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SPONTANEOUS LOSS OF A HIGH MOLECULAR WEIGHT PLASMID AND THE BIOCIDE OF BACILLUS THURINGIENSIS VAR. ISRAELIENSIS

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A rapid loss of biocidal potency of B. thuringiensis var israeliensis against mosquito larvae has been correlated to the appearance of acrystalliferous variants at a high frequency. This irreversible loss is attributed to the loss of a large molecular weight plasmid. Segregation of asporogenic variants has also been noticed although at a much lower frequency.

Bacillus thuringiensis var israeliensis, classified as serotype H14 has been demonstrated to be pathogenic to several species of mosquitoes and black flies (1,2). This organism is being proposed as one of the powerful alternatives to chemical pesticides for the control of these vectors of tropical diseases (3). This and the anti-lepidopteran biocide factors often referred to as δ-endotoxins are located in the parasporal and irregular crystalline body, formed during the process of sporulation of the organism (4,5). The location of the gene(s) governing the formation of the endotoxins of B. thuringiensis strains has been hitherto difficult on account of the lack of proper genetic exchange systems in this organism. Different strains of B. thuringiensis are characterized by highly specific and complex patterns of extra-chromosomal elements ranging from 3 to 100 MD (6,7,8). Possible correlation between one of the high molecular weight plasmids and that of anti-lepidopteran δ-endotoxin formation has been

ABBREVIATIONS

B.t.H14 : Bacillus thuringiensis var israeliensis ; CHR : chromosome ; cry : crystal production ; sp : sporulation.

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reported (8). The cloning of δ-endotoxin gene from different strains of B. thuringiensis has been successfully attempted both in E. coli (9,10,11) and in B. subtilis (11). However, the expression of the biocide gene in either of the cases was only between one to ten percent of the level in the B. thuringiensis strains. On the basis of hybridization studies of such cloned fragments, the δ-endotoxin gene appears to be present both in a high molecular weight plasmid and in the chromosome (10,11).

In our studies on the formation of biocide in B. thuringiensis H14 (B.t.H14) we had observed that there was a continuous decrease of the biocidal potency of the cultures, although they were grown under identical conditions. The results of the studies reported here, point out that this loss of potency of the cultures might be due to the spontaneous loss of one of the high molecular weight plasmids of B.t.H14 and this is accompanied by the appearance of acrystalliferous variants at a high frequency. Repeated culturings also result in the formation of both acrystalliferous and asporogenic variants.

Materials and Methods

Bacillus thuringiensis H14 (cry^+ sp^+) was provided by Dr H. de Barjac, Institut Pasteur, Paris and was grown on KTB medium (synthetic medium supplemented with 10 % tryptose), pH 7.5 (12).

Growth and sporulation was completed by 18 hrs in this medium. To study the colony morphology of acrystalliferous (cry^- sp^+) and/or asporogenic variants (cry^- sp^-), the culture grown in KTB was suitably diluted and plated on nutrient agar plates and incubated for 3-4 days.

Toxicity assays were carried out in triplicates by adding suitably diluted cultures to 25 ml of distilled water containing 25 larvae of Culex tritaenirhynchus per tube and kept at 30°C. Mortality counts were made at the end of 12 hrs and LC_{50} values were expressed as ng protein/ml required to cause 50 % mortality of the larvae.

Plasmids from B.t.H14 and its variants were isolated essentially by the method of Kado et al. (13). The electrophoretic separation of different plasmids was carried out in 0.7 % agarose gel using TEB buffer (Tris 89mM, EDTA 2.5 mM, Boric acid 89 mM pH 8.3) at 15-20 v/cm at 0-5°. DNA bands were visualized by staining the gel with ethidium bromide (1 µg/ml).

Results

Appearance of acrystalliferous and asporogenic variants of B.t.H14 during growth

When the spores of B.t.H14 were grown in KTB medium, at the end of growth and sporulation (18 hrs) about 30 % of the population was found

Table 1

Biocidal level and the frequency of appearance of cry^-sp^+ and cry^-sp^- variants of B.t.H14 during repeated transfers in KTB medium.

Number of growth cell	Percentage of			Biocidal level* ng protein/ml LC_{50}
	cry^-sp^+	cry^-sp^-	cry^+sp^+	
I cycle	30	3	67	160
II cycle	48	3	49	350
III cycle	55	7	38	560
IV cycle	75	16	9	640

* Determined at the end of sporulation of the cultures.

to be of acrystalliferous nature (Table 1). These cry^- sp^+ variants were identified by their colony morphology on NB plates. These colonies appear opaque and raised as opposed to the white and depressed colonies of the wild type (cry^+ sp^+). In the subsequent growth cycles the percentage of cry^- sp^+ variants continued to increase along with the appearance of asporogenic and acrystalliferous phenotypes at lower frequencies (Table 1). The cry^- sp^- variants are easily distinguished by their transparent colony morphology (Fig. 1). The increasing heterogeneity of the culture

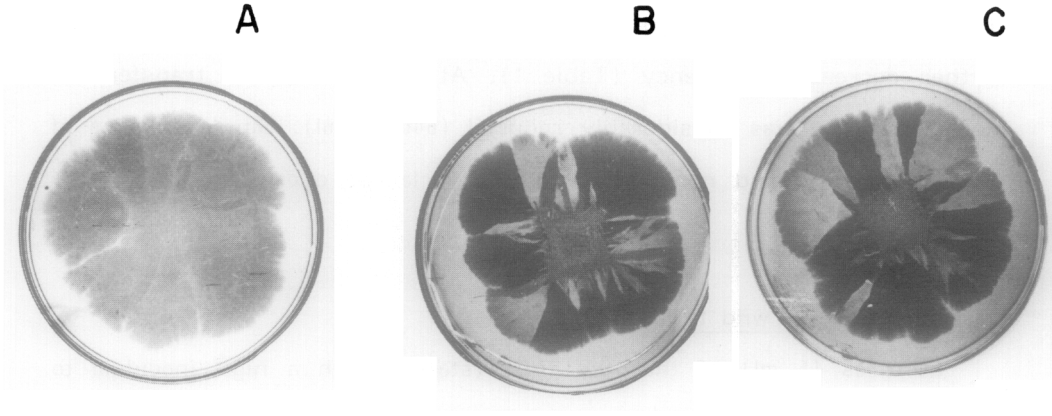

Fig. 1

Heterogeneity of B. thuringiensis H14 cultures after repeated transfers in KTB medium: Heat shocked spores (10^6 spores/5 µl) obtained after each growth cycle in KTB medium were spread on NB plates (4 sq. cm) and incubated at 30° for 3-5 days.
A: 1st transfer ; B: 2nd transfer ; C: 3rd transfer.

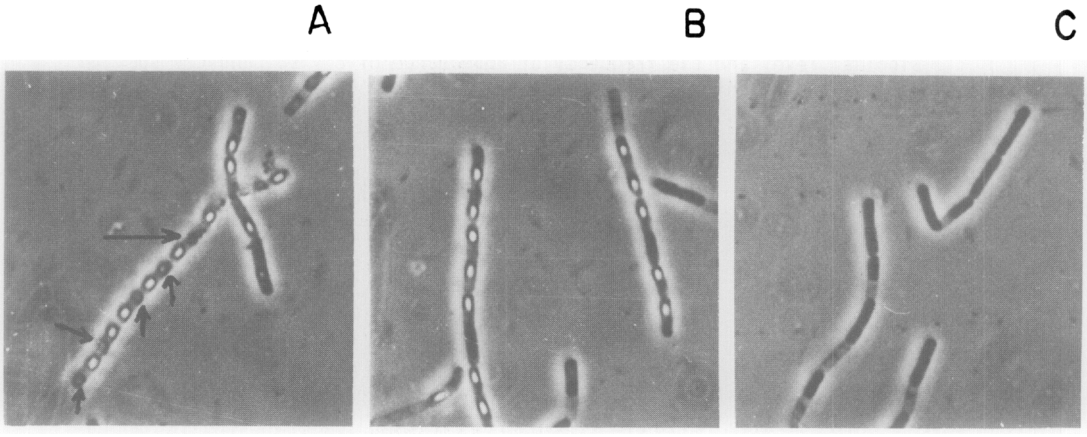

Fig. 2

Phase contrast microscopic pictures of B. thuringiensis H14 and its variants.
A: cry^+sp^+ ; B: cry^-sp^+ ; C: cry^-sp^-.
Arrows indicate the presence of crystals. The spores are seen as bright refractile bodies in panel A and B.

after 2-3 growth cycles can be seen from Fig. 1. Both cry^- sp^+ and cry^- sp^- variants were devoid of any visibly detectable parasporal inclusions (Fig. 2).

Biocidal levels of B.t.H14 cultures

When spores from this culture were transferred to fresh KTB medium, the potency of the culture i.e. LC_{50} value, at the end of sporulation was 160 ng/ml. Subsequent transfers into a fresh medium resulted in the further decrease of potency (Table 1). At the end of 4^{th} transfer the biocidal activity was considerably reduced (640 ng/ml). Single colonies of cry^- sp^+ were found non-toxic to mosquito larvae even upto 2 mg protein/ml.

Plasmid pattern of wild type and its variants

Cultures (5 ml) of the wild type (started with a high inoculum to minimise the initial cry^- variants in the population) containing cry^- sp^+ and cry^- sp^- variants were analysed for their plasmid contents (Fig. 3). It is apparent that the cry^- variants lacked one of the large molecular weight plasmids, which migrated below the chromosome, observed in the

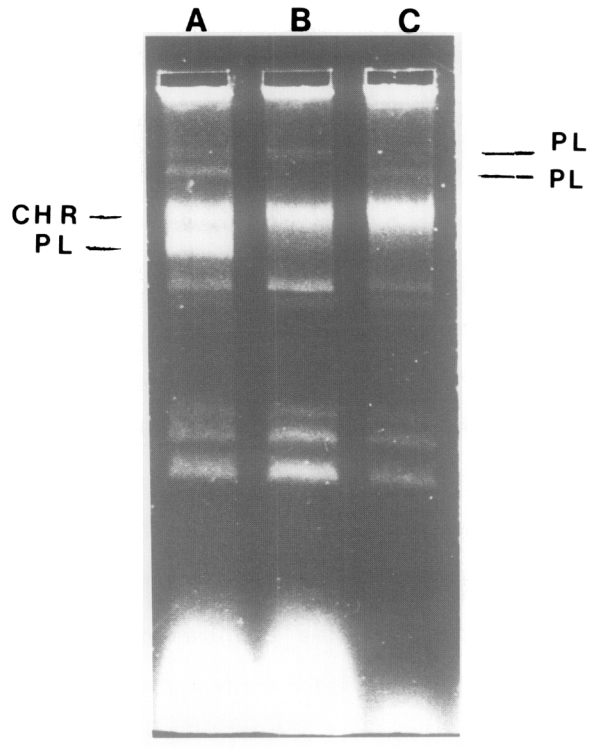

Fig. 3
Plasmid pattern of B. thuringiensis H14 and its variants. Agarose gel (0.7 %) electrophoretic pattern of the plasmids of :
A: cry^+sp^- ; B: cry^-sp^+ ; C: cry^-sp^-.
Chr : chromosome
Pl : Plasmid

wild type. Thus the loss of this plasmid seems to account for the irreversible loss of the crystalline endotoxin. The cry^- sp^- variants show an additional alteration in the high molecular weight plasmid that moved above the chromosome. Identifying the role of these plasmids in the process of sporulation and in the toxin formation is under way.

Discussion

The results outlined in this communication point out that B. thuringiensis var israeliensis (serotype H14) is relatively unstable with respect to the biocidal activity under the growth conditions employed. The progressive loss of the toxicity is related to the appearance of acrystalliferous variants at a relatively high frequency (30 % at the end of one growth cycle). The appearance of spontaneous asporogenic variants was also significant. Similar observations on the loss of biocidal potency,

with the appearance of cry mutants of B.t.H14 have been recently made. In addition, appearance of asporogenic variants of B.t.H14 at a high frequency in semi-synthetic medium is also reported (14).

The irreversible loss of the crystalline δ-endotoxin has been correlated to the loss of a high molecular weight plasmid (Fig. 3). The factors governing spontaneous loss of one of the high molecular weight plasmids of B.t.H14 are not known at present. However, a similar rapid loss of a low copy number and high molecular weight (63.5 MD) composite plasmid RMS201 has been observed in E. coli which has been attributed to the deficiency in transfer of this plasmid to daughter cells (15). Characterization of the high molecular weight plasmid, in order to locate the gene governing the synthesis of δ-endotoxin is presently underway. These studies might contribute to the stabilization of this trait, which is vital for its use as an effective vector control agent.

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