Plasmids of Crystal-forming Bacilli and the Influence of Growth Medium Composition on their Appearance

By L. M. ERMAKOVA, F. P. GALUSHKA, A. YA. STRONGIN,
I. A. SLADKOVA, B. A. REBENTISH, M. V. ANDREEVA AND
V. M. STEPANOV

*Institute of Genetics and Selection of Industrial Microorganisms, P.O. Box 825,
113545 Moscow, U.S.S.R.*

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INTRODUCTION

A number of bacilli have been reported to contain plasmids, e.g. *Bacillus megaterium* (Carlton, 1976), *B. pumilus* (Lovett & Bramucci, 1975), *B. subtilis* (Tanaka, Kuroda & Sakaguchi, 1977; Tanaka & Koshikawa, 1977) and *B. thuringiensis* (Debabov *et al.*, 1977), but the functional role of these plasmids has not been clearly established. Ermakova *et al.* (1977) have found that *B. subtilis* mutant 1000M, derived from strain A-50, can form proteinaceous crystalline inclusions at a frequency of 1 to 2 %. This mutant also has a higher sporulation ability and reduced levels of intra- and extracellular serine protease activity compared with its parent strain. Looking more closely into this phenomenon, we have now found that mutant 1000M carries plasmids, the quantity of plasmid material isolated being dependent on the composition of the growth medium. Comparable data were also found for typical crystal-forming bacteria (*B. thuringiensis*).

METHODS

Strains and media. *Bacillus subtilis* mutant 1000M, derived from strain A-50, was grown in solid and liquid nutrient media (NM) [containing (per litre) 150 ml Hottinger hydrolysate (pancreatic hydrolysate of meat), 70 ml barley malt wort, 0.5 g yeast extract, 10 g NaCl and 5 g glucose] or in minimal Spizizen salts medium (SM; Spizizen, 1958). *Bacillus thuringiensis* var. *galleriae* strain 612 was grown in solid and liquid nutrient media (NM_L) [containing (per litre) 150 ml Hottinger hydrolysate, 0.5 g yeast extract, 0.5 g Bacto-tryptone, 10 g NaCl and 5 mg L-tryptophan] or in minimal salts media SM and BM, supplemented with 0.2 % (w/v) sodium citrate (Nickerson & Bulla, 1974).

Medium-shift experiments. Bacteria subcultured in Hottinger broth [Hottinger hydrolysate, diluted sixfold, containing 0.5 % (w/v) NaCl, pH 7.2 to 7.4] until the end of the exponential growth phase (1×10^8 to 2.5×10^8 cells ml⁻¹) were harvested by centrifugation, carefully washed and resuspended in the same volume of SM or BM. They were incubated for 5 to 6 h at 37 °C and then the bacteria were collected. One portion was used immediately for plasmid isolation; an equal portion was transferred to NM or NM_L, incubated for 2 h at 37 °C (for 1 to 2 generations) and then used for plasmid screening. Samples of both cultures were incubated in the corresponding media for 48 to 72 h at 28 °C in order to visualize spores and proteinaceous crystals. The appearance of crystals and spores was verified by light microscopy. Crystals were stained with Amido black according to Smirnov (1962).

Plasmid DNA. Plasmid DNA was isolated from the cell lysates by ethidium bromide-caesium chloride centrifugation, according to a modification of the procedure of Clewell & Helinski (1969). The sensitivity of plasmid DNA detection was 0.3 µg DNA per tube after photography in ultraviolet light. For electron microscopy, plasmid DNA was prepared according to Davis, Simon & Davidson (1971).

Protease activity. Intracellular serine protease activity was measured in cell extracts using the method of Stepanov *et al.* (1977).

Fig. 1. Electron micrographs of *B. subtilis* 1000M plasmids with molecular weights of (a) 1.6×10^6 , (b) 3.2×10^6 , (c) 4.7×10^6 and (d) 8.7×10^6 . Bar marker represents $0.5 \mu\text{m}$.

Table 1. *Some properties of B. subtilis* 1000M and *B. thuringiensis* var. *galleriae* 612 grown in complex and minimal media

Strain	Growth medium	Spores	Crystals	Plasmids	Protease activity*
<i>B. subtilis</i> 1000M	NM	+	+	+	3.5
	SM	+	—	—	11.0
<i>B. thuringiensis</i> 612	NM ₁	+	+	+	0.25
	BM	+	±†	—	0.70

* Intracellular serine protease activity expressed in units per g wet cells.

† Crystals were formed at a frequency of about 0.01 %.

RESULTS AND DISCUSSION

Electron microscopy revealed the presence of four types of plasmids in *B. subtilis* 1000M grown in NM (Fig. 1). These had molecular weights of 1.6×10^6 , 3.2×10^6 , 4.7×10^6 and 8.7×10^6 (ColEI DNA served as a reference). Ethidium bromide-agarose gel electrophoresis also revealed four types of plasmids. The total amount of plasmid DNA was about 1 % of that of chromosomal DNA. All the plasmids were resistant to restriction endonuclease *Bam*HI treatment but were specifically cleaved by *Eco*RI and *Sal*I. The number of plasmid copies did not exceed a few per cell.

Plasmid DNA could be detected in *B. subtilis* 1000M cells after 13 to 36 h growth in NM. These cells could also sporulate, they formed crystals (at a frequency of 1 to 2 %) and they had a reduced level of intracellular serine protease activity (Table 1). No crystals or plasmid DNA were found in *B. subtilis* cells grown in SM, but these had two- to threefold higher protease activities. The transfer of cells grown in NM into SM resulted in an increase in protease activity and the disappearance of the plasmid DNA fraction.

Bacillus thuringiensis strain 612 contains plasmids with molecular weights of 5.9×10^6 , 10.0×10^6 and 10.9×10^6 when grown in NM₁, as has been found for strain 351 (Debabov *et al.*, 1977). Crystals appeared at a frequency of at least 50 % under these conditions. However, in cells grown in solid or liquid SM or BM, no extrachromosomal DNA was found, and crystal formation was negligible (0.001 to 0.1 %). The transfer of *B. thuringiensis* from NM₁ into SM or BM resulted in an increased protease activity, a reduced ability to

form crystals and the disappearance of plasmid DNA. The reverse transfer from BM or SM into NM₁ led to the appearance of the plasmid DNA fraction.

The results obtained suggest there may be a correlation between the presence of plasmid DNA, the formation of crystals and the level of intracellular protease activity in *B. subtilis* 1000M and in *B. thuringiensis* 612. The medium-shift experiments support the idea that the *Bacillus* plasmid DNA may have a chromosomal origin, perhaps resulting from specific excision and amplification of certain chromosomal DNA segments. Previously reported experiments with *B. megaterium* plasmids (Carlton, 1976) are consistent with this explanation. The existence of the two patterns of organization of genetic material in the strains studied could be attributable to a mechanism of adaptation to the eventual changes in the environment characteristic for at least *B. thuringiensis* – a species occurring in two ecological niches.

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