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Overexpression and Purification of a Fimbria-Associated Adhesin of *Streptococcus parasanguis*

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A *Streptococcus parasanguis* adhesin that blocks the attachment of *S. parasanguis* FW213 to saliva-coated hydroxyapatite (SCHA) has been purified. Previous work demonstrated that the attachment of FW213 to SCHA is mediated by fimbriae and that one component associated with fimbriae is a 36-kDa protein (FimA) that reacts with antifimbria serum in Western blots (immunoblots) and is not present in afimbriated mutants. To obtain amounts of FimA sufficient for adhesion blocking assays, we cloned the gene coding for FimA into an *Escherichia coli* T7 overexpression system. The resulting strain produced large amounts of FimA, as much as 50% of the total cell protein. FimA was purified by elution from sodium dodecyl sulfate-polyacrylamide gels, and its native conformation was reestablished by sodium dodecyl sulfate removal, resolubilization in guanidine hydrochloride, and 50-fold dilution. Some refolded FimA aggregated into dimers and trimers. Preincubation of SCHA with 100 μg of purified, renatured FimA per ml blocked 85% of the binding of FW213. The FimA-SCHA complex was quite stable and could be washed continuously for at least 2 h with only a slight loss of FimA blocking activity. When FimA was added to preformed bacterium-SCHA complexes, it displaced 40% of the bacteria already bound to SCHA. The results suggest that FimA is an adhesin with a high substrate affinity and may prove useful in the development of a therapeutic agent for the prevention of plaque formation and endocarditis initiated by the sanguis streptococci.

Members of the group of microorganisms formerly classified as *Streptococcus sanguis* (*S. sanguis*, *S. gordonii*, and *S. parasanguis*) selectively adhere to the tooth surface and are primary colonizers of dental plaque (3, 15). These organisms and their products may serve as a substrate for the colonizers of mature plaque, which may contain potential oral pathogens. In addition, the sanguis group streptococci are also a major cause of subacute bacterial endocarditis (12). It is possible that the prevention of colonization by these organisms may prevent subsequent disease.

The attachment of *S. parasanguis* FW213 to teeth is mediated by fimbriae (6, 7, 11). Wild-type, fimbriated organisms bind well to saliva-coated hydroxyapatite (SCHA), an in vitro tooth model, whereas afimbriated mutants do not (11).

To investigate which proteins are associated with fimbriae, we prepared a fimbria-specific antiserum and used it to probe an *Eco*RI FW213 library (7, 10). All the colonies that expressed protein reactive with this antifimbria serum contained the same 6-kb fragment of FW213 DNA (10). Western blots (immunoblots) of the lysates of these positive colonies showed a single 36-kDa band. (Workers in our laboratory previously described a 30-kDa fimbrial protein whose relative mobility was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] on gradient gels containing urea [10]. This use of a slightly different technique to determine molecular weight may account for the discrepancy in the apparent molecular weight of the same protein described further in the present report.) A 36-kDa band was also seen in Western blots of FW213 lysates along with some higher-molecular-weight proteins (9, 17). Western blots of afimbriated, nonadherent FW213 mutants consistently lacked the 36-kDa protein (9, 17).

The results described above suggested that the 36-kDa protein (FimA) is a component of fimbriae and possibly a molecule responsible for adherence to teeth (an adhesin). In purified form, such an adhesin might block the binding sites on saliva-coated teeth and inhibit the formation of dental plaque.

To perform blocking studies, we had to purify FimA in milligram amounts. Lysates of the FimA-producing strains isolated directly from the library did not show a unique band at 36 kDa in Coomassie blue-stained SDS-polyacrylamide gels (data not shown). Thus, protein production was low, detectable only by Western blotting. In this study, large amounts of FimA were obtained by overexpression in *E. coli*. After a protein renaturation process, FimA was assessed for its ability to block the adhesion of FW213 to SCHA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this study are shown in Table 1. The plasmids used and constructed are described in Table 2. All *E. coli* strains were grown in 2×YT medium, which contains 10 g of yeast extract, 16 g of tryptone, and 5 g of NaCl per liter. Bacterial medium components were obtained from Difco. *E. coli* was grown at 37°C with aeration, and ampicillin (50 μg/ml) (Sigma) was added when appropriate for plasmid selection or maintenance. FW213 was grown in fresh brain heart infusion (Difco) at 37°C with 5% CO_2. Agar (15 g/liter) was added for plate medium.

DNA methodology. Plasmids were isolated by the method of Birnboim and Doly (1). Restriction endonuclease digestion and agarose gel electrophoresis protocols were those of

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TABLE 1. Relevant characteristics of bacterial strains used in this study

Bacterium	Strain	Construction	Source or reference
E. coli	JM101	*recA*	26
	JM105	*recA*	26
	VT638	JM105(pKK223-3)	This study
	VT640	JM105(pVT640)	This study
	CJ236	*ung-1 dut-1*	19
	GM2929	*dam*	21
	VT581	JM101(pVT581)	This study
	VT781	JM105(pVT781)	This study
	BL21(DE3)	T7 RNA polymerase lysogen	24
	BL21(DE3)(pLys)	BL21(DE3)(pLys)	24
	VT784	BL21(DE3)(pVT781)	This study
	VT786	BL21(DE3)(pLys, pVT781)	This study
	VT789	BL21(DE3)(pLys, pET3a)	This study
S. parasanguis (formerly *S. sanguis*)	FW213		Roger Cole, National Institutes of Health

Sambrook et al. (23). Competent cells were transformed by the $CaCl_2$ heat shock method (5). A 24-mer oligonucleotide was synthesized on a Generator DNA synthesizer (Dupont).

Site-specific mutagenesis of the start site of *fimA* to an *Nde*I site was performed by the procedure of Kunkel et al. (18, 19).

Preparation of ssDNA. Plasmid pVT581 was transformed into *E. coli* CJ236 (19). A transformant was grown to an optical density at 660 nm (OD_{660}) of 0.05 in 10 ml of medium and inoculated with 75 μl of M13KO7 (United States Biochemical, Cleveland, Ohio) stock (3×10^{10} PFU/ml). After 5 min, uridine was added to a final concentration of 25 μg/ml, and after 30 min, kanamycin was added to a final concentration of 70 μg/ml. After 6 h, cells were removed from the phage particles containing single-stranded DNA (ssDNA) by centrifugation at 12,000 × *g* for 15 min at 4°C. Longer incubation times resulted in a loss of the plasmid and a low yield of ssDNA. The phage, located in the supernatant, were centrifuged again to remove all cells, and 8 ml was transferred to a fresh glass tube. A 720-μl quantity of 25% polyethylene glycol 8000–3 M NaCl was mixed gently with the supernatant, and the mixture was incubated for 30 min at 25°C. Precipitated phage particles were harvested by centrifugation for 30 min at 12,000 × *g* and drained thoroughly. The pellet was resuspended in 1.8 ml of TE (23) and reprecipitated with 360 μl of the polyethylene glycol 8000–3 M NaCl solution for 15 min at 25°C. The phage pellet was harvested as described above. Phage protein was removed by extraction with phenol-chloroform as described by Sambrook et al. (23).

TABLE 2. Relevant characteristics of plasmids used in this study

Plasmid	Relevant phenotype	Construction	Source or reference
pTZ19R	Ap^r	*lac* expression vector	United States Biochemical
pVT581	Ap^r	pTZ19R + 2.2-kb fragment containing *fimA*	This study
pVT776	Ap^r	pVT581 with a base-pair substitution creating an *Nde*I site	This study
pKK223-3	Ap^r	*tac* expression vector	Pharmacia
pVT640	Ap^r	pKK223-3 + 6-kb *Eco*RI fragment containing *fimA*	This study
pET3a	Ap^r	T7 expression vector	24
pVT781	Ap^r	pET3a + *Nde*I-*Bcl*I fragment containing *fimA*	This study
pLys	Cm^r	T7 lysozyme vector	24

Mutagenesis reaction. The 24-mer oligonucleotide (1.2 pmol) containing the base-pair change described below was phosphorylated, annealed to the ssDNA template, and used as a primer in a DNA synthesis reaction. The synthesis reaction was carried out essentially as described in the instructions of the Muta-gene M13 in vitro mutagenesis kit (Bio-Rad). The resulting DNA was transformed into *E. coli* JM105. Transformants were screened by *Nde*I digestion of plasmid preparations, which were analyzed on agarose gels.

Construction of a FimA-overproducing strain. Plasmid DNA from a transformant that possessed an *Nde*I site at the *fimA* start site (pVT776) was transformed into a *dam* strain, *E. coli* GM2929, to enable the use of a *Bcl*I site 200 bp downstream of the stop codon of *fimA*. The *Nde*I-*Bcl*I fragment of pVT776 containing *fimA* and the large *Nde*I-*Bam*HI fragment of expression vector pET3a developed by Studier et al. (24) were purified from agarose gels with Geneclean (Bio 101), ligated at 14°C for 15 h with T4 DNA ligase, and transformed into *E. coli* JM105. Transformants were screened by a previously described colony immunoassay (10) with a fimbria-specific antiserum probe. A positive transformant, VT781, harbored plasmid pVT781, containing *fimA* cloned into pET3a in the correct orientation. The FimA expression strain, VT786, was constructed by transforming pVT781 into *E. coli* BL21(DE3)(pLys) (24), which contains the gene for T7 RNA polymerase under the control of the *lac* operon, inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). Plasmid pLys produces a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase. The T7 lysozyme prevents leaky expression of the cloned gene of interest before induction. VT784 contains the same FimA plasmid as VT786 in the same host strain but does not contain pLys.

Protein production. FimA overexpression strain VT786 was grown to an OD_{660} of 0.6, and IPTG was added to a final concentration of 1 mM to induce the expression of T7 RNA polymerase and, in turn, the production of FimA. Cells were harvested after 2 to 3 h by centrifugation at 4,000 × *g* for 10 min. The supernatants were removed, and the cell pellets were stored at −20°C.

Protein analysis. SDS-PAGE was performed as described by Laemmli (20) with 12% acrylamide separating gels and 3% acrylamide stacking gels. Western blotting was carried out by the method of Towbin et al. (25). Protein concentrations were determined as described by Bradford (2) with bovine serum albumin (BSA) as a standard. The relative percentage of FimA in *E. coli* lysates was assessed by laser scanning densitometry of Coomassie blue-stained SDS-polyacrylamide gels.

Purification and renaturation of FimA. Frozen VT786 cell pellets harvested from 4 ml of cell culture were resuspended quickly in 1 ml of SDS-PAGE sample buffer, and the suspension was placed in a boiling water bath for 2 min. The lysate was passed through a 26-gauge needle 20 times to shear DNA and was loaded onto a 2-cm stack of an SDS-polyacrylamide gel 16 cm by 12 cm by 1.5 mm. Electrophoresis was carried out at 200 V until the dye front reached the bottom. A narrow vertical gel slice was stained for 15 min in Coomassie blue and destained for 30 to 60 min to allow localization of FimA in the preparative gel. The remainder of the gel was kept under Saran Wrap during this destaining. A horizontal gel slice containing FimA (36,000 M_r) was stored at −20°C. For elution, the slice was thawed and extruded through a 5-ml syringe into a sample cup of a model 1750 electrophoretic sample concentrator (ISCO). Elution was performed with SDS-PAGE running buffer at 3 W for 6 h. Eluted FimA was harvested in 250 to 300 μl of running buffer and renatured as described by Hager and Burgess (16).

Adhesion inhibition assays. Purified, renatured FimA was dialyzed overnight against 50 mM phosphate buffer (PB) (pH 6.0), which was the buffer used throughout the assays. *S. parasanguis* was streaked for confluency onto a blood agar plate (tryptic soy agar blood agar base with 5% defibrinated sheep blood) and incubated for 12 h anaerobically. A 300-ml sidearm flask containing 40 ml of fresh, filter-sterilized brain heart infusion medium was inoculated with a smooth suspension of bacteria from the blood agar plate to an OD_{470} of 0.05. [^{3}H]thymidine (New England Nuclear) was added to a concentration of 2 μCi/ml, and the culture was grown without agitation at 5% CO_2 until the cells reached an OD_{470} of 0.20. The cells were washed twice and resuspended in 20 ml of PB, and the suspension was sonicated for 1 min in a model 5200 water bath sonicator (Branson Ultrasonics, Danbury, Conn.). Clarified saliva was added as previously described (7) to 40 mg of spheroidal hydroxyapatite beads in a 20-ml glass scintillation vial, and the mixture was incubated for 1 to 3 h at 37°C on a New Brunswick gyratory shaker (3.75 speed setting). Saliva was removed by aspiration, the beads were washed once with 3 ml of buffer, and 1.0 ml of protein solution or control buffer was added. After 1 h of incubation, the protein was removed, and the beads were washed twice immediately, except during the FimA affinity experiments, in which the FimA-SCHA complex was incubated in wash fluid for various times. Following these washes, 1.0 ml of the labelled bacterial suspension was added. After 1 h of incubation with the bacteria, the beads were washed immediately three times and transferred into a fresh scintillation vial with the last wash before aspiration of the last wash. (For the displacement affinity experiment [see Table 4], FimA was added only after incubation of SCHA with bacteria. SCHA with bound bacteria was prepared and washed as usual. One milliliter of a 100-μg/ml preparation of FimA was added and incubated with the SCHA-bacterium complex for 1 h. The beads were washed once and counted as described below.) Ten milliliters of scintillation fluid was added per vial, and the mixture was incubated overnight at 25°C before counting. The percentage of blocking activity for each protein sample was calculated as $(1 - x) \times 100$, where x is the number of sample counts divided by the number of counts in the sample preincubated with buffer only.

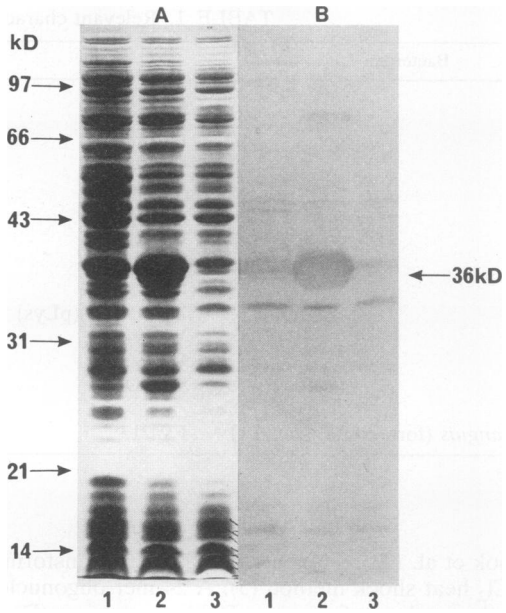

FIG. 1. Protein analysis of lysates of *E. coli* overexpressing FimA. (A) Coomassie blue-stained SDS-polyacrylamide gel of crude *E. coli* lysates. (B) Western blot analysis with a fimbria-specific antiserum probe. Lanes: 1, VT784 (FimA positive); 2, VT786 (FimA positive and pLys positive); 3, VT789 (control) lysates after 2 h of IPTG induction.

RESULTS

Overexpression of FimA. An overexpression system developed by Studier et al. (24) was used to increase protein production and facilitate purification. This system uses a strong promoter of the T7 virus and a strong T7 translation initiation region. In addition to high protein yields, a further advantage of this system is that, unlike the protein product in fusion protein overexpression systems, the protein product contains only those amino acids coded for by the original gene, i.e., this system produces native protein (albeit before any posttranslational processing). This characteristic increases the chance of the protein product retaining its native biological activity. For optimal transcription and translation, the system requires an *Nde*I site precisely at the start codon of the gene of interest. Thus, the first step in placing *fimA* into this overexpression system was the site-specific mutagenesis of the CACATG start region of *fimA* (8) to the CATATG *Nde*I recognition site. The site-specific mutagenesis procedure used was essentially the same as the procedure developed by Kunkel et al. (18, 19), except that a phagemid vector was used to carry *fimA* instead of M13. When this procedure was used, 11 of 20 transformants contained the desired mutation. The cloning of *fimA* into the T7 overexpression system is described in Materials and Methods. The resulting strain (VT786) expressed large amounts of FimA (Fig. 1). Laser scanning densitometry of Coomassie blue-stained SDS-polyacrylamide gels of VT786

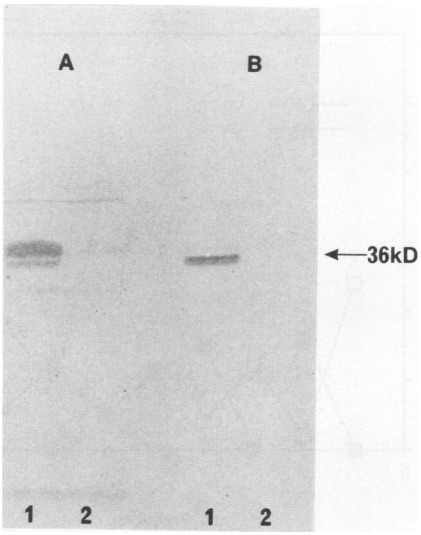

FIG. 2. Western blot analysis of Fim A expressed in *E. coli*. (A) *E. coli* cell lysates. (B) Concentrated growth medium. Lanes: 1, VT640 (FimA positive); 2, VT638 (control).

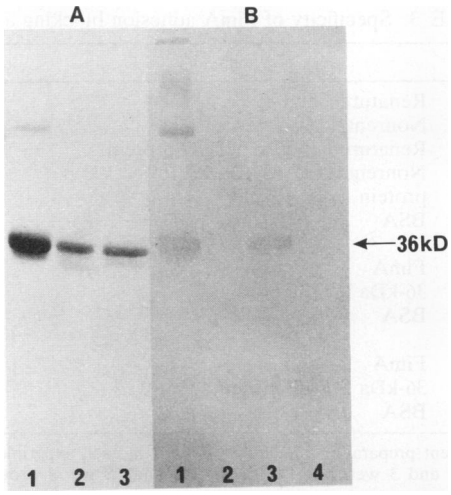

FIG. 3. Protein analysis of purified FimA. (A) Coomassie blue-stained SDS-polyacrylamide gel. (B) Western blot analysis with a fimbria-specific probe. Lanes: 1, purified, renatured FimA; 2, a protein fraction purified from the 36-kDa region of VT789 and renatured (VT789 is the negative control strain that lacks *fimA*); 3, purified FimA before renaturation; 4, purified negative control protein before renaturation (panel B [Western blot] only). The Coomassie blue stain of the last protein is not shown but it is indistinguishable from that in lane 2 of panel A.

lysates demonstrated that FimA accounted for 50% of the total cell protein. Figure 1 also shows that the presence of pLys in the host strain greatly increased the yield of FimA from the cloned gene.

The level of a protein with an M_r of 28,000 was moderately increased, and this protein reacted with the antifimbrial antiserum but did not block adhesion (see Fig. 4). No *E. coli* proteins reacted with our antifimbrial antiserum. Furthermore, *fimA* was the only *S. sanguis* gene present in VT784 and VT786. Hence, the 28-kDa protein seen in the Western blot in Fig. 1 is probably the result of alternative transcription of *fimA* or an abortive translational product that results in a protein that does not block adhesion. A second possibility, but one that is less likely because the band is so clear, is that the 28-kDa protein is a degradation product of FimA.

Secretion of FimA into culture media. The FimA found in VT786 cell lysates appeared as a doublet at 36 and 34 kDa in SDS-PAGE. Concentrated cell growth medium from a different FimA-producing strain, VT640, which does not carry pLys (VT786 underwent too much self-lysis to allow this analysis), also contained FimA but only the lower half of the doublet (Fig. 2). The difference in size between the two parts of the doublet is the same as that of the leader peptide predicted by the nucleotide sequence (8). These data suggest that the predicted leader peptide of FimA is functional and is removed during the transport of FimA through the cell membrane.

Purification of FimA. VT786 cell pellets were prepared as described above. Because of the presence of T7 lysozyme, the thawed cells were easily lysed by brief sonication in PB at 25°C or by suspension in SDS-PAGE sample buffer. In an effort to retain the biological activity of FimA, purification was first attempted with cells lysed in PB rather than with cells lysed in denaturing SDS-PAGE sample buffer. Centrifugation of the PB lysate at 40,000 × *g* for 15 min resulted in the distribution of large amounts of FimA into both the pellet and the supernatant. The FimA pellet could be solubilized with 1.5% SDS or 6 M guanidine hydrochloride. The FimA in the supernatant, however, did not appear as a solubilized monomer, as assessed by sizing column chromatography, isoelectric focusing, or ultrafiltration (data not shown). It appeared instead as a heterogeneous aggregate of $>2 \times 10^6$ kDa that was difficult to separate from contaminating *E. coli* proteins. Although 6 M guanidine HCl could solubilize the FimA aggregate, removal of the guanidine HCl resulted in the precipitation of FimA and precluded its use in further purification techniques. Because of these difficulties, a purification and renaturing procedure described by Hager and Burgess (16) was used instead. This procedure consists of the elution of protein from preparative SDS-polyacrylamide gels, the removal of SDS by acetone precipitation, resolubilization of the purified protein in a small volume of 6 M guanidine HCl, and 50-fold dilution in a buffer containing a reducing agent (dithiothreitol), which presumably allows the primary amino acid chain to refold into its native tertiary structure complete with native disulfide bridges. FimA purified from SDS polyacrylamide gels and renatured by the procedure described above was no longer precipitated when guanidine HCl was removed. This result suggests that the dilution step does facilitate correct protein folding. It is possible that subjecting VT786 lysates to the renaturing procedure alone would allow the use of other purification procedures, such as column chromatography. Interestingly, although the protein was made from a single *S. parasanguis* gene coding for a predicted 34-kDa protein (8) and was isolated from only the 36-kDa region of the gels, after renaturation, some FimA appeared in dimeric and trimeric forms (Fig. 3). Figure 3 also shows that these polymeric forms were not present before renaturation. Approximately 300 μg of renatured protein was isolated from each gel.

Adhesion inhibition assays. Purified, renatured FimA consistently blocked the adhesion of *S. parasanguis* FW213 to SCHA (Tables 3 to 5 and Fig. 4). To demonstrate the uniqueness of the ability of FimA to inhibit the adhesion of *S. parasanguis*, we also assayed the following proteins for blocking activity: (i) control strain VT789 protein isolated

TABLE 3. Specificity of FimA adhesion blocking activity

Expt	Protein[a]	% Blocking
1	Renatured FimA	32
	Nonrenatured FimA	0
	Renatured 36-kDa VT789 protein	17
	Nonrenatured 36-kDa VT789 protein	0
	BSA	8
2	FimA	39
	36-kDa VT789 protein	0
	BSA	0
3	FimA	68
	36-kDa VT789 protein	23
	BSA	14

[a] A different preparation of FimA was used in each experiment. Experiments 1, 2, and 3 were done with 20, 10, and 58 μg of protein per ml, respectively.

from the 36-kDa region in SDS-PAGE and renatured (Table 3 and Fig. 4A), (ii) BSA (Table 3 and below), (iii) FimA that was purified but not renatured (the guanidine HCl solubilization step was omitted) (Table 3), and (iv) nine different fractions of protein purified from 0.5-cm horizontal gel slices from SDS-PAGE of both VT786 and VT789 and renatured (Fig. 4A). Equal amounts of protein were used within each experiment unless otherwise indicated. All experiments were done in triplicate. Although other proteins did display various degrees of background blocking activity, Table 3 shows that FimA blocked at least twice as efficiently as any other protein and that nonrenatured FimA did not block at all. The experiment shown in Fig. 4A, in which nine different groups of proteins from both the FimA-producing strain and the control strain were used, shows a clear peak of blocking activity at 36 kDa in VT786, the FimA-producing strain. Proteins from VT789, the control strain, did not show this peak of activity. Figure 4B shows the presence of FimA in the fractions with peak blocking activity. Gel fractions 1, 2, and 9 yielded the least amount of protein and are not shown in the SDS-PAGE analysis in Fig. 4B, since all the isolated protein was used in blocking experiments. Some of the blocking activity in the upper VT786 fractions was most likely due to the dimeric forms of FimA seen in Fig. 3. The blocking activity of FimA was concentration dependent (Table 4). To be useful clinically, an *S. parasanguis* blocking agent must be able to adhere to the substrate receptor for a reasonable length of time. FimA remains attached to SCHA after at least 2 h of incubation with PB and retains its ability to block FW213 adhesion (Table 5, experiment 1). A final blocking experiment (Table 5, experiment 2) was designed to determine whether soluble FimA could actually displace bacteria already bound to SCHA. A regular blocking assay

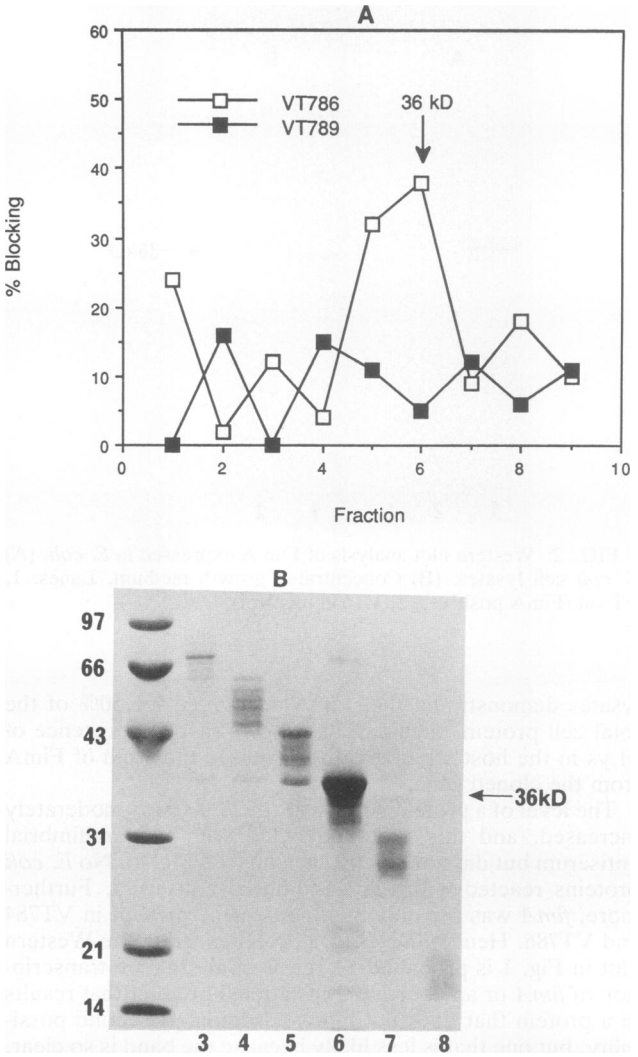

FIG. 4. Fractions of *E. coli* VT786 (FimA positive) and VT789 (control) lysate proteins. (A) Adhesion blocking activity of proteins purified from 0.5-cm horizontal gel slices from SDS-PAGE and renatured. Twenty five micrograms of protein from each fraction was used in the assay. (B) Coomassie blue-stained SDS-polyacrylamide gel of the lysate proteins used in the blocking assay. Equal volumes of each fraction, which did not necessarily contain equal amounts of protein, were loaded onto the gel.

with 100 μg of FimA per ml added before the bacteria resulted in 85% blocking. When 100 μg of FimA per ml was added after the bacteria had already bound and the mixture was incubated for 1 h and washed once, 40% of the attached bacteria were displaced from the SCHA.

TABLE 4. Concentration dependence of adhesion blocking ability of FimA

Protein amt (μg/ml)	% Blocking	
	FimA	BSA
10	33	0
30	57	0
90	73	0

TABLE 5. Affinity of FimA for SCHA

Expt	Wash time (h)	% Blocking
1	0	66
	1	66
	2	54
2	FimA added before	85
	FimA added after	40

DISCUSSION

Adhesion to the host surface is a necessary first step in the pathogenicity of many microorganisms. The streptococcal species that were originally classified as *S. sanguis* are considered initiators of plaque development and serve as substrates for more mature plaque that may contain potential oral pathogens (3, 15). In addition, these organisms also adhere to post-rheumatic fever heart valves and are a major cause of subacute bacterial endocarditis (12). An understanding of the mechanisms that they utilize to adhere may allow a rational approach to therapy design. Molecules that act as adhesins are of particular interest since, with overexpression technology or peptide synthesis, large amounts of free adhesive molecules can be made. Such free adhesins could theoretically block sites to which pathogenic organisms normally attach and thus prevent disease. Adhesins are also promising vaccine candidates. So far, the streptococcal adhesin described here, FimA, shows considerable promise as a means of controlling the colonization of host surfaces by sanguis streptococci.

The purification of large amounts of a protein expressed as a small percentage of the total cell protein can be a long and tedious process. Overexpression technology has provided a means to circumvent many of the difficulties inherent in purifying protein directly from the organism of interest. The overexpression system of Studier et al. (24) used in this study was consistently reliable and provided excellent yields of the desired protein. The presence of pLys in the host greatly increased the yield of FimA. Plasmid pLys produces a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase that prevents leaky expression of the cloned gene of interest. Studier et al. (24) found that inclusion of pLys in the host strain helps stabilize the plasmid containing the cloned gene of interest, especially when the gene codes for a product toxic to *E. coli*.

The only drawback to this initial phase of protein purification was that the overexpressed protein did not appear as a solubilized monomer, as assessed by sizing column chromatography, isoelectric focusing, or ultrafiltration. It appeared instead as a heterogeneous aggregate of $>2 \times 10^6$ kDa. It is possible the aggregation was an artifact of the overexpression system, as many foreign proteins overexpressed in *E. coli* tend to aggregate (22). However, the same aggregation phenomenon has been noted with the cloned subunit of the fimbriae of another gram-positive organism, *Actinomyces viscosus* (4). We were able to solubilize the aggregates with 6 M guanidine HCl. However, the removal of guanidine HCl resulted in FimA precipitation, precluding its use in further assays.

The elution of FimA from SDS-polyacrylamide gels and the renaturation protocol used here proved a simple and efficient means of purifying the overexpressed protein in a biologically active form. Purified FimA that was not renatured was completely inactive in blocking assays. This result suggests that the ability of FimA to bind to a receptor on SCHA and block the adherence of FW213 is dependent on a specific three-dimensional conformation of its peptide chain.

We had previously demonstrated that the polyclonal fimbria-specific antibody used in this study blocked the adhesion of FW213 to SCHA (7). In this study, we showed that purified, renatured FimA recognized by this serum was a specific adhesin that inhibited the attachment of FW213 whole cells to SCHA. SsaB, a cloned adhesin from *S. gordonii* (14), inhibited the binding of *S. gordonii* 12na to SCHA, although the specificity of the protein was not clear, as control proteins were not included. Both *fimA* (8) and *ssaB* (13) have been sequenced and shown to be approximately 73% homologous at the DNA level. As these two genes were cloned from different species of sanguis streptococci, this finding suggests that the gene is well conserved. Southern analysis of several isolates from each of the sanguis streptococci probed with an internal *fimA* fragment confirmed that the gene coding for FimA was present and conserved at 70% or better homology (unpublished data).

The FimA adhesin had a high affinity for its substrate and resisted removal from the substrate by continuous washing for several hours. Furthermore, FimA was able to displace FW213 cells that were already bound to SCHA. Both the washing time and displacement experiments described here suggest not only that FimA is an adhesin but also that it has a high affinity for its substrate on the salivary pellicle.

The polymer forms of FimA seen after renaturation are not dissociated into the 36-kDa monomer by boiling in 1.5% SDS in the presence of 2-mercaptoethanol. The nature of the binding of these subunits is not clear, but the data suggest that binding of the polymeric subunits does not occur via disulfide bonds, as one might expect. The nature and significance of these subunit interactions are not yet understood. The polymeric forms of FimA may be an artifact of the renaturation process. However, polymeric forms were also seen with the SsaB protein isolated from mutanolysin digests of *S. sanguis* 12 (14). Furthermore, SDS-PAGE and immunoblotting of the unheated cloned 54-kDa fimbrial subunit of *A. viscosus* revealed polymeric forms as well (4). The polymerization suggests the possibilities that FimA is a structural part of the fimbria as well as an adhesin and that the renaturation process allows some degree of self-assembly. An adhesin that exists on each structural subunit could allow a great number of substrate binding interactions and strong adherence to the substrate.

Further studies are needed to determine the active binding portion of the 36-kDa protein. In addition, these in vitro results need to be confirmed in vivo.

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REFERENCES

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Carlsson, J., H. Grahnnew, G. Jonsson, and S. Wikner.** 1970. Establishment of *Streptococcus sanguis* in the mouths of infants. Arch. Oral Biol. **15**:1143–1148.
4. **Cisar, J. O., E. L. Barsumian, R. P. Siraganian, W. B. Clark, M. K. Yeung, S. D. Hsu, S. H. Curl, A. E. Vatter, and A. L. Sandberg.** 1991. Immunochemical and functional studies of *Actinomyces viscosus* T14V type 1 fimbriae with monoclonal and polyclonal antibodies directed against the fimbrial subunit. J. Gen. Microbiol. **137**:1971–1979.
5. **Davis, R. W., D. Botstein, and J. L. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Elder, B. L., and P. Fives-Taylor.** 1986. Characterization of monoclonal antibodies specific for adhesion: isolation of an

adhesin of *Streptococcus sanguis* FW213. Infect. Immun. **54:** 421–427.
7. **Fachon-Kalweit, S., B. Elder, and P. Fives-Taylor.** 1985. Antibodies that bind to fimbriae block the adhesion of *Streptococcus sanguis* to saliva-coated hydroxyapatite. Infect. Immun. **48:** 617–624.
8. **Fenno, J. C., D. J. LeBlanc, and P. Fives-Taylor.** 1989. Nucleotide sequence analysis of a type I fimbrial gene of *Streptococcus sanguis* FW213. Infect. Immun. **57:**3527–3533.
9. **Fives-Taylor, P., J. C. Fenno, E. Holden, L. Linehan, L. Oligino, and M. Volansky.** 1991. Molecular structure of fimbria-associated genes of *Streptococcus sanguis*, p. 240–243. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.
10. **Fives-Taylor, P. M., F. L. Macrina, T. J. Pritchard, and S. S. Peene.** 1987. Expression of *Streptococcus sanguis* antigens in *Escherichia coli*: cloning of a structural gene for adhesion fimbriae. Infect. Immun. **55:**123–128.
11. **Fives-Taylor, P. M., and D. W. Thompson.** 1985. Surface properties of *Streptococcus sanguis* FW213 mutants nonadherent to saliva-coated hydroxyapatite. Infect. Immun. **47:**752–759.
12. **Frandsen, E. V. G., V. Pedrazzoli, and M. Kilian.** 1991. Ecology of viridans streptococci in the oral cavity and pharynx. Oral Microbiol. Immunol. **6:**129–133.
13. **Ganeshkumar, N., P. M. Hannam, P. E. Kolenbrander, and B. C. McBride.** 1991. Nucleotide sequence of a gene coding for a saliva-binding protein (SsaB) from *Streptococcus sanguis* 12 and possible role of the protein in coaggregation with actinomyces. Infect. Immun. **59:**1093–1099.
14. **Ganeshkumar, N., M. Song, and B. C. McBride.** 1988. Cloning of a *Streptococcus sanguis* adhesin which mediates binding to saliva-coated hydroxyapatite. Infect. Immun. **56:**1150–1157.
15. **Gibbons, R. J., S. S. Socransky, W. C. DeAraujo, and J. vanHoute.** 1964. Studies of the predominant cultivable microbiota of dental plaque. Arch. Oral Biol. **9:**365–370.
16. **Hager, D. A., and R. R. Burgess.** 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. **109:**76–86.
17. **Holden, E.** 1991. Master's thesis. University of Vermont, Burlington.
18. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
19. **Kunkel, T. A., J. D. Roberts, and R. A. Zabour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Marinus, M. G.** 1987. DNA methylation in *Escherichia coli*. Annu. Rev. Genet. **21:**113–131.
22. **Marston, F. A. O.** 1986. The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. Biochem. J. **240:**1–12.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct the expression of cloned genes. Methods Enzymol. **185:**60–89.
25. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
26. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.