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1 **Effects of saliva or serum coating on adherence of *Streptococcus***
2 ***oralis* strains to titanium**

3
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22 polyacrylamide gel electrophoresis; GCF, gingival crevicular fluid; IPG, immobilized
23 pharmalyte gradient; LC-MS/MS, liquid chromatography-tandem mass spectroscopy.

24

25 **SUMMARY**

26

27 The use of dental implants to treat tooth loss has increased rapidly over recent years.
28 ‘Smooth’ implants showing high long-term success rates have successively been replaced by
29 implants with rougher surfaces, designed to stimulate rapid osseointegration and promote
30 tissue healing. If exposed in the oral cavity, rougher surfaces may promote bacterial adhesion
31 leading to formation of microbial biofilms which can induce peri-implant inflammation.
32 *Streptococcus oralis* is an early colonizer of oral surfaces and has been recovered from
33 titanium surfaces *in vivo*. The purpose of this study was to examine the adherence of clinical
34 strains of *S. oralis* to titanium with smooth or moderately rough surface topography and to
35 determine the effect of a saliva- or serum-derived coating on this process. Adherence was
36 studied using a flow-cell system with confocal laser scanning microscopy, while putative
37 adhesins were analysed using proteomics of bacterial cell wall proteins. This showed that
38 adherence to moderately rough was greater than to smooth surfaces. Serum did not promote
39 binding of any studied *S. oralis* strains to titanium whereas a saliva-coating increased
40 adherence in two of three strains tested. The high level of adherence to the moderately rough
41 surfaces was maintained even in the presence of a saliva coating. The *S. oralis* strains that
42 bound to saliva expressed an LPXTG-linked protein which was not present in the non-
43 adherent strain. Thus strains of *S. oralis* differ in their capacity to bind to saliva-coated
44 titanium and we propose that this is due to differential expression of a novel adhesin.

45

46 INTRODUCTION

47 Osseointegrated implants have been in use for the past forty years and are now an established
48 and widespread treatment for the replacement of lost and missing teeth. The early type of
49 ‘smooth’ implants (average height deviation, $S_a < 0.5 \mu\text{m}$) which showed high long-term
50 success rates (Astrand *et al.*, 2008; Lekholm *et al.*, 2006) have largely been replaced by new
51 generations of implants with rougher surfaces, designed to stimulate osseointegration and
52 therefore promote more rapid tissue healing. Such implants may have topographical and/or
53 chemical surface modifications as well as coatings with biocompatible materials. Most
54 currently available implants have minimal- to moderately-rough surfaces with an S_a of 1-2
55 μm and studies in animal models have shown this to give a better initial bone response than
56 smooth surfaces (Buser *et al.*, 1999; Wennerberg *et al.*, 1997). However under conditions
57 where titanium is exposed in the oral cavity, rougher surfaces may also promote bacterial
58 adhesion leading to the formation of microbial biofilms which are difficult to remove. Plaque
59 accumulation can induce an inflammatory reaction in the mucosal tissues surrounding the
60 implant which can lead to destruction of the supporting bone (peri-implantitis) and eventually
61 to implant failure (Serino & Strom, 2009).

62 Immediately upon exposure to protein-containing fluids, implant surfaces become covered
63 with a protein-rich pellicle and in the oral cavity this is derived from saliva as well as GCF
64 and/or serum. Studies of pellicle formation have suggested that the salivary proteins amylase,
65 immunoglobulin A and proline-rich proteins (Edgerton *et al.*, 1996; Lima *et al.*, 2008) as
66 well as albumin and fibronectin can bind to titanium (Lima *et al.*, 2008). Pellicle proteins
67 serve as receptors for the attachment of microorganisms which initiate the formation of a
68 microbial biofilm (Murray *et al.*, 1992, Kolenbrander *et al.*, 2010). The major early
69 colonizers of dental biofilms are streptococci such as *Streptococcus oralis*, *Streptococcus*

70 *mitis* and *Streptococcus gordonii* which express adhesins recognizing specific motifs on host
71 proteins (Schachtele *et al.*, 2007; Jakubovics & Kolenbrander, 2010). *S. gordonii*, expresses
72 the SspA/B and Hsa adhesins which recognize sialylated structures on, for example, salivary
73 agglutinin (Jakubovics *et al.*, 2005) as well an amylase-binding protein (AbpA) (Rogers *et*
74 *al.*, 2001) and has been shown to bind to these proteins *in vitro* (Murray *et al.*, 1992).
75 However, although *S. oralis* has been shown to bind to multiple salivary proteins including
76 MUC7, proline-rich proteins and amylase (Murray *et al.*, 1992), the adhesins responsible
77 have not yet been identified.

78 *S. oralis* is known to colonize titanium surfaces *in vivo* (Leonhardt *et al.*, 1995) and in this
79 study, we have used a flow-cell model to investigate the effect of roughness of titanium
80 surfaces on adherence of three fresh clinical isolates, two from sites with clinical signs of
81 peri-implantitis and one from supragingival plaque. The effects of saliva- or serum-coating
82 on the level of adherence have also been investigated. Cell wall proteins from the different *S.*
83 *oralis* strains have been identified in order to identify putative adhesins which may be
84 involved in recognition of salivary proteins.

85

86 **METHODS**

87 **Characterization of bacterial strains.** Three fresh clinical isolates of *S. oralis*; one from
88 dental plaque (LA11) and the other two (89C and 192B) from peri-implant infection sites,
89 were used in this study. Identification as *S. oralis* was based on positive phenotypic tests for
90 N-acetylglucosaminidase and sialidase, and negative tests for D-fucosidase. The identity of *S.*
91 *oralis* was also confirmed by sequencing of the *gdh* gene as well as the *ddl* gene (89C and
92 192B) or the *sodA* gene (LA11) (Hoshino *et al.*, 2005).

93 **Bacterial culture conditions.** The three strains (LA11, 89C and 192B) were grown on blood
94 agar in 5 % CO₂ in air at 37 °C. Colonies were transferred to Bacto Todd–Hewitt broth (TH)
95 (Becton Dickinson & Co) and grown overnight in 5 % CO₂ in air at 37 °C. The suspension
96 was transferred to fresh TH broth and incubated at 37 °C until the mid-exponential growth
97 phase was reached (OD_{600nm} ≈ 0.5). Cultures were then centrifuged (4000 g) for 10 minutes at
98 37 °C, and the pellets suspended in half the original volume of TH to double the cell
99 concentration prior to the biofilm experiments.

100 **Titanium surfaces.** The titanium surfaces used in the study were of commercially pure,
101 grade IV titanium (ELOS Pinol A/S). The plates (99.25 x 25.25 x 0.8 mm) were either turned
102 or blasted with 250-500 µm Al₂O₃ particles (distance 25mm, pressure 5 bar), cleaned with
103 detergent, rinsed with distilled water and sterilized using γ-irradiation. Surface topography
104 was investigated using an optical interferometer (MicroXam™, PhaseShift, Tuscan, USA)
105 with a high pass Gaussian filter (50 x 50 µm) to distinguish roughness from errors of form
106 and waviness. The Surfscan software was used to compute three 3D parameters: S_a, µm, a
107 special parameter - density of summits (S_{ds}, summits/µm²) and a hybrid parameter -
108 developed interfacial area ratio (S_{dr}, %) (Stout *et al.*, 1993). Images were produced using
109 SPIP™ (Scanning Probe Image Processor). Water contact angles on the smooth titanium

surfaces were measured using the sessile drop technique with a goniometer from Sinterface Technologies, Berlin. For the saliva and serum-coated surfaces (see below), contact angles were measured after 10, 30 and 60 minutes of drying at 30 °C.

Flow-cells. The flow-cell system was similar to that described by Welin-Neilands & Svensäter (2007), except that the glass plates were replaced by two titanium surfaces, one smooth and one moderately rough, separated by a 1.6 mm rubber spacer. A laminar flow of 42 ml h⁻¹ was used and all experiments were carried out at 37 °C.

Coating of surfaces with saliva and serum. Stimulated whole saliva, pooled from six individuals was prepared according to the method described by Palmer *et al.*, 2001. Briefly, dithiothreitol (Sigma) was added to give a final concentration of 2.5 mM and the saliva gently stirred on ice for 10 min. After centrifugation (30000 g, 20 min, 4 °C), the supernatant was retained and 3 volumes of distilled water added prior to passing through a 0.2 µm filter. The filtrate was kept at -20 °C until use. Human serum (Lonza Group Ltd) was diluted to 5 % using distilled water. Saliva or serum were introduced into the flow-cells through a three-way tap and the surfaces allowed to coat overnight at room temperature. The presence of proteins on the titanium surfaces after this treatment was confirmed using gradient SDS-PAGE gels after desorption with 0.5 % SDS (data not shown).

Adhesion assay. For each experiment the same bacterial suspension (exponential growth phase cells suspended in TH broth) was introduced simultaneously into three flow-cells. In one of the flow-cells the surfaces were uncoated, while in the other two, the surfaces were coated with either 25 % saliva or 5 % serum. After 2 hours, the surfaces were washed for a further hour with TH broth to remove loosely attached cells. Adhered cells were stained using the Live/Dead *BacLight* staining kit (Molecular Probes) and then visualised using an Eclipse

TE2000 inverted CLSM (Nikon Corporation). Experiments were carried out three times using independent bacterial cultures.

Image analysis and statistics. Ten images were taken at random points on each of the surfaces. Single images were taken on the smooth titanium plates while multi-layer stacks composed of 15 2D images were acquired on the moderately rough surfaces. Image analysis to determine biofilm biovolume was performed using the bioImage_L software package (Chavez de Paz, 2009). The results were analyzed using the Mann Whitney U Test and one-way ANOVA with the Bonferroni post-test. A confidence interval of 95 % was chosen and p values below 0.05 were considered significant.

Preparation of cell wall proteins. Mid-exponential growth phase cells were washed with PBS and centrifuged at 2000 g for 10 min at 4 °C. The pellet was then re-suspended in 0.2 % sulfobetaine (3-10) and shaken for 1 h at 100 rpm, 28 °C before being centrifuged at 6000 g for 10 min at 4 °C. After washing three times in ultrapure water, the cells were re-suspended in spheroplasting buffer (20mM Tris-HCl, pH 6.8 containing 10mM MgCl₂ and 26% w/v raffinose), 100 U ml⁻¹ mutanolysin (Sigma) added and the sample incubated for 75 min at 37 °C followed by 20 min at 60 °C. Samples were placed on ice before being centrifuged at 12000 g for 20 min at 4 °C. The supernatant was then dialysed against ultrapure water and freeze-dried. The resulting material was dissolved in 2DE rehydration buffer [8 M urea, 2 % CHAPS, 10 mM DTT, 2 % IPG buffer (GE Healthcare Life Sciences)] and stored at -20 °C until subjected to 2DE.

2DE. The protein concentration in the extracts was determined using a 2D Quant kit (GE Healthcare Life Sciences). A volume corresponding to 20 µg protein was diluted with rehydration buffer and placed in a swelling cassette with the 18 cm pH 4-7 linear IPG strips (GE Healthcare Life Sciences) on top. Rehydration was carried out at room temperature for

30 h under silicone oil. Isoelectric focusing was carried out using a Multiphor II (GE Healthcare Life Sciences) with cooling water at 15 °C supplied by Pharmacia Multitemp II. The focusing was initiated at 150 V for 1 h and continued at 300 V for 3 h, 600 V for 3 h, 1200 V for 12 h and finally 3500 V for 20 h. After focusing, the IPG strips were stored at –80 °C. Before being run in the second dimension, the IPG strips were equilibrated first in 50mM Tris buffer pH 6.8 containing 2 % SDS, 26 % glycerol and 16 mM DTT for 15 min and then in 50 mM Tris buffer pH 6.8 containing 2 % SDS, 26 % glycerol, 250 mM iodoacetamide and 0.005 % bromophenol blue for another 15 min. The equilibrated IPG strips were embedded on top of 7 % polyacrylamide gels (20 x 20 x 0.1 cm) using 0.5 % (w/v) molten agarose. SDS-PAGE was performed at a constant current of 15 mA gel⁻¹, 10 °C, overnight in a PROTEAN II xi cell (Bio-Rad) with rainbow high-range molecular mass standards (GE Healthcare Life Sciences) run on the acidic side of the IPG strips. Gels were stained with Coomassie brilliant blue or silver according to the relevant protocols from GE Healthcare Life Sciences. All gels were run in triplicate and only protein spots occurring in all gels were considered further.

Identification of proteins on 2DE gels using LC-MS/MS. Proteins of interest were excised manually from Coomassie brilliant blue stained gels and tryptic peptides subjected to LC, followed by MS/MS as described previously (Davies *et al.*, 2009). Mass lists were used as the input for Mascot MS/MS Ions searches of the NCBI database using the Matrix Science web server (www.matrixscience.com).

RESULTS

Characteristics of titanium surfaces. The interferometer analysis of surface topography revealed that the turned surfaces were smooth, with an S_a of 0.1 μm , whereas the blasted surfaces demonstrated a moderate surface roughness (S_a of 1.4 μm) (Fig. 1). Measurements of surface parameter S_{ds} , revealed similar values for both surfaces (≈ 150000 summits μm^{-2}) while S_{dr} for turned surface was low ($2.8 \% \pm 0.2$) compared to that for the blasted surfaces ($58 \% \pm 9.2$). Since S_a and S_{ds} both contribute to S_{dr} and the density of summits was almost the same for both surfaces, the difference in developed interfacial surface area ratio can be attributed to differences in the surface roughness. These data show that, due to the greater degree of roughness of the blasted surface, the total surface area available for bacterial adhesion was 1.5 times greater than that on the smooth surfaces.

The wettability and hydrophobicity of uncoated as well as the saliva- and serum-coated smooth titanium surfaces were investigated using contact angle measurements. The mean contact angle ($\pm$ standard error) for sessile water drops on the uncoated smooth surfaces was $74.2^\circ \pm 4.9$, indicating that the surface is rather hydrophilic (Förch *et al.*, 2009). For the saliva and serum coated surfaces, the mean contact angle measured after 30 minutes was similar to that obtained after 60 minutes incubation at 30 °C suggesting that the values had reached a plateau after 30 minutes. The mean contact angle ($\pm$ standard error) for surfaces coated with saliva or serum were $36^\circ \pm 1.0$ and $31^\circ \pm 1.2$, respectively demonstrating that the surfaces became more hydrophilic when coated with saliva or serum, but there was no major difference in surface energy between the two coatings.

Effect of surface roughness on adherence to titanium surfaces and differences between

strains. As revealed by the representative CLSM images shown in Fig. 2, all bacteria showed a high level of viability (>99%). On uncoated surfaces, all strains of *S. oralis* demonstrated a greater level of bacterial adherence to moderately rough surfaces than to smooth ones. The images were subjected to image analysis in order to quantify the number of adhered cells on each surface. In order to enable comparison of adherence to the two topographically different surfaces (S_{ar} : smooth surface, 2.8 %; moderately rough surface, 58 %) the number of attached cells was calculated after correction for the differences in actual surface area. The results obtained confirmed that the level of adherence for strain LA11 on moderately rough surfaces was five times greater than that on smooth surfaces. Similarly, for strains 89C and 192B, the bacterial adherence to moderately rough surfaces was greater than that to the smooth ones. For both strains 89C and 192B, the mean number of attached cells on the moderately rough surfaces was approximately twice that on the uncoated smooth ones. For all three strains, the differences between adherence to moderately rough and smooth surfaces were significant (strain 89C, $p < 0.05$; strains LA11 and 192B, $p < 0.0001$)(Fig. 3). Thus although all three strains of *S. oralis* adhered at higher levels to moderately rough than to smooth surfaces, the actual levels of adherence varied between them with strain 89C showing the greatest, and strain 192B the lowest, mean number of attached cells (Fig. 3).

Effect of saliva- or serum on adherence of different *S. oralis* strains to titanium surfaces.

Initial experiments using moderately rough surfaces revealed marked differences between adherence of the three strains in the presence of 25 % saliva or 5 % serum coatings. Representative CLSM images of bacteria stained with Live/Dead *BacLight* are presented in Fig. 4. For strains LA11 and 89C, coverage on saliva-coated surfaces was much greater than

on those coated with serum whereas for strain 192B, there was no obvious difference between them. Image analysis confirmed that mean number of attached cells for strain LA11 on moderately rough surfaces coated with saliva was 7 times greater than that on the same surfaces coated with serum. The mean number of adhered cells in the presence of the saliva coating was also significantly increased ($p < 0.0001$) over that on the uncoated surface, whereas for serum there was no significant change (Fig. 3). Strain 89C also demonstrated a significantly ($p < 0.0001$) higher mean level of adherence to surfaces coated with saliva than to uncoated surfaces or those coated with serum (Fig. 3). In contrast to strains LA11 and 89C, the mean number of adhered cells for strain 192B was generally low and was not significantly greater on saliva-coated surfaces than on uncoated or serum-coated ones. Similar results were observed on the smooth surfaces, where saliva enhanced adherence for strains LA11 and 89C, but not strain 192B. As for the moderately rough surfaces, serum coating had no significant effect on level of adherence for any of the strains (Fig. 3).

Identification of cell wall proteins from *S. oralis* strains. Since the *S. oralis* strains showed differences in their binding capacity to saliva, the presence of potential adhesins was investigated by comparing cell wall protein preparations (which include LPXTG-linked adhesins) from the different strains. In the analysis of the 2DE gels, special attention was paid to differences in proteins in the M_w greater than 200 kDa. In strain LA11, two protein spots with M_w of around 200 kDa were identified (Fig. 5a). Both these were also present in cell wall extracts from strain 89C (Fig. 5b) but not strain from 192B (Fig. 5c). Using LC-MS/MS, these spots were shown to correspond to a novel protein comprised of 1060 amino acids (SOR_0366). Analysis of structural elements within the amino acid sequence revealed the presence of a membrane-spanning sequence and a sequence of positively charged amino acids at the C-terminal end. An LPXTG-motif was also present in the C-terminal domain,

suggesting that this corresponds to a cell wall anchored adhesin. The N-terminal extracellular domain contained three tandem repeats of an 84-amino acid sequence as well as a number of degenerate repeats. At the extreme N-terminus, there was a unique, alanine-rich region containing 424 amino acids. This structural pattern is similar to that of cell surface adhesins from other streptococcal species (Davies *et al.*, 2009).

DISCUSSION

In this study, we have investigated the early stages of biofilm formation on dental implants using a flow-cell model to evaluate the adherence of *S. oralis* strains to titanium surfaces with different topography. Coverage by the *S. oralis* strains was 2-5-fold greater on the moderately rough than on the smooth surface (Fig. 2&3). This result was obtained after compensation for the greater developed interface area ratio (S_{dr}) (*i.e.* the percentage enlargement if the surface was flattened out) of the moderately rough as compared to the smooth surface. Thus the greater total surface area of the moderately rough surface cannot wholly account for the greater level of adhesion suggesting that the topography of the surface, with peaks and troughs, provided the bacteria with protection from removal by shear forces (Quirynen & Bollen, 1995). Without correction for S_{dr} , the actual numbers of bacteria adhering to the moderately rough surfaces was 3-8 fold greater than to the smooth ones. These results are consistent with findings from other *in vitro* studies using the early colonizer, *Streptococcus sanguinis*, (Pereira da Silva *et al.*, 2005) as well as for plaque accumulation on titanium dental implants *in vivo* (Burgers *et al.*, 2010; Elter *et al.*, 2008; Quirynen & Bollen, 1995).

Serum-derived proteins had no significant effect upon the adherence of any of the three *S. oralis* strains investigated as compared to the uncoated surfaces. This in agreement with other

studies showing that serum had either no effect or a small inhibitory effect upon adherence of other Gram positive bacteria including *S. mitis*, *Staphylococcus aureus* and coagulase negative staphylococci (Muller *et al.*, 2007; Paulsson *et al.*, 1993). Strains LA11 and 89C, but not strain 192B, adhered at significantly greater levels on saliva-coated surfaces than on either uncoated, or serum-coated ones. The greatest overall increase in adherence was seen for strain LA11 on the smooth surface where the presence of saliva increased bacterial coverage more than 12-times compared to the uncoated surface. These data suggest therefore that the *S. oralis* strains were representatives of two groups: one group which bound well to salivary proteins (strains LA11 and 89C) and one which showed little or no binding (strain 192B). Interestingly, binding capacity could not be related to the site of isolation since the two isolates from individuals with peri-implantitis fell into different groups. In a previous *in vitro* study, using a similar model to that used here, (Edgerton *et al.*, 1996) a fresh isolate of *S. oralis* recovered from titanium implant plaque showed lower adherence to saliva-coated than to uncoated titanium, in agreement with the pattern seen here for strain 192B. On the contrary, it has been reported that other strains of *S. oralis* bind well to a range of saliva-coated dental materials (Meier *et al.*, 2008), as seen for LA11 and 89C. Other oral streptococci such as *S. mitis*, *S. sanguinis* or *S. mutans* have also been reported to show different binding in different studies with some reporting decreased levels (Ahn *et al.*, 2002; Lima *et al.*, 2008; Pratt-Terpstra *et al.*, 1989; Mei *et al.*, 2011) in the presence of saliva and others reporting enhanced adherence (Nikawa *et al.*, 2006). Thus, overall the data suggest that differences between strains of oral streptococci may be as great as those between streptococcal species.

After coating, the surface hydrophobicity of the titanium surfaces was similar irrespective of whether the protein layer was derived from serum or saliva and thus the greater adherence of

299 *S. oralis* strains LA11 and 89C to saliva than to serum-coated surfaces cannot be attributed to
300 differences in the substratum surface free-energy. Differences in binding of *S. mitis* and *S.*
301 *gordonii* to serum and saliva-coated surfaces has been proposed to be due to differential
302 expression of adhesins (Muller *et al.*, 2007). In this study, we have shown the presence of an
303 LPXTG-linked adhesin with an approximate M_w of 200 KDa in strains LA11 and 89C,
304 which was not present in strain 192B. *In silico* analysis of the *S. oralis* genome reveals the
305 presence of three putative LPXTG-linked adhesins containing 1707, 1359 and 1060 amino
306 acids respectively. Both the putative 1707 and 1060 amino acid adhesins have been predicted
307 in *S. oralis* strain Uo5 whereas the 1359 amino acid is the only putative adhesin in strain
308 ATCC 35037. The protein identified in *S. oralis* strains LA11 and 89C, corresponds to the
309 1060 amino acid-containing adhesin and to our knowledge this is the first time that this
310 putative adhesin has been identified at the protein level. We suggest that this adhesin is
311 responsible for mediating specific interactions between the *S. oralis* strains and salivary
312 molecules on titanium surfaces. Salivary proteins to which *S. oralis* has previously been
313 shown to bind include MUC7, proline-rich proteins and amylase (Murray *et al.*, 1992) but
314 further studies are now required to identify the components that interact with this novel
315 adhesin.

316
317 While surface topography undoubtedly influences plaque accumulation, this process is also
318 affected by the thin film of salivary proteins and/or proteins from GCF that coat dental
319 implants exposed to the oral environment. In this study, the overall adherence of LA11 and
320 89C to the moderately rough surfaces coated with saliva was more than twice that on the
321 smooth saliva-coated surfaces. This clearly demonstrates that surface topography is, at least
322 to some degree, maintained in the presence of a saliva-coating. Thus, as proposed by Mei *et*
323 *al.*, 2011, the greater surface area of the saliva-coated moderately rough surface gives rise to

a larger contact area and an increased number of possible binding sites as compared to the smooth surface.

In conclusion, the data suggest that implants with moderately rough surfaces, that have been developed to promote osteoblast adhesion (Anselme & Biggerelle, 2005) and differentiation of osteoblast progenitor cells (Wall *et al.*, 2009) have, in themselves, a greater propensity for retention of adhered bacteria. Bacterial adherence on these surfaces would be compounded by the presence of a salivary film to which bacterial species/strains which express adhesins for salivary molecules can bind. Taken together, these factors would make moderately rough surfaces a less appropriate alternative from the perspective of plaque accumulation, than smooth ones.

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Figure legends

Figure 1. Interferometry images of the titanium surfaces.

Figure 2. CLSM images showing adherence of three strains of *S. oralis* on titanium surfaces after 2 hours. Bacteria were visualized using the BacLight Live/Dead stain.

Figure 3. Graphs showing mean values $\pm$ se of adherence for three strains of *S. oralis* to uncoated moderately rough and smooth titanium surfaces as well as in the presence of a saliva- or serum- coating. Data from three independent experiments were analyzed using the Mann Whitney U test (** = $p < 0.01$ and **** = $p < 0.0001$).

Figure 4. 3-D CLSM images illustrating adherence of three strains of *S. oralis* on moderately rough surfaces coated with either saliva or serum. The bars in all panels represent 40 μ m.

Figure 5. 2DE of cell wall proteins from three strains of *S. oralis*. Arrows indicating LPXTG-linked protein. Reference proteins are circled: 1, DnaK; 2, GroEL; 3, trigger factor.

Turned

Blasted

LA 11

89 C

192 B

Moderately rough
surface

Smooth surface

Moderately rough
surface

Smooth surface

Figure 4

Figure 5