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THE ROLE OF THE DNA UPTAKE MACHINERY IN THE VIRULENCE OF *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*

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We're all capable of the most incredible change.
We can evolve while still staying true to who we are.
We can honour who we've been and choose who we want to be next.

-The Doctor

UNIVERSITY OF TURKU
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ABSTRACT

Biofilm infections pose a significant health threat owing to their increased resistance to antibiotics. The oral inflammatory disease periodontitis is caused by a multispecies biofilm. One species associated with periodontitis is the opportunistic pathogen *Aggregatibacter actinomycetemcomitans*. It produces various virulence factors that contribute to its pathogenicity and survival in the host. The species has been shown to bind and internalize human cytokines, which affect the biofilm composition. Cytokine uptake has the potential to alter host immune responses and bacterial gene expression. Recently it has been suggested that cytokine and DNA uptake could be linked in a naturally competent *A. actinomycetemcomitans* strain.

This study aimed to provide new knowledge on cytokine binding and uptake, and their proposed connection with DNA uptake in the naturally competent *A. actinomycetemcomitans* strain D7S. The focus was on two previously studied cytokine-binding outer membrane proteins, the secretin HofQ and the lipoprotein BilRI, along with a type IV pilin homolog, PilA. The functions of PilA have not been previously studied, apart from the discovery that *pilA* is an essential competence gene. However, type IV pili have been recognized as important virulence factors. Another aim was to study how cytokines alter bacterial gene expression.

This study shows that *hofQ* and *bilRI* are involved in the competence of *A. actinomycetemcomitans*, and that PilA interacts with human cytokines, similar to HofQ and BilRI. The results support the hypothesis that the uptake of cytokines and DNA may occur via the same mechanism. The gene expression studies did not show differences between cytokine-treated and control cultures. However, removal of *pilA* or *hofQ*, which are essential for competence, altered gene expression in the competent strain. The changes suggested decreased biofilm formation and virulence.

In addition to cytokine interactions, PilA interacted with human leukocytes. The virulence properties of PilA make it a potential drug target. Meanwhile, BilRI might be involved in survival outside the host, as the activity of the *bilRI* promoter was increased at lowered temperatures. However, future studies are required to clarify the full potential of PilA and BilRI as virulence factors and the link between cytokine and DNA uptake.

KEYWORDS: Aggregatibacter actinomycetemcomitans, biofilms, host–pathogen interactions, DNA transformation competence, bioinformatics

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TIIVISTELMÄ

Biofilmien aiheuttamat infektiot, kuten tulehduksellinen suusairaus parodontiitti, ovat merkittävä terveysuhka, sillä antibiootit eivät toimi niitä vastaan tehokkaasti. Yksi parodontiitin mikrobeista on opportunistinen patogeeni *Aggregatibacter actinomycetemcomitans*, jonka monet virulenssitekijät edistävät patogeenisyyttä. Laji pystyy sitomaan ja ottamaan sisäänsä sytokiineja, mikä vaikuttaa biofilmin koostumukseen. Sytokiinien sisäänotto voi muokata isännän immuunipuolustuksen toimintaa ja bakteerin geenien ekspressiota. Sytokiinien sisäänotto on myös yhdistetty DNA:n sisäänottoon transformaatiokykyisessä *A. actinomycetemcomitans* kannassa.

Tutkimuksen tavoitteena oli selvittää sytokiinien ja DNA:n sisäänoton yhteyttä. Tutkimuksen keskiössä oli kaksi sytokiineja sitovaa ulkokalvon proteiinia, kanavaproteiini HofQ ja lipoproteiini BilRI, sekä tyypin IV piluksen kaltainen PilA. Aikaisemmin *pilA*-geenin on osoitettu olevan välttämätön transformaatiolle, mutta proteiinin funktiota ei ole tutkittu. Tyypin IV pilukset ovat kuitenkin tärkeitä virulenssitekijöitä monilla bakteereilla. Tavoitteena oli myös tutkia sytokiinien vaikutusta bakteerin geenien ekspressioon.

Tutkimuksessa osoitettiin, että *hofQ* ja *bilRI* vaikuttavat transformaatiokykyyn, ja että PilA sitoo sytokiineja HofQ:n ja BilRI:n tavoin. Tulokset tukevat aikaisempaa hypoteesia siitä, että sytokiinien ja DNA:n sisäänotto voisi tapahtua samalla mekanismilla. Geenien ekspressiotutkimuksissa ei havaittu eroa sytokiini-käsiteltyjen ja kontrollikasvatusten välillä, mutta *pilA*- tai *hofQ*-geenin poistaminen vaikutti transformaatiokykyisen kannan geenien ekspressioon. Muutokset geenien ekspressiossa viittasivat heikentyneeseen biofilmin muodostukseen ja taudin-aiheuttamiskykyyn.

Sytokiinien lisäksi myös leukosyyttien ja PilA:n välillä havaittiin vuoro-vaikutuksia. Tutkimuksen tulokset viittaavat siihen, että PilA on mahdollinen virulenssitekijä ja siten uusien mikrobilääkkeiden kohdeproteiini. Sen sijaan BilRI edistää mahdollisesti bakteerin selviämistä isännän ulkopuolella, sillä *bilRI*-geenin promoottorialueen aktiivisuus kasvoi, kun lämpötilaa laskettiin. Lisätutkimuksia kuitenkin vaaditaan selvittämään PilA:n ja BilRI:n virulenssiominaisuuksia ja sytokiinien ja DNA:n sisäänoton yhteyttä.

ASIASANAT: Aggregatibacter actinomycetemcomitans, biofilmit, isännän ja patogeenin väliset vuorovaikutukset, DNA-transformaatiokyky, bioinformatiikka

Table of Contents

Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AHL	acylated homoserine lactone
AI-2	autoinducer-2
Bap	biofilm-associated protein
BilRI	bacterial interleukin receptor I
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
cAMP	cyclic AMP
c-di-GMP	cyclic dimeric GMP
cDNA	complementary DNA
CDT	cytolethal distending toxin
COG	Cluster of Orthologous Groups
cryo-EM	cryoelectron microscopy
CUP	chaperone/usher pili
dsDNA	double-stranded DNA
DUS	DNA-uptake sequence
ECP63	embryogenic cell protein 63
eDNA	extracellular DNA
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohemorrhagic *Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
emHofQ	extramembranous part of HofQ
EMSA	electrophoretic mobility shift assay
EPS	extracellular polymeric substances
Flp	fimbrial low-molecular-weight protein
GO	Gene Ontology
GRAVY	grand average of hydropathicity
HACEK	group consisting of bacteria from the genera *Haemophilus*, *Aggregatibacter*, *Cardiobacterium*, *Eikenella* and *Kingella*
HBSS	Hank's balanced salt solution
gHBSS	supplemented with gelatine

HGT	horizontal gene transfer
HRP	horseradish peroxidase
HU	histone-like DNA-binding protein
IDP	intrinsically disordered protein
IgG	immunoglobulin G
IL	interleukin
IPTG	isopropyl β-D-thiogalactopyranoside
K_d	dissociation constant
LEA	late embryogenesis abundant
LPS	lipopolysaccharide
MAA	monomeric autotransporter adhesin
mTSB	modified TSB (TSB supplemented with yeast extract and glucose)
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY-HSQC	nuclear Overhauser effect spectroscopy-heteronuclear single quantum coherence spectroscopy
OD	optical density
OMP	outer membrane protein
OMV	outer membrane vesicle
OPG	osteoprotegerin
PAGE	polyacrylamide gel electrophoresis
PAL	peptidoglycan-associated lipoprotein
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PBS-N	supplemented with sodium azide
PBS-T	supplemented with Tween-20
PCR	polymerase chain reaction
PDB	Protein Data Bank
PGA	poly-*β*-1,6-*N*-acetyl-d-glucosamine
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
PMSF	phenylmethylsulfonyl fluoride
PNAG	poly-*N*-acetylglucosamine
PQS	*Pseudomonas* quinolone signal
Psl	polysaccharide synthesis locus
PTS	phosphotransferase system
qPCR	quantitative polymerase chain reaction
QS	quorum sensing
RANKL	receptor activator of nuclear factor κB ligand
RFP	red fluorescent protein

RNAseq	RNA sequencing
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
SSP	secondary structure propensity
TAA	trimeric autotransporter adhesin
tad	tight adherence
TBS	Tris buffered saline
TBS-T	supplemented with Tween-20
THP-1	a human acute monocytic leukemia cell line
TNF	tumor necrosis factor
TRFIA	time-resolved fluorometric immunoassay
TSA	tryptic soy agar
TSA-H	supplemented with horse serum
TSA-B	supplemented with sheep blood
TSB	tryptic soy broth
USS	uptake signal sequence

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Maula T, **Vahvelainen N**, Tossavainen H, Koivunen T, Pöllänen MT, Johansson A, Permi P, Ihalin R. Decreased temperature increases the expression of a disordered bacterial late embryogenesis abundant (LEA) protein that enhances natural transformation. *Virulence*, 2021; 12(1): 1239–1257.

II **Vahvelainen N**, Bozkurt E, Maula T, Johansson A, Pöllänen MT, Ihalin R. Pilus PilA of the naturally competent HACEK group pathogen *Aggregatibacter actinomycetemcomitans* stimulates human leukocytes and interacts with both DNA and proinflammatory cytokines. *Microbial Pathogenesis*, 2022; 173(Pt A): 105843.

III **Vahvelainen N**, Kovesjoki L, Maula T, Ihalin R. Deletion of competence genes represses expression of genes associated with anaerobic respiration/metabolism in *Aggregatibacter actinomycetemcomitans*. *Preprint*. bioRxiv 2023.05.18.541267

1 Introduction

1.1 Bacterial biofilms

Biofilms are the predominant growth state of prokaryotes in natural settings, as up to 80% of prokaryotes on Earth are estimated to live in biofilms [1]. Biofilms are sessile, organized multicellular structures consisting of cells embedded in an extracellular polymeric substance (EPS) matrix. The EPS matrix is produced by biofilm cells and mainly consists of water, polysaccharides, proteins, lipids, and extracellular DNA (eDNA). Notably, the EPS matrix can account for as much as 90% of the biofilm mass. The matrix provides protection against desiccation, antibiotics, and host immune cells, among other factors. Therefore, biofilm cells are more tolerant to environmental stress than planktonic, free-living single cells. [2]

Most natural biofilms are multispecies communities and can contain species from the same kingdom (e.g., only bacteria) or different kingdoms (e.g., bacteria and fungi). Bacterial biofilms have been widely studied using the human pathogen *Pseudomonas aeruginosa* as a model organism, although biofilms formed by other species including *Vibrio cholerae, Escherichia coli* and *Staphylococcus aureus* have also been well characterized [3–6].

1.1.1 Biofilm formation and dispersion

The most widely used model of biofilm development consists of five steps: (i) reversible attachment, (ii) irreversible attachment, (iii) maturation I, (iv) maturation II, and (v) dispersal [7]. According to the model, biofilm formation is initiated when planktonic cells attach to a surface. However, later, it was discovered that nonsurface-associated microbial aggregates embedded in the EPS matrix also exhibited properties characteristic of biofilms [8]. Therefore, a new model that includes both traditional surface-associated biofilms and nonsurface-associated biofilms (aggregates), has recently been suggested [9]. The new model presents three major events in the biofilm lifecycle: (i) aggregation, (ii) growth, and (iii) disaggregation. However, biofilm formation and development have been extensively studied using surface-adhered biofilms, and currently it is unknown how surface-associated and nonsurface-associated biofilms differ in transcriptional level [9].

Thus, the following paragraphs mainly refer to studies performed with surface-associated biofilms.

Each phase in biofilm development is characterized by specific gene expression and protein production, which are induced by specific regulators [7,10–12]. First, the transition from a planktonic to a biofilm lifestyle occurs as a response to environmental cues that trigger transcriptional changes. For example, contact with a surface, nutrient limitation, host-derived signals, and antimicrobials can induce biofilm formation [13–17]. Levels of environmental sugars, iron, and inorganic phosphate can either induce or inhibit biofilm formation [18–28]. In particular, the sugar-transporting phosphoenolpyruvate phosphotransferase system (PTS) has been linked to biofilm formation [29–32]. Environmental cues activate transcription factors, which in turn induce the expression of a specific gene pool. Known transcription factors for biofilm formation include the alternative sigma factor RpoS, curli subunit gene D (CsgD), and the universal bacterial second messenger cyclic dimeric GMP (c-di-GMP) [33–36]. Another second messenger, c-di-AMP, has also been associated with biofilm formation [37]. Additionally, small RNAs can regulate biofilm formation [22,38,39].

Regarding surface-associated biofilms, the initial attachment is mediated by bacterial surface organelles [40–42]. Motile species, such as *P. aeruginosa* use flagella to overcome surface repulsion and initiate attachment; however, the expression of flagella is repressed upon biofilm formation [41,43]. Tight adherence to the surface is acquired with various adhesins, such as long, polymeric fimbrial adhesins, short, mono- or trimeric nonfimbrial adhesins, and nonproteinaceous adhesins (reviewed in [44]). Fimbrial adhesins, or pili, include chaperone/usher pili (CUP), alternative CUP, type IV pili, and curli fibers. Many species possess several types of pili, which vary in assembly pathways, sizes, and functions, to facilitate attachment to various surfaces under different conditions [42,45]. Along with biofilm formation, pili can be involved in, for example, motility and DNA transfer [46]. Nonfimbrial adhesins involved in biofilm formation include adhesins secreted via the type 1 or type 5 secretion system, such as biofilm associated proteins (Bap), monomeric autotransporter adhesins (MAAs), trimeric autotransporter adhesins (TAAs) and hemagglutinin-like adhesins. Additionally, nonproteinaceous polysaccharide adhesins can promote adhesion. Aggregative polysaccharides include cellulose, alginate, and propylene glycol alginate [47–49].

Because bacterial cells are in close contact with each other in biofilms, cell–cell communication mediates biofilm formation. Quorum sensing (QS) is a form of cell–cell communication that relies on small secreted molecules, called autoinducers, such as acylated homoserine lactones (AHLs), quinolone derivatives, such as the *Pseudomonas* quinolone signal (PQS), or S-adenosylmethionine derivatives, such as autoinducer-2 (AI-2) [50–52]. Bacterial cells detect the accumulation of

autoinducers at a high cell density, which allows bacterial cells to regulate their gene expression [53]. Many bacterial species require QS for efficient biofilm formation, although the impact of QS may be dependent on growth conditions [54–59]. Moreover, in certain species, QS negatively regulates biofilm formation, or has no effect on biofilm formation [60–62]. In biofilms, QS plays a role in the structural development and generation of eDNA [63–65], which is necessary for biofilm formation because eDNA has been determined to be an integral part of the EPS [66].

Along with eDNA, cells in a developing biofilm generate other EPS components. The production of the EPS is often positively regulated by the second messenger c-di-GMP, in addition to the abovementioned QS [67]. Overall, the mature biofilm structure varies greatly depending on the species residing in the biofilm, available nutrients, and physical environment [68–70]. This adaptive development has led to failure to detect genes that would be constitutively expressed in all biofilms, apart from the regulation of adhesins and EPS-encoding genes (e.g., reviewed in [71]).

The final step in the biofilm lifecycle is dispersion or disaggregation. Dispersion is an active process for the removal of cells from the biofilm, unlike detachment, which occurs, for example, as a result of shear stress. Similar to biofilm formation, biofilm dispersion can be induced by various environmental cues, including nutrient, oxygen, and iron availability, nitric oxide, and hydrogen peroxide [72–76]. As mentioned above, QS can trigger dispersion at a high cell density in certain species [60]. The signals leading to dispersion induce the activation of transcriptional regulators, which in turn induce the expression of detachment agents or inhibit positive regulation of biofilm formation [77]. In general, detachment agents are enzymes that degrade the components of the EPS matrix, such as proteases, nucleases, and polysaccharide-degrading glycoside hydrolase and alginate lyase [78–83]. Low levels of c-di-GMP have also been observed in dispersed cells [75]. Dispersed cells are phenotypically diverse and exhibit properties of both biofilm and planktonic cells [84]. Notably, they have an enhanced ability to form biofilms compared to planktonic cells, which can ease colonization of new habitats [84].

1.1.2 Biofilm heterogeneity

Biofilms are naturally heterogeneous (reviewed in [85]). In multispecies biofilms, species are distributed in different locations based on their optimal oxygen, nutrient and light preferences. However, heterogeneity is not limited to multispecies biofilms, as single-species biofilms may consist of diverse subpopulations [86]. In general, heterogeneity results from chemical gradients in a mature biofilm. Oxygen, nutrients, metabolites, and signal molecules diffuse in different concentrations within the biofilm, creating various microenvironments [87]. Biofilm cells adapt to gradients by regulating gene expression, which leads to physiological heterogeneity,

even at the micrometer scale. In addition to physiological heterogeneity, genetic alterations, such as mutations, genetic recombination and random variations in gene expression can create genetically diverse subpopulations within the biofilm [88–91].

Transcriptional analyses performed with monospecies *P. aeruginosa* biofilms have revealed that the metabolic activity and gene expression vary between the top (in the air interface) and bottom (surface-adhered) parts of the biofilm [92,93]. In general, the top part of the biofilm is more metabolically active. Expression of genes involved in cell growth and division, stress responses, or belonging to the QS and *rpoS* regulons is higher in the top than in the bottom part of the biofilm. The bottom cells also have high levels of ribosome hibernation factors, although the ribosomal RNA (rRNA) levels are consistent throughout the biofilm. Thus, it has been suggested that while cells in the top part of the biofilm are metabolically active and dividing, the bottom cells are already dormant and metabolically inactive [93]. Moreover, dormant cells have increased tolerance toward antimicrobials [93].

1.1.3 Biofilms in disease

Biofilms are associated with approximately 80% of bacterial infections in humans [94,95] and are found in various infection sites in the body, from wounds to the brain and heart [96]. Common biofilm infections include lung infections caused by *P. aeruginosa* in cystic fibrosis patients, and chronic otitis media (middle ear infection) in children. Moreover, infections of the heart valves (endocarditis) are associated with biofilms [97]. Biofilms can also be formed on medical devices, such as catheters, and prostheses [98]. Benign, commensal biofilms also inhabit the human body, for example, dental plaque and intestinal gut microbiotas along with other mucosal microbiotas. However, commensal biofilms may become pathogenic after the introduction of certain pathogenic species or increased growth of opportunistic pathogens [99,100]. This is the case, for example, in the oral infections caries and periodontitis, of which the latter will be discussed in chapter 1.4.1.

It is not unusual for biofilm-associated infections to become chronic and recurring because the EPS matrix provides protection against the human immune system and antimicrobial drugs [96,101]. The host immune system recognizes bacterial cells by pathogen-associated molecular patterns (PAMPs) that are present on the bacterial surface, such as lipopolysaccharide (LPS), peptidoglycan, membrane proteins, and flagellin [102]. Biofilms prevent efficient PAMP recognition because the PAMPs are covered by the EPS matrix, rather than being exposed. However, the immune system can recognize certain components of the EPS matrix, such as exopolysaccharides and eDNA, which have been aptly named biofilm-associated molecular patterns [103]. Certain EPS matrix components, such as the exopolysaccharides Psl (encoded by the polysaccharide synthesis locus) and alginate

of *P. aeruginosa* and poly-*N*-acetylglucosamine (PNAG) of *Staphylococcus epidermidis*, can also provide protection against opsonization, complement-mediated killing, and macrophage killing [104–107]. Moreover, the size of bacterial aggregates in a biofilm prevents phagocytosis, because polymorphonuclear leukocytes (PMNs) are unable to engulf particles larger than their own size (10 μm) [108]. Although recognition of biofilms activates the host immune system, leukocytes are often not capable of completely removing cells, which leads to the development of persister cells that can cause recurring symptoms [109].

Biofilm cells are up to 100–1,000 times more tolerant to certain antibiotics, antiseptics, and biocides than their planktonic counterparts (reviewed in [110]). The biofilm age and cell density positively contribute to antimicrobial tolerance, as older, thicker biofilms are more tolerant to antimicrobials than young biofilms [110]. The EPS matrix hinders the penetration of antimicrobial drugs into the biofilm by binding or inactivating them [111,112]. The slow diffusion rate allows bacterial cells in the biofilm to adapt to the changing environment by activating specific gene expression [108]. Owing to biofilm heterogeneity, a subpopulation of biofilm cells may have slower metabolism, which increases tolerance [113]. Spontaneous or stress-induced mutations can also occur in biofilm cells. The varying antibiotic concentrations within the biofilm allow the emergence of both high- and low-level resistant mutants, which results in a higher overall number of resistant cells in biofilms than in planktonic cultures [114]. Moreover, antibiotic resistance genes efficiently spread by horizontal gene transfer (HGT) in high-cell-density biofilms [115].

Treatment with antibiotics alone rarely leads to the complete eradication of biofilms. Early and aggressive antibiotic treatment could be effective against biofilm infections; however, infections are usually observed only after the biofilm has matured, and antibiotic treatment would be inadequate. Recommended treatments focus on mechanical removal of the biofilm mass along with antimicrobial therapy. However, novel approaches to treating biofilm infections have been developed. The polysaccharide matrix and bacterial surface fibers, amyloids, have emerged as novel drug targets to combat biofilm infections. Moreover, targeting QS and c-di-GMP signaling could induce biofilm dispersion and ease its removal, while antimicrobial or repelling coatings on medical devices could prevent biofilm formation. (Reviewed in [116,117].)

1.2 DNA uptake and natural transformation in bacteria

As mentioned in the previous chapter, antibiotic resistance genes can spread easily in biofilms owing to the high cell density. These genes are distributed via HGT mechanisms, which include conjugation, transduction, and transformation, although

genetic material can also be transferred via nanotubes and (outer) membrane vesicles (reviewed in [118]). Unlike the other mechanisms, transformation does not require cell–cell contact or transfer of the genetic material via phages or membrane vesicles. Instead, cells that are capable of transformation, *i.e.*, naturally competent, can bind and internalize free DNA from their environment. The new genomic material can then be integrated into the cell genome via homologous recombination. This requires sequence homology between the new genetic material and genomic DNA. In addition to acquiring genetic material for evolution or to repair damage, internalized DNA can be used as a nutrient. The consequences of DNA uptake depend on the sequence homology between the internalized and genomic DNA, the presence of DNA damage in the genome, and the nutritional status of the cell [119].

Currently, approximately 80 bacterial species are known to be naturally competent for transformation [120]. These species comprise both gram-positive and gram-negative bacteria, as well as pathogenic and nonpathogenic species. However, not all strains within one species are necessarily competent [121]. Competence is rarely a consistent trait, but it is regulated during different growth phases. Environmental cues can induce competence or regulate the level of competence in constitutively competent species such as *Neisseria* spp. [122]. DNA damage, starvation, cell–cell communication, or carbon source availability are known to affect competence (reviewed in [123]). Moreover, biofilm growth and QS can enhance the efficiency of transformation [124–127], although natural transformation is not limited to biofilm-forming species.

Even though the current knowledge implies that competence is a relatively rare trait, it is possible that more than 80 known species are capable of natural transformation. Because various environmental cues affect competence, some species might not appear competent in laboratory settings. Genetic analyses show that competence gene homologs are found in various species, which suggests that competence is an ancestral trait [128,129]. However, evolutionary studies have revealed the loss of competence, resulting from the inactivation of some competence genes due to mutations [130,131]. Mutations have resulted in the evolution of new, noncompetent strains within a species. For example, within *Aggregatibacter actinomycetemcomitans*, a member of the family *Pasteurellaceae*, competent strains have become a minority, despite the advantages that competence may present [121].

1.2.1 Mechanism of DNA uptake and transformation in gram-negative species

In gram-negative species the uptake of DNA occurs in two phases, as the nucleic acid must be transported across two membranes (reviewed in [120]) (**Figure 1**). First, the double-stranded DNA (dsDNA) is transported across the outer membrane

to the periplasmic space. The uptake of DNA from the environment is mediated by type IV pili or similar structural elements in all gram-negative species, except the gastric pathogen *Helicobacter pylori* [132]. The polymeric pilus fiber protruding from the outer membrane binds the extracellular dsDNA. The pilus assembly machinery then starts to disassemble the polymer, which results in the retraction of the pilus. The retracting pilus pulls the bound dsDNA into the periplasmic space through an outer membrane secretin channel. Pilus retraction has been recognized as an essential mechanism in DNA uptake [133].

From the periplasmic space, one strand of DNA is transported across the inner membrane to the cytosol. A periplasmic DNA-binding protein likely guides the dsDNA to an inner membrane transport protein, which translocates one strand to the cytosol. The other strand is degraded by a nuclease. The translocated single-stranded DNA (ssDNA) is protected from degradation by cytosolic ssDNA-binding proteins. Finally, a recombinase catalyzes homologous recombination, provided that the ssDNA shares sequence homology with the genomic DNA.

A type IV or structurally similar pilus is almost invariably required for DNA uptake [134]. Studies with various species have shown that mutant strains that are devoid of pilin proteins or pilus assembly proteins are not capable of DNA uptake and are thus noncompetent [133,135–138]. However, competence does not always correlate with a piliated phenotype, as certain noncompetent mutant strains express long pili [135,139]. Moreover, some competent cells do not exhibit a piliated phenotype, yet expression of the pilin protein is required for DNA uptake [140]. Therefore, it has been proposed that DNA uptake could be mediated by a shorter pilus structure, similar to that of pseudopili of gram-positive species [141–143]. Although invisible to transmission electron microscopy, shorter pili can span the outer membrane and bind eDNA that is in the close vicinity of the cell membrane.

1.2.2 DNA uptake specificity

The majority of competent species take up any DNA from the environment. However, certain species are fastidious about the source of DNA. *Neisseriaceae* and *Pasteurellaceae* family members have a strong preference for DNA that originates from the same or closely related species. DNA preference is based on a specific short sequence that is abundant in the species genome. The *Neisseriaceae* DNA uptake sequence (DUS) has been identified to be 10 or 12 nucleotides long (ATGCCGTCTGAA) [144,145], although several variants of the DUS have been found in *Neisseriaceae* species [146]. In *Pasteurellaceae* the corresponding uptake signal sequence (USS) was originally identified in *Haemophilus influenzae* [147]. However, later, two different USSs, both 9 nucleotides long, were identified within *Pasteurellaceae* species: the original *H. influenzae* (*Hin*)-USS (AAGTGCGGT) and

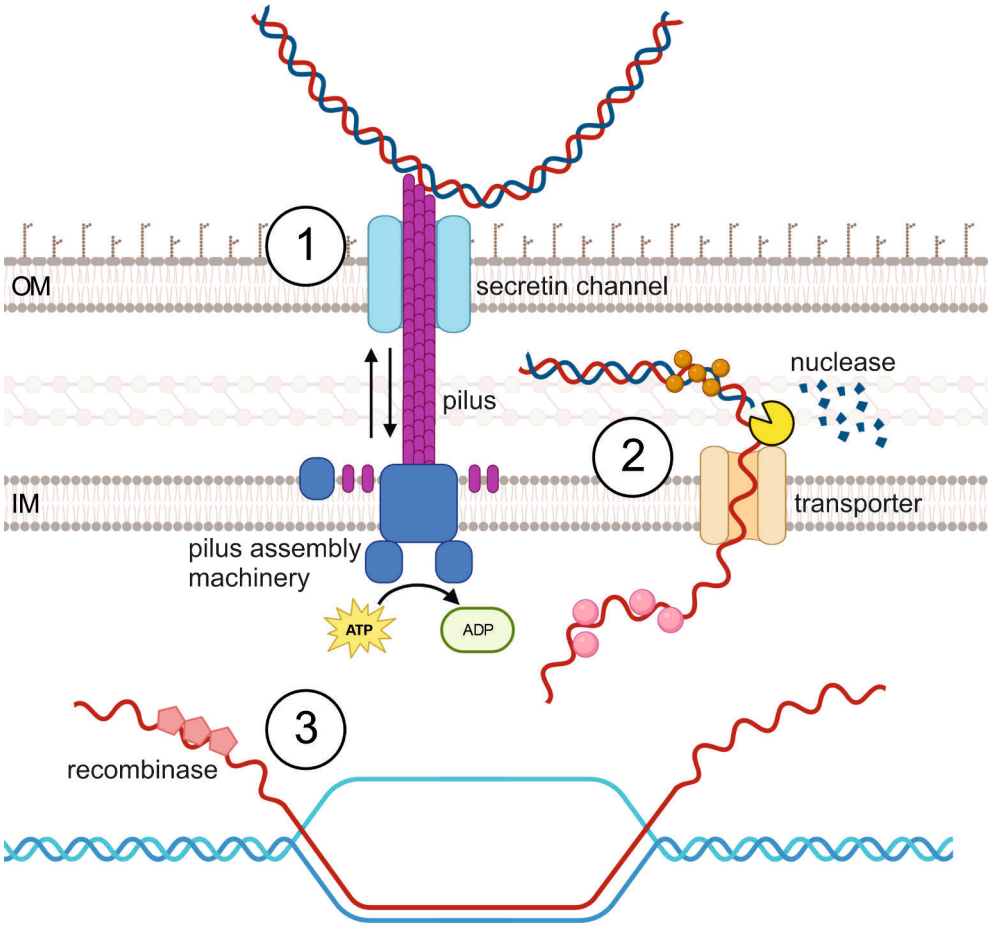

Figure 1. **Mechanism of DNA uptake, followed by natural transformation, in gram-negative bacteria.** 1) Extracellular dsDNA is bound by a type IV pilus fiber or a similar structural element that protrudes from the outer membrane (OM) through a secretin channel. Retraction of the pilus pulls the bound DNA into the periplasmic space. Assembly and disassembly of the pilus polymer is mediated by the pilus assembly machinery in the inner membrane (IM) of bacteria. 2) Cytosolic DNA-binding proteins direct dsDNA to an inner membrane transport protein. One strand of the DNA is translocated into the cytosol and protected from degradation by ssDNA-binding proteins. The other strand is degraded by a nuclease. 3) The homologous ssDNA strand is integrated into the bacterial genome via homologous recombination. (Created with BioRender.com.)

the *Actinobacillus pleuropneumoniae* (*Apl*)-USS (ACAAGCGGT) [129]. The frequency of the DUS in the *Neisseria meningitidis* genome and the USS in the *H. influenzae* genome is on average 1/kb [148]. Thus, collectively, approximately 1% of the whole genome is composed of the uptake sequence.

The biased DNA uptake suggests that the uptake machinery recognizes the uptake sequence. The recognition mechanism of the USS by members of *Pasteurellaceae* is unclear, but the minor pilus protein ComP of *N. meningitidis* has been shown to bind DUS-containing DNA with a higher affinity than non-DUS-DNA [149,150]. The specific recognition of the DUS was later suggested to depend on conserved structural features [151]. Instead, nonspecific DNA binding by type IV pilins occurs via electrostatic interactions between the electropositive surface of the pilin and the negatively charged DNA molecule [150,152].

1.3 Bacterial virulence

Currently, approximately 1,500 bacterial species have been recognized as human pathogens, accounting for approximately 7% of all known species. However, new pathogenic species are discovered at a rapid pace [153]. The high number of pathogenic species reflects the high diversity among pathogens. Bacterial infections can vary from mild to life threatening, and the prevalence of a species in human infections can vary from common to rare. Additionally, not all pathogens are obligate, that is, invariably cause infections, as many pathogenic species are opportunistic. Opportunistic pathogens act as commensals, *i.e.*, they do not cause any symptoms until they sense certain changes in the host. For example, immunocompromised individuals are more vulnerable to infections caused by opportunistic pathogens. Moreover, interactions between bacterial cells within or between species often promote virulence [154,155].

Pathogenic bacteria produce several virulence factors that aid in colonization, invasion, and immune evasion. Adhesive membrane compounds promote attachment to host cells [156], and secreted toxins damage host cells [157]. Moreover, pathogens have developed virulence properties that allow them to manipulate or evade the host immune response. Invasion of host cells protects pathogens from immune defense cells, and interference with cytokine signaling hinders the immune response [158]. Biofilm formation and HGT contribute to virulence, as they increase fitness and promote survival of bacterial cells [2,159].

Factors that increase survival under environmental stresses, such as antimicrobial resistance and tolerance to temperature changes, can enhance virulence [160,161]. A less studied protein group, late embryogenesis abundant (LEA) proteins, possibly contributes to bacterial survival. LEA proteins have been mainly characterized in plants, where they provide protection against dehydration and freezing [162]. However, LEA proteins have also been found in bacteria, among which they are likely distributed via HGT [163]. The known bacterial LEA proteins are involved in the resistance against environmental stresses, such as cold, desiccation, and oxidation [164–170].

1.3.1 Outer membrane components and pathogenicity

Outer membrane components of gram-negative bacteria are often associated with virulence, especially when they are released in outer membrane vesicles (OMVs) [171]. Outer membrane proteins (OMPs), lipoproteins, and LPS can interact with host cells, act as proinflammatory agents, and interfere with host defense. Bacterial outer membrane components often act as PAMPs that are recognized by human immune cells. Recognition of pathogens leads to the activation of the immune response, including secretion of cytokines and oxidative compounds, and the activation of phagocytic leukocytes (reviewed in [102,172]).

LPS is an abundant outer membrane component in gram-negative bacteria. It consists of a lipid core that forms the outer monolayer of the outer lipid bilayer and a long, heterogeneous polysaccharide. The polysaccharide moieties of LPS interact with each other, creating a protective barrier against hydrophilic molecules and antimicrobial compounds [173,174]. LPS is highly immunogenic. It induces a strong immune response in human leukocytes, including the secretion of proinflammatory cytokines and other immunomodulatory compounds [175]. Interestingly, LPS has been shown to bind the chemokine interleukin (IL)-8 [176].

OMPs of several gram-negative species can bind human cytokines [177–181]. Binding and uptake of cytokines allow bacterial cells to sense the inflammatory state of the host and to adapt to the inflammatory environment. Sensing host immune activation via cytokines can enhance bacterial growth and induce the expression of virulence factors [179,182]. Moreover, internalized cytokines can bind to promoter sites in genomic DNA, which leads to altered gene expression [178].

1.3.2 Type IV pili in virulence

As mentioned in previous chapters, type IV pili are involved in biofilm formation and DNA uptake during natural transformation. However, type IV pili also have various other functions and are thus important virulence factors for many pathogens [183]. Many species possess more than one type IV pilus system, which are involved in different functions [46]. Moreover, different pilin proteins, the subunits that form the polymeric pilus fiber, modify the function of the pilus. For example, *N. meningitidis* ComP is a minor pilin protein that can bind DNA, unlike the major pilin protein PilE [150], which is involved in colonization and cytokine uptake [178,184].

The diverse functions of type IV pili include the adherence, twitching motility, microcolony formation, DNA uptake during transformation, DNA exchange by conjugation, and serving as a receptor for bacteriophages (reviewed in [46,183]). Additionally, certain type IV pili are linked to electron transfer, cytokine uptake, and manipulation of the host immune system [178,185,186]. Because pili protrude from the outer membrane of the cell, they can be recognized by the host immune system;

thus, some pilin subunits have been shown to be immunogenic [187–190]. However, some species, such as *Neisseria* spp., can evade immune system recognition by regulating the level of pilus expression (phase variation), altering the sequence of the expressed pili (antigenic variation), or masking the pilin structure by glycosylation [188,191,192].

According to the current classification, type IV pili are divided into type IVa, IVb and IVc/tight adherence (tad) pili based on differences in the amino acid sequence and protein structure; previously, type IVc/tad pili were considered a subgroup of the type IVb group because of their structural similarities [46,193–196]. Moreover, the functions of pili differ between these types. The features of the three groups are presented in **Table 1**.

Table 1. Comparison of the features of type IVa, IVb, and IVc/tad pili.

Group	Polypeptide length*	Signal peptide length*	First residue of a mature pilin*	Functions
IVa	150–160 aa	<10 aa	phenylalanine (methylated)	DNA uptake (transformation) [134], motility [197,198]
IVb	180–200 aa	15–30 aa	variable	adhesion, colonization, microcolony formation, secretion, DNA uptake (other than transformation) [199]
IVc/tad	40–50 aa	15–30 aa	variable	adhesion, microcolony formation [196,200]

*references: [46,195,199]
aa: amino acid

1.4 The oral pathogen *Aggregatibacter actinomycetemcomitans*

Aggregatibacter actinomycetemcomitans, which was previously known as *Bacterium actinomycetem comitans* and *Actinobacillus (Haemophilus) actinomycetemcomitans*, is the type species of the genus *Aggregatibacter*, established in 2006 [201]. The genus name refers to bacteria that aggregate with others, while the name "actinomycetemcomitans", literally meaning "accompanying an actinomycete", was originally derived from commonly observed coinfections with *Actinomyces* species. *A. actinomycetemcomitans* belongs to the class Gammaproteobacteria and family *Pasteurellaceae*, which consists of gram-negative genera, including both commensal and pathogenic genera, such as *Haemophilus*, *Actinobacillus* and *Pasteurella*. [201]

A. actinomycetemcomitans is a small (0.4–0.5 μm×1.0–1.5 μm), nonmotile rod, although in laboratory settings the cells can resemble cocci [202]. Primary cultures form tightly adherent colonies with rough edges; however, subsequent culturing can

lead to the formation of smooth-edged nonadherent colonies that form a uniform suspension in a liquid medium [203]. The phenotypic difference between rough and smooth colonies is caused by the presence or absence of expression, respectively, of bundled fimbriae, which are responsible for nonspecific attachment [196].

A. actinomycetemcomitans is a facultative anaerobe, which allows it to survive under aerobic conditions although it grows best in an anaerobic environment or in ambient air supplemented with 5% CO_2 [202]. The natural habitat of *A. actinomycetemcomitans* is the oral cavity of humans and nonhuman primates [204,205]. It can be transmitted from person to person, most likely through saliva as a transport vehicle [206]. Transmission can occur vertically (parent–child), or horizontally (between spouses or siblings), of which the former is more common [207].

1.4.1 *A. actinomycetemcomitans* in disease

As an opportunistic pathogen, *A. actinomycetemcomitans* can be found in both periodontally healthy individuals and those suffering from periodontal diseases [208,209]. *A. actinomycetemcomitans* is associated with the inflammatory periodontal disease periodontitis. More specifically, the species is linked to a form of periodontitis characterized by a molar-incisor pattern and rapid progression (formerly, localized aggressive periodontitis) [210]. *A. actinomycetemcomitans* was initially thought to cause periodontitis, but later, longitudinal studies challenged this view, as several individuals harboring *A. actinomycetemcomitans* did not develop the disease [211,212]. Nevertheless, *A. actinomycetemcomitans* has an important role in the development of periodontitis by inducing dysbiosis and thus creating a favorable environment for the overgrowth of pathogenic organisms [213].

Periodontitis is caused by a dysbiotic multispecies biofilm, mainly consisting of gram-negative species. In periodontal health, the subgingival biofilm formed on the tooth, also known as a dental plaque, is in homeostatic balance with the host. The homeostatic biofilm mainly consists of commensal species whose growth is regulated by the host immune system. A shift from homeostasis to dysbiosis occurs as the growth of pathogenic species increases and, correspondingly, the number of commensal species decreases, which often results from poor oral hygiene. Moreover, commensal species, such as *Streptococcus gordonii*, can act as accessory pathogens that promote the growth and virulence of pathogenic species such as *A. actinomycetemcomitans* and *Porphyromonas gingivalis*. Inflammation has been found to play an important role in the microbial shift. Inflammation promotes the growth of pathogenic species, which in turn can produce compounds that induce inflammation. This positive reinforcement creates a feedforward loop that can result in chronic periodontitis. Advancement of periodontitis leads to progressive

destruction of the tooth-supporting gingival tissue and alveolar bone, which can ultimately result in the detachment of teeth (**Figure 2A**). [99,214]

In addition to periodontal diseases, *A. actinomycetemcomitans* can spread to other anatomical locations and cause severe infections throughout the body [215]. *A. actinomycetemcomitans* belongs to the HACEK group, which consists of microbes that are a normal part of the oral-pharyngeal microbiota but can cause rare infective endocarditis [216]. The HACEK group includes bacteria from the genera *Haemophilus*, *Aggregatibacter*, *Cardiobacterium*, *Eikenella* and *Kingella*. Less than 2% of infective endocarditis cases are caused by these species [217–219]. *A. actinomycetemcomitans* has also been associated with brain abscesses [220], rheumatoid arthritis [221], pulmonary infections [222], and endophthalmitis [223], among other infections. However, *A. actinomycetemcomitans* strains found in extraoral infections likely originate from the oral cavity and have spread as a result of poor oral hygiene and recurring periodontal infections [220].

1.4.2 Host-microbe interactions in periodontitis

In healthy gingival tissues, leukocytes and cytokines are present in low levels to maintain the homeostasis [99,214,224]. Disruption of homeostasis leads to overactivation of the immune response, which can precede tissue destruction. *A. actinomycetemcomitans* induces the production of proinflammatory cytokines in gingival epithelial cells, fibroblasts, PMNs and peripheral blood mononuclear cells [225–228]. The host response to periodontitis involves increased production of proinflammatory cytokines belonging to the IL-1, IL-6, and tumor necrosis factor (TNF) families, which play a role in leukocyte activation, tissue destruction, and bone resorption [229]. Of these cytokines, IL-6 is a diagnostically accepted salivary biomarker for periodontitis [230]. *A. actinomycetemcomitans* cells and DNA induce the production of IL-6 and TNF-α in mouse macrophages [231]. This immune response is dependent on the Toll-like receptors TLR2 and TLR4, which also mediate the production of IL-8 induced by *A. actinomycetemcomitans* [232]. The host response in periodontitis is summarized in **Figure 2B**.

Accumulation of neutrophil chemoattractant molecules, especially the chemokine IL-8, in the gingival tissues enhances the migration of neutrophils to the inflammation site [233]. Neutrophils release reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, to destroy pathogens. However, excessive ROS production leads to tissue destruction, including the apoptosis of fibroblasts, as the concentration of ROS exceeds the concentration of antioxidant compounds [234].

The released cytokines also induce the production of receptor activator of nuclear factor κB ligand (RANKL) in host cells [214]. Activated T and B lymphocytes and gingival epithelial cells are the primary sources of RANKL in periodontal tissues

[235,236]. Moreover, *A. actinomycetemcomitans* induces the production of RANKL in gingival tissue cells [237]. The excessive production of RANKL disrupts bone homeostasis. RANKL has two receptors: the osteoclast receptor RANK and a soluble decoy receptor osteoprotegerin (OPG). In bone homeostasis, OPG inhibits the RANKL-RANK interaction on pre-osteoclasts, which would otherwise result in the maturation of pre-osteoclasts into bone-degrading osteoclasts. The increased concentration of RANKL raises the RANKL/OPG ratio, which leads to an increased number of mature osteoclasts. The mature osteoclasts break down bone tissue causing the characteristic bone loss in periodontitis [238].

1.4.3 Virulence properties of *A. actinomycetemcomitans*

A. actinomycetemcomitans produces several virulence factors, such as toxins, LPS, OMVs, fimbriae, adhesins, and cytokine-binding proteins (reviewed in [239,240]). Virulence factors contribute to the colonization and biofilm formation, natural competence for transformation, the ability to bind and internalize human cytokines, and the ability to evade the host response. Moreover, the peptidoglycan-associated lipoprotein (PAL) of *A. actinomycetemcomitans* elicits antibody production in the host [241] and stimulates cytokine production by leukocytes similar to LPS and exotoxins [227,228,242,243]. Therefore, *A. actinomycetemcomitans* can promote disease progression by stimulating host cells. The virulence potential varies between strains owing to the great genetic variability of *A. actinomycetemcomitans* [244]. Some important *A. actinomycetemcomitans* virulence characteristics are discussed in more detail in the next paragraphs.

Biofilm formation and tight adherence

In natural settings, *A. actinomycetemcomitans* grows in a multispecies biofilm. Biofilm formation by clinical isolates is also observed *in vitro* [196,245]. Biofilm formation and adhesion to host tissues are essential for the colonization and pathogenesis of *A. actinomycetemcomitans* [246,247]. The matrix of *A. actinomycetemcomitans* biofilms mainly consists of proteinaceous adhesins, eDNA, and the polysaccharide poly-*β*-1,6-*N*-acetyl-d-glucosamine (PGA) [248,249]. Biofilm formation requires the production of AI-2, which is a quorum sensing signal molecule encoded by *luxS* [250]. Dispersion of the *A. actinomycetemcomitans* biofilm is induced by increased expression of dispersin B (*dspB*), which is a glycosyl hydrolase that breaks down PGA [82,251].

Nonspecific adherence of *A. actinomycetemcomitans* requires the expression of type IVc/tad pili, whose major subunit is the fimbrial low-molecular-weight protein (Flp) [196]. A characteristic of Flp pili is their tendency to pack into bundles of

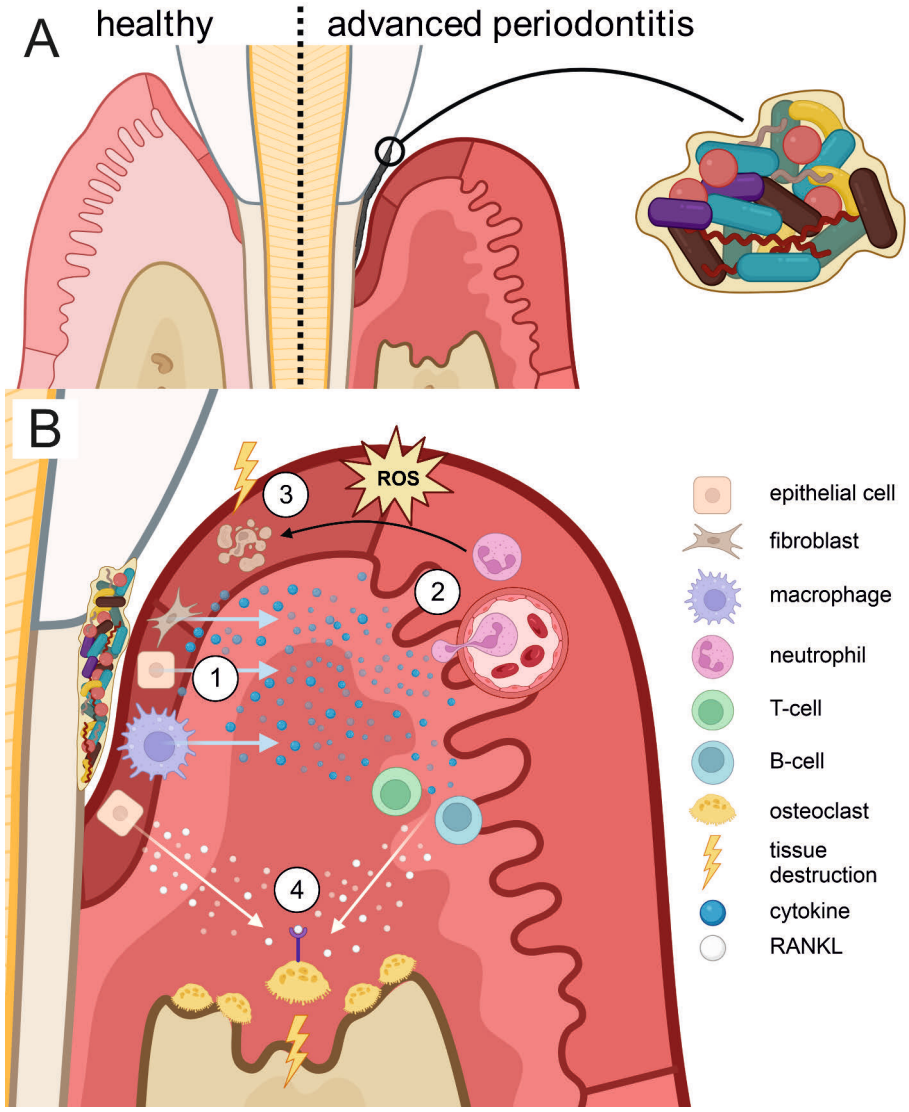

Figure 2. **A. Progression of periodontitis.** In periodontitis, a dysbiotic multispecies biofilm, mainly consisting of gram-negative species forms in the gingival pocket between the tooth and the gingiva. Advanced periodontitis is characterized by the destruction of the gingival tissue and alveolar bone. **B. Host response in periodontitis.** 1) The pathogenic biofilm induces the production of proinflammatory cytokines in gingival epithelial cells, fibroblasts, and macrophages. 2) Accumulation of chemoattractive cytokines enhances the migration of neutrophils from the blood circulation to the inflamed gingival tissue. 3) The activated neutrophils produce reactive oxygen species (ROS) to destroy pathogens. Excessive ROS production leads to tissue destruction and apoptosis of gingival fibroblasts. 4) Cytokines stimulate nuclear factor κB ligand (RANKL) production in host cells. In periodontitis, the main source of RANKL is activated T- and B-cells. Moreover, pathogens stimulate gingival epithelial cells to produce RANKL. Excessive RANKL binds to the RANK receptors on pre-osteoclasts. The mature osteoclasts degrade bone tissue, which leads to bone resorption. (Created with BioRender.com.)

multiple parallel pilus fibers. The genes necessary for Flp pili production and assembly belong to the *tad* (tight adherence) locus in the *A. actinomycetemcomitans* genome. The *tad* locus comprises 14 genes, of which at least 13 are essential for pilin production and assembly [196,252–254]. The major pilin subunit is encoded by *flp-1* [196]. Immediately downstream is a similar gene, *flp-2*, which is dispensable for pilin formation [254]. The pre-pilin peptidase encoded by *tadV* is required for the maturation of the Flp pilin subunits and pseudopilin subunits encoded by *tadE* and *tadF* [255]. Protein products of the genes *rcpCAB*, *tadZABCD* and *tadG* are predicted to form the Flp pilus assembly machinery [200].

Toxins

A. actinomycetemcomitans produces two toxins, which are secreted to the surrounding environment causing damage to host immune cells. Oral microbes rarely produce exotoxins, which makes *A. actinomycetemcomitans* atypical in its niche [256]. The two *A. actinomycetemcomitans* exotoxins are a leukotoxin and a cytolethal distending toxin (CDT). Both contribute to the pathogenesis of periodontal diseases [257,258].

The most intensively studied *A. actinomycetemcomitans* virulence factor is its leukotoxin (reviewed in [259]). It targets a variety of human leukocytes that express lymphocyte function-associated receptor 1 [260]. Leukotoxin can kill PMNs, T cells, natural killer cells, monocytes, and erythrocytes [261–264]. Leukotoxin production can therefore protect *A. actinomycetemcomitans* against PMN-mediated phagocytosis. The concentration of leukotoxin in the bacterial population plays a crucial role, as highly leukotoxic strains are more capable of killing PMNs [265]. Moreover, leukotoxin is highly immunogenic and induces, for example, secretion of the proinflammatory cytokines IL-1β and IL-18 in macrophages [243].

The leukotoxin operon is part of the core genome of *A. actinomycetemcomitans* [266]. The operon consists of four genes, *ltxCABD*, whose protein products are needed for posttranslational acylation and thus activation (*ltxC*) of the toxin (*ltxA*) and its translocation and secretion (*ltxBD*) [267–269]. A 1,000-bp promoter site regulates leukotoxin expression. Isolates containing a specific 530-bp deletion in the leukotoxin promoter site (JP-2 genotype) are highly leukotoxic [270]. Moreover, isolates with an 886-bp insertion or a 640-bp deletion in the leukotoxin promoter site produce more leukotoxin than isolates with an intact promoter site [271,272].

CDT likely plays a role in breaking down the gingival tissue (reviewed in [256]). CDT targets human gingival epithelial cells, gingival fibroblasts, and periodontal ligament cells, as well as a subset of immune defense cells [227,273–277]. The CDT operon comprises three genes, *cdtABC* [278]. Expression of all three protein subunits encoded by the genes is necessary for the production of an active, heterotrimeric

toxin [279]. The cytotoxic subunit CdtB is translocated into target cells, where it exhibits DNase activity. The resulting DNA damage leads to cell cycle arrest, inhibited proliferation, and ultimately, cell death. Moreover, CDT induces cytokine secretion by peripheral blood mononuclear cells, human gingival epithelial cells, and gingival fibroblasts [226,227]. CDT also induces the production of RANKL in gingival tissue cells [237]. Unlike leukotoxin, a complete CDT operon is not found in all *A. actinomycetemocomitans* strains, although it is present in the majority of studied oral isolates [280,281].

LPS

LPS is abundant on the surface of *A. actinomycetemcomitans* cells, which is typical of gram-negative species. Based on the structure of the variable part of LPS, O-antigen, *A. actinomycetemcomitans* strains are divided into seven serotypes (a through g) [282–285]. Some *A. actinomycetemcomitans* strains are, however, nonserotypable [286]. The seven serotypes differ in prevalence and virulence. Serotypes a, b, and c are typically most commonly found among isolates [287]. Serotype b has been associated with aggressive periodontitis and high leukotoxicity, suggesting that it is more virulent than the other serotypes [288,289].

A. actinomycetemcomitans LPS is immunogenic. It stimulates cytokine production in a variety of human cells, such as PMNs, epithelial cells, and dendritic cells [228,290–292], and increases the production of ROS in neutrophils [293]. Notably, the serotype b LPS is more immunogenic than LPSs from other serotypes [292,294]. *A. actinomycetemcomitans* LPS can also potentially modulate the host immune response by binding the proinflammatory cytokine IL-8, which is a chemoattractant for neutrophils [176]. Moreover, LPS increases leukotoxin secretion by *A. actinomycetemcomitans* [295].

Interactions with host cytokines

A. actinomycetemcomitans can bind and internalize the human cytokines IL-1β, IL-6 and IL-8 [296–298]. Biofilms grown in the presence of IL-1β have a decreased metabolic activity [296]. The presence of IL-1β or IL-8 results in significantly lowered amounts of eDNA in the biofilm matrix [298,299]. Moreover, the binding of proinflammatory cytokines can disturb the host immune response and create a more suitable environment for *A. actinomycetemcomitans*.

Some cytokine-binding proteins of *A. actinomycetemcomitans* have been identified. Intracellular ATP synthase subunit β and histone-like DNA-binding protein HU interact with IL-1β [296,297]. Bacterial interleukin receptor I (BilRI) binds various cytokines, such as IL-1β, IL-8, IL-10, INF-γ and TNF-α, and is

involved in the uptake of IL-1β [181,298]. The outer membrane secretin HofQ binds various cytokines, with the strongest affinity to IL-8 [299]. Both HofQ and IL-8 interact with DNA [299,300]. Therefore, a hypothesis was presented that DNA uptake during natural transformation could be linked to cytokine uptake in *A. actinomycetemcomitans*.

1.4.4 Natural competence of *A. actinomycetemcomitans*

It is estimated that approximately 30% of *A. actinomycetemcomitans* strains are naturally competent. Although members of *Pasteurellaceae* have been suggested to have a competent ancestor, the competence of some *A. actinomycetemcomitans* strains has been lost during evolution, leading to the emergence of noncompetent strains [129,130]. Competence has been mainly associated with serotype a, d and e strains, while serotype b and c strains are considered noncompetent [121]. Similar to other *Pasteurellaceae*, competent *A. actinomycetemcomitans* strains preferentially internalize DNA originating from closely related species. The genome of *A. actinomycetemcomitans* contains repeats of the *Hin*-type USS (AAGTGCGGT). The presence of the *Hin*-USS greatly enhances the transformation efficiency, regardless of the overall sequence of the transforming DNA [301].

Natural competence is mediated by various genes, and mutations in any of them can cause the loss of competence [130]. Direct involvement of the regulatory gene *tfoX (sxy)*, type IV pilus-resembling cluster *pilABCD*, and DNA transformation-related *urpA* in the competence of *A. actinomycetemcomitans* has been shown experimentally [135,302,303]. However, based on the competence regulon of *H. influenzae* [304], it is very likely that naturally competent strains of *A. actinomycetemcomitans* also require the DNA uptake-related genes *comABCDE* (of which *comE* is also known as *hofQ* [300]), *comEAFE1*, and *rec2*, and the transformation-related gene *comM* [129,130]. Moreover, the expression of the regulatory gene *tfoX (sxy)* is affected by the *pga* gene cluster, which also contributes to biofilm formation [305]. The competence of *A. actinomycetemcomitans* can be induced by cyclic AMP (cAMP), suggesting that it could be regulated by catabolic repression [301]. However, the expression of fimbriae encoded by the *tad* cluster is not linked to competence, as nontransformable strains, including the nontransformable deletion mutant strain Δ*pilA::spe*^{r}, exhibit a fimbriated phenotype [135,301]. Additionally, a nonfimbriated mutant of a competent strain has been shown to retain competence [301].

2 Aims of the Study

The general aim of this study was to characterize the cytokine- and DNA-binding outer membrane proteins of naturally competent strains of the oral opportunistic pathogen *A. actinomycetemcomitans*. Furthermore, this study aimed to investigate the roles of these proteins in host-microbe interactions. Based on previous studies, the hypotheses of this study were that the DNA uptake machinery participated in cytokine uptake, that the type IV pilin homolog PilA was a potential virulence factor and that cytokine uptake led to altered gene expression in *A. actinomycetemcomitans*.

The specific aims of this study were as follows:

1. To investigate the roles of the outer membrane lipoprotein BilRI and secretin HofQ in natural transformation.
2. To investigate the link between DNA uptake and cytokine uptake.
3. To investigate the virulence and immunogenic properties of the outer membrane lipoprotein BilRI and the type IV pilin homolog PilA.
4. To study the effects of cytokines and inhibition of DNA uptake on gene expression in *A. actinomycetemcomitans*.

3 Materials and Methods

3.1 Bacterial strains and growth conditions (I–III)

The bacterial strains used in this study included clinical *A. actinomycetemcomitans* isolates, mutants derived from the clinical *A. actinomycetemcomitans* strain D7S, and commercial *E. coli* strains XL1-Blue (Stratagene) and BL21-CodonPlus (DE3)-RIL (Stratagene).

All *A. actinomycetemcomitans* strains used are listed in **Table 2** along with their serotypes and colony morphology. The most commonly used *A. actinomycetemcomitans* strains were D7S and its derivatives, of which Δ*bilRI*, Δ*bilRI::spe*^{r} and Δ*hofQ* were constructed previously by our research group [298,299]. Construction of the D7S Δ*bilRI::dsred* strain is described in detail in chapter 3.1.1 and in original publication I. Prof. Casey Chen (University of Southern California, Los Angeles, CA, USA), Prof. Emerita Sirkka Asikainen (Umeå University, Sweden), and Docent Rolf Claesson (Umeå University, Sweden) are thanked for kindly providing *A. actinomycetemcomitans* strains.

A. actinomycetemcomitans strains were stored at -80 °C in skim milk or a culture medium containing 20% glycerol. Cells were revived by culturing for 3–4 days on tryptic soy agar supplemented with either sheep blood (TSA-B: 3.7% tryptic soy agar (TSA), 0.3% agar, 5% defibrinated sheep blood) or horse serum (TSA-HS: 3% tryptic soy broth (TSB), 0.3% yeast extract, 1.5% agar, 5% heat-inactivated horse serum). Plate-grown *A. actinomycetemcomitans* cells were collected into the culture medium or a buffer solution. The number of *A. actinomycetemcomitans* cells was determined based on the optical density (OD) at 600 nm measured in a homogeneous cell suspension (OD_{600} of 1 is equal to 1×10^9 *A. actinomycetemcomitans* cells per milliliter [205]). For biofilm cultures, desired numbers of cells were transferred into 50-ml cell culture flasks (Cellstar®, Greiner bio-one, Germany) in 5 ml of culture medium or, on a smaller scale, into wells of a 96-well plate in 200 µl of medium. Unless otherwise mentioned, *A. actinomycetemcomitans* was grown in a candle jar at 37 °C.

Table 2. ***A. actinomycetemcomitans*** **strains used in this study.**

strain	serotype	colony morphology	used in	reference
D7S	a	rough	I, II, III	[301,306]
D7S Δ*bilRI*	a	rough	I, III	[298]
D7S Δ*bilRI::dsred*	a	rough	I	I
D7S Δ*bilRI::spe*^{r}	a	rough	I	[298]
D7S Δ*flp1-flp2::spe*^{r}	a	smooth	I	[307]
D7S Δ*hofQ*	a	rough	III	[299]
D7S Δ*pilA::spe*^{r}	a	rough	II, III	[135]
D11S	c	rough	II	[308]
NCTC9710	c	smooth	I	(ATCC 33384)
SA492	d	smooth	I	[309]
S23A	b	smooth	I	[310]
Tr.GU 17-4	f	smooth	I	[286]
173	e	smooth	I	[281]

3.1.1 Construction of the *A. actinomycetemcomitans* D7S Δ*bilRI::dsred* mutant strain (I)

The *A. actinomycetemcomitans* D7S Δ*bilRI::dsred* mutant strain was constructed to investigate the activity of the *bilRI* gene promoter region. The *bilRI* gene (D7S_RS10570, RefSeq genome NC_017846.2) was replaced with the synthetic *dsred-M1* gene encoding a red fluorescent protein (RFP) by using a construct comprising the *dsred-M1* gene flanked by DNA sequences upstream and downstream from *bilRI* in the *A. actinomycetemcomitans* D7S genome.

The synthetic *dsred-M1* gene (*dsred*$^{A.a}$) (Eurofins Genomics, Ebersberg, Germany) and the spectinomycin resistance cassette (*spe*^{r}) flanked by *loxP* sites (cloned from the pLox2-Spe^r plasmid [311], a gift from Prof. Casey Chen (University of Southern California, Los Angeles, CA, USA)) were inserted into the pUC19 plasmid [312] to create a *dsred*$^{A.a}$*_spe*^{r} construct. The upstream and downstream regions of *bilRI* were amplified from the *A. actinomycetemcomitans* D7S genome by polymerase chain reaction (PCR). The *dsred*$^{A.a}$*_spe*^{r} construct digested from the pUC19 plasmid was ligated between the PCR-amplified sequences to create a linear DNA construct. The construct was transformed into *A.*

actinomycetemcomitans D7S using a natural transformation assay, as described in [301]. The replacement of *bilRI* by *dsred*$^{A.a}$*_spe*^{r} in the transformed *A. actinomycetemcomitans* cells was confirmed by colony PCR. The *spe*^{r} cassette was then removed using a site-specific Cre/*loxP* gene deletion system [311,313] to create a markerless Δ*bilRI::dsred*$^{A.a}$ strain. Colony PCR was performed to confirm the deletion of *bilRI*, the insertion of *dsred*$^{A.a}$, and the removal of *spe*^{r}.

3.2 Cloning, expression, and purification of recombinant proteins (I, II)

All recombinant proteins produced in this study are listed in **Table 3** Genes encoding BilRI and the extramembranous part of HofQ (emHofQ) were previously amplified from the genome of *A. actinomycetemcomitans* strain D7S [181,300]. Genes encoding IL-8, PilA (all variants) and DsRed-M1 were ordered as synthetic genes with codons optimized for *E. coli* (Eurofins Genomics).

The genes were cloned and inserted into pET15b expression vector for N-terminal 6×His-tag protein expression or pET36b expression vector for C-terminal 8×His-tag protein expression. The pET15b expression vector contains a thrombin cleavage site next to the His-tag site, which was used to produce proteins without a His-tag. Cloning was performed with FastDigest™ (Thermo Scientific) restriction enzymes and T4 DNA ligase and verified by sequencing (Eurofins Genomics). Verified expression constructs were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL for protein production.

The *E. coli* BL21-Codon-Plus-DE3(RIL) cells containing the expression construct were grown in Terrific Broth (12 g/l tryptone, 24 g/l yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) supplemented with the required antibiotics or, for DsRed production, in 2xTY (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) supplemented with the required antibiotics. Protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at OD_{600}=1.0–2.2. After induction, bacterial cultures were grown at 37 °C for 2.5–4 h, at 30 °C for 16–18 h, or at 16 °C for 16–18 h (**Table 3**). Cells were harvested by centrifugation (6,400×g, 10 min, 4 °C) and stored at -20 °C. The production and purification of ^{15}N,^{13}C-labeled BilRI is described in detail in [314].

To purify the recombinant proteins, cells were resuspended in Buffer A (20 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole, pH 7.5–8.0) supplemented with 10 mM $MgCl_2$, 1 µg/ml DNase I and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by ultrasound sonication (10 µM amplitude, 4×15–30 s), and soluble proteins were separated from the cell debris by centrifugation (48,000×g, 30 min, 4 °C). Except DsRed, the recombinant proteins were purified by Ni^{2+} affinity chromatography, followed by size exclusion chromatography. The supernatant

containing a soluble protein was loaded onto a 5-ml Hi-trap column (GE Healthcare) charged with Ni^{2+}, and the matrix was rinsed with Buffer A. When needed, the His-tag was cleaved with 200 U of thrombin (in 3 ml of Buffer A) overnight at room temperature. The His-tagged proteins were eluted by increasing the imidazole concentration whereas the thrombin-digested proteins were eluted with Buffer A. Elution fractions containing protein (detected by absorbance at 280 nm) were loaded onto a gel filtration column (HiLoad 26/600 Superdex 200 pg, GE Healthcare) balanced with phosphate-buffered saline (PBS: 2.7 mM KCl, 1.8 mM K_2HPO_4, 140 mM NaCl, 10 mM Na_2HPO_4, pH 7.4). The fractions containing the desired protein, as well as the size and purity of the recombinant proteins, were confirmed by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), and the protein concentrations were measured using a method described by Lowry et al. [315]. The purified proteins were stored at -80 °C in aliquots.

DsRed was purified by Co^{2+} affinity chromatography. A PD-10 gravity flow column was filled with 3 ml of the TALON Super Flow cobalt matrix (GE Healthcare) balanced with Buffer A. A filtered cell lysate was loaded onto the column and the matrix was rinsed with Buffer A. The N-terminal 6×His-tag was cleaved with thrombin as described above before elution of DsRed. Fractions containing DsRed could be determined visually based on the visible red color.

Table 3. Recombinant proteins used in this study.

protein	tag	residues	production temperature	used in	production described in
BilRI	-	21–181	37 °C	I	I, [176]
^{15}N,^{13}C labeled BilRI	-	21–181	16 °C	I	[314]
emHofQ	- C8-His	27–195 27–195	37 °C 37 °C	II II	[300] [299]
IL-8	- N6-His	28–99 23–99	37 °C 37 °C	II II	[298] [299]
PilAD7S	N6-His	1–134	16 °C	II	II
PilAD7S(Δ1–23)	N6-His	24–134	16 °C	II	II
PilAD7S(Δ1–27)	- N6-His	28–134 28–134	16 °C 16 °C	II II	II II
RFP (DsRed.M1)	-	1–225	30 °C	I	I

3.3 Protein characterization (I, II)

3.3.1 Sequence analysis (I, II)

The NCBI Basic Local Alignment Search Tool (BLAST) [316] was used to search for potentially different natural variants of BilRI and PilA among the completely or partially sequenced *A. actinomycetemcomitans* strains, which exhibit high clonal variability [244]. BLAST was also used to search for LEA proteins homologous to BilRI. Multiple sequence alignments were performed with Clustal Omega [317]. The alignments were edited and/or visualized with the BioEdit sequence alignment editor (Informer Technologies, Inc.).

The amino acid sequences of BilRI (RefSeq: WP_005542451.1) and PilA (GenBank: AAM88344.1) from *A. actinomycetemcomitans* strain D7S were used as templates for the BLAST searches. The searches were performed against *A. actinomycetemcomitans* (taxid: 714) to find natural variants. The sequence of BilRI was searched against all organisms to find LEA proteins homologous to BilRI. To observe similarities between *A. actinomycetemcomitans* PilA and known type IV pili, the sequence of PilAD7S was aligned with the sequences of *N. meningitidis* PilE (Protein Data Bank (PDB): 5KUA), enterohemorrhagic *E. coli* type IV pilin (PDB: 6VG9), *H. influenzae* PilA (GenBank: AAX87353), *Neisseria gonorrhoeae* PilE (UniProtKB: P02974), *P. aeruginosa* PilA (UniProtKB: P02973), *Francisella tularensis* PilE1 (GenBank: CAG45522) and *Dichelobacter nodosus* FimA (GenBank: X52403).

3.3.2 Structural characterization (I, II)

The structure of the intrinsically disordered BilRI was studied with nuclear magnetic resonance (NMR), as described in detail in [314] and original publication I. The predicted 3-dimensional structures of the PilAD7S and PilAD7S(Δ1–27) monomers and the PilAD7S homooligomer were computationally modeled as described in original publication II.

3.3.3 RFP expression in the *A. actinomycetemcomitans* Δ*bilRI::dsred* mutant strain under various growth conditions (I)

The expression of RFP DsRed.M1 under the *bilRI* promoter in the *A. actinomycetemcomitans* D7S Δ*bilRI::dsred* mutant strain was used to investigate the *bilRI* promoter activity under different growth conditions. The initial screening was performed on a small scale in 96-well plates (data not shown). The conditions

included the addition of salts (NaCl and KCl), a sugar (sucrose), alcohols (ethanol, glycerol, and sorbitol), or H_2O_2; different growth media, temperatures (17–37 °C), and incubation times (6–72 h); and exposure to drought or oxygen. The fluorescence intensities of the treated biofilms were measured using 544 nm excitation and 595 nm emission filters (Hidex Sense microplate reader, Hidex Oy, Turku, Finland). Based on the initial screening, the assay was scaled up for selected growth conditions.

For larger-scale studies, *A. actinomycetemcomitans* D7S Δ*bilRI::dsred* was grown in modified TSB (mTSB: 30 g/l TSB, 6 g/l yeast extract, 8 g/l glucose) in 50-ml cell culture flasks or on mTSB agar (mTSB + 15 g/l agar). A total of 1.2×10^8 cells per bottle or 1.44×10^8 cells per plate were grown for 24 h, after which the growth conditions were modified. The cells were grown for an additional 72 h and collected in the growth medium (flask cultures) or into mTSB (plate cultures) by scraping. The cells were pelleted, washed with water, and lysed by sonication (10 µm amplitude, 3×10 s). Soluble proteins were separated from the cell debris by centrifugation (16,000×g, 2 min, 4 °C). The samples were concentrated with an Eppendorf Concentrator Plus (Eppendorf AG, Hamburg, Germany), and the protein concentration was measured using a method described by Lowry et al. [315].

Samples containing 50 µg of total protein were run on a native polyacrylamide gel (10% Criterion TGX stain-free protein gel, Bio-Rad) using recombinant DsRed.M1 as a positive control. In-gel fluorescence detection was performed with a ChemiDoc MP imaging system (Bio-Rad) using the Alexa 546 channel. Gel images were analyzed using ImageLab (version 6.0.1, Bio-Rad). Red fluorescence signal intensities were normalized against the control conditions (growth at 37 °C). The assay was repeated 4–7 times for different growth conditions.

3.3.4 Detection of PilA aggregation by western blotting (II)

Formaldehyde crosslinking was used to investigate the aggregation of recombinant PilA proteins. His-tagged PilAD7S, PilAD7S(Δ1–23) or PilAD7S(Δ1–27) (100–200 ng/µl) was incubated with 1% formaldehyde until the reaction was stopped by the addition of the SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 18% glycerol, 0.01% bromophenol blue, 0.1% β-mercaptoethanol), followed by incubation at 100 °C for 5 min. The crosslinked samples were separated by size using SDS–PAGE (12% Criterion™ TGX™ Precast Midi Protein Gel, #5671044, Bio-Rad). Proteins were transferred onto a nitrocellulose membrane using semidry transfer. Excess binding sites on the membrane were blocked with 2.5% bovine serum albumin (BSA) in Tris-buffered saline (TBS: 25 mM Tris, 150 mM NaCl, pH 7.6) supplemented with 0.05% Tween 20 (TBS-T). The following steps were performed protected from light. The bound proteins were detected by incubation

with His-Probe-conjugated horseradish peroxidase (His-Probe HRP, #15165, Thermo Scientific) (1 µg/ml in TBS-T with 2.5% BSA). Unbound His-Probe HRP was washed off, and the membrane was incubated with the HRP substrate for enhanced chemiluminescence (Pierce™ ECL western blotting substrate; #32106, Thermo Scientific) for 1 min. The light reaction was immediately detected by exposing a light-sensitive film to the membrane. The film was developed with Kodak reagents.

3.4 Natural transformation assays (I, III)

The involvement of the *A. actinomycetemcomitans* outer membrane proteins BilRI and HofQ in natural transformation following the uptake of eDNA was studied using a natural transformation assay [301]. The assay was performed with the naturally competent *A. actinomycetemcomitans* strain D7S (wild type), the markerless deletion mutant strains D7S Δ*bilRI* and D7S Δ*hofQ*, and the deletion mutant strain Δ*bilRI::spe*^{r}, in which the *bilRI* gene was substituted with a spectinomycin resistance cassette.

A linear, blunt-end DNA construct consisting of an antibiotic resistance cassette flanked by DNA sequences complementary to the *A. actinomycetemcomitans* D7S genome was constructed for this assay. The spectinomycin resistance cassette (*spe*^{r}) digested from the pLox2-Spe plasmid [311], and the kanamycin resistance cassette (*kan*^{r}) digested from the pVT1503 plasmid (from Dr. Jan Oscarsson, University of Umeå, Umeå, Sweden) were used as alternative antibiotic resistance cassettes. Upstream and downstream regions of the *bilRI* gene were amplified by PCR from the *A. actinomycetemcomitans* D7S genome, except the upstream region for the *kan*^{r} construct, which was produced synthetically (Eurofins Genomics). The antibiotic resistance cassettes and the PCR-amplified sequences were cloned and inserted into the pUC19 plasmid [312], which was multiplied in the *E. coli* XL1-Blue strain. The construct was digested from the purified plasmids, and the 5' overhangs were filled with Klenow fragment (#EP0051, Thermo Scientific).

A. actinomycetemcomitans cells grown on TSA-HS plates were collected into mTSB. For each strain, two droplets (5–15 µl) containing 2×10^7 cells each were spotted onto a TSA-HS plate. The plates were incubated for 2 h (lids up) to let the droplets dry and the cells attach to the agar. The linear DNA construct (25 ng of DNA in 5–10 µl of sterile water) was added to each spot and mixed carefully with the cells. The plates were incubated for 5 h to allow the cells to take up the DNA. Cells from the two adjacent spots were collected into mTSB and plated on TSA-HS plates containing either spectinomycin (50 µg/ml) or kanamycin (30 µg/ml), depending on the DNA construct used. The plates were incubated for 3–6 days until the transformant colonies were visible and could be counted.

A negative control was included in each assay as follows: a single spot of each strain was incubated with 5–10 µl of sterile water without DNA and plated on selective agar. The DNA construct containing *spe*^{r} was used for the markerless mutants, and *kan*^{r} was used for the Δ*bilRI::spe*^{r} mutant. The wild-type strain was treated with the same construct as the mutant. The assay was repeated 5–8 times in duplicate or triplicate for each deletion mutant strain. The average number of the mutant strain transformants was compared with that of the wild-type strain transformants to determine the change in the transformation efficiency.

3.5 Molecular interactions (I, II)

3.5.1 Protein-cytokine interactions using an ELISA (II)

Interactions between cytokines and recombinant proteins were initially studied using an enzyme-linked immunosorbent assay (ELISA) performed in 96-well plates. Between each step, the wells were washed three times with distilled water or, after the addition of recombinant proteins, with PBS supplemented with 0.05% Tween 20 (PBS-T). The wells were coated with either IL-8 (produced in house) or TNF-α (ReliaTech, Wolfenbüttel, Germany) diluted to a concentration of 120 µM in PBS with 0.05% sodium azide (PBS-N). BSA (30 µM) was used as a negative control. Excess binding sites were blocked with BSA (0.25% in PBS-T). Recombinant His-tagged PilAD7S(Δ1–27) (2.8–14 µM in PBS) was added, and the bound proteins were detected by the addition of His-Probe HRP (2 µg/ml in PBS-T). HRP was detected by the addition of its substrate (1 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, #A9941, Sigma–Aldrich) in 10 mM sodium citrate buffer (pH 4.2) supplemented with 0.03% H_2O_2), which induced color development. The color change was detected by measuring absorbance at 405 nm after 60 min of incubation. The test was performed five times in triplicate. A positive control (His-tagged emHofQ) and a negative control (no protein) were included in each test.

3.5.2 Protein-cytokine interactions using TRFIA (II)

The interaction between recombinant PilAD7S(Δ1–27) and IL-8 was further studied with a more sensitive time-resolved fluorometric immunoassay (TRFIA). The assay was performed in 96-well plates. The wells were washed with PBS-T three times between each step, unless otherwise mentioned. The wells were coated with 86 pmol of recombinant PilAD7S(Δ1–27) (without His-tag). Excess binding sites were blocked with Alternative Block (#6299, ImmunoChemistry Technologies, Bloomington, MN, USA). Recombinant His-tagged IL-8 (0–9.3 µM in DELFIA

Assay Buffer (#4002-0010, PerkinElmer)) was added, and the bound proteins were detected by the addition of 25 ng of the DELFIA® Eu-N1 anti-6xHis antibody (#AD0108, PerkinElmer) in DELFIA® Assay Buffer. The fluorescent Eu-ion was released from the antibody with DELFIA® Enhancement Solution (#1244-105, PerkinElmer), and the fluorescence signal was measured using a VICTOR3 multilabel plate counter (PerkinElmer) after 5 min. The test was performed five times in triplicate. A positive control (recombinant emHofQ without His-tag) was included in each test. The fluorescence background, which was subtracted from the results, was obtained from wells coated with Alternative Block only. An interplate control (6.1 µM IL-8 in DELFIA® Assay Buffer in noncoated wells) was included in each plate to normalize the obtained fluorescence values between tests.

3.5.3 Protein–DNA interactions using an EMSA (I, II)

Interactions between dsDNA and recombinant BilRI or PilAD7S(Δ1–27) were investigated using an electrophoretic mobility shift assay (EMSA) with a linearized USS plasmid [300] as dsDNA. Each recombinant protein (100 µg; without His-tag) was incubated with dsDNA (300 ng) for 30 min at room temperature. The samples were run on an agarose gel and visualized with the Midori Green Advance stain (#MG04, Nippon Genetics Europe) under UV light. Recombinant emHofQ was used as a positive control, and BSA was used as a negative control.

3.6 Antimicrobial susceptibility test (I)

The effect of the deletion of *bilRI* on the antimicrobial susceptibility of the *A. actinomycetemcomitans* D7S strain was tested using Etest® strips (bioMérieux). The *A. actinomycetemcomitans* D7S wild-type and Δ*bilRI* strains were grown on TSA-B plates and collected into PBS. Turbidity of the bacterial suspensions was adjusted to 1 McFarland and 200 µl of suspension was plated onto *Haemophilus* test medium agar plates (Beckton Dickinson, #254,058). One Etest® strip was added per plate (ampicillin, amoxicillin/clavulanic acid, tetracycline, or doxycycline). After 45–48 h of incubation at 35 °C, the minimum inhibitory concentrations (MICs) were measured. The MIC values were interpreted using the CLSI antimicrobial susceptibility testing breakpoint table (M100, 2017; table for *H. influenzae*). The test was performed 5–9 times.

3.7 Host recognition studies (I, II)

3.7.1 Ethics statement

Venous blood samples were collected from adult volunteers who provided written informed consent. Samples were collected from both *A. actinomycetemcomitans*-positive periodontitis patients and healthy individuals. Individuals who had severe health problems, had been treated with antimicrobials in the previous three months, were on immunosuppressive medication, or were pregnant or breastfeeding were excluded from this study. Permission to collect and use blood samples was obtained from the Ethics Committee of the Hospital District of Southwest Finland.

3.7.2 Human serum, plasma, and leukocyte collection (I, II)

Human venous blood samples were collected by a laboratory nurse at the Community Dental Health Care Center of Turku (Institute of Dentistry, University of Turku) or by a physician at the Unit for Specialized Oral Care in the Helsinki Metropolitan Area and Kirkkonummi (Helsinki, Finland). The presence of *A. actinomycetemcomitans* in subgingival biofilm samples of the patients with periodontitis was confirmed by PCR or culture. The patient samples were collected at the beginning of the periodontal treatment.

Serum/plasma isolation

The sera of the *A. actinomycetemcomitans*-positive (n=21) or healthy (n=14) individuals were isolated from whole-blood samples by centrifugation (1,000×g, 15 min). The sera were stored at -80 °C in aliquots to avoid freeze–thaw cycles. All plasma was collected from one healthy volunteer. Venous blood was collected into ethylenediaminetetraacetic acid (EDTA)-treated tubes to prevent coagulation. The plasma was collected by centrifugation (1,000×g, 10 min) and stored at -20 °C in aliquots to avoid freeze–thaw cycles.

Leukocyte isolation

Leukocytes were isolated from whole-blood samples collected into EDTA-treated tubes from 7 healthy individuals. The isolation was performed as described in [318]. One milliliter of EDTA-anticoagulated blood was mixed with 10 ml of a lysis solution (0.83% NH_4Cl, 0.084% $NaHCO_3$, 0.037% EDTA) to lyse red blood cells. Leukocytes were collected by centrifugation (400×g, 10 min). The leukocyte pellet was resuspended in 1–3 ml of Hank's balanced salt solution (HBSS: 1 mM $CaCl_2$, 5

mM KCl, 0.4 mM KH_2PO_2, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 140 mM NaCl, 4 mM $NaHCO_3$, 6 mM glucose) supplemented with 1 mg/ml gelatin (gHBSS). The leukocytes were used immediately after isolation.

3.7.3 Leukocyte ROS production (I, II)

A. actinomycetemcomitans-induced ROS production in human leukocytes was measured using luminol-enhanced chemiluminescence as described in [318]. The *A. actinomycetemcomitans* D7S wild-type, D7S Δ*bilRI*, D7S Δ*pilA::spe*^{r}, and D11S wild-type strains were grown on TSA-B plates and collected into gHBSS. A suspension containing 1.25×10^9 cells in 100 µl of gHBSS or 100 µl of gHBSS as negative a control was added to a white 96-well plate and mixed with 3 µl of human serum/plasma to opsonize the cells. Luminol was added to a final concentration of 0.4 µM (in 250 µl), and the volume was adjusted to 200 µl with gHBSS. Finally, 50 µl of freshly isolated leukocytes in gHBSS was added to each well. Chemiluminescence signals were recorded every 2 min on a VICTOR3 multilabel plate reader (Perkin Elmer) until the peak signal values were obtained.

When studying ROS production induced by the D7S wild-type and Δ*bilRI* strains, 12 patient sera and 11 control sera were used for opsonization. The leukocytes were isolated from the same healthy volunteer for every individual test. When studying ROS production induced by the D7S wild-type, D7S Δ*pilA::spe*^{r} and D11S wild-type strains, plasma from the same healthy volunteer was used for opsonization, but leukocytes were isolated from a different healthy volunteer every time (n=6–7). All tests were performed in technical triplicates, and the average peak values were compared.

3.7.4 Macrophage stimulation (I, II)

Human macrophages were stimulated with recombinant BilRI or PilAD7S(Δ1–27) or with isolated *A. actinomycetemcomitans* LPS [237] to investigate how these bacterial agents affected macrophage viability and cytokine secretion. THP-1 human acute monocytic leukemia cells (ATCC® TIB-202™) [319] were differentiated into macrophage-like phenotypes by incubating them with 50 nM phorbol 12-myristate 13-acetate (PMA; #5.00582, Sigma–Aldrich) for 24 h. The THP-1 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma) and 100 U/ml penicillin–streptomycin (#P4333, Sigma) at 37 °C under 5% CO_2, unless otherwise stated. The assays were performed 4–5 times in triplicates.

THP-1 monocytes (2×10^5–1×10^6, passages 12–24) were differentiated into macrophages and incubated in a fresh medium for 24 h. The cells were washed with PBS (#D8537, Sigma) and incubated with a medium containing a stimulation agent

(diluted to 0.1 ng/ml in RPMI) for 6, 20 or 24 h. After incubation, the medium was collected and stored at -20 °C. Cytokine concentrations (IL-6, IL-8, and TNF-α) in the medium samples were measured with the Single-Analyte ELISArray kit (Qiagen).

After medium collection, the viability of macrophages was immediately measured using a neutral red uptake assay [320]. The cells were incubated with 100 µl of the neutral red dye diluted to 40 µg/ml in RPMI. After 90 min of incubation, the cells were washed with PBS to remove excess dye, and the absorbed neutral red dye was released from the cells with a lysis solution (EtOH:MQ:acetic acid at 50:49:1 (v/v)). The amount of the released dye was determined by measuring the absorbance at 540 nm.

3.7.5 Screening for specific antibodies in patient sera (I, II)

Patient sera were screened for specific antibodies against *A. actinomycetemcomitans*, BilRI, or PilA using an ELISA. First, 96-well plates were coated with either an *A. actinomycetemcomitans* suspension consisting of equal amounts of serotype a-f strains (D7S *Δflp1-flp2::spe* (a), S23A (b), NCTC9710 (c), SA492 (d), 173 (e), and Tr.GU 17-4 (f)) or recombinant BilRI or PilA (500 ng) in PBS-N. BSA (500 ng) was used as a negative control. Excess binding sites were blocked with a blocking buffer (0.25% BSA in PBS-T). Sera from periodontitis patients or healthy controls were diluted 1/100 (anti-Aa) or 1/20 (anti-BilRI and anti-PilA) in the blocking buffer and added to the wells. After secondary blocking, the bound human immunoglobulin G (IgG) antigens were detected with a peroxidase-coupled Fc-specific anti-human IgG antibody (#A1070, Sigma Aldrich), which was diluted 1/6,000 in PBS-T. Peroxidase was detected by the addition of ABTS (1 mM in 10 mM sodium citrate buffer (pH 4.2) supplemented with 0.03% H_2O_2). The induced color development was measured using a spectrophotometer at 405 nm.

The test was performed in triplicate for 21 patient sera and 13 (anti-Aa and anti-BilRI) or 14 (anti-PilA) control sera. Serum dilutions were selected based on initial tests, which contained several dilutions from 1/20 to 1/2,500. Nonspecific binding to BSA was subtracted from the results.

3.8 Gene expression studies (III)

3.8.1 Biofilm cultures

The *A. actinomycetemcomitans* D7S wild type and deletion mutants Δ*bilRI*, Δ*hofQ*, and Δ*pilA::spe*^{r} were grown on TSA-B plates and collected into mTSB. A total of 0.5×10^9 cells in mTSB were cultured in cell culture flasks for 20 h. The formed

biofilms were rinsed with RPMI (RPMI-1640 medium (#R7509, Sigma) supplemented with 0.6 g/l L-glutamine (#G7513, Sigma)) and incubated in RPMI for 4 h. The medium was then replaced with RPMI supplemented with IL-1β (10 ng/ml, ReliaTech) or an equivalent volume (5 µl) of sterile water. After 2 h of incubation, the cells were scraped off into the RNA*later*® solution (#AM7020, Ambion) and collected by centrifugation (6,000×g, 10 min, 4 °C). The cell pellets were stored in RNA*later* at 4 °C until RNA isolation. The culture was performed three times in duplicate.

3.8.2 RNA isolation

RNA isolation was performed with the RiboPure™ Bacteria kit (#AM1925, Ambion). Cells were first washed with nuclease-free water (#AM9938, Ambion) to remove excess RNA*later*. The cells were resuspended in a phenol reagent and lysed by vortexing with zirconia beads. The beads were removed by centrifugation, and the lysate was mixed with 0.3 volumes of chloroform (without isoamyl alcohol; #C2432, Sigma). After 10 min of incubation the phases were separated by centrifugation, and the top aqueous phase was carefully collected and mixed with ethanol. RNA was further purified with spin-column purification as follows: RNA was bound to a filter and washed three times to remove impurities before being eluted with a low-ionic-strength solution. The purified RNA samples were treated with DNase I (2 h at 37 °C), and the absence of genomic DNA was confirmed by 16S rRNA PCR (forward primer: 5'-GAA GCT AAC GTG ATA AAT CG-3'; reverse primer: 5'-TCG AAT TAA ACC ACA TGC TC-3'). The RNA concentration was measured using a spectrophotometer at 260 nm.

3.8.3 RNAseq and data analysis

Total RNA sequencing (RNAseq), including sample quality checks and library preparation, was performed at the Finnish Functional Genomics Centre (Turku, Finland). Sample quality checks were performed using an Agilent Bioanalyzer 2100. Library preparation was performed according to the library preparation protocol (TruSeq® Stranded Total RNA Reference Guide, Illumina (1000000040498)) with the Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit (Illumina). Paired-end sequencing (read length: 100 bp) was performed using an Illumina NovaSeq 6000 S4 v1.5 instrument.

RNAseq data were analyzed using the CSC Chipster v.4 analysis software [321]. The quality of the raw data was checked with the FastQC-based [322] MultiQC tool [323] and the strandedness of the reads was checked with RseQC [324]. The raw data contained 90–160 million reads per sample. To simplify data analysis, subsets

containing 10 million reads per sample were generated with a seqtk-based Chipster tool [325]. The reads were mapped to the *A. actinomycetemcomitans* D7S-1 genome (RefSeq: NC_017846.2) with Bowtie2 [326], and the aligned reads per gene were counted with HTSeq [327] (assembly ASM16361v3, RefSeq: GCF_000163615.3). DeSeq2 [328] was used for the normalization of counts, differential expression analysis, and principal component analysis. The cutoff for Benjamini–Hochberg adjusted p value was set at 0.05 for significant differential gene expression. The genes whose expression was changed at least 2-fold ($\log_2$ fold change ≥ 1 or ≤ -1) were selected for Gene Ontology (GO) enrichment analysis. Protein identifications (RefSeq Protein ID) were retrieved from NCBI and imported to the UniProt (release 2022-05) Retrieve/ID mapping tool to generate a list of GO terms. The protein IDs were also searched against the Cluster of Orthologous Groups (COG) database.

3.8.4 Validation of the RNAseq results by qPCR

The RNAseq data were validated using a two-step reverse transcription (RT)–quantitative polymerase chain reaction (qPCR) using an iCycler iQ5 real-time PCR system (Bio-Rad). Two housekeeping genes were used as references for measuring the relative expression of six genes that were selected based on the RNAseq results. All primers are listed in **Table 4**. Primer specificities were confirmed by melting curve analyses and/or agarose gel electrophoresis.

Table 4. Primers used for qPCR.

target	forward (5'→3')	reverse (5'→3')
clpX	GAGGTGGCATTGGAATTCAC	AGGATGGCAAGTCGTACATG
gapdh	ATTCAACTCACGGTCGTTTC	CGACTAAGTTGCCGTCTTTC
aspA	CGTGGGACAGGAATTCAAAGC	GTTCAACCCGGTACCAATCG
nrfB	ACCGCAGTTGGACAATCAAC	TGGGTGCCACGGAATTTACC
D7S_RS06105	GCACACAACTTGCCGGATATG	CCTTGTTCTTCTGCCGGTTTC
D7S_RS09470	GGCAGTGCGATCAAGTTCTG	ACTGCCGGTTTCGGTTTAAATG
vgrG	ATCATCTCCCGTGGCATTGG	GATATCAGCGCGACGAACAG
nrdD	ACGACAACGTGGCACAAATC	CGATGTAGCCGAGGGAAATG

The complementary DNA (cDNA) for qPCR was synthesized from 500 ng of RNA with the LunaScript® RT SuperMix kit (New England Biolabs, #E3010). A negative control without reverse transcriptase (no-RT control) was included within each sample. One microliter of cDNA or no-RT control (each diluted 1:5 in nuclease-

free water) was added as a template to the qPCR mixture containing 10 µl of 2x Luna Universal qPCR Master Mix (New England Biolabs, #M3003), 1 µl of both primers (5 µM) and 7 µl of nuclease-free water. The qPCR protocol was as follows: Cycle 1 (1×): 95 °C for 60 s; Cycle 2 (40×): Step 1: 95 °C for 15 s, Step 2: 60 °C for 30 s + plate read; Melting curve: from 60 °C to 95 °C in 0.5 °C increments for 30 s each.

All cDNA samples were run in triplicate and no-RT control samples were run in duplicate. A no template control was included for each primer pair in triplicate. An interplate control containing 5 ng of a cDNA mixture as a template for the *gapdh* primers was included in each plate in triplicate. The average Ct value from the interplate control wells was used to normalize Ct values between plates. Relative gene expression was calculated with the Pfaffl method [329] using the geometric mean of the two housekeeping genes as reference [330,331]. The average Ct value of wild-type samples was used as the control for calculating ΔCt values. Primer efficiencies were calculated from cDNA standard curves created with serial 2-fold dilutions.

The correlation between RNAseq and qPCR was calculated using a linear correlation model (Pearson correlation coefficient). The relative gene expression values compared against the wild type, obtained from qPCR data, were used as is. DeSeq2-normalized counts obtained from the RNAseq data were compared against the average count value of wild-type samples to obtain values comparable to the qPCR results. The linear correlation model was visualized using the ggpubr package in R [332].

3.9 Statistics

Statistical analyses in this study were mainly conducted using nonparametric tests because of small sample sizes (n=3–21). Pairwise analyses were conducted using the Mann–Whitney U test with Bonferroni correction, when necessary. Groups of three or more were analyzed using the Kruskal–Wallis test with pairwise post hoc analysis, when necessary. Correlations between two variables were measured with Pearson correlation coefficient. The P value cutoff for statistical significance was set at 0.05. Statistical analyses were performed using the IBM SPSS Statistics (versions 26 and 27) or R (version 4.1.2) [333] and R Studio (version 2022.12.0.353) [334] software.

4 Results

4.1 Characterization and expression of the outer membrane lipoprotein BilRI (I)

4.1.1 Highly conserved BilRI resembles LEA proteins

The amino acid sequences of BilRI from 81 partially or completely sequenced *A. actinomycetemcomitans* strains (retrieved from GenBank on October 15th, 2020) were compared to find naturally occurring variants. BilRI was present in all the studied strains, and the sequences were, in most cases, over 95% identical (Figure S1; Original publication I). The protein variants could be divided into groups based on amino acid sequence similarities, and genetically closest strains (based on genomic BLAST) expressed most similar variants of BilRI (**Figure 3**). A similar observation was made for the type IV pilin homolog PilA (described in chapter 4.2.1). The majority of amino acid differences between the BilRI variants did not significantly affect side chain properties. Thus, the hydrophobicity or hydrophilicity of the side chain did not drastically change. BilRI is a highly hydrophilic protein, which was confirmed by calculating the grand average of the hydropathicity (GRAVY) index [335]. The GRAVY indices for mature BilRI variants varied between –0.639 and –0.773. The BilRI variant used in this study came from strain D7S and had a GRAVY index of –0.707.

Further BLAST searches showed that *A. actinomycetemcomitans* D7S BilRI was homologous to several bacterial LEA proteins from *Pasteurellaceae* species (**Figure 4**). BilRI was also similar to LEA proteins from various plant species, although their sequences were significantly longer (Figure S3; Original publication I). Most of the plant LEA proteins were either embryogenic cell protein 63 (ECP63) or At3g53040.

All the bacterial LEA proteins as well as the plant proteins ECP63 and At3g53040 belong to the LEA_4 protein family (Pfam: PF02987) [336]. LEA_4 proteins have a characteristic repeating 11-mer motif [162,337] and regularly repeating lysines (K), alanines (A) and aspartic acid/glutamic acid (D/E) [338]. The amino acid sequence of *A. actinomycetemcomitans* D7S BilRI contains 11-mer repeats ending in the repeating KDA motif, which, along with the sequence homology, indicates that BilRI likely belongs to the LEA_4 group.

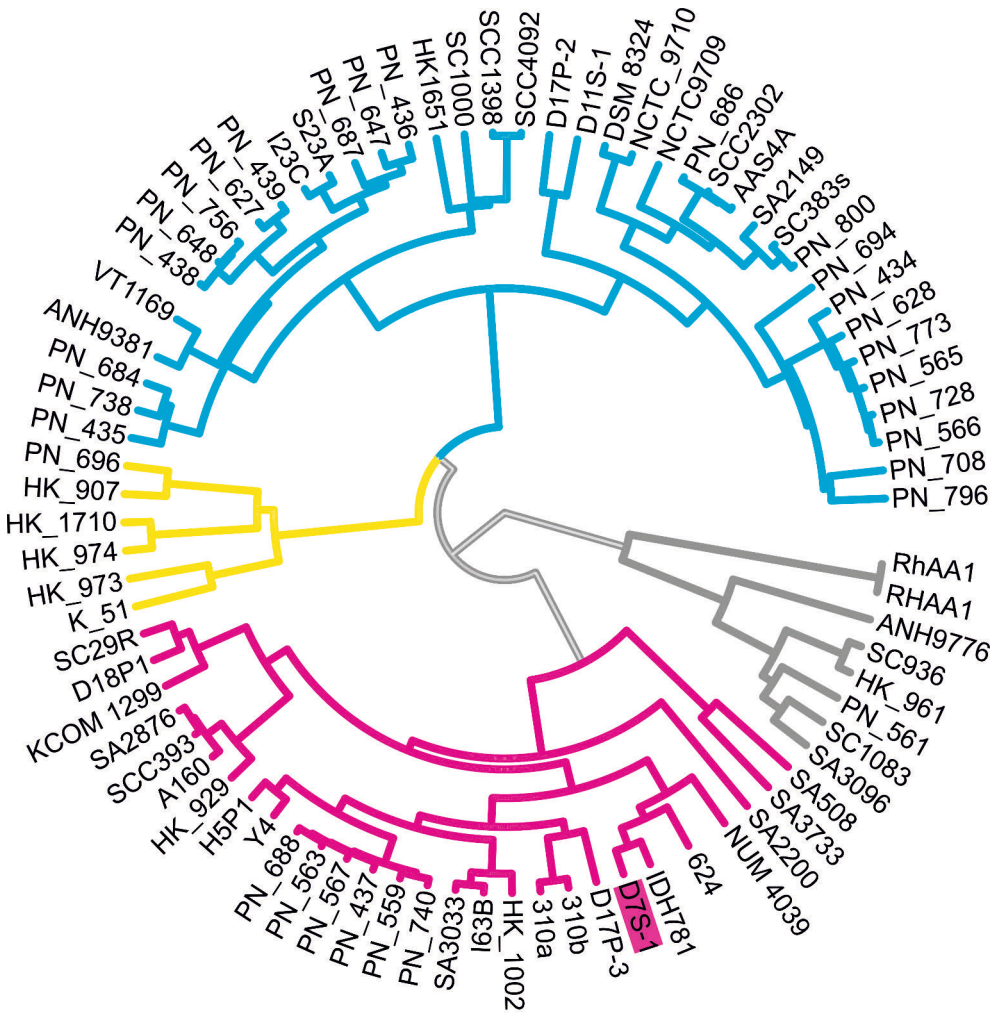

Figure 3. **Distribution of protein variants of BilRI and PilA among *A. actinomycetemcomitans* strains.** Genetically closest *A. actinomycetemcomitans* strains express the most similar BilRI and PilA variants. The identities of the protein variants among the strains of each of the four clades are indicated with respective colors. The most commonly used strain in this study, D7S, is highlighted. The dendrogram was built based on the genomic BLAST results (retrieved from NCBI in October 2020).
*excluding strains ANH9381, SCC1398 and SCC4092
**excluding strain SA3096

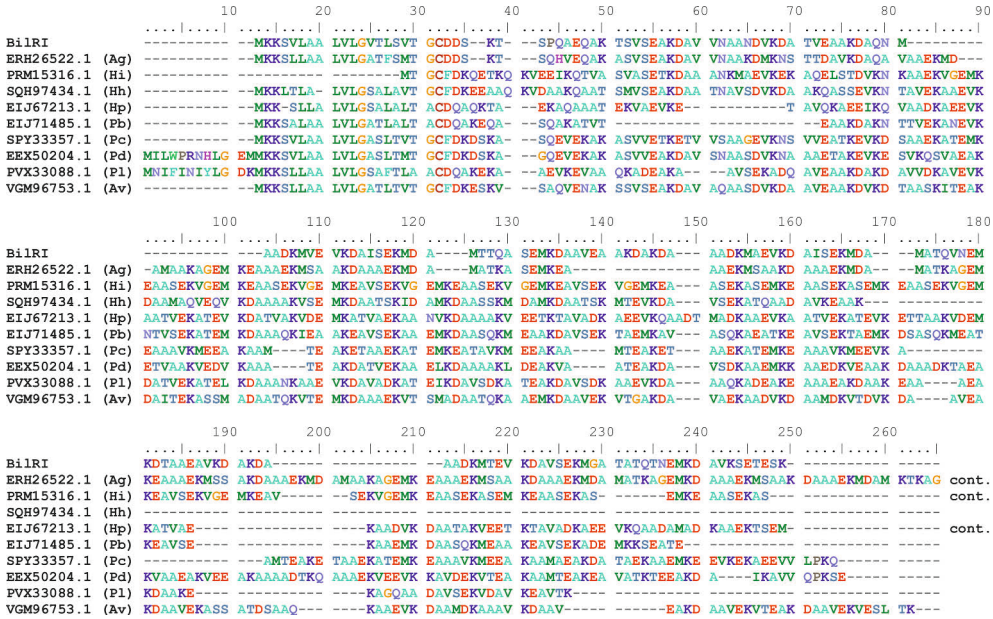

Figure 4. **BilRI is homologous to bacterial LEA proteins.** The amino acid sequence of BilRI from *A. actinomycetemcomitans* strain D7S was aligned with bacterial LEA proteins from gram-negative species of the family *Pasteurellaceae* (Ag: *Aggregatibacter* sp., Hi: *H. influenzae*, Hh: *Haemophilus haemolyticus*, Hp: *Haemophilus parahaemolyticus*, Pb: *Pasteurella bettyae*, Pc: *Pasteurella canis*, Pd: *Pasteurella dagmatis*, Pl: *Pasteurella langaaensis*, Av: *Avibacterium* sp.). (Adapted from Fig. 1; Original publication I.)

4.1.2 Deletion of *bilRI* does not affect antimicrobial susceptibility

The wild-type *A. actinomycetemcomitans* D7S was susceptible to all tested antibiotics, which included beta-lactams (penicillin and amoxicillin/clavulanic acid) and tetracycline-group antibiotics (tetracycline and doxycycline). The Δ*bilRI* strain showed similar susceptibility to all antibiotics as the wild-type strain (Figure 4; Original publication I), which indicates that BilRI does not contribute to the antibiotic susceptibility of *A. actinomycetemcomitans*.

4.1.3 Expression of *bilRI* increases at lower temperatures

The RFP DsRed was used as a reporter protein for *bilRI* expression in the *A. actinomycetemcomitans* D7S *ΔbilRI::dsred* mutant strain. Based on initial screening (data not shown), lowered temperatures were chosen for more thorough studies because they affected DsRed expression the most. Additionally, because BilRI showed similar sequence properties to those of LEA proteins, its expression was studied under conditions mimicking drought; instead of culturing cells in liquid

growth medium in a cell culture flask, some cultures were grown on agar to be exposed to air.

The *bilRI* promoter was activated at temperatures lower than 37 °C (**Figure 5A**). The red fluorescence signal increased 2–3 times when the temperature was lowered to 27 °C (p=0.002). Cells grown at 17 °C expressed slightly, but not significantly, more DsRed than control cultures. There was no significant difference in the amount of DsRed between the plate- and flask-grown *A. actinomycetemcomitans* cultures. The amount of DsRed was calculated based on the red fluorescence intensity (**Figure 5B**). After incubation at 27 °C, approximately 60 ng of DsRed was detected in the sample containing 50 µg of total protein.

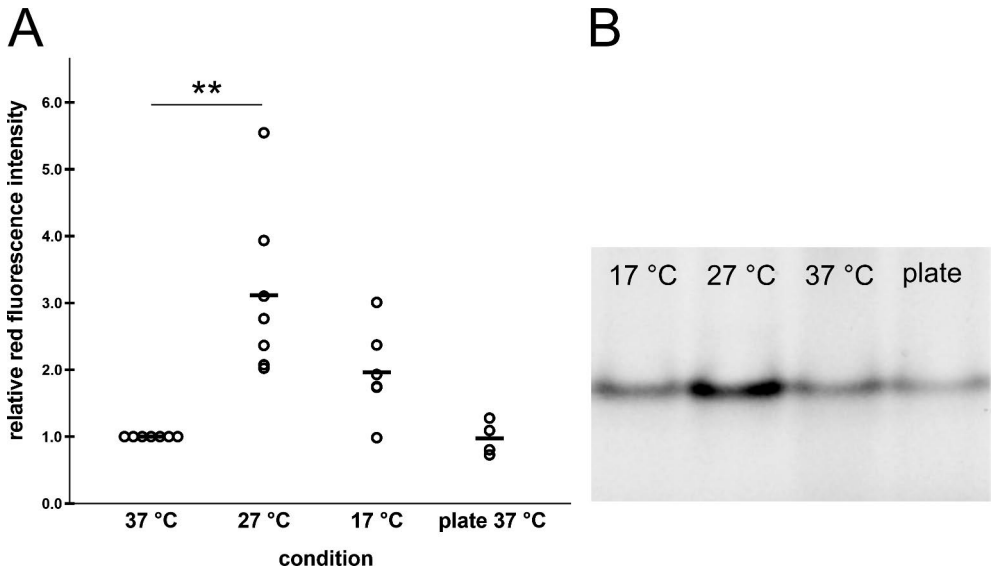

Figure 5. **The *bilRI*-promoter was activated at lowered temperatures. A.** Activity of the *bilRI*-promoter was determined using the red fluorescence protein DsRed as a reporter protein in the *A. actinomycetemcomitans* D7S *ΔbilRI::dsred* mutant strain. Incubation at 27 °C resulted in significantly higher (**, p=0.002) expression of DsRed compared to growth at 37 °C. Plate-grown cells, mimicking growth under dry conditions, expressed similar levels of DsRed as the biofilm culture in a liquid growth medium at the same temperature. Fluorescence intensities were normalized against control cultures (37 °C). The data is presented as the relative red fluorescence intensities from 4–7 individual experiments. The mean relative red fluorescence intensity value for each condition is marked with a horizontal line. **B.** The amount of DsRed in each sample was calculated based on the detected red fluorescence signal. (Adapted from Fig. 6; Original publication I.)

4.1.4 Structural description of BilRI

Previous NMR studies and amino acid sequence analysis have shown that BilRI is an intrinsically disordered protein (IDP) [298]. IDPs lack an ordered 3-dimensional structure, but their binding to a molecular partner can induce various conformational changes [339]. In the current work, the structure of BilRI was further examined by analyzing NMR spectra from a recently developed chemical shift assignment [314], along with the $\{^{1}H\}$-^{15}N heteronuclear nuclear Overhauser effect (NOE), T_1 and ^{1}H, ^{15}N nuclear Overhauser effect spectroscopy-heteronuclear single quantum coherence spectroscopy (NOESY-HSQC).

The amino acid sequence of BilRI contains three 30 residue-long repeating regions (residues 51–80, 91–120, and 131–160 [181]). The repeats caused overlaps in the NMR spectra, which hampered analyses. Nevertheless, a reasonable amount of data was retrieved (Figure 2a; Original publication I). Secondary structure predictions based on the amino acid sequence [340–342] predicted a helical secondary structure with high confidence, but the experimental data showed low helicity according to the secondary structure propensity (SSP) score. The SSP score for BilRI varied between –0.13 and 0.23, in contrast to the scores of a fully formed strand (–1) or a helix (+1) [343]. However, the three repeating regions showed a helical tendency, especially in their N-terminal parts, whose residues had positive SSP scores. Additionally, the regularly repeating hydrophobic residues could predict a helical structure (Figure 2b; Original publication I).

The tertiary contacts within the BilRI molecule were examined by 3D NMR. The regions that had positive SSP scores exhibited properties typical of helical structures. However, the overlapping peaks caused by the repeating regions made analysis difficult. The protein backbone dynamics were also studied, and the obtained spectra revealed that the BilRI backbone was very flexible. The N-terminal parts of the repeat regions showed slightly less flexibility than the rest of the protein.

In natural settings, BilRI is located in the outer membrane, along with other molecules, such as LPS. Binding to LPS could induce folding of BilRI and the formation of suspected helical structures. This was tested using an NMR titration assay. Increasing concentrations of purified LPS were mixed with BilRI and changes in the spectrum were monitored (Figure S4; Original publication I). The observed interactions between LPS and BilRI occurred in residues that were located outside of the repeat regions. The spectra obtained from the repeat regions were not changed by the addition of LPS, which indicated that LPS did not affect the helicity of BilRI.

4.2 Characterization of the type IV pilin homolog PilA (II)

4.2.1 Highly conserved PilA has several natural variants

The amino acid sequences of PilA were searched for 95 partially or completely sequenced *A. actinomycetemcomitans* strains (retrieved from GenBank on March 10th, 2021). PilA was found in every strain. There were 18 different protein variants that shared at least 90% sequence identity (**Figure 6**). As was observed for BilRI (chapter 4.1.1), genetically close strains expressed similar PilA variants (**Figure 3**).

Two protein variants were clearly the most common. These variants, referred to as PilAD11S and PilAD7S after one of the strains in which they are expressed, were present in 36 (38%) and 28 (29%) strains, respectively. PilAD7S was found in several serotype a and d strains, including the naturally competent serotype a strains D7S, 624 and D17P-3 [130]. PilAD11S is expressed in the majority of serotype b and c strains, which have not been associated with natural competence [121]. The two variants differ by only two amino acids (**Figure 6**). While the change from serine in PilAD11S to threonine in PilAD7S (residue 113) does not majorly affect side chain properties, the effect of the change from the negatively charged large aspartic acid to the small nonpolar glycine (residue 126) is more notable.

4.2.2 PilA shares sequence similarity with type IVa major pilins of gram-negative species

The amino acid sequence of *A. actinomycetemcomitans* PilA was studied to determine the class of the type IV pilin to which PilA belongs. The relatively long signal peptide (16 residues) would indicate type IVb, while the size of the mature protein (134 amino acids) indicates type IVa instead. Furthermore, the first residue of mature PilA is phenylalanine (F), and the hypervariable C-terminal region, flanked by two cysteines, is small (11 residues), which both indicate type IVa (distinctive features of type IVa and IVb pili are reviewed in [46]).

To confirm the predicted classification, the sequence of PilAD7S was aligned with those of two type IVa major pilins whose structures have been solved, namely, *N. meningitidis* type IVa pilin PilE (PDB: 5KUA [344]) and enterohemorrhagic *E. coli* (EHEC) type IVa pilin (PDB: 6GV9 [345]) (**Figure 6A**). The sequences were most similar at the N-terminus, which is a conserved region in type IVa pilins [198]. Therefore, the first 50 amino acids of mature PilAD7S were aligned with several type IVa pilins from gram-negative species (**Figure 6B**). The N-terminal sequences were highly similar, which further indicated that *A. actinomycetemcomitans* PilA is a type IVa major pilin.

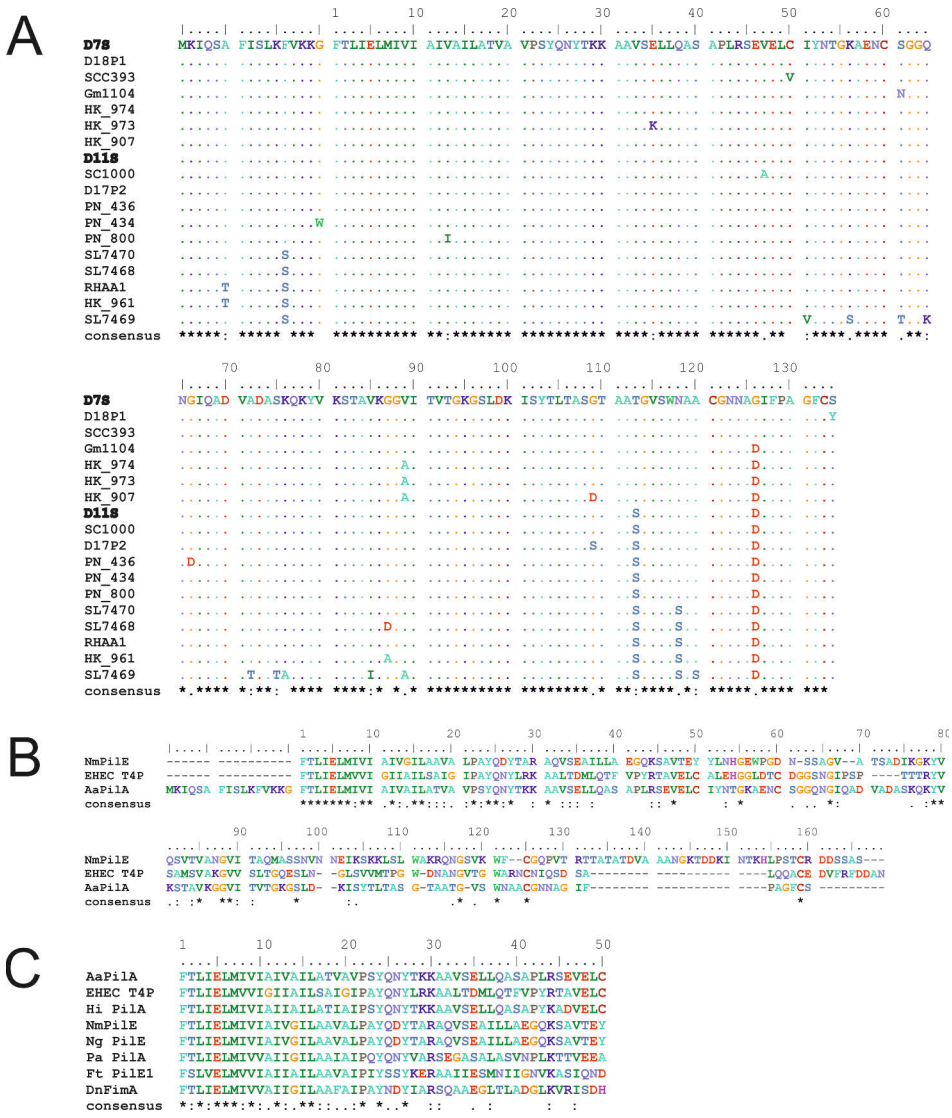

Figure 6. **Highly conserved *A. actinomycetemcomitans* PilA is homologous to type IV pili.** **A.** The 18 different protein variants of PilA, which were found in a total of 95 partially or completely sequenced *A. actinomycetemcomitans* strains, were aligned. The numbering follows the mature protein sequence, starting with phenylalanine (F) at position 1. The first 16 amino acids form the signal peptide. The two most common variants are labelled in bold. **B.** Alignment of *A. actinomycetemcomitans* PilA (AaPilA) from strain D7S, *N. meningitidis* PilE (NmPilE) and EHEC type IV pilin (T4P). **C.** The first 50 amino acids of a mature *A. actinomycetemcomitans* PilA were aligned with those of type IVa pilins from gram-negative species. (Hi: *Haemophilus influenzae*, Ng: *Neisseria gonorrhoeae*, Pa: *Pseudomonas aeruginosa*, Ft: *Francisella tularensis*, Dn: *Dichelobacter nodosus*). The consensus sequence indicates the similarity of residues: an asterisk (*) indicates a fully conserved residue; a colon (:) indicates conservation between groups of strongly similar properties (>0.5 in the Gonnet PAM 250 matrix); and a period (.) indicates conservation between groups of weakly similar properties (≤0.5 in the Gonnet PAM 250 matrix). (Adapted from Fig. 1; Original publication II.)

4.2.3 The hydrophobic N-terminus causes aggregation in recombinant PilA

Recombinant PilAD7S proteins were analyzed by SDS–PAGE to confirm their sizes and purity (**Figure 7A**). Mature PilAD7S has a highly hydrophobic N-terminus, which decreases the solubility of the protein and is the likely cause of the formation of aggregates (**Figure 7B**). Thus, efficient production of mature PilAD7S without a His-tag was difficult because the recombinant protein formed complexes with *E. coli* proteins (data not shown). Similar complexes were not observed with His-tagged recombinant mature PilA (**Figure 7A**). Although in natural settings major pilin proteins similar to PilA form large multimeric pilus filaments, the observed protein aggregates are likely nonnative because a complex molecular machinery is needed for pilus formation [198,346].

To avoid unwanted aggregation, N-terminally truncated PilAD7S(Δ1–23) and PilAD7S(Δ1–27) were produced. Formaldehyde crosslinking showed that the truncated variants formed significantly smaller complexes than the full-length protein (**Figure 7B**). The sizes of the recombinant proteins (N6-His-tagged/without His-tag) were: 15.6/14.1 kDa for PilAD7S, 13.2 kDa for PilAD7S(Δ1–23) and 12.7/11.2 kDa for PilAD7S(Δ1–27). The small formaldehyde link (12 Da) did not significantly increase the sizes of protein complexes.

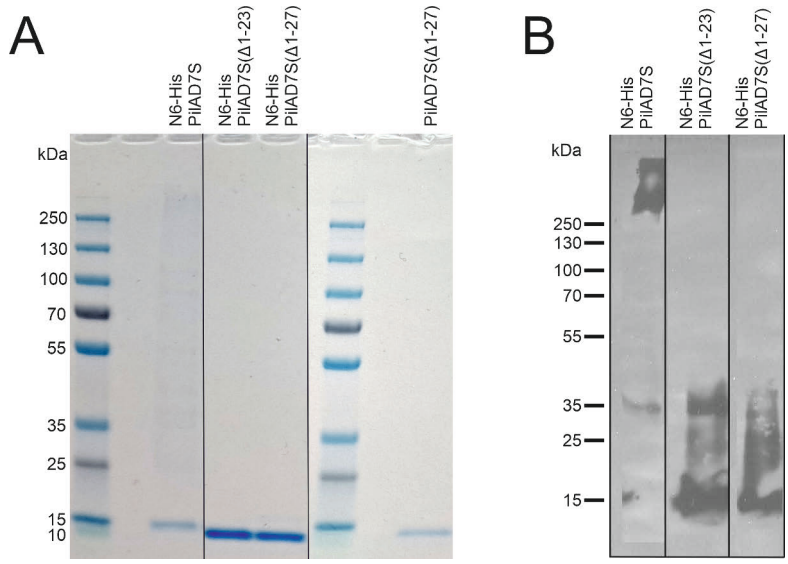

Figure 7. **The integrity of the recombinant PilA proteins was assessed by SDS–PAGE and western blotting. A.** The purity and sizes of the produced PilA proteins were confirmed by SDS–PAGE. **B.** Protein aggregates formed by full-length and two N-terminally truncated PilA proteins were fixed by formaldehyde crosslinking. The protein aggregates were detected with a probe that recognized the N-terminal His-tags on the recombinant proteins. (Adapted from Fig. 3; Original publication II.)

The N-terminally truncated PilAD7S(Δ1–27) protein was used in some experiments to avoid potential interferences caused by aggregation. Similar N-terminally truncated recombinant pilin proteins have been successfully used in both functional and structural studies [150,347,348]. While N-terminal truncation prevents aggregation, it leaves the functional C-terminal domain intact.

4.2.4 Predicted 3D-structure of PilA

Computational modeling was used to predict the 3-dimensional structure of *A. actinomycetemcomitans* PilA. Predicted structures were modeled for both mature PilAD7S and N-terminally truncated PilAD7S(Δ1–27). Both predicted monomeric structures consisted of the same structural elements, namely, an N-terminal α-helix connected to four antiparallel β-strands via a loop structure and a hypervariable segment in the flexible C-terminus (**Figure 8**). The interfaces between the α-helix and one of the β-strands mainly involved hydrophobic residues (valine, leucine, and isoleucine). The predicted structures of PilAD7S and PilAD7S(Δ1–27) were highly similar, as they were superimposed with a root mean square deviation of 0.78 Å for the Cα main chain atoms.

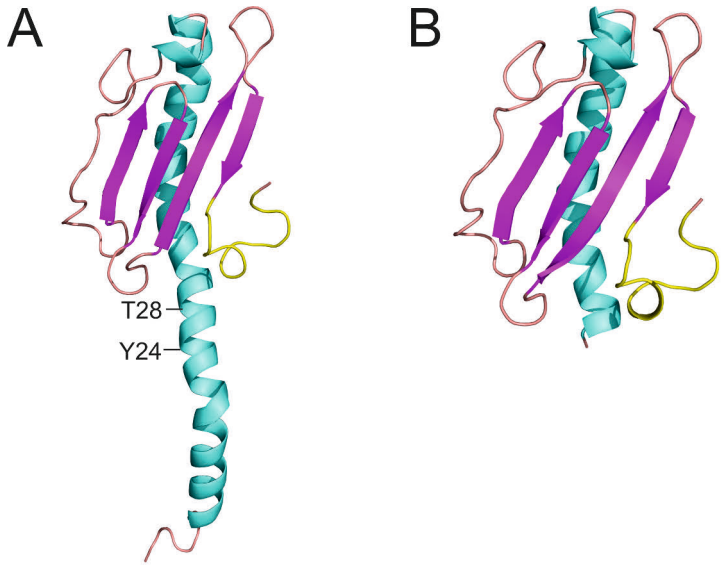

Figure 8. **Computationally predicted 3D-structures of PilA. A.** The predicted structure of a mature PilAD7S monomer. The protein has an N-terminal α-helix, four antiparallel β-sheets, and a C-terminal hypervariable D-region. The first residues of the N-terminally truncated recombinant PilAD7S(Δ1–23) and PilAD7S(Δ1–27) proteins are indicated in the figure with labels. **B.** The predicted structure of PilAD7S(Δ1–27) contains essentially the same secondary structural elements as the full-length mature protein, except for a truncated N-terminal α-helix. (Adapted from Fig. 2; Original publication II.)

The monomeric structure of PilAD7S was used to build a 14-homooligomeric pilus filament. The cryoelectron microscopy (cryo-EM) map of the most structurally similar protein homolog, a type IVa pilus from EHEC [345], was used as a reference. The resulting oligomeric structure resembles the cryo-EM structure of another PilA homolog, *N. meningitidis* PilE [344] (Figure S1; Original publication II). The neighboring N-terminal α-helices interact with each other and form bonds that are essential for the pilus filament assembly. The conserved glutamate (E5) and threonine (T2) form an intermolecular hydrogen bond, and the glutamate interacts with tyrosine residues (Y24 and Y27) on the neighboring helix. Moreover, the pilus assembly is likely stabilized by a hydrogen bond between S97 and Y52, which are also conserved residues in *A. actinomycetemcomitans* PilA.

4.3 Natural transformation and DNA binding (I–III)

4.3.1 Genetic organization of *bilRI* and competence genes (I)

Competence genes of the naturally competent *A. actinomycetemcomitans* strain D7S are distributed in different orientations and sites in the genome (**Figure 9A**). The standalone gene *bilRI* [298] is located downstream from known competence genes. The closest competence gene to *bilRI* is *comE1*, which is located 390 kb from *bilRI* but in opposite direction. The *pilA* gene, followed by *pilBCD* in the same gene cluster, is the closest competence gene in the same orientation as *bilRI*. However, the *pil* cluster is likely regulated by its own promoter [135].

4.3.2 BilRI and HofQ are required for efficient natural transformation (I, III)

Because OMPs are needed for the initial steps of transformation [119], the involvement of the outer membrane lipoprotein BilRI and the outer membrane secretin HofQ in the natural competence of *A. actinomycetemcomitans* D7S was studied. HofQ is homologous to the competence protein ComE of *H. influenzae* and binds dsDNA, which suggests that it may have a role in natural competence [300].

The linear DNA insert that was used to study natural transformation contained sequences up- and downstream from the *bilRI* gene. Homologous recombination would thus take place in the genomic region where the deletion had occurred in the Δ*bilRI* mutant. Therefore, the Δ*bilRI::spe*^{r} mutant, in which the deleted gene was replaced with the antibiotic cassette, was also used in the natural transformation assay to better mimic the original size of the locus for homologous recombination. The deletion of *bilRI* significantly decreased the transformation efficiency in both deletion mutants ($p=0.011$), but transformation was not completely inhibited (**Figure**

9B). However, the deletion of *hofQ* resulted in a nontransformable strain (p=0.0002) (**Figure 9C**).

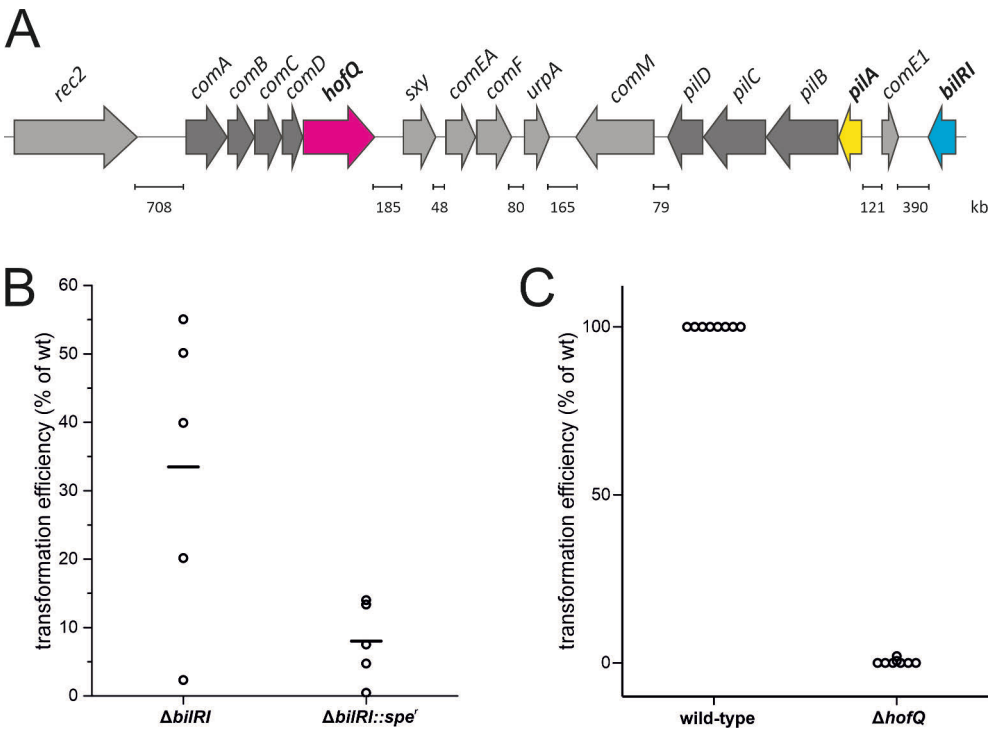

Figure 9. **BilRI and HofQ are involved in natural transformation of *A. actinomycetemcomitans*** **A.** The standalone gene *bilRI* is located downstream from competence genes in the *A. actinomycetemcomitans* D7S genome. Both *hofQ* and *pilA* are part of a cluster regulated by a single operon. **B.** The transformation efficiency of the mutant strains Δ*bilRI* and Δ*bilRI::spe*^{r} were compared to that of the naturally competent wild-type strain (wt). The data points represent the mean transformation efficiency from five individual experiments for each mutant strain. The overall mean transformation efficiency is marked with a horizontal line. **C.** The transformation efficiency of the mutant strain Δ*hofQ* was compared to that of the naturally competent wild-type strain. The data is presented as mean values from eight individual experiments. (Adapted from Fig. 5; Original publication I and Fig. 1; Original publication III.)

4.3.3 Linear *A. actinomycetemcomitans* DNA interacts with PilA but not with BilRI (I, II)

The first step of genetic transformation is the binding of extracellular DNA to the cell surface [119]. Since BilRI contibutes to the competence of *A. actinomycetemcomitans* strain D7S it may potentially bind DNA. Additionally, PilA, which is required for natural transformation [135], likely surfaces in the extracellular

space similar to competence pili [141–143] and may also be involved in the direct binding of DNA.

Competent *A. actinomycetemcomitans* strains preferably internalize DNA that contains a specific USS [301]. Interactions between linear USS-containing dsDNA and recombinant N-terminally truncated PilAD7S(Δ1–27) or recombinant BilRI were tested by observing mobility shifts in an agarose gel. Recombinant emHofQ has previously been shown to bind dsDNA [300]. Therefore, it was used as a positive control. PilAD7S(Δ1–27) interacted with DNA, but BilRI did not (**Figure 10**).

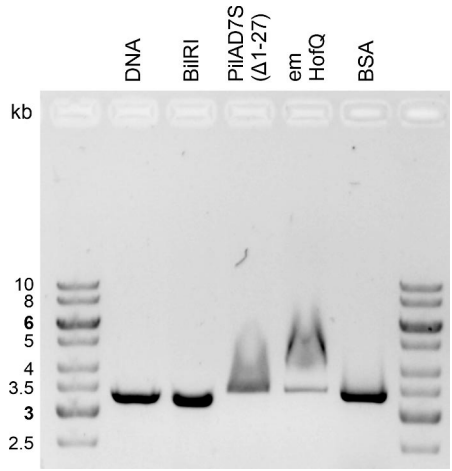

Figure 10. **PilA interacted with *A. actinomycetemcomitans* specific dsDNA.** Interactions between linear dsDNA (300 ng) and recombinant proteins (100 µg) were studied using an EMSA. The DNA contained an *A. actinomycetemcomitans* specific uptake signal sequence (USS). Recombinant emHofQ was used as a positive control and BSA was used as a negative control. A sample containing no protein (leftmost) was used as a control for DNA migration.

4.4 PilA binds proinflammatory cytokines (II)

N. meningitidis employs the type IV pilin subunit PilE, a protein homolog of *A. actinomycetemcomitans* PilA, in the binding and uptake of cytokines [178]. Therefore, the involvement of PilA in the binding of the proinflammatory cytokines TNF-α and IL-8 was studied. Owing to the formation of unwanted protein aggregates (chapter 4.2.3), it was not feasible to use the full-length recombinant PilA. Instead, the N-terminally truncated PilAD7S(Δ1–27) was used to determine the dissociation constants (K_d) for cytokine binding. Interactions between TNF-α and His-tagged PilAD7S(Δ1–27) were studied using an ELISA, and interactions between His-tagged IL-8 and PilAD7S(Δ1–27) were studied using a more sensitive TRFIA. The K_d values were calculated from binding curves using the Hill equation. The obtained K_d values were 7.16±0.40 µM for TNF-α and 0.67±0.18 µM for IL-8 (**Figure 11**).

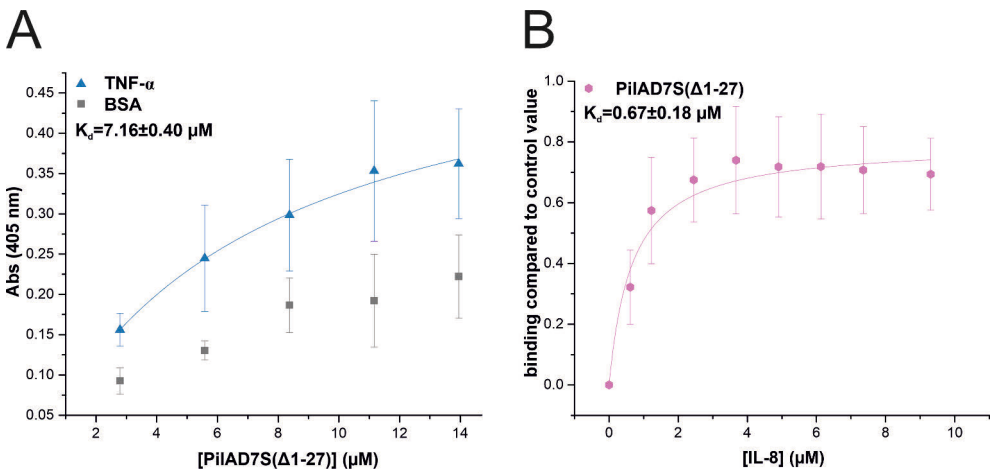

Figure 11. **PilA interacted with proinflammatory cytokines. A.** The interaction between TNF-α and the recombinant N-terminally truncated PilAD7S(Δ1–27) was studied with ELISA. Equal amounts of TNF-α were bound to the wells and PilA was used in various concentrations (2.8–14 μM). BSA was used as a negative binding control. The K_d value was calculated from the binding curve. Data is presented as the mean values±standard deviation from five independent experiments. **B.** The interaction between IL-8 and PilAD7S(Δ1–27) was studied with TRFIA. Equal amounts of PilA were bound to the wells and IL-8 was used in various concentrations (0–9.3 μM). The K_d value was calculated from the binding curve. Data is presented as the mean values±standard deviation from five independent experiments. The values were normalized with an interplate control. (Adapted from Fig. 4; Original publication II.)

4.5 PilA, but not BilRI, exhibited immunogenic properties (I, II)

Bacterial OMPs encounter host cells, such as leukocytes, during the immune response. Therefore, both BilRI and PilA are potentially involved in host-microbe interactions. The possible roles of BilRI and PilA in host-microbe interactions were studied with isolated human leukocytes, commercial human monocyte cells differentiated into macrophage-like phenotypes, and human sera using both whole *A. actinomycetemcomitans* cells and recombinant proteins.

4.5.1 Leukocyte ROS production was induced by PilA, but not by BilRI (I, II)

Because excessive leukocyte ROS production causes tissue destruction in periodontitis, the potential of *A. actinomycetemcomitans* to stimulate ROS production was studied with isolated human leukocytes. The role of BilRI in leukocyte stimulation was studied by incubating leukocytes with the D7S wild type or Δ*bilRI* mutant. However, there was no significant difference in ROS production induced by the two strains. Opsonization with *A. actinomycetemcomitans*-positive

patient sera slightly increased the ROS production compared to that with healthy sera, but the increase was not statistically significant (**Figure 12A**).

The role of PilA in leukocyte stimulation was studied using two wild-type strains, D7S and D11S, that express the two most common natural variants of PilA, and the deletion mutant Δ*pilA::spe*^{r}, from which the respective gene was deleted. There was no significant difference in the ROS production induced by the two wild-type strains. Meanwhile, the deletion mutant D7S Δ*pilA::spe*^{r} could not induce ROS production as efficiently as the wild-type D7S (p=0.005) or D11S (p=0.008) strain (**Figure 12B**). These results indicate that PilA plays a role in leukocyte stimulation, but BilRI does not.

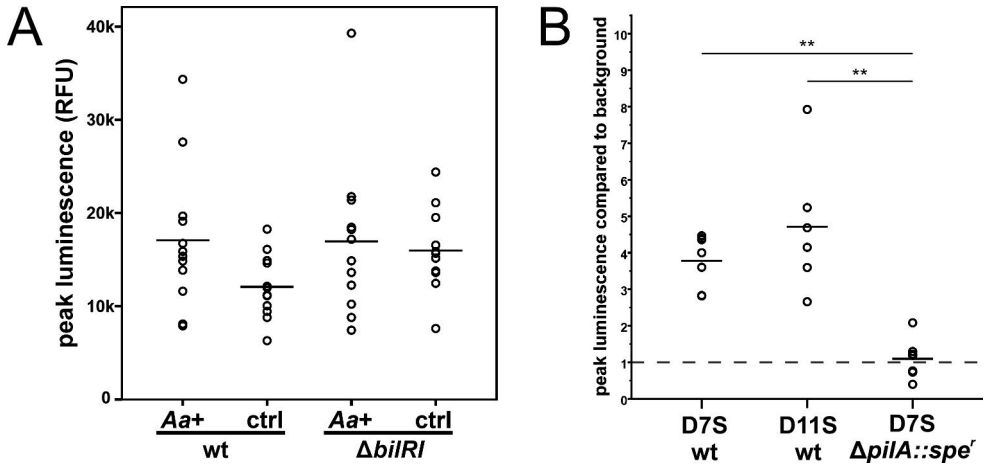

Figure 12. **PilA stimulated leukocyte ROS production. A.** ROS production by human leukocytes induced by *A. actinomycetemcomitans* cells was measured as luminol-enhanced chemiluminescence. The bacterial cells were opsonized with serum from either *A. actinomycetemcomitans*-positive patients (*Aa*+) or healthy control individuals (ctrl). The data represents peak luminescence values from 12 (patient sera) or 11 (control sera) individual experiments. The mean values for each set of experiments are marked with horizontal lines. **B.** ROS production was measured in the presence of two wild-type (wt) *A. actinomycetemcomitans* strains, D7S and D11S, and the deletion mutant D7S Δ*pilA::spe*^{r}. All the bacterial cells were opsonized with plasma from the same healthy individual. Significant difference (**, p≤0.005) was obtained when comparing D7S Δ*pilA::spe*^{r} to either of the wt strains. The data represents peak luminescence values compared to background signal (dashed line). The data was collected from 6–7 individual experiments and the mean values for each set of experiments are marked with horizontal lines. (Adapted from Fig. 3; Original publication I, and Fig. 4: Original publication II.)

4.5.2 Recombinant PilA or BilRI did not stimulate human macrophages to secrete cytokines (I, II)

Because macrophages secrete cytokines in response to pathogen recognition, human THP-1 cells, which were differentiated into macrophage-like phenotypes, were

stimulated with *A. actinomycetemcomitans* proteins and LPS. Stimulation of the differentiated THP-1 cells with recombinant mature BilRI for 20 h did not increase the amount of IL-6 in the culture medium. Similarly, the N-terminally truncated recombinant PilAD7S(Δ1–27) did not induce the secretion of any of the three cytokines after 6 or 24 h of stimulation. Neither BilRI nor PilA were cytotoxic, unlike *A. actinomycetemcomitans* LPS, which was used as a positive control. LPS induced the secretion of IL-6 and TNF-α. The amounts of IL-6 and TNF-α were normalized against relative cell viability to obtain the cytokine concentration per viable cell. The results are presented in **Table 5**. IL-8 secretion did not significantly change after stimulation with either the recombinant proteins or LPS (data not shown). Meanwhile, in the medium collected from the control cultures, the amount of IL-8 was higher (130–160 ng) than that of IL-6 (3–7 ng) or TNF-α (17–38 ng).

Table 5. Cytokine secretion by differentiated THP-1 cells in response to bacterial agents. Differentiated THP-1 cells were incubated with recombinant BilRI, recombinant PilAD7S(Δ1–27), or *A. actinomycetemcomitans* LPS and the amount of secreted cytokines was measured at different time points. (Adapted from Fig. 3; Original publication I, and Table 2; Original publication II.)

treatment	**time**	**relative cell viability**	**relative [IL-6]**	**[IL-6] / viability**
control	20 h	1.00±0.00	1.00±0.00	1.00±0.00
BilRI	20 h	0.97±0.08	0.93±0.07	0.99±0.15
LPS	20 h	0.60±0.12 *	19.39±13.61	31.19±22.94 *
treatment	**time**	**relative cell viability**	**IL-6 (pg)**	**IL-6 / viability**
control	6 h	1.00±0.00	3.16±1.95	3.16±1.95
PilAD7S(Δ1–27)	6 h	1.09±0.08	2.13±1.75	1.98±1.61
LPS	6 h	0.66±0.33	36.92±24.26	65.39±39.43 *
treatment	**time**	**relative cell viability**	**IL-6 (pg)**	**IL-6 / viability**
control	24 h	1.00±0.00	3.82±4.89	3.82±4.89
PilAD7S(Δ1–27)	24 h	1.07±0.10	2.03±1.60	2.02±1.71
LPS	24 h	0.59±0.27 *	0.59±0.27	0.59±0.27 *
treatment	**time**	**relative cell viability**	**TNF-α (pg)**	**TNF-α / viability**
control	6 h	1.00±0.00	34.49±60.54	34.49±60.54
PilAD7S(Δ1–27)	6 h	1.09±0.08	48.19±68.19	48.83±72.76
LPS	6 h	0.66±0.33	1130.44±727.70	1840.16±729.46 *
treatment	**time**	**relative cell viability**	**TNF-α (pg)**	**TNF-α / viability**
control	24 h	1.00±0.00	9.4±16.86	9.40±16.86
PilAD7S(Δ1–27)	24 h	1.07±0.10	6.76±10.89	7.14±11.85
LPS	24 h	0.59±0.27 *	1083.71±963.96	1924.31±1256.12 *

Values are presented as the mean±standard deviation from 4–5 individual experiments.
* differs significantly from the control sample ($p<0.05$)

4.5.3 PilA, but not BilRI, elicits specific antibody production in *A. actinomycetemcomitans*-positive patients (I, II)

Because the outer membrane lipoprotein PAL of *A. actinomycetemcomitans* is immunoreactive [241], it was hypothesized that BilRI, another lipoprotein, could also elicit antibody production in *A. actinomycetemcomitans*-positive periodontitis patients. Although there were significantly higher levels of antibodies binding to whole *A. actinomycetemcomitans* cells (p=0.007) in the patient sera than in the healthy control sera (**Figure 13A**), there was no significant difference in BilRI-specific antibodies between the patient and healthy control sera (**Figure 13B**). Therefore, the amount of specific antibodies against PilA were measured to study whether it can elicit antibody production. The patient sera contained increased amounts of antibodies against recombinant PilA (p=0.034) (**Figure 13C**). Additionally, the mean absorbance value obtained from the BilRI-specific antibody measurement was close to zero, unlike the mean absorbance values obtained from the *A. actinomycetemcomitans*- and PilA-specific antibody measurements. These results indicate that *A. actinomycetemcomitans*-binding antibodies are present in the patient sera and that PilA, unlike BilRI, elicits specific antibody production.

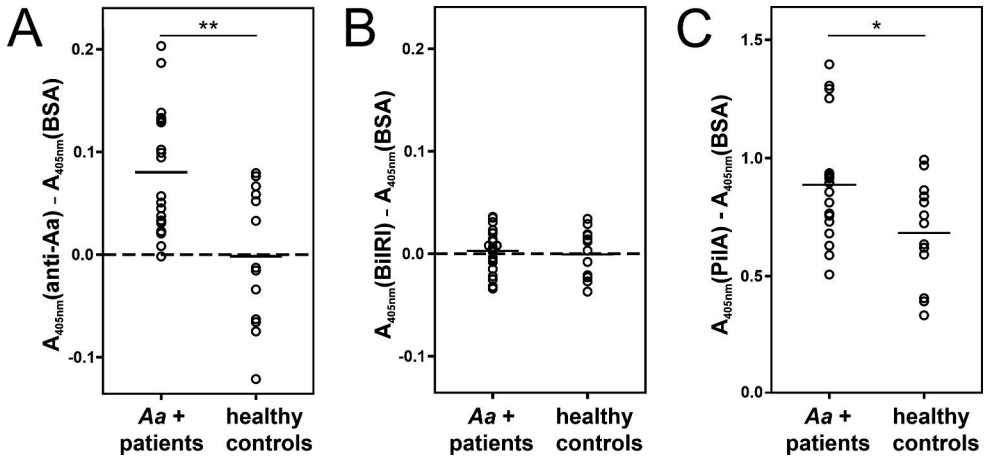

Figure 13. ***A. actinomycetemcomitans*- and PilA-specific antibodies were present in the sera of *A. actinomycetemcomitans*-positive patients.** The presence of antibodies was studied with ELISA. Microplate wells were coated using equal amounts of **A.** whole *A. actinomycetemcomitans* cells, **B.** recombinant BilRI or **C.** recombinant PilA. *A. actinomycetemcomitans*-positive (*Aa*+) patient sera or healthy control sera were added to the wells, and the bound human IgG-antibodies were detected with an anti-IgG antibody. Significant amounts of specific antibodies in the *Aa*+ patient sera were detected from *A. actinomycetemcomitans*- (**, p=0.007) and PilA-coated (*, p=0.034) wells. BSA was used as a negative binding control and the signal obtained from binding to BSA was subtracted from the results. The data was collected from 21 (patient sera) or 13–14 (control sera) individual experiments. The mean values for each set of experiments are marked with horizontal lines. (Adapted from Fig. 3; Original publication I, and Fig. 4; Original publication II.)

4.6 Gene expression in naturally competent *A. actinomycetemcomitans* D7S and its single-gene deletion mutants (III)

4.6.1 IL-1β did not induce changes in gene expression in *A. actinomycetemcomitans*

Because *A. actinomycetemcomitans* D7S can bind and internalize IL-1β, which leads to altered biofilm composition, RNAseq was performed to study the effect of IL-1β on bacterial gene expression in *A. actinomycetemcomitans* biofilms. However, the RNAseq revealed that cytokine treatment did not change gene expression in any of the strains used in this experimental setup. Principal component analysis showed no significant differences between IL-1β-treated and control cultures (**Figure 14A**).

4.6.2 Deletion of *hofQ* or *pilA* changed gene expression in *A. actinomycetemcomitans* D7S

Biofilms of the deletion mutants D7S Δ*bilRI* and D7S Δ*hofQ* have different compositions than that of the biofilm of the parental wild-type strain [298,299]. Therefore, RNAseq was used to study differences between wild-type *A. actinomycetemcomitans* D7S and deletion mutant biofilms at the transcriptional level. The Δ*pilA::spe*^{r} deletion mutant strain was included in the experiments to study how the inhibition of DNA uptake affects gene expression in *A. actinomycetemcomitans*. All the abovementioned deletion mutant strains exhibit a rough (fimbriated) phenotype similar to that of the wild-type strain [135,298].

Genes whose expression changed at least 2-fold ($p<0.05$) compared to that in the wild-type strain were considered differentially regulated in the deletion mutants. In the Δ*bilRI* mutant, the only differentially expressed gene was *bilRI*, with a negative fold change. Accordingly, the genes with the highest negative changes in the *ΔhofQ* and *ΔpilA::spe*^{r} mutants were also the corresponding deleted genes. However, deletion of *hofQ* or *pilA* resulted in changes in the expression of several other genes. In total, 34 genes were differentially regulated in the Δ*hofQ* mutant (26 downregulated and 8 upregulated), and 154 genes were differentially regulated in the Δ*pilA::spe*^{r} mutant (66 downregulated and 88 upregulated). Altogether 14 differentially regulated genes were shared between the two strains (12 downregulated and 2 upregulated) (**Figure 14B**). The majority (172 of 188) of the regulated genes encode proteins. The remaining 16 genes encode RNA molecules, such as a transfer RNA, or proteins that are inactive because of mutations (annotated as pseudoproteins).

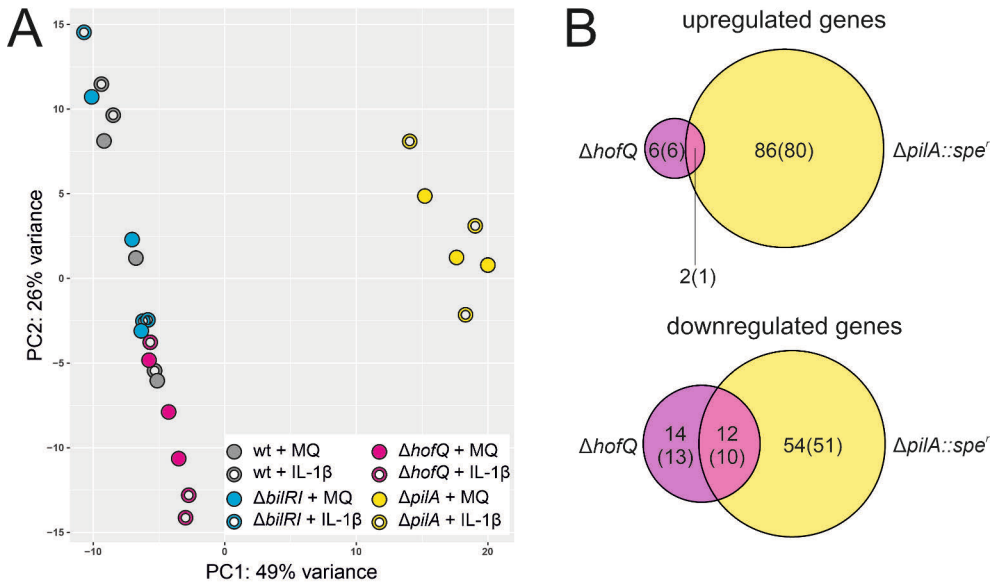

Figure 14. **Deletion of *hofQ* or *pilA* altered *A. actinomycetemcomitans* gene expression. A.** Principal component analysis of the RNA sequencing data was performed with DeSeq2 tool in the CSC Chipster data analysis software. The RNA was isolated form either IL-1β-treated or control (MQ) cultures. The culturing was performed three times for each *A. actinomycetemcomitans* D7S strain (wild type (wt), Δ*bilRI*, Δ*hofQ* and Δ*pilA::spe*^{r}). **B.** Gene expression in the deletion mutants Δ*hofQ* and Δ*pilA::spe*^{r} was altered compared to that in the parental wild-type strain. In the Δ*hofQ* mutant, 34 genes were differentially regulated, and in the Δ*pilA::spe*^{r} mutant, 154 were differentially regulated. The two strains shared 14 regulated genes in total. The majority of the differentially regulated genes encode proteins (numbers in parentheses). (Adapted from Fig. 2; Original publication III.)

GO terms were generated for the protein products of the regulated genes to find enriched terms among the sets of the differentially regulated genes. Altogether, 83 GO terms were found for 24 protein-encoding genes regulated in the Δ*hofQ* mutant, and 291 GO terms were found for 98 protein-encoding genes regulated in the Δ*pilA::spe*^{r} mutant. GO terms from all three categories (biological process, cellular component, and molecular function) were found in both strains. The most enriched molecular function term for both up- and downregulated genes in both strains was metal ion binding (GO:0046872) (**Figure 15**), which is a common function for various enzymes that use metal ions as cofactors, such as oxidoreductases, transferases, and ligases [349]. Cellular component terms revealed the subcellular localization of the regulated proteins. In both the Δ*hofQ* and Δ*pilA::spe*^{r} mutants the most common cellular component terms were cytoplasm (GO:0005737) and plasma membrane (GO:0005886) (**Figure 15**).

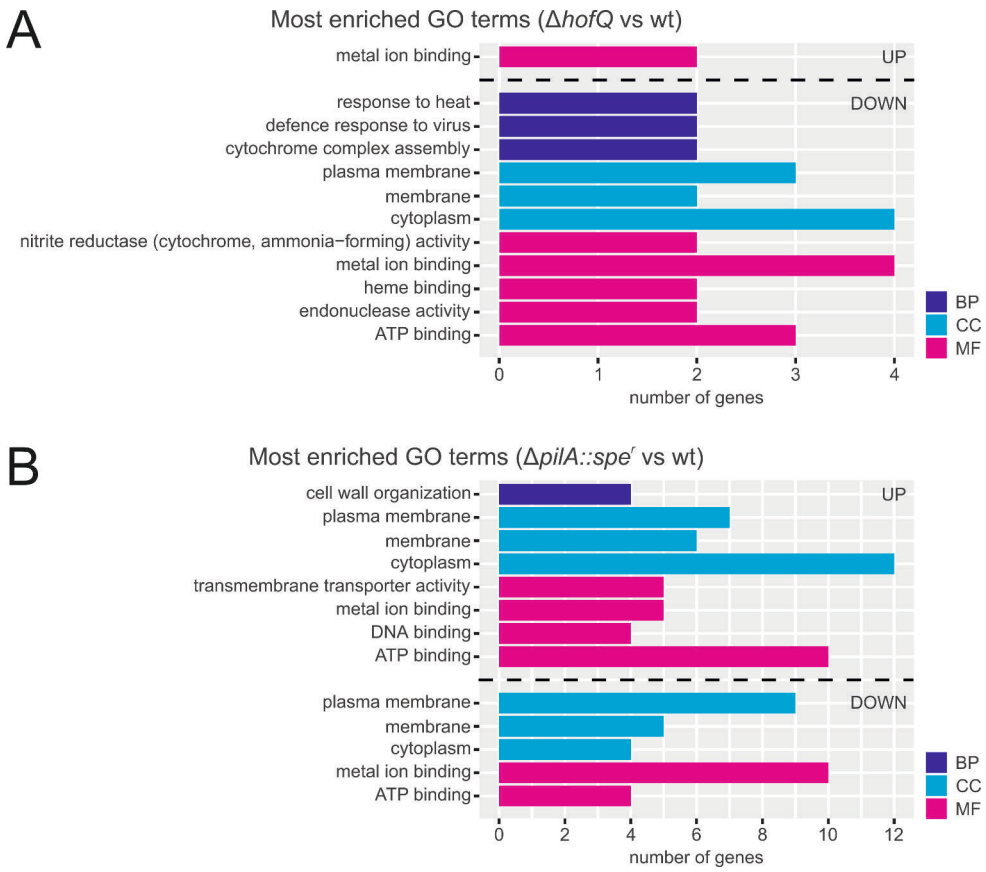

Figure 15. **Gene Ontology (GO) enrichment analysis of the protein products of the differentially regulated genes in the deletion mutants Δ*hofQ* and Δ*pilA::spe*^{r}.** GO terms are classified into three categories: biological process (BP), cellular component (CC) and molecular function (MF). **A**. GO terms that were associated with at least two up- or downregulated proteins in the Δ*hofQ* deletion mutant. **B.** GO terms that were associated with at least four up- or downregulated proteins in the Δ*pilA::spe*^{r} deletion mutant. (Adapted from Fig. 3; Original publication III.)

The protein products of the differentially regulated genes were also assigned to COG categories (**Figure 16**). Twenty-six proteins from the Δ*hofQ* mutant and 103 proteins from the Δ*pilA::spe*^{r} mutant were found in the COG database. The most common categories for the downregulated proteins in the Δ*hofQ* mutant were E (amino acid metabolism and transport), O (posttranslational modification, protein turnover, chaperone functions) and S (function unknown). The upregulated proteins were almost equally distributed among five different categories. In the Δ*pilA::spe*^{r} mutant, the most common categories among the downregulated proteins were C (energy production and conversion) and G (carbohydrate metabolism and transport). The most common categories among the upregulated proteins were M (cell

wall/membrane/envelope biogenesis) and O (posttranslational modification, protein turnover, chaperone functions).

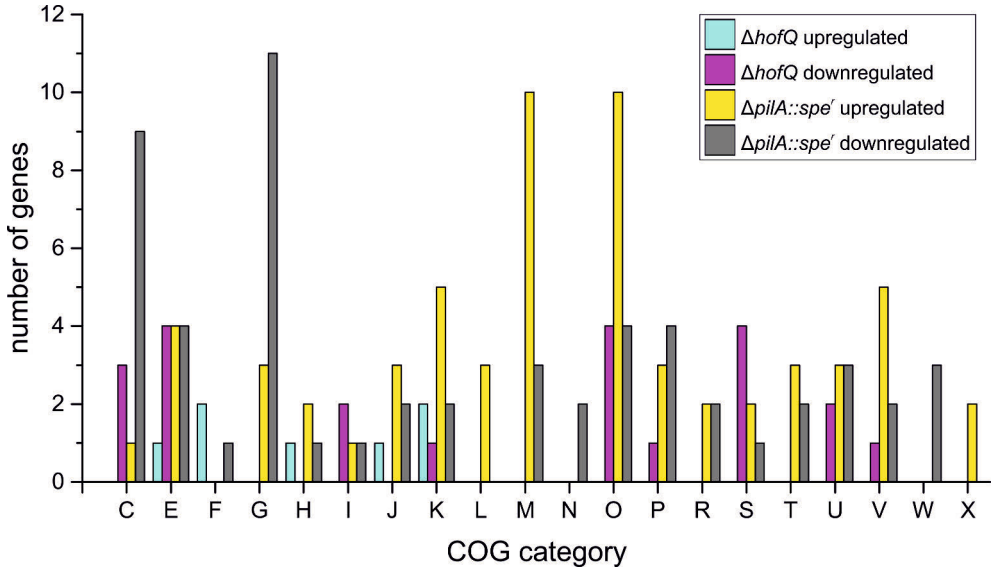

Figure 16. **Distribution of differentially regulated genes whose protein products were found in the Cluster of Orthologous Genes (COG) database in COG categories.** C: energy production and conversion, E: amino acid metabolism and transport, F: nucleotide metabolism and transport, G: carbohydrate metabolism and transport, H: coenzyme metabolism, I: lipid metabolism, J: translation, K: transcription, L: replication and repair, M: cell wall/membrane/envelope biogenesis, N: cell motility, O: posttranslational modification, protein turnover, chaperone functions, P: inorganic ion transport and metabolism, R: general functional prediction only, S: function unknown, T: signal transduction, U: intracellular trafficking and secretion, V: defense mechanisms, W: extracellular structures, X: mobilome: prophages, transposons. (Adapted from Fig. 3; Original publication III.)

4.6.2.1 Genes associated with anaerobic respiration and anaerobic metabolism were downregulated in the Δ*hofQ* and Δ*pilA::spe*^{r} mutants

Several downregulated genes in the deletion mutants Δ*hofQ* and Δ*pilA::spe*^{r} were associated with anaerobic respiration or anaerobic metabolism. The two strains had seven of these genes in common, including the periplasmic nitrite reductase operon *nrfABCD*, genes encoding the heme exporter protein CcmD and cytochrome c membrane protein NapC, and the aspartate ammonia-lyase gene (*aspA*). These genes are required for anaerobic respiration [350] except *aspA*, whose protein product catalyzes the conversion from aspartate to fumarate during anaerobic growth [351]. Additionally, three genes associated with anaerobiosis were downregulated in the Δ*hofQ* mutant, namely, the gene encoding the c-type cytochrome biogenesis protein

CcmI, which is associated with anaerobic respiration, and two genes whose protein products participate in anaerobic arginine catabolism (D7S_RS08420–08425).

Five additional genes associated with anaerobic respiration were downregulated in the Δ*pilA::spe*^{r} mutant, namely, the periplasmic quinol-oxidizing system *napGH*, dimethylsulfoxide reductase subunit b (*dmsB*), and genes encoding the cytochrome c subunit NapB and cytochrome c peroxidase [350,352]. Other protein products of the downregulated genes associated with anaerobiosis were L-asparaginase II, encoded by *ansB* [353], 3-keto-L-gulonate-6-phosphate decarboxylase UlaD [354], malate dehydrogenase, encoded by *mdh* [355], and the iron-sulfur cluster insertion protein ErpA [356].

A couple of genes associated with anaerobic conditions were instead upregulated in both mutant strains. In the Δ*pilA::spe*^{r} mutant, two genes encoding formate dehydrogenase subunits α (*fdnG*) and β (*fdxH*), which are associated with anaerobic respiration [352], were upregulated. In the Δ*hofQ* mutant, the *nrdD* and *nrdG* genes, which are associated with anaerobic DNA synthesis [357], were upregulated. Moreover, the cytochrome bd operon gene *ygbE* was downregulated in the Δ*hofQ* mutant. In facultative anaerobes, cytochrome bd is activated at low oxygen levels; however, the expression of *ygbE* is not required for oxidase activity, and its function remains unclear [358].

4.6.2.2 Gene expression in the Δ*hofQ* mutant indicates increased transcription and translation

Along with the upregulation of the abovementioned *nrdD* and *nrdG* genes, which are involved in DNA synthesis, analysis of the differentially regulated genes in the Δ*hofQ* mutant implied an increase in translation and transcription. An IMP dehydrogenase (*guaB*), which is involved in the *de novo* synthesis of guanine nucleotides, and a ribosome maturation factor (*rimP*) were upregulated, whereas the transcriptional repressor *metJ* was downregulated. Moreover, a gene (D7S_RS03355) whose protein product is homologous to the *E. coli* transcription antiterminator CspE [359] was upregulated.

The remaining two upregulated genes in the Δ*hofQ* mutant were type IV secretion system tip protein-encoding *vgrG* and the γ-glutamyltransferase gene *ggt*, whose protein products are putative virulence factors [360,361]. Other downregulated genes encode proteins involved in membrane lipid synthesis, membrane transport and protection against mobile genetic elements, along with uncharacterized or hypothetical proteins. Additionally, 6S rRNA was downregulated in the Δ*hofQ* mutant.

4.6.2.3 Virulence-associated genes were downregulated in the Δ*pilA::spe*^{r} mutant

Certain genes associated with virulence were downregulated in the Δ*pilA::spe*^{r} mutant, including genes encoding glycosyltransferase family 9 proteins, involved in LPS synthesis [362], the leukotoxin activator LtxC [269], and the major fimbrial subunit Flp-1. The quorum sensing gene *luxS*, which is required for biofilm formation, along with *flp-1* [196,250], was downregulated. The transformation regulator *tfoX (sxy)* [303] was downregulated, but only two competence-associated genes were differentially regulated, namely, the deleted gene *pilA* and a member of the same cluster, *pilC* [135], which were both downregulated. Apart from the several downregulated genes, a putative virulence factor, the type IV secretion system tip protein-encoding gene *vgrG*, was upregulated, along with *lpxH*, which is involved in the biosynthesis of lipid A of LPS.

The other downregulated genes in the Δ*pilA::spe*^{r} mutant encode proteins that are involved in various functions, such as sugar transport, ribose metabolism, glycogen biosynthesis, protein modification, DNA transposition and protection against mobile genetic elements, along with uncharacterized or hypothetical proteins.

4.6.2.4 Stress response and cell membrane biogenesis were induced in the Δ*pilA::spe*^{r} mutant

The alternative sigma factors RpoE (σ^E or σ^{24}) and RpoH (σ^{32}) control gene expression under cellular stress induced by extracytoplasmic stress such as heat [363–365]. Both RpoE and RpoH were upregulated in the Δ*pilA::spe*^{r} mutant. Multiple genes identified as a part of the RpoE or RpoH regulon were upregulated, including sigma factor regulators, chaperones, and proteases. However, three genes included in the RpoE regulon were downregulated instead, namely, L-asparaginase (*ansB*), N-acetylneuraminate anomerase (*nanQ*) and a gene encoding CRISPR-associated helicase/endonuclease Cas3 (D7S_RS00925). Interestingly, three regulon genes were downregulated in the Δ*hofQ* mutant, namely, genes encoding the chaperones ClpB and DnaJ and the protease HslV. All three were upregulated in the Δ*pilA::spe*^{r} mutant. Additionally, two putative stress response genes were upregulated in the Δ*pilA::spe*^{r} mutant, namely, those encoding a Sel1-repeat family protein (D7S_RS00715), which is associated with cellular stress [366], and a YoeB family toxin (D7S_RS10315), which is associated with thermal stress [367].

Several genes associated with cell membrane synthesis and integrity were upregulated in the Δ*pilA::spe*^{r} mutant. These genes encode proteins involved in peptidoglycan synthesis and cell wall formation, lipid asymmetry maintenance, and

the Tol-Pal system. Additionally, two genes whose protein products are homologous to the cell division -associated proteins RlpA [368] and ParA [369] were upregulated.

Other upregulated genes in the Δ*pilA::spe*^{r} mutant included genes whose protein products were involved in iron uptake, type I secretion, carbohydrate metabolism, amino acid and protein metabolism, translation regulation, queuosine synthesis and DNA transposition, along with uncharacterized or hypothetical proteins.

4.6.2.5 The genome of the Δ*pilA::spe*^{r} mutant contained a prophage-like element

In total, 88 genes were upregulated in the Δ*pilA::spe*^{r} mutant. However, ten of these genes belonged to a 15-gene cluster at locus D7S_RS06500–06570, which was not transcribed in the wild-type, Δ*bilRI*, and Δ*hofQ* strains. PCR analysis revealed that the 15-gene cluster was absent from the genome of the strains other than Δ*pilA::spe*^{r} (data not shown). A previous study by Kittichotirat et al. has characterized this gene cluster as part of a genetic island unique to the D7S strain [266].

Only five of the 15 genes have a known function, namely, a host cell division inhibitor (D7S_RS06515), phage antirepressor (D7S_RS06525), transcriptional regulator (D7S_RS06550), phage regulatory protein (D7S_RS06555), and recombinase/integrase (D7S_RS06570). Proteins encoded by the genes at loci D7S_RS06525 and D7S_RS06570 belong to the COG category X (Mobilome: prophages, transposons). Moreover, the upstream region of the gene cluster contains a 24-nucleotide-long sequence that is almost identical to the *attL* attachment site of *E. coli* prophage CP4-57 [370,371]. Taken together, these observations suggest that the 15-gene cluster, which is present only in the Δ*pilA::spe*^{r} mutant, could be a prophage [372].

4.6.3 Validation of the RNAseq results by qPCR

The expression of six genes was studied using qPCR to validate the RNAseq results. Four of the selected genes (*aspA*, *nrfB*, D7S_RS06105 and D7S_RS09470) were downregulated in both the Δ*hofQ* and Δ*pilA::spe*^{r} mutants; one gene (*vgrG*) was upregulated in both mutants, and one gene (*nrdD*) was upregulated only in the Δ*hofQ* mutant. Gene expression levels were normalized against two housekeeping genes: *clpX* (ATP-dependent Clp protease ATP-binding subunit ClpX) which is expressed constitutively in biofilms [373], and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase), which is a commonly used reference gene [374,375].

The qPCR results were largely similar to the RNAseq results. Gene expression levels in the Δ*bilRI* mutant were most similar to those in the wild-type strain. Although RNAseq did not show differential regulation of any of the six genes in the

bilRI mutant, the qPCR results showed significant downregulation of *aspA* (p=0.0495) (**Figure 17A**). The expression of all six genes was differentially regulated in the Δ*hofQ* and Δ*pilA::spe*^{r} mutants; however, only in five (Δ*hofQ*) and three (Δ*pilA::spe*^{r}) cases was the difference statistically significant (p=0.0495) (**Figure 17A**). The correlations between RNAseq and qPCR results varied from moderate to extremely high (Pearson's R=0.59–0.96, p<0.05), which further validated the RNAseq results (**Figure 17B**).

RNAseq showed that the *vgrG* gene was upregulated over 2-fold in the Δ*hofQ* and Δ*pilA::spe*^{r} mutants. The qPCR results showed instead lower, nonsignificant upregulation. The *vgrG* gene has eight highly similar copies in the *A. actinomycetemcomitans* D7S genome, and RNAseq indicated upregulation of a specific copy (D7S_RS12360). Owing to the high similarity of the eight genes, qPCR primers likely also targeted the *vgrG* genes in other loci, which could distort the results.

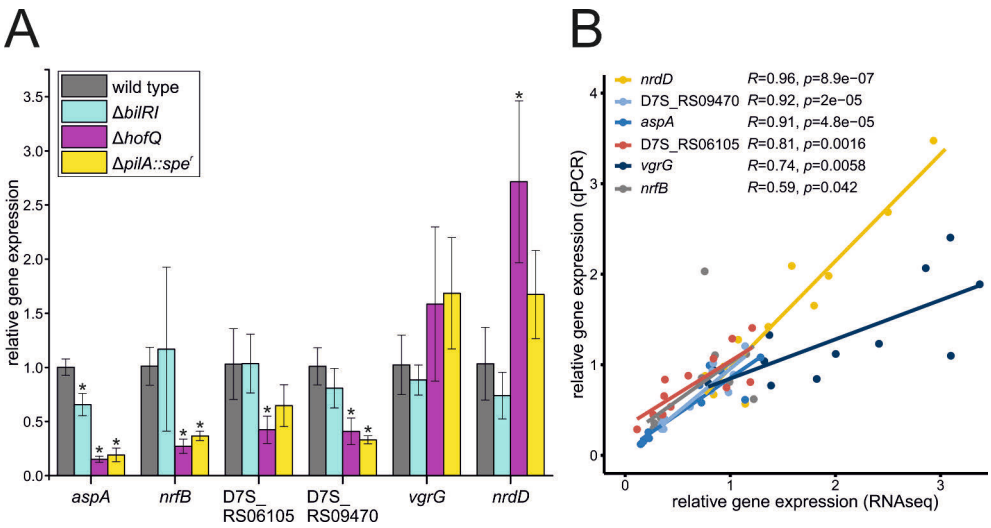

Figure 17. **The RNAseq results were validated by qPCR. A.** The relative gene expression of six genes was studied using qPCR. Compared to those in the wild-type strain, one gene was significantly differentially expressed (*) in the Δ*bilRI* mutant (p=0.0495), five genes were significantly differentially expressed in the Δ*hofQ* mutant (p=0.0495), and three genes were significantly differentially expressed in the Δ*pilA::spe*^{r} mutant (p=0.0495). The gene expression levels were normalized against two housekeeping genes. The data (mean±standard deviation) is presented as relative gene expression compared to that of the wild-type strain. The data was obtained from three individual experiments. **B.** Correlation between the RNAseq and qPCR results was measured with Pearson's correlation coefficient (R). The relative gene expression values are compared against gene expression in the wild-type strain. The data was collected from three individual qPCR and RNAseq experiments for each of the four strains (wild type, Δ*bilRI*, Δ*hofQ* and Δ*pilA::spe*^{r}). (Adapted from Fig. 4; Original publication III.)

5 Discussion

5.1 Proteins associated with competence interact with human cytokines suggesting a link between cytokine and DNA uptake

This study confirmed that the outer membrane secretin HofQ was required for competence in *A. actinomycetemcomitans*, as mutants devoid of the *hofQ* gene were nontransformable. Previously, HofQ was predicted to participate in DNA uptake during natural transformation owing to its ability to bind DNA [300]. It is likely that HofQ forms a membrane channel through which DNA passes the outer membrane. A pilus structure that binds eDNA is also required for DNA uptake [134]. The gene *pilA* encoding the putative type IV pilus subunit was among the first identified competence genes of *A. actinomycetemcomitans*; Wang et al. [135] showed that the removal of the *pilA* gene resulted in a nontransformable strain. In the current study, PilA was shown to be conserved in *A. actinomycetemcomitans*, although several protein variants were found among different strains. Interestingly, all known naturally competent *A. actinomycetemcomitans* strains [130] share the same PilA variant (referred to as PilAD7S). Furthermore, the amino acid sequence and computationally modeled 3D-structure of PilAD7S were found to resemble those of type IVa pilus subunits, which are associated with DNA uptake [134]. The importance of *hofQ* and *pilA* in natural competence was supported by the observation that recombinantly produced HofQ and PilA could bind the USS-containing dsDNA.

The membrane lipoprotein BilRI was not essential for natural transformation, although its removal significantly decreased the transformation efficiency. However, BilRI did not bind DNA, suggesting its indirect involvement. As BilRI is a membrane protein, it is possible that it affects the composition or properties of the *A. actinomycetemcomitans* outer membrane. Removal of the *bilRI* gene could thus change membrane properties and hinder, but not completely inhibit, the transfer of DNA through the *A. actinomycetemcomitans* outer membrane.

Although a couple of genes, in addition to *hofQ* and *pilA*, have been recognized as essential for competence of *A. actinomycetemcomitans* [302,303], the details of DNA uptake remain unclear. It is likely that eDNA binds to the PilA polymer and passes the outer membrane through HofQ. Translocation across the inner membrane

is mediated by Com proteins in other gram-negative species [120]. Therefore, *A. actinomycetemcomitans* Com proteins, which are located near the transformation regulator *tfoX (sxy)* (**Figure 9A**), could participate in translocating ssDNA into the cytosol.

PilA was shown to bind the proinflammatory cytokines IL-8 and TNF-α. The binding of these cytokines to PilA was relatively loose, which suggests transient interactions. Previously, cytokine interactions have been studied with two outer membrane proteins of *A. actinomycetemcomitans*, BilRI and HofQ, along with LPS [176,298,299]. To date, the only cytokine that has been shown to interact with all the studied proteins and LPS is IL-8, although BilRI–IL-8 interactions were too weak to determine the K_d. The affinity for IL-8 increases from BilRI (undetermined) to LPS (1.2–17 μM) to PilA (0.7±0.2 μM) to HofQ (43±4 nM). Because HofQ is a channel protein, it is possible that its moderate affinity promotes the uptake of IL-8, while components with lower affinity for IL-8 sequester the cytokine on the cell surface.

A link between cytokine uptake and DNA uptake was proposed after the discoveries that HofQ could bind both cytokines and DNA and that IL-8 could bind to DNA [299]. The recognition of HofQ as an essential part of the DNA uptake machinery strengthens this previous hypothesis. Additionally, PilA was shown to bind human cytokines. A PilA homolog, the type IV pilus major pilin PilE of *N. meningitidis*, has been associated with both DNA and cytokine uptake [178], which opens up the possibility that PilA could also be involved in both functions. Furthermore, BilRI has previously been shown to be involved in the binding and uptake of cytokines [298]. In this study, BilRI was shown to affect the transformation efficiency of *A. actinomycetemcomitans*. The involvement of all three proteins, HofQ, PilA and BilRI, in both cytokine interactions and competence further suggests that these two functions could occur simultaneously or through the same uptake route (**Figure 18**).

The effects of cytokines on *A. actinomycetemcomitans* biofilms have been previously studied but not at the transcriptional level. The expression of *S. aureus* virulence genes has been shown to be altered by IL-1β [182]. Moreover, the DNA-binding protein HU of *A. actinomycetemcomitans* has been shown to bind IL-1β [297]. In many species, HU regulates the expression of various genes [376], which leads to the suggestion that the uptake of IL-1β could alter gene expression. Therefore, IL-1β was added to *A. actinomycetemcomitans* biofilm cultures and gene expression was studied by RNAseq. The experimental setup used in this study did not show differences in gene expression between cells from IL-1β-treated and control biofilms. However, the hypothesis that cytokine internalization affects the gene expression of *A. actinomycetemcomitans* should not be discarded, as IL-1β and IL-8 have been shown to affect the composition of the extracellular matrix [298,299].

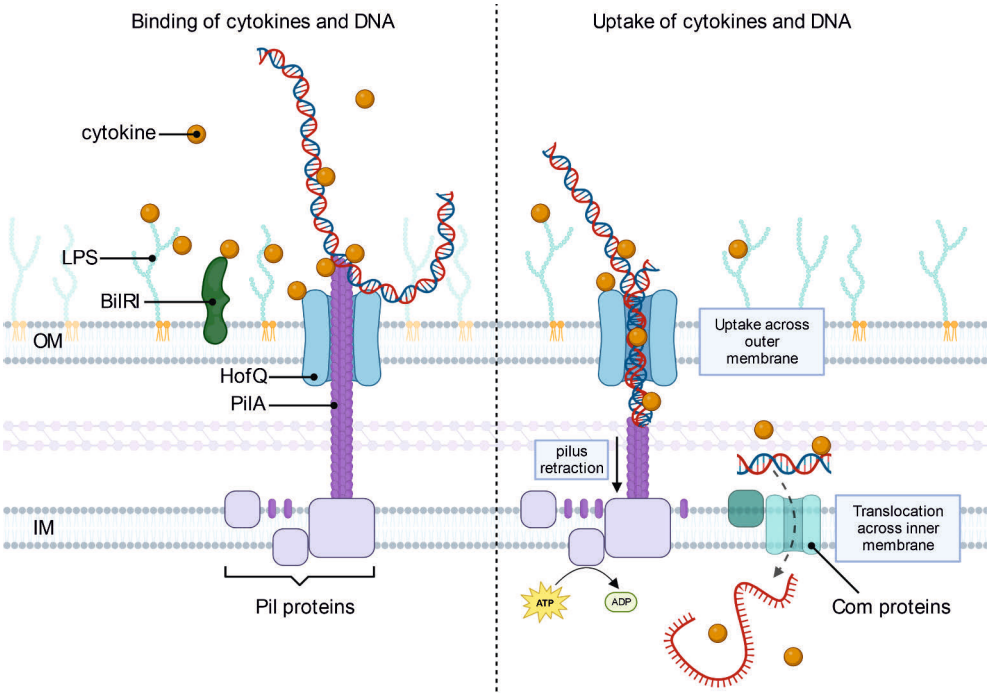

Figure 18. **Model of simultaneous uptake of cytokines and DNA by *A. actinomycetemcomitans*.** Outer membrane (OM) components sequester cytokines from the environment (left). LPS, BilRI, HofQ, and PilA, a subunit of the pilus polymer protruding from the outer membrane likely through HofQ, have been shown to bind human cytokines. HofQ and PilA bind dsDNA and their expression is necessary for natural competence. Moreover, IL-8 and DNA interact with each other. Retraction of the pilus pulls the bound DNA and cytokines into the periplasmic space (right). It is unclear how the translocation of DNA into the cytosol occurs, but Com proteins in the inner membrane (IM) are likely involved [120]. However, whether cytokines pass the inner membrane through the same route is unknown. Assembly and disassembly of the PilA polymer is mediated by other Pil proteins, which are encoded by genes in the same locus as *pilA* [135]. (Created with BioRender.com.)

5.2 The type IV pilin subunit PilA is a potential virulence factor of *A. actinomycetemcomitans*

A. actinomycetemcomitans was found to elicit specific antibody production in periodontitis patients, as patient sera contained significantly higher amounts of *A. actinomycetemcomitans*-binding antibodies than the sera isolated from healthy individuals. Previous studies have revealed that sera from periodontitis patients contain specific antibodies against the outer membrane lipoprotein PAL and major OMPs of *A. actinomycetemcomitans*, such as the 29-kDa OMP [241,377–379]. Meanwhile, CDT subunit A and HofQ do not elicit specific antibody production [299,380]. This study showed that PilA-specific antibodies could be found in periodontitis patient sera. Specific antibodies against BilRI were not detected. The

disordered structure of BilRI may contribute to its poor immunoreactivity [381], although the importance of disordered antigenic epitopes has been discussed in a comprehensive study by MacRaild et al. [382]. Their study revealed that disordered epitopes were typically small (approximately 9 residues) and mutations within the epitopes were frequently associated with a significant decrease in the binding affinity. The amino acid sequence of BilRI contains repeats consisting of 10 amino acids, which are not, however, completely identical [181]. The sequence variation could potentially contribute to the poor immunoreactivity of BilRI, but this remains to be clarified in further studies.

Sera of patients with periodontitis were also used to opsonize *A. actinomycetemcomitans* cells prior to measuring the phagocytic activity of leukocytes induced by the bacterium. The phagocytic activity was detected and quantified as ROS production. Effective phagocytosis of *A. actinomycetemcomitans* by leukocytes requires opsonization with human IgG antibodies, along with an active complement [383]. Opsonization with the patient sera better induced ROS production than opsonization with the healthy control sera, which is not surprising, considering the amount of *A. actinomycetemcomitans* specific antibodies in each pool of sera. Previous studies have suggested that human IgG antibodies that enhance phagocytosis target LPS, fimbriae, and various OMPs [377–379,384–386].

Deletion of *bilRI* did not affect the phagocytic activity of leukocytes. However, the deletion mutant Δ*pilA::spe*^{r} induced significantly weaker ROS production than the parental wild-type strain D7S, which indicates that PilA has immunogenic properties. The wild-type strains D7S and D11S, which express the two most common variants of PilA, induced ROS production in a similar manner. Thus, the two amino acid difference between the two protein variants does not seem to affect leukocyte stimulation. Previously, purified *A. actinomycetemcomitans* LPS was shown to prime human leukocytes, leading to the increased ROS production [293]. However, lipid A was required for priming, which is not exposed in native, membrane-bound LPS. Moreover, the LPS was from serotype b, which suggests that the serotype a and c strains used in this study might not be as effective in terms of increasing ROS production.

Recombinant BilRI and PilA were used to stimulate human THP-1 cells, which were differentiated into a macrophage-like phenotype. However, neither protein was able to induce secretion of IL-6, which is a potential diagnostic salivary biomarker of periodontitis [387]. Furthermore, PilA did not induce secretion of the proinflammatory cytokines IL-8 and TNF-α, which are involved in periodontitis [229]. Various *A. actinomycetemcomitans* molecules, including LPS, leukotoxin, and PAL, have been shown to induce cytokine production in PMNs [228,242,243]. It is possible that these proinflammatory molecules are more abundant on the *A. actinomycetemcomitans* surface and are thus more effective in inducing cytokine

production than BilRI and PilA. Moreover, BilRI and PilA were not cytotoxic to human macrophages, unlike *A. actinomycetemcomitans* LPS.

Overall, *A. actinomycetemcomitans* BilRI did not show any immunogenic properties. However, the recombinant BilRI used for the stimulation of ROS and cytokine production contained only the polypeptide part of the lipoprotein, while native BilRI contains an additional lipid component [181]. Lipoproteins from gram-negative species have been shown to stimulate macrophages by binding to their receptors with the lipid component [388]. It is therefore possible that native BilRI might function in a similar manner.

PilA did exhibit immunogenic properties, which makes it a potential virulence factor of *A. actinomycetemcomitans*. It was found to stimulate the production of ROS and specific antigens in human leukocytes. However, recombinant PilA could not induce cytokine secretion in macrophages. The recombinantly produced PilA used in this study was an N-terminally truncated monomeric subunit. Owing to the highly hydrophobic N-terminus, the purification of the full-length mature PilA was challenging. The N-terminus promoted the aggregation of PilA subunits with each other and with *E. coli* proteins. Although pilin subunits interact with each other in native settings, the assembly of a functional pilus polymer requires a specific protein machinery [198]. Therefore, it is highly unlikely that the observed PilA-aggregates were in a native form. However, N-terminally truncated recombinant pilin proteins have been shown to be sufficient for both functional and structural studies because the functional domain of type IV pilins is located in the globular C-terminus [150,347,348]. Monomeric pilin subunits that were used in this study, might not exhibit completely similar functions to those of native pilus polymers.

5.3 Deletion of *hofQ* or *pilA* may impair biofilm formation by *A. actinomycetemcomitans*

Deletion of *hofQ* or *pilA* altered the global gene expression in *A. actinomycetemcomitans* strain D7S, but deletion of *bilRI* did not. In total, 34 genes were differentially regulated in the Δ*hofQ* mutant, 154 genes were differentially regulated in the Δ*pilA::spe*^{r} mutant, and only one gene (*bilRI*) was differentially regulated in the Δ*bilRI* mutant. In each deletion mutant strain, the gene with the greatest negative change, *i.e.*, the gene whose expression decreased the most, was the corresponding deleted gene. This expected observation confirms the integrity of the deletion mutant strains used.

Several of the differentially regulated genes in the Δ*hofQ* and Δ*pilA::spe*^{r} mutant strains were associated with anaerobic respiration or anaerobic metabolism. Certain enzymes in the anaerobic respiratory pathway use molybdenum as a cofactor [350]. Therefore, it is not surprising that the genes encoding the molybdopterin synthase

subunit (*moaD*) and molybdate-binding protein (*modA*) were also downregulated Additionally, a gene encoding a YdcF family protein (D7S_RS05750) was downregulated in the Δ*pilA::spe*^{r} mutant. The protein is homologous to *E. coli* YdcF, which is possibly involved in anaerobic respiration [389], and shares sequence similarities with the conserved protein domain family NfrG (COG4235) of cytochrome c-type biogenesis proteins. However, a search against the COG database suggested that the YdcF family protein of *A. actinomycetemcomitans* was involved in membrane biogenesis.

Decreased expression of genes associated with anaerobic respiration or anaerobic metabolism has been linked to low iron availability [390]. Correspondingly, genes associated with iron uptake were upregulated in the Δ*pilA::spe*^{r} mutant, which resembled gene expression during iron limitation [390]. Moreover, iron-induced activation of QseBC, a two-component signaling system, leads to increased expression of genes associated with anaerobic respiration and metabolism [391]. The QseBC regulon has been suggested to play a role in virulence and help the bacterium adapt to the host environment, such as an anaerobic niche [391]. Moreover, QseBC is required for biofilm formation and virulence of *A. actinomycetemcomitans* [392]. Therefore, downregulation of genes associated with anaerobic growth, as observed in the Δ*hofQ* and Δ*pilA::spe*^{r} mutants, could indicate decreased virulence and colonization ability.

As mentioned above, the gene expression profile of the Δ*pilA::spe*^{r} mutant resembled the transcription pattern observed during iron limitation [390]. Iron limitation is known to promote biofilm dispersion by inducing the expression of the *dspB* gene, which encodes the PGA-hydrolyzing enzyme dispersin B [390]. The RNAseq data revealed that the expression of *dspB* in the Δ*pilA::spe*^{r} mutant was slightly upregulated (fold change: +1.6, $p<0.05$), which indicated increased dispersion compared to that of the wild-type biofilm. Moreover, *flp-1*, which encodes the major pilin subunit responsible for nonspecific adherence, and *luxS*, which encodes the autoinducer-2 molecule that mediates QS, were downregulated in the Δ*pilA::spe*^{r} mutant. Both *flp-1* and *luxS* are essential for efficient biofilm formation [196,250], which implies that the biofilm formation by the Δ*pilA::spe*^{r} mutant might be impaired. Biofilm formation has also been linked to the PTS in some species [29–32]. In the Δ*pilA::spe*^{r} mutant, two genes encoding ascorbate-specific PTS subunits were downregulated, while one gene, encoding a galactitol-specific subunit, was upregulated. However, the involvement of these genes in the biofilm formation by *A. actinomycetemcomitans* has not been studied. As both *flp-1* and *luxS* were downregulated and *dspB* was upregulated, the biofilm formed by the Δ*pilA::spe*^{r} mutant might not be as robust as that formed by the wild-type strain. The gene expression observed in the Δ*hofQ* mutant did not show similar changes. However,

6S rRNA (*ssrS*) was downregulated, which could negatively affect biofilm formation [393].

Another noteworthy difference between the Δ*pilA::spe*^{r} mutant and wild-type strain was the expression of stress response genes. The alternative sigma factors *rpoH* and *rpoE*, which are expressed in response to extracellular stresses, were upregulated in the Δ*pilA::spe*^{r} mutant. The RpoE regulon in other gram-negative species contains genes involved in preserving membrane integrity and genes involved in virulence [394–396]. Several genes associated with membrane biogenesis were upregulated in the Δ*pilA::spe*^{r} mutant. However, only one virulence gene belonging to the RpoE regulon, *lpxH*, which is involved in the biosynthesis of LPS, was upregulated. In contrast, two putative LPS synthesis genes were downregulated. Moreover, the virulence of the Δ*pilA::spe*^{r} mutant was likely decreased, according to the gene expression analysis. The downregulation of the *luxS* gene indicates decreased leukotoxicity, because *luxS* has been shown to induce leukotoxin expression [397]. Although the leukotoxin-encoding gene *ltxA* was not differentially regulated, the *ltxC* gene, whose protein product is required for leukotoxin activation, was downregulated. Therefore, the Δ*pilA::spe*^{r} mutant might be less leukotoxic than the wild-type strain.

RpoH and proteins belonging to its regulon are more abundant in the top layers of a *P. aeruginosa* biofilm than in the bottom layers [93]. Owing to the heterogeneous nature of biofilms, even monospecies biofilms consist of subpopulations that express certain genes at different levels [85]. In this study, the entire biofilm population was harvested. Therefore, the results represented the average gene expression in the biofilm cells. The upregulation of *rpoH* and its regulon in the Δ*pilA::spe*^{r} mutant could thus indicate that the Δ*pilA::spe*^{r} biofilm had a larger proportion of "top-layer" biofilm cells than the wild-type biofilm. In other words, the Δ*pilA::spe*^{r} biofilm might not be as thick and robust as the wild-type biofilm. Although *rpoH* was not differentially regulated in the Δ*hofQ* mutant, the upregulated genes in this mutant could indicate a similar effect on biofilm formation. All seven upregulated genes are involved in transcription or translation, which suggests increased metabolic activity. Studies with *P. aeruginosa* biofilms have shown that metabolically active cells are more likely to be found in the top layers of biofilms than in the bottom layers [93].

Overall, changes in the gene expression in the Δ*hofQ* and Δ*pilA::spe*^{r} mutants indicate decreased and/or impaired biofilm formation compared to that of the parental wild-type strain. However, deletion of *bilRI* did not result in any significant changes in gene expression. The results of this study are in accordance with previous studies of *A. actinomycetemcomitans* biofilm formation. Deletion of *hofQ* has been shown to result in decreased biofilm formation [299], while the Δ*bilRI* mutant formed biofilms as efficiently as the wild-type strain [298]. Biofilm formation is

strongly associated with antibiotic resistance, and many OMPs have been shown to be involved in antibiotic resistance in various gram-negative species [398,399]. Deletion of *bilRI* did not change the antibiotic susceptibility of *A. actinomycetemcomitans*, but HofQ has been shown to be involved in the resistance to β-lactam antibiotics [299]. The involvement of PilA in biofilm formation has not been studied, but deletion of *pilA* has been shown not to affect fimbriae expression [135]. Interestingly, *hofQ* and *pilA* are indispensable for natural transformation, but deletion of *bilRI* does not completely abolish transformation. Taken together, the results indicate that the expression of essential competence genes could be linked to the virulence of naturally competent *A. actinomycetemcomitans* strains.

It is worth noting that a prophage-like element was discovered in the genome of the Δ*pilA::spe*^{r} mutant but not in any of the other three strains used in the gene expression study. Although *A. actinomycetemcomitans* D7S was used as a common parental strain, the Δ*pilA::spe*^{r} mutant was received as a gift from Prof. Chen, unlike the Δ*bilRI* and Δ*hofQ* mutants, which were generated from the exact same wild-type cell stock that was used as a control in this study (**Table 2**). The presence of the prophage-like element in the RefSeq genome (NC_017846.2) suggests that this element has been spontaneously excised from the D7S strain at some point and is therefore not present in the genome of the wild-type strain used. Prophages can affect biofilm formation and virulence of bacterial cells [400–402]. Therefore, it remains unclear whether all of the observed changes in the gene expression in the Δ*pilA::spe*^{r} mutant resulted from the deletion of *pilA* or whether the prophage affected the gene expression in the mutant strain. However, there were some similarities between the expression profiles of the Δ*pilA::spe*^{r} and Δ*hofQ* mutants. Most notably, several genes associated with anaerobic respiration were downregulated in both mutant strains. These similarities suggest that the deletion of *pilA* induced some changes in the global gene expression of the naturally competent *A. actinomycetemcomitans* D7S. Nevertheless, the effect of *pilA* on *A. actinomycetemcomitans* gene expression needs to be confirmed.

5.4 BilRI might have a role in survival during transmission

The results of this study show that BilRI resembles LEA proteins. Sequence analyses of BilRI revealed homology with the plant LEA proteins EPC63 and At3g53040, which have mainly been studied in *Arabidopsis thaliana* (mouse-ear cress). ECP63 is involved in desiccation tolerance [403], while At3g53040 might provide protection against abiotic stresses [404]. Bacterial LEA proteins have not been as extensively studied but have been found to be also involved in protection against abiotic stresses. For example, *Deinococcus radiodurans* DrLEA3 has been linked to

protection from desiccation and oxidation [165,169], and *Zymomonas mobilis* Zmo0994 enhances tolerance to ethanol [170]. Overall, LEA proteins are known to protect cells from drying during anhydrobiosis and enhance tolerance to cold [405,406]. Using the red fluorescence reporter gene *dsred*, the activity of the *bilRI* promoter was shown to increase at lower temperatures but not under dry conditions.

BilRI, which has previously been identified as an IDP [298,314], was confirmed to exhibit IDP properties through extensive NMR studies. LEA proteins have been widely characterized as intrinsically disordered and highly hydrophilic [163], and the low GRAVY index of BilRI (–0.707) suggested its high hydrophilicity. Secondary structure predictions indicated that the three repeat sequences of the BilRI polypeptide exhibited helical properties. Some LEA proteins respond to dehydration by forming α-helical structures, which can form electrostatic interactions with lipid membranes [407,408]. Therefore, it is possible that BilRI could interact with membrane or matrix components, which could induce the formation of the predicted helical structures. The natural environment of BilRI was stimulated by the addition of *A. actinomycetemcomitans* LPS, but the obtained NMR spectra did not show any indication of folding or conformational changes in the predicted helical regions.

Some nematodes and larvae have been suggested to express LEA proteins while outside the host [409–411]. LEA proteins could protect parasitic species from environmental stresses and prolong their survival until a new host is found. *A. actinomycetemcomitans* is known to be transmitted from person to person [206], and this bacterium is likely to be subjected to environmental stresses while outside the host. BilRI, which exhibits LEA-like properties, might protect the pathogen against stressful conditions, such as lower temperatures, during transmission.

5.5 Future prospects and challenges

The link between the cytokine uptake and DNA uptake of *A. actinomycetemcomitans* should be further studied. Because HofQ and PilA interact with cytokines, they could provide a route for the cytokines across the outer membrane. However, the route for cytokine translocation across the inner membrane is unclear, as interactions between cytokines and inner membrane channel proteins participating in the DNA translocation have not been studied. Recombinant production of transmembrane proteins is, however, challenging, and studying the role of these proteins in cytokine uptake may require different approaches.

Another factor possibly affecting the cytokine uptake via the DNA uptake route is that the competence of *A. actinomycetemcomitans* can be regulated by catabolite repression [301]. Therefore, it is possible that the proteins involved in DNA uptake are not constitutively expressed, which can also control the ability to sequester cytokines. Detailed studies of the regulation and expression of these genes would

help to understand how competence and cytokine uptake are linked. Moreover, further studies are needed to clarify how cytokine binding and internalization modulates *A. actinomycetemcomitans* gene expression. This study showed no differences between IL-1β-treated and control cultures. Different growth conditions or longer incubation with the cytokine could provide additional information on the effect of cytokine uptake.

Because the type IV pilin homolog PilA is highly conserved in *A. actinomycetemcomitans*, it likely has important functions. Although PilA is essential for natural competence, most of the studied *A. actinomycetemcomitans* strains are noncompetent. This implies that PilA has other roles in addition to natural transformation. This study only focused on the one variant of PilA, which is found in the naturally competent strains. Thus, it would be interesting to study, for example, the other very common variant that is found in the noncompetent serotype b and c strains.

The multiple roles of BilRI shown in this study likely owe to its intrinsically disordered nature. However, further studies are required to clarify the functions of BilRI. The exact role of BilRI in the DNA uptake and natural transformation remains unclear, but comprehensive studies on how BilRI affects the outer membrane structure could provide additional information. Since BilRI is found in all sequenced *A. actinomycetemcomitans* strains (to date), its role in natural transformation could be studied using other naturally transformant strains than the D7S strain used in this study. Moreover, the different variants of the protein could possibly contribute differently to the transmission potential of *A. actinomycetemcomitans*. When it comes to host-microbe interactions, recombinant BilRI did not show any stimulation of the host cells. However, studies of the full immunogenic potential of BilRI would require purification of native BilRI from the *A. actinomycetemcomitans* outer membrane, which could be challenging due to the low expression levels shown in this study.

6 Conclusions

This study focused on three proteins of the oral opportunistic pathogen *A. actinomycetemcomitans*, namely, the outer membrane lipoprotein BilRI, the outer membrane secretin HofQ, and the type IV pilus subunit PilA. Previous studies of BilRI and HofQ revealed their importance in cytokine sequestration and uptake. Moreover, HofQ has been hypothesized to participate in natural competence owing to its DNA-binding ability, while PilA has been directly linked to natural competence. The current study further investigated the involvement of these proteins in natural transformation, cytokine binding, and host-microbe interactions, which provided new knowledge about their functions.

All three proteins, PilA, HofQ, and BilRI, are involved in the natural transformation of the competent *A. actinomycetemcomitans* strain D7S. They also interact with human proinflammatory cytokines. Therefore, the results of this study strengthen the previously proposed hypothesis that cytokine and DNA uptake by competent *A. actinomycetemcomitans* might occur simultaneously and/or through the same uptake route. The binding and uptake of cytokines may disturb the host immune response, as some cytokines, such as IL-8, attract neutrophils to the inflammation site. The uptake of cytokines has been shown to affect *A. actinomycetemcomitans* biofilm physiology. Initially, this study aimed to provide a deeper knowledge on the effect of IL-1β on gene expression in *A. actinomycetemcomitans*, but the experimental setup failed to provide detectable changes at the transcriptional level. However, gene expression studies suggested that deletion of either of the essential competence genes *hofQ* and *pilA* changed the physiology and pathogenicity of the bacterial biofilm. Because both HofQ and PilA likely participate in DNA uptake during natural transformation, it is possible that inhibiting the DNA uptake by a naturally competent *A. actinomycetemcomitans* strain leads to impaired biofilm formation.

The virulence-associated properties of *A. actinomycetemcomitans* PilA were shown for the first time in this study. PilA is highly homologous to type IVa pilin proteins, which are involved in a variety of functions in gram-negative species. PilA exhibited immunogenic properties, suggesting that it is involved in host-microbe interactions. Type IV pili have been considered potential drug targets owing to their

virulent properties [412]. Small molecules that inhibit the assembly of the pilus fiber, also referred to as pilicides, have the potential to decrease the virulence of the bacterium [412,413]. Optionally, since *A. actinomycetemcomitans* PilA interacts with host molecules, potential antimicrobial drugs could be developed to target the binding sites on the surface of PilA. Thus, its binding to human receptors and cytokines could be inhibited, which could allow the host immune response to work more efficiently. Comprehensive studies on the regulation and expression of *A. actinomycetemcomitans* PilA could provide information on how to combat bacterial virulence.

The results of this study suggest that BilRI may enhance bacterial survival during transmission, *i.e.*, outside the host, as BilRI is expressed at lowered temperatures. Comprehensive studies using native BilRI, which resides on the *A. actinomycetemcomitans* outer membrane, could elucidate the role of lipoproteins in transmission. Moreover, native BilRI could exhibit immunogenic properties. This study only showed that the polypeptide part of the lipoprotein did not stimulate antigen-producing leukocytes or induce cytokine secretion in macrophages.

Overall, this study provides new knowledge about the virulence mechanisms of the oral pathogen *A. actinomycetemcomitans*. Currently, bacterial infections are treated widely with antibiotics. Antibiotic resistance and biofilm formation present a threat to health care because they reduce the effectiveness of antibiotics. Therefore, the development of novel antimicrobial drugs is important to prevent severe bacterial infections. Moreover, the use of antimicrobial drugs that aim to block virulence factors, instead of antibiotics, may hinder the emergence of new antibiotic-resistant strains [414].

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