**Shewanella oneidensis** MR-1 Restores Menaquinone Synthesis to a Menaquinone-Negative Mutant

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Received 10 February 2004/Accepted 14 May 2004

The mechanisms underlying the use of insoluble electron acceptors by metal-reducing bacteria, such as **Shewanella oneidensis** MR-1, are currently under intensive study. Current models for shuttling electrons across the outer membrane (OM) of MR-1 include roles for OM cytochromes and the possible excretion of a redox shuttle. While MR-1 is able to release a substance that restores the ability of a menaquinone (MK)-negative mutant, CMA-1, to reduce the humic acid analog anthraquinone-2,6-disulfonate (AQDS), cross-feeding experiments conducted here showed that the substance released by MR-1 restores the growth of CMA-1 on several soluble electron acceptors. Various strains derived from MR-1 also release this substance; these include mutants lacking the OM cytochromes OmcA and OmcB and the OM protein MtrB. Even though strains lacking OmcB and MtrB cannot reduce Fe(III) or AQDS, they still release a substance that restores the ability of CMA-1 to use MK-dependent electron acceptors, including AQDS and Fe(III). Quinone analysis showed that this released substance restores MK synthesis in CMA-1. This ability to restore MK synthesis in CMA-1 explains the cross-feeding results and challenges the previous hypothesis that this substance represents a redox shuttle that facilitates metal reduction.

The metal-reducing bacterium **Shewanella oneidensis** (formerly **Shewanella putrefaciens**) MR-1 has the greatest anaerobic respiratory versatility described to date. In addition to utilizing a variety of soluble compounds (e.g., fumarate, nitrate, nitrite, dimethyl sulfoxide [DMSO], trimethylamine N-oxide [TMAO], thiosulfate, and others), it also uses insoluble iron (Fe) and manganese (Mn) oxides as terminal electron acceptors (16, 22, 24, 28). This respiratory versatility is reflected in the numerous electron transport components encoded in its genome (6). The in vivo role of some of these electron transport components has been determined. For example, two components of the cytoplasmic membrane (CM), menaquinone (MK) and the tetraheme cytochrome CymA, are required for the use of several electron acceptors, including Mn(IV), Fe(III), fumarate, nitrate, DMSO, and anthraquinone-2,6-disulfonate (AQDS) (15, 17, 25, 27, 32, 37). While MK is required for thiosulfate reduction (32), CymA is not (25).

The use of extracellular insoluble electron acceptors, such as Mn(IV) and Fe(III) oxides, implies that, in addition to the requirement for the CM components MK and CymA, the cells must have a mechanism for shuttling electrons across the outer membrane (OM) to the cell surface. Two different mechanisms have been proposed as possible explanations. The first is the localization of electron transport components to the OM (2). There is significant evidence to support this mechanism in MR-1. When grown under anaerobic conditions, 80% of the membrane-bound cytochrome content is localized in the OM (14). The OM cytochromes are integral OM proteins, have c-type hemes, and can be reoxidized by Mn(III) and Fe(III) oxides (14, 18). Electron transport inhibitors block the reduction of the OM cytochromes by formate (18). Two of the OM cytochromes, OmcA and OmcB, have been purified (26, 28). Their genes encode decaheme cytochromes c with lipoprotein consensus motifs (26, 28), and both OmcA and OmcB are exposed on the cell surface (21), an arrangement that could facilitate direct contact with extracellular electron acceptors. Gene replacement mutants demonstrated a role for both OmcA and OmcB in Mn(IV) reduction (28–30). OmcA may participate in Fe(III) reduction (1), but it is not essential for Fe(III) citrate reduction (29). The absence of the OM protein MtrB results in the inability to properly localize and insert OmcA and OmcB into the OM, as well as a significant deficit in Mn(IV) and Fe(III) reduction (20).

Alternatively, the excretion of redox shuttling molecules has been proposed. MR-1 releases into the medium an unidentified compound that restores the ability of an MK-negative mutant to reduce AQDS (32). This compound is nonproteinaceous and is not MK or the MK precursor 1,4-dihydroxy-2-naphthoic acid, but it is redox active in the presence of H₂ and palladium, suggesting that it could have a quinone-type moiety (32). It was proposed that this compound represented an extracellular redox shuttle that allowed MR-1 to respire extracellular electron acceptors (32). The experiments presented in that study (32), however, did not distinguish this possibility from the possibility that this compound restored the ability of the MK-negative mutant to synthesize MK. The experiments described here are consistent with the latter possibility, i.e., that this diffusible compound represents a precursor that restores MK synthesis and thus the ability to use all electron acceptors that are dependent on MK.

### MATERIALS AND METHODS

Chemicals and reagents were obtained and prepared as described previously (17, 27).
Bacterial strains and growth conditions. A list of the bacteria used in this study is presented in Table 1. Experiments were conducted at room temperature under anaerobic conditions in a Coy anaerobic chamber (4 to 5% H$_2$; balance, N$_2$). Media were preequilibrated under anaerobic conditions before use. For some strains, the media were supplemented with kanamycin (50 $\mu$g ml$^{-1}$).

Cross-feeding experiments. The ability of MR-1 and its derived strains to cross-feed the MK-negative mutant CMA-1 was examined on plates by using the procedure described by Newman and Kolter (32). M1 defined medium (24) was supplemented with 15 mM lactate, vitamin-free Casamino Acids (0.1 g liter$^{-1}$), and one of following electron acceptors: 20 mM Fe(III) citrate, 20 mM fumarate, 20 mM TMAO, 5 mM DMSO, 3 mM thiosulfate, 2 mM nitrate, or 5 mM AQDS.

The ability of MR-1 and its derived strains to reduce Fe(III) citrate was determined by using plates that were overlaid with 0.1% (wt/vol) ferrozine plus 1.2% (wt/vol) SeaKem LE agarose (BioWhittaker, Rockland, Maine) in 50 mM HEPES (pH 7). The magenta color resulting from ferrozine-Fe(II) complexes formed immediately, and the plates were photographed as soon as the agarose overlay solidified.

### TABLE 1. Bacteria and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td>Shewanella</td>
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<td></td>
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<tr>
<td>MR-1</td>
<td>Manganese-reducing strain from Lake Oneida, N.Y., sediments</td>
<td>22</td>
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<tr>
<td>OMC1A</td>
<td>omcA mutant derived from MR-1; omcA::Km$^r$</td>
<td>28</td>
</tr>
<tr>
<td>OMCB1</td>
<td>omcB mutant derived from MR-1; omcB::Km$^r$</td>
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</tr>
<tr>
<td>MTRB1</td>
<td>mtrB mutant derived from MR-1; mtrB::Km$^r$</td>
<td>20</td>
</tr>
<tr>
<td>CMA-1</td>
<td>Acridine orange-generated mutant of MR-1 that lacks menaquinone</td>
<td>15</td>
</tr>
<tr>
<td><strong>E. coli</strong> TOP10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F' menC $\Delta$(menA::Kmr)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pCR2.1-TOPO</td>
<td>3.9-kb vector for cloning PCR products; Ap$^r$</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCRmenB</td>
<td>pCR2.1-TOPO with the menD gene from MR-1 plus 34 and 223 bp of 5' and 3' DNA</td>
<td>This work</td>
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<td>This work</td>
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<td>23-kb broad-host-range cosmID; Te$^c$ Km$^r$ Tra$^+$</td>
<td>10 (ATCC 37156)</td>
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<td>pVKmenB</td>
<td>EcoRI menB fragment from pCRmenB cloned into the EcoRI site of pVK100; used to complement CMA-1</td>
<td>This work</td>
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Extraction of hydrophobic compounds from spent medium. Hydrophobic compounds were collected from spent medium by using a procedure similar to that described by Newman and Kolter (32) but with one additional step. Two-liter cultures of MR-1 and its derived mutants were grown anaerobically for ca. 40 h in M1 defined medium supplemented with 15 mM lactate, 30 mM fumarate, and vitamin-free Casamino Acids (0.1 g liter$^{-1}$). The cells were harvested by centrifugation for 20 min at 4°C in a Beckman JA-10 rotor at 5,000 rpm (4,420 x g). The supernatant fraction was passed through a 0.2-$\mu$m-pore-size filter to remove remaining cells; this step was not included in the original procedure of Newman and Kolter (32), but its inclusion here prevented the accumulation of brick red material (cytochromes from nonpelleted cells) in the C$_{18}$ column (see below). Each liter of filter-sterilized spent medium was passed through a 60-$\mu$m C$_{18}$ reversed-phase column (Prep-Sep; Fisher Scientific), which collected a prominent band of dark yellow material. Aliquots of methanol-water (50:50 [vol/vol]) were used to elute material from the C$_{18}$ column, and the resulting fractions were tested for the ability to restore electron acceptor use to MK-negative mutant CMA-1. The yellow fraction had the desired activity and was used for subsequent studies. This fraction was filter sterilized with a 0.2-$\mu$m-pore-size Corning Costar cellulose acetate syringe filter and was stored overnight at 4°C before use. A vehicle control (50% methanol) was similarly filter sterilized.

Determination of growth and Fe(III) reduction in liquid cultures. Inocula were pregrown aerobically on Luria-Bertani agar (pH 7.4) (35), cells were suspended in sterile M1 defined medium, and the inoculum densities were adjusted to equalize the turbidity (optical density at 500 nm [OD$_{500}$]; Beckman DU-64 spectrophotometer). The inoculum was added to establish an initial calculated turbidity (OD$_{500}$) of ca. 0.015. Growth was assessed by measuring culture turbidity at 500 nm, and the medium was also supplemented with C$_{18}$-recovered spent medium extract (see above) or an equivalent volume of 50% methanol (vehicle control). Since recovery by the C$_{18}$ extraction method could not be quantitated but was probably less than complete, the amount of C$_{18}$-recovered material added to each milliliter of fresh medium...
corresponded to that obtained from 4 ml of spent medium. The addition of this C18-recovered material or the methanol control to liquid medium resulted in a final methanol concentration of 0.4 to 0.8% (vol/vol), depending on the batch of C18-recovered extract.

Fe(III) reduction experiments were conducted with LM basal medium (16) supplemented with 15 mM lactate, 15 mM formate, and 10 mM Fe(III) citrate. Fe(II) was quantified by a ferrozine extraction procedure (13, 23).

Quinone content of cells. Quinones were extracted from cells essentially as described previously (12). The cells were harvested by centrifugation, and the cell pellets were washed in cold 20 mM MgCl2–0.1 M triethanolamine HCl (pH 7.2); the washed pellets were resuspended in this buffer (1.0 ml of buffer per 0.6 g of wet cell weight). Per 0.6 g of cells, 6.0 ml of methanol-acetone (1:1 [vol/vol]) was added to the cell suspension, followed by vigorous agitation and incubation at room temperature for 30 min. Petroleum ether (2.0 ml) was added, followed by vigorous agitation. Following 1 min of centrifugation at 3,500 rpm (2,800 × g) at 15°C to break the phases, the upper layer was removed; the lower layer was reextracted with another 2.0 ml of petroleum ether, followed by centrifugation and recovery of the upper layer. The upper layers were pooled and evaporated under a stream of nitrogen at 37°C. The dried residue was dissolved in chloroform-methanol (2:1 [vol/vol]).

Quinones were resolved by thin-layer chromatography (TLC) essentially as described previously (8) on Merck Kieselgel 60 F254 plates (20 by 20 cm; 0.25 mm)

FIG. 1. Cross-feeding experiments examining anaerobic growth with the soluble electron acceptors fumarate, nitrate, and TMAO or no electron acceptor. The MK-negative strain CMA-1 was inoculated along the two upper arms of each triangular pattern, and the indicated strains were streaked along the bottom of each. The streak lines did not overlap but were separated by a distance of ca. 3 mm. The images are color reversals of the originals, so that growth is indicated by areas of dark blue against a yellow-orange background. CMA-1 can grow on TMAO, as indicated by its growth along the entire inoculation line. CMA-1 grows on fumarate or nitrate only when it is near the other strains, indicating successful cross-feeding. The variation in the color on the plates is the result of the particulate nature of the ProBlue (colloidal Coomassie blue) stock solution, which results in working solutions that exhibit variability in the intensity with which they stain the agar.
thick) developed with petroleum ether-diethyl ether (9:1 [vol/vol]). The plates were examined under reflective UV light (254 nm) and photographed with a digital camera.

DNA manipulations. The menB, menC, and menD genes in the MR-1 genome are found at genomic loci S04739, S04575, and S04573, respectively (www.tigr.org). These genes, as well as flanking upstream DNA and downstream DNA, were amplified from the genomic DNAs of MR-1 and CMA-1 with high-fidelity Platinum Pfx polymerase (Invitrogen, Carlsbad, Calif.). To create overhangs from the blunt ends, the PCR products were briefly incubated with Taq DNA polymerase, and the agarose-purified fragments were cloned into pCR2.1-TOPO, creating pCRmenB, pCRmenC, and pCRmenD. The DNA sequences of both strands of each were determined at the Medical College of Wisconsin Protein and Nucleic Acid Core Facility by using an ABI 3100 capillary sequencer.

For genetic complementation of CMA-1, the menB-containing insert from pCRmenB was excised with EcoRI and cloned into the EcoRI site of pVK100, creating pVKmenB. Plasmids pVKmenB and pVK100 were electroporated into CMA-1 by a previously described protocol (19).

RESULTS

A previous report (32) had noted that a soluble substance released by MR-1 cells could restore the ability of an MK-negative mutant to reduce AQDS. It was proposed that this compound represented an extracellular redox shuttle that allows MR-1 to respire extracellular electron acceptors (32). The alternative possibility is that this substance restores MK synthesis to the MK-negative mutant and thereby restores its ability to reduce AQDS. If this substance serves as an extracellular redox shuttle, then it could facilitate AQDS and possibly even Fe(III) reduction by an MK-negative strain, but it would not facilitate the use of soluble electron acceptors that require MK as part of the electron transport chain (e.g., fumarate, DMSO, nitrate, and thiosulfate). Alternatively, if this substance restores MK synthesis to an MK-negative strain, then it should simultaneously restore the ability to use all electron acceptors that are dependent on MK. Experiments were conducted to further explore these two possibilities.

Cross-feeding experiments. The ability of MR-1 and its derived mutants to release a substance that facilitates the use of various anaerobic electron acceptors by MK-negative mutant CMA-1 was examined with solid medium. These cross-feeding experiments were the same as those described by Newman and Kolter (32), except that a variety of electron acceptors were tested. Because of its inability to synthesize MK, CMA-1 cannot use a variety of electron acceptors, including fumarate, nitrate, DMSO, thiosulfate, AQDS, and Fe(III). Wild-type MR-1 grew on fumarate and excreted a substance that allowed CMA-1 to grow on fumarate as long as it was in close proximity to MR-1 (Fig. 1A). Analogous results were obtained with nitrate (Fig. 1B) and with DMSO and thiosulfate (data not shown). Like MR-1, gene replacement mutants of MR-1 that are deficient in OM cytochromes (OMCA1 and OMCB1) or OM cytochrome localization (MTRB1) also restored the growth of CMA-1 on fumarate and nitrate (Fig. 1A and B) and on thiosulfate and DMSO (data not shown). Since MK is not required for growth on TMAO (15), CMA-1 grew on TMAO along its entire inoculum line (Fig. 1C). None of the strains could grow in the absence of an electron acceptor (Fig. 1D).

Strains MR-1 and OMCA1 also restored the ability of CMA-1 to reduce Fe(III) and AQDS (Fig. 2) and to grow on these electron acceptors. However, OMCB1 and MTRB1 did not grow on or reduce Fe(III) or AQDS and therefore did not restore significant use of these electron acceptors by CMA-1 (Fig. 2). Interestingly, however, a small amount of AQDS reduction was seen over the CMA-1 streaks in close proximity to OMCB1 and MTRB1 (Fig. 2). This finding suggests that OMCB1 and MTRB1 can minimally cross-feed CMA-1 even when they themselves do not reduce AQDS.

Together, these findings provide evidence that the substance...
released from growing strains is capable of restoring the ability of CMA-1 to use multiple anaerobic electron acceptors that require MK.

**Extracts from spent medium restore electron acceptor use.** A previous study (32) noted that the factor that restores AQDS reduction could be recovered from the spent medium of MR-1 by a C18 column. This procedure was used to recover such a factor(s) from the spent media of MR-1, OMCA1, OMCB1, and MTRB1. The data for the extracts from MR-1 and OMCB1 spent media are shown in Fig. 3 and 4. Analogous results were obtained with extracts from OMCA1 and MTRB1 spent media (Fig. 5). These extracts were able to restore the anaerobic growth of CMA-1 on fumarate and nitrate (Fig. 3A, 3B, 5A, and 5B). Since MK is not required for TMAO utilization, all strains grew well on TMAO (Fig. 3C and 5C). In each instance, there was no growth from the C18-recovered material in the absence of added cells (Fig. 3 and 5), demonstrating that the C18-recovered material was sterile. None of the strains grew in the absence of an electron acceptor, whether or not the C18-recovered material was present (Fig. 3D and 5D). This finding is important because it shows that the amounts of the C18-recovered material used here were not able to support growth in the absence of a known electron acceptor. Consistent with the growth data, the C18-recovered material restored the ability of CMA-1 to reduce Fe(III) (Fig. 4). Experiments which monitored growth by increases in cell pellet weight also showed that the C18-recovered material from the spent media of MR-1, OMCA1, OMCB1, and MTRB1 restored the growth of CMA-1 on Fe(III), fumarate, nitrate, and AQDS (data not shown).

The MK restorative activity of the C18-recovered extracts was labile over time when the extracts were stored at 4°C in...

**FIG. 3.** Anaerobic growth of strain MR-1 or CMA-1 with fumarate (A), nitrate (B), TMAO (C), or no electron acceptor (D) in the presence of extracts from strain MR-1 or OMCB1. Growth was measured as the increase in optical density (O.D.) over time. The cultures were supplemented with C18 column-collected extracts from spent medium of strain MR-1 (C18 MR1) or OMCB1 (C18 OMCB1) or an equivalent volume of control solvent (MeOH; 50% methanol). The C18-recovered extracts were stored for 4 days at 4°C before use in these experiments. All values represent the means and standard deviations for three parallel but independent cultures of each strain. For points lacking apparent error bars, the bars were smaller than the points.
FIG. 4. Reduction of Fe(III) citrate under anaerobic conditions, as determined by the formation of Fe(II) over time. The cultures were supplemented with C18-column collected extracts from spent medium of strain MR-1 (C18 MR1) or OMCB1 (C18 OMCB1) or an equivalent volume of control solvent (MeOH; 50% methanol). The C18-recovered extracts were stored for 2 days at 4°C before use in these experiments. All values are the means and standard deviations for three parallel but independent cultures of each strain. For points lacking apparent error bars, the bars were smaller than the points.

50% methanol. The extracts retained maximal activity for the first 2 days of storage. Activity gradually declined in extracts that were stored from 3 to 7 days, as evidenced by progressively longer times to the onset of restoration of growth of CMA-1 on fumarate. Extracts stored for 7 days at -20°C were only modestly more active than those stored at 4°C. The activity of extracts stored for more than 7 days was not examined.

Together, these results indicate that a somewhat labile substance recovered from the spent media of growing strains is able to restore the ability of CMA-1 to use multiple electron acceptors that require MK.

Restoration of MK synthesis. The data in Fig. 1 to 5 indicate that MK-positive strains (e.g., MR-1, OMCA1, OMCB1, and MTRB1) release one or more factors that restore the ability of CMA-1 to synthesize MK. To further examine this hypothesis, the quinone content of CMA-1 cells grown with or without the C18-recovered material was examined. In agreement with previous reports (15, 27), wild-type MR-1 contained ubiquinone, MK, and methyl-MK (Fig. 6, lane 1), whereas CMA-1-synthesized ubiquinones but not MK (Fig. 6, lane 3). The C18-recovered extract of MR-1 spent medium restored the synthesis of MK to CMA-1, but methyl-MK was not detected (Fig. 6, lane 2). The MK content in CMA-1 grown with the C18-recovered extract was much lower than that of the wild type; i.e., material from a larger amount of cells had to be loaded in order to detect MK (Fig. 6). However, this amount of MK was sufficient to restore the use of various electron acceptors (Fig. 3 to 5); this result is consistent with the previous observation that subwild-type levels of MK in another MR-1-derived strain were sufficient to support the use of these electron acceptors (27).

Identification of the defect in CMA-1. Since strain CMA-1 was made by acridine orange mutagenesis (15), experiments were done to identify the mutation responsible for the MK deficiency. Since 1,4-dihydroxy-2-naphthoic acid (DHNA), an intermediate in the MK pathway (Fig. 7), restores MK synthesis to CMA-1 (15), the focus was on genes prior to the DHNA gene in the pathway. The DNA sequences of the menC and menD genes from CMA-1 were the same as those from MR-1. However, compared to the menB gene from MR-1, the menB gene from CMA-1 had a single base deletion of a cytosine (base 494 of the menB open reading frame). This deletion is consistent with the ability of acridine orange to cause frameshift mutations and would result in a complete change in the amino acid sequence after residue 165 (of a total of 300 for MenB) and multiple subsequent premature stop codons, with the first at residue 189.

To confirm whether the menB frameshift mutation in CMA-1 is responsible for its electron acceptor phenotype, the ability of wild-type menB to restore electron acceptor use to CMA-1 was examined. While CMA-1/pVK100 could not grow on nitrate or fumarate, CMA-1/pVKmenB grew well on these electron acceptors, although it was 1 day slower than MR-1/pVK100 (Fig. 8A and B). No strain could grow without an electron acceptor (Fig. 8D), whereas all strains could grow with TMAO (Fig. 8C), since MK is not required for TMAO utilization.

To confirm that pVKmenB restored the ability of CMA-1 to synthesize MK, quinone extracts of the various strains were examined by TLC. Quinones extracted from 0.2 g of wet cells of MR-1/pVK100 showed both MK and ubiquinone species, as in Fig. 6. While MK could not be visualized on TLC plates loaded with quinones extracted from 0.2 g of CMA-1/pVKmenB cells, spots which migrated in a manner similar to that of a MK-4 standard were clearly evident when quinone extracts were derived from 1.1 g of CMA-1/pVKmenB cells (data not shown). This finding indicates that pVKmenB restores MK synthesis to CMA-1, albeit at a lower level than in the wild type. This smaller amount of MK in CMA-1/pVKmenB likely explains the slower growth of CMA-1/pVKmenB on MK-dependent electron acceptors, such as nitrate and fumarate (Fig. 8). This notion is consistent with the decreased growth rates of previously described strains MR-1A and MR-1B, which also had significantly less MK than the wild type (27). As noted before, MK species were not detected in quinones extracted from 1.1 g of CMA-1/pVK100 cells.

The UV spectra of the material recovered from the CMA-1/pVKmenB MK spots (Fig. 9A) are consistent with those for MK from MR-1/pVK100 (Fig. 9B). The MK spectra are in contrast to those of ubiquinones recovered from CMA-1/pVKmenB (Fig. 9C) and MR-1/pVK100 (data not shown).

DISCUSSION

A previous report (32) noted that MR-1 releases a substance that can restore the ability of a MK-negative strain to reduce AQDS. This substance is insensitive to proteases, is smaller than 10 kDa, and can be captured from spent medium on a C18 solid-phase extraction column (32). It was proposed that this substance could represent a quinone-type electron shuttle that facilitated the respiration of extracellular minerals (32). The findings here, however, indicate that this substance restores the
ability of MK-negative strains to synthesize MK. The restoration of MK extends beyond just AQDS or Fe(III) reduction and provides for the ability to use all tested electron acceptors that are MK dependent. The previous results (32) can therefore be explained by the restoration of MK synthesis and do not provide sufficient evidence for an excreted electron shuttle. Excreted redox shuttles in spent medium cannot account for the ability of washed cells to instantaneously generate respiratory energy in response to pulses of electron acceptors, including Mn(IV) and Fe(III) (24).

DHNA, a precursor in the latter portion of the MK synthesis pathway, restores MK synthesis and the ability to use MK-dependent electron acceptors to the MK-negative mutant used in these studies (15) as well as that used by Newman and Kolter (32). However, Newman and Kolter (32) noted that the restorative substance excreted by MR-1 does not comigrate with DHNA but likely has a molecular mass of <300 Da. We concur that it is not DHNA. The UV absorption spectrum (200 to 320 nm) of the C18-recovered material showed a single peak with an absorbance maximum at 268 nm. While this spectrum is a composite of all of the compounds in the extract, it did not resemble that of DHNA, MK 4, flavin adenine dinucleotide, or flavin mononucleotide. In our experiments, mass spectrometry analysis of gradient high-pressure liquid chromatography eluates of the C18-recovered material indicated the presence of numerous compounds, many with m/z values of <300 Da. Masses expected for known precursors in the MK synthesis pathway were not apparent, but there were several ions in the...

FIG. 5. Anaerobic growth of strain MR-1 or CMA-1 with fumarate (A), nitrate (B), TMAO (C), or no electron acceptor (D) in the presence of extracts from strain OMCA1 or MTRB1. Growth was measured as the increase in optical density (O.D.) over time. The cultures were supplemented with C18 column-collected extracts from spent medium of strain OMCA1 (C18 OMCA1) or MTRB1 (C18 MTRB1) or an equivalent volume of control solvent (MeOH; 50% methanol). The C18-recovered extracts were stored for 1 day at 4°C before use in these experiments. All values represent the means and standard deviations for three parallel but independent cultures of each strain. For points lacking apparent error bars, the bars were smaller than the points.
C<sub>18</sub>-recovered extract of MR-1 spent medium that were not seen in the extract of CMA-1 spent medium. It is currently unknown which, if any, of these species unique to the MR-1 spent medium may, alone or together, contribute to the restoration of MK synthesis in CMA-1.

While MR-1 synthesizes both MK and methyl-MK, a previous report noted that DHNA restores MK but not methyl-MK to CMA-1 (15). Similarly, the factor recovered from MR-1 spent medium restores MK but not methyl-MK to CMA-1 (Fig. 6). Since the use of MK-dependent electron acceptors is restored in both instances, it is likely that MK is sufficient and that methyl-MK is not necessary. It is possible, however, that methyl-MK also participates in the electron transport chains for these electron acceptors in wild-type cells.

Since OMCB1 and MTRB1 cannot grow on or reduce Fe(III) or AQDS, cross-feeding with these electron acceptors could not be determined directly on plates (Fig. 2). However, testing of the C<sub>18</sub>-recovered extract from the spent medium of fumarate-grown strains demonstrated that they still released the substance that restored the ability of CMA-1 to grow on or reduce all electron acceptors, including AQDS and Fe(III). Their inability to reduce Fe(III) and AQDS therefore is not due to an inability to release this material and demonstrates that the OM protein MtrB and the OM cytochrome OmcB are not required for the release of this material. It also implies that this released substance may not be a redox shuttle, as originally proposed (32), because if it were, then OMCB1 and MTRB1 would be expected to retain a significant ability to reduce Fe(III). While it is possible that MR-1 releases a redox shuttle, the data in these experiments suggest that it does not account for the vast majority of Fe(III) or AQDS reduction by these strains.

It was previously noted that Vibrio cholerae can similarly restore AQDS reduction to an MK-negative mutant of MR-1 (32). We are not aware of reports on the ability of V. cholerae to respire metals, so its need to excrete a putative redox shuttle to facilitate metal respiration is not evident. However, analysis of the genomic sequence of MR-1 indicated that its genome is more similar to that of V. cholerae than to any other available genome (6). Approximately one-third of the genes of MR-1 are most similar to those of V. cholerae, and V. cholerae has the only known genome with extensive regions of gene order similar to those in MR-1 (6). The Men proteins of MR-1 also show marked similarity to those of V. cholerae (61.6, 52.2, 58.2, and 50.8% similarity for MenB, MenC, MenD, and MenE, respectively). It is therefore plausible that V. cholerae similarly excretes a compound that can restore MK synthesis to MR-1. However, the gene products that are responsible for the synthesis or excretion of the MK restorative substance have not yet been identified.

It should be noted that the MK mutant used in our studies (CMA-1) (15) is not the same as that used in the previous report (H2) (32). CMA-1 was generated by acridine orange...
and has a 1-base deletion frameshift mutation in menB (see above). Such a frameshift would not be expected to have polar effects, and menB is not part of an operon with other men genes. The fact that wild-type menB could restore the abilities of CMA-1 to synthesize MK and to use MK-dependent electron acceptors (Fig. 8 and 9) confirms that the menB frameshift mutation in CMA-1 is responsible for its phenotype. In contrast, H2 was generated by transposon mutagenesis, which apparently interrupted menC (32). Given the polar nature of transposon insertions, other open reading frames (e.g., menE) immediately downstream in the same operon could also have been affected. Although effects on menE expression were not reported for H2 (32), it is possible that H2 is functionally menC and menE deficient. However, the mutations in CMA-1 and H2 both affect genes prior to the DHNA gene in the MK synthesis pathway (Fig. 7). They are functionally similar in this regard because DNHA can restore the ability of both mutants to use anaerobic electron acceptors that are dependent on MK (15, 32). They are also similar in that the unidentified compound released by MR-1 restores the ability of both strains to reduce AQDS (15, 32). The previous report on strain H2 (32) did not examine the restoration of other electron acceptors, whereas our study shows that the excreted substance also restores the ability of CMA-1 to grow on many electron acceptors that are dependent on MK. The two mutants are also similar with respect to most other electron acceptor deficiencies noted, including Mn(IV), fumarate, thiosulfate, DMSO, and Fe(III) (15, 25, 32). However, it was noted that H2 could grow on nitrate, albeit at a reduced level relative to the wild type (32), although no data or experimental details were provided. In contrast, quantitative data reported here (Fig. 3 and 5) show that CMA-1 does not exhibit detectable growth on nitrate after 3 days, even though the wild type exhibits maximal growth after 1 day. These data agree with previous data that showed that rates of anaerobic nitrate reduction by CMA-1 were <2% those of the wild type when monitored for 4 days (15). It has also been noted that a defect in the cytochrome CymA, which is likely downstream from MK

FIG. 8. Anaerobic growth of strains MR-1/pVK100, CMA-1/pVK100, and CMA-1/pVKmenB with fumarate (A), nitrate (B), TMAO (C), or no electron acceptor (D). Growth was measured as the increase in optical density (O.D.) over time. All values represent the means and standard deviations for three parallel but independent cultures of each strain. For points lacking apparent error bars, the bars were smaller than the points.
in the electron transport chain, leads to an inability to use nearly all MK-dependent electron acceptors, including nitrate (17, 25, 27). It is unclear why H2 would retain some ability to grow on nitrate even though it is deficient in the use of other MK-dependent electron acceptors and is apparently blocked earlier in the MK pathway than is CMA-1.

It has been noted that *Sphingomonas* can convert exogenously added naphthalene-2-sulfonate to 4-amino- and 4-ethanolamino-1,2-naphthoquinones, which can then serve as redox shuttles for the reduction of azo dyes under anaerobic conditions (9). However, other than the plate experiments which contained AQDS, sulfonates or quinone sulfonates were not included in the MR-1 experiments, and yet material which restored MK synthesis to CMA-1 was generated in both the plate and the spent medium experiments.

Exogenously added redox shuttles (e.g., AQDS and methyl viologen) can facilitate the reduction of hematite ($\alpha$-$Fe_2O_3$) by *S. putrefaciens* CN32 (34), and AQDS facilitates the reduction of insoluble Fe(III), U(VI), and Tc(VII) by *Deinococcus radiodurans* (5). The addition of AQDS similarly stimulates the reduction of FeOOH and Fe(III)-smectite by MR-1, but there is little to no accompanying stimulation of cell growth, as assessed by cell numbers or total cellular carbon levels (11). A recent report noted that exogenously added AQDS enhanced Fe(OH)$_3$ reduction by MR-1 at nearly all time points but enhanced growth only at a single matched time point early in the growth period (7). Thus, while exogenously added AQDS may stimulate Fe(III) reduction by MR-1, any growth enhancement is transient.

Redox shuttles may be released by other Fe(III)-reducing bacteria. While *Geobacter sulfurreducens* and *G. metallireducens* do not excrete redox shuttles (31, 38), such redox shuttles may be produced by certain Fe(III)-reducing enrichment cultures (38). The species composition of these enrichments was not reported. *Geothrix fermentans* was reported to release a soluble substance of $<12$ kDa that is capable of mediating Fe(III) reduction (31). A relatively water-soluble but unidentified fluorescent compound is a possible candidate as a potential redox shuttle in *G. fermentans* (31).

Some bacteria may release complexation factors that enhance Fe(III) reduction by creating a more bioavailable Fe(III) ligand species (3). It is not clear whether MR-1 synthesizes Fe(III) chelators, but it is unlikely that such solubilization factors would contribute to its ability to reduce insoluble Mn(IV), because solubilization of Mn(IV) oxides requires reduction to Mn(II) (4).

The use of MK or an MK derivative as a redox shuttle to generate respiratory energy from Fe(III) or AQDS reduction would imply that MK or MK derivatives are the terminal electron donors to Fe(III) or AQDS. This scenario is not likely, because other components downstream of MK are also required for the reduction of AQDS, Fe(III), and other electron acceptors. For example, both the CM cytochrome CymA and MK are required for the use of several electron acceptors, including Fe(III), fumarate, nitrate, and AQDS (17, 25, 27, 32). Recent evidence indicates that CymA can mediate electron flow from MK to fumarate reductase (36, 37). This finding implies that CymA is downstream of MK. The fact that MK, but not CymA, is required for thiosulfate reduction (25) further indicates that CymA is downstream of MK. It is therefore

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**FIG. 9.** UV absorption (ABS) spectra for quinones recovered from TLC plates and extracted in 100% ethanol. Each extract was scanned relative to a reference cuvette containing 100% ethanol. The spectra for MK spots from CMA-1/pVKmenB (A) and MR-1/pVK100 (B) are typical for MKs (27, 33) and are in marked contrast to that of ubiquinone from CMA-1/pVKmenB (C). The TLC plates from which the material was recovered were loaded with quinone extracts isolated from either 0.2 g (MR-1/pVK100) or 1.1 g (CMA-1/pVKmenB) of wet cell weight of anaerobic fumarate-grown cells.
difficult to envision how MK or MK-like compounds would be used twice during electron transport to Fe(III) and AQDS, once to reduce CymA and then again downstream of CymA as electron shuttles. If MK or MK-like compounds were used as the primary mediators of extracellular electron acceptor reduction, then downstream components such as CymA should not be required.

In summary, MR-1 and various strains derived from MR-1 restore the ability of an MK-negative strain to use multiple electron acceptors that are dependent on MK. This ability is not limited to cells grown on AQDS and can be explained by the restoration of MK synthesis to the MK-negative strain. The hypothesis that this restorative substance represents a redox shuttle that allows MR-1 to respire minerals (32) can therefore be explained by the restoration of MK synthesis.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant MCB-0318575 to C.R.M.

We are grateful to Kaseem Nithipatikom, Marilyn Isbell, and members of the Department of Pharmacology and Toxicology Mass Spectrometer Facility for mass spectrometry analyses.

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