Identification of Highly Potent Competence Stimulating Peptide-Based Quorum Sensing Activators in *Streptococcus mutans* Through the Utilization of *N*-Methyl and Reverse Alanine Scanning

Chowdhury Raihan Bikash, and Yftah Tal-Gan*

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

Abstract

Quorum sensing (QS) controls the pathogenic behavior of *Streptococcus mutans*, a primary cause of dental caries. *S. mutans* uses the competence stimulating peptide (CSP) to control mutacin production, a bacteriocin utilized by *S. mutans* to outcompete different commensal bacteria in mixed biofilm environments. In this study, we performed an *N*-methyl scan of an 18-CSP-based scaffold lacking the first two amino acid residues that were shown to be dispensable, to gain important mechanistic insight as to the role of backbone amide protons in the interaction between CSP and the ComD receptor. We then utilized the reverse alanine approach to develop CSP-based analogs with enhanced activities. The two most potent analogs were found to induce bacteriocin production at sub-nanomolar concentration using an interspecies inhibition assay. Overall, our analysis revealed that the 18-CSP sequence is not optimized and can be improved by replacement of multiple positions with alanine. Our results further suggest that the hydrophobic residues in *S. mutans* 18-CSP are involved in both receptor binding and activation.

Keywords

Quorum sensing; *Streptococcus mutans*; Competence Stimulating Peptide (CSP); Bacteriocin production.

* To whom correspondence should be addressed. ytalgan@unr.edu

Streptococcus mutans, an oral Gram-positive human pathogen, plays a major role in dental caries.[1] *S. mutans* forms robust biofilms and utilizes an impressive arsenal of anti-bacterial agents to eliminate competing bacterial species during oral colonization. Through carbohydrate metabolism, *S. mutans* produces and secretes lactic acid, resulting in acidic micro-environment within the biofilms that suppresses the growth of non aciduric commensal bacteria such as *Streptococcus sanguinis* and *Streptococcus gordonii*.[2, 3] Additionally, *S. mutans* utilizes cationic bacteriocins, termed mutacins, to lyse competing bacterial species and acquire foreign genetic material.[4, 5] To this end, *S. mutans* uses a conserved quorum sensing (QS) system, a cell-density dependent gene regulation method,[6, 7] termed the competence regulon. The competence regulon in *S. mutans* regulates biofilm formation, stress response, mutacin production, and the development of genetic competence (a process that allows an organism to internalize DNA from its environment), all of which are essential processes for *S. mutans* to thrive in the oral cavity.[8] *S. mutans* uses two peptide pheromones: the competence stimulating peptide (CSP) and SigX inducing peptide (XIP) as QS regulators of the competence regulon.[9, 10] The CSP signal regulates biofilm formation and mutacin production, while the XIP signal induces genetic competence.[10, 11] In the ComABCDE pathway, *comC* encodes a propeptide, ComC, that is cleaved and secreted by a dedicated ABC transporter, ComAB, to generate a 21-mer peptide (21-CSP). A membrane-localized protease, SepM, further cleaves 21-CSP at the *C*-terminus to form the active 18-CSP signal. 18-CSP then binds to the cognate transmembrane histidine kinase receptor, ComD, resulting in the activation of a cytoplasmic response regulator, ComE, through phosphorylation. Activated ComE upregulates the *comABCDE* genes and stimulates the production of mutacins, biofilm formation, and indirectly competence development (**Figure 1**).[12] Thus, targeting the competence QS circuitry, specifically the CSP:ComD interaction, could lead to attenuation of the infectivity of *S. mutans*.

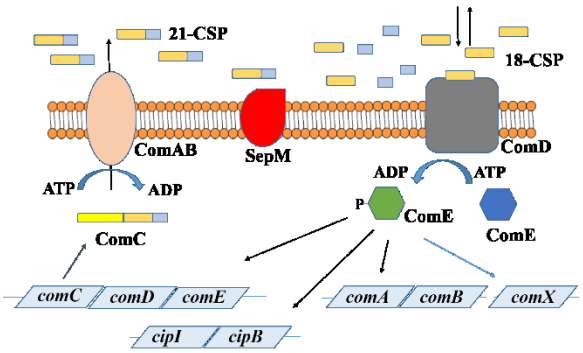


Figure 1. QS circuit in *S. mutans*. ComC, the 21-CSP propeptide, is processed and transported through the ABC transporter, ComAB. 21-CSP is further processed by SepM, a membrane protease, to form the active 18-CSP. 18-CSP binds to the cognate transmembrane histidine kinase ComD receptor, leading to activation of ComE, a response regulator. Activation of ComE results in autoinduction of the *S. mutans* QS circuitry genes and leads to upregulation of QS-related phenotypes. Blue arrow indicates the indirect activation of *comX* by ComE.

In our previous study, we have conducted systematic structure-activity relationship (SAR) studies of both the 21-CSP and 18-CSP scaffolds and identified important structural features of CSPs that are important for 21-CSP:SepM and 18-CSP:ComD interactions.[13] Furthermore, our SAR analysis revealed the minimal sequence of 18-CSP that is required for effective ComD binding and activation (18-CSP-des-S1G2). In this study, we set to (1) investigate the role of amide protons in 18-CSP:ComD interaction; and (2) design CSP-based QS modulators with enhanced activities. To this end, we performed a full *N*-methyl scan of the 18-CSP-des-S1G2 scaffold as well as utilized the reverse alanine approach on 18-CSP-des-S1, the most potent truncated analog of 18-CSP. Our results revealed two highly potent QS activators that are approximately 8-fold more potent than the native 18-CSP signal. Lastly, we utilized a phenotypic interspecies inhibition assay to validate the enhanced activities of our two most potent CSP analogs.

***N*-methyl Scan.** In Our initial SAR study we reported that the first two residues of 18-CSP (**P1**) were dispensable, resulting in 18-CSP-des-S1G2 (**P2**) exhibiting similar potency to **P1** (**Table 1**).[13] Therefore, in this study, we used **P2** as our starting scaffold to investigate additional structural features that are required for effective ComD binding and activation, namely the role of amide protons. To this end, we conducted a full *N*-methyl scan of **P2** and evaluated the backbone-modified CSP analogs for their ability to modulate QS in *S. mutans*. The peptides were first screened for their ability to activate the ComD receptor at high concentration (10 μM, **Figure S-1**). EC_{50} values were determined for peptides that exhibited high induction in the initial screening (>75% compared to **P1**, **Table 1**), whereas peptides that exhibited low induction in the initial screening (<50% compared to **P1**) were further evaluated for their ability to competitively inhibit ComD activation by **P1** (**Figure S-3**). The initial screening revealed that none of the peptides were capable of inhibiting QS. Moreover, with the exception of positions Q17 and A18, modification to the central or *C*-terminal regions (positions R9 – T16) resulted in complete loss of activity. Interestingly, position Q17 exhibited higher tolerance toward *N*-methylation (76-fold reduction in potency) whereas A18 exhibited only a modest reduction in potency (9-fold change; **Table 1**). When comparing the results of the *C*-terminus to those of the *N*-terminus, a similar trend appears: that is, the end residues are most tolerant toward modification (S3 and A18, 0.85 and 9 fold change, respectively) and as we move toward the central region of the peptide, *N*-methylation becomes less tolerated (L4 and Q17, 5.7 and 76 fold change, respectively; S5 and T16, 19-fold change and complete loss of activity, respectively). Combined, these results suggest that the activity change is due to a conformational restriction or change, as opposed to loss of direct binding interaction (H-bonds). The closer the modification is to the ends, the smaller the conformational restriction is. Finally, when comparing the results of the *N*-terminus region (S3 – F8) to that of the *C*-terminus region (R9 – A18), it is clear that the *C*-terminus plays a more significant role in binding the ComD receptor, as modifications in this region resulted in more significant reduction in potency. This trend is in agreement with our previous SAR data.[13]

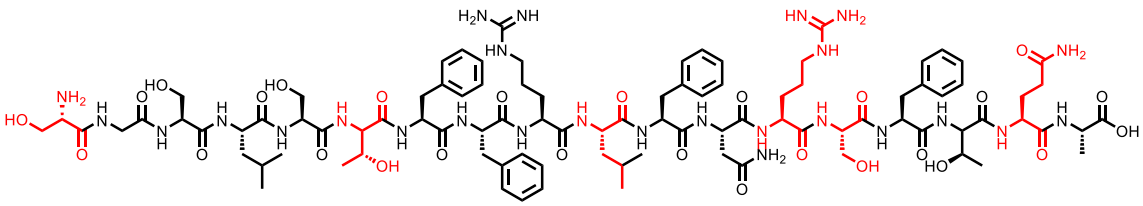
Table 1. EC_{50} values of the *N*-methyl 18-CSP-des-S1G2 analogs against the ComD receptor[a]
18-CSP: SGSLSTFFRLFNRSFTQA

Name	Percent activation	EC_{50} (nM)[b]	95% CI[c]	Fold change[d]
18-CSP[13] (**P1**)	100%	6.2	3.7 – 10.0	
18-CSP-des-S1G2[13] (**P2**)	100%	7.9	4.1 – 15	
18-CSP-des-S1G2-*N*-Me-S3 (**P3**)	97%	6.7	3.2 – 14	0.85
18-CSP-des-S1G2-*N*-Me-L4 (**P4**)	89%	45	36 – 56	5.7
18-CSP-des-S1G2-*N*-Me-S5 (**P5**)	81%	150	100 – 200	19
18-CSP-des-S1G2-*N*-Me-T6 (**P6**)	81%	>1,000	--	--
18-CSP-des-S1G2-*N*-Me-F7 (**P7**)	88%	760	600 – 950	96
18-CSP-des-S1G2-*N*-Me-F8 (**P8**)	86%	170	96 – 310	22
18-CSP-des-S1G2-*N*-Me-R9 (**P9**)	45%	--[e]	--	--
18-CSP-des-S1G2-*N*-Me-L10 (**P10**)	29%	--[e]	--	--
18-CSP-des-S1G2-*N*-Me-F11 (**P11**)	0%	--[e]	--	--
18-CSP-des-S1G2-*N*-Me-N12 (**P12**)	11%	--[e]	--	--
18-CSP-des-S1G2-*N*-Me-R13 (**P13**)	5%	--[e]	--	--
18-CSP-des-S1G2-*N*-Me-S14 (**P14**)	12%	--[e]	--	--
18-CSP-des-S1G2-*N*-Me-F15 (**P15**)	17%	--[e]	--	--
18-CSP-des-S1G2-*N*-Me-T16 (**P16**)	38%	--[e]	--	--
18-CSP-des-S1G2-*N*-Me-Q17 (**P17**)	100%	600	350 – 1000	76
18-CSP-des-S1G2-*N*-Me-A18 (**P18**)	87%	71	49 – 100	9.0

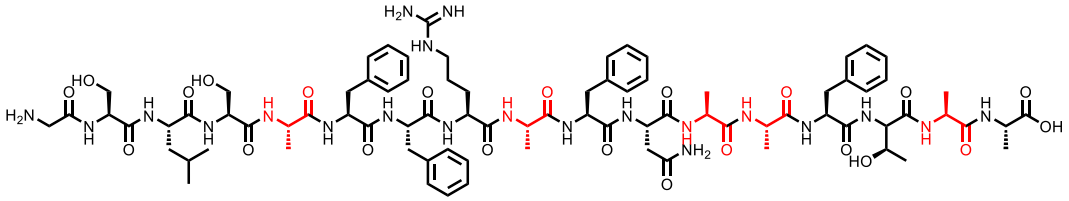
[a] See experimental section for details on reporter strain. See supporting information for methods, primary screening assay results and plots of agonism dose response curves. All assays were performed in triplicate. [b] EC_{50} values determined by testing peptides over a range of concentrations. [c] 95% confidence interval. [d] Ratio where each analog's EC_{50} is divided by **P2** EC_{50}; a value <1 indicates a better activator than the parent **P2**. [e] EC_{50} not determined due to the analog's low induction in primary agonism screening assay.

Reverse Alanine Scan. Our previous SAR study of **P1** revealed that the hydrophobic residues at the central region of the peptide are critical for ComD receptor binding.[13] Moreover, we could not identify one residue in **P1** that is critical for receptor activation, meaning that mutating this residue leads to CSP-based competitive inhibitors. We reasoned that an analog, bearing only the minimally required residues for effective binding, might lose its ability to activate the ComD receptor and would thus exhibit inhibitory properties. We therefore decided to utilize the reverse alanine approach and designed a series of five **P1** analogs where only the critical residues are maintained, while the other residues are mutated to alanine. For this set of analogs, we chose to use the 18-CSP-des-S1 (**P19**) scaffold, as this scaffold was found to exhibit a slightly increased potency compared to **P1** (0.77-fold change; **Table 2**).[13] Within the series, starting with 18-CSP-des-S1-S5AT6AR9AL10AR13AS14AQ17A (**P24**) as the minimal core, in each of the analogs we included back additional residues from the original sequence (**Table 2**). Unexpectedly, all five peptides were found to activate the ComD receptor (**Figure S-2** and **Table 2**). Importantly, with the exception of **P24**, all the analogs exhibited increased activity compared to the parent **P19** scaffold. Specifically, two analogs, 18-CSP-des-S1-T6AL10AR13AS14A (**P21**) and 18-CSP-des-S1-T6AL10AR13AS14AQ17A (**P23**), exhibited a 6-fold increase in potency compared to **P19** and an 8-fold increased activity compared to the native **P1** (**Figure 2**). Combined, these results suggest that the *S. mutans* CSP has a significantly different mechanism of action compared to its *S. pneumoniae* counterpart, and that in the *S. mutans* case, the hydrophobic residues are involved in

both receptor binding and activation, as opposed to the *S. pneumoniae* CSP, where the hydrophobic residues are only involved in receptor binding, while Glu1 is driving receptor activation.[14-17]



P1: SGSLSTFFRLFNRSFTQA



P23: GSLSAFFRAFNAAFTAA

Figure 2. Structural representation of the native 18-CSP (**P1**) and the optimized CSP analog 18-CSP-des-S1-T6AL10AR13AS14AQ17A (**P23**) with the modified residues highlighted in red.

Table 2. EC_{50} values of the reverse alanine 18-CSP-des-S1 analogs against the ComD receptor[a]
18-CSP: SGSLSTFFRLFNRSFTQA

Name	EC_{50} (nM)[b]	95% CI[c]	Fold change[d]
18-CSP[13] (**P1**)	6.2	3.7 – 10.0	
18-CSP-des-S1[13] (**P19**)	4.8	2.3 – 10.0	
18-CSP-des-S1-L10AR13AS14A (**P20**)	1.7	1.3 – 2.1	0.35
18-CSP-des-S1-T6AL10AR13AS14A (**P21**)	0.82	0.53 – 1.3	0.17
18-CSP-des-S1-T6AR9AL10AR13AS14A (**P22**)	4.2	2.6 – 7.1	0.87
18-CSP-des-S1-T6AL10AR13AS14AQ17A (**P23**)	0.80	0.59 – 1.1	0.17
18-CSP-des-S1-S5AT6AR9AL10AR13AS14AQ17A (**P24**)	9.2	4.1 – 20.5	1.9

[a] See experimental section for details on reporter strain. See supporting information for methods, primary screening assay results and plots of agonism dose response curves. All assays were performed in triplicate. [b] EC_{50} values determined by testing peptides over a range of concentrations. [c] 95% confidence interval. [d] Ratio where each analog's EC_{50} is divided by **P19** EC_{50}; a value <1 indicates a better activator than the parent **P19**.

Interspecies Inhibition Assay. *S. mutans* uses CSP signaling to control its bacteriocin production during competition with commensal oral streptococci. We therefore set to utilize bacteriocin production as a phenotypic marker to test the ability of our most potent CSP-based analogs, **P21** and **P23**, to activate QS. To this end, we used an interspecies inhibition assay between *S. mutans* and *Streptococcus anginosus*. *S. anginosus* ATCC 33397 was spread plated on a THY agar plate, then wells were created and *S. mutans* SMCC3 (*ΔcomC*), treated with various concentrations (100, 10, 5, 1, 0.25 and 0.1 nM) of either **P1**, **P21**, **P23**, **P11**, **P13** or DMSO (negative control), was added in triplicate. The plate was incubated for 24 h followed by visual inspection of zones of inhibition (**Figure S-4** and **S-5**). Without the addition of **P1** or its active analogs (**P21** or **P23**), or

with the addition of inactive CSP analogs (**P11** or **P13**), SMCC3 was unable to inhibit the growth of *S. anginosus*. At high CSP concentrations (100 to 5 nM), a clear zone of inhibition was observed for all the active CSP analogs. However, at low CSP concentrations, a smaller (1 nM) to no zone of inhibition (0.25 nM and 0.1 nM) was observed for **P1**. Consistent with our reporter assay results, **P21** and **P23** exhibited superior activities at these concentrations with an observed reduction in the zone of inhibition only at 0.25 nM (**Figure 3** and **S-4**). Overall, the interspecies inhibition assay validated the enhanced potencies of **P21** and **P23** at inducing QS in *S. mutans*. To our knowledge, these CSP analogs are the most potent QS activators known to date and can be used as chemical tools to study the role of QS in interspecies interactions between oral streptococci.

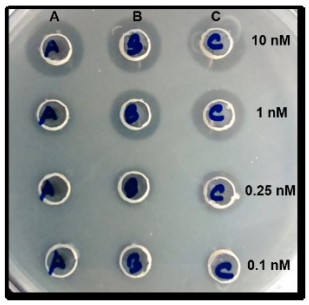


Figure 3. Interspecies inhibition assay between *S. mutans* and *S. anginosus*. *S. mutans* SMCC3 (Δ*comC*) was tested for its ability to inhibit the growth of *S. anginosus* ATCC 33397 when SMCC3 was first treated with varying concentrations (10, 1, 0.25, 0.1 nM) of: (A) **P1**, (B) **P21**, or (C) **P23**. A reduced zone of inhibition is observed for **P1** (A) at 1 nM compared to (B) and (C). No zone of inhibition is observed for **P1** (A) at 0.25 nM, while only a reduced zone of inhibition is observed for (B) and (C) at the same concentration.

Dental caries, a growing and costly human disease remains a prevalent global health concern.[18] *S. mutans*, a major contributor to dental caries, utilizes the CSP-mediated QS circuit to produce mutacins during interspecies competition with commensal oral streptococci. In this study, we report a systematic SAR analysis of the 18-CSP signal, with an emphasis on the role of amide protons in the interaction between the CSP signal and the ComD receptor. Our results indicate that *N*-methylation at the peptide termini is more tolerated than *N*-methylation at the central region, likely due to reduced conformational restriction. Moreover, the use of the reverse alanine approach revealed that the 18-CSP sequence is not optimized and can be improved by replacement of multiple positions with alanine. The reverse alanine results further suggest that the hydrophobic residues in *S. mutans* 18-CSP are involved in both receptor binding and activation, as opposed to the CSP signals in *S. pneumoniae*, where the hydrophobic residues play a role only in receptor binding. Lastly, an interspecies phenotypic assay was used to validate and highlight the improved potencies of **P21** and **P23**, the most potent *S. mutans* QS modulators reported to date.

Acknowledgements

This work was supported by grants from the National Institutes of Health (R35GM128651), the National Science Foundation (CHE-1808370), and by the Nevada INBRE through a grant from the NIH (GM103440). The *S. mutans* SAB249 (pCipB::*lacZ*) reporter strain was a generous gift

from R. A. Burne (University of Florida). The *S. mutans* SMCC3 (*ΔcomC*) strain was a generous gift from D. G. Cvitkovitch (University of Toronto). The *S. anginosus* ATCC 33397 strain was a generous gift from L. McGee (CDC *Streptococcus* Lab).

References:

1. Aas JA, Griffen AL, Dardis SR, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. *J Clin Microbiol.* 2008;46(4): 1407-1417.
2. Marsh PD, Moter A, Devine DA. Dental plaque biofilms: communities, conflict and control. *Periodontology 2000.* 2011;55(1): 16-35.
3. Moye ZD, Son M, Rosa-Alberty AE, et al. Effects of carbohydrate source on genetic competence in Streptococcus mutans. *Appl Environ Microbiol.* 2016;82(15): 4821-4834.
4. Kreth J, Merritt J, Shi W, Qi F. Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. *Mol Microbiol.* 2005;57(2): 392-404.
5. Merritt J, Qi F. The mutacins of Streptococcus mutans: regulation and ecology. *Mol Oral Microbiol.* 2012;27(2): 57-69.
6. Miller MB, Bassler BL. Quorum sensing in bacteria. *Annu Rev Microbiol.* 2001;55(1): 165-199.
7. Ng W-L, Bassler BL. Bacterial quorum-sensing network architectures. *Annu Rev Genet.* 2009;43: 197-222.
8. Senadheera D, Cvitkovitch DG. Quorum sensing and biofilm formation by Streptococcus mutans. *Bacterial Signal Transduction: Networks and Drug Targets.* Springer; 2008:178-188.
9. Son M, Ahn SJ, Guo Q, Burne RA, Hagen SJ. Microfluidic study of competence regulation in Streptococcus mutans: environmental inputs modulate bimodal and unimodal expression of comX. *Mol Microbiol.* 2012;86(2): 258-272.
10. Mashburn-Warren L, Morrison DA, Federle MJ. A novel double-tryptophan peptide pheromone controls competence in Streptococcus spp. via an Rgg regulator. *Mol Microbiol.* 2010;78(3): 589-606.
11. Khan R, Rukke HV, Høvik H, et al. Comprehensive transcriptome profiles of Streptococcus mutans UA159 map core Streptococcal competence genes. *Msystems.* 2016;1(2): e00038-00015.
12. Hossain MS, Biswas I. An extracelluar protease, SepM, generates functional competence-stimulating peptide in Streptococcus mutans UA159. *J Bacteriol.* 2012;194(21): 5886-5896.
13. Bikash CR, Hamry SR, Tal-Gan Y. Structure–Activity Relationships of the Competence Stimulating Peptide in Streptococcus mutans Reveal Motifs Critical for Membrane Protease SepM Recognition and ComD Receptor Activation. *ACS Infect Dis.* 2018;4(9): 1385-1394.
14. Yang Y, Koirala B, Sanchez LA, Phillips NR, Hamry SR, Tal-Gan Y. Structure–Activity Relationships of the Competence Stimulating Peptides (CSPs) in Streptococcus pneumoniae Reveal Motifs Critical for Intra-group and Cross-group ComD Receptor Activation. *ACS Chem Biol.* 2017;12(4): 1141-1151.
15. Yang Y, Cornilescu G, Tal-Gan Y. Structural Characterization of Competence-Stimulating Peptide Analogues Reveals Key Features for ComD1 and ComD2 Receptor Binding in Streptococcus pneumoniae. *Biochemistry.* 2018;57(36): 5359-5369.

16. Koirala B, Lin J, Lau GW, Tal-Gan Y. Development of a Dominant Negative Competence Stimulating Peptide (dnCSP) that Attenuates Streptococcus pneumoniae Infectivity in a Mouse Model of Acute Pneumonia. *ChemBioChem.* 2018: In press.
17. Zhu L, Lau GW. Inhibition of competence development, horizontal gene transfer and virulence in Streptococcus pneumoniae by a modified competence stimulating peptide. *PLoS Pathog.* 2011;7(9): e1002241.
18. Pepperney A, Chikindas ML. Antibacterial peptides: opportunities for the prevention and treatment of dental caries. *Probiotics Antimicrob Proteins.* 2011;3(2): 68.

TOC