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Characterization of cytochrome mutants for pellicle formation in *Shewanella onedensis* MR-1

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Abstract: Biofilm systems are effective for biosorption of metal ions. *Shewanella oneidensis* MR-1, a Gram-negative facultative anaerobe, is a natural pellicle-like biofilm former. The mechanisms of pellicle formation by *S. oneidensis* MR-1 have not yet been understood. 17 *S. oneidensis* MR-1 deletion mutants, including 12 c-type cytochromes were generated and tested if they were involved in pellicle formation. The results show that Δ*SO4666*, Δ*SO1777*, Δ*SO1782*, Δ*SO2361* and Δ*SO2363* have varying deficiency in pellicle formation. The deletion mutant Δ*SO4666* cannot form a pellicle under non-shake conditions, suggesting that it may play an important role in pellicle formation by *S. oneidensis* MR-1. Overall, these data suggest a very complex picture of aerobic respiration by *S. oneidensis* MR-1.

Key words: cytochrome; mutant; electron transport; pellicle; biofilms

1 Introduction

Considerable studies have been focused in recent years upon the field of biosorption for the removal of metal ions from aqueous effluents[1]. Biofilms promise to be suitable systems for the treatment of metal ions since microorganisms that absorb metals stabilize the extracellular polymeric matrix by the combined action of chemical, physical and physiological phenomena that are, in some instances, linked to phenotypic variation among the constituent biofilm cells[2]. *Shewanella oneidensis* MR-1, a facultative Gram-negative anaerobe with a remarkable respiratory versatility, is a natural biofilm former. In recent years, this organism has attracted a great deal of interest due to its potential applications in bioremediation of metal contaminants in the environment. *S. oneidensis* MR-1 has been extensively studied, including the development of the canonical biofilms [3−9].

S. oneidensis MR-1 possesses a complex electron-transport system. Cytochromes, heme-containing proteins are the main components of the respiratory electron transport chains[10]. Approximately, 42 cytochrome c genes are annotated based on sequence analysis and 41 are likely to be functional in comparison with five to seven in *E. coli* and other enteric bacteria [11], and most of them are more or less mobile electron transfer proteins on the periplasmic side of the membrane[12]. A fraction of the *Shewanella* cytochrome genes have been characterized at the molecular level under anaerobic condition[13]. Since c-type cytochromes are essential for energy metabolism, their mutation will also directly affect the aerobic electron transport network. However, the role of most cytochromes under aerobic conditions remains unknown.

In this work, the mechanisms of pellicle formation in *S. oneidensis* MR-1 are explored. We generated and characterized 12 cytochrome mutants and 5 other mutants related to protein secretion systems and regulatory proteins, and found that some cytochromes show deficiency in pellicle formation under aerobic conditions. These data represent the first identification of genes specifically related to pellicle formation and an

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important step towards understanding the aerobic respiratory systems in *S. oneidensis* MR-1 on a genomic scale.

2 Experimental

2.1 Bacterial strains, plasmids, media, and growth conditions

S. oneidensis MR-1 and *Escherichia coli* strain WM3064 used for mutagenesis were grown using Luria-Bertani (LB) medium (pH 7.2) at 30 ℃ and 37 ℃, respectively.

2.2 Mutagenesis

A list of the mutants used in this study is provided in Table 1. *S. oneidensis* MR-1 cytochrome mutants were constructed either by fusion PCR method as described by WAN et al[14] or by a cre-lox recombination method described by MARX and LIDSTROM[15]. Other deletion mutation strains were constructed using the fusion PCR method illustrated previously[16, 17]. All deletions were verified by PCR and DNA sequencing. Each mutant could be readily distinguished by a 20 bp marker[14].

2.3 Complementation of Δ*SO4666* mutant

For complementation of Δ*SO4666*, DNA fragment containing *SO4666* and its native promoter was generated by PCR amplification with MR-1 genomic DNA as the template using primers *SO4666*-COM-F (AGGATCCGCAATGTCTGGTGCACTGAT) and *SO4666*-COM-R (AGGATCCACTTATGTTGCGGC-TGACG). This fragment was digested with BamHI and ligated to BamHI-digested pBBR1MCS-5 to form pBBR-*SO4666*[18], which was electroporated into WM3064. Introduction of pBBR-*SO4666* into Δ*SO4666* was done by mating with WM3064 hosting pBBR-*SO4666*, and gentamycin-resistant colonies were selected. The presence of pBBR-*SO4666* in Δ*SO4666* was confirmed by plasmid purification and restriction enzyme digestion.

2.4 Pellicle formation, measurement of growth, and quantification of pellicle

A fresh colony grown overnight on a LB plate was used to inoculate 50 mL LB and incubated in a shaker (200 r/min) to an OD_{600} of 0.8 at the room temperature. This culture was then diluted 500-fold with fresh LB, resulting in the starting cultures. Throughout the study, all starting cultures of *S. oneidensis* strains were prepared in such a way. Aliquots of 30 mL starting cultures were transferred to 50 mL Pyrex beakers equipped with side-arm stop-cocks (Lab-made). The beakers were kept still for pellicle formation at the room temperature. To

Table 1 Summary of evaluated set of cytochrome mutants, selected transport or regulator protein mutants

Functional category	Open reading frame targeted	Gene product deleted (gene)
Electron transport	*SO0479*	Octaheme cytochrome c, involved with sulfur cycle
	SO0610	Ubiquinol-cytochrome c reductase (PetC)
	SO0714	Periplasmic monoheme cytochrome c4, involved in sulfite oxidation
	SO0716	Periplasmic monoheme cytochrome c (SorB), involved in sulfite oxidation
	SO0845	Diheme cytochrome c (NapB), involved in nitrate reduction
	SO1777	Periplasmic decaheme cytochrome c MtrA (MtrA), involved in metal oxide reduction
	SO1780	Outer membrane decaheme cytochrome c (MtrF)
	SO1782	Periplasmic decaheme cytochrome c (MtrD)
	SO2361	Cytochrome c oxidase, cbb3-type, subunit Ⅲ (CcoP)
	SO2363	Cytochrome c oxidase, cbb3-type, subunit Ⅱ (CcoO)
	SO4360	MtrA-like decaheme cytochrome c
	SO4666	Cytochrome c (CytcB)
Protein secretion	*SO4319*	Type Ⅰ secretion membrane fusion protein, EmrA
	SO0168	General secretion pathway protein F, Type Ⅱ secretory pathway, GspF
Regulatory	*SO0624*	Catabolite gene activator, Crp
	SO2356	Electron transport regulator A, EtrA
	SO3988	Aerobic respiration control protein, ArcA

separate cells in pellicle and underneath, cultures were withdrawn through the stop-cock even for collecting planktonic cells and cells from the pellicle were gathered[19]. To quantify the pellicles formed by the *S. oneidensis* wild-type and mutation strains, cells from pellicles were collected, suspended in 30 mL fresh LB, violently vortexed, and measured at 600 nm by a spectrometer.

2.5 Quantitative-PCR (q-PCR) estimation of biomass density during competition

Each of 12 cytochrome mutants was first grown separately to the late stationary phase in the LB medium, and then all of them were mixed together each with equal biomass, which was defined as the starting sample (C_0). C_0 sample was diluted by 100-fold into a flask or beaker containing 50 mL of fresh medium and grown to the late stationary phase, which was defined as a circle. Three sets of experiments differing in growth conditions, pellicle or planktonic growth under static condition and agitated culture, were performed in triplicate. The cells in pellicle and planktonic in beaker were separated by the stop-cock as we mentioned before. In agitated growth competition experiment, cytochrome mutants were co-inoculated into LB and agitated at the rate of 200 r/min for 10 cycles. The biomass of C_0, circle 5 (C_5) or circle 10 (C_{10}) were collected by centrifugation and followed by DNA extraction. A certain series diluted gDNA of each 12 cytochrome mutants was used to make the standard curve by SYBR Green I fluorescence dye based q-PCR. The primers used are listed in Table 2. The PCR reactions were performed in an iQ^{TM}5 thermal cycler (BioRAD Company) and the program used was: 5 min at 94 ℃; 40 cycles at 94 ℃ for 15 s, 55 ℃ for 30 s. The biomass densities for each competitor were estimated by SYBR q-PCR using same amount of total DNA of each circle. To compare the relative fitness in each competition group, the biomass densities of C_5 or C_{10} of each mutant were compared to that of C_0. The density of certain strain was not increased or decreased if the value of $\log_2[(C_5/C_0)R_5]$ or $\log_2[(C_{10}/C_0)R_{10}]$ equal to 0.

3 Results and discussion

3.1 Pellicle formation assay

In the microbial world, existence within surface-associated structured multicellular communities is the prevailing lifestyle. *Shewanella* is a ubiquitous microbe suitable to live in redox interfaces in freshwater and marine, wetlands, and sediment environments where biofilm formation may provide a selective advantage. Besides canonical biofilm, *Shewanella oneidensis* MR-1 forms a natural pellicle-like biofilm. Two different types of cells, pellicle and planktonic co-exist in the static cultures. At the liquid-air interface, cells attach to the wall and then spread across the surface to form a layer of complex, the pellicle (Fig.1). Dissolved oxygen(DO) readings from the unshaken cultures show that DO remains stable at 0.04 mg/L. This suggests that the planktonic cells are grown under microaerobic conditions.

A comparison of pellicle formation at 60 h from mutants and *S. onedensis* MR-1 wild type is shown in Fig.2, where the level of pellicle formed by WT strain is set to 100%, and a few deletion mutants have significant effects on pellicle formation. Specifically, Δ*SO4666* (cytochrome c4, CytcB) showed a severe deficiency in pellicle formation. Unlike the flat, uniform biofilms formed by the WT strain, the Δ*SO4666* strain remained as turbid cultures composed of independent planktonic cells that were not held together by a matrix after 5-day growth. However, the complemented strain of the *SO4666* deletion mutant could be fully recovered for pellicle formation. Additionally, 4 other cytochrome mutants, including Δ*SO1777* (periplasmic decaheme cytochrome c, MtrA), Δ*SO1782* (periplasmic decaheme cytochrome c, MtrD), Δ*SO2361* (cytochrome c oxidase, cbb3-type, subunit Ⅲ, CcoP), and Δ*SO2363* (cytochrome c oxidase, cbb3-type, subunit Ⅱ, CcoO) showed a partial deficiency in pellicle formation. For example, Δ*SO1777* formed 68.74%±4.58% of pellicle compared to the WT strain. Similarly, Δ*SO1782*, Δ*SO2361*, and Δ*SO2363* generated less than 85% of the pellicle compared to the WT strain (Fig.2). This suggests that heme-containing proteins are in close association

Fig.1 Pellicle formed by *S. onedensis* MR-1 in glass beaker at room temperature

Table 2 Primers used in q-RCR assays

Mutant	Sequence (Forward/reverse)
Δ*SO0479*	GTTTATGTCGTTCAGTGAAC/CCAGTCGGTGTAGCAGCAAT
Δ*SO0610*	CTTTGGCTTCTTTGTGGCGC/TAATTGGCAAGACTTCAGGG
Δ*SO0714*	GGTGTAACTTAATGTTATC/GACGATGGTGCCGTATCTCT
Δ*SO0716*	AAGAGCCTCACAAGCGTGCA/CTTCGGCATAAACCGCTGTG
Δ*SO0845*	TGCTCCTGCTCTAGGTGAAA/GAGTTTTTAAACGTCTATGT
Δ*SO1777*	GCGACAGGTTTAACTCAGCG/GACGGCCAAGCCTGTGTTGG
Δ*SO1780*	GGCACCTGTTCATCGTCGTT/CATCAGCCGCGACTACAATG
Δ*SO1782*	TACGTGGCTTGCGGCTCATT/GGAAAAATGGACATGGATAT
Δ*SO2361*	GCGGTCAATTACGTGCTGTC/CCTTGTGTTTTCTGATGAGG
Δ*SO2363*	GACCAGTGTGGCGTCATCAT/GCTGAAGCTAAAGCGGCTTA
Δ*SO4360*	ATGAGCCCGTGACTGCGATT/TGCCTGAAAGCAAAATCCTGC
Δ*SO4666*	GTCCTCAGTTCTATCCGTGG/CGACTGCAAGACGGTGCAAA

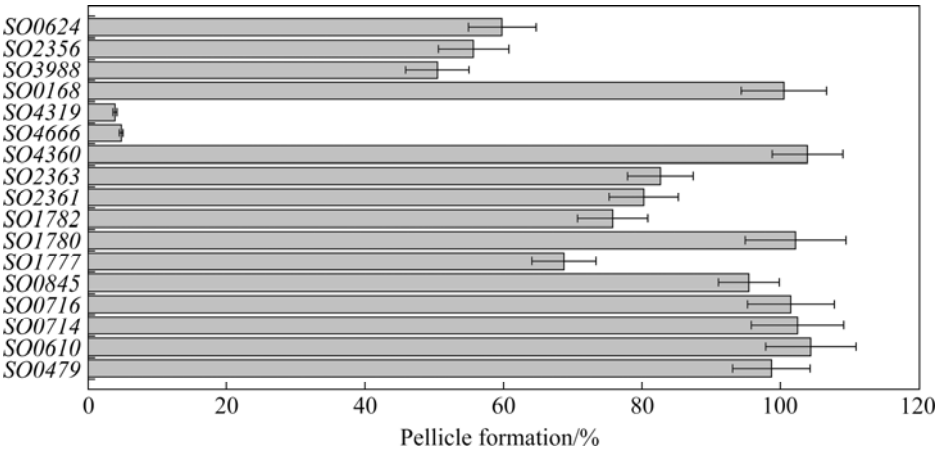

Fig.2 Relative pellicle formation by 17 (including 12 cytochrome) mutants compared to *S. onedensis* MR-1 wild type

with pellicle formation. Δ*SO1780* mutant strain showed no reduced ability of pellicle formation although it shared the same operon with *SO1782*. Mutants involved in sulfur redox reaction (Δ*SO0479*, Δ*SO0714*, and Δ*SO0716*), or nitrate reduction (Δ*SO0845*) had no effect on pellicle formation. Among 12 cytochrome mutants tested in this study, no mutant showed significant higher pellicle formation ability than the WT strain.

Besides *SO4666*, other genes were found to be involved in pellicle formation. A previous study showed AggA (*SO4320*), type I secretion protein, increased adhesive properties of *Shewanella* strain[8]. Our data showed that an interruption of *SO4319* (type I secretion membrane fusion protein, EmrA) resulted in severe deficiency in pellicle formation. However, another transporter mutant Δ*SO0168* (general secretion pathway protein F, Type II secretory pathway, GspF) sustained the ability of pellicle formation. These observations suggest that the type I secretion system is critical for pellicle formation. Consistent with previous work, the regulator mutants lacking *arcA*, *etrA*, or *crp* generated less than 60% of the pellicle produced by the WT[7, 20].

3.2 Static growth competition assay

In order to further understand the degree of adaptation to the environment, q-PCR was used to determine the competition ability of cytochrome mutants. In a good agreement with the independent results, the growth competition among 12 cytochrome mutants under the conditions supportive of pellicle growth revealed that Δ*SO4666* was undetectable at C_5. At C_5, the biomass of Δ*SO1777*, Δ*SO1782*, Δ*SO2361* and Δ*SO2363* decreased rapidly ($R_5 < -6$) compared to C_0. At C_{10}, the biomass of Δ*SO1777*, Δ*SO1782*, and Δ*SO2363* was even more dramatically decreased with the $R_{10} < -10$ (Fig.3). A superior growth characteristic of *SO0610* mutant (ubiquinol-cytochrome c reductase, PetC) along the course of the experiment was also observed. As it expected that slower-growing strains are overwhelmed by the faster-growing strains. The results demonstrated that *SO4666* may play an important role in pellicle formation in *S. oneidensis* MR-1.

A growth competition in another set of experiment revealed that slower-growing strains in pellicle such as

Δ*SO4666*, Δ*SO1777*, Δ*SO1782*, Δ*SO2361* and Δ*SO2363* also grew slowly under planktonic growth condition and Δ*SO0610* was able to better withstand the rigorous competitive pressures (Fig.4). However, the population variations due to differences in growth rate were less apparent compared to pellicle growing conditions. At C_{10}, the biomass of Δ*SO4666*, Δ*SO1777*, Δ*SO1782*, Δ*SO2361* and Δ*SO2363* was decreased with the R_{10} of −6.66±0.60, −6.40±0.38, −6.37±0.38, −5.10± 0.40, and −2.47±0.35, respectively. The competition ability of Δ*SO4666* was similar to that of Δ*SO1777*, Δ*SO1782*, Δ*SO2361* and Δ*SO2363* strains under planktonic growing condition although Δ*SO4666* mutant was undetectable in pellicle competition set (Fig.3). It is suggested that different mechanisms may exist in pellicle formation. SO2361 and SO2363 mediate the final step of electron transfer reactions between cytochrome c and the molecular oxygen and concomitantly pumping protons across the inner membrane. SO1780 and SO1782 function as intermediate electron carriers in the Fe (Ⅲ) electron transport pathway[21]. It is possible that Δ*SO2361*, Δ*SO2363*, Δ*SO1777*, Δ*SO1782* reduce pellicle formation by affecting growth rates under static growth conditions. Δ*SO4666* not only affects planktonic growth but also blocks the pellicle matrix formation at the air-liquid interface.

3.3 Agitated growth competition assay

S. oneidensis MR-1 has been extensively studied in anaerobic metal reduction while little is known about energy metabolism under aerobic condition. Interestingly, the results from agitated growth competition studies showed that Δ*SO4666* had a distinct competitive advantage over other strains, which is similar to Δ*SO0714* and Δ*SO0716* (Fig.5). Under aerobic growing conditions, Δ*SO1782*, Δ*SO2361* and Δ*SO2363* mutants were undetectable after C_{10} competition and the biomass of Δ*SO1777*, Δ*SO0610* and Δ*SO4360* decreased sharply. The results suggest that these genes may be important for aerobiosis processes, and that *S. oneidensis* MR-1 may have a quite complex electron transport network.

Cytochrome C4 encoded by *SO4666* is the least abundant of the *Shewanella* soluble cytochromes, and it is primarily membrane-bound like that of the *Pseudomonas*[22]. However, the function of *SO4666* is unclear. A previous study revealed that during uranium

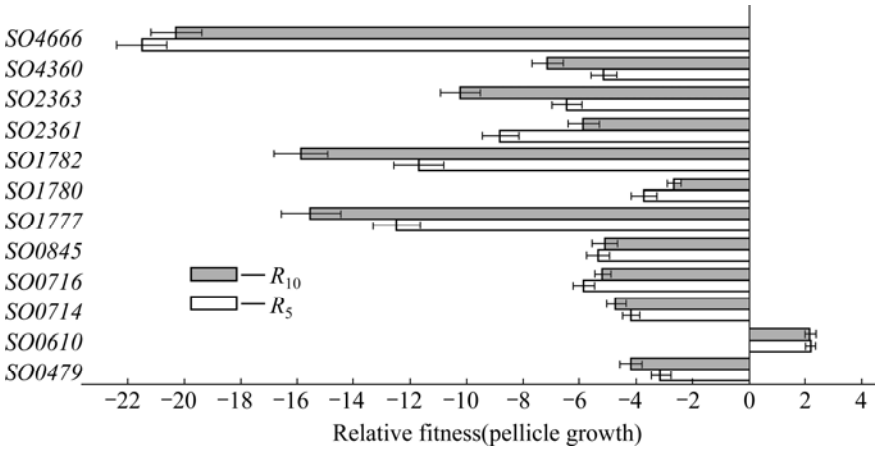

Fig.3 Pellicle growth competition among 12 cytochrome mutants

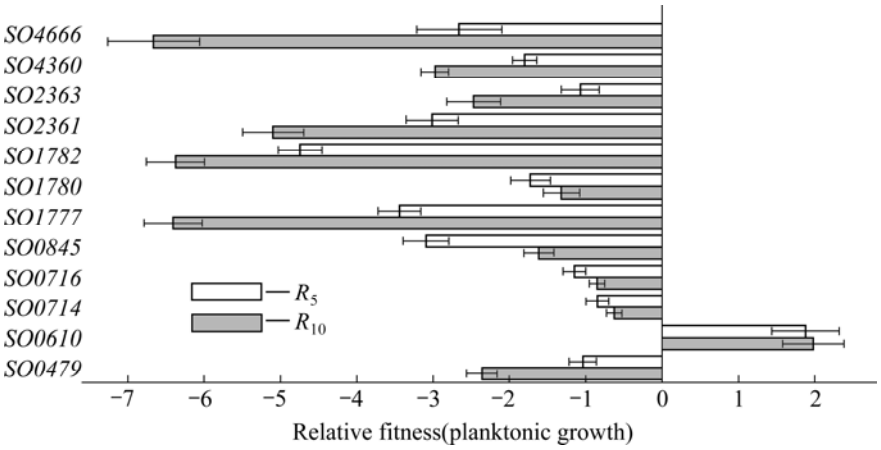

Fig.4 Planktonic growth competition among 12 cytochrome mutants

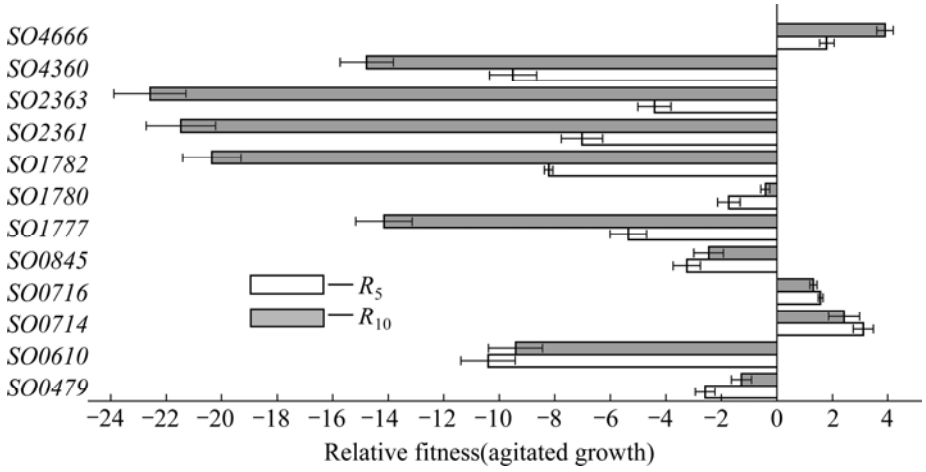

Fig.5 Agitated growth competition among 12 cytochrome mutants

bioremediation, a cluster of three heme-containing proteins of MR-1, MtrA-C (SO1776—SO1778) was directly localized with extracellular polymeric substance (EPS), which contained biogenic UO_2 nanoparticles[23]. EPS matrix of pellicle exhibits glycocalyx-like properties and contains multiple elements of polysaccharide, DNA, and humic acid. Giving the complexity of the c-type cytochromes in MR-1, we hypothesize that the extracellular material of pellicle may be comprised, at least in part, of cytochromes or outmembrane-derived cytochromes, such as SO4666. Clearly, further studies will be required to fully understand the role of cytochromes and other factors in pellicle formation to facilitate the biosorption of metal ions.

4 Conclusions

1) The Δ*SO4666* mutant shows a severe deficiency in pellicle-like biofilm formation at the air-liquid interface, and is not detectable at C_5 in pellicle growth competition among 12 cytochrome mutants. In addition, the biomass of Δ*SO4666* decreases after planktonic growth competition. The results demonstrate that *SO4666* may play an important role of pellicle formation in *S. oneidensis* MR-1.

2) The mutants Δ*SO1777*, Δ*SO1782*, Δ*SO2361* and Δ*SO2363* reduce pellicle formation, and their biomass decreases after planktonic or pellicle growth competition, indicating that those genes may be involved in pellicle formation.

3) *S. oneidensis* MR-1 possesses a quite complex aerobic electron transport network. Δ*SO1782*, Δ*SO2361* and Δ*SO2363* mutants are undetectable and the biomass of Δ*SO1777*, Δ*SO0610* and Δ*SO4360* decreases sharply after agitated growth competition, which suggests that these genes may be important for aerobiosis processes. Δ*SO4666* has a competitive advantage over other strains in agitated growth competition.

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