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Environmental pH Sensing: Resolving the VirA/VirG Two-Component System Inputs for *Agrobacterium* Pathogenesis

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Agrobacterium tumefaciens **stands as one of biotechnology's greatest successes, with all plant genetic engineering building on the strategies of this pathogen. By integrating responses to external pHs, phenols, and monosaccharides, this organism mobilizes oncogenic elements to efficiently transform most dicotyledonous plants. We now show that the complex signaling network used to regulate lateral gene transfer can be resolved as individual signaling modules. While pH and sugar perception are coupled through a common pathway, requiring both low pH and sugar for maximal virulence gene expression, various VirA and ChvE alleles can decouple pH and monosaccharide perception. This VirA and ChvE system may represent a common mechanism that underpins external pH perception in prokaryotes, and the use of these simple genetic elements may now be extended to research on specific responses to changes in environmental pH.**

The hydrogen ion concentration represents a critical environmental constraint for all macromolecular structures, physiological networks, and biological energy conversions. Accordingly, the ability to sense and respond to changes of pH is essential for all organisms. In contrast to the vast number of genes discovered to be pH regulated, the actual proteins responsible for sensing H^+ are poorly defined. Only a few systems, such as the PhoQ/PhoP system in *Salmonella enterica* serovar Typhimurium (3), the ChvG/ChvI system in *Agrobacterium* sp. (26), and the chemotactic proteins Tar and Tsr (43), are known to be involved in sensing either extracellular or intracellular pH. Enteric bacteria, such as *Escherichia coli* and *S. enterica* serovar Typhimurium, have evolved sophisticated acid tolerance response systems to adapt to adverse pH environments (4, 18), but the pH sensing mechanisms remains ill defined. This deficiency both hinders development of a system-wide understanding of pH responses and limits our capability to further regulate and exploit these elements as pH sensors.

The response to extracellular pH is particularly critical for many pathogenic and symbiotic relationships in which a change in hydrogen ion concentration can mark a transition from a free-living mode to the host environment. *Agrobacterium tumefaciens*, a soil pathogen that causes plant tumors via the transfer of oncogenic DNA into host cells, uses pH as a part of its arsenal of host indicators. Low pH (approximately 4.8 to 5.5), monosaccharides (including glucose), and phenols (e.g., acetosyringone [AS]) are all required to maximally induce the expression of the virulence (*vir*) genes that mediate the transfer of DNA into the plant genome (6, 42, 54). Two genes, the VirA/VirG two-component sensor-transducer system, regulate the production of both the transferred oncogenic DNA and the DNA transfer machinery from the tumor-inducing Ti plasmid (22, 50, 52). VirA, a membrane-localized histidine sensor kinase, is autophosphorylated upon perceiving signals characteristic of host wound sites and transfers the phosphoryl group to VirG (32). Phosphorylated VirG acts as a transcription activator that induces the expression of the remaining genes of the *vir* regulon.

VirA contains two membrane-spanning segments, a periplasmic (P) domain, and a large cytoplasmic C terminus that has been subdivided into linker (L), kinase (K), and receiver (R) domains (12). The linker domain has been implicated in phenol sensing (12). Monosaccharides are perceived through the periplasmic domain of VirA via chromosomal sugar binding protein ChvE, and their perception enhances the sensitivity and maximal response to phenolic compounds (1, 37, 38). Optimal *vir* gene induction, however, does not occur at neutral pHs (40) but rather requires an acidic environment. Part of this external pH dependence is attributed to enhanced *virG* expression, as mediated by the acidic pH-inducible P2 promoter (13, 28, 49). However, replacing wild-type P1 and P2 promoters of *virG* with the *lac* promoter does not create a pH-independent virulence response (15), indicating that other pH-regulated inputs are mediated through the VirA/VirG system.

Chang and Winans (12) demonstrated that the kinase domain of VirA could activate *vir* gene expression independent of pH, while VirA truncation mutants with only the linker and kinase domains were still pH responsive. They concluded that the cytoplasmic linker domain of VirA was responsible for pH input. However, a later report (14) showed that an allele product of VirA, of which the linker domain was truncated, maintaining the periplasmic and kinase domains, still responded to a low-pH stimulus. Other investigators constructed fusion proteins with the periplasmic domain replaced by one of the *E. coli* membrane chemoreceptors, Tar (30). One of the hybrid proteins achieved high induction even at pH 7.0, further suggesting that the periplasmic domain of VirA is critical for pH sensing. Here we exploit the modularity of the histidine sensory kinases to establish that the periplasmic domain of VirA is involved in pH perception. Further, we show that the pH response is directly coupled with sugar signaling through

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[*F′ proAB lacI*^{q}*ZΔM15* Tn*10* (Tc^r)]	Stratagene
DH5α	*recA1 endA1 hsdR17 supE44 gyrA96 relA1*Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZΔM15*)	Invitrogen
A. tumefaciens		
A348	A136 containing pTiA6NC	19
A136	Strain C58 cured of pTi plasmid	48
DL8	A136 *chvE* deletion, Km^r	This study
Plasmids		
pBluescript II KS(+/−)	Cloning vector, ColE1 Ap^r	Stratagene
pJZ4	5.1-kb fragment containing P_{virB}-*lacZ-nptII* cloned into pMON596, Inc P Spec^r	53
pRG109	P_{N25}-His$_6$-*virG* in pJZ4, Spec^r	This study
pRG108	P_{N25}-His$_6$-*virG*(N54D) in pJZ4, Spec^r	This study
pYW47	P_{N25}-His$_6$-*virG* in pYW15, IncW Ap^r	47
pAM13	P_{N25}-His$_6$-*virG*(N54D) in pYW15, IncW Ap^r	32
pSM102	*occQ*::*lacZ,* IncP Ap^r	39
pVRA8	*virA* from pTiA6 in pUCD2, pBR322*ori,* IncW Ap^r	25
pMutA	*virA*(G665D) in pUCD2, pBR322*ori,* IncW Ap^r	29
pYW15	Broad-host-range expression vector, IncW Ap^r	47
pFF4	P_{N25}-*virA* in pQE50, ColE1 Ap^r	This study
pFQ25	P_{N25}-*virA* and P_{N25}-*virG,* IncW Ap^r	This study
pYW21	P_{N25}-His$_6$-*virA*(285-829) in pYW15, Ap^r	45
pYW39	P_{N25}-His$_6$-*virA*(285-829)(*G665D*) in pYW15, Ap^r	47
pAM23	P_{N25}-His$_6$-*virA*(285-711) in pYW15, Ap^r	32
pAM28	P_{N25}-His$_6$-*virA*(285-711) *(G665D)* in pYW15, Ap^r	32
pRG100	P_{N25}-His$_6$-*virA*(426-829) *(G665D)* in pYW15, Ap^r	This study
pRG118	P_{N25}-His$_6$-*virA*(426-711) *(G665D)* in pYW15, Ap^r	This study
pRG119	P_{N25}-His$_6$-*virA*(426-711) in pYW15, Ap^r	This study
pRG120	P_{N25}-His$_6$-*virA*(426-829) in pYW15, Ap^r	This study
pRG135	*virA*(1-711) in pYW15, Ap^r	This study
pRG157	*chvE* from A136 in pUC19, ColE1 Ap^r	This study
pRG159	Km^r gene cloned in BamH I site of *chvE* in pRG157, ColE1 Ap^r Km^r	This study
pRG162	*virA*(E210V) in pBluescript II KS (+/−), ColE1 Ap^r	This study
pRG165	P_{N25}-*chvE* in pYW15, Ap^r	This study
pRG166	P_{N25}-*chvE*(T211M) in pYW15, Ap^r	This study
pRG168	*virA* and P_{N25}-*chvE* in pYW15, Ap^r	This study
pRG169	*virA* and P_{N25}-*chvE*(T211M) in pYW15, Ap^r	This study
pRG170	*virA*(E210V) and P_{N25}-*chvE* in pYW15, Ap^r	This study
pRG171	*virA*(E210V) and P_{N25}-*chvE*(T211M) in pYW15, Ap^r	This study

ChvE-VirA association. This knowledge allows the multiple inputs to be separated and several alleles of ChvE and VirA to be developed as pH-specific response modules.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* strains XL1-Blue and DH5α were used for routine cloning. *A. tumefaciens* strains were grown at 28°C in Luria-Bertani (LB) medium or induction medium (11) containing either glucose or glycerol. The *chvE* deletion strain, DL8, was constructed as follows. The *chvE* gene was cloned from genomic DNA of A136 by PCR amplification and then inserted into pUC19 to create pRG157. A kanamycin resistance gene was then cloned from pUC4K (Pharmacia) into the BamHI site within the *chvE* gene of pRG157. The resulting plasmid, pRG159, was electroporated into A136. The correct marker exchange mutant, DL8, was selected from kanamycin-resistant and ampicillin-sensitive colonies and further confirmed by colony PCR.

Plasmid constructions. The plasmids used in this study are listed in Table 1. The coliphage T5 promoter (P_{N25}) was used to drive the expression of *virG* independent of environmental factors like pH and phosphate concentration (47). The NcoI fragments containing either P_{N25}-His$_6$-*virG* (where His$_6$ stands for a six-histidine tag) from pYW47 or P_{N25}-His$_6$-*virG*(N54D) from pAM13 were treated with Klenow fragment and ligated with pJZ4 (P_{virB}-*lacZ*) that was treated with KpnI and Klenow fragment, resulting in pRG109 and pRG108.

The coding region of the *virA* gene was placed behind P_{N25} to create pFF4, and P_{N25}-*virA* was subsequently released for insertion into a pYW47 derivative plasmid to give pFQ25. The VirA mutant construct comprising amino acids (aa) 426 through 829 with a G665D mutation in the kinase domain [VirA(426-829)(G665D)] was amplified by PCR from pYW39 with primers 5′-ATTCAGCTTC TTGAACTCGCCACC-3′ and 5′-GCGGTACCCTACGTCTTGATTTTGGTT AG-3′ (KpnI), followed by digestion with KpnI and ligation with EcoICRI- and KpnI-digested pYW15 to generate pRG100. pRG118, pRG119, and pRG120 were made by replacing the HindIII fragment of pRG100 with HindIII fragments from pAM23 (coding for stop codon after aa 711), pAM28 (coding for wild-type kinase domain and stop codon after aa 711), and pYW45 (coding for wild-type kinase and receiver domains of VirA) (46), respectively. The VirA with the truncated receiver domain (aa 1 to 711) was released from pCH355 (14) as a KpnI fragment and inserted into KpnI-digested pYW15 to make pRG135.

To place *chvE* behind the P_{N25} promoter, *chvE* was amplified from pRG157 by PCR oligonucleotides 5′-CACAGAATTCATTAAAGAGGAGAAATTAACT ATGAAGTCCATTATTTCG-3′ (EcoRI) and 5′-CGTCTTGGTGATGTTCC GCATTTC-3′ and cloned into pCR2.1-TOPO by TOPO cloning (Invitrogen). Subsequently, *chvE* was released as an EcoRI fragment and ligated into pYW15 to create pRG165. For site-directed mutagenesis of *virA*(E210V) and *chvE*(T211M), the QuickChange site-directed mutagenesis kit (Stratagene) was used. Primers 5′-CGCATACTTGCACGTGTAGGTCCCATTATCTTATCG-3′ and 5′-CGATAAGATAATGGGACCTACACGTGCAAGTATGCG-3′ were used to introduce the E210V mutation into the *virA* gene, resulting in pRG162. Primers 5′-GAGCCTGGGCCATTGCCGGATCCC-3′ and 5′-GGGATCCGG CAATGGCCCAGGCTC-3′ were used to introduce the T211M mutation into *chvE* of pRG165, creating pRG166. The sequences of all PCR products were confirmed by DNA sequencing. The wild-type *virA* gene and *virA*(E210V) were cut from pVRA8 and pRG162 by use of KpnI and cloned into the KpnI sites of pRG165 and pRG166 to generate pRG168, pRG169, pRG170, and pRG171.

Immunoblot analysis. *A. tumefaciens* cells were grown overnight in 30 ml of LB medium with the appropriate antibiotics. The bacterial pellets were washed with

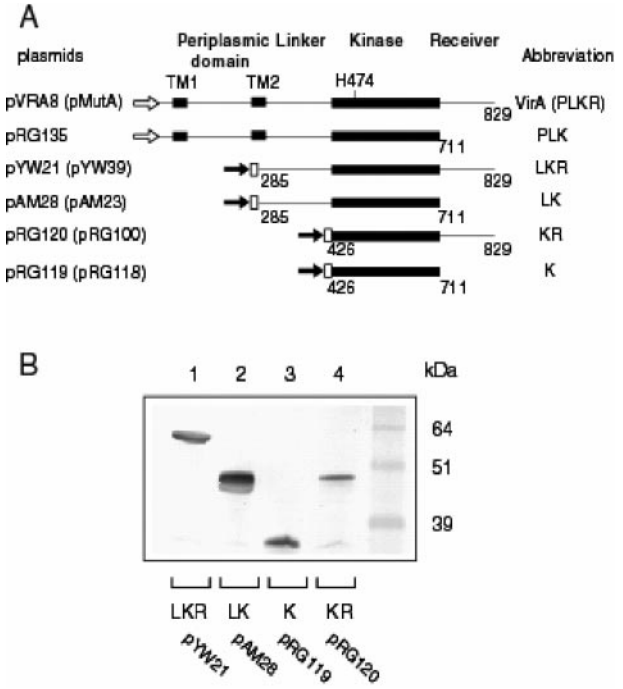

FIG. 1. Protein expression of VirA truncation mutants. (A) Schematic representation of VirA constructs. The plasmids expressing VirA constructs are listed on the left; in parentheses are the corresponding plasmids of VirA constructs with a G665D mutation in the kinase domain. Open arrow, *virA* promoter; solid arrow, P_{N25} promoter; white box, His_6 tag. (B) Western blot analysis of VirA constructs. Clarified lysates from bacteria carrying indicated plasmids were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% Bis-Tris NuPage (Invitrogen) gels followed by Western blot analysis using anti-RGS-His monoclonal antibody.

phosphate-buffered saline and lysed by brief sonication on ice. Clarified lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in polyacrylamide gels (Invitrogen), followed by electro-blotting onto nitrocellulose membranes using the Mini Trans-Blot transfer apparatus (Bio-Rad). Visualization of His_6-tagged proteins was achieved by Western blot using anti-RGS-His monoclonal antibody (QIAGEN).

β-Galactosidase assays for *vir* gene induction. pRG109 or pRG108 carrying the β-galactosidase reporter P_{virB}-*lacZ* and P_{N25}-*virG* or P_{N25}-*virG*(N54D) was used to assay *vir* gene expression in Ti-cured *Agrobacterium* strains. Cells were grown in 20 ml of LB medium to an optical density at 600 nm (OD_{600}) of 0.4 to 0.6 in the presence of the appropriate antibiotics. The cell mass was pelleted by centrifugation for 10 min at 7,000 × *g* at 4°C and washed with phosphate-buffered saline. The pellet was diluted to an OD_{600} of ~0.1 into tubes containing a total of 1 ml of induction media (50 mM MES [2-(4-morpholino)-ethanesulfonic acid], 1× AB salts) supplemented with 1% glycerol (11) and cultured at 28°C with shaking at 225 rpm for 15 h. β-Galactosidase activity was determined as described previously (31) with readings of optical densities at 600 and 415 nm using the EL800 microplate reader (BIO-TEK Instruments).

RESULTS

***virA* truncation mutants.** *virG* was placed behind a constitutive P_{N25} promoter (47) to eliminate pH activation of the P2 *virG* promoter (28, 49). pRG109, containing P_{N25}-*virG* and P_{virB}-*lacZ*, together with plasmids expressing various *virA* truncation mutants, was transformed into *A. tumefaciens* A136, a strain lacking pTi, to assay for *lacZ* expression. Among the constructs evaluated in Fig. 1A, VirA(285-829) (LKR), VirA(285-711) (LK), VirA(426-829) (KR), and VirA(426-711) (K) were fused with an N-terminal His_6 tag. Western blot analyses using anti-His monoclonal antibody showed that all these VirA truncation proteins were stably expressed (Fig. 1B) and the change of pH from 5.5 to 7.0 did not alter the levels or apparent stability of these proteins (data not shown).

The pH- and AS-dependent responses of *virA* allele products are shown in Fig. 2A. Consistent with previous reports implicating the linker domain's involvement in AS sensing (12, 14), the kinase alone (K) displayed AS-independent activity while LKR and LK remained AS-inducible. When the pH was reduced from 7.0 to 5.5, all strains showed an increase of *vir* expression, but a far greater increase was observed for the full-length VirA strain. Figure 2B presents pH responses as ratios of the activities at pH 5.5 and pH 7.0. The pH 5.5/pH 7.0 ratio of the full-length VirA strain was around 10, while VirA mutants lacking the periplasmic domain (LKR, LK, KR, and K) had ratios between 1.2 and 2.0. The pH ratio appeared to be independent of the promoter, as both autoinducing wild type and P_{N25} had similar increases. Moreover, removal of the inhibitory receiver domain (12, 14) (PLK) significantly elevated both the basal and induced activity (Fig. 2A) beyond the linear range of the β-galactosidase assay under these conditions. Here the pH 5.5/pH 7.0 ratio was at least 6, significantly higher than the ratios for LKR, LK, KR, and K, strongly supporting a role of the periplasmic domain in pH sensing.

Therefore, all the periplasmic-domain-truncated variants of

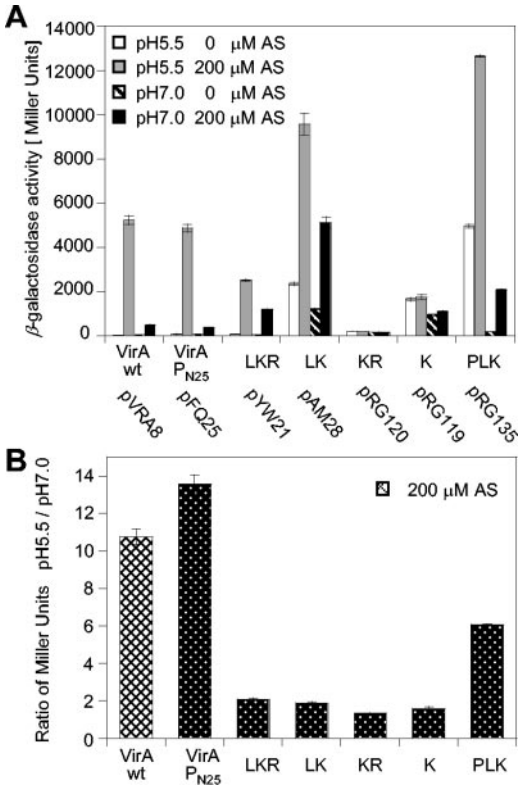

FIG. 2. pH responses of VirA constructs. *A. tumefaciens* A136 carrying the indicated VirA constructs together with pRG109 containing P_{virB}-*lacZ* and P_{N25}-*virG* was cultured in induction medium supplemented with 14 mM glucose and assayed for β-galactosidase activity. (A) Expression of P_{virB}-*lacZ* at pH 5.5 with 0 μM AS, pH 5.5 with 200 μM AS, pH 7.0 with 0 μM AS, and pH 7.0 with 200 μM AS. (B) Ratio of the β-galactosidase activity, pH 5.5/pH 7.0.

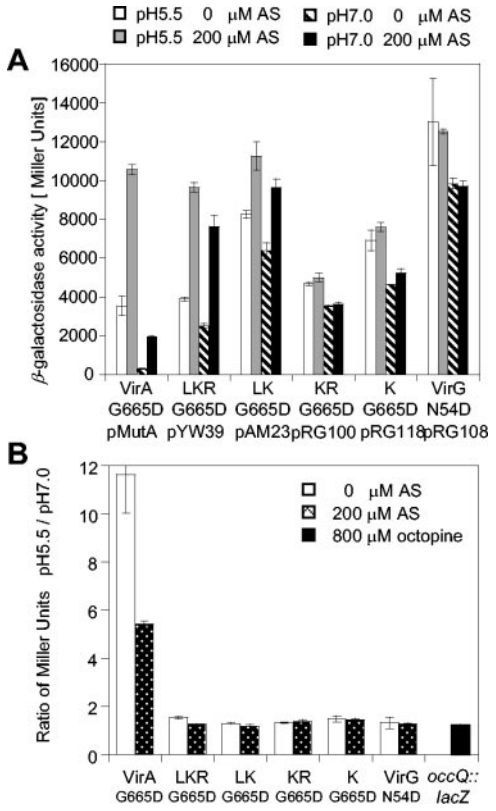

FIG. 3. pH response of VirA(G665D) constructs. *A. tumefaciens* A136 carrying the indicated VirA constructs and pRG109 was cultured as described above in the presence of 14 mM glucose. (A) Expression of P_{virB}-*lacZ* at pH 5.5 with 0 μM AS, pH 5.5 with 200 μM AS, pH 7.0 with 0 μM AS, and pH 7.0 with 200 μM AS. (B) Ratio of the β-galactosidase activity at pH 5.5 to that at pH 7.0 with 0 and 200 μM AS.

VirA displayed a much smaller pH 5.5/pH 7.0 ratio around 2. This weak pH response may be consistent with the previous conclusion that linker-domain-containing constructs (LKR and LK) still responded to low pH (12). However, the octopine reporter *occQ*::*lacZ* (39) and the product of the VirG(N54D) allele, which activates *vir* expression independent of either VirA or AS (21, 35), also showed a similar twofold increase (Fig. 3). Thus, this weak pH response appears not specific for VirA but more consistent with a small global pH effect, possibly reflecting the overall control of expression from pTi during pathogenesis. Further, the products of VirA alleles carrying a G665D substitution, which are known to give both basal and AS-inducible *vir* expressions (29), had pH responses similar to those of the wild-type VirA variants, for which a high pH 5.5/pH 7.0 ratio was associated only with VirA allele products containing an intact periplasmic domain (Fig. 3).

Interdependence of pH and sugar perception. Analysis of these VirA truncation allele products implicated a common role for the periplasmic domain in pH and monosaccharide perception. To investigate whether these were distinct or interdependent perception events, cells containing wild-type VirA were induced with the indicated concentrations of AS at pH 5.5 and 7.0 in the presence and absence of 13 mM glucose (Fig. 4). Glucose enhanced the maximal induction at pH 5.5 >5-fold; however, this increase was dependent on both glucose and acidic pH (Fig. 4A). At pH 7.0, no sugar-enhanced increase was observed; without glucose, the maximal induction by an acidic pH was twofold, consistent with the global pH dependence observed in Fig. 2. In addition, glucose perception has been associated with an increase in both the maximal induction and the sensitivity of the VirA/VirG system to AS (2). In Fig. 4B, the dosage-dependent data plotted in Fig. 4A are expressed as percentages of maximal activity in order to emphasize this sensitivity difference. A 10-fold shift of AS sensitivity was apparent only when both glucose and acidic pH were present.

The periplasmically localized sugar binding protein ChvE is required for glucose sensing in *Agrobacterium* (10, 37). Disruption of *chvE* in mutant strain DL8 abolished the pH or sugar response (Fig. 5A and B). When P_{N25}-*chvE* was moved into strain DL8, the maximal induction (Fig. 5C) and AS sensitivity (Fig. 5D) were both rescued, but this was the case only when both glucose and acidic-pH stimuli were present. Given the similar responses to pH and sugar for A136 carrying a wild-type *chvE* (Fig. 4) and DL8/pRG168 carrying P_{N25}-*chvE* (Fig. 5C and D), the wild-type *chvE* promoter was not critical for coupling the responses to pH and sugar. Taken together, these data suggest that pH and sugar sensing are mechanistically coupled.

Decoupling pH and sugar perception. Genetic evidence provides the strongest support for a direct interaction between ChvE and the periplasmic domain of VirA. VirA allele prod-

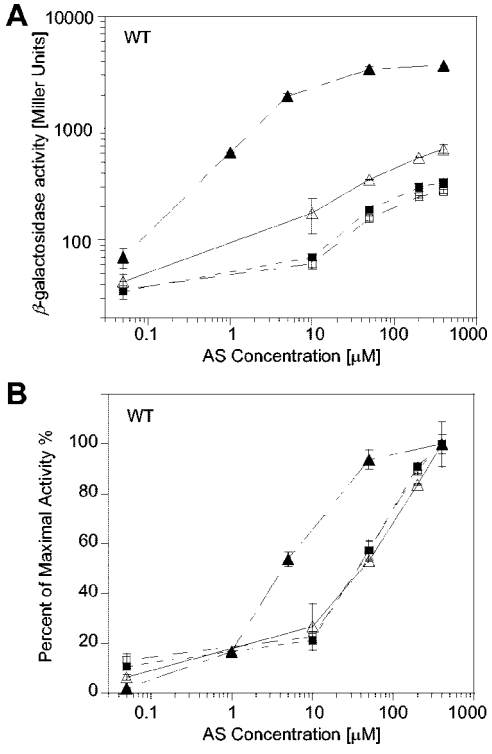

FIG. 4. P_{virB}-*lacZ* expression by wild-type VirA as a function of AS concentrations. *A. tumefaciens* A136/pRG109 containing pVRA8 was cultured for 16 h in media supplemented with 1% glycerol at pH 5.5 (Δ), pH 5.5 with 14 mM glucose (▲), pH 7.0 (□), and pH 7.0 with 14 mM glucose (■). β-Galactosidase activity calculated as Miller units (A) and expressed as percentages of the maximal activity (B).

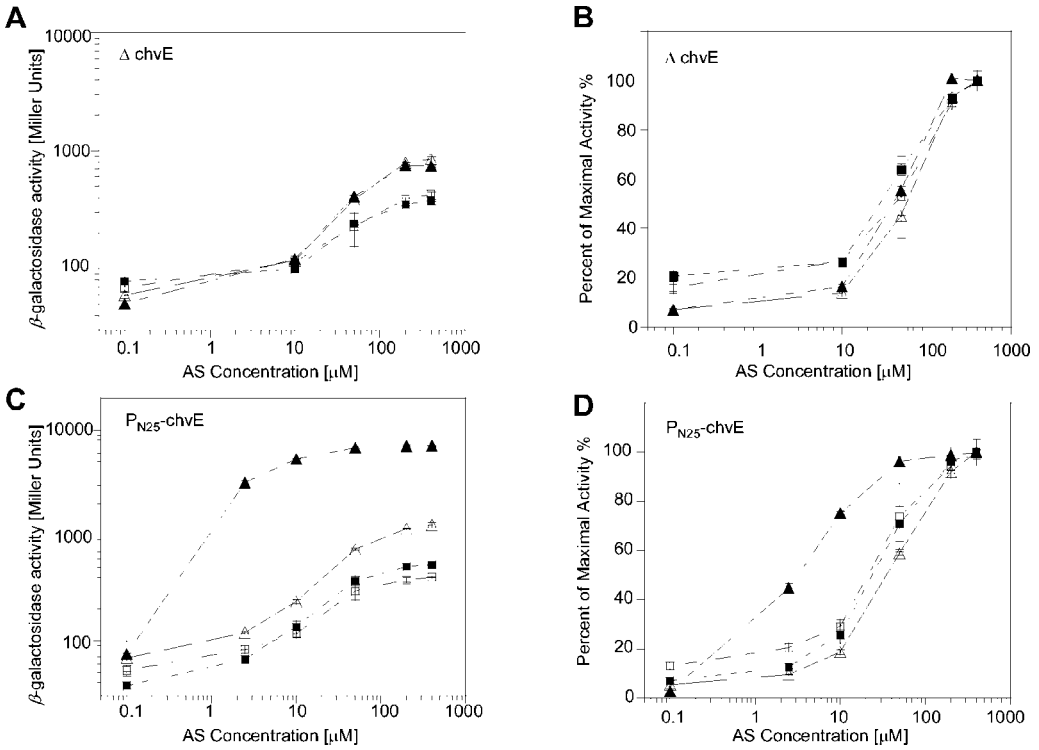

FIG. 5. P_{virB}-*lacZ* expression by wild-type VirA as a function of AS concentrations in a background in which *chvE* was disrupted. *A. tumefaciens* DL8/pRG109 containing pVRA8 (A and B) or pRG168 (C and D) was cultured as described at pH 5.5 (Δ), pH 5.5 with 14 mM glucose (▲), pH 7.0 (□), and pH 7.0 with 14 mM glucose (■). β-Galactosidase activity calculated as Miller units (A and C) and expressed as percentages of the maximal activity (B and D).

ucts containing an E210V substitution in the periplasmic domain are not responsive to glucose (37, 41), but a compensating ChvE(T211M) allele product was discovered that fully restored sugar perception (37). Consistent with these reports, VirA(E210V) didn't respond to glucose, even with P_{N25}-*chvE*, while DL8/pRG171 containing VirA(E210V) and P_{N25}-*chvE* (T211M) had a wild-type pH or sugar response (data not shown), mediating both an increased maximal induction and an enhanced AS sensitivity.

While the precise arrangement of these compensating interactions between VirA and ChvE remains unclear in the absence of greater structural resolution, the importance of these residues to perception motivated investigation of the pH or sugar response of wild-type VirA with P_{N25}-*chvE*(T211M). DL8/pRG169, expressing the wild-type VirA and ChvE(T211M), displayed maximal induction at pH 5.5 without glucose (Fig. 6A). The acidic pH alone induced a >10-fold increase over pH 7.0 induction. Glucose alone was also sufficient to increase the maximal activity at a neutral pH; similar increases in AS sensitivity are also apparent in Fig. 6B. Without glucose at pH 7.0, the AS sensitivity of VirA with ChvE(T211M) was similar to that of VirA with the wild-type ChvE as shown in Fig. 5D.

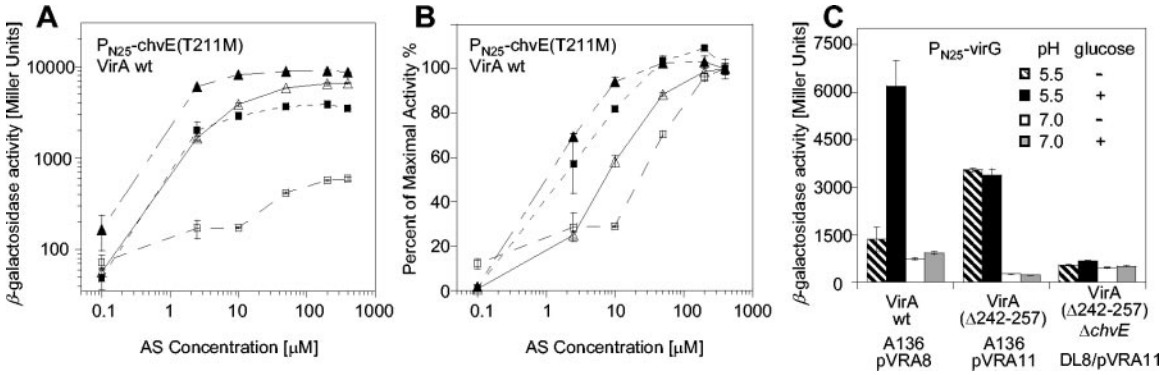

FIG. 6. pH and sugar responses of VirA and ChvE allele products. P_{virB}-*lacZ* expression by DL8/pRG109 containing pRG169 with wild-type VirA and P_{N25}-*chvE*(T211M) at pH 5.5 (Δ), pH 5.5 with 14 mM glucose (▲), pH 7.0 ([squo]), and pH 7.0 with 14 mM glucose (■), in terms of β-galactosidase activity calculated as Miller units (A) and expressed as percentages of the maximal activity (B). (C) P_{virB}-*lacZ* expression by wild-type VirA and VirA with aa 242 through 257 deleted (Δ242-257) with 200 μM AS.

Therefore, either adding glucose or dropping the pH to 5.5 increased AS sensitivity. The pH and sugar responses are no longer coupled for these allele products; either glucose or low pH alone is a sufficient inducer signal.

Residues of VirA outside of the predicted ChvE binding site have also been implicated in sugar signaling. For example, allele products carrying E255Q or lacking amino acids 242 through 257 of VirA are no longer sugar responsive (2). In strain A136 carrying a wild-type copy of *chvE*, VirA with aa 242 through 257 deleted was not responsive to glucose, but the activity at pH 5.5 was more than 10-fold higher than that at pH 7.0 (Fig. 6C). When this VirA allele is placed in the DL8 strain, in which *chvE* is disrupted, pH induction is no longer observed. Therefore, the pH response is dependent not on sugar but on *chvE* achieving a highly specific, sugar-independent pH response element.

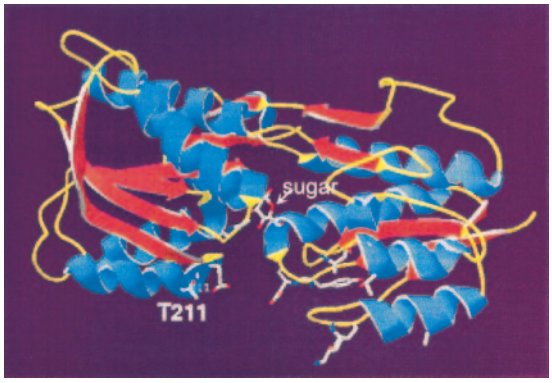

FIG. 7. Simulated structure of ChvE. The structural data from GBP and RBP were used as templates to generate the coordinates for ChvE by use of Swiss-Prot. The resulting structure of ChvE contains two domains joined by three hinges with the sugar binding site located within the domain cleft. Based on the RBP and GBP models, the indicated side chains are involved in protein-protein interactions.

DISCUSSION

Environmental pH sensing represents a critical regulatory module that interconnects with virtually every component of the cellular network, controlling responses as diverse as cell growth, energy interconversion, taxis, symbiosis, and pathogenesis. Pathogens have evolved simple, efficient and, very precise host sensing modules (33). For example, the tumor-inducing plasmid of *Agrobacterium* encodes a two-component sensor kinase, VirA, which mediates virulence by responding to environmental pH. While VirA responds to diverse structural inputs, we have exploited the modularity of this two-component sensor kinase to resolve the pH-signaling module and generate elements that respond specifically to extracellular pH.

Agrobacterium is shown here to maintain a global acidic pH response, generally increasing pTi gene expression by approximately 1.2- to 2.0-fold when the environmental pH drops from 7.0 to 5.5. Acidic pH is known to increase the expression of *virG* through the P2 promoter (49, 51), and the previously discovered ChvG/ChvI two-component system (26) may well play an important role in promoter activation (13), but little is currently known about its mechanism. This global pH response may also have complicated previous assignments of the cytoplasmic domain of VirA in pH sensing (12).

In addition to this global pH response, distinct pH activation is associated specifically with the periplasmic domain of VirA. Removal of the periplasmic domain elevated *vir* induction over that of full-length VirA at pH 7.0, consistent with the periplasmic domain limiting the activity of VirA at pH 7.0. Banta et al. and others showed that the removal of aa 63 through 240, the presumed sugar binding region in the periplasmic domain, resulted in higher *vir* expression in the absence of sugars, supporting a model in which the ChvE-sugar complex relieves repression by the periplasmic domain (2, 10, 38). Thus, the periplasmic domain of VirA may play an inhibitory role, and the perception of sugars and pH relieves this inhibition to achieve the host-specific response.

This pH response is mechanistically coupled with sugar perception. Either the absence of sugars (Fig. 4) or the disruption of *chvE* (Fig. 5A and B) abolishes pH signaling, a response rescued by the in *trans* expression of *chvE* (Fig. 5C and D). Such pH and sugar perceptions may be coupled by either pH-induced expression of *chvE* or a conformation change required for optimal ChvE-VirA interaction. *chvE* expression is regulated by a subset of inducing sugars through GbpR, a LysR family transcriptional regulator (16, 36), and there is no evidence for the existence of a pH-regulated promoter for either *chvE* or *gbpR*. Moreover, replacing the wild-type *chvE* promoter with a constitutive P_{N25} promoter does not decouple pH sensing and sugar sensing. However, we cannot exclude the possibility that extracellular pH alters the turnover of the periplasmic ChvE protein, resulting in the dependence of sugar response on environmental pH.

Specific substitutions in ChvE override the requirement for both low pH and sugar for optimal *vir* expression. The ability of ChvE(T211M) to rescue the sugar response of VirA(E210V) might suggest that residue 211 is critical to the VirA-ChvE interaction, possibly by increasing the binding affinity to VirA so that either sugar- or low-pH-induced changes are sufficient for perception. Therefore, pH or sugar sensing is most likely mediated through a direct interaction between VirA and ChvE.

Figure 7 shows the homology model built on the crystal structure templates of ribose binding protein (RBP) and glucose-galactose binding protein (GBP). T211 is predicted to reside on the surface of ChvE, close to the "lips" of two domains in RBP and GBP where mutations that alter the protein-protein interactions with chemoreceptors and membrane transport proteins have been found (5, 7, 17). The binding affinity of sugar substrates can also be altered by mutations in this region, even though it is distinct from the buried sugar binding site (34, 44). Moreover, GalR, one of the LacI-GalR family of bacterial repressor proteins that has an N-terminal DNA binding domain and a C-terminal domain homologous to those of sugar binding proteins, displayed both pH- and galactose-dependent behavior of DNA binding (8). Further analysis suggested that pH and galactose could both modify the structure of the sugar binding domain of GalR to affect dimer formation and alter DNA binding affinity (9, 23). In addition, CcpA, another member of the GalR family of proteins, requires both an acidic pH and its sugar substrate glucose-6-phosphate to bind its regulatory DNA element (20). Therefore, it is not surprising that both a certain pH and sugars are required to activate ChvE for optimal interaction with VirA.

Taken together, pH sensing through VirA relies on the VirA-ChvE interaction and is coupled with the perception of other signals. This pH sensing mechanism, the first to be described in microorganisms, may be common. For example, both external pH and iron are sensed by the PmrA/PmrB two-component system of plant pathogen *Erwinia carotovora* subsp. *carotovora* to contribute to bacterial virulence (24). This ability to switch on only in the presence of two signals could greatly increase response precision. Similar arguments have been made for phenol perception by VirA (46), in which multiple inputs "ratchet" in the full response. However, the additive response of the VirA-ChvE interaction may not be solely at the level of perception. Removal of aa 242 through 257 from VirA leads exclusively to a pH sensor. This deletion could alter the ChvE binding site, decoupling pH and sugar perception, but the amino acids deleted abut the second membrane-spanning segment of VirA, suggesting that signal transmission through the membrane may be altered. Thus, sugar and pH transmission events may be processed separately. We are now positioned to resolve these mechanistic questions, to extend the use of these signaling modules in heterologous hosts (27), and to evolve different pH responses and alternate signal inputs for other regulatory networks.

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