Journal of proteome research

Article

pubs.acs.org/jpr

Aggregatibacter actinomycetemcomitans Growth in Biofilm versus Planktonic State: Differential Expression of Proteins

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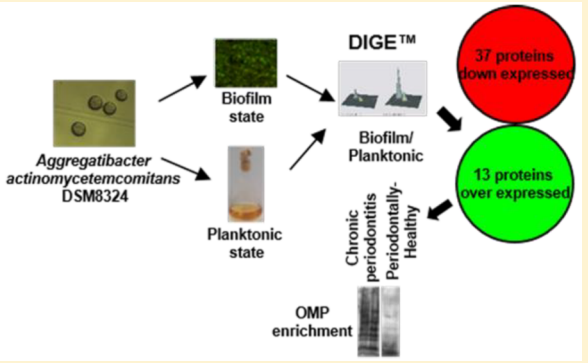
Supporting Information

ABSTRACT: *Aggregatibacter actinomycetemcomitans* (*Aa*) is a pathogenic bacterium residing in the subgingival plaque biofilm strongly associated with the pathogenesis of periodontitis. The aim of this investigation was to study the protein differential expression of *Aa* when growing on biofilm compared with planktonic state using proteomic analysis by the 2D-DIGE system. Eighty-seven proteins were differentially expressed during biofilm growth (1.5-fold, $p < 0.05$), with 13 overexpressed and 37 down-expressed. Those repressed were mainly proteins involved in metabolism, biosynthesis, and transport. The overexpressed proteins were outer membrane proteins (OMPs) and highly immunogenic proteins such as YaeT (OMP), FtsZ, OMP39, OMP18/16, the chaperone GroEL, OMPA, adenylate kinase (Adk), and dihydrolipoamide acetyltransferase. The enrichment fractions of the OMPs from biofilm and planktonic states were obtained, and these proteins were analyzed by Western blotting with human serum from a periodontitis patient and one healthy control. These immunogenic proteins overexpressed in the biofilm may represent candidate virulence factors.



KEYWORDS: *Aggregatibacter actinomycetemcomitans, biofilm, proteomic, 2D-DIGE, outer membrane proteins*

INTRODUCTION

Aggregatibacter actinomycetemcomitans (*Aa*) is a small nonmotile Gram-negative facultative anaerobic, microaerophilic, and capnophilic coccobacillus that grows singly, in pairs, or in small clumps.[1] Its natural habitat is the oral cavity of humans and other mammals.[2−4] Within the human oral cavity, it has been isolated from a range of habitats including supragingival and subgingival plaque, saliva, cheek and buccal mucosa, gingivae, tongue (dorsal and lateral surfaces), hard palate, and tonsils.[2,5] Although this microorganism can be detected in the oral cavity of periodontally healthy individuals, it has been strongly associated with aggressive forms of periodontitis, mainly those affecting adolescents and young adults.[6−8] It has been also associated with nonoral infections such as endocarditis.[9]

The potential pathogenicity of this bacterium has been attributed to the presence of a variety of virulence factors that enhance its survival in the oral cavity by evading the defense host strategies. These include the ability to attach to extracellular matrix proteins[10] and epithelial cells;[11,12] its resistance to antibacterial compounds;[13,14] the secretion of bacteriocins;[15,16] the expression of proteins that inactivate host defense cells and defensive pathways, such as a leukotoxin;[17] a chemotactic inhibitor;[18] a collagenase;[19] a cytotoxin;[20] Fc-binding proteins;[21] immunosuppressive factors;[22,23] endotoxin[24] or surface-associated material;[25,26] as well as the ability to invade epithelial cells[27] and tissues.[28] All of these pathogenic characteristics may occur in vivo when *Aa* colonizes and grows within the subgingival biofilm. In this state (biofilm) microorganisms may develop gene expression patterns different from those of their planktonic cells and hence may exhibit increased resistance to antimicrobial compounds, environmental stresses, and host immune defense mechanisms.[29]

Biofilm formation is a complex process that requires the coordinate expression and simultaneous regulation of many proteins for reversible and irreversible attachment, formation of microcolonies, and development of a stable community and 3D structure.[30] These sessile bacterial communities can better withstand host immune responses and antimicrobial compounds, such as antibiotics, than their nonattached individual planktonic counterparts. It is likely that biofilms evade antimicrobial challenges by multiple mechanisms,[29] and it is therefore important to study the protein expression by comparing bacteria in biofilms with planktonic cells. Proteins can be studied through transcriptomic analysis, although there is not always a clear correlation with their functional activity, and hence proteomic analysis is recommended.[31]

In this investigation, we aimed to study the differential expression of the proteins of *A. actinomycetemcomitans* DSM8324,

Received: March 6, 2017
Published: July 14, 2017

DOI: 10.1021/acs.jproteome.7b00127
J. Proteome Res. 2017, 16, 3158−3167

when growing in biofilms as well as in planktonic state, by using 2D difference gel electrophoresis (DIGE) and mass spectrometry identification.

EXPERIMENTAL SECTION

Bacterial Strain and Culture Conditions

A standard reference strain of *A. actinomycetemcomitans* (DSMZ 8324) was selected and grown on blood agar plates (Blood Agar Oxoid No 2; Oxoid, Basingstoke, U.K.), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg/L hemin (Sigma, St. Louis, MO), and 1.0 mg/L menadione (Merck, Darmstadt, Germany) under anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 24−72 h, and then transferred to a protein-rich medium containing brain-heart infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 2.5 g/L mucin (Oxoid), 1.0 g/L yeast extract (Oxoid), 0.1 g/L cysteine (Sigma), 2.0 g/L sodium bicarbonate (Merck), 5.0 mg/L hemin (Sigma), 1.0 mg/L menadione (Merck), and 0.25% (v/v) glutamic acid (Sigma) (modified BHI medium) until reaching an exponential growth phase.

Planktonic cell cultures were obtained by inoculating 30 mL of the modified BHI medium with bacteria suspension in a 50 mL sterile plastic tube until reaching 10^7 colony forming units (CFU)/mL and incubated at 37 °C during 24 h under anaerobic conditions.

Biofilms were formed on sterile plasma-coated hydroxyapatite slides (7.5 cm × 2.5 cm) (Clarkson Chromatography Products, Williamsport, PA) immersed in 100 mL of modified BHI medium with the same bacteria suspension containing 10^7 CFU/mL within a Petri dish of 140 mm diameter and incubated at 37 °C for 24 h under anaerobic conditions.

Confocal Laser Scanning Microscopy

Confocal laser scanning microscope (CLSM) was used to study the structure of the *A. actinomycetemcomitans* biofilms obtained. Fully hydrated biofilms were visualized with a fixed-stage Ix83 Olympus inverted microscope coupled to an Olympus FV1200 confocal system (Olympus; Shinjuku, Tokyo, Japan) and 63× water-immersion lenses (Olympus). Specimens were stained with LIVE/DEAD BacLightTM Bacterial Viability Kit solution (Molecular Probes B. V., Leiden, The Netherlands) at room temperature. The 1:1 fluorocrome ratio and 12 ± 1 min of staining time were used to obtain the optimum fluorescence signal at the corresponding wavelengths (Syto9:515−530 nm; PI: > 600 nm).

Protein Extraction

To extract the total proteins from biofilm growing cells, biofilms were scraped from the slide and dispersed into 1 mL of phosphate-buffered saline (PBS), where organisms were recovered by centrifugation. Cell pellets were maintained frozen until sample preparation. To extract the total proteins from planktonic cells, the bacterial culture (~30 mL) was centrifuged, and the pellet was maintained frozen until sample preparation. In both cases, biofilm and planktonic, the pellets were resuspended in PBS buffer containing 100 mM DTT (GE Healthcare, Uppsala, Sweden), 0.5 mM PMSF (Sigma), 1× EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), and nuclease mix (GE Healthcare). Biofilm and planktonic cells were broken using a tip sonicator Labsonic (B. Braun Biotech International, Melsungen, Germany) with 20 cycles of 10 s each (100% ultrasonic amplitude). To avoid overheating, the samples were kept on ice during the ultrasonic process. Then, they were centrifuged to remove intact cells and residual debris.

Four pooled samples from both biofilm and planktonic state were processed for the proteomic experiments. Each pool corresponded to one biological replicate and was processed in the same manner. In brief, the proteins were precipitated with 10% (w/v) TCA (Sigma) by incubation on ice 3 h. Then, the precipitated proteins were recovered by centrifugation (9000 rpm, 10 min, 4 °C), cleaned with the wash buffer from the 2D-Clean Kit (GE Healthcare), and resuspended in a solubilization buffer consisting of 25 mM Tris (GE Healthcare), 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), and 4% (w/v) CHAPS (GE Healthcare). The proteins were solubilized by vortexing for 3 h at room temperature and by sonication on ultrasonic bath (Branson 1510 Ultrasonic Cleaner, Branson Ultrasonics Corporation, Danbury, USA), four times during 5 min. The samples were kept on ice between the time intervals. The protein concentration was determined using 2D-Quant Kit (GE Healthcare). The pH of the sample was then adjusted to pH 8.5 on ice in preparation for CyDye labeling.

DIGE Experimental Design

Four hundred pmol of CyDye in 1 μL of anhydrous *N*,*N*-dimethylformamide (DMF) (Sigma) was used per 50 μg of protein for the labeling. After 30 min of incubation on ice in the dark, the reaction was quenched with 10 mM lysine (Sigma) and incubated for 10 min. In these experiments, the samples from biofilm and planktonic cells were labeled with Cy3 or Cy5, and to avoid any possible bias derived from the labeling efficiency, half of the samples of each condition were labeled with Cy3 dye and the other half with Cy5 dye. The Cy2 dye was used to label the internal standard sample. Samples were combined according to the experimental design described in Table 1. An equal volume of 2× sample buffer (8 M urea, 4% (w/v) CHAPS, 130 mM DTT, 2% pharmalytes pH 3−11 NL (GE Healthcare)) was added for the cup loading.

Table 1. Experimental Design for DIGE Analysis, Indicating Gel Number, CyDye Labelling and Four Replicates for Each Condition (biofilm and planktonic) (Indicated as "1 to 4")[a]

gel number	Cy2	Cy3	Cy5
1	pooled standard	biofilm 1	planktonic 4
2	pooled standard	planktonic 3	biofilm 2
3	pooled standard	biofilm 3	planktonic 1
4	pooled standard	planktonic 2	biofilm 4

[a]Pools of samples, both of biofilm as planktonic, were labelled with Cy3 or Cy5. An internal standard should be incorporated within each gel. The internal standard comprises pooled aliquots from all biological samples within the experiment and is labelled with Cy2.

The 2-DE was performed using GE Healthcare reagents and equipment. The first-dimension isoelectric focusing (IEF) was performed on 18 cm 3−11 NL pH range IPG strips, previously rehydrated with rehydration buffer (8 M urea, 4% (w/v) CHAPS, 13 mM DTT, 1% pharmalytes pH 3−11 NL. IEF was performed at 20 °C using the following program: 120 V for 1 h (120 Vh), 300 V for 3 h (900 Vh), 300−1000 V for 6 h (3900 Vh), 1000−8000 V for 3 h (13 500 Vh), 8000 V for 7 h 30 min (60 000 Vh), and 500 V for 12 h. After this, strips were equilibrated first for 15 min in reducing solution (6 M urea, 50 mM Tris-HCl pH 6.8, 30% v/v glycerol, 2% w/v SDS and 2% w/v DTT) and second for 15 min in alkylating solution

DOI: 10.1021/acs.jproteome.7b00127
J. Proteome Res. 2017, 16, 3158−3167

(6 M urea, 50 mM Tris-HCl pH 6.8, 30% v/v glycerol, 2% w/v SDS and 2.5% iodoacetamide (Sigma)). Second dimension SDS-PAGE was run on 11.5% (w/v) acrylamide gels in low fluorescence glass plates. Electrophoresis was carried out at 20 °C, 1 W/gel for 20 h, using Ettan DALTsix unit.

Preparative gels from planktonic and biofilm pool samples were prepared. For the first-dimension separation on pH 3−11 NL IPG strips, 500 μg of protein sample was focused with the following program: 120 V for 1 h (120 Vh), 300 V for 3 h (900 Vh), 300−1000 V for 6 h (3900 Vh), 1000−8000 V for 3 h (13 500 Vh), 8000 V for 8 h 45 min (70 000 Vh), and 500 V for 12 h.

Image Acquisition and DIGE Analysis

Proteins were visualized using a Typhoon 9400 scanner (GE Healthcare) with CyDye filters. For the Cy3, Cy5, and Cy2 image acquisition, the 532/580, 633/670, and 488/520 nm excitation/emission wavelengths were used, respectively, and 100 μm as pixel size. Image cropping and filtering were carried out with Image Quant v.5.1 software (GE Healthcare). Analyses for detection of different abundance between spots from different replicates were performed with the DIA (Differential In gel Analysis) module of the DeCyder 6.5 package (GE Healthcare). Matching and normalization of the internal standard spot maps using the Biological Variance Analysis (BVA) module corrected intergel variability. The internal standard image gel with the greatest number of spots was used as a master gel. BVA module was also employed for comparative cross-gel statistical analyses based on spot normalized volume ratio (Cy3:Cy2 and Cy5:Cy2).

For comparing the planktonic versus biofilm proteomic output, the average ratios and unpaired Student's t test were calculated. To reduce false-positives in the p-value calculation, the false discovery rate (FDR) was applied. Those protein spots with 1.5-fold as a threshold in the average ratio and with p values under 0.05 were considered as significantly differentially expressed.

Protein Identification

Total protein profile was detected by staining the DIGE gels with Colloidal Coomassie staining. The gel spots of interest were manually excised from the gels. Proteins selected for analysis were in-gel reduced, alkylated, and digested with trypsin according to Sechi et al.[32] In brief, the samples were reduced with 10 mM DTE in 25 mM ammonium bicarbonate for 30 min at 56 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 15 min in the dark. Finally, samples were digested with 12.5 ng/μL sequencing-grade trypsin (Roche) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. After digestion, the supernatant was collected and 1 μL was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μL of a 3 mg/mL of α-cyano-4-hydroxy-cinnamic acid matrix (Sigma) in 50% acetonitrile was added to the dried peptide digest spots and allowed again to air-dry at room temperature.

MALDI-TOF MS analyses were performed in a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) at the Genomics and Proteomics Center, University Complutense, Madrid (Spain). The MALDI-TOF/TOF operated in positive reflector mode with an accelerating voltage of 20 000 V. All mass spectra were calibrated internally using peptides from the auto digestion of trypsin.

Proteins ambiguously identified by peptide mass fingerprints were subjected to MS/MS sequencing analyses using the 4800 plus Proteomics Analyzer. They were selected from the MS spectra suitable precursor for MS/MS analyses by CID (atmospheric gas was used) 1 kV ion reflector mode and precursor mass Windows ±4 Da. The plate model and default calibration were optimized for the MS/MS spectra processing.

For protein identification, NCBInr (40 910 947 sequences; 14 639 572 021 residues) and Actinomycete (77 445 sequences; 20 877 593 residues) with taxonomy restriction to *A. actinomycetemcomitans* were searched using MASCOT 2.3 (www.matrixscience.com) through the Global Protein Server v 3.6 from ABSciex. Search parameters were: (i) carbamidomethyl cystein as fixed modification and oxidized methionine as variable modification; (ii) peptide mass tolerance, 50 ppm (PMF)−80 ppm (MSMS or Combined search); (iii) 1 missed trypsin cleavage site; and (iv) MS/MS fragments tolerance, 0.3 Da.

The parameters for the combined search (peptide mass fingerprint plus MS/MS spectra) were the same as described above. In all protein identification, the probability scores were greater than the score fixed by mascot as significant with a $p < 0.05$.

Outer Membrane Preparation, SDS-PAGE, and Western Immunoblotting

The outer membrane proteins were prepared from *A. actinomycetemcomitans* biofilm and planktonic samples by a slightly modified version of the method of Haase et al.[33] In brief, cells from biofilm and planktonic samples were resuspended in 10 mM HEPES (Sigma) (pH 7.4) containing 0.5 mM PMSF (Sigma) and 1× EDTA-free protease inhibitor cocktail (Roche). The suspension was sonicated with tip sonicator Labsonic (B. Braun) with 20 cycles of 10 s each (100% ultrasonic amplitude). To avoid overheating the material, the samples were kept on ice during the ultrasonic process. Then, cell debris were removed by centrifugation for 10 min at 2000g at 4 °C. The total membrane pellet obtained by ultracentrifugation of the supernatant at 105 000g for 1 h (Beckman Ti50.3 rotor; Beckman Coulter, Brea, CA) at 4 °C was then suspended in 1% sodium lauroylsarcosine (Sarkosyl, Sigma) in 10 mM HEPES (pH 7.4) and gently agitated for 1 h at room temperature. The outer membrane-enriched fraction was pelleted by ultracentrifugation at 105 000g for 1 h at 4 °C, then resuspended in 300 μL of distilled water, and the protein was quantified using Bradford protein assay.

Outer membrane proteins from biofilm and planktonic samples (20 μg) were denatured by heating in SDS buffer containing 0.5 M Tris-HCl pH 6.8, 5% (w/v) SDS, 25% (v/v) glycerol, 0.2% (w/v) bromophenol blue, and 20% (w/v) DTT for 5 min at 95 °C. Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis using the Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad Laboratories, Hercules, CA). A prestained molecular mass standard (Precision Plus Protein, Bio-Rad) was included in each run. The SDS-PAGE bands were excised from the colloidal Coomassie blue-stained gel and in-gel digested. The MALDI-TOF identification was performed as previously described.

For the immunoblot analysis, proteins were electrotransferred onto nitrocellulose membranes of pore size 0.45 μm (Amersham Protran; GE Healthcare) at 100 mA for 1 h using 1× Transfer Buffer (38.6 mM glycine, 41.3 mM Tris, 0.04% SDS, 20% methanol). Successful transfer was controlled using the ECL DualVue Western blotting markers (GE Healthcare). Nonspecific reactivity was blocked for 1 h at room temperature

DOI: 10.1021/acs.jproteome.7b00127
J. Proteome Res. 2017, 16, 3158−3167

with 5% (w/v) nonfat skim milk in TTBS (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.03% (v/v) Tween 20 (AppliChem Panreac, Darmstadt, Germany). The membranes were probed 2 h at room temperature with a serum (final dilution 1:400 in TTBS with 1% (w/v) nonfat skimmed milk) taken from a patient suffering generalized moderate chronic periodontitis, positive for A. *actinomycetemcomitans* in gingival crevicular fluid. She had the following clinical characteristics: probing pocket depths (45.5% were shallow (1−3 mm); 43.2% were intermediate (4−5 mm) and 12% (≥6 mm)), bleeding on probing: 32.72% and plaque: 61.70%. Her microbiological profile was: *A. actinomycetemcomitans* (9.6×10^4 cfu/mL), *Fusobacterium nucleatum* (7×10^3 cfu/mL), and *Tannerella forsithya* (1.5×10^4 cfu/mL). As a control, a serum collected from a periodontally healthy patient, with absence of periodontal pathogens, was used (1:400). The clinical data of this healthy patient were: probing pocket depths (96.4% were shallow (1−3 mm); 3.6% were intermediate (4−5 mm) and 0% (≥6 mm)), bleeding on probing: 13.09% and plaque: 10.71%. After washing (TTBS), as secondary antibody, antihuman IgG (Fc-specific) peroxidise conjugate (Sigma) was used (1:2000 in TTBS with 1% (w/v) nonfat skimmed milk) during 2 h at room temperature. Immunoreactive bands were visualized using a chemiluminescence detection system ECL (ECL Western blotting detection reagents, GE Healthcare) on autoradiographic medical X-ray film (GE Healthcare).

RESULTS AND DISCUSSION

In Vitro Biofilm Formation

Before performing the experiments, different bacterial concentrations from both biofilm and planktonic cell incubation were analyzed at different times using culture standard techniques until optimal conditions were achieved (data not shown).

Figure 1 shows a representative confocal micrograph depicting 2D projection images of the biofilm at different time points (24, 48, and 96 h). Viability of the biofilms obtained was analyzed using CLSM images stained with LIVE/DEAD BacLight Bacterial Viability kit solution, clearly identifying the viable and nonviable bacteria as green- and red-stained cells, respectively.

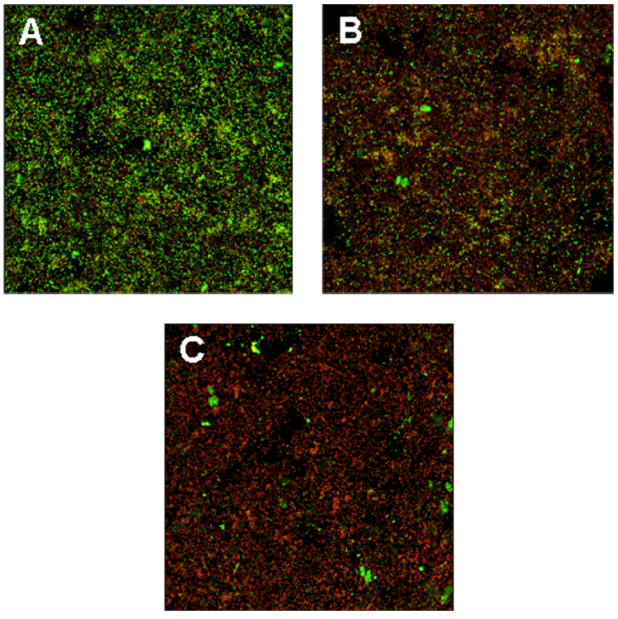


Figure 1. Confocal micrographs representing a 2D maximum projection of the series along a fixed axis of the biofilms after 24(A), 48 (B), and 72 h (C) of growth. BacLight Live/Dead stain was used to assess the vitality of cells.

Different Protein Expression in Biofilm versus Planktonic State

Biofilms developed on coated hydroxyapatite slides provided small quantities of protein that were insufficient for the proteomic analysis. Hence, pooled samples were used, similarly to planktonic samples.

Two-dimensional electrophoresis presents several advantages over other techniques in proteomic studies such as direct visualization of the protein maps, thus allowing easy identification of protein isoforms and posttranslational modifications as well as comparison with other existing protein maps databases.[34] Moreover, the DIGE system has shown a high sensitivity.[35,36] To the best of our knowledge, this is the first investigation on the differential protein expression of *Aa* using the DIGE system. Table 1 depicts the experimental design. In these experiments two samples from a different replicate (Cy3 and Cy5) and an aliquot of the internal standard pool (Cy2) were separated by 2-DE in each of the four gels (Figure 2A).

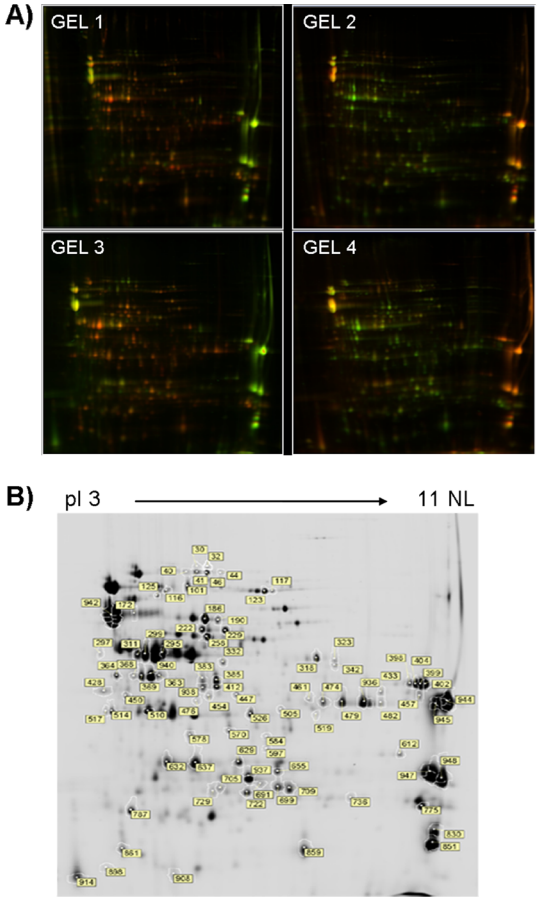


Figure 2. 2D-DIGE analysis identified proteins differentially expressed in biofilm and planktonic states. (A) Cy3 and Cy5 merged images of the 2D-DIGE: the four gels corresponding with the four biological replicates. (B) 2D-DIGE scan of *A. actinomycetemcomitans* sample: gel master image with the proteins of interest (proteins differentially expressed) with the corresponding number master.

DOI: 10.1021/acs.jproteome.7b00127
J. Proteome Res. 2017, 16, 3158−3167

Eighty-seven protein spots displaying statistically significant differences between biofilm and planktonic grown (1.5-fold ($p < 0.05$)) in their level of protein expression cells, which were visually confirmed not to be artifacts, were identified (Figure 2B), and a total of 79 protein spots were excised from the gel. The excised proteins were subjected to trypsin digestion and MS (MALDI-TOF/TOF) study of the corresponding peptide fragments, being able to identify 71 proteins (Table 2). The spectra obtained were found in Supporting Information Figure S1. The nonidentified proteins (8) did not have a enough amount of available protein. These 71 identified spots rendered 50 unique proteins, with several having multiple isoforms (i.e., spot 186 and spot 190), and, in some cases, the protein fractions resulted in the same proteins with the same isoelectric points but with smaller sizes (spot 612; spot 948) (Figure 2B). The numbers on the gel master in Figure 2B correspond to the numbers in Table 2, which summarizes the identified proteins with their average ratio (biofilm/planktonic), statistical data (t test), GI accession (sequence identification), protein name, and MASCOT data (% sequence coverage and protein score). These proteins were classified according to their biological function into down-expressed or overexpressed.

Down-Expressed Proteins in Biofilm Growth

Most of the statistically significant differences findings occurred in *A. actinomycetemcomitans* growing in biofilms and were associated with down-regulation of protein expression. Thirty-seven proteins from a total of 50 differential proteins, were identified (Table 2). Examination of the functional categories of proteins confirmed that this downturn in protein expression represented a regulated state of lower metabolism, biosynthesis (amino acid, coenzyme, cofactor or fatty acid biosynthesis) and transport, what is consistent with the reduced metabolic activity that occurs in mature biofilms.[37,38] These results are consistent with other studies also analyzing differences in protein expression when bacteria are forming biofilms.[39−41] In the case of the proteins involved in transport, down regulations

Table 2. Proteins Differentially Expressed *A. actinomycetemcomitans* in Biofilms and Planktonic States[a]

			biofilm/planktonic			
master gel no.	GI accession	protein name	t test	av. ratio	% sequence coverage	protein score
Metabolism						
101	491742098	dihydrolipoamide acetyltransferase	0.0023	1.89	24	341
117	491741753	transketolase	0.0019	−1.6	33	108
123	491741753	transketolase	0.0027	−1.52	42	182
186	491692275	phosphoenolpyruvate carboxykinase	0.00012	−2.08	65	285
190	491692275	phosphoenolpyruvate carboxykinase	0.00036	−1.6	22	101
222	518348158	phosphomannomutase	0.002	−1.94	44	246
229	518348158	phosphomannomutase	0.0015	−1.75	48	217
258	491724857	aspartate ammonia-lyase	0.0025	−1.66	36	472
295	491738229	enolase	0.00017	−1.93	59	175
299	491738229	enolase	0.000084	−2.07	57	150
311	491738229	enolase	0.00019	−1.77	20	64
363	491730152	phosphoglycerate kinase	0.00041	−1.82	35	82
368	491730152	phosphoglycerate kinase	0.00022	−1.51	39	74
369	491730152	phosphoglycerate kinase	0.00005	−1.8	47	101
940	491730152	phosphoglycerate kinase	0.000031	−2.56	62	224
383	491730150	fructose-bisphosphate aldolase	0.000078	−1.63	8	126
385	491730150	fructose-bisphosphate aldolase	0.000041	−2.4	60	201
412	491730150	fructose-bisphosphate aldolase	0.000022	−2.76	55	147
938	491730150	fructose-bisphosphate aldolase	0.000052	−2.16	26	77
450	491741811	PTS mannose transporter subunit IIAB	0.0033	2.11	39	159
474	491707379	glyceraldehyde-3-phosphate dehydrogenase	0.00022	−1.78	18	112
479	491707379	glyceraldehyde-3-phosphate dehydrogenase	0.00026	−1.69	25	302
482	491707379	glyceraldehyde-3-phosphate dehydrogenase	0.000099	−2.49	15	141
936	491707379	glyceraldehyde-3-phosphate dehydrogenase	0.000084	−2.39	75	174
722	491729956	phosphoglyceromutase	0.00014	−1.54	75	201
787	491693918	inorganic pyrophosphatase	0.0011	−1.52	33	308
861	491709275	PTS glucose transporter subunit IIA	0.000022	−2.32	59	151
937	491741684	triosephosphate isomerase	0.001	−1.57	40	510
Metabolism of Purines						
116	491741998	3′-nucleotidase	0.00017	−1.85	47	226
Transcription						
364	491742573	DNA-directed RNA polymerase subunit alpha	0.0015	2	52	120
898	491714067	transcription elongation factor GreA	0.00044	−1.75	44	64
125	491729383	prolyl-tRNA synthetase	0.0019	−1.92	34	107
332	491690654	tyrosyl-tRNA synthetase	0.00019	−2.35	41	103
Amino Acid Biosynthesis						
318	491689753	serine hydroxymethyltransferase	0.0002	−1.95	48	165
570	491699752	dihydrodipicolinate synthase	0.00029	−2.32	38	164
578	491691360	2,3,4,5-tetrahydropyridine-2-carboxylate *N*-succinyltransferase	0.0005	−1.53	49	136

DOI: 10.1021/acs.jproteome.7b00127
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Table 2. continued

master gel no.	GI accession	protein name	biofilm/planktonic *t* test	biofilm/planktonic av. ratio	% sequence coverage	protein score
Cofactors Biosynthesis						
510	491693650	thioredoxin reductase	0.00058	−1.85	59	152
Coenzyme Biosynthesis						
584	491690606	pyridoxamine kinase	0.00017	−3	59	130
597	491690606	pyridoxamine kinase	0.000031	−4.52	37	172
Fatty-Acid Biosynthesis						
629	491714182	enoyl-ACP reductase	0.0012	−1.7	29	75
736	491742684	3-ketoacyl-ACP reductase	0.00045	−1.62	14	192
ADP Biosynthesis						
691	491738131	adenylate kinase	0.0018	1.6	43	135
Cellular Processes, Chaperones						
172	491780231	chaperonin GroL	0.00017	2.08	50	189
942	491688234	molecular chaperone GroEl	0.00053	2.02	81	345
655	491720860	peptidyl-prolyl *cis*−*trans* isomerase	0.000084	−1.96	49	101
Cellular Processes, Cell Division						
297	491735878	FtsZ cell division protein	0.0034	1.53	52	135
Cellular Processes, Detoxification						
447	491688058	peptide methionine sulfoxide reductase	0.00036	−1.98	53	197
454	491692871	asparaginase	0.0014	−1.71	47	166
632	443551738	peroxiredoxin like protein	0.00065	2.11	48	383
637	443551738	peroxiredoxin like protein	0.0028	1.6	73	147
859	491692661	superoxide dismutase	0.002	−1.98	69	94
OMP						
41	503933512	YaeT outer membrane protein assembly complex	0.00018	3.02	23	121
46	503933512	YaeT outer membrane protein assembly complex	0.00029	2.76	42	215
398	491755504	outer membrane protein 39	0.00016	3.75	54	128
399	491755504	outer membrane protein 39	0.00018	3.98	65	215
402	491755504	outer membrane protein 39	0.00025	3.53	75	276
404	491755504	outer membrane protein 39	0.000084	4.08	61	185
612	491755504	outer membrane protein 39	0.0005	3.57	54	144
830	491761969	outer membrane protein 18/16	0.0017	4.11	75	180
851	491761969	outer membrane protein 18/16	0.000078	3.52	79	252
944	491728526	outer membrane protein A	0.000087	3.93	76	311
945	491728526	outer membrane protein A	0.00041	4.27	76	310
947	491728526	outer membrane protein A	0.000096	4.06	39	108
948	491728526	outer membrane protein A	0.000052	3.72	69	213
Transport						
342	491712350	TolB translocation protein	0.000078	2.89	41	152
428	601112850	spermidine/Putrescine ABC transporter substrate-binding protein	0.000087	3.65	48	127
457	491732394	iron ABC transporter substrate-binding protein	0.0049	−1.86	37	112
514	491737575	putrescine/spermidine ABC transporter substrate-binding protein 2	0.000031	−2.11	56	268
517	491756829	putrescine/spermidine ABC transporter substrate-binding protein	0.00062	−1.76	25	62
519	491722381	iron ABC transporter substrate-binding protein	0.000052	−2.25	73	91
526	491774383	thiamin/thiamine pyrophosphate ABC transporter	0.00049	−2.14	44	117
699	491691137	amino acid ABC transporter substrate-binding protein	0.0019	−2.35	69	143
705	491691137	amino acid ABC transporter substrate-binding protein	0.0021	−2.18	69	154
709	491780558	metal ABC transporter, nickel uptake transporter family	0.000084	−2.2	45	284
729	491727698	arginine ABC transporter substrate-binding protein	0.000022	−1.91	53	121
775	429155223	toluene tolerance protein	0.00062	−1.92	52	103
908	491718896	iron transporter	0.00036	−2.32	44	98
Unknown						
461	491744613	hypothetical protein	0.0025	−1.79	23	88

[a]Average ratios and Student's *t* test *p* values are calculated using Decyder software v6.5.

may be due to the limited availability of nutrients within the biofilm, what has been described as particularly acute within the depths of established biofilms.[38] It is also concurrent with other investigations describing a decrease in the expression of a membrane protein in iron deficient conditions.[42]

Overexpressed Proteins in Biofilm Growth

Because the bacteria forming biofilms have been shown to be more virulent than when growing under planktonic conditions,[29] overexpressed proteins in biofilm and their possible role as virulence factors were also analyzed.

DOI: 10.1021/acs.jproteome.7b00127
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Twenty-four overexpressed proteins were identified that corresponded to 13 unique proteins (Table 2). These were mainly outer membrane proteins (OMPs), proteins having an immunogenic properties, and proteins conferring virulence.

Among the identified overexpressed proteins:

●*Dihydrolipoamide acetyltransferase* is involved in metabolism and has been described as strongly immunogenic for *Mycoplasma mycoides* subsp. *capri*,[43] so much that this protein is used as a diagnostic marker for "MAKePS" syndrome caused by that bacterial species.[43]

●*Adenylate kinase.* This enzyme participates in ADP biosynthesis. Xiao et al. have identified the adenylate kinase (ADK, Rv0733) of *Mycobacterium tuberculosis* as an antigen that induces high cellular and antibody responses in active tuberculosis patients.[44] Also, others authors have associated the adenylate kinase from *Streptococcus pneumoniae* as an essential element for growth.[45]

●*Chaperone GroEl.* Surface-associated material has been identified as the molecular chaperone, GroEl,[46] and this protein appears to act directly with bone-resorbing cells such as osteoclasts.[47,48] Through this mechanism, this protein has been associated with bone resorption and has been identified as a virulence factor.[49,50] Because a characteristic feature of periodontitis is alveolar bone resorption, the finding of this chaperone overexpressed in *A. actinomycetemcomitans* biofilms may implicate this protein as a virulence factor of this bacterial species. In fact, other studies have demonstrated that this bacterium is associated with bone resorption through different mechanisms, such as by its lipopolysaccharide,[24,51] proteolysis-sensitive factor in microvesicles,[52] and surface-associated material.[53]

●*Cell division protein (FtsZ).* This essential protein FtsZ belongs to the cytoskeletal family because it forms a cytokinetic ring at midcell, and it is involved in the division machinery that orchestrates cell division.[54] Several authors have highlighted the immunogenic character of this protein in *A. actinomycetemcomitans*[55] and in other bacteria.[56]

●*Peroxiredoxin-like protein.* Peroxiredoxins are low-efficiency peroxidases using thiols as reductants, and they are considered to determine virulence of mycobacteria and trypanosomatids.[57] Kaihami et al. have revealed the role of a 1-Cys peroxiredoxin protein as a novel virulence factor belonging to the *Pseudomonas aeruginosa* arsenal against host defenses, thus allowing this bacterium to protect from other stresses under various environmental conditions.[58] Similarly, other authors have also described the immunogenic role of the peroxiredoxin 2 family protein in *A. actinomycetemcomitans*.[55]

●*Outer membrane proteins.* The outer membrane proteins (OMPs) are surface proteins that serve essential functions for the cell, including nutrient uptake, cell adhesion, cell signaling, and waste export. For pathogenic strains, many of these OMPs also serve as virulence factors for nutrient scavenging and evasion of host defense mechanisms.[59] They have also been attributed to immunogenic properties.[55,60−67] In this investigation, we have identified the following OMPs: OMP39, OMP18/16, OMPA, and YaeT (Table 2).

With the goal of confirming the immunogenicity of these proteins extracted from *A. actinomycetemcomitans*, an enrichment of these proteins in both states (biofilm and planktonic) was extracted; different OMPs, membrane proteins, and chaperone GroEl were obtained (Figure 3A, Table 3). The spectra obtained can be found in Supporting Information Figure S2. Their immunogenic responses were tested undertaking immunoblotting. For that, the same amounts of the enrichment fraction of these proteins (20 μg) were loaded in the gel, irrespective of whether *A. actinomycetemcomitans* was growing in biofilm or planktonic, and then incubated against serum from a patient with periodontitis (Figure 3B, lanes 1 and 2) and one healthy control (Figure 3B, lanes 3 and 4). It was observed that a strong immune response was elicited (in biofilm and planktonic states) with the serum from the patient with chronic periodontitis positive for *A. actinomycetemcomitans* (Figure 3B, lanes 1 and 2) when compared with the periodontally healthy subject negative for *A. actinomycetemcomitans* (Figure 3B, lanes 3 and 4). The immune response was similar under the biofilm and planktonic conditions: the protein profiles identified were analogous (Table 3), and the amount of the protein in the gel is identical (Figure 3A, lanes 2 and 3), although the measured expression of these proteins was different when assessed by DIGE (overexpressed in the biofilm).

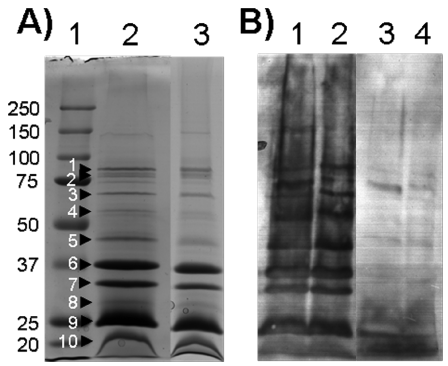

Figure 3. Outer membrane proteins (OMPs) extracted from biofilm and planktonic grown cells of *A. actinomycetemcomitans* DSM8324. (A) OMPs were separated by SDS-PAGE using 10% polyacrylamide gel, then stained with Colloidal Coomassie. Lane 1, molecular marker; Lane 2, extract from biofilm; Lane 3, extract from planktonic. The bands excised from the gel and identified by MS (MALDI-TOF/TOF) were indicated through arrows and numbers. (B) Western blotting of the different OMPs extracts using human serum from a patient with periodontitis and a patient healthy. Lane 1, extract from biofilm incubated with human serum from a patient with periodontitis; Lane 2, extract from planktonic incubated with human serum from a patient with periodontitis; Lane 3, extract from biofilm incubated with human serum from a patient healthy; Lane 4, extract from planktonic incubated with human serum from a patient healthy.

Table 3. Proteins Identified by Mass Spectrometry from Enrichment Fractions of the Outer Membrane Proteins (OMPs) from Biofilm and Planktonic Grown Cells[a]

no. band	GI accession	protein name
1	503933512	YaeT outer membrane protein assembly complex
2	502578142	LptD outer membrane protein LPS-assembly
	491729990	PgaA poly-β-1,6-*N*-acetyl-D-glucosamine export
3	491689683	TonB
4	491780231	GroEl molecular chaperone
5	491725647	OMPA-like
	491701003	membrane protein (TdeA)
6	491755504	OMP39
7	491725647	OMPA-like
8	491725647	OMPA-like
9	491725647	OMPA-like
10	491761969	OMP18/16

[a]Number of the bands is indicated in Figure 3A.

DOI: 10.1021/acs.jproteome.7b00127
J. Proteome Res. 2017, 16, 3158−3167

These results were similar to results reported by other authors describing antigenic specificity to *A. actinomycetemcomitans*,[53,68−73] although this is one of the first investigations where extraction and immunogenicity of OMPs of this bacteria forming the biofilm are reported. Under in vivo conditions, we would expect that these proteins will be overexpressed in the oral biofilm.

CONCLUSIONS

Seventy-eight differential proteins were identified by comparing biofilm versus planktonic growth of *A. actinomycetemcomitans*, where 13 proteins were overexpressed in biofilm growth while 37 were down expressed. The majority of the overexpressed proteins in the biofilm were outer membrane proteins and proteins that presented an immunogenic character in this bacteria and other bacteria. Further research must be done to clarify the role of these specific proteins in the virulence of *A. actinomycetemcomitans* and their potential use in both diagnosis and treatment of periodontitis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00127.

Figure S1. Spectra obtained from MALDI-TOF/TOF mass spectrometer (proteins differentially expressed). Figure S2. Spectra obtained from MALDI-TOF/TOF mass spectrometer (proteins extracted from OMPs enrichment). (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by CDTI (Center for the Development of Industrial Technology) of the Spanish Ministry of Science and Innovation and the company Dentaid (Barcelona, Spain), contract number 332/2011. A.L.-P. and M.C.S. received funding from CDTI (Center for the Developmentof Industrial Technology) of the Spanish Ministry of Science and Innovation and the company Dentaid (Barcelona, Spain), contract number 332/2011. O.P. received funding from the Clinical Research Foundation (CRF) for the Promotion of Oral Health, Brienz, Switzerland. DIGE and mass spectrometry analysis were carried out in the Proteomics Facility UCM, a member of ProteoRed-ISCIII network. We acknowledge the technical assistance of Alfonso Cortés from the Centre of Microscopy and Cytometry from the University Complutense, Madrid. We thank Rubén León and Vanessa Blanc for scientific support.

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DOI: 10.1021/acs.jproteome.7b00127
J. Proteome Res. 2017, 16, 3158−3167