A novel intrinsically disordered outer membrane lipoprotein of *Aggregatibacter actinomycetemcomitans* binds various cytokines and plays a role in biofilm response to interleukin-1β and interleukin-8

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Abbreviations and acronyms: BilRI, bacterial interleukin receptor I; BSA, bovine serum albumin; CFUs, colony forming units; ClfA, clumping factor A; DsrA, Ducreyi serum resistance A; eDNA, extracellular DNA; ELISA, enzyme-linked immunosorbent assay; FgbA, fibrinogen binder A; FID, free induction decay; HGF, human gingival fibroblast; HGK, human gingival keratinocyte; HSQC, heteronuclear single quantum coherence spectroscopy; IDP, intrinsically disordered protein; IL, interleukin; IFN, interferon; IPTG, isopropyl β-D-1-thiogalactopyranoside; *ltxP*, leucotoxin promoter; MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NMR, nuclear magnetic resonance; OPG, osteoprotegerin; PAMP, pathogen-associated molecular pattern; PGA, poly-N-acetylglucosamine; PMSF, phenylmethylsulfonyl fluoride; RANK, receptor activator of nuclear factor κ-B; RANKL, RANK ligand; RT, room temperature; sIL-6R, soluble IL-6 receptor; TGF, transforming growth factor; TNF, tumour necrosis factor; TSA, tryptone soy agar; TSB, tryptone soy broth

ABSTRACT

Intrinsically disordered proteins (IDPs) do not have a well-defined and stable three-dimensional fold. Some IDPs can function as either transient or permanent binders of other proteins and may interact with an array of ligands by adopting different conformations. A novel outer membrane lipoprotein, bacterial interleukin receptor I (BilRI) of the opportunistic oral pathogen *Aggregatibacter actinomycetemcomitans* binds a key gatekeeper proinflammatory cytokine interleukin (IL)-1β. Because the amino acid sequence of the novel lipoprotein resembles that of fibrinogen binder A of *Haemophilus ducreyi*, BilRI could have the potential to bind other proteins, such as host matrix proteins. However, from the tested host matrix proteins, BilRI interacted with neither collagen nor fibrinogen. Instead, the recombinant non-lipidated BilRI, which was intrinsically disordered, bound various pro/anti-inflammatory cytokines, such as IL-8, tumour necrosis factor (TNF)-α, interferon (IFN)-γ and IL-10. Moreover, BilRI played a role in the *in vitro* sensing of IL-1β and IL-8 because low concentrations of cytokines did not decrease the amount of extracellular DNA in the matrix of *bilRI*⁻ mutant biofilm as they did in the matrix of wild-type biofilm when the biofilms were exposed to recombinant cytokines for 22 hours. BilRI played a role in the internalization of IL-1β in the gingival model system but did not affect either IL-8 or IL-6 uptake. However, *bilRI* deletion did not entirely prevent IL-1β internalization, and the binding of cytokines to BilRI was relatively weak. Thus, BilRI might sequester cytokines on the surface of *A. actinomycetemcomitans* to facilitate the internalization process in low local cytokine concentrations.

Introduction

Intrinsically disordered proteins (IDPs) go against the *structure-defines-function* paradigm given that they lack a well-defined three-dimensional fold; yet, they are elementary components in a myriad of cellular processes.[1] The proportion of IDP increases when moving from simple microorganisms to more complex eukaryotes, suggesting an evolutionary advantage of having flexible proteins that may possess several functions. For instance, the proteome of *Escherichia coli* has been predicted to contain approximately 15% proteins having more than 30 amino acid disordered segments, whereas in *Saccharomyces cerevisiae*, the ratio is approximately 50-60%.[2] In eukaryotes, many IDPs have roles in signal transduction, where they may bind to multiple ligands with variable affinities.[3]

The oral opportunistic pathogen *Aggregatibacter actinomycetemcomitans* can be found from multispecies biofilms in diseased periodontal pockets of patients suffering from aggressive or chronic forms of periodontitis.[4-6] Among the diverse changes in the host response to multispecies biofilms, periodontal diseases are characterized by alterations in the levels of various inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and IL-8, and the anti-inflammatory cytokine IL-10.[7] The highly leucotoxic JP2 genotype of *A. actinomycetemcomitans* has been suggested to be an important aetiological agent in disease initiation,[8] where the inflammatory reaction is caused by inflammophilic dysbiotic multispecies bacterial biofilm whose existence may be favoured by the micromilieu in inflammation.[9] This biofilm grows attached to the tooth surface and invades between the tooth and gingival tissue towards the junctional epithelium.[10] The host tissue, including alveolar bone, is mainly destroyed by the host response to the pathogenic biofilm. *A. actinomycetemcomitans* may have systemic effects on host health because it has been linked to the aetiology of cardiovascular diseases,[11, 12] endocarditis,[13] and brain abscesses.[14] Thus, its pathogenic properties may have broader significance to human health than merely oral health.

Human pathogens have several strategies to disturb and evade the host innate immune defence systems. Bacterial cells may grow as protective communities known as biofilms, in which the extracellular matrix provides protection from antibodies, antibiotics and cellular immune defence cells, such as macrophages.[15] Adhesive type IV Flp-pili, poly-N-acetylglucosamine (PGA) and

77 extracellular DNA (eDNA) are the main biofilm matrix components of *A. actinomycetemcomitans*.[16, 17] Of these, long bundled Flp-pili protein fibre plays the most important role in autoaggregation, nonspecific adherence, biofilm formation and virulence in a rat model.[17-20]

80 Various pathogens possess receptors that bind host inflammatory cytokines.[21-24] The binding of cytokines to bacteria may change the properties of the bacteria, such as their biofilm formation [25, 26] and virulence gene expression,[21, 23, 27] and may also manipulate complex host inflammatory reactions, leading to debilitated host defence against colonizing or invading pathogens. We have shown that *A. actinomycetemcomitans* is able to bind the central proinflammatory cytokine IL-1β [26] and to internalize IL-1β [26, 28] and that intracellular IL-1β binds to at least two bacterial proteins [26, 28]. In addition, IL-1β decreases the metabolic activity of *A. actinomycetemcomitans* biofilms.[26] In our recent study, we have identified an outer membrane lipoprotein of *A. actinomycetemcomitans*, bacterial interleukin receptor I (BilRI),[24] which is most likely one of the first-line binders of IL-1β on the extracellular side of the bacterium. Whether this novel outer membrane protein is involved merely in the response of *A. actinomycetemcomitans* biofilm to IL-1β or whether it could bind host proteins and cytokines other than IL-1β was not known. Thus, the aims of the present study was to resolve the three-dimensional structure of BilRI, to investigate the host protein- and cytokine-binding capacity of the *Pasteurellaceae*-specific BilRI and to study the phenotype and response to cytokines of a single-gene-deletion mutant of *bilRI*.

95 Our results indicate that BilRI is not a specific receptor of IL-1β *in vitro* and binds to other inflammatory cytokines, such as IL-8 and IL-10. We also found that BilRI is an IDP, which most likely explains the existence of several ligands. *bilRI* deletion did not completely prevent cytokine internalization, but it significantly decreased IL-1β uptake and impeded the response of biofilm to low concentrations of IL-1β and IL-8. Because the binding of cytokines to the BilRI was relatively weak, BilRI might function as a non-specific cytokine concentrator on the surface of *A. actinomycetemcomitans* that facilitates the internalization process, especially in low concentrations of cytokines.

Results

BilRI is an intrinsically disordered protein

The proton (^{1}H) spectrum of BilRI measured at 600 MHz exhibits features typical of a disordered protein, including a collapsed chemical shift dispersion in the amide proton region (8.2 ± 0.3 ^{1}H ppm) and the lack of shielded methyl protons, i.e., clustering of methyl protons to so-called random coil shift, 0.7 ppm (Fig. 1A). To confirm these observations, we also performed a two-dimensional 1H, 15N heteronuclear single-quantum coherence (15N HSQC) experiment at the 800-MHz 1H frequency of BilRI (Fig. 1B). To slow down the chemical exchange of labile amide protons with solvent protons, we measured the 15N HSQC spectrum of BilRI under mildly acidic conditions (pH 5). This spectrum more clearly highlights the same features already visible in the corresponding 1H spectrum, i.e., poor dispersion of amide proton chemical shifts, indicating that BilRI remains disordered in solution and under slightly acidic conditions.[29]. The amino acid sequence analysis supported this finding, showing high numbers of charged and polar residues and a low number of hydrophobic bulky amino acids (Fig. 1C). Moreover, the BilRI sequence had a low complexity, i.e., biased amino acid composition: it did not have any aromatic amino acids, such as phenylalanine, tyrosine and tryptophan, and 48% of the sequence is made up of three residues: alanine, lysine and aspartate (Fig. 1C). All of the above-mentioned amino acid sequence features are typical for IDPs.

Recombinant BilRI binds to various cytokines but not to the host matrix proteins collagen and fibrinogen

A microplate assay showed that recombinant BilRI bound to various cytokines, of which the binding to IL-8 was high compared with the binding of BilRI to the negative control protein bovine serum albumin (BSA; p=0.008; paired-samples T-test; Fig. 2A). However, the binding to IL-6-coated wells was weak and almost as inefficient as the binding to BSA, which was used as a blocking agent in the assay (Fig. 2A). We decided to use C-tagged recombinant BilRI in our binding assays because binding to IL-1β was originally shown with a similar protein.[24] However, we also tested an N-tagged variant of BilRI, which did not show increased binding to IL-1β, IL-8, or IL-6 compared with the C-

tagged protein (data not shown). BilRI did not bind to fibrinogen- (Fig. 2B) or to collagen (Fig. 2C)-coated wells. Moreover, BilRI binding to IL-8 was weaker than the fibrinogen-binding of positive control protein clumping factor A (ClfA) of *S. aureus* and the collagen-binding of positive control protein YadA of *Yersinia enterocolitica* (Fig. 2C).

Viable biofilm of wild-type A. actinomycetemcomitans bound IL-8 and IL-6

When wild-type *A. actinomycetemcomitans* biofilm was co-cultured together with an organotypic gingival mucosa in the absence of antibiotics, the biofilm sequestered both IL-8 and IL-6 (Fig. 3A). However, when the co-culture was performed in the presence of antibiotics, which decreased the viability of the biofilm,[28] the immunohistological staining of the biofilm with anti-IL-8 and anti-IL-6 was faint (Fig. 3A). However, the epithelium contained more cytokines in the presence than in the absence of antibiotics (Fig. 3A). In addition, the growth medium contained slightly elevated amounts of IL-6 and IL-8 (Fig. 3B) when antibiotics were used in the biofilm-gingival tissue co-culture, suggesting that the cytokines leaked from the system when not sequestered by the viable biofilm. However, due to inter-sample variance, the difference was not statistically significant. In similar organotypic gingival tissue – biofilm co-cultures with a slightly thinner keratinocyte layer [28] *A. actinomycetemcomitans* cells efficiently internalized IL-1β (Fig. 3C).

Deletion of stand-alone gene bilRI altered the biofilm matrix composition in rich medium

The prokaryotic operon database (ProOpDB, http://operons.ibt.unam.mx/OperonPredictor) [30] predicted that *bilRI* is a stand-alone gene. When cultured on blood agar plates, the single-gene-deletion mutant of *bilRI* produced typical colonies with a rough colony morphology (Fig. 4A). Although the *bilRI*$^-$ mutant colonies were slightly more adherent to the agar than the wild-type colonies, cell suspensions [31] could be produced similarly from both strains (Fig. 4B). However, BilRI overexpression resulted in a tiny colony size, and only small amounts of bacteria could be harvested from the plates. Nonetheless, an even cell suspension could be attained (Fig. 4B). The *bilRI*$^-$ mutant formed as much biofilm as the wild-type strain in rich medium, whereas the overexpression of BilRI in *A. actinomycetemcomitans* almost completely disappeared the cell's capacity to form biofilm ($p=0.0003$, paired-samples T-test with Bonferroni corrections) (Fig. 4C). In biofilm, the cell

morphology of *bilRI*⁻ mutants did not differ from the morphology of the wild-type strain (Fig. 4D). BilRI overexpression appeared to cause outer membrane lysis (Fig. 4D), explaining the tiny colonies (Fig. 4A) and small cell size (Fig. 4D). In rich medium, the young biofilm, *i.e.*, the biofilm that had not started to detach by releasing cells into the medium,[32] of the *bilRI*⁻ mutant strain contained more total protein in proportion to the biofilm mass than the wild-type *A. actinomycetemcomitans* strain ($p=0.009$; Mann-Whitney U-test) (Fig. 5A). In contrast, the *bilRI*⁻ mutant biofilm contained less eDNA than the wild-type strain ($p=0.021$; Mann-Whitney U-test) (Fig. 5B). Because some outer membrane proteins of *A. actinomycetemcomitans* or close relative species bind to host proteins, such as collagen and fibrinogen, we studied the binding of the *bilRI*⁻ mutant to these host proteins. *bilRI* deletion did not decrease the binding of *A. actinomycetemcomitans* to either fibrinogen- or collagen-coated wells (Fig. 5C). In contrast, the *bilRI*⁻ mutant bound collagen slightly more efficiently than the corresponding wild-type strain, but the difference was not statistically significant ($p=0.275$; Mann-Whitney U-test, Fig. 5C).

BilRI played a role in IL-1β internalization

Because the viable biofilm of wild-type *A. actinomycetemcomitans* bound both IL-8 and IL-6, the uptake of these cytokines and the role of BilRI in their uptake were studied by incubating *A. actinomycetemcomitans* wild-type and *bilRI*⁻ biofilms with gingival keratinocyte monolayers. Previously reported IL-1β uptake [28] was used as a positive control. The wild-type *A. actinomycetemcomitans* biofilm cells internalized IL-8, IL-6, and IL-1β (Fig. 6A) in these conditions. When *bilRI* was deleted from the *A. actinomycetemcomitans* genome, the amount of IL-1β inside and attached to the biofilm cells, which were co-cultured with human gingival epithelial cells, was significantly lower than for corresponding wild-type cells ($p=0.007$; Mann-Whitney U-test, Figs. 6A and 6B). However, the *bilRI*⁻ mutant cells did not differ from the wild-type cells in their IL-8 and IL-6 uptake efficiencies ($p=0.649$ and $p=0.128$, respectively; Mann-Whitney U-test, Fig. 6A and B).

Deletion of bilRI abolished biofilm response to IL-1β and IL-8

When exposed to low concentrations of IL-1β and IL-8, the matrix composition of the wild-type biofilm changed, *i.e.*, the amount of eDNA decreased, whereas the amount of PGA, total protein and

183 total biofilm mass remained unchanged (Table 2). The deletion of *bilRI* rendered the biofilm

184 unresponsive to IL-1β and IL-8, as determined by measuring the composition of the biofilm (Table 2).

Discussion

Although IL-1β was the cytokine that was originally used in the identification of BilRI, it was only moderately bound by this bacterial protein compared with the other tested cytokines. Our novel finding that BilRI is an IDP could explain the existence of multiple ligands. The results of the nuclear magnetic resonance (NMR) studies, which indicated the absence of a specific fold, were supported by the amino acid analysis showing high numbers of charged and polar residues and a low number of hydrophobic bulky amino acids, a composition that is typical for IDPs.[1] In addition, the BilRI sequence had low complexity: it lacks all aromatic amino acids, such as phenylalanine, tyrosine and tryptophan, and 48% of the sequence is made up of three residues: alanine, lysine and aspartate. The unadorned peptides of IDPs are often involved in molecular interactions, in which they may bind the ligand with variable affinities. Thus, an IDP can function as a scavenger/effector protein if it has a strong affinity or as a chaperone/recognition motif if it has a weak affinity for its ligands.[33] Our results confirmed that BilRI had relatively weak affinity for the cytokines, suggesting that it might function as a cytokine concentrator in the outer membrane of *A. actinomycetemcomitans* binding cytokines only temporarily before sending them forward to the next binding motif in the internalization chain. This hypothesis was supported by the observation that the deletion of *bilRI* did not completely inhibit the internalization of IL-1β but significantly decreased the uptake efficacy.

Various molecules released by the *A. actinomycetemcomitans* biofilm are known to induce IL-8 and IL-6 production in human whole blood.[34] Because IL-8 showed the highest affinity to BilRI, it was selected for further studies to investigate whether it affects the composition of *A. actinomycetemcomitans* biofilm and is internalized by the biofilm cells in a BilRI-dependent or BilRI-independent manner. *A. actinomycetemcomitans* responded to BilRI in a manner dependent on low concentrations (10 ng/ml) of IL-1β and IL-8 by decreasing the eDNA amount in biofilm. This was the only observed change in the biofilm composition because the cytokines did not alter either the PGA or total protein amounts. Moreover, the total biofilm mass did not change in response to the cytokines. In general, eDNA is suggested to play an important role in the early stages of biofilm development, enhancing adhesion to the surface and stabilizing the young biofilm (for a review, see ref. [35]).

Although eDNA protects at least young biofilms from antimicrobial agents,[36] host defence factors,[37] and mechanical stress,[38] it may also compromise the bacterial viability by acting as a pathogen-associated molecular pattern (PAMP) [39] and boosting the innate immune defence. The observed decrease in the amount of eDNA in response to IL-1β and IL-8 could impede immune defence by reducing the amount of potential PAMPs.

In a gingival epithelial cell co-culture model, the IL-8 and IL-6 uptake efficiencies were not affected by *bilRI* deletion. This observation was expected in the case of IL-6, which did not bind to BilRI *in vitro*. The BilRI-independent uptake of IL-8 might be explained by the high concentration of IL-8 in the system. For example, our organotypic gingival tissue culture system produces approximately 200 ng IL-8 in 24 h compared with 200 pg of IL-1β [28] during the same time period. The *A. actinomycetemcomitans* biofilm virtually bathes in IL-8, which may allow efficient IL-8 uptake without a cell surface concentrator. In our test systems, the IL-8 concentration always exceeded that of IL-6. In the *in vivo* environment of periodontitis-associated biofilm, a similar surplus of IL-8 is observed with approximately one hundred times more IL-8 than IL-6 in gingival crevicular fluid.[40]

The deletion of *bilRI* exerted only minor effects on the phenotype of *A. actinomycetemcomitans*, which were mainly observed as a change in the composition of the biofilm matrix. However, the overexpression of BilRI caused lysis of the outer membrane. In addition, our previous study showed that *E. coli* cells are more prone to cell lysis when expressing BilRI under a strong promoter.[24] Due to the vulnerability of the outer membrane, the expression of outer membrane proteins of Gram-negative bacteria needs to be precisely regulated.[41] We decided to use a constitutively expressed strong *ltxP* promoter instead of the endogenous *bilRI* promoter, which may be more strictly regulated, to ascertain efficient complementation. Moreover, we were interested in investigating how the overproduction of BilRI affects the phenotype. BilRI was not involved in binding collagen and fibrinogen, although the wild-type *A. actinomycetemcomitans* cells clearly bound the proteins. Both experiments with the *bilRI*⁻ mutant and purified BilRI showed similar results. Our findings are partly contradictory to those obtained in previous study conducted by Bauer and co-workers,[42] which showed that a similar protein of *H. ducreyi*, which was named fibrinogen-binding protein A (FgbA), interacts with human fibrinogen. The incapability of C-tagged BilRI to interact with fibrinogen cannot be explained by the

location of the histidine tag because N-tagged BilRI showed similar results (data not shown) and the control protein FgbA, which was N-tagged, could not bind fibrinogen. More recent studies have confirmed that another protein, *i.e.*, ducreyi serum resistance A (DsrA), a trimeric autotransporter, is, in fact, the main binder of fibrinogen in *H. ducreyi* and that FgbA does not play a central role in fibrinogen binding.[43] Our results are in line with those obtained in the more recent later study because we also found that the slightly truncated form of FgbA, which can be found in some strains of *H. ducreyi*, does not bind to fibrinogen. However, FgbA undoubtedly promotes *H. ducreyi* virulence; thus, the major functions of FgbA and similar proteins, such as BilRI, are worth studying.

The differential affinity and capacity to uptake various cytokines may provide the pathogen with the means to modulate the host inflammatory response and the cytokine balance. In healthy periodontal tissue, IL-8 forms a concentration gradient with higher concentrations in the coronal parts of the junctional epithelium, near the bacterial biofilm.[7] During acute inflammation, neutrophils are the first innate immune cells to enter the site. However, their activity, *i.e.*, the release of reactive oxygen species and proteases, causes severe tissue damage if not limited by regulative actions. IL-6 signalling is known to suppress chemokines, such as IL-8, which attract neutrophils and directly causes neutrophil apoptosis.[44] The immune system redirects from innate to acquired immunity by replacing the neutrophils with monocytes and T cells. IL-6 is involved in this process by inducing the production of chemokines that attract monocytes (for a review, see ref. [45]), augmenting monocyte differentiation into macrophages,[46] recruiting T cells [47] and impeding their apoptosis [48]. Periodontitis is characterized by progressive bone loss in tooth supportive tissues, which is associated with a high receptor activator of nuclear factor κ-B (RANK) ligand (RANKL) / osteoprotegerin (OPG) ratio, *i.e.*, RANKL causes bone destruction by binding RANK, which leads to the induction of osteoclast production.[49] However, OPG can inhibit osteoclastogenesis by sequestering RANKL.[50] Various cytokines, such as IL-1β, IL-6, IL-11, IL-17 and TNF-α, increase the expression of RANKL over OPG (for a review, see ref. [49]). IL-6 activates osteoclastogenesis together with soluble IL-6 receptor (sIL-6R) [51] by provoking RANKL expression [52]. Thus, high IL-6 concentration in the inflammatory milieu resolves the acute inflammation reaction that is detrimental to the host tissue by enhancing the clearance of neutrophils and moves the balance to acquired immunity by increasing the recruitment of

monocytes and T cells. [44-48] However, bone homeostasis is skewed in the direction of osteoclastogenesis and bone degradation due to the increased RANKL/OPG ratio.[52] By decreasing local IL-6 amounts in inflammation, *A. actinomycetemcomitans* could decelerate the clearance of acute inflammation and could extend the time of the neutrophil-skewed immune reaction.

In conclusion, the role of intrinsically disordered BilRI is most likely to concentrate small proteins, such as different host cytokines, on the surface of *A. actinomycetemcomitans*, which facilitates the efficient uptake of cytokines using as yet unknown machinery. The affinity of BilRI to the cytokines is relatively weak when compared with, for example, the binding of *Y. enterocolitica* YadA to collagen. The weak affinity is most likely needed for the proficient transfer of the cytokine to the next binding protein in the chain of internalization. Because periodontitis is an inflammatory disease caused by multispecies biofilm, cytokine binding and uptake might not benefit only *A. actinomycetemcomitans*. By binding and internalizing cytokines, *A. actinomycetemcomitans* could help other species in a periodontal biofilm to persist in an inflammatory environment.[53] The uptake of cytokines by opportunistic pathogens may disturb the balance of cytokines with low local concentrations, whereas the effect on cytokines with high local concentrations, such as IL-8, might be only marginal. Moreover, in low cytokine concentrations, the role of BilRI, a potential cytokine concentrator, might be emphasized in facilitating the uptake of cytokines at the surface of *A. actinomycetemcomitans*.

Materials and methods

Cloning and expression of recombinant proteins: BilRI, FgbA, ClfA, IL-8, YadA

To study the interaction of BilRI with various cytokines, the *bilRI* gene was cloned into the pET36b expression vector, which inserts an 8-histidine long tag into the C-terminal end (Novagen, Darmstadt, Germany) using the forward primer 5'-ATT CATATG GATGACAGCAAAACTTCACC-3' and the reverse primer 5'- ATA CTCGAG TTTGCTTTCAGTTTCGC-3' during PCR. The underlined sequences are the NdeI and XhoI restriction sites, respectively. The *bilRI* gene was amplified from a previously produced expression vector,[24] which contained the gene from D7S. The plasmid was transformed into bacterial cells from the BL21 CodonPlus (DE3)-RIL *E. coli* protein expression strain (Stratagene, San Diego, CA, USA).

The recombinant BilRI containing amino acids 21-181 was expressed in Terrific broth medium (12 g/L tryptone, 24 g/L yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) containing 30 µg/mL kanamycin. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the OD_{600nm} was 1.2. Cells were grown for 3 h under induction, after which they were harvested by centrifugation (6 400×g, 10 min, 4°C), and cell pellets were stored at -20°C.

To purify the intracellular recombinant protein, 8-10 g of cells were defrosted and suspended to 30 mL in binding buffer (20 mM NaH_2PO_4/Na_2HPO_4, 800 mM NaCl, 20 mM imidazole, pH 7.5) including DNAse I (Roche, Mannheim, Germany), 5 mM $MgCl_2$ and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor. Cells were sonicated 5x15 s with a 100 Watt MSE ultrasonic disintegrator. Cell debris was removed by centrifugation (48 000×g, 25 min, 4°C), and the clarified supernatant containing the recombinant BilRI protein was loaded in a balanced 5-mL HisTrap HP (GE Healthcare, Uppsala, Sweden) column. The unbound material was washed out with 5% elution buffer (20 mM NaH_2PO_4/Na_2HPO_4, 800 mM NaCl, 500 mM Imidazole, pH 7.5), and the His-tagged BilRI was eluted with 50% elution buffer. The eluate was loaded into a size-exclusion chromatography Superdex 200 26/60 (GE Healthcare) column in PBS_1 (2.7 mM KCl, 1.8 mM KH_2PO_4, 140 mM NaCl, and 10 mM Na_2HPO_4, pH 7.4). The recombinant BilRI does not include any

tryptophans or any other aromatic residues; therefore, it is nonvisible at 280 nm. However, the protein could be detected in fractions based on both A_{220nm} readings and Bio-Safe™ Coomassie (Bio-Rad, Hercules, CA, USA)-stained SDS-PAGE (Thermo Fisher Scientific Precise™ 4-20% Tris-Glycine Gels). According to the analysis, BilRI-containing protein fractions were concentrated using an Amicon Ultra-15 Centrifugal Filter Unit with an Ultracel-10 membrane (Millipore, Billerica, MA, USA), and the protein amount in the final concentrate was determined using the method by Lowry et al.[54] Protein purity was verified with SDS-PAGE, and the homogeneity was determined by native PAGE (PhastGel Gradient, 8-25, GE Healthcare).

Synthetic DNA with optimized codon usage for *E. coli* expression was ordered from Eurofins Genomics for FgbA, including the gene for residues 20-105 from *Haemophilus ducreyi* HMC112, the fibrinogen-binding segment of *Staphylococcus aureus* strain NCTC 8325 ClfA, the gene fragment for residues 230-542 and the cDNA of the coding residues 23-99 of human IL-8. N-terminal NdeI and C-terminal XhoI restriction sites were added for all three synthetic genes. DNA fragments were ligated into the pET15b plasmid (Novagen, Darmstadt, Germany), and DNA sequences were verified with sequencing.

FgbA was expressed, purified and identified by the same method as BilRI because it lacks all aromatic residues. ClfA was expressed and purified similarly to FgbA, except the binding and elution buffers contained 300 mM NaCl. The ClfA concentration was measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) using an $A_{280}^{0.1\%}$ of 0.999.

IL-8 was purified as a mature protein. The N-terminal His-tag was cut by digesting in a HisTrap column with 200 NIH units of thrombin (MP Biomedicals, Santa Ana, CA, USA) at RT overnight. Digested IL-8 was eluted with binding buffer, and the protein was purified from other proteins by size-exclusion chromatography. After concentration with an Amicon Ultra-15 Centrifugal Filter Unit with an Ultracel-10 membrane (Millipore), the protein concentration was determined with $A_{280\ nm}$ using an $A_{280}^{0.1\%}$ of 0.863. The IL-8 molecular mass was verified with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) (Bruker). Analysis yielded a mass of 8381.99 Da (+H form) (+/-1 Da) for IL-8, whereas the theoretical mass with two cysteines

was 8381.67 Da, indicating that the recombinant IL-8 had 72 amino acids, suggesting that the endogenous thrombin site of IL-8 was exposed.

The plasmid pHN-1 and the *E. coli* M15(pREP4) strain (Qiagen, Hilden, Germany) for the collagen-binding fragment of *Yersinia enterocolitica* adhesin (YadA) expression were kind gifts from Professor Mikael Skurnik (University of Helsinki, Finland). YadA expression and purification were performed as published by Nummelin et al.[55] except that the size-exclusion chromatography buffer was PBS_1. All proteins were deep-frozen with liquid nitrogen and stored at -85°C. All recombinant protein preparations has high purity, as observed in the Coomassie-stained 4-20% Tris-glycine SDS-PAGE gel (Fig. 7).

NMR spectroscopy studies of BilRI structure

NMR spectra were collected at 298 K using either Varian INOVA 600 MHz or INOVA 800 MHz NMR spectrometers (Agilent, Santa Clara, CA, USA), both equipped with cryogenically cooled ^{1}H, ^{13}C, ^{15}N triple-resonance probeheads with z-gradient coils. For ^{1}H NMR spectra, measured at 600 MHz, the recombinant BilRI was diluted in 95%/5% H_2O/D_2O, 50 mM NaCl, pH 7 buffer in a Shigemi microcell (250 μL). The final BilRI concentration was 4.6 mM. The ^{1}H spectrum was sampled with 20,438 complex points using 64 transients per free induction decay (FID), resulting in an acquisition time of 500 ms in the ^{1}H dimension. The two-dimensional ^{1}H-^{15}N HSQC spectrum of BilRI at pH 5 was measured at the 800 MHz ^{1}H frequency using 128 and 852 complex points in ^{15}N and ^{1}H dimensions, corresponding to acquisition times of 49 ms and 85.2 ms, respectively. A total of 256 transients per FID were used to assure sufficient signal accumulation. The total experimental time was 18 h. Spectra were processed with VnmrJ (Agilent, Santa Clara, CA, USA) and analysed with Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco, CA, USA) software packages.

Cytokine-binding assay for recombinant BilRI

Because BilRI produced unwanted spontaneous dimers when the cysteine at position 20 was included in the recombinant protein, the construct that was used in the cytokine-binding assays contains neither

366 the signal sequence (the first 19 amino acids) nor the C20. Moreover, the recombinant BilRI
367 contained an eight-histidine long tail in the C-terminus to allow detection with His-Probe (Thermo
368 Fisher Scientific).
369 A total of 100 ng of each cytokine (IL-1β/IL-6/IL-8/IL-10/tumour necrosis factor [TNF]-α/interferon
370 [INF]-γ/transforming growth factor [TGF]-β1) diluted in PBSN buffer (0.05% sodium azide in PBS_1)
371 was incubated in a Nunc MaxiSorp 96-well plate (Affymetrix, Santa Clara, CA, USA) at RT
372 overnight. Wells were washed three times with ion-exchanged water, after which the wells were
373 blocked with blocking buffer (0.25% BSA, 0.02% sodium azide in PBS-T) at 37°C for 3 h. The wells
374 were again washed as above, and 400 ng of C-His-tagged $BilRI_{21-181}$ was added to the wells and
375 incubated at 4°C overnight. The wells were washed four times with PBS-T using Delfia Platewash
376 (Perkin Elmer, Turku, Finland). His-Probe-HRP™ (Thermo Fisher Scientific) was diluted to 1:5000,
377 and 50 µl of the dilution was incubated in the wells at RT for 15 min. The wells were washed again
378 four times with PBS-T as described above, and detection was performed with 2,2′-azino-bis(3-
379 ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich) in citrate buffer (10 mM
380 sodium citrate and 0.03% H_2O_2, pH 4.2). The A_{414nm} value was read using a Multiscan Go plate reader
381 (Thermo Fisher Scientific).

382 ***Collagen- and fibrinogen-binding assay for recombinant BilRI (EuLISA)***

383 The binding of BilRI to type V collagen and fibrinogen was determined in a microplate assay
384 modified from the method described by Yu et al. [56] Type V collagen from human plasma (Sigma-
385 Aldrich) was dissolved in 0.5 M acetic acid, and fibrinogen from human placenta (Sigma-Aldrich)
386 was dissolved in 0.85% NaCl at 37°C with gentle mixing for 5 h and filtered through a 0.2-µm
387 syringe filter. Collagen and fibrinogen were diluted in PBS_1, and a total of 1 µg of each was incubated
388 in the wells of a Nunc MaxiSorp 96-well plate (Affymetrix) at 4°C overnight. Equal amounts of BSA
389 (Sigma-Aldrich) and IL-8 (production described above) were used as negative and positive controls,
390 respectively.
391 Unbound proteins were removed by washing once with PBS_1 using a Delfia Platewash (Perkin
392 Elmer). The wells were blocked with 200 µg of BSA in PBS_1 at RT for 1-2 h and washed as above. A

total of 1 μg of C-His-tagged $BilRI_{21-181}$ was diluted in Delfia Assay Buffer (Perkin Elmer) and incubated in wells at RT for 1 h. YadA (0.8 μg), FgbA (1.6 μg) and ClfA (0.5 μg) were used as positive collagen or fibrinogen binders. The production of these proteins is described above. The wells were washed 3 times with PBS_1 using Delfia Platewash (Perkin Elmer). Then, 25 ng of DELFIA® Eu-N1 Anti-6xHis antibody (Perkin Elmer) in 50 μL of Delfia Assay Buffer was incubated in wells at RT for 1 h. The wells were washed as in the previous step. Detection was performed measuring time-resolved fluorescence using a Victor^3 multilabel plate reader (Perkin Elmer) after a 5-min incubation in DELFIA® Enhancement Solution (Perkin Elmer).

Binding of IL-8 and IL-6 by the viable A. actinomycetemcomitans biofilm

A. actinomycetemcomitans biofilms were co-cultured in a gingival mucosa model as described by Paino et al.[28] In brief, in the model, human gingival fibroblasts (HGFs) [57] and spontaneously immortalized human gingival keratinocytes (HGKs) [58] were cultured at an air-liquid interphase to obtain the three-dimensional tissue organization. First, HGFs (passages 13-18) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Paisley, UK) were suspended in collagen solution (PureCol®, Advance Biomatrix, AZ, USA), and an aliquot containing 1.5×10^5 fibroblasts was transferred to cell culture inserts and grown for 1 day submerged in Green's medium.[59] To obtain the epithelial layer in the top layer of the connective tissue, 4×10^5 HGKs (passage 18-22) were added on top of the fibroblast-collagen layer. The epithelial cells were cultured submerged for 1 day, and the tissue model was then lifted in the air-liquid interface and allowed to mature for 5 days, after which the separately grown *A. actinomycetemcomitans* biofilm was added on top of the tissue culture model. The biofilms were co-cultured with the tissue models for 24 h. Culture medium was collected before and after the 24 h co-culture and stored at -80°C. In half of the cultures, penicillin (63.4 IU/ml) and streptomycin (63.4 μg/ml) were used in culture media to decrease biofilm viability. The co-cultures were fixed with 10% formalin solution overnight, and the sectioning of paraffin-embedded samples was performed using standard histological techniques.

Before staining with anti-IL-8 and anti-IL-6 antibodies, the specimens were mechanically deparaffinized, and heat-mediated antigen retrieval was conducted in 10 mM citrate buffer (pH 6.0)

with microwaving. The staining was performed with Dako TechMate™ 500 Plus Autostainer (Dako, Glostrup, Denmark) using 20 μg/mL of primary polyclonal rabbit IgG against IL-8 (NBP2-16958; Novus Biologicals, Cambridge, UK) and IL-6 (NBP2-16957; Novus Biologicals) and a Dako REAL™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Code K5001; Dako) as instructed by the producer. The histological samples were imagined with Leica DM RXA light microscope using Leica HC PL APO 20x / 0.70 objective.

The culture media samples collected prior to the co-culture indicating the basal level of cytokine expression, along with the samples collected after 24 h co-culture, were analysed with IL-8- and IL-6-specific enzyme-linked immunosorbent assay (ELISA) kits (SABiosciences, Qiagen, Germantown, MD, USA). Because the volume of the medium varied slightly between different experiments, the amount of cytokine that was excreted into the medium was calculated as a total amount (ng) leaked into the culture medium in 24 h.

Prediction of the size of the mRNA expressing BilRI

The Prokaryotic Operon Database (ProOpDB, http://operons.ibt.unam.mx/OperonPredictor) [30] was used to predict whether *bilRI* is a stand-alone gene or belongs to an operon. Because *A. actinomycetemcomitans* strain D7S was not deposited into the database, *A. actinomycetemcomitans* strain D11S was used. The hypothetical protein D11S_0933 of *A. actinomycetemcomitans* D11S-1 (GenBank accession number CP001733.1) has an identical amino acid sequence to the BilRI protein of *A. actinomycetemcomitans* D7S. In addition, the genes surrounding the gene encoding BilRI are similar in both strains. Downstream of *bilRI* is a gene encoding septum site-determining protein MinC, whereas genes encoding the SixA phosphohistidine phosphatase, phosphoglucosamine mutase and dihydropteroate synthase are found upstream of *bilRI*.

Markerless bilRI-deletion mutant

A single-gene-deletion mutant of *bilRI* was produced from *A. actinomycetemcomitans* strain D7S.[60] The strain was recovered from -80°C frozen storage cultures by culturing on modified tryptone soy agar (TSA) plates consisting of 3 % tryptone soy broth (TSB, Lab-M, Lancashire, UK), 0.3 % yeast extract (Lab-M), 1.5 % agar and 5 % heat-inactivated horse serum (HyClone, SH30074.03, Thermo

Fisher Scientific) in a candle jar at 37°C for 2.5 days. In addition, two types of TSB media were used. TSB_1 contained 3 % TSB and 0.6 % yeast extract. TSB_2 was additionally supplemented with 0.8% separately autoclaved glucose. Whenever necessary, the cultures were supplemented with the appropriate antibiotics: either 50 µg/mL spectinomycin or 6 µg/mL tetracycline.

The plasmids used for mutant generation were generous gifts from Professor Casey Chen (University of Southern California, Los Angeles, CA, USA). The pLox2-Spe plasmid contained a *spe* cassette flanked by *loxP* sites.[61] The pAT-Cre plasmid contained the *cre* recombinase and *tet(O)* genes.[62, 63] The plasmids were amplified in *Escherichia coli* strain TOP10 (Invitrogen).

The gene encoding BilRI (NC_017846.1 AaD7S_02241) was deleted using the Cre-*loxP* mediated recombination method optimized for *A. actinomycetemcomitans.*[61, 62] The primer sequences used for PCR product generation in the target-gene-deletion mutant are listed in Table 1. First, a 2960-bp PCR product containing the *bilRI* gene was amplified from the genome of *A. actinomycetemcomitans* D7S using bilRI_nest primers. The PCR product was then used to generate two PCR fragments flanking the *bilRI* gene in both the downstream and upstream directions. The primer pair ycgL_FD/bilRI_RD_BamHI was used to amplify the downstream region, and primer pair phoGlu-R/sixA_FD_SalI was used for the upstream region. The PCR fragments and pLox2-spe-plasmid were digested with BamHI and/or SalI restriction enzymes (FastDigest restriction enzymes, Thermo Fisher Scientific). After fragment isolation, the ligation was completed by incubating the amplicons and the *spe*-cassette fragment (130 ng each) in the presence of T4 DNA ligase (Thermo Fisher Scientific). The natural transformation was performed according to a previously described method.[60, 64] In brief, suspensions of plate-grown *A. actinomycetemcomitans* cells were prepared in TSB_1, and the bacterial cell number was estimated according to the method described by Karched et al.[31] Then, 2×10^7 colony-forming units (CFUs) were plated on TSA-plates, and the cells were grown in a candle jar at 37°C for 2 h after mixing the recipient cells with the ligation mix (250 ng of DNA). After culturing for 5 h, the cells were scraped off the plate, resuspended in 150 µL of TSB_1 and plated on a spectinomycin-supplemented TSA plate. Colony PCR was used to confirm the presence of a deletion in the *bilRI* gene site in the *A. actinomycetemcomitans* D7S genome. Using this method, a loopful of bacteria was

suspended in lysis buffer (20 µg/mL proteinase K, 2.5% glycerol in 10 mM Tris-HCl, pH 8.0). Twenty microliters of the resulting suspension were added to a PCR reaction using bilRI_nest-primers, and the correct 3550-bp PCR product was detected. The pAT-Cre plasmid was then transformed into electrocompetent primary *bilRI*-deletion mutant cells by electroporation (5 ms, 1250 V) using a BTV ECM399 electroporation apparatus (BTX Instrument Division, Harvard Apparatus, Inc., Holliston, MA, USA) to remove the *spe*-cassette. After culturing the cells in TSB_2 for 2 h, the cells were plated on TSA-plates supplemented with tetracycline and grown for a few days until visible colonies were formed. The selected colonies were further plated onto TSA-plates with no antibiotics and with tetracycline and spectinomycin and then grown for 4 days. Colonies sensitive to both antibiotics were considered potential markerless *bilRI* mutants. Colony PCR using minc_F_1 (5'-CGCGCTATCAACCGACTAAA-3') and SixA_R_2 primers (5'-TTTATCTCGGTGATGAGCGC-3') was used to select products of the correct size (2100 bp), and the products were further verified by sequencing the flanking regions of the *bilRI* gene in both directions by Eurofins Genomics (Ebersberg, Germany). Moreover, the absence of *bilRI* in the *bilRI*$^-$ mutant was verified by PCR using genomic DNA as the template and primers [24] that amplify the whole *bilRI* gene, including the signal sequence.

Restoration of BilRI expression in the bilRI-deletion mutant

Because we did not succeed in restoring the *bilRI* gene to the markerless *bilRI* deletion mutant despite many attempts, we decided to restore BilRI expression using an *A. actinomycetemcomitans*/*E. coli* shuttle plasmid under a constitutively expressed leucotoxin promoter (*ltxP*). The *bilRI* gene was amplified from *A. actinomycetemcomitans* strain D7S by PCR using the 5'-ATACTCGAGTTTAGGAGTAACGATG-3' forward primer and the 5'-TTTCTGCAGTTATTTGCTTTCAGTT-3' reverse primer, which contained XhoI and PstI restriction sites, respectively. The *bilRI* gene was inserted into *ltxP* from the pVT1296 plasmid [65] by ligating the *bilRI* PCR product into XhoI- and PstI-digested pVT1296. The final *ltxP-bilRI* construct was moved to the pPK1-based [66] pVT1503 plasmid [67] by cutting the pVT1296-based construct with PstI, blunting the ends with Klenow, cutting *ltxP-bilRI* from the plasmid with KpnI, and ligating *ltxP-bilRI* to KpnI-

and EcoRV-digested pVT1503 (KanR). The correct insert size was confirmed through KpnI-EcoRI double digestion of the final expression plasmid pVT1503-*ltxP-bilRI*. The *bilRI*-containing product of the KpnI-EcoRI-digested expression plasmid pVT1503-*ltxP-bilRI* was ligated into the KpnI-EcoRI-digested pUC19 plasmid (New England Biolabs, Ipswich, MA, USA) and sequenced (Eurofins Genomics). The expression plasmid pVT1503-*ltxP-bilRI* was then transformed into a markerless *bilRI*-deletion mutant through natural transformation as described above. This time, 300 ng of DNA was mixed with cells and supplemented with 1 mM $CaCl_2$ to improve the transformation efficiency.[68] After incubation, the transformants were screened on TSA plates supplemented with 30 μg/mL kanamycin to select a BilRI-overexpressing variant containing the pVT1503-based expression plasmid.

Effect of BilRI on biofilm formation

A. actinomycetemcomitans D7S wild-type, *bilRI*$^-$ mutant and BilRI-overexpressing strains were compared to determine the effect of BilRI on biofilm formation, which was measured through crystal violet staining.[32] Briefly, *A. actinomycetemcomitans* D7S wild-type and *bilRI*$^-$ strains were grown on TSA-blood-plates (37 g/L TSA [Lab-M], 3 g/L agar, 5% defibrinated sheep blood) in a candle jar at 37°C for three days. A uniform cell suspension was prepared from plate-grown bacteria in TSB_2 medium, and the cell density was determined by measuring the optical density.[31] The cell suspension was added to the wells of a 48-well microtitre plate such that each well contained $2.5x10^7$ CFUs in a total volume of 0.5 mL. Seven replicates of each strain were prepared. The plate was incubated in a candle jar at 37°C overnight. The medium was removed with suction, 200 μL of Gram-staining reagent (20 mg/mL crystal violet, 8 mg/mL ammonium oxalate, and 20% ethanol) was added to each well, and the samples were incubated at RT for 10 min. The Gram stain was removed with suction, and the wells were washed seven times with ultrapure water. After 200 μL of 95% ethanol was added to the wells, the plates were incubated at RT for 10 min. The amount of released stain was measured by transferring 100 μL of liquid from each well to a 96-well microtitre plate, and the A_{620nm} value was measured using a Multiscan Go plate reader (Thermo Fisher Scientific).

Effect of BilRI on biofilm composition

Because the *bilRI*⁻ mutant formed similar amounts of biofilm as the wild-type *A. actinomycetemcomitans* strain, we further analysed the biofilm composition of the wild-type and *bilRI*⁻ mutant strains. However, because the *bilRI*⁻ mutant in which BilRI expression was restored with a plasmid loses its viability when expressing elevated amounts of the outer membrane protein BilRI (Figs. 4A-4D), its biofilm composition could not be studied. Biofilm cultures were generated as described above with the exception that the biofilms were grown in 50-mL cell culture bottles (Cellstar #690160, Greiner Bio-One, Frickenhausen, Germany) by adding 2.5×10^8 CFUs in a total volume of 5 mL of TSB_2 medium. The biofilms were grown in a candle jar at 37°C overnight. Samples were collected from the culture medium and cultured on blood agar plates to ensure that the biofilms were not contaminated. The TSB_2 medium was removed, the biofilms were washed three times with 10 mL of PBS_1, and the biofilm was scraped into 3 mL of PBS_1 with an inoculation loop. The samples were divided into three 1-mL aliquots, the biofilm mass of centrifuged (12,000×g, 15 min) pellets from each sample was weighed, and the amounts of total protein and eDNA were estimated using the methods described below.

For the total protein measurement, the pre-weighed biofilm pellets were suspended in 200 µL of ultrapure water with mild sonication (2×5 s, 5-µm amplitude, 100-Watt MSE ultrasonic disintegrator), and the volume was then doubled by adding sodium dodecyl sulphate (SDS) to a final concentration of 2% in 0.5× PBS_1. The samples were boiled for 10 min, the insoluble fraction was separated through a short centrifugation, and the total protein amount in the supernatant was measured using the method described by Lowry et al.[54]

For eDNA extraction,[69] the pre-weighed biofilm pellet was suspended in 0.9% NaCl to obtain 9 mg/mL, and the suspension was homogenized using mild sonication, as described above. The suspension was supplemented with 1× Glyko Buffer 2 (New England Biolabs) and 250 units of PNGase F (New England Biolabs). After the mixture was incubated at 37°C for 30 min, proteinase K (Thermo Fisher Scientific) was added to a final concentration of 5 µg/mL, the samples were incubated at 37°C for 30 min. The samples were filtered through a 0.2-µm polyethersulfone (PES) membrane (VWR) before the amount of eDNA was determined with propidium iodide, as described by Rose et

555 al.[70] Briefly, 25 μL of biofilm extract was mixed with an equal volume of 6 μM propidium iodide in a
556 white 96-well plate (Thermo Fisher Scientific). The plate was incubated in the dark at RT for 15 min
557 before the fluorescence was read using a Hidex Sense Microplate reader (Hidex, Turku, Finland) with
558 a 535-nm excitation filter and a 620-nm emission filter.

559 ***Binding of bilRI mutant cells on collagen and fibrinogen***

560 The binding of *A. actinomycetemcomitans* to type V collagen and fibrinogen was determined using a
561 microplate assay modified from the methods described by Yu et al [56] and Tang and Mintz[71]. Collagen
562 and fibrinogen solutions were prepared as in the collagen- and fibrinogen-binding assay for
563 recombinant BilRI. A total of 1 μg of collagen in sodium bicarbonate buffer (16 mM sodium
564 carbonate, 34 mM sodium bicarbonate, and 0.02% sodium azide, pH 9.6) or 25 ng of fibrinogen in
565 $PBSN_1$ (0.05% sodium azide in PBS_1) was added to the wells of a Nunc MaxiSorp 96-well plate
566 (Affymetrix). The plate was coated at 4°C overnight. Liquid was removed from the wells by
567 decanting, and the wells were washed four times with ion-exchanged water. The wells were blocked
568 with 1 mg of BSA in PBS_1 at RT for 2 h, and the wells were then washed as described above. Wild-
569 type *A. actinomycetemcomitans* and the *bilRI* mutant were collected from TSA-blood plates, a
570 uniform bacterial suspension in PBS_1 was prepared, and the number of bacterial cells was estimated as
571 described above. One hundred microliters of bacterial suspension ($1.25x10^6$, $2.5x10^6$, $5x10^6$ and $1x10^7$
572 CFUs) were added, and the mixture was incubated in a candle jar at 37°C for 1 h. After the liquid was
573 removed from the wells by suction, the plate was washed three times with 200 μL of PBS-T (PBS_1
574 with 0.05% Tween-20). A volume of 100 μL of anti-serotype A antibody [72] (1/1000, diluted in PBS-T
575 supplemented with 0.25% BSA) was added to each well, and the plate was incubated at RT for 1 h.
576 The wells were washed four times with PBS-T using a Delfia Platewash (Perkin Elmer). After 100 μL
577 of anti-rabbit IgG–horseradish peroxidase (HRP) antibody (Promega, 1/9000, diluted into PBS-T) was
578 added to each well, the plate was incubated at RT for 1 h. The wells were washed as in the previous
579 step, and detection was conducted as in the investigation of BilRI binding to cytokines but measured
580 at A_{405nm}.

581 ***Role of BilRI in the binding and internalization of IL-8 and IL-6 by the biofilm cells***

582 A clinical wild-type *A. actinomycetemcomitans* strain D7S, the *bilRI*⁻ mutant strain and the BilRI-
583 overexpressing strain were revived from frozen milk stocks or TSB_2 containing 20% glycerol through
584 growth on TSA-blood plates for 4 days. Bacterial suspensions were prepared in TSB_2 medium, and
585 the number of bacterial cells was estimated as described above. Then, 2.5 mL of suspension ($5x10^8$
586 CFUs) was added to sterile hydrophilic PES membranes (Supor®-200; diameter of 25 mm; 0.2-µm
587 pore size; Pall Corporation, Ann Arbour, MI, USA) in a 6-well culture plate followed by incubation in
588 a candle jar at 37°C for 24 h. To remove non-adherent bacteria, the membranes were briefly washed
589 twice with 0.85% NaCl prior to a 24-h incubation in RPMI-1640 medium (Sigma-Aldrich)
590 supplemented with 0.6 g/L L-glutamine (Sigma-Aldrich).
591 In parallel to biofilm formation, spontaneously immortalized HGKs [58] were maintained in
592 keratinocyte SFM growth medium (#17005-075, Gibco®, Thermo Fisher Scientific, Paisley, UK)
593 containing the supplement provided by the manufacturer. Briefly, the HGKs (passages 12-16) were
594 grown to confluence in 175-cm^2 cell culture flasks with a medium change every 4–5 days. The same
595 day on which the biofilms were incubated with RPMI-1640 medium, the confluent epithelial cells in
596 flasks were reseeded into six-well plates ($4x10^5$) and grown for 24 h. Prior to co-culturing, the
597 biofilms were gently washed with PBS_2 (10 mM Na_2HPO_4 and 150 mM NaCl, pH 7.4). Then, the
598 biofilms were placed on top of HGKs, and the co-cultures were incubated at 37ºC in 5% CO_2 for 24 h.
599 After the co-culture with gingival epithelial cells, the biofilms were fixed initially in 4 %
600 paraformaldehyde with 2.5 % sucrose in 0.1 M phosphate buffer pH 7.4 at RT for 6 h. Then, the
601 biofilms were moved to 4ºC, and an extra 1-h fixation was applied in the same fixative. After the
602 fixation was completed, the co-cultures were stored in 2.3 M sucrose in PBS_2 at 4ºC. For immuno-
603 electron microscopy (immuno-EM) detection, small spherical samples (with a diameter of 2 mm)
604 were taken from the co-cultures using a biopsy punch (Miltex, Lake Success, NY, USA).
605 The immuno-EM detection of IL-1β, IL-8 and IL-6 in the spherical biofilm samples was performed as
606 described previously.[28] Briefly, the samples were stored in 2.3 M PBS_2 at 4°C before freezing in
607 liquid nitrogen and cryosectioning. The sections were incubated in 0.2% gelatin-PBS_2 followed by
608 0.1% glycine-PBS_2. The primary antibodies, rabbit anti-IL-1β (NB600-633; Novus Biologicals),
609 rabbit anti-IL-8 (NBP2-16958; Novus Biologicals), and rabbit anti-IL-6 (NBP2-16957), were diluted

610 in 1% BSA-PBS_2 and incubated with the samples for 60 min. After washing with 1% BSA-PBS_2, the
611 bound antibodies were detected by incubating with protein A-gold complex (10 nm) diluted in 0.1%
612 BSA-PBS_2.[73] Negative controls were prepared similarly, except primary antibodies were omitted from
613 the protocol. The labelled sections were embedded in methylcellulose and examined with a Philips
614 CM100 transmission electron microscope (FEI Company, Eindhoven, The Netherlands). Two
615 independent repetitions of the experiments were performed, of which the amounts of gold labels in
616 39-104 labelled cells were counted from 9-18 representative pictures.

617 ***Effect of IL-1β and IL-8 on biofilm composition of A. actinomycetemcomitans***

618 Because it was impossible to control the amounts of cytokines in the organotypic mucosa co-culture
619 model, we studied the effects of IL-1β and IL-8 on the composition of the biofilm matrix by exposing
620 *A. actinomycetemcomitans* biofilms to similar amounts (10 ng/mL) of recombinant cytokines in 50-
621 mL tissue culture bottles or 48-well standard tissue culture-treated plates.
622 *A. actinomycetemcomitans* D7S wild-type and *bilRI*⁻ mutant strains were grown on TSA-blood plates
623 for four days. An even cell suspension was prepared in TSB_2 medium, and the number of bacterial
624 cells was estimated as described above. The cell suspension was added to 50-mL cell culture bottles
625 (Cellstar #690160, Greiner Bio-One, Frickenhausen, Germany) to obtain a cell density of $1x10^9$ CFUs
626 in 5 mL of TSB_2. After a 5-h incubation in a candle jar at 37°C, the medium was discarded, and the
627 attached biofilms were washed with 9 mL of RPMI-1640 medium (Sigma-Aldrich) supplemented
628 with 0.6 g/L L-glutamine (Sigma-Aldrich). Five millilitres of the same medium was added to the
629 culture bottles and supplemented with 10 ng/mL IL-1β or IL-8. One bottle per strain was prepared
630 without cytokines as a control. The biofilms were grown in a candle jar overnight at 37°C. The
631 following morning, the medium was replaced by fresh medium supplemented with 10 ng/mL
632 cytokines when needed. The biofilms were grown for an additional 5 h and then collected as described
633 above. The eDNA and protein amounts in the biofilms were determined from pre-weight cell pellets
634 as described above.
635 To determine the effect of cytokines on the PGA amount and total biofilm formation, biofilms were
636 prepared using a protocol similar to that described above with the exception that they were grown on a

48-well microtiter plate instead of culture bottles and $3.8x10^7$ CFUs were added to each well. Each sample was prepared in triplicate. After the biofilms were grown for 5 h in TSB_2 and for 22 h in RPMI-1640 (supplemented with L-glutamine and cytokines as described above), the biofilms were washed with ultrapure water and stained with Congo red to determine the PGA amount in the biofilms using the method described by Izano et al.[16] with some modifications. Briefly, the biofilms were stained with 200 µL of 1% Congo red dye (Sigma-Aldrich) diluted in ultrapure water. The stain was incubated for 2 min, and the wells were washed twice with ultrapure water. The bound dye was solubilized with 200 µL of 50% DMSO (Sigma-Aldrich) at RT for 1 h. The absorbance was measured using a Multiscan GO plate reader (Thermo Fisher Scientific) at 405 nm. To determine the overall biofilm formation, identically prepared biofilms were alternatively stained with Crystal violet stain as described above.

Statistics

The binding of recombinant BilRI to various cytokines was analysed through related-samples Friedman's two-way analysis of variance on ranks, and this was followed by an analysis of BilRI binding to IL-8 through a paired-samples T-test (IBM SPSS Statistics 22). Due to the small sample size, which was always less than ten, the differences in the biofilm composition, binding capacities and uptake efficiencies of various cytokines of wild-type and *bilRI* mutant strains were analysed using the nonparametric Mann-Whitney U-test (IBM SPSS Statistics 22). The effects of cytokines on the biofilm amount and composition were analysed using a Kruskall-Wallis test followed by paired Mann-Whitney U-tests with Bonferroni corrections (IBM SPSS Statistics 22) when needed. Differences were regarded as statistically significant at $p < 0.05$.

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Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interests.

References

[1] Uversky VN. Intrinsically disordered proteins from A to Z. Int J Biochem Cell Biol 2011; 43:1090-103; PMID: 21501695; DOI: 10.1016/j.biocel.2011.04.001 [doi].

[2] Tompa P, Dosztanyi Z, Simon I. Prevalent structural disorder in E. coli and S. cerevisiae proteomes. J Proteome Res 2006; 5:1996-2000; PMID: 16889422; DOI: 10.1021/pr0600881 [doi].

[3] Wright PE, Dyson HJ. Intrinsically disordered proteins in cellular signalling and regulation. Nat Rev Mol Cell Biol 2015; 16:18-29; PMID: 25531225; DOI: 10.1038/nrm3920 [doi].

[4] Zambon JJ. Actinobacillus actinomycetemcomitans in human periodontal disease. J Clin Periodontol 1985; 12:1-20; PMID: 3882766.

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

[6] Teles RP, Gursky LC, Faveri M, Rosa EA, Teles FR, Feres M, Socransky SS, Haffajee AD. Relationships between subgingival microbiota and GCF biomarkers in generalized aggressive periodontitis. J Clin Periodontol 2010; 37:313-23; PMID: 20447254; DOI: 10.1111/j.1600-051X.2010.01534.x; 10.1111/j.1600-051X.2010.01534.x.

[7] Darveau RP. Periodontitis: A polymicrobial disruption of host homeostasis. Nat Rev Microbiol 2010; 8:481-90; PMID: 20514045; DOI: 10.1038/nrmicro2337.

[8] Haubek D, Johansson A. Pathogenicity of the highly leukotoxic JP2 clone of Aggregatibacter actinomycetemcomitans and its geographic dissemination and role in aggressive periodontitis. J Oral Microbiol 2014; 6:10.3402/jom.v6.23980. eCollection 2014; PMID: 25206940; DOI: 10.3402/jom.v6.23980 [doi].

[9] Hajishengallis G. The inflammophilic character of the periodontitis-associated microbiota. Mol Oral Microbiol 2014; 29:248-57; PMID: 24976068; DOI: 10.1111/omi.12065 [doi].

[10] Bosshardt DD, Lang NP. The junctional epithelium: From health to disease. J Dent Res 2005; 84:9-20; PMID: 15615869.

[11] Hyvärinen K, Mäntylä P, Buhlin K, Paju S, Nieminen MS, Sinisalo J, Pussinen PJ. A common periodontal pathogen has an adverse association with both acute and stable coronary artery disease. Atherosclerosis 2012; 223:478-84; PMID: 22704805; DOI: 10.1016/j.atherosclerosis.2012.05.021; 10.1016/j.atherosclerosis.2012.05.021.

[12] 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; PMID: 15662025; DOI: 10.1161/01.ATV.0000155018.67835.1a.

[13] Das M, Badley AD, Cockerill FR, Steckelberg JM, Wilson WR. Infective endocarditis caused by HACEK microorganisms. Annu Rev Med 1997; 48:25-33; PMID: 9046942; DOI: 10.1146/annurev.med.48.1.25 [doi].

[14] Rahamat-Langendoen JC, van Vonderen MG, Engström LJ, Manson WL, van Winkelhoff AJ, Mooi-Kokenberg EA. Brain abscess associated with Aggregatibacter actinomycetemcomitans: Case report and review of literature. J Clin Periodontol 2011; 38:702-6; PMID: 21539594; DOI: 10.1111/j.1600-051X.2011.01737.x [doi].

[15] Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002; 15:167-93; PMID: 11932229.

[16] Izano EA, Sadovskaya I, Wang H, Vinogradov E, Ragunath C, Ramasubbu N, Jabbouri S, Perry MB, Kaplan JB. Poly-N-acetylglucosamine mediates biofilm formation and detergent resistance in Aggregatibacter actinomycetemcomitans. Microb Pathog 2008; 44:52-60; PMID: 17851029; DOI: 10.1016/j.micpath.2007.08.004.

[17] Inoue T, Shingaki R, Sogawa N, Sogawa CA, Asaumi J, Kokeguchi S, Fukui K. Biofilm formation by a fimbriae-deficient mutant of Actinobacillus actinomycetemcomitans. Microbiol Immunol 2003; 47:877-81; PMID: 14638999.

[18] 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; PMID: 11359562; DOI: mmi2422 [pii].

[19] Schreiner HC, Sinatra K, Kaplan JB, Furgang D, Kachlany SC, Planet PJ, Perez BA, Figurski DH, Fine DH. Tight-adherence genes of Actinobacillus actinomycetemcomitans are required for virulence in a rat model. Proc Natl Acad Sci U S A 2003; 100:7295-300; PMID: 12756291; DOI: 10.1073/pnas.1237223100.

[20] Wang Y, Chen C. Mutation analysis of the flp operon in Actinobacillus actinomycetemcomitans. Gene 2005; 351:61-71; PMID: 15837433; DOI: 10.1016/j.gene.2005.02.010.

[21] Wu L, Estrada O, Zaborina O, Bains M, Shen L, Kohler JE, Patel N, Musch MW, Chang EB, Fu YX, et al. Recognition of host immune activation by Pseudomonas aeruginosa. Science 2005; 309:774-7; PMID: 16051797; DOI: 10.1126/science.1112422.

[22] Zav'yalov VP, Chernovskaya TV, Navolotskaya EV, Karlyshev AV, MacIntyre S, Vasiliev AM, Abramov VM. Specific high affinity binding of human interleukin 1 beta by Caf1A usher protein of Yersinia pestis. FEBS Lett 1995; 371:65-8; PMID: 7664886; DOI: 0014-5793(95)00878-D [pii].

[23] Mahdavi J, Royer PJ, Sjölinder HS, Azimi S, Self T, Stoof J, Wheldon LM, Brännström K, Wilson R, Moreton J, et al. Pro-inflammatory cytokines can act as intracellular modulators of commensal bacterial virulence. Open Biol 2013; 3:130048; PMID: 24107297; DOI: 10.1098/rsob.130048 [doi].

[24] Paino A, Ahlstrand T, Nuutila J, Navickaite I, Lahti M, Tuominen H, Välimaa H, Lamminmäki U, Pöllänen MT, Ihalin R. Identification of a novel bacterial outer membrane interleukin-1Beta-binding protein from Aggregatibacter actinomycetemcomitans. PLoS One 2013; 8:e70509; PMID: 23936223; DOI: 10.1371/journal.pone.0070509; 10.1371/journal.pone.0070509.

[25] 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; PMID: 16769197; DOI: S0882-4010(06)00058-1 [pii]; 10.1016/j.micpath.2006.04.005 [doi].

[26] Paino A, Tuominen H, Jääskelainen M, Alanko J, Nuutila J, Asikainen SE, Pelliniemi LJ, Pöllänen MT, Chen C, Ihalin R. Trimeric form of intracellular ATP synthase subunit beta of Aggregatibacter actinomycetemcomitans binds human interleukin-1beta. PLoS One 2011; 6:e18929; PMID: 21533109; DOI: 10.1371/journal.pone.0018929.

[27] 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; PMID: 17307379; DOI: 10.1016/j.micinf.2006.12.018.

[28] Paino A, Lohermaa E, Sormunen R, Tuominen H, Korhonen J, Pöllänen MT, Ihalin R. Interleukin-1beta is internalised by viable Aggregatibacter actinomycetemcomitans biofilm and locates to the outer edges of nucleoids. Cytokine 2012; 60:565-74; PMID: 22898394; DOI: 10.1016/j.cyto.2012.07.024.

[29] Hellman M, Tossavainen H, Rappu P, Heino J, Permi P. Characterization of intrinsically disordered prostate associated gene (PAGE5) at single residue resolution by NMR spectroscopy. PLoS One 2011; 6:e26633; PMID: 22073178; DOI: 10.1371/journal.pone.0026633 [doi].

[30] Taboada B, Ciria R, Martinez-Guerrero CE, Merino E. ProOpDB: Prokaryotic operon DataBase. Nucleic Acids Res 2012; 40:D627-31; PMID: 22096236; DOI: 10.1093/nar/gkr1020 [doi].

[31] 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; PMID: 16904783; DOI: S0167-7012(06)00190-4 [pii]; 10.1016/j.mimet.2006.06.004 [doi].

[32] Kaplan JB, Meyenhofer MF, Fine DH. Biofilm growth and detachment of Actinobacillus actinomycetemcomitans. J Bacteriol 2003; 185:1399-404; PMID: 12562811.

[33] Tompa P, Schad E, Tantos A, Kalmar L. Intrinsically disordered proteins: Emerging interaction specialists. Curr Opin Struct Biol 2015; 35:49-59; PMID: 26402567; DOI: S0959-440X(15)00124-4 [pii].

[34] 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; PMID: 19038023; DOI: 10.1186/1471-2180-8-206.

[35] Okshevsky M, Meyer RL. The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. Crit Rev Microbiol 2015; 41:341-52; PMID: 24303798; DOI: 10.3109/1040841X.2013.841639 [doi].

[36] Chiang WC, Nilsson M, Jensen PO, Hoiby N, Nielsen TE, Givskov M, Tolker-Nielsen T. Extracellular DNA shields against aminoglycosides in pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 2013; 57:2352-61; PMID: 23478967; DOI: 10.1128/AAC.00001-13 [doi].

[37] Jones EA, McGillivary G, Bakaletz LO. Extracellular DNA within a nontypeable Haemophilus influenzae-induced biofilm binds human beta defensin-3 and reduces its antimicrobial activity. J Innate Immun 2013; 5:24-38; PMID: 22922323; DOI: 10.1159/000339961 [doi].

[38] Peterson BW, van der Mei HC, Sjollema J, Busscher HJ, Sharma PK. A distinguishable role of eDNA in the viscoelastic relaxation of biofilms. MBio 2013; 4:e00497-13; PMID: 24129256; DOI: 10.1128/mBio.00497-13 [doi].

[39] Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, Wagner H, Lipford GB. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U S A 2001; 98:9237-42; PMID: 11470918; DOI: 10.1073/pnas.161293498 [doi].

[40] Khalaf H, Lonn J, Bengtsson T. Cytokines and chemokines are differentially expressed in patients with periodontitis: Possible role for TGF-beta1 as a marker for disease progression. Cytokine 2014; 67:29-35; PMID: 24680479; DOI: 10.1016/j.cyto.2014.02.007 [doi].

[41] Lee SY, Choi JH, Xu Z. Microbial cell-surface display. Trends Biotechnol 2003; 21:45-52; PMID: 12480350.

[42] Bauer ME, Townsend CA, Doster RS, Fortney KR, Zwickl BW, Katz BP, Spinola SM, Janowicz DM. A fibrinogen-binding lipoprotein contributes to the virulence of Haemophilus

ducreyi in humans. J Infect Dis 2009; 199:684-92; PMID: 19199547; DOI: 10.1086/596656; 10.1086/596656.

[43] Fusco WG, Elkins C, Leduc I. Trimeric autotransporter DsrA is a major mediator of fibrinogen binding in Haemophilus ducreyi. Infect Immun 2013; 81:4443-52; PMID: 24042118; DOI: 10.1128/IAI.00743-13 [doi].

[44] Kaplanski G, Marin V, Montero-Julian F, Mantovani A, Farnarier C. IL-6: A regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol 2003; 24:25-9; PMID: 12495721; DOI: S1471490602000133 [pii].

[45] Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011; 1813:878-88; PMID: 21296109; DOI: 10.1016/j.bbamcr.2011.01.034 [doi].

[46] Chomarat P, Banchereau J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nat Immunol 2000; 1:510-4; PMID: 11101873; DOI: 10.1038/82763 [doi].

[47] McLoughlin RM, Jenkins BJ, Grail D, Williams AS, Fielding CA, Parker CR, Ernst M, Topley N, Jones SA. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proc Natl Acad Sci U S A 2005; 102:9589-94; PMID: 15976028; DOI: 0501794102 [pii].

[48] Curnow SJ, Scheel-Toellner D, Jenkinson W, Raza K, Durrani OM, Faint JM, Rauz S, Wloka K, Pilling D, Rose-John S, et al. Inhibition of T cell apoptosis in the aqueous humor of patients with uveitis by IL-6/soluble IL-6 receptor trans-signaling. J Immunol 2004; 173:5290-7; PMID: 15470075; DOI: 173/8/5290 [pii].

[49] Cochran DL. Inflammation and bone loss in periodontal disease. J Periodontol 2008; 79:1569-76; PMID: 18673012; DOI: 10.1902/jop.2008.080233 [doi].

[50] Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 1998; 95:3597-602; PMID: 9520411.

[51] Tamura T, Udagawa N, Takahashi N, Miyaura C, Tanaka S, Yamada Y, Koishihara Y, Ohsugi Y, Kumaki K, Taga T. Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. Proc Natl Acad Sci U S A 1993; 90:11924-8; PMID: 8265649.

[52] Hashizume M, Hayakawa N, Mihara M. IL-6 trans-signalling directly induces RANKL on fibroblast-like synovial cells and is involved in RANKL induction by TNF-alpha and IL-17. Rheumatology (Oxford) 2008; 47:1635-40; PMID: 18786965; DOI: 10.1093/rheumatology/ken363 [doi].

[53] Hajishengallis G. Periodontitis: From microbial immune subversion to systemic inflammation. Nat Rev Immunol 2015; 15:30-44; PMID: 25534621; DOI: 10.1038/nri3785 [doi].

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

[55] Nummelin H, El Tahir Y, Ollikka P, Skurnik M, Goldman A. Expression, purification and crystallization of a collagen-binding fragment of yersinia adhesin YadA. Acta Crystallogr D Biol Crystallogr 2002; 58:1042-4; PMID: 12037311; DOI: S0907444902005231 [pii].

[56] Yu C, Ruiz T, Lenox C, Mintz KP. Functional mapping of an oligomeric autotransporter adhesin of Aggregatibacter actinomycetemcomitans. J Bacteriol 2008; 190:3098-109; PMID: 18310342; DOI: 10.1128/JB.01709-07 [doi].

[57] Oksanen J, Hormia M. An organotypic in vitro model that mimics the dento-epithelial junction. J Periodontol 2002; 73:86-93; PMID: 11846204.

[58] 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; PMID: 9878537; DOI: 10.1006/bbrc.1998.9641.

[59] Green H, Fuchs E, Watt F. Differentiated structural components of the keratinocyte. Cold Spring Harb Symp Quant Biol 1982; 46 Pt 1:293-301; PMID: 6179694.

[60] Wang Y, Goodman SD, Redfield RJ, Chen C. Natural transformation and DNA uptake signal sequences in Actinobacillus actinomycetemcomitans. J Bacteriol 2002; 184:3442-9; PMID: 12057937.

[61] Cheng YA, Jee J, Hsu G, Huang Y, Chen C, Lin CP. A markerless protocol for genetic analysis of Aggregatibacter actinomycetemcomitans. J Formos Med Assoc 2014; 113:114-23; PMID: 24530245; DOI: 10.1016/j.jfma.2012.05.005 [doi].

[62] Fujise O, Wang Y, Chen W, Chen C. Adherence of Aggregatibacter actinomycetemcomitans via serotype-specific polysaccharide antigens in lipopolysaccharides. Oral Microbiol Immunol 2008; 23:226-33; PMID: 18402609; DOI: 10.1111/j.1399-302X.2007.00416.x [doi].

[63] Sternberg N, Sauer B, Hoess R, Abremski K. Bacteriophage P1 cre gene and its regulatory region. evidence for multiple promoters and for regulation by DNA methylation. J Mol Biol 1986; 187:197-212; PMID: 3486297; DOI: 0022-2836(86)90228-7 [pii].

[64] 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; PMID: 12909361.

[65] Lippmann JE, Froeliger EH, Fives-Taylor PM. Use of the Actinobacillus actinomycetemcomitans leukotoxin promoter to drive expression of the green fluorescent protein in an oral pathogen. Oral Microbiol Immunol 1999; 14:321-5; PMID: 10551160.

[66] Sreenivasan PK, Fives-Taylor P. Isolation and characterization of deletion derivatives of pDL282, an Actinobacillus actinomycetemcomitans/Escherichia coli shuttle plasmid. Plasmid 1994; 31:207-14; PMID: 8029328; DOI: S0147-619X(84)71022-5 [pii].

[67] Mintz KP, Moskovitz J, Wu H, Fives-Taylor PM. Peptide methionine sulfoxide reductase (MsrA) is not a major virulence determinant for the oral pathogen Actinobacillus actinomycetemcomitans. Microbiology 2002; 148:3695-703; PMID: 12427959; DOI: 10.1099/00221287-148-11-3695 [doi].

[68] Hisano K, Fujise O, Miura M, Hamachi T, Matsuzaki E, Nishimura F. The pga gene cluster in Aggregatibacter actinomycetemcomitans is necessary for the development of natural

competence in Ca(2+) -promoted biofilms. Mol Oral Microbiol 2014; 29:79-89; PMID: 24450419; DOI: 10.1111/omi.12046 [doi].

[69] Wu J, Xi C. Evaluation of different methods for extracting extracellular DNA from the biofilm matrix. Appl Environ Microbiol 2009; 75:5390-5; PMID: 19561191; DOI: 10.1128/AEM.00400-09 [doi].

[70] Rose SJ, Babrak LM, Bermudez LE. Mycobacterium avium possesses extracellular DNA that contributes to biofilm formation, structural integrity, and tolerance to antibiotics. PLoS One 2015; 10:e0128772; PMID: 26010725; DOI: 10.1371/journal.pone.0128772 [doi].

[71] Tang G, Mintz KP. Glycosylation of the collagen adhesin EmaA of Aggregatibacter actinomycetemcomitans is dependent upon the lipopolysaccharide biosynthetic pathway. J Bacteriol 2010; 192:1395-404; PMID: 20061477; DOI: 10.1128/JB.01453-09 [doi].

[72] 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.

[73] Slot JW, Geuze HJ. A new method of preparing gold probes for multiple-labeling cytochemistry. Eur J Cell Biol 1985; 38:87-93; PMID: 4029177.

Figure Legends

Figure 1. BilRI is an IDP lacking a specific fold without a binding ligand. A) ^{1}H spectrum of BilRI (pH 7.0, 25°C) at 600 MHz. The lack of signal dispersion in the ^{1}H methyl (< 1 ppm) and amide proton ($^{1}H^{N}$, 7-8 ppm) regions is indicative of the disordered nature of BilRI in solution. B) ^{1}H-^{15}N correlation spectrum (^{15}N-HSQC) of BilRI (pH 5.0, 25°C) at 800 MHz. A two-dimensional ^{1}H-^{15}N correlation map highlights the poorly dispersed $^{1}H^{N}$ region, confirming observations of the intrinsic disorder of BilRI, based on a ^{1}H spectrum at 600 MHz at pH 7. C) Amino acid sequence analysis confirmed the IDP nature of BilRI. The high proportions of either polar (blue) or charged (magenta) and the low numbers of bulky hydrophobic (yellow) amino acids are typical for IDPs.

Figure 2. BilRI bound to various human inflammatory cytokines but not to fibrinogen or collagen. A) Recombinant BilRI containing an 8-histidine-long C-terminal tag bound to various recombinant human cytokines in a microplate assay. BSA served as a negative control and was used as a blocking agent in the assays. The bound BilRI was detected with HRP-labelled HisProbe™. The BilRI binding to IL-8 was high compared to the binding to the control protein BSA (**:p=0.008, paired-samples T-test). B) Recombinant BilRI containing an 8-histidine-long C-terminal tag did not bind to fibrinogen-coated wells in a microplate assay when detected with europium-labelled antibody against the histidine tag. Although the positive control recombinant ClfA of *S. aureus* bound fibrinogen efficiently, the recombinant FgbA, which has been reported to bind fibrinogen,[42] did not show positive binding in the assay. Only BilRI binding to IL-8 showed a statistically significant positive difference compared with the BSA control (p=0.028, Mann-Whitney U-test) from the test experiments. C) Recombinant BilRI containing an 8-histidine-long C-terminal tag did not bind to collagen-coated wells in the microplate assays when detected with europium-labelled antibody against the histidine tag. The positive control recombinant YadA of *Y. enterocolitica* bound collagen efficiently. Only BilRI binding IL-8 showed a statistically significant positive difference compared with control BSA (p=0.028, Mann-Whitney U-test) from the test experiments.

Figure 3. Viable wild-type *A. actinomycetemcomitans* biofilm bound IL-8 and IL-6, and internalized IL-1β when co-cultured with organotypic gingival mucosa. A) *A. actinomycetemcomitans* wild-type

biofilm bound both IL-8 and IL-6 when co-cultured with organotypic gingival mucosa in the absence of the antibiotics penicillin and streptomycin. In the presence of these antibiotics, the biofilm bound less IL-8 and IL-6 whereas the epithelium contained elevated amounts of IL-8 and IL-6. B) The amount of IL-8 produced was approximately ten times the amount of IL-6 in the organotypic gingival mucosa tissue culture model. The co-culture system released slightly more IL-8 and IL-6 to the culture medium when stimulated with *A. actinomycetemcomitans* biofilm in the presence of antibiotics than in the absence of antibiotics. Due to the standard deviation between the samples, the difference was not statistically significant. N=3. C) In the organotypic gingival tissue culture model, which produced approximately 200 pg of IL-1β to the culture medium during a 24-h incubation with viable *A. actinomycetemcomitans* wild-type biofilm,[28] the uptake efficiency of IL-1β was estimated by counting the number of gold particles in anti-IL-1β-stained immuno-EM samples.

Figure 4. The outer membrane lipoprotein BilRI was not essential for the formation of typical *A. actinomycetemcomitans* rough-type colonies, biofilm or cell size and shape. BilRI overexpression-induced lysis of the outer membrane resulted in tiny colonies and significantly reduced biofilm amounts. A) On blood agar plates, the *bilRI*⁻ mutant formed typical rough-type colonies, whereas the BilRI-overexpressing strain (*bilRI* rev) formed very tiny colonies (circled in white). B) Uniform cell suspensions could be produced similarly with the wild-type and *bilRI*⁻ mutant strains following the method described by Karched et al.[31] Because the BilRI-overexpressing strain *bilRI* rev grew slowly on agar plates, it was difficult to harvest a sufficient cell mass to obtain a dense cell suspension. C) The *bilRI*⁻ mutant formed as much biofilm as the wild-type strain after 20-24 hours, as estimated through Crystal violet staining.[32] The overexpression of BilRI almost completely eliminated the capacity of the strain (*bilRI* rev) to form biofilm (***:p=0.0003, paired-samples T-test with Bonferroni corrections). D) Transmission electron microscopy revealed that the shape and size of the *bilRI*⁻ mutant cells resembled those of wild-type cells. The overexpression of BilRI (*bilRI* rev) lysed the bacterial outer membrane, resulting in a smaller cell size. Arrows indicate the *A. actinomycetemcomitans* cells in images in which other structures, such as the filter disc, are visible.

Figure 5. *A. actinomycetemcomitans bilRI*⁻ mutant cells differed from wild-type cells in the composition of the biofilm and their capacity to bind collagen and fibrinogen. A) The *bilRI*⁻ mutant biofilm contained more total protein than the corresponding *A. actinomycetemcomitans* wild-type strain. N=7, ** p=0.009 (Mann-Whitney U-test). B) The *bilRI*⁻ mutant biofilm contained less eDNA than the corresponding *A. actinomycetemcomitans* wild-type strain. N=4, * p=0.021 (Mann-Whitney U-test). C) *A. actinomycetemcomitans bilRI*⁻ mutant cells did not differ significantly from the wild-type cells in terms of binding to collagen-coated or fibrinogen-coated wells.

Figure 6. *A. actinomycetemcomitans* wild-type and *bilRI*⁻ mutant strains internalized all tested inflammatory cytokines: IL-1β, IL-8 and IL-6. The outer membrane lipoprotein BilRI had a role in the uptake of only IL-1β in the test system. A) Both *A. actinomycetemcomitans* wild-type and *bilRI*⁻ mutant biofilm cells internalized IL-1β, IL-8 and IL-6 when incubated for 24 h with human gingival keratinocyte monolayers. Cytokine uptake was studied with anti-cytokine IgG antibodies combined with protein A-gold labelling and transmission electron microscopy. B) Deletion of the *bilRI* gene decreased only IL-1β uptake (p=0.007, Mann-Whitney U-test), while IL-8 and IL-6 uptake levels were not affected. The uptake efficiencies were estimated by counting the amounts of gold labelling in the positively stained cells.

Figure 7. The produced recombinant proteins YadA (23 kDa), ClfA (36 kDa), FgbA (12 kDa), IL-8 (9 kDa) and BilRI (18 kDa) were pure, as observed in a Coomassie-stained SDS-PAGE gel. A total amount of 1 μg of each protein was run in the gel.

978 # Tables

979 **Table 1.** The nucleotide sequences of primers that were used in producing the markerless *bilRI*
980 deletion mutant of *A. actinomycetemcomitans* D7S.

Primer name	Sequence
bilRI_nest-F	5`- GTATGGTGCCTGACTTTCGG-3
bilRI_nest-R	5´-TTATGGTGGATCACCTTGGT-3´
ycgL-FD	5`CCAAGGCTGGAAAGCGATATT-3´
bilRI_RD_BamHI	5'-CTAGGATCCTGAAAGCAAATAAAAAAGCAGTCTA-3'
phoGlu-R	5´-GCGACCAAGCCTTATTTA -3´
sixA_FD_SalI	5'- CGT GTC GAC TTA ATA TAG GTC AAA ATT TAT CT -3'

981 **Table 2.** Effect of the cytokines IL-1β and IL-8 (22 h incubation) on the amount and composition of
982 pre-formed *A. actinomycetemcomitans* D7S wild-type (wt) and *bilRI*⁻ mutant biofilms. The data are
983 shown as the means ±SD from four independent experiments. The statistically significant differences
984 ($p \leq 0.05$, Mann-Whitney U-test with Bonferroni corrections) between the cytokine-treated and
985 cytokine-untreated biofilms are given in parenthesis.

		Percentage of the control (without cytokines)			
Strain	Cytokine	Biofilm mass	eDNA	PGA	Total protein
D7S wt	IL-1β	94 ± 7	56 ± 16 (0.018)	89 ± 8	86 ± 17
D7S wt	IL-8	96 ± 12	63 ± 23 (0.028)	88 ± 15	88 ± 19
D7S *bilRI*⁻	IL-1β	92 ± 5	106 ± 26	94 ± 7	89 ± 9
D7S *bilRI*⁻	IL-8	104 ± 11	103 ± 22	89 ± 9	94 ± 32

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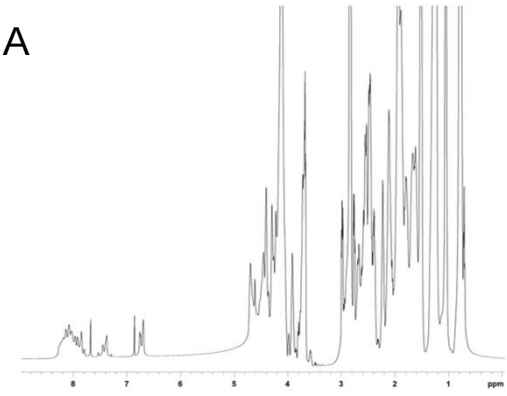
A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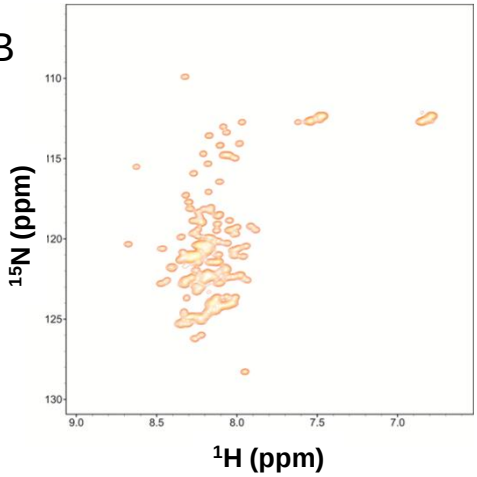
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8.0
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^{1}H (ppm)

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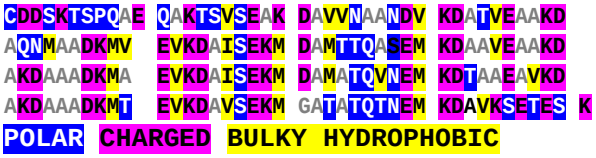
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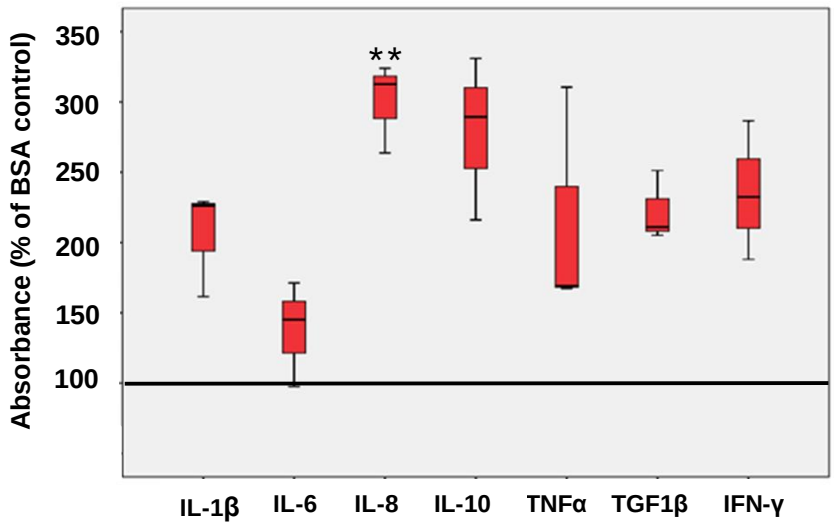
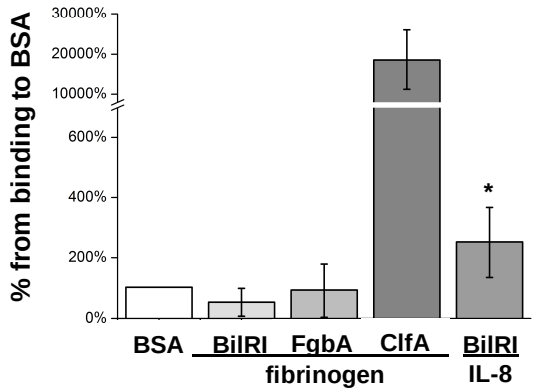
AQNMAADKMV EVKDAISEKM DAMTTQASEM KDAAVEAAKD
AKDAAADKMA EVKDAISEKM DAMATQVNEM KDTAAEAVKD
AKDAAADKMT EVKDAVSEKM GATATQTNEM KDAVKSETES K
POLAR CHARGED BULKY HYDROPHOBIC

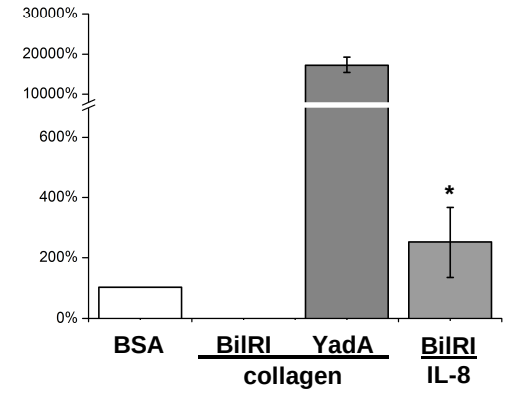
A

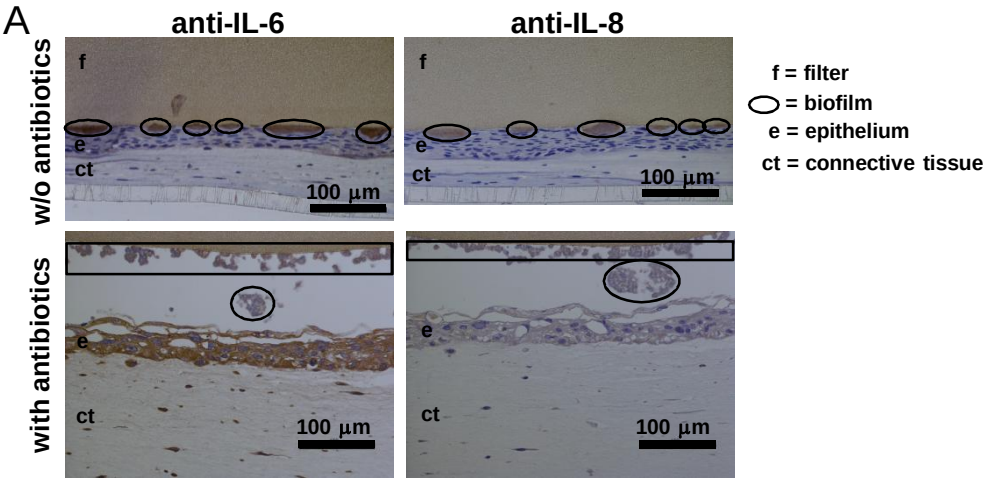

Absorbance (% of BSA control)
350
300
250
200
150
100
**
IL-1β
IL-6
IL-8
IL-10
TNFα
TGF1β
IFN-γ

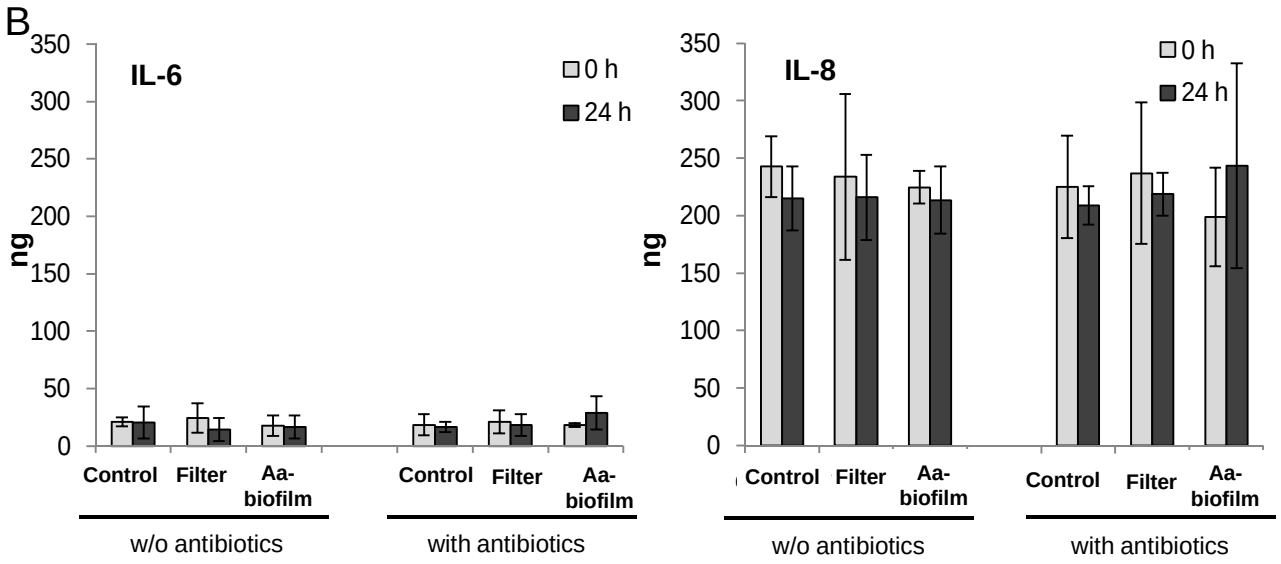
B

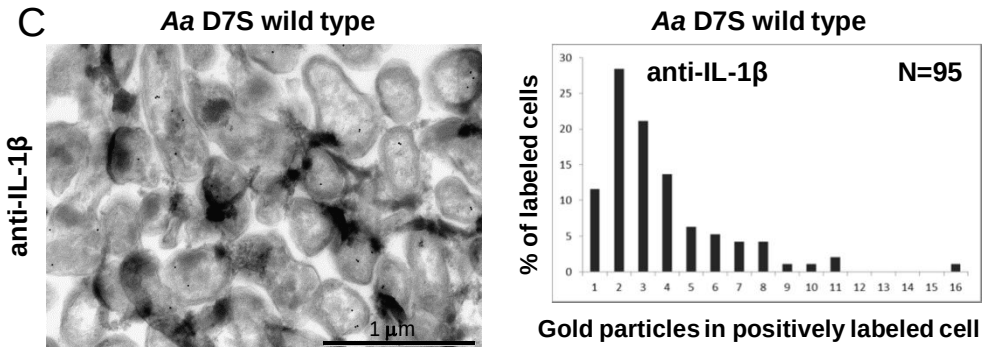

% from binding to BSA
30000%
20000%
10000%
600%
400%
200%
0%
*
BSA
BilRI
FgbA
ClfA
BilRI
fibrinogen
IL-8

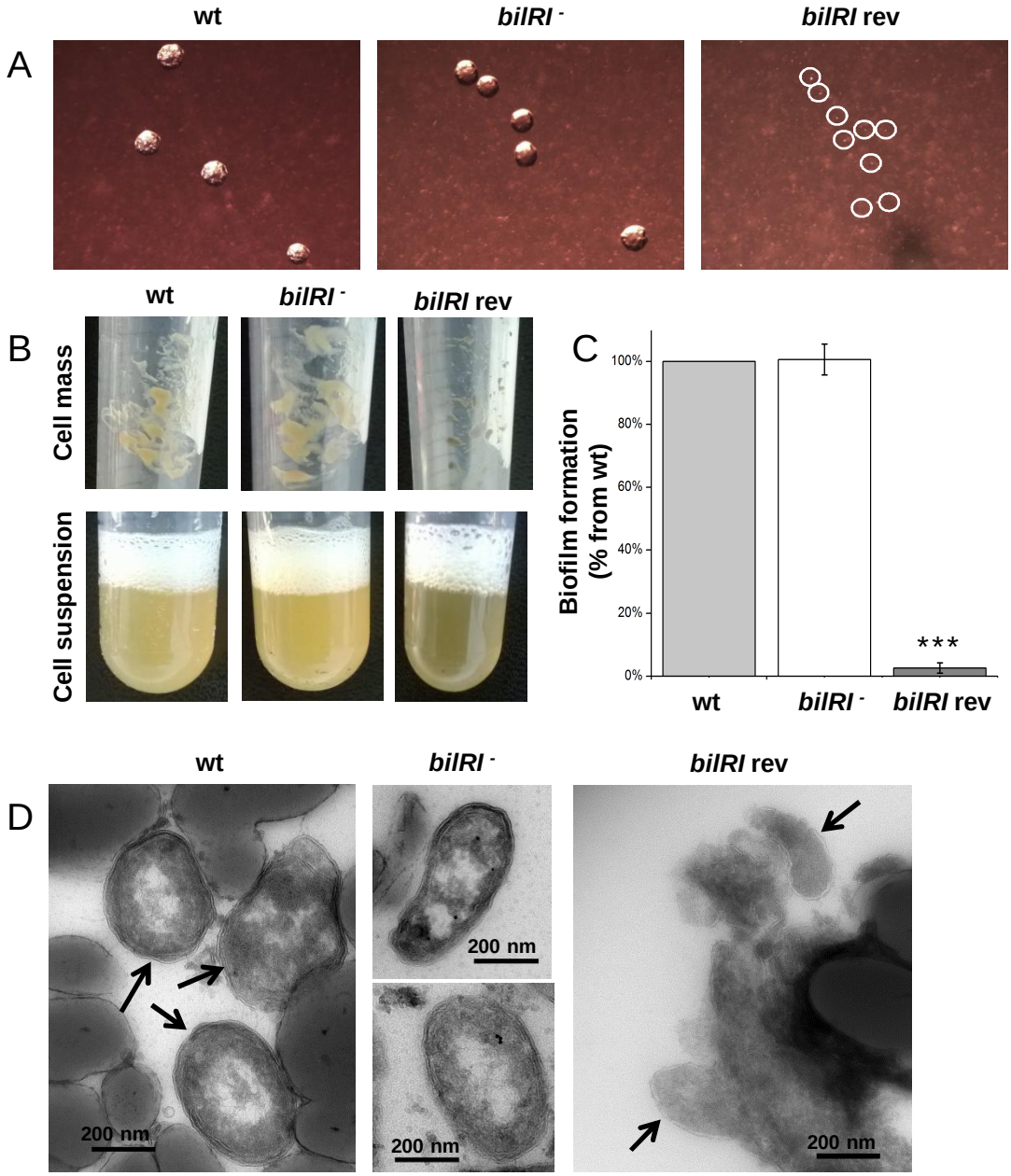
C

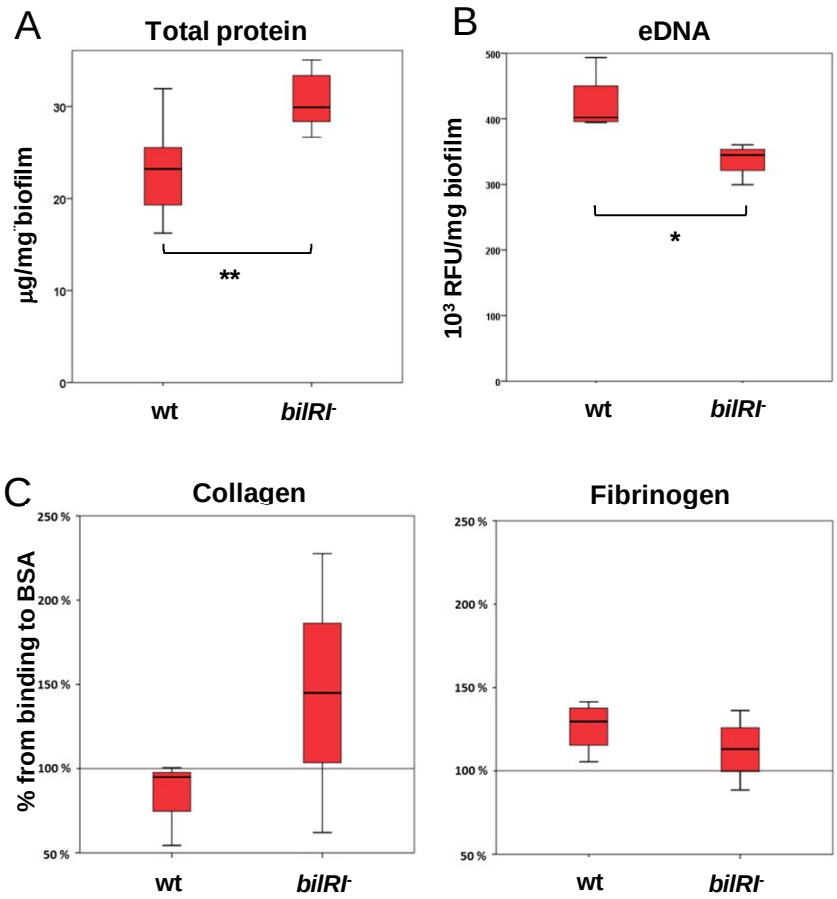

30000%
20000%
10000%
600%
400%
200%
0%
*
BSA
BilRI
YadA
BilRI
collagen
IL-8

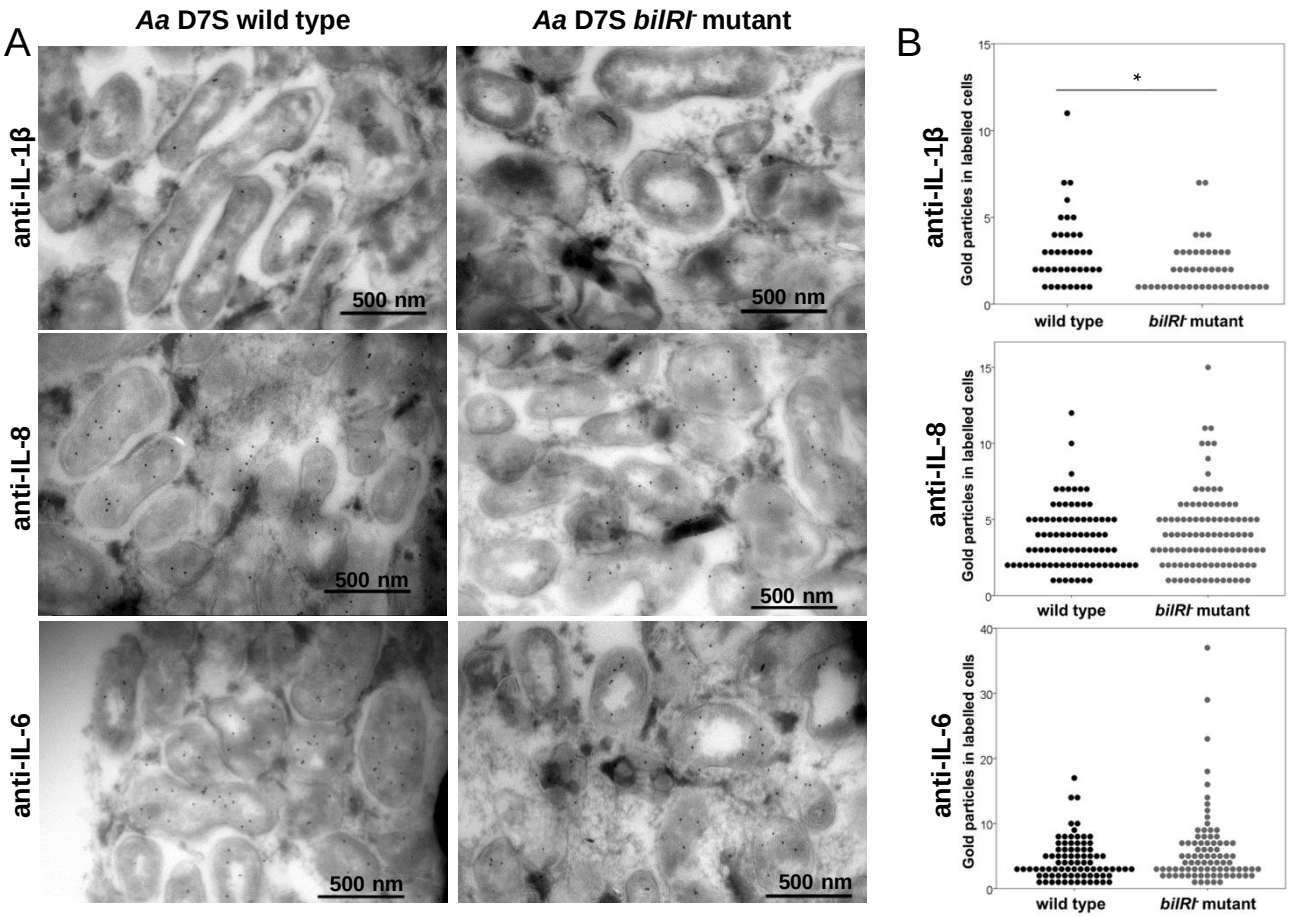

A
anti-IL-6
anti-IL-8
w/o antibiotics
with antibiotics
f
e
ct
100 µm
f = filter
= biofilm
e = epithelium
ct = connective tissue

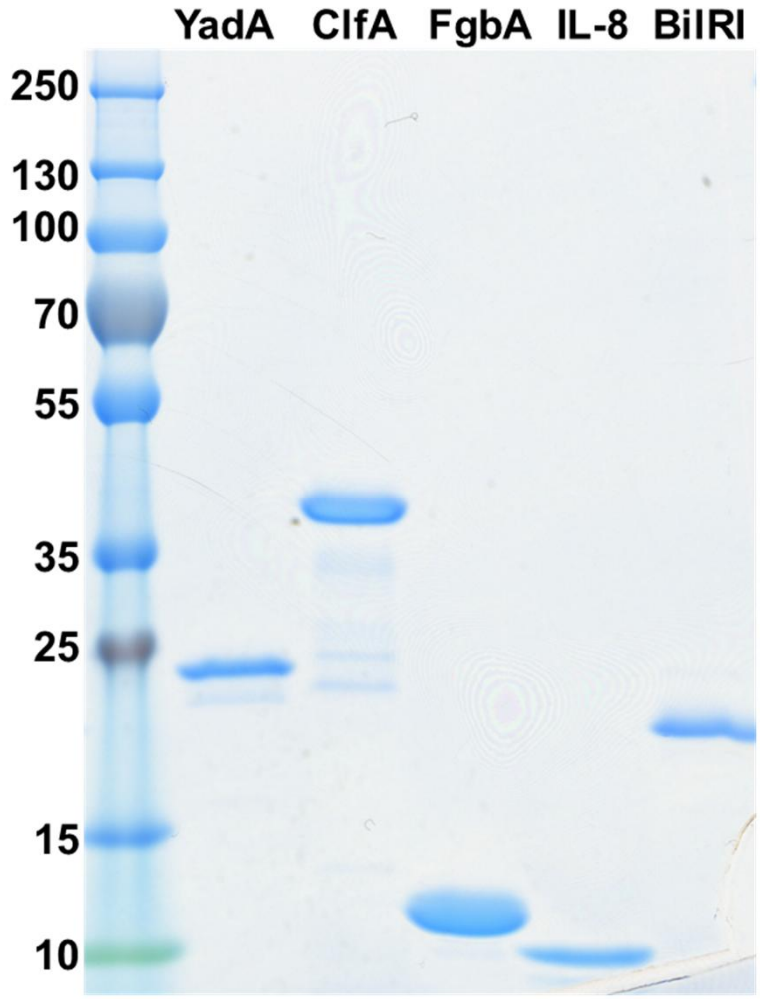

B
IL-6
IL-8
0 h
24 h
ng
350
300
250
200
150
100
50
0
Control
Filter
Aa-biofilm
w/o antibiotics
with antibiotics


C
Aa D7S wild type
anti-IL-1β
1 µm
Aa D7S wild type
anti-IL-1β
N=95
% of labeled cells
Gold particles in positively labeled cell


A
wt
bilRI -
bilRI rev
B
wt
bilRI -
bilRI rev
Cell mass
Cell suspension
C
Biofilm formation (% from wt)
100%
80%
60%
40%
20%
0%
***
wt
bilRI -
bilRI rev
D
wt
bilRI -
bilRI rev
200 nm
200 nm
200 nm
200 nm


A
Total protein
µg/mg biofilm
30
20
10
0
**
wt
bilRI-
B
eDNA
10³ RFU/mg biofilm
500
400
300
200
100
0
*
wt
bilRI-
C
Collagen
% from binding to BSA
250 %
200 %
150 %
100 %
50 %
wt
bilRI-
Fibrinogen
250 %
200 %
150 %
100 %
50 %
wt
bilRI-


A
Aa D7S wild type
Aa D7S bilRI- mutant
anti-IL-1β
anti-IL-8
anti-IL-6
500 nm
B
anti-IL-1β
anti-IL-8
anti-IL-6
Gold particles in labelled cells
*
wild type
bilRI- mutant


YadA
ClfA
FgbA
IL-8
BilRI
250
130
100
70
55
35
25
15
10