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A Nonribosomal Peptide Synthetase Gene (*mgo*A) of *Pseudomonas syringae* pv. *syringae* Is Involved in Mangotoxin Biosynthesis and Is Required for Full Virulence

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***Pseudomonas syringae* pv. *syringae*, which causes the bacterial apical necrosis of mango, produces the antimetabolite mangotoxin. We report here the cloning, sequencing, and identity analysis of a chromosomal region of 11.1 kb from strain *P. syringae* pv. *syringae* UMAF0158, which is involved in mangotoxin biosynthesis. This chromosomal region contains six complete open reading frames (ORFs), including a large gene (ORF5) with a modular architecture characteristic of nonribosomal peptide synthetases (NRPS) named *mgo*A. A Tn*5* mutant disrupted in *mgo*A was defective in mangotoxin production, revealing the involvement of the putative NRPS gene in the biosynthesis of mangotoxin. This derivative strain impaired in mangotoxin production also showed a reduction in virulence as measured by necrotic symptoms on tomato leaflets. Mangotoxin production and virulence were restored fully in the NRPS mutant by complementation with plasmid pCG2-6, which contains an 11,103-bp chromosomal region cloned from the wild-type strain *P. syringae* pv. *syringae* UMAF0158 that includes the putative NPRS gene (*mgo*A). The results demonstrate that *mgo*A has a role in the virulence of *P. syringae* pv. *syringae*. The involvement of an NRPS in the production of an antimetabolite toxin from *P. syringae* inhibiting ornithine acetyltransferase activity is proposed.**

Additional keywords: Mangifera indica.

Pseudomonas syringae is an epiphytic plant-pathogenic bacterium that infects a large variety of plants and produces a wide spectrum of phytotoxic compounds (Bender et al. 1999). Among them the best characterized are the lipodepsipeptidic toxins, syringomycins and syringopeptins, and the antimetabolite toxins, phaseolotoxin and tabtoxin (Bender et al. 1999). In general, the phytotoxins produced by *P. syringae* pathovars are non-host specific (Mitchell 1984). Although not essential for pathogenicity, they generally act as virulence factors and, hence, are involved in disease symptom production in many plants (Bender et al. 1999; Durbin 1991; Gross 1991). The genes involved in the production of these toxins mainly are clustered in the bacterial chromosome and, in certain cases, as for lipodepsipeptides, they include nonribosomal peptide synthetase (NRPS) systems (Bender et al. 1999; Scholz-Schroeder et al. 2003).

Antimetabolite toxins are produced by several pathovars of *P. syringae* and include metabolites of diverse chemical structure that are effective at very low concentrations (Mitchell 1991). Two of the better known of these phytotoxins are tabtoxin and phaseolotoxin. Tabtoxin is a monocyclic β-lactam with chlorosis-inducing activity (Mitchell 1991) that irreversibly inhibits glutamine synthetase (Thomas et al. 1983). The biosynthesis of tabtoxin is not completely elucidated. However, a proposed biosynthetic model of tabtoxin formation resembles that of lysine (Bender et al. 1999). Other authors have suggested that tabtoxin biosynthesis branches off from the lysine biosynthetic pathway before the formation of diaminopimelate (Roth et al. 1990; Unkefer et al. 1987). Phaseolotoxin is a sulfodiaminophosphinyl moiety linked to a tripeptide consisting of ornithine, alanine, and homoarginine (Bender et al. 1999), which produces chlorosis by inhibition of ornithine carbamoyl transferase (Patil et al. 1972) and ornithine decarboxylase (Bachmann et al. 1998). The biosynthetic precursors for this toxin have not been identified (Märkisch and Reuter 1990). It has been demonstrated that the homoarginine and ornithine residues of phaseolotoxin are synthesized by a transamidination reaction from arginine and lysine (Hernández-Guzmán and Álvarez-Morales 2001; Zhang and Patil 1997). A nonribosomal thiotemplate mechanism may be required for biosynthesis of phaseolotoxin, but it has yet to be demonstrated (Bender et al. 1999).

Our group reported *P. syringae* pv. *syringae* as the agent for bacterial apical necrosis of mango (*Mangifera indica* L.) (Cazorla et al. 1998). In addition, *P. syringae* pv. *syringae* causes disease in a large variety of plants (Hirano and Upper 1990). *P. syringae* pv. *syringae* strains isolated from mango, as well as those isolated from other plants hosts such as tomato, have been demonstrated to produce a novel antimetabolite toxin named mangotoxin in addition to lipodepsipeptides (Arrebola et al. 2003; Cazorla et al. 2003). Mangotoxin inhibits ornithine acetyl transferase, a key enzyme in the biosynthetic pathway of ornithine and arginine. Although the molecular structure of mangotoxin remains unknown, a preliminary biochemical characterization showed that the toxin has features of a small oligopeptide, resembling those of other antimetabolite toxins (Arrebola et al. 2003).

Syringomycins are a representative of the cyclic lipodepsinonapeptide class of *P. syringae* phytotoxins, which are composed of a polar peptide head and a hydrophobic 3-hydroxy

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Nucleotide sequence data reported are available in the GenBank database under accession numbers DQ532433 to DQ532440 for the interrupted genes in the eight mutants described, DQ532441 for the genomic sequence in pCG2-6, and DQ532442 for the *mgo*A gene (ORF5).

fatty acid tail (Segre et al. 1989). This family of toxins induces necrosis in plant tissues, and early studies of its mode of action established that the plasma membrane of host cells is the primary target (Backman and DeVay 1971). These toxins are synthesized via an NRPS (Grgurina and Benincasa 1994; Lu et al. 2002; Raaijmakers et al. 2006; Scholz-Schroeder et al. 2001), which catalyzes the activation and addition of amino acids into the peptide chain (Konz and Marahiel 1999; Marahiel et al. 1997).

NRPS consists of an arrangement of modules with at least three different types of domains: the adenylation domain (A) that activates an amino acid monomer; a thiolation domain (T), also named peptidyl carrier protein (PCP), that tethers the growing peptide; and a condensation domain (C) that catalyses the formation of the peptidic bound between the activated amino acid and the growing peptide (Finking and Marahiel 2004). In addition to the basic subset of core domains, each NRPS could include a domain responsible for the initiation of chain assembly and a chain termination or reductase (R) domain (Finking and Marahiel 2004; Marahiel 1997; Schwarzer et al. 2003). Thus, NRPS systems are used simultaneously as templates, because the amino acid to be incorporated is determined by the module, and as biosynthetic machinery, because the module harbors all necessary catalytic functions (Finking and Marahiel 2004). In bacteria, NRPS modules usually are distributed over several genes organized in an operon. However, in some cases, an NRPS with only one single module is able to produce small active oligopeptides (Finking and Marahiel 2004). The syringopeptin synthetase C gene (*syp*C), involved in syringomycin biosynthesis currently is the largest bacterial NRPS known, comprising a total of 12 modules (Scholz-Schroeder et al. 2003). In the genus *Pseudomonas*, many bacterial peptides, including secondary metabolites, are synthesized by NRPS systems (Konz and Marahiel 1999).

The wild-type strain *P. syringae* pv. *syringae* UMAF0158 produces the antimetabolite mangotoxin. In this work, we describe the cloning and DNA sequencing of a putative NRPS gene, which we named *mgoA*, present in the chromosome of this strain, and report on the role of *mgoA* in mangotoxin biosynthesis and virulence of *P. syringae* pv. *syringae*.

RESULTS

Isolation of mangotoxin defective mutants of *P. syringae* pv. *syringae* UMAF0158.

To detect putative genes involved in mangotoxin production, a mini Tn*5* mutagenesis was carried out. Derivative mutants from strain *P. syringae* pv. *syringae* UMAF0158 were obtained by conjugation experiments using as donor the *Escherichia coli* strain S17λpir containing the miniTn*5*km2 and *P. syringae* pv. *syringae* UMAF0158 as a recipient. From a collection of 2,592 exconjungants, 18 derivative strains defective in mangotoxin production were isolated by the *E. coli* growth inhibition assay, and 8 of them were selected based on their lipodepsipeptidic toxin production and growth on minimal medium for more detailed analysis (Table 1). Five mutants displayed an increased production of lipodepsipeptidic toxin (Table 2). Also, four derivative *P. syringae* pv. *syringae* strains displayed altered growth on minimal medium, showing lower bacterial counts after the fourth day of growth when compared with the wild-type strain *P. syringae* pv. *syringae* UMAF0158 (Table 2). Only three derivative strains impaired in mangotoxin production (*P. syringae* pv. *syringae* UMAF0158-3γH1, -6γF6, and -5αC5) displayed growth characteristics and production of lipodepsipeptidic toxins similar to the wild-type strain (Table 2).

Sequencing of the flanking DNA of the miniTn*5*km2 insertions showed that five of the derivative mutants were located in DNA with a high similarity to the global regulatory genes *gac*A and *gac*S, or to genes encoding hypothetical proteins of *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 (Table 2). The insertion on the mutants *P. syringae* pv. *syringae* UMAF0158-4βA2 and *P. syringae* pv. *syringae* UMAF0158-5αC5 was located in regions that did not show identity with sequenced genomes from *P. syringae* strains, but they showed partial identity with a protein involved in the outer membrane biosynthesis of *Burkholderia dolosa* AUO158 and a carboxylase of *B. pseudomallei* 668, respectively. Finally, in mutant *P. syringae* pv. *syringae* UMAF0158-6γF6, the insertion was located in DNA with high similarity to an NRPS gene from *P. syringae* pv. *syringae* B728a (Psyr 5011, 95%), *P. syringae* pv. *tomato* DC3000

Table 1. Bacterial strains and vectors used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Pseudomonas syringae pv. *syringae*		
UMAF0158	Wild type isolated from mango, Nf^r	Cazorla et al. 1998
UMAF0158-2βB7, UMAF0158-2βH4 UMAF0158-3αE10, UMAF0158-3γH1 UMAF0158-4βA2, UMAF0158-5αC5 UMAF0158-6γD7, UMAF0158-6γF6	miniTn*5*km2 mutants of UMAF0158, defective in mangotoxin production, Km^r, Nf^r	This study
UMAF0158-4βE6	miniTn*5*km2 mutant, nondefective in mangotoxin production, Km^r, Nf^r	This study
UMAF2-6A, UMAF2-6D, UMAF2-6E	UMAF0158-6γF6 complemented strains, containing plasmid pCG2-6, production of mangotoxin restored, Km^r, Amp^r, Nf^r	This study
Escherichia coli		
CECT 831	Indicator strain in the bioassay for antimetabolite toxins production.	CECT[b]
S17λ*pir*	*rec*A, *thi*, *pro*, *hsd*R$^-$M$^+$, RP4:2-Tc:Mu:Km Tn*7*, λ*pir*, Tp^r, Sm^r	de Lorenzo and Timmis 1994
DH5α	*rec*A *lac*ZΔM15	Hanahan 1985
		GIBCO-BRL, Paisley, U.K.
ER1647	F$^-$ *fhu*A2 Δ(*lac*Z)r1, *sup*E44, *hsd*S ($r_{k12}^-m_{k12}^-$), Sm^r, Tet^r	Novagen, Darmstadt, Germany
BM25.8	*sup*E *thi* Δ (lac-proAB), *hsd*R ($r_{k12}^-m_{k12}^+$), Cm^r, Km^r	Novagen, Darmstadt, Germany
Plasmids		
pUT(miniTn*5*Km2)	*mob*RP4, delivery vector for miniTn*5*Km2, Km^r	de Lorenzo and Timmis 1994
pBluescript KS	Cloning vector, Amp^r	Stratagene, La Jolla, CA, U.S.A.
pBlueSTAR-1	Derived from λBlueSTAR-1 vector by autosubcloning	Novagen, Darmstadt, Germany
pCG2-6	UMAF0158 genomic DNA cloned in pBluesSTAR-1, which complements mangotoxin production of derivative strain *P. syringae* pv. *syringae* UMAF0158-6γF6	This study

[a] Km^r, kanamycin resistance; Amp^r, ampicillin resistance; Nf^r, nitrofurantoin resistance; Sm^r, streptomycin resistance; Tet^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Tp^r, thiamphenicol resistance.
[b] CECT: Spanish Type Culture Collection.

(PSPTO 5457, 88%), and *P. syringae* pv. *phaseolicola* 1448A (PSPPH 5090, 88%) (Table 2).

Mutant *P. syringae* pv. *syringae* UMAF0158-6γF6 was selected for further experiments because it produced levels of lipodepsipeptidic toxins and showed growth characteristics similar to those of the wild-type strain *P. syringae* pv. *syringae* UMAF0158, and because of the high similarity of the disrupted gene to those encoding for NRPS involved in toxin production in other microorganisms.

Analysis of a chromosomal region encoding for an NRPS.

In order to search for the gene or genes involved in mangotoxin production, a phage library from *P. syringae* pv. *syringae* UMAF0158 DNA was constructed and screened using as a probe the right flank of the disrupted gene in the defective mutant *P. syringae* pv. *syringae* UMAF0158-6γF6. Of 3,000 plaques screened, eight phagemids hybridized to the probe and, hence, were selected for further analysis. Several plasmids with different sizes were isolated; then, the biggest in size (13.2 kb) of them (pCG2-6, Table 1) was selected and electroporated into the mangotoxin-defective mutant UMAF0158-6γF6, to confirm the role of this genetic trait in mangotoxin production.

Three different complemented *P. syringae* pv. *syringae* strains (UMAF2-6A, UMAF2-6D, and UMAF2-6E) obtained from three independent experiments of complementation had restored mangotoxin production, which was demonstrated by both ornithine acetyltransferase (OAT) activity assay and *E. coli* growth inhibition bioassay (Table 3), in comparison with the wild-type strain and the mangotoxin-defective mutant *P. syringae* pv. *syringae* UMAF0158-6γF6 (Table 3).

By Southern blot analysis on the complemented strains, only one hybridization signal of the DNA fragment from *P. syringae* pv. *syringae* UMAF0158 cloned in pCG2-6 was associated with chromosomal but not to plasmid DNA, revealing integration of the plasmid sequence in the chromosome of the derivative strain. Sequence analysis of the plasmid pCG2-6, which restored mangotoxin production in the derivative strain UMAF0158-6γF6, showed a DNA insert of 11,103 bp. Six complete open reading frames (ORFs) were defined within the 11.1-kb fragment (Fig. 1), designated as ORF1 to ORF6, followed by genes encoding from the rRNA 5S and 23S. Also, an incomplete ORF (ORF –1, 472 bp) was observed at the beginning of the DNA fragment (Fig. 1). Analysis of deduced amino acid sequences of these ORFs indicated that all ORFs had a high similarity to genes sequenced in strains *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *tomato* DC3000, and *P. syringae* pv. *phaseolicola* 1448A, and were arranged in the same order (Fig. 1). The analysis of predicted sites for putative promoters resulted in the presence of three sites, upstream of ORF2 (P = 0.87), ORF3 (P = 0.95), and 5S rDNA (P = 0.87) (Fig. 1). Similar predicted sites for putative promoters were found in the homologous sequence of *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000, allocated upstream of the correspondent genes homologous to ORF2, ORF3, and 5S rDNA.

The miniTn*5*km2 insertion in the mutant *P. syringae* pv. *syringae* UMAF0158-6γF6 was located in the nucleotide 3,014 of the ORF5 sequence (3,447 bp). This ORF5 was named gene *mgo*A. A detailed analysis of the 1,148 amino acids from the deduced product of the *mgo*A gene in comparison with other peptide synthetases determined that it contains an amino acid activation module (Marahiel et al. 1997) composed of conserved motifs for aminoacyl adenylation, a peptidyl carrier protein region with a thiolation domain and a condensation domain (Stein and Vater 1996) (Fig. 2). The aminoacyl adenylation domains encompass 451 amino acids, containing nine conserved core

Table 3. Inhibition of *Escherichia coli* growth and specific inhibition of ornithine N-acetyl-transferase (OAT) activity by cell-free culture filtrates of mangotoxin-producing and nonproducing *Pseudomonas syringae* pv. *syringae* strains in liquid minimal medium (PMS)

Culture filtrates of:	Diameter of inhibition zone (mm)	OAT activity (nkat/gfw)[a]
PMS[b]	Null	6.75 ± 1.36
PCMB[c]	…	1.21 ± 0.81
P. syringae pv. *syringae*		
UMAF0158	22 ± 1.8	1.14 ± 0.98
UMAF0158-6γF6	Null	5.57 ± 0.97
UMAF0158-2βH4	Null	5.80 ± 1.08
UMAF2-6A	21 ± 1.3	1.45 ± 1.08
UMAF2-6D	21 ± 1.7	1.45 ± 0.95
UMAF2-6E	22 ± 1.6	1.14 ± 0.77
UMAF0158-4βE6	20 ± 0.5	1.69 ± 1.15

[a] Average and standard deviation values obtained from five independent replicated experiments; nkat/gfw = nanokatals/g of fresh weight.
[b] Negative inhibition control
[c] PCMB = p-chloromercuribenzoic acid; specific inhibitor of OAT activity.

Table 2. Characterization of eight transposon mutants, derived from the wild-type strain of *Pseudomonas syringae* pv. *syringae* UMAF0158, impaired in mangotoxin production, and analysis of the flanking regions of the mini*Tn*5Km2 insertion by comparison with the sequenced genome of *P. syringae* pv. *syringae* B728a

Strains[b]	Mangotoxin[c]	LDP toxins[d]	PMS counts[a] 3 days	PMS counts[a] 5 days	Putative function[e]	Identity (%)[f]	Accession no.
UMAF0158 (WT)	+	+	8.0 ± 0.5	8.4 ± 0.2	…	…	…
UMAF0158-2βB7	–	++	8.5 ± 0.1	5.3 ± 1.4	GacA	94	DQ532433
UMAF0158-2βH4	–	++	8.6 ± 0.0	5.4 ± 0.1	GacS	93	DQ532434
UMAF0158-3αE10	–	++	8.5 ± 0.0	8.2 ± 0.2	GacS	93	DQ532435
UMAF0158-3γH1	–	+	8.5 ± 0.2	8.2 ± 0.3	Hypothetical protein	97	DQ532436
UMAF0158-4βA2	–	++	8.5 ± 0.3	4.0 ± 1.0	OM biosynthesis[g]	…	DQ532437
UMAF0158-5αC5	(–)	+	8.4 ± 0.0	8.6 ± 0.1	Carboxylase[h]	…	DQ532438
UMAF0158-6γD7	–	++	7.2 ± 1.6	4.0 ± 1.4	GacS	96	DQ532439
UMAF0158-6γF6	–	+	8.6 ± 0.1	8.3 ± 0.5	Nonribosomal peptide synthetase	95	DQ532440

[a] Bacterial counts ($\log_{10}$ CFU/ml) on Pseudomonas minimal medium (PMS) broth at 22ºC after 3 and 5 days.
[b] Derivative strains of *P. syringae* pv. *syringae* UMAF0158; WT = wild type.
[c] Diameter of inhibition halo: + = 15 to 20 mm; – = absent; and (–) = slight production, lower than 8 mm.
[d] Lipodepsipeptidic (LDP) toxin production: + = diameter of inhibition halo ranging between 9 and 11 mm, ++ = diameter of inhibition halo ranging between 21 and 34 mm.
[e] Putative function of the disrupted gene.
[f] Identity at nucleotide level (%) in respect to the sequenced genome of *P. syringae* pv. *syringae* B728a.
[g] No identity was observed with *P. syringae* pv. *syringae* B728a, but it showed identity (34% at amino acid level) with a protein involved in the outer membrane biosynthesis of *Burkholderia dolosa* AUO158.
[h] No identity was observed with *P. syringae* pv. *syringae* B728a, but it showed identity (43% at amino acid level) with a carboxylase of *B. pseudomallei* 668.

sequences (Fig. 2). Some of these conserved sequences include the conserved motif YTSGTTGxPKG (A3), the highly conserved YGPTE motif (A5), and the YRTGD motif (A7) (Fig. 2). The aminoacyl adenylation domains are followed by the thiolation domain of the PCP region (Fig. 2), which contains the conserved thioester binding motif LGGHS(T). The condensation domain (C) followed the thiolation domain, with QDY as the unique condensation core present on ORF5 protein, and it is highly conserved in the *P. syringae* strains analyzed (Fig. 2). The analysis of the substrate-binding region of the module by the NRPS predictor software showed that the residues around the substrate were FWYSFDSSIIDPFLMTGGDHNLYGPTEA TVLVTA, and they have a prediction of valine, leucine, isoleucine, 2-amino butiric acid, and isovaleric acid-like as the substrate amino acids (score 2.24560058905).

As expected, a carboxy-terminal reductase I domain of 189 amino acid residues was observed at the C-terminal end of the deduced protein. This region contains three core sequences, including a binding domain to NADP(H) (R1) and the highly conserved RPG (R6) domain (Fig. 2). The different domains and motifs present in the putative NRPS encoded in *mgo*A showed a high level of identity with those present in other bacteria (Fig. 2). Only minimal differences were observed among adenylation, condensation, and reductase domains present in different *Pseudomonas* spp.

Symptom development and bacterial multiplication on tomato leaflets.

To explore the involvement of the putative NRPS encoded by *mgo*A (ORF5) in the virulence of *P. syringae* pv. *syringae* UMAF0158, we examined the development of disease symptoms and in planta multiplication of three bacterial strains (*P. syringae* pv. *syringae* UMAF0158-6γF6, the mangotoxin-defective mutant which contains a transposon insertion in ORF5; the mangotoxin-producing strain *P. syringae* pv. *syringae* UMAF0158; and the complemented strain *P. syringae* pv. *syringae* UMAF2-6A), using detached tomato leaflets. Leaflets were inoculated with a high bacterial dose by placing six 10-µl droplets of a bacterial suspension (approximately 10^8 CFU/ml) on the principal lateral veins and piercing through them with an entomological pin. Analysis of bacterial growth in planta over 10 days revealed no significant differences between the strains assayed (Fig. 3).

The necrotic lesions produced on tomato leaflets by the inoculated strains were evaluated daily (Fig. 4). The strain *P. syringae* pv. *syringae* UMAF2-6A produced disease symptom levels similar to those observed in the wild-type strain *P. syringae* pv. *syringae* UMAF0158, but strain *P. syringae* pv. *syringae* UMAF0158-6γF6 caused clearly reduced symptoms when they were evaluated as the number of inoculation points where necrotic lesions were developed (Fig. 4). The mangotoxin-producing strains, wild-type and complemented, produced severe disease symptoms (necrotic areas with a diameter larger than 2 mm) in more than 70% of the inoculation points within 6 days after inoculation, increasing every day, reaching more than 90% at the end of the assay (10 days); whereas the defective mutant *P. syringae* pv. *syringae* UMAF0158-6γF6 produced significant lesions in more than 50% of the inoculation points only after 10 days (Fig. 4A), and, in general, less severe symptoms were observed (Fig. 4B).

The virulence also was evaluated as a function of bacterial dose size (Table 4). The median effective dose that caused necrotic lesions in 50% of inoculations (ED_{50}) was estimated for the different strains from the straight line produced by adjusting the bacterial-inoculated dose (log_{10}-transformed) against the response data on a Weibull scale obtained from two independent assays. The ED_{50} of the mangotoxin-defective mutant UMAF0158-6γF6 ($ED_{50} = 5.3 \times 10^7$ CFU) was more than one order of magnitude higher than those of the mangotoxin producer strains UMAF0158 (1.8×10^6 CFU) and UMAF2-6A (8.0×10^5 CFU) and the derivative strain UMAF0158-4βE6 (1.9×10^6 CFU), which contains a miniTn*5*km2 insertion that did not alter mangotoxin production (Table 4).

DISCUSSION

The cloning and DNA sequence of a putative NRPS gene present in the chromosome of the mangotoxin-producing strain

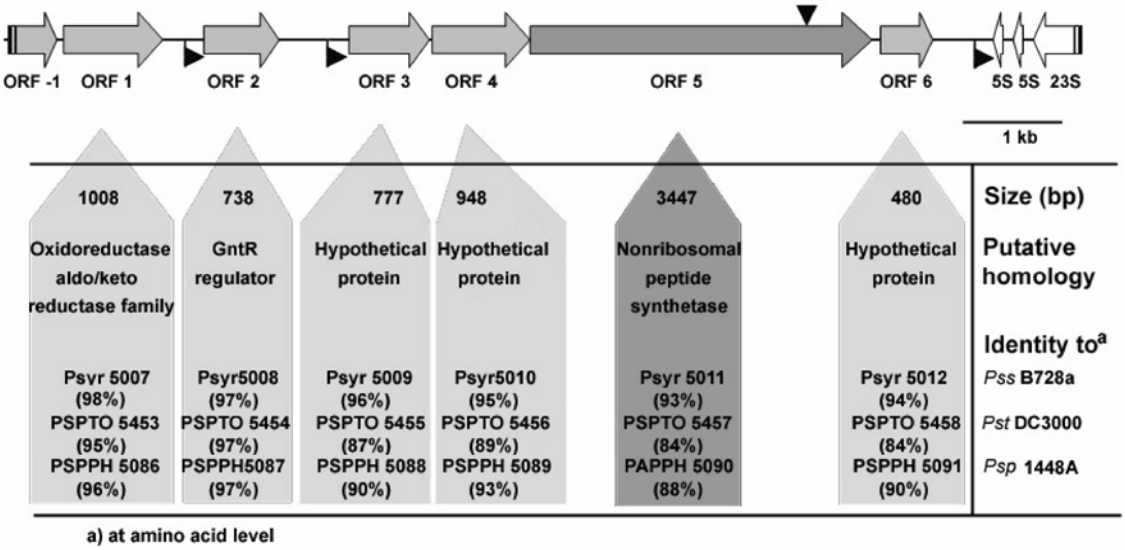
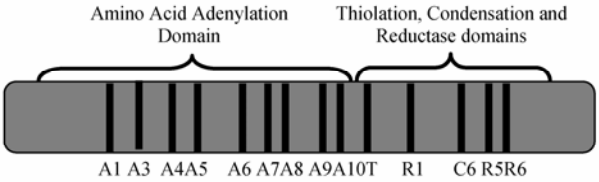


Fig. 1. Arrangement of the open reading frames (ORFs) present in the chromosomal DNA fragment of 11,103 bp cloned into the plasmid pCG2-6. This plasmid was obtained from a phage library of the wild-type strain *Pseudomonas syringae* pv. *syringae* UMAF0158 and restores the mangotoxin production of the derivative mutant UMAF0158-6γF6. The line represents the sequenced genomic insert of *P. syringae* pv. *syringae* UMAF0158. Putative ORFs are indicated by thick arrows on the line. The insertion of the mini Tn*5*km2 in the derivative mutant UMAF0158-6γF6, located in ORF5 is shown in the figure (▼). Predicted sites for putative promoters were analyzed by the BPROM and NNPP 2.2. software, and are also marked (►). Accession number of sequence from DNA fragment cloned into pCG2-6 is DQ532441. Size and putative homology of the different ORFs and identity to homologous genes sequenced in *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *tomato* (*Pst*) DC3000, and *P. syringae* pv. *phaseolicola* (*Psp*) 1448A are indicated.

P. syringae pv. *syringae* UMAF0158 have been performed, and the role of this NRPS gene in mangotoxin biosynthesis and virulence has reported.

Analysis of the disrupted genes in eight derivative strains impaired in mangotoxin production showed that, as observed with other compounds secreted by *Pseudomonas* spp. (Heeb and Hass 2001), the global regulator system *gac*A/*gac*S is involved in the production of mangotoxin (Table 2). In this sense, it has been described recently that the *sal*A gene, a member of the GacS/GacA system, is required for bacterial



Strains	% Identities	Core	Sequence	Core	Sequence	Core	Sequence
	consensus	A1	L(T/S)YxEL	A3	LAYxxYTSG(S/T)TGxPKG	A4	FDxS
UMAF0158	--		L S YRGL		PCMALYTSG T TGQPKG		FDSS
B728a	93		L S YRGL		PCMALYTSG T TGQPKG		FDSS
DC3000	86		F S YGEL		PCMALYTSG T TGQPKG		FDSS
PfO-1	68		I S YGEL		PCMALYTSG T TGHPKG		FDSS
ATCC33913	32		--		TAYVLYTSG S TGVPKG		FDIA
USDA110	36		M A ADAQ		LAYVIYTSG S TGRPKG		FDVS
CFT073	28		L T YAEL		QAYLLFTSG S TGEPKG		FDIS
MA4680	28		N P DLYD		LAYVLHTSG S TGRPKG		FDTS
ACTT14579	26		L T YEEL		VAYMIYTSG S TGNPKG		FDAS
		A5	NxYGPTE	A6	GELxIxGxG(V/L)ARGYL	A7	Y(R/K)TGDL
			NLYGPTE		GELYIVGPG V CLGYV		Y R TGDI
			NLYGPTE		GELYIVGPG V CLGYV		Y R TGDI
			NLYGPTE		GELYIVGPG V CLGYI		Y R TGDI
			NLYGPTE		GELFIVGPG V CLGYL		Y R SGDM
			QLYGPTE		GELYIGGAG V AKGYR		Y R TGDR
			NLYGPTE		GELCIGGAG V ARGYL		Y R TGDL
			NLYGPTE		GELYIAGDG V AQGYD		Y R TGDI
			NTYGPTE		GELHLLGDN L AIGYR		Y R TGDL
			NVYGPTE		GELYIDSVG L AKGYF		Y K TGDI
		A8	GRxDxQVKIRGxRIELGEIE	A9	LPxYM(I/V)P	A10	NGK(V/L)DR
			GRRDNQVKIRGFRVEPEEIE		LPDYM H P		NGK V DR
			GRRDNQVKIRGFRVEPEEIE		LPDYM H P		NGK V DR
			GRRDNQVKIRGFRVEPEEIE		LPDYM H P		NGK V DR
			GRRDNQVKIRGFRVEPEEIE		LPDYM Q P		NGK V DR
			GRADGQLKIRGHRVEPAEIE		LPASM I P		NGK L DR
			GRNDDQVKIRGFRIEPGEIE		LPDYM V P		NGK L DR
			GRKDSQIKLRGYRIELGEIE		LPDYM V P		NGK I DR
			GRSDTEFKISGHRVHPAEVE		LPAAM I P		SGK I DR
			HRKDDQVKIRGHRIELGEIQ		LPDYM V P		NKK I DR
		T	DxFFxxLGG(H/D)S(L/I)	C	(H/N)QD(Y/V)PFE	R1	V(L/F)(L/V)TG(A/V)(T/N)G(Y/F)LG
			MKLFN-LGG H S I		V QD Y PFE		V I V TG A N G F LG
			DSFFN-LGG H S I		V QD Y ESL		V I V TG A N G F LG
			ESFFN-LGG H S I		V QD Y ETL		V I V TG A N G F LG
			ESFFN-LGG H S I		V LD Y ESL		V I V TG A N S F VG
			DNFFE-LGG D S L		np		np
			DNFFE-LGG H S L		R GL V NLL		G L S PL Q D G I LF
			MKLFN-LGG H S L		np		np
			DDVFD-LGG H S L		M RE Y ATL		I L L TG A T G F VG
			DDFFT-IGG H S L		F GD V KQF		V F L TG A T G Y LG
		R5	GYxxSKWxxE	R6	RPG		--
			GYNLSKWVAE		RPG		--
			GYNLSKWVAE		RPG		--
			GYNLSKWVAE		RPG		--
			GYNLSKWVGE		RPG		--
			np		np		--
			SKDPERLAAQ		RTG		--
			np		np		--
			GYQQSKWAAE		RLG		--
			VYSNSKLQAE		RAG		--

Fig. 2. Diagrammatic representation of *mgo*A (open reading frame [ORF]5), displaying the amino acid activation module illustrated by the aminoacyl adenylation, thiolation, and condensation domains characteristic of a nonribosomal peptide synthetase (NRPS), and showing a comparison of conserved sequences in adenylation (A) condensation (C) thiolation (T), and reductase (R) domains (Marahiel et al. 1997) of different NRPS from several microorganisms. Conserved amino acids are indicated with bold letters; lack of the domain is denoted as np. Accession number for ORF 5 gene is DQ532442. % Identity = percentage of deduced protein from ORF5 of *Pseudomonas syringae* pv. *syringae* UMAF0158 with the NRPS gene from other bacteria; *P. syringae* pv. *syringae* B728a (Psyr 5011), *P. syringae* pv. *tomato* DC3000 (PSPTO 5457), *P. fluorescens* PfO-1 (Pflu 3964), *Xanthomonas campestris* pv. *campestris* ATCC33913 (XCC3867), *Bradyrhizobium japonicum* USDA 110 (Blr 2108), *Escherichia coli* CFT073 (C2459), *Streptomyces avermitilis* MA 4680 (SAV 603), and *Bacillus cereus* ATCC14579 (BC0423). Consensus sequences are outlined with boxes.

virulence, syringomycin and syringopeptin production, and putative NRPS expression in *P. syringae* pv. *syringae* B301D (Lu et al. 2005; Raaijmakers et al. 2006; Wang et al. 2006). It is remarkable to note the overproduction of lipodepsipeptides by several defective mutants in mangotoxin production, mainly affected in *gac*A/*gac*S genes. This fact could suggest a possible coordinated regulation of the production of both toxins, which present different production patterns, so that syringomycins are produced preferentially in rich media, in contrast with mangotoxin and other antimetabolite toxins, which are produced in minimal medium.

The mutant *P. syringae* pv. *syringae* UMAF0158-6γF6 was selected for further studies due to the similarity of the gene disrupted by the miniTn*5*km2 insertion with an NRPS, a key enzyme involved in the production of different antibiotics and phytotoxins (Finking and Marahiel 2004). To study the role of the disrupted NRPS gene, a chromosomal fragment of 11.1 kb of the wild-type *P. syringae* pv. *syringae* UMAF0158, cloned into the plasmid pCG2-6, was recovered; which restored the mangotoxin production in the defective mutant (Table 3). The sequence of the 11.1-kb DNA insert revealed the presence of six complete ORFs and genes encoding rRNA (Fig. 1). The ORFs are located close to each other on the same DNA strand and ORFs 3 to 6 could constitute an operon because they are located between two putative promoters. The disrupted gene in *P. syringae* pv. *syringae* UMAF0158-6γF6 corresponded to ORF5, with the insertion of the miniTn*5*Km2 at nucleotide 3,014 of this ORF (Fig. 1). This gene was named *mgo*A, and showed a very high identity to an NRPS, the system involved in synthesis of antibiotic peptides via the nonribosomal thiotemplate mechanism of biosynthesis (Finking and Marahiel

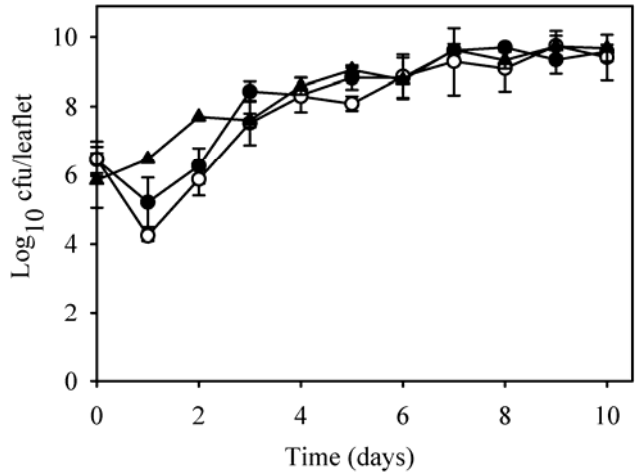


Fig. 3. Time course of bacterial growth in tomato leaflets maintained in vitro and inoculated with mangotoxin-producing and nonproducing strains: the wild-type strain *Pseudomonas syringae* pv. *syringae* UMAF0158 (●); *P. syringae* pv. *syringae* UMAF0158-6γF6, a mini Tn*5*km2 mutant defective in mangotoxin production (○); and *P. syringae* pv. *syringae* UMAF2-6A, a derivative strain from *P. syringae* pv. *syringae* UMAF0158-6γF6 harboring pCG2-6 and showing mangotoxin production restored (▲). Data points are the means of four experiments, and error bars indicate the standard deviations.

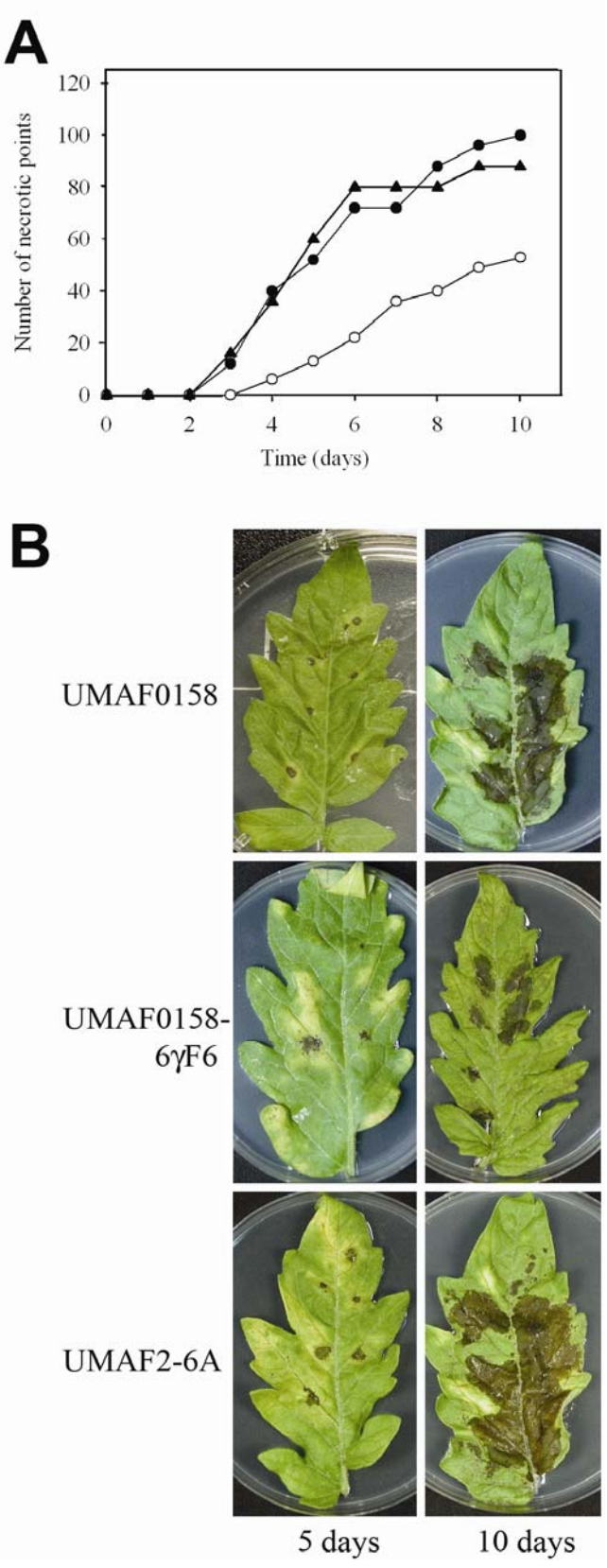


Fig. 4. Symptom development on tomato leaflets inoculated with mangotoxin-producing and nonproducing *Pseudomonas syringae* pv. *syringae* strains. The assayed strains were the wild-type *P. syringae* pv. *syringae* UMAF0158 (●); *P. syringae* pv. *syringae* UMAF0158-6γF6, a mini Tn*5*km2 mutant defective in mangotoxin production (○); and *P. syringae* pv. *syringae* UMAF2-6A, a derived strain from *P. syringae* pv. *syringae* UMAF0158-6γF6 by complementation with the plasmid pCG2-6 and showing mangotoxin production restored (▲). **A,** Development of the necrotic symptoms was followed during 10 days after inoculation and the cumulative number of inoculation points showing a surrounding necrotic area larger than 2 mm in diameter was recorded every day after inoculation from a total of 120 inoculated points with every strain (30 inoculation points × 4 experiments). **B,** Representative symptoms on tomato leaflets at 5 and 10 days after inoculation.

2004). Several NRPS gene clusters have been described for biosynthesis of syringomycin, syringopeptin, or syringolin by *P. syringae* strains (Amrein et al. 2004; Guenzi et al. 1998; Scholz-Schroeder et al. 2001, 2003).

Analysis of the predicted amino acid sequence from MgoA indicated that it contains only one amino acid activation module typical of a functional NRPS, and it is composed of 14 conserved domains, including aminoacyl adenylation, condensation, thiolation, and additional reduction domains, with a remarkable identity level to other bacterial NRPS, proportional to the phylogenetic distance between them (Fig. 2). The aminoacyl adenylation domains, found at the N-terminal end, seem to be responsible for recognizing and adenylating an amino acid substrate via ATP hydrolysis (Challis et al. 2000; Marahiel et al. 1997). The unstable adenylate then is covalently bound to the thiolation domain of the PCP region, located at the C-terminal portion of the amino acid activation module, in a thioester linkage via a 4′-phosphopantheine cofactor to the conserved serine located within the conserved thioester-binding motif LGGHS(T) of this domain (Conti et al. 1997; Fernández-Moreno et al. 1992, Gocht and Marahiel 1994; Schlumbohm et al. 1991; Stein and Vater 1996). In this thiolation domain, the most relevant differences in the amino acid sequence between the putative NRPS deduced from ORF5 of *P. syringae* pv. *syringae* UMAF0158 and its homologous putative NRPS from *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 were observed; therefore, the initial four amino acids of this domain of *P. syringae* pv. *syringae* UMAF0158 were different from those *P. syringae* strains and similar to those of *E. coli* CFT073 (Fig. 2). The condensation domains are conventionally fused to the amino terminal end of modules accepting acyl groups from the preceding module, and they are absent in modules activating the first acyl constituent to be incorporated (Konz and Marahiel 1999) although, in the predicted MgoA protein, it was located around the middle of the protein. The condensation domain recently was demonstrated to be necessary for peptide bond formation (De Crécy-Lagard et al. 1995; Stachelhaus et al. 1998). Three potential reductase domains also have been identified at the carboxy terminal end of the predicted ORF5 protein, containing an NADP(H)-binding site R1, characteristic of a diverse group of reductive enzymes (Pospiech et al. 1996; Suvarna et al. 1998). The R domains terminate many NRPS genes, and they are proposed to cleave the covalent protein-small molecule thioester bonds to release aldehides, which undergo a variety of metabolic fates (Dworkin 1996).

We suggest that an NRPS system encoded in the *mgo*A gene and described in this article could be involved in the biosynthesis of mangotoxin by introducing only one predicted amino acid (Ile, Leu, Val, Abu, or Iva) to a previous amino acid or oligopeptide, in a way similar to that described for other compounds (Amrein et al. 2004; Byford et al. 1997; Rausch et a. 2005). Mangotoxin structure remains unknown; however, preliminary characterization showed that it is a very small molecule with chromatographical and biochemical characteristics similar to other well-known antimetabolite toxins produced by *P. syringae*, such as tabtoxin and phaseolotoxin, which are di- and tripeptide (Arrebola et al. 2003). Therefore, the precursor molecule for mangotoxin would be an individual amino acid or a short oligopeptide. Similarly, NRPS systems has been described in *Pseudomonas* spp. and involved in the biosynthesis of small oligopeptides with antimicrobial properties, such as syringolin (Amrein et al. 2004) and ACV-oligopeptide (Byford et al. 1997). The deduced NRPS from *mgo*A of *P. syringae* pv. *syringae* UMAF0158 showed a high identity to different NRPS genes (Fig. 2), especially from *Pseudomonas* spp. and, in particular, from *P. syringae* strains, such as *P. syringae* pv. *syringae* B728a (Psyr 5011), *P. syringae* pv. *tomato* DC3000 (PSPTO 5457), and *P. syringae* pv. *phaseolicola* 1448A (PSPPH 5090). However, in spite of the fact that *P. syringae* pv. *syringae* B728a has a putative NRPS gene (Psyr5011) embedded in a cluster highly similar to the chromosomal DNA cloned into the plasmid pCG2-6, *P. syringae* pv. *syringae* B728a fails to produce mangotoxin. Whether or not the lack of mangotoxin production by *P. syringae* pv. *syringae* B728a could be related to differences in the specificity of the NRPS or a lack of functionality (95% similarity to *mgo*A) remains to be investigated; however, when *P. syringae* pv. *syringae* B728a was transformed with pCG2-6, it remained without produce mangotoxin (data not shown).

However, this putative NRPS (MgoA) is not the unique protein responsible for the production of mangotoxin, and it could be involved in initial biosynthetic steps of mangotoxin or another oligopeptide produced by *P. syringae*. However, it is clear that the *mgo*A gene is not involved in the lipodepsinonapeptide biosynthesis (Table 2). On the other hand, we also obtained evidences of other genes, not present in *P. syringae* pv. *syringae* B728a (genes disrupted in mutants 4βA2 and 5αC5) (Table 2) which also could be involved in specific processes for mangotoxin production, suggesting specific reactions for mangotoxin production encoded out of the predicted operon containing the putative NRPS of *mgo*A.

The role of NRPS in the production of bacterial phytotoxins involved in virulence or plant pathogenesis also has been reported from other plant-pathogenic bacteria, such as *Xanthomonas albilineans* (Royer et al. 2004) or *Streptomyces acidiscabies* (Healy et al. 2000). In this sense, we established a clear relationship between the activity of *mgo*A and the virulence of *P. syringae* pv. *syringae* UMAF0158 on tomato leaflets. Artificial inoculation experiments showed that the mangotoxin-defective mutant *P. syringae* pv. *syringae* UMAF0158-6γF6 and the mangotoxin-producer *P. syringae* pv. *syringae* UMAF0158 and *P. syringae* pv. *syringae* UMAF2-6A strains grew at similar rates and reached similar population densities in inoculated tomato leaflets (Fig. 3). Similar results have been described for a *P. syringae* pv. *tomato* DC3000 *avrE1* mutant that is reduced in its ability to produce lesions but not in its ability to grow in host tomato leaves (Badel et al. 2006). However, the disease symptoms caused by the mangotoxin-defective mutant strain *P. syringae* pv. *syringae* UMAF0158-6γF6 clearly were less severe than those produced by the wild-type and the complemented mangotoxin-producing strains. These findings are similar to those previously demonstrated for other toxins, such

Table 4. Effective dose of mangotoxin-producing and nonproducing *Pseudomonas syringae* pv. *syringae* strains to induce necrotic symptoms in 50% of the inoculations on tomato leaflets (ED_{50})

Strains	Mangotoxin production	Characteristics	ED_{50}
P. syringae pv. *syringae*			
UMAF0158	+	Wild type, produces mangotoxin	1.8×10^6
UMAF0158-6γF6	–	Contains a mini*Tn*5km2 insertion in a putative nonribosomal peptide synthetase	5.3×10^7
UMAF2-6A	+	UMAF0158-6γF6 complemented with the plasmid pCG2-6	8.0×10^5
UMAF0158-4βE6	+	mini*Tn*5km2 mutant used as control	1.9×10^6

as coronatine (Mittal and Davis 1995) and other antimetabolite toxins from *P. syringae* (Engst and Shaw 1992; Peet et al. 1986). In inoculated tomato leaflets, the wild-type and complemented strains caused necrotic symptoms which began to be apparent on the third day post inoculation, and reached approximately 80% severely affected inoculation points on the tenth day post inoculation. In contrast, the mangotoxin-defective *P. syringae* pv. *syringae* UMAF0158-6γF6 produced tissue necrosis in a lower number of inoculation sites (approximately 40% inoculation points affected on the tenth day post inoculation) and, in this case, the symptoms began to be apparent on the fourth day post inoculation. Thus, the mangotoxin-defective mutant displays a clear delay in the initiation and development of the necrotic symptoms, in comparison with the mangotoxin-producing strains (Fig. 4). However, necrotic symptoms are still present at lower levels in mangotoxin-defective strains, mainly due to the production of other compounds related to the virulence (such as lipodepsipeptidic toxins) not affected by the Tn*5* insertion in those mutants. The role of the putative NRPS-encoding gene in the production of mangotoxin and its involvement in the symptoms development also was revealed by the ED_{50} experiments. The median effective dose (ED_{50}) is a value directly related to the virulence of the inoculated strains (Cazorla et al. 1998; Ercolani 1984). The ED_{50} determined after artificial inoculations on tomato leaflets showed clearly higher values for *P. syringae* pv. *syringae* UMAF0158-6γF6 than for *P. syringae* pv. *syringae* UMAF0158 and *P. syringae* pv. *syringae* UMAF2-6A, revealing a higher virulence for the mangotoxin-producing strains than for the mutant with the disrupted NRPS gene, defective in mangotoxin production (Table 4).

The putative NRPS gene (*mgo*A) that we have identified in this study is the first gene described for *P. syringae* pv. *syringae* which is directly involved in the biosynthesis of mangotoxin, and also is associated with virulence in the mangotoxin-producing strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and mutagenesis.

The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* pv. *syringae* strains were grown in King's medium B (KMB) at 22°C, and *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C. Transposon mutagenesis of *P. syringae* pv. *syringae* UMAF0158 was carried out by mating experiments on nitrocellulose membrane (0.22 μm) onto LB agar at 27°C for 2 h using *E. coli* S17λ*pir* containing the Tn*5*-derived minitransposon pUT-mini Tn*5*km2 as donor strain (de Lorenzo and Timmis 1994). Derivative strains were isolated after 48 h at 22°C on KMB amended with kanamycin (30 μg/ml) and nitrofurantoin (100 μg/ml) as selective agents.

The growth of *P. syringae* pv. *syringae* UMAF0158 derivative strains were characterized on Pseudomonas minimal medium (PMS) broth at 22°C. Bacterial counts were determined every day on KMB at 22°C after 48 h.

Detection of *P. syringae* pv. *syringae* toxins.

The lipodepsipeptidic toxins produced by *P. syringae* pv. *syringae* strains were demonstrated by the growth inhibition bioassay on potato-dextrose agar (PDA) using *Geotrichum candidum* (Gross and DeVay 1977) and *Rhodotorula pilimanae* (Iacobellis et al. 1992) as indicator strains.

The antimetabolite toxin production was assayed by the indicator technique previously described (Gasson 1980) with minor modifications (Arrebola et al. 2003), which relies on the inhibition of growth of *E. coli* CECT 831 on PMS (Gasson 1980). Briefly, a double layer of indicator microorganism was made using strain CECT831 of *E. coli*. After solidification, strains of *P. syringae* pv. *syringae* to be tested were stabbed and plates were incubated at 22°C for 24 h and 37°C for an additional 24-h period. To assess the biochemical step that is the putative target of the toxin, the same plate bioassay was carried out, but adding to the double layer 100 μl of a 6-mM solution of *N*-acetyl-ornithine or L-ornithine (Arrebola et al. 2003). Testing of toxic filtrates from *P. syringae* pv. *syringae* strains was done by growing bacteria in liquid PMS for 48 h at 22°C; the supernatants then were filtered through 0.22-μm nitrocellulose membranes (Arrebola et al. 2003) and tested by *E. coli* growth inhibition bioassays as described above.

OAT enzymatic assay.

OAT activity experiments were carried out using crude protein extracts from tomato leaves (Arrebola et al. 2003). In the standard assay, 25 μl of 6 mM *N*-acetyl-L-ornithine and 25 μl of 6 mM L-glutamine were mixed with 50 μl of crude protein extract of tomato leaves, together with 25 μl of the culture filtrate to be tested. After 1 h of incubation at 37°C, the reaction was stopped by adding three volumes of ninhydrin reagent and heated at 100°C for 1.5 min. Two volumes of 0.7 M NaOH then were added and the absorbance at 470 nm recorded after 20 min of incubation at room temperature (Arrebola et al. 2003). The amount of ornithine was determined colorimetrically by using a standard curve prepared for this purpose as reference (Dénes 1970; Vogel and McLellan 1970). A 0.2-mM solution of p-chloromercuribenzoic acid, a chemical inhibitor of OAT (Dénes 1970), and sterile liquid PMS were used as controls.

DNA manipulation and sequencing.

DNA isolation was performed by a standard protocol (Sambrook and Russell 2001). Plasmid mini-preps were done using the Qiaprep spin miniprep kit (Qiagen GmbH, Düsseldorf, Germany). For sequence analysis of the regions flanking the miniTn*5* insertions, the primers "KS" (5′TCGAGGTCG ACGGTATC3′) and "Tn*5*K2-P4" (5′GGCAGAGCATTACG CTGACT3′) were used. For the construction of a genomic library of the wild-type *P. syringae* pv. *syringae* UMAF0158, isolation of genomic DNA was carried out using the Puregene commercial kit (Gentra Systems, Minneapolis, MN, U.S.A.). The genomic library was constructed using λBlueSTAR Vector System (Novagen, Darmstadt, Germany), and genomic DNA of *P. syringae* pv. *syringae* UMAF0158 digested with *Sau*3A, following the manufacturer's recommendations. Automated DNA sequencing of genomic clones was carried out by Newbiotechnic S.A. (Sevilla, Spain).

Isolation and characterization of wild-type genomic clones.

To obtain genomic clones containing the Tn*5* insertions, Southern blot analysis was performed. *Pst*I-digested genomic DNA from selected mutants was hybridized with a Tn*5* fragment as probe to determine the sizes of fragments containing that Tn*5* element. Genomic DNA fragments containing the appropriate size range were ligated into pBluescript II KS(+) and transformed into *E. coli* DH5α. After plating the transformants onto selective media, kanamycin-resistant colonies were isolated and the transposon-containing inserts were purified. This DNA was used as a probe to screen the wild-type *P. syringae* pv. *syringae* UMAF0158 genomic DNA phage library. To obtain phagemid clones containing a chromosomal fragment harboring genes of mangotoxin, a 256-bp fragment from mangotoxin-defective mutant *P. syringae* pv. *syringae* UMAF0158-6γF6, flanking the Tn*5* insertion, was used as a probe in phage library screening on the phage plaques formed on the lysogenic

E. coli ER1647 strain. Of 3,000 plaques screened, eight phagemids hybridized to the probe.

Phage infections on the nonlysogenic *E. coli* BM25.8 were performed in order to obtain independent plasmids, and one of them, the plasmid pCG2-6, was selected for further studies.

Bioinformatics.

Database searches were performed with the BLAST 2.0 service of the National Center for Biotechnology Information (Bethesda, MD, U.S.A.). Amino acid sequences were aligned with ClustalW service of the EMBL European Bioinformatics Institute (Cambridge, U.K.). For mapping the chromosomal fragment cloned in plasmid pCG2-6, a comparison analysis with *P. syringae* pv. *syringae* B728a genome was carried out, and it was confirmed by using Artemis software (Sanger Institute, Cambridge, U.K.). The putative promoter site prediction was performed analyzing the sequences out of the ORF with two bioinformatic applications, BPROM software (Softberry Inc., Mount Kisco, NY, U.S.A.) and NNPP 2.2. software (Berkeley Drosophila Genome Project-BDGP, Berkeley, CA, U.S.A.).

Analysis of the predicted substrate-binding region and substrate amino acid for ORF5 was performed with the NRPS predictor software (Rausch et al. 2005).

Plant material, bacterial inoculation, and disease symptom evaluation.

Inoculation experiments were performed using tomato plants (*Lycopersicon esculentum* Mill.) cv. Hellfrucht Frühstamm. Plants were grown in greenhouse under natural light. Prior to inoculation, tomato leaflets from 6- to 8-week-old plants were detached, disinfected in 0.1% (wt/vol) $HgCl_2$ solution, and placed in petri dishes with their petioles immersed in Murashige and Skoog (MS) agar medium (Sigma-Aldrich, St. Louis) (Pérez-García et al. 1995). Inoculations were conducted with the *P. syringae* pv. *syringae* strains UMAF0158, UMAF0158-6γF6, and UMAF2-6A, which produces levels of lipodepsipeptidic toxins similar to the wild-type strain (Table 1). Detached leaflets were inoculated by placing six 10-µl droplets of approximately 10^8 CFU/ml bacterial suspensions on the principal lateral veins. Then, inoculations were performed by piercing through the droplets with an entomological pin. The leaflets were maintained at 22°C under a 16-h photoperiod. Five tomato leaflets were used to evaluate symptoms for each assayed strain. Leaflets treated with sterile water and maintained in MS medium were included in all experiments as a control. All experiments were repeated four times.

Appearance of necrotic areas at inoculation points were recorded daily over a 10-day period by visual analysis. Necrotic area surrounding the inoculation point larger than 2 mm was considered positive. In parallel, two inoculated leaflets were used every day to determine the bacterial densities. Tomato leaflets were homogenized in sterile phosphate-buffered saline. Then, bacterial populations were determined by serial dilutions on plates of KMB after incubation at 27°C during 48 h.

To estimate the ED_{50} of *P. syringae* pv. *syringae* strains, inoculation with bacterial suspensions of different cell densities (approximately 10^2 to 10^7 CFU per inoculation point) were carried out as described below. Suspensions of each strain at different bacterial concentrations were prepared, and six leaflets (six inoculation points per leaflet) were inoculated with each bacterial suspension. The number of inoculated points showing necrotic symptoms was recorded after 10 days. Each experiment was repeated four times. Then, a dose-response curve was produced by plotting the bacterial dose ($\log_{10}$-transformed) against a Weibull transformation of the proportion of diseased inoculation points ($\log_{10}[-\log_e(1 - R)]$). A straight line was fitted to the data points that were not equal to a 0 or 100% response and the value of dose corresponding to 50% of inoculated points showing necrotic symptoms (ED_{50}) was obtained (Shortley and Wilkins 1965).

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