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Proteomic Analysis of *Acinetobacter baumannii* in Biofilm and Planktonic Growth Mode

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Recently, multidrug-resistant clinical isolates of *Acinetobacter baumannii* have been found to have a high capacity to form biofilm. It is well known that bacterial cells within biofilms are highly resistant to antibiotics, UV light, acid exposure, dehydration, and phagocytosis in comparison to their planktonic counterparts, which suggests that the cells in a biofilm have altered metabolic activity. To determine which proteins are up-regulated in *A. baumannii* biofilm cells, we performed a proteomic analysis. A clinical isolate of *A. baumannii* 1656-2, which was characterized to have a high biofilm forming ability, was cultivated under biofilm and planktonic conditions. Outer membrane enriched *A. baumannii* 1656-2 proteins were separated by two-dimensional (2-D) gel electrophoresis and the differentially expressed proteins were identified by MALDI-TOF mass spectrometry. The proteins up-regulated or expressed only in biofilm cells of *A. baumannii* are categorized as follows: (i) proteins processing environmental information such as the outer membrane receptor protein involved in mostly Fe transport, a sensor histidine kinase/response regulator, and diguanylate cyclase (PAS-GGEDF-EAL domain); (ii) proteins involved in metabolism such as NAD-linked malate dehydrogenase, nucleoside-diphosphate sugar epimerase, putative GalE, ProFAR isomerase, and *N*-acetylmuramoyl-L-alanine amidase; (iii) bacterial antibiotic resistance related proteins; and (iv) proteins related to gene repair such as exodeoxyribonuclease III and GidA. This proteomic analysis provides a fundamental platform for further studies to reveal the role of biofilm in the persistence and tolerance of *A. baumannii*.

***Keywords*:** proteomics, biofilm, planktonic cells, 2-dimensional gel electrophoresis, *A. baumannii*

Biofilms are structured communities of bacteria encapsulated within a polymeric matrix called either extracellular polymeric substance or exopolysaccharide (EPS) (Monds and O'Toole, 2009). In medical settings, there are two major types of biofilm-associated infection: device-related infections and non-device-related chronic inflammatory conditions including cystic fibrosis, chronic obstructive pulmonary disease, otitis media, and prostitis (Ehrlich *et al*., 2004). Bacterial biofilm infections are particularly problematic because biofilm bacteria are generally much more tolerant to antibiotics than their planktonic (free swimming) counterparts. The mechanism of bacterial biofilm resistance to antibiotics is still under investigation but several explanations have been raised so far: (i) the biofilm EPS may act as an adsorbent, reducing the amount of antibiotics; (ii) the biofilm structure act as penetration barriers; (iii) the biofilm bacteria up-regulate efflux pumps, decreasing intracellular antibiotic concentration; and (iv) the presence of a non-dividing, dormant bacterial subpopulation which is protected from antibiotic-induced killing (Ehrlich *et al*., 2004). Besides antibiotic tolerance, bacterial cells within biofilms are also highly resistant to UV light, metal toxicity, acid exposure, dehydration, and phagocytosis. The physiological and molecular basis of the increased tolerance of cells within biofilms still remains to be investigated.

Acinetobacter baumannii is a non-motile, non-fermentative Gram-negative bacterium that can be isolated from hospital environmental sources (Villegas and Hartstein, 2003). This bacterium causes various human infections including urinary tract infection, secondary meningitis, wound or burn infection, and pneumonia (Chen *et al*., 2001; Davis *et al*., 2005). The nosocomial infections caused by this bacterium have become a severe problem worldwide due to the magnitude of its antimicrobial resistance (Bergogne-Bérézin and Towner, 1996; Dijkshoorn *et al*., 2007). In addition, *A. baumannii* is known to be able to persist in dry conditions and after treatment with disinfectants (Bergogne-Bérézin and Towner, 1996; Wisplinghoff *et al*., 2007). A few studies have suggested that the ability to form biofilms may contribute to the unique survival or physiological properties of *A. baumannii*. Villers *et al.* (1998) showed that *A. baumannii* in biofilms is indicative of enhanced antibiotic resistance. Lee *et al.* (2008) showed that clinical isolates of multidrug-resistant (MDR) *A. baumannii* showed a high ability to form biofilm, which was positively associated with a capability to adhere to human bronchial epithelial cells. Recently, Loehfelm *et al*. (2008) characterized biofilm-associated proteins (Bap, 854 kDa) from *A. baumannii* and Tomaras *et al.* (2008) characterized a two-component regulatory system from *A. bau-*

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mannii ATCC 19606^T that controls biofilm formation and cellular morphology. At present, however, very little is known about the underlying mechanism of biofilm formation in *A. baumannii* and the exact role of biofilm in the persistence and antibiotic tolerance of *A. baumannii*.

To understand the underlying mechanism of the persistence and tolerance of biofilm cells of *A. baumannii*, we investigated the differentially expressed proteins of *A. baumannii* cultivated under biofilm and planktonic conditions. For the proteomic study, a clinical isolate of *A. baumannii* 1656-2, which was characterized to have a high biofilm forming ability in our previous study (Lee *et al*., 2008), was cultivated under biofilm and planktonic conditions. Outer membrane enriched *A. baumannii* 1656-2 proteins were prepared and separated by two-dimensional (2-D) gel electrophoresis. The differentially expressed proteins of *A. baumannii* 1656-2 were then identified by MALDI-TOF/TOF analysis. The recent completion of full genome sequences of *A. baumannii* strains (Smith *et al*., 2007; Adams *et al*., 2008; Iacono *et al*., 2008) allowed us to identify *A. baumannii* proteins using the public database.

Materials and Methods

Bacterial strain and growth conditions

A. baumannii clinical isolate 1656-2 was used throughout this study. This organism was isolated from the sputum of a hospitalized patient and characterized as a MDR strain with a high ability to form biofilm in our previous study (Lee *et al*., 2008). For biofilm cultures, *A. baumannii* was grown in 100 mm polystyrene petri dishes containing 20 ml of TSB (Tryptic Soy Broth) (Difco, USA) medium at 37°C for 24 h without shaking. The supernatant was then removed and the plates were washed in 10 mM HEPES buffer (pH 7.4). Biofilm cells were detached by scraping, then resuspended in 10 mM HEPES buffer. The cells were pelleted by centrifugation at approximately 5,000×g for 5 min and washed with 10 mM HEPES. For planktonic cultures, *A. baumannii* was grown in 500 ml Erlenmeyer flasks containing 100 ml of TSB medium at 37°C for 24 h in a rotary shaker (180 rpm). Planktonic cells were pelleted and washed as described for the biofilm cultures above.

Extraction of the outer membrane enriched proteins

The outer membrane enriched proteins were prepared as described by Rapp *et al.* (1986) with slight modification. The pellets from the *A. baumannii* grown in biofilm and planktonic cultures were suspended in 5 ml of 10 mM HEPES buffer (pH 7.4), extracted by sonication (Sonifer 450, Branson Ultrasonics, USA) for 5 min in an ice bath and centrifuged at 1,700×g for 20 min. The supernatants obtained were ultracentrifuged at 100,000×g for 1 h at 4°C (Sorvall OTD-75B, USA). The pellets were solubilized by treatment with 1 ml of 10 mM HEPES containing 2% sodium lauryl sarcosinate and incubated at room temperature for 30 min. After treatment, outer membrane enriched proteins were collected by ultracentrifugation at 100,000×g for 1 h at 4°C and the resulting pellet was solubilized in elution buffer (8 M urea, 4% CHAPS, 40 mM Tris, 0.5% ampolyte, 10 mM DTT). The total protein levels were determined using the Bio-Rad Protein Assay 20 reagent (Bio-Rad, USA).

2-D gel electrophoresis

The outer membrane enriched proteins were separated in the first dimension using broad range IPG strips (pH 3∼10) (Bio-Rad). One miligram of protein was loaded on each IPG strip. IEF was performed in a Protein IEF Cell (Rio-Rad) using a stepwise voltage gradient to 80 kVh. Strips were then equilibrated for 10 min in 50 mM Tris-HCl (pH 8.8), containing 6 M urea, 2% SDS, 30% glycerol, and 1% DTT, and then followed by another 10 min in the same solution to which 5% iodoacetamide was added instead of DTT. The strips were placed on top of 9∼16% gradient SDS-PAGE gels. After electrophoresis to separate the proteins in the second dimension, protein spots were visualized by Coomassie blue (GBB-G250, Bio-Rad) staining. Gel comparison analysis was using Image Master Platinum 5.0 software (GE Healthcare, Sweden). An analysis set was created to find the spots with a minimum 2-fold increase or decrease between the biofilm and planktonic samples.

Protein identification by MALDI-TOF/TOF mass spectrometry (MS)

For protein spot identification, gel slices of interest were excised from the 2-D gel and digested using in-gel tryptic digestion. Briefly, excised gel slices were washed with 50 mM ammonium bicarbonate buffer, pH 7.8 and acetonitrile (ACN) (6:4). Gel slices were then dehydrated by vacuum drying and rehydrated with 50 mM ammonium bicarbonate buffer containing 5 μl of 12.5 ng/μl sequencing-grade modified trypsin (Promega, USA) and incubated at 37°C for 12 h. Tryptic peptides were extracted and desalted from the gel matrix using the GELoader tip (Eppendorf, Germany) packed with resin solution, Poros R2 and Oligo R3 (PerSeptive Biosystems) (2:1) in 70% ACN. The column was equilibrated by adding 20 μl of 2% formic acid in 70% ACN. The peptide solution was added to the column and then the column was washed with 20 μl of 2% formic acid in 70% ACN. One microliter of a matrix solution, 10 mg/ml α-cyano-4-hydroxycinnamic acid (Aldrich, USA) in 2% formic acid and 70% ACN, was added and the eluted peptides and matrix mixture were directly spotted onto the MALDI plate (Opti-TOF^{TM}, Applied Biosystems). The peptide mass fingerprinting was analyzed by MALDI-TOF/TOF MS (4700 Proteomics Analyzer, Applied Biosystems, USA) and was used to search Swiss-Prot and NCBInr using MASCOT software (Matrix Science). A 95% confidence level threshold was used for MASCOT protein scores. The subcellular location of proteins identified was determined by PSORTb version 2.0 (http://www.psort.org/psortb/).

Reverse transcription (RT)-PCR

Total RNA was isolated from *A. baumannii* grown in biofilm and planktonic mode for 24 h with an RNeasy kit (QIAGEN, Germany) according to the manufacturer's instructions. The RT reaction was done using a First Strand cDNA Synthesis kit (Fermentas, USA). Briefly, 0.5 μg of total RNA and 1 μl of random hexamer primer were incubated at 65°C for 5 min, and then M-MuLV reverse transcriptase (40 units) was added and incubated at 25°C for 5

min, followed by 37°C for 60 min. The reaction was terminated by heating at 70°C for 5 min. The resulting cDNA was amplified by PCR with *Taq* polymerase (TaKaRa, Japan). The cDNA was normalized to the 16S rRNA gene. A specific primer set was used to detect bla_{PER-1} (forward primer; 5'-ATGAATGTCATTATAAAAGC-3' and reverse primer; 5'-AATTTGGGCTTAGGGCAAGAAA-3'), outer membrane receptor protein, mostly Fe transport coding gene (forward primer; 5'-ACTACAACCTTTATGGGTTGCG-3' and reverse primer; 5'-ACTTCATGCTCATACGAACCCA-3') and the 16S rRNA transcript levels (forward primer; 5'-TGGCTCA GATTGAACGCTGGCGGC-3' and reverse primer; 5'-TAC CTTGTTACGACTTCACCCCA-3'). PCR products were analyzed by agarose gel electrophoresis.

Results and Discussion

Proteins extracted from the planktonic and the biofilm cells were subjected to 2D electrophoresis. In Coomassie blue stained gels, 17 protein spots were found with a minimum two-fold change in expression level and numbered with a black arrowhead (Fig. 1). Of 17 spots, 11 were up-regulated and 6 were down-regulated in biofilm *A. baumannii*. Apart from these spots, 6 protein spots were found in either only planktonic or only biofilm cells and numbered with a white arrowhead (Fig. 1).

Proteins significantly up-regulated in biofilm cells

Among 11 protein spots which were up-regulated in the biofim cells, 8 proteins were identified by searching the *A. baumannii* database and 3 proteins were found to significantly match the database of other bacteria (Fig. 1 and Table 1).

NAD-linked malate dehydrogenase (MDH, spot 11) is an oxidoreductase which catalyzes the conversion of malate into oxaloacetate using NAD as a cofactor and plays an essential role in the tricarboxylic acid (TCA) cycle. In *E. coli*, MDH is known to be highly regulated to adapt to changing conditions such as aerobic and anaerobic cell growth and is also known to be involved in biofilm growth (Trémoulet *et al*., 2002).

Protein spot 15 was significantly matched with an outer membrane receptor protein involved in iron (Fe) transport (TonB-dependent). Iron is an important environmental signal that controls a large number of gene expression pathways, some of which code for important bacterial virulence factors (Rhodes *et al*., 2007). Under iron starvation conditions, aerobic bacteria synthesize and secrete highly ferric siderophores into the environment. The ferric siderophore complexes are transported into the cells by the Iron Regulated Outer Membrane Proteins system (IROMPs) which consists of one specific outer membrane receptor, one periplasmic protein, and several inner-membrane-associated proteins (i.e. the ExbB:ExbD:TonB complex) (Vallenet *et al*., 2008). A siderophore named acinetobactin has been identified in some clinical isolates of *A. baumannii* (Yamamoto *et al*., 1994; Vallenet *et al*., 2008) and a gene cluster of 18 genes involved in the biosynthesis and transport of acinetobactin has been identified in the *A. baumannii* ATCC 19606^T strain (Mihara *et al*., 2004). In the current study, one outer membrane iron receptor protein was more significantly up-regulated in biofilm (about 7.5 times more) than in planktonic culture and also transcription level of this protein coding gene was greater in the cells grown in biofilm compared to those in planktonic culture (Fig. 2A), suggesting an important role for iron in the biofilm formation of *A. baumannii*. Indeed, Tomaras *et al.* (2003) reported that biofilm formation of *A. baumannii* ATCC 19606^T was controlled by iron. Under iron-limiting conditions, a significant increase in biofilm was found in *A. baumannii* ATCC 19606^T when compared to that obtained with cells cultured under iron- rich conditions (Tomaras *et al*., 2003).

Two proteins (spots 19 and 22), PER-1 and aminoglycoside acetyltransferase (6') type I enzyme, were identified as

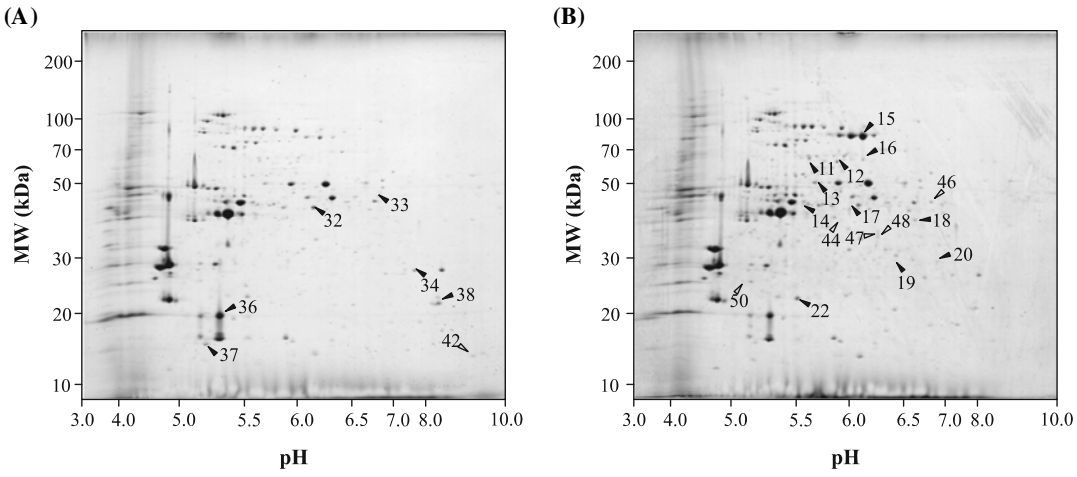

Fig. 1. 2-D gel electrophoresis patterns of *A. baumannii* from outer membrane enriched proteins. The proteins were separated in the first dimension by IEF (pH range 3~10) and in the second dimension by SDS-polyacrylamide gel electrophoresis. (A) Protein pattern in the planktonic culture. (B) Protein pattern in the biofilm culture. Spots indicated by a black arrowhead are proteins with a significantly increased level in each culture mode. White arrowheads indicate represented spots specific to each culture mode.

Table 1. Proteins with increased expression levels in biofilm *A. baumannii*, identified by MALDI-TOF/TOF MS

Spot no.	Protein	Average ratio	Mascot score	Accession no.	Organism	Calculated p*I*	Mol. wt.	% Coverage	Peptides matched	PSORTb
Matched proteins from the database of *A. baumannii*										
11	NAD-linked malate dehydrogenase	2.054	93	gi169797658	*A. baumannii* AYE	5.32	63,069	29	12	Unknown
13	Putative protein (DcaP-like)	2.009	140	gi126642784	*A. baumannii* ATCC 17978	5.79	44,818	44	14	Outer membrane
14	Hypothetical protein	2.369	62	gi184159021	*A. baumannii* ACICU	5.67	44,858	29	13	Outer membrane
15	Outer membrane receptor protein, mostly Fe transport	7.468	287	gi184156512	*A. baumannii* ACICU	5.90	78,008	51	28	Outer membrane
18	Nucleoside-diphosphate sugar epimerase	2.482	162	gi184156395	*A. baumannii* ACICU	5.96	37,496	49	18	unknown
19	beta-Lactamase PER-1	5.643	98	gi155674975	*A. baumannii*	5.90	33,478	29	12	Periplasmic
20	Exodeoxyribonuclease III	2.132	176	gi213159082	*A. baumannii* AB0057	6.21	31,815	52	15	Cytoplasmic
22	Aminoglycoside acetyltransferase (6') type I	2.042	124	gi114809968	*A. baumannii*	5.68	18,957	66	11	Cytoplasmic
Matched proteins from the database of other bacteria										
12	Putative transcriptional regulator	2.866	94	gi22299660	*Thermosynechococcus elongatus* BP-1	7.14	40,916	36	12	Cytoplasmic
16	aldo/keto Reductase	3.321	78	gi33860998	*Prochlorococcus marinus* subsp. pastoris str. CCMP1986	8.87	43,562	41	13	Unknown
17	Sensor histidine kinase/ response regulator	3.092	82	gi16125949	*Caulobacter crescentus* CB15	5.42	59,990	19	12	Cytoplasmic membrane

responsible for antibiotic resistance and were also highly induced during biofilm culture. PER-1 is a class A type extended-spectrum beta-lactamase (ESBL) which is responsible for the resistance of Gram-negative bacteria to extended-spectrum beta-lactam antibiotics. Aminoglycoside acetyltransferase (6') type I enzyme is known to catalyze the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the 6' amino group of the aminoglycoside, which is the predominant cause of bacterial resistance to aminoglycosides (Magnet and Blanchard, 2005). In our previous

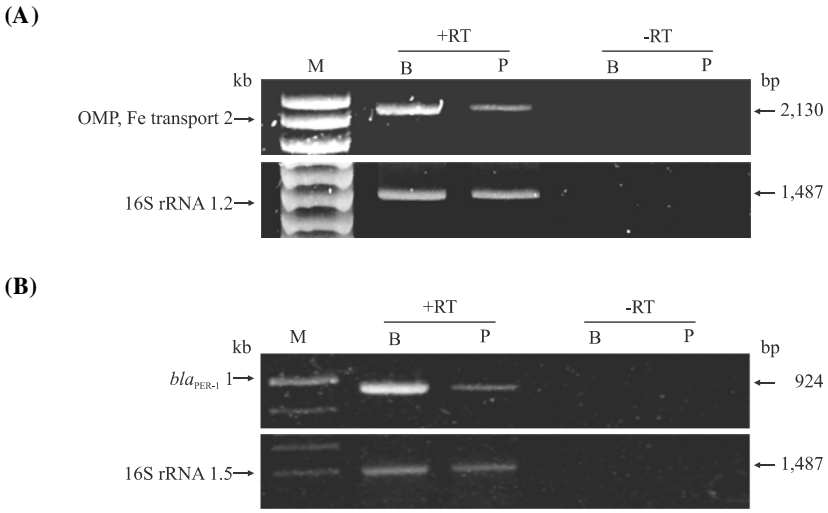

Fig. 2. Detection of outer membrane receptor protein, mostly Fe transport coding gene (A) and bla_{PER-1} (B) transcripts. RT-PCR from total RNA isolated from *A. baumannii* grown in biofilm (B) and in planktonic mode (P). 16S rRNA gene was used to normalize the cDNA. Lane +RT and –RT indicate with and without reverse transcriptase, lane M contains size marker, a size marker is indicated on the left, and the predicted lengths of the expected transcripts are indicated on the right.

Table 2. Proteins found only in biofilm *A. baumannii*, identified by MALDI-TOF/TOF MS

Spot no.	Protein	Mascot score	Accession no.	Organism	Calculated p*I*	Mol. wt.	% Coverage	Peptides matched	PSORTb
Matched proteins from the database of *A. baumannii*									
44	Putative UDP-galactose 4-epimerase (GalE-like)	78	gi169148594	*A. baumannii*	5.62	38,251	33	11	Cyto-plasmic
50	Phosphoribosylformimino-5-amino-imidazole carboxamide ribonucleotide (ProFAR) isomerase	61	gi126389130	*A. baumannii* ATCC 17978	4.96	26,181	52	9	Cyto-plasmic
Matched proteins from the database of other bacteria									
46	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	96	gi42561530	*Mycoplasma mycoides* subsp. mycoides SC str. PG1	7.96	70,982	31	17	Cyto-plasmic
47	Diguanylate cyclase with PAS/PAC sensor	72	gi94970507	*Acidobacteria bacterium* Ellin345	7.60	34,356	51	15	Cyto-plasmic
48	*N*-acetylmuramoyl-L-alanine amidase	71	gi37520091	*Gloeobacter violaceus* PCC 7421	9.73	64,209	33	12	Cyto-plasmic membrane

study, the presence of $bla_{\text{PER-1}}$ in *A. baumannii* was associated with its high biofilm forming ability and the transcription level of $bla_{\text{PER-1}}$ was positively correlated with the amount of biofilms formed by *A. baumannii* (Lee *et al*., 2008). In the current study, PER-1 was produced about 5.6 times more in biofilm cells than in planktonic cells. RT-PCR analysis showed that transcription level of the $bla_{\text{PER-1}}$ gene was greater in the cells grown in biofilm compared to those in planktonic culture (Fig. 2B). Based on these data, it is possible to speculate that the acquisition of the $bla_{\text{PER-1}}$ gene in *A. baumannii* may enhance its ability to form biofilm and suggests that biofilm bacteria may have a regulatory system for the efficient expression of the $bla_{\text{PER-1}}$ gene. The mechanism underlying the $bla_{\text{PER-1}}$ gene involvement in biofilm formation and the control of $bla_{\text{PER-1}}$ gene expression are currently under investigation.

The protein spot 20 was identified as exodeoxyribonuclase III, encoded by the *xth* gene of *A. baumannii*. Recently, Boles and Singh (2008) found that *Pseudomonas aeruginosa* within biofilms incur DNA damage due to endogenous oxidative stress. Souza *et al.* (2006) suggested that exodeoxyribonuclase III plays an important role in base excision repair (BER) and a DNA oxidative stress repair mechanism in *E. coli*. Therefore, exodeoxyribonuclase III may play a role in the repair of DNA damage incurred by endogenous oxidative stress in *A. baumannii* within biofilms.

In biofilm cells of *A. baumannii*, the signaling related proteins were up-regulated. Aldo/keto reductase (spot 16) is regulated by the general stress transcription factor, σ^{B} (Petersohn *et al*., 1999) in *Bacillus subtilis*, and plays an important role in adaptation to growth under acid conditions in *Helicobacter pylori* (Cornally *et al*., 2008). A sensor histidine kinase (spot 17) is part of a two-component signal transduction system (TCSTS) that is a ubiquitously distributed communication interface in bacteria. TCSTS allow adaptational responses to a huge variety of environmental stimuli. Recent studies have shown that biofilm formation is also controlled by TCSTS in many bacteria such as *E. coli* (Vidal *et al*., 1998; Dorel *et al*., 1999), *P. aeruginosa* (O'Toole *et al*., 2000), *Vibrio cholerae* (Yildiz *et al*., 2001), and *A. baumannii* (Tomaras *et al*., 2008). Tomaras *et al.* (2008) characterized a TCSTS from *A. baumannii* ATCC 19606^{T}, which

Table 3. Proteins with decreased expression levels in biofilm *A. baumannii,* identified by MALDI-TOF/TOF MS

Spot no.	Protein	Average ratio	Mascot score	Accession no.	Organism	Calculated p*I*	Mol. wt.	% Coverage	Peptides matched	PSORTb
Matched proteins from the database of *A. baumannii*										
32	Hypothetical protein	2.203	132	gi184159021	*A. baumannii* ACICU	5.67	44,858	30	14	Outer membrane
33	Outer membrane protein, OprE3	2.049	101	gi213155602	*A. baumannii* AB0057	6.09	47,833	35	12	Outer membrane
34	Putative signal peptide	2.234	130	gi126642491	*A. baumannii* ATCC 17978	6.75	24,389	64	13	Unknown
36	Putative outer membrane, protein W	2.016	137	gi213155740	*A. baumannii* AB0057	5.56	21,213	63	11	Outer membrane
37	Bacterioferritin	2.662	117	gi169152923	*A. baumannii*	5.02	18,137	61	14	Cyto-plasmic
Matched proteins from the database of other bacteria										
38	Lipoate-protein ligase A, putative	2.116	76	gi21674164	*Chlorobium tepidum* TLS	6.53	26,718	31	8	Cyto-plasmic

Table 4. Proteins found only in planktonic *A. baumannii*, identified by MALDI-TOF/TOF MS

Spot no.	Protein	Mascot score	Accession no.	Organism	Calculated p*I*	Mol. wt.	% Coverage	Peptides matched	PSORTb
Matched proteins from the database of *A. baumannii*									
42	Putative outer membrane protein	67	gi169152833	*A. baumannii*	9.30	22,559	40	7	Outer membrane

was named *bfmRS* operon and controls biofilm formation and cellular morphology. The up-regulation of signaling proteins in the biofilm culture of *A. baumannii* identified in the current study implies that *A. baumannii* in biofilms produce diverse signaling proteins to efficiently survive and adapt to undesirable environments.

Proteins expressed only in biofilm cells

Five proteins were identified as present only in biofilm cells. Two proteins were found in the *A. baumannii* database and 3 proteins were matched with proteins in the database of other bacteria (Table 2).

UDP-galactose 4-epimerase (GalE) (spot 44) catalyzes the conversion of UDP-galactose to UDP-glucose. UDP-glucose and UDP-galactose are used as building blocks for EPSs or capsular polysaccharides (CPSs) in a wide range of bacteria (Whitfield and Paiment, 2003). GalE protein was known to be one of the essential factors in biofilm formation on abiotic surfaces, and development of the biofilm requires the production of EPSs composed of UDP-glucose and UDP-galactose in *Vibrio cholerae* (Nesper *et al*., 2001). GalE also plays an important role in antibiotics susceptibility. For example, a *Porphyromonas gingivalis galE* mutant was more susceptible to some antibiotics (benzylpenicillin, oxacillin, cefotaxime, imipenem, and vancomycin) than the wild type due to its shorter lipopolysaccharide (LPS) O antigen (Nakao *et al*., 2006).

Phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide (ProFAR) isomerase (HisA, spot 50) is involved in histidine biosynthesis, which plays an important role in both the *de novo* synthesis of purines and nitrogen metabolism (Fani *et al*., 2007). It is not clear yet how these metabolisms are involved in biofilm formation in *A. baumannii*.

tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA (glucose-inhibited division protein A) (spot 46) is highly conserved among a wide range of eubacteria and eukaryotes (Yim *et al*., 2006). The function of GidA is known to be involved in translational control through modification of tRNAs during errors in gene expression, such as translational misreading (Brégeon *et al*., 2001). GidA has been also reported to be a global regulator in the plant pathogen *Pseudomonas syringae*; thus *gidA* mutants affect antibiotic production, swarming, presence of fluorescent pigment, and virulence (Kinscherf and Willis, 2002). tRNA modification by GidA may be important for the persistence of *A. baumannii* in biofilms by DNA repair or by regulation of certain genes, but the exact role of GidA in biofilm bacteria remains unknown.

A. baumannii diguanylate cyclase (phosphodiesterase) protein (spot 47) contains an EAL domain (associated with cyclic-di-GMP phosphodiesterase activity), a GGDEF domain (associated with a cyclic-di-GMP-synthesizing diguanylate cyclase activity), and a PAS domain that act as light, oxygen, and redox sensors (Galperin, 2004). The intracellular signaling molecule, cyclic-di-GMP, has been implicated in the regulation of bacterial behaviors, including motility and biofilm formation (Nakhamchik *et al*., 2008). In several species, proteins containing GGDEF domains have been associated with the biosynthesis of EPS, formation of biofilms, surface motility, and regulation of gene expression (Ryjenkov *et al*., 2005).

N-acetylmuramoyl-L-alanine amidase (spot 48) is a peptidoglycan hydrolase that breaks covalent bonds in bacterial cell walls (Oshida *et al*., 1995). *Staphylococcus epidermidis* AtlE has autolytic activity and a *N*-acetylmuramoyl-L-alanine amidase domain. The production of extracellular DNA by AtlE activity promotes surface attachment of the remaining population in biofilms. Qin *et al.* (2007) suggests that AtlE-mediated cell lysis may occur in established biofilms because extracellular DNA was found in biofilms of the *S. epidermidis* wild-type but not in microcolonies of the *atlE* mutant.

Proteins significantly down-regulated in biofilm cells

From the database of *A. baumannii*, a hypothetical protein, two OMPs (OprE3 and OmpW), a signal peptide, and iron storage protein bacterioferritin were identified and were significantly down-regulated in biofilm cells (Table 3). Lipoate-protein ligase A, identified from the database of other bacteria, was also down-regulated (Table 3). At present, the function of these proteins in *A. baumannii* biofilm remains unclear. In addition, a putative OMP was expressed only in planktonic cells (Table 4).

In summary, this study identified differential protein expression profiles of *A. baumannii* cultivated under biofilm and planktonic conditions. Among the differentially expressed proteins, 23 proteins were identified using MALDI-TOF/TOF MS; 17 proteins had a minimum two-fold change in expression level and 6 proteins were expressed only in either biofilm or planktonic mode. The proteins up-regulated or expressed only in biofilm cells of *A. baumannii* are associated with processing environmental information, metabolism, bacterial antibiotic resistance, and gene repair. Most of these proteins are known to have a role in biofilm formation and the direct or indirect establishment of specific biofilm traits. The physiologic and phenotypic relevance of these proteins are under investigation.

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References

Adams, M.D., K. Goglin, N. Molyneaux, K.M. Hujer, H. Lavender, J.J. Jamison, I.J. MacDonald, K.M. Martin, T. Russo, A.A. Campagnari, A.M. Hujer, R.A. Bonomo, and S.R. Gill. 2008. Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J. Bacteriol.* 190, 8053-8064.

Bergogne-Bérézin, E. and K.J. Towner. 1996. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin. Microbiol. Rev.* 9, 148-165.

Boles, B.R. and P.K. Singh. 2008. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proc. Natl. Acad. Sci. USA* 105, 12503-12508.

Brégeon, D., V. Colot, M. Radman, and F. Taddei. 2001. Translational misreading: a tRNA modification counteracts a +2 ribosomal frameshift. *Genes Dev*. 15, 2295-2306.

Chen, M.Z., P.R. Hsueh, L.N. Lee, C.J. Yu, P.C. Yang, and K.T. Luh. 2001. Severe community-acquired pneumonia due to *Acinetobacter baumannii*. *Chest* 120, 1072-1077.

Cornally, D., B. Mee, C. MacDonaill, K.F. Tipton, D. Kelleher, H.J. Windle, and G.T. Henehan. 2008. Aldo-keto reductase from *Helicobacter pylori* - role in adaptation to growth at acid pH. *FEBS J*. 275, 3041-3050.

Davis, K.A., K.A. Moran, C.K. McAllister, and P.J. Gray. 2005. Multidrug-resistant *Acinetobacter* extremity infections in soldiers. *Emerg. Infect. Dis*. 11, 1218-1224.

Dijkshoorn, L., A. Nemec, and H. Seifert. 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat. Rev. Microbiol.* 5, 939-951.

Dorel, C., O. Vidal, C. Prigent-Combaret, I. Vallet, and P. Lejeune. 1999. Involvement of the Cpx signal transduction pathway of *E. coli* in biofilm formation. *FEMS Microbiol. Lett.* 178, 169-175.

Ehrlich, G.D., F.Z. Hu, and J.C. Post. 2004. Microbial Biofilms, p. 332-358. *In* M. Ghannoum and G.A. O'Toole (eds.). Role for biofilms in infectious disease. ASM Press, Washington, D.C., USA.

Fani, R., M. Brilli, M. Fondi, and P. Lió. 2007. The role of gene fusions in the evolution of metabolic pathways: the histidine biosynthesis case. *BMC Evol. Biol.* 7 (Suppl 2), S4.

Galperin, M.Y. 2004. Bacterial signal transduction network in a genomic perspective. *Environ. Microbiol.* 6, 552-567.

Iacono, M., L. Villa, D. Fortini, R. Bordoni, F. Imperi, R.J. Bonnal, T. Sicheritz-Ponten, G. De Bellis, P. Visca, A. Cassone, and A. Carattoli. 2008. Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the European clone II group. *Antimicrob. Agents Chemother.* 52, 2616-2625.

Kinscherf, T.G. and D.K. Willis. 2002. Global regulation by *gidA* in *Pseudomonas syringae*. *J. Bacteriol.* 184, 2281-2286.

Lee, H.W., Y.M. Koh, J. Kim, J.C. Lee, Y.C. Lee, S.Y. Seol, D.T. Cho, and J. Kim. 2008. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. *Clin. Microbiol. Infect.* 14, 49-54.

Loehfelm, T.W., N.R. Luke, and A.A. Campagnari. 2008. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. *J. Bacteriol.* 190, 1036-1044.

Magnet, S. and J.S. Blanchard. 2005. Molecular insights into aminoglycoside action and resistance. *Chem. Rev.* 105, 477-498.

Mihara, K., T. Tanabe, Y. Yamakawa, T. Funahashi, H. Nakao, S. Narimatsu, and S. Yamamoto. 2004. Identification and transcriptional organization of a gene cluster involved in biosynthesis and transport of acinetobactin, a siderophore produced by *Acinetobacter baumannii* ATCC 19606^{T}. *Microbiology* 150, 2587-2597.

Monds, R.D. and G.A. O'Toole. 2009. The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol.* 17, 73-87.

Nakao, R., H. Senpuku, and H. Watanabe. 2006. *Porphyromonas gingivalis galE* is involved in lipopolysaccharide O-antigen synthesis and biofilm formation. *Infect. Immun.* 74, 6145-6153.

Nakhamchik, A., C. Wilde, and D.A. Rowe-Magnus. 2008. Cyclic-di-GMP regulates extracellular polysaccharide production, biofilm formation, and rugose colony development by *Vibrio vulnificus*. *Appl. Environ. Microbiol.* 74, 4199-4209.

Nesper, J., C.M. Lauriano, K.E. Klose, D. Kapfhammer, A. Kraiss, and J. Reidl. 2001. Characterization of *Vibrio cholerae* O1 El tor *galU* and *galE* mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. *Infect. Immun.* 69, 435-445.

Oshida, T., M. Sugai, H. Komatsuzawa, Y.M. Hong, H. Suginaka, and A. Tomasz. 1995. A *Staphylococcus aureus* autolysin that has an *N*-acetylmuramoyl-$_{L}$-alanine amidase domain and an endo-β-*N*-acetylglucosaminidase domain: cloning, sequence analysis, and characterization. *Proc. Natl. Acad. Sci. USA* 92, 285-289.

O'Toole, G.A., K.A. Gibbs, P.W. Hager, P.V. Phibbs, Jr., and R. Kolter. 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. *J. Bacteriol.* 182, 425-431.

Petersohn, A., H. Antelmann, U. Gerth, and M. Hecker. 1999. Identification and transcriptional analysis of new members of the σ^{B} regulon in *Bacillus subtilis*. *Microbiology* 145, 869-880.

Qin, Z., Y. Ou, L. Yang, Y. Zhu, T. Tolker-Nielsen, S. Molin, and D. Qu. 2007. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. *Microbiology* 153, 2083-2092.

Rapp, V.J., R.S. Munson, Jr., and R.F. Ross. 1986. Outer membrane protein profiles of *Haemophilus pleuropneumoniae*. *Infect. Immun.* 52, 414-420.

Rhodes, E.R., S. Menke, C. Shoemaker, A.P. Tomaras, G. McGillivary, and L.A. Actis. 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.

Ryjenkov, D.A., M. Tarutina, O.V. Moskvin, and M. Gomelsky. 2005. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J. Bacteriol.* 187, 1792-1798.

Smith, M.G., T.A. Gianoulis, S. Pukatzki, J.J. Mekalanos, L.N. Ornston, M. Gerstein, and M. Snyder. 2007. New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev.* 21, 601-614.

Souza, L.L., I.R. Eduardo, M. Pádula, and A.C. Leitão. 2006. Endonuclease IV and exonuclease III are involved in the repair and mutagenesis of DNA lesions induced by UVB in *Escherichia coli*. *Mutagenesis* 21, 125-130.

Tomaras, A.P., C.W. Dorsey, R.E. Edelmann, and L.A. Actis. 2003. Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: Involvement of a novel chaperone-usher pili assembly system. *Microbiology* 149, 3473-3484.

Tomaras, A.P., M.J. Flagler, C.W. Dorsey, J.A. Gaddy, and L.A. Actis. 2008. Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology* 154, 3398-3409.

Trémoulet, F., O. Duché, A. Namane, B. Martinie, and J.C. Labadie. 2002. A proteomic study of *Escherichia coli* O157:H7 NCTC 12900 cultivated in biofilm or in planktonic growth mode. *FEMS Microbiol. Lett.* 215, 7-14.

Vallenet, D., P. Nordmann, V. Barbe, L. Poirel, S. Mangenot, E. Bataille, C. Dossat, S. Gas, A. Kreimeyer, P. Lenoble, S. Oztas, J. Poulain, B. Segurens, C. Robert, C. Abergel, J.M. Claverie,

D. Raoult, C. Médigue, J. Weissenbach, and S. Cruveiller. 2008. Comparative analysis of Acinetobacters: three genomes for three lifestyles. *PLoS ONE* 3, e1805.

Vidal, O., R. Longin, C. Prigent-Combaret, C. Dorel, M. Hooreman, and P. Lejeune. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. *J. Bacteriol.* 180, 2442-2449.

Villegas, M.V. and A.I. Hartstein. 2003. *Acinetobacter* outbreaks, 1977-2000. *Infect. Control. Hosp. Epidemiol.* 24, 284-295.

Villers, D., E. Espaze, M. Coste-Burel, F. Giauffret, E. Ninin, F. Nicolas, and H. Richet. 1998. Nosocomial *Acinetobacter baumannii* infections: microbiological and clinical epidemiology. *Ann. Intern. Med.* 129, 182-189.

Whitfield, C. and A. Paiment. 2003. Biosynthesis and assembly of Group 1 capsular polysaccharides in *Escherichia coli* and related extracellular polysaccharides in other bacteria. *Carbohydr. Res.* 338, 2491-2502.

Wisplinghoff, H., R. Schmitt, A. Wöhrmann, D. Stefanik, and H. Seifert. 2007. Resistance to disinfectants in epidemiologically defined clinical isolates of *Acinetobacter baumannii*. *J. Hosp. Infect.* 66, 174-181.

Yamamoto, S., N. Okujo, and Y. Sakakibara. 1994. Isolation and structure elucidation of acinetobactin, a novel siderophore from *Acinetobacter baumannii*. *Arch. Microbiol.* 162, 249-254.

Yildiz, F.H., N.A. Dolganov, and G.K. Schoolnik. 2001. VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of *vps* biosynthesis genes and EPS(ETr)-associated phenotypes in *Vibrio cholerae* O1 El Tor. *J. Bacteriol.* 183, 1716-1726.

Yim, L., I. Moukadiri, G.R. Björk, and M.E. Armengod. 2006. Further insights into the tRNA modification process controlled by proteins MnmE and GidA of *Escherichia coli*. *Nucleic Acids Res.* 34, 5892-5905.