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Exploring the competence stimulating peptide (CSP) *N*-terminal requirements for effective ComD receptor activation in group1 *Streptococcus pneumoniae*

Yifang Yang, Yftah Tal-Gan*

Department of Chemistry, University of Nevada, Reno, 1664 North Virginia Street, Reno, Nevada 89557, United States

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ABSTRACT

The competence stimulating peptide (CSP) plays a key role in the regulation of pneumococcal quorum sensing (QS), a communication system that is critical to the infectivity of pneumococci. CSP functions through binding and activating a transmembrane receptor, ComD. Molecules that can modulate pneumococcal QS through intercepting CSP:ComD interaction may serve as new generation of antibacterial agents to treat pneumococcal infections. In this work, we systematically modified the *N*-terminus of CSP1, a region that is essential to ComD activation, to identify detailed structural features of the *N*-terminus that are responsible for its function. Our results revealed structural features that are optimal to achieve receptor activation and structure-activity trends that improve our understanding of CSP:ComD interaction, all of which will contribute to the design of novel pneumococcal QS modulators with higher potency and improved pharmacological properties.

1. Introduction

Streptococcus pneumoniae is a Gram-positive, commensal bacterium that asymptomatically colonizes the nasopharynx. However, *S. pneumoniae* is also an opportunistic pathogen that can cause acute diseases such as community acquired pneumonia, meningitis and sepsis. [1] *S. pneumoniae* mainly affects young children and patients with compromised immune system. It was estimated that in 2011 *S. pneumoniae* was responsible for approximately 2 million severe episodes of pneumonia and 411,000 deaths worldwide in children younger than 5 years.[2] Antibiotics such as penicillin were mostly effective against pneumococcal infections when they were first introduced. However, following extensive usage of these antibiotics in the clinic, antibiotic resistant pneumococci, including multidrug resistant pneumococci, had emerged worldwide, resulting in increased number of deaths, hospitalizations and medical costs. [3] One major contributor to the development of antibiotic resistance is the bactericidal or bacteriostatic effect of antibiotics, which results in strong selective pressure for resistance development. Therefore, to tackle the threat of drug resistance, one strategy is to develop alternative therapies that attenuate the infectivity of the bacteria without affecting the fitness of the bacteria, thus minimizing the selective pressure for resistance. [4–7] A peptide-mediated quorum sensing (QS) circuitry in *S. pneumoniae* has emerged as an excellent target for the design of anti-virulence therapy to treat pneumococcal infections due to its extensive involvement in pathogenicity while being nonessential for the bacterial survival.

Bacteria utilize QS systems to coordinate their gene expression in response to their population. [8–11] *S. pneumoniae* utilizes a peptide-mediated QS system to regulate competence, virulence factor production and biofilm formation. [12–14] Since the first phenotype to be associated with this QS circuitry was competence, a state where bacteria are able to lyse non-competent bacteria and take up DNA from the extracellular environment, the system was termed the competence regulon and the signaling peptide that drives this circuitry was termed the competence stimulating peptide (CSP). [12] The precursor peptide of CSP, ComC, encoded by the *comC* gene, is cleaved and exported out of the cell by an ABC transporter (ComAB; See Fig. 1). [15] CSP accumulates in the extracellular environment as the cell density increases. Once the concentration of CSP reaches a threshold, the peptide can effectively bind and activate a transmembrane histidine kinase receptor called ComD. On activation, ComD then activates its response regulator ComE through phosphorylation. [16] Once activated, ComE can bind the promoter region of numerous genes and activate their transcription. The genes that are activated by ComE include *comX*, *comAB*, and *comCDE*. *comX* encodes a sigma factor that leads to the initiation of competence. [17–19] Activation of *comAB* and *comCDE* leads to upregulation of the QS components, resulting in a positive feedback loop that ensures the synchronization of gene expression. The majority of *S.*

* Corresponding author.
E-mail address: ytalgan@unr.edu (Y. Tal-Gan).

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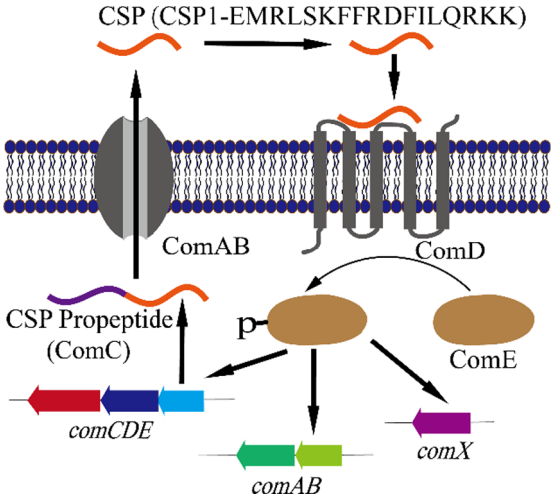


Fig. 1. *S. pneumoniae* CSP-mediated QS circuit. CSP is made from a propeptide, ComC, which is encoded by the *comC* gene. An ABC transporter, ComAB, then cleaves the propeptide and exports the mature CSP out of the cell. As the population of the bacteria grows, the concentration of CSP increases. Once the concentration of CSP reaches a threshold, CSP can effectively bind and activate a transmembrane histidine kinase receptor, ComD. On activation, ComD activates its response regulator, ComE, through phosphorylation. Activated ComE can trigger the transcription of numerous genes including *comX*, which is responsible for the induction of competence. The sequence of CSP1 is shown.

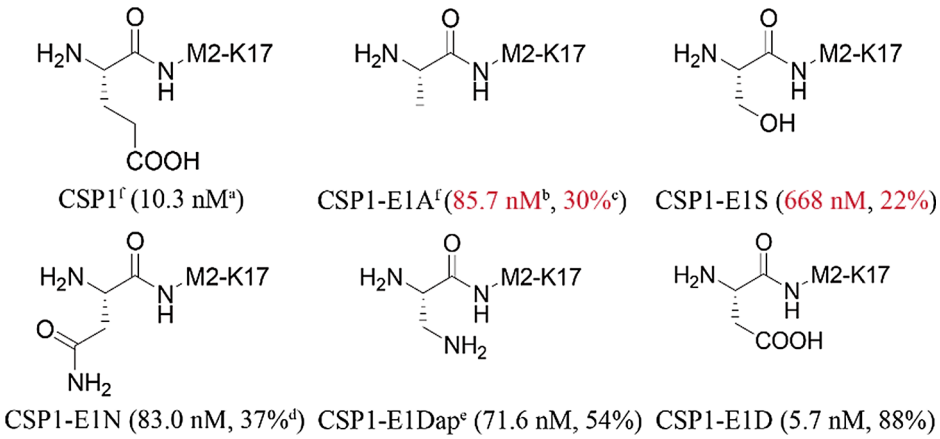
pneumoniae strains produce one of two variants of CSP, namely CSP1 and CSP2, with their cognate receptors ComD1 and ComD2, respectively. Therefore *S. pneumoniae* strains can be divided into two specificity groups, group1 and group2. [20] This CSP-mediated QS system can be modulated by peptide analogs to attenuate virulence factor production *in vitro* and *in vivo* infectivity, as demonstrated by Lau and coworkers for group1 *S. pneumoniae*, as well as by us for group2 *S. pneumoniae*. [21–22]

To improve the potency and pharmacological properties of CSP-based QS modulators, we have performed extensive structure-activity relationship (SAR) studies on both native CSPs. [23] For example, our alanine and D-amino acid substitution studies revealed several key residues that are important for receptor binding, activation and specificity. Our circular dichroism (CD) spectroscopy studies indicated that CSP1 adopts an α-helix conformation in membrane mimicking conditions, and that this conformation is required for bioactivity. Furthermore, our two-dimensional structural NMR spectroscopy studies revealed two distinct hydrophobic patches that are critical for effective ComD1 and ComD2 binding. [24] Despite the efforts we have made to understand the SAR of the native CSPs, little is known regarding the mechanism by which the first residue, glutamate, drives receptor activation. An in-depth understanding of the Glu1 interactions that lead to receptor activation could facilitate the design of novel pneumococcal QS modulators with improved potencies.

In 2011, Zhu et al. reported that replacement of Glu1 in CSP1 with alanine resulted in an analog, CSP1-E1A, that competitively inhibits the activity of CSP1 in triggering *comX*, suggesting that Glu1 is critical to receptor activation, but nonessential to receptor binding. [21] In 2012, Duan et al. expended the studies of Glu1 and confirmed that a negative charge at this position is important to receptor activation, since the incorporation of neutral or positively charged side chain residues at this position, such as Gln, Leu, or Arg, resulted in a substantial reduction in bioactivity compared to the incorporation of a different negatively charged residue (Asp). [25] Nevertheless, this limited study did not explore how other parameters such as the size of the side chain residue or the presence and charge of the *N*-terminal amine affect the activity of CSP1. Here, we report a comprehensive study focused on identifying the structural features of the *N*-terminus of CSP1 that are important for receptor activation. To this end, we replaced Glu1 with different proteogenic and non-proteogenic amino acids that vary in both polarity and size. Furthermore, we also modified the functionality and charge of the *N*-terminal amine through either acetylation or the incorporation of a succinyl group. Our results revealed several trends that shed light on the mechanism by which CSP1 activates the ComD1 receptor.

2. Results and discussion

We first set out to assess the effect polarity of the side chain has on the activity of CSP1. To this end, we chose five amino acid residues to replace Glu1 with, all bearing side chain functionalities with similar size: we included amino acids bearing uncharged polar side chain residues (Ser and Asn), amino acids bearing positively or negatively charged side chain residues (2,3-Diaminopropanoic acid (Dap) and Asp), and an amino acid bearing a nonpolar side chain residue (Ala). Comparison of the bioactivities of the resulting analogs revealed that CSP1-E1D, which has a negatively charged side chain at the first position, has the highest potency in inducing *comX* expression (See Fig. 2). This observation is consistent with the study by Duan et al. except that our results show that CSP1-E1D has a potency and maximal induction level of *comX* comparable to CSP1, whereas in Duan's study CPS1-E1D was reported as being able to induce *comX* to levels comparable to only 35% of those observed for CSP1 [25]. The observed difference between the two studies can be attributed to the low concentration of the peptide used in Duan's study as well as the different conditions that were used to conduct the bioassays in the two studies. The other four analogs exhibited significantly lower activity in inducing *comX* expression, reaffirming that a negative charge at the first position is optimal for receptor activation. Nonetheless, the negative charge was not essential to receptor activation, as both CSP1-E1Dap and CSP1-E1N were capable of



CSP1[f] (10.3 nM[a]) CSP1-E1A[f] (85.7 nM[b], 30%[c]) CSP1-E1S (668 nM, 22%)

CSP1-E1N (83.0 nM, 37%[d]) CSP1-E1Dap[e] (71.6 nM, 54%) CSP1-E1D (5.7 nM, 88%)

Fig. 2. Structure and bioactivity of the first set of CSP1 analogs. [a] The concentrations in black are EC_{50} values determined by testing peptides over a range of concentrations. [b] The concentrations in red are IC_{50} values determined by testing peptides over a range of concentrations in the presence of CSP1. [c] The percentage in red is the average of ComD1 activation by CSP1 in the presence of 10 μM inhibitor. [d] The percentage in black is the average of ComD1 activation by 10 μM of the CSP1 analog. [e] Dap stands for 2,3-Diaminopropionic acid. [f] EC_{50}/IC_{50} values taken from Ref. [23]. See the Supporting Information for detail of reporter strains, methods, and plots of agonism and antagonism dose response curves. All assays performed in triplicate.

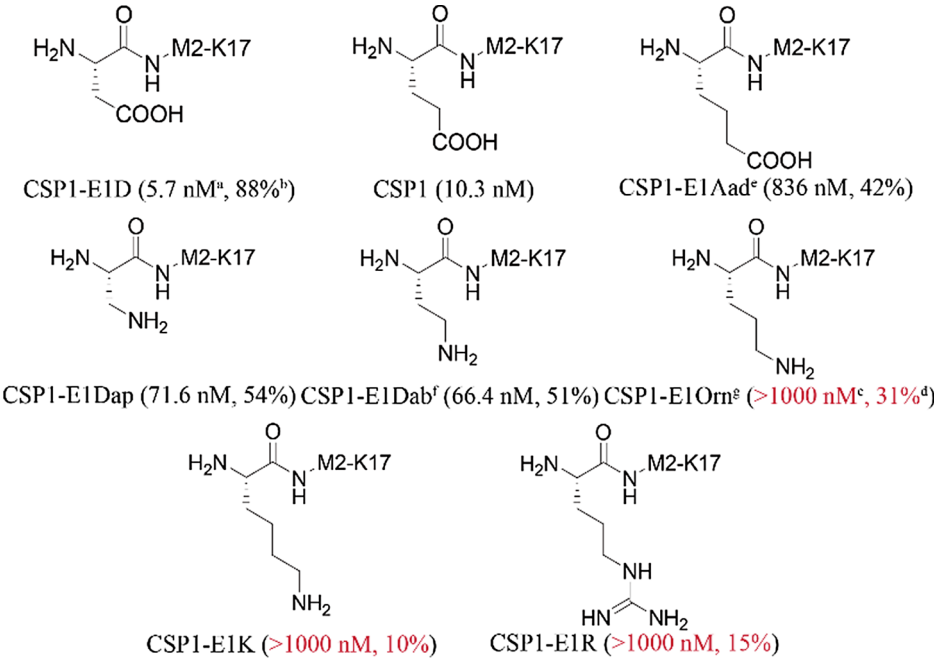


Fig. 3. Structure and bioactivity of the second set of CSP1 analogs. [a] The concentrations in black are EC_{50} values determined by testing peptides over a range of concentrations. [b] The percentage in black is the average of ComD1 activation by 10 μM of the CSP1 analog. [c] The concentrations in red are IC_{50} values determined by testing peptides over a range of concentrations in the presence of CSP1. [d] The percentage in red is the average of ComD1 activation by CSP1 in the presence of 10 μM inhibitor. [e] Aad stands for α-Aminoadipic acid. [f] Dab stands for 2,4-Diaminobutyric acid. [g] Orn stands for Ornithine. See the Supporting Information for detail of reporter strains, methods, and plots of agonism and antagonism dose response curves. All assays performed in triplicate.

inducing *comX* expression: 54% and 37% of maximal induction of *comX* compared to CSP1, respectively (Fig. 2). The last two analogs, CSP1-E1A and CSP1-E1S, failed to activate the ComD receptor but were capable of competitively inhibiting the activity of CSP1 by over 70%, suggesting that both peptides can still bind the receptor effectively. Overall, our results indicate that the presence of a highly polar or charged side chain at the first position is only needed to maintain the ability of the peptide to activate the ComD receptor, but not to bind the receptor.

Next, we set out to assess the effect the size of the side chain has on CSP1 activity. We therefore replaced Glu1 with amino acids that have the same functional groups but vary in side chain length. The first set of analogs included CSP1-E1D, CSP1 (bearing Glu in the first position), and CSP1-E1Aad (Aad, α-Aminoadipic acid), all bearing a carboxyl group on the side chain but varying in the number of methylenes comprising the alkyl side chain (See Fig. 3). CSP1-E1D and CSP1, which have alkyl chains comprised of one and two methylene groups, respectively, displayed similar potency. However, CSP1-E1Aad, which has an alkyl chain comprised of three methylene groups, exhibited a significantly reduced potency (42% of maximal induction of *comX* and over 80-fold reduction in EC_{50} compared to CSP1), suggesting that a larger side chain has a negative effect on the overall activity of the peptide. The second set of analogs included CSP1-E1Dap, CSP1-E1Dab, CSP1-E1Orn and CSP1-E1K, all bearing an amine side chain functionality but again vary in the length of the alkyl chain (See Fig. 3). CSP1-E1Dap and CSP1-E1Dab, which have alkyl chains comprised of one and two methylene groups, respectively, displayed similar activity as partial agonists. However, CSP1-E1Orn and CSP1-E1K, which have longer alkyl chains (three and four carbon chains, respectively), exhibited significant reduction in *comX* activation, but interestingly were both able to competitively inhibit the activity of CSP1, indicating that with a similar polarity profile, a larger side chain is tolerated for initial receptor binding but is devastating for receptor activation. Further validation of this trend was achieved by replacing Glu1 with arginine to afford CSP1-E1R, bearing a bulky positively-charged side chain, which was also observed to competitively inhibit ComD1 activation by CSP1. Lastly, comparison of the two sets of analogs, specifically CSP1-E1D and CSP1 with CSP1-E1Dap and CSP1-E1Dab, all are capable of activating the ComD receptor, further validated the importance of a negatively-charged side chain, since for analogs with the same length of alkyl chain, the analog bearing a negatively-charged side chain exhibited higher potency (Fig. 3). Combined, our results indicate that a less bulky, preferably negatively-charged, side chain at the first position is optimal for receptor activation.

Finally, we examined the function of the *N*-terminal amine in the activity of CSP1. First, we capped the *N*-terminal amine through acetylation. The resulting peptide, Ac-CSP1 was neither able to activate ComD1 nor inhibit the activity of CSP1, indicating that the acetylation leads to complete loss of receptor binding (See Fig. 4). We then added a negatively charged group at the *N*-terminal amine by capping the amine using a succinyl group. The resulting peptide, Succ-CSP1, still could not activate ComD1 or inhibit the activity of CSP1 (See Fig. 4). A third analog, Succ-CSP1-des-E1, whose structure differs from the structure of CSP1-E1D only in the absence of the *N*-terminal amine group, was also unable to activate ComD1 and only exhibited very weak competitive inhibition of CSP1 (See Figs. 2 and 4). Together, these results suggest that the presence of the *N*-terminal amine, likely due to the presence of a positive charge, is critical to receptor binding.

To further test this hypothesis and evaluate the orientation requirements for both the positive and negative charges at the *N*-terminal residue, we designed several CSP1 analogs where the negative and positive charges are flipped, that is the side chain residue bears the positive charge (amine group) while the *N*-terminus bears the negative charge, by capping it using the succinyl group. The resulting analogs: Succ-CSP1-E1Dap, Succ-CSP1-E1Dab, Succ-CSP1-E1Orn, Succ-CSP1-E1K and Succ-CSP1-E1R, displayed improved receptor binding compared to Succ-CSP1, by exhibiting either partial agonism or antagonist activity, confirming our hypothesis that a positive charge at the first residue is required for receptor binding (See Fig. 4). Furthermore, comparison of Succ-CSP1-E1Orn to its non-succinated counterpart, CSP1-E1Orn (See Fig. 3), revealed that the succinated analog exhibit improved receptor activation and is acting as a partial agonist, providing additional validation as to the importance of a negative charge at the *N*-terminus of CSP1 to effective receptor activation.

Interestingly, when comparing the succinated analogs, Succ-CSP1-E1Dap, Succ-CSP1-E1Dab, and Succ-CSP1-E1K, to their non-succinated counterparts, CSP-E1Dab, CSP1-E1Dap, and CSP1-E1K, it appears, based on the maximal percent inhibition, that the succinated analogs have weaker receptor binding affinities compared to the non-succinated counterparts. This observation suggests that the *N*-terminal amine and

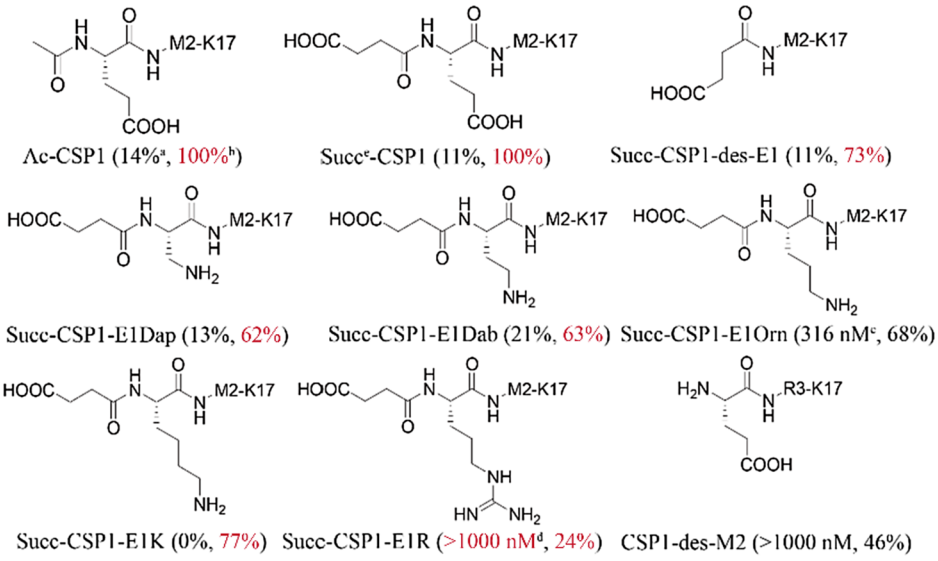


Fig. 4. Structure and bioactivity of the third set of CSP1 analogs. [a] The percentage in black is the average of ComD1 activation by 10 μM of the CSP1 analog. [b] The percentage in red is the average of ComD1 activation by CSP1 in the presence of 10 μM inhibitor. [c] The concentrations in black are EC_{50} values determined by testing peptides over a range of concentrations. [d] The concentrations in red are IC_{50} values determined by testing peptides over a range of concentrations in the presence of CSP1. [e] Succ stands for succinyl group. See the Supporting Information for detail of reporter strains, methods, and plots of agonism and antagonism dose response curves. All assays performed in triplicate.

the side chain of Glu1 have localized interactions with the ComD receptor that do not accommodate drastic positioning changes. The positioning requirement was further validated when we tested an additional CSP1 analog, CSP1-des-M2, lacking the Met2 residue, which we have previously reported to be dispensable. Although CSP1-des-M2 has the same *N*-terminal amine and negatively charged side chain at the first residue as CSP1, this analog exhibited significantly reduced activity compared to CSP1 (See Fig. 4). Moreover, both Duan et al. and our lab have shown that the replacement of Glu1 in CSP1 with its stereoisomer, D-Glu, resulted in a peptide, CSP1-e1, that could neither activate the ComD receptor nor inhibit receptor activation by CSP1. [23,25] Together, these results suggest that the interactions between the Glu1 residue, specifically the *N*-terminal amine and the negatively charged side chain, and the ComD receptor are strictly localized and therefore positional changes of these key functionalities in CSP1 may result in the loss of critical interactions with the ComD receptor or introduce steric clashes that damage these interactions.

In conclusion, by systematically modifying the side chain and the *N*-terminal amine of the first residue in CSP1, we revealed some trends that may shed light on the mechanism by which the Glu1 residue interacts with and activates the ComD1 receptor. First, the side chain is critical to the ability of the peptide to activate the receptor but is not required for the peptide to bind the receptor. Specifically, a relatively small, negatively charged side chain is optimal for receptor activation. However, other highly polar functionalities can also achieve receptor activation, although to a lesser degree. Second, the *N*-terminal amine, specifically the presence of a positive charge, is critical to receptor binding. Though a positive charge on either the *N*-terminus or the side chain functionality is sufficient to retain receptor binding, optimal binding is achieved when the positive charge is positioned at the *N*-terminus in the form of a free amine. Lastly, the interactions between the Glu1 residue, both side chain and *N*-terminal functionalities, and the ComD receptor are highly localized and cannot accommodate significant positional changes to the Glu1 functionalities. The improved understanding of the CSP1:ComD1 interactions that drive receptor activation could be applied to design novel *S. pneumoniae* QS modulators with enhanced potencies.

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Notes

The authors declare no competing financial interest.

Appendix A. Supplementary material

Supplementary data to this article including details of peptide synthesis and characterization, Beta-Galactosidase assays, and dose-response curves can be found online at https://doi.org/10.1016/j.bioorg.2019.102987.

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