Multiple Two-Component Systems Modulate Alkali Generation in *Streptococcus gordonii* in Response to Environmental Stresses

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ABSTRACT

The oral commensal *Streptococcus gordonii* must adapt to constantly fluctuating and often hostile environmental conditions to persist in the oral cavity. The arginine deiminase system (ADS) of *S. gordonii* enables cells to produce, ornithine, ammonia, CO$_2$ and ATP from arginine hydrolysis, augmenting the acid tolerance of the organism. The ADS genes are substrate-inducible and sensitive to catabolite-repression, mediated through ArcR and CcpA, respectively, but the system also requires low pH and anaerobic conditions for optimal activation. Here, we demonstrate that the CiaRH and ComDE two-component systems (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 repression by oxygen. The known anaerobic activator of the ADS, Fnr-like protein (Flp), appeared to act independently of the Vic TCS. 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. This study provides the first evidence that TCS can regulate the ADS of bacteria in response to specific environmental signals and reveals some notable differences in the contribution of CiaRH, ComDE and VicRK to viability and stress tolerance between the oral commensal *S. gordonii* and the oral pathogen *Streptococcus mutans*. 
INTRODUCTION

*S. gordonii* colonizes the oral cavity very early in life (9, 26, 52) and its presence in oral biofilms is generally associated with oral health (1, 6, 48, 49). Colonization by *S. gordonii* is believed to be beneficial to the host in large part because of the contribution of this microorganism to pH homeostasis in oral biofilms through the hydrolysis of arginine in saliva and the diet (10). The pH of the oral cavity fluctuates in response to the diet and diurnal rhythms of the host, and adaptation to acidic conditions is important for the survival of *S. gordonii* and other oral biofilm organisms (58).

Residents of oral biofilms also experience other environmental stresses, including modest temperature fluctuations, substantial changes in nutrient source and abundance, and wide variation in oxygen tension and redox potential as oral biofilms mature (25). Despite these challenging conditions, *S. gordonii* is able to persist as a significant proportion of the biofilm populations of the oral cavity (19, 56), and, like some other viridans streptococci, can cause endocarditis (5, 23, 42). Thus, adaptations by *S. gordonii* to conditions in multiple intraoral sites, or in blood and infected heart valves, are essential for persistence.

The arginine deiminase system (ADS), which is present in *S. gordonii* and a number of other abundant commensal oral streptococci, is a three enzyme pathway that converts arginine to ornithine, CO$_2$ and ammonia, with the concomitant generation of ATP (12). The ADS augments acid tolerance in *S. gordonii* by neutralizing the cytoplasm and environment, and the ATP generated can be used for growth, anabolism and to extrude protons (10, 13, 14, 37). A strong association with the arginolytic potential of human dental biofilms and resistance to caries has been documented (45, 60), and organisms that are ADS positive are believed to have a beneficial impact on oral microbial ecology. Mechanistically, the presence of the ADS in oral biofilms is believed to provide a selective advantage to those organisms that possess it and to moderate biofilm
acidification, both of which favor the persistence of a microflora that is compatible with
dental health and discourage the emergence of aciduric organisms associated with
dental caries.

In *S. gordonii*, the genes for the three enzymes of the pathway, arginine deiminase
(*arcA*), ornithine carbamyltransferase (*arcB*) and carbamate kinase (*arcC*) are co-
transcribed in an operon with *arcD* (arginine:ornithine antiporter) and *arcT* (arginine
aminopeptidase). Induction of the ADS genes (*arcABCDT*) by arginine is mediated by a
transcriptional activator encoded by the divergently-transcribed *arcR* gene, located
immediately 3’ to the ADS operon. Immediately 5’ to the ADS operon is a gene for an
Fnr-like protein (*flp*) (13), which activates expression from the *arcA* promoter (*P_{arcA}*)
under anaerobic conditions. Carbohydrate catabolite repression (CCR) of the operon
by preferred carbohydrate sources, such as glucose, is exerted primarily through CcpA
(13, 14). In addition, ADS expression is optimal under acidic conditions, but the basis
for pH-dependent control of transcription has not been investigated (37). Other
complexities in ADS regulation exist in *S. gordonii*, including that queosine modification
of tRNA may impact translation of the genes (37), ADS expression is higher in stationary
phase (37), and other mechanisms for post-transcriptional regulation of expression
appear to exist (37). Because of the spectrum of control mechanisms governing ADS
production and the wide distribution of this system in abundant commensal organisms
and pathogens, analysis of ADS expression has provided many insights into genetic
regulation in streptococci. There is also a high degree of relevance of ADS regulation
to oral biofilm ecology, oral health and disease (10). In spite of substantial progress
understanding control of the ADS, critical gaps remain in our knowledge of how this
system is regulated.

Transcriptional control of gene expression by two component signal transduction
systems (TCSs) is a common mechanism used by bacteria to modulate cell behaviors in
response to environmental conditions (57). A TCS is composed of a histidine kinase that usually detects an environmental signal and a response regulator that can be phosphorylated by the sensor kinase (24). TCSs are involved in the stress responses of many bacteria, including Escherichia coli (15, 50), Pseudomonas aeruginosa (51, 59), Staphylococcus aureus (18, 59), and Streptococcus mutans (4, 8, 28, 54). In S. gordonii, the ComDE TCS was shown to regulate development of genetic competence (22, 40) and was required for efficient in vitro biofilm formation (39). The BfrAB TCS of S. gordonii affects biofilm development and the expression of multiple ABC transporters (27, 64, 65). In addition to ComDE, the CiaRH and VicRK TCS of oral streptococci and some pathogenic streptococci seem particularly important for regulation of traits associated with colonization, growth in the host and pathogenesis (4, 34, 36, 54). Here we investigate whether the CiaRH, ComDE and VicRK TCS are able to influence the expression of the ADS in response to pH and oxidative stress. In addition, we begin to explore whether these TCS, which play key regulatory roles in a variety of critical cellular functions in low G+C Gram-positive bacteria (17, 44, 46), affect traits of S. gordonii that are known to be important for establishment and persistence in the oral cavity.

MATERIALS AND METHODS

Bacterial strains, growth conditions and reagents. S. gordonii DL1 and its derivatives were maintained and passaged in Brain Heart Infusion medium (BHI; Difco Laboratories, Detroit, MI) at 37 °C in 5% CO₂. Recombinant S. gordonii DL1 strains carrying a promoterless chloramphenicol acetyltransferase (CAT) gene (cat) fused to the arcA (P_{arcA}-cat) promoter (37) were selected and maintained on BHI agar supplemented with erythromycin (Em) at 5 µg ml⁻¹. Strains carrying insertion/deletion mutations in the ciaRH, comDE or vicRK genes were selected on BHI agar with kanamycin (Km; 250 µg ml⁻¹). Escherichia coli strains were grown in Luria-Bertani (LB) medium supplemented...
with tetracycline (Tet; 12 µg ml⁻¹), if needed. Preparation of competent cells and
transformation of S. gordonii were done as previously described (32). Chemical
reagents and antibiotics were obtained from Sigma (St. Louis, MO). To monitor
arginine deiminase (AD) expression, batch cultures of strains of S. gordonii were grown
in a low-carbohydrate tryptone-yeast extract (TY) medium (62) supplemented with 25
mM galactose and 10 mM arginine to an optical density at 600 nm (OD₆₀₀) of 0.5. For
aerobic growth, overnight cultures of S. gordonii strains were diluted 1:50 into a 250-ml
conical flask containing 50 ml of BHI and cultures were grown on a rotary shaker (150
rpm) at 37°C to an optical density at 600 nm (O.D.₆₀₀) of 0.5. For anaerobic growth,
cultures were similarly diluted and incubated, but the medium was overlaid with mineral
oil (1).

Construction of mutant strains. Strains used in this study are listed in Table 1 and
primers used for deletion mutagenesis are listed in Table 2. To make deletions of the
genes of interest, 5’ and 3’ flanking regions of each gene were amplified from chromosomal
DNA from S. gordonii DL1, ligated together using BamHI sites designed into each primer
set, and cloned into the pGEM-T Easy vector (Promega, Madison, WI). These plasmids
were digested with BamHI and a non-polar kanamycin (NPKm) resistance gene (3), which
lacks its own promoter, was inserted (Table 1). The desired mutagenic plasmids were
selected after PCR amplification using vector-originated M13 primers, isolated and used to
transform S. gordonii / PₐrcA cat. To construct strains lacking the vicRK and flp genes, the
mutagenic plasmid containing regions flanking vicRK was transformed into a Flp-deficient
mutant of S. gordonii / PₐrcA cat (13). Transformants were selected on BHI agar with Km.
In all cases, double-crossover mutants of each gene were confirmed by PCR and DNA
sequencing, including sequencing the flanking regions to ensure no unwanted mutations
were inadvertently introduced. To construct ciaRH, comDE and vicRK complemented strains, the DNA fragments of ciaRH, comDE and vicRK with their respective promoter regions were amplified using primers described in Table 2 and cloned into the shuttle vector pDL278 (31) to create plasmids pDL-ciaRH, pDL-comDE and pDL-vicRK, respectively. The ligation mixtures were transformed into E. coli and transformants were selected on LB plates with spectinomycin (Spc; 100 µg ml\(^{-1}\)). The integrity of the constructs was confirmed by restriction enzyme digestion and DNA sequencing. The plasmids were recovered from E. coli and introduced into ciaRH-, comDE- and vicRK-deficient mutants of S. gordonii /P\(_{arcA}\)-cat by natural transformation. Transformants were selected on BHI agar with spectinomycin (Spc; 1000 µg ml\(^{-1}\)) and screened for the correct plasmid content.

Growth kinetics. Growth of all strains in BHI (pH 7.0) or BHI that was acidified to pH 5.0 with HCl (BHI/HCl), under aerobic or anaerobic conditions (1), was monitored using a Bioscreen C (Growth Curves USA, NJ) Microbiology Reader with multi-well disposable microtiter plates. An aliquot (3 µl) of cell suspension from an overnight culture was inoculated in at least triplicate into each well containing 300 µl BHI (pH 7.0) or BHI/HCl (pH 5.0) fresh medium. Inocula were adjusted to the same OD\(_{600}\) before dilution. To assess the ability of cells to grow in the presence of oxidative stressors, overnight cultures of cells were transferred to pre-warmed BHI and grown at 37°C in a 5% CO\(_2\) atmosphere to OD\(_{600}\) = 0.5. The cells were then diluted into fresh BHI containing 25 mM paraquat (methyl viologen; Sigma) and the impact of the agents on bacterial growth was monitored in a Bioscreen C at 37°C under aerobic or anaerobic conditions.

Biochemical and acid stress tolerance assays. CAT activity was measured as previously described (37) and expressed as nanomoles of chloramphenicol acetylated (minute x milligram of total protein)\(^{-1}\). AD activity was measured by a method detailed
elsewhere (41) and expressed as micrograms of citrulline produced (minute x mg total protein)$^{-1}$. The concentration of protein was determined by using a Bradford protein assay (Bio-Rad, Hercules, Calif) with bovine serum albumin as the standard. The ability of bacterial cells to withstand acid killing was assessed as previously described (61).

RESULTS

ComDE and CIARH affect low pH induction of the ADS genes. To examine whether the CIA, COM or VIC TCSs were involved in the regulation of the ADS in S. gordonii, the entire ciaRH, comDE and vicRK operons were replaced by a non-polar kanamycin (Km) cassette to create strains SgciaRH, SgcomDE and SgvicRK, respectively. To evaluate the function of individual components of each TCS, ciaR, ciaH, comD, comE, vicR and vicK were disrupted by non-polar insertions (Table 1). In all cases, the non-polar Km insertion was confirmed to allow efficient read-through to the downstream genes by real-time PCR (data not shown). All mutations were confirmed by PCR analysis and DNA sequencing of the regions flanking the insertion site of the marker to ensure that no mutations had been introduced into flanking genes. Although repeated attempts to generate a vicR mutant in S. mutans strains NG8 and UA159 were unsuccessful (54), both VicR- and VicRK-deficient strains of S. gordonii were isolable.

The cia, com and vic mutants of S. gordonii carrying the arcA promoter (P_{arcA}) fused to a chloramphenicol acetyltransferase (cat) gene were grown to mid-exponential phase in TY medium that was acidified with HCl to pH 5.5 or buffered at pH 7.0 using 50 mM potassium phosphate buffer (53). CAT activity and arginine deiminase activity were measured as outlined in the methods section. In the SgWT background, cells expressed 3-fold higher CAT activity from P_{arcA} at pH 5.5 than at pH 7.0. A similar phenotype was observed in the vic mutants (Fig. 1A), whereas only a 1.1- to 2-fold induction of P_{arcA} by low pH could be detected in the cia and com mutants (Fig. 1A). Complementation of the
SgciaRH or SgcomDE strains with plasmid-borne ciaRH or comDE genes, respectively, restored induction by low pH to a level comparable to that observed in SgWT (Fig. 1A). Measurements of AD activity (Fig. 1B) supported an involvement of the Com and Cia systems, but not Vic, in low-pH induction of the ADS. Both the histidine kinases (CiaH and ComD) and response regulators (CiaR and ComE) of these TCSs were required for activation of ADS gene expression by low pH (Fig. 1).

**Contribution of VicRK to anaerobic induction of the ADS.** The VicK sensor kinase of *S. mutans* contains a PAS domain (55) and was found to be involved in regulation of oxygen-responsive genes in *S. mutans* (2, 54). To examine whether the Vic system affected expression of the ADS in response to oxygen in *S. gordonii*, vic mutants and SgWT were cultured to mid-exponential phase in TY medium containing 25 mM galactose and 10 mM arginine, under aerobic or anaerobic conditions, and CAT and AD activity were measured. In the SgWT background, cells expressed 5-fold higher CAT activity from $P_{arcA}$ under anaerobic conditions compared to aerobic conditions (Fig. 2A). A 3-fold induction in $P_{arcA}$ expression in anaerobic conditions was detected in both SgvicR and SgvicK strains (Fig. 2A), and only a 2-fold induction was observed in the SgvicRK strain (Fig. 2A). Both the histidine kinase VicK and the response regulator VicR were shown to contribute to anaerobic induction of the transcription of the *arc* operon in *S. gordonii*. CAT activity was consistent with AD activity, demonstrating that the response of the organism to aeration occurred mainly at the transcriptional level (Fig. 2B). Introduction of vicRK on a plasmid into the SgvicRK strain resulted in restoration of the 5-fold induction of the ADS under anaerobic conditions that was observed with strain SgWT (Fig. 2A, 2B). Importantly, no difference in the response of ADS expression to aeration was noted between the SgWT strain and strains lacking one or both components of the CiaRH or ComDE TCS (data not shown).
The Vic system appears to act independently of Flp. Flp (Fnr-like protein) activates arc operon expression in response to low oxygen tension in *S. gordonii* (13). To examine if the Flp and the Vic system acted independently in the anaerobic induction of the arc operon, the strain SgvicRK-flp, in which the *flp* and *vicRK* genes were deleted, was examined. Inactivation of *flp* resulted in 10- and 3.3-fold decreases in CAT activity compared to the results seen with SgWT grown under anaerobic and aerobic conditions, respectively (Fig. 2A). Loss of both Flp and VicRK resulted in 15- and 5-fold lower CAT activity than in SgWT cultured under anaerobic or aerobic conditions, respectively (Fig. 2A). Thus, Flp and VicRK may act independently in the anaerobic induction of the ADS in *S. gordonii*. Measurements of AD activity showed the same trend as the gene fusion results, although the modest differences in fold-induction between CAT and ADS in the SgvicRK-flp strain (Fig. 2B) add further support that post-transcriptional events can modulate AD enzyme activity (37).

CiaRH, ComDE and VicRK contribute to acid tolerance. Given the participation of the Vic, Com and Cia TCS in modulation of the ADS in response to pH and oxidative stress, we explored whether these systems contribute to tolerance of environmental stresses by *S. gordonii*. The various strains were cultured in BHI (pH 7.0) or BHI/HCl (pH 5.0) broth and growth was monitored spectrophotometrically. At pH 7.0, the growth curves of the SgciaR and SgvicK strains were similar to that of the wild-type strain (Fig. 3A, 3C), whereas longer lag phases were noted in the SgciaH, SgciaRH, SgvicR and SgvicRK strains (Fig. 3A, 3C). In addition, all *com* mutants displayed a decreased final optical density after 15 h of incubation compared to that of SgWT (Fig. 3B). When the medium was adjusted to pH 5.0, all *cia* and *com* mutants, as well as the SgvicR strain could not grow (Fig. 3A, 3B, 3C), and a decreased final optical density was evident for
the SgvicRK strain compared with SgWT (Fig. 3C). However, similar growth curves were observed for the SgvicK and SgWT strains (Fig. 3C).

After 45 min of exposure to pH 2.8, the survival rate of the ciaRH, comDE and vicR mutants was more than 3-logs lower than that of SgWT (Fig. 4A). SgvicRK showed a 1-log lower survival rate than the wild-type strain, whereas no significant differences in the survival rates of the SgvicK and SgWT strains were noted (Fig. 4A).

To examine whether S. gordonii was able to mount a classical acid tolerance response (ATR), characterized by an increase in resistance to acid killing after initial exposure to mildly acidic conditions, SgWT was preincubated in BHI medium that was adjusted to pH 5.0 with HCl for 2 h to allow acid adaptation, then incubated in pH 2.8 buffer to monitor the rate of acid killing. After 45 minutes, a 1-log higher survival rate of cells preincubated at pH 5.0 was observed, compared to cells that were not acid-adapted (Fig. 4A, 4B). Interestingly, the ability to resist acid killing of the SgvicR mutants, which showed a substantial deficiency in acid tolerance in unadapted cells, was restored to a level comparable to that of SgWT after acid adaptation (Fig. 4B). Also of note, the com mutants could not mount an effective ATR, as evidenced by the lack of enhanced survival after pre-exposure to acidic conditions, but the cia mutants could. In all cases, though, the cia and com mutants were less resistant to acid killing when compared with the wild-type strain, provided that the strains were pre-treated in the same manner (Fig. 4B).

The requirement of VicRK for oxidative stress tolerance in S. gordonii. To examine whether VicRK in S. gordonii contributed to oxidative stress tolerance, the vic mutants and SgWT were cultured in BHI medium under aerobic and anaerobic conditions, and the growth curves of the cells were monitored. In the SgWT and SgvicK strains, similar growth curves were noted for aerobic and anaerobic cultures (Fig. 5A), whereas the SgvicR and...
SgvicRK strains grew more slowly and achieved slightly lower final optical densities when cells were cultured under aerobic conditions, compared to anaerobic conditions (Fig. 5A).

When the cells were cultured with 25 mM paraquat, similar growth curves were noted for the SgWT and SgvicK strains, under both aerobic and anaerobic conditions (Fig. 5B).

However the SgvicR strain had a greatly extended lag phase and achieved about half the final OD of the SgWT strain when cultured with paraquat under anaerobic conditions (Fig. 5B). Also, the SgvicR strain could barely grow aerobically in the presence of paraquat (Fig. 5B) and the SgvicRK strain achieved about half the final OD of the SgWT strain when cultured with paraquat under aerobic or anaerobic conditions (Fig. 5B).

**DISCUSSION**

*S. gordonii* is a particularly effective colonizer of the oral cavity and is present in significant proportions in healthy supra- and sub-gingival biofilms. To persist, this organism must adapt to often adverse and fluctuating environmental conditions, particularly variations in oxygen tension, acidification of the surroundings, and transitions between nutrient-limitation and -excess due to intermittent feeding by the host. A substantial effort has been focused on the adaptation strategies of caries and periodontal disease pathogens, but comparatively little information is available on these traits in the commensal flora associated with dental health. Such information is needed for the design of new strategies to control oral diseases by fostering the persistence of oral biofilms that are compatible with health. To our knowledge, this is the first report demonstrating a role for TCS in low-pH and oxygen-dependent activation of the arginine deiminase genes in bacteria, yielding insights into the molecular basis for differential expression of one of the two major alkali-generating systems in dental biofilms. Our results also disclose significant differences and similarities in the functions of key TCS components in the modulation of ammonia production, an important protective
mechanism against environmental acidification, and in the general stress tolerance
properties of S. gordonii and S. mutans, an established oral pathogen.

The CiaRH, ComDE and VicRK TCS have been studied in some detail in S.
mutans (4, 8, 28, 54) and contribute in various ways to acid tolerance, biofilm formation
and virulence gene expression. Recently, we determined that the Cia and Com TCS
also were involved in activation of the agmatine deiminase system (AgDS) of S. mutans.
The AgDS is highly similar to the ADS: generating ammonia, CO₂ and ATP from the
decarboxylated derivative of arginine, agmatine, with putrescine instead of ornithine as
an end product. Both systems are substrate-inducible, catabolite repressible and low
pH-inducible (13, 20, 37, 38), but the expression of the ADS (13), not the AgDS, is
sensitive to oxygen (data not shown). The AgDS is believed to augment acid tolerance
while concomitantly disposing of exogenous agmatine, which is inhibitory to the growth
of S. mutans and other oral streptococci (20, 21). Interestingly, only the histidine
kinases CiaH and ComD were required for AgDS induction in S. mutans, whereas the
histidine kinases (CiaH and ComD) and the response regulators (CiaR and ComE) were
required for ADS induction by low pH in S. gordonii. In S. mutans, the transcription of
the comDE genes is influenced by CiaR (4), but there is no evidence yet for regulation of
comDE by CiaRH in S. gordonii. Still, it is possible that differences in cross-regulation
between CiaRH and ComDE could be one explanation for the disparate influences of
components of these TCSs in the regulation of alkali generation in S. mutans and S.
gordonii.

The transcription of the ADS operon of S. gordonii was found to be optimally
induced under anaerobic conditions (13) and this study showed contributions of both the
Vic system and Flp to optimal expression of the ADS in response to the redox
environment. Although our results suggest that Flp and Vic act independently (Fig 2A,
2B), further proof is needed to confirm that there is no interaction between Flp and the
Another notable finding here is that inactivation of *comDE* resulted in an inability of *S. gordonii* to mount an ATR (Fig 4A, 4B), whereas mutants lacking *cia* and *vic* genes could undergo acid adaptation, as evidenced by enhanced resistance to killing at a lethal pH acquired during pre-exposure to mildly acidic conditions (Fig 4A, 4B). In fact, even though *vic* mutants were markedly more acid sensitive than the wild-type strain in the absence of acid adaptation, induction of the ATR in the *vic* mutants restored acid tolerance almost to the levels observed in SgWT cells that had been pre-adapted to low pH (Fig 4A, 4B). Thus, the ComDE system plays a critical role in adaptation to low pH in *S. gordonii*, whereas the CiaRH and VicRK systems primarily impact constitutive acid tolerance. Similar roles for ComDE, CiaRH and VicRK vis-à-vis constitutive acid tolerance and the ATR were observed in *S. mutans* UA159 (manuscript submitted, 4).

Notably, mutants lacking *comC, -D* or –*E* in *S. mutans* BM71 displayed an attenuated ATR, but still acquired enhanced resistance to acid killing after adaptation at a mildly acidic pH (35).

The Vic system of *S. mutans* is critical for modulation of gene expression in response to aeration and regulates a variety of genes and phenotypes (2, 34), including autolysis. In *S. mutans*, strains lacking VicK showed modified adherence, biofilm formation and genetic competence development, and it is believed that VicR can directly regulate the expression of several virulence-associated genes, including *gtfBCD, ftf*, and *gbpB* (2, 4, 8, 28, 33, 54). Interestingly, lack of VicR in *S. gordonii* resulted in slower growth, but also in a higher final yield, perhaps indicating a role for the Vic system in control of autolytic behavior. Consistent with this idea, the *vic* mutants of *S. gordonii* formed clumps after incubation in BHI medium, a trait that was not observed in wild-type *S. gordonii*, but which has been associated with altered autolysis (3). In fact, VicR-deficient strains of *S. gordonii* do show substantial changes in autolytic behavior (data...
not shown) and the underlying mechanisms are under investigation.

VicR-deficient *S. gordonii* were also more sensitive to growth in air or in the presence of the superoxide anion-generating compound paraquat (Fig. 3D), but VicK-deficient strains did not show any obvious defects in tolerance of oxidative stresses (Fig. 3D), perhaps indicating the potential for other sensor kinases to modulate the phosphorylation state of VicR. Consistent with the proposed role in responses to oxygen, the *S. gordonii* VicK protein contains a PAS domain, which can function in sensing of redox state (46). Collectively, these results indicate that the Vic system in *S. gordonii* may function similarly to the Vic system of *S. mutans* and other WalRK homologs of gram-positive bacteria (16) by sensing redox and monitoring the integrity of the cell envelope. However, it should be reiterated that a vicR mutant of *S. gordonii* is viable, whereas efforts to generate a deletion mutation of vicR in *S. pneumoniae* and *S. mutans* have thus far been unsuccessful (2, 4, 8, 28, 33, 54). In addition, VicRK was required for low-pH induction of the AgDS in *S. mutans*, but not the ADS in *S. gordonii*.

Further analysis of the spectrum of genes and activities under the control of the Vic system in *S. gordonii* should prove useful for understanding how evolution may have shaped the functions of the Vic TCS in the oral pathogen *S. mutans* and the oral commensal *S. gordonii*.

The differences between *S. gordonii* and *S. mutans* in terms of the participation of various TCS components in modulation of alkali generating capacity and in the phenotypes elicited by inactivation of the TCS components is of particular interest. The evolutionary divergence of these TCS may be due to the ecological and physiologic differences in the two species, and, in the case of the comDE genes, a result of the genes originating from different ancestral genes (11, 63). In particular, *S. mutans* is aciduric and the organism does not generate substantial amounts of H₂O₂ when grown in aerobic environments (7, 29), whereas *S. gordonii* is considered only weakly acid.
tolerant (7), in the absence of arginine, and has an active H$_2$O$_2$-forming NADH-oxidase (47). In addition, evolutionary differences in the competence regulons of streptococci have been described by Martin et al. (43), including that ComDE of S. gordonii are more similar to ComDE of S. pneumoniae, than to those annotated in S. mutans. In fact, the S. mutans ComDE proteins appear to have evolved independently from the bacteriocin regulators BlpRH (43). Thus, the fundamental differences in acid tolerance and oxygen metabolism between these two organisms may be associated, at least in part, with the observations that individual mutations in the TCS studied here impact ADS, AgDS and stress tolerance in various ways. Moreover, based on the behaviors of mutants lacking individual or double mutations in the TCS, it is also reasonable to predict that the abilities of the sensor kinases of these two organisms to “cross-regulate” (30) response regulators has also diverged.

In summary, this study reveals additional complexities in the regulation of the ADS, sheds new light on the molecular mechanisms of stress tolerance by S. gordonii, and illustrates important differences of the roles and interactions of key TCSs in S. gordonii and closely related streptococci. S. gordonii has been suggested to play an important role in maintaining pH homeostasis in oral biofilms (10). Recently, a clinical study demonstrated that there were higher levels of salivary ADS activity in caries-free subjects compared to caries-active subjects (45). Therefore, the ADS has significant potential as an avenue to prevent dental caries in humans (10), so understanding how to optimize ADS expression in dental biofilms could be very beneficial. Continued investigation of CiaRH, ComDE and VicRK TCS of S. gordonii will be essential to develop a comprehensive understanding of the role of these systems in pathogenic and commensal streptococci.
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REFERENCE


Table 1. Strains used in this study.

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<td>$\Delta$vicK / S. gordonii / $P_{\text{arcA}}$-cat</td>
<td>This study</td>
</tr>
<tr>
<td>SgvicR</td>
<td>$\Delta$vicR / S. gordonii / $P_{\text{arcA}}$-cat</td>
<td>This study</td>
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<tr>
<td>SgvicRK</td>
<td>$\Delta$vicRK / S. gordonii / $P_{\text{arcA}}$-cat</td>
<td>This study</td>
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<td>SgvicRK/vicRK</td>
<td>$\Delta$vicRK / $pDL$-vicRK / S. gordonii / $P_{\text{arcA}}$-cat</td>
<td>This study</td>
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<tr>
<td>Sgflp</td>
<td>$\Delta$flp / S. gordonii / $P_{\text{arcA}}$-cat</td>
<td>(13)</td>
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<td>SgvicRK-flp</td>
<td>$\Delta$vicRK / $\Delta$flp / S. gordonii / $P_{\text{arcA}}$-cat</td>
<td>This study</td>
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</table>
Table 2. Primers used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence*</th>
<th>Application source</th>
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<tbody>
<tr>
<td>ComE-BamHI-3'</td>
<td>5'- CGGGATCCATTCTCGAAGAC- 3'</td>
<td>Deletion of comE</td>
</tr>
<tr>
<td>ComD-5'-2</td>
<td>5'- GCAACCGGAAATTAACGCTG - 3'</td>
<td>Deletion of comE</td>
</tr>
<tr>
<td>ComE-BamHI-5'</td>
<td>5'- ACTGGTGACAAAGCGGGATCCACTGG- 3'</td>
<td>Deletion of comE</td>
</tr>
<tr>
<td>ComE-3'</td>
<td>5'- CCAAGTTTTATCTGTTCTTG- 3'</td>
<td>Deletion of comE</td>
</tr>
<tr>
<td>ComE-3'-3</td>
<td>5'- GCCCTCTTGCTGTGC- 3'</td>
<td>Deletion of comD</td>
</tr>
<tr>
<td>ComD-BamHI-5'</td>
<td>5'- GAAGTAGGATCTCAACAAAGG- 3'</td>
<td>Deletion of comD</td>
</tr>
<tr>
<td>ComD-5'</td>
<td>5'- CCCCTCCTCATCAATTAATGCG- 3'</td>
<td>Deletion of comD</td>
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<tr>
<td>ComD-BamHI-3'</td>
<td>5'- GACGGCAACGGATCCACCAGTAACCTGC- 3'</td>
<td>Deletion of comD</td>
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<td>Pcom-BamHI-5'</td>
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<td>Cloning of comDE into pDL278</td>
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<tr>
<td>ComE-SphI-3'</td>
<td>5'- CCAGTTTATCTCGCAGATGCTACGACAG- 3'</td>
<td>Cloning of comDE into pDL278</td>
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<td>CiaR-5'-1</td>
<td>5'- GTCCGGTCTCGTGGATATAATCATTCCG- 3'</td>
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<tr>
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<td>5'- CATCTGGGATGCTCTGATACGATAGAC- 3'</td>
<td>Deletion of ciaR</td>
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<td>CiaH-3'-2</td>
<td>5'- GATTCGACTGGTTTGCTGC- 3'</td>
<td>Deletion of ciaR</td>
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<tr>
<td>CiaR-BamHI-5'</td>
<td>5'- GATCCGGATCCGGGCGTTGCG- 3'</td>
<td>Deletion of ciaR</td>
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<tr>
<td>CiaH-BamHI-3'</td>
<td>5'- TGGTGTGGTTACGATCTTTGCAGAGG- 3'</td>
<td>Deletion of ciaH</td>
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<tr>
<td>CiaH-3'</td>
<td>5'- CCCAGATTCTGCTATCGCCCCACC- 3'</td>
<td>Deletion of ciaH</td>
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<tr>
<td>CiaR-BamHI-3'</td>
<td>5'- GATGACTTTGCGGATGTCATGCGGCA- 3'</td>
<td>Deletion of ciaH</td>
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<tr>
<td>CiaH-BamHI-3'-2</td>
<td>5'- GCCGGATTCCTAGCCATGCCACCCACC- TC- 3'</td>
<td>Deletion of vicR</td>
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<tr>
<td>VicR-5'-2</td>
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<td>Pcia-HindIII-5'</td>
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<td>CiaH-SalI-3'</td>
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<td>Deletion of vicR</td>
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<td>VicR-BamHI-3'</td>
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<td>Deletion of vicR</td>
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<td>VicK-3'</td>
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<td>Deletion of vicR</td>
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<td>VicR-5'-2</td>
<td>5'- CAAGGGTGCTTTTATCGATCTGCGGAGGCA- 3'</td>
<td>Deletion of vicR</td>
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<tr>
<td>VicR-BamHI-3'-2</td>
<td>5'- GCCGGATTCCTAGCCATGCCACCCACC- TC- 3'</td>
<td>Deletion of vicR</td>
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<td>VicK-3'-2</td>
<td>5'- CGGAAAAAGGCGATCCGGCCAG- 3'</td>
<td>Deletion of vicR</td>
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<td>Pvc-BamHI-5'</td>
<td>5'- GCTATCTTTACGCGGATCCGGCGCAAC- 3'</td>
<td>Cloning of comDE into pDL278</td>
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<td>Vic-SphI-3'</td>
<td>5'- TACCGGTAGTACGAGATGCTG - 3'</td>
<td>Cloning of comDE into pDL278</td>
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<td>Flp-5'</td>
<td>5'- CCAGTTTTATATGCGGAGTGTTGA- 3'</td>
<td>Deletion of Flp (13)</td>
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<tr>
<td>Flp-3'</td>
<td>5'- GCAGTTTTATATGCGGAGTGTTGA- 3'</td>
<td>Deletion of Flp (13)</td>
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<tr>
<td>Flp-Smal-S</td>
<td>5'- CTCTTTTTTTTCTGGAGAAGCCGGGTATCGGCTTCT- 3'</td>
<td>Deletion of Flp (13)</td>
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<tr>
<td>Flp-Smal-AS</td>
<td>5'- GAGAAAAAGGCGATCCGGCGCGCGGTTTCTGCGG- 3'</td>
<td>Deletion of Flp (13)</td>
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</table>

* Boldface indicates engineered restriction sites.
Figure 1. CAT (A) and Arginine Deiminase (B) activities of *S. gordonii* carrying *P*$_{arcA}$*$cat$ and its derivatives (Table 1) cultured in TY broth containing 25 mM galactose with 20 mM arginine that had been acidified to pH 5.5 with HCl (TY/HCl), or TY buffered at pH 7.0 (TY/KPB) to mid-exponential phase. The values of the columns are the average of a minimum of nine separate cultures for each strain and condition. The standard deviations for CAT activities (A) of pH 7.0 group from left to right is 20.46, 40.79, 12.02, 11.86, 23.19, 9.48, 0.95, 16.83, 29.79, 22.95, 15.60, 8.23; and those of pH 5.5 group from left to right are 26.13, 30.18, 1.61, 37.76, 27.10, 40.02, 12.80, 20.16, 37.42, 52.80, 16.90, 8.44. The standard deviations for AD activities (B) of pH 7.0 group from left to right are 2.78, 1.40, 3.23, 1.94, 7.07, 2.41, 0.95, 4.94, 3.63, 8.33, 0.84, 2.22; and those of pH 5.5 group from left to right are 17.22, 2.31, 1.60, 6.47, 3.22, 2.99, 1.35, 1.25, 1.12, 1.80, 1.67, 2.38. Solid bars depict activities from pH 7.0 cultures and open bars from pH 5.0 cultures. * Indicates statistically significant differences between SgWT and mutants grown under identical culture conditions. *P* < 0.05; Student’s *t* test.

Figure 2. CAT (A) and Arginine Deiminase (B) activities of the mutants of *S. gordonii* carrying *P*$_{arcA}$*$cat$ and its derivatives (Table 1) cultured in TY broth containing 25 mM galactose with 20 mM arginine, under aerobic or anaerobic conditions, to mid-exponential phase. The values of the columns are the average of a minimum of nine separate cultures for each strain and condition. The standard deviations for CAT activities (A) of aerobic group from left to right are 25.00, 10.13, 18.14, 19.28, 7.04, 3.60, 7.81; and those of anaerobic group from left to right is 34.12, 3.60, 44.30, 6.36, 38.95, 2.51, 24.13. The standard deviations for AD activities (B) of aerobic group from left to right are 5.20, 0.50, 2.40, 2.61, 3.23, 15.23, 1.78; and those of anaerobic group from left to right are 5.17, 1.11, 1.89, 9.79, 1.29, 1.28, 7.78. Solid bars depict activities from
aerobic cultures and open bars from anaerobic cultures. * Indicates statistically significant differences between SgWT and mutants grown under identical culture conditions. \( P < 0.05; \) Student’s \( t \) test.

Figure 3. Growth of strains SgWT and cia mutants including SgciaR, SgciaH, SgciaRH (A), com mutants including SgcomD, SgcomE, SgcomDE (B), vic mutants including SgvicR, SgvicK and SgvicRK (C) in BHI (pH 7.0) or acidified BHI with HCl (pH 5.0).

Optical density at 600 nm was determined every 15 min for 24 h using a Bioscreen C.

Figure 4. Acid tolerance assay. (A) S. gordonii carrying P_{arcA}-cat and its derivatives were grown in BHI medium adjusted to pH 7.0 to an OD_{600} of 0.3, washed with 0.1 M glycine buffer, pH 7.0, and subjected to acid killing by incubating the cells in 0.1 M glycine buffer, pH 2.8. (B) The wild type and mutants of S. gordonii were grown in BHI medium adjusted to pH 7.0 to OD_{600} = 0.2, then cells were harvested and resuspended in fresh BHI medium adjusted to pH 5.0. Following two additional hours of incubation, cells with an OD_{600} of 0.3 were prepared for acid killing as described above. In all cases, survival rate was determined by plating in triplicate on BHI agar plates. The results are expressed as percent survival rate versus time at pH 2.8. The data presented is representative of at least 9 individual replicates for each strain.

Figure 5. Growth of strains SgWT and vic mutants including SgvicR, SgvicK and SgvicRK cultured in BHI medium without (A) or with 25 mM paraquat (B), under aerobic or anaerobic conditions. Optical density at 600 nm was determined every 30 min for 24 h or 48 h using a Bioscreen C.
Fig. 1
Fig. 2

A

Arginine Deiminase Activity

B

CAT Activity

\[ A = \text{Aerobic}, \quad B = \text{Anaerobic} \]
Fig. 3
Fig. 3C
Fig. 4