Expression of Streptococcus sanguis Antigens in Escherichia coli: Cloning of a Structural Gene for Adhesion Fimbriae

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Chromosomal DNA from Streptococcus sanguis FW213 was partially digested with EcoRI and ligated into the positive-selection cloning vector pOP203(A2+). The ligation mixture was used to transform Escherichia coli K-12, and 4,500 transformants were examined. The tetracycline-resistant colonies had inserts averaging 3.2 kilobases. The entire colony bank was screened by colony immobilization with polyclonal rabbit serum raised against S. sanguis FW213 whole cells. Thirty recombinant colonies produced stable positive reactions of various intensities, indicating that S. sanguis antigens could be expressed in E. coli. Restriction endonuclease digestion of these clones suggested that 26 of the clones were unique. Only two clones, VT616 and VT618, gave positive reactions with fimbria-specific antisera. That the gene coding for the antigen was located on the plasmid was confirmed by demonstrating that the presence of the plasmid was linked to antigen production. Western immunoblot analyses of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels showed that both clones produced a fimbrial peptide of Mr, 30,000. The two recombinant plasmids were shown by Southern analysis and restriction mapping to contain the 6-kilobase EcoRI fragment inserted in opposite orientations. Southern hybridization confirmed that this fragment is present in S. sanguis genomic DNA. The Mr, 30,000 protein gene was expressed in both orientations, suggesting that the fimbrial promoter is located on the 6-kilobase fragment. These results show that at least one streptococcal fimbrial gene can be cloned and expressed in E. coli.

Adherence to and colonization of host tissue are primary steps in bacterial disease. Dental caries and periodontal diseases are the most prevalent infections of humans. Both disease processes are initiated by the formation of dental plaque (8, 13, 14, 17, 29). Streptococcus sanguis is a gram-positive bacterium that is found in high numbers in dental plaque (15). It has been shown to be an initiator of the formation of dental plaque (1, 12). For these reasons, an understanding of the molecular mechanisms involved in the adhesion of this organism is of great interest.

Recent studies have suggested that the adhesion of S. sanguis is complex, involving hydrophobic (24), lectin (23), cell wall protein (19, 27), and fimbria-mediated interactions (7, 9–11). Immunological analysis and analysis of mutants have implicated the peritrichous fimbriae on S. sanguis FW213 in adhesion of this organism to the in vitro tooth model, saliva-coated hydroxyapatite (7, 9–11). Similar analyses have implicated the type 1 fimbriae of Actinomyces viscosus in adhesion to saliva-coated hydroxyapatite (2, 31). The precise mechanism(s) of adhesion in both systems remains unknown.

Little is known about the biochemical nature of these gram-positive fimbriae. Part of the difficulty has arisen from the complications in obtaining purified material because of the extremely hydrophobic nature of the fimbriae. In addition, methods that involve dissociation and depolymerization of fimbriae from gram-negative organisms are ineffective with gram-positive fimbriae (6; manuscript in preparation).

This study was undertaken to determine whether an S. sanguis gene encoding the fimbrial subunit could be cloned and expressed in Escherichia coli. Such cloning will allow us to identify and characterize the putative fimbrial subunits and precursors and begin a biochemical and genetic analysis of adhesion.

MATERIALS AND METHODS

Bacterial strains. The strains used or constructed in this study are described in Table 1. E. coli strains were grown in L broth (5) or Z medium, which contains (per liter) tryptone, 10 g; yeast extract, 1 g; NaCl, 8 g; glucose, 1 g; and 16 N NaOH, 0.2 ml. E. coli cells for bacteriophage lambda infections were grown in NZYM medium with maltose, as described by Maniatis et al. (21). Tetracycline was added to the media at a concentration of 10 μg/ml, and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added when induction was desired. Streptococcal strains were grown in brain heart infusion broth supplemented with 3% yeast extract.

Materials. Brain heart infusion broth was purchased from Difco Laboratories, Detroit, Mich. Restriction endonucleases, lambda DNA, and T4 DNA ligase were purchased from Bethesda Research Laboratories, Gaithersburg, Md. Alkaline phosphatase-conjugated, affinity-purified goat antimouse and goat anti-rabbit immunoglobulin G (heavy and light chain) were obtained from Dynatech Diagnostics, Windham, Maine. Supplies for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), crossed immunoelectrophoresis, and dot immunoblots were purchased from Bio-Rad Laboratories, Richmond, Calif. IPTG was purchased from Sigma Chemical Co., St. Louis, Mo.

Plasmid and chromosomal DNA isolation. Covalently closed-circular plasmid DNA was amplified, extracted by SDS lysis, and isolated on cesium chloride-ethidium bromide gradients as described by Clewell (4). Small quantities of plasmid DNA for screening transformants was isolated by the minilysose procedure as described by Macrina et al. (20). Chromosomal DNA was isolated by the method of Marmur (22). Purified DNA and restriction endonuclease diges
TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or phenotype</th>
<th>Plasmid (size, kb)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli SK1592</td>
<td>hsdR4 Gal'</td>
<td>None</td>
<td>S. Kushner, UGA</td>
</tr>
<tr>
<td>E. coli D1204</td>
<td>lac+</td>
<td>pOP203(A2+)/Tc'</td>
<td>L. Gold and R. Winter, UCO</td>
</tr>
<tr>
<td>E. coli JA228</td>
<td>argH hsdR'</td>
<td>None</td>
<td>J. Carbon, UCSB</td>
</tr>
<tr>
<td>S. sanguis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. sanguis FW213</td>
<td>AD+ Fim+</td>
<td>pVT213 (10.4)</td>
<td>R. Cole, NIH</td>
</tr>
<tr>
<td>S. sanguis VT321</td>
<td>AD+ Fim−</td>
<td>pVT213 (10.4)</td>
<td>D. Thompson (10)</td>
</tr>
</tbody>
</table>

* hsdR. Host-specific restriction deficient; lac+ hyperlactose repressor-producing mutation carried by Fluc exogeneate; Tc'. Growth on media containing >10 μg of tetracycline per ml; argH, arginine requiring; AD', ability to adhere to in vitro tooth model; Fim, type 1 adhesion fimbiae.

were analyzed by agarose gel electrophoresis. Size standards were linear phage lambda DNA digested with HindIII.

DNA enzymology. Restriction endonuclease digestions were performed as described by Maniatis et al. (21). Partial DNA digestions were carried out with an appropriate enzyme dilution at 37°C for 1 h (21). DNA ligation reactions were performed with T4 DNA ligase at 4°C for 18 h.

Construction of the gene library in pOP203(A2+). S. sanguis DNA (50 μg) was partially restricted with 0.3 U of EcoRI for 1 h at 37°C. The reaction was stopped by addition of EDTA to 20 mM and incubation at 68°C for 15 min. The DNA was extracted with phenol and then chloroform, precipitated with ethanol, and fractionated on a sucrose 10 to 30% gradient in an SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) as described by Maniatis et al. (21). Fractions (0.5 ml) were collected and analyzed by electrophoresis in 0.7% agarose, and fractions containing DNA fragments 10 to 20 kilobases (kb) in size were pooled and concentrated by ethanol precipitation.

Plasmid pOP203(A2+), a positive selection expression vector that has the Q8 lymph gene (A2+) inserted under the control of a lactose promoter, was isolated from E. coli D1204 as described above. The plasmid was digested to completion with EcoRI (a unique site within the lysine gene) and precipitated with ethanol.

For ligation, 1.6 μg of EcoRI-digested S. sanguis DNA (10 to 20 kb) and 5.4 μg of EcoRI-cut pOP203(A2+) DNA were incubated overnight at 4°C. Ligation was confirmed by agarose gel electrophoresis.

E. coli SK1592 cells were grown in L broth and made competent for transformation by the CaCl2 heat shock method (5), with cells harvested at an A600 of 0.25. Competent SK1592 cells were cryogenically stored at −70°C and used in transformation experiments as needed. Competent SK1592 cells were transformed with 1 μg of ligated DNA by standard methods (21). The cells were then spread onto the surface of L plates containing 10 μg of tetracycline per ml and 0.5 mM IPTG. Plates were incubated overnight at 37°C. Twenty colonies were picked at random to determine the fraction of clones containing plasmids with inserts and to estimate the average size of the inserts. Restriction analysis of plasmids extracted by the minilysate technique from the 20 representative clones showed that all clones had plasmids with inserts. The average insert size was 3.2 kb. Assuming that the genome of S. sanguis and S. mutans are equivalent in size (5 × 106 base pairs), we estimated, by the Clarke and Carbon formula (3), that a 3,000 clone library was greater than 99.9% complete.

Antibodies and antigens. Polyclonal antibodies (PAb) raised against whole S. sanguis cells were made as previously described (7). Antibodies specific for fimbiae (AdAb) were prepared as previously described (7). When used in experiments, all the antibodies were partially purified by several cycles of 45% ammonium sulfate precipitation.

Colonies immunoassays. All recombinant clones were transferred with toothpicks onto sterile nitrocellulose disks placed on L agar plates containing 10 μg of tetracycline per ml. The plates were incubated overnight at 37°C. The filters were placed over chloroform vapor for 20 min to lyse the cells. The blots were blocked with 3% bovine serum albumin for 2 h at room temperature. A 1:1,000 dilution of PAb in 3% bovine serum albumin–10% fetal calf serum–phosphate-buffered saline was incubated with the blots for 2 h at room temperature. The blots were given six 10-min washes in phosphate-buffered saline and reacted with anti-rabbit immunoglobulin G (heavy and light chains), conjugated with horseradish peroxidase, for 2 h at room temperature. The blots were given six more 10-min washes in phosphate-buffered saline and equilibrated with 10 mM Tris (pH 7.4). They were developed with 25 μg of o-diansidine per ml in 0.01% H2O2 and 10 mM Tris (pH 7.4). Three milliliters of methanol was used to solubilize the o-diansidine. We stopped the reaction by rinsing the blots in warm water.

Western immunoblotting. Washed cells from 7 ml of L broth were suspended in 100 μl of lysis buffer (0.25 M Tris hydrochloride, 4% SDS, 40% glycerol, 0.004% bromphenol blue, 4% β-mercaptoethanol) and heated to 100°C for 10 min. The lysates were centrifuged in a Fisher Microfuge for 10 min, and 50 μl of the supernatant fluid was subjected to SDS-PAGE in 5 to 15% gradient polyacrylamide gels by the method of Laemmli (18) modified by the addition of 6 M urea. Antigens were electrophoretically transferred from SDS-PAGE gels to nitrocellulose (30) at 4°C in a Hoefer transphor unit (TE50; Hoefer Scientific Instruments, San Francisco, Calif.) containing 5 liters of buffer (25 mM Tris, 192 mM glycine, 20% methanol). Transfer was performed overnight at 20 V or for 2 h at 90 V. A permanent marker was used to spot the resolving gel/stacking gel interface and the band fronts of the prestained molecular weight markers when the gel was separated from the nitrocellulose. The blots were developed as described for immunoassays.

Restriction analyses and Southern hybridization. Restriction endonuclease site maps were determined and Southern blots were performed as described by Maniatis et al. (21).

RESULTS

Construction of S. sanguis genomic library. Plasmid pOP203(A2+), a pMB9 Tc' derivative which contains the lactose promoter-operator fused to the Q8 phage A2 lysin gene, was used as our cloning vector (25). When the A2 gene is expressed, it is lethal to the E. coli host (32). The gene can be insertionally inactivated by cloning of DNA fragments into any one of several unique restriction sites within the gene. This strategy allows positive selection of tetracycline-resistant recombinants in the presence of IPTG, an inducer of the lac operon. Chromosomal DNA was isolated from S. sanguis FW213, partially digested with EcoRI, size fraction-
ated by sucrose gradient centrifugation, and ligated into the EcoRI site of pOP203(A2+). The ligation mixture was used to transform E. coli SK1592, and 4,500 presumptive recombinant colonies were picked from the tetracycline-IPTG plates and subcultured. Analysis of 20 randomly selected colonies showed that all contained plasmids greater than 7 kb in size. Restriction with EcoRI revealed that these plasmids had distinct restriction patterns and that the average size insert was 3.2 kb (data not shown). On the basis of the Clarke and Carbon (3) formula, this library was greater than 99.9% complete.

Expression of *S. sanguis* antigens. All 4,500 recombinant colonies were screened by colony immunoassays with PAb, the antiserum made against whole FW213 cells that are Ad+ and Fim*. Thirty colonies produced positive reactions of various intensities after growth on nitrocellulose, in situ lysis, and probing with PAb, suggesting that a number of *S. sanguis* antigens can be expressed in *E. coli* (Fig. 1A). When these 30 clones were probed with fimbria-specific antibody, only two clones, VT616 and VT618, were positive (Fig. 1B, row 3). That the gene coding for the antigen is located on the recombinant plasmids was confirmed by isolating the plasmids from VT616 and VT618 and transforming both SK1592 and JA228 (*E. coli* hosts) in two separate experiments. Colony immunoassays showed that antigen production and plasmid presence were linked (Fig. 1B, row 6). SK1592 and JA228 were applied as negative controls (Fig. 1B, row 1).

**EcoRI restriction analysis of PAb**\(^{+}\) **clones.** Plasmid DNA from the 30 PAb\(^{+}\) clones was cut with EcoRI and electrophoresed in 1.2% agarose gels. The plasmid sizes and patterns of EcoRI restriction sites suggested that 26 of the 30 clones were unique (Table 2). VT616 and VT618 appeared to be the same 6-kb fragment, VT608 and VT609 appeared to be the same 2.8-kb insert, VT619 and VT265 appeared to be the same 1.0-kb fragment, and VT627 and VT628 appeared to be the same 4-kb insert. The identity of the fragments was confirmed by HindIII restriction endonuclease analysis.

**Characterization of immunoreactive proteins.** The putative fimbrial clones were induced with IPTG, and extracts of them were electrophoresed in SDS-PAGE gels, electrophoretically transferred to nitrocellulose, and probed with AdAb in a Western immunoblots assay. The clones, VT616 and VT618, produced an *M* \(_{\text{r}}\) 30,000 protein that, in agreement with the colony immunoassays, reacted in fimbria-specific antibody (Fig. 2). The protein was produced whether the cells were induced or not, suggesting that the pOP203(A2+)
lactose operator-promoter was not necessary for expression of the gene. Equal numbers of cells of clone VT618 (three experiments) produced more protein when induced than when not induced, suggesting that transcripts were being made from the lactose promoter as well as from some unknown promoter. There was no reaction with the host, SK1592. The relationship between the 30-kilodalton cloned protein and S. sanguis fimbriae is shown in Fig. 2. One component of the fimbrial preparation was indistinguishable in size from the immunoreactive products of the clones. In addition to this component, a number of larger polypeptides at 43, 53, 72, 74, and 76 kilodaltons, as well as a faint high-molecular-weight smear with a distinguishable band at 200 kilodaltons, were observed. Reactions with monoclonal antibodies and tandem immunoelectrophoresis indicated that all these bands were antigenically identical (data not shown).

**Molecular analyses of PAb** + **clones.** Chromosomal DNA from S. sanguis FW213 and plasmid DNA from some of the PAb** + clones were cleaved with EcoRI and probed with plasmid pVT618 by Southern hybridization (Fig. 3A and B). A 6-kb EcoRI fragment from S. sanguis FW213 chromosomal DNA and recombinant plasmid DNA from VT616 showed homology with the 6-kb insert from pVT618, providing proof that the pVT618 insert was streptococcal DNA and that the insert in pVT616 was related to the insert in pVT618. All clones showed homology with the 7.2-kb pOP203(A2+*) vector. Restriction endonuclease cleavage maps of pVT616 and pVT618 are shown in Fig. 4. The inserts in both plasmids were 6 kb in size and had no EcoRI sites. Restriction digestion with other enzymes revealed that both inserts had two BamHI sites, one HindIII site, one SstI site, two SphI sites, and no PvuII sites, further confirming the Southern analysis data that these two inserts are the same fragment of DNA. Restriction mapping, however, indicated that these two inserts had been cloned in reverse orientation. Since the 30-kilodalton protein was expressed in both orientations, the fimbrial promoter must be on the 6-kb EcoRI fragment.

**DISCUSSION**

The results presented in this paper demonstrate that S. sanguis genes can be cloned into and expressed by E. coli K-12. Certain genes from other streptococci, notably S. mutans (16, 25, 26) and S. pyogenes (28), have been found to express in E. coli. We initially screened an S. sanguis genomic library with a broad-spectrum polyclonal antibody that recognized a number of S. sanguis antigens. At the antibody concentration used for screening, only 1% of the clones produced material that was consistently cross-reactive with antibody to S. sanguis FW213 whole cells. Restriction enzyme analyses suggested that 26 of the 30 clones were unique.

Two of the clones, VT616 and VT618, expressed a structural gene for a subunit of the adhesion-related fimbriae of S. sanguis. These adhesion-related fimbriae have been named type 1 fimbriae for the following reasons: (i) there are several
types of fimbriae on S. sanguis (9), and there is therefore a need for specific designations; (ii) the adhesion fimbriae have been shown to be Ag1 (7) in crossed immunoelectrophoretic gels of blended extracts; and (iii) the adhesion-related fimbriae of A. viscosus are called type 1 (31).

The fimbria-specific clones produced a protein of 30 kilodaltons. This protein comigrated with the 30-kilodalton protein from S. sanguis type 1 fimbriae. Restriction mapping and Southern analysis of VT616 and VT618 showed that both clones contained the same 6-kb EcoRI fragment cloned in opposite orientations. The 6-kb fragment was inserted in a region of the vector where expression of the gene might be enhanced because of the nearby lactose promoter. Both clones produced the protein without induction of lactose. Furthermore, the 39-kilodalton protein was expressed when the gene was cloned in reverse orientation. These data suggested that the fimbrial promoter had been cloned on the 6-kb fragment. VT618 produced more of the 30-kilodalton protein when induced, suggesting that transcripts were being made from the lactose promoter as well as the fimbrial promoter.

Many higher-molecular-mass polypeptides were noted in the Western immunoblot of type 1 fimbriae. Type 2 fimbriae of A. viscosus have been similarly purified and cloned, and a similar pattern of multiple bands also has been noted (6). These are the only two reports on the biochemistry of gram-positive fimbriae, but the data do suggest that gram-positive fimbriae are very distinct from gram-negative fimbriae. Why the fimbrial epitope appears in multiple bands on SDS-PAGE gels is unknown. Several reasonable suggestions are (i) fimbriae are only partially dissociated in SDS, β-mercaptoethanol, and urea; (ii) the fimbrial peptides may be glycosylated, resulting in a smear; (iii) cell wall material may still be attached to the peptides; and (iv) fimbriae may be composed of nonidentical subunits with an epitope in common. Incomplete dissociation can be ruled out, since negative gels produced the same pattern of multiple bands as did denaturing gels (data not shown), suggesting that the multiple bands were not due to incomplete dissociation. Glycosylation remains a viable alternative, since all the bands were sensitive to periodate (manuscript in preparation). We are presently testing the fimbrial preparations with endoglycosidase H to resolve this alternative. Attachment of cell wall material is less likely, since treatment of the fimbrial preparation with mutanolysin had no effect on the number or size of the multiple bands (in preparation). The question of whether fimbriae are composed of nonidentical subunits awaits genetic analysis and sequencing of the fimbrial gene.

This cloned fimbrial gene product will be used to epidemiologically assess the relatedness of the S. sanguis fimbrial genes. It is also the first step in understanding the regulation and control of the fimbriae in S. sanguis and can be used to make mutants by marker exchange. In addition, this cloned product will permit production of the protein in high quantity for further biochemical analysis.

ACKNOWLEDGMENTS

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