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PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*

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Genomic diversity in 21 strains of *Bacillus cereus* and 10 strains of *Bacillus licheniformis* was investigated by random amplified polymorphic DNA (RAPD) analysis, which samples the whole genome, and by two PCR fingerprinting techniques sampling the hypervariable spacers between the conserved 16S and 23S rRNA genes of the rRNA gene operon (ITS-PCR) and regions between tRNA genes (tDNA-PCR). RAPD analysis showed a remarkable diversity among strains of *B. cereus* that was not observed with the rRNA and tRNA intergenic-spacer-targeted PCR, where all the strains showed practically identical fingerprints. A wide variability among the *B. cereus* strains was also observed in the plasmid profiles, suggesting that the genetic diversity within *B. cereus* species can arise from plasmid transfer. One contribution to the diversity detected by RAPD analysis was determined by the presence of large extrachromosomal elements that were amplified during RAPD analysis as shown by Southern hybridization experiments. In contrast to the strains of *B. cereus*, the 10 strains of *B. licheniformis* were grouped into two clusters which were the same with all the methods employed. The 16S rRNA genes were identical in all 10 strains when examined using single strand conformation polymorphism analysis after digestion with *AluI* and *RsaI*. From these data we hypothesize two different evolutionary schemes for the two species.

Keywords: PCR fingerprinting, rRNA gene spacers, tRNA intergenic regions, genomic variability, *Bacillus cereus*, *Bacillus licheniformis*

INTRODUCTION

In group 1 of the Bacilli (*Bacillus sensu stricto*), as defined by Ash *et al.* (5) on the basis of the 16S rRNA sequences, some species are clustered together with a strict phylogenetic relationship. This is the case for the so called '*Bacillus cereus* group' (21, 22) which encompasses the practically relevant species *Bacillus anthracis* (9, 20–22), *Bacillus cereus* (25), *Bacillus mycoides* (38) and *Bacillus thuringiensis* (1, 7, 8).

Abbreviations: AP-PCR, arbitrarily primed PCR; DIG, digoxigenin; ITS-PCR, intergenic 16S–23S transcribed spacer PCR; RAPD, random amplified polymorphic DNA; SSCP, single strand conformation polymorphism; tRNA-PCR, tRNA intergenic region PCR.

Although considered as different species on the basis of DNA similarities below 70% (33, 34), the members of the *B. cereus* group show many common characteristics. For example they all show a very high similarity level (from 99·5 to 100%) of the 16S and 23S rRNA sequences (3, 4). *B. mycoides*, which is the most distant species in the *B. cereus* group [according to DNA/DNA hybridization studies (34)], harbours only seven to nine scattered nucleotide differences in the 1446 bp sequence of the 16S rRNA gene (4). Other workers have shown a high similarity in the genome structure of *B. cereus* and *B. thuringiensis* and have proposed to assign the two species to a unique taxonomic unit (10, 12, 13, 33). Moreover a very high phenotypic similarity characterizes these bacteria (29, 34, 41, 42, 56).

The degree of intraspecific diversity is variable in the four species. *B. anthracis* has been demonstrated to be a clonal species with all isolates showing almost complete similarity using fingerprinting techniques (20, 22). In contrast, *B. cereus* and *B. thuringiensis* isolates are characterized by a high degree of intraspecific diversity. Differences in chromosome size, varying from 2·4 to 6·3 Mb, together with several rearrangements have been found in different strains of *B. cereus* by physical chromosome mapping (14). The genome of *B. cereus* strains can be organized into one large chromosome or into a smaller one with stably maintained large plasmids (14). Part of the chromosomal genome, corresponding to the 2·4 Mb chromosome, is more conserved and common to all *B. cereus* and *B. thuringiensis* isolates analysed (14). The genetic diversity of *B. cereus* isolates is enhanced by extrachromosomal elements, including large plasmids that have been found in many strains (10–14, 26).

Comparison of the 16S rRNA sequences showed that other group 1 species are characterized by a strict phylogenetic relationship (5). For example *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus pumilus* form a distinct clade. *B. licheniformis* is a typical soil bacterium and is used in the fermentation industry to produce a variety of products such as bacitracin, penicillinase, alkaline phosphatase, α-amylase, protease and surfactant (23, 32, 47). Some strains produce site-specific restriction endonucleases (31, 36).

The intraspecific diversity in *B. licheniformis* has been described in population studies of wild isolates. By analysing a *B. licheniformis* population by multilocus enzyme electrophoresis and phenotypic analysis, Duncan *et al.* observed a high level of diversity between the isolates that were clustered in two main groups with panmictic structure (16). The panmixia observed within the groups was attributed to the capability of *B. licheniformis* to exchange genetic material with other micro-organisms, such as the closely related species *B. subtilis* (16, 17).

In the last 10 years PCR-based fingerprinting has been widely used for typing and assessing the genetic diversity of many micro-organisms. Depending on the primer/s and the amplification conditions employed, the results allow discrimination between organisms at the level of genera, species or strains (48).

In theory, with arbitrarily primed PCR (AP-PCR) (50) or random amplified polymorphic DNA (RAPD) analysis (54), amplification of DNA fragments randomly scattered on the genome can be accomplished using one primer at low stringency, leading to identification of genetic traits with a specificity at different taxonomic ranks (50, 54). These techniques have been applied to a wide range of bacteria (e.g. 2, 46, 45).

Other fingerprinting amplifications are directed towards regions with quite a high level of phylogenetic conservation. For example the intergenic 16S–23S ribosomal transcribed spacers, even if defined as 'hypervariable' with respect to the adjacent genes, usually only show significant variations at the genus or species level (27). Intergenic 16S–23S transcribed spacer PCR (ITS-PCR) fingerprinting is generally used to show spacer length polymorphism in the different rRNA gene operons of the genome, showing intraspecies variations in some cases (25, 52). Other structural genes that are highly conserved between species and which are useful to infer phylogenesis are the tRNA genes (48, 49). Bacterial tRNA genes are generally clustered (with up to 10 genes per cluster) and are separated by spacers whose length and sequence are subjected to a higher degree of variation (49, 51). Welsh & McClelland have developed a PCR fingerprinting technique (tDNA-PCR) that uses consensus tRNA gene primers to amplify tRNA intergenic regions, generally generating species-specific band patterns (51, 52).

With the aim of evaluating if the genetic variability of *B. cereus* is scattered over the whole genome, including the more conserved parts, or is confined only to the more variable part of the genome, we compared DNA fingerprinting obtained with PCR-based methods that sample (a) the whole genome including extrachromosomal elements (RAPD analysis) and (b) hypervariable parts of conserved regions such as rRNA and tRNA gene spacers (ITS-PCR and tDNA-PCR, respectively). We also checked if the variability measured as RAPD derives only from the chromosomal DNA or also from plasmids. The data for *B. cereus* were compared with those from a differently organized bacterium, *B. licheniformis*.

METHODS

Bacterial strains, culture conditions and DNA extraction. A total of 33 *Bacillus* strains were used in this study (Table 1). All the strains were routinely grown in nutrient broth or agar media (Difco) with incubation at 30 °C. *B. licheniformis* strains were always incubated at 37 °C. The isolated strains were identified with an API 50 CHB kit (BioMérieux).

To obtain DNA, 200 µl of an overnight culture was centrifuged at 13000 ***g*** for 3 min. The pellets were washed with 200 µl TE (10 mM Tris/HCl, pH 8, 1 mM EDTA), resuspended in 1 ml 20% (w/w) Chelex-100 sodium form beads (100–200 mesh, Bio-Rad) in 0·1% SDS, 1% (v/v) Nonidet-P40, 1% (v/v) Tween-20 aqueous solution and finally incubated at 100 °C for 30 min (15). After centrifugation the supernatant was recovered and used for PCR experiments.

RAPD analysis, ITS-PCR and tDNA-PCR conditions. Primers OPG-5, OPG-8, OPG-11 and OPG-16 from Operon Technologies were used for RAPD analysis (Table 2). The reactions were carried out in 500 µl thin wall sterile tubes by adding 2·5 µl 10× buffer (Promega), each dNTP at a concentration of 100 µM, 2·5 mM $MgCl_2$, 0·4 µM primer, 1 U *Taq* polymerase (Promega), 1 µl DNA solution and enough sterile water to bring the total reaction volume to 25 µl. The reaction mixture was covered with 25 µl sterile wax (MJ Research). Control reaction mixtures lacking template DNA were also prepared. The amplification reactions were performed in a PTC-100 thermal cycler (MJ Research). The temperature profile was as follows: initial

Table 1. *Bacillus* species and strains analysed in this study

Species	Strain	Source*	Origin
B. cereus	31^T	DSMZ	Unknown
	318	DSMZ	Garden soil
	336	DSMZ	Garden soil
	345	DSMZ	Unknown
	487	DSMZ	Unknown
	626	DSMZ	Unknown
	2896	DSMZ	Unknown
	6127	DSMZ	Unknown
	46321	DSMZ	Unknown
	CER-1	A. Galli	Unknown
	CER-3	A. Galli	Candies
	CER-4	A. Galli	Rice
	CER-5	A. Galli	Rice
	CER-6	A. Galli	Tomato sauce
	BC-1	E. Zanardini	Ca' d'oro marble (Venice)
	BC-2	A. Galli	Unknown
	BC-5	A. Galli	Unknown
	MY-1	A. Galli	UHT milk†
	CO-1	A. Galli	Pasteurized milk
	CO-2	A. Galli	Unknown
	PO-1	A. Galli	UHT milk†
B. circulans	CIR-1	A. Galli	Unknown
B. licheniformis	14580^T	ATCC	Unknown
	3.2	MIM	Soil (Shandong, China)
	17.1	MIM	Soil (Shandong, China)
	61.1	MIM	Soil (Senarang, Java)
	75.2	MIM	Soil (Tokyo, Japan)
	283.A	MIM	Soil (Abu-Simbel, Egypt)
	Fl4	MIM	Soil (Alagna, Italy)
	Fl11	MIM	Soil (Alagna, Italy)
	MP3	MIM	Soil (unknown)
	4-40	MIM	Soil (unknown)
B. subtilis	9799	ATCC	Unknown

* ATCC, American Type Culture Collection, Rockville, MD, USA; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; MIM, Microbiologia Industriale Milano, Milano, Italy; A. Galli and E. Zanardini, DISTAM, Università degli Studi, via Celoria 2, 20133 Milano, Italy.

† UHT milk, ultra-high-temperature-treated milk.

Table 2. Primers used for PCR fingerprinting

Primer	Sequence (5′–3′)	Nucleotides
OPG-5	CTGAGACGGA	10
OPG-8	TCACGTCCAC	10
OPG-11	TGCCCGTCGT	10
OPG-16	AGCGTCCTCC	10
S-D-Bact-1494-a-S-20	GTCGTAACAAGGTAGCCGTA	20
L-D-Bact-0035-a-A-15	CAAGGCATCCACCGT	15
T3A	AGTCCGGTGCTCTAACCAACTGAG	24
T5B	AATGCTCTACCAACTGAACT	20
S-D-Bact-0008-a-S-20	AGAGTTTGATCCTGGCTCAG	20
S-D-Bact-1495-a-A-20	CTACGGCTACCTTGTTACGA	20

denaturation at 94 °C for 4 min followed by 40 cycles each consisting of 94 °C for 1 min, 35 °C for 1 min and 72 °C for 2 min with a final extension step at 72 °C for 5 min.

ITS were amplified in 50 µl reactions with a Gene Amp PCR 2400 system (Perkin Elmer) using 1·25 U Dynazyme II DNA polymerase (Finnzymes OY), 5 µl of the provided 10 × Mg-free PCR reaction buffer, 200 µM each dNTP, 2·5 mM $MgCl_2$, 0·3 µM each primer and 2 µl bacterial DNA. The primers used, named according to the nomenclature used in the Oligonucleotide Probe Database (OPD) (53), were forward S-D-Bact-1494-a-S-20 and reverse L-D-Bact-0035-a-A-15 (Table 2). The following temperature profile was used: initial denaturation at 94 °C for 4 min followed by 30 cycles each consisting of 94 °C for 1 min, 55 °C for 7 min and 72 °C for 2 min with a final extension step at 72 °C for 7 min (27). The temperature ramp between the denaturation and the annealing steps was reduced by 50% with respect to the default ramp, while that between the annealing and the extension steps was reduced by 95%.

tDNA-PCR was performed in a MJ Research PTC-100 thermal cycler with a final volume of 50 µl made of 1·25 U *Taq* polymerase (Promega), 5 µl of the provided 10 × Mg-free PCR reaction buffer, 200 µM each dNTP, 1·5 mM $MgCl_2$, 1 µM each primer T3A and T5B (51; Table 2) and 2 µl bacterial DNA. A 'hot start' protocol was used: the PCR mixture without *Taq* polymerase and overlaid with mineral oil (30 µl) was incubated at 75 °C for 3 min. The enzyme was then added and the mixture processed with the following thermal programme: initial denaturation at 94 °C for 4 min followed by 35 cycles each consisting of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min with a final extension step at 72 °C for 10 min (51).

Ten microlitres of the PCR fingerprinting products was electrophoresed in agarose gel (1·5, 2 and 3% for RAPD, ITS-PCR and tDNA-PCR, respectively) in TBE buffer (89 mM Tris/boric acid, 2 mM EDTA) (40) at 4·2 V cm^{-1}, stained in a solution containing 0·5 µg ethidium bromide ml^{-1} and photographed under UV light. Pharmacia 50 and 100 bp ladders were used as size standards.

Single strand conformation polymorphism (SSCP) analysis of endonuclease-digested 16S rDNA. The 16S rDNA gene from all the *B. licheniformis* strains was amplified by PCR with a PTC-100 MJ Research thermal cycler with a final volume of 100 µl containing 2 µl bacterial DNA solution, 10 µl 10 × PCR buffer (Promega), 200 µM each dNTP, 2 µM $MgCl_2$, 0·5 µM each primer (forward S-D-Bact-0008-a-S-20 and reverse S-D-Bact-1495-a-A-20, Table 2) and 2 U *Taq* polymerase (Promega). Thirty-five cycles were performed with the following temperature profile: initial denaturation at 94 °C for 4 min, primer annealing at 55 °C for 1 min and primer extension at 72 °C for 2 min. Final extension was at 72 °C for 7 min. The amplified band was visualized on a 1·5% (w/v) agarose gel stained with ethidium bromide. Aliquots (18 µl) of the amplified 16S rDNA were separately digested with 10 U of both *Alu*I and *Rsa*I (Pharmacia) in a final volume of 40 µl. Digestions were carried out at 37 °C overnight to obtain DNA fragments with a size (< 500 bp) suitable for SSCP analysis (19, 24, 30, 35). The success of DNA restriction was checked by 1·5% agarose gel electrophoresis. For SSCP analysis the digestion products were mixed with the gel loading buffer (40%, w/v, sucrose, 0·1 mM EDTA, 0·5% SDS, 0·05% bromophenol blue), denatured for 10 min at 95 °C and immediately cooled in an ice bath. Twelve microlitres were applied to a 6% polyacrylamide gel (acrylamide/bisacrylamide, 29:1) with 10% glycerol and electrophoresed in 1 × TBE buffer on a minislab gel (80 × 73 × 1 mm, Bio-Rad) at room temperature (30). The gel was stained with ethidium bromide and photographed on a UV transilluminator.

Plasmid extraction and Southern hybridization with RAPD. Plasmids were extracted by the alkaline lysis method from 1·5 ml of an overnight culture (40). For *B. cereus* 6127, DNA was also extracted by omitting NaOH to avoid denaturation of the chromosomal DNA (28). A plasmid profile was obtained by separating the bands on a 0·8% agarose gel. The bands were visualized by ethidium bromide staining. λ DNA digested with *Eco*RI and *Hin*dIII and the supercoiled DNA ladder (both from Sigma) were used as size standards. The agarose gel with plasmid DNA from strains 6127 (extracted both with and without NaOH), 2896, 487, CER-5, CER-6 and CO-1 was rinsed in 250 mM HCl for 15 min at room temperature to depurinate the DNA before Southern blotting. DNA was blotted overnight on positively charged nylon membrane (Amersham) by capillary transfer (40), fixed in UV light for 4 min and hybridized overnight at 42 °C in 50% (v/v) formamide with the corresponding DIG-labelled RAPD obtained with primer OPG-5. Two stringent washes were performed at 55 or 68 °C for 15 min. Prehybridization, hybridization, washes and chemiluminescent detection were performed using the DIG DNA labelling and detection kit following the instructions of the supplier (Boeringher Mannheim) (6).

The RAPD probes were DIG-labelled by PCR using the same conditions described in the protocol for RAPD analysis. DIG-labelled dUTP from the Boeringher Mannheim kit was used to label the RAPD (6).

Analysis of band pattern similarities. The amplified fragments detected in the agarose gel were treated as discrete characters and recorded in a data matrix by scoring 1 for the presence and 0 for the absence of a fragment. Similarity between strains was determined by the band-sharing coefficient (F) calculated by the formula of Jaccard (44), $F = N_{xy}(N_{xy} + N_x + N_y)$, where N_{xy} is the number of common amplified fragments between two strains x and y and N_x and N_y are the number of fragments in strains x and y, respectively. The generated matrix was subjected to clustering by the unweighted pair group method with arithmetic means (UPGMA) (44). The NTSYS-PC computer program (version 1.30) was used in data analysis (39).

RESULTS

RAPD analysis

RAPD analysis was used to group and type 21 strains of *B. cereus* and another 12 *Bacillus* strains from *B. licheniformis*, *B. subtilis* and *Bacillus circulans*. As an example, the RAPD patterns using primer OPG-5 are reported in Fig. 1. Only a few strains of *B. cereus* showed some similarity (e.g. strains 318 and 336 and strains 6127 and 46321). In general a high level of variability was observed. Analogous results were obtained with the other primers.

With primers OPG-5, OPG-8, OPG-11 and OPG-16, *B. licheniformis* strains were always clustered in two main groups corresponding to two different pattern types (Fig. 1). Analysis of a wider collection of 80 *B. licheniformis* strains isolated from soils sampled from many countries all over the world by RAPD with

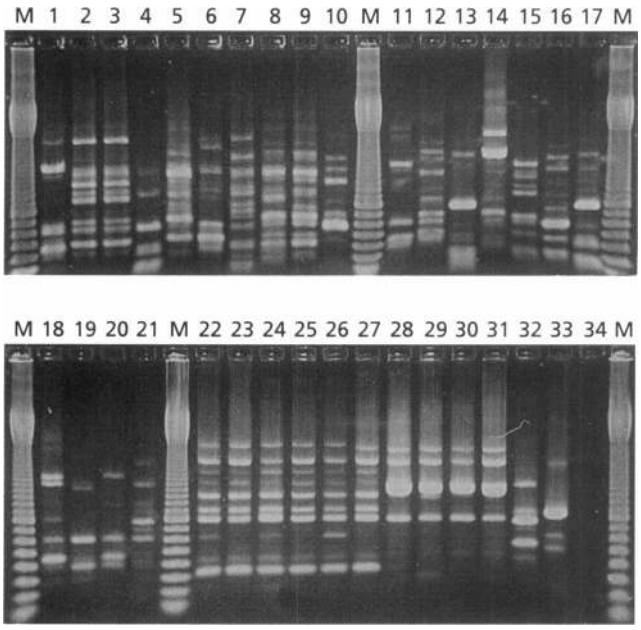

Fig. 1. RAPD analysis of *Bacillus* strains with primer OPG-5. Lanes: M, 100 bp ladder; 1–21, *B. cereus* 31^T, 318, 336, 345, 487, 626, 2896, 6127, 46321, CER-1, CER-3, CER-4, CER-5, CER-6, BC-1, BC-2, BC-5, MY-1, CO-1, CO-2, PO-1; 22–31, *B. licheniformis* 14580^T, 61.1, FI4, FI11, MP3, 4-40, 3.2, 17.1, 75.2, 283.A; 32, *B. subtilis* 9799; 33, *B. circulans* CIR-1; 34, control without DNA template.

primer OPG-5 showed analogous results, grouping the strains in few pattern types (data not shown).

The dendrogram obtained from the clustering analysis of the RAPD fingerprinting is reported in Fig. 2. This allowed the four species studied to be separated into different clusters. The RAPD similarity between *B. cereus* strains was between 15 and 50%, while that between *B. licheniformis* strains was higher, between 40 and 95% and between 39 and 82% for strains grouped in clusters I and II, respectively, and 22% between the two clusters.

Plasmid profile analysis and Southern hybridization with RAPD

To investigate the reasons for the wide variability observed in *B. cereus* by RAPD, we decided to analyse the plasmid content of the isolates to evaluate if the presence of extrachromosomal elements could be a factor influencing the RAPD fingerprinting. More than the half of the strains examined, i.e. strains 487, 2896, 6127, 46321, CER-3, CER-5, CER-6, BC-2, MY-1, CO-1, CO-2 and PO-1 showed various extrachromosomal bands (Fig. 3). The size of these plasmids was between 1 kbp (the smallest band of strain 2896) and 40 kbp (the largest band of strain CER-6). Strains 487, 2896, CER-5, CER-6 and CO-1 showed bands with an estimated size larger than 20 kbp. Some of the strains showed common bands, indicating that they probably harboured plasmids of the same size (e.g. strains CER-3, MY-1 and CO-2, and strains 6127 and 46321). RAPD fingerprinting grouped these strains quite close together, suggesting a certain genotypic relationship (Fig. 2).

The wide variability of the RAPD fingerprinting and plasmid profile lead us to suppose that some of the RAPD could derive from extrachromosomal DNA. We chose six strains, harbouring only small plasmids (< 20 kb; strain 6127) or both small and large plasmids (> 20 kb; strains 487, 2896, CER-5, CER-6 and CO-1), to investigate the influence of plasmids on RAPD analysis by Southern hybridization experiments. The plasmid profile of these strains was hybridized with the corresponding DIG-labelled RAPD after Southern transfer to a nylon membrane (Fig. 4). Strain 6127, which showed only plasmids smaller than 20 kb, was blotted both with and without chromosomal DNA which was maintained in the DNA preparation by omitting NaOH. A hybridization signal was obtained with only the chromosomal band for strain 6127, only in the lane where plasmids were extracted without NaOH. The low hybridization signal was due to the high temperature (68 °C) used in the stringency washes. For the other hybridizations the stringency washes were performed at 55 °C. Under these conditions, signals were obtained only with the largest plasmid bands of strains CER-5 and CER-6 (estimated sizes 40 and 35 kbp, respectively), indicating that in presence of chromosomal DNA, RAPD amplicons can also be obtained from plasmid DNA.

ITS-PCR and tDNA-PCR analysis

To evaluate if the polymorphism observed in *B. cereus* by RAPD fingerprinting was also reflected in conserved regions of the genome, we performed ITS- and tDNA-PCR fingerprinting on the same strains used for RAPD analysis. The results of ITS-PCR fingerprinting of *B. cereus* showed identical patterns in all the strains tested (Fig. 5a). Three major bands were observed with the strongest one having an estimated length of about 240 bp. The estimated length of the other bands was 450, 550 and 650 bp. These results show that the length of the spacer regions between the 16S and the 23S rRNA genes are constant for all the strains of *B. cereus* tested.

ITS-PCR fingerprinting of *B. licheniformis* strains showed two major pattern types, mainly differing in terms of the presence/absence of two bands of about 330 and 360 bp, respectively (Fig. 5b). The analysed strains were grouped in the same order as that observed by RAPD analysis.

The results of tDNA-PCR fingerprinting using primers T3A and T5B are shown in Fig. 6. The 21 strains of *B. cereus* were very homogeneous showing the same banding pattern with amplicon length ranging from 150 to about 1000 bp (Fig. 6a). The 10 *B. licheniformis* strains showed two pattern types which grouped the

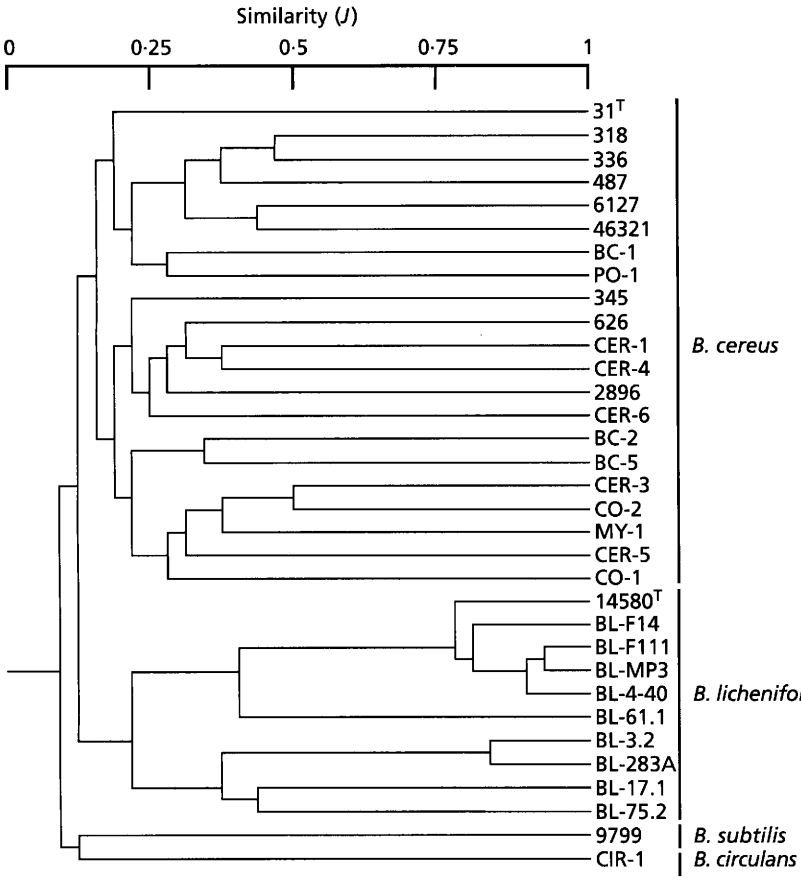

Fig. 2. Dendrogram obtained by UPGMA clustering of RAPD patterns obtained with primers OPG-5, OPG-8, OPG-11 and OPG-16. The Jaccard algorithm was used for the data analysis.

strains in the same way as RAPD analysis and ITS-PCR (Fig. 6b). The main differences between the two pattern types were observed in a single band of 550–600 bp and in two bands of around 250 bp. The signals observed under 100 bp were not significant and were probably primer concatamers due to the high primer concentration (1 μM). They were also observed in the negative controls in four separate experiments performed with independently prepared template DNAs using separate new reagents. Seal *et al.* observed similar small bands using analogous primers for the amplification of *Ralstonia solanacearum* strains (43).

16S rDNA analysis by endonuclease digestion and SSCP

To check if the highly conserved 16S rRNA gene displayed sequence differences between the two groups of *B. licheniformis* strains, distinguished by the fingerprinting methods, the PCR-amplified 16S rRNA gene was analysed by SSCP. This method has been reported to detect from 70 to 95% of point mutations in sequences of 200 bp or less (19). To obtain fragments of the appropriate length the amplified fragments were digested with *Alu*I and *Rsa*I. *Alu*I gave five bands with estimated sizes of about 70, 140, 180, 260 and 800 bp, while *Rsa*I gave four bands with estimated sizes of about 110, 400, 450 and 500 bp. The results of the SSCP analysis of the restriction patterns are shown in Fig. 7. No apparent differences were observed in the band patterns, suggesting that all the strains had the same overall sequence.

DISCUSSION

Studies reported in the literature on PCR fingerprinting of *B. cereus* show a wide genotypic heterogeneity among isolates (8, 21, 22, 45). In a study to differentiate *B. anthracis* from the other members of the *B. cereus* group using PCR fingerprinting, Henderson *et al.* (22) showed a high degree of polymorphism both among the six strains of *B. cereus* studied and the related species *B. thuringiensis* and *B. mycoides*. In contrast, all the strains of *B. anthracis* were practically identical and tightly clustered together. In another study to evaluate the strain diversity in *B. cereus* by RAPD analysis using five different primers, 25 strains were grouped into 22 different pattern types (45). Similar results, showing polymorphic AP-PCR fingerprints, have also been obtained with the different serovars of *B. thuringiensis* (8). All these investigators clearly indicated the heterogeneity of the whole *B. cereus* genome. This was confirmed by the RAPD and plasmid profile data reported here.

The wide extent of diversity observed using RAPD can be partially explained by the presence of extrachromosomal plasmid DNA that can be annealed by the

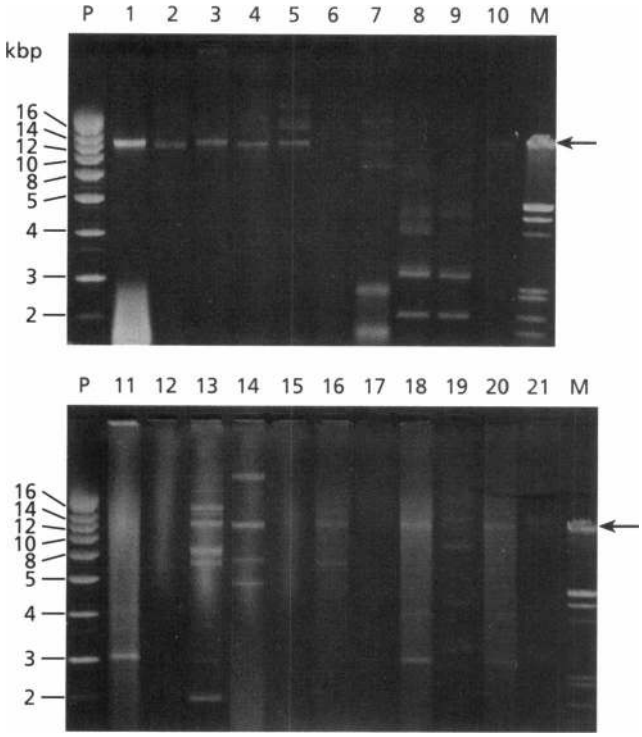

Fig. 3. Plasmid profile found in the 21 strains of *B. cereus*. Lanes: M, λ DNA digested with *Eco*RI and *Hin*dIII; P, supercoiled DNA ladder; 1–21, *B. cereus* 31^T, 318, 336, 345, 487, 626, 2896, 6127, 46321, CER-1, CER-3, CER-4, CER-5, CER-6, BC-1, BC-2, BC-5, MY-1, CO-1, CO-2, PO-1. The arrows on the right indicate the position of the chromosomal DNA bands.

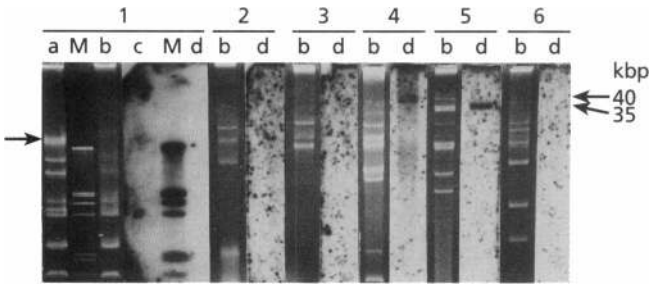

Fig. 4. Southern hybridization of *B. cereus* plasmids with DIG-labelled RAPD obtained by RAPD analysis of total DNA with primer OPG-5. Lanes: M, λ DNA digested with *Eco*RI and *Hin*dIII; a and c, plasmid profiles from *B. cereus* 6127 obtained by DNA extraction omitting NaOH (a) and Southern hybridization with DIG-labelled RAPD (c); b and d, plasmid profiles obtained by DNA extraction including NaOH (b) and Southern hybridizations with DIG-labelled RAPD products (d). 1–6, *B. cereus* strains 6127, 2896, 487, CER-5, CER-6 and CO-1. The arrow on the left indicates the position of the chromosomal DNA bands. The numbers on the right indicate the estimated sizes of the plasmid bands showing hybridization signals.

primer during PCR, giving some fragments which are amplified only in the strains harbouring that plasmid. In a study to evaluate the influence of plasmids on

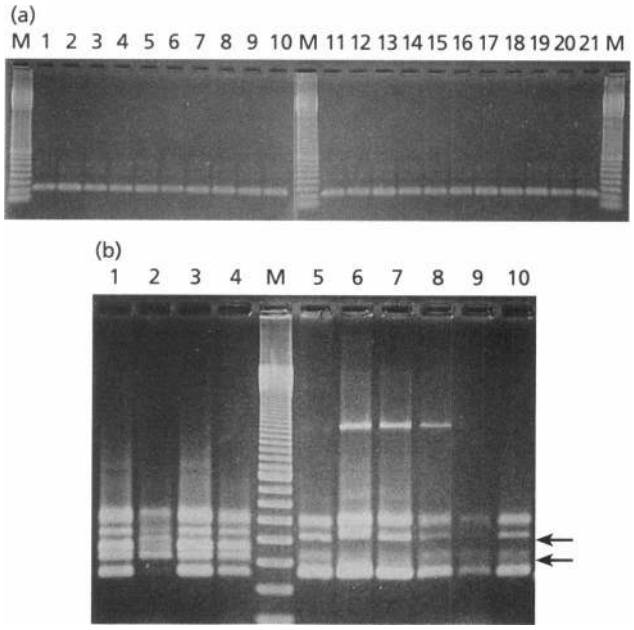

Fig. 5. ITS-PCR of the *B. cereus* (a) and *B. licheniformis* (b) strains analysed in this study. Lanes: M, 100 bp ladder; (a) 1–21, *B. cereus* 31^T, 318, 336, 345, 487, 626, 2896, 6127, 46321, CER-1, CER-3, CER-4, CER-5, CER-6, BC-1, BC-2, BC-5, MY-1, CO-1, CO-2, PO-1; (b) lanes 1–10, *B. licheniformis* 3.2, 17.1, 75.2, 283.A, 14580^T, 61.1, FI4, FI11, MP3, 4-40. The arrows indicate the band length polymorphisms between the two main pattern types found in *B. licheniformis*.

RAPD analysis, Elaichouni *et al.* (18) concluded that the amount of chromosomal DNA is so high in the routine preparation of DNA for PCR that it does not allow the amplification of plasmids, even if the plasmids can be amplified when they are alone, generating different band patterns with the same primers and the same conditions for RAPD analysis. The same conclusions were made by Brousseau *et al.* (8). We found that these conclusions are correct when plasmids are smaller than 20 kbp. Our data and those from other researchers (8, 10–14, 26) show that *B. cereus* isolates are characterized by the presence of various small and large extrachromosomal elements. When plasmids larger than 20 kbp are present, the extrachromosomal DNA can compete with that from the chromosome, resulting in partial amplification of the plasmid. The Southern hybridization experiments reported here confirmed this hypothesis, since only the largest extrachromosomal bands gave hybridization signals with the RAPD probes. This indicates that the strain to strain RAPD variability of *B. cereus* can be partially explained by the presence or absence of plasmid DNA.

The ITS-PCR patterns obtained were consistent with those obtained by Wunschel *et al.* from seven *B. cereus* strains (56). The small differences observed in amplicon length are due to the different primer positions on the 16S and 23S rRNA gene sequences. These results

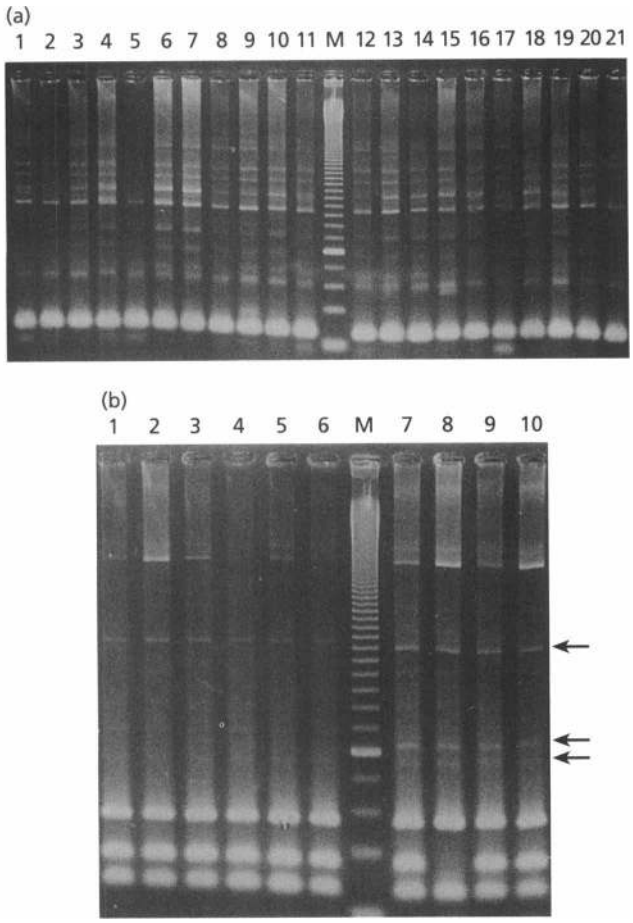

Fig. 6. tDNA-PCR of the *B. cereus* (a) and *B. licheniformis* (b) strains analysed in this study. Lanes: M, 50 bp ladder; (a) lanes 1–21, *B. cereus* 31^T, 318, 336, 345, 487, 626, 2896, 6127, 46321, CER-1, CER-3, CER-4, CER-5, CER-6, BC-1, BC-2, BC-5, MY-1, CO-1, CO-2, PO-1; (b) lanes 1–10, *B. licheniformis* 14580^T, 61.1, FI4, FI11, MP3, 4-40, 3.2, 17.1, 75.2, 283.A. The arrows indicate band length polymorphisms between the two main pattern types found in *B. licheniformis*.

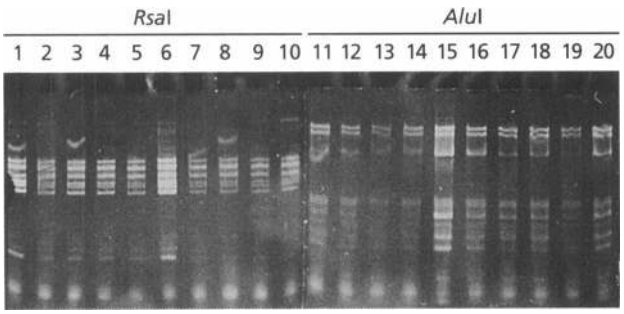

Fig. 7. SSCP analysis of the 16S rDNA digested with *Rsa*I and *Alu*I from the 10 strains of *B. licheniformis*. Lanes: 1–10 and 11–20, *B. licheniformis* strains 14580^T, 61.1, FI4, FI11, MP3, 4-40, 3.2, 17.1, 75.2, 283.A.

suggest that in *B. cereus* the 16S–23S ITS are well-conserved in terms of length, in contrast, for example, with those in *B. licheniformis* for which at least two different ITS fingerprints have been shown. Two different ITS-PCR patterns have also been observed in *B. subtilis* (56). The apparent homogeneity observed in *B. cereus* with ITS- and tDNA-PCR does not reflect the diversity observed by RAPD fingerprinting. Kolstø and coworkers observed a wide variability in the genome organization of *B. cereus* from PFGE and hybridization data (10–14). These authors supposed that a high rate of DNA rearrangement could have occurred in *B. cereus*, leading to an evolution of strains with differences in chromosome size from 2·4 to 6·3 Mb (14). The presence of rather large plasmids in those strains with the 2·4 Mb chromosome could complement these differences (14). The authors observed an overall coincidence of the 2·4 Mb chromosome between strains, which was reported as a rather constant chromosomal component and which includes most of the rRNA gene operons (14). It is well known that both the rRNA operons and the tRNA clusters are generally located in a limited part of the genome around the origin of replication. In *B. subtilis*, for example, it is in the half from 225° to 45° of the circular map (49). The data reported here support the idea that the heterogeneity observed with whole-genome-sampling fingerprinting techniques might not derive from this conserved part but rather from the rest of the genome, which is probably more susceptible to insertions, transpositions or other changes in genomic organization. Both ITS- and tDNA-PCR fingerprinting suggest that the evolution of the rRNA operons and of the tRNA clusters in *B. cereus* does not reflect that of the whole DNA.

The division of *B. licheniformis* strains into two main groups according to RAPD analysis was confirmed by ITS- and tDNA-PCR fingerprinting, indicating that this species evolved with at least two different lineages. Within each lineage the organization of the 16S–23S rRNA gene spacers as well as that of the spacers between the tRNA genes had an evolution similar to that of the whole genome. Even if the *B. licheniformis* strains examined were isolated from very different soils recovered from countries with very different climates (Table 1), the low genomic heterogeneity observed among the *B. licheniformis* isolates remains to be confirmed for strains isolated from other ecological niches. Stephan *et al.* examined 46 *B. licheniformis* strains isolated from food matrices (37 strains isolated from spoiled blood sausage, 3 strains isolated from pig's blood and 6 strains of unknown origin) using RAPD analysis (46). They also observed a quite low heterogeneity, grouping the strains into 5 and 10 pattern types depending on the primer used. The two lineages that we have found in *B. licheniformis* by PCR fingerprinting were strictly related, as shown both by DNA/DNA homology experiments (37) and SSCP analysis of endonuclease-digested 16S rDNA. For all 10 *B. licheniformis* strains included in this study, the

DNA/DNA homology with the type strain was always higher than 70% (37) and SSCP analysis showed that all these strains harboured the same 16S rDNA sequence.

From the data reported we hypothesized that two different evolutionary schemes characterized the two species. *B. cereus* evolved more recently with wide rearrangement of the genome, without significant variation of the hypervariable spacers between the conserved 16S and 23S rRNA genes. *B. licheniformis* is characterized by a more ancient differentiation with respect to the hypervariable spacers between the conserved genes in the rRNA operon and tRNA gene clusters. In contrast with *B. cereus*, the distinct lineages of *B. licheniformis* evolved clonally.

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