



# Identification of *Streptococcus gallolyticus* subsp. *gallolyticus* (Biotype I) Competence-Stimulating Peptide Pheromone

Anthony Harrington,<sup>a</sup>  Yftah Tal-Gan<sup>a</sup>

<sup>a</sup>Department of Chemistry, University of Nevada, Reno, Reno, Nevada, USA

**ABSTRACT** *Streptococcus gallolyticus* subsp. *gallolyticus*, a member of the group D streptococci, is normally found in the bovine rumen and human gut. It is an opportunistic pathogen that was recently determined to be a bacterial driver of colorectal cancer, in addition to causing other diseases, such as infective endocarditis, bacteremia, neonatal meningitis, and septicemia. As an emerging pathogen, not much is known about this bacterium, its virulence mechanisms, or its virulence regulatory pathways. Previous studies suggest that *S. gallolyticus* subsp. *gallolyticus* uses a ComRS pathway, one of many *Streptococcus* quorum-sensing circuitries, for competence. However, thus far, the ubiquitous ComABCDE pathway has not been studied, nor has its regulatory role in *S. gallolyticus* subsp. *gallolyticus*. We therefore sought to study the *S. gallolyticus* subsp. *gallolyticus* ComABCDE quorum-sensing pathway and have identified its peptide pheromone, which is termed the competence-stimulating peptide (CSP). We further determined that this peptide regulates the production of bacteriocin-like inhibitory substances (BLISs), a phenotype that has been linked with the ComABCDE pathway in both *Streptococcus pneumoniae* and *Streptococcus mutans*. Our data show that *S. gallolyticus* subsp. *gallolyticus* TX20005 produces a 21-mer CSP signal, which differs from CSP signals of other *Streptococcus* species in that its active form begins three residues after the double-glycine leader signal of the ComC precursor peptide. Additionally, our data suggest that this peptide might not be related to competence induction, as opposed to CSP signaling peptides in other *Streptococcus* species. This study provides the first evidence that *S. gallolyticus* subsp. *gallolyticus* utilizes quorum sensing to eliminate competitors, presenting a potential pathway to target this emerging human pathogen.

**IMPORTANCE** *Streptococcus gallolyticus* subsp. *gallolyticus* is an emerging human pathogen known as a causative agent of infective endocarditis, and recently, of colorectal cancer. In this work, we revealed a functional quorum-sensing circuitry in *S. gallolyticus* subsp. *gallolyticus*, including the identification of the central signaling peptide pheromone, competence-stimulating peptide (CSP), and the regulatory role of this circuitry in the production of bacteriocin-like inhibitory substances (BLISs). This work uncovered a mechanism by which this bacterium outcompetes other bacterial species and thus provides a potential tool to study this opportunistic pathogen.

**KEYWORDS** *S. gallolyticus*, competence-stimulating peptide, quorum sensing

**S***treptococcus gallolyticus* subsp. *gallolyticus*, a member of the group D streptococci belonging to the bovis group, was originally classified as *Streptococcus bovis* biotype I, until it was later changed to *Streptococcus gallolyticus*, which consists of three subspecies, *S. gallolyticus* subsp. *gallolyticus* (biotype I), *S. gallolyticus* subsp. *pasteurianus* (biotype II.2), and *S. gallolyticus* subsp. *macedonicus* (1–4). Since the late 1970s, a strong association has been established between patients suffering from bacteremia or endocarditis caused by *S. gallolyticus* subsp. *gallolyticus* and colorectal cancer (CRC) (5–11). Recently, *S. gallolyticus* subsp. *gallolyticus* has been shown to promote colon

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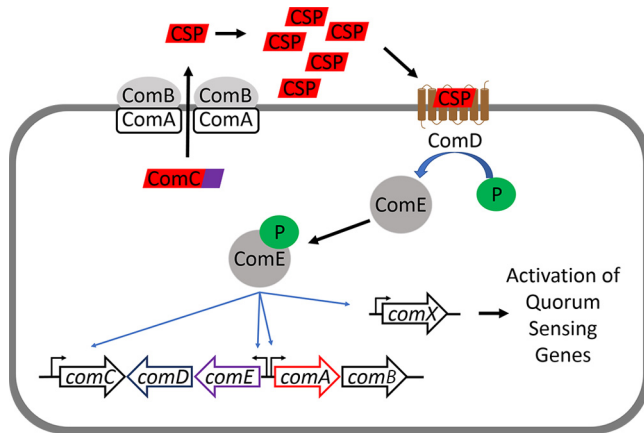
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Address correspondence to Yftah Tal-Gan, ytalgan@unr.edu.



**FIG 1** Streptococcus ComABCDE QS pathway. ComC is processed intracellularly and secreted by ComAB as the mature CSP. Upon reaching a threshold concentration, CSP binds and activates its cognate receptor, ComD. Activation of ComD leads to ComE phosphorylation, which results in the autoinduction of the QS circuitry (ComABCDE) and the expression of *comX*, the master regulator of QS-dependent phenotypes.

cancer cell proliferation and tumor growth, establishing that *S. gallolyticus* subsp. *gallolyticus* is a bacterial driver of CRC; however, the exact mechanism remains unknown (12, 13).

The competence quorum-sensing (QS) regulon was first discovered in *Streptococcus pneumoniae*, where it was shown that a signaling peptide, termed competence-stimulating peptide (CSP), induces *S. pneumoniae* to become competent (14–16). This QS circuitry consists of five components, collectively termed ComABCDE, and is centered on the CSP signaling peptide. The CSP signaling peptide is derived from its precursor peptide, ComC, and contains an N-terminal double glycine leader signal sequence that gets cleaved by the ABC transporter, ComAB, prior to being excreted into the environment (17). Upon reaching a threshold concentration, the CSP activates a two-component signal-transduction system (TCSTS) involving a histidine kinase (ComD) and a DNA-binding transcriptional response regulator (ComE). ComD activation leads to phosphorylation of ComE, resulting in the expression of QS genes (*comABCDE*) and the alternative sigma factor, SigX/ComX, a master regulator of competence genes (Fig. 1) (17, 18). This competence circuitry (ComABCDE) has been shown to be conserved in 12 *Streptococcus* species that are naturally transformable, with variations in the CSP sequence between species and, in a few cases, within different strains of the same species (phenotypes) (16, 19–21). The competence regulon in *S. pneumoniae* and *Streptococcus mutans* was also found to be linked to bacteriocin production (17). A different QS circuit, ComRS, was discovered in two species of the salivarius group, *Streptococcus thermophilus* and *Streptococcus salivarius* (22). In these species, a propeptide pheromone, ComS, gets secreted into the environment and cleaved either during or after its secretion to produce a 7-amino-acid signaling peptide, termed sigma inducing peptide (XIP) (23). XIP is imported into the intracellular environment, where it binds to a transcriptional regulator, ComR. The binding of XIP to ComR then upregulates the expression of ComX, leading to induction of competence. This pathway was found to exist in some species of the mutans, pyogenic, and bovis groups, suggesting that this alternative QS circuitry is also conserved among streptococci (24).

The genomes of many bovis group species, including those of a few strains of *S. gallolyticus* subsp. *gallolyticus*, are publicly available, and analysis of the *S. gallolyticus* subsp. *gallolyticus* genome suggests that it contains both *comABCDE* and *comRS* operons. Sequencing of the *S. gallolyticus* subsp. *gallolyticus* genome showed that it contains a *comABCDE* operon; however, the *comC* gene was previously claimed to be an inactive allele, with no further justification provided on why the authors concluded that this gene is inactive (25). Nonetheless, no further studies were conducted to confirm the presence of these operons or identify their functions (26–28). It was

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Sgg_ATCC_43143    KNKDFLTVGPF-DWLKKNHK-PTKHA
Sgg_UCN34        KNKDFLTVGPF-DWLKKNHK-PTKHA
Sgg_TX20005     KNKDFLTVGPF-DWLKKNHK-PTKHA
Sgg_NTS_3130195 KNKDFLTVGPF-DWLKKNHK-PTKHA
Sgg_NTS_3110609 KNKDFLTVGPF-DWLKKNHK-PTKHA
Sgg_NTS_3130765 KNKDFLTVGPF-DWLKKNHK-PTKHA
Sgg_DSM_16831   KNKDFLTVGPF-DWFKKHQGKSQKHM
Spne_TIGR4      ---EMRISRIILDVFLFLRKK-----
Spne_D39        ---EMRISKFFRDFIILQRKK-----
Sint_NCDO2227   ---DSRTRMGF-DFSKLFGK-----
    
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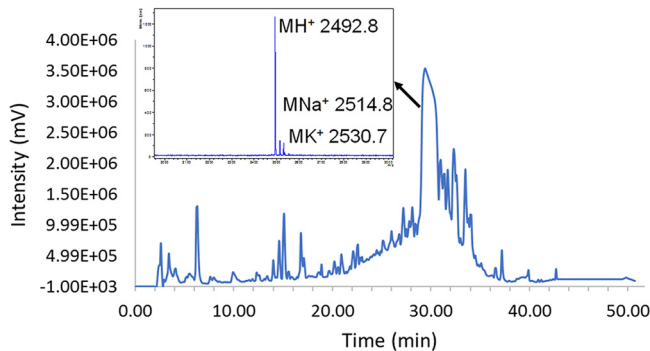
**FIG 2** Multiple-sequence alignment of ComC. The ComC sequences of several *S. gallolyticus* subsp. *gallolyticus* strains, with double-glycine leader signal removed, were aligned. The ComC sequences of *S. pneumoniae* D39, *S. pneumoniae* TIGR4, and *S. intermedius* NCDO2227 were used as reference sequences.

suggested through genomic analysis that the bovis group uses the ComRS circuitry for competence. This hypothesis was later confirmed experimentally in two species, *Streptococcus infantarius* and *Streptococcus macedonicus* (25). Additionally, it was shown that the recombinant Strep II-tagged ComR of *S. gallolyticus* subsp. *gallolyticus* strain UNC34 can bind DNA probes containing the promoter regions of both *comS* and *comX* in the presence of its cognate XIP, suggesting that *S. gallolyticus* subsp. *gallolyticus* probably uses the ComRS pathway for competence (24). However, no studies to date were able to demonstrate competence in *S. gallolyticus* subsp. *gallolyticus*. The purpose of this work was to study the ComABCDE circuitry in *S. gallolyticus* subsp. *gallolyticus* by predicting the CSP sequence, isolating the CSP, and determining its regulatory role. Our results show that *S. gallolyticus* subsp. *gallolyticus* uses a 21-mer CSP that is involved in QS-dependent production of bacteriocin-like inhibitory substances (BLISs).

## RESULTS AND DISCUSSION

**Multiple sequence alignments of ComC sequences.** A prediction of the *S. gallolyticus* subsp. *gallolyticus* CSP was made by performing a multiple-sequence alignment (MSA) of ComC sequences of several *S. gallolyticus* subsp. *gallolyticus* strains, using the ComC protein sequences of *S. pneumoniae* (strains D39 and TIGR4) and *Streptococcus intermedius* strain NCDO 2227 as reference sequences. The MSA predicted *S. gallolyticus* subsp. *gallolyticus* to have a 24-mer CSP containing a positively charged, N-terminal amino acid that differs from the negatively charged, N-terminal amino acids of the reference sequences (see Fig. S1 in the supplemental material). After removing the double glycine leader signal from the original MSA, we performed another alignment to look for any similarities between the predicted *S. gallolyticus* subsp. *gallolyticus* CSP and the reference CSP sequences. This analysis indicated that the fourth residue of the predicted *S. gallolyticus* subsp. *gallolyticus* CSP is likely the first residue of the mature CSP, as it follows the trend in the reference CSPs of having a negatively charged, N-terminal amino acid (Fig. 2). We therefore hypothesized that *S. gallolyticus* subsp. *gallolyticus* produces and utilizes a 21-mer CSP. Lastly, we noticed that the *S. gallolyticus* subsp. *gallolyticus* strain DSM 16831 sequence differs from those of the other strains in eight positions and is one amino acid longer (22 mer), suggesting the presence of pherotypes in this species, similarly to those of *S. pneumoniae* (16, 29). To support our hypothesis, we designed primers to amplify the intergenic region in front of the *comC* gene and the 3' coding strand region of the *comD* gene from *S. gallolyticus* subsp. *gallolyticus* TX20005. We PCR amplified and sequenced the *comC* gene, which matched with the results of the uploaded genome of *S. gallolyticus* subsp. *gallolyticus* TX20005 (GL397173.1). Furthermore, the translated sequence matched the ComC sequences of *S. gallolyticus* subsp. *gallolyticus* TX20005 (EFM28801.1) and other *S. gallolyticus* subsp. *gallolyticus* strains (Fig. 2).

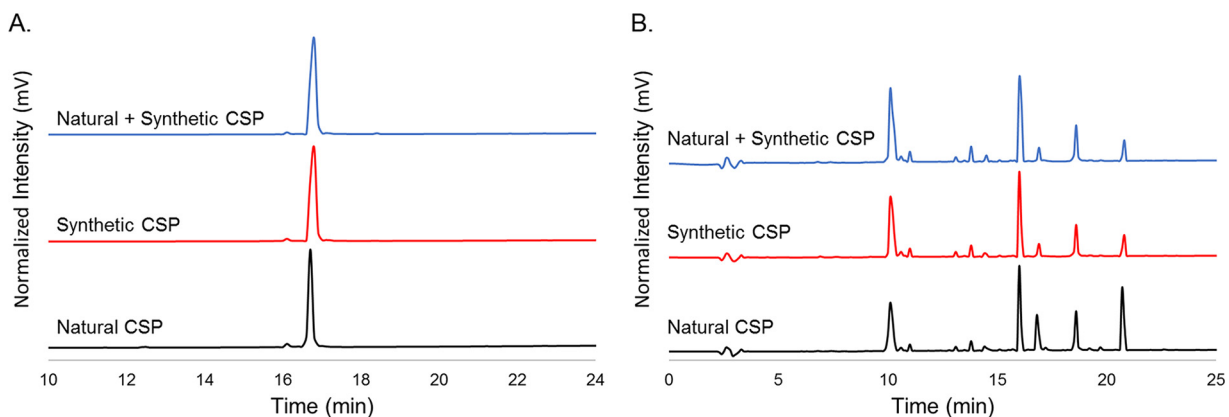
**Isolation of predicted CSP peptide from spent, cell-free supernatants.** Our next step in identifying the *S. gallolyticus* subsp. *gallolyticus* CSP was to isolate it from bacterial supernatants. Following precipitation of the excreted crude peptide mixture from cell-free



**FIG 3** Isolation and identification of *S. gallolyticus* subsp. *gallolyticus* CSP. RP-HPLC chromatogram trace of crude protein extract from *S. gallolyticus* subsp. *gallolyticus* cell-free supernatants and MALDI-TOF MS of a fraction collected between 29.5 to 30.5 min (major peak).

supernatants of *S. gallolyticus* subsp. *gallolyticus* TX20005, we purified various peptides/proteins using reversed-phase high-performance liquid chromatography (RP-HPLC). We observed an intense peak eluting between 29.5 to 30.5 min that contained a mass of 2,492.8 Da, as determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Fig. 3). This mass corresponds to the predicted 21-mer CSP sequence (mass without the first three N-terminal residues, 2,491.9 Da). This analysis provided the first support for our hypothesis that the mature CSP in *S. gallolyticus* subsp. *gallolyticus* is a 21-mer peptide bearing a negatively charged residue at the N terminus. Overall, we were able to recover approximately 2.5 mg of this peptide from 50 ml of overnight-grown *S. gallolyticus* subsp. *gallolyticus* spent supernatants, corresponding to a 20  $\mu$ M concentration.

**Chemical synthesis of predicted CSP and comparison to the naturally isolated CSP.** We decided to synthesize the predicted CSP sequence using microwave-assisted 9-fluorenyl methoxycarbonyl (Fmoc)-based solid-phase peptide synthesis and obtained 2 mg of purified peptide (3.8% yield of purified peptide, >95% purity). We compared the synthetic peptide to the peptide isolated from cell-free supernatants to confirm the identity of the *S. gallolyticus* subsp. *gallolyticus* CSP. HPLC analysis of both peptides revealed a single peak with the same retention time (Fig. 4A; see also Fig. S2 and S3 in the supplemental material). Furthermore, a combined fraction of both the synthetic and isolated peptides resulted in a single peak in the HPLC with the same retention time (Fig. 4A; see also Fig. S4 in the supplemental material). In addition, positive electrospray ionization (ESI+) high-resolution mass spectrometry (HRMS) of both peptides exhibited similar ionization trends (+2, +3, and +4 charged states; all exact



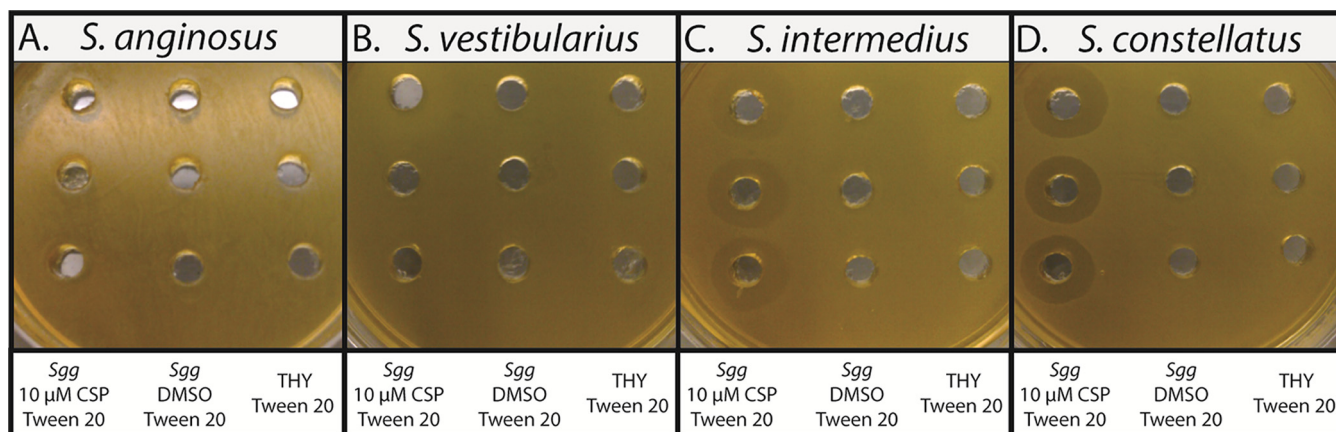
**FIG 4** Comparison of synthetic and isolated CSPs. (A) Overlay with offsets of analytical RP-HPLC chromatograms of purified natural, synthetic, and natural and synthetic CSP. (B) Overlay with offset of analytical RP-HPLC chromatograms of chymotrypsin digestion of natural, synthetic, and natural and synthetic CSP.

masses [EM] within 5 ppm error) (see Fig. S8 and S9 in the supplemental material). We then submitted both the naturally isolated and synthetic peptides to the Nevada Proteomics Center for tandem MS analysis. This analysis confirmed that the amino acid sequence is DFLIVGPFDWLKKNHKPTKHA; however, uncertainty remained in regard to the order of the first two amino acids (see Fig. S10 to S13 in the supplemental material). Thus, we treated both samples with chymotrypsin, a protease that selectively cleaves the amide bonds following an aromatic residue (phenylalanine, tyrosine, or tryptophan), with the aim of confirming the order of the first two residues by obtaining either an LIVGPF fragment (DF) or a DLIVGPF fragment (FD). After treating both naturally isolated and synthetic *S. gallolyticus* subsp. *gallolyticus* CSPs with chymotrypsin, we observed similar chromatograms by analytical RP-HPLC analysis (Fig. 4B; see also Fig. S14 and S15 in the supplemental material), and we observed a mass of 645.39 Da using ESI+ HRMS for both peptides, corresponding to an amino acid sequence of LIVGPF. Together, these results confirmed that the CSP sequence of *S. gallolyticus* subsp. *gallolyticus* is DFLIVGPFDWLKKNHKPTKHA. Lastly, we performed structural analysis of both peptides using circular dichroism and found that *S. gallolyticus* subsp. *gallolyticus* CSP has a weak  $\alpha$ -helical propensity in membrane-mimicking conditions (approximately 10% helicity) (see Fig. S16 and S17 in the supplemental material) (30).

**Phenotypic assays.** After confirming the CSP sequence in *S. gallolyticus* subsp. *gallolyticus*, we set out to determine its regulatory role. Since these peptides are termed competence-stimulating peptides and have been shown to induce competence in several *Streptococcus* species (14, 21), we first attempted to induce competence in *S. gallolyticus* subsp. *gallolyticus* using the synthetic CSP and pALH122, a plasmid that contains an erythromycin-resistance gene. All our attempts to induce competence were unsuccessful. We tried plating the mixtures grown in Todd-Hewitt agar supplemented with 0.5% yeast extract (THY) containing CSP and plasmid, using various concentrations of synthetic CSP (100 nM, 500 nM, and 10  $\mu$ M) at different time points (30 min, 1 h, 2 h, 3 h, 4 h, and 16 h) with no transformants. We decided to try the transformation of *S. gallolyticus* subsp. *gallolyticus* using a chemically defined medium to help induce competence with the synthetic CSP, as was shown for *S. mutans* (31–33). We repeated the experiment, growing *S. gallolyticus* subsp. *gallolyticus* in a chemically defined medium, M9 supplemented with 1% glucose and 1% biotin, prior to plating, and again we observed no transformants.

After our attempts to induce competence failed, we turned to assessing biofilm formation, as this phenotype was also correlated to the competence regulon in *S. pneumoniae*, *S. intermedius*, *S. gordonii*, and *S. mutans* (34–38). To this end, we performed a crystal violet biofilm quantification assay. Our results show no significant difference in biofilm formation with various concentrations of CSP (10  $\mu$ M, 1  $\mu$ M, and 0.1  $\mu$ M) compared to our negative control (dimethyl sulfoxide [DMSO]) (data not shown). These results do not necessarily rule out any role of this circuitry in regulating these phenotypes; rather, they indicate that for the conditions we tested, this QS circuitry failed to play a significant role.

We next tested the role of the competence regulon in bacteriocin production. To this end, we performed an interspecies inhibition assay, where we tested the ability of *S. gallolyticus* subsp. *gallolyticus* to inhibit the growth of several *Streptococcus* species, namely, *Streptococcus intermedius* F0413, *Streptococcus vestibularius* F0396, *Streptococcus mutans* ATCC 25175, *Streptococcus anginosus* ATCC 33397, *Streptococcus constellatus* ATCC 27823 and *Streptococcus agalactiae* MNZ938, following treatment of *S. gallolyticus* subsp. *gallolyticus* with either CSP (10  $\mu$ M or 100 nM) or DMSO (negative control). Our results revealed similar CSP-mediated inhibition for both concentrations of CSP against *S. intermedius* F0413, *S. anginosus* ATCC 33397, *S. constellatus* ATCC 27823, and *S. vestibularius* F0396, whereas neither self-inhibition nor inhibition against *S. agalactiae* MNZ938 or *S. mutans* ATCC 25175 was observed (see Fig. S18 in the supplemental material). Moreover, treatment with only *S. gallolyticus* subsp. *gallolyticus* CSP (no bacteria) did not induce any growth inhibition against any of the species, indicating that *S. gallolyticus* subsp. *gallolyticus* CSP is not toxic to the bacteria tested



**FIG 5** Images comparing results from *S. gallolyticus* subsp. *gallolyticus* interspecies inhibition assay, where *S. gallolyticus* subsp. *gallolyticus* is incubated with CSP (10 μM) or DMSO, followed by addition of Tween 20, sterile filtration, and testing of the supernatants against *S. anginosus* ATCC 33397 (A), *S. vestibularius* F0396 (B), *S. intermedius* F0413 (C), and *S. constellatus* ATCC 27823 (D). Inhibition was observed in panels C and D but not in panels A and B.

and that the CSP-mediated inhibition was due to a regulatory role it possesses. To verify the removal of all *S. gallolyticus* subsp. *gallolyticus* cells, we attempted the experiment again, using filtered supernatants, and observed no inhibition. We performed another experiment, where we grew *S. gallolyticus* subsp. *gallolyticus* without CSP and only added it to the centrifuged supernatants prior to placing the supernatants in the agar wells; in this case, we observed inhibition zones. Lastly, we tested the centrifuged supernatants for the presence of *S. gallolyticus* subsp. *gallolyticus* cells and found that the supernatants contained a few bacterial cells. Together, these experiments confirm that the observed inhibition is CSP-dependent. Moreover, the results indicate that either the inhibitory agent (presumably bacteriocin) is cell associated or that the observed inhibition is mediated by a different mechanism requiring direct cell contact. In a previous study, Mantovani et al. showed that a *Streptococcus bovis* bacteriocin is cell associated and requires detergents to be released from the cell surface (39). We therefore repeated the interspecies inhibition assay against the four species that were found to be inhibited by *S. gallolyticus* subsp. *gallolyticus* (*S. intermedius* F0413, *S. anginosus* ATCC 33397, *S. constellatus* ATCC 27823, and *S. vestibularius* F0396), using only one CSP concentration (10 μM), but this time, following centrifugation, we washed the overnight cells with phosphate-buffered saline (PBS) containing Tween 20 to remove any cell-associated bacteriocin-like inhibitory substances (BLISs) (40), prior to treatment with either CSP or DMSO. Following the 4 h of incubation, we added Tween 20 prior to sterile-filtration of the supernatants and addition to the agar plates containing the *Streptococcus* species. Interestingly, CSP-dependent inhibition was observed against only two species (*S. intermedius* F0413 and *S. constellatus* ATCC 27823), while the growth of the other two species (*S. anginosus* ATCC 33397 and *S. vestibularius* F0396) was not affected (Fig. 5). We concluded that *S. gallolyticus* subsp. *gallolyticus* utilizes two distinct mechanisms to outcompete other *Streptococcus* species and that both mechanisms are CSP-dependent; *S. intermedius* F0413 and *S. constellatus* ATCC 27823 are inhibited through a secreted BLIS, while *S. anginosus* ATCC 33397 and *S. vestibularius* F0396 are inhibited through an unknown mechanism that involves direct cell contact. Finally, to further characterize the BLIS, we washed CSP-treated cells with PBS, added 0.1% Tween 20, filter-sterilized the solution, and treated with ammonium sulfate to precipitate peptide and protein contents. Then, following resuspension of the precipitate, we observed interspecies inhibition, suggesting that the BLIS is peptide/protein based. We isolated the BLIS using RP-HPLC (see Fig. S19 in the supplemental material) and found that only one fraction exhibited interspecies inhibition against *S. intermedius* F0413 and *S. constellatus* ATCC 27823, suggesting that one BLIS is responsible for the observed inhibition.

There is a continuous debate regarding the role of the ComABCDE circuitry in both competence and bacteriocin production. Our results provide supporting evidence that the ComABCDE pathway is likely related to the production of a BLIS in *S. gallolyticus* subsp. *gallolyticus*. Our data suggest that this BLIS is a bacteriocin; however, additional studies are needed to determine the identity of the secreted agent/s that are responsible for the CSP-mediated growth inhibition. These studies are ongoing in our lab and will be reported in due course.

In summary, we demonstrated that *S. gallolyticus* subsp. *gallolyticus* has a functional *comC* gene that produces a 21-mer CSP. Furthermore, our results indicate that the CSP precursor peptide is unique compared to other known *Streptococcus* species CSP precursor peptides, in that its active sequence begins three residues after the double glycine leader signal, a phenomenon that has never been observed in other *Streptococcus* species (16). These findings suggest that either the ComAB differs in *S. gallolyticus* subsp. *gallolyticus* compared to those of other streptococci or that *S. gallolyticus* subsp. *gallolyticus* possesses an extracellular protease, such as SepM, which was found to process the C termini of CSPs in other streptococci prior to binding to the ComD receptor (41). Additionally, our bioinformatics analysis suggests that *S. gallolyticus* subsp. *gallolyticus* strains might be divided into two phenotypes with distinct CSP sequences. Finally, we found that *S. gallolyticus* subsp. *gallolyticus* is capable of inhibiting the growth of other *Streptococcus* species through two distinct CSP-dependent mechanisms: (i) the production of a BLIS and (ii) direct cell contact. This observation could explain the ability of *S. gallolyticus* subsp. *gallolyticus* to outcompete other bacteria and occupy particular niches related to infective endocarditis and/or CRC.

## MATERIALS AND METHODS

All chemical reagents and solvents were purchased from Sigma-Aldrich or Chem-Impex and used without further purification. Double-distilled water (ddH<sub>2</sub>O) was purified using a Millipore Analyzer feed system. Solid-phase 2-(4-(chloromethyl)phenoxy)acetamide (Cl-MPA) ProTide resin was purchased from CEM Corporation. 9-Fluorenyl methoxycarbonyl (Fmoc)-protected L- $\alpha$ -amino acids were purchased from Advanced ChemTech.

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using a Shimadzu ultra fast liquid chromatography (UFLC) system equipped with a CBM-20A communications bus module, two LC-20AT pumps, an SIL-20A autosampler, an SPD-20A UV-visible (UV-Vis) detector, a CTO-20A column oven, and an FRC-10A fraction collector. All RP-HPLC solvents (ddH<sub>2</sub>O and HPLC-grade acetonitrile [ACN]) contained 0.1% trifluoroacetic acid (TFA). Preparative RP-HPLC was performed using a Phenomenex Kinetex 5- $\mu$ m, 100- $\text{\AA}$  C<sub>18</sub> column (250  $\times$  10 mm), whereas analytical RP-HPLC was performed using a Phenomenex Kinetex 5- $\mu$ m, 100- $\text{\AA}$  C<sub>18</sub> column (250 by 4.6 mm). Fmoc solid-phase peptide synthesis was performed on a Discover Microwave and a Liberty1 automated peptide synthesizer (CEM Corporation). PCR amplification was performed in an Eppendorf Mastercycler gradient 5331 PCR machine (Eppendorf). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) data were obtained by mixing 0.75  $\mu$ l of sample with 0.75  $\mu$ l of matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid dissolved in ddH<sub>2</sub>O-ACN [1:1] with 0.1% TFA) on an MSP 96 polished steel target plate (Bruker Daltonics) and allowing it to air dry. Data were obtained using a Bruker Microflex spectrometer equipped with a 60-Hz (337-nm wavelength) nitrogen laser and a reflectron. MALDI-TOF MS data were obtained using the reflectron's positive ion mode with the following settings: ion source 1, 19 kV; ion source 2, 15.9 kV; lens, 8.75 kV; reflector, 20 kV; up to 300-Da matrix suppression; 200 laser shots per sample; and detector gain, 1,594 V. Exact mass (EM) data were obtained on an Agilent Technologies 6230 time of flight mass spectrometer (an HRMS system) with the following settings for positive electrospray ionization (ESI+) mode: capillary voltage, 3,500 V; fragmentor voltage, 175 V; skimmer voltage, 65 V; Oct 1 RF Vpp, 750 V; gas temperature, 325°C; drying gas flow rate = 3 liters/min; and nebulizer, 25 lb/in<sup>2</sup>.

**Bacterial growth conditions.** *S. gallolyticus* subsp. *gallolyticus* TX20005, *S. intermedius* F0413, *S. vestibularis* F0396, and *S. agalactiae* MNZ938 freezer stock cultures were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources), while *S. mutans* ATCC 25175, *S. anginosus* ATCC 33397, and *S. constellatus* ATCC 27823 freezer stock cultures were obtained from the *Streptococcus* Lab at the Centers for Disease Control and Prevention. The following procedure was followed for each obtained isolate: a freezer stock was streaked onto a plate of Todd-Hewitt agar supplemented with 0.5% yeast extract (THY). The plate was incubated for 12 to 24 h in a CO<sub>2</sub> incubator (37°C with 5% CO<sub>2</sub>). Fresh colonies were picked and Gram-stained to ensure purity. After purity was confirmed, colonies (2 to 5) were picked and inoculated into a sterilized culture tube containing 3 ml of sterile THY broth and incubated statically in a CO<sub>2</sub> incubator for 8 to 24 h.

**PCR amplification of RNA polymerase B and the *comC* gene.** PCR amplification was conducted using established protocols (42, 43); see the supplemental material for details.

**Sequencing and verification of PCR-amplified *rpoB* and *comC* genes.** Sequencing and verification of PCR products were conducted at the Nevada Genomics Center, using established protocols (44, 45).

The sequenced partial *rpob* gene sequences were used to confirm the identity of our isolates to the species level; see the supplemental material for details.

**Predicting the *S. gallolyticus* subsp. *gallolyticus* competence-stimulating peptide sequence.** The following ComC protein sequences were downloaded from the NCBI database for various *S. gallolyticus* subsp. *gallolyticus* strains and for *S. pneumoniae* and *S. intermedius* for multiple-sequence alignment: WP\_009854999.1, AQP43123.1, CBI14504.1, EFM28801.1, OCW49321.1, KJE98669.1, OAV84346.1, WP\_000799689.1, AAK76284.1, and CAA04354.1. The sequences were aligned in Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software, using ClustalW with the default settings. The aligned sequences were then trimmed from the N terminus to the first residue after the double glycine leader signal, and another alignment was performed under the same conditions. This alignment was used to predict the possible CSP sequence. Multiple sequence alignments were then exported and edited using Boxshade 3.21 ([https://www.ch.embnet.org/software/BOX\\_form.html](https://www.ch.embnet.org/software/BOX_form.html)).

**Isolation of crude peptides from bacterial supernatants.** Overnight *S. gallolyticus* subsp. *gallolyticus* culture (25 ml) was centrifuged at  $4,600 \times g$  for 10 min. The supernatants were then filtered through a sterile 0.22- $\mu\text{m}$  polyethersulfone (PES) filter into a sterile, empty 50-ml centrifuge tube. Ammonium sulfate was added to the filtered supernatants to give a 20% (wt/vol) concentration and mixed by inversion until all of the ammonium sulfate went into solution. The solution was stored at 4°C for 45 min, followed by centrifugation at  $4,600 \times g$  for 15 min. The supernatants were discarded and the remaining pellet was dissolved in 10 ml of ddH<sub>2</sub>O-ACN (1:1) and freeze-dried. The lyophilized material was purified by RP-HPLC.

**Bacteriocin-like inhibitory substance extraction and isolation.** Overnight *S. gallolyticus* subsp. *gallolyticus* culture (500 ml) was centrifuged at  $4,600 \times g$  for 10 min. The supernatants were discarded, whereas the cell pellet was resuspended in 25 ml of sterile PBS. The resuspended cells were centrifuged at  $4,600 \times g$  for 10 min, and the supernatants were discarded. This process was repeated 3 times. The washed cells were resuspended in 10 ml of sterile PBS containing 10  $\mu\text{l}$  Tween 20 and shaken vigorously for 30 min. The mixture was centrifuged at  $4,600 \times g$  for 10 min, and the supernatants were filtered through a 0.22- $\mu\text{m}$  PES filter. Ammonium sulfate was added (40% [wt/vol]) and the mixture was stored at 4°C for 1 h and centrifuged at  $4,600 \times g$  for 30 min. The supernatants were discarded, and the remaining pellet was dissolved in 5 ml of H<sub>2</sub>O and freeze-dried. RP-HPLC purification was performed, and the various fractions were freeze-dried prior to being resuspended in 250  $\mu\text{l}$  of sterile water and tested in the interspecies inhibition assay against *S. intermedius* F0413 and *S. constellatus* ATCC 27823.

**Peptide purification.** Peptide purification was conducted using RP-HPLC and established procedures (46); see the supplemental material for details.

**Solid-phase peptide synthesis.** Peptide synthesis was conducted using established protocols (47–49); see the supplemental material for details.

**Peptide mapping.** Samples of synthetic *S. gallolyticus* subsp. *gallolyticus* CSP and naturally isolated *S. gallolyticus* subsp. *gallolyticus* CSP were prepared in a 20  $\mu\text{M}$  concentration in ddH<sub>2</sub>O and provided to the Nevada Proteomics Center for peptide mapping by ESI-tandem MS. The amino acid sequences of multiple charge states were processed using Scaffold 4 software.

**Chymotrypsin digestion.** Chymotrypsin (Worthington Biochemical Corporation) was dissolved in 1 mM HCl solution to a final concentration of 1  $\mu\text{g}/\text{ml}$ . Both naturally isolated and synthetic *S. gallolyticus* subsp. *gallolyticus* CSPs were dissolved to a final concentration of 1 mg/ml in PBS (pH 7.6) containing 1 mM CaCl<sub>2</sub>. A mixture containing 1:20 chymotrypsin-*S. gallolyticus* subsp. *gallolyticus* CSP was prepared and incubated at 37°C for 12 to 16 h. Samples were analyzed by analytical HPLC to confirm cleavage of the peptide. The chymotrypsin-*S. gallolyticus* subsp. *gallolyticus* CSP mixtures were purified using RP-HPLC to remove buffer salts and freeze-dried prior to being resuspended in ddH<sub>2</sub>O for fragment analysis by ESI+HRMS to determine the correct sequence of the *S. gallolyticus* subsp. *gallolyticus* CSP.

**Circular dichroism spectroscopy.** Circular dichroism spectra were acquired as previously described (46); see the supplemental material for details.

**Crystal violet biofilm assay.** Biofilm quantification using crystal violet was performed as previously described (50); see the supplemental material for details.

**Plasmid extraction.** Plasmid extraction and quantification were conducted using established protocols (51); see the supplemental material for details.

**Transformation assay.** A 1:10 dilution of overnight culture was made in sterilized THY and incubated statically in a CO<sub>2</sub> incubator (37°C with 5% CO<sub>2</sub>) for 4 to 6 h (optical density at 600 nm [OD<sub>600</sub>], ~0.25). Six sterile microcentrifuge tubes for each experimental time point (30 min, 1 h, 2 h, 3 h, 4 h, and 16 h) received 154  $\mu\text{l}$  of sterile medium (THY or M9 with 1% glucose and 1% biotin) and 40  $\mu\text{l}$  of the fresh culture (for a total of 36 tubes). The tubes were incubated at 37°C with shaking at 50 rpm for 30 min prior to addition of CSP, plasmid, or DMSO. For each time point, 2 tubes (one THY and one M9) received 2  $\mu\text{l}$  of 1 mM CSP with 4  $\mu\text{l}$  of sterile water, 2 other tubes (one THY and one M9) were treated with 4  $\mu\text{l}$  of pALH122 (40 ng/ $\mu\text{l}$ ) with 2  $\mu\text{l}$  of DMSO, and the final 2 tubes (one THY and one M9) were treated with 2  $\mu\text{l}$  of 1 mM CSP and 4  $\mu\text{l}$  of pALH122. The microcentrifuge tubes were incubated at 37°C with shaking at 50 rpm. Six tubes (three THY and three M9), consisting of CSP only, plasmid only, and CSP plus plasmid conditions were removed at the appropriate time point, and the content of each tube was spread plated onto two agar plates (100  $\mu\text{l}$  for each plate), one plate of THY agar and one plate of THY agar with erythromycin (10 ng/ $\mu\text{l}$  final concentration). The plates were incubated for 24 to 96 h in a CO<sub>2</sub> incubator (37°C with 5% CO<sub>2</sub>) prior to being inspected for colonies. The procedure was repeated using working stocks of 50  $\mu\text{M}$  and 10  $\mu\text{M}$  CSP to give final concentrations of 500 nM and 100 nM CSP, respectively.

**Interspecies inhibition assay.** Three sterile microcentrifuge tubes received 445  $\mu\text{l}$  of sterile THY and 50  $\mu\text{l}$  of *S. gallolyticus* subsp. *gallolyticus* overnight culture. A fourth sterile microcentrifuge tube



received 495  $\mu$ l of sterile THY and 5  $\mu$ l of CSP (10  $\mu$ M final concentration). The three *S. gallolyticus* subsp. *gallolyticus*-containing microcentrifuge tubes were incubated aerobically for 30 min at 37°C with shaking at 50 rpm. Each tube then received either 5  $\mu$ l of CSP (10  $\mu$ M or 100 nM final concentration) or DMSO (negative control), followed by 4 h of incubation in a CO<sub>2</sub> incubator (37°C with 5% CO<sub>2</sub>). The tubes were then centrifuged at 14,000  $\times$  g for 5 min to pellet as many cells as possible. The supernatants were then transferred to a sterile microcentrifuge tube and centrifuged again at 14,000  $\times$  g for 5 min. The process was repeated one more time, and the supernatants were used for the interspecies inhibition assay.

For each isolate tested against *S. gallolyticus* subsp. *gallolyticus*, 250  $\mu$ l of overnight culture was spread plated onto a THY agar plate, and the plate was allowed to dry for 10 min. After the plate was dry, wells were made by using the larger diameter of a sterile 200  $\mu$ l pipette tip, and the agar plugs were picked out using the smaller-diameter tip. Wells were made in triplicates for each of the following conditions: *S. gallolyticus* subsp. *gallolyticus* supernatants and DMSO, THY and 10  $\mu$ M CSP, *S. gallolyticus* subsp. *gallolyticus* supernatants and 10  $\mu$ M CSP, and *S. gallolyticus* subsp. *gallolyticus* supernatants and 100 nM CSP. A volume of 85  $\mu$ l was transferred to three wells for each condition and incubated overnight in a CO<sub>2</sub> incubator (37°C with 5% CO<sub>2</sub>). After overnight incubation, the plates were inspected for zones of inhibition around the wells. The experiment was repeated for 3 consecutive days. The same process was repeated, with the exception that the supernatants were filtered with a 0.45- $\mu$ m polytetrafluoroethylene (PTFE) filter for one trial and a 0.22- $\mu$ m PES filter for another trial, prior to being placed in the wells. These experiments were also repeated for 3 consecutive days. In a different trial, *S. gallolyticus* subsp. *gallolyticus* cells were grown under the same conditions, but with no added CSP during the incubation; only after 3 consecutive high-speed centrifugations or filtration using a 0.22- $\mu$ m PES filter, were 5  $\mu$ l of CSP added to reach the final concentration of 10  $\mu$ M. A volume of 85  $\mu$ l of these two supernatants was transferred to three wells and incubated overnight in a CO<sub>2</sub> incubator (37°C with 5% CO<sub>2</sub>), prior to inspection for zones of inhibition. These experiments were also repeated for 3 consecutive days. Finally, the same procedure was repeated, using either 10  $\mu$ M final concentration CSP or DMSO, with the exception that at the end of the 4 h of incubation, 0.5  $\mu$ l of Tween 20 was added to the supernatants and shaken vigorously for 10 min, followed by centrifugation at 14,000  $\times$  g. The supernatants were filtered through a sterile 0.22- $\mu$ m PES filter and used for the interspecies inhibition assay. These experiments were also repeated for 3 consecutive days.

**Accession number(s).** The *comC* nucleotide and protein sequences of *S. gallolyticus* subsp. *gallolyticus* TX20005 were deposited in GenBank under the assigned accession numbers [MF964227](#) and [AWK22889](#).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00709-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 1.8 MB.

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