The VicRK Two-Component System Regulates *Streptococcus mutans* Virulence

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Abstract:

Streptococcus mutans is considered the predominant etiological agent of dental caries with the ability to form biofilm on the tooth surface. And, its abilities to obtain nutrients and metabolize fermentable dietary carbohydrates to produce acids contribute to its pathogenicity. The responses of *S. mutans* to environmental stresses are essential for its survival and role in cariogenesis. The VicRK system is one of the 13 putative TCS of *S. mutans*. The conserved

functions of the VicRK signal transduction system is the key regulator of bacterial oxidative stress responses, acidification, cell wall metabolism, and biofilm formation. In this paper, it was discussed how the VicRK system regulates *S. mutans* virulence including bacterial physiological function, operon structure, signal transduction, and even post-transcriptional control in its regulon. Thus, this emerging subspecialty of the VicRK regulatory networks in *S. mutans* may strengthen our understandings aimed at providing a basis for the prevention of dental caries.

Introduction

Dental plaque is a multi-species microbial biofilm that form on the tooth surfaces. Dental caries is promoted by environmental changes (e.g., changes in pH) that cause ecological shifts to plaque residents that favor the proliferation of aciduric bacteria (Kazor *et al*., 2003; Paster *et al*., 2001; Paster *et al*., 2002). Among the hundreds of bacterial species that colonize and persist in the oral cavity, *Streptococcus mutans* is among the few species that have been consistently linked with caries formation (Loesche *et al*., 1986). In addition, the ability of *S*. *mutans* to synthesize extracellular polysaccharides that promote formation of the plaque biofilm also contributes to its pathogenicity.

Two-component signal transduction systems (TCS) are among the regulatory networks that are essential for bacterial adaptation, survival, and virulence in response to changes in the external environment. Typically, signal transduction is accomplished via two regulatory elements comprising a membrane-associated histidine kinase (HK) and a cytoplasmic response

regulator (RR). Upon exposure to an environmental change such as pH, osmolarity, or oxidation-reduction potential, the HK becomes autophosphorylated at a conserved histidine residue. Following the transfer of this phosphate group to a RR, the regulator can control transcription of target genes by binding to their promoter regions (Stein *et al*., 2002; Davies *et al*., 1998; Mascher *et al*., 2006). The most widely distributed essential TCS, the WalK/WalR system, was originally described in *Bacillus subtilis* (Fukuchi *et al*., 2000). This system has since been reported as essential for cell viability in several closely related pathogens (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *S. mutans*, and *Streptococcus pyogenes*), and referred to under various designations (YycG/YycF, VicK/VicR, MicA/MicB) (Martin *et al*., 1999; Lange *et al*., 1999; Wagner *et al*., 2002). And, attempts to inactivate the *walRK* locus in *Listeria monocytogenes*, *Enterococcus faecalis, Staphylococcus epidermidis* and *Str. pneumoniae* were unsuccessful, indicating that it is likely to be essential in these bacteria (Lange *et al*., 1999, Kallipolitis *et al*., 2001; Hancock *et al*., 2004; Dhiman *et al*., 2014).

The VicRK TCS is one of 13 such systems found in *S. mutans* UA159 (Senadheera *et al*., 2005). Based on sequence homology, the VicRK genes encode a surface-associated HK (VicK) and an intracellular RR (VicR). The *vic* operon comprises three genes encoding the following: VicR, a RR of the OmpR family; VicK, its cognate HK; and VicX, a putative protein sharing 55% identity to YycJ in *B. subtilis* (Fukuchi *et al*., 2000). A putative transcriptional start site for the *vic* operon was mapped 16 bp upstream of the ATG codon of *vicR*. VicK, an atypical PAS domain-containing HK, can be autophosphorylated *in vitro*, and

VicR functions as a phospho-acceptor protein *in vitro* (Ajdic *et al*., 2002; Senadheera *et al*., 2007; Durso *et al*., 2014). YycFG (VicRK) TCS has been the topic of many recent studies among these quorum-sensing systems. In streptococci, the YycFG (VicRK) system regulates processes involved in cell wall metabolism, nutrient uptake, osmotic protection, cell division (Barendt *et al*., 2009), cell wall biogenesis (Moraes *et al*., 2014), antibiotic-resistance (Li *et al*., 2009) and resistance to complement immunity(Alves *et al*., 2017). Furthermore, *vicRK* genes in 242 *S. mutans* clinical isolates were sequenced, locus 470 showed higher missense mutation in high-severity caries group than control group, indicating The locus 470 missense mutation of the vicK gene may be related to caries in children with *S. mutans* (Zhuang *et al*., 2018). The VicRK system has attracted considerable interests in *S. mutans*, yielding insights into its physiological function, its potential activating signal and virulence (Liu *et al*., 2006). In this review, we summariz how the VicRK system regulates *S. mutans* virulence including bacterial physiological function, operon structure, signal transduction, and even post-transcriptional control in its regulon. The current knowledge on the system of the VicRK regulatory networks in *S. mutans* may strengthen our understandings of bacterial virulence and provided new aspects for the management of dental caries.

VicRK-dependent phenotype

The WalK/WalR system has been reported as essential for cell viability in several closely related pathogens (*Sta. aureus*, *Str. pneumoniae*, *S. mutans*, and *Str. pyogenes*) and referred to under various designations (YycG/YycF,

VicK/VicR, MicA/MicB). The WalK/WalR (YycFG) TCS is highly conserved and specific to low G+C Gram-positive bacteria. *B. subtilis* and *L. monocytogenes* have a 6-gene operon (*walR*, *walK*, *yycH*, *yycI*, *yycJ*, *yycK*), *E. faecalis* lacks the last gene, while only three are present in streptococci (*walR*, *walK*, *yycJ*; Figure. 1).

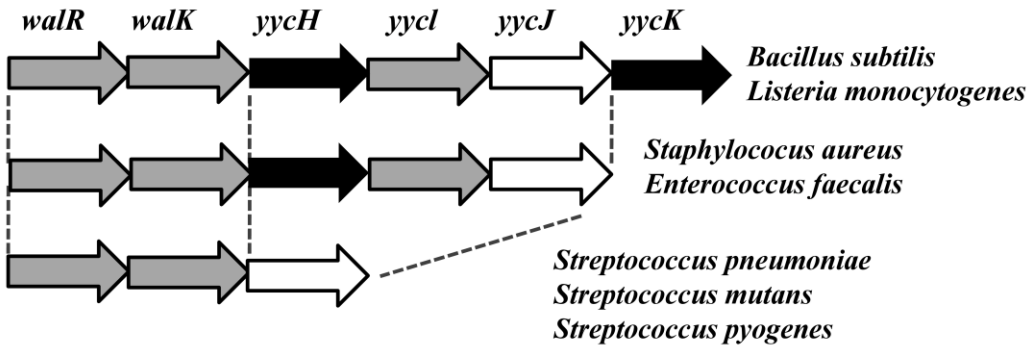

Figure 1. Genetic map of the *walRK* (*vicRK*) operon.

Oxidative stress response

After the initial colonization stage, *S. mutans* undergoes continuous dynamic challenges to which it must respond and adapt. There is much evidence suggesting a relation between oxidative stress and biofilm formation (Sampathkumar *et al*., 2006; Loo *et al*., 2004) because oxidative stress response genes were up-regulated during biofilm growth. D.M. Deng *et al* (Deng *et al*., 2004) constructed a strain which expressed of a green fluorescent reporter-protein (GFP) under control of the autoregulated *vicRK* promoter, showing increased fluorescence intensity under oxidative stress. After a clean knockout of the *vicK* gene, the mutant proved to be more sensitive to H_2O_2 than the wild-type parent strain, leading to the hypothesis that there was a link

between oxidative/redox stress and the VicRK TCS of *S. mutans*.

Additionally, oxygen is required by several oral bacteria for respiration and energy generation. Although oral streptococci do not possess a full electron transport chain and cannot carry out oxidative phosphorylation, these organisms maintain a high capacity to metabolize oxygen, primarily through NADH oxidase enzymes (Higuchi *et al*., 1993; Marquis *et al*., 1995). It has been shown that exposure of *S. mutans* to oxygen strongly inhibits biofilm formation and alters cell surface biogenesis. The results of transcription profiling showed that roughly 5% of the genes of the organism are differentially expressed in response to aeration. Consistent with this observation, the ability of *S. mutans* to form biofilms is severely impaired by exposure to oxygen. Real-time reverse-transcription polymerase chain-reaction revealed that transcription of *gtfB*, but not *gtfC*, was responsive to oxygen and that aeration causes major changes in the amount and degree of cell association of the Gtf enzymes. Moreover, inactivation of the VicK sensor kinase affected the expression and localization the GtfB and GtfC enzymes. These studies provide insights into the complex transcriptional and posttranscriptional regulatory networks in *S. mutans* that modulate virulence gene expression in response to changes in oxygen availability (Ahn *et al*., 2007).

Acid production and acid survival

S. mutans can rapidly metabolize dietary sucrose, producing lactic acid as a metabolic end product, which can lower the plaque pH and erode the tooth enamel, thereby initiating dental decay (cariogenesis). Studies have shown that if *S. mutans* cultures are pre-exposed to an adaptive acidic environment

(approximately pH 5.0 to 5.5), the survival rates of the bacteria are dramatically enhanced at approximately 3.5 of pH value (Hamilton *et al*., 1998; Hamilton *et al*., 1991; Quivey *et al*., 2000). The regulation of acid production and the tolerance to low pH of *S*. *mutans* have gained considerable attention since both properties contribute substantially to the virulence of this organism. Senadheera *et al.* (Senadheera *et al*., 2009) reported that the VicK sensor kinase of *S. mutans* was involved in both the acidogenicity and the aciduricity. When cultures were supplemented with glucose, the glycolytic rate of a *vicK* null mutant was significantly decreased, compared with the wild type, suggesting the impaired acid production for the mutant. When *S. mutans* wild-type UA159 and *vicK* deletion mutant strains grown at neutral and low pH values, global transcriptional analysis using DNA microarray revealed that loss of VicK significantly affected expression of 89 transcripts at pH 5.5. Using a cut-off of two-fold change, 38 genes were significantly up-regulated while 51 genes were suppressed. The results provide insights into the acid-inducible regulon of *S. mutans*, and the affected transcripts included genes with putative functions in transport and maintenance of cell membrane integrity, implying a novel role for VicK in regulating intracellular pH homeostasis in *S. mutans* (Senadheera *et al*., 2009).

Exopolysacharrides synthesis

In part, the virulence of the dental caries pathogen *S*. *mutans* relies on the sucrose-dependent synthesis of and interaction with glucan, a major component of the extracellular matrix of tooth biofilms. Glucan, including water-insoluble glucan (WIG) and water-soluble glucan (WSG), undergo structural modifications resulting from the effects of glucosyltransferases (GtfB/C/D) and

fructosyltransferase (Ftf) during glucan synthesis (Rolla *et al*., 1983). A *vicK* deletion mutant was isolated by Senadheera *et al* (Senadheera *et al*., 2009), while a *vicR* (putative RR) null mutation was apparently lethal. Compared with the wild-type UA159, the *vicK* mutant biofilm formed in sucrose-supplemented medium was easily detachable. The rate of total dextran formation by *vicK* mutant was remarkably reduced compared to the wild type. Furthermore, a recombinant VicR fusion protein was shown to bind the promoter regions of the *gtfB*, *gtfC*, and *ftf* genes. Study also showed that addition of high-fructose corn syrup-5, a substitute for sucrose, up-regulated the expression of *vicR* and *gtfB* and reduced the microhardness of teeth (Sun. *et al*., 2014). Animal studies conducted using *vicK* mutant in a specific-pathogen free (SPF) rat model resulted in a significant reduction in *vicK* mutant CFU counts compared to that of the wild-type UA159.

Additionally, it has been suggested that other surface proteins, such as glucan-binding proteins (Gbps), with affinity for glucan contribute to *S. mutans* biofilm growth by mediating bacterial interaction with extracellular glucan (Banas *et al*., 2003. A conditional knockdown mutant that expressed *gbpB* antisense RNA under the control of a tetracycline-inducible promoter was constructed in wild type strain UA159 (Duque *et al*., 2011). It was established that GbpB depletion impaired initial phases of sucrose-dependent biofilm formation. Additionally, GbpB was directly regulated by VicR, and exogenous native GbpB partially restored the biofilm phenotype. Several cellular phenotypes were significantly affected by GbpB depletion, including altered bacterial cell shape, decreased autolysis, increased cell hydrophobicity, and sensitivity to antibiotics

and osmotic and oxidative stresses.

Cell wall biosynthesis

Defining the specific functions of genes involved in cell wall biogenesis is important for clarifying the transition mechanism of *S. mutans* from planktonic to biofilm growth in the presence of sucrose. Using transcriptomic analysis comparing the wild-type UA159 strain with a *vicK* deletion mutant together with electrophoretic motility shift assays, Stipp *et al.* (Stipp *et al*., 2013) identified genes directly regulated by both VicR and GcrR (CovR) with putative functions in cell wall biogenesis, including *gbpB*, *wapE*, *smaA*, *SMU.2146c*, and *lysM*. Deletion mutants of genes (*wapE*, *lysM*, *smaA*) promote significant alterations in biofilm formation, including increased fragility, defects in microcolony aggregation, and atypical cell morphology. Additionally, VicR, CovR and VicRK/CovR targets (*gbpB*, *wapE*, *smaA*, *SMU.2146c*, *lysM*, *epsC*) are up-regulated in UA159 during biofilm initiation, in a sucrose-dependent manner. These results support a model in which VicRK and CovR coordinate cell division and surface biogenesis with the extracellular polysaccharides synthesis for formation of structurally stable biofilm.

Cell division

In *S. mutans*, *vicK*-defective mutant also forms long chains. However, GbpB, homologues of PcsB, seems to work as a glucan-binding protein in *S. mutans*. Abnormal cell morphology but no long chain was observed after bringing down expression of GbpB. Cristiane Duque *et al*. (Duque *et al*., 2011) constructed an antisense RNA to disturb the expression of *gbpB* and this strain formed chains of

shorter cocci but not long chains, which is different to a *vicK*-defective mutant. Previous study demonstrated that a 60-kilodalton immunodominant glycoprotein contributed to cell wall integrity and maintains cell shape in *S. mutans* (Chia *et al*., 2001).

Bacteriocin production and autolysin

Bacteriocins are peptides that have antimicrobial activity against other strains or related species. Although bacteriocin producers are resistant to their own bacteriocins through the action of immunity proteins, production of these bacteriolytic or bacteriostatic molecules can be costly due to constitutively expressed plasmid carriage, and possible lethality of production. *S. mutans* is a primary pathogen associated with dental caries and its bacteriocin (mutacin) production ability is thought to play an important role in maintaining competitiveness in the multispecies oral biofilm. The signaling networks that regulate bacteriocin production and transport as well as immunity to bacteriocins involve peptide pheromone sensing pathways which is very similar to those involved in genetic competence (Kleerebezem *et al*., 2001). Currently, two classes of mutacins have been identified: Lantibiotics are ribosomal synthesized and undergo extensive posttranslational modifications that create the unusual amino acids lanthionine or b-methyllanthionine, whereas non-lantibiotics consist of either one or two small unmodified peptides (Qi *et al*., 2001). It has been demonstrated that a putative inducible repressor, *irvA*, seems to be involved in the gene regulation pathway of the LuxS-mediated lantibiotic, mutacin I(Tsang *et al*., 2006). In this study, it was found that all these genetic loci (*vicK, pttB hk03, hrcA, adhE, Smu1281,* and *ciaH*) influenced mutacin I production at the

transcriptional level. It has been demonstrated that these multiple inputs can be divided into two pathways: *irvA*-dependent and *irvA*-independent. Mutations in genes such as *luxS*, *hk03*, *vicK*, and *pttB* activate the expression of a common regulator, *irvA*. Thus, current studies suggest that VicRK signals may modulate the transcription of the mutacin operon.

Senadheera *et al.* (Senadheera *et al*., 2012) showed that the VicRK modulates bacteriocin production and cell viability, in part by direct modulation of competence-stimulating peptide (CSP) production in *S. mutans*. VicR binding to the *comC* coding region has been confirmed since a well-conserved VicR binding site was identified within the *comC* open reading frame. Global transcriptome and real-time transcriptional analysis of the VicK-deficient strain revealed significant modulation of several bacteriocin-related genes, including *nlmAB*, *nlmC*, and *nlmD*, suggesting a role for the VicRK in producing mutacins IV, V, and VI (Qi *et al*., 2001; Qi *et al*., 1999; Qi *et al*., 1999). The results may reveal a novel regulatory link between the VicRK and ComDE systems to modulate bacteriocin production and autolysis of *S. mutans.*

Also, the *atlA* gene in *S. mutans* encodes an autolysin required for biofilm maturation and biogenesis of a normal cell surface (Ahn *et al*., 2006). The formation of long chains, a characteristic of *atlA* mutant, was less evident in cells grown with aeration. Deletion of the *vicK* gene also led to inhibition of processing of *atlA*, and the mutant was more resistant to autolysis (Qi *et al*., 1999; Ahn *et al*., 2007). When grown under aerobic conditions, the *vicK* mutant also showed significantly increased biofilm formation compared to wild-type UA159 strain.

Taken together, these investigations illustrate the crucial role of AtlA and VicK in orchestrating growth on surfaces and envelope biogenesis in response to autolysis.

Carbohydrate substrates metabolism

Although other microorganisms may also be involved in dental plaque*, S. mutans* is a key contributor in the formation of biofilms associated with sucrose ingestion (Beighton *et al*., 2005; Kanasi *et al*., 2010). *S. mutans* produces several extracellular sucrose-metabolizing enzymes, such as glucosyltransferases and fructosyltransferase. The cooperative action of these enzymes is essential for sucrose-dependent cellular adhesion and biofilm formation. A global RR (VicR) plays important roles in *ftf* and *gtf* expression in response to environmental changes. Real-time reverse-transcription polymerase chain-reaction were used to quantify the relative levels of *ftf*, *gtfB*, *gtfC*, *gtfD* and *vicR* transcription of *S. mutans* in the presence of various dietary carbohydrates: sucrose, D-glucose, D-fructose, D-glucitol (D-sorbitol), D-mannitol and xylitol. The expression of *vicR* was induced only at the presence of xylitol at late exponential phase and declined at early exponential phase. Taken together, these findings show that dietary carbohydrates have a major influence on the transcription of *ftf*, *gtfB*, *gtfC* and *gtfD*, but less on *vicR* in *S. mutans* (Shemesh *et al.,* 2006).

To avoid the role of sucrose on the production of virulence factors by *S. mutans*, sugar substitutes are commonly consumed because of their potential to lower or block the production of acids and interfere with biofilm formation (Rozen

et al., 2001; Rozen *et al*., 2004). The presence of sugars or sugar substitutes profoundly affected the expression of *spaP, gtfB, gtfC, gbpB, ftf, vicR* and *vicX* in either biofilm or planktonic cells. The substitution of sucrose induced a down-regulation of *vicR* and *vicX* in sucrose-dependent colonization in biofilm cells. However, sucralose but not sorbitol fulfilled the purpose of reducing the cariogenic potential, since it induced the biofilm formation with the lowest biomass, did not change the pH value of the medium (Durso et al., 2014).

Biochemistry of VicRK operon structures and gene function

Structural features of the VicK kinase in S. mutans

Prokaryotic organisms commonly regulate gene expression in response to environment changes using TCS (Parkinson *et al*., 1993; Zhulin *et al*., 1997). Such systems typically comprise a membrane associated sensory kinase and a cytoplasmic RR. Wang *et al* (Wang *et al*., 2013) reported the crystal structure of a complete cytoplasmic portion of sensory kinase VicK from *S. mutans*. The overall structure of VicK is a long-rod dimer that anchors four connected segment domains: HAMP, Per-ARNT-SIM (PAS), DHp, and catalytic and ATP binding domain. The HAMP, a signal transducer, and the PAS domain, major sensor, adopt canonical folds with dyad symmetry. In contrast, the dimer of the DHp and ATP binding domains is asymmetric because of different helical bends in the DHp domain and spatial positions of the ATP binding domains. Moreover, a conserved proline, is adjacent to the phosphoryl acceptor histidine. This structure contributes to helical bending, which is essential for the autokinase and phosphatase activities. Together, the elegant architecture of VicK with a signal transducer and sensor domain suggests a model where DHp helical bending and

an ATP binding domain swing movement are likely coordinated for autokinase activation (Wang *et al*., 2013).

DNA binding sites of VicR

The binding sequence for the VicR homolog in *B. subtilis* and *Sta. aureus*, WalR, has been well characterized. The consensus sequence consists of two hexameric half sites separated by a non-conserved 5 base pair (Howell *et al*., 2003). The upstream regions of virulence genes (*gtfB, gtfC* and *ftf*) in *S. mutans* were analyzed previously and were found to be match to the WalR consensus in the promoter regions (Senadheera *et al*., 2005). Ayala *et al.* (Ayala *et al*., 2014) investigated the biochemical characteristics of the *S. mutans* protein VicR. They dissected the DNA binding requirements of the recognition sequences for VicR in its unphosphorylated and phosphorylated forms. In *S. mutans*, CovR (RR of GbpC) is an orphan RR that is involved in biofilm formation, sucrose mediated adhesion and the acid tolerance response (Dunning *et al*., 2008). In addition, CovR regulates virulence traits that overlap with the VicRK TCS and it has been identified a perfect match to the WalR consensus binding site (TGTTATagaacTGTAAT) 94 bp upstream of the start codon on the forward strand. The VicR consensus sequence of *nlmC, bmsH, gtfC, gbpB, gcrR* and *plsX* are located on the coding strand of each gene. In contrast, the consensuses for *gtfB, wapA, atlaA, relP, glnQ,* and *copY* are found on the non-coding strand (Ayala *et al*., 2014).

The role of vicX in S. mutans virulence

Despite our knowledge of the various physiological properties that are

subject to control by VicRK, a putative role for the third gene (*vicX*) in the tricistronic operon is not well known. Senadheera *et al.* (Senadheera *et al*., 2007) constructed a nonpolar deletion mutation in the *vicX* coding region in *S. mutans* UA159. The growth kinetics of the *vicX* mutant (SmuvicX) showed that there was considerable sensitivity to paraquat-induced oxidative stress. Interestingly, *vicX*-deficient cells grown in a glucose-supplemented medium exhibited significantly increased *gtfB*/*C* expression compared with expression in the wild type. In the absence of *vicX*, the ability of *S. mutans* to grow under oxidative stress conditions and its ability to take up foreign DNA are drastically compromised (Senadheera et al., 2007). What's more, a decrease of WIG synthesis and WIG/ WSG were observed in *vicX*-deficient cells (Lei *et al*., 2015). However, the information regarding the structural features and biochemistry characteristics of the VicX is still limited.

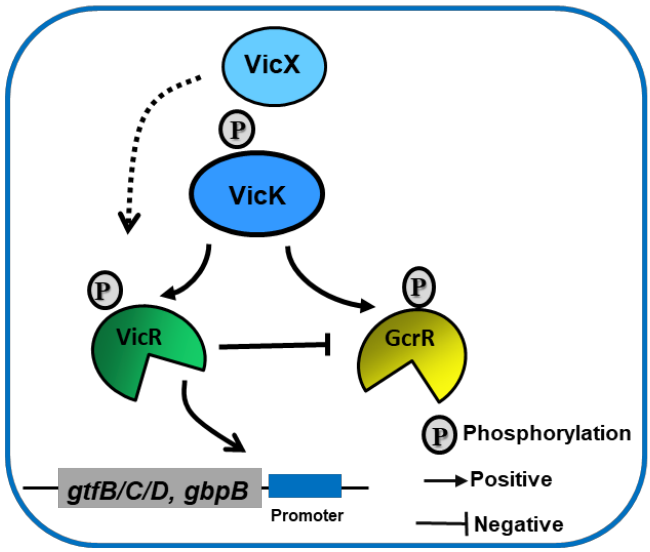

Figure 2. The interaction of VicRK TCS in *S. mutans*.

VicRK-mediated signal transduction in sensing and co-regulating cell physiology

VicK and CovR (GcrR)

Previously, we discussed the role of the *S. mutans* VicRK TCS in modulating biofilm formation, oxidative stress and acid tolerance responses. Using *in vitro* phosphorylation assays, Downey *et al* (Downey *et al*., 2014) demonstrated that activating its cognate RR protein, the sensor kinase, VicK, can transphosphorylate a non-cognate stress regulatory RR, CovR, in the presence of manganese. Overlapped DNA binding specificities for VicR and CovR were observed in native promoters, consistent with these proteins being part of the same transcriptional regulon. Their findings supported the regulatory complexities observed with the *S. mutans* manganese-dependent response, which involves cross-talk between non-cognate signal transduction systems (VicRK and CovR) to modulate stress response pathways (Figure 2).

VicRK and ADS

The oral commensal *Streptococcus gordonii* must adapt to constantly fluctuating and often hostile environmental conditions to persist in oral biofilms through the hydrolysis of arginine in saliva (Burne *et al*., 2000). The arginine deiminase system (ADS) of *S. gordonii* enables bacterial cells to produce, ornithine, ammonia, CO_2, and ATP from arginine hydrolysis, augmenting the acid tolerance of the organism. It has been demonstrated that the CiaRH and ComDE TCS are required for low-pH-dependent expression of ADS genes in *S. gordonii*. Further, the VicRK TCS is required for optimal ADS gene expression under anaerobic conditions and enhances the sensitivity of the operon to oxygen (Liu *et al*., 2009). The authors observed mutants of *S. gordonii* lacking components of the CiaRH,

ComDE, or VicRK grew more slowly in acidified media and were more sensitive to killing at lethal pH values and to agents that induce oxidative stress. Simultaneously，the VicRK, ComDE, and CiaRH two-component systems affect AgDS gene expression in response to acidic stresses in *S. mutans*(Liu *et al*., 2009). These studies reveal some notable differences in the contribution of CiaRH, ComDE, and VicRK to stress tolerance between the *S. gordonii* and *S. mutans*.

VicRK and CRISPR-Cas Systems

CRISPR-Cas (clustered regularly interspaced short palindromic repeats) systems provide adaptive microbial immunity against invading viruses, plasmids, and transposable elements (Barrangou *et al*., 2008; Brouns *et al.*, 2008). *S. mutans* UA159 has two CRISPR-Cas systems: CRISPR1 (type II-A) and CRISPR2 (type I-C). Using plasmid transformation experiments, Serbanescu *et al.* (Serbanescu *et al*., 2015) demonstrated that the CRISPR1-Cas system inhibits transformation of *S. mutans* by plasmids. Functional analysis of *cas* deletion mutants revealed that in addition to a role in plasmid targeting, both CRISPR systems also contribute to the regulation of bacterial physiology in *S. mutans*. Compared to wild-type cells, the *cas* deletion mutant strain displayed diminished growth under oxidative stress, enhanced growth under low pH, and reduced survival under heat shock conditions. Transcriptional analysis revealed that VicRK differentially modulates expression of *cas* genes within CRISPR-Cas systems, suggesting that VicRK might coordinate the expression of two CRISPR-Cas systems.

VicRK and c-di-AMP signaling pathway

Cyclic di-AMP (c-di-AMP) is an emerging second messenger in bacteria (Oppenheimer-Shaanan *et al*., 2011). The level of c-di-AMP is modulated by activity of diadenylate cyclase (CdaA) that produces c-di-AMP and phosphodiesterase (PDE) that degrades c-di-AMP (Romling *et al*., 2008). Cheng *et al.* (Cheng *et al*., 2015) observed genes with increased or decreased expression in the *cdaA* mutant were clustered in cellular polysaccharide biosynthetic or oxidoreductase activity, respectively. Previous studies have also found that VicRK is involved in cell wall and a *vicK* knockout mutant in *S. mutans* would likely result in enhanced cell death, increased lysis (Senadheera *et al*., 2009) and increased sensitivity to oxidative stress (Deng *et al*., 2004). This study suggests a probable interplay between c-di-AMP signaling and TCS in this bacterium. Interestingly, it has been revealed that only CabPA deficiency inhibited both the increased biofilm formation and the up-regulated expression of GtfB observed in the *pdeA* mutant (Peng *et al*., 2005). In addition, CabPA but not CabPB interacted with VicR, a known transcriptional factor that regulates expression of *gtfB*, suggesting that a signaling link between CabPA and GtfB through VicR. They performed a pull-down assay using recombinant CabPA, CabPB and VicR, and confirmed that VicR directly interacted with CabPA not CabPB *in vitro*. Take together, these studies revealed a new role of c-di-AMP in mediating biofilm formation through a CabPA/VicR/GtfB signaling network in which c-di-AMP mediates GtfB expression and biofilm formation through the interaction between CabPA and VicR in *S. mutans.*

Interaction with LiaRS in S. mutans

Previous study shows that expression of *vicR* is pH-dependent and LiaRS signal system in *S. mutans* controls acid adaption and envelope stress response. These evidences suggest a model which LiaS senses external stimulus and autophosphorylation, then passing a phosphate group to LiaR and phosphorylated LiaR regulates expression of *vicR* (Tremblay *et al*., 2009).

Response of VicRK system to natural products treatment

Carolacton is a newly identified secondary metabolite with high inhibitory activity by inducing damage of *S. mutans* biofilms. Carolacton was also found to induce dose-dependent damage of *S. mutans* biofilms over a wide concentration range (Kunze *et al*., 2010). The sub-networks modulated by the VicR could putatively be related to the physiological effect of carolacton, and the microarray data show that expression of *vicRK*, one of the earliest responding TCSs to carolacton treatment, was down-regulated. The network approach revealed *vicRK* regulators and interactions as part of the response mechanisms of *S. mutans* biofilm cells to carolacton.

Although several antiplaque agents have been used, some studies reported that several natural products derived from herbs, such as *Mentha longifolia* L., *Aralia continentalis* and *Curcuma longa L*, showed inhibitory effects on dental biofilms. Jeong *et al.* (Jeong *et al*., 2013) isolated a single chemical compound from *A. continentalis* for investigation of anticariogenic properties. Inhibitory effects of the extracted chemical compound on growth, acid production, biofilm formation, and adherence of *S. mutans* were evaluated. Real-time PCR analysis was performed and showed that the expression of *gtfB, gtfC, gbpB, spaP, brpA,*

relA, and *vicR* were significantly decreased in *S. mutans* after treatment with the compound.

Another natural product extracted from *Chrysanthemum boreale* was also evaluated for antibacterial activity against *S. mutans*. This essential oil exhibited significant inhibition of growth, adherence capacity, and acid production. Furthermore, real-time PCR analysis showed that gene expression of several virulence factors such as *gtfB*, *gtfC*, *gtfD*, *gbpB*, *spaP*, *brpA*, *relA*, and *vicR* was significantly decreased in a dose-dependent manner. These results suggest that natural products may inhibit growth, adhesion, acid tolerance, biofilm formation, and related virulence factors of *S. mutans* through the VicRK regulatory mechanism.

Small regulatory noncoding RNA control of *vic* operon

Recently, we identified and confirmed a noncoding antisense RNA (ASvicR) with the potential to attenuate the functions of an essential RR, VicR, that covered the *vicR* coding sequence, including the DNA binding and dimerization domains. Furthermore, it has previously been shown that the overexpression of ASvicR resulted in a reduction in biofilm formation and acid production (Lei *et al*., 2018). A novel category of miRNA-size small RNAs (msRNAs) targeting *vicR*, which reduced dental caries by interfering with bacterial exopolysaccharide metabolism, has been described in *S. mutans*. Notably, a putative RNase III-encoding gene (*rnc*) that may play a critical role in the specific cleavage of dsRNA structures located downstream from the *vicRKX* operon (Mao *et al*., 2016). Thus, this emerging subspecialty of bacterial regulatory RNAs may

reshape our understanding of *S. mutans* gene regulation from its transcriptional level.

Anti-TCS drugs

TCSs play a role in the virulence of *S. mutans*, making them attractive targets for drug development. Anti-TCS drugs that repress virulence have mostly targeted the sensory domains of their target HKs, and since such domains are unique to each HK, anti-TCS drugs should potentially be effective only to a specific TCS (Gotoh *et al*., 2010). One of the targeted TCSs for HK inhibitors is the WalK/WalR (formerly YycG/YycF) system, which plays a key role in cell wall metabolism (Watanabe *et al*., 2003; Watanabe *et al*., 2008)]. The effect of Walkmycin C (WKM C), a HK inhibitor, against *S. mutans* has been evaluated. This compound inhibited the *in vitro* autophosphorylation activity of three purified *S. mutans* HKs: VicK, CiaH, and LiaS. This inhibitory effect suggests that blocking the autophosphorylation of multiple HKs may inhibit phosphotransfer to VicR from VicK and other HKs. In the presence of WKM C, *S. mutans* formed abnormal biofilms and showed a defect in competence. When pretreated with the inhibitor, cells showed an increase in acid sensitivity. These results indicated the possibility of developing HK inhibitors into anti-virulence drugs for *S. mutans*.

Concluding remarks and future directions

S. mutans has few alternative sigma factors encoding genes in its genome (Ajdic *et al*., 2002), therefore TCSs are considered to play a central role in stress tolerance (Lemos *et al*., 2008). It will be important to further perform structure-function studies on the sensory domains of VicR which binds to the

promoters of *gtfBCD* and positively regulates their expression. Both the unknown structural features of the VicK HK and its interaction with RRs are worthy of further study. Furthermore, little is known on the structural features and biochemistry characteristics of the VicX hydrolase. With the increasing number of bacteria that are rapidly becoming resistant to antibiotics, the VicRK system provide promising targets for a new class of antimicrobials and effective dental caries therapy.

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Disclosure statement

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