

Characterization of hydrogen-uptake activity in the hyperthermophile *Pyrodictium brockii*

(hydrogenase/*Bradyrhizobium japonicum*/archaeobacteria)

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ABSTRACT *Pyrodictium brockii* is a hyperthermophilic archaeobacterium with an optimal growth temperature of 105°C. *P. brockii* is also a chemolithotroph, requiring H₂ and CO₂ for growth. We have characterized *P. brockii* hydrogen-uptake activity with regard to temperature, ability to couple hydrogen oxidation to artificial electron acceptor reduction, sensitivity to O₂, and cellular localization. The hydrogen-uptake activity was localized predominantly in a particulate fraction, was reversibly inhibited by O₂, and coupled H₂ uptake to the reduction of positive potential artificial electron acceptors. Comparisons between these results and those of the well-studied hydrogen-uptake hydrogenase from the mesophile *Bradyrhizobium japonicum* showed the two enzymes to be similar despite the very different natural environments of the organisms. However, the optimum temperature for activity differed greatly in the two organisms. We have also used immunological and genetic probes specific to the 65-kDa subunit of *B. japonicum* hydrogenase to assay crude extracts and genomic DNA, respectively, from *P. brockii* and found the enzymes to be similar in these respects as well. In addition, we report a formulation for artificial seawater capable of sustaining the growth of *P. brockii*.

The discovery of microorganisms capable of growth at extreme thermal conditions has fostered an expanding interest in the effects of temperature on biological systems (1). In addition to several important applications that have been considered for organisms and their associated biomolecules at elevated temperatures (1, 2), a variety of fundamental scientific questions have arisen concerning the biochemical basis for life under extreme conditions. Understanding the intrinsic nature of enzyme thermostability at high temperatures, for example, may be a key to gaining further insight into several issues related to protein structure and function. By comparing enzymes obtained from organisms growing at high temperatures with their mesophilic counterparts, the subtleties of protein thermostability should become clearer.

Pyrodictium brockii is a hyperthermophilic (grows above 100°C) sulfur-metabolizing archaeobacterium with a reported optimal growth temperature of 105°C (3, 4). To date, this is the highest reported optimal growth temperature for an organism grown in pure culture. *P. brockii* is a chemolithotroph, fixing CO₂ for cell mass and oxidizing H₂ for energy generation (5). The necessity of H₂ for growth and metabolism suggests that a hydrogen-uptake hydrogenase is present in *P. brockii*.

The work described here involved a comparison between the hydrogen-uptake system of *P. brockii* with that of the mesophilic bacterium *Bradyrhizobium japonicum*. Among the issues addressed were optimal temperature for hydrogen uptake, ability to use artificial electron acceptors, sensitivity

to oxygen, and cellular localization. Furthermore, the hydrogenases from the two bacteria were compared on a structural (immunological) and genetic (DNA) level. In addition, we describe a formulation for artificial seawater capable of sustaining the growth of *P. brockii*.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals were of reagent grade or better and were obtained from Sigma, Alfa Chemical (Danvers, MA), or J. T. Baker Chemical (Phillipsburg, NJ). Horseradish peroxidase-conjugated goat anti-rabbit antibodies and all enzymes for DNA manipulation were purchased from Boehringer Mannheim. Enzymes and reagents used in nick-translation were obtained from Bethesda Research Laboratories. [α -³²P]dCTP was purchased from New England Nuclear. SeaKem agarose was obtained from FMC (Rockland, ME). Nitrocellulose was purchased from Schleicher & Schuell (Keene, NH). All gases were purchased from Potomac Airgas (Baltimore, MD).

Bacterial Strains. All experiments using *P. brockii* were done with strain DSM 2708, originally obtained from Deutsche Sammlung von Mikroorganismen (Göttingen, F.R.G.) *B. japonicum* strain SR473 has been described (6).

Media and Growth Conditions. The growth conditions described for *P. brockii* (5) were followed with some modifications. Instead of using Chesapeake Bay water for growth, an artificial seawater (ASW) was used. ASW was a composite of three separate solutions. The first solution was mixed in 500 ml of deionized H₂O and contained 23.9 g of NaCl, 4.0 g of Na₂SO₄, 0.7 g of KCl, 0.2 g of NaHCO₃, 0.1 g of KBr, 30 mg of H₃BO₃, and 3.0 mg of NaF. The second solution was mixed in 455 ml of deionized H₂O and contained 10.8 g of MgCl₂·6H₂O, 1.5 g of CaCl₂·2H₂O, and 25.0 mg of SrCl₂·6H₂O. These two solutions were slowly combined while stirring. To this was added 5 ml of resazurin (2 mg/ml), 10 ml of a 100× trace elements stock, and 20 ml of 50× additional salts stock. The 100× trace element solution consisted of (per liter) 1.5 g of nitroacetic acid, 3.0 g of MgSO₄·7H₂O, 1.0 g of NaCl, 0.1 g of FeSO₄, 0.1 g of CoSO₄, 0.1 g of CaCl₂, 0.1 g of ZnSO₄, 10 mg of CuSO₄, 10 mg of KAl(SO₄)₂, 10 mg of H₃BO₄, 10 mg of Na₂MoO₄·2H₂O, and 25 mg of NiCl₂·6H₂O (pH 7.0). The 50× additional salts stock contained (per liter) 12.5 g of NH₄Cl, 7.0 g of K₂HPO₄, and 50 g of sodium acetate. Once the medium was mixed, yeast extract at 2 g/liter was added and the solution was brought to pH 5.7 with HCl (to prevent precipitation) and then autoclaved.

P. brockii was grown by placing 100 ml of ASW in a 150-ml serum bottle. To this was added 0.4 g of precipitated, purified sulfur (Alfa Chemical) and 0.1 M sulfuric acid. The appropriate amount of 0.1 M sulfuric acid was determined by titration of an aliquot of ASW plus Na₂S to pH 5.5. This titration was repeated for each batch of medium. The medium

Abbreviation: ASW, artificial seawater.

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was then placed in a 98°C oil bath and heated for ≈15 min. After heating, the medium was sparged for 5 min with a gas mixture of 20% CO₂/80% H₂. Four milliliters of 2.5% (wt/vol) Na₂S was then added and the bottles were sparged for an additional minute. The bottles were then sealed with a rubber serum stopper and aluminum cap, and 2 ml of a previously grown culture were added. The bottles were then pressurized to 40 psi (1 psi = 6.9 kPa) with the 20% CO₂/80% H₂ gas mixture and placed back in the oil bath. Cells were allowed to grow under quiescent conditions for ≈24 hr. Cells typically reached a concentration of 10⁷ cells per ml as determined by epifluorescence microscopy (5). *B. japonicum* was grown in modified Bergersen's medium as described (7).

Crude Extract Preparation. Crude extracts from *P. Brockii* were prepared by first passing the cell culture through a glass Millipore filter support (without a filter) to remove the large sulfur particles. The cells were then harvested at 12,000 × *g* for 10 min. The pellet was washed twice with 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl and resuspended in the same buffer. Typically, the cells from 1 liter of growth medium were resuspended in a final volume of 3 ml. The cells were then rapidly frozen in a dry ice/ethanol bath. Samples were occasionally stored at -80°C overnight after the rapid freeze. Upon thawing, the cells were homogenized by using an ice-cold ground-glass homogenizer, and then broken by a single pass through a French press at 20,000 psi. This crude extract was subsequently kept on ice. Crude extracts from *B. japonicum* were prepared in the same manner. Protein concentrations were determined by the BCA assay (Pierce) using bovine serum albumin as a standard.

Anaerobic preparations were made by a slightly modified version of the above procedure. Cells were removed from the growth vials by using a N₂-sparged syringe and were placed in sealed centrifuge tubes under a constant stream of N₂. Cells were washed and resuspended in the same phosphate buffer as before only with the addition of 2 mM sodium dithionite. The resuspended cells were not frozen, but were broken with one pass through the French press. Prior to breaking, the pressure cell was thoroughly sparged with N₂. The broken cells were collected under a continuous stream of N₂ and subsequently stored in a sealed serum bottle under N₂.

Cell Fractionation. Sedimentable and soluble fractions were separated by centrifugation of the crude extract at 12,000 × *g* for 10 min. The pellet and supernatant from this step are referred to as P1 and S1, respectively. Fraction S1 was subsequently centrifuged at 100,000 × *g* for 2 hr. The pellet and supernatant from this step are referred to as P2 and S2.

Hydrogenase Assay. Hydrogen-uptake activity was determined amperometrically as described (8–10). The temperatures were maintained by connecting a circulating water bath to the electrode's water jacket. All assays were done in 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl. The buffer was kept at the assay temperature to prevent bubble formation in the electrode chamber. All electron acceptor and O₂ inhibition studies were done at 80°C.

NaDodSO₄/PAGE. NaDodSO₄/PAGE was done on 10% polyacrylamide gels according to the method of Laemmli (11).

Immunoblotting and Antigen Detection. The procedures followed for Western blots and the antibodies used have been described (12).

DNA Isolation and Detection. *P. Brockii* genomic DNA was isolated according to standard procedures (13). Restriction digests, gel electrophoresis, transfer to nitrocellulose membranes, and probe preparation were all done according to standard procedures (14, 15). *P. Brockii* genomic digests were probed with ³²P-labeled nick-translated pKM-15, a probe containing 1.2 kilobases of *B. japonicum* DNA that is specific for the 65-kDa subunit of the *B. japonicum* hydrogenase. pKM-15 was isolated from a *B. japonicum* gene bank in λgt11

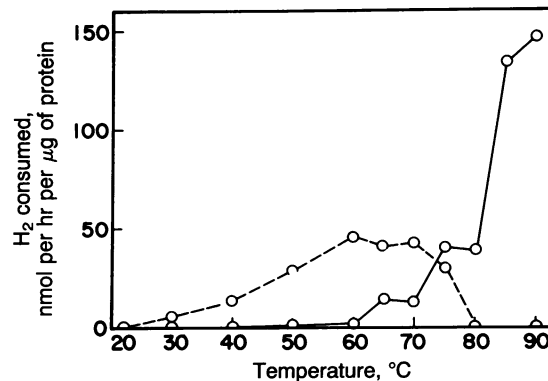


FIG. 1. Temperature profile for hydrogen-uptake activity in crude extracts from *P. Brockii* (solid line) and *B. japonicum* (dashed line). The specific activities shown are determined from methylene blue (200 μM)-dependent H₂-uptake rates in the absence of O₂.

by cross-reaction with antibodies specific for the 65-kDa subunit of *B. japonicum* hydrogenase.[§] Hybridization was carried out for 20 hr at 32°C in the presence of 50% (vol/vol) formamide. The filter was then washed twice in 2× SSC at room temperature (13).

RESULTS

Most of the following results on *P. Brockii* hydrogenase are presented in comparison with the mesophilic bacterium *B. japonicum*. *B. japonicum* is a soil bacterium that can live symbiotically with soybeans (*Glycine max*), and strain SR473 is a mutant that is constitutive for the expression of the normally repressed hydrogen-uptake hydrogenase (6). The optimal growth temperature for *B. japonicum* is ≈28°C.

Temperature Curve. One of the more interesting questions that can be asked about the *P. Brockii* hydrogenase is how thermostable is it? To address this question, we conducted hydrogen-uptake experiments at various temperatures for extracts of both *P. Brockii* and *B. japonicum*. The results are presented in Fig. 1. The *P. Brockii* extract showed little hydrogen-uptake activity below 60°C. Interestingly, the hydrogen-uptake activity for the *B. japonicum* hydrogenase was maximal at 60°C but was completely undetectable by 80°C, a point at which *P. Brockii* hydrogenase functioned well. Temperatures above 90°C were attempted; however, due to equipment limitations and technical difficulties, no reliable data were obtained.

Artificial Electron Acceptors. Table 1 shows a comparison between *P. Brockii* and *B. japonicum* membrane-containing extracts of their ability to couple H₂ oxidation to artificial electron acceptor reduction. In general, electron acceptors with a positive potential worked well with the *P. Brockii* hydrogen-uptake hydrogenase, while those with a negative potential worked poorly or not at all. The results indicate that the *P. Brockii* enzyme, like the *B. japonicum* hydrogenase, functions at a relatively high oxidation-reduction potential. When *P. Brockii* crude extracts were exposed to reduced methyl viologen and reduced benzyl viologen, no hydrogen evolution was seen (data not shown). These results suggest that *P. Brockii* has only hydrogen-uptake hydrogenase activity.

Oxygen Stability. The hydrogen-uptake hydrogenase of *B. japonicum* is a fairly oxygen-stable enzyme (16, 17). Similarly, we found that the *P. Brockii* hydrogen-uptake hydrogenase is also fairly oxygen stable (Fig. 2). In the presence of ≈125 nmol of oxygen, the *P. Brockii* hydrogenase had no

[§]Maier, R. J., Kim, H., Moshiri, F. & Novak, P. D., Second International Symposium on the Molecular Biology of Hydrogenase, Sept. 18, 1988, Helen, GA.

Table 1. *P. Brockii* and *B. japonicum* hydrogenase activity with various electron acceptors

| Electron acceptor | E_0' , mV | Relative activity, % | |
|--------------------------------------|-------------|----------------------|---------------------|
| | | <i>P. Brockii</i> | <i>B. japonicum</i> |
| Methylene blue (200 μ M) | 11 | 100 | 100 |
| Phenazine methosulfate (400 μ M) | 80 | 40 | 182 |
| Ferricyanide (1.5 mM) | 360 | 30 | 141 |
| DCIP (200 μ M) | 217 | 55 | 53 |
| Cytochrome <i>c</i> (100 μ M) | 250 | 6 | 18 |
| FMN (1 mM) | -122 | 2 | <1 |
| Benzyl viologen (1 mM) | -360 | 5 | <1 |
| Methyl viologen (1 mM) | -440 | 2 | <1 |

Relative activity is activity relative to methylene blue by membrane-containing fractions. The data from *B. japonicum* have been published (16) and are presented only for comparison. DCIP, dichloroindophenol.

activity, although it appeared to be capable of some function in the presence of very small amounts of oxygen. Upon the removal of all oxygen (see Fig. 2), the enzyme was able to restore full activity. It should be noted that the restoration of full activity was not possible in all preparations. This may be due to an increased oxygen lability in the presence of excess dithionite in the reaction chamber. At no time, however, was oxygen able to destroy irreversibly all of the hydrogen-uptake activity of *P. Brockii* crude extracts. This suggests that *P. Brockii* hydrogenase, like *B. japonicum* hydrogenase (16), is reversibly inhibited by oxygen.

P. Brockii crude extracts prepared anaerobically did not differ significantly in their hydrogen-uptake activity from that of aerobically prepared crude extracts; specific activities of *P. Brockii* crude extracts at 80°C averaged ≈ 150 nmol of H_2 consumed per hr per μ g of protein, and anaerobically prepared crude extracts averaged ≈ 170 nmol of H_2 consumed per hr per μ g of protein. Like the aerobically prepared extracts, the anaerobically prepared crude extracts were not able to evolve hydrogen in the presence of either reduced methyl viologen or reduced benzyl viologen (data not shown).

Cell Fractionation. To localize the hydrogen-uptake activity in *P. Brockii*, the cells were fractionated into sedimentable

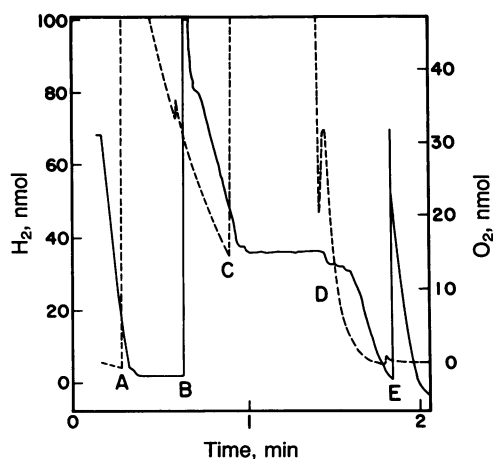


FIG. 2. Amperometric dual-pen chart recording showing the effect of O_2 on methylene blue-dependent H_2 -uptake activity of *P. Brockii* crude extracts. H_2 levels are shown by the solid line and O_2 levels are shown by the dashed line. The specific activity before point A was 220 nmol of H_2 consumed per hr per μ g of protein. At point A, 62 nmol of O_2 was injected followed by 74.5 nmol of H_2 at point B. The rate prior to point C was 79 nmol of H_2 consumed per hr per μ g of protein. At point C, an additional 125 nmol of O_2 was added. At point D, 800 nmol of sodium dithionite was added. At point E, 74.5 nmol of H_2 was again added. The specific activity at this point was 182 nmol of H_2 consumed per hr per μ g of protein.

Table 2. Fractionation of *P. Brockii* crude extracts

| Sample | Specific activity | Total protein, μ g | Total activity, $\times 10^{-3}$ | % of total crude activity | Fold purification |
|--------|-------------------|------------------------|----------------------------------|---------------------------|-------------------|
| Crude | 137 | 7870 | 1077 | 100 | 1.0 |
| S1 | 1195 | 896 | 1070 | 99 | 8.7 |
| P1 | 110 | 4800 | 529 | 49 | 0.8 |
| S2 | 403 | 96 | 39 | 4 | 3.0 |
| P2 | 1238 | 804 | 995 | 93 | 9.1 |

Specific activity is nmol of H_2 consumed per hr per μ g of protein and is based on methylene blue-dependent H_2 -uptake rates in the absence of O_2 . Total activity is specific activity multiplied by total protein. Fold purification is based on specific activity.

and soluble fractions (Table 2). Fraction P1 consisted of high molecular weight material, including unbroken cells. Fraction S1 probably consisted of subcellular components, such as membrane vesicles and soluble materials. Fraction P2 contained sedimentable material (probably membranes) whereas the soluble material most likely remains in fraction S2. The data indicate that most of the hydrogen-uptake activity remained in the sedimentable fraction, with a yield of 93% and a 9.1-fold purification compared to the crude extract. Although we have not definitively shown that fraction P2 is composed predominantly of membranes, the analogous *B. japonicum* fraction does contain mostly membranes (9). This localization of hydrogen-uptake activity in a sedimentable membrane-containing fraction is consistent with the idea of a membrane bound hydrogenase, since H_2 -uptake hydrogenases are typically membrane associated (18). It is interesting that despite preparation under aerobic conditions, fraction P2, the sedimentable fraction, contains 93% of the original activity.

Immunoblotting. Crude extracts from *P. Brockii* were subjected to Western blot analysis to see if they were immunologically similar to *B. japonicum* hydrogenase. Blots using antibodies raised against the 65-kDa subunit of *B. japonicum* hydrogenase (12) did cross-react with crude extracts from *P. Brockii* (Fig. 3A). The reaction was weak, but reproducible. The cross-reactive material seemed to be slightly larger than the 65-kDa subunit of *B. japonicum* hydrogenase. Antibodies raised against the 33-kDa subunit of

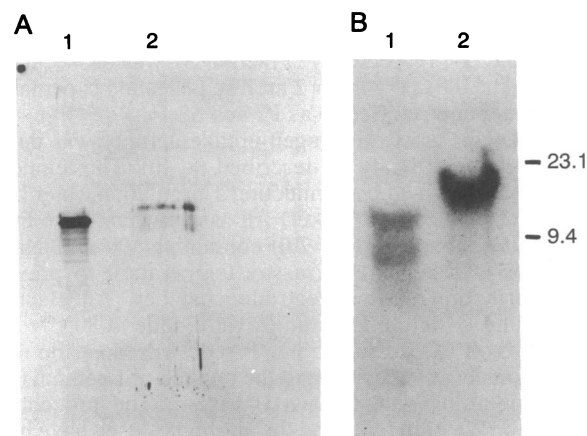


FIG. 3. Western and Southern blots of *P. Brockii*. (A) Western blot of *P. Brockii* crude extract. Lanes: 1, purified *B. japonicum* hydrogenase; 2, *P. Brockii* crude extracts. Both lanes were probed with antibodies specific for the 65-kDa subunit of *B. japonicum* H_2 -uptake hydrogenase. (B) Southern blot of *P. Brockii* genomic DNA digests. Lanes: 1, *P. Brockii* genomic DNA digested with *EcoRI*; 2, *P. Brockii* genomic DNA digested with *HindIII*. These lanes were probed with a 1.2-kilobase fragment of *B. japonicum* DNA specific for the 65-kDa subunit of the H_2 -uptake hydrogenase. DNA sizes are from λ digested with *HindIII* and are given in kilobase pairs.

B. japonicum hydrogenase did not cross-react with *P. Brockii* crude extracts (data not shown).

Southern Analysis. Since the two proteins appeared to be similar on a functional and structural level, comparison at the DNA level was an obvious step. Hybridizing *P. Brockii* genomic DNA digests with a probe (pKM-15) specific to the *B. japonicum* hydrogenase large subunit revealed that the two enzymes are somewhat homologous at the DNA level as well as at the structural level (Fig. 3B).

DISCUSSION

Despite the vast differences in the ecology and physiology of the two microorganisms, the hydrogen-uptake hydrogenases of *P. Brockii* and *B. japonicum* are rather similar in many ways. The two activities are similar in localization (sedimentable), electron acceptor specificity, and inability to evolve appreciable amounts of H₂. In addition, both the enzymes are reversibly inhibited by O₂, and full activity is obtained in aerobically prepared samples. The relationship of the *P. Brockii* enzyme to O₂ is similar to that of many H₂-uptake hydrogenases (19–21) but contrasts that of the strict anaerobe *Clostridium pasteurianum* (22).

As with the *B. japonicum* hydrogenase, the *P. Brockii* hydrogenase is sedimentable, thus it is associated with the particulate material. However, the mechanism by which *P. Brockii* coordinates the uptake of hydrogen to generate energy for growth and metabolism is not yet clear. H₂-uptake hydrogenases function in the membrane presumably to facilitate electron flow to other membrane bound carriers (i.e., quinones and cytochromes). Unlike *B. japonicum*, *P. Brockii* respire sulfur through a form of chemolithotrophy described as hydrogen-sulfur autotrophy (1, 2). As such, during growth of this organism, large amounts of sulfide are generated (5). It is interesting to note that in *P. Brockii*, the uptake of hydrogen and the reduction of sulfur are not necessarily associated with growth (5). For *P. Brockii*, the specific electron transport components between H₂ and S⁰ are not known. Potential metabolic models for the *P. Brockii* hydrogenase system might be found in some species within the genus *Desulfovibrio*. The *Desulfovibrio* genus is made up of strictly anaerobic sulfur-reducing bacteria that have reasonably well characterized hydrogenases and electron-transport proteins (23). Therefore, detailed comparisons between *Desulfovibrio* electron transport proteins involved in H₂ oxidation and S⁰ reduction and those analogous ones in *P. Brockii* may be useful in the investigation of the thermostability of proteins. In any case, elucidating the specific electron transport components between H₂ and S⁰ and the mechanistic features of this system may reveal much about the bioenergetic aspects of life at extreme temperatures.

Not only do the enzymes from the hyperthermophile *P. Brockii* and the mesophile *B. japonicum* appear to be functionally similar, but they seem to share some structural similarities. The ability of the *B. japonicum* probes to cross-react with both the protein and DNA of *P. Brockii* is especially exciting. Since the genes have homology, the *B.*

japonicum hydrogen-uptake gene probes should allow the isolation of the corresponding *P. Brockii* genes; a comparison of the two enzymes at the amino acid level should then be possible. Even though the H₂-uptake hydrogenases of *P. Brockii* and *B. japonicum* have some structural similarities, their temperature versus activity profiles differ markedly. Studying the specific structural differences between the two enzymes will shed some light on how hydrogenases can differ structurally but still be similar functionally and may help to elucidate what makes the *P. Brockii* protein so thermostable.

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