DNA Competition Studies within the *Bacillus cereus* Group of Bacilli

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SUMMARY

The relatedness between strains of bacilli in the *Bacillus cereus* group has been studied by DNA/DNA competition using the membrane filter technique. Strains of *B. cereus*, *B. thuringiensis* and of *B. anthracis* form a closely related group, in which *B. thuringiensis* can be distinguished from *B. cereus*. *Bacillus megaterium* includes a wider range of strains, some of which are closely related to *B. cereus*, *B. thuringiensis* and *B. anthracis*.

INTRODUCTION

In their detailed investigation of the aerobic spore-forming bacteria, Smith, Gordon & Clark (1952) distinguished *Bacillus anthracis* and *B. thuringiensis* from *B. cereus* on the basis of their toxicity to mammals and insects respectively. In *B. thuringiensis* the toxicity has been associated with the formation of a crystalline protein inclusion (Hannay & Fitz-James, 1955; Angus, 1956). The pathogenicity of *B. thuringiensis* to Lepidoptera has led to its commercial use as an agent of biological control (Angus, 1968). Different strains have shown marked differences in their toxicity to test insects, and several investigators have evolved methods for identification of strains of *B. thuringiensis* (de Barjac & Bonnefoi, 1962, 1967; Norris, 1964). Characterization of surface antigens (Norris & Burges, 1965), comparison of biochemical properties, and electrophoretic separation of esterases give results that are in fairly close agreement and point to some ten to twelve subdivisions within the group. These studies were confined to the crystal-forming bacteria which, with *B. anthracis*, were excluded from a recent biochemical comparison of Group I bacilli (Lemille, de Barjac & Bonnefoi, 1969).

The present work was undertaken using DNA/DNA competition to study the relatedness between strains of *Bacillus thuringiensis* and between *B. thuringiensis* and other members of Group I of aerobic spore-formers (Smith *et al.* 1952), in particular *B. cereus* and *B. anthracis*. Some of these results have been communicated to the 3rd meeting of the North West European Microbiological Group (Somerville & Jones, 1971).

METHODS

Organisms. The origins of the strains used are shown in Table 1. No characterization other than morphological examination was carried out for the strains of *Bacillus* apart from *Bacillus thuringiensis*. Both strains of *B. anthracis* were non-pathogenic. Crystals from four strains of *B. thuringiensis* (serotype 1, 2, 3 and 9) were shown to be distinct and all of the strains of this species could be distinguished by disc electrophoresis of solutions of purified crystals in the presence of sodium lauryl sulphate (B. N. Herbert & H. J. Somerville, unpublished). Two strains of *B. megaterium* (SF96 and SF163) were distinguished from others by atypical germination characteristic (J. Wolf, personal communication).

Table 1. *Strains and their sources*

Strain		Source
<i>Bacillus thuringiensis</i> var. <i>berliner</i>	Serotype 1	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>finitimus</i>	Serotype 2	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>alesti</i>	Serotype 3	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>alesti anduze</i>	Serotype 3	E. A. Steinhaus
<i>B. thuringiensis</i> var. <i>kenyae</i>	Serotype 4	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>sotto</i>	Serotype 4	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>galleriae</i>	Serotype 5	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>subtoxicus</i>	Serotype 6	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>entomocidus</i>	Serotype 6	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>aizawa</i>	Serotype 7	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>morrison</i>	Serotype 8	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>tolworth</i>	Serotype 9	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>darmstadiens</i>	Serotype 10	Institut Pasteur, Paris
<i>B. thuringiensis</i> var. <i>thompson</i>	Serotype 11	Institut Pasteur, Paris
<i>B. cereus</i>	203a	Bacteriology Dept., University of California, Los Angeles
<i>B. cereus</i>	T	Unilever, C. Davidson, G. W. Gould
<i>B. cereus</i> var. <i>mycoides</i>		Bacteriology Dept., University of California, Los Angeles
<i>B. cereus</i>	A30	Institut Pasteur, Paris
<i>B. megaterium</i>	KM	Bacteriology Dept., University of California, Berkeley
<i>B. megaterium</i>	SF96	University of Leeds, J. Wolf
<i>B. megaterium</i>	SF163	University of Leeds, J. Wolf
<i>B. megaterium</i>	64a	Bacteriology Dept., University of California, Los Angeles
<i>B. megaterium</i>	8508	National Collection of Industrial Bacteria, Aberdeen, Strain 8508
<i>B. anthracis</i>	A3	Institut Pasteur, Paris
<i>B. anthracis</i>	Sterne	Cutter Laboratories, Berkeley, California
<i>B. licheniformis</i>		Bacteriology Dept., University of California, Berkeley
<i>B. subtilis</i>	SB19	Bacteriology Dept., University of California, Los Angeles, W. R. Romig
<i>B. pumilis</i>	5242	Institut Pasteur, Paris

Growth. Cultures were maintained on slopes of nutrient agar (Oxoid). For the preparation of DNA, organisms were grown on 1% peptone (Oxoid), 0.3% Marmite, 0.5% NaCl, 0.2% glucose (pH 6.6). The medium was prepared in 1 l quantities in 3 l Erlenmeyer flasks and each flask was inoculated with 50 ml of an overnight culture grown on the same medium. The culture was shaken at 30 °C on a rotary shaker at 220 rev./min.

For preparation of radioactive DNA, cultures were grown on 0.4% Casamino acids (Difco), 0.1% glucose, 0.03 M-phosphate buffer (pH 7.0) in modified Hutner's mineral base (Hegeman, 1966). About 30 to 40 min before harvesting 20 µCi [¹⁴C]adenine (Radiochemical Centre, Amersham, Buckinghamshire) were added to the culture.

DNA isolation. Bacteria were harvested by centrifuging when cultures reached an extinction (660 nm) of 1.0 to 1.5. After resuspension in saline-EDTA (0.075 M-NaCl, 0.05 M-EDTA, pH 7.2) to about 10% (wet wt/vol.) the cells were incubated with 1 mg lysozyme (British Drug Houses, Godalming, Surrey)/ml at 37 °C for 30 min. Sodium lauryl sulphate was then added to 1% (w/v) and the mixture heated at 60 °C for 5 to 10 min (Marmur, 1961). The rest of the isolation procedure was that of Marmur (1961) as used by Ballard, Palleroni, Doudoroff, Stanier & Mandel (1970), with the omission of the treatment with pronase. The

mixture with detergent was cooled on ice, one-half vol. liquified phenol added, and the mixture shaken for 10 min. After centrifuging, the aqueous layer was removed and the phenol layer was shaken with approximately 2 vol. of saline-EDTA for 10 min. After shaking and centrifuging, the aqueous layers were pooled and re-extracted with phenol. The aqueous phase was then precipitated with 2 vol. of absolute ethanol and the precipitate collected on a glass rod. The DNA was dissolved in one-tenth strength SSC (SSC is 0.15 M-NaCl, 0.01 M-Na citrate, pH 7.0) and dialysed against one-tenth strength SSC for 15 h at 4 °C. The solution was then incubated at 37 °C for 2 h with 50 µg ribonuclease (A grade, bovine pancreatic, Calbiochem, La Jolla, California, U.S.A.)/ml. The salt concentration was then raised to one SSC and the DNA deproteinized by repeated extraction with chloroform, isoamyl alcohol (24:1, v/v) (Marmur, 1961). The DNA was then reprecipitated with ethanol as above, and dissolved in one-tenth strength SSC. All preparations, excepting radioactive ones, were precipitated with isopropanol (Marmur, 1961). The DNA was stored at 4 °C in the presence of chloroform.

All DNA preparations had a ratio of extinction (260 nm:280 nm) of greater than 1.8; the concentration was estimated from the extinction at 260 nm.

Sheared DNA of mol. wt approx. 500000 was prepared by passage through a French pressure cell at 10000 to 15000 lb/in² (McCarthy & Bolton, 1963). Sheared DNA was stored at -20 °C.

DNA/DNA hybridization. The methods used were those developed by Johnson & Ordal (1968) as used by Palleroni and co-workers (Ballard *et al.* 1970; Palleroni, Doudoroff, Stanier, Solánes & Mandel, 1970). DNA (50 µg/ml, 87 ml) was denatured by boiling for 10 min in one-tenth strength SSC and was quickly cooled by diluting to 5 µg/ml with cooled six-times strength SSC. The solution was passed slowly (60 ml/min) through a 15 cm Millipore filter. When the denatured DNA had passed through, 30 ml of 0.02 % (w/v) bovine serum albumen (Armour Pharmaceutical Co, Eastbourne, Sussex), 0.01 % (w/v) polyvinylpyrrolidone (Calbiochem), 0.02 % (w/v) Ficoll (Pharmacia, Uppsala, Sweden) in 2 × SSC were added and, after standing for 10 min, the filter was sucked dry. After drying in air overnight small filters (10.5 mm diam.) were punched from the larger filter.

For DNA hybridization studies vials (12 mm × 50 mm) were used. To these were added the sheared radioactive DNA (0.05 ml one-tenth strength SSC containing about 1 µg DNA) and the sheared competitor DNA (0.15 ml one-tenth strength SSC containing about 150 µg DNA). The tubes were stoppered and heated in a boiling water bath for 10 min then cooled quickly on ice. To each tube 0.05 ml 9.6 strength SSC was added followed by a 10.5 mm filter carrying unsheared reference DNA. Incubations were carried out for 12 or 16 h and at 61 ± 1 °C.

Results are expressed as competition (McCarthy & Bolton, 1963) defined (Ballard *et al.* 1970) as % competition = $(X - Y)/(X - Z) \times 100$, where X = radioactivity bound to filter in absence of competitor, Y = radioactivity bound in presence of heterologous competitor, Z = radioactivity bound with homologous competitor. Each determination was the average of two duplicates. Duplicate determinations were within 10 % of the full scale of competition in all results quoted.

RESULTS

In general, for the 12 to 16 h incubation some 20 to 35 % of added radioactive DNA was bound to the homologous DNA on the filter in the absence of competitor. The amount of DNA retained on the filter was not estimated. The radioactivity bound in the presence of homologous competitor was about 40 to 50 % of that bound in the absence of competitor.

Table 2. Competition between DNA preparations from reference strains of bacilli

Competitor strain	Competition (%)										
	<i>Bacillus thuringiensis</i>						<i>B. cereus</i>				
							<i>B. cereus</i>				
	Serotype 1	Serotype 2	Serotype 3	Serotype 4	Serotype 7	Serotype 11	T	<i>B. cereus</i> 203a	<i>B. megaterium</i> sf96	<i>B. megaterium</i> 64a	<i>B. anthracis</i> A3
Reference strain											
<i>Bacillus thuringiensis</i>											
Serotype 1	100	46	73	90	91	—	78	58	—	44	66
Serotype 2	65	100	—	—	57	66	53	64	—	—	79
Serotype 3	78	76	100	76	84	—	—	—	—	40	60
Serotype 4	79	72	78	100	93	—	69	64	20	60	76
Serotype 7	71	57	59	76	100	—	45	32	—	45	68
Serotype 11	88	70	64	96	80	100	75	57	—	79	84
<i>B. cereus</i> T	76	69	66	89	88	70	100	81	—	78	—
<i>B. cereus</i> 203a	—	53	48	54	—	—	—	100	—	—	56
<i>B. megaterium</i> sf96	50	23	—	—	—	—	57	23	100	13	36
<i>B. megaterium</i> 64a	—	—	47	—	—	—	75	64	49	100	47
<i>B. anthracis</i> A3	65, 73	88	67, 76	—	67, 87	—	—	60	48	42	100

Table 3. DNA/DNA competition within *Bacillus thuringiensis* group

Reference strain	No. of competitor <i>Bacillus thuringiensis</i> strains	Competition (%)
Serotype 1	7	88 ± 7
Serotype 2	2	71
Serotype 3	13	84 ± 9
Serotype 4	13	85 ± 8
Serotype 7	8	72 ± 9
Serotype 7*	13	68 ± 10
Serotype 11	10	86 ± 9
Serotype 11†	9	80 ± 10

*† Different preparations of reference DNA used.

Table 4. DNA/DNA competition of *Bacillus thuringiensis* strains with *Bacillus thuringiensis* reference strains

Reference strains: serotypes 1, 2, 3, 4, 7, 11.

Strain	No. of determinations	Competition (%)
Serotype 1	7	64 ± 12
Serotype 2	10	76 ± 12
Serotype 3	7	70 ± 8
Serotype 4	10	84 ± 10
Serotype 5	8	83 ± 12
Serotype 6	11	85 ± 10
Serotype 7	6	83 ± 12
Serotype 8	7	83 ± 12
Serotype 9	8	73 ± 12
Serotype 10	5	75 ± 7

In every case, the greatest competition, set at 100% by the formula used, was observed with homologous competitor. The reciprocal competitions, which can be determined from the values in Table 2, show a difference of up to 40%.

Results of competitions between DNA preparations from *Bacillus thuringiensis* strains are summarized in Tables 3 and 4. The reference strains showed a mean competition with other strains in the range 68 to 88% (Table 3). In Table 4 the mean competition of each strain with different reference strains is shown. In each case competition was measured with at least four of the reference strains listed in Table 1. In this case the mean varied over a similar range (65 to 85%) and examination of the results suggests that differences over this range are not significant; for example, serotype 1 shows a mean competition of 88% when used as reference strain (Table 3) and of 65% when tested as competitor (Table 4).

Although these results indicate that the average competition varied from reference strain to reference strain, results of individual experiments, which are not shown, did not consistently indicate a spectrum of relatedness.

The results of competition of DNA from other bacilli with *Bacillus thuringiensis* reference strains are shown in Table 5. With DNA from the *B. anthracis* strains, competition was similar to that shown by strains of *B. thuringiensis*, whereas with *B. cereus* strains competition was lower and was consistently so in each of the strains studied. With *B. subtilis*, *B. licheniformis* and *B. pumilis* no significant competition was observed. With *B. megaterium* two strains (64a and KM) showed competition similar to that of *B. cereus* whereas the other strains (SF96, SF163 and 8508) did not show significant competition.

Table 5. *DNA/DNA competition with Bacillus thuringiensis reference strains*

Reference strains: serotypes 1, 2, 3, 4, 7, 11.

Organism	No. of determinations	Competition (%)		
		Mean	Confidence limits (95 %)	
			Upper	Lower
<i>Bacillus thuringiensis</i>	76	78	82	75
<i>B. cereus</i> (all strains)	29	58	65	51
<i>B. cereus</i> 203a	8	56	68	43
<i>B. cereus</i> T	5	62	75	49
<i>B. cereus</i> var. <i>mycoides</i>	9	55	65	45
<i>B. cereus</i> A30	7	63	80	45
<i>B. megaterium</i> (64a, KM)	6	63	73	53
<i>B. megaterium</i> (others)	3	13	—	—
<i>B. anthracis</i> (A3, Sterne)	14	77	83	70
<i>B. subtilis</i> SB19	6	14	21	7
<i>B. licheniformis</i>	4	14	—	—
<i>B. pumilis</i> 5242	1	0	—	—

Table 6. *DNA/DNA competition with Bacillus cereus, Bacillus anthracis and Bacillus megaterium as reference strains*

Reference strain	...	...	Competition (%)			
			<i>Bacillus anthracis</i> A3	<i>B. cereus</i> T	<i>B. megaterium</i> SF96	<i>B. megaterium</i> 64a
Competitor strain						
<i>Bacillus thuringiensis</i> S-1			—	74	—	84
<i>B. thuringiensis</i> S-2			88	69	23	—
<i>B. thuringiensis</i> S-3			76	67	—	—
<i>B. thuringiensis</i> var. <i>kenyae</i> S-4			57	90	—	—
<i>B. thuringiensis</i> var. <i>sotto</i> S-4			92	80	—	—
<i>B. thuringiensis</i> S-6			93	—	—	—
<i>B. thuringiensis</i> S-7			87	—	—	—
<i>B. thuringiensis</i> S-8			—	94	—	—
<i>B. thuringiensis</i> S-9			92	91	16	—
<i>B. thuringiensis</i> S-10			65	65	—	—
<i>B. thuringiensis</i> S-11			—	70	—	—
<i>B. cereus</i> 203a			81	82	23	69
<i>B. cereus</i> T			—	100	57	70
<i>B. cereus</i> var. <i>mycoides</i>			74	80	—	—
<i>B. cereus</i> A30			79	79	—	—
<i>B. megaterium</i> KM			31	—	17	55
<i>B. megaterium</i> 64a			42	78	13	100
<i>B. megaterium</i> SF96			—	—	100	45
<i>B. megaterium</i> SF163			48	—	78	40
<i>B. megaterium</i> 8508			—	16	—	41
<i>B. anthracis</i> A3			100	94	36	47
<i>B. anthracis</i> Sterne			100	56	19	—
<i>B. licheniformis</i>			—	—	13	26
<i>B. subtilis</i> SB19			0	13	32	31
<i>B. pumilis</i> 5242			—	—	3	16

The results of competition with strains other than *Bacillus thuringiensis* are shown in Table 6. With *B. cereus* T as reference strain no clear distinction was evident between *B. cereus*, *B. anthracis* and *B. thuringiensis*; although the two *B. anthracis* strains differed widely (56 and 94% competition), distinction between these strains on this basis would require more extensive experiments. The two *B. megaterium* strains tested (8508 and 64a) were again clearly different. *Bacillus anthracis* Sterne showed 100% competition with *B. anthracis* A3 as reference and was the only strain to do so; here the three *B. megaterium* strains tested were in a relatively narrow range (31 to 48%). With *B. megaterium* 64a as reference and four strains of *B. cereus*, *B. thuringiensis* and *B. anthracis* as competitors, average competition was 68% and the range 47 to 85%. With *B. megaterium* SF96 as reference a similar comparison with six strains gave an average of 29% competition (range 16 to 57%).

DISCUSSION

The results of the DNA/DNA competition experiments reported must be regarded with some caution as it is accepted that variation in experimental values can arise from several factors, including the presence of impurities in DNA preparations. Using similar methods Johnson & Ordal (1968) reported a $\pm 12\%$ variation in the DNA bound per filter. With the present strains we have found that there was variation also in the total amount of DNA retained on the large filter with 20 to 50% of the denatured DNA passing through with different DNA preparations from the same strain. With different preparations of DNA from several of the strains used results of hybridizations were reproducible to within 5 to 10% of the full scale of competition. However, wide divergence was observed in reciprocal competitions (Table 2). Such variations have been observed by other workers and, although no full explanation has been forthcoming, variations in hybridization have been attributed to the presence of impurities in the DNA, and the variable loss of immobilized DNA from the filter during hybridization (De Ley & Tijtgat, 1970; Palleroni & Doudoroff, 1971). Identity of reciprocal values is not necessary since the total complement of DNA may vary from one organism to another (McCarthy & Bolton, 1963).

The binding of DNA has been shown to depend on temperature, with non-specific binding occurring at temperatures more than 25 °C below the melting temperature of the DNA (Marmur & Doty, 1961; Johnson & Ordal, 1968). Although no T_m determinations were carried out in this work, guanine plus cytosine (G/C) contents in the range 32 to 37% have been reported for DNA from *Bacillus thuringiensis*, *B. anthracis* and *B. cereus*, and about 41 to 47% for *B. subtilis*. *Bacillus subtilis* 168, from which *B. subtilis* SB19 is derived (Nishihara & Romig, 1964), has reported G/C contents of 42 and 43% (for compilations of data on G/C contents see Hill, 1966; Normore & Brown, 1970; Shapiro, 1970). These values indicate a clear distinction between *B. subtilis* and the *B. cereus* group and suggest that 61 °C is close to the optimum temperature for the reference strains used in this study. Marmur & Doty (1962) demonstrated the relationship between base composition and T_m and reported the following relevant G/C contents and T_m values: *B. cereus* (33% G/C, T_m 83 °C), *B. megaterium* (37% G/C, T_m 85 °C), *B. thuringiensis* (34% G/C, T_m 83.6 °C) and *B. subtilis* 168 (43% G/C, T_m 87.5 °C). In the present work, a few of the reported hybridizations were repeated at both 55 °C and 68 °C without altering the pattern of relatedness.

Despite the above reservations and the limited number of strains studied some conclusions can be drawn. All the strains examined from the large-cell subgroup of Group I of the genus *Bacillus* (Smith *et al.* 1952) showed significant competition with other members of the group. The crystal-forming bacteria form a closely related group in which no clear spectrum or

subdivision is evident from the present results. Of particular interest is that serotype 2, formerly known as *Bacillus finitimus* (Heimpel, 1967) appears to be a typical member of the group. This strain differs from the others in that crystal formation occurs inside, rather than outside, the exosporium (Somerville, James, Ruffell & Norris, 1971). The group of crystal-forming bacteria appears to be distinct from *B. cereus* on the basis of the accumulated results with *B. thuringiensis* reference strains. Although the two strains of *B. anthracis* were non-pathogenic there is little doubt of their identity and the results both with *B. anthracis* A3 reference strain and with the *B. anthracis* strains as competitors suggest a closer relationship with *B. thuringiensis* than with *B. cereus*.

The limited results with *Bacillus megaterium* indicate that this group includes strains with a much wider range of relatedness and it seems possible that some (typified by 64a and KM in the present study) may be closer to *B. thuringiensis* and *B. cereus* than to other strains of *B. megaterium*. The results with *B. thuringiensis* reference strains clearly indicate two groups within *B. megaterium* consisting of strains SF96, SF163 and 8508 which are not so closely related to *B. thuringiensis* as strains 64a and KM. The *B. cereus* group appears not to be closely related to other members of Group I of the genus *Bacillus*. This is not surprising in view of the knowledge that members of this genus are united largely by their ability to form spores, a property which may not reflect a large proportion of the chromosome.

Previous results from nucleic acid hybridization studies with *Bacillus* species are in agreement with the present findings. Doi & Igarashi (1965) studied the direct binding of *Bacillus subtilis* ribosomal RNA and messenger RNA fractions from both *B. subtilis* and *B. cereus* to DNA from various bacilli. Whereas *B. subtilis* ribosomal RNA bound to heterologous DNA at about 50% of the level of bindings to homologous DNA, messenger RNA did not bind extensively to heterologous DNA. Similar results were obtained with messenger RNA preparations from log phase and sporulating cells. Takahashi, Saito & Ikeda (1966) studied the binding of labelled *B. subtilis* DNA to DNA of different bacilli using the DNA agar method. Compared with homologous bindings some 0 to 20% binding was observed with strains of *B. cereus* and *B. megaterium*, with one *B. megaterium* strain showing 30 to 50% hybridization.

The present results and those of Takahashi *et al.* (1966) suggest that a more extensive study of the *Bacillus megaterium* group would be invaluable and might indicate where overlap with the *B. subtilis* group occurs.

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