The presence of \textit{sboA} and \textit{spaS} genes and antimicrobial peptides subtilosin A and subtilin among \textit{Bacillus} strains of the Amazon basin

Renata Voltolini Velho$^1$, Ana Paula Basso$^1$, Jeferson Segalin$^2$, Luis Fernando Costa-Medina$^1$ and Adriano Brandelli$^1$

$^1$Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.
$^2$Unidade de Química de Proteínas e Espectrometria de Massas, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Abstract

This report demonstrates the usefulness of PCR for the genes \textit{spaS} and \textit{sboA} as a means of identifying \textit{Bacillus} strains with a potential to produce subtilin and subtilosin A. One collection strain and five \textit{Bacillus} spp. isolated from aquatic environments in the Amazon basin were screened by PCR using primers for \textit{sboA} and \textit{spaS} designed specifically for this study. The sequences of the PCR products showed elevated homology with previously described \textit{spaS} and \textit{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 \textit{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 \textit{Bacillus} strains with the essential genes for producing subtilosin A and subtilin.

Keywords: \textit{Bacillus}, bacteriocin, polymerase chain reaction, subtilin, subtilosin A.

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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 Triptone 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’-CATCCTCGATCACAGACTTACATG-3’) and sboA-r (5’-CGCGCAAGTAGTCGATTTCTAACAC-3’) were used to amplify a 734 bp sboA fragment corresponding to the B. subtilis subtilosin gene cluster (AJ430547), whereas primers spas-f (5’TGTCTATGTTACAGCGGTGATC-3’) and spas-r (5’-AGTGCAAGGAGTCAGAGCAAGGTA-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 following 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 automated 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 subtilosin A, subtilin and isoforms of these peptides (Heinzmann et al., 2006; Abriouel et al., 2011). Typical m/z values for subtilosin 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 subtilosin A and subtilin. With the exception of *B. subtilis*, the presence of subtilosin 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, subtilosin 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.

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**References**


**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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