CHARACTERIZATION OF CYTOKINE BINDING AND ITS IMPLICATIONS IN THE PHYSIOLOGY OF AGGREGATIBACTER ACTINOMYCETEMCOMITANS

Tuuli Ahlstrand
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“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.”

- Thomas A. Edison

To my family
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ABSTRACT

Bacterial pathogens cause severe infections in humans. The emergence of multiresistant bacteria requires the development of new antimicrobial strategies. Bacteria utilize various virulence mechanisms to promote their survival in the host organism. Among these virulence mechanisms, we may find novel targets for antimicrobial treatment.

This thesis concerns a virulence mechanism of an oral bacterium that may disturb the human immune response and potentially increase bacterial virulence. The model organism for this study is Aggregatibacter actinomycetemcomitans, a gram-negative opportunistic pathogen that forms biofilms on the surface of teeth and causes an inflammatory oral disease called periodontitis. Bacteria living in biofilms are more resistant to antimicrobial compounds and the mechanisms associated with their virulence are poorly known. Previously, it was discovered that A. actinomycetemcomitans binds and uptakes human inflammatory cytokines, which may modulate the local inflammatory milieu and weaken the host defense.

This thesis consists of four parts, each published as a separate article in scientific journals. In the beginning of this thesis I discovered a novel cytokine-binding outer membrane protein, BilRI, in A. actinomycetemcomitans. BilRI was located on the outer membrane of A. actinomycetemcomitans and interacted with the human cytokine IL-1β. The second article showed that BilRI binds multiple cytokines and has an intrinsically disordered structure. In the third article, I showed that a major bacterial cell wall component, lipopolysaccharide, interacted with certain cytokines. This interaction was shown in many A. actinomycetemcomitans serotypes using intact bacterial cells, outer membrane vesicles and isolated lipopolysaccharides. Further, some information was obtained about the location of the interaction site. In the fourth article, I found that a channel protein of A. actinomycetemcomitans, HofQ, binds human cytokines, such as IL-1β and IL-8. This protein has been previously associated with DNA uptake, providing a possible link between the naturally occurring uptake of extracellular DNA and the cytokine uptake mechanism in bacteria.

This thesis showed the cytokine binding of A. actinomycetemcomitans outer membrane molecules and how they affected the physiology of the pathogen. Further research is needed regarding the roles of these molecules in the virulence of A. actinomycetemcomitans.
TIIVISTELMÄ


LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred in the text by Roman numerals I-IV.


*equal contribution by first authors


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Amino Acid Residue</th>
<th>Full Name</th>
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<tr>
<td>A</td>
<td>Ala</td>
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</tr>
<tr>
<td>C</td>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>D</td>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>E</td>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>F</td>
<td>Phe</td>
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<tr>
<td>G</td>
<td>Gly</td>
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</tr>
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<td>H</td>
<td>His</td>
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</tr>
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<td>I</td>
<td>Ile</td>
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<tr>
<td>M</td>
<td>Met</td>
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</tr>
<tr>
<td>N</td>
<td>Asn</td>
<td>Asparagine</td>
</tr>
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<td>P</td>
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<td>R</td>
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<td>S</td>
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</tr>
<tr>
<td>V</td>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>W</td>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Y</td>
<td>Tyr</td>
<td>Tyrosine</td>
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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BilRI</td>
<td>bacterial interleukin receptor I</td>
</tr>
<tr>
<td>$B_{\text{max}}$</td>
<td>maximum binding</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BS3</td>
<td>bis(sulfosuccinimidyl)suberate</td>
</tr>
<tr>
<td>CDT</td>
<td>cytolethal distending toxin</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>DUS</td>
<td>DNA uptake sequence</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>eDNA</td>
<td>extracellular DNA</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>emHofQ</td>
<td>extramembranous part of HofQ</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substance</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HGK</td>
<td>human gingival keratinocyte</td>
</tr>
<tr>
<td>HGT</td>
<td>horizontal gene transfer</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IDP</td>
<td>intrinsically disordered protein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon $\gamma$</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl $\beta$-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IrmA</td>
<td>interleukin receptor mimic protein A</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>Kdo</td>
<td>3-deoxy-D-manno-octulosonic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OMV</td>
<td>outer membrane vesicle</td>
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<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PA-I</td>
<td>type I <em>Pseudomonas aeruginosa</em> lectin</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PGA</td>
<td>poly-N-acetylglucosamine</td>
</tr>
<tr>
<td>PMB</td>
<td>polymyxin B</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>QS</td>
<td>quorum sensing</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of NF-κB ligand</td>
</tr>
<tr>
<td>RFU</td>
<td>relative fluorescence unit</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RTX</td>
<td>repeats-in-toxin</td>
</tr>
<tr>
<td>scFv</td>
<td>single-chain antibody fragment</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>STI</td>
<td>soybean trypsin inhibitor</td>
</tr>
<tr>
<td>TFP</td>
<td>type IV pilus</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TRFIA</td>
<td>time-resolved fluorometric immunoassay</td>
</tr>
<tr>
<td>UPEC</td>
<td>uropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>USS</td>
<td>uptake signal sequence</td>
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1. INTRODUCTION

Bacteria are everywhere. They live on all surfaces in nature, in deep oceans and even in extremely hot, cold, and dry conditions. They are living inside us humans, many as commensals that cohabitate peacefully with our own cells, but some are pathogenic and capable of causing infections.

Bacterial infections are one of the most common diseases in humans. The severity of the disease depends on the pathogen as well as the host. When the immune system of an individual is compromised because of some other medical condition, the susceptibility to severe bacterial infections will increase. Additionally, the lethality of the infection increases in an immunocompromised host. In some cancers, the immune functions in the cancerous tissue microenvironments are dysregulated, which leads these tissues to be more susceptible to infections (Mantovani et al., 2008).

Virulence factors are bacterial components that help bacteria survive and reproduce in the host as well as use the host for nutrition. These components include molecules for adhesion, nutrition acquisition and harming the host cells. In addition to virulence factors, bacteria have many additional structural and functional properties that support their survival in the host. Most bacteria live in biofilms, which are sessile bacterial communities formed on any surface in nature. Biofilms cause chronic infections and are more resistant to host clearance (Hall-Stoodley & Stoodley, 2009). The unique mechanisms underlying the virulence of biofilms require further research.

In this era of increasing multiresistant bacterial strains, it is necessary to develop new ways to fight infections without promoting the development of antibiotic resistance. The various bacterial virulence mechanisms may provide new targets for antimicrobial therapy. Only detailed molecular knowledge of virulence mechanisms and how they affect the overall bacterial function will enable the development of effective novel antimicrobial strategies. While many traditional antibiotics target more general bacterial structures and functions, and are effective for multiple pathogens, new strategies may require a species-targeted approach. This approach naturally requires more detailed knowledge of each bacterium and its possible targets. The clinical challenge of this approach is that effective treatment can be started only after the exact pathogen is known. More targeted treatment, however, is less likely to promote the development of resistant strains, since the treatment affects only certain pathogens and is not used widely as are broad-range antibiotics.

The host immune responses are mediated by inflammatory signaling molecules such as cytokines, chemokines and interferons. For instance, these molecules attract immune cells to the infection site or induce them to secrete other inflammatory
molecules. Cytokines, such as interleukin (IL)-1β, play a vital role in regulating the host immune responses (Parkin & Cohen, 2001).

In this thesis, I will focus on the study of a virulence-associated mechanism that includes the binding and uptake of human cytokines. The model organism for this study is an oral opportunistic pathogen, *Aggregatibacter actinomycetemcomitans* (Zambon, 1985). This pathogen is linked to the onset of an aggressive form of periodontitis, presently renamed molar-incisor pattern periodontitis with rapid progression (Papapanou et al., 2018), which is a chronic inflammatory oral disease. *A. actinomycetemcomitans* grows as a biofilm that has increased resistance to host clearance. *A. actinomycetemcomitans* toxins induce cytokine production from human immune cells. In periodontitis, the inflammatory response is prolonged because the host is unable to destroy the biofilm. The overactivated host immune response instead destroys the teeth-supporting tissue and leads to resorption of the bone.

In previous studies, *A. actinomycetemcomitans* was found to have the ability to bind and uptake the human proinflammatory cytokine IL-1β (Paino et al., 2011). Furthermore, this binding decreases the metabolic activity and increases the biofilm formation of *A. actinomycetemcomitans*. These effects are likely facilitated by the binding of IL-1β to intracellular proteins (Paino et al., 2011, 2012).

This thesis focuses on finding and characterizing membrane protein(s) and other components of *A. actinomycetemcomitans* that interact with human cytokines. The other aim is to evaluate the role of these proteins in bacterial virulence-associated functions and survival in a hostile environment.
2. REVIEW OF THE LITERATURE

2.1 Bacterial biofilms

Biofilms are multicellular bacterial communities that are enclosed by a self-produced matrix, and attached to a surface or an interface. Their phenotype is also altered compared with freely suspended planktonic cells in regard to gene expression and growth rate (Donlan & Costerton, 2002). The majority of the bacteria in all ecosystems live in biofilms that can form on virtually all moist surfaces in nature. In some ways, the structural qualities of biofilms resemble those of tissues in higher organisms: they are multicellular organized structures enclosed with channels that allow the flow of nutrients, metabolites and waste to different parts of the biofilm (reviewed in Flemming et al., 2016). A large amount of the current knowledge of biofilms is concerning gram-negative *Pseudomonas aeruginosa* as it is the most widely used organism in biofilm studies.

Biofilms are structurally very heterogeneous while constantly changing due to internal events as well as external signals. The development of biofilms follows a certain pattern: initial attachment to the surface is followed by the formation of microcolonies and maturation into matrix-enclosed biofilm. The mature biofilms release single planktonic cells that are dispersed into the environment to colonize new sites. To move from one phase to another, the activation of certain genes is required (McDougald et al., 2012). In natural environments, biofilms are usually composed of several bacterial species that coaggregate. Biofilms may have both aerobic and anaerobic bacteria that inhabit different layers. For example, the oral cavity is a specific niche that harbors versatile multispecies biofilms in which oxygen-requiring aerobes occupy the surfaces, while anaerobic bacteria thrive in the oxygen-limited inner parts of the biofilm. Each species in the biofilm contributes to the biofilm composition. The biofilm can be a mutualistic community where the species are recycling nutrients and genes with their neighboring cells. However, in many biofilms, there is ruthless competition among species over nutrients and space. Some species may even release antimicrobial compounds that are harmful to their neighbors in order to promote their survival (Nadell et al., 2016).

Quorum sensing (QS) is a multicellular behavior that regulates many physiological processes, such as movement (swimming, swarming, twitching), biofilm formation and maturation. It is also linked to the production of virulence factors, aggregation, and production of antimicrobials and secreted products (reviewed in (Whiteley et al., 2017)). The QS system is a population response that is switched on after a population reaches a certain level, “quorum”, of bacteria. The quorum level is sensed by bacteria
via the accumulation of small signal molecules, inducers. The common signal molecules for the QS systems in gram-negative bacteria are acyl-homoserine lactones (Parsek & Greenberg, 2000; Papenfort & Bassler, 2016) and quinolone derivatives (Pesci et al., 1999), both of which are found for instance in *P. aeruginosa*. In the oral pathogen *A. actinomycetemcomitans*, a QS signal molecule called autoinducer-2 as well as the two-component QS signal QseBC regulate biofilm formation and virulence (Novak et al., 2010; Weigel et al., 2015).

2.1.1 Extracellular matrix of biofilms

Bacteria in biofilms secrete molecules to form an extracellular matrix (EM) (reviewed in Flemming & Wingender, 2010). The main function of the EM is to maintain the biofilm structure and to protect biofilm cells from outside threats, such as antimicrobials. EM may also function as a buffer to cells in the biofilm so that they do not need to adapt to the ever-changing environments in nature. Additionally, EM may act itself as a reservoir of carbon and energy. EM is a dynamic environment with multiple microenvironments, and the overall composition of EM differs between different bacteria and even between different time points. The biofilm EM is formed from polysaccharides, DNA and proteins supplemented with other substances from the environment.

The EM of bacterial biofilms is formed mainly of extracellular polymeric substance (EPS), which contains a mixture of large exopolysaccharides (Costerton et al., 1999); for example, alginate is a major exopolysaccharide in *P. aeruginosa* biofilms (Davies et al., 1993). The polysaccharide matrix is often viscous and quite elastic; it endures shear stress from the environment and reshapes instead of breaking apart. EPS production is affected by the available nutrients, especially carbon (Sutherland, 2001). EPS probably plays a role in antibiotic resistance in biofilms, as it may slow the penetration of antibiotics to the biofilm and inactivate some antibiotics (Donlan, 2000).

Extracellular DNA (eDNA) is also an important structural component in many bacterial biofilms. It forms a network by interacting with polysaccharides and proteins in the EPS (Wu & Xi, 2009). The eDNA in biofilms was previously presumed to be present solely as a product of cell lysis, but it has been shown to be deliberately secreted from cells either as bare DNA fragments or inside membrane-derived vesicles (Hamilton et al., 2005; Jakubovics et al., 2013). eDNA in EM may resemble bacterial genomic DNA (Allesen-Holm et al., 2006; Wu & Xi, 2009). However, bacteria have also been shown to utilize the DNA of lysed polymorphonuclear leukocytes (PMNs) in the infection site (Walker et al., 2005).
Thus, unsuccessful eradication attempts of the host can sometimes help the bacteria to strengthen their biofilms.

eDNA is important for biofilm formation, especially in the initial stages. The presence of DNase I in the growth medium prevents *P. aeruginosa* biofilm formation in younger biofilms (12-60 hours) but not in established biofilms (84 hours) (Whitchurch, 2002). Treatment with DNase I could therefore be used to prevent the formation of biofilms. The production of eDNA has been linked to the QS system in *P. aeruginosa*. While the basal level of eDNA in *P. aeruginosa* biofilm was not dependent on QS, the great majority of eDNA production was directly linked to QS-regulating genes (Allesen-Holm et al., 2006).

eDNA can have a shielding role on bacteria in biofilms by binding positively charged antibiotics, such as aminoglycosides. It has been shown that eDNA increases the tolerance of *P. aeruginosa* biofilm to the aminoglycosides (Chiang et al., 2013). In addition to having structural roles in biofilm EM, eDNA also plays an important role as a genetic material reservoir. The genes associated with virulence and antimicrobial resistance can spread in the biofilm population through horizontal gene transfer (HGT), a process by which bacteria exchange genetic material (von Wintersdorff et al., 2016). The role of eDNA in natural competence is described in the next section.

Biofilm EM contains many proteins and enzymes that interact with other EM molecules (Sutherland, 2001). They may also act as adhesins that facilitate the binding of the bacterial cells to the EM and to each other. Carbohydrate-binding proteins called lectins are abundant in oral biofilms and are especially important in initial biofilm formation to coaggregate the bacteria of different species.

### 2.1.2 Natural competence and DNA uptake in biofilms

Many bacterial species spontaneously bind DNA from their surroundings and transport it to their cytosol; this is called natural competence. Natural competence is a mode of HGT, together with conjugation (through direct contact between the donor and recipient cells) and transduction (gene transfer by phages) (von Wintersdorff et al., 2016). Since the EM of bacterial biofilms is loaded with eDNA, constant exchange of genetic material occurs in the biofilms. Bacteria use the DNA as a nutrient (Finkel & Kolter, 2001), or if the uptaken sequences have enough similarity with the chromosomal DNA, various species can incorporate the DNA into their genome through homologous recombination. Natural competence is considered an advantage to bacteria. In addition to the nutrition that it provides, it may serve as a way for bacteria to receive genes that improve their survival, such as genes encoding antibiotic resistance or virulence factors.
The transport of linear DNA into gram-negative bacteria occurs in two stages (reviewed in Chen & Dubnau, 2004; Mell & Redfield, 2014). DNA is first transported through the outer cell membrane (DNA uptake) and then across the inner membrane (DNA translocation). The double-stranded DNA (dsDNA) is pulled through an outer membrane secretin pore by a retracting type IV pilus (TFP). The inner membrane of naturally competent strains has conserved membrane pores that will translocate one strand from the periplasm into the cytoplasm, while the other strand will become degraded and used as building blocks in DNA replication. The DNA transport in gram-positive bacteria occurs via similar mechanisms except that these bacteria do not require the first transport step because they lack an outer membrane (Chen et al., 2005).

The effect of uptaken DNA on the genome varies. If homologous recombination occurs between very similar strains, there might be no change at all, or a recently acquired new mutation might disappear. In heterogeneous populations, new gene combinations are possible, even though a newly acquired gene does not always promote fitness and can even contain lethal mutations (Redfield et al., 1997). In fact, the introduction of new genes into a species is not very efficient via homologous recombination since it requires a certain level of homology. However, homologous recombination is a very efficient mechanism for spreading a novel gene that is first introduced into the genome by another, non-homologous mechanism. (Mell & Redfield, 2014).

There are bacteria that will take up any eDNA, while others prefer to uptake DNA homologous to their genome. The uptake signal sequences (USSs), or the DNA uptake sequences (DUSs), are short species-specific sequences that are taken up more efficiently by a certain species and are overrepresented in the genomes of that species (Smith et al., 1999). Two widely studied USS sequences are the 9-bp-long USS of *Haemophilus* (Danner et al., 1980) and the 10-bp-long *Neisseria* USS (Goodman & Scocca, 1988).

### 2.1.3 Resistance mechanisms in biofilm diseases

Biofilms are more resistant to antimicrobials than planktonic bacteria (reviewed in Hall & Mah, 2017). This resistance is derived from a combination of mechanisms that vary from species to species, and these mechanisms differentially affect each antimicrobial. The EM that surrounds the bacteria in the biofilms may limit the penetration of some antimicrobial agents into the biofilm. Further, various EM components may directly interact with antimicrobials. Polysaccharides, such as Psl in *P. aeruginosa* biofilm, can sequester certain antibiotics, preventing interaction with their targets (Billings et al., 2013). eDNA has been shown to increase the tolerance
for various antimicrobials (Chiang et al., 2013). Anionic eDNA may induce survival-associated genes by modifying the local environment (by chelating cations or decreasing pH), or it can promote more efficient distribution of resistance genes through HGT. In *Salmonella enterica* biofilm, eDNA chelated Mg\(^{2+}\) ions, which increased the expression of the polymyxin resistance operon (Johnson et al., 2013). The secreted enzymes in EM can directly degrade antimicrobial substances; for instance, the β-lactamases produced by *P. aeruginosa* make these bacteria resistant to β-lactam antibiotics (Bagge et al., 2004). The second biofilm characteristic that decreases the effect of antibiotics is the reduced growth rate of the bacterial cells in biofilms. Many antibiotics target the synthesis of cell wall or proteins and therefore have the greatest effect on fast-growing cells. The biofilm contains a substantial population of cells in a slow- or non-growing state due to nutrition and oxygen limitations. Some of these non-growing cells are so-called persister cells, which are in a metabolically inactive, dormant state and can survive very high doses of antimicrobials (Lewis, 2010). Additionally, the metabolic state of bacteria varies greatly among biofilms, so there is a great possibility that some bacteria will always avoid antimicrobial effects and serve as a reservoir for the biofilm to re-establish. Additionally, antimicrobial efflux pumps, QS and genetic diversity (through HGT and an elevated mutation rate) have been shown to play a role in the antimicrobial resistance in biofilms (Hall & Mah, 2017).

Bacterial biofilms are also more resistant to clearance by the host immune system. Bacterial presence attracts phagocytes to the site of a biofilm and while failing to ingest the biofilm-protected bacteria, the phagocytes still release phagocytic enzymes that instead damage the host tissue around the biofilm (Fig. 1) (Costerton et al., 1999). Antibodies are usually not effective on biofilms since they are unable to penetrate the EM.

Biofilms are associated with many chronic inflammatory diseases because they are difficult to eradicate by antimicrobials or by the host immunity (Costerton et al., 1999). *P. aeruginosa* biofilms are commonly associated with persistent bacterial infections in the lungs of cystic fibrosis patients. Periodontitis is a severe chronic infection caused by oral biofilms (plaque) that will be discussed later in this review. Biofilm-associated diseases are seldom diagnosed in their early stages because slow-growing biofilms show no overt symptoms. Biofilms are also readily formed on the surfaces of indwelling medical devices, such as mechanical heart valves, prosthetic joints, catheters, and contact lenses, and cause local or systemic infections (Donlan, 2001).
Antibiotic treatment for a biofilm-borne infection can reduce symptoms temporarily because it will kill the planktonic cells shed from the biofilm. However, the symptoms will gradually return if there is any remaining biofilm. The most effective way to eradicate the biofilm is mechanical or surgical removal. Recently, the focus has been to discover new ways to treat biofilms by inhibiting unique mechanisms for biofilms, such as the formation of the EM or the QS system.

Figure 1. Biofilm formation on medical devices. (a) Human immune response (neutrophils and antibodies) together with antibiotics clear the planktonic bacteria. (b) Biofilm starts to form on the surface of the medical device. Antibodies and antibiotics are no longer able to penetrate the biofilm matrix, so the clearance is ceased. (c) Neutrophils are unable to ingest bacteria but are still releasing their lytic enzymes. (d) Biofilm growth and lytic enzymes start to destroy the tissue, but the biofilm remains. Planktonic cells that are released from the biofilm can colonize new sites once they escape the defenses. Figure from Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science*, 284(5418), 1318–1322. Reprinted with permission from AAAS.
2.2 Bacterial virulence

Bacteria may cohabit in their environment peacefully as a commensal, or they might be pathogenic and cause diseases in the host organism. Pathogens are called opportunistic when they are acting as a commensal until they sense certain environmental cues, for example, signs that the host immunological state is getting weaker, and only then start expressing their virulence properties. However, many diseases are not caused by a single pathogen but rather by a dysbiosis in the indigenous multispecies biofilm; the divide of these species into commensals and pathogens is not straightforward. Instead, these species can be described, for instance, as the keystone pathogens (pathogens with low abundance but great pathogenic effect), pathobionts (commensals that can be triggered into pathogenic by changes in the host immunity) and accessory pathogens (commensals that support the virulence of pathogenic species) (Hajishengallis & Lamont, 2016).

There are various ways that virulence is conveyed in bacteria. Bacteria produce molecules called virulence factors that boost the bacteria in their efforts to colonize a niche, to escape or inhibit host immune responses, to obtain nutrition from the host and to survive in the host without killing the host (Finlay & Falkow, 1989). Virulence factors include toxins that can damage host cells, adhesins that aid in bacterial adhesion to the host or to extracellular components, invasins that promote invasion and evasins that help bacteria evade host immunity. Toxins may also disturb essential host functions to aid other virulence factors in invasion or evasion. The sequestering of host cytokines, which is the main topic of this thesis, is a mechanism that bacteria use to evade host immunity.

Virulence factors also include factors that help bacteria acquire nutrition from the host and factors that are needed for biofilm formation or for the QS system (Papenfort & Bassler, 2016). These factors work together dynamically to initiate and maintain infection. Virulence is often altered via HGT, since many virulence genes and antibiotic resistance genes are located in plasmids that are readily dispersed throughout the population. For instance, in pathogenic E. coli, most of the virulence-associated genes are encoded by mobile genetic elements (Croxen et al., 2013). Many virulence factors are multifunctional so-called moonlighting proteins (Henderson & Martin, 2011). Virulence is expressed differently in different species, and all virulence behaviors in bacteria may not have even been discovered yet.
2.2.1 Lipopolysaccharide

The outer leaflet of the outer membrane in gram-negative bacteria is mainly assembled out of lipopolysaccharide (LPS) (Fig. 2). It plays an important role in maintaining membrane structure and acting as a barrier to hydrophobic compounds such as antibiotics and detergents. It helps bacteria to survive in harsh environmental conditions and to resist antibiotics/antimicrobials (Papo & Shai, 2005). While LPS is essential for most gram-negative species, some viable LPS-deficient mutant strains have been discovered in *Neisseria*, *Acinetobacter* and *Moraxella* species (reviewed in Zhang et al., 2013). LPS is one of the main components of the outer membrane vesicles (OMVs) that bacteria release from their outer membrane (Mashburn-Warren & Whiteley, 2006). OMVs are small membrane-enclosed particles that are coated with lipids and LPS and can contain various virulence factors, such as toxins.

The general LPS structure is conserved in all gram-negative species and consists of a lipid part (lipid A) and a polysaccharide part formed from core- and O-polysaccharides (reviewed in Erridge et al., 2002; Raetz & Whitfield, 2002) (Fig. 3). Lipid A has a backbone of two acylated glucosamines bound by four to six lipid chains. These hydrophobic lipid chains attach LPS to the lipid membrane. The structure of lipid A is fairly conserved, but there is some variation between bacterial
families. Lipid A is responsible for endotoxic activity, and its properties determine the level of its toxicity.

Figure 3. The general structure of LPS. The lipid A part of LPS is composed of one di-polysaccharide conjoined with lipid chains. The inner and outer core regions are formed out of diverse polysaccharides, while 3-deoxy-D-manno-octulosonic acid (Kdo) is fairly conserved as the polysaccharide that connects the core with lipid A. The O-antigen polysaccharide is composed of repeated oligosaccharide units. Figure was modified from Maeshima & Fernandez 2013.

Lipid A is attached to the core polysaccharide part of LPS, which is divided into inner core and outer core regions (Fig. 3). The inner core is usually made of uncommon sugars such as L-glycero-D-manno-heptose and 3-deoxy-D-manno-octulosonic acid (Kdo). In the majority of studied LPS molecules, Kdo is the starting sugar of the chain and the one bound to lipid A (Erridge et al., 2002). The smallest saccharide part of LPS is found in *Haemophilus influenzae*: only one Kdo is attached to lipid A (Helander et al., 1988). It seems that while bacteria can survive without the O-antigen and with a truncated core polysaccharide, this Kdo residue is most likely essential for the bacterial structure. The outer core is generally more diverse than the inner core and is made out of more conventional sugars, such as glucose, galactose and N-acetyl glucosamine.

The hypervariable O-polysaccharide region is made from repeated oligosaccharide units (Fig. 3). The oligosaccharide units are usually formed from 1 to 8 glycosyl residues and can be repeated in the chain from a few up to 50 times (Erridge et al., 2002). The diversity of the O-polysaccharide is substantial even within one species and derives from the complex structure of the O-polysaccharide. The oligosaccharide units can be formed from different monosaccharides with various modifications (e.g. glycosylation) and are connected to each other in various ways. Furthermore, in the chain of oligosaccharide units, the amount and position of the repeated
oligosaccharide units vary greatly, and the chain can be branched (reviewed in Erridge et al., 2002; Raetz & Whitfield, 2002). However, not all LPS molecules have O-polysaccharides. The O-polysaccharide is located furthest from the membrane surface, so it is the natural target for host recognition and immune responses. Therefore, it is also called the O-antigen. Since the structure of O-polysaccharide varies even within bacterial species, it has been used as a factor to divide bacterial species into different serotypes. The number of serotypes varies; for example, \textit{E. coli} has over 180 different O-antigen serotypes (Fratamico et al., 2016).

LPS, also called the endotoxin, is a highly immunogenic compound that induces an inflammatory response, for instance, the production of cytokines, from multiple human immune cells. A substantial amount of lipid A is released when the bacteria are lysed, and it activates the host immune responses even at picomolar concentrations. Lipid A is bound by the LPS-binding protein and CD14 opsonic receptor. The CD14 receptor does not have an intracellular part, so subsequent signaling requires the binding of this complex to toll-like-receptor 4 (TLR4) in the animal cell membrane (Hoshino et al., 1999; Aderem & Ulevitch, 2000). TLR4 is an innate immunity receptor that is expressed, for example, in macrophages and human gingival fibroblasts (Wang et al., 2000; McGettrick & O’Neill, 2007), but its expression has also been characterized in various cancer cell types (Pandey et al., 2018). TLR4 activation in macrophages leads to the production of proinflammatory mediators, such as IL-1\( \beta \) and tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)). The detection of lipid A leads to a vast immune response that can cause fever, diarrhea, and even septic shock. The proteins involved in LPS synthesis are promising targets for the development of new antibiotics. One example of this is a synthetic peptide that targets \textit{P. aeruginosa} LptD, a LPS assembly protein, which displays antimicrobial properties against \textit{P. aeruginosa} spp. but not towards other gram-negative or gram-positive species (Srinivas et al., 2010).

2.2.2 Bacterial moonlighting proteins in virulence

Proteins that perform more than one biologically relevant function within one polypeptide chain are called moonlighting proteins (Jeffery, 1999). The term “moonlight” is used to describe people working multiple jobs and is adopted here to refer the multiple jobs of a protein. Moonlighting means that a protein can perform multiple distinct functions without any proteolytic modifications, gene rearrangements or fusions. Under certain conditions, a monofunctional protein may acquire a secondary function (and may still maintain the primary function as well). In some moonlighting proteins, the functional regions are overlapping, while other proteins have those regions far apart, which enables them to perform multiple functions simultaneously. A moonlighting protein may have one or multiple
moonlighting functions. (Henderson, 2016). The presence of multiple functions in one protein is cost-effective for bacteria because they do not have to express so many proteins or have so many corresponding genes to replicate.

There are various ways that moonlighting proteins switch between their functions (reviewed in Jeffery, 1999) (Fig. 4). Many bacterial moonlighting proteins may perform different functions in the intracellular environment than in the outer membrane or as a secreted protein. Some moonlighting proteins have different functions as monomers and multimers, when expressed in different cell types or when bound to a cofactor. Additionally, they might have different functions when bound to a protein complex, such as a ribosome, rather than on their own. Some proteins can sense changes in their environment, such as pH or the concentration of a ligand, and change their conformation and thus their function accordingly.

Figure 4. Moonlighting mechanisms of proteins. (A) Moonlighting protein may have different functions in different organisms or B) in different cell types of the same organism. C) Protein may have different functions intracellularly compared to when secreted, or D) whether they are located in the cytosol or bound to a membrane. E) Proteins may also harbor different functions when forming multimers or F) complexes with other proteins or G) when they are bound to a substrate, cofactor or other ligand. H) Moonlighting proteins may have multiple binding sites for different ligands in different parts of their polypeptide chain.

Many of the identified moonlighting proteins play roles in cellular metabolism (glycolysis, tricarboxylic acid cycle, etc.) or act as chaperones (proteins that aid the folding of other proteins) (reviewed in Jeffery, 2018). The secondary (moonlighting) functions for these proteins are typically linked to adhesion or secretion of these proteins as signal molecules.
Bacteria utilize some of the cytosolic (housekeeping) proteins on their cell surface to bind the host or EM components. The first protein that was identified within this group of moonlighting proteins, was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in group A streptococci. GAPDH has cytosolic enzymatic activity but is also localized to the membrane and binds to plasmin, fibronectin and lysozyme (Pancholi & Fischetti, 1992). GAPDH has since become the archetypal example of moonlighting proteins, as it has been discovered in a variety of bacteria and has a variety of roles (in adhesion, DNA repair, evasion and many more) (Tristan et al., 2011).

Moonlighting proteins have been identified in all organisms, including humans. Currently, almost 400 moonlighting proteins have been characterized and all the found moonlighting proteins are collected in the Moonlighting proteins database (MoonProt, http://www.moonlightingproteins.org, Chen et al., 2018). It is interesting that many metabolic moonlighting proteins are evolutionarily very old and conserved from prokaryotes to eukaryotes (Henderson & Martin, 2014). Proteins with one function may have evolved into multifunctional proteins by modifying the parts of proteins that are not critical for the primary function.

Many bacterial moonlighting proteins have one or multiple functions related to virulence. They act in all the main virulence factor classes: as adhesins, invasins, evasins, and toxins or in nutrition acquisition. However, for the currently known virulence-associated moonlighting proteins, the most common virulence function is adhesion (Henderson, 2016). These virulence-associated moonlighting functions could have evolved through coincidental virulence, which means that virulence traits originally evolved to protect bacteria from predatory protozoans in their natural environment (Adiba et al., 2010). Moonlighting proteins seem to play important biological roles in bacteria because they are commonly utilized by several different bacteria, and many moonlighting proteins bind their ligands with high affinity (Henderson, 2016).

2.2.3 Bacterial proteins interacting with human cytokines

Cytokines are signal molecules that are produced by host cells to initiate and regulate immune responses. In particular, cytokines play important roles in response to infections. To the best of our knowledge, bacteria may sense cytokines in their environment and obtain clues about the immunological conditions of the host. Based on this information, bacteria may alter their virulence properties. The expression of virulence properties consumes energy, so it is beneficial for the bacteria to sense their environment for these cues and act only when they sense threats to their survival. Some host immune molecules might directly activate virulence, and it has been
suggested that cytokines may be broken into peptides that directly act as transcription modulators (Kanangat et al., 2001). Human proinflammatory cytokines have been shown to enhance the growth of bacteria and affect bacterial virulence. Direct binding on bacterial cells has also been observed. Most of the binding was observed with IL-1β, and the reported binding occurred with gram-negative *Escherichia coli* and *Acinetobacter sp.* and with gram-positive *Staphylococcus aureus* (Porat et al., 1991; Meduri et al., 1999; Kanangat et al., 2001). IL-1β is one of the cytokines that activate the immune response; therefore, binding IL-1β may disturb the inflammatory cascade.

Virulent strains of *E. coli* bind IL-1β, and bacteria grow faster when supplemented with IL-1β. The enhancement of growth by IL-1β occurs only in the logarithmic phase of growth, resulting in the bacteria reaching the stationary phase faster (Porat et al., 1991). IL-1β has also been shown to enhance the growth of fresh isolates of *Acinetobacter sp.* and *S. aureus* (Meduri et al., 1999). *S. aureus* binds IL-1β to its surface, and IL-1β decreases the expression of leukotoxins and increases the levels of virulent adhesins that bind to EM molecules in *S. aureus* (Kanangat et al., 2001, 2007). Thus, under high IL-1β concentrations, bacteria modulate their gene expression profile to survive within the host rather than to fight. In a later study, *S. aureus* biofilms were found to bind more IL-1β than did planktonic cells of the same strain. Growth enhancement of the biofilm but not the planktonic culture was observed with IL-1β, as well as TNF-α and macrophage inflammatory protein-1α (McLaughlin & Hoogewerf, 2006). This suggests that the biofilm cells upregulate the genes that respond to cytokines.

TNF-α has been shown to bind gram-negative *Shigella flexneri*, *E. coli* and *Salmonella typhimurium*. *S. flexneri* bound TNF-α with high affinity (Kd = 2.5 nM) when studied using radiolabeled TNF-α (Luo et al., 1993). The same study also showed TNF-α binding to gram-positive *S. aureus*, *Listeria monocytogenes* and *Streptococcus mitis*, but this binding was significantly less than that to the tested gram-negative bacteria. Moreover, IL-6 (but not IL-1β or TNF-α) has been shown to enhance the growth of fresh isolates of gram-negative *P. aeruginosa* (Meduri et al., 1999). The growth enhancement of *S. aureus* was also observed with IL-6 and TNF-α when the bacteria were grown in chemically defined synthetic medium (Meduri et al., 1999).

All the above-mentioned studies have shown the effect of cytokines at the cellular level without identifying the actual molecules on bacteria interacting with the cytokines. However, the bacterial proteins discovered to directly interact with cytokines are reviewed in the next paragraphs.
Caf1A of *Yersinia pestis*

*Yersinia pestis* is a gram-negative coccobacillus that can cause plague. One of its major virulence features is its large capsule. The main component of the *Y. pestis* capsule is F1 (fraction 1), which is highly immunogenic. The interaction between radiolabeled recombinant IL-1β and Caf1A, the capsule F1 usher protein, was first shown using *E. coli* cells that expressed the *Y. pestis* capsule operon *f1* from a plasmid. Caf1A was identified as the interaction partner by immunoblotting and further verified by the purified Caf1A inhibiting recombinant IL-1β binding to the cells (Zav’yalov et al., 1995). The capsule subunit Caf1 has structural similarity to human IL-1β and can bind the IL-1 receptor (on mouse fibroblasts), inhibiting IL-1β binding to it (Abramov et al., 2001).

The *f1* operon is responsible for the production and surface assembly of the F1 capsule. This operon consists of four genes: *caf1*, *caf1A*, *caf1M* and *caf1R*. Caf1 is the structural subunit of the capsule (Galyov et al., 1990), and the other three genes are responsible for its assembly, transport and transcription regulation, respectively (Galyov et al., 1991; Karlyshev, Galyov, Abramov, et al., 1992; Karlyshev, Galyov, Smirnov, et al., 1992). Fibrillar F1 capsule assembly occurs with the chaperone/usher pathway using the donor strand exchange mechanism (Zavialov et al., 2003). The Caf1M chaperone in the periplasm aids the folding of the Caf1 subunit and presents it to the Caf1A usher. Caf1A adds Caf1 to the forming capsule fiber and serves as the channel for the fiber to go through (Fig. 5). The N-terminal part of Caf1A has been shown to interact with the Caf1:CafM complex in *Y. pestis* capsule assembly (Di Yu et al., 2012).

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**Figure 5. Schematic presentation of the *Y. pestis* capsule fiber assembly.** (1) The fiber subunit Caf1 is correctly folded in the periplasm by the Caf1M chaperone. (2) Caf1M:Caf1 complex binds with the N-terminal domain (N) of Caf1A usher protein. (3) Caf1A aids the Caf1 to polymerize with the preceding Caf1 subunit. After that Caf1M is released back to the periplasm. Caf1A periplasmic domains are marked in the figure: middle domain (M), N-terminal domain (N), C-terminal domains (C1 and C2). Figure from Di Yu et al. 2012.
OprF of *Pseudomonas aeruginosa*

*P. aeruginosa* is a gram-negative opportunistic pathogen causing severe lung infections, especially in patients with cystic fibrosis. The major non-specific outer membrane porin OprF of *P. aeruginosa* has multiple roles contributing to biofilm formation, adhesion to eukaryotic cells and formation of OMVs, and it also acts as a passage for small molecules (reviewed in (Chevalier et al., 2017)). However, the major role of 38-kDa (kilodalton) OprF is to maintain cell shape by anchoring the outer membrane to the periplasmic peptidoglycan layer with its C-terminal peptidoglycan-binding domain (Rawling et al., 1998). OprF also plays a major role in the overall virulence of *P. aeruginosa* since the absence of OprF disturbs the attachment to eukaryotic cells, altering the function of the type III signal secretion system and decreasing the QS system-induced virulence factors (Fito-Boncompte et al., 2011). The absence of OprF also leads to increase in the levels of secondary messenger cyclic-di-GMP, which increases the biofilm formation (Bouffartigues et al., 2015).

OprF was shown to bind human interferon γ (IFN-γ) (Wu et al., 2005; Maccarini et al., 2017), which induced the expression of type I *P. aeruginosa* (PA-I) lectin, a central virulence factor (Wu et al., 2005), but did not have any effect on the channel functions of OprF (Maccarini et al., 2017). PA-I lectin is an adhesin that facilitates the contact of bacterial cells with epithelial cells. It significantly disturbs the barrier function of the host intestinal epithelium, leading to increased permeability of bacterial cytotoxins through the epithelium (Laughlin et al., 2000). The expression of PA-I lectin from the *lacA* gene is regulated by the QS system regulators (Winzer et al., 2000). Accordingly, IFN-γ also induces the expression of various QS-associated molecules in *P. aeruginosa* (Wu et al., 2005).

OprF belongs to an OmpA protein family, of which the most studied protein is outer membrane porin OmpA of *E. coli* (Krishnan & Prasadarao, 2012). The three-dimensional OmpA structure was used as a homologous model when the structure of OprF was modeled. Initially, OprF was presumed to form a wide channel like the OmpA, but the fact that the diffusion rate for various molecules was very low challenged that assumption. Sugawara and co-workers discovered that OprF actually exists in the membrane in two stable conformers: abundantly as the closed, two-domain conformant and rarely (~5 %) as the open, one-domain conformant (Sugawara et al., 2006) (Fig. 6A).
In the closed conformant, the N-terminal region forms a barrel of eight β-sheets (Fig. 6B), and the C-terminal region forms a globular domain (Fig. 6C) in the periplasmic space. The globular domain is the part of the protein that attaches the peptidoglycan. In the open form, the C-terminal part is also inserted in the membrane, forming a large channel of approximately 16 β-sheets. (Sugawara et al., 2006).

**Figure 6. Two putative conformers of *P. aeruginosa* OprF** (A) Schematic representation of two conformers of OprF. The two-domain closed conformer is composed of an N-terminal β-barrel of 8 β-sheets and the C-terminal globular domain. The open conformer, on the other hand, forms only one domain, a large β-barrel of 16 β-sheets. The open conformer can also form multimers. 3D structures of the closed channel conformer domains have been solved: (B) the N-terminal barrel (Protein Data Bank (PDB):4RLC) and (C) the C-terminal globular domain (PDB:5U1H). Domains are colored blue to orange from the N- to C-terminus. Figure was adapted from Sugawara et al., 2006 and Högbom & Ilalin, 2017.
PilQ and PilE of *Neisseria meningitidis*

*N. meningitidis* is a gram-negative opportunistic pathogen that is found in the human respiratory tract as a commensal but can cause serious diseases such as meningitis and sepsis (Hill et al., 2010). The TFP subunit PilE and secretin PilQ of *N. meningitidis* have been shown to bind TNF-α and IL-8 (Mahdavi et al., 2013). TFP is an important virulence factor in *N. meningitidis* since it mediates movement and adhesion to the host (Hill et al., 2010).

PilE is the pilus subunit that polymerizes to form the long chain through the channel formed by the PilQ secretin (Fig. 7, PilE and PilQ circled in figure) (Craig et al., 2004). The structure of PilQ was modeled by Berry and co-workers using a combination of nuclear magnetic resonance (NMR), electron microscopy and homology modeling (Berry et al., 2012).

Induction with TNF-α or IL-8 did not affect the growth of *N. meningitidis*. However, IL-8 and TNF-α were localized in the *N. meningitidis* cytoplasm, where TNF-α directly binds genomic DNA within specific genes (Mahdavi et al., 2013). The binding of cytokines to promoter areas may alter their structure, either promoting or preventing the binding of RNA-polymerase or transcription factors. Overall, induction with TNF-α or IL-8 altered the expression of 20-45% of *N. meningitidis* genes. The majority of genes had unknown function, but those with known functions were responsible for adhesion, cell envelope, energy metabolism and bacterial

![Figure 7. The type IV pilus (TFP) of *Neisseria spp.*](image)
survival (Mahdavi et al., 2013). Most of these genes are likely important for virulence and pathogenicity, suggesting that IL-8 and TNF-α could increase the virulence of \( N.\) \textit{meningitidis}. PilQ has been shown to bind DNA, and the deletion of \textit{pilQ} made \( N.\) \textit{meningitidis} deficient in transformation, implying the importance of PilQ in bacterial competence (Assalkhou et al., 2007; Lång et al., 2009). Additionally, the mutant strains with unglycosylated pili displayed less binding to cytokines, which indicates a role of PilE glycosylation in this interaction (Mahdavi et al., 2013).

\textbf{IrmA of \textit{Escherichia coli}}

Uropathogenic \textit{E. coli} (UPEC) causes urinary tract infections. IL receptor mimic protein A (IrmA) in UPEC was initially identified as a potential vaccine antigen C1275 (Moriel et al., 2010). A more detailed study of the virulence properties of IrmA revealed that it interacted with several cytokines (Moriel et al., 2016).

IrmA is a secreted protein that exists as a stable domain-swapped dimer in aqueous solution. IrmA has a fibronectin III-like (FNIII) domain with seven antiparallel \( \beta \)-sheets (Moriel et al., 2016) (Fig. 8). IrmA has no significant sequence similarity to other proteins with known functions. However, structural similarities can be observed with many proteins that have FNIII-like domains, such as extracellular domains of cytokine receptors: IL-2 receptor and IL-4 receptor, and, to a lesser extent, the IL-10 receptor. The microplate assay showed that IrmA bound IL-2, IL-4 and IL-10 (Moriel et al., 2016).

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{irmA.png}
\caption{3D structure of \textit{E. coli} IrmA dimer. IrmA monomer (c. 13 kDa) is formed from seven antiparallel \( \beta \)-sheets. Two monomers form a domain-swap dimer where a short \( \beta \)-strand (red) at the C-terminal end extends into the other monomer. Domains are colored blue to red from the N- to C-terminus. Figure was adapted from Högboom & Ihalin, 2017.}
\end{figure}

The \textit{irmA} gene is prevalent in 70 % of sequenced UPEC strains, suggesting a potential role in pathogenesis. It is regulated by the global regulatory protein OxyR (Moriel et al., 2016). IrmA was also shown to be immunogenic: patients infected with \textit{irmA}-positive UPEC had higher antibody titers against IrmA in their plasma than did non-infected individuals (Moriel et al., 2016). IrmA may participate in the manipulation of immune responses during urinary tract infections.
2.3 *Aggregatibacter actinomycetemcomitans*

*A. actinomycetemcomitans* is a gram-negative coccobacillus that belongs to the Pasteurellaceae family (Zambon, 1985). *A. actinomycetemcomitans* was formerly known as *Actinobacillus actinomycetemcomitans* but reclassified in 2006 to form the new *Aggregatibacter* genus with *Aggregatibacter aphrophilus* and *Aggregatibacter segnis* (Norskov-Lauritsen & Kilian, 2006). *Haemophilus* species are the closest relatives of the *Aggregatibacter* genus.

*A. actinomycetemcomitans* is an opportunistic pathogen that inhabits the human oral cavity; the surface of teeth and periodontal pockets. It forms tenacious biofilms in gingival pockets/dento-gingival junctions with other oral bacteria (Fig. 9). *A. actinomycetemcomitans* is a facultatively anaerobic bacterium that grows well in an anaerobic environment or in the presence of 5% carbon dioxide but can survive, while growing poorly, in the presence of oxygen (Zambon, 1985). *A. actinomycetemcomitans* forms small star-shaped colonies when grown on agar. The primary cultures are adherent phenotypes with bundled fimbriae (rough) but become non-adherent (smooth) after repeated culturing (Zambon, 1985).

![Figure 9. Formation of oral biofilm in periodontal pockets.](image)

*Figure 9. Formation of oral biofilm in periodontal pockets.* Biofilm forms in the junction between the tooth and the gingiva. The progression of periodontal disease is characterized by the attachment loss of the junctional epithelium and the formation of the gingival pocket. Figure was adapted from Pöllänen et al. 2012.

*A. actinomycetemcomitans* can be found as a part of normal oral microbiota in periodontally healthy individuals. However, it is strongly associated with oral inflammatory disease, periodontitis and especially periodontitis with a molar-incisor pattern and rapid progression (Papapanou et al., 2018), formerly known as localized aggressive periodontitis. Periodontitis is characterized by the progressive destruction
of the tooth-supporting tissues and bone. Advancement of the disease will eventually lead to tooth loss.

Periodontitis is a multifactorial disease that is initiated by polymicrobial biofilms of mainly gram-negative oral bacteria, such as Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola (Haffajee & Socransky, 1994; Lamont et al., 2018). The onset of periodontitis is linked to both dysbiosis of the oral microbiota and to the elevated host inflammatory response (Darveau, 2010; Lamont et al., 2018). Dysbiosis occurs when a number of oral commensals is decreased while the number of pathogenic species is increased. Inflammation promotes the growth of certain pathogens, which increases the dysbiosis. The pathogens, on the other hand, produce compounds that promote inflammation, creating a feedforward loop that sustains both the dysbiosis and the inflammation (Lamont et al., 2018). Upon dysbiosis, the commensal species may support the virulence of pathogens. One of the oral commensals, Streptococcus gordonii, has been shown to promote the pathogenesis of A. actinomycetemcomitans by producing lactate that A. actinomycetemcomitans utilizes as an energy source (Ramsey et al., 2011). The initiation and progression of periodontitis also depends on host susceptibility and factors such as defects in immune responses, systemic diseases or smoking (Haffajee & Socransky, 1994).

There are constant low levels of cytokines and chemokines present in healthy periodontium and these are highly important mediators for maintaining a healthy balance in the periodontium. Imbalance will lead to increased cytokine production, leukocyte activity and tissue destruction. A. actinomycetemcomitans is able to stimulate the production of proinflammatory cytokines from gingival epithelial cells or fibroblasts (Agarwal et al., 1995; Dongari-Bagtzoglou & Ebersole, 1996; Uchida et al., 2001). These cytokines will then induce the production of the receptor activator of NF-κB ligand (RANKL). Bone resorption is due to increased levels of RANKL compared to osteoprotegerin (OPG), which is an inhibitor of the RANK-RANKL interaction that leads to the release of bone-degrading enzymes (Darveau, 2010).

As mentioned before, the role of cytokines and chemokines, especially IL-1β, IL-6 (CXCL6), IL-8 (CXCL8), IFN-γ and TNF-α, in the tissue is pivotal for bone destruction in periodontitis (Graves, 2008). IL-1β is an important gatekeeper cytokine in inflammation that initiates the immune cascade and induces the production of other inflammatory markers as well as activates many leukocytes (Dinarello, 2018). IL-8 is a chemotactic agent for neutrophils and some other granulocytes (Rossi & Zlotnik, 2000). It stimulates immune cells in the infection site.

A. actinomycetemcomitans is also linked to the etiology of non-oral infections, such as cardiovascular diseases (Haraszthy et al., 2000; Kozarov et al., 2005; Hyvärinen et
Review of the literature

al., 2012), endocarditis (Kaplan et al., 1989; Lützen et al., 2018), and brain abscesses (Rahamat-Langendoen et al., 2011). *A. actinomycetemcomitans* belongs to the HACEK group of gram-negative pathogenic bacteria that includes *Haemophilus parainfluenzae*, *Aggregatibacter* species, *Cardiobacterium* species, *Eikenella corrodens*, and *Kingella* species. The HACEK group of bacteria includes causative agents of infective endocarditis and is responsible for 1-3% of cases (Paturel et al., 2004; Revest et al., 2016). *A. actinomycetemcomitans* species that were found in other organs were most likely originated from the oral cavity, suggesting that poor dental conditions and recurring periodontal infections can lead to non-oral infections (Rahamat-Langendoen et al., 2011).

2.3.1 Virulence properties of *A. actinomycetemcomitans*

Biofilm formation

Biofilm formation is an important virulence factor of *A. actinomycetemcomitans*, as mutants unable to form biofilms are unable to colonize or infect. The biofilm formation and integrity of *A. actinomycetemcomitans* have been shown to rely on polysaccharides, eDNA and proteinaceous adhesins (Inoue et al., 2003; Izano et al., 2008).

TFP, called Flp-pili (fimbriae), are the major proteinaceous adhesins in the *A. actinomycetemcomitans* biofilm (Inoue et al., 2003; Izano et al., 2008). Flp-pili are found in freshly isolated *A. actinomycetemcomitans* but are lost upon culturing in the laboratory after 30-40 generations (Rosan et al., 1988). The major subunit of the pili is the 6.5-kDa Flp1 protein (Inoue et al., 1998), which assembles helically to form the long pili strand (Kachlany et al., 2001). In *A. actinomycetemcomitans*, the pili are rarely observed as individual strands but rather form bundled fibrils of multiple parallel pili strands (Kachlany et al., 2000, 2001). The tight adherence (tad) locus, containing over 10 *flp*, *rcp* and *tad* genes, is required for Flp-pili assembly (Clock et al., 2008). The pili facilitate cell attachment to each other and the initial non-specific attachment to surfaces (Kachlany et al., 2001). Flp-pili are not essential for biofilm formation *in vitro* (Inoue et al., 2003), but *flp* mutant biofilms are more loosely packed than are wild type biofilms and are dispersed when treated with agents that break DNA or polysaccharides (Inoue et al., 2003). Additionally, other non-pili-associated outer-membrane macromolecules regulate biofilm formation, such as the outer membrane collagen binding protein EmaA (Danforth et al., 2019).

The most abundant polysaccharide in *A. actinomycetemcomitans* biofilms is poly-N-acetylglucosamine (PGA). It is a polysaccharide containing hexosamine and is produced by all four genes in the *pgaABCD* locus (Izano et al., 2008). PGA is tightly
attached to bacterial cells and mediates adhesion between them in biofilms. PGA mutant strains are able to form biofilms, but they are more susceptible to dispersion by DNase I or proteinase K treatment (Izano et al., 2008). PGA may also protect *A. actinomycetemcomitans* biofilm by acting as a general diffusion barrier limiting the penetration of various antimicrobial agents to the biofilm (Izano et al., 2007, 2008) and protecting *A. actinomycetemcomitans* cells from being killed by macrophages (Venketaraman et al., 2008).

**Toxins**

*A. actinomycetemcomitans* secretes two exotoxins that are potent virulence factors: leukotoxin and cytolethal distending toxin. Both toxins may have been acquired via HGT (Kaplan & Fine, 1998; Mayer et al., 1999).

Leukotoxin belongs to the family of repeats-in-toxins (RTX), a large group of exoproteins produced by gram-negative bacteria that are characterized by calcium ion-binding repeated nonapeptide sequences (reviewed in Linhartová et al., 2010). Leukotoxin is a pore-forming lipoprotein that selectively lysys human leukocytes by making pores in the membrane and rupturing the cells. Leukotoxin targets lymphocyte function-associated receptor 1, which is expressed only in circulating leukocytes (Lally et al., 1997), thus leaving human epithelial and endothelial cells unharmed.

Leukotoxin kills PMNs (neutrophils, eosinophils, basophils), monocytes, erythrocytes and some lymphocyte populations (reviewed in Johansson, 2011). In monocytes/macrophages, leukotoxin activates the intracellular protease caspase-1, which cleaves the inactive pro-IL-1β and leads to IL-1β secretion (Kelk et al., 2003, 2005). In addition to killing human cells, leukotoxin also protects *A. actinomycetemcomitans* cells from macrophage killing and phagocytosis (Johansson et al., 2000; Venketaraman et al., 2008).

Leukotoxin operon *ltxCABD* codes four proteins with distinct functions. The *ltxA* protein is the actual toxin, and *ltxC* performs the post translational acylation of the toxin, which makes the toxin active. The *ltxB* and *ltxD* proteins, together with a third protein called TdeA, transport the toxin out of the cell via a type I secretion system (Lally et al., 1991; Crosby & Kachlany, 2007). Additionally, the MorC protein, which maintains membrane morphology, is associated with leukotoxin secretion (Gallant et al., 2008). The differences in the expression levels of leukotoxin in certain strains are due to modifications in the promoter area that are located upstream of the *ltxC*. For instance, the 530 bp deletion (so-called JP2 genotype) and the 640 bp deletion in the leukotoxin promoter site have been linked to highly leukotoxic phenotypes (Brogan et al., 1994; Claesson et al., 2015). The leukotoxin expression levels in *A. actinomycetemcomitans*...
**actinomycetemcomitans** isolates in the oral cavity have been shown to correlate with the severity of periodontitis (Höglund Åberg et al., 2014).

Cytolethal distending toxin (CDT) is produced by the *cdtABC* operon in *A. actinomycetemcomitans*, and all three genes are required for toxin production. CDT causes swelling of mammalian cells and blocking of their cell cycle, leading to apoptosis (Sugai et al., 1998). Additionally, CDT induces the production of IL-1β, IL-6 and IL-8 from peripheral blood mononuclear cells (Akifusa et al., 2001). In human periodontal ligament cells and gingival fibroblasts, CDT has been shown to arrest proliferation and increase RANKL expression (Belibasakis et al., 2002, 2004, 2005). The increase in RANKL induces bone resorption, which features in periodontitis.

**Lipopolysaccharide**

*A. actinomycetemcomitans* strains are divided into seven distinct serotypes (a to g) based on the different LPS O-antigen polysaccharides (Zambon et al., 1983; Kaplan et al., 2001; Lakio et al., 2003; Takada et al., 2010). Additionally, there are some strains that lack the O-antigen and are therefore non-serotypable (Kanasi et al., 2010). Overall, serotypes a, b and c are the most prevalent among the identified strains, while serotypes d and e are rare (Saarela et al., 1992). Information on the prevalence of the more recently discovered serotypes f and g is sparse. Serotype b is most commonly associated with periodontal disease, suggesting higher pathogenicity than other serotypes, and serotype c is found in periodontally healthy subjects (Asikainen et al., 1991). In the non-oral *A. actinomycetemcomitans* strains, the most commonly presented serotypes are b and c (Paju et al., 2000; Pietiäinen et al., 2018). Lipid A of *A. actinomycetemcomitans* is formed out of glucosamine disaccharide with four attached fatty acid chains: two 3-hydroxymyristic acids and two 3-myristoylmyristic acids (Masoud et al., 1991).

*A. actinomycetemcomitans* LPS plays a role mediating leukotoxin secretion; a defect in the O-antigen polysaccharide leads to increased membrane-bound leukotoxin and a decrease in secreted leukotoxin (Tang et al., 2012). *A. actinomycetemcomitans* LPS (strain Y4, serotype b) induced the production of IL-1β, IL-8, TNF-a and interleukin 1 receptor antagonist from PMNs (Yoshimura et al., 1997). Serotype b is able to prime dendritic cells to activate more tissue-destructive T-lymphocyte responses (Díaz-Zúñiga et al., 2015).
DNA uptake and the natural competence for transformation

There are both naturally competent and non-competent strains of *A. actinomycetemcomitans* (Jorth & Whiteley, 2012). The sequence analysis of several *A. actinomycetemcomitans* strains (genomes approximately 2.1-2.3 Mb) showed HGT, such as genomic islands (Jorth & Whiteley, 2012; Kittichotirat et al., 2016). The mechanisms of competence in *A. actinomycetemcomitans* are very similar to those of the widely studied *Pasteurellaceae* family member *H. influenzae*. *A. actinomycetemcomitans* preferentially uptakes DNA with a specific USS that is very similar to that of *H. influenzae*. The competence of *A. actinomycetemcomitans* can be induced by cyclic AMP (Wang et al., 2002).

Some of the genes that are linked to the natural competence in *A. actinomycetemcomitans* have been identified. The gene cluster pilABCD is essential for the natural competence in *A. actinomycetemcomitans* since deleting any of those genes leads to a non-transformable phenotype (Wang et al., 2003). While the pilABCD operon is homologous to TFP-like gene clusters in other bacteria, it does not take part in fimbriae biosynthesis in *A. actinomycetemcomitans* (Wang et al., 2003). The regulatory gene tfoX turns on the competence genes in most *A. actinomycetemcomitans* strains (Bhattacharjee et al., 2007). Furthermore, the deletion of the urpA gene, which is located upstream of the flp operon, results in the loss of natural competence in *A. actinomycetemcomitans* (Tanaka et al., 2012). The urpA gene is non-homologous to any other known competence gene found in other bacteria. The encoded UrpA protein (uptake-related protein A) is predicted to be a cytoplasmic protein with cell surface association that has a function related to the uptake of DNA (Tanaka et al., 2012).

### 2.3.2 *A. actinomycetemcomitans* interactions with cytokines

As previously discussed, many human proinflammatory cytokines play a significant role in the progression of periodontitis. *A. actinomycetemcomitans* biofilms have been shown to bind and internalize IL-1β. Fimbriated strains bind IL-1β more efficiently than do non-fimbriated strains, but none of the tested *tad* locus proteins directly affect the binding (Paino et al., 2011). The binding requires a viable biofilm; when the biofilm viability is compromised by adding antibiotics to the culture medium, the binding and internalization of IL-1β are omitted, and IL-1β is detected in the culture medium (Paino et al., 2012).

IL-1β in the growth medium increased the biofilm mass in *A. actinomycetemcomitans* D7S (serotype a) and SA1151 (serotype c) but decreased the metabolic activity of *A. actinomycetemcomitans* D7S (Paino et al., 2011). Planktonic *A. actinomycetemcomitans* preferentially upt...
*Actinobacillus actinomycetemcomitans* D7SS cells also showed a decrease in metabolic activity when incubated in the presence of IL-1β, except when the *rcpA* gene was deleted (Paino et al., 2011). Although RcpA was hypothesized to be the putative IL-1β receptor in *A. actinomycetemcomitans* because it has a similar role in Flp1 fimbria assembly as does IL-1β binding Caf1A in *Y. pestis* (Zav’yalov et al., 1995), binding between the N-terminal extramembranous domain of RcpA and IL-1β was not observed. Instead, IL-1β binds the trimeric form of F1F0 ATP-synthase subunit β (Paino et al., 2011). This might explain the effect of IL-1β on metabolic activity. The following studies also showed that the DNA-binding histone-like HU protein of *A. actinomycetemcomitans* bound IL-1β (Paino et al., 2012). The HU protein is conserved in bacteria, and in many bacteria, it regulates a substantial amount of genes (Oberto et al., 2009). Therefore, IL-1β binding to HU might have the potential to alter gene expression in *A. actinomycetemcomitans*.

Based on these results, IL-1β binding might help bacteria evade host immune responses, as has been observed (reviewed in section 2.2.3).
3. HYPOTHESIS AND AIMS OF THE STUDY

Hypothesis

Based on previous knowledge, I hypothesized that the outer membrane of *A. actinomycetemcomitans* could harbor molecules that could bind cytokines. Moreover, there must be a specific route for their internalization since IL-1β has been found inside *A. actinomycetemcomitans*. The HofQ secretin in *A. actinomycetemcomitans* might interact with cytokines, as the HofQ homolog PilQ has been shown to play a role in the internalization of cytokines in *N. meningitidis* (Mahdavi et al., 2013).

Aims

The aim of this thesis was to investigate outer membrane proteins and other components that were associated with cytokine binding and uptake mechanisms using *A. actinomycetemcomitans* as a model organism. In addition, this thesis aimed to provide new knowledge on how the crosstalk between *A. actinomycetemcomitans* and the host signal molecules might change bacterial physiology, such as the biofilm formation and antimicrobial resistance, at the infection site.

Specific aims of the thesis:

1. To discover and characterize the first-line cytokine-binding molecule(s) from the *A. actinomycetemcomitans* membrane
2. To identify the interaction areas in the first-line cytokine-binding molecules and cytokines
3. To discover the route for cytokine internalization
4. To uncover the effects of the studied proteins and the binding of cytokines on bacterial physiology
4. MATERIALS AND METHODS

4.1 Bacterial strains
The bacterial strains used in this study were mainly clinical *A. actinomycetemcomitans* isolates, of which the strain D7S was the most commonly used. All of the used *A. actinomycetemcomitans* strains are listed in Table 1. Commerically available *E. coli* strains XLI-blue (Stratagene), TOP10 (Invitrogen/Thermo Fisher Scientific), BL21-Codon-Plus-(DE3)RIL (Stratagene) and C41(DE3)RIL (Lucigen) were used in this study as well as *P. aeruginosa* strain Boston 41501 (ATCC 27853).

4.2 Cloning and expression of recombinant proteins [I - IV]
The recombinant proteins that were produced during the study are listed in Table 2. Detailed descriptions of all purifications are found in the original publications [I - IV]. Most of the proteins were produced with histidine tags to facilitate purification and further utilization as markers for detection in downstream applications.

Genes for the bacterial interleukin receptor I (BilRI) and extramembranous part of HofQ (emHofQ) were amplified from the *A. actinomycetemcomitans* D7S genome using specifically designed primers. Genes for the emHofQ mutants, IL-8, IL-1β, FgbA and ClfA were ordered as codon-optimized synthetic genes. Constructs for YadA production were kind gifts from Prof Mikael Skurnik from the University of Helsinki, Finland.

The genes were cloned into the pET36b expression vector to produce proteins with C-terminal 8xHis-tags or into the pET15b vector to produce proteins with N-terminal 6xHis-tags. The proteins were expressed in the *E. coli* BL21-Codon-Plus-(DE3)RIL protein expression strain using Terrific Broth medium supplemented with antibiotics. Expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) when the cell density reached the target level ($A_{600nm}=0.8-1.3$). Bacterial cultures were grown at 37°C for 2.5-3 hours before harvesting, except the emHofQ mutants grown at 16°C overnight. Proteins were separated from the cell lysate using a HisTrap HP affinity column and eluted using 250-500 mM imidazole. When needed, the His-tags were excised with thrombin. The eluted proteins were further purified with size-exclusion chromatography. Protein purity was checked with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration was determined with the Lowry method (Lowry et al., 1951). Diverging from the expression described above, IL-1β was produced in the pET-28bTEV plasmid, and the His-tag was cut with TEV protease.
Table 1. *A. actinomycetemcomitans* strains used in this thesis.

<table>
<thead>
<tr>
<th>strain</th>
<th>serotype</th>
<th>colony</th>
<th>used in</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>173s</td>
<td>e</td>
<td>smooth</td>
<td>III</td>
<td>(Höglund Åberg et al., 2013)</td>
</tr>
<tr>
<td>D7S</td>
<td>a</td>
<td>rough</td>
<td>I - IV</td>
<td>(Chen et al., 2010; Wang et al., 2002)</td>
</tr>
<tr>
<td>D7S <em>bilRI</em> overexpr.</td>
<td>a</td>
<td>rough</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>D7S <em>AbiRI</em></td>
<td>a</td>
<td>rough</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>D7S <em>AhofQ</em></td>
<td>a</td>
<td>rough</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>D7S <em>AhofQ::Spe</em></td>
<td>a</td>
<td>rough</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>D7SS</td>
<td>a</td>
<td>smooth</td>
<td>III</td>
<td>(Wang et al., 2003)</td>
</tr>
<tr>
<td>D7SAflp1-flp2::Spe</td>
<td>a</td>
<td>smooth</td>
<td>III</td>
<td>(Wang &amp; Chen, 2005)</td>
</tr>
<tr>
<td>HK1651</td>
<td>b</td>
<td>smooth</td>
<td>III</td>
<td>(ATCC 700685)</td>
</tr>
<tr>
<td>IDH781</td>
<td>d</td>
<td>rough</td>
<td>III</td>
<td>(Haubek et al., 1995; May et al., 2016)</td>
</tr>
<tr>
<td>NCTC 9710</td>
<td>c</td>
<td>smooth</td>
<td>III</td>
<td>(ATCC 33384)</td>
</tr>
<tr>
<td>O75U</td>
<td>d</td>
<td>smooth</td>
<td>III</td>
<td>(Claesson et al., 2017)</td>
</tr>
<tr>
<td>S23A</td>
<td>b</td>
<td>smooth</td>
<td>III</td>
<td>(Huang et al., 2013)</td>
</tr>
<tr>
<td>SA1151</td>
<td>c</td>
<td>rough</td>
<td>I</td>
<td>(Asikainen et al., 1995; Ihalin &amp; Asikainen, 2006)</td>
</tr>
<tr>
<td>SA1151s</td>
<td>c</td>
<td>smooth</td>
<td>III</td>
<td>(Asikainen et al., 1995; Ihalin &amp; Asikainen, 2006)</td>
</tr>
<tr>
<td>SA1216</td>
<td>c</td>
<td>smooth</td>
<td>III</td>
<td>(Asikainen et al., 1995)</td>
</tr>
<tr>
<td>SA1398</td>
<td>b</td>
<td>rough</td>
<td>I</td>
<td>(Ihalin &amp; Asikainen, 2006)</td>
</tr>
<tr>
<td>SA2146</td>
<td>b</td>
<td>rough</td>
<td>III</td>
<td>(Saarela et al., 1992)</td>
</tr>
<tr>
<td>SA2292</td>
<td>c</td>
<td>rough</td>
<td>III</td>
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<td>smooth</td>
<td>III</td>
<td>(Asikainen et al., 1995)</td>
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<tr>
<td>SA3139</td>
<td>N/A</td>
<td>smooth</td>
<td>III</td>
<td>(Kanasi et al., 2010)</td>
</tr>
<tr>
<td>SA492</td>
<td>d</td>
<td>smooth</td>
<td>III</td>
<td>(Saarela et al., 1992)</td>
</tr>
<tr>
<td>SUNYab 75</td>
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<td>smooth</td>
<td>III</td>
<td>(ATCC 43717)</td>
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<tr>
<td>Tr.GU 17-4</td>
<td>f</td>
<td>smooth</td>
<td>III</td>
<td>(Kanasi et al., 2010)</td>
</tr>
<tr>
<td>Y4</td>
<td>b</td>
<td>smooth</td>
<td>III</td>
<td>(ATCC 43718)</td>
</tr>
</tbody>
</table>
Materials and methods

Table 2. Recombinant proteins produced for this study.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tag</th>
<th>Residues</th>
<th>Used in</th>
<th>Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>BilRI</td>
<td>C- His</td>
<td>20-181</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>C- His</td>
<td>21-181*</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>N- His</td>
<td>21-181*</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>emHofQ</td>
<td>C- His</td>
<td>27-195</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- † 27-195</td>
<td>IV</td>
<td>(Tarry et al., 2011)</td>
</tr>
<tr>
<td>emHofQ mutants (L1, L2, L1L2)</td>
<td>C- His</td>
<td>27-195</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td>- † 28-99</td>
<td>II - IV</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>N- His</td>
<td>23-99</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td>- ‡ 117-296</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>FgbA</td>
<td>N- His</td>
<td>20-105</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>ClfA</td>
<td>N- His</td>
<td>230-542</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>YadA</td>
<td>N- His</td>
<td>26-241</td>
<td>II</td>
<td>(Nummelin et al., 2002)</td>
</tr>
</tbody>
</table>

* BilRI variant without residue C20 was produced, as this residue caused unwanted dimer formation.
† N-His-tag cleaved off with thrombin, ‡ N-His-tag cleaved off with TEV protease

4.3 Isolation of cell components for the interaction studies

4.3.1 LPS extraction from *A. actinomycetemcomitans* [III]

LPS was extracted from different *A. actinomycetemcomitans* strains as well as from one *P. aeruginosa* and one *E. coli* strain. Cells were lysed using sonication, and the cell debris and the intact cells were removed by centrifugation. The cell membranes were harvested using high-speed ultracentrifugation. Inner membranes were dissolved by sodium lauroyl sarcosinate, and the insoluble outer membranes were collected by ultracentrifugation. LPS was dissolved from the outer membranes using buffer containing 2-mercaptoethanol and SDS, and residual proteins were digested with proteinase K. LPS was precipitated with sodium acetate and ice-cold ethanol and collected by centrifugation. The precipitation step was repeated using only ethanol. The LPS pellet was dissolved in ultrapure water and frozen for further use.

Analysis of the extracted LPS

The amount of LPS in isolated samples was determined by using a commercial kit (ToxiSensor™ Chromogenic LAL Endotoxin Assay Kit). Since LPS likely contains some residual EM components, the quantities of proteins, DNA and PGA in the samples were determined as described in section 4.6.4. The amounts of phosphorus in the LPS samples were examined using the method of Rouser and co-workers (Rouser et al., 1970).
4.3.2 Isolation of outer membrane vesicles [III]
OMVs were isolated from *A. actinomycetemcomitans* by ultracentrifugation. Bacteria were grown for 3 days, harvested and centrifuged. Supernatants were filtered through 0.45-μm and 0.2-μm filters. OMVs were collected by ultracentrifugation, and formed pellets were washed twice and frozen. The presence of bacterial contamination was checked by cultivating samples of OMVs in blood agar plates.

4.3.3 Collection of human sera [IV]
Venous blood samples were collected from 12 adult periodontitis patients who had *A. actinomycetemcomitans* detected in their subgingival biofilm samples prior to their periodontal treatment. Control blood samples were collected from twelve orally and systemically healthy dentistry students. All samples were collected with written informed consent by the healthcare professionals at the Community Dental Health Care Center of Turku or at the Unit for Specialized Oral Care in Helsinki Metropolitan Area and Kirkkonummi.

4.4 Characterization of BilRI [I - II]

4.4.1 Identification of BilRI by mass spectrometry [I]
An electrophoretic mobility shift assay (EMSA) was used to detect the IL-1β binding membrane proteins from *A. actinomycetemcomitans*. The membrane protein fraction was first isolated from the D7S strain of *A. actinomycetemcomitans* and then solubilized in the mild detergent CHAPS, co-incubated with human recombinant IL-1β and run on native-PAGE. Pure membrane protein fraction and IL-1β were used as controls. An additional control experiment was performed by replacing IL-1β with a soybean trypsin inhibitor (STI), a protein with a similar size. Immunoblotting was used to detect IL-1β using an anti-IL-1β antibody. All proteins in the samples were visualized by silver staining from the equivalent gel. The lane containing the interacting proteins was cut from the silver-stained gel, and the proteins were identified by high-performance liquid chromatography (HPLC) followed by tandem mass spectrometry (MS/MS).

4.4.2 NMR spectroscopy to study the structure of BilRI [II]
The structure of BilRI was studied with nuclear magnetic resonance (NMR). The $^1$H NMR spectra from 4.6 mM of recombinant BilRI$_{21-181}$ were measured at 600 MHz using a Varian INOVA 600 MHz NMR spectrometer (Agilent). The two-dimensional $^1$H-$^{15}$N HSQC spectra of BilRI at pH 5 were collected at 800 MHz using predetermined complex points for each dimension. The spectra were measured using...
Materials and methods

4.4.3 Effect of BilRI expression on *E. coli* [I]

Full-length BilRI in the pET36b vector was expressed in the *E. coli* C41(DE3)RIL strain, and production was induced with IPTG. The location of expressed BilRI was determined by isolating the cytosolic, inner membrane and outer membrane fractions from the cells. The fractions were run on SDS-PAGE, and the presence of BilRI was detected by silver staining and HPLC-MS/MS. The purity of the outer membrane fractions was inspected by detecting the absence of the inner-membrane-located heme with a peroxidase substrate that interacts with it.

The effect of the membrane-expressed BilRI on membrane stability was studied by inspecting the leakage of DNA from the cells after a freeze-thaw cycle. The relative amount of DNA in the samples was visualized on an agarose gel using a fluorescent DNA-binding stain and UV light. The amount of leaked DNA was compared with that in *E. coli* in which BilRI production was not induced. Adding glycerol (20-50 % (v/v)) to the cells before freezing decreased the cell breakage.

The IL-1β binding capacity of BilRI-expressing *E. coli* was studied by flow cytometry. Induced and harvested cells were fixed with formaldehyde solution. The cells were co-incubated with biotinylated IL-1β or with biotinylated control protein STI and stained with avidin-fluorescein isothiocyanate. The samples were analyzed with a flow cytometer using excitation at 488 nm, and the emission signal was collected at 525 nm. The proportion of IL-1β-binding cells in the bacterial population was analyzed by comparing the fluorescence values with an identical sample set that was stained with a viability stain that stains nucleic acids in both live and dead bacteria.

4.4.4 BilRI-recognizing antibody fragment screening [I]

A synthetic M13 phage display library (Huovinen et al., 2013) was used to screen for BilRI-recognizing single-chain antibody fragments (scFvs). Recombinant BilRI was immobilized on magnetic polymer beads. Two rounds of incubation with the phage library were performed: first with the 5x10^{12} colony forming units (CFU) of phages and the second with the 5x10^{10} CFU. Bound phages were eluted with trypsin, cloned into pLK06H-vectors and produced as scFv-AP fusion proteins in *E. coli* XL1-blue. A sandwich-type immunoassay was used to test the binding of antibody fragments to BilRI. Ten active clones were identified, and western blotting was performed to verify the activity.
4.4.5 BilRI expression in *A. actinomycetemcomitans* [I]

Biofilm cultures from *A. actinomycetemcomitans* D7S and clinical *A. actinomycetemcomitans* isolates were grown in rich culture medium overnight and in poor medium for an additional 3 hours before harvesting. Cells were broken down by sonication, and BilRI expression was analyzed from the cell lysates by SDS-PAGE followed by immunoblotting using anti-BilRI antibody clone 16B8.

The surface exposure of BilRI was examined by treating intact *A. actinomycetemcomitans* D7S cells with proteinase K, which breaks down all the proteins that are exposed from the cell membrane. After 5 or 21 hours of treatment at 37°C, proteinase K was inactivated. The antibiotics chloramphenicol and globomycin were used to inhibit the protein synthesis and maturation of outer membrane lipoprotein during the treatment. Cells were lysed with sonication, and the presence of BilRI was analyzed by immunoblotting as above.

4.5 Molecular interactions

4.5.1 Interaction studies in ligand-binding microplate assays [I, II, IV]

A ligand-binding microplate assay was used as an initial screening method for possible interactions between certain molecules. The assays were performed in 96-well plates that were coated with one interaction partner followed by the blocking of excess binding sites with bovine serum albumin (BSA). The interacting His-tagged protein was incubated in the wells, and the bound proteins were detected using conjugate binding to their His-tail (HisProbe™-HRP, Thermo Fisher Scientific). The addition of substrate (ABTS) to horseradish peroxidase (HRP) led to a color reaction in the solution that was measured with a spectrophotometer at 405 or 414 nm. The results are presented as percentages of the signal obtained from the negative binding control BSA.

BilRI interaction with various cytokines [I, II]

The binding of BilRI to IL-1β and a set of other cytokines was investigated using a ligand-binding microplate assay. A total of 100 ng of cytokine (IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-γ or TGF-β1) was bound to the wells. After the blocking step, 5 μg of recombinant C-His BilRI_{20-181} (in study I) or 400 ng of recombinant C-His BilRI_{21-181} (in study II) was added. BSA was used as a negative binding control, and additional controls, STI and the N-terminal domain of *A. actinomycetemcomitans* protein RcpA, were used in study I.
The interaction of collagen/fibrinogen with D7S wild type or D7S ΔbilRI [II]

The interaction of BilRI with collagen or fibrinogen was studied at the cellular level using a microplate binding assay. *A. actinomycetemcomitans* D7S wild type or D7S ΔbilRI (section 4.6.2) cells were bound on microtiter wells that were coated with 1 μg of type V human collagen or 1 μg of human fibrinogen. After washing away the unbound bacterial cells, the bound cells were detected with anti-serotype A antibody and anti-rabbit IgG-HRP antibody combined with the HRP-substrate.

emHofQ interaction with various cytokines [IV]

The binding of emHofQ to a set of cytokines (IL-1β, IL-8, IL-6, TNF-α, IFN-γ) was performed by adding 6 pmol of cytokines to the wells, and after blocking, 490 nM of C-His emHofQ was added.

4.5.2 Interaction studies with TRFIA [II - IV]

A more sensitive microplate assay, time-resolved fluorometric immunoassay (TRFIA), was used to measure the interaction in a concentration-dependent manner. Assays were performed in 96-well plates, similarly to the ligand binding assay (section 4.5.1), but the bound proteins were detected using europium-labeled anti-His antibody (DELFIA, Perkin Elmer). Chelating detergent solution was added to dissociate the europium ion from the antibody, and fluorescence of the europium ion was measured using a time-resolved fluorometer. The results (except for those for collagen and fibrinogen) were presented by plotting the relative fluorescence units (RFUs) as a function of the concentration of His-tagged protein. The apparent dissociation constants of the interactions were calculated from the binding curves using a one-site binding model.

BilRI interaction with collagen or fibrinogen [II]

TRFIA was used to study BilRI binding to human EM molecules collagen and fibrinogen. The microtiter wells were coated with 1 μg of type V human collagen or 1 μg of human fibrinogen. BSA was added as a blocking agent, followed by incubation with 1 μg of C-His BilRI_{21-181}. Proteins that were previously shown to bind fibrinogen (FgbA, ClfA) or collagen (YadA) were used as positive binding controls. The results are presented as percentages of the negative binding control BSA.

Interaction of BilRI and LPS with IL-8 [III]

To investigate the affinity of the IL-8-BilRI interaction, the wells were coated with 6 pmol of IL-8 and blocked with BSA. Recombinant N-His BilRI_{21-181} was added to wells in concentrations ranging from 0 to 56 μM.
Materials and methods

To enhance the binding of LPS to the bottom of the microtiter wells, the wells were first coated with poly-L-lysine. The LPS extractions (section 4.3.1) from various *A. actinomycetemcomitans* strains (12x10³ EU/well) were incubated in the wells, after which the remaining binding sites were blocked using a commercial blocking agent. Controls were prepared without any LPS. Recombinant N-His IL-8 was added in concentrations ranging from 0 to 46 μM. The inhibitory effect of polymyxin B (PMB) on this interaction was studied with an equivalent method, though using only 15 μM IL-8 and incubating 1-50 mg/ml PMB in the wells after the blocking step. The maximum solubility of PMB to water was 50 mg/ml.

*A. actinomycetemcomitans* cells interacting with IL-8 [III]

The interaction of IL-8 with whole *A. actinomycetemcomitans* cells of serotypes a-f was studied in a similar experiment with fixed *A. actinomycetemcomitans* cells bound to the microtiter wells, and N-His IL-8 was added at a concentration of 15 μM.

**EmHofQ interaction with IL-1β and IL-8 [IV]**

The binding affinity of emHofQ to IL-1β and IL-8 was studied using TRFIA. Wells were coated with 6 pmol of IL-8 or IL-1β and blocked with a commercial blocking reagent. The C-His emHofQ was added in concentrations ranging from 10 to 740 nM.

The method for studying the interaction of emHofQ mutants (L1, L2 and L1L2) with IL-1β or IL-8 was the same, except that the amount of cytokines was 60 pmol during the coating step and the concentration range of C-His emHofQ mutants was 5 nM to 2 μM.

**4.5.3 Interaction studies with surface plasmon resonance [IV]**

Surface plasmon resonance was used to study the binding of IL-1β or IL-8 to emHofQ that was bound on the surface. The C-His emHofQ was coupled on the surface of an NTA chip. IL-8 was injected at concentrations ranging from 1 to 15000 nM, and binding was monitored with a Biacore device. An uncoupled chip was used as a reference. The peak values (RU) of each injection were plotted as a function of IL-8 and the dissociation constants were calculated using a one-site binding model.

**4.5.4 Exploring the interaction regions with chemical crosslinking [IV]**

The interaction site of emHofQ and IL-8 was studied by chemical crosslinking. The crosslinking agent bis(sulfosuccinimidyl)suberate (BS3) was used to chemically link the lysine residues in the studied proteins that were in close proximity to one another. The crosslinked protein samples were then run through SDS-PAGE and visualized by silver staining. The band corresponding to the crosslinked proteins, i.e., observed only when IL-8, emHofQ and BS3 were co-incubated, was excised, destained and digested...
with dimethylated porcine trypsin. Digested samples were analyzed with HPLC-MS/MS. Detected peptides were identified, the effects of the crosslinking agent were detected, and peptides were compared with the sequences of IL-8 and emHofQ.

4.5.5 Interaction studies with EMSA [III, IV]

Interaction of cytokines with LPS and OMVs [III]

The interactions of certain cytokines with LPS and OMVs were studied using EMSA. The isolation of LPS and OMVs is described in sections 4.3.1 and 4.3.2, respectively. The cytokines (IL-8, IL-1β or IFN-γ) were co-incubated with LPS, run on native-PAGE and visualized by silver staining. The interaction of IL-8 with LPS in isolated OMVs was studied by a similar experiment.

Interaction of IL-8 and DNA [IV]

The interaction of IL-8 with dsDNA was studied with EMSA. For this, 31 μg/ml linearized plasmid containing a USS was incubated with recombinant IL-8 in a concentration range from 20 ng/ml to 100 μg/ml. BSA was used as a negative binding control. Samples were supplemented with fluorescent DNA stain, run on an agarose gel and visualized under UV light.

4.6 Effect of BilRI and HofQ on *A. actinomycetemcomitans* biofilm [II, IV]

4.6.1 Organotypic gingival mucosal tissue co-culture model [II]

The organotypic gingival mucosa tissue co-culture model was developed to mimic in composition and arrangement the gingival tissue in contact with *A. actinomycetemcomitans* biofilm (Paino et al., 2012). First, the human gingival fibroblasts were combined with a collagen solution, transferred into cell culture inserts and grown for one day submerged in the medium. Spontaneously immortalized human gingival keratinocytes (HGKs) were added on top of the fibroblast-collagen layer, and the model was cultured in submersion for another day. Then, the model was lifted on a customized metal grid and cultured on liquid-air interphase for 5 days. *A. actinomycetemcomitans* biofilms were grown separately on membrane discs and added to the top of the model so that the biofilm side was adjacent to the human cells. Antibiotics, streptomycin and penicillin were added to half of the cultures to decrease the biofilm viability. The biofilms were co-cultured with the tissue culture model for 24 hours, after which the co-cultures were collected and fixed. Samples from the culture medium were collected before and after the 24-hour incubation and then frozen.
4.6.2 Preparation of D7S ΔbilRI [II] and D7S ΔhofQ [IV] deletion mutants

Site-specific deletion of bilRI or hofQ genes was performed using a Cre/loxP system (Cheng et al., 2014; Fujise et al., 2008) to obtain markerless deletion into the A. actinomycetemcomitans D7S genome. A schematic of the method can be found in study IV (Fig. 7 in study IV). The pLox2-Spe and pAT-Cre plasmids used for the method were a kind gift from Professor Casey Chen from the University of Southern California.

A single gene deletion of bilRI or hofQ was introduced into the genome using two primer pairs that were designed to flank the gene in question (bilRI or hofQ) from upstream and downstream. These sequences were ligated into a part of the pLox2-Spe plasmid, which contains a Spe<sup>R</sup> cassette with loxP cutting sites on each side. The linear DNA was transformed into A. actinomycetemcomitans D7S via natural transformation. Successful transformants that had the loxP-Spe-loxP fragment in place of the bilRI or hofQ gene were selected from the spectinomycin plates and verified with PCR. The loxP-Spe-loxP fragment was removed by the pAT-Cre plasmid, which contained cre recombinase and the tet(O) gene (Fujise et al. 2008). Cre recombinase catalyzed the recombination reaction between the loxP sites. The loss of both the loxP-Spe-loxP fragment and the pAT-Cre plasmid was checked by observing the sensitivity of the colonies to spectinomycin and tetracycline. Colonies sensitive to both antibiotics were considered markerless deletion mutants, and the deletions were verified by sequencing.

Restoring the bilRI gene to the genome of A. actinomycetemcomitans D7S ΔbilRI was not successful, so expression was restored by a plasmid containing bilRI constitutively expressed by the leukotoxin promoter. However, high bilRI expression led to poor cell viability.

4.6.3 Binding and uptake of IL-6, IL-8 and IL-1β by the biofilm [II]

The binding of IL-6 and IL-8 by A. actinomycetemcomitans biofilms was determined by immunohistological staining. Biofilms were co-cultured with the gingival tissue culture model (4.6.1) for 24 hours. The medium samples, which were collected before and after the 24-hour co-culture, were analyzed with IL-6- and IL-8-specific enzyme-linked immunosorbent assay (ELISA) kits to determine the expression levels of those cytokines with and without the bacterial biofilms. The co-cultures were collected and fixed overnight in 10 % formalin solution, embedded into paraffin and sectioned using standard histological techniques. De-paraffinized sections were treated with anti-IL-6 and anti-IL-8 antibodies. Bound antibodies were detected using the Dako REAL™ Detection System (Dako), and the samples were imagined under a light microscope.
The uptake of IL-6, IL-8 and IL-1β by *A. actinomycetemcomitans* biofilms was studied by immunoelectron microscopy. Biofilms were co-cultured with monolayers of HGKs for 24 hours. Then, the biofilms were fixed with 4 % paraformaldehyde for 7 hours and stored at 4°C until they were cut into cryo-sections and treated with anti-IL-6, anti-IL-8 or anti-IL-1β antibodies. Primary antibodies were detected with protein A-gold complexes (10 nm). Sections were examined with a transmission electron microscope. Labels were counted from 39-104 intact bacterial cells from 9-18 representative pictures. The primary antibodies were omitted from negative controls.

4.6.4 Characterization of biofilm formation and composition [II, IV]

The effects of BilRI and HofQ on *A. actinomycetemcomitans* biofilm formation and composition were studied by comparing the D7SΔbilRI and D7SΔhofQ biofilms with wild type D7S biofilms grown under identical conditions. Biofilm formation was determined with crystal violet staining. Biofilms were grown overnight on 48-well plates, and then gram staining reagent was applied to the top of the biofilms. Excess stain was removed by washing, the bound stain was released with ethanol, and the absorbance was measured at 620 nm. The level of the most abundant *A. actinomycetemcomitans* polysaccharide, PGA, was determined from biofilms grown as described above. Congo red stain was added to the top of the biofilm, and after a washing step, the bound stain was detached with dimethyl sulfoxide. Absorbance was measured at 405 nm.

For protein and eDNA determination, the biofilms were grown in cell culture bottles. The biofilm growth was mechanically detached, moved to microcentrifuge tubes and centrifuged. The proteins in the pellet were solubilized with SDS, and the insoluble material was separated by centrifugation. The protein concentration was determined with the Lowry method. For eDNA extraction, the cell pellets were treated with agents disrupting the polysaccharides and the proteins. Then, the samples were filtered through a membrane filter and placed in microtiter wells. The DNA levels in the samples were determined by adding propidium iodide and measuring the formed fluorescence using 535 nm excitation and 620 nm emission filters. The measured protein or eDNA amounts were proportioned to the mass of each biofilm sample.

The effect of cytokines on wild type D7S biofilm formation and composition was studied by adding 10 ng/ml IL-8 or IL-1β to nutrient-poor culture medium. The amounts of biofilm components were compared with those in the biofilms that were grown without cytokine supplementation. Experiments were performed with D7SΔbilRI and D7SΔhofQ strains to evaluate how BilRI and HofQ affect the biofilm’s response to cytokines.
4.7 Studies on the transcription unit [II, IV]
Operon prediction databases were utilized to predict whether *bilRI* and *hofQ* are stand-alone genes or transcribed as part of an operon. The Prokaryotic Operon Database (ProOPDB) was used to study the *bilRI* gene and the Database of prokaryotic Operons (DOOR) to study the *hofQ* gene.

The transcription of *hofQ* was further studied by RNA analysis. RNA was extracted from *A. actinomycetemcomitans* D7S and D7S Δ*hofQ* using a RiboPure™-Bacterial Kit (Ambion). Samples were treated with DNase I to remove DNA, and PCR with 16S primers was performed to confirm that the samples were DNA-free. Reverse transcription PCR was used to confirm the *hofQ* deletion from the D7S at the transcriptional level, to determine whether *hofQ* is part of a transcription unit and to study *hofQ* expression in different culture conditions. The amplicons were visualized in agarose gel using fluorescent DNA stain and UV light.

4.8 Exploring the potential role of HofQ in antimicrobial susceptibility and in host recognition [IV]

4.8.1 Antimicrobial susceptibility test [IV]
The antimicrobial susceptibility of *A. actinomycetemcomitans* D7S and D7S Δ*hofQ* was tested by plating the bacteria on *Haemophilus* test medium agar plates (Beckton Dickinson) and supplementing each plate with Etest-strips (BioMérieux) containing one of the tested antibiotics: ampicillin, amoxicillin/clavulanic acid, doxycycline or tetracycline. The minimum inhibitory concentration (MIC) values were read from the test strips after 45-48 hours of incubation.

4.8.2 Measurement of ROS production by leukocytes [IV]
The production of reactive oxygen species (ROS) from human leukocytes exposed to *A. actinomycetemcomitans* cells was examined by a luminol-amplified chemiluminescence experiment. The bacterial suspension was co-incubated with human plasma/serum (collection described in section 4.3.3), which opsonizes the bacteria, and with luminol, which produces chemiluminescence in the presence of ROS. Freshly isolated human leukocytes were added to the sample, after which the measurement of chemiluminescence was immediately started. The chemiluminescence was measured for 2 hours, and the peak value of each individual reaction was recorded.

4.8.3 Anti-HofQ antibodies in patient sera examined by ELISA [IV]
To study the possible immunogenic properties of emHofQ, the amounts of emHofQ-specific antibodies were determined from serum samples (4.3.3) using ELISA. The
relative antibody amounts from sera collected from the individuals who tested positive for *A. actinomycetemcomitans* were compared with those from serum samples collected from healthy individuals. The emHofQ protein was used to coat the microtiter wells, and excess binding sites were blocked with BSA followed by incubation with serum samples. Bound serum was detected with anti-human IgG-peroxidase antibody combined with the peroxidase substrate, and absorbance was measured at 405 nm.

4.9 Ethics statement [I – II, IV]

Blood samples for this study were collected from periodontitis patients who tested positive for *A. actinomycetemcomitans* and from healthy individuals with written informed consent. Permission to collect and use these samples was obtained from the Ethics Committee of the Hospital District of Southwest Finland.

4.10 Statistics [I - IV]

All the studies consisted of a small sample size (n=3-10). Therefore, most of the statistical tests used were non-parametric. The tests that were used were the Paired samples T-test (I, II), Friedman’s 2-way analysis of variance (II), Mann-Whitney U-test (with Bonferroni corrections when needed) (II, IV), Spearman’s rank-order correlation (III, IV) and Kruskal Wallis test (IV). All statistical analyses were performed using IBM SPSS Statistics 22 software. Statistical significance is considered at p-values less than 0.05.
5. RESULTS

5.1 Characterization of an outer membrane cytokine-binding protein

5.1.1 Identification and structural characteristics [I]

Discovery of IL-1β binding protein

The binding of IL-1β to the *A. actinomycetemcomitans* biofilm and its interactions with intracellular *A. actinomycetemcomitans* proteins were shown by Paino and co-workers (Paino et al., 2011, 2012). To discover the first-line IL-1β binding protein from the outer membrane, the membrane protein fraction isolated from *A. actinomycetemcomitans* D7S was co-incubated with IL-1β. The protein band with potential IL-1β binder was observed in EMSA (Fig. 10A). The band containing IL-1β together with the membrane proteins was significantly more intense in the silver-stained gel than was the lane with IL-1β on its own (Fig. 10A, circled). The anti-IL-1β antibody detected equal amounts of IL-1β in both samples (Fig. 10B). Therefore, the intense band likely contained other proteins besides IL-1β, and those might interact with IL-1β. No interaction was observed between the membrane proteins and the control protein STI (I: Fig. 1C).

![Figure 10](image)

**Figure 10.** Putative *A. actinomycetemcomitans* membrane proteins interact with IL-1β. (A) Membrane proteins (MPs) isolated from *A. actinomycetemcomitans* D7S were incubated with IL-1β, the samples were run on native-PAGE, and the gel was silver stained to visualize the proteins. The intensity of the lane where MPs and IL-1β were co-incubated (marked with red circle) was significantly higher than that of the lane containing only IL-1β. (B) An immunoblot with the same samples showed equal amounts of IL-1β in the samples, with or without the MPs. The presence of IL-1β was detected with an anti-IL-1β antibody, and it was not observed in the MP sample. The figure was adapted from Fig. 1 from study I.
The lane of interest was excised, and the subsequent HPLC-MS/MS analysis identified a putative protein of *A. actinomycetemcomitans* with a high identity rate. The analyzed sample also contained IL-1β. The putative protein was previously uncharacterized and was named bacterial IL receptor I (BilRI).

The structural characteristics of BilRI

The amino acid sequence of BilRI was analyzed with the SignalP 4.1 server, which predicted the presence of a signal sequence at the beginning of the sequence (Fig. 11, marked in red). The signal sequence guides the protein to the outer membrane. The BilRI has a 40-amino-acid-long region that is repeated three times with little variance in its amino acid sequence (marked with alternating colors, Fig. 11). Another special characteristic of the sequence is that there are no aromatic amino acids in BilRI, and it is mostly made of small polar and charged amino acids (II: Fig. 1C).

![Figure 11. The amino acid sequence of BilRI (181 amino acids).](image)

The database search of homologous sequences revealed almost identical sequences within the *Aggregatibacter* genus (I: Fig. 2B) and similar sequences in other bacteria, such as in *Haemophilus influenzae* and *Pasteurella multocida*, but none from species outside the *Pasteurellaceae* family (I: Fig. 3). The only homologous protein with a previously known function was the fibrinogen-binding FgbA protein in *Haemophilus ducreyi* (Bauer et al., 2009).

The three-dimensional structure of BilRI was studied with NMR, which showed that BilRI did not adopt a stable fold. The one-dimensional NMR $^1$H spectra and the two-dimensional $^1$H-$^{15}$N correlation spectra showed poor signal dispersion (II: Fig. 1A-B), which led to the conclusion that BilRI was an intrinsically disordered protein (IDP). IDPs lack a stable three-dimensional fold on their own but can have various conformations when interacting with other molecules (Uversky, 2011). The intrinsically disordered nature of BilRI was also shown in its amino acid sequence.
that contained only a few hydrophobic amino acids, which are required for the formation of a stable hydrophobic protein core.

5.1.2 BilRI expression and its localization on the outer membrane [I]

The localization of BilRI was first studied in *E. coli*, where the recombinant full-length *bilRI* was expressed from the plasmid. The expressed product was observed in the isolated outer membrane protein fraction of *E. coli* in SDS-PAGE (I: Fig. 4A), and the overexpression of *bilRI* on the *E. coli* membrane made the cells more susceptible to breaking when frozen without glycerol (I: Fig. 4C-E). The IL-1β binding of the *bilRI*-expressing *E. coli* cells was measured with flow cytometry using fluorescently labeled IL-1β. The *bilRI*-expressing cells bound IL-1β more efficiently than the cells in which the expression was not induced (I: Fig. 5B).

In *A. actinomycetemcomitans* D7S, BilRI was detected in the outer membrane protein fraction by the anti-BilRI antibody (I: Fig. 6A-B). The proteinase K treatment of the intact D7S cells deleted the BilRI from the outer membrane fraction, suggesting that BilRI was facing the extracellular space. BilRI was detected as two forms of different sizes in the outer membrane fractions and as three forms in the inner membrane fraction of *A. actinomycetemcomitans* (I: Fig. 6A-C). The outer membrane forms were hypothesized to be mature (35 kDa) and immature lipidated (70 kDa) forms of BilRI, while the inner membrane, which also contained the 70-kDa form, contained additional 17-kDa and 19-kDa forms suggested as the mature unlipidated and the proprotein forms of BilRI, respectively.

5.2 Characterization of molecular interactions

5.2.1 BilRI interacted with cytokines [I-III]

To study BilRI and its interactions with other proteins *in vitro*, BilRI was produced as a recombinant protein. The recombinant BilRI was produced without its signal sequence and with a histidine tail to aid in purification and detection. Microplate assays demonstrated BilRI interaction with IL-1β in study I (I: Fig. 5C). No significant binding was observed between IL-1β and another *A. actinomycetemcomitans* outer membrane protein, RcpA. In study II, an additional set of cytokines was included in the microplate assay. The interactions between BilRI and IL-8, IL-10, IFN-γ, TGF-1β, TNF-α and IL-6 were observed (Fig. 12A). Compared with the negative binding control BSA, the interaction of BilRI with IL-6 was the weakest, and the interaction with IL-8 was the strongest. The interaction between BilRI and IL-8 was studied in a concentration-dependent manner with TRFIA to obtain quantitative information about the binding. The specific binding between BilRI and IL-8 was observed compared with the BSA, but the measured...
values remained in the linear part of the binding curve, even with a BilRI concentration of 56 μM (Fig. 12B). Since the maximum binding (B_max) was not reached, determination of the dissociation constant (K_d) was not possible.

Since BilRI was shown to interact with multiple structurally unrelated cytokines and its sequence is homologous to the fibrinogen binder FgbA, its binding to the abundant extracellular molecules collagen and fibrinogen, was studied. Recombinant BilRI did not bind human collagen or fibrinogen when tested with a microplate assay (II: Fig. 2B). The lack of binding to collagen and fibrinogen was also observed at the cellular level, as the A. actinomycetemcomitans cells deficient in BilRI did not show any change regarding the binding of collagen or fibrinogen (II: Fig. 5C).

5.2.2 IL-8 interactions with non-proteinaceous components [III-IV]

IL-8 has a positive charge at neutral pH (pI-value of 9.3), while DNA, which is abundant in the EM of bacterial biofilm, is mainly negatively charged. The binding of recombinant IL-8 on the USS-containing dsDNA was shown in EMSA. The formation of DNA-IL-8 complexes was observed as immobile bands in the agarose

![Graph A](image1.png)

**Figure 12. Recombinant BilRI interacted with human cytokines.** (A) The binding of recombinant BilRI to various cytokines was detected in a microplate assay. The interaction between BilRI and IL-8 was high compared with that of the control (p=0.008, paired samples T-test). Data is presented as percentages compared with the binding control BSA (=100 %). The mean values ± SD from three independent experiments are presented. (B) The concentration-dependent IL-8-BilRI interaction was studied with TRFIA using 6 pmol of IL-8 and 0-56 μM of BilRI. The binding of BilRI to IL-8 was significantly higher than that of the control (BSA), but the maximum binding was not reached. Data is presented as the mean values ± SD from three independent experiments. Figure was adapted from Figs. 2A in study II and 1C from study III.
Results

No interaction was observed between negatively charged IL-1β (pI 5.9, data not shown) and DNA, suggesting that the interaction is likely electrostatic. The interaction of DNA and IL-8 has also been shown by Perks & Shute (Perks & Shute, 2000).

The observation of electrostatic interactions between IL-8 and DNA originated the study of possible interactions between IL-8 and LPS, which is a bacterial outer membrane component with negatively charged phosphate groups. The interaction between IL-8 and LPS was also studied with EMSA. The recombinant IL-8 enhanced the mobility of LPS, which was extracted from different *A. actinomycetemcomitans* strains, in native gel (Fig. 13B). A similar phenomenon was observed between LPS and IFN-γ (pI 9.5) but not between LPS and IL-1β (III: Fig. 1). The lack of interaction with negatively charged IL-1β supports the theory that the interaction between LPS and IL-8 or IFN-γ might be at least partly due to opposite charges.

The concentration dependency of the IL-8-LPS interaction was studied with TRFIA. Specific binding was observed between IL-8 and LPS extracted from *A. actinomycetemcomitans* serotypes a to f, and a saturated level of binding was reached with each tested LPS (Fig 14). The obtained *K*<sub>d</sub> values of these interactions ranged from...
from 1.2 to 17 μM (Table 3), and they were not correlated with the serotype. Therefore, the binding affinities were not serotype specific. To elucidate whether the interaction between IL-8 and LPS is a universal phenomenon among gram-negative bacteria, the LPS extracted from *E. coli* and *P. aeruginosa* was subjected to the same experiment. Subsequently, IL-8 also bound the LPS of *E. coli* and *P. aeruginosa* but with lower affinity than most of the tested *A. actinomycetemcomitans* LPS (Fig. 14, the last panel).

Figure 14. LPS of different *A. actinomycetemcomitans* serotypes interacted with IL-8. The interaction between IL-8 and LPS isolated from *A. actinomycetemcomitans* serotypes a-f was studied with TRFIA. LPS was used in equal amounts, and IL-8 was used in concentrations from 0 to 46 μM. The controls were prepared without any LPS. All tested LPS variants interacted with IL-8. The $K_d$ and $B_{max}$ values were calculated from the binding curves and listed in Table 3. LPS from other bacterial species moderately bound IL-8. Data is presented as the mean values ± SD from three independent experiments. Figure was adapted from Fig. 2 in study III.
Table 3. The \( K_d \) and \( B_{\text{max}} \) values of the IL-8 interaction with bacterial LPS.

<table>
<thead>
<tr>
<th>Strain</th>
<th>serotype</th>
<th>( K_d ) (( \mu \text{M} ))</th>
<th>( B_{\text{max}} ) (kRFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA1216</td>
<td>c</td>
<td>1.2 ± 0.3</td>
<td>1 200 ± 54</td>
</tr>
<tr>
<td>S23A</td>
<td>b</td>
<td>2.4 ± 0.6</td>
<td>1 400 ± 75</td>
</tr>
<tr>
<td><em>D7SAflp1-flp2::Spe</em></td>
<td>a</td>
<td>2.8 ± 0.5</td>
<td>1 500 ± 62</td>
</tr>
<tr>
<td>173s</td>
<td>e</td>
<td>3.8 ± 1.3</td>
<td>1 400 ± 120</td>
</tr>
<tr>
<td>SA492</td>
<td>d</td>
<td>4.0 ± 1.2</td>
<td>760 ± 62</td>
</tr>
<tr>
<td>Y4</td>
<td>b</td>
<td>4.1 ± 0.7</td>
<td>1 700 ± 77</td>
</tr>
<tr>
<td>SA1151s</td>
<td>c</td>
<td>4.2 ± 1.4</td>
<td>470 ± 42</td>
</tr>
<tr>
<td>O75U</td>
<td>d</td>
<td>4.6 ± 1.2</td>
<td>1 000 ± 74</td>
</tr>
<tr>
<td>Tr.GU 17-4</td>
<td>f</td>
<td>4.9 ± 1.2</td>
<td>1 400 ± 100</td>
</tr>
<tr>
<td>SUNYab 75</td>
<td>a</td>
<td>6.9 ± 0.6</td>
<td>1 300 ± 38</td>
</tr>
<tr>
<td>SA3138</td>
<td>a</td>
<td>7.8 ± 0.9</td>
<td>1 200 ± 47</td>
</tr>
<tr>
<td>HK1651</td>
<td>b</td>
<td>17 ± 3.1</td>
<td>1 200 ± 99</td>
</tr>
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<td>NCTC 9710</td>
<td>c</td>
<td>17 ± 2.0</td>
<td>1 100 ± 58</td>
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<td><strong>Other</strong></td>
<td></td>
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</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td>11 ± 3.0</td>
<td>1 000 ± 100</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td>17 ± 4.8</td>
<td>1 100 ± 130</td>
</tr>
<tr>
<td><strong>control (without any LPS)</strong></td>
<td></td>
<td>3.8 ± 0.8</td>
<td>340 ± 18</td>
</tr>
</tbody>
</table>

The binding of IL-8 to intact *A. actinomycetemcomitans* cells and to the OMVs of *A. actinomycetemcomitans* was studied with TRFIA and EMSA, respectively. OMVs are released from the outer membrane and contain plenty of LPS. The fixed *A. actinomycetemcomitans* cells from serotypes a to f bound IL-8 at equal levels (III: Fig. 2C). IL-8 altered the mobility of OMVs from serotypes a-e (III: Fig. 4a) in the native-PAGE. The same effect was observed with OMVs from the nonserotypable *A. actinomycetemcomitans* strain SA3139 (III: Fig. 4b), which lacks the O-antigen, suggesting that O-antigen plays no role in LPS binding to IL-8. Alternatively, the negative charges in the phosphate groups in the inner core of LPS are possible binding partners for the positively charged IL-8. The phosphorus contents in different LPS samples were analyzed and compared with the binding affinities. Although the LPS with the highest phosphorus content had the highest binding to IL-8, no correlation was observed between the amount of phosphorus and the binding affinities in the whole sample set (III: Fig. 5). To explore the role of the lipid A part of LPS in the interaction, the lipid A-binding antibiotic PMB, was added to the microplate assay. PMB had a small inhibitory effect on the LPS-IL-8 interaction under the tested conditions. However, even when the highest PMB concentration of 50 mg/ml was used, approximately 80 % of the binding was still detected (Fig. 15). This result suggests that the lipid A part of LPS may function in the interaction but is not the primary interaction site between IL-8 and LPS.
Extracted LPS samples will unavoidably contain proteins, polysaccharides and eDNA as impurities. They might affect the binding of IL-8, so the amounts of those impurities were determined from each sample. No correlation was found between the levels of proteins or eDNA in LPS samples and their binding affinity to IL-8 (III: Fig. 5). The amount of polysaccharide PGA in the samples was below the detection limit.

5.2.3 emHofQ interacted with cytokines [IV]

HofQ is a transmembrane outer protein of *A. actinomycetemcomitans* that plays a role in DNA translocation. The structure of the extramembranous part of HofQ (emHofQ) was determined by Tarry and co-workers (Tarry et al., 2011), who also showed that emHofQ directly interacted with DNA. The uptake of DNA has been demonstrated in *A. actinomycetemcomitans* (Wang et al., 2003), and while the actual mechanism of that is not yet discovered, the DNA uptake in *Neisseria* species has been proposed to occur through a secretin homologous to HofQ (Assalkhou et al., 2007; Krüger & Stingl, 2011). Since the HofQ homolog, called PilQ, in *N. meningitidis* was shown to bind cytokines (Mahdavi et al., 2013), the cytokine binding of HofQ was explored. The recombinant emHofQ with a C-terminal His-tag was produced, and its binding to an array of cytokines was studied in a microplate assay. The steady-state binding of IL-8, IL-6, IFN-γ and IL-1β to emHofQ was observed (Fig. 16A). The binding of emHofQ to IL-8 (the high binder) and IL-1β (the low binder) was studied with
Results

TRFIA to obtain quantitative values for the interaction. The $K_d$ values for emHofQ-IL-8 and emHofQ-IL-1β interactions were $43 \pm 4$ nM and $140 \pm 20$ nM, respectively (Fig. 16B). In vivo, emHofQ is immobilized in the outer membrane. Therefore, surface plasmon resonance was used to study the binding of IL-8 to the immobilized emHofQ. The binding affinity was approximately 50 times weaker than in the static environment, with a $K_d$ value of $2.4 \pm 1.3$ μM (IV: Fig. 1C). The affinity of IL-1β to the immobilized emHofQ was not obtained because the binding curve did not reach equilibrium, even with very high IL-1β concentrations (data not shown).

The interaction sites of emHofQ and IL-8 were explored using the crosslinking method. The two studied proteins were covalently crosslinked together from the lysine residues that are in close proximity to one another. Analysis of the crosslinked peptides identified by HPLC-MS/MS revealed that Lys139 in emHofQ was in close proximity to Lys15 in IL-8. The previously solved emHofQ homodimer structure was utilized to demonstrate the location of Lys139 in the loop area between the secondary structure elements $\alpha 3$ and $\beta 5$ in the C-terminal domain of emHofQ (Fig. 17). The crosslinking experiment of emHofQ and IL-1β did not provide any information about

Figure 16. emHofQ interacted with various cytokines. (A) The binding of recombinant emHofQ to multiple cytokines was detected in a microplate assay. High binding was observed between IL-8 and emHofQ compared with the control ($p=0.029$, Mann-Whitney U-test). Data (mean values $\pm$ SD) is presented as percentages of the emHofQ binding to control protein BSA ($=100\%$) and obtained from four independent experiments. (B) Concentration-dependent binding was observed between emHofQ and IL-8, as well as between emHofQ and IL-1β in TRFIA. Equal amounts of cytokines were bound to wells, and emHofQ was used in concentrations from 0 to 740 nM. BSA was used as a negative binding control. Data is presented as the mean values $\pm$ SD obtained from three independent experiments. Figure was adapted from Fig. 1 in study IV.
the interaction since the interaction between them was probably too weak (data not shown).

Figure 17. K139 in the emHofQ type I KH domain was close to the interaction site with IL-8. The emHofQ-IL-8 interaction site in emHofQ (PDB:2Y3M) is localized in the loop area containing K139 by the crosslinking experiment. The loop is flanked by α3 and β5 secondary structure elements (marked in the figure). Monomer A of emHofQ is colored blue-red from the N- to C- terminus and a part of monomer B is shown in gray. Figure was adapted from Fig. 2 in study IV.

Further verification of the interaction site was performed with mutational analysis. Three emHofQ mutant proteins were produced as His-tagged recombinant proteins. The first mutant, named emHofQ-L1, had three amino acids (His136, Phe137, Lys139) substituted with alanine in the emHofQ loop structure (loop 1), where the interaction was located (Fig. 18, the yellow loop). The emHofQ-L2 mutant had alanine substitutions of two amino acids (Asp165, Arg166) in the second loop (loop 2) right next to the L1 (Fig. 18, the green loop). The third emHofQ mutant had all five of the above-mentioned mutations and was called emHofQ-L1L2. The binding affinities of these emHofQ mutants to IL-8 and IL-1β, were assessed with TRFIA and compared with those of the non-mutated emHofQ (referred herein as the emHofQ-wt). All emHofQ mutants bound both tested cytokines with lower affinity (Fig. 19). A two- to fourfold decrease was observed in the emHofQ-IL-8 interaction, and a four- to fivefold decrease was observed in the emHofQ-IL-1β interaction. The $K_d$ and $B_{max}$ values from all interactions are listed in Table 4.

Figure 18. The amino acids in emHofQ that were chosen for the mutational analysis. A total of five one-amino-acid substitutions were made in two loop areas in the emHofQ (PDB:2Y3M) type I KH domain. Three mutations were made in loop 1 (L1, in yellow): H136A, F137A and K139A and two in loop 2 (L2, in green): D165A and R166A. Monomer A structures of emHofQ are colored in grey and monomer B structures in purple. Figure was adapted from Fig. 2 in study IV.
Figure 19. Mutations in the type I KH domain of emHofQ decreased its binding to IL-8 and IL-1β. The binding of emHofQ to IL-8 or IL-1β to the emHofQ mutant proteins L1, L2 and L1L2 was studied with TRFIA. The amounts of IL-8 or IL-1β were kept constant and the emHofQ proteins were added in concentrations ranging from 0 to 2 μM. The detected binding was slightly lower in all the emHofQ mutants compared with the emHofQ-wt (the black curve). BSA was used as the negative control (IV: Fig. 2C). Data was obtained from three independent experiments and presented as the mean values ± SD. Figure was adapted from Fig. 2 in study IV.

Table 4. The $K_d$ and $B_{max}$ values of the cytokine – emHofQ variant interactions.

<table>
<thead>
<tr>
<th>emHofQ variant</th>
<th>IL-8</th>
<th>IL-1β</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ ± SD (nM)</td>
<td>$B_{max}$ ± SD (kRFU)</td>
<td>$K_d$ ± SD (nM)</td>
</tr>
<tr>
<td>WT</td>
<td>44 ± 7</td>
<td>930 ± 60</td>
<td>90 ± 170</td>
</tr>
<tr>
<td>L1</td>
<td>190 ± 60</td>
<td>1,000 ± 150</td>
<td>450 ± 160</td>
</tr>
<tr>
<td>L2</td>
<td>110 ± 10</td>
<td>900 ± 80</td>
<td>330 ± 70</td>
</tr>
<tr>
<td>L1L2</td>
<td>200 ± 40</td>
<td>1,100 ± 90</td>
<td>440 ± 70</td>
</tr>
</tbody>
</table>

Although the cytokine binding of *N. meningitidis* PilQ was previously shown (Mahdavi et al., 2013), its cytokine-binding region has not yet been established. The type I KH domain of emHofQ shares 26 % sequence identity with the N1 domain of PilQ. This level of sequence identity suggests these proteins have a shared fold. The overall structure of PilQ has been modeled (Berry et al., 2012), and in that model (PDB: 4AV2) the N1 domain of PilQ adopts the same fold as the type I KH domain of emHofQ (IV: Fig. 2D), suggesting that the N1 domain might function in the cytokine binding of *N. meningitidis* PilQ.
5.3 Effects of BilRI and HofQ on biofilm [II, IV]

5.3.1 Genetic organization of bilRI and hofQ genes

The bilRI gene is flanked by the septum site-determining protein minC downstream and the phosphohistidine phosphatase sixA and phosphoglucosamine mutase glmM upstream (Fig. 20A). Using the operon prediction databases, bilRI was predicted to be a stand-alone gene, which seems logical, as the distance between the upstream sixA and bilRI is over 200 nucleotides and the downstream minC is transcribed in a different direction. The hofQ was predicted to be part of the comABCD operon (Fig. 20B) located upstream. However, reverse transcription PCR showed that hofQ is co-transcribed with only the flanking comD gene (IV: Fig. 4C).

![Figure 20. Genetic organization of A. actinomycetemcomitans D7S gene regions flanking bilRI and hofQ genes. (A) The bilRI gene is preceded by minC and followed by sixA and glmA. The arrows show the transcription direction and the proportional sizes of the genes. The distance between bilRI and sixA is marked in the figure. (B) The hofQ gene is preceded by the comABCD operon (black) and followed by the shikimate kinase. Figure was adapted from Fig. 4 in study IV.](image)

5.3.2 Deletion of bilRI or hofQ altered the biofilm composition [II, IV]

The bilRI or the hofQ gene was deleted from A. actinomycetemcomitans D7S with the Cre-LoxP system, and the deletion was verified at the genetic level by sequencing. The deletion of hofQ was further verified at the transcriptional level by reverse transcription PCR (IV: Fig. 4A). Deletion of bilRI did not have a large impact on the A. actinomycetemcomitans D7S phenotype. The D7S ΔbilRI strain was grown in similar rough colonies on agar plates (II: Fig. 4A). However, the bilRI-overexpressing strain (D7S bilRI rev) grew very poorly on agar plates, and the biofilm formation was completely abolished (II: Fig. 4C). Electron microscope images revealed that the
outer membranes of the D7S *bilRI* rev cells were completely ruptured (Fig. 21), explaining the poor growth and survival of these cells.

![Image](image_url)

**Figure 21.** Deletion of *bilRI* did not impact the cell morphology of *A. actinomycetemcomitans* D7S, but its overexpression lysed the outer cell membranes. D7S wild type (wt) and D7S Δ*bilRI* (*bilRI*- ) strains shared similar sizes and morphologies when fixed cells were observed by electron microscopy. In contrast, the cells of the D7S *bilRI* rev strain were smaller, and only one cellular membrane was observed. Arrows indicate *A. actinomycetemcomitans* cells in pictures where parts of the membrane filter are present. Figure was adapted from Fig. 4 in study II.

The effect of *bilRI* and *hofQ* on the formation of *A. actinomycetemcomitans* biofilm was studied by staining the D7S, D7S Δ*bilRI* and D7S Δ*hofQ* biofilms with crystal violet. All of the main biofilm components (eDNA, PGA and proteins) were separately analyzed in equivalent biofilms. The D7S Δ*bilRI* biofilm mass was comparable to the wild type D7S biofilm, but the D7S Δ*hofQ* biofilm mass was only half that of the wild type D7S biofilm mass (Table 5). The D7S Δ*bilRI* biofilm had significantly higher amounts of proteins and PGA and less eDNA relative to the wild type D7S biofilm, while the D7S Δ*hofQ* biofilm had significantly lower levels of all the main biofilm components (Table 5).

**Table 5. Effects of the *bilRI* or *hofQ* deletions on the D7S biofilm.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biofilm mass</th>
<th>eDNA</th>
<th>PGA</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>D7S Δ<em>bilRI</em> (II)</td>
<td>101 ± 5</td>
<td>80 ± 5 (0.021*)</td>
<td>117 ± 5 (0.029*)</td>
<td>134 ± 13 (0.009*)</td>
</tr>
<tr>
<td>D7S Δ<em>hofQ</em> (IV)</td>
<td>45 ± 22</td>
<td>57 ± 12 (0.029*)</td>
<td>78 ± 9 (0.029*)</td>
<td>54 ± 13 (0.029*)</td>
</tr>
</tbody>
</table>

* p-value (Mann-Whitney U-test), PGA = poly-N-acetylglucosamine
The amounts of biofilm components in the D7S biofilm were altered when the growth medium was supplemented with either IL-1β or IL-8 for 22 hours compared with the control biofilm without the cytokines (Table 6). The experiment with the D7S strain was performed in both studies II and IV to obtain a reliable, equivalent control for the D7S \( \Delta\text{bilRI} \) (study II) and the D7S \( \Delta\text{hofQ} \) (study IV) strains. The greatest and most consistent change in the wild type D7S biofilm was the decrease in eDNA in response to both tested cytokines. The deletion of \( \text{bilRI} \) or \( \text{hofQ} \) omitted this change. Cytokine supplementation did not significantly alter the overall biofilm mass or amounts of PGA or proteins in any of the tested strains (Table 6).

### Table 6. Effects of cytokines on the D7S wild type (wt), \( \Delta\text{bilRI} \) or \( \Delta\text{hofQ} \) biofilms.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Strain</th>
<th>Biofilm mass</th>
<th>eDNA</th>
<th>PGA</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D7S wt</td>
<td>94 ± 7</td>
<td>56 ± 16 (0.018*)</td>
<td>89 ± 8</td>
<td>86 ± 17</td>
</tr>
<tr>
<td></td>
<td>(II)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D7S ( \Delta\text{bilRI} ) (II)</td>
<td>103 ± 7</td>
<td>70 ± 14 (0.029*)</td>
<td>105 ± 5</td>
<td>85 ± 11</td>
</tr>
<tr>
<td></td>
<td>(IV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D7S wt</td>
<td>96 ± 12</td>
<td>63 ± 23 (0.028*)</td>
<td>88 ± 15</td>
<td>88 ± 19</td>
</tr>
<tr>
<td></td>
<td>(II)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D7S ( \Delta\text{bilRI} ) (II)</td>
<td>96 ± 2</td>
<td>65 ± 18 (0.029*)</td>
<td>88 ± 19</td>
<td>90 ± 20</td>
</tr>
<tr>
<td></td>
<td>(IV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p-value (Mann-Whitney U-test), PGA = poly-N-acetylglucosamine

Viable \( \text{A. actinomycetemcomitans} \) biofilm was previously shown to bind IL-1β when it was co-cultured with organotypic gingival tissue culture (Paino et al., 2011). In this study, the binding of IL-6 and IL-8 to the viable biofilm was detected using anti-IL-6 and anti-IL-8 antibodies. In the presence of penicillin and streptomycin, the biofilm viability was compromised, and less IL-6 and IL-8 were observed bound to the biofilm (II: Fig. 3A). The role of BilRI in the uptake of cytokines was analyzed by immunoelectron microscopy from the immunostained bacterial samples that were co-incubated with gingival keratinocytes that naturally produce cytokines. Less bound anti-IL-1β antibody was observed inside the D7S \( \Delta\text{bilRI} \) cells than inside the wild type D7S cells (II: Fig. 6). Therefore, the deletion of \( \text{bilRI} \) from \( \text{A. actinomycetemcomitans} \) D7S decreased the transport of IL-1β inside the bacterial cells. The uptake of IL-6 and IL-8, however, was not affected (II: Fig. 6).
5.4 The potential role of HofQ in host recognition and in antimicrobial susceptibility [IV]

Outer membrane proteins are potential targets for host recognition and the onset of immune responses. ROS production from human leukocytes is induced, for example, by bacterial products. The possible role of HofQ in this induction was studied with an experiment in which isolated human leukocytes were exposed to *A. actinomycetemcomitans* D7S or D7S ΔhofQ, and the subsequent ROS production by the leukocytes was measured. No significant difference was detected between the signal levels induced by the two strains (Fig. 22A). The pretreatment of bacteria with *A. actinomycetemcomitans*-positive sera resulted in slightly higher ROS production upon exposure to the wild type D7S or D7S ΔhofQ, but no significant difference between the strains was detected (Fig. 22B). The only statistically significant increase in ROS production was observed between the D7S ΔhofQ that was pretreated with *A. actinomycetemcomitans*-positive sera and the same strain that was treated with the control sera (p=0.005, Mann-Whitney U-test, Fig 22B).

**Figure 22. HofQ did not alter ROS production.** (A) The ROS production from the leukocytes in the presence of bacteria was measured as luminol-amplified chemiluminescence. Bacteria were pretreated with plasma collected from a healthy individual. The deletion of hofQ from D7S did not significantly affect ROS production compared with the D7S wild type (WT). Data (mean ± SD) is presented as percentages from the D7S WT. (B) ROS production was elevated when the bacteria were pretreated with the *A. actinomycetemcomitans*-positive (Aa+) serum instead of the healthy control serum. The ROS production with the Aa+ sera was slightly higher when stimulated with D7S ΔhofQ than when stimulated with D7S WT, while only a significant difference was observed between the Aa+ and control sera with the D7S ΔhofQ (p=0.005, Mann-Whitney U-test). The mean values of each sample set are marked with a line. Figure was adapted from Fig. 6 in study IV.
The presence of anti-emHofQ antibodies in *A. actinomycetemcomitans*-positive serum samples was studied with ELISA. The emHofQ was immobilized, and bound antibodies were detected with anti-human antibodies. No significant difference in antibody levels was discovered between the *A. actinomycetemcomitans*-positive serum samples and samples obtained from healthy individuals (IV: Fig. 6C).

The effect of HofQ on the antimicrobial susceptibility was tested with a panel of four antibiotics: amoxicillin/clavulanic acid, ampicillin, doxycycline and tetracycline. Deletion of *hofQ* did not alter the susceptibility of *A. actinomycetemcomitans* to doxycycline or tetracycline but made the bacteria more susceptible to β-lactam antibiotics amoxicillin/clavulanic acid and ampicillin (Fig. 23).

**Figure 23.** The deletion of *hofQ* made *A. actinomycetemcomitans* more susceptible to β-lactam antibiotics. The susceptibility of *A. actinomycetemcomitans* D7S wild type (wt) and D7S ΔhofQ to amoxicillin/clavulanic acid (amc), ampicillin (amp), doxycycline (dox) and tetracycline (tet) was tested by growing the bacteria in *Haemophilus* test plates with antibiotic test strips. After 45-48 hours, the MIC values were read from the strips. The D7S ΔhofQ was less resistant to the β-lactam antibiotics amc and amp, while the susceptibility to dox and tet was not altered. Figure was adapted from Fig. 5 in study IV.

**5.5 Summary of the results**

In this study, a novel outer membrane protein, BilRI, was discovered in *A. actinomycetemcomitans*. The sequence of BilRI was unique, consisting of repeated sequences and no aromatic amino acids. BilRI did not adopt a stable three-dimensional fold, hence being an IDP. In *A. actinomycetemcomitans*, BilRI was localized in the outer membrane facing the extracellular space. The recombinant BilRI bound various cytokines with low affinity but not the human EM components collagen or fibrinogen.

IL-8 interacted with DNA and the abundant outer membrane component LPS. The LPS extracted from six *A. actinomycetemcomitans* serotypes bound IL-8 with different affinities, but the affinities were not coupled with the serotypes. The binding
of IL-8 was not clearly correlated with any of the tested LPS regions: the O-antigen, the lipid A part or the phosphates in the inner core.

Channel protein secretin HofQ of *A. actinomycetemcomitans* was proposed to play a role in DNA uptake. The recombinant emHofQ bound multiple cytokines, of which IL-8 and IL-1β with moderate affinity. This suggests a possible link between DNA uptake and cytokine uptake. The KH I-type domain in the C-terminal end of emHofQ participated in the interaction with IL-8 and IL-1β. EmHofQ did not possess immunogenic properties.

Deletion of *hofQ* or *bilRI* from *A. actinomycetemcomitans* had a little effect on the phenotype. The deletion of *bilRI* decreased the internalization rate of IL-1β, and the deletion of *hofQ* made the bacterium more susceptible to β-lactam antibiotics. Both deletions decreased the amount of eDNA in the *A. actinomycetemcomitans* biofilms and abolished the cytokine-induced decrease of eDNA in the biofilm.

**Figure 24. Summary of the results.** This thesis showed that human cytokines interacted with the flexible outer membrane protein BilRI, the abundant outer membrane component LPS, dsDNA and the extramembranos part of HofQ in *A. actinomycetemcomitans*. The HofQ secretin was previously connected with DNA uptake, providing a possible link between DNA and cytokine uptake systems. Cytokines have been observed inside cells, but the internalization route is still unknown. The competence-associated Pil and Com proteins might play a role in the cytokine internalization in *A. actinomycetemcomitans*. 
6. DISCUSSION

Bacteria utilize multiple virulence mechanisms to survive in a hostile environment. Binding inflammatory mediators may be beneficial to bacteria (Meduri et al., 1999; Kanangat et al., 2001, 2007; Wu et al., 2005; Mahdavi et al., 2013). The effect of cytokines on the overall survival of bacteria has not been studied much, but IL-1β was shown to increase the growth rate of *E. coli*, *Acinetobacter* sp. and *S. aureus* (Porat et al., 1991; Meduri et al., 1999). It also seems that in the presence of IL-1β, *S. aureus* expresses a phenotype that promotes survival in the host, rather than fighting against the host (Kanangat et al., 2007). By sequestering the cytokines at the site of inflammation, the bacterial biofilm potentially alters the concentrations of inflammatory markers locally. This could significantly obstruct the host immune response and halt bacterial clearance because many responses require appropriate cytokine levels. For example, the formation of an IL-8 gradient at the infection site is highly important for neutrophil migration, and disturbance of the gradient will greatly weaken the immune responses.

In the quest for the outer membrane interaction partner of IL-1β in *A. actinomycetemcomitans*, a small uncharacterized lipoprotein was discovered and named BilRI. BilRI was the first cytokine-binding outer membrane protein identified in *A. actinomycetemcomitans* and was the first identified in all oral pathogens. BilRI had no previously known function, unlike all the other cytokine-binding proteins that have been discovered in other species. These already had at least one known function other than cytokine binding, such as assembly protein of the capsule (Zav’yalov et al., 1995), multifunctional porin (Wu et al., 2005) or secretion path for the TFP (Mahdavi et al., 2013).

The highly soluble BilRI did not crystallize so the approach for determining the three-dimensional structure of BilRI was NMR instead of X-ray crystallography. The NMR spectra showed poor signal dispersion, which strongly indicated that BilRI was an IDP. IDPs are common in nature and play versatile roles in signaling, regulation and assembly (Uversky, 2011). Determining the three-dimensional structure of BilRI could have resulted in the discovery of structurally homologous proteins that could have given us information about its possible functions. However, information about the common properties of intrinsic proteins may also provide some concepts that could apply to BilRI. IDPs are flexible with no rigid structures, while binding to their interaction partner will trigger them to fold into an ordered form. IDPs can interact with multiple binding partners (even simultaneously) and are often seen in reversible signaling interactions that need high-specificity and low-affinity binding (Uversky, 2011). Indeed, BilRI interacted to some extent with all of the tested cytokines: IL-8,
Discussion

IL-6, IL-1β, IL-10, TNF-α, TGF-1β and IFN-γ. Even the strongest observed interaction, between BilRI and IL-8, was still too weak for the binding constant to be determined in TRFIA. Since the binding affinity of BilRI to all tested cytokines was low, the interactions are probably reversible and temporary in nature. Our studies localized BilRI to the outer cell membrane of A. actinomycetemcomitans facing the extracellular space. This result fits well with our hypothesis that BilRI interacts with extracellular cytokines. The flexibility of an IDP brings it many advantages for interactions: it can increase the speed and spatial reach of interactions (Uversky, 2011). Therefore, BilRI with its flexible IDP structure could have many unknown important functions.

In this study, human cytokine interaction with bacterial LPS was shown for the first time. The interaction between LPS and cytokines was likely based on the net charges of the molecules since only the tested molecules with positive charge in neutral pH, i.e., IL-8 and IFN-γ, but not the negatively charged IL-1β, interacted with LPS. Interaction of IL-8 was observed with LPS isolated from six A. actinomycetemcomitans serotypes. The LPS structures on these serotypes differ in their O-antigen polysaccharide structures. The fact that IL-8 binds to all serotypes and even OMVs from a nonserotypable strain that lacks the O-antigen suggests that the interaction does not occur in the O-polysaccharide region. The PMB antibiotic, which binds the lipid A part of LPS, inhibited the interaction between IL-8 and LPS only lightly, suggesting that the interaction site of LPS and IL-8 might be somewhat different than that of PMB. The affinity of PMB to A. actinomycetemcomitans lipid A, however, is not known. While the core region of LPS has several phosphate groups that may bind positively charged IL-8, the amount of phosphorus in the samples did not correlate with the binding affinities. Compounds that affect the relative charges of molecules could be utilized to investigate this interaction further. Overall, our results were inconclusive about the interaction site between IL-8 and LPS. The results suggested that neither the O-antigen, lipid A nor the negative phosphate groups in the core are the primary interaction site between LPS and IL-8.

The extramembranous part of the A. actinomycetemcomitans HofQ porin, emHofQ, interacted with various cytokines in the static setting. Moderate binding affinities for emHofQ interactions with IL-8 and IL-1β were obtained. The K_d values for the interactions were 43 nM and 140 nM, respectively. The immobilized emHofQ bound IL-8 in a liquid flow with approximately 50 times lower affinity (2.4 μM). The disrupting forces present in the dynamic environment could explain the lower binding affinity. The setting in the dynamic experiment, however, more accurately represented the biological situation where the HofQ is membrane bound and IL-8 is diffusing freely. Moderate binding affinities were expected because the interaction
between emHofQ and cytokines is likely temporary before cytokines are passed forward in the uptake machinery. The HofQ homolog PilQ of *N. meningitidis* has been shown to bind IL-8 and TNF-α using mutant strains, but no binding constants have been reported (Mahdavi et al., 2013). Zav’yalov and co-workers obtained high binding affinity (K_d = 140 ± 14 pM) between IL-1β and the usher protein ClfA1 of *Y. pestis* (Zav’yalov et al., 1995). However, the experiment was not performed with purified proteins but by expressing recombinant ClfA1 in the outer membrane of *E. coli*, so it is impossible to compare these binding constants.

The interaction site between IL-8 and the *A. actinomycetemcomitans* emHofQ was studied with crosslinking, which identifies the lysine residues in the two proteins that are in close proximity to one another. In emHofQ, the detected lysine was Lys139, which was located in the C-terminal type I KH domain. The mutational assay was conducted with two, three or five amino acids substituted in the loop containing Lys139 and/or from the neighboring loop. That assay was designed to give a rough estimate of the interaction region, not to provide information about the roles of individual amino acids in the interaction. All mutations decreased the binding affinity of emHofQ to both IL-8 and IL-1β; therefore, these two loop regions contributed to the interaction. According to our studies, loop 1 has a greater role in the interaction because the mutations in that loop decreased the K_d more than mutations in loop 2. At the same time, these loop regions are in very close proximity, and the amino acids in them might interact. Therefore, changes in one of the loops might also change the conformation of the other. The interesting result was that the mutations in the loops also decreased emHofQ binding to IL-1β. This indicates that the type I KH domain in the emHofQ interacts with both IL-1β and IL-8. Structural comparison of the emHofQ of *A. actinomycetemcomitans* and the PilQ of *N. meningitidis* showed a homologous type I KH domain in the PilQ. Our results suggest that the type I KH domain is a possible cytokine-binding site in PilQ.

The overall structure of HofQ secretin has not been determined, but the structures of other homologous secretins could provide a clue. Secretins are usually formed out of 12 or 14 subunits that form channels across the outer membrane, and the usual channel diameter is approximately 6-8 nm (Bayan et al., 2006). Tarry and co-workers showed the structural homology between emHofQ and the EscC and GspD proteins from *E. coli* (Tarry et al., 2011). Homology was observed between emHofQ and the periplasmic regions of EscC and GspD (Korotkov et al., 2009; Spreter et al., 2009), suggesting that the emHofQ part is on the periplasmic side of the outer membrane rather than towards the EM.

The *A. actinomycetemcomitans* emHofQ was previously shown to bind linearized dsDNA both with and without the presence of USS (Tarry et al., 2011). Further, it
was suggested that HofQ is a possible channel transferring the DNA inside the cells because homologous secretins in the TFP system play a role in DNA uptake (Chen & Dubnau, 2004; Assalkhou et al., 2007). I showed that the interaction between IL-8 and dsDNA is likely electrostatic between negatively charged DNA and positively charged IL-8. The binding of human placental DNA and IL-8 has been shown by Perks and Shute (Perks & Shute, 2000), which supports our finding. IL-8 binding to DNA may have an important biological effect because eDNA is abundantly available in the extracellular space as one of the main components of the biofilm matrix (Whitchurch, 2002; Izano et al., 2008).

The possible roles of BilRI or HofQ in different aspects of *A. actinomycetemcomitans* physiology were studied using deletion mutants. The deletion of *bilRI* of *hofQ* did not significantly alter the phenotype or growth of *A. actinomycetemcomitans*. This result suggested that neither of these proteins are essential to the species and that their functions may be compensated by other proteins or that their essentiality did not manifest in the tested conditions. The overexpression of *bilRI*, on the other hand, decreased the membrane integrity and overall viability in both *E. coli* and *A. actinomycetemcomitans*. The deletion of *bilRI* decreased the uptake of IL-1β but not of IL-6 or IL-8. In the gingival fluid, the concentration of IL-1β is considerably lower than those of IL-6 and IL-8. The *in vitro* gingival tissue model produced approximately 200 ng of IL-8, 20 ng of IL-6 and 200 pg of IL-1β during the same time period (Paino et al., 2012). Based on this knowledge, the role of BilRI was suggested to be important when the cytokine concentrations in the environment are low. Overall, the immunostained bacterial samples did not have sufficient negative controls in this study, so the unspecific binding of the cytokine antibodies could not be disregarded. However, the samples in these experiments were identically prepared, so it could be suspected that the unspecific background in all the samples is approximately the same level.

The deletion of *hofQ* significantly decreased the formation of biofilm and all of its main components. Biofilm formation is an important virulence factor of *A. actinomycetemcomitans*, and HofQ clearly plays a pivotal role in that. The HofQ homolog PilQ in *N. meningitidis* has been shown to play a crucial role in natural transformation since its deletion disrupted *N. meningitidis* transformation (Lång et al., 2009). In our studies, the Δ*hofQ* strain was not transformable, suggesting that HofQ could also affect the competence of *A. actinomycetemcomitans*.

Many antibiotics enter cells through secretins or porins, leading to the hypothesis that in the absence of the HofQ, the penetration of antibiotics would be decreased and therefore the resistance to antibiotics increased. The susceptibility of D7S Δ*hofQ* to tetracyclines was not altered compared with that of the wild type D7S. However, the
resistance of D7S ΔhofQ to tested β-lactams decreased. This unexpected discovery could be explained by the notion that some EM components, such as the eDNA, are also found to decrease the effectivity of certain antibiotics (Chiang et al., 2013; Cavaliere et al., 2014). The deletion of hofQ decreased the amounts of all matrix components, including the eDNA, and increased its susceptibility to β-lactams. Those results suggest that matrix components, especially eDNA, may protect biofilm cells from β-lactams.

However, the mechanism concerning the antibiotic susceptibility of A. actinomycetemcomitans do not possess great clinical significance since the primary treatment for periodontitis is a mechanical removal of biofilm from tooth surfaces. Antibiotics can be used as an adjunct therapy to that but their effectivity and benefit are clearly shown only in some patient groups (Pretzl et al., 2018). Since the host inflammatory response is contributing to the development of periodontitis, promising results have recently been obtained by treating periodontitis with anti-inflammatory substances (anti-IL-23 antibodies) (Moutsopoulos et al., 2017). It has been shown that the periodontal keystone pathogen P. gingivalis can induce host inflammation, promoting dysbiosis and at the same time inhibiting the killing functions of immune cells (Maekawa et al., 2014). The cytokine binding of A. actinomycetemcomitans might also have a role in regulating host inflammation.

Bacterial outer membrane proteins are potential targets for recognition by human immune cells. For example, the cytokine-binding protein IrmA in E. coli is immunogenic (Moriel et al., 2016). In our studies, we did not discover any immunogenic effects of HofQ. The healthy control sera had very low levels of anti-A. actinomycetemcomitans antibodies compared with the A. actinomycetemcomitans-positive patient sera (unpublished results). The amounts of anti-emHofQ antibodies detected in A. actinomycetemcomitans-positive patient sera, however, were not higher than those of healthy controls, and the deletion of hofQ did not affect the stimulatory power that A. actinomycetemcomitans had on the leukocytes. These results are consistent with the view that emHofQ is likely facing the periplasmic space and therefore, that no antibodies against it will be formed.

In this study, I showed that BilRI interacted with various human cytokines but not with the extracellular molecules collagen or fibrinogen. BilRI homolog FgbA in H. ducreyi has shown binding to fibrinogen (Bauer et al., 2009), although another study proposed a completely different protein, DsrA, as the primary fibrinogen binder in H. ducreyi (Fusco et al., 2013). Our results suggest that the role of BilRI is more likely associated with the evasion mechanism (binding to human cytokines) than with cellular attachment to the EM. However, our experiments were mainly performed in a
static environment in a microplate, so no conclusion can be drawn about the interaction in a more dynamic environment or in vivo.

The notion that emHofQ interacts with DNA and IL-8 and that these molecules also interact with each other, suggested a possible link between the DNA and cytokine uptake mechanisms. The secretin channels are usually approximately 6-8 nm wide (Bayan et al., 2006) and can easily fit dsDNA (2.5 nm). On the other hand, the maximal diameter of IL-8 is approximately 4 nm (Högboom & Ihalin, 2017), so the IL-8 could possibly fit to the secretin channel bound with the DNA. Overall, the cytokine binding of emHofQ was an important discovery, even though it has not yet been verified if HofQ is the actual channel internalizing the cytokines.

LPS is highly abundant in the outer membrane of bacteria, so its binding to cytokines might have a greater biological impact than BilRI, which has a low presence on the membrane. LPS also plays an important role in the initiation of immune responses. The idea that LPS would also dampen the immune response by binding IL-8 and other cytokines is intriguing. The stable IL-8 gradient, which guides neutrophils and macrophages in the healthy gingival epithelium, is lost during periodontal infection (Tonetti et al., 1998). Our findings may suggest that the oral bacteria might play a role in disturbing the IL-8 gradient.
7. CONCLUDING REMARKS AND FUTURE PROSPECTS

Cytokine binding of *A. actinomycetemcomitans* outer membrane proteins BilRI and HofQ and of the outer membrane component LPS is shown in this thesis. The interactions were inspected with various methods and the binding affinities of emHofQ and LPS to certain cytokines were obtained as well as information about the interaction sites. The HofQ secretin has previously been associated with DNA binding and uptake; thus, this thesis suggests a possible connection between the uptake of DNA and the cytokine uptake mechanism. BilRI was shown to bind cytokines with low affinity, and its role in the cytokine uptake mechanism is most likely minor. This thesis also showed that LPS likely plays a role in cytokine binding, which is a notable discovery, as LPS already has many other important functions in bacterial virulence.

This thesis provides new knowledge of the virulence mechanism of an oral pathogen. On a larger scale, it offers a novel perspective of how the bacteria can interact with host molecules. However, the results obtained in this thesis just scratch the surface of this bacterial evasion mechanism and reveal a research area that could be explored further. Not much is known about BilRI and HofQ proteins, nor about their functions in general or their relation to bacterial virulence. If BilRI and HofQ are moonlighting proteins, like many virulence-associated proteins, they might have multiple functions in different parts of the cell or in different environmental conditions. The moonlighting functions of the proteins are difficult to discover intentionally, and most of those functions have been found serendipitously. Analysis of the expression levels of *bilRI* and *hofQ* in different culture conditions may provide new ideas for their potential roles. Other functions could be found especially from BilRI, as its role in cytokine binding seems vague and because it is an IDP that has a flexible structure enabling it to perform different functions. The suspected role of HofQ as a channel that transfers cytokines inside the cell also needs to be investigated. If HofQ is not the route for cytokines, then other possible internalization methods need to be explored.

The cytokine uptake mechanism in *A. actinomycetemcomitans* most likely involves many molecules in the cytosol, on the membrane and even in the biofilm EM that need to be discovered. For instance the Pil and Com proteins of *A. actinomycetemcomitans* are considered as being involved in the same secretion system as is HofQ (Zijenge et al., 2012), therefore their participation on the cytokine binding and uptake may be explored.

Cytokine binding has already been shown in many bacterial species, and it might be discovered in even more species. Additionally, the cytokine binding of LPS from
other gram-negative species would be an interesting study target. In nature, bacteria usually occur as multispecies biofilms. To gain insight into how the cytokine uptake mechanism might work in nature, more complex experimental setup with multiple bacterial species is required.

The effects of human cytokines on bacterial virulence are still relatively little studied. Cytokines are important host regulators in the infection site, and it seems that bacteria can utilize those for their own benefit; to regulate their gene expression or the inflammatory milieu. This thesis concentrated on some of the consequences that the cytokine binding and uptake have on the bacteria. In the future, it would be interesting to study how the cytokine uptake of bacteria affects the host. Tissue culture models, such as the organotypic gingival mucosal tissue model used in this thesis, could be utilized to investigate some of those effects for host cells.

Bacterial cytokine-binding mechanism could have potential clinical implications for the treatment or prevention of bacterial infections, but extensive research about the mechanisms is still needed. Detailed information should be obtained about how the cytokine-binding effects both bacteria and the host as well as the structure of the active sites of cytokine-binding bacterial molecules. Only after sufficient information is gathered, the possibility for the development of effector molecules could be evaluated.
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