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 Bacillus subtilis strain to synthesize 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 stationary and sporulation master regulator Spo0A.

Keywords: Bacillus Subtilis, Bacteriocin, Subtilosin, Probiotic

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...
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 known 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 modifications 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 BSPI.

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 DNA 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 (Qiagen, 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 BSPI on bacteria isolated from swine intestinal content

| Strain                  | Antimicrobial activity*               |
|-------------------------|--------------------------------------|
| 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 salivarius| -                                    |
| Lactobacillus fermentum | -                                    |
| Lactobacillus acidophilus| -                                 |
| Clostridium perfringens | +                                    |
| Clostridium perfringens P41 | +++                             |
| Listeria monocytogenes HCIP59.53 | +                               |
| Listeria ivanovii DCIP107777 | +++                              |

*All strains are from DSM Nutritional Products collection, Basel, Switzerland

+ 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) |
| BSP1ΔsboAX        | sboAX::Cm<sup>R</sup>        | This study |
| 168ΔsboAX         | sboAX::Cm<sup>R</sup>        | His study |
| AH7496 PY79       | derivative (sboA::PsboAPY79-cfp::Cm<sup>R</sup>) | This study |
| AH7497            | BSP1 derivative (sboA::PsboABSP1-cfp::Cm<sup>R</sup>) | This study |
| **Plasmids**      |                              |           |
| pCS57             | bla PsboA PY79-cfp cat        | This study |
| pCSS8             | bla PsboA 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        | TTTTGGCCCCAGGGCCCAATGAACTCTCCCTCTTTTTTTGT | SfiI |
| Sbo3        | TTTTGGCCCCAGGGCCCAATGAACTCTCCCTCTTTTTTTGT | SfiI |
| Sbo4        | CAAGTTTGGGCAAAGAGGCTTTC |          |
| sboA-380F   | GGGGTACCCGATGCCATCTTCTTGGGCTCGAGCGAGCGATGTT | KpnI |
| sboACFP+38R | GTTCTTACCTATAAGCTGCTCGAGCGATGTT |          |
| CFP-24F     | AAGCTTACATAAGGAGGAC |          |
| CFP+750R    | CGCGGATATTCTATTATAAG | BamHI |

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<sup>-1</sup> 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 (Δψ) was monitored using the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide (DiSC₃(5), Anaspec, Ferment, 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, Sigma-Aldrich). After stabilization of the fluorescence signal (excitation 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 the cell suspension. DiSC₃(5) fluorophore (1 µM, final concentration, Sigma-Aldrich) was added to dissipate any residual Δψ. 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 sbol- 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-chloramphenicol 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 ΔsboAX 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 BSP1ΔsboAX and 168ΔsboAX, 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 and CFP+750R, which generated a KpnI 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 BamHI 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 KpnI restriction site and sboA-CFP+38R (containing a tail for cfp). The resulting strains were denominated BSP1ΔsboAX and 168ΔsboAX, respectively.

2.8. Fluorescence Microscopy

For visualization of the PsboA-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 ΔΨm by 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 ΔsboAlX derivative compared to the parental strain (Fig. 2C). Similar results were obtained for a ΔsboAlX 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 (Psbo) 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 Psbo-cfp in LB at the end of the logarithmic phase of growth, compared to the laboratory strain. The fraction of BSP1 cells expressing Psbo-cfp was almost two times higher (Fig. 3B) and the average fluorescence signal was also higher for the BSP1 population (Fig. 3C) bottom panels.
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 (pH 3 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 DiSC3 (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.
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 BSP1∆sboAX deletion mutant (dotted 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 ΔsboAX 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 PsboA-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 Psbo-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 synthesized 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 gastrointestinal tract (Hong et al., 2005). These include bacteriocin and bacteriocin-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 protease-sensitive and heat-labile bacteriocin, polyfermenticin, 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 Salmonella 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 gastrointestinal 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 ATP-dependent 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 stationary and sporulation master regulator Spo0A.

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