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## Elucidating the Role and Structure-Activity Relationships of the *Streptococcus oligofermentans* Competence Stimulating Peptide

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### Abstract

*Streptococcus oligofermentans* is an early colonizer of the oral microbiome with documented bactericidal activity against the oral pathogen *Streptococcus mutans*. *S. oligofermentans* has been observed to possess the typical *comABCDE* competence regulon found within most oral streptococci, however the competence stimulating peptide (CSP) responsible for QS activation and the regulatory role of the competence regulon have yet to be explored. Herein, we have both confirmed the identity of the *S. oligofermentans* CSP and utilized a wide range of phenotypic assays to characterize its regulatory role in competence, biofilm formation, and hydrogen peroxide formation. To determine the importance of each amino acid residue in CSP:ComD binding, we performed systematic replacement of amino acid residues within the *S. oligofermentans* CSP and developed a luciferase-based reporter system to assess the ability of these mutated analogs to modulate the competence regulon. Additionally, we performed CD analysis on mutated CSP analogs to determine the correlation between peptide secondary structure and QS activation. To further explore *S. oligofermentans*' potential as a biotherapeutic against *S. mutans* infection, lead QS activators and inhibitors were used in interspecies competition assays to assess the effect of QS modulation on interactions between these two species. Lastly, we have documented a lack of *S. oligofermentans* induced cytotoxicity, highlighting the potential of this native flora as a biotherapeutic with minimal health risks.

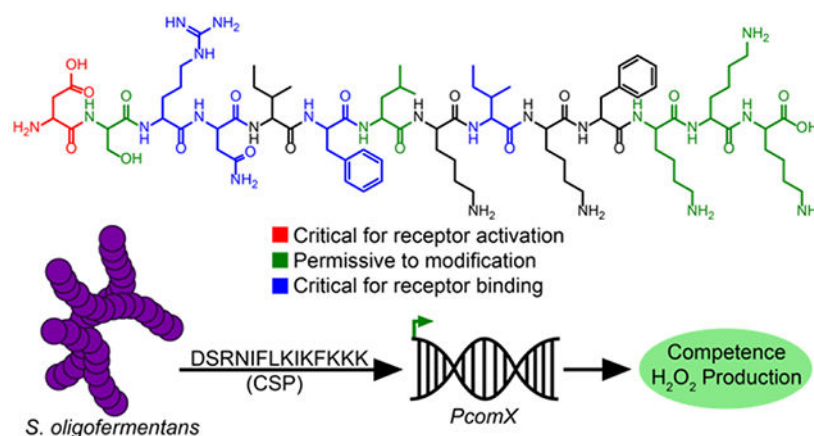
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Supporting Information

Additional experimental procedures, peptide characterization, primary reporter assay data, dose-response curves for CSP analogs, and supplementary figures. This material is available free of charge via the internet at <https://pubs.acs.org/doi/10.1021/acscchembio.1c00746>.



## Keywords

Quorum sensing; streptococcus; peptide; biotherapeutic; antibiotic resistance

## Introduction

The human oral cavity is colonized by a diverse array of microorganisms, including at least six different distinct groups of streptococci.<sup>1–3</sup> Many of these oral streptococci act as opportunistic pathogens and several species, including *Streptococcus mutans*, are primary causative agents of dental caries, a condition that in the US alone results in over \$100 billion dollars a year in medical costs.<sup>4–7</sup> As streptococci are among the earliest colonizers of the oral microenvironment, they play an important role in the development and final composition of the oral microbiome.<sup>1, 8–9</sup> In many cases, streptococci, such as *S. mutans*, produce bacteriocins or other chemical compounds, as well as complex biofilms, all of which can impede the growth of other members of the oral microbiome, aiding in their colonization of the microenvironment.<sup>10–11</sup> Of particular interest is the ability of *S. mutans* to outcompete other oral streptococci through the production of high amounts of lactic acid, acidifying the surrounding microenvironment and resulting in the killing of competing bacteria.<sup>10–12</sup>

A relatively newly described member of the oral microbiome is *Streptococcus oligofermentans*, a member of the mitis group of streptococci that was first isolated from caries-free patients in China.<sup>13–14</sup> Compared to other oral streptococci, *S. oligofermentans* is relatively non-pathogenic, having only been observed in low incidence among patients with bacterial endocarditis.<sup>15</sup> Despite its lack of bacterial pathogenesis, *S. oligofermentans* plays an important role within the oral microbiome, making it of particular interest to study. Specifically, there has been shown to be a decrease in the amount of *S. mutans* in the presence of *S. oligofermentans* within the oral microbiome.<sup>5, 16–19</sup> Tong and coworkers elucidated that *S. oligofermentans* possesses lactic acid oxidase (*lox*) genes that convert lactic acid produced by *S. mutans* into hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), contributing to the hydrogen peroxide-mediated killing of *S. mutans*.<sup>5</sup> This relatively unique quality suggests that it may be possible to utilize *S. oligofermentans* as a biotherapeutic against *S. mutans*,

preventing the formation of dental caries. However, the expression of this desired quality within *S. oligofermentans* is relatively understudied, and there is little information of how *S. oligofermentans* regulates other phenotypes that have been recognized to play an important role in the colonization of the oral microbiome by other streptococci.

It has been well established that many oral streptococci use a process known as quorum sensing (QS) to regulate the expression of important phenotypes including biofilm formation, bacterial competence, and bacteriocin production to aid in colonization of the oral microbiome.<sup>20–25</sup> QS is a method of communication used by both gram-positive and gram-negative bacteria to coordinate the expression of group behavior phenotypes when the bacterial cell density reaches a threshold concentration.<sup>20–23, 25</sup> Gram-negative bacteria typically use small molecule *N*-acyl-homoserine lactones (AHLs), whereas gram-positive bacteria utilize autoinducing peptides (AIPs) as their primary signaling molecule to active QS.<sup>25–27</sup> In streptococci the main QS circuitry, termed the competence, or ComABCDE, regulon, is comprised of five different components, and is centered around an AIP known as the competence stimulating peptide (CSP).<sup>28–29</sup> Typically, the CSP pro-peptide (ComC) is processed and secreted by an ABC transporter (ComAB) as the mature CSP signal.<sup>28</sup> Once a minimal threshold level of the CSP signal is reached in the extracellular environment, the CSP binds and activates the transmembrane histidine-kinase receptor (ComD).<sup>28</sup> This binding event triggers phosphorylation of a response regulator (ComE), resulting in further expression of QS genes (*comABCDE*) and the expression of the alternative sigma factor ComX, the master regulator of competence associated genes and phenotypes (Figure 1).<sup>28–29</sup>

The native CSP sequence of *S. oligofermentans* was previously deduced from genomic analysis, but its identity was not confirmed through isolation from bacterial supernatants. Moreover, the proposed native 14-amino acid *S. oligofermentans* CSP has been shown to upregulate bacterial competence in a dose-dependent manner through exogenous dosing of the synthetic peptide.<sup>14</sup> However, the role of the competence regulon in *S. oligofermentans* ability to modulate its microenvironment and the expression of other bacterial phenotypes has yet to be explored. In this study we set out to confirm the identity of the *S. oligofermentans* CSP by isolating it from *S. oligofermentans* supernatants, characterize the regulatory role of the competence regulon within *S. oligofermentans*, and to gain a deeper understanding of the molecular mechanisms that drive CSP:ComD binding and subsequent activation of the QS circuitry. To this end, we employed sequencing, protein extraction, and high-resolution mass spectrometry to elucidate the structure of the *S. oligofermentans* CSP. Following confirmation of the CSP sequence, we utilized several phenotypic assays to determine what role the *S. oligofermentans* competence regulon plays in the phenotypic expression of bacterial competence, biofilm formation, hydrogen peroxide production, and virulence factor production. To assess the structure activity relationships (SARs) behind CSP:ComD interactions, we performed a full alanine screen of the *S. oligofermentans* CSP and developed a luciferase based reporter gene assay to measure the degree of QS activation that results from CSP:ComD binding and subsequent ComD activation for all CSP analogs. We then performed structural analysis of all alanine screen analogs using circular dichroism (CD) spectroscopy to further correlate CSP structure and function. Finally, we designed and synthesized a library of second generation truncated and double mutant CSP derivatives based on trends observed in our initial findings. Our analysis revealed that in

*S. oligofermentans* the expression of several key bacterial phenotypes is controlled by the competence regulon. Additionally, we have both identified several residues within the *S. oligofermentans* CSP that are critical for ComD binding and activation and developed the first CSP derived inhibitors of the *S. oligofermentans* competence regulon.

## RESULTS AND DISCUSSION

### Prediction and isolation of the *S. oligofermentans* CSP from cell-free supernatants

Tong and coworkers have previously predicted the *S. oligofermentans* CSP based on genomic data, synthesized the predicted 14-amino acid CSP with the sequence DSRNIFLKIKFKKK, and exhibited that the synthetic peptide is capable of inducing competence in *S. oligofermentans*.<sup>14</sup> To ensure that our tested *S. oligofermentans* strain contained the same CSP sequence, we performed PCR amplification and sequencing of the *comC* gene as previously described,<sup>14</sup> obtaining a predicted sequence that corresponded to that of the previously reported 14-amino acid peptide (Figure 2). Several studies have reported that ComC will sometimes undergo additional processing beyond cleavage at the double glycine repeat to afford a mature CSP signal, either through cleavage of the exported CSP by an extracellular SepM protease,<sup>6</sup> or through further processing by the ComAB transporter.<sup>30</sup> To ensure that this predicted CSP sequence correlated to that of the mature CSP afforded by processing of ComC by the ComAB transporter, we attempted to isolate the processed CSP from bacterial supernatants. Following precipitation of the excreted crude peptide mixture from cell-free supernatants of *S. oligofermentans*, we fractionated the total peptides/proteins using reverse-phase high-performance liquid chromatography (RP-HPLC) and observed masses similar to that of the predicted CSP (Figure 3). Further purification of this fraction allowed us to obtain a purified peptide fraction (>97%) with a mass corresponding to the expected mass for the predicted 14-mer CSP, suggesting that this is indeed the mature peptide sequence.

### Comparison of isolated and synthetic CSP

To further confirm the identity of the *S. oligofermentans* CSP, we synthesized the predicted CSP sequence using Fmoc solid-phase peptide synthesis (see the Supporting Information for full experimental details). We then compared the purified synthetic peptide to the purified peptide isolated from cell-free supernatants. HPLC analysis of both individual peptides revealed a dominant peak with the same retention time, and combined fractions of these peptides resulted in a single peak that possessed the same retention time as the individual fractions (Figure 4). In addition, ESI-TOF MS analysis of the isolated and synthetic peptides exhibited corresponding exact masses within 5 ppm error of the expected mass of the 14-mer CSP (Figure 4). Lastly, we performed MS/MS analysis of the isolated CSP. The MS/MS results validated the connectivity of the CSP sequence (Figure S-7). Together, these results confirmed that the CSP sequence for *S. oligofermentans* is the 14-amino acid peptide, DSRNIFLKIKFKKK.

### Phenotypic assays

After confirming the CSP sequence for *S. oligofermentans*, we set out to determine its regulatory role. Since it has been well established in several species of streptococci that the

competence regulon QS pathway is responsible for the regulation of competence, biofilm formation, and virulence factor production, we set out to determine if these processes were under QS control in *S. oligofermentans*. Additionally, we aimed to determine whether hydrogen peroxide production, the primary means by which *S. oligofermentans* regulates *S. mutans* populations within the oral microbiome, was under QS control.

Previous work from Tong and coworkers revealed that bacterial competence was enhanced in response to CSP addition in a dose-dependent manner.<sup>14</sup> To observe the effect of exogenous CSP on bacterial competence and confirm these results, we performed transformation assays on *S. oligofermentans* using four different concentrations of synthetic CSP, 10,000 nM, 1,000 nM, 100 nM, 10 nM, and control with no exogenous CSP. (Figure 5). We observed a dose dependent correlation between CSP concentration and bacterial competence, as the number of transformants was reduced with decreasing CSP concentration, supporting Tong's earlier observation that competence in *S. oligofermentans* is under QS control.

As the competence regulon has been determined in other streptococci to regulate biofilm formation, a trait associated with enhanced bacterial pathogenesis, we sought out to determine whether biofilm formation in *S. oligofermentans* was under QS control. To this end, we performed a crystal violet biofilm quantification assay to assess the formation of bacterial biofilms. Interestingly, our results revealed that under treatment of different exogenous concentrations of CSP (10,000 nM, 1,000 nM, 100 nM, or 10 nM), there was no significant difference in the amounts of biofilms formed compared to untreated wild type *S. oligofermentans* (Figure 6). As other mitis group streptococci utilize the competence regulon to control biofilm formation, these results cannot entirely rule out the *S. oligofermentans* QS circuitry playing a role in the expression of this phenotype. Rather, our results indicate that the competence regulon failed to play a significant role in the tested conditions.

A major aspect of *S. oligofermentans* that makes it of particular interest is its ability to convert lactic acid to hydrogen peroxide, weaponizing it against other pathogenic oral streptococci including *S. mutans*. To determine whether this desired capability was under QS control, we assessed the effect of different concentrations of exogenous CSP (10,000 nM, 1,000 nM, 100 nM, or 10 nM) on the ability of *S. oligofermentans* to produce hydrogen peroxide in THY media containing 10 mM lactic acid, a concentration that Tong and coworkers had previously observed to cause maximal hydrogen peroxide production.<sup>5</sup> For CSP concentrations of 100, 1,000, and 10,000 nM, we observed a statistically significant increase in hydrogen peroxide production compared to wild type bacteria untreated with exogenous CSP (Figure 7). These results suggest that the competence regulon is responsible, to some degree, for hydrogen peroxide production in *S. oligofermentans*, perhaps through downstream upregulation of the *lox* genes that encode for the lactic acid oxidase that is necessary for hydrogen peroxide production. These results further suggest that hydrogen peroxide production in *S. oligofermentans* can be modulated through activation of its QS circuitry, potentially providing an approach by which *S. oligofermentans* can be utilized as a potent biotherapeutic against *S. mutans* and other pathogenic oral streptococci.

## Development of *S. oligofermentans* luciferase reporter strain

In an effort to gain a deeper understanding of the activity of the native CSP and determine the role of each CSP residue in CSP:ComD binding, a luciferase based *S. oligofermentans* reporter strain was constructed to directly quantify the degree of *comX* expression upon CSP:ComD binding using an approach similar to that performed by Salvadori and coworkers.<sup>31</sup> First, the primer pair SOComXfwd-SOComXrev (Table S-1) was used to amplify the *comX* promoter region within *S. oligofermentans*. Both the amplified *comX* and the plasmid pFW11-*luc* (Spec<sup>R</sup>) were then restriction digested using BamHI and NheI. The digested products were then ligated and cloned into *Escherichia coli* as previously described by Green and coworkers.<sup>32</sup> The purified pFW11-*luc* plasmid containing the *S. oligofermentans comX* promoter region was then transformed into *S. oligofermentans*. Incorporation of the reporter plasmid was validated by observing an increase in luminescence following treatment of *S. oligofermentans* reporter cultures with CSP and 15 µg/mL D-luciferin, compared to a control untreated with CSP (see the Supporting Information for full experimental details).

## Design and synthesis of *S. oligofermentans* CSP alanine screen analogs

To elucidate the SAR for each residue within the 14-amino acid *S. oligofermentans* CSP, we performed a full alanine screen of the CSP sequence. All alanine screen CSP analogs were constructed using Fmoc solid-phase peptide synthesis on 4-benzyloxymethyl alcohol (Wang) resin. Following on-resin synthesis of each alanine screen CSP, peptides were cleaved from the solid support, and purified to homogeneity by semipreparative RP-HPLC (see the Supporting Information for full experimental details and peptide characterization).

## SAR of the *S. oligofermentans* CSP

To determine the role of each sidechain residue of the 14-amino acid *S. oligofermentans* CSP in ComD receptor binding, the activity of synthesized alanine screen peptide analogs was assessed using luciferase reporter gene assays. The *S. oligofermentans* CSP can be divided into three distinct regions, the *N*-terminus, the central region, and the *C*-terminus. Alanine screening of the CSP *N*-terminus revealed that residues Arg3 and Asp1 play critical roles in receptor binding and activation, respectively. Alanine replacement of residue Asp1 resulted in the conversion of the *S. oligofermentans* CSP into a ComD inhibitor (Table 1). This observation is consistent with other mitis group streptococci such as *S. pneumoniae*, as the negatively charged first residue of the CSP has been shown to be critical for receptor activation.<sup>33</sup> Interestingly, alanine substitution of residue Arg3 in other mitis group streptococci including *S. pneumoniae*<sup>33</sup> and *S. mitis* (unpublished data) has resulted in a complete loss of activity. In the case of the *S. oligofermentans* CSP, while substitution of Arg3 with alanine resulted in a significantly reduced activity compared to the native CSP, the resulting peptide was not completely inactive. Lastly, alanine substitution of Ser2 resulted in a peptide with comparable activity to the native sequence (Table 1). When compared to other mitis group streptococci, these results suggest that the binding site of the *S. oligofermentans* CSP *N*-terminus within ComD is more permissive to CSP side chain modification than its homologues among other mitis group streptococci, as Ser2 modification is permissible and Arg3 substitution does not completely abolish CSP activity,



a first for the mitis group streptococci. Similar to previous findings for other mitis group streptococci, the first negatively charged *N*-terminal amino acid plays a critical role in receptor activation, suggesting the presence of a highly conserved motif at this position.

There is no clear correlation between side chain composition and receptor binding or activation within the core region of the *S. oligofermentans* CSP. Substitutions to residues Asn4 and Phe6 resulted in peptides that were completely inactive, while substitution to residue Ile9 resulted in a significant loss of activity, suggesting that these residues are critical for receptor binding (Table 1). Alanine substitutions of residues Ile5, Lys8, Lys10, and Phe11 were relatively tolerated resulting in analogs with only a slight decrease in activity compared with the native CSP (3-4.5-fold decrease), whereas substitution of residue Leu7 produced a peptide with activity comparable to that of the native CSP, suggesting that these residues do not play an important role in receptor binding or activation (Table 1). It was previously observed that signal:receptor recognition in *S. pneumoniae* was mediated by a hydrophobic patch within the CSP core region.<sup>33</sup> A similar trend is not observed within the *S. oligofermentans* CSP as modification of several side chains that facilitate both polar and hydrophobic interactions lead to a loss of activity, whereas the substitution of other side chains produced CSP analogs with activity comparable to the native sequence. Overall, these findings suggest that the receptor binding site for the core region of the *S. oligofermentans* CSP is likely more promiscuous than that of the *N*-terminal region.

The *C*-terminus of the peptide appears to not have a role in either receptor binding or activation as replacement of Lys12, Lys13, or Lys14 with alanine resulted in analogs with comparable activities to the native CSP signal (Table 1). These results indicate that the entire *C*-terminus of the peptide is dispensable, however the high conservation of a three amino acid charged tail on all mitis group CSPs indicates this region plays an important evolutionary role, likely to increase the overall hydrophilic properties of these peptides to aid in their solubility.

### Circular Dichroism analysis of CSP alanine screen analogs

Our SAR analysis of the *S. oligofermentans* CSP revealed several residues that are critical for receptor binding or activation; however, this analysis was lacking key information as to how these different modifications affected the overall peptide structure. To gain additional structural information and correlate it to bioactivity, we set out to assess the overall structure of the native *S. oligofermentans* CSP and its alanine screen analogs using Circular Dichroism (CD) spectroscopy. We assessed all CSP analogs in both aqueous (PBS buffer, pH 7.4) (Figure S-6) and membrane mimicking conditions (20% TFE in PBS, pH 7.4) (Figure 8). In aqueous solution the *S. oligofermentans* CSP was found to be unfolded, but in membrane mimicking conditions this peptide was found to possess a helical conformation. We quantified the percent helicity of the CSP using the mean residue ellipticity at 222 nm as previously reported and found that 18.9% of the CSP sequence is helical (Figure 8). Further evaluation of the alanine analogs of the *S. oligofermentans* CSP in membrane mimicking conditions revealed that there is no correlation between the degree of helicity and peptide activity, a finding that was atypical for a mitis group streptococcus, as other members of the group have displayed a clear increase in activity in analogs with a higher

degree of helicity.<sup>33</sup> Alanine substitution of Asn4 produced a peptide that was completely inactive, yet it possessed a degree of helicity higher than the native peptide and the second highest helicity of all tested analogs (Figure 8). Interestingly, replacement of Arg3 was the only alanine substitution that resulted in an analog that did not form an alpha-helix in membrane mimicking conditions, instead exhibiting a spectrum more indicative of a beta-sheet (Figure 8). It has been widely established that the bioactive conformation of a CSP is alpha-helical, and as peptide CSP-R3A exhibited some degree of receptor activation it is likely that the more hydrophobic nature of this peptide forces it to aggregate in solution at higher concentrations, resulting in the beta-sheet observed in this study, while it still likely binds the ComD receptor as an alpha helix.

### Second-generation CSP analogs

Our initial analysis of the *S. oligofermentans* CSP revealed that the *N*-terminus may be involved in receptor activation, while the *C*-terminus may be completely dispensable. To further explore these trends in an effort to create enhanced activators and inhibitors of the *S. oligofermentans* competence regulon, we designed a second-generation library of CSP analogs encompassing sequential truncations of either the three *N*-terminal residues, or the three *C*-terminal residues of the native CSP signal. Truncation of one, CSP-des-D1, or two, CSP-des-D1S2, sequential amino acids of the peptide *N*-terminus produced weak inhibitors of the *S. oligofermentans* competence regulon (Table 2). Truncation of the entire peptide *N*-terminus, CSP-des-D1S2R3, produced a peptide that was completely inactive, highlighting the importance of the CSP amino terminus in receptor binding and activation. Moving on to the *C*-terminus, we determined that truncation of one or two residues produced two peptides with activities comparable to that of the native peptide, CSP-des-K14 and CSP-des-K13K14, whereas truncation of three residues exhibited a more significant decrease in activity. Interestingly, removal of two or three residues from the *C*-terminus significantly increased the hydrophobic properties of these peptides, as these analogs were found to be harder to dissolve in aqueous solutions, supporting our claim that the purpose of this charged lysine tail is to increase the hydrophilicity of the *S. oligofermentans* CSP.

As truncation of either the *N*- or *C*-terminal regions of the *S. oligofermentans* CSP was insufficient to produce enhanced QS activators, we hypothesized that double alanine mutation of residues found to be permissive to modification during the initial alanine screening could produce CSP analogs with enhanced receptor binding. Our initial alanine screen revealed that replacement of residues Ser2, Leu7, Lys13, and Lys14 with alanine afforded analogs with activities equivalent to that of the native signal. Double alanine mutant combinations were made with substitutions at two of these positions, with the intent of producing an enhanced activator of the *S. oligofermentans* competence regulon. Double mutation with residues Ser2, Lys13, and Lys14 all produced CSP analogs with activities similar to that of the native peptide (Table 2). Interestingly, double substitutions incorporating alanine replacement of residue Leu7 displayed a complete loss in agonistic activity, despite the fact that substitution of Leu7 alone was able to activate the competence regulon at a comparable concentration to the native peptide (Table 2).



We previously observed that mutation of the negatively charged residue Asp1 to alanine produced a QS inhibitor, a trend consistent with other mitis group streptococci. In an effort to produce enhanced inhibitors of the competence regulon, double alanine mutant analogs were made with substitutions of Asp1 and the residues Ser2, Leu7, Lys13, or Lys14. Double mutation with Asp1 and Ser2 or Lys13 produced weak QS inhibitors with activities comparable to replacement of Asp1 alone (Table 2). Importantly, double mutation of Asp1 and Lys14 produced the strongest *S. oligofermentans* QS inhibitor, with a near two-fold improvement in activity compared to replacement of Asp1 alone (Table 2). Dual alanine mutation of positions Asp1 and Leu7 produced a peptide that was unable to interact with the ComD receptor, similar to what was observed for the double mutant activators (Table 2). These results further confirm that while Leu7 alone does not play an important role in ComD binding or activation, mutation of this position likely induces a conformational change that is not tolerable with other mutations of the CSP sequence, leading to a complete loss of ComD binding or activation.

### Determining the role of QS modulation in interactions between *S. oligofermentans* and *S. mutans*

Previous studies have highlighted how *S. mutans* strives to achieve dominance of the oral microbiome by either acidifying the microenvironment through the production of lactic acid or using its QS circuitry to coordinate the expression of mutacin, a bacteriocin that can kill off many competing oral microbes.<sup>5, 12, 34–35</sup> It has been well documented that *S. oligofermentans* and *S. mutans* compete within the microbiome, and that the *S. oligofermentans* mediated killing of *S. mutans* is primarily conducted through the conversion of lactic acid into hydrogen peroxide,<sup>5</sup> a process that our phenotypic studies have suggested is under control of the competence regulon. As a result of these findings, we hypothesized that by modulating the *S. oligofermentans* QS circuitry we would be able to tune its response to *S. mutans* by either enhancing the hydrogen peroxide mediated killing of *S. mutans* in the presence of a QS activator (native CSP) or decreasing it in the presence of a QS inhibitor (CSP D1AK14A). Thus, we tested the effectiveness of QS modulators on *S. oligofermentans* in the presence of two different *S. mutans* UA159 strains, one with intact QS circuitry (SMCOM3), and a *comC* knockout unable to produce mutacin (SMCC3).

In regard to the *comC* knockout strain SMCC3, we fully expected *S. oligofermentans* to be able to inhibit the growth of *S. mutans* due to a lack of mutacin production. Our experiments in several trials showed slight zones of inhibition on *S. mutans* when in the presence of *S. oligofermentans* alone, with added lactic acid, or with an added QS activator, or no zone of inhibition when in the presence of a QS inhibitor or added lactic acid and QS activator or QS inhibitor (Figure 9). Furthermore, we predicted that *S. oligofermentans* would be inhibited by the *S. mutans* UA159 strain SMCOM3, as the mutacin produced by this strain has been shown to be bacteriocidal against many species of streptococci. Interestingly, in many of the co-culture conditions the growth of neither *S. mutans* nor *S. oligofermentans* was inhibited. The only case in which a clear zone of inhibition was observed in several trials was with the pre-treatment of *S. oligofermentans* with both QS inhibitor and lactic acid, perhaps suggesting that the dual activity of mutacin and an inability to process lactic acid as a byproduct of QS inhibition was sufficient to prevent the growth of *S. oligofermentans* in the

presence of *S. mutans* (Figure 9). Altogether, we were unable to directly observe conclusive evidence for enhanced *S. oligofermentans* mediated killing of *S. mutans*. Nonetheless, since there are many different factors that can influence the interspecies interactions between streptococci, our results cannot entirely rule out QS modulation of *S. oligofermentans* as an effective approach to counter *S. mutans* infection. Rather, our results indicate that QS modulation did not have any observable effect in the tested conditions, and that further experiments are required to fully explore the effect of QS on these interactions.

### Assessing the cytotoxicity of *S. oligofermentans* and CSP analogs

One of the major advantages of *S. oligofermentans* is its ability to act as a biotherapeutic, outcompeting more pathogenic bacteria within the oral microbiome to promote human health. As many streptococci are known to produce virulence factors associated with cytotoxicity in both human and animal models, we sought to ensure that there was no observable *S. oligofermentans* or CSP induced cytotoxicity, which would negatively impact the capacity of this proposed approach.<sup>35–37</sup> To this end, hemolysis assays against defibrinated rabbit red blood cells (RBCs) conducted in both THY media alone and THY media containing *S. oligofermentans* were utilized to assess the degree of cytotoxicity attributed to *S. oligofermentans* or any tested peptides. In THY media alone there was no significant increase in hemolysis in the presence of any of the tested CSP analogs, whereas a significant degree of hemolysis was observed in the .01% Triton X positive control (Figure 10). These results suggest that treatment with CSP alone has no significant cytotoxic effect against the RBCs. Additionally, we observed that there was no significant effect on *S. oligofermentans* hemolysis upon treatment with the native peptide (CSP), a strong activator (CSP S2A), or a strong inhibitor (CSP D1AK14A) (Figure 10), highlighting both that *S. oligofermentans* is not likely to cause any significant degree of cytotoxicity, and that any potential cytotoxicity is not the product of a QS-mediated response.

### Summary and Conclusions

It has been well established that streptococcal QS is used to mediate important bacterial phenotypes, and that it plays a critical role in the complex web of inter- and intra-species interactions that occur within the oral microbiome. Herein we have confirmed the identity of the *S. oligofermentans* CSP as the 14 amino acid peptide DSRNIFLKIFKKK and demonstrated its involvement in the regulation of bacterial competence and hydrogen peroxide production through activation of the competence regulon.

Through alanine screening of the *S. oligofermentans* CSP we were able to gain several insights into the SAR between CSP amino acid side chain moieties and interactions with the ComD receptor. We have demonstrated that the *N*-terminus of the CSP is critical for receptor binding and activation, and similarly to other mitis group streptococci, it was observed that mutation of the first negatively charged residue to alanine produced a QS inhibitor. Interestingly, we observed that there is no clear trend of activity for mutations to the central region of the CSP, as mutation of both polar or hydrophobic residues produced inactive and active CSP analogs. Moreover, we have demonstrated that the *C*-terminal region of the CSP signal can be removed without significantly impacting ComD receptor binding or activation.

This finding was further confirmed by a retention of peptide activity following truncation of this region. Additionally, through multiple alanine mutation of the CSP we were able to produce the most potent *S. oligofermentans* QS inhibitor, CSP D1AK14A, which displays a near two-fold greater degree of inhibitory activity compared to CSP D1A.

Comprehensive structural analysis of all alanine screen CSP analogs revealed that, unlike what has been previously observed in other mitis group streptococci, there is no clear trend between alpha helicity and peptide activity for the *S. oligofermentans* CSP. Additionally, there was no clear trend that would suggest that the CSP may be able to tune the effectiveness of *S. oligofermentans* against *S. mutans* in interspecies competition assays, suggesting that future efforts should focus on further probing of these interactions. Hemolysis assays demonstrated that CSPs themselves are relatively non-toxic, and that *S. oligofermentans* does not produce any virulence factors that could result in undesired toxicity. Overall, this study suggests that *S. oligofermentans* QS modulators are promising tools for probing interspecies interactions among oral streptococci, or as potential non-toxic additives to help increase the potency of *S. oligofermentans* as a biotherapeutic against *S. mutans* infection.

## Materials and Methods

### General:

Synthesis of peptides was performed through Fmoc solid phase peptide synthesis (SPPS). Peptides were purified to 95% as confirmed by analytical reverse phase high-performance liquid chromatography (RP-HPLC). Masses of purified peptides were confirmed with high-resolution ESI-TOFMS. See Tables S-2–S-3 for peptide masses and purities. See supporting information for full experimental details.

### Isolation of Crude Peptides from Bacterial Supernatants:

The native *S. oligofermentans* CSP was extracted from bacterial supernatants using previously described methods with minor modifications.<sup>9</sup> See Supporting Information for full experimental details.

### Development of *S. oligofermentans* reporter strain:

The *S. oligofermentans* luciferase-based reporter strain was constructed using previously described methods with some modifications.<sup>31</sup> See Supporting Information for full experimental details.

### Luminescence Assay:

**Activation Assays.**—The ability of synthetic CSP analogs to induce activation of the competence regulon was determined using the *S. oligofermentans* luciferase reporter strain. First, a single colony of the *S. oligofermentans* luciferase reporter strain was grown at 37 °C with 5% CO<sub>2</sub> in 10 mL THY media (pH 7.3) containing 100 µg/mL spectinomycin for 18 hours. Following incubation, the bacteria were diluted 1:10 in fresh THY media, and incubated for 30 min at 37 °C with 5% CO<sub>2</sub>. During incubation, clear bottom white 96-well microtiter plates were prepared for activation assays. Initially, activation screening

was performed at a high CSP concentration (10,000 nM) for all CSP analogs. For each experimental sample, 2  $\mu$ L of a 1 mM CSP stock solution in dimethyl sulfoxide (DMSO) was added in triplicate to the clear bottom white 96-well microtiter plate. A total of 2  $\mu$ L of DMSO was added in triplicate as a negative control, and 2  $\mu$ L of a 1 mM stock of the native CSP was added in triplicate as a positive control. In the dark, 2  $\mu$ L of a 15 mg/ml D-luciferin stock was added to each well. Following addition of CSP and D-luciferin, 196  $\mu$ L of the diluted bacterial culture was added to each well, and the plate was incubated for 30 min at 37 °C. Following incubation, the OD<sub>600</sub> and luminescence of each experimental well was measured. Final results were reported as percent activation, which is the ratio between the final blank and OD<sub>600</sub> corrected luminescence of experimental values and the positive control. Analogs that exhibited activity > 75% compared to the positive control (see Figures S-2–S-3) were further evaluated using a dose-dependent assay in which peptide stock solutions were diluted with DMSO in serial dilutions (1:2 or 1:3) and assayed as described above. EC<sub>50</sub> values, the concentration of the peptide to achieve a half-maximal response, for each activator were calculated using GraphPad Prism. Experiments were performed in triplicate on three separate days.

**Inhibition Assays.**—Analogs that exhibited a low degree of activation in the initial screening (<50% activation) were evaluated for competitive inhibition of the competence regulon. The ability of synthetic CSP analogs to competitively inhibit *comX* expression by outcompeting CSP for the receptor binding site was evaluated using the same assay conditions as the activation assays, except inhibition screening introduced the native CSP to each well at a set concentration (300 nM) that was chosen to afford complete activation of the competence regulon, as determined from dose-dependence curves created for the native CSP. As a positive control, 2  $\mu$ L of CSP and 2  $\mu$ L of DMSO were added to the same well in triplicate. As a negative control, 4  $\mu$ L of DMSO was added in triplicate. Then 2  $\mu$ L of 15 mg/mL D-luciferin was added to each experimental or control well, after which 194  $\mu$ L of bacteria was added, and the plate was incubated for 30 min at 37 °C. Following incubation, the OD<sub>600</sub> and luminescence of each experimental well were measured. Final results were reported as percent activation, which is the ratio between the final blank and OD<sub>600</sub> corrected luminescence of experimental values and the positive control. Analogs that exhibited activity < 50% compared to the positive control (see Figures S-4–S-5) were considered competitive inhibitors of the QS circuitry. These peptides were further evaluated in dose-dependent assays where peptide stock solutions were diluted with DMSO in a 1:2 serial dilution and assayed as described above. Inhibitor IC<sub>50</sub> values, the concentration of inhibitor required to reduce the QS activation by half, were determined using GraphPad Prism. Experiments were performed in triplicate on three separate days.

### Transformation Assay:

A single colony of *S. oligofermentans* was grown at 37 °C with 5% CO<sub>2</sub> in 10 mL THY media (pH 7.3) for 18 hours. Following incubation, *S. oligofermentans* was diluted 1:25 into fresh THY media. After static incubation at 37 °C with 5% CO<sub>2</sub> for 30 min, CSP was added at final concentrations of 10,000 nM, 1,000 nM, 100 nM, or 10 nM, along with 300 ng pDL278 (Spec<sup>R</sup>). Parallel assays without CSP or without both CSP and plasmid were used as controls to assess the indigenous competence or antibiotic resistance of *S.*

*oligofermentans* in the tested conditions. After 2 h incubation at 37 °C with 5% CO<sub>2</sub>, 20 µL of the culture was plated on THY agar containing 400 µg/mL spectinomycin and incubated at 37 °C with 5% CO<sub>2</sub> for 48 hours to identify positive transformants. Experiments were performed in triplicate on three separate days.

#### **Biofilm Formation Assay:**

Biofilm formation was assessed using previously described methods with minor alterations.<sup>38</sup> See Supporting Information for full experimental details.

#### **Hydrogen Peroxide Production Assay:**

Hydrogen peroxide production was assessed using previously described methods.<sup>39</sup> See Supporting Information for full experimental details.

#### **Interspecies Competition Assays:**

A single colony of *S. oligofermentans* was inoculated into 10 mL THY media (pH 7.3) and grown for 18 h at 37 °C with 5% CO<sub>2</sub>. Concurrently, single colonies of *S. mutans* SMCOM3 (UA159, Spec<sup>R</sup>) and SMCC3 (UA159, *comC*, Spec<sup>R</sup>) were separately inoculated into 10 mL THY media (pH 7.3) containing 100 µg/mL spectinomycin and were grown at 37 °C with 5% CO<sub>2</sub> for 18 h. Following growth of the overnight cultures, bacteria were then diluted 1:10 in fresh THY media, and the following experimental conditions were arranged with a total final volume of 500 µL: *S. oligofermentans*, *S. mutans* SMCOM3, *S. mutans* SMCC3, *S. oligofermentans* + 10 mM lactic acid, *S. oligofermentans* + 300 nM native CSP, *S. oligofermentans* + 2,500 nM CSP D1AK14A, *S. oligofermentans* + 300 nM native CSP + 10 mM lactic acid, *S. oligofermentans* + 2,500 nM CSP D1AK14A + 10 mM lactic acid. Cultures were then incubated for 2 h at 37 °C with 5% CO<sub>2</sub>. Immediately following incubation, a 10 µL portion of each *S. oligofermentans* experimental condition was then spotted adjacently to a 10 µL portion of either *S. mutans* strain on a THY agar plate. Spotted media was allowed to absorb into the plate for 30 min at RT, followed by incubation at 37 °C with 5% CO<sub>2</sub> for 24 h. Following incubation, competition between bacteria was assessed by observing the presence of a zone of inhibition on the spotted cultures. Experiments were performed in triplicate on three separate days.

#### **Hemolysis Assay:**

Hemolysis was assessed using previously described methods with minor alterations.<sup>40</sup> See Supporting Information for full experimental details.

#### **Circular Dichroism (CD) Spectroscopy:**

CD spectra were recorded using an Aviv Biomedical CD spectrometer (model 202-01). All the measurements were performed with a peptide concentration of 100 µM in 1x PBS buffer with 0% or 20% trifluoroethanol (TFE). Measurements were performed using a quartz cuvette (Starna Cells) with a pathlength of 0.1 cm at 25 °C. Samples were scanned once at 3 nm min<sup>-1</sup> with a bandwidth of 1 nm and a response time of 20 s over a wavelength range (195 to 260 nm). Single scans were acquired and corrected for their respected solvent

concentrations, then converted to mean residue ellipticity (MRE) values using the following equation:

$$MRE = \left( \frac{\theta}{10 * c * l} \right) / n$$

$\theta$  is the observed ellipticity in millidegrees,  $c$  is the molar peptide concentration,  $l$  is the pathlength in centimeters, and  $n$  is the number of residues in the peptide sequence. Percent helicity ( $f_H$ ) was calculated for all peptide analogs using the following equation:

$$f_H = \frac{[\theta_{222}]}{[\theta_{\infty}]_{222} \left( 1 - \frac{x}{n} \right)}$$

$[\theta_{222}]$  is the mean residue ellipticity of the peptide at 222 nm,  $[\theta_{\infty}]_{222}$  is the assumed mean residue ellipticity for a peptide with 100% helicity ( $-44,000 \text{ deg cm}^2 \text{ dmol}^{-1}$ ),  $n$  is the number of residues within the tested peptide sequence, and  $x$  is an empirical correction for end effects.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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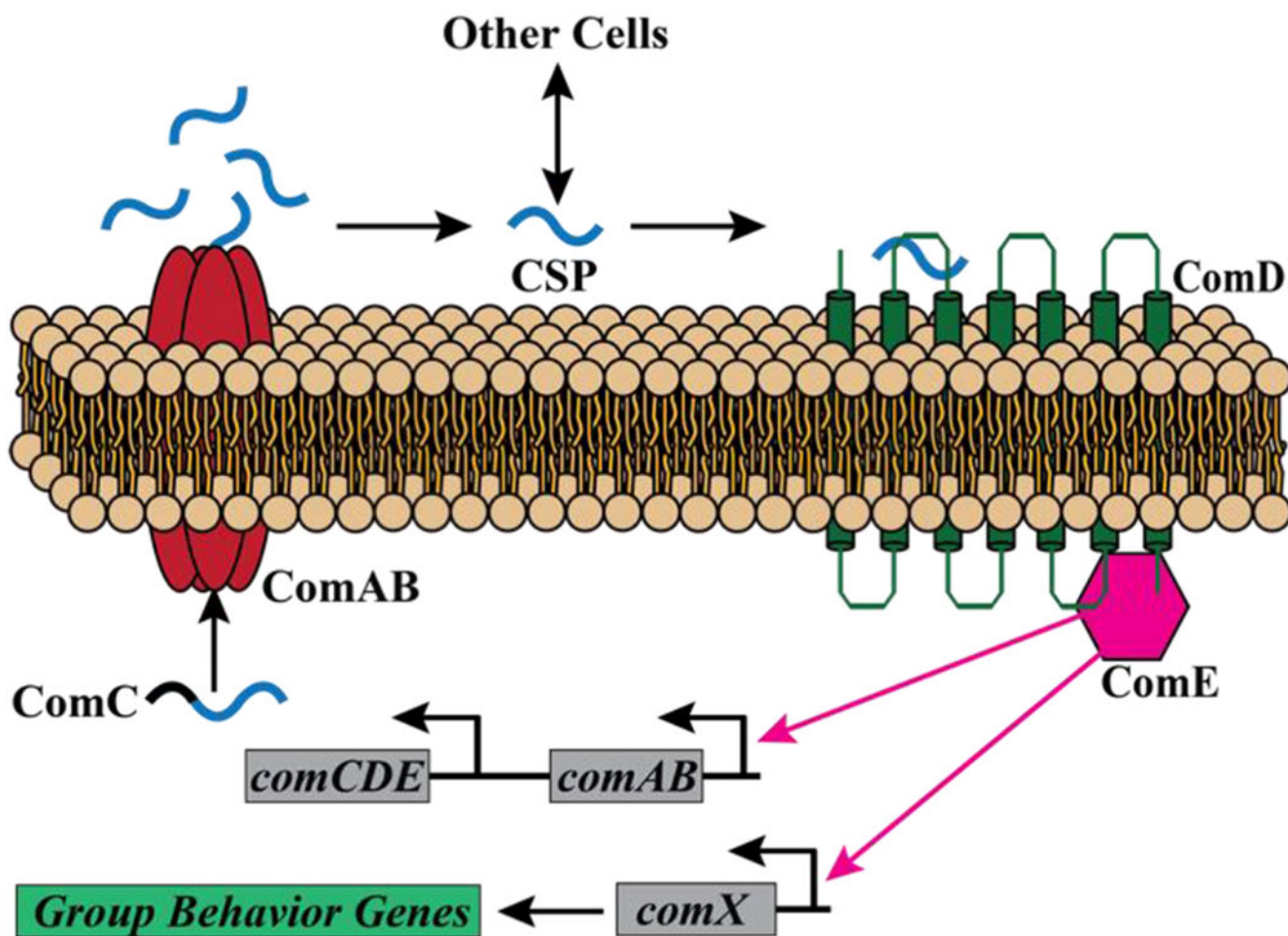
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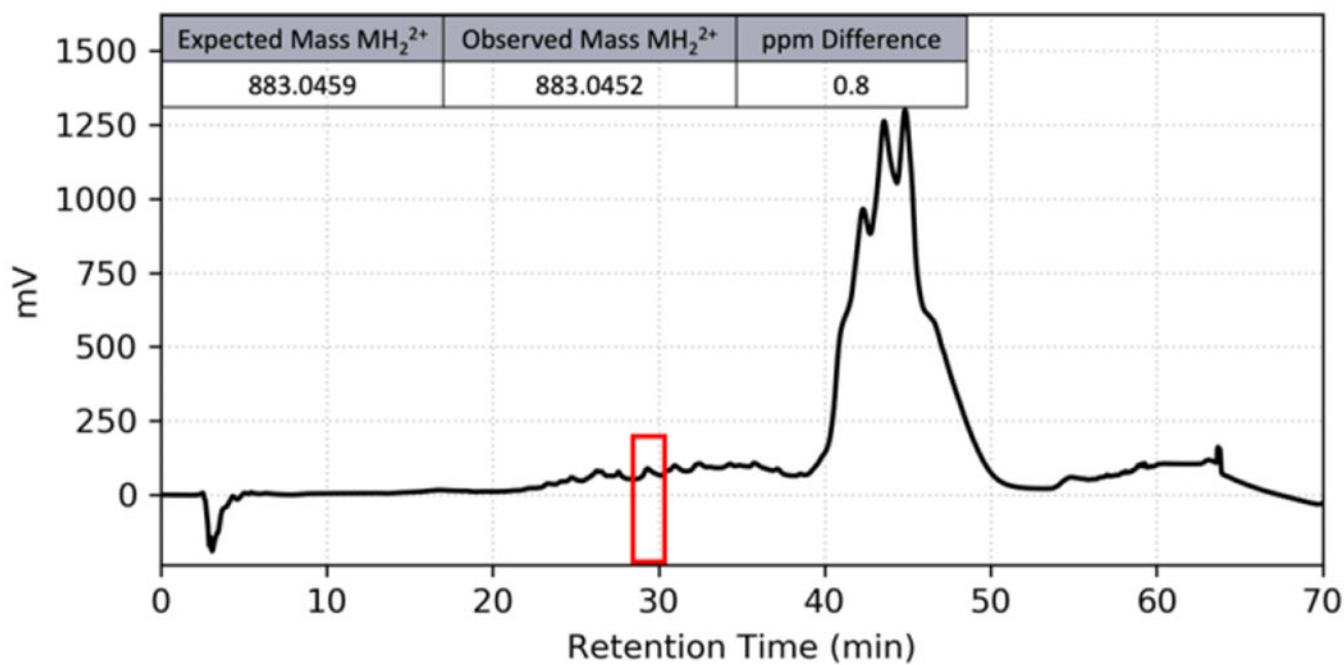
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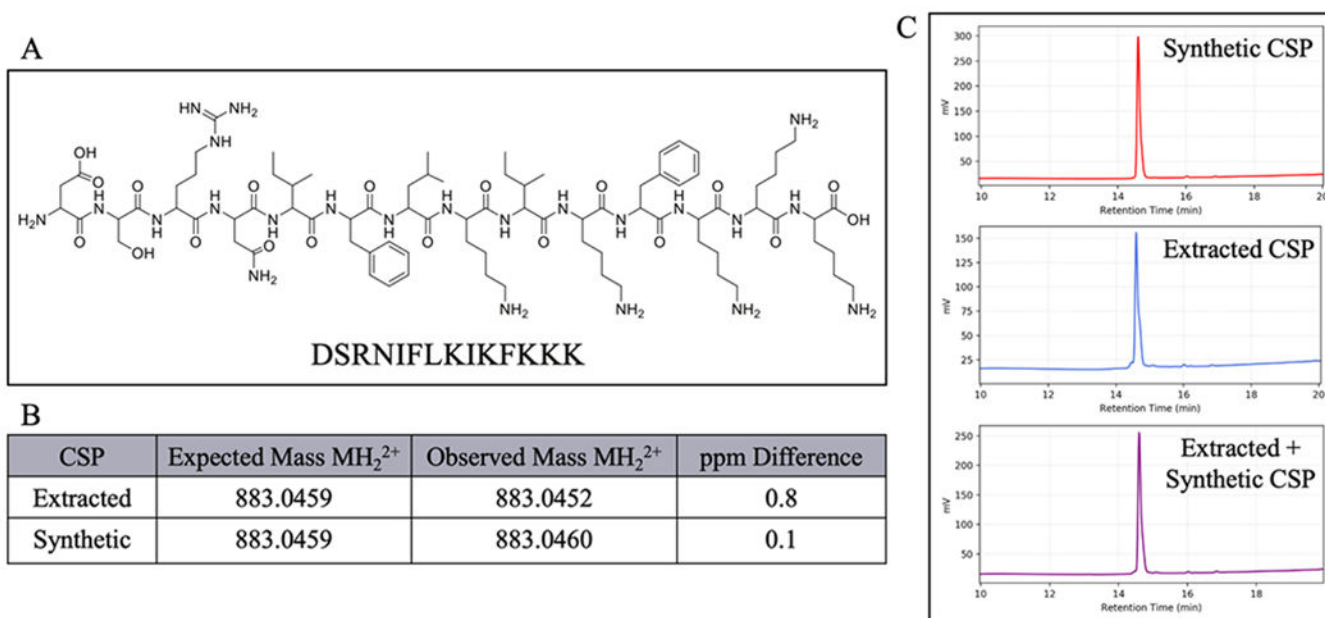


**Figure 1.** The general streptococcal quorum sensing pathway. ComC is processed and transported to the extracellular environment as the CSP by ComAB. Upon reaching a minimal threshold concentration the CSP binds to its cognate histidine kinase receptor ComD. Activation of ComD leads to phosphorylation of ComE, a response regulator, resulting in further activation of the *comABCDE* operon and expression of *comX*, the master regulator of group behavior genes.





**Figure 3.** Isolation and detection of the *S. oligofermentans* CSP from crude cell-free supernatants. RP-HPLC chromatogram of total proteins isolated from the supernatant sample and High Resolution ESI-TOF MS of the fraction collected from 28 to 30 min (red). See the Supporting Information for full experimental details.



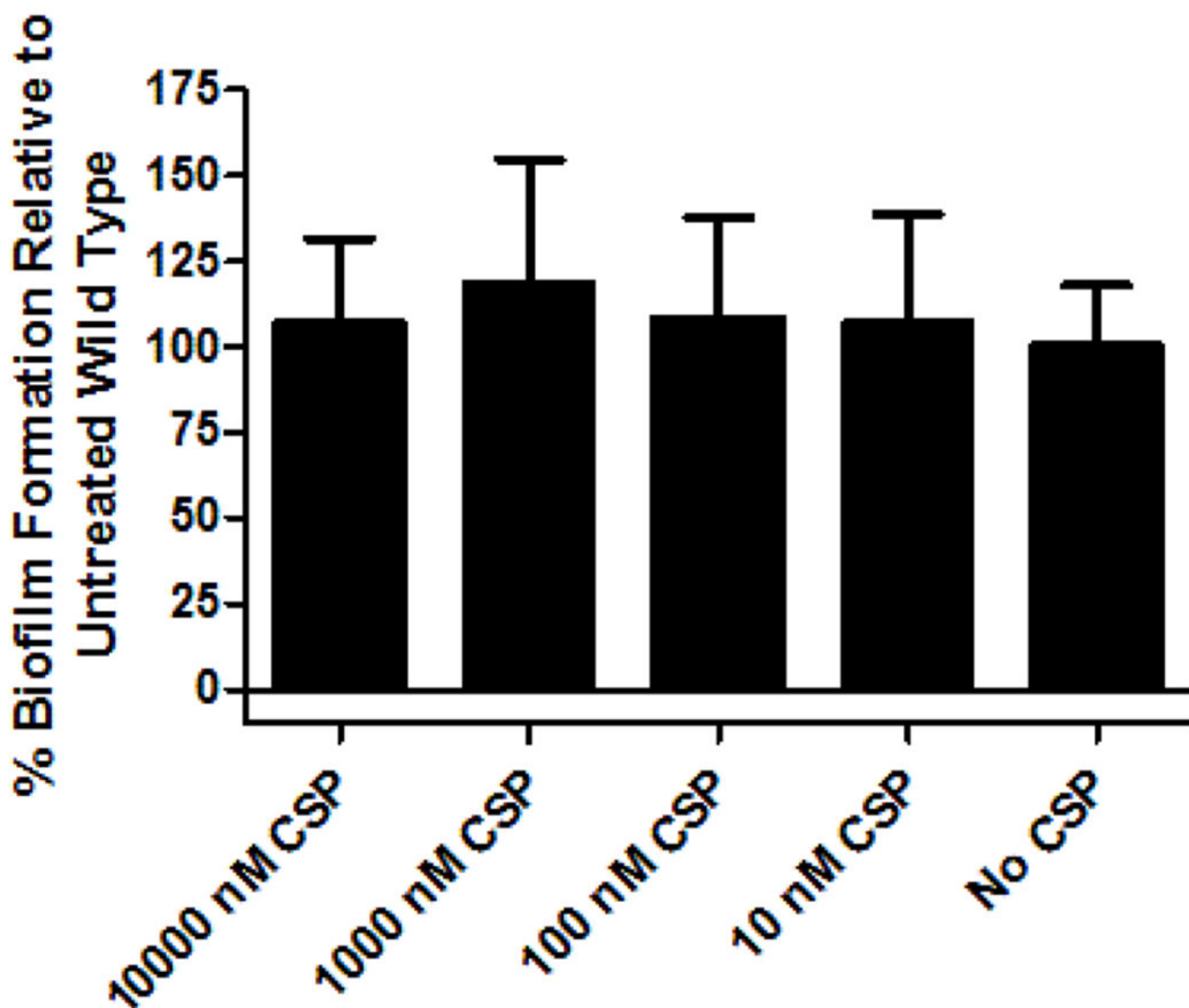
**Figure 4.**

Comparison of purified synthetic and isolated *S. oligofermentans* CSP. (A) Proposed structure of the 14 amino acid *S. oligofermentans* CSP. (B) Comparison of masses of synthetic and extracted peptides by ESI-TOF MS (C) Comparison of analytical RP-HPLC chromatograms of purified natural, synthetic, and natural and synthetic CSP.

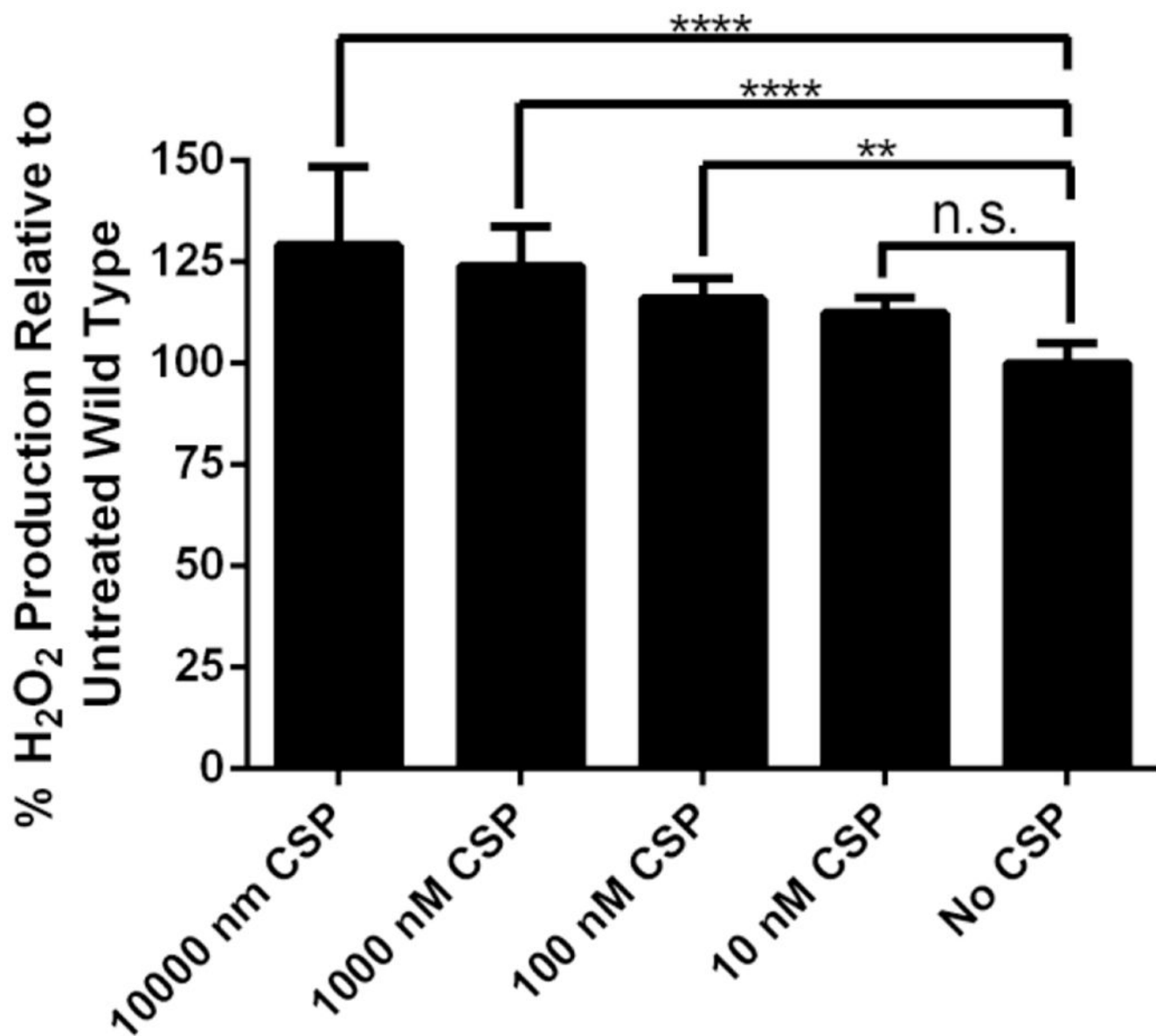




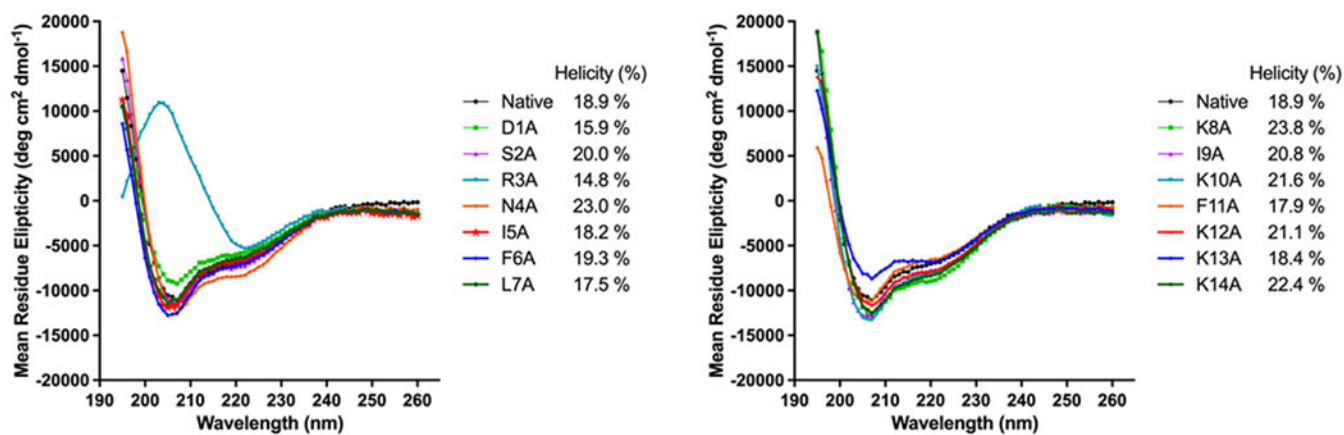
**Figure 5.** *S. oligofermentans* competence is dependent upon CSP concentration. Synthetic CSP was added at concentrations of 10,000 nM (A), 1,000 nM (B), 100 nM (C), 10 nM (D), or no exogenous CSP (E). Experiment was repeated three times in triplicate for a total of nine experiments. See Materials and Methods for full experimental details.



**Figure 6.** Biofilm formation of *S. oligofermentans* as a function of CSP concentration. The competence regulon of *S. oligofermentans* does not appear to have a clear effect on biofilm formation. Experiment was repeated three times in triplicate for a total of nine experiments. See the Supporting Information for full experimental details.

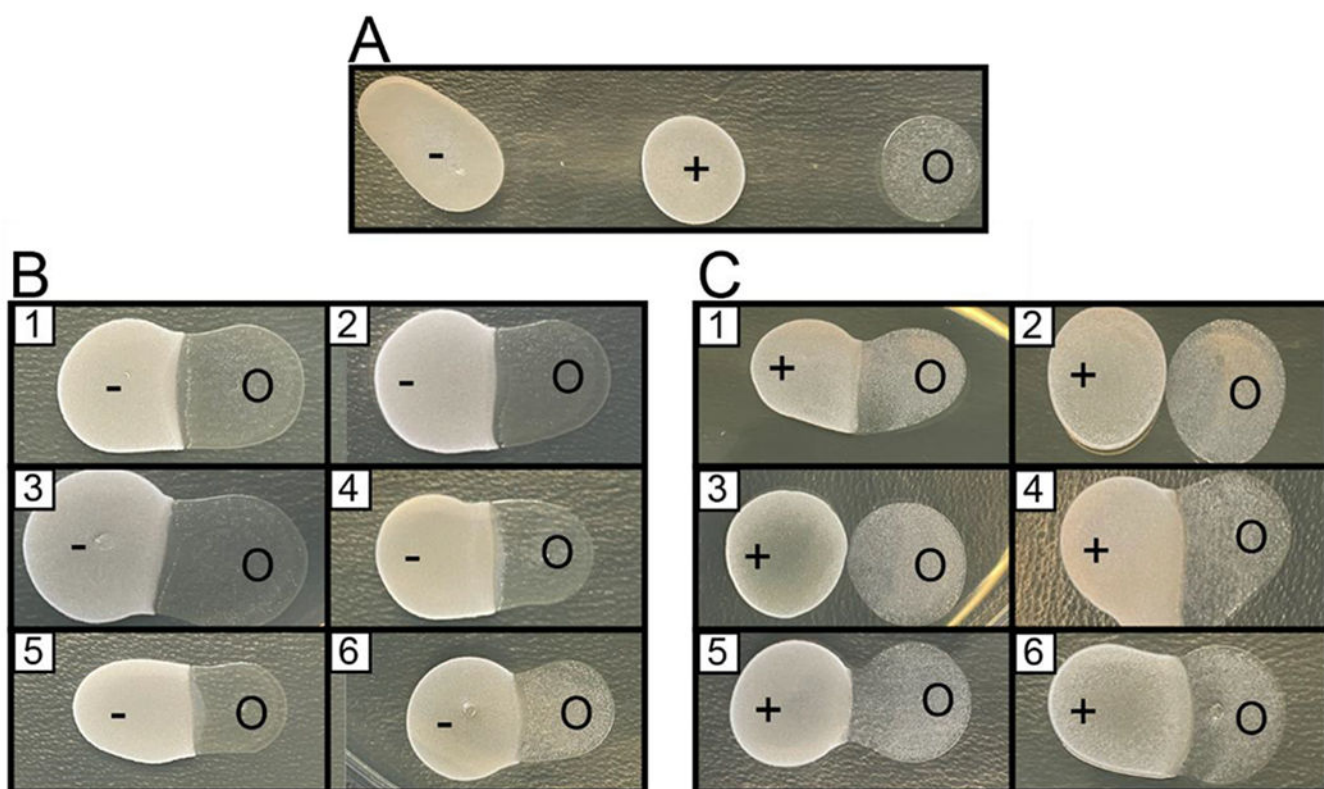


**Figure 7.** Hydrogen peroxide formation of *S. oligofermentans* as a function of CSP concentration. Induction of the competence regulon through the addition of exogenous CSP produces a statistically significant increase in the degree of hydrogen peroxide production. Statistical significance was determined using a one-way ANOVA with Bonferroni's correction; n.s., not significant; \*\*, P 0.01; \*\*\*\*\*, P 0.0001. Experiment was repeated three times in triplicate for a total of nine experiments. See the Supporting Information for full experimental details.



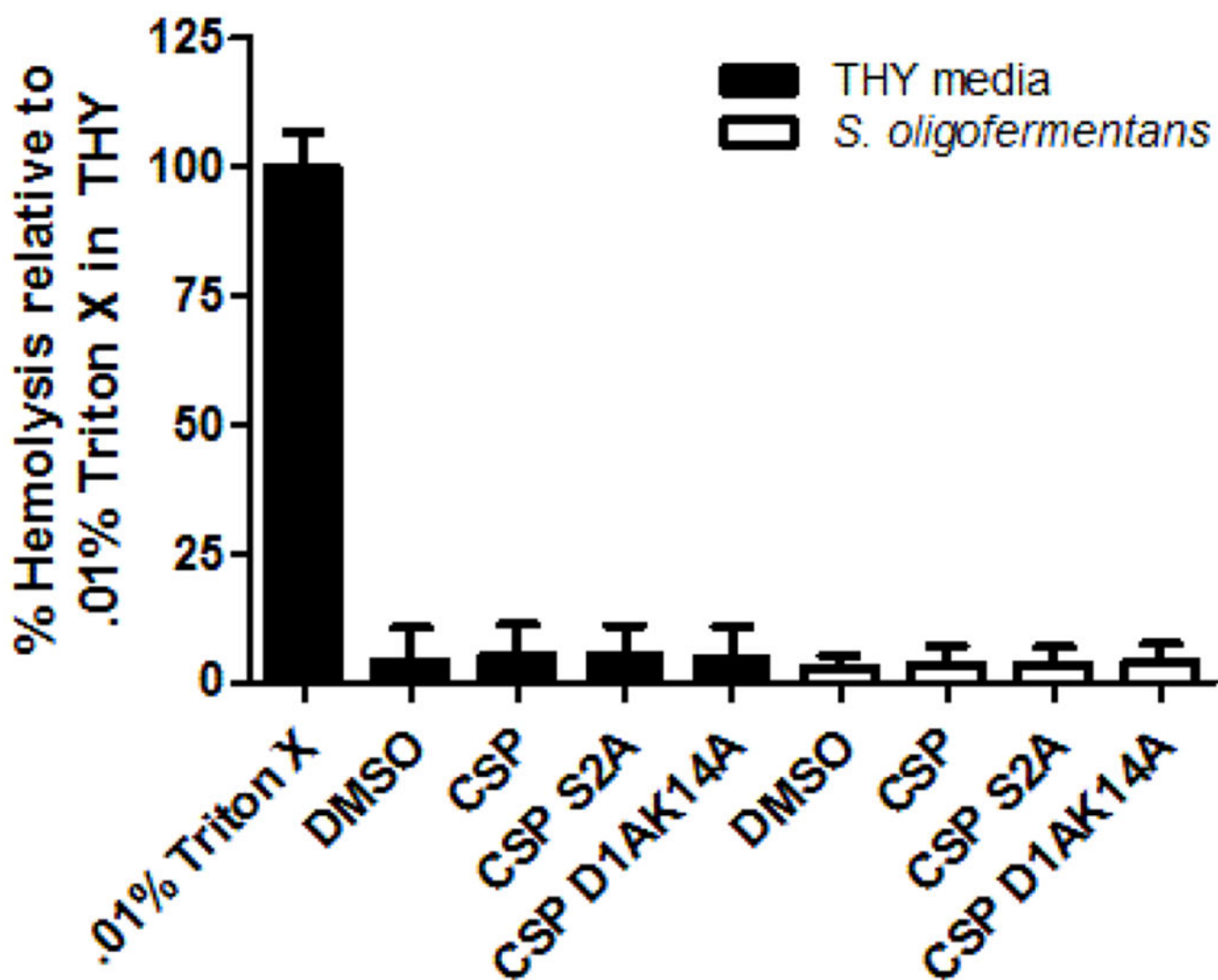
**Figure 8.**

CD spectra of *S. oligofermentans* alanine screen analogs in membrane mimicking conditions. Most of the alanine screen analogs adopt an alpha helical confirmation in membrane mimicking conditions. However, there does not appear to be a correlation between the degree of alpha helicity and activity.



**Figure 9.**

Interspecies competition between *S. oligofermentans* and *S. mutans*. A. Individual colonies of *S. mutans* SMCC3 (UA159, *comC*, *Spec<sup>R</sup>*) (-), SMCOM3 (UA159, *Spec<sup>R</sup>*) (+), and wild type *S. oligofermentans* (O). B. Competition between SMCC3 (left) and *S. oligofermentans* (right). C. Competition between SMCOM3 (left) and *S. oligofermentans* (right). In both B and C, *S. oligofermentans* was exposed to the following conditions prior to co-inoculation with either *S. mutans* strain: 1. THY media; 2. THY media + 10 mM lactic acid; 3. THY media + 300 nM native CSP; 4. THY media + 2,500 nM CSP D1AK14A; 5. THY media + 10 mM lactic acid + 300 nM CSP; 6. THY media + 10 mM lactic acid + 2,500 nM CSP D1AK14A. Experiment was repeated three times in triplicate for a total of nine trials. See Materials and Methods for full experimental details.



**Figure 10.** Hemolytic activity of CSP derived QS modulators on defibrinated rabbit red blood cells in the presence of THY media (black) or *S. oligofermentans* cultures (white). Experiments were performed in triplicate on three separate days. See the Supporting Information for full experimental details.



**Table 1.**EC<sub>50</sub> or IC<sub>50</sub> values of alanine screen analogs against the ComD receptor<sup>a</sup>

| Name     | EC <sub>50</sub> or IC <sub>50</sub> * (nM) <sup>b</sup> | 95% CI <sup>c</sup> |
|----------|--|---------------------|
| CSP      | 65.7   | 40.4 - 107          |
| CSP-D1A  | 957*   | 881 - 1040*         |
| CSP-S2A  | 105  | 72.1 - 153          |
| CSP-R3A  | >1000  | >1000               |
| CSP-N4A  | --   | --                  |
| CSP-I5A  | 270  | 159 - 457           |
| CSP-F6A  | --   | --                  |
| CSP-L7A  | 109  | 72.9 - 163          |
| CSP-K8A  | 231  | 176 - 304           |
| CSP-I9A  | >1000  | >1000               |
| CSP-K10A | 207  | 113 - 381           |
| CSP-F11A | 288  | 188 - 439           |
| CSP-K12A | 115  | 100 - 131           |
| CSP-K13A | 107  | 52 - 219            |
| CSP-K14A | 156  | 123 - 199           |

<sup>a</sup>See the Materials and Methods section for experimental methods. See Supporting Information for detail of reporter strain and plots of agonism or antagonism dose response curves. All assays performed in triplicate.

<sup>b</sup>EC<sub>50</sub> or IC<sub>50</sub> values determined by testing peptides over a wide range of concentrations.

<sup>c</sup>95% confidence interval.

<sup>d</sup>EC<sub>50</sub> not determined due to the analog's low induction in primary agonism screening assays. See Supporting Information for details.

**Table 2.**EC<sub>50</sub> or IC<sub>50</sub> values of truncated or double mutation analogs against the ComD receptor<sup>a</sup>

| Name              | EC <sub>50</sub> or IC <sub>50</sub> * (nM) <sup>b</sup> | 95% CI <sup>c</sup> |
|-------------------|--|---------------------|
| CSP               | 65.7   | 40.4 - 107          |
| CSP-des-D1        | >1000*   | >1000*              |
| CSP-des-D1S2      | >1000*   | >1000*              |
| CSP-des-D1S2R3    | --   | --                  |
| CSP-des-K14       | 179  | 96.4 - 332          |
| CSP-des-K13K14    | 190  | 108 - 333           |
| CSP-des-K12K13K14 | 416  | 165 - 1050          |
| CSP-D1AS2A        | 881*   | 538 - 1440*         |
| CSP-D1AL7A        | --   | --                  |
| CSP-D1AK13A       | >1000*   | >1000*              |
| CSP-D1AK14A       | 537*   | 294 - 980*          |
| CSP-S2AL7A        | --   | --                  |
| CSP-S2AK13A       | 211  | 161 - 275           |
| CSP-S2AK14A       | 201  | 117 - 345           |
| CSP-L7AK13A       | --   | --                  |
| CSP-L7AK14A       | --   | --                  |
| CSP-K13AK14A      | 166  | 112 - 244           |

<sup>a</sup>See the Materials and Methods section for experimental methods. See Supporting Information for detail of reporter strain and plots of agonism or antagonism dose response curves. All assays performed in triplicate.

<sup>b</sup>EC<sub>50</sub> or IC<sub>50</sub> values determined by testing peptides over a wide range of concentrations.

<sup>c</sup>95% confidence interval.

<sup>d</sup>EC<sub>50</sub> not determined due to the analog's low induction in primary agonism screening assays. See Supporting Information for details.