Assignment of the δ-endotoxin gene of *Bacillus thuringiensis* var. *israelensis* to a specific plasmid by curing analysis

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Received 20 April 1983

Plasmid analysis of *Bacillus thuringiensis* var. *israelensis* revealed the presence of at least 9 plasmids. A high frequency of plasmid loss occurred when this organism was grown at elevated temperature (42°C). Analysis of over 100 isolates cured of one or more plasmids by this method revealed that loss of a 72 MDa plasmid was invariably accompanied by loss of the ability to synthesize the insecticidal δ-endotoxin protein. Deletion of any of the other plasmids had no effect on δ-endotoxin production. These results indicate the presence of a plasmid-coded copy of the structural gene for the insecticidal δ-endotoxin in *B. thuringiensis* var. *israelensis*.

Bacillus thuringiensis *var.* israelensis *δ-Endotoxin* *Gene* *Plasmid* *Plasmid curing* *Insecticide*

1. INTRODUCTION

Strains within the Gram positive bacterial species *Bacillus thuringiensis* are characterized by the production of protein δ-endotoxins that are toxic to a wide variety of *Lepidopter an* and some *Dipteran* larvae [1]. The δ-endotoxins are synthesized during sporulation as parasporal protein inclusions [2,3]. Most of the 20 serotypes of *B. thuringiensis* are toxic to *Lepidoptera* and synthesize a bipyramidal crystalline endotoxin that is refractile when viewed by phase contrast microscopy [4]. The *B. thuringiensis* var. *israelensis* endotoxin is much more irregular in shape however [5] and is extremely toxic to the larvae of certain *Diptera*, notably mosquitoes and blackfly [6]. Because of their potential as biological insecticides the study of these δ-endotoxins is of considerable scientific and commercial interest.

Recent studies [7–14] have demonstrated the presence of a complex array of plasmids in most of the insecticidal *B. thuringiensis* strains. Mutant strains cured of one or more plasmids are frequently no longer able to synthesize the δ-endotoxin. Direct evidence for the existence of plasmid-borne toxin genes in *B. thuringiensis* strains HD-1, HD-73, HD-2 and HD-8, was obtained by analysis of a large number of acrystalliferous mutants [7,8]. These findings were subsequently confirmed with the aid of the high frequency plasmid transfer system [14,15]. For *B. thuringiensis* var. *kurstaki* and var. *berliner*, genetic studies [16,17] have demonstrated the existence of a chromosomal copy of the endotoxin gene in addition to the plasmid-borne copy. *B. thuringiensis* var. *israelensis* has been variously reported to contain 5 [9], 7 [10] or 8 [14] covalently closed circular (CCC) plasmids and preliminary reports have implicated at least 3 different plasmids in δ-endotoxin synthesis: a 72 MDa plasmid [18], a 4.8 MDa plasmid [11] and a plasmid of indeterminant size [10]. In [12] only 3 or 4 plasmids in various parental *B. thuringiensis* var. *israelensis* strains were detected. From analysis of 6 acrystalliferous derivatives they assigned the δ-endotoxin gene to a 4.0–4.4 MDa plasmid.

We report here the isolation of 107 strains of *B. thuringiensis* var. *israelensis* which have been

Published by Elsevier Science Publishers B.V.
00145793/83/$ 3.00

cured of one or more plasmids. Under the conditions used the parent strain was found to contain 9 readily detectable plasmids ranging from 110–3.2 MDa. Analysis of plasmid patterns in the cured strains indicated that a 72 MDa plasmid is involved in δ-endotoxin synthesis in this organism.

2. MATERIALS AND METHODS

2.1. *Origin of strains*

B. thuringiensis var. *israelensis* IPS 78 [6] (Dr H. de Barjac, Institute Pasteur, Paris); *B. thuringiensis* var. *israelensis* 4Q2 and 4Q3 (Dr D.H. Dean, *Bacillus* Genetic Stock Centre, Columbus OH); *B. thuringiensis* var. *israelensis* HD500, HD654, HD655 and HD659 (Dr H.D. Burges, Glasshouse Crops Research Institute, Littlehampton, Sussex).

2.2. *Isolation of cured derivatives of* B. thuringiensis *var.* israelensis

Strains cured of one or more plasmids were isolated by growing the parent strain on nutrient agar at 42°C for 24 h. Plates were subsequently transferred to 30°C until sporulation was complete. Individual colonies were then examined microscopically for the absence of the typical crystal. The majority of Spo^+ Cry^- colonies selected in this way showed an atypical colonial morphology compared to the parental strain, as described for other *B. thuringiensis* serotypes [8]. A small number of Spo^+ Cry^- strains were isolated by restreaking from confluent areas of the 42°C plates as in [8]. Spo^+ Cry^+ strains lacking one or more plasmids were isolated by analysis of a random selection of Spo^+ Cry^+ colonies from the 42°C plates.

2.3. *Toxicity assay*

The toxicity of individual strains was assayed by the method in [19], modified to enable the activity of single colonies to be tested. A loopful from a streak of sporulated cells grown on nutrient agar was suspended in 200 μl distilled water by brief agitation in a sonic bath, added to the cup containing *Aedes aegypti* larvae, and gently swirled. [*Aedes aegypti* eggs were kindly supplied by Mr D. Funnell (Shell Res. Ltd).]

2.4. *Agarose gel electrophoresis*

Plasmid patterns of Spo^+ Cry^- and Spo^+ Cry^+ cells were generated essentially as in [20] and incorporating the modifications suggested in [8,15].

Lysozyme and RNase were 2 mg/ml and 200 μg/ml, respectively. Cells were loaded onto the gel either after incubation at 37°C for 30–40 min to generate protoplasts, or after 5–10 min incubation at 20°C (section 3). The sodium dodecyl sulphate solution was used at 6% final conc. Electrophoresis was in 0.5% agarose gels with Tris–borate buffer [7] using the conditions in [15]. Gels were stained for 30 min in 2 μg/ml EtBr solution before being photographed.

The plasmid M_r-values were determined to an accuracy of ± 2 MDa using *B. thuringiensis* HD-2 plasmids as standards [7].

3. RESULTS AND DISCUSSION

By growing the parent strain at elevated temperature, 107 cured derivatives were isolated, of which the majority (86) were Spo^+ Cry^- and the remainder (21) were all identified as Spo^+ Cry^+. Nearly all the Spo^+ Cry^- cells formed colonies of unusual morphology compared to the parental strain when allowed to sporulate on nutrient agar. All the Spo^+ Cry^- mutants formed apparently normal spores, as judged by their appearance under phase contrast. In addition, all Spo^+ Cry^- mutants were shown to be non-toxic to *A. aegypti* larvae. With Spo^+ Cry^+ isolates, 100% larval mortality was obtained within 60 min under the conditions of assay. The assay was found to be sufficiently sensitive to detect a small percentage of Spo^+ Cry^+ cells (~10%) present amongst a population of Spo^+ Cry^- cells.

Fig.1,2a,b show the high- and low-M_r plasmids of a number of representative Spo^+ Cry^- mutants, in addition to the wild type pattern (track 2, fig.1; track 1, fig.2a,b) and the pattern from several Spo^+ Cry^+ cells that have lost one or more plasmids (tracks 10, 13, 16 in fig.2a,b). These patterns are combined in diagram form in fig.3. In our hands, different periods of lysozyme/RNase incubation were found to be necessary to display the total plasmid complement of *B. thuringiensis* var. *israelensis* because the high-M_r plasmids (50–110 MDa) of this organism were not reproducibly observed if the cells were incubated in the lysozyme/RNase solution at 37°C for a time (30–40 min) sufficient to generate protoplasts.

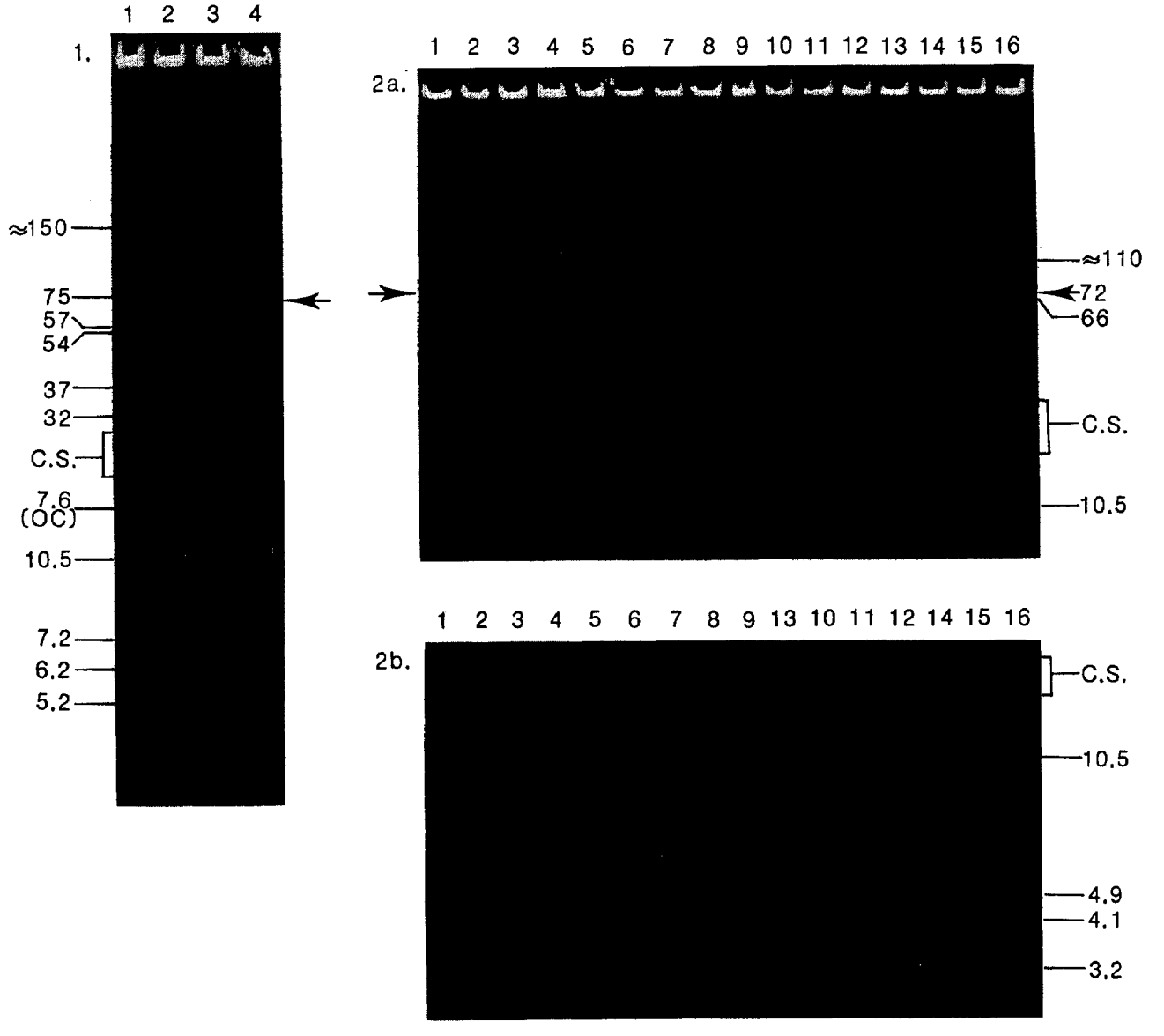

Fig.1. Agarose gel electrophoresis showing plasmids of wild type *Bacillus thuringiensis* var. *thuringiensis* (HD2) and *B. thuringiensis* var. *israelensis* wild type and cured derivatives: (1) wild type HD2; (2) wild type *B. thuringiensis* var. *israelensis*; (3,4) cured Spo$^+$ Cry$^+$ derivatives of *B. thuringiensis* var. *israelensis*. The M_r-values (in MDa) of the HD2 plasmids are shown on the left margin as size references: OC, open circular form of plasmid; C.S., chromosomal smear. The arrow identifies the 72 MDa *israelensis* plasmid.

Fig.2. (a) High-M_r plasmids of wild type *B. thuringiensis* var. *israelensis* and cured derivatives of this organism analysed by agarose electrophoresis after short incubation time (see text): (1) wild type; (2–9,11,12,14,15) cured derivatives that are Spo$^+$ Cry$^-$; (10,13,16) cured derivatives that are Spo$^+$ Cry$^+$. The arrows identify the 72 MDa *israelensis* plasmid.

Fig.2. (b) Low-M_r plasmids of wild type *B. thuringiensis* var. *israelensis*, and identical cured derivatives to those in fig.2a, analysed after longer periods of incubation (see text): (1) wild type; (2–9,11,12,14,15) cured derivatives that are Spo$^+$ Cry$^-$; (10,13,16) cured derivatives that are Spo$^+$ Cry$^+$. Plasmid M_r-values shown on the right of fig.2a and 2b were calculated from a calibration curve constructed using HD2 wild type as a size reference (fig.1); C.S., chromosomal smear.

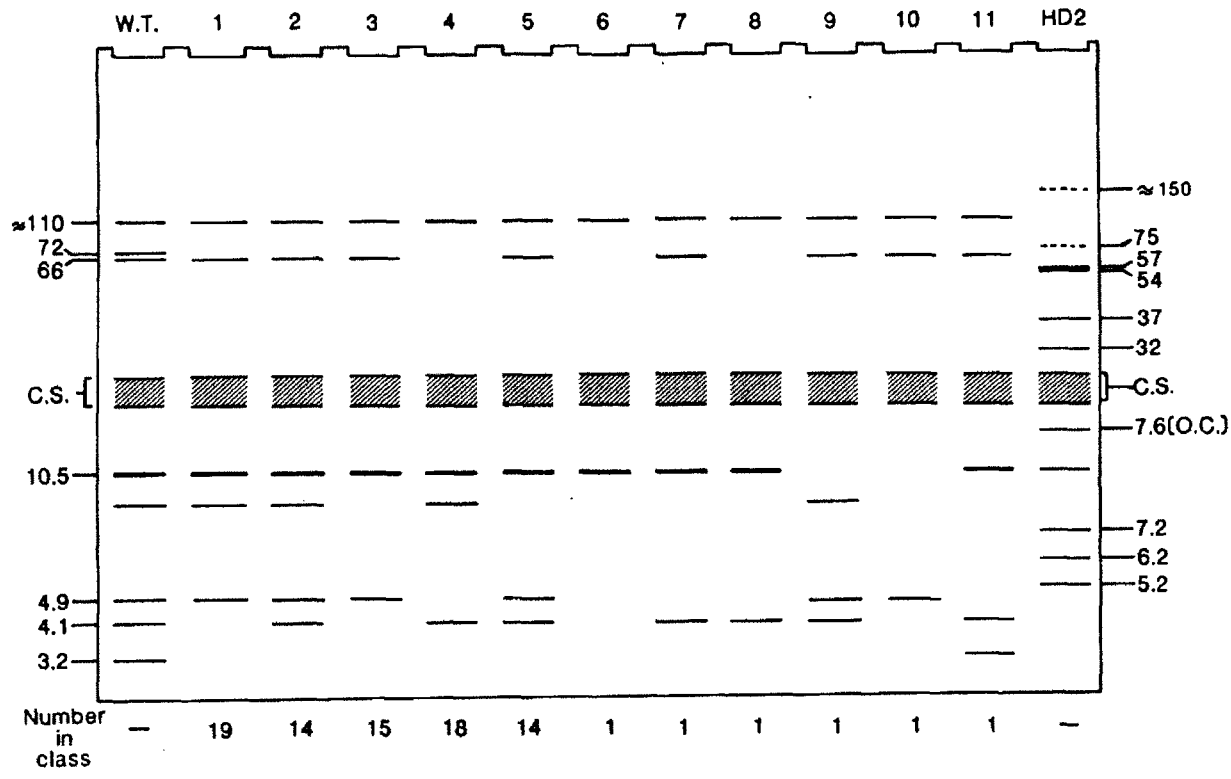

Fig.3. Diagram summarising the plasmid patterns of *B. thuringiensis* var. *israelensis* wild type and of the 11 classes containing the 86 Spo$^+$ Cry$^-$ mutants found. The plasmid pattern of the HD2 wild type is also shown. M_r-values of the wild type *israelensis* and HD2 plasmids are shown on the left and right margins, respectively. OC, open circular form of plasmid; C.S., chromosomal smear. The 150 MDa and 75 MDa HD2 plasmids denoted by broken lines were difficult to discern on some gels.

When the incubation conditions were altered to 5–10 min at 20°C the high-M_r plasmids were readily visible upon subsequent electrophoresis despite the fact that no protoplasts could be observed microscopically at this time. Paradoxically, using these modified incubation conditions, the lower-M_r plasmids ($\leq$20 MDa) appeared not to be released into the gel as efficiently as the large ones. To obtain reproducible plasmid profiles for these plasmids below the chromosomal smear (fig.2b), it was necessary to generate protoplasts by incubation for the longer period.

The explanation for these findings is unknown at present, but the phenomenon was also observed in plasmid screening of *B. thuringiensis* HD1 (not shown). In similar screening of *B. thuringiensis* HD2 however, plasmids located above the chromosomal smear were reproducibly observed even after prolonged incubation at 37°C (not shown). Conceivably, during the longer incubation period endogenous DNases in certain *B. thuringiensis* strains may cleave the plasmids to the open circular (OC) or linear (L) forms which fail to electrophorese into the agarose. The reason for the differential behaviour of the high- and low-M_r plasmids is not apparent, but differences have been reported [21] in the efficiency of recovery of these two categories by various extraction procedures.

The 86 Spo$^+$ Cry$^-$ mutants could be classified into 11 groups on the basis of their plasmid pattern (fig.3): 6 groups contain only 1 representative; 5 groups contain the majority of the mutants. All of the Spo$^+$ Cry$^-$ mutants, without exception, lack a high-M_r plasmid that is invariably present in Spo$^+$ Cry$^+$ cells. The M_r of this plasmid has been determined to be 72 MDa, using HD-2 as a standard [8] (fig.1, track 1). Complementary to this lack of the 72 MDa plasmid in all Spo$^+$ Cry$^-$ strains is its continued presence in the 21 cured Spo$^+$ Cry$^+$ strains. These data are strong evidence for the involvement of the 72 MDa plasmid in the synthesis of the parasporal insecticidal δ-endotoxin of *B. thur-*

ingiensis var. *israelensis*. This is in agreement with the preliminary report [18], confirmed in [18a], but does not support the suggested involvement of either a low-M_r plasmid (4.0–4.8 MDa) [11,12] or a plasmid which migrates directly below the chromosomal DNA [10]. However, in [10] only 3 cured strains were described and no indication of plasmid M_r-values was given. In [12], only 6 cured strains were analysed and their electrophoresis/extraction procedure revealed only 3 or 4 of the 9 plasmids observed in the parent strain ([18], this report).

The possibility remains that the 72 MDa plasmid codes for a transmissible positive regulatory element involved in crystal synthesis [8]. Studies are now in progress to investigate this using the high frequency plasmid exchange system described for *B. thuringiensis* serotypes [14,15].

In an attempt to discover the generality of our assignment of the toxin gene to the 72 MDa plasmid we have recently obtained Spo^+ Cry^- derivatives of the 6 other strains of var. *israelensis* listed in section 2. For each of these strains the Cry^- phenotype was correlated with loss of a plasmid of M_r 72 ± 2 MDa.

In view of the marked differences in insecticidal specificity, crystal toxin shape, toxin M_r and in vitro toxicity [22,23] between *B. thuringiensis* var. *israelensis* and other *B. thuringiensis* serotypes, it is interesting to note that no homology between var. *israelensis* plasmids and plasmids from a range of lepidoptera-toxic *B. thuringiensis* strains were reported using a fragment of the cloned plasmid borne lepidoptera toxin gene as a hybridization probe [24].

ACKNOWLEDGEMENTS

We thank L. Futter, L. Jewitt and D. Guttridge for their assistance in the preparation of this manuscript. The research was supported by the Agricultural Research Council to D.J.E. and the Medical Research Council to E.S.W.

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