**Streptococcus mutans Competence-Stimulating Peptide Inhibits Candida albicans Hypha Formation**

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The oral cavity is colonized by microorganisms growing in biofilms in which interspecies interactions take place. *Streptococcus mutans* grows in biofilms on enamel surfaces and is considered one of the main etiological agents of human dental caries. *Candida albicans* is also commonly found in the human oral cavity, where it interacts with *S. mutans*. *C. albicans* is a polymorphic fungus, and the yeast-to-hypha transition is involved in virulence and biofilm formation. The aim of this study was to investigate interkingdom communication between *C. albicans* and *S. mutans* based on the production of secreted molecules. *S. mutans* UA159 inhibited *C. albicans* germ tube (GT) formation in cocultures even when physically separated from *C. albicans*. Only *S. mutans* spent medium collected in the early exponential phase (4-h-old cultures) inhibited the GT formation of *C. albicans*. During this phase, *S. mutans* UA159 produces a quorum-sensing molecule, competence-stimulating peptide (CSP). The role of CSP in inhibiting GT formation was confirmed by using synthetic CSP and a comC deletion strain of *S. mutans* UA159, which lacks the ability to produce CSP. Other *S. mutans* strains and other *Streptococcus* spp. also inhibited GT formation but to different extents, possibly reflecting differences in CSP amino acid sequences among *Streptococcus* spp. or differences in CSP accumulation in the media. In conclusion, CSP, an *S. mutans* quorum-sensing molecule secreted during the early stages of growth, inhibits the *C. albicans* morphological switch.

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The oral cavity is colonized by many different microbial species, where most reside in biofilms. Because of its multispecies nature, the oral microbial community is one of the best biofilm models for studying interspecies interactions (17). The gram-positive bacterium *Streptococcus mutans* shows a high prevalence in dental biofilms, and it is considered to be the major etiological agent involved in human dental caries (21). The fungal species *Candida albicans* constitutes a minor part of the total microbial flora (19) and can be isolated as a commensal from the oral cavity of 50% to 60% of healthy adults (33). However, in immunocompromised individuals (for example, due to human immunodeficiency virus infection or as a result of chemotherapy) and elderly patients, this fungus often leads to candidiasis (24). *C. albicans* is a polymorphic fungus that can exist in three morphotypes: budding yeast, pseudohypha, and true hypha (5). The morphological switch from yeast to hyphal cells is important in many processes, such as virulence (22) and biofilm formation (10, 18), and is therefore the subject of many studies.

Bacteria and yeasts are often found together in vivo, and there is growing evidence that interspecies, and even interkingdom, interactions occur within these populations (7). These interactions can be mediated through signaling molecules (40), as recently described for the interaction between *C. albicans* and *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen (15). N-3-oxo-C12 homoserine lactone (HSL), a signaling molecule involved in bacterial quorum sensing, completely represses *C. albicans* hypha formation without altering the growth rate. Although many gram-negative bacteria produce HSLs with shorter acyl chains (e.g., C4-HSL), the inhibition of *C. albicans* hypha formation is caused specifically by long-chained HSL molecules. In addition, related, non-HSL molecules with long acyl chains, such as dodecanol and farnesol, also inhibit the hypha formation of *C. albicans* (8).

A recent report described the coculturing of *C. albicans* and *S. mutans* in model oral biofilms on hydroxyapatite (26). It was shown that *S. mutans* increased the growth of *C. albicans* by stimulating coadhesion while simultaneously suppressing the formation of hyphae. *S. mutans* is a gram-positive bacterium and does not produce HSL-type molecules, and the nature of the interaction with *C. albicans* is presently unknown. In this study, the interaction between *S. mutans* and *C. albicans* was investigated by studying the effect of secreted molecules of *S. mutans* on *C. albicans* hypha formation.

**MATERIALS AND METHODS**

**Strains and growth conditions.** All bacterial and fungal strains used in this study are shown in Table 1. *C. albicans* was plated onto yeast nitrogen base (YNB) (pH 7.0; BD Becton, Dickinson, and Company, Sparks, MD) supplemented with 0.5% glucose and 1.5% (wt/vol) agar from a 15% (vol/vol) glycerol stock maintained at -80°C and incubated 48 h at 30°C. *Streptococcus* spp. were cultured on blood agar, except for the *S. mutans* UA159 ΔcomC mutant, which was cultured on brain heart infusion (BHI; Oxoid, Basingstoke, United Kingdom) agar plates supplemented with erythromycin (10 μg/ml). YNB broth was used to grow *C. albicans* cells overnight at 30°C while shaking at 120 rpm. Cultures of *Streptococcus* spp. were grown statically in BHI broth overnight in
5% CO₂ at 37°C and supplemented with erythromycin (10 μg/ml) when appropriate. To obtain spent-medium samples, Streptococcus spp. were grown in 200 ml BHI broth; 10 ml of medium was taken after 4, 6, 8, and 24 h and centrifuged at 10,000 x g for 5 min; the pH was checked and, if lower than 7, was set to 7; and cultures were filtered sterilized and stored at −20°C. Cocultures of C. albicans and S. mutans were grown in medium containing 70% YNB and 30% BHI broth (vol/vol). Where indicated, spent medium replaced the 30% fresh-BHI fraction. Competence-stimulating peptide (CSP), with an amino acid sequence of NH₂-GGCGCGCTGTCGGAGATCC-COOH (2) and with a purity of >95%, was purchased from Ansynth Service BV, Roosendaal, The Netherlands. A 12 mM stock of synthetic CSP was prepared by dissolving the peptide in demineralized water and stored at −20°C.

**Construction of the S. mutans UA159 ∆comC strain.** To delete the comC gene, we used a previously described PCR ligation mutagenesis method (20): the flanking regions of the comC gene were amplified from S. mutans UA159 genomic DNA with PCR primer pairs comCu/comCd and comCd/comCdt (see Table 2 for details). The erythromycin resistance gene (erm) gene was amplified from an ermAM cassette using primer pair ermF/ermR. These three PCR fragments were digested with the appropriate restriction enzymes and ligated. The ligation mix was used as a template for the amplification of the knockout construct using the primers comCu/comCdt. The resulting PCR product was purified and transformed into S. mutans UA159 cells. The comC knockout strain was obtained via double crossover, resulting in the replacement of a major part of the chromosomal comC gene by the erm gene. The insertion of the erm gene was verified by PCR. The lack of CSP production by the comC knockout strain was confirmed by its incapability of bacteriocin production in competition assays (36).

**Saliva preparation and coating.** Freeze-dried saliva was prepared as described previously (35). Briefly, stimulated saliva (by chewing paraffin) was collected from healthy volunteers, put on ice, and centrifuged twice at 10,000 x g for 5 min at 10°C. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM to prevent protein degradation. Afterwards, the solution was again centrifuged at 10,000 x g for 5 min, dialyzed against demineralized water overnight at 4°C, and freeze-dried before storage at −80°C. For experiments, lyophilized saliva was dissolved in adhesion buffer (1 mM CaCl₂, 2 mM potassium phosphate, 50 mM KCl) at a concentration of 1.5 mg/ml. This solution was centrifuged at 10,000 x g for 5 min at 10°C, and the supernatant was checked for the absence of bacteria by microscopy and subsequently used to coat 12-well tissue culture polystyrene plates (Costar; Corning, Inc.) overnight. Before the start of experiments, saliva was removed, and the wells were washed once with adhesion buffer. All volunteers gave their informed consent to saliva donation in accordance with the rules set out by the ethics committee at the University Medical Center Groningen.

**GT assay.** As a measure of filamentation, a germ tube (GT) assay was performed using 12-well tissue culture polystyrene plates as described previously (15), with minor modifications. Saliva-coated culture plates filled with 1 ml of mixed medium were inoculated with S. mutans SC5314 cells to a final optical density at 600 nm of 0.1 (corresponding to 1 x 10⁶ cells/ml). When appropriate bacteria were added to 1 x 10⁷ cells/ml. The plates were incubated for 3.5 h at 37°C while shaking at 80 rpm. The morphological state of S. mutans was analyzed by microscopy using a 20x objective lens on an inverted microscope (Olympus, Tokyo, Japan). For selected experiments, ThinCerts tissue culture polystyrene inserts (Greiner Bio-One) with a 0.4-μm pore size were used to physically separate yeasts from bacteria.

**Fluorescent microscopy of GFP expression.** C. albicans HB12 cultures carrying green fluorescent protein (GFP) under the control of the hwp1 promoter (31) were grown with the addition of 1 μM CSP in a GT assay. HB12 expresses GFP only in true hyphate and not in pseudohyphate or yeast cells. Fluorescence microscopy was performed with a Leica DM4000B fluorescence microscope. Cells were observed with a 20x objective. For visualization of all cells, calcfluor white (Fluorescence Brightener 28; Sigma-Aldrich) staining was applied in a final concentration of 3.6 μM. Separate images were taken using filter sets for GFP and UV. Overlay images were created using Leica Application Suite, version 2.8.0.

**Statistical analysis.** Differences in percentages of GT formation were tested using Student's t test with the threshold of a P value of <0.05 for statistical significance.

### RESULTS

**Effect of saliva and S. mutans UA159 on C. albicans SC5314 GT formation.** Upon the induction of GT formation at 37°C, C. albicans showed a higher fraction of germinating cells in saliva-coated wells (52%) than in uncoated wells (20%) (Fig. 1). Coculture with S. mutans UA159 resulted in only 35% of all cells being germinated. This inhibition of the fraction of germinating cells from 52% to 35% was not significant (P = 0.06) for saliva-coated and uncoated wells (20% to 16%; P = 0.25). When C. albicans and S. mutans UA159 cells were physically separated from each other by polystyrene inserts, GT formation was significantly inhibited in saliva-coated wells (from 52% to 17%; P = 0.007) but not in uncoated wells (from 20% to 9%; P = 0.06), indicating that physical contact is not involved in this inhibition of GT formation. The effect of S. mutans UA159 on C. albicans is best visualized after saliva coating, and hence, these conditions were applied for all following experiments.

**Effect of spent medium of S. mutans on C. albicans GT formation.** Spent medium obtained from S. mutans UA159 and S. mutans ∆comC cultures at different phases during growth (4-, 6-, 8-, and 24-h-old cultures) was tested for its ability to affect the germination of C. albicans. Spent medium from 4-h-old culture of S. mutans UA159 inhibited C. albicans GT formation (from 50% to 8%), whereas spent medium of 6-, 8-, and 24-h-old cultures did not show any inhibition (Fig. 2A). CSP, a quorum-sensing molecule involved in the stimulation of competence (27), encoded by comC (14), is produced by S. mutans during the beginning of the exponential growth phase (25). Spent medium from the S. mutans ∆comC strain did not show any inhibition, whereas the addition of 1 μM CSP to 4-, 6-, and 8-h-old cultures inhibited GT formation to 4%, 38%, and 31% of their respective controls.
respectively (Fig. 2A). It should be noted that the pH of the spent medium in the GT assay was always set to 7.

To chemically complement the \textit{comC} mutation, synthetic CSP was added to 4-h-old spent medium of the \textit{S. mutans} \textit{UA159} \textit{comC} strain at various concentrations. When 1 \textmu{}M CSP was added, the effect was very similar to that of 4-h-old wild-type spent medium, showing a rate of GT formation of 8\% (Fig. 2B). Together with the reduction in the concentration of CSP to 0.1 or 0.01 \textmu{}M, the inhibitory effect was reduced (16\% and 32\% GT formation, respectively).

\textbf{Effect of synthetic CSP on \textit{C. albicans} GT formation.} The effect of increasing concentrations of synthetic CSP, ranging from 0.1 to 5 \textmu{}M, is shown in Fig. 3A. Synthetic CSP at 0.1 \textmu{}M significantly inhibited GT formation by 60\% compared to the control. Using 1.0 \textmu{}M CSP, an inhibition of GT formation by 90\% compared to the control was obtained, indicating that the inhibition of GT formation is dependent on the concentration of synthetic CSP added to the culture; however, concentrations up to 5.0 \textmu{}M did not enhance the effect. Synthetic CSP did not inhibit the growth of \textit{C. albicans} at the concentrations tested (data not shown). The effect of synthetic CSP on \textit{C. albicans} GT formation was confirmed using \textit{C. albicans} HB12, expressing GFP under the control of the hypha-specific \textit{HWP1} promoter. \textit{HWP1} is expressed only in germinating cells and not in pseudohyphae or yeast cells. A decrease in the number of green fluorescent (germinating) cells and an increase in the number of pseudohyphae and yeast cells compared to the control were observed in the presence of 1 \textmu{}M CSP (Fig. 3B and C).

\textbf{Effect of synthetic CSP on preformed GT.} To investigate whether synthetic CSP can revert preexisting hyphae back to yeast cells, \textit{C. albicans} was grown without (control) or in coculture with \textit{S. mutans} \textit{UA159} either without or with an insert physically separating both species. The morphology of \textit{C. albicans} was determined after 3.5 h of growth. Wells were uncoated (gray bars) or coated with saliva (black bars). The results are averages of data from four independent experiments, with at least 300 cells counted per sample for each experiment. An asterisk indicates a significant ($P < 0.05$) difference compared to the control.
rates of GT formation of 74% and 45%, respectively. None of the 24-h-old spent media, corrected for pH, showed inhibition.

**DISCUSSION**

*S. mutans* can affect GT formation of *C. albicans* in cocultures even if the bacteria and the fungi are physically separated. In addition, filter-sterilized spent medium of *S. mutans* inhibited GT formation. These results indicate that *S. mutans* secretes one or more diffusible molecules that affect *C. albicans* hypha formation. The inhibition of GT formation occurs through several mechanisms, one of which involves the secreted quorum-sensing molecule CSP. This is supported by several lines of evidence: (i) there is a strong inhibitory effect of 4-h-old spent medium on GT formation (CSP was previously described to be produced specifically in the early exponential phase [25]), (ii) synthetic CSP inhibits GT formation in a concentration-dependent manner, and (iii) a mutant of *S. mutans* that is unable to produce CSP showed a reduced inhibition of GT formation compared to the wild-type strain but could be restored to wild-type levels of GT inhibition by the addition of synthetic CSP.

Saliva was present in all experiments to better resemble the conditions in the oral cavity, where saliva affects the behavior of microorganisms. In the present study, it was shown that saliva induces the GT formation of *C. albicans*. CSP did not affect GT formation induced by serum (data not shown), which may indicate that saliva is a weaker inducer of hypha formation than serum. This is in line with data from a previous report showing that contact-stimulated hypha formation was more pronounced in the presence of serum than in the presence of saliva (23). Biofilms of *S. mutans* and *C. albicans* grown under comparable conditions on hydroxyapatite, polymethylmethacrylate, and soft denture liner discs showed that the presence of *S. mutans* leads to the suppression of hypha formation in *C. albicans* without affecting the extent of biofilm formation (26). The extent of *C. albicans* biofilm formation, when grown in 96-well tissue culture polystyrene plates with saliva or serum coating, was not significantly affected by synthetic CSP (data not shown). A more detailed study of the effect of CSP and other secreted molecules of *S. mutans* is needed to obtain a deeper understanding of the mechanism governing this interaction in mixed-species biofilms.

Another quorum-sensing molecule secreted by *Streptococcus* spp., autoinducer 2 (AI-2) (39), has been implicated in interactions within mixed *C. albicans* biofilms. The presence of *S. gordonii* cells appeared to induce more extensive hypha formation but not in a *luxS* mutant (3). There was no significant effect of exogenous dihydroxypentanedione (precursor to AI-2) on hypha formation. However, because many different bacterial species produce slightly different AI-2 derivatives (9), the role of AI-2 in *S. mutans-C. albicans* interactions should be carefully investigated in the future.

A maximal inhibition of GT formation (Fig. 3 A) was realized by the addition of 1 μM synthetic CSP. This CSP concentration is commonly used to induce competence, and it was speculated to be a physiologically relevant concentration (29, 30). Several attempts to determine CSP concentrations in the spent media used in the current study were unsuccessful. We also note that there are no reports of this in the literature.
although many groups have studied CSP-related phenomena. In a paper where a reporter gene was used, there was also no CSP concentration mentioned (32). Unfortunately, there is only speculation regarding the physiological concentrations of CSP produced by *S. mutans* (28). Based on our chemical complementation of the ΔcomC strain and a comparison between the response of *C. albicans* to spent medium and the response to synthetic CSP, we predict that the natural concentration of CSP can reach an order of magnitude of 1 μM. Synthetic CSP is not toxic to *C. albicans*, as it does not inhibit growth, nor does it induce the expression of genes within the general stress response (11), such as *CTA1* and *HSP12* (data not shown).

**FIG. 4.** Effect of synthetic CSP on germinating cells. (Top) GT formation after 2 h of incubation. (Bottom) Lack of germination 2 h after the addition of 1 μM synthetic CSP (right) compared to a control without synthetic CSP (left).

**FIG. 5.** Effect of spent media of several *Streptococcus* spp. on GT formation of *C. albicans*. Shown are data for spent media of various *Streptococcus* spp. grown for 4 h (black bars) or 24 h (gray bars). The pH of all samples was set to 7 prior to the GT assay. The results are averages of data from two experiments, with at least 300 cells counted per experiment. An asterisk indicates a significant (P < 0.05) difference compared to the control.
The suppressing effect on \textit{C. albicans} GT formation was obtained only with 4-h-old \textit{S. mutans} UA159 spent medium, coinciding with maximal CSP production (25). The pH of the medium is one of the factors that is known to affect GT formation in \textit{C. albicans} (4), and \textit{S. mutans} is known to produce significant amounts of acid during growth (13). GT formation was significantly inhibited with spent medium from 8- and 24-h-old cultures of \textit{S. mutans} UA159 and the \(\Delta comC\) strain, which was caused by acidification (pH 4.8) (data not shown), whereas this inhibition disappeared when the spent medium was set to pH 7. However, after 4 h, the pH of the spent medium had not yet changed and was still 7 (data not shown); therefore, pH is not causing the inhibitory effect on GT formation.

The richness of BHI medium affects GT formation; the addition of 10% or more BHI medium in YNB significantly inhibited GT formation (data not shown). An exhaustion of BHI medium in spent-medium experiments would therefore stimulate GT formation. Both 6-h-old and 8-h-old spent media of \textit{S. mutans} UA159 and the \(\Delta comC\) strain induced GT formation slightly, by 15%. This effect could be explained by the exhaustion of BHI medium. Alternatively, the effect could be explained by the production of an inducer of GT formation in both strains, possibly AI-2, as reported previously for \textit{S. gordonii} (3). In addition, the induction of GT formation using 6- and 8-h-old spent media illustrates the reduction of the level of CSP production in \textit{S. mutans} UA159, as described previously (34). Spent media of 4-h-old cultures from \textit{S. mutans} ATCC 22175, \textit{S. mutans} NS, and several other streptococcal strains also inhibited GT formation albeit to different extents. This may indicate some level of redundancy toward CSP sequences recognized by \textit{C. albicans}, as different streptococcal strains and species produce CSP with different amino acid sequences (38). Interestingly, an induction of GT was found for \textit{S. mitis} ATCC 33399, which may indicate the production of GT-inducing factors. Twenty-four-hour-old cultures of all species did not show an inhibition of GT formation.

A number of C12 acyl chain-containing molecules that affect the morphological transition of \textit{C. albicans} have been described. Farnesol is produced and sensed by \textit{C. albicans} (16) to levels as high as 58 \(\mu\)M (37). N-3-Oxo-C12 HSL, produced by \textit{P. aeruginosa} (15), and dodecanol (8), which mimics farnesol, were the first bacterial signaling molecules described to inhibit the yeast-to-hypha transition in \textit{C. albicans}. Recently, cis-2-dodecenoic acid, produced by \textit{Burkholderia cenocepacia} (6), was shown to inhibit the morphological transition at much lower concentrations than farnesol and N-3-oxo-C12 HSL. Because of the structural similarities, it is possible that the regulation of morphological transitions occurs through a shared pathway. CSP of \textit{S. mutans} is a small, 22-amino-acid-containing peptide that is structurally unrelated to the bacterial signaling molecules known to affect the \textit{C. albicans} yeast-to-hypha transition. The discovery of \textit{S. mutans} CSP as a signaling molecule involved in interactions with \textit{mutans} CSP adds a structurally unrelated molecule to the list of known bacterial signaling molecules affecting \textit{C. albicans}.

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