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Bacterial two-hybrid analysis of the *Shewanella oneidensis* MR-1 multi-component electron transfer pathway

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ABSTRACT

Understanding cellular systems requires profound analysis of the protein interaction networks. Protein interaction mapping is performed mainly by co-purification strategies or two-hybrid systems. Recently, we expanded the tools for analyzing protein-protein interactions in bacteria with a two-hybrid technique based on beta-galactosidase complementation, and demonstrated its potential to explore interactions of membrane systems and to study transient interactions between redox partners (Borloo et al. 2007a). We demonstrate here the functionality of this assay to reveal the interactions within the complex electron transfer chain of the dissimilatory metal reducing bacterium Shewanella oneidensis MR-1. Specifically, we identified the cytoplasmic membrane-bound CymA as a key component, after which the electron transport chain is found to bifurcate towards several periplasmic proteins. It again congregates at the crucial MtrA, which in turn forms an electron transfer complex with the outer membrane localized MtrB and the terminal ferric reductases MtrC and OmcA. These data are complemented by mutant screening and confirm previous kinetic analyses (Borloo et al., 2007b).

Keywords: *Shewanella oneidensis*; bacterial two-hybrid; protein interactions; beta-galactosidase.

Abbreviations

NTA nitrilo triacetic acid; **FR** fumarate reductase; **IPTG** isopropyl-β,d-thiogalactopyranoside; **ONPG** o-nitrophenyl-β,d-galactose; **NIF** non-interacting fusion proteins; **IF** interacting fusion proteins.

1. Introduction

Several bacterial strains have been found capable of reducing heavy metals and to link this process to energy generation in order to grow and survive [1,2]. One of the most potent and striking examples of such strains is the Gram-negative facultative anaerobe Shewanella oneidensis MR-1 [3]. During anaerobic metal respiration, S. oneidensis MR-1 applies a sophisticated multi-component cascade, often referred to as the 'metal respiratory system' or the 'Mtr respiratory pathway'. This cascade transfers electrons from the cytoplasmic electron pool over the cytoplasmic membrane and periplasm, ultimately to the outer membrane localized terminal metal reductase(s). Many proteins, most of which are multi-haem cytochromes c, have been identified and characterized in the past as playing important roles in dissimilatory metal reduction [4,5]. Among these proteins is the centrally positioned cytoplasmic membrane-attached tetrahaem cytochrome c CymA [5-7] that collects excess electrons from carbon sources and shuttles them to MtrA, a decahaem periplasmic c-type cytochrome [8]. MtrA facilitates electron transfer to the outer membrane decahaem cytochromes c MtrC and OmcA [9], which are dependent on the non-haem integral outer-membrane protein MtrB for proper localization in the outer membrane [10]. MtrC and OmcA in turn reduce the terminal electron acceptors, which range from organic flavins to iron or manganese oxide, onto chelated iron complexes.

Despite the fact that the Mtr respiratory pathway has already been quite well-documented, some prominent questions remain. Curiously, the Shewanella oneidensis MR -1 genome encodes over 40 c-type cytochromes. It includes

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a number of paralogues, in particular and most interestingly to this study, MtrC (MtrF), MtrA (MtrD) and MtrB (MtrE). It has been shown that the MtrC-OmcA pair is largely responsible for chelated and insoluble iron reduction [11-13], but that MtrF is modular with MtrC and even more active than OmcA during flavin reduction in the absence of MtrC [14]. Additionally, Coursolle and Grainick [14] proved that, upon deletion of MtrA, alternative electron flow routes are recruited and that primarily MtrD, and to a lesser extent DmsE (the periplasmic component of the DMSO respiratory system), at least in part take over its function. Furthermore, involvement of the periplasmic iron-induced flavocytochrome IfcA [15], the small tetrahaem cytochrome c (CctA) [16,17], fumarate reductase [18] and the MtrA paralogue MtrD have been shown to be implicated in the Mtr respiratory pathway, indicating that also periplasmic electron shuttling is modular. Even though the key players in anaerobic dissimilatory metal reduction have already been identified, the exact organization of the electron transport chain, that is, which protein interacts with which protein and in what order and stoichiometry, has not yet been elucidated.

In this work we provide crucial insights in the organization of the chelated iron respiration cascade by: i) assaying several well-selected S. oneidensis MR-1 insertion mutants (i.e. which have been shown to be part of the electron transport chain) for their Fe(III)-NTA reduction capacity, thereby verifying and evaluating their importance or redundancy in the involved electron transport chain, and comparing them to other mutant studies, and ii) applying a recently developed β-galactosidase based bacterial two-hybrid system [19] in order to assess the interaction behavior of all previously identified components of the electron transport chain. Finally, we propose a comprehensive model that represents the S. oneidensis MR-1 Mtr respiratory pathway used during chelated metal reduction.

2. Material and Methods

2.1 *Bacterial Strains, Plasmids, Media and Growth Conditions*

S. oneidensis MR-1 was originally isolated from Oneida Lake sediments [5] and was obtained from the LMG culture collection (LMG 19005; Ghent, Belgium). The strain MR-1R, used in this study, is a spontaneous rifampicin-resistant mutant of strain MR-1 that was isolated in-house. The *S. oneidensis* MR-1 *ccmE*, *cymA*, *STC*, *ifcA*, *FR*, *mtrA*, *mtrB*, *mtrD*, *mtrE*, *mtrF*, *omcA* and *omcB* disruption mutants, as well as the *omcA*/*omcB*, *mtrA*/*mtrD*, *mtrB*/*mtrE*, *mtrB*/*mtrF* and *omcB*/*mtrF* double mutants, were obtained during a previous study [20]. *Escherichia coli* strain XL-1 Blue (Stratagene, La Jolla, CA, USA) was used in all subcloning steps, whereas recombination deficient strain JM109 (The Coli Genetic Stock Center, New Haven, CT, USA) was applied in all subsequent cloning experiments. β-Galactosidase activity tests were performed using *E. coli* strain MC1061 (The Coli Genetic Stock Center), which lacks the entire *lacZ* locus.

The pCOLADuet-1 vector (Km^R) was obtained from Novagen (Darmstadt, Germany). The pB2HΔα (Cm^R) and pB2HΔω (Cb^R) plasmids were obtained from a previous study [19].

Media and growth conditions for MR-1R and all *S. oneidensis* mutant cultures were as described previously [11]. *E. coli* cultures were grown aerobically in Luria Bertani broth on a rotary shaker (200 rpm) at 37°C [21]. Growth media were supplemented with appropriate antibiotics when necessary, including chloramphenicol (Cm) at 25 μg/mL, carbenicillin (Cb) at 100 μg/mL, and kanamycin (Km) at 25 μg/mL. When required, IPTG (Duchefa, Haarlem, The Netherlands) was added to a final concentration of 20 mM.

2.2 *DNA Manipulations*

To allow cytochrome c maturation in E. coli, the ccm genes were cloned from the pEC86 vector into the pCOLADuet-1 vector by XbaI-SalI restriction digest. A list of the synthetic oligonucleotides used in this study is presented in Table 1. Oligonucleotides included the restriction sites used to clone the DNA fragments into the vectors. Restriction digests, cloning and DNA electrophoresis were performed using standard techniques. DNA ligations were performed using T4 DNA Ligase (Promega, Madison, WI, USA). Isolation of plasmid DNA was accomplished using the QIAprep Plasmid Midi Kit 100 (Qiagen, Hilden, Germany). All DNA constructs were confirmed by DNA sequencing (GENOME Express, Meylan, France).

2.3 *Fe(III)-NTA Reduction Assay*

S. oneidensis MR-1R and all mutant strains were assayed for Fe(III)-NTA reduction activity according to previous reports [11], with the exception that 1.5 mM Fe(III)-NTA was used. Figure 1 presents the results of triplicate experiments.

2.4 *β-Galactosidase-based Bacterial Two-Hybrid Assay*

All steps in the application of the two-hybrid system, with the exception of protein sample preparation and the enzymatic β-galactosidase assay, were carried out as previously described [19].

Protein sample preparation was carried out as follows. Cells were centrifuged for 10 minutes at 10,000 x *g*. The pellet was resuspended in 1 mL phosphate buffered saline (PBS, pH 7.4) with Complete Protease Inhibitor (Roche Diagnostics Corporation, Indianapolis, IN, USA) (1 tablet per 200 mL sample), followed by sonication for 30 seconds using a Branson Digital Sonifier Model 250-D at 10% of its maximum force. The sample was subsequently clarified by centrifugating for 30 seconds at 10,000 x *g* in a tabletop centrifuge (Eppendorf, Hamburg, Germany). The obtained super-

Table 1. *S. oneidensis* MR-1 proteins applied in this study.

Protein	Description	Oligonucleotide name and sequence[a]	
CymA	Tetrahaem cytochrome c	A1	5′-GCATGCAGGAGGACAGCTATGAACTGGCGTGCACTATTTAAACCC-3′
		A2	5′-GGATCCTCCTTTTGGATAGGGGTGAGCGAC-3′
IfcA	Tetrahaem flavocytochrome c	B1	5′-GCATGCAGGAGGACAGCTATGTTGAATACCAAATTATTACCGTTA-3′
		B2	5′-GGATCCTTTAATAGAATTAGCTACTTGTTC-3′
STC	Tetrahaem cytochrome c	C1	5′-GCATGCAGGAGGACAGCTATGAGCAAAAAACTATTAAGTGTGCTT-3′
		C2	5′-GGATCCCTTCTTCAGAACAGACGCAGAAGT-3′
FR	Tetrahaem flavocytochrome c	D1	5′-GCATGCAGGAGGACAGCTATGTTCACAAGAAAGATTCAAAAAACA-3′
		D2	5′-GGATCCATTATCTTTAGCGAATTTAGCGGC-3′
MtrA	Decahaem cytochrome c	E1	5′-GCGGCCGCAGGAGGACAGCTATGAAGAACTGCCTAAAAATGAAAAACCTACT-3′
		E2	5′-GGATCCGCGCTGTAATAGCTTGCCAGATGG-3′
MtrB	75 kDa, outer membrane	F1	5′-GCATGCAGGAGGACAGCTATGAAATTTAAACTCAATTTGATCACT-3′
		F2	5′-GGATCCGAGTTTGTAACTCATGCTCAGCAT-3′
MtrD	Decahaem cytochrome c	G1	5′-GCATGCAGGAGGACAGCTATGCTTACATTAATGTTATCGATTCTC-3′
	40 kDa, periplasm	G2	5′-GGATCCTCTCTGCAGCAACTTGCCGGATGG-3′
MtrE	77 kDa, outer membrane	H1	5′-GCGGCCGCAGGAGGACAGCTATGCA AATAGTGAATATATCGACTCCT-3′
		H2	5′-GGATCCCATTTGGTAGCTTAAGGTCAAACC-3′
MtrF	Decahaem cytochrome c	I1	5′-GCATGCAGGAGGACAGCTATGAATAAGTTTGCAAGCTTTACCACG-3'
		I2	5′-GGATCCGTTTATTGGATGGACTTTGAGTAC-3′
OmcA	Decahaem cytochrome c	J1	5′-GCGGCCGCAGGAGGACAGCTATGATGAAACGGTTCAATTTCAATACC-3′
	83 kDa, outer membrane	J2	5′-AGATCTGTTACCGTGTGCTTCCATCAATTG-3′
OmcB	Decahaem cytochrome c,	K1	5′-GCATGCAGGAGGACAGCTATGATGAACGCACAAAAATCAAAAATCGCAC-3′
		K2	5′-GGATCCCATTTTCACTTTAGTGTGATCTGC-3′

[a] Underlined regions indicate the added restriction endonuclease sites to facilitate cloning: *Bam*HI for A2, B2, C2, D2, E2, F2, G2, I2 and K2; *Sph*I for A1, B1, C1, D1, F1, G1, I1 and K1; *Not*I for E1, H1 and J1; *Bgl*II for J2

natant was used in the assay.

β-Galactosidase activity was assayed quantitatively at room temperature by following ONPG hydrolysis and 2-nitrophenol formation at 415 nm in a Bio-Rad model 680 microplate reader (Bio-Rad, Hercules, CA), in a total volume of 200 μL using β-Galactosidase Assay Buffer (Pierce, Rockford, IL, USA). Half of the volume was protein sample while the other half consisted of the β-galactosidase substrate ONPG. Spectrophotometric measurements were started immediately after mixing. All values were subsequently normalized toward the total protein content per sample. The experiment was repeated five times for every fusion protein couple.

During analyses, an internal NIF set was used consisting of the fusion protein couple AtpE-Δα and AtpB-Δω [19]. The β -galactosidase activity value of this NIF set was 3.49 ± 2.62 nmol/(min.mg) and is arbitrarily represented here as 1.00 ± 0.75. The activity levels of the analyzed fusion protein couples were subsequently expressed relative toward this NIF set (Table 2).

2.5 *Statistical Analysis*

In the application of the two-hybrid system, statistical analysis was used to attribute IF and NIF status to different sets of fusion protein couples. Since only comparisons between the mean values of small data sets were made, the student *t*-test was applied. Since we expected the IF sets to have at least an equal or larger mean value (β-galactosidase activity) than the NIF sets, on the one hand, combined with the fact that a larger mean value for the NIF sets compared to the IF sets can only be attributed to chance, on the other hand, it was appropriate to choose a one-tail P-value. For all two-hybrid experiments, $n = 5$ and the alpha level (α-level) was set at 0.05. All statistical analyses were carried out using GraphPad Prism Version 4.00 and on-line GraphPad statistical software (GraphPad Software, Inc., San Diego, CA, USA).

2.6 *Chemical Cross-linking*

S. oneidensis MR-1R was grown to mid-log phase in mini-

Table 2. β-Galactosidase based two-hybrid analysis results [a]

	CymA	IfcA	STC	FR	MtrA	MtrD	MtrB	MtrE	OmcA	OmcB	MtrF
CymA	+										
IfcA	+	-									
STC	-	-	-								
FR	+ +	+ +	+	+ +							
MtrA	+ +	+ +	+ +	+ +	+ +						
MtrD	+	+ +	-	+ +	+ +	+ +					
MtrB	-	-	-	-	+ +	+	-				
MtrE	-	-	-	-	+	-	-	-			
OmcA	-	-	-	-	+ +	+ +	+ +	+ +	-		
OmcB	-	-	-	-	+	+ +	+ +	+ +	+ +	-	
MtrF	-	-	-	-	-	+ +	-	+	-	-	-

[a] "+" or "+ +" was given when $P < 0.05$ compared to the NIF set values. "+", meaning interaction, when NIF ± SEM < value ± SEM < 10 and "+ +", meaning strong interaction, when 10 < value ± SEM

mal medium [11] with 20 mM lactate and 50 mM ferric citrate as the electron donor and acceptor, respectively. Cells were centrifuged at 16,000 x *g* for 1 minute and then washed with an equal volume of phosphate buffered saline (PBS, pH 7.0) before suspending them in PBS to an optical density of 0.4 at 550 nm. Cells were cross-linked for 1 hour at room temperature with 1% formaldehyde. Following cross-linking, the total membrane fraction was prepared for both cross-linked and non-cross-linked cells, as described previously [19]. Equal amounts of the resulting samples were loaded on a 12% SDS-PAGE gel for analysis. After running the gel, it was stained specific for haem containing proteins as described by Thomas *et al.* [22]. The protein content of the resulting bands was subsequently analyzed by MALDI-TOF mass spectrometry.

2.7 *SDS-PAGE, Western Blotting and Haem Staining Analyses*

Protein content was verified by denaturing protein gel electrophoresis (SDS-PAGE) in 12% gels according to the method of Laemmli [24]. Western blotting using an antibody specific for β-galactosidase from *E. coli* (Invitrogen; Carlsbad, CA, USA) was performed by blotting the proteins to a Hybond ECL membrane for 4 hours at 40 V followed by an overnight non-fat dry milk blocking step at 4°C, extensive washing with PBS + 0.1% Tween20, and incubating the membrane with the anti-β-galactosidase antibody for 1 hour at room temperature. The washing steps were repeated and the membrane was subsequently incubated with horse raddish peroxidase (HRP) coupled to an antibody raised against the anti-β-galactosidase antibody. HRP chemiluminescence was then used to detect the fusion proteins containing the β-galactosidase fragments. Staining specific for haem containing proteins was carried out as described by Thomas *et al.* [22]. For all analyses, equal amounts of total protein (approx. 20 μg) were consistently loaded on the gels.

2.8 *Miscellaneous Procedures*

Protein concentrations were determined by the Bradford assay [23] using the Bio-Rad Protein Assay Solution (Bio-Rad, Hercules, CA, USA). Image acquisition was carried out using software packages Corel® version 9 and Paintshop Pro version 5 (Corel, Berkshire, UK).

3. Results

3.1 *Fe(III)-NTA Reduction Activity of the Insertion Mutants.*

When comparing to *S. oneidensis* MR-1R, the *STC* insertion mutant has its Fe(III)-NTA reduction capacity being diminished by approximately 40% (Fig. 1). Disrupting the *omcA*, *FR*, *ifcA*, *mtrD* and *mtrF* genes leads to a slight drop in Fe(III)-NTA reduction activity, of approx. 10%. Whereas the *omcB* and *cymA* mutants still bear some residual reduction activity (10% of the MR-1R level at most), disrupting *mtrA* and *mtrB* almost completely abolishes Fe(III)-NTA reduction. Figure 1 also shows that when two (often modulatory) genes are simultaneously interrupted, particularly the *omcA*/*omcB*, *mtrA*/*mtrD*, *mtrB*/*mtrE*, *mtrB*/*mtrF* and *omcB*/*mtrF* double mutants, no significant levels of reduction activities are observed either. Importantly, a *ccmE* mutant (CcmE, SO_0259), which is defective in cytochrome *c* maturation, also lacks Fe(III)-NTA reduction activity

3.2 *β-Galactosidase-based Bacterial Two-Hybrid Assay*

To assess their protein-protein interaction behaviour, *cymA*, *FR*, *ifcA*, *STC*, *mtrA*, *mtrB*, *mtrD*, *mtrE*, *mtrF*, *omcA* and *omcB* were all separately cloned both into the pB2HΔα and pB2HΔω constructs as described previously [19]. A list of

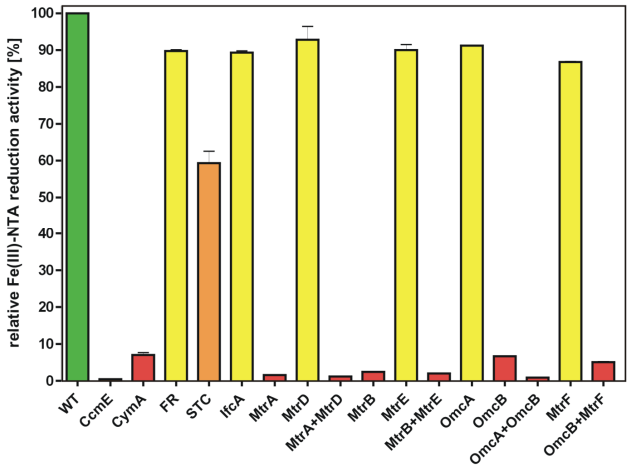

Figure 1. *Fe(III)-NTA reduction assay of the mutants.* Bar representation of the Fe(III)-NTA reduction activities of the single and double insertion mutants applied in this study. Values are presented relative to the reduction capacity of S. oneidensis MR-1R, for which the level was set at 100%. Error bars indicate the standard error of the mean (SEM).

these proteins and their as of yet known properties is provided in Table 1.

Correct synthesis of the fusion proteins was controlled by Western blotting (immunoblot) for those containing MtrB and MtrE, and haem staining for those carrying haem-proteins. Figure 2 shows the expression results for pB2HΔα-containing fusion proteins. Haem staining reveals successful haem attachment for all haem-carrying fusion proteins after proper translocation to the periplasm (Fig. 2*A*). The immunodetection using anti-β-galactosidase antibodies verified that the MtrB- and MtrE-containing chimeras are properly

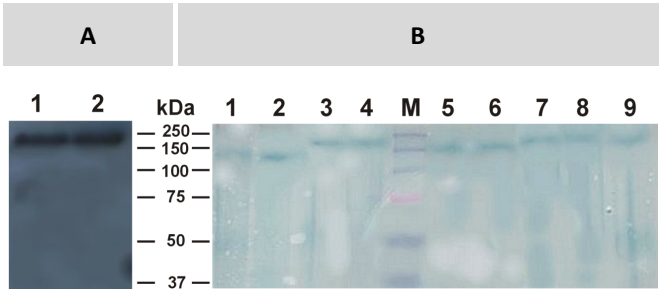

Figure 2. *Controls regarding the proper synthesis of the fusion proteins.* A. Western blot using an antibody specific for β-galactosidase, equal amounts of protein were loaded (~20 µg total protein). The molecular weight marker is indicated at the centre of the figure. Lane 1, pB2HΔαΩmtrB (193 kDa); Lane 2, pB2HΔαΩmtrE (195 kDa). B. Specific staining for haem proteins, equal amounts of protein were loaded (~20 µg total protein). The molecular weight marker is indicated at the centre of the haem-stained gel. Lane 1, pB2HΔαΩcymA (139 kDa); Lane 2, pB2HΔαΩSTC (132 kDa); Lane 3, pB2HΔαΩifcA (183 kDa); Lane 4, pB2HΔαΩFR (181 kDa); Lane 5, pB2HΔαΩmtrA (161 kDa); Lane 6, pB2HΔαΩmtrD (158 kDa); Lane 7, pB2HΔαΩmtrF (192 kDa); Lane 8, pB2HΔαΩomcA (201 kDa); Lane 9, pB2HΔαΩomcB (195 kDa).

synthesized at low, *quasi* physiological levels when IPTG is added to the growth medium (Fig. 2*B*).

Our two-hybrid analysis, the results of which are presented schematically in Figure 3, and in a complete though condensed form in Table 2, reveals the interaction of the cytoplasmic membrane-anchored CymA with itself and with the periplasm-located IfcA, FR and MtrA. The latter two display a strong interaction with CymA, whereas the MtrA-paralogue MtrD surprisingly yielded no positive signal. The periplasmic pool, consisting of the multi-haem c-type cytochromes IfcA, STC, FR, MtrA and MtrD, furthermore reveal an extensive divergence of interactions, that is, besides IfcA interacting with FR, both of these proteins interact strongly with MtrA and its paralogue MtrD. In contrast, STC seems to be more limited in its interaction behaviour since it only interacts with FR and MtrA. Of the periplasmic proteins, FR and MtrA both exhibit positive interaction values with all other periplasm-directed cytochromes, and with themselves. However, given their interaction profiles with the outer membrane localized proteins MtrB, MtrE, OmcA and OmcB, apparently only MtrA, and to a lesser extent MtrD, are capable of making the link to the membranous protein pool. MtrB and its paralogue MtrE were additionally identified as to strongly interact with both OmcA and OmcB. Finally, although not found to form homo-oligomers, a convincingly positive interaction signal was also observed when the terminal ferric reductases OmcA and OmcB were co-expressed.

3.3 *Chemical cross-linking.*

The membrane fractions of both formaldehyde cross-linked and non-cross-linked *S. oneidensis* MR-1R cells were applied to SDS-PAGE and visualized by haem-staining. The gel showed two prominent bands of 80 and 73 kDa (Fig. 4) corresponding to OmcA and OmcB, respectively. Addition of 1% formaldehyde to undisrupted cells resulted in the appearance of a new haem band corresponding to a molecular weight of approximately 200 kDa, as shown in Figure 4. MALDI-TOF mass spectrometric analysis identified the cross-linked proteins as OmcA, OmcB and MtrB, which is in accordance with the observed molecular weight of the proteins on the haem-stained gel.

4. Discussion

The fundamental importance of cytochromes *c* in anaerobic metal reduction has been recognized for quite some years [4, 25, 26] and is here confirmed through our experiments in that a *S. oneidensis* MR-1 mutant defective in cytochrome *c* maturation (*ccmE* mutant) does not hold any significant Fe (III)-NTA reduction activity (Fig. 1).

The central position of CymA (Fig. 3), being cytoplasmic membrane-anchored and faced to the periplasm, already suggests its pivotal role in the electron transfer process. This is again reflected by the lack of any substantial Fe(III)-NTA

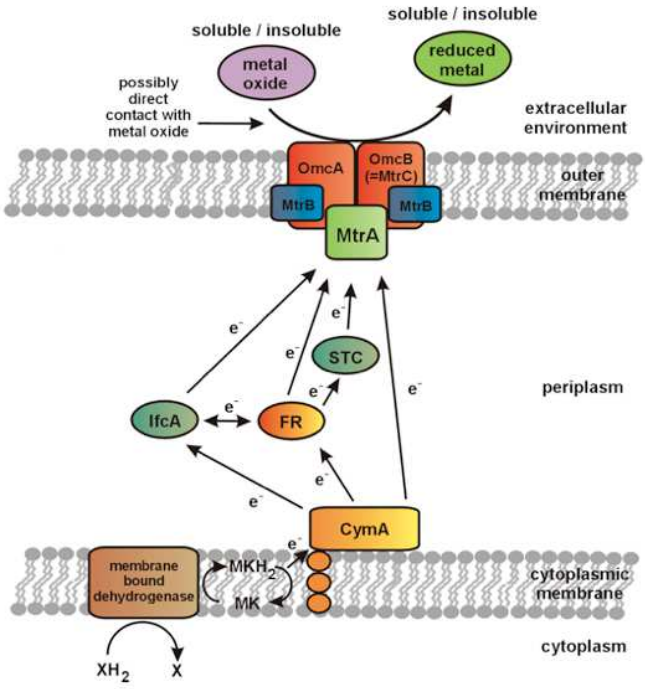

Figure 3. *Model for the metal reduction mechanism by S. oneidensis MR-1.* The arrows indicate the possible routes of electron transfer starting from the cytoplasm, via the cytoplasmic membrane, periplasm and outer membrane, and ending at the reduction of the targetted metal species. See text for further details.

reduction capacity of the *cymA* mutant. The fact that it interacts with all periplasmic components of the electron transport chain, except with STC, is decisive in our conclusion that CymA, apparently as a homo-oligomer, forms the crucial link between the menaquinone pool in the cytoplasmic membrane and the several periplasmic electron transfer proteins, which is in agreement with previous studies [14,27]. From CymA onwards, the electron transport chain further branches off towards three periplasmic electron transporters, i.e. IfcA, FR and MtrA (Fig. 3), of which the latter seems to take the leading role, given the effect of disrupting the *mtrA* gene on Fe(III)-NTA reduction (Fig. 1). The redundancy of IfcA and FR in the electron transfer cascade is reflected by i) the minor decrease in Fe(III)-NTA reduction activity that is observed when disrupting the respective genes (Fig. 1), and ii) the fact that both IfcA and FR interact with each other and, more importantly, with MtrA (and its paralogue MtrD). This indicates that after the bifurcation, the electron transport chain again congregates at some point, more particularly, at the key component MtrA, as was also proposed by Coursolle and Gralnick [14].

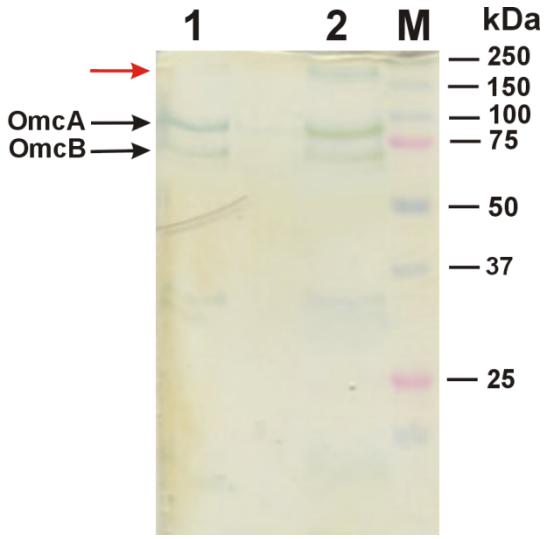

Figure 4. *Heme-stain after the formaldehyde cross-linking experiment.* Total membrane fractions originating from non-cross-linked S. oneidensis MR-1R cells (Lane 1), and formaldehyde cross -linked cells (Lane 2). Black arrows specify the positions of OmcA and OmcB, whereas the red arrow indicates the cross-linked high-molecular weight species observed only in Lane 2. The molecular weight marker is indicated on the right in units of kDa.

In *Shewanella frigidimarina* the iron-induced flavocytochrome c_3 is coded by the IfcA gene, and although it displays fumarate reductase activity it is only induced when *S. frigidimarina* is grown on soluble Fe(III) [15]. Since IfcA might behave similarly in *S. oneidensis* MR-1, its redundancy in the electron transport chain seems plausible. Previous reports that Ifc_3 forms dimmers in the psychrophilic *S. frigidimarina* [14] cannot be supported by our study of the *S. oneidensis* MR-1 IfcA.

Besides its previously reported [17] and in the present study confirmed interaction with CymA, the soluble fumarate reductase (FR) is one of two periplasmic proteins (the other being MtrA) that interacts with the small tetrahaem cytochrome *c* (STC), indicating yet another point where the electron transfer cascade splits up. This non-central position of STC, together with the limited effect in Fe (III)-NTA reduction observed from the *STC S. oneidensis* MR-1 mutant strain, makes us deduce that STC plays a secondary role in anaerobic metal respiration.

From the results given in Table 2 it is clear that MtrA, and to a lesser extent its paralogue MtrD, functions as the central electron courier in the periplasm, possibly as a homo-oligomer and as part of a greater outer membrane-associated complex, thus corroborating the observations made by Ross *et al.* [27,28]. This, together with the total loss of Fe(III)-NTA reduction activity in the *mtrA* mutant (but not in the *mtrD* mutant), is in contrast to earlier reports which stated that membrane fractions of a *mtrA* deletion mutant were still able to reduce Fe(III)-oxides [2].

Making a bridge from the periplasm to the outer membrane, we find MtrA again playing a decisive role in that it clearly interacts with the outer membrane situated β-barrel porin MtrB. These findings are in line with those of Ross *et al.* [28] and Hartshorne *et al.*[9], the latter of whom attribute a sheath-like role to MtrB, enabling it to transfer electrons from the periplasmic MtrA to the outer membrane -situated MtrC (= OmcB). Besides such crucial task and the fact that it serves as a component responsible for anchoring

or orienting proteins in/to the outer membrane [10], chances that MtrB and MtrE would serve as an electron transporter are inexistent, since they carry no haem or any other redox-active moieties. The presence or absence of MtrB thus indirectly affects the ability of *S. oneidensis* reducing certain electron acceptors**,** a statement that is corroborated by the results of our Fe(III)-NTA reduction assays with the *mtrB* mutant (Fig. 1). Surprisingly, the *mtrE* mutation merely caused a ~10% drop in Fe(III)-NTA reduction activity. Apart from MtrA and MtrB interacting, both proteins, and their paralogues MtrD and MtrE, displayed a positive interaction signal with both outer membrane localized decahaem cytochromes *c* OmcA and MtrC (= OmcB), and the MtrC paralogue MtrF. Also hinted at other studies [9,28], these results strengthen the theory that MtrA, MtrB, OmcA and MtrC might form a multi-component complex that would be responsible for metal reduction. It should be noted at this point that, using the techniques that we applied ,no assumptions can be made concerning the stability of these interactions, since electron transfer only requires transient interactions (approx. 10^{-9} s). Nevertheless, from our two-hybrid and cross-linking experiments we can deduce that the two outer-membrane decahaem cytochromes *c* OmcA and MtrC interact and very likely form a stable complex, as was previously also suggested by Shi *et al.* [29]. Our results support their suggestion that OmcA and MtrC interact in a 2:1 ratio.

Even though the results of our two-hybrid analyses suggest that MtrD, MtrE and MtrF play significant roles in the electron transport chain, the Fe(III)-NTA reduction assays refute this conclusion, since inactivating the respective genes brings the reduction activities down by only approximately 10% (Fig. 1). However, this seems logical, since their paralogues MtrA, MtrB and MtrC, respectively, are still present and take over their roles; a feature referred t as the 'modularity' by Coursolle and Gralnick [14]. The lack of reduction activity in the *mtrA*, *mtrB* and *omcB* mutants, in contrast, may find its origin in the low or non-expression of their respective paralogues.

5. Concluding Remarks

In conclusion, we have shown that our bacterial two-hybrid system is a powerful tool in protein-protein interaction studies. As the case of S. oneidensis MR-1 exemplifies, anaerobic metal reduction was found to require cytoplasmic membrane, periplasmic and outer membrane components, the most important of which being CymA, MtrA and OmcA, MtrC and MtrB, respectively. It has previously been shown that OmcA and OmcB both function as terminal ferric reductases, the latter (MtrC) being the principal enzyme (Fig. 1) [11]. We believe therefore that the electron transport chain ends at the OmcA/MtrC complex, accompanied by MtrA and MtrB, and that the ultimate step in metal reduction is the transfer of electrons to the targetted metal species, as is depicted in Figure 3.

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