



Short Communication

The presence of *sboA* and *spaS* genes and antimicrobial peptides subtilosin A and subtilin among *Bacillus* strains of the Amazon basin

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Abstract

This report demonstrates the usefulness of PCR for the genes *spaS* and *sboA* as a means of identifying *Bacillus* strains with a potential to produce subtilin and subtilosin A. One collection strain and five *Bacillus* spp. isolated from aquatic environments in the Amazon basin were screened by PCR using primers for *sboA* and *spaS* designed specifically for this study. The sequences of the PCR products showed elevated homology with previously described *spaS* and *sboA* genes. Antimicrobial peptides were isolated from culture supernatants and analyzed by mass spectrometry. For all samples, the mass spectra revealed clusters with peaks at m/z 3300-3500 Da, corresponding to subtilosin A, subtilin and isoforms of these peptides. These results suggest that the antimicrobial activity of these strains may be associated with the production of subtilosin A and/or subtilin. The PCR used here was efficient in identifying novel *Bacillus* strains with the essential genes for producing subtilosin A and subtilin.

Keywords: *Bacillus*, bacteriocin, polymerase chain reaction, subtilin, subtilosin A.

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Many peptide antibiotics possess bactericidal, fungicidal and immunomodulatory activities and frequently occur as secondary metabolites produced by microorganisms. Among *Bacillus* species, some antimicrobial peptides are gene encoded and synthesized ribosomally while others are produced non-ribosomally by the multienzyme thiotemplate mechanism (Cotter *et al.*, 2005).

Subtilosin A is the only anionic, circular antimicrobial peptide produced by wild strains of *Bacillus subtilis* (van Belkum *et al.*, 2011). The production of mature subtilosin A requires the expression of eight (*sboA*-*albABCDEFG*) of the nine genes identified in subtilosin A-producing strains of *B. subtilis*. These clustered genes are transcribed from a promoter residing upstream of the *sboA* gene and their products are involved in the posttranslational modification and processing of presubtilosin, secretion and immunity. The *albABCDEFG* genes are believed to constitute an operon that encodes the proteins that function in presubtilosin processing and subtilosin ex-

port. The *sboA* gene encodes presubtilosin, a 43-amino acid peptide (Zheng *et al.*, 2000; van Belkum *et al.*, 2011).

Subtilin is a lanthionine-containing peptide antibiotic (lantibiotic) very similar to nisin, which is the most important member of the group of linear lantibiotics and is used as a food preservative. The biosynthesis of this bacteriocin is based on the expression of ten genes that are organized in a gene cluster *spaBTCSIFEGRK*. The *spaS* gene encodes the prepeptide of subtilin in which amino acids are enzymatically modified by the products of genes *spaB* and *spaC*. The modified precursor is transported through the cytoplasmic membrane by the ABC transporter encoded by *spaT*, which appears to be associated with a membrane complex containing the enzymes SpaB and SpaC (Kiesau *et al.*, 1997; Stein *et al.*, 2003).

The Amazon basin is a source of enormous biological diversity for microorganisms with potential value for biotechnological applications (Chies *et al.*, 2002). Some investigations have undertaken screening for useful microorganisms (Cladera-Olivera *et al.*, 2004; Motta *et al.*, 2004). The aim of this work was to screen for the subtilosin A and subtilin genes among *Bacillus* strains isolated from the intestine of Amazon basin fishes, using a simple, efficient PCR with specific primers for *sboA* and *spaS*.

Bacterial isolates were kindly provided by Dr. Spartaco Astolfi Filho (Universidade Federal do Amazonas, Manaus, AM, Brazil). The strains were *Bacillus* sp. P11 (GenBank accession no. DQ387864), *Bacillus* sp. P34 (AY962472) and *Bacillus licheniformis* P40 (AY962473), isolated from the fish *Leporinus* sp., and *Bacillus* sp. P7 (DQ387865) and *Bacillus subtilis* P45B (AY962474) isolated from *Piaractus mesopotamicus* intestines. *B. subtilis* ATCC 19659 and *B. cereus* ATCC 14579 were used as reference strains. The bacteria were maintained at -21 °C in BHI broth containing 20% (v/v) glycerol and were cultivated in Tryptone soy broth (TSB; Mast Diagnostics, Merseyside, UK) agar plates for 24 h at 37 °C and then in TSB for 24 h at 37 °C before use.

DNA was extracted from overnight cultures using the Promega Wizard SV Genomic DNA kit (Promega, Madison, WI, USA). The specific primers used for PCR amplification of the *sboA* and *spaS* genes were developed using Vector NTI primer design software (Invitrogen, Carlsbad, CA, USA). Primers *sboA-f* (5'-CATCCTCGATCACAGACTTCACATG-3') and *sboA-r* (5'-CGCGCAAGTAGTCGATTCTAACAC-3') were used to amplify a 734 bp *sboA* fragment corresponding to the *B. subtilis* subtilosin gene cluster (AJ430547), whereas primers *spas-f* (5'-TGTCATGGTTACAGCGGTATCGGTC-3') and *spas-r* (5'-AGTGCAAGGAGTCAGAGCAAGGTGA-3') were used to amplify a 566 bp *spaS* fragment corresponding to the *B. subtilis* subtilin gene cluster (U09819). Each 50 µL of PCR mix contained 5 µL of *Taq* buffer 10x, 3 µL of 25 mM MgCl₂, 0.4 µL of 25 mM dNTPs, 0.5 µL of *Taq* polymerase (5 U/mL; Invitrogen), 1.25 µL of 20 µM primer, 50 ng of genomic DNA and 36.1 µL of Milli-Q H₂O. PCR was done using a Mastercycler Personal thermocycler (Eppendorf AG, Hamburg, Germany) under the fol-

lowing conditions: denaturation for 1 min at 94 °C, annealing for 30 s at 50 °C (*sboA*) or 55 °C (*spaS*) and elongation for 1 min at 72 °C for a total of 35 cycles for both subtilosin A and subtilin.

Electrophoretic analyses of the PCR products revealed 734 bp and 566 bp fragments for the *sboA* and *spaS* primers, respectively (Figure 1). No PCR products were observed for *B. cereus* ATCC 14579 that was used as a negative control (data not shown). The PCR products were sequenced in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil) using an automatic sequencer (ABI-PRISM 3100 Genetic Analyzer; Applied Biosystems). The sequence data were collected using the software Data Collection ver. 1.0.1 (Applied Biosystems) and a BLAST algorithm was used to retrieve homologous sequences from GenBank (National Center for Biotechnology Information) with the software CLUSTAL W v.1.8 (Thompson *et al.*, 1994).

All sequences of the 734 bp fragments showed elevated homology (minimum identity: 98%) with the gene encoding presubtilosin (*sboA*). Similarly, the sequences of the 566 bp fragments had high homology (minimum identity: 97%) with the gene encoding presubtilin (*spaS*). Differences in the sequences corresponded to silent mutations. The genes *sboA* and *spaS* are critical for production of the antimicrobial peptides subtilosin A and subtilin, respectively (Zheng *et al.*, 2000; Stein *et al.*, 2003).

Antimicrobial peptides were isolated as described by Kawulka *et al.* (2004). Samples concentrated in a vacuum centrifuge (SpeedVac SC100, Savant, USA) were dissolved in ethanol and analyzed by mass spectrometry in a MALDI-TOF mass spectrometer (MALDI-Micro MX PSD, Micromass, Manchester, UK) operated in reflection mode with a matrix of α -cyano-4-hydroxycinnamic acid.

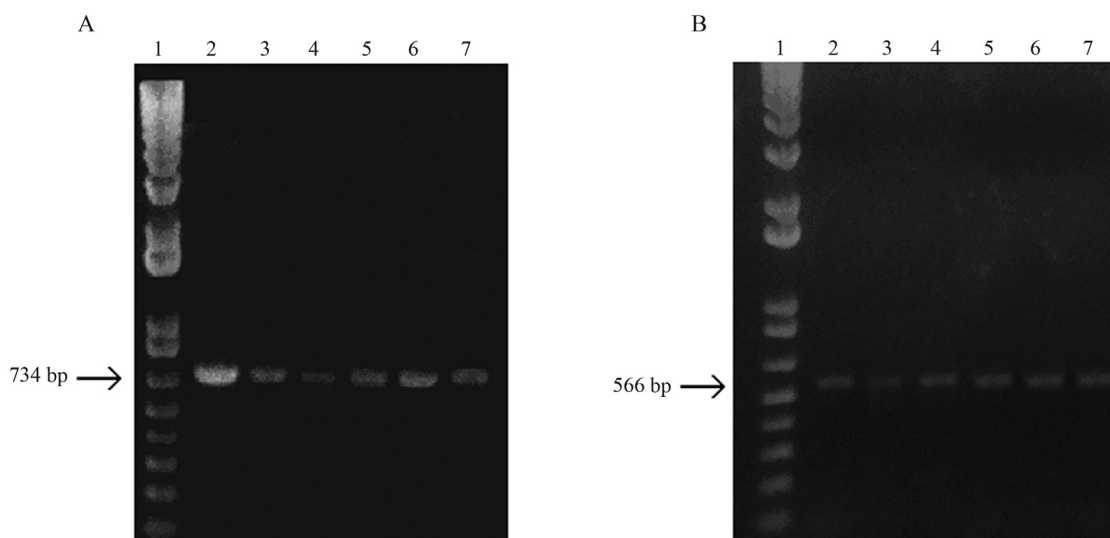


Figure 1 - PCR product profiles of the (A) *sboA* and (B) *spaS* genes. 1 - Molecular weight marker (1 kb PLUS, Invitrogen), 2 - *Bacillus subtilis* ATCC 19659, 3 - *Bacillus* sp. P7, 4 - *Bacillus* sp. P11, 5 - *Bacillus licheniformis* P40, 6 - *Bacillus subtilis* P45B, 7 - *Bacillus* sp. P34. Samples were run in 1% agarose gels.

Antimicrobial activity was determined by the disk diffusion method, essentially as described elsewhere (Motta *et al.*, 2007). *Listeria monocytogenes* ATCC 7644 was used as the indicator organism.

The antimicrobial peptides were isolated from the cell-free culture supernatant of *Bacillus* spp. All strains showed antimicrobial activity against *L. monocytogenes*. The mass spectra revealed major peaks at m/z 3300-3500 Da (Figure 2), corresponding to subtilisin A, subtilin and isoforms of these peptides (Heinzmann *et al.*, 2006; Abriouel *et al.*, 2011). Typical m/z values for subtilisin A (3399), subtilin (3319), succ-subtilin (3419) and its K^+ adduct (3457) were observed in the spectra. A major peak at m/z 3442 corresponding to ericin S (Bierbaum and Sahl, 2009) was also detected. Strain P7 presented a minor peak at m/z 3347, possibly associated with the recently discovered lantibiotic entianin (Fuchs *et al.*, 2011). Strain P34 showed a more complex spectrum (Figure 2C), with some peaks that could not be attributed to known antimicrobial

peptides from *Bacillus* spp. These results agree with the fact that *Bacillus* may produce a diversity of antimicrobial peptides that vary according to the strain (Abriouel *et al.*, 2011).

The results of this work indicate that PCR with specific primers for *sboA* and *spaS* is a valuable means of screening for *Bacillus* spp. and strains that produce subtilisin A and subtilin. With the exception of *B. subtilis*, the presence of subtilisin A had previously been reported only in a strain of *B. amyloliquefaciens* (Sutyak *et al.*, 2008). *Bacillus* sp. P7 and P11 belong to the *B. subtilis*, *B. amyloliquefaciens* and *B. velesensis* cluster (Giongo *et al.*, 2007), whereas strain P34 appears to be a novel *Bacillus* species (Motta *et al.*, 2007). PCR-based methods are valuable tools for the rapid screening of class II bacteriocin-producing isolates in environmental samples (Yi *et al.*, 2010). PCR has also been used to detect the bacteriocins nisin, pediocin and enterocin A in lactic acid bacteria isolated from traditional Thai fermented foods (Suwanjinda *et al.*, 2007).

The continuous emergence of antibiotic resistance has led to increased interest in bacteriocins. These peptides are considered as the ultimate candidates for food preservation and some clinical applications because their range of activity is often limited and specific (Cotter *et al.*, 2005). Like nisin, subtilisin A and subtilin have a proven track record of efficacy against *L. monocytogenes* (Stein *et al.*, 2004; Burkard *et al.*, 2007). Our results provide another option that should be investigated by the food industry.

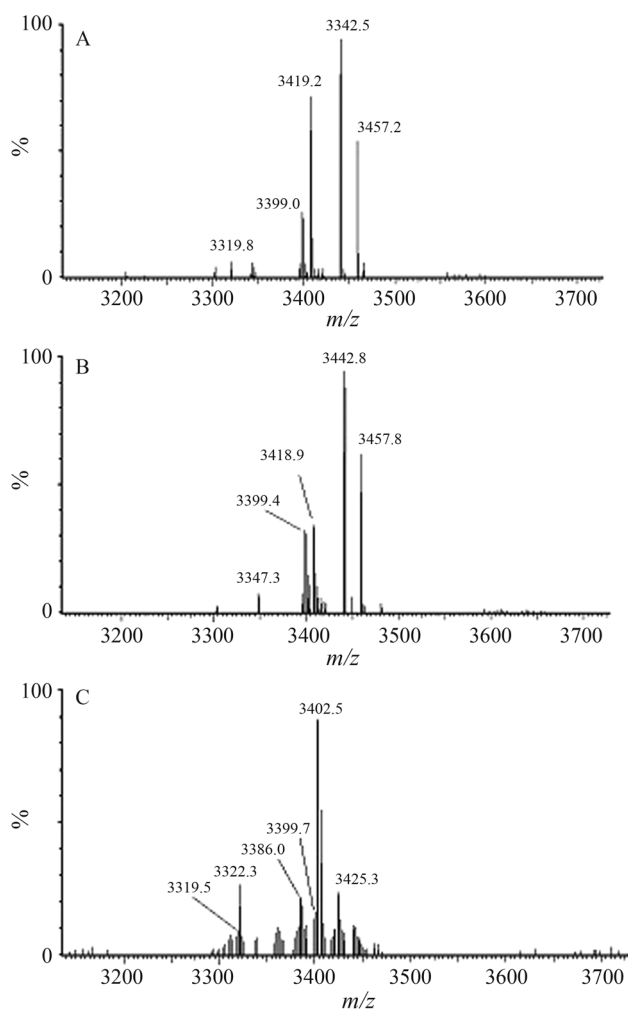


Figure 2 - Typical mass spectra of antimicrobial peptides extracted from culture supernatants of *Bacillus* spp. (A) *B. subtilis* ATCC 19659, (B) *Bacillus* sp. P7, (C) *Bacillus* sp. P34.

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