A Rapid Procedure for Screening and Isolation of Various Sizes of Plasmid DNA in Serovars of *Bacillus thuringiensis*

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李泳根·로베트 엠 파우스트·姜錫權·페트리시아 이 멕콜리·케롤 엘 메이어-다울링(1985) *Bacillus turingiensis* 變種들로부터의 Plasmid DNA 抽出 및 分離

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ABSTRACT The use of a modified procedure for the isolation of extrachromosomal DNA of low to high molecular weight, followed by agarose gel electrophoresis of the crude lysates, provided a simple screening procedure for detecting plasmids ranging in molecular weights from approximately 1 to more than 135 megadaltons from serovars of *Bacillus thuringiensis*. The procedure provides for a relatively large-volume stable lysate for isolation of plasmids for restriction endonuclease mapping and cloning procedures. The method was used for screening of plasmids in 6 differenentially effective serovars of *B. thuringiensis* toxic to dipteran and lepidopteran insects. Relatively large plasmid DNAs of masses above 50 megadaltons (Mdal) were isolated from all of the serovars examined using this technique. The number of extrachromosomal DNAs detected in serovars of *B. thuringiensis* was 8 for *israelensis*, 10 for *kurstaki*, 13 for *aizawai*, 2 for *de ndrolimus*, 1 for *finitimus*, and 6 for *yunnanensis*. Smaller plasmid DNAs were isolated in four of the six serovars that ranged in mass down to approximately 2 Mdal.

The gram-positive becterium *Bacillus thuringiensis* is responsible for the production of several insecticidal toxins; one of these toxins, the δ-endotoxin, is of commercial importance because of its lethal effects on many lepidopteran as well as to some dipteran larvae (de Barjac, 1978; Faust and Bulla, 1982). The δ-endotoxin is a glycoprotein that appears during the sporulation phase as a crystalline inclusion, which is a bipyramidal and phase refractile body (Bulla et al., 1977; Faust and Bulla, 1982). It has recently been shown that some of the genetic information related to toxin expression is carried on plasmids harbored by the various serovars of *B. thuringiensis* (Schnepf and Whiteley, 1981; Wong et al., 1983; Kronstad et al., 1983; Kronstad et al., 1983; Held et al., 1982; Klier et al., 1982; Klier et al., 1983; Kronstad and Whiteley, 1984).

Improved procedures have been devised for the isolation and analysis of covalently closed circular DNA molecules(CCC-DNA) and applied to the study of serovars of *B. thuringiensis* and other *Bacillus* species. In early studies with *B. thuringiensis* plasmid DNA was generally isolated by the sodium dodecyl sulfate(SDS)-NaCl lysis procedure of Guerry, LeBlanc and Falkow (1973), or modified methods there of giving a decreasing plasmid yield with increasing plasmid size indigenous to the strain under study. Plasmids greater than 100 megadaltons(Mdal) in size are recovered poorly or not at all (Currier and Nester, 1976; Hansen and Olsen, 1978). Later methods encompassed the use of controlled alkaline denaturation and allowed for relatively higher yields of large plasmids, generally freed of most chromosomal DNA (Currier and Nester, 1976; Hansen and Olsen, 1978; Birnboim and Doly, 1979; Kronstad et al., 1983).

A reasonably simple method for screening of plasmid content on agarose gels, described by Eckhardt (1978) and modified by Gonzalez et al., (1981) for *B. thuringiensis* eliminates the requirement for isolation of the plasmid DNA initially. The method employs electrophoresis of SDS through a layer of protoplasts in the agarose gel well of a vertical apparatus. Presumably, the cells will then gently lyse leaving the chromosomal DNA largely intact at the gel origin, while the plasmid DNA's separate as bands in the gel. Since the only manipulation of the cells prior to gel electrophoresis is the protoplasting, isolation of the various indigenous plasmids in the gel, including plasmids greater than 100 Mdal, is usually achieved. The potential confusion of large

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covalently closed circular(CCC) DNA plasmids with open-circular (OC) forms of smaller plasmids can be minimized by applying high voltages to the agarose gel, at which OC molecules larger than 10 Mdal are excluded from the gel (Gonzalez and Carlton, 1980).

Unfortunately, many strains of *B. thuringiensis* are highly resistant to lysozyme treatment and we have often found inconsistent lysis on the gel resulting in an incomplete plasmid profile with some serovars. We report here the use of a modified large-volume procedure based on the Eckhardt technique for isolation of CCC-DNA of low and high molecular weight from *B. thuringiensis* servars, followed by agarose gel electrophoresis of SDS-treated lysates. The technique provides a simple screening method for detecting plasmids with molecular weights ranging from about 1 to more than 100 Mdal and consistently yields a relatively large-volume lysate for isolation of *B. thuringies* plasmids for restriction endonuclease mapping and cloning procedures.

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MATERIALS AND METHODS

Bacterial strains: *B. thuringiensis* serovars *kurstaki* (serotype 3a, 3b), *dendrolimus* (serotype 4a, 4b), and *finitimus* (serotype 2) are maintained as subcultures in the Insect Pathology Laboratory, USDA, Beltsville, MD. *B. thuringiensis* serovar *yunnanensis* (no H antigen) was the gift of T. Iizuka, Hokkaido University, Japan. *B. thuringiensis* serovar *aizawai*(serotype 7) was the gift of K. Aizawa, Kyushu University, Japan. *B. thuringiensis* serovar *israelensis*(serotype 14, strain 4Q1) and *Escherichia coli* DEC 110 were obtained from D. Dean, Ohio State University, Columbus, Ohio.

Cell growth and preparation of lysates for agarose gel electrophoresis: Only the final procedure is described here. Cells were grown in 250ml of Spizizen's minimal phosphate medium (Spizizen, 1958)supplemented with 0.5% glucose (added as a sterile 50% solution) and 0.1% Difco[1] vitamin-free casamino acids or yeast extract at 30°C with shaking for 9~10h (OD 600≒0.360). The cells were harvested by centrifugation at 700g; washed once with cold TES buffer (30mM Tris base, 5mM disodium EDTA, 50 mM NaCl, pH 8.0) and resuspended in 8ml of TES buffer containing 20% sucrose and 2mg/ml egg white lysozyme-200μg/ml RNase A(Sigma) in a polypropylene centrifuge tube. The cell suspension is vortexed vigorously and incubated at 37°C for 30 to 60 min(until spheroplasts are generated), then lysed by the addition of 16ml of 6% sodium-dodecyl sulfate (SDS) in electrophoresis buffer (89mM Tris base, 2.5mM disodium EDTA, 89mM boric acid, pH 8.3) containing 5% sucrose with gentle mixing.

Agarose gel electrophoresis of DNA: Plasmid DNA's in the cleared lysate were resolved on vertical 0.5% agarose gels (low electroendosmosis, FMC Corp.) in Tris-borate electrophoresis buffer. The samples (200μl) were adjusted with 20μl of 0.25% bromophenol blue in 50% glycerol (made in electrophoresis buffer). The DNA preparations were applied to the sample slots in 10~70μl volumes.

Gels were run at constant current in three stages: 1h, 45min at 3.0mA, then 30min at 7.0mA, and finally 3.5h at 28mA (~120V). The gels were stained in electrophoresis buffer with 2μg of ethidium bromide/ml for 30min, destained indistilled water for 4~5h, and photographed on a transilluminator (Model TM-36, Ultraviolet Products, Inc., San Gabriel, CA) with Polaroid type high-speed No. 55 film and a Kodak No. 23A red filter. The plasmid Mr-values were determined to an accuracy of ±2 Mdal by reference to the *B. thuringiensis* serovar *israelensis* plasmids previously sized by Ward and Ellar (1983) and Gonzalez and Carlton (1984). At least three different gels were run for each strain.

RESULTS

A modified large-volume procedure based on the Eckhardt technique suitable for screening and isolation of plasmid DNA of low and high molecular we-

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ight from a variety of *B. thuringiensis* serovars is described. Typical results from a number of experiments are shown in Fig. 1 and estimates of the numbers and masses of the plasmid DNAs are summarized in Table 1.

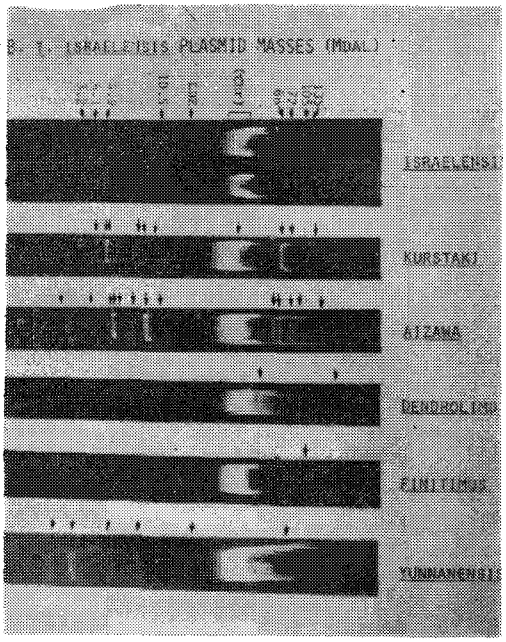

Fig. 1. Representative comparison of extrachromosomal DNA molecules from various serovars of *Bacillus thuringiensis* on 0.5% vertical agarose gels subjected to electrophoresis. Direction of migration is downward. Black pointers indicate the positions of the DNA molecules as revealed on the original photographic negatives. The (Cqr) abbreviation indicates the position of chromosomal DNA. The molecular weights (in megadaltons, Mdal) of the standard (*B. thurinsgiensis* serovar *israelensis* 4Q1) are indicated on the left margin. The abbreviation LDE represents a linear DNA element detected in the *B. thuringiensis* serovar *israelenis* standard.

Table 1. Some *Bacillus thuringiensis* serovars and their extrachromosomal DNA resolved on agarose gels

Serovar	Mean masses (megadaltons)[a]	Total number
israelensis[b]	135, 105, 72, 66, 10.5(LDE^{-10})4.9, 4.1,	8(9)
kurstaki	>3.2 135, 110, 52, 29, 10, 9.3, 7.5, 4.9, 4.7, 4.0	10
aizawai	>135, 84, 75, 63, 57, 11.1, 8.5, 7.0, 6.1, 5.2, 4.9, 3.8, 2.6	13
dendrolimus	>135, 48.4	2
finitimus	110	1
yunnanensis	75, 17.8, 7.8, 5.1, 2.8, 2.3	6

[a] Relative masses(megadaltons, Mda) of extrachromosomal DNAs on agarose gels were determined from a standard curve constructed from plasmid DNA standards isolated from *B. thuringiensis* serovar *israelensis* 4Q1.

[b] Relative masses for this serovar were previously determined by Ward and Ellar (1983) and Gonzalez and Carlton (1984).

Based on agarose gel electrophoresis profiles, the number of extrachromosomal DNAs detected in serovars of *B. thuringiensis* was eight for *israelensis*, ten for *kurstaki*, thirteen for *aizawai*, two for *dendrolimus*, one for *finitimus*, and six for *yunnanensis*. Serovar *israelensis* also contains a plasmid-like linear DNA element(LDE) that has been verified by electron microscopy or on cesium-chloride gradients and is removed during deproteinization with chloroform(Gonzalez and Carlton, 1984; Faust, unpublished data). Approximate masses of the extrachromosomal DNA molecules in the serovars were estimated by reference to the *israelensis* plasmids as mentioned previously. Relatively large plasmid DNAs of masses above 50 Mdal were isolated from all of the serovars examined using this techuique. Relatively smaller plasmid DNAs were isolated in four of the six serovars (*israelensis, kurstaki, aizawai, yunnanensis*) ranging in mass down to approximately 2 Mdal by our method. All of the plasmids described by various other reports(Ward and Ellar, 1983; Faust et al., 1983; Gonzalez and Carlton, 1984) for the *B. thuringiensis* serovar *israelensis* strain were also isolated.

DISCUSSION

Recent studies have demonstrated the presence of a complex array of plasmids in most of the serovars of *B. thuringiensis* (Gonzalez and Carlton, 1980; Gonzalez et al., 1981; Lerecus et al., 1982; Faust et al., 1983; Jarrett, 1983; Miteva, 1978; Kronstad et al., 1983; Stahly et al., 1978; Faust et al., 1979; Iizuka et al., 1981 a,b; Ward and Ellar, 1983). Indeed, the literature gives a good indication not only of the ubiquity of plasmids in the many different natural isolates, but also of their variability in both number and size distribution among serovars and among strains belonging to the same serovar. Comparison of the plasmid profiles of strains of different serovars in these reports reveal that the similarity of plasmids is greater within serovars than between them, although extensive divergent patterns is evident at both the serovar and strain levels. It also appears that the plasmid content of *B. thuringiensis*

serovars are in a state of constant and continual change. These frequent changes in plasmid patterns may arise by "a combination of events including deletion and insertion of plasmid DNA segments, curing of entire plasmids, recombination events between plasmids and the *B. thuringiensis* chromosome, and by transfer of plas mids between strains"(Carlton and Gonzalez, 1984). Additionally, some of the various isolation techniques used by investigators may result in an incomplete pictures of the plasmid content of *B. thuringiensis* strains. For example, we recently analyzed several crystal positive (Cry^+) strains of *B. thuringiensis* serovar *israelensis*(Faust et al., 1983) and found them to contain similar plasmid arrays of three to four small plasmids (4.0 to 13.0 Mdal). Cry^- mutants were isolated after growth of a parent *B. thuringiensis* serovar *israelensis* strain (ONR 60A /WHO 1887-1) at 42°C. The Cry mutants had lost a 4.0~4.3 Mdal plasmid which suggested that this plasmid may be involved with toxic parasporal cystal production. Results from other laboratories, including the results reported in this communication, suggest that larger plasmids are present in Cry^+ *B. thuringiensis* serovar *israelensis* strains. Thus, larger plasmids in serovar *israelensis* may not have been detected by the previous technique used; if a loss of larger plasmids occurred, in addition to the 4.0~4.3 Mdal plasmid, their loss by curing may have not been noticed. In any event the technique presented here yields results similar to other reports using the Eckhardt technique for serovar *israelensis* (Ward and Ellar, 1983; Gonzalez and Carlton, 1984) and is applicable for resolving both large and small plasmids in a number of other serovars that have in our laboratory resulted in inconsistent plasmid arrays when the standard Eckhardt procedure has been applied.

Our aim was to develop a method for rapid detection and identification of plasmids in a variety of *B. thuringiensis* serovars based on a modification of the Eckhardt procedure. We required a methed suitable for screening for plasmids, regardless of the serovar used, yielding consistent lysis, and which could detect consistantly plasmids of a wide range of molecular weights. We also wanted a technique that resulted in a relatively large volume lysate useful for continuous plasmid isolations. The technique as described here fulfill these requirements. Other advantages are speed (the extraction procedure can be performed within 8 hr by one person), the feasibility of processing many strain samples simultaneously, and plasmids that can be extracted from the agarose gels (see Maniatis et al., 1982) pure enough for restriction enzyme analysis and cloning procedures (restriction enzyme analysis of the 4.9, 4.1, and 3.2 Mdal plasmids in *B. thuringiensis* serovar *israelensis* has been completed using this isolation technique and will be reported elsewhere).

The initial experiments were done with *E. coli* DEC 110 (Macrina et al., 1978, data not shown; plasmid masses of 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, and 1.4 Mdal) and *B. thuringiensis* subsp. *israelensis* 4Q1 (Ward and Ellar, 1983; Gonzalez and Carlton 1984; plasmid masses of 135, 105, 72, 66, 10.5, 4.9, 4.1, 3.2 Mdal) carrying plasmids of known number and size. The method used for detection of these known plasmids proved to be rapid and relatively reproducible, although it was found necessary to grow the cells no more than 9~10hr and to use higher concentrations of lysozyme and up to 6% SDS to obtain clear lysates in all the strains examined over that described in the standard Eckhardt procedure. Also, in Eckhardt's original lysis protocol both a SDS mixture and an overlay mixture is used, and the agarose gel slots are sealed with molten agarose. As modified by Gonzalez et al., (1981) the protocol uses a 2% SDS mixture pipetted into each slot of a vertical agarose gel which is allowed to stand for 20min. Then, the spheroplast suspension is pipetted under the SDS mixture, without mixing the two layers, and electrophoresis is begun immediately without sealing the agarose. In our protocol a 6% SDS mixture is added to the spheroplast sample, mixed gently, then after a cleared lysate has formed(within 5 min), a small quantity of the preparation is mixed with the tracking dye solution and immediately added to the slots and electrophoresis begun. No sealing of the gel slots is necessary and samples can be added to the gel slots with assurance that lysis has indeed occurred. We

have found that the remaining lysate sample will keep up to 4~5 days without noticeable loss of its plasmid content and the sample can be used repeatedly. Lastly, we found that the growth media described here to be vital for optimum lysis and consistant isolation of all plasmids inherent in a strain. The use of media or components such as nutrient broth, tryptose phosphate or tryptone resulted either in poor lysis or low yields of plasmids when good lysis did occur. Since some serovars grew poorly with the vitamin-free casamino acid supplement, yeast extract was generally favored as a medium supplement in the procedures. We also found it useful to clean the gel slots with the dye-glycerol Solution prior to adding the DNA sample and to destain the gel for several hours in distilled water before photographing. These procedures resulted in a much improved resolution of the plasmids.

Understanding the biological significance of the variety of plasmids in *B. thuringiensis* will require further genetic studies using plasmid curing and transfer and plasmid mutation and restriciion enzyme analysis. It is hoped that the plasmid detection procedure described in this study will provide simple physical chemical complements to these genetic experiments. In ecological and genetic studies this efficient screening procedure will permit examination of a large number of strains and new isolates for genetic studies as they become available.

摘 要

*Bacillus thuringiensis*變種들로부터 Extrachromosomal DNA를 抽出分離코저 從來의 方法을 補完하여 適用한바 分子量의 크기가 1 Megadalton에서 135 Megadalton에 이르는 plasmid들을 分離함에 보다 效果的이었고 또 이 plasmid들을 利用, 制限酵素에 依한 遺傳子配列作成 및 gene Cloning을 하는데 比較的 安定된 많은 量의 細菌溶解物을 얻을 수 있었다.

파리목과 나비목에 各其 毒性이 다른 *Bacillus thuringiensis* 6個 變種으로부터 plasmid들을 分離한 結果 分子量이 큰 50 Megadalton 以上의 plasmid들이 供試된 모든 變種으로부터 抽出되었으며 이들 plasmid의 數를 보면 *israelensis*로부터 8個 *kurstaki*로부터 10個 *aizawaü*로부터 13個 *dendrolimus*로부터 2個, *finitimus*로부터 1個 그리고 *yunnanensis*로부터 6個가 各各 檢出되었다. 供試된 變種中 4個의 變種으로부터는 2 Megadalton 以下의 적은 plasmid들도 抽出되었다.

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