The Iturin-like Lipopeptides Are Essential Components in the Biological Control Arsenal of *Bacillus subtilis* Against Bacterial Diseases of Cucurbits

Houda Zeriuoh, Diego Romero, Laura García-Gutiérrez, Francisco M. Cazorla, Antonio de Vicente, and Alejandro Pérez-García

Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora” (IHSM-UMA-CSIC), Bulevar Louis Pasteur-Campus Universitario de Teatinos s/n, 29071 Málaga, Spain

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The antibacterial potential of four strains of *Bacillus subtilis*, UMAF6614, UMAF6619, UMAF6639, and UMAF8561, previously selected on the basis of their antifungal activity and efficacy against cucurbit powdery mildew, was examined. Among these strains, UMAF6614 and UMAF6639 showed the highest antibacterial activity in vitro, especially against *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum*. These strains produced the three families of lipopeptide antibiotics known in *Bacillus* spp.: surfactins, iturins, and fengycins. Using thin-layer chromatography analysis and direct bioautography, the antibacterial activity could be associated with iturin lipopeptides. This result was confirmed by mutagenesis analysis using lipopeptide-defective mutants. The antibacterial activity was practically abolished in iturin-deficient mutants, whereas the fengycin mutants retained certain inhibitory capabilities. Analyses by fluorescence and transmission electron microscopy revealed the cytotoxic effect of these compounds at the bacterial plasma membrane level. Finally, biological control assays on detached melon leaves demonstrated the ability of UMAF6614 and UMAF6639 to suppress bacterial leaf spot and soft rot; accordingly, the biocontrol activity was practically abolished in mutants deficient in iturin biosynthesis. Taken together, our results highlight the potential of these *B. subtilis* strains as biocontrol agents against fungal and bacterial diseases of cucurbits and the versatility of iturins as antifungal and antibacterial compounds.

Phytopathogenic bacteria are responsible for significant economic losses in valuable agronomical crops worldwide (Agrios 2005). Recurrent species related to relevant diseases belong to a selective group of gram-negative genera: *Agrobacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. Bacterial diseases are generally treated with chemicals, especially those based on copper compounds. This traditional management practice is threatened by the development of resistance in popu-

lations of the target pathogens (Bender et al. 1990; Cazorla et al. 2002). Thus, there is a need for the development of new management methods that combine efficacy with a low environmental impact (Jacobsen et al. 2004; Ji et al. 2006). Recently, biological control agents have received a great deal of attention because of their versatility in modes of action, efficacy, and potential to be combined with classical chemical management methods (Fravel 2005; Shoda 2000).

Members of the *Bacillus* genus are among the beneficial bacteria that can be exploited as biopesticides in plant health care (Pérez-García et al. 2011). The features contributing to their success are the production of a vast array of secondary metabolites with potential antimicrobial activity (Krebs et al. 1998; Romero et al. 2007b; Wulff et al. 2002) and the formation of spores that ensure permanence in diverse habitats under hostile environmental conditions and also ease the formulation of feasible long-term commercial products (Broggini et al. 2005). For example, 5 to 8% of the *Bacillus subtilis* and *B. amyloliquefaciens* genomes are devoted to the synthesis of secondary metabolites with antifungal, antibacterial, and nematocidal activity (Chen et al. 2009; Stein 2005).

Lipopeptides probably represent the most common class of compounds produced by *Bacillus* spp. (Stachelhaus et al. 2002). These amphiphilic compounds share a common structure consisting of a lipid tail linked to a short cyclic oligopeptide. The lipopeptides are classified into three different families depending on the amino acid sequence: surfactins, iturins (mycosubtilin, iturin A, and bacillomycin), and fengycins (Ongena and Jacques 2008). All of these lipopeptides are produced in a variety of isoforms, which are responsible for the differences in their biological activities (Stein 2005). The surfactins are powerful biosurfactants, with exceptional emulsifying, foaming, antiviral, and antimycoplasmal activities but little or no marked antibacterial or antifungal activities (Peypoux et al. 1999; Tendulkar et al. 2007). The fengycins (synonymous with plipastatins) show strong fungitoxic activity, specifically against filamentous fungi (Ongena and Jacques 2008; Vanittanakom et al. 1986). The iturins display strong antifungal activity against a wide range of yeasts but only limited antibacterial activity (Ongena and Jacques 2008; Thimon et al. 1995). In addition to this direct antimicrobial activity, lipopeptides might also contribute to plant disease control by suppressing biofilm formation by pathogens or by inducing a global defense response in the plant known as induced systemic resistance (ISR) (Bais et al. 2004; Ongena et al. 2007). In general, the activity of lipopeptides from the *Bacillus* spp. is more commonly described against fungal patho-

Corresponding author: A. Pérez-García; E-mail: aperez@uma.es; Telephone: +34 952131890; Fax: +34 952136645.

Current address of D. Romero: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston 02115, U.S.A.

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gens. However, some reports have demonstrated the antagonistic activity of *Bacillus* spp. against phytopathogenic bacteria such as *Xanthomonas campestris* (Etcheagaray et al. 2008; Massomo et al. 2004; Wulff et al. 2002).

One of the most realistic applications of biocontrol agents is their inclusion in integrated control programs with other management techniques; however, this practice requires a deep characterization and understanding of their modes of action. In previous studies, we reported four strains of *B. subtilis* as potential biocontrol agents against the powdery mildew pathogen *Podosphaera fusca* (Romero et al. 2004), one of the most important fungal pathogens of cucurbits (Pérez-García et al. 2009). We also demonstrated the relevant role of the iturins and fengycins in the antifungal activity and control of cucurbit powdery mildew by these strains (Romero et al. 2007b). Furthermore, these strains performed reasonably well in controlling the powdery mildew disease under greenhouse conditions and demonstrated their potential to be fermented for large-scale production (Romero et al. 2007c). The aim of this work was to expand our understanding of the activities and modes of action of these *B. subtilis* strains by studying their antibacterial activity against the most important bacterial pathogens of cucurbits (Zitter et al. 1996). We present data indicating the efficacy of these biocontrol agents in controlling the bacterial leaf spot and soft rot pathogens of cucurbits, *X. campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum*. We also demonstrated that iturin A and bacillomycin have antibacterial activity and that the cytotoxic effect is targeted to the bacterial plasma membrane. This study combines the accumulated knowledge on these naturally occurring compounds and shows how they contribute to the performance of *B. subtilis* strains as plant-associated bacteria. Additionally, this study indicates the potential of these particular *B. subtilis* strains as biocontrol agents against bacterial and fungal pathogens of cucurbits and the versatility of the iturins as antifungal and antibacterial compounds.

RESULTS

Antibacterial activity of *B. subtilis* strains.

To determine the antibacterial potential of the four *B. subtilis* strains previously characterized as biocontrol agents of cucurbit powdery mildew, the antibacterial activity was first tested against selected strains of five cucurbit pathogenic bacteria by the in vitro dual culture technique (Table 1). The four *B. subtilis* strains inhibited the growth of at least two of the target bacteria but only the strain UMAF6639 was capable of efficiently targeting all of the assayed pathogens; the only exception was *P. syringae* pv. *lachrymans* NCPPB537, a strain that was not affected by any of the *B. subtilis* strains tested. Further analysis of the antibacterial activity using the cell-free filtrates of the four *B. subtilis* strains was performed against *X. campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum*. As expected, the results showed a generalized inhibitory effect against these bacterial pathogens, confirming the higher antibacterial activity of *B. subtilis* UMAF6614 and UMAF6639 (data not shown).

Biochemical characterization of the bactericidal activity.

To gain insight into the chemical nature of the putative molecules responsible for the antibacterial activity, the cell-free filtrates of the two selected *B. subtilis* strains were subjected to different stability treatments. The antibacterial activity of the supernatants showed a clear resistance to high temperatures and degradation by pronase or proteinase K. In contrast, the antibacterial activity was affected by treatments with both extremely acidic and alkaline pH and was completely extracted with *n*-butanol. Finally, the ultrafiltration tests, using a filter with a 3-kDa molecular weight cut-off, showed that the antibacterial activity was present at similar levels in both filterable and nonfilterable fractions (data not shown). The consistency of these findings with those observed for the same bacterial strains and fractions but using a fungal strain as an indicator (Romero et al. 2007b) suggested

Table 1. Inhibitory activity of *Bacillus subtilis* strains against selected cucurbit pathogenic bacteria^y

<i>B. subtilis</i> strains	Cucurbit pathogenic bacteria ^z				
	<i>Acidovorax avenae</i> subsp. <i>avenae</i>	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	<i>Pseudomonas syringae</i> pv. <i>lachrymans</i>	<i>Xanthomonas campestris</i> pv. <i>cucurbitae</i>	<i>X. campestris</i> pv. <i>melonis</i>
UMAF6614	–	10 ± 2	–	43 ± 1	30 ± 2
UMAF6619	–	–	–	25 ± 1	14 ± 2
UMAF6639	10 ± 2	10 ± 1	–	40 ± 2	28 ± 2
UMAF8561	–	–	–	26 ± 2	25 ± 2

^y Antibacterial activity is expressed as diameter of inhibition zone in millimeters on nutrient agar plates according to the dual-culture bioassay.

^z Each value represents the mean ± the standard error of three independent experiments; – indicates no inhibition halo was observed.

Table 2. Bacterial strains used in this study

Bacterial strains	Relevant characteristics	Reference or source ^z
<i>Bacillus subtilis</i>		
<i>B. subtilis</i> UMAF6614	Producer of bacillomycin, fengycin, surfactin	Romero et al. 2007b
<i>B. subtilis</i> UMAF6619	Producer of iturin A, fengycin, surfactin	Romero et al. 2007b
<i>B. subtilis</i> UMAF6639	Producer of iturin A, fengycin, surfactin	Romero et al. 2007b
<i>B. subtilis</i> UMAF8561	Producer of bacillomycin, fengycin, surfactin	Romero et al. 2007b
6614:: <i>bamA</i>	UMAF6614 <i>bamA</i> ::pFen2-2; bacillomycin deficient	Romero et al. 2007b
6614:: <i>fenB</i>	UMAF6614 <i>fenB</i> ::pFen2-2; fengycin deficient	Romero et al. 2007b
6639:: <i>ituD</i>	UMAF6639 <i>ituD</i> ::pItu2-2; iturin A deficient	Romero et al. 2007b
6639:: <i>fenB</i>	UMAF6639 <i>fenB</i> ::pFen2-2; fengycin deficient	Romero et al. 2007b
Cucurbit pathogenic bacteria		
<i>Acidovorax avenae</i> subsp. <i>citrulli</i> NCPPB3679	Causal agent of bacterial fruit blotch	NCPPB
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i> NCPPB2349	Causal agent of bacterial soft rot	NCPPB
<i>Pseudomonas syringae</i> pv. <i>lachrymans</i> NCPPB537	Causal agent of angular leaf spot	NCPPB
<i>Xanthomonas campestris</i> pv. <i>cucurbitae</i> NCPPB2597	Causal agent of bacterial leaf spot	NCPPB
<i>Xanthomonas campestris</i> pv. <i>melonis</i> NCPPB3434	Causal agent of bacterial leaf spot	NCPPB

^z NCPPB = National Collection of Plant Pathogenic Bacteria, United Kingdom.

that lipopeptides could be the active compounds responsible for the antibacterial activity.

To confirm this hypothesis, the antibacterial activities of butanolic extracts from cell-free culture filtrates of the wild-type strains of *B. subtilis*, UMAF6614 and UMAF6639, and derivative mutants that are deficient in the production of iturin (iturin A or bacillomycin) or fengycin lipopeptides from a previous study (Table 2), were subjected to thin-layer chromatography (TLC) and bioautography analysis using *X. campestris* pv. *cucurbitae* and *P. carotovorum* as bioindicators. These results are shown in Figure 1. In this figure, only the results obtained for strain UMAF6639 are presented because the results obtained for strain UMAF6614 were similar. The TLC plates stained with 10% H₂SO₄ confirmed the presence of bands corresponding to the three main groups of lipopeptides (surfactins, iturins, and fengycins) at the expected retention factor (Rf) values, and the absence of those bands in the corresponding lipopeptide-deficient mutants, fengycins for the *fenB* mutant and iturins for the *ituD* (Fig. 1A) and *bamA* mutants (data not shown). The bioautographs showed large inhibition zones for the wild-type strains at Rf values of approximately 0.35 to 0.45, typically associated with iturin (iturin A or bacillomycin) lipopeptides; notably, these inhibition zones were larger for *X. campestris* pv. *cucurbitae* than for *P. carotovorum*. The iturin (*ituD* and

bamA) mutants showed no production of iturin A and bacillomycin, respectively, and did not show bactericidal activity, whereas the fengycin *fenB* mutants showed small areas of inhibition associated with iturin bands against *X. campestris* but not against *P. carotovorum* (Fig. 1B).

The production of the three different families of lipopeptides—surfactins, fengycins, and iturins (iturin A or bacillomycin)—in the cell-free culture filtrates of the *B. subtilis* strains UMAF6614 and UMAF6639 was confirmed by reverse-phase high-performance liquid chromatography (RP-HPLC) analysis. This technique also allowed the precise quantification of each lipopeptide in liquid cultures (Table 3). In vitro, these strains produced similar amounts of fengycins, whereas surfactin production was different; UMAF6639 produced fourfold

Table 3. Quantification of the in vitro production of lipopeptides by the *Bacillus subtilis* strains UMAF6614 and UMAF6639^y

Lipopeptide family	Production (mg/liter)	
	UMAF6614	UMAF6639
Fengycins (fen)	31.05	31.28
Iturins (itu) ^z	49.80	32.50
Surfactins (stf)	11.25	46.93
Ratio (fen/itu/srf)	2.8/4.4/1	1/1/1.5

^y Lipopeptides were purified from 3-day-old medium optimal for lipopeptide production liquid cultures and quantified by reverse-phase high-performance liquid chromatography.

^z Data include the production of bacillomycin by *B. subtilis* UMAF6614 and iturin A by *B. subtilis* UMAF6639.

Table 4. Antibacterial activity of lipopeptide-enriched solutions from cell-free culture filtrates of *Bacillus subtilis* wild-type strains and lipopeptide-deficient derivative mutants against *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum*^x

Treatments	Inhibition zone ^y	
	<i>X. campestris</i> pv. <i>cucurbitae</i>	<i>P. carotovorum</i> subsp. <i>carotovorum</i>
<i>B. subtilis</i> strains		
<i>B. subtilis</i> UMAF6614	37 ± 2	8 ± 1
6614:: <i>fenB</i>	22 ± 2	—
6614:: <i>bamA</i>	—	—
<i>B. subtilis</i> UMAF6639	38 ± 2	9 ± 1
6639:: <i>fenB</i>	20 ± 2	—
6639:: <i>ituD</i>	—	—
Purified lipopeptides (1 mg/ml) ^z		
Bacillomycin	22 ± 1	12 ± 1
Fengycin	—	—
Iturin A	45 ± 2	15 ± 3
Surfactin	—	—

^x Antibacterial activity is expressed as diameter of inhibition zone in millimeters on nutrient agar plates.

^y Each value represents the mean ± standard error of three independent experiments; — indicates no inhibition halo was detected.

^z Purified fractions of the different lipopeptides were also included in the analysis.

Fig. 1. Thin-layer chromatography (TLC) and bioautographic analysis of lipopeptide-enriched solutions from the cell-free culture filtrates of *Bacillus subtilis* UMAF6639 and lipopeptide-deficient derivative mutants. **A**, Butanolic extracts (100 μ l) from the wild-type strain *B. subtilis* UMAF6639 (wt), the fengycin-deficient mutant (*fenB*), and the iturin A-defective transformant (*ituD*) were fractionated by TLC and revealed with 10% H₂SO₄. **B**, Bioautographic analysis against *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum*. Developed chromatograms were overlaid with a thin layer of Luria-Bertani agar inoculated with *X. campestris* or *P. carotovorum* cells (10⁸ CFU/ml). Pictures were taken after 24 h of incubation at 25°C. The retention factor (Rf) values of the different lipopeptide families, surfactins (srf), iturins (itu), and fengycins (fen) are indicated on the left. The bacterial growth inhibition zones, produced by iturin A activity from *B. subtilis* UMAF6639, are indicated by arrows. Similar results were obtained for the *B. subtilis* strain UMAF6614 and its fengycin- and bacillomycin (iturin)-defective derivatives mutants (data not shown).

more lipopeptides than UMAF6614. Regarding the iturins, these strains produced different compounds and at different levels. Thus, bacillomycin was more abundantly produced by UMAF6614 than iturin A by UMAF6639. Interestingly, the relative production of these lipopeptides was also different in these strains. In UMAF6614 the iturins (bacillomycin) were the predominant lipopeptides whereas, in UMAF6639, the three lipopeptide families were produced at similar levels.

Assessment of the bactericidal activity of lipopeptides.

The lipopeptide-enriched extracts from the *B. subtilis* wild-type strains UMAF6614 and UMAF6639 and their lipopeptide-defective mutants were further assessed for antibacterial activity using the in vitro inhibition bioassay against *X. campestris* pv. *cucurbitae* and *P. carotovorum* (Table 4). As previously observed, the extracts obtained from the supernatants of the wild-type strains showed remarkable inhibitory activity towards *X. campestris* pv. *cucurbitae*. Likewise, mutants deficient in the production of iturin A or bacillomycin completely lacked any detectable bactericidal activity. However, mutants impaired in fengycin production showed a slight decrease in their suppressive effect compared with the wild-type strains. Similarly, *P. carotovorum* was also inhibited by extracts of the wild-type strains but, in this case, no inhibition of bacterial growth was achieved by either mutant. The in vitro inhibition bioassay was also performed against *X. campestris* pv. *cucurbitae* and *P. carotovorum* using purified solutions of each lipopeptide at concentrations of 1 mg/ml (Table 4). The results showed that iturin A retained the highest antibacterial activity, followed by bacillomycin. In contrast, neither surfactin nor fengycin showed detectable bactericidal activity at the assayed concentration.

A more accurate assessment of the inhibitory effect of the *B. subtilis* lipopeptides was performed using a microplate spectrophotometric bioassay employing *X. campestris* pv. *cucurbitae* as an indicator (Table 5). In this case, the ability of *X. campestris* pv. *cucurbitae* to grow in the presence of lipopeptide-enriched solutions was analyzed by recording the absorbance at 492 nm after 1 h of incubation at 25°C. As expected, the butanolic extracts from the culture filtrates of the wild-type strains retained the strongest antibacterial activities, with inhibitory effects remaining detectable at 1,000-fold dilutions. The antibiotic activity of fengycin mutants was slightly reduced compared with the wild-type strains, showing inhibitory effects at 100-fold dilutions but becoming undetectable at 1,000-fold. By contrast, the undiluted solutions of the iturin A or bacillomycin mutants showed slight inhibitory effects, and

no inhibition was observed with the diluted solutions, reaching cell density values almost identical to those of the untreated control. The same bioassay was used to examine the bactericidal activity of solutions of purified lipopeptides (Table 5). As expected, surfactin had no effect on bacterial growth, whereas fengycin showed some inhibitory activity that diminished at 0.01 mg/ml. By contrast, the iturins displayed the strongest antibacterial activity. Thus, iturin A proved to be the most active lipopeptide, inhibiting bacterial growth at the lowest concentration assessed (0.001 mg/ml). Bacillomycin displayed an inhibitory activity at 0.01 mg/ml that became almost undetectable at 0.001 mg/ml.

Microscopic analyses of the cytotoxic activities of lipopeptides.

Lipopeptides are known to interact with biological membranes. Therefore, it was reasonable to hypothesize that their mechanism of action involved the bacterial plasma membrane. The extent of bacterial membrane damage elicited by the lipopeptides was microscopically evaluated by visualizing the entrance of molecular fluorescent probes into *X. campestris* pv. *cucurbitae* cells (Fig. 2). This double staining technique is based on the differential ability of probes with different molecular weights to traverse membranes and bind to intracellular nucleic acids, rendering the products easily detectable by fluorescence microscopy. The incubation of cell suspensions from *X. campestris* pv. *cucurbitae* with the probes did not result in an increase of dead cells (red fluorescence) and, accordingly, a vast proportion of the cell population remained alive (green fluorescence) as visualized by phase-contrast (Fig. 2A) and fluorescence microscopy (Fig. 2B and C). In contrast, a large proportion of red fluorescent cells were observed after 1 h of exposure to the lipopeptide extracts from the wild-type strain of *B. subtilis* UMAF6639 (Fig. 2D to F) as a consequence of the extensive accumulation of the red probe into the bacterial cells in response to perturbations induced at their plasma membranes. The influx of the probes into cells after treatments with extracts of iturin-defective *ituD* mutants was comparatively lower than those caused by the wild type and, consequently, the ratios of dead (red) to alive (green) cells were similar (Fig. 2J to L). Cell images similar to those of the wild type were obtained after exposure to the extracts of the fengycin-deficient *fenB* mutants (Fig. 2G to I). Similar experiments were performed with solutions of purified lipopeptides. The bacterial cells were intensely stained with the red probe after the addition of solutions of purified iturin A (Fig. 2P to R),

Table 5. Effect of lipopeptide-enriched solutions from cell-free culture filtrates of *Bacillus subtilis* wild-type strains and lipopeptide-deficient derivative mutants on the growth of *Xanthomonas campestris* pv. *cucurbitae*^x

Treatments	Dilution factor of lipopeptide-enriched solutions ^y				
	1:0	1:5	1:10	1:100	1:1000
Untreated	0.45 ± 0.04	...	...	...	...
<i>B. subtilis</i> strains					
<i>B. subtilis</i> UMAF6614	0.16 ± 0.06	0.17 ± 0.01	0.27 ± 0.01	0.33 ± 0.01	0.38 ± 0.01
6614:: <i>fenB</i>	0.23 ± 0.03	0.26 ± 0.02	0.33 ± 0.02	0.36 ± 0.01	0.41 ± 0.01
6614:: <i>bamA</i>	0.39 ± 0.01	0.43 ± 0.01	0.42 ± 0.01	0.43 ± 0.01	0.44 ± 0.02
<i>B. subtilis</i> UMAF6639	0.16 ± 0.06	0.18 ± 0.01	0.22 ± 0.01	0.30 ± 0.01	0.37 ± 0.02
6639:: <i>fenB</i>	0.23 ± 0.06	0.25 ± 0.02	0.33 ± 0.02	0.35 ± 0.01	0.40 ± 0.01
6639:: <i>ituD</i>	0.40 ± 0.04	0.44 ± 0.01	0.43 ± 0.01	0.44 ± 0.01	0.45 ± 0.02
Purified lipopeptides (1 mg/ml) ^z					
Bacillomycin	0.23 ± 0.01	0.27 ± 0.01	0.30 ± 0.01	0.37 ± 0.01	0.42 ± 0.01
Fengycin	0.31 ± 0.01	0.36 ± 0.04	0.37 ± 0.02	0.41 ± 0.01	0.45 ± 0.01
Iturin A	0.18 ± 0.02	0.24 ± 0.01	0.28 ± 0.01	0.32 ± 0.01	0.35 ± 0.01
Surfactin	0.42 ± 0.06	0.46 ± 0.02	0.45 ± 0.01	0.44 ± 0.02	0.46 ± 0.01

^x Growth of *X. campestris* was measured by scoring the absorbance at 492 nm after incubation at 25°C for 1 h with the corresponding lipopeptide solution.

^y Data values represent the mean ± the standard error of three independent experiments.

^z Serial dilutions of purified lipopeptide solutions were also included in the analysis.

while similar numbers of dead and living cells were typically observed after treatment with fengycin (Fig. 2M to O). Finally, the surfactin-treated samples showed the same accumulation of probes as the untreated controls, which was indicative of the weak cytotoxic effect of this lipopeptide at the bacterial membrane level (data not shown).

Visualization of the cellular damage caused by lipopeptides on *X. campestris* pv. *cucurbitae* at the ultrastructural level was followed by negative staining and transmission electron microscopy (TEM) analysis of the bacterial cells exposed to lipopeptide-enriched solutions for different periods of time (Fig. 3). The untreated cells showed a typical electro-dense cytoplasm (Fig. 3A and B). After 1 h of exposure, the first cytotoxic effects of the lipopeptides could be clearly observed, which were initiated with a general disorganization of the cytoplasm that became less electro-dense (Fig. 3C and D). After 8 h, the disintegration of the plasma membrane became evident, resulting in a partial vesiculation of the membrane fragments into small vesicles (Fig. 3E and F). Finally, after 24 h of exposure to lipopeptides, the lysis of the bacterial cell and passive efflux of the cytoplasmic contents were clearly observed (Fig. 3G and H).

To compare the cytotoxic effects of the different lipopeptides, cell suspensions of *X. campestris* pv. *cucurbitae* and *Pseudomonas syringae* pv. *lachrymans* were separately exposed for 1 h to solutions of different concentrations of purified lipopeptides and then negatively stained and subjected to TEM analysis (Fig. 4). All of the lipopeptides were cytotoxic against *X. campestris* pv. *cucurbitae* at 1 mg/ml (Fig. 4C, E, and G), with the exception of surfactin, which induced no apparent effects on bacterial cells (Fig. 4B). Iturin A and bacillomycin were clearly the most toxic compounds, producing visible cytotoxicity at the lowest assayed concentration (0.01 mg/ml) (Fig. 4F and H), whereas fengycin lost the cytotoxic effect at 0.1 mg/ml (Fig. 4D). By contrast, no cytotoxic effect was observed on *P. syringae* pv. *lachrymans* cells, which remained intact after exposure to the different lipopeptides, even at the higher concentration (1 mg/ml) (Supplementary Fig. 1S).

Biological control of bacterial pathogens.

Finally, we examined whether the antibacterial activity observed in vitro could be reproduced in vivo on melon leaves and, therefore, be responsible for the control of bacterial pathogens. Prior to performing these bioassays, values for the number of cells able to induce 90% of disease severity to the inoculated leaves (ID_{90}) were determined for *X. campestris* pv. *cucurbitae* NCPPB2597 and *Pectobacterium carotovorum* NCPPB2349 at 7×10^3 and 1×10^4 CFU, respectively. At these doses, typical necrotic and chlorotic symptoms could be observed on the leaves inoculated with these bacterial pathogens (Fig. 5). Once the suitable bacterial doses were established, the cell-washed suspensions of the different antagonistic *B. subtilis* and lipopeptide-defective mutants were evaluated for suppressive activity against both bacterial diseases (Table 6). The wild-type strains UMAF6614 and UMAF6639 significantly reduced the disease severities (67 to 84% of disease reduction) compared with the untreated controls. As previously observed

in the in vitro assays, the mutants impaired in the production of bacillomycin or iturin A completely lost the ability to control both diseases, and these leaves developed symptoms similar to those observed in the untreated leaves (Fig. 5). However, the biocontrol abilities of the fengycin mutants were slightly decreased (41 to 50% of disease reduction) compared with the wild-type strains and, as illustrated in Figure 5, yellowing and weak necrosis was observed.

To associate the observed biocontrol activity with a direct antagonistic effect of lipopeptides on the population density of the phytopathogenic bacteria on melon leaves, *X. campestris* and *P. carotovorum* populations on melon leaves were measured at the end of these experiments (Table 6). The populations of both phytopathogenic bacteria decreased significantly (more than two orders of magnitude) following treatments with the wild-type *B. subtilis* strains UMAF6614 and UMAF6639 in comparison with the untreated controls. However, population levels similar to those observed in the untreated controls were recorded when the mutants impaired in the production of bacillomycin or iturin A were applied. Finally, treatment with the mutants impaired in the production of fengycin induced a slight but significant decrease in the populations of both bacterial pathogens.

DISCUSSION

Intense research on different pathosystems has shown that biological control is an interesting strategy that might be applied in the management of plant diseases (Fravel 2005). More interestingly, the fact that the intrinsic biological skills of certain microorganisms can be physiologically manipulated offers great possibilities for success in disease control (Asaka and Shoda 1996). For the proper use of a given biocontrol agent, a deep understanding of its biocontrol mechanisms is required. Biological properties commonly associated with biocontrol agents have included the production of antimicrobial compounds and the ability to colonize the ecological niches where they are expected to develop their disease suppression activity. Thus, the *Bacillus* spp. are among the most promising biological control agents; indeed, there is evidence supporting the feasibility of this genus against diverse pathogens and on different crops (Arguelles-Arias et al. 2009; Kildea et al. 2008; Krebs et al. 1998; Pérez-García et al. 2011; Wulff et al. 2002). However, the key features contributing to their success and how they interact remain largely unknown.

The protective effect provided by *Bacillus* spp. against plant pathogens has often been associated with the production of different antimicrobial compounds (Asaka and Shoda 1996; Montesinos 2007; Ongena et al. 2005; Romero et al. 2007b, Stein 2005). Previously, we identified specific *B. subtilis* strains by their wide antifungal spectrum and ability to control cucurbit powdery mildew (Romero et al. 2004, 2007b,c). These *B. subtilis* strains also produced different families of lipopeptides compounds that are essential for the antifungal and biocontrol abilities of the bacteria. Thus, *B. subtilis* UMAF6614 and UMAF6639 produce fengycin, surfactin, and iturins (bacillomycin and iturin A, respectively), although at

Fig. 2. Cytotoxic effect of lipopeptides from *Bacillus subtilis* UMAF6639 on *Xanthomonas campestris* pv. *cucurbitae*. Cell suspensions of the bacterial pathogen were incubated for 1 h with lipopeptide-enriched solutions from cell-free culture filtrates of the *B. subtilis* UMAF6639 wild-type strain or lipopeptide-deficient derivative mutants. After incubation, fluorescent molecular probes were added and cells were visualized by phase-contrast and fluorescence microscopy to investigate the proportion of living (green) and dead (red) cells. Solutions of purified lipopeptides adjusted to 1 mg/ml were used as controls. Untreated *X. campestris* cells were observed by **A**, phase-contrast microscopy and **B** and **C**, fluorescence microscopy. *X. campestris* cells after treatments with lipopeptide-enriched solutions from *B. subtilis* UMAF6639 wild-type strain (wt) were examined by **D**, phase-contrast microscopy and **E** and **F**, fluorescence microscopy. Similar pictures were taken after treatments with lipopeptide-enriched solutions from the **G** to **I**, fengycin-deficient mutant *fenB*; **J** to **L**, iturin-A-defective transformant *ituD*; and solutions of **M** to **O**, purified fengycin or **P** to **R**, iturin A (P-R) at 1 mg/ml. Bars: 40 μ m.

different levels, in vitro. Furthermore, these strains are also able to produce lipopeptides on melon leaves in situ (Romero et al. 2007b).

The antimicrobial activity of the lipopeptides from *Bacillus* spp. has been generally described against fungi, with only a

few reports addressing their effects on bacteria (Bais et al. 2004; Etchegaray et al. 2008; Massomo et al. 2004; Sharga and Lyon 1998; Wulff et al. 2002). The present work was aimed at studying the antimicrobial spectrum of some strains of *B. subtilis* selected on the basis of their antifungal properties,

Fig. 3. Ultrastructural effects of lipopeptide-enriched solutions from cell-free culture filtrates of *Bacillus subtilis* UMAF6639 on *Xanthomonas campestris* pv. *cucurbitae*. Cell suspensions of the bacterial pathogen were incubated with butanolic extracts from *B. subtilis* UMAF6639 during different periods of time and examined by transmission electron microscopy (TEM). **A to B**, TEM micrographs of untreated *X. campestris* cells; **C to H**, TEM micrographs of *X. campestris* cells exposed to lipopeptide-enriched solutions from *B. subtilis* UMAF6639 for 1 h (**C** and **D**), 8 h (**E** and **F**), or 24 h (**G** and **H**). Bars: 0.1 μ m (**A**, **C**, **E**, and **G**) and 0.2 μ m (**B**, **D**, **F**, and **H**).

by analyzing their potential to target bacterial diseases of cucurbits. Based on their antibacterial potential in vitro, two *B. subtilis* strains were selected (UMAF6614 and UMAF6639) and their antagonistic activities were investigated in detail

against two important bacterial pathogens of cucurbits, *X. campestris* pv. *cucurbitae* and *P. carotovorum* subsp. *carotovorum*. Through TLC and bioautographical analysis, using lipopeptide-defective derivatives and purified lipopeptides, the antibacterial

Fig. 4. Ultrastructural effects of purified lipopeptides from *Bacillus subtilis* UMAF6639 on *Xanthomonas campestris* pv. *cucurbitae*. Cell suspensions of the bacterial pathogen were separately exposed to the different lipopeptides at concentrations ranging from 0.01 to 1 mg/liter for 1 h and observed by transmission electron microscopy (TEM). **A**, TEM micrograph of an untreated *X. campestris* cell; **B** to **H**, TEM micrographs of *X. campestris* cells treated with **B**, surfactin at 1 mg/ml; **C**, fengycin at 1 mg/ml; **D**, fengycin at 0.1 mg/ml; **E**, iturin A at 1 mg/ml; **F**, iturin A at 0.01 mg/ml; **G**, bacillomycin at 1 mg/ml; or **H**, bacillomycin at 0.01 mg/ml. Bars: 0.1 μ m (**A**, **B**, **D**, and **F**) and 0.2 μ m (**C**, **E**, **G**, and **H**).

Fig. 5. Effect of lipopeptides from *Bacillus subtilis* UMAF6639 on the suppression of disease symptoms elicited by *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum* on melon leaves. Detached leaves were first treated with washed cells of the wild-type strain UMAF6639 or lipopeptide-deficient mutants and then inoculated with the bacterial pathogens **A**, *X. campestris* or **B**, *P. carotovorum*. Treatments are listed as follows: untreated, nontreated control; wt, *B. subtilis* UMAF6639 wild-type strain; *fenB*, fengycin-deficient derivative; and *ituD*, iturin A-defective transformant. Panels are compositions of photographs of melon leaves treated as described and inoculated with the corresponding bacterial pathogen taken 10 or 15 days after inoculation with *P. carotovorum* or *X. campestris*, respectively.

activity could be unequivocally associated with the iturin lipopeptides iturin A and bacillomycin, produced by UMAF6639 and UMAF6614, respectively. The antifungal activity of iturins is well known; however, only a few studies have reported the bactericidal effect of iturins (Etcheagaray et al. 2008; Monteiro et al. 2005). The results presented here show conclusively the main role of iturins in the antibacterial activity of *B. subtilis* strains UMAF6614 and UMAF6639.

Lipopeptides are reported to act mainly by antagonistic interactions with the plasma membranes of target microorganisms (Etcheagaray et al. 2008; Magnet-Dana and Peypoux 1994; Romero et al. 2007a; Straus and Hancock 2006). In our study, a detailed microscopic analysis was performed to characterize the cytotoxic effects of *B. subtilis* lipopeptides on bacterial cells using *X. campestris* pv. *cucurbitae* as a target bacterium. The alterations in the integrity and permeability of the plasma membrane were initially analyzed using a double fluorescence staining technique, which revealed the strongest effects by iturins. These observations were supported by TEM analysis, which confirmed the ability of the iturin lipopeptides to induce ultrastructural damage to bacterial cells by disrupting the plasma membrane. Although the activity of these antibiotics has been mainly characterized as antifungal (Romero et al. 2007a; Stein 2005), their amphiphilic nature may allow them to ingress many diverse biological membranes (Carrillo et al. 2003; Makovitzki et al. 2006), as shown here for bacterial membranes. This ability to target fungal and bacterial pathogens makes lipopeptides one of the most attractive candidate compounds among the next generation of plant health products (Joshi and McSpadden Gardener 2006).

Previous attempts to elucidate the mode of action of lipopeptides have revealed that the formation of micelles and the dissociation of these oligomers are required for their interaction with the bacterial membranes. These requirements may ultimately lead to variations in the antimicrobial activities of lipopeptides (Straus and Hancock 2006). In fact, their efficacy and specificity may be manipulated by changing the amino acids of the peptide moiety or altering the length of the fatty acid tail (Makovitzki et al. 2006; Montesinos 2007). Thus, the strongest bactericidal activity observed for the iturin family of lipopeptides in this study suggests that this family possesses a suitable primary structure that endows them with the optimum physicochemical properties to efficiently ingress into the membranes of a broad spectrum of microorganisms (Stein 2005; Yoshida et al. 2001). By contrast, lipopeptides other than iturins may have difficulties in penetrating cell wall layers outside the cytoplasmic membrane. Thus, the comparatively larger size of fengycin could weaken its ability to access into the bacterial membranes, resulting in low toxicity as previously documented

Table 6. Severity of disease symptoms and populations levels of *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum* on melon leaves after treatments with vegetative cells of *Bacillus subtilis* wild-type strains and lipopeptide-deficient derivative mutants^w

Treatments	<i>X. campestris</i> pv. <i>cucurbitae</i>			<i>P. carotovorum</i> subsp. <i>carotovorum</i>		
	Severity ^x	Reduction ^y	Bacterial counts ^z	Severity	Reduction	Bacterial counts
Untreated	91 a	...	7.6 a	96 a	...	7.8 a
<i>B. subtilis</i> UMAF6614	30 c	67	5.1 e	25 c	74	5.4 c
6614:: <i>bamA</i>	89 a	2	6.9 ab	93 a	3	7.4 ab
6614:: <i>fenB</i>	51 b	44	6.5 bc	53 b	45	6.9 b
<i>B. subtilis</i> UMAF6639	25 c	72	5.3 de	15 c	84	4.9 c
6639:: <i>ituD</i>	88 a	3	6.9 ab	90 a	6	7.2 ab
6639:: <i>fenB</i>	54 b	41	6.0 cd	48 b	50	6.6 b

^w Data within each column are the means of three independent experiments. Values followed by the same letter in each column are not significantly different at $P = 0.05$ according to Fisher's least significant difference test.

^x Disease severity was assessed as percentage of necrotic tissue according to a scale of symptoms specific for each bacterial pathogen.

^y Percentage of disease reduction achieved by treatments referred to values of disease severity on untreated leaves.

^z Population levels of *X. campestris* and *P. carotovorum* in leaf tissue were estimated by serial dilutions and colony counts on plates of selective medium and expressed as log CFU per g of leaf tissue.

(Makovitzki et al. 2006). Similarly, surfactin did not show antibiotic activity in our study. This lipopeptide is not recognized as an antimicrobial but has been characterized as a powerful biosurfactant, a quality related to its tendency to aggregate into large micelles in aqueous solutions (Carrillo et al. 2003; Straus and Hancock 2006). This ability, which may be advantageous for the bacteria in a specific ecological context, has proven to be futile or disadvantageous regarding antagonistic interactions due to its impaired ingress into the bacterial membrane, such as occurs with fengycin-like large lipopeptides (Mukherjee and Das 2005).

All of the antibacterial effects of iturin lipopeptides observed *in vitro* were also confirmed by *in vivo* experiments. The biocontrol assays demonstrated the ability of *B. subtilis* strains UMAF6614 and UMAF6639 to provide protection against the bacterial leaf spot and soft rot agents *X. campestris* pv. *cucurbitae* and *P. carotovorum* subsp. *carotovorum*. Both *B. subtilis* strains caused a clear and significant reduction of the symptoms produced on melon leaves, which was a direct consequence of the reduction of the bacterial populations in leaf tissues. These experiments provided additional evidence for the key role of iturins. First, iturin-defective mutants completely lost their protective and antibacterial abilities. Second, fengycin-defective but iturin-producing mutants retained significant antibacterial and biocontrol activities. In previous reports, the antagonism of these strains toward the fungal pathogen *Podosphaera fusca* was associated with both iturins and fengycins that retained the highest antifungal activity (Romero et al. 2007a,b). The differential antimicrobial efficacy of iturins and fengycins could be influenced by the composition of the target membranes, as previously suggested (Makovitzki et al. 2006; Malina and Shai 2005), or the previously mentioned difficulties to penetrate cell wall layers of different natural origins.

Another important and necessary attribute of biocontrol agents is the efficient colonization of plant surfaces where they are intended to function. *Bacillus* spp. are among the most commonly found gram-positive bacteria on diverse plant surfaces (McSpadden Gardener 2004). The *B. subtilis* strains analyzed in this study have been reported to efficiently colonize plant surfaces, remaining on melon leaves at levels similar to those originally applied, even at 16 days after their application. Moreover, the fact that they were mainly found as vegetative cells rather than dormant spores clearly emphasizes their colonization fitness upon melon leaves (Romero et al. 2004, 2007c). These findings, together with the role recently attributed to mycosubtilin (another iturin-like lipopeptide) and surfactin as enhancers of the spreading qualities of the producing-cells (Leclère et al. 2006), suggest an additional role for the iturins and, consequently, emphasize their definitive contribution to plant protection.

Our findings offer an overview that supports the potential of the *B. subtilis* strains UMAF6614 and UMAF6639 as promising candidates for integrated control programs of cucurbit diseases. Their primary mode of action is attributed to the production of antibiotic lipopeptides—specifically, the family of iturins—which efficiently antagonize bacterial and fungal pathogens by inducing perturbations at their plasma membranes. In this study, no cytotoxic effect was observed with the surfactins, at least toward the bacterial species tested. However, the involvement of surfactin in the biocontrol ability of these strains should not be ruled out. The strong surfactant quality of surfactin has been associated with the ability of bacilli to colonize diverse plant surfaces or to form stable biofilms (Ahimouh et al. 2000; Bais et al. 2004; López et al. 2009), which is a quality that could be exploited against other important pathogens (Torres et al. 2007).

MATERIALS AND METHODS

Bacterial strains and culture conditions.

The bacterial strains used in this study are listed and described in Table 2. Fresh bacterial cultures were obtained from frozen stocks before each experiment. The *B. subtilis* strains and their derivatives were grown at 37°C in nutrient agar (NA), nutrient broth (NB), medium optimal for lipopeptide production (MOLP), or 868 agar medium (Ahimou et al. 2000). The cucurbit-pathogenic bacteria *Acidovorax avenae* subsp. *citrulli* NCPPB3679, *Pectobacterium carotovorum* subsp. *carotovorum* NCPPB2349, *Pseudomonas syringae* pv. *lachrymans* NCPPB537, *X. campestris* pv. *cucurbitae* NCPPB2597, and *X. campestris* pv. *melonis* NCPPB3434 were grown in NA at 25°C. Antibiotics and sodium deoxycholate, when required, were added to the culture media at the following concentrations: chloramphenicol, 5 µg/ml; kanamycin, 10 µg/ml; spectinomycin, 100 µg/ml; and sodium deoxycholate, 250 µg/ml.

In vitro evaluation of antibacterial activity.

The *B. subtilis* strains were screened for their ability to inhibit bacterial growth using the dual-culture technique (Han et al. 2005). The *B. subtilis* strains, previously grown on 868 medium, were inoculated by a sterilized toothpick onto NA plates on which the different target plant-pathogenic bacteria had previously been spread. The diameter of the inhibition zone was measured after 24 h of incubation at 25°C.

The antibacterial activity of the *B. subtilis* cell-free culture filtrates was roughly analyzed as previously described (Arrebola et al. 2003). The *B. subtilis* strains were grown on NB medium at 37°C with agitation for 3 days. After centrifugation at 4,000 × g for 10 min, the supernatants were filtered through 0.2-µm nitrocellulose membranes (Millipore Corporation, Bedford, MA, U.S.A.). The antibacterial activity of these filtrates was evaluated against *X. campestris* pv. *cucurbitae* NCPPB2597 and *Pectobacterium carotovorum* subsp. *carotovorum* NCPPB2349 as indicators. Briefly, 7-mm wells were made in the NA plates, filled with filtrate samples (properly mixed with the sterile melted media), and overlaid with a double layer of medium containing the indicator bacterium. The plates were incubated at 25°C for 24 h and the inhibition zones were measured from the border of the well to the perimeter of visible bacterial growth.

Biochemical characterization and chromatographic detection of antibacterial activity.

The cell-free supernatants from the *B. subtilis* strains were obtained as described above and subjected to the following stability tests (Romero et al. 2007b): resistance to heat, extreme pH, enzymatic degradation, solubility in different organic solvents, and estimation of molecular size using a Centricon centrifugal filter device (3 kDa) (Millipore Corporation). After treatment, the presence or absence of antibacterial activity was tested using the bioassay against *X. campestris* pv. *cucurbitae* NCPPB2597, as described above.

The chromatographic detection of antibacterial activity was performed as described by Hofemeister and associates (2004). The crude cell-free culture filtrates from the *B. subtilis* wild-type strains and derivative mutants were extracted three times with one-fourth of the volume of *n*-butanol, as described elsewhere (Romero et al. 2007b). After the butanol layers were completely evaporated, the remaining residues were dissolved in 10% methanol. These methanolic solutions were separated by TLC on normal-phase HPTLC silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) and developed at room temperature with chloroform-methanol-water (65:25:4, vol/vol). When the solvent had migrated within 1 cm of the top

of the chromatogram, the sheets were air dried and the organic compounds were detected by spraying the TLC sheets with water or 10% H₂SO₄ (Hiraoka et al. 1992; Razafindralambo et al. 1993). For bioautography analysis (chromatographic detection of antibacterial activity), the TLC sheets were developed under identical conditions, placed in 120-mm petri plates, overlaid with a thin layer of Luria-Bertani (LB) agar inoculated with *X. campestris* pv. *cucurbitae* NCPPB2597 or *P. carotovorum* subsp. *carotovorum* NCPPB2349 cells at 10⁸ CFU/ml, and inspected for areas of bacterial growth inhibition after 24 h of incubation at 25°C.

Determination of lipopeptide production.

The quantification and relative production of the lipopeptides produced in vitro by the *B. subtilis* strains UMAF6614 and UMAF6639 were estimated after purification as described in a previous study (Romero et al., 2007b). Briefly, the strains were grown in MOLP at 37°C for 72 h, and the cell-free supernatants were obtained by centrifugation. The lipopeptides were extracted from supernatants with *n*-butanol as described above. Subsequently, the samples were evaporated and the remaining precipitates were extracted with methanol. The lipopeptides present in these methanolic fractions were separated by RP-HPLC using a 4.6-mm-diameter by 250-mm-long ultrasphere analytical reverse-phase C18 column (Beckman Instrument Inc., Fullerton, CA, U.S.A.) and solutions of 0.05% trifluoroacetic acid in acetonitrile and in MilliQ water, with a flow rate of 1 ml/min (Cazorla et al. 2007). The quantitative determinations were carried out at 214 nm using purified standards of fengycin, bacillomycin, iturin A, and surfactin lipopeptides (Romero et al. 2007b).

Assessment of bactericidal activity.

The bactericidal activities of the lipopeptide-enriched solutions from *B. subtilis* wild-type strains, their lipopeptide-deficient derivatives, and purified lipopeptides were evaluated against *X. campestris* pv. *cucurbitae* NCPPB2597 according to a microplate bioassay developed to determine cell density as an indicator of bacterial growth (Malina and Shai 2005). For this assay, sterile 96-microwell plates (Nunc A/S, Roskilde, Denmark) were used. The butanolic extracts, obtained as described above, were serially diluted. Aliquots of 50 µl of these solutions were mixed with 50 µl of bacterial cell suspension adjusted to 10⁷ CFU/ml in LB medium. The plates were incubated for 1 h at 25°C, and the bacterial cell density was determined by measuring the absorbance at 492 nm with a microplate reader (Bio-Whittaker, Inc. Walkersville, MD, U.S.A.). Solutions of purified lipopeptides, adjusted to concentrations ranging from 0.001 to 1 mg/ml, were also assayed.

Fluorescence microscopy.

Fluorescence microscopy was used to examine the bacterial membrane integrity after exposure to *Bacillus* lipopeptides using the two-color fluorescence staining technique provided by the LIVE/DEAD BacLight bacterial viability kit (Invitrogen Ltd., Paisley, U.K.). Cells from the *X. campestris* pv. *cucurbitae* NCPPB2597 were grown in LB at 25°C overnight, washed twice, and resuspended in 10 mM sodium phosphate buffer (pH 7.4) at a concentration of 10⁷ CFU/ml. Aliquots of 20 µl of the bacterial cell suspension were combined with 20 µl of the butanolic extracts obtained from the *B. subtilis* UMAF6639 wild-type strain or lipopeptide-deficient derivative mutants and incubated for 1 h at 25°C. After incubation, 40 µl of the molecular probes, prepared as recommended by the manufacturer, were added, and the cell suspensions were incubated for 15 min at 25°C with agitation in the dark. Then, aliquots of 5 µl were poured onto slides and examined by phase-contrast mi-

croscopy to observe the entire bacterial cell population, and fluorescence microscopy (excitation wavelength of 485 nm and emission wavelengths of 520 or 630 nm) to visualize dead (red) or living (green) cells within the population. Solutions of purified lipopeptides, adjusted to 1 mg/ml, were used as controls.

TEM.

TEM analysis was used to determine the effects of *B. subtilis* lipopeptides on bacterial cells at the ultrastructural level. Aliquots of 20 µl containing *X. campestris* pv. *cucurbitae* NCPPB2597 cells at a concentration of 10⁷ CFU/ml were treated with 20 µl of butanolic extracts from *B. subtilis* UMAF6614 or UMAF6639 strains obtained as described above, and incubated for 1, 8, or 24 h at 25°C. After incubation, drops containing bacteria were deposited onto carbon-coated grids and negatively stained with 2% phosphotungstic acid (Makovitzki et al. 2006). The effects of the purified lipopeptides were also investigated using this technique. Solutions of purified lipopeptides adjusted to concentrations ranging from 0.01 to –1 mg/ml were tested against *X. campestris* pv. *cucurbitae* for 1 h at 25°C and examined by TEM according to the same methodology. *Pseudomonas syringae* pv. *lachrymans* NCPPB537 cells were also included in the analysis as a negative control.

Biological control assays.

Prior to performing the biological control experiments, the virulence of the bacterial strains *X. campestris* pv. *cucurbitae* NCPPB2597 and *Pectobacterium carotovorum* subsp. *carotovorum* NCPPB2349 was assessed by deducing their corresponding ID₉₀ values. Biological control assays were carried out on detached melon leaves (*Cucumis melo* ‘Rochet’) using the double petri plate system and the bioassay as previously described (Romero et al. 2003, 2004). Prior to inoculation with the bacterial pathogen, the cell suspensions, at a concentration of the biocontrol agents (*B. subtilis* wild-type strains UMAF6614 or UMAF6639 or their lipopeptide-deficient derivative mutants) of 10⁸ CFU/ml, were sprayed on the melon leaves. After the leaf surface was completely air dried, the pathogenic bacteria were inoculated at the corresponding ID₉₀ value by placing 10-µl droplets of the proper bacterial suspension onto the melon leaves and piercing through the droplets with an entomological pin (Arrebola et al. 2007). After inoculation, the leaves were incubated at 25°C under a 16-h photoperiod. After 10 to 15 days of incubation, the disease symptoms were recorded according to a 0-to-3 scale of values specific for each pathogen based on the diameter of chlorotic and necrotic symptoms. The disease severity was calculated as $[(0a + 1b + 2c + 3d)/3N] \times 100$, where *a*, *b*, *c*, and *d* were the number of inoculation points corresponding to the scale values 0, 1, 2, and 3, respectively, and *N* was the total number of inoculation points assessed (*a* + *b* + *c* + *d*).

Quantification of bacterial populations in leaf tissue.

To further clarify the antibacterial activity of *B. subtilis* lipopeptides in planta, the population levels of the pathogenic bacteria *P. carotovorum* pv. *cucurbitae* NCPPB2597 and *X. campestris* subsp. *carotovorum* NCPPB2349 were determined in leaf tissue following treatment with *B. subtilis* wild-type strains UMAF6614 or UMAF6639 or their lipopeptide-deficient derivative mutants. Disks were taken from inoculated leaves with a sterilized cork borer and then homogenized in 10 ml of sterile phosphate-buffered saline. The populations of bacterial pathogens were determined by serial dilutions on plates of selective NA medium supplemented with 0.025% sodium deoxycholate to inhibit the growth of *B. subtilis* and

other gram-positive bacteria (Leifson 1935). The colony counts for *P. carotovorum* and *X. campestris* were established after incubation at 25°C for 24 or 48 h, respectively.

Statistical analysis.

The data were analyzed using the statistics software SPSS 8.0 (SPSS Inc., Chicago). The one-way analysis of variance was applied, and the means for each treatment were separated by Fisher's least significant difference test ($P = 0.05$).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The *Bacillus* Genetic Stock Center (BGS): www.bgsc.org
 The GenoList World-Wide Web server:
genodb.pasteur.fr/cgi-bin/WebObjects/GenoList