Transcriptome Analyses of Metabolic Enzymes in Thiosulfate- and Hydrogen-Grown Hydrogenobacter thermophilus Cells

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Hydrogenobacter thermophilus is a chemolithoautotroph that utilizes not only hydrogen (H₂) but also thiosulfate as sole source of energy and assimilates carbon dioxide via the reductive tricarboxylic acid (RTCA) cycle. We systematically carried out transcriptome analysis of metabolic enzymes in both H₂- and thiosulfate-grown H. thermophilus cells. The analysis indicated that the expression of hydrogenase genes is repressed under thiosulfate oxidation conditions as compared with H₂ oxidation conditions. This was confirmed by enzyme assay. In contrast, some genes for sulfur metabolism, including sox genes, showed almost the same expression levels under both conditions. In addition, the genes for the RTCA cycle showed high expression levels under both conditions. It was suggested that sulfur metabolism and the RTCA cycle function as forms of basal metabolism, and H₂ oxidation is inducible. Switching of H₂ oxidation can be advantageous for the lifestyle of this bacterium in nature.

Key words: thermophiles; thiosulfate oxidation; hydrogen oxidation; transcriptome; reductive tricarboxylic acid cycle

A geothermal area is a habitat for diverse thermophilic microorganisms. Many of these organisms are capable of growth on inorganic substances, using autotrophic and lithotrophic forms of metabolism. Such microbes contribute to biogeochemical cycling. One example, H. thermophilus, is a thermophilic, chemolithoautotrophic bacterium, isolated from a hot spring in Izu, Japan. In the phylogenetic tree of the 16S rRNA sequence, this bacterium is located on the deepest branch in the domain Bacteria, belonging to the order Aquificales.

H. thermophilus is able to utilize hydrogen (H₂) or thiosulfate as sole source for reducing power. Four gene clusters for Ni,Fe-hydrogenases (hyn, hox, hup1, hup2) have been found in the genome of this bacterium. Among these, Hox and Hyn have been characterized biochemically. The former was purified and the latter was examined as to gene expression.

Two different biochemical pathways for thiosulfate oxidation, the S4I pathway and a pathway catalyzed by the sulfur oxidation (Sox) system, have been proposed. Although sox genes (soxY, Z, A1, A2, X, and B) have been found in the genome of H. thermophilus, the genes for the S4I pathway were not identified. Thiosulfate oxidation by the Sox system is performed by several periplasmic proteins. In this pathway, at least three proteins (SoxAX complex, SoxB, and SoxYZ complex) are indispensable. At the first step of thiosulfate oxidation by the Sox system, thiosulfate is oxidatively bound to the cysteinyl-SH group of SoxY, in which two-electron oxidation is catalyzed by SoxAX. SoxY-bound thiosulfonate is then hydrolyzed by SoxB, generating SoxY(-S)-SH, sulfate, and a proton. Biochemical experiments on the thiosulfate oxidation activity of H. thermophilus cells have been performed by Sano et al., and activity was observed in the soluble cell-free extract of H₂-grown H. thermophilus (without thiosulfate).

As to anabolic metabolism, the bacterium was extensively analyzed biochemically, and was found to utilize several unique pathways. For instance, H. thermophilus fixes carbon dioxide (CO₂) as sole carbon source through the reductive tricarboxylic acid (RTCA) cycle. The key enzymes of this pathway have been investigated in detail. The bacterium does not grow on any sugar, and hence the sugars for biomolecules and cell components are to be synthesized through gluconeogenesis. In addition, the glutamine synthase (GS)-glutamate 2-oxoglutarate amidotransferase (GOGAT) system has been found to function for ammonium assimilation in this bacterium. H. thermophilus GOGAT was the first example of a ferredoxin-dependent type of nonphotosynthetic organism. All of these anabolic gene sets have been found in the H. thermophilus genome.

Although biochemical data and genome information related to H. thermophilus energy metabolism have accumulated, the expression patterns of the responsible genes have not been analyzed systematically. In this study, genome information on H. thermophilus was compared with that on other Aquificales species, and transcriptome analyses of the bacterium grown under both H₂ and thiosulfate oxidation conditions were carried out.

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Materials and Methods

Genome comparison. Genome data obtained from the National Center for Biotechnology Information (NCBI) nucleotide data-base were used. The NCBI genome IDs are as follows: Hydrogenobacter, 1909; Thermocrinis, 1933; Aquifex, 1049; Hydrogenobaculum, 1494; Hydrogentiriga, 1536; Sulfurhydrogenibium, 1153; and Persephonella, 1151.

Bacterial strain and growth condition. H. thermophilus TK-6 (IAM 12695, DSMZ 6534) was cultivated at 70 °C with CO2 as sole carbon source in an inorganic medium19 in a 100-mL vial under H2 or thiosulfate oxidation conditions. For thiosulfate oxidation growth, the medium was supplemented with 10 mM sodium thiosulfate. The head space gas in the vial was replaced with gas mixtures of H2:O2:CO2 (75:10:15, v/v) for H2 oxidation conditions, and with N2:O2:CO2 (75:10:15, v/v) for thiosulfate oxidation conditions. For observation of growth profiles, the preculture was inoculated into 10 mL of inorganic medium in a 100-mL vial and cultivated as described above. The optical density at 540 nm was adjusted to 0.068 for thiosulfate oxidation conditions. For H2 oxidation growth, the medium was supplemented with 10 mM sodium thiosulfate. The head space gas in the vial was replaced with gas mixtures of H2:O2:CO2 (75:10:15, v/v) for H2 oxidation conditions, and with N2:O2:CO2 (75:10:15, v/v) for thiosulfate oxidation conditions. For observation of growth profiles, the preculture was inoculated into 10 mL of inorganic medium in a 100-mL vial and cultivated as described above. The optical density at 540 nm was adjusted to 0.12 for H2 oxidation, and to 0.068 for thiosulfate oxidation conditions.

Preparation of cell suspension. H. thermophilus cells were cultivated under H2 or thiosulfate oxidation conditions. At the stationary phase, the cells were harvested at 10,000 × g for 15 min and washed once with 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer (pH 7.0). The cells were resuspended in the same buffer, and then disrupted by sonication using a Bioruptor (Cosmobio, Tokyo, Japan) using a 50% duty cycle for 15 min. The resulting mixtures were stored at −80 °C until use, and were used as cell suspensions in hydrogenase assays.

Protein assays. The protein concentrations of the cell suspensions determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin the standard.

Hydrogenase assays. Hydrogenase activity was determined by measuring H2-dependent methyl viologen (MV) reduction.3 The reduction of MV was measured by monitoring the increase in the absorbance at 578 nm, which corresponds to the specific absorption of MV was measured by monitoring the increase in the absorbance at 578 nm, which corresponds to the specific absorption of MV. The reaction mixture contained 757 μM MV at 70 °C for 10 min. The reaction was started by the addition of MV after 4 min of precultivation. The reduction of MV was calculated by assuming an extinction coefficient of 9.7 mM−1 cm−1.18 One unit of hydrogenase activity was defined as the activity that reduces 1 nmol of MV per min.

RNA extraction. H. thermophilus TK-6 was grown in 15 mL and 10 mL of inorganic medium for H2 and thiosulfate oxidation conditions respectively in a 100-mL vial. RNA extraction was performed in two independent cultures. For H2 and thiosulfate oxidation conditions, 15 and 40 mL aliquots of the culture were mixed with 2 and 1 volumes of RNA protect (Qiagen, Hilden, Germany), when the optical density (OD) at 540 nm reached approximately 0.50 and 0.20 respectively to 0.068 for thiosulfate oxidation conditions.

Microarray experiments and data analysis. A customized tiling array with a 4 × 72k format for H. thermophilus TK-6 was designed and manufactured by Roche NimbleGen (Basel, Switzerland) based on the genome sequence. A genomic tiling microarray probe set consisting of 139,450 unique 60-mer oligonucleotide sequences covering the H. thermophilus TK-6 genome was used. Control probes were also included to ensure that there was no intra-quadrant contamination during the hybridization process. The duplicate RNA samples isolated from cells grown under each of the two conditions were used for the microarray analyses. Double-stranded (ds)-cDNA synthesis and labeling were performed following the instructions in the NimbleGen arrays User’s Guide for Gene Expression Analysis (Roche NimbleGen), as described previously.19 A hybridization solution was prepared with a hybridization kit (Roche NimbleGen) following the protocol supplied by the manufacturer, and hybridization was performed using a NimbleGen hybridization system 4. Washing was carried out with a wash buffer kit (Roche NimbleGen) following the manufacturer’s instructions, and the arrays were scanned with an MS 200 Microarray Scanner (Roche NimbleGen) with NimbleGen MS 200 v1.1 software. NimbleScan 2.5 software was used for extraction of the probe signal intensities and processing of the data. Expression data were normalized by quantile normalization.20 A robust multichip average algorithm was used to generate gene expression signal values.21 The mean signal values of each probe set of the duplicates and their relative fold changes between the growth conditions were calculated using ArrayStar v3.0 software (DNASTAR, Madison, WI). Genes that showed a 2-fold or greater difference in expression level with 95% confidence were considered significant.

Results and Discussion

Genome analysis

Conservation of the genes for hydrogenases and Sox proteins was compared using seven Aquificales species whose genome sequences have been determined. As shown in Table 1, Sox proteins are well conserved among all seven species, while hydrogenase genes are distributed among certain species. Based on molecular ecological analyses, the Aquificales species appear to be dominant members of communities in near-neutral hot springs in Japan,23 Iceland,23 and Yellowstone National Park,24,25 and in shallow marine hydrothermal systems,26 where sulfur compounds are available. All seven species are known to be capable of utilizing thiosulfate as an electron donor,27−29 and hence it is plausible that such species conserve sulfur oxidation enzymes.

In addition, the genes for anabolic enzymes were also compared among the Aquificales species. Key enzymes for the RTCA cycle and glutamogenesis and grhS gene are well conserved among these organisms, indicating the metabolic importance of the RTCA cycle, glutamogenes, and the GS-GOGAT system.

Growth of H. thermophilus

To compare growth profiles under H2 and thiosulfate oxidation conditions, H. thermophilus was cultivated under each of the various conditions. The growth curves are shown in Fig. 1. The growth rate of the thiosulfate-grown cells was much lower than that of the H2-grown cells. H2 oxidation appears to be more efficient than thiosulfate oxidation in obtaining reducing power for the growth of this bacterium. In addition, because sulfate is generated by thiosulfate oxidation, a decrease in the pH of the culture might be another cause of the lower growth rate under thiosulfate oxidation conditions.

Transcriptome analysis

A tiling microarray for H. thermophilus TK-6 was custom designed according to the genome sequence and was used to determine gene expression levels in cells grown under H2 and under thiosulfate oxidation conditions. The genes that showed 2-fold or greater difference between the conditions tested with 95% confidence are shown in Supplemental Table 1 (see Biosci. Biotechnol. Biochem. Web site).
Table 1. Distribution of the Genes for Energy- and Anabolic-Metabolism among Six Aquificales Species

<table>
<thead>
<tr>
<th>Species</th>
<th>hupL1</th>
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</table>

Distribution of the genes for four hydrogenases (hupL1, hupL2, hynL, and hosL), a Sox protein (soxY), a key enzyme of the RTCA cycle (citryl-CoA lyase, ccl), a key enzyme of gluconeogenesis (phosphoenolpyruvate synthase, ppsA), and glutamine 2-oxoglutarate amidotransferase (ghs) are shown. Circle denotes the presence of a given gene in the genome of a given species. The presence of each gene was confirmed by BLAST search (National Center for Biotechnology Information) with the gene of *H. thermophilus* as query.

Genes for Sox proteins (soxYZA1A2XB) were highly expressed under both growth conditions (Fig. 2A). This expression pattern indicates that thiosulfate oxidation can occur constitutively even in *H*₂-grown *H. thermophilus* cells, as biochemically observed previously (Sano et al.). Furthermore, in addition to *sox* genes, several other genes encoding enzymes for sulfur metabolism, sulfide-quinone reductase (sqr), sulfur reductase (sreABC), and sulfite:cytochrome c oxidoreductase (sorAB), showed relatively high expression values under both conditions (Fig. 2C).

In contrast to the constitutive high expression of *sox* genes, expression of three of the four hydrogenase gene clusters (*hox*, *hup1*, and *hup2*) was highly enhanced under *H*₂ oxidation conditions and significantly repressed under thiosulfate oxidation conditions (Fig. 2B). This was confirmed by enzyme assay measuring the hydrogenase activity of both *H*₂- and thiosulfate-grown cells of *H. thermophilus*. The hydrogenase activity of the sonicated cell suspension is shown in Fig. 3. The Hydrogenase activity of the thiosulfate-grown cells was 4.4-fold lower than that of the *H*₂-grown cells. These results suggest that hydrogenases are inducible in this bacterium.

Many of the genes predicted to encode efflux pumps and transporter proteins showed relatively high expression levels under thiosulfate oxidation conditions (Supplemental Table 1). Under such conditions, sulfate is generated in the medium by thiosulfate oxidation, and thus cells might be forced to maintain cellular pH at a physiological level. For pH maintenance, efflux pumps may be important. This might be one of the reasons for the high expression of many of the efflux pump and transporter genes.

Several genes encoding denitrification enzymes, nitrate reductase (*nar*), nitrite reductase (*nir*), and nitric oxide reductase (*nor*), showed relatively low expression levels under thiosulfate oxidation conditions as compared with *H*₂ oxidation conditions (the fold change values of *narG*, *nirS*, and *norB* were −4.2, −4.2, and −4.4 respectively; Supplemental Table 1). These might be due to low oxygen consumption activity in the thiosulfate-grown cells. The cells for transcriptome analysis were batch-cultured, and hence the amount of oxygen in the gas phase was limited. Since the reducing power from thiosulfate oxidation appeared to be lower than that from *H*₂ oxidation, the thiosulfate-grown cells might be unable to reduce oxygen as much as the *H*₂-grown cells. Thus the remaining amount of oxygen in the gas phase under thiosulfate oxidation conditions might be greater than that under *H*₂ oxidation conditions. It has been reported of many denitrification bacteria that denitrification enzymes are upregulated under low oxygen conditions. A relatively high cellular oxygen concentration in thiosulfate-grown cells might cause low expression levels of denitrification genes.

We focused on the expression patterns of the genes for the RTCA cycle as well as the genes for gluconeogenesis and the GS-GOGAT system as central metabolic pathways, which are connected with the RTCA cycle. The genes for the RTCA cycle enzymes, malate dehydrogenase (*mdh*), fumarate hydratase (*fumA, fumB*), fumarate reductase (*frdA, frdB, frdC, frdD, frdE*), succinic-CoA synthetase (*sucCD*), two types of 2-oxoglutarate: ferredoxin oxidoreductase (OGOR) (*korAB, forABGD*, 2-oxoglutarate carboxylase (*cfAB*), isocitrate dehydrogenase (*icd*), aconitate hydratase/aconitase (*aanA*), citryl-CoA synthetase (*ccsA, ccsB*), citryl-CoA lyase (*ccl*), pyruvate:ferredoxin oxidoreductase (POR) (*porABGD*), and pyruvate carboxylase (*pycA, pycB*), for gluconeogenesis, phosphoenolpyruvate synthase (*ppxA*), phosphoglycerate mutase (*pgmA*), 3-phosphoglycerate kinase (*pgk*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), triosephosphate isomerase (*timA*), putative fructose 1,6-bisphosphate aldolase/ phosphatase, and bifunctional phosphoglucone/phosphomannoseisomerase (*pgi*), and for the GS-GOGAT system, glutamine synthetase (*glnA*) and ferredoxin-dependent glutamate synthase (*ghs*), showed relatively
high expression levels under both conditions. The expression levels of 34 of the 38 genes were higher than the average expression level for all the genes. The high expression levels of these enzymes suggest the importance of central metabolism for H. thermophilus under both H$_2$ and thiosulfate oxidation conditions.

The fdx1 gene for ferredoxin1 (Fd1), which functions as an electron donor for OGOR and POR of the RTCA cycle,$^{12}$ showed high expression levels under both conditions, suggesting that Fd1 is utilized as an electron donor in H$_2$-grown cells as well as in thiosulfate-grown cells. Fd1 reduction is an essential process for H. thermophilus to utilize the RTCA cycle. Several mechanisms to reduce low-redox-potential Fd (about $-490$ mV) were reviewed by Fuchs recently.$^{31}$ In the review, five kinds of Fd-reducing mechanisms are introduced, although no complete gene sets for any those mechanisms were found in the genome of H. thermophilus. There may be a characteristic mechanism for Fd1 reduction in H. thermophilus cells.

From the results obtained in this study, one can gain insight into the relationship between observed expression patterns and the life of H. thermophilus in nature. The sox genes showed constitutive high expression levels under both H$_2$ and thiosulfate oxidation conditions, whereas the hydrogenase genes were significantly repressed under thiosulfate oxidation conditions. In addition, the genes for anabolic metabolism, such as the RTCA cycle, gluconeogenesis, and ammonium assimilation, also showed constitutive high expression levels under both conditions. This bacterium was isolated in a hot spring, and hence abundant sulfur compounds, such as thiosulfate, might have been available. Although H. thermophilus obtains reducing power more efficiently by H$_2$ oxidation than by thiosulfate oxidation, cells which constitutively express the enzymes for sulfur metabolism might be advantageous for survival in sulfur-rich environments. H. thermophilus is presumed to utilize sulfur metabolism and anabolic pathways in basal metabolism, and to utilize H$_2$ oxidation when H$_2$ is available. Constitutive high expression of the genes for basal metabolism may contribute to the survival of H. thermophilus in an environment in which inorganic compounds are available, while inducible H$_2$ oxidation enables the bacterium to grow at a high rate when H$_2$ is available. Therefore, switching of H$_2$ oxidation can be advantageous for H. thermophilus.

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References