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

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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) [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 (1x10⁸ to 2.5x10⁹ 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 NMₐ, 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 Smirnoff (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 \textit{B. subtilis} 1000\textsuperscript{M} plasmids with molecular weights of (a) $1.6 \times 10^6$, (b) $3.2 \times 10^6$, (c) $4.7 \times 10^6$ and (d) $8.7 \times 10^6$. Bar marker represents $0.5 \mu m$.

Table 1. \textit{Some properties of B. subtilis 1000\textsuperscript{M} and B. thuringiensis var. galleriae 612 grown in complex and minimal media}.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>Spores</th>
<th>Crystals</th>
<th>Plasmids</th>
<th>Protease activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. subtilis} 1000\textsuperscript{M}</td>
<td>NM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>SM</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>11.0</td>
</tr>
<tr>
<td>\textit{B. thuringiensis} 612</td>
<td>NM\textsubscript{1}</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>+</td>
<td>±\dagger</td>
<td>-</td>
<td>0.70</td>
</tr>
</tbody>
</table>

* 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 \textit{B. subtilis} 1000\textsuperscript{M} grown in NM (Fig. 1). These had molecular weights of $1.6 \times 10^6$, $3.2 \times 10^6$, $4.7 \times 10^6$ and $8.7 \times 10^6$ (ColEl 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 \textit{BamHI} treatment but were specifically cleaved by \textit{EcoRI} and \textit{SalI}. The number of plasmid copies did not exceed a few per cell.

Plasmid DNA could be detected in \textit{B. subtilis} 1000\textsuperscript{M} 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 \textit{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.

\textit{Bacillus thuringiensis} strain 612 contains plasmids with molecular weights of $5.9 \times 10^6$, $10.0 \times 10^6$ and $10.9 \times 10^6$ when grown in NM\textsubscript{1}, as has been found for strain 351 (Debabov \textit{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 \textit{B. thuringiensis} from NM\textsubscript{1} 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 NM 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* 1000~ 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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REFERENCES


