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

Kumaraswamy Jeyaram [a,*], Wahengbam Romi [a], Thangjam Anand Singh [a], Gbenga Adedeji Adewumi [b], Khundrakpam Basanti [a], Folarin Anthony Oguntoyinbo [b]

[a] *Microbial Resources Division, Institute of Bioresources and Sustainable Development (IBSD), Takyelpat Institutional Area, Imphal-795001, Manipur, India*
[b] *Department of Microbiology, Faculty of Science, University of Lagos, Akoka, Lagos, Nigeria*

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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

* Corresponding author. Tel.: +91 385 2446122x29; fax: +91 385 2446121.
E-mail address: jeyaram.ibsd@nic.in (K. Jeyaram).

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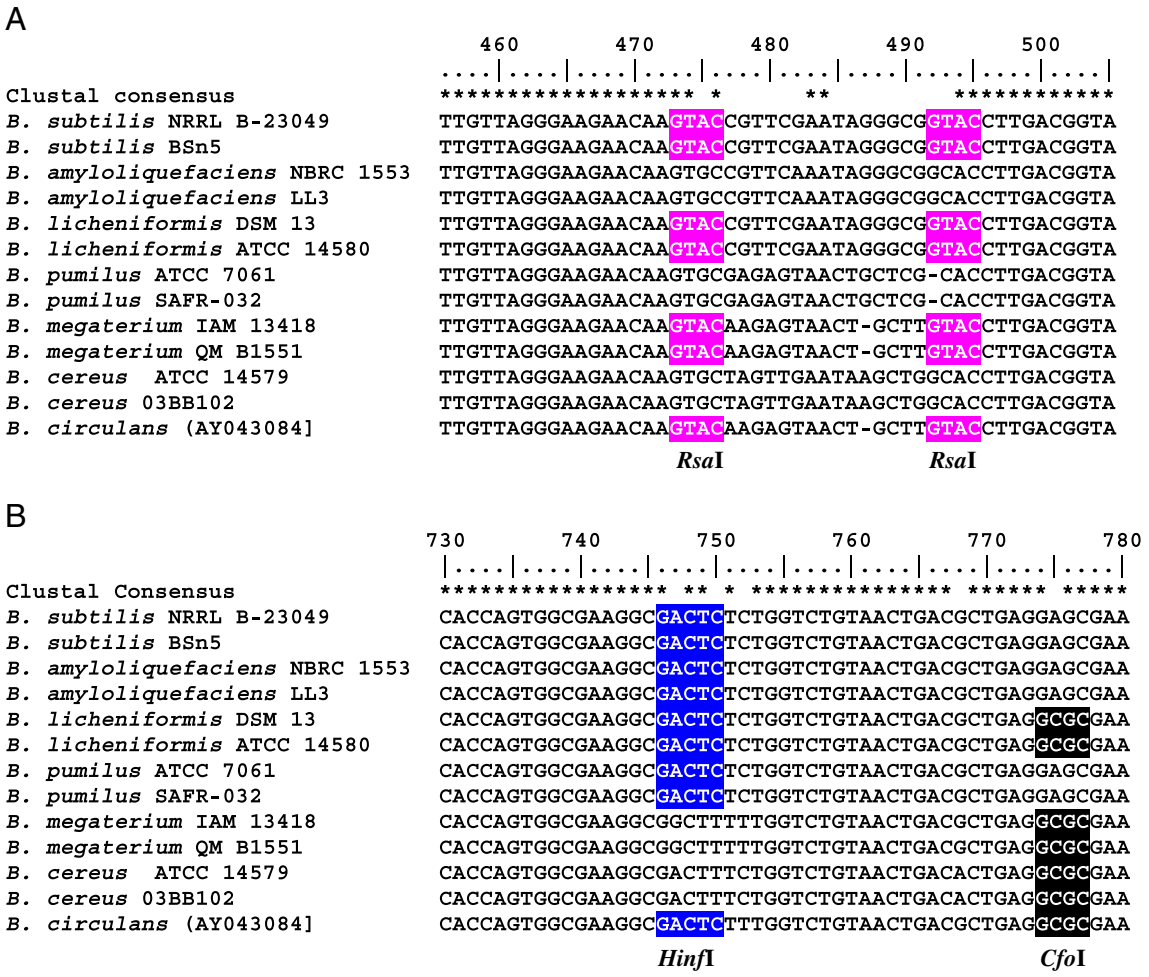

Fig. 1. Alignment of representative 16S rRNA gene sequences from the type strains and genome data sourced from NCBI GenBank and RDP database. The restriction recognition site for *Rsa*I in V3 region (A), and recognition site for *Cfo*I and *Hinf*I in between V4 and V5 region (B) identified for distinct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

Table 1
Theoretical restriction fragments size and gel detectable restriction fragments size of 16S rRNA gene RFLP analysis of closely related species of *Bacillus subtilis* group.

Bacillus species	Theoretical restriction fragment size (in base pairs) calculated based on *in silico* analysis of 16S rRNA gene sequences from RDP and NCBI GenBank				Gel detectable restriction fragments size (in base pairs) during agarose gel electrophoresis of restriction fragments of 16S rRNA gene			
	Genome/Type strain (Accn. No.)	*Rsa*I	*Cfo*I	*Hinf*I	Reference strain	*Rsa*I	*Cfo*I	*Hinf*I
Bacillus subtilis	Bsn5 (CP002468.1)	501, 465, 406, 157, 19, 11	869, 441, 249	605, 372, 313, 220, 25	MTCC 2451	505, 460, 410, 140	891, 431, 262	607, 373, 345, 230
Bacillus amyloliquefaciens	LL3 (CP 002634.1)	501, 486, 406, 145	869, 429, 240	605, 372, 316, 220, 25	MTCC 1270	505[a], 415, 144	881, 431, 246	607, 375, 345, 230
Bacillus licheniformis	ATCC 14580 (CP 000002)	435, 346, 406, 151, 19, 11	435, 346, 337, 242 182, 4	605, 372, 337, 207, 25	MTCC 429	515, 470, 415, 146	451, 358[a], 253, 195	607, 373, 345, 230
Bacillus pumilus	ATCC 7061 (AY876289)[b]	501, 436, 406, 91	868, 375, 191	605, 371, 287, 146, 65, 25	SD3B14	505, 425[a], 145	864, 430, 256	607, 373, 345, 230
Bacillus megaterium	QM B1551 (CP001983.1)	458, 406, 357, 145, 144, 8, 11	578, 428, 348, 182, 4	978, 318, 219, 25	MTCC 428	450, 404 338, 135[a]	570, 411, 354, 175	980, 345, 230
Bacillus cereus	ATCC 14579 (AE016877)	495, 406, 355, 110	586, 394 346, 182, 4	977, 345, 165, 25	MTCC 430	507, 412, 361, 148	594, 406, 353, 193	980, 345, 230
Bacillus circulans	Type strain (AY043084)	430, 406, 357, 146, 128, 18, 11	412, 348, 336, 214, 182, 4	607, 371, 310, 183, 25	MTCC 490	453, 413, 365, 148[a]	429, 345[a], 217, 183	607, 373, 345, 230

[a]Band with double intensity.
[b]Size of 16S rRNA gene analysed was only 1434 bp.

Differentiation of *B. cereus* group from *B. subtilis* group can be easily achieved but differentiation within group has always been problematic (Wunschel et al., 1995). It has been reported that 16S rRNA gene-RFLP based amplified ribosomal DNA restriction analysis (ARDRA) using universal primer cannot differentiate *B. licheniformis* from *B. pumilus* and *B. subtilis* from *B. amyloliquefaciens* (Vaerewijck et al., 2001), except differentiating *B. subtilis* group from *Bacillus cereus* group (Oguntoyinbo et al., 2010). Several researchers attempted to differentiate these closely related species of *B. subtilis* group and its subgroups by precise molecular weight determination of 16S-23S rRNA intergenic spacer region by using electrospray quadrupole mass spectrometry (ESI-Q-MS) (Johnson et al., 2000), RFLP analysis (Shaver et al., 2002), single strand conformation polymorphism (SSCP) analysis of shortest ITS region (Daffonchio et al., 1998), RAPD-PCR and species-specific PCR (Kwon et al., 2009). Wu et al. (2006) developed a *Bacillus* group specific PCR combined with RFLP, which differentiated *B. licheniformis* from *B. subtilis* group. In the present study, a simple ARDRA by PCR amplification of 16S rRNA gene using universal primers followed by restriction digestion using *Rsa*I, *Cfo*I and *Hinf*I distinctly differentiated closely related *B. amyloliquefaciens, B. licheniformis* and *B. pumilus* from *B. subtilis sensu stricto*. But the differentiation of *B. pumilus* from *B. amyloliquefacins* was difficult and the difference showed by *Taq*I enzyme was not convincing. ITS-RFLP analysis by PCR amplification of 16S-23S rRNA gene-ITS region using primer pair 16-1A (5'-GAATCGCTAATCG-3') and 23-1B (5'-GGGTTCCCCCATTCGGA-3') (Moreira et al., 2005) followed by restriction digestion with *Cfo*I (Promega) distinctly differentiated *B. pumilus* from *B. amyloliquefaciens*. But ITS-PCR-RFLP analysis showed very high intra species variation in *Bacillus* species particularly in *B. subtilis* group (Jeyaram et al., 2008; 2010). The length variation in ITS region is mainly due to the type and number of tRNA genes interspersed (Osorio et al., 2005) and the

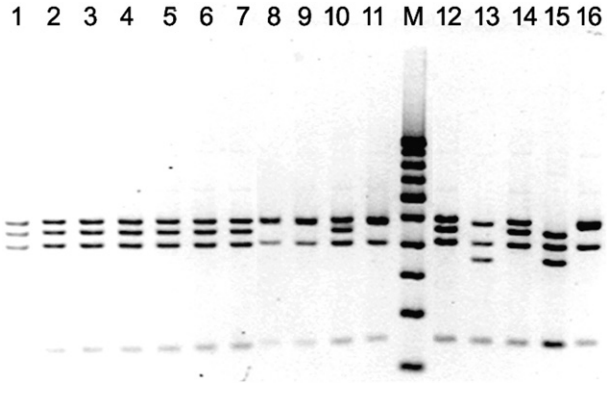

Fig. 2. Distinct differentiation of *B. amyloliquefaciens* from *B. subtilis* by 16S rRNA gene–RFLP using *Rsa*I restriction endonuclease. Lane 1–7 & 10: *Bacillus subtilis* isolates MH1B3, MH13B4, MH12B5, MH13B4, MH12B3, MH13B1, MH6B18 and MH10B5; Lane 8, 9 & 11: *B. amyloliquefaciens* isolates MH18B1, MH26B4 and MH2B5; Lane 12: *B. sub*tilis MTCC 2451; Lane 13: *B. cereus* MTCC 430; Lane 14: *B. licheniformis* MTCC 429, Lane 15: *B. circulans* MTCC 490, Lane 16: *B. amyloliquefaciens* MTCC 1270; Lane M: 100 bp DNA ladder (Promega).

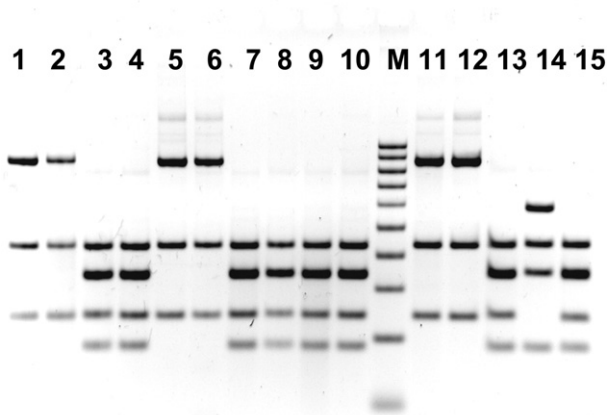

Fig. 3. Distinct differentiation of *B. licheniformis* from *B. subtilis* by 16S rRNA gene–RFLP using *Cfo*I restriction endonuclease. Lane 1, 2, 5 & 6: *Bacillus subtilis* isolates MH10B5, MH1B3, U146A & U166BB; Lane 3, 4, 7, 8, 9 & 10: *B. licheniformis* isolates MH7B9, MH5B2, U186, U126, U112 & U170A; Lane 11: *B. sub*tilis MTCC 2451; Lane 12: *B. amyloliquefaciens* MTCC 1270; Lane 13: *B. licheniformis* MTCC 429; Lane 14: *B. cereus* MTCC 430; Lane 15: *B. circulans* MTCC 490, Lane M: 100 bp DNA ladder (Promega).

Table 2
Identification by API 50CHB, 16S rRNA gene-RFLP analysis and 16S rRNA gene sequencing.

Bacillus strain	Identification by API 50CHB	Identification by 16S rRNA-RFLP	Identification by 16S rRNA gene sequencing		
			Closest relative	% similarity	Accession Number
MH6B18	*B. subtilis/ B. amyloliquefaciens*	*B. subtilis*	*B. subtilis*	99%	GQ268319.1
MH12B3	*B. pumilus*	*B. subtilis*	*B. subtilis*	99%	JN255700
MH10B5	*B. subtilis/ B. amyloliquefaciens*	*B. subtilis*	*B. subtilis*	100%	JN255701
U166BB	*B. subtilis/ B. amyloliquefaciens*	*B. subtilis*	*B. subtilis*	99%	JN255719
U146A	*Brevibacillus agri*	*B. subtilis*	*B. subtilis*	100%	JN255713
U4EBA	*B. subtilis/ B. amyloliquefaciens*	*B. subtilis*	*B. subtilis*	99%	JN255703
MH18B1	*B. subtilis/ B. amyloliquefaciens*	*B. amyloliquefaciens*	*B. amyloliquefaciens*	99%	JN558839
MH26B4	*B. subtilis/ B. amyloliquefaciens*	*B. amyloliquefaciens*	*B. amyloliquefaciens*	100%	JN255702
U97	*B. pumilus*	*B. amyloliquefaciens*	*B. amyloliquefaciens*	99%	JN255708
U259	*B. subtilis/ B. amyloliquefaciens*	*B. amyloliquefaciens*	*B. amyloliquefaciens*	98%	JN255730
U103	*B. licheniformis*	*B. amyloliquefaciens*	*B. amyloliquefaciens*	96%	JN255709
MH7B9	*B. licheniformis*	*B. licheniformis*	*B. licheniformis*	99%	JN558837
MH5B2	*B. megaterium*	*B. licheniformis*	*B. licheniformis*	98%	JN558838
U126	*B. licheniformis*	*B. licheniformis*	*B. licheniformis*	100%	JN255711
U186	*B. licheniformis*	*B. licheniformis*	*B. licheniformis*	98%	JN255725

difference in intensities of band is due to the number of *rrna* operons present (Moreira et al., 2005). The validity of ITS-PCR-RFLP analysis for species level differentiation has not been verified for most of the *Bacillus* species.

In conclusion, the simple ARDRA protocol described in this article allows an economical, rapid, reliable and reproducible alternative to misleading phenotype-based grouping of closely related species of *B. subtilis* group.

Supplementary materials related to this article can be found online at doi:10.1016/j.mimet.2011.08.011.

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