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Investigating the *Streptococcus sinensis* competence regulon through a combination of transcriptome analysis and phenotypic evaluation

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Abstract

Streptococcus sinensis is a recently identified member of the Mitis group of streptococci. This species has been associated with infective endocarditis; however its mechanisms of pathogenesis and virulence are not fully understood. This study aimed to investigate the influence of the competence-stimulating peptide (CSP) and the competence regulon quorum-sensing circuitry (ComABCDE) on subsequent gene transcription and expression, as well as resultant phenotypes. In this study we confirmed the native CSP identity, ascertained when endogenous CSP was produced and completed a transcriptome-wide analysis of all genes following CSP exposure. RNA sequencing analysis revealed the upregulation of genes known to be associated with competence, biofilm formation and virulence. As such, a variety of phenotypic assays were utilized to assess the correlation between increased mRNA expression and potential phenotype response, ultimately gaining insight into the effects of CSP on both gene expression and developed phenotypes. The results indicated that the addition of exogenous CSP aided in competence development and successful transformation, yielding an average transformation efficiency comparable to that of other Mitis group streptococci. Additional studies are needed to further delineate the effects of CSP exposure on biofilm formation and virulence. Overall, this study provides novel information regarding *S. sinensis* and provides a substantial foundation on which this species and its role in disease pathogenesis can be further investigated.

INTRODUCTION

Bacterial competence is a physiological state bacteria enter to uptake and integrate exogenous DNA into their genomes. This process plays a vital role in both horizontal gene transfer (HGT) and genetic recombination events [1–3]. Bacterial competence also contributes to capsule polysaccharide variation and acquisition of antibiotic resistance genes to aid in bacterial survival under stressed conditions [1]. Bacterial competence has contributed to vast evolutionary development and speciation, thereby aiding the continued survival and proliferation of a multitude of bacterial species, including streptococci.

Competence has been thoroughly investigated in *Streptococcus pneumoniae*, 1 of 20 different species in the Mitis group of streptococci [1, 4–6]. The Mitis group of streptococci mainly comprises commensal inhabitants of the human naso-oropharyngeal tract, with some species being opportunistic pathogens [7]. Competence induction in *S. pneumoniae* was originally described as a quorum-sensing (QS) mechanism by which endogenous CSP production and secretion regulates the bacteria's ability to uptake exogenous DNA [5, 8]. Further investigation of QS and the competence regulon, ComABCDE,

Fig. 1. Depiction of the ComABCDE QS circuit regulating competence in Mitis group streptococci.

suggests that QS also takes part in the regulation of many downstream genes and resultant proliferative phenotypes, such as biofilm formation and the production of virulence factors [9–12].

The ComABCDE QS pathway comprises the *comABCDE* genes, where *comC* encodes a precursor peptide that is cleaved and exported outside the cell by an ABC transporter, ComAB (Fig. 1). The excreted and processed peptide, now termed the competence-stimulating peptide (CSP), binds ComD, a transmembrane histidine kinase receptor, when its concentration reaches a certain threshold in the local environment [13]. The CSP : : ComD binding results in phosphorylation of a response regulator, ComE. The phosphorylation of ComE has been postulated to promote self-dimerization and the subsequent upregulation and expression of other genes, including the *comABCDE* regulon and an alternative sigma factor, *comX* [14–16]. The activation of ComX leads to a transient cascade event where multiple genes are upregulated or downregulated, giving rise to various QS phenotypes, such as competence [15, 17, 18]. Due to the energetic cost and stress associated with the competence state, this phenotype is only maintained for ~30–40 min [1, 19, 20].

Global transcriptome analysis during competence has been investigated in four Mitis group species: *S. pneumoniae*, *Streptococcus gordonii*, *Streptococcus mitis* and *Streptococcus sanguinis* [21–24]. Competence induction by the ComABCDE pathway has two stages: activation of early competence genes, which are controlled by phosphorylated ComE, and activation of late competence genes, which are controlled by ComX [20, 21]. Early competence genes include *comABCDE*, *comW* and *comX*, along with a few other genes that are species specific [25]. The function of ComW was initially unknown, but later research has clarified its role as an early competence gene and additional QS regulator [18]. Researchers confirmed that ComW aids in the stabilization of ComX and is required for the induction of late competence genes in *S. pneumoniae* [18]. Late competence genes, often under the direct regulation of ComX, include many genes that are involved with DNA uptake, recombination and DNA repair.

In the QS model, all of which is dependent on the initial production of CSP, early competence genes are upregulated as soon as ComE is phosphorylated, and maximal expression occurs within 10 min. Late competence genes are upregulated approximately 5 min following ComE phosphorylation, and maximal expression occurs within 15 min [20, 21].

The competence regulon in *S. pneumoniae* has served as a model for investigating competence in the Mitis group of streptococci. While competence has largely been attributed to QS, alternative theories also suggest that pneumococcal competence, and potentially competence in other closely related streptococcal species, may instead rely on a cell–cell contact mechanism [26]. In

this model, pneumococcal competence is reliant upon paracrine CSP transmission. Additionally, the rate of competence spread is dictated by the contact between activated and latent cells, rather than the free diffusion of CSP in the extracellular environment, as is demonstrated in QS [26].

In *S. pneumoniae*, competence shutoff is not fully understood; however, it is well established that a separate late competence gene termed DNA-processing protein A (DprA) aids in this process [19, 27, 28]. DprA, under the control of ComX, binds phosphorylated ComE and prevents it from upregulating early competence genes [28]. Researchers also found that unabridged expression of *dprA* is required for complete competence shutoff [29]. An additional hypothesis for competence shutoff proposes that some unknown factor, referred to as ComZ, interacts with ComW to destabilize ComX and downregulate the expression of late competence genes [30]. Overall, the two-phase competence induction is conserved in the Mitis group of streptococci, although knowledge of other conserved key genes important for both competence induction and shutoff is lacking.

Presently, CSP-regulated competence has been shown to contribute to enhanced pathogenicity and virulence, as competent bacteria maintain the ability to uptake genes for antibiotic resistance [3, 4, 31, 32]. Such ability is well conserved among streptococcal species, although there are fine nuances between species that lend to unique phenotypes and group behaviours that have yet to be defined. Investigating streptococcal competence, as well as the effects of the competence regulon on the whole genome and proliferative phenotypes, is imperative to gain a more thorough understanding of these bacteria. Moreover, such knowledge will help establish a strong basis by which bacterial virulence and pathogenicity can be further investigated and potentially modulated. Because the QS competence regulon is known to play a significant role in bacterial competence, we set out to investigate the competence regulon and its effects in a recently reclassified species, *Streptococcus sinensis*.

S. sinensis, a bacterium belonging to the Mitis group of streptococci, was further examined and reclassified in 2002, where it was isolated from the blood of a Chinese woman admitted to the hospital for infective endocarditis [33]. *S. sinensis* naturally resides within the human oral cavity and it is currently unknown how this bacterium enters the bloodstream to cause bacteraemia and infective endocarditis [34]. Phylogenomic analysis of *S. sinensis* and other Mitis group streptococci revealed that *S. sinensis*, *Streptococcus cristatus* and *Streptococcus oligofermentans* are closely related [7, 35]. Presently, research related to *S. sinensis* has been limited to proper identification of this species. As such, investigation into *S. sinensis* Forsyth1A can serve as a model for this species. Moreover, understanding of *S. sinensis* Forsyth1A has the potential to aid in the investigation of closely related species and elucidate their mechanisms of pathogenesis and virulence. We therefore set out to evaluate the ComABCDE QS circuit in *S. sinensis* Fortsyth1A and determine its regulatory role in pathogenesis.

METHODS

All chemical reagents and solvents were purchased from Sigma-Aldrich or Chem-Impex and used without further purification. Water (ddH$_2$O) was purified using a Millipore Analyzer Feed System. All synthetic peptides were synthesized using preloaded Wang-Lysine resin (N$^\alpha$-Fmoc-N$^\epsilon$-Boc-L-lysine 4-alkoxybenzyl alcohol resin, 0.343 meq g^{-1}). 9-fluorenylmethoxycarbonyl (Fmoc) protected L-α-amino acids were purchased from Advanced ChemTech.

Bacterial strains

S. sinensis Forsyth1A was provided as a lyophilized powder from the Forsyth Institute by Dr Margaret Duncan [36].

Growth conditions

The lyophilized powder was resuspended in sterile Todd–Hewitt media supplemented with 0.5% yeast extract (THY) upon arrival and incubated for 12–24 h in a CO$_2$ incubator (37 °C with 5% CO$_2$). The suspension was streaked onto a THY agar plate and incubated for 12–24 h in a CO$_2$ incubator (37 °C with 5% CO$_2$). Fresh colonies were picked, Gram stained and checked for purity prior to making freezer stocks using THY broth and 20% glycerol solution. The new freezer stocks were used for all other experiments.

Species level identification

S. sinensis Forsyth1A identity was confirmed to the species level by polymerase chain reaction (PCR) amplifying a 740 base pair (bp) region unique to streptococci followed by Sanger sequencing using a method described previously [37, 38].

Isolation and characterization of competence-stimulating peptide (CSP)

Approximately 1 ml of an overnight culture of *S. sinensis* Forsyth1A was inoculated into 30 ml of sterile THY in a sterile 50 ml conical centrifuge tube. The culture was grown for 36 h in a CO$_2$ incubator (37 °C with 5% CO$_2$). The culture was centrifuged at 3000 *g* for 10 min and the supernatant was filter-sterilized using a 0.22 µM PES filter in a sterile 50 ml conical centrifuge tube. Ammonium sulfate was added to a final concentration of 40% w/v followed by incubation at 4 °C for 2 h. The solution was centrifuged at 3000 *g* for 20 min and the supernatant was discarded. The crude peptide/protein pellet was dissolved in 10 ml of DI

water and lyophilized. The lyophilized powder was resuspended in 10 ml of water : ACN (90:10) and purified using preparative RP-HPLC. Fractions were analysed for masses corresponding to the predicted CSP sequence, DFRKVRLPRLFGK (1631 Da). Fractions that showed a mass matching the predicted CSP were lyophilized and checked for purity using analytical RP-HPLC. The identity of the fractions containing the purified natural CSP was confirmed using HRMS and sent out for peptide mapping at the Nevada Proteomics Center (Fig. S2).

Solid-phase peptide synthesis

Peptide synthesis and purification was conducted using previously established methods [39, 40]. See the Supplementary Material for full experimental details.

Development of *S. sinensis* reporter strain

The *S. sinensis* Forsyth1A luciferase-based reporter strain was constructed using previously described molecular cloning methods with some modifications [41]. Briefly, the promoter of the *S. sinensis comX* was ligated with the starting codon of the firefly luciferase gene in the pFW11-ffluc backbone using the Gibson Assembly Cloning kit (NEB, Ipswich, MA, USA). PCR amplification was performed with Q5 DNA polymerase (NEB, Ipswich, MA, USA) per the manufacturer's recommendations. All constructs were verified via DNA sequencing and the observation of luminescence following exposure to both endogenous and exogenous CSP.

Temporal determination of endogenous CSP production

The temporal production and secretion of endogenous CSP in *S. sinensis* was determined using previously described methods with some modifications [42]. See the Supplementary Material for full experimental details.

DNA extraction and whole-genome sequencing

Genomic DNA for both Illumina sequencing and Oxford Nanopore Technology (ONT) Minion sequencing was prepared by making a 1:20 dilution of overnight cultures of *S. sinensis* Forsyth1A in 5 ml of sterile THY broth and incubating the freshly diluted culture statically for 6–8 h in a CO_2 incubator (37 °C with 5% CO_2).

For Illumina sequencing, genomic DNA was extracted from pelleted cultures (4000 *g* for 10 min) using the E.Z.N.A Bacterial DNA kit (Omega Bio-tek, Inc., Norcross, USA) according to the manufacturer's instructions. The DNA was submitted to the Nevada Genomics Center (Reno, USA) for multiplexed sequencing library preparation using an Illumina Nextera XT DNA Library Preparation kit and Illumina Sequencing using an Illumina mid output v2 (2×75) sequencing kit and flowcell on an Illumina NextSeq 500 (Illumina, Inc., San Diego, CA, USA). Quality control for the genomic DNA and prepared library was conducted using an Agilent High Sensitivity DNA Chip on an Agilent 2100 BioAnalyzer System (Agilent, Inc., Santa Clara, CA, USA).

For ONT Minion sequencing, genomic DNA was extracted from pelleted cultures (4000 *g* for 10 min) using a modified phenol : chloroform extraction protocol designed for obtaining high-molecular weight genomic DNA [43]. The pelleted cells were gently resuspended by pulse vortexing (5 s pulses) in 5 ml of modified buffer TLB pH 8.0 [10 mM Tris–Cl, 25 mM EDTA, 0.5% (w/v) SDS, 20 µg ml^{-1} RNase A and 20 µg ml^{-1} lysozyme] and incubated at 37 °C for 1 h. An equal volume of Tris–EDTA (pH 8.0) saturated phenol (TE phenol) was added to the lysate and mixed by hand inversion (20 inversions min^{-1}) for 10 min. The mixture was centrifuged at 5000 *g* for 10 min. The aqueous layer was transferred to a 15 ml centrifuge tube containing 5 ml of TE phenol chloroform (1:1) using a cutoff P1000 pipette tip that mimics a wide-bore pipette tip and mixed by hand inversion (20 inversions min^{-1}) for 10 min. The mixture was then poured into a new 15 ml centrifuge tube containing DOW Corning High Vacuum Grease in place of phase lock gel and centrifuged at 5000 *g* for 10 min. The top aqueous layer was transferred to a new 15 ml centrifuged tube using a cutoff P1000 pipette tip that mimics a wide-bore pipette tip containing 10 ml of ice-cold 100% ethanol and mixed by hand inversion (10 inversions) and incubated at −20 °C for 30 min. The solution was centrifuged at 5000 *g* for 15 min and gently decanted to give a white glassy pellet. The pellet was gently dislodged with 0.5 ml 70% ethanol solution and gently transferred using a cutoff P1000 pipette tip that mimics a wide-bore pipette tip into a 1.5 ml Lo-Bind microcentrifuge tube. The sample was centrifuged on a microcentrifuge at 10000 *g* for 10 min. The 70% ethanol solution was gently pipetted off the pellet without disturbing the pellet. The pellet was dried on a heat block set at 40 °C (15 min). The dried pellet was dissolved by adding 50 µl of nuclease-free water and incubating on a heat block set at 40 °C for 10 min. Isolated genomic DNA of *S. sinensis* Forsyth1A was barcoded for multiplexed sequencing using the Oxford Nanopore 1D ligation sequencing kit (SQK-LSK109) with a native barcoding expansion kit (EXP-NBD103). The ONT protocol library prep was performed according to the manufacturer's instructions with the following exceptions: the Axygen AxyPrep Mag PCR Clean-Up kit was used instead of Agencourt AMPure XP beads and the QuantiFluor dsDNA System was used with a Biotek Synergy H1 multimode plate reader (BioTek Instruments, Inc., Winooski, VT, USA) to quantify DNA instead of a Qubit. The sequencing ran for 8 h on the Minion using MinKNOW (v3.1.19) for basecalling.

De novo genome assembly

Raw Illumina reads were analysed using FastQC (v0.11.8), adapter removal and quality trimming was performed using Trimmomatic software (v.0.38) [44]. Raw ONT FASTQ format files were demultiplexed using EPI2ME Agent (v2.59.1896509) and those sequences containing the appropriate barcode were collected into one FASTQ output file (specifically, ONT native barcode 'BC12'). Unicycler software (v0.4.7) was used to create a hybrid assembly using both Illumina and ONT data [45]. Different methods of assembly were compared using bold, conservative and normal modes. Assembly using the bold mode produced the best draft genome with the fewest number of contigs. Assembly quality was assessed using QUAST (v5.0.0) [46] with *S. sinensis* HKU4 (GCF_000767835.1) as a reference genome. This assembly was submitted to the National Center of Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline and DFAST for annotation (BioProject: PRJNA526114; BioSample: SAMN11268212; SRA: SRX5584543 and SRX5962323) [47, 48].

Culturing and harvesting of *S. sinensis* Forsyth1A for RNA sequencing experiments

On the evening prior to the day of the experiment, 100 µl of *S. sinensis* Forsyth1A freezer stock was inoculated into 20 ml of sterile THY in a 50 ml sterile centrifuge tube and incubated statically in a CO_2 incubator (37 °C with 5% CO_2). On the morning of the experiment, the overnight culture of *S. sinensis* Forsyth1A was transferred to 980 ml of sterile THY +1% sterile horse serum contained in a sterile 1 l media bottle. The culture was incubated statically in a CO_2 incubator (37 °C with 5% CO_2) for 2 h. After 2 h incubation (OD_{600} ~0.05–0.15), 25 ml aliquots were dispensed for each time point into labelled sterile 50 ml centrifuge tubes containing 20 µl of either DMSO or synthetic CSP (800 nM final concentration). The tubes were incubated for 3 and 20 min with shaking at 200 r.p.m. at 37 °C. After the specified time point had passed, 3 ml of cold stop solution (5% phenol in absolute ethanol) was added to each tube and the tube was inverted several times before being centrifuged at 4 °C (5000 *g*) for 10 min. The supernatants were discarded, and the cell pellets were snap-frozen using dry ice and ethanol. The cell pellets were stored at −80 °C until total RNA extraction commenced. The RNA-seq experiment was conducted using two biological replicates for each condition (CSP and DMSO) for each time point (3 and 20 min) (Fig. S7).

Total RNA isolation

For each sample, the frozen cell pellet was thawed on ice. The thawed cell pellet was resuspended in 600 µl of lysis solution (sodium acetate 30 mM, sodium dodecyl sulfate 10 mM and ethylenediaminetetraacetic acid 1 mM, pH 5.5), 20 mg of sterile glass beads (0.1 mM) was added to the suspension and vortexed for 3–5 min. The solution was centrifuged (10000 *g* at 4 °C) for 2 min and the supernatant was transferred to a new sterile 1.5 ml microfuge tube containing 500 µl of acidic phenol (pH 5.5). Total RNA was extracted using phenol–chloroform extraction followed by DNase treatment (PureLink DNase Set, Thermo Fisher Scientific) according to the manufacturer's specifications. Another round of phenol–chloroform extraction was performed to yield total RNA. The extracted RNA samples were resuspended in 16 µl of nuclease-free water and purity was determined using a NanoDrop.

RT-qPCR

For each condition, a volume corresponding to 500 ng of RNA (according to NanoDrop quantification values) was used for cDNA synthesis. cDNA was created using Maxima Reverse Transcriptase (Thermo Fisher Scientific), random hexamers (Thermo Fisher Scientific), RiboLock RNase Inhibitor (Thermo Fisher Scientific) and 10 mM dNTP mix (Thermo Fisher Scientific) following the manufacturer's protocol. A 1:5 dilution of the generated cDNA was made using RNase-free water. The primers used are detailed in Table 1.

Table 1. Primers used in RT-qPCR

Primer name	Nucleotide sequence
gyrB Fwd	5′-GCTTTCCTCAATCGTGGTCTA-3′
gyrB Rev	5′-CGTAACTAGAGATTCCGCCTTC-3′
comX5 Fwd	5′-TATCCGCAAACAAGAGAGTCAA-3′
comX5 Rev	5′-ATTTCACGTGCCTTCAGTTTATG-3′
cinA2 Fwd	5′-GACCTTGCGATTGTCCACTAA-3′
cinA2 Rev	5′-CAGATAAAGGCTGTCCCTCAAA −3′
comE2 Fwd	5′-GTTGCTCGCTTTATTCGTCATC-3′
comE2 Rev	5′-AATCGAGAGCGGATACCTTATATTT −3′

The qPCR experiments were performed using i*Taq* Universal SYBR Green Supermix (Bio-Rad). The total volume of each qPCR reaction was 10 µl with the following components: 5 µl of SYBR Green Supermix, 3 µl of 1:5 diluted cDNA, 1 µl of forward primer (10 µM) and 1 µl of reverse primer (10 µM). The qPCR assays were performed on a CFX 96 system (Bio-Rad) using the following parameters: 95 °C 2 min, 40 cycles of 95 °C for 15 s, 53 °C for 30 s and 72 °C for 60 s. A melt curve analysis was performed at the end of the amplification starting at 65 °C and increasing at 0.5 °C increments to 95 °C.

Library preparation and Illumina sequencing

The total extracted RNA samples were fluorometrically quantified using the QuantiFluor RNA System (Promega) on a Biotek Synergy H1 multimode plate reader (BioTek Instruments, Inc., Winooski, VT, USA). Library preparation was constructed with a Universal Prokaryotic RNA-seq, Prokaryotic AnyDeplete kit (Tecan Genomics, CA, USA) using 120 ng of total RNA input per sample. Library preparations were sent to the Nevada Genomics Center for library quality and quantity checking (Bioanalyzer High Sensitivity Analysis) and for Illumina sequencing on a NextSeq500 using a NextSeq 500/550 Mid Output kit (v2.5 150 cycles).

Read mapping and quantification and differential gene analysis

Raw Illumina reads were analysed using FastQC (v0.11.8), adapter removal and quality trimming was performed using Fastp (v0.20.0) and reanalysed using MultiQC (v1.8). Trimmed reads were then mapped to the *S. sinensis* Forsyth1A genome (RefSeq Accession: GCF_019090945.1) using Bowtie 2 (v2.3.0). Read quantification was performed using subread featurecounts (v2.0.0). Differential analysis using DESeq2 (v1.0.0) was conducted in RStudio (v. 3.6) [49]. Volcano plot was created using Enhanced Volcano (v1.4.0).

Average nucleotide identity (ANI) analysis

The following genome assemblies were downloaded from the NCBI database: GCF_000767835.1 (*S. sinensis* HKU4), GCF_001578725.1 (*S. sinensis* DD04) and GCF_902460385.1 (*Streptococcus* sp. Marseille-P7376). ANI analysis was performed using fastANI (v1.3) [50].

Transformation assay

A single colony of *S. sinensis* was grown at 37 °C with 5% CO_2 in 10 ml of THY media (pH 7.3) for 18 h. The bacteria were initially incubated overnight to accomplish appropriate growth concentrations. Following incubation, the overnight *S. sinensis* culture was diluted 1:10 in fresh THY media and underwent static incubation at 37 °C with 5% CO_2 for 1 h to achieve late-lag/early-exponential phase growth. The CSP was added at final concentrations of 10000, 1000, 100 and 10 nM, along with 300 ng pDL278 (Spec[R]). pDL278 is a replicative plasmid obtained from Addgene (Addgene plasmid #46882; https://www.addgene.org/46882/). Parallel assays without the CSP or without both the CSP and plasmid were used as controls to assess the indigenous competence or antibiotic resistance of *S. sinensis* under the tested conditions. After 6 h of incubation at 37 °C with 5% CO_2, 100 µl of the culture was plated on THY agar containing 300 µg ml⁻¹ spectinomycin and incubated at 37 °C with 5% CO_2 for 48 h to identify positive transformants. Experiments were performed in triplicate on three separate days.

Transformation efficiency

A single colony of *S. sinensis* was grown at 37 °C with 5% CO_2 in 10 ml of THY media (pH 7.3) for 18 h. Following incubation, 100 µl of the overnight culture was used to inoculate 900 µl of fresh THY media and underwent static incubation at 37 °C with 5% CO_2 for 1 h . CSP was added at final concentrations of 10000 nM, 1000 nM, 100 nM and 10 nM, along with 300 ng pDL278 (Spec[R]). After 6 h of incubation at 37 °C with 5% CO_2, 40 µl aliquots were spread onto either THY plates or THY plates with 300 µg ml⁻¹ spectinomycin. Those spread onto non-selective plates were first diluted by a factor of 10^6. Plates were incubated at 37 °C with 5% CO_2 for 24 h to 36 h to identify positive transformants. Transformation efficiency was calculated using the following equation:

$$\text{Transformation efficiency (\%)} = \frac{\text{c.f.u. transformants}}{\text{Total c.f.u.}} * 100\%$$

Biofilm formation assay

Biofilm formation was assessed using a previously described method with minor alterations [51, 52]. See the Supplementary Material for full experimental details.

Haemolysis assay

Virulence factor production and associated haemolysis were assessed using a previously described method with minor alterations [51]. See the Supplementary Material for full experimental details.

RESULTS

For this work we elected to investigate a previously unidentified streptococcal clinical isolate obtained from the Forsyth Institute. This particular strain was isolated from an adult patient suffering from aggressive periodontitis in the late 1970s [36]. In that study, researchers identified the infective bacterium as a *S. mitis* derivative. We sequenced the RNA polymerase *β* subunit (rpoB) gene, a unique marker specific to different bacterial species, and confirmed that this species was in fact a strain of *S. sinensis* [37, 53].

A genomic search revealed that the only *S. sinensis* genome that is publicly available through the NCBI database is that of *S. sinensis* HKU4^{T} (GenBank assembly accession: GCA_000767835.1) [35]. Evaluation of *S. sinensis* HKU4 genome revealed that it has the following predicted CSP sequence, DFRKVRLPRLFGK, based on the sequence preceding the double glycine motif in its ComC protein sequence (GenBank accession: KGM37872.1).

PCR amplification of the *comCDE* gene revealed that the *S. sinensis* strain received from the Forsyth Institute instead demonstrated a different *comC* gene with a unique CSP signal, DSRRLNFGGFIKFFGK [54]. As such, this new strain was termed *S. sinensis* Forsyth1A. Protein BLAST of the *S. sinensis* Forsyth1A ComC sequence matched with *Streptococcus* sp. DD04 and *Streptococcus* sp. Marseille-P7376 [55].

To evaluate the activity of the competence regulon in *S. sinensis* Forsyth1A and validate the identity of the predicted CSP sequence, the supernatant of *S. sinensis* Forsyth1A was screened for a peptide with a mass corresponding to our hypothesized sequence (DSRRLNFGGFIKFFGK; Fig. 2a). The endogenous CSP was isolated from the cell-free supernatant of a *S. sinensis* culture grown to stationary phase. Comparison of the naturally isolated peptide with a synthetic CSP synthesized via solid-phase peptide synthesis (SPPS) was used to further confirm the CSP sequence (Figs 2b and S1).Tandem mass spectometry (MS/MS) analysis of the natural CSP validated its connectivity and identity as well (Fig. S2). Together, these results confirmed that the predicted CSP sequence for *S. sinensis* is the 16 amino acid peptide, DSRRLNFGGFIKFFGK. This sequence was found to demonstrate homology with the CSP sequences of other, closely related streptococci (Fig. 3).

While the detection of endogenous CSP was prevalent after the culture had reached stationary phase, it was of interest to further determine when endogenous CSP production was initiated, and thus when competence was initiated. To accomplish this, we developed a luciferase-based reporter in which p*comX* was placed upstream of the gene for luciferase, thereby allowing for the quantification of *comX* activity via luminescence detection. Wild-type *S. sinensis* has one copy of *comX*. The wild-type strain was then transformed with this plasmid construct and monitored for luminescence. The results indicated that endogenous CSP production stimulated luminescence in *S. sinensis* transformed with the luciferase-based reporter system, whereas luminescence was not exhibited in the wild-type strain (Fig. S3). Furthermore, luminescence, and therewith CSP production, is initiated at an OD_{600} of approximately 0.230. These OD_{600} values correspond to the early- and mid-exponential growth phases, respectively. Luminescence, and suspected CSP production, tapers as the cultures near the late-exponential and stationary phases. Overall, our results support the notion that endogenous CSP production leads to ComE phosphorylation and the subsequent upregulation of p*comX*, as visualized by luminescence.

Prior to global transcriptome analysis of the *S. sinensis comABCDE* regulon, whole-genome sequencing was performed using a hybrid approach combining Illumina sequencing and Oxford Nanopore sequencing data (GenBank assembly accession: GCA_019090945.2). The draft genome obtained is 2.12 Mbp and comprises 61 contigs with 1997 coding sequences (Fig. S4). Average nucleotide identity (ANI) analysis was performed comparing Forsyth1A against DD04, HKU4 and Marseille-P7376 genomes, revealing that isolates Forsyth1A, DD04 and HKU4 meet the species boundary ANI cutoff value of 94% (Table 2). Strain Marseille-P7376 had an 87.95 ANI with Forsyth1A, which is below the suggested species boundary ANI cutoff value, suggesting that it belongs to a different species of the Mitis group of streptococci [50].

Whole-genome mining of Forsyth1A revealed many genes associated with virulence and competence (Table 3) [17, 56–58]. Virulence gene comparison of Forsyth1A against HKU4 and DD04 revealed that the three strains share the same genes (Table S1). Moreover, both Forsyth1A and DD04 have the same capsular gene profile compared to HKU4.

RT-qPCR analysis revealed that *S. sinensis* Forsyth1A follows the same pattern, common in the Mitis group of streptococci, where activation of the *comX* gene occurs within 5 min after addition of synthetic CSP and declines to baseline expression after 20 min (Figs 4 and S5).

RNA-seq analysis of *S. sinensis* with either a 3 min treatment of synthetic CSP or DMSO control was performed. Analysis revealed that 147 genes were significantly upregulated while 127 genes were downregulated (log fold change >1, *P*-value <0.05, FDR value <0.05) (Fig. 5). The early competence genes, *comABCDE*, were upregulated, but the predicted *comW* gene was not significantly upregulated (Table 4). Many late competence genes associated with DNA uptake and integration were also upregulated (*comYA, comYB, comYC, comYD, comGF, celA, comEC, coiA, recX, recN, rmuC*), suggesting that activation of the competence regulon aids in the induction of competence in *S. sinensis*. An RT-qPCR analysis of *comX*, *comE* and *cinA* was used to validate the differential gene expression trends (Fig. 6).
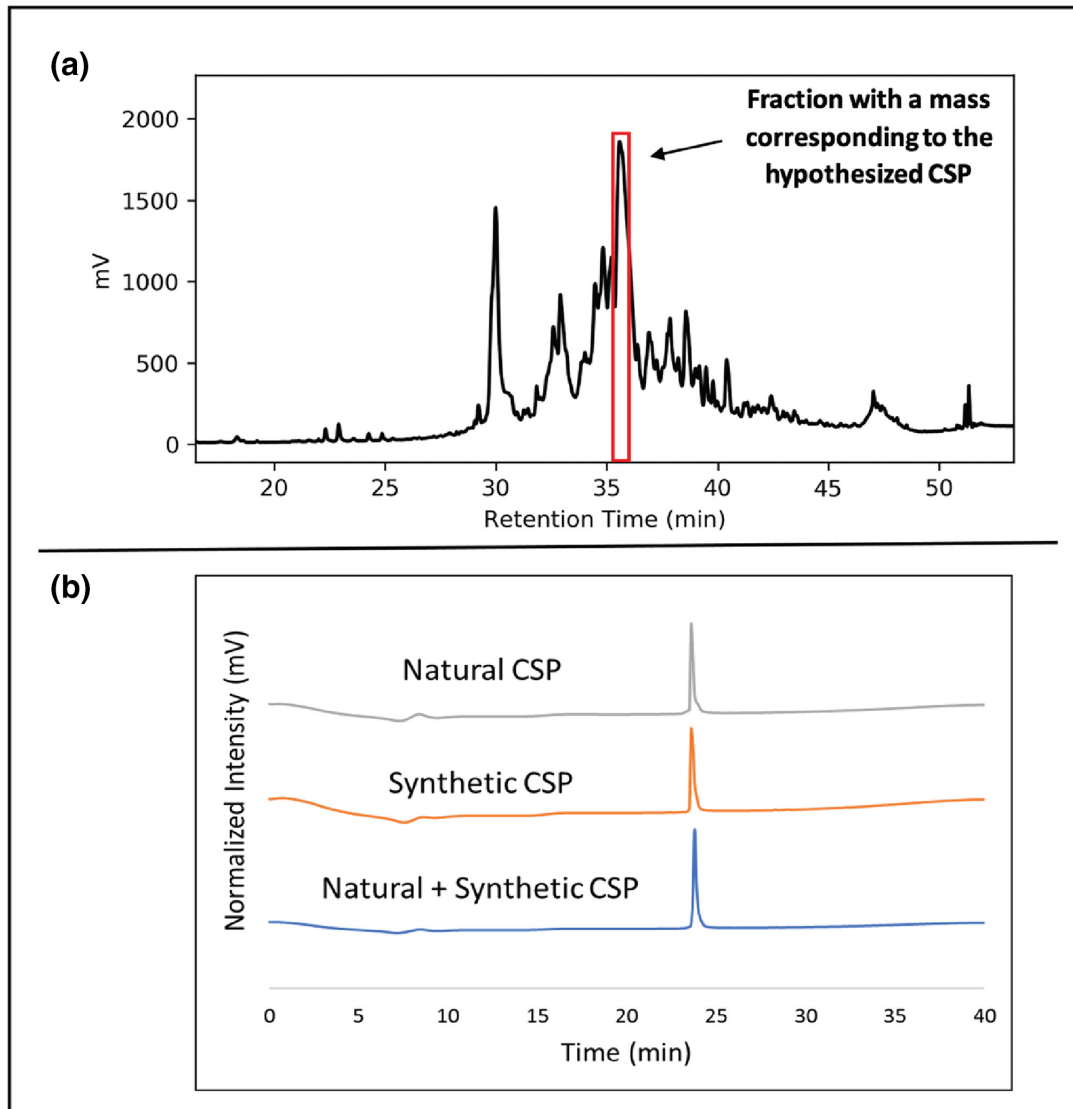
Fig. 2. (a) Preparative RP-HPLC trace of *S. sinensis* Forsyth1A spent supernatant following 30% ammonium sulfate precipitation. (b) Analytical RP-HPLC trace comparing naturally isolated *S. sinensis* Forsyth1A CSP to synthetic CSP.
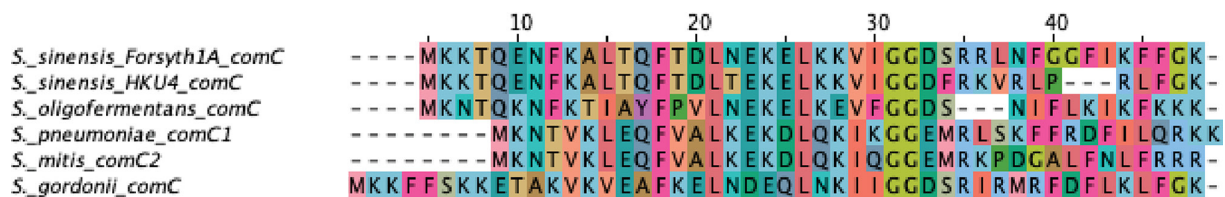
Fig. 3. CLUSTALW alignment of the ComC proteins of several Mitis group streptococci. ComC is presented as a propeptide that is typically cleaved following the double-glycine motif to render the mature CSP signal.

Due to the limited knowledge about *S. sinensis*, many of the genes that were affected by inducing the competence regulon are of unknown function; however, a few genes identified as virulence-related and metabolic-related were affected by synthetic CSP treatment (Table 4) [56]. For example, high-temperature requirement A serine protease (*htrA*), which is induced during competence development and has been shown to play a role in virulence phenotypes in *S. pneumoniae*, was upregulated [59, 60]. Another example is catabolite control protein A (*ccpA*), a regulatory metabolic protein that directs selective metabolism of

Table 2. Average nucleotide identity results comparing the genomes of different *S. sinensis* strains

Isolate comparison	ANI value
Forsyth1A vs HKU4	96.64%
Forsyth1A vs DD04	95.06%
Forsyth1A vs Marseille-P7376	87.95%

available sugars and has been found to play a role in competence in two Mitis group streptococci: *S. cristatus* and *S. gordonii* [61, 62]. Transcriptome analysis of *S. gordonii* during competence revealed that *ccpA* was downregulated during the competence state, while a similar observation was made with *S. sinensis* Forsyth1A (Table 5) [23].

Phenotypic assays following the exogenous addition of synthetic CSP were utilized to assess the correlation between the increase of mRNA transcript abundance as determined by RT-qPCR and related proliferative phenotype expression. It has been well established in several streptococcal species that the competence regulon either completely or partially aids in the regulation of specific phenotypes, including competence, biofilm formation and virulence factor production [63, 64]. As such, experiments were conducted to evaluate whether these processes are indeed regulated by the ComABCDE regulon in *S. sinensis*.

As described previously, bacterial competence is enhanced by exogenous CSP exposure in many streptococcal species [5, 65–67]. To evaluate this effect in *S. sinensis* Forsyth1A, transformation assays were completed in which cultures of the wild-type strain were incubated with the synthetic native peptide and plasmid-containing genes for antibiotic resistance. Conditions included final peptide concentrations of 10000 nM, 1000 nM, 100 nM and 10 nM, as well as a control with no exogenous CSP. A correlation between the addition of exogenous CSP and bacterial competence was observed, suggesting the successful internalization of plasmid DNA via induced transformation (Fig. 7). While the addition of CSP was needed for successful transformation, there was no significant difference between the varying concentrations of added exogenous CSP.

Alternatively, competence was not achieved when *S. sinensis* Forsyth1A was incubated with the HKU4 CSP pherotype, DFRKVRL-PRLFGK, under the same experimental conditions (Fig. S6). Overall, our results suggest that the competence regulon does in fact regulate competence in *S. sinensis* and is specific to the given pherotype.

At this point we also sought to determine the transformation efficiency for *S. sinensis* to aid in additional characterization of this strain. Comparison of total transformants grown on selective media to total colonies grown on non-selective media yielded transformation efficiencies of approximately 1.14×10^{-4}, 5.61×10^{-5}, 3.88×10^{-5} and 3.70×10^{-5}%; these values correspond to final exogenous CSP concentrations of 10000 nM, 1000 nM, 100 nM and 10 nM, respectively. Previous studies have indicated that the transformation efficiencies for many streptococci, including *S. mitis*, tend to be low, often <1% [68–70]. The transformation efficiency of *S. sinensis* is comparable to these values. Further experimentation is needed to optimize this value and define conditions that allow for enhanced transformation efficiency in *S. sinensis*.

The competence regulon has also been shown to aid in the regulation of biofilm formation in many streptococcal species [63, 71]. This trait is associated with increased bacterial pathogenesis and virulence, and as such it is of great interest to delineate the relationship that exists between the competence regulon and biofilm formation. Here, a crystal violet biofilm quantification assay was used to assess the formation of bacterial biofilms [51, 52]. Cultures were exposed to exogenous CSP in a dose-dependent manner, yielding final concentrations of 10000 nM, 1000 nM, 100 nM and 10 nM synthetic CSP. The data are normalized to the amount of biofilm produced in the wild-type system (no addition of exogenous CSP). The data indicated that there is no significant difference in the quantity of biofilm formed when incubated with CSP, when compared to the untreated wild-type strain of *S. sinensis* Forsyth1A (Fig. 8). The OD_{600} and OD_{595} of wild-type *S. sinensis* cultures were used to quantify biofilm growth. Cultures of *S. sinensis* exhibited significant levels of biofilm formation compared to media-only samples (Table S2). This assay alone cannot fully determine the involvement of the competence regulon in the regulation of biofilm formation. However, this does indicate that the competence regulon does not affect biofilm formation under the described experimental conditions. CSP may instead influence biofilm formation in the context of polymicrobial interactions that take place in the microbiome.

Lastly, many streptococcal species, including many closely related Mitis group species, are known to produce virulence factors to promote their own survival and pathogenesis in both human and animal models [72]. For example, it has been shown that *S. pneumoniae* produces a haemolytic toxin and virulence factor, pneumolysin, known to aid in the development of pneumococcal pathogenesis [73]. As such, it was of interest to determine whether *S. sinensis* produced similar virulence factors capable of haemolysis. This phenotype was assessed using a haemolysis assay in which the lysis of defibrinated rabbit erythrocytes was quantified. Cultures of *S. sinensis* Forsyth1A exposed to varying concentrations of synthetic CSP were incubated with red blood cells (RBCs). The results indicate that the presence of CSP does not directly influence haemolysis when normalized to the 0.01% Triton X control (Fig. 9). This assay alone does not fully assess for virulence factor production and subsequent effects on RBCs.

Table 3. List of genes found in the *S. sinensis* Forsyth1A genome that are associated with competence, biofilm formation and virulence

| Gene function	Genes	Annotation	Locus
Adherence	pavA	Fibronectin-binding proteins	FOA32_000330
	lmb	Laminin-binding protein	FOA32_001121
	srtA	Sortase A	FOA32_001697
	slrA	Streptococcal lipoprotein rotamase A	FOA32_000620
	plr/gapA	Streptococcal plasmin receptor	FOA32_001298
	srtB	Sortase B	FOA32_001612
Biofilm and capsule production	eno	Streptococcal enolase	FOA32_000730
	cap3	UDP-glucose-6-dehydrogenase	FOA32_000241
	galE	UDP-glucose-4-epimerase	FOA32_07480
Manganese uptake	psaA	Pneumococcal surface antigen A	FOA32_000346
Protease	htrA	Serine protease	FOA32_001484
	cppA	Carbon catabolite protein A	FOA32_000227
	tig/ropA	Trigger factor	FOA32_001645
Competence	comA	ABC transporter	FOA32_001444
	comB	ABC transporter	FOA32_001445
	comC	Competence-stimulating peptide	FOA32_001482
	comD	Histidine kinase	FOA32_001481
	comE	Response regulator	FOA32_001480
	comX	Alternative sigma factor	FOA32_000049
	comW	Competence protein	FOA32_19470
	comYA	Competence protein	FOA32_000391
	comYB	Competence protein	FOA32_000390
	comYC	Competence protein	FOA32_000389
	comYD	Competence protein	FOA32_000388
	comGF	Competence protein	FOA32_000386
	celA	Competence protein	FOA32_000664
	comEC	Competence protein	FOA32_000665
	coiA	Competence protein	FOA32_000685
	tfoX	Competence protein	FOA32_000685
	cinA	Competence–damage inducible protein	FOA32_001005
	recX	Recombination protein	FOA32_001238
	rmuC	DNA recombination protein	FOA32_001890
	recN	Recombination protein	FOA32_000639
	ydel/omp	Bacteriocin protection protein	FOA32_001270
	pepF	Oligoendopeptidase F	FOA32_001503
	recF	Recombination protein	FOA32_001471
	recO	Recombination protein	FOA32_001839
	radA	DNA repair protein	FOA32_001984
	recJ	Recombination protein	FOA32_000543
	mecA	Adapter protein	FOA32_000035
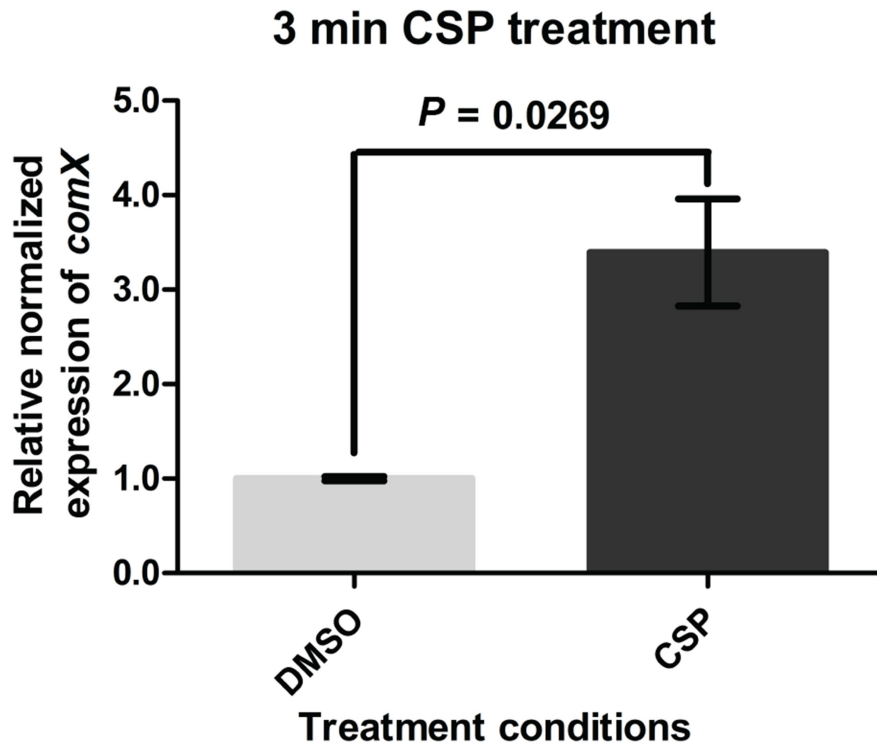
Fig. 4. RT-qPCR results after 3 min synthetic CSP treatment. Treatment with exogenous CSP for 3 min leads to activation of the *comX* gene. After a 3 min incubation, stop solution was added to each sample to stop transcription. Data are normalized to expression of *gyrB*. The RT-qPCR experiment was conducted using two biological replicates for both the CSP and DMSO conditions.
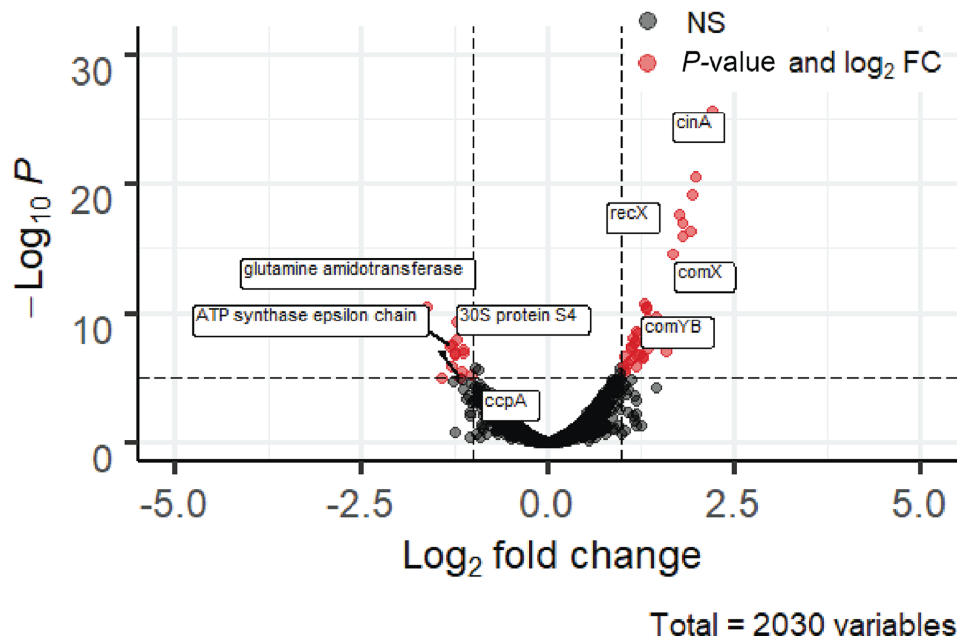
Fig. 5. Volcano plot of differentially expressed genes comparing treatment with DMSO (negative control) to synthetic CSP. Grey, non-significant genes expressed; red, genes with both a log fold change >2 and cutoff *P*-value >10^{-6} (statistically significant genes).

Table 4. Differential expression results highlighting various genes associated with competence and virulence being upregulated when comparing DMSO (negative control) to synthetic CSP

| Locus tag	Gene	Annotation	Log$_2$ change	*P*-adj
FOA32_000391	*comYA*	Competence protein YA	1.1362	1.96E-05
FOA32_000390	*comYB*	Competence protein YB	1.4176	7.27E-08
FOA32_000389	*comYC*	Competence protein YC	1.3002	1.21E-05
FOA32_000388	*comYD*	Competence protein YD	0.6785	2.42E-02
FOA32_000386	*comGF*	Competence protein GF	1.1461	2.46E-06
FOA32_000664	*celA*	Competence protein	1.4683	1.05E-03
FOA32_000665	*comEC*	Competence protein	1.8266	3.21E-15
FOA32_000685	*coiA*	Competence protein	0.8556	2.51E-03
FOA32_001444	*comA*	ABC transporter	1.1788	1.51E-06
FOA32_001445	*comB*	ABC transporter	0.8518	3.91E-04
FOA32_000049	*comX*	Transcriptional regulator of competence-specific genes	1.8639	5.05E-11
FOA32_001005	*cinA*	Competence–damage inducible protein A	1.9992	1.53E-18
FOA32_001482	*comC*	Competence-stimulating peptide	1.4634	1.08E-07
FOA32_001481	*comD*	Histidine kinase	0.8049	3.67E-03
FOA32_001480	*comE*	Response regulator	0.9189	4.93E-04
FOA32_001238	*recX*	Recombination protein	1.8172	2.60E-14
FOA32_001890	*rmuC*	Recombination protein	1.3208	6.69E-08
FOA32_000639	*recN*	DNA repair protein	1.0128	6.99E-03
FOA32_000971	*cap3*	UDP-glucose-6-dehyrogenase	0.6498	1.74E-03
FOA32_001484	*htrA*	Serine protease	0.5213	4.87E-02
FOA32_001961	*pbp 2A*	enicillin-binding protein 2A	0.9618	8.45E-06
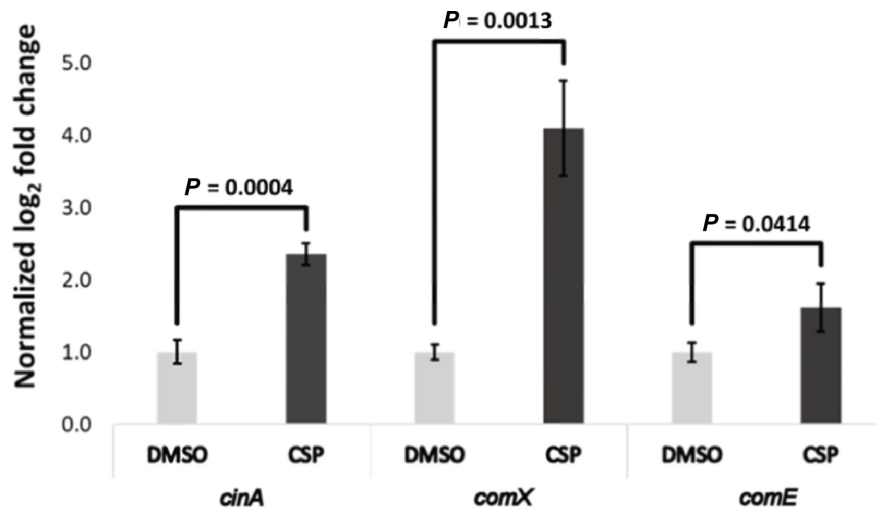
Fig. 6. RT–qPCR analysis of original RNA samples used in the RNA sequencing experiment as validation of differential gene expression analysis. After a 3 min incubation, stop solution was added to each sample to stop transcription. Data are normalized to expression of *gyrB*. The RT-qPCR experiment was conducted using two biological replicates for both the CSP and DMSO conditions.

Table 5. Differential expression results highlighting various genes associated with competence and virulence being downregulated when comparing DMSO (negative control) to synthetic CSP

Locus tag	Gene	Annotation	log$_2$ FC	p-adj
FOA32_000227	*ccpA*	Catabolite control protein A	−0.7463	6.89E-03
FOA32_001808	-	PTS fructose transporter subunit IIA	−0.9372	1.16E-04
FOA32_001809	-	PTS fructose transporter subunit IID	−0.9665	8.87E-05
FOA32_000099	-	PTS glucose transporter subunit IIABC	−0.6123	3.76E-02
FOA32_001341	-	PTS mannose/fructose/sorbose transporter subunit IIC	−1.1371	4.67E-06
FOA32_000475	*crcB*	Putative fluoride ion transporter	−0.8183	2.49E-02
FOA32_000035	*mecA*	Adapter protein	−0.8028	1.49E-02
FOA32_000761	*amy*	Alpha-amylase	−1.2753	4.96E-04
FOA32_001226	*ftsA*	Cell division protein	−0.6769	2.98E-02
FOA32_000717	*ftsX*	Cell division protein	−0.9199	1.40E-02
FOA32_001028	*malR*	LacI family transcriptional regulator	−0.6059	4.17E-02
FOA32_000006	*rsfS*	Ribosomal silencing factor	−1.4111	4.93E-04
FOA32_001645	*tig*	Trigger factor	−0.6221	4.44E-02
FOA32_001049	-	Type VII secretion protein EssA	−0.9494	1.12E-02
FOA32_001443	*rpsD*	30S ribosomal protein S4	−1.2171	7.21E-08
FOA32_001630	*rpsH*	30S ribosomal protein S8	−0.9610	2.05E-03
FOA32_001505	*rplM*	50S ribosomal protein L13	−1.2907	6.32E-05
FOA32_001635	*rplO*	50S ribosomal protein L15	−1.0335	7.64E-04
FOA32_000259	*infC*	Translation initiation factor	−1.1224	8.05E-06
FOA32_000143	-	Membrane protein	−0.9259	1.34E-02
FOA32_000586	-	Membrane protein	−0.8700	1.59E-02
FOA32_000860	*scrB*	Membrane protein	−0.6828	1.84E-02
FOA32_001191	*yidC_1*	Insertase YidC	−1.2089	1.24E-05

The upregulation of genes associated with virulence factor production may instead have a greater effect on interspecies interactions and potential competition that occur in the microbiome rather than direct effects on RBCs.

Ultimately, our results indicated that the *S. sinensis* competence regulon does have an effect on competence development, but does not directly impact biofilm formation or virulence factor production under the described testing conditions. As such,

(a) **(b)** **(c)** **(d)** **(e)**
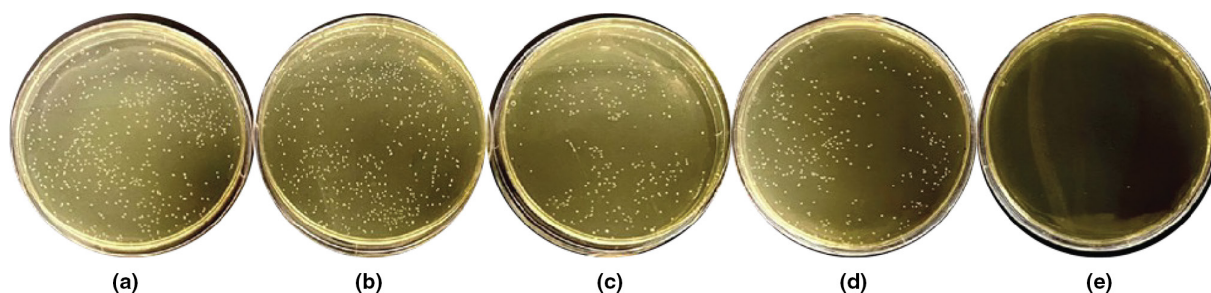
Fig. 7. *S. sinensis* transformation assay. *S. sinensis* demonstrates dependence on the addition of exogenous synthetic CSP for the successful uptake of added plasmid (pDL278). The synthetic CSP was added at final concentrations of 10 000 nM (a), 1000 nM (b), 100 nM (c) and 10 nM (d). The control includes no exogenous CSP (e). See the Methods section for full experimental details.
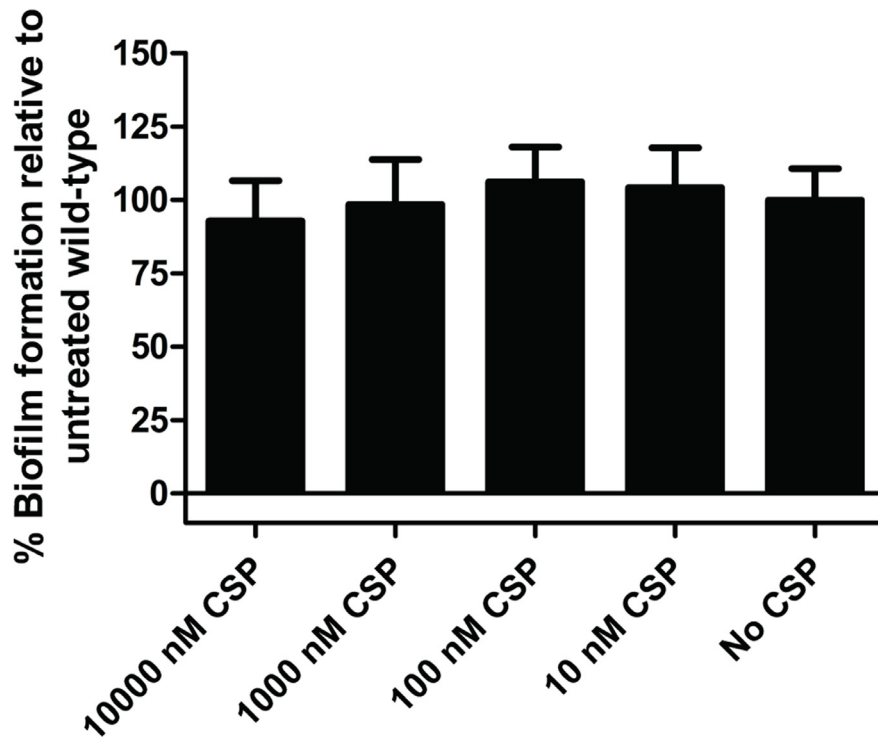
Fig. 8. Effect of exogenous CSP on *S. sinensis* biofilm formation. The addition of exogenous CSP at varying concentrations does not significantly affect biofilm formation in *S. sinensis*. The results thereby indicate that the competence regulon QS circuitry in *S. sinensis* does not seem to have an effect on biofilm formation. See the Supplementary Material for full experimental details.

further experimentation is needed to clarify the effect of CSP on subsequent *S. sinensis* phenotypes, both within and outside of a polymicrobial community.

DISCUSSION

Investigation into the *S. sinensis* ComABCDE QS pathway revealed that this pathway is functional in strain Forsyth1A, demonstrating that this member of the Mitis group of streptococci is also naturally competent. *S. sinensis* likely has at least two CSP pherotypes, DFRKVRLPRLFGK (strain HKU4) and DSRRLNFGGFIKFFGK (strains DD04 and Forsyth1A). The genome of *S. sinensis* Forsyth1A was sequenced and whole-genome comparison with other isolates suggests that Forsyth1A is more closely related to strain DD04 compared to strain HKU4. This strain demonstrated a transformation efficiency of <0.001%, which is consistent with other streptococcal species. *S. sinensis* also demonstrated initial production of endogenous CSP in early- to mid-exponential phase, as detected via luminescence.

This is the first in-depth transcriptome investigation into the competence regulon in *S. sinensis*. Through this analysis, many competence-related genes were observed to be differentially expressed after 3 min incubation with synthetic CSP. *S. sinensis* competence regulation differs from *S. pneumoniae* and *S. mitis* in that it does not exhibit the upregulation of *comW* during competence [21, 74]. Further experiments are needed to confirm the basal expression level of *comW*, as well as *comW* expression during competence shutoff. It is probable that this gene is differentially expressed in *S. sinensis* while still playing a regulatory role in bacterial competence, but additional research is needed to ascertain such information.

A separate gene, *htrA*, demonstrated increased expression following CSP induction in *S. sinensis*. Previously, researchers had found that the presence of HtrA appears to be required for appropriate transformation in *S. pneumoniae* [60]. Furthermore, it was recently shown that in *S. pneumoniae*, *S. gordonii* and *S. mitis*, HrtA degrades the CSP signal and certain competence proteins involved with DNA uptake to assist with competence termination [75]. The observed increase in expression of *htrA* in *S. sinensis* suggests that it may be playing a role in regulating competence in Mitis group streptococci. Again, additional experimentation examining the expression levels and activity of HtrA throughout the process of competence induction and shutoff will be needed to further delineate its role as a potential competence regulator.
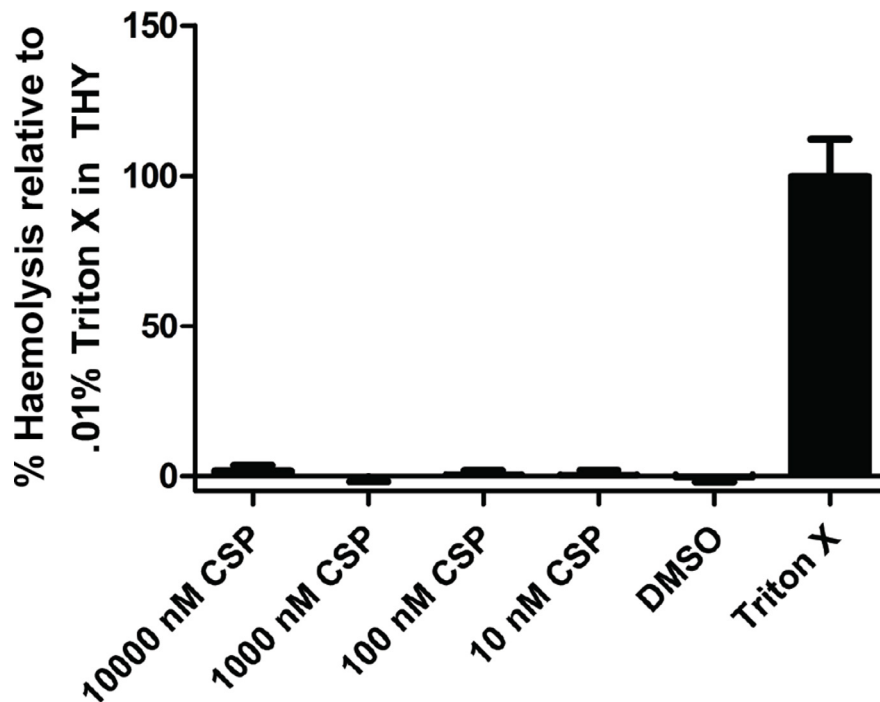
Fig. 9. Effect of exogenous CSP on *S. sinensis* virulence factor production. The addition of exogenous CSP at varying concentrations does not significantly affect lysis of RBCs by *S. sinensis*. See the Supplementary Material for full experimental details.

The expression of another known competence regulatory element, CcpA, was downregulated during the 3 min CSP treatment transcriptome snapshot. This result directly indicates that its expression is somewhat suppressed or reduced during early competence development. Researchers had previously found that CcpA indirectly aids in the regulation of early competence genes, including *comCDE*, in Mitis group streptococci [62, 76]. For example, it was found that an *S. oligofermentans* Δ*ccpA* mutant had higher expression levels of phosphorylated ComE, which is known to bear great influence on other competence genes, such as *comX* [61]. As such, *ccpA* is potentially downregulated in early competence to allow for the adequate expression of early competence genes that are needed for successful transformation. Nonetheless, these findings highlight the need to perform additional competence transcriptome analyses on members of the Mitis group of streptococci to fully elucidate the complexity of this phenotype.

Lastly, the increased expression of genes related to competence, biofilm formation and virulence was of particular interest, as these have potential to influence proliferative phenotypic response. It was found that the *S. sinensis* Forsyth1A competence regulon does play a role in the development of bacterial competence and subsequent transformation. However, incubation with CSP does not have an effect on biofilm formation or virulence as it relates to haemolysis. These results do not fully explain the dynamic relationship that exists between the competence regulon QS circuitry and these phenotypes. Rather, these results suggest that there is no direct influence of the CSP on biofilm formation and virulence in the described tested conditions. Instead, the upregulated genes may instead have an effect on various interspecies interactions that take place in polymicrobial communities.

Future studies are needed to clarify the effects of CSP exposure on *S. sinensis* Forsyth1A phenotypes in the context of mixed microbial interactions, thereby providing even more insight into the role of the native CSP on the competence regulon. Overall, this study sheds light on a newly identified species, *S. sinensis* Forsyth1A, and provides a robust foundation for future investigation of this species, as well as its interactions and effects on other members of the oral microbiome.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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