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

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INTRODUCTION

Hydrogen gas (H₂) 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 H₂ 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 fermenting carbohydrates to produce hydrogen (H₂) or, if elemental sulfur (S⁰) 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 almost no obvious phenotype. This study has now been extended to include biochemical analyses and growth studies using the ∆SHI and ∆SHII deletion strains together with strains lacking a functional MBH (∆mbhL). Hydrogenase activity in cytoplasmic extracts of various strains demonstrate that SHI is responsible for most of the cytoplasmic hydrogenase activity. The ∆mbhL strain showed no growth in the absence of S⁰, confirming the hypothesis that, in the absence of S⁰, MBH is the only enzyme that can dispose of reductant (in the form of H₂) generated during sugar oxidation. Under conditions of limiting sulfur, a small but significant amount of H₂ was produced by the ∆mbhL strain, showing that SHI can produce H₂ from NADPH in vivo, although this does not enable growth of ∆mbhL in the absence of S⁰. We propose that the physiological function of SHI is to recycle H₂ and provide a link between external H₂ 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

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Table 1 | Pyrococcus furiosus strains used in this study.

| Strain            | Genotype  | Deleted ORF(s) | Reference or source   |
|-------------------|-----------|----------------|-----------------------|
| COM1c (MW0004)    | ΔpyrF::pyrF | None          | Lipscomb et al. (2011) |
| ΔSHI (MW0022)     | ΔpyrF::pyrF ΔshIβγδα | FF0891–FF0894 | This work             |
| ΔSHII (MW0023)    | ΔpyrF::pyrF ΔshIβγδα | FF1329–FF1332 | This work             |
| ΔSHIΔSHII (MW0016)| ΔpyrF::pyrF ΔshIβγδα | FF0891–FF0894, FF1329–FF1332 | This work             |
| Δmbhl (MW0024)    | ΔpyrF::mbhlΔ::PpeppyrF | FF1114, FF1433 | This work             |
| ΔSHIΔSHIIΔmbhl (MW0025) | ΔpyrF::mbhlΔ::PpeppyrF | FF1114, FF0891–FF0894, FF1329–FF1332, FF1434 | This work             |
H₂ 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). H₂S 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. H₂ production was measured using a gas chromatograph (GC-8A, Shimadzu, Columbia, MD, USA). Hydrogenase activity in cell-free extracts was determined by H₂ 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 ΔSHI ΔSHII. 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 ΔSHI ΔSHII 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 H₂-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 S⁰, limiting S⁰ (0.5 g/L), or sufficient S⁰ (2 g/L). Both mutants containing a deletion of mbhL displayed no detectable growth in the absence of S⁰, but had no growth defect in the presence of sufficient S⁰ (Figure 2). In the presence of limiting S⁰ (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 (ΔSHI ΔSHII ΔmbhL) did not produce any detectable H₂ under any of the growth conditions (Figure 3). With sufficient S⁰ (2 g/L) only a very small amount of H₂ 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 S⁰ (B), and supplemented with 2 g/L S⁰ (C). The symbols represent: closed circles, COM1c; open squares, ΔSHI ΔSHII; open triangles, ΔmbhL; closed diamonds, ΔSHI ΔSHII ΔmbhL.
was grown with limiting S⁰, a small but significant amount of the parental strains (can also produce H₂ from NADPH). Analyzed by SHI, showing that this cytosolic “uptake” hydrogenase was shaken during growth. We observed significant amounts of S⁰ left in the cell’s metabolism even though the cultures are continuously (Figures 2 and 3). Interestingly, when ΔmbhL was grown with limiting S⁰, a small but significant amount of H₂ was produced (ca. 20% compared to the parental strains). Therefore, the H₂ produced in the ΔmbhL strain must be catalyzed by SHI, showing that this cytosolic “uptake” hydrogenase can also produce H₂ from NADPH in vivo. A concentration of 0.5 g/L S⁰ (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 S⁰ 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 S⁰ 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 H₂, 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 H₂ in wild-type cells and that no alternative electron pathway is available to *P. furiosus* that can allow for growth in the absence of S⁰. In addition, no other enzyme (for example MBX) is capable of producing H₂ in vivo, as shown by the lack of H₂ 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 H₂ evolving conditions (Kanai et al., 2005, 2011; Santangelo et al., 2011). We propose that the in vivo function of SHI is to recycle H₂ for the formation of NADPH needed for biosynthesis. Although in the double deletion mutant lacking both SHI and SHIII 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 (Chandrawan et al., 2011). The relative amount of H₂ 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 H₂ production (ca. 10%; Kanai et al., 2011), which is more or less in agreement with our results, while another reported over a fivefold increase in relative H₂ production (Santangelo et al., 2011). Both studies used similar growth media and H₂ measurement methods, and it is not clear why these studies give such different results. In general, *T. kodakaraensis* displays growth yields and H₂ 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 H₂ 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 H₂ 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% H₂ recycling (Kanai et al., 2004, 2008). In the laboratory setting when these organisms are grown in nutrient rich conditions, H₂ 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 SHIII deletion strains. When SHI was first described, it was proposed to be responsible for the production of H₂, 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 H₂ 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 ∆mbhL 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\(^0\)” 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\(_2\)S. This type of mixed H\(_2\) and S\(^0\) metabolism has also been observed for Staphylococcus 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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