Relationship between thigmotropism and *Candida* biofilm formation in vitro.

Abstract

The biofilm formation of the oral fungal pathogen *Candida* on denture acrylic strips coated with saliva or serum were examined in relation to the ability to induce hyphae by thigmotropic reaction, using *C. albicans* (4 isolates), *C. glabrata* (3 isolates) and *C. tropicalis* (3 isolates). Both the degree of biofilm formation and the amount of hyphae exhibiting thigmotropism varied depending upon both the species and strains of *Candida*. Although no signioficant correlation between the amount of hyphae induced by thigmotropic reaction of fungal isolates and biofilm formation on uncoated control specimans ($r=0.577$; p.0.05), the ability of hyphae induced by thigmotropic reaction significantly correlated with the amount of both saliva- and serum-admixed biofilms ($r=0.734$; $p<0.05$ and $r=0.793$; $p<0.01$, respectively).

Taken together our in vitro data suggested that the hyphal induction by thigmotropic reaction is of importance in candidal biofilm formation on saliva- or serum-coated acrylic surfaces.

Introduction

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 (3,4,8). 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 (4).

Despite the realization that successful candidal colonization of denture surface is an important step in the pathogenesis of this condition (22), the role played by saliva or serum pellicles during the colonization process and subsequent multilayer biofilm formation is poorly understood. The relationship between the salivary or serum pellicle on denture surfaces and candidal colonization is a complex subject, since 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 (1,10,14), 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. (20). There is, however, no data on the formation of biofilms by non-*albicans* species such as *C. glabrata* and *C. tropicalis*.

Besides the interactions with proteinaceous pellicles, dimorphism and/or germ tube formation is thought to be one of the most important factors in biofilm formation, since there are several ways in which germ tube or hyphae formation contributes to candidal persistence (2,5). Recently, Sherwood et al. (1992) demonstrated thigmotropism (contact sensing) of hyphae of this fungus (25) and Gow et al. (1994) characterized the nature of their touch-sensitive responses (11). The phenomenon should be of importance in the biofilm formation of fungi on denture acrylic surfaces. However, at present, there is no information available on the relationship between the biofilm formation and thigmotropism.

Thus the purpose of the present study was to analyze the relationship between ability of biofilm formation on saliva- or serum-coated acrylics and thigmotropism of 10 *Candida*

isolates belonging to *C. albicans* (4 isolates), *C. grabrata* (3) and *C. tropicalis* (3) species.

Materials and Methods

Microorganisms and growth conditions

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 (26).

A loopful of the yeast was inoculated in yeast nitrogen base medium (Difco, Detroit, USA) containing 250mM glucose and grown aerobically at 37℃ (19). 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 (18,19).

Fabrication of acrylic strips

Heat-cured denture acrylic sheets (50 × 50 × 0.7mm) were fabricated according to conventional prosthodontic techniques (21,24). 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.

Saliva and serum

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 (6). Human serum was purchased from Sigma Chemical Co.(St Louis, MO, USA). Whole saliva and serum were stored at -25℃ before use.

Biofilm assays

The colonization assay was conducted as follows. The acrylic strips were coated with whole saliva (saliva), 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 μ l 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 μ l 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 (20).

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.

Quantification of thigmotropism

The amount of hyphae induced by thigmotropic reaction was quantified by our previous method (21). A chemotaxicell (pore size 3um; surface area 0.48 cm^2;Kurabo, Osaka, Japan; 25) was placed on 20 % (v:v) serum agar, made with human serum (Sigma Chemical Co. St Louis, MO, USA), pre-warmed to 37°C, so that the filter of Chemotaxicell is in contact with the surface of agar (25). Subsequently, 150 ul of yeast suspension (1×10^7 cells/ml) was

dispensed into each Chemotaxicell and the whole assembly was incubated at 37°C for 72hrs. Afterwards each filter with penetrant/adherent yeasts was carefully detached from Chemotaxicell, washed ultrasonically for 10min and then washed manually further three times for a total of 60 seconds with distilled water, to remove the loosely attached organisms. The filter with penetrant hyphal mass was then immersed in 1.0 ml of the extraction-reagent (benzalkonium; 27) and allowed to react for 15min in an ultrasonicator. The total ATP in resultant reagent was then quantified using a bioluminescence apparatus (ATPA-100, TOA Electronics Ltd., Tokyo, Japan) as described previously (1,20).

The assays were carried out on two independent occasions, with quadruplicated samples on each occasion. The numerical data obtained were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 5 and 1 % levels.

Results

Biofilm formation

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 serum coated acrylics. However no such effect was noted with *C. albicans* GDH 20 and *C. grabrata* GDH 1407 isolates despite the protein pellicle. 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*.

Quantification of thigmotropism

When the property of thigmotropism was evaluated by estimating the degree of hyphal

extension through the filter pores,*C. albicans* GDH 18 exhibited the highest degree of thigmotropism followed by *C. albicans* GDH20, *C. albicans* GDH16, *C. tropicalis* GDH 1362, *C. albicans* GDH 19, *C. tropicalis* GDH 0465, and lastly *C. tropicalis* IFO 1070 (Fig. 2). A relatively good correlation was observed between germ tube forming ability and thigmotropism of seven isolates of *C. albicans* and *C. tropicalis* ($r=0.854$; $p<0.05$). As to the isolates of *C. glabrata*, no hyphal emergence was observed (data not shown). However, little ATP activity was detected in the filter, and SEM observation revealed this to be due to the nonspecific adsorption of little number of blastospores on the filter surface (not shown). This value was therefore the background 'noise' of the assay.

Although no signioficant correlation between the amount of hyphae induced by thigmotropic reaction of fungal isolates and biofilm formation on uncoated control specimans ($r=0.577$; p.0.05), the amount of hyphae induced by thigmotropic reaction significantly correlated with the activity of both saliva- and serum-admixed biofilms ($r=0.734$; $p<0.05$ and $r=0.793$; $p<0.01$, respectively, Fig. 3).

Discussion

A number of experimental approaches have been made to examine the mechanisms of *C. albicans* adherence to solid surfaces, such as denture acrylic (7,9,15,16,17,23,24,28). It should also be noted that the phenomenon of adherence may represent only the first step in the colonization process (13) 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 that both saliva and serum pellicle facillitated the fungal biofilm formation, and scanning electron microscopic study revealed that this was due to the multilayer, including blastospore-blastospore co-adhesion, germ tube, hyphal and pseudohyphal emergence and blastospore-hyphal coadherence, phenomena not observed in the uncoated controls. In addition we also have shown that the strand-like polymeric adhesive materials obseved on hyphal surface mediated the hyphal adhesion to acrylic surfaces with saliva- or serum-admixed biofilms.

Hawser & Douglas (1994)(12), reported hyphal induction relative to variations in substrates. It is therefore likely that during biofilm formation, hyphal emergence, particularly thigmotropic reaction may be involved and thus a deeper understanding *Candida* biofilm formation necessitates clarification of intertwined relationships.

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, on protein coated acrylic strips. 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. Similarly, the protein coats apparently promoted the biofilm formation of *C. albicans* GDH 20 and *C. grabrata* GDH 1407, however the results did not giva a level of significance (Fig. 1; $p>0.05$). Although in the case of *C. glabrata* GDH 2269 and all three isolates of *C. tropicalis,* the uncoated control specimen exhibited significantly higher activity than saliva-admixed biofilm, but the activity of control biofilm did not exceeded that of the serum-admixed biofilm.

Although no signioficant correlation between the amount of hyphae induced by thigmotropic reaction of fungal isolates and biofilm formation on uncoated control specimans ($r=0.577$; p.0.05), the ability of hyphae induced by thigmotropic reaction significantly correlated with the amount of both saliva- and serum-admixed biofilms ($r=0.734$; $p<0.05$ and $r=0.793$; $p<0.01$, respectively, Fig. 3). The results were consistent with our previous findings, that germ tube, hyphal and pseudohyphal emergence and blastospore-hyphal coadherence were involved in the formation of saliva- or serum-admixed biofilm and the phenomena not observed in the uncoated controls (20). Thus the results tend to suggest that hyphal emergence induced by the touch-sensitive reaction against substrates should be of importance in pellicle-admixed biofilm formation. However, whether this phenomenon simply contributes to fungal colonization in vivo is still unexplained, because there should be the antagonism against

Candida by bacteria in the oral cavity. Hence further work is required to clarify the interactions of the fungi with commensal bacteria.

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Legends to Figures

Fig. 1

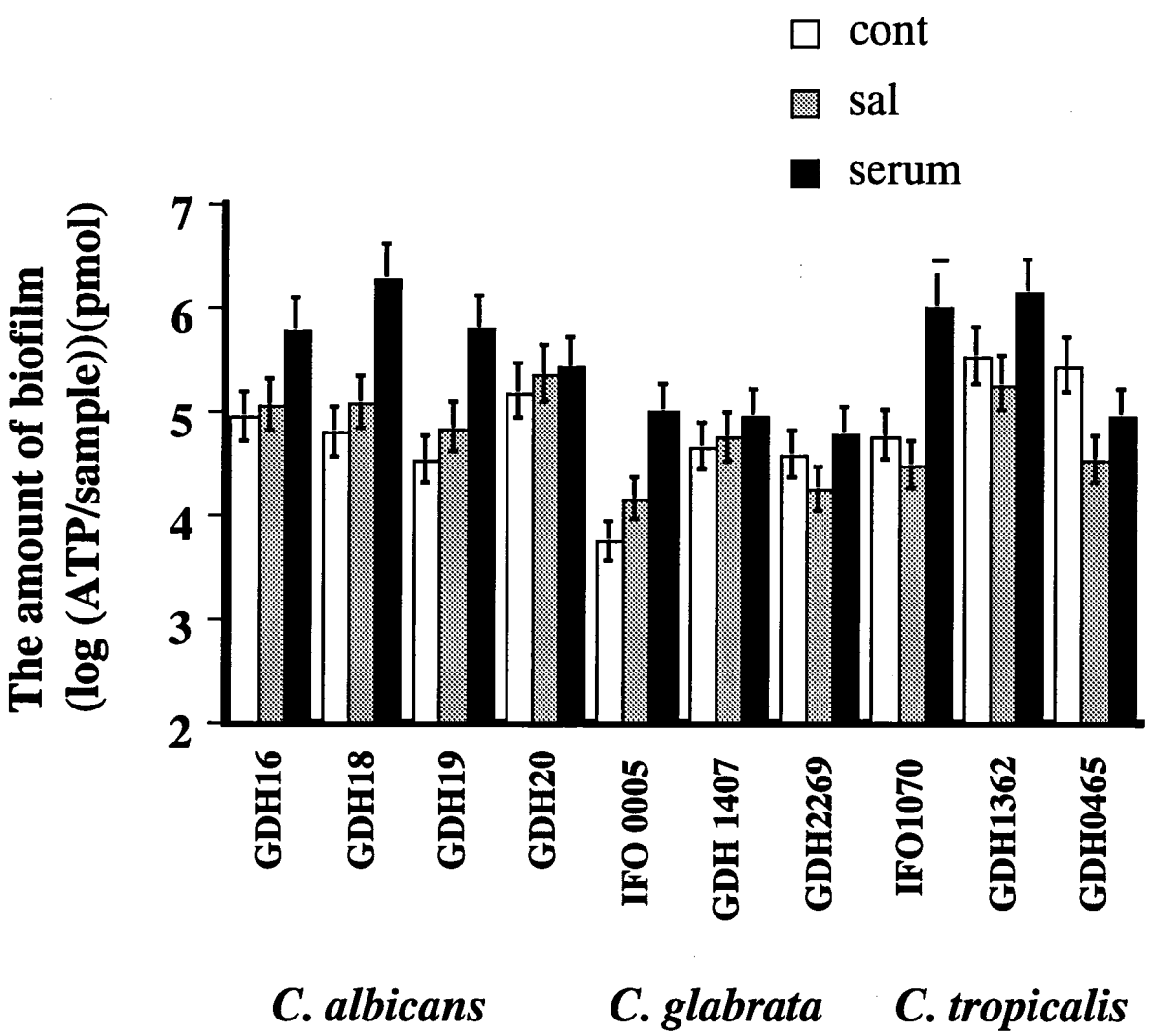
Three-days biofilm activity of *Candida* spp. on uncaoted (cont), whole saliva coated (sal), or neat serum coated (ser) acrylic specimens. The assays were carried out on two independent occasions, with quadruplicated samples on each occasion.

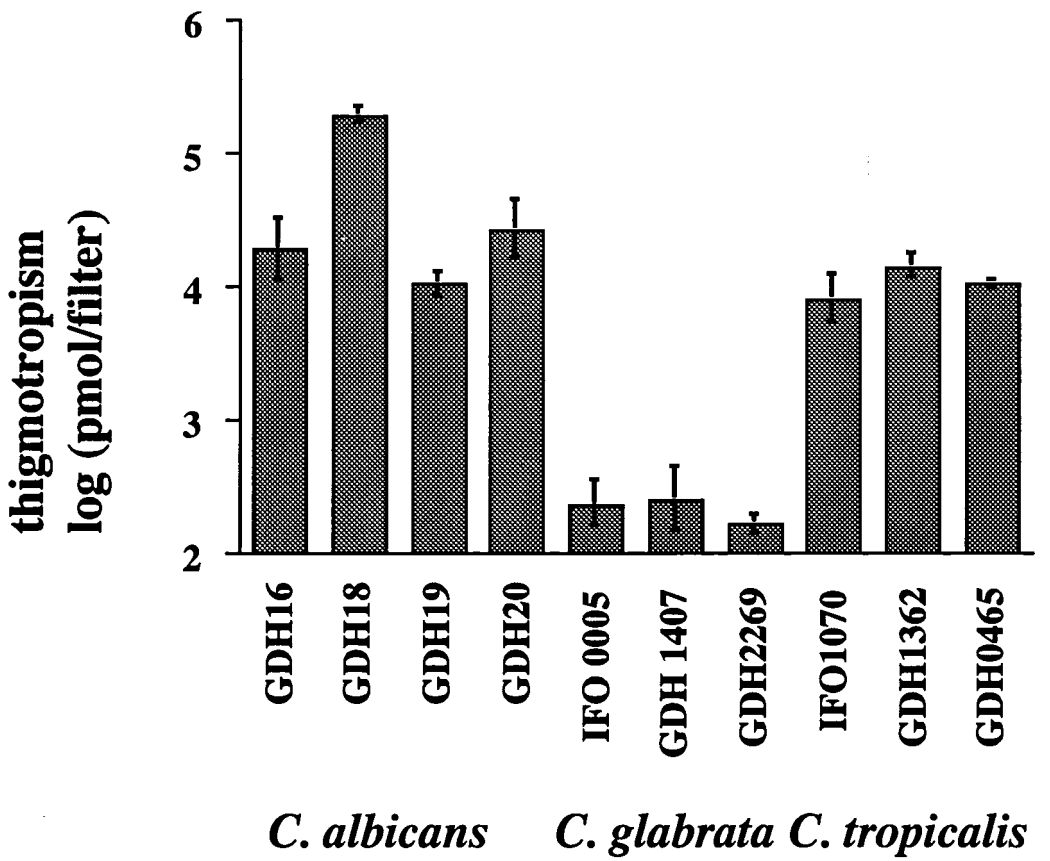
Fig. 2

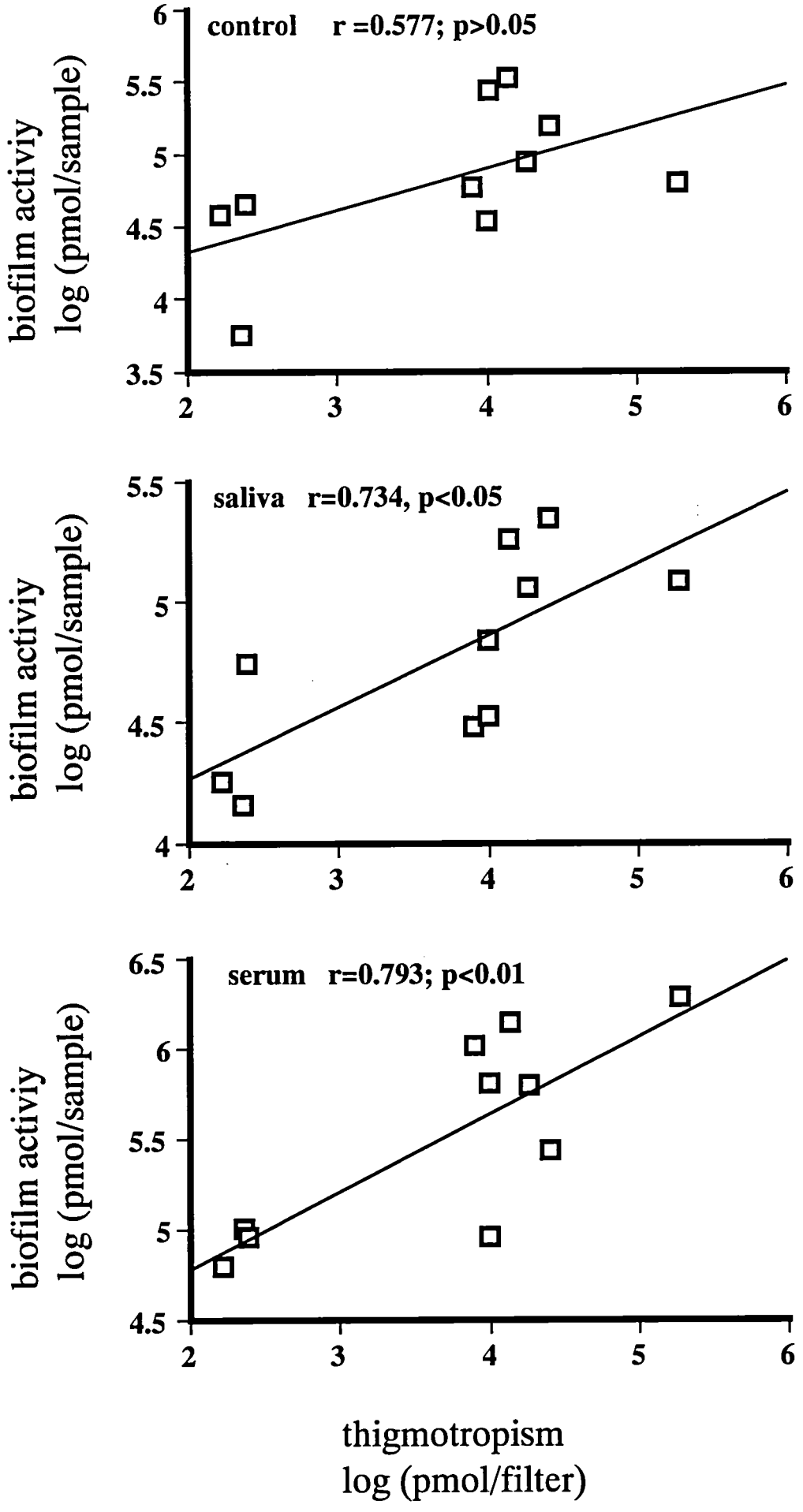
The ATP content of penetrant hyphae retained on the filter of the Chemotaxicell. This penetrant was considered a measure of thigmotropism. (The surface area of filter of Chemotaxicell for culture was 0.48 cm^2.) The assays were carried out on two independent occasions, with quadruplicated samples on each occasion.

Fig. 3

Relationship between thigmotropism against the amount of biofilm formation on control, saliva- and serum-coated acrylics.


cont
sal
serum
The amount of biofilm
(log (ATP/sample))(pmol)
7
6
5
4
3
2
GDH16
GDH18
GDH19
GDH20
IFO 0005
GDH 1407
GDH2269
IFO1070
GDH1362
GDH0465
C. albicans
C. glabrata
C. tropicalis


thigmotropism
log (pmol/filter)
6
5
4
3
2
GDH16
GDH18
GDH19
GDH20
IFO 0005
GDH 1407
GDH2269
IFO1070
GDH1362
GDH0465
C. albicans
C. glabrata
C. tropicalis


control r =0.577; p>0.05
saliva r=0.734, p<0.05
serum r=0.793; p<0.01
biofilm activiy
log (pmol/sample)
thigmotropism
log (pmol/filter)