Mutants of *Ralstonia* (*Pseudomonas*) *solanacearum* Sensitive to Antimicrobial Peptides Are Altered in Their Lipopolysaccharide Structure and Are Avirulent in Tobacco

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Ralstonia solanacearum **K60 was mutagenized with the transposon Tn*5*, and two mutants, M2 and M88, were isolated. Both mutants were selected based on their increased sensitivity to thionins, and they had the Tn*5* insertion in the same gene, 34 bp apart. Sequence analysis of the interrupted gene showed clear homology with the *rfaF* gene from *Escherichia coli* and *Salmonella typhimurium* (66% similarity), which encodes a heptosyltransferase involved in the synthesis of the lipopolysaccharide (LPS) core. Mutants M2 and M88 had an altered LPS electrophoretic pattern, consistent with synthesis of incomplete LPS cores. For these reasons, the *R. solanacearum* gene was designated *rfaF*. The mutants were also sensitive to purified lipid transfer proteins (LTPs) and to an LTP-enriched, cell wall extract from tobacco leaves. Mutants M2 and M88 died rapidly *in planta* and failed to produce necrosis when infiltrated in tobacco leaves or to cause wilting when injected in tobacco stems. Complemented strains M2* and M88* were respectively obtained from mutants M2 and M88 by transformation with a DNA fragment harboring gene *rfaF*. They had a different degree of wild-type reconstituted phenotype. Both strains retained the rough phenotype of the mutants, and their LPS electrophoretic patterns were intermediate between those of the wild type and those of the mutants.**

Plants are exposed to a variety of microorganisms that have the potential to cause disease, but they are resistant to most of them through several defense mechanisms, such as hypersensitive cell death, cell wall reinforcement, and synthesis of antimicrobial compounds (3). Different families of plant proteins have direct antimicrobial properties; these include thionins (12–14, 30), glucanases and chitinases (24, 26, 50, 52), defensins (33, 56), ribosome-inactivating proteins (24), thaumatin-like proteins (19), zeamatins (58), osmotins (60), lipid transfer proteins (15, 28, 31, 51, 55), and desintegrin-like proteins (34). The observation of *in vitro* antimicrobial properties is not by itself sufficient to assign a defense role to a given protein. Additional data are required to support such a role. For example, gene expression patterns and *in planta* concentrations of the protein before or after infection must be congruent with the proposed function, and a decrease of symptoms in transgenic plants overexpressing the protein should occur.

A prediction that has not yet been tested is that an alteration of symptoms should occur in mutants of the pathogen with altered resistance levels to one or more defense proteins. A successful infection by a plant pathogen implies the existence of mechanisms to withstand or circumvent the action of toxic compounds present or induced in the plant, but little is known about them. Because the bacterium *Ralstonia solanacearum* K60 showed a high degree of natural resistance to thionins and lipid transfer proteins (LTPs), it was chosen to investigate both the relevance of plant antipathogenic peptides in plant-pathogen interactions and the pathogen defense mechanisms against them. This bacterium is the causal agent of bacterial wilt, one of the most destructive plant diseases, and attacks a wide range of species, including tobacco and potato (4). Thionins and LTPs have been isolated from different tissues in many plant species (for reviews, see references 14 and 15) and have been shown to inhibit *in vitro* growth of many bacterial and fungal pathogens (2, 12, 30, 31). Their concentration ranges *in vivo* are congruent with a protective role (29, 31), induction of their genes in response to pathogens has been observed (16, 29), and transgenic plants overexpressing these genes showed enhanced resistance to pathogens (5, 32).

We report here that two Tn*5* mutants of *R. solanacearum* K60 with increased sensitivity to thionins are affected in gene *rfaF*, which encodes a putative heptosyltransferase involved in lipopolysaccharide (LPS) biosynthesis, heptose being a component of the core region of LPS in *R. solanacearum* (1). These mutants are also sensitive to LTPs and are avirulent, while complemented strains that are resistant or partially resistant to thionins are fully virulent or partially virulent, respectively, in tobacco.

MATERIALS AND METHODS

Microbiological methods. Bacterial strains and plasmids used in this work are described in Table 1. Strains of *Escherichia coli* were cultivated at 37°C in LB medium (27). *R. solanacearum* strains were grown in either CPG medium (21) or selective Sm-1 medium (17). Antibiotics were added to the media at the following concentrations: tetracycline, 15 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; and chloramphenicol, 50 μg/ml. Bacterial sensitivity to plant peptides was determined in enzyme-linked immunosorbent assay plates as described by Molina et al. (31). *R. solanacearum* K60 was mutagenized by insertion of transposon Tn*5* by using as a suicide plasmid pSUP2021 (53), which was introduced by conjugation as described by Rothmel et al. (46). Transconjugants were screened for thionin sensitivity in Sm-1 plates containing 200 μg of purified wheat thionin per ml.

Antimicrobial peptide purification. Thionins were purified as described by Ponz et al. (44). One kilogram of wheat flour was extracted with petroleum-ether (boiling point, 40 to 60°C), concentrated in a rotoevaporator, mixed with 3 volumes of 0.5 M HCl in ethanol, and centrifuged at 5,000 × *g* for 20 min. The pellet was washed twice with cold ethanol and twice with ether and then subjected to preparative electrophoresis in a column of 15% acrylamide in 0.1 M acetic acid. A tobacco extract enriched in lipid transfer proteins was prepared as described by Molina et al. (31). Frozen leaf material (20 g) was ground to powder in liquid nitrogen, using a mortar and pestle, and washed once with 80 ml of

TABLE 1. Bacterial strains and plasmid used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
S-17.1	*thi pro* r_K m_K^+ *rec A res* RP4-2-Tc::Mu-Km::Tn-7	53
XL-1	*recA endAI hsdR17 thi-1 recAI supE44 gyrA96 relAI(lac)*[*F' proAB lacZ MIS* Tn*10* (Tet^r)]	Stratagene
R. solanacearum		
K60	Wild type	22
M2	K60 *rfaF*::Tn*5* Km^r	This work
M88	K60 *rfaF*::Tn*5* Km^r	This work
M2$^+$	M2 complemented with pPs3 Km^r Amp^r	This work
M88$^+$	M88 complemented with pPs3 Km^r Amp^r	This work
Plasmids		
pSUP2021	*mob*$^+$ Amp^r Km^r Cm^r	53
pUC18	Amp^r, subcloning vector	61
pT7T3-18U	pUC18 with T7 and T3 promoters	Pharmacia
pJB313	Amp^r	1a
pPs1	1.8-kb insert flanking Tn*5* insertion in M2	This work
pPs2	4.1-kb insert which spans Tn*5* insertion site of both M2 and M88	This work
pPs3	pPs2 insertion recloned in pJB313	This work

buffer (0.1 M Tris-HCl, 10 mM EDTA [pH 7.5]) and twice with 80 ml of distilled H_2O. The resulting pellet was then extracted with 50 ml of 1.5 M LiCl at 4°C for 1 h, and the extract was dialyzed against 5 liters of H_2O, using a Spectra/Por 7 membrane (molecular weight cutoff, 2,000; Spectrum Medical Industries Inc.), and freeze-dried. Lipid transfer protein LTP2 from barley leaves, LTP1 from *Arabidopsis thaliana*, and the antibody against LTP1 were a generous gift of A. Molina (Madrid, Spain).

DNA manipulation and sequencing. Cloning of the DNA fragment harboring Tn*5* was performed by a ligation-mediated PCR technique (35). Chromosomal DNA from the M2 mutant was digested with the endonuclease *Alu*I, primer extended with Sequenase, using the 25-mer oligonucleotide 5'ACGGATCCAG GAGGTCACATGGAAG3' (positions 60 to 76 of Tn*5*), and blunt-end ligated with an adapter composed of oligonucleotides 5'CTGCAGGTCGACTCTAGA GGATCC3' and 5'GGATCCTCTAGAGT3'. Two rounds of PCR amplification were performed, using as primers the first of the two adapter oligonucleotides and oligonucleotide 5'GAGGATCCCCGTTCAGGACGCTACT3' (positions 17 to 41 of Tn*5*). The amplified product, a 1.8-kb fragment flanking the Tn*5* insertion, was cloned in the pUC18 vector. The cloned insert (plasmid pPs1) was used as a probe to screen a DNA genomic library from wild-type *R. solanacearum*. This library was constructed by ligating 3.5- to 6-kb, *Eco*RI-digested DNA fragments into plasmid pT7T3-18U/*Eco*RI (Pharmacia) and electroporating them into *E. coli* XL-1 Blue (Stratagene). A positive clone (pPs2) with a 4.1-kb insert was identified. Standard molecular cloning techniques used in this study (small- and large-scale plasmid DNA purification, restriction enzyme digestion, agarose gel electrophoresis, DNA subcloning, Southern blot and hybridization, and colony screening by hybridization) were performed as described by Sambrook et al. (49). DNA sequencing of both strands was done by the chain termination method on double- or single-stranded DNA templates, using a Sequenase version 2.0 sequencing kit as instructed by the manufacturer (U.S. Biochemical).

LPS extraction and fractionation. LPS from wild-type *R. solanacearum* K60 and from mutant and complemented strains was purified as described by Tsai and Frasch (57). Bacteria were grown overnight in solid medium, collected, and diluted in water to an absorbance of 0.4 at 420 nm. A 1.5-ml sample of this suspension was centrifuged, and the pellet was treated with 50 μl of lysis buffer (2% sodium dodecyl sulfate [SDS], 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris [pH 6.8]) and heated at 100°C for 10 min. Proteinase K (10 μl of a 2.5-mg/ml solution) was added, and the mixture was kept at 60°C for 1 h. Fractionation was carried out by SDS-polyacrylamide gel electrophoresis (PAGE) on a 4 to 20% acrylamide gradient gel (Bio-Rad), and the gels were silver stained. The same extract was used to analyze the heptose content by the HCl-cysteine method described by Osborn (38).

Plant assays. Pathogenicity of wild-type *R. solanacearum* K60 and of mutant and complemented strains was assessed as follows. Tobacco plants (cv. Wisconsin 38) were grown in growth chamber at 25°C and 75% relative humidity until they were about 20 cm high. Leaf inoculations were performed on the fully expanded leaves by infiltrating 20 μl of bacterial suspension containing 10^5 CFU/ml in 10 mM $MgCl_2$ essentially as described by Kao and Sequeira (21). For wilting assays, plants were stem inoculated with 50 μl of a bacterial suspension containing 10^5 CFU/ml in 10 mM $MgCl_2$.

To monitor bacterial growth *in planta*, 20 μl of a bacterial suspension containing 10^2 CFU was infiltrated in tobacco leaves as described by Cook and Sequeira (8). Leaf disks (1 cm^2) from infiltrated tissue were cut with a cork borer at different times and ground with a tissue homogenizer in 500 μl of 10 mM $MgCl_2$. Bacterial CFU/milliliter in the homogenate was determined by dilution plating.

Nucleotide sequence accession number. The nucleotide sequence of *rfaF* was originally deposited in the EMBL database under accession no. X95498.

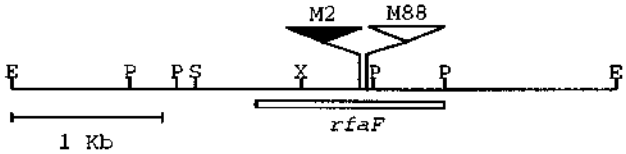

FIG. 1. Restriction map of the 4.1-kb insert of plasmid pPs2. The position of gene *rfaF* in the insert is indicated. The insertion points of Tn*5* in mutants M2 and M88 are indicated by triangles. E, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xho*I.

RESULTS

Isolation of Tn*5* mutants. To produce Tn*5* insertion mutants, *R. solanacearum* K60 was mated with *E. coli* S-17, which carried the suicide plasmid pSUP2021 (53). Transconjugants were screened in solid medium containing purified wheat thionin (200 μg/ml). In each of two independent experiments, one thionin-sensitive mutant was obtained out of about 5,000 Km^r bacteria. The two mutants, named M2 and M88, appeared to have a single Tn*5* insertion, as judged by Southern blot analysis using a Tn*5* fragment as probe (data not shown). These mutants had essentially the same growth rate in CPG medium as the wild type. A DNA fragment from mutant M2 harboring Tn*5* was amplified by a ligation-mediated PCR technique (35) and cloned in the pUC18 vector (see Materials and Methods). This DNA was used as a probe to screen a DNA genomic library from wild-type *R. solanacearum*, constructed in the pT7T3-18 vector. A positive clone (pPs2) with a 4.1-kb insert was identified, and its restriction map was determined (Fig. 1). The open reading frame (ORF) around the Tn*5* insertion point was sequenced and designated *rfaF*, as will be justified later (Fig. 1). The Tn*5* insertion site of mutant M88 was found within the same ORF, 34 bp downstream from that in mutant M2.

Characterization of a putative *rfaF* gene. The amino acid sequence deduced from the *rfaF* nucleotide sequence has 369 residues and an estimated isoelectric point of 11.8. A search using the TFASTA algorithm (41) against all sequences in the EMBL databank led to the identification of two homologous sequences, RfaF from *E. coli* (48) and RfaF from *Salmonella typhimurium* (54), both of which correspond to a heptosyltransferase that is involved in LPS biosynthesis. An alignment of these sequences shows that RfaF from *R. solanacearum* has 66% similarity with the other two (Fig. 2).

To complement the insertion mutants, plasmid pPs3 was constructed by recloning the 4.1-kb insert from clone pPs2 into the vector pJB313, which was suitable for expression in *Pseudomonas* spp. (1a), and introduced into the mutant bacteria by electroporation; these new strains were designated M2* and M88*.

Changes in LPS. To ascertain the possible role of the *R. solanacearum rfaF* gene in LPS biosynthesis, LPS was purified from the wild type, from mutant strains M2 and M88, and from strains complemented with the *rfaF* gene, M2* and M88*.

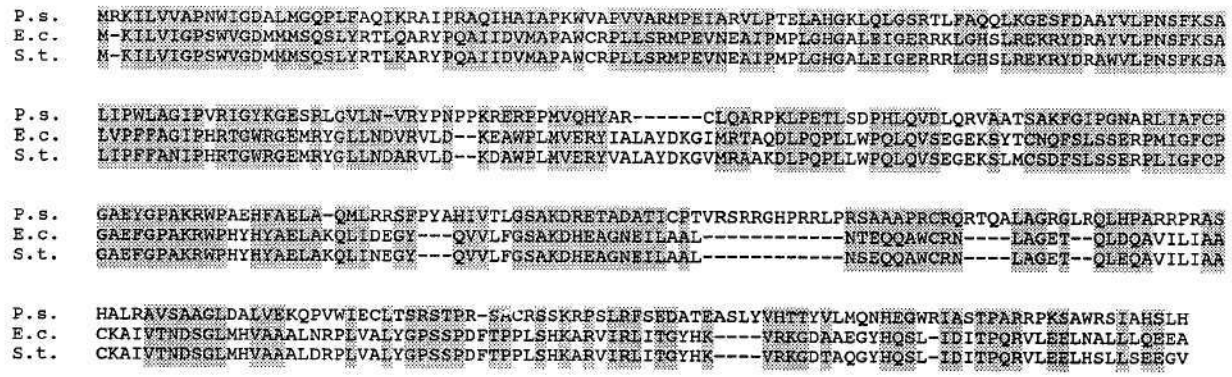

FIG. 2. Alignment of the deduced RfaF amino acid sequence from *R. solanacearum* (P.s.) with those from *E. coli* (E.c.) and *S. typhimurium* (S.t.). Shadowed letters indicate either identical residues or conservative changes. The alignment was performed by using the CLUSTAL IV program (20). GenBank accession numbers for *rfaF* from *E. coli* and *S. typhimurium* are U06472 and X62530, respectively.

Purified LPS was subsequently fractionated by SDS-PAGE (Fig. 3). Electrophoretic bands *a*, *b*, and *c* were present in the wild-type pattern and missing in those of mutants M2 and M88, whereas band *d* was present in the mutant patterns but absent in the wild-type pattern. Complemented strains had all four bands, indicating partial restoration of the wild-type phenotype. This could be due to an insufficient expression level of the complementing gene, to polar effects of the mutations that persisted after complementation, or to interference of the truncated RfaF proteins with the functional proteins expressed from the complementing plasmid. It has been previously proposed that bands *a*, *b*, and *c* represent LPS molecules with different numbers of repeating units in their O side chains (1, 18, 40). Band *d* would then represent incomplete core molecules, to which the O side chains cannot be attached because of the absence of the heptoses. The faint low-mobility bands appearing in all lanes are contaminants, probably due to incomplete protease hydrolysis. Complemented strains M2* and M88* had the rough phenotype, essentially indistinguishable from that of mutants M2 and M88, which indicated that the partial restoration of LPS biosynthesis in these strains, which can be deduced from the LPS electrophoretic patterns, was not sufficient to regain the smooth phenotype of the wild type. To further investigate the functional involvement of the mutated gene in LPS biosynthesis, the heptose content was measured in the wild type and mutant M2. It was found that M2 contained no heptoses, whereas the wild type contained 1.5×10^{-10} micromoles of total heptose per cell. These data, together with the sequence homology relationships, justified its designation as *rfaF*.

Sensitivity of mutants to antimicrobial peptides. A quantitation of thionin sensitivity for wild-type, mutant, and complemented strains of *R. solanacearum* K60 was carried out in liquid medium (Fig. 4). Mutants M2 and M88 were equally sensitive, whereas strain M2* was practically as resistant as the wild type, and strain M88* showed an intermediate level of resistance. These observations indicated that complete restoration of resistance was possible without full recovery of LPS biosynthesis. They also suggested that a slight displacement of the Tn*5* insertion site had a significant effect on either the interference produced by the truncated gene products or the severity of the polar effects in strains M2* and M88*. Mutants M2 and M88 were found to be also sensitive to purified LTP2 from barley and to an extract of cell wall proteins from tobacco leaves that was enriched in LTPs (Fig. 4).

Plant-pathogen interactions. The relevance of gene *rfaF* in plant-pathogen interactions was investigated by inoculation of wild-type, mutant, and complemented strains in tobacco cv. Wisconsin 38, a natural host in which *R. solanacearum* K60 causes necrotic lesions when infiltrated in the underleaf and rapid wilting when injected in the stem. Mutants M2 and M88 died rapidly when infiltrated in the leaves, whereas complemented strains M2* and M88* were able to grow, although more slowly than the wild type (Fig. 5). Wild-type bacteria, as well as strains M2* and M88*, caused necrotic lesions after 7 days when infiltrated in the underleaf, while mutants M2 and M88 produced no necrosis (Fig. 6). Plants that were stem inoculated with the wild-type bacterium totally collapsed after 10 days, whereas no symptoms were observed after inoculation with mutants M2 and M88 (Fig. 7). Symptoms produced by strain M2*, which was fully peptide resistant, were essentially identical to those produced by the wild type, while strain M88*, which showed intermediate resistance, caused wilting of only the upper leaves (Fig. 7).

DISCUSSION

Two lines of evidence support the idea that gene *rfaF* encodes a heptosyltransferase required for normal LPS synthesis, necessary for resistance to thionins in *R. solanacearum*: the low frequency with which thionin-sensitive mutants have been recovered, and the coincidence within the same ORF of the Tn*5* insertions in the two independent mutants (sensitive mutants affecting other genes were not found). These observations suggest that the sensitive phenotype is independent of polar effects; however, a partial association of resistance with other genes polarly affected by the *rfaF* mutations cannot be completely excluded. In *E. coli*, gene *rfaF* belongs to operon 1 of the *rfa* locus (47), which includes genes *rfaD*, *rfaF*, *rfaC*, and *rfaL*, all of which except the last are required for inner core

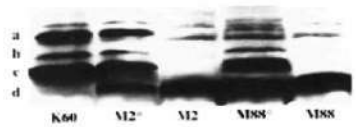

FIG. 3. SDS-PAGE fractionation of LPS from *R. solanacearum* K60, mutants M2 and M88, and complemented strains M2* and M88*. Gels were silver stained. Major bands are designated *a* to *d*.

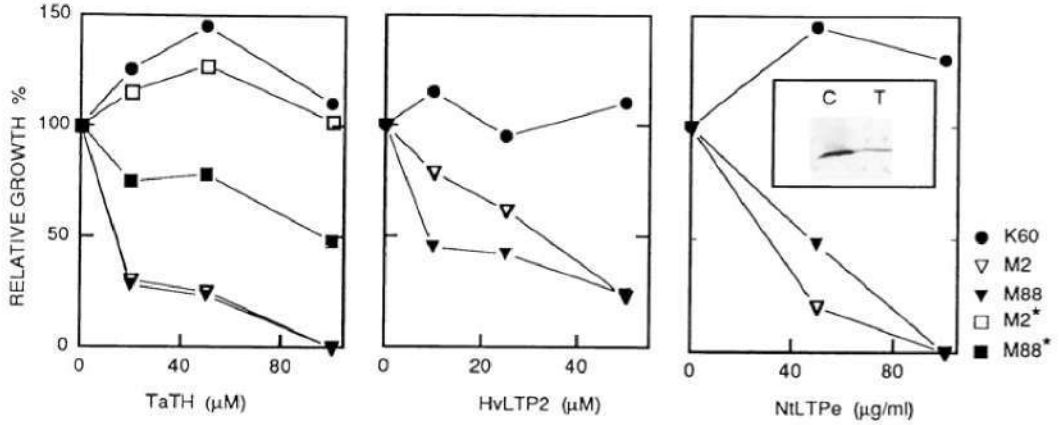

FIG. 4. Sensitivity of *P. solanacearum* K60, mutants M2 and M88, and complemented strains M2* and M88* to wheat thionins (TaTH), barley LTP2 (HvLTP2), and an LTP-enriched extract from tobacco leaves (NtLTPe). Growth was monitored by measuring absorbance at 494 nm and expressed as a percentage of growth in the absence of the inhibitory agent. The inset is a Western blot of NtLTPe (lane T) and LTP1 from *Arabidopsis* (lane C) obtained with an anti-LTP1 antiserum.

synthesis. Whether the organization of this locus is similar in *R. solanacearum* is currently under investigation. It is well established that bacterial LPS is a permeability barrier that confers resistance to antimicrobial agents and that its alteration results in increased sensitivity to hydrophobic and cationic compounds (for a review, see reference 37). Thus, Macías et al. (25) studied mutant strains of *S. typhimurium* with altered LPS and found that their sensitivity to magainin, a cationic antibacterial peptide from insects, increased with the depth of the structural alteration.

That LPS is a protective barrier and not the target of thionin toxic action is indicated by the LTP sensitivity of mutants M2 and M88. Although the mechanisms of toxicity of thionins and LTPs are not well understood, current evidence suggests that these peptides do not have a common target. Thus, thionins are known to alter membrane permeability, to inhibit macromolecular biosynthesis, especially protein synthesis (6, 7), and to form disulfide links with certain other proteins in a selective way (10, 42, 43), while LTPs do not share most of these properties. It has been proposed that positively charged thionins interact with negatively charged phospholipids in the membrane (59), but the involvement of a specific receptor has not been ruled out (39). Heptoses are components of the core region of LPS in *R. solanacearum* (1). These molecules probably are the main sites for insertion of phosphates, as is the case for *Salmonella* (45), and so it would be plausible to speculate that these anionic groups can act as traps for the basic antimicrobial polypeptides.

Results from the plant inoculation experiments were consistent with the proposed defense role of these plant antimicrobial peptides, as the severity of symptoms produced was correlated with *in vitro* sensitivity to these peptides: sensitive mutants M2 and M88 were avirulent, resistant complemented strain M2* was fully virulent (although this strain is less able to grow *in planta*), and partially resistant complemented strain M88* showed milder symptoms. However, more experiments will be necessary to prove a cause-effect relation between increased peptide sensitivity and avirulence. LPS mutants are probably pleiotropic; thus, their avirulent phenotype could be due to sensitivity to other toxic compounds (like phytoalexins) from the plant or to a completely different mechanism. It should be noted that mutants M2 and M88 have growth rates

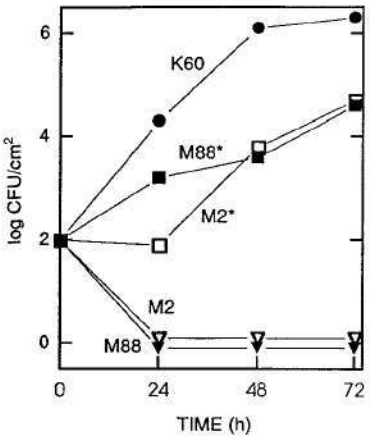

FIG. 5. Growth of *R. solanacearum* K60, mutants M2 and M88, and complemented strains M2* and M88* in 6-week-old leaves of tobacco cv. Wisconsin 38. After inoculation of the leaf, bacterial populations were estimated at different times by excising and grinding 1 cm^2 of tissue and plating appropriate dilutions in agar plates.

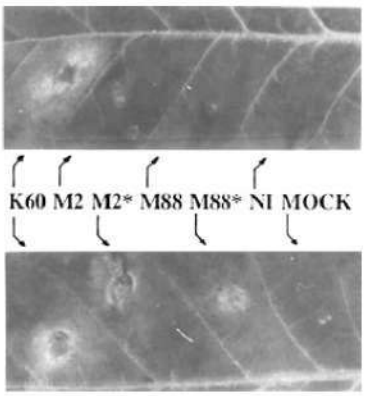

FIG. 6. Leaf symptoms produced by *R. solanacearum* K60, mutants M2 and M88, and complemented strains M2* and M88* on tobacco cv. Wisconsin 38 after infiltration with 20 μl of a bacterial suspension containing 10^5 CFU/ml. Symptoms were recorded after 7 days. NI, not inoculated; MOCK, inoculated with 10 mM Mg_2Cl.

FIG. 7. Wilting symptoms produced by *R. solanacearum* K60, mutants M2 and M88, and complemented strains M2* and M88* on tobacco cv. Wisconsin 38. Symptoms were recorded 10 days after stem inoculation of 50 μl of bacterial suspension containing 10^5 CFU/ml. MOCK, inoculated with 10 mM Mg_2Cl.

in rich media similar to that of the wild type; therefore, the strikingly different behaviour *in planta* between the wild type and mutants cannot be explained by a difference in growth rate.

Mutants M2 and M88 did not elicit higher levels of pathogenesis-related gene expression (data not shown), which was in agreement with the previous observation that LPS-defective mutants of *X. campestris* pv. campestris were weaker inducers of β-1,3-glucanase than the wild type (9, 36).

Although no *rfaF* mutants have been so far found in phytopathogenic bacteria, other LPS-altered mutants with reduced virulence have been reported. For example, the above-mentioned mutants of *X. campestris* pv. campestris characterized by Dow et al. (11) show diminished ability to grow *in planta* and partially reduced pathogenicity. Other mutants of *R. solanacearum* affected in the *ops* cluster, which is involved in the synthesis of both LPS and extracellular polysaccharides, are avirulent in eggplants (8, 21), and the *opsX* mutant of *X. campestris* pv. citrumelo is avirulent in citrus plants but remains virulent in bean (23).

Considerable natural variation of the *in vitro* sensitivity to thionins and LTPs has been observed within given bacterial and fungal species (29). This implies that a successful infection might result not only from the plant failing to recognize a particular pathogenic strain, and therefore failing to activate the defense system (compatible interaction), but also from the ability of a specific strain of pathogen to resist the action of a wide range of plant antimicrobial compounds to which other pathogenic strains are susceptible. Other genes responsible for the above-mentioned natural variation merit future investigation.

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