Cloning and Expression of an Adhesin Antigen of *Streptococcus sanguis* G9B in *Escherichia coli*

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A genomic library of *Streptococcus sanguis*, strain G9B, was constructed and expressed in *Escherichia coli* using a λ gt11 expression vector. The amplified library was probed with polyclonal anti-G9B IgG and 13 antigen-positive clones were isolated. A lysate of one clone, designated PP39, absorbed the adhesion-inhibitory activity of anti-G9B IgG. This clone contained an insert of approximately 2000 bp and expressed unique 200 and 53 kDa proteins that reacted with monospecific anti-adhesin antibody. The 200 kDa protein also reacted with anti- β -galactosidase IgG, indicating that it is a fusion protein of which 84 kDa represents the streptococcal adhesin. The 84 and 53 kDa proteins are similar in size to the major polypeptides in a streptococcal antigen complex which is associated with the adhesion of G9B to saliva-coated hydroxyapatite. The 53 kDa fragment may result from post-translational cleavage of the recombinant polypeptide.

INTRODUCTION

The binding of *Streptococcus sanguis* to the salivary pellicle that coats tooth surfaces is believed to be the first phase of dental plaque formation (Gibbons & van Houte, 1971; Hamada & Slade, 1980). Saliva-coated hydroxyapatite (SHA) is used as an *in vitro* model for investigating bacterial attachment to the pellicle and for characterizing bacterial adhesins. Most of these studies suggest that attachment of *S. sanguis* to SHA involves more than one adhesin and salivary receptor (Cowan *et al.*, 1986, 1987; Doyle *et al.*, 1982; Gibbons *et al.*, 1983; Morris & McBride, 1984).

With the exception of a study by Liljemark & Bloomquist (1981), in which a partially purified cell wall extract of *S. sanguis* was found to block attachment of whole bacteria to SHA, most studies have relied on indirect methods to demonstrate the presence of adhesins in extracts of the streptococcal surfaces (Bergey *et al.*, 1986; Curtiss *et al.*, 1985; Douglas & Russell, 1984; Elder & Fives-Taylor, 1986; Fachon-Kalweit *et al.*, 1985; Fives-Taylor, 1982; Fives-Taylor & Thompson, 1985; Morris *et al.*, 1985, 1987). The role of fimbriae and/or fibrils (Handley *et al.*, 1985) has been explored by several groups. Fives-Taylor & Thompson (1985) isolated a number of non-adherent mutants of *S. sanguis*, strain FW213, in which the fimbriae were absent. In one of the mutants a 150 kDa antigen associated with the fimbriae was also missing (Elder & Fives-Taylor, 1986). Morris *et al.* (1985, 1987) isolated hydrophilic variants of *S. sanguis* which did not adhere as well as the parent hydrophobic strain. These variants did not have the fibrils found in the parent strain and apparently had very few proteins in their cell wall compared to the parent (Morris *et al.*, 1987). In studies comparing *S. sanguis* G9B with a non-adherent mutant (Adh⁻) (Lamont *et al.*, 1988a, b), no differences in fibrils between parent and mutant were observed

Abbreviations: HA, hydroxyapatite; SHA, saliva-coated hydroxyapatite.

(P. S. Handley, personal communication). Ganeshkumar *et al.* (1988) showed that a 36 kDa *S. sanguis* protein cloned in *Escherichia coli* blocked attachment of the organism to SHA. This protein did not react with anti-fibril serum, although it appeared to be present on the surface of the streptococcus. The results of these studies make it unclear whether it is the fimbriae or fibrils *per se*, or a component of these appendages or some other cell wall component, which is responsible for the specificity of attachment to SHA.

In recent studies (Lamont *et al.*, 1988b), we have identified an antigen(s) which is associated with the specific attachment of *S. sanguis* G9B to SHA. We refer to this antigen(s) as the 80 kDa complex because a polypeptide of this size is the dominant of three antigens detected by immunoblotting. In order to obtain the quantities of this antigen necessary for further characterization, we shotgun cloned the G9B chromosome into *E. coli* using the λ gt11 phage expression vector (Young & Davis, 1983; Young *et al.*, 1985). The goal of these initial studies was to isolate a clone expressing the 80 kDa antigen from the *E. coli* library.

METHODS

Organisms and media. *S. sanguis* strain G9B was originally isolated from dental plaque and is a biotype I (Facklam, 1977) and serotype 1 strain (Rosan, 1973). Immediately after isolation the cultures were lyophilized and frozen in trypticase (BBL Microbiology Systems)/yeast extract (Difco) medium (TY) with 15% (v/v) glycerol. The organisms have been subcultured only infrequently to maintain stocks. The bacteria were grown in TY with 0.5% (w/v) glucose as a carbon source at 37 °C for 18 h (Rosan *et al.*, 1982). *E. coli* strain Y1090 (Δ lacU169 *proA*⁺ Δ lon *araD139 strA supF* [*trpC22::Tn10*] *hsdR hsdM*⁺ (pMC9); pMC9 = pBR322 - *lacI*⁺) was obtained from Promega Biotec. The strain was maintained on Luria-Bertani (LB) broth supplemented with 50 µg ampicillin ml⁻¹ (Young & Davis, 1983; Young *et al.*, 1985).

Barbital extraction. Streptococcal surface molecules were extracted by shaking in 2 mM-barbital buffer, pH 8.6, as reported previously (Lamont *et al.*, 1988a).

Immunoblotting. SDS-PAGE gels (10%, w/v, acrylamide) were run (Laemmli, 1970), blotted onto nitrocellulose (Towbin *et al.*, 1979) and probed with antibody as described previously (Lamont *et al.*, 1988a, b). Samples were heated at 100 °C for 3 min in 0.05 M-Tris/HCl, pH 8.6, containing 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) 2-mercaptoethanol prior to electrophoresis.

Antisera. Antisera were produced in rabbits against formalinized whole cells of *S. sanguis* G9B using procedures described previously (Rosan, 1973, 1976, 1978). The concentration of the antisera was adjusted to obtain optimal visualization of as many antigens as possible by immunoblotting. The IgG fractions of normal rabbit sera and the antisera used in these studies were purified by affinity chromatography over a protein A-Sepharose 4B column (Lamont *et al.*, 1988a). All of the studies reported here used the same IgG pools.

Antibodies reacting specifically with the native 80 kDa antigen complex were prepared by affinity purification from immunoblots (Olmsted, 1981; Lamont *et al.*, 1988a). Polyacrylamide gels of *S. sanguis* G9B barbital extract were run and blotted onto nitrocellulose as described above except that SDS was omitted from all buffers and the samples were not boiled. A strip of the transfer was developed with antiserum to the denatured 80 kDa antigen (Lamont *et al.*, 1988a). The strip was aligned with the rest of the blot and the band containing the native 80 kDa antigen complex was excised. This strip was incubated with G9B antiserum diluted 1 in 100 in PBS (phosphate buffered saline: 0.01 M-sodium phosphate, pH 7.4, 0.15 M-NaCl) containing 0.1% (v/v) Tween 20. After washing, the antibody bound to the strip was eluted with 0.05 M-glycine/HCl buffer, pH 2.3, and dialysed against 0.05 M-Tris in 0.15 M-NaCl, pH 8.6. Monoclonal antibody (IgG) against β -galactosidase was obtained from Promega.

Absorption of antibodies with *E. coli*. Antibodies cross-reacting with *E. coli* were removed from streptococcal antibody preparations by absorption with *E. coli* lysates obtained by freeze-thawing (Maniatis *et al.*, 1982). The lysate (10 mg) was dissolved in 5 ml PBS and adsorbed to nitrocellulose filters (8.5 cm diameter) for 1 h at room temperature with shaking. The filters were washed with PBS and treated with blocking buffer [PBS with 10% (v/v) new-born calf serum and 0.2% (v/v) Triton X-100]. After rinsing with PBS, the filters were used immediately or stored at -20 °C after drying on Whatman filter paper. The IgG to be absorbed was diluted to 7 µg ml⁻¹ in blocking buffer. Volumes of 5 ml were added to the nitrocellulose filters and shaken for 45 min at room temperature. The absorbed IgG was removed and reabsorbed five times before use.

Construction of the *S. sanguis* genomic library. To obtain genomic DNA, *S. sanguis* G9B was grown overnight in TY, 1 ml was subcultured in the same medium (250 ml) containing 200 µCi [³H]thymidine (Amersham) and the cells were grown to early exponential phase (OD₆₆₀ 0.3). The cells were harvested and washed once in TES (10 mM Tris/HCl pH 8, 100 mM-NaCl, 1 mM-EDTA) and lysed with 1 ml mutanolysin (525 units) (Sigma) containing 250 units T₁ RNAase (Sigma). The solution was heated at 80 °C for 15 min to destroy DNAase.

A genomic library was constructed in λ gt11 (Promega Biotech) according to established procedures (Young & Davis, 1983; Young *et al.*, 1985). In brief, 100 µg DNA was sonicated and 2-9 kb fragments were isolated by

elution from low-melting-point agarose (Sea Plaque, FMC Bioproducts) following electrophoresis. Internal *EcoRI* sites were methylated using *EcoRI* methylase (NE Biolabs) and fragments were made blunt-ended with T4 DNA polymerase as described by Maniatis *et al.* (1982). The DNA was dissolved in 4.5 µl of a 100 mM-Tris, pH 7.5, buffer containing 100 mM-MgCl₂, 100 mM-DTT, 4.5 µg *EcoRI* linkers, 10 mM-ATP and 2 units T4 ligase; the final volume was 46.4 µl. Cohesive ends were made by digestion with *EcoRI* (Bethesda Research Laboratories). Linkers were separated by passage through a Sepharose Cl-4B column (5 ml) which had been calibrated with [³H]thymidine-labelled fragments of *S. sanguis* DNA. The final yield was approximately 1 µg of 2–9 kb fragments of *S. sanguis* DNA. Ligation with λgt11 arms and *in vitro* packaging were carried out according to the manufacturer's specifications. The library was plated on LB plates containing 50 µg ampicillin ml⁻¹ and 160 µg isopropyl β-D-thiogalactopyranoside ml⁻¹ (IPTG: Boehringer Mannheim) with 0.7% (w/v) semi-solid top agar containing IPTG as well as 400 µg 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside ml⁻¹ (X-Gal, Sigma) as an indicator of β-galactosidase activity. The plates were incubated overnight at 37 °C.

Screening libraries with antibody. A nitrocellulose disk was applied to plates containing 400–500 plaques and incubated for 15 min at room temperature. The disks were marked to allow for future realignment. The nitrocellulose was washed for 30 min with 5 ml blocking buffer and incubated with 5 ml of the absorbed anti-G9B IgG (7 µg ml⁻¹) for 1 h with shaking. After washing three times, the primary antibody reaction was detected as described above for immunoblots.

Antigen-positive plaques were picked, diluted in 100 µl phage dilution buffer (100 mM-NaCl, 10 mM-Tris, pH 7.9, 10 mM-MgSO₄), mixed with an equal volume of the indicator strain and plated on semi-solid agar (Maniatis *et al.*, 1982). After overnight growth at 37 °C, the soft agar containing nearly confluent plaques was scraped into centrifuge tubes and shaken with 0.5 ml chloroform at 37 °C. The phage were recovered by centrifugation at 5875 g at 10 °C.

Recovery of expressed protein. The concentration of phage and host *E. coli* was adjusted to obtain nearly confluent lysis on LB plates containing ampicillin and IPTG. The semi-solid agar was scraped into a centrifuge bottle and 0.1 ml chloroform added for each plate used. Following incubation at 37 °C for 15 min the lysate was centrifuged at 4000 g for 10 min at 4 °C to remove agar and residual cells. The supernatant was centrifuged at 107000 g for 2 h to remove phage particles, dialysed against H₂O, and lyophilized.

Characterization of recombinant clones. The phage DNA and control λgt11 DNA were recovered by CsCl density-gradient centrifugation (Maniatis *et al.*, 1982).

Adhesion assay and antibody inhibition of adhesion. The adhesion assay has been described previously (Eifert *et al.*, 1984). In brief, [³H]thymidine-labelled bacteria suspended in buffered KCl (2 mM-K₂HPO₄, 5 mM-KCl, 1 mM-CaCl₂, pH 6) were incubated with SHA or control uncoated hydroxyapatite (HA). Samples of the cells in equilibrium with the beads were removed to determine the number of free cells; after washing, the beads with attached bacteria were also removed. The numbers of free and bound cells were determined by scintillation spectroscopy.

Antibody inhibition of adhesion was determined by incubating the anti-G9B IgG (6 µg ml⁻¹) with an equal volume of [³H]thymidine-labelled G9B cells (2 × 10⁹ cells ml⁻¹) for 18 h at 4 °C with continuous rotation. The mixture was centrifuged (7000 g, 10 min, 4 °C) and the cells washed once in buffered KCl. The cell pellet was suspended in the original volume of KCl and sonicated for 15 s prior to use in the adhesion assay. The final concentration of G9B used in the assay was 1 × 10⁹ cells ml⁻¹.

Absorption of adhesion-inhibitory activity from *S. sanguis* antibodies. The expressed proteins were tested for adhesin activity by absorption of adhesion-inhibitory activity from the anti-G9B IgG. The proteins were dissolved in buffered KCl and anti-G9B IgG (6 µg ml⁻¹) was added. The mixture was rotated overnight at 4 °C and any resulting precipitate removed by centrifugation (10000 g, 20 min, 4 °C). The supernatant containing absorbed antibody was collected and examined for adhesion-inhibitory activities as described above.

RESULTS

Identification of clones containing S. sanguis antigens

When screened for β-galactosidase activity, approximately 50% of the plaques were colourless, indicating insertional inactivation by *S. sanguis* DNA of the gene encoding β-galactosidase. Initially 3000 recombinant plaques were screened with anti-G9B IgG. Thirteen plaques expressed proteins which reacted to some extent with the antibody. Thus, approximately 0.5% of the recombinants were producing detectable *S. sanguis* antigens. The antigen-positive plaques were amplified and each was checked for β-galactosidase activity by growth on LB ampicillin agar containing IPTG and X-Gal. Antigen production was confirmed by seeding a lawn of *E. coli* Y1090 with 10 µl of phage suspension obtained from each of the

Fig. 1. Absorption of adhesion-inhibitory activity from anti-G9B IgG antibody by antigen-positive plaques: PP1, PP2, PP3, PP29 and PP39 (400 μ g protein). NRIgG is normal rabbit IgG (6 μ g ml⁻¹), G9B IgG is purified anti-G9B IgG (6 μ g ml⁻¹), BBX is a positive control of anti-G9B IgG (6 μ g ml⁻¹) absorbed with 400 μ g protein of barbital extract of G9B. Binding of *S. sanguis* G9B to SHA is expressed as a percentage of the value obtained with the NRIgG control: [(percentage of cells bound in test sample)/(percentage of cells bound in control)] $\times$ 100. The binding of 1×10^9 G9B cells treated with NRIgG was $40\% \pm 5\%$.

positive plaques; controls from negative plaques and parent phage were included. The positive plaques were subcloned and again screened for antigenic activity. Some plaques which were weakly positive in the initial screening did not react following amplification; others still gave weak reactions with anti-G9B IgG. However, one subclone, PP39, gave a strong reaction in both the initial screening and subsequent amplification. None of the antigen-positive plaques expressed β -galactosidase activity. The parent λ gt11 plaque was positive for β -galactosidase and did not react with the anti-G9B IgG.

Absorption of adhesion-inhibitory activity

Lysates of the antigen-positive plaques were tested for the ability to absorb the adhesion-inhibitory activity of anti-G9B IgG; the results are shown in Fig. 1. At the concentration of cells used in these studies, approximately 40% of the labelled G9B cells adhere to SHA (Eifert *et al.*, 1984). Treating the cells with normal rabbit IgG (NRIgG) did not affect adhesion (Fig. 1). In contrast, treating the cells with anti-G9B IgG (G9B IgG) inhibited attachment by approximately 70%. Inhibition has been shown to be specific for SHA and does not occur if HA is used as the substrate (Rosan *et al.*, 1985). The antibody-inhibitory activity was absorbed with a barbital extract of G9B (BBX), which serves as a positive control for adhesin activity. Of the 13 antigen-producing clones, 11 showed no antibody-absorption activity, one (PP3) absorbed antibody to an extent causing 70% loss of inhibitory activity, and one (PP39) absorbed antibody to an extent causing 94% loss of inhibitory activity. The results suggested that PP39 expressed proteins most closely related to the *S. sanguis* adhesin and therefore this clone was selected for further study.

Immunoblots of cloned proteins

Both parent λ gt11 and PP39 lysates were examined by immunoblotting (Fig. 2). PP39 produced unique 200 and 53 kDa proteins which reacted with anti-G9B IgG (absorbed with *E. coli*). This clone also reacted with anti-80 kDa antibodies which inhibited binding of G9B to SHA to the same extent as anti-G9B IgG. No reaction was observed with normal rabbit IgG (not shown). In addition, the 200 kDa antigen reacted with monoclonal anti- β -galactosidase

Fig. 2. Immunoblot of plaque lysates. (a) Lysate of PP39 (2 µg protein); (b) lysate of parent phage λgt11 (2 µg protein). The blots have been treated with antibody diluted 1 in 100: lane 1, anti-G9B; lane 2, anti-80 kDa antigen complex.

antibodies, indicating that it is a fusion protein produced from the *lac* promoter and containing antigenic determinants of the *lacZ* gene product. The anti-β-galactosidase antibodies reacted with a 116 kDa band (consistent with the size of β-galactosidase) in the parent λgt11 lysate (not shown). Thus, PP39 appeared to be producing a fusion protein of 200 kDa and a smaller protein of 53 kDa, which could be a breakdown product. Both of these proteins reacted with the monospecific antibodies that inhibit adhesion of G9B to SHA.

Preliminary restriction map of cloned adhesin gene

Phage DNA isolated from clone PP39 was digested with *EcoRI*. The resulting fragments, which were separated by agarose gel electrophoresis, indicated that the *S. sanguis* DNA insert contained two internal *EcoRI* sites. Therefore, a larger fragment was cut with *BamHI* to yield a 22 kb fragment that contained the inserted DNA. This fragment was digested with *EcoRI*, *KpnI*, *HindIII* and *SstI* to map the inserted DNA. Analysis of these DNA fragments resulted in the map shown in Fig. 3. The streptococcal insert DNA is about 2000 kb in length and contains two internal *EcoRI* sites upstream of a *KpnI* site. Expression of the adhesin gene is directed from the *lac* promoter, indicating that the streptococcal promoter is not contained in this clone.

DISCUSSION

Investigations of *S. sanguis* adhesion to SHA have been hampered by the difficulty of obtaining large amounts of active surface adhesins from the organism. The production of adhesin and related proteins by recombinant DNA methodologies is one means of obviating this problem. The observations presented in this paper indicate that a 2 kb DNA insert from *S. sanguis* G9B coding for a protein showing adhesin activity has been cloned and expressed as a fusion protein in *E. coli*. This protein absorbs the SHA adhesion inhibitory activity of G9B IgG. Given that approximately 116 kDa of the fusion protein would represent β-galactosidase, then the streptococcal protein would be approximately 84 kDa. The coding capacity of the insert would be sufficient to code for a protein of this size. The 53 kDa polypeptide may result from post-translational cleavage. Proteins of 80 and 52 kDa (80 kDa antigen complex) were previously found to be associated with adhesin activity in *S. sanguis* G9B (Lamont *et al.*, 1988a, b). Further evidence that these cloned proteins are related to the 80 kDa complex is provided by a positive reactivity with monospecific anti-80 kDa antibodies which inhibit adhesion of G9B to SHA.

The restriction map of the G9B adhesin gene is distinct from the maps of an adhesin gene

Fig. 3. Partial restriction map of a 22 kb *Bam*HI fragment containing the segment of adhesin gene (*adh*) from *S. sanguis* G9B cloned into the λ gt11 expression vector. The first *Bam*HI site is located 5.53 kb downstream of the left end of the phage chromosome (Thanh *et al.*, 1985).

from *S. sanguis* 12 (Ganeshkumar *et al.*, 1988), and of the 30 kDa protein cloned from FW213 by Fives-Taylor *et al.* (1987). The latter appears to be similar in size to a protein found in fimbriae isolated from *S. sanguis* FW213. We have found a cross-reaction between an antigen of FW213 and the adhesin of G9B. However, none of the polypeptides of the 80 kDa antigen complex were detected in FW213, suggesting that the cross-reaction was due to other antigenic determinants (unpublished observations). These could be the glycosylated determinants which may form part of the adhesin in *S. sanguis* (Lamont *et al.*, 1988*b*). The restriction map of the FW213 adhesin gene is also distinct from that of a 36 kDa protein from *S. sanguis* 12, described by Ganeshkumar *et al.* (1988). Thus, at least three distinct adhesin genes have been identified in *S. sanguis*.

Since no obvious differences in numbers and types of fibrils between G9B and its nonadherent mutant (Adh⁻) have been found (P. S. Handley, personal communication), these appendages *per se* may not be responsible for the adhesion of *S. sanguis* to SHA. In addition, Wyatt *et al.* (1987) have found no correlation between the fibril density of a large number of *S. sanguis* strains and their adhesion to SHA. Adhesion among these strains may be more closely related to proteins associated with the fibrils or the longer fimbriae (Handley *et al.*, 1985) but not covalently bound to them. This would account for the observations that surface molecules, including those with adhesin activity, can be extracted readily from oral streptococci by procedures which are not usually thought to disrupt covalent linkages (Lamont *et al.*, 1988*a*; Ogier *et al.*, 1984; A. S. Bleiweis, personal communication). The availability of *S. sanguis* adhesin genes will provide probes as well as the ability to produce sufficient quantities of adhesins and antibody reagents, which should aid in elucidating the specific functional domains of the adhesin molecules, their location on the cell surface and their salivary receptors.

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