Mycosubtilin Overproduction by *Bacillus subtilis* BBG100 Enhances the Organism's Antagonistic and Biocontrol Activities

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A *Bacillus subtilis* derivative was obtained from strain ATCC 6633 by replacement of the native promoter of the mycosubtilin operon by a constitutive promoter originating from the replication gene *repU* of the *Staphylococcus aureus* plasmid pUB110. The recombinant strain, designated BBG100, produced up to 15-fold more mycosubtilin than the wild type produced. The overproducing phenotype was related to enhancement of the antagonistic activities against several yeasts and pathogenic fungi. Hemolytic activities were also clearly increased in the modified strain. Mass spectrometry analyses of enriched mycosubtilin extracts showed similar patterns of lipopeptides for BBG100 and the wild type. Interestingly, these analyses also revealed a new form of mycosubtilin which was more easily detected in the BBG100 sample. When tested for its biocontrol potential, wild-type strain ATCC 6633 was almost ineffective for reducing a *Pythium* infection of tomato seedlings. However, treatment of seeds with the BBG100 overproducing strain resulted in a marked increase in the germination rate of seeds. This protective effect afforded by mycosubtilin overproduction was also visualized by the significantly greater fresh weight of emerging seedlings treated with BBG100 compared to controls or seedlings inoculated with the wild-type strain.

Members of the *Bacillus subtilis* family produce a wide variety of antibacterial and antifungal antibiotics. Some of these compounds, like subtilin (41), subtilosin A (2), TasA (34), and sublancin (27), are of ribosomal origin, but others, such as bacilysin, chlorotetain, mycobacillin (41), rhizocticins (19), bacillaene (28), difficidin (40), and lipopeptides belonging to the surfactin, iturin, and fengycin families (41), are formed by nonribosomal peptide synthetases and/or polyketide synthases. The latter compounds are amphiphilic cyclic peptides composed of 7 α -amino acids (surfactins and iturins) or 10 α -amino acids (fengycins) linked to one unique β -amino fatty acid (iturins) or β -hydroxy fatty acid (surfactins and fengycins). The length of the fatty acid chain varies from C₁₃ to C₁₆ for surfactins, from C₁₄ to C₁₇ for iturins, and from C₁₄ to C₁₈ in the case of fengycins. Different homologous compounds for each lipopeptide family are thus usually coproduced (1, 16). Iturins and fengycins display strong antifungal activity and inhibit the growth of a wide range of plant pathogens (11, 17, 20, 22, 35). Surfactins are not fungitoxic by themselves but have some synergistic effects on the antifungal activity of iturin A (23).

B. subtilis ATCC 6633 produces subtilin (21), subtilosin (33), rhizocticin (19), and two lipopeptides, surfactin and mycosubtilin, a member of the iturin family (21). Production of surfactin requires the *urf* operon encoding the three subunits of surfactin synthetase that catalyze the thiotemplate mechanism

of nonribosomal peptide synthesis to incorporate the seven amino acids into the surfactin lipopeptide. The mycosubtilin gene cluster consists of four open reading frames, designated *fenF*, *mycA*, *mycB*, and *mycC*, controlled by the same promoter, *P_{myc}* (Fig. 1) (9). The subunits encoded by the three *myc* genes contain the seven modules necessary to synthesize the peptide moiety of mycosubtilin. The N-terminal multifunctional part of *mycA* shows strong homology with fatty acid and polyketide synthases.

The production of surfactin is activated by a regulatory system coupled to the accumulation of cell-derived extracellular signals at the end of exponential growth (7), while iturin synthesis is induced during the stationary phase (16).

Of the biological control alternatives to chemical pesticides used for reducing plant diseases, the application of nonpathogenic soil bacteria living in association with plant roots is promising. Treatment with these beneficial organisms was in many cases associated with reduced plant diseases in greenhouse and field experiments. These bacteria can antagonize fungal pathogens by competing for niche and nutrients, by producing low-molecular-weight fungitoxic compounds and extracellular lytic enzymes, and, more indirectly, by stimulating the defensive capacities of the host plant (10, 26, 30, 35). On the basis of the wide diversity of powerful antifungal metabolites that can be synthesized by *B. subtilis* strains, it was suggested that antibiotic production by these strains plays a major role in plant disease suppression (4, 32, 35, 38). These bacteria were reported to be effective for controlling many plant or fruit diseases caused by soilborne, aerial, or postharvest pathogens (4, 22, 35, 37, 39). Some of these strains are currently used in

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FIG. 1. Replacement in *B. subtilis* ATCC 6633 of the original *P_{myc}* promoter by the *P_{repU}-neo* cassette using homologous recombination between genomic DNA of the strain and hybrid plasmid pBG106. (A) Recognition of homologous regions located (i) after the termination region of the *pbp* gene (coding for a penicillin-binding protein) located upstream of the mycosubtilin operon (for convenience, the cassette generated by PCR in this region was designated $\epsilon''pbp''$) and (ii) immediately downstream of the *P_{myc}* promoter (cassette $\epsilon fenF$). Four genes, *fenF*, *mycA*, *mycB* and *mycC*, constitute the mycosubtilin operon and code for a malonyl coenzyme A transacylase and three peptide synthetases, respectively. *yngL*, gene coding for an unknown function; *PrepU*, promoter of the replication gene of pUB110; *neo*, gene conferring resistance to neomycin/kanamycin from pUB110 (15). An asterisk indicates the site newly created after ligation between the *BspEI*- and *XmaI*-compatible cohesive ends. (B) Construct obtained for the genomic DNA of the strain following homologous recombination (generated by the inability of pUC19 to replicate in *Bacillus* spp., together with the selective pressure for resistance to neomycin). The mycosubtilin operon came under control of the *P_{repU}* constitutive promoter.

commercially available biocontrol products (3, 5). However, most studies have focused primarily on the degree of disease reduction, and mechanisms of suppression in soil have not been as extensively investigated.

In this study, the native promoter of the mycosubtilin operon from *B. subtilis* ATCC 6633 was replaced by the *P_{repU}* promoter from staphylococcal plasmid pUB110, which was shown previ-

ously to be strong and constitutive in *B. subtilis* (36). Growth and lipopeptide production by the derivative were compared to growth and lipopeptide production by the wild type, and the antimicrobial and hemolytic activities of the derivative and the wild type were also compared. The effect of early overproduction of mycosubtilin in the biocontrol of damping-off caused by *Pythium aphanidermatum* in tomato seedlings was also evaluated.

TABLE 1. Strains and plasmids

Strain or plasmid	Description ^a	Source or reference
Bacterial strains		
<i>Escherichia coli</i> DH5 α	$\Phi 80dlacZ\Delta M15$ <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> ($r_k^- m_k^+$) <i>supE44 relA1 deoR</i> $\Delta(lacZYA-argF)U169$ <i>phoA</i>	Promega, Madison, WI
<i>Bacillus subtilis</i> ATCC 6633	Produces mycosubtilin, surfactin, subtilin, subtilosin, and rhizoctinins	9
<i>B. subtilis</i> BBG100	ATCC 6633 derivative overproducing mycosubtilin, Nm ^r	This study
<i>Erwinia chrysanthemi</i> 3937		14
<i>Micrococcus luteus</i>		Lab stock
<i>Pseudomonas aeruginosa</i> 7NSK2		12
Fungi		
<i>Botrytis cinerea</i>	Wild type	Lab stock
<i>Fusarium oxysporum</i>	Wild type	Lab stock
<i>Pythium aphanidermatum</i>	Wild type	26
Yeasts		
<i>Pichia pastoris</i>	Wild type	Lab stock
<i>Saccharomyces cerevisiae</i>	Wild type	Lab stock
Plasmids		
pUC19	Cloning vector, Ap ^r	New England Biolabs, Beverly, MA
pBG101	0.5-kb <i>fenF</i> PCR fragment inserted into pUC19, Ap ^r	This study
pBG102	0.7-kb <i>pbp</i> PCR fragment inserted into pUC19, Ap ^r	This study
pBG103	0.5-kb <i>SalI-EcoRI</i> <i>fenF</i> fragment from pBG101 inserted into pBG102, Ap ^r	This study
pBEST501	pGEM4 carrying the <i>P_{repU}</i> promoter and <i>neo</i> gene from pUB110, Nm ^r	15
pBG106	<i>P_{repU}-neo</i> fragment inserted into pBG103, Ap ^r Nm ^r	This study

^a Ap^r, resistance to ampicillin; Nm^r, resistance to neomycin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The microorganisms and plasmids used in this study are listed in Table 1. *B. subtilis* strains were grown at 30°C in either Landy medium (20) or medium 863 (1). *Escherichia coli* DH5 α was cultured at 37°C in Luria-Bertani (LB) medium supplemented, when required, with the following antibiotics: ampicillin (50 μ g ml⁻¹; Sigma, St. Louis, MO), neomycin (20 μ g ml⁻¹; Serva, Heidelberg, Germany), and streptomycin (25 μ g ml⁻¹; Sigma). The yeast strains were grown at 28°C in medium 863 (1), and the fungal strains were cultured at 30°C on potato dextrose agar (Biokar Diagnostics, Beauvais, France).

Molecular biology procedures. Total genomic DNA was extracted from *B. subtilis* ATCC 6633 and purified using genomic tips 20/G together with the corresponding buffers purchased from QIAGEN (Hilden, Germany). Plasmid DNAs were prepared from *E. coli* using either Miniprep Spin or Maxiprep kits (QIAGEN). Screening for hybrid plasmids in various *E. coli* transformants was done by the "boiling" procedure of Holmes and Quigley (13). For restriction endonuclease digestion, ligation, and transformation of *E. coli* by CaCl₂ thermal shock we used standard procedures (31). *B. subtilis* ATCC 6633 was transformed by electroporation using the method of Dennis and Sokol (8).

For construction of the pUC19-derived plasmid dedicated to promoter exchange by homologous recombination in *B. subtilis*, the *pbp* and *fenF* fragments were generated by PCR using *Taq* polymerase "Arrow" from Qiogene (Montreal, Canada). The primers were designed by using the previously published sequence of the mycosubtilin operon from strain ATCC 6633 (PubMed nucleotide accession no. AF184956) (9). The following primers were used: (i) for *pbp*, forward primer 5'-TTAGAAGAGCATGCAAAAATG-3' (the underlined artificial SphI site was generated by substitution of the two bases in boldface type) and reverse primer 5'-CCCTCCAATCTTTTGAACG-3'; and (ii) for *fenF*, forward primer 5'-GACATGTATCCGTTCTAGAAGATTG-3' (the underlined artificial XbaI site was generated by substitution of the two bases in boldface type) and reverse primer 5'-ATCGGCCATTCAGCATCTC-3'. The PCR conditions consisted of an initial denaturation step at 95°C for 2 min, followed by 30 cycles of 30 s at 95°C, 30 s at 45°C, and 30 s at 70°C. The final extension step was at 70°C for 2 min.

The two PCR-generated cassettes were purified from 2% agarose gels using a QIAquick kit (QIAGEN), treated with proteinase K (50 μ g ml⁻¹) for 1 h at 37°C, and subjected to deproteinization using a phenol-chloroform procedure. The *fenF* fragment was XbaI and BspEI double digested and introduced between the XbaI and XmaI sites of pUC19 to obtain pBG101. After SphI and Mph1103I double digestion, the *pbp* fragment was inserted into SphI and PstI sites of pUC19, generating pBG102. Then, after EcoRI and SalI double digestion, the *fenF* fragment was inserted at the corresponding sites of pBG102. The resulting construct was designated pBG103. After XbaI digestion, the *P_{repU-neo}* fragment was extracted from pBEST501 (15) and inserted into the XbaI site of pBG103. This construct, designated pBG106 (Fig. 1), was then used to transform *B. subtilis* ATCC 6633, which was plated on LB agar containing neomycin to select recombinants and incubated at 37°C.

Lipopeptide purification and identification. Cultures were centrifuged at 15,000 $\times$ g for 1 h at 4°C. For lipopeptide extraction, 1-ml samples of supernatants were purified on C₁₈ Maxi-Clean cartridges (Alltech, Deerfield, IL) used according to the recommendations of the supplier. Lipopeptides were eluted with 5 ml of pure methanol (high-performance liquid chromatography grade; Acros Organics, Geel, Belgium). The extract was dried, and the residue was dissolved in methanol (200 μ l) before analysis by high-performance liquid chromatography using a C₁₈ column (5 μ m; 250 by 4.6 mm; VYDAC 218 TP; VYDAC, Hesperia, CA). Each family of lipopeptides was separately analyzed with the acetonitrile-water-trifluoroacetic acid solvent system (40:60:0.5 [vol/vol/vol] and 80:20:0.5 [vol/vol/vol] for iturins and surfactins, respectively). Samples (20 μ l) were injected, and compounds were eluted at a flow rate of 1 ml min⁻¹. Purified iturins and surfactins were purchased from Sigma (St. Louis, MO). The retention time and second derivatives of UV-visible spectra (Waters PDA 996 photodiode array detector; Millenium Software) of each peak were used to identify the eluted molecules.

Lipopeptide extracts were further analyzed by matrix-assisted laser desorption/ionization—time of flight mass spectrometry (MS). A saturated solution of α -cyano-4-hydroxy-cinnamic acid was prepared in a 3:1 (vol/vol) solution of CH₃CN and H₂O containing 0.1% trifluoroacetic acid. The cell culture supernatant was diluted 10-fold with an α -cyano-4-hydroxy-cinnamic acid-saturated solution. Then 0.5 μ l of this solution was deposited on the target. Measurement was performed using a UV laser desorption-time of flight mass spectrometer (Bruker Ultraflex tof; Bruker Daltonics) equipped with a pulsed nitrogen laser (λ

= 337 nm). The analyzer was used at an acceleration voltage of 20 kV. Samples were measured in the reflectron mode.

Evaluation of antimicrobial and hemolytic activities. Supernatants from *B. subtilis* cultures obtained from various media were filter sterilized with 0.2- μ m-pore-size membranes and treated or not treated for 1 h at 37°C with protease (type XIV; final concentration, 10 μ g ml⁻¹; Sigma) to neutralize subtilin and subtilisin activities.

Antimicrobial activities of supernatant samples from both wild-type and modified strains were tested by plate bioassays. The bacterial and yeast strains to be tested were grown in LB medium and 863 medium, respectively. Overnight bacterial cultures (2 ml) were diluted (10⁻²) and inoculated by flooding 2 ml onto LB medium plates. The excess liquid was removed, and the plates were allowed to dry under a laminar flow hood for 30 min. In tests performed with yeast strains, 4 ml of semisolid 863 medium (0.8% agar) containing 100 μ l of a diluted cell suspension (10⁻¹) were spread onto 863 medium plates. In both cases, 200- μ l portions of supernatant samples were deposited in 10-mm-diameter wells created in the solidified media using sterile glass tubes. The plates were incubated at either 30°C or 37°C depending on the strain tested. A similar method was used to test supernatant samples for their antifungal activities against filamentous fungi. Mycelial plugs (5 mm) were deposited in the center of the plates at equal distances from the wells. The plates were incubated at 28°C, and inhibition zones were measured after 1 to 3 days. To evaluate the hemolytic activities of the various supernatants, 200- μ l samples were dispensed into wells made in blood agar plates (with 5% defibrinated sheep blood; Eurobio, Les Ulis, France). Hemolytic activity was visualized by development of a clear halo around the wells after incubation at 37°C. In all cases, two replicate plates were used for each strain on each medium, and the experiment was repeated once.

Determination of MIC. Serial half-dilutions of filter-sterilized culture supernatants, containing known concentrations of mycosubtilin, were prepared up to 1/1,024 using 863 medium. After inoculation with 100 μ l of a diluted *Saccharomyces cerevisiae* culture (about 10⁵ cells ml⁻¹), the test tubes were incubated at 30°C. The MIC was determined by taking into account the higher dilution at which no growth of the test organism was visible.

Biocontrol assays with tomato. For preparation of a bacterial inoculum, *Bacillus* strains were grown at 30°C for 24 h in Landy medium. Cells were harvested by centrifugation at 35,000 $\times$ g for 20 min, and the cell pellet was washed twice with sterile saline water (0.85% NaCl). Vegetative cell suspensions were then diluted in order to obtain the desired bacterial concentration for seed treatment. The origin of the fungal pathogen *P. aphanidermatum*, maintenance of this organism, and preparation of suspensions used in the bioassays have been described previously (24).

In the damping-off assays, tomato seeds (*Lycopersicon esculentum* L. cv. Merveille des Marchés) were germinated in a peat substrate (Brill Substrate GmbH & Co. KG, Georgsdorf, Germany), referred to below as "soil." Prior to sowing, seeds were washed three times (5 min each) with sterile distilled water and soaked for 10 min in the appropriate bacterial suspension at a concentration of approximately 4 $\times$ 10⁸ CFU ml⁻¹ or in NaCl 0.85% in the case of control plants. In every experiment, 200 seeds were used for each treatment. The seeds were sown in large plastic trays containing soil previously infected with *P. aphanidermatum* by mixing with a suspension of mycelial fragments. The final concentration of the pathogen in the substrate for plant growth was 10⁵ propagules g (dry weight) of soil⁻¹. The trays were incubated in a growth cabinet set to maintain the temperature at 28°C at 95% relative humidity with a photoperiod of 16 h. Seedling emergence was recorded after 12 days, and the number of healthy plantlets was compared to the number of seeds.

RESULTS

Construction of the BBG100 mutant by allelic exchange. Several transformation experiments with *B. subtilis* ATCC 6633 and pBG106 led to isolation of 15 Nm^r colonies. Genomic DNA of these clones and the wild-type strain were purified. Direct observation of the restriction endonuclease (HindIII and PstI) profiles did not reveal any major difference (data not shown). Replacement of the natural promoter by the constitutive promoter *P_{repU}* associated with the *neo* gene was demonstrated by PCR amplification of genomic DNA with the *epbp* forward and *efenF* reverse primers. For one of the different colonies tested, a ~2.8-kb fragment was obtained instead of the ~1.5-kb fragment obtained with the wild type. The corre-

TABLE 2. Biomass and lipopeptide production by the wild-type ATCC 6633 strain and the BBG100 derivative after 72 h of growth^a

Prepn	Biomass (g liter ⁻¹)	Lipopeptide production (mg liter ⁻¹)	
		Mycosubtilin	Surfactin
Wild type in flask	3.23 (0.13)	17 (0.5)	15 (4.1)
BBG100 in flask	3.19 (0.24)	203 (12.6)	10 (3.4)
Wild type in bioreactor	3.25 (0.35)	4.35 (5.1)	1.15 (1.2)
BBG100 in bioreactor	4.45 (1.4)	66 (0.7)	3.35 (4.03)

^a The values are means from two experiments, and the values in parentheses are standard deviations.

sponding modified strain, designated BBG100, was compared to the wild type for the lipopeptide production level and biological activities.

Mycosubtilin overproduction by BBG100. Mycosubtilin production was monitored upon growth of both strains in agitated Erlenmeyer flasks and 3-liter bioreactors for 3 days (Table 2). Although the absolute levels of mycosubtilin were different in the shake flasks and the bioreactors, 12- to 15-fold increases were observed after 72 h in the BBG100 culture supernatant under the two different growth conditions. Greater production of mycosubtilin was observed in the flask with BBG100 (63.6 mg/g of cells). As expected, surfactin synthesis was not affected by replacement of the promoter since the levels of production by BBG100 and the wild type were similar under both growth conditions. The lower concentrations found in the bioreactors than in the shake flasks were probably due to the low aeration rate used in the bioreactors in order to limit liquid extraction by foaming. This resulted in lower oxygen transfer compared to that in the well-agitated flasks and thus in a reduced rate of production of lipopeptides since the synthesis of these molecules is positively influenced by oxygen (16).

The time courses of the evolution of biomass concentration and the pHs during the 72 h of growth in the fermentors were also very similar for the two strains. Typically, acidification of the medium was observed during the early exponential growth phase, and this acidification was due to the production of organic acids from glucose. This was followed by a neutralization step during the second growth phase related to the consumption of these acids and by a slight alkalization due to the use of glutamic acid as a carbon source by the cells (data not shown). It is thus likely that BBG100 had a physiological behavior similar to that of the wild type.

Analysis of lipopeptide production during the first 8 h of growth in the bioreactor revealed early synthesis of mycosubtilin by BBG100 (Fig. 2). Significant amounts of mycosubtilin were produced after 4 h of incubation when the cells entered the exponential growth phase. Despite a similar biomass level, mycosubtilin production by the wild type was not observed during the first 8 h, as expected since the synthesis of such compounds is known to occur only at the beginning of the stationary phase.

Matrix-assisted laser desorption ionization—time of flight mass spectrometry analyses of lipopeptide extracts allowed identification of several homologues of surfactins and mycosubtilins produced by both strains (Fig. 3). Signals attributed to protonated forms of mycosubtilin and surfactin and their Na⁺

FIG. 2. Early stage of growth (solid symbols) and mycosubtilin production (open symbols) of *B. subtilis* ATCC 6633 (□) and its BBG100 derivative (Δ) in a bioreactor. OD 600 nm, optical density at 600 nm.

and K⁺ adducts are summarized in Table 3. However, MS peaks showing higher intensity were detected in the extract from BBG100; a signal at *m/z* 1095.54 corresponded to the M+K⁺ ion of the C₁₅ homologue of mycosubtilin, and, more interestingly, there was a signal at *m/z* 1137.6 which could not be attributed to known ions of surfactin or mycosubtilin.

Biological activities. BBG100 and the wild type were compared for their antagonistic properties against a wide range of microorganisms. Supernatants from both strains did not inhibit the growth of *Erwinia chrysanthemi*, *E. coli*, and *Pseudomonas aeruginosa* even after 10-fold concentration. When tested on *Micrococcus luteus*, however, the two supernatants generated similar growth inhibition zones that completely disappeared upon treatment with protease type XIV, which neutralizes bacteriocin-like activities. By contrast, BBG100 culture supernatant induced growth inhibition zones significantly larger than those observed for the wild-type supernatant when it was tested against three phytopathogenic fungi, *Botrytis cinerea*, *Fusarium oxysporum*, and *P. aphanidermatum*, and two yeasts, *Pichia pastoris* and *S. cerevisiae* (Table 4). Protease treatment of the supernatants slightly reduced the antifungal activity against *P. aphanidermatum*.

Serial dilutions of culture supernatants from both strains were tested independently for their inhibitory effects on the growth of *S. cerevisiae*. An eightfold-higher dilution of the BBG100 supernatant than of the wild-type supernatant was necessary to obtain the MIC of mycosubtilin. In both cases, this MIC was determined to be 8 μg ml⁻¹. The data confirmed that the antagonistic activity against yeast of both supernatants was essentially due to mycosubtilin.

When tested for lytic activity on blood corpuscles, the supernatant from BBG100 yielded greater hemolytic areas than the supernatant of the wild type (Fig. 4).

Protection against *Pythium* damping-off of tomato seedlings. Biocontrol assays were conducted with the tomato/*Pythium*

FIG. 3. Matrix-assisted laser desorption/ionization—time of flight spectra of lipopeptides produced by *B. subtilis* ATCC 6633 (A) and BBG100 (B).

pathosystem to compare the ability of wild-type strain ATCC 6633 with that of BBG100 for reducing seedling infection. As shown in Table 5, pretreatment of tomato seeds with vegetative cells of the wild-type strain failed to have any protective effect but appeared to be conducive to disease development. However, inoculation with the lipopeptide-overproducing derivative prior to planting led to enhanced seedling emergence that was consistently observed in four independent experiments, while strong differences were observed in disease incidence. Whether they were previously inoculated with the wild-type, with the BBG100 strain, or with no strain (healthy control), the germination rates of seeds in the absence of pathogen did not vary significantly and were in most cases between 90% and 95% (Table 5). The protective effect of BBG100 was also illustrated by an increase in the size and vigor of emerging plantlets compared to the size and vigor of diseased controls or plants inoculated with the wild type (Fig. 5). In one representative experiment, the mean value for the fresh weight of individual plants (aerial part, harvested after 18 days of incuba-

tion) was significantly greater following seed treatment with the BBG100 strain (0.79 g/plant) than it was for nonbacterized plants (0.31 g/plant) or for plants inoculated with wild-type strain ATCC 6633 (0.23 g/plant).

DISCUSSION

In this work, we replaced the native promoter of the mycosubtilin operon of *B. subtilis* ATCC 6633 by a constitutive promoter which governs the replication gene *repU* from *S. aureus* plasmid pUB110. This led to isolation of the BBG100 derivative, which had a 15-fold increase in the mycosubtilin production rate. The P_{repU} promoter was previously reported

TABLE 3. Calculated mass values of $M+H^+$, $M+Na^+$, and $M+K^+$ ions corresponding to identified homologues of surfactins and mycosubtilins in culture extracts from *B. subtilis* ATCC 6633 and the BBG100 overproducing derivative

Lipopeptide	Mass value		
	$M+H^+$	$M+Na^+$	$M+K^+$
Surfactin C ₁₃	1008.66	1030.64	1046.61
Surfactin C ₁₄	1022.67	1044.66	1060.63
Surfactin C ₁₅	1036.69	1058.67	1074.65
Mycosubtilin C ₁₅	1057.57	1079.55	1095.52
Mycosubtilin C ₁₆	1071.58	1093.56	1109.54
Mycosubtilin C ₁₇	1085.6	1107.58	1123.55

TABLE 4. Growth inhibition activities of supernatants obtained from growth of the ATCC 6633 wild-type strain and the BBG100 derivative

Strain	Antagonistic activity ^a			
	Wild type		Strain BBG100	
	Supernatant	Protease treated	Supernatant	Protease treated
<i>E. chrysanthemi</i>	—	—	—	—
<i>E. coli</i>	—	—	—	—
<i>P. aeruginosa</i>	—	—	—	—
<i>M. luteus</i>	+++	—	+++	—
<i>B. cinerea</i>	+/-	+/-	+++	+++
<i>F. oxysporum</i>	+/-	+/-	++	++
<i>P. aphanidermatum</i>	+/-	—	++	+
<i>P. pastoris</i>	—	—	++	++
<i>S. cerevisiae</i>	+/-	+/-	+++	+++

^a The intensity of the antagonistic activity was rated on the basis of the size of growth inhibition zones from the wells in which supernatant samples were deposited to the edge of the spreading fungal mycelium or cell colony. —, 0 mm; +/-, 1 to 4 mm; +, 5 to 7 mm; ++, 8 to 9 mm; +++, 10 mm or more.

FIG. 4. Hemolytic activities of supernatants obtained after growth of the wild-type strain in Landy medium (a) or 863 medium (c) and of strain BBG100 in Landy medium (b) or 863 medium (d).

to enhance the biosynthesis of iturin A, another antifungal lipopeptide structurally very similar to mycosubtilin, by about threefold in *B. subtilis* RB14 (36).

When tested against different bacteria, yeasts, and fungi, the supernatant of the wild-type strain showed very good antagonistic activity only against *M. luteus*. This activity, which was also detected with the supernatant of the modified strain, completely disappeared upon pretreatment with protease. Thus, the antibiotic activity could be attributed to some protease-sensitive compounds, like subtilin and subtilisin, known to be produced by this strain (21, 33). The very weak antifungal activity displayed by the wild-type strain suggested that rhizoctinins and mycosubtilin are produced in very small amounts. The slight reduction in antagonistic activity against *P. aphanidermatum* observed after proteolytic treatment could have resulted from amino acids or oligopeptides liberated by the treatment and known to neutralize the biological activity of rhizoctin (19). By contrast, P_{repU} -governed mycosubtilin overproduction in *B. subtilis* BBG100 led to clearly enhanced fungitoxic activities, showing that this lipopeptide plays a crucial role in the antagonism developed by the strain.

When applied to seeds or mixed with soil, some *B. subtilis* strains were reported to provide crop protection mostly due to direct control of soilborne pathogens through efficient production of various fungitoxic metabolites (3, 29, 32). By use of the tomato/*P. aphanidermatum* pathosystem, this study demonstrated that overproduction of mycosubtilin by *B. subtilis*

FIG. 5. Plantlets obtained from seeds treated either with wild-type *B. subtilis* strain ATCC 6633 (A), with the mycosubtilin-overproducing derivative BBG100 (B), or with water (C) (disease control) in *P. aphanidermatum*-infested soil 18 days after sowing.

ATCC 6633 may confer some biocontrol potential to a strain that does not naturally protect plants. Based on the mean values calculated from pooled data, the germination rate of seeds treated with the mycosubtilin overproducer was 31% greater than that of control seeds and 48% greater than that of seeds treated with the wild type. As mycosubtilin displays strong antifungal activity in vitro against *P. aphanidermatum*, it is obvious that the 15-fold-higher rate of in vitro production of this compound is involved in the protective effect developed in vivo by the modified strain. Early and higher production of the lipopeptides probably enhances the biological effect of the strain by immediately reducing plant pathogen growth. The role played by these molecules is reinforced by the fact that other possible biocontrol mechanisms are seemingly not involved. For example, some *B. subtilis* strains were reported to reduce disease incidence indirectly by triggering systemic resistance in the plant (25). We performed some experiments with tomatoes preinoculated at the root level with either wild-type strain ATCC 6633 or the mycosubtilin-overproducing derivative before challenge with the pathogen *B. cinerea* on leaves. This procedure is used to reveal disease suppression due to induction of resistance in the host plant by bacteria. However, none of the strains had a protective effect under these conditions, showing that they do not have any plant resistance-inducing activity (data not shown). In the same line, growth promotion activity *sensu stricto* could also probably not be used to explain the beneficial effect of the mycosubtilin overproducer. The size and robustness of plants inoculated with the modified strain were greater than the size and robustness of disease controls and were very similar to the size and robustness of untreated controls when they were grown in a soil not infested with the pathogen (data not shown). In contrast to its overproducing derivative, wild-type strain ATCC 6633 did not have any protective effect on tomato seedlings. Surprisingly, strain ATCC 6633 even appeared to be conducive to the disease. However, when grown in the absence of pathogen, tomato plantlets inoculated with the wild type were similar to the control plants, suggesting that the strain did not have any phytotoxic effects *per se*.

Mass spectrometry analyses of supernatants from *B. subtilis* ATCC 6633 and BBG100 revealed the presence of two main molecular ions corresponding to the homologous mycosubtilins with C_{16} or C_{17} fatty acid chains. These homologues are

TABLE 5. Effect of strain ATCC 6633 and of the overproducing derivative BBG100 on the reduction of damping-off of tomato plants caused by *P. aphanidermatum*^a

Pathogen	Treatment Bacterium	Seedling emergence (%)			
		Expt 1	Expt 2	Expt 3	Expt 4
—	None	ND ^b	96	95	ND
—	ATCC 6633	ND	90	92	ND
—	BBG100	ND	93	88	ND
+	None	38	48	59	8
+	ATCC 6633	31	25	42	6
+	BBG100	53	59	69	34

^a Two hundred seeds were used for each treatment in every experiment, and the number of healthy plantlets was counted 12 days after planting.

^b ND, not done.

considered to be more biologically active than the iturins, which have shorter hydrocarbon side chains (C_{14} and C_{15}) (11). It has been shown that fungitoxicity increases with the number of carbon atoms in the fatty acid chain; i.e., C_{17} homologues are 20-fold more active than the C_{14} forms. This was also shown by the similarity of the in vitro antagonistic activity of BBG100 and the in vitro antagonistic activity of other *Bacillus* strains that produce larger amounts of iturinic compounds with shorter fatty acid chains (16, 35).

The overproduction of mycosubtilin by the BBG100 derivative was also accompanied by qualitative changes in the pattern of lipopeptides. Interestingly, a signal at m/z 1137.7 was clearly enhanced. The corresponding compound is probably structurally similar to iturins since its appearance followed the purification of mycosubtilin. In addition, it should correspond to a K^+ adduct since MS/MS analysis did not reveal any fragmentation (data not shown). Bacillomycin F with a C_{17} fatty acid chain is the sole iturin form that could correspond to this molecular weight. However, a single insertion of the new promoter was confirmed in the mycosubtilin operon. Thus, overexpression of bacillomycin synthetases is obviously not involved. This signal could thus be attributed to a modified mycosubtilin with either a C_{18} fatty acid chain or a peptide moiety containing a Thr instead of a Ser. In both cases, this molecule represents a new form of mycosubtilin. Indeed, such a long fatty acid chain was never encountered in iturin-like lipopeptides, and amino acid residue replacement has never been demonstrated with iturin derivatives. However, the last phenomenon may occur, as shown in the case of the nonribosomal surfactin synthetase which possesses adenylation domains able to activate different amino acid residues with similar side chains (18). Similarly, the mycobactin synthetase contains an adenylation domain that may recognize both L-serine and L-threonine (6). Such low specificity could thus also be observed in mycosubtilin synthetase. Further structural investigations are being performed to confirm this hypothesis.

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