Pilus hijacking by a bacterial coaggregation factor critical for oral biofilm development

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The formation of dental plaque, a highly complex biofilm that causes gingivitis and periodontitis, requires specific adherence among many oral microbes, including the coaggregation of *Actinomyces oris* with *Streptococcus oralis* that helps to seed biofilm development. Here, we report the discovery of a key coaggregation factor for this process. This protein, which we named coaggregation factor A (CafA), is one of 14 cell surface proteins with the LPXTG motif predicted in *A. oris* MG1, whose function was hitherto unknown. By systematic mutagenesis of each of these genes and phenotypic characterization, we found that the *Actinomyces*/*Streptococcus* coaggregation is only abolished by deletion of *cafA*. Subsequent biochemical and cytological experiments revealed that CafA constitutes the tip of a unique form of the type 2 fimbria long known for its role in coaggregation. The direct and predominant role of CafA in adherence is evident from the fact that CafA or an antibody against CafA inhibits coaggregation, whereas the shaft protein FimA or a polyclonal antibody against FimA has no effect. Remarkably, FimA polymerization was blocked by deletion of genes for both CafA and FimB, the previously described tip protein of the type 2 fimbria. Together, these results indicate that some surface proteins not linked to a pilus gene cluster in Gram-positive bacteria may hijack the pilus. These unique tip proteins displayed on a common pilus shaft may serve distinct physiological functions. Furthermore, the pilus shaft assembly in Gram-positive bacteria may require a tip, as is true for certain Gram-negative bacterial pili.

sortase | pilus assembly | interbacterial interaction | cell wall-anchored proteins

In Gram-positive pathogens, many virulence factors that mediate bacterial adherence, biofilm formation, and other pathogenic processes are covalently attached on the cell surface (1). Most of these virulence factors are anchored to the cell wall by a cysteine-transpeptidase enzyme called sortase, first discovered in *Staphylococcus aureus* (2). The cell wall anchoring mechanism of surface proteins catalyzed by sortase is conserved in Gram-positive bacteria. Substrates of sortase contain not only an N-terminal signal peptide needed for export across the cytoplasmic membrane but also a C-terminal cell wall sorting signal (CWSS) required for cell wall anchoring that is composed of an LPXTG motif, followed by a hydrophobic region and a positively charged cytoplasmic tail (3). Sortase recognizes this LPXTG motif, cleaves between the threonine and glycine residues, and joins the cleaved polypeptide to the stem peptide of the cell wall lipid II precursor that is ultimately incorporated into the cell envelope (4). In a number of Gram-positive pathogens studied to date, some LPXTG-containing proteins are assembled into covalently linked polymers known as pili (or fimbriae) by a unique class of "pilus-specific" sortases first described in *Corynebacterium diphtheriae* (5), and subsequently in *Enterococcus faecalis*, *Bacillus cereus*, streptococci, and *Actinomyces oris* among others (6–11).

A. oris is a Gram-positive pathogen that plays a pivotal role in the development of dental plaque (12). The *A. oris* genome encodes three sortases, two of which are organized into separate gene clusters, each containing cognate CWSS-harboring substrates that form an antigenically distinct fimbria. The *fimQ-fimP-srtC1* gene cluster encodes the type 1 fimbria, which is composed of FimP polymerized into the fimbrial shaft and FimQ located at the tip (11). Similarly, the *fimB-fimA-srtC2* gene cluster specifies the type 2 fimbria, which is assembled from the shaft fimbrillin FimA and the tip fimbrillin FimB (11, 13). Although all fimbrillins harbor a CWSS, the shaft fimbrillins (FimA and FimP) also contain an N-terminal pilin motif harboring a conserved lysine residue, which participates directly in the cross-linking reaction that joins each pilin subunit to another (14, 15). According to our current model, using type 2 fimbriae as an example (16), the pilus-specific sortase SrtC2 joins FimB and FimA by cross-linking the threonine residue of the FimB LPXTG motif to the lysine residue of the FimA pilin motif. Elongation of the fimbrial structure is permitted when the available FimA monomers are added to this dimeric FimB-FimA intermediate. An elongated fimbrial polymer is ultimately anchored to the cell wall, preferentially by the housekeeping sortase, SrtA, that is involved in anchoring all surface proteins to the cell wall.

The two antigenically distinct fimbriae of *A. oris* perform distinct functions in pathogenesis. In the case of type 1 fimbriae, the tip fimbrillin FimQ mediates bacterial binding to the salivary proline-rich proteins that coat the tooth surface (17). Strikingly, when *fimQ* is deleted, the assembly of type 1 fimbriae is nearly abolished. This suggests that FimQ acts to nucleate the assembly of FimP into a fimbrial shaft. Although little is known about the determinants that govern the ordered assembly of pilins into a proper pilus structure in Gram-positive bacteria, the incorporation of a designated pilin into the pilus tip appears to rely upon the specific CWSS of the tip pilin (18). Unexpectedly, the tip fimbrillin FimB was found to be dispensable not only for assembly of the type

Significance

The development of dental plaque biofilm requires specific and sequential molecular interactions between oral bacteria-colonizing host surfaces. Coaggregation between early colonizers is crucial to establish an environment suitable for late colonizers. Here, we describe that a surface protein in a Gram-positive bacterium that is not genetically linked to the fimbrial gene clusters hijacks a specific fimbrial polymerization apparatus to be displayed on the fimbrial tip. This tip-localized protein not only functions as the bona fide cell-to-cell adhesion factor for mediating coaggregation between the early colonizers *Actinomyces oris* and *Streptococcus oralis* but also serves as an initiator of fimbrial assembly.

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2 fimbrial shaft, which is made of FimA, but also for binding of the type 2 fimbriae to receptor polysaccharides (RPSs) on the surface of streptococci, resulting in the coaggregation of this bacterium with *Actinomyces* (13). This interbacterial interaction, which is critical for the development of the oral biofilm (19), is attributed to the shaft fimbrillin FimA because its absence abrogated bacterial coaggregation, biofilm formation, and hemaglutination (13). Indeed, structural studies revealed that FimA contains three IgG-like modules that are commonly found in Gram-positive pilins (15), and the recombinant FimA protein was able to bind to the surface of epithelial cells and *S. oralis* as well as to asialofetuin, a glycoprotein that contains the RPSs for *Actinomyces* interaction (15). Paradoxically, whereas antibodies raised against the purified type 2 fimbriae have been reported to block bacterial coaggregation (20), polyclonal antibodies directed against recombinant FimA or FimB did not display this inhibitory activity (Fig. 1). One possibility is that the type 2 fimbriae might contain an additional unknown component that serves this adhesive function. The present study was designed to test this intriguing hypothesis.

A. oris MG1 harbors many predicted cell wall-anchored proteins that contain the LPXTG motif, as is true of many Gram-positive bacteria. To date, nothing has been described regarding the function of these putative cell surface proteins of *A. oris*. Here, we report the unexpected discovery that one of these predicted cell surface proteins, named coaggregation factor A (CafA), is incorporated at the tip of FimA polymers, thus resulting in a novel form of the type 2 fimbriae that do not contain the classical tip fimbrillin FimB. Importantly, we show that CafA is a bona fide and critical coaggregation factor that mediates interbacterial interactions. This provides a previously unidentified example of a virulence factor hitchhiking a fimbrial organelle for display on the bacterial surface. Remarkably, homologs of CafA are widespread, hinting that the mechanism of pilus hitchhiking for surface display of virulence factors may be common in Gram-positive bacteria.

Results

Identification of a Unique CafA of *Actinomyces* That Mediates Bacterial Coaggregation and Hemagglutination. The fact that certain antibodies against type 2 fimbriae can block RPS-mediated bacterial coaggregation with *S. oralis* (20) but polyclonal antibodies against FimA do not (Fig. 1*B*) suggested that type 2 fimbriae may contain an unidentified surface antigen that mediates coaggregation. This prompted us to survey the *A. oris* MG1 genome systematically for additional surface proteins that may perform the adhesive function in the cell-to-cell interactions. A total of 14 ORFs, named *acaA–acaN* (aca for *Actinomyces* cell wall-anchored proteins) predicted to encode LPXTG-containing proteins not linked to the fimbrial gene clusters were identified (Table S1). Subsequently, we deleted each of these *aca* genes individually in the parental strain MG1 (*Materials and Methods*) and assessed the relative proficiency of the individual mutants for coaggregation with *S. oralis* 34 (So34; RPS-positive) (13). In these experiments, equivalent numbers of *A. oris* and *S. oralis* cells were mixed together, and coaggregation was visually determined. *S. oralis* OC1 lacking RPS was used as a control. Results showed that with the exception of the Δ*acaF* deletion mutant, all other mutant strains coaggregated normally with So34 (Fig. S1*A*), demonstrating that the product of the *acaF* gene is essential for coaggregation. We thus renamed *acaF* as *cafA* (caf for coaggregation factor), which is genetically distant from the type 2 fimbrial operon (Fig. S1*B*).

Deletion of *cafA* produced an RPS-dependent coaggregation defect identical to that seen with the deletion of *fimA*. This defect was rescued by expression of *cafA* on a complementing plasmid (Fig. 1*A*, *Left*). Furthermore, deletion of *cafA* also abolished receptor-mediated hemagglutination (Fig. 1*A*, *Right*), the property that previous studies have attributed to the shaft fimbrillin FimA of the type 2 fimbriae (13). Notably, in contrast to *fimA* deletion, which eliminates type 2 fimbriae from the *Actinomyces* cell surface and affects biofilm formation (13),

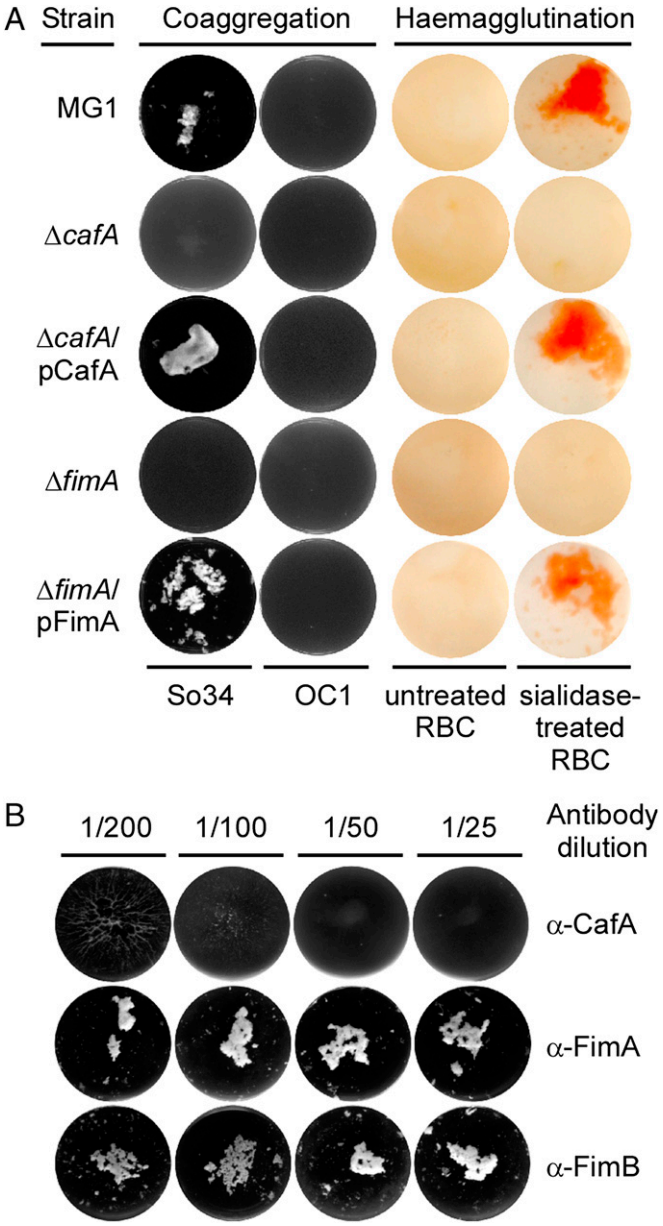

Fig. 1. Requirement of CafA for receptor-mediated cell-to-cell interactions. (*A*) *A. oris* MG1 and its isogenic derivatives were examined for coaggregation with RPS-positive (So34) and RPS-negative (OC1) *S. oralis*. For hemagglutination, RBCs were treated with sialidase or mock-treated before incubation with *A. oris* cells. (*B*) *A. oris* cells were pretreated with increasing concentrations of antibodies raised against CafA, FimA, or FimB before examination for coaggregation.

deletion of *cafA* showed none of these defects (Fig. S1*C*). Hence, CafA appears to be selectively required for the coaggregation of *Actinomyces* with *Streptococcus* and hemagglutination.

Next, to examine whether CafA-mediated coaggregation involves a direct interaction between CafA and the *Streptococcus* receptors, we generated polyclonal antibodies against the mature CafA protein, which lacks its N-terminal signal peptide and the C-terminal CWSS. We then treated *A. oris* MG1 cells with various concentrations of polyclonal antibodies against CafA, FimA, or FimB before subjecting them to the coaggregation assay. As shown in Fig. 1*B*, CafA antibodies blocked *A. oris* coaggregation with *S. oralis* in a concentration-dependent manner, whereas the antibodies against FimA or FimB failed to show any measurable

inhibition in this assay. Conversely, incubation of *S. oralis* cells with increasing amounts of the CafA protein, not the FimA protein, also prevented streptococcal coaggregation with *A. oris* MG1 (Fig. S2). Of note, coaggregation was not observed between *S. oralis* and *A. oris* lacking *cafA* (i.e., Δ*cafA*) when the CafA protein was added exogenously (Fig. S2). We thus conclude that CafA is the specific and predominant adherence factor that is required for *A. oris* coaggregation with *S. oralis*.

CafA Is a Component of the Type 2 Fimbrial Structures Assembled on the Bacterial Surface. Based on above results, one might predict that CafA is a simple cell wall-associated adhesin that mediates the interbacterial interaction between *Actinomyces* and *Streptococcus*. However, the fact that the cell aggregation phenotype of the Δ*cafA* mutant mirrors that of the Δ*fimA* mutant made us wonder whether CafA is a component of the type 2 fimbriae. To examine this unprecedented scenario, we used the polyclonal anti-CafA antibody to detect surface expression of CafA through Western blotting and immunoelectron microscopy (IEM). In our quantitative Western blotting experiments, cell wall extracts of *Actinomyces* isolated by muramidase treatment were precipitated by trichloroacetic acid and dissolved in SDS-containing sample buffer. Protein samples representing equivalent amounts of the cell cultures were then separated on gradient gels and immunoblotted with specific antibodies (α-CafA, α-FimA, or α-FimP). Excitingly, we observed high-molecular weight polymers of CafA ($CafA_P$) in the MG1 strain, reminiscent of the heterogeneous lengths of fimbrial polymers detected by α-FimA (compare Fig. 2 *A* and *B*, lane WT). In addition to the $CafA_P$, monomers (predicted molecular mass of 100 kDa) and possibly dimeric forms of CafA could be detected migrating around 115 and 200 kDa, respectively (Fig. 2*A*). These various forms of CafA were specific to the protein because they were not observed in the Δ*cafA* mutant and they were restored in the complementing strain (Fig. 2*A*, lanes Δ*cafA* and Δ*cafA*/pCafA). Significantly, formation of the high-molecular weight CafA polymers depended on the ability of bacteria to assemble FimA polymers (Fig. 2 *A* and *B*, lanes Δ*fimA* and Δ*fimA*/pFimA). Consistent with this, CafA polymers were not detected in a lysine-substituted FimA mutant (15) that cannot polymerize the type 2 fimbriae due to its inability to form the cross-linking isopeptide bond (Fig. 2 *A* and *B*, lane Δ*fimA*/pFimA-K198A). Importantly, no reduction in CafA polymers was observed in a strain lacking *fimB*, which encodes the canonical type 2 tip fimbrillin FimB (Fig. 2 *A* and *B*, lane Δ*fimB*). Finally, there was a significant reduction of FimA polymers when *cafA* was deleted (Fig. 2*B*, lane Δ*cafA*). The observed phenotypes were specific for the type 2 fimbriae, with no effect on the type 1 fimbriae (Fig. S3). Consistently, deletion of the genes that encode components and machinery for the type 1 fimbriae did not affect the polymerization of FimA and assembly of type 2 fimbriae (11). We conclude that CafA is a covalently linked component of the type 2 fimbriae.

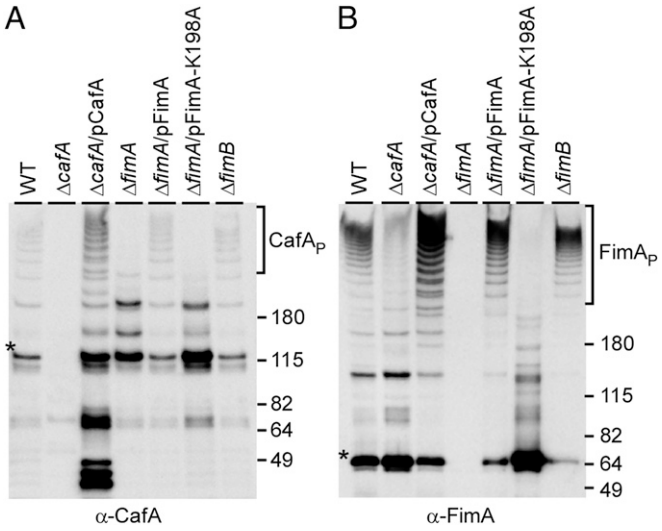

Fig. 2. Polymerization of CafA and its association with FimA polymers. W fractions of *A. oris* MG1 (WT) and its isogenic derivatives grown to midlog phase were isolated by muramidase treatment. Equivalent protein samples were separated on 3–12% Tris·glycine gradient gels and detected by immunoblotting with α-CafA (*A*) or α-FimA (*B*). Protein monomers (*), polymers (P), and molecular mass markers (kilodaltons) are indicated.

CafA Forms a Distinct Fimbrial Tip Independent of FimB. To examine how CafA is incorporated into the type 2 fimbriae, we analyzed fimbrial assembly by IEM accordingly (15). In these experiments, *Actinomyces* cells were incubated with specific antibodies (α-CafA and α-FimA), followed by labeling of antibody-bound cells with IgG-conjugated gold particles, and were viewed with a transmission electron microscope. Reminiscent of a typical picture obtained for labeling FimB-containing fimbriae (11, 13), the gold particles labeling CafA were detected at the outer ends of fibers extending from the bacterial envelope in MG1 cells, whereas such signals were absent on mutant bacteria in which either *cafA* or *fimA* was deleted (Fig. 3 *A*, *B*, and *D*); note that the fibers visible in the Δ*fimA* mutant are known to be type 1 fimbriae (11, 13). The missing CafA-specific gold label in strains Δ*cafA* and Δ*fimA* was restored when each protein in the corresponding mutants was expressed by *trans*-complementation using respective recombinant plasmids (Fig. 3 *C* and *E*).

To obtain further evidence that CafA is located at the tip of the type 2 fimbriae, we used double-labeling IEM, whereby fimbrial components were differentially labeled with different sizes of gold particles (11). In the parental MG1 cells, CafA stained with 18-nm gold particles was seen at the tip of FimA structures stained with 12-nm gold particles (Fig. S4*A*, black arrowheads). In the absence of *cafA*, only FimA-labeled structures were detected (Fig. S4*D*). Consistent with our previous report of FimB fimbrial tip localization (11), FimB labeled with 18-nm gold particles was seen at the tip of FimA structures regardless of whether CafA was present or not (Fig. S4 *B* and *E*, white arrowheads), suggesting that FimB and CafA are not colocalized on the same fimbrial structures. Consistent with this conclusion, the 18-nm gold particles labeling CafA were mostly well separated from 12-nm gold particles that specifically labeled FimB (Fig. S4 *C* and *F*).

To address unequivocally whether CafA and FimB are cross-linked to separate FimA polymers, we engineered recombinant CafA and FimB proteins, with each having a His tag inserted upstream of the LPXTG motif for pull-down assays. Each construct was introduced into a corresponding deletion mutant. The cell wall extracts of these strains were isolated by muramidase treatment, and CafA and FimB proteins were purified by affinity chromatography. The eluates collected were then blotted with specific antibodies (i.e., α-CafA, α-FimA, α-FimB) to determine the nature of the purified proteins (Fig. 4). As expected, purified CafA polymers were positive for CafA- and FimA-reactive signals; importantly, these polymers did not contain FimB. Conversely, purified FimB polymers contained FimA but not CafA. Collectively, these results demonstrate that the fimbrial shaft FimA forms two distinct heterodimeric fimbrial structures, one harboring FimB and the other CafA as tip fimbrillins.

Structural Determinants of CafA Required for Its Fimbrial Assembly and Coaggregation. CafA is predicted to harbor two CnaB-like domains at the C terminus, named Cna1 and Cna2 (Fig. 5*A*). First identified in *S. aureus*, CnaB domains have been suggested to serve as "stalks" to orient receptor-binding regions of proteins away from the cell surface (21). To assess whether CnaB folds are important for CafA surface display and/or function, truncations of the two CnaB domains were generated and the resulting constructs were examined by immunoblot analysis and coaggregation assays. For fimbrial polymerization analysis, protein

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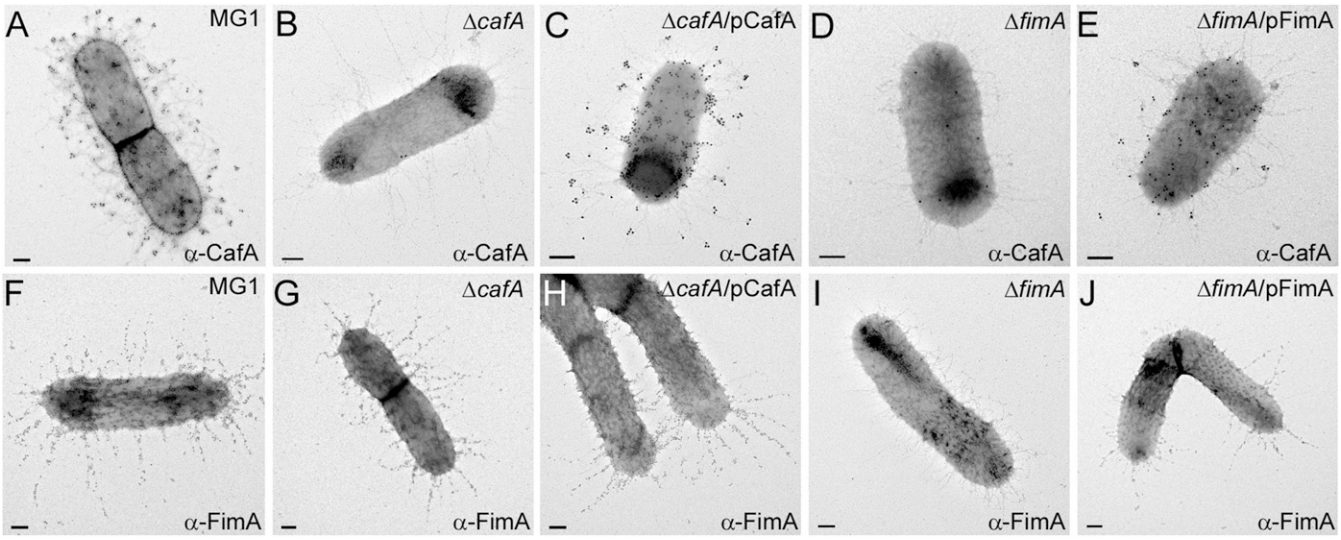

Fig. 3. Localization of CafA on the type 2 fimbrial structures. Cells were immobilized on nickel-carbon grids and stained with antibodies against CafA (*A–E*) or FimA (*F–J*), followed by goat anti-rabbit IgG conjugated to 12-nm gold particles. Grids were stained with 1% uranyl-acetate and viewed with an electron microscope. (Scale bars: 0.2 μm.)

samples collected from the culture medium (M) and cell wall (W) fractions of various *A. oris* strains were subjected to immunoblotting with α-CafA and α-FimA as mentioned previously. Compared with the strain that expressed WT CafA, which produced CafA polymers and monomers found in the M and W fractions, a mutant carrying a version of CafA with a deletion of Cna1 failed to assemble CafA polymers but, instead, secreted CafA into the culture medium in the form of degradation products (Fig. 5*B*; compare lanes pCafA and ΔCna1). In contrast, deletion of Cna2 did not affect CafA incorporation into the fimbriae (Fig. 5*B*, lanes ΔCna2). A close inspection of the protein sequence revealed that the Cna1 domain contained a pair of cysteine residues (Fig. 5*A*). Mutations of these residues to alanine also caused secretion of CafA degradation products into the culture medium, a phenotype that is comparable to the deletion of Cna1 (Fig. 5*B*, lanes Δ1N and C713A/C722A). Consistently, the pilus polymerization defects by mutations of the Cna1 domain and cysteine residues C713/C722 paralleled the coaggregation defect of these *A. oris* mutants with *S. oralis* (Fig. S2).

Interestingly, we noted that pilus polymerization in strains Δ*cafA* and ΔCna1 was different from that in strains pCafA and ΔCna2. In the former, no pilus polymers were found in the culture medium (Fig. 5*C*), suggesting that the tip fimbrillin may affect the process of pilus polymerization, similar to the phenotype of the tip fimbrillin FimQ mutant affecting assembly of the type 1 fimbriae (17). Given the fact that FimA forms two independent fimbrial structures, each with a distinct tip fimbrillin (i.e., FimB, CafA) (Fig. 4), we hypothesized that the presence of either tip fimbrillin would compensate for the loss of the other in fimbrial assembly. To investigate this, we examined pilus assembly of FimA by IEM using individual deletion mutants of *cafA* and *fimB* as well as a double mutant of *cafA* and *fimB* in the type 1 fimbria-negative background (denoted as 1^-). Remarkably, although the number of FimA-labeled fimbriae appeared to be reduced in the absence of *cafA* or *fimB* (Fig. S5*A*, compare panel $1^-,2^+$ with panels 1^-,Δ*cafA* and 1^-,Δ*fimB*), no FimA-labeled fimbriae were detected in the absence of both CafA and FimB (Fig. 5*D*, panel 1^-,Δ*cafA*/Δ*fimB*). This was confirmed by the lack of FimA polymerization in the Δ*cafA*/Δ*fimB* mutant as detected by Western blotting (Fig. S5). Evidently, the necessity of the tip fimbrillins in fimbrial assembly is a general feature in *Actinomyces*, whereas the tip pilins are dispensable for the assembly of the three antigenically distinct types of pili in *C. diphtheriae* (5, 22, 23).

Discussion

In this work, we report the discovery of a bacterial coaggregation factor that hijacks the tip of a pilus fiber for specific interactions between two pioneer bacteria that seed the development of dental plaque, the most complex biofilm known to date. This factor, termed CafA, is one of the 14 predicted cell surface proteins of *A. oris* (strain MG1) not linked to the two fimbrial gene clusters and whose function had not been assessed. To address the function of these predicted proteins, we deleted each of the respective protein-coding genes individually and then investigated whether any one of the mutants affected the known coaggregation process between *A. oris* and *S. oralis*. Only one deletion mutant, Δ*cafA*, showed a coaggregation defect, leading to the discovery of a previously unidentified adhesin specifically involved in a key step in the initiation of oral biofilm development. Significantly, as determined by BLAST analysis, CafA is a highly

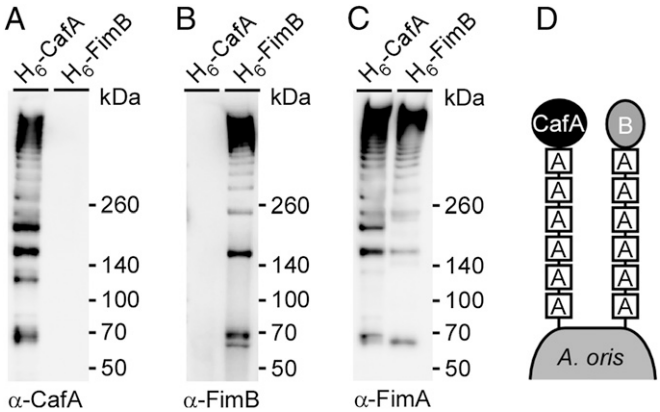

Fig. 4. Distinct fimbrial polymers formed by CafA and FimA independent of FimB and FimA polymers. Cell wall extracts of *A. oris* strain Δ*cafA* or Δ*fimB* expressing CafA or FimB, respectively, with a "6×-His tag inserted upstream of the LPXTG motif, were used for affinity chromatography. Purified proteins were subjected to immunoblotting with α-CafA (*A*), α-FimB (*B*), or α-FimA (*C*). (*D*) Schematic representation shows that *A. oris* assembles two distinct fimbrial structures made of FimA, forming the fimbrial shaft, and CafA or FimB, each constituting the fimbrial tip.

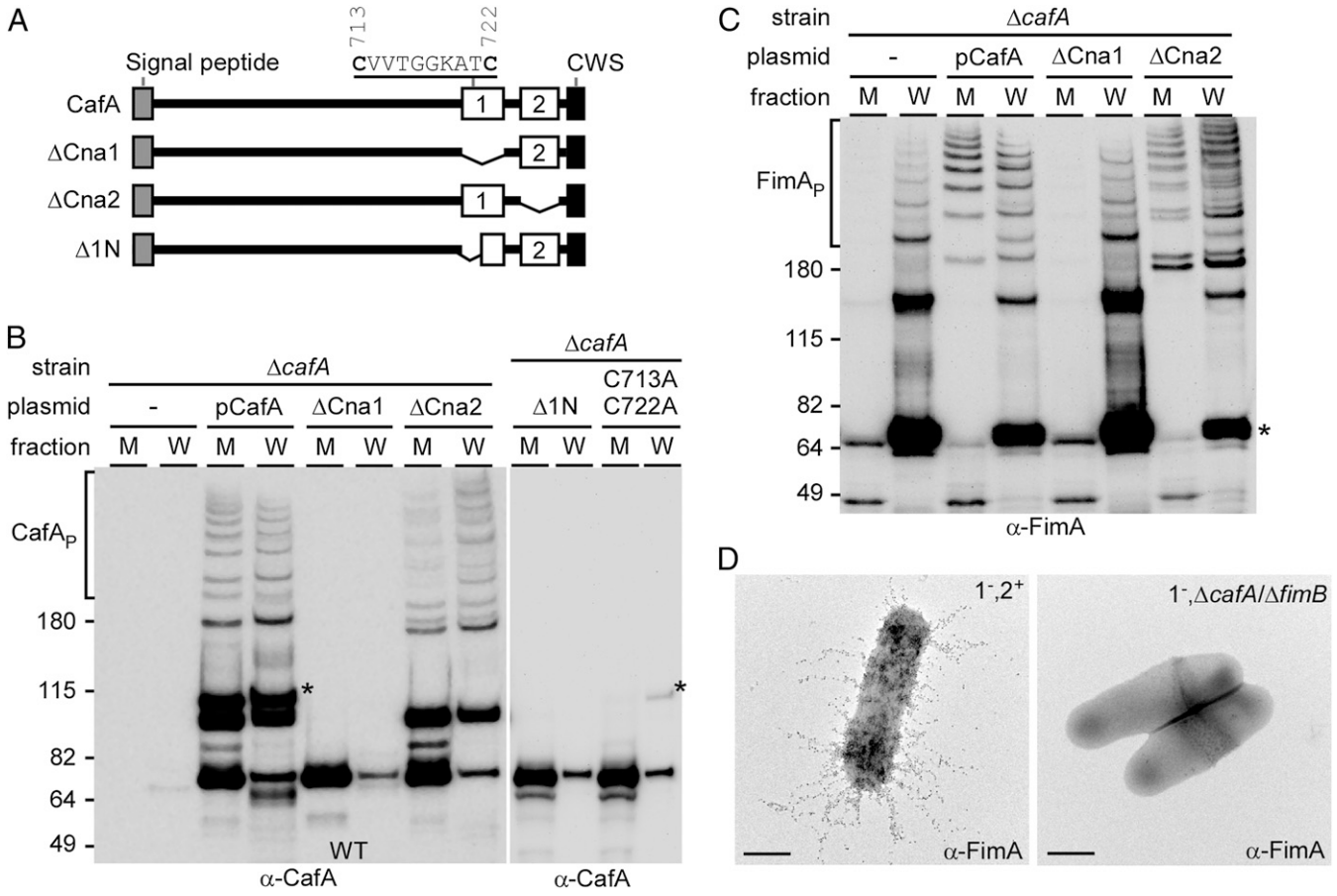

Fig. 5. Structural determinants of CafA required for fimbrial assembly and cell-to-cell interaction. (*A*) Diagram of CafA with a signal peptide, two CnaB-type domains with domain 1 containing a pair of cysteine residues, and a CWSS (CWS). Truncated derivatives of CafA lacking individual CnaB-type domains are shown. Protein samples collected from M and W fractions of *A. oris* strains carrying specific CafA mutations were analyzed by immunoblotting with α-CafA (*B*) or α-FimA (*C*). Polymers (P), monomers (*), and molecular mass markers (kilodaltons) are indicated. (*D*) Bacterial strains were immobilized on nickel-coated carbon grids and stained with α-FimA, followed by goat anti-rabbit IgG conjugated to 12-nm gold particles. (Scale bars: 0.2 μm.)

conserved protein found in many *Actinomyces* species. Our findings thus have implications for potential therapeutic intervention.

Because constant sheer forces pose a physical challenge for bacteria colonizing the oral cavity, a tight cell-to-cell interaction or coaggregation between various colonizing bacteria may not only present a metabolic advantage over planktonic cells but directly facilitate the development of oral biofilms (24). Over 30 y ago, Cisar and coworkers (20, 25) identified the type 2 fimbriae as essential determinants for *Actinomyces* coaggregation to oral streptococci. This was based on the observations that an *Actinomyces* mutant lacking type 2 fimbriae failed to coaggregate with *S. oralis* (25) and that this cell-to-cell interaction was blocked by certain antibodies raised against the type 2 fimbriae (20). More recently, following the sequencing of the *Actinomyces* MG1 genome, genetic and biochemical work done in our laboratory revealed that the type 2 fimbriae are composed of a shaft protein FimA and a tip fimbrillin FimB that are genetically linked together in a fimbrial gene cluster encoding the fimbria-specific sortase (11). Gene deletion experiments led us to conclude that the receptor-mediated coaggregation may require the shaft protein FimA but not the tip protein FimB (13, 15). Although our in vitro experiments suggested a direct interaction between recombinant FimA of *Actinomyces* with surface receptors of oral *Streptococcus* (15), it was rather unusual that the polyclonal antibodies we raised against FimA (or another antiserum that was generated by the Cisar laboratory against the type 2 fimbria (20), which cross-reacts with FimA) do not prevent this receptor-mediated coaggregation process (Fig. 1*B*). The current work has essentially solved this paradox: The key adhesin that allows coaggregation is not FimA, but CafA, whose antibody does prevent bacterial coaggregation. Most importantly, we showed that CafA forms a unique tip of the type 2 fimbriae. Thus, the type 2 fimbriae are assembled in two distinct forms: one that contains FimB, whose function remains to be investigated, and the other harboring CafA, which is indeed the pilus that takes part in the coaggregation process.

Two pieces of evidence lend further support to the surprising conclusion that CafA is the actual coaggregation factor for *A. oris*. First, when *cafA* is deleted from the bacterial chromosome, the *A. oris* mutant cells fail to adhere to either *S. oralis* or RBCs, which are known to share a common RPS with that of *S. oralis* (Fig. 1*A*). Second, the coaggregation is not observed when the receptors are absent from the cell surface of *S. oralis* and RBCs (Fig. 1*A*) and CafA antibody or soluble CafA blocks the binding of bacterial surface-linked CafA to these receptors (Fig. 1*B* and Fig. S2*A*). Thus, CafA directly and specifically interacts with the cell receptors. Interestingly, CafA function is essential for bacterial coaggregation but not for biofilm formation in the presence of sucrose (Fig. S1). This CafA-independent but sucrose-dependent biofilm development is attributed to FimA, which explains the independent ability of FimA to bind polysaccharides in vitro (15), as well as the resistance of coaggregation to the polyclonal antibody raised against FimA.

An unprecedented observation reported in this paper is that CafA is associated with FimA structures, forming a distinct tip independent of the canonical tip FimB, whose gene is linked to the fimbrial gene cluster. This raises a significant question: What

makes CafA unique among all other cell surface proteins to become a component of the type 2 fimbriae? Of the 14 putative surface proteins with the CWSS encoded by the *A. oris* MG1 genome (Table S1), it is only CafA that shows the highest homology to FimB (Expect value of 5×10^{-79} and 36% identities, based on their primary sequences). We hypothesize that CafA may fold into a tertiary structure similar to that of FimB to provide the essential determinants recognized by the pilus-specific sortase SrtC2 for fimbrial tip localization. Although this hypothesis remains to be tested experimentally, it is noteworthy that the C-terminal CWSSs of FimB and CafA are highly similar to each other, specially the presence of the FLIAGxxV motif that is absent from other Aca proteins (Fig. S1*B* and Table S1). This is consistent with our proposal that the CWSS is a major determinant of pilins to serve as the tip that nucleates the assembly of the pilus shaft (14). Interestingly, in this paper, we have also provided compelling evidence that the tip serves as an essential component to initiate fimbrial shaft assembly in *Actinomyces*: The deletion of both *cafA* and *fimB* results in the absence of FimA polymers on the cell surface (Fig. 5*D*). Whether or not this reflects a general rule for pilus assembly in Gram-positive bacteria is an important question that remains to be addressed in future. However, the essentiality of a tip fimbrillin in fimbrial assembly has also been observed in the case of the type 1 fimbriae of *A. oris* (17) and pili of *Streptococcus suis* (26), suggesting a conserved mechanism for fimbrial polymerization in these organisms. It is important to point out that nothing is known at present about how the pilus tip dictates and nucleates the assembly of the shaft or what governs the order in which a pilus polymer is assembled from the various monomeric pilin precursors. Equally puzzling is how expression of FimB or CafA affects fimbrial assembly or whether the expression of either is subject to regulation. Although there is no apparent regulatory element associated with the *fimB* gene cluster, several genes encoding AraC-type transcriptional regulators are linked to *cafA* and transcribed in the opposite direction from *cafA*. It remains to be investigated whether these regulators are genetically linked to *cafA* and control CafA expression and, hence, fimbrial incorporation.

Lastly, an intriguing question emerging from this work is why might there be a reason to display CafA on the bacterial surface via long fimbrial structures. We found that CafA can be anchored as monomers to the cell wall when FimA is present or absent (Figs. 2 and 3), yet the CafA monomeric molecules fail to mediate bacterial coaggregation (Fig. 1*A*). The simplest interpretation of this finding is that *Actinomyces* must have evolved pilus hijacking as a strategy to lengthen the reach of the coaggregation factors to ensure an efficient contact with the cognate receptors on the surface of the cocolonizers of the oral cavity. In the absence of this long-distance interaction, the two interacting partners will have to come into close contact with each other, which must be much less efficient stochastically than the long-distance contacts.

Materials and Methods

Strains, plasmids, and primers used in this study are listed in Tables S2–S4 of the *Supporting Information*, which contains information on recombinant plasmids, gene deletion, protein purification, cell fractionation and Western blotting, IEM, biofilm formation, and coaggregation assays. *A. oris* was grown in heart infusion (HI) broth or on HI agar plates. *S. oralis* was grown in HI broth supplemented with 1% glucose, whereas *Escherichia coli* cells were cultivated in Luria broth. When required, kanamycin was added at a concentration of 50 μg/mL.

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