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Protective Role of *tolC* in Efflux of the Electron Shuttle Anthraquinone-2,6-Disulfonate

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Extracellular electron transfer can play an important role in microbial respiration on insoluble minerals. The humic acid analog anthraquinone-2,6-disulfonate (AQDS) is commonly used as an electron shuttle during studies of extracellular electron transfer. Here we provide genetic evidence that AQDS enters *Shewanella oneidensis* strain MR-1 and causes cell death if it accumulates past a critical concentration. A *tolC* homolog protects the cell from toxicity by mediating the efflux of AQDS. Electron transfer to AQDS appears to be independent of the *tolC* pathway, however, and requires the outer membrane protein encoded by *mtrB*. We suggest that there may be structural and functional relationships between quinone-containing electron shuttles and antibiotics.

Microbial respiration using insoluble minerals as terminal electron acceptors is an important environmental process, influencing both the fate and transport of organic and inorganic pollutants (12, 13). It is also believed to be one of the most ancient forms of respiration (13, 37). Microbes may use various strategies to respire minerals (21). These strategies include those that require direct contact (e.g., electron transfer occurs through outer membrane proteins that contact the mineral surface [15]) and those that do not. The latter pathway is driven by extracellular electron transfer, where natural materials, such as humic substances (e.g., allomelanins derived from the decomposition of organic matter), and microbially excreted small molecules, such as quinones, may serve as shuttles to carry electrons from the cell to the mineral surface (14, 20, 22). Quinones are also known to be the redox-active moieties of humic substances (31). Although significant progress has been made in identifying the molecules involved in the direct-contact pathway, much remains to be learned regarding the molecular genetics of extracellular electron transfer.

Since previous studies have shown that *Shewanella oneidensis* strain MR-1 (19, 38) is able both to respire humic acids and to produce electron-shuttling molecules (22; M. Dubiel and D. K. Newman, unpublished results), we performed a small genetic screening of 500 transposon-generated mutants to explore the mechanistic basis of the reduction of the humic acid analog anthraquinone-2,6-disulfonate (AQDS) (35). Transposon mutagenesis was performed as previously described (22), and isolates were screened to identify mutants that were apparently unable to use AQDS as an electron acceptor during anaerobic growth on lactate. Isolates were subcultured from aerobically grown cultures in Luria-Bertani (LB) medium (18) into lactate minimal medium (19) containing 10 mM AQDS and incubated anaerobically. Reduction of AQDS was scored by monitoring the appearance of orange color in the medium over time, indicating the production of the reduced form, anthrahydroquinone-2,6-disulfonate (AHDS). Two mutants that were completely defective in the reduction of 10 mM AQDS, strains AQ-38 and AQ-41, were identified. Their aerobic and anaerobic growth rates are indistinguishable from those of the wild-type strain in the absence of AQDS (data not shown), which implies that the defect in AQDS reduction is not due to a general growth defect. Neither of the mutants can reduce humic acids (Aldrich Chemicals, catalog no. H1-675-2) in an assay coupling humic acid reduction to iron oxide reduction (14). In qualitative assays for the reduction of amorphous iron (III) hydroxide [$Fe(OH)_3$] and manganese (IV) oxide (MnO_2) (22), performed to distinguish mutants pleiotropically defective for reduction of multiple electron acceptors from mutants with specific defects in AQDS reduction, the two mutants behave differently. While AQ-38 is unable to reduce either mineral, AQ-41 is normal with respect to both Fe(III) and Mn(IV) reduction (data not shown).

A PCR-based technique (25), involving transposon-specific and arbitrary primers to amplify DNA flanking the transposon, was used to identify the genes into which the transposon had inserted in these strains. Longer DNA segments containing these flanking sequences were identified in the nearly complete *S. oneidensis* MR-1 genome sequence database that is available from The Institute for Genomic Research website (http://www.tigr.org) and analyzed using the ORF Finder utility that is available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The results showed that AQ-38 has a mutation in the *mtrB* gene, which in *S. oneidensis* MR-1 encodes an outer membrane protein that is known to be required for solid Fe(III) and Mn(IV) reduction (2). The 24 bases immediately flanking the transposon insertion in strain AQ-38 are 100% identical to bp 14311 to 14334 of the published *S. oneidensis* MR-1 *mtr* region (GenBank accession number AF083240 at www.ncbi.nlm.nih.gov). Insertion of the transposon following bp 14310 of the *mtr* region sequence would truncate the MtrB protein after amino acid 264 out of 698 total residues in this protein. The metal-reduction-deficient phenotype of AQ-38 is consistent with that of the *mtrB* transposon insertion mutant described previously (2), although the location of the insertion point in that mutant was

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never reported. Because *mtrB* appears to be at the end of an operon (2, 3), it seems likely that the transposon insertion affects only this gene. Thus, our result suggests that at least part of the pathway leading to the reduction of minerals is the same as that leading to the reduction of humic substances. Given that some fraction of humic substances is not likely to be taken up by the cell due to their large molecular weight or sorption to mineral surfaces (17), it seems reasonable that outer membrane proteins would be involved in their reduction.

The 30 bases immediately flanking the transposon insertion in strain AQ-41 are 100% identical to bp 3049332 to 3049362 of the *S. oneidensis* MR-1 genome. The open reading frame (ORF) from *S. oneidensis* strain MR-1 containing this sequence encodes a protein that is most closely related to a *Vibrio cholerae* TolC homolog (VC2436) and to the TolC protein from *Escherichia coli* strain K-12. The *S. oneidensis* protein exhibits 48% identity and 65% similarity at the amino acid level to *V. cholerae* VC2436 versus 43% identity and 63% similarity to the *E. coli* TolC protein by a BLAST2 pairwise alignment. The transposon inserted after bp 1000 of the ORF, which would truncate the protein product after amino acid 333 out of 680 total residues. Our analysis of the *S. oneidensis* genome segment encoding the TolC homolog indicates that this ORF is apparently monocistronic, since both flanking ORFs are encoded on the opposite DNA strand. Thus, the transposon insertion in AQ-41 is unlikely to have caused a polar effect.

To determine whether other TolC homologs might be present in *S. oneidensis* strain MR-1, the *E. coli* TolC protein sequence and the protein sequence from the ORF disrupted in strain AQ-41 were used to search the *S. oneidensis* MR-1 genome in a BLAST analysis. In addition to the ORF disrupted in AQ-41 (which we refer to as the *S. oneidensis tolC* gene hereafter), two other ORFs were identified. The protein encoded by the first ORF shows 22% identity and 36% similarity at the amino acid level to the entire TolC protein from *E. coli*. This protein is most closely related to the protein encoded by ORF NMB1714 from *Neisseria meningitidis* (30% identity and 53% similarity at the amino acid level); this ORF encodes a putative multidrug efflux pump channel protein. Other outer membrane and efflux proteins related to TolC were similarly related to this *S. oneidensis* protein as determined by the BLAST and Cognitor functions of the ORF Finder program. The second ORF encodes a protein that exhibits 21% identity and 39% similarity at the amino acid level to the entire sequence of the *S. oneidensis* TolC protein but has only 23% identity and 44% similarity to 166 amino acids near the carboxyl terminus of the 495-amino-acid TolC protein of *E. coli*. This protein is most closely related to a putative agglutination protein in *V. cholerae* (ORF VC1621) (42% identity and 64% similarity at the amino acid level), although it is also related to other outer membrane and efflux proteins similar to TolC.

In *E. coli*, *tolC* encodes an outer membrane transport protein (24, 27) that is part of several efflux pump systems, including the AcrAB-TolC efflux pump (5, 16). This is an energy-dependent efflux system belonging to the resistance/nodulation/cell division family (26). Mutational and other analyses suggest that this system may be the major efflux system in *E. coli* involved in resistance to a variety of toxic molecules, including antibiotics, dyes, and detergents (1, 23, 30, 32, 36, 40). We have identified homologs with high similarities to AcrA and AcrB in the *S. oneidensis* MR-1 genome sequence database (44% identity and 58% similarity to *E. coli* AcrA and 64% identity and 78% similarity to *E. coli* AcrB at the amino acid level with ORFs from the *S. oneidensis* MR-1 genome database), suggesting that this organism uses a similar efflux pathway for protection against toxic compounds. Therefore, it seemed likely that the inability of strain AQ-41 to reduce AQDS was due to a toxic effect of AQDS as opposed to a specific defect in AQDS reduction.

A toxicity test proved this hypothesis (Fig. 1A and B). Cells from aerobically grown cultures in LB medium were subcultured into either 96-well microtiter plates (for AQDS reduction determination) or 12-mm-diameter test tubes (for cell number determinations by dilution and plating) containing LB medium plus 0, 1, or 10 mM AQDS. Under static aerobic growth conditions, strain AQ-41 grew as well as wild-type strain MR-1 in LB medium containing no AQDS or 1 mM AQDS. The anaerobic environment that developed at the bottom of the microtiter plate wells allowed both strains to reduce AQDS to AHDS at this concentration, which demonstrates that AQ-41 is not defective in AQDS reduction. When the AQDS concentration was increased to 10 mM, the growth of AQ-41 was severely hampered. Similar results were observed under strictly anaerobic conditions. This experiment could not resolve whether AQDS itself was the toxic compound; it is also possible that a toxic by-product was produced during the reduction of AQDS or that the reduced product, AHDS, was the active toxic compound.

To investigate whether the toxic effect was independent of the reduction of AQDS, we performed a similar toxicity test with *E. coli* K-12 derivatives ZK95 (strain MC4100; genotype, F^- *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 thi-1 recA1*) and the *tolC* mutant ZK796 (MC4100 *tolC*::Tn*10*) (42), using the optical density at 600 nm (OD_{600}) from the microtiter plate wells to follow growth. *E. coli* is not able to reduce or respire AQDS, as both the parent strain (ZK95) and ZK796 showed no change of color in the medium (Fig. 1C). However, growth of the *E. coli tolC* mutant was completely inhibited when exposed to 10 mM AQDS, while no growth inhibition was observed with 1 mM AQDS (Fig. 1D). This indicates that AQDS itself is the major toxic compound for *E. coli* K-12 and that the protective effect of *tolC* is probably due to its role in the efflux of toxic compounds. Since *tolC* is required for the increased resistance of both *E. coli* K-12 and *S. oneidensis* MR-1 to AQDS, it seems likely that a similar mechanism is operating in both organisms.

To confirm that AQDS accumulates inside the cells of the *S. oneidensis tolC* mutant and kills them, dense suspensions (10^9 cells/ml) of exponentially growing LB cultures of wild-type *S. oneidensis* strain MR-1 and strain AQ-41 were prepared and resuspended in a nongrowth medium (2.5 g of bicarbonate/liter) containing 10 mM AQDS. Over a period of several days of aerobic incubation at room temperature (22 to 25°C), viable cell numbers dropped at a faster rate in the AQ-41 cultures than in the wild-type cultures. Similarly, the total concentration of AQDS (including AHDS) in the culture supernatants decreased at a faster rate for AQ-41 than for the wild-type strain (data not shown). These results imply that AQDS enters the cells by an unknown pathway and kills the cells if it accumulates inside beyond a critical concentration. While a basal

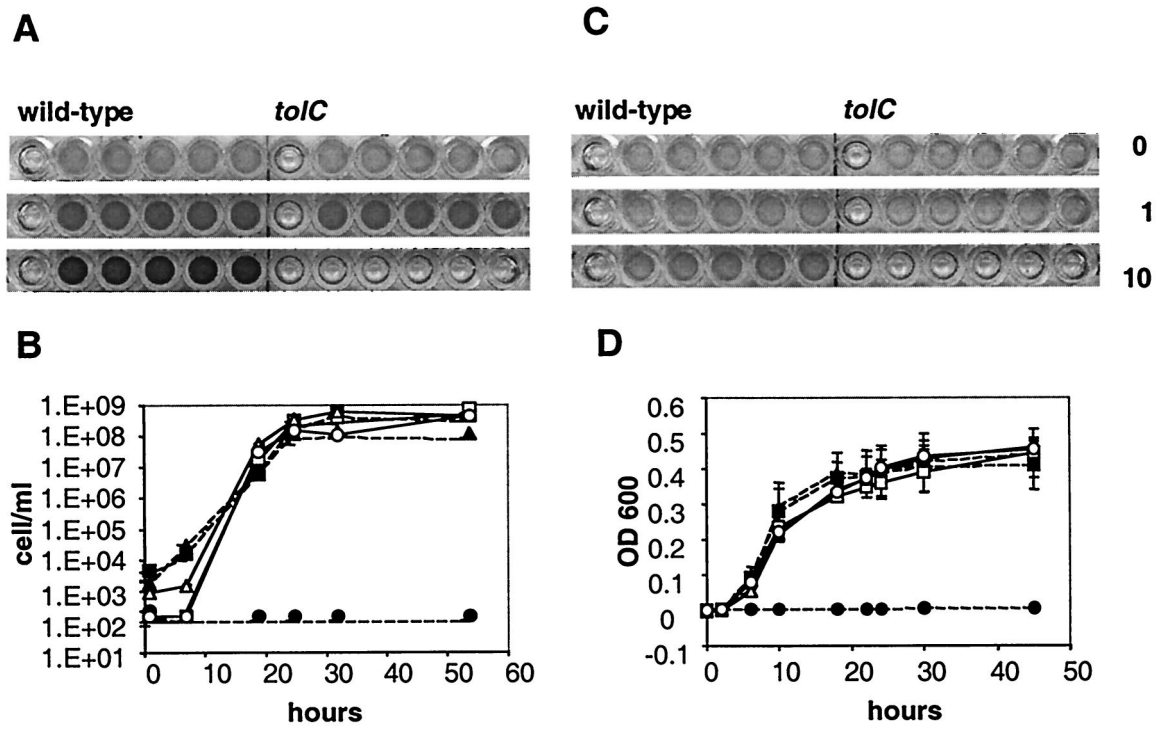

FIG. 1. The toxicity test for AQDS. (A) Microtiter plate assay comparing AQDS reduction by *S. oneidensis* strain MR-1 (wild type) to that by the AQ-41 (*tolC*) mutant for different concentrations. Dark-colored wells indicate production of AHDS. The well at the left end of each panel shows an uninoculated control, and the remaining five wells are replicates. Both strains can reduce 1 mM AQDS, but the *tolC* mutant cannot reduce 10 mM AQDS. (B) Wild-type *S. oneidensis* shows similar growth rates in LB medium containing 0 to 10 mM AQDS, whereas the *tolC* mutant cannot grow in the presence of 10 mM AQDS. □, wild type at 0 mM; △, wild type at 1 mM; ○, wild type at 10 mM; ■, *tolC* mutant at 0 mM; ▲, *tolC* mutant at 1 mM; ●, *tolC* mutant at 10 mM. (C) Microtiter plate assay comparing AQDS reduction by the *E. coli* wild-type strain to the *tolC* mutant for different concentrations. The well at the left end of each panel shows an uninoculated control, and the remaining five wells are replicates. Neither strain can reduce AQDS at any concentration, and shading in the wells is indicative of cell growth. (D) The wild-type *E. coli* strain shows similar growth rates in LB medium containing 0 to 10 mM AQDS, whereas the *tolC* mutant cannot grow in the presence of 10 mM AQDS. Symbols are as for Fig. 1B.

level of nonspecific efflux may permit AQ-41 cells to tolerate AQDS at low concentrations, a *tolC*-dependent secretion pathway appears to be the primary efflux route for this compound in wild-type MR-1.

Given that AQDS can be toxic at high levels, we asked whether transcription of the *tolC* gene was sensitive to the concentration of AQDS in the medium. The transposon used to generate strain AQ-41 (41) created a *lacZ* transcriptional fusion to the *tolC* gene, allowing us to monitor its expression by the production of β-galactosidase (18) from exponentially growing aerobic LB cultures in the presence of nonlethal AQDS concentrations. Although the *tolC* gene appeared to be expressed constitutively at low levels in the absence of AQDS, its expression increased reproducibly by about a factor of two when AQDS concentrations rose to high levels in the medium (5 mM). To verify that the induction of *tolC* gene expression was not simply a general response to the presence of an inhibitor, we tested expression in the presence of various concentrations of gentamicin (0 to 0.5 μg/ml), a hydrophilic aminoglycoside antibiotic. Aminoglycosides do not appear to be transported by TolC-linked efflux pathways (32). As expected, *tolC* gene expression did not vary as a function of the presence of gentamicin, suggesting that the induction by AQDS reflected exposure to a compound transported by a TolC-linked efflux pathway. TolC has previously been reported to be a minor outer-membrane protein (39). Accordingly, the low level of expression of the *tolC* gene is not surprising, and the small fold increase in gene expression in response to increasing AQDS concentration may be significant.

AQDS, like many electron-shuttling compounds, has a structure similar to aromatic antibiotics, such as tetracycline, doxorubicin, and pyocyanin (9) (Fig. 2A). To test whether these compounds share similar efflux pathways, we performed disk-diffusion toxicity tests with the AQ-41 mutant and these compounds. When we placed a disk containing low levels of tetracycline or doxorubicin (15 μl of 15 μg/ml solutions; i.e., 225 ng of antibiotic) on an LB plate inoculated to provide a lawn of *S. oneidensis* cells, the wild-type strain MR-1 could grow up to the border of the disk, whereas the AQ-41 mutant could not grow within a 5-mm radius around the disk (Fig. 2B). AQ-41 also showed increased sensitivity to pyocyanin in microtiter plate toxicity assays (M. E. Hernandez and D. K. Newman, unpublished results). The similar effects of tetracycline, doxorubicin, pyocyanin, and AQDS on strain AQ-41 (i.e., increased toxicity) suggest that, under the conditions tested, these molecules all utilize TolC-linked efflux systems for transportation out of the cell.

Similar disk diffusion assays were used to examine the sen-

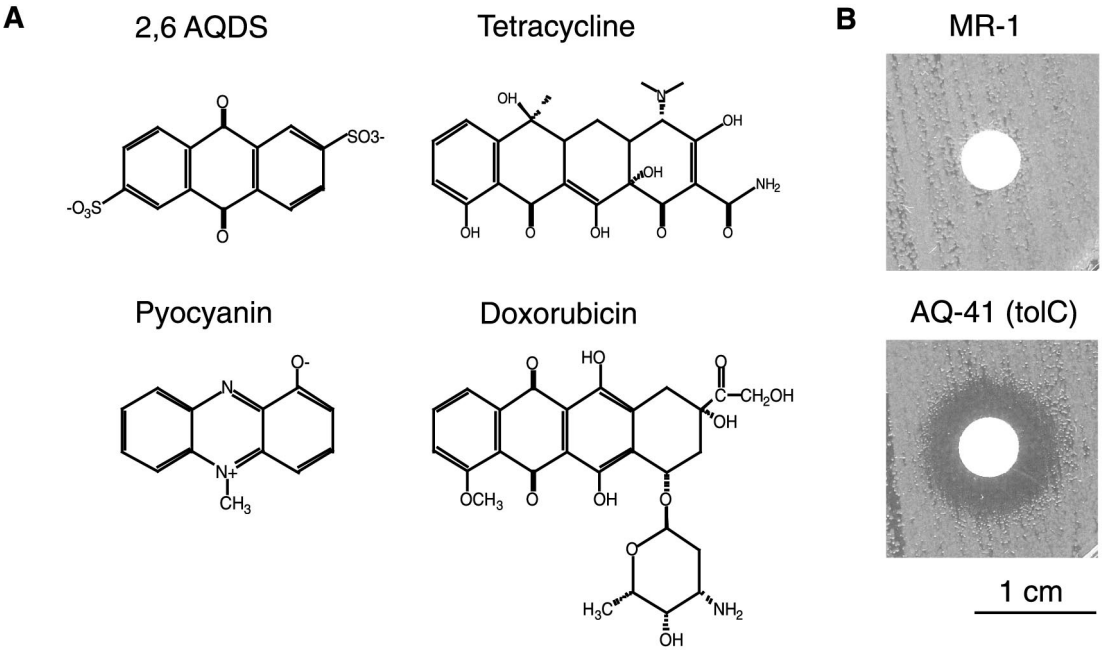

FIG. 2. Structural and toxicity similarities between AQDS and antibiotics. (A) Structures of AQDS, doxorubicin, pyocyanin, and tetracycline. (B) Representative sensitivities of wild-type *S. oneidensis* strain MR-1and the *tolC* mutant to low levels of tetracycline. Assays with doxorubicin gave essentially indistinguishable results.

sitivities of strain AQ-41 and the *E. coli* ZK796 *tolC* mutant to several other compounds for which increased sensitivity of *E. coli tolC* mutants has been demonstrated (32). Both *tolC* mutants exhibited similar sensitivities to the antibiotics chloramphenicol and rifampin when compared to their respective parent strains. This was evident by the fact that the parent and mutant strains were able to withstand approximately the same concentrations of these antibiotics (i.e., the threshold levels of chloramphenicol or rifampin required to produce a visible zone of clearing in the mutant and parent strains were ≤2-fold different from one another; data not shown). However, both *tolC* mutant strains exhibited increased sensitivities to the compounds sodium dodecyl sulfate, ethidium bromide, and nalidixic acid (i.e., the threshold levels of these compounds required to produce a visible zone of clearing in the mutant and parent strains were ≥5-fold different from one another; data not shown). The *E. coli* ZK796 *tolC* mutant also was more sensitive to doxorubicin and pyocyanin, as seen for strain AQ-41. These results are consistent with previously reported toxicity profiles for *E. coli tolC* strains (32) and suggest that TolC-mediated efflux pathways are similar in *S. oneidensis* and *E. coli*.

The requirement for *tolC* to confer resistance to low levels of tetracycline and other antibiotics has also been reported recently for *E. coli* strain W3110 (32). Under high tetracycline concentrations, however, the *tet* genes (which encode efflux pumps) are required to confer resistance to this antibiotic (4, 33); thus, a TolC efflux system is a determinant of the cells' intrinsic resistance to tetracycline. Interestingly, *tet* genes alone do not appear to promote increased efflux and resistance to AQDS, as the *tolC* mutant of *E. coli* used in this study was generated by a transposon encoding tetracycline resistance and was as sensitive to AQDS as AQ-41. This implies that significant AQDS efflux from the cell requires specific efflux systems.

The fact that the *tolC* gene is required for the efflux of AQDS, pyocyanin, tetracycline, and doxorubicin suggests that there may be hitherto-unrecognized links between electron shuttles and redox-active antibiotics. The structural similarities between redox-active antibiotics and known electron shuttles not only explain why they all utilize TolC-linked efflux systems but also indicate that they may have similar functions (9). For example, pyocyanin, a phenazine blue pigment that is typically considered to be an antibiotic due to the generation of toxic intermediates during its oxidation (7, 8), may also function as an electron shuttle during aerobic growth of *Pseudomonas aeruginosa* (6, 10). Indeed, the same underlying chemistry that makes redox-active antibiotics toxic to some cells (11) may in some contexts (such as respiratory growth with limiting terminal electron acceptors) be beneficial to other cells. The recent identification of novel antibiotics from the soil that have properties similar to those of humic substances (28, 29, 34) prompts us to take a closer look at their ecological functions. While much work remains to be done to prove this hypothesis, it seems plausible that the production of redox-active molecules that serve as both electron shuttles and antibiotics may be a widespread survival strategy for bacteria in the environment.

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