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RESEARCH HIGHLIGHT

Structural and functional insights into a quorum-sensing signal peptide receptor, the ComD histidine protein kinase of *streptococcus mutans*

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Quorum sensing activation by signal peptide pheromones (SP) in Gram-positive bacteria depends on a membrane-associated histidine kinase receptor, which senses the signal and triggers the signaling cascade for various cell density-dependent activities. However, relatively little is known of peptide pheromone-receptor interactions in these bacteria, largely because of technical challenges in working with membrane-associated proteins in these bacteria. Recently, we have described a genetic approach and several analysis methods to studying membrane topology and structure-function interaction of a quorum sensing pheromone receptor ComD in a Gram-positive bacterium *Streptococcus mutans*. Using these methods, we confirm that the membrane-spanning domain of the ComD protein forms six transmembrane segments and three extracellular loops, loopA, loopB and loopC. By mutational analyses of these three extracellular loops, we demonstrate that both loopC and loopB are required for signal recognition and quorum sensing activation, while loopA plays little role in signal detection. In particular, a deletion or substitution mutation of four residues NVIP within loopC abolishes signal recognition for quorum sensing activation. Consistent with these findings, the loopC and loopB mutants are completely or partially defective in bacteriocin production. We conclude that both loopC and loopB are required to form the signal peptide receptor and the residues NVIP of loopC are essential for signal recognition and quorum sensing activation in *S. mutans*.

Keywords: quorum sensing; signal peptide pheromone receptor; histidine protein kinase; membrane topology; *phoA-lacZ* dual fusion reporter; streptococci

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Bacteria in nature are frequently exposed to various threats such as stressful environments, nutrient starvation or killing by antimicrobial compounds [1]. To survive these threats, bacteria must be able to sense, respond and cope with such life-threatening challenges in natural environments or in the hosts. Two-component signal transduction systems (TCSTSs) are the most prevalent form of signal transduction mechanisms that allow bacteria to sense, respond and adapt these threats [2-4]. Completed microbial genomes show that each bacterial genome contains many TCSTS systems, which

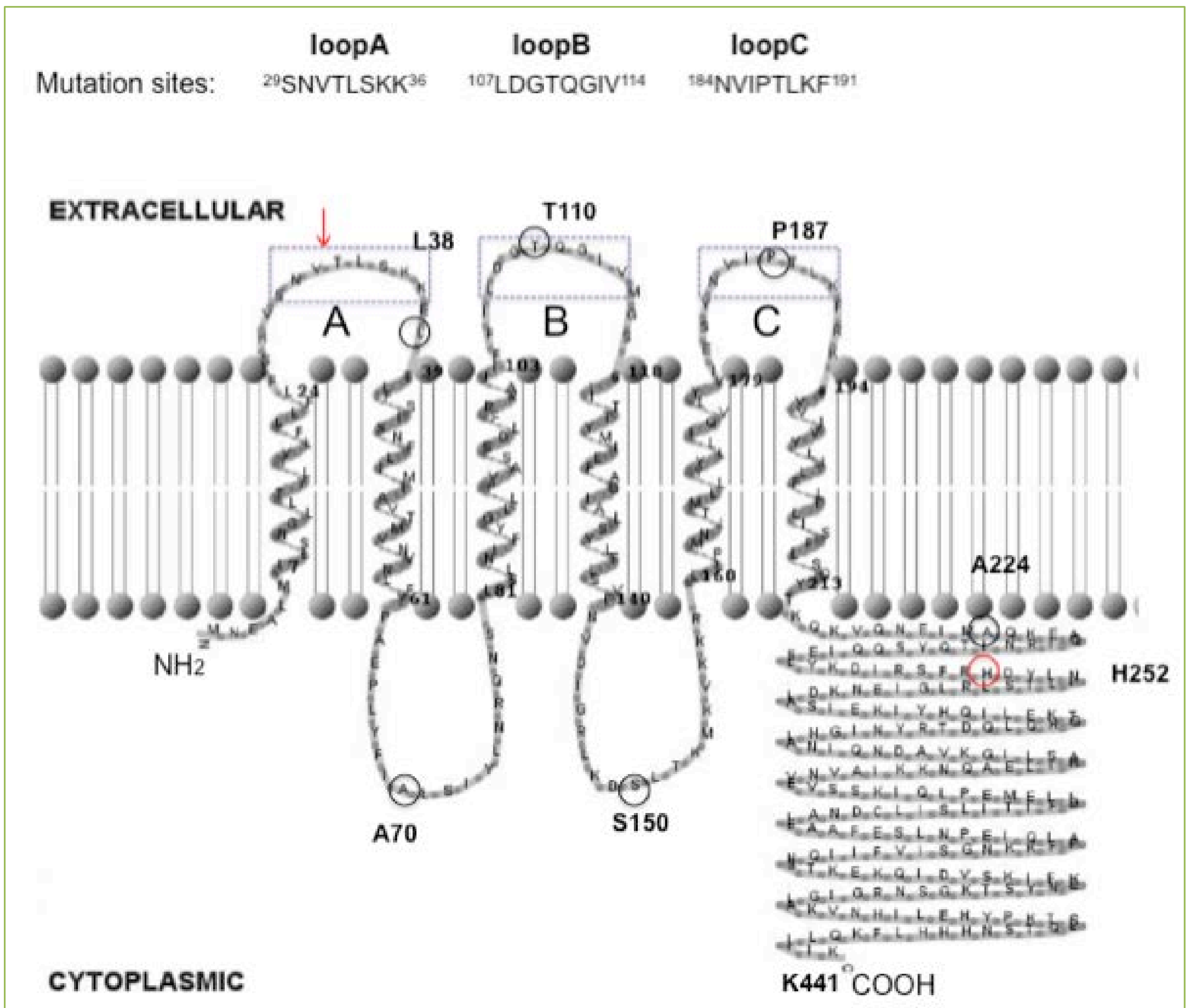

Figure 1. A hypothetical topology model of the ComD receptor protein in *S. mutans*. The membrane-spanning domain of ComD protein from *S. mutans* UA159 is predicted to form six transmembrane segments (TMSs) with three extracellular loops, loopA, loopB and loopC, and two intracellular loops. Small open circles indicate insertion locations by a dual *phoA-lacZ* fusion reporter in frame after selected codons corresponding to the amino acid residues L38, A70, T110, S150, P187 and A224. Open rectangles indicate the amino acid residues of loopA, loopB and loopC involved in the construction of in-frame deletion or substitution mutants (the precise amino acid residues at mutation sites are indicated above). The conserved histidine residue (His252) in the *C*-terminal domain of the ComD protein inside the cytoplasm is also indicated.

play important roles in signal transduction for various physiological processes, stress adaptation and virulence [4-6]. Many TCSTSs have been found to function as global regulators by initiating signaling cascades, in which large sets of genes can be switched on or off [4]. These signal transduction systems provide the major means by which bacteria communicate with each other and the outside world. Although many TCSTSs are identified to regulate diverse physiological activities, relatively little is known of how a given TCSTS recognizes its extracellular signal and initiates the signaling cascade for gene expression. In many cases, signals sensed by a TCSTS are chemically undefined, so that signal recognition mechanisms of many TCSTSs remain poorly understood. Among various TCSTSs in bacteria, however, quorum-sensing signal pheromones (SP) are the best-studied signal molecules that can be specifically sensed by their cognate histidine kinase receptor proteins [7]. These signal transduction systems may provide an excellent opportunity to study signal molecule-receptor interactions in bacteria. To advance our understanding of these interactions, we recently investigated the membrane topology and functionality of such a peptide pheromone receptor ComD in

a Gram-positive bacterium, *Streptococcus mutans*, the primary cariogenic pathogen in human dental caries [8].

In Gram-positive bacteria, most known peptide pheromone receptors fall into the HPK_{10} subfamily, which includes AgrC from *Staphylococcus* [9], ComD from *Streptococcus* [10] and PlnB from *Lactobaccillus* [11]. These signal pheromone-activated histidine protein kinases, which regulate competence development, bacteriocin production and virulence factors, have been widely studied with the respect to the events following phosphorylation of their cognate response regulators [12, 13]. However, relatively little is known of quorum sensing signal molecule-receptor interactions. No report has directly described ComD receptor kinase proteins, which are widely distributed among the members of the Genus *Streptococcus* [7, 8, 10]. Based on known peptide pheromone receptors [3, 6, 10], membrane-spanning regions of the HPK_{10} subfamily consist usually of 5-8 transmembrane segments (TMSs). As the first step, we obtained a hypothetical topology model of ComD protein from *S. mutans* strains by using several topology prediction methods [8]. With these methods, we predicted that the membrane-spanning region of ComD protein of *S. mutans* forms six transmembrane segments (TMSs) with three extracellular loops and two intracellular loops (**Fig. 1**). To validate this topology model, we constructed six *comD-phoA-lacZ* dual fusion reporters, which represented six in-frame insertion sites (L38, A70, T110, S150, P187, A224) of the membrane-spanning region of the ComD protein. We then examined these fusion strains for the reporter activities by growing them on LB agar plates containing dual indicators. One was a blue chromogenic substrate (X-Phos) to allow detection of phosphatase activity (PhoA) and another was a red chromogenic substrate (Salman-Gal) that allows detection of β-galactosidase activity (LacZ). The strains expressing $ComD_{L38}$-Pho/Lac (GF-L38), $ComD_{T110}$-Pho/Lac (GF-T110) and $ComD_{P187}$-Pho/Lac (GF-P187) exhibited the higher levels of PhoA activity (blue color), which indicate their extracellular location of the reporter fusion points. The strains expressing $ComD_{A70}$-Pho/Lac (GF-A70), $ComD_{S150}$-Pho/Lac (GF-S150) and $ComD_{A225}$-Pho/Lac (GF-A224) exhibited the higher levels of LacZ activity (pink color), which indicate their cytosolic location of the reporter fusion points. The results from this dual reporter system clearly confirmed the topology of the ComD receptor protein as predicted by the hypothetical model, validating the membrane locations of extracellular loopA, loopB and loopC of the ComD protein.

Upon the establishment of the membrane topology of ComD protein, we further explored the contribution of extracellular loopA, loopB or loopC on signal recognition and quorum-sensing activation, since the data obtained may have important implications for ligand-receptor interaction and design of quorum sensing inhibitors. To test the hypothesis that the ComD extracellular loops might act as the receptor for SP recognition, we genetically constructed three sets of *S. mutans* strains that allowed us to investigate the effects of individual extracellular loop, loopA, loopB or loopC, on SP recognition and quorum sensing activation. The first set of the strains included a series of in-frame deletion mutants or alanine substitution mutation mutants. These mutants and control strains (both $ComD^+$ and $ComD^-$ controls) were then transformed with a *luxAB* reporter plasmid, generating the second set of *lux* reporter strains. These reporter strains allowed us to examine SP-activated quorum sensing activities by luciferase reporter assays. However, one of the major concerns in mutational analysis of the ComD protein was whether a partial deletion or mutation of these extracellular loops affected translocation or insertion of the mutant proteins into the membrane. It was important to track the mutant proteins in the membrane fractions of these strains, otherwise, it would be difficult to interpret the results regarding the effects of a deletion or point mutation of one of these extracellular loops on SP recognition. To resolve this problem, we constructed the third set of the *S. mutans* strains that constitutively expressed a His-tagged mutant loopA, loopB or loopC *in trans* to detect and monitor the mutant ComD proteins by Western blotting. The results from Western blot analysis revealed that all the mutants contained a strong reactive band (≈ 51-kDa) in their membrane fractions, except the *comD* deletion mutant ($ComD^-$). The positive reactive bands in these strains were consistent with the size (50.5 kDa) of the wild type ComD protein (ComD+). The results suggest that a point deletion or substitution mutation of the extracellular loops does not affect translocation or insertion of these mutant proteins into the cytoplasmic membrane. Thus, all the mutants and control strains constructed should be valid for evaluating the effects of a deletion or mutation of one of these extracellular loops on SP recognition and quorum sensing activation in *S. mutans*.

Next, we determined whether a deletion or mutation of loopA, loopB or loopC affected SP recognition and quorum sensing activation by examining the lux reporter strains in response to SP. All the reporter strains were constructed in the mutant backgrounds as well as control strains ($ComD^+$ and $ComD^-$) by transforming one of *luxAB* reporter plasmids, pGF-PcipB and pGF-PnlmAB, respectively. Therefore, each reporter strain carried a shuttle vector that contained the promoterless *luxAB* fused to a SP-inducible promoter of two bacteriocin-encoding genes, *cipB* and *nlmAB* in *S. mutans* [14]. These two bacteriocin-encoding genes were chosen for constructing the reporter strains, because their promoters (P*cipB* and P*nlmAB*) contain the consensus ComE binding

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                           TMS1                 loopA                  TMS2
UA159  MNEALMILSNGLLTYLTVLFLLFLFSKVSNVTLSKKELTLFSISNFLIMIAVTMVNVNLF
GS-5   ------------------------------------------------------------
KK23   ------------------------------------------------------------
R221   ------------------------------------------------------------

                                      TMS3                 loopB
Ua159  YPAEPLYFIALSIYLNRQNSLSLNIFYGLLPVASSDLFRRAIIFFILDGTQGIVMGSS
GS-5   -------------------------------------------------N-----D--
KK23   -------------------------------------------------N-----D--
R221   -------------------------------------------------N-----D--

                 TMS4                                      TMS5
UA159  IITTYMIEFAGIALSYLFLSVFNVDIGRLKDSLTKMKVKKRLIPMNITMLLYYLLIQV
GS-5   ----------------------------------------------------------
KK23   ----------------------------------------------------------
R221   ----------------------------------------------------------

              loopC                   TMS6
UA159  LYVIESYNVIPTLKFRKFVVIVYLILFLILISFLSQYTKQKVQN 220
GS-5   -----------T-------------------------------- 220
KK23   -----------V-------------------------------- 220
R221   -----------V-------------------------------- 220
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Figure 2. A sequence alignment among four *S. mutans* strains shows natural point mutations either within loopB (T_{110} > N_{110} and G_{116} > D_{116}) in strain GS-5 or within both loopB (T_{110} > N_{110} and G_{116} > D_{116}) and loopC (T_{178} > V_{178}) in strains KK23 and R221. Only 1-220 amino acid residues of the membrane-spanning domain of the ComD proteins are shown. The six transmembrane segments (TMSs 1-6) are underlined. The extracellular loopA, loopB and loopC are bold. −: indicates the same residues.

site and are directly controlled by the ComE response regulator [14-16]. We then examined specific lux reporter activities of these strains in response to SP. The results revealed that a deletion within loopA, either four residues (SNVT) or eight residues (SNVTLSKK), showed little effect on the response of these two promoters to SP. This suggests that extracellular loopA appears to be not directly involved in SP recognition. However, a deletion of four residues LDGT of loopB resulted in a dramatic reduction in the luciferase reporter activities compared to the reporter activities in the $ComD^+$ control strains. Even more dramatically, a deletion of residues NVIP of loopC completely abolished the response of these promoters to SP, suggesting that the residues NVIP of loopC may be essential for SP recognition. To further confirm this suggestion, we constructed two more loopC mutants that had an alanine substitution mutation (AA/NV) and (AA/IP) within loopC. The results showed that both substitution mutants failed to respond to SP, strongly suggesting that the residues NVIP of loopC are truly essential for SP recognition. Together, the results suggest that both extracellular loopC and loopB are required for SP recognition and the residues NVIP of loopC are essential for SP detection and quorum sensing activation in *S. mutans*.

By sequence alignments of ComD proteins (Fig. 2), we also identified several *S. mutans* strains that had one or two residue substitution mutations, either within loopB such as in strain GS-5 (Asn_{110} > Thr_{110} and Asp_{116} > Gly_{116}), or within both loopB and loopC such as in strains KK23 and R221 (Asn_{110} > Thr_{110}, and Val_{178} > Thr_{178}) (8). We were curious to know whether these strains might be defective in recognition and response to SP for quorum sensing activation. We included these strains and their genetically engineered derivatives to examine the effects of these point mutations on SP recognition and quorum sensing activation. Surprisingly, all the strains exhibited wild type levels of the luciferase reporter activities in response to SP, except the ComD deletion mutants ($ComD^-$) derived from these strains. The results suggest that the single residue mutation within loopB or within both loopB and loopC did not appear to affect SP recognition and quorum sensing activation in these *S. mutans* strains.

It has been well recognized that quorum-sensing signal molecules produced by many bacteria often induce species-specific or even strain-specific activities at nano-molar concentrations [9]. This feature has provided an excellent opportunity to explore structure-activity

relationships between a signal pheromone and its cognate receptor [17]. For example, the AIP-AgrC quorum sensing system in *S. aureus* is one of the best-studied model systems that show highly strain-specific activities to induce quorum sensing response [18]. The sequence variations of the AIPs from different *S. aureus* strains have led to identification of at least four specificity groups within *S. aureus*. All strains within one group produce the same AIP, which only activates quorum sensing and the virulence within its own specificity group, but not in other groups. Instead, an AIP from one group can competitively inhibit quorum sensing and its controlled activities in other groups [18]. In *S. pneumoniae*, extensive screening of pneumococcal isolates reveals two major CSP variants that are highly specific to interact with their respective receptors, ComD1 and ComD2 [19, 20]. These studies have led to the proposal that *S. pneumoniae* strains can be divided into different pherotypes based on the specificity of their signal molecules or competence-stimulating peptide (CSP) [19]. Similar studies have been carried out to identify quorum-sensing signaling peptide variants of *S. mutans* strains and clinical isolates. Seven *comC* alleles encoding three distinct mature SPs are identified among 36 geographically diverse *S. mutans* strains [21]. In contrast to *S. pneumoniae*, however, all three SP variants function equivalently to induce quorum sensing, bacteriocin production and genetic competence [16, 17, 22]. There is no evidence of signal pheromone specificity or pherotype in *S. mutans*. In our study, two ComD receptor variants that have a point mutation either within loopB or within both loopB and loopC reveal that these single substitution mutations do not appear to affect SP recognition and quorum sensing activation. Neither do these mutations significantly affect SP-induced bacteriocin production, suggesting that the ComD receptor in *S. mutans* displays relatively low specificity to sense and transduce the signal for quorum sensing activation within the species. Such less constraint specificity in SP-ComD interaction in *S. mutans* clearly differs from those in *S. pneumoniae*. In fact, the structural and functional divergences in ComCDE quorum sensing systems between *S. mutans* and *S. pneumoniae* have been recognized for years. Phylogenetic analysis of the Genus *Streptococcus* reveals that ComCDE system in *S. mutans* is more closely related to the BlpCRH quorum sensing system that directly controls bacteriocin production in *S. pneumoniae* [23]. The sequence alignments also show that ComD proteins of *S. mutans* only shares 22% of identity and 44% of similarity with those of *S. pneumonia* [8]. This suggests that ComD proteins of *S. mutans* may not necessarily share the same membrane topology and signal recognition domain with the ComD proteins in *S. pneumoniae*. Despite no experiments to test this difference, *in silico* analysis of ComD proteins among streptococcal species appears to support this suggestion that the ComCDE system in *S. mutans* likely combines the action of two orthologous systems, the ComCDE and BlpCRH, in *S. pneumoniae,* which are well known to be involved in competence development and bacteriocin production, respectively [23]. What is the ecological implication of such less constraint specificity of the signal peptide-receptor interaction in *S. mutans* is unclear. However, we speculate that the less constraint specificity may provide *S. mutans* with more flexibility or even an advantage to sense the signal molecules in densely packaged dental biofilms, where *S. mutans* needs to compete with closely related colonizers, such as various species of streptococci, by population-wide production of bacteriocins in dental biofilms consisting of diverse species of microbes.

In summary, our study demonstrates that the membrane domain of the *S. mutans* ComD receptor protein forms six transmembrane segments and three extracellular loops, loopA, loopB and loopC. Structural and functional analyses of these extracellular loops reveal that both loopC and loopB are required for signal recognition and quorum sensing activation, while loopA plays little role in signal detection. In addition, single sequence variations within loopB or loopC exist in some *S. mutans* strains, but these strains do not show any detectable defects in SP detection. Thus, unlike other pheromone receptors in the HPK_{10} subfamily, the ComD receptor in *S. mutans* shows more flexibility to sense and transduce the signal for quorum sensing, which may provide *S. mutans* with an advantage for intra-species communication in its natural ecosystem, dental biofilms.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Abbreviations

TCSTS: two-component signal transduction system; HPK: histidine protein kinase; SP: signal peptide; CSP: competence-stimulating peptide; TMS: transmembrane segment.

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