Differentiation among strains and serotypes of *Bacillus thuringiensis* by M13 DNA fingerprinting

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The inter- and intraserotypic variations of *Bacillus thuringiensis* were studied by M13 DNA fingerprinting. Strain-specific patterns were obtained. The degree of homology was evaluated on the basis of pairwise comparisons and calculation of similarity indexes. Some strains belonging to the same serotype showed highly similar patterns, but others differed significantly. A high degree of polymorphism was established among the serotypes. These results provide evidence that the classification of *B. thuringiensis* strains on the basis of flagellar antigens does not always adequately reflect their genetic relatedness. DNA fingerprinting could help in future numerical taxonomic analysis of this species.

Introduction

The insect pathogen *Bacillus thuringiensis* is classified on the basis of flagellar antigens into more than 20 serotypes (de Barjac & Bonnefoi, 1973; Aronson *et al.*, 1986). Usually, isolates within serotypes differ in their general biochemical characteristics, plasmid patterns, and the shape, stability and host range activity of the entomocidal crystals. Some of the serotypes are divided into subserotypes. Krywienzyk (1977) extended this classification by including crystal antigens.

The investigation of genetic relatedness within the species *B. thuringiensis* was started by DNA–DNA competition experiments (Somerville & Jones, 1972; Kaneko *et al.*, 1978) and concentrated later on comparison of plasmids and endotoxin genes using cloned toxin sequences as probes (Kronstad *et al.*, 1983; Prefontaine *et al.*, 1987). No strong correlation between flagellar and crystal serotype and endotoxin genotype has been observed. The need for a new classification system based on the degree of genetic homology has been emphasized (Luthy, 1986; Priest *et al.*, 1988).

We recently reported the presence of hypervariable nucleotide sequences in several Gram-positive bacteria, including *B. thuringiensis*, detected by M13 DNA fingerprinting (Miteva *et al.*, 1990). This technique, based on Southern hybridization of restriction-enzyme-digested genomic DNA with a probe containing a specific repeated sequence, reveals patterns which are highly variable from one individual to another (Jeffreys

et al., 1985; Vassart *et al.*, 1987). This allows differentiation between closely related species and between individual organisms and strains (Ryskov *et al.*, 1988). A major advantage of this technique is that the whole genome is examined. Here, we applied DNA fingerprinting with M13 DNA as a probe for studying of inter- and intraserotypic variations of different *B. thuringiensis* strains.

Methods

Strains. The strains of *B. thuringiensis* used are listed in Table 1. They were grown in Nutrient Broth (Difco) at 28 °C for 16 h.

Total DNA isolation and restriction. High-molecular-mass DNA was isolated as described previously (Miteva *et al.*, 1990). DNA samples (10 µg) were digested with the appropriate restriction enzyme (Boehringer or Amersham) in the reaction buffer of the supplier, using 5–10 U per µg DNA. Digestions were allowed to proceed to completion for 12–18 h at 37 °C. The fragments were separated by electrophoresis in 25 cm long 1% (w/v) agarose gels (Sigma) at 8 V cm⁻¹ and stained with ethidium bromide.

Hybridization. DNA from the agarose gels was blotted onto nitrocellulose filters (Hybond-C, Amersham) according to Southern (1975). Single-stranded M13mp8 DNA was labelled by the primer extension method to a specific activity of 1.0×10^{-9} c.p.m. µg⁻¹ (1.5×10^7 c.p.m. per filter) using [³²P]dCTP, sequencing primer and a commercial kit from Amersham. Hybridization was performed in 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, 5 mM-EDTA at 57 °C or 51 °C for 16–20 h (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0; 1 × Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone). The filters were washed in

Table 1. *Bacillus thuringiensis* strains

All the strains have been maintained as a local laboratory collection for over 15 years.

Subspecies and strain designation	Flagellar serotype	Source*
<i>thuringiensis</i>		
B	1	Institut Pasteur ¹
Berliner	1	PPI ²
Steinhaus 1715	1	I. R. Pendleton ³
Leith	1	I. R. Pendleton
N	1	Local isolate (Grigorova, 1964)
058	1	Institut Pasteur
4058/A	1	I. R. Pendleton
I		J. Y. Shethna, Bangalore, India
<i>finitimus</i>		
fin-2	2	H. J. Somerville ⁴
<i>kurstaki</i>		
Euxoae	3a	I. R. Pendleton
HD1	3a3b	P. Luthy, Zürich, Switzerland
HD1-Dipel	3a3b	E. Videnova, Sofia, Bulgaria
<i>sotto</i>		
T-84-A	4a4b	I. R. Pendleton
<i>kenyae</i>		
S-4-2	4a4c	H. J. Somerville
Rhodesia	4a4c	I. R. Pendleton
<i>galleriae</i>		
Slough	5	I. R. Pendleton
Galleria	5a5b	PPI
Beira	5	I. R. Pendleton
<i>subtoxicius</i>		
SubVIA	6	H. J. Somerville
<i>entomocidus</i>		
EntVIB	6a6b	H. J. Somerville
Limassol	6	I. R. Pendleton
<i>aizawai</i>		
ai-VII3	7	H. J. Somerville
1 HA VII	7	I. R. Pendleton
Pill 122	7	I. R. Pendleton
<i>morrisoni</i>		
8m	8a8b	H. J. Somerville
morrisoni	8a8b	Institut Pasteur
<i>israelensis</i>		
H14	14	Institut Pasteur
H14-99	14	AcrySTALLiferous, plasmidless mutant (Miteva <i>et al.</i> , 1986)

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2 × SSC, 0.1% SDS for 30 min at the same temperature as used for hybridization and exposed to X-ray films at -70 °C.

Data analysis. The approach of Jeffreys *et al.* (1985) and Nybom *et al.* (1990) was used. In the comparative analysis, several autoradiographs of different intensity were used to evaluate the number and intensity of the bands. The similarity index, *D*, was calculated for each pair of patterns A and B according to the equation $D_{AB} = 2 \times \text{no. of shared fragments} / (\text{no. of fragments in A} + \text{no. of fragments in B})$. It reflects the probability that a fragment in A is also present in B. The probability

that A and B share all common fragments (P_C) was calculated as the mean D_{AB} for a given serotype raised to the power of the mean number of fragments per genome. Standard deviations were calculated where appropriate.

Reproducibility of the method. To obtain reproducible band patterns it was important to use high-molecular-mass DNA and to achieve total cleavage and perfect resolution of the fragments in the agarose gels. The stability of the patterns was proved by repeating the whole procedure after subcultivation (five or more passages on agar). Two or more blots from each digestion were prepared and analysed. The original *EcoRI* and *HaeIII* + *EcoRI* genomic fingerprints were found to be maintained in all cases.

Results and Discussion

Twenty-eight strains belonging to thirteen serotypes and subserotypes were studied (Table 1). Total DNA preparations were digested to completion with one or two restriction endonucleases. After blotting and hybridization with radioactively labelled M13 DNA, the profiles were compared pairwise and the similarity index, *D*, was calculated. Taking into consideration our previous observations that Gram-positive micro-organisms need less stringent conditions for hybridization than Gram-negative ones (Miteva *et al.*, 1990) we performed the hybridization of each filter at 57 °C and at 51 °C. At the lower temperature the number of informative bands was approximately doubled, hence the number of differences increased (see Fig. 3). However, for most of the strains the similarity indexes obtained at the two temperatures were quite close (Table 2). Even on the basis of the lower number of bands under higher stringency conditions the data were still sufficient to detect differences not only between serotypes but within them as well, and to give reproducible quantitative results.

We tried to find some correlation between the enzymes used and the polymorphic patterns. For this purpose restriction endonucleases recognizing a different number of nucleotides were used. Double digestions gave a larger number of bands, usually of lower intensity, than did single digestions (Fig. 1). The molecular size of the fragments obviously decreased when double digestions and enzymes recognizing four nucleotides were used (Fig. 2). The different intensity of the observed bands could be explained by the lower number and arrangement of the repeats homologous to the consensus M13 sequences in a given fragment. Usually several cleavage patterns had to be examined in order to differentiate between the strains and the serotypes. In all cases the number and distribution of the hybridization bands of a sample digested with the same enzyme or pair of enzymes reproducibly generated a unique pattern.

Table 2. Analysis of the intraserotypic variations of *EcoRI*+*HaeIII*-digested DNA samples of *B. thuringiensis* under two hybridization stringency conditions

The index of similarity, *D*, is a mean of all pairwise comparisons. Data are means of the analysis of autoradiographs from two hybridization series. SD values are given in parentheses.

Serotype/ no. of strains	Hybridization temp. (°C)	No. of informative fragments	No. of fragments per genome	Index of similarity (<i>D</i>)	Probability (<i>P_c</i>)
1/2	57	24	6.0 (1.42)	0.84 (0.09)	3.5 × 10 ⁻¹
	51	43	10.7 (0.96)	0.93 (0.03)	2.1 × 10 ⁻¹
3/3	57	50	8.3 (2.80)	0.45 (0.36)	1.3 × 10 ⁻⁴
	51	96	16.0 (4.40)	0.45 (0.70)	2.8 × 10 ⁻⁶
4/3	57	42	7.0 (1.37)	0.62 (0.22)	3.5 × 10 ⁻²
	51	69	11.5 (3.56)	0.57 (0.24)	1.5 × 10 ⁻³
5/3	57	38	6.3 (2.04)	0.60 (0.28)	4.0 × 10 ⁻²
	51	69	11.5 (1.52)	0.62 (0.24)	4.1 × 10 ⁻³
6/3	57	32	5.3 (1.89)	0.57 (0.24)	5.1 × 10 ⁻²
	51	70	11.6 (0.80)	0.53 (0.31)	6.3 × 10 ⁻⁴
7/3	57	38	6.3 (2.33)	0.58 (0.23)	3.2 × 10 ⁻²
	51	86	14.3 (4.55)	0.66 (0.21)	2.6 × 10 ⁻³
8/2	57	24	4.0 (0.82)	0.41 (0.07)	2.8 × 10 ⁻²
	51	55	9.2 (0.50)	0.61 (0.33)	1.1 × 10 ⁻²
14/2	57	19	4.7 (1.50)	0.62 (0.07)	1.1 × 10 ⁻¹
	51	33	8.2 (0.50)	0.79 (0.05)	1.4 × 10 ⁻¹

Comparison of strains within a serotype

For this purpose, two or more strains belonging to the same serotype were studied. The indexes of similarity (*D*) presented in Table 3 were obtained by pairwise comparisons of the strain genomes within a serotype; they showed a wide range of values (from 0.11 to 0.9). The mean *D* values were also calculated for each serotype (Table 3).

The investigation of serotype 1, represented by eight strains, revealed a high level of identity. Strains B, 1715, N, 4058, I and Leith showed identical patterns when digested with *MspI* + *HaeIII* (Fig. 1), *ClaI* + *KpnI*, or *HindIII* + *MspI* (data not shown). Only strain 058 differed significantly, which resulted in a very low index of similarity (0.1–0.3) when this strain was compared to any of the above strains. The significant genomic variations of this strain, originating from Czechoslovakia, could be the result of a different evolutionary pathway. They also prove that the flagellar serotyping does not always correlate with the degree of genetic homology.

A detailed analysis of the serotype 1 strains B and Berliner was undertaken. Our previous results showed identical patterns for these strains (Miteva *et al.*, 1990). Here we applied more enzymes, double restrictions and less stringent conditions. The results were still very similar after single digestions with *PstI*, *PvuII*, *BglII* or *BamHI* and with four combinations of two enzymes – *PvuII* + *HaeIII*, *PstI* + *PvuII* (Fig. 2), *EcoRI* + *HaeIII* (Fig. 3, lanes 1 and 2), and *EcoRI* + *PstI* (data not

shown). In all cases differences were detected in only one or two bands. The similarity index ranged from 0.75 to 0.93 (Table 3). Again no differences were found with *EcoRI* (Fig. 4, lanes 1 and 2). These results obviously reflect a very high genetic homology of most of the strains from serotype 1. They also show that many enzymes should be used in such cases.

In further experiments, two or three strains from each serotype were studied by pairwise comparisons (Figs 3–5). In some cases, two of the three strains showed highly similar patterns (*D* 0.8–0.9), while the third one differed significantly, which led to lower mean *D* values for the whole serotype (Table 3). This did not depend on the enzyme used. The *D* values for each serotype obtained after digestion with different restriction endonucleases were in most cases very close. It should be noted that the strains with highly homologous profiles belong to the same subserotype such as 3a3b (Fig. 3, lanes 4 and 5; Fig. 4, lanes 4 and 5; Fig. 5, lanes 2 and 3) and 4a4c (Fig. 3, lanes 7 and 8; Fig. 4, lanes 7 and 8; Fig. 5, lanes 5 and 6), while the strains demonstrating stronger variations came from other subserotypes (3a and 4a4b) respectively (Fig. 3, lanes 3 and 6; Fig. 4, lanes 3 and 6; Fig. 5, lanes 1 and 4). This finding indicates a certain correlation between the division of strains on the basis of flagellar serotyping and the degree of their genetic homology. The results also show, however, that significant genetic variability exists among some strains within the *B. thuringiensis* serotypes.

With the aim of identifying minor genomic differences

Fig. 1

Fig. 1. Southern hybridization of ^{32}P -labelled M13 DNA to DNA from *B. thuringiensis* subsp. *thuringiensis* (serotype 1) after digestion with *MspI* + *HaeIII* under low-stringency conditions (51 °C). Strains: lane 1, B; lane 2, Steinhaus 1715; lane 3, N; lane 4, 058; lane 5, 4058; lane 6, I; lane 7, Leith. The molecular sizes indicated on the right were determined from *HindIII*-digests of phage λ DNA.

Fig. 2

Fig. 2. Southern hybridization of ^{32}P -labelled M13 DNA to DNA from *B. thuringiensis* subsp. *thuringiensis* serotype 1 (strains B and Berliner), digested with different restriction enzymes under low-stringency conditions (51 °C). Even-numbered lanes, strain B; odd-numbered lanes, strain Berliner. Lanes 1 and 2, *PstI*; lanes 3 and 4, *PvuII*; lanes 5 and 6, *BglII*; lanes 7 and 8, *BamHI*; lanes 9 and 10, *PvuII* + *PstI*; lanes 11 and 12, *PvuII* + *HaeIII*.

we compared strain H14 with an acrySTALLIFEROUS plasmidless mutant of the same strain designated as H14-99. No differences were detected after *EcoRI* and *PstI* digestions (data not shown) but the enzyme combination *EcoRI* + *HaeIII* yielded specific patterns with some band position differences (Fig. 3, lanes 20 and 21) This observation raises the question whether DNA fingerprinting allows genome differentiation at the strain level or further. More investigations of other mutants will be necessary to answer this question.

Comparison among serotypes

As shown above, the variations between strains within the same serotype were sometimes quite significant. This

made difficult not only the comparison of such strains but also that of the serotypes. For most of the strains belonging to different serotypes, DNA digested with the same enzyme possessed one or more common bands, sometimes more intense than the other bands in the profile. This was better observed in single enzyme digestions (Fig. 4). Since cleavage was always performed to completion with excess enzyme, the appearance of these strong bands could be due to the presence of species-specific sequences containing multiple copies of M13 repeats. The strain representing serotype 2 was an interesting exception, also displaying an intense band but of smaller size (Fig. 4, lane 22). Very low *D* values were obtained when this strain was compared to strains from other serotypes (Table 4). Serotype 2 of *B.*

Fig. 3. Southern hybridization of ^{32}P -labelled M13 DNA to *EcoRI* + *HaeIII* digests of DNA from *B. thuringiensis* strains at two different stringencies. (a) Temperature of hybridization and washing 57 °C. (b) The same nitrocellulose filter was reprobed at 51 °C. The restriction conditions and the probe were the same as in Fig. 1. The serotypes are given in parentheses in the list below and above the lanes in the figure. Lanes: 1, B (1); 2, Berliner (1); 3, Euxoae (3a); 4, HD1 (3a3b); 5, HD1-Dipel (3a3b); 6, T-84-A (4a4b); 7, S-4-2 (4a4c); 8, Rhodesia (4a4c); 9, Slough (5); 10, Galleria (5a5b); 11, Beira (5); 12, SubVIA (6); 13, EntVIB (6a6b); 14, Limassol (6); 15, ai-VII3 (7); 16, 1 HA VII (7); 17, Pill 122 (7); 18, 8m (8a8b); 19, morrisoni (8a8b); 20, H14 (14); 21, H14-99 (14); 22, fin-2 (2).

thuringiensis is unique in its ability to form the parasporal inclusion inside and not outside the exosporium.

The patterns for serotypes 4, 5 and 7 obtained after digestion with *XbaI* contained a small number of fragments with minor differences in their size (Fig. 5). Similar results were obtained with other single enzyme digestions.

The picture changed when double digestions were performed. This resulted in a larger number of bands, generating unique patterns that still showed common bands (Fig. 3). To gain more information, several double

digestions were analysed, including typical strains from each serotype (Fig. 3b). One strain typical for each serotype was chosen among the highly similar pairs. These representative strains were compared pairwise. The results presented in Table 4 show that the strains from different serotypes displayed a marked degree of polymorphism, giving relatively low similarity indexes. At the same time serotypes 3 and 7, 3 and 8, 5 and 7, and 7 and 8 possessed very similar patterns (*D* 0.71–0.75). These values were higher than those obtained when strains from the same serotype were compared. This

Fig. 4. Southern hybridization of ^{32}P -labelled M13 DNA to *Eco*RI digests of DNA from *B. thuringiensis* strains under high-stringency conditions (57 °C). The serotypes are given in parentheses in the list below, and above the lanes in the figure. Lanes: 1, B (1); 2, Berliner (1); 3, Euxoae (3a); 4, HD1 (3a3b); 5, HD1-Dipel (3a3b); 6, T-84-A (4a4b); 7, S-4-2 (4a4c); 8, Rhodesia (4a4c); 9, Slough (5); 10, Galleria (5a5b); 11, Beira (5); 12, SubVIA (6); 13, EntVIB (6a6b); 14, Limassol (6); 15, ai-VII3 (7); 16, 1 HA VII (7); 17, Pill 122 (7); 18, 8m (8a8b); 19, morrisoni (8a8b); 20, H14 (14); 21, H14-99 (14); 22, fin-2 (2).

Fig. 5. Southern hybridization of ^{32}P -labelled DNA to *Xba*I digests of DNA from *B. thuringiensis* strains under low-stringency conditions (51 °C). The serotypes are given in parentheses in the list below, and above the lanes in the figure. Lanes: 1, Euxoae (3a); 2, HD1 (3a3b); 3, HD1-Dipel (3a3b); 4, T-84-A (4a4b); 5, S-4-2 (4a4c); 6, Rhodesia (4a4c); 7, Slough (5); 8, Galleria (5a5b); 9, Beira (5); 10, SubVIA (6); 11, EntVIB (6a6b); 12, Limassol (6); 13, ai-VII3 (7); 14, 1 HA VII (7); 15, Pill 122 (7); 16, 8m (8a8b); 17, morrisoni (8a8b).

Table 3. Similarity indexes of all pairwise comparisons of *B. thuringiensis* strains within the serotypes

Serotype/ no. of strains	Mean <i>D</i> value for the serotype			
	<i>Eco</i> RI	<i>Xba</i> I	<i>Eco</i> RI + <i>Pst</i> I	<i>Eco</i> RI + <i>Hae</i> III
1/2*	1.00	—	0.94	0.93
3/3	0.11	0.23	0.28	0.21
	1.00	0.77	0.86	0.93
	0.11	0.55	0.20	0.21
4/3	0.55	0.23	0.58	0.36
	0.82	0.59	0.83	0.87
	0.59	0.44	0.33	0.48
5/3	0.36	0.72	0.47	0.54
	0.44	0.40	0.25	0.44
	0.83	0.83	0.95	0.92
6/3	0.35	0.22	0.31	0.30
	0.90	0.31	0.67	0.92
	0.33	0.19	0.31	0.35
7/3	0.26	0.20	0.26	0.51
	0.67	0.67	0.83	0.90
	0.30	0.14	0.23	0.57
8/2	0.38	0.50	0.48	0.61
14/2	1.00	—	1.00	0.79

* The two strains from serotype 1 were studied with more restriction enzymes. The *D* values for pairwise comparison of these strains were: *Pst*I, 0.93; *Pvu*II, 0.92; *Bgl*II, 0.87; *Bam*HI, 0.75; *Pvu*II + *Hae*III, 0.90; *Pvu*II + *Pst*I, 0.90 (mean value for serotype, 0.88).

Table 4. Similarity among serotypes of *B. thuringiensis*

Pairwise comparisons were made for all possible pairs among the patterns of eight strains, each representing one serotype (Fig. 3*b*).

Serotype	Index of similarity (<i>D</i>)							
	2	3	4	5	6	7	8	14
1	0.40	0.44	0.44	0.50	0.34	0.52	0.50	0.55
2		0.44	0.22	0.20	0.18	0.44	0.25	0.33
3			0.32	0.30	0.49	0.74	0.75	0.40
4				0.55	0.40	0.32	0.27	0.25
5					0.36	0.74	0.42	0.55
6						0.48	0.54	0.20
7							0.71	0.32
8								0.54

provides further evidence that the division of *B. thuringiensis* strains into serotypes does not always adequately reflect their genetic relatedness.

Several factors have played an important role in the genetic divergence of *B. thuringiensis*, such as the presence of many different plasmids in each strain, the conjugation transfer mechanism and the transposon-like inverted repeats flanking the endotoxin genes (Aronson *et al.*, 1986). These considerations could help to explain

the large variations in this species on the basis of a high frequency of DNA rearrangements.

Although the data presented here involve only a few strains from several serotypes, the general conclusion can be made that DNA fingerprinting has a very high resolution, making it possible to differentiate between individual strains of *B. thuringiensis*. The results are encouraging for a future application of hypervariable minisatellite analysis as a rapid and reliable tool for

confirmation of *B. thuringiensis* strain identity on the basis of a unique pattern of the whole genome. This approach, together with other techniques of molecular biology, could contribute to the establishment of a new classification scheme for *B. thuringiensis*. It could also add to the precision of numerical taxonomic analysis of this and other *Bacillus* species.

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