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ChvD, a Chromosomally Encoded ATP-Binding Cassette Transporter-Homologous Protein Involved in Regulation of Virulence Gene Expression in *Agrobacterium tumefaciens*

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A yeast two-hybrid screen searching for chromosomally encoded proteins that interact with the *Agrobacterium tumefaciens* VirB8 protein was carried out. This screen identified an interaction candidate homologous to the partial sequence of a gene that had previously been identified in a transposon screen as a potential regulator of *virG* expression, *chvD*. In this report, the cloning of the entire *chvD* gene is described and the gene is sequenced and characterized. Insertion of a promoterless *lacZ* gene into the *chvD* locus greatly attenuated virulence and *vir* gene expression. Compared to that of the wild-type strain, growth of the *chvD* mutant was reduced in rich, but not minimal, medium. Expression of *chvD*, as monitored by expression of β-galactosidase activity from the *chvD-lacZ* fusion, occurred in both rich and minimal media as well as under conditions that induce virulence gene expression. The ChvD protein is highly homologous to a family of ATP-binding cassette transporters involved in antibiotic export from bacteria and has two complete Walker box motifs. Molecular genetic analysis demonstrated that disruption of either Walker A box, singly, does not inactivate this protein's effect on virulence but that mutations in both Walker A boxes renders it incapable of complementing a *chvD* mutant strain. Constitutive expression of *virG* in the *chvD* mutant strain restored virulence, supporting the hypothesis that ChvD controls virulence through effects on *virG* expression.

Agrobacterium tumefaciens is a gram-negative bacterium capable of transferring DNA from the tumor-inducing (Ti) plasmid into the nucleus of a higher plant cell, where it can be integrated into the chromosomal genome and expressed (for a review, see reference 19). The processes that are involved in this transfer have been extensively studied and include (i) activation of virulence gene expression by plant-derived xenobiotics such as phenolic compounds and monosaccharides, (ii) production and processing of proteins and protein-DNA complexes that will be transferred into the plant cell, and (iii) construction of a complex in the bacterial membrane system that is used to transport these substrates.

Activation of virulence gene expression in *Agrobacterium* is under complex control, and the system has not yet been completely characterized. The central regulatory scheme is comprised of the VirA and VirG proteins, which are highly homologous to two-component systems found in a wide variety of prokaryotes (8, 41). VirA is a membrane-bound histidine kinase that is critical for signal input, and VirG is the response regulator that, after phosphorylation by VirA, can efficiently bind to the *vir* gene promoters and activate gene expression. Multiple signals are involved in this regulatory pathway. In most strains, phenolic compounds are absolutely required for the induction of *vir* gene expression, though the site of signal recognition—e.g., VirA or some other protein—and the mechanism of signal transduction have not been elucidated (9, 26, 33). Monosaccharides such as arabinose or glucose lower the concentrations of phenolic compounds necessary for activation of *vir* gene expression (3, 36). This occurs as a result of the activities of ChvE, a chromosomally encoded sugar-binding protein that interacts with VirA (10, 37). In addition to phenolic compounds and monosaccharides, several other environmental cues are known to affect virulence gene expression through the VirA-VirG system. Low pH stimulates *vir* gen expression by at least two mechanisms: one that is VirA dependent and one that activates the P2 promoter of *virG* independently of VirA (11, 27, 43). Low PO_4 has been shown to upregulate expression of *virG* through effects on the P1 promoter of *virG* (40). Finally, an elevated temperature downregulates *vir* gene induction, probably because of the temperature sensitivity of VirA (30). In none of these cases are the mechanisms of signal recognition or transduction understood.

The VirB proteins comprise a membrane-bound complex that is necessary for T-DNA transport to the plant cell and are homologous to the type IV transporter systems used by many bacteria to export either proteins or DNA-protein complexes (13, 42). Because VirB8 localizes to specific sites in the membrane system (24), we undertook a search for proteins that may serve as VirB8 anchors by using the periplasmic domain of VirB8 (VirB8′) as bait in a *Saccharomyces cerevisiae* two-hybrid screen of the *Agrobacterium* genome. This screen yielded two proteins that are strong interactors with VirB8. Genetic analysis demonstrated that one of these is involved in virulence but that the other is not. The gene affecting virulence, *chvD*, has been previously identified in a screen searching for mutations

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TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics	Reference or source
Strains		
E. coli		
S17-1/λpir	RK2 *tra* regulon, *pir* negative, host for *pir*-dependent plasmid	22
DH5α	$\lambda^-\phi$80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	GIBCO BRL
A. tumefaciens		
A348	C58 chromosomal background carrying pTiA6	18
AB20	A348 *chvD* gene was disrupted by insertion of the pZL101 suicide plasmid, Kan^r	This study
AB21	A348 "*21-5*" gene was disrupted by insertion of the pZL102 suicide plasmid, Kan^r	This study
AB22	A348 *chvD* gene was disrupted by insertion of the pZL103 suicide plasmid, Kan^r	This study
S. cerevisiae EGY 48	*MAT*α *trp1 his3 ura3 lexAops*-LEU2	20
Plasmids		
pBSII	ColE1 Amp^r, cloning vector	Stratagene
pJB20	Broad-host-range plasmid, derivative of pUCD2; IncW Amp^r Tet^r	5
pJB93	A 528-bp *virB8 Eco*RI fragment without a transmembrane domain in pJK202, HIS3 Amp^r	This study
pJG4-5 (pV0)	Cloning prey vector for the construction of activator fusion, TRP1 Amp^r	20
pJK202	Cloning bait vector for the construction of LexA fusions, HIS3 Amp^r	20
pMutA-G665D	IncRi derivative plasmid carrying *virA* (G665D) and wild-type *virG*, Amp^r	29
pSH18-34	Contains Gal1-$LexA_{op}$-*lacZ*, a reporter gene in yeast, URA3 Amp^r	20
pV1	Modified version of pJG4-5 with *Eco*RI site carrying +1 reading frame, TRP1 Amp^r	This study
pV2	Modified version of pJG4-5 with *Eco*RI site carrying +2 reading frame, TRP1 Amp^r	This study
pV01-1	835-bp *Tsp*509I fragment of A348 DNA containing a portion of *chvD* in pJG4-5, TRP1 Amp^r	This study
pV21-5	468-bp *Tsp*509I fragment of A348 DNA in pV2, TRP1 Amp^r	This study
pVK112	R6K suicide vector, *lacZY* for transcriptional fusions, Kan^r	22
pYW12	pJB20 derivative, with *lac* promoter followed by the multiple cloning site from pUC19; Amp^r	39
pYW15c	Broad-host-range expression vector, P_{N25}-MCS-STOP, IncW and pBR322ori, Amp^r	39
pYW48	pYW15 derivative plasmid with P_{N25}::*virG* and wild-type *virA*, IncW Amp^r	39
pZL35	2.7-kb *Eco*RV fragment containing the *chvD* gene from pZL78 cloned into pBSII, Amp^r	This study
pZL39	2.7-kb *Eco*RV *chvD*-containing fragment from pZL78 cloned into pJB20 *Sca*I site, Tet^r	This study
pZL78	7.8-kb *Hin*dIII fragment including *chvD* open reading frame cloned into pBSII, Amp^r	This study
pZL81	2.7-kb PCR fragment containing *chvD* open reading frame and promoter cloned into pYW12 *Kpn*I site; Amp^r	This study
pZL82	pZL81 derivative with mutation in Walker A1 ATP-binding site of *chvD*; Amp^r	This study
pZL83	pZL81 derivative with mutation in Walker A2 ATP-binding site of *chvD*; Amp^r	This study
pZL84	pZL81 derivative with mutations in both Walker A ATP-binding sites of *chvD*; Car^r	This study
pZL101	449-bp *chvD* (nucleotides 335–784) PCR fragment from pV01-1 cloned into *Eco*RI site of pVK112 in the same orientation as *lacZ*; Kan^r	This study
pZL102	398-bp *v21-5* (nucleotides 28–426) PCR fragment from pV21-5 cloned into *Eco*RI site of pVK112; Kan^r	This study
pZL103	449-bp *chvD* (nucleotides 335–784) PCR fragment from pV01-1 cloned into *Eco*RI site of pVK112 in the orientation opposite to that of *lacZ*; Kan^r	This study

that affect the capacity of the *virG* promoter to be activated at low pH and low PO_4 (43). This gene, originally discovered in a transposon mutagenesis screen of *A. tumefaciens* chromosomes, was not isolated. In this study, the intact *chvD* gene was cloned and sequenced and its expression, role in *vir* gene expression, and effect on growth in rich medium were characterized.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the plasmids, bacterial strains, and yeast strains used in this study. Plasmids were introduced into *Escherichia coli* and *A. tumefaciens* by electroporation.

DNA manipulations. Standard methods were used for plasmid isolation, PCR amplification, restriction digestion, and DNA gel electrophoresis and blotting. Genomic DNA was extracted from *A. tumefaciens* strain A348 by the method described in reference 12. For Southern hybridization, probe DNA was labeled with digoxigenin-11-UTP (Boehringer Mannheim) during PCR amplification and signal was detected using the chemiluminescent CPD-*Star* detection system according to the recommended procedure of the manufacturer (Boehringer Mannheim).

***vir* gene induction, growth, and virulence assays.** The bacterial growth media and growth conditions as well as procedures for *vir* gene induction have been described previously (9). To determine the difference in growth activities among wild-type A348, AB20, and its derivatives, the strains were grown in liquid AB minimal medium (43) with antibiotic at 25°C overnight. Equal amounts of cells (based on their optical densities at 600 nm [OD_{600}s]) were then transferred to L broth, and the OD_{600} of each culture was determined every 4 h. Virulence assays were performed according to the method of Banta et al. (4). Briefly, overnight cultures (adjusted to an OD_{600} of 0.5) of *Agrobacterium* were cocultivated with *Nicotiana tabacum* cv. Havana 425 leaf squares on hormone-free MS medium (32) supplemented with 300 μM acetosyringone (AS). After 2 days, the 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. After 10 days, the tumors on each leaf piece were counted and photographed.

Immunoblotting. Equal numbers of cells grown in AB induction medium (43) with or without AS (Sigma) were collected, resuspended in sample buffer, and boiled for 8 min. Ten microliters of sample was loaded, and proteins were separated by sodium dodecyl sulfate–10 to 12% polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane by electrotransfer, and probed with antibodies directed against VirA (39), VirE2 (7), and VirB8 (15) by chemiluminescent-detection procedures described previously (5).

Yeast two-hybrid vector construction, genomic libraries, and library screening. The pJG4-5 prey vector was mutagenized so that the *Eco*RI cloning site for fusion protein production existed in altered reading frames. This was accomplished by cloning double-stranded oligonucleotides, with an altered *Eco*RI cloning site that carried a different reading frame, into pJG4-5 digested with *Eco*RI and *Xho*I. For vector 1 (pV1), the mutagenic oligonucleotide consisted of oligonucleotides 1a (5′AATTGGGAATTCGGCCGAC3′) and 1b (5′TCGAGT CGGCCGAATTCCC3′); for vector 2 (pV2), it consisted of oligonucleotides 2a (5′AATTGAATTCGGCCGAC3′) and 2b (5′TCGAGTCGGCCGAATTC3′). Each modified vector was confirmed by DNA sequence analysis. Genomic libraries in these prey vectors were constructed with A348 genomic DNA partially digested with *Tsp*509I, a 4-bp cutter that yields the same 5′ overhangs as *Eco*RI. The digested sample was size selected by purifying the 500- to 3,000-bp fragments from a preparative agarose gel. This DNA was cloned into the *Eco*RI sites of the three pJG4-5-derived prey vectors and transformed into *E. coli* DH5α with 39,200 colonies recovered from the library in pJG4-5, 40,400 colonies from pV1, and 21,300 colonies from pV2. Plasmid pJB93, a bait plasmid, was constructed by amplifying a 528-bp PCR fragment encoding amino acid residues 56 to 231 of *virB8* on plasmid pJW239 (6) with primers JB7-8 (5′CGAATTCCCCGTTGAG CAGGCTTCTGCCC3′) and JB8-8 (5′CGAATTCATGGTGCGCCCTGGCCT AC3′). This fragment, lacking the N-terminal transmembrane domain of VirB8 (16), was cloned into the *Eco*RI site of plasmid pJK202, yielding pJB93, and is referred to as VirB8′. For the two-hybrid library screen, the prey plasmids were isolated with the three libraries and transformed into *S. cerevisiae* strain EGY48 that contains pSH18-34, a reporter plasmid carrying the *Gal1-lex*$_{op}$*-lacZ* gene cluster, and the VirB8′ bait plasmid pJB93. The pJG4-5 empty vector was also transformed into EGY48(pJB93, pSH18-34) as a control. The basic protocol and all strains and plasmids used in the assay are as described by Golemis et al. (20). Positive clones in the library screens were identified as only those colonies passing both the β-galactosidase production and the leucine prototrophy screens. After sequence analysis, two clones were chosen for further characterization. pV01-1 was chosen because it had homology to a previously identified virulence gene, *chvD* (43), and pV21-5 was chosen because it had an approximately 15-kDa peptide fused to the activator domain of the prey vector.

Insertion mutagenesis of *chvD* and *v21-5*. The suicide plasmid pVK112 carries a unique *Eco*RI site followed by a promoterless *lacZ* reporter gene as well as a kanamycin selection marker (22). It also contains the vegetative origin of R6K but lacks the plasmid's *pir* gene and can therefore replicate only in strains that contain *pir* on a separate replicon, such as *E. coli* S17-1/λ*pir*, but not in *Agrobacterium*. pZL101 contains a 449-bp internal *chvD* PCR fragment derived from pV01-1 cloned such that the *chvD* fragment is in the same orientation as the promoterless *lacZ* gene, while pZL103 carries the same fragment cloned into pVK112 in the opposite orientation. pZL102 contains a 398-bp internal *v21-5* PCR fragment cloned in the same orientation as the *lacZ* gene of the pVK112. Homologous recombination of these plasmids in the respective *Agrobacterium* chromosomal locus creates an insertion mutation at the gene along with a *lacZ* transcriptional fusion (22). pZL101 and pZL102 were electroporated into A348, and the cells were plated on kanamycin (100 μg/ml) plus X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The blue colonies are the candidate mutants in which the chromosomal *chvD* gene or *v21-5* gene was disrupted by insertion of pZL101 or pZL102, yielding strain AB20 or AB21, respectively. In the case of pZL103, white, kanamycin-resistant colonies were obtained and yielded strain AB22. The mutants were confirmed by PCR with one primer annealing to the *lacZ* gene (AlacZ, 5′CCGCCACATATCCTGATCTT3′) and another primer being external to the 5′ terminus of the *chvD* or *v21-5* fragments cloned into pVK112 (for *chvD*, 5′CCGCTACAACGAATTGATGA3′, and for *v21-5*, 5′TTCGTTCTCCCGGGTACTGC3′). The integration events were also checked by plasmid rescue in which the genomic DNA of each putative mutant was digested with restriction enzymes (*Bst*EII, *Not*I, and *Xmn*I) that do not cut within the plasmids. These fragments were circularized with T4 DNA ligase and electroporated into S17-1/λ*pir*. Primers external and internal to the *chvD* gene were used on these rescued plasmids to determine the precise insertion sites by sequence. Finally, the genomic DNAs of these two mutants were digested with *Bam*HI and *Xmn*I, Southern blotted, and probed with PCR fragments from *chvD, v21-5*, and *neo* (from pVIK112). The predicted extra hybridization band or larger band was seen from the mutant but not from wild-type A348, confirming the insertion.

Cloning of the full-length *chvD* gene. *Bam*HI, *Cla*I, *Eco*RV, *Hin*dIII, and *Sma*I digests of A348 genomic DNA were electrophoresed on an agarose gel and transferred by capillary blotting to a Hybond-N membrane (Amersham). A 450-bp *chvD* probe was obtained by incorporation of alkali-labile digoxigenin-11-dUTP (Boehringer Mannheim) during PCR amplification from pV01-1 with the primers ChvD1 (5′CCGACGAAACGGCGGATGA3′) and AchvD-1 (5′CCTTCTGGCGCGAGGCGTC3′). The hybridization was carried out under stringent conditions as recommended by the manufacturer, and detection was performed with a chemiluminescent CPD-*Star* detection system purchased from Boehringer Mannheim. A single 7.8-kb hybridization band from the *Hin*dIII digest was detected. A preparative *Hin*dIII digest was electrophoresed, and the DNA in the 7.0- to 8.5-kb range was isolated and cloned into pBluescript II (pBSII) and then transformed to DH5α. Approximately 2,000 transformants were screened by colony hybridization using the *chvD* probe, and a single colony containing the 7.8-kb *Hin*dIII fragment was identified. The plasmid carrying this fragment was designated pZL78.

Sequence analysis of *chvD*. A 2.7-kb *Eco*RV fragment encompassing the whole *chvD* gene was subcloned from pZL78 into pBSII, yielding pZL35, and into the broad-host-range vector pJB20, yielding pZL39. pZL78 and pZL35 were sequenced using T3 and T7 primers and primers from known *chvD* sequences (AChvD4, 5′CCTCATCCGCCGTTTCGTCG3′; ChvD3, 5′GTTATGACGAG CTGGTGGAAG3′). Additional new primers were designed on the basis of determined *chvD* sequence results and used to obtain additional sequences upstream and downstream of the *chvD* gene. The DNA sequences were compiled and analyzed through BLAST searches (2).

Construction of mutations in the NTP-binding sites of *chvD*. Mutations in the *chvD* Walker A ATP-binding sites were generated by overlap extension PCR mutagenesis (31). PCR was carried out with *Pfu* DNA polymerase (Stratagene) and primers designated as follows: chvD35, 5′CGGGGTACCCCGAAAACAT CCTGCCAGAGC3′; chvd35-stop, 5′CGGGGTACCCCGACAAGAGGATCA GCGCGT3′; walkA1, 5′ACGGCGCCGGTGAATCGACCGTTCT3′; AwalkA1, 5′AGAACGGTCGATTCACCGGCGCCGT3′; walkA2, 5′ACGGTGCAGGT GAGACCACACTGTT3′; and AwalkA2, 5′AACAGTGTGGTCTCACCTGCA CCGT3′. Primer chvD-35 and primer chvD35-stop contain 9 nucleotides for constructing a *Kpn*I site (underlined). A 2.5-kb PCR fragment was amplified from pZL35 and cloned into pYW12, yielding pZL81. Primers walkA1 and AwalkA1 contain a mutation in the first ATP-binding site, and primers walkA2 and AwalkA2 contain a mutation in their second ATP-binding sites, which were underlined in the primers. The first Walker A box ATP-binding site (Gly-Pro-Asn-Gly-Ala-Gly-Lys-Ser) between amino acid residues 39 and 46 was mutated by the primers walkA1 and AwalkA1. The Lys-45 codon was changed to Glu with four primers and two rounds of PCR. The first-round PCR had two reactions and used pZL35 as the template; one reaction was with primers chvD-35 and Awalk1, and the other was with primers chvD-stop and walkA1. These two PCR products were run on a 1% agarose gel, isolated, and then combined in a new PCR mixture with primers chvD-35 and chvD-stop. The amplified fragment that had the mutation in the Walker A ATP-binding site was digested by *Kpn*I and cloned into pYW12 to make pZL82. The Lys-45 change to Glu was confirmed by sequencing. In the same way, pZL81 was mutagenized at the second Walker A ATP-binding site between amino acid residues 350 and 357 (Gly-Pro-Asn-Gly-Ala-Gly-Lys-Thr) with primers walkA2 and AwalkA2, resulting in Lys-356 being changed to Glu in plasmid pZL83. pZL84, containing *chvD* with mutations at each of these two ATP-binding sites, was constructed with walkA2 and AwalkA2 and pZL82 as the template. The absence of random mutations was verified by sequence analysis.

Nucleotide sequence accession number. The GenBank accession number of the 2.7-kb fragment containing *chvD* and the surrounding sequence is AY027490.

RESULTS

Construction and screening of an *Agrobacterium* genomic library in the yeast two-hybrid system. VirB8, encoded by pTiA6, is a membrane-bound protein with a large C-terminal periplasmic domain. This region of the protein, referred to here as VirB8′, includes amino acids 164 to 692 of the VirB8 protein. Earlier studies using the yeast two-hybrid system (reference 17 and our unpublished observations) demonstrated interactions between VirB8′ and itself, VirB9, and VirB10. Given its apparently critical role in the construction of the VirB complex (24), we sought to determine whether VirB8′ also interacted with other *Agrobacterium* proteins. For these studies we constructed genomic libraries using total DNA from *A. tumefaciens* strain A348, which carries the two *Agrobacterium* chromosomes, the Ti plasmid, and the so-called cryptic plasmid pAtC58 (18). A partial *Tsp*509I digest was size se-

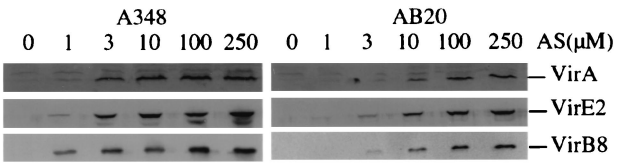


FIG. 1. *vir* gene expression in *A. tumefaciens* strains A348 (wild type) and AB20 (*chvD* mutant). Bacterial cells were induced with the indicated concentrations of AS, and immunoblots were probed with antibodies directed against the indicated Vir proteins.

lected (0.5 to 3 kb) and cloned into three different prey vectors (see Materials and Methods) that contain an *Eco*RI cloning site in each open reading frame. Screening of these libraries with VirB8′ in the bait yielded 10 to 15 colonies per vector that were positive for β-galactosidase activity and were leucine prototrophic, as is expected for interactors. Western analysis demonstrated that five of these interactors had significant fusions (>5 kDa) to the activator domain of the prey vector and were characterized further. The plasmids carrying positive interactors were isolated, transformed into *E. coli*, and used for sequence analysis.

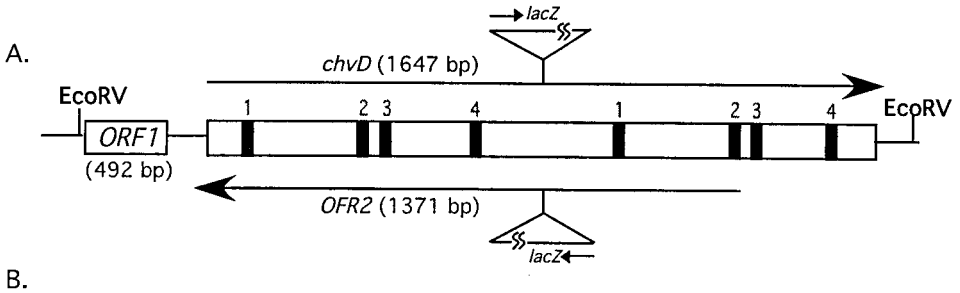
Characterization of positive interactors. Sequence analysis of the positive interactors showed that three had identical 835-bp inserts in pV0 (= pJG4-5) and that two had identical 478-bp inserts in pV2. BLAST searches showed that the inserts pV01-1, pV01-4, and pV01-5 had 100% homology with the partial sequence available for chromosomal virulence gene D (*chvD*) in *A. tumefaciens* but that the inserts in pV21-2 and pV21-5 had no homologies in the database. As described in Materials and Methods, internal fragments of approximately 400 bp from either pV01-1 or pV21-5 were cloned into pVIK112, a plasmid that carries a promoterless *lacZ* gene and cannot replicate in *Agrobacterium* (22), yielding pZL101 or pZL102, respectively. The resultant plasmids must recombine into either the chromosome or resident plasmids in order to confer antibiotic resistance on the cell. Recombination of this plasmid into the appropriate target site was confirmed (see Materials and Methods), and the strains were tested for virulence and their capacity to express β-galactosidase. With AB21 (insert from pV21-5), the *lacZ* gene was expressed in both rich (L broth) and minimal AB medium, indicating insertion into a functional operon, but there was no effect on virulence.

The insertion of pZL101 into the *chvD* locus resulted in a strain (AB20) that expressed β-galactosidase under all conditions tested, and phenolic compounds that induce the *vir* genes did not affect this expression either positively or negatively (data not shown). A second insertion at the *chvD* locus, with pZL103, inserted *lacZ* in the opposite orientation. The strain carrying this insertion, AB22, did not exhibit β-galactosidase activity under any of the conditions tested. Both AB20 and AB22 were, as expected, attenuated in virulence when they were tested on either Kalanchoe or tobacco (see below). A severe reduction in the capacity of AB20 to induce the expression of virulence genes in response to AS was observed (Fig. 1). Finally, both mutant strains exhibited a pronounced reduction in growth rate compared to that of the wild type in L broth but not minimal AB medium (data not shown; see below).

Cloning and sequencing of *chvD*. Because the mutations in AB22 and AB20, as well as the original *chvD* mutation in strain A348 *chvD1* (43), were generated via insertional mutagenesis, the possibility existed that one or more downstream open reading frames of a potential operon are responsible for the observed phenotypes. To address this issue, the entire *chvD* gene was cloned and sequenced as described in Materials and Methods. Briefly, Southern blots of chromosomal DNA, digested with a variety of restriction enzymes and probed with the 449-bp fragment of *chvD*, revealed a 7.8-kb *Hin*dIII fragment that carried at least part of the *chvD* gene. This fragment was cloned into pBSII, and a 2.7-kb *Eco*RV fragment of this plasmid was shown to carry the *chvD*-homologous sequences. The 2.7-kb *Eco*RV fragment was subcloned into pBSII and sequenced. Analysis of these data, as well as some additional upstream sequences from the 7.8-kb *Hin*dIII fragment, demonstrated that the *chvD* open reading frame is 1,647 bp (549 amino acids). BLAST searches demonstrated that the ChvD protein is homologous to a variety of ATP-binding cassette (ABC) transporters, particularly members of a group of bacterial ABC transporters involved in antibiotic export (Fig. 2). For example, it shares 31% identity and 48% similarity with the carbomycin resistance (*carA*) gene of *Streptomyces thermotolerans* (35) and 28% identity and 45% similarity with the pristinamysin resistance (*vgaB*) gene of *Staphylococcus aureus* (1). Finally, the ChvD protein contains two complete sets of ABC consensus sequences but no obvious hydrophobic domains, which would be expected if it were inserted into the membrane.

Two additional potential open reading frames have been identified on the 2.7-kb *Eco*RV fragment carrying *chvD*. First, 5′ to the *chvD* translation start site is a 492-bp open reading frame (ORF1) with 245 bp separating these two coding regions (Fig. 2). No significant hydrophobic domains are predicted for this protein, and BLAST searches did not reveal significant homologies to any known proteins. Second, the antisense strand of *chvD* encodes a 497-amino acid open reading frame (ORF2), if we assume that GTG (at position 2082 of the 2.7-kb *Eco*RV fragment) is the start codon (Fig. 2). The predicted protein has two potential membrane-spanning domains, based on prediction programs of Czero et al. (14) and Hoffman and Stoffel (21), but BLAST searches did not reveal significant homology to known proteins.

Complementation of the *chvD* mutant strain AB20. Two strategies were used to test the role of *chvD* in virulence and *vir* gene expression. The first was to clone the 2.7-kb *Eco*RV fragment described above into the broad-host-range IncW vector pJB20, yielding pZL39, and to determine whether it could complement the *chvD* mutation in strain AB20. Strain AB20 or AB20(pJB20) induced significantly fewer tumors than the wild-type strain, A348 (Fig. 3A to C). In contrast, AB20(pZL39), containing the wild-type *chvD* gene, exhibited completely restored virulence (Fig. 3D). These results demonstrate that no open reading frame downstream of *chvD* is involved in the attenuated virulence phenotype. A second complementation strategy was used to determine whether the *chvD*-mediated effect on virulence and *vir* gene expression lies upstream or downstream of the VirA-VirG signaling cascade. In this case, pYW48, containing *virG* constitutively expressed from the P_{N25} promoter of the T5 coliphage (39) or pMutA-G665D, contain-



B.

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                                   Walker A1
CarA: 11   ITKRYQDHVVLDRIGFTIKPGEKVGVIGDNGSGKSTLIKLIAGREQPDNXXXXXXXXXXX 70
           + K Y    VL+ +   +  P  K+G++G NG+GKST++K++AG ++
ChvD: 12   LNKSYGAKKVLENVHLSFYPDAKIGILGPNGAGKSTVLKIMAGLDKEYTGEAWLAEGATL 71

CarA: 71   XYLAQTLELPLEATVQXXXXXXXXXXXXXXXGMRRTEAELAERPYQTGQDPELAGLLESY 130
            YL Q  +L    TV                ++     G
ChvD: 72   GYLEQEPKLDENKTVMENVMEGVASKTAIVDRYNELMMNYSDETADEG------------ 119

CarA: 131  AALVDRYQARGGYEADSRVEIAXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXSEPEL 190
           A L D   ++  ++ +++VE+A                                 S+P+L
ChvD: 120  AKLQDMIDSQNLWDLENQVEMAMDALRCPPGDSAVTGLSGGERRRVALCKLLL--SQPDL 177
                Walker B1
CarA: 191  LLLDEPTNDLDDRAVDWLEEHLRKHKGTVVAVTHDRLFLDRLTTTILEIDSGKVMRYGNG 250
           LLLDEPTN LD   + WLE+HLR + G V+ +THDR FLD +T  ILE+D G+ + Y
ChvD: 178  LLLDEPTNHLDAETIAWLEKHLRDYPGAVMMITHDRYFLDNVTGWILELDRGRGIPYEGN 237

CarA: 251  YEGYLAAKAAERQRRLLEYEQWRAELDRSRDLIASNVARLDAIPRKLPFAVFGAGQFRMR 310
           Y  YL AKA   Q+   E    +  + R ++ IAS+                      +
ChvD: 238  YSAYLQAKAKRMQQEAREDASRQKAISREQEWIASS----------------------PK 275

CarA: 311  GRGHGAMVRIRNAKERVARLTENPVAPPPEPLTFTAEITTEAAQSRETVAELTGVRVGDR 370
            R   +  RIR   E V    EN      + +   AE         E + +      GDR
ChvD: 276  ARQTKSKARIRRYDELV-EAAENRRPGDAQIVIPVAERLGRVVIEAENLTK----SYGDR 330
                          Walker A2
CarA: 371  LSVDSL--HLGPGERLLVTGPNGAGKTTLLRVLSGELEPDSGSLLVSGRV--GHLRQEQT 426
           + +++L   L PG  + V GPNGAGKTTL R+++G+ +PDSGS+ V   V  G++ Q +
ChvD: 331  VLIENLTFKLPPGGIVGVIGPNGAGKTTLFRMITGQEQPDSGSVTVGETVHLGYVDQSRD 390

CarA: 427  PWRPGMTVLQAFSSG----RAGDIDEHTEALLSLGLFSPDDLRQRVQDLSYGQRRRIELA 482
              TV +  S G    + G  + ++ A      F   D +Q+V +LS GQR R+ LA
ChvD: 391  TLAGDKTVWEEISGGNDIIKLGKFEVNSRAYCGAFNFKGGDQQQKVGNLSGGQRNRVHLA 450
                     Walker B2
CarA: 483  RLVTEPVDLLLLDEPTNHLSPALVEELEEALTGYQGTVVVVTHDRRMRSRFNGAHLTLQD 542
           +++    ++LLLDEPTN L    +  LEEAL  + G  V+++HDR    R    H+  +
ChvD: 451  KMLKAGGNVLLLDEPTNDLDTETLAALEEALEAFAGCAVIISHDRMFLDRL-ATHILAFE 509

CarA: 543  G 543
           G
ChvD: 510  G 510
```

FIG. 2. (A) Map of ChvD indicating sites of Walker A boxes (box 1), the LSGG signatures (box 2), Walker B boxes (box 3), and the conserved histidine found in most ABC transporters (box 4). The orientation of the *lacZ* gene from the inserted plasmids pZL101 (upper) and pZL103 (lower) are also shown. (B) BLAST comparison of the amino acid sequences of ChvD and CarA, where the middle line indicates identical (letters) and conserved (+) amino acids.

ing a constitutively active form of VirA (29), was electroporated into AB20, and the resultant strain was tested in a tobacco leaf explant tumor assay. Full virulence was restored in both cases (Fig. 3E and F), demonstrating that the effects of *chvD* activity are not necessary if VirG is constitutively expressed or activated, consistent with a model in which *chvD* is involved in controlling *virG* expression (43). In terms of growth in L broth, AB20(pZL81), carrying the wild-type *chvD* gene, exhibited restored growth compared to that of AB20 with the vector pYW12 while the AB20(pYW48) strain, which contains constitutively expressed *virG* and complements the virulence phenotype, continued to exhibit the reduced growth phenotype (Fig. 4). Similarly, pMutA-G665D did not restore the capacity of AB20 to grow optimally in L broth (data not shown).

Mutagenesis of *chvD* ATP-binding sites. An important question is whether the ATPase activity of ChvD is necessary for the effects on virulence. Therefore, the Walker A1 and Walker A2 sites of *chvD* (Fig. 2) were mutagenized either singly or together and tested for effects on growth in rich medium, virulence, and *vir* gene expression. The capacity for the AB20 strain to grow in L broth was partially disrupted when ChvD contained the Walker A box mutations (Fig. 4). However, mutation in either Walker A box site individually (pZL82 and pZL83) resulted in *chvD* genes that could still fully complement the AB20 mutant strain for virulence and *vir* gene expression. The strains induced as many tumors (Fig. 5) and induced virulence gene expression (Fig. 6) as well as the wild-type strain A348. When the *chvD* gene carried both mutations

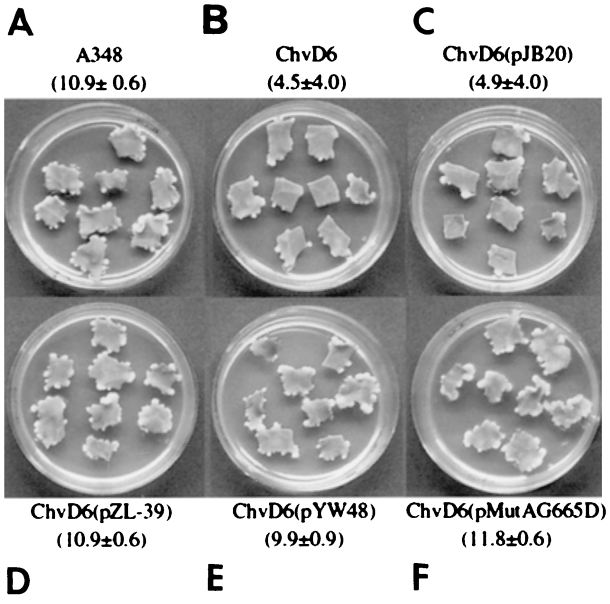

FIG. 3. Tobacco leaf virulence assay. Complementation of *chvD* mutant strain AB20. (A) A348 (wild type); (B) AB20; (C) AB20(pJB20); (D) AB20(pZL39); (E) AB20(pYW48); (F) AB20(pMutA-G665D). The mean numbers of tumors/explant ± standard errors (n = 14) for the experiments are shown.

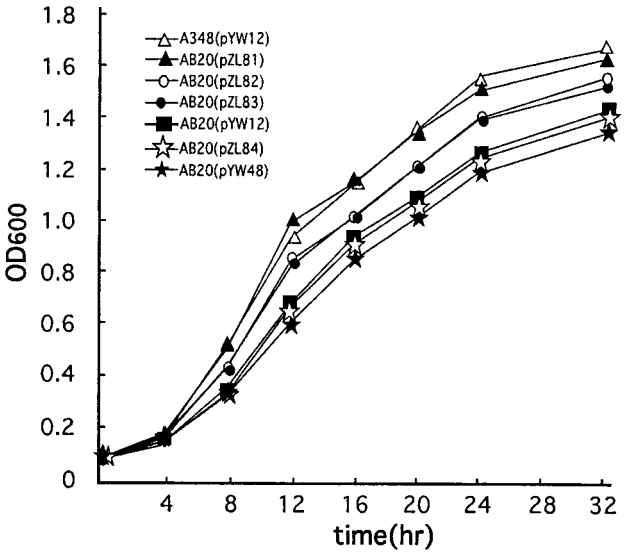

FIG. 4. Growth in L broth of wild-type A348(pYW12) and the AB20 mutant complemented with the following plasmids: pYW12 (vector), pYW48 (constitutively expressed *virG*), pZL81 (wild-type *chvD*), pZL82 (*chvD* Walker A1 mutation), pZL83 (*chvD* Walker A2 mutation), and pZL84 (*chvD* Walker A1 and A2 mutations). Mean OD_{600}s (n = 3) ± standard errors (error bars not visible) are shown.

(pZL84), the resultant gene could not complement the AB20 strain in any of these three assays (Fig. 4 to 6).

DISCUSSION

Phenolic compounds, sugars, and low pH and low PO_4 concentrations are all involved in maximizing expression of the virulence genes on the Ti plasmid of *A. tumefaciens*. This complex set of environmental signals is integrated by the VirA-VirG two-component system, with input arriving from both known and as yet unidentified proteins (41, 8, 9). The experiments presented here identify *chvD* as encoding an ABC transporter homologue and demonstrate that it is a critical member of the virulence regulatory pathway, most likely affecting the capacity of *virG* to be expressed.

ChvD is most closely related to a class of bacterial ABC transporters that are involved in antibiotic resistance, including, for example, *carA* of *Streptomyces thermotolerans* (35) and *vgaB* of *Staphylococcus aureus* (1). These have been classified in the ABC-A2 subset of ABC transporters (38). The ATP-binding proteins of these transporters are characterized by having two complete sets of Walker A and B boxes and ABC signature motifs. They have no membrane-spanning domains but, rather, interact with membrane-spanning proteins that are the apparent pore involved in the export process. As in the case of ChvD, the membrane-spanning proteins of this subfamily of ABC transporters are often not part of the same operon as the ATP-binding protein (38). Preliminary studies have not yet identified any antibiotics that are more lethal to mutant strain AB20 than to the wild-type strain. However, strains carrying a disruption in the *chvD* gene grow poorly in comparison to the wild type in rich medium but equivalent to the wild type in minimal medium. This phenotype is consistent with the hypothesis that this ABC transporter system has an activity that can export potentially inhibitory compounds, within the L broth, that gain entry into the cell. Supporting evidence for this concept is that mutation at the conserved lysine in either Walker A box of ChvD results in genes that can partially restore the mutant growth phenotype. Usually, however, such mutations in the ATP-binding site of ABC transporter systems

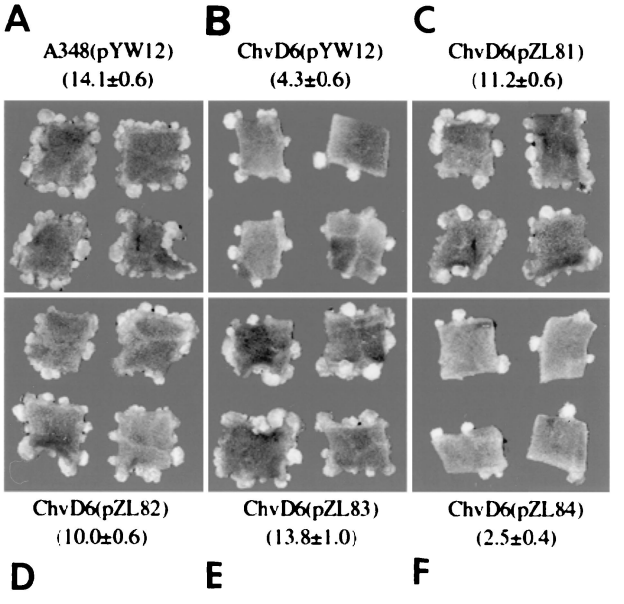

FIG. 5. Tobacco leaf virulence assay of strain AB20 complemented with *chvD* containing mutations at the Walker A ATP binding sites. (A) Wild-type A348(pYW12); (B) AB20(pYW12); (C) AB20(pZL81); (D) AB20(pZL82); (E) AB20(pZL83); (F) AB20(pZL84). The mean numbers of tumors/explant ± standard errors (n = 14) for all experiments are shown.

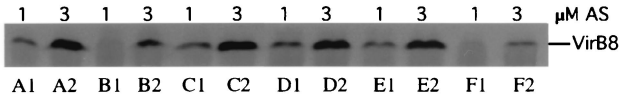

FIG. 6. *vir* gene expression in Walker A box ATP-binding site mutants. Shown are the results of an immunoblot analysis of VirB8 expression in strains induced with either 1 or 3 μM AS. A lanes, wild-type A348(pYW12); B lanes, AB20(pYW12); C lanes, AB20(pZL81); D lanes, AB20(pZL82); E lanes, AB20(pZL83); F lanes, AB20(pZL84).

completely disrupt their transport activities reflecting the fact that the biochemical mechanism of almost all ABC transporter ATP-binding proteins requires two functional ATP-binding sites for transport activity (34). Biochemical characterization will be required to determine whether the single point mutations in ChvD completely disrupt its ATPase activity or, alternatively, whether the activity of the mutant ChvD reflects dimerization to provide sufficient ATP-binding sites for partial activity.

The relationship between the transport activity of ChvD and virulence is uncertain. Disruption of two other *Agrobacterium* chromosomal virulence proteins, ChvI and ChvG, also results in poor growth in rich medium (12, 28) and lowered virulence. The relationship between this apparent two-component control system and ChvD is not known. Of significant interest is that, despite their effects on growth, mutations at the conserved lysine of either Walker A box in the ChvD protein did not disrupt this protein's effects on virulence or *vir* gene expression. Assuming that the growth phenotype reflects ChvD-mediated transport activity, these results suggest that the role of ChvD in regulation of *vir* gene expression and virulence is not dependent on a fully functional transport activity. Such dual functions have been observed, for example, in ChvE, which is the periplasmic sugar-binding protein of an ABC transporter sugar uptake system in *A. tumefaciens* but also affects, independently of sugar uptake, *vir* gene expression (23). Similarly, the *ecsA* (ATP-binding protein) and *ecsB* (membrane-spanning protein) genes of *Bacillus subtilis* have been proposed to work coordinately to regulate transcription independently of transport activities (25). However, when both Walker boxes of ChvD carried mutations at the conserved lysine, *vir* gene expression, virulence, and growth in L broth were completely disrupted. Perhaps some level of transport activity is necessary for ChvD activity in terms of virulence. However, because antibody directed against ChvD is not yet available, the possibility that the two Walker A box mutations within the protein render it unstable has not been addressed.

Complementation analysis demonstrated that a 2.7-kb fragment encoding the ChvD protein is capable of restoring optimal *vir* gene expression, virulence, and growth in L broth of the *chvD* mutant to wild-type levels. This result demonstrates that the *chvD* gene is not followed by a downstream open reading frame involved in controlling these phenotypes. It is unlikely that ORF2, encoded by the opposite stand of the *chvD* coding region, is responsible for any of the phenotypes investigated here. First, the insertion of a transcriptional *lacZ* fusion in this orientation (strain AB22) did not result in β-galactosidase activity, indicating that, under the conditions tested, this strand is not transcribed. Second, while mutation at the Walker A1 site of ChvD (K→E) resulted in a mutation in ORF2 (P→L), the mutation at the Walker A2 site of ChvD (K→E) was silent in Orf2 (L→L). The strain carrying this Walker A2 mutation [AB20(pZL83)] had a mutant phenotype with regard to growth in L broth (Fig. 4), thus demonstrating that Orf2 must not be involved in this phenotype. Third, the AB20 strain carrying a version of ChvD with both Walker box mutations (pZL84) was not complemented in either growth or virulence assays, in contrast to the strains with individual mutations (pZL82 and pZL83), which exhibited partially restored growth in L broth and wild-type virulence. Since ORF2 in pZL82 is identical to ORF 2 in pZL84, these results demonstrate that the observed phenotypes are the result of ChvD activities or disruptions thereof.

The original *chvD* mutant strain, A348 *chvD1*, was isolated based on the reduced ability of a *virG*::*lacZ* fusion to be expressed in the presence of low PO_4 concentrations (43). To further clarify the position of ChvD activity in the pathways regulating *vir* gene expression and the potential role of its transport function in virulence, two different plasmids carrying a mutant form of *virG* or *virA* were tested for their capacity to complement the *chvD* mutant strain AB20. The first of these, pYW48, carries wild-type *virG* expressed from the constitutive P_{N25} promoter of the T5 coliphage (39) along with wild-type *virA*, while the second, pMutA-G665D, carries wild-type *virG* expressed from its own promoter and a constitutively active form of VirA (29). In both cases, virulence was restored whereas the strains continued to exhibit the mutant phenotype in terms of the capacity for growth in L broth. Thus, ChvD-mediated transport activity does not affect regulatory processes downstream of *virG* expression. Rather, these results are consistent with the previously published data demonstrating that a transposon insertion into *chvD* downregulates the activity of the *virG* promoter (43).

The modified pJG4-5 prey vectors described here allow insertion of sequences into the *Eco*RI site of the activation protein fusion in all three open reading frames. This finding is particularly useful in the construction of genomic libraries from any prokaryotic organism. Using libraries of *Agrobacterium* genomic DNA in such vectors resulted in the isolation of fusion proteins that interacted with the periplasmic domain of VirB8, a result that led to the ultimate isolation and characterization of the *chvD* gene. At this point, however, the biological relevance of this interaction is not clear. First, the domain of VirB8 used in the screen of the yeast two-hybrid *Agrobacterium* genomic library is periplasmic (16) whereas the predicted location of ChvD is cytoplasmic. Second, strains carrying deletions of VirB8 exhibit wild-type expression from a plasmid carrying a *virB* promoter driving *lacZ* (A. Yin and A. N. Binns, unpublished data), indicating that VirB8-ChvD interaction is not required for ChvD's stimulating activity. This does not eliminate the possibility, however, that degradation or breakdown products of VirB8 in the cytoplasm may yield peptides that interact with ChvD and repress its activity, thereby reducing *vir* gene expression.

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