Adhesion of Candida albicans to oral streptococci is promoted by selective adsorption of salivary proteins to the streptococcal cell surface

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INTRODUCTION

Candida albicans is an opportunistic human pathogen that colonizes the oral cavities of a large proportion of the population without causing disease (Odds, 1988; Cannon et al., 1995a; Cannon & Chaffin, 1999). Under conditions of host immunosuppression, however, C. albicans can cause a number of mucosal infections, including oral candidiasis and Candida vulvovaginitis (Odds, 1988). Oral candidiasis is the collective term for a number of distinct clinical oral pathologies that include acute pseudomembranous candidiasis, erythematous candidiasis, chronic hyperplastic candidiasis, chronic atrophic candidiasis and angular cheilitis (Odds, 1988; Samaranayake, 1992). Chronic atrophic candidiasis (denture-induced stomatitis) is the most common clinical manifestation of oral candidiasis and occurs in up to 60% of denture wearers (Odds, 1988; Lynch & Tenn, 1994).

The oral cavity comprises diverse micro-environments containing a range of surfaces to which microbial cells can adhere, including the teeth, epithelial mucosa and the nascent surfaces created as micro-organisms bind to existing biofilms (Whittaker et al., 1996). All these surfaces are coated with salivary glycoproteins that are selectively bound (Douglas & Russell, 1984;
Scannapieco et al., 1989; Nikawa & Hamada, 1990; Edgerton et al., 1993; Newman et al., 1993; Rudney et al., 1995). Co-adhesion (the binding of planktonic microbial cells to surface-bound microbial cells) is an important mechanism in microbial cell growth and survival in the oral cavity, and may be modulated by salivary molecules.

C. albicans has been isolated from dentures (Arendorf & Walker, 1979; Wright et al., 1985), dental plaque (Hodson & Craig, 1972; Arendorf & Walker, 1980) and from sites of periodontal disease (Slots et al., 1988; Rams & Slots, 1991). Furthermore, adhesion of C. albicans to oral surfaces including buccal epithelial cells and acrylic (Verran & Motteram, 1987; Branting et al., 1989) has been shown to be influenced by oral bacteria. Therefore, there is good in vivo and in vitro evidence for Candida interactions with bacterial biofilms. Holmes et al. (1995b) demonstrated that C. albicans cells co-aggregate with, and co-adhere to, certain species of oral streptococci, and for some species, e.g. Streptococcus gordonii, salivary proteins enhance the interactions (Holmes et al., 1995a). Salivary-protein-enhanced co-adhesion of oral bacteria has been frequently described (see Jenkinson & Lamont, 1997) and the salivary-enhanced binding of Streptococcus mutans to Actinomyces naeslundii. Streptococcus sanguinis and Streptococcus mitis is believed to be a significant factor in the development of early plaque (Lamont & Rosan, 1990).

Several studies (Gibbons & Hay, 1988; Gibbons et al., 1991) have demonstrated that the acidic proline-rich proteins (aPRPs) can promote the adherence of A. naeslundii and S. gordonii cells to apatitic surfaces. These studies concluded that the acidic PRP-1 protein was cryptic in nature due to the fact that it did not bind to the cell surface of A. naeslundii LY7 cells, but rather is only recognized as a receptor for A. naeslundii LY7 and S. gordonii Blackburn cells when immobilized onto an apatitic surface (Gibbons & Hay, 1988; Gibbons et al., 1991). However, subsequent studies have shown that oral streptococci, when incubated with parotid saliva, adsorb salivary protein components including the aPRPs and some basic PRPs (bPRPs IB-1 to IB-9) onto their surfaces in a species- and strain-specific manner (Newman et al., 1993). Therefore, it is possible that some streptococci are able to recognize alternate forms of PRPs, either soluble or bound. This is analogous to the suggestion that streptococci are able to bind and differentiate between soluble and immobilized forms of salivary agglutinin (Jenkinson & Demuth, 1997) and allows bacterial cell adhesion to salivary pellicles despite the excess soluble forms of the receptor in saliva.

Since we have previously identified the bPRP IB-6 and three other PRPs as possible pellicle receptors for C. albicans ATCC 10261 adhesion (O’Sullivan et al., 1997), we investigated the hypothesis that selective adsorption of bPRPs by streptococci may enhance C. albicans adhesion within oral biofilms.

**METHODS**

**Strains and growth media.** The streptococcal strains used included S. gordonii NCTC 7869 (Channon), S. gordonii DL1 (Challis) (Pakula & Walczak, 1963), S. sanguinis ATCC 10556, S. mitis NCTC 10712, Streptococcus oralis 34 (from P. E. Kolenbrander, National Institutes of Health, Bethesda) and S. mutans NG8 (from A. S. Bleiweis, University of Florida, Gainesville). C. albicans ATCC 10261 was the yeast strain used in this study. Streptococcal cells were grown on TSBY agar (1:1.2 g tryptone, 1 g yeast extract, 5 g agar, 5 g glucose, 20 g agar) or in BHY (1:1.27 g brain heart infusion, 5 g yeast extract) medium at 37 °C in screw-capped tubes or bottles as stationary cultures (McNab et al., 1994). Late-exponential-phase cultures (OD₆₀₀ 2, approx. 10⁹ cells ml⁻¹) were centrifuged (6000 g, 4 °C, 10 min) to pellet bacterial cells, which were then washed and prepared for assays as described below. C. albicans ATCC 10261 cells were grown in glucose salts biotin medium (GSB; Holmes & Shepherd, 1988) at 30 °C with vigorous shaking and collected from exponential-phase cultures by centrifugation (4000 g, 10 min, 4 °C) (O’Sullivan et al., 1997).

**Collection and fractionation of parotid saliva.** Stimulated parotid saliva samples were collected from six donors using a modified Carlson–Crittenden device (Shannon et al., 1962) and stored on ice. The following proteinase inhibitors were added to the saliva samples (final concentrations): PMSF (1 mM); bisulphite (10 mM); benzamidine/HCl (2 mM); pepstatin A (2.9 μM) and aprotonin (0.3 μM). Samples were then pooled and clarified by centrifugation (12000 g, 4 °C, 15 min). For fractionation, saliva (60 ml) was freeze-dried, suspended in water and applied to a Sephacryl S200 column (XL 26/100; Pharmacia). Fractions were collected and characterized as previously described (O’Sullivan et al., 1997).

**Adhesion of C. albicans to saliva-coated hydroxylapatite (HA) beads.** C. albicans cells were radioactively labelled with [³⁵S]methionine [New England Nuclear; 4·3×10⁵ Bq mmol⁻¹ (1175 Ci mmol⁻¹)] to a specific radioactivity of 0.036±0.017 c.p.m. per cell as described previously (O’Sullivan et al., 1997). Labelled cells were washed and suspended in KCl buffer (2 mM KH₂PO₄, 2 mM K₂HPO₄, 5 mM KH₂PO₄, 1 mM CaCl₂, pH 6-5) and numbers of C. albicans cells adhering to parotid-saliva-coated HA beads (20 mg; Macrosorb C, Phase Sep) were measured as described by Cannon et al. (1995b).

**SDS-PAGE and electroblotting.** SDS-PAGE was performed according to the method of Laemmli (1970) through 12·5% (w/v) acrylamide gels. The apparent molecular mass of proteins was estimated using pre-stained protein markers (15–200 kDa; Gibco-BRL). Sample protein concentrations were determined using a modified Bradford assay (Bio-Rad). Proteins were electroblotted (Towbin et al., 1979) onto nitrocellulose membranes (Hybond-C; Amersham) for use in blot overlay assays. Transfer conditions were 100 V, 1·5 h, at 4 °C and membranes were stored in plastic wrap at 4 °C until use.

**Blot overlay assay.** The binding of C. albicans ATCC 10261 cells to electrophoretically separated parotid saliva proteins, immobilized on nitrocellulose membranes, was determined as previously described (O’Sullivan et al., 1997). Salivary proteins on blot membranes were first visualized by staining them with 0·2% (v/v) Ponceau S in 1% (v/v) acetic acid for 5 min, followed by four washes with 1% (v/v) acetic acid. The membranes were destained with two washes (30 min, 20 °C) of PBS (1:1: 8·5 g NaCl, 0·3 g KH₄PO₄, 0·6 g Na₂HPO₄, pH
Saliva did not, however, significantly affect the binding of yeast cells to S. mutans or to homologous C. albicans cells (Fig. 1). Preliminary, as well as subsequent, experiments showed that this salivary stimulation of the numbers of Candida binding to streptococci was dose-dependent (saturating at approximately 1-4 pg salivary protein per S. gordonii cell), and in all instances was maximal at the concentration of salivary proteins (equivalent to approximately 2 pg per bacterial cell) used in the experiment shown in Fig. 1. These results indicated that streptococcal cells selectively adsorbed salivary protein receptors for C. albicans adhesion and therefore we proceeded to determine the nature of these receptors.

**Adsorption of salivary proteins by streptococcal cells**

To identify the protein components depleted from saliva by streptococci, adsorbed saliva samples were analysed by SDS-PAGE and the candidal adhesion receptors remaining were determined by blot overlay. Incubation of saliva with S. gordonii NCTC 7869 (Fig. 2) or DL1 (data not shown) cells caused a depletion in protein bands of apparent molecular masses 72, 42, 32, 28, 24, 16.5 and 15 kDa, compared with saliva samples incubated under identical conditions without bacteria added.

(\(P < 0.005\)) increased binding of C. albicans cells to S. sanguinis ATCC 10556 and S. oralis 34 cells (Fig. 1).

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**RESULTS**

**Effect of parotid saliva on C. albicans co-adhesion**

The co-adhesion of C. albicans ATCC 10261 cells to immobilized streptococcal cells was measured in a microtitre well assay. Substantial proportions of input yeast cells (24–49 %) adhered to S. gordonii, S. oralis or S. sanguinis cells, while much fewer cells bound to S. mutans cells (Fig. 1). C. albicans demonstrated low-level binding to immobilized homologous candidal cells. S. oralis 34 and S. sanguinis ATCC 10556 supported greater numbers of C. albicans bound (up to 49% of input cells) compared with S. gordonii DL1 cells, confirming previous results (Holmes et al., 1995a). Precipitation of streptococcal cells with parotid salivary proteins (equivalent to approximately 2 pg protein per microbial cell) increased markedly the binding of C. albicans to S. gordonii (2.3-fold) and also significantly
S. oralis 34 or S. sanguinis ATCC 10556 cells also removed the 72 or 42 kDa protein bands, respectively (Fig. 2a, lanes 3 and 5). By contrast, incubation of saliva with cells of S. mitis NCTC 10712, S. mutans NG8 or C. albicans ATCC 10261 had no significant effects on the protein profiles (Fig. 2). S. gordonii binds α-amylase with high affinity from fluid-phase parotid saliva (Rogers et al., 1998; Scannapieco et al., 1989), but under the assay conditions described the putative α-amylase was not completely removed by any strain tested (see Fig. 2). This was confirmed by demonstrating the presence of α-amylase activity, using starch agar plates (Douglas et al., 1990), in saliva samples that had been pre-incubated with streptococcal cells (data not shown).

We have previously identified four salivary bPRPs (denoted band A, 17 kDa; band B, 20 kDa; band C, 24 kDa; and band D, 27 kDa) that are bound by C. albicans cells in blot overlay assays (O’Sullivan et al., 1997). In addition, C. albicans cells also bind to the bPRP Ps-2 (43 kDa). Following incubation of parotid saliva samples with S. gordonii cells, no binding of C. albicans cells to band C or band D was observed (Fig. 2b) and the 43 kDa (Ps-2) receptor for C. albicans present in untreated saliva (Fig. 2) was also reduced. Binding of C. albicans cells to band A (17 kDa, bPRP IB-6; O’Sullivan et al., 1997) and band B (20 kDa) was also reduced (Fig. 2b). Pre-incubation of parotid saliva with S. mitis NCTC 10712 or S. sanguinis ATCC 10556 also removed the 43 kDa C. albicans ATCC 10261 adhesion receptor Ps-2 (Fig. 2b, lanes 4 and 5). Incubation of parotid saliva with S. oralis, C. albicans ATCC 10261 (Fig. 2b) or with S. mutans (data not shown) did not alter the binding profiles of C. albicans in blot overlay assays. These results demonstrated that the various streptococcal species adsorbed different salivary proteins to their cell surfaces, and suggested that the adsorption of bPRPs by S. gordonii could account for the C. albicans–S. gordonii co-adhesion-promoting ac-

### Fig. 2. Selective adsorption of salivary proteins by oral streptococci. Clarified parotid saliva was incubated with streptococcal or C. albicans ATCC 10261 cells at 20 °C for 1 h. Microbial cells were sedimented by centrifugation and supernatants (20 μl per lane) were subjected to SDS-PAGE. Gels were either stained with Coomassie blue R250 or electroblotted onto nitrocellulose membranes which were then incubated with radiolabelled C. albicans cells (103 × 107 cells ml⁻¹). (a) Coomassie-stained gel; (b) autoradiogram of corresponding blot overlay. Micro-organisms used to adsorb salivary proteins were: C. albicans ATCC 10261, lane 1; S. gordonii NCTC 7869, lane 2; S. oralis 34, lane 3; S. mitis NCTC 10712, lane 4; S. sanguinis ATCC 10556, lane 5; KCl buffer control, lane 6. A–D are bands previously identified as receptors for C. albicans adherence (O’Sullivan et al., 1997) and Ps-2 is the basic PRP-2. The experiment was carried out three times and representative results are shown.

![Fig. 2](image-url)
Adhesion of *Candida albicans* to oral streptococci

Proteins bound by streptococci also support adhesion of *C. albicans* to experimental pellicle

To determine if the same salivary components that promote binding of *C. albicans* to streptococci also promote adherence of *Candida* cells to saliva-treated HA beads, parotid saliva samples were pre-treated with streptococcal cells and then used to coat HA beads. There was a >50% reduction in the numbers of *C. albicans* cells binding to HA beads coated with saliva that had been pre-incubated with *S. gordonii* cells, compared with untreated saliva (Fig. 3). A reduction (28%) was also noted in numbers of yeast cells adhering to *S. oralis*-treated saliva-coated beads, but there was no significant effect on the numbers binding to HA beads coated with saliva that had been pre-incubated with *S. mitis, S. mutans* or *C. albicans* (Fig. 3).

As expected, parotid saliva that had been pre-incubated with *S. gordonii* cells was significantly (*P* < 0.05, t-test) reduced in its ability to promote *C. albicans–S. gordonii* DL1 co-adherence (Fig. 4). By contrast, parotid saliva pre-incubated with *S. oralis* 34 cells, or with *C. albicans* ATCC 10261, was not significantly reduced in stimulation of co-adherence (Fig. 4). These data confirm that the major enamel pellicle receptors for *C. albicans* are also subject to high-affinity adsorption to the cell surface of *S. gordonii*.

Fig. 4. Effect of pre-adsorbing parotid saliva on the ability to support *C. albicans* cell adhesion to immobilized microorganisms. Saliva was pre-adsorbed with streptococcal or *C. albicans* cells and then used in microtitre assays to measure adhesion of *C. albicans* cells to fresh, immobilized, microorganisms. Numbers are the percentage of the input *C. albicans* ATCC 10261 cells (2.6 ± 10⁶ per well) bound, and are the means of quadruplicates. White bars, unadsorbed saliva; black bars, pre-adsorbed saliva. *Significant decrease in adhesion (*P* < 0.05, Student’s t-test).

Promotion of *C. albicans–streptococcal* co-adhesion by parotid salivary protein fractions

To confirm that the bPRPs within parotid saliva were capable of directly promoting *C. albicans* adhesion to *S. gordonii*, adhesion-promoting activities in salivary protein fractions obtained following size-exclusion chromatography were measured (Fig. 5). Pools designated 1 through 6 contained salivary proteins as previously characterized (O’Sullivan et al., 1997), with pool 4 being enriched in bPRP bands A–D (Fig. 5b). The greatest stimulation of *C. albicans* adhesion to *S. gordonii* was associated with pool 4, while protein pools 3 or 5, which also contained lesser amounts of bPRPs, also promoted adhesion. Pool 1, which contains protein aggregates excluded from the column, also stimulated a high level of *C. albicans* adhesion to *S. gordonii*. The ability of pools 1, 2 and 6 to promote *C. albicans–S. gordonii* co-adhesion indicates that other proteins, or protein complexes, within saliva also act as receptors for
C. albicans adherence. However, the magnitude of the adherence stimulation obtained with pool 4, containing all four bPRPs, indicates that the bPRPs are of greatest significance.

**DISCUSSION**

Colonization of the oral cavity by C. albicans is a crucial step in the sequence of events leading to an infection. Salivary proteins and glycoproteins can act as receptors for binding of C. albicans cells to enamel surfaces (Cannon et al., 1995b) and to denture acrylic surfaces (Vaslas et al., 1992; Edgerton et al., 1993; Nikawa et al., 1993) and can modulate the binding of yeast cells to buccal epithelial cells (Kimura & Pearsall, 1978; Samaranyake & MacFarlane, 1982). In addition, C. albicans binding to oral viridans streptococci (Jenkinson et al., 1990; Holmes et al., 1995a, b, 1996) and to Actinomyces (Grimaudo et al., 1996) may be enhanced by salivary components (Holmes et al., 1995a; Grimaudo et al., 1996). We have now determined that the basic PRPs present in human parotid salivary secretions are not only active in providing receptors for adhesion of C. albicans to enamel pellicles, but also that these salivary components are adsorbed by streptococci and act to promote adhesion of C. albicans ATCC 10261 to bacterial cells.

Salivary proteins and glycoproteins are present in multiply active conformations within the human oral cavity. In the fluid phase, salivary components may be present as discrete molecules or more usually as macromolecular complexes often involving mucins (Iontcheva et al., 1997). A number of these components are selectively adsorbed onto enamel, and the aPRPs and statherin change conformation upon binding to enamel (Moreno et al., 1984) thus unmasking receptors for adhesion of streptococci and Actinomyces species (Gibbons & Hay, 1988; Gibbons et al., 1990, 1991). This ability of oral micro-organisms to bind immobilized salivary proteins is of considerable ecological significance. Adhesins that have evolved to bind immobilized conformations of salivary components provide a mechanism for attachment of microbial cells to oral surfaces despite the presence of excess soluble forms of the receptors within saliva. The bPRPs appear to comprise another group of salivary proteins that act as receptors for microbial cell adhesion when immobilized. Interestingly, the streptococci, in particular S. gordonii, are able to adsorb these proteins to their surfaces and the bound forms then act as receptors for C. albicans adhesion. Conversely, C. albicans cells are unable to adsorb bPRPs from saliva, although small amounts of other macromolecules are adsorbed by yeast cells (Edgerton et al., 1993). Thus C. albicans demonstrates a unique adhesive mechanism whereby adhesion may occur to surface-bound forms of bPRPs despite the presence of excess fluid-phase bPRPs in saliva.

Streptococci are early colonizers of salivary pellicles and their ability to bind salivary proteins and glycoproteins is important in plaque development. Of the streptococcal species tested, S. gordonii cells bound salivary proteins to the greatest extent, but the components of saliva bound by the streptococcal species varied quite considerably. The changes to the salivary protein profiles observed following incubation with streptococcal cells were highly reproducible, and evidence suggested that the alterations resulted from adsorption of the proteins to the bacterial cell surface rather than for other reasons such as proteolysis. Even in the presence of a cocktail of protease inhibitors, bPRP bands A, C and D were adsorbed by S. gordonii within 15 min determined by the overlay assay (data not shown). Previously, S. gordonii cells have been reported to bind to PRPs IB-1 to IB-9 and Ps-1 in blot overlay assays (Newman et al., 1996). The adsorption of bPRPs by S. gordonii occurs via a high-affinity mechanism since maximal adsorption of bPRPs from the fluid phase occurred under conditions that did not result in the complete removal of z-amylase, which is a high-affinity binding reaction (Scannapieco et al., 1989). Pool 4 promotes high binding of C. albicans to immobilized S. gordonii cells and it is possible that all four bPRPs are recognized, when presented on the surface of the bacterial cells, with a concomitant increase in binding activity. As a result of adsorption of bPRPs by streptococci, the PRP-depleted saliva was much less efficient at supporting C. albicans adhesion to HA beads, confirming that similar forms of the adhesion receptors are presented by enamel and by streptococci.

S. gordonii is found at multiple sites within the human oral cavity and is associated with numerous intra- and inter-generic bacterial co-aggregations which are postulated to be involved in the formation of oral biofilms (Kolenbrander & London, 1993; Holmes et al., 1996). Therefore, adhesion of C. albicans cells to salivary components adsorbed to S. gordonii cells would potentially increase the number of available sites for C. albicans adhesion and colonization. In support of this suggestion, it has been observed that C. albicans cells are more usually found associated with plaque-coated areas of enamel (Arendorf & Walker, 1980). It could be envisaged that presentation of bPRPs on the surface of streptococci might also tend to co-aggregate C. albicans and promote clearance from the oral cavity. However, streptococcal cells also bind to aPRP 1-coated enamel (Gibbons et al., 1991). Thus direct co-aggregation of C. albicans with streptococci, or co-aggregation via bPRP, followed by binding of streptococci to an oral surface could also provide a means by which C. albicans colonization is promoted.

Currently, the molecular nature of the receptor that binds bPRPs to the streptococcal cell surface is not known. However, two protein families on the streptococcal cell surface are known to bind to other salivary proteins. The antigen I/II proteins (Jenkinson & Demuth, 1997) bind salivary agglutinin glycoprotein while AbpA (Rogers et al., 1998) binds z-amylase. The antigen I/II proteins SspA and SspB are also involved in the direct binding of S. gordonii to C. albicans cells (Holmes et al., 1996). We have observed that mutants
deficient in the production of SspA and SspB are reduced, by approximately 20%, in their ability to adsorb bPRPs (unpublished observations). However, since adsorption of bPRPs by wild-type cells appears to be a rapid and high-affinity process, simply reduced adsorption by the mutants implies that the SspA and SspB proteins are not the major bPRP adhesins. Therefore, it seems likely that the binding of bPRPs to the streptococcal cell surface may occur through a multi-modal mechanism.

In summary, C. albicans cells do not appear to bind salivary bPRPs in the fluid phase. However, adsorption of these proteins to a surface such as enamel, Streptococcus, or to nitrocellulose in vitro exposes receptors that are then recognized by C. albicans cells. This ability to adhere to only surface-bound peptides would enable C. albicans cells to adhere to multiple oral cavity surfaces in the presence of fluid-phase saliva and thus enhance its colonization potential.

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