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AggA is required for aggregation and increased biofilm formation of a hyper-aggregating mutant of *Shewanella oneidensis* MR-1

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Shewanella oneidensis COAG, a hyper-aggregating mutant of MR-1, was isolated from a rifampicin-challenged culture. Compared to the wild-type, COAG exhibited increased biofilm formation on glass carrier material. The role of surface-located proteins in the process of COAG auto-aggregation was confirmed by different proteolytic treatments of the aggregates. All of the tested proteolytic enzymes resulted in deflocculation within 3 h of incubation. In order to examine the altered expression of outer-membrane proteins in COAG, membrane-enriched cell preparations were analysed by proteomics and the protein pattern was compared to that of MR-1. From the proteomics results, it was hypothesized that the agglutination protein AggA, associated with the secretion of a putative RTX protein, was involved in the hyper-aggregating phenotype. These results were confirmed with a DNA microarray study of COAG versus MR-1. An insertional mutation in the *S. oneidensis* COAG *aggA* locus resulted in loss of the hyper-aggregating properties and the increased biofilm-forming capability. The insertional mutation resulted in strongly decreased attachment during the initial stage of biofilm formation. By complementing this mutation with the vector pCM62, expressing the *aggA* gene, this effect could be nullified and biofilm formation was restored to at least the level of the MR-1 wild-type.

INTRODUCTION

The attachment of bacteria to a surface often results in the formation of complex biofilm structures. This involves molecular events that encompass mechanisms for adhesion, aggregation and community expansion (O'Toole *et al.*, 2000), and in many cases this process is facilitated by auto-aggregation factors (Schembri *et al.*, 2003a). These factors also allow bacteria to auto-aggregate, a phenomenon readily observed under the microscope as a clumping of cells and, macroscopically, as flocculation and settling of cells from liquid suspensions (Schembri *et al.*, 2001). Different auto-aggregation factors exist in different strains: surface adhesins (Schembri *et al.*, 2003b), type IV pili (Schreiber *et al.*, 2002; Bechet & Blondeau, 2003; Bieber *et al.*, 1998), curli (Olsen *et al.*, 1989) and aggregating adherence fimbriae (Nataro *et al.*, 1993; Czeczulin *et al.*, 1997).

A number of theories have been proposed for the auto-aggregation of micro-organisms, including factors such as substrate gradients, slow growth of the organism, physical/chemical stress, and predation (Farrell & Quilty, 2002). In some cases, auto-aggregation of bacteria can be induced and even become a permanent feature. *Pseudomonas putida* CP1 exhibits auto-aggregation during the degradation of mono-chlorophenols and phenol, and it has been shown that this is a response to the toxicity of the added substrates (Farrell & Quilty, 2002). Bossier *et al.* (2000) observed that after inoculation of the non-aggregative *Ralstonia eutropha*-like strain AE815 into activated sludge, reisolation on selective medium yielded mutant strain A3, which was characterized by auto-aggregative behaviour.

In our laboratory, an auto-aggregating mutant of *Shewanella oneidensis* strain MR-1 has been obtained, *S. oneidensis*

Abbreviations: AI, aggregation index; 2D, two-dimensional; Rif, rifampicin.

The results of gel electrophoresis of *aggA* PCR amplification products are shown in Supplementary Fig. S1, and an overview of the 11 identified protein spots and their relative spot intensities, and a complete list of all the genes that showed significant upregulation in the COAG strain in at least one sampling event, are shown in Supplementary Tables S1 and S2, respectively, with the online version of this paper.

COAG, which exhibits increased biofilm formation. *S. oneidensis* MR-1 is a facultative aerobic Gram-negative bacterium with remarkably diverse respiratory capabilities, including the reduction of several metal ions. As a result, the biological activities of *S. oneidensis* have considerable implications with regard to direct bioremediation of both metal and organic pollutants (Heidelberg *et al.*, 2002). However, the activities of such a versatile bacterium also have negative effects. In the case of *S. oneidensis*, unwanted and often hard-to-control effects include the deterioration and destruction of human commodities. For instance, biofilm growth coupled to iron respiration can affect the chemistry of the environment around steel, leading to microbially induced corrosion (MIC). Since *S. oneidensis* and related *Shewanella* species have been isolated from corroding steel pipelines (Semple & Westlake, 1987; Dubiel *et al.*, 2002), a mechanistic understanding of the role of *Shewanella* biofilm formation and metabolic activity may help to facilitate corrosion control (Lee & Newman, 2003). The capacity of *S. oneidensis* to attach to and detach from solid surfaces and to form biofilms has already been described in a number of applications and studies (Bagge *et al.*, 2001; Little *et al.*, 1998; Thormann *et al.*, 2004, 2005). It has recently been suggested that two type IV pilin gene clusters, MSHA (mannose-sensitive haemagglutinin) and *tapABCD*, could be involved in this process (Heidelberg *et al.*, 2002), in the same way that MSHA plays a role in biofilm formation by *Vibrio cholerae* (Chiavelli *et al.*, 2001). This hypothesis is further developed in the work of Thormann *et al.* (2004), in which it is shown that defects in MSHA type IV pilus biosynthesis and pilus retraction result in severe defects in adhesion to surfaces. Furthermore, motility genes are found to be involved in the three-dimensional development of biofilms. In other studies, exopolymeric substances and surface proteins have been suggested to be involved in transient contact between several *Shewanella* species and Fe(III)oxides during Fe(III) respiratory activity (Caccavo *et al.*, 1997; Lower *et al.*, 2001).

The goal of this study was to examine the properties of *S. oneidensis* COAG with regard to biofilm formation and hyper-aggregating behaviour. With proteomics and microarray analysis, surface proteins were screened for their possible relation to the auto-aggregating properties of this strain. By means of insertional mutation, the role of AggA in the auto-aggregation of COAG was elucidated. It was shown that AggA is a necessary auto-aggregation factor for the increased cell-to-cell and cell-to-surface attachment of the hyper-aggregating COAG mutant.

METHODS

Isolation of *S. oneidensis* COAG. *S. oneidensis* MR-1 was inoculated into test tubes containing 10 ml Luria–Bertani (LB) medium and propagated using increasing concentrations of rifampicin (Rif). Cells able to grow in test tubes containing 100 mg Rif l^{-1} were plated as a dilution series on LB agar plates containing 100 mg Rif l^{-1}. Colonies were resuspended in LB medium with 100 mg Rif l^{-1}. One of several series of this Rif^R selection procedure produced a Rif^R *S. oneidensis* derivative of MR-1 with an irreversibly auto-aggregative phenotype, and this strain was termed *S. oneidensis* COAG. No growth defect was observed in COAG when compared to MR-1. This phenotype was maintained in the absence of Rif.

Construction of the insertional mutant AGGA1. *S. oneidensis* COAG was used as a parental strain to generate an *aggA* null allele by integrative disruption with the suicide plasmid pKNOCK-Km^r (Alexeyev, 1999). Briefly, a 768 bp *aggA* internal fragment was amplified by PCR with primers AGG183F (5′-TCAGGCAATTGC-TGGTTACA-3′) and AGG951R (5′-ACCACCTACGTTTTGGCTTG-3′). The PCR fragment was digested with *Sal*I and *Pst*I, yielding a 538 bp fragment, which was ligated into the pKNOCK-Km^r vector according to standard procedures (Sambrook & Russell, 2001). The resulting construct was then transformed into *Escherichia coli* S17-1/λ_{pir} and subsequently transferred into *S. oneidensis* COAG by conjugation. Six transconjugants were picked from LB + Rif (100 mg l^{-1}) + kanamycin (25 mg l^{-1}) agar plates, and disruption of the *aggA* locus was verified by PCR amplification with primers AGG183F and AGG1122R (5′-GTAGGCATTCCATGCCAGAT-3′).

Complementation of the *aggA* insertional mutation. *S. oneidensis* AGGA1, a COAG mutant with an *aggA*::pKNOCK-Km^r insertional mutation, was complemented with the pCM62 cloning vector (Marx & Lidstrom, 2001) expressing the *aggA* gene. Briefly, the complete *aggA* fragment was amplified from *S. oneidensis* COAG with primers AGGf (5′-TTTGGATCCTTTTAAGGGCTAAGGACACA-GC-3′) and AGGr (5′-GCGAATTCGTATGGAGCAACCCGCTAAA-3′). Both primers were provided with the recognition sequence for *Bam*HI and *Eco*RI. The *aggA* PCR fragment was ligated (sticky end) into the pCM62 vector according to standard procedures (Sambrook & Russell, 2001). The resulting vector, pCM62, with a 1510 bp *Bam*HI–*Eco*RI DNA fragment containing the *aggA* gene from *S. oneidensis* COAG, was designated pAG11. This construct was then transformed into *E. coli* S17-1/λ_{pir} and subsequently transferred into *S. oneidensis* AGGA1 by conjugation. Six transconjugants were picked from LB + Rif (100 mg l^{-1}) + kanamycin (25 mg l^{-1}) + tetracycline (12·5 mg l^{-1}) + X-Gal (40 μg ml^{-1}) + IPTG (1 mM) agar plates, and complementation with *aggA* was verified by PCR amplification with primers AGGf and AGGr. The results of gel electrophoresis of the PCR products are shown in Supplementary Fig. S1. Expression of *aggA* in the complemented AGGA1(pAG11) was verified by RT-PCR of extracted mRNA with internal *aggA* primers AGGA617F (5′-GGGCTAATGCGAATGTGATT-3′) and AGGA951R (5′-ACCACCTACGTTTTGGCTTG-3′) according to the manufacturer's instructions (Qiagen) and standard procedures (Sambrook & Russell, 2001).

Determination of aggregation index. Determination of the aggregation index (AI) was done according to Malik & Kakii (2003). Briefly, 10 ml of an *S. oneidensis* culture in M9 medium was rigorously vortexed to destroy aggregates, and the OD_{total} of this suspension was determined. Another 10 ml of the culture was centrifuged, without vortex treatment, for 2 min at 650 ***g***, providing $OD_{supernatant}$.

AI was defined as $(OD_{total} - OD_{supernatant})/OD_{total}$ (Equation 1).

Enzymic treatment of the aggregates. *S. oneidensis* COAG was grown in culture flasks containing 50 ml LB + 100 mg Rif l^{-1}. Enzymic treatments were repeated in triplicate. All enzymes except alcalase were added in a 1 : 250 (w/w) enzyme : cell dry weight ratio, and incubation with the aggregates was at 37 °C for 3 h with shaking (150 r.p.m.) at neutral pH. Alcalase was added at a concentration of 0·06 AU l^{-1}, according to the manufacturer's instructions (ORFFA Health and Nutrition, Londerzeel, Belgium). As a control, a treatment with an inactive proteolytic enzyme, pepsin at neutral pH, was carried out under identical incubation conditions.

Flow-cell experiments. Biofilms were established in 1/10 LB medium, maintaining the original NaCl concentration of 5 g l^{-1}, at

25 °C on a glass carrier (glass flow-through tubes, internal diameter 3 mm, external diameter 4 mm), using a twelve-channel autoclaved flow-cell system. In the case of the complemented AGGA1(pAG11) mutant, X-Gal (40 mg l^{-1}) and IPTG (1 mM) were added to the medium. The tubing consisted of marprene tubing (Watson-Marlow, Brussels, Belgium) in the pump head and Masterflex 96400-16 silicone tubing (Cole-Parmer Instrument Co. Ltd, London, UK) in the flow-cell system. The flow cell was inoculated with 1 ml of an overnight LB culture at OD_{610} 1·5. One hour of stagnation was applied to allow initial adhesion of the bacterial cells onto the glass. After initial attachment of the cells, the medium was pumped through the flow channels at a rate of 0·7 ml min^{-1} using a peristaltic pump (Watson-Marlow). The biofilm was sampled in the early development stage (6 h) and as a mature biofilm (50 h). The glass biofilm carrier was homogenized and the attached biofilm was suspended in 0·85 % saline. A dilution series was plated on LB agar plates with appropriate antibiotic selections, and c.f.u was calculated per square millimetre of carrier material. Every biofilm sample was obtained from an independent experiment in triplicate.

Microscopy of the aggregates. Aggregates were analysed by standard phase-contrast microscopy on a Zeiss Axioskop II microscope. The microscope was equipped with a Peltier cooled single-chip digital colour charge-coupled device (CCD) camera (Hamamatsu) connected to a PC to obtain digital images.

Microarray experimental design. *S. oneidensis* COAG cultures were set up in triplicate ($n=3$) in 1000 ml culture flasks with 150 ml LB broth, and aerobic conditions were maintained by shaking at 100 r.p.m. The transcriptional profile of the COAG cells in these cultures was compared to that of *S. oneidensis* MR-1, grown under identical conditions, at several time-points. There were four sampling events, at intervals of at least 2 h. The first samples were taken in the early exponential phase (OD 0·5) from both *S. oneidensis* MR-1 and COAG cultures, and this sampling event was termed t_0. Consecutive samples were taken at 2, 4 and 8 h after t_0, and referred to as sampling events t_2, t_4 and t_8, respectively. Gene expression analysis was performed using three independent microarray experiments with fluorescent dye reversal, each slide containing two replicate arrays, and at four sample-taking events, yielding a maximum of 48 data points per gene.

RNA isolation and preparation of fluorescein-labelled cDNA. Cells of both wild-type and COAG strains of *S. oneidensis* were harvested by centrifugation, and total cellular RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Ambion) to digest residual chromosomal DNA, and then purified with the Qiagen RNeasy Mini kit prior to spectrophotometric quantification at 260 and 280 nm. Fluorescein-labelled cDNA copies of total cellular RNA extracted from wild-type and mutant cells were prepared essentially as described previously (Thompson *et al.*, 2002). Two sets of duplicate reactions were carried out in which the fluorescent dyes were reversed during cDNA synthesis to minimize gene-specific dye effects. The labelled cDNA probe was purified and concentrated as described previously (Thompson *et al.*, 2002).

Microarray hybridization and scanning. The PCR product-based whole-genome ORF arrays for *S. oneidensis* MR-1 were constructed as described previously (Gao *et al.*, 2004).

Quantitative analysis of hybridization intensities and normalization. TIFF scanned images (16-bit) were analysed with the software package ImaGene 5.5 (Biodiscovery Inc., Los Angeles, CA) to determine spot intensities. Noise and spots of poor signal quality were removed from the data matrix (Hegde *et al.*, 2000). Data in this output were normalized (LOWESS) with the software package Genespring 6.0 (Silicon Genetics, Redwood City, CA). Data were subjected to two filters: a filter on confidence, selecting only significant differences between data points of wild-type and mutant as determined by a Student's t test ($P \leqslant 0{\cdot}05$), and a filter selecting only gene expression values with a greater than twofold ratio between the two fluorescent signals (Schena *et al.*, 1996).

Two-dimensional (2D) PAGE of membrane-enriched preparations of *S. oneidensis*. In order to generate membrane-enriched preparations of *S. oneidensis*, pellets of MR-1 and COAG, harvested from an aerobic LB culture in the mid-exponential growth phase, were sonicated for 3×30 s in carbonate buffer on ice (Molloy *et al.*, 1998). The samples were diluted and stirred on ice for an additional 3 h. Membranes were collected by ultracentrifugation at 4 °C. Pellets were resuspended in water and recentrifuged at 4 °C. This washing step was repeated two additional times. The final pellet was resuspended in detergent-containing solubilization buffer (0·1 % Triton X-100) and treated by sonication on ice to enhance solubilization. Insoluble particles were then removed by centrifugation, and the cleared supernatant was treated with tributyl phosphine (TBP) and iodoacetamide before loading of the samples onto high-resolution non-equilibrium pH gradient electrophoresis (NEPHGE) first-dimension gels. Total protein (120 μg per gel, as determined by a protein assay using BSA as reference) was used for triplicate gels of each sample. For the second dimension, glass-plate-immobilized 15 % SDS-PAGE gels (20×30 cm) were used. After electrophoresis, the gels were stained with SYPRO Ruby (Invitrogen).

Image acquisition and analysis of 2D gels. SYPRO Ruby-stained gels were scanned using a Fuji FLA-3000 fluorescence scanner (Raytest, Straubenhardt, Germany). Pictures were imported into the 2D evaluation software Melanie 3 (Genebio, Geneva, Switzerland), matched spots were analysed for significant intensity differences, and COAG upregulated (Student's t test value $\geqslant 0{\cdot}95$) spots were selected.

Protein identification by mass spectrometry. Selected protein spots were picked using an Ettan spot picker robot (Amersham Biosciences) and digested with trypsin. For desalting and matrix crystallization, a GYROS SP1 micro CD system (GYROS AB, Uppsala, Sweden) was used. Mass fingerprints were used to search the NCBI database using MASCOT (Matrix Science Ltd, London, UK) and identify the proteins.

RESULTS

Characterization of aggregate formation by *S. oneidensis* COAG

COAG is a mutant of *S. oneidensis* MR-1 and was obtained from a rifampicin (Rif) enrichment culture. To investigate hyper-aggregating properties, COAG cells were harvested from LB broth and inoculated in M9 mineral medium with 100 mg Rif l^{-1} at an inoculum concentration of 0·1 % (v/v). The aggregation index (AI, see Equation 1) in M9 was determined every hour and compared to the growth phase (Fig. 1a). The AI represents the fraction of cells associated in aggregates. Aggregates started to form in the early exponential phase, after which the aggregate size steadily increased, and aggregates remained present in the late stationary phase. This was confirmed by microscopic and visual examination. The AI of the *S. oneidensis* MR-1 wild-type was determined and was found to be below 0·01 for more than 24 h. The following proteolytic enzymes were added to a stationary-phase COAG culture in LB broth and lowered the AI

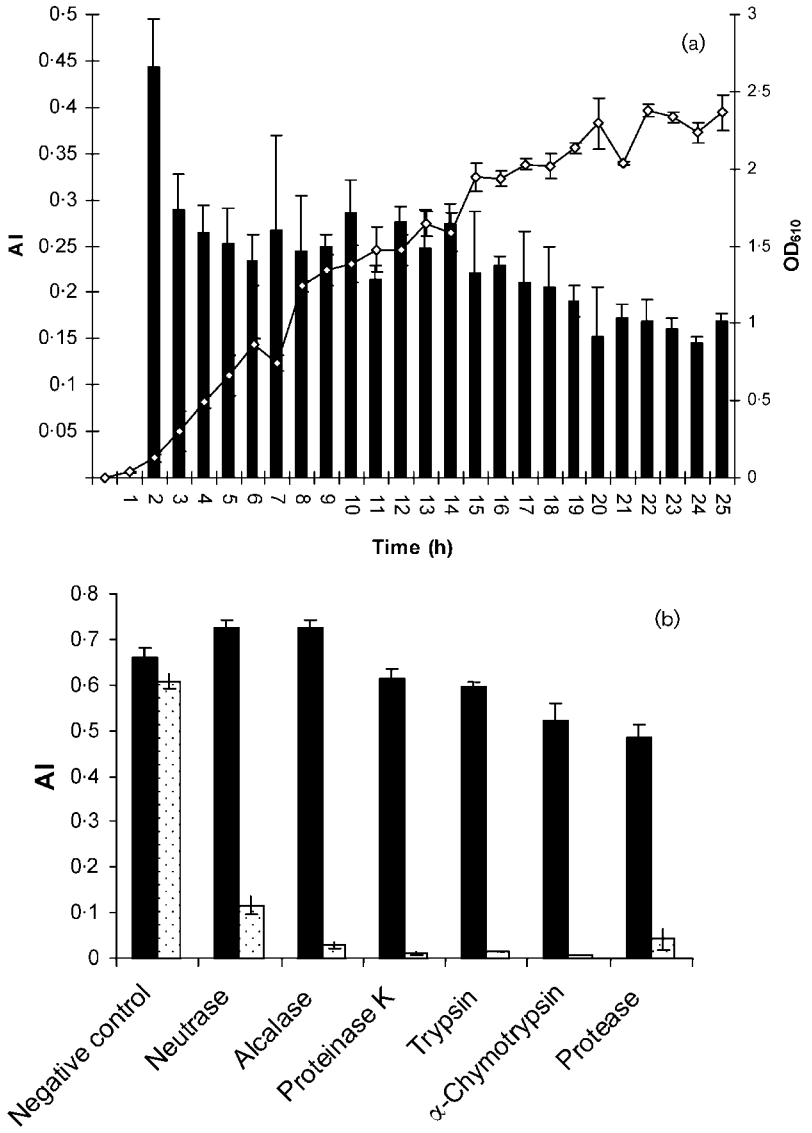

Fig. 1. (a) Aggregation index (AI, black bars) in an *S. oneidensis* COAG culture as a function of time; the OD_{610} of the culture (◇) was also measured at every sampling. All measurements were done independently and in triplicate. The AI of MR-1 wild-type did not exceed 0·01 over 24 h. (b) Effect of digestion by different enzyme preparations on the stability of *S. oneidensis* COAG aggregates. Conditions appropriate to each enzyme were employed. Inactive pepsin served as a negative control. Black bars, AI before treatment; grey bars, AI after treatment.

significantly (Fig. 1b): proteinase K, of the serine proteases α-chymotrypsin and trypsin, neutrase, alcalase, and a commercial protease mix. A phase-contrast image of COAG aggregates is shown in Fig. 2.

Microarray and 2D PAGE analysis of COAG differential outer-membrane expression compared to MR-1

By proteomics analysis, outer-membrane proteins were screened for their possible correlation to the auto-aggregating phenotype. In order to examine the altered expression of outer-membrane proteins in COAG, membrane-enriched cell preparations were analysed by proteomics and the protein pattern was compared to that of MR-1. Sixteen protein spots were found to be induced in COAG, of which 11 could be identified. In parallel, a DNA microarray with the complete genome of *S. oneidensis* MR-1 (4758 predicted protein-encoding ORFs) (Gao *et al.*, 2004) was used to monitor the altered gene expression of COAG with respect to MR-1. For each condition, a total of six independent hybridizations were performed, including three biological replicate experiments with fluorescent-dye reversal. Following spot intensity quantification and data normalization, 249 genes showed significant upregulation in COAG grown aerobically in LB broth, in at least one sampling event (mean fold change $\geqslant 2{\cdot}0$ and $P \leqslant 0{\cdot}05$). An overview of the 11 identified protein spots and their relative spot intensities, and a complete list of all the genes that showed significant upregulation in COAG in at least one sampling event, are shown in Supplementary Tables S1 and S2, respectively.

Only upregulated outer-membrane proteins for which the increased expression of the corresponding genes was confirmed by microarray analysis were considered in this study. The gene encoding the agglutination protein AggA, part of the putative RTX toxin operon, was overexpressed in COAG, and this was confirmed by 2D PAGE, in which induction of

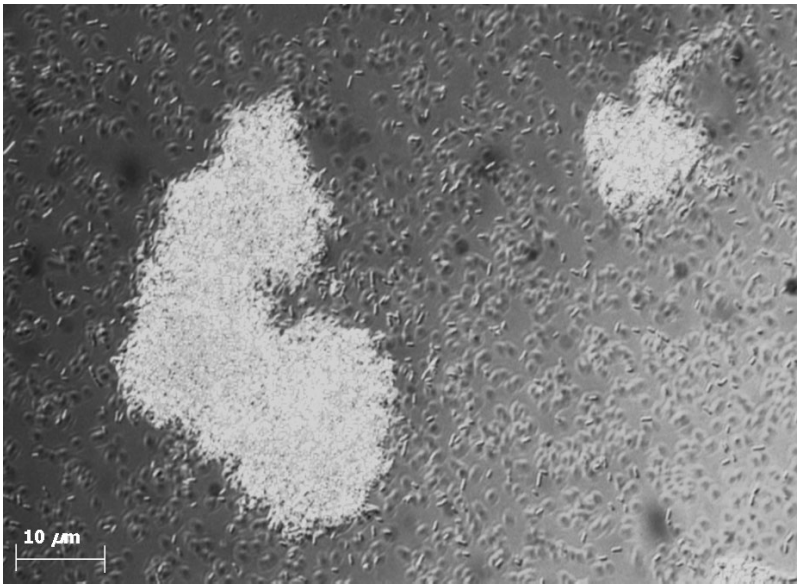

Fig. 2. *S. oneidensis* COAG aggregates visualized by phase-contrast microscopy, surrounded by suspended cells.

the three monomers of this protein in COAG was observed (Tables 1 and 2). The transcription study revealed that the other genes in the RTX operon were significantly upregulated in COAG as well. The SO4317 gene, encoding a putative RTX toxin protein, was significantly overexpressed at all sampling time-points. The proposed components of the secretion system for this putative RTX protein, consisting of *rtxB* (SO4318), encoding an ATP-binding protein in the inner membrane, SO4319, encoding an HlyD-family membrane-fusion protein, and *aggA* (SO4320), encoding the outer-membrane component of the secretory system, were all found to be upregulated in the auto-aggregating mutant (Table 1). The expression levels of the outer-membrane component *aggA* steadily increased at consecutive sampling time-points. The upregulation of *rtxB* and SO4319 was found to be significant (mean fold-change $\geqslant 2$ and $P \leqslant 0{\cdot}05$) at the earlier growth stages (t_0 and t_2).

The outer-membrane protein precursor MtrB, involved in the incorporation of several cytochromes, and possibly also other proteins, into the *S. oneidensis* MR-1 outer membrane, was found by 2D PAGE to be upregulated in COAG (Table 2), and the transcription of *mtrB* was found to be increased up to 4·3-fold (Table 1).

Analysis of the *aggA* mutant strain

To establish the role of AggA in hyper-aggregation and increased biofilm formation, the *aggA* gene was inactivated in COAG by integrative disruption with the suicide plasmid pKNOCK-Km^r. Disruption of the *aggA* locus in six transconjugants was verified by PCR amplification with primers AGG183F and AGG1122R. A product of approximately 940 bp was amplified from the parental COAG DNA, whereas from all the transconjugant *aggA*::pKNOCK-Km^r DNA, a 3·3 kb product was amplified, confirming the integration of the plasmid into the *aggA* gene. Supplementary Figure S1 illustrates the agarose gel loaded with the different PCR products. The aggregating property of the *S. oneidensis* COAG transconjugants with *aggA* integrative

Table 1. Genes from the RTX operon and genes encoding an outer-membrane protein that show significant overexpression in COAG aggregates compared to the MR-1 wild-type in at least one of the sampled growth stages (t_0, t_2, t_4 and t_8)

Fold changes are represented as mean ± standard deviation. Significant fold-changes in expression (fold-change $\geqslant 2{\cdot}0$ and $P \leqslant 0{\cdot}05$) are shown in bold type. SO no., the *S. oneidensis* gene locus number (according to the TIGR genomic database).

Gene symbol	SO no.	Gene product	GOAG versus MR-1 fold-change at:			
			t_0	t_2	t_4	t_8
mtrB	1776	Outer-membrane protein precursor MtrB	**4·31 ± 0·45**	1·66 ± 0·69	1·80 ± 1·20	1·72 ± 1·42
–	4317	RTX toxin, putative	**2·86 ± 0·78**	**2·60 ± 0·66**	**2·86 ± 0·85**	**4·72 ± 1·59**
rtxB	4318	Toxin secretion ATP-binding protein	1·75 ± 0·51	**2·57 ± 0·63**	1·41 ± 0·52	1·25 ± 0·29
–	4319	HlyD-family secretion protein	**2·01 ± 0·54**	1·67 ± 0·51	1·31 ± 0·53	1·61 ± 0·83
aggA	4320	Agglutination protein	1·18 ± 0·43	1·31 ± 0·55	1·41 ± 0·42	**2·97 ± 1·38**

Table 2. Relevant spot intensities of three replicate 2D PAGE gels for MR-1 and COAG

The spot volume as a percentage of the total volume of all spots is given for both MR-1 and COAG, and also for each gel. Upregulated COAG (Student's *t* test value $\geqslant$0·95) spots were selected. In the case of the trimeric agglutination protein AggA, relative spot intensities for each monomer are given.

Identified protein	Monomer no.	Spot volume					
		MR-1			COAG		
		Gel no. 1	Gel no. 2	Gel no. 3	Gel no. 1	Gel no. 2	Gel no. 3
Agglutination protein (trimeric)	1	0·105	0·120	0·130	0·165	0·185	0·210
	2	0·240	0·250	0·255	0·360	0·415	0·465
	3	0·280	0·290	0·300	0·500	0·610	0·720
Outer-membrane protein precursor MtrB	–	0·080	0·085	0·095	0·154	0·165	0·185

disruption was analysed by microscopy and the AI was determined for all of them. For all transconjugants, the hyper-aggregating property was completely lost, and this was due to insertional mutation of *aggA* in COAG. The AI of the *aggA* insertion mutant was not significantly different from that of the MR-1 wild-type. From these results, it follows that the AggA agglutination protein plays a central role in the aggregation by COAG.

Biofilm formation

COAG was able to form a dense biofilm on a glass carrier within 6 h, and its biofilm-forming capacity was considerably higher than that of MR-1. Even a mature MR-1 biofilm did not reach this density (Fig. 3). After 50 h, COAG biofilms consisted of $(1{\cdot}5 \pm 0{\cdot}1) \times 10^6$ c.f.u. mm^{-2}, whereas MR-1 biofilms only reached a cell density of $(4{\cdot}4 \pm 0{\cdot}6) \times 10^4$ c.f.u. mm^{-2}. Biofilm formation was analysed for one of the *S. oneidensis* COAG transconjugants with *aggA* integrative disruption, designated AGGA1, in a flow-cell set-up with three independent replicates. Due to an insertional mutation in the *aggA* locus, AGGA1 had lost the increased biofilm-forming properties of COAG. In the early biofilm development phase (6 h), in which initial cell-to-surface attachment takes place, AGGA1 biofilm density was significantly lower than that of both MR-1 and COAG. After 50 h, the cell density in AGGA1 biofilms had reached that of MR-1 biofilms. By complementation of the insertional mutation in AGGA1 with the cloning vector pCM62 expressing the *aggA* gene from COAG, the strong inhibition of initial cell-to-surface attachment could be nullified. The complemented AGGA1(pAG11) mutant exhibited a biofilm cell density of $(1{\cdot}3 \pm 0{\cdot}03) \times 10^4$ c.f.u. mm^{-2} within 6 h, and this corresponded well to the biofilm-forming capacity of MR-1.

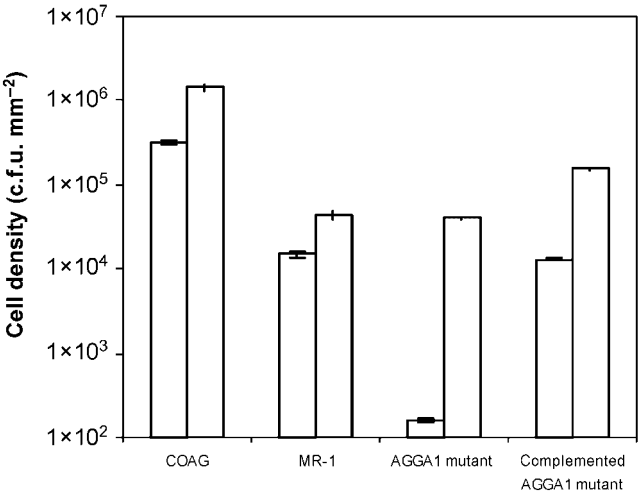

Fig. 3. Biofilm formation of *S. oneidensis* MR-1, COAG, AGGA1 and the complemented AGGA1(pAG11) strains on a glass carrier in a flow-cell set-up. Every biofilm sample was obtained from an independent experiment in triplicate. In some cases, the standard deviation (error bars) was too small to be visible on the figure. White bars, sampling at 6 h; grey bars, sampling at 50 h.

DISCUSSION

Shewanella oneidensis COAG was isolated from a rifampicin enrichment culture and is resistant to 100 mg Rif l^{-1}. Phenotypically, this strain is characterized by auto-aggregation and by an increased biofilm-forming capacity. Other Rif^R mutants isolated from *S. oneidensis* MR-1 Rif enrichment cultures lacked the phenotypic properties of COAG. Rif^R mutants usually carry single-nucleotide mutations in the beta subunit of the prokaryotic RNA polymerase. This can generate pleiotropic phenotypes, because some of the mutations may overlap with the ppGpp-binding site of this subunit, thus influencing a considerable number of functions (Artsimovitch *et al.*, 2004). As a consequence, the hyper-aggregating phenotype could be accompanied by changes in a large collection of genes, which may or may not be related to the auto-aggregating property. Therefore, the microarray and proteomics analyses have been focused on altered expression of outer-membrane proteins and proteins directly associated with them, in order to identify candidate surface proteins that could be related to the hyper-aggregation and increased biofilm formation of *S. oneidensis* COAG.

The role of surface-located proteins in the process of COAG auto-aggregation, and in the formation of organized multicellular structures in general, was confirmed by different proteolytic treatments of the aggregates. All of the proteolytic enzymes tested resulted in deflocculation within 3 h of incubation. In a similar test, Malik *et al.* (2003) have studied the effect of the addition of trypsin and actinase E on aggregate structure, and have found that protein structures mediate co-aggregation between *Acinetobacter johnsonii* and *Microbacterium esteraromaticum*. To further substantiate the involvement of surface-located proteins in COAG aggregation, we investigated differential expression of cell membrane proteins by 2D PAGE of membrane-enriched preparations of COAG and MR-1. The results from the proteomics analysis were compared to those of COAG and MR-1 differential gene expression observed by transcriptional analysis with DNA microarrays. The predicted transport component of a putative RTX toxin operon, agglutination protein AggA, was found to be upregulated in COAG. DNA microarray analysis confirmed that all genes from this operon were upregulated in COAG. The role of other aggregation factors, such as type IV-pili, which have been suggested by Heidelberg *et al.* (2002) to be important during *S. oneidensis* attachment to solid surfaces, could not be established in our study, since no significant difference was observed with regard to these structures in the parent and mutant (results not shown). In *Pseudomonas fluorescens*, a similar ATP-binding cassette (ABC) transporter system for the secretion of a large protein has been reported to be involved in biofilm formation (Hinsa *et al.*, 2003). For the latter organism, the putative *lapEBC*-encoded ABC transporter participates in the secretion of the large protein LapA (large adhesion protein A), and this system is required for transition from reversible to irreversible attachment during biofilm formation. LapB is the predicted inner-membrane protein of 74 kDa, analogous to the predicted 79 kDa RtxB ATP-binding protein of *S. oneidensis*. RtxB is proposed to be the inner-membrane-anchored ATPase of an ABC transporter involved in export of the putative RTX toxin. In *P. fluorescens*, LapC has been described as an HlyD-family membrane-fusion protein, part of the proposed ABC transporter, and in *S. oneidensis*, the SO4319 gene product is predicted to be an HlyD-family secretion protein as well. Finally, the predicted *P. fluorescens* outer-membrane protein LapE, with sequence similarity to the *P. putida* AggA adhesin, has been suggested to promote stable adhesion of *P. fluorescens*, possibly in association with LapA. This is comparable to our observation that overexpression of the *S. oneidensis* AggA outer-membrane protein was associated with the altered cell-adhesion properties of COAG.

To further establish the importance of AggA in COAG aggregation and increased biofilm formation, the effect of insertional inactivation of the *aggA* gene on the phenotypic properties under aerobic growth conditions was investigated. The *aggA* mutation reversed the AGGA1 phenotype to that of the original MR-1 strain, with loss of hyper-aggregation and the reacquisition of a biofilm-forming capability that was not significantly higher than that of the MR-1 wild-type. Interestingly, the initial cell density was significantly lower in AGGA1 biofilms than in both MR-1 and COAG biofilms. This indicates the importance of AggA in the initial phase of biofilm formation, cell-to-surface attachment. A short period of exposure of COAG to proteolytic enzymes resulted in digestion of the outer-membrane agglutination protein, and this also resulted in loss of aggregation. These experiments indicate the strong involvement of the upregulated *aggA* gene and the associated putative RTX operon in COAG hyper-aggregation, and the necessity of the AggA outer-membrane protein to the COAG phenotype with its increased adhesive properties. These findings were further confirmed by complementation of the AGGA1 mutant with pCM62 expressing the *aggA* gene from COAG. In this cloning vector, *aggA* was under the control of a *lacZ* promoter, and as such could not be upregulated in the same manner as in COAG, but the inhibitory effect of the *aggA* null allele in mutant strain AGGA1 on the initial phase of biofilm formation could nevertheless be eliminated by this complementation. Biofilm-forming capacity was restored to the level of the MR-1 wild-type. Thus, it was shown that auto-aggregation by *S. oneidensis* COAG, coupled to increased biofilm formation at the stages of both initial surface attachment and biofilm maturation, requires upregulation of the AggA agglutination protein. The AggA outer-membrane protein controls cell-to-surface attachment in *S. oneidensis*, since insertional mutation of the *aggA* gene strongly inhibited the initial surface colonization, but biofilm formation could be restored to the level of the MR-1 wild-type by complementation with a cloning vector expressing the *aggA* gene under the control of a *lacZ* promoter. The auto-aggregating phenotype could not be restored by this complementation, and required AggA overexpression.

A recent study has suggested the involvement of MSHA, the mannose-sensitive haemagglutinin type IV pilus, during the initial adhesion of *S. oneidensis* to abiotic surfaces, observed after 1 and 4 h (Thormann *et al.*, 2004). Also, disruptions of motility genes (*flhB*, *fliK* and *pomA*) and of a gene involved in pilus retraction (*pilT*) affect biofilm formation and the progression of biofilm development into a pronounced three-dimensional architecture. Although none of these elements was found to be upregulated in *S. oneidensis* COAG, it is possible that the three-dimensional development of COAG biofilms is under the influence of several of these factors. Our data suggest, however, that during initial attachment, other outer-membrane factors are involved as well. Although initial attachment by *S. oneidensis* COAG was found to be strongly inhibited by *aggA* disruption, the mature biofilm nevertheless developed with a decreased density. It is possible that other surface structures described in the study of Thormann *et al.* (2004) may have substituted for part of the role of AggA in this process.

The characterization of the adhesive properties of COAG provided new insights into cell-to-cell and cell-to-surface attachment by *S. oneidensis*. The importance of AggA in

biofilm formation by the *S. oneidensis* MR-1 wild-type has, to our knowledge, not been described in the literature. However, it was shown in our research that AggA is a necessary factor for the increased surface and cell adhesion of the hyper-aggregating COAG mutant, and that cell-to-surface attachment can be inhibited and restored by, respectively, disruption and complementation of *aggA*. Similarly, the MSCRAMM (microbial surface components that recognize adhesive matrix molecules) adhesin LapA is a cell-surface protein that enables *P. fluorescens* to attach to plastic, glass and quartz sand (Hinsa *et al.*, 2003). In *P. putida*, the *lapA* gene is of importance during attachment to plastics, glass and corn seeds (Espinosa-Urgel *et al.*, 2000). The similarities between the function and ABC-transporter structure of the operons containing *aggA* and *lapA*, coupled to the necessity of AggA to the increased adhesive properties of COAG, the *aggA* upregulation in COAG, and the control that can be exerted over the initial stages of biofilm formation by disruption and complementation of *aggA*, strongly suggest that the MR-1 wild-type also partially depends on *aggA*, and possibly the putative RTX operon, for attachment to surfaces and biofilm formation. Further research is required to formulate any definite statements about MR-1, although recent research has shown that AggA is over-expressed in biofilms of the MR-1 wild-type compared to MR-1 planktonic cells (De Vriendt *et al.*, 2005).

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