Characterization of *Bacillus thuringiensis* and related bacteria by ribosomal RNA gene restriction fragment length polymorphisms

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Ribosomal RNA gene restriction patterns have been determined for 43 strains of *Bacillus thuringiensis* representing 10 serovars and eight reference strains of *B. anthracis*, *B. cereus* and *B. mycoides*. Strains within a *B. thuringiensis* serovar produced highly related or identical ribotype patterns: in particular, 12 strains of serovar *israelensis*, five strains of serovar *kurstaki*, two strains of serovar *galleriae* and three strains of serovar *aizawa* produced ribotype patterns consistent with serotype designations. Moreover, variety *tenebrionis* (serotype 8a8b), a coleopteran pathogen, could be distinguished from the more common lepidopteran pathogens of this serotype (serovar *morrisoni*) by ribotyping. The correlation of ribotype patterns with serotype suggests a clonal population structure for *B. thuringiensis*.

Keywords: *Bacillus thuringiensis*, ribotyping, 16S rRNA genes

INTRODUCTION

Bacillus thuringiensis is a Gram-positive soil bacterium characterized by the synthesis of a crystal protein or parasporal body which is toxic against various insects or other invertebrates. Toxin genes are assigned to at least six major classes (Höfte & Whiteley, 1989); *cryI* and *cryII*, active principally against lepidoptera (butterflies and moths), *cryIII* toxic to coleoptera (beetles), *cryIV* toxic to mosquitoes and blackflies, *cryV* toxic to lepidoptera and coleoptera (Tailor *et al.*, 1992) and *cryVI* active against nematodes (Feitelson *et al.*, 1992; Lereclus *et al.*, 1993). Within these classes numerous subclasses of genes are recognized (Lereclus *et al.*, 1993).

The taxonomy of *B. thuringiensis* is similarly complex. Strains may be typed according to their reaction with flagellar antisera and allocated to a serovar (de Barjac & Frachon, 1990). This classification has provided a sound basis for strain characterization and in some instances there is a correlation between serotype and pathogenicity; serotype 14 strains (serovar *israelensis*), for example, are almost invariably toxic to mosquitoes (de Barjac, 1990). Strains of other serovars, such as serotype 8 (serovar *morrisoni*), however, may be toxic to diptera (Padua *et al.*,

1986), lepidoptera (de Barjac & Frachon, 1990) or coleoptera (Keller & Lagenbruch, 1993) depending on the toxin genes they contain. As toxin genes are, with few exceptions, located on conjugative plasmids (Aronson, 1993), it may be unrealistic to expect a clear relationship between toxicity and serotype.

At the species level the situation is no clearer. *Bacillus anthracis*, *B. cereus*, *B. mycoides* and *B. thuringiensis* are considered a single phenetic group (Gordon *et al.*, 1973; Priest *et al.*, 1988). This view has been supported by DNA sequence homology studies (Somerville & Jones, 1972; Kaneko *et al.*, 1978) which show 50–100% homology between strains of the four species but few clear patterns. Moreover, strains of these four species have highly conserved 16S rRNA sequences (Ash *et al.*, 1991). Nevertheless, the important pathogenic attributes of *B. anthracis* and *B. thuringiensis* are considered sufficient to maintain separate species status for these bacteria and their relatives, *B. cereus* and *B. mycoides* (Claus & Berkeley, 1986).

Ribotyping, in which a Southern blot of restriction-enzyme-digested chromosomal DNA is hybridized to a probe based on the prokaryotic 16S rRNA gene has provided a useful approach to bacterial typing at the species and subspecies level (Grimont & Grimont, 1991). In this study we have examined numerous strains of *B.*

Abbreviation: DIG, digoxigenin.

Table 1. Strains used, their sources and rRNA gene restriction patterns

Strain	Source*	Serotype	Serovar	Ribotype
<i>B. anthracis</i> ASC1	1	—	—	BAH1
<i>B. anthracis</i> ASC6	1	—	—	BAH2
<i>B. anthracis</i> ASC68	1	—	—	BAH3
<i>B. anthracis</i> ASC69	1	—	—	BAH4
<i>B. cereus</i> S60 (DSM 31)	2	—	—	BCH1
<i>B. cereus</i> S68	2	—	—	BCH2
<i>B. cereus</i> S71	2	—	—	BCH3
<i>B. mycoides</i> S83	2	—	—	BMH1
<i>B. thuringiensis</i> S331	2	1	<i>thuringiensis</i>	BTH1
<i>B. thuringiensis</i> S337	2	1	<i>thuringiensis</i>	BTH1
<i>B. thuringiensis</i> S336	2	1	<i>thuringiensis</i>	BTH2
<i>B. thuringiensis</i> S461	2	1	<i>thuringiensis</i>	BTH1
<i>B. thuringiensis</i> HD-1	3	3a3b3c	<i>kurstaki</i>	BTH3
<i>B. thuringiensis</i> HD-73	3	3a3b3c	<i>kurstaki</i>	BTH3
<i>B. thuringiensis</i> S466	2	3a3b3c	<i>kurstaki</i>	BTH3
<i>B. thuringiensis</i> S467	2	3a3b3c	<i>kurstaki</i>	BTH3
<i>B. thuringiensis</i> CCT0434	5	3a3b3c	<i>kurstaki</i>	BTH3
<i>B. thuringiensis</i> TO3A002	4	3a3b3c	<i>kurstaki</i>	BTH4
<i>B. thuringiensis</i> S332	2	3a3c	<i>alesti</i>	BTH5
<i>B. thuringiensis</i> TO3001	4	3a3c	<i>alesti</i>	BTH5
<i>B. thuringiensis</i> S468	2	4a4b	<i>sotto (sotto)</i>	BTH6
<i>B. thuringiensis</i> TO4001	4	4a4b	<i>sotto (sotto)</i>	BTH7
<i>B. thuringiensis</i> TO4A001	4	4a4b	<i>sotto (dendrolimus)</i>	BTH8
<i>B. thuringiensis</i> S469	2	4a4b	<i>sotto (dendrolimus)</i>	BTH6
<i>B. thuringiensis</i> TO5001	4	5a5b	<i>galleriae</i>	BTH9
<i>B. thuringiensis</i> TO5002	4	5a5b	<i>galleriae</i>	BTH9
<i>B. thuringiensis</i> TO6002	4	6	<i>entomocidus</i>	BTH10
<i>B. thuringiensis</i> S474	2	6	<i>entomocidus (subtoxicius)</i>	BTH11
<i>B. thuringiensis</i> S476	2	7	<i>aizawai</i>	BTH12
<i>B. thuringiensis</i> S477	2	7	<i>aizawai</i>	BTH12
<i>B. thuringiensis</i> TO7002	4	7	<i>aizawai</i>	BTH12
<i>B. thuringiensis</i> TO8001	4	8a8b	<i>morrisoni</i>	BTH13
<i>B. thuringiensis</i> TO8002	4	8a8b	<i>morrisoni</i>	BTH13
<i>B. thuringiensis</i> CCT2265	5	8a8b	<i>morrisoni</i>	BTH13
<i>B. thuringiensis</i>	6	8a8b	<i>tenebrionis</i>	BTH14
<i>B. thuringiensis</i> TO9001	4	9	<i>tolworthi</i>	BTH15
<i>B. thuringiensis</i> TO9002	4	9	<i>tolworthi</i>	BTH16
<i>B. thuringiensis</i> S478	2	9	<i>tolworthi</i>	BTH17
<i>B. thuringiensis</i> IPS82	4	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2257	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2258	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2259	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2260	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2264	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2339	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2340	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2341	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2342	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2343	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2344	5	14	<i>israelensis</i>	BTH18
<i>B. thuringiensis</i> CCT2338	5	NT†	unassigned	BTH19

* Sources are: 1, P. C. B. Turnbull, CAMR, Salisbury, Wiltshire, UK; 2, laboratory collection (see Priest *et al.*, 1988, for details); 3, J. Iizuka, Faculty of Agriculture, Hokkaido University, Sapporo, Japan; 4, Collection of Entomopathogenic Bacteria, Institut Pasteur, Paris; 5, Collection de Culturas Tropical, Campinas, Brazil; 6, F. Audrade, Instituto de Biologia, Universidade Estadual de Campinas, Brazil.

† Strain could not be typed using existing H-antisera.

thuringiensis and reference strains of *B. anthracis*, *B. cereus* and *B. mycoides* using this approach.

METHODS

Strains and growth conditions. The strains used and their origins are listed in Table 1. They were maintained on nutrient agar slopes and as cell and spore suspensions in 20% (v/v) glycerol at -20°C . They were grown in Spizizen salts supplemented with 0.1% yeast extract and 0.4% glucose for 4.5 h ($\text{OD}_{600} < 0.7$) at 37°C for DNA preparation.

DNA extraction, digestion and Southern hybridization. DNA from the *B. anthracis* strains was kindly provided by P. C. B. Turnbull (PHLS, Porton Down, Salisbury). DNA was prepared from all other strains according to Kronstad *et al.* (1983) and Southern blots prepared as described previously (Aquino de Muro *et al.*, 1992).

Preparation of probe DNA and detection of hybrids. The probe was amplified from *B. subtilis* Marburg DNA using oligonucleotide primers directed to the extremities of the 16S rRNA gene; forward, 5'-GGATCCAGAGTTTGATCCTGGCTCAGGACG-3' (nucleotides 6-31) and reverse, 5'-GGATCCTACCTTGTACGACTTCACCCCAG-3' (nucleotides 1509-1484). The amplification reaction was supplemented with 5 μl digoxigenin (DIG)11-dUTP (0.07 mM final concentration) (Boehringer) and the concentration of dTTP was reduced to 200 μM from the standard 250 μM as used for the other dNTPs. The amplification programme comprised 1 cycle at 94°C for 10 min; 30 cycles of $55^{\circ}\text{C}/2$ min, $72^{\circ}\text{C}/3$ min, $94^{\circ}\text{C}/2$ min, and a final cycle of $55^{\circ}\text{C}/2$ min, $72^{\circ}\text{C}/10$ min. The mixture was precipitated with ethanol in the presence of sodium acetate and resuspended in 500 μl water; 15 μl amounts were used as the probes.

Agarose gel electrophoresis of labelled amplified DNA showed a marked reduction in mobility due to the incorporation of the DIG label.

After reassociation, hybridized DNA was detected using an antibody reaction linked to alkaline phosphatase according to the manufacturer's instructions (Boehringer).

Amplification of toxin gene DNA. Chromosomal DNA from strains of *B. thuringiensis* serovar *israelensis* was amplified using primers Dip2A (5' GGTGCTTCCTATTCTTTGGC) and Dip1B (5' ATGGCTTGTTTCGCTACATC), specific to regions 740-760 and 3328-3348 of the *cryIV/A* and *cryIV/B* genes as described by Carozzi *et al.* (1991). The reaction mix contained no DIG label and the concentration of dTTP was increased to 250 μM . For use as a hybridization probe, the PCR product was labelled with random hexanucleotide primers and DIG 11-dUTP according to the manufacturer's instructions (Boehringer).

Analysis of ribotype patterns. Hybridization profiles were divided into 30 segments and the presence/absence of a band in each segment scored visually. A table of plus/minus attributes based on the presence/absence of bands was prepared and analysed using the Jaccard similarity coefficient and average linkage cluster analysis (Sneath & Sokal, 1973).

RESULTS AND DISCUSSION

Ribotype patterns derive from the distribution of restriction enzyme sites within and between rRNA genes. Since there are no *Hind*III sites in the published sequence for the 16S rRNA genes of these bacteria (Ash *et al.*,

1991), the banding patterns result from sites in the flanking regions and each band represents a single or multiple 16S genes. There are 10 rRNA operons in *B. subtilis* (Jarvis *et al.*, 1988) and in strains where there are more than 10 ribotype bands (e.g. serovar *tolworthi*) this may indicate *Hind*III sites in the 16S rRNA gene or more rRNA operons than *B. subtilis*. Although great care was taken to avoid partial digestion of the DNA, pure chromosomal DNA is difficult to extract from these lysozyme-resistant cells, and partial digestion of slightly impure DNA cannot be discounted.

There were no common ribotype patterns between strains from different species. Common bands at about 2.5 and 4 kb, however, indicated a core area of homology in the rRNA gene clusters of *B. anthracis*, *B. cereus*, *B. mycoides* and *B. thuringiensis* (Figs 1 and 2). This is consistent with the highly conserved 16S rRNA gene sequences of these bacteria (Ash *et al.*, 1991). When the ribotype patterns were subjected to cluster analysis, the four *B. anthracis* strains were assigned to a single group but the *B. cereus* strains were dispersed throughout the dendrogram (Fig. 3). This reflects the high genetic and phenotypic similarity of the *B. anthracis* strains (Thorne, 1993) compared with the phenetic dissimilarity of the *B. cereus* strains which may be associated with soil, both emetic and diarrhoeal types of food poisoning or wound infections. Such strains were recovered in three different subclusters following numerical analysis of phenotypic features (Priest *et al.*, 1988) and although only strains associated with emetic-type food poisoning were included here, such organisms are phenotypically dissimilar to, and share limited DNA sequence homology with the type strain which was isolated from soil (Logan *et al.*, 1987; C. J. Ash, personal communication).

The diversity of rRNA gene restriction patterns displayed by the 43 *B. thuringiensis* strains was greater than for all other species examined by this technique. Staphylococcal strains average about three *Hind*III ribotype patterns per species, although 25 *S. aureus* strains were allocated to nine ribotypes (De Buyser *et al.*, 1989). Similarly, *Listeria* species generally contain two or three *Hind*III ribotypes (Jaquet *et al.*, 1992) and *Bacillus sphaericus* insect pathogens belong to a single ribotype (Aquino de Muro *et al.*, 1992). Of Gram-negative species, all strains of *Brucella* belong to a single genomic species and show a uniform ribotype (Verger *et al.*, 1987) while *Legionella* species can be identified by their individual ribotypes (Saunders *et al.*, 1988).

The 19 ribotypes recorded here therefore indicate extensive genetic heterogeneity and, if we had studied strains from additional serotypes, it is likely that more ribotypes would have been revealed. This heterogeneity has also been indicated by DNA pairing studies in which strains of different serotypes often share less than 60% sequence homology (Somerville & Jones, 1972), a value below the 70% level usually adopted as the minimum for strains of the same species (Grimont & Grimont, 1988). Electrophoretic patterns of whole cell proteins (unpublished) and numerical phenetic analysis (Priest *et al.*, 1988) also indicate heterogeneity within *B. thuringiensis*.

Fig. 1. Representative *Hind*III-generated rRNA gene restriction (ribotype) patterns for strains of *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. mycoides*.

Correlation of ribotype with serotype

There was a strong correspondence between ribotype and serotype for the strains studied here. This was particularly notable for 11 isolates of serovar *israelensis* from Brazil (CCT strains) which shared an identical ribotype pattern with the reference strain IPS82 (Figs 1 and 2). This may be a reflection of the narrow catchment area from which the strains were isolated and if we were to examine strains from a wider geographical spread more variation may have been observed. However, the inclusion of the reference strain suggests that this taxon is homogeneous and this is supported by virtually identical whole-cell protein electrophoresis profiles (Kaji *et al.*, 1994). Other serovars with conserved or identical ribotype patterns included *aizawai*, *alesti*, *galleriae*, *kurstaki*, *morrisoni*, *thuringiensis* and *tolworthi*. Interestingly the coleopteran pathogen, variety *tenebrionis* could be distinguished from the serologically identical lepidopteran/dipteran pathogen, serovar *morrisoni*, by ribotyping.

An alternative typing system for *B. thuringiensis* strains based on M13 DNA as a probe and double digestion of

the target chromosomal DNA produces more complex banding patterns than those shown here but again the results reveal a general correspondence between serotype and genetic fingerprint (Miteva *et al.*, 1991). In particular, serovars *thuringiensis*, *kurstaki* and *kenyae* (not included here) were genetically homogeneous by M13 typing. Similarly, arbitrarily primed PCR typing revealed that strains belonging to the same serovar produced closely related or identical banding patterns (Brousseau *et al.*, 1993). Enzyme distributions also indicate a clonal structure to *B. thuringiensis*. Esterase patterns show almost complete agreement with serotyping (Norris, 1964) and in a small study using multilocus enzyme electrophoresis two strains of serovar *israelensis* produced identical zymograms and of three strains of serovar *kurstaki*, two were identical and the third highly related (Zahner *et al.*, 1989). Although most serovars were assigned to individual zymovars, strains of serovars *finitimus*, *entomocidus* and *galleriae* produced identical zymogram patterns, suggesting that they belong to the same clone. This is difficult to reconcile with the results presented here, but more strains must be studied by these methods before

Fig. 2. Diagrammatic representation of *Hind*III-generated ribotype patterns for *B. anthracis*, *B. cereus*, *B. mycoides* and *B. thuringiensis* strains.

definite conclusions about allozyme distribution can be made.

RFLPs of toxin genes from serovar *israelensis*

We investigated the structure and arrangement of the 130 kDa *cryIV* toxin genes of the serovar *israelensis* strains initially by hybridizing Southern blots of total *Eco*RI or *Hind*III digested DNA with the *cryIV* gene probe. This revealed bands of about 11.0 and 9.4 kb (*Eco*RI) and 5.3,

3.6 and 2.3 kb (*Hind*III) in all Brazilian isolates and the reference strain IPS82 (data not shown). There was no hybridization between the probe and DNA from any other strain included in the study. When the PCR amplified toxin gene products from the serovar *israelensis* strains were digested with *Sau*3A, five bands corresponding to the predicted sizes from the 130 kDa toxin gene sequence (Yamamoto *et al.*, 1988) of 717, 555, 432, 249 and 186/180 bases, together with a faint band of about 600 bases (which may have been the result of partial

Fig. 3. Dendrogram showing the relationships between ribotype patterns following analysis using the S_j similarity coefficient with average linkage (UPGMA) cluster analysis.

digestion) were observed in every instance (data not shown). This shows that the *cryIV_A* gene and surrounding DNA is fairly highly conserved in these isolates.

Evolutionary considerations

B. thuringiensis is genetically heterogeneous as indicated by ribotyping and DNA pairing (Sommerville & Jones, 1972), and yet classifications based on serotyping and ribotyping are largely congruent. In other serologically

rich species, such as *Salmonella*, lack of congruence between the clonal structure of the species, as judged by multilocus enzyme electrophoresis, and serological classification, has been used as evidence for lateral transfer of flagellin genes between strains by transduction (Smith *et al.*, 1990). Similarly, horizontal gene exchange by transformation explains antigenic variation of the class 1 outer membrane protein in *Neisseria meningitidis* (Feavers *et al.*, 1992). Chromosomal gene exchange by transduction (Heierson *et al.*, 1983) and transformation (Heierson *et al.*,

1987) occurs in *B. thuringiensis* but the correlation of ribotype with serotype indicates limited chromosomal gene exchange during the evolution of these bacteria. Alternatively, the rRNA and flagellin genes may be closely linked and almost invariably transferred together, but this possibility seems unlikely.

It is interesting to compare *B. thuringiensis* with the other crystalliferous insect pathogen in the genus, *B. sphaericus*. The latter comprises an independent phylogenetic lineage of species status (Aquino de Muro & Priest, 1993) in which strains of identical ribotype (but varying flagellar serology and phage type) carry chromosomal genes for mosquito pathogenic crystal proteins. These genes are highly conserved and the toxins of similar, limited host range (reviewed by Baumann *et al.*, 1991). It would appear that *B. sphaericus* has evolved to occupy a defined habitat in which toxicity to the larvae of *Culex* and some other mosquito species provides a definite competitive benefit.

B. thuringiensis, on the other hand, is a member of a collection of organisms including *B. anthracis*, *B. cereus* and *B. mycoides* which together are only slightly broader than a single species as defined phenetically (Priest *et al.*, 1988), by 16S rRNA sequences (Ash *et al.*, 1991) or by DNA sequence homology (Sommerville & Jones, 1972). They carry transmissible plasmids, which in most cases are responsible for pathogenicity to a range of invertebrates (Feitelson *et al.*, 1992) and, in the case of *B. anthracis*, mammals and man (Thorne, 1993). These plasmids carrying the toxin genes are transmissible between serovars and even species in the laboratory (Gonzalez *et al.*, 1982) and in the field (Jarrett & Stephenson, 1990). Perhaps the plasmids are largely responsible for the extensive clonality of *B. thuringiensis*. Plasmids could drive evolutionary change by achieving a stable residence in a *B. thuringiensis* cell thus enabling it to colonize a particular insect, habitat, or both. This would lead to the establishment of a clone of such cells containing their associated plasmid (*B. thuringiensis* serovar *israelensis* for example). Plasmid transfer into a new strain and recombination with resident plasmid(s) would give rise to new toxins (Aronson, 1993). Selection of these new host/plasmid combinations would give rise to new clonal types (as judged by ribotyping) which would become fixed in the population. The result is a rapidly evolving complex of clones in which chromosomal divergence indicated by 16S rRNA gene sequence or DNA sequence homology has yet to 'catch up' and reach the point at which species are currently recognized.

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