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Transcriptome Profiling and Functional Analysis of *Agrobacterium tumefaciens* Reveals a General Conserved Response to Acidic Conditions (pH 5.5) and a Complex Acid-Mediated Signaling Involved in *Agrobacterium*-Plant Interactions▽

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Agrobacterium tumefaciens **transferred DNA (T-DNA) transfer requires that the virulence genes (*vir* regulon) on the tumor-inducing (Ti) plasmid be induced by plant phenolic signals in an acidic environment. Using transcriptome analysis, we found that these acidic conditions elicit two distinct responses: (i) a general and conserved response through which *Agrobacterium* modulates gene expression patterns to adapt to environmental acidification and (ii) a highly specialized acid-mediated signaling response involved in *Agrobacterium*-plant interactions. Overall, 78 genes were induced and 74 genes were repressed significantly under acidic conditions (pH 5.5) compared to neutral conditions (pH 7.0). Microarray analysis not only confirmed previously identified acid-inducible genes but also uncovered many new acid-induced genes which may be directly involved in *Agrobacterium*-plant interactions. These genes include *virE0*, *virE1*, *virH1*, and *virH2*. Further, the *chvG-chvI* two-component system, previously shown to be critical for virulence, was also induced under acid conditions. Interestingly, acidic conditions induced a type VI secretion system and a putative nonheme catalase. We provide evidence suggesting that acid-induced gene expression was independent of the VirA-VirG two-component system. Our results, together with previous data, support the hypothesis that there is three-step sequential activation of the *vir* regulon. This process involves a cascade regulation and hierarchical signaling pathway featuring initial direct activation of the VirA-VirG system by the acid-activated ChvG-ChvI system. Our data strengthen the notion that *Agrobacterium* has evolved a mechanism to perceive and subvert the acidic conditions of the rhizosphere to an important signal that initiates and directs the early virulence program, culminating in T-DNA transfer.**

Agrobacterium tumefaciens can transfer and integrate an oncogenic transferred DNA (T-DNA) from its tumor-inducing (Ti) plasmid into a wide variety of susceptible dicotyledonous plants. The T-DNA becomes integrated into the plant genome, and expression of the transferred genes leads to synthesis of the phytohormones auxin and cytokinin, resulting in neoplastic growth and the formation of crown gall tumors, a serious problem in many horticultural crops and stone fruits (46). T-DNA transfer is initiated when the *vir* regulon becomes activated through the VirA-VirG two-component regulatory system (33, 64). VirA is a membrane-bound histidine sensor kinase, and VirG is a cytoplasmic transcriptional activator. Upon perceiving phenolic signals (e.g., acetosyringone) in the rhizosphere, VirA undergoes autophosphorylation on a conserved histidine and subsequently transfers the phosphoryl group to a conserved aspartate of VirG (12, 33). Phosphorylated VirG stimulates the transcription of 30 identified members of the *vir* regulon, including itself, by specifically binding to a conserved 12-bp AT-rich sequence (*vir* box) in the promoter regions (33, 52).

Previous studies have demonstrated that the VirA periplasmic domain is required for sensing monosaccharides (sugars produced by plants) through coupling with the sugar binding protein ChvE (4, 11, 12, 22, 53), while the VirA linker domain recognizes plant-derived phenolic signals (12). Interestingly, *vir* regulon induction occurs only under acidic conditions, at around pH 5.5, which is typically the environmental pH of the rhizosphere (20, 28), the site of *Agrobacterium* infection of plant hosts. It has been found that VirA is also involved in coupling the perception of the phenolic signal with the acid signal during *vir* regulon induction (12, 22), although the molecular mechanism underlying this signal pathway is still a mystery. There is also evidence suggesting that phenolic compounds activate the *virG* distal promoter (P1), while acid conditions alone induce the *virG* proximal promoter (P2), thereby raising the level of VirG (13, 43). The activation of the *virG* P2 promoter by an acidic signal also appears to involve a chromosome-encoded two-component system, ChvG-ChvI (39, 44).

In addition to the *vir* regulon, a number of chromosomally encoded genes (*chv* genes) are also important for virulence (4, 11, 14, 44, 53). *chv* genes play important roles both in the physiology of the organism growing in the absence of its plant hosts and in the interaction of *Agrobacterium* with its plant hosts (4, 11, 14, 44, 53). One of the most interesting and important *chv* gene systems is the *chvG-chvI* system. This sys-

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tem is essential for tumor formation and bacterial growth under acidic conditions (14, 39, 44). None of the *chv* genes are induced by phenolic signals or regulated by the VirA-VirG system.

Bacteria are subject to a wide range of pHs in their environments, and extracellular pH is an important factor influencing bacterial physiology (58). During a pH shift, bacteria can rapidly mount a complex cellular response to maintain the intracellular pH near neutrality, a process referred to as pH homeostasis (21, 30). *Agrobacterium* has been isolated from soils in all parts of the world. The ability to tolerate and adapt to various acidic conditions is critically important for the ability of *Agrobacterium* to infect plants in the mildly acidic rhizosphere (8, 20, 28, 39). Genome sequencing revealed that *Agrobacterium* has a relatively large genome (5.67 Mb) (70), and most strikingly, it contains almost 500 regulatory genes (9% of the total predicted open reading frames), including 52 two-component regulatory systems. This large complement of regulatory elements presumably gives *Agrobacterium* the ability to sense, respond to, and adapt to a dynamic and changing acidic rhizosphere. In addition to their involvement in induction of the *vir* regulon, acidic conditions also induce other *Agrobacterium* determinants required for virulence, such as *aopB* encoding an outer membrane protein (32) and *pckA* encoding phosphoenolpyruvate carboxykinase (40). Moreover, salicylic acid, a plant signal important in regulating plant defense, activates the quormone degradation system in *Agrobacterium*, which also is involved in virulence. Intriguingly, salicylic acid activates this system only under acidic conditions (74). These lines of evidence highlight the fact that signal perception and exchange of *Agrobacterium* with its plant hosts occur primarily under mild acidic conditions, and acidic conditions play critical roles in setting in motion the entire virulence program. However, how *Agrobacterium* senses and appropriately responds to acidic conditions in the rhizosphere is still unclear. To gain some insight into the complex acid signaling process, it is necessary to understand how *Agrobacterium* modulates gene expression at a global level as a response to mild acid conditions.

In this study, we used whole-genome microarrays to obtain the transcriptional profiles of wild-type *Agrobacterium* cells in the exponential phase grown under acidic conditions (pH 5.5) and neutral conditions (pH 7.0). Our data revealed that 152 genes were differentially expressed approximately twofold or more under the experimental conditions tested. The extent and complexity of the *Agrobacterium* responses to pH 5.5 were reflected in the wide distribution of genes that play a role in the general adaptative response, *Agrobacterium*-plant signaling, or directly contribute to *Agrobacterium* virulence.

MATERIALS AND METHODS

Bacterial strains, media, antibiotics, and general growth conditions. *Escherichia coli* strain DH5α was grown in LB medium at 37°C. *A. tumefaciens* strain C58 was used in this study, and it bears the nopaline-type pTi plasmid. *Agrobacterium* was grown aerobically at 28°C with shaking (200 rpm) in MG/L complex medium or modified AB minimal medium (10). Unless otherwise indicated, acid conditions or medium refers to pH 5.5, whereas neutral conditions or medium refers to pH 7.0. The defined AB minimal medium consisted of AB salts (10), 0.02× AB buffer, 0.5 mM phosphate, and 50 mM sodium 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.5) or morpholinopropanesulfonic acid (MOPS) (pH 7.0), with 0.5% glucose as the carbon source. Antibiotics were used at the following concentrations: for *Agrobacterium*, 100 μg/ml carbenicillin and 50 or 100 μg/ml gentamicin; and for *E. coli*, 100 μg/ml ampicillin and 10 μg/ml gentamicin,. The chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a concentration of 40 μg/ml.

Cell growth for microarray experiments. Seven independent biological replicates of *Agrobacterium* sp. strain C58 picked from well-isolated colonies on MG/L agar plates were grown overnight (16 to 18 h) in 3 ml of MG/L broth at 28°C. The following day, cells from each independent biological replicate were split into two equal aliquots (1.5 ml), pelleted, and washed thoroughly (five times) in fresh and prewarmed (at 28°C) AB minimal medium at either pH 5.5 or pH 7.0. Following washing, cells were subcultured in 15 ml of fresh and prewarmed (at 28°C) AB minimal medium buffered with either MES (pH 5.5) or MOPS (pH 7.0), using an initial optical density at 600 nm (OD_{600}) of around 0.15. After 7 h of incubation (mid-logarithmic phase of growth), the OD_{600} for cells growing in the acidic medium was approximately 0.7, whereas the OD_{600} of the culture growing under neutral conditions was approximately 0.8. Cells were then harvested for RNA isolation.

RNA isolation. Four milliliters of each of the cultures described above was mixed with 8 ml of RNA Protect bacterial reagent (Qiagen) and processed as recommended by the manufacturer. Cells were then immediately harvested by centrifugation at 4°C and 16,000 × *g* for 5 min, and the pellet was quickly frozen in a dry ice-ethanol bath and then stored at −70°C until it was used for RNA isolation. Total bacterial RNA was isolated using a Qiagen mini RNA isolation kit. Any contaminating DNA was removed by treatment with RNase-free DNase I (Ambion) by on-column DNase digestion. RNA integrity was checked by performing 1.5% agarose gel electrophoresis with Tris-acetate buffer, and the RNA concentration was determined spectrophotometrically at 260 nm (Beckman DU350 spectrophotometer). The absence of residual DNA was further confirmed by the lack of a product after 25 cycles of PCR with primers specific for the *virG* and *chvA* genes.

Microarray design and processing. Unique 60-mer oligonucleotides representing each of the 5,419 predicted *A. tumefaciens* open reading frames were selected using the Featurama program designed by the Institute for Systems Biology in Seattle, WA. All of the designed oligonucleotides were commercially synthesized in situ on glass slides (1 by 3 in.) with a total of 8,000 features by Agilent Technologies (High Point, NC). Each microarray slide contained duplicate sets of probes (i.e., two technical replicates) for 2,983 genes (55% of the 5,419 open reading frames) at different locations. Each microarray experiment reported below represented seven biologically independent replicates for each growth condition (either pH 5.5 or pH 7.0). Single-stranded cDNA was generated from 30 μg of total RNA using random hexamer primers and Superscript II (Invitrogen). Aminoallyl-modified dUTP was incorporated into the cDNA at a ratio of aminoallyl-modified dUTP to dTTP of 4:1. Indirect labeling was accomplished by incubating aminoallyl-modified cDNA with Cy3 or Cy5 monoreactive dye (Amersham). RNA from the cells grown at pH 7.0 was fluorescently labeled with Cy5, and RNA from the cells grown under acidic conditions (pH 5.5) was labeled with Cy3. The two differently labeled cDNA populations were mixed and hybridized simultaneously to the array slides, and the arrays were hybridized and washed according to manufacturer's instructions (Agilent publication G4140-90030, version 4.1, April 2004). Data acquisition was performed using an Agilent G2565AA microarray scanner, and data were extracted using Agilent's feature extraction software. Fluorescence data were processed using GenePix 6.0 image analysis software (Molecular Devices).

Microarray data analysis. Initial data handling and visualization were done with the Matlab software (The MathWorks, Natick, MA); all remaining data analysis was done in the "R" statistical computing environment using the "samr" package in Bioconductor (24). Array data were inspected for systematic intensity-dependent and spatial variation, as described by Cui and Churchill (17). This assessment yielded no indication of systematic spatial trends on the arrays and only a small amount of systematic intensity-dependent variation. Therefore, the "loess" procedure for removing systematic intensity-dependent variation (73) was used to normalize the array data. Normalized data were analyzed to identify candidate differentially expressed genes by the methodology generally referred to as "significance analysis of microarrays" (67). First, a test statistic was computed for each gene to evaluate the evidence for differential expression. This test statistic is similar to the statistic for the *t* test, but it has a modified denominator. Second, a reference distribution was estimated by permutation. Specifically, the labels of the pH 5.5 and 7.0 samples were shuffled, and the test statistics were recomputed with each permutation to generate a permutation null distribution. The computed test statistic for each gene was then compared against this reference distribution to get a nonparametric *P* value gauging the evidence that the gene is differentially expressed. For our chosen critical value of the test statistic, we estimated the proportion of "false discoveries" on the resulting gene list. The

TABLE 1. PCR primers used in this study

Primer[a]	Sequence[b]
Primers used to generate promoter-*gusA* gene fusions	
Atu6178(*virG*)-F	GTCAAGCTTACCGCTGAGCACCTGCTACAG
Atu6178-R	TGACTCGAGATCGACGACCACGACATCGAC
Atu1131(*aopB*)-F	GTCAAGCTTGCGAGATCGGAGGCGATTGCCC
Atu1131-R	TGACTCGAGTTGACCGTACCACCGAGGTAGGC
Atu2160-F	GTCAAGCTTGGTATTCAAGGTGGCGCAGGCTG
Atu2160-R	TGACTCGAGACCACGGTCGTAGCGCGGACGATA
Atu4343(*impA*)-F	GTCAAGCTTCCAGCAGCCAATGCGGTATATCCA
Atu4343-R	TGACTCGAGCTTCTGCGCGTGCCTGGTTTCTTTC
Primers used to knock out genes	
Atu0290-F	TCACTGCAGTCAGGCGAATGCGGCAAATGGTTG
Atu0290-R	CAGTCTAGACAATCGGCCTTGACGATCTCGTAG
Atu0841-F	TCACTGCAGGTTCCACACCCATCTCCTGCGAA
Atu0841-R	CAGTCTAGAAGAAGCGTCAGCGTGCCGCCATTG
Atu0944(*cscA*)-F	CAGTCTAGAACCGGCATCACCGTCGTCCATAC
Atu0944-R	TCACTGCAGTGGATCTGCTGCTCGGGAATACGG
Atu2055-F	CAGTCTAGAGCGATCCAGTCGGTCGTGGTCATG
Atu2055-R	TCACTGCAGTGGTGACGTTGTCGCCGACATTGG
Atu2224(*aldA*)-F	CAGTCTAGACGGTCGCCTATCATTTCCATGAAC
Atu2224-R	TCACTGCAGGCAAAGAAGATGTTCGGCGACTTG
Atu2470-F	CAGTCTAGATCGCCGTCTTCCTCGTCATCATC
Atu2470-R	TCACTGCAGATGGAGAACCAGAGCTTGCGTTTG
Atu3274-F	CAGTCTAGAGCCGATGTTCAGGCCGCCATTTCG
Atu3274-R	TCACTGCAGCTGGGTATCGGCCTCGATGGTCAA
Atu4130(*acsA*)-F	CAGTCTAGACGATGGCACACGACGCGAGATAAG
Atu4130-R	TCACTGCAGCGACGAAATCCGCAAGCGTCAAAC
Atu5278(*katN*)-F	GACTCTAGAGATCCTGAAGGCGCTCCCAAAGAT
Atu5278-R	CATCTGCAGCTCTTCGCCCAGCGTCTGTTCGAAC

[a] The suffix F indicates a forward primer, and the suffix R indicates a reverse primer.
[b] The underlining indicates restriction enzyme sites.

advantages of this approach include protection against false positives through the modified t-statistic and lessened reliance on parametric assumptions through permutation testing. Genes with significant P values and with $\log_2$ ratios of around 1.0 or more are reported here.

Genetic techniques and DNA methods. Plasmid isolation, restriction analysis, agarose gel electrophoresis, and DNA ligation were performed according to standard protocols (61). Plasmid DNA was introduced into *Agrobacterium* recipient cells by electroporation with a Bio-Rad Gene Pulser set at 25 RiF (capacitance), 400 fQ (pulse controller), and 2.5 kV. To generate promoter-*gusA* transcriptional gene fusions, each intergenic region containing the promoter of interest was PCR amplified from *Agrobacterium* strain C58 genomic DNA. The PCR primers are listed in Table 1. PCR-amplified promoter fragments (approximately 500 bp) were digested with appropriate restriction enzymes and then cloned into a modified pJP2 vector (54) in which the promoterless *gusA* (*uidA*) gene was replaced by the gene from pFUS1 (57). The corresponding gene fusion plasmids were verified by sequencing and subsequently introduced into *Agrobacterium* by electroporation.

Gene expression measurement. For the β-glucuronidase activity assay, *Agrobacterium* cells harboring the appropriate plasmid-borne promoter-*gusA* gene fusions were grown overnight in MG/L medium supplemented with carbenicillin (100 μg/ml). Cells were washed with acidified (pH 5.5) or neutral (pH 7.0) AB minimal medium five times and inoculated into fresh, prewarmed (28°C) AB medium buffered at pH 5.5 and pH 7.0, respectively, using an initial OD_{600} of 0.15. Bacteria were incubated for 7 to 8 h before they were assayed. β-Glucuronidase assays were performed with *p*-nitrophenyl glucuronide substrate, and activities (in Miller units) were determined by using the previously described protocol (75). Unless otherwise indicated, the data presented below are the averages of three independent determinations.

Construction of *Agrobacterium* null mutants. To disrupt an *Agrobacterium* gene, a 300- to 400-bp internal region of the gene was PCR amplified from strain C58 genomic DNA (with PCR primers listed in Table 1) and cloned into the suicide vector pUCP30T (GenBank accession number U33752; kindly provided by Herbert P. Schweizer, Colorado State University) to generate recombinant plasmids for mutagenesis. These gentamicin-resistant recombinant plasmids were introduced and recombined in *Agrobacterium* wild-type strain C58 by electroporation, and transconjugants were isolated, purified, and confirmed by PCR amplifying the conjunction region, followed by sequencing (data not shown).

Virulence assay with *Kalanchoe daigremontiana* leaves. The virulence of *Agrobacterium* strains was tested by inoculating wound sites on *K. daigremontiana* leaves as described previously (4). Wild-type strain C58 and avirulent strain A136 lacking the Ti plasmid served as controls for the tumorigenesis assay. The virulence assay was repeated at least three times for each strain using separate leaves. The tumors were assessed 14 days after inoculation.

RESULTS

***Agrobacterium* cell growth at pH 7.0 and 5.5.** To understand the responses of *Agrobacterium* to acidic growth conditions, we first compared the growth rates of *Agrobacterium* at acidic and neutral conditions (Fig. 1). We found that the growth rate was slightly lower at pH 5.5 than at pH 7.0. The organism still exhibited steady-state exponential growth. Thus, the acidic (pH 5.5) growth conditions conferred mild acid stress on the cells.

Overall changes in the gene expression profile under acidic conditions. To obtain a global view of the effects of acidic conditions on *Agrobacterium* gene expression, we compared the transcriptomes of *Agrobacterium* cells cultured at pH 5.5 and pH 7.0 in AB minimal medium (see Materials and Methods). Microarray analyses were performed as described in Materials and Methods. Data for genes with low P values and with average fold changes of approximately 2 or more based on seven biological replicates were considered both statistically and biologically significant. There was a very good correlation between duplicate probes on the array (Fig. 2A). Overall, 152 genes were identified as genes that were differentially ex-

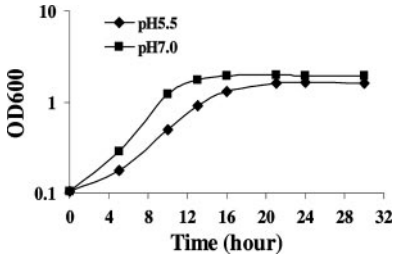

FIG. 1. *Agrobacterium* growth under acidic (pH 5.5) and neutral (pH 7.0) conditions. Cells were grown in MG/L medium overnight, washed five times with 0.85% NaCl, and inoculated into fresh AB minimal medium at the indicated pHs with an initial OD_{600} of around 0.05. The OD_{600} values are the means of three independent experiments.

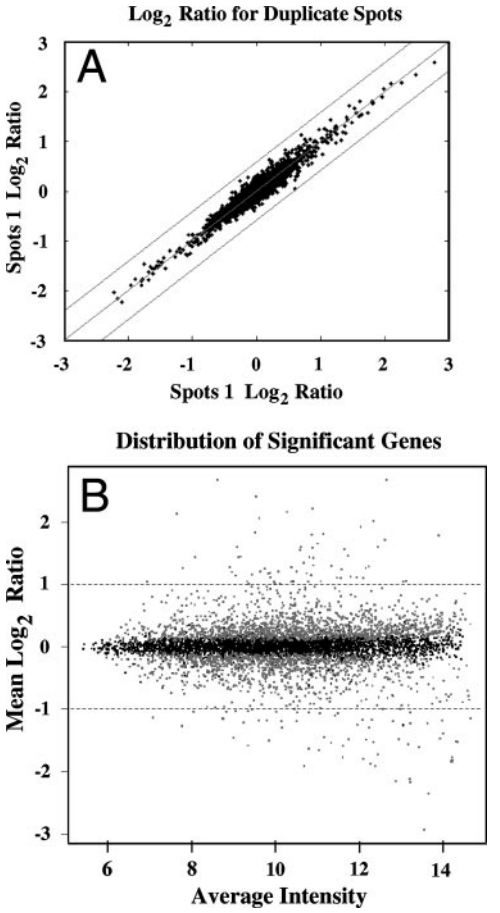

FIG. 2. Statistical analysis of microarray data. (A) Repeatability of the duplicate probes on the array. (B) Modest number of genes differentially expressed under acidic and neutral conditions.

pressed. The modest number of acid-regulated genes obtained from the microarray analysis further indicates that pH 5.5 represented only mild acid stress conditions (Fig. 2B). The acid-regulated genes (either induced or repressed) were classified according to predicted gene functions using Gene Ontology categories (27, 70) (Tables 2 and 3).

Seventy-eight genes were significantly induced (approximately twofold or more) under acidic conditions (Table 2). These genes fell into nine functional categories. Figure 3 summarizes the percentages of differentially induced genes. The largest category includes 17 genes mainly concerned with the synthesis of the cell envelope (Fig. 3). Of the 30 *vir* regulon members identified, 5 were significantly induced under acidic conditions. Another major group that was differentially expressed was the 15 transporter genes predicted to function in transporting sugars and peptides. Of special interest was the observation that acidic conditions stimulated the expression of a putative nonheme catalase and the *imp* gene cluster, which encodes a putative type VI secretion system (T6SS) (Table 2) (47). Five genes commonly associated with a stress response, as well as four regulatory genes, were induced. Further, the *chvG-chvI* two-component signal transduction system was specifically induced in response to acid conditions (Table 2).

The microarray analysis revealed that the expression of 74 genes was repressed around twofold or more under acidic conditions (Table 3). Numerous genes encoding ribosomal proteins were repressed. In addition, many genes encoding proteins involved in amino acid synthesis and uptake, carbohydrate uptake or metabolism, respiration and electron transfer, flagellum synthesis and chemotaxis, energy metabolism, and cofactor synthesis were also repressed. The repression of genes involved in amino acid synthesis, energy metabolism, and respiration likely is consistent with the slight growth inhibition observed under acidic conditions.

Experimental validation of microarray data by analysis of transcriptional gene fusions. To confirm the data obtained by microarray analyses, we examined the expression of six genes using transcriptional gene fusions (Fig. 4). Cells were grown under conditions similar to those used in the microarray experiment. Genes were chosen based on their expression levels in the microarray experiments or their known functions. *virG*, *chvI*, and *aopB* were chosen because they have been implicated in virulence (13, 14, 32). The *chvA* gene was included as a control, since microarray analysis showed that its expression was not altered under acidic and neutral conditions. Atu2160 is a conserved hypothetical gene that exhibited modest induction ($\log_2$ ratio, 1.35). *impA* is the first gene of the *imp* gene cluster that is induced under acidic conditions (Table 2). *gusA* transcriptional gene fusions of *virG*, *impA*, *aopB*, and Atu2160 were generated as described in Materials and Methods. The *chvI*::*gusA* and *chvA*::*gusA* gene fusions were constructed previously (74). In agreement with previous reports (13, 32), both microarray experiments and gene fusion analysis showed that *virG* and *aopB* were expressed preferentially in acidic conditions (Fig. 4). Gene fusion studies also confirmed that the *chvI*, Atu2160, and *impA* genes were induced by acidic conditions, while the expression of *chvA* was not altered (Fig. 4).

Acid conditions induce a general and conserved response in *Agrobacterium*. Previous studies demonstrated that different bacteria share some general and conserved adaptative responses to environmental acidic conditions, such as decreased macromolecule synthesis, reduced respiration and metabolism, and activated cellular repair and protection, although the underlying regulatory mechanisms may be different (21, 30, 31, 60, 63, 66). Microarray analyses revealed that acid conditions also trigger general and conserved responses through which *Agrobacterium* fundamentally modulates its gene expression patterns to adapt to environmental acidification. These general

TABLE 2. Acid-induced genes

Gene	Log_2 ratio	Designation	Annotation or description
Stress response genes			
Atu0044	1.04	*hslV*	Heat shock protein, ATP-dependent protease
Atu0365	1.43	*rmuC*	RmuC domain cellular repair protein
Atu0782	1.27	*csbD*	CsbD-like stress protein
Atu5052	1.82	*ibpA*	Small heat shock protein
Atu5449	1.26	*ibpA*	Heat shock protein
Cell envelope genes			
Atu0290	1.45		Rare lipoprotein A
Atu1131	1.76	*aopB*	Outer membrane protein
Atu2222	1.02		Glycosyl transferase family protein
Atu2321	1.04		(Penicillin binding) D-alanyl-D-alanine carboxypeptidase
Atu2611	1.27		Putative phosphatase protein
Atu3325	1.64	*exoQ*	Exopolysaccharide production protein ExoQ
Atu3327	2.76	*exoY*	Galactosyltransferase protein
Atu4014	1.71	*exsI*	Transcriptional regulator, ExsI protein
Atu4049	2.25	*exop*	Succinoglycan biosynthesis polymerization/transport ExoP
Atu4050	2.05	*exoN*	UTP-glucose-1-phosphate uridylyltransferase
Atu4052	1.81	*exoM*	Succinoglycan biosynthesis protein
Atu4054	1.38	*exoL*	Succinoglycan biosynthesis protein
Atu4055	1.39	*exoK*	Endo-1,3-1,4-beta-glycanase
Atu4056	1.73	*exoH*	Succinoglycan biosynthesis protein
Atu4057	1.06	*exoT*	Succinoglycan biosynthesis transport protein
Atu4058	1.66	*exoW*	Succinoglycan biosynthesis protein
Atu4060	1.24	*exoU*	Succinoglycan biosynthesis glycosyltransferase
Metabolism and cofactor synthesis genes			
Atu0035	0.95	*pckA*	Phosphoenolpyruvate carboxykinase
Atu0607	2.06	*aceA*	Isocitrate lyase
Atu0811	1.05	*mqo*	Malate:quinone oxidoreductase
Atu0881	1.59		Cytochrome c_{556} precursor (cytochrome c_{556})
Atu0944	1.43	*cscA*	Sucrose-6-phosphate hydrolase
Atu2224	1.81	*aldA*	NAD-dependent aldehyde dehydrogenase
Atu4020	1.89		Sulfatase
Atu4130	1.24	*acsA*	Acetoacetyl-coenzyme A synthetase
Atu4740	1.47		Zinc-containing alcohol dehydrogenase superfamily
Amino acid synthesis and degradation gene			
Atu0841	1.83		Amino peptidase family protein
Hypothetical protein genes			
Atu0377	2.16		Hypothetical protein
Atu1150	1.14		Hypothetical protein
Atu1219	1.22		Hypothetical protein
Atu1221	2.16		Hypothetical protein
Atu1682	1.19		Hypothetical protein
Atu1805	1.49		Hypothetical protein
Atu2065	2.77		Hypothetical protein
Atu2160	1.35		Hypothetical protein
Atu2198	1.22		Hypothetical protein
Atu2711	1.05		Hypothetical protein
Atu3082	1.08		Hypothetical protein
Atu3506	1.07		Hypothetical protein
Atu5515	1.15		Hypothetical protein
Atu6118	1.11		Hypothetical protein
Conserved hypothetical protein genes			
Atu2203	0.99		Conserved hypothetical protein
Atu2249	1.05		Conserved hypothetical protein
Atu2257	1.03		Conserved hypothetical protein
Atu2272	1.43		Conserved hypothetical protein
Atu4768	1.12		Conserved hypothetical protein
Atu4499	1.03		Conserved hypothetical protein
Atu5278	1.15		Conserved hypothetical protein
Atu5516	1.43		Conserved hypothetical protein

Continued on following page

TABLE 2—*Continued*

Gene	Log_2 ratio	Designation	Annotation or description
Transporter genes			
Atu2055	1.14		Multidrug efflux pump, HlyD family
Atu2470	1.23		TRAP-T family transporter, inner membrane subunit
Atu2471	1.57		Possible TctA subunit of tripartite tricarboxylate transport
Atu3273	1.03		Multidrug efflux RND membrane fusion protein MexE
Atu3274	2.47		Probable RND efflux transporter
Atu4017	1.22	*kgtp*	MFS permease (alpha-ketoglutarate)
Atu4322	1.04	*rbsC*	Ribose/xylose/arabinose/galactoside ABC-type transporter
Atu4334	1.04	*impJ*	Type VI secretion protein
Atu4335	1.03	*impI*	Type VI secretion protein
Atu4337	1.12	*impG*	Type VI secretion protein
Atu4338	1.07	*impF*	Type VI secretion protein
Atu4340	1.23	*impD*	Type VI secretion protein
Atu4341	1.17	*impC*	Type VI secretion protein
Atu4342	1	*impB*	Type VI secretion protein
Atu4343	1.12	*impA*	Type VI secretion protein
Regulatory protein genes			
Atu0033	0.88	*chvG*	Two-component sensor kinase
Atu0034	0.92	*chvI*	Two-component response regulator
Atu4006	1.32		Helix turn helix, Fis type
Atu4319	1.31		Transcriptional regulator, AraC-type DNA-binding protein
vir genes			
Atu6150	1.02	*virH1*	Cytochrome P-450 monooxygenase
Atu6151	1.2	*virH2*	Cytochrome P-450
Atu6178	1.59	*virG*	Two-component response regulator
Atu6188	1.03	*virE0*	Protein with unknown function
Atu6189	0.99	*virE1*	Chaperone protein

and conserved responses are reflected in the differentially expressed genes involved in (i) motility and chemotaxis; (ii) cellular protection or repair, including a putative nonheme catalase; (iii) regulatory and signal transduction; (iv) amino acid and protein synthesis; (v) cell metabolism and respiration; and (vi) the cell envelope, including exopolysaccharide synthesis. In addition, acidic conditions also initiated a highly specialized acid-mediated signaling response specifically involved in *Agrobacterium*-plant interactions.

Motility and chemotaxis. Chemotaxis is the movement of bacteria toward or away from certain chemical signals in their environment (36). In *E. coli* and *Salmonella enterica* serovar Typhimurium, acidic conditions (pH 5.5) repressed cell motility (18, 30, 45). Of the 74 genes repressed by acidic conditions, 8 are involved in motility (flagella) and chemotaxis (Table 3), including *flaA* (Atu0545) and *flaB* (Atu0543) encoding the structural subunits of the flagellum. These data agree with a previous observation that wild-type strain C58 rarely produced flagella when it was grown in induction broth (pH 5.5) (16). Not only were the two genes for flagellum synthesis repressed, but six genes associated with chemotaxis were also repressed. These genes are three alleles of *mcpA* (Atu0387, Atu2223, and Atu6132), *cheY2* (Atu0520), *mcpX* (Atu0373), and *mclA* (Atu1912). All six genes encode methyl-accepting chemotaxis proteins. Obviously, if flagella are not being synthesized, the cell has no need to synthesize proteins concerned with their function (chemotaxis). The reduced expression of flagellar genes is also consistent with the conserved energy consumption under acidic conditions, since flagellum biosynthesis is extremely energetically expensive for the cell (42).

Cellular protection and repair process. Microarray analysis showed that a number of genes that play a role in cellular protection and repair processes were upregulated under acidic conditions. One such protein was RumC (Atu0365), a regulator of homologous recombination and the SOS response (18). In addition, several genes involved in the degradation of proteins, peptides, and glycopeptides and cellular protection were also induced significantly. These genes include two genes encoding heat shock proteins (*ibpA* and *csbD*), three stress response genes (Atu0044, Atu5052, and Atu5449), and a gene encoding a putative nonheme catalase, KatN (Atu5278). It is known that in *Streptococcus mutans*, *E. coli*, and *Lactococcus lactis*, acidic conditions (pH 4.8 to 6.0) also increase cellular repair activities (26, 59, 66), suggesting that acidic conditions induce a conserved cellular repair response in unrelated bacteria. The elevated activities of cellular repair and protection under acidic conditions further suggest that the cells recognize pH 5.5 as a mild stress signal and modulate their gene expression pattern to adapt to these acid conditions.

Acid conditions induce Atu5278 encoding a putative nonheme catalase (KatN). Accumulating evidence suggests that the bacterial acid response is connected to the oxidative stress response (2). In *Staphylococcus aureus*, catalase was induced under acidic conditions (8). In *E. coli* and *Brucella melitensis*, acid stress cross protects against H_2O_2 challenge (45, 65). For plant-associated bacteria, the ability to defend against oxidative stress is of vital importance during bacterium-plant interactions (51, 71). Catalases detoxify H_2O_2, a major component of the oxidative stress imposed on a cell. In bacteria, many factors influence the levels of catalase; these factors include

TABLE 3. Acid-repressed genes

Gene	Log_2 ratio	Designation	Annotation or description
Cell envelope genes			
Atu0482	−1.05		Polysaccharide deacetylase
Atu0887	−1.15		Acetyltransferase
Atu3191	−2.03		Outer membrane protein
Stress response gene			
Atu3121	−1.02	*cspA*	Cold shock family protein
Metabolism and cofactor synthesis genes			
Atu0727	−1.12		Ferredoxin:oxidoreductase, flavin adenine dinucleotide/NAD(P) binding
Atu0771	−1.01	*coxC*	Cytochrome *c* oxidase, subunit III
Atu1529	−0.99	*fixH*	Nitrogen fixation protein
Atu1534	−1.29	*fixp*	Cytochrome *c* oxidase, cbb_3-type, subunit III
Atu1535	−1.78	*fixQ*	cbb_3-type cytochrome oxidase
Atu1536	−1.95	*fixO*	cbb_3-type cytochrome oxidase component
Atu1537	−2.23	*fixN*	cbb_3-type cytochrome oxidase component
Atu1601	−1.51	*hemN*	Oxygen-independent coproporphyrinogen III oxidase
Atu1997	−1.52		Sulfite reductase (NADPH) flavoprotein alpha component
Atu2073	−1.07		Electron transport protein SCO1/SenC
Atu2283	−1.62		Pseudoazurin; copper binding electron transfer protein
Atu2613	−1.04	*hemA*	5-Aminolevulinate synthase
Atu3977	−1.8		Large exoprotein involved in heme utilization or adhesion
Atu4091	−1.55	*cydA*	Cytochrome *bd* ubiquinol oxidase subunit I
Atu4092	−1.47	*cydB*	Cytochrome *d* ubiquinol oxidase subunit II
Atu4823	−1.06	*acaB*	Acetyl-coenzyme A acetyltransferase
Genes for biodegradation and modification of organic compounds			
Atu1814	−1.47		Epoxide hydrolase
Atu2394	−1.58		Aminoglycoside acetyltransferase
Atu3664	−1.23	*ephA*	Epoxide hydrolase
Amino acid synthesis and degradation genes			
Atu0053	−1.84		Small-subunit 16S rRNA
Atu0057	−1.52		Small-subunit 23S rRNA
Atu2109	−1.17		RNase P RNA (*rnpB*)
Atu2542	−1.53		Small-subunit 23S rRNA
Atu2547	−1.78		Small-subunit 16S rRNA
Atu3292	−1.26	*alr*	Alr, alanine racemase
Atu3937	−1.84		Small-subunit 16S rRNA
Atu3941	−1.55		Small-subunit 23S rRNA
Atu4180	−1.89		Small-subunit 16S rRNA
Atu4186	−0.99		Small-subunit 5S rRNA
Hypothetical genes			
Atu0515	−1.06		Hypothetical protein
Atu0544	−1.27		Hypothetical protein
Atu0896	−1.19		Hypothetical protein
Atu0904	−1.48		Hypothetical protein
Atu0955	−1.05		Hypothetical protein
Atu0983	−1.88		Hypothetical protein
Atu1031	−1.49		Hypothetical protein
Atu1467	−1.01		Hypothetical protein
Atu1637	−1.05		Hypothetical protein
Atu1667	−1.53		Hypothetical protein
Atu1975	−0.99		Hypothetical protein
Atu3190	−1.01		Hypothetical protein
Atu3321	−1.04		Hypothetical protein
Atu3837	−1.1		Hypothetical protein
Atu3838	−1.03		Hypothetical protein
Atu4185	−1.75		Hypothetical protein
Conserved hypothetical genes			
Atu1049	−2.94		Conserved hypothetical protein
Atu1468	−1.21		Conserved hypothetical protein
Atu4443	−1.19		Conserved hypothetical protein

Continued on following page

TABLE 3—*Continued*

Gene	Log_2 ratio	Designation	Annotation or description
Transporter genes			
Atu1577	−1.27		Amino acid ABC transporter, periplasmic binding
Atu1717	−1.23	*fadL*	Long-chain fatty acid transport protein
Atu2744	−1.77		TRAP dicarboxylate transporter
Atu3253	−1.04		TRAP dicarboxylate transporter
Atu3575	−1.33	*xylH*	Xylose transport permease protein
Atu3821	−1.23	*rbsB*	ABC-type sugar transport, periplasmic component
Atu4667	−1.65		ABC-type multidrug transport, ATPase component
Atu4687	−1.06		Amino acid ABC transporter, periplasmic binding
Atu5268	−1.45	*dctp*	TRAP dicarboxylate transporter
Genes for extracellular appendages and function			
Atu0373	−1.07	*mcpX*	Methyl-accepting chemotaxis protein
Atu0387	−1.03	*mcpA*	Methyl-accepting chemotaxis protein
Atu0520	−1.08	*chey2*	Chemotaxis protein CheYII
Atu0543	−1.24	*flaB*	Flagellum-associated protein
Atu0545	−2.35	*flaA*	Flagellum-associated protein
Atu1912	−1.19	*mclA*	Methyl-accepting chemotaxis protein
Atu2223	−1.65	*mcpA*	Methyl-accepting chemotaxis protein
Atu6132	−1.25	*mcpA*	Methyl-accepting chemotaxis protein
Nucleic acid synthesis and metabolism genes			
Atu0069	−1.24	*nrdI*	Nrdl protein involved in ribonucleotide reduction
Atu0070	−1.07	*nrdE*	Ribonucleoside-diphosphate reductase 2 alpha chain
Atu0071	−1.54	*nrdF*	Ribonucleoside-diphosphate reductase 2 beta chain
Atu3003	−1.68		Transposase
Atu3181	−2.17	*cowB*	Cobalamin synthesis protein/P47K family protein

H_2O_2 (2), the stationary phase of growth (2), phosphate starvation (75), and cell density (1). In *Agrobacterium*, catalase (KatA) has been implicated in virulence, and *katA* was induced by plant tissue and acidic conditions (pH 5.5) (71, 72). However, our microarray analysis did not detect induction of *katA* under acid conditions. These apparently inconsistent observations could have resulted from several factors, including differences in the experimental growth conditions. In our microarray experiments, cells were grown in liquid medium, whereas the cells were grown on agar plates in the previous study (72). Instead of detecting the acid inducibility of *katA*, microarray analysis revealed that acid conditions induced the transcription of Atu5278 located on the At plasmid (the second plasmid in *A. tumefaciens* C58). The Atu5278 protein is 70% identical to the *Sinorhizobium meliloti* SMc00371 protein, 40% identical to *Bradyrhizobium* sp. KatN, and 33% identical to the *Rhodopseudomonas palustris* Mn catalase.

To confirm the acid inducibility of Atu5278 (putative *katN*), we generated a *gusA* transcriptional gene fusion to the promoter of Atu5278 as described in Materials and Methods. Expression assays showed that there was >threefold induction of an Atu5278::*gusA* fusion under acidic conditions (723 Miller units of β-glucuronidase activity at pH 5.5 and 198 Miller units at pH 7.0). To gain some insight into whether Atu5278 plays any functional role in the acid response and pathogenicity, we

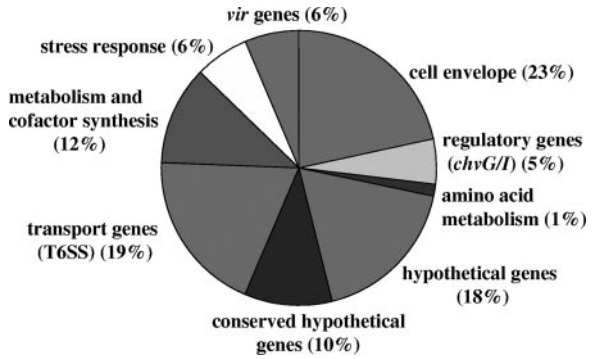

FIG. 3. Acid-induced genes classified into nine categories.

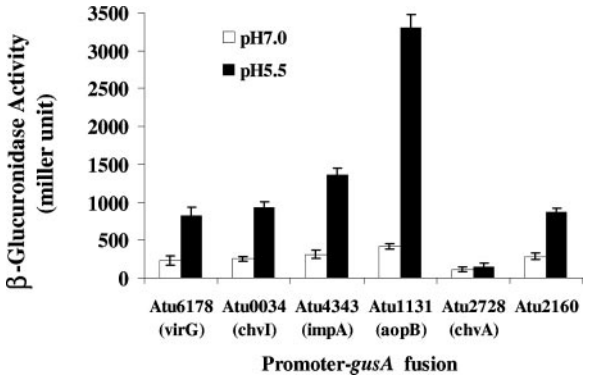

FIG. 4. Confirmation of microarray data by using transcriptional gene fusions. The expression of plasmid-borne *gusA* transcriptional gene fusions in *Agrobacterium* grown under acidic and neutral conditions was determined as described in Materials and Methods. The data are the averages ± standard deviations of three independent determinations.

constructed an Atu5278 mutant as described in Materials and Methods. The Atu5278 mutant grew as well as the wild-type parent strain under acidic conditions (data not shown), suggesting that the putative *katN* gene is not essential for growth in acidic liquid medium. In addition, the *katN* mutation did not affect virulence on *K. daigremontiana* leaves. However, wild-type *Agrobacterium* pregrown in acidic liquid medium exhibited increased resistance to subsequent H_2O_2 challenge, whereas the Atu5278 mutant pregrown in a similar acid medium was more sensitive to subsequent H_2O_2 challenge (Z. C. Yuan, P. Saenkham, and E. W. Nester, unpublished data). Therefore, it appears that the oxidative stress response is one of the conserved responses to acid conditions in *Agrobacterium*, *E. coli*, *B. melitensis*, and *S. aureus*. Further study is needed to characterize the putative nonheme catalase.

Genes involved in regulation and signal transduction. In bacteria, acidic conditions activate a number of regulatory and signal transduction genes which appear to play roles in an acid environment. In *Helicobacter pylori*, acidic conditions induce the regulatory factor *fur* (*f*erric *u*ptake *r*egulator), which regulates iron homeostasis (6). In *S. enterica* serovar Typhimurium and *E. coli*, an external acid pH (pH 4.5 to 5.8) induces expression of the RNA polymerase σ^s factor encoded by *rpoS* (55). In *Agrobacterium* and in other α-proteobacteria that have been sequenced, no RpoS homolog has been identified. No sigma factors were induced by acid conditions in *Agrobacterium*. In *S. enterica* and *E. coli*, the *phoP-phoQ* two-component system was induced by acid conditions and is involved in virulence and acid tolerance (5, 23, 41). In *Agrobacterium*, the annotated *phoP-phoQ* (Atu4712-Atu4711) operon has not been characterized, but microarray analysis suggested that the expression of the *phoPQ* operon is not affected by mild acidic conditions. However, two functionally undefined transcriptional regulatory genes located in the linear chromosome, Atu4006 (encoding a Fis-type transcriptional regulator) and Atu4319 (encoding an AraC-type DNA binding protein), were induced by acid conditons. In addition, microarray analysis confirmed that *virG*, the transcriptional regulator of the VirA-VirG system, was induced by acid conditions. Further, microarray analysis revealed that the *chvG-chvI* system was acid inducible, which is discussed below. The acid inducibility of these regulatory genes suggests that the acid response in *Agrobacterium* may be more complex than we originally thought, which also implies that *Agrobacterium* uses distinct mechanisms to adapt to acid conditions. This may also reflect a pattern of intricate signaling between *Agrobacterium* and its plant hosts.

Macromolecule synthesis and degradation. Microarray analysis also revealed that several genes involved in basic cellular processes, such as amino acid and nucleic acid synthesis, were repressed (Table 3). Eight ribosomal protein genes were significantly repressed under acidic conditions (Table 3). These genes were four genes concerned with 16S rRNA synthesis (Atu0053, Atu2547, Atu3937, and Atu4180), three genes concerned with 23S rRNA synthesis (Atu0057, Atu2542, and Atu3941), and one gene concerned with 5S rRNA synthesis (Atu4186). A gene encoding RNA polymerase (Atu2109) was also repressed. No ribosomal protein-encoding gene was upregulated under acidic conditions. Moreover, genes encoding amino acid transporters were also repressed (Atu1577, Atu1717, and Atu4687). Further, three genes participating in ribonucleotide reduction were also repressed. These genes were the genes in the putative operon comprising *nrdI* (Atu0069), *nrdE* (Atu0070), and *nrdF* (Atu0071). These data suggest that the ability to synthesize nucleic acid and protein was decreased under acid conditions, which may be consistent with the slightly lower growth rate of cells at pH 5.5. It is noteworthy that repression of genes encoding ribosomal proteins and amino acid synthesis in response to acidic pH was also observed in other gram-negative bacteria, including *Shewanella oneidensis* (pH 4) (37), *S. enterica* serovar Typhimurium (30), and *E. coli* (pH 4.8 to 6.0) (66). Thus, it appears that these bacteria share some conserved features that may reflect general mechanisms used to repress macromolecule synthesis when organisms are adapting to an environmental acidic pH.

Alterations of cell metabolism and respiration in response to acid conditions. Increasing evidence suggests that, as general and conserved responses, bacterial metabolism and respiration are altered under acidic conditions (59, 66). *E. coli gadA* and *gadB* (encoding glutamate decarboxylase) are induced by acidic conditions (pH 5.5) (29, 45). However, in *Agrobacterium*, there is no evidence of genes with sequence similarity to *gadAB*, suggesting that *Agrobacterium* may utilize alternative mechanisms to adapt to an acidic environment. In the rhizosphere, bacteria are subjected to a wide variety of low-molecular-weight natural products produced by plants and other soil organisms (19). A recent study reported that the exudates of several unwounded plants consist primarily of organic acids of the tricarboxylic acid (TCA) cycle, particularly citric, malic, and succinic acids, as well as the sugars glucose and fructose and the amino acid tryptophan (35). Further, the bacteria that colonize root tips most efficiently are the bacteria that utilize citrate as a major carbon source (35). Thus, it is perhaps not surprising that many acid-induced genes are involved in the transport and metabolism of citrate and other members of the TCA cycle, including genes encoding isocitrate lyase (Atu0607), malate:quinone oxidoreductase (Atu0811), sucrose-6-phosphate hydrolase (Atu0944), the TRAP-T family transporter (Atu2470), the TctA subunit of the tricarboxylate transport family (Atu2471), the dicarboxylate MFS transporter (Atu4017), and acetyl-coenzyme A synthase (Atu4130). Consistent with the recent observation that *pckA* was acid inducible (40), microarray analysis revealed that transcription of *pckA* was induced around twofold (Table 2). In addition to the organic acids used as a source of carbon and energy, the consumption of organic acids by the TCA cycle results in alkalinization and thus may help maintain internal pH homeostasis (29).

In contrast to the acid-induced genes involved in selective uptake of TCA cycle intermediates, *Agrobacterium* repressed nine genes involved in transporting multidrugs, amino acids, and fatty acids (Atu1577, Atu1717, Atu2744, Atu3253, Atu3575, Atu3821, Atu4667, Atu4768, and Atu5268) (Table 3). The reduced uptake of multidrugs might be consistent with the reduced cell ability to detoxify these multidrugs under acid conditions. The reduced transport of amino acids and fatty acids is consistent with the slightly lower growth rate of cells under acidic conditions. The slight growth inhibition under acidic conditions is also reflected in the repression of another set of genes involved in respiration, energy metabo-

lism, and electron transfer. These genes include *fixN*, *fixP*, *fixQ*, *fixH*, *hemA*, and *hemN*, as well as *coxC* (Table 3). It is noteworthy that respiration and electron transfer is one of the endogenous sources of H_2O_2 accumulation and oxidative stress (2, 59). Therefore, decreased respiration and electron transfer might help reduce acid-related oxidative stress.

Alterations in the cell envelope, including exoploysaccharide synthesis. The bacterial cell envelope is a partial shield against environmental stress and is also the first cell structure that interacts directly with an acidic environment (18, 31, 34, 66, 68). Further, it is the structure that likely interacts with the plant surface. Many bacteria change their envelope in response to an acid environment; these bacteria include *S. oneidensis* (pH 4) (37), *S. mutans* (pH 5.0) (26), and *E. coli* (pH 5.5) (30, 34, 66). Microarray analysis revealed that the expression of 17 cell envelope genes was induced in cells grown at pH 5.5; this is 24% of the total number of acid-induced genes (Fig. 3). This may reflect the fact that *Agrobacterium*, like other bacteria, evolved the ability to respond to mild acid conditions by synthesizing an altered cell envelope. These 17 genes are 5 genes directly involved in synthesis of the cell envelope (Atu0290, Atu1131, Atu2321, Atu2222, and Atu2611) and 12 *exo* genes that participate in the synthesis and metabolism of succinoglycan. Atu0290 encodes the rare compound lipoprotein A, whose function needs to be identified. Previous studies have identified Atu1131 (*aopB*) as an acid-inducible gene (32). Atu2321 encodes a penicillin binding protein involved in peptidoglycan synthesis and cell wall metabolism (3). Atu2222 is putatively involved in glycosyl transferase reactions which could modify the lipopolysaccharide of the cell wall. Atu2611 encodes a putative phospholipid phosphatase that may be involved in a modification of the phospholipid profile of the envelope.

Succinoglycan was initially identified in *S. meliloti* as a calcofluor-stained exopolysaccharide required for invasion of alfalfa roots (38). In *S. meliloti*, exopolysaccharide biosynthesis is influenced by a wide variety of factors, including a low phosphate concentration (76), the ppGpp-mediated stringent response (69), and the ExoS-ChvI two-component regulatory system, which is orthologous to the *Agrobacterium* ChvG-ChvI system (15). Nine of the acid-induced *exo* genes are located in a single cluster. These genes are Atu4049 (*exoP*), Atu4050 (*exoN*), Atu4052 (*exoM*), Atu4054 (*exoL*), Atu4055 (*exoK*), Atu4056 (*exoH*), Atu4057 (*exoT*), Atu4058 (*exoW*), and Atu4060 (*exoU*) (Fig. 3). In addition, three genes that are involved in exopolysaccharide synthesis but are far from this main cluster were also acid induced. These genes are *exoQ* (Atu 3325), *exoY* (Atu 3327), and *exoI* (Atu4014). To our knowledge, this is the first observation that *exo* genes are induced by environmental acid conditions.

***Agrobacterium* T6SS is induced under acid conditions.** An interesting finding of this work was the observation that the *imp* gene cluster is induced by acid (Table 2), which was confirmed by demonstrating that acidic conditions significantly induced the expression of an *impA*::*gusA* gene fusion (Fig. 4). The *imp* gene cluster was first identified in *Rhizobium leguminosaum* as a cluster encoding secreted proteins that impair nitrogen fixation in peas (7). The *imp* cluster in *R. leguminosarum* comprises 14 genes, including genes involved in protein phosphorylation (encoding either a kinase or a phosphatase) (7). Other genes in this cluster show similarity to genes involved in bacterial type III secretion. Four proteins secreted by wild-type cells were not secreted by an *imp* mutant. Interestingly, the secreted proteins blocked effective nodulation on pea plants, suggesting that they participated in the interaction with plants. Recent studies with *Vibrio cholerae* and *Pseudomas aeruginosa* further established that the *imp* orthologous genes encode a new bacterial secretion system now designated the T6SS (47, 56). Importantly, this T6SS is required for extracellular secretion of proteins lacking canonical hydrophobic amino-terminal signal sequences (47). In *P. aeruginosa*, an *imp* cluster gene designated *icmF1* encodes a protein secretion apparatus which secretes a hexameric protein that forms rings. This secretion apparatus may also function in chronic *Pseudomonas* infections in cystic fibrosis (47). In addition, in *S. enterica*, a *sciS* (*icmF* homolog) knockout mutant was hypervirulent in mice (50). Interestingly, T6SS also plays an important role in *Burkholderia* virulence (62).

In *Agrobacterium*, the entire *imp* cluster also contains 14 open reading frames, *impABCDEFGHIJK-icmF-impMN* (from Atu4330 to Atu4343), which are contiguous in the linear chromosome of the sequenced *A. tumefaciens* C58 genome (70). The acid-inducible *imp* genes identified by microarray analysis include *impJ* (Atu4334), *impI* (Atu4335), *impG* (Atu4337), *impF* (Atu4338), *impD* (Atu4340), *impC* (Atu4341), *impB* (Atu4342), and *impA* (Atu4343). In silico analysis revealed that in addition to the genera already mentioned, many gram-negative proteobacteria, including animal and plant pathogens, also have orthologous *imp* clusters. These organisms include members of the genera *Bradyrhizobium*, *Burkholderia*, *Rhodobacter*, *Shewanella*, and *Xanthomonas*. This *imp* cluster was also found in *Agrobacterium* strain S4, but it is not present in *S. meliloti* and *Agrobacterium* sp. strain K84. The significance of its presence in closely related genera or species is not clear. Nevertheless, the acid-induced expression pattern of the *imp* genes represents a novel model for transcriptional regulation of T6SS. Whether the T6SS plays any role in acid adaptation or *Agrobacterium*-plant interactions needs to be determined.

Functional analysis of knockout mutations of acid-inducible genes. Prior to infecting plants, *Agrobacterium* must survive, propagate, and interact with wounded plants in the rhizosphere. In addition, acid conditions (pH 5.5) is an essential signal for *Agrobacterium vir* regulon induction. We considered the possibility that certain acid-inducible genes likely contribute to growth at pH 5.5 or are involved in *Agrobacterium*-plant interactions in an acidic rhizosphere. To explore these possibilities, nine acid-induced genes were chosen for functional analyses. These genes were Atu0290 (rare lipoprotein A), Atu0841 (amino peptidase family protein), Atu0944 (*cscA*; sucrose-6-phosphate hydrolase), Atu2055 (multidrug efflux pump), Atu2224 (*aldA*; NAD-dependent aldehyde dehydrogenase), Atu2470 (TRAP-T family transporter), Atu3274 (RND efflux transporter), Atu4130 (*acsA*; acetoacetyl-coenzyme A synthetase), and Atu5278 (*katN*; putative nonheme catalase). Knockout mutants were constructed as described in Materials and Methods. A mutation in each of these nine genes did not affect the growth at pH 5.5 noticeably (data not shown), indicating that none of these genes is essential for growth under mild acidic conditions. Nor was the colony morphology of any of the mutants growing on solid acidic medium altered compared to the colony morphology of the wild-type strain. More-

over, when inoculated onto *K. daigremontiana* leaves, all of the mutants formed normal-size tumors compared to the wild-type strain tumors (data not shown). Thus, these acid-inducible genes appear not to be involved in *Agrobacterium*-plant interactions. However, we cannot rule out the possibility that these genes are involved in pathogenicity in the natural environment of the rhizosphere. It is also conceivable that individual mutations had no effect either on growth at pH 5.5 or virulence because other genes with redundant functions are present in the cell.

Acid-inducible genes directly involved in *Agrobacterium*-plant interactions. Microarray analysis revealed that not only does *Agrobacterium* share general and conserved responses to environmental acid conditions with other bacteria, as described above, but it also has a highly specialized response to perceive the environmental acid as an important signal dedicated to *Agrobacterium*-plant interactions. In addition to confirming the previously identified acid-inducible genes involved in *Agrobacterium*-plant interactions, such as *aopB*, *virG*, and *pckA* (13, 32, 40), the present microarray experiments uncovered many additional acid-inducible genes associated with *Agrobacterium*-plant interactions. These genes include *virE0*, *virE1*, *virH1*, *virH2*, and the *chvG-chvI* system (Table 2).

***Agrobacterium chvG-chvI* two-component system is induced under acid conditions.** The *Agrobacterium chvG-chvI* two-component system is of special interest because it is a global pH-sensing and regulatory system which controls acid-inducible genes involved in virulence (39). These genes include the chromosomal gene *katA*, which encodes a catalase detoxifying H_2O_2 (72), *aopB* (32), and *pckA* (40). Mutations in the *chvG-chvI* loci, apparently pleiotrophic, conferred a number of distinctive properties on the cell. *chvG-chvI* mutants cannot grow in a minimal acidic medium, a complex medium, or minimal media containing the antibiotics tetracycline, novobiocin, and carbenicillin, as well as several detergents (14). The importance of the *chvG-chvI* regulatory system was further underscored by its role in the interaction of other α-proteobacteria with their hosts. In *S. meliloti*, the synthesis of succinoglycan, which is required for the nodulation of alfalfa, is under the control of the ExoS-ChvI system, which is a homolog of the ChvG-ChvI system (15). Moreover, the *S. meliloti* ChvG-ChvI system is essential for viability, since neither gene could be deleted (49). In *Brucella*, BvrS and BvrR, the homologs of ChvG and ChvI, control the synthesis of several outer membrane proteins required for virulence (25). Although the ChvG-ChvI system plays important roles in the interactions of plant and animal pathogens with their hosts, its regulatory pattern has not been reported. Using microarray analysis, we demonstrated that the *chvG-chvI* system was activated by acid conditions (Table 2). The upregulation of *chvI* was also confirmed by transcriptional analysis of a *chvI*::*gusA* gene fusion (Fig. 4). This evidence suggests that the *Agrobacterium chvG-chvI* system, like most other two-component systems, is self-activated. Further identification of the regulated targets of the ChvG-ChvI system (*chvI* regulon) would provide more insight into to how *Agrobacterium* modulates its gene expression in an acidic rhizosphere and initiates the virulence program.

Acidic conditions alone induce five *vir genes*. Surprisingly, microarray analyses also identified *virE0*, *virE1*, *virH1*, and *virH2* as acid-inducible genes, and this was confirmed by transcriptional gene fusion studies (Table 4). Previous studies showed that VirE1 and VirE2 are transferred into plant cells independent of the T-DNA and are required for virulence (48). *virE0* has not been described previously, and whether it plays any role in pathogenicity is not clear. The small VirE1 protein is a chaperone which is required for exit of the VirE2 protein into the host cell (77). Why *virE0* and *virE1* are acid inducible is not clear, especially since neither *virE2* nor *virE3* is acid inducible. VirH1 and VirH2 share high homologies with cytochrome P-450 monooxygenases (9). Both genes are under the control of VirA-VirG, but mutations in either one or both have no significant effect on virulence, as determined by laboratory assays (9). In addition, the VirH2 protein can demethylate and mineralize many phenolic *vir* gene inducers and thereby reduce their toxic activity against bacterial growth and destroy their *vir* gene-inducing activity (9). The fact that acid alone induces expression of both *virH1* (Atu6150) and *virH2* (Atu6151) suggests that many phenolic compounds are exuded by the plant and that it behooves *Agrobacterium* to detoxify these compounds.

TABLE 4. Acid-induced gene expression is VirA-VirG independent[a]

Gene fusion	Expression in:			
	Wild-type strain		Δ*virG* mutant	
	pH 7.0	pH 5.5	pH 7.0	pH 5.5
impA::*gusA*	314 ± 69	1,130 ± 108	387 ± 59	1,236 ± 83
virG::*gusA*	206 ± 57	759 ± 71	232 ± 45	817 ± 62
aopB::*gusA*	511 ± 144	4,121 ± 239	473 ± 129	3,979 ± 213
virE0::*gusA*	152 ± 31	364 ± 46	201 ± 24	385 ± 37
virH1::*gusA*	137 ± 26	391 ± 39	156 ± 19	412 ± 33
chvI::*gusA*	255 ± 41	1,051 ± 75	271 ± 33	1,192 ± 81
virB::*gusA*	83 ± 14	116 ± 17	97 ± 21	126 ± 30

[a] The expression of plasmid-borne *gusA* transcriptional fusions in *Agrobacterium* wild-type and *virG* deletion mutant strains grown under acidic and neutral conditions was determined as described in Materials and Methods. The data are averages ± standard deviations for three independent determinations.

Previous studies have demonstrated that the ChvG-ChvI system is involved in the activation of the proximal *virG* promoter P2 under acidic conditions. In addition, mutations in *chvI* significantly reduced the expression of *virG* (44). However, whether ChvG-ChvI regulates VirG transcription directly or indirectly is not clear (39, 43, 44). The present microarray study confirmed that *virG* is induced under acidic conditions. Using a *virG*::*gusA* reporter gene construct, we observed a similar extent of *virG* induction at pH 5.5 (Fig. 4). This evidence, together with the observation that ChvG-ChvI was self-activated under acid conditions (Fig. 4) and the previous evidence that ChvG-ChvI is involved in the regulation of *virG* expression under acid conditions (39, 44), strongly suggests that the induction of *virG* under acid conditions is directly regulated by the ChvG-ChvI two-component system and supports the hypothesis that the ChvG-ChvI-governed acid signaling increases the level of VirG in *Agrobacterium*. The observation that a *chvI* mutation significantly reduced, but did not completely abolish, the expression of *virG* might have resulted from a leaky mutation of *chvI* or cross talk among signal transduction pathways (14, 39, 44).

Acid-induced gene expression is VirA-VirG independent. The VirA-VirG regulatory system controls the expression of the entire *vir* regulon, including *virE0*, *virH1*,and *virA-virG* itself (22, 33, 52). In addition, acid conditions alone induce many genes involved in *Agrobacterium*-plant interactions, including *aopB*, *chvG-chvI*, *virE0*, *virH1*, and especially *virG*. To explore whether acid-induced expression of these plant-associated genes was regulated by the VirA-VirG system, we compared the expression of *chvI*, *virG*, *aopB*, *impA*, *virE0*, and *virH1* under acid and neutral conditions in wild-type and *virG* deletion mutants. The data indicate that the induction of these genes under acid conditions alone did not involve the VirA-VirG system, since their expression levels were similar in the wild type and the *virG* mutant (Table 4). This evidence, together with the fact that in the absence of plant-derived phenolic compounds other members of the *vir* regulon, such as *virB*, were not induced under acid conditions (Table 4), suggests that acid signaling occurs prior to, and is independent of, the VirA-VirG-mediated phenolic signaling during *Agrobacterium*-plant interactions.

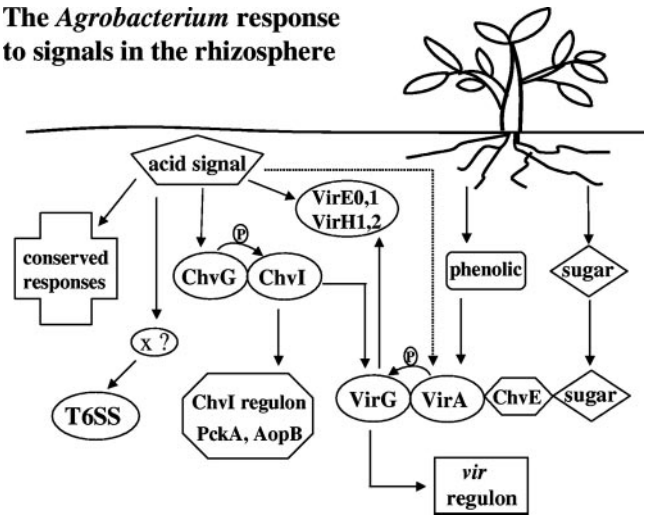

FIG. 5. Schematic diagram of the *Agrobacterium* response to signals (acid, phenolic compounds, and sugars) in the rhizosphere.

DISCUSSION

This report describes the global responses of *Agrobacterium* that are elicited when it is grown under acid conditions (pH 5.5) compared to the responses when it is grown under neutral conditions (pH 7.0). Our results revealed that acid conditions elicited two classes of responses, general and conserved adaptative responses and highly specific signaling responses involved in *Agrobacterium*-plant interactions. The conserved adaptative responses include the induction of genes related to cellular repair, uptake systems, and the cell envelope, including exopolysaccharide synthesis. The conserved responses also include the repression of genes involved in cell metabolism, respiration and electron transfer, chemotaxis, and macromolecule synthesis. To fit into its unique ecological niche, *Agrobacterium* evolved special strategies to perceive and respond to the acid conditions as an important signal in the rhizosphere. Based on the present data, as well as previous data, Fig. 5 outlines the major signaling pathways associated with *Agrobacterium*-plant interactions in the rhizosphere. Interestingly, under acidic conditions, the expression of the putative T6SS was significantly elevated, although additional studies are needed to understand the regulatory mechanism and the function of T6SS during *Agrobacterium*-host interactions. To the best of our knowledge, this is the first observation that a bacterial T6SS is regulated by an environmental signal at the transcriptional level.

Our initial interest in studying the transduction of environmental acid signals originated from previous observations that acid conditions were a prerequisite for activating the *vir* regulon and that acid alone induced *virG* expression to a limited extent (13). In addition, previous studies proposed that VirA couples acid and phenolic signaling during *vir* regulon induction through an unknown mechanism (12, 22) (Fig. 5). The present report demonstrates that the *chvG-chvI* two-component system is induced by acid conditions. Importantly, our results, together with previous observations (44), further suggest that the ChvG-ChvI system functions upstream of the VirA-VirG two-component system during *Agrobacterium*-plant signaling and favor a model involving a three-step sequential signaling process for activating the *vir* regulon (Fig. 5). We envision that when *Agrobacterium* transits from the bulk soil to the rhizosphere, a general and conserved response is triggered by the environmental acid signal, which induces the expression of the *chvG-chvI* system. In the second stage, the ChvG-ChvI system directly induces a basic level expression of *virG*, the transcriptional regulator of the *vir* regulon. However, at this stage, the VirG protein still requires phosphorylation by VirA. Once phenolic signals are available, the VirA protein senses the phenolic compounds, undergoes autophosphorylation, and phosphorylates VirG, which subsequently promotes the maximum expression of the entire *vir* regulon, including *virA-virG* itself. The first two steps of *vir* regulon activation, induction of the ChvG-ChvI system and subsequently the VirG regulator, are elicited by the acid signal alone, whereas the third step is promoted by the phenolic signal and involves phosporylation of the VirA-VirG system. This cascade of regulatory elements outlines a hierarchical signaling network in which the ChvG-ChvI-mediated environmental acid signaling integrates with the VirA-VirG-governed phenolic signaling pathways to coordinately activate the *Agrobacterium* virulence program in the rhizosphere. In addition, this hierarchical regulatory scheme ensures that there is maximum expression of the *vir* regulon when a susceptible plant is available for infection, yet it ensures that *Agrobacterium* saves energy, because the 30-member *vir* regulon is not activated, even in an acidic environment, unless plant-derived phenolic compounds are available. This unique three-step signaling process likely reflects an exquisite evolutionary result, in which *Agrobacterium* perceives and subverts the acidic environment as a critically important regulatory signal to initiate and direct the early responses during *Agrobacterium*-plant interactions. Aldose monosaccharides (e.g., arabinose), although not essential for *vir* regulon induction, can enhance sensitivity to phenolic inducers through the complex VirA-VirG-ChvE signal transduction system (4, 11, 53) (Fig. 5).

This study also extended our current knowledge by demon-

strating for the first time that *virE0*, *virE1*, *virH1*, and *virH2* are induced under acid condition (Table 2 and Fig. 5). Although these four *vir* genes were directly activated by the VirA-VirG system to a much greater extent with plant-derived phenolic signals, their induction by an acid signal alone was not mediated through the VirA-VirG system (Table 4). Since the expression of other *vir* genes, such as the *virB* operon, was not affected by acidic conditions (Table 4), it is reasonable to believe that, parallel to the induction of *virG* by acidic conditions, these four *vir* genes may have additional unidentified roles in recognizing and responding to environmental acidic signals.

Microarray analyses also revealed that a considerable number of hypothetical genes with unidentified functions were regulated by acid conditions (Tables 2 and 3). Some of these genes may play roles in the general acid response or participate in *Agrobacterium*-plant interactions. However, it is possible that some of these hypothetical genes are indirectly regulated by acid conditions and correlated with the slightly lower growth rate of the cells under acid conditions.

The evidence that the plant defense signal salicylic acid modulates *Agrobacterium* quorum sensing only under acid conditions (74), the fact that the *vir* regulon is activated only under acid conditions, and the observation that acid conditions alone induce the expression of several virulence factors, including *chvG-chvI*, highlight the fact that signal perception and exchange during *Agrobacterium*-plant interactions occur predominantly under the acidic conditions of the rhizosphere. Therefore, studies aimed at understanding the molecular mechanisms underlying *Agrobacterium*-plant interactions should mimic the acidic conditions of the rhizosphere, although identification of the mechanism and signaling pathway(s) by which external pH is sensed by bacteria is still a lingering challenge.

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