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Mutational analyses of the enzymes involved in the metabolism of hydrogen by the hyperthermophilic archaeon *Pyrococcus furiosus*

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Pyrococcus furiosus grows optimally near 100˚C by fermenting carbohydrates to produce hydrogen (H_2) or, if elemental sulfur (S^0) is present, hydrogen sulfide instead. It contains two cytoplasmic hydrogenases, SHI and SHII, that use NADP(H) as an electron carrier and a membrane-bound hydrogenase (MBH) that utilizes the redox protein ferredoxin. We previously constructed deletion strains lacking SHI and/or SHII and showed that they exhibited no obvious phenotype. This study has now been extended to include biochemical analyses and growth studies using the ΔSHI and ΔSHII deletion strains together with strains lacking a functional MBH (ΔmbhL). Hydrogenase activity in cytoplasmic extracts of various strains demonstrate that SHI is responsible for most of the cytoplasmic hydrogenase activity. The ΔmbhL strain showed no growth in the absence of S^0, confirming the hypothesis that, in the absence of S^0, MBH is the only enzyme that can dispose of reductant (in the form of H_2) generated during sugar oxidation. Under conditions of limiting sulfur, a small but significant amount of H_2 was produced by the ΔmbhL strain, showing that SHI can produce H_2 from NADPH *in vivo*, although this does not enable growth of ΔmbhL in the absence of S^0. We propose that the physiological function of SHI is to recycle H_2 and provide a link between external H_2 and the intracellular pool of NADPH needed for biosynthesis. This likely has a distinct energetic advantage in the environment, but it is clearly not required for growth of the organism under the usual laboratory conditions. The function of SHII, however, remains unknown.

Keywords: hydrogenase, energy metabolism, sulfur, ferredoxin, *Pyrococcus furiosus*, thermophile, anaerobe

INTRODUCTION

Hydrogen gas (H_2) plays an important role in anaerobic metabolism as the majority of anaerobes contain the enzyme hydrogenase responsible for the reversible interconversion of molecular hydrogen, protons, and electrons. Hydrogenases can be grouped into three classes based on the metal composition of their active site: [NiFe]-hydrogenases, [FeFe]-hydrogenases, and the more recently defined [Fe]-hydrogenases, so far restricted to certain methanogenic organisms (Vignais et al., 2001; Shima and Thauer, 2007). Organisms in the bacterial domain contain both [NiFe]- and [FeFe]-hydrogenases, while archaeal organisms are known to utilize [NiFe]- and [Fe]-hydrogenases (Vignais and Billoud, 2007). Almost all of the anaerobic archaea contain one or more [NiFe]-hydrogenases, implying that H_2 metabolism plays an important role in the extreme environments in which many of these organisms are found (Vignais and Billoud, 2007).

Pyrococcus furiosus is a well-studied hyperthermophile belonging to the order Thermococcales. It grows optimally at 100°C by peptide and carbohydrate fermentation with H_2, organic acids, and CO_2 being the main fermentation products (Fiala and Stetter, 1986; Kengen and Stams, 1994; Driskill et al., 1999; Adams et al., 2001). *P. furiosus* degrades glucose via a modified Embden–Meyerhof glycolytic pathway that utilizes the low potential electron carrier protein ferredoxin (Fd) in place of NAD for all oxidative steps, resulting in no overall substrate level ATP yield (Kengen et al., 1994; de Vos et al., 1998; Verhees et al., 2004). Because of the low redox potential of Fd (−480 mV), H_2 production (−420 mV) with Fd is thermodynamically favorable (Park et al., 1991; Smith et al., 1995; Hagedoorn et al., 1998). Therefore, all reducing equivalents generated in sugar metabolism can be disposed of as H_2, with production of up to 4 mol of H_2 per mol glucose oxidized (Verhees et al., 2004). The enzyme responsible for H_2 formation is a unique membrane-bound hydrogenase (MBH) complex, which uses the energy from this exergonic reaction to create an ion gradient across the membrane that drives ATP synthesis, resulting in the generation of an estimated 0.3 mol of ATP per mol H_2 (Sapra et al., 2003). This system is one of the simplest forms of respiration. Besides MBH, *P. furiosus* also contains two cytoplasmic hydrogenases (SHI and SHII), and these homologous enzymes each consist of four subunits. Based on kinetic studies, SHI and SHII are proposed to be involved in H_2 recycling to provide NADPH for biosynthesis (Ma and Adams, 2001b; van Haaster et al., 2008).

Pyrococcus furiosus can utilize carbohydrates for growth in either the presence or absence of elemental sulfur (S^0; Fiala and Stetter, 1986; Adams et al., 2001). In the presence of S^0, *P. furiosus* undergoes a major metabolic shift to utilize S^0 as an electron

acceptor, thereby switching production from H_2 to H_2S. When S^0 becomes available, there is a dramatic decrease in the expression of all three hydrogenase operons correlated with a decrease in hydrogenase activity, and concomitantly, there is a large increase in expression of genes related to S^0 reduction, such as those encoding the membrane-bound oxidoreductase (MBX) as well as the NAD(P)H-linked sulfur reductase (NSR; Adams et al., 2001; Schut et al., 2007). The response to S^0 is mediated at least in part by the redox switch containing transcriptional regulator SurR (Lipscomb et al., 2009; Yang et al., 2010). MBX is highly homologous to MBH and is thought to function analogously to MBH by using Fd as an electron acceptor and conserving energy via formation of an ion gradient (Silva et al., 2000; Schut et al., 2007; Bridger et al., 2011). NSR has been proposed to reduce S^0 synergistically with MBX in which MBX can provide NADPH using Fd as electron donor (Schut et al., 2007; Bridger et al., 2011).

In a previous study it was shown that strains containing deletions of either SHI or SHII or both have no growth phenotype under the conditions tested, suggesting that other enzyme(s), independent of H_2, can guide electrons from carbohydrate oxidation to feed the NADPH pool (Lipscomb et al., 2011). This study has now been extended to include strains containing a deletion of the active subunit of MBH (mbhL) either alone or in combination with deletions of the genes encoding both soluble hydrogenases. Herein, we address the physiological function of all three hydrogenases in *P. furiosus* both in the absence and presence of sufficient and limiting concentrations of S^0.

MATERIALS AND METHODS

STRAINS AND GROWTH CONDITIONS

The *P. furiosus* strains used or constructed in the study are listed in **Table 1**. Growth medium was prepared as previously described (Adams et al., 2001), and contained maltose (5 g/L) as the primary carbon source, supplemented with 0.5 g/L yeast extract, with or without addition of S^0 at 0.5 or 2 g/L. Growth experiments were carried out in biological duplicates in 150-mL serum bottles with 75 mL medium, with incubation at 95°C and shaking at 150 rpm. Cultures for cell-free extracts were grown in a 20-L custom fermenter, and cytoplasmic fractions were prepared by ultra-centrifugation as described previously (Adams et al., 2001).

CONSTRUCTION OF GENE DELETIONS

Gene splicing by overlap extension and PCR (SOE–PCR; Horton et al., 1989) was used to construct a PCR product containing 0.5 kb regions upstream and downstream of *mbhL* on either side of a genetic marker. The destination strains (COM1 and ΔSHIΔSHII) contain a deletion of the *pyrF* gene; therefore, a cassette containing *pyrF* expressed by the PEP synthase promoter (123 bp) was used as the marker. The 500-bp flanking regions were amplified from wild-type gDNA using the two primer sets WN011 (GTCATAAAACTAAATGATGAGCATTTGACTTCATTTCTTCTCCCTC), WN013 (TTGGAGAAGAGAATTGCCCAAC), and WN012 (AGAATGGAGCTCAAGATAAATATGAAAATTGTATATGGAGTTATTGG), WN014 (AGACATCAACACACTGCTTACAC). The deletion construct for *mbhL* was transformed into *P. furiosus* COM1 and ΔSHIΔSHII selecting uracil prototrophy on solid defined medium as previously described (Lipscomb et al., 2011). To obtain *mbhL* deletions, solid medium was supplemented with 8 mM polysulfide (equivalent to approximately 1 g/L S^0) or 2 mM polysulfide with solid S^0 powder sprinkled on the surface of the plate. The *pyrF* gene deleted in the COM1, ΔSHI, ΔSHII, and ΔSHIΔSHII strains was restored to the wild-type by transformation with a PCR product of the wild-type *pyrF* locus containing ∼0.5 kb on either side of the gene. This was done to ensure all strains were uracil prototrophic so growth could be achieved in identical media. DNA was extracted from transformants as previously described (Lipscomb et al., 2011) and screened for deletion by PCR amplification of the locus using primers outside the homologous flanking regions used to construct the deletions. Transformants were further colony purified by serial passage on solid medium. Mutants were confirmed by sequence analysis of the *mbhL* region and qPCR analyses (see below).

RNA ISOLATION AND qPCR ANALYSES

Total RNA from various *P. furiosus* strains was obtained, and cDNA was synthesized as previously described (Lipscomb et al., 2011). Quantitative PCR was carried out using an Mx3000P instrument (Agilent) and the Brilliant SYBR green qPCR master mix (Agilent) with primers specific to the genes encoding SHI and SHII beta subunits (PF0891 and PF1329), *mbhK* (PF1433), *mbhL* (PF1434), *mbhM* (PF1435), and *pyrF* (PF1114), along with the constitutively expressed genes encoding the pyruvate ferredoxin oxidoreductase (POR) gamma subunit (PF0971) and DNA Polymerase (PF0983) as internal controls. Successful gene deletions were verified by the absence of a specific qPCR signal.

CELL PROTEIN, H_2S, AND H_2 ANALYSES

To monitor cell growth, the Bradford method (Bradford, 1976) was used to estimate total cell protein concentration from 1 mL culture samples, with bovine serum albumin as the standard. For H_2S and

Table 1 | *Pyrococcus furiosus* strains used in this study.

Strain	Genotype	Deleted ORF(s)	Reference or source
COM1c (MW0004)	Δ*pyrF::pyrF*	None	Lipscomb et al. (2011)
ΔSHI (MW0022)	Δ*pyrF::pyrF* Δ*shI*βγδα	PF0891–PF0894	This work
ΔSHII (MW0023)	Δ*pyrF::pyrF* Δ*shII*βγδα	PF1329–PF1332	This work
ΔSHIΔSHII (MW0016)	Δ*pyrF::pyrF* Δ*shI*βγδα Δ*shII*βγδα	PF0891–PF0894, PF1329–PF1332	This work
ΔmbhL (MW0024)	Δ*pyrF* Δ*mbhL::*P_{pep}*pyrF*	PF1114, PF1434	This work
ΔSHIΔSHIIΔmbhL (MW0025)	Δ*pyrF* Δ*shI*βγδα Δ*shII*βγδα Δ*mbhL::*P_{pep}*pyrF*	PF1114, PF0891–PF0894, PF1329–PF1332, PF1434	This work

H_2 analyses, headspace, and medium samples (500 μL each) were taken at 6 and 9 h during growth and transferred anaerobically into the double-vial system as previously reported (Schut et al., 2007). H_2S production was assayed by the methylene blue method (Chen and Mortenson, 1977), and abiotic sulfide production was subtracted from the experimental samples using control bottles containing uninoculated medium. H_2 production was measured using a gas chromatograph (GC-8A, Shimadzu, Columbia, MD, USA). Hydrogenase activity in cell-free extracts was determined by H_2 production using sodium dithionite (5 mM) as the electron donor with methyl viologen (1 mM) as the electron carrier as described previously (Ma and Adams, 2001b).

RESULTS

CHARACTERIZATION OF SHI AND SHII MUTANTS

We showed previously that strains containing deletions of either of the two cytosolic NADP-linked hydrogenases (SHI and SHII) alone or together did not produce any growth phenotype under the conditions tested (Lipscomb et al., 2011). In order to verify the effect of disruption of SHI and SHII on hydrogenase activity in the cytoplasm, we prepared cell-free extracts from ΔSHI, ΔSHII and ΔSHIΔSHII. The amount of hydrogenase activity (using the artificial electron carrier methyl viologen) in cytoplasmic fractions was not significantly affected in the ΔSHII strain but was much lower in the ΔSHI strain (<10% of that produced in the parental strains), while in the ΔSHIΔSHII strain, no hydrogenase activity could be detected (**Figure 1**). These data indicate that SHI is responsible for the majority of hydrogenase activity in the cytoplasm and confirms that the activity of the MBH is strictly associated with the membrane (Sapra et al., 2000; Silva et al., 2000).

CHARACTERIZATION OF *mbhL* MUTANTS

We have constructed strains containing a deletion of the catalytic subunit (mbhL) of the membrane-bound ferredoxin-linked H_2-producing hydrogenase (MBH), either alone or in combination with deletions of SHI and SHII. Growth of these strains was compared on maltose-based medium containing minimal yeast extract with either no S^0, limiting S^0 (0.5 g/L), or sufficient S^0 (2 g/L). Both mutants containing a deletion of *mbhL* displayed no detectable growth in the absence of S^0, but had no growth defect in the presence of sufficient S^0 (**Figure 2**). In the presence of limiting S^0 (0.5 g/L), the MBH disruption strains exhibited ∼40% less final protein at the end of log phase, although growth rate was similar to the parental strains initially. The strain devoid of all three hydrogenases (ΔSHIΔSHIIΔmbhL) did not produce any detectable H_2 under any of the growth conditions (**Figure 3**). With sufficient S^0 (2 g/L) only a very small amount of H_2 was produced in the parental strains and in ΔmbhL (<5% of that produced in the

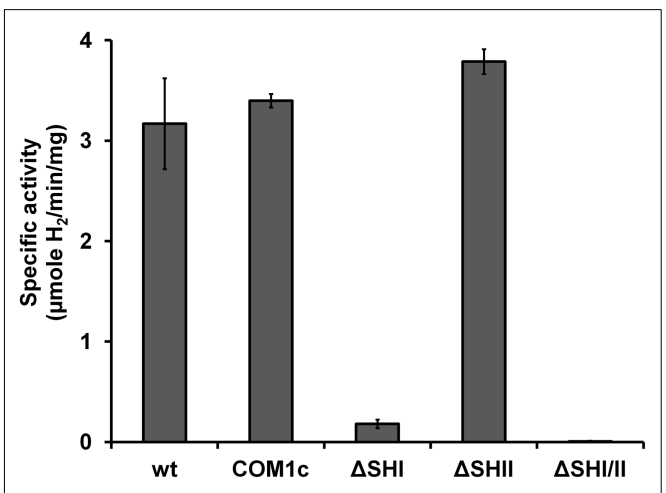

FIGURE 1 | Hydrogenase activity (using methyl viologen as electron carrier) in cytoplasmic fractions obtained from *P. furiosus* cultures of hydrogenase disruption mutants and parental strains. See **Table 1** for strain definitions.

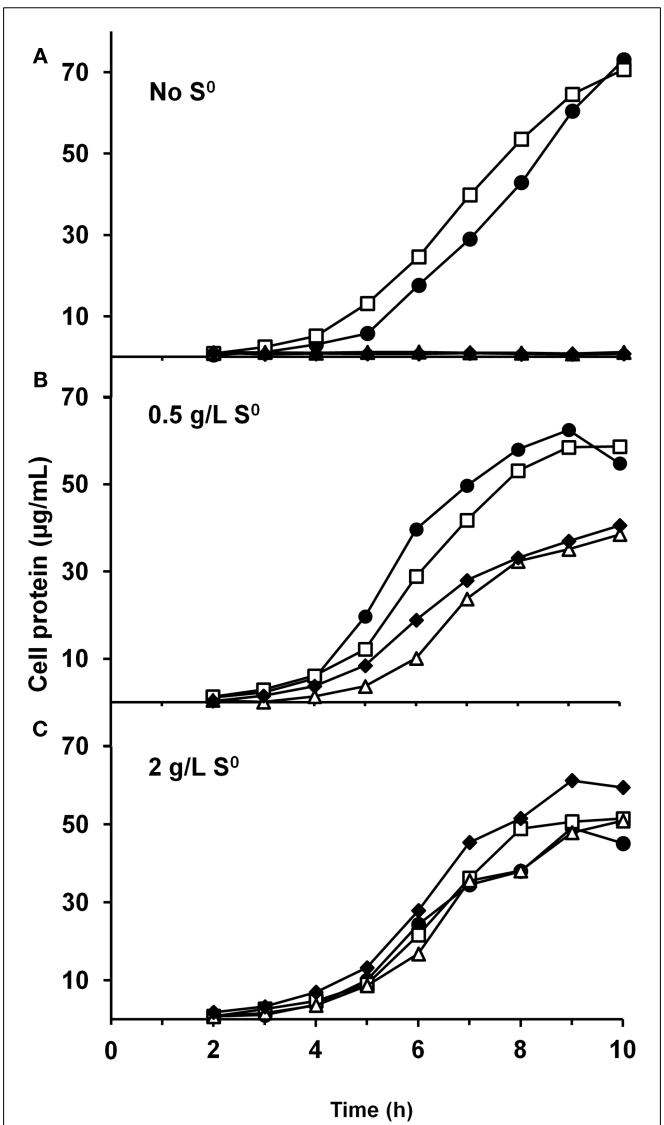

FIGURE 2 | Growth characteristics of *P. furiosus* strains grown in maltose-based medium (A), supplemented with 0.5 g/L S^0 (B), and supplemented with 2 g/L S^0 (C). The symbols represent: closed circles, COM1c; open squares, ΔSHIΔSHII; open triangles, ΔmbhL; closed diamonds, ΔSHIΔSHIIΔmbhL.

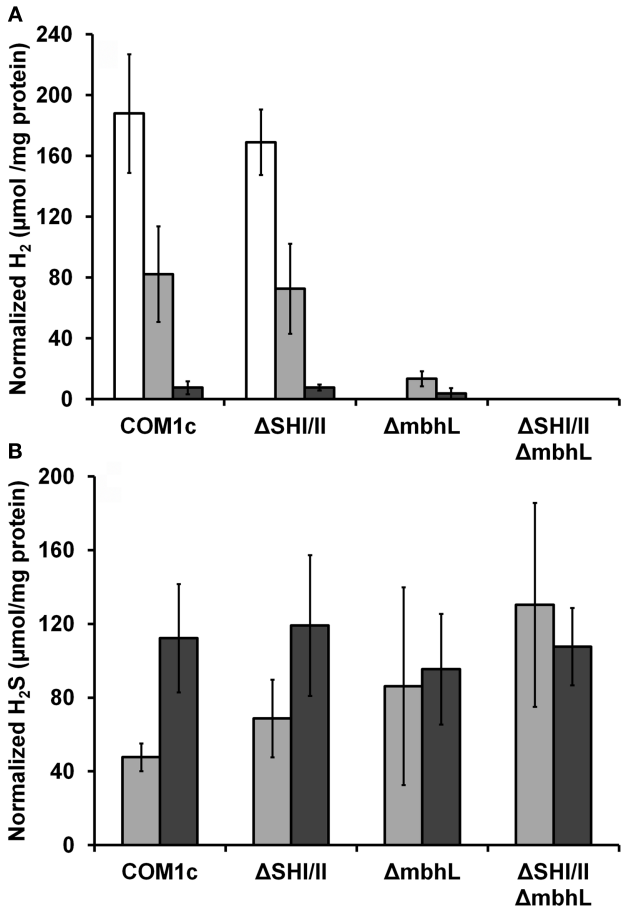

FIGURE 3 | Comparison of H_2 (A) and H_2S (B) production by *P. furiosus* strains. White bars, cells grown without S^0 (no H_2S is produced); light gray bars, cells grown with 0.5 g/L S^0; dark gray bars, cells grown with 2 g/L S^0. The amounts of H_2 and H_2S produced are given with respect to the total cell protein in the culture.

absence of S^0, **Figure 3**; Schut et al., 2007). With limiting S^0, both S^0 is reduced and H_2 is produced (c.a. 50% compared to no S^0) in the parental strains (**Figures 2** and **3**). Interestingly, when ΔmbhL was grown with limiting S^0, a small but significant amount of H_2 was produced (c.a. 20% compared to the parental strains). Therefore, the H_2 produced in the ΔmbhL strain must be catalyzed by SHI, showing that this cytosolic "uptake" hydrogenase can also produce H_2 from NADPH *in vivo*. A concentration of 0.5 g/L S^0 (equivalent to ca. 15 mM) appears to be limiting for the cell's metabolism even though the cultures are continuously shaken during growth. We observed significant amounts of S^0 left in suspension in the culture medium after growth. Since only up to 3 mM rather than 15 mM sulfide is produced in these cultures, it appears that not all S^0 in the medium is accessible to the cells.

DISCUSSION

The glycolytic pathway of *P. furiosus* only uses a low potential ferredoxin that is linked to MBH, for the disposal of all reducing equivalents as H_2, with simultaneous production of an ion gradient for energy generation (Sapra et al., 2003; Verhees et al., 2004). From this study involving all hydrogenases in *P. furiosus*, it is clear that MBH is the only enzyme that produces H_2 in wild-type cells and that no alternative electron pathway is available to *P. furiosus* that can allow for growth in the absence of S^0. In addition, no other enzyme (for example MBX) is capable of producing H_2 *in vivo*, as shown by the lack of H_2 formation in the strain lacking all three hydrogenases. Similar observations were made for the related archaeon *Thermococcus kodakaraensis*, in which the disruption of its MBH also did not allow growth under H_2 evolving conditions (Kanai et al., 2005, 2011; Santangelo et al., 2011). We propose that the *in vivo* function of SHI is to recycle H_2 for the formation of NADPH needed for biosynthesis. Although in the double deletion mutant lacking both SHI and SHII no hydrogenase activity was observed in the cytoplasmic fraction of cellular extracts, no growth phenotype was observed when either or both of these hydrogenases are absent (Lipscomb et al., 2011). In this case other enzymes must provide the pool of NADPH, and a potential candidate is the ferredoxin:NADPH oxidoreductase (FNOR) described previously (Ma and Adams, 2001a). In a previous study, an SHI overexpression strain was constructed, and this strain also did not display any obvious phenotype, although it contained almost an order of magnitude more SHI activity (Chandrayan et al., 2011). The relative amount of H_2 produced in the strain lacking both SHI and SHII was not significantly different than that in the parental strains (**Figure 3**). However, conflicting results have been reported with a *T. kodakaraensis* deletion strain lacking its SHI. One study found only a small increase in H_2 production (c.a. 10%; Kanai et al., 2011), which is more or less in agreement with our results, while another reported over a fivefold increase in relative H_2 production (Santangelo et al., 2011). Both studies used similar growth media and H_2 measurement methods, and it is not clear why these studies give such different results. In general, *T. kodakaraensis* displays growth yields and H_2 production rates similar to what has been observed in *P. furiosus* and other Thermococcales (Kanai et al., 2005; Verhaart et al., 2010).

In the natural environment, the use of H_2 recycling could have a distinct energetic advantage because the cytosolic hydrogenases could provide reductant in the form of NADPH without interfering with the energy balance through electron transport phosphorylation. However, we predict that there is a low level of H_2 recycling in *P. furiosus* when grown with maltose as the carbon source, especially considering the low growth yields on this substrate (25 g cdw/mol glucose utilized; Kengen and Stams, 1994). Assuming all major cellular components (protein, nucleic acids, and lipids) are synthesized *de novo*, we estimate about 6% H_2 recycling (Kanehisa et al., 2004, 2008). In the laboratory setting when these organisms are grown in nutrient rich conditions, H_2 recycling would not be important to the overall growth of the organism, and this may be the reason why there is a lack of phenotype for the SHI and SHII deletion strains. When SHI was first described, it was proposed to be responsible for the production of H_2, but the subsequent discovery of MBH called this into question (Ma et al., 1993; Sapra et al., 2000; Silva et al., 2000). The generation of H_2 from NADPH is thermodynamically unfavorable; however, *in vitro* this reaction can be easily demonstrated (Ma and Adams, 2001b; Verhaart et al., 2010). In this study, we have now shown that

this reaction can actually take place *in vivo*, although at a low level since SHI cannot compensate for the absence of MBH to allow growth of the ΔmbhL mutant in the absence of S^0.

Members of the order Thermococcales are characterized by the ability to use S^0 as an electron acceptor (Kelly and Adams, 1994). We have previously shown that peptides can only be utilized by *P. furiosus* (and likely most Thermococcales) in the presence of S^0, and we have concluded that S^0 is the preferred electron acceptor (Adams et al., 2001; Schut et al., 2007). When S^0 is made available to the cell, a rapid switch from H_2 production to S^0 metabolism occurs, and this is orchestrated at least in part by the redox sensitive SurR regulator (Schut et al., 2007; Lipscomb et al., 2009; Yang et al., 2010). However, *P. furiosus* does not appear to possess a high affinity S^0 binding system such as that described for *Wolinella succinogenes* (Sud; Klimmek et al., 1998). From the results presented herein it appears that the addition of sufficient S^0 to a maltose-based medium seems to consistently reduce the overall cell yield (by c.a. 10–20%). At 0.5 g/L, S^0 appears to be limiting to the cells, but the overall concentration (15 mM "S" atoms) should be sufficient to provide the sole electron sink. In this case, the cells are able to utilize both H_2 and S^0 metabolism simultaneously and produce both H_2 and H_2S. This type of mixed H_2 and S^0 metabolism has also been observed for *Staphylothermus marinus* which also contains orthologous MBH and MBX gene clusters (Hao and Ma, 2003; Anderson et al., 2009). Altogether, this suggests that *P. furiosus* has a relatively low affinity for S^0, and that, when growing on carbohydrates, it might actually prefer to generate H_2 rather than utilizing the poorly soluble S^0 as an electron acceptor.

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