Immunoproteomics of *Actinobacillus actinomycetemcomitans* outer-membrane proteins reveal a highly immunoreactive peptidoglycan-associated lipoprotein

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In a search for novel bioactive cell surface structures of periodontal pathogens, it was found that sera from two patients with *Actinobacillus actinomycetemcomitans*-associated infections reacted strongly at 17 kDa on immunoblots of *A. actinomycetemcomitans* outer-membrane protein (OMP) preparations. The 17 kDa antigen was also recognized by anti-CsgA (*Escherichia coli* curli major subunit) antibody. The 17 kDa *A. actinomycetemcomitans* protein was identified as peptidoglycan-associated lipoprotein (PAL; AaPAL) by two-dimensional immunoblotting and subsequent sequence analysis by mass spectrometry and bioinformatics tools. AaPAL was an OMP and a lipoprotein, and it had an OmpA-like domain. In a group of middle-aged subjects ($n=26$), serum reactivity to AaPAL was associated with the presence of periodontitis but not with the oral detection of *A. actinomycetemcomitans*. Both human sera and rabbit antisera against three different types of antigens, the gel-purified AaPAL, *A. actinomycetemcomitans* whole-cell antigens, and CsgA, recognized putative PALs of oral haemophili in addition to AaPAL. The results demonstrated that the novel AaPAL is a conserved bacterial lipoprotein. It is expressed *in vivo* and is strongly immunoreactive. The antigenic cross-reactivity found between AaPAL and oral haemophili may enhance local and systemic immuno-inflammatory reactions in periodontitis.

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INTRODUCTION

Periodontitis is a chronic bacterial infection, in which overgrowth of subgingival Gram-negative bacteria leads to chronic inflammation and gradual degradation of tooth-supporting tissues. A number of studies have reported that levels of circulating antibodies against periodontal pathogens, as well as levels of proinflammatory cytokines and acute-phase reactants, are higher in patients with periodontitis than in healthy controls (Fredriksson *et al.*, 1999;

Loos *et al.*, 2000; Pussinen *et al.*, 2002). Moreover, levels of these parameters in a given group of patients decline following treatment of periodontitis (D'Aiuto *et al.*, 2005; Mattila *et al.*, 2002; Pussinen *et al.*, 2004). Interestingly, in patients with infections caused, for example, by *Chlamydia pneumoniae* and *Helicobacter pylori*, similar immuno-inflammatory findings have been linked to an increased risk of cardiovascular disease (Epstein, 2002).

Actinobacillus actinomycetemcomitans is a small Gram-negative coccobacillus that is implicated in aggressive forms of periodontitis (Asikainen & Chen, 1999; Zambon, 1985). The oral cavity is its natural habitat, but it can also translocate from the oral cavity into the blood circulation, as evidenced by the occurrence of non-oral *A. actinomycetemcomitans* infections. These infections include infective endocarditis and abscesses in various body sites, for example, brain and lung (Paju *et al.*, 2003; Paturol *et al.*, 2004). The evoked systemic immune responses may be directed at invading, viable cells, and/or released cell components of *A. actinomycetemcomitans*. The established

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Abbreviations: 1D, one-dimensional; 2D, two-dimensional; MALDI-TOF MS, matrix-assisted laser desorption time-of-flight mass spectrometry; OMP, outer-membrane protein; PAL, peptidoglycan-associated lipoprotein; Q-TOF MS, quadrupole time-of-flight mass spectrometry; RT, room temperature.

virulence determinants of *A. actinomycetemcomitans* include leukotoxin, lipopolysaccharide and fimbriae (Fives-Taylor *et al.*, 1999; Kachlany *et al.*, 2001; Zambon *et al.*, 1983). However, there is only limited knowledge of the identity and functions of the outer-membrane proteins (OMPs) of this species. The available data show that patients with periodontitis are commonly seroreactive to certain *A. actinomycetemcomitans* OMPs, such as the OMP18/16 and the heat-modifiable OMP29 (Ebersole *et al.*, 1999; Komatsuzawa *et al.*, 2002; Wilson, 1991a; Wilson & Hamilton, 1995). Additionally, *A. actinomycetemcomitans* OMP100 induces production of proinflammatory cytokines from murine macrophages and is involved in the adhesion and invasion of *A. actinomycetemcomitans* into epithelial cells (Asakawa *et al.*, 2003).

In the present study, we have identified and characterized a novel 17 kDa *A. actinomycetemcomitans* OMP, which was identified as a peptidoglycan-associated lipoprotein (PAL). Our results indicate that the *A. actinomycetemcomitans* PAL (AaPAL) is a strongly immunoreactive antigen in patients with periodontitis and that it is also highly immunogenic in the rabbit.

METHODS

Bacteria and culture conditions. *A. actinomycetemcomitans* and haemophilus (Table 1) were cultured on blood agar plates (5% defibrinated horse blood, 5 mg haemin l⁻¹, 10 mg vitamin K l⁻¹, Columbia agar base) incubated in air supplemented with 5% CO₂, at 37 °C for 3 days. *E. coli* was cultured on CFA agar plates (1% Casamino acids, 0.15% yeast extract, 0.005% MgSO₄, 0.0005% MnCl₂, 20% agar, pH 7.4) (Evans *et al.*, 1977) incubated in air at room temperature (RT) for 2 days.

Subjects, sera, and bacterial sampling. The initial immunoblot experiments for finding novel bioactive molecules of *A. actinomycetemcomitans* included serum samples from two dentate subjects with *A. actinomycetemcomitans*-associated infections: a patient with suspected infective endocarditis (age 48 years, male), whose blood samples were repeatedly *A. actinomycetemcomitans* culture-positive; and a periodontitis patient (age 57 years, male) with *A. actinomycetemcomitans* culture-positive subgingival samples. A serum sample from a periodontally healthy, subgingivally *A. actinomycetemcomitans* culture-negative subject (age 55 years, female) was used for comparison. Additional serum samples were included to determine the frequency of seroreactivity to AaPAL in dentate subjects with ($n=22$; age range 38–66 years, mean $\pm$ SD 56.0 $\pm$ 8.0 years; five females, 17 males) or without ($n=4$; age range 48–55 years, mean $\pm$ SD 51.5 $\pm$ 3.1 years; four females) periodontitis. All sera, except the serum sample from the endocarditis patient (1:8000), were used at a dilution of 1:4000 in immunoblotting.

Periodontitis was classified according to the guidelines of Armitage (1999). Periodontal health was defined as lack of alveolar bone loss in a full dentition (28 teeth per subject), although gingival bleeding after probing could occur.

In all study subjects, bacterial samples were taken from both saliva and subgingival sites and cultured for *A. actinomycetemcomitans*, as reported previously (Von Troil-Linden *et al.*, 1995). All subjects gave informed consent for sample collection and analyses.

Rabbit antisera. Three types of rabbit antisera were included in the study.

(i) Polyclonal antibodies against *E. coli* CsgA were prepared as described by Hammar *et al.* (1996). Briefly, a maltose binding protein (MalE)–CsgA fusion protein was expressed in IPTG-induced *E. coli* XL-1 Blue/pZB-aIII. Cells were pelleted, and periplasmic contents were released by osmotic-shock treatment (Neu & Heppel, 1965). The fusion protein was purified from osmotic-shock fluid obtained after spheroplasts had been removed by an amylose resin column and maltose (20 mM) elution. The purified fusion protein was used to

Table 1. Bacterial strains, characteristics and origins

Species	Strain	Characteristics	Origin	Reference
<i>A. actinomycetemcomitans</i>	SA269	Rough-colony isolate, serotype d	Subgingival plaque, localized juvenile periodontitis, 14-year-old Caucasian female, Finland	Asikainen <i>et al.</i> (1990)
<i>A. actinomycetemcomitans</i>	D7S	Rough-colony isolate, fimbriated, serotype a	Subgingival plaque, generalized aggressive periodontitis, 28-year-old African-American female, USA	Wang <i>et al.</i> (2003)
<i>A. actinomycetemcomitans</i>	D7SS	Smooth-colony isolate, non-fimbriated, serotype a	Spontaneous laboratory variant of strain D7S	Wang <i>et al.</i> (2003)
<i>A. actinomycetemcomitans</i>	D7SS-p	pal-deficient mutant, serotype a	D7SS	M. Karched and others, unpublished results
<i>H. aphrophilus</i>	CCUG 3715			
<i>H. paraphrophilus</i>	CCUG 1802			
<i>H. influenzae</i>	CCUG 23945			
<i>E. coli</i>	MC4100			Olsén <i>et al.</i> (1993)
<i>E. coli</i>	MC4100-1	csgA-deficient mutant	MC4100	Olsén <i>et al.</i> (1993)

immunize a rabbit. To remove anti-MaE antibodies, antiserum was adsorbed to MaE coupled to Sepharose 4B (Pharmacia). To remove non-specific antibodies, the unadsorbed serum was collected and extensively adsorbed against cell extracts of an *E. coli* *csgA* deletion-mutant strain. The antibodies were used at a dilution of 1:4000 in immunoblotting.

(ii) Antiserum against gel-purified AaPAL was raised in a rabbit as follows. The *A. actinomycetemcomitans* (SA269) whole-cell lysate was separated by SDS-PAGE using an 8–16% gradient, single-well preparative gel (Criterion, Bio-Rad) with an 800 µl protein sample (0.4 mg ml⁻¹) (determined by the method of Lowry *et al.*, 1951) at constant voltage (100 V, 1 h). A parallel gel was run for immunoblotting in the same Criterion cell. The immunoblot membrane showing a 17 kDa band reactive with anti-CsgA was superimposed on the gel to excise a piece to raise antiserum in a rabbit (AgriSera). The antiserum was called anti-AaPAL, and was used at a dilution of 1:100 000 in immunoblotting.

(iii) Antisera against whole cells of *A. actinomycetemcomitans* strains representing five serotypes (serotype a, ATCC 29523; serotype b, ATCC 43718; serotype c, NCTC 9710; serotype d, IDH 781; serotype e, IDH 1705) (Asikainen *et al.*, 1991; Saarela *et al.*, 1992) were used to study whether the membrane-bound AaPAL acted as an immunogen in various serotypes. The antisera were used at a dilution of 1:100 000 or 1:10 000 in immunoblotting.

OMP extraction. Bacterial outer membrane fraction was extracted as described by Wilson (1991b). The bacteria were harvested (10 mM HEPES, pH 7.4), centrifuged, resuspended in HEPES, and then sonicated. After centrifugation, the supernatant was subjected to OMP extraction. Cytoplasmic membrane was solubilized in 1% (w/v) Sarkosyl (Sigma) (RT, 30 min). The insoluble fraction containing the outer membrane was separated by ultracentrifugation, and the pellet was resuspended in buffer A [1% (w/v) octylglucoside (Sigma) in TE buffer (50 mM Tris, 5 mM EDTA, pH 8.0)] (RT, 30 min). The insoluble fraction was recovered by ultracentrifugation and the pellet was resuspended in buffer A supplemented with 0.5 M NaCl (RT, 30 min). The octylglucoside/NaCl-insoluble fraction contained the OMPs. After centrifugation, the pellet was solubilized in 20 mM sodium phosphate buffer (pH 7.5) containing 0.1% (w/v) SDS (RT, 30 min).

SDS-PAGE and immunoblot. The methods employed were modified from Collinson *et al.* (1991). Protein samples (10 µg) (Lowry *et al.*, 1951) were mixed with sample buffer [final concentrations: 2% (w/v) SDS, 25% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol, 0.01% bromophenol blue (Merck), 62.5 mM Tris/HCl (pH 6.8)], boiled (10 min), and separated on 8–16% gradient gels (Criterion) in electrophoresis buffer (0.1% SDS, 192 mM glycine, 25 mM Tris, pH 8.3) at constant voltage (100 or 150 V) (Criterion cell). A pre-stained molecular mass standard (Precision Plus Protein, Bio-Rad) was included in each run. The gels were stained with GelCode Blue stain (Pierce).

For the immunoblot analysis, proteins were electrotransferred onto PVDF membranes (Perkin Elmer) using 192 mM glycine, 20% methanol, 25 mM Tris (pH 8.3), or a discontinuous buffer system [60 mM Tris and 40 mM CAPS with either 15% methanol (anode buffer only) or 0.1% SDS (cathode buffer only)] in the case of large-scale testing of sera, and constant current (140 mA) (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad). Non-specific reactivity was blocked (RT, 1 h) with 5% (w/v) non-fat skimmed milk (Merck) in TTBS (0.05% v/v, Tween-20, 150 mM NaCl, 100 mM Tris, pH 7.5). The membranes were probed (RT, 1 h) with primary antibodies diluted in TTBS with 0.5% (w/v) non-fat skimmed milk. After washing (TTBS), the reaction was detected by peroxidase-conjugated donkey anti-rabbit IgG (1:10 000) (Jackson ImmunoResearch Laboratories) (RT, 1 h) or

rabbit anti-human IgG (1:10 000) (Dako) and visualized using chemiluminescence (SuperSignal, Pierce) on autoradiographic medical X-ray film (BioMax MR, Kodak, or Cronex 5, Agfa-Gevaert). The exposed films were scanned (GS-700 imaging densitometer, Bio-Rad) for the analysis and documentation.

Two-dimensional (2D) gel electrophoresis and immunoblot.

The *A. actinomycetemcomitans* (SA269) OMP preparation was treated with 99% formic acid on ice for 10 min (Romling *et al.*, 1998). Dried (SpeedVac) preparation was resuspended in 100 µl solubilization buffer (0.3% w/v, SDS, 200 mM DTT, 28 mM Tris/HCl, 22 mM Tris base). After intensive vortexing, 400 µl of 2D sample buffer [9.9 M urea, 4% (v/v) Igepal CA630, 2.2% ampholytes (pH 3–10; Amersham Biosciences), 100 mM DTT, 2% (w/v) CHAPS] was added. After vortexing (37 °C), 5 µg protein (Lowry *et al.*, 1951) was loaded in each gel for silver staining or immunoblotting. For isoelectric focusing, 180 mm pH 3–10 non-linear-gradient IPG strips were used (Amersham Biosciences) on a Multi-Phor II apparatus. Focusing conditions were 150 V for 2 h, 300 V for 2 h, a gradual increase from 300 to 3500 V for 5 h, and final focusing at 3500 V for 10–12 h (20 °C). In the second dimension, proteins were separated on linear 12% SDS-PAGE gels (15 °C), and silverstained or transferred to a PVDF membrane (Immobiline Pseq, Millipore) using a discontinuous buffer system (39 mM glycine, 0.0375% w/v, SDS, 20% v/v, methanol, 48 mM Tris). The electroblots were immunostained as for one-dimensional (1D) immunoblots.

Proteomics identification. *A. actinomycetemcomitans* (SA269) OMP preparations were processed and run in 2D gel electrophoresis as above, and stained with colloidal Coomassie brilliant blue (Bio-Rad). A parallel gel was analysed by immunoblot with anti-CsgA. The immunoblot was superimposed on the gel to excise plugs from three immunoreacting 16–18 kDa spots. The plugs were destained (0.2 M ammonium bicarbonate in 50% acetonitrile) and washed (50 mM ammonium bicarbonate in 50% acetonitrile) (37 °C, 1 h). The proteins were dried, rehydrated, and trypsinized (0.1 µg µl⁻¹). After concentration, the peptides were analysed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) (Swedish Institute for Infectious Disease Control, Solna, Sweden, and Mass Spectrometry Facility at the Microbiology and Tumor Biology Center, Karolinska Institute, Sweden). Peptide sequence information was obtained using quadrupole time-of-flight mass spectrometry (Q-TOF MS) (Swegene Proteomics Centre, University of Gothenburg, Sweden).

Bioinformatics. The *A. actinomycetemcomitans* genome sequence (<http://www.genome.ou.edu/act.html>) was used for gene identification using the peptide sequences obtained from the MS/MS analysis. The BLAST program at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/Database/index.html>) was used for protein identification. T-COFFEE (<http://igs-server.cnrs-mrs.fr/Tcoffee/>) was used to align the test amino acid sequence with known sequences. Additional tools, such as CLUSTALW (<http://www.ch.embnet.org/software/ClustalW.html>) and Multalin (<http://prodes.toulouse.inra.fr/multalin/multalin.html>), were used to verify the T-COFFEE results. Tools on the ExPASy server (<http://www.expasy.org/>), such as SWISS-PROT, ScanProsite, PSORT (Prediction of Protein Localization Sites) and SignalP, were used to study protein sequence characteristics, and the Protein Information Resource (PIR; <http://pir.georgetown.edu/>) and the Database of Bacterial Lipoproteins (DOLOP; <http://www.mrc-lmb.cam.ac.uk/genomes/dolop/dolop.htm>) to study the lipoprotein properties of the test protein.

pal-deficient mutant. A *pal*-deficient mutant strain (D7SS-p) was constructed by the gene-replacement technique (Wang & Chen, 2005), using *A. actinomycetemcomitans* D7SS as the parental strain.

Briefly, a 580 bp sequence upstream (primers PAL-Sm, 5'-CTTTCC-CGGGACATAACGG, and PAL-D1, 5'-CTCACGTGGTGATTCT-CCTTGTATTATTAG) and a 630 bp sequence downstream (primers PAL-D2, 5'-AACACGTGGTGAAGAAAAACCGGCAGT, and PAL-Hd, 5'-GCTGAAAGCTTATAGTGTAAGAG) of the *pal* ORF were PCR amplified. The amplicons were purified, digested with *Dra*III (New England Bio-Labs) and ligated to a spectinomycin-resistance cassette. The ligation mix was used directly to transform strain D7SS. The mutant was verified by PCR using primers PAL-Sm and PAL-Hd.

Statistics. Results are shown as mean $\pm$ SD. Relationships between two categorical variables were examined by two-sided Fisher's exact test (SPSS). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Patient sera and antiserum against *E. coli* CsgA recognized a 17 kDa *A. actinomycetemcomitans* antigen

Sera from two patients with *A. actinomycetemcomitans*-associated infections produced strong and dominant signals at 17 kDa on the electroblots of *A. actinomycetemcomitans* OMP preparations (Fig. 1a, b), whereas serum from a healthy subject produced no signals (Fig. 1c). Despite the standardized amounts of protein employed in the samples run side-by-side in gel electrophoresis, the molecular mass of the band from the wild-type strain (SA269) was repeatedly slightly lower than that of the smooth-colony variant (D7SS) (Fig. 1a, b, lanes 1 and 2). This difference was seen with human sera (Fig. 1a, b), but not with the rabbit anti-sera (Figs 2 and 6a). Despite the strong immunoblot signal at approximately 17 kDa (Fig. 1a), the Coomassie-blue-stained gel demonstrated only faint bands in that region (Fig. 1d). Quite the opposite reactivity was found for the

Fig. 1. Reactivity of human sera with *A. actinomycetemcomitans* OMP preparations. Immunoblots with sera from (a) a patient with *A. actinomycetemcomitans*-positive periodontitis, (b) a patient with *A. actinomycetemcomitans* blood culture-positive endocarditis, and (c) a periodontally healthy, *A. actinomycetemcomitans*-negative subject. (d) GelCode blue staining of the SDS-PAGE gel. *A. actinomycetemcomitans* strains: lane 1, D7SS; lane 2, SA269. M, molecular mass marker.

Fig. 2. Anti-CsgA antibody cross-reacts with a 17 kDa *A. actinomycetemcomitans* OMP. 1D immunoblots of the OMP preparations from the *A. actinomycetemcomitans* strains D7SS (lanes 1 and 2) and SA269 (lanes 3 and 4), and the *E. coli* strain MC4100 (lanes 5 and 6). The protein samples were prepared without (-; lanes 1, 3 and 5) or with (+; lanes 2, 4 and 6) formic acid treatment.

relatively strong ~ 34 kDa bands in the Coomassie-stained gel (Fig. 1d). However, the present patients' sera reacted only poorly at the ~ 34 kDa region in the immunoblots (Fig. 1a, b). Interestingly, the ~ 34 kDa protein may correspond to the heat-modifiable 29 kDa *A. actinomycetemcomitans* protein that in SDS-PAGE migrates with an apparent molecular mass of 34 kDa following solubilization at elevated temperatures (Wilson, 1991b). That our results demonstrated an immunodominant reactivity at 17 kDa instead of at 34 kDa may be related to differences in the study patients, in their individual immune responses, in the methods, and/or in the *A. actinomycetemcomitans* strains used as antigens in the immunoassays. In the earlier studies using ELISA analysis, the 34 kDa protein was a major target of the antibody response in young patients with localized juvenile periodontitis (Wilson, 1991a; Wilson & Hamilton, 1995). Furthermore, different *A. actinomycetemcomitans* serotypes were used in each study: Wilson (1991a) and Wilson & Hamilton (1995) utilized serotype b, whereas serotypes a and d were used in the present study.

In a parallel approach, we hypothesized that the *A. actinomycetemcomitans* cell surface components that share antigenicity with established virulence factors of other bacterial species might also be bioactive in *A. actinomycetemcomitans*. Therefore, we tested the reactivity of *A. actinomycetemcomitans* OMP preparations with a specific antibody against the major subunit (CsgA) of *E. coli* curli (Chapman *et al.*, 2002; Hammar *et al.*, 1996). Since curli fibres are highly stable structures, the *E. coli* OMP preparations were subjected to treatment with 99% formic acid (Romling *et al.*, 1998). Concurrently, the *A. actinomycetemcomitans* OMP preparations were treated similarly. The results revealed that, in contrast to *E. coli*, the *A. actinomycetemcomitans* OMP preparations, whether formic acid treated or untreated, entered the gel (Fig. 2). Thus, our results confirmed the

findings of others (Collinson *et al.*, 1991) that *E. coli* curli are resistant to depolymerization (Fig. 2), and also demonstrated that the 17 kDa *A. actinomycetemcomitans* protein has different solubility characteristics. Furthermore, anti-CsgA reacted with the *A. actinomycetemcomitans* OMP preparations only at 17 kDa (Fig. 2). This is also the apparent molecular mass of CsgA (Chapman *et al.*, 2002) and the molecular mass range at which anti-CsgA reacted strongly on the *E. coli* OMP immunoblot (Fig. 2). The additional signals in *E. coli* (Fig. 2) at approximately 36 and 69 kDa probably represented oligomeric forms of CsgA.

Identification of the 17 kDa *A. actinomycetemcomitans* antigen

We used an immunoproteomics approach to identify the 17 kDa *A. actinomycetemcomitans* antigen. The silver-stained 2D gel of the *A. actinomycetemcomitans* (SA269) OMP preparation showed a wide spectrum of proteins (Fig. 3a), among which anti-CsgA recognized three (pI 5.3, 5.6, 6.1) protein species between 16 and 18 kDa (Fig. 3b). These three proteins had identical mass spectra by MALDI-TOF MS (Fig. 3c, table), suggesting that they were isoforms of the same protein. Q-TOF MS/MS identified the three peptide sequences GTPEYNMALGER, YNTVYFGFDK and AVQNFLTAK, which were used in the homologue search. Using TBLASTN with the *A. actinomycetemcomitans* (strain HK1651) genomic database at Oklahoma University (Advanced Centre for Genomic Technology, Oklahoma University, OK; <http://www.genome.ou.edu/act.html>), the above three peptide sequences resulted in hits within a

200-base sequence. With the Translate tool at ExPASy, we translated 500 bases both upstream and downstream of the 200-base sequence. A single protein sequence of 155 amino acids was identified. The same 155 amino acid sequence at the Oklahoma University *A. actinomycetemcomitans* genomic database also gave a complete match with a single protein. This sequence, with BLASTP at NCBI, gave a hit with peptidoglycan-associated lipoprotein (PAL) and was identified as the PAL of *A. actinomycetemcomitans* (AaPAL). Although low-molecular-weight bands have been observed in *A. actinomycetemcomitans* OMP preparations before (Bolstad *et al.*, 1990; Komatsuzawa *et al.*, 2002; Wilson & Hamilton, 1995), the only published sequence is from OMP18/16 (Komatsuzawa *et al.*, 2002). However, as shown in the present study, OMP18/16 had no homology with the PAL of *A. actinomycetemcomitans* (Fig. 5d).

PAL belongs to the TOL-PAL system, which is well-conserved in Gram-negative bacteria (Walburger *et al.*, 2002). Six genes, *ybgC-tolQ-tolR-tolA-tolB-pal*, of the TOL-PAL system were found in a region of 4668 bases in the *A. actinomycetemcomitans* genome (at OU) (Fig. 4a). The TOL-PAL system plays an important role, for example, in the maintenance of outer-membrane integrity (Cascales *et al.*, 2002), proper functioning of certain nutrient-uptake systems across the cytoplasmic membrane (Llamas *et al.*, 2003), import of macromolecules, such as colicins (Lloubes *et al.*, 2001), and possibly in cell-envelope biogenesis (Cascales & Lloubes, 2004). PALs from other species are known to be highly immunogenic (Burnens *et al.*, 1995; Frey *et al.*, 1996; Spinola *et al.*, 1996). Therefore, AaPAL,

Fig. 3. 2D immunoblot of an *A. actinomycetemcomitans* OMP preparation using anti-CsgA followed by molecular mass fingerprinting with MALDI-TOF MS. (a) Silver-stained 2D gel of the *A. actinomycetemcomitans* (SA269) OMP preparation; (b) 2D immunoblot with anti-CsgA antibody; (c) MALDI-TOF MS analysis of the three 16–18 kDa protein species [marked as 1, 2 and 3 in (b)]. The three prominent mass peaks from the three protein species (shown as Protein-1, Protein-2 and Protein-3 in the table) were similar to each other. One (Protein-3) of the three respective graphs is shown.

Fig. 4. The relative localization of the *tol-pal* gene cluster in *A. actinomycetemcomitans* and sequence analysis of AaPAL. (a) The genes of the TOL-PAL system were found in the *A. actinomycetemcomitans* genomic database; (b) the nucleotide and amino acid sequences of AaPAL with an analysis of the regions. The *tolB* gene is immediately followed by a Shine–Dalgarno region (S-D; *aggag*, bold and underlined type) of the *pal* gene. The encoded methionine starts the *pal* gene (M; position 1). A hydrophobic region (position 7–16; underlined) is followed by a lipobox (LAAC; grey). The signal peptidase cleavage site with cysteine (position 20; upward arrow) and the sorting amino acid (position 21; G, italic and underlined type), and a region with sequence similarity to *E. coli* OmpA (position 87–131) are shown.

belonging to the same family of proteins, might be expected to elicit a humoral immune response, as also suggested by our results shown in Fig. 1.

Characterization of the AaPAL sequence

The *pal* gene was immediately downstream of the *tolB* gene (Fig. 4a). However, unlike in other bacterial species, it was not possible to identify the gene of a periplasmic protein, YbgF, downstream of *pal*. AaPAL was classified as an OMP by the absence of aspartate at position 21 (Terada *et al.*, 2001). The N-terminal leader sequence of 20 amino acids consisted of two basic residues (lysine) and a hydrophobic stretch of 10 residues (ALLVAGSVAV) followed by a lipobox, LAAC (Fig. 4b). The cleavage site of the signal peptide was between residues 19 and 20. AaPAL also presented a C-terminal OmpA-like domain (from residues 87 to 131) (Fig. 4b), as do the PALs of several other Gram-negative bacteria, including *Haemophilus influenzae* P6 (Bogdan & Apicella, 1995). Eliminating the signal sequence,

the predicted molecular mass of the gene product without the lipid portion was 14.7 kDa and the pI was 5.69.

The AaPAL sequence was highly similar to the PAL sequences of the other Gram-negative species studied. Alignment of the AaPAL sequence with the PAL sequences from *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Haemophilus ducreyi* and *H. influenzae* resulted in overall amino acid sequence identity and similarity values of 52 and 78 %, respectively (Fig. 5a). Corresponding values for the AaPAL and *E. coli* PAL sequences were 53 and 79 % (Fig. 5b), and for the AaPAL and *E. coli* CsgA sequences 16 and 46 % (Fig. 5c). The sequence of another low-molecular-mass *A. actinomycetemcomitans* OMP (OMP18/16) was 15 % identical and 48 % similar to the AaPAL sequence (Fig. 5d).

To gain insight into possible interactions of AaPAL with other components in the cell envelope, the AaPAL sequence was compared with the published sequence data of the PALs of other species. The C-terminal SYGK/E motif, a ‘TolA

Fig. 5. The AaPAL sequence in comparison with the sequences of the PALs from selected members of the *Pasteurellaceae* (a), and of *E. coli* (b), *E. coli* CsgA (c) and *A. actinomycescomitans* OMP18/16 (d). CLUSTAL format for T-COFFEE software was used for all sequence alignment analyses. (a) Multiple alignment of AaPAL, HiPAL (*H. influenzae* PAL; accession no. NP_438542), PmPAL (*P. multocida* PAL; accession no. Q51886), ApPAL (*A. pleuropneumoniae* PAL; accession no. CAA61413), and HdPAL (*H. ducreyi* PAL; accession no. AAP96526). (b) Alignment of AaPAL with EcPAL (*E. coli* PAL; accession no. NP_415269), (c) EcCsgA (*E. coli* CsgA; accession no. NP_287176), and (d) OMP 18/16 (accession no. ABO64944). Colour coding or indicator for sequence similarity: red or *, identical amino acid residues in all sequences in the alignment; blue or :, conserved substitutions; green or ., semi-conserved substitutions.

box', required for the binding of PAL to TolA in *E. coli* (Cascales & Lloubes, 2004), was also present in AaPAL (residues 129–132) (Fig. 5b, box). Similarly, TolB and the peptidoglycan-binding sequence of *E. coli* PAL (Cascales & Lloubes, 2004; Clavel *et al.*, 1998) closely matched a region (residues 97–124) in the aligned AaPAL sequence (Fig. 5b, box). The AaPAL sequence also contains the VYF and KNRR motifs (Fig. 5b, boxes) required for interaction of PAL with cell-envelope proteins in *E. coli* (Cascales & Lloubes, 2004). This suggests that AaPAL interacts with other outer-membrane components in a similar manner to the PALs of other species. Preliminary crystallographic studies of *E. coli* PAL have suggested that the VYF, SYGK/E and KNRR motifs are located in exposed loops or β -sheet structures,

and that the peptidoglycan-binding motif is contained within a long α -helix (C. Abergel, personal communication). The sequence similarity between AaPAL and the PALs of other species implies that AaPAL might also possess potent bioactivity in infections.

AaPAL was immunogenic in rabbit

To gain knowledge of the bioactivity of AaPAL, we raised antiserum against the gel-purified AaPAL, as described in Methods. The produced anti-AaPAL, like anti-CsgA (Fig. 2), recognized a 17 kDa antigen in the OMP preparations of the *A. actinomycescomitans* test strains (Fig. 6a). Interestingly, both the wild-type SA269 and the

Fig. 6. Immunoblot analysis of *A. actinomycetemcomitans* OMP preparations using anti-AaPAL. (a) The strains in the 1D immunoblot were *A. actinomycetemcomitans* D7SS (lane 1) and SA269 (lane 2), and *E. coli* MC4100 (lane 3) and MC4100-1 (lane 4). (b) The 2D immunoblot shows three 16–18 kDa immunoreactive spots (pI 5.3, 5.6 and 6.1, respectively).

spontaneous smooth-colony variant D7SS demonstrated bands which migrated in a similar manner in SDS-PAGE. This differed from the result with human sera (Fig. 1). Therefore, it seems unlikely that the presence or absence of fimbriae in the wild-type and the smooth variant, respectively, influenced the migration of the 17 kDa band in the gel (Fig. 1).

The 2D immunoblot analysis of the *A. actinomycetemcomitans* (SA269) OMP preparation disclosed three protein species of the same molecular masses and isoelectric points (16–18 kDa molecular mass, pI 5.3, 5.6 and 6.1) (Fig. 6b) as those detected when anti-CsgA was used as primary antibody (Fig. 3b). This suggests that anti-AaPAL and anti-CsgA recognized the same *A. actinomycetemcomitans* protein, AaPAL.

When anti-AaPAL was used in the 1D immunoblot analysis of the *E. coli* OMP preparation, the signal appeared at 19 kDa (Fig. 6a), instead of the 17 kDa when anti-CsgA was used (Fig. 2). Since the apparent molecular mass of *E. coli* PAL is 19 kDa (Lazzaroni & Portalier, 1992), the finding could result from anti-*E. coli* PAL contamination in anti-CsgA. However, this seems unlikely, because the immunizing antigen and the antibody produced were extensively

purified for anti-CsgA production, and in addition, in earlier testing, the antibody showed no reactivity at 19 kDa (Hammar *et al.*, 1995, 1996). Thus, it is conceivable that anti-AaPAL cross-reacted with *E. coli* PAL due to the high similarity level between the PAL sequences of *A. actinomycetemcomitans* and *E. coli* (Fig. 5b).

Sera from patients with periodontitis recognized AaPAL

The OMP preparations of *A. actinomycetemcomitans* D7SS and a *pal*-deficient mutant strain D7SS-p were used as antigens in the immunoblot analyses of serum samples obtained from 26 subjects. Several sera produced a 17 kDa signal on the immunoblots of D7SS (Fig. 7), whereas none reacted with D7SS-p. The patients with periodontitis were more frequently (15/22, 68%; $P=0.02$) AaPAL immunoblot-positive than the periodontally healthy subjects (0/4, 0%). It appeared that half of the patients with periodontitis (11/22, 50%) had a positive subgingival and/or salivary *A. actinomycetemcomitans* culture, whereas all healthy subjects presented with a negative culture. Interestingly, the detection of *A. actinomycetemcomitans* from oral samples was not related to the immunoblot result: 3/11 (27%) culture-positive patients were immunoblot-negative, and 8/15 (53%) culture-negative patients were immunoblot-positive.

All 22 patients with periodontitis were middle-aged or older and exhibited a chronic type of periodontitis (Armitage, 1999). However, it is believed that *A. actinomycetemcomitans* is particularly associated with aggressive forms of periodontitis in young individuals (Slots & Ting, 1999). Therefore, it is conceivable that in the periodontal infection process of our patients, the subgingival levels and the aetiological significance of this bacterium were low. This could partly explain the AaPAL immunoblot-negative result among the *A. actinomycetemcomitans* culture-positive subjects. It remains to be seen whether in patients with aggressive periodontitis the seroreactivity to AaPAL is directly associated with the detection of subgingival *A. actinomycetemcomitans*.

AaPAL shared antigenic determinants with putative PALs of oral haemophili

Although in the present AaPAL immunoblot-positive but *A. actinomycetemcomitans* culture-negative subjects a

Fig. 7. Immunoblot analysis of *A. actinomycetemcomitans* OMP preparations using sera from 15 patients with periodontitis. All sera recognized a 17 kDa band in *A. actinomycetemcomitans* strain D7SS (lanes 1), but not in the *pal*-deficient mutant strain D7SS-p (lanes 2). Letters below the lanes are patient identification codes.

false-negative culture result for oral *A. actinomycetemcomitans* (Asikainen & Chen, 1999) was a possibility, it was equally likely that the patients' sera reacting with AaPAL contained cross-reacting antibodies to PALs of other oral and/or non-oral Gram-negative species. To test this hypothesis, we subjected sera from two patients with *A. actinomycetemcomitans*-associated infections and from a healthy subject to further analyses. The sera were used for immunoblotting of the OMP preparations of Gram-negative species related or unrelated to *A. actinomycetemcomitans* (Fig. 8a). The results showed that, in a similar manner to the rabbit antisera against CsgA and AaPAL (Fig. 8a i, ii), the patients' sera reacted strongly with *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *H. influenzae* (Fig. 8a iii, iv), probably targeting the PALs of these species. To date, the only sequence data available for the PALs of these species is for *H. influenzae* PAL (15 kDa) (Deich *et al.*, 1988). On the other hand, the patients' sera did not react with a non-oral, phylogenetically distant species, *E. coli*, at the expected size of its PAL (19 kDa) (Lazzaroni & Portalier, 1992). The serum of the healthy subject did not react with any bacterial species tested (Fig. 8a v).

The above findings may indicate that in periodontitis with inflamed, pathologically deepened periodontal pockets, oral bacteria that are not directly involved in the disease yet present in dental plaque gain access to the blood circulation through micro-ulcerated pocket epithelium. This may lead to elevated systemic antibody production against these species. However, the systemic exposure to these antigens is probably low in healthy periodontal conditions, since the total number of plaque bacteria is only a fraction of that in periodontitis (Moore & Moore, 1994) and the tissue area for bacterial intrusion is radically smaller. Therefore, it seems

possible that the detected AaPAL seroreactivity in patients with natural *A. actinomycetemcomitans*-positive infections could be due to AaPAL-specific antibodies and also to antibodies against antigenic epitopes shared by AaPAL and the PALs of the oral phylogenetic relatives of *A. actinomycetemcomitans*.

Rabbit immunization with *A. actinomycetemcomitans* whole-cell antigens elicits production of antibodies against AaPAL

Although AaPAL is a strong target of the IgG antibody response in human periodontitis (Fig. 7), it remained unclear (Fig. 8a), whether the antibodies were produced against AaPAL and only cross-reacted with the related species, or even vice versa. Thus, to clarify whether the membrane-bound AaPAL could act as an immunogen, as is probable in natural infections (Fig. 6), we immunized rabbits with whole *A. actinomycetemcomitans* cells. Additionally, it was considered that since some *A. actinomycetemcomitans* serotypes have been related to disease and others to health (Asikainen *et al.*, 1991), their immunogenic potential might also differ. Therefore, we chose the most common *A. actinomycetemcomitans* serotypes (a–e) as immunization antigens. The results showed that all the rabbit antisera raised recognized a 17 kDa band in *A. actinomycetemcomitans* (Fig. 8b). However, the antisera also cross-reacted with the phylogenetically related bacterial species at molecular masses (Fig. 8b) expected to represent PALs of these species. Interestingly, like the patient sera from natural *A. actinomycetemcomitans* infections (Fig. 8a iii, iv), the rabbit antisera against whole *A. actinomycetemcomitans* cells did not recognize the putative 19 kDa *E. coli* PAL (Fig. 8b). The results did, however, confirm that in all tested

Fig. 8. Immunoblot analysis of the OMP preparations from *A. actinomycetemcomitans*, its phylogenetic relatives, and a non-relative, using rabbit and human sera. (a) Immunoblots with anti-CsgA antibody (i), anti-AaPAL antiserum (ii), and sera from a patient with *A. actinomycetemcomitans* culture-positive endocarditis (iii), a patient with *A. actinomycetemcomitans* culture-positive periodontitis (iv), and a periodontally healthy subject (v). The bacterial strains are *A. actinomycetemcomitans* D7SS (lane 1) and D7SS-p (lane 2), *H. aphrophilus* (lane 3), *H. paraphrophilus* (lane 4), *H. influenzae* (lane 5) and *E. coli* MC4100 (lane 6). (b) Immunoblots with antisera against whole cells of five *A. actinomycetemcomitans* serotypes (a–e). The bacterial strains are *A. actinomycetemcomitans* D7SS (lane 1), *H. aphrophilus* (lane 2), *H. paraphrophilus* (lane 3), *H. influenzae* (lane 4) and *E. coli* (lane 5).

serotypes, the membrane-bound AaPAL can act as an immunogen in rabbits (Fig. 8b).

To compare the immunoreactivity of AaPAL with that of the other *A. actinomycetemcomitans* OMPs, we subjected the OMP preparations of strains D7SS (serotype a) and D7SS-p (serotype a) separately to immunoblotting with each of the anti-serotype-specific antisera (a–e) (Fig. 9). While none of the five antisera produced a 17 kDa band in D7SS-p, all produced it in D7SS (Fig. 9). Antisera against serotypes b, c and d each recognized a dominant band at 17 kDa, whereas antisera against serotypes a and e additionally detected strong bands of higher molecular mass. However, the reactivity of the serotype-specific antisera other than anti-serotype a may have been affected by the sourcing of the immunoblot antigens from serotype a strains (D7SS and D7SS-p).

Apart from being a strong immunogen, AaPAL can be expected to play an important role in *A. actinomycetemcomitans* pathogenesis, due to its bioactivity as a bacterial lipoprotein, for example. In several bacterial species, lipoproteins have demonstrated strong pathogenic potential (Adu-Bobie *et al.*, 2004; Rolhion *et al.*, 2005; Sha *et al.*, 2004); for instance, they are cytotoxic, and cause apoptosis in mammalian cells (Aliprantis *et al.*, 1999; Hasebe *et al.*, 2004). Bacterial lipoproteins also trigger the production of proinflammatory cytokines (Fisette *et al.*, 2003; Hirschfeld *et al.*, 1999). It seems that the proinflammatory signalling occurs through Toll-like receptor 2 (Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999) and is mediated by the glyceride and acyl groups attached to the N-terminal cysteine (cysteine-tripalmitoyl) of the lipoproteins. Additionally, *in vivo* studies on the PALs of other species have shown, for example, that a *pal*-deficient mutant of *H. ducreyi* causes less-serious infections in human volunteers than the parental strain (Fortney *et al.*, 2000), and that PAL released to the blood

circulation by *E. coli* is an important bacterial mediator in Gram-negative sepsis (Hellman *et al.*, 2002).

The results of our study indicate that AaPAL is expressed *in vivo* and that it is an immunoreactive bacterial lipoprotein. We have also demonstrated that oral haemophili share antigenicity with AaPAL. Based on these novel findings, we postulate that the humoral immune response to the PALs of *A. actinomycetemcomitans* and oral haemophili might be of significance in inducing harmful systemic effects in patients with periodontitis. As the role of bacterial lipoproteins in bacterial pathogenesis is becoming increasingly recognized (Adu-Bobie *et al.*, 2004; Fadl *et al.*, 2005), the identification and characterization of a previously unknown PAL from an important periodontal pathogen may carry biological significance and have future clinical implications.

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Fig. 9. Immunoblot analysis of *A. actinomycetemcomitans* OMP preparations using rabbit antisera against whole-cell antigens of five *A. actinomycetemcomitans* serotypes (a–e). Lane 1, *A. actinomycetemcomitans* D7SS; lane 2, the *pal*-deficient mutant D7SS-p.

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