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Deletion of alternative pathways for reductant recycling in *Thermococcus kodakarensis* increases hydrogen production

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Summary

Hydrogen (H₂) production by *Thermococcus kodakarensis* compares very favorably with the levels reported for the most productive algal, fungal and bacterial systems. *T. kodakarensis* can also consume H₂ and is predicted to use several alternative pathways to recycle reduced cofactors, some of which may compete with H₂ production for reductant disposal. To explore the reductant flux and possible competition for H₂ production *in vivo*, *T. kodakarensis* TS517 was mutated to precisely delete each of the alternative pathways of reductant disposal, H₂ production and consumption. The results obtained establish that H₂ is generated predominantly by the membrane-bound hydrogenase complex (Mbh), confirm the essential role of the SurR (TK1086p) regulator *in vivo*, delineate the roles of sulfur (S⁰) regulon proteins and demonstrate that preventing H₂ consumption results in a substantial net increase in H₂ production. Constitutive expression of TK1086 (*surR*) from a replicative plasmid restored the ability of *T. kodakarensis* TS1101 (ΔTK1086) to grow in the absence of S⁰ and stimulated H₂ production, revealing a second mechanism to increase H₂ production. Transformation of *T. kodakarensis* TS1101 with plasmids that express SurR variants constructed to direct the constitutive synthesis of the Mbh complex and prevent expression of the S⁰-regulon was only possible in the absence of S⁰ and, under these conditions, the transformants exhibited wild-type growth and H₂ production. With S⁰ present, they grew slower but synthesized more H₂ per unit biomass than *T. kodakarensis* TS517.

Keywords

archaeal genetics; H₂ production; metabolic regulation; transcription; bio-energy; hyperthermophile

Introduction

Thermococcus kodakarensis is a hyperthermophilic, heterotrophic *Archaeon* that degrades environmentally abundant polymers (starch, chitin) to sugars from which ATP is generated by a modified Embden-Meyerhof pathway [Atomi *et al.*, 2004; Fukui *et al.*, 2005; (Fig. 1)]. Glycolysis generates reduced ferredoxins (Fd_{red}) that must be re-oxidized (Fd_{ox}) to maintain metabolic flow and conserve energy for growth. Based on analyses of culture products (Kanai *et al.*, 2005), the activities of enzymes and complexes purified from *T. kodakarensis* and closely related *Thermococcales* (Chou *et al.*, 2007; Clarkson *et al.*, 2010; Kobori *et al.*, 2010; Lee *et al.*, 2006; Lipscomb *et al.*, 2009; 2011; Ma *et al.*, 2000; Ma and Adams, 2001a; Sapra *et al.*, 2000; 2003; Schut *et al.*, 2007; Yang *et al.*, 2010), and the genome annotation (Fukui *et al.*, 2005), *T. kodakarensis* has several different systems to accomplish the regeneration of Fd_{ox} from Fd_{red}. Most of these predicted electron disposal pathways use

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sulfur (S°) as the terminal electron acceptor, generating H_2S , but there are also pathways that result in alanine or H_2 production (Fig. 1).

Given the demands for renewable energy and the concerns for carbon emissions from fossil fuels, the ability of *T. kodakarensis* to generate substantial amounts of H_2 under hyperthermophilic conditions has already attracted attention (Kanai *et al.*, 2005). Intriguingly, with S° present, H_2 production is reduced suggesting a hierarchy of potentially competing pathways of reduced co-factor oxidation (Chou *et al.*, 2007; Lipscomb *et al.*, 2009; Schut *et al.*, 2007). Based on this hypothesis, it seemed that H_2 production by *T. kodakarensis* might be increased if the potentially competing pathways of reduced co-factor oxidation were eliminated, S° -repression of H_2 -production was prevented and reductant flow was channeled to the H_2 generating pathway. *T. kodakarensis* strains have therefore been constructed with the genes encoding the alternative pathways of reductant disposal deleted (see below; Fig. 1) and the H_2 -producing complex constitutively synthesized. The consequences of these genetic manipulations on growth and H_2 production by *T. kodakarensis* in the presence and absence of S° are reported.

As illustrated in Fig. 1, with S° present, at least three routes to H_2S production are predicted (Chou *et al.*, 2007; Fukui *et al.*, 2005; Kanai *et al.*, 2005; Lipscomb *et al.*, 2009; Ma *et al.*, 2000; Ma and Adams, 2001a; Sapra *et al.*, 2000; 2003; Schut *et al.*, 2007; van Haaster *et al.*, 2008; Yang *et al.*, 2010). An NADPH sulfur reductase (NSR; TK1299; Fig. 1; brown) is thought to reduce S° to H_2S with regeneration of NADP in collaboration with a membrane bound oxidoreductase complex (Mbx; TK1214–26; Fig. 1; green) that oxidizes Fd_{red} (Schut *et al.*, 2007). The *T. kodakarensis* genome also encodes an NADPH:polysulfide oxidoreductase [TK1481; Fig. 1; pink; (Fukui *et al.*, 2005; Kobori *et al.*, 2010)] and a homologue of a *Pyrococcus furiosus* cytoplasmic hydrogenase (TK2069–72; Fig. 1; light blue) that can generate H_2S (Ma and Adams, 2001a; van Haaster *et al.*, 2008).

In the absence of S° , at least two pathways are available to re-oxidize reduced cofactors. Alanine is produced from pyruvate by an alanine aminotransferase (TK1094; Fig. 1; grey) that concomitantly generates 2-oxoglutarate from glutamate. Regeneration of glutamate by a glutamate dehydrogenase (TK1431) then oxidizes NADPH to NADP (Fukui *et al.*, 2005). Alanine concentrations in the effluent have been shown to be higher than in the input medium in continuous cultures of *T. kodakarensis* (Kanai *et al.*, 2005). In the second pathway, a membrane-bound hydrogenase complex (Mbh; TK2080–93; Fig. 1; yellow) is thought to oxidize Fd_{red} with the reduction of protons, generating and releasing H_2 . This reaction also exports protons ($2e^- + 3H^+ = H_2 + H^+$) resulting in a proton gradient that can fuel ATP synthesis via an A_0A_1 -ATPase (Sapra *et al.*, 2000; 2003).

The roles of each of the predicted pathways of reductant disposal have been probed by the precise and markerless deletion of gene(s) encoding components of that pathway. The results establish that the Mbh complex is the predominant and possibly only H_2 -generating system available to *T. kodakarensis*, and that Mbh expression is essential for growth on pyruvate in the absence of S° . As elimination of some of the reductant metabolizing pathways did result in increased H_2 production, reductant flow *in vivo* can therefore be genetically manipulated to increase H_2 production by *T. kodakarensis*.

Results

Construction of deletion strains

The selection/counter-selection procedure (Santangelo *et al.*, 2010; Santangelo and Reeve, 2010) illustrated for the deletion of TK1094 (Fig. 2), was repeated to generate all the *T. kodakarensis* strains listed in Table 1. For each strain, the genes/operons shown in Fig. 3

were precisely deleted. Plasmids incapable of autonomous replication in *T. kodakarensis* were constructed and amplified in *E. coli* that carried the target gene(s) adjacent to a cassette that directed the constitutive expression of TK0254 (*trpE*) and TK0664. The target gene(s) and expression cassette were flanked by a duplication of ≥ 500 bp of *T. kodakarensis* genomic DNA that naturally was located immediately 5' or 3' to the target locus. These plasmids were used to transform *T. kodakarensis* TS517 ($\Delta pyrF$; $\Delta trpE::pyrF$; $\Delta TK0664$) and transformants were selected by colony growth on medium lacking tryptophan (Santangelo *et al.*, 2010). The transformants had either the entire donor plasmid DNA integrated into the *T. kodakarensis* genome via a single homologous recombination, or only the target gene(s), expression cassette, and flanking sequences integrated as a result of two crossover events. The growth of a representative transformant resulting from two crossovers, identified by diagnostic PCR and sequencing, was confirmed to be sensitive to 6-methyl purine (6-MP^S), a phenotype conferred by the expression of TK0664. Colony growth on plates containing 6MP selected clones that were spontaneously 6MP^R, and diagnostic PCR analyses of genomic DNA from 6MP^R isolates confirmed that both the target gene(s) and expression cassette had been deleted by recombination in the flanking, duplicated chromosomal region. To confirm the precise and markerless deletion of the target gene(s) and cassette, at least two diagnostic PCRs were performed (Figs. 2 and S1). In one PCR, and for multi-gene operon deletions in several PCRs, the primers used were complementary to sequences within the target gene(s) (primers B and C; Fig. 2). In the second, the PCR primers were complementary to sequences flanking the target gene(s) (primers A and D; Fig. 2). Failure to generate a PCR product with the internal primers was consistent with the loss of the target gene/operon from its normal location, and confirmed its absence from anywhere in the *T. kodakarensis* genome. The size(s) of the PCR product generated with primers that hybridized to DNA flanking the target genes(s) confirmed the precise location and size of the deletion. The exact structure of each deletion in all of the strains used for further study (Table 1) was fully confirmed by both multiple diagnostic PCRs (Fig. S1) and sequencing. Rarely, *T. kodakarensis* strains have been selected through this procedure in which chromosomal rearrangements or regional duplications have occurred and, when essential genes have been targeted for deletion, strains have been isolated that appear to contain two distinct chromosomes, one lacking and one retaining the target gene (unpublished results). None of these phenomena were observed in constructing strains used in this study. In each case, only the target gene(s) or operon was deleted, precisely from the start codon of the first ORF through and including the stop codon of the last ORF.

An important feature of the deletion protocol is that each strain so constructed has not only the desired deletion, but retains the $\Delta pyrF$; $\Delta trpE::pyrF$; $\Delta TK0664$ genotype and so can be subjected to repetitions of the procedure to construct strains with multiple targeted deletions. *T. kodakarensis* TS1104 ($\Delta TK2080-93$; $\Delta TK1094$) was so derived from *T. kodakarensis* TS1103, *T. kodakarensis* TS1113 ($\Delta TK1324-5$, $\Delta TK1684-5$) from *T. kodakarensis* TS1107, *T. kodakarensis* TS1110 ($\Delta TK1299$; $\Delta TK2069-72$) from *T. kodakarensis* TS1109, and *T. kodakarensis* TS1111 ($\Delta TK1214-26$, $\Delta TK1299$) from TS1105 (Table 1; Fig. 3). As the deletion strains are tryptophan auxotrophs ($\Delta trpE::pyrF$) and mevinolin-sensitive, derivatives containing replicative expression plasmids were generated by transformation with plasmids that confer tryptophan prototrophy, by expression of a plasmid-borne TK0254 (*trpE*), and mevinolin resistance (Mev^R) by expression of a hydroxymethylglutaryl-coenzyme A reductase encoding gene (*PF1848*) cloned from *P. furiosus* (Santangelo *et al.*, 2008b).

Growth and H₂ production

Construction of the deletion strains confirmed that none of the genes targeted for deletion was essential for viability, consistent with the hypothesis of functionally redundant

mechanisms to re-oxidize reduced co-factors. The consequences of each deletion on growth and H₂ production were measured for cells growing in media containing artificial seawater (ASW) salts, yeast extract and tryptone (YT) plus S^o (S^o medium), sodium pyruvate (Pyr medium), or both S^o and Pyr (S^o+Pyr medium). In most cases, growth was robust, with doubling times <1h and final cell densities were ~2-fold higher in S^o+Pyr medium than in S^o medium (Fig. 4). Where the growth of a strain was notably changed by the genetic construction, this is described specifically below together with the details of H₂ production by that strain.

The H₂ present in the headspace gas in each culture was determined by GC analysis with samples taken at regular intervals throughout the exponential growth phase and during the subsequent stationary phase. From the data accumulated, both the rates of H₂ production and the amounts of H₂ produced were determined. From these measurements, the amount of H₂ produced per unit biomass was calculated and, for convenience, this is designated the Ω value. With a few exceptions that are discussed individually below, the Ω value remained constant for each strain throughout the exponential growth phase and well into stationary phase. Therefore, in most cases, a single Ω value was sufficient to describe and compare the H₂ production capacity of each strain under each growth condition (Fig. 5). Changes did occur in the Ω values coincident with the spontaneous cell lysis that occurs routinely, at later times, during extended periods of stationary phase incubation (see below).

Before describing the results obtained specifically with each strain, the following were consistent and general observations: H₂ was produced by cells grown in S^o medium but only in low amounts that are not further discussed; H₂ was produced at the highest rate during exponential growth; Ω values are not reported for cultures that had no appreciable growth after 12 h incubation. For most, but not all strains, H₂ production in Pyr medium was higher than in S^o+Pyr medium.

TS1103 (Δ TK2080–2093)—This strain lacks the Mbh complex that in *P. furiosus* has been shown to directly reduce protons, generating H₂ using electrons obtained from Fd_{red} (Sapra *et al.*, 2000; 2003). Deletion of TK2080–93 resulted in a strain that could not grow in Pyr medium (Fig. 4), and essentially eliminated H₂ production in all media tested (Fig. 5). H₂ production by *T. kodakarensis* is apparently therefore almost exclusively catalyzed by the Mbh complex, and this process and/or proton extrusion is essential for growth on Pyr in the absence of S^o. *T. kodakarensis* TS1103 grew normally in S^o and S^o+Pyr media (Fig. 4), consistent with H₂S production then being sufficient for cofactor re-oxidation. As noted, low but detectable amounts of H₂ were produced by all strains that contained the TK2080–93 operon when grown in S^o medium consistent with a constitutive, basal-level expression of Mbh.

TK1101 (Δ TK1086)—A transcription regulator, designated SurR, has been identified and characterized from *P. furiosus* (Lipscomb *et al.*, 2009; Yang *et al.*, 2010). In the absence of S^o, SurR activates transcription of the Mbh operon *in vitro* and represses transcription of genes in the S^o regulon. *T. kodakarensis* has an almost identical homologue encoded by TK1086 (Fukui *et al.*, 2005). The predicted requirement for SurR (TK1086p) to activate Mbh expression *in vivo* was fully supported by the phenotypes of *T. kodakarensis* TS1101 (Δ TK1086). Consistent with an inability to synthesize the Mbh complex, *T. kodakarensis* TS1101 did not generate H₂ in any medium (Fig. 5), and was unable to grow on pyruvate (Pyr medium) in the absence of S^o (Fig. 4). Consistent with the loss of a repressor of the S^o regulon, growth of *T. kodakarensis* TS1101 in S^o and S^o+Pyr media was unimpaired (Fig. 4).

TS1106 (Δ TK2069–2072)—Addition of S° to *P. furiosus* cultures reduced transcription of two operons that encode closely-related cytosolic hydrogenases that catalyze both H_2 and H_2S synthesis and, in reverse, can oxidize H_2 (Chou *et al.*, 2007; Ma and Adams, 2001a; Schut *et al.*, 2007; van Haaster *et al.*, 2008). Neither enzyme is essential for *P. furiosus* viability (Lipscomb *et al.*, 2011) but, as S° addition reduces their expression, active role(s) in H_2 metabolism rather than in H_2S production seem most likely. *T. kodakarensis* has only one homologue of these enzymes, encoded by TK2069–72 (Fukui *et al.*, 2005). *T. kodakarensis* TS1106 (Δ TK2069–72) cultures grew at the same rate as *T. kodakarensis* TS517 cultures but, in Pyr and S° +Pyr media, grew to lower final cell densities than *T. kodakarensis* TS517 (Fig. 4). Despite the lower cell densities, H_2 accumulated in the headspace of these *T. kodakarensis* TS1106 cultures to concentrations that far exceeded those produced by *T. kodakarensis* TS517 (Fig. 5). A likely explanation is that *T. kodakarensis* TS1106 is unable to re-consume H_2 generated by the Mbh complex and, under the laboratory growth conditions, H_2 recovery is required for maximum growth yield. With H_2 uptake eliminated, much higher Ω values were calculated for *T. kodakarensis* TS1106 than for *T. kodakarensis* TS517. Furthermore, the Ω value increased throughout the growth of *T. kodakarensis* TS1106 cultures and continued to increase during the subsequent stationary phase consistent with H_2 production without growth (Fig. 6).

TS1105 (Δ TK1214–1226)—Based on sequence conservation, this 13-gene operon encodes proteins that are homologues of the subunits of the Mbh complex. Expression of this operon is rapidly and substantially increased by S° addition and results in the assembly of a membrane-located complex, designated the Mbx complex. The Mbx complex is thought to functionally replace the Mbh complex when S° is available (Chou *et al.*, 2007; Schut *et al.*, 2007) but it remains to be determined if the Mbx complex generates a proton or ion gradient. Also, although electrons transferred by the Mbx complex from Fd_{red} to NADP could generate NADPH used directly to reduce S° to H_2S , this also has yet to be established. In *T. kodakarensis* TS1105 the entire ~10 kbp Mbx operon is deleted (Fig. 3). In S° medium, this strain grew ~2.5-fold slower and reached final densities <25% of that of *T. kodakarensis* TS517 (Fig. 4). In Pyr and S° +Pyr media, *T. kodakarensis* TS1105 grew and produced H_2 essentially the same as *T. kodakarensis* TS517 (Figs. 4 and 5) indicating that Mbh expression was then sufficient for all necessary co-factor re-oxidation.

TS1109 (Δ TK1299), TS1110 (Δ TK1299, Δ TK2069–2072) and TS1111 (Δ TK1299, Δ TK1214–1226)—TK1299 encodes an NADPH: S° reductase (NSR; Fukui *et al.*, 2005), a very close homologue of the NSR isolated and characterized from *P. furiosus* (Schut *et al.*, 2007). Synthesis of the *P. furiosus* NSR was stimulated by S° addition leading to the proposal that it might generate H_2S using NADPH supplied by the Mbx complex (Chou *et al.*, 2007). If so, this is not an obligatory collaboration in *T. kodakarensis* as the phenotype of *T. kodakarensis* TS1109 (Δ TK1299) differed significantly from that of *T. kodakarensis* TS1105 (Δ TK1214–26), the strain with the Mbx operon deleted. The NSR deletion did not result in a decreased ability to grow in S° medium (Fig. 4). *T. kodakarensis* TS1109 cultures also grew to the same cell density as *T. kodakarensis* TS517 in Pyr and S° +Pyr media but accumulated ~2 to 3-fold higher concentrations of H_2 in the headspace resulting in much higher Ω values (Fig. 5). Possibly, with both S° and Pyr present, Fd_{red} can be oxidized by either the Mbh or Mbx system but with NSR deleted more reductant is channeled through the Mbh complex increasing H_2 production.

T. kodakarensis TS1111 (Δ TK1214–26; Δ TK1299) has both the Mbx complex and NSR deleted. As with *T. kodakarensis* TS1105 (Δ TK1214–26), cultures of *T. kodakarensis* TS1111 grew slower and to lower cell densities in S° medium (Fig. 4). In Pyr and S° +Pyr media, H_2 production by *T. kodakarensis* TS1111 resulted in Ω values much higher than those of *T. kodakarensis* TS517 but essentially the same as those of *T. kodakarensis* TS1109

(Δ TK1299) (Fig. 5). Deletion of the Mb_x complex did not therefore further increase the flow of reductant through the Mb_h complex. In *T. kodakarensis* TS1110 (Δ TK1299; Δ TK2069–72), both the cytoplasmic hydrogenase and NSR are deleted. As with *T. kodakarensis* TS1105 (Δ TK2069–72) and TS1109 (Δ TK1299), *T. kodakarensis* TS1110 grew normally in S° medium but grew slower and to lower cell densities in S°+Pyr medium where both substrates could otherwise facilitate cofactor re-oxidation (Fig. 4). Although reaching lower cell densities, the H₂ produced by *T. kodakarensis* TS1110 cultures resulted in high Ω values, very similar to those of *T. kodakarensis* TS1106 (Δ TK2069–72) (Fig. 5). This is consistent with the prevention of H₂ consumption, by deletion of the cytosolic hydrogenase (Δ TK2069–72), dominating in terms of net H₂ accumulation.

TS1102 (Δ TK1260–1261)—Microarray hybridizations revealed that S° addition to *P. furiosus* cultures dramatically increased the transcription of two genes, designated *sipA* and *sipB* (S°-induced proteins A and B), predicted to encode a nucleotide pyrophosphohydrolase and transcription regulator (Chou *et al.*, 2007; Clarkson *et al.*, 2010). The homologous genes in *T. kodakarensis* (TK1260 and TK1261) appear to form an operon that was deleted in *T. kodakarensis* TS1102. The deletion (Δ TK1260–61) had no detectable effects on growth in the three media tested but did result in a slight but reproducible reduction in H₂ synthesis in Pyr medium. In S°+Pyr medium, H₂ production by *T. kodakarensis* TS1102 was indistinguishable from that of *T. kodakarensis* TS517 (Fig. 5).

TS1100 (Δ TK1094) and TS1104 (Δ TK1094; Δ TK2080–2093)—*T. kodakarensis* excretes alanine, and alanine biosynthesis from pyruvate is catalyzed by an alanine aminotransferase encoded by TK1094 (Fukui *et al.*, 2005; Kanai *et al.*, 2005). This reaction also converts glutamate to 2-oxoglutarate, and glutamate dehydrogenase (GDH) is employed to regenerate glutamate in a reaction that oxidizes NADPH to NADP that can then be used by a ferredoxin:NADP oxidoreductase to convert Fd_{red} to Fd_{ox} and so maintain the EM pathway (Fig. 1). *T. kodakarensis* TS1100 (Δ TK1094) exhibited no detectable differences from *T. kodakarensis* TS517 in growth or H₂ production (Figs. 4 and 5). As the three media investigated all contain yeast extract and tryptone, alanine was supplied exogenously eliminating the need for TK1094p activity for alanine production for protein synthesis.

In *T. kodakarensis* TS1104 (Δ TK1094; Δ TK2080–93) both the alanine aminotransferase and Mb_h complex are deleted. Although the absence of the Mb_h complex precluded H₂ production, this combination of deletions had no detrimental effects on the ability of *T. kodakarensis* to grow in S° or S°+Pyr media. *T. kodakarensis* TS1104 grew at the same rate and reached the same final cell densities in S° and S°+Pyr media as *T. kodakarensis* TS517.

TS1107 (Δ TK1684–1685), TS1108 (Δ TK1325–1326) and TS1113 (Δ TK1325–1326, Δ TK1684–1685)—Based on the genome annotation (Fukui *et al.*, 2005), and homologies with enzymes purified from *P. furiosus* (Ma and Adams, 2001b), *T. kodakarensis* has at least two cytosolic ferredoxin:NADP oxidoreductases that can oxidize Fd_{red} and so could regenerate and supply Fd_{ox} to the EM pathway. These enzymes have two subunits, apparently encoded in operons that were individually deleted in *T. kodakarensis* TS1108 (Δ TK1325–26) and *T. kodakarensis* TS1107 (Δ TK1684–85) and both were deleted in *T. kodakarensis* TS1113 (Δ TK1325–26; Δ TK1684–85). All three strains grew and produced H₂ in the three media essentially the same as *T. kodakarensis* TS517 (Figs. 4 and 5). Neither enzyme is therefore essential for growth or H₂ production under the laboratory conditions investigated.

Plasmid expression of SurR (TK1086p)

T. kodakarensis TS1101 (Δ TK1086) does not grow in Pyr medium (Fig. 4). As argued above, this is consistent with SurR (TK1086p) being required to activate the synthesis of the Mbh complex that is necessary for growth on Pyr through H₂ production (Lipscomb *et al.*, 2009). To confirm this conclusion, TK1086 was cloned into a replicative plasmid [pLC70; (Santangelo *et al.*, 2008b)] positioned immediately downstream from a constitutive promoter (Santangelo *et al.*, 2008a; Santangelo *et al.*, 2009) and the resulting plasmid (pTS703) was transformed into *T. kodakarensis* TS517, TS1101 (Δ TK1086) and TS1103 (Δ TK2080–93). The presence of pTS703, but not the parental plasmid pLC70, restored the ability of *T. kodakarensis* TS1101 to grow and generate H₂ in Pyr medium (Fig. 7A). Surprisingly, the presence of pTS703 not only restored but increased H₂ production by *T. kodakarensis* TS1101 and also by the parental strain, *T. kodakarensis* TS517 in S^o+Pyr medium. The Ω values measured for *T. kodakarensis* TS1101 (pTS703) and *T. kodakarensis* TS517 (pTS703) were 2- and 3-fold higher, respectively, than for *T. kodakarensis* TS517 and *T. kodakarensis* TS517 (pLC70) (Fig. 7B). Possibly, the constitutive over-expression of SurR from pTS703 maximizes Mbh expression while minimizing expression of the S^o regulon, and this results in increased H₂ production in S^o+Pyr medium. Consistent with the deletion of the Mbh complex, the presence of pTS703 in *T. kodakarensis* TS1103 (Δ TK2080–93) did not restore growth in Pyr medium or stimulate H₂ production in any medium.

Growth and H₂ stimulation by SurR (C24A), (C27A) and (C24A+C27A) variants

SurR is a redox sensitive regulator (Yang *et al.*, 2010). *In vitro*, SurR binding to regulatory sequences activated transcription of the operon encoding Mbh and inhibited transcription of genes in the S^o regulon. Under redox conditions that result in an intramolecular disulfide bond in SurR, between cysteines 24 (C24) and 27 (C27), SurR binding to regulatory DNA sequences was dramatically reduced. Replacing both cysteines with alanines resulted in a SurR variant (C24A+C27A) that could not form the disulfide bond and this variant bound to the regulatory DNA sites *in vitro* under all redox conditions. The presence of such a SurR variant *in vivo* was therefore predicted to result in constitutive Mbh expression, and non-inducible repression of the S^o regulon. To test this prediction, the TK1086 sequence in pTS703 was changed to generate plasmids pTS705, pTS709 and pTS710 (Table 1) that encode the SurR (C24A+C27A), (C27A) and (C24A) variants, respectively (Table 1). Transformation of *T. kodakarensis* TS517 with the parental plasmid, pTS703, generated many transformants but very few transformants were obtained with pTS705, pTS709, and pTS710. In almost every case, the plasmid recovered from such a transformant had the wild-type TK1086 sequence and so had apparently undergone recombination with the chromosomal copy of TK1086. In the rare exceptions, when the plasmid recovered from a *T. kodakarensis* transformant retained one or both of the alanine codons, sequencing revealed the acquisition of an additional mutation that would inactivate SurR or prevent SurR expression from the plasmid. Transformation of *T. kodakarensis* TS1101 (Δ TK1086) with plasmids pTS705, pTS709, and pTS710 resulted in even fewer transformants, and every plasmid isolated from a *T. kodakarensis* TS1101 transformant had a mutation in the promoter, ribosome binding site or coding sequence of TK1086. Most of these spontaneous mutations caused frame-shifts and/or introduced nonsense codons that severely shortened the TK1086 open reading frame.

Given these results, it seemed that expression of the SurR variants was incompatible with the survival of *T. kodakarensis* transformants using the established transformation procedure. One possibility was that constitutive repression of the S^o regulon resulted in increased sensitivity to S^o and/or oxygen (Kobori *et al.*, 2010) and so the transformation procedure was repeated using a S^o-free medium and strictly anaerobic conditions. This

resulted in many pTS705, pTS709 and pTS710 transformants of *T. kodakarensis* TS1101, and sequencing plasmids isolated from representative transformants (Table 1) confirmed the presence of the intact TK1086 sequence with the cysteine to alanine mutation(s). As established for pTS703, the presence of pTS705, pTS709 or pTS710 also restored the ability of *T. kodakarensis* TS1101 to grow on Pyr medium (Fig. 7A), and to generate H₂ in both Pyr and S^o+Pyr media (Fig. 7B). The growth of *T. kodakarensis* TS1101 [pTS709; (C27A)] closely resembled that of *T. kodakarensis* TS1101 (pTS703) in all three media, but *T. kodakarensis* TS1101 [pTS710; (C24A)] and *T. kodakarensis* TS1101 [pTS705; (C24A +C27A)] grew more slowly in S^o and S^o+Pyr media. Given these differences in growth, the C24A and C27A substitutions may result in different structural and so possibly regulatory consequences, in addition to preventing disulfide bond formation. Surprisingly, as also observed for *T. kodakarensis* TS517 (pTS703) and TS1101 (pTS703), the *T. kodakarensis* TS1101 strains containing pTS705, pTS709 and pTS710 generated more H₂ and had higher Ω values when grown in S^o+Pyr than in Pyr medium.

Discussion

Most biofuel research to date has been focused on bacterial and fungal systems although heterotrophic, hyperthermophilic *Archaea* offer the attractive advantages of greater substrate solubility, and much less concern for contamination if an industrial scale process is run at high temperature. With this in mind, it has already been established that H₂ production (per unit biomass) by *T. kodakarensis* can exceed the most productive bacterial systems (Kanai *et al.*, 2005) and, as a marine organism, a large production system employing *T. kodakarensis* would not require fresh water. Furthermore, *T. kodakarensis* can degrade and use chitin as a substrate for growth and H₂ production (Tanaka *et al.*, 1999; 2001; 2003; 2004), and chitin is the largest global reservoir of bio-polymerized carbon (Benner *et al.*, 1992).

Based on growth studies, biochemical investigations and bioinformatics, *T. kodakarensis* appears to have several alternative mechanisms to oxidize reduced cofactors, including the reduction of protons and so production of H₂ (Fig. 1). In the experiments reported here, we took advantage of the genetic technologies now established for *T. kodakarensis* (Santangelo and Reeve, 2010) to determine if H₂ production by this hyperthermophilic *Archaeon* could be manipulated, and possibly increased by deleting the alternative pathways predicted for reductant flow. As summarized in Table 2, the results obtained support the premise that there are competitive pathways and provide evidence that reductant flow *in vivo* is malleable and can be channeled to H₂ production by genetic manipulations. The results establish that Mbh (TK2080–92), a membrane-bound hydrogenase complex is predominantly, and perhaps exclusively, responsible for H₂ production and that although this complex is essential for growth in the absence of S^o, its deletion had no detrimental effects with S^o present. The results obtained also argue that much of the H₂ produced by the Mbh complex is re-assimilated and oxidized by the cytoplasmic hydrogenase (TK2069–72). When this hydrogenase was deleted, and so energy conservation via H₂ oxidation was lost, *T. kodakarensis* TS1106 grew only to low cell densities in laboratory culture (Fig. 4). *T. kodakarensis* TS1106 cultures, however, continued to produce H₂ during the subsequent stationary phase. This discovery that H₂ generation by *T. kodakarensis* can be unlinked from growth is intriguing, and is potentially a very important observation for bioprocess development.

With S^o present, synthesis of the Mbh complex is reduced and expression of the Mbx complex (TK1214–26) is induced (Chou *et al.*, 2007). Consistent with the predicted importance of the Mbx operon under these conditions (Schut *et al.*, 2007), *T. kodakarensis* TS1105 (Δ TK1214–1226) had reduced growth in S^o medium but grew to the same extent as *T. kodakarensis* TS517 in S^o+Pyr nad Pyr medium. The activity of the Mbh complex present

in cells grown with both S° and Pyr is apparently sufficient to fully support growth in the absence of Mb_x. The S°-induced NADPH:S° reductase (NSR; TK1299p) (Schut *et al.*, 2007) appears to play a role in directing reductant flow under these conditions. With NSR deleted, *T. kodakarensis* TS1109 (Δ TK1299) generated more H₂ than *T. kodakarensis* TS517 when grown in S°+Pyr medium consistent with reductant that would have passed through the Mb_x/S°-dependent system being channeled to the Mb_h complex.

Our results are fully consistent with the proposal that SurR activates expression of the H₂ producing system(s) in the absence of S° (Lipscomb *et al.*, 2009) and demonstrate further that the presence of SurR is actually essential for H₂ production *in vivo* and for S°-independent growth. In the absence of SurR, expression of the S° regulon should be fully de-repressed but this did not confer more rapid growth or growth of *T. kodakarensis* TS1101 to higher cell densities in S° containing media. *T. kodakarensis* TS1101 (pTS703), in which TK1086 (*surR*) is constitutively expressed from a replicating plasmid, grew robustly and generated H₂ in the absence of S°, consistent with complementation of the chromosomal Δ TK1086 mutation. Growth in Pyr medium and H₂ production by *T. kodakarensis* TS1101 were also restored by plasmid expression of the SurR (C24A), (C27A) and (C24A+C27A) variants but there were significant differences in the growth of *T. kodakarensis* TS1101 (pTS705), (pTS709) and (pTS710) cultures in S° and S°+Pyr (Figs 7A). Consistent with results from *P. furiosus* (Chou *et al.*, 2007; Lipscomb *et al.*, 2009; Schut *et al.*, 2007), H₂ production by *T. kodakarensis* TS517 was reduced by the presence of S°, but this was not the case for *T. kodakarensis* TS517 (pTS703) or *T. kodakarensis* TS1101 (pTS703), (pTS705), (pTS709) or (pTS710) (Fig. 7B). The constitutive expression of SurR and the SurR variants from these plasmids most likely results in higher than usual concentrations of these regulators and this could result in increased levels of Mb_h expression *in vivo* and so increased H₂ production. The S°-dependent differences in growth of the *T. kodakarensis* strains expressing (pTS705), (pTS709) or (pTS710) was unexpected (Fig. 7A); it argues for an additional level of structural/regulatory control and further research attention.

Some of the results obtained do not support, but also do not negate, a simple model of competition for reductant flow (Table 2). For example, deletion of the alanine aminotransferase encoded by TK1094 was predicted to eliminate one pathway of reduced cofactor re-oxidation (Fig. 1; Kanai *et al.*, 2005) but this had no detectable effects on growth and H₂ production. The most likely explanation is that *T. kodakarensis* has several enzymes that can convert pyruvate to alanine, and deletion of more genes is probably necessary to effectively eliminate this route of reduced cofactor re-oxidation. Similarly arguing for the presence of additional functionally-equivalent enzymes, deletion of both of the identified ferredoxin:NADPH oxidoreductases had no detectable effects on growth or H₂ production (Table 2). An experimentally challenging observation is that *T. kodakarensis* TS1102 (Δ TK1260–61) exhibited no growth defects even though transcription of *sipA* and *sipB* is very highly induced by S° addition (Chou *et al.*, 2007). This strongly suggests that SipA and SipB play key role(s) in growth and/or metabolism with S° present (Clarkson *et al.*, 2010) but we saw no evidence for such importance.

Conclusions

The results presented demonstrate that the metabolism of *T. kodakarensis* can be genetically manipulated to increase H₂ production and H₂ accumulation, and presumably additional pathways to H₂ production from other bioprocess-attractive substrates can be added by genetic engineering. Deletion of the cytosolic hydrogenase (TK2069–72) prevents H₂ consumption and revealed that H₂ production can be uncoupled from growth. Deletion of this enzyme generated *T. kodakarensis* TS1106, a strain that accumulates ~6-fold more H₂ per unit biomass but grows to lower cell densities on pyruvate than the parental strain.

Plasmid over-expression of SurR also increased H₂ production, and resulted in H₂ production that is not inhibited by the presence of S[°]. Expression of the SurR C24A and C27A variants was incompatible with survival of cells through the established transformation protocol. This was a surprise, but plasmids isolated from some of the few transformants that did form colonies on S[°]-containing plates have acquired missense mutations in TK1086. Presumably, these are mutations that suppress the toxicity of the SurR (C24A) and SurR(C27A), and they offer a new and direct approach to determining how the structure and function of SurR are related *in vivo*.

Experimental procedures

Reagents and enzymes

Except where noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO), restriction enzymes and thermophilic DNA polymerases were from New England Biolabs (Waltham, MA), and plasmid and PCR-purification kits from Zymo Research (Orange, CA).

Strains and plasmids

The construction and relevant genetic features of the *T. kodakarensis* strains and plasmids used are listed in Table 1. Standard molecular biology procedures were used for PCR amplifications, plasmid construction, to transform *E. coli* DH5 α , select transformants and isolate plasmid preparations. DNA molecules PCR amplified from *T. kodakarensis* genomic DNA (primer sequences are available from T.J.S. upon request) were cloned into pTS535 or pTS414, as described (Santangelo *et al.*, 2010, 2008b; Santangelo and Reeve, 2010). The selection and counter selection procedure used to generate markerless deletions has been described in detail (Santangelo and Reeve, 2010), and is fully illustrated in Fig. 2. The procedure was repeated to generate *T. kodakarensis* strains with deletions at two loci. Briefly, plasmids were constructed that when transformed into *T. kodakarensis* TS517 (Δ pyrF; Δ trpE::pyrF; Δ TK0664), made competent for DNA uptake as previously described (Santangelo *et al.*, 2007), allowed growth on plates lacking tryptophan. The Δ trpE mutation was complemented by expression of the TK0254 (*trpE*)-TK0664 cassette. Expression of TK0664 from the cassette conferred 6MP^S. Cells of a representative transformant were spread on plates containing 6MP. Genomic DNA was isolated from spontaneous 6MP^R clones and the presence of the desired deletion and loss of the expression cassette were confirmed by diagnostic PCR and sequencing.

Plasmids, capable of replication and expression in *T. kodakarensis* were generated from the pLC70 shuttle vector (Table 1) and transformed, as described (Santangelo *et al.*, 2008b), into *T. kodakarensis* strains with transformants selected by both tryptophan prototrophy and resistance to mevinolin. Plasmid preparations were re-isolated from *T. kodakarensis* strains, amplified in *E. coli* and re-sequenced to confirm the identity and precise sequence of each plasmid (see below). Plasmid pTS703 was generated from pTS414 (Santangelo *et al.*, 2008b) by replacing *rpoL* in pTS414 with TK1086 (*surR*). Plasmids pTS705, pTS709 and pTS710 were generated from pTS703 by using the Quikchange procedure (Agilent Technologies, Santa Clara, CA).

Media and growth conditions

T. kodakarensis cultures were grown anaerobically at 85°C either in a nutrient-rich artificial sea water (ASW-YT) medium that contained 5 g/l yeast extract (Y), 5 g/l tryptone (T) and the required trace minerals and vitamins, or in minimal (ASW-AA) medium that contained only the nutritionally required amino acids plus trace minerals and vitamins (Santangelo *et al.*, 2007). Where noted the media were supplemented with 2 g/l S[°] (S[°] medium) or 5 g/l sodium pyruvate (Pyr medium), or both S[°] and pyruvate (S[°]+Pyr medium). When needed,

media were solidified by the incorporation of 1% Gelrite. To obtain transformants with pTS705, pTS709 and pTS710, S⁰ and polysulfides were omitted and Pyr was added to the medium. The containers in which the plates were incubated anaerobically were repeated flushed with 100% N₂. Growth of liquid cultures was monitored by measuring changes in the optical density at 600nm (OD₆₀₀) using a Genesys 20 spectrophotometer equipped with a COD vial cell holder. Multiple, independent cultures of each strain were grown, and grown on different days; differences in growth rates of duplicate cultures varied <5%.

Measurement of H₂ production

Identical culture conditions were used for growth-rate and H₂ production measurements. The headspace gas was replaced with 100% N₂ at one bar. Aliquots (100 µl) of the headspace were removed and injected into a GC 14 gas chromatograph (Shimadzu, Columbia, MD) equipped with a TCD using a carboxen-1000, 60/80 mesh, 1/8" × 15' column (Supelco, St. Louis, MO). N₂ was supplied as the carrier gas at a rate of 40 ml/min. The injector and column were maintained at 180°C and 30°C, respectively. H₂ production per OD₆₀₀ (Ω values) were calculated at the time of headspace sampling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Pathways proposed for ATP synthesis and reduced cofactor recycling in *T. kodakarensis*. A modified Embden-Meyerhof glycolytic pathway generates ATP and reduced ferredoxin(s) (Fd_{red}; red). The alternative pathways proposed to regenerate Fd_{ox} (brick red) are illustrated with enzyme/complexes identified by name and the encoding *T. kodakarensis* gene number(s). The same colors are used to report the results obtained with each enzyme/complex in Figs. 4 through 7. Electron transfer to protons by the membrane bound hydrogenase (Mbh) complex (yellow; TK2080–93) generates H₂ (red square). Mbh activity also produces a proton-gradient that can be exploited for ATP-production. A cytosolic hydrogenase (cyan; TK2069–72) can consume H₂ to generate NADPH, and NADPH may also be produced by ferredoxin-NADPH oxidoreductases (blue; TK1684–5, purple; TK1325–6). An NADPH:sulfur reductase (brown; TK1299) and NADPH:polysulfide reductase (pink; TK1481) are predicted to generate H₂S when S⁰ is available. A membrane bound oxidoreductase (Mbx) complex (green; TK1214–26) is predicted to functionally replace the Mbh complex when S⁰ is present, although no efflux function for Mbx has been established. SurR (red; TK1086) is a redox-sensitive transcription regulator. When reduced, SurR activates transcription of the Mbh operon and represses transcription of the S⁰ regulon. Transcription of the genes (TK1260–61) encoding SipA and SipB (orange) is highly induced by the presence of S⁰. A glutamate-dependent alanine aminotransferase (grey; TK1094) converts pyruvate to alanine, generating 2-oxoglutarate from glutamate, and the regeneration of glutamate by glutamate dehydrogenase (TK1431) oxidizes NADPH to NADP.

Fig. 2.

Deletion of TK1094 from the *T. kodakarensis* TS517 genome. (A) Plasmid pLC120 was constructed, as illustrated, with the PCR-amplified regions of the *T. kodakarensis* genome flanking the [TK0254–TK0664] expression cassette and used to transform *T. kodakarensis* TS517 ($\Delta pyrF$; $\Delta trpE::pyrF$; $\Delta TK0664$). The genome organization in a representative transformant (intermediate strain), selected by growth in the absence of tryptophan, was confirmed by diagnostic PCR. The intermediate strain was plated on a medium containing 6MP, and 6MP^R clones were isolated. A representative clone (designated *T. kodakarensis* TS1100; Table 1) was confirmed (see below) to have the desired $\Delta TK1094$ mutation. As illustrated, a recombination between sequences directly repeated in the intermediate strain genome deleted both the expression cassette and TK1094. (B) Ethidium-bromide stained agarose gel electrophoresis of the PCR products generated by primers A plus D, and B plus C from *T. kodakarensis* TS1100 genomic DNA. The sites to which the primers hybridized are indicated above and below the *T. kodakarensis* TS517 genome organization in (A). Consistent with the presence of the TK1094 deletion, a PCR product was not generated using primers B and C, and the PCR product generated with primers A and D was shorter, by the length of the TK1094 deletion, than the PCR product generated using these primers with the parental *T. kodakarensis* TS517 genomic DNA. As shown in Fig. S1, the same diagnostic PCR procedure was used to confirm the presence of the desired deletion(s) in the genomes of all the *T. kodakarensis* strains used in this study (Table 1).

Fig. 3.
T. kodakarensis genes deleted for this study. The genes precisely deleted from the *T. kodakarensis* TS517 genome to generate the *T. kodakarensis* strain(s) listed above each genome organization are shown in black. The organizations are drawn to scale with the length of a 1 kb region noted as a size reference.

Fig. 4. Growth of the *T. kodakarensis* strains in ASW-YT media containing S° , $S^\circ + \text{Pyr}$, or Pyr . Increases in the optical density (OD₆₀₀) were measured over time. The results shown for each strain, in the color listed below the graphs, are the moving averages ($P=2$) of the data collected from at least two independent cultures. *T. kodakarensis* TS1101, TS1103, and TS1104 exhibited essentially no growth when incubated in Pyr medium. OD₆₀₀ measurements below 0.01 are noted as points on the X-axis.

Fig. 5.

Comparison of H₂ production by *T. kodakarensis* strains. The H₂ present in the headspace of a culture during exponential growth was divided by the culture density (OD₆₀₀) to calculate the Ω value. The solid and hatched columns are Ω values from cultures grown in S°+Pyr and Pyr medium, respectively. The Ω values shown for *T. kodakarensis* TS1106 and TS1110 were measured near the end of exponential growth (see Fig. 6). As indicated, there was essentially no detectable H₂ in the headspace of cultures of *T. kodakarensis* TS1101, TS1103 and TS1104.

Fig. 6.

Comparison of H₂ production by cultures of *T. kodakarensis* TS517 and TS1106. The Ω values shown were calculated from the H₂ present in the headspace of *T. kodakarensis* TS517 (black bars) and TS1106 (blue bars) cultures grown in Pyr medium sampled during early, middle and late exponential growth (hrs 5 to 9), at the end of exponential growth (hr 11) and in stationary phase (hr 13). In Pyr medium, *T. kodakarensis* TS1106 (Δ TK2069–72) cultures grew to a lower final cell densities than *T. kodakarensis* TS517 cultures (see Fig. 4), but H₂ production was apparently unlinked from growth and the Ω values increased throughout the culture incubation.

Fig. 7A

Fig. 7B

Fig. 7.

Growth and H_2 production by *T. kodakarensis* TS1101 (Δ TK1086) strains containing pTS703 (◇), pTS705 (Δ), pTS709 (○) or pTS710 (□). (A) Growth of each plasmid-containing *T. kodakarensis* TS1101 strain in (ASW-YT) S° , S° +Pyr and Pyr media supplemented with 20 μ M mevinolin. (B) The Ω values measured for *T. kodakarensis* TS517 and TS1101 cultures are shown as bars adjacent to the Ω values (bars on grey background) measured for these strains containing plasmids pLC70, pTS703, pTS705, pTS709 or pTS710. The solid and hatched bars report the Ω values for cultures grown in S° +Pyr and Pyr medium, respectively. As indicated by the very small bar, *T. kodakarensis* TS1101 cultures grew in S° +Pyr medium but did not accumulate measurable H_2 . The adjacent space notes that *T. kodakarensis* TS1101 did not grow in Pyr medium.

Table 1

Strains	Origin and genetic features	Source
TS517	Δ TK2276 (<i>pyrF</i>), Δ TK0254::TK2276 (<i>trpE::pyrF</i>)	Santangelo <i>et al.</i> , 2010
TS1100	TS517 with Δ TK1094 (alanine aminotransferase)	This study
TS1101	TS517 with Δ TK1086 (SurR transcription regulator)	This study
TS1102	TS517 with Δ TK1260-1 (SipA & SipB)	This study
TS1103	TS517 with Δ TK2080-93 (membrane bound hydrogenase operon)	This study
TS1104	TS1103 with Δ TK1094 (alanine aminotransferase)	This study
TS1105	TS517 with Δ TK1214-26 (membrane bound oxidoreductase operon)	This study
TS1106	TS517 with Δ TK2069-72 (cytosolic hydrogenase operon)	This study
TS1107	TS517 with Δ 1684-5 (ferredoxin:NADP oxidoreductase operon I)	This study
TS1108	TS517 with Δ 1325-6 (ferredoxin:NADP oxidoreductase operon II)	This study
TS1109	TS517 with Δ TK1299 (NADPH sulfur reductase)	This study
TS1110	TS1109 with Δ TK2069-72 (cytosolic hydrogenase operon)	This study
TS1111	TS1105 with Δ TK1299 (NADPH sulfur reductase)	This study
TS1113	TS1107 with Δ TK1325-6 (ferredoxin:NADP oxidoreductase operon II)	This study
Plasmids		
pLC70	<i>trpE</i> ; P_{gdh} -PF1848	Santangelo <i>et al.</i> , 2008b
pTS703	pLC70 with P_{hmdB} -TK1086	This study
pTS705	pLC70 with P_{hmdB} -TK1086 C24A, C27A	This study
pTS709	pLC70 with P_{hmdB} -TK1086 C27A	This study
pTS710	pLC70 with P_{hmdB} -TK1086 C24A	This study

Table 2
Summary of the hypothesized and observed phenotypes of the *T. kodakarensis* deletion strains

Strain ¹	Activity deleted ²	Hypothesis	Phenotype
TS517	—		H ₂ generated in Pyr and S°+Pyr media H ₂ production decreased by the presence of S°
TS1100	alanine amino-transferase	Prevents reductant flow to alanine and so increases H ₂ production	No detectable effects on growth No increase in H ₂ production
TS1101	SurR transcription regulation	H ₂ producing system inactive H ₂ S producing system constitutively synthesized	No H ₂ production. No growth in Pyr medium Growth in S° and S°+Pyr media
TS1101 (pTS703)	SurR regulation (complemented by SurR expression from plasmid pTS703)	Plasmid expression of SurR should restore SurR transcription regulation, H ₂ production, and S°-independent growth	H ₂ produced in all media. Growth in Pyr medium Increased H ₂ production in S°+Pyr medium
TS1102	SipA and SipB.	Highly induced by S° suggesting participation in reductant flow to H ₂ S; elimination may increase H ₂ production	No detectable effects on growth No detectable effects on H ₂ production.
TS1103	Mbh; membrane bound hydrogenase	Complex responsible for H ₂ generation that facilitates growth on Pyr; deletion should reduce H ₂ production	No H ₂ production No growth in Pyr medium Growth in S° and S°+Pyr media
TS1105	Mbx; membrane bound oxido-reductase	Complex participates in Fd _{oxd} oxidation catalyzing S° reduction to H ₂ S; facilitates growth on S°; deletion may increase H ₂ production	Growth and H ₂ production in Pyr and S°+Pyr media unchanged. Much reduced growth in S° medium
TS1106	cytosolic hydrogenase	Uptake hydrogenase that consumes H ₂ generated by the Mbh complex; elimination should increase H ₂ accumulation	Reduced growth in Pyr and S°+Pyr media Increased and continuous H ₂ accumulation No detectable effects on growth in S° medium
TS1109	NADPH; S° oxido-reductase	Reductant flow to S° decreased, and so increased H ₂ production	No detectable effects on growth Increased H ₂ production in S+Pyr
TS1113	Ferredoxin:NADP oxido-reductases I & II	Reductant flow to NADP decreased, and so increased H ₂ production	No detectable effects on growth. No detectable effects H ₂ production

¹The genes deleted in each strain are listed in Table 1.

²The reactions/activities deleted are illustrated in Fig. 2