PilB from *Streptococcus sanguinis* is a bimodular type IV pilin with a direct role in adhesion

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Type IV pili (T4P) are functionally versatile filamentous nanomachines, nearly ubiquitous in prokaryotes. They are predominantly polymers of one major pilin but also contain minor pilins whose functions are often poorly defined and likely to be diverse. Here, we show that the minor pilin PilB from the T4P of *Streptococcus sanguinis* displays an unusual bimodular three-dimensional structure with a bulky von Willebrand factor A-like (vWA) module “grafted” onto a small pilin module via a short loop. Structural modeling suggests that PilB is only compatible with a localization at the tip of T4P. By performing a detailed functional analysis, we found that 1) the vWA module contains a canonical metal ion–dependent adhesion site, preferentially binding Mg\(^{2+}\) and Mn\(^{2+}\), 2) abolishing metal binding has no impact on the structure of PilB or pilination, 3) metal binding is important for *S. sanguinis* T4P–mediated twitching motility and adhesion to eukaryotic cells, and 4) the vWA module shows an intrinsic binding ability to several host proteins. These findings reveal an elegant yet simple evolutionary tinkering strategy to increase T4P functional versatility by grafting a functional module onto a pilin for presentation by the filaments. This strategy appears to have been extensively used by bacteria, in which modular pilins are widespread and exhibit an astonishing variety of architectures.

**Significance**

Type IV pili (T4P) are functionally versatile filamentous widespread in prokaryotes, implicated in a variety of functions such as adhesion, twitching motility, DNA uptake, etc (1). T4P are helical polymers consisting of type IV pilins, usually one major pilin and several minor (low abundance) ones, assembled by conserved multiprotein machineries. These defining features are shared by a superfamily of filamentous nanomachines known as type IV filaments (T4F) (1), ubiquitous in prokaryotes (2).

T4P have been intensively studied for decades in diderm bacteria because they play a central role in pathogens’ life-threatening infective endocarditis (IE), has emerged as a monoderm model for deciphering T4P biology (3). Our comprehensive functional analysis of *S. sanguinis* T4P (16) revealed that they are canonical T4A pilins. Indeed, filaments are 1) assembled by a multiprotein machinery similar to diderm T4A pilus species but simpler with only 10 components, 2) retracted by a PilT ATPase, generating tensile forces similar to diderm species, and 3) powering intense twitching motility, leading to spreading zones around bacteria growing on plates, visible by the naked eye. Subsequently, we performed a global biochemical and structural analysis of *S. sanguinis* T4P (17), showing that 1) they are heteropolymers composed of two major pilins, PilE1 and PilE2, rather than one as usually seen, 2) the major pilins display classical type IV pilin three-dimensional (3D) structure, and 3) the filaments contain a low abundance of three minor pilins (PilA, PilB, and PilC), which are required for pilation.

The present study was prompted by a perplexing observation—that the minor pilin PilB harbors a protein domain that has been extensively used by bacteria, in which modular pilins are widespread and exhibit an astonishing variety of architectures.

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extensively studied in eukaryotic proteins where it mediates adhesion to a variety of protein ligands (18)]. This suggested that PilB might be an adhesin, promoting T4P-mediated adhesion of *S. sanguinis* to host cells and proteins. Therefore, since both the molecular mechanisms of T4P-mediated adhesion and the exact role of minor pilins in T4P biology remain incompletely understood (1), we performed a structure/function analysis of PilB, which is reported here. This uncovered a widespread strategy for minor pilins to enhance the functional properties of T4P.

**Results**

**PilB Displays a Modular Pilin Architecture.** PilB, one of the three minor pilins in *S. sanguinis* T4P (17), exhibits a canonical N-terminal class III signal peptide, the defining feature of type IV pilins (4). This sequence motif consists of a seven-residue leader peptide composed predominantly of hydrophilic and neutral amino acids (aa), ending with a Gly (Fig. 1A). This leader peptide, which is processed by the prepilin peptidase PilD (17), is followed by a stretch of 21 predominantly hydrophobic aa, except for a negatively charged Glu in position 5 (Fig. 1A). Processed PilB is unusually large for a pilin, with a predicted molecular mass of 50.5 kDa (Fig. 1B). For comparison, the two major pilins of *S. sanguinis* T4P, PilE1 and PilE2 (16), have typical pilin sizes of 14.7 and 14.1 kDa, respectively (Fig. 1B). The larger size of PilB is due to the presence of a C-terminal domain (Fig. 1B) readily detectable by bioinformatics, which belongs to the von Willebrand factor A-like domain superfamily (InterPro entry IPR036465). We will refer to this domain as vWA. The prototypical vWA domain is found in the von Willebrand factor (vWF), a human blood protein required for hemostasis (19), the physiological process that prevents/stops bleeding. vWA domains, which are found in more than 300,000 proteins in the three domains of life, have been extensively studied in eukaryotic proteins where they mediate adhesion to a variety of protein ligands (18). They have been much less studied in bacteria. Of note, the vWA domain in PilB is predicted to contain a metal coordination site known as MIDAS [metal ion-dependent adhesion site (20)] (Fig. 1A), which is important for ligand binding in several eukaryotic vWA-containing proteins (21).

The above type IV pilin architecture is unusual for two reasons. First, in contrast to classical pilins that consist only of a pilin module (4) defined by a short N-terminal IPR012902 motif (Fig. 1B), PilB apparently has an additional module. Second, the extra C-terminal module in PilB corresponds to a well-defined functional domain not specific to T4P biology, which has not been previously seen in pilins. Specifically, the second module—vWA—is often associated with adhesion to protein ligands (18, 21). This is what we call a modular architecture and why we refer to PilB as a modular pilin.

Took together, these findings suggest that PilB is a modular pilin in which a functional module has been grafted during evolution onto a pilin moiety in order to promote T4P-mediated adhesion of *S. sanguinis* to protein ligands.

**Crystal Structure of PilB Reveals a Bimodal Pilin in which a Small Type IV Pilin Module Is Linked to a Bulky vWA Module via a Short Loop.** High-resolution structural information was required to confirm that PilB is composed of two modules but also to understand how modular pilins are polymerized in the filaments and how they modulate T4P functionality. We therefore endeavored to solve the 3D structure of PilB by X-ray crystallography. To facilitate protein purification, we used a synthetic gene codon optimized for expression in Escherichia coli and produced a recombinant PilB protein in which the N-terminal 35 aa (encompassing the leader peptide and hydrophobic α-1N) (Fig. 1A) were replaced by a hexahistidine tag (6His) (17). This is a commonly used approach in the field to promote protein solubility since the truncation of α-1N has minimal structural impact on the rest of the protein (22). The resulting 48.4-kDa 6His-PilB protein was soluble and could be purified using a combination of affinity and gel-filtration chromatography. The protein readily crystallized in multiple conditions, and after optimizing the best diffracting crystals, we collected a complete dataset on crystals forming in the space group *P6_1* (Table 1). After phase determination, done using crystals produced in the presence of seleno-methionine (SeMet), we solved a high-resolution structure (2.26 Å) of native 6His-PilB. As can be seen in Fig. 2A, this structure reveals a clear bimodular architecture with a small pilin moiety (highlighted in blue) linked, via a short nine-residue loop (gray), to a bulky vWA moiety (in red).

While bioinformatics could only predict that the extreme N terminus of PilB corresponds to a class III signal peptide motif, our structure reveals that the first 180 residues of processed PilB clearly display a type IV pilin fold (4) and thus indeed correspond to a pilin module (Fig. 2B). The pilin module exhibits a long N-terminal α-helix packed against, not one β-sheet as usual.
but two consecutive β-sheets consisting of six and three β-strands respectively, which together form the globular head of the pilin. The 432918 topology of the first β-sheet, that is, the order of the β-strands (Fig. 2B), is unusual since the β-strands are not contiguous along the protein sequence. Moreover, the last portion of this β-sheet forms a Ψ-loop (23) in which two antiparallel strands (β-8 and β-9) are linked via β-1 in between, connected to both of them by hydrogen bonds. This motif occurs rarely in proteins (23).

As for the vWA module (Fig. 2C), the structure strengthens the bioinformatic predictions. The vWA moiety of PilB adopts a canonical vWA fold (20, 24), with a central β-sheet (composed of five parallel and one antiparallel β-strands) surrounded on both sides by a series of α-helices (Fig. 2C). Consequently, the vWA module of PilB shows high structural similarity to many vWA-containing proteins with which it shares little sequence identity. For example, the vWA module of PilB is very similar to the third vWA domain of human vWF (25) (SI Appendix, Fig. S1), with an RMSD of 1.72 Å when the Ca atoms of the two structures are superposed. As in eukaryotic vWA-containing proteins (20, 24), PilB exhibits a MIDAS located on top of the central β-sheet (Fig. 2C). However, in contrast to these proteins, the MIDAS in PilB is flanked by two protruding “arms,” which is reminiscent of the RrgA adhesin from Streptococcus pneumoniae (26). While the first arm is perhaps unremarkable, the second folds into a four-stranded β-sheet (Fig. 2C). The MIDAS motif in PilB, which is formed by residues conserved in vWA-containing proteins, noncontiguous in the sequence (Fig. 4A) but in close proximity in the 3D structure (Fig. 2C), is functional since it coordinates a metal ion in the crystal. We have modeled the metal as Mg2+ because of its abundance in the growth medium and the high affinity of PilB for it (see Fig. 4).

The Ser206, Ser208, Thr291, and Asp319 residues in the MIDAS motif of PilB show high structural similarity to many vWA-containing proteins, the MIDAS sometimes coordinating Mn2+ as well (20, 24). The MIDAS shows high structural similarity to many vWA-containing proteins, the MIDAS sometimes coordinating Mn2+ as well (20, 24).

An important biological implication of the PilB structure is that modular pilins PilE1/PilE2 are held together by interactions between their α-1N helices (Fig. 3A), which was based on the cryo-EM structure of Neisseria meningitidis T4P (9). Despite its unusual modular structure, PilB can be readily modeled into T4P, its pilin module establishing extensive hydrophobic interactions via its α-1N with the α-1N of neighboring major pilins (Fig. 3A). This suggests that PilB will assemble into filaments in the same way as classical pilins (9, 10). However, PilB can only be accommodated at the tip of the filaments because the bulky vWA module sits on top of the pilin module in the PilB structure and essentially prevents other pilin subunits from being modeled above it (Fig. 3B). Accordingly, when PilB is modeled in the body of the filament (SI Appendix, Fig. S3A), it exhibits important steric clashes with neighboring major pilins (SI Appendix, Fig. S3B).

Together, these structural findings show that PilB is a bimodular protein composed of two clearly distinct structural modules. The pilin module adopts a canonical type IV pilin fold (4), explaining how modular pilins are polymerized into T4P, most probably at their tip. The second module, which is linked to the end of the pilin module via a short loop, adopts a vWA fold (20, 24) with a clearly defined MIDAS that coordinates a metal. Since the vWA motif in many eukaryotic proteins is involved in adhesion to protein ligands (18, 21), our structure strengthens our working hypothesis that PilB might be an adhesin.

Table 1. Crystal structures data collection and refinement statistics

| Proteins | PilB | PilB<sub>D319A</sub> |
|-----------|------|-----------------|
| (PDB)     | (7B7P) | (7BA2) |
| Maximum resolution (Å) | 2.26 | 3.00 |
| Space group | P6<sub>1</sub> | P6<sub>1</sub> |
| Unit cell parameters | | |
| a, b, c (Å) | 124.38, 124.38, 140.74 | 120.95, 120.95, 151.25 |
| α, β, γ (°) | 90, 90, 120 | 90, 90, 120 |
| Number of observations | 329,504 | 208,399 |
| Number of unique observations | 57,372 | 25,101 |
| R<sub>merge</sub> (%) | 6.2 | 16.6 |
| I/σ (l) | 13.4 | 7.6 |
| CC 1/2 | 0.999 | 0.998 |
| Resolution range used for refinement | 56.88 to 2.26 | 75.63 to 3.00 |
| Completeness (%) | 99.4 | 93.8 |
| R factor (%) | 20.5 | 24.1 |
| Free R factor (%) | 23.7 | 28.3 |
| Ramachandran favored (%) | 93.4 | 90.1 |
| Ramachandran allowed (%) | 6.4 | 8.6 |
| Ramachandran outliers (%) | 0.2 | 1.3 |
| RMSD from ideal values | | |
| bond length (Å) | 0.008 | 0.005 |
| bond angles (°) | 0.974 | 1.273 |

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PilB using ThermoFluo. This fluorescent-based method, which measures changes in thermal denaturation temperature, is a commonly used approach for quantifying protein–ligand interactions (27). We determined the affinity of purified PilB for Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$ (Fig. 4B). While no binding was detected to Ca$^{2+}$, we found that PilB binds Mg$^{2+}$ and Mn$^{2+}$ efficiently in the micromolar range, with estimated $K_d$ of 70 and 54 μM, respectively. To confirm that metal binding involves the MIDAS motif, we produced PilBD319A in which the key MIDAS residue Asp319 (Fig. 2C) was changed into an Ala by site-directed mutagenesis. Binding assays on crystals diffracting to a resolution of 3 Å performed with PilBD319A showed that changing this single residue abolishes the metal-binding ability of PilB for both Mg$^{2+}$ and Mn$^{2+}$ (Fig. 4B). These findings show that the MIDAS in PilB is functional and preferentially binds Mg$^{2+}$ and Mn$^{2+}$.

Next, to determine whether metal presence/absence might impact the 3D structure of PilB, we solved the structure of PilBD319A by X-ray crystallography. The PilBD319A protein readily crystallized in the same condition as the wild-type (WT) protein. We collected a complete dataset on crystals diffraction to a resolution of 3 Å (Table 1) and solved the structure of PilBD319A (Fig. 5A) by molecular replacement. The structure of PilBD319A (Fig. 5A) clearly shows that no metal is occupying the mutated MIDAS pocket on top of the central β-sheet (Fig. 5B), which is consistent with metal-binding assays. Although PilBD319A resolution is significantly lower than WT, it nevertheless allows for meaningful structural comparison. When the vWA modules were compared, we found that they are essentially identical, the Cα atoms superposing onto each other (Fig. 5C) with an RMSD of merely 0.45 Å, including the two arms flanking the MIDAS pocket. This shows that metal binding by MIDAS has no detectable structural impact on PilB.

Next, we explored whether MIDAS-mediated metal binding by PilB is important for piliation and/or T4P-powered twitching motility, both of which were previously shown to be abolished in a ΔpilB mutant (16). We therefore constructed an unmarked S. sanguinis mutant in which the endogenous pilB gene was altered by site-directed mutagenesis to produce PilBD319A. We first tested whether the pilBD319A mutant retains the ability to assemble T4P using filament purification (16). As can be seen in Fig. 6A, in which purified T4P were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue, the pilBD319A mutant is unpiliated. This is evidenced by the presence of the two bands corresponding to major pili PilE1 and PilE2, which are absent in a nonpiliated ΔpilD control (Fig. 6A). Moreover, the amount of T4P that can be purified from the pilBD319A mutant and WT strain appear comparable. We then tested whether the pil in the pilBD319A mutant are able to mediate twitching motility (16). For the WT strain, twitching motility is evidenced by spreading zones around bacteria grown on agar (Fig. 6B). Spreading zones were absent for the pilBD319A mutant (Fig. 6B). This shows that the MIDAS-mediated metal-binding ability of PilB, while dispensable for piliation, is important for T4P-mediated twitching motility.

Fig. 2. Crystal structure of PilB. (A) Orthogonal cartoon views of the PilB structure in which the two distinct modules have been highlighted in blue (pilin module) and red (vWA module), while the short loop connecting them is in gray. The orange sphere represents a magnesium ion. (B, Left) Close-up cartoon view of the pilin module colored in rainbow spectrum from blue (N terminus) to red (C terminus). (B, Right) Topology diagram of the pilin module structure. (C, Left) Close-up cartoon view of the vWA module in which the β-strands composing the central β-sheet are highlighted in red, while the surrounding α-helices are highlighted in yellow. The connecting loops are in gray, except for the two “arms” on top of the structure (colored in orange and blue), which surround the MIDAS. (C, Right) Diagram of the magnesium coordination by the conserved MIDAS residues. Coordinating oxygen atoms are shown, with dashed lines corresponding to hydrogen bonds.
Together, these findings show that the MIDAS in PilB is a functional metal-binding site, dispensable for piliation and protein folding but essential for T4P functionality.

**4T4P-Mediated Adhesion to Eukaryotic Cells Requires PilB, which Specifically Binds Several Human Proteins.** Since vWA is involved in adhesion in many eukaryotic proteins (18, 21), our original hypothesis was that PilB might mediate *S. sanguinis* adhesion to host cells and/or proteins, which we aimed to test next. First, we determined whether T4P might be involved in its well-known ability of *S. sanguinis* to adhere to host cells (28). After testing a few eukaryotic cell lines, we opted for Chinese hamster ovary (CHO) cells because the WT strain adheres very efficiently to them. When CHO cells were infected by *S. sanguinis* at a multiplicity of infection (MOI) of 10, 31.6 ± 9.1% of the bacterial inoculum adhered to the cells (Fig. 6C). In contrast, a nonpiliated ΔpilD mutant showed a significantly reduced adhesion, with an 18-fold decrease relative to the WT (Fig. 6C). Next, we tested our original assumption that PilB might be an adhesin by quantifying the adhesion of the pilBD319A mutant. As can be seen in Fig. 6C, although the pilBD319A mutant is piliated, its adhesion to CHO cells is dramatically impaired, with a 33-fold decrease when compared to the WT. These findings show that *S. sanguinis* T4P are multifunctional filaments important for adhesion to eukaryotic cells and that PilB plays an important role.

Since the vWA domain in multiple eukaryotic proteins has been shown to mediate cell–extracellular matrix (ECM) interactions (21), we reasoned that PilB might recognize similar ligands because it exhibits a canonical vWA module (SI Appendix, Fig. S1). We tested this hypothesis by performing binding assays with purified PilB using enzyme-linked immunosorbent assay (ELISA). In brief, we coated 96-well plates with selected putative ligands, added serial dilutions of purified 6His-PilB, and detected binding using an anti-6His antibody. We tested binding to fibrinogen and the ECM proteins fibronectin, elastin, and laminin. While PilB exhibits no binding to bovine serum albumin (BSA) that was used as a negative control (Fig. 7A), we observed dose-dependent binding to fibrinogen and fibrinogen but not to the other ECM proteins that were tested (elastin and laminin). Specific binding to fibrinectin and fibrinogen was in the high nanomolar range, with calculated $K_d$ of 494 and 865 nM, respectively (Fig. 7A). Under these in vitro experimental conditions, metal coordination by the MIDAS is dispensable for binding to fibrinectin and fibrinogen since PilBD319A binds these ligands as well as PilB (SI Appendix, Fig. S4). Finally, to
confirm the prediction that binding of PilB to the above ligands is mediated by its vWA module, we produced and purified PilBvWA corresponding only to the vWA module (Fig. 1A). We found that PilBvWA binds to fibronectin and fibrinogen (Fig. 7B), with calculated $K_d$ of 997 and 337 nM, respectively, which were comparable to PilB. These findings confirm that the adhesive ability of PilB is due to its vWA module.

Taken together, these findings show that S. sanguinis T4P are multifunctional filaments mediating adhesion to eukaryotic cells and that PilB is a bona fide adhesin using its vWA module to bind several human protein ligands it shares with eukaryotic vWA-containing proteins.

Discussion

T4P are an important research topic because of their virtual ubiquity in prokaryotes and their ability to mediate several key biological processes (1). Furthermore, the molecular mechanisms of T4P-mediated functions and the exact role of minor pilins remain incompletely understood. Therefore, in this report we focused on T4P—the prototypical T4P (1)—in the recently established monoderm model S. sanguinis (15) and performed a structure/function analysis of the unusual minor pilin PilB, which we predicted might play a role in T4P-mediated adhesion. This led to several notable findings discussed below and confirmed predictions that the

![Fig. 5. 3D crystal structure of PilBD319A. (A) Close-up cartoon view of the vWA module in PilBD319A. (B) Comparison of electron density maps in the MIDAS pocket for the PilBD319A (Upper) and PilB (Lower) structures. (C) Superposition of the vWA modules of PilB with bound Mg$^{2+}$ (gray) and PilBD319A (orange). The two structures superpose with an RMSD of 0.45 Å.](Image)

![Fig. 6. Phenotypic characterization of a S. sanguinis mutant expressing PilBD319A with an inactive MIDAS. (A) Piliation was quantified by purifying T4P from cultures adjusted to the same OD$_{600}$, purified T4P (identical volumes were loaded in each lane) were separated by SDS-PAGE and stained with Coomassie blue. A molecular weight marker (MW) was run in the first lane. MWs are indicated in kDa. (B) Twitching motility was assessed by a macroscopic motility assay. Bacteria were streaked on plates, which were incubated several days at 37°C in a humid atmosphere and then photographed. Twitching motility is characterized by spreading zones around colonies. (C) Adhesion of S. sanguinis to eukaryotic cells was quantified by incubating bacteria (MOI = 10) with CHO cells for 1 h. After removing nonadherent bacteria by several washes, bacteria adhering to cells were enumerated by performing CFU counts. The results are expressed as adhesion relative to WT (set to 1) and are the average ± SDs from five independent experiments. For statistical analysis, one-way ANOVA followed by Dunnett's multiple comparison tests were performed (****p < 0.0001).](Image)
study of T4P in monoderms has the potential to shine new light on these filaments (14, 15).

The first important finding in this study is that modular type IV pilins—T4F subunits in which an N-terminal pilin module is fused via a short linker to a functional module with a direct role in a T4F-mediated function—are widespread and extremely diverse. Modular pilins are likely to be tip exposed in the filaments because of their peculiar architecture. However, a location in the body of the filament cannot be excluded provided that the linker is flexible enough. While previous 3D structures of a few large minor pilins suggest that they are modular pilins, their second modules do not correspond to protein domains readily identifiable by available bioinformatic tools. For example, in CoFB from enterotoxigenic E. coli (ETEC) T4bP, there are two additional structural domains linked to the C terminus of the pilin module by a flexible linker, a β-repeat domain followed by a β-sandwich domain (30). CoFB, which forms a trimer predicted to be exposed at the tip of ETEC T4bP (31), appears to be an adapter for a secreted protein CoFJ (32) that has a direct role in adhesion. TcpB, from Vibrio cholerae T4bP toxin-coregulated pilus, is a tip-located minor pilin forming trimers with a structure very similar to CoFB (33), which probably has a similar function. Incidentally, CoFB is also the receptor for the bacteriophage CTXφ (33). In ComZ from Thermus thermophilus T4aP, the additional structural domain is a large β-solendom inserted not at the end of the pilin module but into the β-sheet (34), and is thought to mediate binding of extracellular DNA during transformation (34). This modular architecture is not restricted to T4P as it is also observed for a minor pilin from another T4F, GspK from type II secretion systems (T2SS) (35). In GspK, the additional structural domain is an α-domain of unclear function inserted into the β-sheet of the pilin module. GspK has been proposed to be at the tip of T2SS pseudopil, together with two other nonmodular minor pilins (GspL and GspI) with which it interacts to form a heterotrimer (35). These examples suggest that we have probably underestimated the global distribution of modular pilins, which are likely to be much more widespread because in many of them, the additional modules are not yet defined by protein signatures in the databases. However, what is clear from our global analysis is that the functions associated with these modular pilins are potentially extremely diverse. Although a “common theme” appears to be the interaction of T4F with a variety of ligands—including proteins (via vWA in PilB, and the β-repeat/β-sandwich module in CoFB), carbohydrates (via a variety of lectin domains including the concanavalin A–like lectin/glucanase domain in PilC), or DNA (the role of the β-solendom module in ComZ)—other previously unreported T4F functions are possible. This is suggested by the modular architectures IPR012902-IPR030392 or IPR012902-IPR011493, in which the second module is a predicted peptidase belonging to S74 and M26 families, respectively.

The functional characterization of the vWA module in PilB including its MIDAS, showing that it is a bona fide adhesin, is another significant achievement of this study. First, the vWA domain, which is ubiquitous in the three domains of life and has been extensively studied in eukaryotes (18, 21), has been much less studied in bacteria. Second, T4P-mediated adhesion remains among the least understood T4P functions (1). Our functional analysis of the

![Fig. 7](image_url) Dose-dependent binding of PilB to various protein ligands. Increasing concentrations of purified PilB was added to constant concentrations of immobilized putative ligands, and binding was quantified by ELISA. BSA served as negative control. The results are the average ± SDs from at least three independent experiments. (A) Binding of PilB to fibrinogen, fibronectin, elastin, and laminin. (B) Binding of PilBvWA, consisting only of the vWA module, to fibrinogen and fibronectin.

![Fig. 8](image_url) Global distribution of modular pilins. The 15 most widespread modular pilin architectures in the InterPro database are presented. The numbers in parenthesis represent the number of proteins displaying that architecture. The representative proteins depicted, drawn to scale, are from the following species: 1) Candidatus Magasanikibacteria (UniProtKB/Trembl protein A0A0G0IUS5), 2) Photobacterium luminescens (A0A022P442), 3) Desulfuribacillus stiiuliensens (A0A1E5L295), 4) Candidatus Falkowibacteria (A0A1J4T672), 5) Candidatus Wolfebacteria (A0A0G1WFE5), 6) Clostridiales bacterium (A0A01018HM7), 7) Corynebacterium glutamicum (A0A1Q6Q8B1), 8) Thermosulfidibacter takaik (A0A0S3QO3H2), 9) Candidatus Gracilibacteria (A0A1J5F7A7), 10) Candidatus Sacharibacteria (A0A1Q3NL9Q), 11) Planctomycetes bacterium (A0A1GZ2HU9), 12) Actinoplanes awajinensis subsp. mycochloracinus (A0A1017V4A), 13) Actinoplanes derwentensis (A0A1H2D7E9), 14) Desulfobacteriales bacterium (A0A1V1WS0E), and 15) Parcubacteria group bacterium (A0A2D6FV5).
vWA module in PilB significantly extends what was known for prokaryotic vWA-containing proteins and highlights important similarities and differences with eukaryotic vWA-containing proteins. Our 3D structure shows that the vWA module in PilB exhibits striking similarity to the vWA domain in eukaryotic proteins (20, 24), with a canonical MIDAS coordinating a metal. The main difference is that the MIDAS in PilB is flanked by two pro-truding arms, similar to what has been described for RsgA from S. pneumoniae (26). Interestingly, RsgA is a subunit with intrinsic adhesive properties (36) of sortase-assembled pilus in monodomers (37), which are unrelated to T4P. The parallel between RsgA and PilB denotes a case of convergent evolution in which two unrelated types of pili have evolved a similar strategy to mediate adhesion. Testing metal binding by the MIDAS in PilB, which was previously done only for eukaryotic vWA-containing proteins (38), highlights important similarities. MIDAS shows no significant binding to Ca\(^{2+}\) and a slight preference for Mn\(^{2+}\) over Mg\(^{2+}\), although the difference in affinity is much smaller than in eukaryotic proteins (38). Metal binding can be abolished by altering the MIDAS motif, which has no impact on PilB structure (38, 39). Abolishing metal binding has no detectable effect on pilation, which is analogous to what has been reported for vWA-containing adhesins of sortase-assembled pili (40, 41), but it impairs T4P-mediated twitching motility. It is unclear at this stage whether the lack of mobility of the pilin is a mutant is not reduced T4P-mediated adhesion to the agar, which would be consistent with PilB role in adhesion, or to impaired filament retraction (11). We also provide evidence that the vWA module of PilB binds several human protein ligands that it shares with eukaryotic vWA-containing proteins such as integrins and/or vWF (19, 21). However, unlike in these proteins where binding is often impaired when the MIDAS is inactivated (20), binding to fibronectin and fibrinogen is unaffected in a PilB319A mutant. This either suggests that the MIDAS is not implicated in binding these specific ligands, which has been described for vWF binding to collagen (25), or that our in vitro binding assay is not sensitive enough to detect subtle but significant differences in binding.

The finding that PilB plays a key role in adhesion of S. sanguinis to host cells and structures via its vWA module has implications for the pathogenesis of this species in particular and for our understanding of T4P-mediated adhesion in general. Our finding that PilB binds with the MIDAS module to host proteins might play a role in IE (42), a life-threatening infection often caused by S. sanguinis. Indeed, during IE, bacteria that have gained access to the bloodstream adhere to preexisting sites of valvular damage where ECM proteins are exposed, and a blood clot is present containing large amounts of platelets, fibrinogen/fibrin, and fibronectin (43). Our finding that PilB adheres directly to two of these proteins, but additional ligands cannot be excluded, suggests that PilB might be important at this early stage in IE, which could be tested in future studies. Our findings, which arguably make PilB the best-characterized T4P adhesin alongside PilC/PilY1 found in diermrr T4aP (44–48), have general implications for our understanding of T4P-mediated adhesion. The vWA module in PilB, which is most likely exposed at the pilus tip, is ideally placed to maximize bacterial adhesion to host protein receptors. T4P spring-like properties—gonococcal T4P can be stretched three times their length (49)—are expected to help bacteria that are bound via a tip-located adhesin to withstand adverse forces in their particular environment (e.g., blood flow in a heart valve). This is likely to apply to other modular pilins as well, which harbor different modules predicted to function in adhesion. The parallel with the best-characterized T4P adhesin PilC/PilY1 is obvious. This protein, which is not a pilin, is an adhesin that has been proposed to be presented at the T4P tip (45) via its interaction with a tip-located complex of four widely conserved minor pilins (50). All PilC/PilY1 have in common a C-terminal IPR008707 \(\beta\)-propeller domain while their N-termini are different (51). Since this is analogous to the situation with modular pilins, we wondered whether it could be an indication of a modular design for PilC/PilY1. This indeed seems to be the case since a search of the InterPro database (29) for all the proteins with an IPR008707 domain shows that 68 different PilC/PilY1 modular architectures are detected (Dataset S2). Strikingly, many of the N-terminal modules in PilC/PilY1 are shared with modular pilins, including vWA that was identified in PilY1 from Pseudomonas aeruginosa (52). These observations suggest that the same tinning strategy has been used by both bacterial pili and that this has increased the functional versatility of T4P. In both instances, a “carrier” module for presentation at the tip of the adhesin (either a pilin, or an IPR008707 domain that interacts with a tip-located complex of minor pilins) has been fused to variety of “effectors” modules directly involved in diverse functions.

In conclusion, by performing a detailed structure/function analysis of the minor pilin PilB from S. sanguinis, we have shed light on several aspects of T4P biology. Our findings are not only of relevance for S. sanguinis, most notably for colonization of its human host, they have general implications for T4P by uncovering a prevalent strategy used by these widespread filamentous nanomachines to promote their well-known exceptional functional versatility (1). The resulting conceptual framework paves the way for further investigations, which will further improve our understanding of these fascinating filaments.

**Materials and Methods**

**Strains and Growth Conditions.** Strains and plasmids used in this study are listed in SI Appendix, Table S1. For cloning, we used E. coli DH5\(\alpha\). For protein purification, we used E. coli BL21(DE3) or E. coli BL21 B834(DE3) (SI Appendix). Chemically competent E. coli cells were prepared as described (33). DNA manipulations were done using standard molecular biology techniques (54). PCR were done using high-fidelity DNA polymerases (Agilent). Primers used in this study are listed in SI Appendix, Table S2. The pET-28b (Novagen) derivative, pET28-pilB36-461, for expressing 6His-PilB36-461 was described previously (17). In this plasmid, the portion of a synthetic pilB gene codon optimi- ted for expression in E. coli, encoding the soluble portion of PilB, was fused to a noncleavable N-terminal 6His tag. Similarly, we constructed pET28-pilBD319A for expressing PilBvWA. To construct pET28-pilBD319A, for expressing 6His-PilB319A, we introduced a missense mutation in pET28-pilBD319A using QuickChange site-directed mutagenesis (Agilent).

The WT S. sanguinis 2908 strain and deletion mutants (\(\Delta pilB\) and \(\Delta pilB\)) were described previously (16). S. sanguinis genomic DNA was prepared using a high-speed homogenization (ON) lysis buffer and treated with DNAse I and RNase A before purification using the XpertMAG DNA from Gram-Positive Bacteria kit (G-Biosciences). Strain 2908, which is naturally competent, was transformed as described (16, 55). The unmarked S. sanguinis pilB319A mutant was constructed using a previously described two-step gene editing strategy (55) (SI Appendix).

**Protein Purification.** To purify native PilB, PilBD319A, and PilBvWA proteins, the corresponding pET-28b derivatives were transformed in E. coli BL21(DE3). Expression and purification are detailed in SI Appendix. To purify SeMet-labeled PilB for phasing, the corresponding pET-28b derivative was transformed in E. coli BL21 B834(DE3). Expression and purification are detailed in SI Appendix.

**Crys- tallization and Structure Determination.** Purified proteins in 50 mM Hepes (pH 7.4) and 200 mM NaCl were concentrated to 50 mg/mL and tested for crystallization using sitting-drop vapor diffusion with 100-nL drops of protein solution in mother liquor. A range of conditions were tested (Table S3), which yielded a number of hits, mainly in high-salt conditions. Crystallization conditions were optimized to yield larger and better diffracting crystals. All data were collected and processed using the Diamond Light Source beamline i03 and integrated in Mosflm, using the 3D pipeline in xia2 (56). Initial molecular replace- ment was performed with Phaser (57) on the 2.26-Å resolution PilB dataset using a low-resolution partial model produced from the SeMet data using autoSHARP (58). Manual building in Coot (59) was performed on the high-resolution dataset, and the full model was then used for molecular replace- ment in the low-resolution datasets. All structures were produced using Coot and phenix.refine (60) and validated using MolProbity (61).

Raynaud et al. PIIB from Streptococcus sanguinis is a bimodular type IV pilin with a direct role in adhesion
Asymmetric Metal Binding by Purified PilB. The metal-binding specificity of PilB was tested using ThermoFluor, a fluorescent-based method measuring changes in thermal denaturation temperature (Tm). Assays were done in a 96-well plate (Applied Biosystems) format. In the wells, we added to a final volume of 40 μL 1) 0 to 1 mM range of concentrations of MgCl2, MnCl2, and CaCl2, 2) 20 μM purified PilB or PilB315A, and 3) 1/5,000 dilution of SYBR Orange (Thermo Fisher Scientific). Plates were then analyzed using a temperature gradient, from 25 to 99 °C, on a StepOnePlus RT-qPCR machine (Applied Biosystems). The data were exported in MATLAB and analyzed in GraphPad. Analyses were performed with Prism (GraphPad Software). Kd were calculated using nonlinear regression fits, applying saturation binding equation (One site – Total and nonspecific binding) using Ca2+ as nonspecific binding control.

Asymmetric Protein–Ligand Binding by Purified PilB. Binding of PilB, PilB315A, and PilB315T to a variety of eukaryotic proteins was tested by ELISA as follows. Purative ligand proteins (elastin from human skin, fibrinogen from human plasma, laminin from human placenta, and fibronectin from human plasma) (all from Sigma) were resuspended in carbonate-bicarbonate buffer (Sigma) at 5 μg/mL. A total of 50 μL was dispatched into the wells of MaxiSorp plates and adsorbed O/N at 4 °C. Wells were washed three times with phosphate-buffered saline (PBS) (Gibco) and blocked during 1 h with 3% BSA (Pro-Hub). After washing with PBST (PBS containing 0.05% Tween 20), serial twofold dilutions of PilB (from 40 to 0.625 μg/mL) were added to the wells and incubated for 2 h at 37 °C. After five washes with PBST, we added 50 μL anti-ε1Hs RTM antibody (Abcam) at 1/500 dilution in PBS and incubated for 1 h at room temperature (RT). After five washes with PBST, we added 50 μL Amersham ECL anti-rabbit IgG horseradish peroxidase-linked whole antibody (GE Healthcare) at 1/500 dilution in PBS and incubated for 1 h at RT. After five washes with PBST, we added 100 μL of a 3.3,5,5,5-Tetramethylbenzidine solution (Thermo Scientific) and incubated the plates during 20 min at RT in the dark. Finally, we stopped the reaction by adding 100 μLwell of 0.18 M sulfuric acid before reading the plates at 450 nm using a plate reader. Analyses were performed with Prism (GraphPad Software). Kd were calculated using nonlinear regression fits, applying saturation binding equation (One site – Total and nonspecific binding) using BSA or gelatin as nonspecific binding control.

Assembling Piliation of S. sanguinis. S. sanguinis T4P were purified as described (16, 17) (SI Appendix). Pili were resuspended in pilus buffer, separated by SDS-PAGE, before gels were stained with Bio-Safe Coomassie (Bio-Rad).

Assembling Twitching Motility of S. sanguinis. Twitching motility was assessed on agar plates as described (16) (SI Appendix). Plates were photographed using an Epson Perfection V700 photo scanner.

Assembling Adhesion of S. sanguinis to Eukaryotic Cells. We tested adhesion of S. sanguinis to CHO cells (Public Health England) as follows. Cells were replicated in flasks in Dulbecco’s Modified Eagle Medium (Gibco) containing 1× minimum essential medium nonessential aa mix (Gibco) and 5% FBS (Gibco) and seeded at 100,000 cells/cm² in 24-well plates, which were incubated O/N at 37 °C in the presence of 5% CO₂. The next day, cell monolayers were gently rinsed with PBS and infected at an MOI of 10 with bacteria grown in Todd Hewitt broth (TH). In brief, bacteria were grown for a few hours to optical density (OD600) 0.5 units, adjusted at the same OD, pelleted by centrifugation at 1,100 g during 10 min, and resuspended in PBS. Bacteria in the inoculum were quantified by performing colony-forming unit (CFU) counts on TH plates. After 1 h of infection at 37 °C, cell monolayers were gently rinsed four times with PBS before cells with adherent bacteria were scraped in distilled water. Adherent bacteria were then quantified by performing CFU counts. Statistical analyses were performed with Prism. Comparisons were done by one-way ANOVA, followed by Dunnett’s multiple comparison tests. An adjusted P < 0.05 was considered significant (**P < 0.05, ***P < 0.01, ****P < 0.001).

Bioinformatics. Protein sequences were routinely analyzed using DNA Strider (62). Prediction of protein domains, their global distribution, and associated architectures was done by using InterProScan (29) to interrogate the InterPro database. This database was also used to download all the protein entries discussed in this paper. Molecular visualization of protein 3D structures was done using PyMOL (Schrödinger). The PDBsum Generate (63) server was used to provide a-at-glance overviews—secondary structure, topology diagram, protein motifs, and schematic diagram of metal–protein interactions—of the 3D structures determined during this work. The Dal (64) server was used for comparing protein structures in 3D. Protein 3D structures were downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) server. The 3DSS (65) server was used to superpose 3D protein structures with the STAMP algorithm (66).

Data Availability. 3D structures have been deposited in the PDB and are available under accession codes 7877P (PilB) and 78B2 (PilB315A). All the datasets generated during this study are included in this paper and SI Appendix.

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