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Streptococcus gordonii comCDE (competence) operon modulates biofilm formation with *Candida albicans*

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Candida albicans is a pleiomorphic fungus that forms mixed species biofilms with *Streptococcus gordonii*, an early colonizer of oral cavity surfaces. Activation of quorum sensing (QS; intercellular signalling) promotes monospecies biofilm development by these micro-organisms, but the role of QS in mixed species communities is not understood. The *comCDE* genes in *S. gordonii* encode a sensor–regulator system (ComDE), which is activated by the *comC* gene product (CSP, competence stimulating peptide) and modulates expression of QS-regulated genes. Dual species biofilms of *S. gordonii* Δ*comCDE* or Δ*comC* mutants with *C. albicans* showed increased biomass compared to biofilms of *S. gordonii* DL1 wild-type with *C. albicans*. The Δ*comCDE* mutant dual species biofilms in particular contained more extracellular DNA (eDNA), and could be dispersed with DNase I or protease treatment. Exogenous CSP complemented the *S. gordonii* Δ*comC* transformation deficiency, as well as the Δ*comC*-*C. albicans* biofilm phenotype. Purified CSP did not affect *C. albicans* hyphal filament formation but inhibited monospecies biofilm formation by *C. albicans*. The results suggest that the *S. gordonii comCDE* QS-system modulates the production of eDNA and the incorporation of *C. albicans* into dual species biofilms.

INTRODUCTION

Streptococci are major constituents of the oral microbiome (Dewhirst *et al*., 2010) and mitis group streptococci, such as *Streptococcus gordonii*, *Streptococcus oralis* and *Streptococcus mitis*, are prominent amongst early-stage colonizers of the oral cavity (Nyvad & Kilian, 1990). These species of streptococci interact with a range of other oral micro-organisms, thus establishing a foundation for the building of biofilm communities (Wright *et al*., 2013). *S. gordonii* forms biofilms with *Porphyromonas gingivalis* (Lamont *et al*., 2002), *Actinomyces oris* (Palmer *et al*., 2003), *Fusobacterium nucleatum* (Foster & Kolenbrander, 2004) and *Aggregatibacter actinomycetemcomitans* (Liu *et al*., 2011) in dual or mixed species systems, and also produces dual species biofilms with the fungus *Candida albicans* (Bamford *et al*., 2009; Dutton *et al*., 2014).

C. albicans is a pleiomorphic fungus that can colonize mucosal surfaces and prosthetic materials (e.g. dentures and catheters) throughout the human body. The incidence of candidiasis continues to expand, and *Candida* is the fourth leading cause of nosocomial bloodstream infections in the USA (Wisplinghoff *et al*., 2004). In the UK, there was an increase in candidiasis infections of 37 % in the period 2003–2007, and *C. albicans* is reported to cause 48 % of all *Candida*-related bloodstream infections (Public Health England, 2013). The interactions of oral streptococci with *C. albicans* are of particular interest because there is evidence that the bacteria can enhance biofilm formation by *C. albicans* (Xu *et al*., 2014a) and fungal pathogenicity (Xu *et al*., 2014b). *S. gordonii* cells adhere to hyphal filaments of *C. albicans* via cell surface adhesin SspB interacting with hyphal cell wall protein Als3 (Nobbs *et al*.,

Abbreviations: CSP, competence stimulating peptide; eDNA, extracellular DNA; EPS, extracellular polymeric substance; QS, quorum sensing; RFP, red fluorescent protein; sCSP, scrambled CSP; Sp, spectinomycin.

Three supplementary figures and two supplementary tables are available with the online Supplementary Material.

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2010; Silverman *et al.*, 2010). The SspB–Als3 interaction is involved in biofilm development in which bacteria and fungi cooperate at the cellular and molecular level to generate a robust mixed microbial community (Whitmore & Lamont, 2011). Community composition may be regulated by extracellular signalling molecules such as autoinducer-2 (AI-2) (McNab *et al.*, 2003; Bamford *et al.*, 2009), farnesol (Bamford *et al.*, 2009; Lu *et al.*, 2014), *N*-acetyl-D-glucosamine (Naseem *et al.*, 2011) and homoserine lactones (Hogan *et al.*, 2004). In addition, the quorum-sensing (QS) molecule, competence stimulating peptide (CSP), produced by *Streptococcus mutans* inhibits hypha formation by *C. albicans* (Jarosz *et al.*, 2009). Paradoxically, *C. albicans* induces the CSP associated QS-system of *S. mutans* in dual species biofilms (Sztajer *et al.*, 2014).

The induction of competence for DNA uptake and transformation in *S. gordonii* involves a complex regulatory network similar to that in *Streptococcus pneumoniae* (Vickerman *et al.*, 2007) and shares some similarities to that in *S. mutans* (Son *et al.*, 2012). Competence regulation in these organisms employs a ComABCDE system that generates the CSP (*comC* gene product). In *S. gordonii*, pre-CSP is a 50 aa polypeptide that is cleaved at a double glycine to generate the mature 19 aa residue CSP (Håvarstein *et al.*, 1996). This peptide is then exported out of the cell via an ABC-binding cassette transporter encoded by *comA* and *comB*. Once a critical concentration of environmental CSP is reached, it is detected by the two-component system ComDE, comprising a membrane-bound histidine kinase (ComD), which phosphorylates the intracellular response regulator ComE (Håvarstein *et al.*, 1996). ComE activates a signalling cascade that includes upregulation of the master regulator SigX (designated ComR in *S. gordonii*; Heng *et al.*, 2006), an alternate sigma factor that controls expression of late competence genes encoding DNA uptake and recombination machinery (Lee & Morrison, 1999; Piotrowski *et al.*, 2009).

In this paper we investigated the role of competence induction and CSP formation by *S. gordonii* in the formation of dual species biofilms with *C. albicans*. We show that competence for transformation is a factor playing an important role in the development of early-stage biofilms of *S. gordonii* and *C. albicans*. In addition, evidence is presented showing that CSP produced by *S. gordonii* inhibits biofilm formation by *C. albicans*; therefore, CSP could normally act as a check on *C. albicans* load in dual species biofilms.

METHODS

Strains and growth conditions. Strains and plasmids utilized are listed in Table S1 (available in the online Supplementary Material). *S. gordonii* wild-type strain DL1 (Challis) and isogenic mutants were cultured in BHY medium [Brain Heart Infusion Broth (LabM) containing 5 g yeast extract l^{-1}] under stationary conditions in a candle jar at 37 °C. *C. albicans* strains were maintained on Sabouraud glucose agar (LabM) and suspension cultures were grown in YPD medium [10 g yeast extract l^{-1}, 20 g neopeptone l^{-1}, 2 % (w/v) glucose] at 37 °C with vigorous aeration. A defined medium (YPT-Glc) supported the growth of *S. gordonii* and *C. albicans* and comprised 20 mM NaH_2PO_4 (pH 7.0), 1× yeast nitrogen base, 1 g Bactotryptone l^{-1} and 0.4 % (w/v) glucose (Dutton *et al.*, 2014). Antibiotics were used at the following concentrations: 100 μg ampicillin ml^{-1}; 100 μg spectinomycin (Sp) ml^{-1}; 5 μg erythromycin ml^{-1}. Competence development by *S. gordonii* was induced in BHY medium containing 1 % fetal calf serum and 0.1 % (w/v) glucose according to Haisman & Jenkinson (1991). Exogenous synthetic CSP (DVRSNKIRLWWENIFFNKK) was included where appropriate in the concentration range 0.1–10 μg CSP ml^{-1}.

Generation of the *S. gordonii* Δ*comCDE* mutant. Deletion of the entire competence locus in *S. gordonii* was achieved by allelic exchange with spectinomycin resistance cassette *aad9*. A schematic representation of the mutagenesis strategy is shown in Fig. S1. In brief, a chromosomal DNA fragment (540 bp) immediately upstream of *comC* and a fragment (603 bp) downstream from *comE* were amplified by PCR with the primer pairs comCDE.F1/comCDE.R1 and comCDE.F2/comCDE.R2 (Table S2), respectively, using Expand Long Template PCR System (Roche). The flanking sequences were ligated by PCR, generating an amplimer (*comCDE*flank) with a central unique *Bam*HI site that was cloned into pGEM-T (Promega) in *E. coli* JM109. The *aad9* cassette with its own promoter and transcription terminator (1082 bp) was amplified from pFW5 (Podbielski *et al.*, 1996) with terminal *Bam*HI sites and cloned into the unique *Bam*HI site within the vector pGEM-T+*comCDE*flank. The resulting construct (pGEM-T+*comCDE*flank–*aad9*; 2272 bp) was confirmed by restriction enzyme digestion (*Sac*I and *Pst*I) and DNA sequencing. The *comCDE*flank–*aad9* fragment was then amplified by PCR and transformed into *S. gordonii* with selection for Sp resistance. Transformants were screened by PCR with the primer pair comCDE.F1/comCDE.R2 and a representative transformant, confirmed by DNA sequencing of the chromosomal PCR product, was designated UB2346.

Generation of the Δ*comC* mutant. In a similar way as described above, flanking DNA fragments of *comC* were prepared by PCR with the primer pairs comCDE.F1/comC2.R1 (690 bp) and comC2.F2/comC2.R2 (626 bp) (Fig. S2 and Table S2). The *aad9* cassette (devoid of promoter and transcriptional terminator sequence) was PCR-amplified from the pFW5 vector using the primers aad9.2F and aad9.2R (752 bp) (Table S2). The *comC* flanking fragments and *aad9* cassette were joined together by overlapping PCR using the primers comCDE.F1 and comC2.R2 (Fig. S2). The resulting amplimer (*comC*flank–*aad9*; 2114 bp) was cloned into the pGEM-T vector in *E. coli* and confirmed by sequencing. The fragment was then reamplified, transformed into *S. gordonii* and Sp-resistant transformants were screened by PCR using the primer pair comCDE.F1/comC2.R2 and identified based on size of the PCR product (2069 bp compared to 1469 bp in wild-type strain). A representative transformant was selected, the chromosomal region sequenced to confirm authenticity, and the strain was designated UB2660.

Preparation of saliva. Unstimulated whole saliva was collected from a minimum of six healthy adults who provided written informed consent, as approved by the National Research Ethics Committee Central Oxford C (#08/H0606/87+5). Saliva samples were pooled on ice, treated with 2.5 mM DTT for 10 min and then centrifuged to clarify (10 000 ***g***, 10 min). The supernatant was removed, diluted to 10 % with distilled water and filter-sterilized through a 0.45 μm nitrocellulose membrane.

Preparation of microbial cells. *S. gordonii* strains were grown in BHY medium for 16 h at 37 °C. Cells were harvested by centrifugation (5000 ***g***, 10 min), suspended in YPT-Glc and adjusted to

OD_{600} 0.05 (2.0×10^7 cells ml^{-1}). *C. albicans* was grown in YPD for 16 h at 37 °C, cells were harvested by centrifugation (5000 ***g***, 5 min), and suspended in YPT-Glc at OD_{600} 0.1 (1×10^6 cells ml^{-1}).

Biofilm formation. Sterile glass coverslips (13 or 19 mm diameter) in wells of 24- or 12-well polystyrene plates (Greiner) were incubated with 10 % saliva for 16 h at 4 °C. Saliva was removed and the coverslips were washed twice with PBS [0.01 M K_2HPO_4-KH_2PO_4 (pH 7.0), 2.7 mM KCl, 0.137 M NaCl]. For monospecies biofilms, cells (0.5 or 1 ml *S. gordonii* or *C. albicans*) were added to wells containing saliva-coated coverslips and incubated for 1 h at 37 °C with gentle agitation (50 r.p.m.) in a humid environment. Non-adhered cells were removed, fresh YPT-Glc (0.5 or 1 ml) was added to each well and biofilms were grown for a further 6 h. Coverslips were then recovered, washed twice with PBS and air-dried. For dual species biofilms, *C. albicans* cells (0.5 or 1 ml) were added to wells of 24- or 12-well plates containing saliva-coated coverslips, and the plates were incubated for 1 h at 37 °C. Non-adherent *C. albicans* were then removed by aspiration, *S. gordonii* cells (0.5 or 1 ml accordingly) were added and the plates were incubated again for 1 h at 37 °C. Non-adhered cells were then removed from the wells, fresh YPT-Glc (0.5 or 1 ml) was added to each well and plates were incubated for a further 6 h. Coverslips were then removed, washed with PBS and air-dried.

Biofilm assays. Biofilms were stained with 0.1 % safranin, washed with distilled water until excess stain was removed and air-dried. Coverslips were then inverted and mounted onto microscope slides, and biofilms visualized with a Leica DMLB light microscope with attached colour view camera, using CellD imaging software (Olympus Soft Imaging Solutions). Biomass quantification required release of safranin stain with 10 % acetic acid for 15 min, transfer of 0.1 ml portions to a 96-well plate and measurement at A_{490} (Lembke *et al.*, 2006) with an iMark microplate reader (Bio-Rad). All studies were performed in triplicate and mean biomass levels calculated from three independent experiments.

To estimate the numbers of *S. gordonii* cells present in dual species biofilms the biomass was removed from the coverslip with a cell scraper into YPT-Glc medium, the coverslip was vortex mixed in PBS and the suspensions were combined. The suspension was serially diluted 10-fold in YPT-Glc and plated onto BHYN agar containing 30 μg nystatin ml^{-1} (to prevent growth of *C. albicans*). Plates were incubated for 24 h at 37 °C and colonies were counted to estimate *S. gordonii* c.f.u. per biofilm.

For enzyme treatment of biofilms, coverslips with deposited biofilms were incubated with 100 U lyticase ml^{-1}, 4.2 U proteinase K ml^{-1}, 100 U DNase I ml^{-1} or 0.002 U neuraminidase ml^{-1} for 1 h at 37 °C. Activity of neuraminidase was confirmed with 2-*O*-(*o*-nitrophenyl)-α-D-*N*-acetylneuraminic acid substrate (Brittan *et al.*, 2012). No enzyme and heat-inactivated (30 min at 80 °C) enzyme controls were included. Biofilms were then estimated for biomass with safranin stain or examined by CLSM as described below.

To measure cell-free DNA, biofilms were scraped from coverslips as described above into 0.5 ml TE buffer (10 mM Tris/HCl, pH 7.5 and 1 mM EDTA), centrifuged (10 000 ***g***, 5 min) and supernatant DNA concentration was calculated from A_{260} measurements using a UV–Vis spectrophotometer (Shimadzu). Nucleic acid purity was indicated by the A_{260}/A_{280} value, where a ratio between 1.7–2.0 was accepted as pure.

S. gordonii CSP (DVRSNKIRLWWENIFFNKK) was synthesized commercially (GenicBio) along with a scrambled CSP (sCSP; DKRFKWWILKVFNSNEINR) with identical amino acid residue composition but random sequence. To assess the effects of these peptides on *C. albicans* biofilms or on dual species biofilms, YPT-Glc medium was supplemented with up to 10 μg peptide ml^{-1}. Biomass values for non-attached cells were calculated by collecting the planktonic phases into microcentrifuge tubes, harvesting by centrifugation (5000 ***g***, 7 min) and mixing the pellet with 0.002 % safranin. After 5 min the cells were centrifuged, washed several times in PBS and then suspended in 10 % acetic acid to solubilize the stain. The suspensions were clarified by centrifugation and then A_{490} measurements were utilized to determine relative biomass.

CLSM. Biofilms on coverslips were fixed with 0.5 ml paraformaldehyde (4 %) in PBS for 1 h at room temperature. Coverslips were rinsed with PBS and stained with FITC for 30 min in the dark (Dutton *et al.*, 2014). In some experiments, *C. albicans* SBC156, derivative of SC5314 expressing red fluorescent protein (RFP) (Milne *et al.*, 2011) was utilized. Biofilms were visualized using a Leica TCS-SP5 confocal imaging system attached to a Leica DMIRBE inverted microscope. Images were observed using ×40 or ×63 oil immersion objective lens with 488 nm excitation wavelength to excite FITC or at 561 nm to excite RFP. Z-slices were obtained every 0.5 μm. The data were analysed using Volocity image analysis software (Improvision).

Wide field microscopy. To visualize extracellular DNA (eDNA), biofilms were fixed with paraformaldehyde as described above, washed twice with PBS and blocked for 45 min with 2 % BSA in PBS. Biofilms were incubated for 45 min with mouse anti-dsDNA antibody (1 : 1000 dilution; Abcam), washed with 0.5 % BSA in PBS and then incubated for 45 min in the dark with goat anti-mouse Alexa594 (1 : 1000 dilution; Life Technologies). Biofilms were washed and then the coverslips were placed face down onto Vectashield mounting medium on a glass slide. Phase-contrast and Alexa 594 fluorescence images were captured using a Leica DMI6000 microscope with a ×40 (NA 1.25) oil immersion lens, DFC365FX CCD camera and Leica acquisition software. For all microscopy studies images were recorded from at least five fields taken at random.

Statistics. Data were analysed using GraphPad Prism, version 5.0. All data are presented as the mean ± SD of at least three independent experiments. For normally distributed data, comparisons were tested with Student's *t*-test. The two-tailed Mann–Whitney U-test was used for comparisons between groups. P values <0.05 were considered statistically significant.

RESULTS

Effects of competence (*comCDE*) operon deletion on early biofilm formation

The effects of *comC* or *comCDE* deletions on biofilm formation have been investigated in *S. mutans* (Li *et al.*, 2002), but not in *S. gordonii*. Biofilms of Δ*comC* or Δ*comCDE* mutants produced after 6 h incubation were compared with those produced by wild-type *S. gordonii* DL1 (Fig. 1). The streptococcal strains each formed a contiguous layer of cells over the surface of each of the coverslips, but the *com* operon mutant biofilms had more compact morphologies. Dual species biofilms of *S. gordonii* and *C. albicans* generally had denser morphologies and increased biomass compared to the single species biofilms (Fig. 1) (Bamford *et al.*, 2009). The Δ*comC* and Δ*comCDE* mutant strains had significantly increased dual species biofilm biomass values compared to wild-type (Fig. 1). In the Δ*comC*–*C. albicans* biofilms, the hyphal filaments appeared to be clustered (Fig. 1).

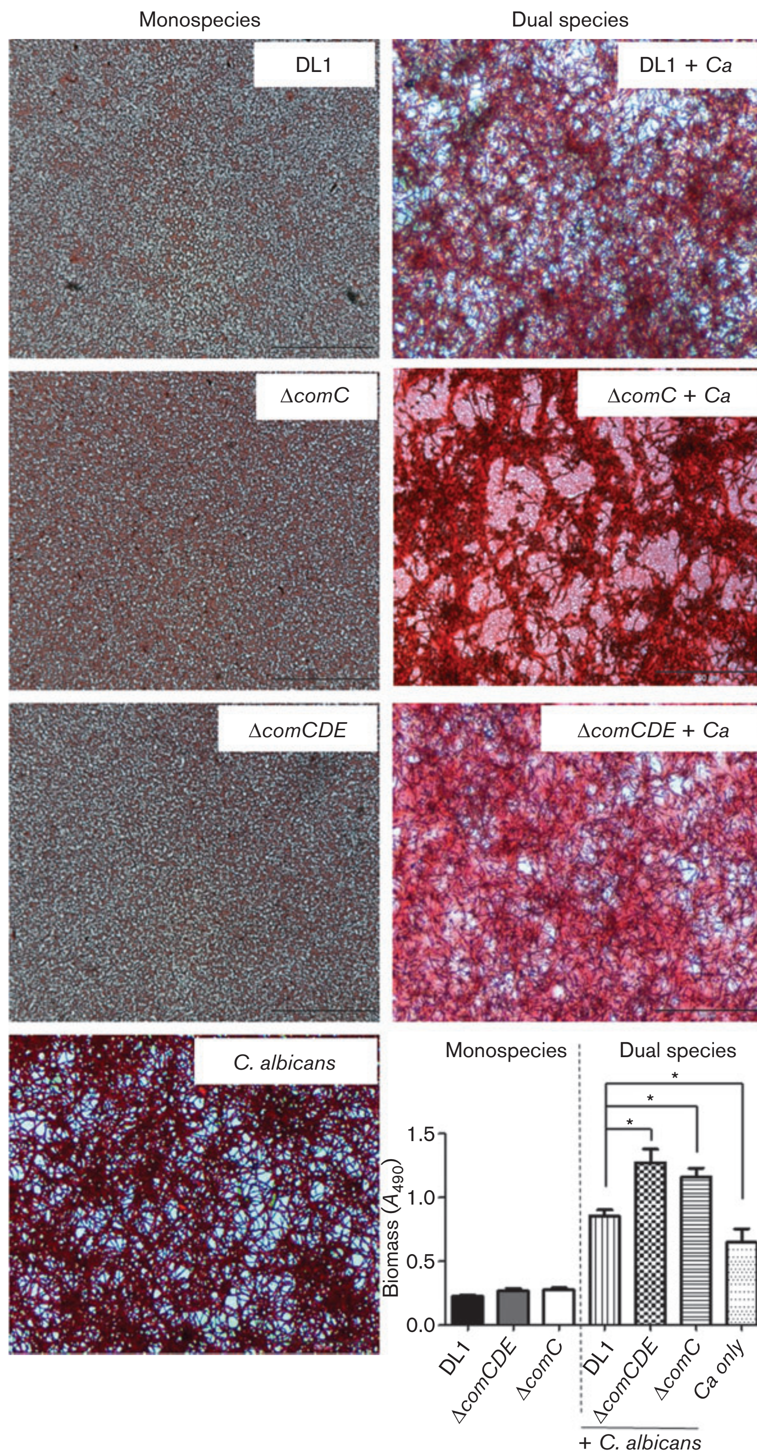

Fig. 1. *S. gordonii* monospecies or dual species biofilms with *C. albicans*. Biofilms were grown on saliva-coated coverslips for 6 h, safranin-stained and viewed by transmitted light microscopy. Biomass measurements (A_{490}) were taken following release of stain with acetic acid. DL1, *S. gordonii* wild-type; *Ca*, *C. albicans*. *$P<0.05$ ($n=3$). Bar, 50 μm.

In *S. gordonii* monospecies biofilms, the Δ*comC* and Δ*comCDE* mutants both formed more closely knitted mats than *S. gordonii* wild-type (Fig. 2). Biofilm depth (thickness) was measured as 5.5–6.0 μm for *S. gordonii* monospecies biofilms. In dual species biofilms for CLSM we utilized a *C. albicans* SC5314 derivative expressing RFP (Table S1). In these biofilms the salivary pellicle surface was colonized by *S. gordonii* and a network of *C. albicans* hyphal filaments (Fig. 3a). By comparison with monospecies *C. albicans* biofilms (Fig. 3d), more true hyphae or longer hyphal filaments were observed in the dual species biofilms. The *S. gordonii* Δ*comC* and Δ*comCDE* mutants formed denser dual species biofilms compared to *S. gordonii* wild-type biofilms (Fig. 3b, c) and the Δ*comCDE*–*C. albicans* biofilm in particular produced matrix (Fig. 3c). By subtracting the green (FITC) channel using the imaging

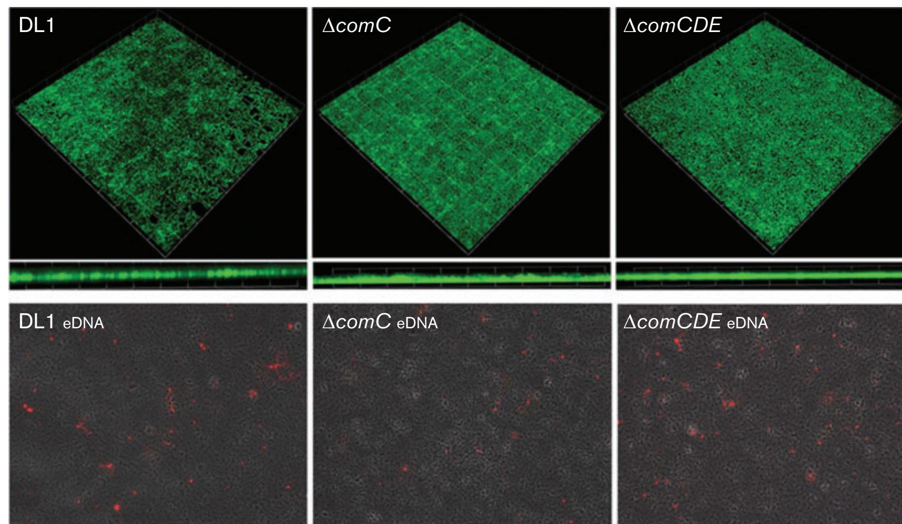

Fig. 2. Architecture and eDNA deposition patterns of 6 h monospecies biofilms of *S. gordonii* DL1 wild-type, Δ*comC* mutant and Δ*comCDE* mutant. Uppermost panels show CLSM 3D (*xyz* stack) and *xz* (thickness) images of biofilms stained with FITC. Maximum thickness (depth) measurements for a representative biofilm were: *S. gordonii* DL1, 6.6 μm; *S. gordonii* Δ*comC,* 5.5 μm; *S. gordonii* Δ*comCDE,* 5.6 μm. Grid square, 25 μm. Lower panels show wide field images of biofilms producing patches of eDNA immunofluorescently labelled with Alexa594 (red).

software it was possible to see that the *C. albicans* hyphae that formed within the *com* mutant–*C. albicans* biofilms were less evenly spread across the surfaces and more coalesced (Fig. 3b, c). The average thickness values for the biofilms (Fig. 3) were ordered Δ*comC*– or Δ*comCDE*–*C. albicans*>wild-type–*C. albicans*>*C. albicans*. There were no statistically significant differences between c.f.u. values (per coverslip) for *S. gordonii* wild-type and Δ*comCDE* mutant in monospecies ($6.4\pm0.8\times10^6$ versus $5.1\pm0.7\times10^6$ c.f.u.) or dual species ($1.5\pm0.3\times10^8$ versus $2.2\pm0.2\times10^8$ c.f.u.) biofilms. This implied that the differences in biomass between dual species biofilms with these strains may primarily reflect changes in relative proportions of *C. albicans* and matrix. Unfortunately, meaningful c.f.u. values for *C. albicans* could not be obtained because of the filament morphology and aggregation. Overall, these results suggested that deletion of the *com* genes relieved some form of repression mechanism normally operating on dual species biofilm formation.

Extracellular polymeric substance (EPS) production

The matrix materials present within streptococcal biofilms include eDNA (Liu & Burne, 2011; Taff *et al.*, 2013; Xu & Kreth, 2013), proteins (Liao *et al.*, 2014) and polysaccharides (when grown in the presence of sucrose) (Falsetta *et al.*, 2014). *C. albicans* biofilms contain ~50 % protein, as well as mannan, glucan and eDNA (Zarnowski *et al.*, 2014). Incubation of monospecies biofilms of *S. gordonii* with DNase I, proteinase K or lyticase (*β*-1,3-glucan hydrolase) resulted in small but not statistically significant effects on biomass (Fig. 4). By contrast, significant reductions in biomass were seen for dual species biofilms with either wild-type or Δ*comCDE* mutant following incubation with the three enzymes (Fig. 4). Lyticase treatment reduced biomass by >50 % (Fig. 4a), while proteinase K digestion reduced biomass by >80 % (Fig. 4b). This is consistent with proteins representing a large proportion of biofilm matrix (Flemming & Wingender, 2010). Controls of heat-inactivated enzymes had no significant effects upon biofilm biomass nor did neuraminidase treatment (Fig. 4).

Incubation of dual species biofilms of *S. gordonii* wild-type–*C. albicans* or Δ*comCDE*–*C. albicans* with DNase I led to 25 or 75 % reductions, respectively, in biomass (Fig. 4) and this was supported by CLSM of the biofilms (Fig. 5). Both *S. gordonii* and *C. albicans* components were removed from DNase I-treated biofilms (Fig. 5), although more streptococci (green) remained in the *S. gordonii* wild-type–*C. albicans* biofilms (Fig. 5c). The *C. albicans* only biofilms were also disrupted by DNase I treatment (Fig. 5e).

eDNA within biofilms

To estimate eDNA concentrations within biofilms, matrix fractions associated with each biofilm were collected by washing them with TE buffer and measuring A_{260}. No statistically significant differences in eDNA concentrations were observed for monospecies biofilms of *S. gordonii* DL1 wild-type and Δ*comCDE* mutant (Fig. 6). However, the Δ*comCDE*–*C. albicans* dual species biofilms contained more eDNA than the *S. gordonii* DL1–*C. albicans* biofilms (Fig. 6).

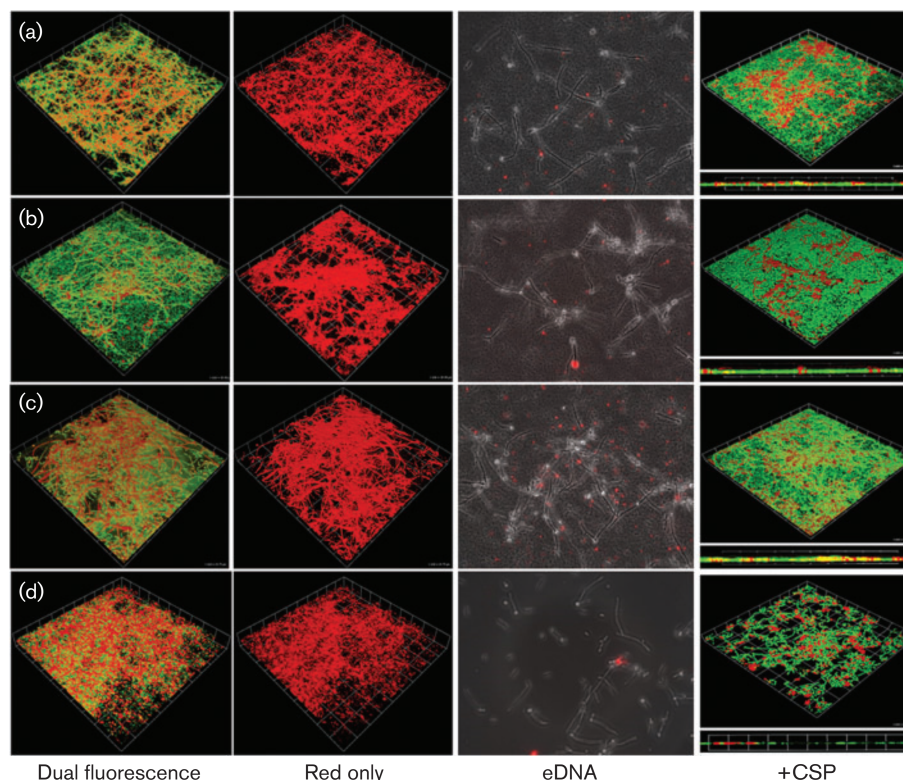

Fig. 3. Architecture and eDNA deposition patterns of 6 h dual species biofilms of *S. gordonii* with *C. albicans* expressing RFP. (a) *S. gordonii* DL1 wild-type–*C. albicans*, (b) Δ*comC*–*C. albicans*, (c) Δ*comCDE*–*C. albicans*, (d) *C. albicans.* Biofilms were stained with FITC. Dual fluorescence: images taken using both laser channels to visualize *C. albicans* RFP^+ (red) and FITC-labelled *S. gordonii* (green). Red only: images taken with the red channel only, showing the *C. albicans* biofilm component. Panels are 3D (*xyz* stack) images. Maximum thickness (depth) measurements for a representative biofilm were: (a) 13.6 μm, (b) 17.1 μm, (c) 18.1 μm, (d) 7.3 μm. Grid square, 25 μm. eDNA: wide field images of biofilms producing patches of eDNA immunofluorescently labelled with Alexa594 (red). +CSP: effect of *S. gordonii* CSP on *C. albicans* monospecies or dual species biofilms. Each panel comprises a 3D (*xyz*) and *xz* (thickness) CLSM image of biofilms grown on saliva-coated coverslips for 6 h at 37 °C in the presence of 10 μg CSP ml^{-1}. Grid square, 38.9 μm (a, b, c) and 25 μm (d).

To visualize eDNA in biofilms we utilized anti-dsDNA antibodies and immunofluorescence. The Δ*comCDE* mutant dual species biofilms contained multiple deposits of eDNA compared with the DL1– or Δ*comC*–dual species biofilms (Fig. 3). Overall, the eDNA deposits were enhanced in the dual species biofilms compared to monospecies biofilms (Fig. 2) and there was little difference between monospecies biofilms of the *S. gordonii* strains. *C. albicans* secreted little or no eDNA in monospecies or dual species biofilms under the experimental conditions (Fig. 3).

Effect of CSP on dual species biofilms

The increased biomass observed for *S. gordonii* Δ*comC*–*C. albicans* compared to wild-type–*C. albicans* biofilms (Fig. 1) was reduced to equivalent DL1 wild-type–*C. albicans* biomass in the presence of 10 μg CSP ml^{-1}, but not with sCSP (Fig. 7). The biomass of Δ*comCDE*–*C. albicans* dual species biofilms was also reduced by addition of CSP (Fig. 7). Inhibition of biofilm formation by *C. albicans* alone in the presence of CSP may be seen in CLSM images (Fig. 3) and this inhibition was supported by biomass measurements (Fig. 7). Exogenous CSP complemented the Δ*comC* mutant deficiency in developing competence for DNA-mediated transformation (Fig. S3c), but did not complement the Δ*comCDE* mutant (Fig. S3c). Thus, the effect of CSP on dual species biofilm formation was due to, at least in part, a direct effect of CSP on the biofilms, since the Δ*comCDE* mutant was unable to respond to CSP signalling.

Effects of CSP on *C. albicans*

The addition of synthetic *S. gordonii* CSP to *C. albicans* biofilms showed a dose-dependent inhibition of *C. albicans* monospecies biofilm formation (Fig. 8). In the presence of 2 μg CSP ml^{-1} there was 35 % reduction in biomass, while at 10 μg CSP ml^{-1} biomass was reduced ~60 % (Fig. 8b). In separate experiments, CSP did not inhibit planktonic growth of *C. albicans* or hypha formation. After 4 h incubation of *C. albicans* in YPT-Glc medium, with or without

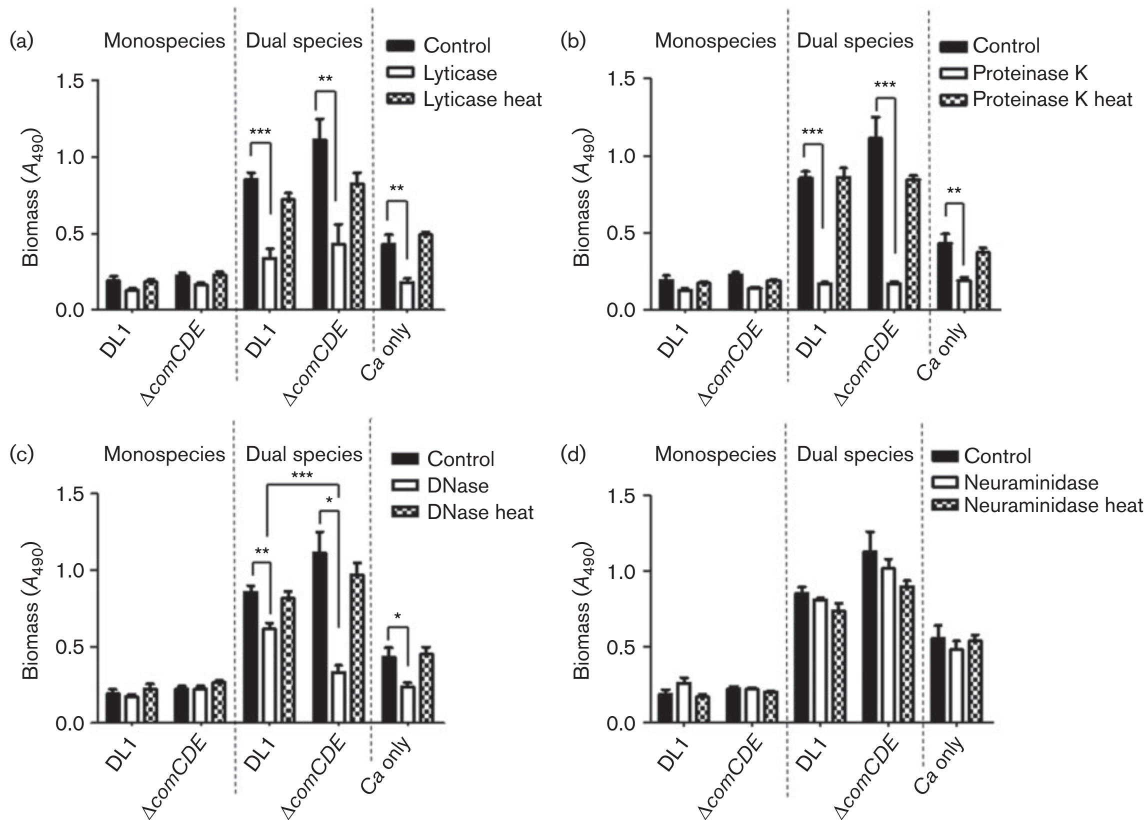

Fig. 4. Enzymic treatments of monospecies or dual species biofilms. Biofilms were grown for 6 h on saliva-coated coverslips, washed and incubated with enzyme for 1 h at 37 °C. Total biomass was quantified by safranin staining and A_{490} measurements following release of stain with acetic acid. (a) 100 U lyticase ml^{-1}, (b) 4.2 U proteinase K ml^{-1}, (c) 100 U DNase I ml^{-1} and (d) 0.002 U neuraminidase ml^{-1}. Filled column, control/no treatment; open column, enzymic treatment; hatched column, treatment with heat-killed enzyme. DL1, *S. gordonii* wild-type; *Ca*, *C. albicans*. *P* values: *$<$0.05, **$<$0.01, ***$<$0.001 (n=4).

10 μg CSP ml^{-1}, 80 % of cells formed hyphae and mean hyphal length was 23 ± 4.0 μm. No significant effects on biomass production or biofilm architecture were seen with equivalent concentrations of sCSP (Fig. 8a, b). We then measured biomass of material present in the planktonic phases of these biofilms by safranin stain assay. There was a threefold increase in planktonic phase biomass of biofilm cultures grown with 10 μg CSP ml^{-1} compared to those grown with sCSP at the same concentration (Fig. 8c). Therefore, reduced biomass of *C. albicans* biofilms formed in the presence of CSP could be accounted for by increased biomass in the planktonic phase. This indicated that CSP inhibited biofilm development by affecting attachment or promoting dispersal of *C. albicans* cells. Overall these results suggested that the production of CSP by *S. gordonii* modulated incorporation of *C. albicans* into dual species biofilms.

DISCUSSION

Mitis group streptococci are early colonizers of oral cavity surfaces and form the basis of many of the polymicrobial communities found in the mouth and nasopharynx (Aas *et al.*, 2005; Kanasi *et al.*, 2010; Shak *et al.*, 2013; Xu *et al.*, 2014a). Such communities may also contain *C. albicans*, which is carried by ~40 % of the healthy adult population (Clayton & Noble, 1966), and it is suggested that synergistic interactions between *C. albicans* and streptococci can promote carriage as well as disease (Dutton *et al.*, 2014; Falsetta *et al.*, 2014; Xu *et al.*, 2014b). The establishment and development of such communities involves small molecule signalling between microbial components, which maximizes metabolic efficiency and controls competition of species. The major signalling molecules produced by Gram-positive cocci are peptides, including CSPs and bacteriocins. There is evidence that peptides produced by *S. mutans* (Jarosz *et al.*, 2009) and *Enterococcus faecalis* (Cruz *et al.*, 2013) are inhibitory to filamentation by *C. albicans*. The ComCDE systems in *S. gordonii*, *S. pneumoniae* and *S. mutans* function as QS autoregulatory control systems, modulating gene expression patterns in different ways for the three streptococcal species (Vickerman *et al.*, 2007; Mashburn-Warren *et al.*, 2010; Son *et al.*, 2012; Merritt & Qi, 2012). Since *S. gordonii* and *C. albicans* form fully

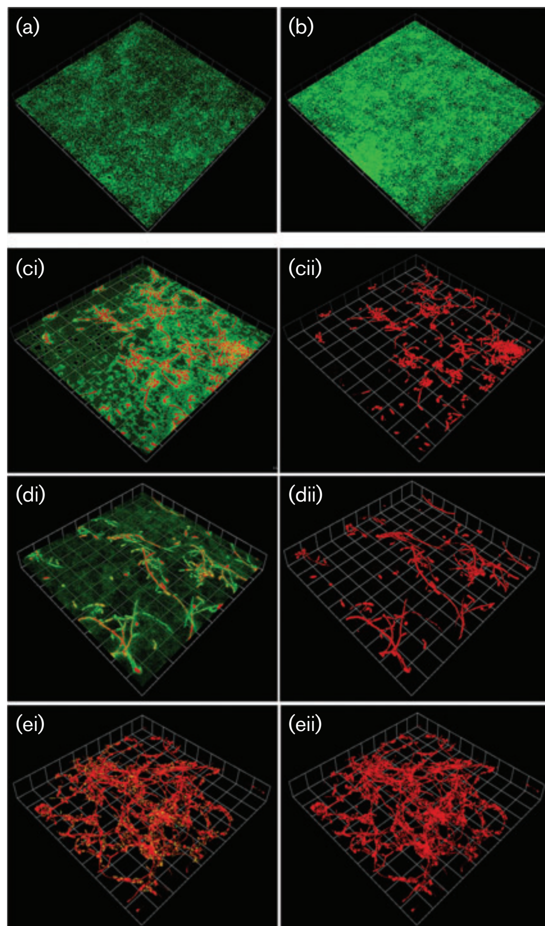

Fig. 5. Representative CLSM micrographs of monospecies or dual species biofilms following incubation with DNase I. Biofilms of *S. gordonii* DL1 wild-type or Δ*comCDE* mutant, with or without *C. albicans* expressing RFP, were grown for 6 h on saliva-coated coverslips, washed and incubated with DNase I for 1 h at 37 °C and stained with FITC. (a) *S. gordonii* DL1 wild-type (maximum thickness measurements for a representative biofilm, 6.18 μm), (b) *S. gordonii* Δ*comCDE* (5.23 μm), (c) *S. gordonii* DL1–*C. albicans* RFP^{+} (8.59 μm), (d) *S. gordonii* Δ*comCDE*–*C. albicans* RFP^{+} (6.12 μm) and (e) *C. albicans* RFP^{+} (5.58 μm). (c–e) (i) Left side are images of RFP and FITC laser channels combined; (ii) corresponding right side images show the RFP channel only (*C. albicans*). Grid square, 25 μm.

integrated dual species biofilm communities (Dutton *et al.*, 2014) we investigated the possible role of the ComCDE system in their regulation.

We generated two deletion mutants in the *comCDE* system. The Δ*comC* mutant carried an *aad9* cassette encoding spectinomycin resistance, engineered such that read-through into *comDE* occurred. This was confirmed by showing that the non-transformable phenotype of the mutant was complemented by addition of CSP (Fig. S3c). The other mutant carried a deletion across *comCDE* and was non-responsive to CSP (Fig. S3c). When grown in dual

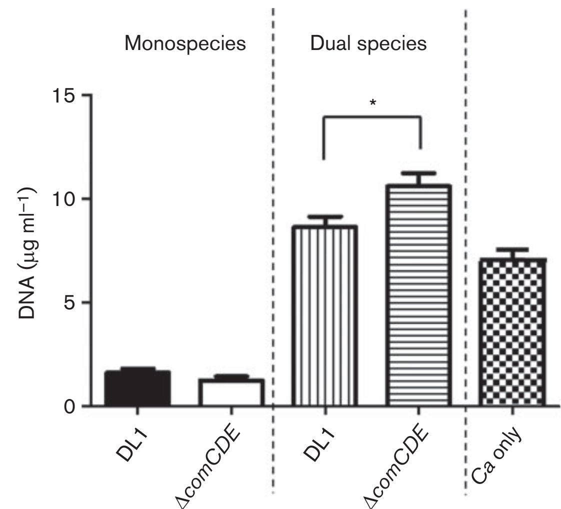

Fig. 6. Concentrations of soluble eDNA extracted from monospecies biofilms or *S. gordonii* dual species biofilms with *C. albicans*. DNA was extracted as described in Methods and measured at A_{260}. DL1, *S. gordonii* wild-type; *Ca*, *C. albicans*. *$P<0.05$ ($n=3$).

species biofilms for 6 h, both *com* mutant–*C. albicans* dual species biofilms were increased in biomass over and above the *S. gordonii* wild-type–*C. albicans* biofilms. The *com* mutants alone formed biofilms that were slightly denser than *S. gordonii* wild-type biofilms but there were no significant differences with respect to depth, biomass or

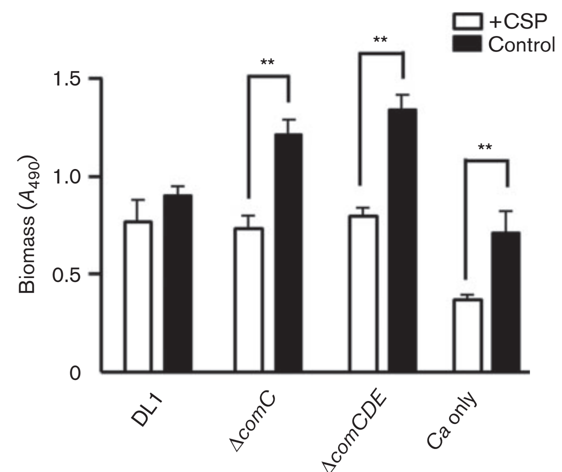

Fig. 7. Effect of *S. gordonii* CSP on biomass of *S. gordonii* and *C. albicans* monospecies or dual species biofilms. Biofilms were grown on saliva-coated coverslips for 6 h, with 10 μg CSP ml^{-1} (+CSP) or 10 μg sCSP ml^{-1} (Control), safranin-stained and biomass measurements (A_{490}) were taken following release of stain with acetic acid. DL1, *S. gordonii* wild-type; *Ca*, *C. albicans*. **$P<0.01$ ($n=3$).

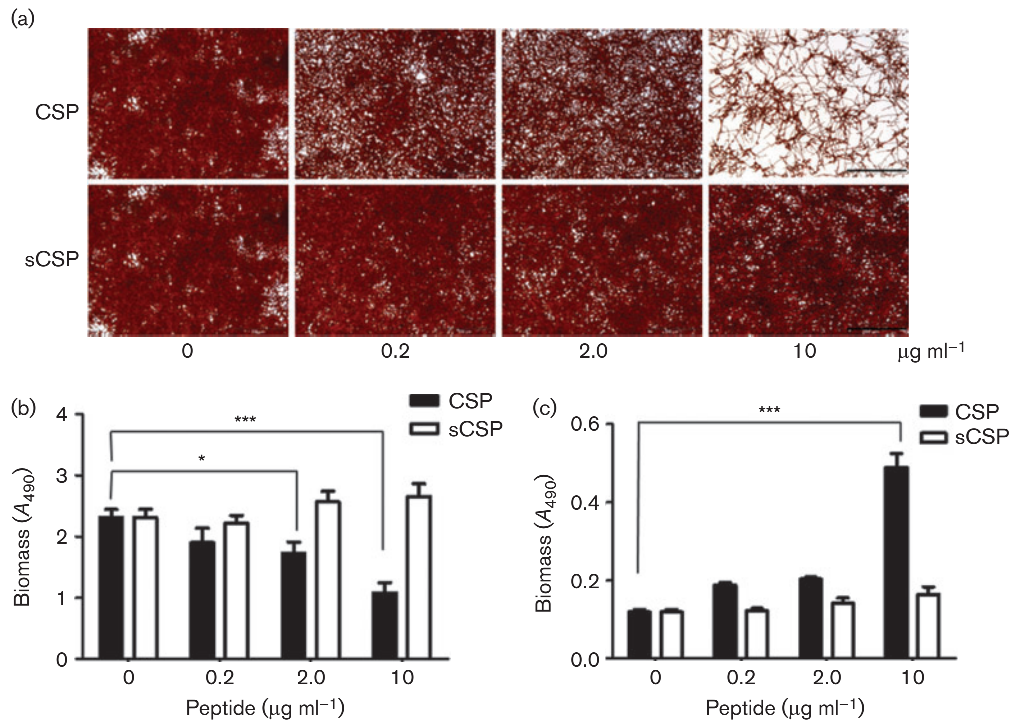

Fig. 8. Effects of *S. gordonii* CSP or sCSP on *C. albicans* biofilm formation. (a) Transmitted light micrographs of *C. albicans* biofilms grown for 6 h on saliva-coated coverslips in the presence of CSP or sCSP at concentrations indicated. Bar, 200 μm. (b) *C. albicans* biofilm biomass quantified by safranin stain assay following growth in the presence of CSP or sCSP. (c) Corresponding *C. albicans* planktonic phase biomass following growth with CSP or sCSP, determined by safranin stain assay. *$P<0.05$, ***<0.001 ($n=4$).

growth rates (Fig. S3a, b) in planktonic phase. This is unlike the effects of *comCDE* mutations in *S. mutans* that resulted in the formation of abnormal biofilms (Li *et al.*, 2002).

The dual species biofilms of Δ*comCDE*–*C. albicans* had visible differences in matrix architecture and more extensive arrays of *C. albicans* hyphal structures. This led us to believe that elevated EPS and numbers of *C. albicans* were responsible for increased biomass. The explanation was in part supported by showing that the biofilms could be ~70 % disrupted by incubation with DNase I. We also obtained evidence that the Δ*comCDE*–*C. albicans* biofilms contained a greater amount of eDNA and that they produced significantly more eDNA deposits. Incubation of the biofilms with lyticase led to ~50 % reduced biomass, suggesting the presence of *β*-glucans, which are derived from *C. albicans* cell wall turnover (Xie *et al.*, 2012). The EPS of *C. albicans* biofilms comprises >50 % protein, branched-chain mannans, mannan–glucan complexes and a small proportion (~5 %) of eDNA (Zarnowski *et al.*, 2014). The EPS of *C. albicans* biofilms is known to contribute to antifungal drug resistance (Taff *et al.*, 2013).

Major components of EPS in oral streptococcal biofilms are α-linked glucans, fructans, proteins and eDNA. Glucan and fructan polymers are produced by the activities of glycosyltransferases, which hydrolyse glucosidic linkages in disaccharides (e.g. sucrose) or trisaccharides and transfer the appropriate monosaccharide to form α-linked polymers. Since we utilized a monosaccharide as carbon and energy source in our studies, no glucans would be produced (Ricker *et al.*, 2014). It has been suggested that release of eDNA by streptococcal cells during early stages of biofilm formation could involve a specialized active release mechanism (Xu & Kreth, 2013) via lysis-independent membrane vesicles (Liao *et al.*, 2014). Our experiments do not enable us to distinguish between release or lysis as mechanisms for eDNA deposition. We have also not been able to determine the relative proportions of eDNA in dual species biofilms derived from *S. gordonii* versus *C. albicans*, but our evidence suggests eDNA emanates mainly from the streptococci. In development of competence, extensive cell lysis occurs to release DNA (Wei & Håvarstein, 2012; Okshevsky & Meyer, 2013; Xu & Kreth, 2013), but the Δ*comCDE* mutant did not develop competence, even when CSP was supplied. We also have no evidence for significant differences in eDNA production by *S. gordonii* wild-type and *com* mutant strains. The production of increased eDNA by the Δ*comCDE* mutant therefore is a response to the presence of *C. albicans*.

The CSP from *S. gordonii* inhibited *C. albicans* biofilm formation, but did not inhibit hyphal filament formation

in planktonic phase. Thus, under normal conditions in which both micro-organisms are growing in proximity, we suggest that CSP directly modulates *C. albicans* biofilm through interfering with cell–cell adherence or activating dispersal. The mechanism by which CSP interacts with *C. albicans* is also unknown, but shows some specificity, since sCSP had no significant effect on the biofilms. Unlike *S. mutans* CSP (Jarosz *et al.*, 2009), which has bacteriocin activity, the *S. gordonii* CSP does not inhibit *C. albicans* hypha formation. Neither peptide shows any direct antifungal activity and the CSPs have no sequence homology (*S. gordonii* DVRSNKIRLWWENIFFNKK and *S. mutans* SGSLSTFFRLFNRSFTQALGK). In future studies we aim to determine the optimal component of *S. gordonii* CSP that will inhibit biofilm formation. Such a peptide or mimetic might be effective in controlling *C. albicans* biofilm formation or carriage.

In summary, we provide evidence that *S. gordonii* CSP can potentially control *C. albicans* in dual species biofilms. Localized concentrations of CSP within biofilms in which the bacteria and fungi are in close contact (Dutton *et al.*, 2014) could be biologically effective in this respect. The production of eDNA is known to play a crucial role in *C. albicans* biofilm formation (Martins *et al.*, 2010; Sapaar *et al.*, 2014) and in addition to the results presented here, eDNA production has been shown to enhance dual species biofilms of *C. albicans* and *Staphylococcus epidermidis* (Pammi *et al.*, 2013). The deletion of *comCDE* from *S. gordonii* leads to dual species biofilms containing elevated amounts of eDNA. This is probably a response to the increased presence of *C. albicans* in the absence of CSP production. Thus, CSP can regulate composition of the dual species biofilm community by modulating EPS production and *C. albicans* retention.

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