Supplementary Methods S1

Construction of *S. mutans* Δ*comS* and Δ*comC* mutants

For the construction of a *comS* and *comC* deficient strain, the PCR ligation mutagenesis approach [1] was used to replace the *comS*-gene by an erythromycin resistance cassette and *comC* by a chloramphenicol resistance cassette via double homologous recombination. Primers ComSP1, ComSP2 and ComCP1, ComCP2 (see table S1) were used to amplify approximately 1 kb of the 5′ flanking region of *comS* and *comC* and to introduce [2] an AscI restriction site. To amplify the 3′ flanking region, the primers ComSP3, ComSP4 and ComCP3, ComCP4 were used, thereby introducing a restriction site for FseI. The erythromycin resistance cassette was amplified from a previously constructed gene deletion mutant [3] using primers ERMFor and ERMRev, containing the restriction sites for AscI and FseI, respectively. The chloramphenicol resistance cassette was amplified from pC194 using primers CATFor and CATRev, containing the restriction sites for AscI and FseI, respectively. After digestion with the appropriate restriction enzyme(s), the three amplicons were ligated together and transformed in *S. mutans* UA159 according to the method of Li *et al.* [4]. Mutants were selected on THBY agar plates (Becton, Dickinson and Company, Sparks, MD, USA) containing erythromycin (10 µg/ml) or chloramphenicol (10 µg/ml) and verified by PCR using primers flanking *comS* and *comC*, respectively (ComSP1 with ComSP4 and ComCP1 with ComCP4). The deletion of *comC* was additionally confirmed by sequencing.

Preparation of conditioned media and induction of SigX

During the incubation time of single and dual species biofilms the culture supernatants were withdrawn, centrifuged (5000 rpm, 20 min at 4°C) and sterile filtered by applying 0.22 µm filters (Roth, Karlsruhe, Germany) and frozen at -20°C. Conditioned media were used for

induction experiments of the *sigX* promoter in biofilms of the reporter strain. Briefly, *S. mutans* $SMP_{sigX}GFP$ was incubated for 6, 10 and 24 h as single biofilm. The medium was removed, and the supernatants (500 µl) of 6, 8 10, 12 and 24 h single and dual species biofilms were loaded onto the reporter strain biofilms. After 2 h of incubation the induction of *sigX* was measured as described above. The experiment was done in four replicates. To test the dispersion-inducing activity of conditioned media *S. mutans* biofilms were grown in sterile filtered conditioned media of dual and single species biofilms (10 and 12 h). After incubation for 4, 6, 8 and 10 h biofilms were analysed by electron microscopy.

Quantitative reverse transcription PCR (qRT-PCR)

RNA from the same sample which was used for microarray experiments was reverse transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Karlsruhe, Germany). Oligonucleotide primers for qRT-PCR were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3/). The PCR reaction was performed using the Light cycler 480 system (Roche, Manheim, Germany) and the reaction mixtures were prepared using the Quantitect SYBR green PCR kit (Qiagen, Hilden, Germany). Primers used for qRT-PCRare shown in table S2. Annealing temperature was 60°C. Elongation time was 30 s. Genomic DNA of *S. mutans* UA159 was used as a standard. To check the specificity, the primers were tested with *C. albicans* DNA. Non template control was used as a negative control. Changes in the level of gene transcription were calculated according to the method described by [5]. The gyrase A gene (SMU.1114) revealing constant transcription in microarray experiments was used as a reference gene. The changes in transcription were compared to the level of transcription of *S. mutans* growing alone. All quantitative real-time PCR measurements were done in duplicates.

Removal of extracellular DNA from *S. mutans* biofilms

The protocol was modified from [6]. Following removal of culture supernatants the biofilms growing in 24-well microtitre plates were resuspended in 1 ml 0.85% NaCl (4 wells) and centrifuged (13000 rpm, 5 min, 25°C). The pellets were resuspended in 99.5 µl of 50 mM phosphate buffer (pH 7.5) and 0.5 µl PNGase F (250U/100µl) was added for hydrolysis of EPS. After incubation for 1 h with shaking (500 rpm) at 37°C 1 µl of proteinase K (28.8 µg/ml) was added and incubated again at 37°C for 30 min. After centrifugation the pellets were collected for isolation of genomic DNA.

Isolation of genomic DNA of *S. mutans* from biofilms

To disrupt the cells the pellets were resuspended in 200 µl buffer (10 mM Tris, 1 mM EDTA, pH 8) containing mutanolysin and lysozyme (500 U/ml, 15 mg/ml, respectively) and incubated for 1 h at 37°C with shaking (300 rpm). Afterwards Proteinase K (25 µl of a 28.8 µg/ml solution) was added and incubated at 56°C until complete lysis (1 h at 300 rpm). To degrade RNA, 20 µl of RNAse (20 mg/ml in water) was added and incubated for additional 5 min at 25°C. Afterwards 200 µl buffer B3 and 210 µl ethanol were added and the samples were loaded onto the column for DNA purification (NucleoSpin Tissue Kit, Macherey-Nagel, Düren, Germany) and treated as described in the manufacturers protocol. Pure genomic DNA was eluted in BE buffer and applied as a template in q-PCR reactions.

Isolation of genomic DNA of *C. albicans* from biofilms

Isolation of DNA from *C. albicans* was carried out according to the protocol of [7]. Briefly, following removing of EPS the biofilm cells were resuspended in 200 µl spheroblast buffer and 20 µl of zymolase (1.5 U/µL) was added. The sample was incubated at 37°C for 60 min without shaking and afterwards centrifuged at 13000 rpm for 10 min. The resulting pellet was resuspended in 200 µl breaking buffer and subsequently 0.25 g glass beads and 200 µl phenol/chloroform/isoamyl alcohol (Carl Roth, Karlsruhe, Germany) were added. The

samples were vigorously mixed for 3 min and before vortexing once again the 200 µl of 10 x TE buffer was added. Following centrifugation at 13000 rpm for 5 min the aqueous layer was transferred to the 2 ml tubes and 1 ml of 99% ethanol was added and gently mixed for DNA precipitation. After centrifugation at 13000 rpm, 25°C the supernatant was discarded and pellet resuspended in 500 µl 1 x TE buffer. Subsequently, the RNA was digested by adding 30 µl of 1 mg/ml RNase A and following incubation at 37°C for 15 min genomic DNA was precipitated by adding 10 µl of 3 M sodium acetate pH 5.5 and 1 ml of 99% ethanol. The samples were microfuged at full speed for 5 min at room temperature. After removing supernatant the pellets were dried and diluted in 50 µl of TE buffer.

Microarrays

After removal of the supernatants, the single and dual species biofilms grown in 24 well microtitre plates (Fisher Scientific, Schwerte, Germany) were covered with 300 µl RNA protect (Qiagen, Hilden, Germany). The biofilms were scraped off with sterile one-way cell scrapers, transferred to sterile Eppendorf tubes and centrifuged (4°C, 5 min at 13000 rpm). For each sample four biological replicas were prepared. The pellets were stored at -70°C until RNA extraction. For RNA isolation the pellets were resuspended in lysis buffer containing 10 mM Tris, 1 mM EDTA, pH 8.0, 2.5 mg/ml lysozyme (Sigma-Aldrich, Taufkirchen, Germany) and 50 U/ml mutanolysin (Sigma-Aldrich, Taufkirchen, Germany) and incubated at 25°C for 1 h [8]. Afterwards the cells were vortexed for 3 min in the presence of 50 µg sterile, acid-washed glass beads (diameter 106 µm; Sigma-Aldrich, Taufkirchen, Germany). RNA extraction was performed using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer`s instructions. Traces of genomic DNA were removed by RNAse free DNaseI (Qiagen, Hilden, Germany) applying the on-column digestion protocol. The absence of genomic DNA was checked by using it as a template for PCR. If neccessary, additionally the DNA digestion protocol in solution was applied. The concentration of RNA was measured

with a Nanodrop 1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). The quality and integrity of RNA was checked with a Bioanalyzer (Agilent Technologies, Böblingen, Germany). RNA samples were labeled either with Cy3 or Cy5 applying the ULS fluorescence labeling kit (Kreatech Diagnostics, Amsterdam, Netherlands). The degree of labeling (DOL) was calculated according to the manufacturer`s instructions. Seven hundred nanograms of Cy3 or Cy5 labeled RNA were fragmented and hybridized to the microarrays according to the Agilent protocols. The design of a whole-genome microarray containing all open reading frames as well as all intergenic regions of *Streptococcus mutans* UA 159 was described previously [9].

At least three biological of labeled samples were used for array experiments. The arrays were scanned using the Agilent microarray scanner. Raw data were extracted and background corrected by using Agilent Feature Extraction software (v. 10.7). Afterwards the data were processed using the LIMMA package [10,11] of the Bioconductor package written in R language (http//www.r-project.org). We compared the transcriptome of single *S. mutans* biofilms to the transcriptome of *S. mutans* growing with *C. albicans* in dual species biofilms. Genes with a log2fold of change ≥ 1.5 and *P* value of ≤ 0.05 were selected for further analysis. The genes were assessed into functional groups according to clusters of orthologous groups (COG). Microarray data have been submitted to the GEO database at NCBI with the accession number GSE52543.

The microarray data were confirmed by quantitative reverse transcription PCR (qRT-PCR). The experimental methods for qRT-PCR, removal of extracellular DNA from *S. mutans* biofilms, extraction of genomic DNA of *S. mutans* from biofilms and extraction of genomic DNA of *C. albicans* from biofilms are described in Supplementary Methods S1.

Quantitative PCR for determination of cell numbers in biofilms

To quantify the number of *S. mutans* and *C. albicans* cells quantitative PCR was performed using the LightCycler®480 (Roche Diagnostics, Mannheim, Germany) and the Quantitect SYBR green PCR kit (Qiagen, Hilden, Germany). Genomic DNA after removal of extracellular DNA (eDNA) was used as a template. Specific primers for the 16S rRNA gene of *S. oralis* [12] and the 18S rRNA gene of *C. albicans* [13] were used. No cross-hybridization of the primers was observed. The primers for *S. mutans* UA159 were forward primer GATACATAGCCGACCTGAG and reverse primer TCCATTGCCGAAGATTCC, and the annealing temperature was 59°C. The primers for *C. albicans* were forward primer GGATTTACTGAAGACTAACTACTG and reverse primer GAACAACAACCGATCCCTAGT, and the annealing temperature was 59°C. The q-PCR was performed using the thermocycling conditions recommended for the SYBR green PCR Master Mix (95°C for 30 s and 40 cycles of 30 s at 95°C and 30 s at 59°C). DNA concentrations (ng/ml) were calculated based on standard curves obtained by using 10-fold serial dilutions of bacterial DNA isolated according to the protocol described above. To convert nanograms of DNA to numbers of cells, the following weights and genome sizes were used: 2.083 fg per 2 Mbp genome for *S. mutans* [12] and 30.49 fg per 15 Mbp genome for *C. albicans*. It was taken into account that *S. mutans* has five 16S rRNA genes per genome, and that *C. albicans* has one 18S rRNA gene per genome and is diploid. Genomic DNA quantified using Quant-iT PicoGreen (Molecular Probes, Eugene, OR) was used as a standard. The data were compared to the standard curves for genomic DNA of both microorganisms. All experiments were done in two biological and two technical replicas.

Mono- and disaccharide analysis by GC/MS

Aliquots (10 µl) of the sterile filtered spent media were dried followed by trimethylsilylation using a mixture of pyridine and N,O-Bistrimethylsilyltrifluoracetamide in a ratio of 2:1 and heated to 70° C for 1 hour. The resulting products were diluted tenfold with cyclohexane and

analyzed by GC/MS using a Thermo-Finnigan GCQ ion trap mass spectrometer (Finnigan MAT Corp., San Jose, CA) running in the positive-ion electron impact (EI) mode equipped with a 30-m DB5 capillary column. Oligosaccharide compounds were identified by comparison of their elution times and mass spectra with those of standard saccharides. Unknown monosacchrides were identified by their intense fragment ions at m/z 204 and 217, which are characteristic for trimethylsilylated sugar residues.

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