Chapter 18

Genetic and Proteomic Studies of Sulfur Oxidation in *Chlorobium tepidum* (syn. *Chlorobaculum tepidum*)

Leong-Keat Chan, Rachael Morgan-Kiss, and Thomas E. Hanson*
College of Marine and Earth Studies and Delaware Biotechnology Institute, University of Delaware, 15 Innovation Way, Newark, DE 19711 USA

Summary

The oxidation of reduced sulfur compounds is perhaps the most poorly understood physiological process carried out by the green sulfur bacteria (the *Chlorobiaceae*). My laboratory is testing models of sulfur oxidation pathways in the model system *Chlorobium tepidum* (ATCC 49652 syn. *Chlorobaculum tepidum* (Imhoff, 2003)) by the creation and analysis of mutant strains lacking specific gene products. The availability of a complete, annotated genome sequence for *C. tepidum* enables this approach, which will specify targets for biochemical analysis by indicating which genes are important in an organismal context. This is particularly important when several potentially redundant enzymes are encoded by

*Corresponding author, Phone: +(302) 831 3404, Fax: +(302) 831 3447, E-mail: tehanson@udel.edu

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the genome for a particular reaction, such as sulfide oxidation. Additionally, we are using proteomics approaches to define the subcellular locations of proteins involved in sulfur oxidation pathways. The results produced by this research will refine models of anaerobic sulfur oxidation pathways and their integration into the global physiology of the *Chlorobiaceae*.

I. Introduction

The *Chlorobiaceae* are anoxygenic phototrophs characterized by the oxidation of reduced sulfur compounds as electron donors for photosynthetic electron transport and anabolic metabolism (Overmann, 2000). The *Chlorobiaceae* participate in global elemental cycles through carbon dioxide (CO_2) assimilation, dinitrogen (N_2) fixation, and the oxidation of reduced sulfur compounds (sulfide, thiosulfate and elemental sulfur) in anaerobic environments. The *Chlorobiaceae* thus provide a valuable ecosystem service: the transformation of compounds, like sulfide, that can be potently toxic to a wide variety of aerobic organisms, including humans, to relatively innocuous forms.

Features that make the *Chlorobiaceae* of general biological interest include metabolic pathways like the reductive tricarboxylic acid (rTCA) cycle of autotrophic CO_2 fixation (Buchanan and Arnon, 1990) and the formation of specific symbiotic associations with other bacteria (Overmann and van Gemerden, 2000). The existence of the rTCA cycle, now considered one of the most ancient metabolic cycles, was first proposed based on experiments with *Chlorobium limicola* f sp. *thiosulfatophilum* (DSM 249 syn. *Chlorobaculum thiosulfatophilum* (Imhoff, 2003)) (Evans et al., 1966). The *Chlorobiaceae* also possess unique quinones, carotenoids (Powls et al., 1968; Jensen et al., 1991; Takaichi et al., 1997; Cho et al., 1998), and a complex light harvesting apparatus called the chlorosome (Frigaard and Bryant, 2004) that is shared with the *Chloroflexaceae*, the green gliding bacteria. The chlorosome is thought to allow the *Chlorobiaceae* to grow at extremely low light intensities, which they appear to be uniquely suited for among phototrophs. Strains of *Chlorobiaceae* have been isolated from extremely light limited environments including deep sea hydrothermal vents (Beatty et al., 2005) and anoxic basins in the Black Sea (Overmann et al., 1992; Manske et al., 2005), supporting this hypothesis.

The wide distribution and gross physiological attributes of the *Chlorobiaceae* indicate that they are significant players in the global sulfur cycle, but few details are known about their direct contributions to that cycle or the enzymes involved. The *Chlorobiaceae* are found at high densities in diverse anoxic environments where dynamic sulfur cycling occurs including geothermal hot springs (Castenholz et al., 1990; Wahlund et al., 1991), freshwater hypolimnia (Butow and Bergsteinbendan, 1992; Baneras et al., 1999; Tuschak et al., 1999; Jung et al., 2000; Vila et al., 2002), and estuaries (Imhoff, 2001). Phylogenetic signatures similar to the *Chlorobiaceae* are found in the open ocean (Gordon and Giovannoni, 1996), another active sulfur transformation environment (Gonzalez et al., 1999), albeit an aerobic one. Clearly, understanding sulfur oxidation in the *Chlorobiaceae* has general implications for global sulfur cycling in anaerobic environments and biotechnological applications in wastewater and industrial gas stream treatment schemes (Kim and Chang, 1991; Basu et al., 1994; An and Kim, 2000; Henshaw and Zhu, 2001).

Prior studies of sulfur oxidation in the *Chlorobiaceae* have produced more contradictions than generalities (Paschinger et al., 1974; Shahak et al., 1992; Prange et al., 1999; Blöthe and Fischer, 2001; Prange et al., 2002; Verté et al., 2002). Some of these discrepancies may be explained by valid strain differences, but a coherent and detailed picture of the enzymes involved in sulfur oxidation in the *Chlorobiaceae* has yet to emerge. A summary of the state of knowledge of sulfur oxidation in *C. tepidum*, with appropriate comparisons to other systems follows. The conservation of sulfur oxidation

Abbreviations: IVTM – in vitro transposition mutagenesis; SQR – sulfide:quinone oxidoreductase; Dsr – dissimilatory sulfite reductase; Gm – gentamycin; PCR – polymerase chain reaction; MS – mass spectrometry; LMW – thiol low molecular weight organic thiol; CoA – coenzyme A

genes in multiple *Chlorobiaceae* strains as well as characterized strain preferences can be found in the chapter by Frigaard and Bryant elsewhere in this volume.

A. Organization of Putative Sulfur Oxidation Genes in C. tepidum

Many of the predicted *C. tepidum* sulfur oxidation genes are physically associated on the genome as parts of gene and operon clusters, which we term Sulfur Islands, ranging from 3 to 26 genes in size (Fig. 1). Clustering may indicate a common evolutionary origin and functional significance for clustered genes. Potential transposase genes are associated with the Sox operon and Sulfur Island I, supporting the hypothesis that some of these genes were inherited as complete clusters by horizontal transfer (Eisen et al., 2002).

Two related models for *C. tepidum* sulfur oxidation pathways have been proposed based on the presence of between 52 and 94 genes (Fig. 1) encoding proteins that are recognizably similar to sulfur oxidation systems in other organisms (Eisen et al., 2002; Hanson and Tabita, 2003). These genes account for 2–4% of the 2,288 predicted genes encoded by *C. tepidum* (Eisen et al., 2002), a significant genetic investment for the organism. The lower bound is the number of open reading frames with recognizable similarity to sulfur oxidation genes in other microbes (shaded ORF's only in Fig. 1). The upper bound is the total number of genes that are associated with these recognized genes in sulfur islands (shaded and white ORF's in Fig. 1). The uncertainty in the number of genes for these pathways reflect the difficulty in correctly assigning gene or protein function in the absence of experimental verification. These numbers are also likely underestimates as they do not include genes of assimilatory sulfur metabolism or low molecular weight (LMW) thiol biosynthesis, which may play important roles in facilitating or regulating sulfur metabolism in *C. tepidum*. The genes for the former are fairly obvious, while the latter are completely unknown probably owing to the fact that the *Chlorobiaceae* contain structurally novel LMW thiols (Fahey et al., 1987; Fahey, 2001). This suggests that model systems like *C. tepidum* have much to tell us about microbial processes controlling biogeochemical cycles (Friedrich et al., 2001).

When it was isolated (Wahlund et al., 1991), *C. tepidum* was found to utilize sulfide and thiosulfate as electron donors for photosynthesis. A typical profile of these compounds as well as elemental sulfur through a batch culture growth curve clearly shows that *C. tepidum* consumes sulfide first, followed by elemental sulfur and finally thiosulfate (Fig. 2). Sulfate accumulation only begins once the oxidation of elemental sulfur has started, indicating that it is an obligate intermediate in the conversion of sulfide to sulfate in *C. tepidum*. Brief discussions of the *C. tepidum* genes potentially responsible for the oxidation of sulfide, elemental sulfur and thiosulfate follow.

1. Sulfide Oxidation

C. tepidum oxidizes sulfide (HS^-) to elemental sulfur (S^0) that is accumulated extracellularly (Brune, 1989; Brune, 1995). Two enzymes are likely candidates for sulfide oxidation, sulfide: quinone oxidoreductase (SQR) and a flavocytochrome *c* sulfide dehydrogenase. The *C. tepidum* genome contains genes for both activities and functionality for both has been implicated by different studies in different strains of *Chlorobiaceae* (Shahak et al., 1992; Verté et al., 2002).

Studies of other phototrophs have failed to provide evidence for flavocytochrome *c* activity in sulfide oxidation. A mutant strain of the purple sulfur bacterium *Allochromatium vinosum* lacking flavocytochrome *c* was competent for sulfide oxidation via SQR (Reinartz et al., 1998). Furthermore, *Rhodobacter capsulatus* contains SQR, but no flavocytochrome *c*, and efficiently oxidizes sulfide (Schütz et al., 1997; Schütz et al., 1999). Conversely, *Rhodopseudomonas palustris*, which contains flavocytochrome *c* and lacks SQR (Larimer et al., 2004), is inhibited by very low levels of sulfide (>0.25 mM) (Hansen and van Gemerden, 1972). These observations suggest that SQR is likely the most important route for sulfide oxidation in *C. tepidum* (Fig. 1). *C. tepidum* has been reported to require sulfide for early stages of growth both in batch (Wahlund et al., 1991) and reactor scale (Mukhopadhyay et al., 1999) cultures unlike other *Chlorobiaceae* that can utilize hydrogen gas or ferric iron (Fe^{2+}) as electron donors (Heising et al., 1999).

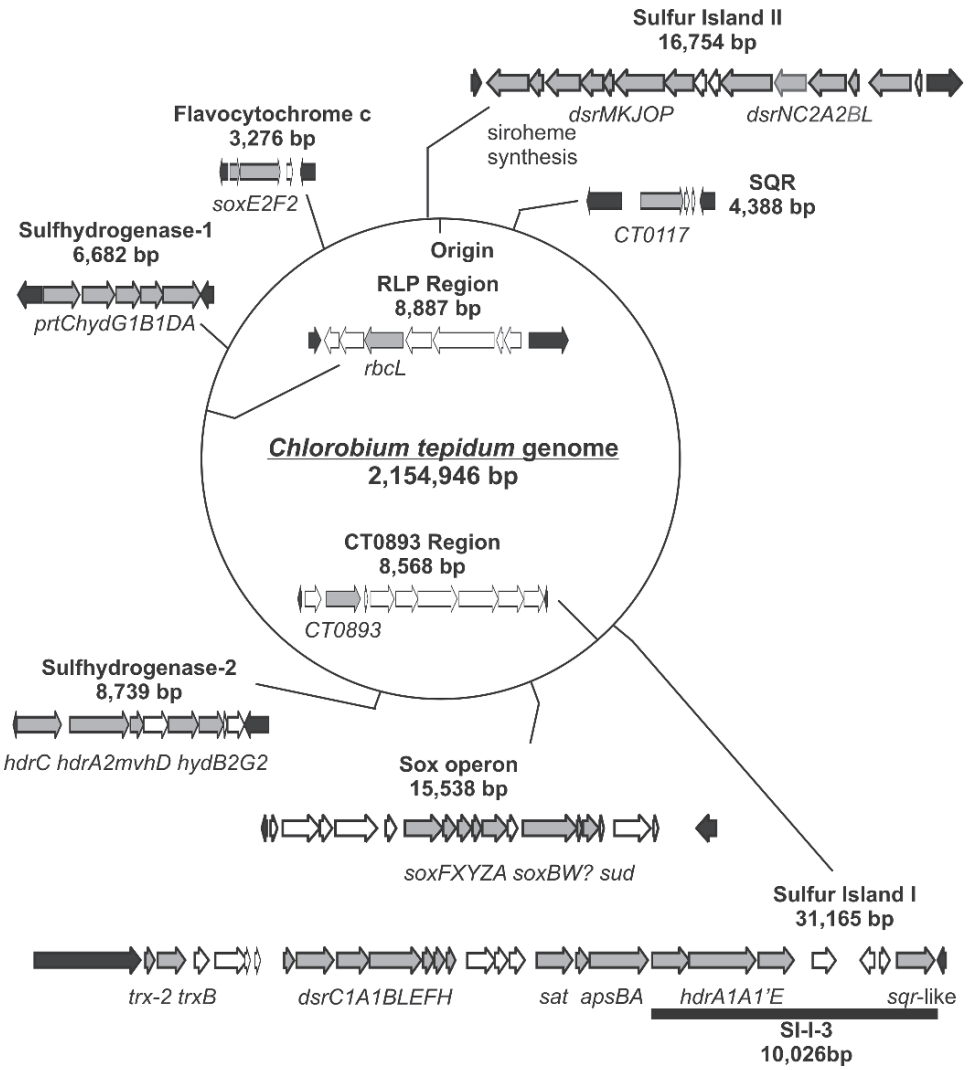

Fig. 1. Sulfur oxidation genes and their locations on the *C. tepidum* genome. The *C. tepidum* genome is depicted as a circle with the origin of replication (nucleotide 1 of the genomic sequence) at the top of the circle. The locations of sulfur oxidation gene clusters are indicated as are the structure of each cluster. Open reading frames are represented by arrows. Grey arrows are ORF's with discernible homology to sulfur oxidizing gene products in other microbes, black arrows indicate the borders of sulfur oxidation gene clusters, and white arrows indicate ORF's with unknown functions in sulfur oxidation. Shaded genes inside the genome have been experimentally implicated in sulfur oxidation in *C. tepidum*, while those outside the genome have been identified strictly by homology.

2. Elemental Sulfur Oxidation

When sulfide levels are low, the *Chlorobiaceae* oxidize extracellular elemental sulfur, the product of sulfide oxidation, to sulfate. The identity of enzymes mediating this process in the *Chlorobiaceae* is not clear (Brune, 1989; Brune, 1995; Pickering et al., 2001; Prange et al., 2002), though speculation exists. The *C. tepidum* genome annotation (Eisen et al., 2002) predicts that a sulfhydrogenase/polysulfide reductase activity will oxidize H_2 and reduce elemental sulfur to sulfide for subsequent oxidation via SQR (Ma et al., 1993). However, *C. tepidum* is normally grown in the absence of H_2 under which conditions it is proficient at elemental sulfur oxidation (Wahlund et al., 1991; Hanson and Tabita, 2001). Furthermore, studies on *C. limicola* suggest that elemental sulfur

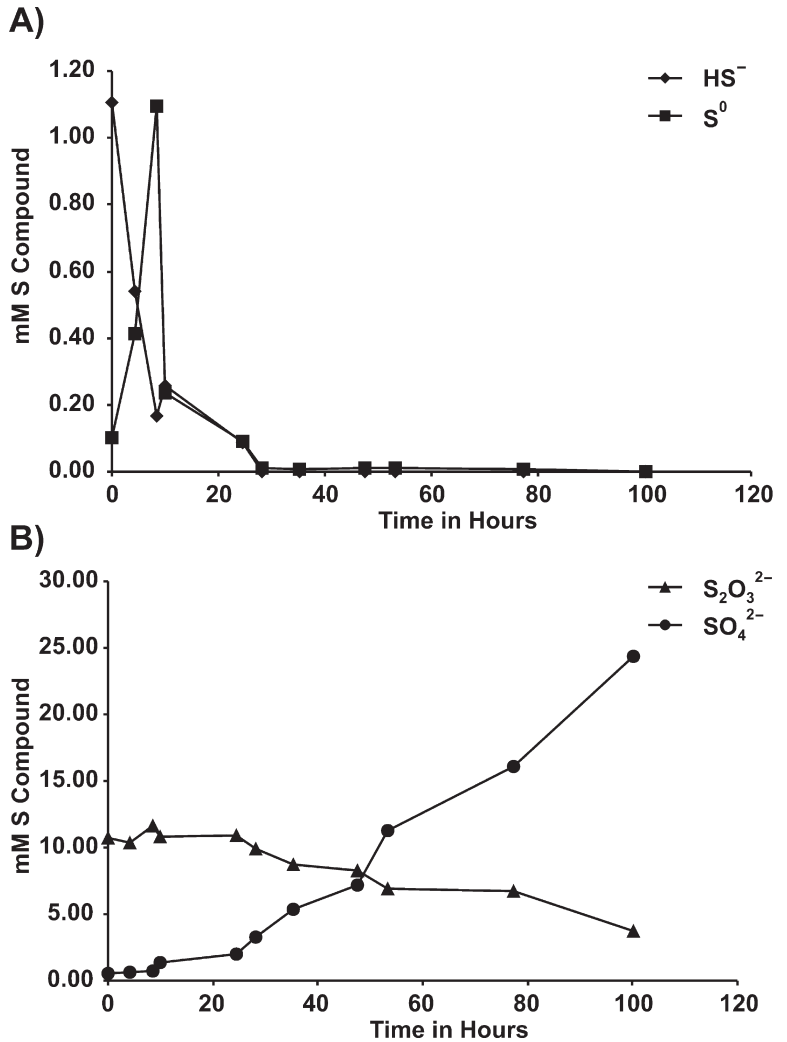

Fig. 2. Typical profiles of sulfur compounds in batch cultures of *C. tepidum*. The concentrations of each of four compounds are plotted as a function of time in a photomixotrophic culture of *C. tepidum* WT2321. Data points are the average of three independent cultures. (A) Sulfide (HS^-) and elemental sulfur (S^0). (B) Thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}). Note that sulfate is much higher than other compounds as two moles of sulfate are produced for each mole of thiosulfate oxidized.

is disproportionated to sulfide and sulfate in a light dependent reaction that is independent of H_2 as a source of reductant (Paschinger et al., 1974).

Other possible routes for phototrophic oxidation of elemental sulfur exist in anoxygenic phototrophic bacteria. Gene products of the dissimilatory sulfite reductase (Dsr) system have been genetically implicated in elemental sulfur oxidation in the purple sulfur bacterium *Allochromatium vinosum* (Pott and Dahl, 1998; Dahl et al., 2005). *C. tepidum* encodes homologs of this system including a duplication of the *dsrCABL* genes (Eisen et al., 2002). One copy of the *dsr* operon along with genes encoding ATP sulfurylase, APS reductase, heterodisulfide reductase, thioredoxin reductase and thioredoxin homologs constitute Sulfur Island I, one of a number of gene clusters encoding potential sulfur oxidizing activities (Fig. 1). A more detailed discussion on the involvement of the *dsr* genes in sulfur oxidation by the *Chromatiaceae* is given by Dahl elsewhere in this volume.

Low molecular weight thiols (LMW thiols) have been postulated to be involved in elemental

sulfur oxidation in phototrophic bacteria that contain Dsr complexes (Brune, 1989; Brune, 1995). Specifically, glutathione amide appears to cycle between the thiol (R-SH) and perthiol (R-S-SH) forms when *A. vinosum* oxidizes stored elemental sulfur globules (Bartsch et al., 1996). A similar process is proposed for *C. tepidum*, but the structure of the thiols and details of their involvement is still unclear (see section B.3 of this chapter).

Chlorobium limicola and *Chlorobium phaeobacteroides* have also been reported to use the metal sulfides MnS and FeS as growth substrates (Borrego and Garcia-Gil, 1995). Metal sulfides are analogous to elemental sulfur in that they are largely insoluble. This capability would presumably enable survival in sulfide poor environments. It is not clear whether *C. tepidum* has this activity or not, nor what genes/proteins would be required to carry out this function.

3. Thiosulfate Oxidation

Some of the *Chlorobiaceae*, including *C. tepidum*, also oxidize thiosulfate ($S_2O_3^{2-}$) to sulfate (Overmann, 2000). The *C. tepidum* genome contains a thirteen gene cluster encoding a system similar to the well characterized Sox sulfur oxidation system of *Paracoccus pantotrophus* strain GB17 (Fig. 1) (Friedrich et al., 2001). However, *C. tepidum* lacks genes encoding the SoxCD protein complex. In *P. pantotrophus*, SoxCD acts as a sulfur dehydrogenase on a SoxY-bound sulfur atom liberating six electrons for use in anabolism or energy conservation (Quentmeier and Friedrich, 2001). As six electrons represents 75% of the available reducing power from thiosulfate, it seems likely that *C. tepidum* possesses an alternative mechanism for the complete oxidation of SoxY-bound sulfur atoms.

B. Gaps and Redundancies in Proposed Sulfur Oxidation Pathways

1. Extracellular Elemental Sulfur Oxidation

Extracellular elemental sulfur is oxidized by *C. tepidum* during growth leading to the production of sulfate. This is a challenging biochemical problem in that elemental sulfur is extremely hydrophobic and insoluble. Clearly, a mechanism must exist that allows *C. tepidum* to access this relatively rich source of reductant, but there are no obvious candidate proteins for either the activation or transport of extracellular elemental sulfur encoded by the *C. tepidum* genome (Eisen et al., 2002). This is a particularly difficult problem if elemental sulfur oxidation proceeds via a periplasmic sulfhydrogenase, as was proposed in the genome annotation.

A similar problem conceptually is the reduction of insoluble electron acceptors such as elemental sulfur, metallics and/or humics by anaerobic and facultatively anaerobic microbes. At least two general strategies have evolved to account for this conundrum (Lloyd, 2003). The first is direct electron transfer from the cell envelope to the acceptor as has been proposed for various metal reducing bacteria. In *Shewanella oneidensis* MR-1, this process is thought to involve cytochromes that span the outer membrane of the organism and provide a direct path for electrons from cytoplasmic or periplasmic reductants to the acceptor (Myers and Myers, 2001). A prediction of this mechanism is that cells should be in intimate physical contact with the substrate to facilitate direct electron transfer. In the case of *C. tepidum*, physical association with elemental sulfur would also facilitate capture of the reduced product, sulfide or polysulfide. However, our observations do not support this proposed strategy of tight associations between the cell and elemental sulfur, since *C. tepidum* does not appear to frequently or conspicuously associate with sulfur globules in cultures that are actively oxidizing elemental sulfur (Chan and Hanson, unpublished).

The second strategy for acquisition of these insoluble sources of sulfur and reducing power is the use of electron shuttling compounds to mediate extracellular electron transfer at sites distant from the cell. One particular example among several is the apparent use of phenazine antibiotics by some soil microbes to facilitate electron transfer to metals or other soil components (Hernandez et al., 2004). A more recent study reported that *S. oneidensis* MR-1 also has the ability to reduce iron at a distance as well as by direct contact (Lies et al., 2005). This concept could be translated to elemental sulfur oxidation via a low molecular weight thiol that could react with exposed –SH or –S-S- groups on the surface of elemental sulfur

globules to generate a linear polysulfide that is tagged with an organic molecule. The organic could then be used as a specific recognition tag for cell surface receptors, which could in effect reserve the elemental resource for use only by organisms that have the specific receptor.

Neither the direct contact nor action at a distance model has been conclusively tested in the *Chlorobiaceae*, but the subcellular fractionation approach discussed in Section III below will test the direct electron transfer model by examining the outer membrane proteome of *C. tepidum* for potential electron transfer proteins.

2. Missing and Duplicated Genes

Duplicated genes include subunits of sulfhydrogenase (*hydBG*, CT1249-50 and CT1891-92), heterodisulfide reductase (*hdrA*, CT0866 and CT1246), sulfide:quinone reductase (CT0117, CT0876, CT1087), polysulfide reductase C and B subunits (CT0495-96 and CT2241-40) and a long duplication of Dsr complex subunits stretching >5 kb at > 99.5% DNA sequence identity (*dsrCABL*, CT0851-54 and CT2250-46). However, one of these copies (CT2250-46) contains an authentic frame shift in the *dsrB* gene (Eisen et al., 2002). Whether both copies of *dsrCABL* are expressed differentially or are indeed functional is currently unknown. Other duplicated or triplicated genes appear to encode viable gene products, though their functions have not been experimentally verified. In particular, the SQR homologs appear to have diverged significantly from one another, raising the question of whether all three can be involved in sulfide oxidation (Chan and Hanson, unpublished data).

Some genes are unexpectedly missing including the previously discussed *soxCD* and *shxV* (Friedrich et al., 2001). The *shxV* gene in *P. pantotrophus* encodes a product similar to CcdA proteins that are involved in the maturation of periplasmic cytochromes. A *P. pantotrophus shxV* mutant is unable to oxidize either thiosulfate or molecular hydrogen, but is not generally defective for periplasmic cytochrome biogenesis (Bardischewsky and Friedrich, 2001). A substitute function for ShxV in *C. tepidum* could be encoded by CT1559. The CT1559 gene product is similar to the plastid encoded *ccsA/ycf*5 gene product of *Chlamydomonas reinhardtii*, which was genetically shown to be involved in cytochrome maturation (Xie and Merchant, 1996). CT1559 is not associated with any other predicted sulfur oxidation genes on the *C. tepidum* genome (Eisen et al., 2002).

3. Other Aspects

The model of sulfur oxidation put forward with the *C. tepidum* genome annotation does not propose any mechanism for the transport and assimilation of reduced sulfur or thiosulfate into methionine and cysteine (Eisen et al., 2002). As *C. tepidum* does not require these amino acids for growth, this process must occur. Cysteine synthase activity has been directly assayed in *C. tepidum* (Hanson and Tabita, 2001) and sufficient levels of activity were found to account for the ability to grow in medium lacking cysteine supplementation. Genes for both methionine and cysteine biosynthesis from sulfide and precursor metabolites have been identified (Eisen et al., 2002). Presumably the sulfide for biosynthesis must be generated in the cytoplasm, but reductive sulfate assimilation has never been observed in the *Chlorobiaceae* (Overmann, 2000) suggesting transport of sulfide by an unknown mechanism as a route for providing this nutrient. It is unclear whether or not cysteine or methionine biosynthetic activities are regulated by exogenous substrate.

Models of sulfur oxidation in *C. tepidum* predict that sulfate will be generated in the cytoplasm. Sulfate accumulates extracellularly (Fig. 2) in *C. tepidum* cultures, implying a transport mechanism as the divalent anion is not likely to cross the cytoplasmic membrane by diffusion. However, the *C. tepidum* genome lacks a clearly identifiable sulfate transporter (Eisen et al., 2002). The lack of a recognizable sulfate transporter also supports the notion that sulfide for sulfur amino acid biosynthesis may be transported from outside the cell when it is available.

As noted briefly above, the *Chlorobiaceae* also lack clearly identifiable genes for the biosynthesis of typical redox buffering LMW thiol compounds, with the exception of CoA, which is used by some Gram positive bacteria. Genes have been identified for the biosynthesis of glutathione, mycothiol, and ergothionine in various microbes (Fahey, 2001), and these have been used to search the *C. tepidum* genome for homologs without any

success (Hanson, unpublished data). *C. limicola* has been shown to contain a LMW thiol named U11, for an unknown compound with a retention time of 11 minutes in an HPLC separation, that does not correspond to the retention time of common thiol compounds (Fahey et al., 1987). *C. tepidum* appears to contain U11 and as many as four other novel LMW thiols (Hanson, unpublished data). Thus, LMW thiol biosynthetic genes should be part of a *Chlorobiaceae* specific suite of genes shared amongst these genomes, but not by other microbes. It will be interesting to see what roles these compounds play in the function and regulation of sulfur oxidation pathways in the *Chlorobiaceae*.

II. Genetic Studies

A. *Genetics in the* Chlorobiaceae

The ability to genetically manipulate members of the *Chlorobiaceae* has been developed to a quite useful level, but it is not by any means mature. It will become increasingly important to develop additional techniques for subtle genetic manipulations and ectopic gene expression as more genomic information is rapidly developed for this group of organisms (see the chapter of Frigaard and Bryant in this volume).

The first instance of genetic manipulation in the *Chlorobiaceae* was reported by John Ormerod in 1987 at the EMBO Workshop on Green and Heliobacteria in Nyborg, Denmark (Ormerod, 1988). This involved the transfer of a spontaneously arising streptomycin resistance allele between two strains of *Chlorobium limicola* strain Tassajara (DSM 249 syn. *Chlorobaculum thiosulfatiphilum* (Imhoff, 2003)) and 8327 (DSM 263 syn. *Chlorobaculum parvum* (Imhoff, 2003)) by natural transformation (the ability of certain microbes to assimilate exogenously provided DNA into their genome by homologous recombination).

Natural transformation is still the preferred method of genetic manipulation the *Chlorobiaceae* and has been used to inactivate genes encoding chlorosome proteins in *Chlorobium vibrioforme* as well (Chung et al., 1998). The ability to inactivate specific genes in *C. tepidum* was reported in 2001 in two separate reports describing the insertional inactivation of genes encoding a nitrogenase subunit (Frigaard and Bryant, 2001) and a RubisCO-like protein (Hanson and Tabita, 2001). Subsequently, gene inactivation has been used with great effect to delineate pathways of bacteriochlorophyll and carotenoid biosynthesis in *C. tepidum* (Vassilieva et al., 2002a; Frigaard et al., 2004a; Frigaard et al., 2004b; Maresca et al., 2004). While gene by gene inactivation is certainly useful, additional genetic approaches to increase the rate of analysis of the *C. tepidum* genome need to be developed and one of these is briefly described below.

B. *In Vitro Transposition Mutagenesis of* C. tepidum *Sulfur Islands*

To take advantage of the large gene clusters encoded by the Sulfur Islands evident in the *C. tepidum* genome (Fig. 1), we have employed in vitro transposition mutagenesis (IVTM). IVTM is a molecular genetic technique that constructs a library of DNA fragments each containing a unique transposon insertion. By using specific fragments of the *C. tepidum* genome produced by PCR, a transposon mutant library can be constructed that is tightly focused on a specific genomic region. Each transposon insertion that occurs in a gene creates a knock-out mutation of that gene, rendering it incapable of producing the protein it normally encodes. Transfer of these mutated fragments back into *C. tepidum* creates mutant strains lacking specific proteins, which can then be analyzed for defects in sulfur oxidation. IVTM was originally developed for the genetic analysis of various enteric and mycobacteria (Rubin et al., 1999; Wong and Mekalanos, 2000) and has been utilized in the genetic analysis of various other microbes as reviewed by Hayes (Hayes, 2003). IVTM has now been commercialized and transposases are available from several companies. IVTM should be adaptable to insert nearly any fragment of DNA that can be PCR amplified as the only requirement is that the transposase recognition sites flank the section of DNA to be inserted. The transposase recognition sites are easily added by including them at the ends of the PCR primers used to amplify the fragment of interest.

C. tepidum Sulfur Island I (Fig. 1) is a 33 kb section of the genome that contains sixteen

genes encoding for potential sulfur oxidation enzymes: thioredoxin and thioredoxin reductase, *trx2* (*CT0841*) and *trxB* (*CT0842*), dissimilatory sulfite reductase and functionally associated proteins, *dsrC1A1BLEFH* (*CT0851-57*), sulfate adenylyltransferase, *sat* (*CT0862*), adenylylsulfate reductase, *apsBA* (*CT0864-65*, known as *aprAB* in many other organisms), heterodisulfide reductase, *hdrA-1A-1'E* (*CT0866-68*), and a sulfide:quinone oxidoreductase (*sqr*)-like (*CT0876*) homolog. Sulfur Island I contains twenty one ORF's encoding hypothetical and conserved hypothetical proteins (*CT0843-50*, *CT0858-61*, *CT0863*, *CT0869-75*, and *CT0877*), well more than half of the thirty seven ORF's in this region. In addition, a predicted transposase is encoded by *CT0878* suggesting that this region of the *C. tepidum* genome has been modified by lateral transfer.

An ~11 kb subsection of Sulfur Island I (SI-I-3, Fig. 3) containing genes *CT0866-CT0876* was chosen to provide a proof of concept that IVTM could be successfully applied to *C. tepidum*. This fragment was amplified by PCR and cloned into *E. coli* followed by IVTM using a synthetic transposon, TnOGm, produced from pTnMod-OGm (Dennis and Zylstra, 1998) by PCR. This transposon was selected because it carries the *aacC1* gentamycin resistance marker that had previously been used to disrupt the *C. tepidum nifD* gene (Frigaard and Bryant, 2001). TnOGm also carries a conditional origin of replication that will allow self cloning of TnOGm insertions in future experiments and is one of a large series of such transposons with varying resistance markers and structures available from Gerben Zylstra at Rutgers University (Dennis and Zylstra, 1998).

Following cloning of the SI-I-3 fragment, IVTM was performed with TnOGm and a library of mutated plasmids was recovered in *E. coli* by virtue of the Gm resistance marker of TnOGm. DNA was purified from this library and then used to transform *C. tepidum*. Randomly selected gentamycin resistant colonies of *C. tepidum* were screened by PCR of the SI-I-3 region to verify that TnOGm insertion had occurred and broadly localize the site of transposon insertion. The specific site of TnOGm insertion in mutant strains displaying growth defects was analyzed by sequencing out from TnOGm in the SI-I-3 PCR product produced from each strain (Fig. 3A). Strains AK and AJ contained transposon insertions in gene *CT0868* encoding an HdrE homolog and *CT0867* encoding a fusion of HdrA and HdrD proteins called HdrA-1′, respectively. These genes will certainly be renamed in light of recent work on homologs in the sulfate reducer *Desulfovibrio desulfuricans* described in the next section. Genes from 3′ of *hdrA-1′* to the 5′ end of the *sqr*-like ortholog (*CT0867-76*) were deleted and replaced with TnOGm in strain C5. Details of the IVTM procedures and conditions will be described fully elsewhere (Chan et al., manuscript in preparation).

C. Physiological Characterization of Mutant Strains

Initial characterization of IVTM generated SI-I-3 mutant strains indicated that the Gm resistance marker in TnOGm appears to be temperature sensitive (Fig. 3B). Both single insertion mutant strains are incapable of growth at 48°C in the presence of Gm, while the insertion deletion strain C5 is clearly defective for growth at 48°C in the presence or absence of Gm. All mutant strains grew well with or without Gm at 42°C indicating that the Gm marker was functional. The wild type was incapable of growth in the presence of Gm at either 42 or 48°C. The temperature sensitivity of a selectable marker can complicate some physiological analyses and will need to be addressed for each marker that will be used in *C. tepidum*. Because of this, physiological measurements are routinely made on cells grown at 48°C, the reported optimum growth temperature for *C. tepidum*, in the absence of Gm selection. Despite the absence of antibiotic selection, the genotype of insertion mutants including the TnOGm mutants is stable under these conditions (Chan et al., manuscript in preparation). Starter cultures for physiological analysis and those for the preparation of frozen permanents are always grown at 42°C under appropriate selection.

Strain C5, the insertion-deletion strain, displayed a markedly temperature sensitive phenotype exhibiting comparable growth in comparison to the wild type at 42°C, but exhibiting a significant growth deficiency at a growth temperature of 48°C (Fig. 3B). The single mutant strains both had more subtle phenotypes, but also displayed reproducible growth yield defects compared to the wild type. All mutant strains

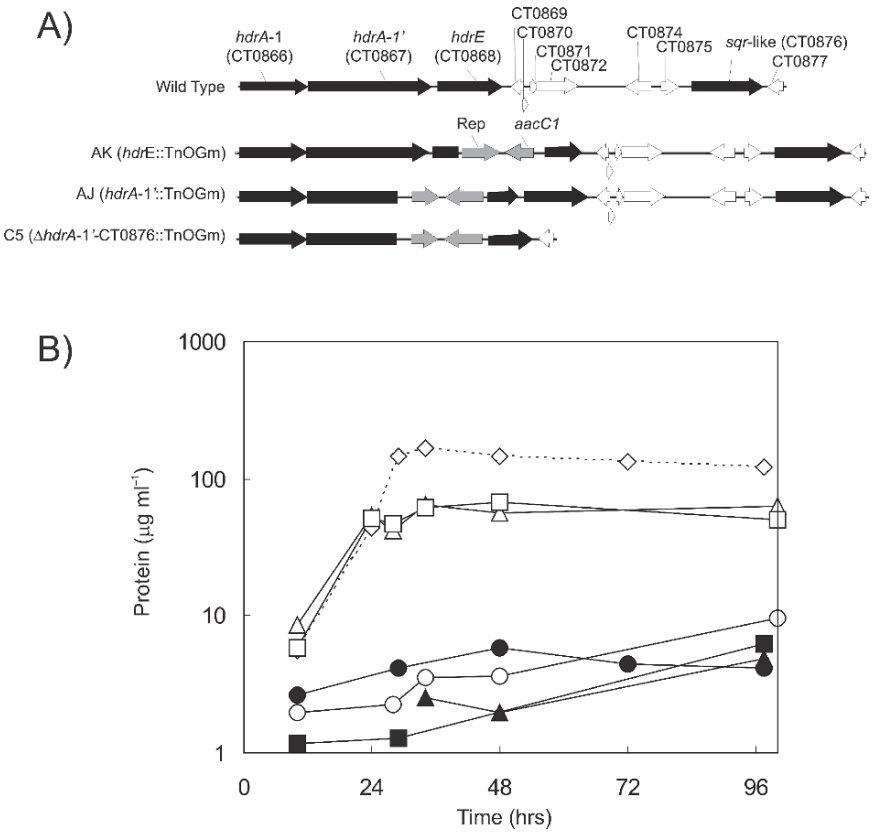

Fig. 3. Single mutations in SI–I–3 result in growth yield defects, while an insertion–deletion results in drastic reductions in growth rate and growth yield. A. Physical map of SI–I–3 (see Fig. 1 for context) and genotypes of relevant mutants. ORF's are depicted as arrows and gene names provided. The genes *hdrA-1*, *hdrA-1'* and *hdrE* encode proteins similar to subunits of the QmoABC complex of *D. desulfuricans* as discussed in the text. White arrows depict ORF's encoding hypothetical and conserved hypothetical proteins, while grey arrows depict regions of the transposon, TnOGm. B. Growth of wild type and SI-I-3 mutant strains. Strains were grown in acetate containing Pf-7 medium as previously described (Hanson and Tabita, 2001) in the presence (filled symbols) or absence (open symbols) of 4 μm Gm ml^{-1}. Symbols: wild type ◊, strain AJ □, strain AK Δ, insertion–deletion strain C5 ○.

exhibited defects in sulfur oxidation, particularly in thiosulfate oxidation. The genes affected include homologs of a membrane protein complex, the quinone interacting membrane bound oxidoreductase (Qmo) as named in *Desulfovibrio desulfuricans* (Pires et al., 2003). In *C. tepidum,* the Qmo homologs (*CT0866-0868*, referred to as *hdrA-1*, *hdrA-1'*, and *hdrE* above and in Fig. 3A) are part of Sulfur Island I and the results indicate that these genes are important for optimal growth of *C. tepidum*. The insertion-deletion strain C5 has also lost a number of hypothetical open reading frames and the first part of a gene encoding one of three SQR homologs in *C. tepidum*. These mutant strains provide the first experimental evidence for specific sulfur oxidation genes being important to the overall function of the *Chlorobiaceae*. The detailed characterization of these strains and their phenotypes is being prepared for publication (Chan et al., manuscript in preparation). While the precise function of the Qmo complex is still not known, it appears to be present in sulfate reducing bacteria and archaea and it has been suggested that it may function to mediate electron transfer between the membrane quinone pool and APS reductase and thereby contribute to energy conservation.

The results taken together strongly suggest that IVTM is a viable route for making insertion mutations in tightly focused regions of the *C. tepidum* genome providing another tool for the genetic dissection of metabolism in the *Chloro-*

biaceae to complement gene by gene inactivation. The development of additional tools, including complementation of mutations, heterologus gene expression, reporter gene development, and others are actively being pursued and should provide yet more tools to understand the biology of these organisms. *C. tepidum* will apparently maintain certain broad host range plasmids (Wahlund and Madigan, 1995) indicating that mutant complementation by ectopic gene expression should be feasible, although this has yet to be demonstrated.

III. Proteomic Studies

A. *Proteomics in the* Chlorobiaceae

Proteomics can be broadly defined as the determination of the entire suite of proteins synthesized by a population of cells in response to prevailing environmental conditions. In practice, the entire suite of proteins is rarely resolved and usually a subset is examined to determine the identity of specific proteins that are differentially synthesized in cells faced with a particular challenge or growth condition. A useful subset of proteomics is subcellular proteomics, or defining the protein complement of specific subcellular components.

The primary tools of proteomics since the coining of the term in 1995 (Patterson and Aebersold, 2003) have been two dimensional polyacrylamide gel electrophoresis (2D PAGE), gel image analysis, and mass spectrometric (MS) methods of protein identification. It should be noted that 2D-PAGE in particular has a long and proven track record in defining protein complements before the term proteomics was coined, for example, see the work of Neidhardt and co-workers in the 1970s and 1980s (Bloch et al., 1980; Phillips et al., 1980; Neidhardt et al., 1989). More recently, 2D-PAGE is being supplanted by liquid chromatographic separations of proteolytically digested cellular extracts followed by direct MS identification of specific peptides (Patterson and Aebersold, 2003). Isotopic and dye based peptide and protein labeling systems have also been developed to facilitate quantitative comparisons of proteomic samples (Patton, 2002).

2D-PAGE has only recently been applied to *C. tepidum* to analyze the proteome under standard growth conditions. Tsiotis and co-workers have examined both the membrane proteome (Aivaliotis et al., 2004a, 2004b, 2006a) as well as cytoplasmic protein complexes (Aivaliotis et al., 2006b). Subcellular proteomics has been applied in a focused manner to examine the protein complement of the chlorosome in *C. tepidum* (Chung et al., 1994; Bryant et al., 2002; Vassilieva et al., 2002b). Both 2D-PAGE and subcellular proteomics have been utilized to study widespread changes in the *C. tepidum* proteome resulting from the loss of the RLP (Hanson and Tabita, 2003).

B. *Subcellular Fractionation of* C. tepidum *to Identify Secreted and Outer Membrane Proteins*

Starting from existing protocols for subcellular fractionation of *C. tepidum* (Vassilieva et al., 2002b; Hanson and Tabita, 2003), my laboratory is now attempting to specifically isolate both the secreted and outer membrane proteome of *C. tepidum* to identify proteins involved in elemental sulfur oxidation. As outlined above, the outer membrane may contain electron transfer proteins if direct cellular contact is the primary mode of elemental sulfur oxidation. Secreted proteins may be involved in either the direct contact or action at a distance model of sulfur oxidation. Genes encoding proteins identified by this approach will be analyzed for their expression patterns followed by mutagenesis as described above.

Prior membrane proteome studies of *C. tepidum* have provided a limited number of outer membrane protein identifications, but ~70% of proteins previously identified in membrane preparations are known or predicted to be cytoplasmic (Aivaliotis et al., 2006a). Acidic glycine extraction to selectively enrich outer membrane proteins was somewhat more successful with ~37% of proteins identified as being either periplasmic or outer membrane proteins.

A variety of methods have been used over the last 30 years to separate inner and outer membrane protein fractions from Gram negative bacteria (Mizuno and Kageyama, 1978; Shen et al., 1989; Buonfiglio et al., 1993; Link et al., 1997; Sagulenko et al., 2001; Yarzabal et al., 2002; Baik

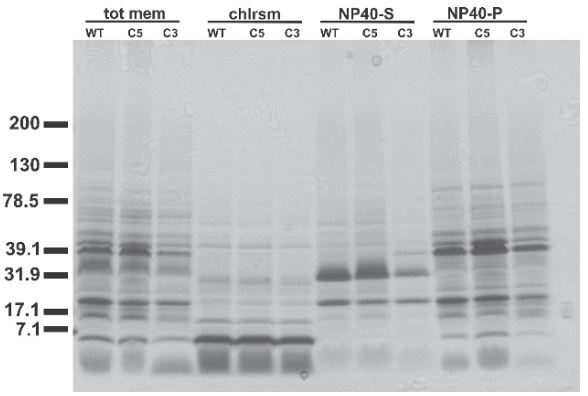

Fig. 4. Subcellular fractionation of *C. tepidum* strains. Chlorosome depleted membranes were prepared via fractionation on sucrose gradients from the wild type (WT) and two IVTM generated mutant strains (C3 and C5) of *C. tepidum* and then fractionated by solubilization with the detergent Nonidet P40 into detergent soluble (NP40-S) and insoluble (NP40-P) fractions. Proteins in each fraction were separated by 1D SDS-PAGE and stained with colloidal Coomassie blue. The NP40–S fraction is enriched in inner membrane proteins while the NP40–P fraction is enriched in outer membrane proteins.

et al., 2004; Huang et al., 2004). A number of these methods were used to fractionate chlorosome depleted membranes of *C. tepidum* (Vassilieva et al., 2002b). Solubilization with the detergent Nonidet P-40, used to extract outer membrane proteins from *Hyphomonas jannaschiana* (Shen et al., 1989), was found to reliably produce distinct banding patterns when NP-40 soluble and insoluble fractions were compared by 1D SDS-PAGE (compare the NP40-S and NP40-P fractions in Fig. 4). Protein identification by mass spectrometry is proceeding from these fractions and preliminary data indicates that NP-40 soluble proteins are predominantly inner membrane proteins including reaction center and ATPase subunits, while the NP-40 insoluble proteins are outer membrane proteins (Snellinger-O'Brien, Morgan-Kiss, Johnston and Hanson, unpublished).

C. Proteomics of Sulfur Oxidation Mutants

The improved fractionation protocol outlined above is currently being applied to *C. tepidum* in our laboratory along with additional fractionation and detection techniques. Heme staining via peroxidase activity (Thomas et al., 1976) has been applied to subcellular fractions from *C. tepidum* WT2321 (Fig. 5) and mutant strains including C5 and an additional mutant strain, C3. The mutation in strain C3 is localized to Sulfur Island II (Fig. 1) and the strain appears to be defective for elemental sulfur oxidation. Current data (Fig. 5) indicate *C. tepidum* synthesizes at least a dozen hemoproteins with distinct subcellular localizations and that strain C5 overexpresses a ~25 kDa outer membrane heme protein relative to WT2321. Two heme stained proteins are apparent in secreted protein fractions at ~15 kDa and 5 kDa, which are distinct from hemoproteins observed in other fractions. The 15 kDa protein appears to be overexpressed in strain C3 while the 5 kDa protein appears to be overexpressed in strain C5. As the identities of these hemoproteins are resolved, they should provide great insight into mechanisms of sulfur oxidation in *C. tepidum*. In addition, there appear to be clear changes in protein expression profiles in the inner and outer membranes in both strains C5 and C3 when total proteins are visualized by Coomassie staining (compare lanes within each fraction across strains in Fig. 4). Identification of the hemoproteins and differentially expressed proteins along with detailed studies of growth and sulfur compound utilization by these particular mutant strains are underway.

IV. Conclusions

A combination of approaches will be required to fully understand anaerobic sulfur oxidation pathways in the *Chlorobiaceae*. The combination of genetic and proteomic approaches outlined above will serve as a starting point to identify the proteins critical for this process in the model system *C. tepidum*. Parallel approaches including comparative genomics as described by Frigaard and Bryant elsewhere in this volume will determine the distribution of these proteins across not only the *Chlorobiaceae*, but in other sulfur oxidizing microbes as well and thus translate to an overall improved understanding of biogeochemical cycling. Biochemical, crystallographic and more refined genetic approaches applied to genes/proteins identified in *C. tepidum*, including gene fusions, site directed mutagenesis and expression of exogenous genes in *C. tepidum* for mutant

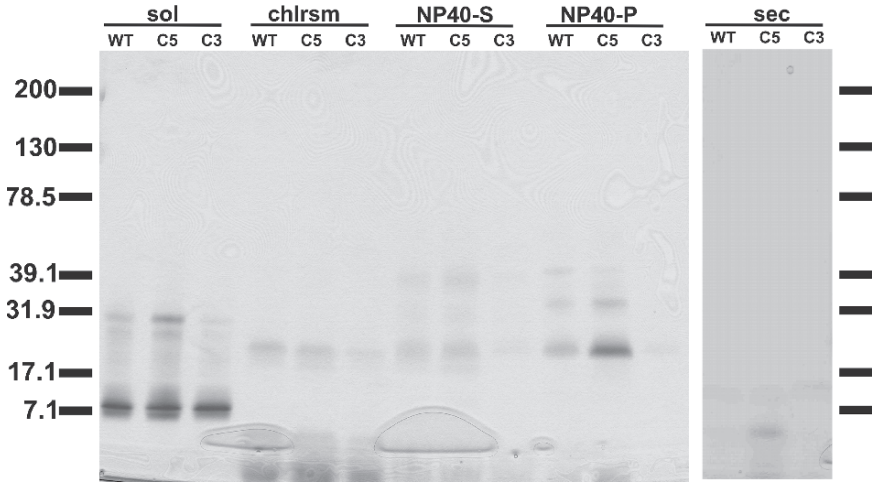

Fig. 5. Cellular distribution of hemoproteins in *C. tepidum* wild type and mutant strains. Cytosolic (sol), chlorosome (chlrsm), inner membrane (NP40–S), outer membrane (NP–40P), and secreted (sec) protein fractions were prepared from cultures of wild type (WT) and two IVTM generated mutant strains (C3 and C5). Proteins were separated by 1D SDS-PAGE without prior sample heating or addition of reducing agent and stained for heme peroxidase activity.

complementation will also bring about a more detailed understanding of anaerobic sulfur oxidation in general and further establish *C. tepidum* as one of the most useful and relevant model systems for the genetics of strictly anaerobic processes.

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