PLASMID STABILITY OF BACILLUS THURINGIENSI S VAR. KURSTAKI (HD-1) DURING CONTINUOUS PHASED CULTIVATION

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SUMMARY

*Bacillus thuringiensis* var. *kurstaki* (HD-1) was grown as a continuous phased culture in a cyclone fermentor. During the time course of the continuous phased cultivation (CPC), the culture was sampled to determine the efficiency of sporulation and parasporal crystal formation. Concurrently, plasmid DNA was extracted and resolved on agarose gels. The plasmid profile remained constant throughout 328 h of cultivation. However, during the same time period, asporogenous, acrystalliferous variants increased from < 1% to > 90% of the cells harvested. Our data suggests that the disappearance of parasporal crystals in *B. thuringiensis* var. *kurstaki* (HD-1) during CPC occurs independent of plasmid copy but may be due to defective sporulation.

INTRODUCTION

The industrial production of the bioinsecticidal β-endotoxin, which is produced concomitantly during sporulation as a parasporal crystal by *B. thuringiensis*, is based upon growth in batch fermentors (Couch and Ross, 1980). Continuous culture technology may provide a more efficient means of producing the β-endotoxin (Khachatourians, 1986; Selinger et al., 1987). The continuous phased culture (CPC) system as proposed by Dawson (1965) was evaluated to determine its suitability for the production of *B. thuringiensis* var. *kurstaki* HD-1. Initial studies indicated that there was selection for asporogenous, acrystalliferous (Spo-Cry-) phase variants when *B. thuringiensis* was grown in CPC (Selinger et al., 1987). The selection of sporulation phase mutants in differentiating organisms, during continuous cultivation, has been described previously for *B. cereus* (Boudreaux and Srinivasan, 1989) and *B. thuringiensis* var. *galleriae* (Blokhina et al., 1984).

In *B. thuringiensis* var. *kurstaki*, the genes coding for the protoxin of the β-endotoxin have been located on the chromosome (Held et al., 1982) as well as on 30 and 47 Md plasmids (Schnepf and Whiteley, 1981; Held et al., 1982; Kronstad et al., 1983). In *B. thuringiensis* var. *israelensis* the loss of certain large plasmids has led to loss of parasporal crystal formation, as well as bioinsecticidal activity (Kamdar and Jayaraman, 1983). In this paper we have studied the development of *B. thuringiensis* var. *kurstaki* in CPC and the fate of its plasmids.

MATERIALS AND METHODS

BACTERIAL STRAIN AND GROWTH MEDIUM

An isolate of *Bacillus thuringiensis* var. *kurstaki* (HD-1), designated strain number GK 1806, was obtained from a commercial preparation of Dipel® (Abbott Laboratories). This was performed by heat shocking (80°C for 10 min.) 0.1 g of Dipel® in 10 ml of nutrient broth [NB; 0.8% Nutrient Broth (Difco) and 0.5% NaCl in distilled H₂O] followed by overnight growth.
incubation at 28°C. A pure culture was obtained from the resulting culture and maintained on nutrient agar stock plates [NA; NB + 2.0% Bacto-Agar (Difco)] at 4°C. All subsequent inoculations into the CPC systems were made from these stock plates. The substrate for the CPC propagations, designated Medium G, was described in Nickerson and Bulla (1974); Glucose, 2.0 g; (NH₄)₂SO₄, 2.0 g; K₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.3 g; MnSO₄·H₂O, 0.05 g; CaCl₂, 0.08 g; ZnSO₄·7H₂O, 0.005 g; CuSO₄·5H₂O, 0.005 g; FeSO₄·7H₂O, 0.0005 g supplemented with monosodium glutamate, 5.0 g and Nutrient Broth (Difco), 0.08 g per litre of distilled water.

ISOLATION OF PLASMID CURED STRAIN

Plasmid cured strains of GK 1806 were obtained by growth in NB plus 0.002% sodium dodecyl sulfate (SDS) for 48 h followed by plating on NA plates. Colonies with atypical morphology were isolated and screened for Spo-Cry− phenotype. Plasmid profiles of these strains were then resolved electrophoretically on agarose gels. One of these isolates has been designated strain GK 1812 and is described here.

CONTINUOUS PHASED CULTURE

A loopful of cells from a NA stock plate was inoculated into 200 ml of medium G in a 500 ml Erlenmeyer flask. The cells were incubated in a reciprocating waterbath (Fisher model 127) for 10-12 h at 30°C and 100 rpm. The resulting culture plus 475 ml of fresh medium were transferred to the continuous phasing apparatus (Dawson, 1965) which is schematically represented as step A in figure 1. The aeration rate was set at 170 ml of air/min and the temperature was maintained at 30°C throughout the propagation. The controller was set to a 4 h phasing cycle program (represented as step B). At the end of each 4 h interval, one half of the culture was harvested (as in step C in figure 1) and replaced with an equal volume of fresh medium as in step A above. The sequence (A-C) was repeated every 4 h for the duration of the propagation. Samples were taken from the harvested culture at a number of intervals and analysed as described below.

PROCESSING OF SAMPLES

Detection of sporogenous, crystalliferous (Spo+Cry+) and oligosporogenous, oligocrystalliferous (Spo−Cry−) colonies. Samples from the CPC fermentor were diluted and spread on NA plates to yield 30-100 colonies per plate. The plates were then incubated at 28°C for 3 d, after which time they were counted and scored for Spo+Cry+ or Spo−Cry− phenotypes.

Dry weight and pH. Ten ml of culture were filtered using preweighed 47 mm Millipore filter disks with a pore size of 0.22 µm (Millipore Corp.). Filter disks were washed twice with distilled water, placed in a vacuum drying oven at 60°C for 16-18 h and reweighed to determine the dry weight of the culture. The filtrate was saved and its pH was measured using a Radiometer PHM82 standard pH meter with combination electrode.

Plasmid analysis. The method described by Birnboim and Doly (1979) and modified by Lereclus et al. (1982) was used to isolate and resolve plasmid DNA from 1.5 ml culture samples containing 1 X 10⁷ to 1 X 10⁸ CFUs/ml. A phenol extraction step was added to remove the excess protein thus improving the resolution of the pDNA on the gels.

RESULTS AND DISCUSSION

The CPC system used in this study of plasmid stability is schematically represented in figure 1. The technique employs nutrient limitation to induce synchronous cell growth. The growth conditions were maintained such that glucose was the limiting factor.