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A family of lysozyme-like virulence factors in bacterial pathogens of plants and animals

(*Agrobacterium/virB* genes/conjugative DNA transfer/invasion/glycosidase)

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ABSTRACT **We describe a conserved family of bacterial gene products that includes the VirB1 virulence factor encoded by tumor-inducing plasmids of *Agrobacterium* spp., proteins involved in conjugative DNA transfer of broad-host-range bacterial plasmids, and gene products that may be involved in invasion by *Shigella* spp. and *Salmonella enterica*. Sequence analysis and structural modeling show that the proteins in this group are related to chicken egg white lysozyme and are likely to adopt a lysozyme-like structural fold. Based on their similarity to lysozyme, we predict that these proteins have glycosidase activity. Iterative data base searches with three conserved sequence motifs from this protein family detect a more distant relationship to bacterial and bacteriophage lytic transglycosylases, and goose egg white lysozyme. Two acidic residues in the VirB1 protein of *Agrobacterium tumefaciens* form a putative catalytic dyad. Each of these residues was changed into the corresponding amide by site-directed mutagenesis. Strains of *A. tumefaciens* that express mutated VirB1 proteins have a significantly reduced virulence. We hypothesize that many bacterial proteins involved in export of macromolecules belong to a widespread class of hydrolases and cleave β-1,4-glycosidic bonds as part of their function.**

The soil bacterium *Agrobacterium tumefaciens* causes crown gall tumors in dicotyledonous plants by transferring a DNA segment (T-DNA) of its large tumor-inducing (Ti) plasmid into the host plant cell nucleus, where it becomes integrated into the plant genome and expresses oncogenes (for reviews, see refs. 1–3). At least eight plasmid-encoded *vir* operons and several chromosomal genes of *Agrobacterium* are required for virulence (1–4). The *vir* genes are transcriptionally activated by the VirA/VirG two-component regulatory system, which responds to signal molecules such as specific phenolic compounds and sugars released by wounded plant cells (5). *virC* and *virD* gene products act in a concerted manner to release single-stranded transferable DNA (T-strand) from the double-stranded Ti plasmids (6–9). The VirE2 protein, a single-stranded DNA-binding protein, is believed to coat the T-strand and facilitate its transfer through the plant cell cytoplasm into the nucleus (10).

There is considerable evidence that the proteins coded by the 11 genes of the *virB* operon are concerned with the formation of a channel for the transfer of T-DNA. These 11 genes are not required for *vir* gene activation or T-strand formation, but every one has been shown to be important in tumorigenesis (11). A comparative sequence analysis has revealed a high similarity between several VirB proteins and gene products required for the export of specific proteins and plasmids. These include pertussis toxin in *Bordetella* (12) and broad-host-range plasmids, such as plasmid pKM101, which contains the full set of VirB homologs (3, 13).

How VirB proteins function in gene transfer is not well understood. The abundance of hydrophobic segments in the amino acid sequences of several VirB proteins has prompted the suggestion that they form pili-like structures in the membrane that serve as a channel to export T-DNA from the cell (1, 13, 14). Immunolocalization and Tn*phoA* analysis confirmed that all of the VirB proteins are membrane-associated *in vivo* (15, 16). VirB4 and VirB11 contain putative nucleotide-binding sites that are indispensable for virulence, suggesting regulatory or energy-converting functions for these proteins (17–19). It is possible that the export of various macromolecules in bacteria is achieved via similar mechanisms, employing membrane transport complexes formed by VirB-like proteins (1, 3, 14, 15).

Here, we describe a protein family that includes a virulence factor of *Agrobacterium*, VirB1, and a number of related plasmid-borne and chromosomal gene products of the family Enterobacteriaceae. These proteins are related to chicken egg white lysozyme at the amino acid sequence level and are likely to adopt a similar tertiary structure. The sequence similarity among a family of bacterial and bacteriophage lytic transglycosylases (20), VirB1, and the related plasmid-encoded proteins has been reported very recently (21). We show that all these proteins, along with chicken egg white lysozyme and goose egg white lysozyme, belong to a single superfamily of enzymes sharing three conserved sequence motifs. Based on these observations, accompanied by three-dimensional (3D) modeling, we propose that VirB1 possesses a lysozyme-like activity. Site-directed mutagenesis of the predicted catalytic residues of VirB1 attenuates *Agrobacterium* virulence, supporting this prediction.

MATERIALS AND METHODS

Sequence Analysis. The nonredundant sequence data base at the National Center for Biotechnology Information, National Institutes of Health, was searched using the BLAST family of programs (22, 23); the BLASTP program was used to search the protein sequence data base, and the TBLASTN program was used to search the nucleotide sequence data base. Multiple protein sequence alignments were constructed with the MACAW program (24). Sequence blocks derived from multiple alignments were used to search the data base for conserved motifs with the MOST program (25), with the ratio of the expected number of retrieved segments to the observed number, $r = 0.005$, as a cutoff.

Protein secondary structure predictions were made by the PHD program, based on a neural-networks algorithm (26). Homology modeling of protein 3D structure was performed using the Swiss-Model server at the GLAXO Research Institute, Geneva, Switzerland (refs. 27 and 28; http://expasy.hcuge.ch/swissmod/PROMODSERVO.html), using the PROMOD program. The 3D profiles (29) were calculated for each of the structures, using the Profile subroutine of the PROMOD

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Abbreviations: Ti, tumor-inducing; T-DNA, transferable DNA; T-strand, single-stranded transferable DNA; 3D, three-dimensional; GlcNAc, *N*-acetylglucosamine.

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program. 3D structures were visualized using the INSIGHTII program (Biosym Technologies, San Diego).

DNA Manipulations. For site-directed mutagenesis, standard techniques were employed (30). The *Escherichia coli* strain DH5α was used for subcloning, *dut*$^-$ *ung*$^-$ strain CJ236 was used for the preparation of the single-stranded, uridine-containing plasmid DNA, and the C2110 strain was used for the selection of IncP broad-host-range plasmids. The plasmid pKJF86, a parent plasmid for all other constructs, contains the portion of the *virB* operon of the plasmid pTiA6 (31, 32), including the *virB* promoter and the *virB1* gene (nucleotides 188-1491; GenBank accession no. M14488), cloned as a *Hin*dIII/*Eco*RV fragment in pUC19. A *Hin*cII/*Sac*II fragment (nucleotides 885-1214) was subcloned into pBluescript SK+ (Stratagene) digested with *Hin*cII and *Sac*II, single-stranded DNA was prepared, and mutations were introduced into the *virB1* gene by the Kunkel method (33). The conserved glutamic acid (residue 32 in the mature lysozyme, residue 53 in the VirB1 precursor) was changed to glutamine using the oligonucleotide 5′-GATAGCTAAGGTGCAGAGTCGCTTTGATCC-3′ (mutant E53Q; mutated residues are underlined) or to alanine using the oligonucleotide 5′-GATAGCTAAGGTGGCCAGTCGCTTTGATCC-3′ (mutant E53A). The conserved aspartic acid (residue 53 in the mature lysozyme, residue 93 in the VirB1 precursor) was changed to asparagine using the oligonucleotide 5′-GGCATTCGCTGAACGTTGGCCTCATGC-3′ (D93N). A nonconserved glutamic acid residue in position 136 in the VirB1 precursor was changed into alanine using the oligonucleotide 5′-GCAGGCGGCGCCACGATTGACG-3′ (E136A). Mutated DNA fragments were completely sequenced to verify the mutations and used to replace the wild-type *Hin*cII/*Sac*II fragment in pKJF86. The mutant derivatives of pKJF86 were digested with *Hin*dIII and ligated into the broad-host-range plasmid pTJS75 (34), partially restricted with *Hin*dIII. The resulting cointegrate plasmids replicate in *Agrobacterium* and express the VirB1 gene (34). For a summary of the wild-type and mutant clones, see Table 1. Purified plasmid DNA was electroporated into *Agrobacterium* strain A348Δ*virB1* (ref. 11; a generous gift of P. J. Christie, University of Texas, Houston) to produce strains that express wild-type or mutant VirB1 protein.

***In Vivo* Activity Assay.** The tumorigenesis assay on *Kalanchoë diagremontiana* leaves was a modification of a previously published procedure (34). A grid of 4 × 4 punctures, 3 mm apart, was applied by a hypodermic needle on a leaf of *K. diagremontiana* and immediately covered with 25 μl of bacterial suspension. Leaf surfaces were washed with distilled water 3 hr after inoculation. Individual tumors were scored visually, and their diameters were measured weekly from 20 to 60 days after inoculation. Each inoculation was performed at least twice with two independent transformants of each mutant construct.

RESULTS

VirB1 and Other Bacterial Virulence Factors Share Sequence Similarity to Chicken Lysozyme. In a search of sequence similarities that might provide clues to the biochemical functions of *Agrobacterium* VirB proteins, we compared VirB protein sequences with protein data bases (22, 23). In addition to the previously described high conservation between most of the *vir* gene products and proteins involved in conjugative plasmid transfer and pertussis toxin export in *Bordetella* (12, 13), the VirB1 protein showed similarity to the TraN protein of the IncP plasmid RP4 (7), the putative product of ORF*19* (also known as ORF169 or ORF X) of the IncFII plasmid R1 (35), and two proteins of enteric pathogens, namely IpgF encoded by invasion plasmids of *Shigella* spp. (36) and IagB encoded by a chromosomal locus of *Salmonella enterica* (37). The similarity between proteins within this group was moderately to highly statistically significant (the probability of matching by chance was $P < 0.05$ for IagB and VirB1 and $P < 10^{-4}$ for IagB and the ORF*19* product). Two putative proteins of the same family are encoded by an uncharacterized ORF (*ygeJ*) at 64.5 min of the *E. coli* chromosome (GenBank accession no. U28375) and by a cosmid DNA clone from a eukaryote, the pathogenic nematode *Wuchereria bancrofti* (38). The TraN and ORF*19* proteins are encoded within the regions of their respective plasmids that are involved in conjugational DNA transfer (7, 35), whereas the *ipgF* gene is found within a locus that is thought to mediate export of the invasion proteins (36). Multiple alignment revealed three blocks of highest sequence conservation, with several invariant amino acid residues in each block (Fig. 1).

An iterative data base search with the BLASTP and TBLASTN programs did not reveal additional highly significant matches; however, limited similarity (P between 0.06 and 0.075) was observed between most of the proteins in the family and chicken egg white lysozyme. Notably, two segments of the highest similarity between VirB1 and lysozyme coincided almost precisely with the conserved blocks within the VirB/TraL/IagB family itself, and the counterpart of the third conserved region was readily identified by multiple sequence alignment (Fig. 1). A block corresponding to motif II in the alignment of closely related IagB, IpgF, and ORF169 proteins was used to iteratively search the data bases for conserved sequence motifs using the MOST program (25). During this search, lysozymes of the chicken egg white (C) group, related mammalian lactalbumin sequences, and VirB/TraL sequences, without false positives, were retrieved. These results indicate that the proteins detected by data base searches are indeed members of a conserved family. Through the length of this alignment, the similarity is 42% between lysozyme and VirB1, and 50% between lysozyme and IagB, as compared with the 55% similarity between lysozyme and the closely related lactalbumin. Importantly, motifs I and II (Fig. 1) each centered around an acidic residue. In lysozyme, both of these residues are directly involved in acid catalysis (40–43).

Chicken Lysozyme-Like Proteins, Bacterial and Bacteriophage Lytic Transglycosylases, and Goose Egg White Lysozyme Belong to the Same Superfamily of Glycosidases. Several polysaccharide-hydrolyzing enzymes (including chicken, goose, and bacteriophage T4 lysozymes; bacterial and phage lytic transglycosylases; and chitinases and chitosanases) have been considered unrelated by sequence while sharing common structural elements (44–46). Recently, a local sequence similarity between the bacterial proteins related to VirB1 and a family of lytic transglycosylases has been reported (21). Iterative data base searches with the BLAST and MOST programs (47), using the members of chicken lysozyme/VirB1 family as queries, confirmed that the Slt transglycosylase of *E. coli* and the related phage proteins share sequence similarity with VirB1 and chicken lysozyme. During these searches, additional related proteins, including several putative proteins from *E. coli*, *Haemophilus influenzae*, and *Synechocystis* sp., and goose egg white lysozyme were retrieved (the similarity between goose lysozyme and Slt transglycosylase sequences has also been noted in ref. 46). Multiple alignment of these proteins resulted in the delineation of three conserved motifs, which coincide with those conserved in the chicken lysozyme/VirB1 family (Fig. 1). The probabilities of motifs I, II, and III occurring by chance, computed by the MACAW program, were below 10^{-12}, 10^{-9}, and 10^{-19}, respectively. Chitinases, chitosanases, and T4 lysozyme have short sequence stretches around the catalytic residues that are similar to the sequences of the above superfamily, but the degree of conservation appears to be too low to produce a statistically significant alignment.

The two groups within the superfamily shown in Fig. 1 each include bacterial and eukaryotic proteins. Despite the conservation of the three motifs in all of the sequences in the superfamily, several notable differences exist between the chicken lysozyme/VirB1 family and the goose lysozyme/

		αB			β			αE		
consensus		UxxxUUxxUxxxESxxxxxxx			xxxxDxGLMQUxxxxx			xxxUxxYNxG		
LYC_CHICK	40	YSLGNWVCAAKFESNFNTQAT	4		DGSTDYGILQINSRWW	41		MNAWVAWRNR	15	P00698
VIB1_AGRT9	40	VATSTLAAIAKVESRFDPLAI	27		RHSLDVGLMQINSRNF	41		RRAISAYNTG	84	P05350
ECU09868_5	40	VSPLTMAYIVGHESSNGPYRI	28		NKSFDMGLAQINSNNL	42		RHALSCYNTG	87	U09868
RP4TRBAO_14	56	IPANILLAIREKEGGKPGQWV	3		NGTYDVGELQFNTAYL	39		WTRAANYHSR	89	M93696
IPGF_SHIFL	29	IPSSLLKAIAEKESGFNKSAV	4		NGSKDYGIMQINDFHS	38		WEAVGAYNAG	34	Q07568
STIAGAB_2	30	IESELLYAIAQQESAMKPGAI	4		DGSTDLGLMQINSFHM	38		WEAVGAYNAG	41	X80892
LCA_PAPCY	20	IALPELICTMFHTSGYDTQAI	3		NESTEYGLFQISNALW	40		IDYWIAHKAL	13	P12065
X191_ECOLI	31	IDPDLLRAISWKESRYRVNAI	4		VTGYGSGLMQVDSQHF	38		WEAVGAYNAG	49	P14499
WUCCHIT	25	VDPYLLYAIANVESGMNPYAV	4		DGTRDVGLMQINSSHF	?				L18869
LYG_ANSAN	60	VEPAVIAGIISRESHAGKVLK	4		DRGNGFGLMQVDKRSH	39		KGGISAYNAG	35	P00718
SLT_ECOLI	492	IPQSYAMAIARQESAWNPKVK	0		SPVGASGLMQIMPGTA	43		IFSSAAYNAG	63	P03810
YAFG_ECOLI	11	NMPMELVLLPIVESAFDPHAT	0		SGANAAGIWQIIPSTG	39		LLTVAAYNSG	38	P32982
YFHD_HAEIN	279	LDWRLLAAVAYQESHWNPDAT	0		SPTGVRGIMMLTKNTA	39		WFALVAYNIG	96	P44587
Y761_HAEIN	201	IDESLILGIMQTESSFNPYAI	0		SYANAIGLMQVVPHTA	50		FAMISAYNSG	58	P44049
SYCSLLLH_44	702	VNPFLVTALIRQESRFEKNIQ	0		SPVGATGLMQIMPATA	39		ALAVASYNAG	60	D64006
TBTA_ALTSM	36	NLPIELALMPLIESDFNASAY	0		SHKHASGLWQLTPAIA	39		YHAIAALNLG	84	P32820
VP07_BPPRD	18	LDPRLVAGVVQTESSGNPRTT	0		SGVGAMGLMQLMPATA	33		NTALLAYHGG	191	P27380
VIVD_BPT7	25	VSYDLLRKVAWTESRFVPTAK	0		SKTGPLGMMQFTKATA	41		LKAALAYNQG	1206	P03726
		H2′			B2 B3			H5		
MOTIFS		I			II			III		

FIG. 1. Conserved sequence elements in the chicken egg white lysozyme family of proteins. The ungapped blocks with the highest sequence conservation are shown; the distances between blocks and the termini of each sequence are indicated by numbers. A unique identifier for each protein is shown in the first column, and the accession numbers in SWISSPROT (starting with P or Q) or in GenBank are shown in the last column. Highly conserved residues are in bold in the alignment. In the consensus line, U stands for a bulky hydrophobic residue (I, V, L, M, W, F, or Y), × stands for any residue, and the two residues shown in bold are the catalytic residues in chicken lysozyme. The α-helices and three-stranded β-sheet in the known structure of lysozyme are shown above the alignment, and the equivalent elements in Slt structure (H, helices; B, sheets; ref. 39) are shown below the alignment. The aspartic acid residue in motif II implicated in catalysis by goose lysozyme is underlined.

transglycosylase family. These include a higher variability in the spacing of motifs I and II in the chicken lysozyme family and the tendency of the goose lysozyme-like proteins to occur as parts of much larger molecules. The goose lysozyme/transglycosylase family of proteins lacks the invariant acidic residue in motif II. The nearby aspartic acid residue that has been implicated in goose lysozyme catalysis (48), is replaced by a serine in transglycosylases. Although both aspartic acid and serine might be involved in nucleophylic attack (49), the catalytic mechanism in transglycosylases remains an open problem.

Structural Modeling Suggests a Lysozyme-Like Fold for the Proteins of the VirB1 Family. The sequence conservation between VirB1 and chicken lysozyme suggested that these

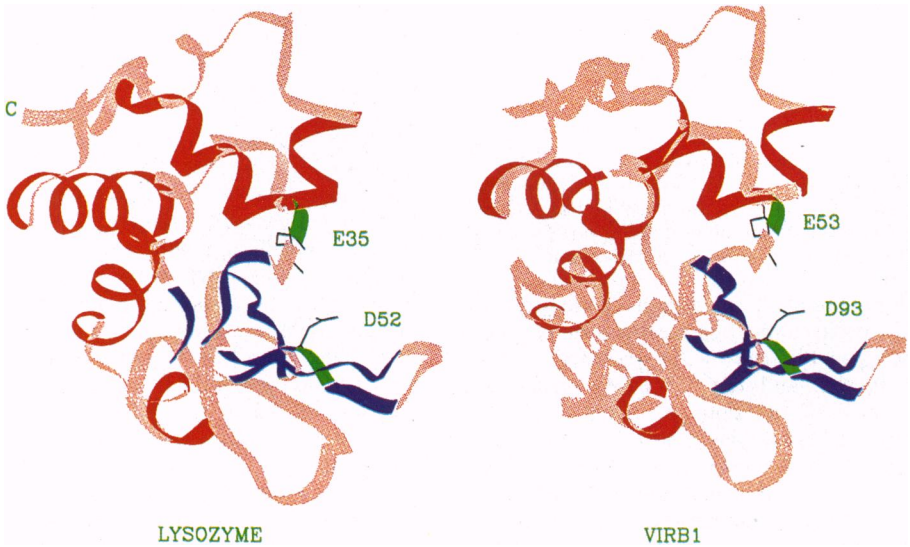

FIG. 2. The 3D model of VirB1. Conserved secondary structure elements are shown in red (α-helices) and blue (β-strands). Positions of the catalytic residues are shown in green. Backbone and side chain atoms of the catalytic dyad are drawn schematically. The 23-residue spacer between motifs I and II in VirB1 contributes to a β-sheet whose topology could not be rebuilt unequivocally.

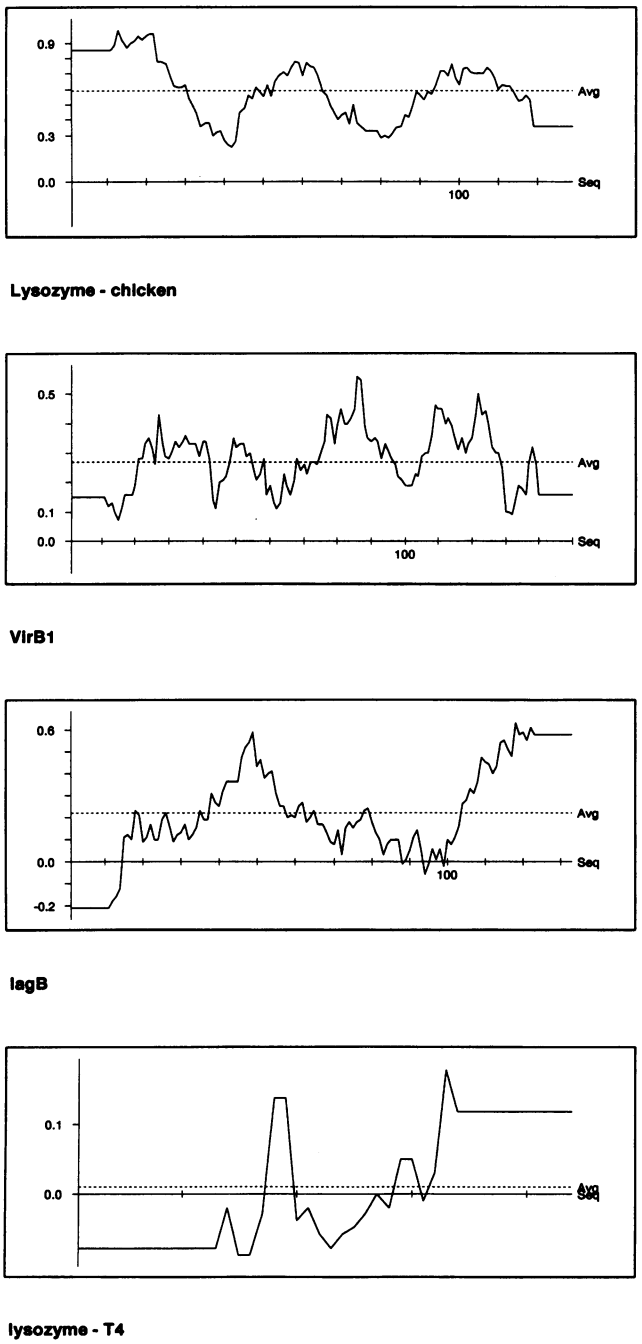

FIG. 3. 3D profiles of chicken lysozyme, VirB1, IagB, and T4 lysozyme models. The horizontal axis indicates the amino acid position within the sequence. Scores for the individual amino acids, determined using a sliding window of 21 residues, are plotted at the vertical axis. The average score for the model is indicated by a dashed line.

proteins may be structurally related. The regions of the highest similarity between the proteins in the family correspond to the conserved core elements of the known 3D structure of the lysozyme molecule (40, 41), and the secondary structure prediction delineated most of the same elements (Fig. 1). Therefore, we employed a knowledge-based 3D modeling approach, based on the PROMOD algorithm (25). The results are shown in Fig. 2. In spite of the insertion in VirB1, the overall topology of the molecule is similar to that of lysozyme, with most of the predicted secondary structure elements occupying equivalent positions. Moreover, the distance between the two putative catalytic residues in VirB1 is predicted to be close to that in lysozyme. This indicates that, like lysozyme, VirB1 may catalyze glycosidic bond hydrolysis via the retaining mechanism, as opposed to the inverting mechanism (49).

Table 1. Virulence in *K. diagremontiana* of *Agrobacterium* strains expressing wild-type or mutant VirB1 proteins, 25 dpi

Shuttle vector expressing VirB1/ mutation in VirB1	No. of tumors out of 32 punctures	
	Exp. I*	Exp. II
Strain A348		
No vector/wild type in Ti plasmid	32	31
pARM143/wild type in Ti plasmid and in the vector	31†	31†
pARM146/wild type in Ti plasmid, *E53A* in the vector	25‡	22‡
Strain A348Δ*B1*		
No plasmid-borne VirB1	3	4
pARM143/wild type	23	26
pARM150/*E53Q*	3	3
pARM146/*E53A*	6	5
pARM144/*D93N*	5	6
pARM148/*E136A*	23	22

*Results of two individual representative experiments are shown.
†Extra large tumors.
‡Very small tumors.

To assess the validity of the tertiary structure prediction, we used the 3D profile approach (29). VirB1, IagB, and lactalbumin were used to model the proteins onto the chicken egg white lysozyme structure. T4 lysozyme that shares only one motif with chicken lysozyme, despite the presence of equivalent structural core elements (44–46), was used as a control. The 3D scores were calculated for each of the models. The models of VirB1 and IagB appear to be valid, with average 3D scores being only about 2-fold lower than the score for lysozyme itself and with scores falling within the range typical for the correct models (29). In contrast, the distantly related bacteriophage T4 lysozyme could not be modeled onto the chicken lysozyme structure (Fig. 3).

The Putative Catalytic Residues in VirB1 Are Required for Virulence. To further test the possibility that VirB1 is a hydrolase with a lysozyme-type acid catalysis, we constructed a series of mutations in the putative catalytic residues of VirB1. Each of the two putative catalytic residues, E53 and D93, was changed to the respective amide; additionally, E53 was changed to alanine. In a control mutant, a distal, nonconserved glutamate (E136) was changed to alanine. The mutant *virB1* genes were placed under the control of the *virB* promoter. The expression cassettes were inserted into a shuttle vector and electroporated into an *A. tumefaciens* strain, in which the *virB1* gene was deleted (11). The virulence of the resulting strains was compared with that of the parent strain by tumor formation on *K. diagremontiana* leaves (Table 1).

After inoculation with the parent strain, A348, tumors developed in 90–100% of the punctures, and reached ≈2 mm in diameter 18–20 days after inoculation. In accord with the previous observations (11), upon inoculation with strain A348Δ*VirB1*, only 5–10% of the punctures developed visible tumors, and these did not reach normal size even 60 days after inoculation. When the wild-type *virB1* gene under its own promoter was introduced into the A348Δ*VirB1* background, the virulence of the resulting strain was partially restored, with normal-size tumors developing in 65–75% of the punctures (Table 1 and Fig. 4). In contrast, the mutants, in which one of the putative catalytic residues was changed, induced tumors in only 10% of the punctures (Table 1 and Fig. 4). The virulence of the mutants was attenuated to the same extent regardless of whether the change was to the amide or to alanine, suggesting

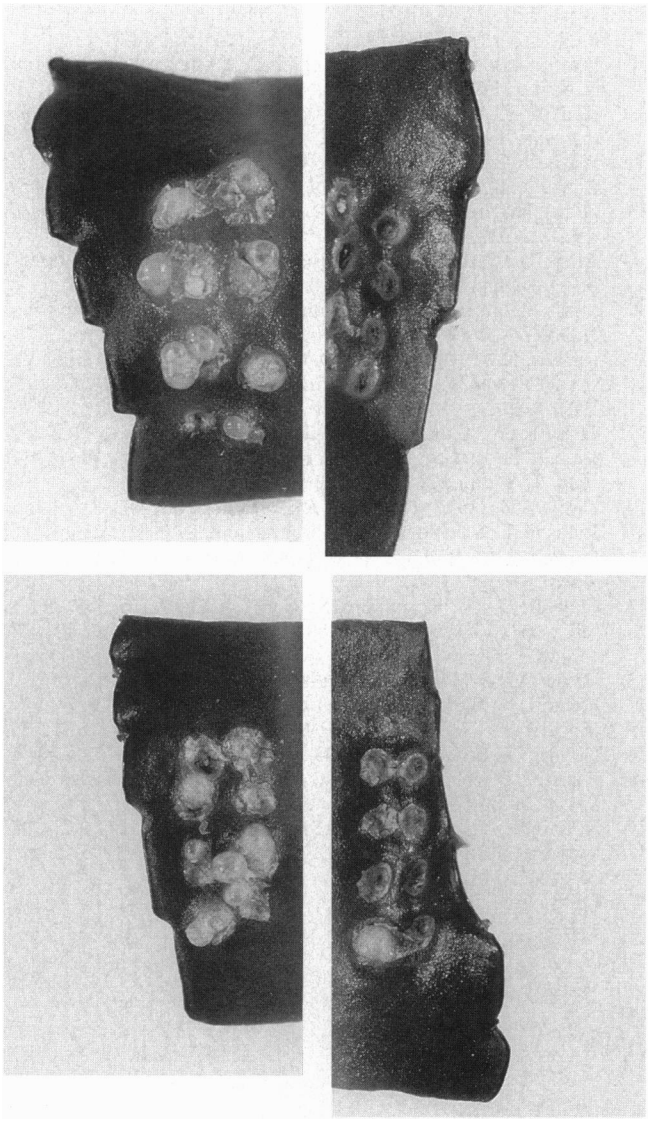

FIG. 4. Tumorigenesis of *A. tumefaciens* strains expressing wild-type and mutant VirB1 proteins on *K. diagremontiana*. (*Upper left*) A348 (parent strain). (*Upper right*) A348Δ*virB1*. (*Lower left*) A348Δ*virB1*::pARM143 (wild-type VirB1). (*Lower right*) A348Δ*virB1*::pARM146 (mutant VirB1).

that the negative charge, at least at position 53, was crucial to restore virulence fully. A control mutation, in which a non-conserved acidic residue downstream of the putative catalytic sites was changed into alanine, developed visible tumors in 60% of the punctures, although these tumors were slightly smaller than in the case of the wild type, reaching the 2-mm size approximately 25 days after inoculation (Table 1).

In a separate assay, the mutant *virB1* genes were introduced into parent strain A348 with the intact *virB1* gene on the Ti plasmid. One strain, A348(*E53A*), produced decreased numbers of very small tumors, suggesting that some of the mutated forms of VirB1 can compete with the wild-type protein, inhibiting its activity (Table 1).

DISCUSSION

In this paper, we describe a family of bacterial proteins that share sequence similarity and a common structural fold with chicken C-type lysozyme. These proteins are associated with the export of macromolecules from the bacterial cell. *virB1*, *traL*, *traN*, and *ORF19* are plasmid-encoded genes that may control the intercellular transfer of plasmid DNA. Genetic evidence supports the contention that VirB (11), TraL (13), and ORF*19* (21) are involved in this process. IagB and IgpF are encoded by loci that may be involved in tissue invasion in enteric bacteria, presumably by mediating the export of proteinaceous invasion antigens (36, 37).

Lysozymes are a broad group of enzymes from bacteria, bacteriophages, and eukaryotes that are defined by their ability to hydrolyze the β-1,4-glycosidic bonds found in the peptidoglycan chains of the cell walls of Gram-positive bacteria (40–43). These chains are alternating polymers of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid; lysozymes also hydrolyze chitin, which is a homopolymer of GlcNAc, and oligosaccharide substrates containing β-1,4-linked GlcNAc (36). Sequence similarity between chicken lysozyme and bacterial proteins is especially prominent around the catalytic residues of lysozyme. Moreover, most of the secondary structure elements that define the structural core of lysozyme seem to be conserved in all of the proteins in the family (Fig. 1). 3D modeling confirms these observations, revealing one deviation from the lysozyme structural fold in the VirB1 protein, namely a different topology of the β-sheet element at one side of the putative catalytic cleft (Fig. 2). This might suggest that VirB1 catalyzes the formation or hydrolysis of β-1,4 bonds within a molecule distinct from a linear polysaccharide.

Mutant VirB1 proteins with changes in the putative catalytic residues have strongly reduced virulence when expressed in the strain of *Agrobacterium*, in which the resident *virB1* gene is completely deleted. A merodiploid that expresses both the wild-type and mutated VirB1 proteins also showed a reduced virulence. The latter effect likely results from competition between the mutant and wild-type VirB1 proteins for participation in the transport process.

What is the role of the predicted β-1,4-glycosidase activity of VirB1 in tumorigenesis? One can speculate that controlled hydrolysis of a glycoside bond in a polysaccharide or in a glycoprotein facilitates the assembly of the membrane channel for T-DNA export (1–3, 21). A related possibility is that VirB1 plays a role in T-DNA transfer by mediating the export of another protein, such as VirE2. Indeed, the latter is known to function in T-DNA transfer when provided extracellularly, either by a complementing bacterial strain or by a transgenic plant (10). However, a strain in which the *virB1* gene has been deleted cannot serve as a source of VirE2 protein (unpublished observations). Thus, the involvement of the β-1,4-glycosidase activity of VirB1 and its homologs in protein export may be the common denominator for their roles in pathogenesis of diverse bacteria.

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