Host ligands and oral bacterial adhesion

*Studies on phosphorylated polypeptides and gp-340 in saliva and milk*

Liza Danielsson Niemi
To my family
Infectious diseases e.g. gastric ulcer, caries and periodontitis, are caused by bacteria in a biofilm. Adhesion of bacteria to host ligands e.g. proteins, polypeptides and glycoproteins, is a key event in biofilm formation and colonization of surfaces such as mucosa and tooth tissues. Thus, host ligands could contribute to the susceptibility to infectious diseases. The general aim of this doctoral thesis was to study the effect of phosphorylated polypeptides and gp-340 in saliva and milk on oral bacterial adhesion and aggregation.

Statherin is a non-glycosylated, phosphorylated polypeptide in saliva. The polypeptide inhibits precipitation and crystal growth of calcium phosphate and mediates adhesion of microorganisms. By using a hybrid peptide construct, the domain for adhesion of Actinomyces isolated from human infections and from rodents was found to reside in the C-terminal end, and the adhesion was inhibitable. With alanine substitution the peptide recognition epitope in the C-terminal end was delineated to Q and TF, where QAATF was an optimal inhibitory peptide. In contrast, human commensal Actinomyces bound to the middle region in a non-inhibitable fashion. Gp-340 is another protein in saliva, and it is a large, multifunctional glycoprotein. Four novel size variants (I-IV) of salivary gp-340 were distinguished within individuals, and their glycoforms were characterized. All four size variants were identical in the N-terminal amino acid sequence and shared core carbohydrates. Low-glyco lung gp-340, high-glyco saliva gp-340, and size variants I-III aggregated bacteria differently. Human milk, which shares many traits with saliva, could inhibit adhesion of Streptococcus mutans to saliva-coated hydroxyapatite (s-HA), a model for teeth, in an individually varying fashion. Human milk caseins, lactoferrin, secretory IgA, and IgG inhibited the binding avidly. By using synthetic peptides the inhibitory epitope in β-casein was mapped to a C-terminal stretch of 30 amino acids. Inhibition by human milk, secretory IgA and the β-casein-derived inhibitory peptide was universal among a panel of mutans streptococci.

The main conclusions are: (i) statherin mediates differential binding of commensal versus infectious Actinomyces strains with small conformation-dependent binding epitopes, (ii) salivary gp-340 has individual polymorphisms that at least affect binding of bacteria, (iii) human milk inhibits S. mutans adhesion to s-HA in an individually varying fashion, and the C-terminal end of human milk β-casein is one inhibitory component. Together these results suggest that the studied host ligands can influence the composition of the oral biofilm. Statherin may protect the host from colonization of bacteria associated with infections. Gp-340 size variants may affect functions related to host innate immune defences such as interactions with a wide array of bacteria, and human milk may have a protective effect in infants from colonization of mutans streptococci.
# TABLE OF CONTENTS

**LIST OF PUBLICATIONS** .............................................................. 9  
**ABBREVIATIONS** ....................................................................... 10  
**INTRODUCTION** ........................................................................ 11  
- INFECTIOUS DISEASES IN THE GASTROINTESTINAL TRACT ............. 11  
- BACTERIAL BIOFILM ECOLOGY – A DETERMINANT FOR HEALTH OR DISEASE ..... 12  
- DENTAL CARIES – AN INFECTIOUS DISEASE INVOLVING BIOFILM ECOLOGY  
  - BALANCE ......................................................................................... 12  
- ADHESION AND BIOFILM FORMATION ............................................. 13  
- ORAL MICROBIAL ECOLOGY ............................................................ 14  
- HOST LIGANDS MEDIATE ADHESION OF ORAL BACTERIA ................. 16  
  - Host ligands in saliva ........................................................................ 17  
  - Host ligands in milk ......................................................................... 21  
- BIOREGULATORY PROTEINS AND PEPTIDES IN SALIVA AND MILK .... 23  
- HOST LIGAND GLYCOSYLATION AND SECRETOR STATUS .................... 24  
- MODEL MICROORGANISMS PRESENT IN ORAL BIOFILMS ....................... 25  
  - Actinomyces .................................................................................... 25  
  - Streptococci .................................................................................... 26  
**AIMS** .......................................................................................... 28  
**MATERIALS AND METHODS** ..................................................... 29  
- BACTERIA AND FUNGUS STRAINS .................................................... 29  
- SALIVA AND MILK ............................................................................. 29  
- PURIFICATION OF SALIVA PROTEINS .............................................. 29  
- SIZE VARIANTS OF GP-340 ............................................................... 30  
- CARBOHYDRATE MAPPING OF GP-340 VARIANTS ................................. 30  
- SEPARATION AND IDENTIFICATION OF MILK PROTEINS .................... 31  
- ADHESION ASSAY ............................................................................ 31  
- ADHESION INHIBITION ASSAY ......................................................... 32  
- PEPTIDES FOR MAPPING BACTERIA–BINDING EPITOPES ...................... 33  
- BINDING OF BACTERIA IN SOLUTION (AGGREGATION ASSAY) .......... 34  
**RESULTS AND DISCUSSION** ..................................................... 35  
- PAPER I. BINDING EPITOPES FOR ACTINOMYCES ON SALIVARY STATHERIN ..... 35  
- PAPER II. VARIANT SIZE- AND GLYCOFORMS OF GP-340 WITH DIFFERENTIAL  
  BACTERIAL AGGREGATION .................................................................... 38
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


Reprints of the original papers were made with permission from the publishers.
ABBREVIATIONS

aa Amino acid
AgI/II Antigen I/II
APRP Acidic proline-rich proteins
BSA Bovine serum albumin
BSSL Bile Salt-Stimulated Lipase
statherin-des-aa<sup>x</sup> A compound obtained by the removal of an amino acid (aa) residue from the polypeptide statherin in the position x.
DMBT1 Deleted in malignant brain tumours 1
ELISA Enzyme-linked immunosorbent assay
gp-340 Glycoprotein 340, formerly called agglutinin
HA Hydroxyapatite
s-HA Hydroxyapatite coated with saliva
m-HA Hydroxyapatite coated with human milk
gp-340-HA Hydroxyapatite coated with gp-340
HIV Human immunodeficiency virus
IgG Immunoglobulin G
Le<sup>a</sup>-Le<sup>y</sup> Lewis a to Lewis y antigens
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S-IgA Secretory immunoglobulin A
sLe<sup>x</sup> sialyl-Lewis x
VNTR Variable number of tandem repeats

Abbreviations for amino acids used in the text:

<table>
<thead>
<tr>
<th>Alanine</th>
<th>Arginine</th>
<th>Asparagine</th>
<th>Aspartate</th>
<th>Glutamate</th>
<th>Glutamine</th>
<th>Glycine</th>
<th>Isoleucine</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala A</td>
<td>Arg R</td>
<td>Asn N</td>
<td>Asp D</td>
<td>Glu E</td>
<td>Gln Q</td>
<td>Gly G</td>
<td>Ile I</td>
<td>Leu L</td>
</tr>
<tr>
<td>Lysine Lys K</td>
<td>Phenylalanine Phe F</td>
<td>Proline Pro P</td>
<td>Serine Ser S</td>
<td>Threonine Thr T</td>
<td>Tryptophan Trp W</td>
<td>Tyrosine Tyr Y</td>
<td>Valine Val V</td>
<td></td>
</tr>
</tbody>
</table>
**INTRODUCTION**

Microbes attach to host tissues by interactions with host ligand molecules (receptors). These interactions are specific, and characteristics of the ligands are crucial for recognition by the bacterial attachment adhesins. Lately, bacterial resistance to antibiotics has increased, making it important to find alternative treatments for bacterial infections. Identifying host ligands and delineating host ligand epitopes for bacterial interactions may lead to construction of peptidomimetic drugs that can prevent colonization of infectious microorganisms and favour commensal ones. Knowledge of host ligands and host ligand polymorphism could also provide explanations for host resistance and host susceptibility towards infectious diseases.

**Infectious diseases in the gastrointestinal tract**

Bacterial infections affect people worldwide, and they constitute major health problems with associated morbidity and mortality. Infectious diseases are among the top ten causes of death both in low income, middle income, and high income countries (World Health Organization, 2008). Many species of pathogenic bacteria are capable of colonizing and infecting the gastrointestinal tract and other areas in the body. These microbes cause diseases such as lower respiratory infections (e.g. *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*) (Guthrie, 2001), diarrhoeal diseases (e.g. *Escherichia coli*, *Salmonella*, *Shigella*, *Campylobacter*, *Vibrio cholerae*) (Niyogi et al., 1994), and tuberculosis (*Mycobacterium tuberculosis*) (Zhang and Yew, 2009). These infections are among the major causes of mortality over the world, and together they caused almost eight million deaths in 2004 (World Health Organization, 2008). Other common infectious diseases in the gastrointestinal tract, caused by less pathogenic bacteria, are peptic ulcers and gastritis caused by *Helicobacter pylori* (Aspholm-Hurtig et al., 2004), periodontitis (e.g. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*) (Asikainen and Chen, 1999), and dental caries associated with Streptococci and Lactobacilli (Marsh, 1994).
**Bacterial biofilm ecology – a determinant for health or disease**

There are approximately ten times as many bacterial cells in the human body as human cells (Cucchiara and Aloi, 2009). However, most of the bacteria are harmless or beneficial to the host. For example, commensal bacteria provide infection resistance, increase the overall immunological capacity of the host, and act as a colonization barrier towards pathogenic microorganism (Salminen et al., 1995; Tlaskalová-Hogenová et al., 2004). Bacteria and other microorganisms form complex communities (biofilms), which once they are established, have a highly specific ecology for the habitat, i.e., the mouth, gut, etc. Many chronic diseases in humans are claimed to involve bacterial biofilms and ecological shifts therein (Donlan and Costerton, 2002). According to Fux et al. (2005), more than 60% of chronic infectious diseases relate to ecology shifts in the associated bacterial biofilms. Examples of such diseases are the oral diseases, dental caries and periodontitis, and also endocarditis, prostatitis, and cystic fibrosis (Fux et al., 2005). Understanding development and maintenance of a health-associated biofilm would be a subject of major therapeutic potential.

**Dental caries – an infectious disease involving biofilm ecology balance**

Dental caries is a highly prevalent chronic infectious disease that involves dental plaque which is a bacterial biofilm on teeth. Caries is prevalent in most countries, but the distribution in the Western populations is skewed. That is, a majority experience few symptoms whereas 15-20% of the populations are highly diseased. Dental caries may cause pain, bone infection, eating problems and social stigmata. The disease is characterized by demineralisation of the tooth tissues occurring at low pH due to bacterial fermentation of carbohydrates. Disease determinants involve both genetic (tooth tissue, taste preference, saliva polymorphism and immune functions) and lifestyle (eating and oral hygiene habits and fluoride exposure) factors (Vieira et al., 2008; Stenudd et al., 2001). In dental caries the tooth biofilm ecology is characterized by a shift towards more acidogenic (acid producing) and aciduric (acid resistant) bacteria, such as mutans streptococci (Streptococcus mutans and Streptococcus sobrinus), lactobacilli, and Candida species. Other somewhat less acidogenic and aciduric bacteria have also been associated with dental caries, e.g., S. mitis biovar 1, Actinomyces naeslundii, A. viscosus, A. odontolyticus, A. israelii, Veillonella species (Brailsford et al., 1998; Tanner et al., 2002; Becker and Paster, 2002; Nyvad
and Kilian, 1990a). Besides prerequisites for attachment of the cariogenic bacteria, \textit{in vivo} proliferation, e.g. of \textit{S. mutans} and \textit{Lactobacillus}, is favoured at low pH, due to the bacteria’s ability to proliferate over other species, at low pH (Marsh, 2006). Thus, frequent or prolonged episodes of low pH, such as after intake of sucrose or other fermentable carbohydrates, may lead to an ecology shift towards higher proportions of, e.g. \textit{mutans} streptococci (Bradshaw et al., 1989; Balakrishnan et al., 2000; Marsh, 2006). This process forms the basis for the present ecological plaque hypothesis of caries, in which several species of bacteria could be classified as “cariogenic” and contribute to the development of dental caries (Marsh, 2006). Thus, “cariogenic” bacteria are normally present in low numbers in the dental plaque biofilm. However, they do not cause disease until major environmental changes occur, such as reduced salivary flow or high and frequent intake of sucrose that changes the ecology of the biofilm (Sbordone and Bortolaia, 2003; Marsh, 2006).

Colonization of the oral cavity starts during birth (normal delivery) and then completes by transmission from the caregiver to the child (Li et al., 2000; Caufield et al., 1993). Early colonization of \textit{mutans} streptococci, as well as avid saliva-mediated adhesion and high numbers of \textit{mutans} streptococci in saliva or the tooth biofilm, are predictors for dental caries (Teanpaisan et al., 2007; Straetemans et al., 1998), whereas acquisition of \textit{S. sanguinis} is associated with healthy teeth (Becker and Paster, 2002). Thus, establishment of a commensal oral flora in early childhood seems highly beneficial.

\section*{Adhesion and biofilm formation}

A biofilm consists of highly structured communities of microorganisms that form on various biotic surfaces, such as mucosa, epithelia, tooth tissues, and implants. It also forms on non-human (abiotic) surfaces such as industrial water pipelines and catheters (Costerton et al., 1999; Kolenbrander, 2000; Whittaker et al., 1996; Dunne, 2002).

Biofilm formation starts with an initial contact between the bacterial cell and the surface via hydrophobic, van der Waals, electrostatic interactions or steric hindrance (Dunne, 2002). Adhesin molecules on the bacteria then lock the contact by binding to specific host molecules (ligands) e.g. proteins, peptides, carbohydrates or lipids. Thus, a key event in biofilm formation is the initial adhesion of bacteria to matching host ligands or partner bacteria (Dunne, 2002; Costerton et al., 1999). Once attached to the surface, other
bacteria bind to the already attached bacteria (co-adhesion) (Costerton et al., 1999; Dunne, 2002; Marsh, 2006). Bacteria in the biofilm produce extracellular polysaccharides, which are a major component of the biofilm matrix (glycocalyx). The glycocalyx is a slime layer, with high viscoelasticity, which surrounds and aids in anchoring the biofilm bacteria (Dunne, 2002). Microbes in the oral biofilm proliferate, and at later stages, different microbial species, such as S. gordonii and Pseudomonas aeruginosa form micro communities within the biofilm organized in mushroom-like or corn-cob formations with channels for water and nutrient transportation and removal of waste (Donlan and Costerton 2002; Costerton et al., 1999). In the mature biofilm approximately 15% of the volume is composed of cells, and 85% is matrix (Donlan and Costerton, 2002). Within the biofilm, bacterial ecology is further regulated by: (i) reduced availability of oxygen facilitating anaerobic species, (ii) nutrient limitations, (iii) gene regulation due to cell-to-cell signalling depending on cell density (quorom sensing), (iv) metabolic communication where excreted metabolites are used as nutrition by a different bacterial cell, such as Veillonella species metabolizing lactic acid from mutans streptococci, and (v) proliferation and detachment from the biofilm (Dunne, 2002; Davies et al., 1998; Costerton et al., 1999; Kolenbrander et al., 2002). Thus, the biofilm reaches a dynamic equilibrium dependent on water, nutrients, removal of waste, pH, oxygen levels, osmolarity, and released cell components from dead bacteria.

By forming biofilms, bacteria are more likely to overcome external influences, such as host immune responses and antibacterial therapeutics (Costerton et al., 1999; Fux et al., 2005). It has been hypothesized that the mechanism behind the resistance to antimicrobial agents involves: (i) failure to penetrate the biofilm, (ii) presence of slow growing, less susceptible bacteria, (iii) bacterial phenotype shift leading to enhanced resistance, and (iv) production of enzymes (Fux et al., 2005; Marsh, 2006; Sbordone and Bortolaia, 2003). Bacteria in biofilms are also more resistant towards mechanical forces due to densely packed bacterial cells in the slime matrix of extracellular polysaccharides produced by the bacteria (Fux et al., 2005). Thus, it is beneficial for the bacteria to organize themselves in biofilms.

**Oral Microbial Ecology**

The oral cavity offers various types of surfaces for bacterial colonization, i.e. the buccal, gingival, palatal, and tongue epithelium, the hard tooth tissues, and various tooth restorative materials. Both the soft and hard tissues and dental materials are coated by a pellicle, composed of salivary components,
gingival exudate, and externally derived (e.g. from foods) proteins, glycoproteins and glycolipids. The pellicle may also offer host ligands for bacterial binding. Formation of the first bacterial monolayer, e.g. on the teeth, is determined by bacterial availability and which host ligands are available for bacterial attachment (Figure 1). Soluble ligands can also help clear bacteria from the oral cavity. Oral biofilms are polymicrobial communities, and more than 700 bacterial taxa have been identified in the oral cavity (Jenkinson and Lamont, 2005; Aas et al., 2005). Some species colonize early and some late in the colonization succession. Early colonizers include *Streptococcus*, *Actinomyces*, *Veillonella* and *Neisseria* species (Jenkinson and Lamont, 2005). *Streptococci*, such as *S. sanguinis*, *S. oralis*, and *S. mitis* (biovar 1) and *A. naeslundii* dominate among bacteria initially colonizing the teeth. It is suggested that *S. sanguinis*, *S. oralis*, and *S. mitis* (biovar 1) constitute 60-90% of the early colonizing flora (Kolenbrander et al., 2002; Nyvad och Kilian, 1987; Nyvad och Kilian, 1990b). Succeeding bacteria attach to adhering host ligands or to the already attached bacteria in a very specific manner (Costerton et al., 1999; Dunne, 2002). This latter phenomenon, co-aggregation or co-adhesion, is described for, e.g. *Actinomyces* co-aggregation groups A-F and *Streptococcus* co-aggregation groups 1-6 (Kolenbrander, 1989; Egland et al., 2001). In the maturing...
biofilm there is an ecological shift from predominantly aerobic bacteria towards more anaerobic bacteria. *Fusobacterium nucleatum*, which can co-aggregate with a wide array of oral bacteria, is suggested to function as a bridge between early and late colonizers unable to co-aggregate with each other (Sbordone and Bortolaia, 2003; Kolenbrander et al., 2002; Jenkinson and Lamont, 2005; Bradshaw et al., 1998). For example, *F. nucleatum* could bridge binding between the aerob-anaerob pair *S. mutans* and *P. gingivalis* (Bradshaw et al., 1998).

Although the oral flora is highly diverse, the teeth are predominately colonized by certain bacteria, e.g. species of the *Streptococcus* and *Actinomyces* families (Li et al., 2004; Rüdiger et al., 2002).

**Host ligands mediate adhesion of oral bacteria**

The bacterial tropism is mainly determined by the bacterial adhesin and the host ligand pairs available at particular host tissues. Bacterial adhesin-host ligand recognition-binding is often highly specific. However, many bacteria express more than one adhesin that enables them to bind to several host ligands. Host ligands (proteins, polypeptides, peptides, glycoproteins, glycolipids or glycosphingolipids) are expressed or attached to the host surface, and the specific binding epitopes can be expressed by several host ligands. Microbe-host interactions have been explored and delineated to minor carbohydrate and peptide recognition motifs. Many tissue cells carry glycosylated structures, and accordingly, a variety of microorganism-binding carbohydrate epitopes have been described. For example, both p-fimbriated (PapG adhesin) *E. coli* (Strömberg et al., 1990) and *S. suis* bacteria (Haataja et al., 1994) bind to the disaccharide Galα1-4Gal in blood group P-related glycolipids. *A. naeslundii* bacteria bind to GalNAcβ in salivary pellicles (Strömberg et al., 1992).

Peptide motifs also act as binding sites for microorganisms. *C. albicans* bind to the ArgGlyAsp (RGD) peptide in iC3b on epithelial cells (Bendel and Hostetter, 1993). The adhesin in type-1 fimbriae of *A. naeslundii* Ly7 binds to ProGln (PQ) in the acidic proline-rich proteins (APRP) in saliva (Gibbons et al., 1991), and *A. viscosus* 19246 binds to ThrPhe (TF) in salivary statherin (Li et al., 1999).

This doctoral thesis focuses on a set of model ligands in human saliva and milk, specifically the phosphorylated statherin in saliva, the highly glycosylated gp-340 in saliva, and the phosphorylated β-casein in milk.
Host ligands in saliva

Approximately, 0.5-1 litre of saliva, with an average of 1 mg/ml of proteins, is produced per 24 hours. Thus, the teeth, and other oral surfaces, are continuously “flushed” by saliva. Saliva contains proteins, peptides, glycoproteins, and glycolipids conferring multiple functions, including lubrication, facilitation of swallowing and speech, buffering capacity, and antimicrobial defence such as bacterial clearance. In healthy conditions the content of free carbohydrates and lipids is low in saliva. Several proteins (host ligands) in saliva carry receptor epitopes (binding sites) for bacterial attachment, some of which bind to bacteria in the fluid phase (e.g. gp-340). This leads to aggregation and clearance of the bacteria from the oral cavity. Other host ligands bind certain bacteria only when surface bound (e.g. statherin, APRPs) (Gibbons and Hay, 1988; Paper I), and other host ligands, e.g. gp-340, bind bacteria both when free in solution and when bound to a surface (Loimaranta et al., 2005). The most abundant proteins in saliva are

Table 1. Major proteins in saliva and human milk with protective functions.

<table>
<thead>
<tr>
<th>Saliva and milk proteins</th>
<th>Protective function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saliva proteins</strong></td>
<td></td>
</tr>
<tr>
<td>Amylase</td>
<td>Bacterial binding</td>
</tr>
<tr>
<td>Cystatins</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>Gp-340</td>
<td>Bacterial and viral binding</td>
</tr>
<tr>
<td>Histatin</td>
<td>Bacteriocidal and fungicidal</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Bacteriostatic, bacteriocidal, bacterial binding</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Gram-positive bacteria lysis</td>
</tr>
<tr>
<td>Mucins</td>
<td>Bacterial binding</td>
</tr>
<tr>
<td>PRP</td>
<td>Bacterial binding</td>
</tr>
<tr>
<td>Salivary peroxidase</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>S-IgA</td>
<td>Bacterial adherence prevention</td>
</tr>
<tr>
<td>Statherin</td>
<td>Bacterial binding</td>
</tr>
<tr>
<td><strong>Milk proteins</strong></td>
<td></td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>Tumour cells apoptosis induction</td>
</tr>
<tr>
<td>BSSL</td>
<td>Lipid digestion</td>
</tr>
<tr>
<td>Caseins</td>
<td>Bacterial binding, Ca²⁺ binding</td>
</tr>
<tr>
<td>Cytokines</td>
<td>Immunomodulatory</td>
</tr>
<tr>
<td>Haptocorrin</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Bacteriostatic, bacteriocidal, bacterial binding</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Gram-positive bacteria lysis</td>
</tr>
<tr>
<td>S-IgA</td>
<td>Bacterial adherence prevention</td>
</tr>
</tbody>
</table>

the PRPs, which make up approx. 25-30% of the salivary proteins, and amylase. Examples of other, less abundant, but biologically active salivary proteins, identified in the pellicle and biofilm, are statherin, gp-340, lactoferrin, secretory immunoglobulin A (S-IgA), and lysozyme (Table 1). Of
these, statherin and gp-340 have been selected as model host ligands in the present thesis.

**Statherin**

Salivary statherin is a non-glycosylated, 43-amino acid residue, tyrosine-rich polypeptide with a charged phosphorylated N-terminal end (Figure 2). It is expressed by secretory cells in the salivary glands and nose (Cole et al., 1999; Schlesinger and Hay, 1977). It is encoded by the \textit{STATH} gene on chromosome 4q13.3 close to the genes for caseins and histatins, which all belong to a “secretory calcium-binding phosphoprotein gene cluster” (Huq et al., 2005). The \textit{STATH} gene is highly homologous, in introns and noncoding exons, to the \textit{HIST} gene encoding the antifungal histatin 1 (Sabatini et al., 1993). Statherin has only been identified in man and monkeys, which implies a late evolutionary appearance. In fact, it is suggested that the statherin ancestral gene could have evolved from the casein genes (Huq et al., 2005). Statherin exhibits diverse biological roles in the oral cavity (Ramasubbu et al., 1993; Schlesinger and Hay, 1977) (Figure 2).

**Figure 2. Functional domains in statherin.**

It (i) mediates binding of bacteria, \textit{F. nucleatum}, \textit{P. gingivalis}, \textit{Actinomyces}, and fungi, \textit{C. albicans} (Johansson et al., 2000; Li et al., 1999; Amano et al., 1996; Sekine et al., 2004). The binding sites for \textit{F. nucleatum} reside in the middle to C-terminal end (aa 21-26 and aa 31-39) (Sekine et al., 2004), whereas Leu<sup>29</sup>Tyr<sup>30</sup> and Tyr<sup>31</sup>Thr<sup>32</sup>Phe<sup>33</sup> are the binding epitopes for \textit{P. gingivalis} (Amano et al., 1996), (ii) inhibits crystal growth and precipitation of calcium phosphate salts from supersaturated saliva (Schlesinger and Hay, 1977), (iii) acts as a lubricant (Ramasubbu et al.,
and (iv) binds with high selectivity to hydroxyapatite in the N-terminal region (Gilbert et al., 2000).

In aqueous solution statherin has a disordered structure, but upon adsorption to hydroxyapatite statherin folds into a secondary structure (Goobes et al., 2006). It is proposed that adsorbed statherin consists of an α-helix in the N-terminal, a poly-proline type II structure, and an α-helix in the C-terminal (Goobes et al., 2006; Long et al., 2001; Naganagowda et al., 1998).

Although statherin is a small unglycosylated polypeptide, it occurs in several polymorphic variants (Jensen et al., 1991). Jensen et al. (1991) showed three different forms of statherin due to alternative RNA splicing and posttranslational modifications: statherin SV1 which lacks the C-terminal phenylalanine residue (statherin-des-Phe\textsuperscript{43}), SV2 which lacks residue 6-15 (statherin-des\textsuperscript{6-15}), and SV3 which is identical to SV2 but lacks the C-terminal phenylalanine (statherin-des\textsuperscript{6-15}, -Phe\textsuperscript{43}). Later Inzitari et al. (2006) analyzed whole saliva from 23 subjects and showed that statherin occurs in even more variants. SV1, SV2 and statherin lacking the N-terminal aspartic acid (statherin-des-Asp\textsuperscript{1}) were found in almost all samples, whereas SV3 was found in one of the 23 saliva samples. Other variants found were mono-phosphorylated and non-phosphorylated statherin, statherin lacking the C-terminal threonine and phenylalanine residues (statherin-des-Thr\textsuperscript{42}, -Phe\textsuperscript{43}), statherin-des\textsuperscript{1-9}, statherin-des\textsuperscript{1-10}, and statherin-des\textsuperscript{1-13}. These variants could be due to proteolytic activity among bacteria, which inevitably exist in whole saliva. However, all variants were also found in parotid saliva (n=3) except for non-phosphorylated statherin and SV3 (Inzitari et al., 2006), which suggests that the variants may not be derived from proteolytic activity by bacteria.

**Glycoprotein-340 (gp-340)**

Salivary gp-340 is a 300-400 kDa, highly glycosylated protein that is a homologue to lung gp-340 (Prakobphol et al., 2000), which is an alternative spliced form of DMBT1 (deleted in malignant brain tumours 1) (Holmskov et al., 1999). Gp-340/DMBT1 is encoded by the DMBT1 gene on chromosome 10q25.3-26.1 and is expressed in salivary glands, lung, trachea, stomach, small intestine, and tears (Holmskov et al., 1999; Mollenhauer et al., 1997; Schulz et al., 2002). Low levels of RNA for gp-340/DMBT1 are found in brain, uterus, testis, prostate, pancreas and mammary glands (Holmskov et al., 1999; Braidotti et al., 2004).
Gp-340 belongs to the Scavenger receptor cystein-rich (SRCR) protein superfamily (Holmskov et al., 1997), which is a family involved in the innate immune system (Ligtenberg et al., 2001, Mollenhauer et al., 2000). Gp-340 contains 13 SRCR domains (involved in protein-protein interactions in the innate immune system) (Bikker et al., 2002) separated by SID (SRCR interspersed domain) except for SRCR domain 4 and 5, and two CUB (Clr/Clq Uegf Bmp1) domains (involved in development) (Bork and Beckmann, 1993) separated by a 14th SRCR domain (Figure 3). There is also a short Thr-region, a Ser-Pro-Thr region and a ZP (Zona Pellucida) domain (Ligtenberg et al., 2001). Gp-340 contains putative glycosylation sites for O-linked carbohydrate chains, highly densely in the SIDs, and 14 possible sites for N-linked carbohydrate chains mainly in the C-terminal CUB-/ZP domains (Holmskov et al., 1999; Mollenhauer et al., 1997). In saliva, gp-340 forms an oligomeric complex with S-IgA (Ericson and Rundegren, 1983).

Gp-340 has many biological functions. It binds surfactant-protein D (SP-D) and A (SP-A) via protein-protein interactions in a calcium-dependent manner (Holmskov et al., 1997; Tino and Wright, 1999). SP-D and SP-A belong to the collectin family, which promotes binding of specific carbohydrates on pathogenic microorganisms, e.g. bacteria, viruses and yeasts, and activates complement cascades (Ligtenberg et al., 2001). Gp-340 interacts with bacteria through carbohydrate and peptide receptors. It is a major host ligand for *S. mutans*, which is both aggregated and attached by gp-340 (Ericsson and Rundegren, 1983; Loimaranta et al., 2005; Prakobphol et al., 2000; Lightenberg et al., 2001). A 16-amino acid peptide from the
SRCR domain (SRCRP2) is suggested to be a receptor for *S. mutans*, and the bacteria-binding motif has been delineated to VEVLXXXXW (Bikker et al., 2002; Bikker et al., 2004). Gp-340 also binds *S. agalactiae*, which is implicated in neonatal meningitis, *H. pylori*, implicated in gastritis, peptide ulcer disease and stomach cancer, and several commensal streptococci (Prakobphol et al., 2000; Loimaranta et al., 2005). Further, gp-340 could interact with polymorphonuclear leukocytes (PMN) through sLe\(^x\) epitopes (Prakobphol et al., 1998; Prakobphol et al., 2000), binds S-IgA (Ligtenberg et al., 2004), lactoferrin (Mitoma et al., 2001), MUC5B (Thornton et al., 2001), induces aggregation of influenza A virus (Hartshorn et al., 2003), and inhibits human immunodeficiency virus (HIV) infectivity by binding viral glycoprotein 120 in a calcium-dependent manner (Wu et al., 2003). Further, gp-340 binds C1q and activates the first complement component (C1) via the classical pathway (Boackle et al., 1993) and stimulates random migration (chemokinesis) of alveolar macrophages (Tino et al., 1999). Homologues of gp-340/DMBT1 have been identified in rat (Ebnerin), mouse (CRP-ductin) and rabbit (Hensin) (Li and Snyder, 1995; Madsen et al., 2003; Takito et al., 1999).

Variation of *DMBT1* has been described in the normal population as well as in tumours. Variable numbers of tandem repeats (VNTRs, variation in length of a repeated nucleotide sequence), in the SRCR/SID region are found in tumours from brain, lung, pancreas and male breast (Mollenhauer et al., 2002). Single nucleotide polymorphisms (SNPs, variation in one single nucleotide) in *DBMT1* (C/T polymorphism in the 5´-region) decreases the promotor activity and is thus hypothesized to be associated with increased breast cancer risk (Tchatchou et al., 2010). However, it is unknown whether these tumour-associated polymorphisms are a cause or effect of the carcinomas.

**Host ligands in milk**

Human milk is the main source of nutrients for the infant and contains also numerous proteins, glycoproteins, glycolipids, polypeptides, and oligosaccharides that could affect colonization of bacteria in the oral cavity (Table 1). Human milk proteins are traditionally divided into caseins and whey proteins. Caseins are found in the pellet when human milk is precipitated with acid and whey proteins are the remaining soluble proteins. Caseins comprise 10-50% of the total protein in human milk and whey proteins comprise 50-90% depending on lactation length (Kunz and Lönnerring, 1992). Caseins are further described below. The major
components of the whey fraction of milk are: (i) α-lactalbumin, (ii) lactoferrin, (iii) lysozyme, and (iv) other proteins. (i) α-lactalbumin, which comprises 10-20% of the total protein, is a 14.1-kDa protein, which is calcium-binding although it is non-phosphorylated and non-glycosylated. α-lactalbumin, forms a protein complex with oleic acid in the infant’s stomach, and that induces apoptosis in tumour cells while normally differentiated cells are unaffected (Gustafsson, 2005). (ii) Lactoferrin is an iron-binding protein with a molecular weight of 80 kDa. Human lactoferrin possesses antiviral potency against HIV (Harmsen et al., 1995) and inhibits attachment of various bacteria, such as H. pylori, to gastric epithelial cells (Orsi, 2004), E. coli to urinary tract epithelium (Hanson, 2004), and S. mutans to dental polymer disks (Berlutti et al., 2004). In addition, lactoferrin per se is bacteriostatic (it inhibits growth of bacteria) by scavenging iron (Lönnerdal, 2003; Soukka et al., 1991) and fungistatic towards C. albicans (Andersson et al., 2000). (iii) Lysozyme is a 15-kDa protein and a major component of the whey protein fraction. It causes lysis of Gram-positive bacteria by hydrolyzing β-1,4 linkages of N-acetylmuramic acid and 2-acetamido-2-deoxy-D-glucose in the outer cell wall (Chipman and Sharon, 1969). Interestingly, lysozyme and lactoferrin enhance each other’s effects, i.e. lactoferrin binds and degrades lipopolysaccharides in the outer cell wall, and lysozyme degrades the inner proteoglycan matrix leading to death of the bacteria (Ellison and Giehl, 1991). Lysozyme also has an antiviral effect against HIV (Harmsen et al., 1995). (iv) Other proteins present in the whey fraction of human milk are the highly glycosylated bile salt-stimulated lipase (BSSL) (Bläckberg and Hernell, 1981), albumin, and the immunoglobulins S-IgA and IgG.

Caseins

Among the many proteins in human milk, caseins were selected for deeper analyses. Caseins, which form micelles or submicelles in the native stage, are present in three forms: α-, β-, and κ-casein. Human milk contains β- and κ-casein, while bovine milk also contains two forms of α-caseins. β-casein constitutes approximately 85% of the human milk caseins, but the β/κ-ratio changes during lactation. β-casein is a 212-aa, 24-kDa, non-glycosylated protein that is phosphorylated (0-5 phosphates) in the N-terminal end with high affinity for hydroxyapatite and binding of Ca$^{2+}$ (Lönnerdal, 2003). β-casein is thought to be an unfolded or random coil structure protein, and based on the aa sequence, it forms little secondary structure due to the many proline residues that are evenly distributed in the protein that disrupt α-helical and β-sheet structures (Bu et al., 2003). However, Syme et al. (2002), found that β-casein contains approximately 10% α-helical structures and 20% β-structure. β-casein is encoded by the CSN2 gene on chromosome
4q13.3 located within the same region as the \textit{STATH} gene (Huq et al., 2005). κ-casein is a glycosylated protein with a molecular weight of 37 kDa. It is present in lower concentrations (<15% of the total caseins) in human milk. κ-casein inhibits adhesion of \textit{H. pylori} to gastric mucosa, probably due to interactions with the carbohydrate moieties (Strömqvist et al., 1995; Hamosh, 1998). It has also been reported that α-, β-, and κ-caseins of bovine origin inhibit adhesion of \textit{S. mutans} to saliva-coated hydroxyapatite (Vacca-Smith et al., 1994).

**Bioregulatory proteins and peptides in saliva and milk**

Innate immunity is an immediate peptide-based host defence mechanism that controls bacterial adhesion and growth before an antigen-specific immune response has been developed (Boman, 1998; Gough and Gordon, 2000). Bioregulatory active peptides are released from “mother” proteins with less biological effect, and their effect is rapid. Their effect is exerted by various mechanisms, such as reduction of microbial growth, promotion of clearance by aggregation, inhibition of adhesion of opportunistic microbes, promotion of adhesion of commensal bacteria, normalization of pH, and induction of bacterial phagocytosis.

The cecropines in insects, which lyse bacterial, but not eukaryotic cells, were the first innate immunity peptides identified (Steiner et al., 1981). By now, several hundred antimicrobial peptides have been identified. Many of the identified innate immunity peptides are present in both saliva and milk, e.g.: β-defensins (killing of Gram-negative and some Gram-positive bacteria and \textit{C. albicans}) (Mathews et al., 1999; Jia et al., 2001); LL37 (broad-spectrum antimicrobial activity against bacteria, \textit{C. albicans} and viruses) (Murakami et al., 2002; Murakami et al., 2005); histatin 5 (from histatin 3, possessing antifungal activity) (Oppenheim et al., 1988; Ruissen et al., 2001; Pollock et al., 1984); and lactoferricin (from the amino terminal region of lactoferrin has bacteriocidal, antiviral and cytotoxic activity against cancer cells) (Orsi, 2004; Gifford et al., 2005; Eliassen et al., 2006). In addition, both saliva and milk contain bioregulatory proteins such as lysozyme (Aimutis, 2004) and lactoperoxidase (Månsson-Rahemtulla et al., 1988; Aimutis, 2004). Salivary gp-340 is an innate immunity glycoprotein with many functions such as interactions with bacteria, viruses, yeasts (Lightenberg et al., 2001; Prakobphol et al., 2000; Loimaranta et al., 2005; Hartshorn et al., 2003; Wu et al., 2003) and stimulation of random migration of macrophages (Tino and Wright, 1999). Salivary statherin is reported to be bacteriostatic (Kochanska et al., 2000) and may contribute to the antimicrobial properties of nasal fluid.
Introduction

(Cole et al., 1999). In addition, the bovine milk casein peptides β-casomorphine-7 and kappacin possess opiate like activity and inhibit bacterial growth, respectively (Kurek et al., 1992; Malkoski et al., 2001).

Thus, there are several proteins and peptides present in both saliva and milk that possess bioregulatory activity and protect the host from infection.

Host ligand glycosylation and secretor status

Many host proteins in human saliva and milk are glycosylated, which is a post-translational modification, in which monosaccharides are sequentially added by glycosyltransferases in the Golgi apparatus and endoplasmic reticulum. There are two main types of glycosylation, N-glycosylation and O-glycosylation. In N-glycosylation the polysaccharide is linked to asparagine and in O-glycosylation the polysaccharide is linked via N-acetylgalactosamine (GalNAc) to serine or threonine. The carbohydrates are highly diverse and differ in structure between tissues, individuals and animal species (Lis and Sharon, 1993). Glycans have many functions, both physicochemical, such as control of protein folding, stabilization of protein conformation, and resistance against proteases (Lis and Sharon, 1993). The glycans also have various biological functions, such as participating in microbial interactions (Aspholm-Hurtig et al., 2004; Strömberg et al., 1990), interaction with viruses, and cell-cell communication (Varki, 2006). The difference in glycosylation could explain the tropism of infections between tissues, individuals and animal species.

Prominent examples of glycosylation diversity between individuals are the ABH and Lewis blood group antigens, crucial to transfusion medicine, among many others. In the H antigens (H type 1 and H type 2), Fucα1-2 is added to the terminal Gal of the type 1 (Galβ1-3GlcNAcβ-R) or type 2 (Galβ1-4GlcNAc-R) precursor chains. Antigens A and B are GalNAcα1-3 and Galα1-3 added to the terminal Gal on the H-antigens. Lewis a antigen (Lea) and Lewis x antigen (Lex) are Fucα1-3/4 added on the GlcNAc of the type 1 (Galβ1-3GlcNAcβ-R) or type 2 (Galβ1-4GlcNAc-R) precursor, respectively. The difucosylated Lewis b antigen (Leb) and Lewis y antigen (Ley) are Fucα1-3/4 added on the GlcNAc of H type 1 and H type 2, respectively. The secretor phenotype, expressing ABH, Leb, and Ley in saliva and milk, is found in 80% of the population of Europe and North America, while the non-secretor phenotype, expressing Lea and Lex but not ABH, Leb, and Ley, is found in the remaining 20% (Marionneau et al., 2001; Henry et al., 1995).
Secretor status glycosylation has been associated with susceptibility towards infectious diseases. Non-secretors are more prone to recurrent urinary tract infections by *E. coli* because the addition of fucose by α-1,2-fucosyltransferase in secretors masks the binding epitope (Stapleton et al., 1992). It is also suggested that non-secretors are more susceptible to candidosis as they harbour higher carriage of *C. albicans* (Ben-Aryeh et al., 1995). Non-secretors are also more caries prone (Arneberg et al., 1976; Holbrook and Blackwell, 1989). Secretors are susceptible to Norwalk virus infection causing gastroenteritis, whereas non-secretors are not (Lindesmith et al., 2003), and the *H. pylori* BabA adhesin binds to Le\(^b\) present in secretors (Ilver et al., 1998).

**Model microorganisms present in oral biofilms**

*Actinomyces*

*Actinomyces* are Gram-positive, pleomorphic bacteria of which several species colonize soft and hard tissues of the mouth. Thus, *A. naeslundii*, *A. viscosus*, *A. odontolyticus*, *A. israelii* and *A. gerencseriae* are identified in the mouth (Li et al., 2004; Aas et al., 2005). *Actinomyces* species are considered to be part of the commensal oral flora. *A. naeslundii* are pioneer colonizers and could be important in stabilizing the early biofilm together with streptococci (Dige et al., 2009). *Actinomyces* species can remove sialic acid epitopes by sialidases and change the environment in the biofilm by modulation of pH (consuming lactic acid and degradation of urea) and removal of oxygen (Dige et al., 2009). *A. naeslundii* genospecies 2 (and also other species) mediate mutualistic growth of the anaerob *P. gingivalis* (Periasamy and Kolenbrander, 2009). However, if invading a broken epithelial cell lining, *Actinomyces* may cause severe infections (Smego and Foglia, 1998). Further, *Actinomyces* has been discussed in relation to root surface caries (Brailsford et al., 1998) and root canal infections (Collins et al., 2000).

*A. naeslundii*, with the two sub-species genospecies 1 and 2, are the predominant *Actinomyces* species in dental biofilms (Whittaker et al., 1996). *A. naeslundii* expresses two antigenically distinct fimbriae (type-1 and type-2) for attachment to host ligands. Type-1 fimbriae of *A. naeslundii* and *A. viscosus*, encoded by the *fimP* gene, mediate binding through protein-protein interaction to saliva APRP and statherin, respectively (Cicar et al., 1991; Hallberg et al., 1998). The type-2 fimbriae, encoded by the *fimA* gene and expressed by both genospecies of *A. naeslundii*, mediate binding to
carbohydrate structures (β-linked galactose and galactosamine) (Hallberg et al., 1998; Ruhl et al., 2004). Some *A. naeslundii* express both type-1 and type-2 fimbriae (e.g. T14V, Ruhl et al., 2004), some only express type-2 (e.g. ATCC 12104, Ruhl et al., 2004), whereas no strain expressing only type-1 has been found.

The taxonomy of *Actinomyces* has changed in the last few decades. The former separation of *A. viscosus* and *A. naeslundii* based on their catalase reactions, was changed when Johnson et al. (1990) investigated DNA-DNA relatedness among oral *Actinomyces*. Johnson et al. proposed that *A. naeslundii* serotype II and III, *A. viscosus* serotype II, and *Actinomyces* serotype NV should be included into *A. naeslundii* genospecies 2. Further, Johnson et al. proposed that *A. naeslundii* genospecies 1 included *A. naeslundii* serotype I and that *A. viscosus* serotype I (a strain isolated from hamster mouth) was named *A. viscosus*. These taxonomy changes were also supported by Putnins and Bowden (1993). Henssge et al., (2009) have further proposed that *A. naeslundii* genospecies 2 should be named *A. oris*, *A. naeslundii* genospecies WVA 963 should be named *A. johnsonii*, and *A. naeslundii* genospecies 1 should be named *A. naeslundii*. In the present thesis the taxonomy by Johnson et al. (1990) is followed.

**Streptococci**

Streptococci are Gram-positive, chain forming, and nearly spherical bacteria. Most Streptococci that colonize the mouth are part of the commensal flora, and several species are dominant in the first monolayer on teeth, such as *S. sanguinis*, *S. oralis* and *S. mitis* (Nyvad and Kilian, 1990b). Still many species of streptococci are linked to infections, such as tonsillitis, otitis, and dental caries (Stenudd et. al, 2001; van Houte, 1993; Brook 1998).

Historically, streptococci have been classified into groups depending on their carbohydrate composition in the cell wall (Lancefield, 1933). Streptococci can also be classified into 6 groups (mutans, mitis, anginosus, pyogenic, bovis and salivarius) based on relatedness of their 16S rRNA (Nobbs et al., 2009). The mutans group streptococci (*S. mutans* and *S. sobrinus*) are implicated in dental caries. Mutans streptococci are both aciduric and acidogenic by producing lactic acid as a by product of carbohydrate metabolism. However, other non-mutans groups (e.g. mitis- and salivarius-group) are also thought to be acidogenic at low pH (van Houte, 1994). Still others, such as *S. sanguinis*, are associated with health (Skovbjerg et al., 2009; Becker and Paster, 2002).
Multiple surface polypeptides (adhesins) are expressed by streptococci for adhesion to host ligands and other microorganisms. Many of the adhesin proteins contain a consensus sequence (LPxTz) in the C-terminal end, through which the adhesin is linked to the cell wall (Nobbs et al., 2009). Oral streptococci express three families of adhesins (Antigen I/II (AgI/II)-, Csh-, and Fap1-family proteins) (Jakubovics et al., 2005). AgI/II is expressed by most streptococci in the oral cavity. Proteins belonging to the AgI/II family are e.g. SspA and SspB in *S. gordonii* (Loimaranta et al., 2005), Pas protein in *S. intermedius*, and SpaP or Pac (also termed B, IF, P1, SR, MSL-1) in *S. mutans* (Oho et al., 2004). The proteins in the AgI/II family are highly conserved. Thus, SpaP and Pac are 97% identical in the amino acid sequence, and SspA and SspB are >96% identical in the C- and N-terminal end but are only 26% identical in the V-region (Jakubovics et al., 2005). Specifically, the alanine-rich N-terminal region of the AgI/II adhesin of *S. mutans* interacts with glycosylated proteins, such as the saliva gp-340 protein (Hajishengallis et al., 1994; Oho et al., 2004). Several other streptococci, including *S. gordonii*, have also been shown to adhere to salivary gp-340 (Kolenbrander, 2000; Prakobphol et al., 2000). In addition, AgI/II are shown to interact with other host ligands (e.g. collagen, laminin, and fibronectin) and other oral microorganisms such as *A. naeslundii, C. albicans, P. gingivalis* (Nobbs et al., 2009).
Aims

The overall aim of the present thesis was to study the effect of phosphorylated polypeptides and gp-340 in saliva and milk on adhesion and aggregation of oral bacteria. The specific aims were the following:

I. delineate the binding epitope in the calcium-binding phosphorylated polypeptide statherin for *A. naeslundii* and *A. viscosus*.

II. investigate the presence of phenotypic variation of saliva gp-340.

III. explore if human milk promotes or prevents *S. mutans* adhesion to saliva-coated hydroxyapatite.

IV. identify human milk components inhibiting adhesion of *S. mutans* to saliva-coated hydroxyapatite and investigate whether gp-340 is expressed by mammary glands.
MATERIALS AND METHODS

This section gives an overview of materials and major methods used in the thesis. For more detailed information see the individual papers.

Bacteria and fungus strains

The following bacteria and fungus strains were used in the studies: A. viscosus strains R28, T6-1600, ATCC 19246, A. naeslundii gsp 1 strains 35334, 29952, 30267, ATCC 12104 and gsp 2 strains T14V, M4356, A. radicidentis strain 42377, S. mutans strains Ingbritt, LT11, NG8, JBP, S. suis KU5, E. coli MS 506, S. sobrinus strains OMZ176, 6715, H. pylori strain 17-1, genetically modified Lactococcus lactis strains expressing Pac or SpaP, and C. albicans strain GDH18.

Saliva and milk

Parotid saliva secretion was stimulated by an acidic lozenge and the secreted saliva was collected into ice-chilled test tubes using sterile Lashley cups. Milk was collected from mothers who had been breast-feeding for 6 to 13 months. The milk was defatted by centrifugation and the pellet and upper cream layer were discarded. Saliva and milk samples were stored at -80°C until used. Depending on the purpose, saliva and milk were used from single donors or pooled from several donors (see further below).

Purification of saliva proteins

Statherin was purified from pooled fresh parotid saliva. The saliva was separated by anion-exchange chromatography, and fractions containing statherin were concentrated before separation by gel filtration. Identity and purity were determined with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and bacteria binding specificity to A. viscosus 19246 (Li et al., 2001).
Gp-340 was purified from fresh parotid saliva, from individual donors (Paper II) or pooled from multiple donors (Papers II and IV), utilizing the ability of *S. mutans* strain Ingbritt to bind gp-340 in solution. Equal volumes of parotid saliva and bacterial suspension of *S. mutans* Ingbritt were mixed and incubated at 37°C for formation of aggregates. After centrifugation and washing, gp-340 was released from the bacteria by repeated treatment with ethylenediamine tetraacetic acid (EDTA). The pooled supernatants were concentrated and proteins separated by gel filtration. The gp-340-containing fractions were pooled and concentrated. The identity and purity of gp-340 was determined with electrophoresis, on 4-20% Tris-HCl gel in reduced and non-reduced form, and by confirming the aggregation and adhesion patterns for three *S. gordonii* strains (DLI, M5, and SK184) (Loimaranta et al., 2005).

**Size variants of gp-340**

Individual gp-340 variants were revealed by separating unreduced parotid saliva proteins from healthy, young individuals (n=7) with SDS-PAGE on 5% precast gels. Gp-340 was detected with mAb143 after immunoblotting. The size variants were confirmed by separating purified gp-340 from the same subjects and staining with mAb143 and glycan detection.

**Carbohydrate mapping of gp-340 variants**

Carbohydrates present on gp-340 size variants were investigated by antibody and lectin binding in an overlay assay. Briefly, parotid saliva and gp-340 separated by SDS-PAGE were blotted onto a hydrophobic membrane (PVDF). After blocking with dried milk or bovine serum albumin (BSA), antibody or lectin was incubated with the membrane, and results were detected using horseradish peroxidase-labeled secondary antibodies or peroxidase-conjugated streptavidin and chemiluminescence.

Enzyme-linked immunosorbent assay (ELISA) and ELISA-like lectin binding were also used for mapping of carbohydrates on gp340. The wells of a microtiter plate were coated with gp-340, and after blocking with BSA, the wells were incubated with primary antibodies directed against blood group- and Lewis antigens (A, B, H, Lea, Leb, Le, Le, and sLe, or biotinylated lectins. Horseradish peroxidase-conjugated secondary antibodies or
peroxidase-conjugated streptavidin and ortho-phenylenediamine substrate were added before measuring absorbance.

Secretor status of the participants was determined by using parotid saliva in ELISA with antibodies against antigen H (cross-reacting with A and B antigen), Le\textsuperscript{a}, Le\textsuperscript{b}, Le\textsuperscript{x}, and Le\textsuperscript{y}.

**Separation and identification of milk proteins**

Defatted milk was separated by gelfiltration, the fractions containing proteins were collected, and dominant proteins were identified by SDS-PAGE and immunoblotting. Primary antibodies against IgG (γ-chain), IgA (α-chain), albumin, lactoferrin, lysozyme, BSSL and gp-340 were used. The fractions were further used to test for bacterial binding and binding inhibition.

**Adhesion assay**

Adhesion of bacteria and *C. albicans* to different ligands was examined using a hydroxyapatite (HA) assay (Gibbons and Hay, 1988). HA beads, a model for tooth enamel, were coated with host ligands (e.g. statherin, hybrid peptide, gp-340, milk or saliva), and the coated beads were washed and blocked with BSA before incubated with radiolabelled microorganisms (Figure 4). Thereafter unbound cells were washed away and the amount of bound microorganisms was determined by scintillation counting in a β-counter. BSA-coated beads (BSA-HA) were used as negative control and

![Figure 4. Principle of HA assay.](image-url) Radiolabelled bacteria and *Candida* cells were bound to test ligands coated onto HA-beads.
binding to standard saliva as positive control. Background binding, i.e. to BSA-HA, was low (1-6%) and constant within the experiments.

**Adhesion inhibition assay**

The ability of test substances to inhibit microorganism binding was tested by (a) simultaneous addition of bacteria and test substance to host ligand-coated HA (Papers III and IV) (Figure 5, panel a), (b) pre-incubation of microorganisms with test substance before adding to host ligand-coated HA (Papers I and IV) (Figure 5, panel b), and (c) pre-incubation of host ligand-coated HA with test substance before microorganisms were added (unpublished results, Paper IV) (Figure 5, panel c). Binding after exposure to test substances was compared with binding of untreated microorganisms/s-HA. Inhibition was calculated by using the equation 100x(binding_{control} - binding_{test})/binding_{control}.

![Figure 5. Schematic illustration of inhibition experiments.](image)
Materials and Methods

The binding of test substances to statherin was investigated using a binding assay. The samples were run in triplicate and both a positive and a negative control (BSA-HA) were included in each experiment.

In Paper I, inhibition of bacterial binding by test substances was also investigated using calcium-phosphate-coated latex beads that were coated with statherin and blocked with BSA. Microorganisms were incubated with test substance (statherin or peptides) and added to the latex beads. Aggregation was scored visually on a scale from 1 to 5 (Hallberg et al., 1998). Latex beads coated with BSA were used as negative control and statherin-coated latex beads with untreated microorganism were used as positive control.

Peptides for mapping bacteria-binding epitopes

For mapping of bacteria-binding epitopes in statherin, synthetic custom peptides and a hybrid peptide construct were used. The hybrid peptides had a common hydroxyapatite binding (phosphorylated) N-terminal end,

![Image of hybrid peptide construction](image)

**Figure 6. Construction of hybrid peptides.** A common phosphorylated N-terminal end with high affinity for hydroxyapatite, consisting of the first 15 aa of statherin, is linked to various test epitopes by a proline residue. The proline residue is flexible and facilitates presentation of the test epitopes when the hybrid peptide adheres to hydroxyapatite. Five hybrid peptides, which together covered aa 16-43 of statherin, were constructed.
consisting of the first 15 amino acids (aa) of statherin, linked to various test epitopes of 5-6 aa via a proline residue (Figure 6) (Gilbert et al., 2000). Together, the test epitopes covered aa 16 to 43 of statherin. Synthetic custom peptides with unmodified endings covering the same amino acids as the test epitopes of the hybrid peptides were used in inhibition experiments.

For mapping of binding epitopes in β-casein, syntetic peptides (30 aa) together covering the sequence of β-casein, were used. In addition, a peptide from lactoferrin, corresponding to a 13-aa stretch in bovine lactoferrin inhibiting S. mutans binding to saliva and gp-340-coated HA (Oho et al., 2002; Oho et al., 2004), was synthesized, and used for binding-inhibition experiments.

**Binding of bacteria in solution (aggregation assay)**

Equal amounts of bacteria (Actinomyces, Streptococcus) or Candida cells and test substance (saliva, milk, milk fractions, purified milk proteins, statherin, or peptides) were incubated at room temperature or 37°C on a glass slide. Aggregation was scored visually on a scale from 1 to 5 (Hallberg et al., 1998).

Aggregation by gp-340 was done by adding gp-340 to washed S. mutans, S. suis, H. pylori, L. lactis or E. coli and OD_{700} was recorded for 90 minutes. Aggregation was expressed as the percentage of OD_{700} at 60 minutes compared to OD_{700} at 0 minutes.
RESULTS AND DISCUSSION

Paper I. Binding epitopes for *Actinomyces* on salivary statherin

The adhesion of *Actinomyces* strains to statherin segments revealed that strains with different origins bind to different statherin segments, which may confer a role of statherin in biofilm diversity. The statherin molecule has three distinct structural domains when bound to hydroxyapatite (Long et al., 2001; Naganagowda et al., 1998; Goobes et al., 2006). An α-helix in the N-terminal region, followed by a polyproline type II structure, and an α-helix (Figure 7). Based on the binding specificity the bacteria could be divided into three distinct binding types (types I, II, and III). *Actinomyces* isolated from human infections and rat/hamster (type I adhesion) bound in the C-terminal α-helix region, whereas commensal *Actinomyces* (type II adhesion) bound in the middle polyproline type II region. The third binding type, *Actinomyces* isolated from human infections and also *C. albicans*, bound to statherin but not to any of the linear statherin segments. This suggests the need for the whole statherin molecule for the correct conformation and presentation of the binding epitope(s). None of the bacteria bound to the hydroxyapatite-binding, helical N-terminal part of statherin.

**Figure 7. Binding of *Actinomyces* strains to statherin.** Commensal strains bound to the middle region of statherin whereas infectious *Actinomyces* strains bound to the C-terminal end. The binding of infectious strains could be inhibited whereas commensal strains could not be inhibited by statherin-derived peptides.
To further delineate the binding epitopes, hybrid peptides were used. The hybrid peptides were constructed as a fusion between the statherin hydroxyapatite-binding N-terminal end and various test epitopes, connected via a proline residue. This directed the epitope out from the surface and facilitated presentation of the epitope. The N-terminal end (aa 1-15) did not mediate binding of any of the tested microorganisms. Together the test epitopes cover the middle- and C-terminal part of statherin. This type of fusion peptide has been used to show that an osteopontin peptide, PGRGDS, mediates dose-dependent adhesion of melanoma cells when bound to hydroxyapatite (Gilbert et al., 2000). By using hybrid peptides, Drobni et al. (2006a) showed that *A. naeslundii*, *A. viscosus*, and *S. gordonii* recognized different peptide motifs in saliva PRP-1.

*Actinomyces* strains classified as type I bound to the most C-terminal test epitopes, PYQPQY (aa 33-38), QQYTF (aa 39-43), and PYQPYQYTF (aa 33-43), whereas type-II strains bound to the two middle YQPVP (aa 21-26) and QPLYPQ (aa 27-32) epitopes. The only common amino acids among the binding motifs are Y and Q, suggesting that these aa could be influential for binding. When substituting some aa in QQYTF with alanine, an amino acid that does not add any features but removes them, it was discovered that the TF dipeptide was crucial for inhibition and desorption of type-I strains, since QQYTF, QAATF and TF, but not QQYAA and PYQPQY, inhibited binding. However, the inhibition and desorption effect was even more efficient when preceding the TF dipeptide with QAA (QAATF instead of QQYTF). This might indicate that both Q and TF are binding sites or that QAA gives the peptide an optimal configuration for the presentation of the TF epitope. The importance of single amino acids flanking a binding epitope has been shown for RGD peptides that inhibit appressorium formation in *Uromyces appendiculatus* (Correa et al., 1996). Appressorium is a hypha structure by which the plant pathogenic fungus, *Uromyces appendiculatus*, infects its host plant. Also for RGRPQ, a peptide released from saliva PRP-1 that inhibits adhesion of *A. naeslundii* to tooth enamel, it has been shown that single amino acids are important for the binding inhibition (Drobni et al., 2006b; Drobni et al., 2006c). In accordance with the findings in Paper I, the Q residue in RGRPQ was crucial for binding inhibition, but the size and hydrophobicity of the amino acids at positions 2 and 4, respectively, were also influential (Drobni et al., 2006c). The latter result was accomplished with QSAR (quantitative structure activity relationship) analyses. QSAR is a multivariate mathematical model in which chemical properties are related to biological activity. The model can help predict peptide design for optimal biological response. This approach could also be useful for optimizing inhibitory peptides from statherin.
**Actinomyces** strains with infectious and rat/hamster origin could be inhibited by peptides in solution whereas commensal strains could not. It has earlier been shown that the FomA porin protein of *F. nucleatum*, a bacterium associated with periodontitis, adheres to the same middle section of statherin as the commensal strains in this study (Nakagaki et al., 2010). However, the binding of *F. nucleatum* could be inhibited by statherin-derived peptides (Sekine et al., 2004). *P. gingivalis*, also associated with periodontitis, is also reported to bind the C-terminal portion of statherin in an inhibitable fashion (Amano et al., 1996). This implies that there is a possible adhesion-regulating property residing within the statherin molecule, where adhesion of commensals is promoted whereas adhesion of potentially infectious strains is inhibited. Bacterial proteolytic activity in the saliva might release peptides from statherin that could inhibit the adhesion of various bacteria, and thus regulate adhesion of bacteria in the oral biofilm in favour of commensals. It is therefore tempting to speculate that statherin is an innate immunity polypeptide, as has been suggested by Cole and coworkers (1999) and by Kochanska et al. (2000), with a possible role in protecting the host from colonization of bacteria associated with infections.

Several different statherin variants (among others SV1, SV2, SV3, Jensen et al., 1991) have been detected in saliva, and Vitorino et al. (2008) have further detected small statherin fragments in the acquired enamel pellicle, among others YPQPYQPQ (aa 29-36) and some N-terminal fragments. According to Helmerhorst et al. (2008), statherin could be cleaved by bacteria-derived glutamine endopeptidase. This endopeptidase preferentially cleaves after a Q residue, with specificity for XPQ. Helmerhorst et al. (2008) found several statherin peptides in whole saliva, among them a peptide cleaved after Q^{35}P^{36}Q^{37}. The remaining YQQYTF (aa 38-43) peptide should then be released, which hypothetically could block adhesion of type I *Actinomyces*. Thus, it would be of interest to test the different statherin variants and released statherin peptides for binding and binding inhibition of the panel of oral bacteria belonging to binding type I-III. Presumably type I *Actinomyces* would not bind SV1, SV3 and statherin des-Thr^{42}, Phe^{43}, and thus individuals with more of these statherin variants would possibly favour colonization of commensal *Actinomyces* strains.

Statherin binds not only hydroxyapatite, but it is also thought to cross-link to buccal epithelial cells (BEC) (Bradway et al., 1992), catalysed by oral transglutaminase to form cross-links between glutamine (Q) and lysine (K) residues (Yao et al., 1999). Statherin has one lysine residue (Lys^{6}) in the N-terminal and seven glutamine residues in the middle and C-terminal part, where cross-linking can form. The reactivity of lysine (K) was higher (100%) than of glutamine (Q) (14%) (Yao et al., 1999). For statherin it has been
shown previously that the C-terminal forms cross-links catalyzed by transglutaminase (Bradway et al., 1992). Cross-linking of the C-terminal would, hypothetically, alter the presentation of the C-terminal. Therefore, it is hypothesized that *Actinomyces* type I from infectious origin and rat/hamster might not be able to adhere to statherin cross-linked to BEC, but still they could bind by co-aggregates. However, this remains to be investigated. In addition, cross-linking between Lys<sup>6</sup> and Gln<sup>37</sup>, facilitated by transglutaminase, forms cyclo-statherin Q-37 detected in whole human saliva (Cabras et al., 2006). How this cyclization affects bacterial binding is unknown.

In the present paper we have suggested that the statherin peptides could adopt different conformations depending on which amino acids that preced the binding epitope. Conformational changes in statherin (Gibbons et al., 1990; Amano et al., 1996) and APRP (Gibbons, 1989) have been shown previously. In particular, salivary proteins, such as APRP, may contain cryptic segments (cryptitopes), i.e. hidden binding epitopes, which only become exposed when adsorbed to hydroxyapatite (Gibbons, 1989). Statherin also seems to adopt different conformations depending on whether it is in solution or bound to hydroxyapatite, since statherin *per se* could not inhibit bacterial binding to statherin or hybrid peptides coated on HA-beads, and statherin was unable to aggregate bacteria in solution. It is reported that statherin has a disordered structure in aqueous solution and that the α-helix in the N- and C-terminal areas form upon adsorption to hydroxyapatite (Goobes et al., 2006). This supports the finding of the different behaviour of statherin in solution versus when attached to hydroxyapatite. Together, these findings suggest that statherin might play a role in biofilm diversity, possibly promoting colonization of commensal bacteria. Statherin *per se* also exists in different polymorphisms and could give rise to bioactive peptides that could bind bacteria differentially. Hypothetically, this could alter the composition of the biofilm towards a more health-associated or more disease-associated biofilm.

**Paper II. Variant size- and glycoforms of gp-340 with differential bacterial aggregation**

In contrast to statherin, gp-340 is a large, heavily glycosylated protein. It is a mucin hybrid type of protein with its tandem repeats of O-glycosylated and serine-, threonine-, and proline-rich SID domains (involved in bacterial interactions) and cystein-rich SSCR domains (for protein-protein interactions). The domains contribute to the complex biological innate
immunity behaviour of gp-340 and to the potential links to caries and Crohn’s disease (inflammatory disease of the gastrointestinal tract).

In this paper, four novel size variants of gp-340 were revealed in parotid saliva and in purified gp-340 from seven donors, as detected by SDS-PAGE and Western blot using a monoclonal antibody (mAb143) directed towards the gp-340 protein core. Variants I, II, and III had single broad bands (345, 375, and 389 kDa, respectively), whereas variant IV had a double band (345 and 287 kDa) (Figure 8). When purified gp-340 from the four variants was stained for carbohydrates the same bands appeared confirming the gp-340 size variants and that they were glycosylated. Purified gp-340 from lung lavage, of a person with pulmonary alveolar proteinosis, also displayed a double band in Western blot with mAb143. Lung gp-340 seemed to have lower carbohydrate content than parotid saliva gp-340 (donor with variant II). A glycan detection kit and carbohydrate composition analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) confirmed a lower and different composition for lung gp-340.

The four size variants were sequenced with Edman degradation and were found to have an identical N-terminal sequence (TGGWIP), which suggests that the N-terminal protein core could be similar among the different size variants. However, differences/deletions could still reside in the middle or C-terminal of gp-340, and thus explain the different size variants detected.

Mollenhauer et al. (1999 and 2002) have described a *DMBT1* allelic variant
(6 kb) with 5 SRCR and 5 SID domains deleted in the middle of the protein. It has been found that variable numbers of tandem repeats (VNTRs) within the SRCR/SID region in DMBT1 form a multi-allele system that could be involved in the variability of DMBT1 in tumours and in healthy subjects (Mollenhauer et al., 2000 and 2002). The polymorphism of gp-340 could thus be explained by allelic variation, VNTRs, or splice variation in the protein core. Post-translational modifications, such as proteolysis or differences in glycosylation, could also be possible explanations for the gp-340 size variants, but this remains to be investigated. It could be speculated that variant IV could be derived from proteolysis of variant I since one of the bands had the same size as variant I. Additionally, variant IV was only detected in one individual.

The different size variants of gp-340 could possibly be related to several biological functions. Jonasson et al. (2007) showed that the gp-340 I protein correlates positively with caries experience and adhesion of S. mutans Ingbritt to s-HA. The phenotype is overrepresented in subjects with Db protein, a caries-susceptibility factor, and subjects positive for both gp-340 I and Db experienced more caries (Jonasson et al., 2007). Thus, it is speculated that gp-340 phenotype I could be a caries-susceptibility factor. The caries-associated gp-340 size variant I was less common (22-26%) than II/III in both Jonasson’s and the present study. Renner et al. (2007) observed that the DMBT1 deletion variant (6 kb) was overrepresented among patients with Crohn’s disease. One may hypothesize that the small 6 kb DMBT1 variant corresponds to the smaller (faster migrating) gp-340 size variant I, however this remains to be investigated further.

All of the size variants of gp-340 from the seven donors were glycosylated but displayed some differences in their glycosylation. The individuals with gp-340 size variants I and IV versus II and III had secretor or non-secretor-like fucosylated patterns, respectively, as shown by fucose-specific lectin (UEA-1), ABH blood group antigens and Lewis antigens binding patterns. However, several core carbohydrates, such as sialylated Galβ1-3GalNAc, α2-6 and α2-3-linked sialic acids, N-glycans, and type 2-lactosamine or polylactosamine structures seemed to be carried by all four variants. The differences in glycosylation between the size variants could be indirectly linked to the size variations because post translational glycosylation normally varies between individuals. However, it could also be directly linked to the size variants based on different or deleted glycosylation sites.

Gp-340 mediates binding of a wide array of bacteria by carbohydrate-lectin binding or protein-protein interactions. Thus, differences in glycosylation could potentially influence the ability to aggregate bacteria. The low and
high-glycosylated lung and parotid saliva gp-340 aggregated bacteria differently (Table 2). The low-glyco- and GlcNAc-lung gp-340 displayed very low aggregation of *S. mutans* LT11, whereas parotid saliva gp-340 had high aggregation, potentially indicating a polylactosamine receptor. By contrast, both lung and saliva gp-340 aggregated *S. mutans* Ingbritt strongly, indicating that the two *S. mutans* strains (Ingbritt and LT11) bind to different receptors. Such a presence of adhesins with different receptor specificities has been described for *S. gordonii* (Loimaranta et al., 2005). In addition, the high-glyco- and GlcNAc-saliva gp-340 aggregated *S. suis* KU5 highly, with binding specificity to sialylpolylactosamine, while lung gp-340 did not aggregate *S. suis* KU5, whereas both lung and saliva gp-340 aggregated *S. suis* 836 highly. The gp-340 size variants I-III showed differential aggregation of *S. suis* KU5 and Leb-binding *H. pylori* 17:1, with preferential aggregation of the highly glycosylated variant II (Table 2). However, *S. mutans* Ingbritt was aggregated by all three size variants to a similar extent indicating that the gp-340 variants share some carbohydrate and peptide receptor motifs. By contrast, adhesion of *S. mutans* Ingbritt to gp-340 variants I-III varied (I>II>III) (Jonasson et al., 2007), suggesting that different epitopes are involved in aggregation and adhesion (Loimaranta et al., 2005).

Table 2. Bacteria aggregation with different gp-340 variants.

<table>
<thead>
<tr>
<th>Species and strains</th>
<th>Aggregationa</th>
<th>Receptor sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High glyco saliva gp-340</td>
<td>Low glyco lung gp-340</td>
</tr>
<tr>
<td><em>S. mutans</em> Ingbritt</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>LT11</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>S. suis</em> KU5</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>836</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>H. pylori</em> 17:1</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

a Aggregation is indicated with + = weak aggregation, ++ = moderate aggregation, +++ = high aggregation, and – = no or very low aggregation.
b Sia2-3PL = sialylα2-3polylactosamine

The conclusion is that saliva harbours different gp-340 size variants among individuals. In addition, individual differences in gp-340 glycosylation were found. These size- and glyco-polymorphisms could affect both *S. mutans* adhesion, development of caries, and interaction with a wide array of bacteria and viruses (including HIV and influenza virus). In this respect, it is notable that the differences in glycosylation between the gp-340 variants possibly could be related to secretor status, which for a long time has been
Results and Discussion

discussed in relation to infections, such as Norwalk virus infection (Lindesmith et al., 2003), *H. pylori* infection (Ilver et al., 1998), candidosis (Ben-Aryeh et al., 1995) and dental caries (Arneberg et al., 1976; Holbrook and Blackwell, 1989). Moreover, gp-340 is a pattern-recognition protein that binds both Gram-positive and Gram-negative bacteria through conserved leucine-rich repeats (Loimaranta et al., 2009). Therefore, the different size variants of gp-340 could affect other biological pattern-recognition functions related to host innate immune defences. Recently, gp-340 polymorphisms have been linked to Crohn’s disease (Renner et al., 2007), and other diseases involving bacterial components, such as dental caries (Jonasson et al., 2007).

Papers III and IV. Effects of human milk and milk components on adhesion of mutans streptococci to saliva-coated hydroxyapatite *in vitro*

Human milk is an exocrine secretion which is very similar to saliva in many aspects. Several proteins and peptides in saliva with innate immunity functions are also present in human milk; examples are lactoferrin, lactoperoxidase, lysozyme, LL37, and defensins (Murakami et al., 2005; Lönnerdal, 2003; Jia et al., 2001). Gp-340 is another protein present in saliva that also possesses innate immunity properties and binds *S. mutans* strongly when the protein is bound to hydroxyapatite. Gp-340 has been found in tears (Schulz et al., 2002; Jumblatt et al., 2006), saliva, lung, small intestine, trachea, and stomach (Holmskov et al., 1999). Low levels of messenger ribonucleic acid (mRNA) of gp-340/DMBT1 have also been detected in mammary glands of non-lactating women (Holmskov et al., 1999), and our own preliminary results suggested that gp-340 might be present in human milk leading to the hypothesis that gp-340 is expressed in lactating women, i.e. in human milk. However, further testing showed that none of four tested antibodies directed against gp-340 could detect gp-340 in human milk separated with SDS-PAGE and Western blotting. This lead to rejection of the hypothesis and the conclusion that the gp-340 protein is not present in human milk or that the levels are too low to be detected by the assay. This finding is supported by the fact that neither milk nor any milk fractions mediated avid binding of *S. mutans*. However, human milk proteins in the size interval of S-IgA, BSSL, lactoferrin, albumin, β-casein, and lysozyme did adhere to hydroxyapatite as determined with SDS-PAGE.

In contrast, human milk could aggregate *S. mutans* weakly, but the extent of aggregation varied between the individuals’ milk and strains. This might
Results and Discussion

indicate that milk proteins bound to hydroxyapatite have an unfavourable conformation that does not present the binding epitope for *S. mutans*, whereas the epitope is exposed in solution. This phenomenon, but reverse when the epitope is exposed in solid phase (adsorbed to hydroxyapatite), but not in solution, has been shown for statherin (Paper I; Amano et al., 1996) and APRP (Gibbons and Hay 1988; Gibbons et al., 1991). In addition, gp-340 contains cryptitopes and binds different bacteria in solution as opposed to when bound to hydroxyapatite (Loimaranta et al., 2005).

The effect of human milk on adhesion of cariogenic *S. mutans* to tooth enamel coated with saliva protein (s-HA) was studied. It has previously been shown that *S. mutans* adhere to certain salivary proteins (e.g. gp-340 and PRP) coated on hydroxyapatite (Ericson and Rundegren, 1983; Gibbons and Hay, 1989), and in this study the binding of *S. mutans* Ingbritt was 19-69% to parotid saliva samples from 21 mothers. The mothers could be divided into three groups depending on *S. mutans* binding to s-HA and binding inhibition by their milk. The mothers in group I displayed high binding of *S. mutans* to s-HA and high inhibition by milk; group II had moderate binding to s-HA and moderate to low inhibition; whereas group III had lower binding to s-HA and enhanced binding by milk (see Figure 2 in Paper III). Milk with high inhibition effect also had a higher aggregation score, and milk that increased binding aggregated *S. mutans* poorly. In the binding experiments described in this paragraph, milk and saliva were from the same mother.

The results above show that there are individual variations in the expression of epitopes for *S. mutans* binding and binding inhibition. To further examine the individual variation, saliva from three mothers (two avidly-binding salivas and one low-binding saliva) were used to cross-test inhibition with milk from all mothers. The adhesion of *S. mutans* to avidly-binding saliva was inhibited by milk from all groups (group I, II, and III). However, milk from all three groups increased *S. mutans* binding to hydroxyapatite coated with low-binding saliva. Thus, the ability to inhibit binding of *S. mutans* seems to depend on individual variations in the composition of the saliva. Therefore, gene variation, alternative splicing, variation in glycosyltransferases, or number of tandem repeats in saliva proteins might contribute to the inhibiting effect. To further examine whether different glycosylation phenotypes were present in groups I-III, blood group of the mothers were determined from the medical records. In group I, 57 % of the mothers had blood group antigen A, whereas 83% in group III had blood group antigen O. Thus, there seems to be a difference in the most common blood group among mothers with avidly-blocking milk (group I) and enhanced-binding milk (group III). It has been reported that blood group
antigens are involved in susceptibility towards infections, e.g. binding of \textit{H. pylori} BabA adhesin to A, B, and O antigens in gastric epithelium (Aspholm-Hurtig et al., 2004). However, due to the small number of subjects involved in this study, these findings only suggest that the blood group glycosylation might be involved, but a more thorough study must be performed to draw any firm conclusions.

These results suggest that the individual properties of saliva, such as polymorphism in the heavily glycosylated \textit{S. mutans} Ingbrit ligand gp-340 in saliva, determine the degree of \textit{S. mutans} inhibition caused by human milk. We have previously shown that gp-340 is a blood group- and Lewis-antigen bearing protein (Paper II). In fact, a pilot evaluation suggested that gp-340 variant I was over represented among mothers belonging to group I (unpublished results). This is in line with the findings that gp-340 variant I correlates positively with caries experience in 12-year-old children and that adhesion of \textit{S. mutans} Ingbrit to saliva-coated hydroxyapatite correlates positively with their parotid saliva (Jonasson et al., 2007). This hypothesis is presently being evaluated in a larger group.

To further investigate which components in human milk that are responsible for the inhibition, human milk from two mothers with high binding/high inhibition (group I) milk was fractionated with gelfiltration. Human milk fractions coated onto hydroxyapatite did not mediate binding of \textit{S. mutans}, which further confirms that gp-340 or any other hydroxyapatite-binding ligand for \textit{S. mutans} is not present in human milk. However, fractions from peaks containing IgG and S-IgA, BSSL, albumin, caseins, lactoferrin, and α-lactalbumin inhibited binding of \textit{S. mutans} to s-HA by more than 50%, and thus were hypothesized as inhibitory components. Purified human milk proteins confirmed that lactoferrin, caseins, S-IgA, and IgG reduced binding of \textit{S. mutans} Ingbrit avidly whereas BSSL and α-lactalbumin reduced the binding moderately to low. However, lysozyme and albumin had no inhibitory effect, suggesting that some other compound(s) in these peaks were responsible for the inhibition. These findings are in accordance with earlier studies, since an inhibitory effect of bovine lactoferrin and bovine caseins on \textit{S. mutans} adhesion to salivary proteins has been shown previously (Vacca-Smith et al., 1994; Oho et al., 2002). Additionally, \textit{S. mutans}-specific S-IgA is present in human milk (Camling et al., 1987) and an inhibitory effect of S-IgA on \textit{S. mutans} adherence to s-HA has been shown (Hajishengallis et al., 1992). However, it is speculated that bacterial IgA1 proteases in the oral cavity might interfere with this anti-adherence effect (Hajishengallis et al., 1992).
By preincubating either s-HA or bacteria with purified milk proteins in the adhesion inhibition assay it was found that lactoferrin, β-casein, α-lactalbumin and IgG all had an effect on the salivary host ligand side, whereas S-IgA and BSSL had an effect on the bacterial side (Figure 9). The effect on the salivary host ligand side could depend on binding of the milk protein to the host ligands. That binding could prevent the bacteria to adhere, or exchange bound salivary host ligands on hydroxyapatite, as described for bovine casein derivatives (caseinoglycomacropeptide and caseinophosphopeptides) that displace albumin from hydroxyapatite (Neeser et al., 1994). As mentioned earlier, the milk proteins per se did not mediate adhesion of S. mutans to hydroxyapatite. However, for lactoferrin, β-casein, and α-lactalbumin, the binding was enhanced when preincubated with bacteria, but the net effect (simultaneous addition) was inhibitory. 

![Figure 9. Adhesion of S. mutans to s-HA or gp-340-HA when milk proteins are preincubated with either s-HA/gp-340-HA or the bacteria. Percent S. mutans binding is compared with control binding of non inhibited S. mutans binding to s-HA or gp-340-HA. (a) Milk proteins with effect on the saliva/gp-340, (b) milk proteins with effect on the bacteria.](image)

For S-IgA and BSSL, the inhibitory effect was on the bacterial side, possibly binding to the adhesin and preventing attachment to salivary host ligand adsorbed to hydroxyapatite. The increased binding when preincubated with s-HA or gp-340-HA was not easily explained. One feasible explanation could be that S-IgA and BSSL alter the presentation of exposed binding epitopes of the hydroxyapatite-adsorbed host ligands, giving a more favourable
presentation of the epitope to the bacterial adhesins. However, the net effect was inhibitory.

Human milk contains both β- and κ-casein, but β-casein constitutes almost 85% of the total caseins in human milk. Therefore, it was hypothesized that β-casein was the strong inhibitor among the caseins. Seven synthetic peptides covering the sequence of β-casein were synthesized and tested for inhibition. A peptide covering the 30 most C-terminal amino acids of β-casein had an inhibitory effect of 87% at a concentration of 0.375 µM, whereas none of the other peptides had any effect, suggesting that a *S. mutans* inhibitory binding epitope resides in the C-terminal portion of β-casein. Lactoferrin was one of the other strong inhibitory components, and it has earlier been shown that a lactoferrin fragment (Lf411, aa 473-538) of bovine milk inhibits aggregation of *S. mutans* to gp-340 (Mitoma et al., 2001). Further, it has been shown that a particular peptide Lf (480-492) of the Lf411 fragment is responsible for the inhibition of PAc (AgI/II adhesin of *S. mutans*) to the PAc-binding domain in salivary agglutinin (Oho et al., 2004). Therefore, a lactoferrin peptide covering aa 501-513, corresponding to aa 480-492 (85% similarity in aa sequence) in lactoferrin from bovine milk, was also tested for inhibition, and inhibited binding of *S. mutans* Ingbritt to s-HA by 66% at a concentration of 500 µM.

Further, adhesion inhibition was compared among six strains of mutans streptococci (*S. mutans* Ingbritt, LT11, NG8, JBP and *S. sobrinus* 6715, OMZ176). All strains were inhibited by human milk, although *S. sobrinus* 6715 was only slightly inhibited. All strains were also inhibited by the most C-terminal β-casein peptide and S-IgA, whereas lactoferrin only inhibited *S. mutans* Ingbritt and no other strain (see Table 1 in Paper IV). It was not expected that the lactoferrin inhibition of the strains would be so diverse.

The first teeth emerge when the infant is around 6 months old, and colonisation of *S. mutans* is thought to occur when the teeth have erupted. This has been suggested to occur in a discrete “window of infectivity” around 19-31 months of age (Caufield et al., 1993). However, by using molecular methods, Tanner et al. (2002) have observed that *S. mutans* and *S. sobrinus* can be detected from tongue and tooth samples in children younger than 18 months (n=57). A relationship between early acquisition of mutans streptococci and dental caries has been seen (Straetemans et al., 1998). The relevance of anti-adhesive mechanisms of human milk is relevant in developing countries where the children usually are breast fed for a longer time than in the Western world. In Sweden full or partial breast feeding of infants is common, but decreases with age (69% at 6 months, 39% at 9 months, and 17% at 12 months in 2006) (The National Board of Health and
Welfare, 2006). Interestingly, human milk coated on hydroxyapatite promotes adhesion of commensal bacteria such as *A. naeslundii* and *S. sanguinis* (unpublished results). Thus, human milk could have a protective effect in infants also in Western countries, since it could still favour acquisition of a commensal bacterial flora, and it also could prevent or delay adhesion of *S. mutans* on the initial primary teeth.
CONCLUSIONS AND FUTURE PERSPECTIVES

This doctoral thesis has focused on studying the interactions between saliva and milk host ligands and oral bacteria with relevance for biofilm formation. Dental caries, a chronic infectious disease, has been thought of as a target disease for selection of model ligands and bacteria for studying such interactions. The main conclusions are the following:

- Bacteria from different origins bind to different segments of statherin. The binding motif for strains with infectious and rodent origin was delineated to a small, linear epitope that seemed to be dependent on conformational presentation. Adhesion of commensal bacteria was promoted whereas adhesion of infectious strains was inhibited. Thus, statherin seems to possess adhesion-regulating properties that may protect the host from colonization of bacteria associated with infections. Since statherin is reported to occur in different forms and proteolytically cleaved peptides are released from statherin, a future aim of interest would be to delineate a possible role of statherin in innate immunity including how the different statherin variants affect adhesion.

- There are individually different size variants of gp-340 in saliva, and in addition there are differences in glycosylation that may be related to secretor status of the host. The size- and glycoforms could aggregate bacteria differently and may affect many biological functions related to host innate immune defences. This would be a highly interesting topic for further studies.

- Human milk could inhibit mutans streptococci adhesion to s-HA in an individually varying fashion, dependent on individual properties of the saliva. Several milk proteins could inhibit adhesion and especially a C-terminal peptide of β-casein was a potent inhibitory component together with a lactoferrin peptide. Thus, human milk may have a protective effect against early acquisition of the caries-associated mutans streptococci in infants. We also have unpublished data showing that human milk mediates binding of health-associated species. Therefore, it would be interesting to explore the role of human milk, and specific components therein, in a complex biofilm model. A possible long-term aim would be to compose baby
formulas mediating such milk protective characteristics together with nutrition.

To sum up, the studied host ligands may affect bacterial colonization and biofilm formation and could possibly also alter the susceptibility towards infections of the host.
Acknowledgements

Det är många personer som har stöttat, uppmuntrat och peppat mig under årens lopp, både arbetskamrater, familj och vänner. Jag vill därför skicka ett stort och varmt TACK till alla som på ett eller annat sätt har hjälpt mig under den här tiden. Speciellt vill jag dock tacka mina arbetskamrater på tandläkarhögskolan:

Min handledare Ingegerd Johansson, som har handlett mig i forskningens värld. Tack för ditt engagemang och din breda kunskap som du har delat med dig av, givande diskussioner och allehanda hjälp samt krav på tidsplaner. Utan dig hade det inte blivit någon avhandling!

Pernilla Lif Holgersson, min bihandledare, för all hjälp i samband med slutförandet av avhandlingen.

Nicklas Strömberg, för brett kunnande, diskussioner och hjälp, särskilt i samband med gp-340 artikeln, samt bra synpunkter på delar av avhandlingen.


Anette Jonasson, Mirva Drobni, Christer Eriksson, det har varit trevligt att lära känna er och dela lab med er. Mirva, tack också för trevligt rumssällskap och ventilationskanal i mitten av resan. Lotta Harnevik, för bra kommentarer på delar av avhandlingen.

Nina Forsgren och Margareta Holmgren, ni var riktigt trevliga rumskompisar!

Hjördis Olsson, tack för diverse administrativ hjälp.

Till alla övriga personer på forskarväningen som har gjort lab, korridorerna, fika- och lunchrasterna trevliga: Elisabeth, Maria, Rolf, Pamela, Inger, Eva, Anna L-B, Karina, Emma, Kjell, Anna B, Mari, Voukko, Lars, Maggan, George, Vincent m.fl. Jag vill även tacka
Thomas Borén och alla medlemmar i hans grupp som under de första åren bidrog till att muntra upp tillvaron.

Tack även till mamma och pappa (och er förståelse över inställd skidresa - ställa in det som verkliga är livet!) och till mina älsklingar Johan och Edvin för att ni har stått ut med mig under de här sista månaderna.

(Liza)
REFERENCES


Aimutis WR. Bioactive properties of milk proteins with particular focus on anticariogenesis. *J Nutr.* 2004;134:989S-995S.


References


Hallberg K, Hammarström KJ, Falsen E, Dahlén G, Gibbons RJ, Hay DJ, Strömberg N. *Actinomyces naeslundii* genospecies 1 and 2 express different binding specificities to N-acetyl-β-D-galactosamine, whereas *Actinomyces odontolyticus* expresses a different binding


**Henssge U, Do T, Radford DR, Gilbert SC, Clark D, Beighton D.** Emended description of Actinomyces naeslundii and descriptions of Actinomyces oris sp. nov. and Actinomyces johnsonii sp. nov., previously identified as Actinomyces naeslundii genospecies 1, 2 and WVA 963. *Int J Syst Evol Microbiol.* 2009;59:509-516.


Takito J, Yan L, Ma J, Hikita C, Vijayakumar S, Warburton D, Al-Awqati Q. Hensin, the polarity reversal protein, is encoded by DMBT1,


