Effect of serum concentration on *Candida* biofilm formation on acrylic surfaces.

Abstract

The biofilm formation of the oral fungal pathogen *Candida* on denture acrylic strips coated with saliva, serum and, saliva/serum pellicle were examined in vitro using *C. albicans* (4 isolates), *C. glabrata* (3 isolates) and *C. tropicalis* (3 isolates). The degree of biofilm activity varied depending upon both the isolate and the pellicle. Thus as compared with the uncoated control, significantly increased biofilm activity on the pellicle (particularly serum)-coated strips, was observed with three isolates of *C. albicans* and another of *C. glabrata* on protein coated acrylics, with increasing concentration of serum in the pellicle (ANOVA; $p<0.01$). Similar trends were observed with one isolate of*C. albicans* and *C. grabrata*, though the effects of pellicles were not significant (ANOVA; $p>0.05$). In contrast, with all 3 isolates of *C. tropicalis* and a single isolate of *C. glabrata,* although the biofilm activity on the protein-free control strips was significantly higher than that of saliva-coated strips, the activity of pellicle admixed-biofilm was increased depending upon the serum-concentration. Taken together our in vitro findings that candidal biofilm formation on acrylic surfaces is essentially promoted with increase in the concentration of serum in pellicle, suggest that inflamation in the oral environment would facilitate the fungal colonization on denture acrylic.

In *Candida*-associated denture stomatitis (syn. chronic atrophic candidiasis), a common infection seen elderly denture wearers, the acrylic denture fitting surface acts as a reservoir of infection (1-3). Although *C. albicans* is by far the predominant isolate in this condition other non-*albicans* species such as *C. tropicalis* and *C. glabrata* (syn. *Torulopsis glabrata*) are frequently isolated both from the acrylic denture surfaces and the palatal mucosa (2).

Despite the realization that successful candidal colonization of denture surface is an important step in the pathogenesis of this condition (4), the role played by saliva or serum pellicles during the colonization process and subsequent multilayer biofilm formation is poorly understood. Indeed components of saliva or serum proteins, such as mucins, fibrinogen and complements specifically bind to *Candida* blastospores and germ tubes (5-8) possibly modifying the biofilm formation. In recent investigations we have demonstrated the specific interactions between mannoprotein adhesin of *C. albicans* and sugar-moiety of salivary proteins, during candidal adherence to protein adsorbed surfaces (9,10), and that salivary and serum pellicles promote fungal colonization on denture lining materials (11). Others have shown that mucinous glycoproteins of human saliva promote adhesion of *C. albicans* to acrylic surfaces by sugar-specific interactions (12). In contrast, some researchers have demonstrated that pretreatment of acrylic strips and/or yeast cells with whole saliva decreases the initial adherence of *C. albicans* to denture acrylic, whereas a serum pellicle promotes adherence (13,14).

It is therefore evident that the relationship between the salivary or serum pellicle on denture surfaces and candidal colonization is a complex subject. This is not surprising as both saliva and serum, which modulate clearance, aggregation, adherence and nutrition of microorganisms are biological fluids of immense complexity contributing to specific and non-specific interactions in fungal colonization. Recently, we developed a bioluminescent adenosine triphosphate (ATP) assay based on firefly luciferase-luciferin system (15-17), and demonstrated the reduced activity of *Candida albicans* biofilms on saliva-coated acrylic strips, although on prolonged incubation, both saliva and serum promoted fungal biofilm activity. (18). There is, however, no data on the formation of biofilms by non-*albicans* species such as *C. glabrata* and *C. tropicalis*. In addition, in the oral cavity, denture surfaces are considered to be coated with pellicles comprising of saliva and serum mixture, depending upon the inflamatory states.

Thus, the purpose of the present study was to investigate *Candida* biofilm formation on saliva, serum, and saliva-serum pellicles-coated acrylic surfaces using 10 *Candida* isolates belonging to *C. albicans*, *C. glabrata* and *C. tropicalis* species.

A total of 10 isolates of *Candida* comprising *Candida albicans* GDH 16, GDH18, GDH 19, GDH 20, *C. glabrata* IFO 0005, GDH 1407, GDH 2269, *C. tropicalis* IFO 1070, GDH 1362 and GDH 0462 were used in the study. All GDH isolates were oral isolates obtained from the routine microbiology services of the Glasgow Dental Hospital and School and the two IFO isolates were purchased from the Institute for Fermentation, Osaka, Japan. All the isolates were identified by sugar assimilation test using the API 20C system (API Products, Biomeroux, Lyon, France) and "germ tube" test (19).

A loopful of the yeast was inoculated in yeast nitrogen base medium (Difco, Detroit, USA) containing 250mM glucose and grown aerobically at 37℃ (9). After overnight culture, the organisms were harvested in the late exponential growth phase, washed twice with 100mM phosphate buffered saline (PBS; pH 6.8) and resuspended to a final concentration of 10^5 cells/ml by using haemocytometeric counts (11,18).

Heat-cured denture acrylic sheets (50 × 50 × 0.7mm) were fabricated according to conventional prosthodontic techniques (18). Briefly, denture acrylic poly(methylmethacrylate) powder and monomer liquid (Bio Resin, Shofu, Kyoto, Japan) were mixed according to manufacturer's directions. The mixture was packed into the flask, processed in water tank at 70℃ for 90min and then 100℃ for 30min, according to Japan Industrial Standard (JIS). A smooth surface was obtained by compressing the mixture onto glass slides. The processed acrylic sheets were cut into 10 × 10 × 0.7mm pieces.

Unstimulated whole saliva was collected by expectoration, on ice, from five healthy adult donors (3 males and 2 females) and an equal amount from each donor was pooled. The saliva was clarified by centrifugation at 12,000g, for 15min at 4°C (20). Human serum was purchased from Sigma Chemical Co.(St Louis, MO, USA). Whole saliva and serum were stored at -25℃ before use.

The colonization assay was conducted as follows. The acrylic strips were coated with whole saliva (saliva), 5%(vol/vol) serum-saliva mixture (5%serum), 20%(vol/vol) serum-saliva mixture (20% serum), 50% (vol/vol) serum-saliva mixture (50% serum), or neat serum (serum) by placing them in wells of Multiwell tissue culture plates (Nunclon^R Delta, Nunc, Kamstrup, Denmark), into which were dispensed 500 μl of the protein solution per well, and incubating for 1 hour at 37 °C. In the control wells, saliva or serum was substituted with an equal volume of sterile distilled water as appropriate. After incubation the protein solution was aspirated, 50 μl of yeast suspension (1×10^5 cells/ml) was inoculated into each well and the whole assembly incubated at 37°C for 2 hrs to promote yeast adherence and colonization.

Subsequently, 2.0 ml of Sabouraud broth was carefully dispensed into each well, and incubated for 72 hrs at 37°C. Afterwards each specimen was carefully removed, washed thoroughly by rinsing three times for a total of 60 seconds with distilled water to remove loosely adherent organisms, and the ATP content was measured as described previously (18). The assays were carried out on two independent occasions, with quadruplicated samples on each occasion. All the numerical data obtained were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 5 and 1 % levels.

The biofilm activity of each isolate of *Candida* initially increased, and plateaued within 48-72h incubation, irrespective of the presence of the pellicles (data not shown). Thus in the present study, 72h-biofilm activity was used as the mature biofilm activity of all *Candida* isolates examined. The results indicated that the extent of biofilm activity varied depending upon both isolates and the quality of proteinaceous pellicles (Fig. 1). As compared with the uncoated control, significantly increased biofilm activity was observed with *C. albicans* GDH 16, GDH 18, GDH 19 and *C. glabrata* IFO 0005 on protein coated acrylics, with increasing concentration of serum in the pellicle (ANOVA; $p<0.01$). Similar trends were observed with *C. albicans* GDH 20 and *C. grabrata* GDH 1407 isolates, though the effects of pellicles were not noted (ANOVA; $p>0.05$). In contrast, the uncoated control specimen exhibited significantly higher activity than the saliva-admixed biofilm, in the case of *C. glabrata* GDH 2269 and three isolates of *C. tropicalis,* the activity of pellicle admixed-biofilm was increased depending upon the serum-concentration.

A number of experimental approaches have been made to examine the mechanisms of *C. albicans* adherence to solid surfaces, such as denture acrylic (12-14,21-25). Even the earliest investigators of this topic, reported the high affinity of *C. albicans* to denture acrylic by non-specific interactions, and modulation of this attachment process due to saliva and serum pellicles (14,26). It should also be noted that the phenomenon of adherence may represent only the first step in the colonization process (27) which, as time progresses, leads to a formation of a thin biofilm and then a multilayer, climax community of yeasts. Recently we adapted a bioluminescent ATP assay to evaluate and quantify biofilm formation in *C. albicans,* and demonstrated a direct correlation between fungal biomass and the ATP content (18).

The biofilm activity of each isolate of *Candida* isolates initially increased, and plateaued within 48-72h incubation, irrespective of the presence of the saliva or serum pellicles (data not shown). Nonetheless the extent of biofilm activity varied depending upon both the isolates and the quality of proteinaceous pellicle (Fig. 1). As compared with the

uncoated control, significantly increased biofilm activity was observed with *C. albicans* GDH 16, GDH 18, GDH 19 and *C. glabrata* IFO 0005, which developed on protein coated acrylic strips, with increasing in the serum concentration of the pellicles. Similar trends were observed with *C. albicans* GDH 20 and *C. grabrata* GDH 1407 isolates, though the protein coats had no significant impact on biofilm formation (Fig. 1; $p>0.05$) (ANOVA; $p>0.05$). These results tend to concur with our previous observations that both salivary and serum pellicles facilitate the development of a *C. albicans* biofilms as compared with acrylic strips devoid of these biological fluids. Similar observations have been reported by Vasilas et al. (1992) (25) and Edgerton et al. (1993) (12) who reported promotion of candidal adhesion due to salivary pellicles on acrylic surfaces. In contrast, the uncoated control specimen exhibited significantly higher activity than the saliva-admixed biofilm, in the case of *C. glabrata* GDH 2269 and three isolates of *C. tropicalis,* though the activity of pellicle admixed-biofilm was increased depending upon the serum-concentration. The former phenomena should be partly due to the higher hydrophobicity of these isolates (data not shown).

In clinical terms, the finding that the increase in serum content in the proteinaceous pellicle promoted the biofilm formation of most isolates of *Candida*, implies that the inflammation induced by denture plaque facilitates the fungal colonization on denture fitting surfaces.

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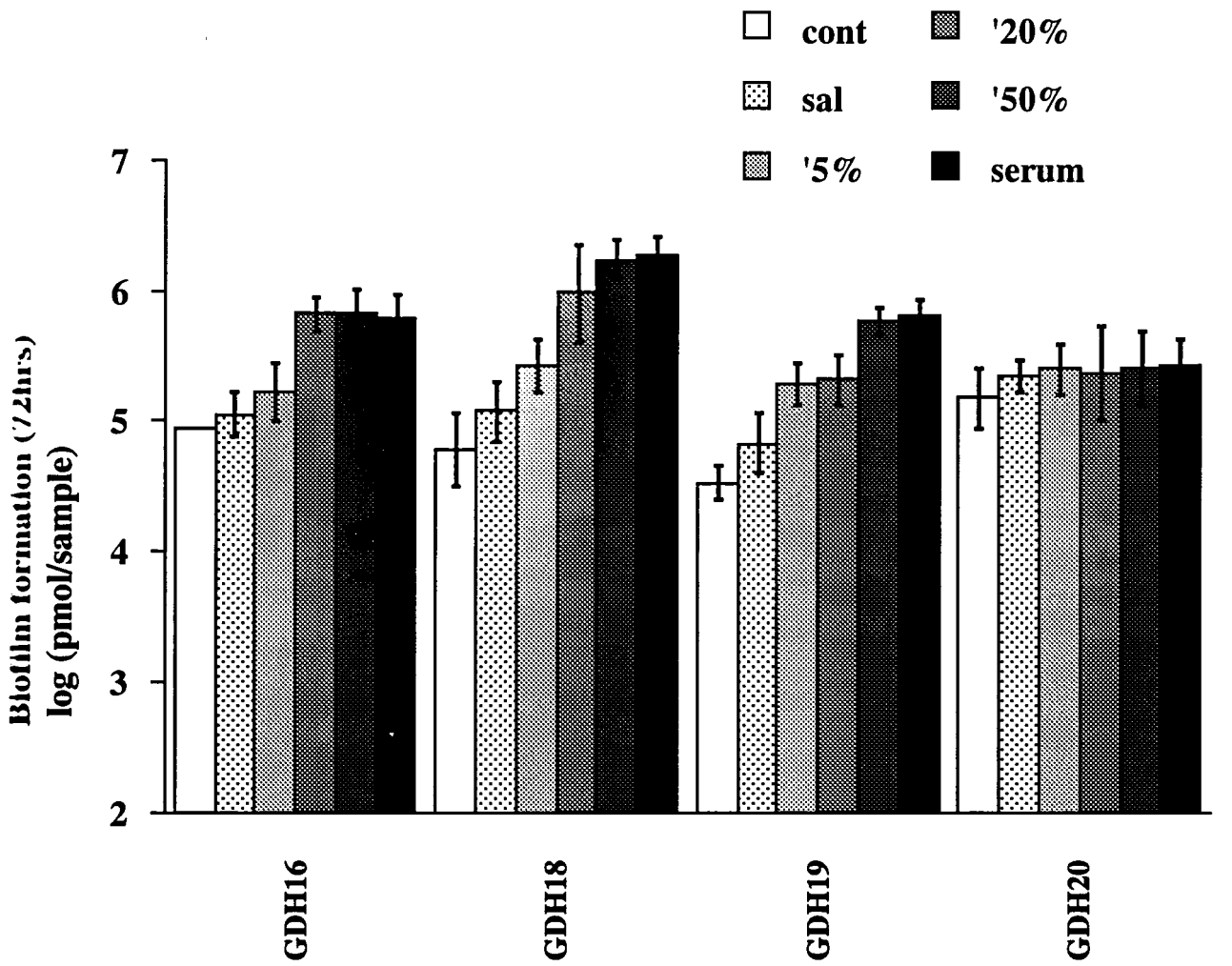
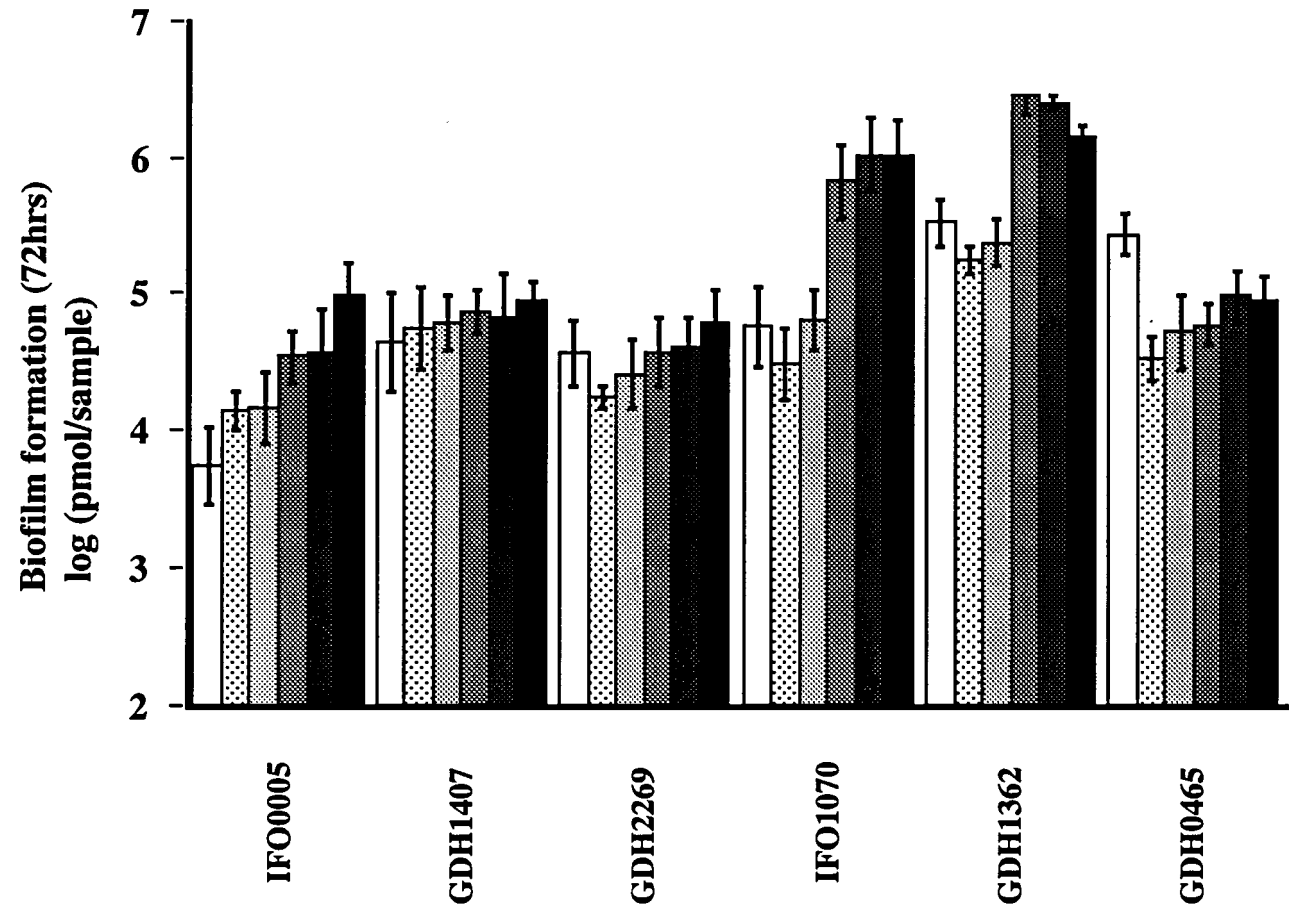
Legend to Figure

Fig. 1

The biofilm activity of *Candida* spp. on uncaoted (cont), whole saliva coated- (sal), 5% (vol/vol) serum-saliva mixture coated ('5%), 20%(vol/vol) serum-saliva mixture coated ('20 %), 50% (vol/vol) serum-saliva mixture coated ('50%), or neat serum coated (serum) acrylic specimens. The assays were carried out on two independent occasions, with quadruplicated samples on each occasion.


cont
'20%
sal
'50%
'5%
serum
Biofilm formation (72hrs)
log (pmol/sample)
7
6
5
4
3
2
GDH16
GDH18
GDH19
GDH20


Biofilm formation (72hrs)
log (pmol/sample)
7
6
5
4
3
2
IFO0005
GDH1407
GDH2269
IFO1070
GDH1362
GDH0465