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Genetic relationship in the '*Bacillus cereus* group' by rep-PCR fingerprinting and sequencing of a *Bacillus anthracis*-specific rep-PCR fragment

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ABSTRACT

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Aims: To evaluate the genetic relationship in the *Bacillus cereus* group by rep-PCR fingerprinting.

Methods and Results: A collection of 112 strains of the six species of the *B. cereus* group was analysed by rep-PCR fingerprinting using the BOX-A1R primer. A relative genetic distinctness was found among the species. Cluster analysis of the rep-PCR patterns showed clusters of *B. thuringiensis* strains quite separate from those of *B. cereus* strains. The *B. anthracis* strains represented an independent lineage in a *B. cereus* cluster. The *B. mycoides, B. pseudomycoides* and *B. weihenstephanensis* strains were clustered into three groups at some distance from the other species. Comparison of sequences of AC-390, a typical *B. anthracis* rep-PCR fragment, from 27 strains of *B. anthracis, B. cereus, B. thuringiensis* and *B. weihenstephanensis,* representative of different clusters identified by rep-PCR fingerprinting, confirmed that *B. anthracis* diverges from its related species.

Conclusions: The genetic relationship deduced from the rep-PCR patterns indicates a relatively clear separation of the six species, suggesting that they can indeed be considered as separate units.

Significance and Impact of the Study: rep-PCR fingerprinting can make a contribution in the clarification of the genetic relationships between the species of the *B. cereus* group.

Keywords: *Bacillus anthracis*, *Bacillus cereus* group, *ywfK*, BOX-A1R, genetic relationship, rep-PCR.

INTRODUCTION

The genetic relationship of the species of the *Bacillus cereus* group is a subject of debate, and is still far from clear. Five species of the group, *B. anthracis, B. cereus, B. mycoides, B. thuringiensis* and *B. weihenstephanensis* (Lechner *et al.* 1998), have a marked impact on human activity, three with negative effects and two with positive. *Bacillus anthracis* and *B. cereus* are well-known pathogens of mammals, including humans, and *B. weihenstephanensis* grows at temperatures as low as 4°C, posing a threat to the conservation of cold-stored food (Mayr *et al.* 1999). The most useful species of the group, *B. thuringiensis,* is a biological insecticide used extensively throughout the world (Schnepf *et al.* 1998), while *B. mycoides* strains can improve plant growth (Petersen *et al.* 1995). A sixth species, *B. pseudomycoides,* has been described (Nakamura 1998).

Given the effect, the five above mentioned bacteria have on human activity, and the wide application of *B. thuringiensis* in the field, a clear evaluation of the taxonomic and phylogenetic relationship of the species is necessary. Several authors have concluded that *B. anthracis, B. cereus* and *B. thuringiensis* belong to a single species, a conclusion

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reached on the basis of multilocus enzyme electrophoresis, the sequencing of discrete protein-coding genes (Helgason *et al.* 2000) and the presence of an S-layer (Mignot *et al.* 2001). An analysis based on the heteroduplexes formed between the 16S–23S rRNA internal transcribed spacers (ITS) has shown that *B. thuringiensis* can be differentiated from *B. cereus,* and that *B. anthracis* represents an independent lineage diverging from *B. cereus* (Daffonchio *et al.* 2000; Cherif *et al.* 2003).

In the rep-PCR fingerprint typing method (Versalovic *et al.* 1991; Martin *et al.* 1994; Versalovic *et al.* 1994), a single primer targets repetitive regions scattered throughout the bacterial genome to give a PCR product profile; such a profile is generally specific to a given strain. Repetitive regions present higher variability than other genomic regions, and can be used to analyse the genetic relationship between strains (Van Belkum *et al.* 1998; Kim *et al.* 2002). In fact, long-range rep-PCR enabled Brumlik *et al.* (2001) to distinguish 105 *B. anthracis* strains from related species of the *B. cereus* group and, recently, rep-PCR plus primer BOX-A1R was used to identify a specific DNA marker for *B. anthracis* (Cherif *et al.* 2002). Also Kim *et al.* (2002) used BOX-A1R-based rep-PCR to establish the genetic relationship between 17 strains of the *B. cereus* group. They found that *B. anthracis* could be separated from the closely related species, but the genetic relationship among the other species of the *B. cereus* group was not well described, because of the small number of strains.

The aim of this work was to establish the genetic relationship between the species of the *B. cereus* group, basing the work on the rep-PCR fingerprinting of 112 strains of the six species. Strains of the species *B. anthracis, B. cereus, B. thuringiensis* and *B. weihenstephanensis* selected from different rep-PCR fingerprinting clusters were further characterized by sequencing AC-390, a rep-PCR fragment typical of *B. anthracis* identified in previous work as homologous to *ymfK* a trascriptional regulator of *B. subtilis* (Cherif *et al.* 2002).

MATERIALS AND METHODS

Bacterial strains, DNA extraction, rep-PCR and pattern cluster analysis

A total of 112 strains of the six species of the *B. cereus* group were used (Table 1). Strain cultivation and DNA extraction procedures are reported elsewhere (Borin *et al.* 1997; Daffonchio *et al.* 1998a,b, 1999a,b, 2000; Cherif *et al.* 2002).

The BOX-PCR were performed using BOX-A1R primer as already described (Urzì *et al.* 2001; Cherif *et al.* 2002).

Computer-assisted analysis of the rep-PCR fingerprinting patterns was performed using the Diversity Database^{Tm} Fingerprinting Software (Bio-Rad, Milan, Italy). The banding patterns were acquired from ethidium bromide-stained gels with the Gel Doc 2000 image system (Bio-Rad) and stored on disk as TIFF files. The 'rolling disk' background subtraction method was applied to each gel and a database containing all the gel images was created. The bands from all the gels were automatically detected and normalized using the 50-bp DNA ladder (Amersham Pharmacia Biotech, Milano, Italy) as the molecular size marker. A band set including all the polymorphic fragments was created, and each band in each lane was compared with the band set. The similarity between strains was determined by the band-sharing coefficient calculated by the formula of Jaccard, and strain clustering was performed by the unweighted pair group method with arithmetic averages (UPGMA), using NTSYS software (Daffonchio *et al.* 2000).

Sequencing of AC-390 fragment

A 342-bp fragment, named AC-390, a rep-PCR marker previously identified in *B. anthracis* and homologous to the *ymfK* gene of *B. subtilis* (Cherif *et al.* 2002), was obtained from selected strains of *B. anthracis, B. cereus, B. thuringiensis* and *B. weihenstephanensis* by PCR and sequenced in both directions using primers YWFK-f (5′-GAAAA-TGGCCGGATGAGT-3′) and YWFK-r (5′-GACGTTG-AAACATTTATGCA-3′) as previously described (Cherif *et al.* 2002).

The obtained sequences were subjected to neighbour-joining analysis to assess the phylogenetic relationship of the species of the *B. cereus* group. The alignment of the sequences was checked manually and corrected, and similarity values were determined using JALVIEW software (http://circinus.ebi.ac.uk:6543/jalview).

RESULTS

rep-PCR polymorphism and genetic relationships in the *B. cereus* group

The entire collection of 112 strains of the six species of the *B. cereus* group (Table 1) was analysed by rep-PCR using the BOX-A1R primer and a protocol optimized in a previous study (Cherif *et al.* 2002). The reproducibility of the rep-PCR patterns was evaluated by repeating PCR for several strains using DNA templates obtained from two independent DNA extractions. An example of this experiments, relative to four *B. cereus* strains (cer4, 487, 6127 and 46321) is shown in Fig. 1. Comparison of the resulting patterns indicated a very high reproducibility of the rep-PCR protocol (Fig. 1, panel A lanes 18–21 and panel D lanes 1–4). Figure 1 shows representative rep-PCR pattern types of the six species. The rep-PCR patterns were shown to be very discriminative and allowed us to identify 97 pattern

Table 1 Strains analysed in this study, rep-PCR pattern type number and relevant characteristics

Species (number of strains)	rep-PCR haplotype number*	Strains with the same rep-PCR haplotype	Strain source†	Relevant characteristic(s) of strain(s) [reference(s)]
Bacillus anthracis (17)	50	779	IPP	pXO1$^-$/2$^+$ (Ramisse *et al.* 1996); strains isolated in France in 1984 (Patra *et al.* 2002)
	51	582	IPP	pXO1$^+$/2$^+$ (Ramisse *et al.* 1996); strains isolated in France (Patra *et al.* 2002)
	52	300	IPP	pXO1$^+$/2$^+$ (Ramisse *et al.* 1996)
	53	256, 282, 846	IPP	pXO1$^+$/2$^+$ (Ramisse *et al.* 1996); strains isolated in the French Pyrenees in 1994 (256, 846) and 1995 (Patra *et al.* 2002)
	54	227, Cepanzo, 832, 957, Davis TE702, 6602	IPP	Strains 227 and 832 were pXO1$^+$/2$^+$, 957 and Cepanzo pXO1$^+$/2$^-$, 6602 and Davis TE702 pXO1$^-$/2$^+$ (Ramisse *et al.* 1996); strains 227 and 957 were vaccinal strains isolated in France (Patra *et al.* 2002); strain 832 was isolated in France in 1979 (Patra *et al.* 2002)
	55	376	IPP	pXO1$^+$/2$^+$ (Ramisse *et al.* 1996)
	56	7700	IPP	pXO1$^-$/2$^-$ (Ramisse *et al.* 1996)
	57	6769	IPP	pXO1$^+$/2$^+$ (Ramisse *et al.* 1996); strain isolated in French Pyrenees and Alps in 1997 (Patra *et al.* 1998)
	58	4229	IPP	pXO1$^-$/2$^+$ (Ramisse *et al.* 1996)
	59	663	IPP	pXO1$^+$/2$^+$ (Ramisse *et al.* 1996); strain isolated in France in 1984 (Patra *et al.* 2002)
Bacillus cereus (19)	6	Myd	DISTAM	Not available
	11	360	DSMZ	Isolated from garden soil
	12	cer1	DISTAM	Not available
	13	bc2	DISTAM	Not available
	14	626	DSMZ	Produces L-leucine dehydrogenase
	15	31^T	DSMZ	*B. cereus* type strain
	16	cer5	DISTAM	Isolated from rice (Daffonchio *et al.* 1998b)
	17	cer3	DISTAM	Isolated from candies (Daffonchio *et al.* 1998b)
	18	my1	DISTAM	Isolated from ultrahigh-temperature milk (Daffonchio *et al.* 1998b)
	19	IO200	LMT	Isolated from Norwegian sea
	20	PO1	DISTAM	Isolated from ultrahigh-temperature milk (Daffonchio *et al.* 1998b)
	21	345	DSMZ	Growth at 7°C (Daffonchio *et al.* 1999a)
	47	46321, 6127	DSMZ	Strain 6127 produces penicillinase
	48	487	DSMZ	Not available
	49	cer4	DISTAM	Isolated from rice (Daffonchio *et al.* 1998b)
	61	351	DSMZ	Isolated from soil
	63	bc1	DISTAM	Isolated from marble, Venice, Italy (Daffonchio *et al.* 1998b)
	74	cer6	DISTAM	Isolated from tomato sauce (Daffonchio *et al.* 1998b)
Bacillus mycoides (21)	1	NRS306	NRRL	Isolated from soil (Nakamura and Jackson 1995)
	2	NRS319	NRRL	Isolated from soil (Nakamura and Jackson 1995)
	3	MycH	DISTAM	Not available
	4	303, TP2	DSMZ, DISTAM	Isolated from soil. Strain 303 grows clockwise on acid and on alkaline media

Table 1 (*Contd.*)

Species (number of strains)	rep-PCR haplotype number*	Strains with the same rep-PCR haplotype	Strain source†	Relevant characteristic(s) of strain(s) [reference(s)]
	5	BmMed	DISTAM	Isolated from alkaline soil, Italy
	8	299	DSMZ	Isolated from soil; growth anticlockwise at pH 7 and clockwise at pH 5
	9	2048^T	DSMZ	*B. mycoides* type strain; growth at 7°C (Daffonchio *et al.* 1999a)
	10	B14828	NRRL	Not available
	76	BmF	DISTAM	Not available
	77	309	DSMZ	Produces a yellow diffusible pigment
	78	384	DSMZ	Growth at 7°C (Daffonchio *et al.* 1999a)
	79	A81	DISTAM	Isolated from soil, Italy
	80	Bif	DISTAM	Isolated from soil, Italy
	81	Ndr	DISTAM	Isolated from soil, Italy
	82	BmS	DISTAM	Not available
	83	B615	NRRL	Isolated from soil (Nakamura and Jackson 1995)
	90	G2	DISTAM	Isolated from garden soil, Italy
	91	Nov2	DISTAM	Isolated from maize rhizosphere, Italy
	92	Nov1	DISTAM	Isolated from maize rhizosphere, Italy
	96	G1	DISTAM	Isolated from garden soil, Italy
Bacillus pseudomycoides (8)	84	A82	DISTAM	Isolated from soil, Italy
	85	BD10	NRRL	Isolated from soil (Nakamura and Jackson 1995)
	86	BD14	NRRL	Isolated from soil (Nakamura and Jackson 1995)
	89	B346	NRRL	Isolated from soil (Nakamura and Jackson 1995)
	93	TP1	DISTAM	Isolated from soil, Italy
	94	$B617^T$	NRRL	*B. pseudomycoides* type strain (Nakamura 1998)
	95	CA	DISTAM	Isolated from soil, Italy
	97	B618	NRRL	Isolated from soil (Nakamura and Jackson 1995)
Bacillus thuringiensis (43)	22	BMG1.6	LMT	Isolated from soil, Tunisia
	23	Bt13	LMT	*B. thuringiensis* subsp. *pakistani*
	24	Bt55	LMT	*B. thuringiensis* subsp. *galleriae*
	25	BX16	LMT	Isolated from hypersaline soil in Tunisia; displays antifungal activity against *Fusarium* sp.
	26	BUPM21, BUPM22, BUPM23, BUPM25, BUPM26	CBS	Isolated from soil, Sfax region, Tunisia
	27	BMG1.9	LMT	Isolated from soil, Tunisia
	28	BMG1.7	LMT	Isolated from forest soil, Tunisia; produces the bacteriocin thuricin 7 (Cherif *et al.* 2001)
	29	Bt33, HD1, Al	DISTAM	Bt33 and HD1 *B. thuringiensis* subsp. *kurstaki*
	30	Bt9	LMT	*B. thuringiensis* subsp. *tolworthi*
	31	Bt7	LMT	*B. thuringiensis* subsp. *aizawai*
	32	Bt1	LMT	*B. thuringiensis* subsp. *thuringiensis*
	33	BUPM30	CBS	Isolated from soil, Sfax region, Tunisia
	34	5724	DSMZ	Isolated from commercial *B. thuringiensis israelensis* product
	35	5725	DSMZ	Isolated from commercial *B. thuringiensis kurstaki* product.
	36	BMG1.1	LMT	Isolated from soil, Tunisia
	37	Bt44	LMT	*B. thuringiensis* subsp. *dendrolimus*
	38	Bt14	LMT	*B. thuringiensis* subsp. *israelensis*

Table 1 (*Contd.*)

Species (number of strains)	rep-PCR haplotype number*	Strains with the same rep-PCR haplotype	Strain source†	Relevant characteristic(s) of strain(s) [reference(s)]
	39	2046^T	DSMZ	*B. thuringiensis* type strain subsp. *thuringiensis*
	40	BMG1.2	LMT	Isolated from soil, Tunisia
	41	Bt10	LMT	*B. thuringiensis* subsp. *darmstadiensis*
	42	BMG1.4	LMT	Isolated from surface water, Tunisia
	43	BMG1.3	LMT	Isolated from surface water, Tunisia
	44	BMG1.5	LMT	Isolated from surface water, Tunisia
	46	BMG1.8	LMT	Isolated from soil, Tunisia
	60	Hc13	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from dead insects (Khyami-Horani *et al.* 1996, 1999)
	62	Bt19	BGSC	Original strain ID: HD868; *B. thuringiensis* subsp. *tochigiensis*; produces the bacteriocin tochicin (Paik *et al.* 1997)
	64	Ht39	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from water (Khyami-Horani *et al.* 1996, 1999)
	65	Hc24	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from chicken faeces (Khyami-Horani *et al.* 1996, 1999)
	66	Hc32	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from infested plant leaves (Khyami-Horani *et al.* 1996, 1999)
	67	Hc35	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from water (Khyami-Horani *et al.* 1996, 1999)
	68	Hc17	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from chicken faeces (Khyami-Horani *et al.* 1996, 1999)
	69	Hc15	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from dead insects (Khyami-Horani *et al.* 1996, 1999)
	70	Hc45	DBS-UJ	Displays entomocidal activity against *Culiseta* sp. larvae. Isolated in Jordan from water (Khyami-Horani *et al.* 1996, 1999)
	71	Hc16	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from dead insects (Khyami-Horani *et al.* 1996, 1999)
	72	Ht51	DBS-UJ	Displays entomocidal activity against *Culiseta* sp. larvae. Isolated in Jordan from soil (Khyami-Horani *et al.* 1996, 1999)
	73	Hc36	DBS-UJ	Displays entomocidal activity against *Culex* sp. larvae. Isolated in Jordan from water (Khyami-Horani *et al.* 1996, 1999)
	75	BUPM33	CBS	Isolated from soil, Sfax region, Tunisia
B. weihenstephanensis (4)	7	10208	WSBC	Growth at 7°C (Lechner *et al.* 1998; Pruss *et al.* 1999); isolated from milk (Mayr *et al.* 1999)
	45	10204^T	WSBC	*B. weihenstephanensis* type strain; growth at 7°C (Lechner *et al.* 1998; Pruss *et al.* 1999); isolated from milk (Mayr *et al.* 1999)

Table 1 (*Contd.*)

Species (number of strains)	rep-PCR haplotype number*	Strains with the same rep-PCR haplotype	Strain source†	Relevant characteristic(s) of strain(s) [reference(s)]
	87	10202	WSBC	Growth at 7°C (Lechner *et al.* 1998; Pruss *et al.* 1999); isolated from milk (Mayr *et al.* 1999)
	88	10201	WSBC	Growth at 7°C (Lechner *et al.* 1998; Pruss *et al.* 1999); isolated from milk (Mayr *et al.* 1999)

*rep-PCR haplotype number represented in Figs 1 and 2.

†Institution from which the strains were obtained. BGSC, *Bacillus* Genetic Stock Center; *B. thuringiensis* strains HD2 and HD868 were kindly provided by D. R. Zeigler; CBS, Centre de Biotechnologie de Sfax, Tunisia; BUPM strains were kindly provided by S. Jaoua; DBS-UJ, Department of Biological Sciences, University of Jordan, Amman, Jordan; DISTAM, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Milan, Italy; DSMZ, Deutsche Sammlung von Mikroorganismen unt Zellkulturen, GmbH, Braunschweig, Germany; IPP, Institute Pasteur, Paris, France; total DNA of *B. anthracis* strain was kindly provided by M. Mock; LMT, Laboratoire de Microbiologie, Faculté des Sciences de Tunis, Tunisia; NRRL, Agricultural Research Service Culture Collection, Peoria, IL, USA; strains were kindly provided by L. K. Nakamura; WSBC, Weihenstephan *Bacillus* Collection, Weihenstephan, Germany; *B. weihenstephanensis* strains were kindly provided by S. Scherer.

types (Table 1). The number of bands per profile varied from 1 to 20 and the apparent molecular weight ranged from 100 to more than 2000 bp.

The *B. anthracis* strains had a relatively uniform rep-PCR pattern with major bands of about 130, 400, 630 and 770 bp. The 400-bp fragment was relatively specific to *B. anthracis* and had a sequence homologous to *ymfK,* a transcriptional regulator of *B. subtilis* (Cherif *et al.* 2002). Only minor band variations were observed between the strains and none of these were correlated with the presence/absence of the pXO1 and pXO2 virulence plasmids. The *B. cereus* strains showed wide polymorph profiles but no signature bands could be identified. Some *B. cereus* strains and most of the *B. thuringiensis* strains had two bands of about 670 and 1070 bp and several bands with an apparent molecular weight higher than 2000 bp. The *B. mycoides* strain showed two main pattern types, one very simple with a main band of variable length around 570 bp, found also in some *B. cereus* and *B. weihenstephanensis* strains, the other more complex with several bands that co-migrated with the corresponding bands in *B. pseudomycoides* and *B. weihenstephanensis*.

Computer-assisted analysis of the rep-PCR profiles was performed using Diversity Database^{Tm} Fingerprinting Software (Bio-Rad). By comparing rep-PCR patterns obtained from independent DNA extractions and PCR, we estimated that the error in clustering as a result of pattern reproducibility was negligible, not exceeding 5%. Figure 2 shows the dendrogram generated by UPGMA cluster analysis. Four major sub-clusters (A.1, A.2, B.1 and B.2) were identified, clusters A and B being separated out quite early. Cluster A grouped the *B. mycoides* (sub-cluster A.1) and *B. cereus* (sub-cluster A.2) strains while cluster B was divided into two major sub-clusters. Sub-cluster B.1 included strains of *B. mycoides, B. pseudomycoides* and *B. weihenstephanensis*. Sub-cluster B.2 showed wide variability and was the most complex, including 70 of the 97 rep-PCR patterns of the strain collection. In sub-cluster B.2 of Fig. 2 were highlighted four groups of patterns at different branching levels, denominated B.2.a to B.2.d and including relevant strains and species. The first group (B.2.a) included only strains of *B. mycoides*. The *B. cereus* and *B. thuringiensis* strains were divided into several groups: the main group of *B. cereus* was the cluster B.2.b, while that of *B. thuringiensis* was cluster B.2.c that included 24 patterns of *B. thuringiensis* (30 strains) and one of *B. weihenstephanensis*. The remaining strains of *B. cereus* and *B. thuringiensis* were grouped in cluster B.2.d, particularly sub-cluster B.2.d.1 that accounted for *B. thuringiensis*, and sub-cluster B.2.d.2, that included *B. cereus*. A branch of sub-cluster B.2.d.2 named B.2.d.3 included all the *B. anthracis* strains.

Genetic relationship based on the sequence of a rep-PCR marker specific to *B. anthracis*

The genetic relationship between *B. anthracis, B. cereus, B. thuringiensis* and *B. weihenstephanensis* was also assessed by sequencing AC-390, a *B. anthracis*-specific rep-PCR marker previously identified as homologous to the transcriptional regulator *ymfK* of *B. subtilis* (Cherif *et al.* 2002). The sequencing was performed in 27 strains of the four species selected from the different branches of the dendrogram in Fig. 2. For the amplification of *ymfK* we used the same primers designed in (Cherif *et al.* 2002). Using these primers, all attempts at obtaining the PCR product from the *B. mycoides* and *B. pseudomycoides* strains failed, indicating that these two species harbour a different sequence at the primer sites.

The genetic relationship of the four species of the *B. cereus* group based on the sequences of the AC-390 fragment

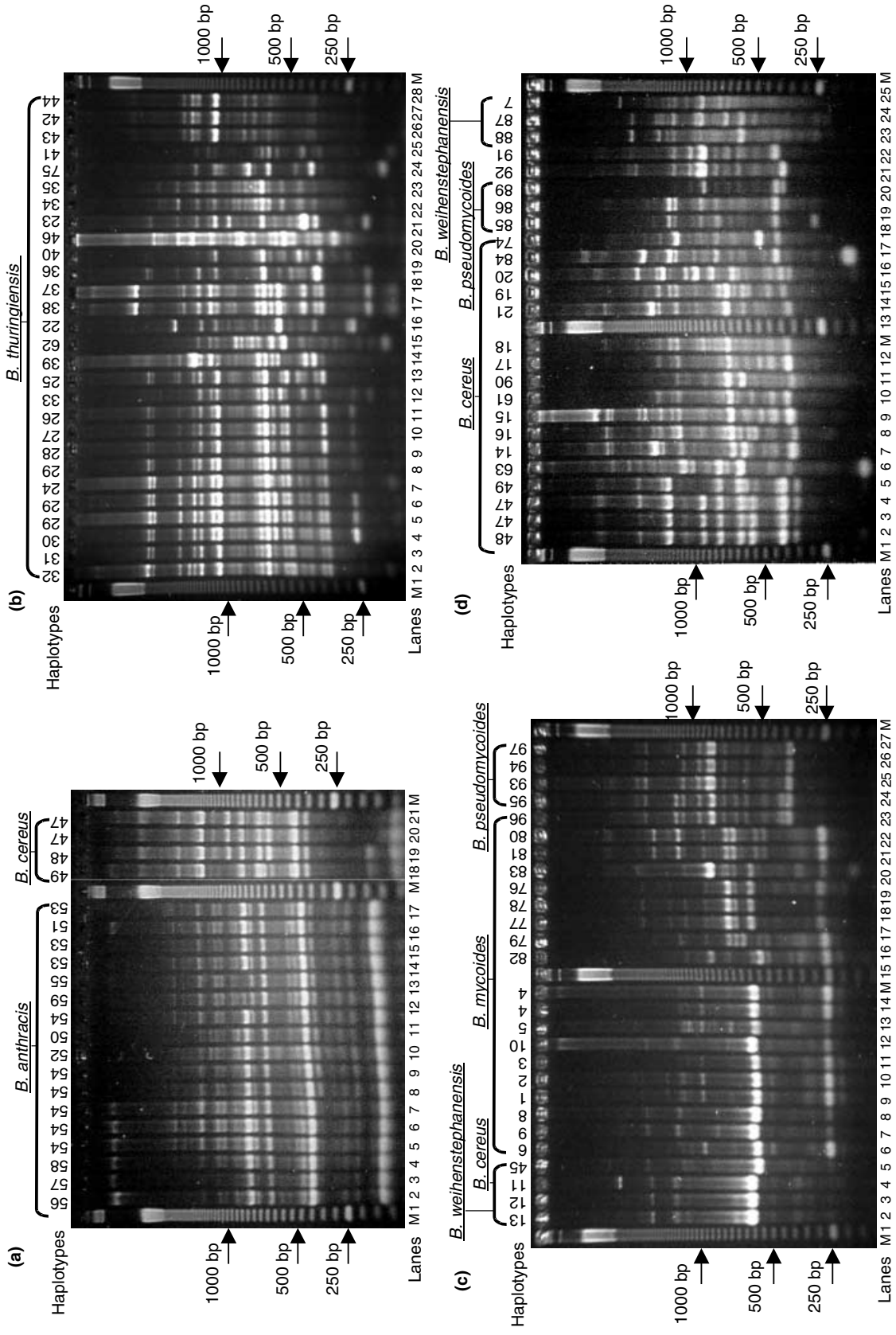
(a)
B. anthracis
B. cereus
Haplotypes
Lanes
1000 bp
500 bp
250 bp
(b)
B. thuringiensis
(c)
B. weihenstephanensis
B. cereus
B. mycoides
B. pseudomycoides
(d)
B. weihenstephanensis
B. cereus
B. pseudomycoides
B. weihenstephanensis

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Fig. 1 Example of rep-PCR pattern variability observed in the *Bacillus cereus* group. Lane M, 50-bp ladder. The 250, 500 and 1000-bp bands of the ladder are indicated. (a) Lanes 1–17, *B. anthracis* 7700 (refer for rep-PCR haplotype in Table 1: B56), 6769 (B57), 4229 (B58), 6602 (B54), Cepanzo (B54), Davis TE702 (B54), 957 (B54), 227 (B54), 300 (B52), 779 (B50), 832 (B54), 663 (B59), 376 (B55), 846 (B53), 256 (B53), 582 (B51), 282 (B53); lanes 18–21, *B. cereus* cer4 (B49), 487 (B48), 6127 (B47), 46321 (B47). (b) Lanes 1–28 *B. thuringiensis*, Bt1 (B32), Bt7 (B31), Bt9 (B30), Bt33 (B29), HD1 (B29), Bt55 (B24), Al (B29), BMG1.7 (B28), BMG1.9 (B27), BUPM21 (B26), BUPM30 (B33), BX16 (B25), 2046^{T} (B39), HD868 (B62), BMG1.6 (B22), Bt14 (B38), Bt44 (B37), BMG1.1 (B36), BMG1.2 (B40), BMG1.8 (B46), Bt13 (B23), 5724 (B34), 5725 (B35), BUPM33 (B75), Bt10 (B41), BMG1.3 (B43), BMG1.4 (B42), BMG1.5 (B44). (c) Lanes 1–3, *B. cereus* bc2 (B13), cer1(B12), 360 (B11); lane 4, *B. weihenstephanensis*, 10204^{T} (B45); lane 5, *B. cereus* myd (B6); lanes 6–23, *B. mycoides* 2048^{T} (B9), 299 (B8), NRS306 (B1), NRS319 (B2), MycH (B3), B14828 (B10), BmMed (B5), TP2 (B4), 303 (B4), BmS (B82), A81 (B79), 309 (B77), 384 (B78), bmf (B76), B615 (B83), Ndr (B81), Bif (B80), G1 (B96); lanes 24–27, *B. pseudomycoides*, CA (B95), TP1 (B93), $B617^{T}$ (B94), B618 (B97). (d) Lanes 1–9, *B. cereus* 487 (B48), 6127 (B47), 46321 (B47), cer4 (B49), Bc1 (B63), 626 (B14), cer5 (B16), 31^{T} (B15), 351 (B61); lane 10, *B. mycoides* G2 (B90); lanes 11–15, *B. cereus* cer3 (B17), my1 (B18), 345 (B21), IO200 (B19), po1 (B20); lane 16, *B. pseudomycoides* A82 (B84); lane 17, *B. cereus* cer6 (B74); lanes 18–22 *B. pseudomycoides* BD10 (B85), BD14 (B86), B346 (B89), Nov1 (B92), Nov2 (B91); lanes 23–25 *B. weihenstephanensis*, 10201 (B88), 10202 (B87), 10208 (B7)

is shown in the neighbour-joining tree of Fig. 3. All but two of the strains of *B. cereus* and *B. thuringiensis* were intermingled in branch A of the tree, confirming that it is very difficult to discriminate these two species by analysing a single locus. In branch B of the *ymfK* neighbour-joining tree, there was the identification of two sequence clusters, namely B1 and B2, in which were placed *B. anthracis* and *B. weihenstephanensis* strains, respectively. In branch B of the tree were also placed *B. cereus* strain PO1 and *B. thuringiensis* strain BUPM33.

DISCUSSION

From the dendrogram topology described in Fig. 2 several considerations can be made: our rep-PCR data confirmed the data of Kim *et al.* (2002) who found *B. anthracis* to diverge from the other species in the genus *Bacillus*; our use of a greater collection of strains of the six *B. cereus* group species gives strength to the findings of their data. *Bacillus anthracis* was found to form a sub-group within the *B. cereus*/*B. thuringiensis* group, indicating that although this species can be considered closely related to *B. cereus* and *B. thuringiensis* it actually diverges from them, sharing only 32% of similarity with the closest *B. cereus* strains (haplotypes 47, 48 and 49 in Fig. 2). These data are also in agreement with the genetic relationship described by ITS fingerprinting (Daffonchio *et al.* 2000) and by the sequencing of the long ITS-containing tRNA (Cherif *et al.* 2003).

Almost all the 19 *B. cereus* strains had different band patterns and were distributed over several branches of the dendrogram (Fig. 2). Some strains were grouped in cluster A together with several strains of *B. mycoides*, others were put into cluster B.2.b, and those remaining went into cluster B.2.d.2, the cluster that included cluster B.2.d.3 of *B. anthracis*. This distribution suggests that the *B. cereus* strains are characterized by a relatively variable genome, possibly due to a relatively frequent horizontal gene transfer within the species itself and with the other species of the group. This hypothesis is substantiated by recent multilocus enzyme electrophoresis data from sympatric *B. cereus* populations that show a panmictic population structure (Vilas-Boas *et al.* 2002).

Of the two main groups of *B. thuringiensis* strains, one included several known subspecies as well as different Tunisian strains isolated from different environments. Almost all the Jordan isolates were grouped together in a separate branch of the dendrogram, indicating that these strains, which all derive from the same geographical regions and that display mosquitocidal activity (Khyami-Horani *et al.* 1996, 1999), are relatively homogeneous. This grouping confirms the findings of a similar relationship revealed by ITS-PCR fingerprinting (Daffonchio *et al.* 2000).

The strains of *B. mycoides, B. pseudomycoides* and *B. weihenstephanensis* were confirmed in their relative genetic distance from the other species of the *B. cereus* group. The rep-PCR data support the distinctness between *B. mycoides* and *B. pseudomycoides*.

The genetic relationship described by the rep-PCR fingerprinting patterns was confirmed by the sequencing of a discrete locus, the AC-390 fragment. The sequence data support the idea that *B. anthracis* diverges from *B. cereus* and *B. thuringiensis* and hence represents an independent genetic lineage in the *B. cereus* group. Also *B. weihenstephanensis* was confirmed to be quite distant from *B. cereus*/*B. thuringiensis*, suggesting that, in general, this bacterium is an intermediate form between *B. cereus*/*B. thuringiensis* and *B. mycoides*. In fact *B. weihenstephanensis* shares colony morphology with *B. cereus*/*B. thuringiensis*, while with *B. mycoides* it shares the psychrotolerant phenotype and several genetic features such as ITS sequences and structure (Daffonchio *et al.* 2000; Cherif *et al.* 2003), signature sequences in several genetic loci like 16S and 23S rRNA (Lechner *et al.* 1998; Pruss *et al.* 1999), and the cold shock protein *cspA* (Francis *et al.* 1998). All attempts at obtaining the AC-390 PCR product from the *B. mycoides* and *B. pseudomycoides* strains failed, confirming the divergence of these two species from the other species of the *B. cereus* group.

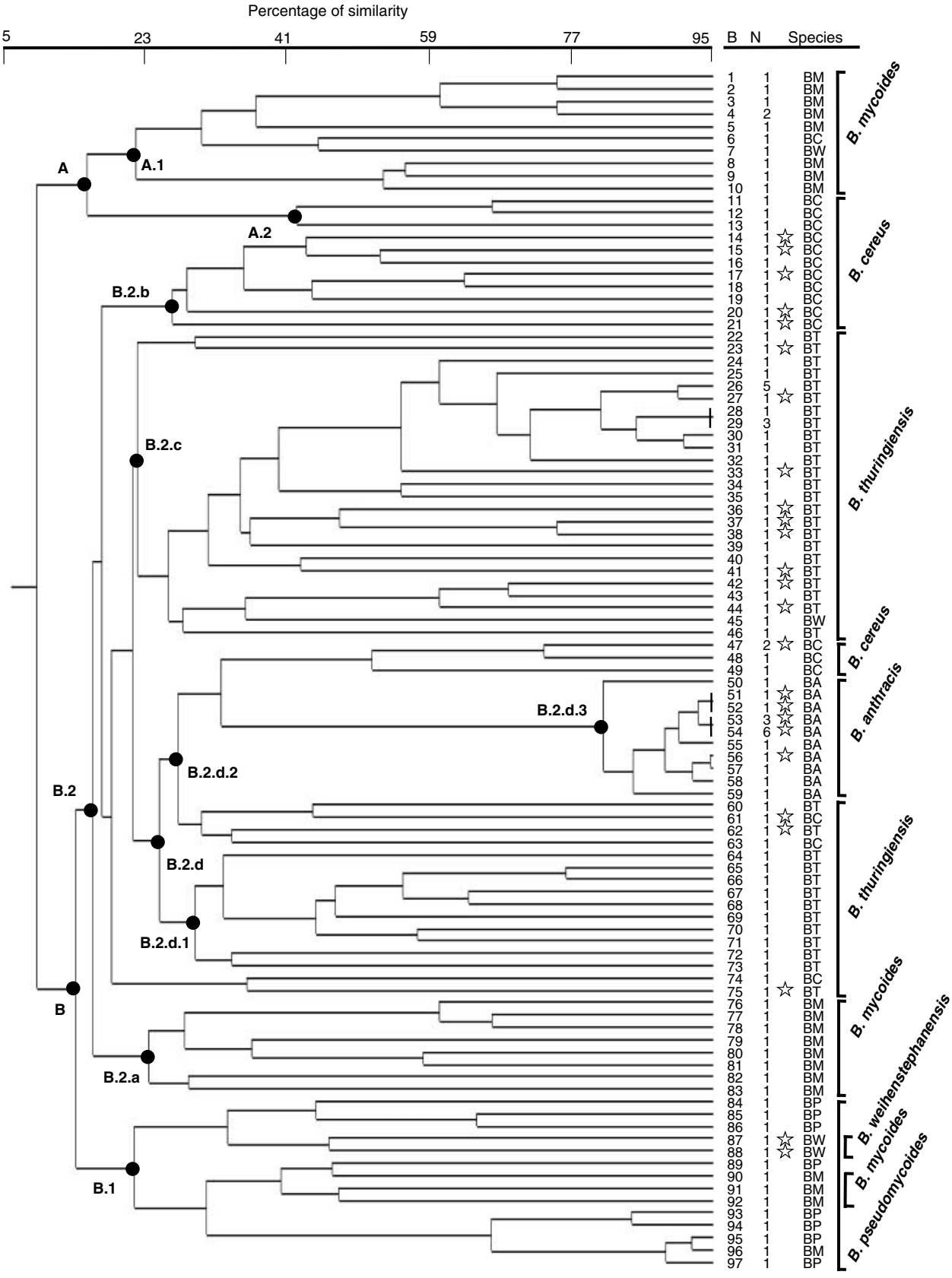
Percentage of similarity
5
23
41
59
77
95
B N Species
A
A.1
A.2
B.2.b
B.2.c
B.2.d.3
B.2.d.2
B.2
B.2.d
B.2.d.1
B
B.2.a
B.1
B. mycoides
B. cereus
B. thuringiensis
B. cereus
B. anthracis
B. thuringiensis
B. mycoides
B. weihenstephanensis
B. mycoides
B. pseudomycoides

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Fig. 2 Genetic relationship between *Bacillus cereus* group strains as described by the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis of the rep-PCR patterns. The percentage of similarity between the rep-PCR patterns was calculated using the Jaccard coefficient. (b) rep-PCR haplotype number (see Table 1); N, number of isolates for each rep-PCR haplotype (see Table 1). Acronyms used to indicate the *B. cereus* group species: BA, *B. anthracis*; BC, *B. cereus*; BM, *B. mycoides*; BP, *B. pseudomycoides*; BT, *B. thuringiensis*; BW, *B. weihenstephanensis*. The dots with letter/number designations (see text) were drawn on the dendrogram nodes where clusters of relevant strains or species are evident. The stars indicate rep-PCR haplotypes for which the *ywfK* marker was sequenced

The AC-390 sequence data confirm the impossibility of discriminating *B. cereus* from *B. thuringiensis* by comparing the sequence of a single discrete genetic locus. Although it has been shown that sympatric populations of *B. cereus* and *B. thuringiensis* have a higher level of recombination within a species than between species, it has been proposed that these two species cannot be considered sexually isolated (Vilas-Boas *et al.* 2002). Interspecies genetic exchange, even at a low level, would determine the wide genetic diversity and the species intermingling observed in the neighbour-joining tree, breaking down the clonal genetic structure in favour of a panmictic one. The impossibility of finding a distinct, exclusive cluster for the different species of the *B. cereus* group, neither in the sequence analysis of the *ywfK* fragment (e.g. the presence of *B. cereus* PO1 and *B. thuringiensis* BUPM33, respectively, close to the clusters where *B. weihenstephanensis* and *B. anthracis* are placed) nor in the rep-PCR fingerprinting analysis, supports the observation (Vilas-Boas *et al.* 2002) that the species in the *B. cereus* group are not completely sexually isolated, and that genetic exchange probably plays a significant role in the structuring and evolution of the populations of these bacteria in the environment.

In conclusion, it has been shown that rep-PCR analysis is a useful tool to describe the genetic diversity and genetic relationship of closely related bacteria such as members of the *B. cereus* group. An analysis of the rep-PCR patterns of these bacteria has confirmed their wide genetic variability, a variability that was further corroborated by the sequences of a rep-PCR marker typical of *B. anthracis* rep-PCR patterns.

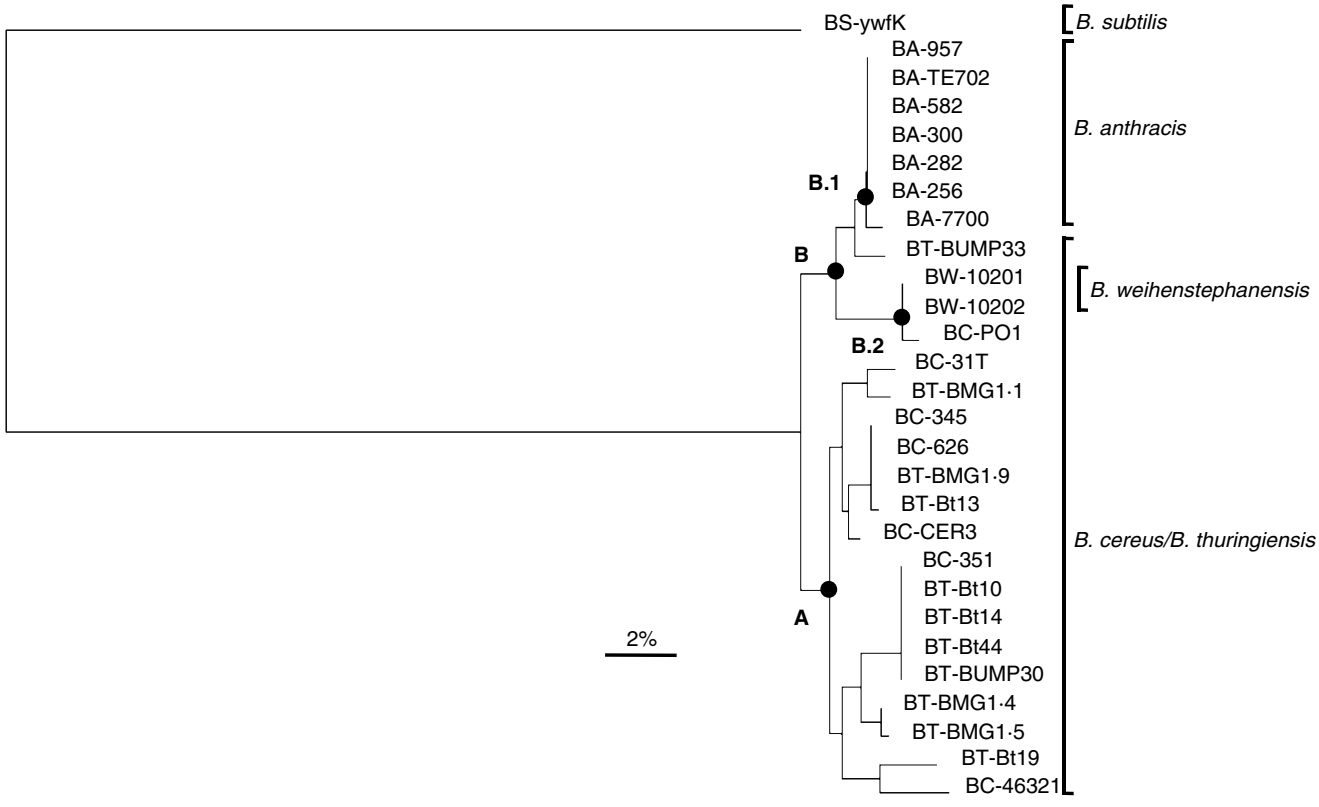

Fig. 3 Phylogenetic relationship between strains of the species of the *Bacillus cereus* group determined by neighbour-joining analysis of the *ywfK* sequences (Cherif *et al.* 2002). Before each strain name the species abbreviation is given (BA, *B. anthracis*; BC, *B. cereus*; BS, *B. subtilis*; BT, *B. thuringiensis*; BW, *B. weihenstephanensis*). The dots with letter/number designations (see text) were drawn on the dendrogram nodes where separated clusters and sub-clusters are evident. Bar indicate 2% of phylogenetic distance

In general, the genetic relationship, from the point of view of the rep-PCR patterns, indicates a relatively clear separation of the six species into quite homogeneous clusters, suggesting that they can indeed be considered as separate units, the level of recombination within each unit being higher than that between the units themselves. In any case, the finding of intermingled stains in several units, revealed by both rep-PCR fingerprinting and *ymfK* sequencing, suggests that a certain degree of recombination does take place between the species (Vilas-Boas *et al.* 2002).

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REFERENCES

Borin, S., Daffonchio, D. and Sorlini, C. (1997) Single strand conformation polymorphism analysis of PCR-tDNA fingerprinting to address the identification of *Bacillus* species. *FEMS Microbiology Letters* **157**, 87–93.

Brumlik, M.J., Szymajda, U., Zakowska, D., Liang, X., Redkar, R.J. and Del Vecchio, V.G. (2001) Use of long-range repetitive element polymorphism-PCR to differentiate *Bacillus anthracis* strains. *Applied and Environmental Microbiology* **67**, 3021–3028.

Cherif, A., Ouzari, H., Daffonchio, D., Cherif, H., Ben Slama, K., Hassen, A., Jaoua, S. and Boudabous, A. (2001) Thuricin 7: a novel bacteriocin produced by *Bacillus thuringiensis* BMG1.7, a new strain isolated from soil. *Letters in Applied Microbiology* **32**, 243–247.

Cherif, A., Borin, S., Rizzi, A., Houzari, H., Boudabous, A. and Daffonchio, D. (2002) Characterization of a repetitive element polimorphism-polymerase chain reaction chromosomal marker that discriminates *Bacillus anthracis* from related species. *Journal of Applied Microbiology* **93**, 456–462.

Cherif, A., Borin, S., Rizzi, A., Houzari, H., Boudabous, A. and Daffonchio, D. (2003) *Bacillus anthracis* diverges from related clades of the *Bacillus cereus* group in 16S-23S ribosomal DNA intergenic transcribed spacers containing tRNA genes. *Applied and Environmental Microbiology* **69**, 33–40.

Daffonchio, D., Borin, S., Consolandi, A., Mora, D., Manachini, P.L. and Sorlini C. (1998a). 16S–23S rRNA internal transcribed spacers as molecular markers for the species of the 16S rRNA group I of the genus *Bacillus*. *FEMS Microbiology Letters* **163**, 229–236.

Daffonchio, D., Borin, S., Frova, G., Manachini, P.L. and Sorlini, C. (1998b) PCR fingerprinting of whole genomes, the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveals a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. *International Journal of Systematic Bacteriology* **48**, 107–116.

Daffonchio, D., Borin, S., Consolandi, A. and Sorlini C. (1999a) Restriction site insertion-PCR (RSI-PCR) for rapid discrimination and typing of closely related microbial strains. *FEMS Microbiology Letters* **180**, 77–83.

Daffonchio, D., Borin, S., Frova, G., Gallo, R., Mori, E., Fani, R. and Sorlini, C. (1999b) A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis*. *Applied and Environmental Microbiology* **65**, 1298–1303.

Daffonchio, D., Cherif, A. and Borin, S. (2000) Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the "*Bacillus cereus* group". *Applied and Environmental Microbiology* **66**, 5460–5468.

Francis, K.P., Mayr, R., von Stetten, F., Stewart, G.S.A.B. and Scherer, S. (1998) Discrimination of psychrotrophic and mesophilic strains of the *Bacillus cereus* group by PCR targeting of major cold shock protein genes. *Applied and Environmental Microbiology* **64**, 3525–3529.

Helgason, E., Økstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I. and Kolstø, A.-B. (2000) *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis* – one species on the basis of genetic evidence. *Applied and Environmental Microbiology* **66**, 2627–2630.

Khyami-Horani, H., Katbeh-Bader, A. and Mohsen, Z.H. (1996) Mosquito larvicidal toxicity of endospore-forming bacilli isolated in Jordan. *Dirasat, Medical and Biological Science* **23**, 140–144.

Khyami-Horani, H., Katbeh-Bader, A. and Mohsen, Z.H. (1999) Isolation of endospore-forming bacilli toxic to *Culiseta longiareolata* (Diptera: Culicidae) in Jordan. *Letters in Applied Microbiology* **28**, 57–60.

Kim, W., Hong, Y.-P., Yoo, J.-H., Lee, W.-B., Choi, C.-S. and Chung, S.-I. (2002) Genetic relationships of *Bacillus anthracis* and closely related species based on variable-number tandem repeat analysis and BOX-PCR genomic fingerprinting. *FEMS Microbiology Letters* **207**, 21–27.

Lechner, S., Mayr, R., Francis, K.P., Pruss, B.M., Kaplan, T., Wiessner-Gunkel, E., Stewart, G.S. and Scherer, S. (1998) *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. *International Journal of Systematic Bacteriology* **48**, 1373–1382.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D.A., Boulnois, G.J. and Claverys, J.-P. (1994) A highly conserved repeat DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research* **20**, 3479–3483.

Mayr, R., Eppert, I. and Scherer, S. (1999). Incidence and identification of psychrotrophic (7°C-tolerant) *Bacillus* spp. in German HTST pasteurized milk. *Milchwissenschaft* **54**, 26–30.

Mignot, T., Denis, B., Couture-Tosi, E., Kolstø, A.-B., Mock, M. and Fouet, A. (2001) Distribution of S-layers on the surface of *Bacillus cereus* strains: phylogenetic origin and ecological pressure. *Environmental Microbiology* **3**, 493–501.

Nakamura, L.K. (1998) *Bacillus pseudomycoides* sp. nov. *International Journal of Systematic Bacteriology* **48**, 1031–1034.

Nakamura, L.K. and Jackson, M.A. (1995) Clarification of the taxonomy of *Bacillus mycoides*. *International Journal of Systematic Bacteriology* **45**, 46–49.

Paik, H.D., Bae, S.S., Park, S.H. and Pan, J.G. (1997) Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp. *tochigiensis*. *Journal of Industrial Microbiology and Biotechnology* **19**, 294–298.

Patra, G., Vaissaire, J., Weber-Levy, M., Le Doujet, C. and Mock, M. (1998) Molecular characterisation of *Bacillus* strains involved in outbreaks of anthrax in France in 1997. *Journal of Clinical Microbiology* **36**, 3412–3414.

Patra, G., Fouet, A., Vaissaire, J., Guesdon, J.-L. and Mock, M. (2002) Variation in rRNA copy number as revealed by ribotyping of *Bacillus anthracis* isolates. *Research in Microbiology* **153**, 139–148.

Petersen, D.J., Shishido, M., Brian Holl, F. and Chanway, C.P. (1995) Use of species- and strain-specific PCR primers for identification of conifer root-associated *Bacillus* spp. *FEMS Microbiology Letters* **133**, 71–76.

Pruss, B.M., Francis, K.P., von Stetten, F. and Scherer S. (1999) Correlation of 16S ribosomal DNA signature sequences with temperature-dependent growth rates of mesophilic and psychrotolerant strains of the *Bacillus cereus* group. *Journal of Bacteriology* **181**, 2624–2630.

Ramisse, V., Patra, G., Garrigue, H., Guesdon, J.-L. and Mock, M. (1996) Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. *FEMS Microbiology Letters* **145**, 9–16.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews* **62**, 775–806.

Urzì, C., Brusetti, L., Salamone, P., Sorlini, C., Stackebrandt, E. and Daffonchio, D. (2001) Biodiversity of *Geodermatophilaceae* isolated from altered stones and monuments in the Mediterranean basin. *Environmental Microbiology* **3**, 471–479.

Van Belkum, A., Scherer, S., van Alphen, L. and Verbrugh, H. (1998) Short-sequence repeats in prokaryotic genomes. *Microbiology and Molecular Biology Reviews* **62**, 275–293.

Versalovic, J., Koeuth, T. and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**, 6823–6831.

Versalovic, J., Schneider, M., De Bruijn, F.J., and Lupski, J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology* **5**, 25–40.

Vilas-Boas, G., Sanchis, V., Lereclus, D., Lemos, M.V.F. and Bourguet, D. (2002) Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. *Applied and Environmental Microbiology* **68**, 1414–1424.