Differentiation of mosquito-pathogenic strains of *Bacillus sphaericus* from non-toxic varieties by ribosomal RNA gene restriction patterns

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(Received 15 October 1991; revised 5 January 1992; accepted 28 February 1992)

DNA from 17 strains of *Bacillus sphaericus*, including representatives of all the established DNA homology groups, was cleaved with *EcoRI* or *HindIII* and fragments were separated by agarose gel electrophoresis. Southern blots of this DNA were hybridized to a radioactively labelled DNA probe prepared from the cloned 16S *rrnB* ribosomal RNA operon of *Escherichia coli*. Banding patterns of the chromosomal DNA digests and the autoradiograms were specific to DNA homology groups I (*B. sphaericus sensu stricto*), IIA (mosquito-pathogenic strains), IIB (*B. fusiformis*) and V, but groups III and IV were not clearly distinguished. This suggests that the mosquito-pathogenic strains represent a separate subspecies.

Introduction

Some strains within the species *Bacillus sphaericus* are toxic towards mosquito larvae and can be used to control these important vectors of human and animal disease. Larval death results from ingestion of a parasporal crystal which is comprised of two proteins, of 51.4 and 41.9 kDa, which are transcribed from adjacent genes (Hindley & Berry, 1987; Baumann *et al.*, 1988). Both proteins are required for toxicity although the mechanism of action is not known (Baumann *et al.*, 1991).

The basis of *B. sphaericus* classification was established by Krych *et al.* (1980), who allocated 50 of 62 strains to five DNA homology groups. These groups have since been validated by numerical classification of phenotypic features (Alexander & Priest, 1990) and enzyme electrophoretic patterns (Singer, 1988). It would seem that these groups represent species, although formal descriptions have not been published. DNA homology group II has been divided into two subgroups: IIA and IIB. All mosquito pathogens (with one possible exception: Guerineau *et al.*, 1991) have been allocated to subgroup IIA (Krych *et al.*, 1980). In two studies, strains within subgroup IIA showed mean DNA sequence homologies of 83% (Krych *et al.*, 1980) and 71%

(Guerineau *et al.*, 1991) while inter-subgroup sequence homologies were 61% and 63% respectively. Although this is strong evidence for separating these taxa, the inter-group sequence homology values are higher than is usual for separate species within the genus *Bacillus* (Priest, 1981) and it is not clear if they should be united or separated. Moreover, with the exception of pathogenicity, there are no definitive phenotypic characters that distinguish these taxa (Alexander & Priest, 1990). The status of these subgroups is therefore unclear.

In this study we have compared insect-pathogenic strains of subgroup IIA with non-toxic strains of subgroup IIB and various other strains using rRNA gene restriction patterns (Grimont & Grimont, 1986).

Methods

Strains and growth conditions. The *B. sphaericus* strains used are described in Table 1. All strains were grown on nutrient agar or in nutrient broth at 30 °C. The *Escherichia coli* strains used for plasmid preparation were grown in L-broth or on L-agar at 37 °C.

Plasmid preparation and labelling of probes. Plasmid pBSE-18 is pUC12 containing a 3.5 kb fragment from *B. sphaericus* BSE-18 (Guerineau *et al.*, 1991). The DNA fragment encodes genes for the 41.9 and 51.4 kDa toxin genes (see Fig. 2c) and is identical in sequence to the 3.5 kb fragment from strain 2362 (Baumann *et al.*, 1991; C. R. Berry, personal communication). *E. coli* JM83 containing pBSE-18 and *E. coli* JA221 containing pKK3535 (Brosius *et al.*, 1981) were grown in 500 ml batches of L-broth to the mid-exponential phase (OD₆₀₀ 0.6) and the plasmids amplified in the presence of chloramphenicol (170 µg ml⁻¹) overnight. Cultures were harvested and plasmids prepared by the

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Abbreviation: DIG, digoxigenin.

Fig. 1. Slot-blot hybridization of *B. sphaericus* chromosomal DNA with the toxin gene probe. Chromosomal DNA from *B. sphaericus* strains 2362, BSE-18, 2297, 1593 and JL-60 is shown at positions A5, B5, A6, C7 and A8 respectively. Strain SSII-1 (position B1), and the other strains listed in Table 1, did not hybridize with the toxin gene probe.

SDS lysis procedure (Maniatis *et al.*, 1982) and purified by caesium chloride/ethidium bromide density-gradient centrifugation. Plasmid pKK3535 was cut with *Pst*I and *Xba*I and the DNA electrophoresed in 0.8% (w/v) low-melting-temperature agarose at 12 V for 16 h. The 2.9 kb fragment from pKK3535 containing the 16S RNA gene from *E. coli* and some surrounding DNA was excised, dissolved in water (3 × volume), boiled for 7 min and labelled directly using random oligonucleotide primers (Feinberg & Vogelstein, 1983, 1984). The pKK3535 probe was labelled with [α - 32 P]dCTP (370 MBq ml $^{-1}$; Amersham); the typical probe activities were 107 c.p.m. (μ g DNA) $^{-1}$. Plasmid pBSE-18 was used intact after random primed labelling with digoxigenin (DIG)-dUTP (BCL).

DNA extraction, digestion and separation of fragments. Chromosomal DNA was isolated from late-exponential-phase cultures as described previously (Alexander & Priest, 1989) and 5–10 μ g was digested with *Eco*RI or *Hind*III (4 units per μ g DNA) for 4 h at 37 °C in the buffer recommended by the manufacturer (NBL, Cramlington, Northumberland). Digests were electrophoresed at 12 V for 16 h in horizontal 0.8% (w/v) agarose gels using Tris/acetate buffer (pH 8) (Maniatis *et al.*, 1982). *Hind*III-generated fragments of λ DNA were used as size markers. After electrophoresis, the gels were stained in ethidium bromide (5 μ g ml $^{-1}$) and photographed.

Southern blot hybridization to 16S rRNA probe. Electrophoresed DNA was denatured and transferred to nitrocellulose membranes by standard procedures (Maniatis *et al.*, 1982). DNA was hybridized to the pKK3535 probe (2.9 kb fragment) at 42 °C for 18 h in 40% (v/v) formamide. Autoradiograms on X-Omat film were exposed with intensifying screens at –70 °C for up to 24 h.

Table 1. Characteristics of *B. sphaericus* strains

Strain*	DNA group†	rRNA (R) group‡		Toxin gene§	Pathogenicity
		<i>Hind</i> III	<i>Eco</i> RI		
ATCC 14577**	I	RI	RI	–	–
BS 84	ND	RI	RI	–	ND
2362	IIA	RIIA	RIIA	+	High
1593**	IIA	RIIA	RIIA	+	High
SSII-1	IIA	(RIIA)	RIIA	–	Low
2297	IIA	RIIA	RIIA	+	High
BSE-18	IIB	RIIA	(RIIA)	+	High
JL-60	ND	ND	ND	+	High
ATCC 7055**	IIB	RIIB	RIIB	–	ND
DSM 493	IIB	(RIIB)	(RIIB)	–	ND
NRS 592**	III	RIII	RIII	–	ND
P1	III	(RIII)	RIII	–	ND
BS 112	ND	(RIII)	RIII	–	–
NRS 400**	IV	RIV	(RIII)	–	ND
NRS 1693	IV	(RIII)	RIII	–	ND
ATCC 13805	IV	(RIII)	(RIII)	–	ND
NRS 1198**	V	RV	RV	–	ND
NRS 1199	V	RV	RV	–	ND

ND, Not determined.

* All strains have been fully described previously (Alexander & Priest 1990) with the exception of JL-60, provided by A. A. Yousten, Virginia, USA.

** Denotes reference strains.

† DNA groups are based on results of Krych *et al.* (1980).

‡ R groups in parentheses indicate slight deviation from the reference pattern.

§ Toxin genes demonstrated by slot-blot hybridization (this paper) and also reported by others (see Baumann *et al.*, 1991 for a review).

|| Pathogenicity reported by Theiry & de Barjac (1989) and A. A. Yousten (personal communication).

Slot-blot hybridization for detection of toxin gene. Slot-blots were prepared with approximately 1 μ g of chromosomal DNA immobilized on nitrocellulose membranes using the Minifold II system (Schleicher & Schuell). Filters were probed with the DIG-labelled pBSE-18 DNA at 42 °C for 16 h in 40% (v/v) formamide. Exactly the same results were obtained using either the total plasmid or the excised 3.5 kb *Hind*III fragment as probe. Filters were washed and the bound DNA detected using the BCL detection kit, which is based on an alkaline-phosphatase-conjugated antibody reaction.

Plasmid preparation from *B. sphaericus*. *B. sphaericus* strains were grown in 100 ml batches of L-broth to the late exponential phase (12 h at 30 °C). Cultures were centrifuged for 10 min at 6000 r.p.m., and the pellet was resuspended in 10 ml TEN buffer (10 mM Tris/HCl pH 7.6, 1 mM-EDTA, 10 mM-NaCl). After centrifugation, 20–30 mg of cells were resuspended in 1 ml SET buffer (20% w/v, sucrose, 50 mM-Tris pH 7.6, 50 mM-EDTA) by thoroughly vortexing for 1 min. The plasmids were prepared as described by Rodriguez & Tait (1983) for *B. subtilis*. Plasmids were electrophoresed at 12 V for 16 h in horizontal 0.8% (w/v) agarose gels using Tris/acetate buffer (pH 8). The respective chromosomal DNA of each strain used for plasmid preparation was digested with *Hind*III for 4 h at 37 °C and electrophoresed in the same gel. The gels were stained in ethidium bromide (5 μ g ml $^{-1}$) and photographed before Southern blotting and hybridization to the DIG-labelled pBSE-18 probe DNA.

Results

Localization of toxin genes

The cloned toxin genes from *B. sphaericus* BSE-18 hybridized strongly to total DNA from high-toxicity

strains of group IIA in slot-blot hybridizations but not to DNA from any other *B. sphaericus* strain, including the low-toxicity strain SSII-1 (Table 1, Fig. 1). Since it did not hybridize to strain SSII-1 it can be concluded that the 3.5 kb fragment is specific for the toxin genes and surrounding DNA.

Fig. 2(a). Agarose gel electrophoresis of undigested plasmids and HindIII-digested chromosomal DNA. Lanes 1-8, plasmid DNA from *B. thuringiensis* var. *israelensis* (B.t.i.) and from the *B. sphaericus* strains shown; lanes 9-14, chromosomal DNA from the *B. sphaericus* strains shown. Fragment sizes are indicated on the right for HindIII-digested fragments of bacteriophage λ DNA. (b) Southern blot hybridization of *B. sphaericus* plasmids and chromosomal DNA with DIG-labelled pBSE-18 probe. Fragment sizes are indicated on the right for HindIII-digested fragments of bacteriophage λ DNA. (c) Map of the 3.5 kb HindIII fragment containing the genes for the 51.4 and 41.9 kDa proteins from *B. sphaericus* strain BSE-18. Abbreviations: E, EcoRI; K, KpnI; H, HindIII. (C. R. Berry, personal communication.)

Fig. 3. (a) Agarose gel electrophoresis of *Hind*III digests of *B. sphaericus* chromosomal DNA. Lanes: 1, ATCC 14577; 2, BS 84; 3, 1593; 4, 2362; 5, BSE-18; 6, ATCC 7055; 7, DSM 493; 8, NRS 592; 9, BS 112; 10, NRS 400; 11, ATCC 13805; 12, NRS 1198; 13, NRS 1199. Fragment sizes are indicated on the right for *Hind*III-digested fragments of bacteriophage λ DNA. (b) Autoradiograph of *Hind*III digests from *B. sphaericus* hybridized to the ³²P-labelled rRNA probe from *E. coli*. Lane designations as in (a). DNA homology groups are shown (ND, not determined).

Fig. 4. (a) Agarose gel electrophoresis of *Eco*RI digests of *B. sphaericus* chromosomal DNA. Lanes: 1, ATCC 14577; 2, BS 84; 3, 1593; 4, 2362; 5, BSE-18; 6, ATCC 7055; 7, DSM 493; 8, NRS 592; 9, BS 112; 10, NRS 400; 11, ATCC 13805; 12, NRS 1198; 13, NRS 1199. Fragment sizes are indicated on the left for *Hind*III-digested fragments of bacteriophage λ DNA. (b) Autoradiograph of *Hind*III digests from *B. sphaericus* hybridized to the 32 P-labelled rRNA probe from *E. coli*. Lane designations as in (a). DNA homology groups are shown (ND, not determined).

Plasmids were prepared from the hybridizing strains of *B. sphaericus*, and from the type strain and strain SSII-1 of *B. sphaericus*, and *B. thuringiensis* var. *israelensis* (B.t.i.), as controls. Plasmid DNA was analysed by agarose gel electrophoresis (Fig. 2a). Large plasmids were detected in strains ATCC 14577, 1593, SSII-1, 2297, 2362, BSE-18 and JL-60. Although it is possible that it represents chromosomal DNA, the large plasmid band is well defined and similar-sized plasmids (75 MDa) have been reported in most of these strains by Singer (1988). Additionally, the non-pathogenic type strain (ATCC 14577) and the mosquito pathogen (2297) contained smaller plasmids. *B. thuringiensis* var. *israelensis* typically contains six plasmids, of 72, 63, 10, 4.9, 4.3 and 3.6 MDa (Himeno *et al.*, 1985), which provides an indication of the sizes of the plasmids in strains ATCC 14577 and 2297. A Southern blot of the gel, which contained *Hind*III-digested chromosomal DNAs as controls, was hybridized with the cloned toxin genes in pBSE-18. None of the plasmid DNAs hybridized, including the putative high-molecular-mass species (Fig. 2b). If this had been chromosomal DNA, hybridization would have been expected, and indeed a faint band can be observed in lane 3 (strain 1593) indicating contamination by chromosomal DNA. However, when total DNA prepared from these strains was digested with *Hind*III, electrophoresed and hybridized distinct bands were revealed at about 4.6 kb for strain 2297 and 3.5 kb for the others. This suggests strongly that the toxin genes are located on the chromosome rather than on plasmids in these *B. sphaericus* strains.

rRNA gene restriction patterns of B. sphaericus strains

Chromosomal DNA from *B. sphaericus* strains was digested with *Hind*III (Fig. 3a) or *Eco*R1 (Fig. 4a) and separated by agarose gel electrophoresis. Visual inspection of the gels revealed distinct banding patterns associated with the DNA homology groups. Moreover, the patterns of group IIA and IIB strains were distinguishable. Southern blots of this DNA were hybridized with a ³²P-labelled DNA fragment representing most of the 16S rRNA gene from *E. coli*. Autoradiographs of the hybridized filters revealed distinct banding patterns for each of the DNA homology groups (Figs 3b, 4b). *Eco*R1 digests produced patterns of 6 to 11 bands ranging from 1 kb to 20 kb while the *Hind*III digests produced more bands, ranging from 2 kb to 20 kb. Patterns were designated according to that produced by the reference strain and are listed in Table 1. Of particular relevance is that toxic strains of group IIA produced unique patterns which were different from group IIB strains and strains of *B. sphaericus sensu stricto*. One anomaly to this classification was strain BSE-18

which, although a high-toxicity strain, had previously been assigned to DNA homology group IIB (Guerineau *et al.*, 1991). The results presented in Figs 3 and 4 show that it should be placed in group IIA. On the other hand, strains of DNA homology groups III and IV were not easily distinguished by this method.

Discussion

The mosquito-pathogenic *B. sphaericus* strains contained large and, for strain 2297, small plasmids. The distribution and sizes of these plasmids were similar to those reported in previous studies (Singer, 1988) with the exception that a plasmid was detected in strain ATCC 14577. However, unlike the other mosquito pathogen, *B. thuringiensis* var. *israelensis*, in which the toxin genes reside on the 72 MDa plasmid (Hofte & Whiteley, 1989), it seems that the toxin genes of *B. sphaericus* are located on the chromosome rather than on the plasmids (Fig. 2a, b). The Southern blots of the plasmids revealed no hybridization but the digests of total DNA showed toxin genes on similar-sized fragments to those described by Baumann *et al.* (1988), with strain 2297 giving an atypical pattern. This chromosomal location might explain the extreme conservation of *B. sphaericus* toxin genes (Berry *et al.*, 1989) compared to the divergence of *B. thuringiensis* toxin genes into various classes pathogenic for Lepidoptera, Coleoptera and Diptera. A plasmid location would allow transfer of the toxin genes by conjugation into strains of the *B. thuringiensis*-*B. cereus* group followed by recombination with resident genes leading to the development of novel sequences, a situation less likely to occur for chromosomally resident *B. sphaericus* toxin genes.

The different location also has a bearing on the systematics of *B. sphaericus* and *B. thuringiensis*. *B. thuringiensis* strains are essentially strains of *B. cereus* that harbour plasmids bearing crystal protein genes. There is no inherent difference between the two species other than the presence of specific plasmids (Somerville & Jones, 1972; Gonzalez *et al.*, 1982; Zahner *et al.*, 1989). Mosquito-pathogenic *B. sphaericus* strains, on the other hand, represent a different species from *B. sphaericus sensu stricto*. Although toxin genes of the 51 and 42 kDa class are not invariably present in the pathogens (see Table 1), all strains examined so far contain the 'low-toxicity' gene (Thanabalu *et al.*, 1991), and the pathogens differ in several other phenotypic characters from *B. sphaericus sensu stricto* (Alexander & Priest, 1990). Moreover, there is low DNA sequence homology between the two taxa (Krych *et al.*, 1980). These differences are borne out by the variation in chromosomal DNA restriction enzyme digests (Figs 3a and 4a). Strains of different species of *Providencia* (Owen *et al.*,

1988), *Helicobacter* (Morgan & Owen, 1990), *Campylobacter* (Hernandez *et al.*, 1991) and several other Gram-negative species (reviewed by Grimont & Grimont, 1991) similarly reveal species-specific banding patterns in this technique.

The distinction of the pathogens from *B. sphaericus sensu stricto* was supported by the rRNA gene patterns, and these also showed that DNA homology groups IIA (pathogens) and IIB (non-pathogens) were different. Indeed, strain BSE-18, previously thought to be a member of group IIB from DNA sequence homology studies (Guerineau *et al.*, 1991), typed unambiguously as a member of group IIA by rRNA patterns (Figs 3*b*, 4*b*). How does this information relate to the classification of group II strains? It is generally accepted that strains within a species should show greater than 70% DNA sequence homology with a thermal instability of hybrids (ΔT_m) less than 6 °C (Grimont & Grimont, 1991). This indicates that strains of groups IIA and IIB, which share about 62% DNA sequence homology, with a ΔT_m of about 7 °C (Krych *et al.*, 1980), cannot be allocated to the same species. Different subspecies within a species usually show ΔT_m values between 2 and 5 °C whereas strains within a subspecies show ΔT_m values close to zero (Grimont & Grimont, 1991). Values for ΔT_m within groups IIA and IIB are less than 2.5 °C (Krych *et al.*, 1980), suggesting subspecies status for these taxa. The rRNA gene restriction patterns shown in Figs 3*b* and 4*b* fully support this classification since patterns for subspecies generated to date such as those for *Serratia odorifera* biotypes I and II (Grimont & Grimont, 1991) and *Campylobacter jejuni* subsp. *jejuni* and subsp. *doylei* (Hernandez *et al.*, 1991) have been subspecies-specific. Since group IIB strains have been named *B. fusiformis* (Priest *et al.*, 1988) this would mean that group IIA strains should be allocated to a subspecies of *B. fusiformis*.

The rRNA gene restriction patterns for strains of DNA homology groups III and IV did not provide definitive patterns. This probably reflects the poor classification of these bacteria and perhaps the incorrect allocation of some strains to DNA homology group IV when their rRNA patterns are similar to those of homology group III. Studies of additional strains from these groups by rRNA gene restriction patterns might clarify the systematics of these bacteria.

M. Aquino de Muro is supported by a post-graduate research scholarship from the Brazilian Ministry of Education – CAPES.

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