Adhesion-related interactions of *Actinomyces* and *Streptococcus* biofilm bacteria

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Till Elliot & Peter

"Even the sceptical mind must be prepared to accept the unacceptable when there is no alternative. If it looks like a duck, and quacks like a duck, we have at least to consider the possibility that we have a small aquatic bird of the family Anatidae on our hands"
Douglas Adams, *Dirk Gently's Holistic Detective Agency*

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This thesis is based on the following papers, referred to in text by their Roman numerals I – IV.

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A host-derived pentapeptide enhancing host-bacteria commensalisms and communication
Submitted and revised [*]*equal contribution*

II. **Mirva Drobni**, Ing-Marie Olsson, Christer Eriksson, Fredrik Almqvist and Nicklas Strömberg
Multivariate design and evaluation of a set of RGRPQ-derived innate immunity peptides
The Journal of Biological Chemistry, Vol. 28, No. 22, pp. 15164-15171, June 2, 2006

III. **Mirva Drobni**[*], Kristina Hallberg[*], Ulla Öhman, Anna Birve, Karina Persson, Ingegerd Johansson and Nicklas Strömberg
Sequence analyses of fimbriae subunit FimA proteins on *Actinomyces naeslundii* genospecies 1 and 2 and *Actinomyces odontolyticus* with variant carbohydrate binding specificities
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IV. **Mirva Drobni**, Anette Jonasson, Nicklas Strömberg and Ingegerd Johansson
Actinomyces and *Streptococcus* species display differential binding epitopes on acidic proline-rich protein PRP-1
Manuscript

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Abstract

Adhesion of bacteria is a key event in biofilm formation and is mediated by bacterial adhesins recognising host or bacterial partner receptors. In oral biofilm formation, primary *Actinomyces* and *Streptococcus* colonizers adhere to salivary pellicle proteins such as proline-rich proteins (PRPs) as well as to mucosal surfaces. Subsequently, *Actinomyces* and *Streptococcus* strains and other bacteria, such as *Veillonella, Fusobacterium* and *Porphyromonas,* adhere to each other. The nature of this community is highly important for the health or disease status, although specific pathogenic species may also have been implicated.
The aim of this thesis was to study key players in early oral colonisation, *Actinomyces* and *Streptococcus* species, and more specifically the nature of their adhesins and ligands. A further aim was to study the function of the salivary PRP proteins and an innate peptide derived thereof on bacterial adhesion, proliferation and regulation of pH, *i.e.* key factors in biofilm formation.

In paper I and II, adhesion, proliferation and pH affecting features of the RGRPQ (arginine-glycine-arginine-proline-glutamine) peptide, derived from PRP-1, were demonstrated. By use of an alanine-scan (I), motifs for adhesion inhibition and desorption of *Actinomyces naeslundii*, and proliferation stimulation, ammonia production and inhibition of sucrose induced pH drop by *Streptococcus gordonii* were indicated. The RGRPQ peptide also stimulated *S. gordonii* colonisation *in vivo*. In paper II, a more sophisticated quantitative structure-activity relationship (QSAR) study, using statistical molecular design (SMD) and multivariate modelling (partial least squares projections to latent structures, PLS), further narrowed down the RGRPQ peptide motifs. The R and Q amino acids were crucial for activity. For proliferation a hydrophobic and large size third position amino acid was crucial, while adhesion inhibition and desorption needed a small hydrophilic second position amino acid. All functions depended on a low polarity hydrophobic fourth position. Accordingly, activities could be optimized separately, with decreased function in the others.

In paper III and IV, focus was on the bacterial adhesins and their binding epitopes. The genes for FimA major subunit proteins of type-2 fimbriae were sequenced from *A. naeslundii* genospecies 1 and 2 and *Actinomyces odontolyticus*, each with unique carbohydrate binding specificities (III). Three major subtypes of FimA proteins were found that correlated with binding specificity, including a novel fimA gene in *A. odontolyticus*. All subtypes contained a pilin, LPXTG and E box motif. In paper IV, multiple PRP binding patterns for *Actinomyces* and *Streptococcus* strains were mapped using a hybrid peptide construct. The two most deviating binding groups deviated in type-1 fimbriae mediated binding to milk and saliva protein ligands.

In conclusion, differences in bacterial adhesins and their ability to utilise salivary proteins may render bacteria tropism for different niches. Peptides derived from protein receptors, such as RGRPQ, may be important modulators of biofilm formation, giving commensal bacteria a competitive edge in the bacterial community.

Sammanfattning

Bakterier beklär de flesta av våra kroppsytor, som huden och olika slemhinnor, t.ex. i mag-tarmkanalen, och de är då organiserade i så kallade biofilmer. Bakteriesamhällena i biofilmerna är normalt ”icke-sjukdomsframkallande” och de verkar i symbios med värden genom att bl.a. blockera infektionsframkallande bakterier. Biofilmbildningen startar med att bakterier fäster till varandra, till ytstrukturer på våra celler eller andra tillgängliga ytor, t.ex. tänder, katetrar eller implantat. Sammansättningen i en biofilm regleras förutom av närvaro av bakterier och tillgång på specifika vidhäftningsstrukturer för den initiala vidhäftningen även av tillgång på näring, syreexponering, pH, salter, och kommunikation mellan bakterier och mellan bakterier och värden. Biofilmer är således komplext reglerade bakteriesamhällen som dock är stabila för de nischer de finns i t.ex. tand och tarmslemhinna. Denna avhandling beskriver, med den orala biofilmen som modell, faktorer som påverkar biofilmbildningen hos en värdorganism.

En för oral biofilmbildning central proteingrupp i saliv är de så kallade sura prolinrika proteinerna (aPRP). I avhandlingen påvisades att ett bioaktivt fragment frisätts av orala streptokocker från aPRP i saliv. Fragmentet (RGRPQ peptiden) kan påverka bakterietillväxt och inbindning, samt höja pH i biofilmen. Således kan vissa Aktinomycesarter hindras från att fästa på en salivklädd tandyta medan tillväxt av vissa hälsoassocierade Streptokocker gynnas. Genom statistiska modelleringar och genom att byta ut olika aminosyror i RGRPQ peptiden påvisades också vilka aminosyror som var viktiga för de olika biologiska funktionerna. Resultaten är intressanta eftersom de visar ett nytt sätt på vilket bakterier i den orala biofilmen kan samverka med värden.

I avhandlingen studerades också variation i det protein (FimA) som bygger upp de sockerbindande vidhäftningsstrukturerna (typ-1 fimbrier) hos Aktinomycesarter. Bland de studerade orala *Actinomyces naeslundii* och *odontolyticus* stammarna identifierades tre varianter av FimA, vilket också var korrelerat till de ytstrukturer de binder till. Den identifierade proteinsekvensen för FimA hos *Actinomyces odontolyticus* var den första hos denna art, och denna kunskap saknades därför tidigare.

Slutligen studerades vilka delar av aPRP proteiner som olika aPRP-bindande munkoloniserande bakteriearter utnyttjar för bindning. Sex mönster/grupper identifierades och bindingsprofilen för de två från varandra mest avvikande grupperna korrelerade med hur de band till andra värdproteiner förutom aPRP.

Förståelsen av bakteriers inbindning är viktig därför att det hjälper oss att se vad det finns för skillnader mellan bra och dåliga bakterier och hur samspelet mellan bakterie och värd ser ut. Ur behandlingssynpunkt har vi också lättare att hitta vägar för bekämpning av infektionssjukdomar när vi förstår detta samspel.
Sammanfattningsvis, så behandlar denna avhandling viktiga faktorer som påverkar hur bakterier bildar biofilmer hos en värd. Detta är viktigt dels för att kunna förstå hur en normal och hälsosam biofilm bildas, men också för att kunna identifiera de faktorer som leder till att den förändras, blir ohälsosam och leder till utveckling av infektionssjukdomar.

Introduction

Biofilm formation

Biofilms and the oral biofilm as a model system

"Communities of microorganisms that are attached to a surface" is a broad and simplified definition of a biofilm (O'Toole, Kaplan, and Kolter, 2000). The main events, important for biofilm formation on a biological surface, are the adhesion of bacteria to host ligands or other bacteria, availability of nutrients for survival and growth, and communication for adjustment of the previous events. Biofilms on host epithelial surfaces are beneficial for the host by blocking adherence and metabolism of pathogens, and without this defence the host would be very vulnerable to infections. This is illustrated in gnotobiotic versus normal mice where significantly higher numbers of pathogenic bacteria are needed to infect mice with normal intestinal flora, compared to germ-free mice (Collins and Carter, 1978).
Biofilms are present on all external epithelial surfaces, and infections may occur at most of these (Costerton, Stewart, and Greenberg, 1999). Bacterial biofilms are present also on inanimate surfaces, such as implants and catheters (O'Toole, Kaplan, and Kolter, 2000), causing severe medical complications. In the biofilm formed on the surfaces of the oral cavity one can find and study all the important components involved in adhesion, growth/metabolism and communication. Therefore the major oral infectious diseases, caries and periodontitis, are interesting study objects, not only because of the specific need to resolve them *per se*, but also as a model system for epithelial and non-cellular surfaces.

Adhesion

Adhesion usually takes place through bacterial surface located adhesins, or fimbriae, which interact with specific target molecules or ligands (Ellen, Lepine, and Nghiem, 1997; Gibbons, 1989). Fimbriae, or pili, can be described as proteinaceous adhesive filaments, protruding from the bacterial surface. They are present both on gram-negative and gram-positive bacteria. *E. coli* P pilus and type 1 pilus (gram-negative) and *Corynebacterium diphtheriae* SpaA pilus (gram-positive), are well described examples (Marraffini, Dedent, and Schneewind, 2006; Sauer et al., 2004). The assembly of fimbriae has been of great interest for the possibility of manipulation and prevention of infectious diseases. Thereby, it was found that separate assembly systems for gram-negative and gram-positive bacteria are at work. For gram-negative bacteria the main system is the chaperone-usher assisted pilus assembly (Sauer et al., 2000), whereas fimbriae assembly in gram-positive bacteria is sortase mediated crosslinking of subunits into the fimbriae stalk (Marraffini, Dedent, and Schneewind, 2006). For the gram-negative *E. coli* P pilus an operon of

11 genes (*papA-K*) code for both structural components as well as chaperone and usher proteins (Hultgren et al., 1993; Sauer et al., 2004). In the gram-positive *C. diphtheriae* four genes are involved in building the structural and sortase proteins (Marraffini, Dedent, and Schneewind, 2006).
The bacterial adhesins interact with host ligands, which can either be proteins, glycoproteins or glycolipids that are secreted or present on the host cell-surface. Whereas cell membrane ligands only mediate binding of bacteria, secreted ligands may both mediate and block bacteria binding, such as seen for saliva gp-340 (Loimaranta et al., 2005). Adhesion and expression of adhesins are affected in several ways. For example, *Porphyromonas gingivalis* is after initial adhesion to *Streptococcus cristatus* affected in a way that decreases its transcription of fimbrial adhesin (*fimA*) and further adhesion and recruitment of *P. gingivalis* to the biofilm is terminated (Xie et al., 2000). Adhesion affecting signalling molecules, such as autoinducers (AIs), have been described to affect the ability to form mixed biofilms involving for example *P. gingivalis* and *S. gordonii* (McNab et al., 2003) although the specific mechanism for this is yet to be resolved.

Metabolism/growth
Availability of nutrients is crucial for bacterial survival and growth in the biofilm. Nutrients either originate from degraded host proteins/glycoproteins, or from external components, such as diet or other bacteria. An important feature of metabolism of host proteins, and other components, is microbial cooperation where different species degrade different parts of the macromolecules, and a shift in microbial biofilm may occur. Further, protein degradation generates ammonia and an elevated pH (Burne and Marquis, 2000), whereas carbohydrate degradation results in acid excretion, *e.g.* lactic acid, and a drop in pH (Bradshaw, McKee, and Marsh, 1989; de Soet, Nyvad, and Kilian, 2000). This favours species which are suited for high or low pH, respectively, such as in the oral cavity where frequent intake of sugar favours acidogenic and acidoduric bacteria in the tooth biofilm (Marsh, 1994; Svensater et al., 1997), causing caries development. A further possible modification is exemplified by *Veillonella*, that can convert lactic acid to weaker acids and thus counteract the acidification (Ng and Hamilton, 1971).

Communication
Recent investigations have displayed that bacteria communicate with each other, and thereby affect for example growth and pathogenicity in an organised manner (Fuqua and Greenberg, 2002) The systems for signalling differ between gram-negative and gram-positive bacteria. Gram-negative bacteria signal through secretion of acyl-homoserine lactones, and the first and well described example is that of *Vibrio fischeri*, which grow to high densities (10^{10} to 10^{11} cells per ml) in the light organ of its host (a squid), and signals to start production of a luminescent product (Fuqua and Greenberg, 2002). Many gram-positive bacteria employ short

linear and cyclic peptides for signalling (Fuqua and Greenberg, 2002; Ji, Beavis, and Novick, 1995; Lazazzera and Grossman, 1998). An example is the *Staphylococcus aureus* octapeptide that controls its toxin production and thus its virulence (Ji, Beavis, and Novick, 1995). Peptide signalling is also used for activation of genetic competence in *Streptococcus pneumoniae* (Havarstein, Coomaraswamy, and Morrison, 1995). In *S. gordonii* genes encoding receptors for peptide signalling are present (Havarstein et al., 1996), showing possible existence of similar systems in streptococci.

Oral microbial ecology

A mature oral biofilm is a multi-species community, with more than 750 bacterial taxa present (Jenkinson and Lamont, 2005) among which *Actinomyces* and *Streptococcus* species comprise a distinct part. Bacterial colonisation of the epithelial surfaces of the mouth starts at birth as bacteria are transmitted in a caregiver-to-child manner (Li and Caufield, 1995). The oral biofilm ecology is modified when the teeth erupt and new binding sites are offered by tooth adhering molecules from *e.g.* saliva and breast milk (Gibbons and Hay, 1988; Wernersson et al., *In press* 2006).
Actinomyces and *Streptococcus* species dominate among the early colonising bacteria, both on the teeth and oral mucosal surfaces (Gibbons, 1989; Hsu et al., 1994; Jenkinson and Lamont, 2005). These first colonisers enable colonisation of other species such as *Veillonella*, *Porphyromonas* and *Fusobacterium*, as well as additional *Actinomyces* and *Streptococcus* species (Jenkinson and Lamont, 2005) (**Figure 1**). From initial colonisation of mainly aerobic strains, the shifting milieu allows anaerobic strains to be introduced into the biofilm. In a very selective manner, bacteria now adhere to each other rather than to host ligands that to a large extent have become inaccessible on the enamel and cell surfaces. This co-aggregation has been mapped in detail for some species such as *Streptococcus*/*Actinomyces*, *Veillonella*/*Streptococcus* and *Actinomyces*, and *Prevotella*/*Actinomyces* species (Hughes et al., 1988; Kolenbrander, 1988; Nesbitt et al., 1993). It has been suggested that for some species, such as *Streptococcus sanguinis* and *Streptococcus mutans* (Caufield, Cutter, and Dasanayake, 1993; Caufield et al., 2000), that colonisation may not occur at any time but rather is limited to "discrete windows of infectivity", and that presence of *S. sanguinis* seems to delay colonisation of *S. mutans* (Caufield et al., 2000). Thus, colonisation is an intricate series of events with specific contacts being made. Due to the selectivity in adhesion points in combination with environmental factors, bacterial species find and establish unique niches of colonisation, unique in site and in co-colonising partners.

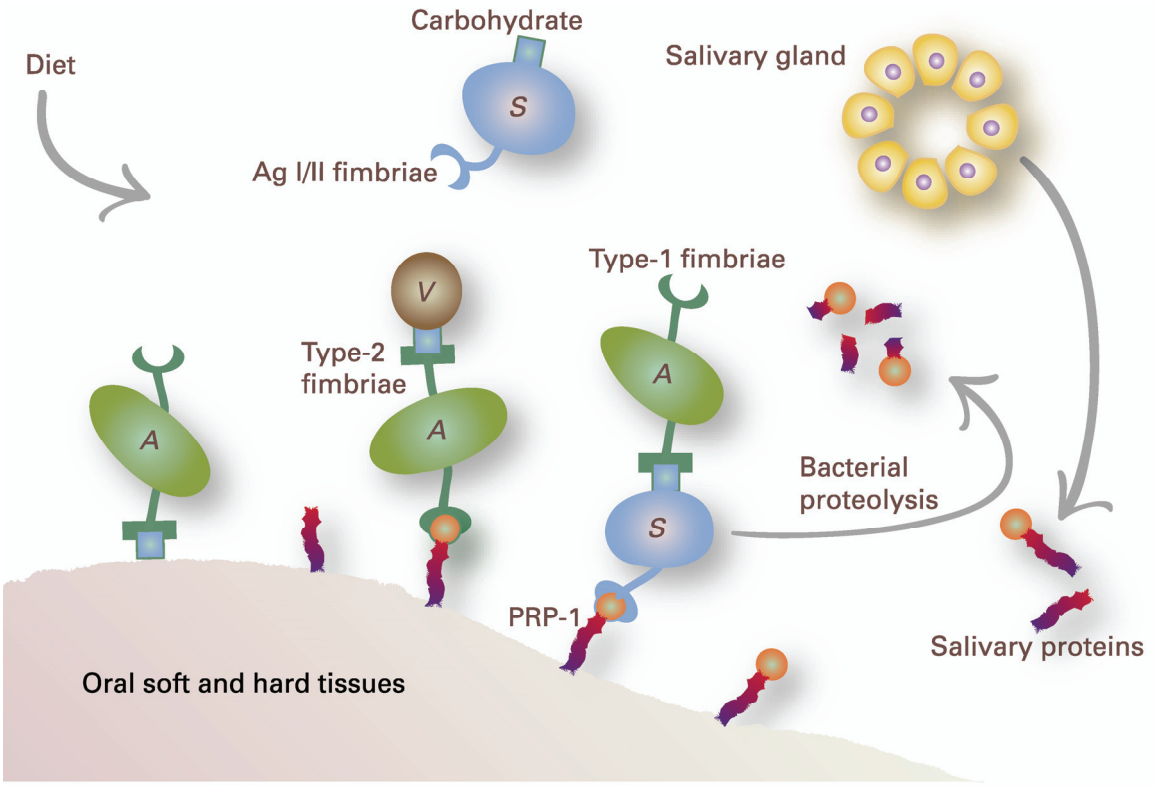


Figure 1. Key faktors of oral biofilm formation

Host factors affecting oral biofilm formation

Saliva and salivary proteins

Saliva, and its components, is of major importance for formation of the oral biofilm and also for maintaining oral health (Dodds, Johnson, and Yeh, 2005; Lamkin and Oppenheim, 1993). Thus, salivary components promote adhesion and clearance of bacteria, and metabolism. Saliva also affects de- and remineralization of teeth, pH-regulation, free radical scavenging, and lubrication of the tissues. Loss of saliva, due to for example radiation therapy or Sjögrens syndrome, enhances the risk for both dental caries and candidosis (Guggenheimer and Moore, 2003; Lacatusu, Francu, and Francu, 1996).

Innate (or non-specific constitutive) immunity is the defence system that is available without any previous challenge from an infectious agent (Boman, 2000). It is immediate and does not require recruitment of the humoral (or specific induced) immune system (Marsh and Martin, 1999). Included in the innate immunity are for example antimicrobial peptides acting directly on bacteria, desquamation of cells with bound bacteria, secreted clearance factors (proteins et c.) as well as the normal microflora limiting adhesion points for pathogenic bacteria (Marsh and Martin, 1999). Antimicrobial peptides are regarded as the main component of the innate immunity, and are present both in plants and animals

with more than 800 described sequences in databases (Boman, 2003). Several salivary proteins/peptides *per se*, as well as through their recruitment of the commensal flora, constitute a powerful innate defence. Proteins controlling colonisation and infection are for example histatins (Oppenheim et al., 1988; Pollock et al., 1984), lactoferrin (van der Strate et al., 2001), proline-rich proteins (PRPs)(Azen and Maeda, 1988; Hay et al., 1994), scavenger gp-340 (Ericson and Rundegren, 1983; Prakobphol et al., 2000), β-defensins (Joly et al., 2004; Krisanaprakornkit et al., 2000), LL37 (Ouhara et al., 2005), and mucins (Bobek and Situ, 2003; Liu et al., 2000). In addition, fragments of proteins, such as from histatins (Oppenheim et al., 1988) and lactoferrin (van der Strate et al., 2001), are shown to have enhanced antimicrobial effects.

Saliva contains approximately one milligram of protein per millilitre. Among the major proteins are the PRPs, amylase and mucins, but a variety of less abundant proteins, glycoproteins and peptides are present. Important functions for salivary proteins include enzymatic activities, tissue coating, lubrication, mineralization and several types of antimicrobial functions (antiviral, -bacterial and -fungal)(Marsh and Martin, 1999). Many, if not most, proteins in saliva are multi-functional, such as the acidic PRPs (aPRPs), statherins, histatins and amylase (Lamkin and Oppenheim, 1993). This could be exemplified by aPRPs and statherin, which both are involved in tooth tissue homeostasis, but also mediate adhesion of bacteria to the tooth surface, and amylase, which facilitates starch degradation and mediate adhesion of some streptococci (Scannapieco, Torres, and Levine, 1995). Further, more than one saliva protein support the same event, such as the aPRPs, statherin, histatins and additional proteins that are all inhibitors of calcium phosphate salt precipitation (Lamkin and Oppenheim, 1993), and many proteins, such as mucins (MG1 and MG2) and statherin, are important for lubrication and viscoelasticity (Wu, Csako, and Herp, 1994).

Acidic proline-rich proteins

Acidic proline-rich proteins (aPRPs) are polymorphic and multifunctional proteins (**Figure 2**). They are encoded by the PRH1 and PRH2 gene loci on chromosome 12 (Azen, 1993), with both allelic and post-translational variants. PRH1 encodes the allelic variants PIF-s, Db-s and $Pa_{monomer}$, that are post-translationally modified to PIF-f and Db-f (cleavage), and Pa_{dimer} (dimerisation of $Pa_{monomer}$,). PRH2 encodes the allelic variants PRP-1 and PRP-2, post-translationally modified (cleavage) to PRP-3 and PRP-4, respectively (Hay et al., 1994; Hay et al., 1988). The PRP-1 and PIF variants are most common (Hay et al., 1994; Lamkin and Oppenheim, 1993). The PRPs are phosphorylated in the N-terminal portion, which confers adherence to tooth tissues/hydroxyapatite and inhibition of calcium phosphate crystal growth and counteracts demineralisation. The middle part contains interaction sites for dietary tannins (polyphenols), which after binding can be cleared out by swallowing. The C-terminal portion, which is conserved in all aPRP variants, is

largely responsible for bacterial binding, and the PQ sequence, which is also repeated throughout this section, is one key epitope described (Gibbons, Hay, and Schlesinger, 1991; Li et al., 1999) (**Figure 2**). APRPs mediate binding of early colonizing commensal viridans-type *Streptococcus* and *Actinomyces* species to the tooth tissues (Gibbons, 1989), but the binding sites are cryptitopic, *i.e.* hidden, in solution due to that the conformation in solution does not expose the bacterial binding site (Gibbons et al., 1990). While the common allelic variant PRP-1 is shown to coincide with caries resistance, the less common variant Db is correlated with caries susceptibility and adhesion of cariogenic mutans streptococci (Stenudd et al., 2001).

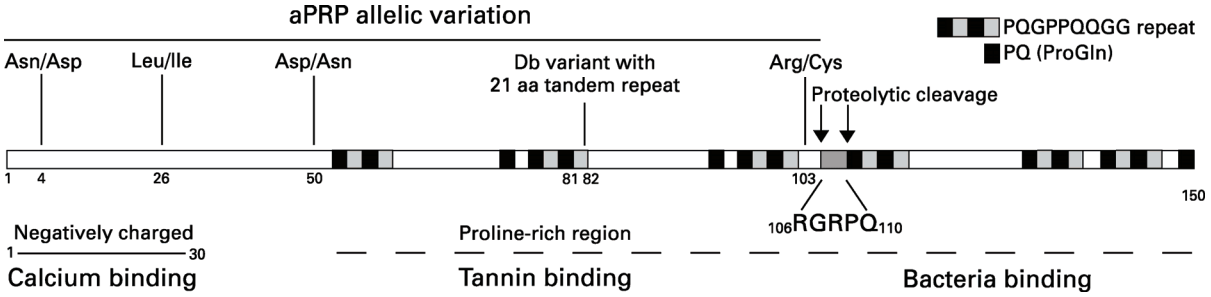


Figure 2. Structural features of PRP-1 and comparison with other aPRPs

Bacterial adhesion in the oral biofilm

Adhesion of bacteria to host and bacterial ligands

Primary adhesion of oral commensal bacteria is directed towards protein and carbohydrate components of the salivary pellicle on teeth and oral epithelial surfaces. This initial "layer" of bacteria in the oral biofilm is dominated by streptococci and *Actinomyces*. *Actinomyces* species adhere to proteins, such as aPRPs and statherin, through their type-1 fimbriae (Cisar et al., 1988; Gibbons and Hay, 1988) and to surface carbohydrate ligands of mucosa and other bacteria through type-2 fimbriae (Cisar et al., 1988; Stromberg and Karlsson, 1990) (**Figure 1**). *Streptococcus* species perform similar interactions through the Ag I/II family, Csh family and Fap1-including family (Jakubovics et al., 2005; Stephenson et al., 2002) of adhesins, which bind to a large range of ligands. *Actinomyces* and *Streptococcus* species then offer adhesion sites for additional bacteria, through co-adhesion/co-aggregation. *Actinomyces* spp. co-aggregate through the type-2 fimbriae (**Figure 1**), recognizing carbohydrate structures on both *Streptococcus* spp. (Palmer et al., 2003) and other species, such as *Fusobacterium* spp. and *Veillonella* spp.. *Streptococcus* spp. have several adhesins involved in co-aggregation, including the Ag I/II family, Csh family and Fap1-including family of adhesins (Elliott et al., 2003; Jakubovics et al., 2005).

The co-aggregation of different species is highly specific and described in detail for some species, such as for the *Actinomyces* - *Streptococcus* co-aggregation groups (Kolenbrander, 1988). Kolenbrander observed that among more than 100 tested

strains of *Actinomyces* and *Streptococcus*, six streptococcal intra-generic coaggregation groups (designated 1-6) and six inter-generic *Actinomyces* groups (designated A-F) were present (Kolenbrander, 1988). Moreover, either one or both coaggregating partners were heat- or protease-inactivated and often inhibited by lactose or other simple sugars. This suggested that coaggregation was mediated by lectins present on one bacteria, recognising carbohydrate structures on the other (Kolenbrander, 1988).

Altering of a commensal biofilm towards a pathogenic

Many infections, including caries and periodontal disease, are caused by bacteria/bacterial communities which are present in low numbers in a health associated biofilm. This means that the bacteria are present in the biofilm already long before an opportunistic manifestation, and the infectious disease. Thus, the infectious diseases in the mouth are chronic infections characterised by an ecology shift in the biofilm, *i.e.* a drift from a healthy towards a pathogenic biofilm (Jenkinson and Lamont, 2005; Marsh, 1994). This opportunistic behaviour may in the oral biofilm be driven by frequent lowered pH as regards the role of acidogenic and aciduric micro organisms in dental caries.

In order to infect, a potential pathogen must find available adhesion receptors and suitable community partners with which it can establish. Such partnership is required for *P. gingivalis* (associated with periodontitis) present at subgingival sites together with Streptococci which may contribute to its invasive properties (Lamont et al., 2002; Love et al., 2000). Consequently, one can also find the bacteria that co-adhere with *P. gingivalis*, such as *Tannerella forsythia* and *Treponema denticola*, at the same locations (Socransky and Haffajee, 2005; Yao et al., 1996). It is important to consider the oral infections as mixed-species infections rather than mainly caused by single species.

Features of Actinomyces and gram-positive bacterial fimbriae

Actinomyes naeslundii, which is further divided into genospecies 1 and 2, express two antigenically different fimbriae, type-1 and type-2. Type-1 fimbriae, more prevalent among *Actinomyes naeslundii* genospecies 2 strains, adhere to protein ligands, and the type-2 fimbriae recognise carbohydrate structures (Cisar, Sandberg, and Mergenhagen, 1984; Cisar et al., 1988; Hallberg et al., 1998). The major fimbrial subunit genes are *fimP* and *fimA*, respectively (Yeung, Chassy, and Cisar, 1987; Yeung and Cisar, 1988), but it is not known if the encoded proteins are merely structural or if they take part in binding of ligands. The *fimP* and *fimA* gene cluster are encoding both structural elements as well as a sortase protein for fimbriae assembly and function (Li et al., 2001; Yeung et al., 1998; Yeung and Ragsdale, 1997). These can be compared to the similar system of the gram-positive *C. diphtheriae* pilus with an operon of four genes including a sortase gene (Marraffini, Dedent, and Schneewind, 2006). The FimP and FimA proteins are approximately 534 amino acids large and show approximately 34 % amino acid

identity to each other (Yeung and Cisar, 1990). They display seven conserved proline-containing regions, regarded to be involved in folding of the protein, and conserved motifs, such as an N-terminal signal peptide and a C-terminal sorting signal with LPXTG motif (Yeung and Cisar, 1990). The conserved motifs are described in *C. diphtheriae* (Schneewind, Mihaylova-Petkov, and Model, 1993; Ton-That, Marraffini, and Schneewind, 2004). Diverse *fimP* and *fimA* genes have been found for *A. naeslundii* genospecies 1 and 2 and *Actinomyces viscosus*, and they have been linked to differential binding patterns that are likely to at least in part determine the differential tropism for *Actinomyces* spp. (Hallberg et al., 1998; Li et al., 1999; Li et al., 2001). In addition, hybridisation studies indicate FimA or FimP related adhesins in at least *A. odontolyticus* (Hallberg et al., 1998).

Aims of this thesis

The summarised aims of this thesis were to…

…identify the postulated saliva PRP-1 derived RGRPQ peptide and investigate its functions as well as structural features relating to its biological activity.

…investigate the diversity of *Actinomyces* FimA fimbrial subunit protein, and the possible relation to deviating binding patterns.

… identify deviations in PRP-1 binding patterns of *Streptococcus* and *Actinomyces* strains, and correlation of these with host ligand binding patterns.

Methodology

Several methods were used throughout this thesis work, and papers included. Here follows an overview of the main methods. For a detailed description, and for methods not described here, see papers I - IV.

Degradation of PRP-1, and detection of RGRPQ (I)

Degradation was studied by incubation of *S. gordonii* SK12 with purified PRP-1. The supernatant obtained after pelleting of bacterial cells was subject to gel filtration, and this revealed a peak that eluted at the same position as a synthetic RGRPQ peptide. This fraction was analysed by mass spectrometry and N-terminal sequencing for description of the content.

Innate immunity-like features of RGRPQ (I and II)

Adhesion inhibition properties were analysed by use of a hydroxyapatite (HA) binding assay. Metabolically labelled (^{35}S-methionine) *A. naeslundii* T14V (Li et al., 2001) was co-incubated with peptide and bound to PRP-1 coated HA beads. The amount of bound bacteria was detected in a β-counter.
Desorption (or reversal of bacteria-bead aggregates) was analysed by adding peptide to *A. naeslundii* T14V aggregated by PRP-1 coated latex beads on a glass slide. The level of desorption was scored visually by the amount of aggregation after addition of peptide.
Ammonia production was detected as pH increase after addition of peptide to *S. gordonii* SK12 in a water system (non-buffering) (Wijeyeweera and Kleinberg, 1989). pH was measured at intervals during approximately 3 hours.
Inhibition of sugar-induced pH drop, was assayed by measuring pH change in a KCl solution after addition of sucrose to a mix of starved *S. gordonii* SK12 and peptide (Takahashi, Kalfas, and Yamada, 1994).
Proliferation (or induction of growth) of *S. gordonii* SK12 was tested by growing bacteria in a chemically defined (including all naturally occuring amino acids) minimal growth media (Terleckyj, Willett, and Shockman, 1975) supplemented with peptide. Absorbance was measured once every hour.

Structure-activity relationship studies (I and II)

Two different approaches were used to map the determinants for the RGRPQ biological features.

The first approach was to make an alanine scan. One by one different amino acids in the RGRPQ peptide were substituted for alanine. The resulting peptides were then run in the assays described above. A preliminary guidance of amino acid determinants for the five RGRPQ features was found.

The second approach was a quantitative structure-activity relationships (QSAR) study. With use of statistical molecular design (SMD) (Linusson et al., 2000; Linusson, Wold, and Norden, 1999) a set of RGRPQ related peptides were designed to have varying properties regarding hydrophilicity, size and charge at positions 2 to 4 simultanously. The peptides were run in growth, adhesion inhibition and desorption assays, and the results analysed with multivariate modelling (partial lest squares projections to latent structures; PLS) (Umetrics, 2002). Thereby, a model for chemical properties affecting the different biological features was retrieved. The model was validated by testing additional peptides, and the observed results were compared with the predicted.

FimA sequencing and DNA and protein analysis (III)

DNA was isolated from bacteria and genes amplified by PCR using FimA primers described previously (Hallberg et al., 1998). Then followed cloning of fragments into vectors from which they could be sequenced. New gene fragments were amplified as the sequence progressed and new primers could be made. The DNA sequence was analysed and the amino acid sequence translated by tools available online. The obtained amino acid sequence was analysed for homology and protein motif similarities using tools at various sites online (see reference list), and by own sequence comparisons.

Immuno-detection of FimA (III)

FimA subunits were detected by Western blot using antisera (R70-3), specific for the *A. naeslundii* 12104 FimA.

Bacterial features relating to FimA sequence diversity (III)

Agglutination of *A. naeslundii* and *A. odontolyticus* strains with antisera (R70-3), was observed in a cyvett as a drop in absorbance. Absorbance was measured once every half hour.

Hemagglutination, *i.e.* bacterial agglutination of erythrocytes, with and without prior treatment with sialidase (cleaves of sialic acid residues located terminally on carbohydrate structures), was tested. Bacteria were mixed with a suspension of 4 % human erythrocytes in PBS (phosphate buffered saline), and hemagglutination was scored visually by estimating the size and appearance of aggregates on a glass slide.

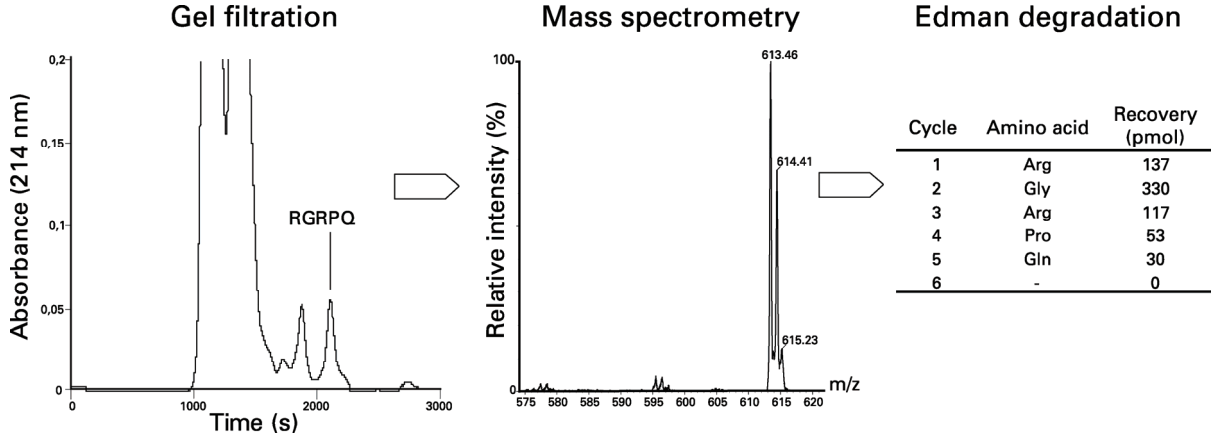
PRP-1 epitope mapping (IV)

Bacterial binding was mapped with the HA adhesion method described above, employing hybrid-peptide constructs (Gilbert et al., 2000; Niemi and Johansson, 2004) with a HA-binding domain and a C-terminal part presenting fragments of PRP-1. Bacteria were metabolically labelled (^{35}S-methionine) and the amount of bound bacteria determined with a β-counter.

Results and Discussions

Proteolytic release of an RGRPQ peptide from PRP-1 (I)

A panel of oral *Streptococcus* species, with defined arginine catabolism and cariogenic properties (de Soet, Nyvad, and Kilian, 2000), where tested for their ability to degrade PRP-1. Abundant degradation was found for species defined as commensal (*S. gordonii*, *S. sanguinis*) but not for potential (*Streptococcus mitis*) or highly cariogenic (*S. mutans*) species exhibiting no arginine but high acid production.
Previous studies suggested that an RGRPQ peptide could be released after bacterial proteolysis of PRP-1, a caries resistans protein (Li et al., 2000; Stenudd et al., 1998). Now, after degradation of purified PRP-1 by *S. gordonii* SK12 a peptide was eluted in the same position in gel filtration as a synthetic RGRPQ peptide (**Figure 3**). The eluted peptide was identified as RGRPQ by mass spectrometry, and sequencing by Edman degradation confirmed this (**Figure 3**). Thus, it was proven that an RGRPQ peptide is released from PRP-1 by bacterial proteolysis.



Cycle	Amino acid	Recovery (pmol)
1	Arg	137
2	Gly	330
3	Arg	117
4	Pro	53
5	Gln	30
6	-	0

Figure 3. Detection of RGRPQ peptide from *S. gordonii* SK12 degradation products.

Since aPRPs are highly abundant proteins in saliva (Scannapieco, 1994) and some 1000 ml of saliva is produced per 24h, the amount of RGRPQ peptide flushing the oral tissues may be considerable. It is a rather common feature that host proteins may be cleaved to highly active peptides with anti-bacterial and other innate immunity properties. Such antibacterial and innate immunity peptides are lactoferricin (from lactoferrin) (van der Strate et al., 2001), histatin 5 (from histatin 3) (Oppenheim et al., 1988), kappacin and caseicin (from casein) (Hayes et al., 2006; Malkoski et al., 2001; Scannapieco, 1994) and LL37 (from LL37/hCAP18) (Bals et al., 1999; Ouhara et al., 2005). The RGRPQ may be an additional

example, although with a previously not described and different mechanism, supporting commensal streptococci.

Biological effects of the RGRPQ peptide (I)

Proliferation in vitro and in vivo

In an *in vitro* study the bacterial generation time was increased about 2.5-fold for *S. gordonii* SK12 incubated in a chemically defined medium with a synthetic RGRPQ peptide (**Figure** 4). In an *in vivo* rat model, a growth stimulatory effect of RGRPQ on *S. gordonii* SK12 was observed when co-infecting the mouth with an *S. gordonii* and an *S. mutans* strain. Thus, though the milieu was favourable for *S. mutans* (with a high sucrose diet causing acidification after metabolism, and desalivation disabling buffering and clearance), *S. gordonii* colonised the rats when RGRPQ was orally administred to the rats twice daily. Thus, RGRPQ can selectively affect proliferation *in vitro* and colonisation *in vivo*, key factors in biofilm formation, although *in vivo* additional functions of RGRPQ will probably be at work.

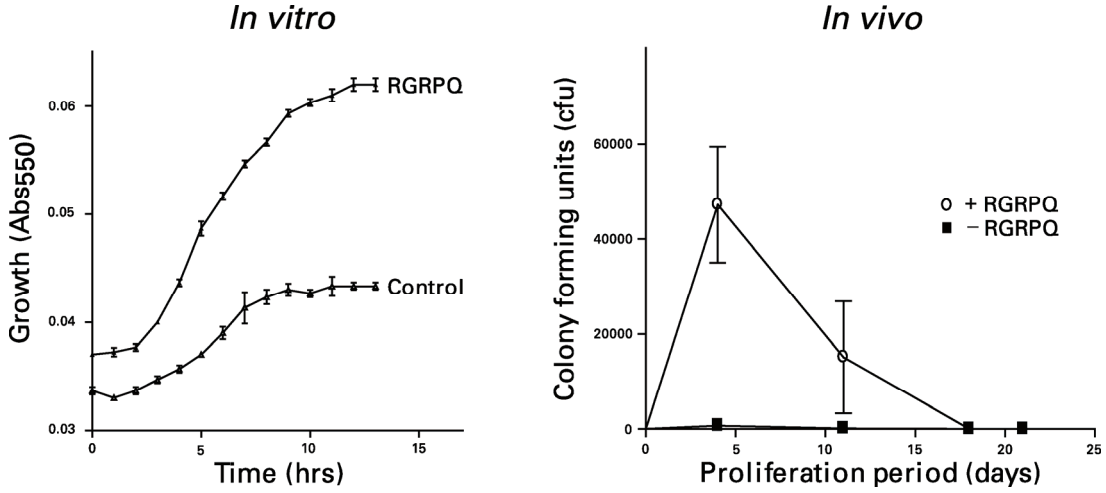


Figure 4. Effect of RGRPQ peptide on growth of *S. gordonii* SK12, *In vitro* and *In vivo*.

pH regulating effects

The inhibition of sucrose-induced pH drop was studied by addition of sucrose to *S. gordonii* incubated with synthetic RGRPQ peptide. In presence of peptide the sucrose characteristic drop in pH was blocked. Ammonia production was also investigated by adding RGRPQ peptide to bacteria, resulting in an elevated pH for the ammonia producing *S. gordonii* SK12 strain.

Inhibition of sucrose-induced pH drop was more prominent for *S. mutans* Ingbritt, lacking ammonia production, than with *S. gordonii* SK12. However, it is not clear if inhibition of sucrose-induced pH drop is a result of blocked sugar uptake or

perhaps blocked sugar metabolism. Regardless of mode of action, a pH regulating effect is a consequence of both ammonia production and blockage of acid production, and it may have implications in regulation of biofilm ecology and possibly development of dental caries.

Adhesion inhibition and desorption of bacteria

Adhesion inhibition was tested through incubation of bacteria with RGRPQ peptide prior to adhesion to PRP-1 coated hydroxyapatite beads, while desorption was tested through adding peptide to aggregates of bacteria and PRP-1 coated latex beads. Both assays displayed high levels of decrease in adhesion (app. 70 % adhesion inhibition and desorption score 4 out of 5) when tested for *A. naeslundii* T14V (**Figure 5**). Adhesion inhibition was also tested for a set of *A. naeslundii* and *S. gordonii* strains. Half of the *A. naeslundii* strains were blocked by RGRPQ, whereas the other *A. naeslundii* and all *S. gordonii* strains remained virtually unaffected.

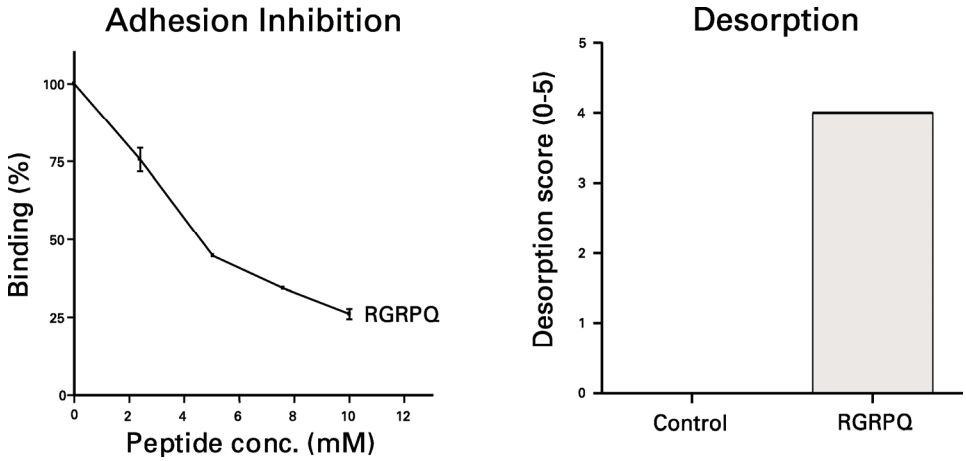


Figure 5. Effect of RGRPQ peptide on adhesion inhibition and desorption of PRP-1 binding *A, naeslundii* T14V.

To further investigate the diversity in binding sites and adhesion inhibition, adhesion was tested for selected strains (*A. naeslundii* LY7, *A. naeslundii* T14V and *S. gordonii* Blackburn) to hybrid peptides with a terminal RGRPQ sequence as well as hybrid peptides with a terminal GQSPQ sequence (PRP-1 C-terminal sequence). *A. naeslundii* LY7, which belonged to the group not inhibited by RGRPQ (or GQSPQ), bound to a lower extent to hybrid-RGRPQ than *A. naeslundii* T14V and *S. gordonii* Blackburn, but equally well to hybrid-GQSPQ. Thus, the RGRPQ and GQSPQ binding *A. naeslundii* T14V strain is inhibited by RGRPQ and GQSPQ, while the *S. gordonii* strain is not. The *A. naeslundii* strains that compete with *S. gordonii* for binding sites may therefore be selectively inhibited by RGRPQ, possibly due to a difference in binding affinity for free peptide in solution.

In summary, the host-derived RGRPQ peptide has features that affect fundamental properties in biofilm formation and ecology; proliferation, local pH and adhesion.

Although the RGRPQ peptide from a theoretical point-of-view may be abundant in oral biofilms *in vivo*, the kinetics, amount or turnover of RGRPQ in oral biofilms *in vivo* is unknown at present. Thus, a novel host beneficial function of aPRP proteins is likely. A comparison of RGRPQ with other host derived innate immunity peptides may suggest two protective systems, one directly inhibiting bacterial growth (*i.e.* histatin-5, LL37 et c.), and the other by stimulating commensal *S. gordonii*. Presence of gastro-intestinal, downstream effects from aPRP/RGRPQ has not been studied but may exist, although the major effect is likely to be in the oral cavity. Finally, the RGRPQ peptide might become a tool for targeted medical treatment of cariogenic biofilms with acidogenic/acidoduric bacteria, both in preventive and therapeutic care.

RGRPQ structure-activity relationships (I and II)

Initially the structure-activity relationship mapping was conducted using mainly an alanine-scan. Different positions in RGRPQ were substituted with alanine and the resulting peptides were tested for activity and compared to RGRPQ. It was found that the pH regulating effects were either not possible to map to any specific amino acid (sucrose-induced pH drop) or it was highly confined to the N-terminal arginine (ammonia production). For proliferation, adhesion inhibition and desorption activities a consensus finding was that the C-terminal Q was crucial. Glycine and proline seemed to have some effect, but these effects differed between proliferation and adhesion inhibition and desorption.
For a more extensive structure-activity relationship study, and with the crucial importance of terminal R and Q in mind, peptides were designed with the three internal GRP positions varied simultaneously. Using chemometrics tools (statistical molecular design, SMD), a set of peptides were varied optimally in the three positions regarding their hydrophilic, size and charge properties. Hence all positions could be analysed for these properties simultaneously using a multivariate statistical method (partial least squares projections to latent structures (PLS).
The results both confirmed and extended previous results. Proliferation activity was indeed different from adhesion inhibition and desorption, that in turn seemed to be virtually identical. All three activities required a hydrophobic and low charged amino acid in position 4 (RGR**P**Q), while position 2 (R**G**RPQ) and 3 (RG**R**PQ) had deviating requirements for proliferation and adhesion inhibition/desorption. High activity in proliferation would be achieved with both positions containing large and hydrophobic amino acids, whereas adhesion inhibiton and desorption would instead reach higher activity with a small and hydrophilic amino acid in position 2 and a large and hydrophilic amino acid in position 3. The RGRPQ peptide is therefore an optimal compromise for achieving high activity simultaneously in all three activities, although for one activity alone a more active peptide could be found. Results showed that proliferation was the feature that could be optimized further to a large extent.

A main feature of SMD in combination with PLS is the possibility to see, not only important properties of individual amino acids, but also if there are amino acids/positions that co-operate for activity. For high proliferation activity the main interactions involved engaging a large amino acid in position 3 in combination with i) high overall peptide lipophilicity, ii) high hydrophobicity of amino acid 3, and iii) low polarity in position 4. Thus, it must be important that position 3 has the required properties and that the other properties adjust to this accordingly.
For adhesion inhibition and desorption, interactions with a small sized amino acid 2 are important in combination with i) low total peptide polar surface area, ii) hydrophilic amino acids 2 and 3. Further, hydrophilic character of amino acid 3 interacts with low total peptide polar surface area. Here, it can be concluded that the main features for high adhesion inhibition and desorption are the small size in position 2 and hydrophilic character in general.
The final conclusion is that while position 4 is important for all activities, position 2 is crucial for adhesion inhibition and desorption and position 3 for proliferation (**Figure 6**). It can again be stated that the RGRPQ peptide is an optimal compromise, but higher activity could be achieved for each of the individual biological features by altering the sequence.

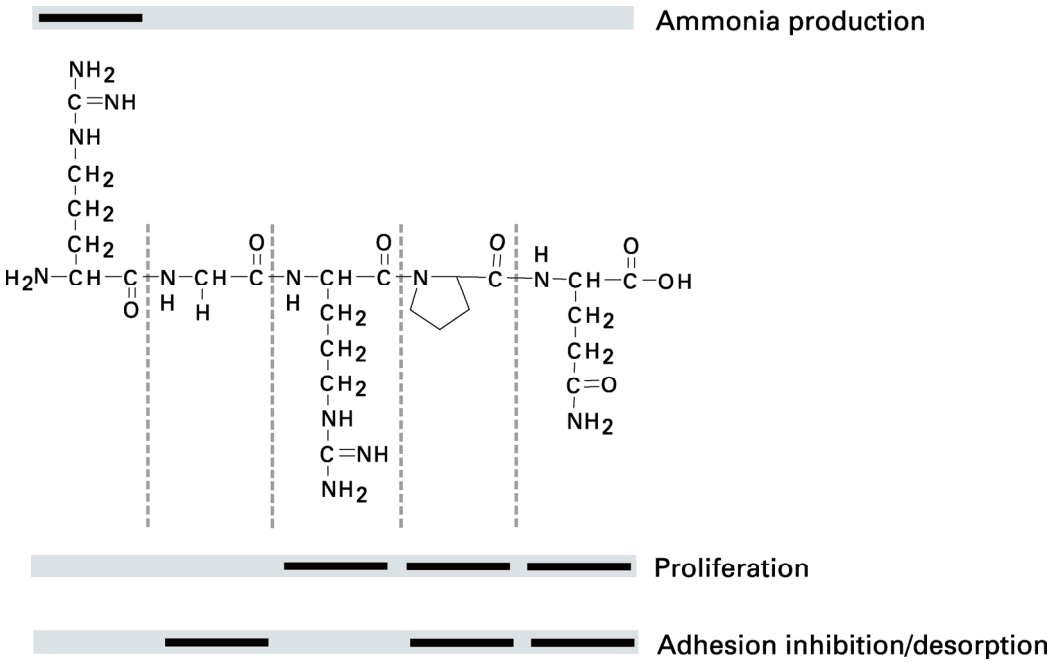


Figure 6. Key determinants of RGRPQ peptide for ammonia production, proliferation and adhesion inhibition/desorption activities

QSAR studies of the RGRPQ peptide shows that it is possible to deliniate the chemical/physical properties rendering it biological activity. This also allows manipulation of activity, favouring either for example growth or adhesion effects. In this way, substances selectively affecting adhesion versus proliferation could be generated and evaluated in biofilm models *in vitro* and *in vivo*. Moreover, on a long

term basis peptidomimetics could be generated for such evaluated RGRPQ properties.

Subtypes of type-2 major subunit protein, FimA, in *Actinomyces* (III)

Sequencing of fimA genes and presence of three FimA protein subtypes
fimA genes from representative strains of *A. naeslundii* genospecies 1 (12104, Pn22E, Pn6N, P5N) and genospecies 2 (T14V, P1N, P1K and L7Y), and *A. odontolyticus* (PK984), with different Galβ and sialic acid binding properties (Hallberg et al., 1998; Loimaranta et al., 2005; Stromberg et al., 1992), were sequenced and the deduced amino acid sequences (FimA proteins) compared. The results clearly indicated the presence of three subtypes of FimA for *A. naeslundii genospecies* 1 and *2,* and *A. odontolyticus*, with different carbohydrate specificities (**Figure** 7). Sequence identity between strains within a subtype was 88.6 - 99.6 %, while it was 62.5 - 66.4 % between subtypes. Thus, within the genus *Actinomyces,* strains with deviating binding properties have FimA major fimbrial subunit proteins that are unique and fall into separate subtypes. Similar to the *E. coli* P-fimbriae PapG adhesins also recognising different carbohydrate epitopes (Hultgren et al., 1993), the three Actinomyces groups may recognise different host ligand (glycoproteins versus glycolipids, **Figure** 7) and evoke different host respons patterns. It should be noted that the *Actinomyces* FimA proteins show closer relation to the SpaH protein of *C. diphtheriae* pilus, than to the closely related FimP protein of *Actinomyces* type-1 pilus (Gaspar and Ton-That, 2006). The FimP has in turn a higher similarity to a protein, SpaD, of an other pilus type of *C. diphtheriae* (Cerdeno-Tarraga et al., 2003; Gaspar and Ton-That, 2006).

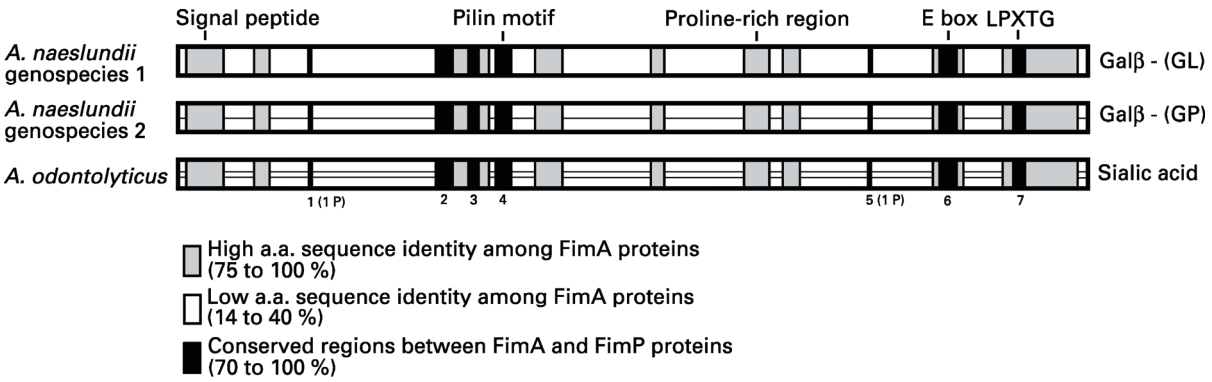


Figure 7. Comparison of FimA protein sequences from *A. naeslundii* genospecies 1 and 2 and *A. odontolyticus* with different binding properties.

Structural features of Actinomyces odontolyticus FimA proteins
The novel FimA protein of *A. odontolyticus* PK984 and the other two FimA subtypes contained all the crucial elements previously described for pilin assembly. These are the pilin, E box and LPXTG motifs, as well as the N-terminal signal peptide and C-terminal cell membrane spanning domain (Navarre and

Schneewind, 1994; Ton-That, Marraffini, and Schneewind, 2004; Ton-That and Schneewind, 2003) (**Figure 7**). The pilin, E box and LPXTG motifs displayed 80 - 100 % sequence identity between the three subtypes, and they were part of the conserved proline-containing domains found in the homologous FimP protein in *A. naeslundii* (Yeung and Cisar, 1990).
Apart from sequences unique and conserved within each subtype (with low sequence identity between subtypes) no specific motifs or segments could be linked to the deviating binding patterns. Based on the presence of the pilin assembly motifs in all three FimA protein subtypes, and the expression of type-2 fimbriae in *C. diphtheriae*, it appears as *Actinomyces* species use the same sortase-mediated pilus assembly system as described for other gram-positive bacteria.

Antigenic properties of Actinomyces FimA proteins
An antisera (R70-3) specific for *A. naeslundii* 12104 (genospecies 1) FimA was used to test reactivity of *A. naeslundii* genospecies 1 and 2, and *A. odontolyticus* strains (whole cells). All *A. naeslundii* genospecies 1 strains (except Pn6N) were agglutinated by the antisera, whereas none of *A. naeslundii* genospecies 2 or *A. odontolyticus* strains were. Thus, the FimA proteins hold different antigenic properties that coincide with FimA protein sequence subtypes and may be related to sequence differences, general folding or exposure of unique segments.
Further, western blots of SDS-separated proteins from whole cell protein extracts of the same strains showed bands recognising R70-3 only for *A. naeslundii* genospecies 1, confirming the deviation between the subtypes. *A. naeslundii* genospecies 2 and *A. odontolyticus* strains, which did not react with antisera, did in fact in a protein sequence dendrogram cluster closer to each other than to *A. naeslundii* genospecies 1 although the sequence identity only differed by a few per cent.

PRP adhesion patterns among *Actinomyces* and *Streptococcus* strains (IV)

PRP peptide recognition groups of Actinomyces and Streptococcus species
A panel of PRP binding *Actinomyces* and *Streptococcus* strains were tested for their PRP-1 binding patterns. Fragments of PRP-1, five to six amino acids long, covering middle to C-terminal portions were linked to the hydroxyapatite binding peptide segment of statherin to construct so called hybrid peptides (Gilbert et al., 2000). This technique has previously been proved usable for mapping of bacterial binding patterns in the salivary polypeptide statherin (Niemi and Johansson, 2004). Based on the binding pattern the strains could be divided into six recognition groups; one each for *A. naeslundii* genospecies 1 and *A. viscosus* strains, and two each for *A. naeslundii* genospecies 2 and *S. gordonii* strains (**Table 1**). While *A. naeslundii* genospecies 1 and one of the two genospecies 2 groups bound peptides with both internal and terminal Q, all groups bound the PRP C-terminal QGQSPQ fragment and one to three of the additional fragments with C-terminal Q (**Table 1**,

see paper IV for a detailed table). The *A. viscosus* group bound mainly to the QGQSPQ fragment. Thus, the binding to aPRPs seem to have different recognition motifs which is correlated to genus, species and subspecies. Further, the importance of Q as a common part of a recognition site was clear. The importance of Q is in accordance with the previously pointed out PQ binding site of *A. naeslundii* LY7 and *S. gordonii* Blackburn to PRP-1, Q in inhibitory peptides of binding *Actinomyces* strains and *Fusobacterium nucleatum* to statherin (Gibbons, Hay, and Schlesinger, 1991; Li et al., 1999; Niemi and Johansson, 2004), as well as from the RGRPQ structure-activity relationship studies described above where Q was crucial for activity (Drobni et al., 2006).

Table 1. Mapping of PRP-1 peptide recognition patterns among PRP-1 binding *Actinomyces* and *Streptococcus* species using a hybrid peptide approach.

			Hybrid peptide adhesion[1]						
			-RGRPQ	-GPPQQ	-GGHQQ	-GPPPPP	-PGKPQG	-PPPQGG	-RPQGPP	-QGQSPQ
Group	Strains	Species							
Actinomyces									
I	n=2	*A. naeslundii gsp 1*	■	■	■	□	□	▒	□	■
II	n=2	*A. naeslundii gsp 2*	■	■	■	□	■	■	□	■
III	n=4		▒	■	▒	□	□	□	□	■
IV	n=3	*A. viscosus*	□	□	▒	□	□	□	□	■
Streptococcus									
V	n=4	*S. gordonii*	■	□	▒	□	□	□	□	■
VI	n=2		▒	□	□	□	□	□	□	▒

[1]Adhesion illustrated as high (black) to none (white). Hybrid-peptide coating concentration 20 μM.

Binding of deviating recognition groups to saliva and milk ligands

Two strains were selected for further analysis of binding patterns to some host ligands. Thus, from two groups with highly deviating PRP-1 recognition patterns; *A. naeslundii* T14V binding fragments with both internal and terminal Q residues, and *A. viscosus* 19246 with strong binding only to the terminal QGQSPQ fragment were selected. In addition, T14V fimbriae mutants lacking either type-1 or type-2 or both, were used. These strains were besides adhesion to PRP-1 tested for adhesion to salivary sIgA and statherin, and β-casein derived from milk. Results showed that strain T14V had a higher binding to PRP-1 and β-casein than statherin and sIgA. Strain 19246 had its highest binding to statherin and bound both PRP-1 and β-casein but not sIgA. Binding to these proteins was inhibitable by either RGRPQ (strain T14V and a type-1 positive/type-2 negative fimbrial mutant) or QQYTF (strain 19246), proving that the interaction was mediated by type-1

fimbriae interacting with the protein ligands. Type-1 negative mutants lacked binding altogether.
Thus, type-1 fimbriae seem to recognise a multitude of host proteins, and it is likely that this is part in defining colonisation niches and host tropism. In fact, strains T14V and 19246 with their deviating binding patterns, display human and rat tropism, respectively (Li et al., 1999; Li et al., 2001). Interestingly, although lack of statherin protein in rat saliva, strain 19246 with rat tropism has preferential statherin binding. This further confirms the recognition of several host proteins by type-1 fimbriae, which in *e.g.* rat saliva may have statherin-resembling ligands. Multiple Q residues, which were found to be crucial for the biological activity of RGRPQ, are present in a large number of salivary proteins, and epitope similarities are likely to occur. Collagen, a component of connective (and other) tissues, contains several Q residues and promotes adhesion of *Actinomyces* (Liu et al., 1991). In wounds and other sites where damage of some sort has occurred, collagen is exposed (e.g. dentinal tubules due to caries) and adheres bacteria that cause infections (Love et al., 2000). Adhesion of commensal bacteria to collagen might protect these tissues from adhesion by pathogenic species. Further, adhesion to milk casein by commensal *Actinomyces* may indicate that establishment of the "healthy" biofilm in infants is facilitated by breast feeding.

Concluding remarks

Key elements in biofilm formation are adhesion, proliferation and communication. Diversity in each of these events guides and sets the limits of which bacteria will be able to prevail in each specific environment or niche. Bacteria may be excluded by lack of correct adhesins, or by competition for ligands. Some bacteria manage to attach but become released due to down-regulation of their adhesins by other bacteria. Further, the metabolism by bacteria strongly influences the physical and chemical properties of the biofilm, such as pH, aerobic condition, red-ox potential et c., and these change as the biofilm matures. Unaffected, the biofilm will find a stable growth called bacterial homeostasis, and the biofilm-host symbiosis becomes a powerful host defence system.

The work included in my thesis, focuses on studying adhesion related factors with relevance for oral biofilm formation and composition. Model bacteria are *Actinomyces* and *Streptococcus* species, abundant bacteria in the oral biofilm. It was previously known that both *Actinomyces* and *Streptococcus* species are members of the initial biofilm, and adhere to the salivary aPRPs with different aPRP variants correlating with health and disease. Different streptococcal species degrade aPRPs and release an array of peptides. One of which is the RGRPQ peptide, harbouring several innate immunity functions.
The RGRPQ peptide is interesting because it is a host-derived peptide with apparent impact on many of the key elements of biofilm formation; *i.e.* it blocks adhesion of *A. naeslundii*, and has beneficial effects on *S. gordonii* growth and local pH. The mechanism of its activities remains to be resolved. However, adhesion inhibition/desorption activity is likely to be a matter of simple competition, and the effects on local pH through ammonia production from arginine has been described. Stimulation of growth may be more complicated. Peptide signalling systems in gram-positive bacteria are described to induce gene transcription through intra- and extra-cellular receptors resulting in activation of various systems, and in *S. gordonii* genes encoding receptors for peptide signalling are present. This could be an explanation for RGRPQ activity, but at present this has not been studied.
From a structure-activity point-of-view, the chemical properties determining activity were studied. The SMD/PLS approach pin-pointed important structure-activity relationships hinted by the alanine-scan, and showed that adhesion inhibition/desorption and proliferation depended on both similar and different chemical entities. Evidently, proliferation activity can be further improved, and adhesion inhibition and desorption have the same determinants. In conclusion, RGRPQ is a compromise for ability to affect several key events in biofilm

formation. On a long-term basis this may form a platform for peptidomimetics of given RGRPQ properties.

Adhesins are important for the composition of biofilms, and this thesis included studies mainly of the *Actinomyces* type-1 and type-2 fimbriae.
Variation among *Actinomyces* species was found in the type-2 major fimbrial subunit protein FimA. Although not certain if this is a structural or adhesin protein, variation between species coincided with deviating binding patterns, and adhesin diversity guides host-tropism and ability to occupy specific niches. These studies of fimbriae extend the knowledge of a common system for pilin formation and assembly in gram-positive bacteria, and emphasis that *Actinomyces* is a good model system for gram-positive bacteria.

APRP binding patterns of *Actinomyces* and *Streptococcus* species were studied, and type-1 fimbriae mediated binding of host proteins by *Actinomyces* species. Differential binding patterns were found, possibly explaining diversity in host tropism and colonisation patterns.
Binding of Q residues, previously described by others, was confirmed in this work. Multiple Q residues are present in PRPs, statherin and caseins, and many other host proteins. With the terminally located O and NH_2-groups, Q may act as both a hydrogen-bond donator and acceptor, and Q has been described in other systems as important in protein-protein interactions.

In conclusion, this thesis approaches the complex features of biofilm formation from different angles by investigating both the host and bacterial determinants. The presence and the features of the host-derived RGRPQ factor has not been seen in any other system before, and an insight into the importance of fimbrial variation in guiding host tropism and formation of ecological niches were revealed. These studies form a basis for continued studies on host derived peptides and their effects on adhesion and metabolism of oral bacteria and oral infectious diseases, as well as similar systems in other biofilms.

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Online tools:

Center for Biological Sequence analysis, SignalP server [http://www.cbs.dtu.dk/services/SignalP/].

ExPASy Proteomics Server, ScanProsite [http://www.expasy.org/tools/scanprosite/].

Genome Net, ClustalW [http://clustalw.genome.jp/].

Molecular Toolkit [http://arbl.cvmbs.colostate.edu/molkit/index.html].

My hits, motif scan [http://myhits.isb-sib.ch/cgi-bin/motif_scan].