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Short communication

Sensitivity to plating of *Escherichia coli* cells expressing the *cryA* gene from *Bacillus thuringiensis* var. *israelensis*

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Summary. The gene (*cytA*) coding for the 27 kDa polypeptide of the *Bacillus thuringiensis* var. *israelensis* mosquito larvicidal δ-endotoxin, was cloned into a plasmid containing the T7 bacteriophage promoter. The plasmid was used to transform an *Escherichia coli* strain containing the T7 RNA polymerase gene *1*, under the control of *lacP*. Loss of colony-forming ability without substantial lysis, associated with immediate inhibition of DNA synthesis, was observed after induction of transformed cells. The *cytA* gene product may kill *E. coli* cells by disrupting their chromosome replicating apparatus.

Key words: *Bacillus thuringiensis* var. *israelensis* – Mosquito larvicidal δ-endotoxin – T7 RNA polymerase – *cytA* gene product – Inhibition of DNA synthesis

Serotype H14 of *Bacillus thuringiensis* (var. *israelensis*) produces parasporal crystals (δ-endotoxin) with highly specific toxicity to mosquito larvae (Bulla et al. 1980). At least four major polypeptides compose the crystals, with molecular weights of about 134, 128, 72 and 27 kDa, as calculated from the derived amino acid sequences of the genes (Hofte and Whiteley 1989). The 27 kDa protein is not the specific mosquito larvicidal component, but rather is hemolytic and shows general cytotoxic effects due to its lipophilic nature (Gill et al. 1987). In an attempt to investigate its mechanism of action, the gene encoding it (*cytA*) was cloned separately into *Escherichia coli* downstream from the *lacP* of plasmid pUC9 (Vieira and Messing 1982). The failure of this approach led us to exploit another, more sophisticated approach, as described below.

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The gene (*cytA*) was first isolated with its original promoter on a 5.4 kb fragment (Ward and Ellar 1986) from our *Eco*RI library of the largest plasmid of *B. thuringiensis* var. *israelensis* (Douek et al. 1990), ligated to pUC9 and cloned into strain JM83 of *E. coli* (Vieira and Messing 1982; Fig. 1). No mosquito larvidical effect was observed with clones containing this fragment in either orientation (pJD16 of Fig. 1, and pJD9). The 2.1 kb *Hae*III-*Eco*RI fragment containing the open reading frame of *cytA* was then ligated into pUC9, which had been cleaved at the *Sma*I and *Eco*RI sites in the polylinker (construction scheme not shown). Analysis of transformants (in JM83) revealed two distinct groups. One type of transformants (about 65% of 60 clones checked) did not express the 27 kDa protein, despite the apparently correct structure of the plasmid (i.e., they contained plasmids yielding the expected 2.1 and 2.6 kb fragments upon *Hin*dIII and *Eco*RI digestion). Post-transcriptional enhancement of *cytA* expression in *E. coli* has been explained by stabilization of its product (the 27 kDa polypeptide) in the presence of a 20 kDa protein (Adams et al. 1989), the open reading frame for which was not included and thus not expressed in the present constructs.

The second group of transformants (about 35%) contained plasmids with various DNA rearrangements (associated with at least 8 different restriction patterns). This result was reproducible, and indicated a strong selection against expression of *cytA* gene product in *E. coli*, even at the low levels expected under the repression conditions employed. Expression of this gene has been reported (unpublished results, cited in Ward and Ellar 1986) to be deleterious to *E. coli* cells.

The gene was therefore cloned under more stringent repression conditions as follows. Plasmid pJD16 (Fig. 1) was cleaved at the two *Acc*I sites, one in the polylinker downstream from *lacP* and the other upstream from the AUG codon of *cytA* itself, and religated after filling-in the non-matching sticky ends. Transformants were isolated on glucose-containing LB plates (with ampicillin). Colonies which did not grow on MacConkey replica

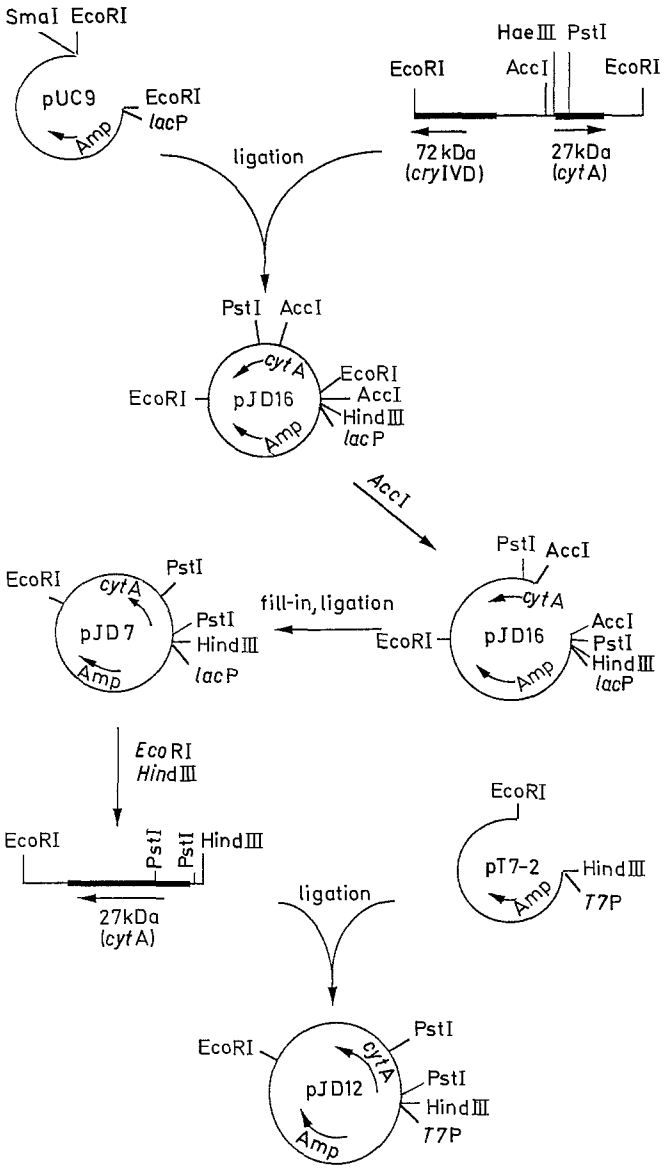

Fig. 1. Construction of plasmid pJD12. The plasmid contains the open reading frame of *cytA* controlled by *T7P*. Only relevant restriction sites are indicated. See text for further details

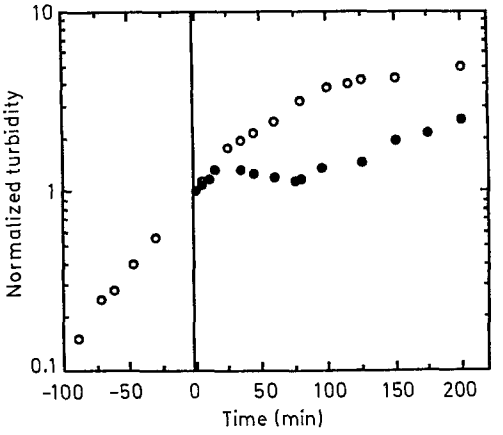

Fig. 2. Growth characteristics of clone BL21(pJD12) before and after induction of *cytA* expression. Relative growth of clone BL21(pJD12) (measured with Klett-Summerson Nephelometer, with red filter) before (○) and after (●) addition of 0.4 mM IPTG (at t=0). Values are normalized to 1.0 at t=0

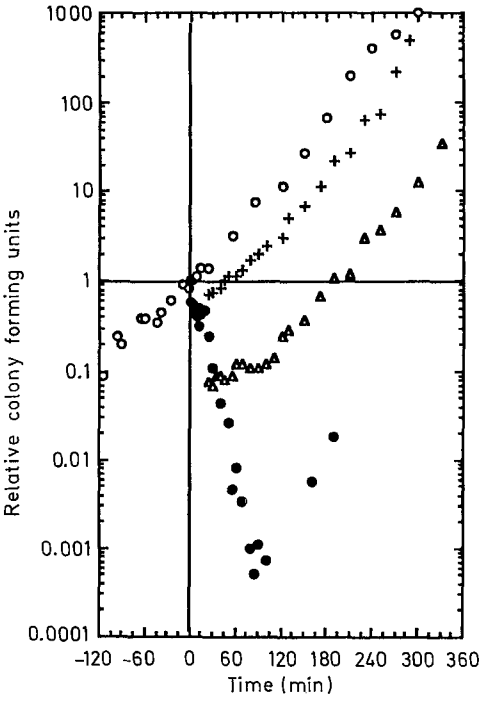

Fig. 3. Plating efficiencies of clone BL21(pJD12) before and after induction of *cytA* expression. Relative numbers of colony-forming units of clone BL21(pJD12), were determined before and after induction as in Fig. 2. Values are normalized to 1.0 at t=0. The exponentially-growing culture was diluted 100-fold at t=0 (○), after 4 min (+) and 6 min (△) into LB, and at t=0 into LB containing IPTG (●)

plates were suspected to express the *cytA* gene product in this *lacP*-inducing medium. The 2.1 kb *Hin*dIII-*Eco*RI fragment containing *cytA* was isolated from one of these clones (plasmid pJD7; Fig. 1), and ligated to plasmid pT7-2 (Studier and Moffatt 1986) preceded by a T7 promoter (*T7P*), to obtain clone pJD12. This promoter is stringently specific for RNA polymerase of phage T7, encoded by T7 gene *1*. This latter gene, under control of *lacP* (Studier and Moffatt 1986), is present as a single copy in the chromosome of *E. coli* strain BL21, which was transformed with pJD12. The resultant combination [named BL21(pJD12)] should have low expression levels of *cytA* when gene *1* is not induced, and produce high levels on polymerase induction. Thus, upon induction of gene *1*, its product T7 RNA polymerase would effectively and specifically transcribe *cytA* from the only *T7P* (Studier and Moffatt 1986) in BL21(pJD12).

The results of such an experiment are displayed in Figs. 2 and 3. Soon after addition of the gratuitous inducer IPTG (0.4 mM) to an LB culture of clone BL21(pJD12), the culture stopped increasing in mass, as estimated by turbidity (Fig. 2). In fact, a small (10–20%) reduction in turbidity was consistently evident between 15–60 min after induction, indicating possible cell lysis.

A more dramatic effect was demonstrated when the colony-forming ability of the induced culture was determined by plating on LB-agar plates (with ampicillin).

Viable cell number decreased sharply after induction by IPTG to a minimum of 0.1% at 90 min (Fig. 3). The increase in cell number after 2 h could be due to a low frequency of pre-existing down-promoter mutants in either *T7P* or *lacP*, which continued to grow and divide while the rest lost viability. The frequency of viable cells was estimated to be 5×10^{-5} from extrapolation of the viability curve back to time 0 (Fig. 3). The relatively high frequency of presumed spontaneous mutants of this type can be explained by high sensitivity of the cells to the *cytA* gene product, which may be produced even under repression of BL21(pJD12), albeit at exceedingly low levels. This interpretation is supported by the instability of this clone (data not shown; Luria and Delbruck 1943), which is therefore maintained in 15% glycerol at −70° C.

The tentative conclusion that the *cytA* gene product causes death of BL21(pJD12) is supported by the use of a control construct (kindly provided by A. Michaels). The *atpB* gene encoding a 56 kDa polypeptide from *Chlamydomonas reinhardtii* chloroplasts had been cloned into the same bacterial strain in an identical manner (Blumenstein et al. 1990). This clone was very stable, and both turbidity and viability of its IPTG-induced cultures reached levels corresponding to 20–40% of those of their non-induced counterparts (results not shown).

The behavior of BL21(pJD12) following induction of *cytA* (loss of colony-forming ability with no substantial lysis (Figs. 2 and 3)), is reminiscent of the 'thymineless-death' phenomenon (Cohen and Barner 1954). Unbalanced growth induced by specific inhibition of DNA synthesis (by either thymine starvation or certain drugs) results in 'sensitivity to plating' (Donachie & Hobbs 1967), similar to that observed here (Fig. 3). No mechanism has been described satisfactorily to explain 'sensitivity of plating'. The similarity between the phenotype associated with *cytA* gene expression and 'thymineless-death' was further substantiated by the following additional observations. The rate of DNA synthesis was reduced soon after onset of induction (Fig. 4). [No reduction was observed during 60 min induction of the control strain (data not shown).] The number of colony-forming units (which decreases sharply after onset of induction) returned to its control value, albeit slowly, following a short pulse-induction (Fig. 3), indicating 'liquid-holding recovery' (Donachie and Hobbs 1967).

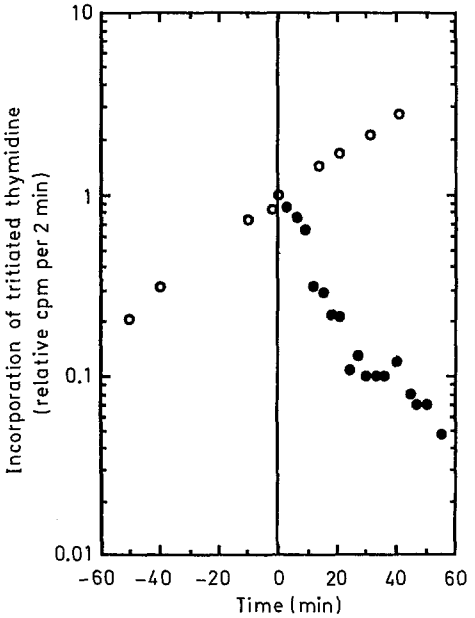

Fig. 4. Rates of DNA synthesis in cells of clone BL21(pJD12) before and after induction of *cytA* expression. Relative rate of DNA synthesis, determined by [^{3}H]-thymidine incorporation, before (○) and after (●) addition of 0.4 mM IPTG (at t = 0). Values are normalized as in Figs. 2 and 3

The rate at which cell division capacity was restored after a brief pulse-induction (Fig. 3) indicates that the 27 kDa polypeptide is not stable in *E. coli*, at least not in strain BL21(pJD12). This observation is in agreement with the conclusion that a 20 kDa polypeptide stabilizes the *cytA* gene product in other *E. coli* strains (Adams et al. 1989; Hofte and Whiteley 1989). This presumed interaction, which apparently protects the 27 kDa polypeptide, might be accompanied by masking of its lethal effect to the host cells by either a direct or an allosteric modification of its presumed active site. This hypothesis should be tested in the *E. coli* strain BL21, where abundant amounts of the 27 kDa polypeptide are expected after induction, by coexpressing the 20 kDa polypeptide.

It is tempting to speculate that in *E. coli* the target of the 27 kDa polypeptide, which is known to interact with phosphatidyl ethanolamine (PE; Thomas and Ellar 1983), could be the chromosome replication complex, the so called 'replisome' (Bleecken 1971), since one of the major membrane phospholipids in *E. coli* cells is PE (Cronan and Rock 1987) and the portion of *E. coli* membrane to which DNA is attached is enriched in PE (Ballesta et al. 1972). Further investigations in this direction are being performed in our laboratory.

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Note added in proof

The first manuscript of this paper was submitted to J. Bacteriol. in July, 1990, and has been witheld there for about seven months, during which time an important, relevant paper (J. Bacteriol. 173:1748–1756, 1991) was submitted by Dr. H.R. Whiteley, since deceased.

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