JOURNAL OF BACTERIOLOGY, Jan. 2006, p. 525–531 Vol. 188, No. 2
0021-9193/06/$08.00+0 doi:10.1128/JB.188.2.525–531.2006

Characterization of Dissimilatory Fe(III) versus NO_3^- Reduction in the Hyperthermophilic Archaeon *Pyrobaculum aerophilum*

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Received 23 August 2005/Accepted 18 October 2005

The hyperthermophilic archaeon *Pyrobaculum aerophilum* used 20 mM Fe(III) citrate, 100 mM poorly crystalline Fe(III) oxide, and 10 mM KNO_3 as terminal electron acceptors. The two forms of iron were reduced at different rates but with equal growth yields. The insoluble iron was reduced when segregated spatially by dialysis tubing, indicating that direct contact with the iron was not necessary for growth. When partitioned, there was no detectable Fe(III) or Fe(II) outside of the tubing after growth, suggesting that an electron shuttle, not a chelator, may be used as an extracellular mediator of iron reduction. The addition of 25 and 50% (vol vol^{-1}) cell-free spent insoluble iron media to fresh media led to growth without a lag phase. Liquid chromatography analysis of spent media showed that cultures grown in iron, especially insoluble iron, produced soluble extracellular compounds that were absent or less abundant in spent nitrate medium. NADH-dependent ferric reductase activity increased approximately 100-fold, while nitrate reductase activity decreased 10-fold in whole-cell extracts from iron-grown cells relative to those from nitrate-grown cells, suggesting that dissimilatory iron reduction was regulated. A novel 2,6-anthrahydroquinone disulfonate oxidase activity was more than 580-fold higher in iron-grown cells than in nitrate-grown cells. The activity was primarily (>95%) associated with the membrane cellular fraction, but its physiological function is unknown. Nitrate-grown cultures produced two membrane-bound, *c*-type cytochromes that are predicted to be monoheme and part of nitrite reductase and a bc_1 complex using genome analyses. Only one cytochrome was present in cells grown on Fe(III) citrate whose relative abundance was unchanged.

Dissimilatory iron reduction was suggested to be widespread among hyperthermophilic archaea and was first demonstrated in *Pyrobaculum islandicum* (14, 36). It has since been characterized in other hyperthermophilic archaea, such as *Ferroglobus placidus*, *Geoglobus ahangari*, and a new species (strain 121) belonging to the family *Pyrodictiaceae* (15, 16). NAD(P)H-dependent ferric reductase activity was measured in *P. islandicum*, primarily (86%) in the soluble protein fraction, but it did not vary in Fe(III) citrate- versus thiosulfate-grown cultures, suggesting that the response was not regulated (7). Furthermore, no *c*-type cytochromes were detected in cell extracts of *P. islandicum* (7).

Frequent topics of study of mesophilic dissimilatory iron-reducing bacteria are their ability to reduce iron by direct and indirect mineral contact and the role of *c*-type polyheme cytochromes. *Shewanella oneidensis*, *Shewanella algae*, *Geobacter sulfurreducens*, *Geobacter metallireducens*, and *Geothrix fermentans* were all capable of dissimilatory iron reduction using Fe(III) citrate (i.e., soluble form), but only *S. oneidensis*, *S. algae*, and *G. fermentans* were capable of reducing Fe(III) oxide (i.e., insoluble form) indirectly without the addition of an artificial electron shuttle (18, 25–27). Indirect iron reduction was proposed to occur through the cycling of extracellular mediators, such as an electron shuttle or a chelator (18, 26, 27). Melanin produced by *S. algae* was shown to act as its own electron shuttle for iron reduction without direct mineral contact (34, 35). In contrast, *G. metallireducens* and *G. sulfurreducens* both required direct contact with insoluble iron (8, 25) and may reduce this iron via conductive pili that attach to iron particles when soluble iron is depleted (8, 30).

In *S. oneidensis*, the *c*-type tetraheme cytochrome CymA was required for both direct and indirect mineral reduction, while the *c*-type decaheme cytochrome OmcB contributed to but was not required for iron reduction (18, 23, 24). In *G. sulfurreducens*, the mRNA levels for another *c*-type polyheme cytochrome (*omcB*) increased significantly when cultures were grown on Fe(III) citrate relative to those grown on fumarate, suggesting that iron reduction was regulated (9, 22). However, these cytochromes are not always required for growth on iron, since they were lacking in the iron reducer *Pelobacter carbinolicus* (21).

In this study, the characteristics of dissimilatory iron reduction in the hyperthermophilic archaeon *Pyrobaculum aerophilum* were determined and compared with those of mesophilic dissimilatory iron reducers and *P. islandicum*. The data suggest that *P. aerophilum* differs from *P. islandicum* in that it appeared to regulate its iron-reducing capability and produced *c*-type cytochromes. *P. aerophilum* may be more similar to *Shewanella* than *Geobacter* functionally in that it does not require direct contact for reduction of insoluble iron.

MATERIALS AND METHODS

Growth conditions. The media for *P. aerophilum* (DSM 7523) growth were modified from the medium described previously (13). The base medium was composed of the following (per liter): 7 g of NaCl, 2.3 g of $MgCl_2 \cdot 6H_2O$, 0.5 g of KH_2PO_4, 0.5 g of KCl, 0.3 g of NH_4Cl, 0.3 g of sodium citrate $\cdot 2H_2O$, 60 mg of $CaCl_2 \cdot 2H_2O$, 50 mg of KBr, 20 mg of H_3BO_3, 50 μl of an iron solution [2 g of $(NH_4)_2Fe(SO_4) \cdot 6H_2O$ per liter and 1 g of $FeSO_4 \cdot 7H_2O$ per liter], 0.1 ml of 10 mM $NaSeO_4$, 10 ml each of Wolfe's mineral solution and Wolfe's vitamin mix (ATCC medium 1045), 1 g of yeast extract (enzymatic; Difco), 1 g of tryptone (Difco), and 0.5 mM of cysteine-HCl. Fe(III) citrate (20 mM), 100 mM poorly

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crystalline Fe(III) oxide, 50 mM hematite, 50 mM goethite, and 10 mM KNO_3 were tested separately as terminal electron acceptors. $FeCl_2$ (1.3 mM) was also added to the iron medium. The insoluble Fe(III) oxide was prepared separately as described previously (20). $FeCl_3$ (108 g) was dissolved in a liter of distilled H_2O, and the pH was adjusted to 7 using 10 N NaOH to precipitate the iron. The solution was spun at 5,000 rpm for 20 min, and the pellet was resuspended in distilled H_2O repeatedly until the supernatant became slightly yellow. The pellet was resuspended to make a 1-liter solution (~1 M) and stored in the dark at 4°C. Stock suspensions of hematite and goethite were provided by the laboratory of Derek Lovley. All media were pH balanced to 6.80 ± 0.05 (room temperature) before the addition of 1 mM potassium phosphate buffer (pH 6.8).

Bottle-grown cultures were degassed and flushed with argon in the headspace and incubated without stirring at 95°C in an oven unless stated otherwise. Fermentor-grown cultures were grown in a 20-liter fermentor with either Fe(III) citrate or KNO_3 as the terminal electron acceptor. The media were flushed with argon at a flow rate of 30 standard cubic centimeters min^{-1}, stirred at 120 to 150 rpm, and heated to 95°C ± 0.1°C. The pH of the iron and nitrate media at 95°C were maintained at 6.0 ± 0.1 and 6.3 ± 0.1, respectively, using an automated pH control system. Experimental bottles were inoculated with a logarithmic-growth-phase culture that had been grown and transferred at least four successive times in bottles on the medium used.

At various times during growth, an aliquot was removed to measure the concentrations of cells and Fe(II). Cell concentrations were measured using a Petroff-Hausser counting chamber and phase-contrast light microscopy. The specific growth rate (k) of the culture was determined by a best-fit curve through the logarithmic portion of the growth data. Fe(II) was determined spectrophotometrically using the ferrozine assay method (28). Cells from the fermentor were harvested when they reached late logarithmic growth phase (~10^8 cells ml^{-1}). The medium was drained from the fermentor through a glass cooling coil bathed in an ice-water slurry into a carboy that had been flushed with argon. The cells were then concentrated to less than 2 liters by ultrafiltration using a hollow fiber cartridge (0.2-μm pore size; Amersham Biosciences) and further concentrated by centrifugation at 10,000 × g for 45 min. The resulting pellets were resuspended in an anoxic chamber in 18 ml of degassed 50 mM Tris buffer (pH 8.0) plus 2 mM each of dithionite (DT) and dithiothreitol, sealed in an argon-flushed serum bottle, and frozen at −20°C.

Iron barrier experiments. Cultures were grown on Fe(III) oxide with and without a barrier to determine whether direct contact with the iron was necessary for growth. The insoluble iron was contained within dialysis tubing (12,000- to 14,000-Da pore size; SpectraPor) in half of the tubes. There was no Fe(III) measured in uninoculated media outside of the tubing after 48 h of incubation at 95°C, suggesting that the barrier maintained its integrity at high temperatures and that the citrate present did not solubilize the iron. At various time points, samples were sacrificed and their concentrations of cells, Fe(II), and total iron were determined. Total iron was measured by reducing 100 μl of sample with 200 μl of freshly prepared 6 N hydroxylamine and 4.7 ml of 0.5 N HCl, incubating the sample overnight in a sealed container, and measuring the Fe(II) concentration as described above.

Spent supernatant analyses. Cultures were grown in media containing Fe(III) citrate, Fe(III) oxide, and nitrate, and uninoculated media with and without Fe(III) citrate were incubated for 24 h at 95°C. The samples were then spun at 10,000 × g for 1 h, and the supernatants were decanted and filtered through a 0.2-μm-pore-size filter. Portions (0, 25, and 50% [vol vol^{-1}]) of this spent medium were added to fresh medium to determine their effect on cell growth. Supernatant profiles were determined using a Hewlett-Packard 1100 series high-performance liquid chromatography separation module equipped with a photodiode array detector. Twenty microliters of sample was loaded onto a Nova-Pak C_{18} column (Waters) at room temperature and 1 ml min^{-1}, rinsed with 0.1% trifluoroacetic acid and 1% acetonitrile for 2 min, and eluted from the column using a 1 to 100% acetonitrile gradient over 45 min.

Protein fractionation. Cell material from the fermentor was separated into cytoplasmic and membrane fractions as described previously (1). All sample transfers and manipulations were performed in an anoxic chamber, and all buffers were degassed and flushed with Ar and contained 2 mM each of DT and dithiothreitol. The cell suspension was thawed, and DNase I was added to a final concentration of 0.0002% (wt vol^{-1}). The cells were then disrupted on ice by sonication. Cell lysis was verified using phase-contrast light microscopy, and a portion of the solution was used as the whole-cell extract (WCE). The remainder was spun at 100,000 × g for 45 min in an ultracentrifuge, and the supernatant was used as the cytoplasmic protein (CYT) fraction. The membrane pellet was resuspended in 50 mM Tris buffer (pH 8.0), homogenized using a glass tissue grinder, and then spun as described above. This procedure was repeated twice. The membrane pellet was then resuspended in 50 mM Tris buffer plus 0.1% sodium dodecyl sulfate (wt vol^{-1}) and used as the membrane-bound protein (MEM) fraction. The protein concentrations of the WCE and CYT fractions were determined spectrophotometrically using a protein determination kit (Bio-Rad) based on the Bradford assay (5). The protein concentrations of the MEM fractions were determined using a kit for detergent-containing samples (DC protein assay kit; Bio-Rad). The latter kit could not be used to determine the protein concentrations of the WCE and CYT fractions from iron-grown cells due to high background reactivity. Bovine serum albumin was used as the protein standard for both kits. Protein fractions that were not used immediately for analysis were frozen in liquid N_2 and stored at −80°C.

Equal amounts of protein (6 μg for silver stain; 50 μg for Coomassie blue stain) from the MEM fractions of Fe(III) citrate- and nitrate-grown cells were boiled for 5 min and separated by electrophoresis in glycine-buffered, 8 and 12% polyacrylamide gels. The proteins were stained with silver using the Silver Stain Plus kit (Bio-Rad) for imaging and with 0.1% Coomassie blue in 40% methanol–10% acetic acid and destained in 20% ethanol–5% acetic acid for mass fingerprinting of proteins. A 120-kDa band from the nitrate fraction was excised from the gel and digested overnight with trypsin (Promega) as described previously (31). The digested peptide fragments were concentrated and desalted using C_{18} ZipTips (Millipore) and eluted onto a matrix-assisted laser desorption ionization (MALDI) disk with α-cyano-4-hydrocinnamic acid in 0.1% trifluoroacetic acid–50% acetonitrile. The fragments were then analyzed using a MALDI-time of flight (TOF) mass spectrometer. The masses of the fragments were then correlated with the *P. aerophilum* genome for protein identification using the web-based program PeptIdent (www.expasy.org/tools/peptident.html).

Enzyme assays. The ferric reductase, 2,6-anthrahydroquinone disulfonate (AHDS) oxidase, and nitrate reductase assays were each performed in rubber stopper-sealed glass cuvettes that had been degassed and flushed with Ar. Ferric reductase activity was determined spectrophotometrically by measuring the formation of Fe(II)-ferrozine complex at 80°C and 562 nm (ε = 27,900 M^{-1} cm^{-1}) in a degassed buffer containing 50 mM morpholineethanesulfonic acid (MES) (pH 6.5) plus 0.1 mM ferric citrate and 0.5 mM ferrozine. The reaction was initiated by the addition of 20 mM NADH or 20 mM NADPH as the electron donor. AHDS oxidase activity was determined spectrophotometrically by measuring the oxidation of AHDS at 80°C and 450 nm (ε = 3,500 M^{-1} cm^{-1}) in degassed 50 mM MES buffer. The assay was initiated by the addition of sample following a 3-min incubation at the assay temperature. AHDS was generated at room temperature from 3 mM 2,6-anthraquinone disulfonate (AQDS) in degassed buffer that had been flushed with H_2 in the presence of palladium pellets. Abiotic oxidation of AHDS was tested for by running the assay at room temperature, by degrading the iron MEM sample with trypsin overnight before running the assay at 80°C, and by spinning the iron MEM fraction again at 100,000 × g for 45 min to remove particulate material from the sample (which was absent from the nitrate MEM fraction).

Nitrate reductase activity was determined spectrophotometrically by measuring the oxidation of 0.3 mM benzyl viologen at 80°C and 598 nm (ε = 7,400 M^{-1} cm^{-1}) using 10 mM KNO_3 as the substrate (3). The assay mixture was run in degassed 100 mM potassium phosphate buffer (pH 7.0). Malate dehydrogenase activity was determined by measuring the aerobic oxidation of 0.4 mM NADH at 80°C and 340 nm (ε = 6,200 M^{-1} cm^{-1}) using 0.4 mM oxaloacetate as the substrate (32). The assay mixture was run in 50 mM EPPS (*N*-[2-hydroxyethyl]piperazine-*N*′-[3-propanesulfonic acid]) buffer (pH 8.4). Activities are expressed in units where 1 U is equivalent to 1 μmol of substrate transformed min^{-1}.

Heme staining of *c*-type cytochromes. Equal amounts of protein (125 μg) from the MEM fractions of iron- and nitrate-grown cells were boiled for 5 min in sample buffer without a reducing agent and separated by electrophoresis in a Tricine-buffered, 10% polyacrylamide gel. Proteins containing *c*-type heme were identified by their peroxidase activity with H_2O_2 and 3,3′,5,5′-tetramethylbenzidine (TMBZ) as described previously (33). The gel was incubated at room temperature in a solution containing 60 mg of TMBZ in 30 ml of methanol and 70 ml of 0.5 M sodium acetate (pH 5.0). After 10 min, 600 μl of 30% (vol vol^{-1}) H_2O_2 was added to initiate the reaction that was terminated by the addition of a 30% isopropanol–70% 0.25 M sodium acetate solution. Heme-containing proteins produced a blue color.

RESULTS

***P. aerophilum* growth and iron reduction rate.** The doubling times of *P. aerophilum* in bottles were 10.7 h (k = 0.065 h^{-1} ± 0.014 h^{-1} [95% confidence interval]) (Fig. 1A) and 4.6 h (k = 0.149 h^{-1} ± 0.013 h^{-1} [data not shown]) for growth on Fe(III)

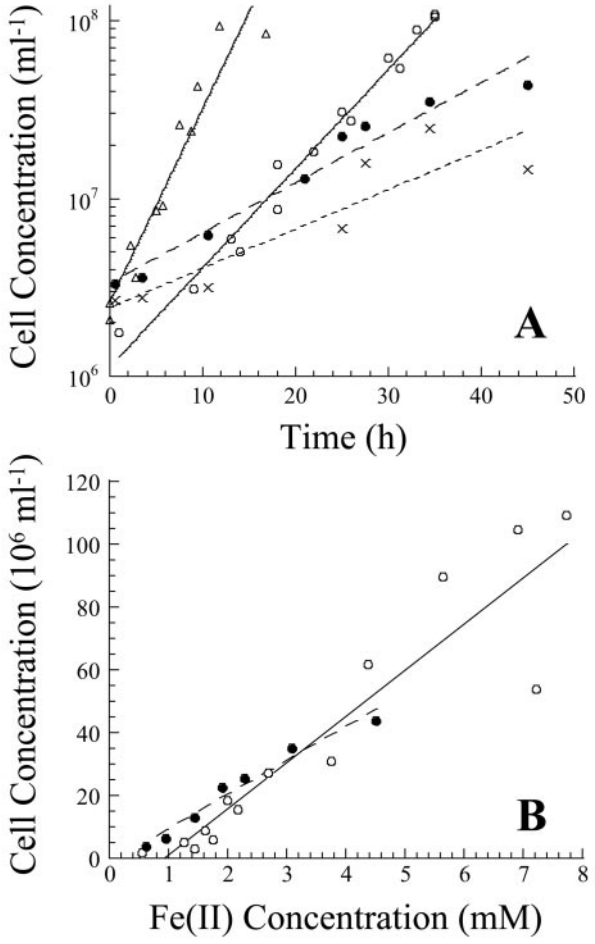

FIG. 1. (A) *P. aerophilum* growth in the 20-liter fermentor on either 10 mM KNO_3 (△, solid line) or 20 mM Fe(III) citrate (○, solid line) and in 25-ml Balch tubes on 100 mM insoluble Fe(III) oxide without (●, – – –) and with (×, - - - - -) containment in dialysis tubing. (B) Growth yields for cultures grown either in the 20-liter fermentor on 20 mM Fe(III) citrate (○, solid line) or in 25-ml Balch tubes on 100 mM insoluble Fe(III) oxide (●, – – –).

oxide and Fe(III) citrate, respectively. The maximum cell concentration for cultures grown on insoluble iron was approximately half that of cultures grown on soluble iron. There was no significant difference in the growth rate for cultures grown on Fe(III) oxide with and without stirring. There was no growth on other forms of Fe(III) oxide, namely, hematite and goethite, after 7 days of incubation. The doubling times in the fermentor during growth on Fe(III) citrate and nitrate were 5.4 h ($k = 0.128\ h^{-1} \pm 0.012\ h^{-1}$) and 2.8 h ($k = 0.251\ h^{-1} \pm 0.057\ h^{-1}$), respectively (Fig. 1A). Fe(II) concentrations increased in a cell concentration-dependent manner on all forms of iron and reached 5 to 8 mM (Fig. 1B). For Fe(III) citrate-grown cultures, there were no significant differences in growth rate, Fe(II) production rate, growth yield, and maximum cell concentration between bottle- and fermentor-grown cells. The cell growth yields for cultures grown on Fe(III) citrate in the fermentor and on Fe(III) oxide in bottles were 1.47×10^7 cells μmol^{-1} of Fe(II) produced ($\pm 0.37 \times 10^7$ cells μmol^{-1} [95% confidence interval]) and 1.09×10^7 cells μmol^{-1} of Fe(II) produced ($\pm 0.24 \times 10^7$ cells μmol^{-1}), respectively (Fig. 1B), and were not significantly different.

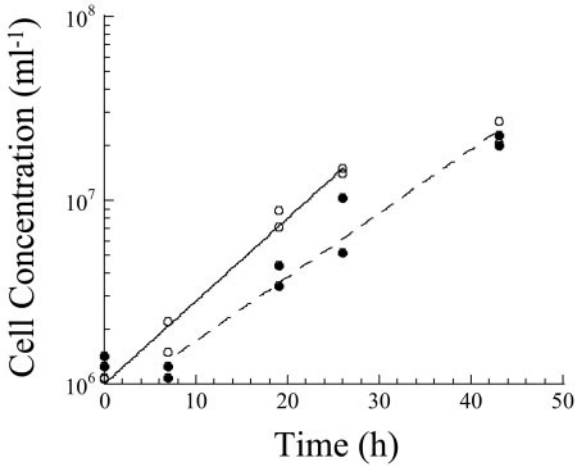

FIG. 2. *P. aerophilum* growth with (○, solid line) and without (●, dashed line) the addition of 25% (vol vol^{-1}) cell-free spent medium to fresh medium.

The doubling time for cultures grown on Fe(III) oxide contained within dialysis tubing was 13.6 h ($k = 0.051\ h^{-1} \pm 0.027\ h^{-1}$) and was not significantly different from the doubling time for growth without the barrier (Fig. 1A). Furthermore, the Fe(II) production rates for cultures with the barrier were not significantly different from those without the barrier (data not shown). There was no increase in Fe(III) or Fe(II) concentrations outside of the dialysis tubing during cell growth. All of the reduced iron remained within the tubing. The addition of 5 and 10 mM sodium citrate to the Fe(III) oxide medium inhibited cell growth. Cells in insoluble iron medium showed no lag phase when 25 and 50% cell-free spent medium was added, while cultures without spent medium showed a lag phase of at least 7 h, although the growth rates were the same in all three media (Fig. 2). The growth kinetics for cultures in 25 and 50% spent medium were nearly identical (data not shown for 50% spent medium). Analyses of cell-free spent media and uninoculated media using liquid chromatography showed six peaks in spent Fe(III) oxide medium with retention times of 2.4, 2.5, 3.5, 3.7, 25.7, and 41.3 min that had significantly higher absorbances than the other samples, and a peak with a 13.9-min retention time with high absorbance in spent Fe(III) oxide and Fe(III) citrate medium (Fig. 3). The peak observed at 1-min retention time was due to the citrate added to the medium.

Cell fractionation. In order to assess the activities of both cytoplasmic and membrane-bound enzymes, the proteins from WCEs were separated into CYT and MEM fractions. Protein assays showed that of the total protein present in the WCE, 64% ± 9% ($n = 4$) was recovered in the combined CYT and 50 mM Tris wash fractions, while the MEM fraction contained 24% ± 8% ($n = 4$) of the total protein. The remaining protein (~10%) was presumably lost during sample transfer and manipulations. Iron- and nitrate-grown cultures contained the same proportions of protein in the various cellular fractions. The washing procedure was effective at removing CYT protein from the MEM fraction as judged

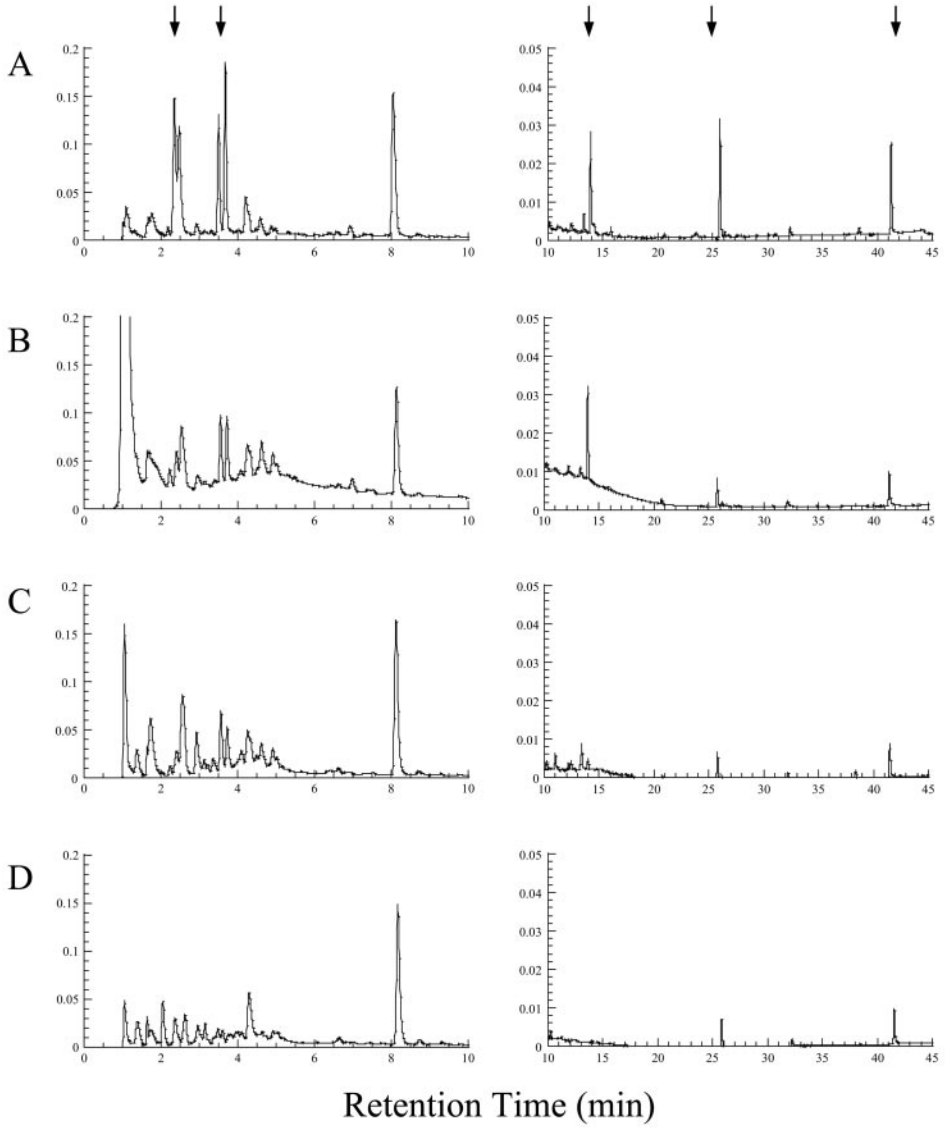

FIG. 3. Absorbances at 280 nm following separation by liquid chromatography of soluble compounds in spent supernatants from cultures grown on 100 mM Fe(III) oxide (A), 20 mM Fe(III) citrate (B), or 10 mM KNO_3 (C) and supernatants from uninoculated media (D). The arrows indicate the peaks of interest.

by the amount of malate dehydrogenase (MDH) activity in the various fractions (Table 1). MDH is a soluble protein (32) and was used as a marker to establish that the wash protocol provided complete separation of CYT proteins from the MEM proteins. Relative to MDH activity in the WCE, most of the MDH activity was recovered in the first CYT fraction (75% ± 22%; $n = 4$) and no activity was measured in the MEM fractions ($n = 4$).

TABLE 1. Specific activities of NADH-dependent ferric reductase, AHDS oxidase, nitrate reductase, and malate dehydrogenase at 80°C in the cellular fractions from cells grown on ferric citrate and nitrate

Fraction	Sp act (U mg^{-1}) (mean ± 1 SD)							
	Ferric reductase		AHDS oxidase		Nitrate reductase		Malate dehydrogenase	
	Iron-grown	Nitrate-grown	Iron-grown	Nitrate-grown	Iron-grown	Nitrate-grown	Iron-grown	Nitrate-grown
WCE	1.09 ± 0.41	0.01 ± 0.00	63.8 ± 12.2	0.11 ± 0.01	1.07 ± 0.45	10.9 ± 2.0	1.07 ± 0.20	0.74 ± 0.03
CYT	0.73 ± 0.22	0.02 ± 0.00	0.24 ± 0.09	0.07 ± 0.00	1.48 ± 0.24	1.61 ± 0.81	1.41 ± 0.38	0.93 ± 0.49
MEM	0.27 ± 0.08	0.03 ± 0.01	33.0 ± 0.51	0.14 ± 0.01	0.22 ± 0.20	23.3 ± 4.7	ND[a]	ND

[a] ND, no activity detected.

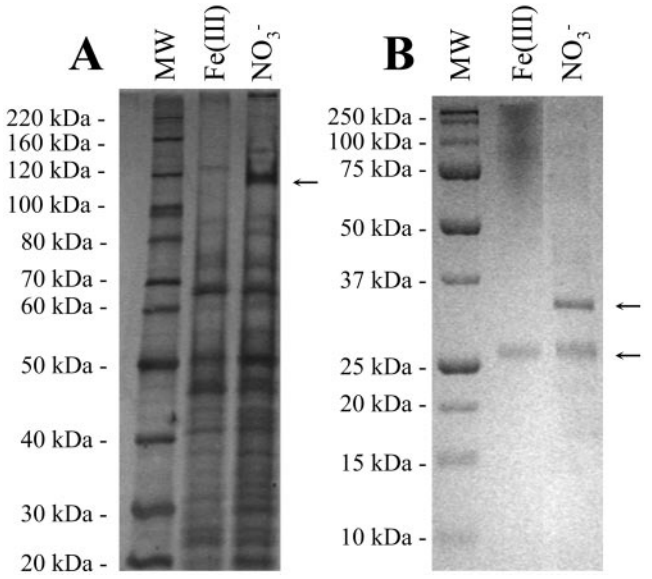

FIG. 4. Gel electrophoresis and silver staining (A) or heme staining (B) of membrane fractions prepared from iron- and nitrate-grown cultures. (A) The protein band used for peptide mass fingerprinting is indicated by the arrow, and the molecular masses of molecular size standard proteins (MW lane) are shown on the left. The gel contained 8% polyacrylamide. (B) The two heme-stained protein bands are identified by arrows, and the molecular masses of prestained standard proteins (MW lane) are shown on the left.

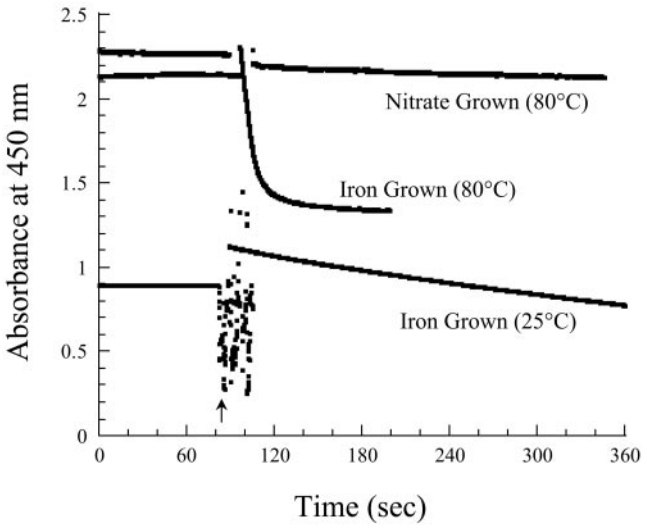

FIG. 5. Absorbance of AHDS at 450 nm and 80 and 25°C during AHDS oxidation assays using membrane fractions prepared from iron- and nitrate-grown cultures. The arrow indicates the point when the sample was added to initiate the reaction.

Membrane protein composition. There were no protein bands in the iron MEM fractions that were higher in relative abundance than those in the nitrate MEM fractions (Fig. 4A). However, there was a highly abundant protein band with a molecular mass of approximately 120 kDa in the nitrate MEM fraction that was virtually absent in the iron MEM fraction. Tryptic digestion and peptide mass fingerprinting of the band resulted in 21 well-defined peaks from the mass spectrometer. Eleven of the fragments matched (±1 Da) those predicted for the nitrate reductase α subunit (NarG, PAE3611), and seven matched those for the nitrate reductase β subunit (NarH, PAE3612). There was only one peptide match overlap between the two proteins. Both fragments are predicted to be membrane bound and part of the dissimilatory nitrate reductase complex (2). Apparently the 5-min boiling and denaturing conditions of the sample buffer were insufficient to completely dissociate these proteins into their respective monomers. Longer boiling times led to the degradation of the proteins (data not shown), perhaps by a membrane-bound subtilisin-type protease found in *P. aerophilum* (aerolysin) that is resilient to the denaturing conditions typically used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (37).

The heme stain suggested that there were two *c*-type cytochrome proteins in the nitrate MEM fraction but only one in the iron MEM fraction (Fig. 4B). The relative molecular masses of these unreduced proteins were 27 kDa for the band found in both fractions and 34 kDa for the second band found in the nitrate fraction. Two putative *c*-type cytochromes are predicted to be in the *P. aerophilum* genome (11). One is a 21.8-kDa subunit of a putative nitrite reductase (PAE3598), and the other is a 19.4-kDa subunit of a putative bc_1 complex (PAE1347). Both cytochromes contain a signal peptide sequence (SOSUIsignal, http://sosui.proteome.bio.tuat.ac.jp/sosuisignal/sosuisignal_submit.html) and are predicted to be associated with the cytoplasmic membrane.

Enzyme activities. The NADH-dependent ferric reductase activity in WCE fractions was 100-fold higher in iron-grown cells than in nitrate-grown cells, suggesting that the iron respiration mechanism is regulated (Table 1). Specific activities in both CYT and MEM fractions from iron-grown cells were lower than those measured in the WCE fraction, suggesting that there was a disruption of the dissimilatory Fe(III) reduction mechanism with the fractionation process. There was also a 10-fold increase in the ferric reductase activity in the iron MEM fraction relative to the nitrate MEM fraction. Rates with NADPH as the electron donor were not significantly different from those with NADH as the donor (data not shown). Nitrate reductase activity decreased 10- and 106-fold in WCE and MEM fractions, respectively, from iron-grown cultures relative to nitrate-grown cultures (Table 1). This corroborated with the nitrate reductase abundance data obtained from the membrane protein composition comparison (Fig. 4A). Nitrate reductase activities in the CYT fractions were unchanged in nitrate- and iron-grown cultures and most likely represented the assimilatory nitrate reductase.

The oxidation rates of AHDS to AQDS at 80°C were 236- and 580-fold higher in MEM and WCE fractions, respectively, from iron-grown cells relative to their nitrate-grown counterparts (Table 1). The reaction was primarily associated with the MEM fraction (>95%) with little activity in the CYT fraction. Since electrons from AHDS can be transferred abiotically to Fe(III), controls were run to determine whether this is a biotic reaction. The oxidation rate in iron MEM fractions decreased >99% when assayed at room temperature (Fig. 5) and 67% at 80°C following overnight digestion with trypsin. There was also a minor black precipitate associated with the iron MEM fraction (but not the nitrate MEM fraction) that could have served as an oxidant. However, all of the AHDS oxidase activity re-

mained within the supernatant following removal of the precipitate by ultracentrifugation from the iron MEM fraction. These data suggest that the reaction was enzymatic rather than abiotic, although the physiological electron acceptor is unknown. Oxidation rates were not significantly different when 0.5 to 1.0 mM DT was used in lieu of H_2 and palladium to reduce the AQDS to AHDS (data not shown).

DISCUSSION

P. aerophilum grew on Fe(III) citrate and Fe(III) oxide but not on hematite and goethite, which was also the case for *P. islandicum* (14). The growth rates and maximum cell concentrations for cultures grown on soluble iron were higher than for those grown on insoluble iron, but the growth yields were the same for both terminal electron acceptors. Therefore, it appeared that the same quantity of electrons are passed to iron per cell doubling regardless of the form of iron used and that the differences in growth rates and maximum cell concentrations may be due to the accessibility of the iron. The total amounts of Fe(II) produced were comparable to those measured for other hyperthermophilic iron-reducing archaea (15, 16).

P. aerophilum reduced Fe(III) oxide when it was segregated spatially by dialysis tubing, suggesting that the organism used an extracellular mediator for insoluble iron reduction. There was no detectable Fe(III) [or Fe(II)] outside of the dialysis tubing following growth, which would be expected if the organism were using a chelator (26, 27). The cultures were grown in media rich in organic compounds that could have served as an extracellular mediator of iron reduction. Low nutrient availability was shown to limit Fe(III) oxide reduction in *Shewanella putrefaciens* (12). However, the addition of cell-free spent medium eliminated the growth lag found without it, suggesting that a *P. aerophilum*-produced extracellular mediator was necessary rather than a compound from the growth medium. Similar results were used to justify the existence of an extracellular mediator in *S. algae* (27). Furthermore, up to seven compounds were found in spent media of Fe(III) oxide-grown cultures that were at lower concentrations or absent from other spent media or the controls, suggesting that they are produced more during insoluble iron reduction. One peak with a retention time of 13.9 min was present in spent Fe(III) oxide and Fe(III) citrate media but was absent in the other samples. Ongoing experiments will determine whether any of these peaks contain compounds that are redox active and capable of serving as an electron shuttle or chelator.

Specific Fe(III) reduction activity in WCE, CYT, and MEM fractions increased approximately 100-, 35-, and 9-fold, respectively, in iron-grown cultures relative to nitrate-grown cultures. At the same time, the specific nitrate reductase activity in the MEM fraction decreased approximately 100-fold in iron-grown cultures relative to nitrate-grown cultures. These results suggest that dissimilatory iron reduction in *P. aerophilum* was regulated and that iron reductase replaced nitrate reductase as the primary terminal electron transfer enzyme. Like *P. islandicum*, the majority of the NAD(P)H-dependent Fe(III) reductase activity in *P. aerophilum* (88%) was measured in the soluble protein fraction. This may be due in part to the substrates used to measure activity. Other potential electron donors (e.g., reduced AQDS, DT, reduced benzyl viologen) were unsuccessful, since they spontaneously and abiotically reduced the iron present. However, there was clearly some level of NADH-dependent Fe(III) reduction occurring in the well-washed membranes that may be a part of the electron transport chain. Future experiments will explore the characteristics of this enzyme. Unusual AHDS oxidase activity was also measured almost entirely (>95%) in the MEM fraction of iron-grown cultures that was nearly absent in nitrate-grown cultures. Its electron acceptor and relationship with iron reduction are unknown but appear to demonstrate that there is some iron reduction-related enzyme or compound present within the membranes that readily transfers electrons from and possibly to soluble electron carriers. Specific nitrate reductase activities were also significantly higher than those reported previously for *P. aerophilum* (2, 3). This may be due to the preparation of the MEM fractions under anoxic conditions and the presence of reducing agents within the buffer.

Polyheme, *c*-type cytochromes are used for electron transport across the cell wall, and certain cytochromes are required for iron reduction in *Shewanella* and *Geobacter* species (4, 6, 17, 19, 23, 24, 29). A search for CXXCH heme-binding motifs throughout the *P. aerophilum* genome (11) showed that 13 open reading frames (ORFs) contained a single copy of this sequence and one other ORF contained two copies. Therefore, there does not appear to be any polyheme cytochromes within this organism. The genome sequence does contain two ORFs that encode for putative *c*-type monoheme, cytochrome-containing proteins. They are each part of operons that encode for a bc_1 complex (PAE1347) and nitrite reductase (PAE3598). Nitrite reductase activity in *P. aerophilum* has previously been localized to the cytoplasmic membrane (3). It appears that the larger *c*-type cytochrome found in the nitrate MEM fraction and not the iron MEM fraction is part of this enzyme complex. The other *c*-type cytochrome found in both fractions may be part of the bc_1 complex. On the basis of its genome sequence (11), *P. aerophilum* appears to generate ATP via oxidative phosphorylation using a membrane-bound NADH dehydrogenase complex (PAE1567 to PAE1582 and PAE2926 to PAE2928), the cytochrome bc_1 complex (PAE1347 to PAE1350), and the H^+-translocating membrane-bound ATP synthase (PAE0661 to PAE0663, PAE0758, PAE1146, and PAE1773). Nitrate reductase and menaquinol:NO oxidoreductase are membrane-bound terminal reductases that have been purified and characterized from *P. aerophilum* and likely help to complete the electron transport pathway in this organism when it is grown on nitrate (2, 10).

In conclusion, both soluble and insoluble forms of iron can be reduced, and direct contact with insoluble iron is not necessary for reduction. Our experimental results suggest that an electron shuttle, not a chelator, was used as the mediator of extracellular iron reduction. In this sense, dissimilatory iron reduction in *P. aerophilum* is similar to that found in *Shewanella* and *Geothrix* species. However, there also appears to be a novel dissimilatory iron reduction mechanism present within this hyperthermophilic archaeon that is regulated but does not use *c*-type polyheme cytochromes. The lack of polyheme cytochromes distinguishes it from iron reduction found in *Shewanella* and *Geobacter* species. Hyperthermophiles may have evolved in relative isolation from the other organisms perhaps due to their existence within so-called extreme environments. Therefore, dissimilatory iron reduction in

P. aerophilum may represent a new category of dissimilatory iron reduction that might be useful by comparison for determining the natural history of this metabolic process.

ACKNOWLEDGMENTS

We thank Kazem Kashefi, Xinlei Qian, and Derek Lovley for their helpful suggestions and provision of hematite and goethite, Kwasi Antwi and Richard Vachet for their liquid chromatographic analyses, and the Mass Spectrometry Facility at the University of Massachusetts for their MALDI-TOF analysis. We also thank the anonymous reviewers of the manuscript for their helpful and insightful comments.

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