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Obligate sugar oxidation in *Mesotoga* spp., phylum *Thermotogae*, in the presence of either elemental sulfur or hydrogenotrophic sulfate-reducers as electron acceptor

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Summary

***Mesotoga prima* strain PhosAc3 is a mesophilic representative of the phylum *Thermotogae* comprising only fermentative bacteria so far. We show that while unable to ferment glucose, this bacterium is able to couple its oxidation to reduction of elemental sulfur. We demonstrate furthermore that *M. prima* strain PhosAc3 as well as *M. prima* strain MesG1 and *Mesotoga infera* are able to grow in syntrophic association with sulfate-reducing bacteria (SRB) acting as hydrogen scavengers through interspecies hydrogen transfer. Hydrogen production was higher in *M. prima* strain PhosAc3 cells co-cultured with SRB than in cells cultured alone in the presence of elemental sulfur. We propose that the efficient sugar-oxidizing metabolism by *M. prima* strain PhosAc3 in syntrophic association with a hydrogenotrophic sulfate-reducing bacterium can be extrapolated to all members of the *Mesotoga* genus. Genome comparison of *Thermotogae* members suggests that the metabolic difference between *Mesotoga* and *Thermotoga* species (sugar oxidation *versus* fermentation) is mainly due to the absence of the bifurcating [FeFe]-hydrogenase in the former. Such an obligate oxidative process for using sugars, unusual within prokaryotes, is the first reported within the *Thermotogae*. It is hypothesized to be of primary ecological importance for growth of *Mesotoga* spp. in the environments that they inhabit.**

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Introduction

For a long time, cultivated members of *Thermotogae*, a deep-branching phylum within *Bacteria* (Huber and Hannig, 2006; Cappelletti *et al.*, 2014), were known to be essentially thermophilic to hyperthermophilic. This situation changed when 16S rRNA gene sequences were reported in many mesothermic environments, suggesting that mesophilic (*Mesotoga*) *Thermotogae* exist (Nesbø *et al.*, 2006). The first mesophilic representative of *Thermotogae* (*Mesotoga* strain PhosAc3) was cultivated in 2011 (Ben Hania *et al.*, 2011). Thereafter, two *Mesotoga* species, *M. prima* strain MesG1.Ag.4.2^T (Nesbø *et al.*, 2012) and *M. infera* strain VNs100^T (Ben Hania *et al.*, 2013) were characterized while *Mesotoga* strain PhosAc3 was recently recognized as a *M. prima* strain (Ben Hania *et al.*, 2015). Phylogenetic analyses of 16S rRNA genes revealed that several distinct uncultured lineages of mesophilic *Thermotogae* may exist and thus that adaptation to mesothermic environments occurred several times independently during the diversification of *Thermotogae* (Nesbø *et al.*, 2010; Ben Hania *et al.*, 2011). Therefore, *Mesotoga* spp. are of noticeable interest to understand bacterial evolution from thermophily to mesophily. To date, beside the genus *Mesotoga*, *Thermotogae* comprise 12 other genera including *Athalassotoga*, *Defluviitoga*, *Fervidobacterium*, *Geotoga*, *Kosmotoga*, *Marinitoga*, *Mesoaciditoga*, *Oceanotoga*, *Petrotoga*, *Pseudothermotoga*, *Thermosipho* and *Thermotoga* (Di Pippo *et al.*, 2009;

Jayasinghearachchi and Lal, 2011; Ben Hania *et al*., 2012; Reysenbach *et al*., 2013; Bhandari and Gupta, 2014; Cappelletti *et al*., 2014; Itoh *et al*., 2016). They all share 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). Members of the phylum *Thermotogae* including the orders *Thermotogales*, *Kosmotogales*, *Petrotogales* and *Mesoaciditogales* (Bhandari and Gupta, 2014; Itoh *et al*., 2016) are usually considered as heterotrophic fermentative microorganisms able to use sugars, polysaccharides or complex organic substrates such as peptone and yeast extract. However, in contrast to all *Thermotogae* and to *M. prim*a strain MesG1.Ag.4.2^T in particular, and despite a very close phylogenetic relatedness with the latter, *M. prima* strain PhosAc3 and *M. infera* strain VNs100^T are able to use sugars only in the presence of elemental sulfur as terminal electron acceptor leading to the production of acetate, CO_2 and sulfide (around 2 moles of acetate and 4 moles of sulfide produced per mole of glucose consumed) with no or only traces of hydrogen production (less than 1 μM) (Ben Hania *et al*., 2013; 2015; Cappelletti *et al*., 2014). This sulfur-dependent metabolism discovered in these two closely related *Mesotoga* species does not concern exclusively sugars but also applies to other substrates tested (e.g. lactate and pyruvate) (Ben Hania *et al*., 2011; 2015) clearly contrasting with typical metabolic features of *Thermotogae* well known as efficient H_2-producing bacteria (over 1 mM) (Cappelletti *et al*., 2014). However, determining if sulfur reduction is an ATP-yielding reaction in *Mesotoga* spp. has not been established so far.

Here we show that co-culturing *M. prima* strain PhosAc3 with hydrogenotrophic sulfate-reducing partners (*Desulfovibrio* and *Desulfotomaculum* spp.) in the presence of sulfate as terminal electron acceptors significantly improved glucose oxidation by the former. Such metabolic process was extended to other *Mesotoga* spp. isolated so far including both *M. prima* strain MesG1.Ag.4.2^T and *M. infera* strain VNs100^T. We demonstrate two major points regarding *Mesotoga* species: (i) their dependence on the presence of elemental sulfur as terminal electron acceptor to use sugar, thus indicating that sugars are oxidized rather than fermented and (ii) the efficient syntrophic association with hydrogenotrophic partners able to replace more efficiently elemental sulfur as biological electron acceptor for sugar oxidation. Our data point out an unusual prokaryotic metabolism within the *Thermotogae*, the syntrophic association with a hydrogenotrophic partner in the absence of elemental sulfur to degrade easily fermentable substrates such as carbohydrates and highlight its possible ecological significance in nature.

Results

Growth of Mesotoga *spp. in pure culture and in co-culture with either sulfate-reducing bacteria or methanogens*

When *M. prima* strain PhosAc3 was cultured with glucose as energy source, only a slight glucose consumption was observed even after 250 days of incubation at 37°C only (Fig. 1A, Table 1). When elemental sulfur was added in the medium, a slow linear degradation of glucose was measured with 6.57 ± 0.19 mM of the glucose consumed

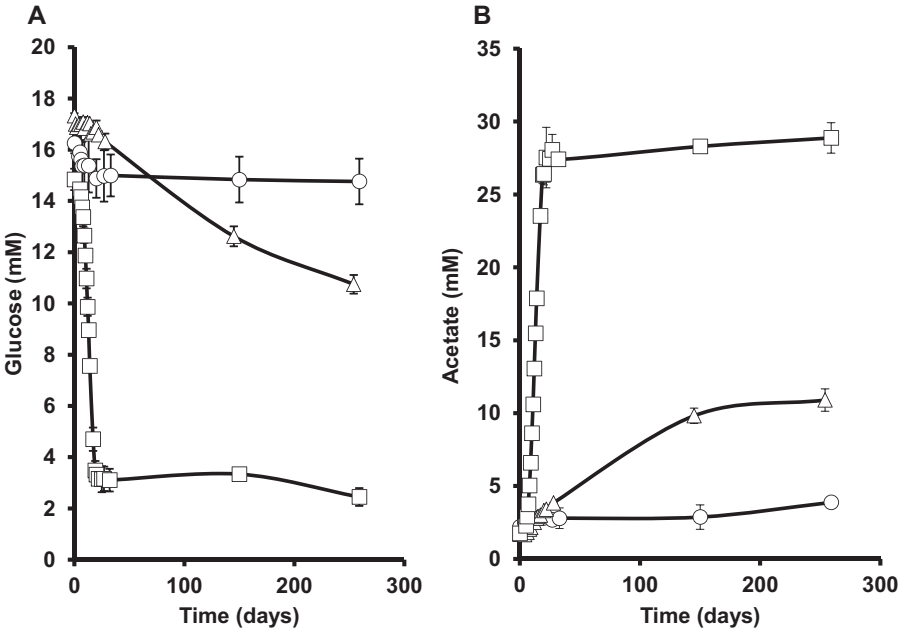

Fig. 1. A. Glucose consumption in pure culture of *M. prima* strain PhosAc3 in either the absence (circle) or presence (triangle) of elemental sulfur (S°) or in co-culture with *D. vulgaris* (square) over the time. B. Acetate production in either pure culture of *M. prima* in the absence (circle) or presence (triangle) of elemental sulfur (S°) or in co-culture with *D. vulgaris* (square) over the time.

Table 1. End-products quantification of the single and co-cultures of *M. prima* PhosAc3 and *M. prima* MesG1Ag4.2T.

	M. prima PhosAc3	*M. prima* PhosAc3 + S°	*M. prima* PhosAc3 + *D. vulgaris*	*M. prima* *MesG1Ag4.2*^{T}	*M. prima* *MesG1Ag4.2*^{T} + S°	*M. prima* *MesG1Ag4.2*^{T} + *D. salexigens*
Sugar consumed (mM)	1.50 ± 0.92	6.57 ± 0.19	12.39 ± 0.33	1.00 ± 0.23	3.27 ± 0.85	6.65 ± 1.88
Acetate produced (mM)	1.67 ± 0.21	9.21 ± 0.13	27.13 ± 0.45	0.70 ±0.41	8.48 ± 1.96	13.64 ± 1.75
H_2S produced (mM)	1.05 ± 0.25	24.40 ± 0.30	9.70 ± 0.85	1.18 ± 0.41	18.03 ± 5.16	14.00 ± 4.52

(around 33%) after 250 days of incubation (Fig. 1A, Table 1). Accordingly, only a slight acetate production was measured in the absence of elemental sulfur while 9.21 ± 0.13 mM acetate was produced in its presence after 250 days. The only detected end-products of glucose metabolism were acetate (Fig. 1B), CO_2 and sulfide (Table 1). No other volatile fatty acid (e.g. formate, butyrate, etc.) nor organic compound (e.g. lactate, ethanol) was detected in any growth conditions. Surprisingly, only trace amount of hydrogen (around 1 μM) were detected in the gas phase during glucose consumption whatever the presence or the absence of elemental sulfur.

These data clearly showed that *M. prima* strain PhosAc3 was unable to ferment glucose while, in the presence of an external electron acceptor (elemental sulfur), it was able to oxidize it although with a low efficiency. The slight glucose consumption in the absence of elemental sulfur was probably due to the presence of an electron acceptor available in yeast extract since hydrogen was detected only in minor quantities (around 1 μM) in the gas phase.

Because *Mesotoga* species are often detected in environments where sulfate-reducing bacteria are present (Nesbø *et al.*, 2010), the capability of *M. prima* strain PhosAc3 to grow in syntrophic association with a sulfate-reducing bacterium (SRB), *Desulfovibrio vulgaris* subsp. *vulgaris*, was tested. As with many other SRB, *D. vulgaris* gains energy for biosynthesis and growth by coupling oxidation of organic compounds or molecular hydrogen to reduction of sulfate to sulfide (Muyzer and Stams, 2008).

When either *M. prima* strain PhosAc3 or *D. vulgaris* subsp. *vulgaris* alone was cultured in a glucose/sulfate medium, no growth was observed (Fig. 2A), confirming that this *Desulfovibrio* species is unable to metabolize sugar. However, when the two bacteria were co-cultured, growth occurred as evidenced by a substantial increase of the optical density (OD) at 580 nm (Fig. 2A). After only 20 days of incubation, 80% of the initial glucose was consumed (~12 mM) with the concomitant acetate production of ~25 mM (Fig. 1A and B). The rate of glucose consumption was 0.8 mM glucose consumed/day. After 250 days of incubation, glucose consumption and acetate production only slightly progressed to give ~13 mM glucose consumed and ~28 mM acetate produced (Fig. 1, Table 1). At this time, around 10 mM sulfide had been produced (Table 1). Electron recovery values were 77% and 85% when *M. prima* strain PhosAc3 was grown in the presence of elemental sulfur and *D. vulgaris* as electron acceptors respectively. These values are in agreement with those expected for an anaerobic sugar-oxidizing metabolism leading to acetate as the only produced fatty acid, where about 20% of the sugar-derived reducing equivalents have been shown to be incorporated into the cells (Thauer *et al.*, 1977; Cord-Ruwish *et al.*, 1986). In contrast, when *M. prima* strain PhosAc3 was grown in the absence of elemental sulfur, electron recovery was lower (60%). In this case however, minor amounts of glucose used by *M. prima* strain PhosAc3 made this value less accurate (Table 1). When another SRB, *Desulfotomaculum gibsoniae* was used instead of *D. vulgaris* in the co-culture, a similar growth was obtained (data not shown). *M. infera* strain VNs100^T which was already reported to use elemental sulfur as electron acceptor for oxidizing glucose (Ben Hania *et al.*, 2013) was also co-cultured successfully with *D. vulgaris* subsp. *vulgaris* as confirmed by the increase of the OD at 580 nm (from 0.08 to 0.19) after 23 days of incubation.

The same kind of experiment was performed with the marine *Mesotoga* genus member, *M. prima* strain MesG1.Ag.4.2^T. However, as glucose was reported to be weakly used by this bacterium, it was replaced by fructose (Nesbø *et al.*, 2012). Similarly to *M. prima* strain PhosAc3, no growth was observed in fructose/sulfate medium and only a poor growth was obtained in the presence of S° (Fig. 2B). When *M. prima* strain MesG1.Ag.4.2^T was co-cultured with *Desulfovibrio salexigens* which was isolated from a marine environment as well, the OD at 580 nm increased during the first 9 days to reach around 0.4 OD_{580nm} unit, thus demonstrating the establishment of the co-culture when growing on fructose. At that time, around 13 mM acetate and 14 mM H_2S were produced (Table 1). A lower amount of acetate was produced in mono-culture in the presence of S°, in agreement with a lower growth under this condition (Table 1, Fig. 2B). Whatever the growth condition, the measured hydrogen concentration in the gas phase was around 1 μM.

To ensure that both *M. prima* strain PhosAc3 and *D. vulgaris* subsp. vulgaris actually grew under co-culture conditions, each cell-type was counted by epifluorescence microscopy after DAPI staining. This was made possible since the two bacteria have distinct cell morphology, easily

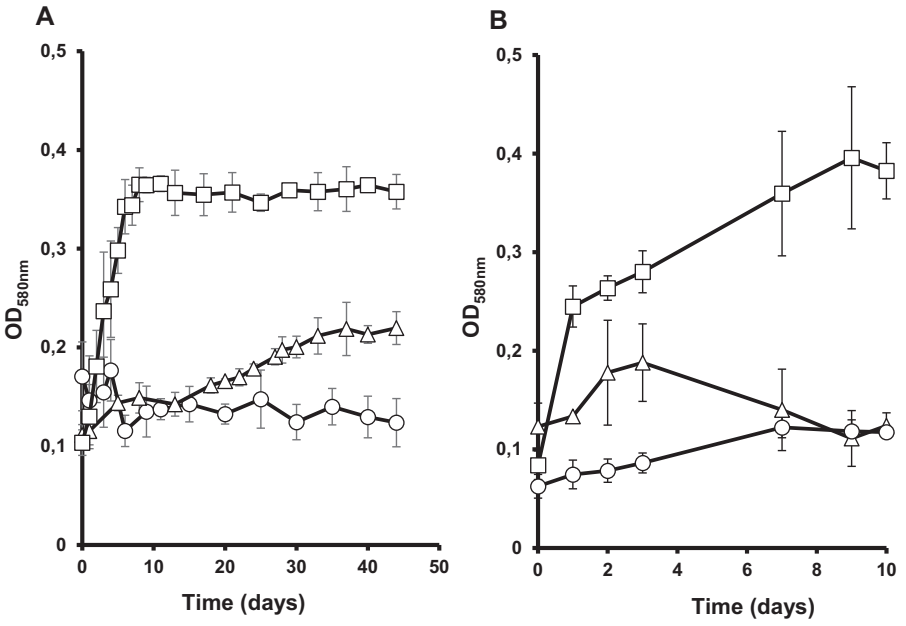

Fig. 2. A. Growth curves of *M. prima* strain PhosAc3 (triangle) and *D. vulgaris* (circle) in pure culture or in co-culture (square) in 20 mM glucose/28 mM sulfate containing medium. B. Growth curves of *M. prima* strain MesG1.A4.2^T in the presence (triangle) or the absence (circle) of S° and in co-culture with *D. salexigens* (square) in 20 mM fructose/28 mM sulfate medium

recognizable: small vibrios for *D. vulgaris* and pleomorphic rods surrounded with the sheath-like structure known as toga for *M. prima* strain PhosAc3 (Fig. 3). It revealed that four and five times more *M. prima* strain PhosAc3 and *D. vulgaris* cells, respectively, were present after 10 days of incubation in comparison to the inoculation time (time 0). At time 0, a *Mesotoga*/*Desulfovibrio* cell number ratio of 5.16 ± 1.06 was counted. This ratio changed very little during the growth time (5.54 ± 1.11, 3.97 ± 0.5 and 3.72 ± 0.91 after 5, 10 and 20 days of incubation respectively), showing that in a well-established co-culture, *Mesotoga* cells were about four times more abundant than *Desulfovibrio* cells.

Our data contrast with that previously obtained on *M. prima* strain MesG1.Ag4.2^T which has been described as a sugar fermentative bacterium producing acetate as major soluble fermentation product (Zhaxybayeva *et al.*, 2009; Nesbø *et al.*, 2012). However, the authors did not provide any information on hydrogen production from sugars but noticed a slight stimulation of growth in the presence of thiosulfate, sulfite or elemental sulfur. In addition, they used a high concentration of yeast extract (5 g l^{-1}) in their culture media (Nesbø *et al.*, 2012). Here, we show that *M. prima* strain PhosAc3 and *M. prima* strain MesG1.Ag4.2^T are able to degrade sugar albeit poorly, in the presence of yeast extract (1 g l^{-1}) and in the absence of elemental sulfur (Table 1). However, due to the production of only traces of hydrogen in such conditions, we can assume that an unknown mineral or organic compound present in the yeast extract serves as electron acceptor as already reported for the sulfur-respiring archaeon *Pyrococcus woesei* (Zillig *et al.*, 1987).

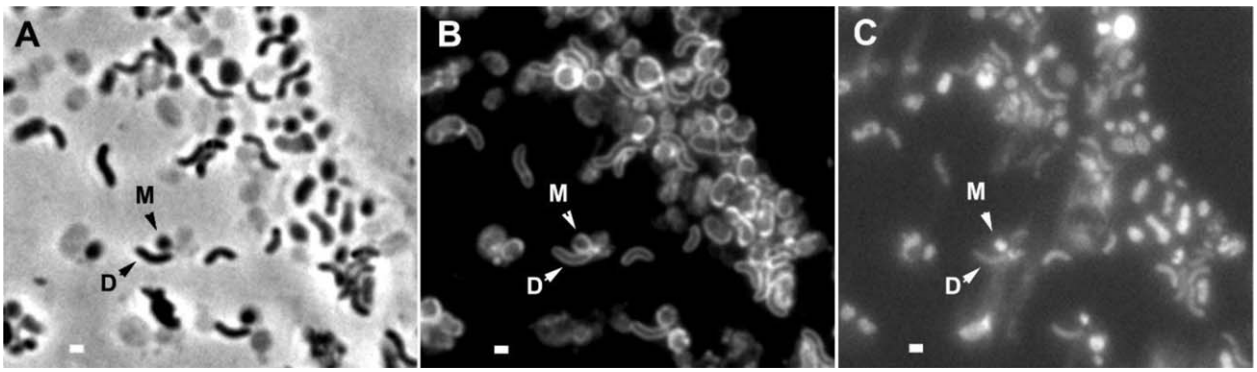

Fig. 3. Microscopy images of *D. vulgaris* and *M. prima* strain PhosAc3 in co-culture.
Exponentially growing co-culture was confined between a coverslip and a thin layer of solid medium culture in a custom hermetic chamber for anaerobic observation. (A) Phase contrast images (DIA), (B) membrane staining (FM4–64) and (C) nucleoid staining (DAPI). Scale bar = 1 μm. M and D arrows show *Mesotoga prima* strain PhosAc3 and *Desulfovibrio vulgaris* subsp. *vulgaris* respectively.

Under the growth condition used for the co-cultures (sugar/sulfate), *Desulfovibrio* spp. were unable to grow. This indicated that, in the co-cultures, *Desulfovibrio* spp. required metabolite(s) or end-product(s) of sugar metabolism released by *Mesotoga* strains. To test this hypothesis and to check whether cell-to-cell contacts were required, *M. prima* strain PhosAc3 and *D. vulgaris* subsp. *vulgaris* were co-cultured using a special set-up where the two bacteria were grown into two compartments separated with a dialysis membrane (Supporting Information Fig. S1). Under these conditions, after 18 days of incubation at 37°C, the OD_{580nm} increased from 0.02 ± 0.01 to 0.31 ± 0.03 in the *M. prima* compartment and from 0.07 ± 0.01 to 0.34 ± 0.03 in the *D. vulgaris* compartment. This clearly indicated that in co-cultures, both bacteria species were able to grow without the requirement of any cell-to-cell contact between them and suggested that *Mesotoga* and *Desulfovibrio* exchanged metabolites, required for their respective growth, able to diffuse across a dialysis membrane. Several attempts to co-culture *Mesotoga* with hydrogenotrophic methanogens as H_2 scavenger (*Methanospirillum hungatei, Methanobacterium congolense* or *Methanobacterium aarhusense*) were only successful using *M. hungatei;* after 67 days of incubation at 37°C, increase of the OD_{580nm} (0.135 ± 0.03) and microscopic observations (data not shown) indicated a growth of both microorganisms while no growth was observed when only one partner was present in the culture medium.

All the data presented above suggested that *M. prima* strain PhosAc3 and *D. vulgaris* growths were tightly coupled through a mutualistic/syntrophic association. Several examples of syntrophic associations involving the exchange of hydrogen or formate between the partners have been documented (Stams and Plugge, 2009). To test the involvement of hydrogen as a possible metabolic intermediate, *Desulfovibrio* and *Mesotoga* were cultured in two separate serum bottles whose headspaces communicated *via* tubing (Supporting Information Fig. S1), allowing only gas to be exchanged. After 24 days of incubation at 37°C, the OD_{580nm} increased from 0.02 ± 0.01 to 0.24 ± 0.03 in the *M. prima* compartment, and from 0.06 ± 0.01 to 0.22 ± 0.02 in the *D. vulgaris* compartment. These results indicated that (a) gaseous substance(s) exchanged between the two partners permitted syntrophic growth between *Mesotoga* and *Desulfovibrio*. Given the previous examples of syntrophic associations, our knowledge of the metabolism of *M. prima* and *D. vulgaris* and the fact that we did detect only traces of H_2 but not that of formate, we suggest that the best candidate as exchanged metabolite was hydrogen. As hydrogen metabolism requires hydrogenases, hydrogenase activity and glucose-dependent H_2 production from *M. prima* strain PhosAc3 cells were thus tested.

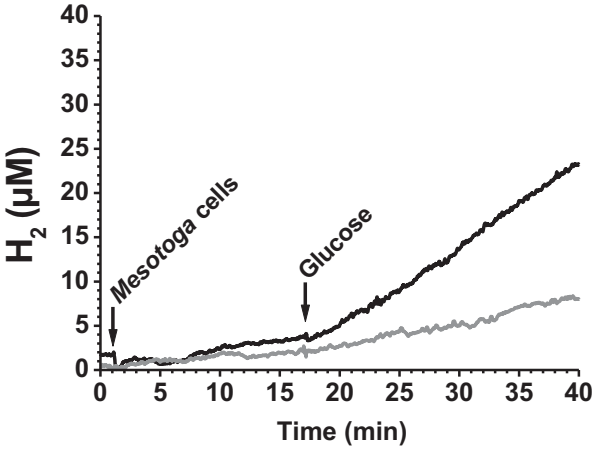

Fig. 4. Hydrogen production by *M. prima* strain PhosAc3 cells. Hydrogen evolution was measured on *Mesotoga* whole washed cells grown either in dialysis tubing co-cultured with *Desulfovibrio vulgaris* subsp. *vulgaris* in glucose/sulfate medium (black line) or grown in pure culture on glucose in the presence of elemental sulfur (grey line). Experiments were carried out in a respirometry chamber (1.9 ml) in Tris-HCl 0.1M NaCl 0.15M buffer (pH 7.5) at 37°C using a specific H_2 microsensor. Addition of cells (1.6 mg total protein for both samples) and glucose (50 mM final concentration) are indicated by arrows.

Hydrogenase activity and hydrogen production in M. prima *strain PhosAc3*

Glucose-dependent hydrogen production by *M. prima* strain PhosAc3, was evaluated using a Clark-type hydrogen microsensor on concentrated cells from either pure culture in the presence of elemental sulfur or co-culture with *D. vulgaris* in dialysis membrane-separated compartments (Fig. 4). A hydrogen production of 1.13 ± 0.25 nmol H_2/min/mg protein using glucose as substrate was measured by *M. prima* strain PhosAc3 when co-cultured with *D. vulgaris* while only 0.42 ± 0.06 nmol H_2/min/mg protein were produced when it was cultured alone in the presence of S°.

Spectrophotometric hydrogenase activity assays using methyl-viologen revealed hydrogenase activities of 33 ± 3 nmol H_2 produced/min/mg total protein and 5920 ± 930 nmol H_2 consumed/min/mg total protein when *M. prima* strain PhosAc3 was co-cultured with *D. vulgaris* while no hydrogenase activity could be detected when it was cultured alone in the presence of elemental sulfur. These data showed that hydrogen production and hydrogenase activity were higher when *M. prima* was cultured in the presence of *D. vulgaris* than in its absence, suggesting that *M. prima* was able to regulate the amount of hydrogenase in the cells, depending on the presence of a H_2 scavenger such as *D. vulgaris*.

Overall, these experimental data clearly showed that *M. prima* strain PhosAc3 was able to oxidize efficiently glucose through an interspecies H_2 transfer with *D. vulgaris*,

Table 2. Enzymes occurrence in *Thermotogae*.

	Bifurcating [FeFe] hydrogenase	Fd-dependent [FeFe] hydrogenase	Na+-NQR complex	Rnf complex
Fervidobacterium nodosum Rt17-B1	+	+	+	+/Short RnfB
Fervidobacterium pennivorans DSM 9078	+	+	+	+/Short RnfB
Pseudothermotoga thermarum DSM 5069	+	+	+	+/Short RnfB
Pseudothermotoga lettingae TMO	+	+	+	+/Short RnfB
Thermosipho africanus TCF52B	+	+	+	+/Short RnfB
Thermosipho melanesiensis BI429	+	+	+	+/Short RnfB
Thermotoga neapolitana DSM 4359	+	+	−	+/Short RnfB
Thermotoga maritima MSB8	+	+	−	+/Short RnfB
Thermotoga petrophila RKU-1	+	+	−	+/Short RnfB
Thermotoga naphthophila RKU-10	+	+	−	+/Short RnfB
Marinitoga piezophila KA3	−	+	−	+/Short RnfB
Petrotoga mobilis SJ95	−	+	+	+/Short RnfB
Kosmotoga olearia TBF 19.5.1	−	+	−	+/Long RnfB
Mesotoga infera VNs100	−	+	+	+/Long RnfB
Mesotoga prima MesG1 Ag 4 2	−	+	+	+/Long RnfB
Mesotoga prima PhosAc3	−	+	+	+/Long RnfB

the latter acting as a hydrogen scavenger. It is thus capable of syntrophic association with *Desulfovibrio* that leads to a more efficient growth, as evidenced by a significant improvement of biomass production and growth rate, than when cultured alone in the presence of elemental sulfur as terminal electron acceptor.

Mesotoga *spp. genome mining regarding hydrogen production and sugar oxidation*

Because hydrogen metabolism is a key pathway for this syntrophic association, the occurrence of genes encoding hydrogenases in the *M. prima* strain PhosAc3 genome was explored. No blast hits were found when using [NiFe]-hydrogenase sequences as input (data not shown), while one [FeFe]-hydrogenase homologue was detected in *M. prima* strain PhosAc3 (MESO_PHOSAC3V1_90124). Close homologues were found in the two other *Mesotoga* members (i.e. *M. prima* strain MesG1.Ag4.2^T (Theba_0443) and *M. infera* strain VNs100 (MESINFAv2 2088)). The phylogenetic analysis of *Thermotogae* [FeFe]-hydrogenases showed that the sequences of *Mesotoga* branched specifically with the ferredoxin-dependent [FeFe]-hydrogenase TM0201 of *Thermotoga maritima* strain MSB8 (Supporting Information Fig. S2). Interestingly, no homologue of the bifurcating trimeric [FeFe]-hydrogenase (TM1424–1426) from *T. maritima* strain MSB8 (Schut and Adams, 2009) was found in any *Mesotoga* strains so far sequenced (Table 2). Bifurcating [FeFe]-hydrogenases play an important function in the link between the carbohydrates oxidation via a classical Embden-Meyerhof pathway and the production of hydrogen in *T. maritima* by using the exergonic oxidation of ferredoxin to drive the endergonic oxidation of NADH to produce H_2 (Schut and Adams, 2009; Buckel and Thauer, 2013). It thus provides a mechanism to regenerate NAD from NADH by producing H_2, even if this reaction is thermodynamically unfavourable. In contrast with *T. maritima*, *M. prima* strain PhosAc3 is unable to ferment glucose; we propose that this metabolic difference is due to the absence of the bifurcating [FeFe]-hydrogenase in *M. prima* preventing the efficient re-oxidation by this enzyme of NADH generated by the NAD-dependent glyceraldehyde 3-phosphate dehydrogenase during glucose oxidation.

A search in *M. prima* strain PhosAc3 genome revealed the presence of two candidates for NADH re-oxidation: the Na^+-translocating NADH-quinone oxidoreductase (Na^+-NQR) complex (MESO_PHOSAC3V1_90501–MESO_PHOSAC3V1_90504) and the type 2 Rnf complex (MESO_PHOSAC3V1_120288–MESO_PHOSAC3V1_120293). It has been proposed that both Na^+-NQR and Rnf complexes would allow the re-oxidation of the NADH by transferring electrons to quinone and ferredoxin, coupled to Na^+ and Na^+/H^+ translocation through the membrane respectively (Hayashi *et al*., 2001; Biegel and Müller, 2010; Biegel *et al*., 2011; Barquera, 2014). While the Rnf complex of type 2 is present in all *Thermotogae*, the Na^+-NQR complex is absent in *Kosmotoga olearia*, *Marinitoga piezophila* and *Thermotoga* species (Table 2). Phylogenetic analysis of the genes encoding Rnf complex of type 2 indicated that this complex was probably present in the ancestor of *Thermotogae* (Supporting Information Fig. S3). In contrast, the Na^+-NQR complex is patchy distributed in *Thermotogae* and the corresponding sequences appeared intermixed with sequences from *Spirochaetes*, *Clostridia* and *Fusobacteria* (Supporting Information Fig. S4), indicating that horizontal gene transfers (HGT) occurred among these lineages and that it could have been secondarily acquired in *Thermotogae*. Sequences alignment of RnfB subunit from *Thermotogae*

revealed that the subunit from *T. maritima* was shorter than that from *M. prima* strain PhosAc3. This shorter sequence bound only one FeS cluster compared to at least two FeS clusters in the larger ones (Supporting Information Fig. S5). This characteristic was shared by all *Thermotogae* sequence so far, except members of the *Mesotoga* and *Kosmotoga* genera (Supporting Information Fig. S5). Interestingly, the presence of a shorter RnfB subunit correlated very well with the presence of a bifurcating [FeFe] hydrogenase in the organism, suggesting that this peculiar sequence feature could be involved in the specific interaction between the two proteins (Table 2).

Discussion

M. prima strain PhosAc3 was the first mesophilic cultivated member within the phylum *Thermotogae* (Ben Hania *et al.*, 2011) which comprised only fermentative thermophilic or hyperthermophilic bacteria (Huber and Hannig 2006; Zhaxybayeva *et al.*, 2009). Here we show that this bacterium is able to consume glucose only in the presence of elemental sulfur, thus clearly indicating that its sugar metabolism is based on an obligatory oxidative pathway, the elemental sulfur being essential to eliminate excess reducing equivalents generated from sugar oxidation. However, because *M. prima* strain PhosAc3 oxidizes very slowly sugar in the presence of elemental sulfur, one could expect that sulfur reduction is not a true respiratory process linked to oxidative phosphorylation. In this respect, elemental sulfur should serve as electron sink. Nevertheless, further experiments are needed to clarify this peculiar point regarding sulfur reduction. It is noteworthy that hydrogen was detected only as traces (around 1 μM in the gas phase) in any tested growth condition. The same type of sugar metabolism was demonstrated for *M. prima* strain MesG1.Ag.4.2^T and *M. infera* strain VNs100^T, the two other known members of the *Mesotoga* genus so far. All these data contrast with energy metabolism of thermophilic and hyperthermophilic *Thermotogae* known to (i) ferment sugars with substantial production of hydrogen (over 1 mM) (Schut and Adams, 2009; Cappelletti *et al.*, 2014) and (ii) reduce elemental sulfur facultatively (Huber and Hannif, 2006; Cappelletti *et al.*, 2014). Strictly following the definition of fermentation as 'an energy yielding process in which an energy substrate is metabolized without the involvement of an exogenous electron acceptor' (Singleton and Sainsbury, 2001), *Mesotoga* spp. should not be considered as sugar-fermenting but rather as sugar-oxidizing microorganisms.

We demonstrate also that *M. prima* strain PhosAc3, *M. prima* strain MesG1.Ag.4.2^T and *M. infera* strain VNs100^T grow more efficiently in syntrophic association with a hydrogenotrophic SRB, which serves as a terminal biological electron acceptor, than as pure culture in the presence of sulfur. Our data strongly suggest the existence of an interspecies hydrogen transfer between these *Mesotoga* species and hydrogenotrophic SRB. It should be noted that while we succeeded to co-culture *M. prima* strain PhosAc3 with all hydrogenotrophic SRB tested, attempts to co-culture it with hydrogenotrophic methanogens were only successful with *M. hungatei* only after several months of incubation. Because sulfate-reducing bacteria are known to have higher affinity for hydrogen than methanogens (Kristjansson *et al.*, 1982; Robinson and Tiedje 1984), it may indicate that the oxidation of substrates by *Mesotoga* requires a very low partial hydrogen pressure that could be better established by SRB than methanogens, with the exception of *M. hungatei* which is known to have the highest affinity for H_2 among methanogens (Robinson and Tiedje, 1984). In this respect, here we provide evidence for *Thermotogae* members to perform an efficient obligatory sugar oxidation through a syntrophic association with a hydrogenotrophic microbial partner.

We propose that the metabolic difference between *Thermotoga* spp. and *M. prima* strain PhosAc3 regarding sugar degradation is related to the absence of a bifurcating [FeFe]-hydrogenase in the latter, preventing it to ferment sugar. In contrast with *T. maritima*, the absence of this enzyme in *M. prima* would prevent the efficient re-oxidation of NADH, generated during glucose oxidation, linked to H_2 production. In the absence of an external electron acceptor, NADH accumulation would thus lead to growth inhibition. During the syntrophic association with a hydrogenotrophic partner, the ferredoxin-dependent [FeFe]-hydrogenase (locus tag MESO_PHOSAC3V1_90124) would produce hydrogen that would be, in turn, metabolized by the hydrogenotrophic partner. It has been proposed that the Rnf complex in *T. maritima* is involved in maintaining the appropriate ferredoxin/NADH ratio for the bifurcating [FeFe]-hydrogenase and other cell processes (Schut and Adams, 2009). Similarly, we advance that the *M. prima* strain PhosAc3 Rnf complex (MESO_PHOSAC3V1_120288–MESO_PHOSAC3V1_120293) would participate in hydrogen metabolism by driving reverse electron flow from NADH, generated by glucose oxidation, to the reduction of ferredoxin which then delivers electrons to the ferredoxin-dependent [FeFe]-hydrogenase. The Na^+-NQR complex (MESO_PHOSAC3V1_90501–MESO_PHOSAC3V1_90504) would also participate in re-oxidation of the reduced NADH generated from glucose oxidation.

Because *M. prima* strain PhosAc3, *M. prima* strain MesG1.Ag4.2^T and *M. infera* strain VNs100^T are all able to oxidize sugars, one can hypothesize that this capacity was inherited from their common ancestor. Actually, like *M. prima* strain PhosAc3, *M. prima* strain MesG1.Ag4.2^T genome encodes homologous ferredoxin-dependent [FeFe]-hydrogenase (Theba_0443) as well as Na^+-NQR

(Theba_0829-Theba_0833) and Rnf (Theba_1343-Theba_1348) complexes but no bifurcating [FeFe]-hydrogenase. We thus propose that the efficient sugar-oxidizing metabolism linked to a syntrophic association with a hydrogenotrophic bacterium can be extrapolated to all members of the *Mesotoga* genus.

The importance of obligate interspecies hydrogen transfer for substrate oxidation has been documented in many occasions with a peculiar emphasis for fatty acids, ethanol as well as aromatic and alicyclic compounds (Sieber *et al*., 2014; Schmidt *et al*., 2016). In contrast, such process involving a hydrogenotrophic partner to degrade easily fermentable substrates as carbohydrates has been reported only a few times, as for *Syntrophococcus sucromutans*, isolated from rumen of steer (Krumholz and Bryant, 1986) and *Bacillus* spp., isolated from Lake Constance sediment (Müller *et al*., 2008). The latter authors, by direct dilution of Lake Constance sediment in mineral agar medium containing *Methanosprillum hungatei*, provided evidence that obligate sugar-degrading *Bacillus* sp. using a hydrogen-scavenging methanogen as terminal electron acceptor outnumbered those fermenting easily these substrates. These *Bacillus* spp. were thus believed to be of high ecological significance in these sediments (Müller *et al*., 2008). The same conclusion may be drawn for *M. prima* strain PhosAc3 since, similarly as for Müller *et al*. (2008), it has been isolated after high dilution steps from anaerobic digester treating phosphogypsum (Ben Hania *et al*., 2011; 2015). It may be extended to other *Mesotoga* strains which have been detected by molecular approaches in various ecosystems including those contaminated by toxic compounds (Nesbø *et al*., 2006; 2010). Association with hydrogenotrophic SRB and possibly with high hydrogen affinity hydrogenotrophic methanogens such as *M. hungatei* would thus be of primary ecological importance for growth of *Mesotoga* spp. and other obligate-oxidizing bacteria in their natural habitats. Interestingly, all these bacteria (*S. sucromutans*, *Bacillus* and *Mesotoga* spp.) produce acetate as the sole organic acid end-product of sugar metabolism. Their ecological role as saccharolytic acetogenic bacteria in association with anaerobic hydrogen scavengers (e.g. sulfate-reducing bacteria), might have been underestimated so far. It thus deserves further investigations especially in sugar containing environments, to enlarge the current view of the microbiology of anaerobic digestion of organic matter (e.g. carbohydrates) that typically gives a predominant role to classical sugar-fermenting bacteria over syntrophic sugar-oxidizing associations.

Experimental procedures

Media and culture conditions

M. prima strain PhosAc3 and *M. infera* strain VNs100^T were grown with glucose (20 mM) as carbon and energy sources at 37°C in medium containing per litre 0.3 g KH_2PO_4; 0.3 g K_2HPO_4; 1.0 g NH_4Cl; 2.0 g NaCl; 0.1 g KCl; 0.1 g $CaCl_2.2H_2O$; 0.5 g $MgCl_2.6H_2O$; 1 g yeast extract; 0.5 g cysteine-HCl; 0.16 g sodium acetate; 1 ml Widdel trace element solution (Widdel and Pfennig, 1981) and 1 ml resazurin 0.1%. When specified, 10 g of elemental sulfur (S^0) per litre of medium was added. Before culture inoculation, 0.2 ml of 10% (wt/vol) $NaHCO_3$, 0.1 ml of 2% (wt/vol) Na_2S-$9H_2O$ and glucose were injected from sterile stock solutions in the culture medium. *Mesotoga prima* strain MesG1.Ag4.2^T was grown in the same culture medium mentioned above containing fructose (20 mM) instead of glucose in the presence of 25 g l^{-1} NaCl. *Desulfovibrio vulgaris* subsp. *vulgaris, D. salexigens* and *D. gibsoniae* were grown in the same culture medium as for *M. prima* strain PhosAc3 with the sugar being replaced by hydrogen (H_2:CO_2; 80:20 vol/vol) as electron donor and elemental sulfur by sodium sulfate (Na_2SO_4) (4 g l^{-1}) as electron acceptor. Twenty-five grams per litre NaCl was added to the culture medium for growth of *D. salexigens*. *Methanospirillum hungatei*, *M. congolense* and *M. aarhusense*, were grown in the same culture medium as for sulfate-reducing bacteria but without sulfate. Co-cultures were performed in the same medium as for culturing *Mesotoga species* alone but without elemental sulfur that was replaced by sodium sulfate (4 g l^{-1}) in the case of co-cultures with sulfate-reducing bacteria.

Cultures and co-cultures were performed under anaerobic conditions in Hungate tubes or in large volume flasks. Co-cultures were established by mixing (10% vol/vol each) exponentially growing (i) *Mesotoga* strain PhosAc3 and either *D. vulgaris* subsp. *vulgaris*, *D. gibsoniae, M. hungatei, M. congolense* or *M. aarhusense*, (ii) *M. infera* strain VNs100^T and *D. vulgaris* subsp. *vulgaris*, (iii) *M. prima* strain MesG1.Ag4.2^T and *D. salexigens*. Fresh medium was inoculated with 10% (vol/vol) of exponentially growing cells and incubated at 37°C. Growth was monitored by measurement of the OD at 580 nm of the culture directly in the Hungate tube. Growth curves and metabolites quantification were obtained from at least 2 independent replicates.

Analytical methods

Soluble sulfides were quantified according to the Cord-Ruwish (1985) method by using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan). Sugars (lactose, glucose), organic acids (acetate, lactate, propionate, butyrate, formate) and ethanol determination was carried out by high performance liquid chromatography (HPLC) as described by Fardeau *et al*. (1997). For hydrogen quantification, 1 ml of culture headspace sample (gas phase) was injected into a Shimadzu 8A TCD-GC system (Shimadzu, Kyoto, Japan) equipped with a concentric CTR1 column (Alltech, Columbia, MD, USA), connected to a computer running WINILAB III software (Perichrom, Saulx les Chartreux, France) (Fardeau *et al*., 1997). Unless otherwise indicated, analytical measures were performed on duplicate culture tubes or bottles.

Cells counting by microscopy

For preparing the microscope observation chamber, all steps were performed in an anaerobic chamber (Coy Laboratory,

Grass Lake, MI, USA). One hundred microlitre of the *M. prima* strain PhosAc3 and *D. vulgaris* subsp. *vulgaris* co-culture were centrifuged for 3 min at 10 000 *g*. DAPI staining was performed by resuspending the cells pellet in 100 μl of 10 mM Tris-HCl (pH 7.6), 8 mM $MgSO_4$ and 1 mM KH_2PO_4 buffer (TPM buffer) containing 5 ng μl^{-1} of 4′,6-diamidino-2-phenylindole (DAPI). After 20 min of incubation in the dark, cells were washed three times with TPM buffer. The DNA was stained under anaerobic conditions to limit the exposure of the cells to air. The cells were placed between a glass coverslip and a thin layer of LS4D-YE medium supplemented with 10 ng μl^{-1} of FM4–64® (Invitrogen) and 1.5% (wt/vol) of Phytagel™ (Sigma-Aldrich) that was previously prepared under anaerobic conditions. The preparation was then transferred in a hermetic microscope observation chamber (Fievet *et al.*, 2015) and put into a standard temperature-controlled inverted microscope. Pictures were acquired with a Nikon TiE-PFS inverted epifluorescence microscope, 100× NA1.3 oil PhC objective (Nikon) and Hamamatsu Orca-R2 camera. Image processing was controlled by the NIS-Element software (Nikon).

Hydrogenase activity measurements by spectrophotometry

Pure cultures of *M. prima* strain PhosAc3 (1 l) were grown in the presence of elemental sulfur until the OD_{580nm} reached 0.28. Cells were harvested by centrifugation (5000 *g* for 30 min), rinsed once with 50 ml of 50 mM Tris-HCl, NaCl 200 mM (pH 7.5) buffer and finally resuspended in 500 μl of 100 mM HEPES buffer (pH 8.0). When *M. prima* strain PhosAc3 was co-cultured with *D. vulgaris* subsp. *vulgaris* in separated compartments, cells were prepared as above from 500 ml of culture. For hydrogenase uptake activities, a rubber-stopper sealed cuvette containing 1 ml of 100 mM HEPES buffer (pH 8.0), 75 μl of 100 mM methyl-viologen was bubbled for 5 min with hydrogen. Then, a defined amount of cells suspension (from 10 to 50 μl) and 5 μl of 1/10 Triton X100 diluted in HEPES buffer was added. The OD at 604 nm was recorded over the time. As controls, methyl-viologen reduction was followed in cuvettes without hydrogen bubbling and without addition of cells.

For H_2 production activity, a rubber-stopper sealed cuvette containing 1 ml of 100 mM HEPES buffer (pH 8.0) was bubbled for 5 min with Argon. A defined amount of cells suspension (from 10 to 50 μl) was then added and the cuvette was bubbled with Argon for 2 min longer. Then, 100 μl of dithionite-reduced methyl-viologen (10 mM) and 5 μl of 1/10 Triton X100 diluted in HEPES buffer were added. The decrease of the OD at 604 nm was recorded over the time. The auto-oxidation kinetic of reduced methyl-viologen was measured in cuvette in the absence of cells. H_2 production activity was obtained by subtracting the auto-oxidation activity from the methy-viologen oxidation activity in the presence of cells.

All buffers, reactives and cells suspensions were kept under anaerobic conditions and continuously flushed with Argon.

H_2 production monitoring by Clark-based microsensor

Hydrogen production was also measured on *M. prima* strain PhosAc3 cells grown on glucose either as mono-culture in the presence of elemental sulfur or as co-culture with *D. vulgaris* in dialysis membrane-separated compartments as described in Supporting Information Fig. S1. Cells were harvested at the end of the exponential phase and washed three times with an anaerobic 0.1 M Tris-HCl, 0.15 M NaCl (pH 7.5) buffer saturated with N_2. The measurements were carried out in a 1.9 ml sealed anaerobic cuvette filled with 0.1 M Tris-HCl, 0.15 M NaCl (pH 7.5) buffer equilibrated at 37°C and containing cells suspension. After 15 min, 50 mM glucose (final concentration) was added and H_2 production was monitored with a H_2-MR 500 μm microsensor plugged to a Microsensor Multimeter (Unisense, Aarhus, Denmark). Electrode was calibrated with known amounts of pure H_2 gas saturated in water at 37°C (750 μM).

Phylogenetic analyses

Two thousand seven hundred seventy-five complete prokaryotic proteomes were downloaded at the NBCI (http://www.ncbi.nlm.nih.gov/). Only one representative proteome per species was kept for analyses. The 1369 corresponding proteomes (including 14 *Thermotogae* species, Supporting Information Table S1) were gathered to build a local database. This database was queried with BLASTP (v2.2.26) (Altschul *et al.*, 1990) to identify homologues of the studied proteins. *Mesotoga prima* strain PhosAc3 or *T. maritima* strain MSB8 protein sequences were used as seed. The 60 protein sequences displaying the highest scores and E-values lower than 10^{-3} were retrieved. Homologues from *M. prima* strain PhosAc3 and *M. infera* strain VNs100^T were downloaded from the MAGE platform at the genoscope (https://www.genoscope.cns.fr). The retrieved homologues were aligned with MAFFT v7 (option L-INS-I) (Katoh and Standley, 2013). The resulting multiple alignments were visually inspected with SEAVIEW v.4.5.4 (Gouy *et al.*, 2010) and trimmed with BMGE v1.1 (Criscuolo and Gribaldo, 2010).

Maximum likelihood phylogenetic trees were inferred with PHYML v.3.1 (Guindon *et al.*, 2010) implemented in the SEAVIEW program (Gouy *et al.*, 2010) using the 'Le and Gascuel evolutionary model' (Le and Gascuel, 2008) and a gamma distribution with four categories of sites to take into account the heterogeneity of site evolutionary rates. The NNI + SPR option was used to explore tree topologies. The robustness of the resulting trees was evaluated using the non-parametric bootstrap procedure implemented in PHYML (100 replicates of the original dataset).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. (A) Compartment-separated co-culture of *M. prima* strain PhosAc3 and *D. vulgaris*. *D. vulgaris* was grown inside a dialysis tube (dialysis membrane Spectra/Por, 12-14K MWCO) that was secluded from the surrounding strain *M. prima* culture by rubber stoppers. The apparatus was incubated in a very slowly moving (30 r.p.m.) rotary shaker at 37°C. (B) Experimental assembly to grow the co-culture in two different vials linked by a tubing.
Fig. S2. Maximum likelihood phylogeny of the [FeFe] hydrogenases in *Thermotogae* (101 sequences, 450 amino acid positions kept after trimming of the alignment). Numbers at nodes are bootstrap values (100 replicates of the original dataset). For clarity, values lower than 50% are not shown. The scale bars indicate the average number of substitutions per site. Colours correspond to taxonomic groups. The complete taxonomy of each strain is indicated on the tree.
Fig. S3. Maximum likelihood phylogenies of the proteins composing the Rnf complex. Numbers at nodes are bootstrap values (100 replicates of the original dataset). For clarity, values lower than 50% are not shown. The scale bars indicate the average number of substitutions per site. Colours correspond to taxonomic groups. The complete taxonomy of each strain is indicated on the tree. A – RfnC–MESO_PHOSAC3v1_120288 (61 sequences, 346 amino acid positions kept after trimming of the alignment). B – RfnD–MESO_PHOSAC3v1_120289 (61 sequences, 265 amino acid positions kept after trimming of the alignment). C – RfnG–MESO_PHOSAC3v1_120290 (54 sequences, 75 amino acid positions kept after trimming of the alignment). D – RfnE–MESO_PHOSAC3v1_120291 (62 sequences, 176 amino acid positions kept after trimming of the alignment). E – RfnA–MESO_PHOSAC3v1_120292 (62 sequences, 185 amino acid positions kept after trimming of the alignment). F – RfnB–MESO_PHOSAC3v1_120293 (62 sequences, 107 amino acid positions kept after trimming of the alignment).
Fig. S4. Maximum likelihood phylogenies of the genes composing the Na+-NQR complex. Numbers at nodes are bootstrap values (100 replicates of the original dataset). For clarity, values lower than 50% are not shown. The scale bars indicates the average number of substitutions per site. Colours correspond to taxonomic groups. The complete taxonomy of each strain is indicated on the tree. A – RfnD-like–MESO_PHOSAC3v1_90501 (62 sequences, 215 amino acid positions kept after trimming of the alignment). B – NrqC–MESO_PHOSAC3v1_90502 (62 sequences, 86 amino acid positions kept after trimming of the alignment). C – NrqD–MESO_PHOSAC3v1_90503 (62 sequences, 191 amino acid positions kept after trimming of the alignment). D – NrqE–MESO_PHOSAC3v1_90504 (62 sequences, 189 amino acid positions kept after trimming of the alignment). E – NrqF–MESO_PHOSAC3v1_90505 (62 sequences, 351 amino acid positions kept after trimming of the alignment).
Fig. S5. Sequence Alignment of the RnfB subunit from Rnf complexes of the *Thermotogae* members. The sequences were aligned with MAFFT. Conserved positions are indicated with colours. Accession number of sequences:

Pseudothermotoga lettingae TMO (YP_001469925), *Pseudothermotoga thermarum* DSM 5069 (YP_004660443), *Petrotoga mobilis* SJ95 (YP_001568014), *Marinitoga piezophila* KA3 (YP_005096257), *Kosmotoga olearia* TBF 19.5.1 (YP_002940621), *Thermotoga naphthophila* RKU-10 (YP_003346384), *Mesotoga prima* MesG1.Ag.4.2 (Theba1348), *Mesotoga prima* PhosAc3 (MESO PHOSAC3v1_120293), *Mesotoga infera* VNs100 (MESINFAv2 2581), *Thermotoga naphthophila* RKU-10 (YP_003346384), *Thermotoga petrophila* RKU-1 (YP_001244271), *Thermotoga neapolitana* DSM 4359 (YP_002533979), *Thermotoga maritima* MSB8 (NP_228063), *Fervidobacterium pennivorans* DSM 9078 (YP_005471998), *Fervidobacterium nodosum* Rt17-B1 (YP_001411073), *Thermosipho melanesiensis* BI429 (YP_001306837) and *Thermosipho africanus* TCF52B (YP_002335587).

Table S1. List of the 2775 prokaryotic proteomes used in the phylogeny analyses.