The ISME Journal (2014) 8, 2256–2271

www.nature.com/ismej

ORIGINAL ARTICLE

Cross-feeding and interkingdom communication in dual-species biofilms of *Streptococcus mutans* and *Candida albicans*

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Polymicrobial biofilms are of large medical importance, but relatively little is known about the role of interspecies interactions for their physiology and virulence. Here, we studied two human pathogens co-occuring in the oral cavity, the opportunistic fungus *Candida albicans* and the caries-promoting bacterium *Streptococcus mutans*. Dual-species biofilms reached higher biomass and cell numbers than mono-species biofilms, and the production of extracellular polymeric substances (EPSs) by *S. mutans* was strongly suppressed, which was confirmed by scanning electron microscopy, gas chromatography–mass spectrometry and transcriptome analysis. To detect interkingdom communication, *C. albicans* was co-cultivated with a strain of *S. mutans* carrying a transcriptional fusion between a green fluorescent protein-encoding gene and the promoter for *sigX*, the alternative sigma factor of *S. mutans*, which is induced by quorum sensing signals. Strong induction of *sigX* was observed in dual-species biofilms, but not in single-species biofilms. Conditioned media from mixed biofilms but not from *C. albicans* or *S. mutans* cultivated alone activated *sigX* in the reporter strain. Deletion of *comS* encoding the synthesis of the *sigX*-inducing peptide precursor abolished this activity, whereas deletion of *comC* encoding the competence-stimulating peptide precursor had no effect. Transcriptome analysis of *S. mutans* confirmed induction of *comS*, *sigX*, bacteriocins and the downstream late competence genes, including fratricins, in dual-species biofilms. We show here for the first time the stimulation of the complete quorum sensing system of *S. mutans* by a species from another kingdom, namely the fungus *C. albicans*, resulting in fundamentally changed virulence properties of the caries pathogen.

The ISME Journal (2014) **8,** 2256–2271; doi:10.1038/ismej.2014.73; published online 13 May 2014
Subject Category: Microbe-microbe and microbe-host interactions
Keywords: biofilm; *Candida*; *Streptococcus*; EPS; quorum sensing

Introduction

It has been estimated that 80% of human infections result from pathogenic biofilms (Harriott and Noverr, 2011). Clinically, biofilm infections represent an overwhelming problem, as the microorganisms embedded in the extracellular matrix are resistant to antibiotics as well as to the host defence. Polymicrobial pathogenic biofilms are not only found in the oral cavity, but also in the respiratory tracts, skin, the reproductive and urinary tract, in patients with chronic lung diseases and on in-dwelling mechanical devices (Peleg *et al.*, 2010).

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Received 20 November 2013; revised 27 March 2014; accepted 28 March 2014; published online 13 May 2014

Candida albicans is the most prevalent opportunistic human pathogenic fungus (Kim and Sudbery, 2011) and can cause infections of mucosal membranes (candidiasis) and the blood stream (candidemia). It is able to form biofilms on mucosal membranes as well as on implants (Cuellar-Cruz *et al.*, 2012). Biofilm formation and virulence of *C. albicans* are connected with the transition from the yeast to the hyphae morphotype, which represents a crucial step towards pathogenesis. Yeast cells colonise predominantly surfaces, whereas the hyphal form of *C. albicans* is invasive (Sudbery, 2011; Gow *et al.*, 2012). Hyphae provide structural integrity to biofilms (Finkel and Mitchell, 2011; Banerjee *et al.*, 2013). *C. albicans* has been found in periodontal pockets in both the chronic and aggressive forms of periodontitis (Urzua *et al.*, 2008).

Streptococcus mutans is another opportunistic pathogen inhabiting the human oral cavity (Ajdic *et al.*, 2002). *S. mutans* belongs to the phylum

Firmicutes and is a Gram positive, facultative anaerobic bacterium that can ferment a large spectrum of dietary sugars. The excreted organic acids result in a strong localized pH drop that can cause lesions of the dental enamel and thus initiate caries development. *S. mutans* is a common inhabitant of the oral cavity and has long been recognized as one of the causes of dental caries, a highly prevalent biofilm-dependent polymicrobial oral infectious disease (Kutsch and Young, 2011; Takahashi and Nyvad, 2011; Burne *et al.*, 2012). Biofilm formation in *S. mutans* is readily induced by dietary sugars that are transformed to extracellular polysaccharides by dedicated enzymes, which are therefore important targets of anti-caries strategies (Bowen and Koo, 2011).

C. albicans and *S. mutans* are found together in early childhood caries (Marchant *et al.*, 2001; de Carvalho *et al.*, 2006; Raja *et al.*, 2010) and on bracket materials (Rammohan *et al.*, 2012). Early childhood caries is a very aggressive form of caries. It has been shown that *C, albicans* is present in 96% of caries-positive children (age group 6–12 years) but only in 24% of caries-free children (Raja *et al.*, 2010). Denture plaque was shown to contain both *S. mutans* and *C. albicans* in 25.5% of healthy individuals (Ribeiro, 2012). However, data showing actual cell–cell contact between those two organisms *in vivo* is currently lacking.

A tight co-aggregation between *C. albicans* and streptococci has been observed (Jenkinson *et al.*, 1990; Metwalli *et al.*, 2013) and could be owing to specific adhesins similar to those found in *S. gordonii* (Silverman *et al.*, 2010) or to a glucan layer formed on the *Candida* cells by the glucosyltransferase B (GtfB) exoenzyme (Gregoire *et al.*, 2011). Interaction between *C. albicans* and streptococci can contribute to enhanced biofilm formation. Oral streptococci produce cell wall-anchored proteins facilitating binding to *C. albicans* (Bamford *et al.*, 2009). The *S. gordonii* cell wall-associated polypeptide SspB3 interacts directly with the *C. albicans* hyphae-specific agglutinin-like sequence 3 (Nobbs *et al.*, 2010). A synergistic partnership between *S. oralis*, *S. sanguinis* and *C. albicans* was observed, where the fungus promoted biofilm formation by the streptococci on abiotic surfaces and on mucosa (Diaz *et al.*, 2012). Thus not only *S. mutans*, but also *C. albicans* might have crucial roles for the cariogenic biofilm succession (Metwalli *et al.*, 2013). This is supported by a recent study showing that an increased load of *Candida* cells correlated with decreased diversity of the saliva microbiome and a shift of the microbial community towards streptococci (Kraneveld *et al.*, 2012).

Quorum sensing signalling has been shown to be involved in interactions between *C. albicans* and bacteria. Hyphae development in *C. albicans* can be inhibited by 3-oxo-C12-homoserine lactone from *Pseudomonas aeruginosa* (Hogan *et al.*, 2004), the *S. mutans* pheromone CSP (competence-stimulating peptide) (Jarosz *et al.*, 2009) or by the diffusible signal factor trans-2-decenoic acid (Vilchez *et al.*, 2010). Conversely, the signalling molecule farnesol from *C. albicans* inhibits the swarming motility of *P. aeruginosa* (McAlester *et al.*, 2008) and induces the Pseudomonas quinolone signal synthesis (Cugini *et al.*, 2010). No report exists of the induction of the quorum sensing signalling circuit of an oral pathogen by a eukaryotic fungus.

The main virulence traits of *S. mutans*—acidogenicity, aciduricity, biofilm formation and mutacin production—as well as its ability to incorporate foreign DNA into its genome (genetic competence) are controlled by quorum sensing. *S. mutans* secretes the so-called AI-2, for which no signalling function could so far be identified in this organism (Sztajer *et al.*, 2008). It also secretes two peptide pheromones, the CSP (Li *et al.*, 2001) and XIP (alternative sigma factor *sigX*-inducing peptide) (Mashburn-Warren *et al.*, 2010; Khan *et al.*, 2012) that induce genetic competence through two different signalling pathways converging on SigX, the only alternative sigma factor of *S. mutans*, which is therefore a master regulator of quorum sensing (Federle and Morrison, 2012).

The overwhelming majority of quorum sensing studies have been performed in mono-species systems. However, the main quorum sensing-controlled traits of *S. mutans*, that is, mutacin synthesis and genetic competence, have their largest impact in multi-species systems because the DNA that is taken up is different from the DNA released from the competent cell, and especially in biofilms, which provide high local concentrations of chemical cues as well as direct cell–cell contact. For example, *S. mitis* has been shown to acquire antibiotic resistance genes by transformation in a multi-species biofilm (Hannan *et al.*, 2010). As *S. mutans* and *C. albicans* have been found together in oral infections and biofilm growth is essential for the virulence of both of them, here we studied morphology, physiology, transcriptomics and genetics of their dual-species biofilms *in vitro* under conditions of robust hyphal growth of *C. albicans* and strong biofilm formation of *S. mutans*. We were particularly interested in the role of cell–cell communication in shaping the physiology of dual-species biofilms, and for this purpose used a reporter strain for the promoter of the alternative sigma factor SigX (Lemme *et al.*, 2011). The data show that both microorganisms profit from growth in dual-species biofilms, and that *C. albicans* has a profound influence on the physiology and quorum sensing-mediated traits of *S. mutans*.

Materials and methods

Strains and culture conditions

Microorganisms used were *S. mutans* UA159 wild type (ATCC 700610, Manassas, VA, USA), *S. mutans* UA159 SMP_{sigX}green fluorescent protein (GFP)

(Lemme *et al*., 2011) and *C. albicans* (DSM 11225, Braunschweig, Germany). Pre-cultures of *S. mutans* strains were grown in THBY medium (Becton, Dickinson and Company, Sparks, MD, USA), if necessary (for the reporter strain and deletion mutants) with erythromycin (3 μg ml^{-1}) at 37 °C aerobically with 5% CO_2 without shaking. *C. albicans* pre-cultures were grown in yeast nitrogen base (YNB) synthetic medium (Difco Laboratories, Detroit, MI, USA) supplemented with maltose (1 g l^{-1}) and glucose (2 g l^{-1}) at 37 °C aerobically with 5% CO_2 without shaking for 16 h. The medium that supported growth of *S. mutans* and *C. albicans* in biofilms (YNBB) contained YNB (6.7 g l^{-1}, Difco Laboratories), 75 mM Na_2HPO_4–NaH_2PO_4 (pH 7.3), *N*-acetylglucosamine (2.5 mM, Sigma-Aldrich, Taufkirchen, Germany), casamino acids (2 g l^{-1}, Becton, Dickinson and Company) and sucrose (5 g l^{-1}). The pH during biofilm growth was in the range of pH 7.0–6.6 (*S. mutans* single biofilm), pH 7.0–6.4 (dual-species biofilm) and pH 7.0–6.95 (*C. albicans* single biofilm).

Biofilm formation

Pre-cultures of *S. mutans* and *C. albicans* were inoculated from single colonies or frozen glycerol stocks, respectively, grown for 16 h, harvested by centrifugation (5000 r.p.m., 20 min, 4 °C) and suspended in YNBB medium to an OD_{600} of 0.1. Equal volumes of each strain (500 μl) were inoculated into the wells of 24-well microtitre plates. Suspensions (1 ml) of one strain only (*S. mutans* or *C. albicans*) were prepared for single-strain inoculums. The microtitre plates (Thermo Fisher Scientific, Rochester, NY, USA) used for biofilm formation were coated with artificial saliva for 1 h at 37 °C to mimick the conditions in the oral cavity (Wong and Sissons, 2001). Natural saliva has a crucial role for the colonization of dental enamel (Scannapieco, 1994). Following the removal of artificial saliva, the inoculum was pipetted into the coated well (1 ml for 24-well microtitre plates and 0.2 ml for 96-well microtitre plates). The plates were incubated aerobically (5% CO_2) at 37 °C. Biofilms were allowed to develop for 4, 6, 8, 10, 12 and 24 h. Growth was monitored by crystal violet staining, quantitative PCR and microscopical analysis.

Induction of the alternative sigma factor SigX

The *S. mutans* reporter strain SMP_{sigX}GFP carrying a plasmid with the promoter of *sigX* fused to GFP was cultivated together with *C. albicans* in dual-species biofilms. Single-species biofilms were prepared in parallel. Sterile medium was used as a control. After removal of the supernatant, the GFP fluorescence intensity was recorded with a Wallac 1420 Multilabel counter (PerkinElmer, Waltham, MA, USA). Fluorescence microscopic analysis of biofilms was carried out using an Olympus BX60 microscope (Olympus, Seelze, Germany) equipped with a colour view II camera and a ×100 immersion oil objective. The filter U-MWIBA3 (excitation 460–495 nm, emission 510–550 nm and dichromatic filter 505 nm) was used.

Two pheromones of *S. mutans* (CSP and XIP) and farnesol of *C. albicans* were tested for induction of *sigX* in biofilms of the reporter strain SMP_{sigX}GFP. The pheromones were added at concentrations between 0.1 and 100 μM, and the biofilm was incubated for 4, 6, 8, 10 and 24 h. Afterwards the planktonic phase of the culture was withdrawn and the fluorescence intensity was recorded with a Wallac 1420 Multilabel counter (PerkinElmer). Fresh YNBB medium was used as a control. In addition, we have tested induction of *sigX* by CSP, XIP and farnesol added to the mature biofilm (8 h). After 2 and 4 h of treatment the fluorescence intensity was recorded.

Field emission scanning electron microscopy

Biofilm samples were fixed with a fixation solution containing 2% glutaraldehyde and 5% formaldehyde in HEPES buffer (100 mM HEPES, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 90 mM sucrose, pH 7.0) and stored at 4 °C. After washing the samples three times with TE buffer (10 mM TRIS, 2 mM EDTA, pH 7.0), they were dehydrated with a graded series of acetone (10%, 30%, 50%, 70%, 90% and 100%) on ice. The 100% acetone step was repeated at room temperature. Samples were then subjected to critical point drying with CO_2 and sputter coated with gold-palladium. The analyses were carried out using a Zeiss Merlin field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at an acceleration voltage of 5 kV using the Everhart-Thornley SE-detector and the inlens SE-detector in a 25:75 ratio. Contrast and brightness were adjusted in Adobe Photoshop CS3.

Staining with concanavalin A

The procedure was adapted from Kolodkin-Gal *et al.* (2012). Biofilms were rinsed with 200 μl of 1 × phosphate-buffered saline buffer and stained with 50 μl Concanavalin A labelled with AlexaFluor 488 (100 μg ml^{-1}) for 40 min at room temparature in the dark. Subsequently the staining solution was withdrawn, the biofilm was washed with 50 μl of 1 × phosphate-buffered saline and then counterstained with 10 μM DAPI (4′,6-diamidino-2-phenylindole) for 15 min at room temperature in the dark. After removal of DAPI the biofilm was analyzed under the fluorescence microscope (Olympus BX 60, Shinjuku, Japan) using the filter U-MWIBA3 for AlexaFluor 488 and U-MINUA2 for DAPI respectively.

Uptake of labelled DNA

The amplicon of the 16S RNA gene of *S. mutans* was used as a source of DNA for the competence test and was obtained as described above. Purified DNA

amplicon (2 μg) was labelled with 4 μl of Cy3 (Kreatech Biotechnology, Amsterdam, the Netherlands) for 15 min at 85 °C. The labelled DNA was purified on the column of the ULS labelling kit (Kreatech Biotechnology) according to the manufacturer's instructions. The degree of labelling was determined by measuring absorbance at 260 and 550 nm. Material with a degree of labelling of 1.5 was used. DNA uptake was tested as described with modifications (Lemme *et al.*, 2011). Biofilms of the *S. mutans* reporter strain $SMP_{sigX}GFP$ alone and together with *C. albicans* were cultivated as decribed for 10 h. The supernatants were removed and biofilm of three wells was suspended in 100 μl of fresh YNBB medium without sucrose. To disrupt cell chains, the biofilm was sonified for 10 cycles of 5 s with 10 s pause at 10% power (Bandelin Electronic, Berlin, Germany) on ice. The Cy3-labelled DNA (final concentration 5 μg ml^{-1}) was added to the biofilm cells and incubated in the dark for 30 min at 37 °C. Excess DNA was digested with DNAse I and the biofilms were analysed by fluorescence microscopy. The red channel was used to visualize Cy3, and the green channel to observe GFP.

Methods for preparation of conditions media, construction of knockout mutants, quantitative PCR, extraction of DNA and RNA, microarray analysis and gas chromatography–mass spectrometry analysis are described in Supplementary Methods S1. Primers and a comparison between qPCR and microarray data are provided in Supplementary Tables S1–S3.

Results

Cultivation medium

We used a chemically defined synthetic medium commonly used for cultivating *C. albicans* as a basis. Sucrose was provided as a carbon source, as it is fermented by both species, and in *S. mutans* induces strong biofilm formation. *S. mutans* is auxotrophic for several amino acids (Ajdic *et al.*, 2002), therefore we added casamino acids. GlcNAc was provided to induce hyphae formation in *C. albicans* (Sudbery, 2011) and the medium was buffered to exclude different pH effects in single and dual-species biofilms. In this medium, designated YNBB, both microorganisms formed biofilms and *C. albicans* grew predominantly in the hyphal form. Phase contrast microscopy (Figure 1a) showed that *C. albicans* and *S. mutans* were in direct cell–cell contact, with many *S. mutans* cells adhering to the hyphae of *C. albicans*.

Growth and morphology of dual-species biofilms of S. mutans and C. albicans

Biofilm biomass increased continuously for both species throughout the 24 h of cultivation, both in mono-culture and in co-culture (Figure 1b). At 10 and 24 h the biomass of dual-species biofilms was almost twice that of single-species biofilms. Quantitative PCR of the 16S rRNA and 18S rRNA gene revealed that cell numbers of both species increased throughout the experiment, in accordance with the biomass data. Cell numbers in dual-species biofilms

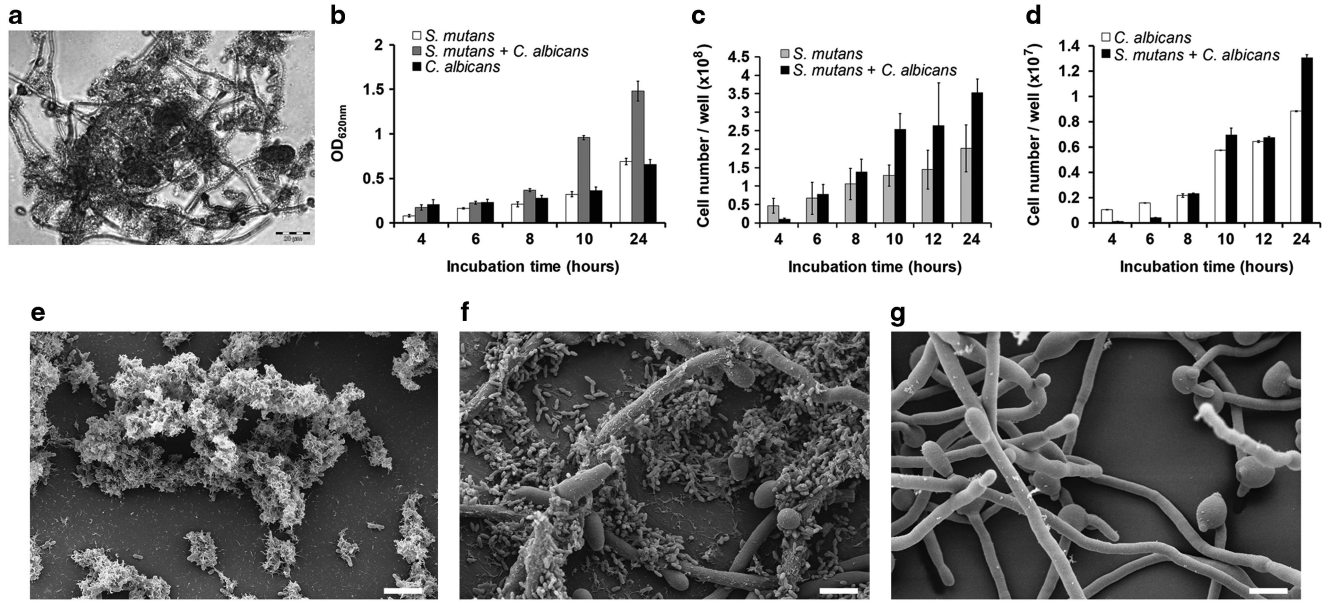

Figure 1 Growth and morphology of *S. mutans* and *C. albicans* in single- and dual-species biofilms. (**a**) Phase contrast micrograph of a dual-species biofilm. *C albicans* mainly grows in the hyphal form; some cells growing in the yeast form are also visible. *S. mutans* attaches to the hyphae of *C. albicans*. (**b**) Biofilm mass determined by crystal violet staining (mean and s.d. from three independent experiments). (**c**) Cell numbers of *S. mutans* (**c**) and *C. albicans* (**d**) determined by quantitative PCR of the 16S rRNA gene and the 18S rRNA gene, respectively. Mean and s.d. of three independent experiments with two technical replicates each are shown. (**e** and **f**) Scanning electron micrographs of 10-h biofilms of *S. mutans* (**e**), dual-species biofilm with *C. albicans* (**f**) and *C. albicans* (**g**). Scale bar (**e**–**g**) 4 μm.

were significantly higher than in mono-species biofilms, both for *S. mutans* and *C. albicans* (Figures 1c and d). The data show that co-cultivation of *S. mutans* and *C. albicans* in dual-species biofilms resulted in better growth of each species suggesting that metabolic interactions may have taken place, which provided additional nutrients.

Scanning electron micrographs (Figure 1e) showed that *S. mutans* formed clumps in mono-species biofilms, which were embedded in extracellular polymeric substance (EPS). By contrast, in dual-species biofilms (Figure 1f) little EPS was visible, and many cells of *S. mutans* appeared to be 'naked', that is, lacking the EPS matrix. The EPS matrix of *S. mutans* consists mainly of glucan and fructan (Trautner *et al.*, 1981) and has been thoroughly studied because it has an important role for caries development (Koo *et al.*, 2013). To visualize the EPS matrix under the fluorescent microscope, we stained the biofilms with the glucan-binding lectin concanavalin A labelled with the green fluorescent dye AlexaFluor 488 (Figure 2). To visualize the DNA of the cells, we used the DNA-binding blue fluorescing dye DAPI. Single-species biofilms of *S. mutans* showed strong green fluorescence of *S. mutans,* indicating that EPS had been formed. No such green fluorescence of *S. mutans* could be observed in dual-species biofilms. The hyphae of *C. albicans* also contain glucan and therefore can clearly be seen on the pictures. These findings confirm the scanning electron micrographs and show that EPS was not formed by *S. mutans* in dual-species biofilms.

The sigX promoter is induced in dual-species biofilms

To determine whether quorum sensing of *S. mutans* was induced in our biofilms, we used the *S. mutans* reporter strain $SMP_{sigX}GFP$. It carries a plasmid where the promoter of the alternative sigma factor SigX is fused to GFP. Upon induction of SigX green fluorescence is observed. It was quantified using a fluorescence plate reader (Figure 3a), by reverse transcriptase (RT)-PCR of *sigX* in the wild type (Figure 3b) and observed directly under the fluorescence microscope (Figure 3c). Strong induction of P_{sigX} was observed in dual-species biofilms with *C. albicans* (Figure 3a), showing a maximum between 8 and 12 h of biofilm growth. There was no detectable activation of P_{sigX} in mono-species biofilms of *S. mutans*. Gene expression of *sigX* was quantified using S*. mutans* wild type by quantitative RT-PCR (Figure 3b). A 73-fold increase of *sigX* expression was observed in co-culture with *C. albicans* at 10 h in comparison with *S. mutans* cultivated alone. At 12 h, *sigX* expression already decreased. Such a fast decrease could not be observed with the $SMP_{sigX}GFP$ reporter strain owing to the stability of gfp. P_{sigX} induction was confirmed by microscopical analysis. The majority of *S. mutans* cells revealed strong green fluorescence (Figure 3c) in co-culture with *C. albicans*. Cells that were attached to *C. albicans* as well as more distant biofilm cells were fluorescing. Such unimodal induction of sigX has previously only been demonstrated in pure cultures of *S. mutans* in a peptide-free chemically defined medium (Son *et al.*, 2012). Here, we show that in a semi-defined medium, unimodal activation of SigX occurs if the two

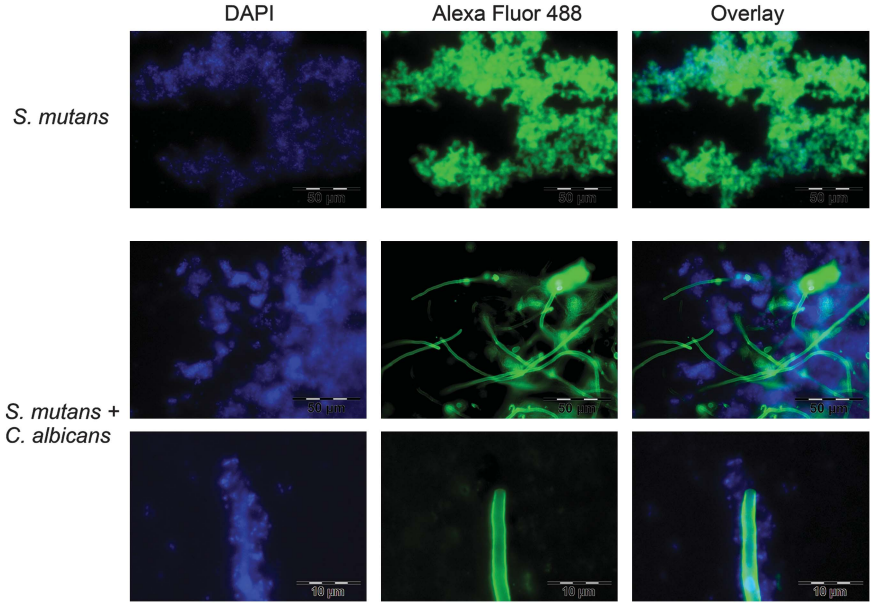

Figure 2 EPS matrix in single- and dual-species biofilms. Biofilms (10-h old) were stained with two fluorescent dyes: The lectin concanavalin A labelled with AlexaFluor 488 binds specifically to termical sugar moieties of glycans and fluoresces green. The dye DAPI (4′,6-diamidino-2-phenylindole) binds to DNA and fluoresces blue. Green and blue fluorescence are shown separately (left and middle panel) and overlaid. Scale bar (upper two panels) 50 µm, (bottom panel) 10 µm.

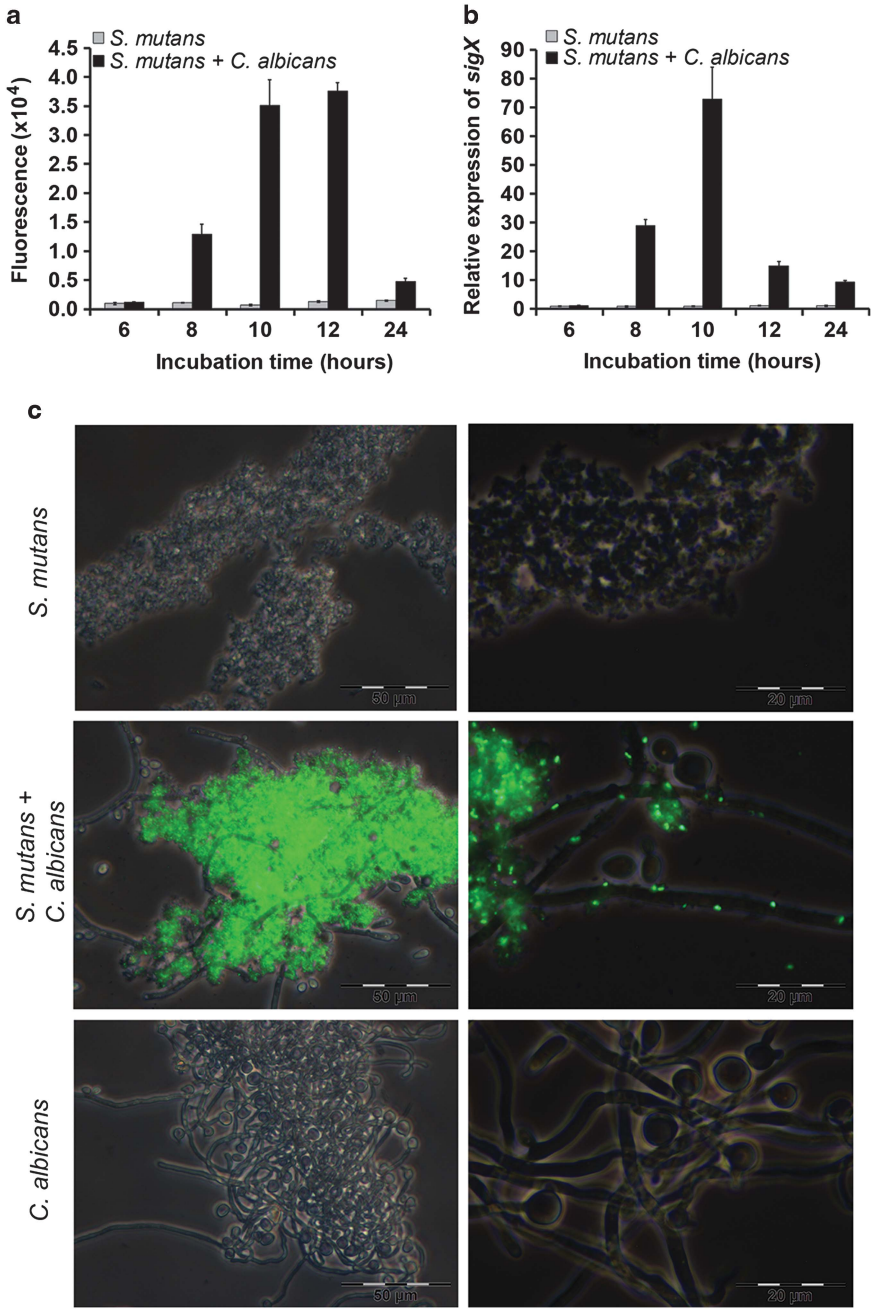

Figure 3 Induction of the alternative sigma factor SigX of *S. mutans* in dual-species biofilms. (**a**) Fluorescence intensity of $SMP_{sigX}GFP$, a gfp-reporter for *sigX* expression in *S. mutans*, grown as a single-species biofilm (grey bars) or together with *C. albicans* as a dual-species biofilm (black bars) quantified using the VictorWallac 1420 fluorescence plate reader. (**b**) Quantitative RT-PCR of *sigX* expression in *S. mutans* wild-type biofilms grown alone (grey bars) or with *C. albicans* (black bars). The data were normalized to *sigX* expression in a *S. mutans* biofilm after 6 h. Mean and s.d. from four independent experiments are shown. (**c**) Fluorescence microscopy of single- and dual-species biofilms after 10 h of growth. Phase contrast and gfp channel are overlaid. Scale bars (**c**, left) 50 µm, (**c**, right) 20 µm.

species *C. albicans* and *S. mutans* are grown together.

Conditioned media of mixed biofilms activate the sigX promoter in reporter strain biofilms and comS is required for this activation

We wanted to know whether (1) an unknown molecule present in the cultivation medium or (2) one of the known quorum sensing signalling molecules of *S. mutans* or *C. albicans*, respectively, might be responsible for the induction of *sigX* in co-culture. Finally, we also tested whether (3) the activating compound was present in the spent medium, or whether activation occurred indirectly by stimulating *S. mutans* to synthesize the inducer. Sterile-filtered supernatants from single- and dual-species biofilms (4, 6, 8, 10, 12 and 24 h) were applied to biofilms of the reporter strain $SMP_{sigX}GFP$

grown for 6, 10 and 24 h, respectively. Supernatants of dual-species biofilms that had been cultivated at least 8 h caused activation of P_{sigX}, whereas single-biofilm culture supernatants either of *S. mutans* or of *C. albicans* had no effect (Figure 4a). This activation was stronger in younger reporter strain biofilms (6 h) than in older ones (10 h), but even 24-h-old reporter strain biofilms could be induced by dual-species biofilm supernatants. The strongest induction was seen for 6-h-old reporter strain biofilms challenged with supernatants from 8-h mixed biofilms. The data show that an external factor produced exclusively in dual-species biofilms was responsible for activating the *sigX* promoter.

We then tested whether reporter strain biofilms could be activated by the known quorum sensing pheromones of *S. mutans*, namely XIP and CSP, or by the quorum sensing signal of *C. albicans*, farnesol. These autoinducers were added at various concentrations to biofilms of the reporter strain, which were then grown for 4, 6, 8, 10 and 24 h. A robust activation of P_{sigX}-GFP after addition of XIP was observed (Figure 4b). The activation was particularly strong for 8- and 10-h-old biofilms and was proportional to the concentration of XIP up to a final concentration of 5 μM XIP. No or only neglectable activation by CSP or farnesol was observed even at concentrations of 10 and 100 μM, respectively. These data suggest that XIP might be responsible for P_{sigX} activation in dual-species biofilms.

To test whether XIP was present in the supernatants or whether it was produced by *S. mutans*, we constructed deletion mutants for *comS* and *comC* and used these mutants for single- and dual-species biofilm growth. Culture supernatants of single-species biofilms of the Δ*comS* mutant as well as from dual-species biofilms of the Δ*comS* mutant with *C. albicans* were unable to induce P_{sigX} in the reporter strain (Figure 4c). By contrast, conditioned media from dual-species biofilms of *S. mutans* Δ*comC* and *C. albicans* clearly induced P_{sigX}. These results suggest that *comS*, but not *comC*, is indispensable for activation of P_{sigX}. Therefore, we hypothesize that *S. mutans* was induced to produce XIP in co-culture with *C. albicans*.

EPS of S. mutans *are not produced in conditioned media from dual-species biofilms*

To test whether a factor suppressing EPS synthesis was secreted into the medium, sterile conditioned media were prepared and *S. mutans* biofilms were cultivated in them. Electron microscopy showed that the EPS matrix was clearly present when *S. mutans* was grown in culture supernatants from 10-h single-species biofilms of *C. albicans* or *S. mutans*, respectively (Figure 5 right and left panels). By contrast, in culture supernatants of 10-h dual-species biofilms, the test biofilm was more dispersed and lacked EPS and the cells of *S. mutans* appeared to be naked, similar to those grown in co-culture with *C. albicans*

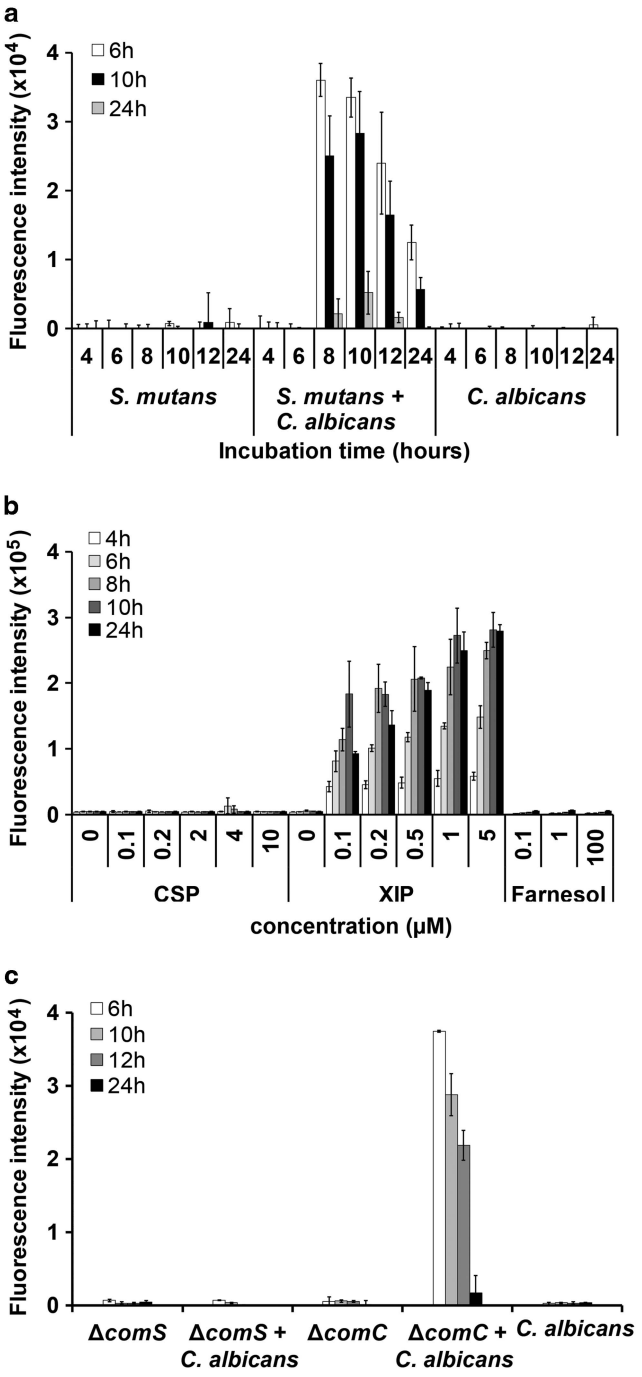

Figure 4 Activation of *sigX-gfp* by culture supernatants and pheromones and role of the autoinducer synthases ComC and ComS. (**a**) Culture supernatants were obtained from biofilms of *S. mutans* and *C. albicans* cultivated separately or together for 4–24 h and added to 6, 10 and 24 h-old-test biofilms of the reporter strain SMP_{sigX}GFP. Fluorescence intensity was determined after 2 h of incubation. (**b**) Activation of *sigX-gfp* in reporter strain biofilms of *S. mutans* by the quorum sensing pheromones CSP (competence-stimulating peptide) and XIP (*sigX*-inducing peptide) produced by *S. mutans* and by farnesol produced by *C. albicans*. The autoinducers were added as pure compounds at the indicated concentrations. Fluorescence was determined after 2–24 h of biofilm growth. (**c**) Same experiment as in (**a**), except that culture supernatants from deletion mutants for *comC* and *comS* of *S. mutans* were tested. *comC* encodes the synthesis of the CSP precursor, whereas *comS* encodes the synthesis of the XIP precursor. Mean and s.d. of four experiments are shown in all cases.

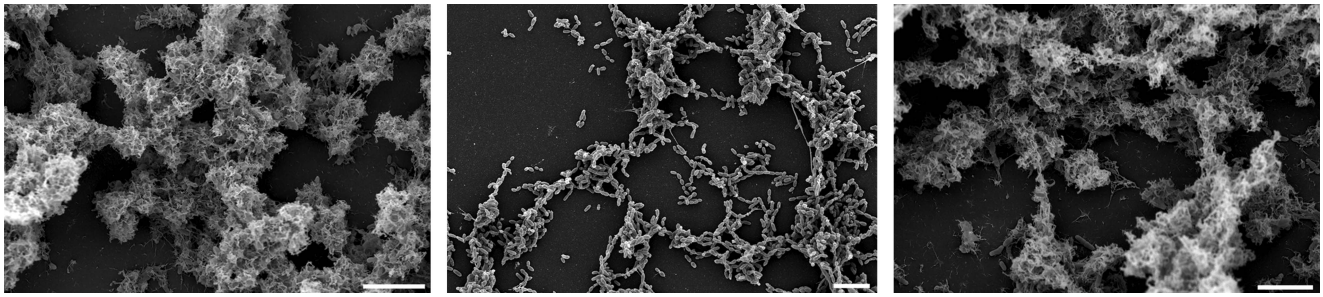

Figure 5 EPS formation of *S. mutans* biofilms in conditioned media. *S. mutans* biofilms were cultivated in conditioned media from 10-h-old single- (left and right panels) and dual-species biofilms (middle panel) for 10 h and analysed by scanning electron microscopy. Scale bar, 5 μm.

(Figure 1f). This effect was already observed after 4 h of growth of test biofilms in dual-species biofilm extract (Supplementary Figure S1). We conclude that supernatants of dual-species biofilms prevented EPS synthesis of *S. mutans*. We tested whether XIP, the quorum sensing molecule excreted by *S. mutans*, might be able to suppress EPS formation, but this was not the case (Supplementary Figure S2).

To determine whether an extracellular enzyme might be present that is able to destroy the polysaccharides constituting the EPS matrix, for example, glucan, we added sterile-filtered culture supernatants from dual-species biofilms (grown for 6, 10, 12 and 24 h) to a 10-h-old established biofilm of *S. mutans*. No significant change in biofilm morphology and EPS thickness occurred as determined by scanning electron microscopy after 2 and 4 h (data not shown).

Transcriptome analysis of S. mutans *growing in single- and dual-species biofilms*

A whole-genome microarray (Xue *et al.*, 2010) was used to analyse the transcriptome of *S. mutans* in dual-species biofilms with *C. albicans* before (6 h), during (10 h) and after (24 h) *sigX* activation in comparison with gene expression of *S. mutans* in mono-species biofilms. We found 510 genes that were differentially expressed. They were sorted into six different groups according to their expression profile using the c-means algorithm (Kumar and Futschik, 2007) (Supplementary Figure S3), and putative biological functions were assigned based on clusters of orthologous groups (Supplementary Figure S4). Several microarray data were confirmed by quantitative RT-PCR (Supplementary Table S3). All transcripts showing high abundance in the microarray data revealed similar or higher gene expression levels in quantitative RT-PCR, in accordance with the higher dynamic range of quantitative RT-PCR, which has often been observed.

The most abundant transcripts belonged to the quorum sensing regulon

At 10 h of growth in mixed biofilms, the expression of 84 genes was significantly increased (groups 3 and 4). Among them the competence-related transcripts were the most abundant ones (Figure 6; Supplementary Figure S5; Supplementary Table S4). Particularly, *comS*, *sigX* and the late competence genes (*comYA* and *comYC*) were the most abundant transcripts and revealed fold change values of 68, 56 and above 200, respectively. These data confirm the biological experiments and indicate that *sigX* was activated by the proximal ComRS system. Genes downstream of *sigX* (late competence genes) were also highly upregulated, including those belonging to the ComY operon, the transformasome, natural transformation (SMU.1001—SMU.1003), and genes encoding enzymes for excision, insertion, inversion and translocation of DNA (SMU. 2085—*recA* and SMU. 2086—*cinA*).

The genes constituting the CSP-driven quorum sensing circuit were also upregulated but much more weakly. The genes of the *comCDE* operon revealed fold change values of 1.8, 3.2 and 3.4, respectively. The genetic competence of *S. mutans* can be modulated by HtrA, an enzyme degrading CSP. The gene encoding this protein (SMU.2164) was downregulated likely resulting in weak degradation of CSP and possible induction of competence development via the CSP pathway as well.

Mutacins were strongly upregulated, especially *nlm*AB (SMU.150 and SMU.151, mutacin IV), *cip*B (SMU.1914c, *nlmC* and mutacinV) and several other putative bacteriocins (Supplementary Figure S5). The mutacin IV immunity protein (SMU.152) and two genes, which display some sequence homology to SMU.152 (SMU.1909 and SMU.1913), were also upregulated. However, the mutacin V immunity protein (SMU.925) was slightly downregulated. In addition to the CSP-driven mutacin synthesis controlled by the ComDE TCS, bacteriocin synthesis is regulated by three other TCS, HdrRM, BrsRM and VicRK (Merritt and Qi, 2012). The HdrRM (SMU.1854 and SMU.1855) and BrsRM (SMU.2080 and SMU.2081) systems did not show significant changes in gene expression. However, the response regulator VicR (SMU.1517) was slightly upregulated in all dual-species biofilms. It was reported that it negatively affects transcription of *comC*, *comDE* and *sigX* (Senadheera *et al.*, 2012) and in this way

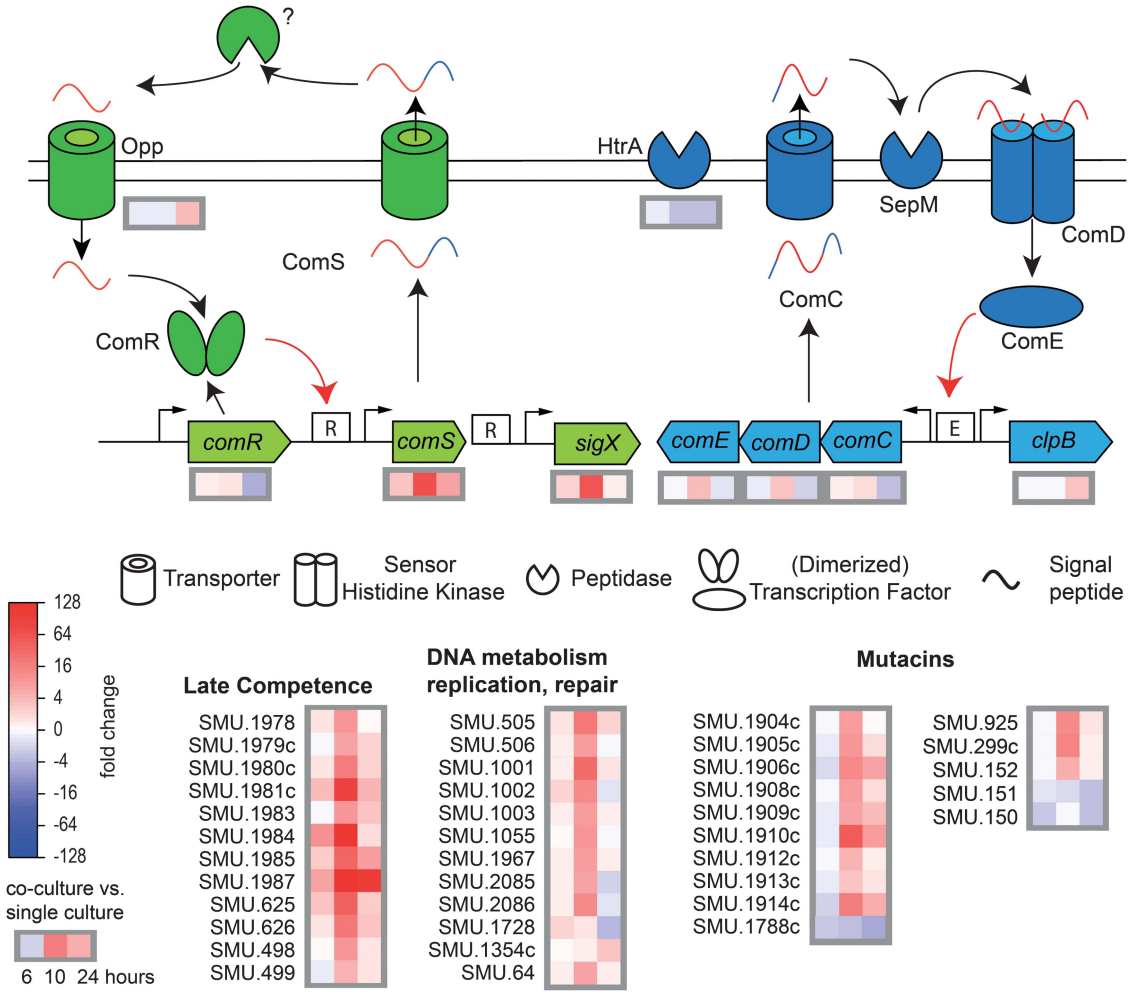

Figure 6 Induction of the quorum sensing regulon and late competence genes in dual-species biofilms. Upper panel: schematic view of the quorum sensing regulon of *S. mutans* (modified from (Perry *et al.*, 2009; Lemme *et al.*, 2011)) and differential gene expression after 6, 10 and 24 h of biofilm growth in dual-species biofilms with *C. albicans* compared with single-species biofilms of *S. mutans* alone. The ComRS system is shown in green, the ComCDE system is shown in blue. Black and red arrows correspond to processing of the signalling peptide and transcriptional regulation by ComR/E, respectively. Pictograms are explained below the scheme. Lower panel: differential gene expression of the late competence genes, genes related to DNA metabolism and repair and mutacins of *S. mutans* in dual-species biofilms.

modulates the CSP-driven signalling system of *S. mutans.* The data show that mutacins were coordinately induced with the development of genetic competence through CSP and XIP, and that even the negative regulator VicR was not able to decrease this effect in dual-species biofilms.

Competent cells of *S. pneumoniae* produce cell wall hydrolases, so-called fratricins, which are secreted by the competent subpopulation and kill the non-competent brothers—hence the name (Berg *et al.*, 2012; Wei and Havarstein, 2012). In *S. mutans*, the gene SMU.836 is 100% identical to *cbpD*, the key fratricin of *S. pneumoniae*. It was recently shown that this cell wall hydrolase actually acts as a fratricin in *S. mutans* (Dufour and Levesque, 2013). Interestingly, it was strongly upregulated in 10-h-old dual-species biofilms, that is, during *sigX* activation, together with the adjacent gene SMU.837, which encodes a putative reductase. To protect themselves against their own fratricins, *S. pneumoniae* cells produce an immunity protein termed ComM (Berg *et al.*, 2011). In *S. mutans* the two genes *murN* (SMU.716) and *murM* (SMU.717) show homology to ComM but they were not upregulated. It remains to be studied whether a subpopulation suffered cell death as a result of the highly expressed murein hydrolases.

To summarize, the complete quorum sensing regulon of *S. mutans* was highly induced in co-culture with *C. albicans*, starting from the two signalling pathways for CSP and XIP, and including the corresponding downstream genes, that is, genetic competence, mutacins and fratricins. To test whether indeed genetic competence was functional, we studied the uptake of fluorescently labelled DNA in biofilms of the reporter strain SMP$_{sigX}$GFP. The middle panel of Figure 7 shows that in co-culture with *C. albicans* the cells of *S. mutans* are not only

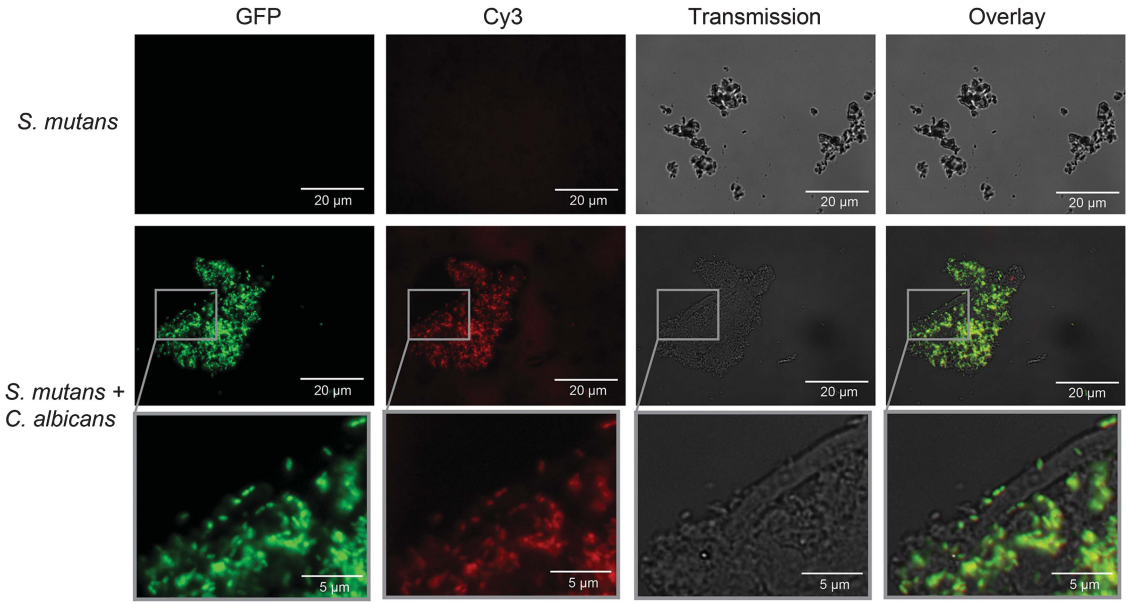

Figure 7 Uptake of DNA in dual-species biofilms of *C. albicans* and *S. mutans*. The reporter strain $SMP_{sigX}GFP$ was cultivated for 10 h alone (top panel) or together with *C. albicans* (middle and bottom panel). DNA labelled with Cy3 was added, and after incubation for 30 min excess DNA was removed by DNAse treatment. See Methods for experimental details. The four rows show (from left to right) the green channel for GPF, the red channel for Cy3, phase contrast and the overlay of red and green channels. Scale bar, 20 µm (top and middle panels) and 5 µm (bottom panel).

fluorescing green, but they have taken up labelled DNA, indicated by red fluorescence of the same cells. Both views can be overlaid resulting in yellow fluorescence. The lower panel in Figure 7 shows a magnification of the indicated window from the middle panel. Single cells of *S. mutans* can clearly be observed as they attach to the hyphae of *C. albicans* or form flocs and are fluorescing both green and red, indicating induction of *sigX* as well as uptake of DNA. None of this can be seen in single-species biofilms of *S. mutans* (upper panel).

Polysaccharide synthesis shifted from the extracellular EPS component glucan to the intracellular storage polymer glycogen

The microarray data suggested fundamental changes in sugar metabolism in dual-species biofilms (Figure 8; Supplementary Table S4). The main component of EPS in *S. mutans* biofilms are glucans and fructans, which are synthesised by extracellular glucosyltransferases (Gtts) and fructosyltransferases, respectively (Bowen and Koo, 2011). The two main glycosyltansferase genes, *gtfB* and *gtfC*, as well as the fructosyltransferase gene *scrK* showed reduced transcript abundance already at 6 h of dual-biofilm growth (Supplementary Table S4). *GtfB* expression was reduced 5.6-fold and 9.7-fold at 10 h and 24 h of dual-biofilm growth, respectively. In addition, expression of the glucan-binding protein GbpC was strongly downregulated (up to 8.1-fold). Sucrose is not only used for extracellular glucan synthesis in *S. mutans*, but also as a carbon source and metabolised intracellularly. However, the genes of the sucrose operon were all downregulated, whereas

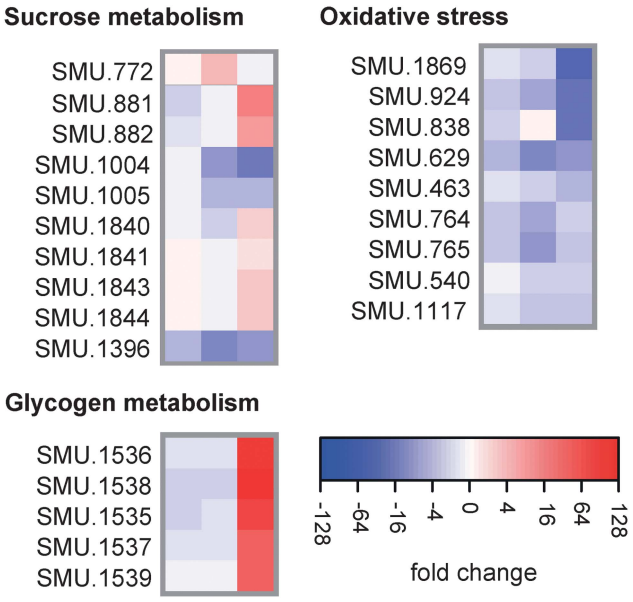

Figure 8 Transcriptional profiling of genes related to sugar metabolism and oxidative stress in dual-species biofilms. Gene expression after 6, 10 and 24 h of biofilm growth of *S. mutans* in dual-species biofilms with *C. albicans* compared with expression in single-species biofilms of *S. mutans* alone.

the sucrose operon repressor ScrR was upregulated. Only in 24-h-old dual-species biofilms, the sucrose phosphorylase GtfA and the sugar-binding transporter MsmK were upregulated (9.2-fold and 6.3-fold, respectively). These data suggest that both extracellular and intracellular sucrose metabolism was suppressed in *S. mutans* in dual-species biofilms.

By contrast, genes encoding enzymes for glycogen synthesis (SMU.1535–SMU.1539) were strongly upregulated in 24-h dual-species biofilms (between 23.3- and 65.9-fold). One can hypothesize that as a result of the high activity of the MsmK transporter an excess of intracellular monosaccharides was present in 24-h-old biofilm cells of *S. mutans*, which most likely were converted to the storage compound glycogen.

Sugar composition in biofilm supernatants

Growth experiments and microarray data suggested major changes in the metabolism of the cultivation medium in dual-species biofilms. Therefore, gas chromatography–mass spectrometry was applied for the analysis of sugars present in the conditioned media (Figure 9; Supplementary Figure S6). Chromatograms from *S. mutans* alone at 10 h showed a reduction in the peaks for sucrose and GlcNAc in comparison with the cultivation medium, indicating that both compounds were metabolised. New peaks appeared (3,4,5 and 6) that were identified as glucose and fructose. They were already present at 4 h of growth and increased in peak area until 24 h of biofilm growth. *C. albicans* alone also reduced both the GlcNAc and the sucrose peak; however, it produced only minute amounts of fructose and an unidentified monosaccharide, which might be a pentose or a deoxy-hexose (peak 7). In dual-species biofilms (10 h), strikingly the sucrose peak, which was prominent in single-species biofilms at that time, had already disappeared, and the GlcNAc peak was substantially reduced in comparison with the single-species biofilms. Monosaccharides were also almost completely depleted in dual-species biofilms at 10 h. After 24 h of biofilm growth, only the putative pentose peak remained in the dual-species biofilm supernatant, whereas single-species biofilms still contained large amounts of monosaccharides in the case of *S. mutans* and GlcNAc in the case of *C. albicans*. The data show that the kinetics of sugar metabolism differed in single- and dual-species biofilms. Sucrose was lacking from the culture medium after 10 h of growth in dual-species biofilms.

Discussion

The data show strong synergism in dual-species biofilms of *S. mutans* and *C. albicans* resulting in increased biofilm mass and cell densities, induction of the complete quorum sensing system of *S. mutans* and lack of the production of EPS, which is fundamental for the cariogenic dental plaque biofilm succession. Thus, in the presence of *C. albicans*, under the conditions used in our experiments, the cariogenicity of *S. mutans* was reduced. At the same time, the induction of quorum

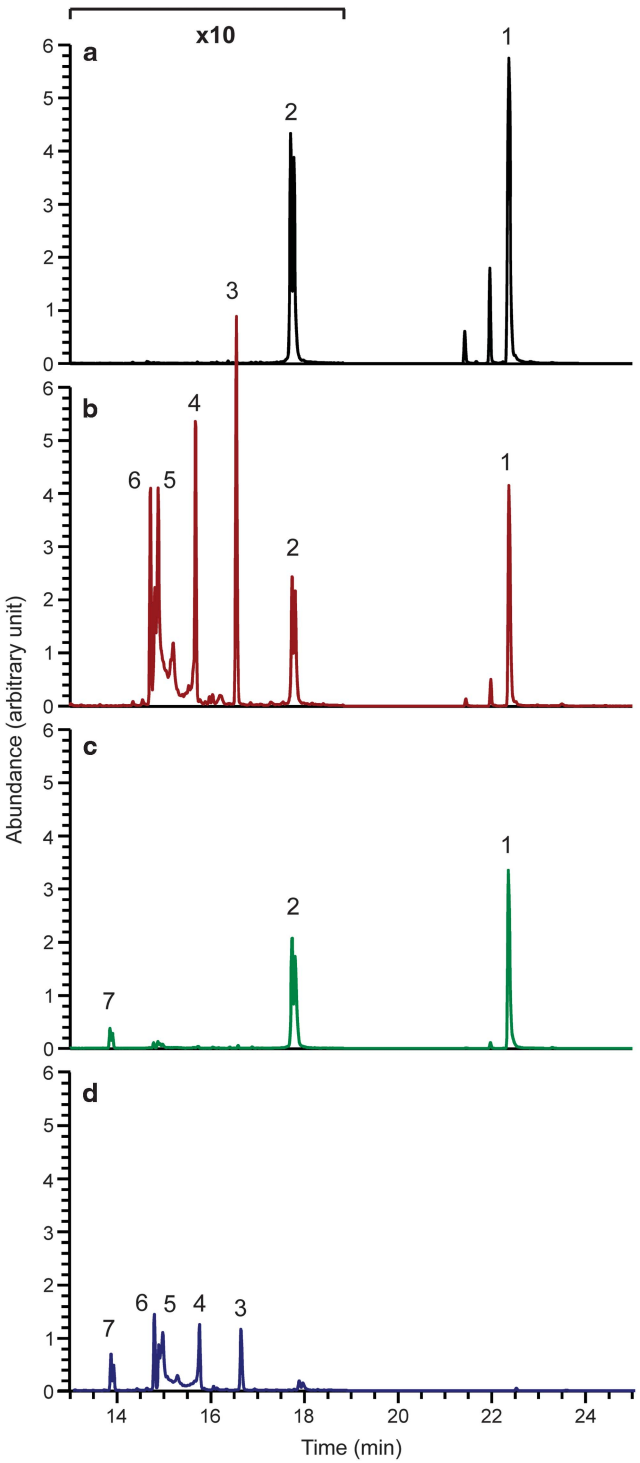

Figure 9 Sugar composition of the cultivation medium after 10 h of biofilm growth. Biofilm supernatants were sterile filtered and analysed by gas chromatography–mass spectrometry. (**a**) Cultivation medium, (**b**) *S. mutans* biofilm supernatant, (**c**) *C. albicans* biofilm supernatant and (**d**) spent medium from dual-species biofilm of *S. mutans* and *C. albicans*. The following peaks were identified: 1, sucrose; 2, *N*-acetylglucosamine; 3 and 4, glucose; 5 and 6, fructose; 7 possibly C5 sugars (pentose). Peak height shows the maximum ion count for this specific mass (arbitrary units). Note the 10-fold enlargement of the y-axis in the left part of the chromatogram.

sensing resulted in phenotypes important for survival (mutacin synthesis) or genetic adaptation (genetic competence). Growth of *C. albicans* in the virulent hyphal mode was enhanced, thus it was a win-win situation for both pathogens. By contrast, the interaction between *P. aeruginosa* and *C. albicans* is detrimental for the fungus. Co-culture with *P. aeruginosa* results in reduced biofilm formation of *C. albicans* (Holcombe *et al.*, 2010) and this could be owing to excreted phenanzines (Morales *et al.*, 2013) or to bacterial lipopolysaccharides (Bandara *et al.*, 2013).

It has been reported that *S. mutans* can improve growth of *C. albicans* biofilms (Pereira-Cenci *et al.*, 2008) and that *C. albicans* profits from lactic acid excreted by *S. mutans* (Metwalli *et al.*, 2013). The gas chromatography–mass spectrometry data show that monosaccharides released by the extracellular glycosyl- und fructosyltransferases of *S. mutans* from sucrose (Ajdic *et al.*, 2002) were depleted from the spent medium in dual-species biofilms at 10 h, and thus may have been taken up by *C. albicans*. Moreover, *C. albicans* may have been more efficient than *S. mutans* in taking up sucrose, as this sugar was depleted in dual-species biofilms after 10 h, and genes for enzymes for both the extracellular and intracellular sucrose metabolism of *S. mutans* were downregulated.

There are 14 phosphotransferase systems for sugar uptake in *S. mutans*, five of which are constitutively expressed independent of the presence of sugars; these five phosphotransferase systems are specific for sucrose (Ajdic and Pham, 2007). A small fraction of sucrose is metabolized by *S. mutans* extracellularly for the synthesis of fructan and glucan, the main polysaccharides comprising the EPS matrix. The responsible glycosyltransferase enzymes (GtfB, GtfC and GtfD) release fructose, whereas the fructosyltransferases (Ftf) release glucose (Ajdic *et al.*, 2002).

However, the transcriptome analysis showed downregulation of *gtfB*, *gtfC* and *scrK* at all stages of growth of the dual-species biofilm. These enzymes are induced by their substrate sucrose (Shemesh *et al.*, 2007), which was initially present at a high concentration in YNBB, and depleted in 10-h-old biofilms. We hypothesize that these extracellular enzymes were initially present in the biofilms, resulting in synthesis of monosaccharides and glucan, but that their transcription was rapidly downregulated due to depletion of sucrose. The half-life of most proteins is in the range of 24 h, whereas that of mRNA is around 5 min (Moran *et al.*, 2013). Excreted glucosyltransferases have been shown to be active on *Candida* surfaces (Gregoire *et al.*, 2011) and thus are likely to have been operating although their transcription was rapidly downregulated.

Interestingly, *S. mutans* failed to produce EPS in co-culture with *C. albicans* or in spent medium from 10-h dual-species biofilms. We first hypothesized that an inhibitor of glucosyltransferases might have been excreted by *C. albicans* potentially providing an interesting anti-caries compound (Koo and Jeon, 2009; Koo *et al.*, 2013). For example, farnesol has been shown to inhibit glucosyltransferases (Koo *et al.*, 2003). However, as the biofilms were mostly composed of hyphae, it is unlikely that farnesol, which inhibits the yeast-to-hyphae transition (Lindsay *et al.*, 2012), was present at sufficiently high concentrations. Another explanation for the lack of EPS synthesis in dual-species biofilms might be that the glucosyl- und fructosyltransferase enzymes were inactive owing to a lack of their substrate. The gas chromatography–mass spectrometry data show that sucrose was depleted in in co-culture with *C. albicans* after 10 h of biofilm growth and no EPS was formed by *S. mutans* cultivated in such spent media. Accordingly, glucosyl- und fructosyltransferase enzymes were downregulated in dual-species biofilms.

The quorum sensing system of streptococci has encountered a paradigm shift in recent years owing to the discovery of a novel competence-inducing peptide, termed XIP, the proximal response regulator *comR* and the gene for the synthesis of the XIP precursor *comS*, all of which are highly conserved in streptococci (Mashburn-Warren *et al.*, 2010; Fontaine *et al.*, 2013). In *S. mutans*, the heptapeptide XIP induces competence in an unimodal way, whereas the previously studied CSP induces competence only in a fraction of the cells while the majority remains uninduced and a small subpopulation undergoes autolysis (Lemme *et al.*, 2011; Dufour and Levesque, 2013). Interestingly, the cultivation medium controls which of those two circuits is activated: XIP is only active in a peptide-free medium; it has been suggested that peptides might block the Opp transporter required for import of the active XIP heptamer (Federle and Morrison, 2012). Conversely, CSP works only in a peptide-rich medium—here it has been suggested that the membrane-bound HtrA enzyme, which can degrade CSP, must be saturated by peptides to reduce CSP degradation (Desai *et al.*, 2012; Federle and Morrison, 2012). In addition, it has been shown for *S. thermophilus* that the ComR response regulator is tolerant to small changes in the sequence of XIP and can even be activated by hydrolysis products of casein, that is, octapeptides present in the medium (Fontaine *et al.*, 2013).

These studies were done with pure cultures of streptococci. To the best of our knowledge the induction of the quorum sensing system of *S. mutans* by another organism, in this case a eukaryotic fungus, has not been observed before. In our data, the *sigX*-driven competence was strongly induced in mixed biofilms of *S. mutans* with *C. albicans* as well as by supernatants from mixed biofilms. If *comS* was knocked out, no induction occurred. How then was *comS* synthesis induced 68-fold in dual-species biofilms?

The YNBB medium contained casamino acids, which are obtained by hydrolysis; thus traces of peptides may have been present; however, no induction of the *sigX* promoter occurred in the sterile medium. *comS* could have been induced by a novel unknown signal secreted only in dual-species biofilms, either by *C. albicans* or by *S. mutans*; several TCS of *S. mutans* have been shown to induce competence but their signal is not known (Okinaga *et al.*, 2010). Alternatively, *C. albicans* may have produced XIP by proteolysis of *S. mutans* proteins and thus triggered the autoinduction of *comS*. *C. albicans* grew in the invasive hyphal mode, excreting an array of hydrolases (Sorgo *et al.*, 2013). The secreted aspartatic proteases (Sap) are the largest group, comprising 10 types, Sap1–Sap10, of which Sap4 to Sap6 were enriched at pH 7.4 in cultures grown in the presence of GlcNAc (Sorgo *et al.*, 2010). More data are needed to confirm this hypothesis and other possibilities cannot be ruled out.

In the dual-species biofilm, the synthesis (*comC*) and sensing (*comDE*) of CSP was only weakly activated, but a high abundance of mutacin transcripts was nevertheless observed. The ComDE TCS directly regulates mutacin synthesis through specific promoter-binding sites for the ComE response regulator (Perry *et al.*, 2009; Hung *et al.*, 2011). Recently, it has been shown that mutacin

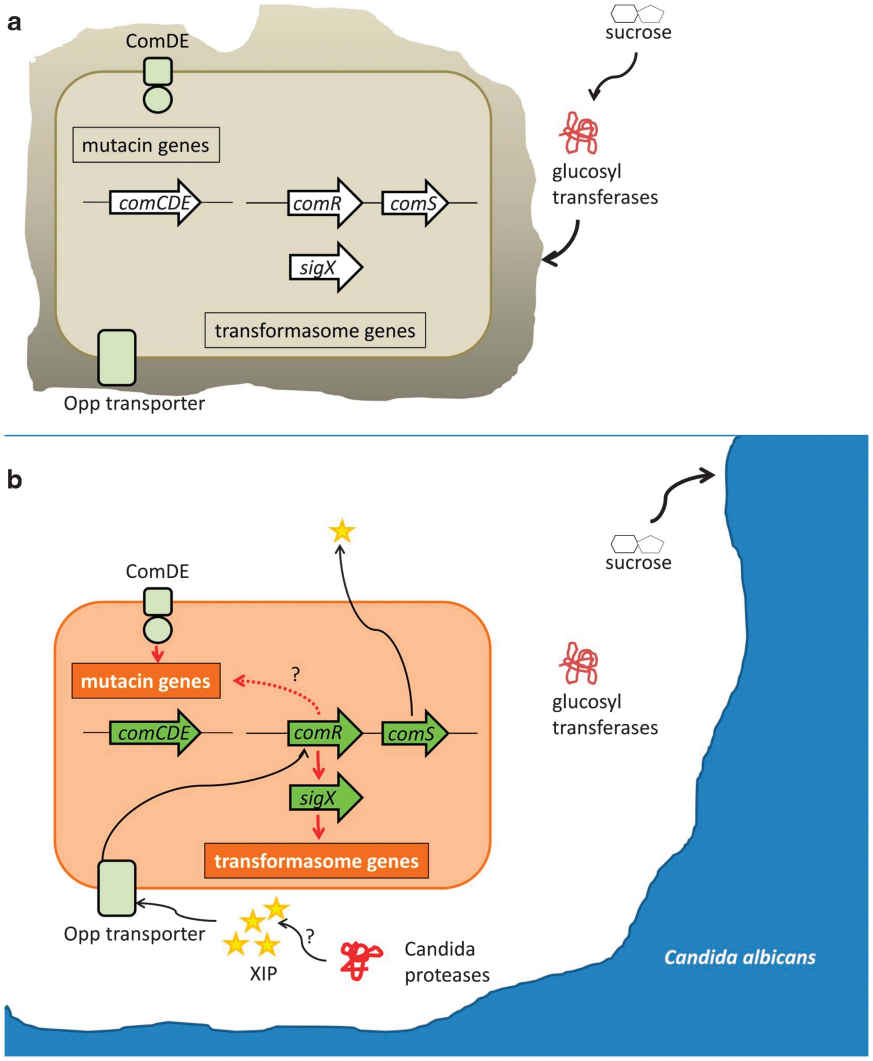

Figure 10 Working hypothesis for cross-feeding and interkingdom communication in dual-species biofilms of *S. mutans* and *C. albicans*. *S. mutans* growing in single culture in a biofilm (**a**) forms EPS from sucrose due to the glucosyltransferase exoenzymes. The quorum sensing genes are not activated. In the presence of *C. albicans* (**b**) sucrose is taken up by *C. albicans* and no EPS is formed by *S. mutans*. Extracellular proteases of *C. albicans* could degrade *S. mutans* proteins resulting in the production of the heptamer XIP, which is the main quorum sensing signal of *S. mutans*. XIP is internalized by the Opp transporter and activates the transcriptional regulator *comR*, thereby triggering activation of the quorum sensing signalling cascade. ComR induces transcription of *comS*, the precursor of XIP, which is processed and exported to yield active extracellular XIP, resulting in a positive feedback loop. ComR induces expression of the alternative sigma factor *sigX*, resulting in transcription of the transformasome genes and genetic competence of the cell. Bacteriocin synthesis is induced either through the response regulator ComE or through ComR. Note that the positive feedback loop established through *comRS* is essential for the observed induction of competence in co-culture, which was not obtained if the *comS* gene was knocked out in *S. mutans*.

synthesis can also be induced by comR. In *S. thermophilus* the ComR-box for binding of ComR is not only found upstream of *sigX* and *comA*, but also upstream of a number of bacteriocin-encoding genes in the locus *blp* (Fontaine *et al.*, 2013). In *S. mutans* two paralogous *comR* loci are present, of which SMU.61 likely controls competence, whereas SMU.381 controls mutacins (Mashburn-Warren *et al.*, 2010). The mutacin-related loci controlled by comR are distinct from those controlled by the *comCDE* TCS and could provide a link between comR induction and mutacin synthesis, which does not require induction through CSP. Figure 10 summarizes our main findings and working hypotheses on cross-feeding and interkingdom communication between *C. albicans* and *S. mutans* in the biofilms studied here.

Conclusion

It is increasingly becoming clear that oral diseases, for example, caries and periodontitis, are polymicrobial and that *C. albicans* has a crucial role for caries progression. Here, we show that interactions with *C. albicans* in biofilms result in changes of *S. mutans* virulence that could not have been anticipated from studying pure cultures. Given the discovery of a conserved core of quorum sensing genes in streptococci, it will be interesting to determine whether *C. albicans* has a similar role for all of those species. *In vivo* data are required to observe these interactions in the human host. The synergism between *C. albicans* and *S. mutans* shown here could potentially have an important role for early childhood caries and other polymicrobial biofilm infections.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank Kathrin Opolka, Ina Schleicher and Celine Krajewski for technical assistance. Funding by the DFG TRR51 for JT, by the European Commission COMBACTE project for MRe and by the German Ministry for Economy ZIM program for SPS is gratefully acknowledged. We greatly appreciate the comments of two anonymous reviewers that gave us a chance to make the manuscript stronger.

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