Role of VicRKX and GlnR in pH-Dependent Regulation of the Streptococcus salivarius 57.I Urease Operon

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ABSTRACT

Ureolysis by Streptococcus salivarius is critical for pH homeostasis of dental plaque and prevention of dental caries. The expression of S. salivarius urease is induced by acidic pH and carbohydrate excess. The differential expression is mainly controlled at the transcriptional level from the promoter 5’ to ureI (p_{ureI}). Our previous study demonstrates that CodY represses p_{ureI} by binding to a CodY box 5’ to p_{ureI} and the repression is more pronounced in cells grown at pH 7 than in cells grown at pH 5.5. Recent sequence analysis revealed a putative VicR consensus and two GlnR boxes 5’ to the CodY box. The results of DNA affinity precipitation assay, electrophoretic mobility shift assay, and chromatin immunoprecipitation-PCR analysis confirmed that both GlnR and VicR interact with the predicted binding sites in p_{ureI}. Isogenic mutant strains (vicRKX null and glnR null) and their derivatives (harboring S. salivarius vicRKX and glnR, respectively) were generated in a recombinant Streptococcus gordonii strain harboring a p_{ureI}-chloramphenicol acetyltransferase gene fusion on gtfG to investigate the regulation of VicR and GlnR. The results indicated that GlnR activates, whereas VicR represses, p_{ureI} expression. The repression by VicR is more pronounced at pH 7, whereas GlnR is more active at pH 5.5. Furthermore, the VicR box acts as an upstream element to enhance p_{ureI} expression in the absence of the cognate regulator. The overall regulation by CodY, VicR, and GlnR in response to pH ensures an optimal expression of urease in S. salivarius when the enzyme is most needed.

IMPORTANCE

Dental plaque rich in alkali-producing bacteria is less cariogenic, and thus, urease-producing Streptococcus salivarius has been considered as a therapeutic agent for dental caries control. Being one of the few ureolytic microbes in the oral cavity, S. salivarius strain 57.1 promotes its competitiveness by mass-producing urease only at acidic growth pH. Here, we demonstrated that the down-regulation of the transcription of the ure operon at neutral pH is controlled by a two-component system, VicRKX, whereas the upregulation at acidic pH is mediated by the global transcription regulator of nitrogen metabolism, GlnR. In the absence of VicR-mediated repression, the α subunit of RNA polymerase gains access to interact with the AT-rich sequence within the operator of VicR, leading to further activation of transcription. The overall regulation provides an advantage for S. salivarius to cope with the fluctuation of environmental pH, allowing it to persist in the mouth successfully.

KEYWORDS: GlnR, Streptococcus salivarius 57.1, two-component system VicRKX, urease, pH regulation

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Urease is a Ni²⁺-dependent metalloenzyme that generally consists of three subunits, α, β, and γ, encoded by ureC, -B, and -A, respectively (1). An exception is found in *Helicobacter pylori*, in which the α subunit (UreA) is encoded by a fusion of the ureA and ureB genes seen in other bacterial urease systems (2). The assembly of a catalytically active urease requires the products of ureE, -F, -G, and -D, known as accessory genes that encode proteins required for the incorporation of nickel into the metallocenter within the active site. Some of the bacterial urease operons contain genes encoding uptake systems for urea and Ni²⁺. For example, a H⁺-gated urea transporter is encoded by ureL in *H. pylori* (3). The *Streptococcus salivarius* strain 57.1 ureMQO genes encode a Ni²⁺-specific ATP binding cassette transporter (4). Although bacterial ureases are highly conserved, the expression of bacterial urease operons is regulated by various mechanisms. For instance, *Bacillus subtilis* and *Sporosarcina ureae* express urease constitutively, whereas the urease expression in *Proteus mirabilis* is activated by a urease-specific activator, UreR, in the presence of urea (5). On the other hand, the expression of the urease operon in *Bacillus subtilis* is regulated by GlnR, TnrA, CodY, and PucR in response to nitrogen availability (6, 7).

Urea is present abundantly in the saliva and crevicular fluid in healthy individuals (8). Thus, ureolysis by bacterial ureases to produce ammonia and CO₂ is the primary alkali source for the oral cavity, which plays a key role in plaque pH homeostasis and dental caries prevention (9). Among the oral microflora, *S. salivarius* is the most dominant and highly ureolytic species (10). Genes encoding a functional urease are arranged as an operon in *S. salivarius* 57.1 (ureLABCEFGDMQO) (4, 11, 12). A previous study using the chemostat culture system indicates that the expression of *S. salivarius* urease is enhanced by acidic growth pH, excess amounts of carbohydrates, and high growth rates (13). Expression analyses demonstrate that the differential expression of the urease operon in response to growth conditions is regulated mainly at the transcriptional level via a ureI-dependent promoter located 5’ to ureL (12). Analysis of the cis elements of pₜₐ₇ₐᵡ₄ₐᵡ reveals that the 21-bp region immediately 5’ to the −35 element of pₜₐ₇ₐᵡ₄ₐᵡ is responsible for the repression of pₜₐ₇ₐᵡ₄ₐᵡ whereas the 40-bp region further upstream participates in the positive regulation of pₜₐ₇ₐᵡ₄ₐᵡ (14). Furthermore, the regulation of pₜₐ₇ₐᵡ₄ₐᵡ in response to pH is also present in the recombinant nonureolytic *Streptococcus gordonii* strain CH1, which harbors a pₜₐ₇ₐᵡ₄ₐᵡ-dependent promoter. For example, a pH-gated urea transporter is encoded by ureIABCEFGDMQO, and ureI, known as the master regulatory system for cell wall metabolism, cell viability, biofilm formation, genetic competence, acid stress response, and oxidative stress response (16, 21–23). This system is essential for the viability of several streptococcal species (16, 23), with the exception of *S. gordonii* CH1 (22).

GlnR, a member of the MerR family of regulators, is the key regulator for nitrogen metabolism in most Gram-positive bacteria (24). The optimal DNA binding activity of GlnR requires feedback-inhibited glutamine synthetase (FBI-GS) in *B. subtilis* (25). Generally, GlnR represses the expression of the GlnR regulon under nitrogen excess (26). A recent study by Chen and colleagues demonstrates that GlnR is activated at acidic growth pH in *Streptococcus mutans*, and the repression of the GlnR regulon at
acidic pH shifts the metabolism from glutamine synthesis to ATP generation to enhance acid tolerance (19).

The regulation by VicR and GlnR of $p_{ureI}$ expression under different growth conditions was investigated in this study. We found that the regulation by VicR and GlnR of $p_{ureI}$ is modulated by the growth pH. GlnR activates the expression of $p_{ureI}$ at acidic pH, whereas VicR represses $p_{ureI}$ activity. In the absence of VicR, the AT tract within the VicR box of $p_{ureI}$ acts as an UP element to further enhance $p_{ureI}$ expression.

RESULTS

Both VicR and GlnR bind directly to the 5′ flanking region of $p_{ureI}$. Recent sequence analysis identified a VicR box and two putative GlnR boxes in $p_{ureI}$ (Fig. 1). The VicR box, 5′-TGTAATGATTGcaaaAT, differs by 3 bases (indicated by lowercase letters) from the consensus derived from $S. mutans$ (16). The 3′ end of the VicR box overlaps the 5′ end of the CodY box by 4 bp. GlnR box 1 (5′-TGTTAGCTTGACTAAt) and GlnR box 2 (5′-TGTCATTTTTGACc) are 3 bases apart and differ from the GlnR box consensus of $S. mutans$ by 1 and 2 bases (indicated by lowercase letters), respectively.

A DNA affinity precipitation assay (DAPA) was performed to investigate whether the endogenous VicR interacts with $p_{ureI}$. A VicR-specific signal was detected with the probe containing the putative VicR box, and the signal was abolished completely when a probe with mutations in the VicR box was used, confirming the binding specificity of VicR (Fig. 2A). As both direct and indirect interaction with the target DNA could lead to a positive result in the DAPA, an electrophoretic mobility shift assay (EMSA) was performed to verify whether VicR binds directly to the predicted VicR box in $p_{ureI}$. A shift of the VicR box-specific probe was observed with 0.8 μM MalE-VicR, and a dose-dependent increase in the intensity of the signal was observed with this probe (Fig. 2B), indicating that MalE-VicR bound directly to the target. When an unlabeled probe in 300-fold excess was used in the reaction mixture, no shift was observed, confirming the binding specificity of VicR to $p_{ureI}$. The recombinant MalE protein alone failed to bind to the probe (data not shown). Finally, the in vivo interaction between VicR and $p_{ureI}$ was verified by chromatin immunoprecipitation (ChIP)-PCR assay, and the result confirmed the interaction between VicR and $p_{ureI}$ (Fig. 2C).

The same approach was used to investigate the interaction between GlnR and the putative GlnR boxes described above. Similarly, specific interactions between endogenous GlnR and the probes containing the putative GlnR box 1 and GlnR box 2, respectively, were observed in the DAPA (Fig. 3A). The binding of GlnR to the putative GlnR boxes was further confirmed by EMSA (Fig. 3B). A shift was seen with probes specific for each of the GlnR boxes, and a dose-dependent enhancement was seen with the probe specific for GlnR box 1 with increasing amounts of MalE-GlnR. Although a signal with high intensity was seen with the probe specific for GlnR box 2 in the presence of 0.8 μM MalE-GlnR, no signal was detected in the reaction mixtures with smaller amounts of MalE-GlnR. Thus, the relative degrees of affinity of GlnR for these two targets remain unclear. When an unlabeled probe in 300-fold excess was used in
the reaction mixture, no shifted band was detected. As above, the recombinant MalE protein failed to interact with both probes, confirming the binding specificity of GlnR (data not shown). Notably, the addition of glutamine and glutamine synthetase to the EMSA reaction mixture did not enhance the binding (data not shown), indicating that, unlike in *B. subtilis*, FBI-GS was not required for the binding activity of *S. salivarius* GlnR.

Finally, the *in vivo* interaction between GlnR and p*ureI* was confirmed by the ChIP-PCR assay (Fig. 3C).

VicRKX represses the transcription of p*ureI*. A vicR-deficient strain is needed to investigate whether VicR is involved in the urease regulatory circuit. However, we failed to generate a vicR-null recombinant strain in *S. salivarius* 57.1. Several studies have shown that VicR is essential for the viability of several oral streptococci (16, 23), and thus, it is possible that VicR plays a similar role in *S. salivarius* 57.1. To circumvent this difficulty, we first investigated the regulatory function of VicRKX in p*ureI* expression by using a promoter fusion with mutations in the predicted VicR box. A derivative of *S. salivarius* strain MC308 was constructed, strain MC308_mVicR_box, in which the sequence of −64 to −59 of p*ureI* (5′-TGTTAAA) in the p*ureI*–cat fusion was mutated to 5′-GTCGAC. Notably, the CodY box in this fusion remains untouched. Both strain MC308 and strain MC308_mVicR_box were cultivated in brain heart infusion (BHI) at pH 7.5 and pH 5.5. A 4-fold increase in CAT activity was observed in strain MC308_mVicR_box compared to its activity in strain MC308 in cells grown at pH 7.5, whereas only a 2.1-fold increase was detected in cells grown at pH 5.5 (Fig. 4A), indicating that the putative VicR box was involved in the negative regulation of p*ureI* and the repression was more evident at pH 7.5.

Since a vicRKX-null strain is available in *S. gordonii* CH1 (22), we examined p*ureI* expression in a vicRKX-null derivative of *S. gordonii* strain SL17 (strain SL17_ΔvicRKX) and its derivative that harbors the vicRKX operon of *S. salivarius* 57.1 (strain...
SL17_CΔvicRKX). Notably, S. gordonii SL17 harbors a single copy of the \( p_{\text{ureI}}-\text{cat} \) fusion on \( gtfG \). In agreement with the \( cis \) element analysis, elevated CAT activity was detected in the \( vicRKX\)-null background at both pH 7.5 and pH 5.5. A wild-type level of CAT activity was observed in strain SL17_CΔvicRKX, confirming that VicR repressed the transcription of \( p_{\text{ureI}} \) (Fig. 4B). It was also noted that the CAT activity of strain SL17_CΔvicRKX was consistently lower (approximately 30%) than that of strain SL17, suggesting that \( S. salivarius \) VicR represses \( p_{\text{ureI}} \) expression more efficiently than \( S. gordonii \) VicR does.

To investigate the potential effects of growth pH and glucose concentration on the regulation of VicR, strains SL17_CΔvicRKX and SL17ΔvicRKX were grown in a chemostat, a culture system that allows tight control of the growth pH and glucose concentration. The CAT activities in cells grown at pH 7 and pH 5.5 with 20 and 100 mM glucose were examined. At pH 7, a 4.7-fold increase in CAT activity was seen in SL17ΔvicRKX compared to the activity in SL17_CΔvicRKX in the presence of 20 mM glucose, but comparable levels of CAT activity were detected between these two strains when cells were grown with 100 mM glucose (Fig. 4C). On the other hand, 1.8- and 1.5-fold increases in activity were seen in cells grown at pH 5.5 with 20 mM and 100 mM glucose, respectively (Fig. 4C). Collectively, the activity of VicR was modulated by both pH and carbohydrate concentration and VicRKX repressed \( p_{\text{ureI}} \) most effectively at neutral pH under glucose limitation.

**GlnR activates \( p_{\text{ureI}} \) more strongly at pH 5.5.** A \( glnR \)-deficient strain is essential to investigate how GlnR regulates urease expression. Unfortunately, multiple attempts...
failed to generate a glnR-null mutant strain in S. salivarius 57.I, indicating that mutations in glnR are also lethal in S. salivarius 57.I. Thus, we initiated the study by using p_{ureI}-cat fusions with mutations in the putative GlnR boxes, since the interaction between GlnR and the putative GlnR boxes 5' to p_{ureI} has been confirmed (Fig. 3). Previous promoter deletion analysis indicates that a 40-bp region 22 bases 5' to the /H11002 35 element of p_{ureI} exhibits a positive effect on p_{ureI} transcription (14). This region contains the 3' portion of GlnR box 2 and the entire GlnR box 1, suggesting that GlnR activates p_{ureI} expression. As a positive effect would be more evident in a repressor-free host, i.e., strain S. salivarius ΔcodY, the activity of all promoter derivatives was examined in the codY-deficient background. In agreement with the hypothesis, mutations in the putative GlnR boxes reduced CAT activity in batch-grown cells at both pH 7.5 and pH 5.5 and the reduction is slightly more evident at pH 5.5 (Fig. 5A), suggesting that GlnR acts as an activator for p_{ureI} expression and its regulatory activity on p_{ureI} is modulated by the growth pH.

**FIG 4** The VicRKK two-component system represses p_{ureI} expression. (A) Effect of the putative VicR box on p_{ureI} activity. The p_{ureI}-cat activities in batch-grown S. salivarius MC308 and MC308_mVicR_box were determined by the CAT assay. Both strains were grown in BHI containing 50 mM KPO4 at pH 7.5 or BHI-HCl at pH 5.5. (B) Effect of VicRKK on p_{ureI} activity. The CAT activities in batch-grown S. gordonii SL17, SL17 ΔvicRKK, and SL17 ΔvicRKK at pH 7.5 and pH 5.5 were examined. (C) Effect of growth pH and glucose concentration on p_{ureI} activity. The CAT activities in chemostat-grown SL17, SL17 ΔvicRKK and SL17 ΔvicRKK were examined in cells grown at pH 7 and pH 5.5, with 20 mM and 100 mM glucose (Glc). The specific activity (Sp. Act.) was calculated as nmol Cm acetylated min^{-1} mg^{-1} total protein. Values are the mean results and standard deviations from three independent experiments. Significant differences between strains were analyzed using one-way ANOVA. ***, P < 0.001.

**FIG 5** GlnR positively regulates p_{ureI}. (A) The p_{ureI}-cat activity in S. salivarius MC308 ΔcodY, ΔcodY mGlnR_box-1, and ΔcodY mGlnR_box-2. All strains were grown in BHI at pH 7.5 and pH 5.5. (B) The p_{ureI}-cat activity in S. gordonii SL17, SL17 ΔglnR, and SL17 ΔglnR. Cells were grown in BHI at pH 7.5 and pH 5.5. (C) Levels of p_{ureI}-cat activity in chemostat-grown S. gordonii SL17, SL17 ΔglnR and SL17 ΔglnR. The specific activities (Sp. Act.) were expressed as indicated in the legend to Fig. 4. The values are the mean results and standard deviations from three independent experiments. Significant differences between strains were analyzed using one-way ANOVA. **, P < 0.01; ***, P < 0.001.
As stated above, the nonureolytic *S. gordonii* strain seems to be a logical alternative host for studying $p_{\text{ure}}$ regulation, and luckily, a *glnR*-null *S. gordonii* derivative could be obtained. Thus, the effect of GlnR on $p_{\text{ure}}$ expression was examined in *S. gordonii* by using a similar approach as for analyzing VicR regulation. The CAT activities in the *glnR*-null *S. gordonii* SL17 (strain SL17_Δ*glnR*) and its derivative harboring the *glnR* gene of *S. salivarius* 57.I (strain SL17_Δ*glnR*) were examined in batch-grown cells at pH 7.5 and pH 5.5. In agreement with the cis element analysis, the CAT activity in strain SL17_Δ*glnR* was lower than the levels in strains SL17 and SL17_Δ*glnR* at both pH 7.5 and pH 5.5, indicating that GlnR activates $p_{\text{ure}}$ expression (Fig. 5B). It was also noticed that the CAT activity of strain SL17_Δ*glnR* was consistently higher (approximately 40%) than that of strain SL17, suggesting that *S. salivarius* GlnR works more efficiently on $p_{\text{ure}}$ than *S. gordonii* GlnR does.

To further investigate the effects of growth pH and carbohydrate concentration on the regulation of GlnR on $p_{\text{ure}}$, strains SL17_Δ*glnR* and SL17_Δ*glnR* were cultivated in a chemostat at pH 7 or pH 5.5 with 20 or 100 mM glucose. At pH 7, comparable expression levels were observed in strains SL17_Δ*glnR* and SL17_Δ*glnR* under glucose limitation (20 mM), whereas a 1.8-fold increase in CAT activity was detected in strain SL17_Δ*glnR* compared to that in strain SL17_Δ*glnR* under glucose excess (100 mM). At pH 5.5, 4-fold and 3.2-fold increases in CAT activity were seen in strain SL17_Δ*glnR* compared to the levels in strain SL17_Δ*glnR* under 20 mM and 100 mM glucose, respectively (Fig. 5C). These results indicated that the activation by GlnR of $p_{\text{ure}}$ was modulated by both carbohydrate concentration and growth pH, and the activation was most pronounced at pH 5.5.

**The VicR box acts as a UP element of $p_{\text{ure}}$.** As the VicR box is rich in AT, it is hypothesized that this region could act as a UP element to enhance the activity of RNA polymerase in the absence of the cognate repressor. Thus, the VicR box in the 5' flanking region of $p_{\text{ure}}$ was mutated in strain SL17_ΔvicRKX to verify the possibility. Notably, the CodY box remains intact in this mutant strain. Mutations in the VicR box downregulated the CAT activity in strain SL17_ΔvicRKX at both pH 7.5 and pH 5.5 (Fig. 6A), suggesting that this region exhibited a positive effect on $p_{\text{ure}}$ expression in the absence of VicR. To further investigate whether this region could interact with the C-terminal domain (CTD) of the RNA polymerase α subunit (α-CTD), EMSA was carried out with a probe of 21 bp covering the entire VicR box. The result indicated that the MalE-tagged α-CTD recombinant protein interacted with the probe, and the shift was abolished when an unlabeled probe in 300-fold excess was included in the reaction mixture (Fig. 6B), confirming that the VicR box acted as a UP element to enhance $p_{\text{ure}}$ expression.

**DISCUSSION**

Urease expression in both *H. pylori* and *S. salivarius* is upregulated during growth at acidic pH; however, *H. pylori* activates the expression at acidic pH by the activity of NikR and ArsR (27–29), which is different from the repression by CodY and VicR at neutral pH in *S. salivarius*. It seems most cost-effective for *H. pylori* to activate urease expression at acidic pH, as the pH of the stomach is generally below pH 5. On the other hand, the pH of oral mucosal pH is close to neutral normally (30), and therefore, *S. salivarius* gains the greatest advantage by repressing urease expression at neutral pH. The repression of *S. salivarius* $p_{\text{ure}}$ by the VicRKX system was most evident in cells cultivated at pH 7 with 20 mM glucose and less evident at pH 5.5, but surprisingly, the repressive effect was absent when cells were cultivated at pH 7 with 100 mM glucose, raising the possibility that the VicRKX system is insensitive to environmental pH under glucose excess. An attempt was made using ChIP-quantitative PCR to verify the DNA binding activity of VicR under different growth pH conditions in batch-grown *S. salivarius* 57.I. More VicR binding was detected in cells grown at pH 7.5 than at pH 5.5 (data not shown), suggesting that VicR is more active at neutral pH. Thus, the absence of repression of $p_{\text{ure}}$ by VicR at pH 7 under 100 mM glucose may result from the repression of an additional regulatory protein that exerts regulation mainly at neutral pH under glucose.
excess, and/or the repression by this regulatory protein is augmented in the absence of VicR under this growth condition. Our recent observations suggested that the catabolite control protein A (CcpA) also participates in the regulation of \( p_{\text{ureI}} \) expression.

Inactivation of \( \text{ccpA} \) led to upregulation of \( p_{\text{ureI}} \) in cells grown at pH 7 with 100 mM glucose, but only marginal upregulation was seen in cells grown at pH 7 with 20 mM glucose (unpublished data). The regulation by CcpA of \( p_{\text{ureI}} \) is not understood currently, as no CcpA binding consensus element is found in the flanking region of \( p_{\text{ureI}} \). However, the effect of CcpA in response to carbohydrate concentration at pH 7 may explain the absence of upregulation in SL17_\( \Delta \text{vicRKX} \) grown at pH 7 with 100 mM glucose.

It is intriguing that \( p_{\text{ureI}} \) was also repressed by the VicRKX system at pH 5.5 regardless of the glucose concentration, if neutral pH is required to activate the system. A study in \( S. \text{gordonii} \) indicates that inactivation of VicR reduces the tolerance of \( S. \text{gordonii} \) for oxidative stresses (22). Furthermore, studies in \( S. \text{mutans} \) have suggested that acidic growth pH could induce the oxidative stress response (31, 32). Specifically, the expression levels of genes encoding enzymes metabolizing reactive oxygen species, e.g., \( \text{sod} \), \( \text{ahpC} \), and \( \text{ahpF} \), are upregulated in chemostat-grown \( S. \text{mutans} \) at pH 5.

**FIG 6** Functional analysis of the VicR box as a UP element to enhance \( p_{\text{ureI}} \) expression. (A) The expression of \( p_{\text{ureI-cat}} \) in \( S. \text{gordonii} \) SL17, SL17_\( \Delta \text{vicRKX} \), and \( \Delta \text{vicRKX} \_m\text{VicR_box} \) strains was detected in cells grown in BHI at pH 7.5 and pH 5.5. The specific activity (Sp. Act.) was expressed as indicated in the legend to Fig. 4. Values are the mean results and standard deviations from three independent experiments. Significant differences between the results for strains SL17_\( \Delta \text{vicRKX} \) and \( \Delta \text{vicRKX} \_m\text{VicR_box} \) were analyzed using one-way ANOVA. ***, \( P < 0.001 \).**

(B) EMSA of \( \alpha-\text{CTD} \) binding to the VicR box in \( p_{\text{ureI}} \). Lane 1, probe only; lanes 2 to 6, 0.25 to 4 \( \mu \text{M MalE-}\alpha-\text{CTD} \) in 2-fold increments; lane 7, 4 \( \mu \text{M MalE-}\alpha-\text{CTD} \) with a specific competitor. All reactions were carried out with 0.01 pmol biotin-labeled probe.
compared to their expression levels in cells grown at pH 7 (31). Thus, the activity of VicR at pH 5.5 may be part of the oxidative stress response.

The results of site-directed mutagenesis of the GlnR boxes and p\textsubscript{ureI} expression in chemostat cultures (Fig. 5) suggest that growth pH modulates the activity of GlnR. Although it is not understood how the acidic pH activates the DNA binding activity of GlnR, the activation by acidic pH is not unique to \textit{S. salivarius}. A recent study in \textit{S. mutans} demonstrates that the repression of GlnR is activated at pH 5.5 (19). Thus, GlnR of oral streptococci is likely to be activated by both excess amounts of the nutrient nitrogen and acidic growth pH. Additionally, the presence of more than one GlnR box in the promoter region is also not unique to p\textsubscript{ureI}. For instance, the \textit{glnRA} operon, the \textit{ureABC} operon, and \textit{tnrA} of \textit{B. subtilis} all possess two GlnR boxes in the promoter regions (7, 18, 33), and cooperative binding of GlnR to the two GlnR boxes, 6 bp apart, has been demonstrated in the promoter of \textit{glnRA} (33). As both GlnR box 1 and GlnR box 2 are required for GlnR-dependent activation (Fig. 5A), these two sites may participate in cooperative binding of GlnR. Furthermore, the positive regulation of GlnR in p\textsubscript{ureI} transcription is similar to the activity of TnrA in \textit{B. subtilis} (26). As a tnrA homolog is absent in most streptococcal species with known genomes (8, 34–36) and studies in \textit{Lactococcus lactis} and \textit{Streptococcus pneumoniae} have shown that GlnR carries out some of the functions that are exerted by TnrA in \textit{B. subtilis} (37, 38), GlnR of \textit{S. salivarius} is likely to function in the same way.

Studies in \textit{S. mutans} have demonstrated that chemostat-grown cells under glucose limitation have an excess of amino acid nutrients at a high growth rate (\(D = 0.4\times^{-1}\)) (39). Reduced expression of \textit{glnRA} was observed in chemostat-grown \textit{S. salivarius} S7.I supplemented with 20 mM glucose compared to that with 100 mM glucose (data not shown), suggesting that the nutrient nitrogen is likely to be in excess under glucose limitation. If this is true, it is expected that the activation of p\textsubscript{ureI} transcription by GlnR should be more pronounced under glucose limitation than under glucose excess. However, we did not observe downregulation of p\textsubscript{ureI} in strain SL17_Δ\textit{glnR} cultivated at pH 7 under 20 mM glucose, suggesting that repressor(s) which are most active at pH 7 with limited sugar supply could mask the activation by GlnR. Based on what we have learned, the repression is likely to be governed by CodY (15) and VicR (Fig. 4B).

A working model for the regulatory network governing urease expression is proposed (Fig. 7). CodY and VicR inhibit urease expression via binding to the cognate operators in the 5’ flanking region of p\textsubscript{ureI} at neutral pH to avoid overalkalinization of the oral cavity. GlnR activates p\textsubscript{ureI} transcription via binding to the GlnR boxes at acidic pH 5.5. Thus, the activity of VicR at pH 5.5 may be part of the oxidative stress response.

The CodY box, VicR box, and GlnR boxes are indicated by black, gray, and white squares, respectively. The model suggests that CodY and VicR repress p\textsubscript{ureI} expression at pH 7.0, whereas GlnR activates the expression at pH 5.5. In the absence of CodY and VicR, the AT tract in both the CodY box and the VicR box could act as a UP element to enhance p\textsubscript{ureI} expression.
pH to enhance acid tolerance. In the absence of the cognate repressors, both the CodY and VicR boxes could act as UP elements to enhance $p_{ure}$ expression. The complex regulation of the urease operon by these regulators links nitrogen metabolism and the acid stress response, which could ensure optimal fitness of *S. salivarius* against environmental stresses.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and general genetic manipulations.** The bacterial strains used in this study are listed in Table 1. *S. salivarius* 57.I, *S. gordonii* CH1, and their derivatives were grown routinely in BHI (Difco) at 37°C under 10% CO$_2$ atmosphere. When necessary, kanamycin (Km) was added to the culture medium at 1,000 and 250 μg·mL$^{-1}$ for recombinant *S. salivarius* and *S. gordonii* strains, respectively. When necessary, 750 μg·mL$^{-1}$ of spectinomycin (Sp) and 5 μg·mL$^{-1}$ of erythromycin (Em) were used for other recombinant streptococcal strains. Recombinant *Escherichia coli* strains were maintained in L broth supplemented, where indicated, with Sp at 50 μg·mL$^{-1}$. To obtain batch cultures grown under neutral or acidic pH conditions, cells were cultivated to mid-exponential phase (optical density at 600 nm [OD$_{600}$] of 0.6) in BHI containing 50 mM KPO$_4$ at pH 7.5 and in BHI that was adjusted to pH 5.5 by the addition of 2 N HCl, respectively. For continuous-culture studies, recombinant *S. gordonii* strains were grown in a Biostat B plus bioreactor (Sartorius Stedim Biotech) at a dilution rate ($D$) of 0.3 h$^{-1}$ (generation time, 2.3 h) in medium containing 3% tryptone and 0.5% yeast extract (TY) (13). Cultures were kept at a specific growth condition for at least 10 generations to reach steady state. Furthermore, when 20 mM glucose was included in the medium, glucose was undetectable in the culture supernatant, whereas approximately 50 mM glucose remained in the culture supernatant when 100 mM glucose was used. Thus, 20 and 100 mM glucose present glucose limitation and excess, respectively.

The oligonucleotides used in this study are listed in Table 2. Synthetic DNA oligonucleotides were purchased from Genomics BioSci & Tech (Taiwan) and Integrated DNA Technologies (Singapore). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (NEB). PCR primers were performed with the high-fidelity DNA polymerase Blend Taq plus (Toyobo).

**Purification of recombinant proteins and generation of polyclonal antisera.** The coding region of VicR was PCR amplified from *S. salivarius* 57.I using primers VicR BamHI S and VicR PstI AS and cloned into pH300 (Qiagen) in *E. coli* M15. The identity of the recombinant plasmid was confirmed by sequencing analysis. The recombinant His-tagged VicR (His-VicR) was induced and purified under denaturing conditions using the standard methods. The identity of the recombinant protein was confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis. The concentration of the purified protein was determined by Bio-Rad protein assay based on the method of Bradford (40). Using a similar approach, the coding region of GlnR was amplified from *S. salivarius* 57.I, using primers GlnR BamHI S and GlnR PstI AS, and cloned into pMAL-c2X (NEB). The purified His-VicR and His-GlnR were used to generate polyclonal antisera in rabbits by LTK BioLaboratories (Taiwan). The titers and specificities of all antisera were tested by immunoblotting.

To purify recombinant VicR and GlnR under the native condition, constructs to produce maltose binding protein-tagged VicR (MalE-VicR) and GlnR (MalE-GlnR) were generated by using pMAL-c2X (NEB). The recombinant proteins were purified by amylose affinity chromatography (NEB) and verified by MALDI-TOF analysis prior to performing the EMSA analysis.

**DAPA and Western blot analysis.** All probes used in DAPA are generated by annealing two biotin-labeled, complementary oligonucleotides. The oligonucleotides were labeled by using the Pierce biotin 3‘-end DNA labeling kit. Mid-exponential phase cultures (OD$_{600}$ of ~0.6) of cultures of *S. salivarius* 57.I were harvested, washed once with an equal volume of 10 mM Tris (pH 7.6), and then resuspended in 1/100 of the original culture volume in the DAPA binding buffer (60 mM KCl, 10 mM Tris-HCl [pH 7.6], 5% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol [DTT]). Concentrated cell suspensions were subjected to...
mechanical disruption in the presence of an equal volume of glass beads (0.1 mm in diameter) by homogenization in a BeadBeater (BioSpec Products) for a total of 120 s at 4°C. Amounts of 1 mg and 500 μg of the total lysate were incubated with 20 nM biotin-labeled DNA probes specific for the VicR box and for the GlnR boxes, respectively, in the DAPA binding buffer. The binding reaction was carried out under rotation at 4°C for 1 h. The DNA-protein complexes were captured by using 50 μl of streptavidin MagneSphere paramagnetic particles (Promega). The mixture was incubated at 4°C for 1 h, followed by five washes with the binding buffer. Finally, the proteins of the DNA-protein complexes were eluted in electrophoresis sample buffer, separated on 12% SDS–PAGE, and then detected by immunoblotting. Anti-VicR and anti-GlnR antisera were used at 1:2,000 and 1:20,000 dilution, respectively. The signals were

### TABLE 2 Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7.1_VicR_XhoI_S</td>
<td>GAAATACTCCGAGTTATTTTACT</td>
<td>PCR for SL17_CΔvicRKX construction</td>
</tr>
<tr>
<td>S7.1_VicX_Sphl_AS</td>
<td>GAGGAATCCGAGGTAGCAAGTCAA</td>
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</tr>
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<td>CH1_GtfG_S</td>
<td>GCTACTCAAGTGGCATACAGG</td>
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</tr>
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<td>CH1_GtfG_BamHI_AS</td>
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<td></td>
</tr>
<tr>
<td>CH1_GtfG_XhoI_S</td>
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<td></td>
</tr>
<tr>
<td>CH1_Spec_AS</td>
<td>CTCCGACAATGAAGTACGCCGCGG</td>
<td></td>
</tr>
<tr>
<td>CH1_PicR_box_1_S</td>
<td>CATCTGAGGCTGCCCCGAGCATCA</td>
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<tr>
<td>CH1_VicR_XhoI_AS</td>
<td>ATCGGATCCGACGACTATGCACTAAG</td>
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</tr>
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<td>S7.1_GlnR_Ncol_S</td>
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<td></td>
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<tr>
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<td>GGAATGTAAGCTATCTATCAGGCA</td>
<td>PCR for His-GlnR construction</td>
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<td>CH1_SG0_012_C</td>
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<td>Inverse PCR to mutate GlnR box 1</td>
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<tr>
<td>CH1_GlnR_XhoI_S</td>
<td>TGGCTAGATCCGACGACTAGT</td>
<td>Inverse PCR to mutate GlnR box 2</td>
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<tr>
<td>CH1_GlnR_Sphl_S</td>
<td>CTGATAGGTCGATGCTAGT</td>
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<tr>
<td>CH1_GlnA1_S</td>
<td>ACATGACGATGCAATCTTAGTA</td>
<td>EMSA probe for analyzing the binding of α-CTD to VicR box</td>
</tr>
<tr>
<td>EMSA_CTS_121_S</td>
<td>CCTGTAATAGTGAAGCTAAC</td>
<td>DAPA and EMSA probe for analyzing the binding of GlnR to the GlnR box</td>
</tr>
<tr>
<td>GlnR_Pstl_S</td>
<td>AGTTCTTCAGCTATTAGATACGCGAAG</td>
<td>PCR for MalE-VicR construction</td>
</tr>
<tr>
<td>GlnR_BamHI_AS</td>
<td>GAGGCCATGGATGGCAAGAGAGAAAGA</td>
<td>Inverse PCR to mutate VicR box in S7.1</td>
</tr>
<tr>
<td>GlnR_box_1_Sall_S</td>
<td>TGGATCCGACGACTATGCACTAAG</td>
<td>Inverse PCR to mutate VicR box in CH1</td>
</tr>
<tr>
<td>GlnR_box_1_Sall_AS</td>
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<tr>
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<tr>
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<tr>
<td>pMAL_VicR_EcoRl_S</td>
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<td>Used in ChIP-PCR</td>
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<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>pMAL_GlnR_Pstl_AS</td>
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<td></td>
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<tr>
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</tr>
<tr>
<td>purel_VicR_box_Sall_S</td>
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<td>Inverse PCR to mutate VicR box in S7.1</td>
</tr>
<tr>
<td>purel_VicR_box_Sall_AS</td>
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<td>Inverse PCR to mutate VicR box in CH1</td>
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<td>purel_320_Small AS</td>
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</tr>
<tr>
<td>VicR_BamHI_S</td>
<td>GGTGACCGAATGCCGAATCTAAGAAGC</td>
<td>DAPA and EMSA probe for analyzing the binding of VicR to the VicR box</td>
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<tr>
<td>VicR_Pstl_AS</td>
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<tr>
<td>VicR_box_S</td>
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<td>DAPA and EMSA probe for analyzing the binding of VicR to the VicR box</td>
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<tr>
<td>VicR_box_S</td>
<td>TGGATCCGACGACTATGCACTAAG</td>
<td>DAPA and EMSA probe for analyzing the binding of VicR to the VicR box</td>
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<td>VikR_box_S</td>
<td>TGGATCCGACGACTATGCACTAAG</td>
<td>DAPA and EMSA probe for analyzing the binding of VicR to the VicR box</td>
</tr>
</tbody>
</table>

*Inserted restriction recognition sites and mutated sequences are underlined.*
detected by horseradish peroxidase-conjugated anti-rabbit IgG (GeneTex) and luminol-based reagents (Merck Millipore).

EMSA and ChIP-PCR. Two biotin-labeled, complementary oligonucleotides containing the target site were annealed and used in EMSA. An amount of 0.01 pmol of the annealed probe was incubated with increasing amounts of recombinant MalE–VicR, MalE–GlnR, and MalE–α-CTD in the EMSA binding buffer [50 mM Tris-Cl (pH 7.4), 50 mM KCl, 2 mM MgCl₂, 100 ng poly(dI-dC), and 0.5 μg bovine serum albumin (BSA)]. All reactions were carried out at 4°C for 30 min, and the products were resolved on 6% nondenaturing polyacrylamide gels. Specific competition was carried out by including the same probe without labeling in a 300-fold excess. The DNA-protein complex was electrotransferred to a piece of Hybond blotting membrane (Amersham), and the signal was detected by the chemiluminescent nucleic acid detection module kit (Pierce).

ChIP-PCR assay was performed as previously described (15). The PCR was carried out by using primers pure₁₋₅_S and pure₁₋₅_AS.

Construction of a pΔerm-cat fusion and its derivatives in S. gordonii CH1 and S. salivarius 57.I. The pΔerm-cat fusion was tagged with an Sp resistance gene (spe) (41) and cloned into the integration vector for S. gordonii CH1, pMJB6 (14), which allows the integration of the promoter fusion at gtfG. The resulting plasmid, pSL16, was introduced into S. gordonii CH1 by transformation (42), and the double-crossover recombination was verified by colony PCR. The resulting strain was designated S. gordonii SL17.

The putative VicR box in the pΔerm-cat fusion was mutated by site-directed mutagenesis. Briefly, the primer pairs pure₁₋₅_VicR_box_SalI_S and pure₁₋₅_VicX_S plus pure₁₋₅_SmaI_AS were used in an inverse PCR using PMC300 (15) (for S. salivarius) and pSL16 (for S. gordonii) as the template, respectively. The PCR products were digested, ligated, and established in E. coli. The resulting plasmids were confirmed by sequencing analysis, and the correct constructs were introduced into S. salivarius 57.I by electroporation (12) and into S. gordonii ΔvicRKX by transformation (42). The double-crossover recombination was verified by colony PCR, and the resulting strains are designated S. salivarius MC308mVicR_box and S. gordonii ΔvicRKX_mVicR_box, respectively.

The GlnR boxes in the pΔerm-cat fusion were mutated by a similar approach. Briefly, the primer pairs GlnR_box1_S plus GlnR_box1_AS and GlnR_box2_S plus GlnR_box2_AS were used in PCR to introduce mutations into GlnR box 1 and 2, respectively.

Construction of the S. gordonii vicRKX-deficient strain and its derivative. All recombinant S. gordonii strains were generated by using PCR ligation mutagenesis (43). To inactivate vicRKX in S. gordonii SL17, the 5’ and 3’ flanking fragments of vicR were generated from S. gordonii CH1 by using the primer pairs CH1_VicR_S plus CH1_VicR_XhoI_AS and CH1_VicR_BamHI_S plus CH1_VicR_AS, respectively. The PCR products were digested, ligated, and established in E. coli. The resulting plasmids were confirmed by sequencing analysis, and the correct constructs were introduced into S. salivarius 57.I by electroporation (12) and into S. gordonii ΔvicRKX by transformation (42). The double-crossover recombination was verified by colony PCR, and the resulting strain was designated S. gordonii SL17ΔvicRKX. The region encoding the 41st to 92nd amino acids of VicR in strain SL17ΔvicRKX was replaced by Δerm.

To generate a vicRKX recombinant strain, two fragments for integrating the vicRKX gene of S. salivarius at gtfG were generated from SL17ΔvicRKX by primer pairs CH1_GtfG_S plus CH1_GtfG_BamHI_AS and CH1_GtfG_XhoI_AS plus CH1_GtfG_SalI_S, respectively. A DNA fragment containing the vicRKX operon was amplified from S. salivarius 57.I by PCR using primers 57.I_VicR_XhoI_S and 57.I_VicX_S plus CH1_GtfG_box1_AS. A DNA fragment containing the nonpolar Em resistance gene (erm) from Tn1666ΔG (45), which does not possess a promoter or a transcription terminator, was also prepared by PCR. All PCR products were digested and ligated into a ligation mixture. The ligation mixture was used to transform strain SL17ΔvicRKX, and the allelic exchange event in the Em-resistant transformants was verified by colony PCR. The resulting strain, SL17ΔvicRKXerm, harbors a copy of erm-tagged vicRKX on gtfG.

Construction of the S. gordonii glnR-deficient and derivative strains. The glnR gene of S. gordonii CH1 was inactivated by the method described above. Briefly, the 5’ and 3’ flanking fragments of glnR were generated from S. gordonii CH1 by primer pairs CH1_SGO_0212_S plus CH1_GlnR_XhoI_AS and CH1_GlnR_SalI_S plus CH1_GlnR_AS, respectively. These two fragments were ligated to the 5’ and 3’ ends of a nonpolar erm fragment, and the ligation mixture was used to transform S. gordonii SL17. The double-crossover recombination was verified by colony PCR, and the resulting strain was designated S. gordonii SL17ΔglnR. The region encoding the 12th to 76th amino acids of GlnR in strain SL17ΔglnR was replaced by a nonpolar erm in this strain.

An S. gordonii glnR-derived strain was generated as described above. Briefly, the 5’ and 3’ flanking fragments were generated from SL17ΔglnR by PCR with the primer pairs CH1_GlnR_S plus CH1_GlnR_Ncol_AS and CH1_GlnR_XbaI_S plus CH1_GlnR_AS, respectively. A DNA fragment containing glnR was generated from S. salivarius 57.I by PCR using primers S7I_GlnR_Ncol_S plus S7I_GlnR_BamHI_AS. A ligation mixture of all three fragments and a DNA fragment containing a nonpolar kan cassette (44) that lacks a promoter and a transcription terminator was prepared and used to transform strain SL17ΔglnR. The ligation was prepared to favor the formation of a construct comprising the 5’ flanking fragment followed by glnR of S. salivarius, the kan fragment, and the 3’ flanking fragment. The correct allelic exchange event in the Km-resistant transformants was verified by colony PCR, and the resulting strain was designated S. gordonii SL17ΔglnR.

CAT assay. Total protein lysates from the concentrated cell suspensions were subjected to mechanical disruption as described previously (14). The CAT activities were determined by the method of Shaw (46), and the specific activities were calculated as nmol of Cm acetylated min⁻¹ mg⁻¹.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA) with GraphPad Prism (version 5) software.
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