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REGULAR ARTICLE

Proteomics of *Shewanella oneidensis* MR-1 biofilm reveals differentially expressed proteins, including AggA and RibB

Kris De Vriendt, Sofie Theunissen, Wesley Carpentier, Lina De Smet, Bart Devreese and Jozef Van Beeumen

Laboratory of Protein Biochemistry and Protein Engineering, Ghent University, Ghent, Belgium

Shewanella oneidensis MR-1 is a Gram-negative, facultative aerobic bacterium, able to respire a variety of electron acceptors. Due to its capability to reduce solid ferric iron, *S. oneidensis* plays an important role in microbially induced corrosion of metal surfaces. Since this requires cellular adhesion to the metal surface, biofilm growth is an essential feature of this process. The goal of this work was to compare the global protein expression patterns of sessile and planktonic grown *S. oneidensis* cells by two-dimensional (2-D) gel electrophoresis. Mass spectrometry was used as an identification tool of the differentially expressed proteins. An IPG strip of pH 3–10 as well as pH 4–7 was applied for iso-electrofocusing. Analysis of the 2-D patterns pointed out a total of 59 relevant spots. Among these proteins, we highlight the involvement of a protein annotated as an agglutination protein (AggA). AggA is a TolC-like protein which is presumably part of an ABC transporter. Another differentially expressed protein is RibB, an enzyme of the riboflavin biosynthesis pathway. Riboflavin is the precursor molecule of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) and may be necessary for the altered respiratory properties of the biofilm cells *versus* planktonic cells. Some proteins that were identified indicate an anaerobic state of the biofilm. This anaerobic way of living affects the energy gaining pathways of the cell and is reflected by the presence of several proteins, including those of a heme-utilization system.

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1 Introduction

Bacteria often grow in close association with solid surfaces to form sessile communities referred to as biofilms [1, 2]. Biofilms are ubiquitous, occurring in environmental, industrial, and medical habitats. They can cause serious problems by corroding pipes, contaminating drinking water, forming dental plaques, and infecting medical implants and devices. The development of a mature biofilm, resulting from studies on *Pseudomonas aeruginosa*, is a stepwise process of attachment, colonization, and spreading. After immobilization to the surface, mushroom-like microcolonies separated by fluid-filled channels are formed, resulting in the typical biofilm architecture. In the final maturation step, the microorganisms spread along the surface and secrete a hydrated matrix consisting of exopolymeric substances and polysaccharides. Compared to their nonadherent counterparts (planktonic cells), biofilms show some distinct features, such as an increased resistance to chemical and antimicrobial treatment [3, 4]. It is now widely believed that an altered gene regulation and expression result in the phenotypical and metabolic differences of bio-

Correspondence: Prof. Dr. Jozef Van Beeumen, Laboratory of Protein Biochemistry and Protein Engineering, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium
E-mail: Jozef.VanBeeumen@UGent.be
Fax: +32-92645338

Abbreviations: FAD, flavin adenine dinucleotide; **FMN**, flavin mononucleotide

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film cells [5]. Proteomic and genomic techniques, such as 2-D gel electrophoresis and microarrays, are used nowadays to identify the proteins and genes that are differentially expressed between planktonic and biofilm cells. Using such an approach, biofilm proteomes of some pathogen-related bacteria have been studied, including *Escherichia coli* [6, 7], *Pseudomonas aeruginosa* [8, 9], *Listeria monocytogenes* [10], and *Bacillus cereus* [11].

In this work, we describe the changes in protein expression of *Shewanella oneidensis* MR-1 when growing as a biofilm. This facultative aerobic Gram-negative microorganism is able to respire on a wide variety of terminal electron acceptors, including fumarate, nitrate, trimethylamine *N*-oxide (TMAO) and oxidized metals, such as Fe(III) and Mn(IV) [12]. Due to its Fe(III)-reducing properties, *S. oneidensis* plays an important role in microbially induced corrosion (MIC) of iron metal surfaces. MIC refers to the influence of microorganisms on the kinetics of corrosion processes and can be considered as a biofilm-related problem. *S. oneidensis* MR-1 can influence corrosion reactions by reducing the corrosion-protective iron oxide layer on the metal surface [13] or by consumption of a protective H_2 layer on the Fe(0) surface [14]. It has been demonstrated that attachment to the metal surface is essential for *S. oneidensis* in order to mediate the reduction of metals [15].

Recently, the *S. oneidensis* MR-1 genome sequence was published [16]. Up to now, proteomic studies of *S. oneidensis* have been focused on its Fe(III)-respiratory properties and on the involvement of electron transport proteins in metal reduction [17, 18]. However, little information is available about the biochemical properties of *Shewanella* biofilms, although this aspect might be particularly important for its metal-reducing capability. Using 2-D gel electrophoresis and differential protein analysis of planktonic and biofilm cells, our goal was to identify proteins with changed expression levels. To distinguish biofilm formation from metal reduction-associated events, we used an inert silicone surface to grow biofilm. Some of the identified proteins are discussed to explain their possible role in biofilm development.

2 Materials and methods

2.1 Materials

Urea, ammonium persulfate, CBB-G250, and agarose were purchased from Amersham Biosciences (Uppsala, Sweden). Iodoacetamide, CHAPS, DTT, and TEMED were obtained from Fluka (Buchs, Switzerland). Immobilized pH gradient strips, SDS, glycine, and carrier ampholytes were from Bio-Rad (Hercules, CA, USA). Acryl/bisacrylamide solution was obtained from National Diagnostics (Atlanta, GE, USA). Silicone tubings were purchased from Degania Silicone (Emek Hayarden, Israel).

2.2 Biofilm and planktonic growth

Planktonic *S. oneidensis MR-1* was grown aerobically overnight in 100 mL Luria Bertani (LB) medium to the stationary phase (OD600 = ±2) in a rotary shaker at 28°C. The cells were collected by centrifugation for 15 min at 4000 rpm (4°C), washed with 50 mM Tris-HCl, pH 8, and stored at −80°C. Biofilm cells of *S. oneidensis MR-1* were grown at room temperature on the inner surface of silicone tubings. A closed circuit of tubings was used, starting and ending in the same flask containing 900 mL LB medium inoculated with *S. oneidensis* MR-1 cells. The total volume in the tubings was 90 mL. In order to collect three replicate samples from a single experiment, T-shaped connection pieces were used to connect three parallel silicone tubings with a length of 50 cm and an internal diameter of 16 mm. Using a peristaltic pump, the bacterial culture was circulated through the tubings at minimal flow (0.7 mL/s). After 48 h, the flask was replaced with a new flask containing fresh LB medium which was further replaced every 24 h. After 7 days, the biofilm was washed twice with 50 mM Tris-HCl, pH 8, for 90 s, to remove unattached or loosely attached cells. The tubings were cut along their entire length and the biofilm was harvested in 50 mM Tris-HCl, pH 8. The cells were centrifuged for 15 min at 4000 rpm (4°C) and stored at −80°C.

2.3 Protein extraction

Proteins were extracted with an extraction solution, consisting of 9 M urea, 4% CHAPS, 1% DTT, and 1 mM PMSF. Per gram of biomass, 2 mL extraction solution was added. The cells were vortexed and sonicated to homogeneity on ice for 30 s. After centrifugation at 14 000 rpm for 30 min the soluble protein fraction was retained. The protein concentration was determined using the Bio-Rad Protein Assay.

2.4 2-D gel electrophoresis and analysis

The IPG strips (17 cm, pH 4–7 and pH 3–10 L) were rehydrated for 7 h *via* a passive rehydration protocol [19]. Rehydration solution contained 8 M urea, 2% CHAPS, 2% carrier ampholytes, and 0.3% DTT. 450 μg of protein was loaded on each IPG strip. IEF was performed at 18°C in a Multiphor II electrophoresis unit (Amersham Bioscience), using a stepwise voltage gradient to 3500 V. This voltage was maintained for 8 h to steady state. Upon completion of the program, the IPG strips were equilibrated for 10 min in 50 mM Tris-HCl, pH 8, containing 6 M urea, 2% SDS, 30% glycerol, and 1% DTT, followed by another 10 min in 50 mM Tris-HCl, pH 8, containing 6 M urea, 2% SDS, 30% glycerol and 5% iodoacetamide. The strips were placed on a vertical SDS-PAGE gel (12.5% T, 2.5% C) and sealed with 0.4% agarose containing a trace of bromophenol blue. The second dimension was performed on a Protean Plus Dodeca Cell sytem (Bio-Rad) at 20 mA/gel, until the bromophenol blue front reached the bottom of the gel. The gels were Coomassie Blue

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(CBB-G250)-stained using a standard procedure. The 2-D gel images were analyzed with the PDQuest 7.1 software of Bio-Rad. Automated spot detection and matching was applied, followed by a manual spot editing to achieve a sufficient correlation between the gels. An analysis set was created to find the spots with a minimum 2-fold increase or decrease between the two replicate groups (biofilm *versus* planktonic). Only spots that differed significantly in abundance ($p < 0.05$) according to the Student's *t*-test were further investigated.

2.5 Protein identification by MS

The excised spots were in-gel digested with trypsin and the peptides extracted as described [18]. The peptide mixture was analyzed using MALDI-TOF-TOF-MS or nano-LC-ESI-MS. For MALDI-TOF-TOF-MS, we used the 4700 Proteomics Analyzer (Applied Biosystems, Framingham, CA, USA), operating in either the MS mode to generate a peptide mass fingerprint (PMF) or in the MS/MS mode for peptide fragmentation. Samples were prepared for analysis by mixing 1 μL of the peptide sample with 1 μL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA, and spotting the mixture on a MALDI target. The instrument was externally calibrated before analysis. Samples that could not be identified using MALDI were loaded on an automated nano-HPLC system (LC Packings, Amsterdam, The Netherlands) with the on-line detection of the separated peptides by an ESI-Q-TRAP mass spectrometer (Applied Biosystems, Framingham, CA, USA), using the nano-LC setup as described previously [20]. For protein identification, an in-house adapted MASCOT server was used for database searching of the *S. oneidensis* MR-1 genome (www.tigr.org).

2.6 Western blot analysis

Antibodies were raised against partially purified recombinant AggA protein (Eurogentec, Liège, Belgium). For Western blot analysis, a protein extract of *S. oneidensis* planktonic cells and one of biofilm cells were subjected to SDS-PAGE, followed by electroblotting to a nitrocellulose membrane (Amersham Biosciences). After blocking the membrane to reduce nonspecific reactivity, it was first incubated with the agglutination protein-reactive antibody, followed by an incubation with a peroxidase-conjugated anti-rabbit antibody (Sigma, St. Louis, MO, USA). The blot was washed with PBS containing 0.1% Tween 20 (PBS-T) and developed using a lumi-light Western Blotting Substrate (Roche, Indianapolis, IN, USA).

3 Results and discussion

3.1 Biofilm growth

Biofilm was grown on silicone tubings for 7 days until visible saturation of the surface. During the first 48 h, the bacteria were able to grow to a sufficient density. During this period 'conditioning' of the tubings was allowed, as this is reported to enhance biofilm formation [21]. Three parallel tubings of 50 cm were used to harvest three replicate samples in one experiment. Typically, 1–1.4 g biomass was collected for each replicate.

In some preliminary work, we used the nonattached cells of the biofilm medium as a control to find differentially expressed proteins between biofilm and planktonic cells. However, these cells gave very similar 2-D patterns to biofilm cells (results not shown). As biofilm formation is a dynamic process of cell attachment and detachment, these cells could be considered as nonreal planktonic cells. They contain probably cells that are detached from the biofilm layer and that retain some biofilm cell characteristics. Steyn *et al.* [8] confirmed this by 2-D PAGE analysis and cited these cells as surface-induced planktonic (SIP) cells. Therefore, we used cells grown in culture flasks as controls. Because biofilm development is a dynamic system, the biofilm used in this study should be regarded as a mixture of mature biofilm cells and newly attached cells. Time-dependent protein analysis will be the subject of further studies.

3.2 2-D maps and analysis

Biofilm and planktonic protein samples were subjected to 2-D electrophoresis. To achieve high reproducibility between gels, samples were prepared in replicates. Out of two biofilm setups, six replicate samples were collected. Together with six planktonic samples, twelve 2-D gels could be generated simultaneously using the Protean Plus Dodeca Cell system (Bio-Rad). Two pH gradients were used for IEF. One set of gels was generated using IPG strips with a linear pH gradient of 3–10, another set using IPG strips with a pH gradient of 4–7. A typical 2-D pattern within the pH ranges 3–10 and 4–7 for biofilm and planktonic *S. oneidensis* is presented in Figs. 1 and 2, respectively.

Subsequently, 2-D maps were analyzed using PDQuest 7.1 software to characterize differentially expressed proteins. Spot detection revealed between 350 and 400 spots per gel. For the differential analysis of the gels covering the pH range 3–10, one biofilm gel was rejected due to poor quality. For the gels covering pH 4–7, one planktonic and one biofilm gel were removed from further analysis. High-reproducible gels with a correlation coefficient of minimum 80% could be achieved. The gels were grouped into two replicate groups (biofilm and planktonic) with an average mean coefficient of variation of 23% for pH 3–10 and 18% for pH 4–7. Only reproducible spots present in four to six gels (when there were six gels in the replicate group) or three to five gels (when there were five gels in the replicate group) were taken into account in the analysis.

3.3 Differentially expressed proteins

On the gels with IEF pH gradient 3–10, 27 spots with a minimum twofold change in expression level were identified. The spots are numbered as shown in Fig. 1. Twelve biofilm upregulated spots and 15 biofilm downregulated spots could be distinguished. Out of the 32 spots found on

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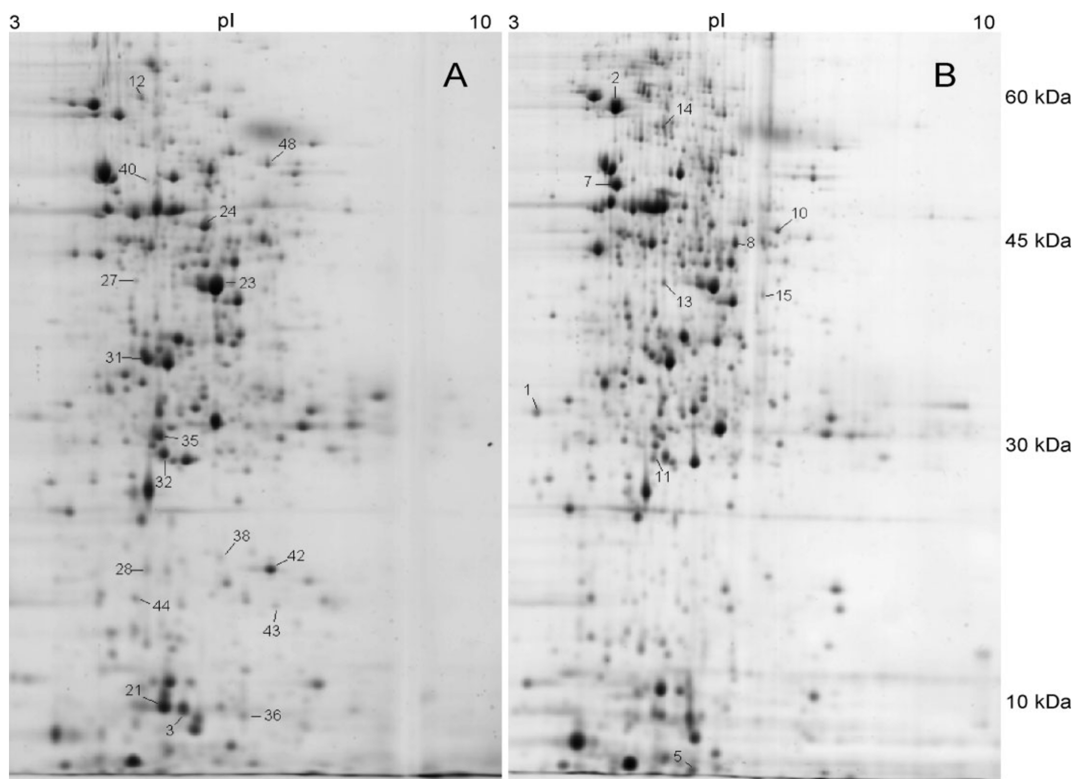

Figure 1. 2-D map of the soluble proteins of *S. oneidensis* (A) in planktonic and (B) biofilm growth state. IEF was performed using an IPG strip pH 3–10. Differentially expressed proteins with a minimum 2-fold change in expression are numbered.

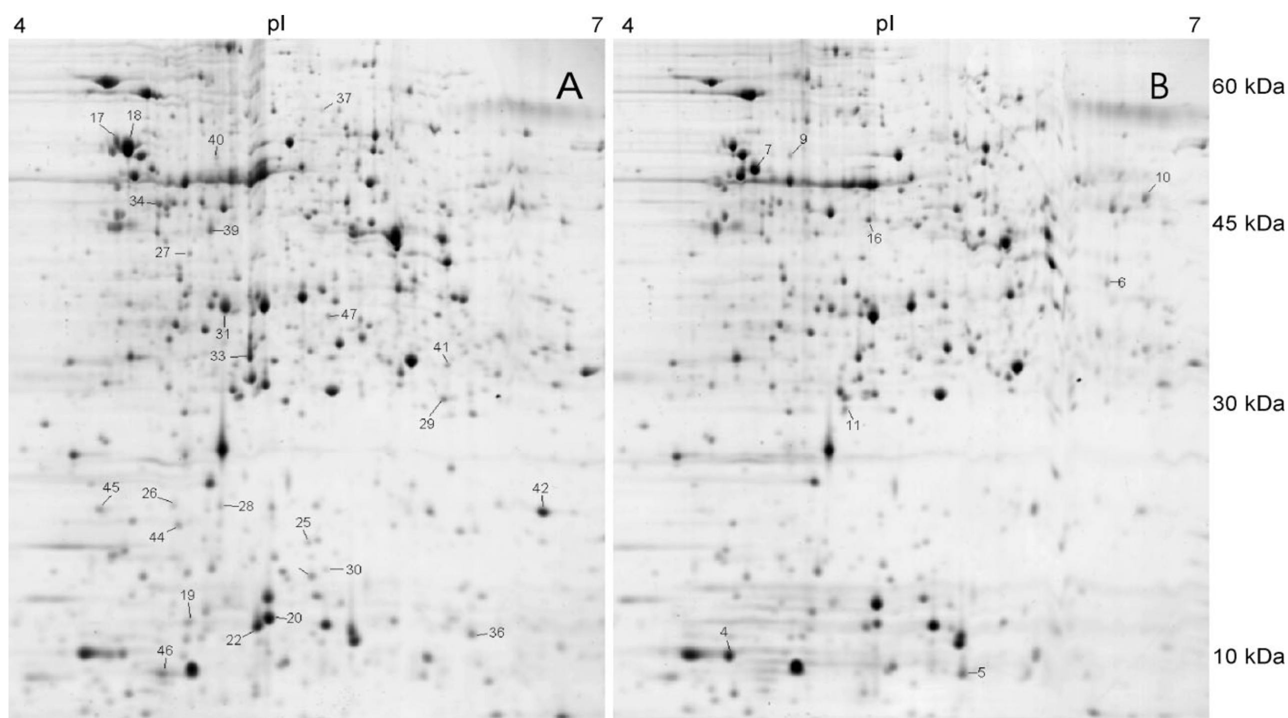

Figure 2. 2-D map of the soluble proteins of *S. oneidensis* (A) in planktonic and (B) biofilm growth state. IEF was performed using an IPG strip pH 4–7. Differentially expressed proteins with a minimum 2-fold change in expression are numbered.

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the gels with IEF pH gradient 4–7, 24 showed a decrease and 8 an increase in abundance in biofilm. Spots are numbered as in Fig. 2. All proteins could be identified using MALDI-MS and nano-LC-ESI-MS as described in Section 2. The proteins with an increased expression in biofilm cells are listed in Table 1. From a total of 16 upregulated spots, 4 proteins were identified on both pH 3–10 and pH 4–7 gels. Table 2 shows the proteins with a decreased abundance in biofilm compared to planktonic cells. Seven of a total of 32 proteins were found in both the pH 3–10 and pH 4–7 gels. Some spots could not be quantified on the pH 3–10 gels because they were not well resolved from neighbouring spots. Due to the zoom-in effect, (partially) overlapping spots were much better resolved on pH 4–7 gels. Therefore, this second pH gradient gave us some additional spots and extended the number of proteins identified from the pH 3–10 gels.

3.3.1 Cellular motility

The motility protein flagellin (spot 31) is downregulated in biofilm (Table 2). Flagellin is the major constituent of the flagellum structure. Motility by flagella and type IV pili is critical in biofilm development. However, the role of the flagellum in biofilm formation differs amongst bacteria. For *Escherichia coli*, the flagellum is important in the initial adhesion phase and in the spreading along the surface [22]. For *Pseudomonas aeruginosa*, this cell structure is necessary for shaping the biofilm and for the development to a mature state [23]. Proteomic analysis of *Pseudomonas putida* biofilm reveals an upregulation of type IV pili proteins, but a downregulation of flagella proteins [24]. In *S. oneidensis*, the flagellum may be important for the attachment to the surface, but the decreased expression of flagellin suggests that the flagellum is of minor importance in a fully developed biofilm.

3.3.2 Quorum sensing

Microorganisms have developed a mechanism to sense the bacterial population so that they can react to their changing environment, a phenomenon called quorum-sensing (QS). Gram-negative bacteria usually produce acylated homoserine lactones (AHLs) as QS signals. These molecules accumulate in function of the cell density and, above a certain threshold,

Table 1. Proteins showing significant upregulation in *Shewanella oneidensis* biofilm when compared to planktonic cells

Spot No.[a]	Accession No.[b]	Protein name	MW (kDa)	p*I*	OD ratio pH 3–10[c]	OD ratio pH 4–7[c]
1	Q8EHS8	Peptidyl-prolyl *cis-trans* isomerase FklB	21.8	4.16	2.2	
2	Q8EEI0	Ribosomal protein S1	61.2	4.65	2.0	
3	Q8EAH5	Ribosomal protein L9	15.7	5.30	2.1	
4	Q8EK75	Ribosomal protein L7/L12	12.5	4.31		2.3
5	Q8EGF6	Cold shock domain family protein	8.9	7.54	+	+
6	Q8EJR1	Alcohol dehydrogenase, zinc-containing	36.3	6.42		+
7	Q8E9G3	Agglutination protein	52.3	4.93	4.9	4.1
8	Q8EIG3	Peptidase B	45.2	5.99	2.3	
9	Not available	Peptidase, M16 family, disrupted reading frame (SO4538)	49.2	5.14		2.1
10	Q8EBN8	Serine hydroxymethyltransferase	45.2	6.58	2.1	3.2
11	Q8EKF2	3,4-Dihydroxy-2-butanone-4-phosphate synthase (RibB)	22.9	4.88	+	+
12	Q8EHL1	Polyribonucleotide nucleotidyltransferase	75.8	4.85	3.1	
13	Q8EA43	Phosphoribosylaminoimidazole-succinocarboxamide synthase	40.0	5.07	2.2	
14	Q8EJQ8	Hypothetical protein	128.8	4.90	2,2	
	Q8ECI8	Prolyl-tRNA synthetase	63.2	5.07		
15	Q8EJM8	Uroporphyrinogen decarboxylase	39.2	6.44	+	
16	Q8EIB4	*S*-Adenosylmethionine synthetase	41.4	5.15		+

a) Spot number as indicated in Figs. 1 and 2
b) Protein entry number of the Swiss-Prot database (http://us.expasy.org)
c) Optical density ratio from the average density of three to six spots in the replicate group. Unique spots present in one replicate group are indicated with +.

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Table 2. Proteins showing significant downregulation in *Shewanella oneidensis* biofilm when compared to planktonic cells

Spot No.[a)]	Accession No.[b)]	Protein name	MW (kDa)	p*I*	OD ratio pH 3–10[c)]	OD ratio pH 4–7[c)]
17	Q8CX48	GroEL	57.1	4.56		3.1
18	Q8CX48	GroEL	57.1	4.56		2.3
19	Q8CX49	GroES	10.2	5.14		+
20	Q8CX49	GroES	10.2	5.14		2.4
21	Q8EB53	Universal stress protein family	15.6	5.17	3.3	
	Q8CX49	GroES	10.2	5.14		
22	Q8EB53	Universal stress protein family	15.6	5.17		3.2
23	Q8EGV1	Alcohol dehydrogenase II	40.0	5.99	2.2	
24	Q8EAG0	Serine protease, HtrA/DegQ/DegS family	46.5	6.28	2.1	
25	Q8EG19	Clp protease, proteolytic subunit	22.1	5.26		4.6
26	Q8EH52	ThiJ/PfpI family protein	19.7	4.73		+
27	Q8ECN4	AcrA/AcrE family protein	36.5	4.87	+	+
28	Q8EI86	Alkyl hydroperoxidase, subunit C	20.9	5.01	+	+
29	Q8EGM4	Glutathione *S*-transferase family protein	25.5	6.14		+
30	Q8EBZ6	Glutathione peroxidase, putative	19.7	5.79		+
31	Q8ECA6	Flagellin	28.5	9.03	2.4	2.7
32	Q8EHK0	Purine nucleoside phosphorylase	25.6	5.11	2.1	
33	Q8E9X9	Uridine phosphorylase	26,8	5.08		2.1
34	Q8EHK3	Thymidine phosphorylase	47.0	4.75		2.1
	Q8EHG3	Adenylosuccinate synthetase, putative	45.6	4.70		
35	Q8EGC4	Enoyl-CoA isomerase/hydratase family protein	27.3	4.98	3.8	
36	Q8CVD5	Azurin	16.3	7.5	+	+
37	Q8E940	Flavoprotein-ubiquinone oxidoreductase, putative	60.2	5.67		+
38	Q8EBH3	OmpA family protein	40.2	4.52	+	
39	Q8E800	Zinc-binding dehydrogenase	40.2	4.85		2.3
40	Q8EJ92	PhoH family protein	52.2	5.06	+	+
41	Q8EB61	Periplasmic hemin-binding protein	33.7	7.16		+
42	Q8EB67	Conserved hypothetical protein	20.6	6.68	4.8	2.6
43	Q8EB68	Hypothetical protein	20.9	6.35	+	
44	Q8EF04	Conserved hypothetical protein	16.8	4.60	+	+
45	Q8EHP3	Conserved hypothetical protein	17.0	4.47		+
46	Q8EB52	Hypothetical protein	14.8	4.66		+
47	Q8E8D4	Adenosine deaminase	36.2	5.67		+
	Q8E8K5	Conserved hypothetical protein	31.8	5.55		
48	Q8E841	Conserved hypothetical protein	63.7	6.58	3.5	

a) Spot number as indicated in Figs. 1 and 2
b) Protein entry number of the Swiss-Prot database (http://us.expasy.org)
c) Optical density ratio from the average density of three to six spots in the replicate group. Unique spots present in only one replicate group are indicated with +.

they trigger the expression of QS regulated genes. QS is an important mechanism for biofilm formation as this cell-cell communication system enables biofilms to respond as an organized group of bacteria [25, 26]. AHL molecules are synthesized by enzymes of the *Lux*I family. The substrates for these enzymes are acyl carrier proteins and *S*-adenosylmethionine [27]. The latter compound is synthesized by *S*-adenosylmethionine synthetase (MetK) starting from L-methionine and ATP. In the present work, MetK is identified as an upregulated enzyme in biofilm (spot 16, Table 1). It is possible that this is due to the activated QS mechanism in biofilm.

3.3.3 AggA, a TolC-like protein

The protein most upregulated in biofilm cells shows a high homology to AggA, an agglutination protein from *P. putida*. The agglutination protein is not only the most upregulated protein in biofilm forming *S. oneidensis* cells but it also displays an increased abundance upon anaerobic growth on Fe_2O_3 when compared to anaerobic growth on fumarate [18]. When growth under anaerobic *versus* aerobic conditions was compared, the AggA was found to be upregulated under the latter conditions [17]. Western blot analysis was

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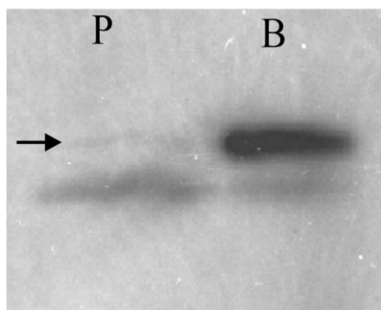

Figure 3. Western blot showing the upregulation of the AggA protein (indicated with arrow) in *S. oneidensis* biofilm (B) when compared to planktonic cells (P).

performed on *S. oneidensis* planktonic and biofilm cells to confirm the upregulation obtained by 2-D map analysis (Fig. 3).

Similarity searches revealed that this protein is widely spread among other bacterial species within the γ-proteobacteria, including several *Vibrio* and *Pseudomonas* species. The agglutination protein from *P. putida* is involved in the adhesion of the bacterial cell to plant roots [28]. Structural prediction based on the amino acid sequence suggests a similarity between *S. oneidensis* agglutination protein and the bacterial membrane protein TolC. The latter forms a trimeric transport channel in the outer membrane and is part of type I secretion systems [29, 30]. Recent studies show that the *Pseudomonas fluorescens* homologue LapE is involved in early biofilm development [31]. Comparison of the AggA chromosomal region in *S. oneidensis* to the LapE operon in *P. fluorescens* suggests that AggA forms an ABC transporter cassette, with two upstream proteins annotated as a toxin secretion ATP-binding protein, RtxB, and a HlyD family secretion protein (Fig. 4). The AggA is predicted to form the outer membrane component of the ABC transporter, while the toxin secretion ATP-binding protein and the HlyD family secretion protein presumably form the cytoplasmatic membrane localized ATPase and the periplasmatic membrane fusion protein, respectively. In *P. fluorescens*, the LapEBC transporter is believed to transport LapA, a large protein (> 800 kDa) upstream of LapEBC. In *S. oneidensis*, the transported protein is predicted to be a putative Rtx-toxin (± 285 kDa) situated upstream of the toxin secretion ATP-binding protein. However, the role of this putative transport system in biofilm remains unclear.

3.3.4 Anaerobic respiration

The oxygen-protective enzyme alkyl hydroperoxidase AhpC was found to be downregulated in biofilm. Moreover, a glutathione-*S*-transferase and a glutathione peroxidase, generally thought to be involved in detoxification processes and oxidative stress also showed a decreased expression. It is presumed that at least the inner cell layers of biofilms grow anaerobically, because oxygen diffusion to these layers is limited [32]. Our results support this hypothesis and are important in the understanding of the biofilm's metabolic networks.

Heme-containing cytochromes are expected to be present in high concentrations when growing anaerobically. *S. oneidensis* is believed to have 42 possible cytochrome *c* genes, which is more than in most other published microbial genomes [33]. In this work, no cytochromes were identified. They are often membrane-associated and are therefore not well recovered on 2-D gels. However, increased protein abundance was detected for uroporphyrinogen decarboxylase (HemE), an enzyme involved in heme biosynthesis. This enzyme may be indirectly linked to the higher cytochrome content in the anaerobic biofilm.

A second highly upregulated protein in biofilm is RibB (3,4-dihydroxy-2-butanone-4-phosphate synthase, spot 11), a key enzyme of the riboflavin synthesis pathway. This molecule is the precursor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), typical cofactors for enzymes involved in reduction processes and for electron transport proteins. However, the role of RibB in biofilm remains obscure. In *Helicobacter pylori*, the bifunctional enzyme RibBA is highly expressed under ferric iron limiting conditions and it is postulated that riboflavin is necessary for the ferric iron reduction by *H. pylori* [34]. RibB has also been found to be greatly expressed in *S. oneidensis* under anaerobic conditions [17] and might therefore be involved in anaerobic reduction pathways. Riboflavin is synthesized starting from guanosine triphosphate (GTP). Two enzymes of the purine metabolism are differentially expressed. The first, phosphoribosylaminoimidazole-succinocarboxamide synthase (spot 13), an enzyme involved in the biosynthesis of purines, is upregulated in biofilm. The second enzyme was identified as a purine nucleoside phosphorylase (spot 32) and takes part in purine degradation. This enzyme is downregulated in biofilm. These results suggest an upregulated synthesis and a downregulated degradation of purines such as GTP. This may be linked to the up-regulation of the riboflavin synthesis as suggested by the increased production of RibB.

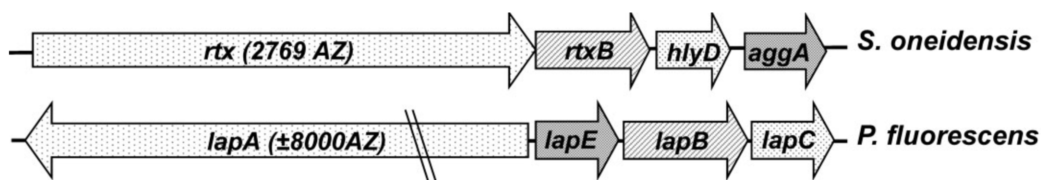

Figure 4. Schematic representation of the *aggA* locus in *S. oneidensis* compared to the *lapBCE* locus in *P. fluorescens*. The AggA protein of *S. oneidensis* shows homology with the LapE locus of *P. fluorescens* and is believed to form an ABC transport system for Rtx, a LapA homologue.

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3.3.5 Heme acquisition

Iron is an essential element for most bacteria. Under iron-limiting circumstances, bacteria can import heme or heme-containing compounds and use these as an iron source. The transport mechanism requires a periplasmatic membrane protein, TonB, and two accessory proteins, ExbB and ExbD. The corresponding genes are part of an operon, including other heme utilization proteins, such as *hutBCD* of *Vibrio cholerae* [35] or *hugBCD* and *hugAWXZ* of *Plesiomonas shigelloides* [36]. In *S. oneidensis* biofilm, we identified three downregulated proteins that are linked to this transport system. The first one (spot 41) is a periplasmatic heme-binding protein, HmuT, a homolog of HutB from *V. cholerae* and HugB from *P. shigelloides*. The other two proteins are annotated as hypothetical proteins (Q8EB67, spot 42 and Q8EB68, spot 43), but show a high homology to HugZ (71% identity) and HugX (61% identity) of *P. shigelloides*, respectively. The function of HugZ and HugX is unclear, although it is suggested that these proteins are needed to prevent heme toxicity [36]. *S. oneidensis* and *P. shigelloides* have the same genetic organization of the heme iron utilization locus, except that HugW is not present in *S. oneidensis*. These results suggest that the heme utilization system is downregulated in biofilm because *S. oneidensis* can no longer use heme as an iron source. Iron is released from the heme by breaking down the porphyrin ring in an oxygen-demanding process. As stated above, we believe that the biofilm is an anaerobic mode of growth and thus limits the capacity to use heme as an iron source.

4 Concluding remarks

Shewanella oneidensis MR-1 biofilm was grown on a silicone surface and its proteome compared to that of planktonic grown *S. oneidensis* using 2-D electrophoresis. Using a combination of IPG strips with pH gradient 3–10 and 4–7, a total of 59 significant spots were identified using mass spectrometric techniques. It is likely that more proteins will have a differential expression pattern, as very acidic and basic proteins as well as membrane proteins are not well recovered from 2-D gels.

The most upregulated protein in *S. oneidensis* biofilm, on pH 3–10 gels as well as on pH 4–7 gels, was identified as an agglutination protein. The existence and the role of this putative TolC-like transport protein and of the secreted protein in biofilm remains to be investigated in future work. Other proteins, such as RibB and detoxification proteins, indicate the anaerobic state of biofilm cells. Due to oxygen limitation, a heme transport system is repressed, as is supported by the identification of three proteins that can be linked to this system. Two of these proteins were annotated as 'hypothetical', but they show high homology with other heme transport-associated proteins. To our knowledge, this work reports the first proteomic analysis of a *S. oneidensis* biofilm, providing initial insight in its cellular biology.

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5 References

[1] Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., Lappin-Scott, H. M., *Annu. Rev. Microbiol.* 1995, *49*, 711–745.

[2] Stickler, D., *Curr. Opin. Microbiol.* 1999, *2*, 270–275.

[3] Williams, I., Venables, W. A, Lloyd, D., Paul, F., Critchley, I., *Microbiology* 1997, *143*, 2407–2413.

[4] Anderl, J. N., Franklin, M. J., Stewart, P. S., *Antimicrob. Agents Chemother.* 2000, *44*, 1818–1824.

[5] Sauer, K., *Genome Biol.* 2003, *4*, 219.1–219.5.

[6] Trémoulet, F., Duché, O., Namane, A., Martinie, B., Labadie, J. C., *FEMS Microbiol. Lett.* 2002, *215*, 7–14.

[7] Schembri, M. A., Kjaergaard, K., Klemm, P., *Mol. Microbiol.* 2003, *48*, 253–267.

[8] Steyn, B., Oosthuizen, M. C., MacDonald, R., Theron, J., Brözel, V. S., *Proteomics* 2001, *1*, 871–879.

[9] Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., Davies, D. G., *J. Bacteriol.* 2002, *184*, 1140–1154.

[10] Trémoulet, F., Duché, O., Namane, A., Martinie, B., The European Listeria Genome Consortium *et al.*, *FEMS Microbiol. Lett.* 2002, *210*, 25–31.

[11] Oosthuizen, M. C., Steyn, B., Theron, J., Cosette, P., Lindsay, D. *et al.*, *Appl. Environ. Microbiol.* 2002, *68*, 2770–2780.

[12] Nealson, K. H., Saffarini, D., *Annu. Rev. Microbiol.* 1994, *48*, 311–343.

[13] Little, B., Wagner, P., Hart, K., Ray, R., Lavoie, D. *et al.*, *Biodegradation* 1998, *9*, 1–10.

[14] De Windt, W., Boon, N., Siciliano, S. D., Verstraete, W., *Environ. Microbiol.* 2003, *5*, 1192–1202.

[15] Myers, C., Nealson, K. H., *Science* 1988, *240*, 1319–1321.

[16] Heidelberg, J. F., Paulsen, I. T., Nelson, K. E., Gaidos, E. J., Nelson, W. C. *et al.*, *Nat. Biotechnol.* 2002, *20*, 1118–1123.

[17] Beliaev, A. S., Thompson, D. K., Khare, T., Lim, H., Brandt, C. C. *et al.*, *OMICS* 2003, *6*, 39–60.

[18] Vanrobaeys, F., Devreese, B., Lecocq, E., Rychlewski, L., De Smet, L. *et al.*, *Proteomics* 2003, *3*, 2249–2257.

[19] Sanchez, J. C., Rouge, V., Pisteur, M., Ravier, F., Tonella, L. *et al.*, *Electrophoresis* 1997, *18*, 324–327.

[20] Devreese, B., Vanrobaeys, F., Van Beeumen, J., *Rapid Commun. Mass Spectrom.* 2001, *15*, 50–56.

[21] Murga, R., Miller, J. M., Donlan, R. M., *J. Clin. Microbiol.* 2001, *39*, 2294–2297.

[22] Pratt, L. A., Kolter, R., *Mol. Microbiol.* 1998, *30*, 285–293.

[23] Klausen, M., Heydorn, A., Ragas, R., Lambertsen, L., Aaes-Jørgensen, A. *et al.*, *Mol. Microbiol.* 2003, *48*, 1511–1524.

[24] Sauer, K., Camper, A. K., *J. Bacteriol.* 2001, *183*, 6579–6589.

[25] Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. *et al.*, *Science* 1998, *280*, 295–298.

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[26] Smith, R. S., Iglewski, B.H., *Curr. Opin. Microbiol.* 2003, *6*, 56–60.

[27] Val, D. L., Cronan, J. E. Jr., *J. Bacteriol.* 1998, *180*, 2644–2651.

[28] Buell, C. R., Anderson, A. J., *Mol. Plant Microbe Interact.* 1992, *5*, 154–162.

[29] Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., Hughes, C., *Nature* 2000, *405*, 914–919.

[30] Buchanan, S. K., *Trends Biochem. Sci.* 2001, *26*, 3–6.

[31] Hinsa, S. M., Espinosa-Urgel, M., Ramos, J. L., O'Toole, G. A., *Mol. Microbiol.* 2003, *49*, 905–918.

[32] DeBeer, D., Stoodley, P., Roe, F. L., Lewandowski, Z., *Biotech. Bioeng.* 1994, *43*, 1131–1138.

[33] Meyer, T. E., Tsapin, A. I., Vandenberghe, I., De Smet, L., Frishman, D. *et al.*, *OMICS* 2004, *8*, 57–77.

[34] Worst, D. J., Gerrits, M. G., Vandenbroucke-Grauls, C. M. J. E., Kusters, J. G., *J. Bacteriol.* 1998, *180*, 1473–1479.

[35] Occhino, D. A., Wyckoff, E. E., Henderson, D. P., Wrona, T. J., Payne, S. M., *Mol. Microbiol.* 1998, *29*, 1493–1507.

[36] Henderson, D. P., Wyckoff, E. E., Rashidi, C. E., Verlei, H., Oldham, A. L., *J. Bacteriol.* 2001, *183*, 2715–2723.

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