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Bacterial decolorization of textile dyes is an extracellular process requiring a multicomponent electron transfer pathway

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Summary

Many studies have reported microorganisms as efficient biocatalysts for colour removal of dye-containing industrial wastewaters. We present the first comprehensive study to identify all molecular components involved in decolorization by bacterial cells. Mutants from the model organism *Shewanella oneidensis* MR-1, generated by random transposon and targeted insertional mutagenesis, were screened for defects in decolorization of an oxazine and diazo dye. We demonstrate that decolorization is an extracellular reduction process requiring a multicomponent electron transfer pathway that consists of cytoplasmic membrane, periplasmic and outer membrane components. The presence of melanin, a redox-active molecule excreted by *S. oneidensis*, was shown to enhance the dye reduction rates. Menaquinones and the cytochrome CymA are the crucial cytoplasmic membrane components of the pathway, which then branches off via a network of periplasmic cytochromes to three outer membrane cytochromes. The key proteins of this network are MtrA and OmcB in the periplasm and outer membrane respectively. A model of the complete dye reduction pathway is proposed in which the dye molecules are reduced by the outer membrane cytochromes either directly or indirectly via melanin.

Introduction

Synthetic dyes are used worldwide in numerous industrial processes. Because dyes are recalcitrant by design, they persist in discharged wastewaters causing environmental health concerns and colour pollution. Several fungi and bacterial species are capable of decolorizing a wide range of dyes (Fu and Viraraghavan, 2001; Pearce *et al*., 2003): the fungal enzymes responsible for colour removal have been identified (Michel *et al*., 1991), but very little is known about the biochemical or molecular mechanisms underlying the bacterial process. Most studies focused on azo dyes of which decolorization is typically initiated by reductive cleavage of the azo bond, followed by relatively fast mineralization of the resulting colourless aromatic amines by aerobic microorganisms (Zimmermann *et al*., 1982). The initial dye reduction, regarded as the rate-limiting step in the overall dye degradation process, is most common under anaerobic conditions. Two types of NAD(P)H-dependent cytoplasmic 'azoreductases' have been described (Chen, 2006), but these enzymes were shown to be unimportant *in vivo*, as their reductase activity was only significant when using cell extracts and not using intact cells (Russ *et al*., 2000; Blümel *et al*., 2002). This can be explained by the fact that many dyes are very polar and/or large molecules which are unlikely to diffuse through the cell membrane. Bacterial dye reduction is therefore hypothesized to be a mainly extracellular process (Keck *et al*., 1997; Kudlich *et al*., 1997; Pearce *et al*., 2003). The fact that decolorization can be catalysed by microbially excreted (Keck *et al*., 1997) or artificial (Kudlich *et al*., 1997; Rau *et al*., 2002) redox mediators is in favour of this hypothesis. Such a system implies the transfer of reducing equivalents from an intracellular electron transport chain to the mediators, which would then reduce the extracellular dye non-enzymatically. Another possibility is that the bacteria establish a link between their intracellular electron transport systems and the extracellular dye via electron transferring proteins in the outer membrane (OM) (Pearce *et al*., 2003), which would then reduce the dye either directly or indirectly via redox mediators. Evidence concerning this extracellular reduction process has yet to be reported.

Recently, the anaerobic decolorization rates of *Shewanella* strains J18 143 (Pearce *et al*., 2006) and *decolorationis* (Xu *et al*., 2006) were shown to be among the highest ever reported. In addition, Hong and colleagues (2007) showed that *S. decolorationis* can grow

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with the dye amaranth as the only electron acceptor and that its purified membrane fraction displays dye reductase activity. Here, we make use of the genome sequence of *Shewanella oneidensis* (Heidelberg *et al*., 2002) to initiate a thorough study in which we identify all genes required for bacterial decolorization. A mutagenesis approach allowed the determination of the reduction pathway for both a diazo (two —N=N— chromophores) and an oxazine dye (—C—N=C— and =C—O—C= chromophores). Our results provide the first fundamental insights in bacterial dye reduction, which can be exploited for the rational design of decolorization methods in the treatment of colour pollution using *Shewanella* species as biocatalysts.

Results and discussion

Effect of different electron donors on anaerobic dye reduction by S. oneidensis *MR-1R*

The ability of MR-1R to decolorize two types of reactive dyes was assayed in defined medium supplemented with a range of electron donors (Fig. 1). The bacterium was found to reduce high concentrations of the dyes at rates that strongly depend on the donor used. A 1% solution of the oxazine dye Levafix Royal Blue E-FR (LRB; C.I. Reactive blue 224) was completely decolorized after 7 h using formate or lactate, although the rate of decolorization was faster with the former. Pyruvate supported dye reduction at a much slower rate. The other electron donors tested resulted in the same insignificant reduction rates as the cell-free and donor-free controls. Formate was also the preferred donor for reduction of a 1% solution of the diazo dye Remazol Black B (RBB; C.I. Reactive black 5), but the decolorization efficiencies were lower than for LRB.

Identification of transposon mutants defective in dye reduction

A library of 40 000 transposon mutants was screened to identify the minimal set of genes required for anaerobic dye reduction. Fifty mutants were found to have significantly slower reduction rates compared with the parental strain (Table S1), suggesting that the Tn*5* transposon interrupted several genes encoding key components of the dye reduction pathway. None of the mutants was found to be dye specific in its deficiency, implying that reduction of both dyes proceeds in an identical manner. The insertions were found to be located in 28 different genes. Based on the annotated functions of these genes, the mutants were divided in five groups as described below.

The first group of 11 mutants had insertions in eight different genes of the *gspC-gspN* operon, which encodes the components of the type II secretion (T2S) system. T2S is broadly conserved in Gram-negative bacteria and is

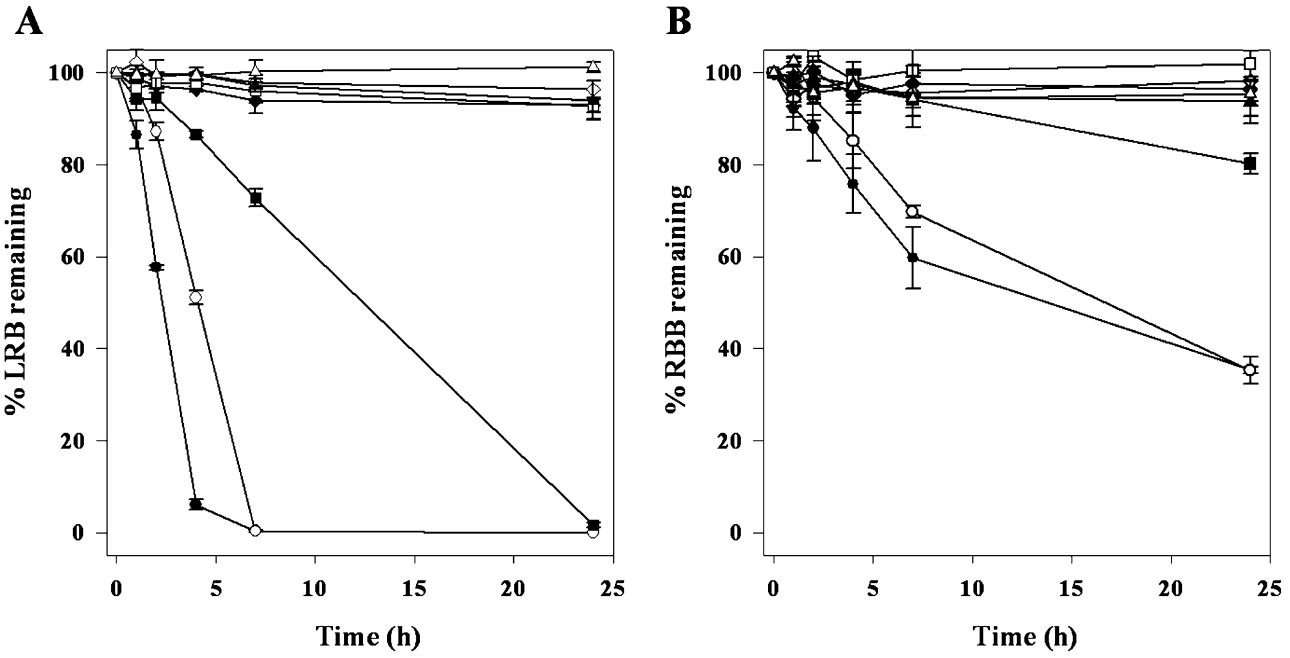

Fig. 1. Effect of different electron donors on dye reduction by *S. oneidensis* MR-1R. Decolorization activity is indicated as per cent of unreduced dye that remains in solution, with formate (●), lactate (○), pyruvate (■), acetate (◆), citrate (◇), glucose (□), no donor (▲) or no cells (△). Error bars represent the data range for triplicate cultures.
A. Decolorization of 1% LRB.
B. Decolorization of 1% RBB.

responsible for the translocation of proteins across the OM (Johnson *et al.*, 2006). In *S. oneidensis*, several *gsp* genes are required for the anaerobic respiration of Fe(III), Mn(IV) (DiChristina *et al.*, 2002) and DMSO (Gralnick *et al.*, 2006). We therefore assume that T2S is also involved in the translocation of proteins necessary for decolorization, which argues for dye reduction being an extracellular process.

The second group pointed out the importance of cytochromes *c* in the dye reduction process, as they had insertions in genes involved in either cytochrome *c* maturation (*ccmE*, *ccmA*, *dsbE*, *ccmH* or *dsbD*) or synthesis of the haem cofactor (*hemN*). The colonies of these mutants were coloured white instead of the typical red colour of the cytochrome-rich parental strain. The involvement of *c*-type cytochromes seems very logical because they are the main components of the extensive electron transport network in *S. oneidensis* (Meyer *et al.*, 2004). Surprisingly, a mutant with insertion in the twitching motility protein PilU was also coloured white, which we believe is the consequence of a polar effect on the downstream *hemH-2* gene. This gene encodes ferrochelatase, which catalyses the last step in the biosynthesis of haem. The deficiency in dye reduction of the PilU mutant is thus most likely caused by the absence of mature cytochromes *c*.

A third group had insertions in *omcB* (*mtrC*), *mtrA* and *mtrB*, which form a gene cluster known to be required for extracellular respiration. MtrB is an OM protein required for the reduction of Fe(III), Mn(IV), V(V) and AQDS (Beliaev and Saffarini, 1998; Beliaev *et al.*, 2001; Shyu *et al.*, 2002; Carpentier *et al.*, 2005). It is believed to act as a chaperone for the proper incorporation of the decahaem cytochromes OmcA and OmcB into the OM of MR-1 (Myers and Myers, 2002a). MtrA, a periplasmic decahaem cytochrome *c*, is a probable electron carrier between the tetrahaem quinol dehydrogenase CymA in the cytoplasmic membrane (CM) and OM cytochromes (Pitts *et al.*, 2003). The identification of the *omcB* mutant was of particular interest as OmcB may potentially act as a terminal dye reductase, given its location in the OM. This protein was also shown to be involved in Fe(III), Mn(IV) and V(V) reduction (Myers and Myers, 2001; 2002a; Myers *et al.*, 2004).

A fourth group had insertions in genes required for the synthesis of redox-active components located in the CM. One mutant had an insertion in *moaC*, which encodes a protein involved in the biosynthesis of the molybdopterin cofactor. Because formate was used as electron donor in the final step of the screening assay, and as formate dehydrogenases (FDHs) are molybdopterin-containing enzymes anchored in the CM, we ascribe the decolorization deficiency of the *moaC* mutant to the lack of an active FDH and thus lack of reducing equivalents for decolorization. Indeed, dye reduction by this mutant could be restored to wild-type (WT) levels using the electron donors lactate or pyruvate (results not shown), which also implies that no other molybdoproteins have significant roles in dye reduction. The *S. oneidensis* genome encodes three heterotrimeric FDHs (Serres and Riley, 2006). The FDH encoded by SO_0101-SO_0103 shows high similarity to the nitrate-inducible FDHs that contain a binding site for menaquinone (MQ) (Jormakka *et al.*, 2002) to which the electrons resulting from formate oxidation are transferred. Menaquinone (vitamin K_2) is a lipophilic naphtoquinone that can freely diffuse in membranes. In MR-1R it is a common intermediate in anaerobic electron transport to multiple terminal acceptors (Myers and Myers, 1993; Saffarini *et al.*, 2002; Carpentier *et al.*, 2005). Our results show an involvement in dye reduction as well, as 11 mutants had insertions in the MQ biosynthetic genes *menA*, *menC*, *menD*, *menE* and *menF*. Another mutant had an insertion in the *aroA* gene encoding a protein involved in the biosynthesis of chorismate, a common intermediate for the biosynthesis of aromatic amino acids, ubiquinone and MQ. The importance of MQ in dye reduction rationalizes why the *aroA* mutant was isolated in the screening. Nine mutants had an insertion in the gene encoding CymA that is responsible for electron transfer from the MQ/quinol pool to periplasmic and OM-bound reductases. A role in nitrate, nitrite, fumarate, Fe(III), Mn(IV) and DMSO respiration has already been established (Myers and Myers, 1997; 2000; Schwalb *et al.*, 2003).

A fifth group of mutants had insertions in genes for which the involvement in dye reduction is not yet clear. Gene SO_3174 encodes a glycosyl transferase with an annotated function in the synthesis of surface polysaccharides. The gene product of SO_1913 is a conserved hypothetical protein with unknown function. We suspected a polar effect on SO_1910 which encodes the MQ biosynthesis protein MenA, but this assumption was proven false as the addition of 0.05 mM vitamin K_2 did not restore the mutant phenotype to that of WT (results not shown). Additional research is required to rationalize the phenotype of these two mutants (see further).

In conclusion, our results demonstrate that dye reduction requires key components from the CM, the periplasm and the OM. In contrast to assumptions in earlier literature, dye reduction is independent of cytoplasmic enzymes. In fact it appears to be an extracellular process requiring electron transfer via a 'redox wire' that at least comprises (formate) dehydrogenase, MQ, CymA, MtrA and OmcB.

Generation of insertion mutants defective in the key components for dye reduction

To confirm that the decolorization deficiency of the transposon mutants was effectively caused by insertion in the genes listed in Table S1, the mutants were reconstructed

from MR-1R by targeted insertional mutagenesis. This reconstruction is required as transposable elements were previously shown to cause spontaneous mutations or large genomic deletions in MR-1 (Myers and Myers, 2002b; Bordi *et al.*, 2003), events that can result in misleading conclusions about the correlation between the site of insertion and the observed phenotype. The following insertion mutants, each named after the inserted gene, were generated: GSPD and CCME to inactivate T2S and cytochrome *c* maturation, respectively, MTRA, MTRB and OMCB to eliminate periplasmic and OM components of the pathway, MENF and AROA to abolish MQ synthesis, CYMA to remove a strategic component of the CM, and M1913 and M3174 to prevent transcription of SO_1913 and SO_3174. Reverse transcription polymerase chain reaction (RT-PCR) analysis showed that each of the interrupted genes was expressed in MR-1R but not in the mutant. Because the *omcB*, *mtrA* and *mtrB* genes constitute an operon, RT-PCR was also employed to determine whether insertional activation of *omcB* and *mtrA* was polar on the downstream genes. This was found not to be the case (Fig. S1A), which is consistent with observations made by Myers and Myers (2002b). SDS-PAGE and haem staining of crude extracts from MR-1R, CCME, MTRA, OMCB and CYMA showed a complete absence of cytochromes in CCME and absence of MtrA, OmcB and CymA in the respective mutants (Fig. S2).

Characterization of the insertion mutants

Decolorization by the different insertion mutants was analysed over a period of 24 h (Fig. 2). As for the corresponding transposon mutants, all insertion mutants had much slower reduction rates compared with the parental strain. The only exception was M3174, which displayed the same phenotype as MR-1R. SO_3174 was thus falsely identified during the Tn*5* mutant library screen, probably due to an unidentified insertion or deletion elsewhere in the genome. The presence of mature cytochromes *c* appears to be vital for the process of decolorization: CCME was unable to reduce LRB or RBB and showed the same phenotype as heat-killed cells. This is consistent with the importance of the cytochromes MtrA, OmcB and CymA. OmcB is located in the OM and can potentially function as a dye reductase. The residual decolorization activity of OMCB, however, suggests that other OM proteins are involved. This is in agreement with the observation that MTRB and the T2S mutant GSPD reduced dyes slower than OMCB, which suggests that OmcA may function as a dye reductase as well. The fact that OmcB and OmcA can form a functional complex with synergistic Fe(III)-NTA reductase activity (Shi *et al.*, 2006) and that they have overlapping roles for V(V) and Mn(IV) reduction (Myers and Myers, 2003; Myers *et al.*, 2004) supports this hypothesis. The contribution to dye reduction of the hypothetical protein encoded by SO_1913 is yet unknown and will be the subject of future studies. SO_1913 is expected to encode an extracytoplasmic protein, as the presence of a signal peptide (probability 1.0) was predicted by SignalP 3.0. Finally, the phenotypes of CYMA, MENF and AROA suggest that the CM components CymA and MQ are indispensable for decolorization. We can speculate that both are central components of the dye

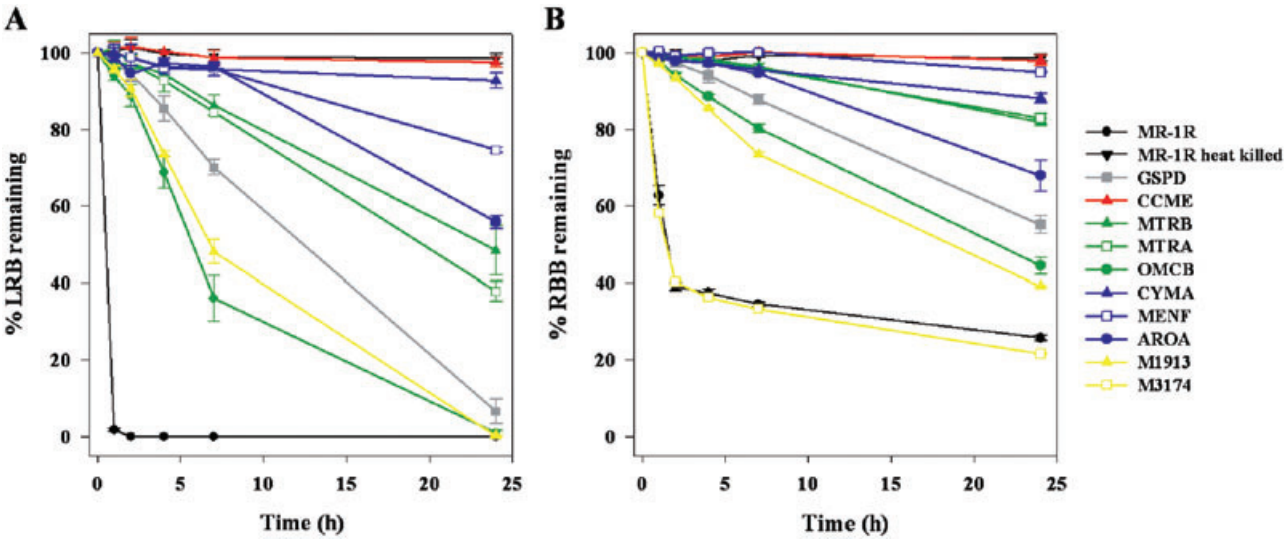

Fig. 2. Differences in decolorization rates of (A) 0.1% LRB and (B) 0.01% RBB by MR-1R and insertion mutants. The legend next to (B) also applies to (A). M3174 is not included in (A) as its phenotype was exactly the same as for MR-1R. For clarity, the following colour code is used: black, MR-1R controls; grey, T2S; red, cytochrome *c* maturation; green, periplasmic and OM proteins; blue, CM components, and orange, other. Error bars represent the data range for triplicate cultures.

reduction pathway, whereas multiple proteins with similar functions are likely to exist downstream.

Complementation of the insertion mutants

Complementation studies were performed to provide additional evidence that the interrupted genes (except SO_3174) code for proteins necessary for dye reduction. The GSPD mutant was also excluded from this study, as the involvement of T2S in dye reduction is indirect. Growth of MENF and AROA in the presence of 0.05 mM vitamin K_2 restored their ability to decolorize LRB and RBB (Table S2). Mutant CCME was complemented with the pEC86 plasmid (Arslan *et al*., 1998) that expresses the *Escherichia coli* cytochrome *c* maturation genes. Wild-type cytochrome *c* levels and decolorization activity were restored in CCME/pEC86 (Table S2, Fig. S2). The remaining insertion mutants were complemented with the WT genes cloned downstream of the *ara*BAD promoter of the pBAD202/D-TOPO vector. As we previously observed that this promoter is leaky in MR-1 (A. Brigé, unpubl. results), the presence of transcripts and proteins can in principle be restored in non-induced cultures of the complemented strains. This strategy proved to be successful, as all complemented strains showed dye reduction rates comparable to MR-1R (Table S2). Only for MTRA/pBAD*mtrA* an initial lag phase was observed. The haem-positive band absent in OMCB, MTRA and CYMA was restored in the corresponding complemented strain (Fig. S2). The complementation of OMCB was unexpected because a previous study has shown that a 21 kb genomic fragment including *omcB* is needed to fully restore OmcB and Mn(IV) reduction (Myers and Myers, 2002b). The reason for this discrepancy is unclear but may be due to the use of different complementation strategies.

Generation and characterization of additional mutants with one or two interrupted genes

To obtain more detailed information about the dye reduction pathway and to verify whether other periplasmic and/or OM proteins might be involved, we created several other mutants, based on the results presented above and on published information concerning the electron transport pathways in MR-1 in general, and anaerobic respiration in particular. An overview of these mutants with one (single mutants) or two interrupted genes (double mutants) is given in Tables 1 and 2, together with their decolorization activities. The use of double mutants in metabolic studies of MR-1 is an unprecedented approach that resulted in some valuable findings. All results and conclusions are presented in the following sections.

Central components of the aerobic respiratory chain are not required for dye reduction

The finding that dye reduction requires MQ, a crucial component in anaerobic respiration (Myers and Myers, 1993; Saffarini *et al*., 2002; Carpentier *et al*., 2005), agrees with the fact that bacterial decolorization is a widespread phenomenon under anaerobic conditions (Pearce *et al*., 2003). We confirmed the apparent insignificance of aerobic metabolism by the inactivation of central components in aerobic respiration. First, the absence of ubiquinones, accomplished by insertion of *ubiA* encoding the first enzyme of the ubiquinone biosynthetic pathway, had no effect on decolorization. Second, insertion in the *nqrF* genes (mutants NQRF-1, NQRF-2 and double mutant NQRF-1/NQRF-2) encoding subunit F of complex I of the electron transport chain did also not affect dye reduction rates. The importance of anaerobic metabolism was further assayed by disruption of the ferric uptake regulator Fur and the electron transport regulator A (EtrA), both pleiotropic gene regulators under anaerobic respiratory conditions in MR-1 (Beliaev *et al*., 2002; Wan *et al*., 2004). Whereas the phenotypes of the ETRA mutant and the parental strain were identical, the FUR mutant was initially slower, suggesting that at least some components of the dye reduction pathway are part of the Fur modulon. It has previously been shown that a *fur* deletion strain has downregulated expression levels of, for example, OmcB and CymA, which may explain the observed phenotype (Wan *et al*., 2004).

MtrA is the main periplasmic protein for electron transport to the OM

Because the MTRA mutant was not completely deficient in dye reduction, it is possible that additional proteins are involved in the intermembrane electron transport required for extracellular dye reduction. *Shewanella oneidensis* contains seven periplasmic cytochromes (Table 1) that could have such a role: SO_4360, *mtrD* and *dsmE* encode proteins similar to MtrA, of which DmsE is needed for DMSO respiration (Gralnick *et al*., 2006). Homologues of STC (Cct) and IfcA-1 are implicated in metal reduction in *Shewanella frigidimarina* (Gordon *et al*., 2000; Reyes-Ramirez *et al*., 2003). The tetrahaem cytochrome *c* SO_3300 displayed significantly elevated transcript levels in several metal-reducing conditions (Beliaev *et al*., 2005). Finally, *fccA* encodes a flavocytochrome c_3 that is essential for fumarate respiration (Gordon *et al*., 1998). The decolorization activities by the seven single mutants and by the double mutants MTRA/MTRD and MTRA/M4360 were comparable to MR-1R and MTRA respectively. Analysis of the double mutants IFCA/STC, MTRA/STC and MTRA/DMSE, however, suggested overlapping roles for MtrA, STC, IfcA-1 and

Table 1. Overview of the genes interrupted by insertional mutagenesis and dye reduction activity of the resulting single mutants.

Locus name	Gene symbol	Putative identification (TIGR cellular role category and/or subcategory)	% Dye remaining[a] LRB (1 h)	LRB (4 h)	RBB (1 h)	RBB (4 h)
Aerobic respiration/transcriptional regulators						
SO_0468	*ubiA*	4-Hydroxybenzoate polyprenyl transferase (Biosynthesis of ubiquinone)	1.7	0	62.5	39.2
SO_0907	*nqrF-1*	NADH:ubiquinone oxidoreductase, β-subunit (Energy metabolism/Electron transport)	3.2	0	69.1	39.5
SO_1108	*nqrF-2*	NADH:ubiquinone oxidoreductase, β-subunit (Energy metabolism/Electron transport)	2.2	0	72.3	37.1
SO_1937	*fur*	Ferric uptake regulation protein (Regulatory functions/DNA interactions)	77.5	1.1	88.0	52.8
SO_2356	*etrA*	Electron transport regulator A (Regulatory functions/DNA interactions)	2.4	0	67.9	39.2
Periplasmic cytochromes						
SO_0970	*fccA*	Fumarate reductase flavoprotein subunit (Energy metabolism/Anaerobic)	1.6	0	57.0	37.2
SO_1421	*ifcA-1*	Fumarate reductase flavoprotein subunit (Energy metabolism/Anaerobic)	2.0	0	64.7	38.9
SO_1427	*dmsE*	MtrA-like decahaem cytochrome *c* (Energy metabolism/Electron transport)	0.4	0	65.4	37.5
SO_1782	*mtrD*	Decahaem cytochrome *c* MtrD (Energy metabolism/Electron transport)	0.8	0	61.1	35.9
SO_2727	*STC*	Cytochrome *c*3 (Energy metabolism/Electron transport)	0.4	0	66.3	40.4
SO_3300		Cytochrome *c* (Energy metabolism/Electron transport)	3.3	0	70.5	39.1
SO_4360		MtrA-like decahaem cytochrome *c* (Energy metabolism/Electron transport)	0	0	67.8	38.6
Outer membrane proteins with (possible) involvement in extracellular respiration						
SO_1779	*omcA*	Decahaem cytochrome *c* (Energy metabolism/ Electron transport)	35.8	0	81.5	38.1
SO_1780	*mtrF*	Decahaem cytochrome *c* MtrF (Energy metabolism/Electron transport)	0.6	0	62.5	39.3
SO_1781	*mtrE*	Outer membrane protein, putative (Cell envelope/ Energy metabolism)	0.7	0	64.9	36.2
SO_1428	*dmsF*	MtrB-like outer membrane protein (Cell envelope)	3.9	0	72.3	38.7
SO_1429	*dmsA-1*	Anaerobic dimethyl sulfoxide reductase, A subunit (Energy metabolism/Anaerobic)	2.5	0	59.2	38.2
SO_3896	*omp35*	Outer membrane porin, putative (Transport and binding proteins/Porins)	3.2	0	76.7	37.2
SO_4358	*dmsA-2*	Anaerobic dimethyl sulfoxide reductase, A subunit (Energy metabolism/Anaerobic)	2.7	0	62.7	38.8
SO_4359		MtrB-like outer membrane protein (Cell envelope)	2.2	0	74.3	38.2
Melanin synthesis						
SO_1962	*4hppd*	4-Hydroxyphenylpyruvate dioxygenase (Energy metabolism/Amino acids and amines)	n/a[b]	n/a	n/a	n/a
Cytoplasmic 'azoreductase'						
SO_4396	*acpD*	Acyl carrier protein phosphodiesterase (Fatty acid and phospholipid metabolism)	0.8	0	63.9	36.9
MR-1R			1.7	0	63.1	39.5

a. For parental strain MR-1R and for each mutant, the dye reduction capacity is given as per cent dye remaining after 1 h and 4 h of incubation with 0.1% LRB or 0.01% RBB. All values given are the average of those obtained from triplicate cultures.
b. Not applicable because assays to determine the contribution of melanin in dye reduction using the 4HPPD mutant were performed in a manner different from the other mutants (see text and Fig. 4).
Genes are grouped according to the function or cellular location of the corresponding gene products.

DmsE (Table 2). IFCA/STC exhibited a lag phase compared with either of its parents, implying that either protein can compensate for the loss of the other. Dye reduction by MTRA/STC and MTRA/DMSE was slower than for MTRA, indicating a minor role for STC and DmsE. This was most apparent for decolorization of RBB (Fig. S3). The combined results suggest that MtrA is the major periplasmic protein in the dye reduction pathway, with the potential to branch off via at least STC, IfcA-1 and/or DmsE.

Table 2. Overview of the generated mutants with two interrupted genes (double mutants), grouped in order of discussion in the text.

Group	Interrupted genes	Double mutant	% Dye remaining[a]			
			LRB (1 h)	LRB (4 h)	RBB (1 h)	RBB (4 h)
Aerobic respiration	SO_0907/SO_1108	NQRF-1/NQRF-2	3.8	0.0	72.3	37.3
Periplasmic cytochromes	SO_1421/SO_2727	IFCA/STC	8.1	0.0	71.1	37.6
	SO_1777/SO_1782	MTRA/MTRD	97.6	93.5	97.5	96.7
	SO_1777/SO_4360	MTRA/M4360	99.5	92.3	99.8	95.6
	SO_1777/SO_2727	MTRA/STC	100	95.8	100	98.2
	SO_1777/SO_1427	MTRA/DMSE	100	96.4	100	98.7
Outer membrane proteins	SO_1779/SO_1778	OMCA/OMCB	97.3	91.4	98.7	94.7
	SO_1776/SO_1781	MTRB/MTRE	95.9	93.0	99.0	95.6
	SO_1776/SO_1428	MTRB/DMSF	98.3	95.8	99.6	98.6
	SO_1776/SO_4359	MTRB/M4359	97.0	94.5	98.8	97.9
	SO_1780/SO_1778	MTRF/OMCB	98.6	95.0	98.9	93.6
	SO_1429/SO_4358	DMSA-1/DMSA-2	3.7	0.0	64.5	38.0
	SO_1429/SO_1776	DMSA-1/MTRB	97.1	94.7	95.0	94.2

a. The values for the parental strain MR-1R and the respective single mutants are given in Tables 1 and S2.

The OM cytochromes OmcA, OmcB and MtrF are potential dye reductases

OmcB is a cell surface-exposed protein (Myers and Myers, 2003) that can contact extracellular dyes directly. As OmcB was previously shown to form a functional complex with OmcA (Shi *et al*., 2006), OMCA and OMCA/OMCB mutants were constructed to determine the effect on dye reduction (Figs 3 and S4, Tables 1 and 2). Analysis of OMCA revealed only a small contribution of OmcA, but the decolorization rates of the double mutant were significantly slower relative to its parents. This suggests that OmcA and OmcB exhibit a synergistic activity for reduction of dyes, a characteristic previously observed for the reduction of Fe(III)-NTA (Shi *et al*., 2006).

Because dye reduction was not completely abolished in OMCA/OMCB, other proteins with dye reductase activity are likely to exist. We screened for possible candidates in gene clusters that encode proteins with a potential involvement in extracellular respiration. The MR-1 genome is known to contain three such clusters in addition to *omcB-mtrAB* (Gralnick *et al*., 2006): *dmsEFABGH*

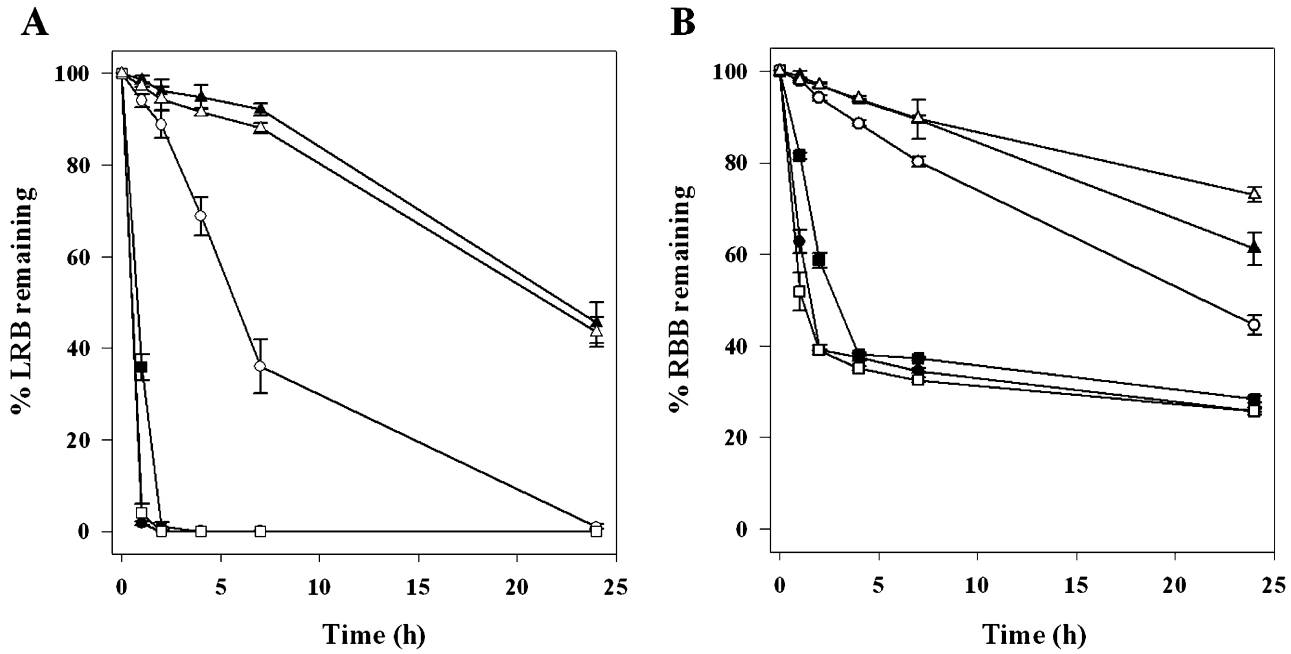

Fig. 3. Comparison of the decolorization rates of (A) 0.1% LRB and (B) 0.01% RBB between MR-1R (●) and mutants MTRF (□), OMCA (■), OMCB (○), MTRF/OMCB (▲) and OMCA/OMCB (△).

(SO_1427-SO_1432) and SO_4357-SO_4362 both encode proteins with similarity to MtrA, MtrB and DMSO reductase subunits, yet only the former is involved in DMSO respiration (Gralnick *et al*., 2006). The *mtrDEF* cluster is similar to *omcB-mtrAB*, with MtrD, MtrE and MtrF being homologues of MtrA, MtrB and OmcB respectively. We created a series of single mutants to analyse the importance of the OM components encoded by these clusters, i.e. the MtrB-like proteins encoded by *dmsF*, SO_4359 and *mtrE*, and the potential reductases encoded by *dmsA-1*, *dmsA-2* and *mtrF* (Table 1). None of the mutations were found to affect dye reduction; only mutants DMSF and M4359 exhibited a small lag phase relative to MR-1R. However, remarkable results were obtained when either *omcB* or *mtrB* was also interrupted in these mutants (double mutants listed in Table 2). Whereas the phenotype of MTRB/MTRE and MTRB/M4359 resembled that of the previously characterized MTRB single mutant, this was not the case for MTRB/DMSF (Fig. S3). This may indicate an involvement for DmsA-1 and DmsA-2 in dye reduction, yet we could not confirm this by analysing DMSA-1/DMSA-2 and DMSA-1/MTRB mutants. The role of DmsF in dye reduction is unclear at this stage. The most profound effect was observed with MTRF/OMCB: although insertional inactivation of the putative decahaem cytochrome *c* MtrF did not affect dye reduction, the deficiencies of MTRF/OMCB were more prominent than for OMCB (Fig. 3). This implies that MtrF and OmcB have overlapping roles and that MtrF, as OmcA, can compensate for the loss of OmcB and probably the OmcAB complex, thus explaining the residual activity of OMCB and OMCA/OMCB respectively. Although we could not distinguish MtrF on haem-stained gels, analysis by RT-PCR showed the presence of the *mtrF* transcript in the OMCB mutant under decolorizing conditions (Fig. S1B), which corroborates our theory. Analysis by haem staining showed the presence of OmcA in MTRF/OMCB (Fig. S4) which may account for the residual activity in this double mutant. In conclusion, it appears that OmcA, OmcB and MtrF can function in concert as the terminal reductases in dye reduction, but with OmcB clearly being the principal reductase. This is the first time that the involvement of MtrF in a respiratory process has been demonstrated, although the evidence provided is only circumstantial. It is possible that MtrF is also involved in metal reduction, which may have been overlooked in previous studies (Myers and Myers, 2002a; Myers *et al*., 2004) as we observed that the presence of OmcB obscures phenotypical analysis of *mtrF* insertion mutants. Analysis of double mutants may provide conclusive evidence.

Finally, Maier and Myers (2004) have demonstrated that the putative OM porin Omp35 affects the anaerobic reduction of fumarate, nitrate and Fe(III) indirectly. The initial dye reduction rates of the OMP35 mutant lagged somewhat behind those of MR-1R, yet the potential role of this OM component in anaerobic respiration is unknown.

Cytoplasmic 'azoreductases' are not involved in bacterial dye reduction

To further support our findings that dye reduction is an extracellular process, we created an insertion mutant in the *acpD* gene (SO_4396) encoding a cytoplasmic protein with 76% similarity to AcpD from *E. coli*. AcpD was identified as an 'azoreductase' (Nakanishi *et al*., 2001), but we could not demonstrate a contribution to dye reduction *in vivo* (Table 1). The term 'azoreductase' for cytoplasmic enzymes is thus misleading in the context of anaerobic decolorization by intact bacterial cells.

A beneficial role for extracellular melanin in dye reduction

Synthetic and metabolically formed quinoid redox mediators can increase bacterial azo dye reduction rates in a non-enzymatic manner (Keck *et al*., 1997; Kudlich *et al*., 1997; Rau *et al*., 2002). Many microorganisms produce a quinone-containing polymer with electrochemical properties called melanin. In *Shewanella algae* BrY, melanin can act as an electron conduit for iron mineral reduction (Turick *et al*., 2002). We reasoned that melanin might also have a catalysing role in dye reduction and therefore compared the decolorization rates between WT MR-1R, melanin-free and melanin-overproducing cells. In *Shewanella* species, homogentisate (HGA) is the primary precursor of melanin synthesis. Homogentisate is formed by the conversion of tyrosine by 4-hydroxyphenylpyruvate dioxygenase (4HPPD, Coon *et al*., 1994) and excess HGA is excreted outside the cells where it auto-oxidizes and self-assembles into melanin. We obtained melanin-free cells by creating an insertion mutant in the *4hppd* gene to abort melanogenesis. Because the production of melanin is related to the concentration of tyrosine (Turick *et al*., 2002), a melanin-overproducing strain was obtained by aerobic growth in the presence of excess tyrosine. Decolorization experiments with LRB and RBB showed that the absence of melanin resulted in initial dye reduction rates that were somewhat slower than those of MR-1R (Fig. 4), implying that dye reduction occurs predominantly in a direct manner by OM reductases. Experiments with the melanin-overproducing strain on the other hand showed that the presence of additional melanin accelerated the reduction of RBB significantly, meaning that dye reduction can also proceed indirectly. In *S. algae*, it was shown that reduced cytochromes can be oxidized by melanin (Turick *et al*., 2002): the OM cytochromes OmcA, OmcB and MtrF could thus provide a means to

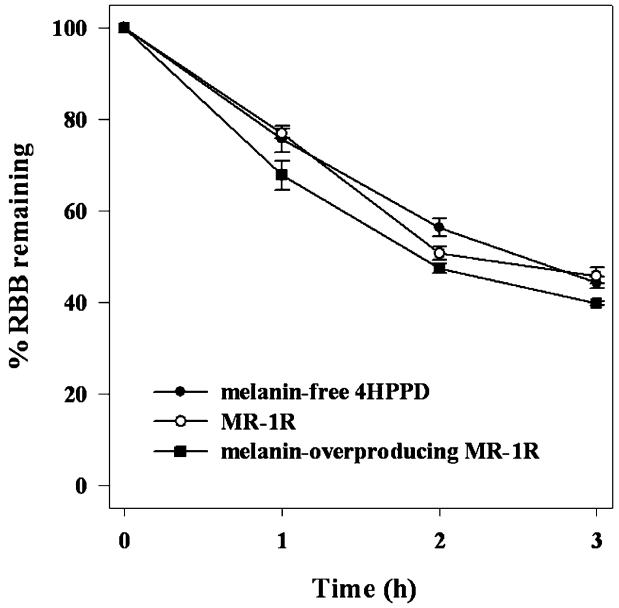

Fig. 4. The contribution of melanin in the reduction of RBB. The melanin-free (mutant 4HPPD) and melanin-producing MR-1R cells exhibit reduced decolorization rates compared with the melanin-overproducing strain. The latter was pre-grown in the presence of excess tyrosine (2 g l^{-1}).

transfer electrons from inside the cell via melanin to distant extracellular dye molecules.

Conclusion

This is the first report in which the major components of the electron transfer pathway required for the anaerobic decolorization of textile dyes have been documented. The identification of terminal reductases in the OM, the importance of type II secretion and the contribution of melanin provide evidence for dye reduction being an extracellular respiratory process. A model of the multicomponent pathway in *S. oneidensis* is given in Fig. 5. When formate is used as the electron donor, electrons enter the MQ/quinol pool via membrane-bound formate dehydrogenase from which they are transferred to CymA. The MtrA and OmcB proteins are the key constituents of the pathway downstream of CymA, yet alternative routes of electron flow to the dyes via a network of cytochromes *c* appear to be present. Given their location in the OM, the multihaem cytochromes MtrF, OmcA and OmcB may fulfil a function as terminal dye reductase. The role of the OmcAB complex is thus not restricted to the reduction of metals. Most identified components are common to the use of multiple electron acceptors, yet the contributions of MtrF and the hypothetical protein encoded by SO_1913 in an extracellular reduction process are new findings.

The observation that dye reduction rates can be enhanced by melanin, a molecule synthesized by *S. oneidensis* itself that can undergo repetitive cycles of reduction and oxidation, is very appealing for the development of more economical bioremediation strategies for the treatment of colour pollution. Finally, it has to be noted that *S. oneidensis* produces electrically conductive nanowires when the supply of electron acceptor becomes limited (Gorby *et al.*, 2006). It was demonstrated that OmcA and OmcB are needed for providing their electrical conductivity, yet it remains to be established whether nanowires also contribute to dye reduction. This would

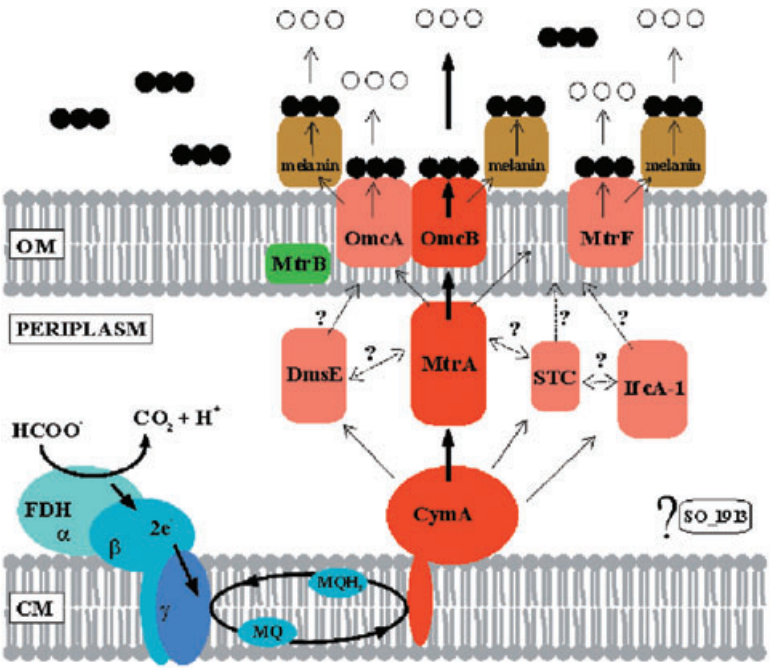

Fig. 5. Model for the decolorization mechanism by *S. oneidensis*. The arrows indicate the possible routes of electron transfer to the extracellular dye RBB resulting in colourless reduction products. The thick arrows represent the principal pathway starting from formate to OmcB via formate dehydrogenase (FDH), menaquinone (MQ), CymA and MtrA. All cytochromes *c* are shown in red. The type II secretion system is not included in the scheme. The contribution of the hypothetical protein encoded by SO_1913 is unknown. See text for details.

definitely be beneficial to obtain high decolorization efficiencies as nanowires not only allow electron transfer to acceptors that are distant from the cell, but they also increase the cell surface area.

Experimental procedures

Random transposon mutagenesis and screening

A transposon mutant library was generated as described in Beliaev and Saffarini (1998) using donor *E. coli* S17-1/pSUP1011 (Simon *et al*., 1983) and recipient *S. oneidensis* MR-1R, a spontaneous rifampicin-resistant mutant of MR-1. About 40 000 mutants were screened in three successive steps for the decolorization rates of LRB or RBB. In the first step, randomly picked colonies were grown overnight in duplicate in 96-well microplates filled with LB broth. Microplates were then transferred to an anaerobic glove box (Coy Laboratories, Grass Lake, MI). Consequently, 0.05% LRB or 0.005% RBB was added to initiate decolorization, which was completed within 10 min by the WT strain. Visual scoring of the individual wells enabled the rapid identification of mutants with decreased decolorization efficiencies. Only those mutants that were at least two times slower than WT were isolated and analysed further. After this initial high-throughput screening, false positives among the isolated mutants were excluded by two successive experiments. First, the selected mutants were inoculated in triplicate in two different 96-well plates and grown overnight. The OD_{600} was measured using a microplate reader and cultures that deviated by more than 10% from the average optical density were disregarded. Mutants that needed more than 20 min to completely decolorize LRB or RBB were selected. Second, WT and the selected mutants were inoculated in triplicate to a final OD_{600} of 0.1 in defined salts medium (Myers and Nealson, 1988) supplemented with 10 mM formic acid and either 0.1% LRB or 0.01% RBB. Decolorization rates were determined spectrophotometrically at the λ_{max} of the two dyes over a period of 24 h, which enabled us to make a final selection of 50 mutants with impaired rates of decolorization. Identification of the interrupted genes was obtained using arbitrary primed PCR (Caetano-Anolles, 1993) and the available genome sequence of *S. oneidensis* (Heidelberg *et al*., 2002).

Generation of insertion mutants

To generate mutants with a single insertion, an internal fragment of the gene of interest was amplified by PCR and cloned in the EcoRV site of the pKNOCK-Km or pKNOCK-Cm suicide vector (Alexeyev, 1999). The resulting plasmid was transformed into *E. coli* S17-1 *λpir* (Simon *et al*., 1983) by electroporation, after which the transformant was mated with *S. oneidensis* MR-1R. Double mutants with two interrupted genes were generated in a similar manner, except that the internal fragment of the second target gene was cloned in the pKNOCK vector with different antibiotic resistance than for the first target gene. Mating experiments were performed with the desired single mutant as the recipient. To confirm the site-specific integration of the vector, colonies were screened by colony PCR using primer pairs specific to the chromosomal DNA flanking the pKNOCK insertion sites. Control reactions using DNA of MR-1R were performed to compare the size of the amplified products.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from WT and mutant strains using the Qiagen RNeasy Mini kit according to the manufacturer's instructions, except that the DNase I treatment was prolonged to 1 h to ensure digestion of all residual chromosomal DNA. RT-PCR was performed with the Titan One Tube PCR System (Roche) using 1 μg of total RNA as template for cDNA synthesis. Appropriate primers were included to verify that the transcript of the target gene was absent in the mutant and present in the WT strain. In those cases where the interrupted gene is located in an operon, RT-PCR was applied to identify possible polar effects on downstream genes. Control reactions with heat-inactivated reverse transcriptase were performed to exclude artefacts from contaminating DNA.

Complementation studies

The MENF and AROA mutants were complemented by the addition of 0.05 mM vitamin K_2 (MQ) to the growth medium (Saffarini *et al*., 2002; Carpentier *et al*., 2005). The cytochrome *c*-deficient phenotype of the CCME mutant was restored with the pEC86 plasmid (Arslan *et al*., 1998) that constitutively expresses the *E. coli ccm* genes. All other mutants were complemented using the pBAD202/D-TOPO vector (Invitrogen). In each case, the WT gene was cloned downstream the *ara*BAD promotor and transformed into the corresponding Cm-resistant insertion mutant. Haem staining (Thomas *et al*., 1976) following SDS-PAGE of protein extracts from WT, mutants and complemented strains was used to check for the presence/absence of cytochromes *c*.

Analysis of dye reduction by WT, insertion mutants and complemented strains

All decolorization experiments were carried out anaerobically and in triplicate using the dyes LRB and RBB. Decolorization activities were determined spectrophotometrically at the λ_{max} of the two dyes (612 nm and 597 nm for LRB and RBB respectively) using cell-free samples taken at regular time intervals. The formula $100 - [(A - B/A) \times 100)]$, with A = initial absorbance and B = observed absorbance, was used to calculate the % dye not reduced in each sample.

Assays to determine the most effective electron donor to obtain maximum decolorization activity were carried out in defined medium (Myers and Nealson, 1988) supplemented with 20 mM of either formate, lactate, acetate, citrate, pyruvate or glucose and with 1% of either LRB or RBB. *Shewanella oneidensis* cultures were pre-grown semi-anaerobically at 28°C in LB medium. After centrifugation, the cells were transferred to an anaerobic glove box and re-suspended to a final OD_{600} of 0.1 in glass tubes filled with the different assay solutions. Control reactions contained either no donor or no cells.

Assays to compare the dye reduction rates between WT, mutant and complemented strains were performed in a similar way, with 10 mM formate as the electron donor and either 0.1% LRB or 0.01% RBB as the acceptor. Measurements were performed at periodic intervals over a period of 24 h. For conciseness, the time-course of decolorization is only given for the most significant mutants. For the other mutants, the values obtained at the 1 and 4 h time points were found to be representative for the observed phenotypes. These values are given in Tables 1 and 2. Assays to determine the contribution of melanin in dye reduction were performed differently. Mutant 4HPPD and MR-1R were pregrown aerobically for at least 48 h in defined medium with 15 mM lactate and formate, either in the absence or in the presence of excess tyrosine (2 g l^{-1}) to stimulate melanin production. The increase in pigmentation was monitored visually and spectrophotometrically at 400 nm. Cultures were transferred to the anaerobic glove box, normalized to an equal amount of cells (corresponding to an OD_{600} of 0.1) and supplemented with 0.01% RBB and 10 mM formate. Dye reduction was monitored at 597 nm. The background was subtracted from the obtained values.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Reverse transcription polymerase chain reaction (RT-PCR) to verify the presence or absence of transcripts from target genes in MR-1R and insertion mutants.
A. RT-PCR using total RNA from MR-1R (lanes 1, 3, 6, 9 and 11) and from the mutants OMCB (lanes 2, 4, 5, 7 and 8) and MTRA (lanes 10, 12 and 13) to analyse potential polar effects in the *omcB-mtrAB* gene cluster. Primer pairs for amplification of the following genes were used: *omcB* (2016 bp, lanes 1 and 2), *mtrA* (1002 bp, lanes 3–5, 9 and 10) and *mtrB* (2094 bp, lanes 6–8 and 11–13). Reactions in lanes 4, 7 and 12 were performed with inactivated reverse transcriptase to verify that products arose from mRNA and not from DNA contamination of the RNA sample. Sizes of the DNA marker (lane 14) are indicated on the right.
B. RT-PCR using total RNA and primers pairs specific for *mtrF* to show the presence of the *mtrF* transcript in MR-1R (lanes 1 and 2) and the OMCB mutant (lanes 3 and 4). The reaction in lanes 2 and 4 was performed with inactivated reverse. Sizes of the DNA marker (lane 5) are indicated on the right.
Fig. S2. Haem-stained SDS-PAGE analysis of total-protein samples from MR-1R (lane 1), insertion mutants CCME (lane 2), MTRA (lane 4), OMCB (lane 6), CYMA (lane 8), and complemented strains CCME/pEC86 (lane 3), MTRA/pBAD*mtrA* (lane 5), OMCB/pBAD*omcB* (lane 7) and CYMA/pBAD*cymA* (lane 9). Sizes of the pre-stained protein marker (lane 10) are indicated on the right. The white triangles indicate the absent or present cytochrome in the mutant or complemented strain respectively.
Fig. S3. Comparison of the reduction rates of 0.01% RBB by mutants MTRA, MTRA/DMSE, MTRA/STC, MTRB and MTRB/DMSF. The reduction rates by the double mutants (open symbols) are slower than those of the respective single mutants (filled symbols).
Fig. S4. Haem-stained SDS-PAGE analysis of total protein samples from MR-1R (lane 1) and from insertion mutants OMCA (lane 2), OMCA/OMCB (lane 3) and MTRF/OMCB (lane 4). Sizes of the pre-stained protein marker (lane 5) are indicated on the right.
Table S1. Overview of the genes identified as transposon insertions sites and characterization of the dye reduction deficiency of the corresponding transposon mutants.
Table S2. Comparison of the decolorization activity by insertion mutants and complemented strains of MR-1R.

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