TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. A I OSA - TOM. 478

ASTRONOMICA - CHEMICA - PHYSICA - MATHEMATICA

VIRULENCE PROPERTIES OF *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* BIOFILM AND CHARACTERISATION OF ITS PUTATIVE CYTOKINE EXPLOITATION

by

Annamari Paino

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2013

From the Department of Biochemistry, University of Turku, Finland; The National Doctoral Programme in Informational and Structural Biology (ISB) and the University of Turku Graduate School (UTUGS), Turku, Finland

Supervised by

Docent Riikka Ihalin, Ph.D.
Department of Biochemistry
University of Turku

Marja Pöllänen, DDS, Ph.D.
Department of Periodontology
Institute of Dentistry
University of Turku, Finland

Reviewed by

Associate Professor Georgios Belibasakis, DDS, Ph.D.
Section of Oral Microbiology and Immunology
Institute of Oral Biology
University of Zurich, Switzerland

Docent Pirkko Pussinen, Ph.D.
Institute of Dentistry
University of Helsinki, Finland

Opponent

Professor Mikael Rhen, Ph.D.
Department of Microbiology, Tumor and Cell Biology
Karolinska Institute, Sweden

ISBN 978-951-29-5602-9 (PRINT)
ISBN 978-951-29-5603-6 (PDF)
ISSN 0082-7002
Painosalama Oy – Turku, Finland 2013

ABSTRACT

Biofilms are surface-attached multispecies microbial communities that are embedded by their self-produced extracellular polymeric substances. This lifestyle enhances the survival of the bacteria and plays a major role in many chronic bacterial infections. For instance, periodontitis is initiated by multispecies biofilms. The phases of active periodontal tissue destruction and notably increased levels of proinflammatory mediators, such as the key inflammatory mediator interleukin (IL)-1β, are typical of the disease. The opportunistic periodontal pathogen *Aggregatibacter actinomycetemcomitans* is usually abundant at sites of aggressive periodontitis. Despite potent host immune system responses to subgingival invaders, *A. actinomycetemcomitans* is able to resist clearance attempts. Moreover, some strains of *A. actinomycetemcomitans* can generate genetic diversity through natural transformation, which may improve the species' adjustment to the subgingival environment in the long term.

Some biofilm forming species are known to bind and sense human cytokines. As a response to cytokines, bacteria may increase biofilm formation and alter their expression of virulence genes. Specific outer membrane receptors for interferon-γ or IL-1β have been characterised in two Gram-negative pathogens. Because little is known about periodontal pathogens' ability to sense cytokines, we used *A. actinomycetemcomitans* as a model organism to investigate how the species responds to IL-1β. The main aims of this thesis were to explore cytokine binding on single-species *A. actinomycetemcomitans* biofilms and to determine the effects of cytokines on the biofilm formation and metabolic activity of the species. Additionally, the cytokine's putative internalisation and interaction with *A. actinomycetemcomitans* proteins were studied. The possible impact of biofilm IL-1β sequestering on the proliferation and apoptosis of gingival keratinocyte cells was evaluated in an organotypic mucosa co-culture model. Finally, the role of the extramembranous domain of the outer membrane protein HofQ (emHofQ) in DNA binding linked to DNA uptake in *A. actinomycetemcomitans* was examined.

Our main finding revealed that viable *A. actinomycetemcomitans* biofilms can bind and take up the IL-1β produced by gingival cells. At the sites of pathogen-host interaction, the proliferation and apoptosis of gingival keratinocytes decreased slightly. Notably, the exposure of biofilms to IL-1β caused their metabolic activity to drop, which may be linked to the observed interaction of IL-1β with the conserved intracellular proteins DNA binding protein HU and the trimeric form of ATP synthase subunit β. A *Pasteurellaceae*-specific lipoprotein, which had no previously determined function, was characterised as an IL-1β interacting membrane protein that was expressed in the biofilm cultures of all tested *A. actinomycetemcomitans* strains. The use of a subcellular localisation tool combined with experimental analyses suggested that the identified lipoprotein, bacterial interleukin receptor I (BilRI), may be associated with the outer membrane with a portion of the protein oriented towards the external milieu. The results of the emHofQ study indicated that emHofQ has both the structural and functional capability to bind DNA. This result implies that emHofQ plays a role in DNA assimilation. The results from the current study also demonstrate that the Gram-negative oral species appears to sense the central proinflammatory mediator IL-1β.

TABLE OF CONTENTS

ABBREVIATIONS

A	Absorbance
aa	Amino acid
ABTS	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
Acp	Accessory protein
AHL	Acyl homoserine lactone
AI	Auto inducer
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
ATCC	American type culture collection
ATP	Adenosine triphosphate
BilRI	Bacterial interleukin receptor I
cBilRI	Cytoplasmic form of BilRI
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
Caf1	Capsule antigen fraction 1
Caf1A	Capsule antigen fraction 1 assembly
cas	CRISPR (see below) associated genes
CD	Cluster of differentiation
Cdt	Cytolethal distending toxin
CFU	Colony forming unit
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CRISPR	Clustered regularly interspaced short palindromic repeats
CRP	Cyclic AMP receptor protein
CSP	Colony-stimulating factor-1
DAB	3,3′-Diaminobenzidine
DAMP	Damage-associated molecular pattern
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Deoxyribonucleic acid
eDNA	Extracellular DNA
ssDNA	Single strand DNA
DPD	4,5-Dihydroxy-2,3-pentanedione
EDTA	Ethylenediaminetetraacetate acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EmaA	Extracellular-matrix protein adhesin-A
EPS	Extracellular polymeric substance
FITC	Fluorescein isothiocyanate
Flp	Fimbrial low molecular-weight pili
GCF	Gingival crevicular fluid
GMC	Gingival mucosa co-culture

HACEK	HACEK-group contains *Haemophilus influenzae, H. parainfluenzae, H. aphrophilus, H. paraphrophilus, Cardiobacterium hominis, Eikenella corrodens* and *Kingella kingae*
HeLa	Human cervical carcinoma cells
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline
HGEC	Human gingival epithelial cell
HGF	Human gingival fibroblast
HGK	Human gingival keratinocyte
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HU	Histone-like protein (from *E. coli* strain U93)
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IHF	Integration host factor
IL	Interleukin
IL-1R	IL-1 receptor
IL-1Ra	IL-1 antagonist
IM	Inner membrane
IPTG	Isopropyl β-D-thiogalactopyranoside
JE	Junctional epithelium
kDa	Kilodalton
LAP	Localized aggressive periodontitis
LB	Luria-Bertani-medium
Lgt	Phosphatidylglycerol/prolipoprotein diacylglycerol tranferase
Lnt	Phospholipid/apolipoprotein transacylase
Lol	Lipoprotein localication machinery
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinase
MP	Membrane protein
MS	Mass spectrometry
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
MW	Molecular weight
NF-kβ	Nuclear factor-kappa B
OD	Optical density
OM	Outer membrane
Omp	Outer membrane protein
OPG	Osteoprotegerin
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pI	Isoelectric point
PG	Peptidoglycan
PGA	Poly N-acetyl-D-glucosamine
PMNL	Polymorphonuclear leukocyte

PMSF	Phenylmethylsulfonyl fluoride
PRR	Pattern-recognition receptor
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B-ligand
RNA	Ribonucleic acid
mRNA	Messager RNA
sRNA	Small RNA
RT	Room temperature
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
scFv	Single-chain variable antibody fragment
SIGIRR	Single immunoglobulin interleukin-1 receptor related
SPII	Signal peptidase II
spe	Spectinomycin
SSC	Side scatter
STI	Soybean trypsin inhibitor
tad	Tight adherence locus
TdT	Terminal deoxytransferase enzyme
TEM	Transmission electron microscopy
Tfp	Type IV pili
T_H	Helper T-lymphocyte
TIR	Toll/IL-1 receptor
TRIF	TIR domain-containing adaptor protein inducing interferon-β
TLR	Toll like receptor
TSA	Tryptic soy agar
TSB	Trypticase soy broth
TSB-YE/Glc	TSB supplemented with yeast extract and glucose
TNF-α	Tumor necrosis factor-α
USS	Uptake signal sequence
WT	Wild type
QS	Quorum sensing
6xHis-tag	Six-histidine tag
8xHis-tag	Eight-histidine tag

ABBREVIATIONS FOR AMINO ACIDS

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methione
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

LIST OF ORIGINAL PUBLICATIONS

This thesis comprises the following publications. I have referred them in the text with their roman numerals.

I Paino, A., Tuominen, H., Jääskeläinen, M., Alanko, J., Nuutila, J., Asikainen, S.E., Pelliniemi, L.J. Pöllänen, M.T., Chen, C., Ihalin, R. (2011) Trimeric Form of Intracellular ATP Synthase Subunit β of *Aggregatibacter actinomycetemcomitans* Binds Human Interleukin-1β *PLoS ONE*, **6**(4):e18929

II Paino, A.Lohermaa E., Sormunen, R., Tuominen, H., Korhonen, J., Pöllänen, M.T., Ihalin, R. (2012) Interleukin-1β is internalised by viable *Aggregatibacter actinomycetemcomitans* biofilm and locates to the outer edges of nucleoids *Cytokine* **60**:565-574.

III Paino, A., Ahlstrand, T., Nuutila, J., Navickaite, I., Lahti, M., Tuominen, H.,Välimaa, H., Lamminmäki, U., Pöllänen, M.T., Ihalin, R. (2013) Identification of Novel Bacterial Outer Membrane Interleukin-1β Receptor from *Aggregatibacter actinomycetemcomitans PLoS ONE* **8**(7):e70509

IV Tarry, M., Jääskeläinen, M., Paino, A., Tuominen, H., Ihalin, R., Högbom, M. (2011) The Extra-Membraneous Domains of the Competence Protein HofQ Show DNA Binding Flexibility and a Shared Fold with Type I KH Domains *J. Mol. Biology* **409**:642-653

1. INTRODUCTION

Bacterial biofilms are surface attached microbe communities in which microbial cells are surrounded by a matrix of extracellular polymeric substances. This biofilm lifestyle is hypothesised to be the reason for most bacterial infections [1]. The structural properties of complex polymicrobial biofilms and the interaction between the community members improve resistance against the host defence system targeted against biofilms (reviewed in [2,3]). Moreover, biofilm cells actively monitor their milieu and dynamically adjust their properties to the prevailing environment. Therefore, bacteria express particular genes only when it is essential. For instance, expression of bacterial virulence genes is regulated by sensing changes in the levels of certain compounds, such as bacterial signalling molecules, host derived compounds, toxic oxidants and iron (see reviews [4-6]). Notably, the bacteria living in biofilms have been suggested to exploit cytokines, the proinflammatory mediators of the host, to modulate their gene expression and biofilm formation [7-9]. Two bacterial cytokine receptors have even been described [8,10]. The outer membrane protein of Gram-negative *Yersinia pestis*, capsule antigen fraction 1 assembly protein (Caf1A), binds human IL-1β [10]. Another Gram-negative bacterium *Pseudomonas aeruginosa* binds IFN-γ through the outer membrane protein OprF [8]. However, the detailed mechanism of bacterial cytokine sensing is still incomplete. Moreover, it is unknown whether bacterial cytokine sequestering manipulates host-cytokine signalling.

Periodontitis is an infectious and inflammatory disease in tooth-supporting tissues, and it is associated with subgingival biofilms. The presence of high cytokine levels in gingival crevicular fluid is an indication of an active host inflammatory immune response during the aggressive form of the disease [11-16]. The host's own immune response against subgingival pathogens mediates periodontal tissue destruction in an indirect manner (see reviews [17,18]). This thesis investigated for the first time if an oral opportunistic pathogen utilises the host inflammatory marker interleukin-(IL)-1β. The ability of a bacterium to disturb host cytokine signalling would partly explain the more quiescent phases of the disease. *Aggregatibacter actinomycetemcomitans* was chosen as a model organism for the study as the species is typical of subgingival biofilms in aggressive periodontitis [19-22], and its genome sequence is known [23]. IL-1β is a key proinflammatory mediator (see reviews [24,25]) that is expressed at high levels in periodontal tissue during aggressive periodontitis [12]. This more detailed molecular mechanism of pathogen-host crosstalk using cytokines will increase the understanding of virulence regulation of pathogens during chronic infections.

2. LITERATURE REVIEW

2.1. Subgingival species in periodontal health and disease

Hundreds of different bacterial species have chosen to live in the oral habitat. It is estimated that each individual may host from 100 to 200 different oral bacteria species (see reviews [22,26]), whereas almost 1200 different taxa have been identified in the entire human oral microbiome using 16S ribosomal RNA gene sequencing [27]. One explanation for the successful colonisation of the oral niche by diverse micro-organisms is their ability to live on the tooth as surface attached communities or biofilms.

In general, the biofilm mode of growth within a disease course is estimated to be a requisite for over 80% of bacterial infections [1]. These biofilm-based infections are typically highly tolerant of the host immune system and antibiotics (reviewed in [2]). For instance, periodontitis is a common inflammatory disease in the tooth-supporting tissues that is induced by bacterial biofilms. The rotation of short active tissue destruction and extended episodes of inactivity is characteristic of periodontal disease [11]. In the aggressive forms of the disease, rapid and severe destruction occurs in tooth-supporting connective tissue and alveolar bone. Severe periodontitis affects approximately 15-20% of adults, and the untreated disease leads to tooth loss among early middle-aged individuals [28].

Periodontitis is a complex inflammatory disease. Therefore, both disease initiation and progression can vary greatly among individuals. Though bacteria are essential for periodontitis initiation, host genetic and environmental factors also have a major impact on disease severity (reviewed in [29]). According to the "ecological plaque hypothesis" by Philip Marsh (see review [30]), a dynamic relationship exists between the environment and the composition of the dental biofilm. Formation of the enhanced biofilm in the subgingival site launches an active immune response. The response targeting the bacteria may cause an alteration in the subgingival milieu that supports the outgrowth of periodontal pathogens [30]. Attempts by the host defence system to combat the bacteria cause tissue destruction in the periodontal area, while the biofilm remains unharmed (reviewed in [31]). Additionally, the genetic and ethnic background of the individuals and lifestyle-associated factors, such as smoking [32], can increase the risk of periodontal diseases (reviewed in [29]). For instance, it has been suggested that individuals with single nucleotide polymorphisms in specific locations in two proinflammatory mediators, tumour necrosis factor (TNF) and interleukin (IL)-1A, are vulnerable to periodontitis if they are also colonised by the combination of three periodontopathogenic species, *Tannerella forsythia* (formerly *Bacteroides forsythus*), *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) [33]. An imbalance in the cytokine networks has been suggested to play a critical role in the progression of periodontitis [34].

Additionally, the microbial profile between periodontal patients fluctuates considerably [22,33]. Subgingival biofilms can be comprised of a complex mixture of archaea, bacteria, fungi and virus species (reviewed in [35]) that are surrounded by the protective extracellular matrix. However, Gram-negative species usually dominate Gram-positive

species in periodontal disease. *P. gingivalis*, *T. forsythia*, *A. actinomycetemcomitans, Fusobacterium, Prevotella, Campylobacter* and *Treponema denticola* are Gram-negative periodontal pathogens frequently identified from the subgingival plaques of periodontal patients using cultivation-based identification techniques [36-38]. Moreover, the use of a modern technique, checkerboard DNA-DNA hybridisation, has verified the presence of high levels of *A. actinomycetemcomitans* in the most diseased sites of generalised aggressive periodontitis subjects [22]. In addition to bacterial species, the fungus *Candida albicans* [39] and herpesviruses [40] are often isolated from subgingival sites of periodontal patients. Due to the complex composition of subgingival flora both in healthy and diseased individuals, it is recommended that one also concentrate on the functional diversity of bacteria rather than only on the phylogenetic diversity when host-microbe interactions are investigated (reviewed in [35]).

The properties of biofilms and thus their ability to cause disease are much more than the sum of the properties of the individual cells that form them. Therefore, even bacterial species that occur in low abundance may have a significant impact on the entire biofilm community composition, diversity and structure [41,42]. The presence of two periodontal pathogens, *A. actinomycetemcomitans* and *P. gingivalis,* influences the gene expression profiles of the nonpathogenic community members in a multispecies biofilm model [43]. Additionally, even different isolates of one bacterial species demonstrate different levels of virulence. For instance, individuals of African ancestry more often accommodate a highly virulent JP strain of *A. actinomycetemcomitans* [44] that produces enhanced amounts of leukotoxin [45,46]. Additionally, this strain has been suggested to increase the risk for early onset periodontitis, as the JP2 strain is often isolated from young patients with localised aggressive periodontitis (LAP, formerly localised juvenile periodontitis) [44,47]. More importantly, recent studies investigating the response of gingival cells to periodontopathogens have shown different cytokine production profiles after exposure to either planktonic or biofilm forms of oral bacteria [48,49]. Despite this result, most of the previous studies investigating the interaction of gingival cells with periodontopathogens have been performed with planktonic bacteria.

2.2. The opportunistic oral pathogen *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans is a Gram-negative, nonmotile, facultative anaerobic coccobacillus. It is rod-shaped and 0.4–0.5 μm x 1.0–1.5 μm in size [50]. *A. actinomycetemcomitans* exhibits enhanced growth under micro-aerophilic conditions [51]. This species is a member of the *Pasteurellaceae* family, which contains many mucosal surface-preferring pathogens of the upper respiratory tract and oral cavity [52]. *A. actinomycetemcomitans* is able to attach firmly to surfaces such as teeth and subgingival crevicular epithelial cells [53,54]. In conjunction with other species, *A. actinomycetemcomitans* forms subgingival biofilms [55-58] and colonises moderate gingival pockets of 5 to 7 mm in depth [59]. *A. actinomycetemcomitans* can also invade the oral epithelium [60,61] and human gingival fibroblasts [62], enhancing the persistence of this pathogen against periodontal treatment.

This opportunistic oral pathogen is included in the periodontopathogens group [20], though the species can occasionally be isolated from healthy sites of the periodontium [21],

and its low occurrence at diseased sites is not unusual [15,22]. However, numerous studies have reported the presence of *A. actinomycetemcomitans* biofilms in diseased sites with aggressive tissue destruction [19-21]. Additionally, a recent study supports the role of this species in periodontitis by demonstrating that high levels of *A. actinomycetemcomitans* accompanied by *Capnocytophaga sputigena* were found at sites of advanced aggressive periodontitis [22]. In addition to periodontal sites, *A. actinomycetemcomitans* can exist in sites that are very far from an oral cavity, such as in atherosclerotic plaques [63-65]. *A. actinomycetemcomitans* is actually linked to atherosclerotic diseases. First, *A. actinomycetemcomitans* belongs to the HACEK group with *Haemophilus influenzae, H. parainfluenzae, H. aphrophilus, H. paraphrophilus, Cardiobacterium hominis, Eikenella corrodens* and *Kingella kingae*. The members of the HACEK group are all Gram-negative bacteria known to cause clinical diseases, such as endocarditis. In reality, 4% of endocarditis may be caused by bacteria belonging to the HACEK group (reviewed in [66]). Moreover, high levels of serum immunoglobulin (Ig)-A antibodies against *A. actinomycetemcomitans* [67,68] and salivary *A. actinomycetemcomitans* are associated with coronary artery disease outcomes [68].

A. actinomycetemcomitans can be divided into seven serotypes (a-g) based on the different composition of the surface polysaccharides of the species [69-73]. It has been suggested that the virulence of the different strains can be serotype-specific [21,47,73]. However, it is known that genomic differences might be greater within strains of the same serotypes than between strains of different serotypes [74]. In fact, the entire genomic sequences of some *A. actinomycetemcomitans* strains are known. For example, the complete genome of the *A. actinomycetemcomitans* D7S-1 strain (serotype a) is composed of 2,308,328 nucleotides and 2,432 predicted coding sequences [23]. The circular genome of *A. actinomycetemcomitans* D11S-1 (serotype c) is 2,105,764 nucleotides in length and contains 2,134 predicted coding sequences [75]. Therefore, different *A. actinomycetemcomitans* strains may display notable genomic diversity both in their genomic content and arrangement [74,76]. Based on the genomic data analysis of eleven *A. actinomycetemcomitans* strains, approximately 20% or less of the genes of each *A. actinomycetemcomitans* strain encode flexible genes, whereas the rest of the genes belong to the core genome [74]. Knowledge of species genetic diversity and exact sequence alterations have explained why some strains of this species possess unique pathogenic potential. For instance, the elevated leukotoxin expression of the JP2 strain is caused by a specific 530-base pair (bp) deletion within the leukotoxin-encoding gene promoter region [46,77]. It is also known that the JP2 strain is unable to use human haemoglobin as its iron source, as the gene encoding haemoglobin-binding protein A is nonfunctional in the strain. The JP2 strain is thus dependent on other mechanisms for the acquisition of this essential compound for bacterial growth. [78]

2.2.1. Molecular players of *A. actinomycetemcomitans* in host-pathogen crosstalk

Toxins and their role in A. actinomycetemcomitans-host interaction

A. actinomycetemcomitans can produce toxins that have a major impact on the viability and proliferation of human cells. Leukotoxin interacts specifically with the leukocyte-specific CD18 antigen subunit of dimeric lymphocyte function-associated antigen-1

receptor [79]. Leukotoxin then kills leukocytes, such as polymorphonuclear leukocytes [80] and monocytes [81], by forming pores in their cell membranes. Additionally, it induces the rapid release of the proinflammatory mediators IL-1β and IL-18 from macrophages and monocytes [82,83].

Cytolethal distending toxin (Cdt) produced by *A. actinomycetemcomitans* has been shown to inhibit host cell proliferation by causing apoptosis and cell cycle arrest in the G2 phase [84] in several mammalian cell lines, including gingival fibroblasts [85,86] and gingival epithelial cells [87]. The ability to produce Cdt, a heterotrimeric AB-type genotoxin, is common in several Gram-negative mucocutaneous bacterial pathogens. This holotoxin is composed of CdtA, CdtB and CdtC components (reviewed in [88]). The CdtB component is the active subunit and functions as DNase [89] and phosphatidylinositol 3-phosphate phosphatase [90]. This DNase toxin likely induces double strand breaks in the DNA, leading to DNA damage response pathway activation in host cells, which eventually causes cell cycle arrest. The delivery of CdtB to host cells is dependent on the CdtA and CdtC components of the holotoxin (reviewed in [88]). Treatment of Chinese hamster ovary cells with labelled Cdt revealed that CdtB is found in the nucleus within 4.5 h after treatment. The labelled CdtC was found on the cell surface and in the cytosol, whereas CdtA remained associated with the outer membrane. The presence of CdtC in the cytosol suggests that CdtC functions as an accessory protein in the delivery of CdtB into host cells [91].

A. actinomycetemcomitans Cdt causes histologically visible damage to the oral epithelium [92-94]. *A. actinomycetemcomitans* Cdt may act by disrupting gingival epithelium cell-cell contacts, as Cdt has been shown to affect the allocation and expression of adherens junction proteins, including E-cadherin, β-catenin and β-actin, in epithelial cells [94]. The structural integrity of the gingival epithelium is essential in preventing attempts by the bacteria to enter the underlying connective tissue [94]. It has also been hypothesised that defects in the proliferation of epithelial cells and periodontal fibroblasts due to Cdt exposure are connected to disturbances in the repair of the epithelial barrier and remodelling of the periodontal connective tissue (reviewed in [95]). Additionally, Cdt can induce receptor activator of nuclear factor kappa B-ligand (RANKL) expression in gingival fibroblasts, periodontal ligament cells [96] and Jurkat T-cells [97], thus contributing to bone destruction. Therefore, Cdt can be considered a highly potent virulence factor of *A. actinomycetemcomitans* in periodontitis.

Adhesins of A. actinomycetemcomitans interact directly with host surface proteins

Adhesins are bacterial cell surface components that mediate bacterial adhesion to eukaryotic cell surfaces through binding to eukaryotic surface receptors. Adhesion is a central event for bacterial colonisation and invasion (reviewed in [98]). Bacterial adhesion to host cells involves complex crosstalk with the host cells. For instance, *Actinobacillus pleuropneumoniae* upregulates the expression of an autotransporter adhesin during its interaction with lung epithelial cells [99]. *A. actinomycetemcomitans* uses fimbrial appendages when it attaches to host tissues through nonspecific binding [55] or non-fimbrial adhesins when it interacts directly with host surface receptors [100]. The three central adhesins of *A. actinomycetemcomitans* and their functions are listed in Table 1.

Table 1. ***Adhesins of A. actinomycetemcomitans that interact with human extracellular matrix components***

Name	Function/Structural homologues	References
Aae	- autotransporter adhesin protein (~130 kDa) - homologue of epithelial cell adhesin (Hap) of *H. influenzae* that interacts with fibronectin, laminin, and collagen IV	[54,101]
Omp100/ApiA	- adhesin that interacts with fibronectin (OmpA monomer size of 32 kDa) - sequence homology with *Yersinia enterocolitica* adhesin YadA	[100,102,103]
EmaA	- extracellular-matrix protein adhesin-A - homotrimeric adhesin (monomer size of ~200 kDa) that binds to collagen (type I, II, III and V) and fibronectin - member of the oligomeric coiled-coil adhesin family of autotransporter adhesins; an orthologue of the *Y. enterocolitica* adhesin YadA	[104-107]

Autotransporter adhesin protein (Aae) was the first characterised *A. actinomycetemcomitans* protein found to be involved in the specific adherence of the species to epithelial cells. Deletion of the *aae* gene from two separate *A. actinomycetemcomitans* strains reduced the mutant strains' ability to bind to epithelial cells compared with wild type (WT) species [54]. Outer membrane protein 100 (Omp100) of *A. actinomycetemcomitans* is also known as surface-expressed *A. actinomycetemcomitans* adhesin. Deletion of the *omp100* gene from the *A. actinomycetemcomitans* Y4 strain led to decreased adhesion and invasion efficiency in mutants. Similarly to the *omp100* gene mutation, use of the anti-Omp100 antibody blocks the bacterial adhesin interaction with human extracellular matrix components and causes a significant decrease in *A. actinomycetemcomitans* adhesion to human cells [103]. Bacterial adhesin Omp100 interacts with human extracellular matrix fibronectin, and human gingival epithelial cells (HGEC) induce the expression of human beta defensins via the mitogen-activated protein kinase (MAPK) pathway as a consequence of this interaction [100]. Moreover, EmaA of *A. actinomycetemcomitans* binds specifically to collagen and fibronectin [104,105]. The deletion of this bacterial adhesin reduces the binding of an *A. actinomycetemcomitans* knockout strain to the exposed collagen of rabbit cardiac valve tissue by five-fold in comparison with a WT bacterium [106].

A. actinomycetemcomitans may adapt to changing environmental conditions by modulating its adhesion properties both at genomic and phenotypic levels; it shows differences in the expression of virulence factors related to bacterial adherence and colonisation when cultured in microaerophilic or anaerobic conditions [108]. The adhesion to epithelial cells is a prerequisite for subsequent bacterial invasion of periodontal tissues. *A. actinomycetemcomitans* can invade epithelial cells and remain viable inside the host cells, although little is known about the exact invasion mechanism [60]. On the contrary, invasion of the periodontal bacterium *P. gingivalis* into host cells is a complex process involving signal cascades and cytoskeletal rearrangements in host cells (reviewed in [109]). One putative explanation for the ability of *A. actinomycetemcomitans* to survive inside eukaryotic cells may be related to the expression of an invasion-associated enzyme

encoded by the *apaH* gene. This enzyme is diadenosine tetraphosphatase, which shows sequence homology with characterised bacterial invasion-associated genes such as *ygdP* of *E. coli* [110]. Interestingly, when cultured under anaerobic conditions, the species increased its expression of *apaH* and *emaA* genes compared with microaerophilic conditions. Similarly, its ability to invade epithelial cells increased under anaerobic conditions [108].

2.2.2. Natural competence in *A. actinomycetemcomitans*

Bacteria are thought to possess complex extracellular DNA (eDNA) uptake systems for various purposes, including providing nutrients and nucleic acid precursors, acquiring genomic diversity and enhancing their genomic stability [111,112]. Natural competence is a transient state in most bacterial species, and its regulation is dependent on factors such as the nutritional state and/or quorum sensing (QS) (reviewed in [113]). Competence regulation in *A. actinomycetemcomitans* has been investigated to a lesser degree than in its close relative species*, H. influenza,* which induces the expression of several competence-related genes during nutrient starvation or a shortage of nucleic acid precursors [114,115]. Competence development in *H. influenzae* is dependent on the expression of competence regulon genes, which are under the control of Sxy and cyclic AMP receptor protein (CRP) regulators [116]. Seventeen of the 26 competence regulon genes are required for successful natural transformation in *H. influenzae* [116]. Alternatively, competence development in Gram-positive *Streptococcus pneumonia* is mediated by a competence-stimulating peptide-dependent QS system [117]. Evidence of QS-dependent competence regulation in Gram-negative bacteria is found only in *Vibrio cholerae*. This species expresses the periplasmic single-stranded DNA (ssDNA) binding protein C*omEA* only at high cell densities, thus regulating DNA uptake in a QS-dependent manner [118,119].

Some *A. actinomycetemcomitans* strains, such as D7S-1, are naturally competent [120]. They import eDNA from the proximal milieu and combine it into their genomes through homologous recombination. It has been suggested that *A. actinomycetemcomitans* can use competence as a mechanism for adjusting to changing environmental conditions and that noncompetent strains arise when the genomic content of current strains is beneficial for the prevailing stable conditions [121]. Based on transformation experiments and bioinformatic analyses, competent *A. actinomycetemcomitans* strains belong to the minority [121,122]. In transformation assays, most serotype a strains are able to perform DNA uptake, whereas noncompetence is more typical in serotype b and serotype c strains [122]. The comparative genomic analysis of *A. actinomycetemcomitans* strains suggests that the genomic variance among species is connected with natural competence [121]. The genomes of competent strains appear to be more dynamic compared with noncompetent strains; noncompetent strains often contain foreign DNA derived from plasmids and phages. Additionally, noncompetent *A. actinomycetemcomitans* strains demonstrate an increased number of deletions or loss of function mutations in genes encoding the system responsible for foreign DNA elimination. This loss of the bacterial adaptive immune system can be beneficial for minor adjustments, as it allows the intake of plasmids and phage DNA [121]. The bacterial adaptive immune system is known as the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas immune system. Cas proteins are encoded by CRISPR-associated genes (*cas*). These Cas proteins

can play a role in events such as CRISPR RNA processing and target DNA cleaving. The first requirement for a functional CRISPR/Cas immune system is the acquisition of short fragments of foreign nucleic acids and the integration of these fragments into the cognate repetitive loci of the host bacterium chromosome. The expression of a variety of diverse CRISPR RNAs in the host leads to complementary base pairing with invading foreign DNA, which causes the elimination of foreign DNA, such as DNA from phages and plasmids (reviewed in [123]).

2.3. Biofilms

2.3.1. Structural components of biofilms

In biofilms, single bacterial cells are embedded inside the self-produced extracellular polymeric substance (EPS) matrix that can comprise up to 90% of biofilm mass (reviewed in [124]). Due to the highly hydrated nature of the EPS, it has received nicknames such as glycocalyx or slime. However, the overall structure of mature biofilms is highly differentiated, and biofilms exhibit heterogeneity at the structural, chemical and biological levels. Biofilm matrix is composed of water; carbohydrate polymers, such as hexosamine that consists of the linear chains of poly N-acetyl-D-glucosamine residues (PGA) in β(1,6) linkage; eDNA; secreted proteins; absorbed nutrients; metabolites; and products of cell lysis. EPS plays a significant role in biofilm attachment to surfaces, aggregation of single cells and stabilisation of community structure (reviewed in [124,125]). Interestingly, the DNA-binding nucleoid-associated proteins IHF and HU (integration host factor and histone-like protein from *E. coli* strain U93, respectively) may play a major role in maintaining the integrity of the eDNA content of the EPS matrix [126,127]. For instance, *H. influenzae* biofilms showed destabilisation after treatment with anti-IHF antibody, and both DNA-binding proteins were required for biofilm formation in *E. coli* [126].

In vivo biofilms, such as subgingival biofilms, can contain hundreds of different bacterial species [26] without considering the virus and yeast species content. These different species and even different bacterial strains make their own contributions to the general biofilm architecture and especially to EPS consistency. Moreover, environmental conditions affect biofilm structure and formation (reviewed in [125]). Several environmental factors, such as temperature, nutritional conditions, oxygen availability, pH and iron level, may all have effects on the expression of biofilm determinants and biofilm formation (reviewed in [128]). The overall architecture of biofilms is a mushroom- or tower-like structure with water channels located between the pillars formed by microbial cell clusters immobilised in EPS (reviewed in [124,125]). The importance of these water channels for biofilm growth has been studied with Gram-positive *Bacillus subtilis*. The biofilm growth of this species appears to be dependent on the presence of structured water channels that allow efficient transportation of nutrients and waste products through the biofilm community [129]. However, little is known about the putative liquid transport mechanism and the presence of specific water channels inside the biofilms formed by Gram-negative bacteria or periodontal pathogens.

Inside a biofilm, compounds occur in gradients between different layers of the biofilm. Therefore, the concentration of essential compounds such as oxygen and nutrients varies.

Accordingly, anaerobic and aerobic micro-organisms prefer to inhabit different layers of biofilms. There can also be great heterogeneity in the metabolic activity of bacteria, as the biofilm may contain very active cells, low activity cells and even metabolically dormant persistent cells (reviewed in [130]). Bacteria have naturally developed different mechanisms to survive in nutrient scarce environments such as biofilms. For instance, slow-growing *A. actinomycetemcomitans* utilises a low-energy carbon source, lactic acid, and inhibits its glucose uptake. *In vitro* biofilm co-culture studies suggest that *A. actinomycetemcomitans* can avoid competition with fast-growing *Streptococcus gordonii* bacteria by using lactate, a secondary product of the energy-rich carbon metabolisation performed by *S. gordonii* [131]. Moreover, the *A. actinomycetemcomitans* biofilm responds to hydrogen peroxide (H_2O_2), another metabolite produced by *S. gordonii.* The presence of H_2O_2 in proximity to the *A. actinomycetemcomitans* biofilm induces the expression of catalase and the serum protein H binding protein ApiA. As result of this sensing, the *A. actinomycetemcomitans* biofilm become more resistant to toxic H_2O_2 through the action of catalase, and the biofilm can inhibit serum complement activity by binding to serum protein H. [132] The polymicrobial interaction between these two oral bacteria thus influences biofilm resistance to innate host immunity.

The structural properties of biofilms increase their resistance to host immune cells, antibodies, and various antimicrobials [147,148], (see review [133]). For instance, Gram-positive *Staphylococcus aureus*, which is associated with many surgical site infections [134], and Gram-negative periodontopathogen *Prevotella intermedia* can form biofilms resistant to phagocytosis [135]. Human macrophages are incapable of phagocytosing biofilm-associated *S. aureus*, and it has been suggested that this resistance is a result of the size and/or physical complexity of the intact biofilm [134]. Additionally, periodontopathogen *P. intermedia* biofilms are resistant to polymorphonuclear leukocytes (PMNL), although a strain unable to form biofilms is internalised by PMNLs [135]. The exopolysaccharide Pel and eDNA of *P. aeruginosa* biofilm have been suggested to both provide structural support for the biofilm and protect against positively charged antimicrobial aminoglycosides by binding these cations [136,137].

Interestingly, cells living in biofilms have been shown to more efficiently take up and integrate free eDNA than their planktonic counterparts [138-141]. For example, the oral Gram-positive bacterium *Streptococcus mutans* uses a biofilm-specific peptide pheromone QS signal transduction system to stimulate the uptake and incorporation of foreign DNA [138]. The transformation efficiency rates can be 10- to 600-fold higher in biofilms than in planktonic cultures [138]. However, the biofilm cultures of Gram-negative oral bacteria have not yet been used in transformation efficiency assays. Nevertheless, actively growing biofilms of Gram-negative *Acinetobacter* species are competent [142]. Additionally, studies with *H. influenzae* have shown that defects in the operons of the essential competence genes *pil* and *com* cause altered biofilm structures with reduced biofilm formation as well as the loss of transformability in mutants [143]. Particularly, all *pil* mutants showed significantly decreased biomass compared to the WT strain. Moreover, expression of both the *pil* and *com* genes was critical for the biogenesis and function of type IV pili (Tfp); this expression occurred only at alkaline pH (pH 8.5 to 9) and was not observed at neutral pH [143,144]. In general, intact Tfp structures are required for pilus-pilus interactions and bacterial cell autoaggregation in microcolony formation during the early phases of biofilm development (reviewed in [145]).

2.3.2. Biofilm lifecycle from single cell attachment to dispersion

The lifecycle of biofilms is a dynamic process composed of different stages that are shown in Figure 1. Microbial cells must first attach to human tissues or to abiotic surfaces. The colonisation of oral bacteria on surfaces, such as salivary pellicles on the teeth or subgingival epithelium, occurs in spatiotemporal order. Some bacterial species function as initial colonisers to which the late colonisers adhere [146]. When bacteria are attached densely enough to surfaces, they can communicate with each other through QS. QS signals control the secretion of EPS, subsequent biofilm maturation and detachment. The regulation of bacterial cell death has been suggested to be important for biofilm maturation, as the genomic DNA released from lysed cells is a central structural component for the developing biofilm matrix [149], (see review [150]). The final stage of biofilm formation includes the active release of cells from the biofilms into the environment and their colonisation of new surfaces [149](see review [151]).

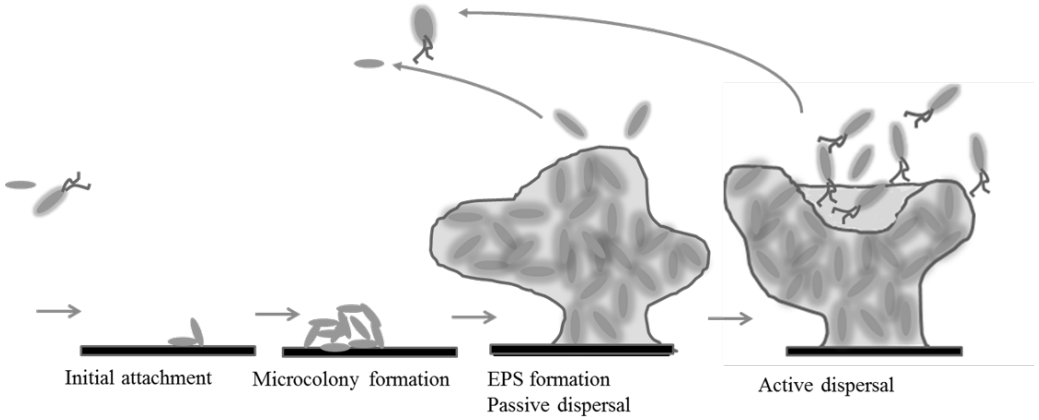


Figure 1. ***Schematic presentation of the different stages of the biofilm lifecycle.*** Biofilm formation initiates with the attachment of a single cell to a surface, followed by microcolony formation and the embedding of cells by their self-produced EPS. During the last stage, biofilms actively disperse cells. Modified picture from McDougald D. *et al*. 2013 [151].

In *A. actinomycetemcomitans,* the initial adhesion to abiotic surfaces is dependent on proteinaceous fimbriae [152,153], polysaccharides [154] and extracellular DNA [155]. The expression of *tight adherence* (*tad*) locus genes is required for nonspecific surface attachment by *A. actinomycetemcomitans* [55] and is essential for biofilm formation [156]. Fourteen different proteins (Flp1-Flp2-TadV-RcpCAB-TadZABCDEFG) encoded by the *tad* locus form the machinery of *A. actinomycetemcomitans* fimbriae production [55,157]. The fimbriae of *A. actinomycetemcomitans* are composed of fimbrial low-molecular-weight pili (flp) components [158], which are secreted through the outer membrane across a secretin channel formed by RcpA proteins. The localisation of RcpA as a multimeric complex in the outer membrane is dependent on TadD lipoprotein [159]. The only gene of the locus that is not required for functional fimbriae expression is *flp2* [157,160]. After 3 hours of contact with porcine lung epithelial cells, the expression of *rcpA, tadB* and biofilm biosynthesis-related genes increased in biofilms formed by the

Pasteurellaceae species *Actinobacillus pleuropneumoniae* [99]. This example implies that bacteria can modulate their biofilm properties as result of host-pathogen crosstalk.

In *A. actinomycetemcomitans*, iron is known to be particularly important in the regulation of both biofilm formation and species virulence. The assumption of iron's significance is supported by the fact that genomes of *A. actinomycetemcomitans* strains contain several putative iron uptake genes [161] and that the species can bind human proteins containing iron, such as haemin, haemoglobin and lactoferrin [162]. Moreover, the regulatory role of iron in *A. actinomycetemcomitans* biofilm formation is strengthened by the finding that iron deficiency increased both the number of colony aggregates and the entire biofilm mass [162,163]. Iron supplementation has been shown to reduce the transcription of genes required for biofilm development in *A. actinomycetemcomitans* [163]. For instance, the expression of fimbria, EPS proteins and lipopolysaccharide (LPS) biosynthesis genes was decreased [163]. This regulation of iron uptake likely occurs via the expression of ferric uptake regulator (Fur) and Fur-responsive noncoding small RNAs (sRNA) [164,165]. Fur has been shown to be involved in the regulation of gene expression of iron uptake-related genes in several Gram-negative and Gram-positive species (reviewed in [166]). The overexpression of a single Fur-regulated RNA reduced biofilm formation and also reduced the expression of leukotoxin secretion protein D in *A. actinomycetemcomitans*. In the presence of iron, cells express both Fur and Fur-responsive sRNAs, which most likely act by base pairing with their target mRNA. [165]

A. actinomycetemcomitans regulates its biofilm dispersal actively via the expression of dispersin B protein. Dispersin B is an enzyme that degrades the adhesive polysaccharide components of the biofilm matrix and causes biofilm dispersion [154,167,168]. Biofilm culture studies have shown that *A. actinomycetemcomitans* biofilms grown in liquid culture showed dispersal and satellite colony formation after 3 days of culture [168]. To successfully survive in hostile environments, the biofilm lifecycle must be highly regulated to enable its formation, renewal and dissemination. Bacteria have thus evolved signalling systems to both temporally and spatially synchronise gene expression in biofilm communities.

2.3.3. Cell-cell signalling between bacteria

QS, a complex cell-cell signalling mechanism, is used to adjust biofilm formation, virulence factor expression and many other biofilm properties under constantly changing conditions. This communication mechanism is density-dependent. Quorum signifies a threshold in attached microbe cell density, which is required to initiate the coordinated expression of QS-regulated genes. The neighbouring cells in biofilms produce and sense small extracellular signalling molecules, such as acyl homoserine lactones (AHLs) (see review [169]), furan derivatives (see review [170]) and small peptides [171], to synchronise their responses to external signals. However, bacterial crosstalk is not entirely universal, as bacteria express diverse signal compounds and their cognate receptors in a species-dependent manner. For example, several periodontal pathogens, including *A. actinomycetemcomitans*, are unable to synthesise AHL signals [172] (reviewed in [173,174]).

Several Gram-negative and Gram-positive bacteria can encode the LuxS enzyme, which acts in the detoxification process of a metabolic intermediate. LuxS converts toxic S-adenosylhomocysteine into 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine

(reviewed in [175]). The synthesised DPD is converted to an autoinducer (AI)-2 species by spontaneous rearrangements. The furan-derived AI-2 signal was initially proposed to be a universal signal in intra- and interspecies communication among the bacterial community [176], but genomic studies have shown that only some species have a LuxPQ or Lsr receptor complex for AI-2 [177]. In fact, LuxS is known to regulate virulence gene expression in enterohemorrhagic *E. coli* [178] and several other species, including the oral pathogens *P. gingivalis* [179] and *A. actinomycetemcomitans* [180]. In *E. coli*, LuxS controls the expression of the two-component QseBC regulatory system, which senses the signalling molecule AI-3 [181]. The QseC component is the signal sensor kinase, and QseB is the response regulator [182].

A. actinomycetemcomitans can respond to AI-2, and it possesses a QS signalling system [183]. Although the signalling system is not required for the growth of planktonic bacteria, it appears to be essential for biofilm growth of the species. The periplasmic proteins LsrB and RspB are putative receptors for AI-2 and mediate the compound's intake in *A. actinomycetemcomitans* [184]. The species induces QseBC expression after sensing AI-2 [180]. Researchers have started to uncover the significance of QS signalling for virulence in the *Pasteurellaceae* family. For example, the mutation of *qseC, lsrB* or *rbsB* in *A. actinomycetemcomitans* resulted in a decrease in the species virulence against mice [180]. Furthermore, alveolar bone loss was significantly reduced in mice infected with these mutant strains compared with mice infected with WT *A. actinomycetemcomitans*. The biofilm mass was also lower in cultures formed by the *qseC* mutant strain than in WT cultures [180]). In *H. influenzae*, deletion of the *rspB* gene resulted in the loss of biofilm load in the middle ear of chinchillas [185].

2.3.4. DNA uptake mechanism

The route of DNA from the extracellular space to the cytoplasm likely contains the following steps: specific signal sequence recognition by a DNA binding receptor, DNA pulling through the secretin channel across the outer membrane to the cytoplasmic membrane and single-stranded DNA entry into the cytoplasm (reviewed in [113,186]). However, many details of DNA transport have yet to be uncovered, though many genes have been determined to be essential for the transport.

The presence of a specific uptake signal sequence (USS) consisting of the 9-bp core sequence 5-AAGTGCGGT-3 is required for efficient DNA transformation in *Pasteurellaceae* species including *H. influenzae, A. actinomycetemcomitans*, *Pasteurella multocida, Mannheimia succiniciproducens*, and *Histophilus somni* (*Haemophilus somnus*) [120,187-189]. This genus-specific core sequences occurs approximately 1,760 times in the genome of the *A. actinomycetemcomitans* HK1651 strain [190]. However, the extracellular surface receptor responsible for USS binding has not yet been characterised in *Pasteurellaceae*. More is known about the specific binding event in *Neisseria* species, as the minor pilin ComP protein prefers to interact with *Neisseria*-specific USS [191]. However, *A. actinomycetemcomitans* does not contain homologues to pilin ComP according to BLASTP searches.

Translocation of the DNA through the outer membrane is accomplished by the Type IV pili secretion system. According to one dominant hypothesis, DNA passes the outer membrane by attaching to the tip of a retracting pilus (reviewed in [192]). The genes *pilABCD* encode the components of the Tfp-system of *A. actinomycetemcomitans*, and

these proteins are essential for its transformation [188]. Recently a completely novel *urpA* gene, which encodes uptake-related protein A (UrpA), was found to be essential for species competence, though its exact function in DNA uptake is not yet known [193]. However, mutational studies have suggested that deletion of *urpA* leads to increased bacterial aggregate formation in *A. actinomycetemcomitans* broth culture; according to bioinformatics analyses, UrpA localises in the cytoplasm [193]. In *H. influenzae*, the proteins encoded by the *com* operon (*comABCDEF)* and the four proteins encoded by the *pil* operon are essential for normal Tfp biogenesis and function [143]. The putative roles of these Pil proteins in DNA uptake have been revealed by homology analyses and functional studies performed with *H. influenzae* and *Neisseria.* The hypothesised gated channel for DNA uptake by *Neisseria* is formed by the outer membrane protein complex, a dodecameric form of PilQ. Based on cryoelectron microscopy studies, this secretin pore was found to have an internal cavity 5.5 nm in diameter [194]. Moreover, the inner cavity domains of *neisserial* PilQ bind both ssDNA and double-stranded DNA in a sequence-independent manner [195]. Proteins homologous to PilQ include the ComE protein in *H. influenzae* [144] and HofQ in *A. actinomycetemcomitans* [23]. In *H. influenzae*, PilA is the major pilin subunit [143]. PilB protein shows homology to hexameric type II secretion and Tfp assembly adenosine 5'-triphosphatases [144]. PilD has homology to prepilin peptidases [144], which are responsible for processing prepilin molecules prior to pili assembly into filaments [196], and PilC has been linked to the regulation of pilus retraction [197]. The DNA transport through the outer membrane appears to be adenosine 5'-triphosphate (ATP)-dependent because the use of ATPase inhibitors or proton motive-force distributors quenched transport in Gram-negative *Helicobacter pylori* [198].

Translocation of DNA through the cytoplasmic membrane is the second step in the DNA uptake process. The process begins when the transforming DNA binds to soluble ComE1 protein located in periplasmic space [199,200]. The binding of ComE1 to incoming DNA has been suggested to exert a force on the DNA, which drives the transfer of ssDNA into the cytoplasm [192]. The enzymes responsible for the conversion of DNA to the single-stranded form have not been described *in vivo*, but it is known that the other strand of DNA must be degraded from the 5′ end before the resulting ssDNA is internalised in the 3′-5′direction across the cytoplasmic membrane [201]. The transportation of ssDNA into the cytoplasm occurs across the integral cytoplasmic membrane channel known as ComA in *Neisseria* [202] and as the DNA internalisation-related competence protein ComEC/Rec2 in *A. actinomycetemcomitans*. This inner membrane channel is highly conserved among competitive bacteria (reviewed in [192]). Inside the cell, the binding of DNA processing protein A to ssDNA protects the molecule against the degradation caused by cellular nucleases [203] and facilitates homologous recombination by the chromosomal recombinase RecA [204]. Successful transformation is also dependent on the ComM protein, which is required for DNA integration into the genome in *H. influenzae* [205]. The predicted model of DNA uptake of *A. actinomycetemcomitans* is summarised in Figure 2, and the different protein components of the DNA uptake machinery of *N. meningitidis, H. influenzae* and *A. actinomycetemcomitans* are described in Table 2.

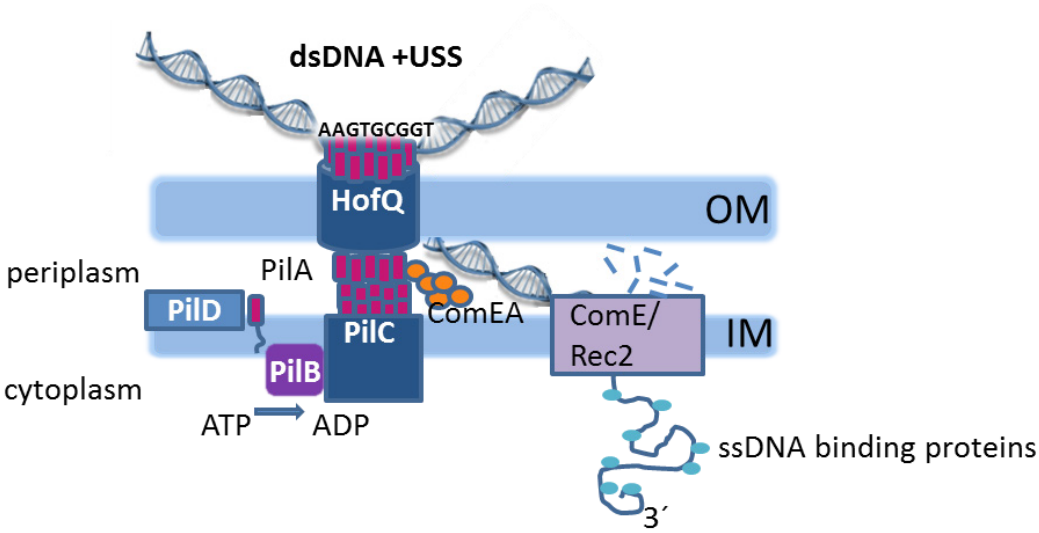


Figure 2. ***Model of DNA uptake in A. actinomycetemcomitans.*** Pil proteins (PilB, PilC and PilD) mediate the assembly of the pseudopilus. PilB is the cytoplasmic assembly ATPase, PilC is the integral inner membrane protein of the pilus assembly complex, and PilD is the peptidase required for prepilin processing. The pseudopilus formed by the assembly complex transvers the outer membrane pore formed by the multimeric HofQ. This DNA uptake machinery binds and specifically takes up DNA containing the USS. Retraction of the pseudopilus and the putative force exerted on the DNA by periplasmic ComEA leads to DNA transfer into the periplasm. The subsequent transfer of ssDNA across the inner membrane ComEC/Rec2 channel and its binding by protective proteins are the last steps prior to homologous recombination. Figure modified from Krüger and Stingl 2011 [192].

Table 2. Homologous DNA uptake machinery proteins in three Gram-negative species. The table includes each protein component's described function in *N. meningitidis* and the predicted subcellular localisation of the competence proteins in *A. actinomycetemcomitas* D7S (*A. a*). Table modified from Carbonnelle *et al.* 2006 [206].

N. meningitidis	***H. influenzae***	***A. actinomycetemcomitans* D7S/ Accession number**		
Protein annotation			**Function in *N. meningitidis*** [192, 206]	**Predicted localisation in *A.a* D7S** [207,208]
PilQ	**ComE**	protein transport protein HofQ/ YP_006286718.1	Secretin channel for Tfp translocation across outer membrane	outer membrane/integral
PilE	**PilA**	putative fimbrial subunit PilA/ YP_006287292.1	Prepilin subunit	outer membrane and pilus
PilF& PilT	**PilB**	type IV pilus assembly protein/ (PilB)/YP_006287291.1	ATPase in pilus assembly/disassembly	cytoplasm
PilG	**PilC**	putative type IV pilin secretion protein (PilC)/YP_006287290.1	Multispanning transmembrane protein	inner membrane
PilD	**PilD**	prepilin peptidase (PilD)/ YP_006287289.1	Prepilin peptidase cleaves prepilin	inner membrane
ComE	**ComE1**	competence protein comEA/ YP_006286960.1	DNA packaging and may establish a driving force for DNA transportation through the OM	periplasm
ComA	**ComF & Rec-2**	DNA internalization-related competence protein ComEC/Rec2 /YP_006286022.1	DNA translocation across inner membrane	inner membrane
RecA	**RecA**	protein RecA/YP_006287021.1	Homologous recombination of incoming DNA	cytoplasm

2.4. Cytokines

2.4.1. Pathogen recognition leads to cytokine production

The innate host immune system provides the first line of defence against pathogens by eliminating pathogens and controlling the launch of a more specific adaptive immune response. A key step in this defence strategy is discrimination between pathogens and commensals before the initiation of a response. Activation of the inflammatory response is regulated by several protective effectors, as unnecessary inflammation can cause severe damage to host tissues (reviewed in [209]). When these regulatory blocks have been bypassed, pathogen elimination is mediated by phagocytes, including macrophages, neutrophils and dendritic cells. Inflammatory mediators, such as IL-1β, are conductors of complex immune responses.

To recognise pathogens, host cells encode divergent pattern recognition receptors (PRRs), which can recognise the conserved molecular features of the microorganism. Some classes of PRRs are membrane-attached receptors, such as Toll-like receptors (TLRs), which recognise pathogen-associated molecular patterns (PAMPs) in extracellular space or inside subcellular compartments, including endosomes and the cytosol. Some cytosolic PRRs can also recognise endogenous damage-associated molecular patterns (DAMPs) released by dying or damaged cells (reviewed in [210-212]). The binding of bacterial products such as lipopolysaccharides to their respective PRRs often trigger an innate immune response through the myeloid differentiation primary-response gene 88 (MyD88)-dependent pathway (see review [210] and Fig 3). This pathway involves nuclear factor (NF)-kB-dependent transcription of pro-IL-1β [210]. Moreover, in the case of TLR3 or TLR4, the binding of pathogen-derived components can lead to activation of the Toll/IL-1 receptor (TIR) domain-containing adaptor protein inducing interferon-β (TRIF)-dependent pathway (see review [210] and Fig 3). The activation of this pathway culminates in the expression of interferon (IFN)-α and -β [210,213].

In the gingival sulcus, the epithelial cells of the junctional epithelium are the first line of defence against bacteria. This mechanical epithelial barrier also acts as a sensor of microbial infection, as it contains microbe-recognising PRRs. The recognition of pathogens induces cytokine production in epithelial cells following inflammasome activation [214,215]. These cytokines are then employed in defence to recruit and activate immune cell populations at the site of infection. For example, human TLR2 and TLR4 mediate the recognition of exogenous ligands such as lipoproteins and lipopolysaccharides from Gram-negative bacteria. TLR2 and TLR4 have been suggested to play a role in controlling *A. actinomycetemcomitans* infection [216,217]. Knocking out these genes in mice caused a significant reduction in the production of TNF-α and interleukin IL-1β in both mutants. Alveolar bone loss was reduced in TLR4 mutants [217], whereas TLR2-deficient mice developed more severe bone loss after *A. actinomycetemcomitans* infection [216]. The TLR4-deficient mice also possessed a higher bacterial load in the periodontal tissues compared to WT infected mice [217]. Moreover, a polymorphism in the human TLR4 gene is associated with periodontal disease susceptibility. The missense mutation Asp299Gly in the TLR4 gene has been found to be a genetic risk factor for susceptibility to chronic periodontitis. In contrast, the missense mutation Thr299Ileu has been found to reduce the risk of aggressive

periodontitis [218]. Interestingly, HGECs deficient in TLR4 do not produce IL-1β after infection with *P. gingivalis* or *A. actinomycetemcomitans* in cell culture studies. Furthermore, the nonfunctionality of TLR4 appears to hinder the release of other proinflammatory cytokines (IL-6, IL-8, TNF-α) from HGECs after bacterial challenge, whereas WT HGECs release these cytokines [214]. Therefore, genetic variations in human PRRs can significantly influence the inflammatory response of the host against bacteria and the resulting onset of periodontitis.

2.4.2. Interleukin (IL)-1β synthesis, cleavage and secretion

The key immune response modulator IL-1β is mainly produced by monocytes [219] and macrophages (see reviews [24,212]); however, it can be produced by other cell lines such as gingival fibroblasts [220] and gingival epithelial cells [215]. This cytokine is synthesised as a 31-kDa precursor and is cleaved to its 17-kDa mature form by caspase-1 of cellular inflammasomes. These cytosolic inflammasomes are multi-protein complexes that act as a scaffold, which is required for caspase-1 activation. They are composed of the "adaptor" protein (ASC, apoptosis-associated speck-like protein containing the required C-terminal caspase domain), the "effector" protein (caspase-1) and the "sensor" protein (NLR protein, nucleotide binding and oligomerisation domain-like receptor, in NLR inflammasomes). The activation of PRRs as a response to PAMPs or DAMPs culminates with inflammasome activation and proteolytic cleavage of pro-IL-1β into its active form (reviewed in [212,221]). However, the regulation of IL-1β production is not identical in monocytes and macrophages. Circulating monocytes possess continuously active caspase-1, and they can thus release IL-1β after TLRs interact with pathogen-associated molecules. In contrast, macrophages require two stimuli before IL-1β is secreted. Binding of the ligand to its cognate TLR induces mRNA transcription from the IL-1β-encoding gene, but a second alarm of ATP released from lysing cells is required for IL-1β secretion [222]. Inflammasome activation as a consequence of ATP release is linked to a change in the ionic environment of the cell. Extracellular ATP activates the P_2X_7 cell membrane receptor, resulting in the opening of the cation-selective ion channel of P_2X_7 (see reviews [223,224]). Mature IL-1β lacks a typical signalling peptide targeting it for secretion by the endoplasmic reticulum and Golgi complex and is thus secreted unconventionally (see reviews [225,226]). The role of inflammasomes in active IL-1β production is described in Figure 3.

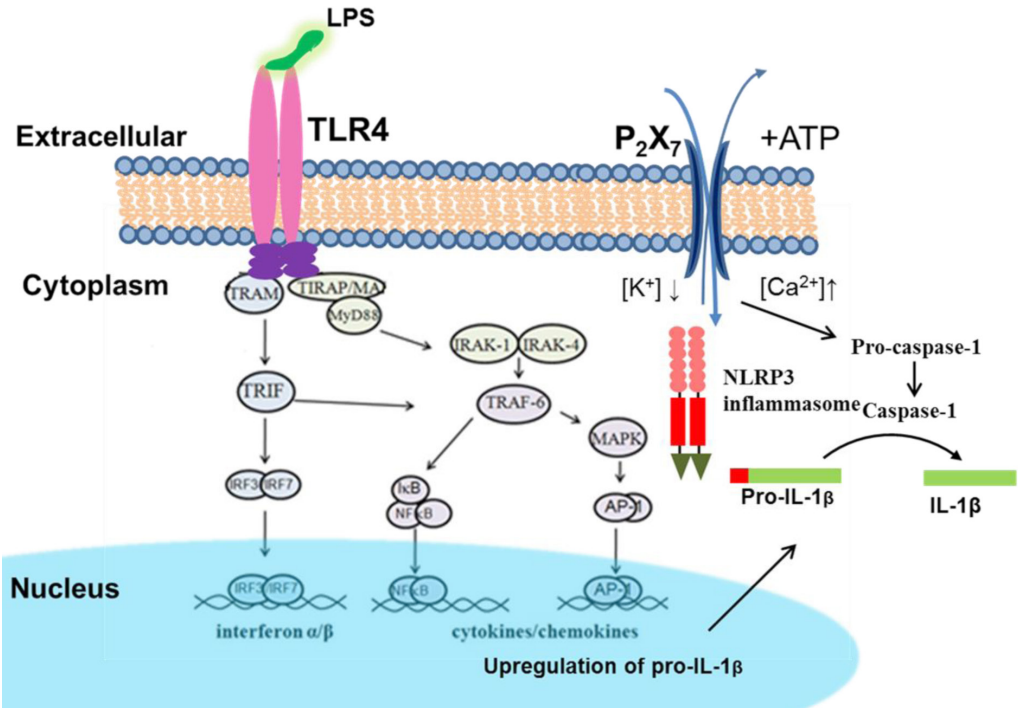


Figure 3. ***The recognition of PAMPs culminates in the immune response, including cytokine and interferon production through a complex signalling pathway.*** An exogenous ligand such as *A. actinomycetemcomitans* lipopolysaccharide (LPS) interacts with human Toll-like receptor (TLR)4 and causes an intracellular signalling cascade. First, the cytoplasmic Toll/IL-1 receptor (TIR) domains (purple) of TLR4 recruit two intracellular adaptor proteins TIRAP and Myd88 prior to the sequential phosphorylation of intracellular kinases. Activation of this Myd88-dependent signalling pathway then activates NF-κβ transcription factor and initiates cytokine expression in the nucleus. However, ligand binding to TLR4 can also lead to recruitment of the adaptor protein TRAM, which regulates the TRIF-dependent pathway. Activation of the TRIF pathway results in the phosphorylation of IRF3 and IRF7 transcription factors and subsequent interferon gene expression (see review [213]). Moreover, active caspase-1 is required to modify the translated pro-IL-1β to its mature form. In macrophages, the activation of the caspase-1 is controlled by cellular inflammasome complexes, such as NLRP3. When human cells are lysed during pathogen infection they release ATP, which is sensed by the neighbouring cells as an extracellular alarm signal. Opening of the P_2X_7 cation channels in the membrane is controlled by ATP. It has been suggested that the opening of this channel leads to fluctuation in the intracellular cation levels following formation of the active inflammasome complex (reviewed in [211,227,228]). Figure adapted from figures from Kondo *et al*. 2012 [227] and McIsaac *et al.* 2012 [213].

In response to *A. actinomycetemcomitans* infection, whole blood cells can enhance the release of IL-1β and TNF-α after being treated with free soluble surface material originating from viable *A. actinomycetemcomitans* (strain D7S) biofilm or planktonic cells of the species. At the transcriptional level, *A. actinomycetemcomitans*-derived material causes a multifold increase in the transcription of proinflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, and macrophage inflammatory protein-1β) in human whole

blood cells [229]. It was recently observed that human mononuclear leukocytes increase the expression of NLRP3 but reduce NLRP6 inflammasome expression when infected with the *A. actinomycetemcomitans* D7SS strain [230]. The secretion of IL-1β from mononuclear cells was enhanced two-fold from baseline during a 3-h challenge with *A. actinomycetemcomitans* [230].

2.4.3. IL-1β function

IL-1α and β are well known cytokines that demonstrate multiple and extensive effects on nearly every type of human cell. In particular, the role of IL-1β as a significant gatekeeper of inflammation has been established. The increased production of this cytokine may partly explain the development of variety of diseases, such as atherosclerosis and some autoimmune disorders (see reviews [24,25]). The potent IL-1β regulates both its own secretion and the secretion of other cytokines from neighbouring cells to adjust the host defence against invaders. In addition to its inflammatory role, IL-1β is also an influential mediator of human cell differentiation, proliferation and apoptosis [231,232]. During pathogen infection, IL-1β influences the release of other major proinflammatory cytokines such as IL-6 and IL-8 [214] from human epithelial cells infected with *P. gingivalis*. *C. albicans* or *S. aureus* infections induce the differentiation of naive T cells into IFN-γ- and IL-10-producing T_H17 cell subsets under the regulation of IL-1β [233]. Additionally, disturbances in IL-1β levels can indirectly contribute to host tissue damage and bone resorption during periodontitis [234-236]. The ability of IL-1β, IL-6, IL-11, TNF-α and IL-17 to regulate and increase the expression ratio of RANKL to osteoprotegerin (OPG) is well described (reviewed in [236]). During inflammation, the level of OPG is therefore low compared with the level of RANKL. RANKL can then freely interact with the RANK receptor located on the osteoclast precursor surface. This RANK-RANKL interaction leads to increased osteoclastogenesis and degradation of the alveolar bone. However, OPG binding to RANKL inhibits it binding to RANK, which reduces the bone loss caused by RANK-RANKL binding. Osteoblast cells, T and B lymphocytes and fibroblasts all express RANKL [236]. The *Macaca fascicularis* primate model of experimental periodontitis suggests that osteoclast formation and periodontal bone loss can be reduced 67% and 60%, respectively, with the use of IL-1 and TNF antagonists [237].

Activation of the inflammatory signalling cascade is initiated by the interaction of extracellular IL-1β with IL-1 receptor type I (IL-1RI) located on the cellular surface. IL-1RI is composed of an extracellular immunoglobulin domain and a Toll/IL-1 receptor (TIR) domain on the cytoplasmic side. The binding of IL-1β to IL-1RI allows the formation of a heterotrimeric receptor complex of IL-1β, IL-1RI and a TIR domain-containing accessory protein (IL-1RAcP). The MyD88 adaptor protein is then recruited to the TIR domain following phosphorylation of the receptor-associated kinases. The signal transduction induces NF-κB activation, which translocates into the nucleus where it induces the expression of inflammatory genes (see Fig 4 and reviews [224,238,239]).

Due to the various consequences of IL-1β production, it is essential to regulate IL-1β function at the gene expression, protein synthesis, propeptide maturation and secretion levels. The rate-limiting step of IL-1β production is the transcription of IL-1β (see review [25]). In IL-1β production, the transcriptional response controls pro-IL-1β synthesis, and caspase-1 modifies pro-IL-1β as previously described. To expand

the control of IL-1β activity even further, human cells express a specific receptor antagonist and a decoy receptor known as IL-1Ra and IL-1RII, respectively. The secreted IL-1Ra competes with IL-1β for IL-1RI binding. However, IL-1Ra binding to IL-1RI does not lead to intracellular signal transduction because the IL-1Ra/IL-1RI complex is unable to recruit the IL-1RAcP required for signalling (reviewed in [224,238,239]). Studies with mice have shown the importance of the correct balance between IL-1Ra and IL-1 for the animal's health. Mice deficient in the gene for IL-1Ra developed a disease that resembles rheumatoid arthritis and arteritis in humans [240].

Membrane-associated IL-1RII competes with IL-1RI to bind IL-1β, which thus blocks cytokine binding to the functional receptor (see reviews [224,238,239]). The cytoplasmic domain of IL-RII is short and does not contain a similar TIR domain as in IL-1RI. Due to this structural difference, IL-1β binding to IL-1RII does not activate the signalling cascade [241]. However, IL-1RII interacts with IL-1RAcP and sequesters free IL-1RAcP from IL-1RI [242]. The disruption of IL-1β homeostasis has severe consequences in humans, including autoinflammatory diseases, and IL-1β blockers, such as IL-1Ra, anti-IL-1, or anti-IL-1-RI antibodies, can be used to treat some of these resulting diseases (see reviews [24,25,226]). Many cell types, including epithelial cells and leukocytes, can express the Toll/IL-1 like receptor 8 (TIR8)/single immunoglobulin interleukin-1 receptor-related (SIGIRR) molecule, which belongs to the family of interleukin-1R-like receptors like IL-RII. TIR8/SIGIRR is recognised as a key regulator of inflammation because it can inhibit IL-1RI signalling, TLR signalling and NF-κβ activation. TIR8 expression has been suggested to disrupt the interaction of IL-1RI with IL-1RAcPs and thus inhibit the activation of NF-κB (reviewed in [243]). Understanding of the role of TIR8 in inflammation regulation has increased in recent years. For example, the overexpression of TIR8 in intestinal epithelial cells is known to diminish NF-κβ-mediated IL-8 responses to enteropathogenic *E. coli* or LPS compared to control cells. In contrast, silencing of the gene encoding TIR8 resulted in an exaggerated IL-8 response after cell stimulation with bacterial flagellin or proinflammatory IL-1β compared with nonsilenced TIR8 control cells [244]. Moreover, *Tir8* knockout mice ($Tir8^{-/-}$) possesses significantly higher lung bacterial loads. Compared to WT control mice ($Tir8^{+/+}$), these mutant mice also demonstrate increased mortality and increased production of proinflammatory cytokines (IFN-γ, TNF-α, IL-1β, and IL-6) in lung tissues after an acute *P. aeruginosa* lung infection [245]. Figure 4 shows a schematic presentation of IL-1β signalling and signalling inhibition by negative regulators. Table 3 summarises the different types of human cells that express IL-1RI, IL-1RII, IL-1Ra and TIR8/SIGIRR.

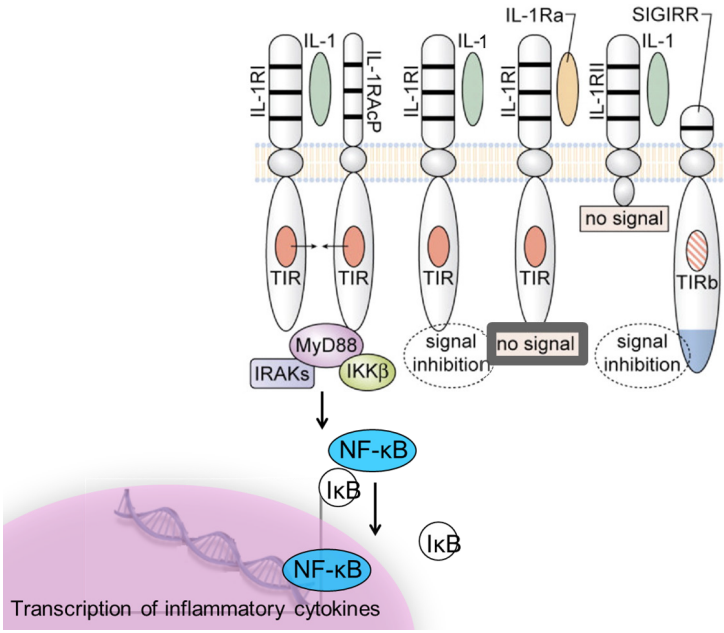


Figure 4. ***The IL-1β signalling mechanism and its inhibition through IL-1RI.*** Briefly, IL-1β binding to IL-1 receptor type I (RI) and the subsequent recruitment of the accessory protein of the receptor (IL-1RAcP) to the complex initiates signalling via the phosphorylation of receptor-associated TIR kinases. After phosphorylation, the cytoplasmic MyD88 adaptor protein is recruited. Intracellular signal transduction includes the phosphorylation of IRAKs and IKKβ, which leads to activation of the NF-κB transcription factor. The activated NF-κB enters the nucleus and induces the expression of inflammatory genes (reviewed in [224,239,246]). Cells also express IL-1RII, which binds IL-1β on the cell surface without causing a signalling cascade. Additionally, IL-1R antagonist (IL-1Ra) can interact with IL-1RI and thus block IL-1β binding. The negative regulator receptor of ILR and TLR signalling TIR8/SIGIRR dampens intracellular signalling pathways (reviewed in [224] and [243]). Figure modified from Dinarello 2011 [224].

Table 3. ***Human cell types expressing the IL-1 receptors IL-1RI, IL-1RII, and IL-1Ra and the IL-1 receptor-like TIR8/SIGIRR receptor.***

IL-1 receptors	Cell types expressing the IL-1 receptor
IL-1RI	- almost all cell types (reviewed in [25])
IL-1RII	- mainly macrophages and B cells but also monocytes and neutrophils (reviewed in [247, 248]) - soluble form of the receptor is released by B cells [249], peripheral blood mononuclear cells [250] and polymorphonuclear cells [251]
IL-1Ra	- macrophages/monocytes, blood polymorphonuclear cells/stimulated neutrophils [252, 253] and hepatocytes [254], and mesenchymal stem cells [255] - intracellular IL-1Ra is constitutively produced by epithelial cells in the skin and the gastro-intestinal tract [256]
TIR8/SIGIRR (TIR8)	- expressed by NK cells, B cells, monocytes, immature myeloid dendritic cells and intestinal epithelial cells - high expression levels detected in tissues of the digestive tract, kidney, liver, lung and lymphoid organs (reviewed in [257])

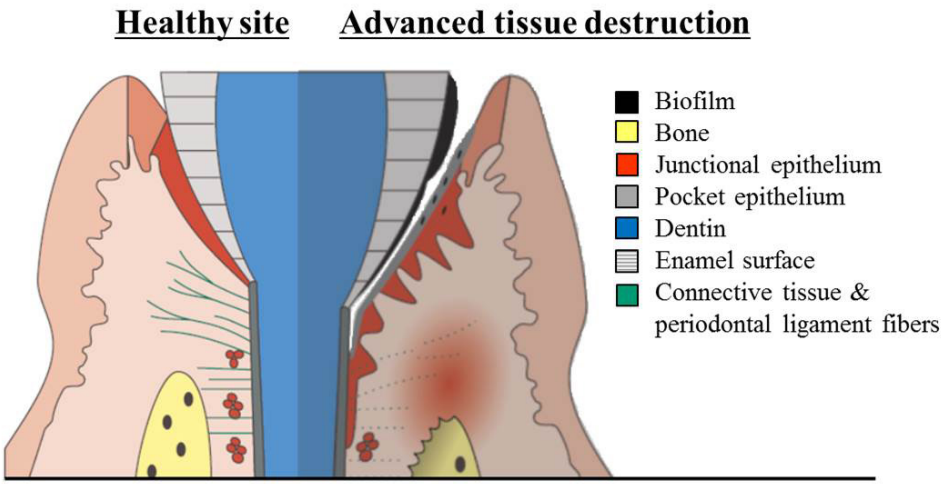
2.4.4. IL-1β and other cytokines in the periodontal environment

In periodontitis, tooth-supporting tissues and alveolar bone are destroyed as a result of inflammation. Figure 5 summarises some major differences in diseased periodontal tissue compared with healthy tissue. According to the definition of periodontitis, the depth of the gingival sulcus increases from ≤3 mm in healthy subjects to ≥5 mm in patients with the disease. Additionally, patients with periodontitis show bleeding when probing the pockets. The main characteristics of LAP, as opposed to chronic periodontitis, include rapid bone loss and disease breakthrough during early adulthood (reviewed in [17,18]). Tissues in the ongoing disease state also show increased local levels of IL-1β in gingival crevicular fluid (GCF) compared with healthy sites. Twenty years ago, it was determined that tissue biopsies taken from deep sites in gingival pockets (depth of 8.5±0.4 mm) contained approximately 4.5-fold higher amounts of IL-1β versus shallow, healthy pockets (depth of 2.8±0.3 mm) [12]. Since then several studies have verified that the levels of IL-1β increase in GCF taken from deep pocket sites [13-16,258]. Samples taken from patients diagnosed with generalised aggressive periodontitis contained the highest levels of IL-1β compared with periodontally healthy subjects or patients with chronic periodontitis [259]. In addition to increased tissue and GCF levels, IL-1β concentrations can also be significantly higher in the saliva of periodontal patients compared with healthy control patients [260]. Table 4 summarises the IL-1β concentrations (ng/ml) determined from periodontal tissue and GCF.

Table 4. ***IL-1β levels in periodontal tissue, GCF and saliva.***

IL-1β levels in periodontal tissues (ng/ml)		
Healthy sites	**Active tissue destruction**	**References**
6.2±4.6	**28.4**±7.2	[12]
IL-1β levels in GCF (ng/ml)		
Healthy sites	**Active tissue destruction**	**References**
44.8±15.0	**86.4**±22.0	[13]
147.8±24.6	**206.0**±20.4	[258]
IL-1β levels in saliva* (ng/ml)		
Periodontally healthy	**Periodontal disease**	**References**
0.5±0.3	**0.7**±0.3	[260]

*The coincident presence of 3 to 5 different periodontal pathogens (*A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia*, and/or *T. denticola*) in saliva was demonstrated.



Healthy site	Periodontitis	References
pH of sulcus≈ 6.9	pH of sulcus≈ 7.8	
Low GCF flow	High GCF flow	Reviewed in [261]
Gram-positive bacteria/ low subgingival biofilm levels	Obligately anaerobic bacteria/ high subgingival biofilm levels	
Minimal inflammation	Strong inflammation(↑IL-1β, IL-6, TNF-α, TLR2, TLR4 and MMPs)	Reviewed in [262, 263]
RANKL/OPG ratio in balance	The increase in the RANKL/OPG ratio culminates with bone loss	Reviewed in [236, 262]

Figure 5. ***The differences in healthy and diseased periodontal sites.*** The healthy dentogingival junction and the same junction during periodontitis are described on the left and right sides of the figure, respectively. In periodontitis, a subgingival pocket has formed between the tooth and gum, and bacteria have colonised the pocket. Moreover, the flow of gingival crevicular fluid (GCF) is enhanced at the infected site, and changes in the pH lead to a more alkaline subgingival environment that is typical during periodontitis. Additionally, the amount and composition of the subgingival biofilm may change dramatically when the disease develops. As a result of immune response activation, the cytokine levels and the osteoclast activator factor (RANKL)/osteoprotegerin (OPG) ratio rise at the diseased sites. The complex host response against pathogenic invaders together with microbe-derived toxins or proteolytic compounds leads to degradation of the epithelial barrier, connective tissue and bone. Despite the aggressive host defence, biofilms may remain viable, and some single pathogens may invade periodontal tissue. MMP: matrix metalloproteinase; TLR: Toll-like receptor. Figure modified from Pöllänen *et al*. 2012 [262].

2.4.5. Role of IL-6, IL-8, IFN-γ and TNF-α in periodontitis

Some studies have reported significantly higher levels of the co-occurrence of secondary cytokines IL-6 and IL-8 with severe forms of periodontal disease [13,264-266]. IL-6 is produced by several types of human cells, including gingival fibroblasts [267], gingival epithelial cells [268] and monocytes [269] as a 21 to 30 kDa phosphorylated and

variably glycosylated molecule [270]. This cytokine has a central role in the regulation of B- and T-cell differentiation and growth [271-275]. Therefore, IL-6 knockout mice have a compromised response to viral and bacterial infections [276]. IL-8 (<9 kDa), a member of the CXC chemokine family, mediates the chemotaxis and activation of neutrophils and other immune cells at the inflammation site. IL-8 is secreted by several cells in gingival crevice, including monocytes [269], macrophages [277], gingival epithelial cells [277,278] and gingival fibroblasts [267]. *In vitro* studies have shown that *A. actinomycetemcomitans* infection induces IL-8 expression and release in gingival cells [268,278] and polymorphonuclear leukocytes [279]. Notably, IL-1β stimulates the production of IL-6 and IL-8 from human gingival cells, and the inhibition of IL-1RI reduces the production of these cytokines [214,280]. Additionally, TNF-α enhances the secretion of IL-6 in gingival fibroblasts [280].

One major cytokine that controls the inflammatory response is TNF-α. This cytokine (a monomer of 17.5 kDa) is mainly secreted by macrophages and monocytes, although gingival fibroblasts and epithelial cells are also known to secrete it after bacterial infection [267,277,279]. Interestingly, TNF-α plays an analogous role in alveolar bone destruction to IL-1β [235], and TNF-α levels in GCF are higher in patients with generalised aggressive periodontitis compared with healthy subjects or patients with chronic periodontitis [259]. Moreover, both IL-1β and TNF-α can induce the expression of matrix metalloproteinases, which can degrade the periodontal connective tissue (see review [281]). In addition to the abovementioned cytokines, a role for IFN-γ in periodontitis development has been suggested. The increased levels of IFN-γ in GCF appear to correlate with the diseased periodontal sites in chronic periodontitis [282]. However, the levels of IFN-γ in GCF do not increase as much as the interleukin levels (IL-1β, IL-6 and IL-8) [15]. IFN-γ is mainly produced by T lymphocytes, but nearly all cells except erythrocytes express the IFN-γ receptor (see review [283]). It has been suggested that this interferon affects macrophage activation, and IFN-γ is essential in the host defence against intracellular pathogens, such as fungi, viruses, and intracellular bacteria [284,285]. Interestingly, the absence of IFN-γ in a mouse model infected with the *A. actinomycetemcomitans* JP2 strain caused the bacterial load to increase and eventually led to the death of the mice [286].

2.4.6. Ability of bacteria to sense host cytokines

IL-1β

Porat *et al.* [287] were the first to suggest that virulent *E. coli* strains can bind human IL-1β, and, as a result of IL-1β binding, these virulent strains enhance their growth. By using a competitive radiolabeled [^{125}I]-IL-1 binding assay, they showed that IL-1β binds to *E. coli* cells and that this binding could be inhibited using competitive IL-Ra. Growth enhancement was observed with final IL-1β concentrations of 10-100 ng/ml. Interestingly, the growth promoting effect appeared to be specific for IL-1β, as treatment with IL-4 or TNF-α did not have any impact on the growth of virulent or avirulent strains [287].

The effect of IL-1β on the growth of other bacterial species was later shown using fresh isolates of *S. aureus* (Gram-positive), *P. aeruginosa* (Gram-negative), and *Acinetobacter* spp. (Gram-negative). All these species showed concentration-dependent

growth enhancement when the bacteria were treated with IL-1β, IL-6 or TNF-α at final concentrations of 0.5-10 ng/ml. However, growth enhancement was lost if the bacteria were passaged more than five times or if IL-1β treatment was performed in the presence of a cytokine neutralising monoclonal antibody [288].

The interaction between biofilm-forming bacteria and IL-1β has been best described with *S. aureus*. The findings of these studies strongly suggest that this opportunistic pathogen can sense host-derived IL-1β. First, it has been shown that *S. aureus* biofilm cultures are more sensitive to IL-1β (0.1-10 ng/ml) than planktonic cultures. IL-1β exposure caused a 2.5-fold increase in the amount of biofilm cells compared to untreated cells, but a similar IL-1β growth dependence was not observed with planktonic cultures. Second, biofilm cells bound more than five-fold greater amounts of IL-1β than planktonic cells according to a flow cytometry assay [9]. It seems that the entire 17.4-kDa mature IL-1β protein with an isoelectric point (pI) of 5.9 is not needed for growth enhancement, as the phenomenon has also been observed with two linear IL-1β peptide fragments (<5 kDa) spanning amino acid (aa) regions 118–147 and 208–240 [7]. Table 5 presents the known effects of different IL-1β fragments on *S. aureus* biofilm or human cells. Moreover, Table 5 includes the calculated pI values and molecular weights of the IL-1β fragments.

Table 5. ***Pro-IL-1β peptide fragments and their suggested effects on S. aureus biofilm and human cells.***

Pro-IL-1β peptide fragments					
aa* region	**Mw* (kDa)**	**pI***	**Effect on *S. aureus* biofilm** [7]	**Effect on human cells/reference**	
118-147	3.4	9.3	- Growth-enhancement	- No biological activity in human glioblastoma cells.	[289]
148-177	3.3	3.8	- No growth-enhancement	- No biological activity in human glioblastoma cells.	[289]
163-171	1.0	4.1	- Not studied	- Immunostimulatory and adjuvant effects without inflammatory and toxic effects	[290,291]
178-207	3.5	6.2	- No growth-enhancement	- No biological activity in human glioblastoma cells.	[289]
208-240	4.1	9.6	- Growth-enhancement	- IL-1β agonist with biological activity in human glioblastoma cells. - Induces intercellular adhesion molecule-1 expression, which can be blocked by IL-1Ra in human glioblastoma and neuroblastoma cells	[289]
241-269	3.1	4.0	- No growth-enhancement	- No biological activity in human glioblastoma cells.	[289]

*Theoretical molecular weight and pI value for human IL-1β peptide fragments were determined by ProtParam analysis [292]. Mature IL-1β spans aa 117-269 of the preprotein.

Most importantly, IL-1β is known to affect the gene expression of *S. aureus*. It has been shown to modulate the gene expression of toxin-encoding genes and some genes responsible for host tissue attachment. For example, high IL-1β levels (25 ng/ml) lead to a significant decrease in the expression of four bicomponent leukotoxin-encoding genes. In contrast, the expression of these genes increased at low IL-1β levels (5 mg/ml). However, the gene expression of fibronectin-binding protein and collagen-binding protein increased in the presence of high or low IL-1β levels compared with control cultures. [293] Kanangat and co-workers [293] have suggested that *S. aureus* may use IL-1β as a cue to change its virulence properties to better meet the requirements for battle between the pathogen and host defence systems.

In an organotypic model of nasal epithelium, both IL-1α and IL-1β inhibited the growth of a nasal carrier strain of *S. aureus* [294]. Though the growth of *S. aureus* was inhibited, the carrier strain was still able to disturb the cytokines response of the nasal epithelial cells. After the infection of nasal epithelial cells with the nasal carrier strain of *S. aureus,* IL-1β levels in culture medium were lower than the respective cytokine levels from infection with noncarrier strains. The results of this study support the hypothesis of human cytokine-responsive bacteria. [294] Although *S. aureus* is most often found on nasal membranes and skin, the species occurrence in the subgingival microbiota has been reported. *S. aureus* strains can inhabit subgingival environments because approximately 6% of the periodontal samples taken from 973 subgingival pockets contained this species [295]. It seems to be typical for nonsmoking patients with aggressive periodontitis, as this species was present in 60.5% of patients in the nonsmoking group [296].

TNF-α

In addition to IL-1β, two other pro-inflammatory cytokines, TNF-α and macrophage inflammatory protein-1α, are known to increase the growth of biofilm cultures. Interestingly, all cytokines may not have similar growth-promoting effects on the species because IL-6 has not shown any influence on biofilm growth [9]. Studies with Gram-negative *Shigella flexneri* have revealed its ability to bind TNF-α and the virulence property alterations that occur after cytokine pretreatment. Based on experiments using [^{125}I]-labelled TNF-α, a single *S. flexneri* cell may contain nearly 300 proteinaceous receptors for TNF-α. As a physiological response, TNF-α pretreatment increases bacterial invasion of human cervical carcinoma (HeLa) cells 20-fold. It seems that cytokine binding to the bacterial cells is required for invasion. Treatment of HeLa cells with antibodies blocking TNF-α binding to the cells can also hinder the enhanced invasion of HeLa cells by TNF-α-treated *S. flexneri*. It was thus suggested that the cytokine-bacterium association may boost bacterial invasion of human cells. The binding of TNF-α is not a unique property of *S. flexneri* strains; Gram-negative *Salmonella typhimurium* and *E. coli* can also bind this cytokine, whereas Gram-positive *Listeria monocytogenes*, *S. aureus* and *Streptococcus mitis* bind considerably less TNF-α. [297]

Bacterial outer membrane receptors for cytokines

The publication of Zav'yalov *et al.* [10] was the first to describe the specific bacterial outer membrane receptor for IL-1β. The studies with *Y. pestis,* the etiologic agent of plague, showed that the outer membrane protein Caf1A specifically binds human IL-1β [10]. The Caf1A protein is encoded by the *f1* operon, which is responsible for the

synthesis and surface assembly of F1 capsule antigen (Caf1). The transportation of the capsule antigen through the outer membrane is dependent on the Caf1A protein [298,299]. The pore-forming Caf1A protein cooperates with periplasmic chaperones to assemble and secrete the polymeric F1 capsule (see review [300]). Sequence alignment demonstrated 28% homology between Caf1 antigen (aa 62-89) and human IL-Ra (aa 57-85) [301]. Moreover, it is known that the Caf1 subunits possess an incomplete Ig-like fold, and the periplasmic CafM chaperone displays Ig-like topology [302]. These incomplete Ig-like folds are typical both for proteins of the chaperone-usher pathway and for adhesins belonging to the MSCRAMM (microbial surface components recognising adhesive matrix molecule) family (see review [303]).

In Gram-negative *P. aeruginosa*, the outer membrane protein OprF was characterised as a specific binder of IFN-γ [8]. This species responded specifically to human IFN-γ because the cytokine caused an induction of *P. aeruginosa* lectin (PA-I or lecA) expression, which is known to be an important factor in the adherence of the species to endothelial cells [304]. The expression of LecA in *P. aeruginosa* as a result of IFN-γ binding to OprF was connected to QS signalling. Therefore, the deletion of genes playing major roles in QS signalling also caused the loss of *lecA* expression induction in *P. aeruginosa* after IFN-γ treatment [8]. Moreover, other tested human cytokines, including IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 and TNF-α, did not have a similar type of effect on lectin expression [8]. Interestingly, none of the cytokines tested in the study had a growth promoting effect on *P. aeruginosa* [8], though another research group has shown the effect of IL-6 and TNF-α on species growth [288].

IL binding compounds in viruses and yeasts

In addition to bacterial species, herpesviruses and the pathogenic yeast *C. albicans* have been observed to disrupt host cytokine communication. Large DNA viruses, such as poxviruses and herpesviruses, have the ability to modulate the host immune system via the production of homologues to host cytokines, chemokines and their receptors (reviewed in [305]). In particular, herpesviruses such as herpes simplex virus, cytomegaloviruses and Epstein-Barr viruses [305] can all be isolated from periodontal lesions and subgingival plaques of patients with aggressive periodontitis [40,306]. Interestingly, Epstein-Barr virus encodes a BARF1 protein, which is a soluble ligand for human colony-stimulating factor-1 (CSF-1), and a recombinant BARF1 protein has been shown to antagonise the proliferative effect of human CSF-1 on mouse macrophage precursor cells [307]. Moreover, Epstein-Barr virus [308] and human cytomegalovirus [309] both encode a viral homologue of IL-10, which inhibits cytokine production in monocytes and macrophages (see review [310]). Vaccinia virus, a member of the poxvirus family, encodes a soluble receptor for IL-1β [311,312]. As these examples suggest, viruses have evolved specific mechanisms to modulate host immune responses to enhance their own survival.

C. albicans commonly occurs in subgingival samples of severe chronic periodontitis patients [39] and has been shown to bind high amounts of IL-2. It has been suggested that the cell wall oligomannosidic N-glycans are responsible for IL-2 binding [313,314]. Furthermore, pre-administration of soluble mannose causes a 60% reduction in IL-2 binding to *Candida* cells [313]. This cytokine-carbohydrate interaction is rational, as the cytokines IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7 [315] and TNF-α [316] use their

carbohydrate-binding domains to recognise specific oligosaccharide ligands in humans (see review [317]). Moreover, IL-1α and IL-β demonstrate different carbohydrate binding activities [315] and different biological impacts, although they share the same target receptor IL-1R, which is expressed on nearly all cells (see review [25]). They both prefer to bind to anionic polysaccharides, but only IL-1β can interact with hyaluronic acid [318]. IL-1α binds N-glycan with two α-2-3-linked sialic acid residues, whereas IL-1β recognises the α-2-3-sialylated β-galactosyl-ceramides containing very long and unusual long-chain bases [319]. In addition to IL-2 binding, the biofilm formation of *C. albicans* was enhanced as a result of co-culture with peripheral blood mononuclear cells (PBMC). The supernatant of *C. albicans* biofilm-PBMC co-cultures contains high levels of IL-1β compared with supernatant derived from PBMC co-cultures infected with planktonic *C. albicans*. Moreover, this cytokine-rich supernatant from *C. albicans* biofilm-PBMC co-cultures induces *C. albicans* biofilm growth [320]. Based on these *Candida* studies, it appears that yeast biofilms can both bind and sense cytokines. If viruses and yeast have captured or evolved genes to specifically modulate human immune pathways, it can be assumed that bacteria have also evolved mechanisms to disrupt host immune processes during their long co-evolution with the human host.

3. AIMS OF THE THESIS

The general aim of the thesis was to investigate whether the biofilms of an opportunistic oral pathogen *Aggregatibacter actinomycetemcomitans* can interact with the human inflammatory mediator IL-1β. A secondary aim was to examine the putative role of the outer membrane secretin protein HofQ in DNA uptake in *A. actinomycetemcomitans* biofilms. We hypothesised that *A. actinomycetemcomitans* might sense IL-1β and possess machinery for cytokine exploitation. Moreover, we hypothesised that the extramembranous portion of emHofQ is involved in DNA binding.

The specific objectives of the thesis were:

- To examine cytokine binding to biofilms and single cells of *A. actinomycetemcomitans* and to determine its effect on the biofilm properties.
- To find a putative bacterial outer membrane receptor for IL-1β and other proteins that interact with IL-1β in *A. actinomycetemcomitans.*
- To characterise DNA binding to the N-terminal portion of the *A. actinomycetemcomitans* outer membrane protein HofQ.

4. MATERIALS AND METHODS

A more detailed description of the experimental procedures can be found in the original publications I-IV.

4.1. *A. actinomycetemcomitans* strains (I, II, III)

Table 6 contains all of the *A. actinomycetemcomitans* strains used in this study. Most of the studies were performed with the clinical isolate D7S-1, which was isolated from an African-American female patient with aggressive periodontitis [120]. The genomic sequence of this strain is known [23]. The WT *A. actinomycetemcomitans* D7S-1 strain and its derivatives, the naturally smooth colony variant D7SS [120] and a single gene mutants of the *tad* locus [321], were a gift from Prof. Casey Chen. The *A. actinomycetemcomitans* clinical strains SA1398 and SA1151 were from Prof. Sirkka Asikainen [321,322]. All gifted strains are described more detailed in paper I (I, Table I). The other clinical strains (RIB04, RIB07, and RIB09) were collected from adult periodontitis patients at the Community Dental Health Care Center of Turku (Institute of Dentistry, University of Turku, Turku, Finland). The isolation and identification of RIB strains were performed at the Helsinki University Hospital Laboratory (HUSLAB, Helsinki, Finland). Additionally, the serotypes of the threeRIB strains were analysed via PCR based serotyping [72,323] after chromosomal DNA isolation from *A. actinomycetemcomitans* cells [324,325].

Table 6. ***A. actinomycetemcomitans strains used in culturing experiments.***

Strain	Serotype	Colony morphology	Paper
D7S-1	a	rough	I, II, III
SA1398	b	rough	I, III
SA1151	c	rough	I, III
D7SS	a	smooth	I
D7SΔflp1-flp2::Spe	a	smooth	I
D7SΔrcpA::Spe	a	smooth	I
D7SΔrcpB::Spe	a	smooth	I
D7SΔtadD::Spe	a	smooth	I
D7SΔtadG::Spe	a	smooth	I
RIB04	b	rough	III
RIB07	c	rough	III
RIB09	a	rough	III

4.2. *A. actinomycetemcomitans* biofilm cultures (I, II, III)

A. actinomycetemcomitans cells were stored at -80°C in milk. They were recovered by culturing on trypticase soy agar (TSA) plates supplemented with 5% defibrinated sheep blood in candle jars at 37°C for 2.5 to 3 days. Even cell suspensions were prepared by scraping the plate cultures into trypticase soy broth (TSB) supplemented with 0.6%

(weight/volume) yeast extract and 0.8% glucose (TSB-YE/Glc) according to the method described by Karched *et al.* [326]. In this method, an optical density (OD) of 1 at 600 nm is equal to approximately 1 x 10^9 *A. actinomycetemcomitans* cells per ml. Bacterial culture was continued with the desired number of cells (0.5-5.0x10^7 cells) in TSB-YE/Glc in 48-well tissue culture plates or in cell culture flasks for 18 h or 24 h. For gingival co-culture model studies, the biofilms were grown on hydrophilic polyethersulfone membranes (Protran® Whatman®) in the wells of cell culture plates, and these cultures were initiated with 1x10^7 bacteria per well. Biofilms formed at the bottom of cell plates were then rinsed twice with 0.85% NaCl solution to remove nonadherent bacteria. After washing, the culture medium was changed to RPMI-1640 medium (R7509, Sigma) supplemented with 4.1 mM L-glutamine (Sigma), and culturing was continued for 3, 6, 18 or 24 h depending on the experiment. This minimal culture medium is suggested be optimal for bacterium-cytokine interaction studies, as it does not include complex organic materials that hinder the biological activities of cytokines [288].

4.3. Cloning, expression and purification of recombinant proteins in *E. coli* (I, II, III, IV)

Genomic DNA was isolated from *A. actinomycetemcomitans* D7S-1, RIB04, RIB07, RIB09 strains using the bacterial DNA extraction method developed by Moncla *et al.* [324]. Genomic DNA of the *A. actinomycetemcomitans* strain ATCC 700685 (HK1651) was commercially available from American Type Culture Collection (700685D-5).

The genes encoding the *A. actinomycetemcomitans* F0F1 ATP synthase subunit beta (*atpsynb*), DNA binding protein HU (*hu*) and the extra-membranous part of HofQ (*emHofQ*; aa 27 to 195) were amplified from the genomic DNA of the *A. actinomycetemcomitans* strain HK1651. The genes encoding the type II/IV secretion system secretin RcpA (*rcpA;* aa 30-192) and the putative bacterial IL-1β receptor I (*bilRI* and *cpbilRI;* aa 19-181) were amplified from the *A. actinomycetemcomitans* strain D7S-1. The residues 1-26 of emHofQ, 1-29 of RcpA and 1-18 of BilRI were excluded from cloning because the signal peptide prediction tool [327] suggested that they are secretion signals. However, the entire recombinant BilRI protein with the signal sequence was also produced in *E. coli* for flow cytometry and membrane fractionation studies. Phusion High-Fidelity DNA Polymerase (Finnzymes) was used in the PCR reactions to amplify the aforementioned DNA sequences. The cloning vectors pET-15b (Novagen) and pET-36b(+) (Novagen) were used for N-terminal 6xHis-tagged recombinant protein production and C-terminal 8xHis-tagged recombinant protein production, respectively. The constructs were prepared by digesting the amplified DNA fragments and suitable cloning vectors with NdeI/XhoI restriction enzymes (Fermentas) and then ligating the digested fragments with T4 ligase (Fermentas). Electroporation was used to transform the cloning vectors into *E. coli* XL1-Blue cells (Stratagene). Competent *E. coli* TOP10 cells (Invitrogen) were used in the cloning of *bilRI*, and construction of the *hu* cloning vector included two separate digestion and ligation rounds. The PCR product of *hu* was first digested with XbaI/XhoI and ligated to the pBluescript (Stratagene) cloning vector digested with the same restriction enzymes. The *hu*-pBluescript construct and the pET-36b(+) cloning vector were then digested with NdeI/XhoI prior to *hu*-insert ligation into pET-36b(+). Finally, the cloning vectors were sequenced in both directions by Eurofins

MWG Operon at the recombinant protein encoding regions to verify the sequences of the produced expression constructs.

The sequenced cloning vectors were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells for cytosolic recombinant protein expression. The one exception was the signal sequence-containing *bilRI* gene in the pET-36b(+) vector, which was transformed into *E. coli* C41(DE3)-RIL cells. This C41 strain is designed for bacterial membrane protein overexpression [328]. Transformants containing a plasmid for cytoplasmic protein production were grown in Terrific broth medium supplemented with the proper antibiotics at 37°C. BilRI membrane protein (BilRI) expression was performed at 37°C in specific culture medium with a composition that is optimised for membrane protein expression in C41(DE3)-RIL cells [328,329]. The expression of soluble recombinant protein or membrane-associated BilRI was induced with isopropyl β-D-thiogalactopyranoside (IPTG) when an OD_{600nm} of approximately 1.2 or 0.8 was reached, respectively. After 2 to 3 h of IPTG induction, BL21-(DE3)-RIL cells were harvested and stored at -20°C. To avoid the lysis of recombinant C41(DE3)-RIL cells, these cells were stored in 50% glycerol at -20°C. Table 8 summarises the theoretical molecular weights and pIs of the produced recombinant proteins.

E. coli cells were lysed using a French Press or ultrasound sonication. The crude extract was loaded onto a Ni^{2+}-charged HisTrap™ HP column (Amersham Biosciences). After the immobilisation of His-tagged proteins, the target proteins were released with increasing concentrations of imidazole. Finally, the eluted protein fraction was further purified using a Superdex-200 16/60 size-exclusion chromatography column (GE Healthcare). The produced recombinant emHofQ protein used in crystallisation studies contained both a thrombin cleavage site and a His-tag in its N-terminus. emHofQ was released from the Ni-column using thrombin exposure, which caused the removal of the N-terminal His-tag. Additionally, a selenomethionine derivative form of emHofQ was used in crystallisation studies. This derivative of emHofQ was expressed using the Doublie method [330].

Table 7. ***Cloning vectors used for recombinant protein production.***

Recombinant protein-encoding gene	Cloning vector	Restriction enzyme pairs	His-tag localisation	Size of the protein without His-tag (kDa)	pI of the protein without His-tag
atpsynb	pET-36b(+)	NdeI/XhoI	C-terminal 8xHis	49.8*#	5.0
emHofQ	pET-15b	NdeI/XhoI	N-terminal 6xHis, thrombin cleavage site	18.8*# (aa: 27-195)	5.4
hu	pBluescript/ pET-36b(+)	XbaI/XhoI NdeI/XhoI	C-terminal 8xHis	9.4*"	10.1
rcpA **(GI:32452623)**	pET-36b(+)	NdeI/XhoI	C-terminal 8xHis	17.9*" (aa: 30-192)	4.8
bilRI **GI:387121899)**	pET-36b(+)	NdeI/XhoI	-	18.9" (aa: 1-181) 17.0 (aa: 19-181)	4.6 4.5
cbilRI **(GI:387121899)**	pET-36b(+)	NdeI/XhoI	C-terminal 8xHis	17.0*" (aa: 19-181)	4.5

* Extra amino acid residues due to C-terminal 8xHis-tag, LEHHHHHHHH, 1.4 kDa or due to N-terminal 6xHis-tag after thrombin treatment, GSHM, 0.4 kDa

\# Computed extinction coefficient and A280 nm values used for protein concentration determination.

" Protein concentration was measured using Lowry´s method for determining protein concentration [331].

4.4. Characterisation of the effects of IL-1β on *A. actinomycetemcomitans* biofilms (I, II)

4.4.1. Biofilm mass estimation using crystal violet (I, II)

Biofilm colonies were grown on the bottom of 48-well cell culture plates as described above. Briefly, biofilm cultures were initiated by adding *A. actinomycetemcomitans* suspension containing $5x10^6$ (I) or $5x10^7$ cells (II) into each well. Even suspensions were prepared by harvesting the plate cultures into TSB-YE/Glc-medium. After culturing biofilms in a candle jar at 37°C for 18 h, the TSB-YE/Glc-medium was removed, and the biofilms were washed with sterile salt solution.

(I) To examine the effect of IL-1β on biofilm mass formation, the biofilm culture was continued in RPMI-1640 medium with glutamine at 37°C for 6 h. In these studies, the medium was supplemented with recombinant human IL-1β (Reliatech GmbH) at a final concentration of 10.0 ng/ml. In control cultures, water was added instead of IL-1β.

(II) The effects of the antibiotics penicillin and streptomycin on the biofilm mass of *A. actinomycetemcomitans* were studied. The biofilms were incubated in RPMI-medium for 24 h following 6 h or 25 h incubation in Green's medium [332,333]. Green's medium contains 65% DMEM (Life Technologies, Inc., Gibco®, cat#52100-039) supplemented with 0.17% $NaHCO_3$ and 18 mM HEPES. In addition to DMEM, Green's medium contains 25% Ham-F-12-medium (Life Technologies, Inc., Gibco®, cat#21765-029), 10% inactivated foetal bovine serum (Life Technologies, Inc., Gibco®), 4 mM L-glutamine (Life Technologies, Inc., Gibco®), 5 µg/ml insulin (Sigma), 0.4 µg/ml hydrocortisone (Sigma), 5 ng/ml epidermal growth factor (EGF) (Sigma), 0.1 nM cholera toxin (Sigma), 1.8 µg/ml adenine (Sigma) and 100 µg/ml ascorbic acid (Sigma). In half of the cultures, Green's medium was supplemented with antibiotics (63.4 U/ml penicillin and 63.4 µg/ml streptomycin).

After culture, the amount of biofilm formed at the bottom of wells was estimated using the crystal violet staining method described by Kaplan *et al.* [168]. Briefly, the biofilm was rinsed twice with water before adding the gram stain (20 mg/ml crystal violet, 8 mg/ml ammonium oxalate, 20% ml ethanol). The gram stain was incubated with the biofilm at room temperature (RT) for 10 minutes, followed by seven water washes to remove unbound stain. After washing, the crystal violet was released from the biofilm by adding 94% ethanol to the wells and incubating the culture plate with shaking at 100 rpm at RT for 10 minutes. Samples were taken from each culture well, and the A_{620nm} was measured using a microplate reader.

4.4.2. Biofilm viability assay (II)

The effects of antibiotics on the viability of *A. actinomycetemcomitans* D7S-1 biofilm were determined by confocal microscopy and crystal violet staining methods [168]. Biofilms were grown in 48-well culture plates or on microscope cover glasses located in 6-well culture plates as described above. The cultures were initiated by pipetting an even bacterial suspension into each well ($1.5x10^8$ cells per well) and culturing the cells in TSB-YE/Glc for 24 h, followed by a subsequent 24-h incubation in RPMI-1640. After these incubations, biofilms

were incubated in Green's medium for 6 h or 25 h. Half of the cultures was grown with a combination of penicillin (63.4 IU/ml) and streptomycin (63.4 µg/ml). After incubation, biofilms were gently rinsed with sterile water and then stained using a FilmTracer™ LIVE/DEAD Biofilm Viability kit (Invitrogen) according to the manufacturer's instructions. Imaging of the samples was performed in the Department of Biology and Cell Imaging Core, Åbo Akademi University (Turku, Finland). The samples were imaged by confocal laser scanning microscopy using a HCX PL APO 63x/1.20 W CORR water immersion objective on a Leica TCS SP5 confocal microscope. Images were analysed using LAS AF scanning software (Leica Microsystems CMS). Three sets of images were taken per specimen, and three independent biological replicates were prepared. The details of the scan settings for specimens and of the stacks scanned are described in paper II.

4.4.3. Metabolic activity of biofilm and planktonic cells (I)

The effect of IL-1β on the metabolic activity of biofilms was investigated using a commercial redox indicator, alamarBlue™ (AbD Serotec). Growing cells cause a chemical reduction of the indicator, which can be detected as a fluorescence change or as a colour change of the culture medium. For these metabolic activity studies, biofilm-forming rough strains were precultured as described above. In this experiment, 0.1 $x10^7$, 0.5 $x10^7$ or 2.5 $x10^7$ cells were used to set up biofilm cultures on plates. After 18 h of preculture in TSB-YE/Glc, biofilms were quickly rinsed with 0.85% NaCl solution, and the culture medium was changed to RPMI-1640 medium. Mature biofilms were then incubated either in the presence of recombinant human IL-1β (10 ng/ml) or in the presence of an identical volume of sterile water in the control cultures. AlamarBlue™ reagent was added as 10% of the sample volume at the same time as the cytokine or water in the control samples. At time points of 0, 0.5, 1, 1.5, 2, 3, 4, and 5 h after the addition of cytokine, the metabolic activity was measured by reading the fluorescence at 544 nm excitation and 590 nm emission. Biofilm culture was continued in candle jars at 37°C between measurements.

In the metabolic activity measurement of the smooth strains, bacteria were scraped off TSA-blood agar plates. Planktonic cultures ($1x10^8$ cells) were grown in TSB-YE/Glc-medium in 14-ml polypropylene round bottom tubes (BD Falcon™ #352006). After culturing the bacteria in candle jars at 37°C for 18 h, the cells were washed with 0.85% NaCl solution. The washed pellets were resuspended in RPMI medium, and $2.4x10^7$ cells were transferred to the wells of a 48-well cell culture plate. Recombinant human IL-1β at a final concentration of 10 ng/ml was added; in control cultures, an equal volume of water was used instead of the cytokine. Finally, alamarBlue™ reagent was added to the cultures, and the fluorescence was measured as described above for the biofilm cultures.

4.5. Characterisation of IL-1β binding to *A. actinomycetemcomitans* biofilms and single cells (I, II, III)

4.5.1. Gingival mucosa co-culture (GMC) model (I, II)

Construction of the GMC model (I, II)

An organotypic GMC model (Fig 6) was used in this study to investigate the localisation of IL-1β in *A. actinomycetemcomitans* biofilms or inside a single bacterial cell. The

ability of the GMC model to produce IL-1β at moderate levels was ideal for the investigation of IL-1β localisation in biofilm cells. Additionally, gingival epithelium-*A. actinomycetemcomitans* biofilm interactions were examined using this model. The GMC model was modified from the *F. nucleatum* biofilm-oral mucosa model of Gursoy *et al*. [334].

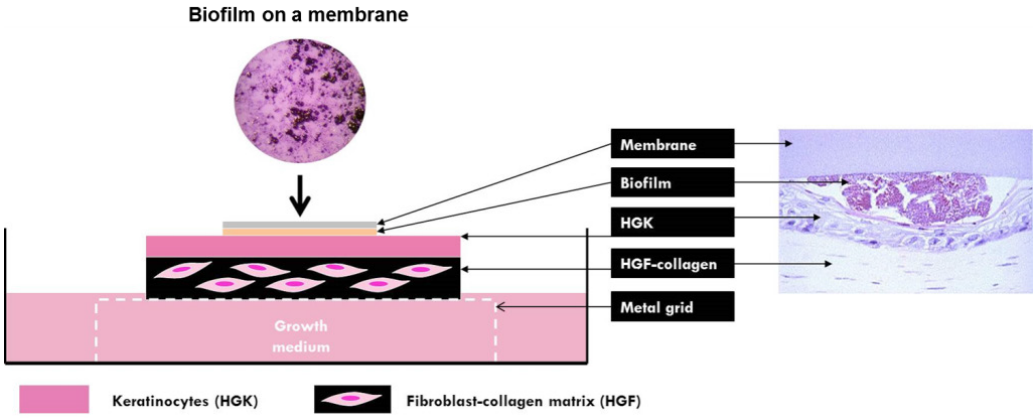


Figure 6. ***Schematic presentation of the gingival mucosa co-culture (GMC) model.*** On the left is a simplified representation of the GMC model. The model consists of human gingival fibroblasts (HGF) cultured in a collagen gel, human gingival keratinocytes (HGK) seeded on the fibroblast-collagen matrix, and 2-day-old *A. actinomycetemcomitans* D7S biofilm in direct contact with the HGFs. The GMC model is grown at the gas-liquid interface. The spherical image of the biofilm at the top of the representation is gram stained only to visualise its appearance. The structure of the GMC can be observed in the haematoxylin and eosin-stained thin section on the right. Figure modified from the models of Oksanen and Hormia 2002 [333] and Gursoy *et al*. 2010 [334].

A summary timetable of the GMC-*A. actinomycetemcomitans* biofilm model construction is represented in Table 8. Additionally, HGFs and HGKs were first grown according to standard cell culture methods in a humidified atmosphere of 95% air and 5% CO_2 at 37°C before construction of the GMC model was initiated. HGFs were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., Gibco®, cat#52100-039) supplemented with 10% foetal bovine serum (Life Technologies, Inc., Gibco®), recombinant epidermal growth factor (EGF, 5 ng/ml, Sigma), ascorbic acid (50 µg/ml, Sigma) and antibiotics (100 IU/ml penicillin and 100 µg/ ml streptomycin, EuroClone). HGKs originally obtained from a healthy human gingival biopsy sample [335] were cultured in keratinocyte-serum free medium (Life Technologies, Inc., Gibco®, cat#17005075) with bovine pituitary extract (50 µg/ml), recombinant epidermal growth factor (EGF, 5 ng/ml, Sigma), penicillin and streptomycin. HGFs (with passage numbers between 13 and 18) and HGKs (with passage numbers between 18 and 22) were passaged by incubation with 0.25% trypsin-EDTA (Life Technologies, Inc., Gibco®).

Model construction was initiated by suspending HGFs in collagen solution (PureCol®, Advanced BioMatrix) and allowing this suspension to solidify inside cell culture inserts (3 µm diameter, ThinCert™, Greiner Bio-One GmbH). The number of HGFs in each insert was $1.5x10^5$. Two hours after casting the fibroblast-collagen gel, Green's medium

[332] was added to the top of each insert, and culturing continued in the presence or absence of penicillin (63.4 IU/ml) and streptomycin (63.4 µg/ml). The composition of Green's medium is described above (see chapter 4.4.1. Biofilm mass estimation).

The next day a keratinocyte layer was seeded above the solid fibroblast-collagen gel. Co-cultures were established with thin or thick HGK layers. A thin keratinocyte layer ($1.50x10^5$ HGKs per co-culture) was used for 8-h bacterial co-cultures, and a thick keratinocyte layer ($4.0x10^5$ HGKs per co-culture) was used for 24-h cultures. The thicker epithelium was used during longer incubations because the thin epithelium appeared too damaged to estimate epithelium thickness after elongated biofilm infections. One exception was made for the 24-h samples for immunoelectron microscopy specimens; in this case, a thin keratinocyte layer GMC sample was used. This longer culture time was chosen for technical reasons, and a thin keratinocyte layer was used because the basal IL-1β levels in the culture medium were higher with thin keratinocyte layers than with thick keratinocyte layers. On third day, the gingival co-culture inserts were lifted onto a metal grid, and mucosa model culturing was continued at the gas-liquid interface. The bottoms of the cell culture inserts were filled with cell culture medium. After growing organotypic gingival cultures on the grid for 5 days, 2-day-old *A. actinomycetemcomitans* D7S-1 biofilms grown on the membrane were placed above the HGK layer. After 8-h or 24-h co-incubations, the culture media were harvested, and the co-cultures were fixed in 10% formalin solution overnight. The experiments were repeated at least three times.

Table 8. ***Timetable for GMC model construction.***

Day	GMC Model	Biofilm
1	HGFs suspended in collagen solution are cultured in cell culture inserts; $1.5x10^5$ HGFs per insert.	
2	HGKs are seeded on top of the collagen-fibroblast matrix; $1.50x10^5$ HGKs per thin culture and $4.0x10^5$ HGKs per thick culture.	
3	Cell inserts are lifted onto a metal grid and grown at the gas-liquid interface.	Bacteria are plated on blood-agar plates.
4		
5	Culture medium change.	
6		Liquid phase biofilm cultures are initiated with $5.0x\ 10^7$ bacteria per well.
7	Culture medium change.	One-day-old biofilms formed on the membrane are washed, and culture medium is changed to minimal medium.
8/ 9	GMC model is infected with a 2-day-old biofilm in the presence or absence of antibiotics for 8 h or 24 h.	

Culture medium analysis (II)

The culture media were collected from each GMC, and the total volumes of these media were quantified. Volume estimates were performed because the total volume of the medium in each well differed slightly due to varying heights of the metal grids. IL-

1β production levels were determined for each GMC by measuring the amount of IL-1β in the collected media samples using a commercial enzyme-linked immunosorbent assay (ELISA) (eBioscience) according to the manufacturer's instructions. The IL-1β detection limit of this assay is 4 pg/ml. Our interassay variation was set at 100±20 pg with thin cultures at 0 h and at 40±15 pg with thick cultures. The IL-1β levels in the analysed culture media were ≥4 pg/ml.

Proportional lactate dehydrogenase (LDH) activities in the culture media of biofilm-containing cultures were compared with the activities of filter-containing control cultures. LDH activities were measured using a Cytotoxicity Detection Kit PLUS (Roche Diagnostics) according to the manufacturer's instructions. LDH activities were compared to determine if *A. actinomycetemcomitans* biofilms caused damage to the plasma membranes of human gingival cells. The amount of cells used ($\geq 0.2\text{-}2 \times 10^4$) was high enough to meet the manufacturer´s requirements for sensitivity.

Gingival keratinocyte cell apoptosis, proliferation and appearance (II)

The apoptosis of HGKs was investigated in thin sections using a TACS® 2 TdT-DAB *In Situ* Apoptosis Detection Kit (Trevigen®) according to the manufacturer's instructions. DNA fragmentation, which is characteristic of apoptosis, can be detected in permeable tissue sections by employing the terminal deoxytransferase (TdT) enzyme. This enzyme catalyses the integration of biotinylated nucleotides at the 3' OH ends of DNA fragments. These biotin labelled fragments are then detected using streptavidin-horseradish peroxidase (HRP) and diaminobenzidine (DAB), the colorimetric substrate of HRP. Additionally, the proliferation of HGKs was examined in the thin sections of GMC. Cell proliferation can be studied by detecting Ki-67 expression in the cells by immunostaining. Proliferating cells express the Ki-67 protein during active phases of the cell cycle (G_1, S, G_2, and mitosis). In contrast, the absence of Ki-67 coincides with the resting phase of the cell cycle (G_0). This immunostaining was performed as previously described by Gursoy *et al*. [334] using the TechMate (DAKO) automated immunostainer and a commercial kit (Universal LSAB™2 Kit/HRP, Rabbit/Mouse, DAKO, cat#KO675). Briefly, to detect the proliferation marker Ki-67, thin section samples were incubated with a monoclonal mouse anti-human Ki-67 antibody (DAKO), with biotinylated secondary linking antibody and with streptavidin-HRP (DAKO) in the abovementioned order. Finally, Ki-67 expression in the tissue samples was visualised by DAB precipitation at the Ki-67 sites, and the specimens were counterstained with haematoxylin. Ki-67-positive cells were counted in the stained sections prepared from 3 to 4 individual biological replicates.

4.5.2. Immunostaining of GMC specimens (I, II)

The GMC samples were fixed in 10% formalin solution overnight, dehydrated using serial washes of increasing alcohol content (70%-100%) and embedded in paraffin wax. These samples were then cut into 4-µm-thick sections from paraffin blocks on poly-L-lysine glass slides using a microtome. Xylene treatment was used to remove paraffin from the thin sections. The sections were rehydrated with a series of washes of decreasing ethanol content (99.6–70%) prior to rehydration in distilled water. Heat-induced epitope retrieval in the tissue sections was performed in citrate buffer (pH 6.0) in a microwave oven before the actual immunostaining protocol.

The commercial NovoLink™ Polymer Detection System kit (Novocastra Laboratories Ltd., cat# RE7290-K) was used to localise IL-1β in the biofilms. Immunostaining was performed according to the manufacturer's instructions with the addition of proteinase K step after heat induction exposure to enhance epitope unmasking. After Proteinase K treatment, sections were incubated with rabbit anti-IL-1β antibody (NB600-633; Novus Biologicals) or with the negative control, rabbit immunoglobulin G (IgG, whole molecule; Jackson ImmunoResearch Laboratories) overnight. The next day, cytokine localisation was determined using the NovoLink polymer and the chromogenic substrate DAB. Finally, the slides were counterstained with haematoxylin and visually analysed to detect the brown precipitate at the antigen site. Additionally, an unconventional electron microscopy protocol [336] was employed with some immunostained specimens to achieve even greater magnification of DAB precipitate localisation in biofilm cells. In that protocol, the immunostained sections on glass slides were first fixed with 5% glutaraldehyde in 0.16 M s-collidine buffer, then incubated in 2% osmium tetroxide in water for 2 h. The sections were then dehydrated, embedded in epoxy resin and sectioned for electron microscopy. After resin removal, the sections were examined with a JEOL JEM-1200EX transmission electron microscope (Japan Electron Optics Laboratory).

4.5.3. Immunoelectron microscopy of GMC (II)

The GMC samples were first fixed in 4% paraformaldehyde supplemented with 2.5% sucrose. The next day, GMC samples were cut to smaller pieces using a biopsy punch and stored in 2.3 M sucrose in PBS. Subsequent steps of the immunostaining protocol were performed at the Biocenter Oulu (Department of Pathology, University of Oulu, Oulu, Finland) as follows. To obtain ultrathin sections for immunolabelling, the specimens were frozen in liquid nitrogen and cut with an ultramicrotome. To label the cryosections, they were first incubated in PBS + 0.2% gelatine, followed by incubation in PBS + 0.1% glycine. The blocked sections were incubated with a diluted primary rabbit anti-IL-1β antibody (NB600–633; Novus Biologicals) solution. Negative control samples were incubated in the absence of the primary antibody. The wash step was performed with PBS + 1% BSA before incubation in PBS supplemented with protein A–gold complex (size 10 nm) and 1% BSA for 30 min [337]. Finally, the sections were embedded in methylcellulose and examined using a Philips CM100 transmission electron microscope (FEI Company). Pictures were taken using a Morada CCD camera (Olympus Soft Imaging Solutions GMBH).

4.5.4. Flow cytometry (I)

Flow cytometry was used to investigate whether *A. actinomycetemcomitans* cells are able to bind IL-1β. The study was performed using the Fluorokine® kit (R&D Systems).

All *A. actinomycetemcomitans* strains, both rough and smooth, were cultured on plates after which the cells were suspended in TSB-YE/Glc. Liquid cultures of biofilm-forming strains (D7S, SA1398 and SA1151) were initiated by adding $5x10^8$ cells to TSB-YE/Glc in 50-ml tissue culture flasks (Cellstar #690160, Greiner Bio-One) and culturing the cells in candle jars at 37°C for 18 h. However, liquid cultures of the planktonic mutant and D7SS strains were initiated with a total of $1.0x10^9$ cells. After overnight cultivation, flow cytometry experiments were performed. The biofilms were rinsed twice and the planktonic cultures were rinsed once with PBS buffer. Planktonic cells and the

detached biofilm cells were fixed with fixing-solution (1% paraformaldehyde, 1% BSA, 0.01% EDTA in PBS) for 2 h at 4°C. Biofilm cells were then filtered through a 100-µm Nylon Cell Strainer filter (BD FalconTM #2360) before adjusting the cell number of each sample to $1x10^8$ cells. Cells were collected by centrifugation at 5,990 *g* for 5 min, and the resulting pellets were suspended in 1 ml of sterile PBS. For labelling, $2.5x10^6$ *A. actinomycetemcomitans* cells were first treated with biotinylated IL-1β or with biotinylated control protein (soybean trypsin inhibitor, STI) for 1 h according to the instructions of the commercial Fluorokine® kit. According to the ProtParam tool [292], STI is 20.1-kDa protein with a pI of approximately 4.5, while IL-1β is a 17.4-kDa protein with a pI of 5.9. After labelling with the biotinylated compounds, the cells were incubated with avidin conjugated to fluorescein isothiocyanate (FITC) for 30 minutes. Unreacted avidin-FITC was removed using the 1x RDF1 buffer included in the kit. Samples diluted to a final volume of 1 ml in 1x RDF1 buffer were analysed with an Epics XL flow cytometer (Coulter Corporation). Forward scatter, side scatter (SSC) and FITC fluorescence at 525 nm were measured upon excitation with a 15-mV, 488-nm argon ion laser. The signals were amplified with the logarithmic mode. Mean fluorescence intensity (MFI) and the percentage of fluorescence-positive bacterial cells were determined separately from approximately 5,000 bacteria per sample.

4.6. Interaction of IL-1β with *A. actinomycetemcomitans* proteins (I, II, III)

4.6.1. Isolation of the membrane protein fraction from *A. actinomycetemcomitans* (I, III)

Two different protocols were used for the isolation of membrane proteins from *A. actinomycetemcomitans*. The first method [338,339] isolates outer membrane and inner membrane proteins into separate fractions (Fig 7). The soluble fraction containing the intracellular proteins of *A. actinomycetemcomitans* (Fig 7, designated as supernatant 2) was used when the interaction of IL-1β with soluble cytoplasmic proteins of the *A. actinomycetemcomitans* D7S-1 strain was investigated (I, II). The outer membrane protein-containing fraction was used as a control sample when localisation of the identified total membrane protein was characterised (III).

The total membrane fraction was isolated as described by Paul-Satyseela *et al*. [339] but using a different buffer composition. *A. actinomycetemcomitans* cells were first scraped off culture plates into PBS (pH 7.4). After collection, the cells were suspended in PBS supplemented with 150 mM sucrose and 1 mM Pefabloc. Cells were lysed by sonication, and the cell debris was separated from the supernatant by centrifugation at 1,700 g. The supernatant was transferred to an ultracentrifuge bottle and underlaid with sucrose (0.9 M sucrose in PBS). The total membrane pellet was isolated using a 3-hour ultracentrifugation step at 150,000 g and stored at −20°C. After thawing, the membrane pellet was suspended in PBS supplemented with 4% CHAPS buffer (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and pellet dissolution was enhanced by incubating in a slowly rotating wheel at 37ºC. The undissolved components were collected by a brief spin, and the supernatant was used for electrophoretic mobility shift assay (EMSA) experiments.

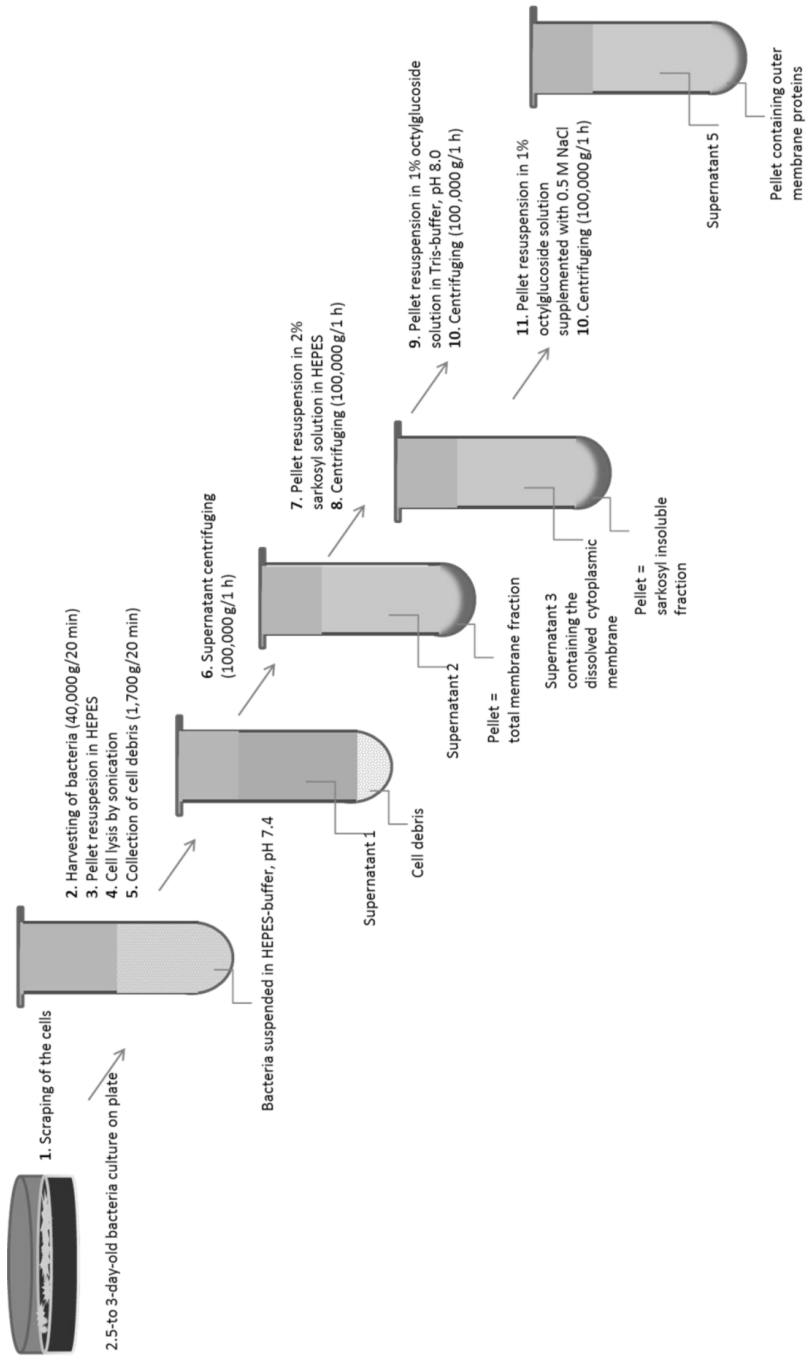


Figure 7. ***Protocol for the extraction of outer and inner membrane fractions of A. actinomycetemcomitans.*** The protocol is described by Wilson 1991 [338] and Paul-Satyaseela *et al.* 2006 [339].

4.6.2. EMSA of the intracellular protein fraction of *A. actinomycetemcomitans* (I, II)

The binding of IL-1β to intracellular proteins of the *A. actinomycetemcomitans* D7S-1 strain was examined by EMSA. Additionally, this method was later used to verify IL-1β interaction with the recombinant DNA binding protein HU to investigate *A. actinomycetemcomitans* nucleoid migration. The intracellular protein fractions were separated by a nondenaturing gel; samples were loaded in duplicate to yield two identical gel halves. One side of the gel was silver stained [340], and the second half was electroblotted onto a nitrocellulose membrane. The BSA-blocked membranes were incubated with biotinylated IL-1β or biotinylated control protein STI. After washing, the membranes were incubated with HRP-linked streptavidin (Sigma). Proteins interacting with biotinylated IL-1β on the blot produced a chemiluminescent signal after addition of the ECL Western blotting substrate (Pierce®, Thermo Scientific). The bands showing higher intensities with IL-1β than with STI were excised from the silver stained gel and identified by mass spectrometry (MS). This protein identification by MS was performed in the Turku Proteomics Facility (Turku Centre for Biotechnology, Turku, Finland). The MS protocol is described in paper I.

We used an EMSA method to study putative effects of IL-1β on the nucleoid binding of HU. Small nucleoid-associated proteins are known to impact chromosomal DNA structure, and we thus hypothesised that the interaction of IL-1β with HU could be observed as altered nucleoid migration. First, recombinant HU (60 pmol) was incubated with IL-1β (0.6 pmol and 6 pmol) or with similar amounts of STI in negative control samples. After this first incubation, nucleoids extracted from *A. actinomycetemcomitans* D7S-1 cells [341] were added to the reaction mix and incubation continued. After incubation, samples were loaded onto a 4–15% Tris–HCl native gel. The differences in protein migration were visualised by silver staining [340]. The ability of purified HU to bind to nucleoids and the presence of DNA contamination in the purified recombinant proteins were tested by running an agarose DNA gel and staining for DNA.

4.6.3. EMSA for the membrane protein fraction of *A. actinomycetemcomitans* (III)

The protein concentration of CHAPS-soluble membrane protein fraction was determined using the method described by Lowry *et al*. [331]. In an EMSA assay, the solubilised membrane protein fraction (2 μg) was incubated with 300 ng of recombinant IL-1β (Reliatech GmbH) or with equal volume of control buffer at RT for 1 h. Then, samples were loaded onto a nondenaturing 4-15% Tris-HCl gel. After electrophoresis the samples were visualised using silver staining [340] and western blotting analysis. In western blotting, the proteins were transferred onto a nitrocellulose membrane in a semi-dry blotter following the membrane blocking with PBS-skimmed milk solution. The IL-1β bands were visualised on nitrocellulose membrane with an Odyssey Infrared Imager (Li-Cor) using rabbit anti-IL-1β antibody (NB600-633; Novus Biologicals) and fluorescently labelled secondary antibody [IRDye 800CW Donkey Anti-Rabbit IgG (H + L) Highly Cross Adsorbed (Li-Cor)]. A silver-stained membrane protein band that bound anti-IL-1β was isolated from a gel and subjected to a mass spectrometry analysis. This analysis was performed in the Turku Proteomics Facility (Turku Centre for Biotechnology, Turku, Finland). Briefly, the sample was digested with trypsin prior to the resulting peptides were processed for coupled high-performance liquid chromatography/electrospray ionisation mass spectrometry/mass spectrometry (HPLC/ESI-MS/MS) analysis. The proteins

identified from MS analysis contained *A. actinomycetemcomitans* or human proteins because the protein hits from other species were filtered out. Additionally, protein hits of less than two peptides were also excluded during protein identification.

4.6.4. Microplate assay to study the interaction of IL-1β with recombinant *A. actinomycetemcomitans* proteins (I, II, III)

A microplate assay was used to verify the interaction between IL-1β and the recombinant proteins ATP synthase subunit β (ATPsynβ), HU and BilRI of *A. actinomycetemcomitans*. These three proteins showed interactions with IL-1β in previously described EMSA analyses. The N-terminal portion of the *A. actinomycetemcomitans* outer membrane protein RcpA (MW=19.3 kDa, aa 30-192) containing the same C-terminal 8xHis-tag as the tested recombinant proteins was used as a negative control in the ELISA studies. The detection method in this interaction study was based on the commercial HisProbe™ reagent (Thermo Scientific). The reagent was used according to the manufacturer's instructions to detect the binding of the labelled probe to the C-terminal 8xHis-tag of recombinant proteins. In this protocol, the wells of a 96-well plate were first coated with similar amounts of BSA, IL-1β or STI negative control protein (Sigma). After the wells were coated, they were rinsed with wash buffer, and nonspecific binding sites in the wells were blocked with 1% BSA solution. Recombinant His-tagged proteins (Table 9) were then added to the wells and allowed to incubate overnight. The following day, the wells were carefully washed before incubation with wash buffer supplemented with HRP-conjugated HisProbe™ reagent. The interaction of recombinant His-tagged proteins with IL-1β was detected after adding HRP substrate ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) solution and measuring the absorbance at 405 nm. The experiments were performed at least in triplicate.

Table 9. ***Protein concentrations (μM) used in the microplate assay investigating the interactions of recombinant proteins with human IL-1β.***

Recombinant protein	IL-1β (μM)§	Recombinant protein (μM) *	Paper
ATPSynβ	0, 6	0, 3, 6, 12, 24, 48, 98	I
HU	0, 6	0, 28	II
cBilRI	0, 0.1	0, 100	III
RcpA (negative control protein)	0, 0.1or 0, 6	0, 3, 6, 12, 24, 48, 98	I, III

§IL-1β concentration used for well coating

*Recombinant 8xHis-tagged protein concentration in the coated well

4.6.5. Characterisation of the IL-1β interaction with BilRI (III)

BilRI-recognising antibody fragments (III)

Selection and screening of BilRI-recognising antibody fragments and the production of active clones were performed by the MolBind Service (Department of Biochemistry, University of Turku, Turku, Finland). Briefly, two synthetic single-chain variable antibody fragment (scFv) phage libraries ScFvM [342] and ScFvP [343] were constructed. M13 phage display, cloning

of the scFvs into the screening vectors and expression of scFv-AP (AP, bacterial alkaline phosphatase) fusion proteins were performed as previously described [342,343]. The activity of BilRI-recognising antibody fragments was tested by a sandwich immunoassay, and ten active clones were identified, selected for further testing and produced as scFv-AP fusion proteins. The ability of the active clones to bind BilRI was also verified through western blotting, and clones 16B8 and 16F7 were selected for use in further analyses.

Bioinformatics (III)

The amino acid sequence of IL-1β-interacting membrane protein was analysed using the SignalP 4.1 server [327] to predict the presence and location of signal peptide cleavage sites. Additionally, the LipoP 1.0 server [344] was employed for the detection of the lipoprotein signal sequence. Subcellular localisation of the protein was estimated using the SosuiGramN tool [345]. Sequence similarity searches were performed with SIB using the BLAST network service. The SIB BLAST network service employs a server developed at SIB and NCBI BLAST 2 software [346]. Sequence alignments were performed using the ClustalW (1.83) program of the SIB T-Coffee multiple sequence alignment package [347].

Expression of BilRI in various clinical isolates of A. actinomycetemcomitans (III)

BilRI protein expression in six clinical *A. actinomycetemcomitans* strains was examined. In addition to the D7S-1, SA1398, and SA1151 strains that have shown IL-1β-binding capacity in our first studies (I), three other clinical strains RIB04, RIB07 and RIB09 were tested. The strains were cultured as biofilms in cell culture flasks and initiated with $5x10^8$ cells. The biofilms were first cultured in TSB-YE/Glc for approximately 18 h and washed twice before culture was continued in minimal nutrient medium for 3 h. Cells were then detached using a cell scraper. Cells were suspended in Laemmli sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at a final concentration of 450 mg/ml, which corresponded to approximately $9x10^9$ CFU/ml. Cells were disrupted by sonication, and aliquots containing $0.3x10^7$ disrupted cells were loaded onto a 10.5-14% Tris-HCl precast gel. Proteins were transferred to a nitrocellulose membrane. After blocking overnight with BSA, the membrane was incubated with an alkaline phosphatase-fused recombinant anti-BilRI antibody clone that was custom-made using the M13 phage display as described above. The membrane was incubated with a biotinylated anti-alkaline phosphatase antibody (NB600-500; Novus Biologicals, Cambridge, UK), with HRP-labelled streptavidin (S2438, Sigma) and with the ECL substrate (Pierce®, Thermo Scientific); careful washes of the membrane with a Tween-containing buffer were performed between each incubation step. BilRI expression by the clinical *A. actinomycetemcomitans* strains was detected by film.

Proteinase K treatment to examine the surface exposure of BilRI in A. actinomycetemcomitans (III)

Surface exposure of lipoprotein BilRI on intact cells was evaluated by Proteinase K treatment. A slightly modified version of a published Proteinase K treatment protocol was employed [348]. *A. actinomycetemcomitans* D7S-1 cells cultured on TSA plates were suspended in PBS and collected via centrifugation at 3,800 g for 10 minutes. The pellets were resuspended in PBS, and the suspensions were filtered through a 100-mm Nylon Cell Strainer (BD FalconTM #2360). Cell density was adjusted to $1.7x10^8$ cells/

ml with Proteinase K buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 40 µg/ml chloramphenicol, pH 7.5) supplemented with 55 µg/ml globomycin (G1424, Sigma). The presence of chloramphenicol inhibited protein synthesis, and globomycin blocked the function of the lipoprotein-specific signal peptidase SPII [349,350] in bacteria. Globomycin treatment of cells was performed in Proteinase K buffer with shaking at 37ºC for 30 minutes, and the subsequent Proteinase K treatment was performed with $4.5x10^7$ bacteria at a final Proteinase K concentration of 2 mg/ml. In the control samples, Proteinase K was replaced with an equal volume of sterile water. Proteolysis reactions were stopped after 0-, 5- or 21-h incubations by the addition of 1 mM PMSF. The treated cells were harvested by centrifugation (3,800 g for 10 minutes) and washed once with Proteinase K-PMSF buffer. Finally, the resulting pellets were suspended in SDS-PAGE sample buffer and lysed via sonication (4x1 min, separated by 1 min incubation breaks on ice). Western blot analysis was performed similarly to the BilRI expression study described above. Proteinase K function was verified using outer membrane protein RcpA as a positive control in the proteolysis analysis. The immunostaining protocol of this control study contained a nitrocellulose membrane blocking with PBS-skim milk solution and an overnight incubation with custom-made rabbit polyclonal anti-RcpA antibody (0.8 µg/ml; Abcell). Additionally, the membrane was incubated with ECL™ Rabbit IgG, HRP-linked whole Ab (NA934, GE Healthcare) before HRP-substrate addition and film development.

Membrane fraction isolation and flow cytometry of E. coli (III)

Recombinant *E. coli* C41(DE3)-RIL cells contained the complete BilRI sequence in a cloning vector. These cells expressed and secreted BilRI to the membranes after IPTG induction. Uninduced cells cultured in the absence of IPTG were used as controls for BilRI-expressing *E. coli* cells. Cell lysis during storage was examined to determine the optimal freezing conditions for membrane protein-overexpressing cells. The effect of glycerol on cell lysis was investigated by storing cells in different concentrations (20%-50%) of glycerol during freezing. Supernatant of the buffer-resuspended cells was collected after a freeze-thaw cycle by centrifugation at 16,000 g for 15 minutes. Supernatant samples were run in a 0.8% agarose gel containing Midori Green DNA Stain (Nippon Genetics Europe). Self-aggregation of the cells was also investigated. Cells were washed twice with HEPES buffer (10 mM HEPES, pH 7.4) after storage and suspended at cell density of OD_{600nm}=1 in HEPES buffer supplemented with 50 mM $CaCl_2$. After a 1-h incubation, self-aggregation of the cells was estimated visually.

Outer membrane (OM) fraction isolation from *E. coli* C41(DE3)-RIL cells containing the pET-36b-BilRI protein expression vector was performed using the protocol of Achtman *et al*. [351] with a slight modification. This method was utilised when the expression or localisation of BilRI in induced *E. coli* cells was investigated. Briefly, the induced cells stored in glycerol were washed twice with PBS before the pellets were suspended in 10 mM Tris (pH 8.0) supplemented with 0.2 mM PMSF. Cell numbers of each sample were adjusted to $2.5x10^9$ using the cell number determination method of Volkmer and Heinemann [352]. According to this method, the concentration of *E. coli* cells cultured in Luria Bertani medium is $7.8\pm0.8x10^8$ cells/ml when the OD_{600nm}=1.0. After fine-tuning the number of cells, the cells were lysed by sonication, and cell debris from unbroken cells was then collected by centrifugation at 1,100 g for 20 minutes. OM fraction isolation continued by centrifuging the resulting supernatant samples at 48,000

g for 1 h. Pellets from the centrifugation were dissolved in water prior to OM extraction with Tris-buffer (11.1 mM, pH 7.6) supplemented with 1.67% sarkosyl. Insoluble outer membranes were collected with a 1.5-hour centrifugation at 48,000 g. Different cellular fractions (soluble cytosolic, sarkosyl-soluble inner and outer membranes) from the extraction were dissolved in 2% SDS. The silver staining method was used to visualise the presence of recombinant BilRI in SDS-dissolved samples. Using the dot blot method coupled to ECL (Pierce®, Thermo Life Science) detection, protein samples were observed on blotted nitrocellulose membrane. The absence of inner membrane-located heme proteins, which react with the ECL reagent, in the extracted outer membrane fraction was used as an indicator of OM fraction purity [353,354] in the dot blot method.

Prior to the initiation of cell labelling for flow cytometry, *E. coli* cells were washed three times with PBS buffer to eliminate the glycerol storage solution. Cells were then fixed and labelled similarly to *A. actinomycetemcomitans* cells for flow cytometry. However, the number of *E. coli* cells was adjusted to $2.5x10^6$ using the *E. coli*-specific cell concentration conversion [352]. Additionally, the avidin-FITC labelling step in the flow cytometry protocol was replaced with Syto9 (LIVE/DEAD ® BacLight™ Bacterial Viability and Counting Kit, L34856) green fluorescent nucleic acid staining for control *E. coli* samples. This staining enabled the determination of the actual proportion of bacteria in samples when a cytometric run was performed with the Beckman Coulter Cell Lab Quanta™SC Flow Cytometer (Beckman Coulter, Inc.). The FITC fluorescence of cells binding biotinylated antigen or Syto9-stained cells was measured at 525 nm after excitation at 488 nm with an argon ion laser. The percentage of fluorescence-positive bacterial cells and MFI were determined separately from approximately 10,000 bacteria per sample by gating the bacterial population according to the green fluorescence/SSC bivariate histogram. To exclude disturbing debris in the green fluorescence/SSC histogram, the discriminant was set to the SSC channel.

4.7. Characterisation of the extramembranous domains of HofQ (IV)

4.7.1. EMSA to examine protein-DNA interactions (IV)

Linearised pUC19-based plasmids were used to test the binding of emHofQ to double-stranded DNA in the EMSA assay. Additionally, the dependency of emHofQ DNA binding on USS was tested. Two separate plasmids were designed; plasmids with and without the USS were constructed as follows. A 760-nucleotide-long region of the aggregation locus of the *A. actinomycetemcomitans* D7S-1 genome (NC_017846.1, bases 1350204 to 1390964) was amplified by PCR. For USS plasmid construction, USS sequence (-5′-AAGTGCGGT, bold)-containing forward and reverse primers were used. The sequence of the forward primer was 5-GCGACATATG**AAGTGCGGT**TTTTTTGTTTAAATCAGTTTCAGTGAGATA (NdeI site underlined), and the sequence of the reverse primer was 5′-AGAAGGATCC**AAGTGCGGT**GTGTTTTTGTGGCGTTTTTA (BamHI site underlined). The constructed USS primer contained two USS sequences at both ends of the 760 bp insert. The following primers were used to construct a plasmid without the USS: forward primer, 5′-TTAACATATGCGGCGGTTTTTTGTTTAAATCAGTTTC A; reverse primer, 5′-TATTGGATCCGTGMTGTTTTTGTGGCGTTTT). Primers for PCR were purchased from Eurofins MWG Synthesis GmbH, and Phusion High-

Fidelity DNA Polymerase was used in the PCR. After PCR, reaction products and the pUC19 plasmid were a double digested with BamHI and NdeI. Restriction products were purified with the Qiaquick Gel Extraction kit prior to fragment ligation (T4 ligase; Fermentas) with the digested pUC19 plasmid and electroporated into competent *E. coli* XL1-Blue cells. Ampicillin (100 μg/ml) was used for transformant selection, which was followed by plasmid amplification and purification using the QIAprep Spin Miniprep kit (Qiagen). Plasmid constructs were verified by sequencing (Eurofins MWG Operon). The correct 0.76-kb USS insert-containing plasmid pUSS (Fig 8) was purified using the Qiagen Plasmid Midi Kit. After construct production, EMSA was performed by incubating the BamHI-linearised plasmids (300 ng) with emHofQ (100 μg), T4 gene 32 protein (10 μg) (New England Biolabs) or BSA (100 μg) for 30 min at RT. Samples were loaded on a 0.8% agarose gel and visualised after the gel electrophoresis using GelRed DNA stain (Biotium) under ultraviolet light.

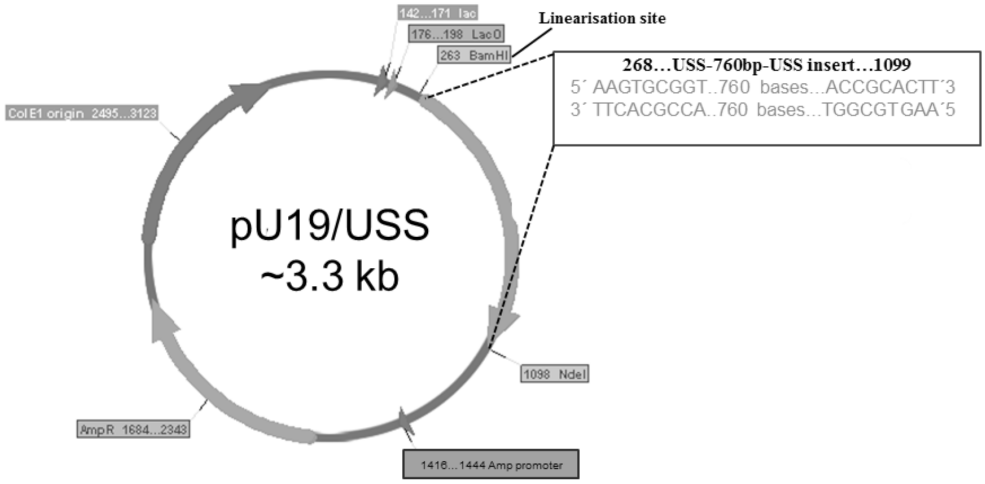


Figure 8. ***The constructed pUC19-based pUSS plasmid.*** pUSS contains two AAGTGCGGT (USS) sequences in a 760-bp insert located between the BamHI and NdeI restriction sites. The BamHI site was used for plasmid linearisation.

4.7.2. Crystallisation and structure determination (IV)

The crystallisation, data collection and structure determination were carried out by a collaborating group at Stockholm University (Sweden). Briefly, crystals were obtained by the sitting drop vapour diffusion method. Correct crystallisation conditions were determined using a Mosquito crystallisation robot (TTP Labtech) in the screening. Crystals formed in a 2 M sodium formate and 0.1 M sodium acetate (pH 4.6) well solution at 21°C after 5 days. Crystals were flash-cooled with liquid nitrogen using 25% glycerol as a cryoprotectant. The native data set was collected at beamline BM30, and multiwavelength anomalous diffraction data were collected at beamline ID14-1 at the European Synchrotron Radiation Facility (Grenoble, France). Detailed statistics of the data collection and structure refinement are reported in paper IV (IV: Table 1).

4.8. Ethics Statement (III)

The collection of subgingival plaque samples was approved by the Ethics committee of the Hospital District of Southwest Finland, Turku, Finland. Subgingival microbial samples from adult periodontitis patients were obtained with informed consent.

4.9. Statistics (I, II, III)

Significant differences were calculated using a Mann-Whitney U-test (I, crystal violet), the paired samples T-test (I, flow cytometry with *tad* locus deletion mutants), Student's t-test (II and III) and Wilcoxon Signed Ranks Test (I, flow cytometry with clinical *A. actinomycetemcomitans* strains). Statistical significance was assigned at $p<0.05$ (I, II, III).

5. RESULTS AND DISCUSSION

5.1. Effects of IL-1β on biofilm mass and *A. actinomycetemcomitans* metabolism (I, II)

Two tested clinical *A. actinomycetemcomitans* strains, D7S-1 (serotype a) and SA1151 (serotype c), showed significantly ($p<0.05$) increased biofilm mass formation when biofilms were cultured in the presence of human recombinant IL-1β (10 ng/ml) for 6 h (I; Fig 1). Biofilm mass estimation was based on the crystal violet staining method [168]. The limitation of this study was that longer incubation times in the presence of IL-1β were not investigated. The incubation time was chosen for the technical reasons. Moreover, three-day-old *A. actinomycetemcomitans* biofilms are known to initiate dispersion [168], which restricted the monitoring of substantially older biofilms.

In addition to the difference in total biofilm mass between the two groups, human IL-1β demonstrated a temporary impact on the metabolic activity of the *A. actinomycetemcomitans* D7S-1 biofilm (I: Fig 2A). IL-1β also decreased the metabolic activity of planktonic *A. actinomycetemcomitans* D7SS (I: Fig 2B), a spontaneous smooth colony mutant of the D7S-1 strain. The metabolic activity of *A. actinomycetemcomitans* was monitored by fluorescence detection after alamarBlue™ reagent reduction. IL-1β transiently decreased the metabolic activity of all three tested biofilm-forming clinical strains (D7S-1, SA1398 and SA1151). IL-1β caused a statistically significant decrease ($p<0.05$) in the metabolic activity of D7S-1 after 1 h of culture (I: Fig 2A). After 3 h of culture, the drop in metabolic activity was not significant (I: Fig 2A). Additionally, studies with the SA1398 and SA1151 biofilms suggested that the metabolic activity decrease is dependent on the IL-1β/cell ratio (data not shown). We thus hypothesise that the noted drop in metabolic activity was only transient, as biofilm cells may quickly consume the supplemented IL-1β. In the present study, the influence of repetitious IL-1β additions on the metabolic activity of the *A. actinomycetemcomitans* biofilm was not examined due to cytokine cost. To test our hypothesis, this type of study should be performed.

Additionally, we studied the putative role of the Tad secretion system in IL-1β uptake by using deletion mutants of the Tad system-encoding genes. This system is responsible for the assembly and export of fimbrial low-molecular-weight protein (Flp) and is encoded by 14 different genes (see review [157]). We examined the metabolic activity change in non-fimbriated Δ*flp1-flp2,* Δ*rcpA,* Δ*rcpB,* Δ*tadD* and Δ*tadG* single gene mutant strains as a result of the IL-1β stimulus [321]. We observed that all mutants, with the exception of Δ*rcp*A, showed a transient metabolic activity decrease in the presence of IL-1β (I: Fig 2B). Furthermore, all non-fimbriated strains, including the Δ*rcpA* mutant, showed a similar weak fluorescence signal formation in the absence of cytokine. This result verifies that the basic metabolism of all mutants was similar; therefore, the observed difference between the ΔrcpA mutant and other non-fimbriated strains was not due to altered basic metabolism (I: Fig 2C). The ability to slow down metabolic processes and cell division usually makes bacteria more resistant to antibiotics and other antimicrobial agents (see review [355]). The tendency of *A. actinomycetemcomitans* biofilms to respond to the human inflammatory mediator IL-β by metabolic activity

decrease may be part of its pathogenic mechanism in periodontitis. To verify this putative connection, a study examining both the expression levels of RcpA in several clinical *A. actinomycetemcomitans* strains and their metabolic response to IL-1β is required.

5.2. Viable *A. actinomycetemcomitans* biofilms bind and take up IL-1β (I, II)

The *A. actinomycetemcomitans* D7S-1 biofilms co-cultured with human gingival cells bound IL-1β produced by the GMC model (Fig 9), as the biofilms showed intense staining during immunolabelling. Labelling with the negative control antibody IgG did not stain the biofilms, suggesting that this staining with IL-1β was not due to nonspecific antibody binding. Moreover, the presence of antibiotics (penicillin and streptomycin) in the co-culture medium prevented IL-1β binding to biofilms. (Fig 9) The ability of cell culture antibiotics to decrease the viability of *A. actinomycetemcomitans* D7S-1 biofilms was verified with LIVE/DEAD staining by confocal microscopy (II: Fig 1A). Additionally, the use of these antibiotics decreased the total *A. actinomycetemcomitans* biofilm mass when cultures were inspected with the crystal violet staining method (II: Fig 1B). The decrease in cell viability was observed after 6 h culturing, and the decrease was more obvious after 25 h. In cell viability assays, the confocal microscope images were used for a qualitative analysis, and crystal violet staining was used for the quantification of biofilms.

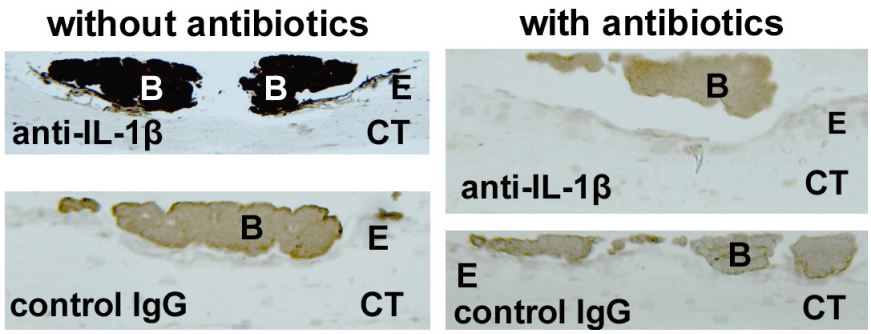


Figure 9. ***Viable A. actinomycetemcomitans biofilm binds IL-1β.*** After 8 h of co-culturing with organotypic gingival mucosa in the absence of antibiotics, two-day-old *A. actinomycetemcomitans* D7S-1 biofilms bound the IL-1β produced by the GMC model. Biofilms cultured with the GMC model containing antibiotics did not demonstrate IL-1β binding. The binding of IL-1β to biofilms was investigated using thin sections prepared from organotypic co-cultures. After the sections were treated with anti-IL-1β antibody or negative control IgG antibody, non-related IgG, immunostaining was completed using a commercial polymer detection method. B: biofilm; E: epithelial cell layer; CT: fibroblast-collagen matrix. Figure from Figure 2A (II).

Using an adapted transmission electron microscopy method, we demonstrated that *A. actinomycetemcomitans* D7S-1 biofilm cells co-cultured with the GMC model were able to take up IL-1β (I: Fig 3). A dark precipitate was localised to both sides of the double membrane and in the vicinity of bacterial nucleoids in anti-IL-1β stained specimens (I: Fig 3G). Additionally, positive control samples treated with the antibody against outer membrane protein RcpA showed intense precipitate formation (I: Fig 3H). In contrast, the control IgG-treated samples did not show these dark precipitates, excluding the

possibility of nonspecific antibody binding (I: Fig 3I). Immunoelectron microscopy was used to detect the precise location of IL-1β in co-culture specimens and to examine how antibiotics affect the IL-1β binding ability of bacterial cells (II: Fig 2C). The major findings of these experiments were that only viable *A. actinomycetemcomitans* biofilm cells could take up IL-1β (II: Fig 2C) and that intracellular IL-1β was often localised to the outer edges of nucleoids (II: Fig 3A). The localisation of IL-1β in specimens derived from antibiotic-free culture is described in Figure 10. Interestingly, specimens from GMC with *A. actinomycetemcomitans* biofilms grown in the presence of antibiotics did not show similar positive staining (II: Fig 2C). As expected, the keratinocyte cell layer of the GMC model was positively stained despite the use of antibiotics during culture (II: Fig 2C). However, the use of antibiotics appeared to have an effect on the appearance of biofilm cells. Single cells from biofilms cultured in the presence of antibiotics were smaller and more damaged according to immunostaining (II: Fig 2C). Furthermore, the LIVE/DEAD staining of biofilms integrated with the biofilm crystal violet staining described above confirmed that antibiotic treatment influenced the viability of *A. actinomycetemcomitans* biofilm cells (II: Fig 1).

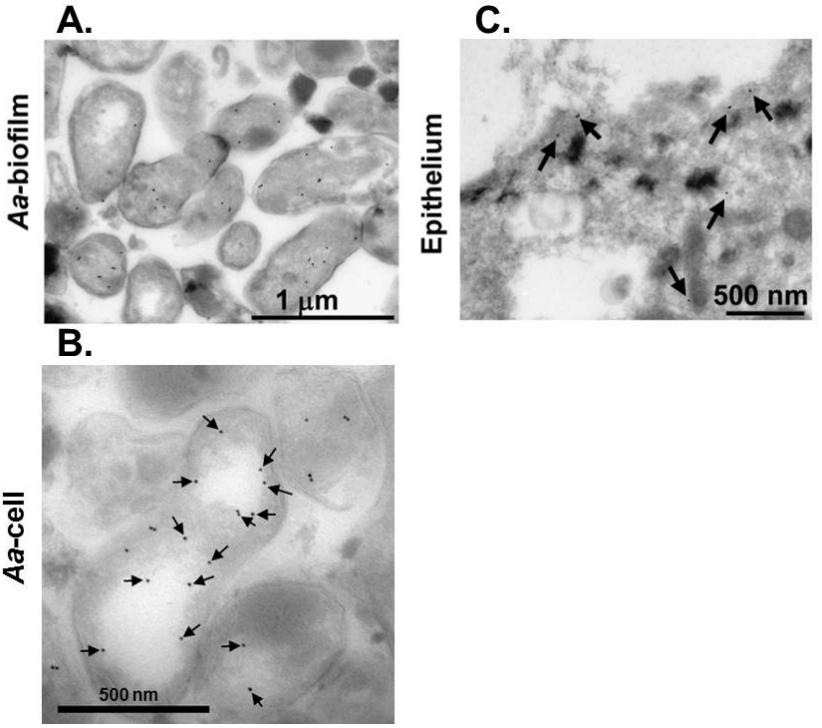


Figure 10. Viable *A. actinomycetemcomitans* D7S-1 biofilm internalises IL-1β. IL-1β localisation in immunoelectromicroscopy specimens prepared from organotypic mucosa co-cultures challenged with *A. actinomycetemcomitans* D7S-1 biofilm for 24 h without antibiotics. (A) Single biofilm cells contain IL-1β in the cytoplasm. (B) The magnification of a single cell shows that IL-1β is localised to the outer edges of nucleoids in some viable *A. actinomycetemcomitans* cells. (C) Based on positive staining, the epithelial cell layer produces IL-1β. Figure modified from Figures 2C and 3A (II).

The detailed mechanism of the internalisation of IL-1β into *A. actinomycetemcomitans* cells remains unsolved. First, we should address the question of how it is possible for a bacterium to take up a compound as large as 17.4 kDa. A previous study by Kanagat *et al*. [7] showed that under >5 kDa peptide fragments of IL-1β, aa residues 118-147 or most importantly aa residues 208-240, had growth-promoting effects in *S. aureus.* In fact, the cytokine may be transferred through the membrane as a shorter fragment. Polyclonal IL-1β antibodies were used in our studies because they may produce more amplified signals at low IL-1β expression levels. Moreover, use of a monoclonal antibody results in a low risk of an unexposed epitope recognition site. The specific epitope recognition site of a monoclonal antibody might have been hidden by the interactions IL-1β of with *A. actinomycetemcomitans*-derived proteins and thus prevent IL-1β detection. Alternatively, the ability of the polyclonal antibody to interact with the entire IL-1β molecule or with a shorter peptide of the cytokine can be observed as a disadvantage of our immunoelectromicroscopy experiment. We cannot be sure whether the entire IL-1β molecule is taken up by the cells. It is known that macromolecules as large as 6 kDa can be passed through *E. coli* porins with molecular weight cut-off filters of 600 Da [356]. A follow-up study with *A. actinomycetemcomitans* biofilms cultured in the presence of specific IL-1β fragments might reveal the size of the cytokine that was taken up. The other important question that needs to be addressed is the quantification of IL-1β uptake. Our experiments did not show the exact amounts of IL-1β uptaken by the cell. The use of labelled [^{125}I]-IL-1β [9] might produce more detailed information about the IL-1β sequestering and uptake capacity of *A. actinomycetemcomitans* biofilms.

The levels of IL-1β in the culture medium from GMC models with a thin keratinocyte layer were significantly higher ($p<0.001$) at 8 h of incubation with *A. actinomycetemcomitans* biofilm in the presence of antibiotics than in the absence of antibiotics according to ELISA analysis (II: Fig 2B). These findings reinforced our initial hypothesis about *A. actinomycetemcomitans* ability to uptake up IL-1β from its environment. However, this difference in IL-1β leakage in nonantibiotic and antibiotic-treated *A. actinomycetemcomitans* co-cultures was not observed when the experiment was performed with thick keratinocyte layers and with 24 h of co-culture (II: Fig 2B). Therefore, the IL-1β production kinetics of our GMC model seems to be highly dependent on the thickness of the gingival cell layers and the duration of the bacterial challenge. Moreover, the complex interactions between the gingival cells and the bacterial biofilm hamper the understanding of IL-1β production kinetics even more. Furthermore, we have not determined the putative basal differences in the IL-1β production levels between HGKs and HGFs using monolayer cultures of these two tested GMC cell lines, and it is not known how each cell line impact on the other's IL-1β production. The finding of lower IL-1β levels after 24-h bacterial challenge is in agreement with a study where HGFs stimulated with LPS from *P. gingivalis* produced significantly more IL-1β during the first 3 to 6 h than after 24 h of infection [220]. However, human gingival epithelial cells are known to steadily enhance their IL-1β production after the first 48 h of stimulation with soluble *A. actinomycetemcomitans* extract [357]. This result is in contrast with our finding that during an elongated challenge, IL-1β levels were considerably lower after 8 h of exposure.

5.3. IL-1β binds single *A. actinomycetemcomitans* cells (I, II, III)

Using flow cytometry, we demonstrated that biotinylated IL-1β binds whole *A. actinomycetemcomitans* cells (I: Fig 4). Cytokine binding was tested with three clinical strains (D7S-1, SA1398 and SA1151), the naturally smooth strain D7SS and the Tad system mutants (Δ*flp-1*-Δ*flp2,* Δ*rcpA,* Δ*rcpB,* Δ*tadD,* and Δ*tadG*). The Δ*flp1-flp2* deletion mutant expressed all components of the Tad system apart from the fimbriae [321] required for nonspecific adherence and biofilm formation [55,158,160,321]. Four other mutants, Δ*rcpA,* Δ*rcpB,* Δ*tadD* and Δ*tadG,* could not form the functional channel for fimbria secretion and thus lost their nonspecific adherence ability [160,321,358]. RcpA forms an outer membrane secretin channel for fimbriae secretion [159]. Outer membrane protein RcpB is also required for bacterial adherence [358]. It has been hypothesised to play a role in assembly of the RcpA complex or to serve as a gate to the secretin pore [160]. The essential role of lipoprotein TadD in the outer membrane localisation of RcpA has been determined [159]. Moreover, the deletion of *tadG* is known to increase the fragility of single *A. actinomycetemcomitans* cells and stimulate the release of extracellular compounds from the bacterium [321]. TadG may also serve as a fimbriae anchor [321].

In our experiment, biotinylated IL-1β bound to all tested *A. actinomycetemcomitans* strains more efficiently than the negative control protein biotinylated STI ($p<0.001$). Therefore, IL-1β binding was not only a property of biofilm-forming isolates, as a relatively higher per cent of non-fimbriated Δ*flp1-flp2* mutant cells was positively stained compared with the three tested biofilm-forming strains (I: Fig 4A). This difference was statistically significant ($p<0.05$) only with strain SA1398 (I: Fig 4A). In contrast, all tested biofilm-forming strains showed higher mean fluorescence intensity per positive stained cell than the planktonic strains. However, a statistically significant ($p<0.05$) difference was observed only with SA1151 (I, Fig 4 B).

In a flow cytometry study, IL-1β binding to whole cells was not significantly reduced ($p<0.05$) in any of the Tad system mutants (I: Fig 4A). This result indicates that none of the proteins (Flp, RcpA, RcpA, TadD or TadG) was a specific binder of IL-1β. However, two deletion mutant strains, Δ*rcpA* and Δ*tadD*, showed enhanced IL-1β binding compared with the control strain, a non-fimbriated Δ*flp1-flp2* mutant. Nevertheless, the difference in IL-1β binding was statistically significant ($p<0.01$) only with the Δ*tadD* mutant (I: Fig 4A). It is worth noting that a deficiency in one *tad* locus gene can affect the abundance, expression and stability of other proteins produced by the locus. For instance, the absence of TadD diminishes the localisation of RcpA and RcpB to the outer membrane [159]. Therefore, the enhanced IL-1β binding to a Δ*tadD* mutant cell might be explained by a coincidental deficiency in the outer membrane RcpA channel. We hypothesise that secretin RcpA forms a channel for IL-1β uptake and that cells can bind more IL-1β in the absence of the channel. The N-terminus of RcpA is hypothesised to form an immunoglobulin (Ig)-like fold found in structures such as IL-1RII (see reviews [157,359]). The outer membrane protein Caf1A from *Y. pestis* has been characterised as an IL-1β-binding bacterial receptor for the cytokine [10]. Caf1A of *Y. pestis* and RcpA of *A. actinomycetemcomitans* play in similar roles in F1 fimbrial capsule production and Flp1 fimbriae subunit assembly [157,298].

However, based on our ELISA analysis, the N-terminal portion of recombinant RcpA (aa 30-192) did not bind to IL-1β (III; Fig 5C).

5.4. IL-1β interacts with the intracellular DNA-binding protein HU and ATP synthase subunit β

The membrane and soluble protein fractions of the *A. actinomycetemcomitans* D7S-1 strain were extracted to further characterise the interaction of IL-1β with *A. actinomycetemcomitans* proteins. In an EMSA, an intracellular protein fraction contained two protein bands bound more effectively to biotinylated IL-1β than to the control protein, biotinylated STI, which has a similar size and charge to IL-1β (I: Fig 3A-B; II: Fig 3B). These two protein bands were extracted from the silver stained native-PAGE gel (I: Fig 3C; II: Fig 5C) and identified by MS-based proteomic analysis. Seven peptides covering 14% of the sequence of ATP synthase subunit β (I: Fig 3D) and three peptides covering 77% of the sequence of DNA-binding protein HU (II: Fig 3D) were identified. Both proteins are conserved among Gram-negative and Gram-positive bacterial species. Intracellular IL-1β localisation (I: Fig 3G and II: Fig 3A) in proximity with the cytoplasmic side of the inner membrane and with the outer edges of nucleoids supports the putative interaction of IL-1β with ATP synthase subunit β and HU, respectively.

The interaction of these two identified intracellular proteins with IL-1β was verified by producing these two proteins in *E. coli* as recombinant proteins. In the case of ATP synthase subunit β, only the trimeric form of the recombinant ATP synthase subunit β bound IL-1β (I: Fig 6A–C), whereas the monomeric form of ATP synthase subunit β interacted only with the negative control protein STI (I: Fig 6D and E). Therefore, IL-1β seems to prefer the trimeric form of ATP synthase subunit β to its monomeric or dimeric form. Interestingly, ATP synthase subunit β is a central component of the ATP-producing F-ATPase, whose catalytic F1 sector contains three β subunits (see review [360]). Moreover, some cationic antimicrobial peptides between 10 to 50 residues in length have been suggested to inhibit the function of the *E. coli* ATP synthase by binding to its β subunit DELSEED-loop [361,362]. Our finding that the trimeric form of ATP synthase subunit β binds IL-1β might also explain the observed drop in metabolic activity after IL-1β treatment (I: Fig 2).

Another *A. actinomycetemcomitans* intracellular protein that interacted with IL-1β was DNA-binding protein HU (HU). HU is a homodimeric protein in the majority of Gram-negative bacteria, including *A. actinomycetemcomitans* [363], and it binds nonspecifically to DNA with a preference for A/T-rich DNA [364]. This protein is one of the factors responsible for bacterial DNA condensation and supercoiling [363,365]. It is known that changes in DNA packaging in *E. coli* have been linked to the alterations in the species gene expression profile [366]. Furthermore, HU regulates promoter activity by controlling the distribution of RNA polymerase in the nucleoid [367]. According to mutation studies, HU can regulate the expression of 8% of the *E. coli* genome [368]. In the oral pathogen *P. gingivalis*, the β subunit of HU regulates the expression of genes with roles in cell wall synthesis, cell division, iron uptake and capsule polysaccharide synthesis [369,370]. This putative interaction between HU and IL-1β could explain why IL-1β caused gene expression modulation in *S. aureus* [293]. By using an ELISA, we

determined that HU bound more efficiently ($p<0.001$) to IL-1β- than to BSA-coated wells (II: Fig 3E). However, HU also bound to STI-coated wells (II: Fig 3E), suggesting that the cationic HU interaction with IL-1β could be due to nonspecific electrostatic interactions.

The ability of recombinant HU to interact with DNA and condense nucleoids was verified two different ways. First, the purified recombinant HU contained small amounts of DNA contamination as HU samples could be visualised in agarose gels stained for the presence of DNA (data not shown). Second, the preincubation of *A. actinomycetemcomitans* nucleoids with recombinant HU protein was required for nucleoid samples to migrate into a 0.5% agarose gel (data not shown). The amount of DNA-bound HU decreased in the native-PAGE EMSA gel if HU was allowed to first react with IL-1β and then with nucleoids (II: Fig 3F). This result indicates that the putative interaction between IL-1β and HU may disrupt the ability of HU to bind DNA. In contrast, preincubation with the control protein STI did not decrease the intensity of DNA-bound HU as much as IL-1β (II: Fig 3F). Therefore, the interaction of HU with IL-1β and STI may not be the same. Furthermore, biotinylated IL-1β more efficiently bound to *A. actinomycetemcomitans* whole cells (I: Fig 4A) than biotinylated STI, suggesting that the internalisation of IL-1β is more likely than the internalisation of STI. The ability of biofilms to bind IL-1β might be partly explained by HU, as HU can also localise in the extracellular milieu of bacteria [126,371,372]. By combining our study results with what is known about HU extracellular location, we can hypothesise that extracellular HU may also sequester IL-1β. This IL-1β binding most likely causes some alterations to biofilm structure because HU acts together with IHF as critical maintenance factors in the structural integrity of the EPS matrix of biofilms [126,127]. However, further studies are required to confirm the specific role of IL-1β in the regulation of bacterial biofilm gene expression and metabolism through its interaction with HU and ATP synthase subunit β. In particular, quantification of bacterial IL-1β uptake must be determined. Our suggestions about the putative effects of IL-1β on the functions of two intracellularly abundant bacterial proteins requires that the internalisation of IL-1β or shorter IL-1β peptide fragments be efficient enough. For example, verification of the interaction between host-derived IL-1β and bacterial HU or ATP synthase subunit β could be performed using a proximity ligation assay in fixed GMC tissue samples.

5.5. IL-1β interacts with a membrane protein of *A. actinomycetemcomitans*

Because we knew that *A. actinomycetemcomitans* binds IL-1β, we wanted to investigate the interaction of the total membrane protein fraction with IL-1β to discover putative cytokine receptors from *A. actinomycetemcomitans*. In an EMSA analysis, the protein band in the blot that interacted with the anti-IL-1β antibody was only visible in lanes containing either IL-1β or the IL-1β-membrane protein (MP) combination (III: Fig 1A). However, the band intensity was the same in both samples (III: Fig 1A), indicating that both samples contained similar amounts of IL-1β. However, the most intense band in the silver-stained gel was present in the lane containing both IL-1β and MP (III: Fig 1B). Moreover, the band was not present in the lane containing only pure MP (III: Fig 1B). Furthermore, this phenomenon was not observed when IL-1β

was replaced with the control protein STI (III: Fig 1C), which has similar size and charge to IL-1β (STI: MW=20.1 kDa and pI=4.5; IL-1β MW=17,4 kDa and pI=5.9). Therefore, it was assumed that the intense band in the IL-1β-MP sample might contain proteins that interact with IL-1β. The intense protein band was identified by MS-based analysis. The band contained IL-1β and one novel protein with no previously determined function. According to a BLAST search, the identified amino acid sequence covered approximately 75% of the protein sequence of a hypothetical protein from the *A. actinomycetemcomitans* D7S-1 strain (III: Fig 1D). We thus designated this *A. actinomycetemcomitans* protein as bacterial interleukin receptor I (BilRI). The bacterial outer membrane proteins Caf1A and OprF have been characterised as the cytokine receptors of *Y. pestis* and *P. aeruginosa*, respectively [8,10]. Caf1A forms a transmembrane channel in the outer membrane and is essential for F1 capsule formation, whereas OprF forms the porin channel for small polar compounds and also plays a role in the adhesion of *P. aeruginosa* to host cells (see reviews [300,373]). However, according to amino acid sequence homology searches, Caf1A demonstrates no sequence homology with BilRI or any other known *A. actinomycetemcomitans* proteins, and OmpA of *A. actinomycetemcomitans* shows only low sequence similarity with OprF. Lipoprotein Lpp, the major outer membrane protein of Gram-negative *Enterobacteriaceae*, has been characterised as a cell surface receptor that specifically recognises several cationic antimicrobial peptides [374].

A BLAST search with BilRI showed close homology with several hypothetical proteins in the *Pasteurellaceae* family (III: Fig 2B and 3), but not in *E. coli*. The notable feature of BilRI and its homologous proteins was the number of charged residues. Thirty-seven per cent of the mature BilRI peptide consisted of charged residues, including 37 negatively charged and 23 positively charged residues. Furthermore, the amino acid sequence of *A. actinomycetemcomitans* BilRI contained 4 different 10-amino acid-long sequences (Fig 10 C; bold green, blue, purple and grey) repeated in the same order in triplicate. A similar type of repeats occurred also in other *A. actinomycetemcomitans* strains, *Aggregatibacter aphrophilus* strains (III: Fig 2B) and the *Pasteurellaceae* family (III: Fig 3). Conservation of the BilRI-encoding gene among *Pasteurellaceae* and its expression in clinical strains of *A. actinomycetemcomitans* suggest that BilRI may have an important function among this bacterial family. Unfortunately, we have not yet determined the significance of BilRI in *A. actinomycetemcomitans*. Characterisation of the *A. actinomycetemcomitans ΔbilRI* mutant might increase our understanding of the role of this protein. For example, our GMC model could be used for cytokine binding experiments, and the rat model for biofilm-mediated oral infection described by Freire *et al*., 2011 [375] would be ideal for determining whether BilRI affects the virulence of *A. actinomycetemcomitans*. Fibrinogen binder A (FgbA) of *Haemophilus ducreyi* is the only *Pasteurellaceae* protein with sequence similarity to have a known function. This lipoprotein contributes to the virulence of *H. ducreyi* and can bind to fibrinogen, suggesting a role in host-pathogen interactions [376]. Moreover, the ability of BilRI to interact with other host-derived compounds, such as fibrinogens, and with other proinflammatory mediators should be examined.

In a bioinformatic analysis, the N-terminus of the protein, including the first 19 amino acids, showed typical features of a bacterial lipoprotein signal sequence [327,344]. A subcellular localisation tool [345] predicted that BilRI is targeted to

the outer membrane in Gram-negative species. Because the lipoprotein biosynthetic pathways in *A. actinomycetemcomitans* have received a little attention, we hypothesised that *A. actinomycetemcomitans* lipoprotein biosynthesis may show similarities to *E. coli*. The modification process of bacterial prelipoprotein is summarised in Figure 11 (Fig 11 A and B). The D7S strain encodes and expresses homologues for the signal peptidase II (SPII, GI:387121531), lipid modification enzymes Lgt (GI:387120487) and Lnt (GI:387121253), and lipoprotein localisation machinery (Lol) of *E. coli* [377] according to BLASTP searches. The discovered homologies for the Lol apparatus in *A. actinomycetemcomitans* included LolA (GI:387121799), LolB (GI:387120279) and the inner membrane lipoprotein-releasing complex, LolCDE (GI:387122018; GI:387122019;GI:387122020). However, the amino acid sequence of BilRI contains two aspartate residues at the + 2 and +3 positions (Fig 11 C), which is a typical inner membrane signal known as the avoidance signal for the Lol system. Some lipoproteins containing this avoidance signal are still translocated to the outer surface of the OM, most likely through the Type II secretion pathway (see review [378]). Most all known lipoproteins in *E. coli* are assumed to be anchored to the outer membrane facing towards the periplasm [379,380]. However, at least one lipoprotein of *E. coli*, Wza, can form the integral outer membrane structures required for capsular polysaccharide export [381,382]. The Wza octamer arrangement inside the transmembrane region is an unusual barrel composed of amphipathic alpha helices [381,382]. The presence of lipoproteins in the outer leaflet of the outer membrane is more common in other Gram-negative species such as *Neisseria meningitides*, which has an incomplete Lol apparatus for lipoprotein transport [379].

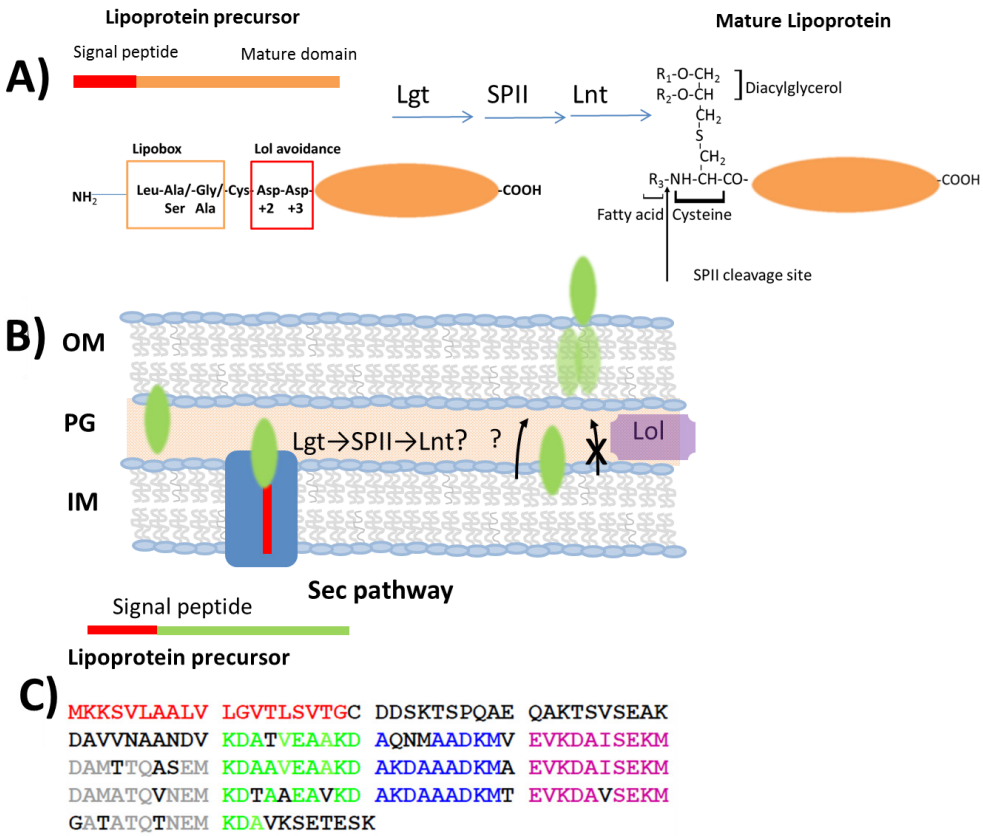


Figure 11. ***Biosynthesis of lipoproteins and the hypothesised biosynthetic pathway of BilRI.*** Briefly, lipoprotein precursors in the cytoplasm typically contain a signal sequence with a conserved sequence called a lipobox (A; yellow outline). Most lipoproteins in *E. coli* are exported from the cytoplasm by the Sec pathway (B). After prelipoprotein export, the first steps in the modification process are the attachment of a diacylglycerol group moiety to the cysteine residue by the diacylglyceryl transferase Lgt and the subsequent cleavage of the signal sequence by lipoprotein signal peptidase (SPII) (A). An additional fatty acid residue is then attached to the N-terminal cysteine residue by the N-acyltransferase Lnt prior to lipoprotein localisation machinery (Lol) export of the lipoprotein from the inner membrane to the outer membrane (A/B). Generally, the periplasmic chaperone LolA is required for the transport of hydrophobic lipoproteins through the hydrophilic periplasm to the outer membrane. Lipoprotein remains in the inner membrane of *E. coli* if it contains an avoidance signal adjacent to the conserved cysteine residue (A, red outlines) (see reviews [378-380]). Additionally, the 19-aa signal sequence of BilRI (C; bold red) contains a typical Sec signal peptide with a positively charged N-terminal region, a central hydrophobic H-region, and a polar C-terminal region with a peptidase cleavage site (see review [383]). In BilRI, the signal sequence is followed by several charged aa residues forming 4 direct tandem repeats of 10 aa (C; bold green, blue, purple and grey). Our hypothesised transport mechanism for BilRI suggests that the Lol system is not used for BilRI transportation (B). According to our experimental data, BilRI localises to the OM and is most likely faces external milieu. Moreover, BilRI exists as larger assemblies than monomeric units on the OM, implying that it might also form integral OM assemblies. Modified from Figure 2 of Okuda S. and Tokuda H. 2013 [380] and Figure 2A (III).

The orientation of mature BilRI protein in the outer membrane of the *A. actinomycetemcomitans* D7S-1 strain was determined by Proteinase K treatment (III: Fig 6) and with two different antibody fragments against BilRI. Globomycin was used to specifically hinder the function of lipoprotein signal peptidase, and chloramphenicol prevented protein synthesis in *A. actinomycetemcomitans*. Therefore, the level of the 19-kDa proprotein form remained constant throughout the experiment. As the Proteinase K treatment of whole cells resulted in the loss of intensity in the bands containing the largest assemblies of BilRI (III: Fig 6A and B), we suggest that some part of BilRI is surface exposed. Moreover, the outer membrane protein fraction of the D7S-1 strain contained two forms of BilRI with sizes of approximately 35 kDa and 70 kDa (III: Fig 6A and B). The larger of the two forms was also found in the inner membrane protein fraction (III: Fig 6A). We hypothesised that the larger form was the immature BilRI assembly containing the lipidated side chain. The smaller assembly of approximately 35 kDa existed only in the outer membrane protein fraction in large amounts (Fig 6A). Therefore, both of these forms are likely surface exposed in the outer membrane because their respective bands could be eliminated by Proteinase K treatment (III: Fig 6A and B). Interestingly, expression of the different forms of BilRI (the proprotein, the unlipidated mature form and the 70-kDa and 35-kDa forms) was observed in *in vitro* biofilm cultures of all tested clinical isolates of *A. actinomycetemcomitans*, which were obtained from periodontitis patients (III: Fig 6C). One potential technique to verify the localisation and amount of BilRI in single *A. actinomycetemcomitans* cells may be immunoelectron microscopy. The exact composition of these different protein forms of BilRI, including their oligomerisation and lipidation states, must be studied in more detail using MS-based technologies.

Overexpression of recombinant BilRI in *E. coli* resulted in less compact pellets after centrifugation (III: Fig 4C), visible cell aggregate formation in the presence of $CaCl_2$ (III: Fig 4F) and increased DNA leakage into the supernatant when bacterial pellets were exposed to a freeze/thaw cycle (III: Fig 4D). However, bacterial cell lysis was notably reduced when cells were frozen in 50% glycerol (III: Fig 4E). The increased fragility of *E. coli* cells was understandable, as the overproduction of outer membrane proteins generally results in enhanced membrane vulnerability in Gram-negative species [384]. Moreover, this result may imply that BilRI forms pore assemblies in the outer membrane similar to the Wza lipoproteins of *E. coli* [381,382]. The interaction between recombinant BilRI and IL-1β was verified using a microplate assay. It showed that BilRI binds to IL-1β (III: Fig 5C). The negative control protein from *A. actinomycetemcomitans*, the N-terminal portion of the outer membrane protein RcpA, did not bind to IL-1β (III: Fig 5C). However, BilRI bound almost identically to IL-1β and STI (III: Fig 5C). This result suggests that the purified soluble form of BilRI may differ structurally from the membrane-associated form of BilRI. Therefore, the interaction of BilRI with IL-1β was also examined with whole *E. coli* cells producing BilRI assemblies in their outer membranes**.** In the flow cytometry analysis, the detected ratio of positively stained *E. coli* cells was similar regardless of whether cells were overexpressing recombinant full-length BilRI protein or not (III: Fig 5A). The putative ability of *E. coli* cells to bind IL-1β was predictable, as *E. coli* has been shown to specifically bind IL-1β [287]. However, significant binding of the control protein STI to *E. coli* cells was not observed (III: Fig 5A). Moreover, the finding that recombinant BilRI-producing *E. coli* cells showed

higher mean fluorescence intensity in positively stained cells than in control cells (III: Fig 5B) indicates more efficient IL-1β binding per cell in BilRI-expressing cells. Our findings indicate that the identified outer membrane lipoprotein BilRI interacts with human IL-1β.

5.6. *A. actinomycetemcomitans* biofilm slightly affects HGKs

The GMC model was used as an *in vitro* tool to mimic conditions that an *A. actinomycetemcomitans* biofilm may encounter when in contact with subgingival dental tissue. Our model was based on Gursoy's model [334], although we used gingival keratinocytes instead of skin keratinocytes. Moreover, different bacterial species and a different biofilm culturing technique were used. In our experiments, biofilms were pregrown in liquid phase and attached to a membrane. However, in the original model, agar plates were first inoculated with the bacterial suspension and then discs were placed on the agar plates to allow biofilm formation on the discs at the solid-gas interface. With these small modifications, our aim was to mimic the subgingival conditions where biofilms are surrounded by gingival crevicular fluid; however, our biofilms were not grown under the flow conditions that prevail *in vivo*.

Upon histological examination, the epithelium of the GMC model co-cultured with the *A. actinomycetemcomitans* D7S-1 strain often appeared damaged and thinned at sites of close contact with the biofilm (II: Fig 4A and 4C). This damaged appearance together with the well-known ability of the Cdt toxin produced by *A. actinomycetemcomitans* to cause apoptosis and necrosis in human cells (see review [95]) led us to examine whether the epithelium undergoes unregulated cell death, necrosis, or programmed cell death (apoptosis) when in contact with *A. actinomycetemcomitans* biofilm cells. Necrosis was investigated by analysing LDH activity in the cell culture medium. We assumed that cell damage in the co-cultured epithelium were not due to necrosis because the LDH activity was low after a 24-h incubation in the absence of antibiotics (data not shown). However, in our model the leakage of LDH from the epithelium into the culture medium might be limited by the collagen-fibroblast matrix. Moreover, Alaoui-El-Azher and co-authors have shown that human immortalised gingival keratinocyte monolayers release significant amounts of LDH 48-h after infection with *A. actinomycetemcomitans* [87].

The HGK proliferation decreased significantly in antibiotic-free medium after a 24-h incubation with a viable *A. actinomycetemcomitans* D7S-1 biofilm (II: Fig 4A and 4B). In the presence of antibiotics, the *A. actinomycetemcomitans* biofilm caused a slight, but not statistically significant, inhibition in HGK proliferation compared with the filter disc control after an 8-h incubation (II: Fig 4B) The stopping of the HGK proliferation during extended incubation with a viable *A. actinomycetemcomitans* biofilm may be caused by *A. actinomycetemcomitans* Cdt. This effective virulence factor expressed by *A. actinomycetemcomitans* causes apoptosis or cell-cycle arrest in several cell types, including T-cells, oral epithelial cells and fibroblast cells [84,86,95,385-387]. The free soluble filtrate extracted from *A. actinomycetemcomitans* D7S-1 biofilm cultures does not contain Cdt, but the species is known to secrete Cdt in outer membrane vesicles, which are delivered to target human cells [229,377,388]. It has been previously suggested that elongated (i.e., more than 24 to 48 h) exposure to the toxin is required

for the inhibition of human gingival epithelial cell proliferation [386]. However, in our study, decreased thickness and damage to the GMC epithelium was observed after 24-h co-culture with a viable *A. actinomycetemcomitans* biofilm. Cdt might explain the damage and reduced epithelium thickness (II: Fig 4C) in GMCs challenged with viable *A. actinomycetemcomitans* biofilm for 24 h compared with the epithelium of filter disc control samples. This finding is consistent with another study in which human gingival tissue samples displayed separation of the keratinised surface layer at the junction of the oral epithelium as the result of an 18-h exposure to *A. actinomycetemcomitans* Cdt [93,94]. Unexpectedly, a slight inhibition in HGK proliferation was already observed after 8 h co-culture with *A. actinomycetemcomitans* (II: Fig 4B). The use of antibiotics may have damaged the *A. actinomycetemcomitans* cell structure and caused the unconventional release of Cdt from cells. However, this Cdt release from *A. actinomycetemcomitans* cells as the result of antibiotic treatment was not investigated. Moreover, *A. actinomycetemcomitans* may also sequester the IL-1β produced by gingival cells. This cytokine binding might interfere with complex cytokine signalling in the host. For instance, HeLa cells undergo apoptosis following IL-1β binding to IL-1R under low oxygen conditions [232]. Interestingly, the use of IL-1Ra inhibited cell death in the same experiment [232].

The apoptosis of epithelial cells challenged with *A. actinomycetemcomitans* D7S-1 biofilm for 8 h increased slightly in the GMC model in the presence and absence of antibiotics (II: Fig 5). Sites that had close contact with the viable biofilm showed lower apoptosis levels during the 8-h incubation based on TUNEL staining for keratinocytes (II: Fig 5; open arrows), but a similar trend was not observed after a 24-h incubation (data not shown). These findings are in contrast with an earlier observation that human gingival epithelial cell multilayers show increased apoptosis 2 and 6 h after infection with an *A. actinomycetemcomitans* suspension [268]. However, there are several differences in these two models that may explain the contrasting results. In the model of Dickinson *et al.*, 2011 [268], epithelial cells were cultured at the gas-liquid interface as in our model. However, our model contained a fibroblast layer below the epithelial cell layer. The presence of fibroblasts is known to influence epithelial cell growth and differentiation [389]. Therefore, the presence/absence of fibroblasts may result in differences in the apoptotic properties of epithelial cells. Moreover, we placed an *A. actinomycetemcomitans* D7S-1 biofilm precultured in minimal medium on top of the epithelial cell layer, whereas an *A. actinomycetemcomitans* VT1169 suspension was used in Dickinson's model. The virulence properties of the different strains or the use of biofilm culture instead of planktonic culture may also explain the observed difference in apoptosis. For example, the cytokine expression profile and apoptosis level of human keratinocyte cells exposed to *S. aureus* biofilms and planktonic cultures are different [390]. Additionally, the regulatory role of IL-1β in proliferation and apoptosis in certain human cell types has been stated [231,232,391]. Therefore, we cannot exclude the possibility that IL-1β binding by the viable biofilm might inhibit apoptosis at close range, which could partially explain the difference between the studies. Obviously, the significance of bacterial IL-1β binding must be studied in more detail.

5.7. Extracellular domains of HofQ show DNA binding

The amino acid sequence of HofQ in competent *A. actinomycetemcomitans* D7S-1 (GI:387120835) was compared with the sequences of ComE from *H. influenzae* (GI:59939227) and PilQ from *N. meningitidis MC58* (GI:15678016) using a BLASTP algorithm. *A. actinomycetemcomitans* HofQ was observed to be approximately 70% identical over 448 amino acids to ComE and 30% identical over 407 amino acids to PilQ. As these homologues of HofQ have been described as outer membrane secretin pores associated with DNA uptake by *neisserial* species and *H. influenzae* [113,143], we assumed that the uncharacterised *A. actinomycetemcomitans* HofQ might play a similar role. Using EMSA, we showed that emHofQ (residues 27-195) could bind DNA (IV: Fig 5). The T4 gene 32 protein (singe-stranded DNA binding) and BSA were used as negative controls. We also investigated the putative interaction of emHofQ with the 9-bp-long core USS, 5-AAGTGCGGT-3, as the DNA uptake machinery of *A. actinomycetemcomitans* is known to prefer DNA containing this sequence [120]. Linearised vectors either with or without the USS were used in the EMSA. DNA binding to emHofQ was not dependent on the presence of the core USS because emHofQ bound to both vector forms (IV: Fig 5). This result was consistent with another study in which PilQ was shown to interact with DNA in a USS-independent manner [195].

The overall structure of crystallised HofQ was a dimer in the asymmetric unit (IV: Fig 1A). However, the particular crystallisation construct used may have caused the dimeric structure, and the oligomerisation state of the full-length protein could thus be different. Each emHofQ polypeptide chain contained two globular domains linked by a disordered segment of approximately 25 residues (IV: Fig 1C; broken lines indicate the disordered regions). Domain 1, which consists of residues 27-100 of emHofQ, showed little structural homology with any other known protein fold unless an alternative domain 1 was formed by means of a domain swap. After the domain swap, domain 1 and 2 of emHofQ displayed both sequence and structural similarity (IV: Fig 3). Domain 2 is comprised of residues 127-191 of emHofQ and contains two α-helices flanked by a three-stranded antiparallel β-sheet (IV: Fig 3 B and 3C). Domain 2 showed structural homology with the N-1 and N-2 domains of secretin peri-GspD from enteropathogenic *E. coli* (IV: Fig 4 A). It is worth noting that a putative DNA binding motif was identified in domain 2 because this domain showed high structural similarity to the nucleotide-binding KH domain of Nova-1 (IV: Fig 4B). Most importantly, a conserved GxxG motif involved in nucleotide binding by KH domains (see review [392]) was also found in emHofQ. The GKGG motif of Nova-1 and the GGSG motif of domain 2 of emHofQ were found in a structurally similar location (IV: Fig 4B). KH domains are known to bind both RNA and DNA with low affinity [393,394]. In KH domains, the GxxG motif forms a protruding loop between two α-helices, while the GGSG motif of emHofQ protrudes from the axis of the preceding helix and is followed by an extended loop (IV: Fig 4B). Sequence alignment of the extramembranous region of *A. actinomycetemcomitans* HofQ with its homologues in six other *Pasteurella* species (*A. pleuropneumoniae, H. ducreyi, H. influenzae, M. haemolytica, M. succiniciproducens* and *P. multicoda*) demonstrates very high sequence similarity for domain 2 compared to domain 1, with strict conservation in many regions (IV: Fig 7). Therefore, domain 2 appears to be functionally involved in DNA uptake and competence; domain 1 may play more of a structural role and perhaps

be involved in gating. Interestingly, the unstructured region of HofQ between β-strands 4 and 5 is not conserved in its sequence between these seven *Pasteurella* species. However, each of these seven homologues contained glycine-rich motifs that align with the GGSG motif in HofQ, with some also containing flanking or internal lysine residues (IV: Fig 7; displayed with a green outline).

5.8. Summary of results

- IL-1β bound both the *A. actinomycetemcomitans* D7S-1 biofilm and single cells of the three tested biofilm-forming *A. actinomycetemcomitans* strains (D7S-1, SA1398 and SA1151). Additionally, single cells of four *tad* locus mutants in *A. actinomycetemcomitans* D7S-1 (Δ*rcpA,* Δ*rcpB,* Δ*tadD* and Δ*tadG)* and the naturally nonadherent *A. actinomycetemcomitans* D7S-1 strain (D7SS) demonstrated the ability to bind IL-1β.

- *A. actinomycetemcomitans* biofilm culture in the presence of IL-1β resulted in elevated biofilm mass accumulation in three tested *A. actinomycetemcomitans* strains (D7S-1, SA1398 and SA1151). In contrast, the metabolic activity of these three *A. actinomycetemcomitans* strains transiently dropped in the presence of IL-1β. Furthermore, the Δ*rcp*A mutant of *A. actinomycetemcomitans* D7S-1 did not show a decrease in metabolic activity in the presence of IL-1β. Decreased metabolic activity was found in the three other tested *tad* locus mutants (Δ*rcpB,* Δ*tadD* and Δ*tadG*).

- The novel outer membrane lipoprotein of *A. actinomycetemcomitans* D7S-1 was designed as bacterial interleukin receptor I (BilRI) and was found to interact with IL-1β. This *Pasteurellaceae*-specific protein was expressed by biofilm cultures of all six tested *A. actinomycetemcomitans* strains, including D7S-1, SA1398 and SA1151. Similarly, the DNA binding protein HU and the trimeric form of ATP synthase subunit β, which are intracellular proteins of *A. actinomycetemcomitans*, were found to bind IL-1β.

- The extramembranous portion of *A. actinomycetemcomitans* outer membrane protein HofQ bound DNA in an USS-independent manner.

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS

During a long co-evolution with humans, micro-organisms have developed several ways to overcome the host immune response. Micro-organisms can avoid elimination by forming very tenacious biofilms in which single community members are surrounded by a protective extracellular matrix. Furthermore, some bacteria, yeast and virus species can even manipulate the signalling crosstalk of the immune system. For example, they can disrupt the host defence by expressing specific receptors against the key coordinating molecules of the innate immune system known as human proinflammatory mediators. Moreover, the ability of some biofilm-forming bacteria to modulate their own gene expression in response to human cytokines has been demonstrated. The purpose of this thesis was to study for the first time the ability of the Gram-negative oral pathogen, *A. actinomycetemcomitans* to sense IL-1β. In the side project, the putative role of the extramembraneous portion of the HofQ protein in DNA uptake was briefly investigated.

A. actinomycetemcomitans is often considered a central pathogen in the onset of aggressive periodontitis. Therefore, we hypothesised that this species may express machinery that senses the presence of IL-1β in the subgingival milieu. The selection of this species as the model organism for this study was supported by its ability to form biofilms and its previously annotated genome, which allowed the identification of IL-1β-interacting proteins in the bacterium. This study demonstrated that the IL-1β produced by gingival cells could enter the cytoplasm of the co-cultured *A. actinomycetemcomitans* biofilm cells. Previously, human cytokines had not been found inside a bacterium. The transient drop in metabolic activity in the biofilms and enhanced total biofilm mass after IL-1β exposure suggested that the species could also sense IL-1β. The novel *Pasteurellaceae*-specific membrane protein, BilRI, and two conserved intracellular proteins, HU and ATP synthase subunit β, were characterised as IL-1β-interacting components in *A. actinomycetemcomitans.* As our results also suggested that BilRI localises to the outer surface of the outer membrane, we hypothesise that BilRI is the first-line binder of IL-1β in *A. actinomycetemcomitans.* However, the putative function of these outer membrane BilRI assemblies as a channel for the uptake of IL-1β has yet to be verified. Moreover, a possible role for the outer membrane secretin RcpA in IL-1β transportation cannot be excluded, as deletion of the protein inhibited the metabolic activity drop in *A. actinomycetemcomitans.* Additionally, the IL-1β transportation mechanism through the periplasm and inner membrane is completely unknown. When the cytokine finally enters the cytoplasm, its interaction with the DNA binding protein HU and an energy production protein, the trimeric form of ATP synthase subunit β, might explain the observed changes in metabolic activity. The interaction of internalised IL-1β with HU could also explain the reported gene expression alterations in a different bacterial species after cytokine treatment. Our results indicated that sequestering of IL-1β by *A. actinomycetemcomitans* might slightly reduce human gingival epithelial cell proliferation. In the study with HofQ, it was noted that both the solved crystal structure and the DNA binding ability of

the extramembranous portion of HofQ supported its role in DNA acquisition in *A. actinomycetemcomitans*.

In this study, we have provided some new insights into an opportunistic periodontal pathogen's ability to respond to the inflammatory micromilieu. We do not yet know the clinical significance of this phenomenon. In theory, it would be beneficial for the bacterium to be able to influence the timing and intensity of the host inflammatory response. The biofilm form of growth might be preferred after sensing high levels of key cytokines. In biofilms, single cells may evade the host defence because of their tolerance. Furthermore, biofilms might sequester host cytokines. As a result of this bacterial cytokine sensing, the host inflammatory response at the site of infection would de-escalate. After a more quiescent phase of infection, some bacteria would again disperse and begin a more active existence. Bacteria would then express their virulence factors and induce an active inflammatory response in the host. The destruction of host tissues would help nourish the biofilms with essential compounds such as iron. During the chronic inflammatory cycle, some dispersed pathogens would find more distant infection sites, while the some community members would remain at the original site and return to the quiescent phase. New follow-up studies have been initiated to gain a more comprehensive understanding of *A. actinomycetemcomitans* cytokine sensing. Whether *A. actinomycetemcomitans* can bind only IL-1β or whether it could also interact with other host cytokines and chemokines needs to be confirmed in further studies. After identification of the putative *A. actinomycetemcomitans* IL-1β receptor, we can test the significance of this receptor in IL-1β sequestering by preparing specific *bilRI* deletion mutants. Additionally, a study on the cytokine specificity of BilRI is being conducted. The purpose of our group is to solve the three-dimensional structure of BilRI. Although sequence homology for BilRI was found only in the *Pasteurellaceae* family, structure determination is essential to determine if other bacterial species express similar cytokine receptors to BilRI.

This study demonstrated that IL-1β is an environmental signal for *A. actinomycetemcomitans* that is transferred through the membranes and into the cytoplasm where it interacts with its targets. It should be noted that the present study only investigated the ability of *A. actinomycetemcomitans* proteins to interact with IL-1β. We have not yet determined the strength of these discovered protein-protein interactions or the actual consequences of these interactions. Figure 12 summarises our current hypothesis about the mechanism by which *A. actinomycetemcomitans* senses IL-1β. This cytokine sensing may increase *A. actinomycetemcomitans* adaptation and subsequent persistence during chronic inflammatory conditions.

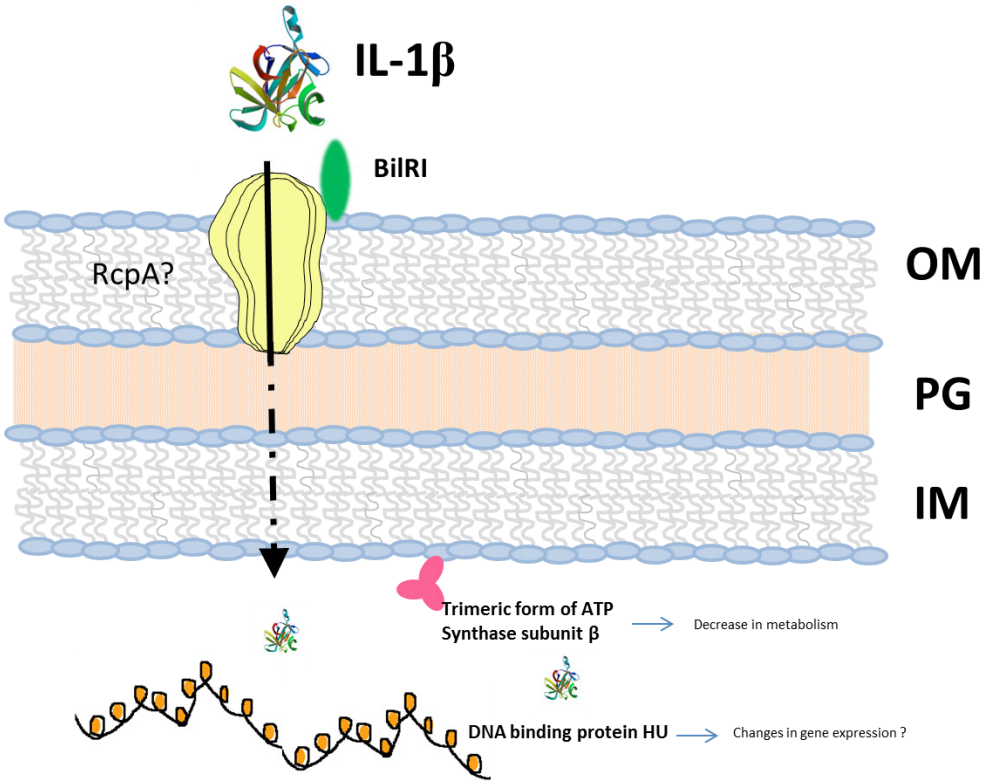


Figure 12. ***Hypothesised model for the entry of IL-1β into the cytoplasm of A. actinomycetemcomitans.*** The extracellular host signalling molecule IL-1β is recognised by the outer membrane bacterial IL-1β receptor I (BilRI). We cannot exclude the possibility of an outer membrane pore formed by the oligomeric lipoprotein assemblies. Moreover, our result implies that the outer membrane secretin channel RcpA may be involved in cytokine uptake. However, the transfer mechanism of IL-1β from the outer membrane to the cytoplasm is still unknown, though we have shown that IL-1β is internalised by *A. actinomycetemcomitans.* Intracellular IL-1β may then affect the metabolic processes of the bacterium through its interaction with ATP synthase subunit β and with the DNA binding protein HU. Its interaction with HU might lead to the modulation of bacterial gene expression. OM=outer membrane, PG=peptidoglycan in periplasmic space, IM=inner membrane.

ACKNOWLEDGEMENTS

The work of this thesis was mainly performed in the Department of Biochemistry, University of Turku. Additionally, some portions of the study were carried out in the Department of Periodontology at the Institute of Dentistry, University of Turku. I would like to thank the present and former heads of the departments, Professors Jyrki Heino and Heikki Kallio in the Department of Biochemistry and Professors Juha Varrela and Pekka Vallittu in the Institute of Dentistry, for providing me with outstanding research facilities.

I would like to warmly thank my supervisors Riikka Ihalin and Marja Pöllänen for guiding me through the fascinating world of biofilms. I would especially thank for Riikka for continuous guidance and encouragement during this study. Your attitude towards work has influenced me profoundly. Moreover, I will never forget those memorable moments in lab and the journeys we have shared. I want to express my gratitude to Marja for sharing her expertise in cell culture modelling and periodontology. It has been a privilege to work with my supervisors.

My thanks are extended to Professors Eija Könönen and Mikael Skurnik for their work as members of my thesis supervisory committee. Our annual thesis meetings always provided new perspectives on my studies.

I thank Docents Georgious Belibasakis and Pirkko Pussinen for taking the time to review my thesis manuscript and for their constructive comments. I also want to acknowledge Professor Mikael Rhen for being the opponent.

I sincerely thank the skilful master students Mari Jääskeläinen, Laura Ahervo, Elina Lohermaa and Tuuli Ahlstrand for their valuable help with performing the laboratory experiments in this thesis. It was fun to share the same lab with you all. I warmly thank Heidi Tuominen for her expertise in protein purification and molecular biology. Additionally, the assistance of Jari Nuutila with flow cytometry, Jari Korhonen with confocal microscopy, and Raija Sormunen with transmission electron microscopy was crucial. Several co-authors contributed the publications contained in this thesis. Therefore, I want to express my gratitude to Jonna Alanko, Sirkka Asikainen, Casey Chen, Martin Högbom, Urpo Lamminmäki, Lauri Pelliniemi, Michael Tarry and Hannamari Välimaa. Special thanks are reserved for Professor Casey Chen for providing me the opportunity to work for 9 months in his research group at the University of Southern California. It was wonderful experience to work in the top laboratory in my field and learn more about genetics. Likewise, I extend my thanks to Katja Sampalahti, Marja-Riitta Uola and Mariia Valkama for their technical assistance with cell culture and immunohistochemical staining. It was always enjoyable to work with Katja, Marja-Riitta and Mariia at the Institute of Dentistry. I thank Juho Mäkelä, Indre Navickaite and Eija Tuominen their experimental contributions. I greatly appreciate Anu Hirvensalo, Satu Jasu, Teija Luotohaara and Jani Sointusalo, the "core biochemistry staff" for their assistance in all practical matters over the years.

I sincerely thank all the Biochemistry and Dentistry staff with whom I have worked during my studies. The ISB administration is thanked for organising social events for

graduate students. In particular, I would like to thank Heidi from the neighbouring lab for being such a good friend, both at the work and outside of the lab.

Last but not least, I would like to thank my family and Antti for their support during my studies.

Financial support from the National Doctoral Programme in Informational and Structural Biology, The Academy of Finland, Turku University Foundation, The Paulo Foundation, The Finnish Cultural Foundation, the Ella & Georg Ehrnrooth Foundation and The Finnish Dental Society Apollonia is gratefully acknowledged.

Turku, December 2013

Annamari Paino

REFERENCES

[1] The National Institutes of Health and Center for Disease Control Prevention. In (http://grants nih gov/grants/guide/pa-files/PA-03-047 html).

[2] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999;284:1318-22.

[3] Richards JJ, Melander C. Controlling bacterial biofilms. Chembiochem 2009;10:2287-94.

[4] Schaible UE, Kaufmann SH. Iron and microbial infection. Nat Rev Microbiol 2004;2:946-53.

[5] Sifri CD. Healthcare epidemiology: quorum sensing: bacteria talk sense. Clin Infect Dis 2008;47:1070-6.

[6] Dubbs JM, Mongkolsuk S. Peroxide-sensing transcriptional regulators in bacteria. J Bacteriol 2012;194:5495-503.

[7] Kanangat S, Bronze MS, Meduri GU, Postlethwaite A, Stentz F, Tolley E et al. Enhanced extracellular growth of Staphylococcus aureus in the presence of selected linear peptide fragments of human interleukin (IL)-1beta and IL-1 receptor antagonist. J Infect Dis 2001;183:65-9.

[8] Wu L, Estrada O, Zaborina O, Bains M, Shen L, Kohler JE et al. Recognition of host immune activation by Pseudomonas aeruginosa. Science 2005;309:774-7.

[9] McLaughlin RA, Hoogewerf AJ. Interleukin-1beta-induced growth enhancement of Staphylococcus aureus occurs in biofilm but not planktonic cultures. Microb Pathog 2006;41:67-79.

[10] Zav'yalov VP, Chernovskaya TV, Navolotskaya EV, Karlyshev AV, MacIntyre S, Vasiliev AM et al. Specific high affinity binding of human interleukin 1 beta by Caf1A usher protein of Yersinia pestis. FEBS Lett 1995;371:65-8.

[11] Haffajee AD, Socransky SS, Goodson JM. Periodontal disease activity. J Periodontal Res 1982;17:521-2.

[12] Stashenko P, Fujiyoshi P, Obernesser MS, Prostak L, Haffajee AD, Socransky SS. Levels of interleukin 1 beta in tissue from sites of active periodontal disease. J Clin Periodontol 1991;18:548-54.

[13] Gamonal J, Acevedo A, Bascones A, Jorge O, Silva A. Levels of interleukin-1 beta, -8, and -10 and RANTES in gingival crevicular fluid and cell populations in adult periodontitis patients and the effect of periodontal treatment. J Periodontol 2000;71:1535-45.

[14] Yoshinari N, Kawase H, Mitani A, Ito M, Sugiishi S, Matsuoka M et al. Effects of scaling and root planing on the amounts of interleukin-1 and interleukin-1 receptor antagonist and the mRNA expression of interleukin-1beta in gingival crevicular fluid and gingival tissues. J Periodontal Res 2004;39:158-67.

[15] Rescala B, Rosalem W,Jr, Teles RP, Fischer RG, Haffajee AD, Socransky SS et al. Immunologic and microbiologic profiles of chronic and aggressive periodontitis subjects. J Periodontol 2010;81:1308-16.

[16] Thunell DH, Tymkiw KD, Johnson GK, Joly S, Burnell KK, Cavanaugh JE et al. A multiplex immunoassay demonstrates reductions in gingival crevicular fluid cytokines following initial periodontal therapy. J Periodontal Res 2010;45:148-52.

[17] Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol 1999;4:1-6.

[18] Armitage GC. Periodontal diagnoses and classification of periodontal diseases. Periodontol 2000 2004;34:9-21.

[19] Slots J. Subgingival microflora and periodontal disease. J Clin Periodontol 1979;6:351-82.

[20] Zambon JJ. Actinobacillus actinomycetemcomitans in human periodontal disease. J Clin Periodontol 1985;12:1-20.

[21] Asikainen S, Lai CH, Alaluusua S, Slots J. Distribution of Actinobacillus actinomycetemcomitans serotypes in periodontal health and disease. Oral Microbiol Immunol 1991;6:115-8.

[22] Teles RP, Gursky LC, Faveri M, Rosa EA, Teles FR, Feres M et al. Relationships between subgingival microbiota and GCF biomarkers in generalized aggressive periodontitis. J Clin Periodontol 2010;37:313-23.

[23] Chen C, Kittichotirat W, Chen W, Downey JS, Si Y, Bumgarner R. Genome sequence of naturally competent Aggregatibacter actinomycetemcomitans serotype a strain D7S-1. J Bacteriol 2010;192:2643-4.

[24] Dinarello CA. Biologic basis for interleukin-1 in disease. Blood 1996;87:2095-147.

[25] Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat Rev Drug Discov 2012;11:633-52.

[26] Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000 2006;42:80-7.

[27] Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH et al. The human oral microbiome. J Bacteriol 2010;192:5002-17.

[28] World Health Organisation (Fact sheet N°318 April 2012).

[29] Rylev M, Kilian M. Prevalence and distribution of principal periodontal pathogens worldwide. J Clin Periodontol 2008;35:346-61.

[30] Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiology 2003;149:279-94.

[31] Benakanakere M, Kinane DF. Innate cellular responses to the periodontal biofilm. Front Oral Biol 2012;15:41-55.

[32] Calsina G, Ramon JM, Echeverria JJ. Effects of smoking on periodontal tissues. J Clin Periodontol 2002;29:771-6.

[33] Laine ML, Moustakis V, Koumakis L, Potamias G, Loos BG. Modeling susceptibility to periodontitis. J Dent Res 2013;92:45-50.

[34] Graves D. Cytokines that promote periodontal tissue destruction. J Periodontol 2008;79:1585-91.

[35] Wade WG. The oral microbiome in health and disease. Pharmacol Res 2012.

[36] Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994;5:78-111.

[37] Slots J. The predominant cultivable organisms in juvenile periodontitis. Scand J Dent Res 1976;84:1-10.

[38] Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL,Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998;25:134-44.

[39] Canabarro A, Valle C, Farias MR, Santos FB, Lazera M, Wanke B. Association of subgingival colonization of Candida albicans and other yeasts with severity of chronic periodontitis. J Periodontal Res 2012.

[40] Saygun I, Kubar A, Sahin S, Sener K, Slots J. Quantitative analysis of association between herpesviruses and bacterial pathogens in periodontitis. J Periodontal Res 2008;43:352-9.

[41] Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. Cell Host Microbe 2011;10:497-506.

[42] Brown JH, Whitham TG, Morgan Ernest SK, Gehring CA. Complex species interactions and the dynamics of ecological systems: long-term experiments. Science 2001;293:643-50.

[43] Frias-Lopez J, Duran-Pinedo A. Effect of periodontal pathogens on the metatranscriptome of a healthy multispecies biofilm model. J Bacteriol 2012;194:2082-95.

[44] Haubek D, Poulsen K, Westergaard J, Dahlen G, Kilian M. Highly toxic clone of Actinobacillus actinomycetemcomitans in geographically widespread cases of juvenile periodontitis in adolescents of African origin. J Clin Microbiol 1996;34:1576-8.

[45] Brogan JM, Lally ET, Poulsen K, Kilian M, Demuth DR. Regulation of Actinobacillus actinomycetemcomitans leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. Infect Immun 1994;62:501-8.

[46] Hritz M, Fisher E, Demuth DR. Differential regulation of the leukotoxin operon in highly leukotoxic and minimally leukotoxic strains of Actinobacillus actinomycetemcomitans. Infect Immun 1996;64:2724-9.

[47] Haubek D, Dirienzo JM, Tinoco EM, Westergaard J, Lopez NJ, Chung CP et al. Racial tropism of a highly toxic clone of Actinobacillus actinomycetemcomitans associated with juvenile periodontitis. J Clin Microbiol 1997;35:3037-42.

[48] Peyyala R, Kirakodu SS, Novak KF, Ebersole JL. Oral microbial biofilm stimulation of epithelial cell responses. Cytokine 2012;58:65-72.

[49] Guggenheim B, Gmur R, Galicia JC, Stathopoulou PG, Benakanakere MR, Meier A et al. In vitro modeling of host-parasite interactions: the 'subgingival' biofilm challenge of primary human epithelial cells. BMC Microbiol 2009;9:280,2180-9-280.

[50] Henderson B, Ward JM, Ready D. Aggregatibacter (Actinobacillus) actinomycetemcomitans: a triple A* periodontopathogen? Periodontol 2000 2010;54:78-105.

[51] Ohta H, Moriki D, Miyagi A, Watanabe T, Kato K, Fukui K. Microaerophilic property of *Actinobacillus actinomycetemcomitans* in fructose-limited chemostat cultures. FEMS Microbiol Lett 1996;138:191-6.

[52] Christensen H, Bisgaard M. Taxonomy and biodiversity of members of *Pasteurellaceae*. In: Kuhnert P, Christensen H, editors. *Pasteurellacea* Biology, Genomics and Molecular Aspects: Caister Academic Press, Norfolk, UK; 2008, p. 1-26.

[53] Fine DH, Furgang D, Kaplan J, Charlesworth J, Figurski DH. Tenacious adhesion of Actinobacillus actinomycetemcomitans strain CU1000 to salivary-coated hydroxyapatite. Arch Oral Biol 1999;44:1063-76.

[54] Rose JE, Meyer DH, Fives-Taylor PM. Aae, an autotransporter involved in adhesion of Actinobacillus actinomycetemcomitans to epithelial cells. Infect Immun 2003;71:2384-93.

[55] Kachlany SC, Planet PJ, Bhattacharjee MK, Kollia E, DeSalle R, Fine DH et al. Nonspecific adherence by Actinobacillus actinomycetemcomitans requires genes widespread in bacteria and archaea. J Bacteriol 2000;182:6169-76.

[56] Periasamy S, Kolenbrander PE. Mutualistic biofilm communities develop with Porphyromonas gingiv-

alis and initial, early, and late colonizers of enamel. J Bacteriol 2009;191:6804-11.

[57] Periasamy S, Kolenbrander PE. Aggregatibacter actinomycetemcomitans builds mutualistic biofilm communities with Fusobacterium nucleatum and Veillonella species in saliva. Infect Immun 2009;77:3542-51.

[58] Sanchez MC, Llama-Palacios A, Blanc V, Leon R, Herrera D, Sanz M. Structure, viability and bacterial kinetics of an in vitro biofilm model using six bacteria from the subgingival microbiota. J Periodontal Res 2011;46:252-60.

[59] Dzink JL, Tanner AC, Haffajee AD, Socransky SS. Gram negative species associated with active destructive periodontal lesions. J Clin Periodontol 1985;12:648-59.

[60] Meyer DH, Sreenivasan PK, Fives-Taylor PM. Evidence for invasion of a human oral cell line by Actinobacillus actinomycetemcomitans. Infect Immun 1991;59:2719-26.

[61] Meyer DH, Lippmann JE, Fives-Taylor PM. Invasion of epithelial cells by Actinobacillus actinomycetemcomitans: a dynamic, multistep process. Infect Immun 1996;64:2988-97.

[62] Arirachakaran P, Apinhasmit W, Paungmalit P, Jeramethakul P, Rerkyen P, Mahanonda R. Infection of human gingival fibroblasts with Aggregatibacter actinomycetemcomitans: An in vitro study. Arch Oral Biol 2012;57:964-72.

[63] Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ. Identification of periodontal pathogens in atheromatous plaques. J Periodontol 2000;71:1554-60.

[64] Kozarov EV, Dorn BR, Shelburne CE, Dunn WA,-Jr, Progulske-Fox A. Human atherosclerotic plaque contains viable invasive Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. Arterioscler Thromb Vasc Biol 2005;25:e17-8.

[65] Gaetti-Jardim E,Jr, Marcelino SL, Feitosa AC, Romito GA, Avila-Campos MJ. Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries. J Med Microbiol 2009;58:1568-75.

[66] Das M, Badley AD, Cockerill FR, Steckelberg JM, Wilson WR. Infective endocarditis caused by HACEK microorganisms. Annu Rev Med 1997;48:25-33.

[67] Pussinen PJ, Nyyssönen K, Alfthan G, Salonen R, Laukkanen JA, Salonen JT. Serum antibody levels to Actinobacillus actinomycetemcomitans predict the risk for coronary heart disease. Arterioscler Thromb Vasc Biol 2005;25:833-8.

[68] Hyvärinen K, Mäntylä P, Buhlin K, Paju S, Nieminen MS, Sinisalo J et al. A common periodontal pathogen has an adverse association with both acute and stable coronary artery disease. Atherosclerosis 2012;223:478-84.

[69] Zambon JJ, Slots J, Genco RJ. Serology of oral Actinobacillus actinomycetemcomitans and serotype distribution in human periodontal disease. Infect Immun 1983;41:19-27.

[70] Saarela M, Asikainen S, Alaluusua S, Pyhälä L, Lai CH, Jousimies-Somer H. Frequency and stability of mono- or poly-infection by Actinobacillus actinomycetemcomitans serotypes a, b, c, d or e. Oral Microbiol Immunol 1992;7:277-9.

[71] Gmur R, McNabb H, van Steenbergen TJ, Baehni P, Mombelli A, van Winkelhoff AJ et al. Seroclassification of hitherto nontypeable Actinobacillus actinomycetemcomitans strains: evidence for a new serotype e. Oral Microbiol Immunol 1993;8:116-20.

[72] Kaplan JB, Perry MB, MacLean LL, Furgang D, Wilson ME, Fine DH. Structural and genetic analyses of O polysaccharide from Actinobacillus actinomycetemcomitans serotype f. Infect Immun 2001;69:5375-84.

[73] Takada K, Saito M, Tsuzukibashi O, Kawashima Y, Ishida S, Hirasawa M. Characterization of a new serotype g isolate of Aggregatibacter actinomycetemcomitans. Mol Oral Microbiol 2010;25:200-6.

[74] Kittichotirat W, Bumgarner RE, Asikainen S, Chen C. Identification of the pangenome and its components in 14 distinct Aggregatibacter actinomycetemcomitans strains by comparative genomic analysis. PLoS One 2011;6:e22420.

[75] Chen C, Kittichotirat W, Si Y, Bumgarner R. Genome sequence of Aggregatibacter actinomycetemcomitans serotype c strain D11S-1. J Bacteriol 2009;191:7378-9.

[76] Kittichotirat W, Bumgarner R, Chen C. Markedly different genome arrangements between serotype a strains and serotypes b or c strains of Aggregatibacter actinomycetemcomitans. BMC Genomics 2010;11:489,2164-11-489.

[77] Huang Y, Kittichotirat W, Mayer MP, Hall R, Bumgarner R, Chen C. Comparative genomic hybridization and transcriptome analysis with a pan-genome microarray reveal distinctions between JP2 and non-JP2 genotypes of Aggregatibacter actinomycetemcomitans. Mol Oral Microbiol 2013;28:1-17.

[78] Hayashida H, Poulsen K, Kilian M. Differences in iron acquisition from human haemoglobin among strains of Actinobacillus actinomycetemcomitans. Microbiology 2002;148:3993-4001.

[79] Dileepan T, Kachlany SC, Balashova NV, Patel J, Maheswaran SK. Human CD18 is the functional receptor for Aggregatibacter actinomycetemcomitans leukotoxin. Infect Immun 2007;75:4851-6.

[80] Tsai CC, McArthur WP, Baehni PC, Hammond BF, Taichman NS. Extraction and partial characterization of a leukotoxin from a plaque-derived Gram-negative microorganism. Infect Immun 1979;25:427-39.

[81] Taichman NS, Dean RT, Sanderson CJ. Biochemical and morphological characterization of the killing of human monocytes by a leukotoxin derived from Actinobacillus actinomycetemcomitans. Infect Immun 1980;28:258-68.

[82] Kelk P, Claesson R, Hanstrom L, Lerner UH, Kalfas S, Johansson A. Abundant secretion of bioactive interleukin-1beta by human macrophages induced by Actinobacillus actinomycetemcomitans leukotoxin. Infect Immun 2005;73:453-8.

[83] Kelk P, Claesson R, Chen C, Sjostedt A, Johansson A. IL-1beta secretion induced by Aggregatibacter (Actinobacillus) actinomycetemcomitans is mainly caused by the leukotoxin. Int J Med Microbiol 2008;298:529-41.

[84] Shenker BJ, McKay T, Datar S, Miller M, Chowhan R, Demuth D. Actinobacillus actinomycetemcomitans immunosuppressive protein is a member of the family of cytolethal distending toxins capable of causing a G2 arrest in human T cells. J Immunol 1999;162:4773-80.

[85] Belibasakis G, Johansson A, Wang Y, Claesson R, Chen C, Asikainen S et al. Inhibited proliferation of human periodontal ligament cells and gingival fibroblasts by Actinobacillus actinomycetemcomitans: involvement of the cytolethal distending toxin. Eur J Oral Sci 2002;110:366-73.

[86] Belibasakis GN, Mattsson A, Wang Y, Chen C, Johansson A. Cell cycle arrest of human gingival fibroblasts and periodontal ligament cells by Actinobacillus actinomycetemcomitans: involvement of the cytolethal distending toxin. APMIS 2004;112:674-85.

[87] Alaoui-El-Azher M, Mans JJ, Baker HV, Chen C, Progulske-Fox A, Lamont RJ et al. Role of the ATM-checkpoint kinase 2 pathway in CDT-mediated apoptosis of gingival epithelial cells. PLoS One 2010;5:e11714.

[88] Jinadasa RN, Bloom SE, Weiss RS, Duhamel GE. Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. Microbiology 2011;157:1851-75.

[89] Lara-Tejero M, Galan JE. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science 2000;290:354-7.

[90] Rabin SD, Flitton JG, Demuth DR. Aggregatibacter actinomycetemcomitans cytolethal distending toxin induces apoptosis in nonproliferating macrophages by a phosphatase-independent mechanism. Infect Immun 2009;77:3161-9.

[91] Damek-Poprawa M, Jang JY, Volgina A, Korostoff J, DiRienzo JM. Localization of Aggregatibacter actinomycetemcomitans cytolethal distending toxin subunits during intoxication of live cells. Infect Immun 2012;80:2761-70.

[92] Ohara M, Miyauchi M, Tsuruda K, Takata T, Sugai M. Topical application of Aggregatibacter actinomycetemcomitans cytolethal distending toxin induces cell cycle arrest in the rat gingival epithelium in vivo. J Periodontal Res 2011;46:389-95.

[93] Damek-Poprawa M, Haris M, Volgina A, Korostoff J, Dirienzo JM. Cytolethal distending toxin damages the oral epithelium of gingival explants. J Dent Res 2011;90:874-9.

[94] Damek-Poprawa M, Korostoff J, Gill R, DiRienzo JM. Cell junction remodeling in gingival tissue exposed to a microbial toxin. J Dent Res 2013;92:518-23.

[95] Smith JL, Bayles DO. The contribution of cytolethal distending toxin to bacterial pathogenesis. Crit Rev Microbiol 2006;32:227-48.

[96] Belibasakis GN, Johansson A, Wang Y, Chen C, Kalfas S, Lerner UH. The cytolethal distending toxin induces receptor activator of NF-kappaB ligand expression in human gingival fibroblasts and periodontal ligament cells. Infect Immun 2005;73:342-51.

[97] Belibasakis GN, Brage M, Lagergard T, Johansson A. Cytolethal distending toxin upregulates RANKL expression in Jurkat T-cells. APMIS 2008;116:499-506.

[98] Chagnot C, Listrat A, Astruc T, Desvaux M. Bacterial adhesion to animal tissues: protein determinants for recognition of extracellular matrix components. Cell Microbiol 2012;14:1687-96.

[99] Auger E, Deslandes V, Ramjeet M, Contreras I, Nash JH, Harel J et al. Host-pathogen interactions of Actinobacillus pleuropneumoniae with porcine lung and tracheal epithelial cells. Infect Immun 2009;77:1426-41.

[100] Ouhara K, Komatsuzawa H, Shiba H, Uchida Y, Kawai T, Sayama K et al. Actinobacillus actinomycetemcomitans outer membrane protein 100 triggers innate immunity and production of beta-defensin and the 18-kilodalton cationic antimicrobial protein through the fibronectin-integrin pathway in human gingival epithelial cells. Infect Immun 2006;74:5211-20.

[101] Fink DL, Green BA, St Geme JW,3rd. The Haemophilus influenzae Hap autotransporter binds to fibronectin, laminin, and collagen IV. Infect Immun 2002;70:4902-7.

[102] Komatsuzawa H, Asakawa R, Kawai T, Ochiai K, Fujiwara T, Taubman MA et al. Identification of six major outer membrane proteins from Actinobacillus actinomycetemcomitans. Gene 2002;288:195-201.

[103] Asakawa R, Komatsuzawa H, Kawai T, Yamada S, Goncalves RB, Izumi S et al. Outer membrane protein 100, a versatile virulence factor of Actinobacillus actinomycetemcomitans. Mol Microbiol 2003;50:1125-39.

[104] Mintz KP. Identification of an extracellular matrix protein adhesin, EmaA, which mediates the adhesion of Actinobacillus actinomycetemcomitans to collagen. Microbiology 2004;150:2677-88.

[105] Ruiz T, Lenox C, Radermacher M, Mintz KP. Novel surface structures are associated with the adhesion of Actinobacillus actinomycetemcomitans to collagen. Infect Immun 2006;74:6163-70.

[106] Tang G, Kitten T, Munro CL, Wellman GC, Mintz KP. EmaA, a potential virulence determinant of Aggregatibacter actinomycetemcomitans in infective endocarditis. Infect Immun 2008;76:2316-24.

[107] Azari F, Radermacher M, Mintz KP, Ruiz T. Correlation of the amino-acid sequence and the 3D structure of the functional domain of EmaA from Aggregatibacter actinomycetemcomitans. J Struct Biol 2012;177:439-46.

[108] Longo PL, Nunes AC, Umeda JE, Mayer MP. Gene expression and phenotypic traits of Aggregatibacter actinomycetemcomitans in response to environmental changes. J Periodontal Res 2013.

[109] Tribble GD, Lamont RJ. Bacterial invasion of epithelial cells and spreading in periodontal tissue. Periodontol 2000 2010;52:68-83.

[110] Bessman MJ, Walsh JD, Dunn CA, Swaminathan J, Weldon JE, Shen J. The gene ygdP, associated with the invasiveness of Escherichia coli K1, designates a Nudix hydrolase, Orf176, active on adenosine (5')-pentaphospho-(5')-adenosine (Ap5A). J Biol Chem 2001;276:37834-8.

[111] Maiden MC. Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria. Clin Infect Dis 1998;27 Suppl 1:S12-20.

[112] Treangen TJ, Ambur OH, Tonjum T, Rocha EP. The impact of the neisserial DNA uptake sequences on genome evolution and stability. Genome Biol 2008;9:R60,2008-9-3-r60. Epub 2008 Mar 26.

[113] Chen I, Dubnau D. DNA uptake during bacterial transformation. Nat Rev Microbiol 2004;2:241-9.

[114] Herriott RM, Meyer EM, Vogt M. Defined nongrowth media for stage II development of competence in Haemophilus influenzae. J Bacteriol 1970;101:517-24.

[115] MacFadyen LP, Chen D, Vo HC, Liao D, Sinotte R, Redfield RJ. Competence development by Haemophilus influenzae is regulated by the availability of nucleic acid precursors. Mol Microbiol 2001;40:700-7.

[116] Sinha S, Mell JC, Redfield RJ. Seventeen Sxy-dependent cyclic AMP receptor protein site-regulated genes are needed for natural transformation in Haemophilus influenzae. J Bacteriol 2012;194:5245-54.

[117] Claverys JP, Havarstein LS. Extracellular-peptide control of competence for genetic transformation in Streptococcus pneumoniae. Front Biosci 2002;7:d1798-814.

[118] Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK. Chitin induces natural competence in Vibrio cholerae. Science 2005;310:1824-7.

[119] Antonova ES, Bernardy EE, Hammer BK. Natural competence in Vibrio cholerae is controlled by a nucleoside scavenging response that requires CytR-dependent anti-activation. Mol Microbiol 2012;86:1215-31.

[120] Wang Y, Goodman SD, Redfield RJ, Chen C. Natural transformation and DNA uptake signal sequences in Actinobacillus actinomycetemcomitans. J Bacteriol 2002;184:3442-9.

[121] Jorth P, Whiteley M. An evolutionary link between natural transformation and CRISPR adaptive immunity. MBio 2012;3:10.1128/mBio.00309,12. Print 2012.

[122] Fujise O, Lakio L, Wang Y, Asikainen S, Chen C. Clonal distribution of natural competence in Actinobacillus actinomycetemcomitans. Oral Microbiol Immunol 2004;19:340-2.

[123] Sorek R, Lawrence CM, Wiedenheft B. CRISPR-mediated Adaptive Immune Systems in Bacteria and Archaea. Annu Rev Biochem 2013.

[124] Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8:623-33.

[125] Branda SS, Vik S, Friedman L, Kolter R. Biofilms: the matrix revisited. Trends Microbiol 2005;13:20-6.

[126] Goodman SD, Obergfell KP, Jurcisek JA, Novotny LA, Downey JS, Ayala EA et al. Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. Mucosal Immunol 2011;4:625-37.

[127] Novotny LA, Amer AO, Brockson ME, Goodman SD, Bakaletz LO. Structural Stability of Burkholderia cenocepacia Biofilms Is Reliant on eDNA Structure and Presence of a Bacterial Nucleic Acid Binding Protein. PLoS One 2013;8:e67629.

[128] Goller CC, Romeo T. Environmental influences on biofilm development. Curr Top Microbiol Immunol 2008;322:37-66.

[129] Wilking JN, Zaburdaev V, De Volder M, Losick R, Brenner MP, Weitz DA. Liquid transport facilitated by channels in Bacillus subtilis biofilms. Proc Natl Acad Sci U S A 2013;110:848-52.

[130] Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nat Rev Microbiol 2008;6:199-210.

[131] Brown SA, Whiteley M. A novel exclusion mechanism for carbon resource partitioning in Aggregatibacter actinomycetemcomitans. J Bacteriol 2007;189:6407-14.

[132] Ramsey MM, Whiteley M. Polymicrobial interactions stimulate resistance to host innate immunity through metabolite perception. Proc Natl Acad Sci U S A 2009;106:1578-83.

[133] Kharazmi A. Mechanisms involved in the evasion of the host defence by Pseudomonas aeruginosa. Immunol Lett 1991;30:201-5.

[134] Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH et al. Staphylococcus aureus biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo. J Immunol 2011;186:6585-96.

[135] Yamanaka T, Furukawa T, Matsumoto-Mashimo C, Yamane K, Sugimori C, Nambu T et al. Gene expression profile and pathogenicity of biofilm-forming Prevotella intermedia strain 17. BMC Microbiol 2009;9:11,2180-9-11.

[136] Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GC et al. The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. PLoS Pathog 2011;7:e1001264.

[137] Chiang WC, Nilsson M, Jensen PO, Hoiby N, Nielsen TE, Givskov M et al. Extracellular DNA shields against aminoglycosides in Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 2013;57:2352-61.

[138] Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG. Natural genetic transformation of Streptococcus mutans growing in biofilms. J Bacteriol 2001;183:897-908.

[139] Molin S, Tolker-Nielsen T. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr Opin Biotechnol 2003;14:255-61.

[140] Eaton RE, Jacques NA. Deletion of competence-induced genes over-expressed in biofilms caused transformation deficiencies in Streptococcus mutans. Mol Oral Microbiol 2010;25:406-17.

[141] Marks LR, Reddinger RM, Hakansson AP. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in Streptococcus pneumoniae. MBio 2012;3:10.1128/mBio.00200,12. Print 2012.

[142] Hendrickx L, Hausner M, Wuertz S. Natural genetic transformation in monoculture Acinetobacter sp. strain BD413 biofilms. Appl Environ Microbiol 2003;69:1721-7.

[143] Carruthers MD, Tracy EN, Dickson AC, Ganser KB, Munson RS,Jr, Bakaletz LO. Biological roles of nontypeable Haemophilus influenzae type IV pilus proteins encoded by the pil and com operons. J Bacteriol 2012;194:1927-33.

[144] Bakaletz LO, Baker BD, Jurcisek JA, Harrison A, Novotny LA, Bookwalter JE et al. Demonstration of Type IV pilus expression and a twitching phenotype by Haemophilus influenzae. Infect Immun 2005;73:1635-43.

[145] Craig L, Pique ME, Tainer JA. Type IV pilus structure and bacterial pathogenicity. Nat Rev Microbiol 2004;2:363-78.

[146] Kolenbrander PE, Palmer RJ,Jr, Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. Nat Rev Microbiol 2010;8:471-80.

[147] Groicher KH, Firek BA, Fujimoto DF, Bayles KW. The Staphylococcus aureus lrgAB operon modulates murein hydrolase activity and penicillin tolerance. J Bacteriol 2000;182:1794-801.

[148] Rice KC, Firek BA, Nelson JB, Yang SJ, Patton TG, Bayles KW. The Staphylococcus aureus cidAB operon: evaluation of its role in regulation of murein hydrolase activity and penicillin tolerance. J Bacteriol 2003;185:2635-43.

[149] Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, Bayles KW. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. Proc Natl Acad Sci U S A PNAS 2007;104:8113–8118.

[150] Bayles KW. The biological role of death and lysis in biofilm development. Nat Rev Microbiol 2007;5:721-6.

[151] McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol 2011.

[152] Kachlany SC, Planet PJ, DeSalle R, Fine DH, Figurski DH. Genes for tight adherence of Actinobacillus actinomycetemcomitans: from plaque to plague to pond scum. Trends Microbiol 2001;9:429-37.

[153] Kachlany SC, Planet PJ, Desalle R, Fine DH, Figurski DH, Kaplan JB. flp-1, the first representative of a new pilin gene subfamily, is required for non-specific adherence of Actinobacillus actinomycetemcomitans. Mol Microbiol 2001;40:542-54.

[154] Kaplan JB, Velliyagounder K, Ragunath C, Rohde H, Mack D, Knobloch JK et al. Genes involved in the synthesis and degradation of matrix polysaccharide in Actinobacillus actinomycetemcomitans and Actinobacillus pleuropneumoniae biofilms. J Bacteriol 2004;186:8213-20.

[155] Izano EA, Sadovskaya I, Wang H, Vinogradov E, Ragunath C, Ramasubbu N et al. Poly-N-acetylglucosamine mediates biofilm formation and detergent resistance in Aggregatibacter actinomycetemcomitans. Microb Pathog 2008;44:52-60.

[156] Inoue T, Shingaki R, Sogawa N, Sogawa CA, Asaumi J, Kokeguchi S et al. Biofilm formation by a fimbriae-deficient mutant of Actinobacillus actinomycetemcomitans. Microbiol Immunol 2003;47:877-81.

[157] Tomich M, Planet PJ, Figurski DH. The tad locus: postcards from the widespread colonization island. Nat Rev Microbiol 2007;5:363-75.

[158] Inoue T, Tanimoto I, Ohta H, Kato K, Murayama Y, Fukui K. Molecular characterization of low-molecular-weight component protein, Flp, in Actinobacillus actinomycetemcomitans fimbriae. Microbiol Immunol 1998;42:253-8.

[159] Clock SA, Planet PJ, Perez BA, Figurski DH. Outer membrane components of the Tad (tight adherence) secreton of Aggregatibacter actinomycetemcomitans. J Bacteriol 2008;190:980-90.

[160] Perez BA, Planet PJ, Kachlany SC, Tomich M, Fine DH, Figurski DH. Genetic analysis of the requirement for flp-2, tadV, and rcpB in Actinobacillus actinomycetemcomitans biofilm formation. J Bacteriol 2006;188:6361-75.

[161] Rhodes ER, Menke S, Shoemaker C, Tomaras AP, McGillivary G, Actis LA. Iron acquisition in the dental pathogen Actinobacillus actinomycetemcomitans: what does it use as a source and how does it get this essential metal? Biometals 2007;20:365-77.

[162] Rhodes ER, Shoemaker CJ, Menke SM, Edelmann RE, Actis LA. Evaluation of different iron sources and their influence in biofilm formation by the dental pathogen Actinobacillus actinomycetemcomitans. J Med Microbiol 2007;56:119-28.

[163] Amarasinghe JJ, Scannapieco FA, Haase EM. Transcriptional and translational analysis of biofilm determinants of Aggregatibacter actinomycetemcomitans in response to environmental perturbation. Infect Immun 2009;77:2896-907.

[164] Haraszthy VI, Lally ET, Haraszthy GG, Zambon JJ. Molecular cloning of the fur gene from Actinobacillus actinomycetemcomitans. Infect Immun 2002;70:3170-9.

[165] Amarasinghe JJ, Connell TD, Scannapieco FA, Haase EM. Novel iron-regulated and Fur-regulated small regulatory RNAs in Aggregatibacter actinomycetemcomitans. Mol Oral Microbiol 2012;27:327-49.

[166] Lee JW, Helmann JD. Functional specialization within the Fur family of metalloregulators. Biometals 2007;20:485-99.

[167] Kaplan JB, Ragunath C, Ramasubbu N, Fine DH. Detachment of Actinobacillus actinomycetemcomitans biofilm cells by an endogenous beta-hexosaminidase activity. J Bacteriol 2003;185:4693-8.

[168] Kaplan JB, Meyenhofer MF, Fine DH. Biofilm growth and detachment of Actinobacillus actinomycetemcomitans. J Bacteriol 2003;185:1399-404.

[169] Schuster M, Greenberg EP. A network of networks: quorum-sensing gene regulation in Pseudomonas aeruginosa. Int J Med Microbiol 2006;296:73-81.

[170] Pereira CS, Thompson JA, Xavier KB. AI-2-mediated signalling in bacteria. FEMS Microbiol Rev 2013;37:156-81.

[171] Lazazzera BA, Grossman AD. The ins and outs of peptide signaling. Trends Microbiol 1998;6:288-94.

[172] Frias J, Olle E, Alsina M. Periodontal pathogens produce quorum sensing signal molecules. Infect Immun 2001;69:3431-4.

[173] Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol Rev 2001;25:365-404.

[174] Shiner EK, Rumbaugh KP, Williams SC. Inter-kingdom signaling: deciphering the language of acyl homoserine lactones. FEMS Microbiol Rev 2005;29:935-47.

[175] Antunes LC, Ferreira RB, Buckner MM, Finlay BB. Quorum sensing in bacterial virulence. Microbiology 2010;156:2271-82.

[176] Schauder S, Shokat K, Surette MG, Bassler BL. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol Microbiol 2001;41:463-76.

[177] Rezzonico F, Duffy B. Lack of genomic evidence of AI-2 receptors suggests a non-quorum sensing role for luxS in most bacteria. BMC Microbiol 2008;8:154,2180-8-154.

[178] Sperandio V, Mellies JL, Nguyen W, Shin S, Kaper JB. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic Escherichia coli. Proc Natl Acad Sci U S A 1999;96:15196-201.

[179] James CE, Hasegawa Y, Park Y, Yeung V, Tribble GD, Kuboniwa M et al. LuxS involvement in the regulation of genes coding for hemin and iron acquisition systems in Porphyromonas gingivalis. Infect Immun 2006;74:3834-44.

[180] Novak EA, Shao H, Daep CA, Demuth DR. Autoinducer-2 and QseC control biofilm formation and in vivo virulence of Aggregatibacter actinomycetemcomitans. Infect Immun 2010;78:2919-26.

[181] Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB. Bacteria-host communication: the language of hormones. Proc Natl Acad Sci U S A 2003;100:8951-6.

[182] Sperandio V, Torres AG, Kaper JB. Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved

in the regulation of flagella and motility by quorum sensing in E. coli. Mol Microbiol 2002;43:809-21.

[183] Fong KP, Chung WO, Lamont RJ, Demuth DR. Intra- and interspecies regulation of gene expression by Actinobacillus actinomycetemcomitans LuxS. Infect Immun 2001;69:7625-34.

[184] Shao H, Lamont RJ, Demuth DR. Autoinducer 2 is required for biofilm growth of Aggregatibacter (Actinobacillus) actinomycetemcomitans. Infect Immun 2007;75:4211-8.

[185] Armbruster CE, Pang B, Murrah K, Juneau RA, Perez AC, Weimer KE et al. RbsB (NTHI_0632) mediates quorum signal uptake in nontypeable Haemophilus influenzae strain 86-028NP. Mol Microbiol 2011;82:836-50.

[186] Maughan H, Sinha S, Wilson, L. & Redfield, R. Competene, DNA uptake and transformation in *Pasteurellaceae*. In: Anonymous ; 2008, p. 79-98.

[187] Danner DB, Deich RA, Sisco KL, Smith HO. An eleven-base-pair sequence determines the specificity of DNA uptake in Haemophilus transformation. Gene 1980;11:311-8.

[188] Wang Y, Shi W, Chen W, Chen C. Type IV pilus gene homologs pilABCD are required for natural transformation in Actinobacillus actinomycetemcomitans. Gene 2003;312:249-55.

[189] Redfield RJ, Findlay WA, Bosse J, Kroll JS, Cameron AD, Nash JH. Evolution of competence and DNA uptake specificity in the Pasteurellaceae. BMC Evol Biol 2006;6:82.

[190] Wang Y, Orvis J, Dyer D, Chen C. Genomic distribution and functions of uptake signal sequences in Actinobacillus actinomycetemcomitans. Microbiology 2006;152:3319-25.

[191] Cehovin A, Simpson PJ, McDowell MA, Brown DR, Noschese R, Pallett M et al. Specific DNA recognition mediated by a type IV pilin. Proc Natl Acad Sci U S A 2013;110:3065-70.

[192] Kruger NJ, Stingl K. Two steps away from novelty--principles of bacterial DNA uptake. Mol Microbiol 2011;80:860-7.

[193] Tanaka A, Fujise O, Chen C, Miura M, Hamachi T, Maeda K. A novel gene required for natural competence in Aggregatibacter actinomycetemcomitans. J Periodontal Res 2012;47:129-34.

[194] Berry JL, Phelan MM, Collins RF, Adomavicius T, Tonjum T, Frye SA et al. Structure and assembly of a trans-periplasmic channel for type IV pili in Neisseria meningitidis. PLoS Pathog 2012;8:e1002923.

[195] Assalkhou R, Balasingham S, Collins RF, Frye SA, Davidsen T, Benam AV et al. The outer membrane secretin PilQ from Neisseria meningitidis binds DNA. Microbiology 2007;153:1593-603.

[196] Lory S, Strom MS. Structure-function relationship of type-IV prepilin peptidase of Pseudomonas aeruginosa--a review. Gene 1997;192:117-21.

[197] Morand PC, Bille E, Morelle S, Eugene E, Beretti JL, Wolfgang M et al. Type IV pilus retraction in pathogenic Neisseria is regulated by the PilC proteins. EMBO J 2004;23:2009-17.

[198] Stingl K, Muller S, Scheidgen-Kleyboldt G, Clausen M, Maier B. Composite system mediates two-step DNA uptake into Helicobacter pylori. Proc Natl Acad Sci U S A 2010;107:1184-9.

[199] Chen I, Gotschlich EC. ComE, a competence protein from Neisseria gonorrhoeae with DNA-binding activity. J Bacteriol 2001;183:3160-8.

[200] Mullen LM, Bosse JT, Nair SP, Ward JM, Rycroft AN, Robertson G et al. Pasteurellaceae ComE1 proteins combine the properties of fibronectin adhesins and DNA binding competence proteins. PLoS One 2008;3:e3991.

[201] Barany F, Kahn ME, Smith HO. Directional transport and integration of donor DNA in Haemophilus influenzae transformation. Proc Natl Acad Sci U S A 1983;80:7274-8.

[202] Provvedi R, Dubnau D. ComEA is a DNA receptor for transformation of competent Bacillus subtilis. Mol Microbiol 1999;31:271-80.

[203] Berge M, Mortier-Barriere I, Martin B, Claverys JP. Transformation of Streptococcus pneumoniae relies on DprA- and RecA-dependent protection of incoming DNA single strands. Mol Microbiol 2003;50:527-36.

[204] Mortier-Barriere I, Velten M, Dupaigne P, Mirouze N, Pietrement O, McGovern S et al. A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. Cell 2007;130:824-36.

[205] Gwinn ML, Ramanathan R, Smith HO, Tomb JF. A new transformation-deficient mutant of Haemophilus influenzae Rd with normal DNA uptake. J Bacteriol 1998;180:746-8.

[206] Carbonnelle E, Helaine S, Nassif X and Pelicic V. A systematic genetic analysis in Neisseria meningitidis defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. *Mol.Microbiol*. 2006; 61:1510-1522.

[207] Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R et al. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics 2010;26:1608-15.

[208] Yu CS, Lin CJ, Hwang JK. Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. Protein Sci 2004;13:1402-6.

[209] Nathan C, Ding A. Nonresolving inflammation. Cell 2010;140:871-82.

[210] Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol 2007;7:179-90.

[211] Bryant C, Fitzgerald KA. Molecular mechanisms involved in inflammasome activation. Trends Cell Biol 2009;19:455-64.

[212] Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. Nat Immunol 2012;13:325-32.

[213] McIsaac SM, Stadnyk AW, Lin TJ. Toll-like receptors in the host defense against Pseudomonas aeruginosa respiratory infection and cystic fibrosis. J Leukoc Biol 2012;92:977-85.

[214] Eskan MA, Benakanakere MR, Rose BG, Zhang P, Zhao J, Stathopoulou P et al. Interleukin-1beta modulates proinflammatory cytokine production in human epithelial cells. Infect Immun 2008;76:2080-9.

[215] Stathopoulou PG, Benakanakere MR, Galicia JC, Kinane DF. Epithelial cell pro-inflammatory cytokine response differs across dental plaque bacterial species. J Clin Periodontol 2010;37:24-9.

[216] Gelani V, Fernandes AP, Gasparoto TH, Garlet TP, Cestari TM, Lima HR et al. The role of toll-like receptor 2 in the recognition of Aggregatibacter actinomycetemcomitans. J Periodontol 2009;80:2010-9.

[217] Lima HR, Gelani V, Fernandes AP, Gasparoto TH, Torres SA, Santos CF et al. The essential role of toll like receptor-4 in the control of Aggregatibacter actinomycetemcomitans infection in mice. J Clin Periodontol 2010;37:248-54.

[218] Ozturk A, Vieira AR. TLR4 as a risk factor for periodontal disease: a reappraisal. J Clin Periodontol 2009;36:279-86.

[219] Hsi ED, Remick DG. Monocytes are the major producers of interleukin-1 beta in an ex vivo model of local cytokine production. J Interferon Cytokine Res 1995;15:89-94.

[220] Tardif F, Ross G, Rouabhia M. Gingival and dermal fibroblasts produce interleukin-1 beta converting enzyme and interleukin-1 beta but not interleukin-18 even after stimulation with lipopolysaccharide. J Cell Physiol 2004;198:125-32.

[221] Vladimer GI, Marty-Roix R, Ghosh S, Weng D, Lien E. Inflammasomes and host defenses against bacterial infections. Curr Opin Microbiol 2013;16:23-31.

[222] Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH et al. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood 2009;113:2324-35.

[223] Miller CM, Boulter NR, Fuller SJ, Zakrzewski AM, Lees MP, Saunders BM et al. The role of the P2X(7) receptor in infectious diseases. PLoS Pathog 2011;7:e1002212.

[224] Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 2011;117:3720-32.

[225] Eder C. Mechanisms of interleukin-1beta release. Immunobiology 2009;214:543-53.

[226] Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1beta secretion. Cytokine Growth Factor Rev 2011;22:189-95.

[227] Kondo T, Kawai T, Akira S. Dissecting negative regulation of Toll-like receptor signaling. Trends Immunol 2012;33:449-58.

[228] Dubyak GR. P2X7 receptor regulation of non-classical secretion from immune effector cells. Cell Microbiol 2012;14:1697-706.

[229] Oscarsson J, Karched M, Thay B, Chen C, Asikainen S. Proinflammatory effect in whole blood by free soluble bacterial components released from planktonic and biofilm cells. BMC Microbiol 2008;8:206.

[230] Belibasakis GN, Johansson A. Aggregatibacter actinomycetemcomitans targets NLRP3 and NLRP6 inflammasome expression in human mononuclear leukocytes. Cytokine 2012;59:124-30.

[231] Fratelli M, Gagliardini V, Galli G, Gnocchi P, Ghiara P, Ghezzi P. Autocrine interleukin-1 beta regulates both proliferation and apoptosis in EL4-6.1 thymoma cells. Blood 1995;85:3532-7.

[232] Friedlander RM, Gagliardini V, Rotello RJ, Yuan J. Functional role of interleukin 1 beta (IL-1 beta) in IL-1 beta-converting enzyme-mediated apoptosis. J Exp Med 1996;184:717-24.

[233] Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M et al. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. Nature 2012;484:514-8.

[234] Ishihara Y, Nishihara T, Maki E, Noguchi T, Koga T. Role of interleukin-1 and prostaglandin in in vitro bone resorption induced by Actinobacillus actinomycetemcomitans lipopolysaccharide. J Periodontal Res 1991;26:155-60.

[235] Graves DT, Cochran D. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol 2003;74:391-401.

[236] Cochran DL. Inflammation and bone loss in periodontal disease. J Periodontol 2008;79:1569-76.

[237] Assuma R, Oates T, Cochran D, Amar S, Graves DT. IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. J Immunol 1998;160:403-9.

[238] Dinarello CA. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. Int Rev Immunol 1998;16:457-99.

[239] Dinarello CA. A clinical perspective of IL-1beta as the gatekeeper of inflammation. Eur J Immunol 2011;41:1203-17.

[240] Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A et al. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. J Exp Med 2000;191:313-20.

[241] Sims JE, Gayle MA, Slack JL, Alderson MR, Bird TA, Giri JG et al. Interleukin 1 signaling occurs exclusively via the type I receptor. Proc Natl Acad Sci U S A 1993;90:6155-9.

[242] Malinowsky D, Lundkvist J, Laye S, Bartfai T. Interleukin-1 receptor accessory protein interacts with the type II interleukin-1 receptor. FEBS Lett 1998;429:299-302.

[243] Riva F, Bonavita E, Barbati E, Muzio M, Mantovani A, Garlanda C. TIR8/SIGIRR is an Interleukin-1 Receptor/Toll Like Receptor Family Member with Regulatory Functions in Inflammation and Immunity. Front Immunol 2012;3:322.

[244] Khan MA, Steiner TS, Sham HP, Bergstrom KS, Huang JT, Assi K et al. The single IgG IL-1-related receptor controls TLR responses in differentiated human intestinal epithelial cells. J Immunol 2010;184:2305-13.

[245] Veliz Rodriguez T, Moalli F, Polentarutti N, Paroni M, Bonavita E, Anselmo A et al. Role of Toll interleukin-1 receptor (IL-1R) 8, a negative regulator of IL-1R/Toll-like receptor signaling, in resistance to acute Pseudomonas aeruginosa lung infection. Infect Immun 2012;80:100-9.

[246] Peyyala R, Ebersole JL. Multispecies biofilms and host responses: "discriminating the trees from the forest". Cytokine 2013;61:15-25.

[247] Mantovani A, Locati M, Vecchi A, Sozzani S, Allavena P. Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines. Trends Immunol 2001;22:328-36.

[248] Franchi L, Nunez G. Immunology. Orchestrating inflammasomes. Science 2012;337:1299-300.

[249] Symons JA, Duff GW. A soluble form of the interleukin-1 receptor produced by a human B cell line. FEBS Lett 1990;272:133-6.

[250] Symons JA, Eastgate JA, Duff GW. A soluble binding protein specific for interleukin 1 beta is produced by activated mononuclear cells. Cytokine 1990;2:190-8.

[251] Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M et al. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science 1993;261:472-5.

[252] Re F, Mengozzi M, Muzio M, Dinarello CA, Mantovani A, Colotta F. Expression of interleukin-1 receptor antagonist (IL-1ra) by human circulating polymorphonuclear cells. Eur J Immunol 1993;23:570-3.

[253] Malyak M, Smith MF,Jr, Abel AA, Hance KR, Arend WP. The differential production of three forms of IL-1 receptor antagonist by human neutrophils and monocytes. J Immunol 1998;161:2004-10.

[254] Gabay C, Smith MF, Eidlen D, Arend WP. Interleukin 1 receptor antagonist (IL-1Ra) is an acute-phase protein. J Clin Invest 1997;99:2930-40.

[255] Ortiz LA, Dutreil M, Fattman C, Pandey AC, Torres G, Go K et al. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. Proc Natl Acad Sci U S A 2007;104:11002-7.

[256] Gabay C, Smith MF,Jr, Arend WP. The human intracellular interleukin 1 receptor antagonist promoter appropriately regulates gene expression in keratinocytes and gastrointestinal epithelial cells in vivo. Cytokine 1999;11:561-70.

[257] Garlanda C, Anders HJ, Mantovani A. TIR8/SIGIRR: an IL-1R/TLR family member with regulatory functions in inflammation and T cell polarization. Trends Immunol 2009;30:439-46.

[258] Silva N, Dutzan N, Hernandez M, Dezerega A, Rivera O, Aguillon JC et al. Characterization of progressive periodontal lesions in chronic periodontitis patients: levels of chemokines, cytokines, matrix metalloproteinase-13, periodontal pathogens and inflammatory cells. J Clin Periodontol 2008;35:206-14.

[259] Ertugrul AS, Sahin H, Dikilitas A, Alpaslan N, Bozoglan A. Comparison of CCL28, interleukin-8, interleukin-1beta and tumor necrosis factor-alpha in subjects with gingivitis, chronic periodontitis and generalized aggressive periodontitis. J Periodontal Res 2013;48:44-51.

[260] Gursoy UK, Kononen E, Uitto VJ, Pussinen PJ, Hyvarinen K, Suominen-Taipale L et al. Salivary interleukin-1beta concentration and the presence of multiple pathogens in periodontitis. J Clin Periodontol 2009;36:922-7.

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

[262] Pöllänen MT, Laine MA, Ihalin R, Uitto VJ. Host-bacteria crosstalk at the dentogingival junction. Int J Dent 2012;2012:821383.

[263] Pöllänen MT, Salonen JI, Uitto VJ. Structure and function of the tooth-epithelial interface in health and disease. Periodontol 2000 2003;31:12-31.

[264] Lee HJ, Kang IK, Chung CP, Choi SM. The subgingival microflora and gingival crevicular fluid cytokines in refractory periodontitis. J Clin Periodontol 1995;22:885-90.

[265] Mogi M, Otogoto J, Ota N, Inagaki H, Minami M, Kojima K. Interleukin 1 beta, interleukin 6, beta 2-microglobulin, and transforming growth factor-alpha in gingival crevicular fluid from human periodontal disease. Arch Oral Biol 1999;44:535-9.

[266] Giannopoulou C, Cappuyns I, Mombelli A. Effect of smoking on gingival crevicular fluid cytokine profile during experimental gingivitis. J Clin Periodontol 2003;30:996-1002.

[267] Imatani T, Kato T, Okuda K. Production of inflammatory cytokines by human gingival fibroblasts stimulated by cell-surface preparations of Porphyromonas gingivalis. Oral Microbiol Immunol 2001;16:65-72.

[268] Dickinson BC, Moffatt CE, Hagerty D, Whitmore SE, Brown TA, Graves DT et al. Interaction of oral bacteria with gingival epithelial cell multilayers. Mol Oral Microbiol 2011;26:210-20.

[269] Bostanci N, Akgul B, Tsakanika V, Allaker RP, Hughes FJ, McKay IJ. Effects of low-dose doxycycline on cytokine secretion in human monocytes stimulated with Aggregatibacter actinomycetemcomitans. Cytokine 2011;56:656-61.

[270] May LT, Shaw JE, Khanna AK, Zabriskie JB, Sehgal PB. Marked cell-type-specific differences in glycosylation of human interleukin-6. Cytokine 1991;3:204-11.

[271] Hirano T, Taga T, Nakano N, Yasukawa K, Kashiwamura S, Shimizu K et al. Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). Proc Natl Acad Sci U S A 1985;82:5490-4.

[272] Tosato G, Seamon KB, Goldman ND, Sehgal PB, May LT, Washington GC et al. Monocyte-derived human B-cell growth factor identified as interferon-beta 2 (BSF-2, IL-6). Science 1988;239:502-4.

[273] Muraguchi A, Hirano T, Tang B, Matsuda T, Horii Y, Nakajima K et al. The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. J Exp Med 1988;167:332-44.

[274] Kopf M, Ramsay A, Brombacher F, Baumann H, Freer G, Galanos C et al. Pleiotropic defects of IL-6-deficient mice including early hematopoiesis, T and B cell function, and acute phase responses. Ann N Y Acad Sci 1995;762:308-18.

[275] Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235-8.

[276] Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T et al. Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature 1994;368:339-42.

[277] Shimada E, Kataoka H, Miyazawa Y, Yamamoto M, Igarashi T. Lipoproteins of Actinomyces viscosus induce inflammatory responses through TLR2 in human gingival epithelial cells and macrophages. Microbes Infect 2012;14:916-21.

[278] Uchida Y, Shiba H, Komatsuzawa H, Takemoto T, Sakata M, Fujita T et al. Expression of IL-1 beta and IL-8 by human gingival epithelial cells in response to Actinobacillus actinomycetemcomitans. Cytokine 2001;14:152-61.

[279] Yoshimura A, Hara Y, Kaneko T, Kato I. Secretion of IL-1 beta, TNF-alpha, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. J Periodontal Res 1997;32:279-86.

[280] Palmqvist P, Lundberg P, Lundgren I, Hanstrom L, Lerner UH. IL-1beta and TNF-alpha regulate IL-6-type cytokines in gingival fibroblasts. J Dent Res 2008;87:558-63.

[281] Sorsa T, Tjaderhane L, Konttinen YT, Lauhio A, Salo T, Lee HM et al. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. Ann Med 2006;38:306-21.

[282] Papathanasiou E, Flavia T, Griffin T, Arguello E, Finkelman M, Hanley J et al. Gingival crevicular fluid levels of interferon-gamma, but not interleukin-4 or -33 or thymic stromal lymphopoietin, are increased in inflamed sites in patients with periodontal disease. J Periodontal Res 2013.

[283] Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol 1993;11:571-611.

[284] Monack DM, Bouley DM, Falkow S. Salmonella typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization. J Exp Med 2004;199:231-41.

[285] Silva MT. Neutrophils and macrophages work in concert as inducers and effectors of adaptive immunity against extracellular and intracellular microbial pathogens. J Leukoc Biol 2010;87:805-13.

[286] Garlet GP, Cardoso CR, Campanelli AP, Garlet TP, Avila-Campos MJ, Cunha FQ et al. The essential role of IFN-gamma in the control of lethal Aggregatibacter actinomycetemcomitans infection in mice. Microbes Infect 2008;10:489-96.

[287] Porat R, Clark BD, Wolff SM, Dinarello CA. Enhancement of growth of virulent strains of Escherichia coli by interleukin-1. Science 1991;254:430-2.

[288] Meduri GU, Kanangat S, Stefan J, Tolley E, Schaberg D. Cytokines IL-1beta, IL-6, and TNF-alpha enhance in vitro growth of bacteria. Am J Respir Crit Care Med 1999;160:961-7.

[289] Hong L, Imeri L, Opp MR, Postlethwaite AE, Seyer JM, Krueger JM. Intercellular adhesion molecule-1

expression induced by interleukin (IL)-1 beta or an IL-1 beta fragment is blocked by an IL-1 receptor antagonist and a soluble IL-1 receptor. J Neuroimmunol 1993;44:163-70.

[290] Tagliabue A, Boraschi D. Cytokines as vaccine adjuvants: interleukin 1 and its synthetic peptide 163-171. Vaccine 1993;11:594-5.

[291] Boraschi D, Tagliabue A. Interleukin-1 and interleukin-1 fragments as vaccine adjuvants. Methods 1999;19:108-13.

[292] Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD et al. Protein Identification and Analysis Tools on the ExPASy Server In: Anonymous ; 2005, p. 571-607.

[293] Kanangat S, Postlethwaite A, Cholera S, Williams L, Schaberg D. Modulation of virulence gene expression in Staphylococcus aureus by interleukin-1beta: novel implications in bacterial pathogenesis. Microbes Infect 2007;9:408-15.

[294] Quinn GA, Tarwater PM, Cole AM. Subversion of interleukin-1-mediated host defence by a nasal carrier strain of Staphylococcus aureus. Immunology 2009;128:e222-9.

[295] Dahlen G, Wikstrom M. Occurrence of enteric rods, staphylococci and Candida in subgingival samples. Oral Microbiol Immunol 1995;10:42-6.

[296] Fritschi BZ, Albert-Kiszely A, Persson GR. Staphylococcus aureus and other bacteria in untreated periodontitis. J Dent Res 2008;87:589-93.

[297] Luo G, Niesel DW, Shaban RA, Grimm EA, Klimpel GR. Tumor necrosis factor alpha binding to bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties. Infect Immun 1993;61:830-5.

[298] Karlyshev AV, Galyov EE, Smirnov OY, Guzayev AP, Abramov VM, Zav'yalov VP. A new gene of the f1 operon of Y. pestis involved in the capsule biogenesis. FEBS Lett 1992;297:77-80.

[299] Dubnovitsky AP, Duck Z, Kersley JE, Hard T, MacIntyre S, Knight SD. Conserved hydrophobic clusters on the surface of the Caf1A usher C-terminal domain are important for F1 antigen assembly. J Mol Biol 2010;403:243-59.

[300] Thanassi DG, Bliska JB, Christie PJ. Surface organelles assembled by secretion systems of Gram-negative bacteria: diversity in structure and function. FEMS Microbiol Rev 2012;36:1046-82.

[301] Zav'yalov V, Denesyuk A, Zav'yalova G, Korpela T. Molecular modeling of the steric structure of the envelope F1 antigen of Yersinia pestis. Immunol. Lett. 1995; 45:19-22.

[302] Zavialov AV, Berglund J, Pudney AF, Fooks LJ, Ibrahim TM, MacIntyre S et al. Structure and biogenesis of the capsular F1 antigen from Yersinia pestis: preserved folding energy drives fiber formation. Cell 2003;113:587-96.

[303] Berisio R, Ciccarelli L, Squeglia F, De Simone A, Vitagliano L. Structural and dynamic properties of incomplete immunoglobulin-like fold domains. Protein Pept Lett 2012;19:1045-53.

[304] Diggle SP, Stacey RE, Dodd C, Camara M, Williams P, Winzer K. The galactophilic lectin, LecA, contributes to biofilm development in Pseudomonas aeruginosa. Environ Microbiol 2006;8:1095-104.

[305] Alcami A. Viral mimicry of cytokines, chemokines and their receptors. Nat Rev Immunol 2003;3:36-50.

[306] Imbronito AV, Okuda OS, Maria de Freitas N, Moreira Lotufo RF, Nunes FD. Detection of herpesviruses and periodontal pathogens in subgingival plaque of patients with chronic periodontitis, generalized aggressive periodontitis, or gingivitis. J Periodontol 2008;79:2313-21.

[307] Strockbine LD, Cohen JI, Farrah T, Lyman SD, Wagener F, DuBose RF et al. The Epstein-Barr virus BARF1 gene encodes a novel, soluble colony-stimulating factor-1 receptor. J Virol 1998;72:4015-21.

[308] Hsu DH, de Waal Malefyt R, Fiorentino DF, Dang MN, Vieira P, de Vries J et al. Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. Science 1990;250:830-2.

[309] Kotenko SV, Saccani S, Izotova LS, Mirochnitchenko OV, Pestka S. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). Proc Natl Acad Sci U S A 2000;97:1695-700.

[310] Ho AS, Moore KW. Interleukin-10 and its receptor. Ther Immunol 1994;1:173-85.

[311] Alcami A, Smith GL. A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. Cell 1992;71:153-67.

[312] Spriggs MK, Hruby DE, Maliszewski CR, Pickup DJ, Sims JE, Buller RM et al. Vaccinia and cowpox viruses encode a novel secreted interleukin-1-binding protein. Cell 1992;71:145-52.

[313] Treseler CB, Maziarz RT, Levitz SM. Biological activity of interleukin-2 bound to Candida albicans. Infect Immun 1992;60:183-8.

[314] Zanetta JP, Bonaly R, Maschke S, Strecker G, Michalski JC. Differential binding of lectins IL-2 and CSL to candida albicans and cancer cells. Glycobiology 1998;8:221-5.

[315] Cebo C, Dambrouck T, Maes E, Laden C, Strecker G, Michalski JC et al. Recombinant human interleukins IL-1alpha, IL-1beta, IL-4, IL-6, and IL-7 show different and specific calcium-independent carbohydrate-binding properties. J Biol Chem 2001;276:5685-91.

[316] Sherblom AP, Decker JM, Muchmore AV. The lectin-like interaction between recombinant tumor necrosis factor and uromodulin. J Biol Chem 1988;263:5418-24.

[317] Cebo C, Vergoten G, Zanetta JP. Lectin activities of cytokines: functions and putative carbohydrate-recognition domains. Biochim Biophys Acta 2002;1572:422-34.

[318] Ramsden L, Rider CC. Selective and differential binding of interleukin (IL)-1 alpha, IL-1 beta, IL-2 and IL-6 to glycosaminoglycans. Eur J Immunol 1992;22:3027-31.

[319] Vergoten G, Zanetta JP. Structural differences between the putative carbohydrate-recognition domains of human IL-1 alpha, IL-1 beta and IL-1 receptor antagonist obtained by in silico modeling. Glycoconj J 2007;24:183-93.

[320] Chandra J, McCormick TS, Imamura Y, Mukherjee PK, Ghannoum MA. Interaction of Candida albicans with adherent human peripheral blood mononuclear cells increases C. albicans biofilm formation and results in differential expression of pro- and anti-inflammatory cytokines. Infect Immun 2007;75:2612-20.

[321] Wang Y, Chen C. Mutation analysis of the flp operon in Actinobacillus actinomycetemcomitans. Gene 2005;351:61-71.

[322] Dogan B, Saarela M, Asikainen S. Genotyping of Actinobacillus actinomycetemcomitans serotype d isolates based on polymerase chain reaction. Oral Microbiol Immunol 1999;14:387-90.

[323] Suzuki N, Nakano Y, Yoshida Y, Ikeda D, Koga T. Identification of Actinobacillus actinomycetemcomitans serotypes by multiplex PCR. J Clin Microbiol 2001;39:2002-5.

[324] Moncla BJ, Braham P, Dix K, Watanabe S, Schwartz D. Use of synthetic oligonucleotide DNA probes for the identification of Bacteroides gingivalis. J Clin Microbiol 1990;28:324-7.

[325] Saarela M, Asikainen S, Chen C, Alaluusua S, Slots J. Comparison of arbitrarily primed polymerase chain reaction and ribotyping for subtyping Actinobacillus actinomycetemcomitans. Anaerobe 1995;1:97-102.

[326] Karched M, Paul-Satyaseela M, Asikainen S. A simple viability-maintaining method produces homogenic cell suspensions of autoaggregating wild-type Actinobacillus actinomycetemcomitans. J Microbiol Methods 2007;68:46-51.

[327] Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 2011;8:785-6.

[328] Miroux B, Walker JE. Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 1996;260:289-98.

[329] Belogurov GA, Malinen AM, Turkina MV, Jalonen U, Rytkonen K, Baykov AA et al. Membrane-bound pyrophosphatase of Thermotoga maritima requires sodium for activity. Biochemistry 2005;44:2088-96.

[330] Doublie S. Preparation of selenomethionyl proteins for phase determination. Methods Enzymol 1997;276:523-30.

[331] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.

[332] Green H, Fuchs E, Watt F. Differentiated structural components of the keratinocyte. Cold Spring Harb Symp Quant Biol 1982;46 Pt 1:293-301.

[333] Oksanen J, Hormia M. An organotypic in vitro model that mimics the dento-epithelial junction. J Periodontol 2002;73:86-93.

[334] Gursoy UK, Pöllänen M, Kononen E, Uitto VJ. Biofilm formation enhances the oxygen tolerance and invasiveness of Fusobacterium nucleatum in an oral mucosa culture model. J Periodontol 2010;81:1084-91.

[335] Mäkelä M, Salo T, Larjava H. MMP-9 from TNF alpha-stimulated keratinocytes binds to cell membranes and type I collagen: a cause for extended matrix degradation in inflammation? Biochem Biophys Res Commun 1998;253:325-35.

[336] Frojdman K, Paranko J, Virtanen I, Pelliniemi LJ. Intermediate filaments and epithelial differentiation of male rat embryonic gonad. Differentiation 1992;50:113-23.

[337] Slot JW, Geuze HJ. A new method of preparing gold probes for multiple-labeling cytochemistry. Eur J Cell Biol 1985;38:87-93.

[338] Wilson ME. The heat-modifiable outer membrane protein of Actinobacillus actinomycetemcomitans: relationship to OmpA proteins. Infect Immun 1991;59:2505-7.

[339] Paul-Satyaseela M, Karched M, Bian Z, Ihalin R, Boren T, Arnqvist A et al. Immunoproteomics of Actinobacillus actinomycetemcomitans outer-membrane proteins reveal a highly immunoreactive peptidoglycan-associated lipoprotein. J Med Microbiol 2006;55:931-42.

[340] O'Connell KL, Stults JT. Identification of mouse liver proteins on two-dimensional electrophoresis gels by matrix-assisted laser desorption/ionization mass spectrometry of in situ enzymatic digests. Electrophoresis 1997;18:349-59.

[341] Zimmerman SB, Murphy LD. Release of compact nucleoids with characteristic shapes from Escherichia coli. J Bacteriol 2001;183:5041-9.

[342] Huovinen T, Syrjänpää M, Sanmark H, Brockmann EC, Azhayev A, Wang Q et al. Two ScFv antibody libraries derived from identical VL-VH framework with different binding site designs display distinct binding profiles. Protein Eng Des Sel 2013;26:683-93.

[343] Brockmann EC, Akter S, Savukoski T, Huovinen T, Lehmusvuori A, Leivo J et al. Synthetic single-framework antibody library integrated with rapid affinity maturation by VL shuffling. Protein Eng Des Sel 2011;24:691-700.

[344] Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci 2003;12:1652-62.

[345] Imai K, Asakawa N, Tsuji T, Akazawa F, Ino A, Sonoyama M et al. SOSUI-GramN: high performance prediction for sub-cellular localization of proteins in gram-negative bacteria. Bioinformation 2008;2:417-21.

[346] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389-402.

[347] Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol 2000;302:205-17.

[348] Loferer H, Hammar M, Normark S. Availability of the fibre subunit CsgA and the nucleator protein CsgB during assembly of fibronectin-binding curli is limited by the intracellular concentration of the novel lipoprotein CsgG. Mol Microbiol 1997;26:11-23.

[349] Inukai M, Takeuchi M, Shimizu K, Arai M. Mechanism of action of globomycin. J Antibiot (Tokyo) 1978;31:1203-5.

[350] Aschtgen MS, Bernard CS, De Bentzmann S, Lloubes R, Cascales E. SciN is an outer membrane lipoprotein required for type VI secretion in enteroaggregative Escherichia coli. J Bacteriol 2008;190:7523-31.

[351] Achtman M, Mercer A, Kusecek B, Pohl A, Heuzenroeder M, Aaronson W et al. Six widespread bacterial clones among Escherichia coli K1 isolates. Infect Immun 1983;39:315-35.

[352] Volkmer B, Heinemann M. Condition-dependent cell volume and concentration of Escherichia coli to facilitate data conversion for systems biology modeling. PLoS One 2011;6:e23126.

[353] Vargas C, McEwan AG, Downie JA. Detection of c-type cytochromes using enhanced chemiluminescence. Anal Biochem 1993;209:323-6.

[354] Shell DM, Chiles L, Judd RC, Seal S, Rest RF. The Neisseria lipooligosaccharide-specific alpha-2,3-sialyltransferase is a surface-exposed outer membrane protein. Infect Immun 2002;70:3744-51.

[355] Lewis K. Persister cells, dormancy and infectious disease. Nat Rev Microbiol 2007;5:48-56.

[356] Housden NG, Wojdyla JA, Korczynska J, Grishkovskaya I, Kirkpatrick N, Brzozowski AM et al. Directed epitope delivery across the Escherichia coli outer membrane through the porin OmpF. Proc Natl Acad Sci U S A 2010;107:21412-7.

[357] Sfakianakis A, Barr CE, Kreutzer DL. Actinobacillus actinomycetemcomitans-induced expression of IL-1alpha and IL-1beta in human gingival epithelial cells: role in IL-8 expression. Eur J Oral Sci 2001;109:393-401.

[358] Haase EM, Zmuda JL, Scannapieco FA. Identification and molecular analysis of rough-colony-specific outer membrane proteins of Actinobacillus actinomycetemcomitans. Infect Immun 1999;67:2901-8.

[359] Daun JM, Fenton MJ. Interleukin-1/Toll receptor family members: receptor structure and signal transduction pathways. J Interferon Cytokine Res 2000;20:843-55.

[360] Nakamoto RK, Baylis Scanlon JA, Al-Shawi MK. The rotary mechanism of the ATP synthase. Arch Biochem Biophys 2008;476:43-50.

[361] Laughlin TF, Ahmad Z. Inhibition of Escherichia coli ATP synthase by amphibian antimicrobial peptides. Int J Biol Macromol 2010;46:367-74.

[362] Ahmad Z, Okafor F, Laughlin TF. Role of Charged Residues in the Catalytic Sites of Escherichia coli ATP Synthase. J Amino Acids 2011;2011:785741.

[363] Rouviere-Yaniv J, Gros F. Characterization of a novel, low-molecular-weight DNA-binding protein from Escherichia coli. Proc Natl Acad Sci U S A 1975;72:3428-32.

[364] Prieto AI, Kahramanoglou C, Ali RM, Fraser GM, Seshasayee AS, Luscombe NM. Genomic analysis of DNA binding and gene regulation by homologous nucleoid-associated proteins IHF and HU in Escherichia coli K12. Nucleic Acids Res 2012;40:3524-37.

[365] Drlica K, Rouviere-Yaniv J. Histonelike proteins of bacteria. Microbiol Rev 1987;51:301-19.

[366] Kar S, Edgar R, Adhya S. Nucleoid remodeling by an altered HU protein: reorganization of the transcription program. Proc Natl Acad Sci U S A 2005;102:16397-402.

[367] Berger M, Farcas A, Geertz M, Zhelyazkova P, Brix K, Travers A et al. Coordination of genomic structure and transcription by the main bacterial nucleoid-associated protein HU. EMBO Rep 2010;11:59-64.

[368] Oberto J, Nabti S, Jooste V, Mignot H, Rouviere-Yaniv J. The HU regulon is composed of genes

responding to anaerobiosis, acid stress, high osmolarity and SOS induction. PLoS One 2009;4:e4367.

[369] Davey ME, Duncan MJ. Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in Porphyromonas gingivalis. J Bacteriol 2006;188:5510-23.

[370] Priyadarshini R, Cugini C, Arndt A, Chen T, Tjokro NO, Goodman SD et al. The nucleoid-associated protein HUbeta affects global gene expression in Porphyromonas gingivalis. Microbiology 2013;159:219-29.

[371] Kim N, Weeks DL, Shin JM, Scott DR, Young MK, Sachs G. Proteins released by Helicobacter pylori in vitro. J Bacteriol 2002;184:6155-62.

[372] Liu D, Yumoto H, Hirota K, Murakami K, Takahashi K, Hirao K et al. Histone-like DNA binding protein of Streptococcus intermedius induces the expression of pro-inflammatory cytokines in human monocytes via activation of ERK1/2 and JNK pathways. Cell Microbiol 2008;10:262-76.

[373] Krishnan S, Prasadarao NV. Outer membrane protein A and OprF: versatile roles in Gram-negative bacterial infections. FEBS J 2012;279:919-31.

[374] Chang TW, Lin YM, Wang CF, Liao YD. Outer membrane lipoprotein Lpp is Gram-negative bacterial cell surface receptor for cationic antimicrobial peptides. J Biol Chem 2012;287:418-28.

[375] Freire MO, Sedghizadeh PP, Schaudinn C, Gorur A, Downey JS, Choi JH et al. Development of an animal model for Aggregatibacter actinomycetemcomitans biofilm-mediated oral osteolytic infection: a preliminary study. J Periodontol 2011;82:778-89.

[376] Bauer ME, Townsend CA, Doster RS, Fortney KR, Zwickl BW, Katz BP et al. A fibrinogen-binding lipoprotein contributes to the virulence of Haemophilus ducreyi in humans. J Infect Dis 2009;199:684-92.

[377] Zijnge V, Kieselbach T, Oscarsson J. Proteomics of protein secretion by Aggregatibacter actinomycetemcomitans. PLoS One 2012;7:e41662.

[378] Tokuda H, Matsuyama S. Sorting of lipoproteins to the outer membrane in E. coli. Biochim Biophys Acta 2004;1694:IN1-9.

[379] Kovacs-Simon A, Titball RW, Michell SL. Lipoproteins of bacterial pathogens. Infect Immun 2011;79:548-61.

[380] Okuda S, Tokuda H. Lipoprotein sorting in bacteria. Annu Rev Microbiol 2011;65:239-59.

[381] Dong C, Beis K, Nesper J, Brunkan-Lamontagne AL, Clarke BR, Whitfield C et al. Wza the translocon for E. coli capsular polysaccharides defines a new class of membrane protein. Nature 2006;444:226-9.

[382] Ford RC, Brunkan-LaMontagne AL, Collins RF, Clarke BR, Harris R, Naismith JH et al. Structure-function relationships of the outer membrane translocon Wza investigated by cryo-electron microscopy and mutagenesis. J Struct Biol 2009;166:172-82.

[383] von Heijne G. Protein targeting signals. Curr Opin Cell Biol 1990;2:604-8.

[384] Lee SY, Choi JH, Xu Z. Microbial cell-surface display. Trends Biotechnol 2003;21:45-52.

[385] Nalbant A, Chen C, Wang Y, Zadeh HH. Induction of T-cell apoptosis by Actinobacillus actinomycetemcomitans mutants with deletion of ltxA and cdtABC genes: possible activity of GroEL-like molecule. Oral Microbiol Immunol 2003;18:339-49.

[386] Kang P, Korostoff J, Volgina A, Grzesik W, DiRienzo JM. Differential effect of the cytolethal distending toxin of Actinobacillus actinomycetemcomitans on co-cultures of human oral cells. J Med Microbiol 2005;54:785-94.

[387] DiRienzo JM, Song M, Wan LS, Ellen RP. Kinetics of KB and HEp-2 cell responses to an invasive, cytolethal distending toxin-producing strain of Actinobacillus actinomycetemcomitans. Oral Microbiol Immunol 2002;17:245-51.

[388] Rompikuntal PK, Thay B, Khan MK, Alanko J, Penttinen AM, Asikainen S et al. Perinuclear localization of internalized outer membrane vesicles carrying active cytolethal distending toxin from Aggregatibacter actinomycetemcomitans. Infect Immun 2012;80:31-42.

[389] Atula S, Grenman R, Syrjanen S. Fibroblasts can modulate the phenotype of malignant epithelial cells in vitro. Exp Cell Res 1997;235:180-7.

[390] Secor PR, James GA, Fleckman P, Olerud JE, McInnerney K, Stewart PS. Staphylococcus aureus Biofilm and Planktonic Cultures Differentially Impact Gene Expression, MAPK Phosphorylation, and Cytokine Production in Human Keratinocytes. BMC Microbiol 2011;11:143.

[391] Nistico S, Paolillo N, Minella D, Piccirilli S, Rispoli V, Giardina E et al. Effects of TNF-alpha and IL-1 beta on the activation of genes related to inflammatory, immune responses and cell death in immortalized human HaCat keratinocytes. Int J Immunopathol Pharmacol 2010;23:1057-72.

[392] Valverde R, Edwards L, Regan L. Structure and function of KH domains. FEBS J 2008;275:2712-26.

[393] Braddock DT, Baber JL, Levens D, Clore GM. Molecular basis of sequence-specific single-stranded DNA recognition by KH domains: solution structure of a complex between hnRNP K KH3 and single-stranded DNA. EMBO J 2002;21:3476-85.

[394] Braddock DT, Louis JM, Baber JL, Levens D, Clore GM. Structure and dynamics of KH domains from FBP bound to single-stranded DNA. Nature 2002;415:1051-6.