New Cell Surface Protein Involved in Biofilm Formation by *Streptococcus parasanguinis*[▽]

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Dental biofilm formation is critical for maintaining the healthy microbial ecology of the oral cavity. Streptococci are predominant bacterial species in the oral cavity and play important roles in the initiation of plaque formation. In this study, we identified a new cell surface protein, BapA1, from *Streptococcus parasanguinis* FW213 and determined that BapA1 is critical for biofilm formation. Sequence analysis revealed that BapA1 possesses a typical cell wall-sorting signal for cell surface-anchored proteins from Gram-positive bacteria. No functional orthologue was reported in other streptococci. BapA1 possesses nine putative pilin isopeptide linker domains which are crucial for pilus assembly in a number of Gram-positive bacteria. Deletion of the 3' portion of *bapA1* generated a mutant that lacks surface-anchored BapA1 and abolishes formation of short fibrils on the cell surface. The mutant failed to form biofilms and exhibited reduced adherence to an *in vitro* tooth model. The BapA1 deficiency also inhibited bacterial autoaggregation. The N-terminal muramidase-released-protein-like domain mediated BapA1-BapA1 interactions, suggesting that BapA1-mediated cell-cell interactions are important for bacterial autoaggregation and biofilm formation. Furthermore, the BapA1-mediated bacterial adhesion and biofilm formation are independent of a fimbria-associated serine-rich repeat adhesin, Fap1, demonstrating that BapA1 is a new streptococcal adhesin.

Biofilm formation is critical for bacterial pathogenesis. Infectious diseases such as dental caries, subacute endocarditis, and otitis media are biofilm-driven infections (6, 7). Molecular details of biofilm formation have been studied extensively using *in vitro* model systems (16, 35). Biofilm development involves an initial adhesion step to immobilize bacteria on a given surface, followed by cell-to-cell interactions to promote microcolony formation. The formation of a complex three-dimensional structure facilitates the development of a mature biofilm (32). Dental biofilms are initiated by the attachment of early colonizers, mainly oral streptococci (31), to the saliva-coated oral surfaces. Oral streptococci interact with a host of later colonizers (22) to promote the formation of a complex microbial biofilm, known as dental plaque. Many bacterial structures such as pili and protein fibers that project from the surfaces (9) and other nonfiber surface proteins are critical for biofilm formation (2, 5, 26, 27, 44).

Streptococcus parasanguinis FW213 is one of the primary colonizers of the tooth surface and is also associated with the pathogenesis of infective endocarditis (3). The long fimbriae on the cell surface, composed by the fimbria-associated protein 1 (Fap1), are involved in bacterial adhesion to an *in vitro* tooth adhesion model and biofilm formation (12, 13). The mutation of *fap1* renders the bacterium defective in both biofilm formation and bacterial adhesion (13); however, it does not completely abolish either activity. Oral streptococci produce an array of adhesion molecules to ensure their survival in the oral

cavity. Three different adhesion systems have been reported for *Streptococcus gordonii* (17, 21, 43). In addition, many functionally redundant proteins exist in genomes of oral streptococci. For instance, the collagen-binding domain Pfam05737 was found in five putative cell wall anchor proteins in *Streptococcus sanguinis* SK36 (11). Moreover, analysis of sequenced genomes has revealed that oral streptococci have large numbers of cell wall-anchored proteins. *S. sanguinis* SK36 (47) and *S. gordonii* CH1 (29), two representative primary colonizers, contain 33 and 20 putative LPXTG cell wall surface-anchored proteins, respectively, implying that oral streptococci have developed comprehensive adhesion mechanisms to survive in the oral cavity (30).

In this study, a large open reading frame, designated *bapA1*, was identified in *S. parasanguinis* FW213. Genetic and functional analyses have revealed that this protein is critical for bacterial adhesion and biofilm formation. BapA1-mediated cell-cell interactions contribute to biofilm formation. BapA1 functions independently from the serine-rich glycosylated adhesin Fap1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli*, *S. parasanguinis*, and *S. sanguinis* strains were cultured under the growth conditions described previously (46).

Molecular cloning techniques. *E. coli* plasmid DNA was isolated using a miniprep DNA preparation kit (Qiagen). The genomic DNA of *S. parasanguinis* was extracted using a Puregene DNA isolation kit (Gentra System). PCR was performed with KOD hot-start DNA polymerase (Novagen) or LA Taq polymerase (TaKaRa), using a GeneAmp PCR system 9700 apparatus (PE Applied Biosystems). Primers used for the amplification of DNA fragments are listed in Table 2. Restriction enzymes and T4 DNA ligase (New England BioLabs) were used according to the manufacturer's instructions. Competent *E. coli* cells were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>E. coli</i>		
Top10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>nupG</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galE15</i> <i>galK16</i> <i>rpsL</i> Str ^r <i>endA1</i> λ ⁻	Invitrogen
BLR(DE3)	F ⁻ <i>ompT</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3) Δ(<i>srl-recA</i>)306::Tn10 Tet ^r	Novagen
<i>S. parasanguinis</i>		
FW213	<i>S. parasanguinis</i> parent strain	4
VT321	FW213 <i>fap1</i> mutant	12
AL805	FW213 <i>bapA1</i> (2499–3159):: <i>aphA3</i> Kan ^r	This study
AL807	FW213 <i>bapA1</i> (2499–3159):: <i>aphA3</i> ::pAL805 Kan ^r Em ^r	This study
AL808	VT321 <i>bapA1</i> (2499–3159):: <i>aphA3</i> Kan ^r	This study
AL809	FW213 <i>bapA1</i> (36–239):: <i>aphA3</i> Kan ^r	This study
AL810	FW213 <i>bapA1</i> (36–239):: <i>aphA3</i> ::pAL805 Kan ^r Em ^r	This study
Plasmids		
pET27b	Commercial expression vector	Novagen
pGEX6p-1	Commercial expression vector	Promega
pGEM-T Easy	Commercial TA cloning vector	Promega
pVPT-gfp	<i>E. coli</i> and <i>Streptococcus</i> shuttle vector	49
pAL801	pET27b- <i>rp</i>	This study
pAL802	pGEX6p-1- <i>mrp</i>	This study
pAL803	pGEX6p-1- <i>hyp</i>	This study
pAL804	pGEM- <i>bapA1</i> (2499–3159)- <i>aphA3</i>	This study
pAL805	pVPT- <i>bapA1</i>	This study
pAL807	pGEM- <i>bapA1</i> (36–239)- <i>aphA3</i>	This study

prepared and transformed by standard techniques (38). *S. parasanguinis* was transformed by electroporation using a gene pulser (Bio-Rad Laboratories) as described previously (10).

Construction of *bapA1* mutants in *S. parasanguinis* FW213. The organization of *bapA1* and its context genes (GenBank accession number JF345716) are schematically displayed in Fig. 1A. The allelic replacement mutagenesis strategy was used to construct two *bapA1* mutants, AL805 and AL809 (Fig. 1B). The first mutant (AL805) was constructed by deleting a 1.9-kb fragment coding for the 2498th to the 3156th amino acids of BapA1. In brief, a 5,186-bp PCR fragment of *bapA1* was first amplified from *S. parasanguinis* using the primer pair Bap-F1/Bap-R1 and then cloned into the pGEM-T Easy vector (Promega). The resulting construct was digested with HindIII to remove a 1.9-kb fragment and ligated in frame with a promoterless kanamycin resistance cassette, *aphA3* (23), to generate pAL804. This plasmid was used to transform *S. parasanguinis* FW213. The second mutant (AL809) was constructed by deleting the majority of *bapA1*. In brief, a 3.0-kb DNA fragment containing 1.7 kb from the 5' end of *bapA1* and a 1.3-kb fragment upstream of *bapA1* was amplified by PCR (primers Bap-F4/Bap-R4) and ligated into the pGEM-T Easy vector. A 609-bp DNA fragment coding for the 36 to 239 amino acid residues of BapA1 was deleted by inverse PCR using a primer pair (Bap-F5/Bap-R5) with an engineered BamHI restriction enzyme site (Table 2). The inverse PCR product was digested with BamHI and ligated in frame with the promoterless *aphA3*. The resulting plasmid, pAL807, was introduced into *S. parasanguinis*, and kanamycin resistance transformants were isolated (Fig. 1B). The replacement of the genomic copy of *bapA1* with the alleles disrupted by double-crossover recombination was confirmed by colony PCR and sequence analysis. The two confirmed mutants, AL805 and AL809, were used in this study.

Construction of complementation strains. The full-length *bapA1* gene was PCR amplified from the genomic DNA of *S. parasanguinis*, using the primer pair Bap-F3/Bap-R3 with engineered SalI and BamHI restriction enzyme sites. The PCR product was digested with SalI and BamHI and cloned into a streptococcus and *E. coli* shuttle vector, pVPT-gfp (49), to generate pAL805. This plasmid was transformed into the *bapA1* mutants AL805 and AL809 to obtain complemented strains AL807 and AL810, respectively.

qRT-PCR. Total RNA was isolated from exponentially grown cultures of *S. parasanguinis* as described previously (42). Residual chromosomal DNA was removed by RNase-free DNase (Promega). The RNA concentration was determined using a Smart Spec Plus spectrophotometer (Bio-Rad). The integrity of the RNA was confirmed by agarose gel electrophoresis. The first strand of cDNA

was reverse transcribed from total RNA using random primers and avian myeloblastosis virus reverse transcriptase (Promega). Reaction mixtures containing no reverse transcriptase served as negative controls. Amplification, detection, and analysis of mRNA expression were carried out using an IQ5 multicolor real-time PCR system (Bio-Rad) with a SYBR green PCR master mix (Bio-Rad). Corresponding oligonucleotide primers (Table 2) were designed using a Plexor primer design system (Promega). The 16S rRNA gene of *S. parasanguinis* FW213 (GenBank accession number DQ163031) was used as an internal standard. Data analysis was performed as described previously (24). Each quantitative real time (qRT-PCR) was carried out with at least three independent RNA samples in triplicate.

Expression and purification of recombinant BapA1 proteins and preparation of a polyclonal antibody. A recombinant BapA1 (rBapA1) from amino acid residues 2498 to 3156 of BapA1 (C terminus of repeat 4 and entire repeats 5 and 6) was produced. In brief, an internal HindIII fragment of *bapA1* containing the sequence encoding amino acid residues 2498 to 3156 was amplified from the genomic DNA of *S. parasanguinis* FW213 by the primer pair Bap-F2/Bap-R2. The PCR product was digested with NcoI and NheI and ligated with pET27b (Invitrogen), creating pAL801. This plasmid was then transformed into *E. coli* BLR(DE3) to generate a recombinant strain, AL802, for protein expression and purification.

Two fragments from the N-terminal region of BapA1, the muramidase-released protein (MRP) homologue and the functionally unknown hypothetical domain (Hyp) (Fig. 1B), were expressed as glutathione S-transferase (GST) fusion proteins in *E. coli*. In brief, a 1,646-bp *mrp* fragment and a 2,080-bp *hyp* PCR fragment were double digested with XhoI and XmaI and cloned into the pGEX6p-1 vector. The resulting plasmids, pAL802 and pAL803, respectively, were transformed into *E. coli* BLR(DE3). rBapA1, GST-MRP, and GST-Hyp were induced and purified using standard protocols.

The purified rBapA1 (1.0 mg) was used to generate polyclonal antisera in rabbits by Calcofine Biologicals, Inc. (Reamstown, PA). The titer and the specificity of the antiserum to rBapA1 were tested by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis, respectively.

Subcellular localization of BapA1. Proteins from different subcellular fractions were prepared by the method described previously (25). Briefly, 10 ml of exponentially grown *S. parasanguinis* cells was harvested and washed with phosphate-buffered saline (PBS) buffer once, followed by two washes with TEP buffer (10 mM Tris, 1 mM EDTA, 1% phenylmethylsulfonyl fluoride, pH 7.5). The cell pellets were resuspended in 200 μl of spheroplasting buffer (10 mM Tris, 2 mM

TABLE 2. Primers used in this study

Primer	Sequence ^a	Origin ^b
Bap-F1	ACGCAGACGGTAACCTCAAG	<i>bapA1</i> 5891–5910
Bap-R1	CGGTGTTGGTGGAAATACAG	<i>bapA1</i> 10142–10161
Bap-F2	TGGCCATGGCTTACGACTCAGTAAC	<i>bapA1</i> 7499–7518
	AGGC	
Bap-R2	CTGGCTAGCGATTGCTGAACCATCTA	<i>bapA1</i> 8458–8478
	CATC	
Bap-F3	TAAGTCGACATGAAAGATATATTTAA	<i>bapA1</i> 1–32
	TAGACGACAGCGCTT	
Bap-R3	ACTGGATCCAAGTTTCCAACACCAAA	<i>bapA1</i> 10467–10496
	CAAAGAGGCCTTG	
Bap-F4	TCTATCGTAGTTACTATGGAGGG	<i>bapA1</i> –1324–1301
Bap-R4	TCAACAGTTGTATCGGTAGA	<i>bapA1</i> 1680–1700
Bap-F5	GACGGATCCGAACGGTGCAACT	<i>bapA1</i> 751–774
Bap-R5	ACTGGATCCGTAATACTGAACAGA	<i>bapA1</i> 55–74
	CACC	
Suf1-F	CGCATGTTTAAATGATGTTAATTTCTA	<i>suf1</i> 469–505
	TGATAAACAAAG	
Suf1-R	CGATCAGGAGTTTCAATCACAACA	<i>suf1</i> 524–551
	TAGG	
Suf2-F	CGGTAATGTTTCAGATCTTCCAAGTG	<i>suf2</i> 36–61
Suf2-R	TACCACTACTGTGCGATCCTTCTC	<i>suf2</i> 109–132
Cbp-F	CCCCAAATTAATCCAATATCGAAGG	<i>cbp</i> 745–773
	TAGG	
Cbp-R	ATTGGTTGGTTTACGATTGACTCCAA	<i>cbp</i> 818–843
Trans-F	AACTGAAGGTTCTTTCCGCTT	<i>tra</i> 6–27
Trans-R	TAGCAGCAAACGTCGCAATCAG	<i>tra</i> 72–93
Fap1-F	GCAAAAGAAACAACCCTGACAAC	<i>fap1</i> 271–298
	TACA	
Fap1-R	TGTGATAAGCTAATCTTTTCTTCCG	<i>fap1</i> 342–372
	CTTT	
FimA-F	ACAGAATTTATCGATGGATTACAGC	<i>fimA</i> 7–36
	AATTC	
FimA-R	CAGCGACAATACCGATGGCAA	<i>fimA</i> 70–89
Sub-F	CAAAAGAAATCACTTATCATACGGTC	<i>sub</i> 2354–2383
	GTC	
Sub-R	AGCAGGAACATGAATAGTTTTTTC	<i>sub</i> 2446–2473
	TCCT	
PrgA-F	AGAAGTCGTCGATCTTGCAAATCT	<i>prgA</i> 1104–1127
PrgA-R	GCAAGTTCTTTACGTTGAGCTGATG	<i>prgA</i> 1131–1155
AbpA-F	ACCAATTGATCCAAAAGAAATATGTT	<i>abpA</i> 125–156
	TCATGG	
AbpA-R	CGAAGAGTTTCTTCTTTGATTCTGTC	<i>abpA</i> 199–228
	TTCA	
16S-F	GGGGTGAAGTCGTAACAAGGTAG	DQ163031 101–123
16S-R	CCCCACAAGACCTCTCAAACTAAAC	DQ163031 189–214

^a Restriction sites are underlined.^b Numbers indicate the nucleotide positions in the coding region of the corresponding genes.

MgCl₂, 26% raffinose, 1 mM phenylmethylsulfonyl fluoride, pH 6.8) with 60 U mutanolysin (Sigma). The suspensions were incubated for 30 min at 37°C with occasional mixing before separation of the spheroplast pellet from the supernatant by centrifugation at 6,000 × g at 4°C for 10 min. An aliquot of 100 µl supernatant was used as the cell wall-associated protein fraction. The pellet was resuspended in 200 µl spheroplasting buffer and used as the cytoplasmic protein fraction.

Biofilm formation assays. The biofilm formation was evaluated by both the microtiter plate method and the plastic coverslip method. The microtiter plate method was adapted from the method of O'Toole and Kolter (32) and modified as previously described (46). Briefly, *S. parasanguinis* cells were cultured in Todd-Hewitt broth and inoculated into the wells of polystyrene flat-bottom 96-well microtiter plates (Nunc 269787) coated with human saliva. Plates were incubated at 37°C under 5% CO₂ for 12 h. The optical density at 470 nm (OD₄₇₀) value was measured to monitor bacterial growth. The planktonic cells were removed by rinsing, and the biofilms were rinsed with distilled H₂O (dH₂O) two times. The adherent bacteria were stained with a 0.5% (wt/vol) solution of crystal violet (Fisher Scientific). The bound dye was released from stained cells using 30% glacial acetic acid, and the amount of biofilm formation was quantified by measuring the absorbance of the solution at 562 nm with a micro-plate reader (Bio-Tek). The biofilm formation assays were repeated 3 times, and the data were analyzed using the TTEST program. For the plastic coverslip assays, an overnight culture at a 1:100 dilution was inoculated into saliva-treated plastic coverslips that were placed in 6-well plates, and the plates were incubated at 37°C under 5% CO₂ for 12 h. Planktonic cells were removed by rinsing with dH₂O,

and the attached cells were stained with SYTO 9 from the LIVE/DEAD BacLight bacterial viability kit (Invitrogen) and examined using a Nikon Eclipse E400 fluorescent microscope.

Adhesion assays. Adhesion of *S. parasanguinis* to saliva-coated hydroxylapatite (sHA) beads, an *in vitro* tooth model, was performed as described previously (9). Briefly, bacteria were labeled with [³H]thymidine and reconstituted in adhesion buffer (50 mM sodium phosphate buffer, pH 6.0). The labeled cells were dispersed by brief sonication, and 1 ml of the suspension was incubated with 40 mg of sHA beads for 1 h at 37°C with constant gentle shaking. The beads and the supernatant were carefully separated and collected by centrifugation at 2,000 rpm for 5 min. After three washes, the bacterial cells bound to the beads and retained in the supernatants were measured separately using a liquid scintillation counter. The experiments were repeated 3 times, and the data were statistically analyzed using the TTEST program.

SDS-PAGE and Western blot analysis. Protein samples were separated on an 8% SDS-polyacrylamide gel and then stained with Coomassie blue R250 stain. For Western blot analysis, the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in PBS for 1 h before they were probed with BapA1 antiserum diluted at 1:1,000 in PBS with 0.1% Tween 20. Horseradish peroxidase-conjugated antirabbit and luminol-based reagents (enhanced chemiluminescence Western blot detection reagents; GE Healthcare) were used to detect BapA1.

Aggregation assays. Overnight cultures of different streptococcal strains were washed with PBS once, adjusted to the same optical density (OD₄₇₀ = 1.9) in test tubes, and kept at 4°C for up to 30 h. The top half of the culture suspensions was sampled, the sample was transferred to a fresh test tube, and the OD value was measured at 470 nm for each sample (14). The experiments were repeated 3 times, and the data were analyzed using the TTEST program.

GST pulldown assays. Fifty milliliters of each of the overnight cultures from the wild-type strain, the *bapA1* mutant AL809, and the complemented strain AL810 was adjusted to an OD₄₇₀ of 1.0 in Todd-Hewitt broth and centrifuged at 5,000 rpm for 5 min, and the culture media were precipitated with 2 volumes of 100% ethanol and then resuspended in 5 ml PBS buffer. One microgram each of GST, GST-MRP, and GST-Hyp immobilized on the glutathione agarose beads was mixed with 400 µl of each culture supernatant. After incubation at 4°C for 1 h, the supernatants were removed by centrifugation at 2,000 rpm for 3 min, and the agarose beads were washed 3 times with modified PBS buffer (500 mM NaCl and 1.5% Triton X-100) and boiled in 1× SDS-PAGE sample buffer to release bound proteins. The supernatants containing the bound proteins were harvested by centrifugation and then analyzed by Western blotting with anti-BapA1 antiserum.

Transmission EM studies. Electron microscopy (EM) studies were conducted to detect the presence of peritrichous fimbriae and short fibrils on the bacterial cell surface. Wild-type, *bapA1*, *fap1* single-mutant, and *bapA1 fap1* double-mutant cells were prepared and treated as described by Ramboarina et al. (37). Briefly, samples were diluted in PBS (100 mM phosphate buffer, pH 7.4, 150 mM NaCl) to the appropriate concentration for electron microscopy studies. A small aliquot (5 µl) was applied to carbon-coated copper grids. The grids were washed with several drops of PBS buffer and negatively stained with a few drops of Nano-W (Nanoprobes). The grids were observed on a Tecnai 12 electron microscope (FEI) equipped with a LaB6 cathode and a 14-µm charge-coupled device (CCD) camera (2,048 by 2,048 pixels; TVIPS, Germany). Micrographs were recorded in the CCD camera at an accelerating voltage of 100 kV and a ×67,000 nominal magnification, which corresponds to a 0.196-nm pixel size on the specimen scale. Images were high-pass filtered to remove the long-range background variations due to the uneven thickness of the stain surrounding the bacterial cell surface (filter radius, 0.001 to 0.0015 in absolute Fourier units).

In-cell ELISA. The wild-type strain, the *bapA1* mutant AL809, the complemented strain AL810, the *fap1* mutant, and the *bapA1 fap1* double mutants were grown to an OD₄₇₀ of 1.0. Bacterial cells were harvested, washed three times with coating buffer (0.1 M Na₂CO₃, 0.1 M NaHCO₃, pH 9.5), and resuspended at 1 × 10⁹ cells ml⁻¹, and 100 µl of cells was then added into the wells of polystyrene flat-bottom 96-well microtiter plates (Nunc 269787). Anti-BapA1 antibody and IRDye 800 goat anti-rabbit antibody (Li-Cor, Lincoln, NE) were used to carry out in-cell assays following the same protocol used for whole-bacterial-cell ELISA experiments (36). The signals were analyzed with an Odyssey infrared imaging system (Li-Cor, Lincoln, NE). Samples were analyzed in duplicate, and the experiment was repeated 3 times.

RESULTS

Identification and sequence analysis of *bapA1* gene. Investigating the genome of *S. parasanguinis* FW213, we identified a

FIG. 1. Schematic diagrams of genes flanking *bapA1* (A) and various *bapA1* derivatives (B). (A) *bapA1* gene locus (GenBank accession number JF345716). Genes flanking the *bapA1* gene are depicted. *dip*, dUTP diphosphatase; *pgm*, phosphoglycerate mutase; *radA*, DNA repair protein; *cah*, carbonic anhydrase; *tra*, ABC transporter. (B) Structural organization of the *bapA1* gene and the BapA1 protein from the wild type and the two *bapA1* mutants (AL805 and AL809). The full-length BapA1 contains 3,462 amino acid (a.a.) residues and is predicted to have a molecular mass of 381 kDa. The 0.1- to 0.7-kb and 7.5- to 9.4-kb fragments labeled on the *bapA1* gene (hatched boxes) represent the DNA fragments replaced by *aphA3* in AL809 and AL805, respectively. The AL805 mutant was truncated after amino acid residue 2446 and generated a 275-kDa protein. The AL809 mutant was truncated after amino acid residue 36. SP, signal peptide; MRP, muramidase-released protein domain; Hyp, hypothetical protein domain; R1 to R6, repetitive domains; CWA, cell wall anchor domain; Str, streptococcal pilin isopeptide linkage domain (TIGR03786); Pro, proline-rich motif; *aphA3*, promoterless kanamycin resistance cassette.

very large open reading frame (10,386 bp) located 13 kb upstream of *fapI*. A putative ribosome-binding site (GGAGA box) and a potential promoter region (TATAAT box) are localized 14 bp and 49 bp upstream of the translation start codon, respectively. The deduced protein sequence has 3,462 amino acid residues (Fig. 1B). Analysis of the protein sequence revealed the presence of a typical Gram-positive bacterium YSIRK-type signal peptide at the N terminus and a conserved cell wall anchor domain at the C terminus (28). A proline-rich stretch immediately precedes the LPETG cell wall anchor motif. The N-terminal region shares 44% homology with a domain of an MRP from *Streptococcus suis* (41), and it is followed by a functionally unknown (Hyp) domain (Fig. 1B). The C-terminal region contains six tandem-repeat domains (Fig. 1B), which exhibit limited homology (40% and 30%) to the collagen-binding surface protein from *S. sanguinis* SK36 (47) and to the aggregation substance from *Enterococcus faecalis* V583 (33), respectively. Nine putative streptococcal pilin isopeptide linkage domains (TIGR03786; Fig. 1B) were identified following the MRP-like domain.

BapA1 is surface localized, and the deletion of the tandem repeats of *bapA1* facilitates the release of the truncated BapA1 into the culture medium. Sequence analysis indicated that BapA1 is a cell surface protein. To determine the function and subcellular localization of this large protein, we generated a nonpolar allelic replacement mutant AL805. In the genome of AL805, a 1.9-kb DNA fragment spanning the 4th to the 6th repeat regions of BapA1 (R4 to R6) was replaced with *aphA3* (Fig. 1B). The insertion created an early stop codon and prematurely terminated the translation of BapA1. The truncated BapA1 lacks the cell wall anchor domain, the C-terminal portion of R4, and the entire R5 and R6 (Fig. 1B). Quantitative

RT-PCR experiments suggested that this mutation did not affect the expression of the downstream gene coding for a putative ABC transporter (data not shown). We analyzed the subcellular distribution of BapA1 to determine if the mutant promotes the release of the truncated protein into the culture medium. A very large (about 380-kDa) and intense protein band was detected in the cell wall fraction of the wild-type strain (Fig. 2A, lane 1). The corresponding protein band was absent in the mutant strain (lane 2); instead, a protein band with a lower molecular mass (about 270 kDa), corresponding to the truncated BapA1 (about 270 kDa), was detected in the culture medium (lane 5). Production of BapA1 was restored in both the cell wall and the culture medium fractions (lanes 3 and 6) in the BapA1 complementation strain, suggesting that BapA1 is responsible for the observed defect. Interestingly, the complemented strain still produced a large amount of truncated BapA1, indicating that the complementation is not complete. To determine if this is related to the mutant that we constructed, another mutant (AL809) that lacks the majority of *bapA1* was constructed and then complemented. The complemented strain (AL810) also only partially restored the production of BapA1 (data not shown).

The protein identity was further verified by Western blot analysis using a polyclonal antibody against the recombinant BapA1 protein (Fig. 2B, lanes 1 to 6). The wild-type BapA1 protein was expressed in both the cell wall and the culture medium fractions (lanes 1 and 4), while the truncated BapA1 was expressed only in the culture medium fractions (lanes 5 and 6). Interestingly, BapA1 was readily detected by Coomassie blue staining (Fig. 2A, lane 1) from the cell wall fraction, suggesting that BapA1 is an abundant protein on the bacterial cell surface.

FIG. 2. Subcellular localization of BapA1. Subcellular fractions of the wild-type (WT) strain, the *bapA1* mutant (AL805), and the complemented strain (AL807) were prepared and analyzed by SDS-PAGE (A) and Western blot analysis (B) to determine the subcellular distribution of BapA1. The positions of BapA1 and the truncated BapA1 are indicated.

BapA1 is required for the formation of short fibrils on the bacterial surface. To further determine the surface presentation of BapA1, we carried out electron microscopy studies. Long fimbriae were detected on the cell surface of both the wild-type and the *bapA1* mutant bacteria. Interestingly only short fibrils were detected in the *fap1* mutant (Fig. 3A). To determine if BapA1 is responsible for the presentation of the short fibrils, we also examined a *fap1* and *bapA1* double mutant by electron microscopy. Neither the long fimbriae nor the short fibrils were present in the double mutant (Fig. 3A), suggesting

that the BapA1 is involved in assembly of the short fibrils. Further in-cell ELISAs using BapA1-specific antibody revealed that BapA1 is present on the cell surface in the wild-type, the *fap1* mutant, and the *bapA1*-complemented strains but it is absent in the *bapA1* mutant and the *bapA1 fap1* double mutant (Fig. 3B). These results suggest that the formation of short fibrils is due to the presentation of BapA1 on the cell surface.

BapA1 is required for biofilm formation. Biofilm formation is critical for the interaction of bacteria with their environment.

FIG. 3. Localization of *bapA1* on the bacterial cell surface. (A) Electron micrographs of whole-cell preparations of wild-type, *bapA1* mutant, *fap1* mutant, and *fap1 bapA1* double-mutant bacterial strains stained with Nano-W. Black arrows, Fap1 fimbriae; white arrows, BapA1 pilus-like structures. Bar = 100 nm. (B) In-cell ELISA analysis of BapA1 on the cell surface of *S. parasanguinis*.

FIG. 4. *In vitro* biofilm assays. Saliva-coated microtiter plates (A) and plastic coverslips (B) were used to grow bacterial biofilms. Cell growth was quantified by measuring OD₄₇₀. The biofilm mass on the binding surface was determined using crystal violet staining and quantified by measuring OD₅₆₀ (A) or by live-cell staining and examined with fluorescent microscopy (B). *, $P < 0.05$.

The ability of the *bapA1* mutant to form biofilms was evaluated. In the microtiter plate assay, the mutant displayed a 90% reduction in biofilm mass accumulation (Fig. 4A, column 2) in comparison with the wild-type strain (Fig. 4A, column 1), although the mutant and the wild-type bacteria grew equally well in the planktonic state (Fig. 4A, columns 4 and 5). Moreover, the complemented strain partially overcame the biofilm defect (Fig. 4A, column 3). Similar results were obtained from the microscopic examination of bacteria grown on saliva-coated coverslips (Fig. 4B). The partial complementation of the biofilm formation phenotype is likely due to the smaller amount of full-length BapA1 expressed on the cell surface compared to the wild-type strain (Fig. 2, lanes 3 and 6). The wild-type bacteria developed a very thick biofilm after 12 h (Fig. 4B, left), while the mutant bacteria formed much thinner biofilms (Fig. 4B, middle). A thick biofilm layer was again observed in the complemented strain (Fig. 4B, right), demonstrating the importance of BapA1 in biofilm formation.

Mutation of BapA1 affects *in vitro* adhesion of bacteria to sHA. *S. parasanguinis* is a primary colonizer of the tooth surface; thus, we examined the contribution of BapA1 to bacterial adhesion in an *in vitro* tooth model. The *bapA1* mutant strain had a greater than 60% reduction in adhesion compared to the wild-type strain (Fig. 5). In contrast, the complemented strain (*bapA1* mutant::pAL805) recovered 57% of the adhesion (Fig. 5), indicating that BapA1 contributes to bacterial adhesion to sHA.

Mutation of BapA1 reduces bacterial autoaggregation. In the process of characterizing BapA1 mutant strains, we also noticed that the wild-type cells form aggregates more pronouncedly than the *bapA1* mutant. After a 30-h incubation, the majority of the wild-type cells have precipitated to the bottom

of the culture tube, whereas the mutant cells remained in suspension (Fig. 6). The complemented strain partially rescued the autoaggregation phenotype (Fig. 6). We also found that wild-type cells formed very tight cell pellets when they were harvested by centrifugation (13,000 rpm for 30 s), while mutant cells formed very loose pellets which are easier to suspend. The complemented strain exhibited an intermediate phenotype. These results demonstrate that BapA1 also plays a role in bacterial autoaggregation.

N-terminal MRP-like domain mediates BapA1-BapA1 interactions. Since the *bapA1* deficiency affected bacterial autoaggregation, we hypothesized that a BapA1-BapA1 interaction mediates bacterial autoaggregation. We have constructed a recombinant GST-BapA1 to examine the interaction between recombinant BapA1 and BapA1 from *S. parasanguinis* culture medium in pulldown assays. Since it is not feasible to overexpress the full-length BapA1 due to its high molecular mass, we

FIG. 5. *In vitro* adhesion assays. [³H]thymidine-labeled *S. parasanguinis* cells were allowed to bind to saliva-coated hydroxylapatite for *in vitro* adhesion analysis. *, $P < 0.05$.

FIG. 6. Autoaggregation of bacterial cells. Cell suspensions of *S. parasanguinis* wild-type, *bapA1* mutant (AL809), and complemented (AL810) strains were allowed to settle down at 4°C for 30 h, and the bacteria remaining in the upper layer of the cell suspensions were quantified by measuring OD₄₇₀. The experiment was repeated 3 times in triplicate. *, $P < 0.05$.

constructed two GST fusion proteins that cover the N-terminal nonrepeat regions of BapA1, MRP, and Hyp. The GST fusion proteins were used to pull down the proteins in the culture medium from the wild-type, the *bapA1* mutant, and the complemented strains. Recombinant GST-MRP was able to pull down full-length BapA1 from the wild-type and the *bapA1* complemented strains but not the truncated BapA1 from the *bapA1* mutant (Fig. 7, lanes 4 to 6). The GST-Hyp (Fig. 7, lanes 7 to 9) and the GST-glutathione agarose beads alone (Fig. 7, lanes 10 to 12) failed to pull down the full-length BapA1. In addition, the recombinant protein spanning the 4th to 6th repeat regions of BapA1 (R4 to R6) did not interact with the full-length BapA1 (data not shown), demonstrating a specific interaction between MRP and BapA1.

Effect of BapA1 on biofilm formation is independent of Fap1. Fap1 is a cell surface adhesin of *S. parasanguinis* FW213. Mutation of *fap1* inhibits biofilm formation as well (13). To determine whether the effect of the BapA1 deficiency is dependent on Fap1, we analyzed the Fap1 profile in the *bapA1* mutant. The production and subcellular distribution of Fap1 were not affected by the BapA1 deficiency (Fig. 8A, lanes 1, 2,

5, and 6). The mutation in *fap1* also had no effect on BapA1 production and BapA1 subcellular distribution (Fig. 8A, lanes 1, 3, 5, and 7). Both *bapA1* and *fap1* mutants had a defect in biofilm formation (Fig. 8B, columns 2 and 3). To further assess the relative contribution of Fap1 and BapA1 to the biofilm formation, a *bapA1 fap1* double mutant, AL808, was examined. As anticipated, the double mutant exhibited a severe defect in biofilm formation (Fig. 8B, column 4).

DISCUSSION

Cell surface-anchored proteins mediate streptococcal adhesion and biofilm formation. In this study, we identified a new cell surface protein, BapA1, which is required for biofilm formation of *S. parasanguinis* FW213. Numerous cell surface proteins from a variety of streptococci contribute to biofilm formation (2, 5, 26, 27, 44), and many of them are highly conserved among diverse streptococci. Interestingly, BapA1 is prevalent only in *S. parasanguinis* isolates and not in strains of the closely related species *S. sanguinis* (data not shown), suggesting that it is a new adhesin of *S. parasanguinis*. Sequence analysis revealed that BapA1 possesses nine putative pilin isopeptide linkage domains. Recent studies have demonstrated that formation of intramolecular isopeptide bonds by the major shaft subunit Spy0128 mediates *Streptococcus pyogenes* pilus biogenesis (19). Interestingly, only one isopeptide linkage domain has been detected in Spy0128. All the members of this family of putative pilin subunits have LPXTG-type cell wall sorting sequences that are presumably targeted by sortases (15). Sortase-mediated isopeptide bond cross-linking stabilizes and strengthens Gram-positive bacterial pili (18, 20) and may mediate bacterial adhesion to host cells (1, 8, 34). In this regard, it is possible that the nine putative pilin isopeptide linkage domains of BapA1 may facilitate the assembly of BapA1 into a pilus-like structure on the bacterial surface. In fact, our electron microscopy and in-cell ELISA studies revealed that the *bapA1* mutant lacks short fibrils on the bacterial surface, supporting the notion that BapA1 is

FIG. 7. MRP domain mediates BapA1 self-interaction. Bacterial culture media from the wild-type, *bapA1* mutant (AL809), and complemented (AL810) strains were incubated with GST-MRP, GST-Hyp, or GST-bound Sepharose beads. BapA1 captured by the fusion proteins was analyzed by Western blot analysis with BapA1 antibody. BapA1, recombinant MRP, and Hyp are labeled with arrows. Culture media harvested from the wild-type strain and the *bapA1* complemented strains were used as positive controls and culture media from the *bapA1* mutant was used as a negative control for BapA1 production (lanes 1 to 3).

FIG. 8. Effect of BapA1 deficiency on Fap1 production and distribution and BapA1-mediated biofilm formation. (A) The distribution of BapA1 and Fap1 in different subcellular fractions was analyzed by Western blot analysis using anti-BapA1 and anti-Fap1 antibody. BapA1 and Fap1 are labeled with arrows. (B) Biofilm formation of wild-type, *bapA1* single-mutant (AL805), *fap1* single-mutant (VT321), and double-mutant (AL808) strains was examined using the microtiter plate method. *, $P < 0.05$.

responsible for the formation of the pilus-like structures observed in the *S. parasanguinis* cell surface.

Multiple streptococcal adhesins have been implicated in the initiation of biofilm formation, and they can interact with each other to form a tightly regulated adhesin network. For instance, inactivation of antigen I/II genes in *S. gordonii* alters the expression of other adhesion genes (48). Mutations in *bapA1* had no impact on the gene expression of many surface proteins, including Fap1, FimA, collagen-binding protein, subtilisin-like protease, a cell surface exclusion protein, amylase-binding protein A, and two putative cell surface proteins of *S. parasanguinis* (data not shown), suggesting that BapA1 plays a distinct role in bacterial adhesion and biofilm formation. Furthermore, the expression level of BapA1 is high in the wild-type bacterium, and the protein is readily detected by Coomassie blue staining. The high level of expression and the presence of nine putative pilin isopeptide linker domains may explain why BapA1 can function by itself and is not involved in the adhesion network. In addition, BapA1 possesses other features that differ from those of the well-characterized adhesin Fap1. Fap1 is a glycoprotein and belongs to a highly conserved serine-rich repeat glycoprotein family. Biogenesis of Fap1 is mediated by specific accessory secretion and glycosylation genes. In this study we also found that (i) BapA1 is not a glycoprotein, (ii) BapA1 biogenesis is not dependent on the Fap1 biosynthetic machinery (data not shown), and (iii) BapA1 deficiency does not affect Fap1 biogenesis and vice versa. In-

terestingly, inactivation of either *bapA1* or *fap1* led to a similar biofilm phenotype: the respective mutants failed to form biofilms. These data are consistent with the notion that BapA1 and Fap1 can function independently of each other. Surprisingly, no obvious synergistic biofilm defect was observed when both *bapA1* and *fap1* were inactivated. It is possible that the sensitivity of the biofilm assays is not sufficient to detect subtle differences between the defects exhibited by the individual and the double mutants.

Like Fap1, the BapA1 deficiency inhibited bacterial biofilm formation. However, the results of this study strongly suggest that BapA1 and Fap1 mediate biofilm formation via different mechanisms. BapA1 may mediate biofilm formation via cell-cell interactions and bacterial autoaggregation. The Fap1 polypeptide, on the other hand, functions as an adhesin to initiate bacterial attachment, and the glycans attached to Fap1 mediate the later steps of biofilm development (46). Although a Fap1-like protein from *Streptococcus pneumoniae* has been implicated in cell-cell interactions (39), Fap1 does not appear to be involved in this type of interaction, since it does not form dimers under physiological conditions (37). Furthermore, the N-terminal region of BapA1 was determined to mediate the BapA1-BapA1 interaction. This region is homologous to the MRP from *Streptococcus suis*. Although the precise function of MRP is unknown, it has been implicated in the pathogenesis of pig disease since avirulent strains of *S. suis* type 2 lack MRP (45). Our studies revealed that the recombinant

MRP-like region of BapA1 is responsible for the BapA1-BapA1 self-interaction, which may be involved in bacterial cell-cell interactions and autoaggregation. Bacterial autoaggregation is known to be involved in bacterial biofilm formation. For instance, a bacterial autotransporter adhesin, TibA, mediates adhesion of enterotoxigenic *E. coli* to a variety of human cells by TibA-TibA interactions. The intercellular TibA-TibA interaction is also responsible for bacterial autoaggregation and biofilm formation (40). AIDA-I (adhesin involved in diffuse adherence 1) and other similar autotransporter adhesins also utilize a self-recognition mechanism to form biofilms which may allow bacteria to switch from free-living to sessile biofilm lifestyles in their native ecological niches (14, 40). All these observations are consistent with our hypothesis that the BapA1-BapA1 interaction contributes to bacterial autoaggregation and biofilm formation.

In conclusion, we have identified a new cell surface protein, BapA1, shown that it forms short fibrils on the bacterial surface, and determined that the self-association of the BapA1 protein mediates bacterial autoaggregation and biofilm formation.

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