Dental plaque is a complex biofilm community, the development of which is spatiotemporally regulated by sequential colonization of specific Gram-positive and Gram-negative oral bacteria (18, 21, 33, 34). Attachment of Gram-positive actinomyces and streptococcal species to the tooth surface is a critical early step in biofilm development (33) because their adherence to the tooth surface allows subsequent binding and colonization of both the bridging-type bacteria, such as *Fusobacterium nucleatum*, and the late colonizers, such as *Porphyromonas gingivalis* (17, 22). The involvement of *Actinomyces* spp. in this process depends on two functionally and antigenically distinct types of cell surface polymeric structures known as fimbriae or pili, which mediate adhesion of actinomyces to dental and mucosal surfaces and interactions with streptococci, as well as other members of the biofilm community (7, 33, 44). Type 1 fimbriae mediate adhesion of actinomyces to the tooth surface through binding of adsorbed salivary proline-rich proteins (PRPs). This interaction was initially revealed by adhesion of *Actinomyces oris* T14V to adsorbed acidic PRP1 (16), a nonglycosylated PRP. Subsequent studies (8) showed that other PRPs, including acidic, basic, and glycosylated proteins, also support type 1 fimbria-mediated adhesion. Extensive homology exists between different PRP sequences, which include repeat regions, and the actual sequence or sequences involved in type 1 fimbria binding have not been identified (8). While type 1 fimbriae recognize protein receptors, type 2 fimbriae recognize specific saccharide motifs present in both streptococcal coaggregation receptor polysaccharides (RPS) and host cell surface glycoconjugates (5–7). Elucidating the mechanism of assembly of these polymers and the precise molecular nature of the underlying host-bacterial and interbacterial interactions is central to our understanding of the development of oral biofilm and the initiation of inflammation at surrounding sites.

The experimental evidence so far (2, 28, 29, 46, 47) has established that the assembly of *Actinomyces* fimbrae occurs by a common sortase-mediated mechanism that was first described in *Corynebacterium diphtheriae* (41) and later in many other Gram-positive pathogens (19, 25, 26, 32, 36, 39). In *C. diphtheriae*, five pilin/fimbrillin-specific sortase genes are organized in three pilus gene clusters (13, 38, 42). Each gene cluster encodes a distinct pilus type comprised of three pilin/fimbrillin-specific sortase genes, *spaA*, *spaB*, and *spaC*, which produces SpaA, forming the pilus shaft; SpaC, located at the tip; and SpaB, at the base, as well as along the pilus structure (24, 42). The pilin-specific sortase SrtA is the sole enzyme that catalyzes polymerization of individual pilins into a high-molecular-weight pilus polymer (37, 42). Surface display of this polymer via its anchoring to the cell...
wall peptidoglycan is catalyzed by the housekeeping sortase SrtF (37), whose gene is located elsewhere in the *C. diphtheriae* genome (42). Importantly, while the deletion of *spaA* abrogates pilus structures, deletion of *spaC* does not, as the tip pilin, SpaC, is dispensable for pilus polymerization (40). Although SpaB is also dispensable for pilus polymer formation, polymers made in the absence of SpaB are secreted into the extracellular milieu (24), much like those made in the absence of *srtF* (37). This is consistent with the role of SpaB as a molecular switch that terminates pilus polymerization and leads to the cell wall anchoring of pilus polymers (24, 25). Intriguingly, SpaB homologs have not been found in some organisms, including *Actinomyces*, suggesting a different termination mechanism for pilus assembly in these microbes. Moreover, unlike the heterotrimeric pilus of corynebacteria, the fimbriae of *Actinomyces* and pili of *Bacillus cereus* (1) are heterodimeric.

As is typical of pili (36, 39, 41), the type 1 and type 2 fimbriae of *A. oris* are each composed of a major protein subunit (12, 45). Moreover, the genes encoding the fimbrial subunits for each fimbria are clustered together with one for a class C sortase (46, 47). Our recent studies in *Actinomyces oris* MG-1, formerly known as *Actinomyces viscosus* MG-1 (11), demonstrated that the type 2 fimbria, encoded by the gene locus *fimB-fimA-srtC2*, is composed of the previously identified FimA, which forms the fimbrial shaft, and a minor subunit, FimB, which is a tip fimbrillin (28). Importantly, we showed that while the formation of the type 2 fimbrial structure absolutely requires both *fimA* and *srtC2*, the tip fimbrillin gene *fimB* is dispensable for fimbrial assembly. Bioinformatics analysis of the MG-1 genome also revealed a type 1 fimbrial gene cluster, *fimQ-fimP-srtC1-fimR* (28), similar to those described in both *Actinomyces naeslundii* T14V (47) and strain ATCC 19246 (23). By electron microscopy, we demonstrated that the type 2 fimbria, encoded by the gene locus *fimB-fimA-srtC2*, is composed of the previously identified FimA, which forms the fimbrial shaft, and a minor subunit, FimB, which is a tip fimbrillin (28). Importantly, we showed that while the formation of the type 2 fimbrial structure absolutely requires both *fimA* and *srtC2*, the tip fimbrillin gene *fimB* is dispensable for fimbrial assembly. Bioinformatics analysis of the MG-1 genome also revealed a type 1 fimbrial gene cluster, *fimQ-fimP-srtC1-fimR* (28), similar to those described in both *Actinomyces naeslundii* T14V (47) and strain ATCC 19246 (23). By electron microscopy, we showed that FimP forms the fimbrial shaft, with FimQ at the tip (28). Studies by Chen et al. (2) showed that a mutant strain lacking the *srtC1* sortase failed to produce FimP polymers and exhibited ~40% reduction in binding to saliva-treated hydroxypatite (SHA). In contrast, a mutant lacking *fimQ* displayed ~75% reduction in SHA binding, suggesting that FimQ is critical for type 1 fimbra-mediated adhesion reduction. Moreover, it was also shown that deletion of *fimQ* reduced the presence of surface-associated FimP, which is noteworthy, as similar findings have not been made in studies of tip pili from other Gram-positive species. However, for *A. oris*, the specific requirements of each fimbrillin for assembly of type 1 fimbriae, as well as for receptor recognition, remained unresolved.

The fact that the *fimQ* mutant exhibits some defects in surface display of the fimbrial shaft FimP, as well as in adherence to SHA (10), and that antibodies raised against the type 1 fimbriae can block bacterial adherence to SHA (28) imply that FimQ may play a positive role in fimbrial assembly and function as a fimbrial adhesin. This is important to establish, as little work has demonstrated the role of tip pili in Gram-positive pilus assembly. We therefore carried out a comprehensive molecular genetic analysis of the *fimQ*-fimP-srtC1-fimR gene cluster. Consistent with the role of FimP as the fimbrial shaft subunit, deletion of *fimP* abrogates the type 1 fimbrial structures whose assembly is dependent on the fimbrillin-specific sortase SrtC1. Remarkably, we find that the tip fimbrillin FimQ plays a necessary role in fimbrial assembly. This is opposite to the situation that we reported for the type 2 fimbriae. Also surprising is our finding, based on genetic analysis, that it is the tip protein, FimQ, rather than the shaft protein, FimP, that serves as the major adhesin of the type 1 fimbriae. In fact, using recombinant proteins, we succeeded in demonstrating in vitro that FimQ is necessary and sufficient to mediate adherence to PRPs, the receptors of the type 1 fimbriae.

**MATERIALS AND METHODS**

*Bacterial strains, plasmids, and media.* The bacterial strains and plasmids used in this study are listed in Table 1. *A. oris* strains were grown in heart infusion broth (HIB) or on HIB agar plates for most experiments and in complex medium containing 0.2% glucose (4) for dot immunoblotting and adhesion assays. *Escherichia coli* strains were grown in Luria broth. When needed kanamycin or streptomycin was added at a concentration of 50 μg/ml (1). Rabbit-raised polyclonal antibodies against recombinant fimbrial proteins were previously obtained (28). Reagents were purchased from Sigma unless otherwise indicated.

**Plasmid construction.** (i) *pFimP.* The promoter region of *fimQ* was generated by PCR with the primer pair Pcom-fimQ-F/Pcom-fimQ-R, and the *fimP* fragment was amplified with primers com-fimP-F/com-fimP-R (Table 2). The *fimQ* promoter amplicon was digested with KpnI and EcoRI. The two fragments were then ligated into the KpnI and EcoRI sites of the *E. coli/Actinomyces* shuttle vector pJRD215 to generate pFimP.

(ii) *pFimQ* and *pFimQΔP,L,PS,G.* A fragment encompassing the promoter region of the *fimQ* gene and its open reading frame was PCR amplified with primers com-fimQ-F/com-fimQ-R. The *fimQ* amplicon was digested with NdeI and EcoRI and ligated into pJRD215 precut with the same enzymes to generate pFimQ. To generate pFimQΔP,L,PS,G, the *fimQ* fragment described above from pFimQ cut with NdeI and EcoRI was cloned into pHT177 (28). The generated plasmid was used as a template for mutagenesis of FimQ by inverse PCR using primers FimQΔP,L,PS,G-F and FimQΔP,L,PS,G-R (Table 2). The PCR product was purified by gel extraction and phosphorylated to facilitate religation of the amplicon into circular plasmids, which were then transformed into *E. coli* DH5α. The mutagenized *fimQ* fragment was digested from the resulting plasmid with NdeI and EcoRI and cloned into pJRD215. The desired mutation was confirmed by DNA sequencing.

(iii) *pSrtC1.* The primer pair Pcom-fimQ-F/Pcom-fimQ-R was used to PCR amplify the promoter region of *fimQ*, whereas primers com-srtC1-F/com-srtC1-R were for the *srtC1* open reading frame. The *fimQ* promoter amplicon was digested with KpnI and NdeI and the *srtC1* amplicon with NdeI and EcoRI. The two fragments were then ligated into the KpnI and EcoRI sites of the shuttle vector pJRD215 to generate pSrtC1.

**Gene deletions in *A. oris.*** (i) Generation of in-frame deletion mutants of *fimP* and *fimQ.* To create an in-frame deletion construct of *fimP* or *fimQ*, 1.5-kb fragments upstream and downstream of *fimP* or *fimQ* were amplified by PCR using primers fimP-up-F/fimP-up-R and fimQ-up-F/fimQ-up-R and fimQ-dn-F/fimQ-dn-R, respectively. The upstream fragment was digested with EcoRI and KpnI, the downstream fragment with KpnI and XbaI, and the deletion vector pCWU2 with EcoRI and XbaI. The upstream and downstream fragments and the vector were ligated together in a single step, and the resulting plasmids, pCWU2-fimP and pCWU2-fimQ, were confirmed by restriction enzyme digestion. The generated plasmids were then introduced into the *A. oris* ΔgalK strain by electroporation. Selection of corresponding in-frame deletion mutants was performed using a previously described method based on GalK as a counterselectable marker (28). To generate *fimQ* and *fimA* double mutants, pCWU2-fimQ was electroporated into the ΔfimA mutant, and selection for the ΔfimQA mutant was performed as mentioned above. The double mutants were characterized by Western blotting with antibodies against FimA or FimQ.

(ii) **Generation of in-frame deletion mutants of *srtC1.*** In-frame deletion mutants of *srtC1* were generated following a published protocol that uses mCherry as a counterselectable marker (43). Briefly, a deletion construct of *srtC1* was generated using the procedure described above with primers SrtC1-up-F/SrtC1-up-R and SrtC1-dn-F/SrtC1-dn-R. The amplified fragments were
TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and description</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>A. oris MG1</td>
<td>Type strain, expressing type 1 and 2 fimbriae</td>
<td>28</td>
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<tr>
<td>A. oris CW1</td>
<td>ΔgalK; an isogenic derivative of MG1</td>
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<tr>
<td>A. oris WU1</td>
<td>ΔfimQ; an isogenic derivative of CW1</td>
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<td>WU1 containing pFimQ</td>
<td>Thistudy</td>
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<tr>
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<td>ΔfimP; an isogenic derivative of CW1</td>
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<td>ΔsrtC1; an isogenic derivative of MG1</td>
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<td>ΔfimA; an isogenic derivative of CW1</td>
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<td>ΔfimQA; an isogenic derivative of CW1</td>
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<td>A. oris WU9</td>
<td>WU6 containing pFimQ_{ALPLSG}</td>
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Plasmids

<table>
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<th>Reference or source</th>
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<tr>
<td>pFimPe</td>
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<td>pCWU2-fimP</td>
<td>pCWU2 allelic replacement of fimP</td>
<td>Thistudy</td>
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<tr>
<td>pCWU2-fimQ</td>
<td>pCWU2 allelic replacement of fimQ</td>
<td>Thistudy</td>
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<tr>
<td>pCWU3</td>
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<td>pFimP</td>
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<td>pFimQ</td>
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</tr>
<tr>
<td>pFimQ_{ALPLSG}</td>
<td>pJRD215 expressing FimQ with deletion of the LPLSG motif</td>
<td>Thistudy</td>
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</tbody>
</table>

cloned into pCWU3 (43) precut with EcoRI and XbaI after digestion with appropriate enzymes. The generated plasmids were then introduced into the A. oris MG-1 strain by electroporation, and selection for in-frame deletion mutants was performed as described previously (43). Deletion mutants were confirmed by Western blotting with anti-SrtC1 (α-SrtC1), as well as Southern hybridization analysis.

**Fimbrial detection.** For cell fractionation and Western blotting, a previously published protocol was adapted (29). Briefly, overnight cultures of *Actinomyces*...
myces were diluted 40-fold in HIB medium and maintained at 37°C. Streptomycin was added to a final concentration of 50 μg ml⁻¹ when necessary. All strains were grown to an optical density at 600 nm (OD₆₀₀) of ~0.2 to 0.3. Normalized aliquots were fractionated into medium (M) and cell pellets by centrifugation. Soluble cell wall (W) fractions were obtained by treating the cell pellets with mutanolysin (300 U ml⁻¹) in SMM buffer (0.5 M sucrose, 10 mM MgCl₂, and 10 mM maleate, pH 6.8) at 37°C overnight, followed by centrifugation. The culture medium and cell wall fractions were subjected to trichloroacetic acid (TCA) precipitation and an aceton wash. The protein samples were then boiled in SDS-containing buffer, separated on 4 to 12% Tris-glycine gradient gels (Invitrogen), and subjected to immunoblotting with corresponding rabbit antiserum (1:10,000 for α-FimP and 1:1,000 for α-FimQ) and detection by chemiluminescence.

For dot immunoblotting, the procedure was followed as previously described (29) with different specific antibodies. Briefly, nitrocellulose membranes were spotted with decreasing numbers of actinomyces; blocked in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 2% skim milk; incubated with rabbit α-FimP serum (1:10,000), α-FimQ serum (1:1,000), or α-FimA serum (1:10,000); washed; incubated with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad); and developed using a metal-enhanced DAB substrate kit (Pierce Biotechnology).

For immunoelectron microscopy, Actinomyces cells grown on HIB agar plates were subjected to immunogold labeling with α-FimP (1:100 dilution) or α-FimQ (1:50), followed by 12-nm gold-goat anti-rabbit IgG (Jackson Immunoresearch) as previously described (29). The samples were finally washed with water before being stained with 1% uranyl acetate and viewed in a JEOL JEM-1400 electron microscope.

**Bacterial adhesion assays.** Actinomyces type 1 fimbrin-mediated aggregation of PRP-coated latex beads was performed as previously described (16) with minor modifications that included the use of parotid saliva-coated latex beads rather than PRP1-coated beads. This modification was based on results from a previous study (8) that showed adhesion of A. oris T14V to a number of parotid saliva components and no adhesion of the type 1 fimbria mutant strain S5951 to any component in parotid saliva. PRP-coated latex beads were prepared in a glass tube by overnight incubation at 4°C of 0.1 ml polystyrene beads (6.8-μm particles; 5% [wt/vol]; Spherotech Inc.) with 20 μl whole parotid saliva (35) in 2 ml of 50 mM sodium carbonate buffer, pH 9.5. Immediately prior to their use in aggregation assays, the beads were pelleted by low-speed centrifugation, suspended in 20 mM Tris-buffered (pH 7.4) saline containing 2 mg/ml bovine serum albumin (TBS-BSA), and dispersed by brief sonication. Aggregation assays were set up in individual wells of 24-well cluster plates by adding 200 μl TBS-BSA, 200 μl coated beads (0.25% [wt/vol]), and 60 μl actinomyces cell suspension (approximately 2 × 10⁵ ml⁻¹), which were prepared for all strains as previously described (29). The plates were incubated for 1 to 2 min on a rotary shaker prior to photography of individual wells, which were illuminated by indirect light against a black background.

**ELISA-based determination of FimP and FimQ binding to salivary PRPs.** Actinomyces fimbrillins FimA, FimP, and FimQ (Table 1) were expressed in E. coli and purified as previously described (28). An enzyme-linked immunosorbent assay (ELISA)-based binding assay was designed to measure the binding affinities of these His-tagged recombinant proteins to adsorbed PRPs from whole parotid saliva. Individual wells of ELISA plates (Greiner Bio-one) were coated with 100 μl of PRPs (1/100 parotid saliva) in PBS buffer (pH 7.4) at 4°C for 18 h. After they were washed and blocked with 100 μl of 1% BSA, the plates were incubated with His-tagged recombinant proteins in 100 μl of 1% BSA for 1 h. Bound recombinant proteins were detected by incubation with Penta-His horsedarhis peroxidase (HRP) conjugate (Qiagen; 1:2,000 dilution), followed by addition of 100 μl chromogenic substrate [0.2% citrate acid, pH 4.1, 0.04% ABTS (2, 2′-azino-bis[3-ethyl benothiazoline-6-sulfonic acid]) 0.1% H₂O₂]. α-FimP was also used for FimP detection. The binding signal was quantified by an automated M1000 plate reader (Tecan) at 405 nm (A₄₀⁵). The experiments were performed in triplicate with His-tagged FimA as a control.

**RESULTS**

Sortase SrtC1 catalyzes the surface assembly of type 1 fimbriae composed of the shaft fimbrillin FimP and the tip fimbrillin FimQ. The type 1 fimbria of A. oris MG-1 is encoded by the gene locus fimQ-fimP-srtC1-fimR (Fig. 1A). To determine the genetic determinants required for the molecular assembly of the type 1 fimbriae, we systematically generated nonpolar, in-frame deletion mutants lacking fimP, fimQ, srtC1, or fimR, using recently developed gaiIk or mCherry allelic exchange systems (29, 43). For analysis of type 1 fimbrial production, cultures of strain MG-1 and its isogenic derivatives grown to early log phase. Equivalent protein samples were separated on a 4 to 12% Tris-glycerine gradient gel and detected by immunoblotting with α-FimP (B) or α-FimQ (C). The positions of monomers (FimP/O₄₆₄) and high-molecular-mass products (FimP/O₄M₆₅₅) of fimbrial polymerization and molecular mass markers are indicated.
As shown in Fig. 1, MG-1 cells produced multiple high-molecular-weight species of fimbrial polymers (designated FimPHMW and FimQHMW) that were stacked at the top of gradient gels, unlike the FimP and FimQ monomers (FimPM and FimQM), which migrated around the 55- and 171-kDa markers, respectively (Fig. 1B and C, lanes WT). Deletion of fimP completely eliminated all FimP-reactive species (FimPHMW and FimPM); although FimQM was detected in this mutant, the production of any FimQHMW species was also abolished by fimP deletion (Fig. 1C, lanes fimP). These defects of the fimP mutant were rescued by expressing FimP from a recombinant plasmid (Fig. 1B and C, lanes fimP/pFimP). Surprisingly, deletion of fimQ significantly reduced the amount of FimPHMW species without affecting the expression of FimP (Fig. 1B and C, lanes fimQ). FimP polymerization was restored in the fimQ mutant by a plasmid expressing FimQ (Fig. 1B and C, lanes fimQ/pFimQ). Thus, FimQ somehow facilitates FimP polymerization. Consistent with the essential role of pilin-specific sortases in pilus polymerization (25), formation of FimPHMW and FimQHMW polymers was completely abolished in the ΔsrtC1 strain (Fig. 1B and C, lanes ΔsrtC1). This defect was solely due to the absence of the fimbria-specific sortase SrtC1, as introduction of a complementing plasmid expressing SrtC1 into the ΔsrtC1 mutant restored fimbrial polymer production (Fig. 1B and C, lanes ΔsrtC1/pSrtC1). Note that significant amounts of FimP and FimQ monomers were still detected in the cell wall fractions in the absence of srtC1 (Fig. 1B and C, lanes ΔsrtC1; compare the M and W fractions). Lastly, no defect in fimbrial polymerization was apparent in strain ΔfimR lacking fimR (Fig. 1B and C, rightmost two lanes), which has been proposed to encode a putative prepilin peptidase (28). Together, these results establish the overall genetic requirements for polymerization of type 1 fimbriae.

Sortase SrtC1 is required for the polymerization of type 1 fimbriae, but not for surface display of monomeric fimbrillins. We next extended the above-mentioned studies by immuno-electron microscopy (IEM) (i) to establish the architecture of the type 1 fimbriae and (ii) to determine whether surface assembly of the type 1 fimbriae and/or fimbrillins is affected by the above-mentioned mutations. Using a previously developed protocol (29), we labeled A. oris cells of various strains with the specific antisera α-FimP and α-FimQ, followed by staining with 12-nm gold particles conjugated with IgG, and the cells were viewed with an electron microscope. With MG-1 cells, FimP signal was observed abundantly along the fimbrial structures, as well as on the bacterial surface (Fig. 2A), whereas FimQ signal was located both on the bacterial surface and at a distance from the cell surface (Fig. 2D). These signals were absent in strains lacking fimP or fimQ (Fig. 3). Deletion of srtC1 in MG-1 abrogated assembly of type 1 fimbriae; however, the mutation did not abolish surface display of FimP and FimQ, nor did it affect the assembly of other fimbriae, i.e., type 2 fimbriae (Fig. 2B and E and data not shown). Complementation of the ΔsrtC1 mutant with the pSrtC1 plasmid restored type 1 fimbrial assembly to the wild-type level (Fig. 2C and F). Altogether,
these results indicate that the type 1 fimbria of MG-1 has a FimP shaft with FimQ in the tip region and that the assembly of this heterodimeric structure requires the fimbria-specific sortase SrtC1. Nonetheless, SrtC1 appeared to be dispensable for surface display of the monomeric fimbrillins FimP and FimQ.

The tip fimbrillin FimQ is involved in fimbrial assembly. Results from Western blotting (Fig. 1) indicated that FimP is essential for the formation of fimbrial polymers and, furthermore, that the presence of FimQ may be important in the assembly of polymeric FimP. To extend these observations to the cell surface, we examined the corresponding strains of Actinomyces oris using IEM as described above. Examination of the ΔfimP mutant revealed abundant clusters of FimQ signal but no labeled fimbriae (Fig. 3A and B), which were restored by expression of FimP from a plasmid (compare Fig. 3C and D to Fig. 2A and D). Remarkably, with the fimQ deletion mutant, FimP fibers were not only less abundant but also shorter than the fimbriae expressed on the wild-type strain MG-1; this defect was rescued by pFimQ (Fig. 3G and H), but not by pFimQH9004LPLSG, which expresses mutant FimQ with deletion of the LPLSG motif (data not shown).

The abundant presence of unlabeled fimbriae on the ΔfimP mutant (Fig. 3) is readily explained by the presence of type 2 fimbriae (28, 29). However, to avoid potential ambiguities in data interpretation, we examined FimP assembly in a ΔfimA mutant devoid of type 2 fimbriae (29). Deletion of fimA did not affect formation of type 1 fimbriae, which were abundant on this mutant (Fig. 4A). However, when fimQ was deleted, the presence of fimbriae was greatly reduced (Fig. 4B). These findings were verified by IEM, which revealed that long fimbrial structures labeled cells with α-FimP on the ΔfimA mutant but only a few short FimP-stained fibers on the ΔfimQA double mutant (Fig. 4C to F).

Altogether, these results firmly establish the positive involvement of FimQ in the assembly of the type 1 fimbriae.

The tip fimbrillin FimQ is required for bacterial adherence to salivary PRPs. Bacterial adhesins frequently occur as tip pilins, which are ideally positioned to interact with host cell receptors (20). However, notable exceptions exist, as indicated by our recent identification of the shaft fimbrillin FimA as the Gal/GalNAc-binding adhesin of Actinomyces type 2 fimbriae (29). To identify the PRP-binding adhesin(s) of type 1 fimbriae, we compared the wild-type and recently described mutant strains of Actinomyces for both aggregation of PRP-coated latex beads (16) and surface expression of specific fimbrillins. The wild-type strain MG-1 and the ΔgalK mutant used to construct fim gene mutants were indistinguishable; both aggregated strongly with PRP-coated latex beads, and both gave comparable reactions in dot immunoblotting for cell surface FimP, FimQ, and FimA (Fig. 5). In contrast, the ΔsrtC1, ΔfimP, and ΔfimQ mutants all failed to aggregate with PRP-coated beads. However, despite their similar nonadhesive phenotypes, these mutants were distinguished by dot immunoblotting with α-FimP and α-FimQ (Fig. 5). Thus, the ΔsrtC1 mutant bound small but detectable amounts of both α-FimP and α-FimQ and the ΔfimQ mutant bound no α-FimP and an amount of α-FimQ that was near the wild-type level, while the ΔfimP mutant bound no α-FimQ and an amount of α-FimP that was significantly below the wild-type level. Each mutant phenotype was fully restored to wild type by expression of the deleted gene from a plasmid. Nonetheless, from these findings, it was unclear whether FimP or FimQ was the PRP-binding adhesin.

Conceivably, the failure of either the ΔfimP or ΔfimQ mutant to adhere might be due to suboptimal cell surface presentation of FimQ on the ΔfimP mutant or to the re-
produced level of FimP on the ΔfimQ mutant. To compensate for these defects, we wondered whether increasing fimbrillin gene expression might enhance bacterial aggregation to PRP-coated beads. To examine this possibility, we introduced a plasmid expressing fimQ into the ΔfimP strain and, conversely, a plasmid expressing fimP into the ΔfimQ mutant strain. Introduction of pFimP into the ΔfimQ mutant did not restore adhesion despite increased cell surface production of FimP (Fig. 5, lane ΔfimQ/pFimP). In sharp contrast, adhesion was restored following the introduction of pFimQ into the ΔfimP mutant, even though the increase in cell surface FimQ production detected by dot immunoblotting was modest at best (Fig. 5, lane ΔfimP/pFimQ). Thus, in these studies, cells that produced only FimQ aggregated with PRP-coated beads, whereas cells that produced only FimP did not.

To further assess the adhesin function of FimQ, we used an ELISA-based assay to measure binding of recombinant FimP and FimQ proteins that lacked both the amino-terminal export signal and the carboxy-terminal cell wall sorting signal. In this assay, ELISA plates were coated with PRP-rich parotid saliva and then incubated with purified His-tagged FimA, FimP, or FimQ at concentrations up to about 30 μg recombinant protein per ml. Binding of His-tagged proteins was determined using penta-His HRP conjugate (Qiagen) and a chromogenic substrate (see Materials and Methods). The results were clear-cut; recombinant FimQ bound to the PRP-coated surface in a dose-dependent manner, whereas recombinant FimP failed to bind (Fig. 6). Recombinant FimA, the major subunit of type 2 fimbriae, also failed to bind, thereby strengthening the identification of FimQ as the PRP-binding adhesin.

FIG. 4. The tip fimbrillin FimQ is required for surface assembly of type 1 fimbriae. (A and B) Cells of ΔfimA or ΔfimQA mutants were stained 1% uranyl acetate before being analyzed by transmission electron microscopy. Cells of the ΔfimA or ΔfimQA mutant were subjected to immunogold labeling with α-FimP (C and D) or α-FimQ (E and F) and goat anti-rabbit IgG conjugated to 12-nm gold particles. Samples were viewed by transmission electron microscopy after being stained with 1% uranyl acetate. Scale bars, 0.2 μm.

FIG. 5. Receptor recognition of A. oris type 1 fimbriae depends on FimQ. Strains were examined for aggregation of PRP-coated latex beads (magnification, ×1.25) and for reactivity with α-FimP, α-FimQ, and α-FimA by dot immunoblotting. The last antibody, which reacted similarly with all strains, was included as a spotting control.

DISCUSSION

The initial attachment of early bacterial colonizers, such as A. oris and various species of oral streptococci, to the tooth surface is critical in the development of dental plaque. For A. oris, this is achieved by the type 1 fimbriae via their interaction with the salivary PRPs that coat the tooth enamel (14–16). Although the role of type 1 fimbriae in this interaction was described in the early 1980s (9, 16), the molecular architecture, genetics, and machinery for assembly of the covalently linked fimbrial structure, which began to emerge from the pioneering work of Yeung (44), have only recently been elucidated (2, 28). Recent advances in this area are due in large part to the development of molecular methods for studies of Gram-positive pili (41) and a
FIG. 6. Binding of recombinant protein FimQ to a PRP-coated surface. Recombinant His-tagged proteins at increasing concentrations were added to ELISA plates coated with PRPs. Binding was quantified by ELISA at an absorbance of 405 nm. The points represent the means, with error bars showing triplicate measurements.

genetic toolbox for Actinomyces (29, 43). From this work, we now know that the type 1 fimbria is encoded by the gene locus fimQ-fimP-srtC1, with FimP constituting the fimbrial shaft and FimQ displayed in the tip region, and that polymerization of these subunits into a high-molecular-weight structure requires the fimbria-specific sortase SrtC1 (2, 28). While the requirement for this sortase for fimbrial polymerization was revealed in earlier studies (2), the function of individual type 1 fimbrijins remained to be deciphered. In this report, we have established that while the sortase-mediated assembly of the type 1 fimbral does not directly affect the synthesis of adhesins, it does associate with FimR. Importantly, FimQ was shown to be the bona fide adhesin of the type 1 fimbral, regardless of whether it is displayed as monomers or aggregated with PRP-coated latex beads (results not shown). This does not, however, exclude the possibility that a quantitative assay for type 1 fimbral polymerization might reveal some inhibition. Instead of pursuing this possibility, we took a genetic approach to the problem of adhesin identification by overexpressing each fimbriin. The results were clear; bacteria that overexpressed fimQ aggregated with PRP-coated latex beads, while those that overexpressed fimP did not (Fig. 5).

Another finding that merits notice is the present identification of FimQ as the PRP-binding adhesin of Actinomyces type 1 fimbral. This finding is consistent with results from previous studies in which certain antibodies against type 1 fimbriae effectively blocked the attachment of actinomyces to saliva-treated hydroxyapatite (10, 30) while others did not, including those known to be FimP specific (3). In the present study, incubation of Actinomyces with α-FimP or α-FimQ did not block subsequent aggregation of PRP-coated latex beads (results not shown). This does not, however, exclude the possibility that a quantitative assay for type 1 fimbral-mediated adhesion might reveal some inhibition. Instead of pursuing this possibility, we took a genetic approach to the problem of adhesin identification by overexpressing each fimbriin. The results were clear; bacteria that overexpressed fimQ aggregated with PRP-coated latex beads, while those that overexpressed fimP did not (Fig. 5).

An important finding from the present study is that the type 1 fimbral mediates adhesion, and it opens a direct approach for identification of the type 1 fimbral mediator. In the assembly of pili on the Gram-positive swine pathogen Streptococcus suis has recently been described (31). Further studies of such systems are needed to clarify the mechanisms that control pilus/fimbrial assembly in Gram-positive bacteria and to define how the process is regulated.

Using newly developed genetic tools in the present study, we generated in-frame, nonpolar deletion mutants of each individual gene in the type 1 fimbral gene locus (Fig. 1A). From characterization of these mutants, we established for the first time that fimP is essential for type 1 fimbral formation. This conclusion is based on the fact that in the ΔfimP mutant no cell wall-linked FimP and FimQ polymers were observed by immunoblotting (Fig. 1) and no fimbrial structures labeled with α-FimP or α-FimQ were detected by IEM (Fig. 3). We also showed that deletion of fimR does not affect the production of FimP and FimQ polymers, which adds to the evidence that fimR (i.e., orfC), which was predicted to encode a preprotein peptidase (23), is not required for type 1 fimbral production. Importantly, we obtained evidence that despite its essential role for surface assembly of the type 1 fimbral, the fimbral-specific sortase SrtC1 is not required for surface display of FimP and FimQ monomers (Fig. 2). In accordance with studies of pilus assembly in other Gram-positive bacteria (25, 26) and cell wall anchoring of protein A in Staphylococcus aureus (27), we assume that cell wall anchoring of monomeric FimP and FimQ in the ΔsrtC1 mutant (Fig. 2) depends on the housekeeping sortase SrtA. However, this assumption has not been confirmed, as several attempts to generate a deletion mutant for the srtA gene of A. oris have so far been unsuccessful.

An important finding from the present study is that the tip fimbrillin FimQ is required for optimal surface assembly of the type 1 fimbral, which is supported by several lines of evidence. First, the formation of cell wall-linked FimP polymers detected by SDS-PAGE analysis was vastly diminished in the ΔfimQ mutant (Fig. 1), as were the number and length of α-FimP-reactive polymers seen by IEM (Fig. 3 and 4). In addition, deletion of fimQ reduced the presence of cell surface FimP by approximately 3-fold, as shown by dot immunoblotting (Fig. 5). Conceivably, the joining of FimP to FimQ may facilitate polymerization of FimP and/or serve as a rate-limiting step in the assembly of type 1 fimbral. Thus, in the absence of FimQ, the rate or efficiency of polymerization may be reduced. While the underlying mechanism of this effect remains to be determined, it is probably not limited to Actinomyces. In fact, the involvement of a tip pilin in the assembly of pili on the Gram-positive swine pathogen Streptococcus suis has recently been described (31). Further studies of such systems are needed to clarify the mechanisms that control pilus/fimbrial assembly in Gram-positive bacteria and to define how the process is regulated.

Importantly, FimQ was shown to be the bona fide adhesin of the type 1 fimbral, regardless of whether it is displayed as monomers or aggregated with PRP-coated latex beads (results not shown). This does not, however, exclude the possibility that a quantitative assay for type 1 fimbral-mediated adhesion might reveal some inhibition. Instead of pursuing this possibility, we took a genetic approach to the problem of adhesin identification by overexpressing each fimbriin. The results were clear; bacteria that overexpressed fimQ aggregated with PRP-coated latex beads, while those that overexpressed fimP did not (Fig. 5). Overexpression of fimQ increased cell surface FimP to the wild-type level, overexpression of fimQ had little effect on cell surface FimQ, which was present at or near wild-type levels in all constructs except the ΔsrtC1 and ΔfimQ mutants (Fig. 5). We do not understand the precise nature of the cell surface difference between the ΔfimP and ΔfimP/pFimQ constructs but expect that it involves a subtle increase in the density or accessibility of FimQ binding sites on the latter construct to a level that is sufficient for adhesion. In addition to evidence from adhesion studies, the existence of a FimQ binding site(s) for PRP was also demonstrated by binding of recombinant FimQ in an ELISA-based assay (Fig. 6). This finding represents a major advance in studies of type 1 fimbral-mediated adhesion, and it opens a direct approach for identification of both the adhesin binding site(s) and sequences in PRPs that are recognized as receptors.

It is generally thought that the function of tip pilins is limited to their role as adhesins and that these minor subunits have little involvement in pilus formation (19, 25, 32,