Characterization of subtilosin gene in wild type Bacillus spp. and possible physiological role

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In a designed study to screen for antimicrobial exhibiting bacteria using molecular aspects, Bacillus species were considered to investigate antibiotic biosynthesis genes. 28 bacterial strains and 3 induced mutants were screened for the presence of subtilosin gene (sbo) and subtilosin through PCR and Mass spectrometry respectively. Sbo gene was detected in 16 out of 28 Bacillus strains. The results from gene sequences deliberated by multiple sequence alignments revealed high-level homology to Bacillus this report provided additional strains to support the idea of subtilosin gene predominance amongst genes, furthermore the utilization of its conserved region as a means of identifying linkages13 that have been elucidated as thioether bonds between cysteine sulphurs and amino acid alpha-carbons14. An acidic isoelectric point differentiates subtilosin from the basic lantibiotics15. In subtilosin, posttranslational linkage of a thiol to the R-carbon of an amino acid residue is unprecedented in ribosomally synthesized peptides or proteins, and very rare in secondary metabolites14,16. The mature product is formed by loss of an unusually short seven amino acid leader peptide, cyclization of the N and C termini, and further modification of Cys, Thr, and Phe residues17. The mature subtilosin peptide is highly resistant to enzymatic proteolysis and is stable to moderate heat and acid treatment. It acts against a variety of Gram-positive bacteria, including pathogens18–21. The production of mature subtilosin is based on the expression of the sbo-alb gene cluster encompassing the subtilosin structural gene sbo and genes involved in posttranslational modification and processing of presubtilosin and in immunity22–24. Expression of the sbo-alb genes occurs under stress conditions25. 16S rRNA gene used for rapid identification of the Bacillus genus was undertaken by Celandroni et al.26. The high 16S rRNA gene sequence similarities between some stains within this genus can even share phenotypic properties27. However, they have been classified as different species based on DNA association values hence, demonstrated the need for a polyphasic approach to the systematics of this genus28,29. This was observed between B. subtilis subsp. subtilis and B. subtilis subsp. spizizenii, which share phenotypic profiles but have segregated based on DNA reassociation values of 58–69%, in addition to minor polymorphisms in the 16S rRNA gene between the type strains30. Further, B. mojavensis and B. subtilis subsp. spizizenii have only a 1-bp difference in the 16S rRNA gene and can only be distinguished from each other by sexual isolation, divergence in DNA sequences of the rpoB and gyrA genes, and fatty acid composition31. Stein et al.32 pre-postulate the coding gene subtilosin gene (sbo) to develop evolutionary divergence in B. subtilis subspecies too. This report is to describe subtilosin production by 16 wild-type B. subtilis strains and B. amyloliquefaciens. The sbo genes of these organisms were sequenced in order to analyze the genetic variation between B. subtilis wild-type strains. Finally, we confirm the association between production of subtilosin A and the detection of sbo gene using PCR screening.
Materials and methods

**Strains and media.** The following 32 different bacterial strains were tested for their sensitivity to the antibiotic using the agar-well diffusion assay: *Bacillus subtilis* 168, *Bacillus subtilis* ATCC, from Bacillus Genetic Stock Center (BGSC) and *Bacillus cereus* (Lab. collection) and 28 environmental isolates (Table 1). *Bacillus fusiformis* was routinely used for sensitivity test. All the strains were regularly maintained on nutrient agar, however, for antibiotic production Landy medium (glucose, 20 g L⁻¹; glutamic acid, 2 g L⁻¹; (NH₄)₂SO₄, 2.3 g L⁻¹; yeast extract, 1 g L⁻¹; K₂HPO₄, 1 g L⁻¹; MgSO₄, 0.5 g L⁻¹; KCl, 0.5 g L⁻¹; CuSO₄, 1.6 mg L⁻¹; Fe₂(SO₄)₃, 1.2 mg L⁻¹; and MnSO₄, 0.4 mg L⁻¹) was used.

**Bacterial identification.** Identification of the isolated strains was carried on by sequence homology of 16S rDNA accompanied by morphological and biochemical characterization. Identification to the species level was defined as a 16S rDNA sequence similarity of ≥ 99% with that of the prototype strain sequence in GenBank; identification at the genus level was defined as a 16S rDNA sequence similarity of ≥ 97% with that of the prototype strain sequence in GenBank. The biochemical profile of test isolates was determined with the API 50 CHB strips following the manufacturer’s instructions (bioMerieux, France). This test allows bacterial strains to be classified according to their ability to ferment 49 different carbohydrates. The results were analyzed with the APILAB Plus software (bioMerieux, France).

**Antibiotic assay.** Samples of culture supernatant containing the antibiotic checked for activity using an agar-well diffusion assay. Fifty µL of *Bacillus fusiformis* liquid culture of 0.3 OD₆₀₀ was spread onto the surface of Petri dish containing L-agar. 50 µL antibiotic sample was transferred into the well made in media plates using a sterile cork borer. The sample was allowed to diffuse into the agar and the plate was inverted and incubated at 37 °C until a lawn of the indicator bacteria appeared on the plate (approximately 10–16 h).

**DNA isolation, extraction and PCR.** Genomic DNA extracted from overnight-inoculated bacterial culture in N-broth at 37 °C with 120 rpm. The extraction carried out using gene extraction kit (Biorad). PCR
The isolated PCR products were sequenced by using an ABI Prism dye terminator cycle sequencing kit (Qiagen). The PCR fragments were excised from agarose gel electrophoresis followed by extraction with a QIAgen gel extraction kit. Amplification of ~1375-bp consisted of 30 cycles as follows: denaturation for 30 s at 94 °C primer annealation for 30 s at 59 °C and extension at 72 °C for 1.2 min. The final extension was at 72 °C for 9.4 min. Subtilosin A production was also noted for correlation.

| S.n. and Strain designation | Source | Homology 16S rRNA | Sbo PCR detection | Final Bacterial identification | Subtilosin A production |
|-----------------------------|--------|-------------------|-------------------|-------------------------------|------------------------|
| 1. B. subtilis 168 | BGSC | B. subtilis subsp. subtilis 168 | +I | B. subtilis subsp. subtilis 168 | + |
| 2. B. subtilis | SOIL | B. subtilis subsp. Subtilis | +I | B. subtilis subsp. subtilis | + |
| 3. B. subtilis | SOIL | B. subtilis subsp. Subtilis | +I | B. subtilis subsp. subtilis | + |
| 4. B. subtilis | SOIL | B. subtilis strain CICC148 | +I | B. subtilis subsp. subtilis | + |
| 5. B. subtilis | SOIL | B. subtilis subsp. Subtilis | +I | B. subtilis subsp. subtilis | + |
| 6. B. subtilis | SOIL/WATER | B. subtilis subsp. Subtilis | +I | B. subtilis subsp. subtilis | + |
| 7. B. subtilis | SOIL/WATER | B. polyfermenticus | +I | B. subtilis subsp. subtilis | + |
| 8. B. subtilis | SOIL | B. subtilis subsp. Subtilis | +I | B. subtilis subsp. subtilis | + |
| 9. B. subtilis | SOIL | B. subtilis subsp. Subtilis | +I | B. subtilis subsp. subtilis | + |
| 10. B. fusiformis | SOIL | B. sp. strain MHS006 | – | B. subtilis subsp. subtilis | – |
| 11. B. subtilis 7 mut (-A) | SOIL | B. subtilis subsp. Subtilis | – | B. subtilis subsp. subtilis | – |
| 12. B. subtilis ATCC6633 | BGSC | B. subtilis subsp. subtilis ATCC6633 | +I | B. subtilis subsp. subtilis ATCC6633 | + |
| 13. B. subtilis | SOIL/DRY | B. subtilis subsp. Spizezini | +II | B. subtilis subsp. spizezini | + |
| 14. B. subtilis | SOIL/DRY | B. subtilis subsp. Spizezini | +II | B. subtilis subsp. spizezini | + |
| 15. B. mojavensis | SOIL/DRY | B. subtilis strain Au53 | +II | B. subtilis subsp. spizezini | + |
| 16. B. mojavensis | SOIL | B. mojavensis | +I | B. subtilis | + |
| 17. B. licheniformis | SOIL | B. mojavensis | – | B. licheniformis | – |
| 18. B. licheniformis | SOIL | B. licheniformis | – | B. licheniformis | – |
| 19. B. licheniformis | SOIL | B. licheniformis | – | B. licheniformis | – |
| 20. B. licheniformis | SOIL/DRY | B. sp. KL-176 | – | B. licheniformis | – |
| 21. B. licheniformis | SOIL/DRY | B. amyloliquefaciens | – | B. licheniformis | – |
| 22. B. licheniformis | SOIL | B. amyloliquefaciens | – | B. licheniformis | – |
| 23. B. licheniformis | SOIL | B. amyloliquefaciens | – | B. licheniformis | – |
| 24. B. licheniformis | SOIL | B. licheniformis strain KL-176 | – | B. licheniformis | – |
| 25. B. licheniformis | SOIL/DRY | B. licheniformis | – | B. licheniformis | – |
| 26. B. amyloliquefaciens | SOIL/DRY | B. amyloliquefaciens | +I | B. amyloliquefaciens | + |
| 27. B. amyloliquefaciens | SOIL | B. amyloliquefaciens | +I | B. amyloliquefaciens | + |
| 28. B. amyloliquefaciens | SOIL | B. amyloliquefaciens | – | B. licheniformis | – |
| 29. B. amyloliquefaciens | SOIL/DRY | B. fusiformis | – | B. licheniformis | – |
| 30. B. pumilus | SOIL | B. pumilus | – | B. licheniformis | – |
| 31. B. amyloliquefaciens | SOIL/WATER | B. fusiformis | – | B. licheniformis | – |

Table 1. Identification of *Bacillus* strains used through 16S rRNA, sbo and biochemical characterization through CHB-50 PCR of. Subtilosin A production was also noted for correlation. a Identification by using CHB-50. b PCR product of the expected size was detected. I and II assigned for subsp. subtilis and spizezini respectively.

amplification of ~1375-bp consisted of sbo and flanking region was performed successfully with TS13C (GAA TTGACACTATCTAGAAATGCCG) and TS14 (ATCCGGTGTTGCGGAATTCGATGA)32. While primers 27F (GAATTTGACACTATCTAGAAATGCCG) and 1522r (ATCCGGTGTTGCGGAATTCGATGA)34 were used to amplify the 16S rRNA gene. Both sets of primers purchased from Gene Link Inc., USA. 0.5–0.1 ng of chromosomal template DNA and 0.25 µM each primer were added to Master mix (Fermantas). The PCR conditions started with heating at 94 °C for 5 min and passed through 30 cycles as follows: denaturation for 30 s at 94 °C primer annealation for 30 s at 59 °C and extension at 72 °C for 1.2 min. The final extension was at 72 °C for 3 min. PCR fragments were excised from agarose gel electrophoresis followed by extraction with a QiAgen gel extraction kit (Qiagen). The isolated PCR products were sequenced by using an ABI Prism dye terminator cycle
The variance of the m/z of ±0.8 Da was considered acceptable.

sbo over the strain was isolated with no detectable zone of inhibition. This (-A) strain was not able to produce subtilosin more

Bank under accession numbers; FJ151503, FJ151504, FJ151505, FJ151506, FJ151507.

and the results revealed the distinction between two subsp. subtilis their sources.

It is well established that, the ribosomal peptide antibiotics are synthesized during active growth, while nonribosomal ones are synthesized at later stages. Theories on the role of antibiotic production are yet to be investigated. The best-accepted theory is that nonribosomal antibiotics may play a role in competition with other microorganisms during the starvation phase or spore germination36-38. While the role of ribosomal peptides remained undefined. Not so obvious the role of such products in the active life cycle. For example the sublancin action. Suggestions of an intrinsic mechanism of gene improvement i.e. utilizing antibiotics as first line

MALDI-TOF–MS. Fractions correlated with Subtilosin A from TLC and Reverse phase HPLC were analyzed using MALDI-TOF–MS. 2 µL of sample mixed with 2 µL matrix solution (2 mg of alpha-hydroxycinnamic acid per ml in acetonitrile-methanol–water (1:1:1) on the target plate. MALDI-TOF–MS spectra were recorded by using a 337-nm nitrogen laser for desorption and ionization. The mass spectrometer operated in the linear mode at an accelerating voltage of 18 kV with an ion flight path that was 0.7 m long. The delay time was 375 ns. Matrix suppression was also used, and the mass spectra were averaged over 50–100 individual laser shots. The laser intensity was set just above the threshold for ion production. External calibration was performed by using the [M + H]⁺ signals of renin, adenocorticotropin hormone, insulin oxidized B, and bovine insulin (Sigma-Aldrich Co.) the results were anticipated as subtilosin A with m/z of 3400.7 and 3406.6. The variance of the m/z of ±0.8 Da was considered acceptable.

Results

Identification of bacterial strains. Strains were identified according to their morphological and biochemical characteristics added by homology to 16S rRNA with the type strains available in NCBI and RIDOM35 the results revealed the distinction between two subsp. subtilis and spizizenii as well as their association with their sources.

Detection and sequencing of sbo gene. Sbo and its flanking region were detected from the environmental strain and Type strains. B. subtilis 168 and B. subtilis ATCC 6633 were used as positive control representing the two classes/subsp. subtilis (class I) and spizizenii (class II) respectively. Results are shown in (Table 1) in which majority of strain designated as B. subtilis were secured sbo class I. All the obtained DNA fragments were sequenced, and the phylogenetic relation was established using Clustal W, EBI (Fig. 2).

Detection and isolation and of Subtilosin. Subtilosin presence was regularly checked on TLC in reference to match the subtilosin produced by B. subtilis 168 further confirmation was carried on using reverse-phase HPLC (Fig. 1A) and MALDI-TOF–MS (Fig. 1B; Table 1).

Mutation analysis. Mutants produced further selected based on inhibitory activity. Out of 200 mutant 1 strain was isolated with no detectable zone of inhibition. This (-A) strain was not able to produce subtilosin more over the sbo was not amplified using PCR. Hence, it was determined to be functional disruption of encoding gene (Fig. 3B).

Nucleotide sequence accession number. The nucleotide sequences reported deposited in NCBI GenBank under accession numbers; FJ151503, FJ151504, FJ151505, FJ151506, FJ151507.

Discussion

It is well established that, the ribosomal peptide antibiotics are synthesized during active growth, while nonribosomal ones are synthesized at later stages. Theories on the role of antibiotic production are yet to be investigated. The best-accepted theory is that nonribosomal antibiotics may play a role in competition with other microorganisms during the starvation phase or spore germination36-38. While the role of ribosomal peptides remained undefined. Not so obvious the role of such products in the active life cycle. For example the sublancin gene cluster is not essential for B. subtilis, however, it contains yet unidentified genes mediating resistance against sublancin action. Suggestions of an intrinsic mechanism of gene improvement i.e. utilizing antibiotics as first line
Figure 2. Phylogenetic diversity of isolated strains using multiple sequence alignments of sequences sbo and flanking region.

Figure 3. (A) Organization of the sboAX locus. The sboA, sboX, and albA region of the sbo-alb gene cluster and flanking region. Arrowheads indicate direction of transcription; (o) is a terminator. (B) PCR amplified fragment of 1375 bp related to sbo gene where M indicates marker (5000, 2000, 850, 400, 100 bp). Positive amplification for lanes 1–3, 5–7 corresponding to different B. subtilis isolates while lane 10 and 11 corresponds to B. amyloliquefaciens and B. subtilis ATCC6633 respectively. Lane 8 is a positive control (B. subtilis 168) and lane 9 is a negative control (B. licheniformis).
of defense for survival rather than a second, question antibiotics as secondary metabolites. Another probability is displayed social behaviors as co-ordinate gene expression and group behavior through different quorum-sensing pathways. It was determined that interaction of subtilosin with the lipid head group region of bilayer membranes in a concentration dependent manner induced a conformational change in the lipid headgroup and disordering in the hydrophobic region of bilayers that ultimately resulted in membrane permeabilization at high peptide concentrations. Such adaption may lead to assume a growth control during prosperous stage. Furthermore, under anaerobic conditions an increased by 4- to 90-fold, anticipated that the cell accumulates inactive precursors of subtilosin, which then undergo oxygen-dependent modifications to yield an active peptide when an aerobic environment is encountered. The widespread occurrence of subtilosin might reflect an important physiological role. A specific function of subtilosin as an antibiotic, killing factor or as a pheromone during anaerobiosis or biofilm growth of B. subtilis could be well thought-out. Never the less the gene encoding subtilosin production has demonstrated a strong biomarker for Bacillus subtilis. The B. subtilis strains have segregated into two subclades, one encompassing strain 168 and the other W23, classified strain 168 as B. subtilis subsp. subtilis and W23-related strains as B. subtilis subsp. spizizenii based on DNA reassociation studies and sbo gene analysis. The W23 and 168 group strains are identical for most phenotypic characteristics. However, cell wall chemistry of the W23 strains and 168 strains were different; the cell wall of the former contained ribitol and glycerol teichoic acids and that of the latter only glycerol teichoic acid. The sbo-gene of B. subtilis encodes the 43-aminoacid residue comprising the prepropeptide of subtilosin. The nucleotide sequences of the sbo genes and flanking regions are identical in strains belonging to the same subspecies, and the sequences differ by three nucleotides in the two subspecies. However, the encoded Sbo prepeptides are identical in all cases. On the other hand sboX, encoded a bacteriocin-like product, a new gene with an unknown function, in strain 168, which resides in an open reading frame overlapping the coding region of sbo (Fig. 3A). Notably, the expression of sboX would result in a 22-amino-acid curtailed peptide in W23- like strains compared to the peptide produced by 168-like strains, which makes it unlikely that sboX is produced by W23-like strains. These observations were further elaborated to support and to evaluate possible evolutionary relationships among the subtilosin producers, however, A correlation between sbo gene and subtilosin production was not established probably due influence of sboX. Previous attempts for sboX insertions were not successful as such insertions might render sboA mRNA unstable and explain the reduced subtilosin production in sboX mutant. Alignments has revealed that the sbo genes is highly conserved with those of B. subtilis subsp. subtilis (96-100% amino acid identity), while the remaining were less conserved (83–88% identity). This high and low level of conservation is important physiological role. A specific function of subtilosin as an antibiotic, killing factor or as a pheromone during anaerobiosis or biofilm growth of B. subtilis could be well thought-out. Never the less the gene encoding subtilosin production has demonstrated a strong biomarker for Bacillus subtilis. The B. subtilis strains have segregated into two subclades, one encompassing strain 168 and the other W23, classified strain 168 as B. subtilis subsp. subtilis and W23-related strains as B. subtilis subsp. spizizenii based on DNA reassociation studies and sbo gene analysis. 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This high and low level of conservation is unprecedented too; for example, thymidylate synthases A (thyA) in B. subtilis subsp. spizizenii ATCC 6633 and W23 and B. subtilis subsp. subtilis (168) exhibit more than 95% amino acid identity. Even the average level of amino acid identity for the DNA gyrase (gyrA) in seven Bacillus type strains was 95.1%. With these results, we can confirm subtilosin gene predominance amongst Bacillus strains isolated from environment and the correlation amongst different sub-species containing homologous genes. Furthermore, this article demonstrated the possibility to utilize Sbo conserved region as a mean of identifying Bacillus spp. that produce subtilosin.

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Competing interests

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Additional information

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