Arch Microbiol (2012) 194:473–482
DOI 10.1007/s00203-011-0782-x

ORIGINAL PAPER

Transcriptome analysis of pellicle formation of *Shewanella oneidensis*

Yili Liang · Haichun Gao · Xue Guo · Jingrong Chen · Guanzhou Qiu · Zhili He · Jizhong Zhou · Xueduan Liu

Received: 16 November 2011 / Revised: 8 December 2011 / Accepted: 12 December 2011 / Published online: 7 January 2012

Abstract Although the pellicle is one of the major growth modes of microorganisms, the metabolic features of pellicle cells and the determinative factors for pellicle formation are largely unknown. In recent years, biofilm development of *Shewanella oneidensis*, an important model organism for bioremediation studies, has been extensively studied. In this paper, a transcriptional profiling of pellicle cells relative to planktonic cells indicated that cells in pellicles were more metabolically active than the planktonic cells. Most notably, up-transcription of general secretion system proteins and iron/heme uptake and transport proteins was observed in pellicle cells. Unexpectedly, neither the *hmuT* nor *hugA* heme transport mutant exhibited a significant defect in pellicle formation. Expectedly, three type I secretion system mutants were severely deficient in pellicle formation, suggesting an essential role of these proteins.

Communicated by Shuang-Jiang Liu.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-011-0782-x) contains supplementary material, which is available to authorized users.

Y. Liang · X. Guo · G. Qiu · X. Liu (✉)
School of Minerals Processing and Bioengineering, Central South University, Changsha 410083, China
e-mail: xueduanliu@yahoo.com

Y. Liang · H. Gao · J. Chen · Z. He · J. Zhou
Institute for Environmental Genomics and Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019, USA

H. Gao
College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310029, People's Republic of China

Keywords Pellicle · *Shewanella oneidensis* · Type I secretion system

Introduction

The air–liquid interface is a selectively advantageous niche for aerobes due to the accessibility to oxygen. Various species of aerobes (e.g. *Bacillus subtilis* and *Pseudomonas fluorescens*) form a biofilm-like structure, pellicle, at air–liquid interface (Kobayashi 2007; Solano et al. 2002; Spiers et al. 2003). In recent years, the subject of microbial pellicles has drawn a lion share of attention since it is easily studied and adaptable for mutant screens (Guvener and McCarter 2003).

As a special form of biofilms, pellicles might be composed of bacterial cells embedded in a matrix formed by extracellular polymeric substances (EPS), which consist of proteins, polysaccharide, humic acid, and DNA (Whitchurch et al. 2002). Secreted proteins and exopolysaccharide productions profoundly influenced pellicle development in a number of organisms (Guvener and McCarter 2003; Kumagai et al. 2011). Transposon insertion mutants of *Vibrio parahaemolyticus* were obtained with defects in genes affecting multiple cell surface characteristics, including extracellular polysaccharide, mannose-sensitive hemagglutinin type 4 pili and secreted proteins or transporters of secreted proteins (Enos-Berlage et al. 2005). FLO11 of yeast is essential for unfavorable pellicle formation of wild pellicle-forming yeasts by mechanisms other than increasing cell surface hydrophobicity (Nakagawa et al. 2011). Pellicle formation by spore-forming bacterium *Bacillus subtilis* follows a distinct developmental pathway. In addition to the EPS component of the matrix, three proteins, encoded in the three-gene operon *yqxM-sipW-tasA*, were identified as involved in matrix assembly in a transposon mutant screen for genes involved in

biofilm formation (Branda et al. 2004). A mutant strain of *Pseudomonas aeruginosa* deficient in the production of exopolysaccharides displayed poor pellicle-forming ability and a growth disadvantage under static conditions compared with the wild-type strain (Yamamoto et al. 2011). In the presence of cellulase, glucose and cellobiose were released from the pellicle of *Asaia bogorensis AJ* strain, suggesting that it is made of cellulose (Kumagai et al. 2011).

Shewanella oneidensis MR-1, a facultative gram-negative anaerobe with a remarkable respiratory versatility, is a natural pellicle former. Because of the potential use of this organism for the bioremediation of metal/radionuclide contaminants in the environment, *S. oneidensis* MR-1 biofilm has been extensively studied, including pellicle development (Bagge et al. 2001; Vriendt et al. 2005; Windt et al. 2006; Teal et al. 2006; Thormann et al. 2004, 2005; Liang et al. 2010). In order to characterize features of the pellicle lifestyle, a microarray analysis on pellicle and planktonic cells of *S. oneidensis* was performed. The results revealed that the pellicle cells were more active in aerobic metabolism. The planktonic cells appeared to be under anaerobic or microaerobic conditions once pellicle formation began. Genes involved in iron/heme uptake and transport or the general secretion pathway were up-regulated in pellicle cells.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. oneidensis* strains were routinely grown in Luria–Bertani (LB) broth or on LB plates at 37°C and the room temperature for genetic manipulation, respectively. When needed, antibiotics were used at the following concentrations: ampicillin at 50 μg/ml and gentamycin at 15 μg/ml.

Pellicle formation, measurement of growth, and quantification of pellicles

A fresh colony grown overnight on a LB plate was used to inoculate 50 ml LB and incubated in a shaker (200 rpm) 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. Aliquots of 30 ml starting cultures were transferred to 50-ml Pyrex beakers. The beakers were kept still for pellicle formation at the room temperature. Cells in pellicle and underneath were separated based on published paper (Liang et al. 2010). To quantify the pellicles formed by the *S. oneidensis* wild-type and mutant strains, cells from pellicles were collected, suspended in 30 ml fresh LB, violently vortexed, and applied to the spectrometer at 600 nm.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli		
WM3064	Donor strain for conjugation; Δ*dapA*	Lab stock
TOP10	F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ80*lacZ*Δ M15 Δ *l acX74 deoR recA1 araD139* Δ *(ara-leu)7697 galU galK rpsL* (Sm^r) *endA1 nupG*	Invitrogen
S. oneidensis strains		
MR-1	Wild-type	Lab stock
JZ3669K	*hugA* deletion mutant derived from MR-1; Δ*hugA*	This study
JZ3673K	*hmuT* deletion mutant derived from MR-1; Δ*hmuT*	This study
JZ4318K	*rtxB* deletion mutant derived from MR-1; Δ*rtxB*	This study
JZ4319K	*emrA* deletion mutant derived from MR-1; Δ*emrA*	This study
JZ4320K	*aggA* deletion mutant derived from MR-1; Δ*aggA*	Liang et al. 2010
Plasmids		
pDS3.0	Ap^r, Gm^r, derivative from suicide vector pCVD442	Gao et al. (2006)

Microarray experiments, data analysis and validation

For microarray analysis, aliquots of 30 ml starting cultures were transferred to 50-ml Pyrex beakers and allowed to develop pellicles at the room temperature. When a complete but thin layer of pellicles (young pellicles) at the interface were formed (about 30 h), the planktonic cultures and pellicles were separated as described above and applied to centrifugation at 8,000 rpm for 3 min at room temperature. The resulting pellets were frozen immediately in liquid nitrogen and stored at −80°C if not immediately used. Construction of whole-genome DNA microarrays, total RNA extraction, cDNA labeling, hybridization, and slide scanning were conducted according to the standard procedure (Gao et al. 2004). LOWESS was used to normalize the data set which subsequently was applied to statistical analysis by analysis of variance (ANOVA) with Benjamini and Hochberg False Discovery Rate as multiple testing correction (Gao et al. 2004, 2008). Raw microarray data were deposited to GEO with the accession number GSE14331.

Quantitative RT-PCR (Q-RT-PCR)

Q-RT-PCR was used to measure the mRNA expression levels of four target genes in pellicle and planktonic cells. Primers for chosen genes were listed in Table 1S.

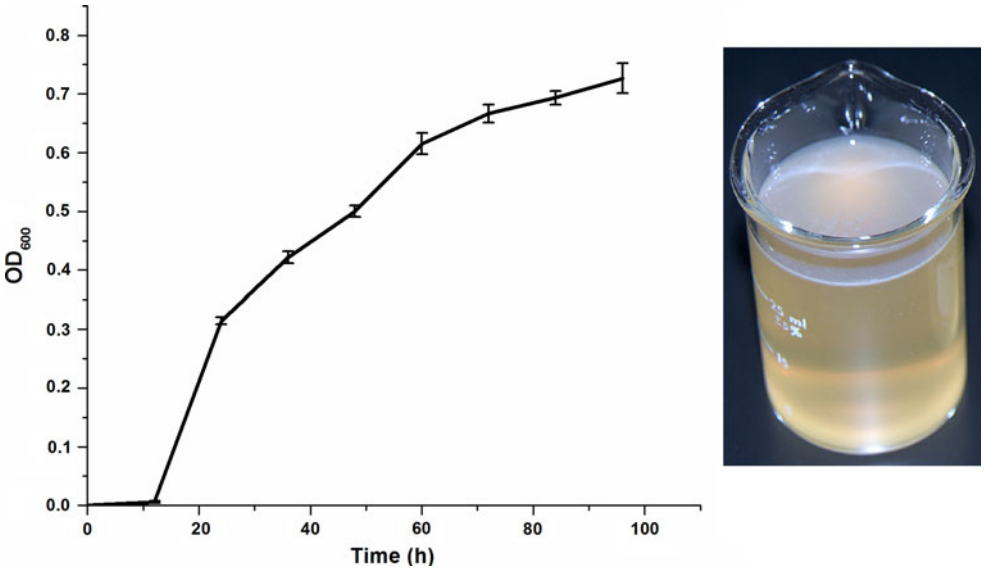

Fig. 1 Growth of *S. oneidensis* pellicle cells in LB medium under aerobic condition

Samples for Q-RT-PCR were prepared and collected the same way that we did for microarray analysis. The reaction was conducted for all primer pairs in an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) as described previously (Gao et al. 2004). To standardize the quantification of the selected target genes, *dsbB* and *ftsA* served as internal controls and were quantified on the same plate as the target genes. The levels of expression of the target genes as determined from the cycle threshold (CT) values were normalized to the level of the average value of *dsdB* and *ftsA* according to the manufacturer's instructions. The expression of each gene was determined from triplicate cultures in a single real-time Q-RT-PCR experiment.

Mutant construction

Deletion mutation strains were constructed using the fusion PCR method illustrated previously (Gao et al. 2006). Primers used for mutagenesis were listed in Table 1S. In brief, two DNA fragments flanking the target gene were generated from *S. oneidensis* genomic DNA by PCR with primers 5F/5R and 3F/3R, respectively. Fusion PCR was then performed to join these two DNA fragments with primers 5F/3R. The resulting single fragment was digested with *Sac*I and ligated into the *SacI*-digested and phosphatase-treated suicide vector pDS3.0. The resultant vectors were electroporated into the donor strain, *E. coli* WM3064 and then moved to *S. oneidensis* by conjugation. Integration of the mutagenesis construct into the chromosome and resolution were performed to generate the final deletion strains. The deletion was verified by PCR and DNA sequencing.

Results

Transcriptional profilings of pellicle versus planktonic cells

When cultured in still LB media under aerobic condition, *Shewanella oneidensis* MR-1 cells attached to the wall and then quickly (about 10 h after inoculation) spread across the surface to form a pellicle-like biofilm at the liquid–air interface (Fig. 1). *S. oneidensis* cells start to make decision to either continue its planktonic living style or turn to form pellicles. To screen for determining factors for the decision, we employed the whole-genome microarray analysis to compare mRNA profiles between the pellicle and planktonic cells in the same cultures when a very thin layer of complex (young pellicle) was formed. To simplify description throughout this article, up- and down-transcribed genes refer to those whose mRNA levels in the pellicle cells were higher and lower than those in the planktonic cells, respectively. The quality of the microarray data was routinely assessed with established methods (Gao et al. 2004), and only qualified data were used for further analysis.

General overview of transcriptomic characterization of pellicle and planktonic cells

In total, 882 genes passed the criterion of $P < 0.05$ in the statistical analysis as described previously (Gao et al. 2008), representing approximately 19.0% of the 4,648 ORFs spotted on the array. These transcription-altered genes covered all functional classes (54% of these genes encoding hypothetical or conserved hypothetical proteins), implicating that changes in these two types of cells were global, at least at the transcriptional level. Up-transcribed

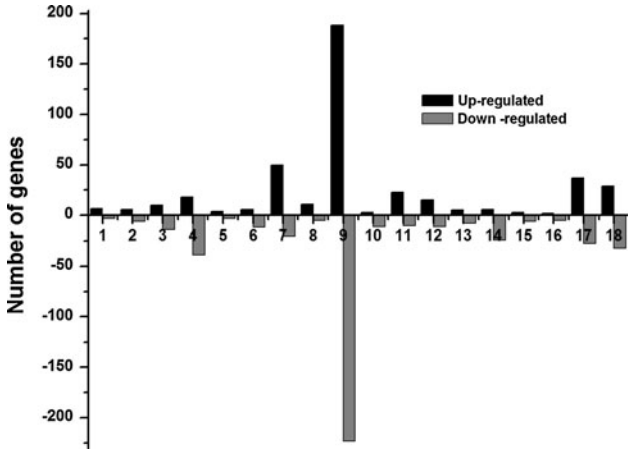

Fig. 2 Differentially expressed genes in pellicle cells grouped by functional classification according to the TIGR *S. oneidensis* genome database (http://www.tigr.org). Columns: *1* amino acid biosynthesis; *2* biosynthesis of cofactors, prosthetic groups, and carriers; *3* cell envelope; *4* cellular processes; *5* central intermediary metabolism; *6* DNA metabolism; *7* energy metabolism; *8* fatty acid and phospholipid metabolism; *9* hypothetical proteins; *10* mobile and extrachromosomal element functions; *11* protein fate; *12* protein synthesis; *13* purines, pyrimidines, nucleosides, and nucleotides; *14* regulatory functions; *15* signal transduction; *16* transcription; *17* transport and binding proteins; *18* unknown function

genes were dominant in the following functional categories: energy metabolism, transport and binding proteins, fatty acid and phospholipid metabolism, protein fate, and protein synthesis, whereas the majority of genes in categories such as cell envelope, cellular processes, DNA metabolism, and regulatory functions were down-transcribed (Fig. 2).

Energy metabolism proteins

It is worth mentioning that almost all of genes encoding TCA enzymes were up-transcribed in pellicle cells (Table 2). In addition, all of 9 *atpA-H* genes encoding subunits of ATP synthase and 12 out of 13 *nuoA-N* genes encoding subunits of NADH dehydrogenase were consistently up-transcribed (Table 2). Consistently, the transcription of a group of genes including *so2361–64*, which encode subunits of cbb_3-type cytochrome *c* oxidase and catalyze the final step of electron transfer between a cytochrome *c* and the molecular oxygen in the respiratory chain, was up-transcribed. All of these data suggested that cells in pellicles were metabolically more active, especially in pathways related to aerobic respiration. The microarray results also demonstrated that the planktonic cells were in fact under microaerobic conditions on the basis of that a number of genes involved in anaerobic respiration were drastically induced in planktonic cells. These included *so2178* (*ccpA*, cytochrome *c551* peroxidase), *so1429–30* (*dmsAB*, dimethyl sulfoxide reductase), *so4512–15* (formate dehydrogenase), and *so0259–63* (*ccm,* heme exporter protein) (Table 2).

Transport proteins

Since EPS, essential biopolymers for the formation of pellicles have to be manufactured mostly by microbes themselves, genes encoding proteins involved in synthesis and transport of EPS are likely to be transcribed differently between the two types of cells. Indeed, expression of a total of 64 (37 up-transcribed and 27 down-transcribed) genes encoding transport proteins was significantly altered. Among these, genes encoding iron or heme uptake and transportation proteins consistently showed the largest transcription differences. These included *so1580* (TonB-dependent heme receptor), *so3030–3* (siderophore biosynthesis proteins and receptor), and *so3667–75* (*so3667-so3668-hugA-tonB1-exbB1-exbD1-hmuT-hmuU-hmuV*, heme/hemin transport proteins) (Table 2). Importance of iron in both canonical biofilm and pellicle formation has been well documented (Banin et al. 2005). Iron or heme from the various resources, such as soluble and insoluble iron-containing chemicals and iron/heme-containing proteins, can be captured by siderophores secreted by cells or by outer membrane receptors (Braun and Braun 2002). These data implicated that iron may have a role in pellicle formation or more iron was biosorpted by pellicle cells.

Three proteins including RtxB, EmrA, and AggA (S04318–4320) are predicted to be the cytoplasmic membrane localized ATP-binding protein, membrane fusion protein and outer membrane protein component, respectively, of a type I secretion system (TISS). Microarray data showed that *so4318–4320* were consistently up-regulated in pellicle cells (Table 2), suggesting certain substrates secreted by TISS may play important role in pellicle formation.

gspF, encoding individual component of the general secretion pathway protein F, was a known gene of *S. oneidensis* MR-1 that participate in the process of electron transfer (Shi et al. 2007; Py et al. 2001). Some best characterized proteins, which are involved in biofilm formation, depend on general secretion pathway to transport across the cytoplasmic membrane (Lasa and Penades 2006). Microarray data showed that *gspF* was up-regulated in pellicle cells (Table 2).

Regulatory proteins

In our study, transcription of 46 (11 up-transcribed and 35 down-transcribed) genes encoding regulatory proteins was significantly different. Although the majority of these genes encode proteins whose function is currently unclear, several genes are worth noting because their products have been

Table 2 Genes with significantly altered transcription and discussed in the text

Locus[a]	Gene	Ratio[b]	Predicted function
Respiration			
TCA cycle			
SO0343	*acnA*	2.12	Aconitate hydratase 1
SO0424	*aceE*	0.61	Pyruvate dehydrogenase complex, E1 component
SO0425	*Ace*	1.28	Pyruvate dehydrogenase complex, E2 component
SO0426	*lpdA*	0.82	Pyruvate dehydrogenase complex, E3 component
SO0432	*acnB*	0.74	Aconitate hydratase 2
SO0770	*Mdh*	1.48	Malate dehydrogenase
SO1483	*aceB*	0.87	Malate synthase A
SO1484	*aceA*	2.29	Isocitrate lyase
SO1926	*gltA*	1.63	Citrate synthase
SO1927	*sdhC*	−0.53	Succinate dehydrogenase, cytochrome b556 subunit
SO1928	*sdhA*	0.56	Succinate dehydrogenase, flavoprotein subunit
SO1929	*sdhB*	0.23	Succinate dehydrogenase, iron-sulfur protein
SO1930	*sucA*	1.03	2-Oxoglutarate dehydrogenase, E1 component
SO1931	*sucB*	1.42	2-Oxoglutarate dehydrogenase, E2 component
SO1932	*sucC*	2.53	Succinyl-CoA synthase, beta subunit
SO1933	*sucD*	1.41	Succinyl-CoA synthase, alpha subunit
SO2222	*SO2222*	−0.23	Fumarate hydratase, class I, anaerobic, putative
SO2629	*Icd*	0.75	Isocitrate dehydrogenase, NADP-dependent
ATP synthase			
SO4746	*atpC*	0.70	ATP synthase F1, epsilon subunit
SO4747	*atpD*	1.12	ATP synthase F1, beta subunit
SO4748	*atpG*	1.17	ATP synthase F1, gamma subunit
SO4749	*atpA*	1.45	ATP synthase F1, alpha subunit
SO4750	*atpH*	1.43	ATP synthase F1, delta subunit
SO4751	*atpF*	1.30	ATP synthase F0, B subunit
SO4752	*atpE*	0.36	ATP synthase F0, C subunit
SO4753	*atpB*	0.94	ATP synthase F0, A subunit
NADH dehydrogenase			
SO1009	*nuoN*	1.33	NADH dehydrogenase I, N subunit
SO1010	*nuoM*	1.63	NADH dehydrogenase I, M subunit
SO1011	*nuoL*	1.53	NADH dehydrogenase I, L subunit
SO1012	*nuoK*	1.19	NADH dehydrogenase I, K subunit
SO1013	*nuoJ*	1.51	NADH dehydrogenase I, J subunit
SO1014	*nuoI*	1.71	NADH dehydrogenase I, I subunit
SO1015	*nuoH*	1.45	NADH dehydrogenase I, H subunit
SO1016	*nuoG*	−1.60	NADH dehydrogenase I, G subunit
SO1017	*nuoF*	0.16	NADH dehydrogenase I, F subunit
SO1018	*nuoE*	0.97	NADH dehydrogenase I, E subunit
SO1019	*nuoCD*	0.27	NADH dehydrogenase I, C/D subunits
SO1020	*nuoB*	1.06	NADH dehydrogenase I, B subunit
SO1021	*nuoA*	0.53	NADH dehydrogenase I, A subunit
Other respiration genes			
SO0259	*ccmE*	−0.61	Cytochrome c biogenesis protein CcmE
SO0260	*ccmD*	−0.23	Heme exporter protein CcmD
SO0261	*ccmC*	−0.75	Heme exporter protein CcmC

Table 2 continued

Locus[a]	Gene	Ratio[b]	Predicted function
SO0262	*ccmB*	−0.96	Heme exporter protein CcmB
SO0263	*ccmA*	−1.05	Heme exporter protein CcmA
SO1429	*dmaA-1*	−3.21	Anaerobic dimethyl sulfoxide reductase, A subunit
SO1430	*dmsB-1*	−3.53	Anaerobic dimethyl sulfoxide reductase, B subunit
SO2178	*ccpA*	−4.36	Cytochrome c551 peroxidase
SO2361	*ccoP*	1.32	Cytochrome c oxidase, cbb3-type, subunit III
SO2362	*ccoQ*	0.48	Cytochrome c oxidase, cbb3-type, CcoQ subunit
SO2363	*ccoO*	0.44	Cytochrome c oxidase, cbb3-type, subunit II
SO2364	*ccoN*	0.97	Cytochrome c oxidase, cbb3-type, subunit I
SO4512	*SO4512*	−1.79	Conserved hypothetical protein
SO4513	*SO4513*	−1.62	Formate dehydrogenase, alpha subunit
SO4514	*fdhB-2*	−1.02	Formate dehydrogenase, iron-sulfur subunit
SO4515	*SO4515*	−1.41	Formate dehydrogenase, C subunit, putative
Transport protein			
SO0168	*gspF*	0.21	General secretion pathway protein F
SO1580	*SO1580*	5.29	TonB-dependent heme receptor
SO3030	*alcA*	3.87	Siderophore biosynthesis protein
SO3031	*SO3031*	3.59	Siderophore biosynthesis protein, putative
SO3032	*SO3032*	3.44	Siderophore biosynthesis protein, putative
SO3033	*SO3033*	3.66	Ferric alcaligin siderophore receptor
SO3253	*SO3253*	−0.016	Flagellar basal-body P-ring formation protein FlgA
SO3667	*SO3667*	3.77	Conserved hypothetical protein
SO3668	*SO3668*	3.89	Conserved hypothetical protein
SO3669[c]	*hugA*	4.36	Heme transport protein
SO3670	*tonB1*	5.27	TonB1 protein
SO3671	*exbB1*	3.57	TonB system transport protein ExbB1
SO3672	*exbD1*	2.46	TonB system transport protein ExbD1
SO3673[c]	*hmuT*	3.65	Hemin ABC transporter, periplasmic hemin-binding protein
SO3674	*hmuU*	2.52	Hemin ABC transporter, permease protein
SO3675	*hmuV*	3.19	Hemin ABC transporter, ATP-binding protein
SO4318[c]	*rtxB*	0.66	Toxin secretion ATP-binding protein
SO4319[c]	*etrA*	0.51	HlyD family secretion protein
SO4320[c]	*aggA*	0.38	Agglutination protein
Regulatory function			
SO1945	*phoQ*	−1.73	Sensor protein PhoQ
SO1946	*phoP*	−2.20	Transcriptional regulatory protein PhoP
SO1558	*phoB*	−3.34	Phosphate regulon response regulator PhoB
SO1559	*phoR*	−0.68	Phosphate regulon sensor protein PhoR
SO3538	*hlyU*	−2.26	Transcriptional regulator HlyU

[a] According to TIGR annotation

[b] log2 ratio (mRNA of pellicle cells/mRNA of planktonic cells)

[c] Subjected to mutational analyses

reported to be involved in biofilm formation in other bacteria, suggesting that these proteins may be involved in *S. oneidensis* pellicle formation as well. These included genes encoding two-component systems PhoP-PhoQ and PhoB-PhoR, and regulatory protein HlyU (Danhorn et al. 2004; Li et al. 2001; Prouty and Gunn 2003) (Table 2). A previous study reported that global regulators ArcA and CRP had strong impacts on biofilm detachment and formation in *S. oneidensis* (Thormann et al. 2005). However, transcription of *arcA* and *crp* was not significantly altered in our transcriptional profilings.

Validation of gene microarray results by reverse transcription-quantitative PCR

Genes encoding transport proteins were significantly altered, and reverse transcription-quantitative PCR (RT-PCR) was employed to measure the mRNA expression levels of

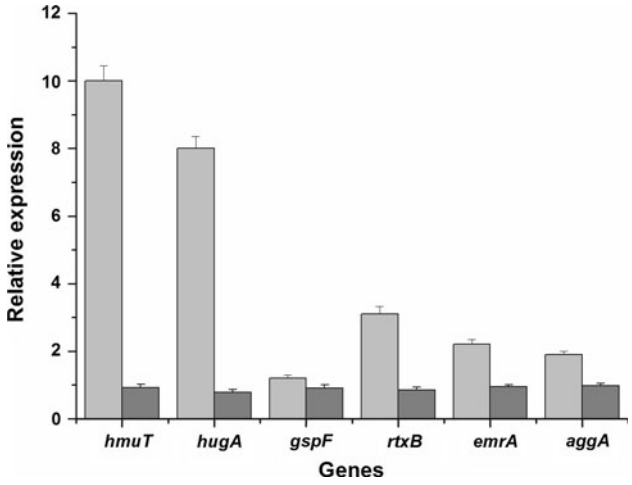

Fig. 3 Q-RT-PCR assays of genes used in the mutational analysis. All strains were grown in static LB under aerobic conditions until the young and manageable pellicles were formed. Q-RT-PCR was used to quantify the mRNA levels of *hmuT*, *hugA*, *gspF*, *rtxB*, *emrA* and *aggA* in the pellicle cells (*light gray*) and planktonic cells (*dark gray*). All data were normalized to the expression of the average of *dsbB* and *ftsA*. Relative expression was determined using the method described in "Materials and methods". Presented are averages of triplicate cultures with the standard deviation indicated by *error bars*

six genes. These genes are *so0168* (*gspF* 0.21-fold (log2), general secretion pathway protein F), *so3669* (*hugA*,4.36-fold, heme transport protein), *so3673* (*hmuT* 3.65-fold, hemin ABC transporter), *so4318* (*rtxB* 0.66-fold, toxin secretion ATP-binding protein), *so4319* (*emrA* 0.50-fold, HlyD family secretion protein), and *so4320* (*aggA* 0.38-fold, type I secretion outer membrane protein, AggA). Two genes, *dsbB* and *ftsA*, were chosen as the internal controls based on that their expression was close to the average of each channel (Cy3 or Cy5) in the microarray analysis. Expression of *hugA*, *hmuT*, *gspF*, *rtxB*, *emrA* and agg*A* in pellicle cells from samples collected independently (from microarray analysis) was induced approximately tenfold, eightfold, 1.2-fold, 3.1-fold 2.2-fold and 1.9-fold, respectively (Fig. 3). In contrast, none of these genes in planktonic cells displayed an expression level significantly different from that of either *dsbB* or *ftsA* (Fig. 3). These results indicated that six genes selected were particularly active in the pellicle cells but not in the planktonic cells. The ratios (pellicle cells/planktonic cells) from Q-RT-PCR were similar to those observed in the microarray analysis, further validating the transcriptomic results.

Mutational analysis of candidate genes

Based on our microarray data and reports published previously, we picked up four genes relevant to protein transport for a mutational analysis to determine their involvement in pellicle formation. These genes are *so3669* (*hugA*), *so3673* (*hmuT*), *so4318* (*rtxB*), *so4319* (*emrA*). Gene *hugA* and gene *hmuT* were not only among those showing the highest induction in pellicles but also reported to be crucial in iron transport and biofilm formation (Rhodes et al. 2007). Three genes encoding TISS proteins were consistently up-regulated in pellicle cells. Our previous data showed that the pellicle was severely deficient in the in-frame deletion mutant of *aggA* which blocked secretion via TISS (Liang et al. 2010).

Mutants carrying in-frame deletion in one of targeted genes were constructed. A relatively comprehensive characterization revealed that mutation *hmuT* or *hugA* did not elicit any distinguishable phenotype regarding a variety of physiological features, including agitated aerobic growth, swimming and swarming, and biofilm and pellicle formation (Fig. 4). This observation suggests that iron/heme transport may not be important in pellicle formation of *S. oneidensis*.

Similar to *aggA* mutant, the other two TISS mutants *so4318* (*rtxB*) and *so4319* (*emrA*) were severely deficient in pellicle formation (Fig. 4). Unlike the wild-type strain, which formed flat, uniform pellicles, each of the three TISS mutants strain culture remained turbid and was composed of independent planktonic cells that were not held together by a matrix. However, the mutant was able to attach to the glass wall at the air–liquid interface, suggesting that TISS is not essential for this step of biofilm formation. The physiological characterization revealed that TISS mutant grew at the rate comparable to that of the parental strain either in the shaking or static conditions. All these data implicate that TISS is required for pellicle formation.

Discussion

The bacterial biofilm, a sessile community with high cell density, is ubiquitous in natural, medical, and engineering environments. As a special form of biofilms, pellicles of facultative bacteria formed at the liquid–air interface can be selectively advantageous given that aerobic respiration with oxygen as the terminal electron acceptor is the most productive. In addition, pellicle confers a number of advantage for survival including nutrient availability with metabolic cooperation and protection from the environment (Li et al. 2001).

Transcriptional profiling has been used to gain insight into the general physiology of the biofilm community and to identify genes that contribute to their formation (Folsom et al. 2010; Hamilton et al. 2009; Stewart and Franklin 2008). In the present study, whole genomic DNA microarray was successfully used to generate the gene expression profiles of a *S. oneidensis* pellicle relative to suspension cells in the same culture, and the results of replicated experiments

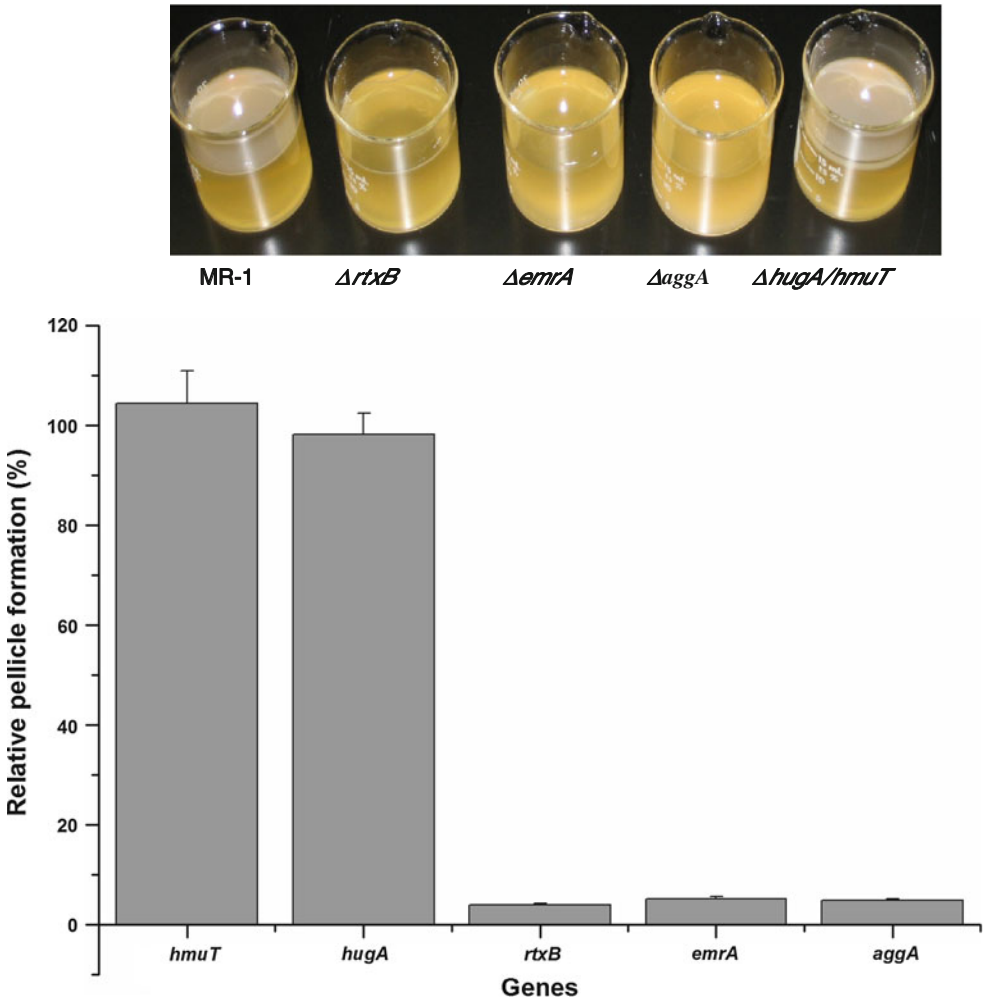

Fig. 4 Relative pellicle formation of mutant strains including *gspF*, *hmuT*, *hugA*, *rtxB*, *emrA* and *aggA*. Mutation *hmuT* or *hugA* did not elicit any distinguishable phenotype in pellicle formation. All the three TISS mutants were severely deficient in pellicle formation

were consistent. Genes clustered in the same operons were found to be induced/repressed together, indicating the data are sound. In addition, Q-RT-PCR analysis validated the transcriptomic results.

One of the main environmental differences between pellicle cells and suspension cells in the present study was the oxygen concentration. Our former data revealed that cells of *S. oneidensis* at the periphery of a pellicle quickly consume oxygen, producing a decreasing gradient from the surface to the interior (Liang et al. 2010). As revealed by the transcriptional analysis, cells of *S. oneidensis* in pellicles were more active in aerobic metabolism while planktonic cells actually lived in microaerobic or anaerobic environments due to the existence of the thick pellicle. Beliaev et al. (2002) have studied anaerobic metabolism with DNA microarrays and found a global change in gene expression between aerobic growth and anaerobic growth with the above anaerobic genes of our studies (e.g. *ccpA*, *dmsAB* and *ccm*) induced during fumarate and nitrate reduction condition. These data indicated that the majority of dissolved oxygen is likely consumed by the cells close to the surface, and planktonic cells underneath alter the expression of respiratory chain components to maximize its efficiency under microaerobic conditions. There was evidence of this in several biofilm transcriptomes (Ren et al. 2004; Beloin et al. 2004; Yamamoto et al. 2011). In addition, the capacity of *S. oneidensis* cells to form pellicles was abolished under anaerobic conditions with fumarate, nitrate, DMSO, TMAO, or ferrous citrate as the electron acceptors, indicating that oxygen is required for the process.

It is particularly worth discussing roles of iron/heme transporter including HmuT HugA in pellicle formation of *S. oneidensis*. As revealed by the microarray and Q-RT-PCR analyses, genes encoding proteins involved in siderophore biosynthesis, iron or heme uptake and transport were highly transcribed in pellicle cells. Irons are important in pellicle formation although not so crucial as Cu (II), Ca (II),

Mn (II), and Zn (II) (Liang et al. 2010). However, pellicle formation of *S. oneidensis* was barely affected by mutations in *hugA* and *hmuT*. When *hugA* or *hmuT* deletion mutant grew in the LB medium, even if the iron was limited, other cations in LB medium could replace the iron, so these two mutant strains could still form the pellicle. In addition, these two genes reside in a large gene cluster encoding iron/heme uptake and transport proteins, and all these genes have been found to respond to the environmental changes consistently (Beliaev et al. 2005; Braun and Braun 2002; Gao et al. 2006). The substantial differences in transcription of these genes between two different types of cells may be resulted from an imbalance in metabolism. In conclusion, the finding that HmuT and HugA have little impacts on pellicle formation demands a further investigation.

The type I secretion apparatus is a rather simple machine containing only three different transport components, two in the inner membrane, which are specific for the passenger protein, and one that forms a general pore in the outer membrane (Holland et al. 2005). Three genes encoding TISS proteins were consistently up-regulated in *S. oneidensis* MR-1 pellicle cells. At the same time, all the three TISS mutants were severely deficient in pellicle formation. In addition, Theunissen et al. subjected MR-1 to random transposon insertion mutagenesis to identify genes contributing to the ability of the organism to form biofilms on polystyrene surfaces. RTX, a novel 285-kDa multi-domain protein, is secreted by TISS to the cell surface, where it is a requisite for solid surface-associated biofilm development (Theunissen et al. 2010). These data suggested that type I secretion pathway is directly related to biofilm formation.

Pellicle formation is a complex process. Many factors, including oxygen, secreted proteins, extracellular organelles such as flagella, and chemical agents supplemented in media such as iron and calcium, played important roles in biofilm formation of *S. oneidensis* MR-1 (Liang et al. 2011). The results presented here provided the comprehensive insights into pellicle formation of *S. oneidensis*. However, the events in transcriptional regulation during pellicle formation are incompletely understood. As we know, the *S. oneidensis* MR-1 cells formed solid surface-associated (SSA) biofilms on the flask wall and another kind of suspension cells in LB when aeration of the media was provided by shaking, which the dissolved oxygen level was as high as the surface growing pellicle cells. Since one of the significant environmental differences between pellicle cells and planktonic cells in the present study was the oxygen concentration, the suspension cells in the agitated medium could be another control. In the future, we can expect the combination of genetics, biochemistry, and microscopy to yield an ever-increasing understanding of the molecular mechanisms of pellicle formation.

Acknowledgments This research was supported by The U.S. Department of Energy under the Genomics: GTL Program through Shewanella Federation, Office of Biological and Environmental Research, Office of Science. This research was also supported by the National Basic Research Program (973 Program: 2010CB630901), Central South University research startup funding (7602110285) and National Natural Science Foundation of China (302001124) to YL.

References

Bagge D, Hjelm M, Johansen C, Huber I, Grami L (2001) *Shewanella putrefaciens* adhesion and biofilm formation on food processing surfaces. Appl Environ Microbiol 67:2319–2325

Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Nat Acad Sci USA 102:11076–11081

Beliaev AS, Thompson DK, Giometti CS, Li GS, Yates J III, Nealson KH, Tiedje JM, Heidelberg JF, Zhou JZ (2002) Gene and protein expression profiles of *Shewanella oneidensis* during anaerobic growth with different electron acceptors. Omics J Integr Biol 6:39–60

Beliaev AS, Klingeman DM, Klappenbach JA, Wu L, Romine MF, Tiedje JM, Nealson KH, Fredrickson JK, Zhou J (2005) Global transcriptome analysis of *Shewanella oneidensis* MR-1 exposed to different terminal electron acceptors. J Bacteriol 187:7138–7145

Beloin C, Valle J, Latour-Lambert P, Faure P, Kzreminski M, Balestrino D, Haagensen JA, Molin S, Prensier G, Arbeille B (2004) Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol Microbiol 51:659–674

Branda SS, Gonzalez-Pastor JE, Dervyn E, Ehrlich SD, Losick R, Kolter R (2004) Genes involved in formation of structured multicellular communities by *Bacillus subtilis*. J Bacteriol 186:3970–3979

Braun V, Braun M (2002) Active transport of iron and siderophore antibiotics. Curr Opin Microbiol 5:194–201

Danhorn T, Hentzer M, Givskov M, Parsek MR, Fuqua C (2004) Phosphorus limitation enhances biofilm formation of the plant pathogen *Agrobacterium tumefaciens* through the PhoR-PhoB regulatory system. J Bacteriol 186:4492–4501

Enos-Berlage JL, Guvener ZT, Keenan CE, McCarter LL (2005) Genetic determinants of biofilm development of opaque and translucent Vibrio parahaemolyticus. Mol Microbiol 55(4):1160–1182

Folsom JP, Richards L, Pitts B, Roe F, Ehrlich GD, Parker A, Mazurie A, Stewart PS (2010) Physiology of *Pseudomonas aeruginosa* in biofilms as revealed by transcriptome analysis. BMC Microbiology 10:294

Gao HC, Wang Y, Liu XD, Yan TF, Wu LY, Alm E, Arkin A, Thompson DK, Zhou JZ (2004) Global transcriptome analysis of the heat shock response of *Shewanella oneidensis*. J Bacteriol 186:7796–7803

Gao H, Yang ZK, Wu L, Thompson DK, Zhou J (2006) Global transcriptome analysis of the cold shock response of *Shewanella oneidensis* MR-1 and mutational analysis of its classical cold shock proteins. J Bacteriol 188:4560–4569

Gao HC, Wang XH, Yang ZK, Palzkill T, Zhou JZ (2008) Probing regulon of ArcA in *Shewanella oneidensis* MR-I by integrated genomic analyses. Bmc Genomics 9 (Suppl 1):S11

Guvener ZT, McCarter LL (2003) Multiple regulators control capsular polysaccharide production in *Vibrio parahaemolyticus*. J Bacteriol 185(8):5431–5441

Hamilton S, Bongaerts RJM, Mulholland F, Cochrane B, Porter J, Lucchini S, Lappin-Scott HM, Hinton JCD (2009) The transcriptional programme of Salmonella enterica serovar Typhimurium reveals a key role for tryptophan metabolism in biofilms. BMC Genomics 10:599

Holland IB, Schmitt L, Young J (2005) Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review). Mol Membr Biol 22(1–2):29–39

Kobayashi K (2007) *Bacillus subtilis* pellicle formation proceeds through genetically defined morphological changes. J Bacteriol 189:4920–4931

Kumagai A, Mizuno M, Kato N, Nozaki K, Togawa E, Yamanaka S, Okuda K, Saxena IM, Amano Y (2011) Ultrafine Cellulose Fibers Produced by *Asaia bogorensis*, an Acetic Acid Bacterium. Biomacromolecules. doi:10.1021/bm2005615

Lasa I, Penades JR (2006) Bap: a family of surface proteins involved in biofilm formation. Res Microbiol 157(2):99–107

Li YH, Lau PCY, Lee JH, Ellen RP, Cvitkovitch DG (2001) Natural genetic transformation of *Streptococcus* mutants growing in biofilms. J Bacteriol 183:897–908

Liang YL, Gao HC, Chen JR, Dong YY, Wu L, He ZL, Liu XD, Qiu GZ, Zhou JZ (2010) Pellicle formation in *Shewanella oneidensis*. BMC Microbiol 10:291

Nakagawa Y, Toda Y, Yamamura H, Hayakawa M, Iimura Y (2011) FLO11 is essential for pellicle formation by wild pellicle-forming yeasts isolated from contaminated wines. J Biosci Bioeng 111(1):7–9

Prouty AM, Gunn JS (2003) Comparative analysis of *Salmonella enterica* serovar typhimurium biofilm formation on gallstones and on glass. Infect Immun 71:7154–7158

Py B, Loiseau L, Barras F (2001) An inner membrane platform in the type II secretion machinery of Gram-negative bacteria. Embo Reports 2(3):244–248

Ren D, Bedzyk LA, Setlow P, Thomas SM, Ye RW, Wood TK (2004) Gene expression in Bacillus subtilis surface biofilms with and without sporulation and the importance of yveR for biofilm maintenance. Biotechnol Bioeng 86:344–364

Rhodes ER, Menke S, Shoemaker C, Tomaras AP, McGillivary G, Actis LA (2007) Iron acquisition in the dental pathogen *Actinobacillus actinomycetemcomitans*: what does it use as a source and how does it get this essential metal? Biometals 20:365–377

Shi L, Squier TC, Zachara JM, Fredrickson JK (2007) Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem c-type cytochromes. Mol Microbiol 65(1):12–20

Solano C, Garcia B, Valle J, Berasain C, Ghigo JM, Gamazo C, Lasa I (2002) Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. Mol Microbiol 43:793–808

Spiers AJ, Bohannon J, Gehrig SM, Rainey PB (2003) Biofilm formation at the air-liquid interface by the *Pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose. Mol Microbiol 50:15–27

Stewart PS, Franklin MJ (2008) Physiological heterogeneity in biofilms. Nat Rev Microbiol 6:199–210

Teal TK, Lies DP, Wold BJ, Newman DK (2006) Spatiometabolic stratification of *Shewanella oneidensis* biofilms. Appl Environ Microbiol 72:7324–7330

Theunissen S, De Smet L, Dansercoer A, Motte B, Coenye T, Van Beeumen JJ, Devreese B, Savvides SN, Vergauwen B (2010) The 285 kDa Bap/RTX hybrid cell surface protein (SO4317) of *Shewanella oneidensis* MR-1 is a key mediator of biofilm formation. Res Microbiol 161:144–152

Thormann KM, Saville RM, Shukla S, Pelletier DA, Spormann AM (2004) Initial phases of biofilm formation in *Shewanella oneidensis* MR-1. J Bacteriol 186:8096–8104

Thormann KM, Saville RM, Shukla S, Spormann AM (2005) Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. J Bacteriol 187:1014–1021

Vriendt D, Theunissen KS, Carpentier W, Smet LD, Devreese B, Beeumen JV (2005) Proteomics of *Shewanella oneidensis* MR-1 biofilm reveals differentially expressed proteins, including AggA and RibB. Proteomics 5:1308–1316

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. Science 295:1487

Windt DW, Gao HC, Kromer W, Van Damme P, Dick J, Mast J, Boon N, Zhou JZ, Verstraete W (2006) AggA is required for aggregation and increased biofilm formation of a hyper-aggregating mutant of *Shewanella oneidensis* MR-1. Microbiology 152:721–729

Yamamoto K, Arai H, Ishii M, Igarashi Y (2011) Trade-off between oxygen and iron acquisition in bacterial cells at the air-liquid interface. FEMS Microbiol Ecol 77(1):83–94