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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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**ABSTRACT**

Intrinsically disordered proteins (IDPs) do not have a well-defined and stable 3-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 protein 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, tumor 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 3-dimensional fold; yet, they are elementary components in a myriad of cellular processes. 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%. In eukaryotes, many IDPs have roles in signal transduction, where they may bind to multiple ligands with variable affinities.

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. 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. The highly leucotoxic JP2 genotype of *A. actinomycetemcomitans* has been suggested to be an important etiological agent...
in disease initiation, where the inflammatory reaction is caused by inflammophilic dysbiotic multispecies bacterial biofilm whose existence may be favored by the micromilieu in inflammation. This biofilm grows attached to the tooth surface and invades between the tooth and gingival tissue toward the junctional epithelium. 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 etiology of cardiovascular diseases, endocarditis, and brain abscesses. 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 defense systems. Bacterial cells may grow as protective communities known as biofilms, in which the extracellular matrix provides protection from antibiotics, and cellular immune defense cells, such as macrophages. Adhesive type IV Flp-pili, poly-N-acetylglucosamine (PGA) and extracellular DNA (eDNA) are the main biofilm matrix components of A. actinomycetemcomitans. Of these, long bundled Flp-pili protein fiber plays the most important role in autoaggregation, nonspecific adherence, biofilm formation and virulence in a rat model.

Various pathogens possess receptors that bind host inflammatory cytokines. The binding of cytokines to bacteria may change the properties of the bacteria, such as their biofilm formation and virulence gene expression, and may also manipulate complex host inflammatory reactions, leading to debilitated host defense against colonizing or invading pathogens. We have shown that A. actinomycetemcomitans is able to bind the central proinflammatory cytokine IL-1β and to internalize IL-1β and that intracellular IL-1β binds to at least 2 bacterial proteins. In addition, IL-1β decreases the metabolic activity of A. actinomycetemcomitans biofilms. In our recent study, we have identified an outer membrane lipoprotein of A. actinomycetemcomitans, bacterial interleukin receptor I (BilRI), 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 3-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.

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 (1H) 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 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 2-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. 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 3 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 Staphylococcus 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 layer28 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 suspensions31 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) (Fig. 4C). In biofilm, the cell morphology of *bilRI* \(^\text{-} \) 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* \(^\text{-} \) 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* \(^\text{-} \) 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* \(^\text{-} \) 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* \(^\text{-} \)
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*<sup>−</sup> biofilms with gingival keratinocyte monolayers. Previously reported IL-1β uptake<sup>28</sup> 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, Fig. 6A and 6B). However, the *bilRI<sup>−</sup>* 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).

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**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*<sup>−</sup> 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*<sup>−</sup> mutant strains following the method described by Karched et al.<sup>31</sup> 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<sup>−</sup>* mutant formed as much biofilm as the wild-type strain after 20-24 hours, as estimated through Crystal violet staining.<sup>32</sup> 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<sup>−</sup>* 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.
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 total biofilm mass remained unchanged (Table 1). The deletion of bilRI rendered the biofilm unresponsive to IL-1β and IL-8, as determined by measuring the composition of the biofilm (Table 1).

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. 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 3 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 ligand.
ligands. 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 efficiency.

Table 1. Effect of the cytokines IL-1β and IL-8 (22 h incubation) on the amount and composition of pre-formed A. actinomycetemcomitans D7S wild-type (wt) and bilRI− mutant biofilms. The data are shown as the means ± SD from 4 independent experiments. The statistically significant differences (p < 0.05, Mann-Whitney U-test with Bonferroni corrections) between the cytokine-treated and cytokine-untreated biofilms are given in parenthesis.

| Strain     | Cytokine | Biofilm mass | eDNA    | PGA     | Total protein |
|------------|----------|--------------|---------|---------|---------------|
| D7S wt     | IL-1β    | 94 ± 7       | 56 ± 16 | 89 ± 8  | 86 ± 17       |
|            |          |              | (0.018) |         |               |
| D7S wt     | IL-8     | 96 ± 12      | 63 ± 23 | 88 ± 15 | 88 ± 19       |
|            |          |              | (0.028) |         |               |
| D7S bilRI− | IL-1β    | 92 ± 5       | 106 ± 26| 94 ± 7  | 89 ± 9        |
| D7S bilRI− | IL-8     | 104 ± 11     | 103 ± 22| 89 ± 9  | 94 ± 32       |

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 labeling 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 labeling in the positively stained cells.
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,66 host defense 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 defense. The observed decrease in the amount of eDNA in response to IL-1β and IL-8 could impede immune defense 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-1β28 during the same time period. The A. actinomyctetemcomitans 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. actinomyctetemcomitans, 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.41 Due to the vulnerability of the outer membrane, the expression of outer membrane proteins of Gram-negative bacteria needs to be precisely regulated.42 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. actinomyctetemcomitans cells clearly bound the proteins. Both experiments with the bilRT 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 Haemophilus 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., dureyi 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 signaling 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 cells47 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. actinomyctetemcomitans 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.\(^5\) 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 GATGACAG-CAAAACCTTCACC-3’ and the reverse primer 5’-ATA CTCTGAG 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,\(^4\) 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 KH₂PO₄, 72 mM K₂HPO₄) containing 30 μg/mL kanamycin. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactoside (IPTG) when the OD₆₀₀nm was 1.2. Cells were grown for 3 h under induction, after which they were harvested by centrifugation (6 400 × g, 15 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 NaH₂PO₄/Na₂HPO₄, 800 mM NaCl, 20 mM imidazole, pH 7.5) including DNase I (Roche, Mannheim, Germany), 5 mM MgCl₂ and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor. Cells were sonicated 5 × 15 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 NaH₂PO₄/Na₂HPO₄, 800 mM NaCl, 500 mM imidazole, pH 7.5), and the Histagged 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 KH₂PO₄, 140 mM NaCl, and 10 mM Na₂HPO₄, pH 7.4). The recombinant BilRI does not include any tryptophans or any other aromatic residues; therefore, it is non-visible at 280 nm. However, the protein could be detected in fractions based on both A₂₈₀nm readings and Bio-Safe\textsuperscript{TM} Coomassie (Bio-Rad, Hercules, CA, USA)-stained SDS-PAGE (Thermo Fisher Scientific Precise\textsuperscript{TM} 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.\(^5\) 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 *H. ducreyi* HMC112, the fibrinogen-binding segment of *S. 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 3 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 A₂₈₀ of 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} 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 (C\textsubscript{12}H\textsubscript{14}N\textsubscript{2}O\textsubscript{1}) for IL-8, whereas the theoretical mass with 2 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 \textit{E. coli} M15(pREP4) strain (Qiagen, Hilden, Germany) for the collagen-binding fragment of \textit{Y. 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.\textsuperscript{55} except that the size-exclusion chromatography buffer was PBS\textsubscript{1}.

All proteins were deep-frozen with liquid nitrogen and stored at \(-85^\circ\text{C}\). All recombinant protein preparations have 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 \(^1\text{H}, ^{13}\text{C}, ^{15}\text{N}\) triple-resonance probeheads with z-gradient coils. For \(^1\text{H}\) NMR spectra, measured at 600 MHz, the recombinant BilRI was diluted in 95\%/5\% H\textsubscript{2}O/D\textsubscript{2}O, 50 mM NaCl, pH 7 buffer in a Shigemi microcell (250 \(\mu\)L). The final BilRI concentration was 4.6 mM. The \(^1\text{H}\) 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 \(^1\text{H}\) dimension. The two-dimensional \(^1\text{H}, ^{15}\text{N}\) HSQC spectrum of BilRI at pH 5 was measured at the 800 MHz \(^1\text{H}\) frequency using 128 and 852 complex points in \(^{15}\text{N}\) and \(^1\text{H}\) 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 analyzed 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 the signal sequence (the first 19 amino acids) nor the C\textsubscript{20}. Moreover, the recombinant BilRI contained an 8-histidine long tail in the C-terminus to allow detection with His-Probe (Thermo Fisher Scientific).

A total of 100 ng of each cytokine (IL-1\(\beta\)/IL-6/IL-8/IL-10/tumor necrosis factor [TNF]-\(\alpha\)/interferon [INF]-\(\gamma\)/transforming growth factor [TGF]-\(\beta\)) diluted in PBS\textsubscript{N} buffer (0.05\% sodium azide in PBS1) was incubated in a Nunc MaxiSorp 96-well plate (Affymetrix, Santa Clara, CA, USA) at RT overnight. Wells were washed 3 times with ion-exchanged water, after which the wells were blocked with blocking buffer (0.25\% BSA, 0.02\% sodium azide in PBS-T) at 37\(^\circ\text{C}\) for 3 h. The wells were again washed as above, and 400 ng of C-His-tagged BilRI\textsubscript{21-181} was added to the wells and incubated at 4\(^\circ\text{C}\) overnight. The wells were washed 4 times with PBS-T using Delfia Platewash (Perkin Elmer, Turku, Finland). Hi-Probe-HRP\textsuperscript{TM} (Thermo Fisher Scientific) was diluted to 1:5000, and 50 \(\mu\)L of the dilution was incubated in the wells at RT for 15 min. The wells were washed again 4 times with PBS-T as described above, and detection was performed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diamonium salt (Sigma-Aldrich) in citrate buffer (10 mM sodium citrate and 0.03\% H\textsubscript{2}O\textsubscript{2}, pH 4.2). The A\textsubscript{414} value was read using a Multiscan Go plate reader (Thermo Fisher Scientific).

**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 \(\mu\)g of each protein was run in the gel.
Collagen- and fibrinogen-binding assay for recombinant BilRI (EuLisa)

The binding of BilRI to type V collagen and fibrinogen was determined in a microplate assay modified from the method described by Yu et al.\(^6\) Type V collagen from human plasma (Sigma-Aldrich) was dissolved in 0.5 M acetic acid, and fibrinogen from human placenta (Sigma-Aldrich) was dissolved in 0.85% NaCl at 37°C with gentle mixing for 5 h and filtered through a 0.2-μm syringe filter. Collagen and fibrinogen were diluted in PBS\(_1\), and a total of 1 μg of each was incubated in the wells of a Nunc MaxiSorp 96-well plate (Affymetrix) at 4°C overnight. Equal amounts of BSA (Sigma-Aldrich) and IL-8 (production described above) were used as negative and positive controls, respectively.

Unbound proteins were removed by washing once with PBS\(_1\) using a Delfia Platewash (Perkin 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\(^®\) 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.\(^2\) In brief, in the model, human gingival fibroblasts (HGFs)\(^57\) and spontaneously immortalized human gingival keratinocytes (HGKs)\(^58\) were cultured at an air-liquid interface to obtain the 3-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 Biomatix, AZ, USA), and an aliquot containing 1.5 × 10⁵ fibroblasts was transferred to cell culture inserts and grown for 1 day submerged in Green’s medium.\(^3\) To obtain the epithelial layer in the top layer of the connective tissue, 4 × 10⁵ 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\(^TM\) 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 analyzed with IL-8- and IL-6-specific enzyme-linked immunosorbent assay (ELISA) kits (SA Bioscience, 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 (pg) 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)\(^3\) 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,
phosphoglucomutase and dihydropteroate synthase are found upstream of \textit{bilRI}.

\textbf{Markerless \textit{bilRI}-deletion mutant}

A single-gene-deletion mutant of \textit{bilRI} was produced from \textit{A. actinomycetemcomitans} strain D7S.\textsuperscript{60} The strain was recovered from \(-80^\circ\text{C}\) 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\(^{\circ}\text{C}\) for 2.5 \text{d}. In addition, 2 types of TSB media were used. TSB\textsubscript{1} contained 3 \% TSB and 0.6 \% yeast extract. TSB\textsubscript{2} was additionally supplemented with 0.8\% separately autoclaved glucose. Whenever necessary, the cultures were supplemented with the appropriate antibiotics: either 50 \mu g/mL spectinomycin or 6 \mu g/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 \textit{spe} cassette flanked by \textit{loxP} sites.\textsuperscript{61} The pAT-Cre plasmid contained the \textit{cre} recombinase and \textit{tet(O)} genes.\textsuperscript{62,63} The plasmids were amplified in \textit{Escherichia coli} strain TOP10 (Invitrogen).

The gene encoding BilRI (NC\_017846.1 AaD7S\_02241) was deleted using the Cre-\textit{loxP} mediated recombination method optimized for \textit{A. actinomycetemcomitans}.\textsuperscript{61,62} The primer sequences used for PCR product generation in the target-gene-deletion mutant are listed in Table 2. First, a 2960-bp PCR product containing the \textit{bilRI} gene was amplified from the genome of \textit{A. actinomycetemcomitans} D7S using \textit{bilRI}\_nest primers. The PCR product was then used to generate 2 PCR fragments flanking the \textit{bilRI} gene in both the downstream and upstream directions. The primer pair ycgL\_FD/\textit{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 \textit{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.\textsuperscript{60,64} In brief, suspensions of plate-grown \textit{A. actinomycetemcomitans} cells were prepared in TSB\textsubscript{1}, and the bacterial cell number was estimated according to the method described by Karched et al.\textsuperscript{31} Then, 2\texttimes\textsuperscript{10}\textsuperscript{7} colony-forming units (CFUs) were plated on TSA-plates, and the cells were grown in a candle jar at 37\(^{\circ}\text{C}\) for 2 \text{h} after mixing the recipient cells with the ligation mix (250 ng of DNA). After culturing for 5 \text{h}, the cells were scraped off the plate, resuspended in 150 \mu L of TSB\textsubscript{1}, and plated on a spectinomycin-supplemented TSA plate. Colony PCR was used to confirm the presence of a deletion in the \textit{bilRI} gene site in the \textit{A. actinomycetemcomitans} D7S genome. Using this method, a loopful of bacteria was suspended in lysis buffer (20 \mu g/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 \textit{bilRI}\_nest-primers, and the correct 3550-bp PCR product was detected. The pAT-Cre plasmid was then transformed into electrocompetent primary \textit{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 \textit{spe}-cassette. After culturing the cells in TSB\textsubscript{2} for 2 \text{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 \text{d}. Colonies sensitive to both antibiotics were considered potential markerless \textit{bilRI} mutants. Colony PCR using \textit{minc\_F\_1} (5\'-CGCGCTATCAACCAGACTAAA-3\') and \textit{SixA\_R\_2} primers (5\'-TTTATCTCGGTGAGC-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 \textit{bilRI} gene in both directions by Eurofins Genomics (Ebersberg, Germany). Moreover, the absence of \textit{bilRI} in the \textit{bilRI} mutant was verified by PCR using genomic DNA as the template and primers\textsuperscript{24} that amplify the whole \textit{bilRI} gene, including the signal sequence.

\begin{table}
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\hline
\textbf{Table 2.} The nucleotide sequences of primers that were used in producing the markerless \textit{bilRI} deletion mutant of \textit{A. actinomycetemcomitans} D7S. & \\
\hline
\textbf{Primer name} & \textbf{Sequence} \\
\hline
\textit{bilRI}\_nest-F & 5\'-GTATGGGCTGCTAATCCTGCG-3\
\textit{bilRI}\_nest-R & 5\'-TTATGGGCTGACCCCTGTT-3\
ycgL\_FD & 5\'-CAAGGCGTGAAGACGGATAT-3\
\textit{bilRI}\_RD\_BamHI & 5\'-CTAGGATCTGAAAGCGAAATAAAGAGCTCTA-3\
\textit{phoGlu-R} & 5\'-GGGCAAGCCGCTTATTT-3\
sixA\_FD\_SalI & 5\'-GGT GTC GAC TTA ATA TAG GAC AAA ATT TAT CT-3\
\hline
\end{tabular}
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\textbf{Restoration of BilRI expression in the \textit{bilRI}-deletion mutant}

Because we did not succeed in restoring the \textit{bilRI} gene to the markerless \textit{bilRI} deletion mutant despite many attempts, we decided to restore BilRI expression using an \textit{A. actinomycetemcomitans}/\textit{E. coli} shuttle plasmid under a constitutively expressed leucotoxin promoter (\textit{ltxP}).

The \textit{bilRI} gene was amplified from \textit{A. actinomycetemcomitans} strain D7S by PCR using the 5\'-
transferring 100 μL of liquid from each well to a 96-well microtitre plate, and the A_{450\text{nm}} value was measured using a Multiscan Go plate reader (Thermo Fisher Scientific).

**Effect of BilRI on biofilm formation**

Because the bilRI mutant formed similar amounts of biofilm as the wild-type *A. actinomycetemcomitans* strain, we further analyzed 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 (Fig. 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 TSB2 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 TSB2 medium was removed, the biofilms were washed 3 times with 10 mL of PBS, and the biofilm was scraped into 3 mL of PBS with an inoculation loop. The samples were divided into 3 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 sulfate (SDS) to a final concentration of 2% in 0.5× PBS. 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 determined using the method described by Lowry et al.

For eDNA extraction, 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 al. Briefly, 25 μL of biofilm extract was mixed with an equal volume of 6 μM propidium iodide in a white 96-well plate
The binding of *A. actinomycetemcomitans* to type V collagen and fibrinogen was determined using a microplate assay modified from the methods described by Yu et al.\textsuperscript{56} and Tang and Mintz.\textsuperscript{71} Collagen and fibrinogen solutions were prepared as in the collagen- and fibrinogen-binding assay for recombinant BilRI. A total of 1 µg of collagen in sodium bicarbonate buffer (16 mM sodium carbonate, 34 mM sodium bicarbonate, and 0.02% sodium azide, pH 9.6) or 25 ng of fibrinogen in PBS\textsubscript{N1} (0.05% sodium azide in PBS\textsubscript{N}) was added to the wells of a Nunc Maxi-Sorp 96-well plate (Affymetrix). The plate was coated at 4°C overnight. Liquid was removed from the wells by decanting, and the wells were washed 4 times with ion-exchanged water. The wells were blocked with 1 mg of BSA in PBS\textsubscript{1} at RT for 2 h, and the wells were then washed as described above. Wild-type *A. actinomycetemcomitans* and the *bilRI*\textsuperscript{−} mutant were collected from TSA-blood plates, a uniform bacterial suspension in PBS\textsubscript{1} was prepared, and the number of bacterial cells was estimated as described above. One hundred microliters of bacterial suspension (1.25×10\textsuperscript{6}, 2.5×10\textsuperscript{6}, 5×10\textsuperscript{6} and 1×10\textsuperscript{7} CFUs) were added, and the mixture was incubated in a candle jar at 37°C for 1 h. After the liquid was removed from the wells by suction, the plate was washed 3 times with 200 µL of PBS-T (PBS\textsubscript{1} with 0.05% Tween-20). A volume of 100 µL of anti-serotype A antibody\textsuperscript{72} (1/1000, diluted in PBS-T supplemented with 0.25% BSA) was added to each well, and the plate was incubated at RT for 1 h. The wells were washed 4 times with PBS-T using a Delfia Platewash (Perkin Elmer). After 100 µL of anti-rabbit IgG–horseradish peroxidase (HRP) antibody (Promega, 1/9000, diluted into PBS-T) was added to each well, the plate was incubated at RT for 1 h. The wells were washed as in the previous step, and detection was conducted as in the investigation of BilRI binding to cytokines but measured at A\textsubscript{405nm}. The number of bacterial cells was estimated as described above. Then, 2.5 mL of suspension (5×10\textsuperscript{8} CFUs) was added to sterile hydrophilic PES membranes (Supor\textsuperscript{®}, 200; diameter of 25 mm; 0.2-µm pore size; Pall Corporation, Ann Arbor, MI, USA) in a 6-well culture plate followed by incubation in a candle jar at 37°C for 24 h. To remove non-adherent bacteria, the membranes were briefly washed twice with 0.85% NaCl prior to a 24-h incubation in RPMI-1640 medium (Sigma-Aldrich) supplemented with 0.6 g/L L-glutamine (Sigma-Aldrich).

In parallel to biofilm formation, spontaneously immortalized HGKs\textsuperscript{58} were maintained in keratinocyte SFM growth medium (#17005–075, Gibco\textsuperscript{®}, Thermo Fisher Scientific, Paisley, UK) containing the supplement provided by the manufacturer. Briefly, the HGKs (passages 12–16) were grown to confluence in 175-cm\textsuperscript{2} cell culture flasks with a medium change every 4–5 d. The same day on which the biofilms were incubated with RPMI-1640 medium, the confluent epithelial cells in flasks were reseeded into 6-well plates (4×10\textsuperscript{5}) and grown for 24 h. Prior to co-culturing, the biofilms were gently washed with PBS\textsubscript{2} (10 mM Na\textsubscript{2}HPO\textsubscript{4} and 150 mM NaCl, pH 7.4). Then, the biofilms were placed on top of HGKs, and the co-cultures were incubated at 37°C in 5% CO\textsubscript{2} for 24 h.

After the co-culture with gingival epithelial cells, the biofilms were fixed initially in 4% paraformaldehyde with 2.5% sucrose in 0.1 M phosphate buffer pH 7.4 at RT for 6 h. Then, the biofilms were moved to 4°C, and an extra 1-h fixation was applied in the same fixative. After the fixation was completed, the co-cultures were stored in 2.3 M sucrose in PBS\textsubscript{2} at 4°C. For immuno-electron microscopy (immuno-EM) detection, small spherical samples (with a diameter of 2 mm) were taken from the co-cultures using a biopsy punch (Miltex, Lake Success, NY, USA).

The immuno-EM detection of IL-1β, IL-8 and IL-6 in the spherical biofilm samples was performed as described previously.\textsuperscript{28} Briefly, the samples were stored in 2.3 M PBS\textsubscript{2} at 4°C before freezing in liquid nitrogen and cryosectioning. The sections were incubated in 0.2% gelatin-PBS\textsubscript{2} followed by 0.1% glycine-PBS\textsubscript{2}. The primary antibodies, rabbit anti-IL-1β (NB600–633; Novus Biologicals), rabbit anti-IL-8 (NBP2–16958; Novus Biologicals), and rabbit anti-IL-6 (NBP2–16957), were diluted in 1% BSA-PBS\textsubscript{2} and incubated with the samples for 60 min. After washing with 1% BSA-PBS\textsubscript{2}, the bound antibodies were detected by incubating with protein A-gold complex (10 nm) diluted in 0.1% BSA-PBS\textsubscript{2}.\textsuperscript{73} Negative controls were prepared similarly, except primary antibodies were omitted from the protocol. The labeled sections were embedded in methylcellulose and examined with a Philips CM100 transmission electron microscope (FEI Company, Eindhoven, The Netherlands). Two independent repetitions of the experiments were performed, of
which the amounts of gold labels in 39-104 labeled cells were counted from 9-18 representative pictures.

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

Because it was impossible to control the amounts of cytokines in the organotypic mucosa co-culture model, we studied the effects of IL-1β and IL-8 on the composition of the biofilm matrix by exposing *A. actinomycetemcomitans* biofilms to similar amounts (10 ng/mL) of recombinant cytokines in 50-mL tissue culture bottles or 48-well standard tissue culture-treated plates.

*A. actinomycetemcomitans* D7S wild-type and *bilRI* mutant strains were grown on TSA-blood plates for 4 d. An even cell suspension was prepared in TSB2 medium, and the number of bacterial cells was estimated as described above. The cell suspension was added to 50-mL cell culture bottles (Cellstar #690160, Greiner Bio-One, Frickenhausen, Germany) to obtain a cell density of $1 \times 10^7$ CFUs in 5 mL of TSB2. After a 5-h incubation in a candle jar at 37°C, the medium was discarded, and the attached biofilms were washed with 9 mL of RPMI-1640 medium (Sigma-Aldrich) supplemented with 0.6 g/L L-glutamine (Sigma-Aldrich). Five milliliters of the same medium was added to the culture bottles and supplemented with 10 ng/mL IL-1β or IL-8. One bottle per strain was prepared without cytokines as a control. The biofilms were grown in a candle jar overnight at 37°C. The following morning, the medium was replaced by fresh medium supplemented with 10 ng/mL cytokines when needed. The biofilms were grown for an additional 5 h and then collected as described above. The eDNA and protein amounts in the biofilms were determined from pre-weight cell pellets as described above.

To determine the effect of cytokines on the PGA amount and total biofilm formation, biofilms were 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 $3.8 \times 10^7$ CFUs were added to each well. Each sample was prepared in triplicate. After the biofilms were grown for 5 h in TSB2 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 analyzed through related-samples Friedman’s 2-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). The effects of cytokines on the biofilm amount and composition were analyzed 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.

**Abbreviations**

- *BilRI*: bacterial interleukin receptor I
- *BSA*: bovine serum albumin
- *CFUs*: colony forming units
- *ClfA*: clumping factor A
- *DsrA*: Dureyi 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
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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RANKL
RANK ligand
RT
room temperature
sIL-6R
soluble IL-6 receptor
TGF
transforming growth factor
TNF
tumor necrosis factor
TSA
tryptone soy agar
TSB
tryptone soy broth

VIRULENCE
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