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**Production of a novel mixture of mycosubtilins by mutants of *Bacillus subtilis***

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## ABSTRACT

Using promoter exchange and gene knock-out strategies, two mutant strains, the so-called BBG116 and BBG125, were constructed from *Bacillus subtilis* wild-type strain ATCC 6633, a surfactin and mycosubtilin producer. Compared to the parental strain, both mutants overproduced constitutively mycosubtilin, while BBG125 had lost the ability to synthesize surfactin. Surprisingly, BBG125 was found to produce about 2-fold less mycosubtilin than BBG116 despite an expected higher availability of the cytoplasmic precursors and cofactors pool for biosynthesis. Further physiological characterization of BBG125 also highlighted: (i) a strong influence of temperature on mycosubtilin biosynthesis in BBG125 with a maximal productivity observed at 22°C, compared to 15 and 30°C; (ii) substantial changes in fatty acid profiles and thereby in mycosubtilin isoforms, compared to the wild-type strain; and (iii) the presence of five novel mycosubtilin isoforms. The antifungal activities of the new mix were higher than or equal to those of purified isoforms.

**Key-words:** *Bacillus subtilis*, mycosubtilin, novel isoforms, antifungal activity, spreading

## 1. Introduction

Mycosubtilin is a potent antifungal lipopeptide of the iturin family synthesized by a limited number of *Bacillus subtilis* (Besson et al., 1978) and *Bacillus* spp. strains.

Mycosubtilin is composed of a seven amino acid cyclic peptide linked by a lactone ring to a  $\beta$ -amino fatty acid of variable chain length (i.e., C15 to C17) and isoforms (*n*-, *iso*- and *anteiso*-) (Jacques, 2011). Antifungal activity of mycosubtilin has been shown to be related to

both the length of the fatty acid component and its isomery (Hbid, 1996). Biosynthesis of mycosubtilin is mediated by a modular multienzymatic complex and the so-called “non-ribosomal peptide synthetases” mechanism (NRPS). This multienzymatic complex is encoded by the hybrid PKS/NRPS *myc* operon of size about 38 kb, which contains four genes (Duitman et al., 1999). Beside this, most of *B. subtilis* strains coproduce other NRPS compounds such as surfactin, a powerful lipopeptide biosurfactant involved in colonization of surfaces (Leclère et al., 2006) and biofilm formation (Chollet-Imbert et al., 2009), other iturins (iturin A and bacillomycins) or lipopeptides from the fengycin and plipastatin family. Despite the fact that multi-lipopeptide producers, notably in *Bacillus amyloliquefaciens*, have been isolated (Jacques et al., 1999; Hofemeister et al., 2004; Koumoutsi et al., 2004), the occurrence of strains naturally producing only mycosubtilin has not been reported so far. Interest in *Bacillus* non-ribosomal lipopeptides both in biocontrol in agricultural area against numerous phytopathogens (e.g., induced systemic resistance in plants) and in potential use as biopesticides, has strongly increased in the recent years (Ongena and Jacques, 2008).

Surfactin synthesis has for a long time been demonstrated to be closely linked to the competence state and sporulation of the cells, and to be subjected to a complex regulatory network involving two groups of pheromones: ComX, on one side, and PhrC, PhrF, PhrG and PhrH on the other side (Hamoen et al., 2003; Hayashi et al., 2006, Jacques, 2011). One of these pheromones, PhrC, would be also involved in the regulation of mycosubtilin biosynthesis. In addition, the strength of the promoters *P<sub>srfA</sub>* and *P<sub>myc</sub>*, which direct expression of the corresponding operons *srfA* and *myc*, is very low (about 200-fold less) in strain ATCC 6633, when compared to the expression of the native *P<sub>srfA</sub>* within the well-known laboratory strain *B. subtilis* 168 (Duitman et al., 2007).

In order to demonstrate the role played by mycosubtilin in the biocontrol activity of plant diseases, we previously reported the construction of a *B. subtilis* ATCC 6633 derivative

overproducing mycosubtilin, resulting from replacement of the native *Pmyc* promoter by a constitutive one linked to an antibiotic resistance marker, i.e., the *PrepU-neo* cassette. This modified strain named BBG100 produced up to 15-fold more mycosubtilin than the parental strain did, and showed a strongly increased antifungal activity *in vivo* in the vicinity of tomato roots (Leclère et al., 2005), favoured by the early constitutive and overproducing status of mycosubtilin production. Moreover, a significant improvement of its behaviour for spreading on various solid media was also observed (Leclère et al., 2006).

However, the common use of either precursors such as fatty acids (Kluge et al., 1988) or cofactors such as phosphopantetheine (Vollenbroich et al., 1994) in the syntheses of both mycosubtilin and surfactin by strain ATCC 6633 may limit the mycosubtilin ratio. In addition, it was recently shown that coproduction of mycosubtilin and surfactin led to the formation of mixed micelles which render their separation quite difficult (Jauregi et al., in press). Thus, as a further step to increase mycosubtilin yield and facilitate its purification, the surfactin (*srfA*) operon was inactivated by disrupting its first *srfAA* gene in a novel constitutive mycosubtilin overproducer. The present paper describes the isolation of such a mutant and the impact of surfactin production suppression on (i) the biosynthesis level and isoforms repartition of mycosubtilin; (ii) the detection of new forms of mycosubtilin; and (iii) the biological activities.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and growth conditions

The microorganisms and plasmids used in this study are listed in Table 1. *B. subtilis* and *E. coli* strains were cultivated at 37°C in Luria-Bertani medium (LB) supplemented, when

required, with various antibiotics: ampicillin sodium salt (Ap; Sigma, St. Louis, MO, USA; 50 µg/mL), chloramphenicol (Cm; Sigma; 10 µg/mL), neomycin trisulfate salt hydrate (Nm; Sigma; 20 µg/mL), spectinomycin, di-HCl (Sp; Sigma, 50 µg/mL) and tetracycline-HCl (Tc; Sigma; 10 µg/mL). *B. subtilis* strains were also grown either in E medium (Clark et al., 1981) or in 100 mM MOPS-buffered modified Landy medium (Guez et al., 2008) containing 2.3 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> + 2 g/L glutamic acid instead of 5 g/L, at 15, 22 or 30°C as stipulated in the text.

Soft B medium was used to assess the invasive behaviour of the strains (Coutte et al., 2010a). The yeast strains were cultured at 28°C in medium 863 (glucose 10 g/L, peptone 10 g/L, yeast extract 10 g/L; pH 5.8), whereas the fungal strains were propagated at 30°C on non-acidified potato-dextrose-agar, pH 6.0 (PDA; Solabia-Biokar Diagnostics, Beauvais, France), to carry out *Bacillus*/fungi confrontations.

For lipopeptide production in defined medium, culture flasks were inoculated at an initial biomass equal to an OD 0.25 at 600 nm (i.e., 0.08 g D.W/L) from an overnight preculture in E medium. Cultures were performed in triplicate at 15, 22 and 30 °C under rotary shaking (160 rpm).

## 2.2. Genetic and molecular biology experiments

### 2.2.1. General genetic techniques

Standard molecular biology procedures, i.e., total genomic DNA extraction, plasmid extraction, PCR, cloning of PCR amplicon and restriction endonuclease digestions were carried out as described previously (Leclère et al., 2005; Coutte et al., 2010a). *B. subtilis* strains RFB102 and BBG116 were transformed by either electroporation (Gene Pulser apparatus from Bio-Rad, Hercules, CA, USA) according to the method of Dennis and Sokol (1995) or natural competence (Dubnau, 1982).

### 2.2.2. Strain construction

The construction of constitutive mycosubtilin-overproducing mutants by double allelic exchange and further mycosubtilin monoproducers needed several steps. First, RFB102 was obtained by transformation of ATCC 6633 with polymers (concatenates) of plasmid ED511, containing  $P_{spac}$ -*comK*-*spc* cassette, followed by selection for amylase-negative spectinomycin-resistant clones. These concatenates were obtained with illustra TempliPhi 100 Amplification Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Then, to construct a constitutive mycosubtilin overproducer mutant, the 2.6 kb  $\epsilon$ *pbp*-*PrepU*-*neo*- $\epsilon$ *fenF* cassette was excised from plasmid pBG106 (Leclère et al., 2005) by a double *SacI*-*SphI* digestion and purified. In parallel, a 451 bp fragment carrying the  $\gamma$  origin of replication sequence (*oriK*) from plasmid R6K (unable to function in *Bacillus* spp.), contained in pTnMod-RKm', was excised from this plasmid using a double *NspI*-*SacI* digestion and purified. These two components were ligated and the product was used to transform *E. coli* CC118( $\lambda$ *pir*) with selection for neomycin resistance, giving rise to plasmid pBG201. Transformation of *B. subtilis* RFB102 with pBG201 linearized by *AatII* led to the isolation of several Nm<sup>R</sup> Spc<sup>R</sup> colonies. Correct recombination was verified by analytical PCR using the primers PBP-FO2 and FENF-REV2 (Table 2). Detection of a ~3.6 kb PCR fragment instead of the ~2.3 kb one found with the parent strain confirmed the promoter exchange. The resulting strain was denominated BBG116.

For the construction of *srfAA* disruption cassette, a 2.2 kb  $\epsilon$ *srfAA* fragment was amplified by PCR from *B. subtilis* 168 genomic DNA using the primers SRF-FO and SRF-REV (Table 2) and subsequently cloned into pGEM-T Easy vector to yield plasmid pBG208. The  $\epsilon$ *srfAA* fragment was then rescued by *EcoRI* digestion and subcloned into *EcoRI*-digested pUC19, to give rise to plasmid pBG210 (4.9 kb).

Beside this, a *tet* gene was amplified by PCR using primers TetP1 and TetT1 and vector pBC16 as a template, before being cloned into pGEM-T Easy to give rise to plasmid pBG209. The *tet* gene from this latter plasmid was rescued by *Eco*RI digestion and subcloned in pBG210 at the *Mfe*I restriction site located within the *εsr*fAA fragment. In a second step, a *cat* gene was obtained by PCR using the primers pC194cmfwd and pC194cmrev (containing an artificial *Bst*EII site) and pC194 as a template before being cloned into pGEM-T Easy to give rise to pBG143. Digestion of this latter plasmid by *Bst*EII generated a *cat* gene-bearing fragment which was inserted at the *Bst*EII site situated at the end of the *tet* gene in pBG212 (without removing the tetracycline-resistance phenotype), giving rise to plasmid pBG144. The mutant BBG116 was then transformed with *Aat*II-linearized pBG144, followed by selection either on LB Cm10 or on LB Tc10 plates, and checking for presence of the associated marker. The resulting strain unable to produce surfactin was called BBG125.

### 2.3. Cellular fatty acids analysis

The cells (from 100 mL cultures) were harvested by centrifugation at 10,000 g for 10 min. The pellets were washed twice with sterile distilled water and transferred into extraction screw-cap tubes. Bacteria were subsequently submitted to saponification and methylation and their fatty acid methyl esters were extracted as described by Miller and Berger (1985).

Methanol, hexane and ter-butylmethylether were of analytical grade (Merck, Darmstadt, Germany).

Analysis of fatty acid methyl esters was performed using a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan) as previously described (Chihib et al., 2003). At least two samples were withdrawn for each challenge condition and each experiment was done in triplicate.



## 2.4. Lipopeptide purification and analyses

### 2.4.1. Purification for HPLC analysis

Lipopeptides were purified from the culture supernatant by solid phase extraction using a 500 mg C18 Maxi-Clean cartridge (Grace Davison-Alltech, Deerfield, IL, USA) according to Guez et al. (2007). Briefly, after extraction of lipopeptide from 1 mL supernatant sample, the cartridge was washed with 8 mL of water. The lipopeptides were then eluted with 8 mL of 100% methanol (HPLC grade, Acros Organics, Geel, Belgium) and the extract was brought to dryness before dissolution in 200  $\mu$ L 100% methanol at 4°C.

Lipopeptides were quantified by high-performance liquid chromatography (Online Degaser, 717 Autosampler, 660S Controller, 626 Pump, 996 PhotoDiodeArray, Waters Corp., Milford, MA, USA) using a C18 column (5  $\mu$ m, 250 x 4.6 mm, 218 TP, VYDAC, Grace Davison-Alltech). Lipopeptides from a 10  $\mu$ L sample were eluted at a flow rate of 0.6 mL/min using an acetonitrile/water/trifluoroacetic acid (TFA) mixture (40:60:0.5; V/V/V) for mycosubtilins, and another acetonitrile/water/ TFA mixture (80:20:0.5; V/V/V) for surfactins. Purified iturin A (Sigma-Aldrich, St Louis, MO, USA), mycosubtilins and surfactins (purified at ProBioGEM as previously described in Coutte et al., 2010b) were used as standards. The retention time and the second derivative of the absorption spectrum between 200 and 400 nm (Diode Array PDA 996, Waters Corp.) were used to identify the eluted molecules (Millenium Software, Waters Corp.).

### 2.4.2. Preparation for MALDI-TOF-MS analysis

A volume of 10 mL at pH 6.5 +/- 1 of the supernatant was purified through a 10 g C18 Extract-Clean cartridge (Grace Davison-Alltech), which was then washed with 50 mL of

water. The lipopeptides were eluted with 10 mL of 100% methanol. The extract was brought to dryness before dissolution in 200  $\mu$ L 100% methanol at 4°C.

A sample volume of 100  $\mu$ L was then injected at 4.5 mL/min and analysed by high-performance liquid chromatography as described above, using a C18 column (5  $\mu$ m, 300 x 10 mm, ACE). A gradient-based method was used for lipopeptide separation. Solvent A (acetonitrile/TFA 99.9:0.1; V/V) was mixed with solvent B (water/TFA 99.9:0.1; V/V). After 4 min in isocratic mode (35% ACN), solvent A/B ratio increased linearly from 35/65 to 50/50 in 53 min, followed by 50/50 to 100 in 3 min, and then 5 min in isocratic mode (35% ACN).

#### 2.4.3. MALDI-TOF mass spectrometry analyses

MALDI-TOF mass spectrometry analyses were done on three different types of samples: (i) raw culture supernatants, (ii) samples purified through ODS cartridges (Grace Davison-Alltech) or (iii) samples purified through ODS cartridges, followed by semi-preparative HPLC. A saturated solution of  $\alpha$ -cyano-4-hydroxy-cinnamic acid (CHCA) was prepared in a 1:2 (V/V) solution of CH<sub>3</sub>CN and H<sub>2</sub>O containing 0.1% TFA. The previous solutions were diluted 10-fold with CHCA-saturated solution. Then, 0.5  $\mu$ L of this solution was deposited on the target. Measurement was performed using a MALDI-TOF spectrometer (Bruker UltraFlex TOF; Bruker Daltonics Inc., Billerica, MA, USA) equipped with a pulsed nitrogen laser ( $\lambda = 337$  nm). The ions were extracted from the ionization source with an acceleration voltage of 20 kV. Samples were measured in the reflectron mode, positive mode. The instrument was externally calibrated with a peptide mix ranging from 1046.54 m/z to 3147.47 m/z (Bruker part. 206195). The spectrum was obtained with 5 x 30 shots on the sample.

#### 2.4.4. Tandem mass spectrometry MS-MS analyses

To determine precisely the different forms of mycosubtilin produced by BBG125 strain, purified samples were analysed by tandem mass spectrometry (MS-MS) with electrospray and ion trap. Measurements were made by direct infusion of the open peptide after treatment with N-bromosuccinimide (Peypoux et al., 1981). The dry peptide was treated with 20  $\mu$ L of a solution containing an equal amount of N-bromosuccinimide in 70% acetic acid for 3 h at room temperature. The excess of reagent was destroyed by 5  $\mu$ L formic acid. The hydrolysate was evaporated to dryness. Time-dependent MS-MS was made with a Bruker Esquire HCT ESI-mass spectrometer. The conditions of the electrospray were: 180  $\mu$ L/h flowrate; ultrascan mass range mode; positive ion polarity; ESI source; 300 °C dry temperature; 15 psi N<sub>2</sub> at nebulizer; 4 L/min dry gas; 4,000 V for HV capillary.

#### 2.5. Analyses of antimicrobial activities, invasive behaviour, and determination of minimal inhibitory concentrations

Antimicrobial activities and invasive behaviour were analysed as described previously (Coutte et al., 2010a). In addition, antifungal activity tests against *B. cinerea* were performed by successive half-dilutions of various solutions of mycosubtilin isoforms (*iso*-C16, *n*-C16, *anteiso*-C17 and *iso*-C17) in 96-well microplates containing a rich medium, according to Besson *et al.* (1979). A mycosubtilin mix (MS comp.) was also tested, composed of (% w/w): *iso*-C16 (26%), Gln3 C17 (1%), *n*-C16 (2%), *anteiso*-C17 (44%), *iso*-C17 (23%) and C18 (1%). Growth was observed after 24 h at 28°C, and the minimum inhibitory concentration (MIC) was determined.

### 3. Results and discussion

3.1. Construction of constitutive mycosubtilin-overproducing mutants by double allelic exchange and generation of a mycosubtilin monoproducer

*B. subtilis* ATCC 6633 is known for its very low transformation frequency by natural competence, probably due to the lack of a functional *comK* gene. To bypass this technical drawback, the two mutants obtained in this study were based on the RFB102 strain, an inducible competent derivative of ATCC 6633, generated by integration of a  $P_{spac}$ -*comK* gene at the *amyE* locus. Thus, in the presence of IPTG, RFB102 harbours a competent phenotype and is less refractory to transformation.

The previously constructed mycosubtilin constitutive overproducer mutant BBG100 (Leclère et al., 2005) was observed to be slightly unstable after repeated cultivations, and to have been generated by a single crossing-over event (Campbell-type recombination; results not shown). Therefore, construction of a novel mutant was undertaken. Natural competence-mediated transformation of *B. subtilis* RFB102 with *AatII*-linearized pBG201 led to isolation of mutant BBG116 (Srf+ Myc++). PCR controls confirmed the occurrence of a double crossing-over event in this  $Nm^R$   $Spc^R$  derivative (data not shown). BBG116 was found to produce up to eight to ten-fold more mycosubtilin than the parent strain RFB102, which are results similar to those obtained with strain BBG100.

After further transformation of BBG116 by natural competence with *AatII*-linearized pBG144, the mycosubtilin monoproducer BBG125, which proved to be stable upon repeated cultivations, was isolated. Insertion of the *cat-tet* cassette within its *srfAA* gene was checked by PCR using the primers SRFAA5-FWD and SRFAA5-REV (Table 2): a ~4.7 kb amplicon was obtained instead of the ~2.3 kb one found with the parent strains (data not shown).

Analysis of mycosubtilin production, carried out after 4 days of incubation at 30°C in Landy modified medium (200 mL in a 1 L flask), revealed that BBG125 synthesized about 2-fold

less mycosubtilin ( $58.9 \pm 6.2$  mg/L) than its parent BBG116 ( $134.1 \pm 11.1$  mg/L; together with  $69.3 \pm 9.1$  mg/L of surfactin). Despite an expected higher availability of precursors/cofactors for mycosubtilin production, these results suggested that a functional surfactin biosynthesis should act positively on mycosubtilin production. Moreover, the fact that the knock-out of *srfAA* gene was located upstream from the second *srfAB* gene, within which the  $P_{srfA}$ -driven regulatory *comS* gene is nested (D'Souza et al., 1994), might induce a negative impact on mycosubtilin production.

### 3.2. Influence of temperature on growth and mycosubtilin production of strain BBG125

#### 3.2.1. Effect of temperature changes on the growth of *B. subtilis* ATCC 6633 and its derivative BBG125

In a previous work (Fickers et al., 2008), the influence of temperature on mycosubtilin production by *B. subtilis* ATCC 6633 was demonstrated. In order to characterize further this influence, the constitutive monoproducer BBG125 was cultivated at three different temperatures (15, 22 and 30°C) under rotary shaking conditions in Erlenmeyer flasks. The growth curve was first compared to that of *B. subtilis* ATCC 6633 under these different conditions. In the experiment performed at 22°C, a diauxic growth was observed for both strains. This diauxic growth results from a consumption of glucose (during the four to nine first hours of culture) accompanied by a strong production of acidic compounds which were re-consumed in a second phase (up to 24 h of culture, data not shown). BBG125 was found to grow slower than ATCC 6633 during the early stage of exponential phase ( $0.17$  and  $0.31$  h<sup>-1</sup>, respectively) until reaching a similar population at the stationary phase. The same slowdown of BBG125 growth compared to ATCC 6633 was observed at every temperature as shown in Table 3. Additionally, the growth of both strains at 15°C was found to be much slower than at

higher temperatures, revealed by a dilatory exponential phase.

### 3.2.2. Effect of temperature on mycosubtilin production in *B. subtilis* ATCC 6633 and its mutant BBG125

Mycosubtilin production was monitored during the growth of both strains by measuring its concentration in the culture supernatant at various time periods. Production of mycosubtilin was found maximal during the stationary phase. Moreover, mycosubtilin specific productivity was more important for BBG125 at 22°C than for ATCC 6633, up to  $132.25 \pm 3.83$  mg/L mycosubtilin being obtained (Table 3). This behaviour was observed from the beginning of the exponential growth phase (27.7 mg/L for BBG125 after 15.3 h of cultivation instead of 7.1 mg/L for ATCC 6633 after 21.6 h of cultivation), demonstrating immediate mycosubtilin production due to the presence of the constitutive  $P_{repU}$  promoter. This increased production could be observed throughout the culture, reaching a maximal productivity up to 4 times higher than for ATCC 6633. Mycosubtilin maximal concentration in BBG125 increased twice when the growth temperature decreased from 30°C to 22°C (Table 3). Under the same conditions, the maximal specific productivity increased 4 times for BBG125. Moreover, when the cells were grown at 15°C, an equivalent specific productivity as at 22°C was observed.

### 3.2.3. Effect of growth temperatures on fatty acid and mycosubtilin patterns

The changes of growth temperature affected the relative amount of homologues of fatty acids in both the wild-type and the mutant strains. When the growth temperature decreased from 30°C to 15°C, the *anteiso* to *iso* fatty acids ratio increased in response to a decrease in the cellular concentration of the *iso* fatty acids (C14, C15, C16 and C17) homologues and an increase in *anteiso* (C15 and C17) homologues. Interestingly, this

behaviour was comparatively more marked for BBG125 (ratios from 3.3 to 24.5) than for ATCC 6633 (ratios from 2.3 to 4.1). In fact, at 30°C, the ratios were similar for both strains but at lower temperatures, the difference between the *anteiso* to *iso* fatty acids ratios of both strains increased. Thus, at 22°C, the ratio was 2.6 times higher for the mutant BBG125 and, at 15°C, the ratio was 5.4 times higher. It was established by Klein et al. (1999), that the growth temperature induced changes in cellular fatty acids profile of *B. subtilis* towards even *iso*, odd *iso* and *anteiso* chain. In fact, in response to a cold stress, *B. subtilis* acclimates by changing the lipid composition of its cell membrane, avoiding a decrease of membrane fluidity. This adjustment is mainly reflected by increasing the proportion of *anteiso* branched fatty acids.

Analysis of culture supernatants by RP-HPLC revealed that the mycosubtilin production of BBG125 showed an increase of the *anteiso* to *iso* mycosubtilin ratio in regard to the ATCC 6633 production at each tested temperature. Moreover, at low temperatures (22°C and 15°C), the difference between the *anteiso* to *iso* mycosubtilin ratio for the two strains was more significant, nearly four times higher for BBG125 at 15°C (10.0 instead of 2.6 for ATCC 6633). Finally, for both strains, an increase in the *anteiso* to *iso* mycosubtilin ratio was observed when the growth temperature decreased, owing to an increase in mycosubtilin *anteiso*-C17 proportion and a slight decrease in *iso* proportions. It has been reported that the *B. subtilis* mycosubtilin production pattern shows an increase in the proportion of the C17 homologues when *B. subtilis* grows at low temperatures (Fickers et al., 2008).

### 3.3. Detection and characterization of new forms of mycosubtilin

The combined HPLC and MALDI-TOF mass spectrometry analyses performed on culture supernatant of BBG125 revealed ten peaks, whose molecular masses are presented in

Table 4. MS-MS analyses were performed for each peak, the results for peaks 8, 6 and 10 being detailed.

The MS1 spectrum of the cyclic product of peak 8 gave peaks at  $m/z$  1085  $[M+H]^+$ , 1107  $[M+Na]^+$  et 1123  $[M+K]^+$  corresponding well to C17 mycosubtilin. The spectrum (MS1) of the open structure of peak 8 (with N-bromosuccinimide) was complicated by the two Br isotopes. Owing to  $^{79}\text{Br}$  and  $^{81}\text{Br}$  isotopes in about natural equal amount, a peak distribution was observed around  $m/z$  1260 corresponding to the expected derivative. For example, the peak at  $m/z$  1257.4 is the  $[M+H]^+$  molecule with two  $^{79}\text{Br}$ , the peak at  $m/z$  1259.4 is the  $[M+H]^+$  molecule with one  $^{79}\text{Br}$  and one  $^{81}\text{Br}$  or  $[M+H]^+$  molecule with two  $^{13}\text{C}$  and two  $^{79}\text{Br}$ , etc... The MS2 spectrum of peak 8 open structure is complicated by the presence of fragments with a loss of 1, 2 or 3  $\text{NH}_3$ , owing to the presence of Asn and Gln amino acids. Increasing  $m/z$  obtained with the fragmentation  $m/z$  1260.4 ion with formula  $\text{NQPSN}_x\text{N}_z$  (with x for fatty amino acid and z for brominated Tyr) is presented in Table 5.

The MS analysis of peak 6 showed that the peptide had an isotopic mass of 1098.6, while the open peptide (with N-bromosuccinimide) had a mass of 1270.4 with two  $^{79}\text{Br}$ . The y fragments were identical to *anteiso*-C17 mycosubtilin while the b fragments were 14  $m/z$  above b fragments of *anteiso*-C17 mycosubtilin. This meant that one of the first two amino acids in the open sequence (N or Q from  $\text{NQPSN}_x\text{N}_z$ ) had been modified. The substitution of an Asn by a Gsn is the sole retained reasonable hypothesis.

The MS analysis of peak 10 showed that the peptide had an isotopic mass of 1098.6, while the open peptide (with N-bromosuccinimide) had a mass of 1270.4 with two  $^{79}\text{Br}$ . The y fragments were 14  $m/z$  above the y fragments of *anteiso*-C17 mycosubtilin. The  $b_n$  fragments, with n less than 6, were identical to *anteiso*-C17 mycosubtilin.  $B_n$  fragments with n equal or over 6, had the same mass plus 14  $m/z$ . These results showed that the molecule should be mycosubtilin with a C18 and not a C17 fatty acid.



On the basis of HPLC elution of the peaks, we deduced the existence of 5 new forms of mycosubtilin: (i) four forms with Gln in position 3: *iso*-C16, *n*-C16, *anteiso*-C17, *iso*-C17 and (ii) one form with C18. The correspondence of these forms with the ten HPLC peaks is presented in Table 4. These forms which are produced in low amounts (about 1% of the total mixture), compared to the main forms already identified, probably result from the ability of some domains of NRPS involved in the biosynthesis of mycosubtilin to recognize different substrates, especially the adenylation domain of module 3 which could be able to activate Asn and Gln residues.

#### 3.4. Biological activities of the mutants compared to parent strain

*B. subtilis* ATCC 6633 and its three derivatives (RFB102, BBG116 and BBG125) were tested for (i) their antagonism versus two fungi (*Botrytis cinerea* and *Fusarium oxysporum*) and the yeast *Saccharomyces cerevisiae*, and (ii) their invasive behaviour on soft B medium. In all cases, mutant BBG116 (Srf+ Myc++) proved to be the most efficient strain, owing to the higher amount of mycosubtilin produced by this strain but probably also by a synergistic effect between surfactin and mycosubtilin (Maget-Dana et al., 1992). The derivative BBG125 (Srf- Myc+) was observed to display the second significant antifungal activity, due to mycosubtilin overproduction, compared to strains ATCC 6633 and RFB102 (data not shown). Thanks to surfactin, a strong biosurfactant implied in colonization of surfaces mechanisms (spreading, swarming,...), the wild-type 6633 was the second best invasive strain (Fig. 1). These results are in agreement with the correlation between enhanced spreading (and thereby colonizing behaviour) and surface-active properties displayed by these two lipopeptides (Leclère et al., 2006).

Against *B. cinerea*, the minimal inhibitory concentrations of (i) individual purified isoforms of mycosubtilin and (ii) a mix of several isoforms, were also determined. The overall results showed that the isoforms mix gave equal or stronger effects, compared to the individual isoforms ones. Indeed, the *anteiso*-C17 isoform and the mix (MS comp.) were the most active against the fungus (MIC of 8  $\mu$ M each), followed by the *n*-C16 and *iso*-C17 isoforms (MIC of 16  $\mu$ M each), and the *iso*-C16 one (MIC of 32  $\mu$ M). The presence of small amounts (1% each) of the new isoforms Gln3 C17 and C18 in the mix significantly improved its activity, suggesting an even more powerful activity for these variants alone.

#### 4. Conclusion

For the first time, the construction of a *B. subtilis* constitutive mycosubtilin monoproducer is reported here. Although mycosubtilin productivity was lowered by the suppression of the capacity of surfactin synthesis, the mutant BBG125 produced a novel mixture of mycosubtilins with five new isoforms. In addition, it displayed substantial changes in both fatty acids profiles and mycosubtilin isoforms, compared to the original strain ATCC 6633. Significant antimicrobial activities were observed against filamentous fungi and a yeast, showing that BBG125 and its surfactin-producing parent (BBG116) were potent candidates as biocontrol agents.

#### Acknowledgements

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**Table 1.**

Strains and plasmids.

Strain or plasmid	Description*	Reference or source
<i>E. coli</i> CC118( $\lambda$ pir)	Host strain for plasmids carrying an R6K-derived replicon; $\Delta(ara-leu)$ <i>araD</i> $\Delta lacX74$ <i>galE galK</i> <i>phoA20 thi-1 rpsE rpoB argE(Am) recA1</i> ( $\lambda$ pir) Rif <sup>R</sup>	Herrero et al. (1990)
<i>E. coli</i> JM109	General purpose cloning strain; <i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i> $\Delta(lac-proAB)$ F' [ <i>traD36 proAB</i> <sup>+</sup> <i>lacI</i> <sup>f</sup> <i>lacZ</i> $\Delta$ M15]	Promega Corp., Madison, WI, USA
<i>Bacillus subtilis</i> 168	Laboratory strain; <i>trpC2 degQ</i> <sup>o</sup> <i>sfp</i> <sup>o</sup> ; Srf <sup>-</sup> Pps <sup>-</sup>	Lab stock
<i>B. subtilis</i> ATCC 6633	Produces mycosubtilin (Myc <sup>+</sup> ), surfactin (Srf <sup>+</sup> ), subtilin, subtilosin and rhizocticins	Duitman et al. (1999)
<i>B. subtilis</i> RFB102	ATCC 6633 derivative with a P <sub>spac-comK</sub> - <i>spc</i> cassette inserted at the <i>amyE</i> locus; Myc <sup>+</sup> Srf <sup>+</sup> Amy <sup>-</sup> Spc <sup>R</sup>	This work
<i>B. subtilis</i> BBG116	RFB102 derivative overproducing mycosubtilin; Myc <sup>++</sup> Srf <sup>+</sup> Amy <sup>-</sup> Spc <sup>R</sup> Nm <sup>R</sup> ; [P <sub>repU-neo</sub> :: <i>myc</i> ]	This work
<i>B. subtilis</i> BBG125	Mycosubtilin monoproducer BBG116 derivative;	This work

Myc<sup>+</sup> Srf<sup>-</sup> Amy<sup>-</sup> Spc<sup>R</sup> Nm<sup>R</sup> Cm<sup>R</sup> Tc<sup>R</sup>;

[*P<sub>repU-neo</sub>::myc; srfAA::cat-tet*]

#### Fungi

*Botrytis cinerea* Wild-type Lab stock

*Fusarium oxysporum* Wild-type Lab stock

#### Yeast

*Saccharomyces cerevisiae* Wild-type Lab stock

#### Plasmids

pGEM-T Easy T-vector; Ap<sup>R</sup>; 3.0 kb Promega Corp.,  
Madison, WI, USA

ED511 *Bacillus* integration vector with the  
*Pspac-comK-spc* cassette; Spc<sup>R</sup>; 7.8 kb Rudner, D.Z.,  
Boston, MA, USA

pBC16 *Staphylococcus/Bacillus* cloning vector;  
Tc<sup>R</sup>; 4.6 kb Bernhard et al.  
(1978)

pC194 *Staphylococcus/Bacillus* cloning vector;  
Cm<sup>R</sup>; 2.9 kb Ehrlich (1977)

pTnMod-RKm' Plasposon; Km<sup>R</sup>/Nm<sup>R</sup>; 4.8 kb Dennis and Zylstra  
(1998)

pUC19 Cloning vector, Ap<sup>R</sup>; 2.7 kb New England  
Biolabs, Beverly,  
MA, USA

pBG106 pUC19 containing *PrepU-neo* inserted Leclère et al. (2005)

	within PCR-amplified flanking regions of <i>myc</i> promoter; Ap <sup>R</sup> Nm <sup>R</sup> ; 5.3 kb	
pBG201	<i>NspI-SacI</i> fragment from pTn <i>Mod-RKm</i> ' ligated to the <i>SphI-SacI</i> [ <i>P<sub>repU-neo</sub></i> ] one from pBG106; Nm <sup>R</sup> ; 3.1 kb	This work
pBG208	<i>εsrfAA</i> amplicon inserted within pGEM-T Easy; Ap <sup>R</sup> ; 5.2 kb	This work
pBG210	<i>εsrfAA</i> amplicon subcloned within pUC19; Ap <sup>R</sup> ; 4.9 kb	This work
pBG209	<i>tet</i> gene amplicon from pBC16 inserted within pGEM-T Easy; Ap <sup>R</sup> Tc <sup>R</sup> ; 4.6 kb	This work
pBG212	pUC19 derivative containing part of <i>srfAA</i> gene from ATCC 6633 interrupted by the <i>tet</i> gene of pBC16; Ap <sup>R</sup> Tc <sup>R</sup> ; 6.5 kb	This work
pBG143	<i>cat</i> gene amplicon from pC194 inserted within pGEM-T Easy; Ap <sup>R</sup> Cm <sup>R</sup> ; 3.7 kb	This work
pBG144	<i>cat</i> gene from pC194 inserted within the end of <i>tet</i> gene in pBG212; Ap <sup>R</sup> Cm <sup>R</sup> Tc <sup>R</sup> 7.2 kb	This work

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\*Ap<sup>R</sup>; Cm<sup>R</sup>; Nm<sup>R</sup>; Spc<sup>R</sup>; Tc<sup>R</sup>: resistances to ampicillin, chloramphenicol, neomycin,

spectinomycin and tetracycline, respectively. Srf- Pps-: unable to synthesize both surfactin and plipastatin. Amy- : unable to degrade starch (amylase negative).

**Table 2**

Characteristics of the pairs of primers used

Gene	Accession number (GenBank or NCBI)	Primer (5' ==> 3')	Sequence	Annealing T° of pairs*/ Restriction sites
<i>myc</i> /6633	AF184956.1	PBP-FO2	AATAACGGACATGCCGAAGTG	57°C
		FENF-REV2	AATAGGCCGACCAAGACGTTTC	
<i>srf</i> AA/168 (1)	NC_000964.3	SRF-FO	ACAGGAATATGCTCAATCGAAG	53°C
		SRF-REV	AAATTCCGCTTCCAGGCTTCTG	
<i>tet</i> /pBC16	NC_001705.1	TetP1	GTTGTATCGAIGATGAAATACTGAAATTTAAACTTAG	49°C/ <i>Cla</i> I**
		TetT1	TTTAAATGGATCTAGAAAGATTTGAAATTCCTGTTAT	<i>Xba</i> I**
<i>cat</i> /pC194	NC_002013.1	pC194cmfwd	AGAAAGCAGACAGGTAACCCCTCCTAA	58°C/ <i>Bsf</i> EII
		pC194cmrev	GCAGGTTAGTGACATTAGGTAACCCGA	<i>Bsf</i> EII
<i>srf</i> AA/168 (2)	NC_000964.3	SRFAA5-FWD	AAGGAATCTCGCAITTCATTTATCG	58°C
		SRFAA5-REV	CTTGGTGTAAGCGGAAATTTCTGTC	

\* Approximately 5°C below T<sub>m</sub> of primers; \*\* The artificial *Cla*I and *Xba*I contained in TetP1 and TetT1 primers were designed for another experiment,

but the specificity of these primers remained sufficient to generate from pBC16 the *tet* amplicon used in this work.

**Table 3.**

Growth rates in the early and late stages of exponential phase, mycosubtilin maximal production and specific productivity of BBG125 at different growth temperatures.\*

T°	Growth rate		Maximal production (mg/L) <sup>†</sup>	Maximal specific productivity (ng/cell) <sup>†</sup>
	Early stage	Late stage		
30°C	0.40 ± 4.4E-02	0.07 ± 6.9E-03	52.70 ± 3.53	33.15 ± 1.48
22°C	0.17 ± 2.2E-03	0.02 ± 1.9E-03	104.83 ± 9.57	132.25 ± 3.83
15°C	0.04 ± 0.1E-03	0.01 ± 0.6E-03	58.44 ± 7.47	103.02 ± 5.9

\*The values are means from three independent experiments. <sup>†</sup>These values were obtained 48 h after the beginning of the stationary phase.

**Table 4.**

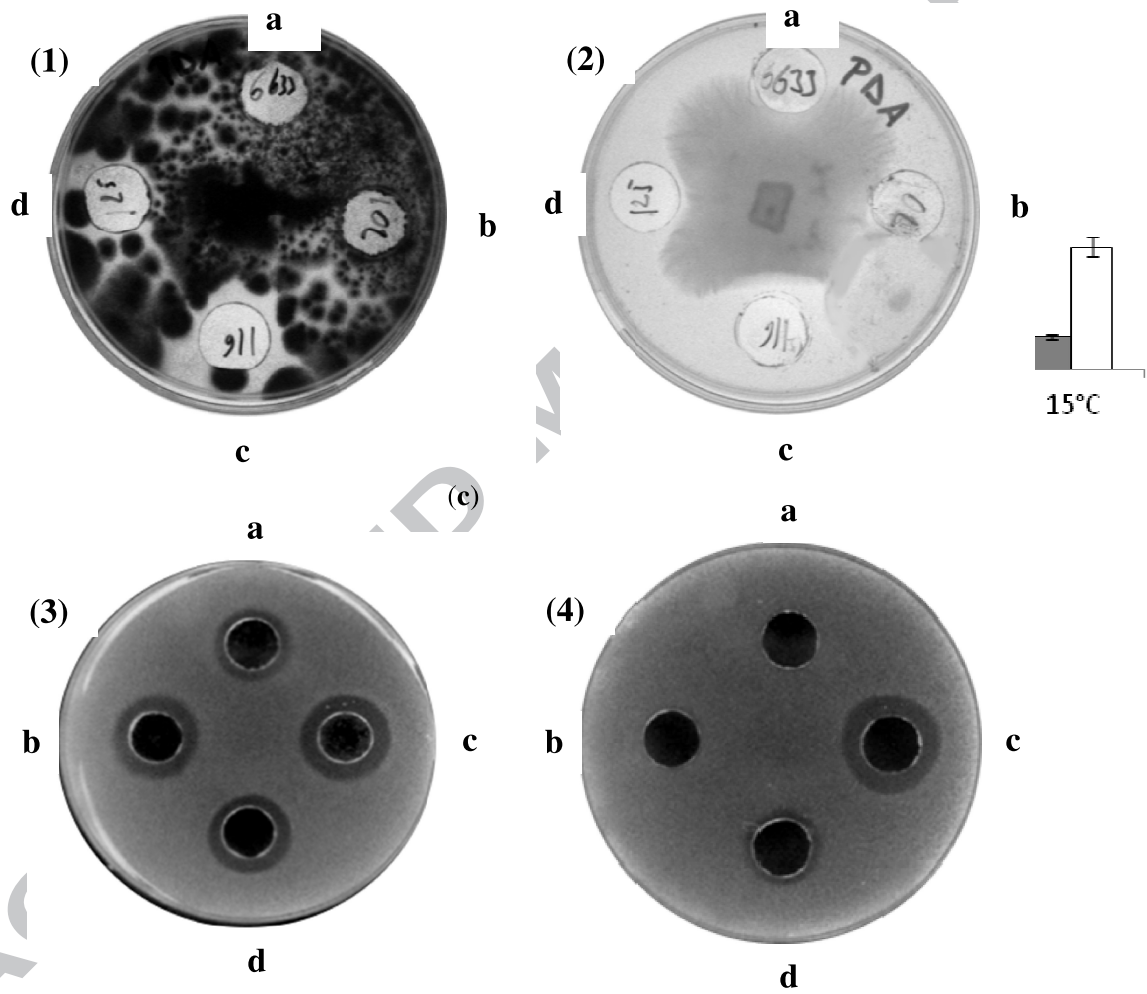
m/z (mass-to-charge ratio) of the 10 peaks detected by combined HPLC and MALDI/TOF analyses and correspondence with mycosubtilin forms.

Peak no.	m/z	Form	Peak no.	m/z	Form
1	1056	<i>n</i> -C15	6	1098	<i>anteiso</i> -C17 Gln
2	1084	<i>iso</i> -C16 Gln	7	1098	<i>iso</i> -C17 Gln
3	1084	<i>n</i> -C16 Gln	8	1084	<i>anteiso</i> -C17
4	1070	<i>iso</i> -C16	9	1084	<i>iso</i> -C17
5	1070	<i>n</i> -C16	10	1098	<i>n</i> -C18

**Table 5.**

Increasing m/z (mass-to-charge ratio) obtained with the fragmentation m/z 1260.4 ion.

<b>m/z</b>	<b>Fragment ion</b>	<b>m/z</b>	<b>Fragment ion</b>	<b>m/z</b>	<b>Fragment ion</b>
<b>382.3:</b>	<b>xN</b>	<b>566.5:</b>	<b>PSN<sub>x</sub></b>	<b>791.5:</b>	<b>b6-NH<sub>3</sub></b>
<b>462.3:</b>	<b>N<sub>x</sub>N-2NH<sub>3</sub></b>	<b>645.3:</b>	<b>PSN<sub>x</sub>N-H<sub>2</sub>O-NH<sub>3</sub></b>	<b>808.4:</b>	<b>b6</b>
<b>479.3:</b>	<b>a5-2NH<sub>3</sub></b>	<b>646.6:</b>	<b>PSN<sub>x</sub>N-2NH<sub>3</sub></b>	<b>831.1:</b>	<b>y4</b>
<b>489.2:</b>	<b>b5-H<sub>2</sub>O-2NH<sub>3</sub></b>	<b>663.5:</b>	<b>PSN<sub>x</sub>N-NH<sub>3</sub></b>	<b>871.5:</b>	<b>b7-3NH<sub>3</sub></b>
<b>506.2:</b>	<b>b5-H<sub>2</sub>O-NH<sub>3</sub></b>	<b>680.5:</b>	<b>PSN<sub>x</sub>N</b>	<b>888.5:</b>	<b>b7-2NH<sub>3</sub></b>
<b>524.2:</b>	<b>b5-NH<sub>3</sub></b>	<b>757.3:</b>	<b>b6-3NH<sub>3</sub></b>	<b>905.5:</b>	<b>b7-NH<sub>3</sub></b>
<b>541.2:</b>	<b>b5</b>	<b>774.5:</b>	<b>b6-2NH<sub>3</sub></b>	<b>1015.2:</b>	<b>y6</b>



FIGURE

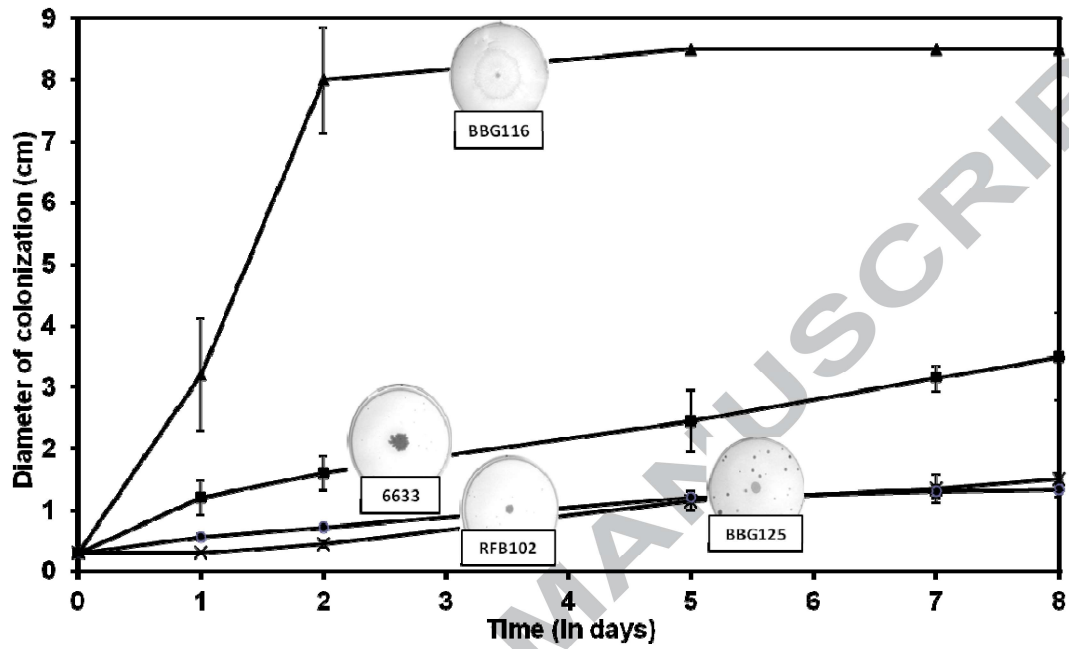


Fig. 1



**LEGEND OF FIGURE**

**Fig. 1. (B)** Comparison of the invasive behaviour of *B. subtilis* ATCC 6633 and its derivatives on soft B medium. ■ ATCC 6633; ● RFB102; ▲ BBG116; × BBG125.

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Highlights

- mutant strain which constitutively produces mycosubtilin only
- new mixture of mycosubtilins containing five new isoforms
- influence of surfactin gene knock-out on mycosubtilin production
- influence of strain modification on fatty acid and mycosubtilin isoform pattern

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