Association of the Encapsulation of *Bacillus anthracis* with a 60 Megadalton Plasmid

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Virulent typical strains (Shikan, Morioka, Shizuoka) and Pasteur vaccine strains (no. 1, no. 2-H, no. 2-17JB) of *Bacillus anthracis* harboured two plasmid species with molecular masses of 110 MDal and 60 MDal. All of the 110 MDal plasmids isolated from the various strains showed indistinguishable patterns of digestion with restriction endonucleases. All the 60 MDal plasmids were also indistinguishable. Strain Davis, which is encapsulated but is asporogenous and avirulent, harboured only the 60 MDal plasmid while three non-encapsulated vaccine strains (34F2, Smith, Mukteswer) harboured only the 110 MDal plasmid. Four non-encapsulated variant strains obtained from the encapsulated strains Shikan, Pasteur no. 1, Pasteur no. 2-17JB and Davis had lost the 60 MDal plasmid, suggesting that encapsulation of *B. anthracis* may be associated with the 60 MDal plasmid.

INTRODUCTION

Bacillus anthracis is normally pathogenic, mainly to herbivora and man, but occasionally to other animals. Encapsulation is considered to be one of the factors that determines virulence of *B. anthracis*, because the capsule plays an important role in protecting the organism from phagocytosis and lytic antibodies (Preis, 1909). On serum agar in the presence of excess CO₂, virulent strains form smooth mucoid colonies consisting of fully virulent encapsulated organisms (Chu, 1952; Sterne, 1937*a, b*). On further incubation, however, rough outgrowth often occurs, subcultures from which give rough and non-mucoid colonies that consist of non-encapsulated bacilli. Non-encapsulated bacilli are uniformly avirulent, and reversion to the virulent encapsulated type has not been observed (Chu, 1952; Sterne, 1937*a, b*). Although the genetic mechanism of this variation is not clear, the so-called Sterne's strain, which is non-encapsulated but has immunizing activity, has been used as a live vaccine for anthrax.

There is now clear evidence that some of the virulence factors of bacteria are mediated by plasmids (Elwell & Shipley, 1980). Mikesell *et al.* (1983) recently reported that *B. anthracis* harbours a large plasmid conferring the production of the toxins. Furthermore, Vodkin & Leppla (1983) proved that this plasmid codes for the protective antigen, which is one of the anthrax toxins. In the course of our studies, we found that *B. anthracis* harbours two plasmids, with molecular masses of 110 MDal and 60 MDal. This paper deals with the distribution of these plasmids in *B. anthracis* strains and the association of capsule formation with the 60 MDal plasmid.

METHODS

Bacterial strains and growth conditions. The *B. anthracis* strains used are listed in Table 1. Bacterial cultures were grown at 37 °C in Penassay broth (Difco) or on nutrient agar.

Plasmid isolation. Plasmid DNA was isolated by the method of Mikesell *et al.* (1983) except that bacterial lysates were deproteinized by extracting twice with phenol/chloroform as described by Kado & Liu (1981). An equal volume of phenol/chloroform (1:1, v/v) was added to the bacterial lysate, and the solution was emulsified by shaking briefly. The partially purified DNA was subjected to electrophoretic analysis for detection of plasmids.

Table 1. *Strains of Bacillus anthracis*

Strain	Relevant characteristics*	Reference or source†
Shikan	C ⁺ , SM, Virulent	Ando <i>et al.</i> (1961a, b)
Morioka	C ⁺ , SM, Virulent	Ando <i>et al.</i> (1961a)
Shizuoka	C ⁺ , SM, Virulent	Ando <i>et al.</i> (1961a)
Pasteur no. 1	C ⁺ , SM, Attenuated	Ando <i>et al.</i> (1961a)
Pasteur no. 2-H	C ⁺ , SM, Attenuated	Ando <i>et al.</i> (1961a, b)
Pasteur no. 2-17JB	C ⁺ , SM, Attenuated	CVL
Davis	C ⁺ , SM, Attenuated, Spo ⁻	Ando <i>et al.</i> (1961a)
34F2	C ⁻ , R, Attenuated	Ando <i>et al.</i> (1961a), CVL
Smith	C ⁻ , R, Attenuated	Ando <i>et al.</i> (1961a)
Mukteswer	C ⁻ , R, Attenuated	Ando <i>et al.</i> (1961a)

* C⁺, Encapsulated; C⁻, non-encapsulated; SM or R, smooth or rough colony when cultivated on serum agar in the presence of CO₂; Spo⁻, asporogenous.

† CVL, Central Veterinary Laboratory, Weybridge, UK.

For further purification, the plasmid DNA was recovered by means of caesium chloride/ethidium bromide density gradient centrifugation as described previously (Ohmae *et al.*, 1981).

Restriction endonuclease digestion. The restriction endonucleases *Bam*HI and *Pst*I were obtained from Takara Shuzo Co., Kyoto, Japan. Plasmid DNA was digested as recommended by the supplier. The sizes of DNA fragments produced after digestion were estimated from their mobilities relative to *Hind*III-digested phage λ DNA fragments.

Analytical gel electrophoresis. Gel electrophoresis was performed by the procedure of Meyers *et al.* (1976). The sample was electrophoresed through a horizontal or vertical 0.8% (w/v) agarose gel at 100 V for 5 h, using Tris/borate buffer (89 mM-Tris, 2.5 mM-EDTA, 8.0 mM-boric acid). The gels were stained for 15 min with ethidium bromide (0.5 μ g ml⁻¹) and then washed for 15 min in distilled water.

Electron microscopical analysis. A modified formamide technique was used (Davis *et al.*, 1971). Plasmid samples were spread from a hyperphase, containing 0.1 μ g DNA ml⁻¹ in 60 mM-Tris/HCl (pH 8.5), 6 mM-EDTA, 50% (v/v) formamide and 60 μ g cytochrome *c* ml⁻¹, onto a hypophase of 18% (v/v) formamide in 10 mM-Tris/HCl (pH 8.5) and 1 mM-EDTA. The cytochrome *c* layer with DNA was picked up on a Parlodion-coated grid, and was stained with uranyl acetate and shadowed with platinum/palladium (80:20). The mean contour length of open circular plasmid molecules was compared with that of plasmid RP4 (36.0 MDal).

Production of non-encapsulated variants. This was done by the method of Chu (1952). The bacterial cultures were grown on a nutrient agar containing 10% (v/v) horse serum, and incubated in 20% (v/v) CO₂ at 37 °C for several days. The rough irregular outgrowths that appeared around the smooth mucoid colonies were picked and purified. Capsule formation was determined microscopically after staining a heat-fixed smear with methylene blue solution for a few seconds (Doetsh, 1981).

RESULTS

Plasmid DNA contents of *B. anthracis* strains. Fig. 1 shows the plasmid contents of various strains of *B. anthracis* as revealed by electrophoretic analysis. Two plasmid species were detected in the three typical strains (Shikan, Morioka, Shizuoka) and the three Pasteur vaccine strains (no. 1, no. 2-H, no. 2-17JB). Strain Davis, which is avirulent and asporogenous but is encapsulated, harboured only the smaller plasmid, while the three non-encapsulated vaccine strains (34F2, Smith, Mukteswer) harboured only the larger plasmid. The molecular masses of these plasmids were estimated to be 110.2 ± 4.9 MDal (SD; 14 molecules measured) and 60.3 ± 2.4 MDal (12 molecules measured) from their contour lengths in the electron microscope.

Plasmid DNA contents of non-encapsulated variants. Non-encapsulated variants were obtained from the encapsulated strains Shikan, Pasteur no. 1, Pasteur no. 2-17JB and Davis by the method of Chu (1952) and were examined, for the presence of plasmids, using electrophoretic analysis. As shown in Fig. 1, all the strains had lost the 60 MDal plasmid.

Restriction endonuclease analysis of *B. anthracis* plasmids. The restriction endonuclease digestion patterns of the 110 MDal and the 60 MDal plasmids were different (Fig. 2). However, the cleavage patterns of the 110 MDal plasmids from the non-encapsulated vaccine strain 34F2 and from the non-encapsulated variant derived from strain 17JB, which had lost the 60 MDal

Fig. 1. Agarose gel electrophoresis of plasmid DNA isolated from various strains of *B. anthracis*. Partially purified plasmid DNA samples were subjected to electrophoresis through a vertical 0.8% agarose slab gel at 100 V for 5 h. The strains used were: Shikan (A), Morioka (B), Shizuoka (C), Pasteur no. 1 (D), Pasteur no. 2-H (E), Pasteur no. 2-17JB (F), Davis (G), 34F2 (H), Smith (I), Mukteswer (J), and non-encapsulated variants of Davis (K), Shikan (L), Pasteur no. 1 (M) and Pasteur no. 2-17JB (N). The dense bands are chromosomal DNA (Chr.).

Fig. 2. Comparison of the restriction cleavage patterns of plasmid DNA isolated from various strains of *B. anthracis*. Purified plasmid DNA was subjected to restriction endonuclease digestion and electrophoresis through a horizontal 0.8% agarose slab gel at 100 V for 5 h. Strains: (A) Davis (60 MDal plasmid), (B) 34F2 (110 MDal plasmid), (C) non-encapsulated variant of strain 17JB (110 MDal plasmid), (D) mixture of Davis and 34F2 (60 MDal and 110 MDal plasmids), (E) 17JB (60 MDal and 110 MDal plasmids), (F) Shikan (60 MDal and 110 MDal plasmids). Phage λ DNA digested with *Hind*III was run in parallel as a size marker (G). Partial digestion products are seen in lanes *Pst*I-A and *Pst*I-B.

plasmid, were indistinguishable. The 110 MDal plasmid yielded at least nine *Bam*HI cleavage fragments (> 15.8, > 15.8, 15.8, 10.6, 6.0, 5.2, 4.9, 4.3, 2.5 MDal) and 15 *Pst*I cleavage fragments (15.5, 14.7, 11.3, 9.7, 9.3, 8.0, 5.0, 4.7, 4.6, 4.5, 4.0, 3.3, 2.8, 2.3, 2.0 MDal). The restriction cleavage pattern of a mixed sample of 34F2 (110 MDal) and Davis (60 MDal) plasmids was the same as those of 17JB (110 MDal and 60 MDal) and Shikan (110 MDal and 60 MDal), suggesting that the 60 MDal plasmids present in various strains are also identical. The 60 MDal plasmid yielded at least five *Bam*HI cleavage fragments (> 15.8, 13.3, 8.0, 1.2, 0.4 MDal) and five *Pst*I cleavage fragments (> 15.8, 8.7, 4.2, 2.8, 1.9 MDal).

DISCUSSION

The present study has shown that most of the strains of *B. anthracis* that we examined contained two plasmid species with molecular masses of 110 MDal and 60 MDal. To isolate the plasmid DNA from *B. anthracis*, we deproteinized bacterial lysates twice with phenol/chloroform; plasmids could only rarely be isolated without this deproteinization step. The restriction endonuclease digestion patterns of the 110 MDal and the 60 MDal plasmids were quite different, indicating that the 110 MDal plasmid is not a dimer of the 60 MDal plasmid. The various 110 MDal plasmids examined were indistinguishable from each other, regardless of their origin, which suggests that they share a common ancestry. Similar results were obtained with the various 60 MDal plasmids, suggesting that these plasmids also share a common ancestry.

Restriction analysis also showed that the 110 MDal plasmids obtained in this study are very similar to plasmid pBA1 which encodes the production of the protective antigen of *B. anthracis* (Vodkin & Leppla, 1983). Mikesell *et al.* (1983) reported that the 110 MDal plasmids, including pBA1, could be cured by serial passages at high temperature (42.5 °C); hence, Pasteur vaccine strains which had been attenuated by repeated subculture at an elevated temperature contained no detectable plasmids. We found that the 110 MDal plasmids of two strains (Shikan, 34F2) could be eliminated by serial passages at 42.5 °C (data not shown), suggesting that the 110 MDal plasmids of these strains may be temperature-sensitive, as reported by Mikesell *et al.* (1983). However, the three Pasteur vaccine strains (no. 1, no. 2-H, no. 2-17JB) examined in this study still harboured two plasmid species of 110 MDal and 60 MDal. Although we have no evidence to explain this discrepancy, it is possible that the 110 MDal plasmid might have been integrated into the host chromosome and that excision then occurred after many subcultures in the laboratory.

With regard to the 60 MDal plasmid in *B. anthracis*, Mikesell *et al.* (1983) have found a plasmid of this size in strain Vollum IB by electron microscopic analysis. However, the presence of 60 MDal plasmids in other strains has not previously been noted. In the present study, we consistently demonstrated the presence of the 60 MDal plasmid in encapsulated *B. anthracis* strains, including virulent and attenuated strains. In contrast, this plasmid was not present in the non-encapsulated strains 34F2, Smith and Mukteswer, which have been used as live vaccine strains in various countries. Moreover, it was found that the non-encapsulated variant strains obtained in this study from the encapsulated strains Shikan, Pasteur no. 1, Pasteur no. 2-17JB and Davis had lost the 60 MDal plasmid. These results strongly suggest that encapsulation of *B. anthracis* is mediated by the 60 MDal plasmid. The lack of a direct selection procedure, however, makes it difficult to introduce the 60 MDal plasmid into non-encapsulated variant strains of *B. anthracis*. Further study is now in progress to confirm the function of the 60 MDal plasmid in *B. anthracis*.

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