

# Phosphoenolpyruvate Carboxykinase Is an Acid-Induced, Chromosomally Encoded Virulence Factor in *Agrobacterium tumefaciens*

Pu Liu, Derek Wood,<sup>†</sup> and Eugene W. Nester\*

Department of Microbiology, Box 357242, University of Washington, Seattle, Washington 98195

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**The *pckA* gene, encoding phosphoenolpyruvate carboxykinase, catalyzes the reversible decarboxylation and phosphorylation of oxaloacetate to form phosphoenolpyruvate. Located on the circular chromosome of *Agrobacterium*, this locus is adjacent to the loci *chvG* and *chvI*, encoding a two-component regulatory system that has been shown to be important in virulence. Using a reporter gene fusion, studies showed that the *pckA* gene is induced by acidic pH but not by acetosyringone. This acid induction is regulated by the *chvG-chvI* regulatory system, which controls acid-inducible genes. A *pckA* mutant had no demonstrable PckA enzyme activity and grew on AB minimal medium with glucose but did not grow on the same medium with succinate as the sole carbon source and was more inhibited in its growth than the wild-type strain by an acidic environment. A *pckA* mutant was highly attenuated in tumor-inducing ability on tobacco leaf disks and was severely attenuated in *vir* gene expression. Although *vir* gene induction was completely restored when a constitutive *virG* gene was introduced into the mutant strain, virulence was only partially restored. These results suggest that avirulence may be due to a combination of the inhibition of this mutant in the acidic plant wound environment and the poor induction of the *vir* genes.**

The transfer of DNA and proteins into host cells by *Agrobacterium* requires that both plasmid- and tumor-inducing (Ti) plasmid-encoded genes participate (for a review, see reference 11). The virulence (*vir*) genes located on the Ti plasmid, defined as those under the control of the two-component regulatory system VirAG, appear to be dedicated to the processing and transfer of the transfer DNA (15). The genes that map to the chromosome (*chv* genes) have dual functions. They play a role in the physiology of the organism growing in the absence of its hosts, as well as in the interaction of *Agrobacterium* with its plant hosts (11). Perhaps the best-understood example of a *chv* gene product is *chvE* (6). This periplasmic protein binds to monosaccharides, which are components of the plant cell wall, and thereby plays a role both in the transport of these sugars into the cell and in the chemotaxis of bacteria toward these sugars. In addition, the ChvE protein, when bound to a sugar synthesized by plant cells, increases the level of induction of *vir* genes by binding to the periplasmic domain of the VirA sensor protein (10). In this way, *Agrobacterium* senses the proximity of a susceptible plant. In addition to the monosaccharides bound to the ChvE protein, *Agrobacterium* recognizes several other signals associated with the plant wound environment. These include acetosyringone (AS) and an acidic environment (pH 5.5) (1, 37). Upstream of each of the *vir* genes is a 12-base-pair consensus sequence, the *vir* box, which is recognized and bound by the response regulator, VirG (9). Although it is likely that all of the genes required for processing and transfer of the

transfer DNA have been identified on the Ti plasmid, the identities and functions of many of the *chv* genes involved in plant cell transformation await elucidation.

Chromosomal virulence genes that have been identified include those that play a role in osmotic stress and attachment (5), *vir* gene induction (12, 23, 27), and avoidance of plant defenses (40). One of the most interesting and significant of these chromosomal virulence genes is a two-component regulatory system, *chvGI*, in which, by homology to other two-component systems, *chvG* codes for the sensor protein and *chvI* codes for a response regulator (7, 25). This system is of special interest because it is a global regulatory system involved in the regulation of certain acid-inducible genes (21). These include the chromosomal gene *katA*, which encodes a catalase that is involved in the detoxification of H<sub>2</sub>O<sub>2</sub>, presumably released during the early interaction of *Agrobacterium* with its host plant (40), and *aopB*, which encodes an outer membrane protein also required for virulence (18). Its function in virulence is not known. In addition, mutations in *chvI* significantly reduce the expression of *virG* (25), which in turn inhibits the expression of the *virB* and *virE* operons. All of these genes under the control of *chvGI* have at least one feature in common. They are all acid inducible, and their levels presumably increase in the environment of a wounded plant.

Mutations in the *chvGI* loci, apparently pleiotropic, confer a number of distinctive properties on the cells. *chvG chvI* mutants cannot grow in media containing the antibiotics tetracycline, novobiocin, and carbenicillin, as well as several detergents (7). This suggests that the mutants may have defects in their cell envelopes. Furthermore, mutants cannot grow on a complex medium, a trait that is shared with another *chv* mutant, *chvD* (23).

Because of the importance of the *chvGI* system in the interaction of several  $\alpha$  proteobacteria with their eukaryotic hosts,

\* Corresponding author. Mailing address: Department of Microbiology, Box 357242, University of Washington, Seattle, WA 98195-7242. Phone: (206) 616-8588. Fax: (206) 543-8297. E-mail: gnester@u.washington.edu.

<sup>†</sup> Current address: Department of Biology, 3307 3rd Ave. W., Suite 205, Seattle Pacific University, Seattle, WA 98119.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>Agrobacterium</i>		
C58	Wild-type strain	14
At11063	C58 <i>chvG</i> <sub>202</sub> :: <i>aac1</i>	7
A136	C58; Ti plasmid cured	35
C58Δ <i>pckA</i>	C58 (Δ <i>pckA</i> )	This study
<i>E. coli</i> strain DH5α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA)U169 φ80dlacZΔM15</i>	Invitrogen
Plasmids		
pSM243cd	<i>virB</i> :: <i>lacZ</i> fusion of pTiA6 in pVK102	36
pSM358cd	<i>virE</i> :: <i>lacZ</i> fusion of pTiA6 in pVK102	36
pWT160	<i>virG</i> :: <i>lacZ</i> fusion	33
pEX18Gm	Gm <sup>r</sup> <i>oriT</i> <sup>+</sup> <i>sacB</i> <sup>+</sup> ; gene replacement vector from pUC18	16
pBBR1MCS4	Broad-host-range vector	20
pAB2002	Vector for <i>lacZ</i> fusion	3
pSY204	<i>virG</i> constitutive construction	19
pLP200	pBBR1MCS-4 with 1.5-kb <i>pckA</i> fragment	This study
pLP201	pWT200 with <i>lacZ</i> -Gm <sup>r</sup> fragment	This study
pEX18Gm- <i>pckA</i>	Construct for deleting <i>pckA</i>	This study

it is important to identify additional target genes of this regulatory system. It is well known that genes under the control of a two-component system often map in the vicinity of the regulatory genes (7). This potential candidate gene downstream of *chvGI* encodes phosphoenolpyruvate carboxykinase (PckA), the first enzyme of gluconeogenesis.

Mutations in the *pckA* locus have profound biological effects in a variety of other bacteria. For example, a *pckA*-deficient *Mycobacterium bovis* BCG mutant was attenuated in infection of both macrophages and mice (22). The *pckA* mutation in *Rhizobium* sp. strain NGR234 resulted in different nodulation phenotypes depending on the host plant (31). In *Sinorhizobium meliloti*, a *pckA* mutant fixed nitrogen at 70% of the level of the wild type, whereas the *pckA* mutant of *Rhizobium leguminosarum* fixed nitrogen at the same efficiency as wild-type cells.

In this paper, we demonstrate that *pckA* is indeed under the control of *chvGI* and that, like other genes under this control system, the expression of *pckA* is induced by acid and is important for maximum virulence. Other properties of the gene and its protein product are described.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. *Agrobacterium tumefaciens* strains were grown in Mg/L or AB minimal medium at 28°C with shaking (4). *Escherichia coli* strain DH5α was grown in LB medium at 37°C with shaking (34). The following antibiotics were used at the indicated concentrations (in μg/ml): for *A. tumefaciens*, carbenicillin (100), kanamycin (100), and gentamicin (50); for *E. coli*, carbenicillin (100), kanamycin (100), and gentamicin (10). *vir* gene induction was analyzed in cells grown in induction broth as previously described (28).

The detergents sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC), and Sarkosyl were used at concentrations (in mg/ml) of 0.2, 2, and 2, respectively (7).

**Construction of *pckA-lacZ* fusion.** To determine whether *pckA* is acid inducible, the *pckA* gene was fused with the *lacZ* reporter gene. To make this construct, two primers, *pckA1* (CGTTCGATCTCGAGTTGCGTTTCCAAAAG CTG) and *pckA2* (GCTAGTTAGAATTCTCATTACAGCCGCGAGCAG), were used to amplify the *pckA* gene from *A. tumefaciens* genomic DNA, using *Taq*-plus polymerase. *XhoI* and *EcoRI* restriction sites were then introduced into primers *pckA1* and *pckA2*, respectively. The 1.5-kb *pckA* PCR product was digested with *XhoI* and *EcoRI* and ligated to a 4.95-kb *XhoI*-*EcoRI* fragment of pBBR1MCS-4 to create pLP200. The 3.5-kb *EcoRI* fragment of pAB2002 con-

taining the *lacZ* and gentamicin resistance genes was cloned into pLP200, which was then digested with *EcoRI*, creating pLP201.

**Construction of the *pckA* in-frame deletion mutant.** The unmarked *A. tumefaciens pckA* deletion mutant was generated as described previously (16). In brief, 1.5-kb regions were amplified from the upstream and downstream regions flanking the region targeted for replacement, using primers that included specific restriction enzyme sites. After restriction enzyme digestion, the upstream and downstream fragments were ligated into the vector pEX18Gm using a directional three-way ligation. The plasmid was introduced into strain C58 by electroporation, and after incubation for 3 h to allow homologous recombination, the cells were plated on LB medium with 5% sucrose for the first selection. Colonies growing on the sucrose plates were streaked onto plates of Mg/L medium and Mg/L medium plus 25 μg/ml gentamicin for the second selection. The deletion mutant cannot grow on Mg/L medium containing 25 μg/ml gentamicin. Putative mutations were verified by sequencing the junction fragment generated by the deletion using PCR fragments that spanned the open reading frame selected for deletion. All experiments that involved a mutation of the *pckA* locus used an in-frame deletion mutation of the entire gene unless otherwise indicated.

**Gene expression measurements.** Expression of the *pckA* gene was measured as β-galactosidase activity using the *pckA*::*lacZ* fusion. The expression of the *virG*, *virB*, and *virE* genes was assayed by using the *virG*::*lacZ*, *virB*::*lacZ*, and *virE*::*lacZ* fusions, respectively (Table 1). For the assay of β-galactosidase, *A. tumefaciens* cells containing the appropriate fusions were grown in AB minimal liquid medium with shaking at 28°C overnight and then transferred to induction broth (4) at pH 5.5 or fresh AB minimal medium (pH 7.0) and incubated for 24 h. The β-galactosidase activity reported was assayed as described previously (28). All β-galactosidase activities represent an average of three independent determinations.

**Virulence assay.** Virulence assays were performed on *Nicotiana tabacum* leaf disks according to the method of Banta et al. (2). Briefly, *A. tumefaciens* cells were grown in liquid Mg/L medium to mid-log phase and harvested by centrifugation, and the cell concentration was adjusted to an optical density at 600 nm (OD<sub>600</sub>) of 0.4 to 0.5 with MS medium (29). The cells were cocultivated with 40 leaf squares of *N. tabacum* in 20 ml of hormone-free MS liquid medium (29) in a petri dish supplemented with 300 μM AS. After 2 days, 40 leaf squares were transferred to hormone-free MS medium containing vancomycin (200 μg/ml) and timentin (200 μg/ml) and cultured at 25°C in the dark. The tumors on each leaf disk were observed after 14 days.

**Complementation.** The plasmid pSY204 (24), containing the constitutive *virG* gene, which induces the *vir* regulon in the absence of AS and sugar and at a neutral pH, was electroporated into C58 containing the *virB-lacZ* or *virE-lacZ* construct.

**Assay of PckA enzyme activity.** Cell extracts were prepared from cells grown in 50 ml AB medium grown to the stationary phase. Cells were harvested by centrifugation in a Sorvall RC-58 centrifuge (10 min; 4°C; 10,000 × g) and then washed twice with cold 20 mM Tris-HCl buffer containing 1 mM MgCl<sub>2</sub> (pH 7.4) and resuspended in the same buffer. The cells were sonicated on ice six times for

TABLE 2. PEP carboxykinase activities under different growth conditions<sup>a</sup>

Conditions	Sp act (nmol of ADP/mg of protein/min)	
	C58	<i>pckA</i> mutant
AB medium	7.0 ± 0.3	0.3 ± 0.2
+ 6 mM MgCl <sub>2</sub>	7.6 ± 0.4	
+ 6 mM MgCl <sub>2</sub> and 6 mM CaCl <sub>2</sub>	8.5 ± 0.4	
+ 1 mM MnCl <sub>2</sub>	7.2 ± 0.3	
+ 1 mM MnCl <sub>2</sub> and 6 mM CaCl <sub>2</sub>	6.8 ± 0.3	
Log phase	6.7 ± 0.2	

<sup>a</sup> *A. tumefaciens* strains were cultured to stationary phase in AB minimal medium (pH 7.0) plus different compounds. The values in the table are means ± standard deviations from at least three independent experiments.

15 seconds each time. The crude extract was clarified by centrifugation (15 min; 13,000 × g), and the supernatant was assayed for PckA activity by a procedure described previously (17).

## RESULTS

**Identification and characterization of the *pckA* gene.** The *pckA* gene encodes the enzyme phosphoenolpyruvate (PEP) carboxykinase, which catalyzes the decarboxylation and phosphorylation of oxaloacetic acid to produce the glycolytic intermediate PEP. This enzyme is the first enzyme in gluconeogenesis. The gene, coding for a predicted protein of 537 amino acids, was initially identified in *Agrobacterium* from the similarity of its nucleotide sequence to those of previously identified genes. The predicted protein encoded by the gene has 52.4% identity to the *E. coli* (26) and 78.3% identity to the *S. meliloti* PckA proteins (30). The PckA enzyme has the specific domain (IGGTSYAGE-KKS; 190 to 202), which is required for its activity (30), and a phosphate-binding site (G--G-GKT; 236 to 243). A divalent or transition metal ion binding site (G--EG) could also be identified in residues 226 to 229. A BLAST search of the C58 genome did not reveal any other copies of this gene (39). Nutritional and biochemical data support the contention that this protein, identified as PckA by a bioinformatics analysis, is the only protein in *Agrobacterium* strain C58 with PckA enzyme activity. This activity is not activated by calcium or cell growth to stationary phase (Table 2), which differs from what has been observed in *E. coli* (26). Furthermore, crude extracts from cells with a deletion of the gene lack PckA enzyme activity (Table 2). Cells with a mutation in the gene cannot grow on succinate as a sole carbon and energy source, presumably because they lack this key enzyme in gluconeogenesis and therefore cannot synthesize the sugars required for macromolecule synthesis. This suggests that an alternative pathway from oxaloacetate to PEP does not exist in *A. tumefaciens* strain C58. The genome sequence indicates that two putative malic enzymes that convert malate to pyruvate are encoded (*atu1652* and *atu3356*), but no evidence for phosphoenolpyruvate synthase, the enzyme that synthesizes PEP from pyruvate, could be seen from a BLAST search of the genome.

The *pckA* gene maps to the circular chromosome and is downstream of genes encoding a two-component system, *chvG* and *chvI* (7, 25, 39), but is transcribed in a direction opposite to that of the regulatory genes (Fig. 1). Downstream of the

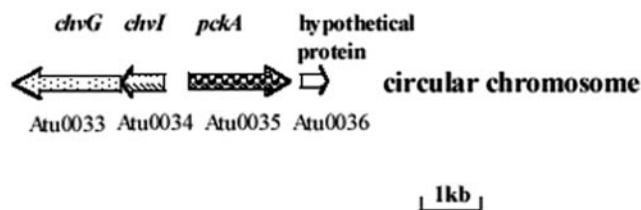


FIG. 1. Location of *pckA* on the circular chromosome of *A. tumefaciens* C58.

*pckA* gene is a locus that by BLAST analysis codes for a conserved hypothetical protein and is transcribed in the same direction as the *pckA* gene.

**Regulation of *pckA* expression.** Since two-component regulatory systems often regulate genes that map nearby, we determined whether *chvGI* regulates the expression of *pckA*. Since a previous study (21) showed that this two-component system regulates many acid-inducible genes in *Agrobacterium*, we first determined whether *pckA* is acid inducible. To test this possibility, *pckA* was fused with the reporter gene *lacZ*; the *pckA-lacZ* fusion was then introduced into strain C58, and the β-galactosidase activity was assayed. As shown in Table 3, *pckA-lacZ* expression increased 4.2-fold when the cells were grown in minimal medium at pH 5.5 compared with growth in the same medium at pH 7.0. Moreover, AS in the induction medium did not affect the expression of *pckA* (data not shown). This observation was expected, since no *vir* box, which is found in the promoter regions of AS-induced genes, could be identified in the upstream region of the gene. These results indicate that *pckA* is an acid-inducible gene.

To determine whether ChvGI plays a role in the regulation of *pckA*, the *pckA-lacZ* fusion was introduced into a *chvG* Tn-*phoA* insertion mutant (7). We found that the expression of *pckA* in this *chvG* mutant was reduced about 90-fold compared to its expression in wild-type cells grown at pH 5.5 (2,468 versus 27) (Table 3). Moreover, the expression of *pckA* in the *chvG* mutant is the same in cells growing at pH 5.5 and pH 7.0 but is 10-fold lower than the expression in the wild-type strain growing at pH 7.0. It appears that *chvG* is very important for the expression of *pckA*.

In *Sinorhizobium meliloti*, the expression of *pckA* is modulated by the carbon source, and arabinose is a stronger inducer of *pckA* expression than glucose (30). To determine if the expression of *pckA* in *Agrobacterium* is also controlled by the carbon source, cells containing the *pckA-lacZ* fusion were cultured in AB minimal medium at pH 7.0 with either glucose,

TABLE 3. Effects of *chvG* on the expression of the acid-inducible gene *pckA*, determined with the *pckA-lacZ* fusion<sup>a</sup>

Strain	β-Galactosidase activity		Change (fold)
	pH 5.5	pH 7.0	
C58 (wild type)	2,468 ± 145	583 ± 45	4.2
<i>chvG</i> mutant	27 ± 8	31 ± 9	0

<sup>a</sup> All strains were grown for 20 to 24 h in AB minimal medium at the pH indicated. The cultures were then assayed for β-galactosidase activity as described in Materials and Methods. The data represent the average of three independent experiments. Data are expressed in Miller units (28).

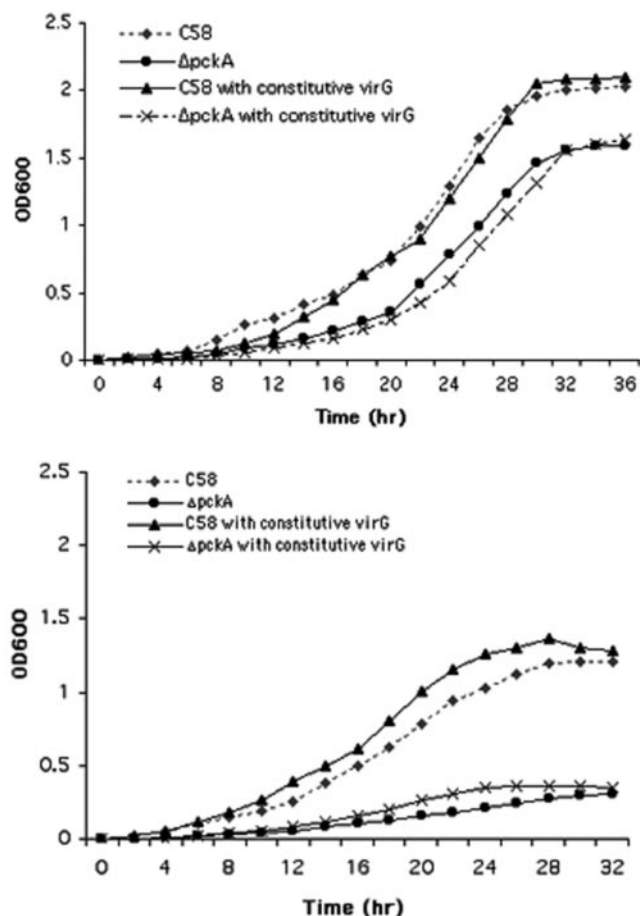


FIG. 2. Growth curve of  $\Delta pckA$  in AB minimal medium. Log-phase cultures of C58, C58 with constitutive *virG*,  $\Delta pckA$ , and  $\Delta pckA$  with constitutive *virG* were diluted in AB minimal medium (top, pH 7.0; bottom, pH 5.5) with a starting  $OD_{600}$  calculated to be 0.001. The cells were grown at 28°C with shaking. The  $OD_{600}$  was measured at 2-h intervals over a 36-h period.

arabinose, glycerol, or sucrose as the sole carbon source. No difference in the expression of *pckA* was seen in cells grown on these different carbon sources (data not shown).

**The *pckA* mutant is inhibited by an acidic environment.** Since *pckA* is an acid-inducible gene, we next determined if a *pckA* deletion mutant was acid sensitive by monitoring the growth behavior of both the *pckA* mutant and its parent strain, C58 (Fig. 2). The *pckA* mutant grew more slowly than its parent at pH 7.0, and this growth defect was even more pronounced at pH 5.5. Furthermore, we grew the *pckA* mutant and wild-type C58 in the acidic medium and adjusted to the same OD and then plated serial dilutions of the bacteria on AB solid medium at pH 7.0; the number of *pckA* mutants growing on AB solid medium was threefold less than that of the wild-type C58. These data suggest that the *pckA* mutant is indeed inhibited by acid. The fact that gene expression is acid inducible suggests that the gene plays a role in overcoming growth inhibition under acidic conditions. Therefore, it is not surprising that a strain lacking the gene would be acid sensitive.

**Virulence of *pckA* mutant.** To determine if the *pckA* locus is important in tumor formation, we inoculated tobacco leaf disks

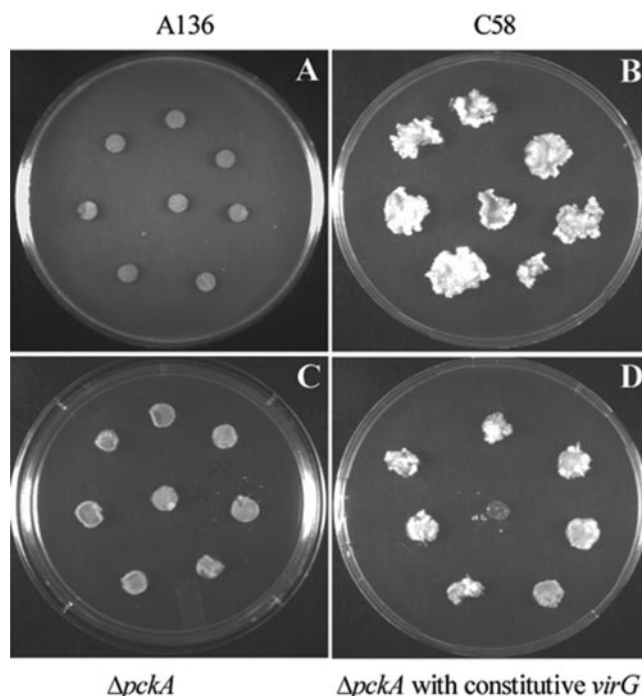


FIG. 3. Virulence assay of *pckA* mutant on tobacco leaf disks. *A. tumefaciens* cells were grown and inoculated onto tobacco leaf disks as described in Materials and Methods. Tumor formation on leaf disks ( $n = 40$ ) is shown.

with the *pckA* deletion mutant, as well as the C58 strain and the C58 strain cured of its Ti plasmid (A136), as described in Materials and Methods. The results are shown in Fig. 3 (compare B and C). The *pckA* deletion mutant was significantly less virulent than the wild-type C58 strain.

***vir* gene induction of mutant.** The attenuated virulence of the *pckA* mutant may be due to the poor growth of the mutant in the acidic plant wound environment, which likely alters the overall physiology of the cell, and/or some other requirement for tumor formation. A readily testable possibility is that the mutation reduces the level of *vir* gene induction in some way. This seemed like a real possibility, since so many other mutations (*ivr211*, *miaA*, and *chvD*), and another gene in carbohydrate metabolism (38), also reduced *vir* gene induction. The plasmid pSM243cd containing the *virB::lacZ* translational fusion was introduced into the *pckA* deletion mutant and the wild-type C58 strain. As shown in Table 4, expression of the *virB-lacZ* fusion was reduced 84% in the *pckA* deletion mutant compared to the wild-type strain. We also introduced a *virE-lacZ* fusion on a plasmid (pSM358cd) into the same strain. Under optimal inducing conditions, the expression of *virE-lacZ* was reduced approximately the same amount, 80%. These results show that the expression of both *virB* and *virE* decreased in the *pckA* mutant to an extent that could likely explain its attenuated phenotype. To further explore this possibility, a *virG-lacZ* translational fusion, pWT160, was introduced into the *pckA* deletion mutant. In the presence of AS, the expression level of *virG-lacZ* decreased 88% compared with the wild type under inducing conditions (Table 4). This greatly reduced induction of the response regulator *virG* could certainly ac-

TABLE 4. *vir* gene induction by AS and acidic pH in a *pckA* deletion mutant

Plasmid	$\beta$ -Galactosidase activity <sup>a</sup>					
	C58		$\Delta pckA$		$\Delta pckA$ with constitutive <i>virG</i>	
	pH 7.0	pH 5.5 + AS	pH 7.0	pH 5.5 + AS	pH 7.0	pH 5.5 + AS
<i>virB::lacZ</i>	3 $\pm$ 2	579 $\pm$ 15	5 $\pm$ 4	95 $\pm$ 11 (83.5%)	523 $\pm$ 56	592 $\pm$ 76
<i>virE::lacZ</i>	11 $\pm$ 2	629 $\pm$ 23	5 $\pm$ 3	126 $\pm$ 10 (80.0%)	579 $\pm$ 67	606 $\pm$ 45
<i>virG::lacZ</i>	7 $\pm$ 5	1,043 $\pm$ 25	21 $\pm$ 5	125 $\pm$ 15 (88%)	907 $\pm$ 134	1,278 $\pm$ 63

<sup>a</sup> *A. tumefaciens* strains were cultured in AB minimal medium (pH 7.0) or induction medium at pH 5.5 and assayed for  $\beta$ -galactosidase activity between 20 and 24 hours. The data are means  $\pm$  standard errors of the mean calculated from three independent experiments. The percentages in parentheses represent the reduction in expression in the  $\Delta pckA$  mutant relative to the C58 wild-type strain grown at the same pH. Data are expressed in Miller units (28).

count for the reduced level of expression of *virB* and *virE* and thereby play a role in the attenuated virulence of the *pckA* mutant.

**Effect of constitutive *virG* on *vir* gene induction, virulence, and growth.** To gain some insight into whether the reduced *vir* gene expression was responsible for the attenuated tumor phenotype and whether this reduction was upstream or downstream of the VirA-VirG signaling cascade, we introduced a constitutive *virG* locus (N54D) on the high-copy-number vector pSY204 into the *pckA* mutant. The mutant *virG* does not require AS, monosaccharides, or acidic conditions to induce the *vir* genes (19). These observations were confirmed (Table 4). However, although *vir* gene induction was raised to the wild-type level (Table 4), the *pckA* mutant still was not as virulent as the parent. Thus, pSY204 can only partially restore virulence (Fig. 3). Moreover, the constitutive *virG* mutation did not change the growth rate of the *pckA* mutant significantly in acidic or neutral glucose salts medium (Fig. 2). These results strongly suggest that reduced *vir* gene expression is only partially responsible for the attenuated avirulence of the *pckA* mutant. Presumably, the acid sensitivity of the mutant may also play a role in its reduced virulence.

**The phenotype of the *pckA* mutant.** A mutation in *chvGI* confers a number of distinct properties on the cell. Since *chvGI* is a positive regulator of *pckA*, some of these properties could be due to reduced expression of this gene. *chvGI* mutants are sensitive to the detergents SDS, DOC, and Sarkosyl. We investigated whether the *pckA* mutant displayed these properties by plating the *pckA* insertion mutant on solid AB minimal media with one of the three detergents (0.1 g/liter SDS, 1 g/liter DOC, or 2 g/liter Sarkosyl). No difference was observed in growth on media with and without these detergents (data not shown). Furthermore, the *chvGI* mutants cannot grow on a complex medium, although they grow well at pH 7.0 on minimal media. The *pckA* mutant grows well on a complex medium, and therefore, this mutation cannot account for the phenotype (data not shown).

## DISCUSSION

This study was initiated in order to identify additional genes that are under the control of the two-component regulatory system, ChvGI, that had previously been shown to be important in the virulence of *Agrobacterium* (7, 25). This regulatory system is especially important to understand because it controls many acid-inducible genes, both chromosomal and on the Ti plasmid, all of which (at least those so far identified) play

some role in the virulence of *Agrobacterium*. Furthermore, it appears to function upstream of the critically important two-component system VirAG, which responds to environmental signals and activates all *vir* genes on the Ti plasmid. Since acidic conditions represent one of the key environmental signals for a plant wound site, the identification and characterization of genes that respond to acid conditions is important to an overall understanding of the physiology of *Agrobacterium*, both as a ubiquitous inhabitant of soils, which often are acidic, and as a plant pathogen, which requires an acidic environment to activate the entire pathogenesis program. The importance of the *chvGI* regulatory system is further underscored by its importance in the interaction of other  $\alpha$  proteobacteria with their hosts. In *S. meliloti*, the synthesis of succinoglycan, which is required for the nodulation of alfalfa, is under the control of ExoS/ChvI, the homologs of ChvGI (8). In *Brucella*, BvrS/BvrR, the homolog of ChvGI, controls the synthesis of two outer membrane proteins, at least one of which is required for virulence (13).

The *pckA* locus falls into the class of acid-inducible genes under the control of ChvGI, which are important in virulence. Although only acid-inducible genes have been found thus far to be under the control of *chvGI*, not all such genes are regulated by *chvGI*. For example, the chromosomally encoded *acvB* locus, which is induced about threefold at pH 5.5 compared to pH 7.0 and which plays a role in virulence, is expressed to the same extent in a wild-type cell and a *chvI* mutant (P. Liu, unpublished observation). A purine biosynthesis gene, *purB*, is also acid inducible but is not under the control of *chvGI* (Y. Liang and P. Liu, unpublished observation).

The relationship between acid inducibility and growth inhibition of mutations at pH 5.5 is variable. A gene that is induced at pH 5.5 is likely to be especially important in a physiological process that occurs at that pH. This process might be related to the growth or survival of the bacteria in acidic soils or in the interaction with wounded plants in an acidic environment. In either case, a mutation in the gene would be magnified by a greater inhibitory effect on growth at the lower pH and perhaps a loss of virulence. This is the situation that has been observed. Thus, mutants of *pckA* and another *chv* gene, *acvB*, are inhibited in their growth at pH 5.5 much more than are their wild-type parents (M. Brodhagen, unpublished observation). It might be expected that a *chv* gene that plays a role in the physiology of *Agrobacterium* in the absence of a wounded plant would have several functions, only one of which is related to virulence. It would not be surprising for a mutation in a gene whose function is important for bacterial physiology under

acidic conditions to result in a greatly reduced rate of growth. On the other hand, mutants of *virG* or *virB* grow as well as the wild-type strain at pH 5.5 and 7.0. A mutation in a *vir* gene dedicated solely to virulence would not be expected to exhibit general physiological changes manifested by a lower growth rate at pH 5.5.

At least two factors apparently play roles in the attenuation of virulence in *pckA* mutants. One may be related to the general physiological consequences of the mutation and the other to effects on a specific interaction with a host plant. The first relates to the inhibition of growth of the *pckA* mutant at pH 5.5, which approximates the acidic conditions at the wound site on a plant. Although wild-type *Agrobacterium* does not grow as well at pH 5.5 as it does at pH 7.0, the mutation in *pckA* amplifies this growth inhibition. This increased generation time certainly must alter the overall physiology of the cell significantly, which in turn might lead to reduced DNA transfer. However, a reduced growth rate does not always reduce virulence. Liu et al. (23) reported that a mutation in *chvD* resulted in an avirulent strain that grew slowly in a complex rich medium but could be complemented with a plasmid that restored virulence without affecting the generation time of the cells. In the present study, the constitutive *virG* locus significantly enhanced the virulence of the *pckA* mutant without significantly affecting its growth rate (Fig. 2 and 3).

The second, and probably more interesting, factor that plays a role in reduced virulence in the *pckA* mutant is the major reduction in the expression of *virG* under acidic conditions with AS. This reduction in *virG* expression, in turn, results in the reduced expression of all *vir* genes. Thus, the induction of *virB* and *virE* is reduced over 80%. The basis for this reduction is not at all clear, but it apparently relates to an early step in the signaling process. Since a constitutive *virG* gene can overcome the reduction in the expression of all three *vir* genes, the *pckA* gene in some way must be involved in the signaling cascade upstream of *virG* gene expression. Furthermore, since the restoration of *vir* gene expression is not accompanied by a complete restoration of virulence, an additional factor(s) must be involved in reducing virulence. One possibility, as already discussed, is the sensitivity of the *pckA* mutant to growth under acid conditions.

The reason(s) why a mutation in *pckA* reduces signal transduction so significantly is not at all clear. This enzyme is the first step in gluconeogenesis, and therefore, the synthesis of sugars would be reduced. Although sugars are critical for *vir* gene induction, especially in strain C58, glucose was supplied, which should have provided the sugar needed for induction. Thus, the *pckA* mutant can be added to the list of chromosomal-gene mutants in *Agrobacterium* that are significantly reduced in *vir* gene induction. These include the *chvD* (23), *ivr211* (27), and *miaA* mutants (12). In none of these cases is the basis for this reduction understood. We have observed recently that a mutation in citrate synthase also results in reduced *vir* gene induction (38). The question of whether the reductions in *vir* gene induction in the two mutations in carbohydrate metabolism share a common basis awaits further study.

One of the interesting features that these studies have revealed is the difference in regulation of *pckA* shown by *Agrobacterium* compared to all of the other prokaryotes studied thus far. There is no evidence for catabolite repression by

glucose, sucrose, or glycerol in *Agrobacterium*, all of which are found in *Rhizobium*. Furthermore, in contrast to *Rhizobium*, there is no evidence from the *lacZ* fusion expression data in *pckA* that the enzyme is induced in the stationary phase of growth (data not shown). This induction in *Rhizobium* requires cyclic AMP (32), but there is no evidence for a cyclic AMP binding site in the promoter region of the *pckA* gene of *Agrobacterium*.

The biological significance of the divalent cation binding site is also uncertain. In *E. coli*, Ca<sup>2+</sup> binds to this site and activates the enzyme allosterically (26). We could not demonstrate that Ca<sup>2+</sup> activates the *pckA* enzyme in *Agrobacterium*. We conclude that the control of *pckA* differs in *Agrobacterium* and in *E. coli*. This may reflect the possibility that this enzyme plays somewhat different roles in *Agrobacterium*, *Rhizobium*, and *E. coli*. In all cases, PckA is the first enzyme in gluconeogenesis, and all three organisms can synthesize sugars from succinate. However, in *Agrobacterium*, it likely plays an additional as-yet-undefined role, which may involve the interaction of *Agrobacterium* with its hosts.

Although we can add the *pckA* gene to the list of genes that are under the control of the ChvGI regulatory system, it is clear that additional genes remain to be identified. This conclusion is based on the phenotypic characterization of mutants of *chvGI* and the mutants known to be under its control. *chvGI* mutants grow poorly under acidic conditions, do not grow on a complex medium, and are inhibited in their growth by detergents; the last phenotype suggests a defect in the cell envelope. Some of the mutants under the control of ChvGI have these properties. The *pckA* mutant grows poorly under acidic conditions, and the *aopB* mutation involves a protein on the surface of the cell. However, whether it confers sensitivity to detergents has not been reported. Since the ChvGI regulatory system plays a crucial role in the acidic signaling process between plants and *Agrobacterium*, it is of considerable interest to identify all of the genes under its control. Probably the most direct approach is to identify genes that are up or down regulated in a *chvI* mutant, using microarray technology. Such experiments are under way.

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