The cymA Gene, Encoding a Tetraheme c-Type Cytochrome, Is Required for Arsenate Respiration in Shewanella Species

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Received 2 November 2006/Accepted 21 December 2006

In Shewanella sp. strain ANA-3, utilization of arsenate as a terminal electron acceptor is conferred by a two-gene operon, arrAB, which lacks a gene encoding a membrane-anchoring subunit for the soluble ArrAB protein complex. Analysis of the genome sequence of Shewanella putrefaciens strain CN-32 showed that it also contained the same arrAB operon with 100% nucleotide identity. Here, we report that CN-32 respines arsenate and that this metabolism is dependent on arrA and an additional gene encoding a membrane-associated tetraheme c-type cytochrome, cymA. Deletion of cymA in ANA-3 also eliminated growth on and reduction of arsenate. The ΔcymA strains of CN-32 and ANA-3 negatively affected the reduction of Fe(III) and Mn(IV) but not growth on nitrate. Unlike the CN-32 ΔcymA strain, growth on fumarate was absent in the ΔcymA strain of ANA-3. Both homologous and heterologous complementation of cymA in trans restored growth on arsenate in ΔcymA strains of both CN-32 and ANA-3. Transcription patterns of cymA showed that it was induced under anaerobic conditions in the presence of fumarate and arsenate. Nitrate-grown cells exhibited the greatest level of cymA expression in both wild-type strains. Lastly, site-directed mutagenesis of the first Cys to Ser in each of the four CXXCH c-heme binding motifs of the CN-32 CymA nearly eliminated growth on and reduction of arsenate. Together, these results indicate that the biochemical mechanism of arsenate respiration and reduction requires the interactions of ArrAB with a membrane-anchored tetraheme cytochrome, which in the non-arsenate-respiring Shewanella species Shewanella oneidensis strain MR-1, has pleiotropic effects on Fe(III), Mn(IV), dimethyl sulfoxide, nitrate, nitrite, and fumarate respiration.

Metal-reducing bacteria can significantly impact the fate and transport of arsenic in sediments and groundwater (2, 10–12, 35). Reduction of iron oxides containing arsenate [HASO$_4^{2-}$; As(V)] can liberate arsenite [H$_3$AsO$_3$; As(III)] into porewaters, leading to the contamination of aquifers and groundwater (4). Arsenate reduction under these conditions is most likely due to As(V)-respiring prokaryotes, which are known to be diverse and to utilize a variety of electron acceptors, including Fe(III) and Mn(IV) (hydr)oxides (17, 24, 25).

In the metal-reducing bacterium Shewanella sp. strain ANA-3, two genes, arrA and arrB, are required for arsenate respiration (28). The gene products, ArrA, a ∼95-kDa molybdenum-containing arsenate reductase subunit, and ArrB, an ∼26-kDa Fe-S-containing subunit, are soluble and localized to the periplasm (1, 15, 16). Based on several biochemical features (e.g., size, Mo cofactor, amino acid sequence similarity, and cofactor binding motifs), ArrA is part of a large family of molybdenum-containing oxidoreductases (e.g., dimethyl sulfoxide, nitrate, polysulfide, and trimethylamine N-oxide [TMAO] reductases) (18). Other prokaryotic molybdenum cofactor-containing oxidoreductases employ a membrane protein, which serves as a site for anchoring the soluble Mo and Fe-S subunits to the cytoplasmic membrane (18). In some cases, the membrane-anchoring subunit also functions to transfer electrons from the quinone pool to the catalytic subunits (6). Several of these membrane-associated subunits have been shown to be c-type cytochromes (16, 20, 26). In the non-As(V)-respiring Shewanella species Shewanella oneidensis strain MR-1, a 21-kDa membrane-associated periplasmic c-type cytochrome, CymA, is required for respiration of fumarate, nitrate, Fe(III), dimethyl sulfoxide, and nitrite (20, 23, 32). CymA is part of the NapC/NirT family of tetraheme cytochromes, which, unlike CymA, provide more specialized functions in other bacteria (20). For example, in Paracoccus denitrificans, the NapC protein transfers electrons from the quinols to the periplasmic nitrate reductase (26). In contrast to most members of the NapC/NirT tetraheme cytochromes, CymA acts as a common branching point in the electron transport chain of Shewanella species by serving as a redox intermediary between the quinone pool and multiple terminal reductases (32).

Because the arr operon of Shewanella sp. strain ANA-3 lacks a gene encoding a membrane subunit for ArrAB, it was predicted that CymA would be involved in the As(V) respiratory pathway in As(V)-respiring Shewanella species. Here, we investigate the functional role of cymA in the As(V) respiratory reduction pathways of several Shewanella species.

MATERIALS AND METHODS

Strains and plasmids. All Escherichia coli and Shewanella strains and plasmids used in this study are described in Table 1. Shewanella sp. strain W3-18-1 was a kind gift from Jizhong Zhou at the University of Oklahoma.

Growth conditions. Standard culturing of ANA-3, CN-32, and E. coli strains was done in Luria-Bertani medium (31) or a minimal medium (described below). The ANA-3 and CN-32 strains were grown at 30°C, and liquid cultures were shaken at 250 rpm. Anaerobic culturing was done in a minimal medium (TME, pH 7) consisting of 1.5 g liter$^{-1}$ NaHPO$_4$, 0.1 g liter$^{-1}$ NaHPO$_4$, 0.1 g liter$^{-1}$ KCl, 0.5 g liter$^{-1}$ yeast extract, 10 mM HEPES, 20 mM lactate, and 10 ml liter$^{-1}$ (each) trace mineral and vitamin solutions (13). The medium was boiled under a stream of N$_2$, anaerobically dispensed into N$_2$-flushed Balch tubes, sealed with...
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or markers, characteristics, and uses</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOPO Top 10</td>
<td>E. coli host for cloning; F- mcrA Δ(mrr-hsdRMS-merBC) ΔlacZΔM15 ΔlacY4 recA1 araD139 Δ(ara-leu)Δ7697 galU galK rpsL (Str*) endA1 mupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>UQ950</td>
<td>E. coli DH5α (pir) host for cloning; F- Δ(argF-lac)169 Δ(8845lacZ58ΔM15) glnV44 (AS)</td>
<td>28</td>
</tr>
<tr>
<td>WM3064</td>
<td>Donor strain for conjugation; thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-1360 Δ(araBAD)Δ567</td>
<td>28</td>
</tr>
<tr>
<td><strong>Shewanella strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shewanella sp. ANA-3</td>
<td>Isolated from an As-treated wooden pier clamping in a brackish estuary (Eel Pond, Woods Hole, MA)</td>
<td>27</td>
</tr>
<tr>
<td>AN-CYMA</td>
<td>Shewanella sp. strain ANA-3; ΔcymA; does not respire As(V) or fumarate</td>
<td>This study</td>
</tr>
<tr>
<td>S. putrefaciens CN-32</td>
<td>Isolated from anaerobic subsurface core sample, New Mexico</td>
<td>8, 34</td>
</tr>
<tr>
<td>CN-CYMA</td>
<td>S. putrefaciens CN-32; ΔcymA; does not respire As(V)</td>
<td>This study</td>
</tr>
<tr>
<td>Shewanella sp. W3-18-1</td>
<td>Isolated from Pacific Ocean marine sediments at 630 m</td>
<td>19</td>
</tr>
<tr>
<td><strong>Plasmids/vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR4-TOPO</td>
<td>4-kb cloning vector; lacZ-ccdB; Km' Amp'</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSMV10</td>
<td>9.1-kb mobilizable suicide vector; oriR6K mobRP4 sacB Km' Gm'</td>
<td>28</td>
</tr>
<tr>
<td>ANpΔcymA</td>
<td>1.2-kb fusion PCR fragment containing ΔcymA from ANA-3 cloned into the SpeI site of pSMV10; used to make the AN-CymA ΔcymA strain</td>
<td>This study</td>
</tr>
<tr>
<td>CNpΔcymA</td>
<td>2-kb fusion PCR fragment containing ΔcymA from S. putrefaciens cloned into the SpeI site of pSMV10; used to make the AN-CymA ΔcymA strain</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1MCS-2</td>
<td>5.1-kb broad-host-range plasmid; Km' lacZ</td>
<td>14</td>
</tr>
<tr>
<td>pANcymA</td>
<td>AN-3 cymA PCR fragment, including the promoter region, cloned into the SpeI site of pBBR1MCS-2</td>
<td></td>
</tr>
<tr>
<td>pCNcymA</td>
<td>S. putrefaciens cymA PCR fragment cloned into the SpeI sites of pBBR1MCS-2</td>
<td></td>
</tr>
<tr>
<td>pH 1(C46S)</td>
<td>pCNcymA plasmid with SXXCH of heme 1</td>
<td>This study</td>
</tr>
<tr>
<td>pH 2(C78S)</td>
<td>pCNcymA plasmid with CXXSH of heme 2</td>
<td>This study</td>
</tr>
<tr>
<td>pH 3(C130S)</td>
<td>pCNcymA plasmid with SXXCH of heme 3</td>
<td>This study</td>
</tr>
<tr>
<td>pH 4(C178S)</td>
<td>pCNcymA plasmid with SXXCH of heme 4</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Butyl rubber stoppers, and autoclave sterilized. Media were supplemented with 10 mM fumarate, nitrate, TMAO, or arsenate or 25 mM hydroxyl ferric oxide from sterile anaerobic stock solutions.</strong></td>
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</table>

**Growth experiments.** Aerobic cultures were grown overnight in TME medium. The optical density (OD) at 600 nm of each culture was brought below 0.6, and they were standardized to each other by the addition of TME medium to ensure that the inoculation levels for all strains were equal. The cells were then inoculated at 1:100 dilution into anaerobic tubes. Growth was monitored by measuring the OD at 600 nm using a Spectronic 20. Control cultures were also grown and monitored in aerobic TME without added electron acceptors.

The ferrozine assay was used to monitor Fe(III) reduction. Under anaerobic conditions, 50 µl of cell culture was added to 450 µl 0.5 M HCl, mixed, and filtered through a 0.2-µm filter (Spin-X column). 10 µl of this mixture was then added to an additional 90 µl 0.5 M HCl in a 96-well microtiter dish. Ferrozine solution (100 µl of 0.1% ferrozine dissolved in 50% [w/v] ammonium acetate solution) was added, and the absorbance at 540 nm was read after 10 min.

Sample absorbances were compared to standard curves of known ferrous chloroform solution (with corresponding accession numbers in brackets). Because of the limited sequence data at the time of this work, two ~400-bp DNA fragments flanking the cymA gene were amplified (the complete ANA-3 genome sequence is now available).

The complementation plasmids pCNcymA and pANcymA were generated by cloning PCR products of the CN-32 and ANA-3 cymA genes into the SpeI site of pBBR1MCS-2. The following primers were used to generate the genes: CNcymA-1-F (SpeI site) for CN-32, ANcymA-1-F (SpeI site) for ANA-3, and cymA-R2, 5'-GGA CTA GTC AAG CAT TTA CTG TTA TGT

The resulting sequence was then used to design primers for inverse PCR and sequencing (ANcymA1PCR-F1, 5'-AGA ACC AGC CAG ACA CTA TG-3' and ANA-CYM1PCR-R1, 5'-GTG TGG TAA GTG GCA GTC TT-3') of BamHI-digested and self-ligated ANA-3 genomic DNA. This PCR product was cloned using the TA TOPO cloning kit, and the resulting plasmids were sequenced using ANA-CymA1PCR-F1 and ANA-CymA1PCR-R1. Primer walking and sequencing were done using the primers ANA-CymA1PCR-F2, 5'-GCT GCA GGA GAA TAA TAG GTT TTA GCG-3' and cymA1PCR-R2, 5'-TAA TAC GAC AAC TGG CTC AA-3'.

Mutagenesis. In-frame, nonpolar deletions of cymA were generated using previously developed methods (28) with primers X-CNcymA-A (5'-GAAGA CGT TCA AAC CGC CAA AAA TAA AA3'), X-CNcymA-B (5'-[TGG TTA AGC ATG CTA GTG GAT GGG] AAA GGA TAA TAG GTT GGG-3'), X-CNcymA-C (5'-[CCC ATC AGC CTA GAT TCA ACA] CCA GTT CAT TAC TCT ATC TCC-3'), and X-CNcymA-D (5'-[GGAGT CTA GTC TCA CGA TGA TCG ACC ACA AAT-3') for cymA in CN2 and X-CNana-CymA-A (5'-GGAGA CGT TGA TGG AAT GTC TCT ACC-3') for cymA in CN2 and X-CNana-CymA-C (5'-[TGT TTA AGC ATG CTA GAT GGG] AAA GGA TAA GTG TTA GGG CTT-3'), and X-CNana-CymA-D (5'-[GGAGA CGT TGA TGG AGG TCA ACA] CCA GTT CAT TAC TCT ATC TCC-3').

All the primers used to generate the genes: CymA-1-F (5'-GAAGA CGT TCA AAC CGC CAA AAA TAA AA3'), CymA-1-R2 (SpeI site) for ANA-3, CN-CymA-1-F (5'-GAAGA CGT TCA AAC CGC CAA AAA TAA AA3'), AN-CYM1-R2 (SpeI site) for ANA-3, and X-CNana-CymA-C (5'-[TGT TTA AGC ATG CTA GAT GGG] AAA GGA TAA GTG TTA GGG CTT-3').
vectors into *Shewanella* sp. strain ANA-3 and CN-32 was performed as previously described (28).

Mutations to the heme binding regions in CN-32 *cymA* were generated using the following modified protocol of the QuikChange Site-Directed Mutagenesis method (Stratagene). Primers CNYcyma-heme-F1 (5'-T ACG GAT CAG TCT 5'-AGT TTC CAC ATC-3') and CNYcyma-heme-F2 (5'-ACC GTT CAA TCT 5'-ACC AGC GAG 5'-ACC CTCA CCA ATC-3') were designed with a cysteine-to-serine substitution (in lowercase); the primers are numbered F1 to F4 to correspond to hemes 1 to 4, respectively. Each heme motif was mutated separately in 25-μl reaction mixtures containing 2.5 μl Pfu Turbo Hotstart Buffer (Stratagene), 0.2 mM deoxynucleoside triphosphate mix, 125 ng primer, 50 ng pCNYcyma plasmid, 1 μl Pfu Turbo Hotstart DNA polymerase (Stratagene), and nuclease-free water. Samples were incubated with the following cycle profile: 95°C for 10 s and 18 cycles of 95°C for 10 s, 55°C for 1 min, and 68°C for 1 min. The reaction mixtures were cooled to room temperature, and 1 μl DpnI (10 units) was added to each reaction mixture and incubated for 1 h at 37°C. Each reaction mixture was transformed into a DH5α-apr strain (UQ950) (28). Plasmids were extracted and sequenced to confirm the correct mutation. The resulting heme-mutated pCNYcyma vectors were then transformed into CN-CYMA by conjugation as previously described. The plasmid strains were designated as described previously (29).

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**Quantification of cymA transcription.** The methods for quantitative reverse transcription-PCR have been described previously (29). ANA-3 cells were grown anaerobically in triplicate 10-ml TME cultures amended with 10 mM nitrate, 10 mM fumarate, or 10 mM As(V). Aerobically prepared cells were grown in 15 ml TME in 250-ml flasks with constant sparging of filtered air. Cells were harvested in mid-exponential growth phase (OD at 600 nm, 0.1). One milliliter of the culture filtrates collected during early- and mid-log and stationary phases of growth confirmed that all As(V) was reduced to As(III) (Fig. 1), with nearly 2:1 molar stoichiometry of As(V) reduction compared to lactate oxidation. All the As(V) (11 mM) was reduced to As(III), while ~6 mM lactate was oxidized to acetate (~4 mM final concentration). These results are consistent with previous growth studies with ANA-3 (27).

We confirmed the involvement of *arrA* in arsenite respiration by generating an *arrA* null mutation in CN-32. Growth on arsenite was eliminated but could be restored by complementation of the wild-type *arrA* gene in *trans* to the CN-32 *arrA* null mutant (data not shown). Strain W3-18-1 also grew with arsenite as a terminal electron acceptor (data not shown). Based on these observations, we concluded that both ANA-3 and CN-32, and most likely W3-18-1, respire arsenite via very similar mechanisms.

**Identification of cymA in ANA-3.** Based on genome sequence data in other arsenate-respiring bacteria (e.g., *Desulfitobacterium hafniense* and *Wolinella succinogenes*), their *arr* operons contain a third gene, *arrC*, which encodes a putative membrane protein that most likely anchors the *arrAB* to the cytoplasmic membrane. However, in the ANA-3, CN-32, and W3-18-1 genomes, no *ArrC* gene homologs were found. We hypothesized that CymA, a membrane-bound tetrahisteme c-type cytochrome, would be required for utilization of arsenate as a terminal electron acceptor in arsenite-respiring *Shewanella*. This predication was based on several studies that demonstrated that *cymA* was required for anaerobic respiration of many electron acceptors in *Shewanella oneidensis* strain MR-1 (20, 32).

We obtained the sequence of ANA-3 *cymA* prior to the recent release of the full genome sequence of ANA-3. PCR primers were designed from conserved regions of DNA multisequence alignments of *cymA* homologs found in other *Shewanella* genomes. The consensus primers generated an ~500-bp PCR product, which was sequenced. The predicted protein exhibited a high degree of similarity to other CymAs from various *Shewanella* species and was 100% identical to the CymA found in the ANA-3 genome sequence that is now available. Figure 2 illustrates the phylogenetic relationship of CymAs and other members of the NapC/NitT family tetrahisteme cytochromes. *Shewanella* CymAs were highly conserved...
and formed a distinct cluster within the tree of the tetraheme cytochrome family.

**Physiological effects of deleting cymA.** Because cymA has such a diverse role in the respiration pathways of *S. oneidensis*, we predicted that similar roles for cymA would be observed in both ANA-3 and CN-32 and in arsenate respiration. To test this prediction, we constructed nonpolar deletions of the cymA gene in ANA-3 and CN-32. To generate a cymA null mutant in ANA-3, additional DNA flanking the cymA gene was sequenced from an inverse PCR product of a genome fragment containing the cymA gene. This was not necessary for CN-32, because the genome sequence was available at the time of this study. Strains lacking cymA were grown anaerobically on a variety of electron acceptors. The results are summarized in Table 2. Compared to wild-type strains of ANA-3 and CN-32 that normally reach stationary phase within 10 h, neither deletion strain was able to grow when As(V) was added as an electron acceptor (Fig. 3A and B). Additionally, AN-CYMA was unable to respire fumarate; however, CN-CYMA showed growth on fumarate similar to that of the wild-type strain when grown under similar conditions. No growth defects were observed in AN-CYMA compared to the wild type when grown on TMAO. The wild-type CN-32 did not utilize TMAO as an electron acceptor. To confirm that the phenotypes of the cymA null mutants were due to the loss of cymA, both deletion strains, CN-CYMA and AN-CYMA, were complemented in trans with pCNcymA (Fig. 4A) or pANcymA (Fig. 4B), respectively. When grown anaerobically on the same substrates, the complemented strains regained the ability to respire As(V). Moreover, heterologous complementation of pCNcymA in

![TABLE 2. Growth characteristics of AN-CYMA and CN-CYMA on known terminal electron acceptors](image)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ANA-3</th>
<th>CN-32</th>
<th>MR-1&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mn(IV)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>As(V)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DMSO</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plus indicates growth on or reduction [Fe(III) and Mn(IV)] of the substrate similar to the wild type. Minus indicates lack of growth on the corresponding substrate. Lactate (20 mM) served as the electron donor and carbon source.

<sup>b</sup> Sources, references 20 and 32.

**AN-CYMA and pANcymA in CN-CYMA showed full recovery of growth on all substrates.** Strains harboring only the vector (pBBR1MCS-2) exhibited the same growth characteristics as their respective genetic backgrounds without the vector. These results provide evidence that cymA is essential to arsenate respiration and support the role of CymA as a uni-
When anaerobic growth conditions were compared, cells grown anaerobically than in cells grown aerobically (Fig. 1). The terminal electron acceptor.

To further understand this mechanism, we investigated various growth conditions that would affect the transcription of cymA. The data points and error bars represent the means and standard deviations of triplicate cultures, respectively.

**DISCUSSION**

Previous work with *Shewanella* sp. strain ANA-3 showed that arsenate respiration was conferred by *arrA* and *arrB* (28). The recent identification of *arrAB* operons in the genome sequences of two other *Shewanella* species strains, strains CN-32 and W3-18-1, suggested that these species should also respire arsenate, which should also be conferred by the *arr* operon. This was confirmed in CN-32 by deleting *arrA*, which eliminated growth on and reduction of arsenate (Fig. 1). *Shewanella* sp. strain W3-18-1 was also able to utilize arsenate.
This observation is not surprising, because the genes are nearly identical; the nucleotide identity is ~90% between the two cymA genes.

Consistent with previous studies of cymA (20, 23, 32), the substrate utilization patterns of *Shewanella cymA* null mutants exhibited pleiotropic effects on Fe(III) and Mn(IV) reduction (Table 2). However, deficiencies in fumarate and nitrate utilization differed among various *Shewanella cymA* null mutants (Table 2). Unlike in ANA-3 and MR-1, deleting cymA in CN-32 did not affect its growth on fumarate. A similar pattern was observed for nitrate respiration. ANA-3 and CN-32 cymA null mutant strains were capable of growing on nitrate; in contrast, MR-1 cymA null mutants cannot respire nitrate (23).

A Blast search for the periplasmic nitrate reductase Nap showed that the CN-32, ANA-3, and W3-18-1 genomes contain two nap loci, whereas MR-1 had only one. This additional nap locus may not involve CymA as an intermediate electron carrier for that particular nitrate reductase. In MR-1, deletion of cymA also resulted in an inability to grow on fumarate (23), but this effect was not observed in CN-32. Additional genome analyses of ANA-3, CN-32, MR-1, and W3-18-1 revealed that CN-32 and W3-18-1 contain additional orthologs of fumarate reductases/succinate dehydrogenases, unlike ANA-3 and MR-1. Similar to the additional nap locus in several *Shewanella*, it is likely that reduction of fumarate by one of these additional fumarate reductases may not involve CymA.

Because cymA is required for growth on arsenate and other terminal electron acceptors, we investigated the environmental conditions that affected its expression. Previous studies had shown that the CymA protein, along with a number of other heme-containing proteins, was present in *Shewanella* membranes isolated under anaerobic growth conditions (7, 21–23). Quantification of cymA gene expression in ANA-3 confirmed that cymA was induced under anaerobic conditions (Fig. 5). The transcription patterns suggest that cymA is regulated in response to several environmental signals; oxygen, which causes repression; and the availability of specific electron acceptors, leading to induction. cymA mRNA was most abundant (data not shown) as an electron acceptor, and it is predicted that deleting its arrA homolog would eliminate growth on arsenate.

One striking feature of the *Shewanella arr* operon is the lack of a gene encoding a membrane-anchoring subunit for ArrAB, "ArrC"; evidence for an ArrC is present in the genomes of several bacteria that contain arr genes (e.g., *Wolinella succinogenes* [locus tag, WS0763; accession no. NP_906979] and *Desulfitobacterium hafniense* strain DCB-2 [locus tag, Dhaf_0249; accession no. ZP_01372403]). This observation raised questions about how the soluble arsenate respiratory reductase, ArrAB, interacts with components of the electron transport chain. In *Shewanella*, this interaction is most likely mediated by the tetraheme c-type cytochrome CymA, which is tethered to the membrane by an N-terminal alpha helix (Fig. 6A). This conclusion is supported by gene deletion and complementation of cymA in two arsenate-respiring *Shewanella* strains, CN-32 and ANA-3 (Fig. 3 and 4). Null mutations of cymA showed that this gene was necessary for As(V) respiration. Heterologous complementation of the ANA-3 cymA null mutant with a CN-32 wild-type cymA gene (and vice versa) restored utilization of arsenate as a terminal electron acceptor in these strains.
in nitrate-grown cells of ANA-3 and CN-32, even though cymA is not essential to nitrate reduction in these strains.

The functional roles of the four hemes of CymA were addressed by examining the involvement of each heme group in arsenate respiration (Fig. 6). It was hypothesized that mutations in any of the four hemes would significantly alter arsenate respiration in Shewanella. One of the cysteines of the CXXCH heme c binding motif was changed to serine. In each of the single heme mutations, growth on arsenate was significantly diminished but not entirely eliminated. We observed an increased lag in growth on arsenate (~25 h) if the mutations occurred in the three distal CXXCH motifs nearest the C terminus of CymA. A mutation in the first CXXCH motif (heme 1) resulted in the least severe growth defect on arsenate (an ~12-h lag) (Fig. 6B). Mutational studies with the CXXCH motifs in monoheme cytochromes have shown varying outcomes for physiology, which were generally attributed to the specific cytochrome maturation machinery employed by the cell (3). In Ccm-based maturation (biogenesis system I), monoheme cytochromes usually do not mature if one of the cysteines in the CXXCH motif is replaced (3, 30). Generally, the physiological effects of a cysteine mutation in CXXCH often eliminate the particular respiration in mutant backgrounds. However, the physiological outcomes of single cysteine mutations in CXXCH in multiheme cytochromes are not well studied. In the Wolinella succinogenes nitrite reductase, NrfH, seven out of eight individual cysteine replacements resulted in elimination of nitrite respiration (33). Interestingly, one of the heme mutants (C66S) contained a heme covalently linked to the apocytochrome. This was attributed to the function of cytochrome biogenesis system II, which is present in several gram-positive bacteria and several epsilon- proteobacteria, including Wolinella succinogenes. Mutations to the histidine in the CXXCH heme binding motifs eliminate heme attachment, which usually eliminates the particular respiration (5, 9). In multiheme cytochromes, mutations in either of the cysteine or histidine residues of one CXXCH motif are likely to affect the structure and conformation of the protein. The absence of one heme could cause distortion in the packing of neighboring hemes, which could lead to secondary effects, such as disrupting a quinone-binding site and/or altering the mid-point potentials of the remaining hemes. It is doubtful that the Ccm biogenesis system (type I) of Shewanella can still attach hemes to variants of CymA containing single cysteine-to-serine mutations (italic) in the heme c binding motif (SXXCH or CXXSH). However, the unaltered CXXCH motifs in CymA may still contain covalently linked hemes. Further investigations should uncover these molecular details.

The interactions of CymA with ArrAB most likely extend to other redox partners (e.g., periplasmic nitrate and fumarate reductases). Such interactions may require conserved domains on both CymA and the cognate redox partner of the terminal reductase. Whether these same interactions and domains occur among similar terminal reductases in non-arsenate respiring Shewanella species strains (e.g., ANA-3 versus MR-1) and more broadly in arsenate-respiring non-Shewanella prokaryotes remains unknown. The conservation of CymA at the sequence and molecular levels (Fig. 2) and its physiological role in anaerobic respiration suggest that Shewanella has evolved to streamline a number of respiratory pathways by simplifying the branching point of the electron transport chain. The origin of CymA and its mechanisms of interaction with other redox partners are largely unknown.

ACKNOWLEDGMENTS

We thank the three anonymous reviewers for their constructive comments on the manuscript during the review process. We also thank Jizhong Zhou at the University of Oklahoma for providing us with Shewanella sp. strain W3-18-1.

REFERENCES


