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

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Abstract

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 substance (EPS) by *S. mutans* was strongly suppressed, which was confirmed by scanning electron microscopy, GC/MS 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 XIP precursor abolished this activity, while deletion of *comC* encoding the CSP 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.

Introduction

It has been estimated that 80 % of human infections result from pathogenic biofilms (Harriott et al., 2011). Clinically, biofilm infections represent an overwhelming problem, since the microorganisms embedded in the extracellular matrix are resistant to antibiotics as well as to the host defence. Polymicrobial pathogenic biofilms are 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).

Candida albicans is the most prevalent opportunistic human pathogenic fungus (Kim et al., 2011) and can cause infections of mucosal membranes (candidiasis) and 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 (Gow et al., 2012;Sudbery, 2011). Hyphae provide structural integrity to biofilms (Banerjee et al., 2013;Finkel et al., 2011). *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 which can ferment a large spectrum of dietary sugars. The excreted organic acids result in a strong localized pH drop which 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 (Burne et al., 2012;Kutsch et al., 2011;Takahashi et al., 2011). Biofilm formation in *S. mutans* is readily induced by dietary

sugars which are transformed to extracellular polysaccharides by dedicated enzymes which are therefore important targets of anti-caries strategies (Bowen et al., 2011).

C. albicans and *S. mutans* are found together in early childhood caries (EEC) (de Carvalho et al., 2006;Marchant et al., 2001;Raja et al., 2010) and on bracket materials (Rammohan et al., 2012). ECC 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) 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 2011). 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 due 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 (ALS3) (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 play 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 PQS 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, mutacin production and 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) (Khan et al., 2012;Mashburn-Warren et al., 2010) which 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 et al., 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*, i.e. mutacin synthesis and genetic competence, have their largest impact in multi-species systems and especially in biofilms, which provide high local concentrations of chemical cues as well as direct cell-cell contact. Since *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 virulence of *S. mutans*.

Materials and Methods

Strains and culture conditions

Microorganisms used were *S. mutans* UA159 wild-type (ATCC 700610), *S. mutans* UA159 $SMP_{sigX}GFP$ (Lemme et al., 2011) and *C. albicans* (DSM 11225). 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) at 37°C aerobically with 5% CO_2 without shaking. *C. albicans* pre-cultures were grown in yeast nitrogen base (YNB) synthetic medium (Difco 291940) supplemented with maltose (1 g/L) and glucose (2 g/L) 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, Difco 291940), 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, Becton, Dickinson and Company, Sparks, MD USA) and sucrose (5 g/L). The pH during biofilm growth was in the range of pH 7.0 to 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 rpm, 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. 1 ml suspensions of one strain only (*S. mutans* or *C. albicans*) were prepared for single strain inoculums. The microtitre

plates (Nuncbrand, Denmark) used for biofilm formation were coated with artificial saliva for 1 h at 37°C to mimick the conditions in the oral cavity (Wong et al., 2001). Natural saliva plays 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 (Perkin Elmer, Waltham, USA). Fluorescence microscopic analysis of biofilms was carried out using an Olympus BX60 microscope (Olympus, Seelze, Germany) equipped with a color view II camera and a 100x immersion oil objective. The filter U-MWIBA3 (excitation 460-495 nm; emission 510 – 550 nm, 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 hours. Afterwards the planktonic phase of the culture was withdrawn and the fluorescence intensity was recorded with a Wallac 1420 Multilabel counter (Perkin Elmer, Waltham, USA). Fresh YNBB medium was used as a control. Additionally we have tested induction of *sigX* by CSP, XIP and farnesol added to the mature biofilm (8 h). After two and four hours 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, 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 in 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 x PBS buffer and stained with 50 µl Concanavalin A labelled with AlexaFluor 488 (100 µg/ml) for 40 min at room temparature in the dark. Subsequently the staining solution was withdrawn, the biofilm was washed with 50 µl of 1 x PBS and then counterstained with 10 µM DAPI 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 labeled 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. 2 µg of the purified DNA amplicon 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 according to the manufacturer`s instructions. The degree of labelling (DoL) was

189 determined by measuring absorbance at 260 nm and 550 nm. Material with a DoL of 1.5 was
190 used. DNA uptake was tested as described with modifications (Lemme et al., 2011). Biofilms
191 of the *S. mutans* reporter strain SMP$_{sigX}$GFP alone and together with C. albicans were culti-
192 vated as decribed for 10 h. The supernatants were removed and biofilm of 3 wells was sus-
193 pended in 100 µl of fresh YNBB medium without sucrose. To disrupt cell chains, the biofilm
194 was sonified for 10 cycles of 5 sec with 10 sec pause at 10% power (Bandelin Electronic, Ber-
195 lin, Germany) on ice. The Cy3 labelled DNA (final concentration 5 µg/ml) was added to the
196 biofilm cells and incubated in the dark for 30 min at 37°C. Excess DNA was digested with
197 DNAse I and the biofilms were analysed by fluorescence microscopy. The red channel was
198 used to visualize Cy3, and the green channel to observe GFP.

199 Methods for preparation of conditions media, construction of knock-out mutants, quantitative
200 PCR, extraction of DNA and RNA, microarray analysis and GC/MS analysis are described in
201 Supplementary Methods S1.

202 # Results

203 ## Cultivation medium

204 We used a chemically defined synthetic medium commonly used for cultivating *C. albicans*
205 as a basis. Sucrose was provided as a carbon source, since it is fermented by both species, and
206 in *S. mutans* induces strong biofilm formation. *S. mutans* is auxotrophic for several amino
207 acids (Ajdic et al., 2002), therefore we added casamino acids. GlcNAc was provided to induce
208 hyphae formation in *C. albicans* (Sudbery, 2011) and the medium was buffered to exclude
209 different pH-effects in single and dual-species biofilms. In this medium, designated YNBB,
210 both microorganisms formed biofilms and *C. albicans* grew predominantly in the hyphal
211 form. Phase contrast microscopy (Fig. 1a) showed that *C. albicans* and *S. mutans* were in di-
212 rect 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 (Fig. 1b). At 10 h 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 were significantly higher than in mono-species biofilms, both for *S. mutans* and *C. albicans* (Fig. 1c and 1d). 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 (Fig. 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 (Fig. 1f) little EPS was visible, and many cells of *S. mutans* appeared to be "naked", i.e. 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 plays 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 labeled with the green fluorescent dye AlexaFluor488 (Fig. 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 if QS 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 if fused to GFP. Upon induction of SigX green fluorescence is observed. It was quantified using a fluorescence plate reader (Fig. 3a), by RT-PCR of *sigX* in the wild-type (Fig. 3b) and observed directly under the fluorescence microscope (Fig. 3c). Strong induction of P$_{sigX}$ was observed in dual-species biofilms with *C. albicans* (Fig. 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 qRT-PCR (Fig. 3b). A 73fold increase of *sigX* expression was observed in co-culture with *C. albicans* at 10 h in comparison to *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 due to the stability of gfp. P$_{sigX}$ induction was confirmed by microscopical analysis. The majority of *S. mutans* cells revealed strong green fluorescence (Fig. 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 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 if (1) an unknown molecule present in the cultivation medium or (2) one of the known QS signaling molecules of *S. mutans* or *C. albicans*, respectively, might be responsible for the induction of *sigX* in co-culture. Finally we also tested if (3) the activating compound was present in the spent medium, or if 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 hours) were applied to biofilms of the reporter strain SMP$_{sigX}$GFP grown for 6 h, 10 h 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 (Fig. 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 if 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 (Fig. 4b). The activation was particularly strong for 8 h 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 µM and 100 µM, respectively. These data suggest that XIP might be responsible for P$_{sigX}$ activation in dual-species biofilms.

To test if XIP was present in the supernatants or if 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 (Fig. 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 if 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 (Fig. 5 right and left panel). 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* (Fig. 1f). This effect was already observed after 4 h of growth of test-biofilms in dual-species biofilm extract (Fig. S1). We conclude that supernatants of dual-species biofilms prevented EPS synthesis of *S. mutans*. We tested if XIP, the QS molecule excreted by *S. mutans*, might be able to suppress EPS formation, but this was not the case (Figure S2).

To determine if an extracellular enzyme might be present able to destroy the polysaccharides constituting the EPS matrix, e.g. 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 h 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 to gene expression of *S. mutans* in mono-species biofilms. We found 510 genes that were differentially expressed. They were sorted into 6 different groups

according to their expression profile using the c-means algorithm (Kumar et al., 2007) (Fig. S3) and putative biological functions were assigned based on clusters of orthologous groups (COG) (Fig. S4). Several microarray data were confirmed by q-RT-PCR (Table S1). All transcripts showing high abundance in the microarray data revealed similar or higher gene expression levels in q-RT-PCR, in accordance with the higher dynamic range of q-RT-PCR which has often been observed.

The most abundant transcripts belonged to the quorum sensing regulon

At ten hours 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 (Fig. 6, Fig. S5; Table S4). Particularly, *comS*, *sigX* and the late competence genes (*com YA*, *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 up-regulated, 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 up-regulated 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 down-regulated likely resulting in weak degradation of CSP and possible induction of competence development via the CSP pathway as well.

Mutacins were strongly up-regulated, especially *nlm*AB (SMU.150 and SMU.151, mutacin IV), *cip*B (SMU.1914c, *nlmC,* mutacinV) and several other putative bacteriocins (Fig. 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 up-regulated. However, the mutacin V immunity protein (SMU.925) was slightly down-regulated. 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 et al., 2012). The HdrRM (SMU.1854, SMU.1855) and BrsRM (SMU.2080, SMU.2081) systems did not show significant changes in gene expression. However, the response regulator VicR (SMU.1517) was slightly up-regulated 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 modulates the CSP driven signaling 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 et al., 2012). In *S. mutans* the hypothetical protein 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 et al., 2013). Interestingly, it was strongly up-regulated in 10 h old dual-species biofilms, i.e. 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 up-regulated. It remains to be studied if 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 down-stream genes, i.e. genetic competence, mutacins, and fratricins. To test if 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 Fig. 7 shows that in co-culture with *C. albicans* the cells of *S. mutans* are not only 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 Fig. 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 (Fig. 8 and 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 et al., 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 (Table S2). *GtfB* expression was reduced 5.6fold and 9.7fold at 10 h and 24 h of dual biofilm growth, respectively. Additionally, expression of the glucan-binding protein GbpC was strongly down-regulated (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 down-regulated, while the sucrose operon repressor ScrR was up-regulated. Only in 24 h old dual-species bio-

384 films, the sucrose phosphorylase GtfA and the sugar binding transporter MsmK were up-
385 regulated (9.2fold and 6.3fold, respectively). These data suggest that both extracellular and
386 intracellular sucrose metabolism was suppressed in *S. mutans* in dual-species biofilms.
387 By contrast, genes encoding enzymes for glycogen synthesis (SMU.1535-SMU.1539) were
388 strongly up-regulated in 24 h dual-species biofilms (between 23.3 and 65.9 fold). One can
389 hypothesize that as a result of the high activity of the MsmK transporter an excess of intracel-
390 lular mono-saccharides was present in 24 h old biofilm cells of *S. mutans* which most likely
391 were converted to the storage compound glycogen.

392 **Sugar composition in biofilm supernatants**

393 Growth experiments and microarray data suggested major changes in the metabolism of the
394 cultivation medium in dual-species biofilms. Therefore GC/MS was applied for the analysis
395 of sugars present in the conditioned media (Fig. 9, Figure S6). Chromatograms from *S. mu-*
396 *tans* alone at 10 h showed a reduction in the peaks for sucrose and GlcNAc in comparison to
397 the cultivation medium, indicating that both compounds were metabolised. New peaks ap-
398 peared (3,4,5,6) which were identified as glucose and fructose. They are already present at 4 h
399 of growth and increased in peak area until 24 h of biofilm growth. *C. albicans* alone also re-
400 duced both the GlcNAc and the sucrose peak; however, it produced only minute amounts of
401 fructose and an unidentified monosaccharide which might be a pentose or a deoxy-hexose
402 (peak 7). In dual-species biofilms (10 h), strikingly the sucrose peak, which was prominent in
403 single-species biofilms at that time, had already disappeared, and the GlcNAc peak was sub-
404 stantially reduced in comparison to the single-species biofilms. Monosaccharides were also
405 almost completely depleted in dual-species biofilms at 10 h. After 24 h of biofilm growth,
406 only the putative pentose peak remained in the dual-species biofilm supernatant, while single-
407 species biofilms still contained large amounts of monosaccharides in the case of *S. mutans*
408 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 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 due 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 GC/MS 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, since 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 down-regulated.

There are 14 phosphotransferase systems (PTS) for sugar uptake in *S. mutans*, five of which are constitutively expressed independent of the presence of sugars; these five PTS systems are specific for sucrose (Ajdic et al., 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, GtfD) release fructose, while the fructosyltransferases (Ftf) release glucose (Ajdic et al., 2002).
However, the transcriptome analysis showed down-regulation 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 mono-saccharides and glucan, but that their transcription was rapidly down-regulated due to depletion of sucrose. The half-life of most proteins is in the range of 24 h while 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 down-regulated.

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 et al., 2013;Koo et al., 2009). For example, farnesol has been shown to inhibit glucosyltransferases (Koo et al., 2003). However, since 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 due to a lack of their substrate. The GC/MS data show that

458 sucrose was depleted in in co-culture with *C. albicans* after 10 h of biofilm growth and no
459 EPS was formed by *S. mutans* cultivated in such spent media. Accordingly, glucosyl- und
460 fructosyltransferase enzymes were down-regulated in dual-species biofilms.

461 The quorum sensing system of streptococci has encountered a paradigm shift in recent years
462 due to the discovery of a novel competence inducing peptide, termed XIP, the proximal re-
463 sponse regulator *comR* and the gene for the synthesis of the XIP precursor *comS*, all of which
464 are highly conserved in streptococci (Fontaine et al., 2013;Mashburn-Warren et al., 2010). In
465 *S. mutans*, the heptapeptide XIP induces competence in an unimodal way, whereas the previ-
466 ously studied CSP induces competence only in a fraction of the cells, while the majority re-
467 mains uninduced and a small sub-population undergoes autolysis (Dufour et al., 2013;Lemme
468 et al., 2011). Interestingly, the cultivation medium controls which of those two circuits is acti-
469 vated: XIP is only active in a peptide free medium; it has been suggested that peptides might
470 block the Opp transporter required for import of the active XIP heptamer (Federle et al.,
471 2012). Conversely, CSP works only in a peptide rich medium – here it has been suggested
472 that the membrane bound HtrA enzyme, which can degrade CSP, must be saturated by pep-
473 tides to reduce CSP degradation (Desai et al., 2012;Federle et al., 2012). In addition it has
474 been shown for *S. thermophilus* that the ComR response regulator is tolerant to small changes
475 in the sequence of XIP and can even be activated by hydrolysis products of casein, i.e. oc-
476 tapeptides present in the medium (Fontaine et al., 2013).

477 These studies were done with pure cultures of streptococci. To the best of our knowledge the
478 induction of the quorum sensing system of *S. mutans* by another organism, in this case a eu-
479 karyotic fungus, has not been observed before. In our data the *sigX* driven competence was
480 strongly induced in mixed biofilms of *S. mutans* with *C. albicans* as well as by supernatants
481 from mixed biofilms. If *comS* was knocked out, no induction occurred. How then was *comS*
482 synthesis induced 68fold 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 ten types, Sap1 to 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 (Hung et al., 2011;Perry et al., 2009). Recently it has been shown that mutacin 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, while 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.

Conclusion

It is increasingly becoming clear that periodontal diseases, e.g. 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 if *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.

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Conflict of interest statement

None of the authors of this study report any conflict of interest.

Supplementary information is available at the ISMEJ´s website.

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Figure legends

Fig. 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 standard deviation (SD) 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 SD of three independent experiments with two technical replicates each are shown. (**e**,**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.

Fig. 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 AlexaFluor488 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 10 µm.

Fig. 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 (gray 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 (gray bars) or with *C. albicans* (black bars). The data were normalized to *sigX* expression in a *S. mutans* biofilm after 6 h. Mean and SD 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.

Fig. 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 to 24 hours 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 to 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, while *comS* encodes the synthesis of the XIP precursor. Mean and SD of four experiments are shown in all cases.

Fig. 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 panel) and dual-species biofilms (middle panel) for 10 h and analysed by scanning electron microscopy. Scale bar 5 µm.

Fig. 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 (Lemme et al., 2011;Perry et al., 2009)) and differential gene expression after 6, 10 and 24 h of biofilm growth in dual-species biofilms with *C. albicans* compared to 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 signaling 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.

Fig. 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 channel. Scale bar 20 µm (top and middle panel) and 5 µm (bottom panel).**Fig. 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 to expression in single-species biofilms of *S. mutans* alone.

Fig. 9. Sugar composition of the cultivation medium after 10 h of biofilm growth. Biofilm supernatants were sterile filtered and analysed by GC-MS. (**a**) Cultivation medium, (**b**) *S. mutans* biofilm supernatant, (**c**) *C. albicans* biofilm supernatant, (**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 10fold enlargement of the y-axis in the left part of the chromatogram.