Isolation of the Antimicrobial Cyclic Peptide Subtilosin a from a Gut-Associated *Bacillus subtilis* Strain

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ABSTRACT

The endospore-forming *Bacillus subtilis* has been used as probiotics over the last 50 years. However, little is known on how *Bacillus* spp act in the gut compared to other well-characterized probiotics such as lactic acid bacteria. It is believed that the competitive exclusion of pathogens results from different mode of action notably the production of antimicrobial compounds such as bacteriocins. Here, we report the characterization of the unexpected ability of a gut-associated *B. subtilis* BSP1 to synthetize the cyclic bacteriocin subtilosin A at high level. Our findings suggest that the BSP1 phenotype could be related, at least in part, to a subsequent increased expression level of the subtilosin A biosynthetic gene cluster sbo-alb in response to a higher activity of the stationery and sporulation master regulator Spo0A.

1. INTRODUCTION

Necrotic enteritis is an enterotoxemia disease caused by *Clostridium perfringens* in chicken, turkey and ducks. It leads to the development of necrotic lesions in the gut wall, resulting in death of poultry (Timbermont *et al.*, 2011). This is a multifactorial disease, whose epidemiology and pathogenesis are not yet fully understood (Garcia and Heredia, 2011). Worldwide, necrotic enteritis is considered one of the most threatening emerging diseases in the broiler industry. Global economic losses as a consequence of necrotic enteritis outbreaks have been estimated at over $2 billion per year, largely attributable to medical treatments and impaired growth performance (Lee *et al.*, 2001). Medical treatments consist most of the time of antibiotic utilization. However, emergence of antibiotic-resistant strains of *C. perfringens* highlights the need of alternative solutions to overcome these sanitary problems.

Probiotics are live microbes, which when administrated in adequate amounts confer a health benefit to the host (Cutting, 2011). Their use as feed additives in the stockbreeding industry is of attracting increased attention as a cost-effective alternative to antibiotics to control animal diseases (Reuter, 2001). Previous studies have suggested that *Bacillus subtilis* can be used to increase and maintain beneficial bacteria in the intestine (Hoa *et al.*, 2000; Thirabunyanon and Thongwittaya, 2012). *B. subtilis* is an endospore-forming bacterium whose spore consists of several protective layers surrounding the nucleoid in the spore core (Henriques and Moran, 2007). Due to their structural organization, spores are extremely resistant to external chemical and physical mugging and are partly

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responsible for their exceptional longevity in the environment (Henriques and Moran, 2000). In regards to their non-pathogenic status, *Bacillus subtilis* have been used as probiotics over the last 50 years. As they might form heat-stable spores, *Bacillus* spp. present several advantages over other probiotic bacteria such as *Lactobacillus* spp. Indeed, *Bacillus* spores present a longer shelf life at room temperature and could cope with the low pH of gastric barrier. Spores probiotics are being extensively used as dietary supplements in human or as growth promoters and competitive agents in animal feed. In the past, it was assumed that most aerobic spore-formers belong to the *B. subtilis* species (Cutting, 2011). However, several of these strains were found to be misidentified and to carry multidrug resistances or to harbor toxic genes (Hoa *et al*., 2000; Duc *et al*., 2006). This highlights the need for a more rigorous selection and a deeper strain characterization in order to obtain a safer utilization. Compared to lactic acid bacteria, little is know about how *Bacillus* spp act as probiotic. It is believed that the competitive exclusion of pathogens results from one or more modes of action, including immune exclusion, competition for adhesion sites or production of antimicrobial compounds, such as bacteriocins (Patterson and Burkholder, 2003; Barbosa *et al*., 2005). Bacteriocins are ribosomally synthetized proteins that elicit bactericidal activity, usually against closely related species (Klaenhammer, 1993). Some bacteriocins, the so-called lantibiotics (class I bacteriocins), can undergo posttranslational modificationsa and contain thioether amino acid lanthionine or methyllanthionine (Bierbaum and Sahl, 2009; Fickers, 2012) whereas class II bacteriocins do not (Nissen-Meyer *et al*., 2009).

It has been suggested in the past, that animal probiotics should preferentially originate directly from the target animal microflora, as their use would be ethically more acceptable and potentially more effective than exogenous strains. In that sense, Barbosa and collaborators isolated 237 presumptive gut-associated spore former *Bacillus* spp strains from broiler chicken feces (Barbosa *et al*., 2005). For thirty-one of them, 16S rRNA gene sequence analysis revealed that they belong to *B. subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus megaterium,* as well as *Bacillus cereus,* of which some strains are considered pathogens because of their association with foodborne illness. All isolates efficiently sporulate in laboratory and the resulting spores were tolerant to simulated gastrointestinal tract conditions. Importantly, some of these isolates also exhibited an antimicrobial activity against food spoilage and/or pathogenic organisms such as *Clostridium perfringens*, *Staphylococcus aureus* and *Listeria monocytogenes*. The study of Barbosa *et al*. (2005) suggests

that some of the *Bacillus* spore-former isolates have the potential to persist in or transiently associated with the complex gut ecosystem. In the present work, we further characterized the antimicrobial activity of one of these *B. subtilis* isolates, the so-called BSP1.

2. MATERIALS AND METHODS

2.1. Bacterial Strains, Culture Media and General Genetic Techniques

Plasmid, recombinant *B. subtilis* and bacteria isolated from swine intestinal content are listed in **Table 1 and 2**, respectively. Oligonucleotide primers used for PCR amplification are listed in **Table 3**. Standard molecular genetic techniques were used (Sambrook *et al*., 1989). Restriction enzymes and T4 DNA ligase were obtained from Fermentas (St. Leon-Rot, Germany). Genomic DNA was purified using the Wizard genomic purification kit (Promega, Madison, WI, USA). PCR amplifications were performed with Fideli Taq polymerase (USB Corporation, Cleveland, OH, USA) and amplified fragments were purified with the Qiagen purification kit (Hilden, Germany). The culture media were Luria-Bertani broth (LB broth, Sigma-Aldrich), brain-heart infusion (BH broth, Difco) and Difco Sporulation Medium (DSM).

Table 1. Activity spectrum of cell-free supernatant of BSP1 on bacteria isolated from swine intestinal content[1]

Strain	Antimicrobial activity[2]
Gram negative	
Escherichia coli 0.94	-
Escherichia fergusonii	-
Klebsiella pneumonia	-
Salmonella typhimurium PF3127	-
Salmonella Derby PF2709	-
Salmonella enteritidis PF1338	-
Enterobacter cloacae	-
Gram positive	
Staphylococcus hyicus	++
Streptococcus bovis	-
Enterococcus faecium	-
Enterococcus durans	+
Lactobacillus salivarus	-
Lactobacillus fermentum	-
Lactobacillus acidophilus	-
Clostridium perfringens	+
Clostridium perfringens P41	+++
Listeria monocytogenes HCIP59.53	+
Listeria ivanovii DCIP78.42	+++++
Listeria ivanovii DCIP107777	+++++

[1]All strains are from DSM Nutritional Products collection, Basel, Switzerland
[2]The different score reflect the different degree of inhibition. +, halo of growth inhibition; multiple +, increased diameter of the inhibition zone; -, no growth inhibition

Table 2. Strains and plasmids used in this study

Strain or plasmid	Relevant Genotype / Phenotype	Reference
B.	*subtilis*	
strains		
PY79	Prototrophic	Zeigler *et al.* (2008)
168	Prototrophic	Zeigler *et al.* (2008)
BSP1	Poultry gastrointestinal isolate #200	Barbosa *et al.* (2005)
BSP1Δ*sboAX*	*sboAX* ::Cm^R	This study
168Δ*sboAX*	*sboAX* ::Cm^R	His study
AH7496 PY79	derivative (*sboA::PsboAPY79-cfp* /Cm^R)	This study
AH7497	BSP1 derivative (*sboA::PsboABSP1-cfp* /Cm^R)	This study
Plasmids		
pCS57	*bla* P*sboA* PY79-*cfp cat*	This study
pCS58	*bla* P*sboA* BSP1-*cfp cat*	This study
pDR200	*bla cfp kan*	Doan *et al.* (2005)
pMS38	*bla cat*	Serrano and Henriques (Unpublished results)
pRFP121	*bla cat*	Fickers (Unpublished results)

unpublished results

Table 3. Oligonucleotide primers used in this study

Primer name	Sequence (5′ - 3′)	Restriction sites
Sbo1	CTTCATTTGTTCCGCAATGTTCA	
Sbo2	TTTTGGCCCAGTGGGCCAATTGAATCCTCCCTTTTTTTGTT	*Sfi*I
Sbo3	TTTTGGCCCCAGCGGCCTGAATCATATGTATAGGGGATGGTATTTT	*Sfi*I
Sbo4	CAAGTTTGGGCAAAAGAGCCTTTTC	
sboA-380F	GGGGTACCCGATGACTTCTTGGTTTCCG	*Kpn*I
sboACFP+38R	GTTCCTCCTTATGTAAGCTTGGCTCCGATCGAGCATGTTG	
CFP-24F	AAGCTTACATAAGGAGGAAC	
CFP+750R	CGCGGATCCTTACTTATAAAG	*BamH*I

Restriction sites are underlined

2.2. Production, Purification and Characterisation of Subtilosin A

For subtilosin A production, BSP1 was grown in shake flasks for 18 h in LB medium at 37°C. Antimicrobial compounds were extracted from the culture supernatant by hydrophobic chromatography using Amberlite XAD-16 (Sigma, 50 gr per liter of culture medium). After washing steps (five bead volume each) with MilliQ water and MilliQ water-methanol mixture (25-75% v/v), subtilosin A was eluted in two bead volume of methanol and concentrated by rotary evaporation under vacuum. Further purifications were conducted by Reverse Phase Highpressure Liquid Chromatography (RP-HPLC) on an Xterra RP18 column (4.6×150 mm, 3.5 μm, Waters, Milford, MA, USA) in a water-acetonitrile eluent system containing 0.1% of trifluoroacetic acid (waters). Molecules were eluted at a flow rate of 1 ml min^{-1} in a 40 min gradient ranging from 0 to 100% of acetonitrile. Eluted compounds were collected and those corresponding to subtilosin A were identified by means of antimicrobial activity tests plates

using *Listeria ivanovii* as a sensitive strain (Halimi *et al.*, 2010). Molecular weight determination of the purified antilisterial peptide (50 ng) was determined by Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry in positive mode on a Ultraflex II apparatus (Bruker Daltonics) using α-cyano-β-hydroxycinnamic acid as a matrix. MALDI-TOF analyses were performed at the GIGA-Proteomics facility (University of Liège, Belgium).

2.3. Antimicrobial Activity

Antimicrobial activity was assessed with a colony overlay assay as described by Barbosa *et al.* (2005). Briefly, overnight cultures of BSP1 grown in LB medium were inoculated as a 5-μL spot (to obtain giant colonies) on LB agar plates and incubated at 37°C for 24 h prior to killing of the cells by exposure to chloroform vapors for 30 min. Plates were then aerated in a laminar flow hood for 20 min to allow the chloroform vapors to dissipate. Plates were finally overlaid with 0.7% LB or BH infusion agar seeded with an overnight culture of the

indicator strain listed in **Table 1**. Zones of growth inhibition of the indicator stains around the *Bacillus* giant colonies observed after 48 h of incubation at 37°C were scored as positive.

2.4. Effect of Temperature, pH and Proteinase K on Antimicrobial Activity

To evaluate protease sensitivity, purified subtilosin A (7 ng mL^{-1}) samples were analyzed by HPLC after 24 h of incubation at 37°C with proteinase K (Sigma-Aldrich, 10 mg mL^{-1} in 10 mM phosphate buffer, pH 7.2). Heat stability was evaluated after incubation for 60 min at various temperatures (30, 50, 75 and 100°C) whereas pH sensitivity was evaluated after one-hour incubation in 10 mM citrate buffer (pH 3), phosphate buffer (pH 6) and CAPS buffer (pH 10). After treatments, the remaining subtilosin A activity was determined by agar diffusion assay using *Listeria ivanovii* as sensitive strain (Halimi *et al*., 2010).

2.5. Measurement of Transmembrane Electrical Potential

Transmembrane electrical potential ($\Delta \psi$) was monitored using the fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide (DiSC3 (5), Anaspec, Fermont, CA, USA) as described elsewhere (Fickers *et al*., 2009). Briefly, an overnight culture of L. ivanovii was pelleted by centrifugation and cells were resuspended in 50mM HEPES buffer (pH 7) to obtain an A600 of 0.1 for the cell suspension. DiSC3(5) fluorophore (1 µM, final concentration) was then added to 1 mL of cell suspension together with glucose (10 µM, final concentration) and nigericin (1 µM, final concentration, SigmaAldrich). After stabilization of the fluorescence signal (excitations and emission wavelengths were set at 622 nm and 670 nm, respectively), purified subtilosin A (10 ng mL^{-1}) was added and the change of fluorescence was monitored by using a LSB50 spectrofluorimeter (Perkin-Elmer, Waltham, MA, USA). Finally, the ionophore valinomycin (1µM, final concentration, Sigma-Aldrich) was added to dissipate any residual $\Delta \psi$. Stock solutions of DiSC3(5) (1 mM), glucose (10 mM), nigericin (1 mM) and valinomycin (1 mM) were freshly prepared before use.

2.6. Disruption of Sbo Operon in BSP1

Primer pairs sbo1-sbo2 and sbo3-sbo4 were designed for the amplification of ~700 bp fragments corresponding to the up- and downstream region of the *sboAX* locus (ORFs A7A1_01574 and A7A1_01575). PCR fragments were then *SfiI* digested, purified and ligated to a chloramphenicol resistance cassette rescued from pRFP121 (Fickers, unpublished) after *SfiI* digestion. The disruption cassette was then amplified by PCR using primer pair sbo1-sbo4, purified and used to transform BSP1 and *B. subtilis* 168 according to Kunst and Rapoport (1995). Transformants were selected on LB-chlorampenicol plates (5 µg mL^{-1}) and correct gene disruption was verified by analytical PCR using primer pair sbo1-sbo4. A ~1.7 kb fragment was obtained for wild-type genotype whereas a ~ 2.5 kb fragment was obtained for the $\Delta sboAX$ strain. For those latter, loss of subtilosin A production was verified either by RP-HPLC analysis and *L. ivanovii* growth inhibition test (Halimi *et al*., 2010). The resulting strains were denominated BSP1$\Delta sboAX$ and 168$\Delta sboAX$, respectively.

2.7. Construction of sbo-cfp Transcriptional Fusion

To construct a fusion of the *sbo* promoter to *cfp* (coding for a cyan fluorescent protein), a ~ 420bp DNA fragment comprising the promoter region of *sboA* (ORF A7A1_0154), its start codon (ATG) and the first 38bp of its coding sequence, was first amplified by PCR from chromosomal DNA of strains *B. subtilis* PY79 and BSP1, using primers sboA-380F, which generated a *Kpn*I restriction site and sboA-CFP+38R (containing a tail for *cfp*). Next, the *cfp* was amplified from pDR200 (Doan *et al*., 2005), using primers CFP-24F and CFP+750R, which generated a *Bam*HI restriction site. An overlapping PCR was then performed between both DNA fragments using primers sboA-380F and CFP+750R, yielding a ~1200bp DNA fragment that carried a *Kpn*I restriction site at the 5′ end and a *Bam*HI restriction site at the 3′ end. The *Kpn*I-*Bam*HI digested PCR fragments were inserted into pMS38 (Serrano and Henriques, unpublished) at its unique restriction sites *Kpn*I and *Bam*HI, yielding pCS57 and pCS58. The resulting P*sboA*-*cfp* transcriptional fusions were then integrated at the *sboA* locus of strains PY79 and BSP1, creating strains AH7496 and AH7497, respectively.

2.8. Fluorescence Microscopy

For visualization of the P$_{sboA}$-*cfp* fluorescence signal, 600 µL of LB bacterial cultures were removed at the appropriated times, centrifuged 30 seconds at 6000 rpm and resuspended in 200 µL of PBS buffer. 2 µL of cell suspensions were mounted on agarose pads (1.7% in H2O) before microscope observation. Phase contrast and fluorescence images were acquired with a Leica DMRA2

Microscope equipped with a 63X magnification objective and a CoolSNAPTM HQ Photometrics camera (Roper Scientific, Tuscon, AZ, USA). Images were acquired with Leica FW4000 software and prepared for publication using Adobe Photoshop. Quantification of the CFP signal was done for 500 cells of each strain using the ImageJ software (http://rsb.info.nih.gov/ij/).

3. RESULTS

3.1. Antibacterial Activity of BSP1

The biological activity of BSP1 was investigated toward an array of bacteria isolated from swine intestinal content. For that purpose, BSP1 was first grown for 24 h on plates as giant colonies before being overlaid with soft agar medium containing the different strains to be tested. As shown in **Table 1**, the antimicrobial activity of BSP1 was specifically directed against Gram-positive bacteria. Indeed, no growth inhibition could be observed around the *Bacillus* giant colonies for the different Gram-negative bacteria tested. Among Gram-positive bacteria, both *L. ivanovii* isolates were found the most sensitive whereas the closely related food poisoning *L. monocytogenes* was weakly sensitive. Beside this, the growth of *C. perfringens,* the most common bacterial agent for gas gangrene, was also inhibited by BSP1. *Staphylococcus hyicus,* a pathogen responsible for swine exudative epidermidis, was also found sensitive, however in a lesser extend.

3.2. Purification and Characterization of the Antimicrobial Compounds

To further characterize this biological activity, the antimicrobial compound was extracted from BSP1 culture supernatant and purified by reverse phase chromatography. The loss of biological activity toward *L. ivanovii,* together with the disappearance of the corresponding peak on HPLC chromatograms upon protease treatment highlight the proteinaceous nature of the purified compound (data not shown). Incubation at different pH showed that the purified antimicrobial compound was more stable at acidic pH. Indeed, its stability decreased as the pH value was increased, especially at alkaline pH. Exposure to increasing temperature led to a decreased biological activity, indicating thus that the antimicrobial compound is temperature sensitive (**Fig. 1A**).

The membrane pore-forming biological mode of action of the purified peptide was highlighted by monitoring the released of potentiometric probe DiSC3 (5) from preloaded *L. ivanovii* cells (**Fig. 1B**). A high fluorescence signal, comparable to that obtained with the ionophore valinomycin, could be observed upon addition of the purified peptide. This indicates the disruption of the $\Delta\psi$ following the formation of transmembrane pore by the peptide.

MALDI-TOF mass spectrometry analysis of the purified antimicrobial compound gave a positive signal at m/z of 3400.527, which is consistent with the molecular mass of the bacteriocin subtilosin A 3400 Da (Stein *et al*., 2004; Liu *et al*., 2012) (**Fig. 1C**). Signals observed at m/z 3422.565 and 3438.545 correspond to the Na+ and K+ adducts, respectively.
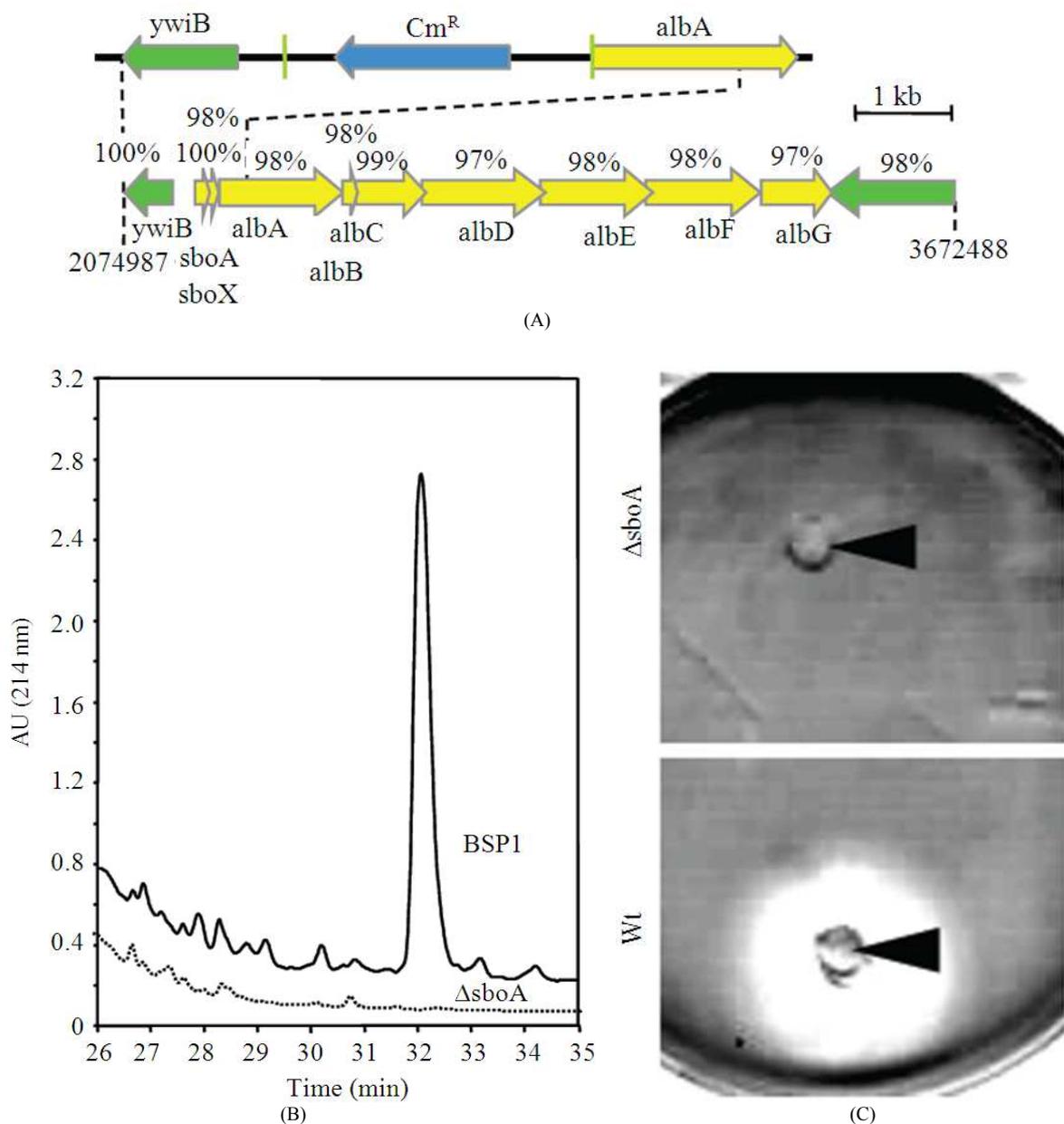
3.3. Genetic Characterization

Inspection of the BSP1 genome sequence revealed the presence of ORFs (A7A1_0154 to A7A1_0162) coding for products with a high level of sequence identity (> 95%) to those of the *sbo-alb* operon of strain 168 (**Fig. 2A**). This suggested that BSP1 could synthesize the bacteriocin subtilosin A. To confirm this, we disrupted the A7A1_0154 and A7A1_0155 ORFs of the subtilosin A cluster encoding the subtilosin A structural gene by insertion of a chloramphenicol resistance cassette (**Fig. 2A**) and repeated the purification scheme. We found that both the eluted peak that contained the antimicrobial compound (**Fig. 2B**) and the antilisterial activity were lost for the Δ*sboAX*-derivative compared to the parental strain (**Fig. 2C**). Similar results were obtained for a Δ*sboAX*derivative of *B. subtilis* 168 (data not shown).

## 3.4. Control of Subtilosin a Production

The level of subtilosin A production was quantified by RP-HPLC in culture supernatants of *B. subtilis* strain BSP1 and 168 grown for 16 h at 37°C. As shown in **Fig. 3A**, the subtilosin A productivity was found six fold higher for BSP1 compared to the laboratory strain. To further characterize this phenotype, the *sbo-alb* expression was quantified in both strains. For that purpose, a transcriptional fusion of the *sbo* promoter (P*sbo*) to a Cyan Fluorescent Protein (CFP) and fluorescence microscopy were used to monitor promoter activity in the cell population. The fluorescence images in **Fig. 3C** show that more cells of BSP1 appeared to express P*sbo-cfp* in LB at the end of the logarithmic phase of growth, compared to the laboratory strain. The fraction of BSP1 cells expressing P*sbo-cfp* was almost two times higher (**Fig. 3B**) and the average fluorescence signal was also higher for the BSP1 population (**Fig. 3C**) bottom panels.

(A)

(B)

(C)
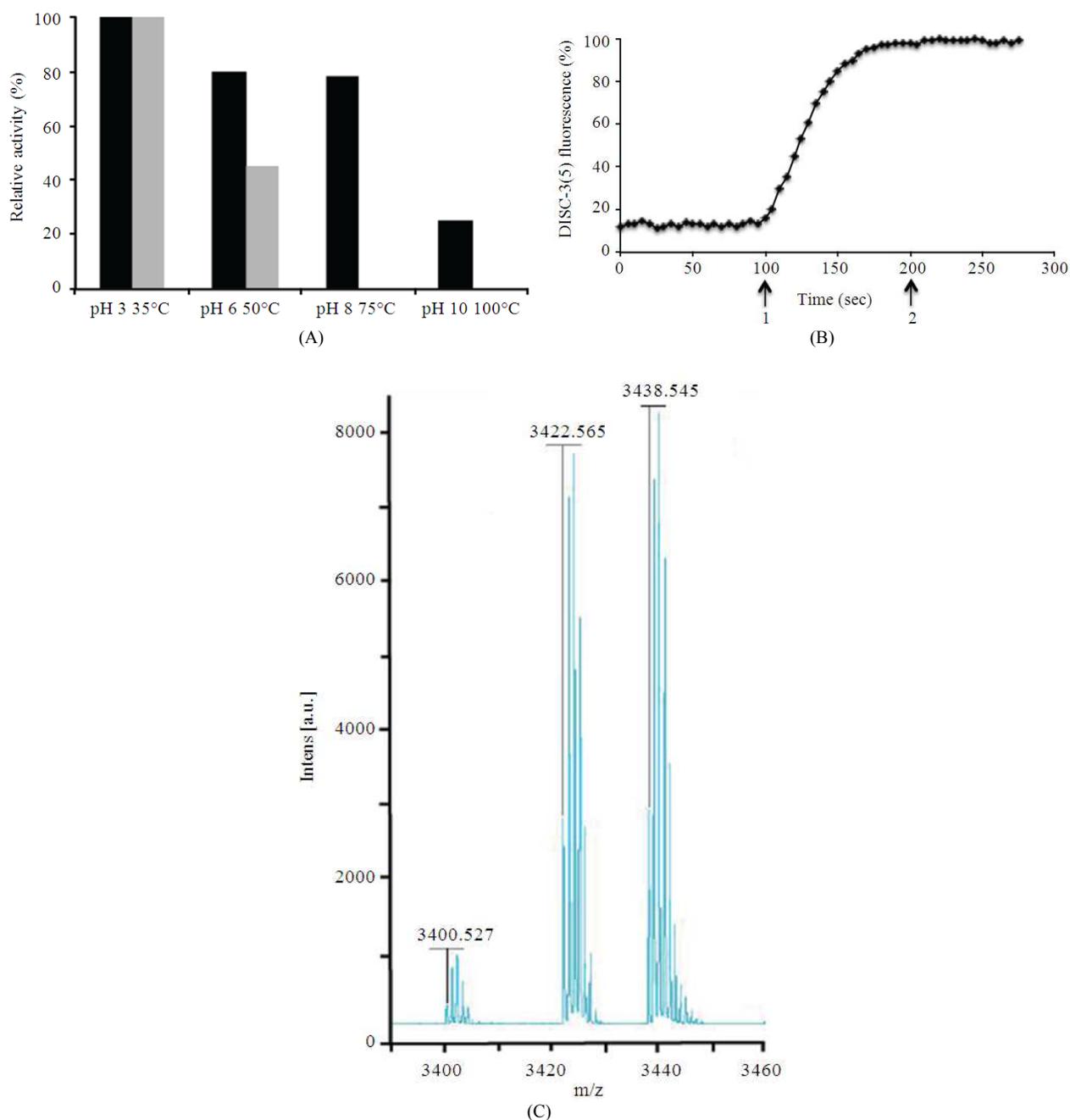
Fig. 1. Characterization of the antimicrobial compound produced by BSP1. Panel A shows the effect of pH (black) and temperature (grey) treatments on the stability of the purified peptide. Values are expressed in relative activity and 100% was set for the higher value of antilisterial activity (pH3 and 35°C, respectively). Values are mean of three independent repetitions and the standard deviations were less than 10%. Panel B shows the dissipation of the transmembrane electrical potential (Δψ). The arrow 1 indicates the addition of purified compound (20 μL) to a DiSC$_3$ (5) preloaded *L. ivanovii* cell suspension whereas arrow 2 indicate addition of the ionophore valinomycin (1 μL). Values were normalized to the fluorescence signal obtained with the ionophore valinomycin under the same conditions. Panel C shows the matrix-assisted laser desorption ionization time of flight spectrum of the purified compound produced by BSP1

(A)

(B)

(C)

Fig. 2. Genetic characterization of the subtilosin gene cluster in BSP1. Panel A shows the structure of the *sboA-alb* cluster of BSP1, which codes for subtilosin A biosynthetic enzymes and confers immunity to the peptide. The cluster is also found at an equivalent locus in strain 168 (the percentage of similarity of the predicted products between the two strains is indicated as well as the coordinates relative to *oriC* for BSP1). Also shown is the structure of a mutant obtained by replacing the *sboA* and *sboX* genes by a chloramphenicol resistance determinant (bordered by the two vertical lines), with the black dotted lines showing the boundaries of the DNA fragments used for recombination. Panel B shows the RP-HPLC chromatogram of culture supernatant of BSP1 (solid line) and the BSP1Δ*sboAX* deletion mutant (doted line). Panel C shows the bactericidal activity of subtilosin A (3.5 µg) purified from wild-type BSP1 strain or of control material purified in parallel from the Δ*sboAX* mutant against a lawn of *L. ivanovii* cells
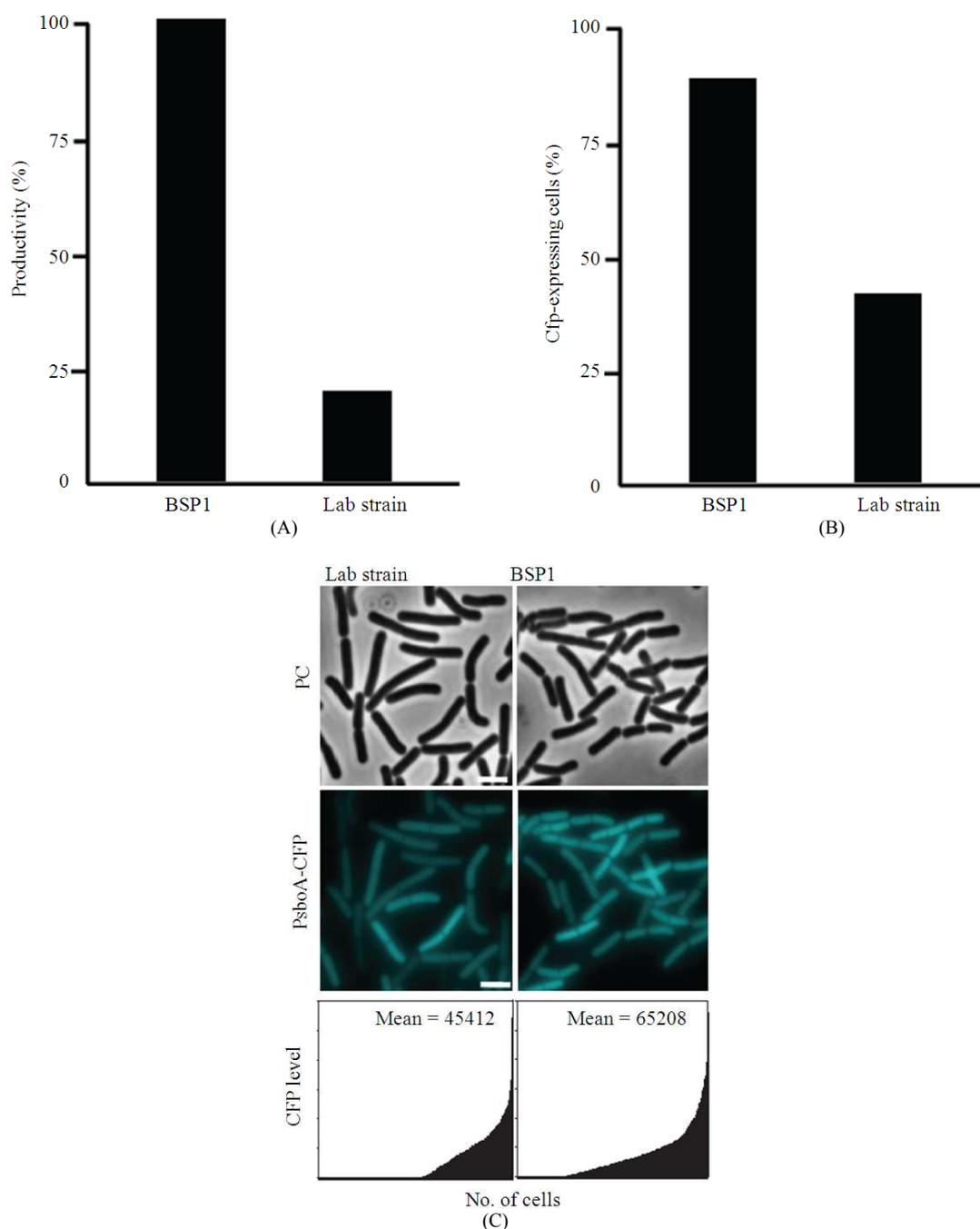
Fig. 3. Comparison of subtilosin productivity and of *sbo-alb* expression in BSP1 and laboratory strain. Panel A shows the relative subtilosin A productivity (quantity of subtilosin A produced per unit of biomass) obtained after 18 of growth in LB medium. Values are mean of three repetitions. Standard deviations were not significant. Panel B shows the percentage of cells of the laboratory strain or BSP1 with detectable expression of P_{sboA}-*cfp*. Panel C shows microscopic examination of cells of the laboratory strain or BSP1, entering stationary phase and expressing a *PsboA-cfp* fusion. The cells were observed by phase contrast (top) and fluorescence microscopy (middle) and the distribution of the signal quantified as a function of cell number (bottom panel)

Essentially the same results were found in cells grown in sporulation medium (data not shown). In control studies, expression of P*sbo-cfp* was abolished by deletion of the *spo0A* gene in line with the requirement for Spo0A for the production of subtilosin A (Strauch *et al*., 2007) (data not shown).

4. DISCUSSION

Members of the genus *Bacillus* are known to produce a wide range of antimicrobial substances, including peptides, lipopeptides and the so-called bacteriocins (Stein, 2005; Fickers, 2012). The production of these compounds, together with sporulation capacity, endow *Bacillus* with potent selective advantages to cope with its natural ecological niches. Bacteriocin are ribosomally synthetized peptide that elicit bactericidal activity through a transmembrane pore-forming mode of action toward pathogens such as *L. monocytogenes*, *Streptococcus pyogenes* or *C. perfringens* (McAuliffe *et al*., 2001). Their antimicrobial activity, their non-toxicity for man and animal commensal gut microflora, render these metabolites very attractive for agro-food and biopharmaceuticals applications (Galvez *et al*., 2007). The primary purpose of this study was to characterize the antimicrobial compound produced by BSP1, a strain isolated from broiler gastrointestinal tract (Barbosa *et al*., 2005). The present results clearly demonstrated that this compound correspond to the bacteriocin subtilosin A, a 35 amino acid cyclic peptide first isolated from *B. subtilis* (Babasaki *et al*., 1985) and subsequently from *Bacillus amyloliquefaciens* (Sutyak *et al*., 2008) and *Bacillus atrophaeus* (Stein *et al*., 2004). This unusual bacteriocin is characterized by the presence of three cross-links between sulfur of cysteine and the α-carbon of the two phenylalanine and threonine, which are distinct from the thioether bridges observed in lantibiotics. This posttranslationally generated architecture renders the molecule stable at extreme pH and highly resistant to endoproteinases in physiological conditions.

The production of antimicrobials by probiotics is considered one of the principal mechanisms that inhibits growth of pathogenic microorganisms in the gastro-intestinal tract (Hong *et al*., 2005). These include bacteriocin and bactriocin-like inhibitory substances. Some *Bacillus* species contained in commercial products were shown to produce antimicrobials. *B. polyfermenticus* SCD carried in Bispan has been shown to produce the proteasesensitive and heat-labile bacteriocin, polyfermenticum, active against Gram-positive bacteria (Lee *et al*., 2001). In poultry studies,

controlled trials have shown that oral administration of *B. subtilis* spores reduced infection by *Samonella enterica* serotype Enteritidis, *C. perfringens* and *Escherichia coli* 078:K80. More precisely, *B. subtilis* 2335, known under the trademark BioPlus 2B, has been shown to produce antibiotics (Pinchuk *et al*., 2001). Beside this, other studies demonstrated that PY79 spores given to day-old chicks 24 prior to challenge with *C. perfringens* resulted in a significant reduction of gastro-intestinal tract infection and fecal shedding (La Ragione and Woodward, 2003). However, subtilosin A, the main antimicrobial compound produced by PY79, was never been demonstrated responsible for this effect.

The higher subtilosin A productivity observed for BSP1 compared to the laboratory strain 168 or PY79 was unexpected. To explain this observation, we hypothesized that this particular phenotype could results, at least in part, from an increased expression of the *sbo-alb* operon. Indeed, the production of mature subtilosin A is based on the expression of the *sbo-alb* gene cluster encompassing the subtilosin A structural gene *sbo* and the *alb* genes involved in posttranslational modifications, processing of presubtilosin A and in immunity mechanism (Zheng *et al*., 1999). The regulation of the *sbo-alb* expression is complex and related with the onset of the stationery phase. Induction occurs during late growth phase in response to carbon sources starvation and oxygen limitation (Nakano *et al*., 2000). Subtilosin A synthesis is negatively regulated by the transition state regulatory protein AbrB which binds directly onto the *sbo-alb* promoter during exponential growth phase (Strauch *et al*., 2007). When cells approach the stationery phase, Spo0A, the stationary phase and sporulation master regulator is phosphorylated through a phosphorelay signal transduction system (Trach *et al*., 1991). The first step is the ATPdependent autophosphorylation of protein kinase Kin family that feed the phosphate group into the phosphorelay. Accumulation of Spo0A-P led to the repression of *abrB* and thus to the derepression of *sbo-alb* operon. Recent investigations highlighted an increased sporulation capacity of BSP1 during growth and demonstrated that this phenomenon was the consequence of an increased activity of Spo0A in the cell population. Therefore the higher level of *sbo-alb* expression and thus subtilosin A productivity, could be explained by this increased activity of Spo0A in *B. subtilis* BSP1 compared to that of the laboratory strain *B. subtilis* 168 and PY79.

5. CONCLUSION

Through this study the gut-associated *B. subtilis* BSP1 has been demonstrated able to synthetize the cyclic bacteriocin subtilosin A at high level compared to laboratory strains. Our findings suggest that the BSP1 phenotype could be related to an increased expression level of the subtilosin A biosynthetic gene cluster sbo-alb in response to a higher activity of the stationery and sporulation master regulator Spo0A.

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