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Hydrogen–Sulfur Autotrophy in the Hyperthermophilic Archaebacterium, *Pyrodictium brockii*

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Introduction

Over the past 25 years, geothermal environments have been the source of a number of micro-organisms that grow at elevated temperatures. As a result, the study of thermophily, once confined to organisms growing up to approximately 60°C, has expanded to include bacteria proliferating well in excess of 100°C. One finds from literature descriptions, both old and new, that the so-called *thermophile* classification covers a temperature range of at least 70°C. For purposes of discussion here, *moderate thermophiles* will apply to micro-organisms growing to 70°C, *extreme thermophiles* to those growing to 100°C and *hyperthermophiles* describing those growing at 100°C and above. At present, the number of identified thermophilic micro-organisms thriving at a particular temperature diminishes as temperature increases, although the relative abundance of organisms growing optimally at elevated temperatures in the biosphere is not known in absolute terms. Furthermore, relatively little is understood concerning the physiology and metabolism of thermophilic organisms. In the past few years, the number of extremely thermophilic and hyperthermophilic isolates known has increased steadily, as has the intensity with which these organisms are being studied. As a result, some patterns have emerged that are useful in the characterization of high-temperature micro-organisms.

Of the extremely thermophilic and hyperthermophilic bacteria isolated to date, most utilize sulfur as part of their metabolism. As growth temperatures

Abbreviations: ADP, adenosine diphosphate; APS, adenylylsulfate; ATP, adenosine triphosphate; EPPS,*N*-(2 hydroxyethyl) piperazine-*N'*-3-propane sulfonic acid; MV, methyl viologen; Pi, orthophosphate; PPi, pyrophosphate.

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approach and exceed 100°C, the sulfur-metabolizing bacteria are obligately anaerobic and reduce significant amounts of certain sulfur compounds to sulfide. The mechanisms and motivations for reductive sulfur biotransformation at elevated temperatures are not understood and may or may not relate to what little is known along these lines for mesophilic micro-organisms. What is clear is that sulfur, often as elemental sulfur, is ubiquitous in geothermal environments, as are dissolved molecular hydrogen, methane, carbon dioxide, hydrogen sulfide and traces of other gases. Micro-organisms inhabiting these niches are known to play a role in the production and/or utilization of these compounds as part of their metabolism.

At high temperatures, both heterotrophic and chemolithoautotrophic bacteria have been shown to produce significant levels of sulfide. It is important to understand the various modes of sulfur transformations (both biotic and abiotic) in geothermal environments to appreciate the metabolisms of indigenous bacteria as well as their interactions. For example, both the hyperthermophilic heterotroph, *Pyrococcus furiosus*, and the hyperthermophilic chemolithotroph, *Pyrodictium brockii*, will produce sulfide during growth. However, the role of sulfur reduction appears to be different for these two bacteria. Blumentals *et al.* (1990) suggested that *P. furiosus* either respires sulfur, or utilizes it as a sink for electrons that could otherwise bottleneck its metabolism. In contrast, sulfur reduction by *P. brockii* represents an unusual metabolic pattern, hydrogen–sulfur autotrophy (Stetter, 1982). This metabolism is characterized by the transfer of electrons from hydrogen to sulfur and the fixation of carbon dioxide; it provides a basis for living processes that rely only on dissolved gases and inorganic compounds and could have served as a primeval mode of life (Fischer *et al.*, 1983). Efforts to understand this metabolism are discussed here.

The genus *Pyrodictium*

Microbial isolates able to grow in mineral media at temperatures in excess of 100°C with H_2 and S^0 as the only potential sources of energy and CO_2 as the only carbon source were first described by Karl O. Stetter in 1982 (Stetter, 1982). These bacteria were isolated from a shallow marine solfatara field off the coast of Vulcano Island, Italy and were later described as a new genus, *Pyrodictium*, comprised of two species: *P. occultum* and *P. brockii* (Stetter, König and Stackebrandt, 1983). Until that time, the most thermophilic species to have been found and studied had been isolated from continental geothermal areas and it was with the expressed intent of seeking organisms capable of growing above the boiling point of water that these submarine locations were sampled. Rod-shaped organisms of the genus *Thermoproteus*, able to grow up to 96°C by hydrogen–sulfur autotrophy, had just been isolated from sites in Iceland (Zillig *et al.*, 1981). Thus, the *Pyrodictium* isolates, capable of growth up to 110°C, represented the most extremely thermophilic organisms yet described and another instance of this autotrophic metabolism based on the reduction of sulfur with hydrogen.

P. occultum and *P. brockii* are disc shaped, between 0·3 and 2·5 μm in

diameter and about 0·2 μm thick and can produce a network of fibers, 0·04–0·8 μm in diameter, and ranging to 40 μm in length if the culture is not agitated (Stetter, König and Stackebrandt, 1983). They grow at temperatures between 80 and 110°C with doubling times ranging from 2 to 9 h, with a reported minimum doubling time of 110 min at 105°C. They tolerate a wide range of salinities, 0·2–12% NaCl, with an optimum around 1·5%, and are moderately acidophilic, preferring pHs between 5 and 7, and growing optimally near pH 5·5. *Pyrodictium* species are strictly anaerobic; no growth is observed unless oxygen is chemically reduced with agents such as sodium sulfide. They are classified as archaebacteria, based on the presence of phytanyl di- and tetra-ethers, the sensitivity of their ribosomes to ADP-ribosylation by diptheria toxin and their lack of muramic acid. The two species, *P. occultum* and *P. brockii*, can be distinguished on the basis of several molecular characteristics, immunological reactions and the influence of organic materials on growth yield. The major S-layer protein of *P. occultum* has been characterized as a glycoprotein of molecular weight (MW) 172 000 whereas the major S-layer protein of *P. brockii* does not appear to be a glycoprotein and has a MW of 150 000 (Stetter, König and Stackebrandt, 1983). The G + C content of their DNA has been reported to be about 62% for *P. occultum* and between 52% and 56% for *P. brockii* (Stetter, König and Stackebrandt, 1983). There was no cross-reactivity between isolates of the two different species, based on enzyme-linked assays using rabbit antisera raised against whole cells of one of the *P. occultum* isolates, PL-19 (Stetter, König and Stackebrandt, 1983). Additionally, it was found that the growth yield of two of the isolates could be increased by about a factor of five through the addition of yeast extract to the medium at a concentration of 2 g l^{-1}; these two isolates were subsequently classified as *P. brockii* (Stetter, König and Stackebrandt, 1983). The growth of the other isolates was slightly stimulated by the presence of yeast extract at 0·2 g l^{-1} and citric acid at 0·01 g l^{-1}, but higher concentrations of yeast extract did not result in increased yields. It should be emphasized at this point that yeast extract cannot serve as sole carbon or energy source for the growth of either species of *Pyrodictium*. It was reported that no growth could be observed in the absence of H_2, with a N_2/CO_2 atmosphere on yeast extract, meat extract, citric acid, starch, glucose, casein, peptone or casamino acids (Stetter, König and Stackebrandt, 1983) and the same result was noted for cultures incubated in sea water with yeast extract (2 g l^{-1}) under an atmosphere of H_2/He, 80%/20% (Parameswaran *et al.*, 1988).

Dissimilatory sulfur metabolism

In order to understand hydrogen–sulfur autotrophy as it applies to the metabolism of *P. brockii*, it is important to appreciate its relationship to other forms of dissimilatory sulfur metabolism in bacteria. The element sulfur is important in two fundamentally different modes of metabolism. First, it is a constituent of proteins, in which the sulfur contained in amino acids, i.e. cysteine and methionine, is built into the covalent structure. Sulfur is also

found in other cellular components such as co-enzyme A. If sulfur-containing amino acids are not available, many micro-organisms possess metabolic pathways for the uptake and reduction of oxidized forms of sulfur, such as sulfate, into these compounds; this process is referred to as assimilatory sulfate reduction. Alternatively, sulfur may be either oxidized or reduced in a dissimilatory metabolic mode. Aerobic sulfur bacteria, e.g. *Thiobacillus* spp., derive energy from the oxidation of reduced sulfur compounds to sulfate. Anaerobic phototrophic bacteria use reduced sulfur compounds as electron donors, in place of water, thereby producing sulfate as opposed to molecular oxygen. These same phototrophs, as well as a variety of chemotrophs, utilize oxidized sulfur compounds as terminal electron acceptors for anaerobic respiratory metabolism. Additionally, several thermophilic species appear to derive a benefit from the reduction of elemental sulfur to hydrogen sulfide, probably through the removal of product inhibition by molecular hydrogen (Fiala and Stetter, 1986; Huber *et al.*, 1986). Thus, in a purely dissimilatory fashion, sulfur can serve either as an electron donor in aerobic metabolism or as an electron acceptor in anaerobic metabolism.

One of the aspects of dissimilatory sulfur metabolism, which makes it both interesting and difficult to understand, is the variety of compounds which can and do participate in this process. This is due, in part, to the number of different valence states that sulfur can assume. The compounds which can be used most widely in these energy-generating dissimilatory modes are: sulfate, sulfite, tetrathionate, trithionate, thiosulfate, elemental sulfur and sulfide. The higher polythionates and polysulfides are also important (Kelly, 1982; Then and Truper, 1983; Blumentals *et al.*, 1990). Typically, micro-organisms are not capable of utilizing the entire spectrum of sulfur species. For example, there is a distinction, although not completely hard and fast, between organisms able to reduce sulfate and those able to reduce only elemental sulfur (Pfennig and Widdel, 1982). *P. brockii* utilizes elemental sulfur as an electron acceptor; it has not been shown to be able to reduce sulfate, sulfite or thiosulfate (Schicho and Kelly, unpublished).

Sulfur in these compounds can serve as either a donor or as an acceptor of electrons but we will restrict further discussion to the dissimilatory reduction of sulfur compounds. The reduction of sulfate to sulfide potentially yields more energy than the reduction of sulfur to sulfide and this process has been studied extensively in several micro-organisms (for relevant reviews *see* Peck and Le Gall, 1982; Odom and Peck, 1984; Truper, 1984; Peck and Lissolo, 1988). The consensus pathway appears to be:

1. the activation of sulfate with ATP, catalyzed by ATP sulfurylase (EC 2.7.7.4);
2. the reduction of adenylylsulfate (APS) to sulfite, catalyzed by APS reductase (EC 1.8.99.2); and
3. the reduction of sulfite to sulfide, catalyzed by bisulfite reductase (EC 1.8.99.1).

$$SO_4^{2-} + 2H^+ + ATP \rightarrow APS + PP_i \quad (1)$$

$$APS + 2e^- \rightarrow AMP + SO_3^{2-} \quad (2)$$

$$HSO_3^- + 6e^- + 6H^+ \rightarrow HS^- + 3H_2O \quad (3)$$

Most sulfate-reducing bacteria are also capable of reducing thiosulfate and polythionates, but the pathways have not been firmly established. The accumulation of trithionate and thiosulfate, possible intermediates in sulfate reduction, has not been demonstrated *in vivo* (Peck and Le Gall, 1982). The growth of some sulfate-reducing bacteria by the reduction of elemental sulfur to sulfide has also been demonstrated (Biebl and Pfennig, 1977). The coupling of the reduction of colloidal sulfur with electrons from hydrogen, to oxidative phosphorylation has been demonstrated in membrane preparations from *Desulfovibrio gigas* (Fauque, Barton and Le Gall, 1980).

Sulfate-reducing bacteria have been shown to grow by utilizing various organic compounds (lactate, pyruvate, ethanol, formate) and/or hydrogen as electron donors with the above-mentioned sulfur compounds as electron acceptors. Since none of these electron donors has a sufficiently low redox potential to drive the reduction of sulfate to sulfite directly (Thauer, Jungermann and Decker, 1977), sulfate must be activated to APS before being reduced to sulfite. In fact, it has been suggested, on the basis of growth yield and enzymological data, that the reduction of sulfate to sulfite requires about 1–2 mole ATP per mole sulfate (Peck and Le Gall, 1982; Nethe-Jaenchen and Thauer, 1984). High levels of the enzyme inorganic pyrophosphatase (EC 3.6.1.1) in *Desulfovibrio* species, support this suggestion. The activation of sulfate to APS is a highly endergonic reaction, the standard free energy change of reaction ($\triangle G_0'$) is +45 kJ mol^{-1}. It has been suggested that the PP_i released in the activation of sulfate, is hydrolyzed by inorganic pyrophosphatase in order to 'pull' reaction (1) in the forward direction (Peck and Le Gall, 1982). This idea is supported by growth yield data for *Desulfovibrio vulgaris* growing on hydrogen and either sulfate, thiosulfate or sulfite; ATP yields calculated from these data suggest that only one ATP is formed per sulfate reduced, whereas three ATP are formed per thiosulfate or sulfite reduced (Badziong and Thauer, 1978; Nethe-Jaenchen and Thauer, 1984). Therefore, it appears that sulfite is energetically the central metabolite, with sulfate and thiosulfate merely sources of sulfite.

In examining the energetics of any metabolic pathway, we should consider the thermodynamics of the substrates involved in order to gain some understanding of the possible transformations and the amount of energy theoretically available. Hydrogen is an important electron donor in both sulfate and sulfur reduction. In fact, the intracellular production of hydrogen from organic substrates has been implicated as a key pathway of electron flow in *Desulfovibrio* species (Odom and Peck, 1981). According to the proposed mechanism, i.e. 'hydrogen cycling', intracellularly produced molecular hydrogen diffuses out through the cytoplasmic membrane where it is then oxidized by the periplasmic hydrogenase. The resultant electrons are transported inward for the reduction of sulfate and sulfite to sulfide while the protons generated in the periplasm are available for proton translocation, ATP synthesis or the transport of ions into or out of the cell. For the purposes

of the present discussion, the relevant redox couples are H^+/H_2 and HSO_3^-/HS^- or S^o/HS^-. The energy available through the transfer of electrons from one redox couple to another is related to the difference in mid-point potentials of the two couples. The differences, $\triangle E_0'$, for these two couples are respectively, 298 mV and 144 mV. Therefore, one would expect organisms reducing sulfite to be able to generate roughly twice as much ATP as those reducing S^o. One must always bear in mind that such calculations serve only as a guide to more detailed investigations because of the large number of variables involved. Such variables include physiological concentrations of the substrates and products, particular aspects of the electron carriers in the electron transport chain, requirements of protons for transport functions and multiple pathways for electron transport. Indeed, even the stoichiometry of the proton-translocating ATP synthetase of both prokaryotes and eukaryotes (F_1F_0 ATPase, EC 3.6.1.34) has been called into question recently (Thauer and Morris, 1984).

Far less has been reported concerning micro-organisms incapable of sulfate reduction but able to reduce other sulfur species in a dissimilatory manner. *Desulfuromonas acetoxidans* is such an organism and was described in 1976 by Pfennig and Biebl. It is unable to utilize sulfate, sulfite, thiosulfate, nitrate or oxygen as a terminal electron acceptor, nor can it grow fermentatively (Pfennig and Biebl, 1976). Shortly thereafter, another organism, called Spirillum 5175, was described as being capable of using sulfite, thiosulfate and elemental sulfur but not sulfate (Wolfe and Pfennig, 1977). Spirillum 5175 is also heterotrophic and incapable of fermentation. A *Campylobacter* species has been described as being able to reduce sulfite, thiosulfate and sulfur but not sulfate. The reduction of these sulfur compounds by this micro-organism was stoichiometrically linked to the oxidation of hydrogen (Laanbroek, Stal and Veldkamp, 1978). *Wolinella succinogenes* has been grown on formate and sulfur (Macy *et al.*, 1986). Recently, a moderately thermophilic sulfur-reducing eubacterium, *Desulfurella acetivorans*, growing by a metabolism similar to *Desulfuromonas acetoxidans* has been described (Bonch-Osmolovskaya *et al.*, 1990). A report of sulfur reduction by methanogenic bacteria may be misleading in terms of the physiology of this transformation, because in all cases some methane was produced in addition to hydrogen sulfide (Stetter and Gaag, 1983). As these authors discussed, the production of H_2S by methanogens may be a mechanism for eliminating O_2 toxicity rather than an energy-generating reaction. Thus among mesophilic and moderately thermophilic species, there have been a limited number of species described as sulfur-reducers, and they are all heterotrophic.

Before discussing further details of microbial sulfur reduction, a few comments on bioenergetics and growth yields are relevant. Any treatment of bioenergetics must eventually deal with the question of available free energy from the transformations thought to be important in the species under consideration. In this case, these reactions are the reduction of sulfate, sulfite and sulfur with hydrogen, and the reduction of sulfur with acetate or formate. For sulfate and sulfite reduction, the free energy changes at standard conditions ($\triangle G_0'$), are $-152{\cdot}2$ and $-171{\cdot}4$ kJ mol^{-1}, respectively (Thauer,

Jungermann and Decker, 1977). For the reduction of sulfur with hydrogen, acetate and formate, the $\Delta G_0'$s are –28·0, –16·7 and –37·2 kJ mol^{-1}, respectively (Thauer, Jungermann and Decker, 1977; Macy *et al.*, 1986). It is currently estimated that the phosphorylation of ADP at physiologically reasonable concentrations (assuming an approximate thermodynamic efficiency of energy conservation of 60–70%) requires about 69·0 kJ mol^{-1} (Thauer, 1988). Until recently, the fact that the $\Delta G_0'$ of a metabolic reaction was less than that currently thought to be required for phosphorylation of ADP (on a mole per mole basis) has caused considerable consternation, and rationalizations have been made involving the effect of physiological concentrations (Thauer, Jungermann and Decker, 1977). However, when considering the phosphorylation of ADP by electron-transport-linked mechanisms, it has become clear that this restriction is not valid (Thauer and Morris, 1984). Energizing the cytoplasmic membrane (in bacteria) by the production of a proton gradient is, of course, the means by which energy from these less exothermic reactions is summed up in order to drive the phosphorylation of ADP. As a result of these developments, fractional stoichiometric ATP gains must be considered valid and an explanation of metabolic reactions with a $\Delta G_0'$ less negative than 69·0 kJ mol^{-1} is no longer necessary. Thermodynamic calculations can still be useful for order of magnitude estimates and can be borne out by experimental determinations of growth yields. For example, growth yields of about 1–2 g(dry wt)/mol(electron acceptor) were found for the growth of *Desulfuromonas acetoxidans* on acetate and sulfur, whereas yields of 8 and 17 g(dry wt)/mol(electron acceptor) were found for *Desulfovibrio vulgaris* growing on hydrogen and sulfate or thiosulfate, respectively (Pfennig and Biebl, 1976; Badziong and Thauer, 1978).

The final step in this energy-generating metabolism is thought to be the reduction of elemental sulfur, presumably catalyzed by an enzyme complex which we will call the 'sulfur reductase'. To date there have been only a few reports of the purification of such an enzyme, one of which involved the purification of c_3-type cytochromes from several sulfate-reducing bacteria (Fauque, Herve and Le Gall, 1979). Three of these species, *Desulfovibrio desulfuricans* Norway 4, *Desulfovibrio gigas* and sulfate-reducing bacterium strain 9974, could grow using elemental sulfur as a terminal electron acceptor, while the fourth, *Desulfovibrio vulgaris* Hildenborough, could not (Biebl and Pfennig, 1977). The sulfur reductase activity of the cytochrome from *D. vulgaris* was shown to be inhibited by levels of sulfide at which the other species still maintained high activity and it was suggested that this result explained the inability of *D. vulgaris* to grow on elemental sulfur. The reduction of sulfur by hydrogen could be catalyzed *in vitro* with any of the cytochromes and purified hydrogenase from *D. gigas*; no other electron carriers were necessary (Fauque, Herve and Le Gall, 1979). Additionally, inside-out membrane vesicles from *D. gigas*, which contained hydrogenase and cytochrome c_3, were shown to be able to drive the phosphorylation of ADP while reducing colloidal sulfur and oxidizing hydrogen (Fauque, Barton and Le Gall, 1980).

A sulfur reductase from *Wolinella succinogenes* has also been purified

(Schröder, Kröger and Macy, 1988). The enzyme, which was isolated as sulfide dehydrogenase activity, had a MW of approximately 200 000 as estimated by density gradient centrifugation and gel filtration. It appeared to be dimeric in structure, since it had subunits of about 85 000 MW by sodium dodecylsulfate gel electrophoresis, and contained equal amounts of iron and sulfide but no heme moiety. The reductase was localized to the membrane fraction and the specific activity of this fraction could be induced by a factor of about 30 when the cells were grown on sulfur as opposed to fumarate. Electron transport from formate to sulfur could be reconstituted in liposomes prepared from soybean phospholipids with only the sulfur reductase and formate dehydrogenase. No other redox active compound was necessary. The sulfur reductase, which was purified by a single chromatofocusing step, was described as being unstable in other purification methods. Another interesting aspect of the work was the finding that colloidal sulfur prepared by the acidification of polysulfides was not as readily reduced by membrane preparations as sulfur that was recovered from the culture after growth.

Zöphel *et al.* (1988) screened several organisms for sulfur reductase activity with an eye toward picking a candidate for more detailed metabolic and enzymatic studies. Three species, *Desulfuromonas, Desulfovibrio baculatus* and the isolate 'Spirillum 5175', were considered. The Spirillum was found to have five- to tenfold higher specific activity in crude extracts than all the others tested. Unfortunately all these cultures were grown on fumarate instead of sulfur even though it had been found that the activity in the Spirillum could be induced by a factor of ten when grown on sulfur. The sulfur reductase activity of the Spirillum was localized to the membrane fraction and both a membrane-bound and a cytoplasmic hydrogenase were detected. As a part of this study, several different preparations of colloidal sulfur were tested for activity with membrane preparations from the Spirillum and significant differences were noted, in agreement with the report on *Wolinella* sulfur reductase (Schröder, Kröger and Macy, 1988).

Sulfur reduction by extremely thermophilic and hyperthermophilic bacteria

After this as prologue, we now turn to the consideration of sulfur reduction by micro-organisms isolated from geothermal environments in which highly reducing conditions are characteristic. Since the early 1980s a number of thermophilic, extremely thermophilic and hyperthermophilic bacteria, which apparently prefer to reduce elemental sulfur, have been isolated and described (Stetter, König and Stackebrandt, 1983; Zillig *et al.*, 1983a, b; Belkin and Jannasch, 1985; Fiala *et al.*, 1986; Stetter, 1986; Jannasch *et al.*, 1988a). A few of the isolates of the hyperthermophilic genus *Pyrobaculum* were found to be able to use sulfite and/or thiosulfate in addition to elemental sulfur as electron acceptors (Huber, Kristjansson and Stetter, 1987). The first extremely thermophilic sulfate-reducer, *Archaeoglobus fulgidus*, was isolated and described recently (Stetter *et al.*, 1987; Stetter, 1988). Like most of its mesophilic counterparts, it can use sulfate, sulfite and thiosulfate but not elemental sulfur as electron acceptors (Stetter *et al.*, 1987). In addition, two

genera of facultative organisms were described, *Desulfurolobus* and *Acidianus* (Segerer *et al.*, 1986; Zillig *et al.*, 1986). Species of these genera are capable of alternately oxidizing elemental sulfur under aerobic conditions, or reducing it under anaerobic conditions. Thus, it appears that, with a few exceptions, we may distinguish between those organisms able to reduce sulfate and sulfite and those only able to reduce elemental sulfur.

Of these high-temperature sulfur-reducing micro-organisms, only a few have been described as autotrophs; *Pyrodictium occultum* and *P. brockii*, species of the genus *Thermoproteus*, *Pyrobaculum islandicum* and the two facultative genera mentioned above (Stetter, König and Stackebrandt, 1983; Segerer *et al.*, 1986; Stetter, 1986; Zillig *et al.*, 1986; Huber, Kristjansson and Stetter, 1987). All the others have been described as obligate heterotrophs (*see Table 1*), but very little is actually known about their metabolisms. In most cases, the reduction of sulfur is a form of anaerobic respiration but in several cases another possibility has been raised. For *Thermodiscus* species, *Staphylothermus* species, *Pyrococcus furiosus* and *Thermotoga* species, optimal growth (rate and yield) can be obtained in the absence of sulfur if measures are taken to remove growth-associated hydrogen from the culture medium (Belkin, Wirsen and Jannasch, 1985; Fiala and Stetter, 1986; Huber *et al.*, 1986; Stetter, 1986; Jannasch *et al.*, 1988b; Malik *et al.*, 1989; Windberger *et al.*, 1989). The production of hydrogen from organic substrates suggests a fermentative type of metabolism and the accumulation of hydrogen becomes inhibitory to growth. However, these thermophilic species have apparently developed a detoxification mechanism in which hydrogen is either converted to H_2S or is not produced at all when sulfur is present (Blumentals *et al.*, 1990). This mechanism allows *P. furiosus* to grow below an atmosphere of pure hydrogen if sulfur is present (Malik *et al.*, 1989).

Hydrogen oxidation in microbial systems

Unlike dissimilatory sulfur metabolism, hydrogen utilization by micro-organisms is a widespread phenomenon. A detailed consideration of all known hydrogenases far exceeds the scope of this review. However, a brief look at a few representative types of hydrogen metabolism may prove useful for later discussion. Since our knowledge of hydrogen metabolism in *P. brockii* is limited, consideration of model systems may provide a useful framework to connect hydrogen–sulfur autotrophy to what is already known about hydrogen metabolism. In addition, these systems may provide some insights into the components and energy considerations of *P. brockii* hydrogen metabolism.

Hydrogenases are enzymes that catalyze the reversible reaction shown below:

$$H_2 \rightleftharpoons 2H^+ + 2e^- \qquad (4)$$

While this equation is simple, the roles hydrogenases play in the metabolism of many species of bacteria is more complicated. For example, hydrogenases are known to exist in strict anaerobes, facultative anaerobes, aerobic

Table 1. Representative sulfur-metabolizing thermophiles; selected sulfur-metabolizing extreme and hyperthermophiles are presented with brief descriptions of their metabolic capabilities; representatives of facultative aerobes, heterotrophs and autotrophs proliferating at elevated temperatures are included

Name, DSM No.	Metabolism	Carbon source	Temp. °C	pH	References
Acidianus infernus 3191	Aerobic, $S^\circ + O_2 \rightarrow SO_4^{2-}$ Anaerobic, $H_2 + S^\circ \rightarrow H_2S$	Obligate autotroph	65–95 opt.90	1·0–5·0 opt. 2·0	Segerer *et al.* (1986)
Archaeoglobus fulgidus 83 4304	Anaerobic, $H_2 + SO_4^{2-} \rightarrow H_2S$	Facultative autotroph	60–95	5·5–7·5	Stetter (1988) Stetter *et al.* (1987)
Desulfurolobus ambivalens 3772	Aerobic, $S^\circ + O_2 \rightarrow SO_4^{2-}$ Anaerobic, $H_2 + S^\circ \rightarrow H_2S$	Obligate autotroph	70–87 opt. 80	1·0–3·5	Zillig *et al.* (1986)
Pyrobaculum islandicum 4184	Anaerobic, $H_2 + S^\circ \rightarrow H_2S$	Facultative autotroph	74–102 opt. 100	5·0–7·0	Huber, Kristjansson and Stetter (1987)
Pyrobaculum organotrophum 3185	Anaerobic, S° Resp.	Obligate heterotroph	74–102 opt.100	5·0–7·0	Huber, Kristjansson and Stetter (1987)
Pyrococcus furiosus 3638	Anaerobic, S° Resp.? and Ferm.?	Obligate heterotroph	70–103 opt. 100	5·0–9·0 opt. 7·0	Fiala and Stetter (1986)
Pyrococcus woesei 3773	Anaerobic, S° Resp.	Obligate heterotroph	opt. 100	opt. 6·0	Zillig *et al.* (1987)
Pyrodictium brockii 2708	Anaerobic, $H_2 + S^\circ \rightarrow H_2S$	Obligate autotroph	80–110 opt. 105	5·0–7·0 opt. 5·5	Stetter, König and Stackebrandt (1983)

Pyrodictium occultum 2709	Anaerobic, $H_2 + S^\circ \rightarrow H_2S$	Obligate autotroph	80–110 opt. 105	5·0–7·0 opt. 5·5	Stetter, König and Stackebrandt (1983)
Staphylothermus marinus 3639 & 3666	Anaerobic, S° Resp.? and Ferm.?	Obligate heterotroph	65–98 opt. 92	4·5–8·5 opt. 6·5	Fiala *et al.* (1986)
Thermococcus celer 2476	Anaerobic, S° Resp. and Ferm.	Obligate heterotroph	opt. 88	opt. 5·8	Zillig *et al.* (1983b)
Thermococcus litoralis 5473 & 5474	Anaerobic, S° Resp.	Obligate heterotroph	55–98 opt. 88	6·0–8·5 opt. 7·2	Neuner *et al.* (1990) Belkin and Jannasch (1985)
Theromodiscus maritimus no DSM No.	Anaerobic, S° Resp. and Ferm.?	Obligate heterotroph	75–98 opt. 90	5·0–7·0 opt. 5·5	Fischer *et al.* (1983) Stetter (1986)
Thermofilum pendens 2475	Anaerobic, S° Resp.	Obligate heterotroph	opt. 85–90	4·0–6·5 opt. 5·2	Zillig *et al.* (1983a)
Thermoproteus neutrophilus 2338	Anaerobic, $H_2 + S^\circ \rightarrow H_2S$	Obligate autotroph	80–96 opt. 88	5·5–7·5	Fischer *et al.* (1983)
Thermoproteus tenax 2078	Anaerobic, $H_2 + S^\circ \rightarrow H_2S$	Facultative autotroph	80–96 opt. 88	2·5–6·0 opt. 5·5	Zillig *et al.* (1981)
Thermotoga maritima 3109	Anaerobic, Fermentative	Obligate heterotroph	55–90 opt. 80	5·5–9·0 opt. 6·5	Huber *et al.* (1986)
Thermotoga neapolitana 4359	Anaerobic, S° Resp.? and Ferm.?	Obligate heterotroph	55–90 opt. 80	5·5–9·0 opt. 7·0	Belkin, Wirsen and Jannasch (1986) Jannasch *et al.* (1998b)

Abbreviations: Resp, respiration; Ferm, fermentation; opt, optimal.

nitrogen-fixing bacteria, photosynthetic bacteria, cyanobacteria, methanogens and thermophiles, as well as in other groups.

Structurally, hydrogenases are somewhat diverse, but are all iron–sulfur proteins. In addition to the iron–sulfur clusters, many, but not all, hydrogenases contain nickel. This has led to a classification of hydrogenases based upon the metal content of the enzyme. Therefore, the hydrogenases are classified into two groups. First are the iron (Fe) hydrogenases, which have been found only in strict anaerobes. The Fe hydrogenases are generally involved in H_2 evolution. The second group of hydrogenases are the nickel–iron (NiFe) hydrogenases, and a selenium-containing subset known as the NiFeSe hydrogenases (Yamazaki, 1982). Despite the fact that this second class is a much more numerous group of enzymes than the Fe hydrogenases (Hausinger, 1987), the NiFe hydrogenases tend to be a fairly uniform group (Adams, 1990). Structurally, all NiFe hydrogenases have at least two subunits. These two subunits may form a 'core' NiFe hydrogenase, since there is a significant degree of homology between many of these hydrogenases (Kovacs *et al.*, 1989; Lorenz *et al.*, 1989; Reeve *et al.*, 1989). It is even possible to functionally substitute a NiFe hydrogenase between species; Tibelius and Yates (1989) have complemented *Azotobacter chroococcum* hydrogenase mutants with hydrogenase genes from *Bradyrhizobium japonicum*. The NiFe hydrogenases also tend to be membrane-bound, and are commonly involved in hydrogen uptake rather than in hydrogen evolution. In fact, many of the hydrogen-uptake-type of hydrogenases cannot be reversed. They do not produce significant amounts of H_2, even in the presence of low-potential electron donors.

In micro-organisms, hydrogenases are characteristically involved in a few fairly well-defined metabolic roles. The uptake-type hydrogenases oxidize H_2 to provide electrons either for reducing power or for energy production via an electron transport system. In aerobes, hydrogen-uptake enzymes may also function in concert with electron transport pathways to protect oxygen-sensitive enzymes from inactivation. The evolution hydrogenases reduce protons to oxidize reduced cellular metabolites such as NADH. Below, we shall briefly examine these roles in some of the better studied hydrogenase systems.

ANAEROBIC EUBACTERIA

In the obligate anaerobes, hydrogenase plays a role in substrate level phosphorylation (Adams, Mortenson and Chen, 1981). Fermentation of sugars results in an accumulation of excess reductant and, in order to maintain the oxidation/reduction cycle of the components of substrate-level phosphorylation, this excess must be eliminated. *Clostridium pasteurianum* is a representative for this sort of metabolism. Hydrogenase I of *C. pasteurianum* is a soluble Fe hydrogenase that reduces protons to H_2 while oxidizing cellular reducing equivalents such as NADH (Chen and Mortenson, 1974). This allows the cells to remove excess reductant while requiring only protons as the electron acceptor. *In vitro*, this enzyme is able to both evolve and

consume large amounts of H_2. Hydrogenase I, like most of the Fe hydrogenases, is very oxygen-labile, and therefore must be studied under strictly anaerobic conditions. *C. pasteurianum* has a second hydrogenase that seems to preferentially oxidize hydrogen *in vivo* (Adams and Mortenson, 1984). Like hydrogenase I, this enzyme is also a Fe hydrogenase. The physiological function of hydrogenase II is not clear.

It is unlikely that removal of excess reductant is important in microorganisms thriving by hydrogen–sulfur autotrophy. The lack of a soluble hydrogenase in *P. brockii* may be further evidence that this organism survives by an autotrophic mode of growth. However, other sulfur-utilizing thermophiles, such as *Pyrococcus furiosus*, may have a fermentative type of metabolism similar to that of *C. pasteurianum*.

The species of the genus *Desulfovibrio* also offers an interesting insight into the role hydrogenase plays in anaerobic cellular metabolism. *Desulfovibrio* are sulfate-reducing bacteria and, as such, their physiology may be especially relevant to the sulfur-utilizing thermophiles. Various species of *Desulfovibrio* contain both types of hydrogenases, NiFe and Fe. One major constraint on the metabolism of *Desulfovibrio* is that substrate level phosphorylation does not provide enough ATP to allow sulfate to be reduced (Odom and Peck, 1984). This means that *Desulfovibrio* must have other energy-generating mechanisms in addition to anaerobic fermentation. One of these mechanisms is to couple the oxidation of H_2 to the reduction of SO_4^{2-}. As mentioned previously, Odom and Peck (1984) have proposed a hydrogen cycling mechanism whereby hydrogen produced by the fermentation of organic substrates is subsequently consumed by a periplasmic hydrogen-uptake hydrogenase. The electrons liberated by the hydrogenase can then be used to reduce sulfate to sulfide, and the protons formed produce a proton gradient for the cell. This sort of mechanism could be important in naturally occurring consortiums, where one species contains a hydrogen generating mechanism, and another is dependent on the utilization of the produced hydrogen.

The example offered by *Desulfovibrio* is important. Although sulfate utilization seems to be exclusive of sulfur utilization, the hydrogen-dependent energy-generating mechanisms present in *Desulfovibrio* may be similar to those in *P. brockii*. The difference is that *Desulfovibrio* may be able to generate more ATP per mole of electrons than does *P. brockii* (*see* the discussion on dissimilatory sulfur metabolism). In addition, the autotrophs are probably not capable of fermenting sugars to form their own H_2. It must come from other, external sources.

AEROBIC NITROGEN-FIXING EUBACTERIA

Under nitrogen limiting or symbiotic conditions, members of this group of bacteria are able to carry out the following reaction:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + 16ADP + 16P_i + H_2 \qquad (5)$$

The nitrogen fixation reaction presents the bacteria with two basic problems.

First, H_2 is an unavoidable side-product of the reaction, and it is also an inhibitor of the nitrogenase enzyme. Secondly, this is a very energy expensive process, both in terms of ATP and the requirement for very low-potential electrons. If the evolved H_2 is not recovered, a significant loss of energy and reducing power occurs.

Two well-studied examples of the nitrogen-fixing bacteria are *Bradyrhizobium japonicum* and members of the genus *Azotobacter*. *B. japonicum* is interesting since it expresses nitrogenase and hydrogenase in symbiosis with soybean plants (*Glycine max*). *B. japonicum* infects the roots of soybeans and forms nodules. Inside these nodules, the bacteria differentiate into bacteroids. It is during this differentiation process that both the nitrogenase and hydrogenase genes are expressed (reviewed by O'Brian and Maier, 1988). By expressing a membrane-bound uptake hydrogenase, *B. japonicum* is able to keep H_2 concentrations below inhibitory levels as well as recover some of the energy and reducing power that would otherwise be lost. There is an additional advantage to having a hydrogen-oxidizing system present. Nitrogenase is an extremely oxygen-labile enzyme. *B. japonicum* bacteroids are largely protected from free O_2 by the presence of the O_2-binding protein, leghemoglobin, in the nodules. However, by respiring H_2 and using O_2 as the terminal electron acceptor, the hydrogenase system is able to offer additional protection against O_2 inactivation of nitrogenase (O'Brian and Maier, 1988, 1989).

The same benefits of hydrogenase seen in *B. japonicum* are also evident in the genus *Azotobacter*. *Azotobacter* are free-living soil bacteria that are capable of nitrogen fixation under nitrogen-limiting conditions. Since *Azotobacter* does not have the environment of the nodule to protect its nitrogenase from oxygen, it must develop other methods. One of the mechanisms it uses is O_2-dependent respiration (Yates *et al.*, 1980). *Azotobacter* has several terminal oxidases; however, not all are involved in hydrogen oxidation (Wong and Maier, 1984). By oxidizing the H_2 at very fast rates while using O_2 as a terminal electron acceptor, *Azotobacter* is able to keep its internal O_2 concentration to levels low enough to permit nitrogen fixation. *Azotobacter* is even capable of using H_2 as the energy source to permit transport and utilization of mannose (Wong and Maier, 1985; Maier and Prosser, 1988), thus allowing the bacteria to grow by the type of metabolism known as mixotrophy.

At first glance, the nitrogen-fixing bacteria do not appear to have much in common with *P. brockii*, but there are similarities that may be important. Under special conditions, *B. japonicum* is capable of expressing hydrogenase and nitrogenase *ex planta* (Maier *et al.*, 1978). The hydrogen-oxidizing system can be coupled to CO_2 fixation via ribulosebiphosphate carboxylase (Hanus, Maier and Evans, 1979; Simpson, Maier and Evans, 1979). This makes *B. japonicum* a facultative autotroph, even though it may never grow autotrophically in nature. This property makes *B. japonicum* an excellent mesophilic model system to compare to *P. brockii*. Also, it is interesting to note that the electron transport chain used by *Azotobacter* to keep its cytoplasm largely anaerobic is thought to produce little energy (Yates *et al.*, 1980), and *P.*

brockii also can uncouple hydrogen oxidation from energy production (Parameswaran *et al.*, 1988). *P. brockii* exhibits uncoupled sulfide production during late log phase and stationary phase for reasons which are not currently understood.

ARCHAEBACTERIA

Hydrogenases are among the few archaebacterial enzymes that have been well studied. This is particularly true among the methanogens. Methanogens have a novel growth mechanism that couples the oxidation of H_2 to the reduction of CO_2 to CH_4 (reviewed by Jones, Nagel and Whitman, 1987). These bacteria are also often capable of chemolithotrophic growth, requiring only H_2 and CO_2. These physiological properties are very suggestive of the presence of hydrogen-uptake hydrogenases, of which there are two fairly well-characterized types (Graf and Thauer, 1981; Fox *et al.*, 1987; Baron and Ferry, 1989; Fiebig and Friedrich, 1989; Reeve *et al.*, 1989). The first is the factor F420-reducing hydrogenase. This is a NiFe hydrogenase that reduces cofactor F420 involved in methanogenesis. The second type of hydrogenase is usually referred to as the methyl viologen-reducing (MV) hydrogenase (Jin, Blanchard and Chen, 1983). This name comes from the fact that the enzyme can reduce the dye methyl viologen, but cannot reduce factor F420 (F420-reducing hydrogenases can reduce both). The methyl viologen hydrogenase is also an uptake-type hydrogenase, although its current role is only poorly understood. Both of these hydrogenases are either soluble, or perhaps peripherally associated with the membrane (Baron, Brown and Ferry, 1987), and are NiFe hydrogenases.

The thermophilic archaebacteria are also known to contain hydrogenases, and hydrogen utilization and production by thermophiles has recently been the subject of a review by Adams (1990). Unfortunately, knowledge of hydrogen metabolism in thermophiles is still very scant. Only the hydrogenases from *Pyrococcus furiosus* and *Pyrodictium brockii* have been studied in any detail, and only the *P. furiosus* enzyme has thus far been purified. The *P. furiosus* hydrogenase seems to represent a new type of hydrogenase. Although reversible *in vitro*, this enzyme is thermodynamically primed to evolve hydrogen under physiological conditions (Bryant and Adams, 1989).

These are only a few of the systems that could provide a model for hydrogen utilization in *P. brockii*. It is possible that *P. brockii* incorporates features found in several of the bacteria discussed. Since hydrogen–sulfur autotrophy represents a novel form of metabolism, it may be derived from new metabolic functions and components, or it may provide a mix of features that have already been seen in other bacteria.

Growth physiology of *Pyrodictium brockii*

EFFECTS OF GAS PARTIAL PRESSURES

Early experiments in our laboratory on the growth of *Pyrodictium occultum* focused on the effects of temperature, gas partial pressure and the addition of yeast extract. In a series of experiments performed in static culture vials

under a gas phase of 80/20, H_2/CO_2 at four temperatures between 98 and 105°C, the effect of adding 0·2 g l^{-1} yeast extract on growth and hydrogen sulfide production was studied. It was found that at the lower temperatures, 98 and 100°C, the addition of yeast extract only slightly stimulated growth and gas production but that the effect was more pronounced at higher temperatures, 103·5 and 105°C (Parameswaran *et al.*, 1987). In these culture vial experiments, the absolute pressure was 4 atmospheres (atm.) and the vials were incubated without agitation (Stetter, König and Stackebrandt, 1983). Because of the low solubility of hydrogen in water and the quiescent culturing conditions, we suspected the possibility of a mass transfer limitation for hydrogen on the growth of *P. occultum*. A possible solution to a gas/liquid mass transfer problem, of course, is to increase gas mixing by agitation, but this did not appear to be an option because of cell fragility. Therefore, it was decided to investigate the effect of increased partial pressures on the system. Using an autoclave reactor described previously (Sturm *et al.*, 1987), it was found that, at 99°C, increasing the pressure of the standard gas mixture (80/20, H_2/CO_2) from 5 atm. to 7 or 9 atm. led to decreases in growth rate and final cell yield (Sturm *et al.*, 1987). Thus it appeared that, rather than being hydrogen-limited under 4–5 atm. of the gas mix, the cultures were closer to the onset of a toxic effect related to the hydrogen partial pressure.

At this point, emphasis was shifted from *P. occultum* to *P. brockii* because of the higher cell yields attainable with *P. brockii* in the presence of yeast extract. Maximal cell densities of less than 1×10^7 per ml are typical for *P. occultum* whereas *P. brockii* normally attains densities approaching 5×10^7 per ml (Parameswaran *et al.*, 1987, 1988). Because of our results for *P. occultum* on the relationship between increasing growth rate and cell yield and increasing gas partial pressures, we investigated the effects of lowered partial pressures of H_2 and CO_2. A series of experiments were undertaken in culture bottles grown quiescently at 98°C in sea-water-based medium with yeast extract at a concentration of 2 g l^{-1}. Bottles were pressurized with mixtures of hydrogen, carbon dioxide and helium to a total absolute pressure of 4 atm. It was found that hydrogen was not limiting to growth (rate or yield) at initial partial pressures of 0·32 atm. and above; the same was found for carbon dioxide at initial partial pressures of 0·04 atm. and above. At the same time, no growth could be observed in the cases where either H_2 or CO_2 was absent, with yeast extract present at a concentration of 2 g l^{-1}. Monod-type saturation parameters (K_s) in terms of partial pressures, of the two gaseous substrates were calculated from the data, the K_s for H_2 was 0·074 and for CO_2 was 0·0061 in atm. (Parameswaran *et al.*, 1988).

Interestingly, this study found that hydrogen sulfide production continued long after the stationary phase had been attained, as long as hydrogen and sulfur remained (Parameswaran *et al.*, 1988). This apparent uncoupling of the energy-generating metabolism from growth was also noted with *Thermoproteus* species growing at 88°C (Fischer *et al.*, 1983). From an engineering standpoint, such a metabolism represents a situation that could be exploited, since high rates of substrate conversion with low biomass yields are an advantage in anaerobic digestion of wastewater (Tempest and Neijssel, 1984).

In fact, we have investigated the use of hyperthermophiles for the desulfurization of coals, which is just such a situation where high biomass yields would be a disadvantage (Schicho *et al.*, 1989). There are many interesting possibilities to consider regarding the apparent uncoupling of the energy-producing pathway.

METABOLIC MODELLING

Microbial metabolism can be considered, on a macroscopic level, to be the conversion of energy and a carbon substrate into biomass. In an idealized case, a plot of the rate of substrate consumption, q, versus the rate of growth of biomass, μ, should be roughly linear, and following the development of S.J. Pirt (Pirt, 1982), this expression may be written:

$$q = \frac{\mu}{Y_G} + m. \tag{6}$$

Thus the slope of such a plot is the reciprocal of the maximal growth yield coefficient, Y_G and the intercept is the maintenance coefficient, m. The maintenance coefficient may be thought of as the substrate required to maintain the cells in a condition such that they are able to grow, and the yield coefficient is related to the substrate actually required for the increase in biomass. The ability of *Pyrodictium* to uncouple the production of H_2S from the synthesis of ATP would be reflected in a reduction of Y_G and will have to be taken into consideration. Various physiological functions have been postulated to contribute to the maintenance coefficient: turnover of macromolecules, motility, production of extracellular enzymes and maintenance of transmembrane ion gradients (Brannan and Caldwell, 1983; Tempest and Neijssel, 1984; Wallace and Holms, 1986).

The effect of temperature on both the maximal yield and maintenance coefficients of both mesophilic and thermophilic species has been investigated, and in several cases it was found that an increase in the maintenance coefficient was the major contributor to decreased cell yields for a given species at higher temperatures (Kuhn, Cometta and Fiechter, 1980; Esener, Roels and Kossen, 1983; Wallace and Holms, 1986). Analysis of the data for maintenance coefficient and temperature on Arrhenius plots:

$$\ln m = C\frac{E_a}{RT} \tag{7}$$

where C is a constant, E_a is the Arrhenius activation energy, R is the gas constant and T is the absolute temperature, allows one to calculate a macroscopic parameter, E_a, related to the effect of temperature on the growth efficiency of an organism. E_as were determined from plots of this type in the three studies just mentioned and were found to vary from a low of 37·7 to a high of 515 kJ mole^{-1} (energy substrate). In one study on *Escherichia*

coli, E_as were also calculated for protein, cell wall and phosphate turnover and were in the range of 44–115 kJ mole^{-1}, while the E_as for maintenance on several substrates were between 212 and 515 kJ mole^{-1} (Wallace and Holms, 1986). Wallace and Holms (1986) suggested that the predominant physiological cause of the increases in maintenance coefficient can be inferred from the magnitude of the activation energy found for maintenance, and concluded from their data that the increases in maintenance coefficient with temperature were not due to the increased turnover of the cellular components studied. At any rate, it appears that the temperature relationship of maintenance coefficients varies widely from species to species, and it will be interesting to see what trends develop as more data become available. Along these same lines, the suspicion that thermophiles would suffer from uniformly low cellular yields due to high maintenance requirements has not been confirmed in all cases (for example *see* Brannan and Caldwell, 1983; Lacis and Lawford, 1985). It remains to be seen what such analyses will reveal about *P. brockii*.

Continuous culture is an effective tool for the determination of metabolic parameters. A fermentation system consisting of a 5·5 liter glass vessel with a stainless steel, hemispherical heating jacket, was initially employed for such experiments. However, we had very little success growing *P. brockii* in this system, in contrast to the relative ease of culturing several other species in the same system. At the same time we had no trouble culturing *P. brockii* in an all-glass system, which has been described elsewhere (Brown and Kelly, 1989). Similar problems with the growth of thermophiles in stainless-steel-containing systems have been reported by other workers for other species (Sonnleitner, Cometta and Fiechter, 1982; Zillig *et al.*, 1983b, 1987). Various attempts to decrease the leaching rate of metal ions into the culture medium and the use of chelators to alleviate their toxicity met with limited success. *Pyrodictium brockii* seems to be uniquely sensitive to one or several of the components of stainless steel, at least among the five extremely thermophilic and hyperthermophilic species which we have grown in our fermentation systems. Experiments to characterize more fully this sensitivity are under way.

Currently, we are beginning to study the behavior of *P. brockii* in continuous culture under hydrogen-limited conditions in the all-glass culturing system mentioned above. The medium used was based on an artificial sea-water mixture supplemented with yeast extract, 2 g l^{-1} (Stetter, König and Stackebrandt, 1983). Elemental sulfur powder was added at least every 12 h at a ratio of 3 g l^{-1}, which should have been in excess with regard to growth. Hydrogen was limited by diluting the gas mix being sparged with nitrogen. Carbon dioxide was held constant at 20%, which is in large excess (Parameswaran *et al.*, 1988). Assuming that the nitrogen, sulfur and phosphorus sources were also not limiting, a reduction in the biomass concentration resulting from a reduction in the hydrogen concentration under these conditions should represent an energy-limited state. As can be seen from the data plotted in *Figure 1*, hydrogen was not limiting at a concentration of 40% for dilution rates up to 0·3 h^{-1}, but it was limiting at a concentration of 20% and dilution rate of 0·3 h^{-1}. Analysis of the effluent gas from the culture indicated the concentration of H_2 to be about 25% when the influent gas was

at 40% and to be about 10% when the sparged gas was at 20%. Thus the finding of a growth rate limitation when the H_2 partial pressure fell to about 0·1 atm. is in good agreement with our K_s for H_2 from batch growth data (*see* pp. 360). We are encouraged, by this preliminary result, that we will be able to quantify some aspects of the metabolism of *P. brockii*, including the determination of growth yield and maintenance coefficients.

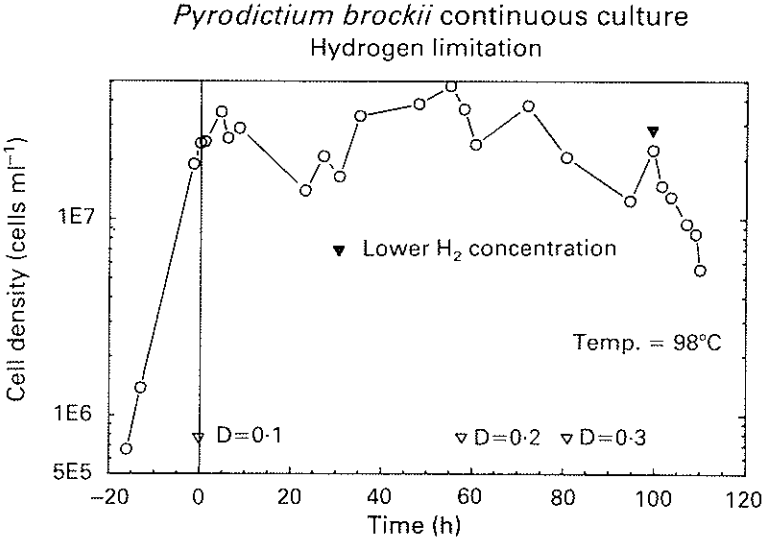

Figure 1. Cell densities are plotted versus time for a continuous culture of *P. brockii* at 98°C in complex medium with elemental sulfur powder. The vertical line indicates initiation of liquid flow, inverted open triangles indicate dilution rate changes, the inverted closed triangle indicates reduction of H_2 concentration in influent gas from 40% to 20%.

Enzymes involved in hydrogen–sulfur autotrophy in *P. brockii*

HYDROGENASE

As yet, there is only an incomplete understanding of the mechanism of hydrogen–sulfur autotrophy in *P. brockii* and comparisons with other autotrophic bacteria may provide insights as to what components serve as the basis of this system. Although *B. japonicum* is probably not a natural autotroph, it is capable of autotrophic growth if placed under the proper inducing conditions (Hanus, Maier and Evans, 1979). Much of our discussion will involve comparisons between the *P. brockii* system and the *B. japonicum* system. We have found that *B. japonicum* is, in fact, a good comparative model system for *P. brockii*, despite the differences in the growth modes and environments of the two bacteria.

The discovery that *P. brockii* was an obligate autotroph in an H_2-rich

atmosphere (Stetter, 1982; Stetter, König, and Stackebrandt, 1983; Parameswaran *et al.*, 1988) suggested that it had a hydrogen-uptake hydrogenase. Our interest in this enzyme stems from two observations:

1. *P. brockii* lives at temperatures far exceeding those of the most commonly studied hydrogenase-containing bacteria; and
2. *P. brockii* is only very distantly related to the most commonly studied bacteria with hydrogen-uptake hydrogenases.

Previous efforts in our lab have centered upon the hydrogen-uptake hydrogenase and the associated electron transport system from the mesophilic eubacteria *B. japonicum* and *A. vinelandii*. The study of a similar hydrogen-oxidizing system in *P. brockii* presents a unique opportunity to study hydrogen-uptake hydrogenases in terms of both thermostability and evolutionary relationships.

The structural information obtained so far on the *P. brockii* hydrogenase has been from immunological studies, genetic relatedness to mesophilic hydrogenases and measurements of the metal content of the enzyme. Immunological comparisons of various hydrogenases has produced some conflicting results. Kovacs *et al.* (1989) found that among several eubacteria, the large subunit of the uptake hydrogenases was more conserved than the small subunit. However, Lorenz *et al.* (1989) found that, at least between several species of *Alcaligenes* and *Paracoccus denitrificans*, the small subunit was the one more conserved. Within this context, it is interesting to note that antibodies raised against the large subunit of the *B. japonicum* hydrogenase (Stults, Moshiri and Maier, 1986) cross-react with *P. brockii* membranes while antibodies raised against the small subunit do not (Pihl *et al.*, 1989). In addition, DNA probes specific to the large subunit of the *B. japonicum* hydrogenase hybridize well to *P. brockii* genomic digests (Pihl *et al.*, 1989; Pihl, Green and Maier, unpublished observations). The degree of homology in the large subunit may be of some importance. Nickel has been strongly implicated in hydrogen activation in hydrogenases (reviewed by Lancaster, 1988). Recent evidence from the periplasmic (NiFeSe) hydrogenase of *Desulfovibrio baculatus* has shown that the selenium of selenocysteine is coordinated to nickel in the active site (Eidsness *et al.*, 1989; He *et al.*, 1989). Since the selenocysteine seems to be associated with the large subunit, this subunit probably contains the active site for hydrogenase. The metal content of the two hydrogenases also serves as a basis for comparison. The *B. japonicum* hydrogenase is known to be a NiFe hydrogenase (Arp and Burris, 1979; Stults, O'Hara and Maier, 1984). Although the exact metal content of the *P. brockii* enzyme is currently unknown, it does seem to contain at least iron (Pihl and Maier, unpublished observations). However, the exact amount of iron present, as well as the presence of other metals, has yet to be determined.

Basic biochemical characterization of the *P. brockii* hydrogen-uptake hydrogenase has revealed that it is fairly similar to the hydrogenase from *B. japonicum* (Pihl *et al.*, 1989). In terms of use of artificial electron acceptors and oxygen sensitivity, the enzymes from the two different organisms are

similar. For example, both the mesophilic *B. japonicum* and hyperthermophilic *P. brockii* membrane-associated enzymes couple H_2 oxidation to the reduction of many positive redox potential artificial electron acceptors. Negative potential electron acceptors function poorly, if at all. Addition of reduced low potential dyes, such as methyl or benzyl viologen, does not cause evolution of appreciable amounts of H_2 from either enzyme. These results suggest that the redox potentials of the two hydrogenases are similar. Also, the presence of oxygen will reversibly inhibit both enzymes (Mutaftschiev, O'Brien and Mayer, 1983; Pihl *et al.*, 1989). The major functional difference noted so far is that the *P. brockii* enzyme is capable of functioning at much higher temperatures than the *B. japonicum* enzyme. Hydrogenases, in general, tend to be fairly thermostable, and the *B. japonicum* hydrogenase is no exception. Despite the fact that the bacterium grows optimally at 29°C, the hydrogen-uptake hydrogenase does not reach maximal activity until 70°C. This, however, is a temperature that is still well below optimal for the *P. brockii* enzyme. Due to equipment limitations, the optimal temperature for *P. brockii* hydrogenase activity has not been determined. Hydrogen-uptake activity could be readily detected in *P. brockii* membranes assayed at 90°C even after incubation at 120°C, despite substantial loss of activity (Pihl and Maier, unpublished observation). Finally, both enzymes are membrane-bound. Despite the vast differences in membrane structure between eubacteria and archaebacteria (Goldfine and Langworthy, 1988), the enzymes can both be easily solubilized from their respective membranes with the detergent Triton X-100. The *P. brockii* enzyme can be solubilized with other detergents as well but, as is typical for membrane-bound proteins, not all detergents are effective (see *Table 2*).

Table 2. Solubilization of hydrogen-uptake activity from *P. brockii* membranes with various detergents; data given are the maximum percentages of starting hydrogen-uptake activity that was solubilized by the detergent; detergents were evaluated at concentrations of 0·01, 0·03, 0·1, 0·3, 1·0 and 3·0 (%w/v)

Detergent	Maximum solubilization (% of starting activity)
Octyl glucoside	18
CHAPS	8
Lubrol PX	46
Triton X-100	90
Brij 58	25
Zwittergent 314	32
Cholate	15
Tween 80	46

QUINONE

In hydrogen–sulfur autotrophy, it is assumed that electrons generated from hydrogen are passed down an electron transport chain, where ultimately sulfur is reduced to hydrogen sulfide. Membranes isolated from *P. brockii*

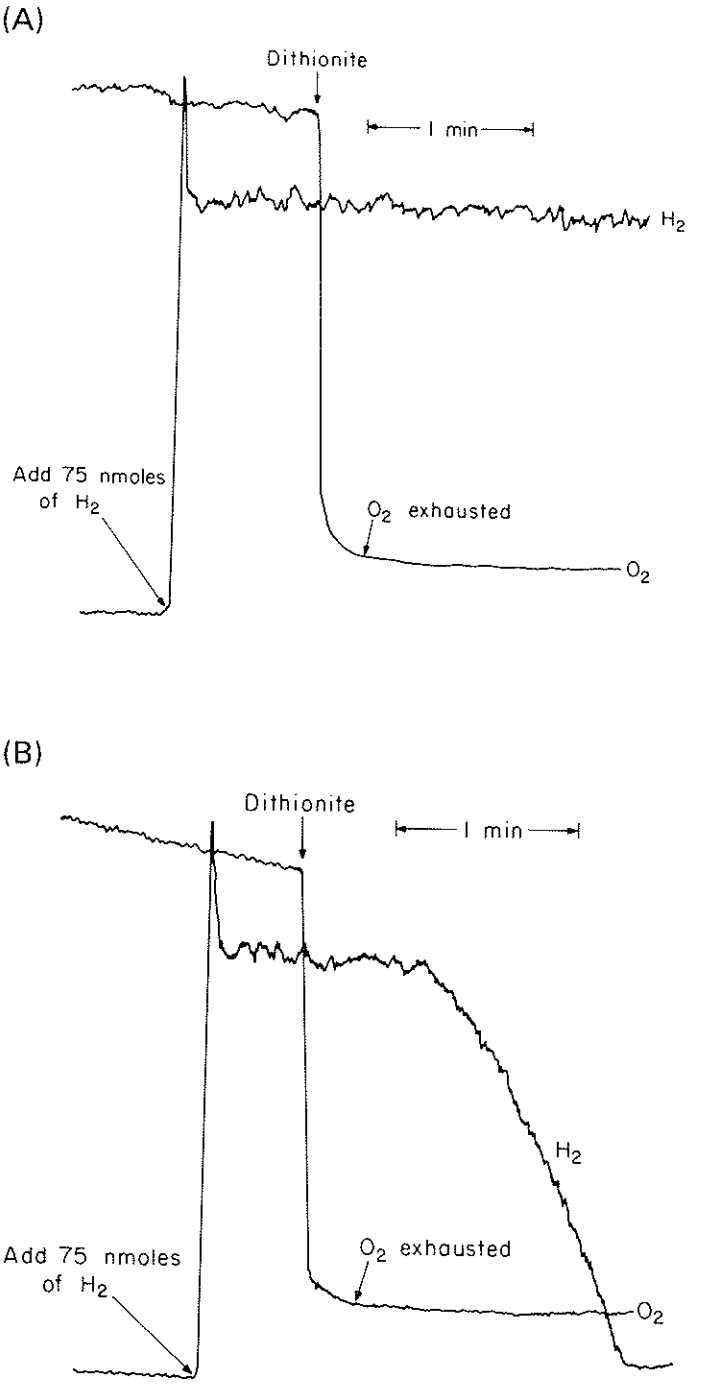

Figure 2. Strip chart recording showing sulfur-dependent hydrogen-uptake in *P. brockii* membranes. Membranes were isolated in 50 mM EPPS (pH 8·0) plus 100 mM NaCl. Hydrogen-uptake was measured amperometrically. (A) Hydrogen uptake in the absence of sulfur; (B) hydrogen uptake in the presence of sulfur.

retain the ability to oxidize hydrogen using sulfur as a terminal electron acceptor, as illustrated in *Figure 2*. Without S^o, the membranes were unable to oxidize H_2 (*Figure 2A*); however the addition of S^o permitted H_2 uptake (*Figure 2B*). This result suggests that all of the components necessary for H_2-dependent electron transport are present in the washed membrane preparations. Preliminary investigations into the components of electron transport between H_2 and S^o suggest that a quinone is involved. The hydrogen-dependent production of hydrogen sulfide by purified membranes can be inhibited by irradiation of the membranes with UV light (Pihl, Schulman and Maier, unpublished observation). Quinones are known to be inactivated by exposure to UV light, and H_2-dependent H_2S production could be restored in membranes by the addition of various quinones (Pihl, Schulman and Maier, unpublished observation). Efforts to purify electron transport components, and identify their order in the chain are currently under way.

CYTOCHROMES

Cytochromes *c* function as major electron carriers in electron transport systems of eukaryotes and prokaryotes. In addition to functioning as intermediate electron carriers between other cytochromes and iron–sulfur proteins, generally dehydrogenases, they sometimes act as terminal oxidases in oxygen-reducing reactions. Small size and stability, along with its widespread occurrence, have made cytochrome *c* an important model both biochemically, in studying protein structure, and genetically, in studying evolutionary relationships at the molecular level. These same properties make cytochrome *c* an excellent candidate for comparative studies on protein structure between mesophilic and thermophilic organisms.

We have found that a cytochrome is associated with *P. brockii* membranes and it can be solubilized from the membrane by use of detergent. Standard difference absorption spectral experiments to identify cytochromes of *P. brockii*-solubilized membranes gave a characteristic *c*-type spectrum with peaks at 553, 522 (*Figure 3*) and 421 nm with dithionite as reducing agent. A spectral shift, up several nm toward the red, has been observed when the cells are grown under certain culturing conditions. This interesting phenomenon is currently being investigated. Hydrogen uptake is associated with sulfur reduction in membranes of *P. brockii* (*see Figure 2*). Therefore, the entire sequence of electron transport components between hydrogen and hydrogen sulfide must be present in the membrane, and we postulate that this cytochrome is involved in the flow of electrons from hydrogenase to elemental sulfur.

The sulfate/sulfur reducing eubacteria, such as *Desulfovibrio*, contain unique *c*-type cytochromes (Odom and Peck, 1984). These cytochromes, which function in sulfate reduction, are multi-heme proteins and have relatively low redox potentials (Pettigrew and Moore, 1987). Cytochrome *c*-551·5 of *Desulfuromonas acetoxidans*, an anaerobic sulfur-reducing mesophilic eubacterium, falls under this classification (Probst *et al.*, 1977).

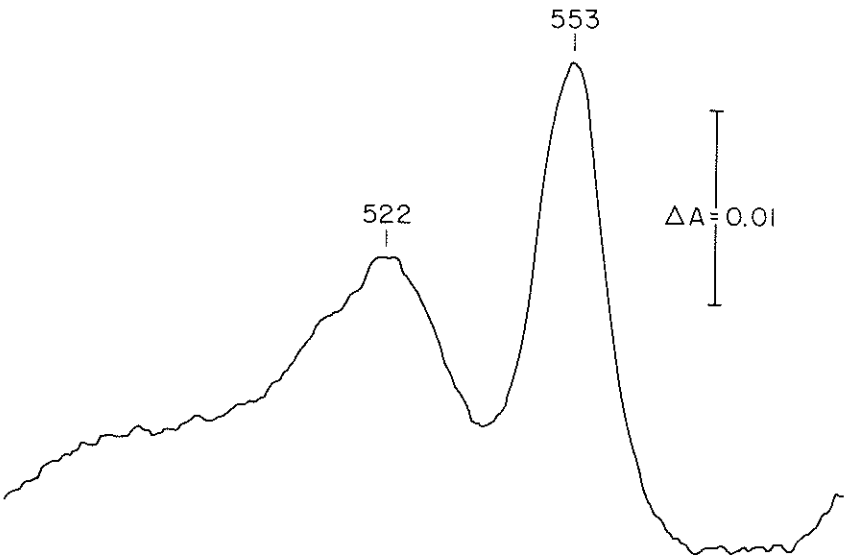

Figure 3. Sodium dithionite-reduced minus air-oxidized difference absorption spectrum of *P. brockii*. Membranes were solubilized in 0·5% Triton X-100 and difference spectra were performed on the supernatant (solubilized) fraction. A 1 ml fraction containing 0·8 mg protein in a 10 mm path length glass cuvette was scanned from 600 to 380 nm.

Comparison of this cytochrome with *P. brockii* cytochrome *c*-553 will provide information on thermophilic protein stability, and evolutionary relationships between thermophiles and mesophiles, as well as insight into sulfur metabolism differences between archaebacteria and eubacteria. Type-*c* cytochromes of thermophilic bacteria have been biochemically and genetically studied (Titani *et al.*, 1985; Pusheva *et al.*, 1988; Sanbongi *et al.*, 1989; Sone, Kutoh and Yanagita, 1989), but none of these organisms are sulfur reducers nor can they grow above 80°C.

SULFUR REDUCTASE ACTIVITY

As mentioned above, we have demonstrated the presence of sulfur reductase activity in membrane preparations from *P. brockii*. Because of the very low solubility of sulfur in water, the physico-chemical form of the sulfur which is actually taken up and reduced by the micro-organisms is uncertain. It is well known that sulfur exists in a number of allotropes, in the form of rings of sizes from S_6 to S_{20} and various different crystalline geometries (Steudel, 1982). The most stable allotrope at common physiological temperatures and pressures is the S_8 ring. At temperatures up to about 96°C, the S_8 ring exists as orthorhombic crystals with a melting point of 112°C; above 95°C the orthorhombic crystals slowly convert to monoclinic crystals which have a

melting point of 120°C (Steudel, 1982). It is also well known that the S_8 ring is subject to ring-opening reactions in aqueous solutions by nucleophilic attack by ions such as bisulfide, resulting in linear polysulfide chains (Pryor, 1962). The possible importance of such molecular species as substrates of sulfur reductases has been discussed by several groups (Cammack *et al.*, 1984; Zöphel *et al.*, 1988). Additionally, the importance of polysulfides in the reduction of sulfur by *Pyrococcus furiosus* has been studied (Blumentals *et al.*, 1990). Following this work, we have observed both growth and hydrogen sulfide production by *P. brockii* when physically separated from sulfur powder by a 6000–8000 MW cut-off dialysis membrane, which suggests that the actual substrate is a soluble species, such as polysulfide (Schicho and Kelly, unpublished).

Discussion

Research interest in thermophilic species spans many areas from basic science to technological application. The structures responsible for protein thermal stability are beginning to be elucidated, and the promise for protein engineering is immense. Progress in all aspects of the study of hyperthermophiles has been hampered by our limited abilities to produce biomass, maximal cell densities rarely exceed 2×10^8 cells ml^{-1}, roughly 0·5 g(wet wt) l^{-1}. Searches for the limiting nutrient or the accumulating toxic product have so far been largely unsuccessful. A better understanding of the metabolisms of these organisms will facilitate other studies and is an absolute prerequisite to industrial application. We have found continuous culture to be an effective tool both for production of biomass and metabolic study of hyperthermophiles. It is especially well suited to hyperthermophiles because of the very low possibility of contamination and the need to produce large volumes of grown culture in order to purify components of interest.

Efforts to determine metabolic parameters, yield and maintenance coefficients, for the growth of *Pyrodictium brockii* are currently under way. Assuming that technical difficulties can be surmounted, we plan to study the temperature relationships of yield and maintenance (along the lines of Wallace and Holms, 1986). It will be very interesting to see if trends develop within the different temperature ranges of growth. One must keep in mind that at temperatures of 100°C and above, it is not just the macromolecular components of a cell that are prone to thermal decomposition but smaller molecules as well, such as NADH, ATP and some amino acids (Bernhardt *et al.*, 1984; Stetter, 1986).

Research into the physiological aspects of hydrogen–sulfur autotrophy may help in determining the maintenance requirements for *P. brockii*. There are several curiosities about hydrogen metabolism in *P. brockii* that may provide clues to adaptation for autotrophic survival at high temperatures. Among these are the fact that archaebacteria other than *P. brockii* seem to lack integral membrane-bound hydrogenases. Since the *P. brockii* hydrogenase seems to interact with a quinone, a membrane-bound hydrogenase may be an integral part of hydrogen–sulfur autotrophy. Quinones often have long

lipid-like side-chains and are only sparingly soluble in water. If these molecules are an integral part of hydrogen–sulfur autotrophy, a membrane-bound hydrogenase may be a requirement. For the most part, archaebacterial hydrogenases are purified from the soluble cellular fraction. In contrast, eubacterial uptake-type hydrogenases are often integral membrane proteins. It should be noted that there is good evidence for the F420 and MV hydrogenases of methanogens being loosely associated with the membrane, but these do not appear to be integral membrane proteins (Baron, Brown and Ferry, 1987; Muth, 1988; Baron and Ferry, 1989). It is established that the hydrogen evolution hydrogenase of *Pyrococcus furiosus* is soluble (Bryant and Adams, 1989). No hydrogenase activity seems to be exclusively associated with the membranes in any archaebacterium except *P. brockii* (Pihl *et al.*, 1989). Whether or not this simply reflects a lack of data on other archaebacteria remains to be seen.

An interesting point, originally made by Adams (1990), is that despite the prevalence of coupling H_2 oxidation to O_2 reduction in the eubacteria, none of the extreme or hyperthermophiles are capable of using O_2 as a terminal electron acceptor in hydrogen metabolism. For hyperthermophiles, this may simply stem from the fact that their native environment is very reducing, and O_2 may exist only transiently. However, some extremely thermophilic organisms such as *Sulfolobus* are obligate aerobes. It seems curious that no extreme thermophile has been isolated which can use O_2 as a terminal electron acceptor in hydrogen metabolism. Perhaps, sulfur plays some unique and as yet unidentified role in thermophilic autotrophy.

One aspect that has been unexplored in *P. brockii* is the effect of pressure upon its energy-generating system. In *Methanococcus jannaschii*, it has been shown that elevated pressure enhances the stability of enzymes involved in methanogenesis (Miller *et al.*, 1988). *M. jannaschii* was isolated at depths much greater than *P. brockii* (Clark and Kelly, 1990) so the effects of pressure may be more pronounced. However, the possibility that enzymatic and transport reactions in *P. brockii* may occur with greater efficiency at elevated pressures should be considered. Developments in engineering may make such studies possible. Recently, Miller *et al.* (1989) described a high-pressure bioreactor that would be very useful in studying the effects of pressure on hydrogen metabolism.

As is evident from the material presented here, there is still much to be done to elucidate the role of hydrogen oxidation and sulfur reduction in the energetics of *P. brockii*. It remains to be seen whether the reduction of sulfur represents a uniquely adapted metabolism for micro-organisms in high-temperature niches. However, the absence of the complications of heterotrophy in *P. brockii* makes it, and related bacteria, attractive systems for understanding life at elevated temperatures.

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