Phenotypic adaptation in early bacterial colonizers on oral surfaces.

- **An *in vitro* study.**

Sylvia Húnfjörd
Jenny Olsson

Mentor: Julia Davies, Prof.

Master thesis, 30 credits
Program of dentistry
February 2016

Malmö University
Faculty of Odontology
205 06 Malmö

ABSTRACT

Oral bacteria, such as the early colonizers; *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis* and *Actinomyces naeslundii* display a wide range of surface adhesins which enable them to bind to receptors in the tooth pellicle. Saliva, gingival crevicular fluid (GCF) and collagen I in cementum on uncovered root surfaces present possible binding sites in the oral cavity onto which microorganisms can adhere and form a biofilm. The aim of this study was to assess whether these selected bacteria can alter their gene expression in response to protein components found on the various surfaces. *In vitro* laboratory assays were conducted, where the four oral species were added to surfaces coated with three substrates; human saliva, human serum and collagen I. The degree of induced proteolytic activity, surface-associated as well as secreted, was subsequently assessed using a FITC-labelled protease substrate, radial diffusion assays on skim milk agar and spectrophotometry. The hypothesis underlying the study was that bacterial species adapt depending on the surfaces they adhere to, thus altering protease expression. Based on the results, small variations could be detected, although no firm conclusion can be drawn regarding proteolytic abilities of the selected bacteria when exposed to the surfaces tested here.

Fenotypförändringar hos tidiga kolonisatörer på orala ytor

- ***In vitro*-studie.**

Sylvia Húnfjörd
Jenny Olsson

Handledare: Julia Davies, Prof.

Masteruppsats, 30 hp
Tandläkarprogrammet
Februari 2016

Malmö Högskola
Odontologiska fakulteten
205 06 Malmö

Sammanfattning

Orala bakterier, såsom de tidiga kolonisatörerna; *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis* och *Actinomyces naeslundii* uttrycker ett brett spektrum av ytadhesiner som möjliggör inbindning till receptorer i tandpellikeln. Saliv, gingivalt exudat (GCF) och kollagen I i cement på blottade rotytor erbjuder möjliga ytor i munhålan där bakterier kan adherera och bilda biofilm. Syftet med denna studie var att undersöka huruvida utvalda bakterier kan förändra genuttryck beroende på innehållet i olika ytor. Laborativa *in vitro*-försök genomfördes där de fyra bakteriearterna tilläts växa på ytor täckta med de tre olika substrat; saliv, serum och kollagen I. Graden av inducerad proteolytisk aktivitet, yt-associerad såväl som utsöndrad, uppskattades därefter med hjälp av ett FITC-konjugerat substrat, radiell diffusionsteknik samt spektrofotometri. Enligt studiens hypotes skulle bakteriearterna anpassa sig beroende på ytan de fäste till, och därigenom ändra metabol aktivitet såsom proteasuttryck. Baserat på resultaten kunde små förändringar noteras. Dock kunde inga bestämda slutsatser dras vad gäller förändrad proteolytisk förmåga hos de utvalda bakterierna exponerade för de olika orala ytorna i studien.

CONTENTS

Abstract.....2

Abstract Swedish.....4

List of abbreviations.....6

Introduction.....7

Pellicle and biofilm formation
Early colonizers
Bacterial niches and metabolism
Hypothesis

Material and Method.....10

Bacterial cultivation and preparation
Preparation of substrates and surface coating
- Saliva
- Human serum & collagen I

Biofilm model
Measurement of proteolytic activity
- FITC-casein
- Skim milk agar
- Radial diffusion assay
- Spectrophotometry

Results.....13

Methodological experiments for measuring proteolytic activity
- FITC-casein
- Skim milk agar
- Radial diffusion assay
- Spectrophotometry

Discussion.....18

Conclusion.....20

Acknowledgements.....20

References.....21

ABBREVIATIONS

Ag I/II	Antigen I/II-family of adhesins
CSLM	Confocal scanning laser microscope
FITC	Fluorescein isothiocyanate
GCF	Gingival crevicular fluid
IgA	Immunoglobulin A, secreted human antibody
MUC5B	Human high-molecular-weight salivary glycoprotein
MUC7	Human small salivary mucin
RFU	Relative fluorescent units
PBS	Phosphate-buffered saline
PRP	Proline-rich salivary protein

INTRODUCTION

Pellicle and biofilm formation

The microbiota in the oral cavity exists in biofilm communities that are formed through a dynamic process where early colonizing species promote binding of new microbes. This often results in a diverse and mature plaque (1). Early colonizers are therefore important in determining the nature of the developing biofilm. In order for oral bacteria to adhere and colonize surfaces in the oral cavity the formation of a pellicle is required (2).

The acquired enamel pellicle comprises proteins and glycoproteins originating from different fluids in the oral environment, which adsorb onto teeth and mucosal surfaces. The composition of the pellicle will differ depending on the site of formation in the oral cavity and depending on the main source of proteins and molecules (3). Research has shown that molecules derived from saliva are the main pellicle component on occlusal and incisal surfaces away from the gingival margins (3). Components derived from saliva include MUC5B, amylase, lysozyme and PRP (4).

An additional source of components contributing to enamel pellicle formation is GCF which originates from blood plasma and enters the oral cavity though the junctional epithelium. Molecules from GCF include γ-globulin, transferrin, albumin, hemoglobin, hemopexin, haptoglobulin and fibrinogen (5). It has been documented that pellicles formed in proximity to the gingival margin comprise largely molecules derived from GCF (3).

In addition to the enamel surfaces of intact teeth, root surfaces may also be exposed in the oral cavity as a result of retraction of the gingiva. Cementum on the dental root surfaces comprises roughly 50 % hydroxyapatite mineral and the remaining 50 % is organic compounds, mainly collagen I (6). The collagens belong to a family of large proteins that form the basis of many tissues in the human body. Thus, a root surface exposed to the oral cavity could present collagen I as a possible binding site for the oral microbiota.

Following pellicle formation, bacteria can adhere with varying affinites to surfaces in the oral cavity. The nature of proteins in the pellicle can affect binding strength (7). Bacterial species possess different surface binding proteins, so-called “adhesins” that interact and mediate attachment to molecules in the varying pellicles (8). The pellicle composition determines what microbiota can colonize the surface and initiate further biofilm formation (9). Thus, one factor determining at what site microbes find their most favorable niche is the components of the acquired pellicle (10). The process of biofilm formation on a pellicle coated surface is initiated by microorganisms commonly referred to as early colonizers (2).

Early colonizers

Early colonizers normally found in the oral cavity are *Streptococcus gordonii, Streptococcus oralis* and *Streptococcus mitis* which all belong to the Gram-positive *Streptococcus mitis* subcategory. These species are known to be facultative anaerobes associated with early plaque accumulation and have been demonstrated to comprise a substantial portion of the oral microflora (11). Another important feature of the *mitis* subcategory is their ability to adapt and cause infections or disease, making them opportunistic pathogens (11). *Actinomyces naeslundii,* belonging to the family of *Actinomycetaceae,* is another type of bacteria frequently found in initial biofilms (11,12). *A. naeslundii* is one of many species in the

Actinomyces subcategory and is a Gram-positive, non-motile, facultative anaerobic and pleomorphic rod (12).

In early plaque there is balance between species growing and interacting with each other in symbiosis. This equilibrium can shift due to exogenic and endogenic factors which alter the structure within the biofilm community (13). Due to the facultative anaerobic properties of early colonizers, consumption of oxygen during plaque development enables them to create an environment rich in carbon dioxide and hydrogen. This will favor species that prefer more anaerobic surroundings (14). Bacterial coaggregation, foodwebs and release of antibacterial substances promote colonization of diverse microbiota (15). An initial biofilm comprising mostly early Gram-positive cocci can therefore undergo an ecological shift favoring growth of Gram-negative anaerobic and possible opportunistic pathogens. This theory is the basis for the so-called "ecological plaque hypothesis" (14).

As mentioned previously, oral bacteria possess various surface proteins that enable attachment to components in the acquired pellicle through electrostatic and hydrophobic interactions. These are dependent on the environment. *S. gordonii* possesses the surface adhesins SspA and SspB that belong to the antigen I/II family (Ag I/II). These proteins are known to show affinity for collagen I and fibrinogen which are found in cementum and GCF respectively (16). Much like *S. gordonii, S. oralis* exhibits adhesins of the Ag I/II family, which have strong affinity for molecules such as glycoproteins derived from saliva (17). Amylase-binding properties observed in *S. mitis,* enable adherence to molecules derived from saliva. Assays have also shown that *S. mitis* binds fairly well to collagen I, although the mechanism behind this binding is still unknown (7). *A. naeslundii* exhibits cell-surface type-1 fimbriae that promote adhesion to PRPs and statherins in the salivary pellicle (18).

Differences between oral microbes and the adhesins they possess will to some extent determine what surface they adhere to. A study has found that *S. gordonii* and *S. mitis* adhere with high affinity to collagen I-coated surfaces compared with *S. oralis* and *A. naeslundii.* Furthermore, *S. gordonii* adheres to a significantly higher degree to serum than the other three species (7).

Bacterial niches and metabolism

Environmental factors of importance to colonizing bacteria are *e.g*. fluctuations in pH, nutrient supply, temperature and oxygen levels (13). Such changes, that consequently alter activity within the biofilm, can be brought on by poor oral hygiene, food intake and conditions such as gingivitis. Oral microbes are sensitive to changes in their environmental surroundings and possess the ability to adapt through alterations in gene expression (13).

Depending on the preferred source of nutrients and differences in pellicle composition, species colonize different sites in the oral cavity. In addition to providing binding sites, the acquired pellicle also functions as a nutrient source for the adhering bacteria (19). Bacteria residing in biofilms show proteolytic activity that enables them to use salivary proteins as a nutrient source (20). Bacterial proteometabolism describes the ability to degrade and metabolize proteins through hydrolysis of peptide bonds resulting in the formation of smaller polypeptides (21). A large range of proteolytic enzymes has been identified. Exopeptidases active outside the bacterial cell membrane degrade large protein fragments into peptides small enough to be transported through the cell membrane for further degradation and metabolism

by endopeptidases within the bacteria (11). Certain exopeptidases are surface-associated while others have been found to be secreted (22). Proteins are large molecules and a wide variety of enzymes are needed in order to degrade them into smaller peptides. Oral bacteria have been shown to benefit from enzymes expressed by co-aggregating species, giving them the ability to use a wider range of molecules as possible nutrients than when relying only on their own limited range of enzymes (20). Previous studies have focused on specific proteases (23,24), but limited data is available regarding how proteolytic activity in early colonizing species can differ in relation to surface adherence.

Hypothesis

The purpose of this study was to analyze four species of common oral bacteria and compare how their proteolytic activity, membrane-associated as well as secreted, varied depending on the surface they adhered to. Three surface substrates were selected: saliva, human serum and collagen I. These were chosen in order to simulate tooth surfaces coated with GCF or saliva derived pellicles and cementum on exposed root surfaces. Primarily due to their early colonization features the following four species were selected: *Streptococcus gordonii, Streptococcus oralis, Streptococcus mitis* and *Actinomyces naeslundii.*

The hypothesis of this study was that phenotype changes could be induced by variations in bacterial environments and depending on the predominant nutrient source (as represented by the surface coating). The four different bacteria were expected to express varying degrees of proteolytic activity when compared to each other. Furthermore, each bacterial species would show differing degrees of protease expression depending on what surface they had adhered to.

MATERIAL AND METHOD

Bacterial cultivation and preparation

The following isolates of oral bacteria were used in the study: *S. oralis, S. mitis, S. gordonii* and *A. naeslundii. S. oralis* LA11 (25) and *S. mitis* biovar 2 (26) were both isolated from dental plaque. *S. gordonii* was of the type strain DL1 and *A. naeslundii* CL a fresh isolate from plaque. These strains were extracted from a milk solution where they had been stored at -80° C. On transfer to horse blood agar (Brain Heart Infusion agar (Acumedia) 52 g, Yeast extract (Acumedia) 5 g, 500 mg Cystein-HCl, defibrinated horseblood 50 ml and hemin solution 10 ml, per litre Aq. dest) they were left for 72 hours for growth in 5 % CO_2 in air at 37° C. Thereafter the bacteria were transferred into Todd Hewitt broth (Todd Hewitt broth (Difco) 30 g/L) and left to grow for 72 hours in 5 % CO_2 in air at 37° C.

Preparation of substrates and surface coating

Mini-flow cells (Ibidi GmbH, Martinsriet, Germany) were coated with three substrates: human saliva (see section 3.2.1), human serum and human collagen I. Flow-cells were also incubated with PBS (NaCl 4.0g, K_2HPO_4 1,21g, KH_2PO_4 0.34g, per litre Aq. dest).

Saliva

Whole, unstimulated human saliva was collected from ten healthy individuals over 30 min into containers placed on ice. Ethical approval for the collection was granted by the Faculty of Odontology, Malmö University. The saliva was pooled, mixed 1:1 with 0.2 M NaCl, and lightly stirred overnight at 4°C. Using a Beckman Coulter Avanti J-E (Beckman JA 20 rotor; Beckman Coulter, Fullerton, CA; 4°C, 20 min, 4400 x g) the sample was centrifuged. The resulting supernatant was subjected to isopycnic density-gradient centrifugation in CsCl/O,1 M NaCl in a Beckman Coulter Optima LE80K Ultracentrifuge [Beckman 50,2 Ti rotor, starting density 1,45 g ml^{-1}, 15°C, 90 h, 36.000 r.p.m as described previously (25)]. Fractions were recovered from the bottom of the tube and analyzed for density by weighing, absorbance (A_{280}) and their content of MUC5B and MUC7.

Fractions not containing bacteria were pooled, dialyzed against PBS and stored at -20°C until used. The saliva was diluted with PBS 1:4 before coating the flow-cells.

Human serum and collagen I

Human serum (Human serum, type AB, heat inactivated; 092938249 MP Biomedicals) was diluted 1:10 and used as a model for GCF. Collagen I (Cohesion, concentration 2.9 mg/ml, CA, USA) was diluted 1:100 with sterile water before coating.

Biofilm model

Under sterile conditions 120 µl of the four coatings (saliva, serum, collagen I or PBS) were pipetted in each Ibidi-channel. The mini-flow cells were left for 24 hours in a laminar flow cabinet to allow coating under sterile conditions. Subsequently, 90 µl of fluid was extracted, thus leaving 30 µl of fluid in each mini-flow cell. Thereafter the channels were rinsed once with 120 µl PBS prior to adding the bacterial solutions. Having determined optical density at 600 nm to approximately 0.2 (corresponding to 10^7 bacteria/ml) of the bacterial solutions, 120 µl of each species were inserted into the channels.

In addition, two assays were performed for comparative reasons. One included analysis of Ibidi-channels coated simply with substrates and no added bacteria. The other assay was conducted coating Ibidi-channels with heat-treated and centrifuged saliva and serum. The treatment consisted of heating the substrates to approximately 70° C for 1h and centrifuging for 10 minutes at 2000 rpm.

Measurement of proteolytic activity

FITC-casein

FITC-casein was used as a general substrate to detect proteolytic activity, both surface-bound and secreted. Briefly, in its native state FITC conjugated to casein is hidden due to saturation of the complex. However, when exposed to hydrolysis by proteases, released FITC will cause emission of green light when excited with laser light (27). In theory, green light emission should be proportional to the level of proteolytic activity (28).

After 24 hours, 90 µl of fluid was extracted and the channels were rinsed three times with 120 µl PBS. In order to have 130 µl of fluid in the channels on addition of 0.7 µl of FITC-casein, 70 µl of PBS was added to each channel. The FITC-casein was left to react with the bacteria in the channels for two hours before adding 0.5 µl of counterstain and then analyzing directly using an Eclipse TE2000 CSLM (Nikon corp., Tokyo, Japan). Photographs were taken from each mini-flow cell.

Skim milk agar

Proteolytic activity can also be studied using skim milk agar plates. Bacterial proteases degrade skim milk in the agar and produce areas of translucency which mirror the degree of activity. Degradation of milk agar in proximity to bacterial colonies reveals surface bound proteases.

Bacterial cultivation, surface coating and microbial adhesion were performed as described above. After leaving the bacteria to adhere to the four, coated surfaces for 24 hours, 150 µl of supernatant was extracted from the mini-flow cells.

Skim milk agar plates were prepared. Briefly; 15.0g agar, 5.0g pancreatic digest of casein, 2.5g yeast extract and 1.0 glucose were added to deionized water and brought to a volume of 1 litre. After autoclaving, 100.0 ml sterile skim milk solution was added before the solution was distributed evenly into petri dishes. 20 µl of each bacteria collected from each of the four surfaces was smeared on half of a milk agar plate. The milk agar plates were placed in 5 % CO_2 in air at 37° C for 48 hours to allow bacterial growth. After 48 hours, the plates were analyzed visually and photographed.

Radial diffusion assay

A radial diffusion assay on skimmed milk plates was used to detect secreted proteases. The remaining bacterial samples were centrifuged for 10 minutes at 2000 rpm resulting in a supernatant and a bacterial pellet. Thereafter 50 µl of the bacterial supernatants were placed in wells punched into milk agar plates under sterile conditions. The plates prepared with the supernatant were placed at 37° C. After 48 hours the plates were analyzed visually and photographed.

Spectrophotometry

A spectrophotometer was utilized to measure bacterial proteases. 50 µl of the bacterial supernatants remaining, from the milk plate agar experiment, were stained with 1 µl FITC-casein and the absorbance at 488nm analyzed. The samples were then placed in a 37° C chamber and one hour later analyzed a second time using the same wavelength. Due to a longer period of interaction between supernatant and FITC-casein, fluorescence was anticipated to increase in proportion to time.

RESULTS

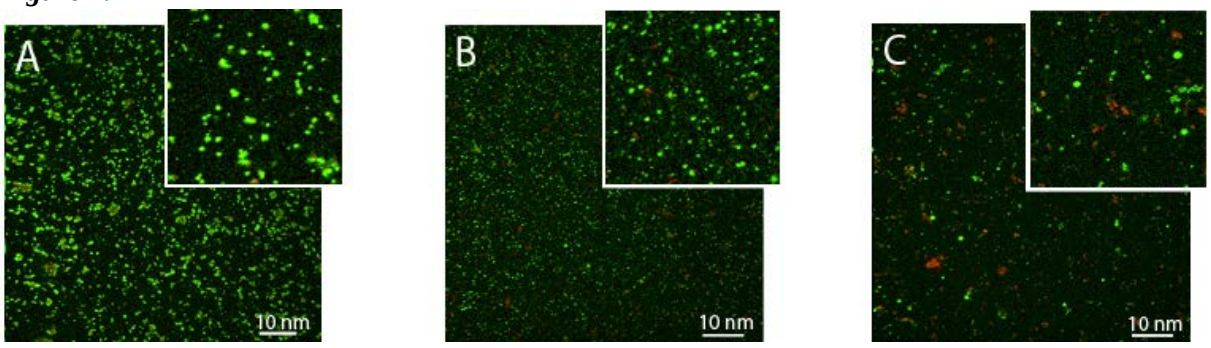
The purpose of this study was to analyze protease expression in four different oral bacterial species and investigate whether adhesion to different surfaces found in the oral cavity could cause phenotypic change, more specifically alterations in proteolytic activity. The experiment was carried out using the following bacteria; *S. gordonii, S. oralis, S. mitis* and *A. naeslundii*. These species adhered to four surfaces-coatings; saliva, human serum, collagen I or an uncoated control. The three methods described in *Material and Method* were used to examine both surface-bound and secreted bacterial protease expression.

Methodological experiments for measuring proteolytic activity

FITC-casein

In order to reveal surface-associated protease expression *in situ,* FITC-casein was added to bacteria adhered to coated mini-flow cells. On analyzing the channels microscopically it became clear that the FITC-casein complex was being activated in areas where no bacteria could be visualized (Fig. 1). Figure 1 shows *S. gordonii* adhered to saliva, human serum and collagen I coated surfaces. Surface-associated proteases produced by the microorganisms should degrade the FITC-casein complex resulting in the emission of green light. Green fluorescent light should therefore be found in proximity to adhered bacteria, stained in red. *S. oralis, S. mitis* and *A. naeslundii* all showed similarities in patterns of green light emission which were not consistent with bacteria stained in red.

Figure 1.



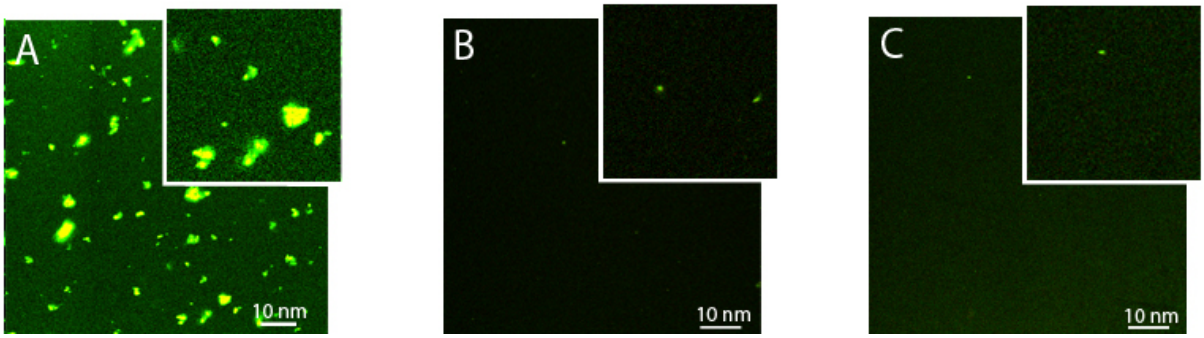
Confocal laser microscope images showing S. gordonii adhered to mini-flow cells coated with; a) saliva, b) serum or c) collagen I, stained with FITC-casein and counterstained.

A trial assay without bacteria was performed to investigate green light emission which was not consistent with the presence of bacteria. Mini-flow cells were coated with saliva, human serum and collagen I and FITC-casein added before analysis with CLSM. As no bacteria were present, no green fluorescence should be present. However, FITC-casein on surfaces coated only with saliva (Fig. 2) was activated, indicating the presence of proteolytic activity.

Further control experiments were then carried out to determine the cause of activation of the substrate. FITC-casein was added to surfaces coated with PBS as well as centrifuged and heat-treated saliva. The purpose of heating the substrate was to degrade possible protease residues, and centrifugation to minimize high density proteins. As a last measure, the same assays as above were performed using a different type of FITC-casein. However, results still showed sporadic fluorescence not coinciding with bacterial cells.

Thus, the method using direct observation was judged unreliable in order to determine surface bound proteases in this study.

Figure 2.



Surfaces coated with A) saliva, B) human serum and C) collagen I stained with FITC-casein. No bacteria present.

Skim milk agar

As the FITC-casein technique did not give adequate results, an alternative method was selected. Bacteria which had been growing on the different substrates in the mini-flow cells were harvested and grown on skim milk agar plates to analyze to what extent colonies could produce proteases. All bacterial species were treated and examined in the same fashion. Results of duplicate experiments are summarized in table 1. In cases where no growth was seen, it was not possible to assess proteolytic activity.

S. oralis harvested from the four different surfaces in the mini-flow cells did not shown any growth on the skim milk agar. Thus it was not possible to determine the level of cell-associated proteolytic activity on any of the surfaces.

Table 1.

	Smear			
	Saliva	Serum	Collagen I	PBS
S. oralis	-/-	-/-	-/-	-/-
S. mitis	+/+	+/+	-/+	-/+
S. gordonii	+/+	+/+	+/+	+/+
A.naeslundii	+/+	+/+	-/+	+/+

Results from analysis of two assays using skim milk agar plates smeared with bacterial samples collected from mini-flow cells coated with various substrates. (-) signifies no bacterial colonization, (+) bacterial colonization without signs of proteolytic activity, (+) bacterial colonization with proteolytic activity in proximity to colonies.

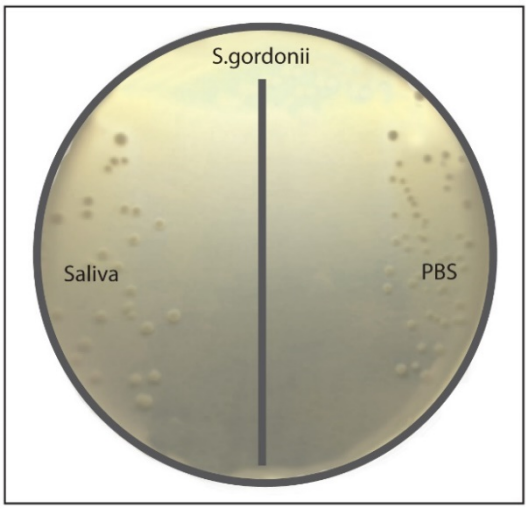
For ***S. mitis***, in all cases where growth was seen, colonies showed proteolytic activity on the milk agar. This demonstrates that *S. mitis* does express surface proteases. However, the fact that activity was present even in the bacteria that had been harvested from the uncoated surface indicates that this expression is not dependent on the nature of the substrate on which *S. mitis* is growing.

Similar to *S. mitis*, ***S. gordonii*** showed proteolytic degradation around bacterial colonies on the milk agar after growth on saliva, serum and collagen I coated surfaces (Fig. 3). Again, a clear zone was seen even after growth on the uncoated surface suggesting that *S. gordonii* expresses proteolytic activity in response to surface contact but that the response is not related to the nature of the surface.

After growth on saliva or serum-coated surfaces, ***A. naeslundii*** showed proteolytic activity in only one out of two assays. A similar result was seen after growth on the uncoated surface. When removed from collagen I coated mini-flow cells, no proteolytic activity was seen in the one experiment where analysis was possible.

When comparing the surface-associated activity of the four species, the results obtained here indicate that *S. mitis* and *S. gordonii* were the most proteolytically active on all surfaces. *A. naeslundii* showed proteolytic activity on saliva and serum, but not collagen I-coated surfaces, suggesting that the nature of the surface influences the activity. Due to lack of growth on the milk agar, no results were obtained for *S. oralis*.

Figure 3.



S. gordonii collected from saliva and PBS coated surfaces and smeared on skim milk agar plates. A transparent zone around colonies developed due to degradation by surface associated proteases.

Radial Diffusion Assay

To determine whether the bacteria produced proteases released into the surrounding medium, bacterial supernatants collected from mini-flow cells coated with various substrates were inserted into wells to allow proteolytic degradation of milk agar. This is seen as a transparent zone (halo) around the site of insertion. Three assays were performed and all bacterial species were treated and examined in the same fashion. Results are summarized in table 2.

S. oralis - Supernatants from *S. oralis* previously adhered to the different surfaces showed no detectable halo in proximity to any well in any of the three assays preformed. However, *S. oralis* revealed no growth when smeared on milk agar and the lack of protease activity can probably therefore be attributed to non-viability.

S. mitis - Supernatants extracted from saliva and collagen I presented negative outcomes in two out of three assays while one assay revealed proteolytic halo in vicinity to bacterial growth in the wells. Supernatant extracted from serum gave positive results in all three assays. However, two positive wells also revealed bacterial colonization. The absence of a proteolytic halo was noted in all three assays with supernatant gathered from bacteria growing on the uncoated surface. To summarize, the results indicate that secreted proteases in *S. mitis* could be induced by access to serum as a surface substrate and main nutrient source.

S. gordonii - One assay showed severe contamination of unknown bacterial growth, causing absence of results. Therefore, only two assays were considered for analysis (Table 2).

Table 2.

	Halo			
	Saliva	Serum	Collagen I	PBS
S. oralis	-/-/-	-/-/-	-/-/-	-/-/-
S. mitis	-/+/-	+/+/+	-/+/-	-/-/-
S. gordonii	+/*/-	+/*/+	-/*/+	-/*/-
A.naeslundii	-/-/*	+/-/-	-/-/-	-/-/-

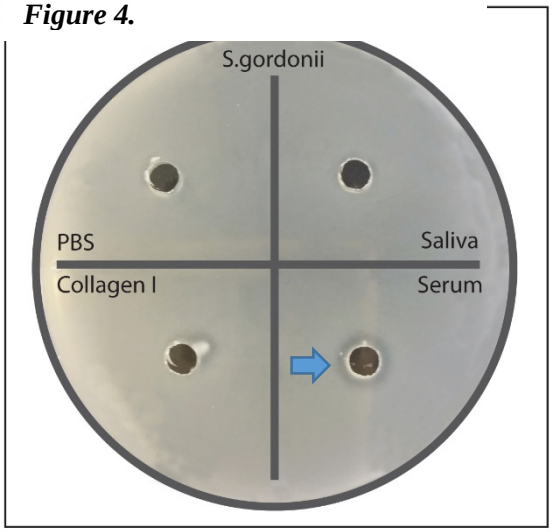
Results from analysis of three assays using skim milk agar plates after insertion of bacterial supernatants into wells. (-) signifies no bacterial colonization, (+) proteolytic in association with bacterial colonies, (+) true proteolytic halo without bacterial growth, (*) contaminated results.

When supernatant collected from saliva was analyzed, a proteolytic halo and bacterial growth was noted in one out of two assays. In one assay degradation could not be detected. Supernatant from a serum covered surface gave positive proteolytic activity in both trials, with one trial also revealing bacterial growth (Fig. 4). Supernatant from collagen I coated surfaces revealed the absence of clearing in one out of two trials, yet clearing was noted together with bacterial colonies in one assay. Furthermore, no clearing and no proteolytic activity was detected in any assay concerning supernatants from PBS. Thus, secreted proteases in *S. gordonii* were stimulated by previous growth on a serum-covered surface.

A. naeslundii – When analyzing supernatants from saliva, collagen I and PBS, no trial showed any clearing in proximity to the wells. One assay with supernatant collected from saliva was excluded due to contamination. A transparent zone was seen in vicinity to the serum well in one out of three trials, while in the two remaining trials no clearing was visible. Expression of secreted proteases in *A. naeslundii* may therefore have been induced by previous growth on a serum-coated surface.

Regarding the four bacteria's ability to produce secreted proteases, *S. mitis*, *S. gordonii* and *A. naeslundii* all revealed proteolytic activity. Interestingly, serum appeared to be the surface coating most capable of inducing enzymatic activity.

Figure 4.



Supernatants of S. gordonii inserted into wells on a skimmed milk agar plate. Arrow in the image shows translucency in proximity to the well containing supernatant collected tom a serum coated surface. Supernatants from bacteria grown on collagen I shows degradation but also bacterial growth. No clearing was visible in wells containing supernatants collected from PBS or saliva coated surfaces.

Table 3.

	Smear				Halo			
	Saliva	Serum	Collagen I	PBS	Saliva	Serum	Collagen I	PBS
S. oralis	-/-	-/-	-/-	-/-	-/-/-	-/-/-	-/-/-	-/-/-
S. mitis	+/+	+/+	-/+	-/+	-/+/-	+/+/+	-/+/-	-/-/-
S. gordonii	+/+	+/+	+/+	+/+	+/*/-	+/*/+	-/*/+	-/*/-
A.naeslundii	+/+	+/+	-/+	+/+	-/-/*	+/-/-	-/-/-	-/-/-

Results from three assays on skim milk agar. The symbols in the smear assay represent; (-) no bacterial growth, (+) bacterial colonization, (+) bacterial colonization and proteolytic activity, (*) contaminated results. The symbols in the halo assay represent: (-) signifies no proteolytic activity, (+) proteolytic activity in association with bacterial colonies, (+) true proteolytic halo without bacterial growth, (*) contaminated results.

Spectrophotometry

This method, similar to the radial diffusion assays, can detect only secreted proteases. In all the experiments the RFU, calculated as the difference between the initial absorbance value and that obtained after 1 hour, was under the cut off value (2000RFU) for reliable results (Table 4).

Table 4.

	Change in absorbance (RFU)			
	Saliva	Serum	Collagen I	PBS
S. oralis	-139	+209	+226	+224
S. mitis	-45	-179	-157	+7
S. gordonii	-369	-463	-214	+68
A.naeslundii	-232	-104	+13	+41

Results of FITC-casein added to bacterial supernatants and analyzed with spectrophotometry. Changes in absorbance over one hour measured in RFU.

Table 5.

	Spectrophotometer 0h				Spectrophotometer 1h			
	Saliva	Serum	Collagen I	PBS	Saliva	Serum	Collagen I	PBS
S. oralis	3490	1936	2118	1754	3351	2145	2344	1978
S. mitis	2349	1425	2303	1794	2304	1246	2146	1801
S. gordonii	2755	2071	1715	1977	2386	1608	1501	2045
A.naeslundii	1768	1792	1961	1903	1536	1688	1974	1944

Results of FITC-casein added to bacterial supernatants and analyzed with spectrophotometry. "Spectrophotometry 0h" shows initial measurements and "Spectrophotometry 1h" shows values one hour after initial measurement.

DISCUSSION

In this study, three methods were used to investigate the level of proteolytic activity in four early colonizers adhered to different surfaces. One method was the use of a general protease substrate linked to a fluorescent reporter. FITC-conjugated casein exposed to hydrolysis by proteases will emit green light (27), and in theory, green light emission should be proportional to the level of proteolytic activity (28). However, this method was shown not to be reliable due to the unexplained activation predominantly on saliva, and to a lesser extent also serum and collagen I coated surfaces. Theoretically, the activation could have been due to residual bacterial-secreted proteases after rinsing of Ibidi flow-cells. However, this explanation was disproven since emission of green light still occurred when FITC-casein was added to substrate-coated surfaces without bacteria. Another possibility was that proteases within the substrates were responsible for activation of FITC-casein. However, none of the control assays performed aiming to eliminate substrate proteases, including the use of a new batch of FITC and treating the substrates with heat and centrifugation could explain or eliminate the undesired activation of FITC-casein. The FITC-casein technique may therefore give misleading results concerning protease activity by bacteria bound to saliva-coated surfaces, as saliva activated the FITC-casein to a much higher degree than serum and collagen I. This is an interesting finding since *Wickström et al*. used a similar technique when analyzing proteolytic activity in *S. mitis* and *S. mutans* (29).

As well as for direct visualization of surface-associated proteolytic activity, the FITC-casein substrate was also used in the spectrophotometric analysis of secreted proteases. The RFU values obtained were very low and in some cases, decreased after one hour. It is thus likely that the data are not reliable and represent simply background noise. A possible explanation for the low measurement is that the levels of proteases secreted by the bacteria were too low in concentration for detection in the assay (30). Ideally, a baseline determining fluorescence of FITC-casein added to pure PBS without secreted proteases should have been established. This would have indicated the degree of background noise, thus providing information for more accurate analysis of results. Interestingly these data are not completely consistent with those from the radial diffusion assay which also assessed secreted proteases. Although no secreted proteases were seen in supernatants collected from saliva-coated, collagen-coated or the uncoated surface, activity was seen in response to the serum-coated surface in *S. mitis*, *S. gordonii* and *A. naeslundii*. This difference is most likely due to the different detection thresholds in the two assays, indicating that the radial diffusion technique is more sensitive to low concentrations of secreted proteases than spectrophotometry.

It has been suggested that *S. oralis* possesses cell-membrane-bound proteases (31). However, *S. oralis* did not colonize milk agar plates in any of the assays, nor exhibit any detectable enzymatic activity. This suggests that the species may not possess enzymes sufficient for survival in an environment strictly composed of saliva, human serum, collagen I or PBS. *In vitro, g*rowth of oral bacteria is enhanced by co-aggregation of various species (32). The conclusion that *S. oralis* lacks the ability to secret proteases is questionable. *S. oralis* may simply not be equipped to survive in monospecies culture in environments selected in this study and thus not able to exhibit proteases.

By expressing proteases, bacteria are able to degrade casein and form nitrogenous compounds leaving a clear zone around the bacterial colonies. The clear zone can be measured to

determine the degree of proteolytic activity. Smearing bacteria on milk agar reveals surface-bound proteases in a similar manner as FITC-casein. Cell-surface-associated protease activity in *S. mitis* biovar 2 is promoted by salivary proteins (29). This correlates to our findings where *S. mitis* appeared to upregulate surface-associated proteases in saliva and serum environments. An earlier publication reports that *S. mitis* displays proteases with the potential to degrade IgA, salivary proteins, casein, albumin and gelatin (24), thus reinforcing the theory that *S. mitis* may possess the ability to upregulate these proteases. In addition, since surface bound activity was noted on non-coated control surfaces, proteases seem to be present at all times on the surface of *S. mitis*.

S. mitis expresses IgA1 proteases (33) capable of degrade one of many protein components in serum. Interestingly, serum was the only surface that induced secreted proteases in *S. mitis.* These findings imply that the species is capable of changes in gene expression, resulting in a higher degree of proteolytic activity, both secreted and surface associated, when it has access to serum. *S. mitis* colonizing a surface close to the gingival margin rich in GCF will therefore be more proteolytic in comparison to when adhering to other surfaces in the oral cavity. The shift from a healthy periodontium to periodontal disease could thus to some extent be attributed to this species. Accessing salivary proteins only upgrades surface-bound and not secreted proteases.

In the radial diffusion assays, certain wells contained bacterial growth. The resulting zone of translucency can therefore not solely be assigned to secreted proteases, but to surface associated enzymes. The results from wells with bacterial growth can still be used to correlate the results from assays on surface-associated activity.

S. gordonii may constitutively exhibit membrane-bound proteases since high activity was noted on PBS, as well as saliva and serum-coated surfaces. Whether the proteolytic activity and possible phenotype changes in *S. gordonii* were influenced by the substrates is therefore difficult to determine. Although the FITC-casein method was disregarded, images taken with CLSM showed higher degrees of activity in bacteria grown on serum-coated surfaces when compared to collagen I, thus correlating with the results from skim milk agar assays. However, the species is able to degrade collagen analogues in a process affected by pH, oxygen and free amino acid concentration (24). The low proteolytic response to collagen I may therefore be due to less favorable levels of pH and aeration. Serum induced secretion of proteases from *S. gordonii*. This agrees with *Harty et al*. who found higher levels of secreted enzymes in *S. gordonii* when grown in a serum culture (34). The conclusion may therefore be drawn that this species becomes more pathogenic from a periodontal point of view when colonizing a pellicle-coated surface close to the gingival margin in the oral cavity.

A. naeslundii has been isolated from periodontal pockets, periapical lesions and caries lesions. This shows the species' ability to adapt and survive in environments that differ in pH, oxygen and nutrient sources (12). Membrane-bound proteolytic activity in *A. naeslundii* did not seem to be affected by the various surfaces. This may point to the species' limited capacity when grown monoculturally and its need for co-aggregation, in order to undergo phenotype change and express optimal enzymatic activity. Human serum was the only substrate inducing secreted proteases. This implies that *A. naeslundii* is able to thrive in sites rich in GCF, correlating to previous findings by Vielkind *et al*, 2015 (12).

Human serum appeared to be the surface coating most capable of causing a change in phenotype, resulting in bacteria with more proteolytic abilities. Sites where proteins are found in abundance, such as the gingival margin, may thus induce and harbor more proteolytically active bacterial phenotypes. This finding is not surprising considering that the selected species are known to be early colonizers that have been linked to gingivitis and early periodontitis (35).

Sources of error

Lack of detected proteolytic activity may not be due to absence of enzymatic activity, but because concentrations were too low to be measured using the methods selected in this study. It should be taken into consideration that all data from skim milk agar was analyzed visually and individual variations in interpretation may have occurred. Furthermore, the bacteria in this study were investigated separately whereas *in vivo* they grow together. Therefore, co-operative effects between the different species may have been overlooked in this work. Yet this study provides valuable baseline data concerning individual species, their proteolytic activity and possible phenotype changes.

CONCLUSION

The hypothesis states that four species of oral bacteria present varying degrees of proteolytic activity depending on whether they were adhered to surfaces coated with saliva, human serum or collagen I. Various molecules and proteins are available as possible nutrients for surface attached microbes depending on surface coating. Each bacterial species was expected to show a varying protease expression depending on what surface they previously had been adhered to. Moreover, different levels of proteolytic activity were expected when comparing various species to each other. The results showed no significant differences between the species and surfaces. However, small variations could be detected, though no firm conclusion could be drawn regarding proteolytic abilities of the selected bacteria when exposed to different surfaces and nutrients. How the ecological balance and maturation of a biofilm in the oral cavity may be influenced cannot be shown based solely on these results, largely due to the fact that this was a monocultural *in vitro* study. Assays performed and presented in this paper may prove helpful in choosing a viable method for future studies on proteolytic activity, thus using resources more cost-effectively. This paper can also serve as a platform for further studies on bacterial properties and the role they play in the development of periodontitis. A deeper knowledge of this disease and how it might be prevented would benefit not only the individual, but also the entire society and dental health care system.

ACKNOWLEDGEMENTS

Special thanks to the Department of Oral Biology at Malmö Högskola. This paper would not have been possible without the kindness, patients and help provided by professor Julia Davies and her esteemed colleagues.

REFERENCES

(1) Oral microbial ecology; current research and new perspectives. Reference & Research Book News 2013;28(2).

(2) Kolenbrander PE, London J. Adhere today, here tomorrow: oral bacterial adherence. J Bacteriol 1993 Jun;175(11):3247-52.

(3) Rudiger SG, Carlen A, Meurman JH, Kari K, Olsson J. Dental biofilms at healthy and inflamed gingival margins. J Clin Periodontol 2002 Jun;29(6):524-30.

(4) Yao Y, Berg EA, Costello CE, Troxler RF, Oppenheim FG. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. J Biol Chem 2003 Feb 14;278(7):5300-08.

(5) Cimasoni G. Crevicular fluid updated. Basel: S. Karger; 1983.

(6) Nanci A. Ten Cate's oral histology: development, structure, and function. St. Louis: Mosby Elsevier; 2008.

(7) Hunfjörd S, Olsson J. Adhesion of the early colonizers *Streptococcus gordonii, Streptococcus mitis, Streptococcus oralis* and *Actinomyces naeslundii* to saliva, human serum and collagen I coated surfaces - an *in vitro* study. 2014.

(8) Handbook of bacterial adhesion: principles, methods and applications. Totowa, N.J.: Humana Press; 2000.

(9) Siqueira WL, Custodio W, McDonald EE. New insights into the composition and functions of the acquired enamel pellicle. J Dent Res 2012 Dec;91(12):1110-18.

(10) Takahashi N. Microbial ecosystem in the oral cavity: Metabolic diversity in an ecological niche and its relationship with oral diseases. Int Congr Ser 2005 9;1284:103-12.

(11) Marsh P. Oral microbiology. Edinburgh: Churchill Livingstone Elsevier; 2009.

(12) Vielkind P, Jentsch H, Eschrich K, Rodloff AC, Stingu CS. Prevalence of *Actinomyces spp*. in patients with chronic periodontitis. Int J Med Microbiol 2015 Aug 21.

(13) Marsh PD, Devine DA. How is the development of dental biofilms influenced by the host? J Clin Periodontol 2011 Mar;38 Suppl 11:28-35.

(14) Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiology 2003 Feb;149(Pt 2):279-94.

(15) Marcotte H, Lavoie MC. Oral microbial ecology and the role of salivary immunoglobulin A. Microbiol Mol Biol Rev 1998 Mar;62(1):71-109.

(16) Jakubovics NS, Brittan JL, Dutton LC, Jenkinson HF. Multiple adhesin proteins on the cell surface of Streptococcus gordonii are involved in adhesion to human fibronectin. Microbiology 2009 Nov;155(Pt 11):3572-80.

(17) Murray PA, Prakobphol A, Lee T, Hoover CI, Fisher SJ. Adherence of oral streptococci to salivary glycoproteins. Infect Immun 1992 Jan;60(1):31-38.

(18) Li T, Johansson I, Hay DI, Stromberg N. Strains of *Actinomyces naeslundii* and *Actinomyces viscosus* exhibit structurally variant fimbrial subunit proteins and bind to different peptide motifs in salivary proteins. Infect Immun 1999 May;67(5):2053-59.

(19) Bowden GH, Hamilton IR. Survival of oral bacteria. Crit Rev Oral Biol Med 1998;9(1):54-85.

(20) Bradshaw DJ, Homer KA, Marsh PD, Beighton D. Metabolic cooperation in oral microbial communities during growth on mucin. Microbiology 1994 Dec;140 (Pt 12)(Pt 12):3407-12.

(21) Dorland WAN. Dorland's illustrated medical dictionary. Philadelphia, PA: Saunders; 2007.

(22) Travis J, Potempa J. Bacterial proteinases as targets for the development of second-generation antibiotics1. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 2000 3/7;1477(1–2):35-50.

(23) Cole MF, Evans M, Fitzsimmons S, Johnson J, Pearce C, Sheridan MJ, et al. Pioneer oral streptococci produce immunoglobulin A1 protease. Infect Immun 1994 Jun;62(6):2165-68.

(24) Juarez ZE, Stinson MW. An extracellular protease of *Streptococcus gordonii* hydrolyzes type IV collagen and collagen analogues. Infect Immun 1999 Jan;67(1):271-78.

(25) Wickstrom C, Svensater G. Salivary gel-forming mucin MUC5B--a nutrient for dental plaque bacteria. Oral Microbiol Immunol 2008 Jun;23(3):177-82.

(26) Dorkhan M, Chavez de Paz LE, Skepo M, Svensater G, Davies JR. Effects of saliva or serum coating on adherence of *Streptococcus oralis* strains to titanium. Microbiology 2012 Feb;158(Pt 2):390-97.

(27) Twining SS. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. Anal Biochem 1984 Nov 15;143(1):30-34.

(28) Neilands J, Wickstrom C, Kinnby B, Davies JR, Hall J, Friberg B, et al. Bacterial profiles and proteolytic activity in peri-implantitis versus healthy sites. Anaerobe 2015 Oct;35(Pt A):28-34.

(29) Kindblom C, Davies JR, Herzberg MC, Svensater G, Wickstrom C. Salivary proteins promote proteolytic activity in *Streptococcus mitis biovar* 2 and *Streptococcus mutans*. Mol Oral Microbiol 2012 Oct;27(5):362-72.

(30) Neilands J, Wickstrom C, Kinnby B, Davies JR, Hall J, Friberg B, et al. Bacterial profiles and proteolytic activity in peri-implantitis versus healthy sites. Anaerobe 2015 Apr 11.

(31) Lo CS, Hughes CV. Identification and characterization of a protease from *Streptococcus oralis* C104. Oral Microbiol Immunol 1996 Jun;11(3):181-87.

(32) Wickstrom C, Herzberg MC, Beighton D, Svensater G. Proteolytic degradation of human salivary MUC5B by dental biofilms. Microbiology 2009 Sep;155(Pt 9):2866-72.

(33) Mitchell J. *Streptococcus mitis*: Walking the line between commensalism and pathogenesis. Mol Oral Microbiol 2011 Apr;26(2):89-98.

(34) Harty DW, Mayo JA, Cook SL, Jacques NA. Environmental regulation of glycosidase and peptidase production by *Streptococcus gordonii* FSS2. Microbiology 2000 Aug;146 (Pt 8)(Pt 8):1923-31.

(35) Essentials of microbiology for dental students. 2nd ed. Oxford: Oxford University Press; 2006.