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Contribution of mangotoxin to the virulence and epiphytic fitness of *Pseudomonas syringae* pv. syringae

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Summary. Mangotoxin is an antimetabolite toxin that inhibits ornithine acetyl transferase, a key enzyme in the biosynthetic pathway of ornithine and arginine and recently reported in strains of *Pseudomonas syringae* pv. syringae (Pss) isolated from mango. Since symptoms on mango tissues are very difficult to reproduce, in this study the role of mangotoxin in Pss virulence was addressed by analyzing the in planta growth and development of disease symptoms on tomato leaflets. Inoculation experiments were carried out following several procedures using the wild-type strain Pss UMAF0158, two *Tn5*-mutant derivative strains defective in mangotoxin production, and their complemented derivative strains in which mangotoxin production is restored. The ability of the mangotoxin-defective mutants to grow in planta was similar, and their epiphytic survival on the tomato leaf surface identical to the wild-type and complemented strains. However, both the disease index data of incidence and the severity of necrotic symptoms indicated that mangotoxin-defective mutants were less virulent, indicating that mangotoxin is a virulence factor. Furthermore, competition experiments showed that the survival values of the wild-type strain were slightly but significantly higher than those of the mangotoxin-defective mutants, suggesting that mangotoxin production would improve the epiphytic fitness of Pss. [**Int Microbiol** 2009; 12(2):87-95]

Keywords: *Pseudomonas syringae* pv. syringae · antimetabolite toxin · bacterial pathogenesis · phytotoxins · virulence factor

Introduction

Pseudomonas syringae has been reported to induce a wide variety of symptoms, such as blights, leaf spots, and gall, on many herbaceous plants and fruit trees, [16,19]. Likewise, *P. syringae* populations exist within diverse epiphytic communities on nearly all terrestrial plant species [17]. *Pseudomonas syringae* pv. syringae has a broader host range than other pathovars, both as an epiphyte and as a pathogen [16]. It is the causal agent of the bacterial apical necrosis of mango, a disease limiting mango fruit production in the Mediterranean area [7].

Many pathovars of *P. syringae* produce a variety of toxins not only in infected plants but also in culture media [5,14,21]. These toxins are secondary metabolites of diverse chemical structures and are effective at very low concentrations [22]. *P. syringae* phytotoxins, although not essential for pathogenicity, generally act as virulence factors that cause or increase disease symptoms (such as chlorosis or necrosis) in infected plants [5,10,14,21]. Toxins with lipodepsipeptidic structure, such as syringomycins and syringopeptins, have been extensively discussed in the literature [3,15]. They are produced by most *P. syringae* pv. syringae strains isolated from a wide range of plant hosts [5]. These toxins are responsible for necrotic symptoms by inducing pore formation and disrupting plant cell membrane functions [10,18].

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Another major group of phytotoxins produced by different *P. syringae* pathovars act as antimetabolite toxins. These oligopeptides inhibit enzymes involved in the biosynthetic pathways of some amino acids [5,21]. The best-known antimetabolite toxins produced by *P. syringae* pathovars are tabtoxin, phaseolotoxin [5], and the more recently described mangotoxin [1,2]. Tabtoxin is a dipeptide that contains tabtoxinine-β-lactam and is produced by *P. syringae* pathovars tabaci, coronafaciens, and garcae [22]. It irreversibly inhibits glutamine synthetase [29] and leads to the accumulation of ammonia, thereby acting as a chlorosis-inducing toxin [5]. Phaseolotoxin, produced by *P. syringae* pv. phaseolicola and pv. actinidiae [23,28], is a sulfodiaminophosphinyl moiety linked to a tripeptide [20]. It induces chlorosis by competitively inhibiting ornithine carbamoyl transferase, a critical enzyme in the arginine biosynthesis pathway [23]. *P. syringae* pv. syringae strains isolated from mango and other plants hosts, including tomato, produce mangotoxin, the focus of this study. Since tomato is a compatible host of Pss UMAF0158 and is a more reliable plant model than mango trees, we monitored the development of disease symptoms and in planta growth in tomato leaflets rather than in mango. Multiplication of the Pss mangotoxin producer strains UMAF0158 and UMAF0158-4βE6, and of the mutants defective in mangotoxin production, UMAF0158-5αC5 and UMAF0158-6γF6, were studied either in PMS minimal media or on tomato leaflets maintained in vitro. The tomato leaflets were inoculated with a relatively high dose of bacteria by creating lesions with a sterile entomological pin, and the total *P. syringae* counts and the *P. syringae* populations growing inside the leaflets (superficially disinfected) were determined each day. Mangotoxin is an oligopeptidic antimetabolite toxin [1,8] that inhibits ornithine acetyl transferase, a key enzyme in the biosynthetic pathway of ornithine and arginine [1]. In a recent work, we reported the involvement of a nonribosomal peptide synthetase (NRPS), encoded by the *mgoA* gene, in the production of mangotoxin. We also showed its putative role, direct or indirect, in the induction of necrotic symptoms by *P. syringae* pv. syringae [2].

Additionally, mangotoxin, as is the case with other antimetabolite toxins produced by *P. syringae* pathovars, shows antimicrobial activity against some bacteria that usually colonize the aerial parts of plants as either epiphytic or pathogenic microorganisms [8,30]. Therefore, antimetabolite toxins might also play a role in the epiphytic environment, favoring bacteria that produce these toxins over other phyllospheric microorganisms.

In this work, the role of mangotoxin in the pathogenesis of *P. syringae* pv. syringae of tomato leaves was determined. The results suggest that mangotoxin is a relevant factor contributing to *P. syringae* pv. syringae virulence. Furthermore, it appears that mangotoxin production is involved in the epiphytic fitness of this bacterium in the phyllosphere.

Materials and methods

The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* pv. syringae (Pss) strains were grown in King's medium B (KMB) at 27ºC. *Escherichia coli* strains were routinely grown in Luria-Bertani medium (LB) at 37ºC. Stable mutants of the wild-type strain Pss UMAF0158 were obtained by transposon mutagenesis (mini-*Tn*5km2) [9] followed by selective isolation on KMB with kanamycin (30 μg/ml) and nitrofurantoin (100 μg/ml), as described in a previous work [2].

A genomic clone of Pss UMAF0158 (pCG1-5) that restored mangotoxin production in the defective mutant Pss UMAF0158-5αC5 (Table 1) was isolated following a procedure similar to that previously described for pCG2-6 and Pss UMAF0158-6γF6 [2]. Briefly, to obtain phagemid clones containing a chromosomal fragment harboring genes involved in mangotoxin production, a 988-bp fragment from mangotoxin-defective mutant Pss UMAF0158-5αC5, flanking the *Tn*5 insertion, was used as a probe to screen a wild-type Pss UMAF0158 genomic DNA phage library, constructed using the λBlueSTAR Vector System [2]. Of the 3000 phage plaques formed on the lysogenic *E. coli* ER1647 strain, eight phagemids that hybridized to the probe were selected. Phage infections of the non-lysogenic *E. coli* BM25.8 were performed to obtain independent plasmids. Plasmid pCG1-5 was selected for further studies and propagated in *E. coli* DH5α for high copy production. Competent cells of the derivative strain Pss UMAF0158-5αC5 were transformed with the plasmid pCG1-5 by electroporation (200 Ω, 25 μFD, 1.6 kV) [2] and transformed colonies were selected on LB with carbenicillin (100 μg/ml). Complementation of mangotoxin production was determined, and the Pss UMAF1-5A strain that contained pCG1-5 and produced mangotoxin was selected for study.

Detection of *Pseudomonas syringae* toxins. The production of syringomycins by Pss strains was determined by the growth inhibition test on potato-dextrose agar (PDA) using *Geotrichum candidum* [15] and *Rhodotorula pilimanae* [18] as indicator strains. Antimetabolite toxin production was assayed by the indicator technique previously described [13], with minor modifications [6]. This technique relies on the inhibition of growth of *E. coli* on *Pseudomonas* minimal media (PMS) [13]. Briefly, a double layer of the indicator microorganism *E. coli* CECT831 was prepared; after solidification, the Pss strains to be tested were stab-inoculated. The plates were initially incubated at 22ºC for 24 h, and then at 37ºC for an additional 24 h [1]. To evaluate mangotoxin activity, the same plate bioassay was carried out with the addition of 100 μl of a 6 mM solution of *N*-acetyl-ornithine or L-ornithine to the double layer of *E. coli* [1].

Plant material. All the experiments were performed using tomato leaflets or tomato plants (*Solanum lycopersicum* Mill.) cv. Hellfrucht Frühstamm. Tomato plants were grown from seeds in a greenhouse under natural light. When detached leaflets were used, tomato leaflets from 10 week-old plants were detached, disinfected in 0.1 % (w/v) $HgCl_2$ solution, and placed in Petri dishes with their petioles immersed in Murashige and Skoog medium (MS, Sigma-Aldrich, USA) [25] prior to inoculation. When leaflets on tomato plants were used, plants were maintained in a growth chamber at 22ºC for a 16-h photoperiod with a relative humidity above 70%.

Bacterial growth and the development of necrotic symptoms on tomato leaflets. Different mangotoxin producing or non-producing Pss strains (UMAF0158, UMAF0158-6γF6, UMAF0158-5αC5, UMAF0158-4βA6, UMAF2-6A, UMAF1-5A; see Table 1) were inoculated on detached tomato leaflets. Bacterial suspensions from exponentially grow-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Mangotoxin production	Syringomycin production	Reference or source
P. syringae pv. syringae				
UMAF0158	Wild-type isolated from mango, Nf^r	+	+	[1]
UMAF0158-5αC5	miniTn5km2 mutant of UMAF0158, Nf^r, Km^r	–	+	[2]
UMAF0158-6γF6	miniTn5km2 mutant of UMAF0158, Nf^r, Km^r	–	+	[2]
UMAF0158-4βE6	miniTn5km2 mutant of UMAF0158, used as control. Nf^r, Km^r	+	+	[2]
UMAF1-5A	UMAF0158-5αC5 complemented strain containing plasmid pCG1-5, Nf^r, Km^r, Amp^r	+	+	This study
UMAF2-6A	UMAF0158-6γF6 complemented strain containing plasmid pCG2-6, Nf^r, Km^r, Amp^r	+	+	[2]
Escherichia coli				
CECT 831	Indicator strain in the bioassay for antimetabolite toxins production			CECT[b]
DH5α	*recA lac*ZΔM15			GIBCO-BRL, Paisley, UK
ER1647	F^- *fhu*A2 Δ(lacZ)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				
pBlueSTAR-1	Derived from λBlueSTAR-1 vector by autosubcloning			Novagen, Darmstadt, Germany
pCG1-5	UMAF0158 genomic DNA (14.0 kb) cloned in pBlueSTAR-1, which restore mangotoxin production of Pss UMAF0158-5 αC5.			This study
pCG2-6	UMAF0158 genomic DNA (11.1 kb) cloned in pBlueSTAR-1, which restore mangotoxin production of Pss UMAF0158-6γF6			[2]

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

ing cultures were adjusted to 10^8 CFU/ml. Detached leaflets were inoculated by placing six 10-μl drops of the bacterial suspension on six different points of each leaflet. Inoculations were then carried out by piercing through the droplets with an entomological pin. The leaflets were maintained in MS media at 22ºC for a 16-h photoperiod. Four to six tomato leaflets were used for each strain and each experimental time point of treatment. Non-infected detached leaflets inoculated with sterile distilled water were included in all experiments as a control. These experiments were repeated four times.

The development of necrotic symptoms at the inoculation points was determined over a 10-day period by two different methods. First, the appearance of necrotic symptoms was recorded daily by visual analysis to evaluate the disease incidence (number of inoculated points showing necrosis). The necrotic area surrounding the inoculation points was scored as follows: 0, no necrotic symptoms; 1, necrotic area < 2 mm in diameter; 2, between 2 and 5 mm; and 3, necrotic area > 5 mm. Second, the total necrotic area induced by the inoculated strains on the last day of the experiment (severity) was digitally analyzed on five leaflets using the computer image software VISILOG 5.0 (Noesis Vision Inc., France).

In parallel, two inoculated leaflets were used each day to estimate total bacterial density. Tomato leaflets were homogenized in sterile phosphate-buffered saline and bacterial counts were determined by serial dilutions on KMB plates after incubation at 27ºC for 48 h. Additionally, the bacterial density inside tomato leaflets was determined on two other inoculated leaflets. The leaflets were superficially disinfected by immersing them in H_2O_2 (3%) for 2 min, followed by treatment with a catalase solution (7.5 μg/ml) for 2 min to inactivate the hydrogen peroxide, and then washed in sterile distilled water for another 2 min [31]. The tomato leaflets were then homogenized and bacterial counts were determined as described above. In addition, a growth curve of the studied Pss strains cultured for 10 days on PMS media at 22ºC was obtained.

Additional experiments were done to determine the ED_{50} of the Pss strains. Bacterial suspensions at different cell densities (10^2–10^7 CFU per inoculation point) were inoculated as described previously [2,7]. Briefly, suspensions of each strain at different bacterial concentrations were prepared, and six tomato leaflets (six inoculation points per leaflet) were inoculated with each suspension as described above. The number of inoculated

points showing necrotic symptoms was recorded after 10 days. Each experiment was repeated four times. A dose-response curve was constructed 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 bacterial dose corresponding to 50% of inoculated points showing necrotic symptoms (ED_{50}) was obtained [7].

Epiphytic survival on tomato plants. The wild-type strain Pss UMAF0158 and the mangotoxin-defective mutants UMAF0158-6γF6 and UMAF0158-5αC5 were separately inoculated on tomato leaves to study epiphytic survival. Additionally, a mixture of the wild-type strain with each of the two studied mutants was used as inoculant. Bacterial suspensions for each strain were adjusted to 10^8 CFU/ml. Ten leaflets of three different tomato plants were inoculated with each strain separately. Two other sets of leaflets were inoculated with a 1:1 mixture (v/v) of the bacterial suspensions of Pss UMAF0158 and UMAF 0158-5αC5, or a mixture of Pss UMAF0158 and UMAF0158-6γF6. Eight drops of 5 µl each were placed on different points of each leaflet without producing lesions; therefore, each leaflet supported a total volume of 40 µl of the bacterial suspension or mixture. The leaflets were surveyed over a 10-day period; one leaflet from three plants per strain or mixture was detached each day, and the bacterial densities per leaflet were determined after homogenization, as described above, and the plating of serial dilutions on KMB (all Pss strains grow) and KMB with kanamycin (30 µg/ml, only the Pss *Tn5*-mutants grow) after 48 h at 27ºC. These experiments were repeated three times.

Statistical analysis. The data were statistically analyzed by analysis of variance followed by Fisher's least significance difference test (P = 0.05) using SPSS software (SPSS Inc., USA). All experiments were performed at least three times.

Results

In this work, a complemented strain (UMAF1-5A) for this mutant was also obtained to explore the role of mangotoxin in the virulence and epiphytic fitness of Pss by analyzing the induction of symptoms and the epiphytic survival of these six strains on tomato leaflets under different experimental conditions.

Bacterial growth and symptom development on tomato leaflets. The bacterial counts obtained from all four assayed strains cultured in PMS and in the inoculated tomato leaflets were very similar, independent of the *Tn5* insertion and the mangotoxin production status of the assayed strains.

The induction of necrotic symptoms after bacterial inoculation was also studied by evaluating different disease variables, such as the incidence and the severity of the necrotic symptoms, and by estimation of the ED_{50} values of the studied strains. To analyze the incidence, the number of spots developing necrotic symptoms after bacterial inoculation was monitored as described in Materials and methods. The number of necrotic spots >2 mm in diameter (categories 2 and 3, Fig. 2B) was recorded daily for 10 days following inoculation, and the results from the different inoculated leaflets in the four repetitions of the experiment were pooled (Fig. 2). For each studied strain, 120 inoculation points were evaluated. The wild-type strain Pss UMAF0158 produced observable disease symptoms 2–3 days after the inoculation, consisting of the development of necrotic spots surrounding

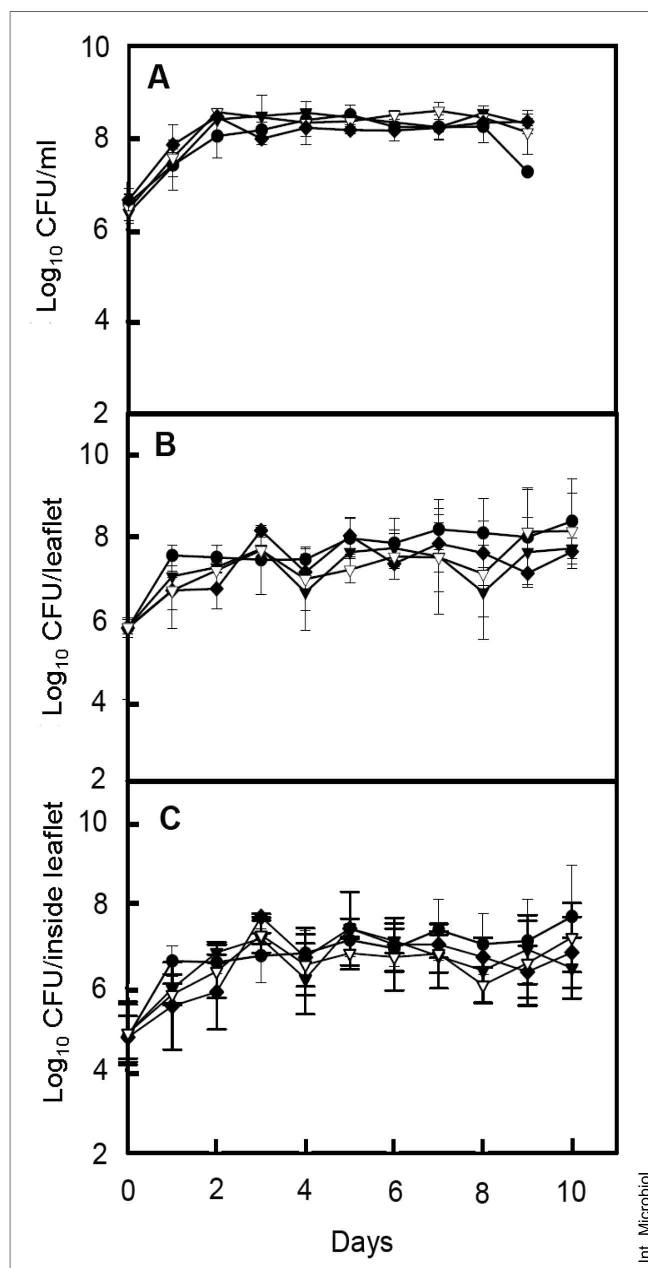

Fig. 1. Time course of in vitro and in planta growth of different *Pseudomonas syringae* pv. syringae strains. (**A**) Growth in minimal medium. (**B**) Total growth in tomato leaflets maintained in vitro. (**C**) Growth inside the tomato leaflets after H_2O_2 surface disinfection. Growth of the wild-type strain of *Pseudomonas syringae* pv. syringae UMAF0158 (closed circles), the mangotoxin-defective Tn*5*-mutants UMAF0158-5αC5 (closed triangles) and UMAF0158-6γF6 (open triangles), and the mangotoxin non-defective mutant UMAF0158-4βE6 (closed diamonds) was followed. Data are the means of four experiments; error bars indicate the standard deviation.

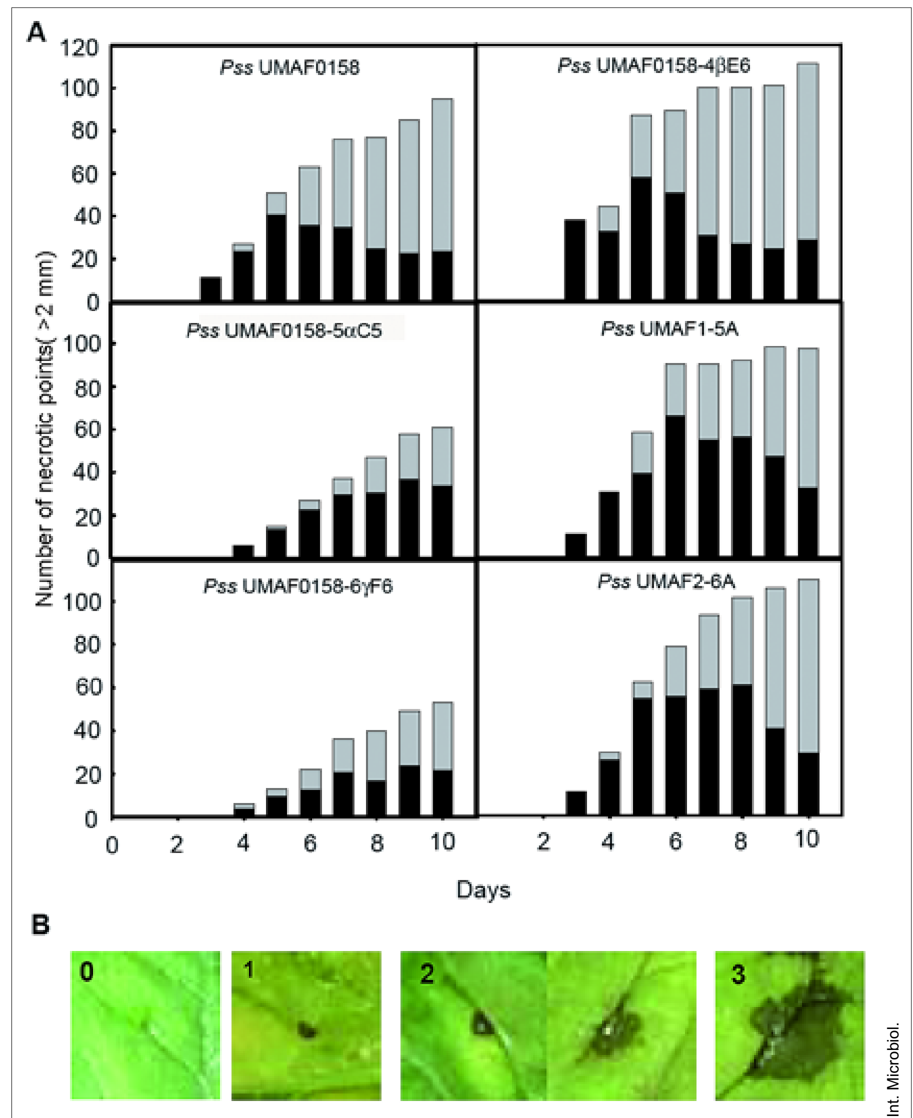

Fig. 2. Development of necrotic symptoms in tomato leaflets inoculated with different *Pseudomonas syringae* pv. syringae strains. The assayed strains were the wild-type UMAF0158, the mangotoxin-defective mutants UMAF0158-5αC5 and UMAF0158-6γF6, and the complemented derivatives UMAF1-5A and UMAF2-6A, with restored mangotoxin production. The mangotoxin non-defective mutant UMAF0158-4βE6 was also inoculated as a control. (**A**) Incidence of necrotic symptoms. The cumulative number of inoculated points developing necrotic symptoms >2 mm in diameter (category 2, grey color, and category 3, black color) was counted each day for a total of 120 inoculated points with each strain (30 inoculation points from 4 experiments). (**B**) Scale of necrotic symptoms. The categories considered to evaluate the necrotic symptoms were the following (diameter): 0, no necrotic symptoms; 1, necrotic area <2 mm; 2, 2–5 mm; 3, necrotic area >5 mm.

the bacterial inoculation point. The number and size of the necrotic spots increased throughout the experiments, with up to 82% of the inoculated points showing necrotic symptoms (>2 mm in diameter) after 10 days and more than 50% at 6 days. When mangotoxin-defective mutants UMAF0158-5αC5 and UMAF0158-6γF6 were inoculated, only approximately 50% of the inoculated points showed necrotic symptoms after 10 days. Likewise, necrotic lesions were not observable until 4 days after inoculation, while after 6 days, <20% of the inoculated points showed necrosis (Fig. 2). The incidence of necrotic symptoms caused by inoculation of the complemented strains (UMAF1-5A and UMAF2-6A) and the ectopic mutant (UMAF0158-4βE6), which also produced mangotoxin, was similar to the described values for wild-type strains, confirming that the reduction in the incidence of necrotic symptoms was associated exclusively with the two strains defective in mangotoxin production. Additionally, necrotic spots appeared earlier in tomato leaflets inoculated with the wild-type strain (observable at 3 days) than in those leaflets inoculated with the mangotoxin-defective mutants (observable after 4 days).

The total necrotic area of five inoculated tomato leaflets (30 inoculated points) from three experiments was determined and used to compare the severity of the disease symptoms among the six assayed strains (Fig. 3). The wild-type strain UMAF0158 produced a total necrotic area >800 mm^2. Similar values were obtained for both of the complemented strains (UMAF1-5A and UMAF2-6A) and the ectopic mutant control (UMAF0158-4βE6), all of which produce mangotoxin. In contrast, the mangotoxin-defective mutants (UMAF 0158-5αC5 and UMAF0158-6γF6) produced a smaller necrotic area (<50 mm^2, Fig. 3). These results show that the

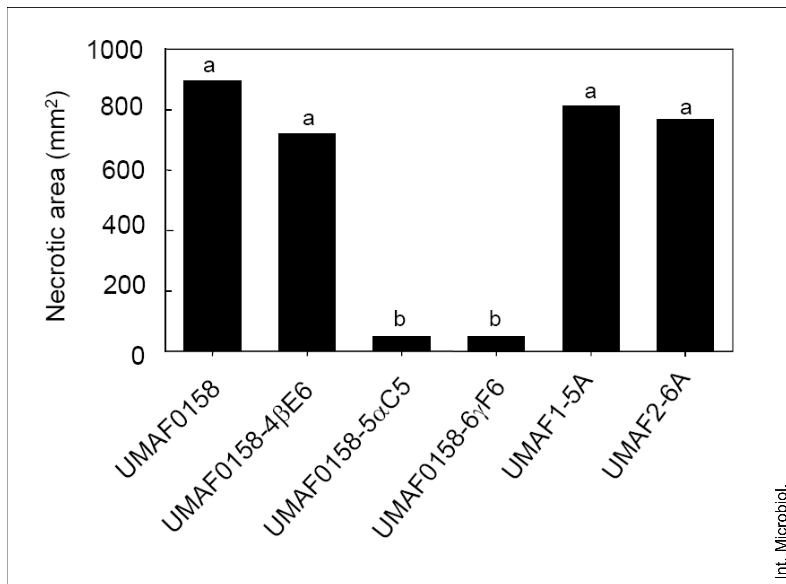

Fig. 3. Severity of necrotic symptoms on tomato leaflets inoculated with different *Pseudomonas syringae* pv. syringae strains. The total necrotic area (mm^2) from 30 inoculated points on tomato leaflets was measured 10 days after inoculation and used to compare the severity of necrotic symptoms produced by the different strains. The assayed strains were the wild-type UMAF0158, the mangotoxin-defective mutants UMAF0158-5αC5 and UMAF0158-6γF6, and the complemented derivatives, UMAF1-5A and UMAF2-6A, with restored mangotoxin production. The mangotoxin non-defective mutant UMAF0158-4βE6 was also inoculated as a control. Different letters denote statistically significant differences at $P = 0.05$, according to analysis of variance followed by Fisher's least significant difference test.

disease severity level induced by the wild-type and other mangotoxin-producing strains was higher than that induced by the mangotoxin-defective mutants. Finally, to confirm these results, an additional set of experiments was carried out in which the median effective dose (ED_{50}) of the assayed strains was estimated. This was accomplished using the straight line produced by plotting the inoculated bacterial dose (log_{10}-transformed) against the response data on a Weibull scale obtained from two different experiments (Fig. 4). The estimated ED_{50} was 1.8×10^6 CFU per inoculation point for the wild-type strain Pss UMAF0158 and 7.8×10^5 and 8.0×10^5 CFU for the complemented strains, UMAF1-5A and UMAF2-6A, respectively. However the estimated ED_{50} values for the mangotoxin-defective mutants UMAF0158-5αC5 and UMAF0158-6γF6 were 4.3×10^7 and 5.3×10^7 CFU per inoculation point, respectively. These results showed that the mangotoxin-defective mutants require a dose that is 1.5 orders of magnitude higher than that required by mangotoxin-producer strains to cause a similar level of disease symptoms.

Role of mangotoxin production in epiphytic fitness. The wild-type strain Pss UMAF0158 and the two derivative mangotoxin defective strains UMAF0158-6γF6 and UMAF0158-5αC5 were inoculated, either separately or

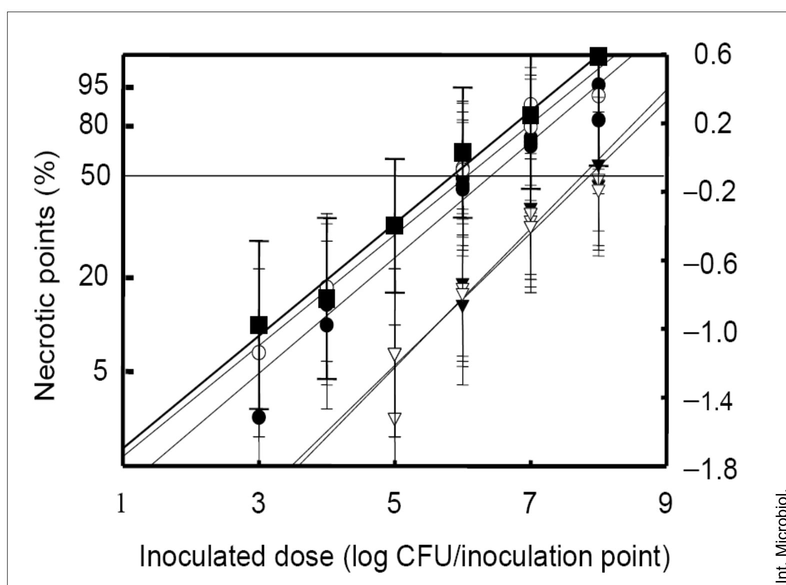

Fig. 4. Relationship between the percentage of inoculated points on tomato leaflets that developed necrotic symptoms and the number of *Pseudomonas syringae* pv. syringae cells inoculated per point (30 inoculated points per dose and strain). From this, the effective doses of the different assayed strains that induce necrotic symptoms in 50% of the inoculations (ED_{50}) were determined. Five Pss strains were compared in this assay: wild-type UMAF0158 (close circle), mangotoxin-defective mutants UMAF0158-5αC5 (close triangle) and UMAF0158-6γF6 (open triangles), and the complemented derivatives with restored mangotoxin production UMAF1-5A (close squares) and UMAF2-6A (open circles). Results were recorded after 10 days. The inoculated dose is plotted (on an exponential scale) against the response proportion of necrotic points in a Weibull scale on the right. The corresponding values of the percentage of response are shown on the left. Confidence limits (50%) corresponding to a sample size of 30 inoculation points are shown.

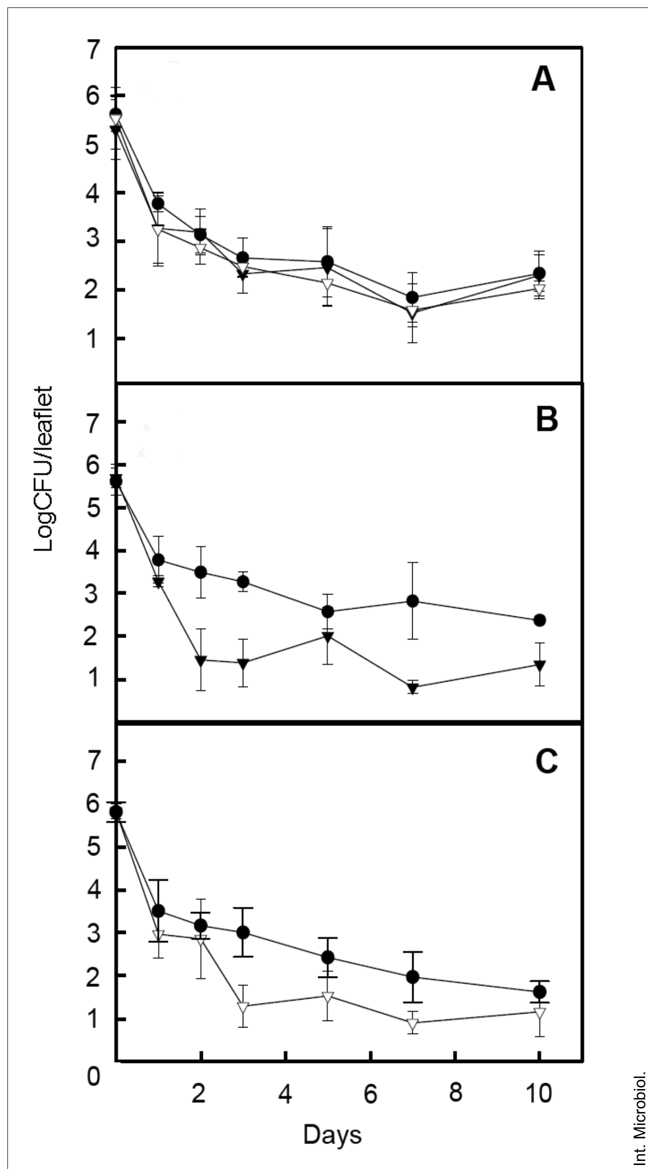

Fig. 5. Epiphytic survival of different *Pseudomonas syringae* pv. syringae strains on tomato leaflets. The wild-type strain UMAF0158 (closed circles) and the derivative mangotoxin-defective strains UMAF0158-6γF6 (open triangles) and UMAF0158-5αC5 (closed triangles) were studied. Bacterial densities (CFU/leaflet) were determined over 10 days on KMB and KMB+ kanamycin plates. (**A**) Experiments were carried out with separate inoculations of each assayed strain on the tomato leaflets. (**B**) The mangotoxin-defective strain UMAF0158-5αC5 (closed triangles) and the wild-type strain UMAF0158 (closed circles) were mixed (1:1) and co-inoculated. (**C**) The mangotoxin-defective strain UMAF0158-6γF6 (open triangles) was similarly co-inoculated (1:1) with the wild-type strain (closed circles).

in mixed suspensions, onto tomato leaflets without producing any lesions, and the population sizes were monitored for 10 days (Fig. 5). The epiphytic survival of all the assayed Pss strains showed very similar bacterial population levels when they were inoculated separately (Fig. 5A), including the mangotoxin non-defective Tn*5* mutant (UMAF0158-4βE6) (data not shown). However, when either Pss UMAF0158 and UMAF 0158-5αC5 (Fig. 5B) or UMAF0158 and UMAF0158-6γF6 (Fig. 5C) were co-inoculated, slight, but significantly higher bacterial population levels were measured for the wild-type strain than for the mangotoxin-defective mutants. This competitive advantage was almost an order of magnitude higher, especially in relation to UMAF0158-5αC5.

Discussion

Generally, a phytotoxin is a product of a pathogen or host-pathogen interaction that directly injures living host protoplasts and influences the course of plant disease or symptom development [10]. Both fungal and bacterial pathogens produce a number of metabolites that are toxic to plant cells; however, this does not necessarily mean that they are important in plant disease. Although most bacterial phytotoxins are not required for pathogenesis, their production frequently results in an increase in disease severity [5,21]. In this sense, the well described antimetabolite toxins tabtoxin and phaseolotoxin have been reported as virulence factors [5]. Tabtoxin, produced by *P. syringae* pv. tabaci and coronafaciens, induces chlorotic and necrotic symptoms [11], and phaseolotoxin, produced by *P. syringae* pv. phaseolicola, is able to produce a systemic chlorosis that has been attributed to the inactivation of OCT [24].

Our group has reported that some strains of *P. syringae* pv. syringae produce mangotoxin, an antimetabolite toxin that inhibits ornithine acetyltransferase. This enzyme is involved in arginine biosynthesis [1] and its inhibition produces defective ornithine and arginine levels. Recently, our group described a gene (*mgo*A) encoding a putative NRPS, which is required mangotoxin biosynthesis [2]. Likewise, the incidence of necrotic symptoms is reduced in a mutant disrupted in *mgo*A and defective in mangotoxin production (Pss UMAF0158-6γF6) [2], revealing that *mgo*A is involved in the virulence of Pss. However, *mgo*A involvement could be associated with a mechanism that is distinct from mangotoxin production, as is the case for other NRPS systems with respect to virulence independent of toxin production [12,26]. In addition, *mgo*A homology is present in other *P. syringae* strains, such as Pss B728a, Ps pv. tomato DC3000 and Ps pv. phaseolicola 1448A, that fail to produce mangotoxin [2]. To detect putative genes involved in mangotoxin production, mangotoxin-defective mutants of Pss UMAF0158 were obtained by transposon mutagenesis in previous studies [1,2]. Interestingly, the mutant Pss UMAF0158-6γF6, which is disrupted in a nonribosomal peptide synthetase (*mgo*A) and

impaired in mangotoxin production, also showed a reduction in virulence that was restored by complementation [2].

To detect putative genes involved in mangotoxin production, mangotoxin-defective mutants of Pss UMAF0158 were obtained by transposon mutagenesis in previous studies [1,2]. Note that the mutant Pss UMAF0158-6γF6, which was disrupted in a nonribosomal peptide synthetase (*mgo*A) and impaired in mangotoxin production, also showed a reduction in virulence that was restored by complementation [2]. To confirm the involvement of mangotoxin in the virulence of Pss, a more detailed study of the induction of symptoms by the wild-type (UMAF0158), the defective *Tn5*-mutant (UMAF0158-6γF6), and the complemented (UMAF2-6A) Pss strains was carried out in this work. An ectopic *Tn5*-mutant that produces mangotoxin (UMAF0158-4βE6), and an additional defective *Tn5*-mutant (UMAF0158-5αC5) that is disrupted in a putative carboxylase, which does not show significant identity with any gene in the sequenced strain Pss B728a, were also included [2]. The results reported herein provide evidence that mangotoxin production is directly related to the virulence of Pss UMAF0158. In addition to the wild-type Pss UMAF0158, which produces mangotoxin, these experiments included two mutants defective in mangotoxin production that were disrupted in different genetic traits, as well as their complemented strains. Tomato leaflets were artificially inoculated by placing droplets of bacterial suspensions on the leaflets and piercing through with an entomological pin. This approach ensured a repetitive and reliable inoculation method for all of the experiments, taking into effect the role that the inoculation method plays when a quantitative estimation of symptoms is necessary [27].

All the assayed strains grew at similar rates and reached similar population densities in and on inoculated tomato leaflets (Fig. 1), showing that the production of mangotoxin is not involved in bacterial growth, either in plant tissues or in culture media. However, the necrotic symptoms produced on the tomato leaflets after Pss inoculation were clearly reduced when the mangotoxin-defective mutants were used; this reduction was observed for all of the experiments and for all of the variables examined, such that mangotoxin production must be a virulence factor. When the incidence of necrotic symptoms was evaluated (Fig. 2), two main trends were observed. Not only was the appearance of visible symptoms in mangotoxin-defective mutants delayed compared with the mangotoxin-producing strains, but the most remarkable trait was the level of the symptoms attained. Ten days after inoculation, the percentage of points inoculated by mangotoxin-defective strains that showed necrosis was 40–50%, whereas in mangotoxin-producing strains the incidence reached 80–90%. In addition, the differences between mangotoxin-producing and non-producing strains were even more obvious when the severity of symptoms was estimated as the total necrotic area induced (Fig. 3). While mangotoxin-producing strains induced a total necrotic area >700 mm^2, the total necrotic area induced by defective mutants was <50 mm^2. Finally, the highest ED_{50} values were those for Pss UMAF0158-5αC5 and UMAF0158-6γF6, around 5×10^7 CFU, whereas those for mangotoxin-producing strains were close to 10^6 CFU (Fig 4). Therefore, we conclude that mangotoxin produced by Pss is involved in the development of necrotic symptoms on tomato leaflets, acting as a relevant virulence factor, as has been previously described for other antimetabolite toxins, such as tabtoxin and phaseolotoxin [5].

Furthermore, most antimetabolite toxins show antimicrobial activity [30] and they may contribute to bacterial competitive ability and epiphytic fitness [4]. The ability of Pss strains to produce mangotoxin might provide these bacteria with a competitive advantage to colonize host plants over other phyllospheric microorganisms. Preliminary results showed an antagonistic activity of Pss mangotoxin-producing strains against other phyllospheric bacteria, such as *Bacillus* spp. and *Erwinia* spp. [8]. In this study, experiments were performed to estimate whether mangotoxin-producing strains had advantages in the competitive colonization of the tomato phyllosphere. These experiments (Fig. 5) revealed similar population densities when the assayed Pss strains were inoculated individually; however, when the wild type strain was co-inoculated with a mangotoxin-defective mutant, the mutants reached lower population densities. In particular, significant differences were observed following co-inoculations with Pss UMAF0158-5αC5, up to ten-fold after 10 days. These results suggest that Pss strains producing mangotoxin are favored in competition with non-producing strains to colonize the phyllosphere. However, a more detailed analysis is required to confirm this conclusion.

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References

1. Arrebola E, Cazorla FM, Durán VE, et al. (2003) Mangotoxin: a novel antimetabolite toxin produced by *Pseudomonas syringae* inhibiting ornithine/arginine biosynthesis. Physiol Mol Plant Pathol 63:117-27
2. Arrebola E, Cazorla FM, Romero D, Pérez-García A, de Vicente A (2007) A nonribosomal peptide synthetase gene (*mgo*A) of

Pseudomonas syringae pv. syringae is involved in mangotoxin biosynthesis and is required for full virulence. Mol Plant-Microbe Interact 20:500-509

3. Ballio A, Barra D, Bossa F, et al. (1991) Syringopeptins, new phytotoxic lipodepsipeptides of *Pseudomonas syringae* pv. syringae. FEBS Lett 291:109-112
4. Beattie GA, Lindow SE (1994) Epiphytic fitness of phytopathogenic bacteria: Physiological adaptation for growth and survival. Curr Top Microbiol 192:1-27
5. Bender C, Alarcón-Chaidez F, Gross DC (1999) *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol Mol Biol Rev 63:266-292
6. Cazorla FM, Olalla L, Torés JA, Codina JC, Pérez-García A, de Vicente A (1997) *Pseudomonas syringae* pv. syringae as microorganism involved in apical necrosis of mango: characterization of some virulence factors. In: Rudolph K, et al. (eds) *Pseudomonas syringae* pathovars and related species. Kluwer Academic Pub., Dordrecht, pp 82-87
7. Cazorla FM, Torés JA, Olalla L, Pérez-García A, Farré JM, de Vicente A (1998) Bacterial apical necrosis of mango in southern Spain: a disease caused by *Pseudomonas syringae* pv. syringae. Phytopathology 88:614-620
8. Cazorla FM, Arrebola E, del Moral E, Rivera E, Olea F, Pérez-García A, de Vicente A (2003) An antimetabolite toxin (mangotoxin) is produced by *Pseudomonas syringae* pv. syringae isolated from mango. In: Iacobellis NS, et al. (eds) *Pseudomonas syringae* and related pathogens: biology and genetics. Kluwer Academic Pub., Dordrecht, pp 175-183
9. de Lorenzo V, Timmis KN (1994) Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5* and Tn*10*-derived minitransposons. Methods Enzymol 235:386-405
10. Durbin RD (1991) Bacterial phytotoxins: mechanism of action. Experientia 47:776-783
11. Engst K, Shaw PD (1992) Identificaction of a *lys*A-like gene required for tabtoxin biosynthesis and pathogenicity in *Pseudomonas syringae* pv. tabaci PTBR2.024. Mol Plant-Microbe Interact 5:322-329
12. Finking R, Marahiel MA (2004) Biosynthesis of nonribosomal peptides. Annu Rev Microbiol 58:453-488
13. Gasson MJ (1980) Indicator technique for antimetabolic toxin production by phytopatogenic species of *Pseudomonas*. Appl Environ Microbiol 39:25-29
14. Gross DC (1991) Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. Annu Rev Phytopathol 29:247-278
15. Gross DC, deVay JE (1977) Production and purification of syringomycin, a phytotoxin produced by *Pseudomonas syringae.* Physiol Plant Pathol 11:13-28
16. Hirano SS, Upper CD (1990) Population biology and epidemiology of *Pseudomonas syringae*. Annu Rev Phytopathol 28:155-177
17. Hirano SS, Upper CD (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*—a pathogen, ice nucleus, and epiphyte. Microbiol Mol Biol Rev 64:624-653
18. Iacobellis NS, Lavermicocca P, Grgurina I, Simmaco M, Ballio A (1992) Phytotoxic properties of *Pseudomonas syringae* pv. syringae toxins. Physiol Mol Plant Pathol 40:107-116
19. Kennelly MM, Cazorla FM, de Vicente A, Ramos C, Sundin GW (2007) *Pseudomonas syringae* diseases of fruit trees. Progress toward understanding and control. Plant Dis 91:4-17
20. Mitchell RE (1976) Isolation and structure of a chlorosis-inducing toxin of *Pseudomonas phaseolicola*. Phytochemistry 15:1941-1947
21. Mitchell RE (1984) The relevance of non-host toxins in the expression of virulence by pathogens. Annu Rev Phytopathol 22:215-45
22. Mitchell RE (1991) Implications of toxins in the ecology and evolution of plant pathogenic microorganisms: Bacteria. Experientia 47:791-803
23. Patil SS, Tam LQ, Sakai WS (1972) Mode of action of the toxin from *Pseudomonas phaseolicola*. I. Toxin specificity, chlorosis and ornithine accumulation. Plant Physiol 49:803-807
24. Peet RC, Lindgren PB, Willis DK, Panopoulos NJ (1986) Identification and cloning of genes involved in phaseolotoxin production by *Pseudomonas syringae* pv. "phaseolicola". J Bacteriol 166:1096-1105
25. Pérez-García A, Cánovas FM, Gallardo F, Hirel B, de Vicente A (1995) Differential expression of glutamine synthetase isoforms in tomato detached leaflets infected with *Pseudomonas syringae* pv. tomato. Mol Plant-Microbe Interact 8:96-103
26. Raaijmakers JM, de Brujin I, Kock MJD (2006) Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: Diversity, activity, biosynthesis and regulation. Mol Plant-Microbe Interact 19:699-710
27. Ruz L, Moragrega C, Montesinos E (2008) Evaluation of four whole-plant inoculation methods to analyze the pathogenicity of *Erwinia amylovora* under quarantine conditions. Int Microbiol 11:111-119
28. Tamura K, Imamura M, Yoneyama K, Kohno Y, Takikawa Y, Yamaguchi I, Takahashi H (2002) Role of phaseolotoxin production by *Pseudomonas syringae* pv. actinidae in the formation of halo lesions of kiwifruit canker disease. Physiol Mol Plant Pathol 60:207-214
29. Thomas MD, Langton-Unkefer PJ, Uchytil TF, Durbin RD (1983) Inhibition of glutamine synthetase from pea by tabtoxinine-β-lactam. Plant Physiol 71:912-915
30. Völksch B, Weingart H (1998) Toxin production by pathovars of *Pseudomonas syringae* and their antagonistic activities against epiphytic microorganisms. J Basic Microbiol 38:135-145
31. Wilson M, Hirano SS, Lindow SE (1999) Location and survival of leaf-associated bacteria in relation to pathogenicity and potential for growth within the leaf. Appl Environ Microbiol 65:1435-1443