Molecules of *Streptococcus gordonii* that bind to *Porphyromonas gingivalis*

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> Interbacterial binding is considered an important colonization mechanism for many of the organisms that inhabit dental plaque. *Porphyromonas gingivalis*, a periodontal pathogen, can adhere to species that comprise early plaque, such as *Streptococcus gordonii*. In this study, the molecules of *S. gordonii* G9B that mediate binding to *P. gingivalis* were investigated. Biotinylated surface molecules of *S. gordonii* were extracted and mixed with *P. gingivalis* cells. Interactive streptococcal components were identified by SDS-PAGE of the *P. gingivalis* cells followed by electroblotting, and visualization of the adsorbed streptococcal molecules with streptavidin-alkaline phosphatase. *S. gordonii* molecules of 45 kDa and a doublet of 62/60 kDa were observed to bind to *P. gingivalis*. Polyclonal antibodies raised to the 62/60 kDa proteins inhibited the binding interaction. These antibodies demonstrated an antigenic relationship between the 62/60 kDa molecules and the 45 kDa protein. Both molecules were also antigenically related to, and may be breakdown products of, a larger molecule of 170 kDa which is antigenically related to the P1 antigen of *S. mutans*. Cloning and expression in *Enterococcus faecalis* of the gene for the P1-like molecule from *S. gordonii* M5 resulted in a phenotype that expressed the 62/60 kDa and 45 kDa antigens and was capable of binding to *P. gingivalis*. These results suggest that a P1-like molecule in *S. gordonii* is involved in adherence to *P. gingivalis*. Processing of the P1-like molecule into smaller fragments of 62/60 kDa and 45 kDa may be required for binding activity.

**Keywords:** *Streptococcus gordonii*, *Porphyromonas gingivalis*, interbacterial binding adhesin, P1

**INTRODUCTION**

The development of dental plaque involves a succession of microbial species (Kolenbrander & London, 1993; Listgarten et al., 1975; Theilade et al., 1966). Early plaque, consisting mostly of Gram-positive facultative organisms such as streptococci and actinomycetes, exists in commensal harmony with the host. Transformation into a potentially pathogenic entity requires the subsequent colonization of mainly Gram-negative anaerobic and facultative species. The early plaque colonizers, predominantly sanguis group streptococci and *Actinomyces viscosus*, can adhere to molecules of the salivary pellicle that coats the enamel surface (Fives-Taylor et al., 1987; Ganeshkumar et al., 1988; Gibbons & Hay, 1988; Lamont et al., 1988b). The colonization of pathogenic organisms, however, is dependent, to some degree, on their ability to adhere to the preformed plaque (Kolenbrander & Andersen, 1986; Lamont et al., 1992; Schwarz et al., 1987; Slots & Gibbons, 1978). This interbacterial binding phenomenon has been extensively studied and many of the species that comprise mature dental plaque are capable of binding to one another (Kolenbrander & Andersen, 1986; Kolenbrander & London, 1993).

*Porphyromonas gingivalis*, a Gram-negative anaerobic organism, is considered an important pathogen in many forms of severe adult periodontitis. This species can be recovered in high numbers from diseased periodontal pockets and possesses a number of virulence factors capable of destroying periodontal tissues (Slots & Listgarten, 1988; van Winkelhoff et al., 1988). *P. gingivalis* is a late colonizer of plaque, and can adhere to many of the antecedent commensal plaque organisms, including *Streptococcus gordonii* (Lamont et al., 1992; Stinson et al., 1991). The molecular mechanisms of this binding in-
teraction are only partially understood. The fimbriae of _P. gingivalis_ mediate binding to the streptococcus, although additional molecules may also be involved (Lamont et al., 1993). Although a number of _S. gordonii_ adhesins that mediate binding to salivary molecules and actinomyces have been described (Fives-Taylor et al., 1987; Kolenderbrand & Anderson, 1990; Lamont et al., 1988a), the molecules responsible for binding to _P. gingivalis_ remain to be determined. The goal of this investigation was to isolate surface molecules from _S. gordonii_ and examine their ability to adhere to _P. gingivalis_.

**METHODS**

**Bacteria and culture conditions.** _P. gingivalis_ strain 33277 and _S. gordonii_ strains G9B and M5 are maintained as frozen stock cultures. _Enterococcus faecalis_ strain S161EB-5 was generated by transformation of _E. faecalis_ S161 with shuttle vector pAM401 (Wirth et al., 1986) containing a 5.3 kb insert encoding the _S. gordonii_ M5 SSP-5 antigen (Demuth et al., 1989). _E. faecalis_ strain S161-401 was generated by transformation of S161 with pAM401 that did not contain a streptococcal insert. _P. gingivalis_ was cultured in Trypticase soy broth (BBL) supplemented with 1 g yeast extract (Difco) 12, 5 mg haemin 12 and 1 mg menadione 12. Streptococci and enterococci were grown in Trypticase peptone broth (BBL) supplemented with 5 g yeast extract 12 and 0.5% glucose. Culture was at 37°C under anaerobic conditions (85% N2, 10% H2, 5% CO2). Numbers of bacteria were determined in a Klett–Summerson photometer. To metabolically label bacteria, 10 μCi (370 kBq) [3H]thymidine (Amersham) ml2 were added to the media. Resulting specific activities ranged between 6 x 106 and 2 x 104 c.p.m. per cell.

**Interbacterial binding assay.** Adherence of _P. gingivalis_ to streptococci and enterococci was determined by the nitrocellulose blot assay described previously (Lamont & Rosan, 1990). Briefly, cells of one partner of the binding pair were suspended in buffered KCl (5 mM KCl, 2 mM K2HPO4, 1 mM CaCl2, pH 6.0) and 1 x 108 bacteria deposited on nitrocellulose paper in a dot-blot apparatus. The blot was washed three times in KCl containing 0.1% Tween 20 (KCI–Tween). The adsorbed bacteria were incubated for 2 h at room temperature with radiolabelled cells of the other test species suspended in KCI–Tween. After washing to remove unbound organisms, the experimental areas of the nitrocellulose were excised and the amount of interbacterial binding quantified by scintillation spectrosopy.

Antibody inhibition of binding was investigated by incubating (1 h, 37°C) the radiolabelled bacteria with antibody prior to assay. Bacteria were then collected by centrifugation (10000 g, 10 min), resuspended in KCI–Tween and adherence measured as described above.

**Biotinylation of _S. gordonii_ surface molecules.** A 100 ml overnight culture of G9B was washed in KCl and suspended in 0.1 M NaHCO3, pH 8.1. N-Hydroxysuccinimidobiotin (Sigma) was added to a final concentration of 1 mg ml–1 of cells in suspension. After 3 h at room temperature the cells were recovered by centrifugation (10000 g, 10 min), washed twice and resuspended in KCl.

**Preparation of surface molecules.** Bacterial surface molecules were prepared from biotinylated and non-biotinylated organisms by the mild sonicnation procedure developed by Kolenderbrand & Andersen (1990). Cells were sonicated in an ice bath for three 30 s intervals with a Visonic 475 ultrasonic disruptor emitting 45 W. The cells were allowed to cool for 1 min between ultrasonic treatments. Bacteria were removed by centrifugation (20000 g, 20 min) and the supernatant, containing surface molecules, was filtered (0.22 μm pore size) and stored at −20°C.

**Binding of biotinylated streptococcal molecules to _P. gingivalis_.** Biotinylated, extracted G9B surface molecules (100 μg protein) were added to 1010 _P. gingivalis_ cells and incubated for 2 h at room temperature. Controls of _P. gingivalis_ cells with non-biotinylated G9B extract were included in all experiments. _P. gingivalis_ cells with any attached streptococcal components were washed and recovered by centrifugation at 10000 g for 10 min. After the final wash, the bacteria were resuspended in 50 μl SDS-PAGE sample buffer and the bacterial suspension (10 μl) subjected to SDS-PAGE and blotted onto nitrocellulose paper as described previously (Lamont et al., 1988a, b). After blocking the membrane with PBS containing 0.1% Tween 20 (PBS–TWEEN), biotinylated streptococcal molecules that bound to the _P. gingivalis_ cells were visualized by developing the blot with streptavidin–alkaline phosphatase conjugate (Bio-Rad) and the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad) according to the manufacturer’s instructions.

**Antiserum and immunoblotting.** Production of rabbit polyclonal antiserum to _S. mutans_ was performed by Dr H. F. Jenkinson, University of Otago, New Zealand, and Dr A. S. Bleiweis, University of Florida, FL, USA. Monospecific polyclonal antibodies to individual streptococcal molecules were prepared by excising the band of interest from Coomassie blue stained 10% (w/v, acrylamide) SDS-PAGE gels of G9B surface extract (50 μg protein). Inoculation of mice and preparation of antiserum was performed by the Berkeley Antibody Co., Richmond, CA, USA. Monospecific polyclonal antibodies to the P1 antigen of _S. mutans_ were provided by Dr H. F. Jenkinson, University of Otago, New Zealand, and Dr A. S. Bleiweis, University of Florida, FL, USA. Antibody–antibody interactions were visualized by immunoblotting, as described above. Bacterial surface extracts (5 μg protein) were separated by SDS-PAGE and electroblotted onto nitrocellulose paper. The membrane was blocked with PBS–TWEEN and incubated with antibody diluted in the same buffer for 1 h at room temperature. After washing, antigen–antibody binding was localized with alkaline-phosphatase-conjugated goat anti-mouse, or anti-rabbit, IgG (Bio-Rad) and developed with the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad) according to the manufacturer’s instructions.

**Chemical analysis.** Protein concentration was estimated by using the Bio-Rad reagent.

**RESULTS**

**Binding of _S. gordonii_ surface extract to _P. gingivalis_.** Mild sonication removed a variety of components from _S. gordonii_ (Fig. 1), many of which reacted with antiserum to whole G9B cells (Fig. 2). To determine which of these molecules bind to _P. gingivalis_, the biotinylated sonic extract was mixed with _P. gingivalis_ cells which were then washed to remove unbound streptococcal molecules. Any interactive streptococcal molecules were identified by subjecting the _P. gingivalis_ cells and associated streptococcal molecules to SDS-PAGE and electroblotting, followed by detection of the biotinylated streptococcal molecules with streptavidin. Fig. 1 shows that a doublet
S. gordonii adhesin for P. gingivalis

Fig. 1. Blot showing binding of S. gordonii G9B molecules to P. gingivalis 33277. Lane 1, biotinylated G9B surface molecules (2 μg protein); lane 2, 33277 cells with adsorbed G9B molecules; lane 3, control of 33277 cells with non-biotinylated G9B molecules.

Fig. 2. Immunoblot of S. gordonii G9B surface extract (5 μg protein) probed with: lane 1, whole G9B antibodies (1:1000); lane 2, 62/60 antibodies (1:1000).

Fig. 3. Immunoblot of S. gordonii G9B surface extract (5 μg protein) probed with: lane 1, 62/60 antibodies (1:1000); lane 2, P1 antibodies (1:500).

of 62/60 kDa along with another molecule of 45 kDa bound to the P. gingivalis cells.

Antigenic relationship between adhesive streptococcal molecules

To investigate the relationship between the S. gordonii molecules that adhered to P. gingivalis, the 62/60 kDa doublet was excised from SDS-PAGE gels and injected into mice. The resulting antibodies were examined by immunoblotting with the G9B sonic extract. As shown in Fig. 2, the anti-62/60 antibodies reacted with the homologous antigen in addition to the 45 kDa antigen (which also adhered to P. gingivalis) and a molecule of 170 kDa. It would appear, therefore, that the 62/60 and 45 kDa antigens, that bind to P. gingivalis, are antigenically related to each other and to a larger molecule of 170 kDa. A possible explanation for this is that the 62/60 and 45 kDa molecules are proteolytic breakdown products of the 170 kDa protein, or that all are components or breakdown products of a larger molecule not seen by SDS-PAGE. When the extraction procedures were repeated in the presence of the proteolytic inhibitors PMSF, TLCK and benzamidine, identical results were obtained. Streptococci, however, possess a wide variety of proteases and complete inhibition of proteolytic activity is not practically attainable. These results, therefore, do not preclude a role for proteolytic enzymes in the generation of the 62/60 and 45 kDa molecules.

The size of the 170 kDa molecule indicated that it may be related to the P1 molecule identified as an adhesin in S. mutans and which has homology with the SSP-5 salivary agglutinin binding molecule found in S. gordonii strain M5. To investigate this possible relationship, antibodies to P1 were reacted with the G9B sonic extract. Fig. 3 shows that P1 antibodies react with the 170 kDa antigen as well as the 62/60 (which did not resolve as separate bands in this experiment) and 45 kDa molecules. The P1 molecule and the 170, 62/60 and 45 kDa molecules are, therefore, antigenically related.

Antibody inhibition of S. gordonii-P. gingivalis binding

To seek further evidence for the role of the 62/60 kDa and antigenically related molecules in mediating binding of S. gordonii to P. gingivalis, the ability of the 62/60 antibodies to inhibit this interaction was investigated. Antibodies raised to the 62/60 molecules inhibited binding of S. gordonii to P. gingivalis by a maximum of 64% (Fig. 4). Normal mouse serum also showed some inhibition of adherence but this was much less than the inhibition caused by the 62/60 serum (Fig. 4).

Binding of recombinant SSP-5 protein to P. gingivalis

The antigenic relationship between the 62/60, 45 and 170 kDa molecules and P1 suggested that the former may represent a homologue of P1 in S. gordonii G9B. Therefore, the ability of SSP-5, a P1 homologue in S. gordonii M5, to bind to P. gingivalis was investigated. E. faecalis S161EB-5, a strain that contains the gene for SSP-5 and expresses the molecule on the cell surface, was able to bind to P. gingivalis, whereas E. faecalis S161-401, which does not
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**DISCUSSION**

Adherence of periodontal pathogens such as *P. gingivalis* to antecedent plaque organisms such as *S. gordonii* is considered an important colonization mechanism (Lamont et al., 1992; Slots & Gibbons, 1978). Although fimbriae have been implicated in mediating binding of *P. gingivalis* (Lamont et al., 1993), the streptococcal molecules involved have not been identified. The surfaces of oral streptococci are complex and contain polysaccharides, teichoic acids and a variety of proteins (Lamont et al., 1988a; Rosan, 1973, 1976). Several of these proteins have been identified as adhesins, both for the enamel salivary pellicle and for other bacteria (Fives-Taylor et al., 1987; Ganeshkumar et al., 1988; Kolenbrander & Anderson, 1990; Lamont et al., 1988a). In this study, surface proteins were removed by mild sonication, a process originally developed to release a streptococcal adhesin for *Actinomyces naeslundii* (Kolenbrander & Anderson, 1990). When biotinylated surface proteins of *S. gordonii* G9B were added to *P. gingivalis*, three molecules were observed to bind: a 45 kDa protein and two proteins of 62 and 60 kDa that ran as a doublet on SDS-PAGE gels. The 60 kDa molecule occasionally did not resolve from the 62 kDa molecule. Thus, the 60 kDa protein may be a partial breakdown product that, under certain experimental conditions, is absent due to complete processing. Antibodies raised to the 62/60 proteins also reacted with the 45 kDa molecule. Thus, these molecules are antigenically related and may be breakdown products of a larger molecule of 170 kDa that also reacted with the antiserum. The 170 kDa molecule itself did not appear to bind to *P. gingivalis*. This may indicate that conformational changes, perhaps caused by processing into smaller fragments, are required to induce binding activity in the streptococcal molecules. Thus, the 170 kDa protein per se is inactive until the 62/60 and/or 45 kDa fragments are generated. Under our experimental conditions the smaller molecules may have been produced by sonication; however, lower molecular mass fragments of streptococcal P1-like molecules have been found when extraction techniques that do not involve sonication were utilized (Demuth et al., 1988; Jenkinson et al., 1993; Wyatt et al., 1988). A more plausible explanation is that the lower molecular mass molecules were generated by proteolytic activity. Oral streptococci produce a number of proteases that can be released from the cells and may degrade surface proteins (Cowman & Baron, 1991; Floders et al., 1987; Lamont & Rosan, 1989; Rodgers et al., 1990). The importance of proteolytic activity in the co-adherence of *P. gingivalis* and *S. gordonii* was demonstrated by Stinson et al. (1992) who found that treating *P. gingivalis* with protease inhibitors reduced binding to streptococci immobilized on CNBr-activated agarose beads. Similarly, Li et al. (1991) reported that proteases are involved in the adherence of *P. gingivalis* to *Actinomyces viscosus*, and proteolytic activity is important in the adherence mechanisms of *Trichomonas vaginalis* and *Candida* (Arroyo et al.,

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**Fig. 4.** Inhibition of *S. gordonii* G9B binding to *P. gingivalis* 33277 by 62/60 antibodies (●) or normal mouse serum control (□). Error bars represent standard errors of the means of three experiments.

**Fig. 5.** Immunoblot of surface extracts from *S. gordonii* M5 (lane 1) and *E. faecalis* S161-EB-5 (lane 2) probed with 62/60 antibodies (1:1000).
1989; Ray & Payne, 1988). Furthermore, the S. gordonii G9B adhesin for salivary pellicle is the substrate for an endogenous protease that may serve to induce conformational changes necessary for activity (Lamont et al., 1988b). The relative contributions of P. gingivalis and streptococcal proteases to their coadherence will be difficult to determine until the proteases produced by S. gordonii are better defined.

Further evidence for the involvement of the 62/60 and antigenically related molecules in adherence was provided by the inhibitory activity of antibodies. Antiserum to the 62/60 antigen inhibited binding of G9B to P. gingivalis, in a dose dependent manner, by up to 64%. The failure to produce complete inhibition may indicate that antibodies raised to the adhesin recovered from SDS-PAGE gels do not recognize possible conformational epitopes involved in binding. Alternatively, more than one streptococcal adhesin/adhesin receptor may be involved in adherence to P. gingivalis. Previous studies have suggested that a number of molecules may be responsible for P. gingivalis binding (Lamont et al., 1993). Binding between P. gingivalis and S. gordonii may, therefore, involve several interactive components of which the P. gingivalis fimbriae and the S. gordonii 62/60 molecules are one example. It has yet to be determined whether the fimbriae and 62/60 molecules interact with each other. The presence of multiple adhesins on bacteria is well established. Indeed, sanguis group streptococci, including S. gordonii may possess several adhesins for both salivary pellicle and Actinomyces (Hasty et al., 1992), and P. gingivalis has more than one surface molecule capable of mediating hemagglutination (Boyd & McBride, 1984; Inoshita et al., 1986; Mouton et al., 1991; Okuda et al., 1986; Prgulsk–Fox et al., 1989).

Normal mouse serum also inhibited binding between P. gingivalis and S. gordonii. Maximum inhibition was about 20% and activity was lost at dilutions greater than 1 in 1000. These results are in agreement with earlier work showing inhibition of P. gingivalis–S. gordonii binding by normal rabbit serum (Stinson et al., 1991). In the oral cavity, therefore, crevicular fluid, which is essentially a serum exudate, may help protect against colonization of P. gingivalis by inhibiting adherence to other bacteria. The 170 kDa and related molecules were found to be antigenically related to the P1 surface molecule of S. mutans. The presence of lower molecular mass breakdown products of P1 has also been demonstrated in S. mutans and sanguis streptococci including S. gordonii (Wyatt et al., 1988). Thus, the 170 kDa protein may be a P1 homologue in G9B. This is supported by studies with SSP-5, a P1-related molecule in S. gordonii M5 (Demuth et al., 1990). This strain of S. gordonii adheres to P. gingivalis in a very similar manner to G9B (Lamont et al., 1992). E. faecalis S161EB-5, that was transformed with the SSP-5 gene, and expresses the SSP-5 protein on the cell surface (Demuth et al., 1989), demonstrated enhanced binding to P. gingivalis. Thus, SSP-5 can mediate binding to P. gingivalis. Furthermore, both M5 and the transformed E. faecalis produced the 62/60 kDa and 45 kDa proteins, which appear to possess the adherence activity. Thus the 170 kDa, 62/60 kDa and 45 kDa molecules may all be encoded by the same gene. This is consistent with the concept that the P1-like gene in some S. gordonii strains produces a high molecular mass molecule of at least 170 kDa that can be broken down into fragments of 62/60 and 45 kDa. These smaller fragments are then capable of binding to P. gingivalis.

The P1-related molecules are involved in a number of other adherence mechanisms. They interact with salivary agglutinin in the fluid phase to mediate bacterial aggregation, and they can react with agglutinin in the salivary enamel pellicle causing bacterial adherence (Demuth et al., 1988, 1989; Lee et al., 1989). In addition, a P1-like molecule in S. gordonii DL-1 can mediate binding to A. naeslundii (Jenkinson et al., 1993), and P1 may effect binding of S. mutans to S. gordonii G9B (Lamont et al., 1991). The functional domains involved in binding to salivary agglutinin have been delineated (Crowley et al., 1993), but those involved in binding to other bacteria are as yet unknown.

In conclusion, we have identified proteins of 62/60 and 45 kDa in S. gordonii G9B that are involved in binding to P. gingivalis 33277. The 62/60 and 45 kDa molecules appear to be part of a larger molecule of 170 kDa that is related to the P1 molecule originally identified in S. mutans. The 170 kDa P1-like molecule may require processing to induce binding activity.

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REFERENCES


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