Structural Insights into Serine-rich Fimbriae from Gram-positive Bacteria

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The serine-rich repeat family of fimbriae play important roles in the pathogenesis of streptococci and staphylococci. Despite recent attention, their finer structural details and precise adhesion mechanisms have yet to be determined. Fap1 (Fimbriae-associated protein 1) is the major structural subunit of serine-rich repeat fimbriae from Streptococcus parasanguinis and plays an essential role in fimbrial biogenesis, adhesion, and the early stages of dental plaque formation. Combining multidisciplinary, high resolution structural studies with biological assays, we provide new structural insight into adhesion by Fap1. We propose a model in which the serine-rich repeats of Fap1 subunits form an extended structure that projects the N-terminal globular domains away from the bacterial surface for adhesion to the salivary pellicle. We also uncover a novel pH-dependent conformational change that modulates adhesion and likely plays a role in survival in acidic environments.

Biofilms offer protection to bacteria from hostile environments, such as adverse temperatures, pH, and attack by other organisms, including their hosts (1). Significant progress has been made toward understanding which gene products are involved in biofilm formation. Fimbriae, which are long, thin filamentous structures that decorate bacterial surfaces, are often employed in the reversible adhesion to surfaces at the early stages of biofilm formation (2). A large number of chronic bacterial infections involve the formation of a biofilm, with the development of dental plaque being one of the most familiar and diverse (3).

The commensal streptococci of the oral cavity play a major role in the foundation of dental plaque (4). These include early colonizers of the tooth surface, viridans Streptococcus sanguinis, and Streptococcus parasanguinis, which serve as substratum for the subsequent adhesion of other microflora, such as cariogenic Streptococcus mutans, and periodontal pathogens Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans (5). In addition, both S. sanguinis and S. parasanguinis have been described as etiological agents in infective endocarditis (6–10) with the formation of streptococcal biofilms being central to the development of disease. Fap1 (Fimbriae-associated protein 1) has been identified as the major structural subunit of S. parasanguinis fimbriae and is essential for fimbrial biogenesis, adhesion, and biofilm formation (11–15). Fap1-like subunits assemble into a new family of fimbriae in Gram-positive bacteria (16), which are characterized by large polypeptides (>200 kDa) comprising an extensive region of glycosylated, serine-rich repeats (SRR)5 flanked by a unique N-terminal region and a C-terminal cell wall-anchoring domain. The SRR family of fimbriae plays direct roles in several bacterial diseases, including the pathogenesis of streptococcal (17) and staphylococcal infective endocarditis (18), streptococcal meningitis (19), invasive pneumococcal disease (20), and neonatal disease (21, 22).

Fap1 from S. parasanguinis provides a model system to study bacterial adhesion and biofilm formation by this poorly understood family of proteins. Furthermore, these adhesins play a major role in early stages of dental plaque formation. Despite recent attention, the finer structural details of SRR fimbriae and their adhesion mechanisms have yet to be determined. Combining multidisciplinary, high resolution structural studies with

The atomic coordinates and structure factors (codes 2x12 and 2x1b) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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5 The abbreviations used are: SRR, serine-rich repeat; HSQC, heteronuclear single quantum coherence; SAXS, small angle x-ray scattering; UA, uranyl acetate; PTA, phosphotungstic acid; MAT, methylamine tungstate; SHA, saliva-coated hydroxyapatite; PDB, Protein Data Bank.
biological assays, we provide new insights into adhesion by Fap1. We propose a model in which the serine-rich repeats of Fap1 subunits form an extended structure that projects the N-terminal globular domains away from the bacterial surface for adhesion to the salivary pellicle. We also uncover a novel pH-dependent conformational change that modulates adhesion and likely plays a role in survival in acidic environments.

EXPERIMENTAL PROCEDURES

Protein Preparation—Recombinant Fap1-NR (residues 106–437), Fap1-NR α (residues 116–231), and Fap1-NR β (residues 231–437) were expressed using pRSETA plasmid (Promega) in the BL21(DE3) Escherichia coli strain (Novagen). Unlabeled proteins were produced in LB media, and isotopically 15N- and 13C-labeled proteins were produced in M9 minimal medium supplemented with 15NH4Cl and [13C]glucose. A 2H/15N/13C triple labeled Fap1-NR sample was produced from Silantes media in E. coli CDN OD1. Selenomethionine-labeled Fap1-NR protein was expressed in methionine auxotroph B834 grown in minimal media supplemented with selenomethionine. All samples were purified using nickel affinity chromatography followed by size exclusion chromatography on a Superdex-75 column (GE Healthcare). NMR samples were concentrated to 0.1–0.2 mM in 50 mM PBS, pH 8, 50 mM NaCl. X-ray diffraction samples were concentrated to ~8 mg/ml and stored at ~72 °C in 20 mM Tris-HCl, pH 7.5, 100 mM KCl. Small angle x-ray scattering samples were gel-filtered in 50 mM PBS, pH 5 or 8, 50 mM NaCl immediately prior to data collection.

NMR Spectroscopy—NMR measurements were performed at 303 K on 15N,13C-labeled Fap1-NR α and Fap1-NR β domain samples in 50 mM PBS buffer, pH 8.0, 50 mM NaCl. NMR experiments for backbone and side-chain assignment on Fap1-NR α were performed on two different Bruker spectrometers, a DRX500 and an Avance II 800, equipped with cryoprobe. Assignments were completed using standard triple-resonance assignment methodology (23). NOE data for structure calculations of Fap1-NR α were obtained from an 800-MHz edited 1H-15N nuclear Overhauser effect spectroscopy-heteronuclear single quantum coherence (NOESY-HSQC) and a 950-MHz edited 1H-15N-13C NOESY-HSQC experiments with a mixing time of 100 ms recorded on a 950-MHz Oxford superconducting magnet. 15N T1 and T2 relaxation times on a 15N-labeled Fap1-NR α sample at pH 8 were recorded at 800 MHz, and the T1/T2 ratio was measured for each residue.

1H-15N and 1H-13C residual dipolar coupling constants were measured using two-dimensional in-phase anti-phase 15N-1H-HSQC (24) and three-dimensional HN(CO)CA experiments, respectively, on 15N,13C-labeled Fap1-NR α sample at pH 8 partially aligned in P1 filamentous phage media. 15N-1H TROSY-HSQC spectra of 15N-1H-labeled Fap1-NR were recorded at pH 5, 6, and 8 at 800 MHz. A series of 15N-1H HSQC on Fap1-NR α at different pH values from pH 5 to 8 were performed at 500 and 950 MHz. 15N-1H HSQC on Fap1-NR β domain was performed at 800 MHz.

1H-15N TROSY NMR experiments (25) were performed at 303 K on a uniformly 15N,13C,2H-labeled Fap1-NR on a Bruker Avance II 800 equipped with a cryoprobe. TROSY versions of HNCA/CB and HN(CO)CA/CB experiments for backbone assignment allowed us to assign ~50% assignment of Fap1-NR spectra. All NMR data were processed using NMRpipe (26) and analyzed with NMRView (27).

NMR Structure Determination—The ARIA protocol for automated NOESY assignment interfaced with the CNS program was used for structure calculation (28–30). Secondary helical structure in Fap1-NR α was first identified using the chemical shift-based dihedral angle prediction software TALOS (31). For residues located in helices, hydrogen bonds, φ and ψ backbone dihedral angles derived from TALOS and residual dipolar couplings, were introduced as restraints in the ARIA structure calculation. 38 DNH and 54 DCαα residual dipolar couplings spread evenly among the three helices were used for structure calculation. Axial Da and rhombic R components were initially estimated using the histogram method from the normalized distribution of these 92 residual dipolar couplings (32). The axial (Da = −3.0 Hz) and rhombic (R = 0.55) components of the alignment tensor were further optimized with the program MODULE (33) based on the precalculated structures. For NOE-derived and hydrogen bond distance restraints, a log harmonic potential was used for the second Cartesian cooling phase of the simulated annealing, and the weight on the restraints was iteratively updated (34). The final average weights were 6.8 kcal/mol/Å2 for NOE restraints and 25 kcal/mol/Å2 for hydrogen bond restraints. To avoid distortion of the peptide group during Cartesian cooling, high force constants were used to maintain planarity, whereas low force constants were employed for residual dipolar coupling restraints. Furthermore, the final structures were carefully inspected for local distortions. 100 conformers were calculated in the final iteration, and 10 lowest energy structures were refined in a shell of water molecules (35). A summary of NMR-derived restraints and statistics on the ensemble of the NMR structure is reported in Table 2. The solution structure of the Fap1-NR α domain was deposited in the Protein Data Bank with PDB ID code 2kub.

Crystallization, Data Collection, and Structure Determination—Crystals of both native and selenomethionine-labeled Fap1-NR were grown at 297 K using the hanging drop vapor diffusion method in 0.1 M Hepes over a range of pH values from pH 6.5 to 8.0, 5% 2-methyl-2,4-pentanediol, 10–12.5% PEG 6K, and 9–12.5 mM spermine-tetra-HCl. Crystals were briefly soaked in the mother liquor supplemented with 20% PEG 6K before cryocooling. Selenomethionine-labeled Fap1-NR data were collected at the selenium peak energy, determined by a fluorescence scan, at beamline ID29 at the European Synchrotron Radiation Facility in Grenoble, France. Data were processed using MOSFLM and scaled with SCALA (36). Two selenium sites were located using SHEXL (37), and then phases were calculated using AUTO-SHARP. Two monomers were located in the electron density, and a model was built into the averaged solvent flattened experimental map using COOT (38), with rounds of refinement in REFMAC5 (39). The final model was refined with NCS restraints and TLS groups, with 5% of the reflections omitted for cross-validation. Statistics of data collection and the final model are shown in Table 1. The coordinates and structure factors have been deposited in the PDB under accession code 2x12.
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Small Angle X-ray Scattering (SAXS)—SAXS data were recorded at the X33 beamline of EMBL (DESY, Hamburg, Germany). Samples of Fap1-NR bufferred at either pH 5 or 8 and at 1.3, 2.7, and 4.5 mg/ml were measured using 120-s exposures to synchrotron radiation. The range of q values measured was from 0.07 to 0.48 Å⁻¹. SAXS data were processed in the standard manner using the program PRIMUS (40). Data points across all three concentrations were merged (points 1–300, 250–600, and 550–2000 were derived from the low, medium, and high concentration samples, respectively). The radius of gyration ($R_g$) and scattering at zero angle ($I(0)$) were calculated from the analysis of the Guinier region by AUTORG. The distance distribution function ($d$) was subsequently obtained from the analysis of the Guinier region by AUTORG. The dis
distance ($R_{max}$) was determined using the program GASBOR utilizing default parameters (41). Models were then aligned and averaged by DAMAVER. The known atomic resolution structures of the α and β domains were docked into the low resolution bead model of Fap1-NR at pH 8 by the program SITUS (42). This was achieved by splitting the bead model into two halves and independently minimizing the fit between the high and low resolution structures.

Site-directed Mutagenesis of Fap1 of S. parasanguinis—Surface regions containing an abundance of exposed hydrophobic amino acid side chains were selected for mutation. The hydrophobic residues together with selected hydrophilic side chains were mutated individually to alanine using the Stratagene QuickChange® mutagenesis system as described previously (43). This included Ile-134, Glu-138, Asp-142, Leu-163, and Val-164 in Fap1-NRα, together with Leu-385, Ile-291, Leu-292, Leu-300, Asn-403, Gln-405, and Ile-411 in Fap1-NRβ. In brief, the full-length fap1 gene cloned into an E. coli and streptococcal shuttle vector pVA838 was used as a template. Site-directed mutagenesis PCR was carried out to construct a series of alanine replacement mutants of fap1 using the mutagenic oligonucleotide primers that corresponded to the changes of selected amino acid residues to alanine residues. The amplified mutant plasmids were transformed into competent E. coli following the instructions by a QuickChange® XL mutagenesis kit (Stratagene, La Jolla, CA). The plasmids with designed mutations were purified; the mutated region was sequenced to ensure the accuracy of the site-directed mutagenesis. The wild-type and mutant plasmids were then transformed into a fap1 mutant of S. parasanguinis. Fap1 expression was monitored by Western blot analysis using an antibody against mature Fap1 (F51).

Adhesion of S. parasanguinis to SHA, an In Vitro Tooth Model—Adhesion of S. parasanguinis to saliva-coated hydroxyapatite (SHA) was performed as described previously (11). In brief, cells of wild-type S. parasanguinis and site-directed mutants were labeled with 2 μCi of [methyl-³H]thymidine/ml. The radiolabeled bacteria were incubated with SHA beads at 37 °C for 1 h to allow bacterial binding to occur and then washed. Supernatants and washed beads were transferred to scintillation vials, and their respective radioactivities were determined and used to calculate adhesion percentage as described previously. In all assays, a wild-type strain FW213 and the fap1-

FIGURE 1. Domain organization of S. parasanguinis Fap1 and adhesion to SHA. a, schematic representation of the domain structure of Fap1. The amino acid positions at which the domains begin and end are indicated. Mature Fap1 is numbered 1–2502. b, Fap1-NR, Fap1-NRα, Fap1-NRβ, and Fap1-minus block bacterial adhesion in a dose-dependent manner. 0, 10, and 100 μg of recombinant Fap1 proteins were preincubated with SHA before adding [³H]thymidine-labeled wild-type S. parasanguinis. Wild-type binding is normalized to 100%, and the fap1 deletion mutant was used as a negative control.

TABLE 1 Crystal data collection and refinement statistics for Fap1-NRβ

| Crystal parameters |       |
|--------------------|-------|
| Space group        | P3 2 1 |
| Cell dimensions    | a = b = 108.67 Å c = 126.25 Å |

| Data collection |       |
|-----------------|-------|
| Beamline        | ESRF ID29 |
| Wavelength      | 0.97910 Å |
| Resolution      | 2.90 Å (2.90-3.06 Å) |
| Unique observations | 19,567 (2832) |
| Rmerge 0.132 (0.47) |
| Completeness     | 99.84% (100.0%) |
| Redundancy       | 7.88 (7.53) |

| Refinement |       |
|------------|-------|
| Rwork 21.10%/25.05% |
| Rfree 5% |
| No. of protein residues | 400 |
| r.m.s.d. stereochemistry | 0.02 Å |
| Bond lengths | 1.8 Å |
| Ramachandran analysis | 2% (Ser-246 A and B chain) |
| Residues in outlier regions | 377 (95.2%) |
| Residues in favored regions | 394 (99.5%) |

* Rwork = Σ |Fobs| - |Fcalc| / Σ |Fobs| where |Fobs| and |Fcalc| are the observed and calculated structure factors, respectively.
* Rfree = Rwork calculated using 5% random data excluded from the refinement.
* Ramachandran analysis was carried out using Molprobity (85).
minus mutant were used as positive and negative controls, respectively.

**Adhesion Blocking Assays**—Wild-type *S. parasanguinis* cells were labeled with 2 μCi of [3H]thymidine/ml. The radiolabeled cells were treated and reconstituted as described previously. Recombinant Fap1-NR, Fap1-NRα, and Fap1-NRβ were expressed and purified as described previously. The purified recombinant proteins were dialyzed overnight against the adhesion buffer and added into SHA beads that were equilibrated with adhesion buffer and then incubated at 37 °C for 1 h on a rotating shaker. The treated SHAs were incubated with labeled *S. parasanguinis* and subjected to the adhesion assay described above.

**Electron Microscopy**—*S. parasanguinis* (FW213) was grown from frozen stocks preserved with 5% dimethyl sulfoxide (44). Frozen cells were streaked onto blood agar plates and incubated aerobically for 12–15 h in 5% CO2 at 37 °C. Broth cultures were prepared by inoculation of single colonies from the blood agar plates into flasks containing Todd-Hewitt Broth (THB) and grown statically under aerobic conditions. 5-ml cell cultures in early exponential phase (A470 ~ 0.4) were centrifuged at 4000 × g to remove the media. Cell pellets were resuspended in 100 μl of PBS (100 mM phosphate buffer, pH 7.4, 150 mM NaCl) at 4 °C. Samples were diluted in PBS to the appropriate concentration for electron microscopy studies. A small aliquot (5 μl) was applied to 400 mesh copper grids coated with a thin carbon film. The grids were washed by running several drops of PBS buffer over them and negatively stained by subsequently running a few drops of the staining solution (1% uranyl acetate (UA; Ted Pella, Redding, CA), 2% phosphotungstic acid, pH 7, with NaOH (Ted Pella), and 2% methylamine tungstate (MAT; Nanoprobes, Yaphank, NY) over the grids. The last drop of stain was left on the grid for 30 s; the excess liquid was wicked off, and the grids were fast air dried. The grids were observed using a dual axis tomography holder (Fischione Instruments, Export, PA) on a Tecnai 12 electron microscope (FEI Co., Hillsboro, OR) equipped with an LaB6 cathode (Kimball Physics Inc., Wilton, NH) operated in point mode, and a 14-μm 2048 × 2048 CCD camera (TVIPS, Germany). The microscope was run under identical conditions as have been used in the past to

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**FIGURE 2.** Three-dimensional structures of the two subdomains Fap1-NRα and Fap1-NRβ from the major nonrepeat region from *S. parasanguinis* Fap1 fimbriae. **a**. ribbon representation of the x-ray structure of Fap1-NRβ. The β-strands are colored in yellow and orange and short helices in red. L1 to L9 refer to loops 1 to 9 in the structure. **b**. topology diagram of the x-ray structure of Fap1-NRα, with the same colored code as in *a*. **c**. superposition of the 10 best NMR structures of Fap1-NRα with the three helices α1, α2, and α3 shown in blue. **d**. ribbon representation of the NMR structure of Fap1-NRα in two different orientations with the three helices α1, α2, and α3 in blue.
obtain images that show Thon rings beyond 0.9 nm resolution in vitreous ice preparations (45). Negatives were recorded at an accelerating voltage of 100 kV and nominal magnification of \( \times 52,000 \) under low dose conditions on 50-163 Kodak film \((\sim 1000 \, \text{e}^{-} / \text{nm}^{2})\). Negatives were scanned with 7 \( \mu \text{m} \) raster size on a Zeiss flat bed scanner (Intergraph, Huntsville, AL). The images were converted for processing with the SPIDERE image processing system (46) and reduced two times to a final pixel size of 0.27 nm at the specimen level. A contrast transfer function correction was performed on the images by calculating the averaged periodogram of each image and fitting to it the theoretical transfer function calculated by varying the defocus value and astigmatism parameters (angle and strength) (47). Each image was corrected by multiplying it with a modified version of its transfer function (47).

Straight filament regions, which showed no overlap, were boxed with the program “helixboxer” in the EMAN image processing package (48). Images containing 55 nm filament sections were windowed using the SPIDERE image processing system (46). The unevenness of the background was removed using the “ramp” command, and the intensities were normalized to zero average (5 pixels strips along the longitudinal edge of the images were used to calculate the average intensity value of the background). A power spectrum was calculated from each image, and an averaged power spectrum was calculated for each stain condition. The averaged power spectra were visualized using the WEB display program (46), and the height of the layer lines was extracted.

RESULTS

Major Nonrepeat Region of Fap1 Blocks S. parasanguinis Adhesion to SHA—Recently, SRR fimbrial proteins known to interact with salivary components and human platelets have also been identified in other medically relevant streptococcal and staphylococcal species (49). Interestingly, the sequences of regions outside the serine repeats are implicated in adhesion and poorly conserved among the family (50–53). To define the minimal region responsible for Fap1 adhesion to the salivary pellicle, the nonrepeat portion from the N terminus of Fap1 (Fap1-NR, residues 106–437; Fig. 1a) was recombinitely produced in E. coli, purified using metal affinity chromatography, and tested for its ability to inhibit S. parasanguinis adhesion to an in vitro tooth model, SHA. Binding assays revealed a dose-dependent decrease in binding in the presence of recombinant Fap1-NR indicating that this region of Fap1 is able to competitively block adhesion (Fig. 1b).

Limited trypsin proteolysis of Fap1-NR released two stable domains corresponding to a C-terminal 25-kDa polypeptide and a smaller 10-kDa N-terminal fragment, confirmed by mass spectrometry (data not shown). To investigate the contribution of these subdomains to adhesion, we produced Fap1-NR\(_{C}\) and Fap1-NR\(_{N}\) separately in E. coli and tested their ability to block S. parasanguinis binding to SHA (Fig. 1b). Each domain shows a dose-dependent reduction in S. parasanguinis adhesion suggesting that they both play important roles in adhesion.

Structure of the Major Nonrepeat Region Fap1-NR—To explore the potential cooperative roles of Fap1-NR\(_{C}\) and Fap1-NR\(_{N}\) and to provide a better understanding of adhesion by Fap1, we embarked on a high resolution structural investigation. We initiated our structural studies using the intact 36-kDa Fap1-NR. Crystals of Fap1-NR were obtained after 1 day, between pH 6.5 and 8.5, with the highest resolution diffraction obtained for crystals grown at pH 8. Structure determination was undertaken using Se-SAD, and the electron density map was refined to 2.9 Å (Table 1 and supplemental Fig. S1). The β domain was built into the experimental electron density map from residues Thr-236 to Asp-435, although the electron density corresponding to all preceding residues was not observable. Crystals were washed and analyzed by SDS-PAGE, which showed no sign of degradation (data not shown); therefore, it was postulated that the lack of electron density for this portion of Fap1-NR results from significant disorder in the N-terminal region, which encompasses Fap1-NR\(_{N}\). The final model contains two identical molecules of the β domain in a head-to-tail arrangement within the asymmetric unit. The dimer interface is small, consisting of Thr-236 to Asp-435, although the electron density corresponded to all preceding residues was not observable. Crystals were washed and analyzed by SDS-PAGE, which showed no sign of degradation (data not shown); therefore, it was postulated that the lack of electron density for this portion of Fap1-NR results from significant disorder in the N-terminal region, which encompasses Fap1-NR\(_{N}\). The final model contains two identical molecules of the β domain in a head-to-tail arrangement within the asymmetric unit. The dimer interface is small, consisting of Thr-236 to Asp-435, although the electron density corresponded to all preceding residues was not observable. Crystals were washed and analyzed by SDS-PAGE, which showed no sign of degradation (data not shown); therefore, it was postulated that the lack of electron density for this portion of Fap1-NR results from significant disorder in the N-terminal region, which encompasses Fap1-NR\(_{N}\). The final model contains two identical molecules of the β domain in a head-to-tail arrangement within the asymmetric unit. The dimer interface is small, consisting of Thr-236 to Asp-435, although the electron density corresponded to all preceding residues was not observable.

### Table 2

NMR structural constraints and structure statistics for Fap1-NR\(_{N}\)

| No. of restraints | Distance restraints |
|-------------------|---------------------|
|                   | Intra-residue \( (i-j=0) \) | 451 |
|                   | Sequential \( (i-j=1) \) | 211 |
|                   | Medium range \( (2 \leq i-j<5) \) | 256 |
|                   | Long range \( (i-j\geq5) \) | 103 |
|                   | Ambiguous | 565 |
|                   | Total | 1586 |
| Dihedral angle restraints \( (\psi/\phi) \) | 138 (69/69) |
| Hydrogen bonds restraints | 49 |
| RDC restraints \( (D_{\text{m}}/D_{\text{an}}) \) | 38/54 |

| Restraints statistics\(^{a}\) |
|-----------------------------|
| r.m.s. of distance violations | NOE restraints | 0.11 \( \pm \) 0.01 Å |
|                             | H-bond restraints | 0.06 \( \pm \) 0.01 Å |
|                             | r.m.s. of dihedral violations | 0.25 \( \pm \) 0.07° |
|                             | RDC Q-factors | 0.19 \( \pm \) 0.04 |
|                             | \( D_{\text{an}} \) | 0.31 \( \pm \) 0.01 Hz |

| r.m.s. from idealized covalent geometry |
|----------------------------------------|
| Bonds | 0.0029 \( \pm \) 0.00008 Å |
| Angles | 0.49 \( \pm \) 0.009° |
| Improper | 1.45 \( \pm \) 0.06° |

| Structural quality\(^{b}\) |
|-----------------------------|
| Ramachandran statistics\(^{c}\) |
| Most favored regions | 99.2 \( \pm \) 0.9% |
| Allowed regions | 0.8 \( \pm \) 0.9% |
| Generously allowed regions | 0 \( \pm \) 0% |
| Disallowed regions | 0 \( \pm \) 0% |
| WHAT-IF Z-score\(^{d}\) | Backbone conformation | 1.90 \( \pm \) 0.18 |
| 2nd generation packing quality | 4.33 \( \pm \) 0.37 |
| Ramachandran plot appearance | 3.02 \( \pm \) 0.39 |
| \( x_{i} y_{j} z_{k} \) rotamer normality | 0.82 \( \pm \) 0.39 |

| Coordinates precision\(^{e}\) |
|-----------------------------|
| All backbone atoms | 0.39 \( \pm \) 0.06 Å |
| All heavy atoms | 0.81 \( \pm \) 0.07 Å |

\(^{a}\) Average values and standard deviations over the 10 lowest energy conformers are shown.

\(^{b}\) Percentage of residues in the Ramachandran plot regions was determined by PROCHECK (86).

\(^{c}\) Ramachandran plot appearance was reported by WHAT-IF (87).

\(^{d}\) Average root mean square (r.m.s.) deviation (r.m.s.d.) over the 10 lowest energy conformer atomic coordinates with respect to the average structure are shown.

\(^{e}\) Average values and standard deviations over the 10 lowest energy conformers are shown.

\(^{f}\) Percentage of residues in the Ramachandran plot regions was determined by PROCHECK (86).

\(^{g}\) Ramachandran plot appearance was reported by WHAT-IF (87).

\(^{h}\) Average root mean square (r.m.s.) deviation (r.m.s.d.) over the 10 lowest energy conformer atomic coordinates with respect to the average structure are shown.
composed of 12 β-strands arranged in two extensive β-sheets built from four anti-parallel β-strands (Fig. 2, a and b). Four additional, short β-strands elaborate one face of the structure, creating an extended twisted β-platform. A striking feature is the presence of large loops protruding at the top (L1–L2–L5) and the bottom (L3–L6–L9) of the β-sandwich and obscuring the face of the extended β-sheet (Fig. 2, a and b, L4–L7–L8). These loops are well ordered and are stabilized through hydrophobic interactions with exposed aromatic residues from the β-sheet core structure. A series of hydrophobic residues within L1–L2–L5 form an exposed ledge on the top of the structure (supplemental Fig. S3).

The absence of electron density for the N terminus of Fap1-NRα may be due to a lack of structural integrity or the presence of multiple, but ordered, conformations arising from flexibility within the 27-amino acid linker. To elucidate the structure of this domain, we used solution state NMR spectroscopy. Two-dimensional ¹H–¹⁵N HSQC spectra display excellent dispersion in backbone amide chemical shifts at pH 8, consistent with a folded polypeptide (supplemental Fig. S4). The ¹⁵N relaxation rates at pH 8 for backbone amides between Glu-128 and Leu-208 reveal a well ordered structure with no flexible loops or residues in chemical exchange, although the termini (residues 116–127 and 209–231) are highly flexible. Furthermore, NMR spectra indicate that Fap1-NRα is monomeric at concentrations up to 300 μM, but it self-associates at higher concentrations (supplemental Fig. S5).

Using a combination of manual and ARIA NMR assignment methods for Fap1-NRα (28, 29), a total of 1586 NOEs were assigned in Fap1-NRα ¹⁵N/¹³C-edited NOESY spectra at pH 8.
The structure determination was supplemented with $\phi$ and $\varphi$ dihedral angles and 92 residual dipolar coupling restraints (Table 2). All areas of secondary structure are well defined (Fig. 2c); the average pairwise root mean squared deviation for the water-refined final structures is $0.39 \pm 0.06 \text{ Å}$ for the backbone atoms and $0.81 \pm 0.07 \text{ Å}$ for the heavy atoms of residues within...
secondary structure. The final structure of the Fap1-NRα exhibits an α-helical bundle topology (Fig. 2c), in which the three α-helices pack against each other in an up-down-up arrangement (α1 residues 129–150, α2 residues 153–178, and α3 residues 184–206). Hydrophobic interactions involving the side chains of leucine, alanine, and isoleucine residues stabilize the inter-helix interactions (Fig. 2d). Comparison of the 15N-1H TROSY HSQC of Fap1-NR with spectra of either Fap1-NRα or Fap1-NRβ indicates that although the majority of peaks for the isolated domains superimpose well with their counterparts for Fap1-NR, a limited number exhibit chemical shifts differences, suggesting the presence of a small interface between the domains (supplemental Fig. S4). Furthermore, differences in 15N relaxation times for α and β domains within Fap1-NR indicate some interdomain flexibility (average T1/T2 ratios are 21 and 27 for α and β domains respectively; supplemental Fig. S5).

pH-dependent Conformational Change within Fap1—The oral cavity experiences a wide pH range, from neutrality at the inter-helix interactions (Fig. 2d). Comparison of the 15N-1H TROSY HSQC of Fap1-NR with spectra of either Fap1-NRα or Fap1-NRβ indicates that although the majority of peaks for the isolated domains superimpose well with their counterparts for Fap1-NR, a limited number exhibit chemical shifts differences, suggesting the presence of a small interface between the domains (supplemental Fig. S4). Furthermore, differences in 15N relaxation times for α and β domains within Fap1-NR indicate some interdomain flexibility (average T1/T2 ratios are 21 and 27 for α and β domains respectively; supplemental Fig. S5).

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**TABLE 3**

| pH 5 | pH 8 |
|-----|-----|
| q range | 0.018–0.464 Å⁻¹ | 0.021–0.464 Å⁻¹ |
| Rg | 35.0 ± 0.2 Å | 36.2 ± 0.1 Å |
| I(0) | 88.5 ± 0.3 | 108.4 ± 0.3 |
| Dmax | 127 Å | 128 Å |
| Estimated molecular mass* | 31 kDa | 38 kDa |
| Mass calculated from sequence | 37 kDa | 37 kDa |

* Normalization against data for BSA was calculated using the formula (I(0)BSA + I(0)Fap1/NR) / I(0)BSA, where I(0)BSA was 188.3.

**Figure 4.** Overall structure of Fap1-NR and mutagenesis. a, SAXS scattering profile of Fap1-NR at pH 5 (red) and pH 8 (blue). b, SAXS-derived electron density for Fap1-NR at pH 8 with the crystal structure of Fap1-NRα and the solution structure of Fap1-NRβ, rigid body fitted into the envelope. The 27-residue linker is shown as a blue dashed line and the N and C termini of Fap1-NR are annotated. c, effects of pH change on the electron density of Fap1-NR. SAXS-derived electron density at pH 8 is blue and at pH 5 is red. d, potential receptor binding surface of Fap1. Two exposed hydrophobic regions were mutant Fap1-NR residues. Additionally, including Ile-134, Glu-138, Asp-142, Leu-163, and Leu-208) proximal to charged groups are also involved in the inter-helix interactions (Fig. 2d). Comparison of the 15N-1H TROSY HSQC of Fap1-NR with spectra of either Fap1-NRα or Fap1-NRβ indicates that although the majority of peaks for the isolated domains superimpose well with their counterparts for Fap1-NR, a limited number exhibit chemical shifts differences, suggesting the presence of a small interface between the domains (supplemental Fig. S4). Furthermore, differences in 15N relaxation times for α and β domains within Fap1-NR indicate some interdomain flexibility (average T1/T2 ratios are 21 and 27 for α and β domains respectively; supplemental Fig. S5).

SAXS provides useful low resolution information on the global structural features of proteins in solution. This technique is sensitive to changes in relative position of domains or positions of secondary structure and is therefore highly suitable for determining the overall structure of Fap1-NR under acidic and alkaline conditions. After exhaustive gel filtration chromatography of recombinant Fap1-NR at pH 8 and 5, SAXS measurements were recorded (Fig. 4a and b; Table 3). The resulting SAXS density indicates that Fap1-NR exists as a curved, extended structure consisting of two domains (Fig. 4b). The relative sizes of Fap1-NRα and Fap1-NRβ domains are consistent with two halves of the density. Comparison of the data at pH 8 and 5 reveals a subtle difference in conformation, as judged by a change in the shape of the pair-distance distribution function (Fig. 4a). Upon reducing the pH, the pair-distance distribution changes to longer distances, for which a plausible interpretation would be an opening of the two-domain structure (Fig. 4c). It is also likely that the extent of interdomain mobility is affected by pH and would contribute to an altered SAXS profile. It is interesting to note that the residues affected by the change in pH within the α domain would be situated adjacent to the 27-residue linker between Fap1-NRα and Fap1-NRβ (Fig. 4d).

Analysis of the atomic resolution details of Fap1-NR reveals two patches on the surfaces of the α and β domains that include a number of exposed hydrophobic side chains (Fig. 4d). The exposed hydrophobic residues together with selected adjacent residues within these regions were selected for mutation; these include five amino acids in Fap1-NRα (Ile-134, Glu-138, Asp-142, Leu-163 and Leu-208) and a number of exposed hydrophobic side chains (Fig. 4d). The exposed hydrophobic residues together with selected adjacent residues within these regions were selected for mutation; these include five amino acids in Fap1-NRα (Ile-134, Glu-138, Asp-142, Leu-163 and Leu-208) and a number of exposed hydrophobic side chains (Fig. 4d). The exposed hydrophobic residues together with selected adjacent residues within these regions were selected for mutation; these include five amino acids in Fap1-NRα (Ile-134, Glu-138, Asp-142, Leu-163 and Leu-208) and a number of exposed hydrophobic side chains (Fig. 4d). The exposed hydrophobic residues together with selected adjacent residues within these regions were selected for mutation; these include five amino acids in Fap1-NRα (Ile-134, Glu-138, Asp-142, Leu-163 and Leu-208) and a number of exposed hydrophobic side chains (Fig. 4d). The exposed hydrophobic residues together with selected adjacent residues within these regions were selected for mutation; these include five amino acids in Fap1-NRα (Ile-134, Glu-138, Asp-142, Leu-163 and Leu-208) and a number of exposed hydrophobic side chains (Fig. 4d). The exposed hydrophobic residues together with selected adjacent residues within these regions were selected for mutation; these include five amino acids in Fap1-NRα (Ile-134, Glu-138, Asp-142, Leu-163 and Leu-208).
measured their ability to adhere to SHA (Fig. 4). As a control, specific Fap1 mutants in this region within site for the salivary pellicle, and we created a series of site-See supplemental Fig. S3. We hypothesized that these residues may form part of a binding domain was also constructed and tested. Western blot analysis revealed that all the mutants expressed Fap1 (data not shown); however, all exhibited deficiencies in bacterial adhesion with the exception E204A.

Overall Structure of Fap1—We analyzed the feasibility of imaging the fimbriae on the bacterial surface of the wild-type strain (FW213) for structure determination by single particle electron microscopy using several negatively stained preparations. Good results were obtained with preparations in 1% UA, 2% PTA, pH 7, and 2% MAT (Fig. 5a). The fimbriae are very flexible structures that bend easily in all the preparations. A close look at individual fimbriae reveals a diameter of ~5.5 nm and an internal substructure with a periodicity of ~6.5 nm. At neutral pH, fimbriae are close together forming rafts, and the internal substructure is more apparent (Fig. 5a, open white arrows). At low pH (UA staining), the tip of the fimbriae come together to form aggregates (Fig. 5a, white arrows), in which the adhesion points are located uniquely at the tip of the fimbriae. Strikingly, despite the fimbriae being more abundant on the cell surface at pH 7.0 using staining reagents PTA and MAT, the tips do not come together. Even though an additional contribution from the electrostatic charges of the different stains at the experimental pH cannot be ruled out at the present time, these data support the notion of a pH-dependent conformational change for Fap1.

Once electron micrographs of whole-mount bacterial preparations in different negative stains had been collected, digitized, and corrected for the contrast transfer function of the microscope, fimbrial regions were extracted from the images following strict criteria; the fimbriae should be straight; they should not overlap other fimbriae, and they should be separated by at least two fimbrial diameters from its nearest neighbor (Fig. 5b). As many images as possible, with a length of ~55 nm, were extracted from the boxed fimbrial regions. After centering the images, an average image (Fig. 5b) and an average power spectrum (Fig. 5c) were calculated for each staining condition. The power spectra, in all stain preparations, show predominant layer lines at 1/6.5 nm and 1/33 nm, which relate to structural repeats in the fimbriae. At the present time, an unambiguous assignment of the Bessel orders is not possible due to the weak scattering power of the fimbrial regions and the uncertainty in the diameter of fimbriae.

DISCUSSION

Recent studies have suggested that Fap1 belongs to a wider family of cell wall-anchored, serine-rich repeat proteins (49) that exhibit features distinct from the pili of other Gram-positive organisms, such as Corynebacteria diphtheriae, group A and group B Streptococcus (GAS and GBS), and Gram-negative bacteria expressing chaperone usher and type IV secretion systems. Typically, these pili are characterized by small subunits noncovalently or covalently linked to each other (54–56), whereas SSR fimbriae are characterized by the presence of extensive glycosylated serine-rich repeats within a large protein subunit (~200 kDa). SSR fimbriae are found on several pathogenic streptococci and staphylococci, which include GspB from Streptococcus gordonii (17), SSR-1 and SSR-2 from Streptococcus agalactiae (21, 22, 57), SrpA from S. sanguinis (50), and Srp from Staphylococcus aureus (18). Many of these proteins mediate specific interactions with human platelets through their N-terminal regions, which share little sequence homology to each other and are implicated in binding to a diverse array of host receptors (20, 57).

We have demonstrated that a 36-kDa fragment from the nonrepeat region of Fap1 (Fap1-NR) harbors the binding properties for colonization of the oral cavity (58). Fap1-NR includes two domains with distinct secondary structure and topology, namely an α-helical domain (residues 116–231, Fig. 1a) and another composed predominantly of β-strands (residues 231–437, Fig. 1a). The protein sequence out-
The larger 33 nm periodicity may be attributed to an independent super-helical extended structure with a principal repeat at 6.5 nm. Extra subunits may be incorporated within a coiled-coil to produce higher oligomeric states, and the model on the right represents multiple polymerized subunits with just two displayed here. The Fap1 model was created from a left-handed helix in which single atoms (representing individual amino acids) were arranged on a helical axis, with additional atoms (representing glycan residues) associated with alternate amino acid residues. This helix was in turn rotated about a central super-helical fiber axis. The SAKS envelope was scaled to the SRR model and placed at the C termini, followed by a short stretch of the helical SRR model representing the minor SRR. An alignment of SRR sequences (middle) from S. parasanguinis Fap1, S. aureus SrpA, S. gordonii M99 GspB, and Lactobacillus johnsonii NCC533 LJ1711 is shown. The consensus SRR dipeptide sequence (SRRc) is SX, where X can be any amino acid.

FIGURE 6. Model for the super-coiled strand of Fap1 fimbriae. The protein is colored as in Fig. 1a with the cell wall anchor colored cyan, the major and minor SRR regions colored dark (protein) and light (glycan) orange, although the major and minor NR regions are colored green. The model on the left represents a super-coiled Fap1 monomer with repeating units of 6.5 nm along the fiber axis of the SRR region and protomer length of ~250–300 nm. Extra subunits may be incorporated within a coiled-coil to produce higher oligomeric states, and the model on the right represents multiple polyimerized subunits with just two displayed here. The Fap1 model was created from a left-handed helix in which single atoms (representing individual amino acids) were arranged on a helical axis, with additional atoms (representing glycan residues) associated with alternate amino acid residues. This helix was in turn rotated about a central super-helical fiber axis. The SAKS envelope was scaled to the SRR model and placed at the C termini, followed by a short stretch of the helical SRR model representing the minor SRR. An alignment of SRR sequences (middle) from S. parasanguinis Fap1, S. aureus SrpA, S. gordonii M99 GspB, and Lactobacillus johnsonii NCC533 LJ1711 is shown. The consensus SRR dipeptide sequence (SRRc) is SX, where X can be any amino acid.

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Insight into Serine-rich Fimbriae from Gram-positive Bacteria

side the nonrepeat region is composed of ~2000 amino acid residues of [S(V/I/E)] dipeptide repeats. Perfect sequence repeats of less than four amino acids tend to form fibrous extended structures that are often super-helical (59), for example the collagen triple helix (60), the zigzag β-structure of the silk I (61, 62), and the β-turn helix of the Plasmodium falciparum circumsporozoite protein (63, 64). The extensive repetition of the serine dipeptides together with our electron microscopy data suggest a model for the fimbriae in which Fap1 strands form a super-helical extended structure with a principal repeat at 6.5 nm and serine side chains exposed for O-glycosylation (Fig. 6). The larger 33 nm periodicity may be attributed to an independent helical stripe of surface glycosylation, the exact pattern of which has yet to be determined. Furthermore, because there is an ambiguity in layer line indexing, fimbriae consisting of a coiled coil arrangement of two or more Fap1 helical strands cannot be excluded. Fap1 fibrinmes are variable in length (300–600 μm range), similar to other SRR fibrils, e.g. SrpA on the surface of Streptococcus cristatus (65). These length variations could be attributed to more than one subunit present per fimbriae, linked in a head-to-tail fashion. Although the classic pilin motif for head-to-tail cross-linking is absent (56, 66), lysines residues present at the N terminus of Fap1 could act as substrates for this cross-linkage.

In this model, the N-terminal nonrepeat domain, Fap1-NR, includes two globular domains that are located at the distal end of the structure for adhesion. Although no significant primary sequence similarity can be detected with proteins of known structure (no e value <1 PHYRE hits (67)), both domains display structural similarity to two separate families of surface proteins from Gram-positive bacteria (68). The structure of the β domain resembles the ligand-binding A-region from the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) family of bacterial adhesins (supplemental Fig. S8a). Specifically, a high degree of similarity is observed with the fibrinogen-binding protein SdrG from Staphylococcus epidermidis (PDB code 1r17; supplemental Fig. S8a) (55, 69, 70), the collagen-binding adhesin from Enterococcus faecalis (PDB code 2x1p; supplemental Fig. S8b) (71, 72), and the clumping factor A from S. aureus (PDB code 1n67; supplemental Fig. S8c) (73–75), typically with a backbone root mean squared deviation of 3.5 Å over ~120 residues. The MSCRAMM ligand-binding region encompasses two similar β domains, in which a ligand peptide docks into an interdomain groove of the open structure and a C-terminal extension closes the complex resulting in a change in relative domain orientation (74). Although the α domain is unrelated to the flanking β-rich domain from the MSCRAMMs, it displays a striking similarity with the imperfect helical repeats of EBH, the giant, extracellular matrix-binding protein from staphylococci (PDB code 2dgj; supplemental Fig. S8d) (76). The lack of electron density for the α domain within the crystal structure of Fap1-NR, coupled with its well defined structure in solution, also implies that some flexibility exists at the interdomain boundary. It is tempting to speculate that the dual similarity with EBH and MSCRAMM surface proteins implies that host extracellular matrix proteins are putative targets for Fap1 and supports the wider role for SRR fimbriae. The interaction between Fap1 and the salivary pellicle also suggests a role for calcium binding; however, no direct interaction was observable in our NMR assays (data not shown).

Fap1 exhibits an unusual pH-dependent conformational change that affects key residues located at the interdomain junction of the two subdomains of Fap1-NR, with concomitant effects on the relative arrangement of these domains that allow Fap1 to adhere more tightly to SHA. The mean resting saliva pH observed in human oral cavities lies in the range 6.5 to 7.1. However, the buffering capacity of saliva is unable to cope with the rapid drop in pH induced by microbial acid production after the ingestion of fermentable carbohydrates. Plaque pH can reach values below 5.0 and remain low for some time before returning to neutrality. Some streptococci, particularly S. mutans, alter their physiology to survive in acidic environments. This is known as the acid tolerance response and includes the production of stress-responsive proteins, increased glycolytic activity, and the regulation of intracellular pH (77, 78). S. parasanguinis and many other oral streptococci are primary colonizers of the oral cavity and will frequently be exposed to acidic stress; however, they are unable to thrive at low pH and
in response shut down their metabolic functions (79). A plausible mechanism for survival would be for S. parasanguinis to modulate adhesion to outcompete acid-tolerant species, a mechanism that has been proposed for other species of bacteria (80–84). Enhanced Fap1-mediated adhesion at low pH would provide a more tenacious attachment to the salivary pellicle and increase the likelihood of S. parasanguinis survival during periods of acidic conditions. Moreover, its role as substrate for biofilm formation would also be enhanced thereby increasing bacterial cell density and providing respite.

Using a combination of low and high resolution methods, we provide new insights into the architecture of Fap1 fimbriae and adhesion by a primary colonizer of the human oral cavity. Furthermore, our conclusions likely extend to other SRR fimbriae from pathogenic organisms and suggest new therapeutic strategies.

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