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Extracellular Identification of a Processed Type II ComR/ComS Pheromone of *Streptococcus mutans*

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The competence-stimulating peptide (CSP) and the *sigX*-inducing peptide (XIP) are known to induce *Streptococcus mutans* competence for genetic transformation. For both pheromones, direct identification of the native peptides has not been accomplished. The fact that extracellular XIP activity was recently observed in a chemically defined medium devoid of peptides, as mentioned in an accompanying paper (K. Desai, L. Mashburn-Warren, M. J. Federle, and D. A. Morrison, *J. Bacteriol.* 194:3774–3780, 2012), provided ideal conditions for native XIP identification. To search for the XIP identity, culture supernatants were filtered to select for peptides of less than 3 kDa, followed by C₁₈ extraction. One peptide, not detected in the supernatant of a *comS* deletion mutant, was identified by tandem mass spectrometry (MS/MS) fragmentation as identical to the ComS C-terminal sequence GLDWWSL. ComS processing did not require Eep, a peptidase involved in processing or import of bacterial small hydrophobic peptides, since *eep* deletion had no inhibitory effect on XIP production or on synthetic XIP response. We investigated whether extracellular CSP was also produced. A reporter assay for CSP activity detection, as well as MS analysis of supernatants, revealed that CSP was not present at detectable levels. In addition, a mutant with deletion of the CSP-encoding gene *comC* produced endogenous XIP levels similar to those of a nondeletion mutant. The results indicate that XIP pheromone production is a natural phenomenon that may occur in the absence of natural CSP pheromone activity and that the heptapeptide GLDWWSL is an extracellular processed form of ComS, possibly the active XIP pheromone. This is the first report of direct identification of a ComR/ComS pheromone.

The ability of bacteria to modify their hereditary material by taking up and incorporating extracellular DNA into their genomes via natural transformation is a widespread phenomenon (17). In streptococci, the mechanisms involved in natural transformation have been described in the most detail in *Streptococcus pneumoniae*, but the phenomenon is also observed in at least 14 other streptococcal species (10, 17, 26).

The pheromones triggering competence for natural transformation in *S. pneumoniae* are the competence-stimulating peptides (CSPs). They are encoded by the *comC* gene of the *comCDE* operon and are thought to be produced as propeptides with a double-glycine leader sequence (11). Cleavage of the leader sequence at the double glycine is concomitant with the export of the mature peptide by the ComA ABC transporter and its accessory protein, ComB (15). Upon reaching a threshold concentration, the CSP mature peptide binds to the ComD histidine kinase. The binding probably leads to autophosphorylation of ComD, followed by the transfer of the phosphate group to the cognate ComE response regulator (12, 30). Phosphorylated ComE is then thought to recognize a direct repeat in the promoter sequences of the *comAB* and *comCDE* operons, as well as in the *sigX* promoter, activating their transcription (42). SigX or ComX is the alternative sigma factor that links the CSP autocatalytic system to competence by activating transcription of competence effector genes involved in DNA binding, uptake, and recombination (22, 35). Other mitis streptococci, as well as streptococci from the anginosus group are thought to use a similar CSP autocatalytic system to activate competence (13, 14, 32, 41).

In the salivarius group, the competence pheromone belongs to a new class of small hydrophobic peptides that most probably bind to the Rgg-like regulator ComR to activate transcription of *sigX*

and the pheromone-encoding gene *comS* (10). The pheromones in the salivarius group belong to the type I class of ComR/ComS pheromone systems (26). Like most other pheromones, the ComR/ComS type I pheromones are predicted to be produced as propeptides that are processed to yield the mature pheromone by a mechanism that has not yet been elucidated.

The *Streptococcus mutans* competence-signaling system is unique in that competence can be triggered by two pheromones. One is the *S. mutans* CSP, which, like the CSP in *S. pneumoniae*, belongs to the class of double-glycine-type leader peptides (24, 31). The other is the recently identified small hydrophobic peptide XIP (for *sigX*-inducing peptide), which belongs to the type II class of ComR/ComS pheromone systems and is characterized by the presence of conserved aspartate and double-tryptophan residues (26).

The *S. mutans* CSP pheromone triggers early events associated with increased expression of the ComDE two-component system, such as upregulation of bacteriocin-related genes, including *cipB* (mutacin V; SMU.1914) and other mutacin loci (19, 20, 29, 39). Comparison of *S. mutans* ComDE with *S. pneumoniae* two-component systems reveals that ComDE is more closely related to the *S. pneumoniae* BlpHR system involved in bacteriocin production than to the *S. pneumoniae* ComDE system involved in competence

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TABLE 1 Strains used in this study

<i>S. mutans</i> strain	Description ^a	Source or reference
UA159	Wild-type transformable strain; sequenced genome; Kan ^s Erm ^s Spc ^s	2
SM059	UA159::Φ(P _{cipB} - <i>luc</i>); Spc ^r	This study
SM065	UA159 Δ <i>comS</i> :: <i>spc</i> ; Spc ^r (from strain MW05 [26])	This study
SM068	UA159::Φ(P _{sigX} - <i>luc</i>); Spc ^r	This study
SM074	SM068 Δ <i>comC</i> :: <i>kan</i> Φ(P _{sigX} - <i>luc</i>); Kan ^r Spc ^r (from strain SM004 [31])	This study
SM089	UA159 Δ <i>comS</i> :: <i>erm</i> ; Erm ^r	This study
SM091	SM068 Δ <i>comS</i> :: <i>erm</i> Φ(P _{sigX} - <i>luc</i>); Erm ^r Spc ^r (from strain SM089)	This study
SM100	SM068 Δ <i>eep</i> :: <i>kan</i> Φ(P _{sigX} - <i>luc</i>); Kan ^r Spc ^r	This study
SM101	SM091 Δ <i>eep</i> :: <i>kan</i> Φ(P _{sigX} - <i>luc</i>); Kan ^r Erm ^r Spc ^r (from strain SM100)	This study

^a Kan, kanamycin; Erm, erythromycin; Spc, spectinomycin.

(25). Increased expression of *sigX* and effector genes of the DNA binding and uptake machinery regulated by *sigX*, however, is also observed, but this is a delayed response, and in contrast to the system in *S. pneumoniae*, no ComE binding site is found in the *S. mutans sigX* promoter. Instead, the *sigX* promoter has a motif that is most probably recognized by ComR, which places the ComR/ComS system at the core of the *S. mutans* competence response (26). Supporting the core role of the ComR/ComS system are the facts that *sigX* expression and competence are abolished in *comR* or *comS* deletion mutants in complex or defined growth medium and that such phenotypes are restored to normal levels in the *comS* mutant in the presence of synthetic XIP, but not CSP (26). This is also supported by the fact that competence development, albeit at lower levels, is frequently observed in mutants with deletion of *comDE* (1, 5, 24, 26, 34) and that deletion of *comC*, at least in *S. mutans* UA159, may result in mutants that are not affected in competence (1).

ComS is most probably produced as a propeptide that is processed into the active XIP pheromone. XIP is imported via an oligopeptide permease system and, inside the cells, is thought to bind to ComR to activate transcription of *comS* and *sigX* (26). Eep metalloproteases of *Enterococcus faecalis*, and of some streptococci, have been implicated in the processing of pheromones (4, 9, 40). In streptococci, Eep also has a purported role in the processing of signal peptides directing oligopeptide permeases to the bacterial membrane (9). A single Eep homologue is found in the *S. mutans* genome (2), but whether it is involved in the possible processing of XIP or in the assembly of permeases, such as the Opp permease system associated with XIP import in *S. mutans*, remains to be determined.

ComR/ComS pheromones are found in at least eight other species of streptococci (9, 10, 26). In all of them, a predicted ComR/ComS binding site is found in the *sigX* promoter sequence, indicating that the system has evolved to regulate competence in a wide range of streptococcal species. In no case, however, has the native structure of the secreted peptides been identified.

Endogenous XIP activity was recently described in the culture filtrates of *S. mutans* grown in a chemically defined medium (CDM) (see the accompanying paper by Desai et al. [8]). The CDM is devoid of peptides, which, together with the high levels of extracellular XIP activity attained, creates ideal conditions for the identification of the native XIP and possibly other peptides. In this study, we used the growth conditions described in the accompanying paper (8) to identify the extracellular native XIP and to investigate the putative involvement of Eep in XIP production or response. The possible link between the accumulation of native extracellular XIP and CSP was also investigated.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. mutans* UA159 and the isogenic mutants used in this study are presented in Table 1. Cultures of *S. mutans* were grown in 5% CO₂ at 37°C in Todd-Hewitt Broth (THB) (Becton, Dickinson and Company, Le Pont de Claix, France) or CDM and stored at -80°C in the same medium supplemented with 15% glycerol. CDM was prepared as previously described (26). The selective antibiotics for *S. mutans* were erythromycin, kanamycin, and spectinomycin at final concentrations of 20 μg ml⁻¹, 500 μg ml⁻¹, and 200 μg ml⁻¹, respectively.

Construction of mutants. The PCR-ligation mutagenesis strategy (21) was used for the construction of deletion mutants, using the oligonucleotide primers listed in Table 2. Mutants with deletions of *comS*, *comC*, and *eep* were constructed. *AscI* or *FseI* restriction sites were incorporated into the 5' ends of the oligonucleotide primers complementary to the gene target used to generate the flanking DNA fragments and into both 5' ends of the resistance cassettes (Table 2). The erythromycin and kanamycin resistance cassettes (kind gifts from D. A. Morrison) were amplified with the primer pairs FP015-FP016 and FP001-FP068, respectively. The *comS* flanking regions were amplified with the primer pairs FP649-FP651 and FP652-FP653 and the *eep* flanking regions with the primer pairs FP658-FP659 and FP660-FP661. The resultant amplicons

TABLE 2 Primers used in this study

Primer	Nucleotide sequence ^a (5' to 3')
FP001	<u>AGGCGCGCCGTTT</u> GATTTTAAATG
FP009	TATGGACCAAGAAATGCTGT
FP012	TGGAGTTGCTTGATTTTCATT
FP015	<u>GGCGCGCCCGGGCCCAAAATTTGTTT</u> GAT
FP016	<u>GGCCGGCCAGTCGGCAGCGACTCATAGAAT</u>
FP037	TCATTTTCTCCCACCAGCTT
FP038	GCGCCTACGAGGAATTTGTA
FP068	<u>AGGCCGGCCTAGGTA</u> CTAAAACAATTCATCCAGTA
FP419	<u>AGGATCCCACCTTCAACAGCTGAAAGTGC</u>
FP420	<u>AAAGTCGACTAAA</u> ACTTCTGTAAACAGCCGG
FP627	<u>CGGCCGTCGACGTCGGATCC</u> ATTCCGGCAT AGCTCAGTTG
FP630	<u>CATTACCTCCTCGAGGATCC</u> AACATTCCCTCTTG TTGCCAAT
FP649	CGATTTTGGCGTTGGTATTT
FP651	<u>AGGCGCGCCATCCTGTTATTCTCCTTTCTTTT</u> TGA
FP652	<u>AGGCCGGCCGAGCTTATAATAGACAGC</u>
FP653	AAGATACCGTTCCGCTCCTT
FP658	TCAAGATGCTGCTTTTCTCCT
FP659	<u>AGGCGCGCCTTCCCGAACCAAAATTCCTG</u>
FP660	<u>AGGCCGGCCATTCTGCTCTTGATGGTGGT</u>
FP661	CTTCATGAGTCGGCCCTAAA

^a Restriction sites are underlined: BamHI, GGATCC; Sall, GTCGAC; *AscI*, GGCGCGC; *FseI*, GGCCGGCC. Sequences homologous to either side of the BamHI restriction site used to linearize pFW5-*luc* are in boldface.

corresponding to the 5' or 3' region flanking the target genes were digested with *AscI* (New England BioLabs) or *FseI* (New England BioLabs), respectively, whereas the resistance cassettes were digested with both enzymes. T4 DNA ligase (Fermentas, St. Leon, Germany) was then used to ligate the upstream or the downstream amplicons of the target genes to either the erythromycin or the kanamycin resistance cassettes in separate reactions. The two ligation products were then mixed and PCR amplified with the distal primers. The resultant amplicons were used to transform *S. mutans*, as described below. The gene deletion in each mutant was confirmed by PCR amplification of the chromosomal DNA extracted from *Erm^r* or *Kan^r* mutants using the distal primers, as well as by using the distal primers in combination with complementary primers internal to the resistance cassette sequences. The correct predicted size of the products was verified by gel electrophoresis. Deletion of *comC* was also verified by DNA sequencing using the primer pairs FP009-FP037 and FP038-FP012 to generate the amplicons that were sequenced using the primer FP009 or FP012.

For the construction of luciferase promoter reporters, the primer pair FP419-FP420 or FP627-FP630 was used for amplification of the *cipB* or *sigX* promoter region, respectively (36). For cloning of the *sigX* promoter, the CloneEZ PCR cloning kit (GenScript, Piscataway, NJ) was used, following the manufacturer's protocol and using *Bam*HI to linearize the pFW5-*luc* vector. For cloning of the *cipB* promoter, *S*all and *Bam*HI restriction sites were incorporated at the 5' ends of the primers. After enzymatic digestion, the plasmid and the amplified promoter regions were ligated, purified, and used to transform One Shot Top 10 chemically competent *Escherichia coli* (Invitrogen). Plasmids with the correct insert were then purified and used to transform the UA159 wild-type strain, with selection in spectinomycin-containing plates. The chromosomal DNAs from *comS*, *eep*, and *comC* deletion mutants were used to transform the *sigX* promoter reporter strain (SM068) (Table 1). The *comS* deletion *P_{sigX}-luc* reporter SM091 and the *P_{cipB}-luc* reporter SM059 were used as indicators for XIP- or CSP-like activity in culture supernatants, respectively.

Transformation. For targeted gene deletion or reporter construction, *S. mutans* was grown in TSB (Oxoid, Hampshire, United Kingdom), and transformation was conducted as previously described (33). Transformants were selected by growth on THB agar plates containing the selective antibiotics.

Bioassay of pheromone activity in culture supernatants. The bioassay described in the accompanying article by Desai et al. (8) was used with slight modifications. The *P_{sigX}-luc* reporter in a *comS* deletion background (SM091) was used for detection of extracellular XIP-like activity, whereas the *cipB* promoter reporter (SM059) was used for detection of extracellular CSP-like activity. Each assay reaction mixture contained 40 μ l of indicator strain (SM091 or SM059), 40 μ l of CDM, 10 μ l of culture supernatant or 10 μ l synthetic peptides diluted in CDM, and 10 μ l of 1 mM D-luciferin (Synchem, Felsberg-Altenberg, Germany) distributed in black 96-well plates with transparent bottoms (Nunc Thermo Scientific). Luminescence and optical density at 600 nm (OD_{600}) were measured by reading the plates in a multidetection microplate reader (Synergy HT; BioTek, Winooski, VT). CDM without synthetic peptides was used to obtain background values that were subtracted from the sample values.

The culture supernatants were collected at different densities during growth. The accumulation of extracellular XIP activity during growth showed a variation between parallel experiments that correlated, at least in part, with the XIP-like activity in the culture used for the inoculum. This carryover of endogenous XIP-like activity introduced variability between the experiments that hampered comparisons between the wild type and the deletion mutants. We then established a protocol for the preparation of supernatants used in comparative activity assays. The protocol used frozen stock cultures in CDM prepared from cultures grown to an OD_{600} of 0.5 that were centrifuged and resuspended to the same OD in fresh CDM. Ten microliters of the stock cultures was used as the inoculum for overnight cultures prepared in 3 ml CDM. The overnight cultures were then centrifuged, and the pellets were resuspended in fresh CDM to an

OD_{600} of 0.3. Under these conditions, expression of *sigX* by the *P_{sigX}-luc* reporter SM068 was reduced to levels close to background. *ComS₁₁₋₁₇* at 50 nM was then used to reactivate the autocatalytic mechanism of endogenous XIP production.

Synthetic peptide. The heptapeptide GLDWWSL (*ComS₁₁₋₁₇*) was custom synthesized by GenScript Corporation with an estimated purity of 98%, as determined by high-performance liquid chromatography (HPLC). The lyophilized *ComS₁₁₋₁₇* was reconstituted with 20 μ l dimethyl sulfoxide (DMSO) (Sigma-Aldrich), to which distilled water was added to give a final *ComS₁₁₋₁₇* concentration of 10 mM. The stock solutions were stored in small aliquots at -20°C . The 18CSP peptide was also synthesized by GenScript and prepared as previously described (31).

LC-MS/MS. *S. mutans* UA159 and the isogenic *comS* (SM065) and *eep* (SM100) deletion mutants were grown at 37°C in CDM under normal atmospheric conditions. At midexponential growth phase ($OD_{600} \sim 1.0$), the culture supernatants were recovered by centrifugation ($10,000 \times g$ for 5 min at 4°C). A sample of the synthetic *ComS₁₁₋₁₇* or 18CSP was used as a standard control. The synthetic peptides were resuspended in CDM to a final concentration of 2 μ M and concentrated following the same protocol that was used for the preparation of the culture supernatants. To deplete high-molecular-weight proteins, aliquots of 1 ml supernatants with 10% acetonitrile (ACN) (Burdick & Jackson, Germany) were loaded on prewashed Nanosep Centrifugal Devices with a molecular mass cutoff of 3 kDa or 10 kDa, followed by centrifugation ($14,000 \times g$ for 15 min at room temperature [RT]). The eluted fractions were then brought to pH 2 using trifluoroacetic acid (TFA) (Sigma-Aldrich) and loaded on Sep-Pak Plus C_{18} silica cartridges (360 mg silica; Waters, Milford, MA) that were prewashed with 80% ACN-0.1% TFA and equilibrated with 0.1% aqueous TFA. After sample loading, the cartridges were washed with 0.1% TFA. For sample elution, a total volume of 1.6 ml of an aqueous solution with 80% ACN-0.1% TFA was passed through the cartridges. Since the bed volume of the C_{18} cartridges was approximately 500 μ l, we collected the eluents in three serial elution fractions with volumes of 400 μ l, 600 μ l, and 600 μ l. Aliquots of 110 μ l corresponding to the different fractions were then dried under vacuum using an Eppendorf (Germany) Concentrator 5301 and resuspended in 5 μ l 1% formic acid before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis or in CDM for analysis of *sigX* promoter induction activity using the luciferase reporter system, as described below. LC-MS/MS analysis was conducted by the proteomic facility at the Biotechnology Centre of Oslo. An LC-MS system consisting of a Dionex Ultimate 3000 RSLC nano-UPLC (Sunnyvale, CA) connected to an Orbitrap linear quadrupole ion trap (LTQ Orbitrap XL) mass spectrometer (ThermoElectron, Bremen, Germany) equipped with a nanoelectrospray ion source was used to analyze the peptides. For liquid chromatography separation, an Acclaim PepMap 100 column (C_{18} ; 3 μ m; 100 \AA ; Dionex, Sunnyvale CA) capillary with 25-cm bed length was used with a flow rate of 300 nl/min. Two solvents, A (0.1% formic acid) and B (aqueous 90% acetonitrile in 0.1% formic acid), were used in the nanocolumn. The gradient went from 7% to 40% solvent B in 17 min and from 40% to 50% solvent B in 3 min, with a total run time of 33 min. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full-scan MS spectra (from m/z 300 to 2,000) were acquired in the Orbitrap with a resolution (R) of 60,000 at m/z 400 and allowed sequential isolation of up to six of the most intense ions for fragmentation on the linear ion trap using collision-induced dissociation (CID) at a target value of 10,000 charges. For accurate mass measurements, the lock mass option was enabled in MS mode. Other instrument parameters were set as previously described (18).

Data search strategy. Peptide identifications were obtained using the MASCOT search algorithm (v2.2; Matrix Science, United Kingdom) (28). All acquired MS/MS spectra were searched against the *ComS* and *ComC* amino acid sequences, followed by a search of possible *ComS* or *ComC* cleavage products in the MS histogram using their corresponding theoretical m/z ion currents. To validate the results, the absence of predicted

ComS or a ComS cleavage product(s) in the supernatant of the *comS* deletion mutant SM065 was examined.

RESULTS

Identification of processed XIP in the supernatants of *S. mutans* cultures. To identify the mature form of the secreted XIP peptide, we first conducted experiments to estimate the concentrations of the active factor in the supernatants of *S. mutans* during growth in CDM, as described in the accompanying paper (8). This medium contains individual amino acids, but no peptides. Concentrations equivalent to 1.5 to 2.5 μM synthetic ComS₁₁₋₁₇ were obtained at the midexponential phase at OD₆₀₀ values between 1 and 2 (data not shown). We selected samples at an OD₆₀₀ of ~ 1 for LC-MS/MS analysis. To estimate the efficiency of the extraction procedures prior to LC-MS/MS analysis, activity recovery from samples using the synthetic ComS₁₁₋₁₇ were compared with that from supernatant samples. Recovery of pheromone activity was similar for the synthetic peptide and for the supernatants. Running the samples through the 3-kDa or 10-kDa molecular mass cutoff centrifugal devices resulted in recovery levels above 90%, whereas recovery following the C₁₈ extraction of the filtered samples was approximately 45%. As expected from the cartridge bed volume, a minor fraction of approximately 3% ComS₁₁₋₁₇-equivalent activity was found in the first eluted fraction of 400 μl , whereas more than 40% of the pheromone activity was in the second fraction of 600 μl . The third fraction had only 2% of the recovered activity. The second elution was therefore chosen for further analysis. Culture filtrates of the *comS* deletion mutant had no detectable activity and were therefore used as a negative control in the identification experiments. LC-MS/MS analysis revealed the presence of a peptide corresponding to the seven C-terminal amino acids of the predicted ComS, as observed in the MS histogram (Fig. 1a) and after fragmentation (Fig. 1b). CID-MS/MS fragmentation showed six fragment masses (b4, b5, b6, y2, y3, and y4) that matched the mass of *m/z* 438.72, which corresponds to the double-protonated form of the heptapeptide (Fig. 1b). In samples of the *comS* deletion mutant SM065, the heptapeptide was not identified by MS (Fig. 1a), supporting the correct identification of the peptide in the wild type. The complete ComS propeptide was not detected by MS/MS fragmentation.

Eep is not required for ComS processing or for the import of extracellular XIP. Eep homologues have been implicated in the processing of SHP-like peptides and possibly also in the processing of signal peptides directing SHP-oligopeptide permeases to the bacterial membranes. The *S. mutans* genome has a single *eep* homologue showing syntenic arrangement with genes neighboring *eep* in *E. faecalis* V583, *Streptococcus gordonii* Challis, *Streptococcus pyogenes* NZ131, *Streptococcus thermophilus* LMD-9, and *Streptococcus uberis* 0140J (Fig. 2). Comparison of Eep in these strains with *S. mutans* UA159 Eep revealed identities of 52%, 66%, 70%, 67%, and 68%, respectively. In *E. faecalis*, Eep processes pheromones involved in plasmid conjugation (3, 4), and in the listed streptococci, it has been associated with pheromone (6, 10, 40) or lipoprotein (7, 9) processing. To assess whether ComS processing involves the Eep homologue in *S. mutans*, samples prepared from the supernatants of an *eep* deletion mutant were run in the LC-LTQ-Orbitrap. The MS results revealed a peak corresponding to a peptide with an *m/z* value similar to that of XIP. The supernatants from the *eep* deletion mutant SM100 were also investigated for the presence of pheromone activity, using the bio-

assay for detection of XIP-like activator, and again, no differences from supernatants from the wild type were observed (Fig. 3). Our results indicate, therefore, that Eep is not required for ComS processing.

In *S. mutans*, XIP is proposed to be imported into the cytoplasm by an oligopeptide permease transporter (26). Since Eep has a putative role in processing lipoprotein signal peptides found in oligopeptide permease subunits (7, 9), we investigated whether import of XIP was affected in *eep* deletion mutants. For this purpose, we measured the expression of *sigX* in response to ComS₁₁₋₁₇ using *comS* mutants that could respond to XIP but not produce it. The results revealed that *sigX* induction levels under stimulated conditions using synthetic XIP were similar in the *eep-comS* mutant SM101 and the *comS* mutant (SM091) (Fig. 4). We conclude that Eep is not required for processing of an active XIP peptide or for mechanisms involved in the import of XIP.

CSP pheromone activity was not detected in culture supernatants. To investigate whether the native CSP pheromone was present in culture supernatants, a bioassay similar to XIP pheromone detection was used, except that the indicator strain, SM059, was a luciferase reporter for the *cipB* promoter. The *cipB* promoter has a ComE binding site and shows early activation in response to synthetic CSP (16, 20). The synthetic 18CSP was used at serial dilutions to test the suitability of the assay and to estimate the activity detection range. 18CSP levels as low as 12 nM could be detected (Fig. 5). Since the test samples correspond to 10% of the total volume in the bioassay, this value corresponds to 120 nM in the original tested samples. The CSP-like activity in the culture supernatants of the *comC* deletion mutant SM074 were compared with the activity in the supernatant of SM068, which has a wild-type background. For both strains, luminescence values for supernatants collected at different densities during growth were below background levels. Exposure of SM068 to 5 nM, 10 nM, or 100 nM 18CSP was then used to test whether low synthetic CSP concentrations could trigger an autocatalytic circuit involving the *comDE* two-component system. However, no differences between SM068 and the *comC* deletion mutant were detected.

We also used LC-MS/MS analysis to search for the presence of the ComC processed peptides in the supernatants of the wild type in which the native XIP was identified. Recovery rates of synthetic CSP activity using a 3-kDa or a 10-kDa centrifugal device were approximately 30% and 40%, respectively, and after the C₁₈ cartridge extraction, they were approximately 20% and 30%. However, no ComC or ComC processed peptides from wild-type supernatant extracts were detected by mass spectrometry.

Synthetic CSP has previously been shown to induce *comS* expression in a subpopulation of *S. mutans* competent cells (23). We investigated, therefore, whether the CSP pheromone was produced under the experimental conditions used, based on the hypothesis that CSP may lead to increased production of XIP. We measured the XIP-like activity in the supernatant of the *comC* mutant SM074 and in the supernatant of SM068, a reporter with a wild-type background. The results showed no differences between the wild type and the deletion mutant. We concluded that endogenous XIP pheromone production is a phenomenon that may occur in the absence of endogenous CSP pheromone activity.

DISCUSSION

S. mutans competence for genetic transformation may be triggered by two pheromones. One is XIP, whose activity was recently

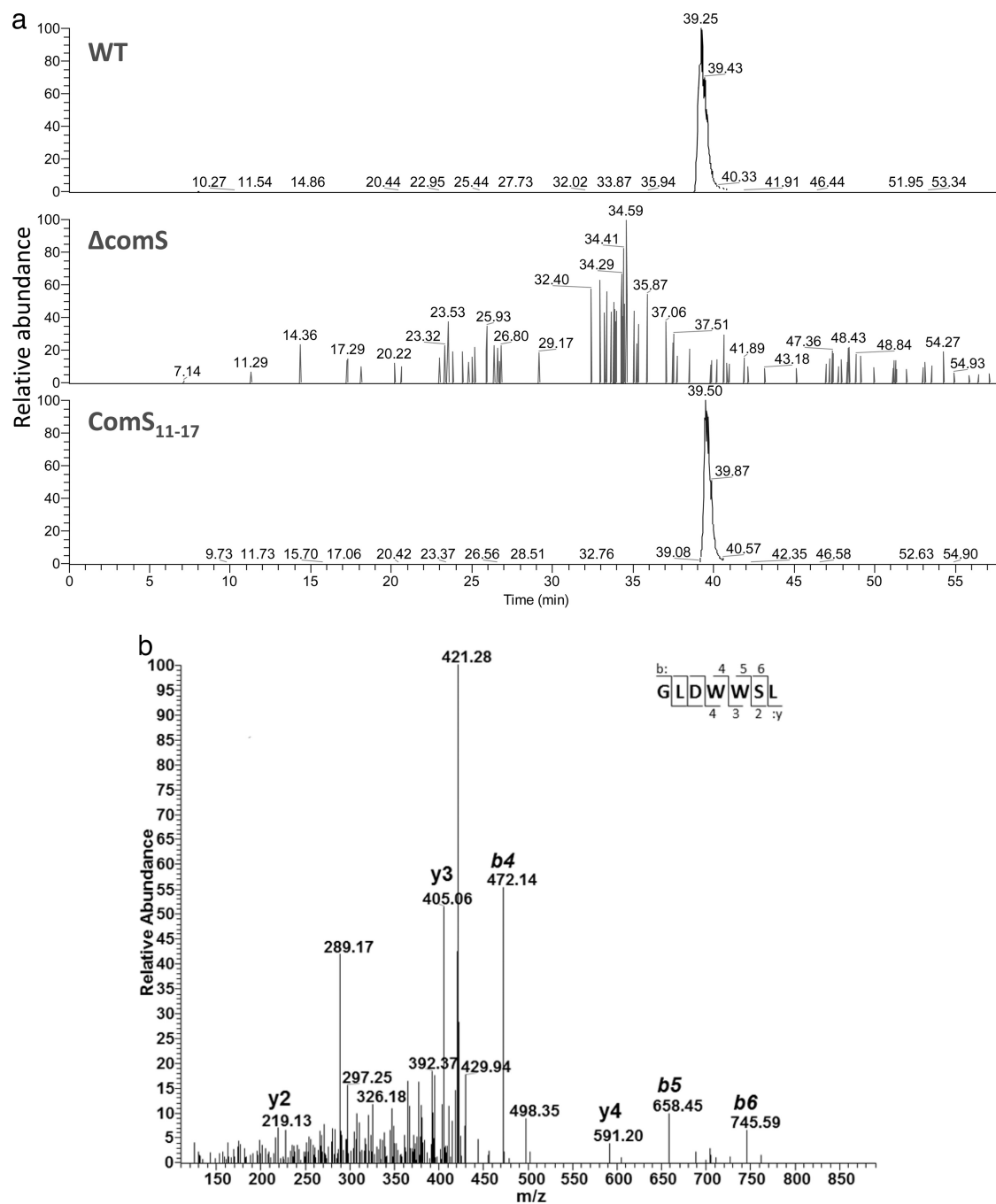


FIG 1 Native XIP detection. (a) Extracted ion chromatograms (XICs) of the m/z range 876.40 to 876.44 of samples from the wild type UA159, the $\Delta comS$ mutant SM065, and the synthetic $ComS_{11-17}$. For relative abundance calculation, the highest peak in the m/z range 876.40 to 876.44 for each sample was set to 100%. (b) CID-MS/MS spectrum showing the protonated b and y ion fragments of the doubly protonated native peptide (m/z 438.72) in the wild-type sample.

described in culture filtrates of *S. mutans* grown in CDM devoid of peptides (8). The other is CSP, which has not been identified in its native form and which may not always be required for competence, since *comC* deletion mutants whose competence levels were not affected have been reported (1). In this study, we identified the heptapeptide GLDWWSL in the supernatants of *S. mutans* and showed that endogenous XIP production occurred in the absence of detectable CSP levels in the supernatants of *S. mutans*. This is

the first report on the direct identification of a ComR/ComS pheromone.

Pheromones have traditionally been identified following the isolation and purification of active fractions from large volumes of bacterial cultures, involving many laborious steps. However, when pheromones are first identified by genomic analysis, the search for posttranslationally processed peptides can be simplified. This is particularly the case for pheromones that are endog-

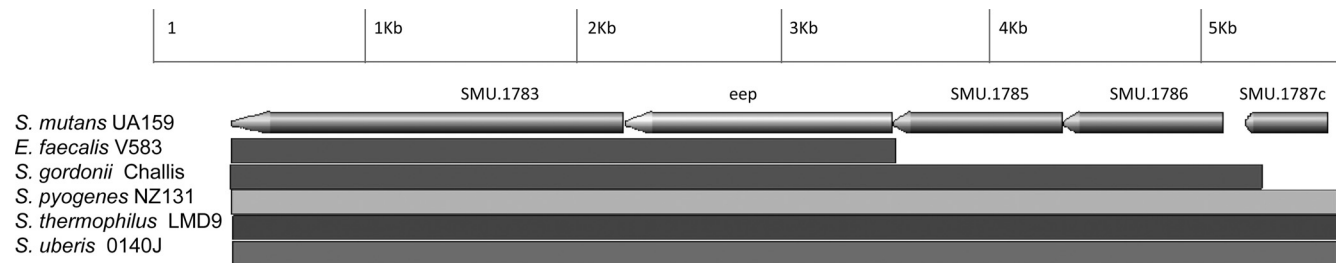


FIG 2 Syntenic region surrounding *eep*. The region shown is 2.2 kb upstream and downstream of the *eep* gene in *S. mutans* UA159. The corresponding syntenic regions in *E. faecalis* V583, *S. gordonii* Challis, *S. pyogenes* NZ131, *S. thermophilus* LMD-9, and *S. uberis* 0140J are shown as horizontal bars. The identities with *S. mutans* within the syntenic regions were 66.31%, 69.3%, 71.51%, 70.98%, and 70.78%, respectively. *Eep* identities were 52%, 66%, 70%, 67%, and 68%, respectively. They represent species or strains in which *Eep* has been associated with pheromone or lipoprotein processing. SMU.1783 encodes a prolyl-tRNA synthetase, SMU.1785 encodes a putative phosphatidate cytidylyltransferase synthase, SMU.1786 encodes a putative undecaprenyl pyrophosphate synthetase, and SMU.1787c encodes a preprotein translocase subunit, YajC. Syntenic region analysis was conducted using GEVO (27).

enously produced in a growth medium devoid of peptides, as in our study. In these instances, MS analytical tools can be used to advantage for identification of peptides of bacterial origin in supernatant extracts. Recently, the SHP 1358 pheromone of *S. thermophilus* was identified using such an approach (9). For the *S. mutans* XIP identification in our study, analysis of the crude extract resulted in few fragments corresponding to XIP in the MS/MS analysis. The analysis of complex mixtures of peptides may be complicated, however, by factors such as ion suppression, in which peptides with a composition that favors ionization may reduce the chances of other peptides to become protonated. Pheromones are also short, and since the aim is to identify the peptide in its native form, trypsinization of samples is omitted. Such factors reduce the power to identify single peptides in complex mixtures, which is based on probability scores, and may have contributed to our difficulties in identifying the processed ComS in crude extracts. This was solved, however, by including a simple filtration step that excluded larger peptides from the supernatants.

Direct identification of intracellular native pheromones bound

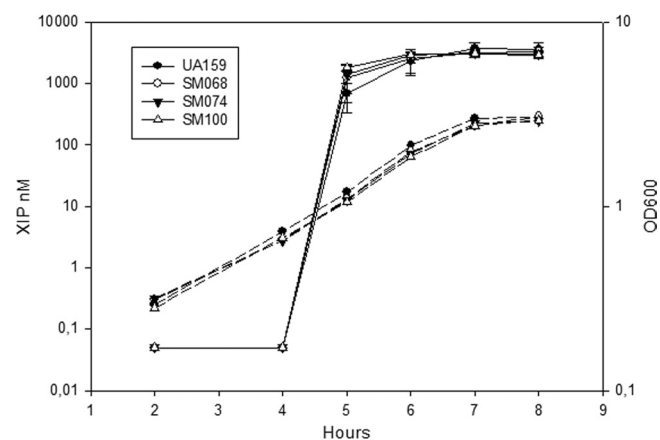


FIG 3 Endogenous XIP activity during growth. Extracellular XIP activity (solid lines) and OD₆₀₀ (dashed lines) were measured at different time points. The following strains were included: UA159, SM068, the *comC* deletion mutant SM074, and the *eep* deletion mutant SM100. The XIP concentration was estimated using a standard curve with serial ComS₁₁₋₁₇ dilutions. The tester strain was the *comS* deletion *P_{sigX}-luc* reporter SM091. The OD₆₀₀ values were measured using 1-cm-path-length cuvettes. The results are averages and standard errors (SE) from three independent experiments for the XIP levels and from one experiment for the growth curve.

to their respective regulators has not yet been described for any Rgg system to which ComR belongs. Without such an approach, it is not possible to draw definitive conclusions about the nature of the native pheromone. However, the findings that the peptide identified in our study corresponded to the seven C-terminal amino acids of ComS previously shown to induce high levels of *S. mutans* transformation in its synthetic form (26) and that active pheromones in Gram-positive bacteria usually represent truncated C-terminal derivatives modified before import (37) indicate that XIP is an active form of the pheromone.

To date, no identified systems for the processing of ComR/ComS pheromones have been reported. Since the *Eep* membrane metalloprotease plays an important role in the processing of *E. faecalis* pheromones and pheromone inhibitors and is probably involved in the processing of SHP pheromones in *S. thermophilus* (9) and *S. pyogenes* (6), we investigated whether *Eep* was involved in *S. mutans* ComS processing. Our results failed, however, to demonstrate its requirement, indicating the presence of an alter-

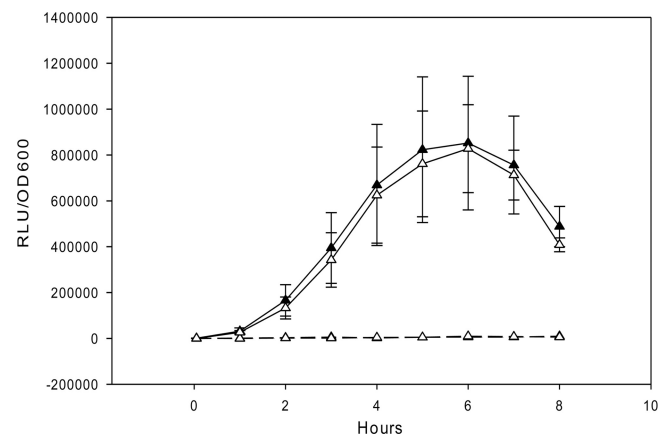


FIG 4 Effect of *eep* deletion on the response to ComS₁₁₋₁₇. *P_{sigX}-luc* reporters were used to measure *sigX* expression in *comS* or *comS-eep* mutants unable to produce their own XIP. The reporter SM091 ($\Delta comS P_{sigX-luc}$) (black triangles) or SM101 ($\Delta comS-\Delta eep P_{sigX-luc}$) (white triangles) was grown in the presence of 1 μ M ComS₁₁₋₁₇ (solid lines) or without ComS₁₁₋₁₇ (dashed line). Relative light unit (RLU) and OD₆₀₀ values were measured in a 96-well plate using a Multidetector microplate reader. The results are averages and SE from three independent experiments for cultures grown in the presence of ComS₁₁₋₁₇ and from one experiment for cultures grown in the absence of ComS₁₁₋₁₇.

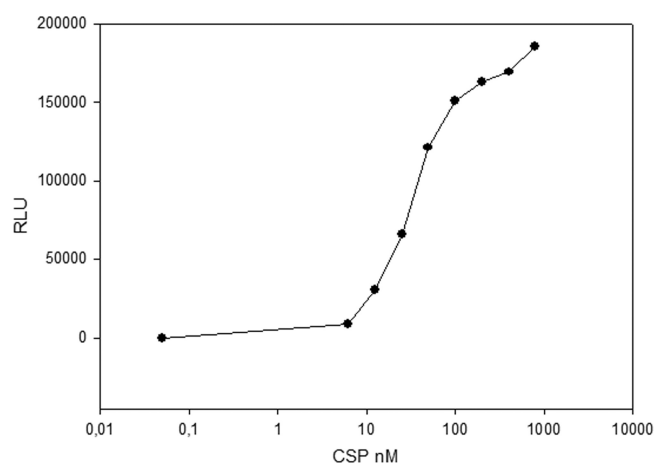


FIG 5 Bioassay for detection of CSP-like activity. The indicator strain SM059 ($P_{cipB-luc}$) was exposed to serial dilutions of synthetic 18CSP. Induction of luciferase activity is presented as RLU.

native system. We also investigated whether the XIP response was affected in the *eep* deletion mutant, since its participation in the assembly of Opp systems has been suggested (9). Although XIP import is thought to be mediated by an Opp system, our results did not demonstrate a role of Eep in the response to XIP. The presence of alternative mechanisms to process lipoprotein signal peptides have, however, been reported in streptococci (7). Therefore, we cannot exclude the possibility that functional redundancy may obscure a role for Eep in the assembly of a functional Opp system.

The recent finding that the ComR/ComS system is at the core of the competence response poses the question of how the second pheromone system represented by CSP is involved in competence stimulation (26). Although this mechanism is still unknown, a recent report that *comS* expression is increased in the presence of synthetic CSP (23) (also supported by our unpublished microarray results), at least in undefined rich media, indicates that CSP may stimulate competence by increasing production of the XIP pheromone. To date, however, there is no direct evidence that native CSP is produced. We therefore investigated whether a CSP-like activator was present in *S. mutans* supernatants, using a $P_{cipB-luc}$ reporter as the indicator strain. The synthetic 18CSP induced luciferase production by the indicator strain at concentrations as low as 12 nM, with maximal levels at approximately 100 nM. This concentration range of synthetic 18CSP activity is similar to that found in cultures of *S. mutans* grown in rich medium (31). No CSP-like activity was identified in the supernatants of wild-type or *comC* deletion strains. MS/MS analysis of wild-type supernatants also failed to demonstrate the presence of ComC processed peptides. Together with the finding that XIP activity in the supernatant of the *comC* deletion mutant was similar to that in the wild type, the results indicate that CSP either is not produced under the conditions investigated or was produced at concentrations that did not stimulate XIP production. Our results support the previous finding that deletion of *comC* has no effect on *sigX* expression or transformation (1). In the previous study, a nutritionally rich medium was used, indicating that the lack of an association between *comC* and *sigX* expression is not restricted to the chemically defined medium used in our study.

ComR/ComS type II pheromones are found in at least eight other species of streptococci, including some with high pathogenic potential, such as *S. pyogenes* (9, 10, 26). It is most likely that, similar to the ComS pheromone in *S. mutans* and to a variety of other pheromones in Gram-positive bacteria, ComS proteins in other streptococci are also processed into mature peptides. The analysis of crude mixtures of peptide extracts by advanced MS/MS methods has already been used for the identification of native peptides from a variety of sources, including, for instance, serum and neuron extracellular fractions (27, 38). In bacteria, such an “omics” approach to the identification of secreted endogenous peptides is in its infancy. Despite the challenges ahead, such as limitations to the identification of bacterial open reading frames (ORFs) encoding small peptides, with *comS* as an example, and of possible uncommon amino acid modifications, this is an exciting field for future investigations.

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