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Interactions between *Streptococcus oralis*, *Actinomyces oris*, and *Candida albicans* in the development of multispecies oral microbial biofilms on salivary pellicle

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Running header: Interkingdom biofilms on salivary pellicle
SUMMARY

The fungus *Candida albicans* is carried orally and causes a range of superficial infections that may become systemic. Oral bacteria *Actinomyces oris* and *Streptococcus oralis* are abundant in early dental plaque and on oral mucosa. The aims of this study were to determine the mechanisms by which *S. oralis* and *A. oris* interact with each other and with *C. albicans* in biofilm development. Spatial distribution of microorganisms was visualized by confocal scanning laser microscopy of biofilms labelled by differential fluorescence or by fluorescence in situ hybridization (FISH). *A. oris* and *S. oralis* formed robust dual-species biofilms, or three-species biofilms with *C. albicans*. The bacterial components tended to dominate the lower levels of the biofilms while *C. albicans* occupied the upper levels. Non-fimbriated *A. oris* was compromised in biofilm formation in the absence or presence of streptococci, but was incorporated into upper biofilm layers via binding to *C. albicans*. Biofilm growth and hyphal filament production by *C. albicans* was enhanced by *S. oralis*. It is suggested that the interkingdom biofilms are metabolically coordinated to house all three components, and this study demonstrates that adhesive interactions between them determine spatial distribution and biofilm architecture. The physical and chemical communication processes occurring in these communities potentially augment *C. albicans* persistence at multiple oral cavity sites.

**Keywords:** human oral cavity; polymicrobial communities; colonization; FISH; coaggregation
INTRODUCTION

The pleiomorphic fungus *Candida albicans* colonizes the oral cavity, GI tract and genitourinary tract where it can remain benign, or cause superficial infection, or invade body tissues to become systemic (Poulain, 2015). Although invasive disease can originate from oral cavity colonization in healthy subjects, it is much more likely to occur in individuals who are immunocompromised either as a result of HIV infection or medical procedures (surgery, radiotherapy, etc.) (Delaloye and Calandra, 2014; Martins *et al.*, 2014). A common oral condition involving *C. albicans* is denture stomatitis (Figueira *et al.*, 2007). Dentures from subjects with stomatitis carry surface biofilms containing networks of hyphal filaments characteristic of *C. albicans* (Douglas, 2003). The biofilms also contain bacterial components (Campos *et al.*, 2008; Salerno *et al.*, 2011) and there is evidence that oral streptococci can enhance growth of *C. albicans* biofilms (Xu *et al.*, 2014a) and augment fungal invasion of oral mucosa (Diaz *et al.*, 2012; Xu *et al.*, 2014b). The presence of *C. albicans* in sub-gingival plaque (Canabarro *et al.*, 2013) and in periodontal disease lesions (Urzúa *et al.*, 2008; Al Mubarak *et al.*, 2013) suggests that the fungus may possibly also have a role in the progression of periodontal disease. Furthermore, *C. albicans* has been found in supra-gingival plaque (Zijnge *et al.*, 2010) and in carious lesions (Marchant *et al.*, 2001; de Carvalho *et al.*, 2006), indicating that interactions with cariogenic bacteria such as *Streptococcus mutans* could potentially be significant in tooth decay (Koo and Bowen, 2014).

In order to colonize the salivary glycoprotein-coated hard or soft surfaces present within the oral cavity, *C. albicans* has acquired a range of adhesins that interact with salivary components (Cannon and Chaffin, 1999; Chaffin, 2008; Nobbs *et al.*, 2010; Demuyser *et al.*, 2014). These include cell wall proteins (CWPs) Hwp1, Hwp2, Rbt1, Eap1, the Iff/Hyr family and the Als family (de Groot *et al.*, 2013). However, *C. albicans* is in competition for adhesion to salivary receptors with several hundred species of bacteria at any given oral cavity site (Jenkinson, 2011) and so interactions with bacteria that promote *C. albicans* colonization are important to define.
Mitis/sanguinis group streptococci and *Actinomyces* species (e.g. *oralis*, *naeslundii*, *johnsonii*) are amongst the first oral bacteria to colonize a fresh salivary glycoprotein-coated surface (Nyvad and Kilian, 1987; Al-Ahmad et al., 2009; Dige et al., 2009). Oral streptococci express a wide range of salivary pellicle adhesins (Nobbs et al., 2009; 2011), while *Actinomyces* type 1 fimbriae mediate interactions with salivary proline-rich proteins and statherin (Gibbons et al., 1988; Clark et al., 1989; Li et al., 2001). *Actinomyces* type 2 fimbriae on the other hand interact specifically with Galβ1-3GalNAc found within the cell surface receptor polysaccharides (RPS) of mitis-group streptococci (Yoshida et al., 2006b) and some salivary glycoproteins (Ruhl et al., 2004). The type 2 fimbriae therefore mediate coaggregation with streptococci expressing RPS (Cisar et al., 1983; Palmer et al., 2003), and also appear to be involved in biofilm formation (Mishra et al., 2010). In vitro studies have shown that mutualism in biofilm formation occurs between strains of *Streptococcus oralis* and *Actinomyces oris* (Palmer et al., 2001). Interactions between these early colonizing bacteria and *C. albicans* could thus impact on the kinds of communities that develop upon oral cavity tissues and prostheses.

Several species of oral streptococci can form mixed-species biofilms with *C. albicans* (Xu et al., 2014a). These biofilms are often more luxuriant than monospecies *C. albicans* biofilms and may contain a higher proportion of hyphal filaments (Dutton et al., 2014; Sztajer et al., 2014; Dutton et al., 2015). While *S. oralis* is able to facilitate invasion of oral mucosal epithelium by *C. albicans* (Diaz et al., 2012), concomitantly *S. oralis* growth is enhanced by the fungus. In addition, the pathogenic properties and invasive potential of streptococci bacteria can be augmented by *C. albicans* (Falsetta et al., 2014; Xu et al., 2014b). Much less is understood about how *Actinomyces* species interact with *C. albicans*, but coaggregation has been reported (Grimaudo et al., 1996; Arzmi et al., 2015), and there is evidence that *C. albicans* proliferation and biofilm development could be inhibited by *Actinomyces* species (Guo et al., 2015).

Microbial synergy in biofilm community development involves primary colonizing species adhering to oral cavity surfaces and thus increasing the availability of receptors for
adhesion by secondary colonizers (Wright et al., 2013). On the basis of studies showing synergy between *S. oralis* and *C. albicans* in biofilm formation (Diaz et al., 2012), and synergy between *S. oralis* and *A. oris* in biofilm formation (Palmer et al., 2001), we have investigated if these interactions are maintained or disrupted in three-species biofilms. The results here demonstrate that *C. albicans* assists incorporation of *S. oralis* and *A. oris* into biofilms independently of the coaggregation interactions between the bacteria.
METHODS

Microbial strains and growth conditions

Bacterial strains utilized were as follows: A. oris T14V (wild type) and A. oris T14V Fim- (mutant lacking type 1 and type 2 fimbriae) (Cisar et al., 1988); S. oralis 34 and S. oralis ΔwchA RPS- (receptor polysaccharide negative isogenic mutant) (Yoshida et al., 2006a).

Bacteria were cultivated anaerobically at 37 °C on BHYN agar (per liter: 37 g Brain Heart Infusion, 5 g yeast extract, 5 g Bacto-Neopeptone, 15 g agar). Suspension cultures were grown in BHY medium (Brain Heart Infusion medium containing 5 g l^-1 yeast extract), in sealed bottles or tubes, and incubated stationary at 37 °C. C. albicans SC5314 was cultivated aerobically on Sabouraud Dextrose (SD) agar (LabM, Heywood, Leics. UK) at 37 °C, and suspensions were grown in YMD medium (per liter: 20 g Oxoid Mycological Peptone, 10 g yeast extract, 20 g dextrose) in conical flasks at 37 °C with shaking (200 r.p.m.) (Dutton et al. 2014). For preparation of cells for experiments, cultures were grown for 16 h at 37 °C, centrifuged (5000 g for 7 min), the cell pellets were suspended and washed twice with YPT medium (1 x yeast nitrogen base, 20 mM NaH_2PO_4-H_3PO_4 buffer pH 7.0, 0.1% tryptone) (Silverman et al., 2010), and suspended in YPT medium at OD_600 1.0.

Bacterial coaggregation

A. oris cells were fluorescently labelled with FITC (fluorescein isothiocyanate) as described by Dutton et al. (2014) and S. oralis cells were labelled with 1.3 mM TRITC (tetrarmethylrhodamine-5 (and 6) isothiocyanate) in 0.05 M Na_2CO_3 containing 0.15 M NaCl for 30 min at 20 °C in the dark with gentle shaking. Bacterial cells were washed three times with YPT and suspended at OD_600 0.5 (2 x 10^8 cells ml^-1) in YPT medium. Equal volumes of bacterial cell suspension were mixed in a glass tube, vortex mixed for 1 min and the tubes allowed to stand for 30 min at room temperature. Samples were taken onto glass microscope slides and visualized by fluorescence microscopy (Leica DMLB).

Planktonic interactions of bacteria with C. albicans
C. albicans cell suspension at OD$_{600}$ 1.0 (approximately $1 \times 10^7$ cells ml$^{-1}$) (0.2 ml) was added to glass tubes containing 1.8 ml warm YPTG medium (YPT supplemented with 0.4% glucose) and incubated for 2 h at 37 °C with shaking (200 r.p.m.) to initiate hyphal filament formation. Fluorescently-labelled bacterial cell suspension (1 ml) was then added to C. albicans cell suspension (2 ml) and incubated for 1 h at 37 °C with gentle shaking. To each cell suspension was then added fluorescent brightener 28 (Calcofluor white, Sigma-Aldrich Co., St. Louis, MO) (1 µl of 0.2 mg ml$^{-1}$ stock solution) to fluoresce the C. albicans. Samples of suspension were applied to glass microscope slides and visualized by fluorescence microscopy (Leica DMLB) (Silverman et al. 2010).

Salivary pellicle formation

Collection of human saliva samples was approved by the National Research Ethics Committee South Central Oxford C (no. 08/H0606/87+5). Saliva was obtained from at least six healthy adult subjects, who provided written informed consent. The samples were pooled, treated with 0.25 M dithiothreitol on ice for 10 min, and centrifuged (8000 $g$ for 10 min). The supernatant was removed, diluted to 10% with sterile water, filter sterilized (0.45-µm pore-size membrane), and portions were stored at -20 °C. Salivary pellicle was formed on glass cover slips or in plastic culture dishes by incubating them with 10% saliva for 16 h at 4 °C.

Biofilm development on cover slips

Salivary glycoprotein-coated glass cover slips (19 mm diam.) were transferred to individual wells of a 12-well tissue culture plate containing 1.9 ml of YPTG medium. C. albicans cell suspension (0.1 ml) was added and incubated for 2 h at 37 °C, over which time fungal cells adhered to the substratum and began to form hyphal filaments. The unattached cells were then aspirated and, for monospecies biofilms, fresh medium (2 ml) was added and incubation continued. For dual species or three-species biofilms, the unattached fungal cells were aspirated, fresh medium (1 ml) was added together with S. oralis cell suspension in
YPTG (1 ml, ~2 x 10⁸ cells ml⁻¹) or A. oris cell suspension (1 ml, ~2 x 10⁸ cells ml⁻¹), or both added separately one immediately after the other, and incubated for 1 h at 37 °C. The bacterial cell suspensions were then removed, replaced with fresh YPTG medium (2 ml) and incubation was continued for up to 5 h. Coverslips were removed, rinsed gently with phosphate-buffered saline (PBS), dried, and stained with 0.5% crystal violet solution. The biofilms were then visualized by light microscopy (Leica DMLB). For biomass estimations the stain was released with 10% acetic acid and optical density at 595 nm (OD₅₉₅) was measured with a microtiter plate reader (Dutton et al., 2014).

**Biofilm development for confocal scanning laser microscopy (CSLM)**

Biofilms (3 ml final volume) were grown in glass-bottom microwell dishes (35 mm diam., 14 mm microwell) (MatTek Corporation, Ashland, Mass.) exactly as described above. For visualizing C. albicans within biofilms, 2 µg Calcofluor ml⁻¹ was incorporated into the YPTG growth medium at the end of the experiment for 10 min to fluorescently label the fungal cells. Biofilms containing single species of bacteria (with or without C. albicans) were fluorescently labelled with FITC (10 µM, 20 min) at the end of the experiments. The dishes were washed twice with PBS and the biofilms were visualized by CSLM with a Leica SP5-AOBS confocal microscope attached to a Leica DM I6000 inverted epifluorescence microscope.

Dual-species or three-species biofilms were also subjected to fluorescence in situ hybridization (FISH) analysis by CSLM. Biofilms grown in glass-bottom microwell dishes were fixed with 4% paraformaldehyde (2 h), washed twice in PBS and then incubated in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 8.0, 0.01% SDS, and 10-30% formamide depending upon the probe) containing 5 µg ml⁻¹ fluorescently-labelled 16 rDNA-specific oligonucleotide probe for 150 min at 55 °C. The oligonucleotide probe sequences were described by Thurnheer et al. (2004) and were labelled (Eurofins Genomic Services Ltd., Wolverhampton, UK) as follows: S. oralis probe MIT447_488 (Alexa 488), A. oris probe ANA103_647 (Alexa 647) and C. albicans EUK516_555 (Alexa 555). Following hybridization the cover slips were incubated in washing buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA,
0.01% SDS, and between 159 and 636 mM NaCl depending upon the formamide concentration used during hybridization) for 15 min at 55 °C. After rinsing briefly in 0.9% NaCl the cover slips were inverted onto a glass slide to be visualized by CSLM. Volocity® software was utilized to prepare three-dimensional (3D) images and Imaris® v7.5 software (Bitplane AG, Zurich, Switzerland) was used to calculate biovolumes (µm³).

Statistical analysis

Data were processed by Prism 6 software at a confidence level of 95% using one-way ANOVA followed by Tukey test respective to the biofilm combinations.

RESULTS

Planktonic interactions of A. oris and S. oralis

Coaggregation between various A. oris strains expressing type 2 fimbriae and S. oralis 34 has been well documented, and involves recognition of RPS on the surface of S. oralis by the type 2 fimbriae (Mishra et al., 2010). To confirm that A. oris T14V, expressing type 1 or type 2 fimbriae (Cisar et al., 1983), coaggregated with S. oralis 34, we performed dual-fluorescence coaggregation assays. The wild type strains formed a wide range of different sized coaggregates, some containing thousands of cells (Fig. 1A,B). An A. oris Fim- strain lacking type 1 and type 2 fimbriae did not coaggregate with S. oralis (Fig. 1C), and an S. oralis RPS- mutant did not interact with A. oris T14V or A. oris Fim- (Fig. 1D,E).

Planktonic interactions of A. oris, S. oralis and C. albicans

A. oris T14V and A. oris Fim- cells adhered to C. albicans hyphal filaments as single cells or clumps (Fig. 2A,B) and no specific regions of the filaments were targeted. Likewise, S. oralis 34 and S. oralis RPS- cells interacted with hyphae (Fig. 2C,D). The RPS- mutant strain formed shorter chains of cells than S. oralis wild type and more individual cells or cell pairs
adhered to the hyphal filaments. In three-species groupings, *A. oris* T14V and *S. oralis* 34 formed coaggregates as expected, and these coaggregates adhered to hyphae, in addition to single cell pairs or chains (Fig. 2E). Lack of fimbriae did not appear to affect the interaction of individual pairs or groups of *A. oris* Fim\(^{-}\) with *C. albicans* hyphae (Fig. 2F), or interaction of the accompanying *S. oralis* cells with hyphal filaments. *S. oralis* RPS\(^{-}\) cells also adhered to hyphal filaments (Fig. 2G) independently of *A. oris* T14V or Fim\(^{-}\) cells (Fig. 2H). A single hyphal filament was able to support adhesion of both actinomyces and streptococcal cells. Therefore the interactions of *A. oris* or *S. oralis* cells with *C. albicans* can occur independently of fimbriae or RPS, respectively, and there is seemingly no direct competition for binding sites on the candidal hyphae.

**Biofilm temporal development**

Development of monospecies biofilms or dual-species biofilms on salivary pellicle over 5-7 h was visualized by light microscopy. *C. albicans* cells that were adhered to the pellicle substratum had formed short hyphae at 2 h, and more extensive networks of hyphal filaments at 5 h and 7 h (Fig. 3A) as previously reported (Dutton et al., 2014). *A. oris* T14V cells formed a confluent biofilm by 5 h, but virtually no biofilm was formed by the *A. oris* Fim\(^{-}\) mutant (Fig. 3A), as reflected by biomass measurements (Fig. 4). When present in biofilms with *C. albicans*, *A. oris* T14V cells were relatively evenly distributed over the pellicle, as well as being associated with *C. albicans* hyphae (Fig. 3A). The *A. oris* Fim\(^{-}\) strain in this case formed a dual-species biofilm with *C. albicans* by adhering to the hyphal filaments. *S. oralis* 34 and RPS\(^{-}\) mutant both formed confluent monospecies biofilms by 5 h, but the RPS\(^{-}\) mutant biomass was significantly lower than strain 34 biomass (Fig. 4). In dual-species biofilms, the presence of the streptococci clearly stimulated formation of extensive networks of hyphal filaments (Fig. 3B). These results confirm that *A. oris* fimbriae and *S. oralis* RPS are not necessary for three-species biofilm formation with *C. albicans*, but that *A. oris* fimbriae are required for attachment to salivary pellicle in the absence of coaggregation partners.
Bacterial biofilm structure and architecture

Monospecies or dual-species bacterial biofilms developed on salivary pellicle were then analyzed by confocal scanning laser microscopy (CSLM). After 6 h, *A. oris* T14V produced a biofilm consisting of growth clusters (Fig. 5A), while the *A. oris* Fim\textsuperscript{−} mutant did not form a biofilm on pellicle (Fig. 5B), as previously noted (Fig. 3A). *S. oralis* 34 formed a dense biofilm of thickness ~15 µm that covered the pellicle surface uniformly (Fig. 5C), while the RPS\textsuperscript{−} mutant formed a more sparse and very much thinner biofilm (Fig. 5D). These images show detail not deduced from the light microscopy images of fixed and stained biofilms (Fig. 3). In dual-species bacterial biofilms the components were resolved by FISH. In *A. oris* T14V-S. *oralis* 34 biofilms, actinomyces and streptococci were interdigitated within a confluent biofilm (Fig. 5E). However, very little growth of the *A. oris* Fim\textsuperscript{−} mutant could be seen amongst *S. oralis* 34 (Fig. 5F) and biomass values of crystal violet-stained biofilms were significantly lower compared to dual species *A. oris* T14V-S. *oralis* 34 biofilms (Fig. 4). *A. oris* T14V formed luxuriant communities on the pellicle amongst the RPS\textsuperscript{−} streptococcal biofilm (Fig. 5G). There were no interspecies interactions occurring in these biofilms, in the absence of RPS, and the two strains coexisted side by side. In dual-species biofilms containing both mutants, no *A. oris* Fim\textsuperscript{−} cells were visible within the less dense biofilm of *S. oralis* RPS\textsuperscript{−} cells (Fig. 5H), and the biomass values of crystal violet-stained biofilms were significantly reduced compared to dual-species *A. oris* T14V-S. *oralis* 34 biofilms (Fig. 4).

Interkingdom biofilm architecture

In biofilms of the bacterial strains together with *C. albicans* the first thing to note is that, in the presence of *C. albicans*, all of the bacterial strains were able to form biofilms. *A. oris* T14V was well integrated with *C. albicans* with both organisms attached to the substratum (Fig. 6A), while *A. oris* Fim\textsuperscript{−} was also well established but not evidently bound to pellicle (Fig. 6B). Streptococcal strains covered the areas of pellicle not occupied by *C. albicans* to form basal layers (Fig. 6C,D), with the *C. albicans* hyphae emanating into the environment.
Biovolume measurements of these biofilms clearly show that A. oris Fim– is incorporated well into biofilms with C. albicans (Fig. 7A). In addition, while C. albicans dominated the overall biovolume in dual-species biofilms, it was possible to detect increase in biovolume of C. albicans when S. oralis 34 cells were present (Fig. 7A).

In three-species biofilms, C. albicans and streptococci always predominated. A. oris T14V and S. oralis 34 colonized the pellicle with the upper surface layers of the biofilms almost completely consisting of C. albicans (Fig. 6E). This occurs presumably as a result of dispersed C. albicans cells recolonizing the biofilm by adhering to S. oralis. Biovolume measurements suggested that ~25% of the volume comprised streptococci while actinomyces comprised <10% of total biovolume (Fig. 7B). In biofilms containing A. oris Fim–, cells of the afimbrial mutant were associated with the C. albicans upper layers over a denser streptococcal basal layer (Fig. 6F). In biofilms containing the S. oralis RPS– mutant, streptococci predominated within the basal layers together with clusters of A. oris T14V, similar to the effect observed in Fig. 5G, and there were fewer C. albicans (Fig. 6G).

Incorporation of A. oris T14V into C. albicans-S. oralis RPS– mutant biofilms on the basis of biovolume measurements was ~10-fold reduced (Fig. 7B) compared with C. albicans-S. oralis 34 biofilms. In three-species biofilms of S. oralis RPS–, A. oris Fim– and C. albicans, the A. oris Fim– mutant was least well-incorporated of all the strains (Fig. 7B) and was associated only with the top layer of C. albicans, while the S. oralis RPS– mutant formed a multi-layer biofilm on the pellicle substratum (Fig. 6H). Taken collectively the results suggest that A. oris, S. oralis and C. albicans are able to form robust polymicrobial biofilms.

Furthermore, C. albicans can promote inclusion of bacteria into a biofilm even when the bacteria themselves do not physically coaggregate, or when they are impaired in attachment to salivary pellicle.

DISCUSSION
S. oralis is an early colonizer of the oral cavity in newborns (Pearce et al., 1995) and of the salivary pellicle in dentate individuals (Diaz et al., 2006). Actinomyces spp. are also found amongst the early colonizers of pellicle and are prominent components of the oral mucosal microbiota (Ellen, 1976). Therefore the presence of these bacterial species will almost always be an important consideration for C. albicans colonization of the oral cavity. A better understanding of these intermicrobial mechanisms and their capacity to influence C. albicans growth and persistence in the oral cavity may help to devise novel strategies to combat the wide range of infections caused by C. albicans in humans of all ages.

It is well documented that S. oralis 34 produces a type 1Gn RPS with the repeating structure [-6Galβ1→6GalNAcβ1→3Galα1-PO4→6GalNAcα1→3Rhaβ1→4Glcβ1→], (Yoshida et al., 2006a), and that the GalNAcβ1-3Gal structure within the repeating unit is recognized by A. oris type 2 fimbriae (Cisar et al., 1988). This mediates interbacterial adhesion, and salivary flow biofilms of A. oris and S. oralis were diminished with type 2 fimbriae negative or RPS- mutants, respectively (Yoshida et al., 2006b). However, in other studies utilizing salivary flow cell biofilms it was shown that S. oralis 34 or A. oris T14V were able only to form dual-species biofilms. Neither strain alone could form a biofilm under salivary flow, unlike the results presented here. Moreover A. oris and S. oralis were deficient in planktonic growth in saliva. Therefore nutrient limitations in these experiments were overcome by forming dual-species biofilms (Palmer et al., 2001) and, under the conditions, interbacterial adhesion was essential for mutual metabolic benefit. By contrast, the experiments conducted in this current paper with a minimal growth medium under static conditions showed that RPS production by S. oralis was not essential for biofilm formation on salivary pellicle. However, biofilms of S. oralis RPS- and A. oris T14V were comprised of patches of actinomyces and streptococci, as opposed to being interdigitated as within the S. oralis-A. oris wild-type biofilms. Thus, when nutritionally-sufficient conditions and available pellicle receptors are present, the ability to employ interbacterial adhesion is not absolutely necessary. On the other hand, afimbrial mutant strain A. oris Fim- was unable to form a monospecies biofilm on salivary pellicle, and unable to form a mixed species biofilm with S.
oralis 34 or RPS, consistent with the absence of both types of fimbriae. The S. oralis RPS-biofilms were patchy and much thinner than wild-type, and so the RPS plays a role in cell-cell interactions and biofilm formation by S. oralis 34.

Interestingly, we did notice some interbacterial coaggregation of A. oris Fim- and S. oralis 34 in the presence of C. albicans (Fig. 2). It is possible that there are additional coaggregation mechanisms between A. oris T14V and S. oralis 34 that are secondary to the fimbriae-RPS interaction. For example, we have recently shown that the S. gordonii SspB (antigen I/II-family) protein binds to a polysaccharide receptor present on A. oris T14V (Back et al., 2015). S. oralis 34 also carries an antigen I/II protein-encoding gene, and so the S. oralis antigen I/II protein might act similarly. In addition, A. oris T14V carries cell-surface sialic acid (Jones et al., 1986), which could be a target for other S. oralis adhesins such as serine-rich repeat proteins (Zhou and Wu, 2009).

Wild-type and mutant strains of A. oris or S. oralis were all able to bind C. albicans hyphal filaments. Fimbriae or RPS were clearly not requirements for interkingdom adhesion. Typically the bacteria, present as single cells, groups or coaggregates, bound to various sites along the C. albicans filaments. It is thought that C. albicans adhesins become clustered as nanodomains following their activation (Lipke et al., 2012) and so these sites possibly represent adhesin clusters. The interaction of S. gordonii cells with C. albicans involves the streptococcal antigen I/II protein SspB binding to Als3 protein present on hyphal filaments (Silverman et al., 2010). Again, since S. oralis 34 contains an antigen I/II family protein-encoding gene, it is possible that this protein drives the S. oralis-C. albicans interaction. Further work generating mutants and heterologous protein-expression strains would be required to confirm this. Als3 appears to be receptor for a range of different bacteria (Demuyser et al., 2015) and could also be the target of A. oris. However, potential adhesins of A. oris aside from type 1 and type 2 fimbriae are currently unknown.

A. oris and S. oralis strains, wild type and mutants, were all able to form biofilms with C. albicans. The afimbriate mutant of A. oris was incorporated via interaction with C. albicans, since A. oris Fim- cells were unable to adhere to pellicle (Fig. 5). The S. oralis RPS-
mutant formed thicker biofilms in the presence of *C. albicans* than in monospecies (Fig. 3 and Fig. 5). One explanation for this is that *C. albicans* directly provides additional nutrients or more conducive environmental conditions for the streptococci. Alternatively, it is possible that RPS is normally an additional nutrient source for *C. albicans* thus providing the fungus with a growth advantage. A wide range of hydrolases are produced by *C. albicans* in the presence or absence of streptococci (Polacheck *et al.*, 1987; Molloy *et al.*, 1994; Bramono *et al.*, 1995; Dutton *et al.*, 2015) and so the RPS could be broken down into sugar residues for uptake by the fungus.

In summary, *C. albicans* and *S. oralis* provide sites on pellicle for colonization by *A. oris*. In the absence of fimbriae production, *A. oris* is compromised in coaggregation with *S. oralis* (via type 2 fimbriae) and in adherence to salivary pellicle (via type 1 fimbriae), but is nevertheless incorporated into biofilms via binding to *C. albicans*. When *C. albicans* and *S. oralis* are mainly occupying pellicle binding sites, the *A. oris* cells tend to be found in the upper layers of the biofilm. Biofilm growth and hyphal filament production by *C. albicans* is enhanced by *S. oralis*. Under these conditions, the bacterial cells mainly occupy the pellicle binding sites and form a lower biofilm layer, while *C. albicans* forms the upper biofilm layers.

We did not detect inhibition of *C. albicans* by *A. oris* in dual-species biofilms (Fig. 6) that has recently been shown for three *Actinomyces* species (Guo *et al.*, 2015). In fact there was growth synergy between *A. oris* and *C. albicans*, and between *S. oralis* and *C. albicans*, resulting in augmentation of total biofilm biomass in each case. We acknowledge that this present study utilized only three specific strains of microorganisms, but our results may be applicable generally to streptococci expressing RPS with Galβ1-3GalNAc or GalNAcβ1-3Gal linkages that are recognized by *Actinomyces* type 2 fimbriae. There are many other coaggregation groupings of *Streptococcus* and *Actinomyces* (Kolenbrander *et al.*, 1983) that remain to be explored in the context of biofilm formation with *C. albicans*. The three-species biofilms that we describe here are thus able to become metabolically coordinated, with the adhesive interactions between the components determining their spatial distribution and overall architecture. Clearly *C. albicans* is well endowed with the ability to form a biofilm
directly on salivary pellicle, or to benefit from two early colonizing bacterial species forming a biofilm into which the fungus can be incorporated and thrive. Since *A. oris* and *S. oralis* are major colonizers of the buccal mucosa, they may also enhance *C. albicans* retention and growth at multiple oral cavity sites.

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The authors declare no conflict of interest


Figure legends

Figure 1 Fluorescence micrographs of planktonic interactions between S. oralis and A. oris wild types and mutant strains. Streptococci were labelled with TRITC (red) and actinomyces with FITC (green), vortex-mixed and incubated for 1 h as described in Material and Methods. Very large aggregates of S. oralis 34 and A. oris T14V developed containing thousands of mixed species cells (Panel A) and smaller aggregates were composed of similar numbers of the two cell types (Panel B). There was no coaggregation observed between S. oralis 34 and A. oris Fim⁻ (Panel C), S. oralis 34 RPS⁻ and A. oris T14V (Panel D), or S. oralis RPS⁻ and A. oris Fim⁻ (Panel E). Scale bars = 20 µm.

Figure 2 Fluorescence micrographs of A. oris and S. oralis wild types or mutant strains with C. albicans. In Panels A-D, bacteria have been labelled with FITC (green) and C. albicans with Calcofluor (blue). A. oris T14V (Panel A) and A. oris Fim⁻ (Panel B) bound to C. albicans as single cells (arrow a) or clumps (arrow b) to hyphae at no specific site. Likewise, S. oralis 34 cells were often in chains (arrow c) that interacted with hyphae (Panel C), as did cell pairs of S. oralis RPS⁻ (Panel D). The RPS⁻ mutant strain formed shorter chains of cells than wild type and more individual cells or cell pairs adhered to the hyphal filaments (arrow d). In three-species experiments (Panels E-H), S. oralis was labelled with TRITC (red), A. oris with FITC (green) and C. albicans with Calcofluor (blue). Wild type strains of A. oris and S. oralis formed coaggregates (Panel E) that adhered to hyphae (arrow e), in addition to single cell pairs or chains (arrow a). Lack of fimbriae did not appear to affect the interaction of individual pairs or groups of A. oris (arrow a) or S. oralis (arrow c) with hyphal filaments (Panel F). Neither did lack of RPS in S. oralis (Panel G) appear to affect streptococcal interactions with hyphal filaments (arrow a), but interestingly there were a few coaggregates with A. oris observed in these experiments (arrow f). Cells of S. oralis RPS⁻ (arrow a) and A. oris Fim⁻ (arrow b) were both able to interact with hyphae (Panel H) but as expected there was no bacterial coaggregation observed. Scale bars = 40 µm.
Figure 3 Light micrographs of dual-species biofilms stained with crystal violet. In biofilms containing *C. albicans*, fungal cells were incubated at 37 °C with the pellicle surface for 2 h, removed and fresh medium was added, with or without bacteria, for 1 h. The medium was then removed, replaced by fresh medium, and biofilms were incubated for up to 7 h. For monospecies bacterial biofilms, bacterial cells were incubated with pellicle for 1 h, removed and replaced with fresh medium, and then incubated for up to 5 h. Panel A, *C. albicans* and *A. oris*. Note the following: there was virtually no biofilm formed by *A. oris* Fim- after 5 h; the presence of *C. albicans* reduced the biofilm coverage by *A. oris* T14V at 7 h; conversely, *C. albicans* provided receptors for biofilm retention of *A. oris* Fim- (inset bottom right hand panel shows coaggregates of *C. albicans* and *A. oris* Fim-). Panel B, *C. albicans* and *S. oralis*. Note the following: there was similar overall coverage of pellicle by *S. oralis* 34 and *S. oralis* RPS- at 5 h, but strain 34 formed more clusters; hyphal filament formation by *C. albicans* was greatly enhanced by *S. oralis* 34 and *S. oralis* RPS- at 7 h. Scale bars = 50 µm.

Figure 4 Biomass values for monospecies or dual-species biofilms grown on salivary pellicle for 6 h at 37 °C. Biofilms were stained with crystal violet which was then released with 10% acetic acid for biomass quantification (OD$_{595}$). Error bars are ± SD for triplicates over two independent experiments (n = 2).

Figure 5 CSLM images of monospecies or dual-species biofilms formed on salivary pellicle after 6 h at 37 °C. Biofilm images are presented in 3D (xyz) and side (xz) views. Panels A-D, bacteria were stained with FITC (green). Panels: A, *A. oris* T14V; B, *A. oris* Fim-; C, *S. oralis* 34; D, *S. oralis* RPS-. *A. oris* T14V produced a biofilm consisting of clusters of growth (A) while the *A. oris* Fim- mutant did not form a biofilm on pellicle (B). Note in (C) that the pellicle surface is covered uniformly by *S. oralis* 34, while the RPS- mutant (D) showed a patchy distribution and a very much thinner biofilm. Panels E-H, dual-species biofilms of *A. oris* (green) and *S. oralis* (red) detected by FISH. Panels: E, *A. oris* T14V + *S. oralis* 34; F, *A. oris* Fim- + *S. oralis* 34; G, *A. oris* T14V + *S. oralis* RPS-; H, *A. oris* Fim- + *S. oralis* RPS-. In (E) the actinomyces and streptococci are interdigitated within a confluent biofilm, while in (F)
few A. oris Fim- cells are visible. In (G), A. oris T14V grows luxuriantly in communities amongst the RPS-streptococci, while in (H) no A. oris Fim- cells are visible within the sparser biofilm of S. oralis RPS mutant. Scale bars = 50 µm.

Figure 6 CLSM images of dual- or three-species biofilms of A. oris and S. oralis wild type and mutant strains with C. albicans. Biofilms were grown in YPTG medium as described in Materials and Methods for 6 h at 37 °C. All biofilms contained C. albicans. In Panels A-D, bacteria are stained green, C. albicans in red. Panels: A, A. oris T14V; B, A. oris Fim-; C, S. oralis 34; D, S. oralis RPS-; Note the distribution of C. albicans and A. oris within the layers of the biofilms (A), and in (B) where A. oris Fim- is clearly incorporated, the predominance of C. albicans within the layer in contact with pellicle. Two distinct cellular layers were seen in combinations of Candida with streptococci (C and D). Panels E-H, three-species biofilms of S. oralis (red), A. oris (green) and C. albicans (blue) detected by FISH. Panels: E, A. oris T14V + S. oralis 34; F, A. oris Fim- + S. oralis 34; G, A. oris T14V + S. oralis RPS-; H, A. oris Fim- + S. oralis RPS-. Note that the wild type strains of bacteria colonized the pellicle together, with hyphal filaments of C. albicans predominantly in the upper layers (E). A. oris Fim- cells were only barely visible in (F) while cells of A. oris T14V colonized the pellicle substratum in (G). C. albicans enabled the A. oris Fim- strain to become incorporated into the biofilm with S. oralis RPS-. Scale bars = 50 µm.

Figure 7 Biovolume measurements of individual components of monospecies or dual-species biofilms (panel A) and a three-species biofilm (Panel B), as shown in Figures 5 and 6. Since these are biovolume measurements from the individual biofilms there are no error bars. 3D Micrograph of C. albicans SC5314 monospecies biofilm is not reproduced in this paper as has been published elsewhere (Dutton et al., 2014). Fluorescence was visualized by CSLM and quantified using Imaris® Bitplane software. Ca, C. albicans; Ao, A. oris; So, S. oralis.
Figure 1
Figure 2
Figure 4
Figure 7