up to 2 %	up to 0.5 %	nd	Growth at 15 % O_2	No growth at 4 %	Strict anaerobes	No growth with 0.2–1 %	No growth at 0.2–8 %	Do not grow aerobically	Strict anaerobes
Electron acceptors										
Elemental sulfur	+/–	+	+	–	+	–	+	+/–	+/–	+
Thiosulfate	+/–	+	+	+	+/–	nd	+/–	+/–	+	+/–
Nitrate	–	nd	–	–	–	nd	–	–	nd	nd
Nitrite	–	nd	nd	–	–	nd	–	–	nd	nd
Sulfite	+/–	–	nd	–	–	nd	+/–	–	nd	nd
Cystine	–	nd	nd	–	+	nd	–	+/–	+/–	nd
Sulfate	–	–	–	+[a]	–	nd	–	–	–	–

(continued)

Table 9.1. (continued)

Characteristic	"*Mesotoga*"	*Defluviitoga*	*Oceanotoga*	*Kosmotoga*	*Marinitoga*	*Geotoga*	*Petrotoga*	*Thermosipho*	*Thermotoga*	*Fervido-bacterium*
Substrate utilization										
Complex organic compounds	+	+	+	+	+/–	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+	+
Alcohols	nd	nd	nd	nd	nd	nd	nd	nd	+	+
Metabolic products	**Acetate**, butyrate, isobutyrate, isovalerate, 2-methyl-butyrate[b]	**Acetate, H_2, CO_2**	**Acetate, H_2, CO_2**, ethanol[b]	**Acetate, H_2, CO_2**, ethanol, isobutyrate, isovalerate[b]	**Acetate, H_2, CO_2**, lactate, isobutyrate, isovalerate[b]	**Acetate, H_2, CO_2**, ethanol[b]	**Acetate, H_2, CO_2**, ethanol[b]	**Acetate, H_2, CO_2**, L-alanine[b]	**Acetate, H_2, CO_2**, lactate, L-alanine[b]	**Acetate, H_2, CO_2**, L-alanine[b]
DNA G+C content (mol%)	45.3	33.6	26.8	42.5	28–29	29.5–29.9	31–39.8	29–33	39.2–50	33.7–40

Data were taken from Ben Hania et al. (2011, 2013); Nesbø et al. (2012) ("*Mesotoga*"); Ben Hania et al. (2012) (*Defluviitoga*); Jayasinghearachchi and Lal (2011) (*Oceanotoga*); Alain et al. (2002); Nunoura et al. (2007); Postec et al. (2005) and Wery et al. (2001) (*Marinitoga*); Davey et al. (1993) (*Geotoga*); L'Haridon et al. (2002); Lien et al. (1998) and Miranda-Tello et al. (2004, 2007) (*Petrotoga*); Antoine et al. (1997); Huber et al. (1989); L'Haridon et al. (2001); Takai and Horikoshi (2000) and Urios et al. (2004) (*Thermosipho*); Balk et al. (2002); Fardeau et al. (1997); Huber et al. (1986); Jannasch et al. (1988); Jeanthon et al. (1995); Ravot et al. (1995); Takahata et al. (2001) and Windberger et al. (1989) (*Thermotoga*) and Andrews and Patel (1996); Friedrich and Antranikian (1996); Huber et al. (1990) and Patel et al. (1985) (*Fervidobacterium*)

nd No data available; – does not enhance growth; + enhanced growth; ± enhanced growth for some, but not all, species

[a] Slight growth enhancement, but no sulfide produced

[b] Metabolic products which are written in bold characters are produced by all species. For those which are not written in bold characters, they are produced depending on species

Petrotoga, *Thermosipho*, *Fervidobacterium*, *Marinitoga*, *Kosmotoga* spp.) (Huber and Hannig 2006). Xylanolytic activity has been detected in *T. maritima*, *T. hypogea*, and *Petrotoga* spp. (e.g. *P. mobilis* and *P. olearia*) (Huber et al. 1986; Davey et al. 1993; Fardeau et al. 1997; Lien et al. 1998). *Marinitoga camini* was shown to degrade chitin and *F. pennovorans* to degrade keratin (Friedrich and Antranikian 1996; Wery et al. 2001).

The alcohols possibly fermented by *Thermotogales* include mannitol (e.g. *T. naphtophila*), and glycerol (e.g. *T. lettingae, T. neapolitana* and *F. nodosum*) (Patel et al. 1985; Takahata et al. 2001; Van Ooteghem et al. 2004; Huber and Hannig 2006). Methanol was poorly fermented by *T. lettingae*, however, in the presence of thiosulfate or elemental sulfur as terminal electron acceptors, or methanoarchaea as hydrogenotrophic partners, methanol was more quickly used (12 days for the oxidative process instead of 30 days for the fermentative process) (Balk et al. 2002). Methanol utilization has also been demonstrated for *T. subterranea*, *T. elfii*, *T. maritima* and *T. neapolitana* by the same authors (Balk et al. 2002).

Regarding organic acids utilization, pyruvate served as energy source for some *Thermotoga* and *Petrotoga* spp. (Huber and Hannig 2006), and there is one report on formate utilization by *Thermotoga lettingae* (Balk et al. 2002). Besides formate, which was used only in the presence of thiosulfate as electron acceptor, *T. lettingae* was the first *Thermotogales* reported to ferment lactate (Balk et al. 2002). Thereafter, the use of lactate has been also evidenced in "*Mesotoga sulfurireducens*" and in *M. infera* where the presence of elemental sulfur as terminal electron acceptor was required thus suggesting that lactate was oxidized, but not fermented by this bacterium (Ben Hania et al. 2011, 2013). *T. lettingae* was also shown to oxidize acetate in the presence of thiosulfate or hydrogenotrophic methanoarchaea as terminal electron acceptors in agreement with results for previously described thermophilic or mesophilic acetate-degrading bacteria coupled to a methanogenic partner or an electron acceptor (Balk et al. 2002).

B. Electron Acceptors

Thermotogales do not only use a wide range of organic substrates as energy sources but have also the ability to reduce sulfur-containing compounds (e.g. elemental sulfur, and thiosulfate, but not sulfate) into sulfide (Table 9.1) (Huber and Hannig 2006). Amongst *Thermotogales* genera, *Geotoga* is the only genus in which no thiosulfate-reducing species was reported (Davey et al. 1993). The ability of *Thermotogales* to reduce elemental sulfur in the presence of sugars was first reported for *T. maritima* and was found stimulatory for its growth (Huber et al. 1986). However, this reductive process was suggested to be a detoxifying process preventing H_2 accumulation rather than an energy-yielding electron sink reaction (Huber et al. 1986; Huber and Hannig 2006). A similar conclusion could have been drawn regarding thiosulfate reduction by *Thermotogales*. However growth experiments with *T. maritima* and *T. neapolitana* in the presence of thiosulfate suggested that thiosulfate reduction could be regarded as an energy-yielding reaction through an oxidative phosphorylation process from sugars (Ravot et al. 1995). Indeed, for both bacteria, despite important improvements of growth observed in the presence of thiosulfate, there was no significant change in the end-products of sugar metabolism (Ravot et al. 1996). Besides thiosulfate and elemental sulfur, Fe(III) was also used as electron acceptor by *T. maritima* and in its presence, hydrogen oxidation was demonstrated (Vargas et al. 1998). While one study indicated that *T. maritima* may gain energy by iron respiration (Vargas et al. 1998), other reports suggested Fe(III) as an additional electron sink together with thiosulfate and elemental sulfur to prevent the inhibitory effect of hydrogen on growth (Huber et al. 1986; Schröder et al. 1994; Huber and Stetter 2001). The ability of *T. maritima* to reduce Fe(III) raises questions on its possible involvement in reducing heavy metals and should merit further attention. In the presence of thiosulfate as terminal electron acceptor, *T. lettingae* was shown to oxidize

hydrogen (Balk et al. 2002). While the possible use of cystine as electron acceptor has been established for *Marinitoga*, *Thermotoga,* and *Thermosipho* species (Huber and Hannig 2006), only *T. lettingae* was reported to use anthraquinone −2,6-disulfonate as electron acceptor (Balk et al. 2002). Results obtained with *Thermotogales* regarding the possible use of sulfur compounds as electron acceptors, indicate they may play a crucial ecological role in mineralizing organic matter in hot ecosystems. This is particularly true for *Thermotogales* originating from shallow or deep sea hydrothermal vents (e.g. *Thermosipho* and *Marinitoga* spp.) where different oxidized forms of sulfur compounds, including elemental sulfur and thiosulfate, are not limiting.

C. End-Products of Metabolism

The major end-products of sugar metabolism by thermophilic *Thermotogales* are acetate, hydrogen, and CO_2 (Table 9.1) (Reysenbach et al. 2001; Huber and Hannig 2006). Surprisingly, while the recent isolated mesophiles "*Mesotoga sulfurireducens*", *M. prima* and *M. infera* were shown to produce mainly/only acetate from sugar utilization, there is no report on H_2 production by these microorganisms (Ben Hania et al. 2011, 2013; Nesbø et al. 2012). Moreover, in contrast to *M. prima* which was described as a fermentative bacterium (Nesbø et al. 2012), *M. infera* was shown to rather oxidize its substrates in the presence of elemental sulfur as terminal electron acceptor (Ben Hania et al. 2013). Further researches on the physiological traits of *Mesotoga* spp. will be therefore of interest to understand the overall metabolic capabilities within the *Thermotogales*. It was found, for example, that acetate was also produced during methanol fermentation by *Thermotoga lettingae* (Balk et al. 2002). Lactate production was detected in particular by *T. maritima* and was dependent on culture conditions (e.g. H_2 partial pressure) (Janssen and Morgan 1992; Schröder et al. 1994). It was also produced by *Marinitoga camini* when growing on sugars (Wery et al. 2001). Ethanol has been measured at many occasions (e.g. *Geotoga*, *Petrotoga*, *Kosmotoga*, and *Oceanotoga* spp.) together with isovalerate, isobutyrate, and/or propionate (e.g. *M. camini*, *K. olearia*), but also alpha-aminobutyrate, hydroxyphenyl-acetate or phenylacetate (Huber and Hannig 2006) as end-products of sugar metabolism. Studies with *T. maritima* indicated that this bacterium used glucose *via* the Embden-Meyerhof glycolytic pathway and, to a lesser extent, *via* the Entner-Doudouroff pathway (Schröder et al. 1994; Selig et al. 1997; Huber and Hannig 2006). However the importance of the use of both pathways in other *Thermotogales* when fermenting sugars is still poorly documented and deserves further investigation. Notably, it was demonstrated in *T. neapolitana* that glucose was taken up *via* an active transport system that was energized by an ion gradient generated by ATP derived from substrate-level phosphorylation (Galperin et al. 1996; Huber and Hannig 2006). It is established, that several *Thermotogales* have a high ratio of acetate produced *versus* sugar consumed, thus indicating that they are good candidates for H_2 production from the biomass approaching the theoretical maximum yield of 4 mol H_2 per mol glucose consumed (Schröder et al. 1994; Van Ooteghem et al. 2004; Eriksen et al. 2011). This is emphasized by the capacities of many species to use various carbohydrates including cellulose, hemicelluloses and starch together with proteinaceous compounds. Besides acetate, lactate and hydrogen, L-alanine was also found as a significant end-product of sugar fermentation by *Thermotoga elfii*, *Fervidobacterium islandicum, F. nodosum*, *F. gondwanense*, and *Thermosipho africanus* with up to 0.52 mol L-alanine produced per mol glucose consumed (*T. africanus*). In contrast, *T. maritima* and *T. neapolitana* were found to be poor L-alanine producers (Ravot et al. 1996). In the presence of thiosulfate, a decrease of the L-alanine/acetate ratio was observed for *F. islandicum*, *T. africanus*, *T. elfii*, *Thermotoga* SEBR 7054, and

Thermotoga lettingae (Ravot et al. 1996; Balk et al. 2002). For all these bacteria, the presence of thiosulfate caused a shift of metabolism with more acetate and less L-alanine being produced from sugars thus enabling them to obtain more ATP from substrate level phosphorylation *via* the formation of acetyl-CoA. It was hypothesized that similarly to the hyperthermophilic archaeon, *Pyrococcus furiosus*, L-alanine production from glucose fermentation resulted from alanine transferase activity coupled with glutamate dehydrogenase activity (Ravot et al. 1996). Therefore, because of a similar type of sugar metabolism by members of the *Thermococcales* (e.g. *Pyrococcus furiosus* and *Thermococcus profundus*), domain *Archaea*, placed as a deep-branching lineage within the phylogenetic tree, L-alanine production by *Thermotogales* has been interpreted as a remnant of an ancestral metabolism (Ravot et al. 1996). Interestingly, L-alanine was also produced when *T. lettingae* grew on methanol as energy source in the presence of thiosulfate or elemental sulfur and this was the first report of L-alanine formation from a C1 substrate. In contrast, in the presence of a methanogenic partner (e.g. *Methanothermobacter thermoautotrophicus*) methanol was completely oxidized to CO_2 (Balk et al. 2002). A complete oxidation of acetate was also observed when *T. lettingae* was cocultured with an hydrogenotrophic methanoarchaea, while L-alanine was produced from acetate in the presence of thiosulfate (Balk et al. 2002).

D. Oxygen Tolerance

Although *Thermotogales* are recognized as strict anaerobes, there are evidences that they may cope with limited amount of oxygen during their growth (Table 9.1). *T. maritima* may grow in the presence of 0.5 % of oxygen (Le Fourn et al. 2008), *T. neapolitana* was shown to grow under oxygen concentrations ranging from 1 to 6 % (Van Ooteghem et al. 2004) (see Sect. IV.A). *Kosmotoga olearia*, an oilfield isolate, was reported to grow in the presence of 15 % oxygen (Di Pippo et al. 2009). This indicates that *Thermotogales* may use biochemical strategy(ies) to face oxidative stress as already reported for other strict anaerobes (e.g. sulfate-reducing bacteria) (Krekeler et al. 1998; Teske et al. 1998; Cypionka 2000; Dolla et al. 2006). *Thermotoga maritima* has been studied in recent years with regard to the strategy(ies) possibly used to deal with O_2 and reactive oxygen species (ROS) such as peroxides. Yang and Ma (2005) have purified and characterized a heterodimeric NADH oxidase catalyzing oxygen to hydrogen peroxide. They proposed this enzyme together with other hydrogen peroxide scavenging enzymes as acting in an oxygen-removing system. Le Fourn and collaborators (2008) using differential proteomics analyses identified a flavoprotein, homologous to the rubredoxin oxygen reductase (FprA) of *Desulfovibrio* sp. that was overproduced when *T. maritima* was cultivated under oxic conditions. They provided evidence that by reducing oxygen to water, this enzyme had a crucial role in protecting the bacterium against oxygen and suggested that NADH oxidase and rubredoxin oxygen reductase were involved in this process (Le Fourn et al. 2008). However, they outlined that the direct reduction of oxygen to water by FprA might be a preferred system as compared to the production of hydrogen peroxide, known to be highly toxic to cells. Later on, Le Fourn et al. (2011) showed that *T. maritima* cells could consume oxygen at a rate of 41.5 nmol min^{-1} per mg of total protein and demonstrated that this bacterium reduced oxygen *via* a three-partner chain involving an NADH oxidoreductase (NRO), a rubredoxin (Rd) together with the rubredoxin oxidoreductase mentioned above (FprA), known as a flavor-diiron protein. They concluded that the genes coding for the three components O_2 reduction system were acquired by the *Thermococcales*, domain *Archaea*, through a single horizontal gene transfer event (Le Fourn et al. 2011). Because of the position of *Thermotogales* and *Thermococcales* within the phylogenetic tree, it has been suggested that such mechanism has been important for the first anaerobes to adjust to the presence of

traces of oxygen on the "primordial" Earth. Oxygen uptake has also been observed when *T. maritima* was grown in a 2.3-L bioreactor under controlled oxygen exposure (Lakhal et al. 2010). Transcriptomic analysis, indicated that when exposed to oxygen for a short time, *T. maritima* had to deal with oxygen but also with the peroxides produced. The oxygen reductase FprA appeared as primary consumer of oxygen, followed by alkyl hydroperoxide reductase and peroxiredoxin-encoding genes as main ROS-scavenging systems when higher concentrations of O_2 were reached (Lakhal et al. 2011). It is noteworthy that the expression of the gene *hyd* that encodes the single hydrogenase of *T. maritima* was drastically affected by the presence of oxygen (Lakhal et al. 2011). These data are in accordance with the known sensitivity of hydrogenases towards oxygen (Vincent et al. 2005). Regarding the anaerobic metabolism of *T. maritima*, batch cultures indicated that it significantly decreased the redox potential (E_h) of the culture medium to about −480 mV (Lakhal et al. 2010) similarly to what was observed for the strict anaerobic methanoarchaea (Fetzer and Conrad 1993). Finally, under oxidative conditions, Lakhal et al. (2010) observed that glucose consumption rate by *T. maritima* decreased with a concomitant shift in glucose metabolism towards lactate production.

From these results, we may conclude that despite the high sensitivity of *T. maritima* to oxygen, this bacterium adapted an adequate strategy to face exposures to this gas (see Sect. IV.B for more details). This may explain why this hyperthermophilic bacterium can survive and even grow in shallow hydrothermal vents where partially oxygenated conditions cannot be precluded.

E. Hydrogen Sensitivity

Hydrogen which accumulates during the fermentation processes of carbohydrates by *Thermotogales*, with the exception of *Mesotoga* spp. (see also § III.D) is known to inhibit the growth of most of them (Huber and Hannig 2006). No growth occurred when cultures of *T. maritima* were pressurized with hydrogen-containing gas (H_2/CO_2 = 80:20; 300 kPa) (Huber et al. 1986). Similar observations were done with *Thermotoga thermarum* and *T. neapolitana* (Windberger et al. 1989), *Petrotoga miotherma*, *Geotoga petraea*, and *Thermosipho melaniensis* (Davey et al. 1993; Antoine et al. 1997). However for all these microorganisms, growth inhibition could be overcome by gassing the headspace with N_2 or N_2/CO_2 or by the addition of S° or thiosulfate (e.g. *Petrotoga mobilis*) which served as an electron acceptor in the culture medium; concomitantly H_2S was formed. Depending on microorganisms (see comments above), the addition of elemental sulfur and/or thiosulfate may or may not have a stimulatory effect on growth. H_2 removal in the gas phase may also result from co-cultures of *Thermotogales* with thermophilic to hyperthermophilic methanoarchaea (e.g. *Methanococcus*, *Methanopyrus* spp.) or other hydrogen oxidizing archaeons (e.g. *Archaeoglobus* or *Ferroglobus* spp.) (Huber and Hannig 2006). Some genera are hydrogen tolerant. *Geotoga subterranea* was not inhibited by the hydrogen concentration mentioned above (Davey et al. 1993). *Marinitoga camini* and *M. piezophila* reached maximum cell concentrations with 0 % H_2 while no growth occurred with 80 % H_2 (Wery et al. 2001; Alain et al. 2002), *M. hydrogenotolerans* can grow in the presence of 100 % H_2 in the gas phase (Postec et al. 2005) and this makes it the most hydrogen tolerant member of the *Thermotogales*. This confirms that *Thermotogales*, depending on species, have different H_2 sensitivities as reported earlier by Ravot et al. (1996).

IV. Hydrogen Production by *Thermotogales* spp.

A. Thermodynamic Features

The organisms described in this chapter are mainly thermophilic or hyperthermophilic anaerobes with temperature optima above 65 °C. As stated in the preceding paragraphs,

many of these, such as *Thermotoga maritima* and *T. neapolitana* are capable of performing fermentative hydrogen production from a wide variety of substrates. Indeed, thermophilic hydrogen production benefits from some general advantages of performing such a process at elevated temperatures thank to a lower viscosity, better mixing, less risk of contamination, higher reaction rates and no need for reactor cooling (Wiegel et al. 1985). As already stated in Chap. 1, to make hydrogen production economically sustainable, organisms are needed that can generate hydrogen or directly or indirectly from biomass. As cellulose and hemicellulose are the most abundant polysaccharides in nature, xylose and glucose are the predominant monomeric sugars available (Kapdan and Kargi 2006). Further, starch and sucrose can be abundantly present in plants as storage material. Interestingly, the bacterial species belonging to *Thermotoga* have the capacity to hydrolyze most of the substrates derived from biomass. For example, as also reported in Chap. 8, both *Caldicellulosiruptor* and *Thermotoga* spp. contain a variety of glycoside hydrolases and transferases stating their metabolic capacity (Vanfossen et al. 2008).

A few thermodynamic considerations clearly indicate that under standard conditions (reactants concentration equal to 1 M, at 25 °C and pH 7.0), glucose oxidation to CO_2 and H_2 has a positive Gibbs energy change (reaction 9.1). This means that H_2 production requires an input of extra energy.

$$\begin{aligned} \text{Glucose} + 12H_2O \rightarrow\ & 6HCO_3^- \\ & + 6H^+ + 12H_2 \end{aligned} \qquad (9.1)$$

$$\Delta G^{0'} = +3.2\,\text{KJ/mol}$$

As shown in Table 9.2, most of the available literature reports that under optimal growth conditions, the oxidation of one hexose molecule will result in the formation of a variable number of hydrogen molecules ($>2 \leq 4$) in addition to acetate and CO_2. The maximum amount of ATP is obtained through production of acetate but this can only occur if all the reducing equivalents are disposed in the form of hydrogen molecules. Apparently, the *a priori* requirement to get a significant hydrogen production yield is to keep a low hydrogen partial pressure through the use of a hydrogen-consuming system (Schink and Stams 2006). Under this latter condition, as shown in reaction 9.2, glucose oxidation to acetate, CO_2 and H_2 has a significantly negative Gibbs energy change.

$$\begin{aligned} \text{Glucose} + 4H_2O \rightarrow\ & \text{Acetate} + 2HCO_3^- \\ & + 4H^+ + 4H_2 \end{aligned} \qquad (9.2)$$

$$\Delta G^{0'} = -206.1\,\text{KJ/mol}$$

In most fermentative hydrogen producers, catabolism *via* the Embden-Meyerhof pathway generates reducing equivalents in the form of NADH at the level of the glyceraldehyde-3-phosphate dehydrogenase (GAP deh) and reduced ferredoxin by the pryruvate:ferredoxin oxidoreductase reaction. Under standard conditions, the mid-point potentials of NAD^+/NADH and $ferredoxin^{ox}/ferredoxin^{red}$ are −320 mV and −380 mV, respectively (Thauer et al. 1977). Recycling of these redox couples can be accomplished by various reactions such as, for example, the production of lactate though reduction of pyruvate by NADH; however, the feasibility of these reactions is *a priori* determined by the standard Gibbs free energy change ($\Delta G^{0'}$) of each specific conversion step. This latter consideration predicts that the production of H_2 by reduction of H^+ with NADH is a thermodynamically unfavorable process as expected by the low mid-point potential ($E^{0'} = -414$ mV) of the H^+/H_2 redox couple. Although the situation is more favorable in the case of ferredoxin ($E^{0'} = -380$ mV) the formation of other products such as ethanol and lactate is thermodynamically more feasible. Thus, although the microbial reduction of protons to lead H_2 generation is a metabolic unexpected process, for certain thermophiles (see Table 9.1) the amount of hydrogen produced is close to

Table 9.2. H_2 production rates and H_2 yields from various sugars conversion by *Thermotoga* spp.

Strain	Carbon source	T°	Conc.	Initial pH	Type of reactor	H_2 production rate ($mmol_{H2}/L/h$)	H_2 yield ($mol_{H2}/mol_{substrate}$)	By-products	References
T. maritima	Glucose	80	10–15 mM	6.5	Batch	10.0	4.0	Acetate	Schröder et al. (1994)
T. elfii	Glucose	65	10 g/L	7.5	Controlled batch	2.7	3.3	Acetate	Van Niel et al. (2002)
T. elfii	Glucose	65	7 g/L	7.5	Batch	–	3.3	Acetate	De Vrije et al. (2002)
T. petrophila	Glucose	80	0.1 % w/v	7.0	Batch	–	3.8	Acetate, lactate	Takahata et al. (2001)
T. naphtophila	Glucose	80	0.1 % w/v	7.0	Batch	–	4	Acetate, lactate	Takahata et al. (2001)
T. maritima	Glucose	80	7.5 g/L	6.5	Batch	8.2	1.7	–	Nguyen et al. (2008a)
T. neapolitana	Glucose	75	7.5 g/L	7.0	Batch	8.7	1.8	–	Nguyen et al. (2008a)
T. neapolitana	Glucose	77	7.0 g/L	7.5	Batch	–	3.2	Acetate butyrate	Nguyen et al. (2010a)
T. neapolitana	Glucose	75	5 g/L	7.5	Controlled batch	4.25	3.2	Acetate, lactate	Ngo et al. (2011b)
T. neapolitana	Glucose	85	2.5 g/L	7.5	Batch	0.9	3.8	Acetate, lactate	Munro et al. (2009)
T. neapolitana	Glucose	80	5 g/L	8.0	Batch	–	2.4	Acetate, lactate	Eriksen et al. (2008)
T. maritima	Arabinose	80	5 g/L	8.0	Batch	0.6	3.2	Acetate, lactate	Eriksen et al. (2011)
T. neapolitana	Arabinose	80	5 g/L	8.0	Batch	1.0	3.8	Acetate, lactate	Eriksen et al. (2011)
T. maritima	Xylose	80	5 g/L	8.0	Batch	0.4	2.7	Acetate, lactate	Eriksen et al. (2011)
T. neapolitana	Xylose	80	5 g/L	8.0	Batch	1.5	3.4	Acetate, lactate	Eriksen et al. (2011)
T. neapolitana	Xylose	75	5 g/L	7.5	Controlled batch	3.9	2.8	Acetate, lactate	Ngo et al. (2012)
T. neapolitana	Xylose	75	5 g/L	7.5	Controlled batch	3.3	2.2	Acetate, lactate	Ngo et al. (2011b)
T. neapolitana	Sucrose	75	5 g/L	7.5	Controlled batch	1.9	5.0	Acetate, lactate	Ngo et al. (2011b)
T. neapolitana	Glycerol	75	5 g/L	7.5	Batch	–	1.0	Acetate, lactate	Ngo and Sim (2011)
T. neapolitana	Cellulose	80	0.5 % w/v	7.5	Batch	–	2.2	–	Nguyen et al. (2008b)

four molecules per molecule of glucose oxidized, suggesting that in these microorganisms the thermodynamic constraints are somehow overcome as the growth conditions are quite far from the standard physiological values. Indeed, the actual Gibbs energy change as a function of both the substrates and products concentrations (see Eq. 9.3) predicts that even the reduction of H^+ by NADH becomes exergonic (−4.7 kJ/mol) if the partial H_2 concentration [$P(H_2)$] is kept as low as 10^{-2} kPa.

$$\Delta G = \Delta G + RT\ln\left([C][D]/[A][B]\right) \quad (9.3)$$

Further, the thermodynamic of the process is affected by temperature at which the reaction takes place according to Eq. (9.4)

$$\Delta G^0 = \Delta H - T\Delta S^0 \quad (9.4)$$

which says that at temperatures higher than standard conditions (25 °C), the Gibbs free energy change for the overall reaction from glucose to acetate (Eq. 9.1) is more favorable. A second consideration that might explain why thermophiles show such unexpected H_2 yields, is based on the fact that as previously shown by Thauer et al. (1977) and Amend and Plyasunov (2001), the hydrogen partial pressure needed to make reaction (9.2) feasible varies from 0.022 kPa at 25 °C to 2.2 kPa at 100 °C. Thus, at room temperature, hydrogen must be rapidly removed to avoid the inhibition of reaction (9.2) while the presence of 10^2 higher hydrogen concentration is tolerated at 100 °C.

Another possible explanation for the hydrogen formation from redox couples having a mid-point potential higher than −414 mV ($E^{0'}$ of H^+/H_2) might be the presence of a reversed electron transport (RET) mechanism linked to membrane bound NAD-dependent hydrogenases. Although this mechanism has never been described in thermophiles, its presence in the genus *Clostridium* (*Cl. tetanomorphum*), where a sodium gradient is used to drive the reduction first of ferredoxin and then for hydrogen production (Boiangiu et al. 2005), does not exclude *a priori* that other fermenting bacteria support the uphill reduction of H^+ by NADH through the use of RET. Alternatively, it has clearly been shown by Schut and Adams (2009) that in cells of *Thermotoga maritima* (*T. maritima*) ferredoxin is a more suitable reducing agent for hydrogen production than NADH.

B. The Hydrogenases of Thermotoga *spp.*

As overviewed in Chaps. 2, 3, 4, and 8, the enzymes responsible for hydrogen production (H_2) combining hydrogen protons and reducing equivalents ($2H^+ + 2e^-$) are the hydrogenases (EC 1.12.99.6 and EC 1.12.7.2) also catalyzing the reversible oxidation of molecular hydrogen. In anaerobic thermophiles, two main types of hydrogenases, based on their metal content, are found: [Fe-Fe] and [Ni-Fe] hydrogenases. Further, hydrogenases can use different types of electron carriers, e.g. NAD, NADP, FAD and ferredoxin (Fd), which are reduced in the glycolytic pathway and in particular during the conversions of both glyceraldehyde-3-P to 3-P-glycerate and pyruvate to acetyl-CoA. In most fermentative hydrogen producers, reduced electron carriers generated in these steps (NADH and Fd_{red}) need to be re-oxidized to keep the glycolytic pathway functioning and this disposal mechanism can be different among the different thermophilic hydrogen producers (Jenney and Adams 2008).

As many other bacterial species, *Thermotoga maritima* uses the Embden-Meyerhof pathways for glycolysis resulting in acetate, lactate, ethanol, CO_2 and H_2. However, recycling of reducing equivalents is performed by a trimeric [Fe-Fe] hydrogenase which uses both NADH and Fd_{red} in a 1:1 ratio to generate hydrogen (see Eq. 9.5).

$$\mathrm{NADH} + 2\,\mathrm{Fd_{red}} + 3\mathrm{H^+} \rightarrow \mathrm{NAD^+} + 2\,\mathrm{Fd_{ox}} + 2\mathrm{H_2} \quad (9.5)$$

This so-called "flavin-based bifurcating enzyme" is coupling the exergonic oxidation of Fd_{red} ($E^{0'}$ of $Fd_{ox}/Fe_{red} = -453$ mV) to drive the unfavorable oxidation of NADH ($E^{0'}$ of $NAD^+/NADH + H^+ = -320$ mV) to produce H_2 ($E^{0'} = -420$ mV) (Schut and Adams 2009). As this mechanism is favored by low hydrogen pressures, in the case of higher H_2 pressures, a switch from acetate to lactate production is seen (Huber et al. 1986). This however does not seem to affect the bifurcating mechanism of the hydrogenase, which presumably remains the same.

The anaerobically purified "bifurcating" hydrogenase of *T. maritima*, the enzyme being inactivated in the presence of even trace amounts of oxygen, is composed of three subunits – HydA (73 kDa), HydB (68 kDa) and HydC (18 kDa) – in a 1:1:1 ratio stoichiometry. The holoenzyme showed an apparent molecular mass of 500 kDa at pH 7.0 and one of 150 kDa at pH 10.0. The enzyme contained loosely bound FMN along with more than 30 Fe per heterotrimer in line with sequence analysis prediction (Buckel and Thauer 2012). Based on this latter approach, HydA subunit should harbor the active site interacting with hydrogen (H_2). This prediction was confirmed by showing that HydA alone, after dissociation of the trimeric-complex with urea, can catalyze the reduction of viologen dyes with H_2. Besides the hydrogen interacting site, HydA contains 3x[4Fe-4S] and 2x[2Fe-2S] iron-sulfur clusters with a 43 % sequence similarity to the monomeric [Fe-Fe] hydrogenase of *Clostridium pasteurianum*. HydA of *T. maritima* differs however in having a C-terminal extension with a [2Fe-2S] binding site which is lacking in the monomeric enzyme from *C. pasteurianum*.

The HydB subunit shows a 70 % similarity to the gene product HndC of the $NADP^+$-reducing [Fe-Fe] hydrogenase from *Desulfovibrio fructosovorans* and 60 % similarity to NuoF of the NADH:ubiquinone oxidoreductase from *E. coli*. Within the sequence, there are two highly conserved stretches, one featuring NAD^+ binding sites and the other recalling orthodox FMN binding sites. The C-terminal part contains Cys motifs that could bind three [4Fe-4S] clusters. At its N-terminus HydB contains four Cys residues that are suggested to be involved in binding a [2Fe-2S] cluster (Verhagen et al. 1999).

The smaller subunit of this trimeric bifurcating hydrogenase, HydC, contains fours Cys residues arranged in a motif which is highly similar (58 %) to motifs in *E. coli* Nuo and *D. fructosovorans* HndA, which are supposed to bind a [2Fe-2S] cluster.

The above reported information, gives a picture of the HydABC complex that is tentatively shown in Fig. 9.2 where a series of assumptions were made, namely: (a) the binding site for reduced ferredoxin (Fd_{red}) at HydC; (b) the presence of a second FMN loosely bound to HydB; (c) the [Fe-Fe] center plus [4Fe-4S] cluster as part of the active site of the hydrogenase in subunit HydA.

It has been proposed that the three genes encoding HydABC in *T. maritima* are arranged in a cluster *hydCBA* that is most likely a transcription unit (Verhagen et al. 1999). Clustered genes for proteins with sequence similarity to HydABC product are also found in many other anaerobic bacteria such as for example *Clostridium ljungdahlii* (Kopke et al. 2010), *Acetobacterium woodii* (Poehlein et al. 2012), and *Moorella thermoacetica* (Pierce et al. 2008), although these gene products have not been characterized. Interestingly, the enzyme complex from the anaerobe *Thermoanaerobacter tengcongensis* is composed of four subunits rather than three. Most likely, this is due to the fact that in this latter species the HydA homolog lacks the C-terminal extension with the [2Fe-2S] cluster so that a fourth subunit, homologous to the C-terminal extension, is required (Soboh et al. 2004).

Another interesting observation recently done by Thauer et al. (2010) is that electron-bifurcating [Fe-Fe] hydrogenases are not found in the Archaea domain which appear to only contain [Ni-Fe]- and/or [Fe]-hydrogenases (Thauer et al. 2010).

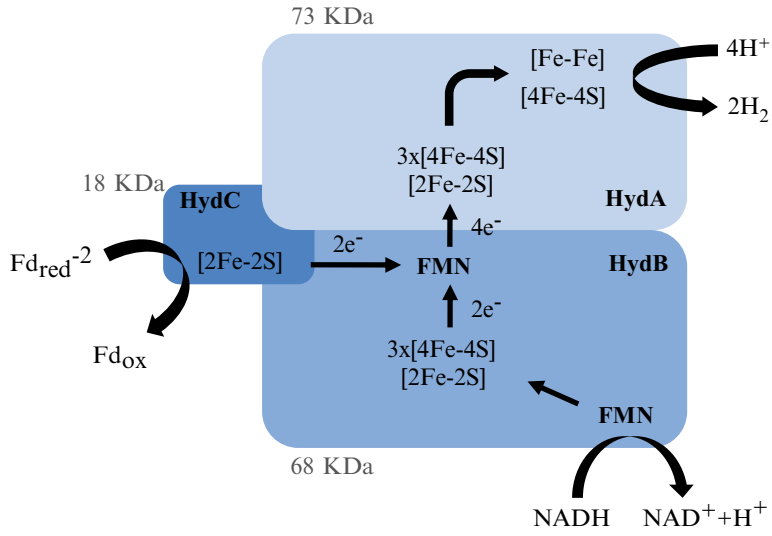

Fig. 9.2. Tentative representation of the structure and function of the HydABC complex from *Thermotoga maritimae* (See text for details).

As mentioned in Sect. III.D, several strains of *Thermotogales*, such as *Petrotoga miotherma*, *Thermosipho africanus*, *Thermotoga elfii*, *Fervidobacterium pennavorans* and *Thermotoga neapolitana*, are able to tolerate microaerophilic growth conditions and efficiently produce H_2 as a by-product of their metabolism (Van Ooteghem et al. 2002). In particular, *T. neapolitana* showed the highest H_2 production (25–30 % v/v) in these conditions (Van Ooteghem et al. 2004). Through the use of a bioinformatics approach it has been shown that the operon structure of *T. neapolitana* is the same as *T. maritima* in both the ordering and spacing of the ORFs (open reading frames) (Tosatto et al. 2008). In details, a high sequence conservation is preserved from a minimum of 75 % to a maximum of 91 % for all gene products with a sequence identity attributed to the [Fe-Fe] hydrogenase subunits of 85–91 %. Notably, the HydA subunits of both species share a 91 % sequence identity, are of the same length, and can be aligned without gaps. At the DNA level, the two sequences share 82 % identity, for a total of 375 mutated nucleotides, comprising three fully mutated codons corresponding to mutations R363E (GAA → AGG), E475S (GAG → TCC) and T539L (ACA → GTG). Taking into account the sequences of *T. petrophila* and *T. maritima* showing that R363 is not conserved between the two species (GAA → AAA), it has been concluded that only two residues, E475S and T539L of the *T. neapolitana* HydA subunit appear to be subjected to strong selection (Tosatto et al. 2008). Apparently, the functional differences between *T. neapolitana* and *T. maritima* reside in these latter subtle changes possibly involved in the conformation of the active site (H-cluster) near the surface of the protein. As suggested by Cohen et al. (2005) in the *C. pasteurianum* [FeFe] hydrogenase crystal structure, there might be two alternative gas diffusion pathways defining a hydrophobic channel toward the H-cluster active site. As suggested in Fig. 9.3, the possibility of a selective effect on gas accessibility based on the size of side-chains and charge distribution on the protein surface at the entrance of the hypothetical gas channels, B (Threonine 539) and A (Serine 475) has been discussed in detail by Tosatto et al. (2008).

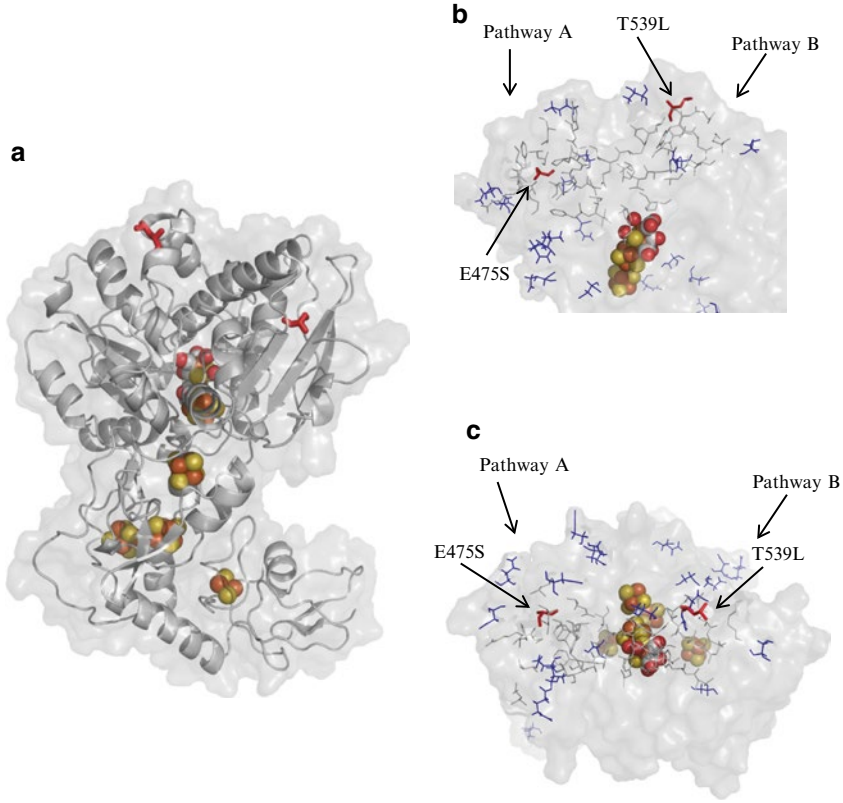

Fig. 9.3. Structural model of *Thermotoga neapolitana* HydA protein. *Panel A*. Model is shown as cartoon beneath a semi-transparent surface. Iron–sulfur clusters are shown as *spheres*. *Panel B*. Close-up of upper half of model. Residues forming part of hydrophobic channel pathways *A* and *B* are shown as sticks. Residues mutating between *T. neapolitana* and *Thermotoga maritima* HydA proteins are shown as *blue lines*. Two mutated residues forming part of hydrophobic channel pathways A (E475S) and B (T539L), are in *red* and indicated by *arrows*. *Panel C*. Same model as in *panel B*, rotated by 90° around the x-axis to show a *top view* of the molecule, where hydrophobic channel entrances are located (Tosatto et al. 2008).

C. Hydrogen Production as a Function of Variable Substrate

As reported above, *Thermotoga* spp. are able to grow on a wide array of simple sugars and polysaccharides, including starch, β-1,4 glucan (cellulose), and hemicellulose (xylan, laminarin and mannan) (Conners et al. 2006). This capacity is consistent with the production of a diverse set of proteins and enzymes that are devoted to the uptake and processing of carbohydrates (Vanfossen et al. 2008). The use of functional genomics-based approaches has provided important insights into the various mechanisms employed by these microorganisms to assimilate and metabolize carbohydrates, and has helped to identify the specific genes and operons involved (Nguyen et al. 2001, 2004). *T. maritima* genome encodes for the largest number of glycoside hydroxylases of any sequenced thermophile (Chhabra et al. 2003). These enzymes specifically hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety and function during the mobilization of complex carbohydrates for subsequent metabolism. T. *neapolitana* produces enzymes such as α-galactosidase, a laminarinase, and two cellulases (endo-1,4-β-glucanases) that

have orthologs in *T. maritima* (Zverlov et al. 1997; Bok et al. 1998; King et al. 1998), but this microorganism also produces several unique glycoside hydrolases. One example is a 1,4-β-D-glucan glucohydrolase (GghA), which hydrolyzes cellotetraose, cellotriose, cellobiose, and lactose (McCarthy et al. 2004). *T. petrophila* slightly differs from *T. maritima* and *T. neapolitana* with respect to the utilization of certain sugars, growing weakly on cellulose and, in the presence of a mixture of monosaccharides, utilizing glucose to a significantly lesser extent than the other two species (Takahata et al. 2001; Frock et al. 2012). Unlike *T. maritima* and *T. neapolitana*, *T. elfii* failed to grow on sucrose and carboxy methyl cellulose (CMC) (Van Niel et al. 2002). *T. maritima* was shown to have a preference for complex carbohydrates, as growth in the presence of monosaccharides was slower than growth in the presence of oligo/polysaccharides (Chhabra et al. 2003).

1. Use of Simple Sugars and Polysaccharides

Thermotoga spp. produce hydrogen from a wide range of organic materials including complex carbohydrates and wastes/biomass rich in sugars. Simple sugars such as glucose and xylose are readily biodegradable and thus preferred as reference substrates for studying biochemical and physiological aspects of the hydrogen production by these bacteria. A wide variety exists among *Thermotogales* with respect to the utilization of sugars for growth and H_2 production that is consistent with the genetic diversity between the strains and the degree of optimization of the process for H_2 production (Frock et al. 2012). Table 9.2 summarizes the results of hydrogen production by *Thermotoga* spp. obtained with different types of simple and complex carbohydrates and different conditions of growth. Under optimal conditions, the oxidation of glucose and xylose will at best result in the formation of 4 mol of H_2 per mole of hexose and 3.33 mol^{-1} mol of H_2 per mole of pentose, respectively. Maximum hydrogen yields, both from hexoses or pentoses, are obtained with acetate as fermentation product. Lower yields are associated to the formation of more reduced end products compared to acetate, such as butyrate, propionate and alcohols (ethanol, butanol) and lactic acid. It would be therefore important to establish the actual bacterial metabolism resulting in acetate as end product (Kaushik and Debabrata 2004).

T. neapolitana converted effectively sucrose to H_2 with H_2 yield of 4.95 $mol_{H2}/mol_{sucrose}$ (Table 9.2) despite the poor fructose based metabolism reported for this strain (de Vrije et al. 2009, 2010). Woodward et al. (2002) reported the list of sugars that were used by *T. maritima* for H_2 production. This strain was shown to preferentially use glucose, fructose, and galactose, whereas mannose and lactose metabolisms mainly produced carbon dioxide (Woodward et al. 2002). *T. neapolitana* and *T. maritima* were able to use the glucose-based complex carbohydrates, starch and cellulose, for hydrogen fermentation, although H_2 yields obtained with raw cellulose (30 $mL_{H2}/g_{cellulose}$) were significantly lower than with starch (180 mL_{H2}/g_{starch}) (Nguyen et al. 2008a). The pretreatment of cellulose with chemical agents (e.g. ionic liquids) that could disrupt the hydrogen-bonding network of the polysaccharide significantly increased its degradability (Nguyen et al. 2008b).

2. Use of Carbon Sources from Varies Waste-Residues

Most studies on hydrogen production in *Thermotoga* have used glucose as carbon source although hydrogen production at a large scale should be based on cheap and renewable substrates, such as industrial/agricultural wastes and residues. These materials have often high contents of hexose and pentoses stored in carbohydrate polymers that are ideal conversion substrates for H_2 generation (Ntaikou et al. 2010). The major criteria that have to be met for the selection of substrates suitable for fermenta-

tive biohydrogen production are availability, cost, carbohydrate content, biodegradability and concentration of inhibitory compounds (Hawkes et al. 2002). As shown in Table 9.3, *T. neapolitana* was widely used in technical processes with variable feedstock sources owing to its many advantageous properties including tolerance of moderate oxygen amount (6–12 %) and resistance to high H_2 partial pressure (Van Ooteghem et al. 2002, 2004). This strain was shown to successfully produce H_2 from lignocellulosic materials such as rice straw, wheat straw and *Mischantus* (de Vrije et al. 2002, 2009; Nguyen et al. 2010b; Eriksen et al. 2011), algal biomasses (Nguyen et al. 2010c; Dipasquale et al. 2012) and food waste materials, such carrot pulp, cheese whey and molasses (Table 9.3) (de Vrije et al. 2010; Cappelletti et al. 2012). Many physical and chemical along with structural and compositional factors in complex feedstock sources often hinder the biological digestibility (Chang et al. 2001). Because of that reason, pre-treatment methods including wet oxidation under alkaline conditions, mechanical pre-treatment, mild and concentrated acid hydrolysis and solvent extractions were required for an efficient utilization of these feedstock sources promoting the accessibility of polysaccharides in the substrates for enzymatic hydrolysis (Ntaikou et al. 2010). Molasses and cheese whey were effectively converted into H_2 by both attached- and suspended-cells of *T. neapolitana* without any pre-treatment needed (Cappelletti et al. 2012). Besides high yields in terms of H_2 production, the *Thermotoga* ability of utilizing different sugars in complex mixtures is essential in making H_2 production from biomass successful. Simultaneous utilization of glucose and xylose has also been observed in *T. neapolitana* (de Vrije et al. 2009), while in this microorganism, catabolite repression of lactose has been demonstrated in the presence of glucose (Vargas and Noll 1996). A preference for glucose was also shown by *T. neapolitana* when a mixture of glucose and fructose was present in the medium although both sugars were consumed at the same time (de Vrije et al. 2010). No mechanism of carbon catabolite repression has yet been defined for *T. maritima*.

In addition to carbohydrate-rich residues, *T. neapolitana* was also shown to produce H_2 by fermenting waste glycerol that is the main byproduct of the large-scale productions of bio-diesel (Ngo et al. 2011a; Ngo and Sim 2011). As compared with mesophilic *Enterobacter aerogenes* fermentation of glycerol, higher H_2 yield was obtained with *T. neapolitana* (2.7 mol_{H2} $mol^{-1}_{glycerol}$ instead of 0.9 mol_{H2} $mol^{-1}_{glycerol}$) (Ito et al. 2005) (Table 9.3).

Non-sugar substrates, such as yeast extract and trypticase that are components of the typical *Thermotoga* growth media, were shown to contribute to 9–12 % of the total H_2 production (d'Ippolito et al. 2010; Cappelletti et al. 2012). These media components represent undefined sources of nitrogen and carbon for bacteria that were shown to increase cell biomass and H_2 production in *T. maritima* and *T. neapolitana* cultures growing on glucose, glycerol, cheese whey and molasses (Nguyen et al. 2008a; d'Ippolito et al. 2010; Ngo and Sim 2011; Cappelletti et al. 2012). van Niel et al. (2002) reported that growth of *T. elfii* was completely dependent on yeast extract, while in the absence of tryptone, lower H_2 yields were obtained. Alternative nitrogen sources such as soybean meal or canola meal alone supported growth but H_2 production rates were reduced (Drapcho et al. 2008).

Because of the complexity and richness of some industrial/agricultural wastes, the utilization of these complex feedstock sources for H_2 production by *Thermotoga* could allow the reduction of the process-associated cost by simplifying the culture medium. A growth medium composed only by NH_4Cl, K_2HPO_4, NaCl, buffer and cysteine-HCl (see Sect. IV.D.b) led to an efficient H_2 production from molasses with *T. neapolitana* (Cappelletti et al. 2012). The omission of vitamin and trace elements solutions, some inorganic elements and nitrogen sources reduced the fermentation cost without a significant lost in H_2 production. Further cost

Table 9.3. H_2 production rates and H_2 yields from various industrial/agricultural wastes conversion by *Thermotoga* spp. described in the literature.

Strain	T°	Feedstock	Main carbon source	Additional treatment	H_2 production rate (mmol/L/h)	H_2 yield	References
T. elfii	65	*Miscanthus*	Glucose, xylose	Mechanical and NaOH	–	1.1 mol_{H2}/mol_{sugar}	de Vrije et al. (2002)
T. neapolitana	80	*Miscanthus*	Glucose, xylose, arabinose	NaOH, $Ca(OH)_2$ and enzymatic hydrolysis	13.1	3.2 mol_{H2}/mol_{hexose}	de Vrije et al. (2009)
T. neapolitana	75	Rice straw	Glucose, xylose	Combined treatment	4.7	2.7 $mmol_{H2}/g_{straw}$	Nguyen et al. (2010b)
T. neapolitana	75	Algal biomass	Starch, dextrins, oligosaccharides	Enzymatic hydrolysis	227.3	2.5 $mol_{H2}/mol_{glucose}$	Nguyen et al. (2010c)
T. neapolitana	80	Carrot pulp	Glucose, fructose, sucrose	Enzymatic hydrolysis	12.5	2.7 mol_{H2}/mol_{hexose}	de Vrije et al. 2010
T. neapolitana	75	Biodiesel manufacturing waste	Glycerol	Removal of methanol/ ethanol and solids	–	2.73 $mol_{H2}/mol_{glycerol}$	Ngo et al. (2011a) and Ngo and Sim (2011)
T. neapolitana	77	Molasses	Sucrose, glucose, fructose	–	1.2	2.95 mol_{H2}/mol_{hexose}	Cappelletti et al. (2012)
T. neapolitana	77	Cheese whey	Lactose, glucose, galactose	–	0.9	2.5 mol_{H2}/mol_{hexose}	Cappelletti et al. (2012)

reductions were achieved by replacing the lab-grade NaCl with non-refined sea salt and the cysteine-HCl with metabisulfite (see Sect. IV.D.b) (Cappelletti et al. 2012).

D. H_2 Production Process and Culture Parameters

Environmental parameters such as pH, hydrogen partial pressure, media components and temperature, are key factors as they influence the metabolism and therefore the fermentation end products. Thus, optimization of these processes and culture parameters is required to give enhanced H_2 yields.

1. Product Inhibition

Strategies for growth under H_2 inhibition conditions have been developed in hyperthermophiles including *T. maritima* and *T. neapolitana*. H_2-producing archea and bacteria can use sulfur compounds such as elemental sulfur, polysulfides, and cystine as alternative electron acceptors (Adams 1990; Drapcho et al. 2008). The use of Fe(III) as electron acceptor was also observed in *T. maritima* when H_2 levels became inhibitory (Vargas et al. 1998). Nevertheless, the metabolic pathways of hydrogen formation are sensitive to H_2 concentrations and are subject to end-product inhibition. Therefore, the H_2 partial pressure is an extremely important factor for hydrogen synthesis. H_2 production is a means by which bacteria re-oxidise reduced ferredoxin and hydrogen-carrying coenzymes, and these reactions are less favourable as the H_2 concentration in the liquid rises (Hawkes et al. 2002). Consequently, H_2 production decreases and the metabolic pathways shift to production of more reduced substrates such as lactate, ethanol, acetone, butanol, or alanine (Levin et al. 2004). Several strategies have been developed to avoid the negative effect of H_2 accumulation. These include vigorous mixing to avoid super-saturation (Lay 2000), utilization of H_2 permeable membrane to remove dissolved H_2 from mixed liquor (Liang et al. 2002) and sparging with inert nitrogen. The application of the latter technique to *T. neapolitana* growing on either glucose or waste glycerol increased the H_2 production by 50–80 % (Nguyen et al. 2010a; Ngo et al. 2011b). However, d'Ippolito et al. (2010) showed that sparging with nitrogen had a little influence on H_2 yield at low ratio between the volumes of culture and headspace. Gas sparging became more important with the increase of the liquid fraction. They observed the highest H_2 yield with a culture/headspace volume ratio of 1:3. An increased ratio between gas and liquid phase volumes was also associated to a decreased synthesis of lactic acid in cultures of *Thermotoga maritima* (Schröder et al. 1994).

In addition to culture/headspace volume ratio, the stirring regime and the substrate concentration were shown to influence H_2 production most probably because of their correlation to the dissolved H_2 concentration (Hawkes et al. 2002). A moderate agitation of the cultures (at 75 rpm) was shown to almost double the H_2 production by *T. neapolitana* cultures in 20 h of growth on glucose (Van Ooteghem et al. 2002). Using the same type of culture and carbon source, H_2 production rate was improved by increasing the stirring speed from 300 to 400 rpm. However, speeds of 500 and 600 rpm did negatively affect this fermentative process (Ngo et al. 2011b).

Considering the influence of the substrate concentration on the H_2 production process, Nguyen et al. (2008a) reported that concentrations of glucose over 15 g/L had inhibitory effects on cell growth and H_2 production in *T. neapolitana* and *T. maritima* strains batch cultures. The growth and H_2 content showed the highest values at the initial glucose concentration of 7.5 g/L for both strains. In *T. neapolitana* cultures growing on xylose, the maximal values of biomass and cumulative H_2 production were obtained at an initial substrate concentration of 5.0 g/L, while higher concentrations of xylose were not favorable for *T. neapolitana* growth and H_2 formation (Ngo et al. 2012). However, at substrate concentration of 5.0 g/L, the converted H_2 yield from xylose was lower than at the initial xylose concentration of 2 g/L

suggesting that the change in xylose concentration remarkably affected not only H_2 production itself but also the substrate utilisation (Ngo et al. 2012). The best performing initial concentration of waste glycerol (3.0 g/L) was less than half than the pure glycerol (7.0 g/L) despite they resulted in comparable H_2 productions (Ngo and Sim 2011). This indicates different potentials of the two types of glycerol sources to release a specific amount of H_2.

2. pH Buffering System

A rapid decrease in pH is observed in cultures of *Thermotoga* spp. fermenting sugars to H_2 causing, in some cases, the process to stop before all the substrate is consumed (Eriksen et al. 2008). Experiments with pH adjustment showed that when cultures were neutralized with either increased initial buffer or injection of $NaHCO_3$ or NaOH, glucose was completely consumed and H_2/acetic acid productions increased proportionally. Both the type of buffer and the initial pH have significant effect on H_2 production process. Organic and inorganic buffer systems have been tested in literature resulting in different H_2 productions depending on the growth conditions and buffers concentration in the culture medium. HEPES resulted to be the best performing buffer when compared to HPO_4:H_2PO_4, Tris-HCl, Mops, Pipes buffers in experiments using *T. neapolitana* batch cultures growing on glucose (Cappelletti et al. 2012). Effective H_2 productions were also obtained with HEPES-buffered medium containing raw material feedstocks such as cheese whey, molasses and glycerol waste as carbon sources for *T. neapolitana* (Ngo et al. 2011a; Cappelletti et al. 2012). The good buffering properties of HEPES might be due to its pK (7.55) that is near the optimum for the growth of *T. neapolitana*. Itaconic acid was also successfully used to overcome pH-induced limitations of H_2-producing *T. neapolitana* cultures growing glucose. The buffering capacity of this carbohydrate was tested after that it was found to be poorly metabolized by this strain (Van Ooteghem et al. 2004). Its applicability for H_2 productions from industrial residues was demonstrated by Ngo and Sim (2011). The addition of itaconic acid into the culture medium of *T. neapolitana* growing on waste glycerol increased the process performance by almost 40 % (Ngo and Sim 2011).

In addition to the buffer, the initial pH value was shown to have a significant effect on growth and H_2 production of *Thermotoga* spp. Anna et al. (1991) indicated that the pH control is crucial to the H_2 formation pathway because of the effects of pH on the hydrogenase activity. The optimum initial pH for *T. neapolitana* suspended-cells ranged from 6.5 to 7.5 depending on both the substrate and the conditions of growth (Ngo et al. 2011b). Interestingly, an higher pH range (7.7–8.5) yielded the highest hydrogen production in experiments with glucose-grown *T. neapolitana* attached-cells on ceramic supports (Cappelletti et al. 2012). An initial pH of 7.0 provided the most promising results in terms of H_2 and acetic acid productions in *T. neapolitana* growing on xylose (Ngo et al. 2012). The optimum initial pH values for growth and hydrogen production from glucose were 6.5–7.0 for *T. maritima* and 6.5–7.5 for *T. neapolitana*, respectively. The best performing initial pH for H_2 production from glycerol by *T. neapolitana* was 7.0–7.5 (Ngo and Sim 2011). The application of initial pH above 8 resulted in a decrease of cumulative H_2 production as well as cell concentration suggesting the influence of pH over the metabolism pathway of the bacteria (Ngo and Sim 2011).

3. Oxygen Exposure

Hydrogen production by *Thermotoga* strains is a hyperthermophilic anaerobic process, thus, high temperatures (70–90 °C) and strictly anaerobic conditions must be initiated and maintained in the reactor vessel during production.

Some researchers have reported that low concentrations of oxygen are tolerated by both *T. neapolitana* (Tosatto et al. 2008) and *T. maritima* (Le Fourn et al. 2008) and an O_2

insensitive hydrogenase have been described in *T. neapolitana* (Käslin et al. 1998). Van Ooteghem et al. (2002, 2004) reported that microaerobic metabolism increased the H_2 yield from *T. neapolitana* up to values higher than the theoretical 4 $mol_{H2}/mol_{glucose}$ possible from fermentative metabolism (Table 9.2); however, this result was not confirmed by other researchers who observed H_2 production after the injection of O_2, but with rate and extent that were lower than those found in cultures without O_2 (Eriksen et al. 2008; Munro et al. 2009).

Prevention of O_2 exposure could be difficult on an industrial scale and requires expensive reducing agents (cysteine-HCl). Anaerobic conditions can be initiated, maintained, and monitored in the reactor vessels by (1) flushing media with nitrogen gas, (2) heating or boiling of the media to remove dissolved oxygen, (3) adding chemical agents such as sodium sulfite or cysteine-HCl to consume residual O_2 in the liquid, (4) adding resazurin to act as visible redox indicator, and (5) maintaining positive pressure in headspace to prevent air contamination (Drapcho et al. 2008). Cysteine-HCl at concentration of 0.5–1 g/L was commonly added to provide reducing conditions in media and consume residual oxygen (de Vrije et al. 2002; Van Ooteghem et al. 2002, 2004). The addition of a reducing agent to H_2 production resulted to be fundamental even when complex feed-stock sources were supplemented to the culture medium. However, to reduce the cost of the process, the utilization of cheaper reducing agents was attempted. For example, the replacement of cysteine-HCl with metabisulfite (MBS) gave promising results in terms of both medium cost (90 % reduction) and H_2 yield in *T. neapolitana* cultures growing on molasses (Cappelletti et al. 2012). Conversely, in the cheese whey tests, the attempt to replace cysteine with MBS led to poor performances (Cappelletti et al. 2012).

4. Growth-Temperature and H_2 Production

Optimum temperature of growth differs among *Thermotoga* species and has to be considered in the bioreactor operating process to maximize H_2 production rates (Munro et al. 2009; Nguyen et al. 2008a). Optimum temperature of growth and H_2 production is 77 °C for *T. maritima*, 88 °C for *T. petrophila* and *T. naphthophila* and only 66 °C for *T. elfii* (Jannasch et al. 1988; Huber and Hannig 2006). Cultures of T. neapolitana grown at 77 and 85 °C exhibited the greatest rate and extent of H_2 production (Munro et al. 2009).

Advantages to using high temperatures for fermentation process include (1) to reduce the likelihood of contamination by H_2-consuming organisms that lessens the need for sterilization of media and equipment, (2) to favour the catalytic activity of hydrogenase of evolving H_2 (Adams 1990) (3) to directly utilize industrial organic wastewaters that are often discharged at elevated temperatures; (4) to avoid cooling down processes usually required by mesophilic fermentations that in large scale generate excess heat (Drapcho et al. 2008).

V. Future Perspectives

This Chapter summarizes our present knowledge on the microbiology, physiology, and biochemistry of the *Thermotogales* order with the aim to better define problems and challenges linked to H_2 production. In this respect, it is worth noting that important improvements have been recently made through optimization of both the bioprocess parameters and *Thermotoga* spp. to be used. Although the identification of suitable feed-stocks for fermentative hydrogen production was done, more research work to improve hydrogen production rates and yields, is required. The use, for example, of *Thermotoga* strains metabolically engineered and the development of a "two stage process" is likely to improve H_2 production. Indeed, this latter approach involves the fermentation of the selected substrate to both hydrogen and organic acids by *Thermotoga* spp. in the first stage and, in a second stage, either an additional energy extraction or the generation of highly-valuable products by exploiting the effluent of the first stage reactor. An alternative approach to improve H_2 production might also be achieved through a specific

bioreactor configuration ameliorating both biomass concentration and substrate conversion efficiency by employing biomass retention systems such as granules, flocs or biofilm-formation supports.

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References

Adams MWW (1990) The metabolism of hydrogen by extremely thermophilic, sulfur-dependent bacteria. FEMS Microbiol Rev 75:219–237

Alain K, Marteinsson VT, Miroshnichenko ML, Bonch-Osmolovskaya EA, Prieur D, Birrien JL (2002) *Marinitoga piezophila* sp. nov., a rod-shaped, thermo-piezophilic bacterium isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol 52:1331–1339

Amend JP, Plyasunov AV (2001) Carbohydrates in thermophile metabolism: calculations of the standard molal thermodynamic properties of aqueous pentoses and hexoses at elevated temperatures and pressures. Geochim Cosmochim Acta 65:3901–3917

Andrews KT, Patel BKC (1996) *Fervidobacterium gondwanense* sp. nov., a new thermophilic anaerobic bacterium isolated from non-volcanically heated geothermal waters of the Great Artesian Basin of Australia. Int J Syst Bacteriol 46:265–269

Anna J, Shigetoshi A, Adams MWW (1991) The extremely thermophilic eubacterium, *Thermotoga maritima*, contains a novel iron-hydrogenase whose cellular activity is dependent upon tungsten. J Biol Chem 266:13834–13841

Antoine E, Cilia V, Meunier J, Guezennec J, Lesongeur F, Barbier G (1997) *Thermosipho melanesiensis* sp. nov., a new thermophilic anaerobic bacterium belonging to the order Thermotogales, isolated from deep-sea hydrothermal vents in the southwestern Pacific Ocean. Int J Syst Bacteriol 47:1118–1123

Balk M, Weijma J, Stams AJ (2002) *Thermotoga lettingae* sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol 52:1361–1368

Ben Hania W, Ghodbane R, Postec A, Brochier-Armanet C, Hamdi M, Fardeau M-L, Ollivier B (2011) Cultivation of the first mesophilic representative ("mesotoga") within the order Thermotogales. Syst Appl Microbiol 34:581–585

Ben Hania W, Ghodbane R, Postec A, Hamdi M, Ollivier B, Fardeau M-L (2012) *Defluviitoga tunisiensis* gen. nov., sp. nov., a thermophilic bacterium isolated from a mesothermic and anaerobic whey digester. Int J Syst Evol Microbiol 62:1377–1382

Ben Hania W, Postec A, Aullo T, Ranchou-Peyruse A, Erauso G, Brochier-Armanet C, Hamdi M, Ollivier B, Saint-Laurent S, Margot M, Fardeau ML (2013) Mesotoga infera sp. nov., a novel mesophilic member of the order Thermotogales, isolated from an underground gas storage in France. Int J Syst Evol Microbiol 63:3003–3008. doi:1099/ijs.0.047993-0

Boiangiu CD, Jayamani E, Brugel D, Hermann G, Kim J, Forzi L, Hedderich R, Vgenopoulou I, Pierik AJ, Steuber J, Buckel W (2005) Sodium ion pumps and hydrogen production in glutamate fermenting anaerobic bacteria. J Mol Microbiol Biotechnol 10:105–119

Bok JD, Yernool DA, Eveleigh DE (1998) Purification, characterization, and molecular analysis of thermostable cellulases CelA and CelB from *Thermotoga neapolitana*. Appl Environ Microbiol 64:4774–4781

Brochier C, Philippe H (2002) Phylogeny: a non-hyperthermophilic ancestor for Bacteria. Nature 417:244

Buckel W, Thauer RK (2012) Energy conservation via electron bifurcating ferredoxin reduction and proton/Na+ translocating ferredoxin oxidation. Biochim Biophys Acta 1827:94–113. doi:10.1016/j.bbabio.2012.07.002

Cappelletti M, Bucchi G, Mendes JDS, Alberini A, Fedi S, Bertin L, Frascari D (2012) Biohydrogen production from glucose, molasses and cheese whey by suspended and attached cells of four hyperthermophilic *Thermotoga* strains. J Chem Technol Biotechnol 87:1291–1301

Chang VS, Nagwani M, Kim CH, Holtzapple MT (2001) Oxidative lime pretreatment of high-lignin biomass. Appl Biochem Biotechnol 94:1–28

Chhabra SR, Shockley KR, Conners SB, Scott KL, Wolfinger RD, Kelly RM (2003) Carbohydrate-induced differential gene expression patterns in the hyperthermophilic bacterium *Thermotoga maritima*. J Biol Chem 278:7540–7552

Cohen J, Kim K, King P, Seibert M, Schulten K (2005) Finding gas diffusion pathways in proteins: application to O_2 and H_2 transport in CpI [FeFe]-

hydrogenase and the role of packing defects. Structure 13:1321–1329

Conners SB, Mongodin EF, Johnson MR, Montero CI, Nelson KE, Kelly RM (2006) Microbial biochemistry, physiology, and biotechnology of hyperthermophilic *Thermotoga* species. FEMS Microbiol Rev 30:872–905

Cypionka H (2000) Oxygen respiration by *Desulfovibrio* species. Annu Rev Microbiol 54:827–848

d'Ippolito G, Dipasquala L, Vella FM, Romano I, Gambacorta A, Fontana A (2010) Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. Int J Hydrog Energy 35:2290–2295

Davey ME, Wood WA, Key R, Nakamura K, Stahl D (1993) Isolation of three species of Geotoga and Petrotoga: two new genera, representing a new lineage in the bacterial line of descent distantly related to the 'Thermotogales'. Syst Appl Microbiol 16:191–200

de Vrije T, De Haas GG, Tan GB, Keijsers ERP, Claassen PAM (2002) Pretreatment of *Miscanthus* for hydrogen production by *Thermotoga elfii*. Int J Hydrog Energy 27:1381–1390

de Vrije T, Bakker RR, Budde MAW, Lai MH, Mars AE, Claassen PAM (2009) Efficient hydrogen production from the lignocellulosic energy crop *Miscanthus* by the extreme thermophilic bacteria *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Biotechnol Biofuels 2:12

de Vrije T, Budde MAW, Lips SJ, Bakker RR, Mars AE, Claassen PAM (2010) Hydrogen production from carrot pulp by the extreme thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Int J Hydrog Energy 35:13206–13213

Di Pippo JL, Nesbo CL, Dahle H, Doolittle WF, Birkland N-K, Noll KM (2009) *Kosmotoga olearia* gen. nov., sp. nov., a thermophilic, anaerobic heterotroph isolated from an oil production fluid. Int J Syst Evol Microbiol 59:2991–3000

Dipasquale L, d'Ippolito G, Gallo C, Vella FM, Gambacorta A, Picariello G, Fontana A (2012) Hydrogen production by the thermophilic eubacterium *Thermotoga neapolitana* from storage polysaccharides of the CO_2-fixing diatom *Thalassiosira weissflogii*. Int J Hydrog Energy 37:12250–12257

Dolla A, Fournier M, Dermoun Z (2006) Oxygen defense in sulfate-reducing bacteria. J Biotechnol 126:87–100

Drapcho CM, Nhuan NP, Walker TH (eds) (2008) Hydrogen production by fermentation. In: Biofuels engineering process technology. McGraw-Hill, New York, pp 269–299

Eriksen NT, Nielsen TM, Iversen N (2008) Hydrogen production in anaerobic and microaerobic *Thermotoga neapolitana*. Biotechnol Lett 30:103–109

Eriksen NT, Leegaard Riis M, Kindby Holm N, Iversen N (2011) H2 synthesis from pentoses and biomass in *Thermotoga* spp. Biotechnol Lett 33:293–300

Fardeau M-L, Olliever B, Patel BKC, Magot M, Thomas P, Rimbault A, Rocchiccioli F, Garcia J-L (1997) *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. Int J Syst Bacteriol 47:1013–1019

Feng Y, Cheng L, Zhang X, Li X, Deng Y, Zhang H (2010) Thermococcoides shengliensis gen. nov., sp. nov., a new member of the order Thermotogales isolated from oil-production fluid. Int J Syst Evol Microbiol 60:932–937

Fetzer S, Conrad R (1993) Effect of redox potential on methanogenesis by *Methanosarcina barkeri*. Arch Microbiol 160:108–113

Friedrich AB, Antranikian G (1996) Keratin degradation by *Fervidobacterium pennavorans*, a novel thermophilic anaerobic species of the order Thermotogales. Appl Environ Microbiol 62:2875–2882

Frock AD, Gray SR, Kelly RM (2012) Hyperthermophilic Thermotoga species differ with respect to specific carbohydrate transporters and glycoside hydrolases. Appl Environ Microbiol 78:1978–1986

Galperin MY, Noll KM, Romano AH (1996) The glucose transport system of the hyperthermophilic anaerobic bacterium *Thermotoga neapolitana*. Appl Environ Microbiol 62:2915–2918

Hawkes F, Dinsdale R, Hawkes D, Hussy I (2002) Sustainable fermentative hydrogen production: challenges for process optimisation. Int J Hydrog Energy 27:1339–1347

Huber R, Hannig M (2006) Thermotogales. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (eds) The prokaryotes. Springer, New York, pp 899–922

Huber R, Stetter KO (2001) Discovery of hyperthermophilic microorganisms. In: Adams MWW, Kelly RM (eds) Methods in enzymology. Academic, San Diego, pp 11–24

Huber R, Langworthy TA, Konig H, Thomm M, Woese CR, Sleytr UB, Stetter KO (1986) *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch Microbiol 144:324–333

Huber R, Woese CR, Langworthy TA, Fricke H, Stetter KO (1989) *Thermosipho africanus* gen. nov., represents a new genus of thermophilic Eubacteria within the Thermotogales. Syst Appl Microbiol 12:32–37

Huber R, Woese CR, Langworthy TA, Kristjansson JK, Stetter KO (1990) *Fervidobacterium islandicum* sp. nov., a new extremely thermophilic eubacterium belonging to the Thermotogales. Arch Microbiol 154:105–111

Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N (2005) Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. J Biosci Bioeng 100:260–265

Jannasch HW, Huber R, Belkin S, Stetter KO (1988) *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch Microbiol 150:103–104

Janssen PH, Morgan HW (1992) Heterotrophic sulfur reduction by *Thermotoga* sp. strain FjSS3.B1. FEMS Microbiol Lett 96:213–217

Jayasinghearachchi HS, Lal B (2011) *Oceanotoga teriensis* gen. nov., sp. nov., a thermophilic bacterium isolated from offshore oil-producing wells. Int J Syst Evol Microbiol 61:554–560

Jeanthon C, Reysenbach AL, L'Haridon S, Gambacorta A, Pace NR, Glenat P, Prieur D (1995) *Thermotoga subterranea* sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir. Arch Microbiol 164:91–97

Jenney FE, Adams MWW (2008) Hydrogenases of the model hyperthermophiles. Incredible anaerobes: from physiology to genomics to fuels. Ann N Y Acad Sci 1125:252–266

Kapdan IK, Kargi F (2006) Bio-hydrogen production from waste materials. Enzyme Microb Technol 38:569–582

Käslin SA, Childers SE, Noll KM (1998) Membrane-associated redox activities in *Thermotoga neapolitana*. Arch Microbiol 170:297–303

Kaushik N, Debabrata D (2004) Improvement of fermentative hydrogen production: various approaches. Appl Microbiol Biotechnol 65:520–529

King MR, Yernool DA, Eveleigh DE, Chassy BM (1998) Thermostable alpha-galactosidase from *Thermotoga neapolitana*: cloning, sequencing and expression. FEMS Microbiol Lett 163:37–42

Kopke M, Held C, Hujer S, Liesegang H, Wiezer A, Wollherr A, Ehrenreich A, Liebl W, Gottschalk G, Durre P (2010) *Clostridium ljiungdahlii* represents a microbial production platform based on syngas. Proc Natl Acad Sci U S A 107:13087–13092

Krekeler D, Teske A, Cypionka H (1998) Strategies of sulfate-reducing bacteria to escape oxygen stress in a cyanobacterial mat. FEMS Microbiol Ecol 25:89–96

L'Haridon S, Miroshnichenko M, Hippe H, Fardeau M-L, Bonch-Osmolovskaya EA, Stackebrandt E, Jeanthon C (2001) *Thermosipho geolei* sp. nov., a thermophilic bacterium isolated from a continental petroleum reservoir in Western Siberia. Int J Syst Evol Microbiol 51:1327–1334

L'Haridon S, Miroshnichenko ML, Hippe H, Fardeau ML, Bonch-Osmolovskaya EA, Stackebrandt E, Jeanthon C (2002) *Petrotoga olearia* sp. nov. and *Petrotoga sibirica* sp. nov., two thermophilic bacteria isolated from a continental petroleum reservoir in Western Siberia. Int J Syst Evol Microbiol 52:1715–1722

Lakhal R, Auria R, Davidson S, Ollivier B, Dolla A, Hamdi M, Combet-Blanc Y (2010) Effect of oxygen and redox potential on glucose fermentation in thermotoga maritima under controlled physico-chemical conditions. Int J Microbiol 2010:896510. doi:10.1155/2010/896510

Lakhal R, Auria R, Davidson S, Ollivier B, Durand M-C, Dolla A, Hamdi M, Combet-Blanc Y (2011) Oxygen uptake rates in the hyperthermophilic anaerobe *Thermotoga maritima* grown in a bioreactor under controlled oxygen exposure: clues to its defence strategy against oxidative stress. Arch Microbiol 193:429–438

Lay JJ (2000) Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnol Bioeng 68:269–278

Le Fourn C, Fardeau M-L, Ollivier B, Lojou E, Dolla A (2008) The hyperthermophilic anaerobe *Thermotoga maritima* is able to cope with limited amount of oxygen: insights into its defence strategies. Environ Microbiol 10:1877–1887

Le Fourn C, Brasseur G, Brochier-Armanet C, Pieulle L, Brioukhanov A, Ollivier B, Dolla A (2011) An oxygen reduction chain in the hyperthermophilic anaerobe *Thermotoga maritima* highlights horizontal gene transfer between Thermococcales and Thermotogales. Environ Microbiol 13:2132–2145

Levin DB, Pitt L, Love M (2004) Biohydrogen production: prospects and limitations to practical application. Int J Hydrog Energy 29:173–185

Liang TM, Cheng SS, Wu KL (2002) Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. Int J Hydrog Energy 27:1157–1165

Lien T, Madsen M, Rainey FA, Birkeland N-K (1998) *Petrotoga mobilis* sp. nov., from a North Sea oil-production well. Int J Syst Bacteriol 48:1007–1013

Magot M, Ollivier B, Patel BK (2000) Microbiology of petroleum reservoirs. Antonie Van Leeuwenhoek 77:103–116

McCarthy JK, Uzelac A, Davis DF, Eveleigh DE (2004) Improved catalytic efficiency and active site modification of 1,4-beta-D-glucan glucohydrolase A from *Thermotoga neapolitana* by directed evolution. J Biol Chem 279:11495–11502

Miranda-Tello E, Fardeau M-L, Thomas P, Ramirez F, Casalot L, Cayol J-L, Garcia J-L, Ollivier B (2004) *Petrotoga mexicana* sp. nov., a novel thermophilic, anaerobic and xylanolytic bacterium isolated from an oil-producing well in the Gulf of Mexico. Int J Syst Evol Microbiol 54:169–174

Miranda-Tello E, Fardeau M-L, Joulian C, Magot M, Thomas P, Tholozan JL, Ollivier B (2007) *Petrotoga halophila* sp. nov., a thermophilic, moderately halophilic, fermentative bacterium isolated from an offshore oil well in Congo. Int J Syst Evol Microbiol 57:40–44

Munro SA, Zinder SH, Walker LP (2009) The fermentation stoichiometry of *Thermotoga neapolitana* and influence of temperature, oxygen, and pH on hydrogen production. Biotechnol Prog 25:1035–1042

Nesbø CL, Dlutek M, Zhaxybayeva O, Doolittle WF (2006) Evidence for existence of "Mesotogas," members of the order Thermotogales adapted to low-temperature environments. Appl Environ Microbiol 72:5061–5068

Nesbø CL, Kumaraswamy R, Dlutek M, Doolittle WF, Foght J (2010) Searching for mesophilic Thermotogales bacteria: "Mesotogas" in the wild. Appl Environ Microbiol 76:4896–4900

Nesbø C, Bradnan D, Adebusuyi A, Dlutek M, Petrus A, Foght J, Doolittle W, Noll K (2012) *Mesotoga prima* gen. nov., sp. nov., the first described mesophilic species of the Thermotogales. Extremophiles 16:387–393

Ngo TA, Sim SJ (2011) Dark fermentation of hydrogen from waste glycerol using hyperthermophilic eubacterium *Thermotoga neapolitana*. Environ Prog Sustain Energy 31:466–473

Ngo TA, Kim MS, Sim SJ (2011a) High-yield biohydrogen production from biodiesel manufacturing waste by *Thermotoga neapolitana*. Int J Hydrog Energy 36:5836–5842

Ngo TA, Kim MS, Sim SJ (2011b) Thermophilic hydrogen fermentation using *Thermotoga neapolitana* DSM 4359 by fed-batch culture. Int J Hydrog Energy 36:14014–14023

Ngo TA, Nguyen TH, Bui HTV (2012) Thermophilic fermentative hydrogen production from xylose by *Thermotoga neapolitana* DSM 4359. Renew Energy 37:174–179

Nguyen TN, Borges KM, Romano AH, Noll KM (2001) Differential gene expression in *Thermotoga neapolitana* in response to growth substrate. FEMS Microbiol Lett 195:79–83

Nguyen TN, Ejaz AD, Brancieri MA, Mikula AM, Nelson KE, Gill SR, Noll KM (2004) Whole-genome expression profiling of *Thermotoga maritima* in response to growth on sugars in a chemostat. J Bacteriol 186:4824–4828

Nguyen TAD, Kim JP, Kim MS, Oh YK, Sima SJ (2008a) Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritima* and *Thermotoga neapolitana* in batch fermentation. Int J Hydrog Energy 33:1483–1488

Nguyen TAD, Kim JP, Kim MS, Oh YK, Sim SJ (2008b) Hydrogen production by the hyperthermophilic eubacterium, *Thermotoga neapolitana*, using cellulose pretreated by ionic liquid. Int J Hydrog Energy 33:5161–5168

Nguyen TAD, Han SJ, Kim JP, Kim MS, Sim SJ (2010a) Hydrogen production of the hyperthermophilic eubacterium, *Thermotoga neapolitana* under N2 sparging condition. Bioresour Technol 101:S38–S41

Nguyen TAD, Kim KR, Kim MS, Sim SJ (2010b) Thermophilic hydrogen fermentation from Korean rice straw by *Thermotoga neapolitana*. Int J Hydrog Energy 35:13392–13398

Nguyen TAD, Kim KR, Nguyen MT, Kim MS, Kim D, Sim SJ (2010c) Enhancement of fermentative hydrogen production from green algal biomass of *Thermotoga neapolitana* by various pretreatment methods. Int J Hydrog Energy 35:13035–13040

Ntaikou I, Antonopoulou G, Lyberatos G (2010) Biohydrogen production from biomass and wastes via dark fermentation: a review. Waste Biomass Valor 1:21–39

Nunoura T, Oida H, Miyazaki M, Suzuki Y, Takai K, Horikoshi K (2007) *Marinitoga okinawensis* sp. nov., a novel thermophilic and anaerobic heterotroph isolated from a deep-sea hydrothermal field, Southern Okinawa Trough. Int J Syst Evol Microbiol 57:467–471

Nunoura T, Hirai M, Imachi H, Miyazaki M, Makita H, Hirayama H, Furushima Y, Yamamoto H, Takai K (2010) Kosmotoga arenicorallina sp. nov. a thermophilic and obligately anaerobic heterotroph isolated from a shallow hydrothermal system occurring within a coral reef, southern part of the Yaeyama Archipelago, Japan, reclassification of *Thermococcoides shengliensis* as *Kosmotoga shengliensis* comb. nov., and emended description of the genus Kosmotoga. Arch Microbiol 192:811–819

Ollivier B, Cayol J-L (2005) The fermentative, iron-reducing, and nitrate-reducing microorganisms. In: Ollivier B, Magot M (eds) Petroleum microbiology. ASM Press, Washington, DC, pp 71–88

Patel BKC, Morgan HW, Daniel RM (1985) *Fervidobacterium nodosum*; gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch Microbiol 141:63–69

Pierce E, Xie G, Barabote RD, Saunders E, Han CS, Detter JC, Richardson P, Brettin TS, Das A, Ljungdahl LG, Ragsdale SW (2008) The complete genome sequence of *Moorella thermoacetica* (f. *Clostridium thermoaceticum*). Environ Microbiol 10:2550–2573

Poehlein A, Schmidt S, Kaster AK, Goenrich M, Vollmers J, Thurmer A, Bertsch J, Schuchmann K, Voigt B, Hecker M, Daniel R, Thauer RK, Gottschalk G, Muller V (2012) An ancient pathway combining carbon dioxide fixation with the generation and utilization of a

sodium ion gradient for ATP synthesis. PLoS One 7:e33439. doi:10.1371/journal.pone.0033439

Postec A, Le Breton C, Fardeau M-L, Lesongeur F, Pignet P, Querellou J, Ollivier B, Godfroy A (2005) *Marinitoga hydrogenitolerans* sp. nov., a novel member of the order Thermotogales isolated from a black smoker chimney on the Mid-Atlantic Ridge. Int J Syst Evol Microbiol 55:1217–1221

Postec A, Ciobanu MC, Birrien J-L, Bienvenu N, Prieur D, Le Romancer M (2010) *Marinitoga litoralis* sp. nov., a thermophilic, heterotrophic bacterium isolated from a coastal thermal spring on Ile Saint-Paul, Southern Indian Ocean. Int J Syst Evol Microbiol 60:1778–1782

Ravot G, Ollivier B, Magot M, Patel B, Crolet J, Fardeau M-L, Garcia J (1995) Thiosulfate reduction, an important physiological feature shared by members of the order Thermotogales. Appl Environ Microbiol 61:2053–2055

Ravot G, Ollivier B, Fardeau M-L, Patel BKC, Andrews KT, Magot M, Garcia JL (1996) L-alanine production from glucose fermentation by hyperthermophilic members of the domains Bacteria and Archaea: a remnant of an ancestral metabolism? Appl Environ Microbiol 62:2657–2659

Reysenbach A-L, Boone DR, Castenholz RW, Garrity GM (2001) Phylum BII. Thermotogae phy. nov. In: Boone DR, Castenholz RW (eds) Bergey's manual of systematic bacteriology. Springer, New York, pp 369–387

Schink B, Stams A (2006) Syntrophism among prokaryotes. In: Schleifer K-H, Stackebrandt E (eds) The prokaryotes. Springer, New York, pp 322–337

Schröder C, Selig M, Schönheit P (1994) Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden-Meyerhof pathway. Arch Microbiol 161:460–470

Schut GJ, Adams MW (2009) The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. J Bacteriol 191:4451–4457

Selig M, Xavier KB, Santos H, Schonheit P (1997) Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium *Thermotoga*. Arch Microbiol 167:217–232

Soboh B, Linder D, Hedderich R (2004) A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. Microbiology 150:2451–2463

Takahata Y, Nishijima M, Hoaki T, Maruyama T (2001) *Thermotoga petrophila* sp. nov. and *Thermotoga naphthophila* sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. Int J Syst Evol Microbiol 51:1901–1909

Takai K, Horikoshi K (2000) *Thermosipho japonicus* sp. nov., an extremely thermophilic bacterium isolated from a deep-sea hydrothermal vent in Japan. Extremophiles 4:9–17

Teske A, Ramsing NB, Habicht K, Fukui M, Kuver J, Jorgensen BB, Cohen Y (1998) Sulfate-reducing bacteria and their activities in cyanobacterial mats of solar Lake (Sinai, Egypt). Appl Environ Microbiol 64:2943–2951

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Microbiol Mol Biol Rev 41:100–180

Thauer RK, Kaster AK, Goenrich M, Schick M, Hiromoto T, Shima S (2010) Hydrogenases from methanogenic archaea, nickel, a novel cofactor, and H2 storage. Annu Rev Biochem 79:507–536

Tosatto SCE, Toppo S, Carbonera D, Giacometti GM, Costantini P (2008) Comparative analysis of the [FeFe] hydrogenase from Thermotogales indicates the molecular bases of resistance to oxygen inactivation. Int J Hydrog Energy 33:570–578

Urios L, Cueff-Gauchard V, Pignet P, Postec A, Fardeau M-L, Ollivier B, Barbier G (2004) *Thermosipho atlanticus* sp. nov., a novel member of the Thermotogales isolated from a Mid-Atlantic Ridge hydrothermal vent. Int J Syst Evol Microbiol 54:1953–1957

Van Niel EWJ, Budde MAW, de Haas GG, van der Wal FJ, Claassen PAM, Stams AJM (2002) Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. Int J Hydrog Energy 27:1391–1398

Van Ooteghem SA, Beer SK, Yue PC (2002) Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. Appl Biochem Biotechnol 98–100:177–189

Van Ooteghem SA, Jones A, van der Lelie D, Dong B, Mahajan D (2004) H2 production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions. Biotechnol Lett 26:1223–1232

Vanfossen AL, Lewis DL, Nichols JD, Kelly RM (2008) Polysaccharide degradation and synthesis by extremely thermophilic anaerobes. Ann N Y Acad Sci 1125:322–337

Vargas M, Noll KM (1996) Catabolite repression in the hyperthermophilic bacterium *Thermotoga neapolitana* is independent of cAMP. Microbiology 142:139–144

Vargas M, Kashefi K, Blunt-Harris EL, Lovley DR (1998) Microbiological evidence for Fe(III) reduction on early Earth. Nature 395:65–67

Verhagen MF, O'Rourke T, Adams MW (1999) The hyperthermophilic bacterium *Thermotoga maritima* contains an unusually complex iron-hydrogenase: amino acid sequence analyses versus biochemical characterization. Biochim Biophys Acta 1412:212–229

Vincent KA, Parkin A, Lenz O, Albracht SPJ, Fontecilla-Camps JC, Cammack R, Friedrich B, Armstrong FA (2005) Electrochemical definitions of O_2 sensitivity and oxidative inactivation in hydrogenases. J Am Chem Soc 127:18179–18189

Wery N, Lesongeur F, Pignet P, Derennes V, Cambon-Bonavita M, Godfroy A, Barbier G (2001) *Marinitoga camini* gen. nov., sp. nov., a rod-shaped bacterium belonging to the order Thermotogales, isolated from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol 51:495–504

Wiegel J, Ljungdahl LG, Demain AL (1985) The importance of thermophilic bacteria in biotechnology. Crit Rev Biotechnol 3:39–108

Windberger E, Huber R, Trincone A, Fricke H, Stetter KO (1989) *Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental solfataric springs. Arch Microbiol 151:506–512

Woodward J, Heyer NI, Getty JP, O'Neill HM, Pinkhassik E, Evans BR (2002) Efficient hydrogen production using enzymes of the pentose phosphate pathway. NREL/CP-610–32405. US Department of Energy, Washington, DC

Yang X, Ma K (2005) Purification and characterization of an NADH oxidase from extremely thermophilic anaerobic bacterium *Thermotoga hypogea*. Arch Microbiol 183:331–337

Zhaxybayeva O, Swithers KS, Lapierre P, Fournier GP, Bickhart DM, DeBoy RT, Nelson KE, NesbØ CL, Doolittle WF, Gogarten JP, Noll KM (2009) On the chimeric nature, thermophilic origin, and phylogenetic placement of the Thermotogales. Proc Natl Acad Sci U S A 106:5865–5870

Zverlov VV, Volkov IY, Velikodvorskaya TV, Schwarz WH (1997) Highly thermostable endo-1,3-β-glucanase (chrysolaminaranase) LamA from *Thermotoga neapolitana*: nucleotide sequence of the gene and characterization of the recombinant gene product. Microbiology 143:1701–1708