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Distinct differentiation of closely related species of *Bacillus subtilis* group with industrial importance

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ABSTRACT

PCR amplification of 16S rRNA gene by universal primers followed by restriction fragment length polymorphism analysis using *Rsa*I, *Cfo*I and *Hinf*I endonucleases, distinctly differentiated closely related *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus pumilus* from *Bacillus subtilis sensu stricto*. This simple, economical, rapid and reliable protocol could be an alternative to misleading phenotype-based grouping of these closely related species.

Note

Closely related species of *Bacillus subtilis* group are of great industrial importance for production of enzymes, antibiotics, fermented foods and vitamins. More than one identification methods have been frequently used to distinguish these closely related species of *Bacillus subtilis sensu stricto, Bacillus amyloliquefaciens, Bacillus licheniformis* and *Bacillus pumilus* (Thorsen et al., 2011). Phenotypic grouping of these closely related species based on morphology, physiology, fatty acid composition and carbohydrate fermentation is very often misleading (Logan and Berkeley, 1984; Wunschel et al., 1995). The 16S rRNA gene based taxonomy is a clear way forward for bacterial identification (Woese, 1987). But analysis based on pair wise alignment of 16S rRNA gene sequences showed limited variation in these closely related species of *B. subtilis* group (e.g. *B. subtilis* and *B. amyloliquefaciens* showed more than 99% similarities), which prevented the resolution of strains and species relationship (Hutsebaut et al., 2006). RFLP analysis of rRNA operons has been reported to discriminate the species in the genus *Bacillus* except closely related members of *B. cereus* group (*B. cereus*, *B. thuringiensis* and *B. mycoides*) and the *B. subtilis* group (*B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*) (Daffonchio et al., 1998). It is very difficult to differentiate these closely related members because of very high sequence homology in the ribosomal operons. The 16S-23S rRNA gene internal transcribed spacer (ITS)-RFLP analysis also not differentiated *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* (Daffonchio et al., 1998). Raman spectroscopy based identification of closely related species of *B. subtilis* group failed to differentiate *B. subtilis* from *B. amyloliquefaciens* (Hutsebaut et al., 2006). Nowadays taxonomy based on multi locus sequence typing (MLST) of house keeping genes has been reported as a promising tool for differentiating closely related *Bacillus* species. In this genomic era (when complete genome data for most of the important species of *B. subtilis* group are available), a simple protocol for reliable differentiation during inventorisation studies and a rapid sensitive protocol for diagnosis of these closely related species are not available (Maughan and Van der Auwera, 2011).

Against this background we developed a simple protocol for distinctly differentiating closely related species of *B. subtilis* group by PCR amplification of 16S rRNA gene by universal primers followed by restriction fragment length polymorphism analysis using three restriction enzymes. The first step involved was selection of restriction enzymes based on their theoretical digestion of 16S rRNA gene sequence of type strains/ genome data sourced from NCBI GenBank (release 183) and RDP database (release 10). An *in silico* analysis using Clustal-X (version 8.1) and Bioedit (version 5.0.9) software was carried out by aligning the sequences of *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. pumilus, Bacillus megaterium, B. cereus* and *Bacillus circulans*. The variable regions which differentiated the closely related species of *B. subtilis* group were identified (Fig. 1). Using Webcutter (version 2.0) software the commonly available restriction enzymes which cut differently in the identified variable regions were selected. The validity of selected enzymes was further verified for their specificity and distinctness through *in silico* restriction digestion analysis

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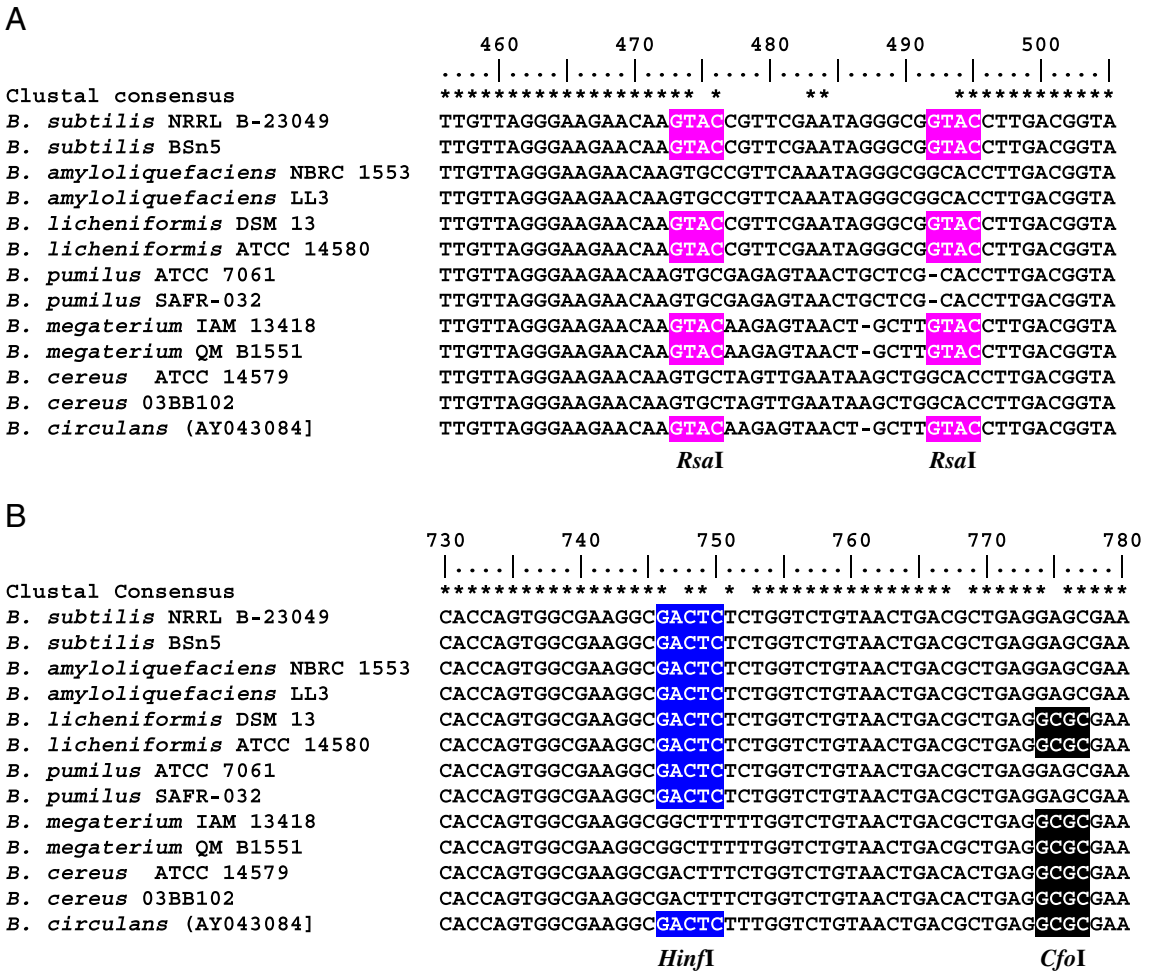

Fig. 1. Alignment of representative 16S rRNA gene sequences from the type strains and genome data sourced from NCBI GenBank and RDP database. The restriction recognition site for *Rsa*I in V3 region (A), and recognition site for *Cfo*I and *Hinf*I in between V4 and V5 region (B) identified for distinct differentiation of closely related species of *Bacillus subtilis* group are highlighted.

of most of the available 16S rRNA gene sequences from the strains of closely related *Bacillus* species. The restriction enzymes *Rsa*I, *Cfo*I and *Hinf*I distinctly differentiated *B. amyloliquecaciens*, *B. licheniformis* and *B. pumilus* from *B. subtilis* (Fig. 1). The selected restriction enzymes were validated *in vitro* for their specificity and accuracy by using reference strains from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The theoretical restriction fragments size calculated by *in silico* analysis were compared with gel detectable restriction fragments size (Table 1) developed during PCR amplification (iCycler, Biorad) of 16S rRNA gene using universal primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCCGCA-3′) (Weisburg et al., 1991) followed by RFLP analysis by digestion with restriction enzymes (Promega) and agarose gel electrophoresis (Biorad). The restriction enzyme *Rsa*I digestion distinctly differentiated *B. amyloliquefaciens* from *B. subtilis* (Fig. 2), *Cfo*I digestion distinctly differentiated *B. licheniformis* from *B. subtilis* (Fig. 3) and *Hinf*I digestion distinctly differentiated *B. subtilis* group from *B. cereus* group. The accuracy of identification by 16S rRNA gene-RFLP analysis was verified by comparing with API 50CHB system of identification (Biomerieux) and 16S rRNA gene sequencing (ABI 3100, Applied Biosystem). A representative data for fifteen *Bacillus* strains isolated from traditional fermented foods are shown in Table 2. The phenotypic identification by carbohydrate fermentation was found to be misleading. The API 50CHB fermentation profile of the above 15 isolates is shown in the supporting information (Table S1).

Realising the need for a simple protocol for grouping hundreds of *Bacillus* isolates to species level accuracy, a simplified nine steps protocol successfully used in our laboratory is given as supporting information (Simplified ARDRA protocol for distinct differentiation of closely related species of *Bacillus subtilis* group). The four critical steps of this protocol are:

- Incubation of *Bacillus* culture at 30 °C reduced the mucilage production. The normal practice of incubating *Bacillus* isolates at 37 °C or 42 °C lead to high mucilage production, which may affect DNA isolation.
- Heat lysis of spheroplast at 95 °C for 20 min yielded cell free DNA lysate with good quality DNA ($A_{260/280}$ ranges from 1.8 to 2.2) and good PCR amplification.
- Annealing temperature at 65 °C during PCR amplification of 16S rRNA gene effectively removed the non-specific amplifications.
- The order of restriction digestion and grouping, first *Hinf*I digestion distinctly differentiated *B. subtilis* group from *B. cereus* group, second *Rsa*I digestion differentiated *B. amyloliquefaciens* from *B. subtilis* group and third *Cfo*I digestion differentiated *B. licheniformis* from *B. subtilis* group.

Using this simple protocol, we successfully differentiated and grouped (with species level accuracy) 482 *Bacillus* isolates from fermented soybean and bamboo shoot products of Northeast India and 280 *Bacillus* isolates from fermented locust bean products of Nigeria.