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Expression of a Tetraheme Protein, *Desulfovibrio vulgaris* Miyazaki F Cytochrome c_3, in *Shewanella oneidensis* MR-1

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Cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki F was successfully expressed in the facultative aerobe *Shewanella oneidensis* MR-1 under anaerobic, microaerophilic, and aerobic conditions, with yields of 0.3 to 0.5 mg of cytochrome/g of cells. A derivative of the broad-host-range plasmid pRK415 containing the cytochrome c_3 gene from *D. vulgaris* Miyazaki F was used for transformation of *S. oneidensis* MR-1, resulting in the production of protein product that was indistinguishable from that produced by *D. vulgaris* Miyazaki F, except for the presence of one extra alanine residue at the N terminus.

Cytochromes c_3 are low-potential tetraheme proteins found almost exclusively in anaerobic bacteria, including sulfate-reducing bacteria in the genus *Desulfovibrio*. These cytochromes (either cell free or in vivo) have many potential uses, including pollutant degradation (6) and bioelectronics (4, 9), so an efficient production system would be very useful. One approach has been to take the cytochrome c_3 (*cyc*) genes from *Desulfovibrio vulgaris* from which it was cloned (5, 17) and express it in other organisms (1, 11, 16). Such an approach has met with considerable difficulty, although some success has been obtained in several systems, including *Rhodobacter sphaeroides* (1) and another species of *Desulfovibrio* (16). However, although expression was obtained, a user-friendly system for protein production has not yet been achieved. Because *Shewanella oneidensis* MR-1 is known to produce cytochrome c_3 (14), and because it is a facultative aerobe that can be easily and rapidly grown to high cell densities, we reasoned that it might be useful as an expression vehicle, and we report here that the gene for cytochrome c_3 from *D. vulgaris* Miyazaki F can be expressed under either aerobic or anaerobic conditions in MR-1.

Cell growth and reagents. *S. oneidensis* MR-1 (formerly called *Shewanella putrefaciens* MR-1 [15]) and its rifampin-resistant strain, TSP-C, were cultured aerobically overnight at 30°C using Luria-Bertani (LB) liquid medium, and rifampin was added at 10 μg/ml for the TSP-C strain. For anaerobic cultures of MR-1, glass bottles with butyl rubber caps containing degassed LB media with 30 mM sodium fumarate as the terminal electron acceptor were used. All enzymes, as well as low- and high-gelling-temperature agaroses, were obtained from TaKaRa Shuzo Co., Ltd. (Kyoto, Japan), while radioactive compounds were purchased from ICN Biomedicals Inc. (Irvine, Calif.) and were used for dideoxynucleotide sequencing. Molecular mass markers for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, Calif.) and Amersham Pharmacia Biotech (Uppsala, Sweden). Both polyvinylidene difluoride membranes (0.2-μm pore size) and horseradish peroxidase color detection reagents (goat anti-rabbit immunoglobulin G secondary antibody conjugated with horseradish peroxidase, 4-chloro-1-naphthol, and hydrogen peroxide) used for Western blotting analysis were obtained from Bio-Rad Laboratories. Albumen was purchased from Seikagaku Corporation (Tokyo, Japan). Columns (SP-Sepharose [2.6 by 10 cm] and Hiload Superdex 75 [2.6 by 60 cm]) were purchased from Amersham Pharmacia Biotech. All other reagent-grade chemicals and antibiotics were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Preparation of plasmids and transfer of the *cyc* genes to MR-1. Two plasmids (pRKM3F and pRKMα) were used in these studies, giving essentially identical results. The former contained the wild-type cytochrome c_3 gene from *D. vulgaris* Miyazaki F, while the latter contained the same gene modified with regard to codon usage by *R. sphaeroides* (K. Ozawa et al., unpublished results). Both plasmids were derivatives of plasmid pRK415 (3) and were constructed as follows.

Plasmid pKM300 containing the *cyc* gene from *D. vulgaris* Miyazaki F (900-bp *Aat*II-*Sph*I fragment in the *Aat*II-*Sph*I site of pUC18) (5) was digested with *Aat*II and *Sph*I. The resultant *Aat*II-*Sph*I fragment (900 bp) was treated with T4 DNA polymerase to produce blunt ends and was ligated into the *Sma*I site of plasmid pUC118. The plasmid containing the cytochrome c_3 gene in the opposite direction to the *lac* promoter was selected. The constructed vector was called pUKM300. Plasmid pUKM300 was then digested with *Xba*I and *Eco*RI, and the 900-bp fragment was isolated from the gel and ligated into the vector pRK415. The ligation product was transformed into *Escherichia coli* JM109 and plated onto LB plates with 15 μg of tetracycline per ml, 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactoside) per ml, and 0.1 mM IPTG (isopropyl-α-D-(−)-thiogalactopyranoside. Recombinant plasmids were isolated from white colonies and analyzed by restriction digestion with *Xba*I and *Eco*RI, resulting in excision of the *cyc* gene as a 900-bp fragment. The recombinant plasmid, pRKM3F, was then used for transformation of MR-1.

Both pRKM3F and pRKMα were transformed into *E. coli* S17-1 (13) and subsequently transferred to *S. oneidensis* TSP-C by conjugation. In order to confirm the presence of the *cyc* gene from *D. vulgaris* Miyazaki F, the soluble protein fractions of *S. oneidensis* MR-1 and the exconjugant *S. oneidensis* TSP-C (pRKMα) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coo-

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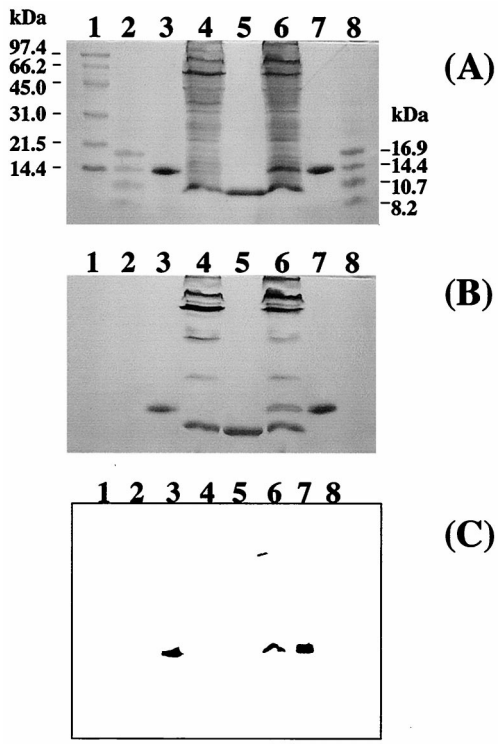

FIG. 1. SDS-PAGE analyses of cytochrome c_3 in *S. oneidensis*. Cell preparations were loaded onto SDS–15% PAGE gels and electrophoresed. (A) Protein staining with CBB; (B) heme staining with *o*-tolidine dihydrochloride heme stain prepared as previously described (2); and (C) antigenically active material via Western blotting with antibody to cytochrome c_3 from *D. vulgaris* Miyazaki F. Lanes 3, 5, and 7 were loaded with ca. 0.005 mg of protein, and lanes 4 and 6 were loaded with ca. 1 mg of protein. Lane 1, high-molecular-mass markers (phosphorylase *b* [97.4 kDa], bovine serum albumin [66.2 kDa], ovalbumin [45 kDa], carbonic anhydrase [31 kDa], soybean trypsin inhibitor [21.5 kDa], and lysozyme [14 kDa]); lanes 2 and 8, low-molecular-mass markers (globin [16.95 kDa], globins I and II [14.4 kDa], globins I and III [10.7 kDa], and globin I [8.16 kDa]); lanes 3 and 7, wild-type cytochrome c_3 from *D. vulgaris* Miyazaki F; lane 4, cell lysate from *S. oneidensis* MR-1; lane 5, wild-type cytochrome c_3 from *S. oneidensis* MR-1; lane 6, cell lysate from *S. oneidensis* TSP-C(pRKMα).

massie brilliant blue R-250 (CBB) (Fig. 1A) for proteins or *o*-tolidine dihydrochloride for hemes (Fig. 1B). Comparison of lanes 4 and 6 in Fig. 1A and B shows that *S. oneidensis* TSP-C(pRKMα) expressed the product of the *D. vulgaris* Miyazaki F cytochrome c_3 gene as a *c*-type cytochrome of 14 kDa. The position of the band of interest in lane 6 was identical to the *D. vulgaris* Miyazaki F cytochrome c_3 marker (lanes 3 and 7) and clearly was different from that of cytochrome c_3 from *S. oneidensis* (lane 5). The 14-kDa band seen in lane 6 was increased approximately twofold when the concentration of tetracycline in the culture of *S. oneidensis* TSP-C(pRKMα) was raised from 15 to 30 μg/ml (data not shown). Heme staining of the gel (Fig. 1B) revealed a variety of *c*-type cytochromes present in *S. oneidensis* MR-1, consistent with previous reports (7). Western blot analysis of these bands was also performed using anti-*D. vulgaris* Miyazaki F cytochrome c_3 serum (Fig. 1C). Cross-reactivity was revealed for the bands in lanes 3, 6, and 7. The 14-kDa band in lane 6 indicates the presence of *D. vulgaris* Miyazaki F cytochrome c_3 in *S. oneidensis* TSP-C, while the absence of cross-reactivity in the negative controls (Fig. 1C, lanes 4 and 5) indicates that the serum has no cross-reactivity with cytochromes produced by MR-1.

Isolation and characterization of recombinant *D. vulgaris* Miyazaki F cytochrome c_3 from *S. oneidensis*. Recombinant *D.*

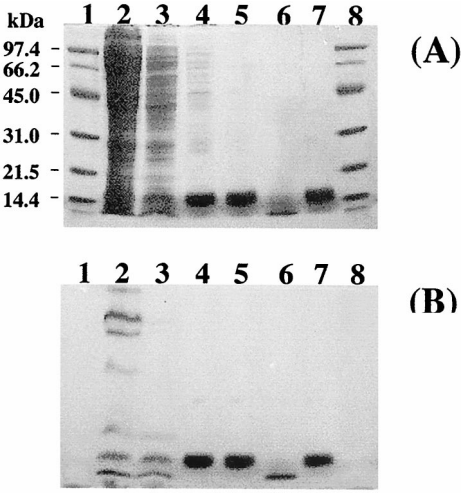

FIG. 2. SDS-PAGE analyses of recombinant cytochrome c_3 in the purification process. Enzyme preparations were loaded onto SDS–15% PAGE gels and electrophoresed. Two different treatments are shown, CBB staining (A) and heme staining (B). Lane 2 was loaded with ca. 1 mg of protein, lane 3 was loaded with 0.3 mg of protein, lanes 4, 5, and 7 were loaded with ca. 0.01 mg of protein, and lane 6 was loaded with 0.005 mg of protein. Lanes 1 and 8, high-molecular-mass markers that are the same as those in lane 1 of Fig. 1; lane 2, supernatant of *S. oneidensis* TSP-C(pRKMα) extract; lane 3, supernatant after dialysis; lane 4, cytochrome c_3 fraction after SP-Sepharose column chromatography; lane 5, cytochrome c_3 fraction after Superdex 75 column chromatography; lane 6, *S. oneidensis* cytochrome c_3 marker; lane 7, *D. vulgaris* Miyazaki F cytochrome c_3 marker.

vulgaris Miyazaki F cytochrome c_3 was purified (Fig. 2, lane 5) from a supernatant obtained after centrifugation of the broken-cell supernatant treated with streptomycin sulfate (0.16 g per g of cells). Purification was carried out at 4°C and pH 7.0. The recombinant cytochrome c_3 was purified in two steps. First, after dialysis against 10 mM sodium phosphate buffer, the supernatant was loaded onto an SP-Sepharose column (2.6 by 10 cm) previously equilibrated with the same buffer. Under these conditions, *D. vulgaris* Miyazaki F cytochrome c_3 (pI = 10.6) binds to the ion-exchange resin, while endogenous *S. oneidensis* cytochrome c_3 (pI = 5.8) is eluted together with other proteins. A gradient of 0 to 500 mM NaCl in 10 mM sodium phosphate buffer was then used to remove the *D. vulgaris* Miyazaki F cytochrome c_3, which was eluted at 150 mM NaCl. Second, the eluted cytochrome c_3 fraction was further purified by gel filtration on fast protein liquid chromatography system (Amersham Pharmacia Biotech) using a Hiload Superdex 75 column (2.6 by 60 cm) equilibrated with 50 mM NaCl–10 mM sodium phosphate buffer. Relative purity was confirmed by the absence of other bands after SDS–15% PAGE using CBB staining and a purity index (A_{552}red/A_{280}ox of ≅3.0.

The UV and visible spectra were recorded with a Shimadzu UV-2200 spectrophotometer at room temperature. The recombinant *D. vulgaris* Miyazaki F cytochrome c_3 showed a peak at 409 nm and a broad band centered at 530 nm in the oxidized state and peaks at 552, 523, and 419 nm in the dithionite-reduced state in 10 mM sodium phosphate buffer (Table 1), identical with those of the wild-type cytochrome c_3 isolated from *D. vulgaris* Miyazaki F (18).

The sequence of the N-terminal 15 amino acid residues of the recombinant *D. vulgaris* Miyazaki F cytochrome c_3 purified from *S. oneidensis*(pRKMα) was determined by sequential Edman degradation. The sequence was identical to that of wild-type cytochrome c_3 (12) isolated from *D. vulgaris* Miyazaki F,

TABLE 1. Properties of cytochromes c_3 from different sources

Host organism	UV spectra[a]	*D. vulgaris* Miyazaki F Ab[b]	N terminus[c]	M_w	pI
D. vulgaris Miyazaki F	409, 419, 523, 552	+	APKAPADGLKM	14,000	10.6
MR-1(pRKM3F)	409, 419, 523, 552	+	AAPKAPADGLKM	14,000	Alkaline
S. oneidensis MR-1	408, 419, 522, 551	−	ADQKLSDFHAES	12,000	5.8

[a] The first value is the peak in the oxidized state, and the other values are the peaks in the dithionite-reduced state.
[b] Interaction with antibody (Ab) against *D. vulgaris* Miyazaki F.
[c] The N-terminal sequences were determined with a Shimadzu PPSQ-10 protein sequencer coupled to a Shimadzu C-R7A analyzer.

except for the addition of an extra alanine at the N terminus, and distinctly different from that reported for cytochrome c_3 from *S. oneidensis* MR-1 (Table 1).

For nuclear magnetic resonance (NMR) studies, the sample was lyophilized three times with 99.9% ^2H_2O and resuspended in deuterated 10 mM sodium phosphate buffer, pH 7.0. One-dimensional ^1H NMR spectra at 600 MHz were recorded at 303 K on a Bruker DRX-600 NMR spectrometer. The NMR spectra of the oxidized form of wild-type and recombinant cytochromes c_3 in the low-field region (10 to 40 ppm, i.e., the region most commonly used to detect chemical or physical changes in cytochromes) were virtually identical to, but easily distinguishable from, that of the cytochrome c_3 from *S. oneidensis* (data not shown). The macroscopic redox potentials of the recombinant cytochrome c_3 were identical to those of the wild-type *D. vulgaris* Miyazaki F cytochrome c_3 (10). Furthermore, the recombinant cytochrome c_3 easily could be reduced by hydrogen in the presence of *D. vulgaris* Miyazaki F hydrogenase, just like the wild-type cytochrome c_3. On the basis of these data, we conclude that the expressed protein is fully functional and identical to the wild-type *D. vulgaris* Miyazaki F cytochrome c_3, except for the additional alanine residue at the N terminus.

Yield of recombinant *D. vulgaris* Miyazaki F cytochrome c_3. Cells were grown under three different conditions: (i) aerobic, with strong aeration in a 5-liter fermentor; (ii) microaerobic, with intermediate aeration (2 liters of culture in a 3-liter Erlenmeyer flask); and (iii) anaerobic, with fumarate as the terminal electron acceptor. Yields of cytochrome c_3 were compared with those obtained during anaerobic growth of *D. vulgaris* (Table 2). On a per-weight basis (milligrams of cytochrome per gram [wet weight] of cells), *D. vulgaris* yielded 0.15 mg, while *S. oneidensis*(pRKMα) yielded 0.29 mg under aerobic conditions and 0.5 mg under microaerobic or anaerobic conditions.

Per liter of culture, *D. vulgaris* yielded 0.3 mg of cytochrome c_3. In comparison, *S. oneidensis*(pRKMα) yielded 1.9 mg aerobically and 1 mg for microaerobic and anaerobic culture. These results reflect the major differences obtained in growth yield under these different conditions. Because of the ease of aerobic growth, high cell yield, and good production of cytochrome c_3, this system offers an easy and efficient vehicle for cytochrome c_3 production.

TABLE 2. Cytochrome c_3 production under different conditions

Host strain (condition)	Cytochrome c_3 concn	
	mg/g of cells	mg/liter of culture
D. vulgaris Miyazaki F (anaerobic)	0.15	0.30
D. desulfuricans G200 (anaerobic)	0.2[a]	
S. oneidensis TSP-C (aerobic)	0.29	1.9
S. oneidensis TSP-C (microaerobic)	0.5	1.0
S. oneidensis TSP-C (anaerobic)	0.5[b]	1.0[b]

[a] Expression of the *D. vulgaris* Hildenborough *cyc* gene (16).
[b] Similar to the amounts obtained under microaerobic conditions, judged from the intensity of the bands on SDS-PAGE.

The system may also have utility for the expression of other multiheme protein genes, because *Shewanella* produces a variety of *c*-type multiheme cytochromes of its own (7, 14), suggesting that it has a very good heme ligase system. MR-1 is capable of reduction of both elemental sulfur and thiosulate (8), an unusual ability even for many anaerobes, and mutants lacking cytochromes *c* are unable to reduce these compounds. Furthermore, even though *D. vulgaris* Miyazaki F and *S. oneidensis* belong to different groups of the *Proteobacteria* (delta and gamma, respectively) based on 16S rRNA analysis, MR-1 was able to transcribe the genes efficiently, with or without codon modification (data not shown).

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