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Construction of cloning vectors for *Bacillus thuringiensis*

(Plasmid copy number; gene expression; δ-endotoxins; segregational stability; replication region; hydroxylamine mutagenesis; *Bacillus subtilis*)

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SUMMARY

The replication region of the *Bacillus thuringiensis* plasmid, pHT1030, was treated with hydroxylamine. Various copy-number mutants were selected and subsequently used to construct shuttle vectors with multiple cloning sites. These recombinant plasmids are very stable and allowed the cloning of a δ-endotoxin-encoding gene in *B. thuringiensis*. Comparison between gene expression level and vector copy-number indicated that a plateau in δ-endotoxin production is reached with a copy-number of about fifteen per equivalent chromosome.

INTRODUCTION

The Gram$^+$ bacterium *B. thuringiensis* is well known for its entomopathogenic properties which are due to crystal proteins produced during the sporulation phase (Lereclus et al., 1989b). The parasporal proteins (δ-endotoxins) are each classified as either CryI, II, III or IV depending on their activity spectrum against insect larvae (Höfte and Whiteley, 1989). Different *cry* genes encoding δ-endotoxins active against various orders of insects have been cloned, and electroporation procedures now make possible the analysis of their expression in their natural hosts and the construction of genetically engineered strains with improved properties for pest control.

In general, most small plasmids originating from Gram$^+$ bacteria replicate by an RC mechanism (cf. Gruss and Ehrlich, 1989, for review), and are frequently structurally unstable when used as cloning vectors (Jannière et al., 1987; Jannière and Ehrlich, 1990; Bron et al., 1991; Mc Dowell and Mann, 1991). It may therefore be advantageous to construct cloning vectors using replication regions from stable resident plasmids of *B. thuringiensis*, which do not replicate by an RC mechanism. This approach has been developed by using replication regions from large plasmids (Baum et al., 1990) and use of the 15-kb plasmid pHT1030 characterized for its segregational stability in *B. subtilis* (Lereclus et al., 1988) and for its different mode of replication than an RC mechanism (D.L. and O.A., unpublished results). A shuttle vector (pHT3101) was constructed with a 2.9-kb DNA fragment carrying the replication and sta-

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Abbreviations: Ap, ampicillin; *B.*, *Bacillus*; *bla*, β-lactamase-encoding gene from pUC19; bp, base pair(s); *cat*, gene encoding Cm acetyltransferase from pC194; Cm, chloramphenicol; Cry, crystal protein; *cry*, gene encoding Cry from *B. thuringiensis*; Δ, deletion; Er, erythromycin; *erm*, Er^R-encoding gene from Tn*1545*; HA, hydroxylamine; kb, kilobase(s) or 1000 bp; *lacZpo*, *lac* promoter-operator; LB, Luria–Bertani (medium); Ω, insertion; *ori*, origin of DNA replication; PAGE, polyacrylamide-gel electrophoresis; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistance/resistant; RC, rolling-circle; SDS, sodium dodecyl sulfate; Tn, transposon.

bility determinants of pHT1030 (Lereclus et al., 1989a). This chimeric plasmid has been successfully used in cloning experiments in *B. subtilis* and *B. thuringiensis* (Débarbouillé et al., 1990; A. Delécluse, personal communication; Lereclus et al., 1989a).

Recently, we have shown that recombinant plasmids carrying the replication region of pHT1030 have a low-copy-number (about 4 per equivalent chromosome) and display properties unlike those of the RC-type plasmids (D.L., unpublished data). Here we report the construction of novel shuttle vectors with various copy-numbers. These plasmids can be used to examine the influence of the plasmid copy-number on the expression of *cry* genes in *B. thuringiensis*.

EXPERIMENTAL AND DISCUSSION

(a) Shuttle vectors with various copy-numbers

(1) Removal of the Hin*dIII site from pHT1035*

Plasmid pHT1035 is a 6.3-kb plasmid bearing the replication region of the *B. thuringiensis* pHT1030 (2.9-kb *Bal*I DNA fragment), a *Hin*cII-*Bal*I DNA fragment containing

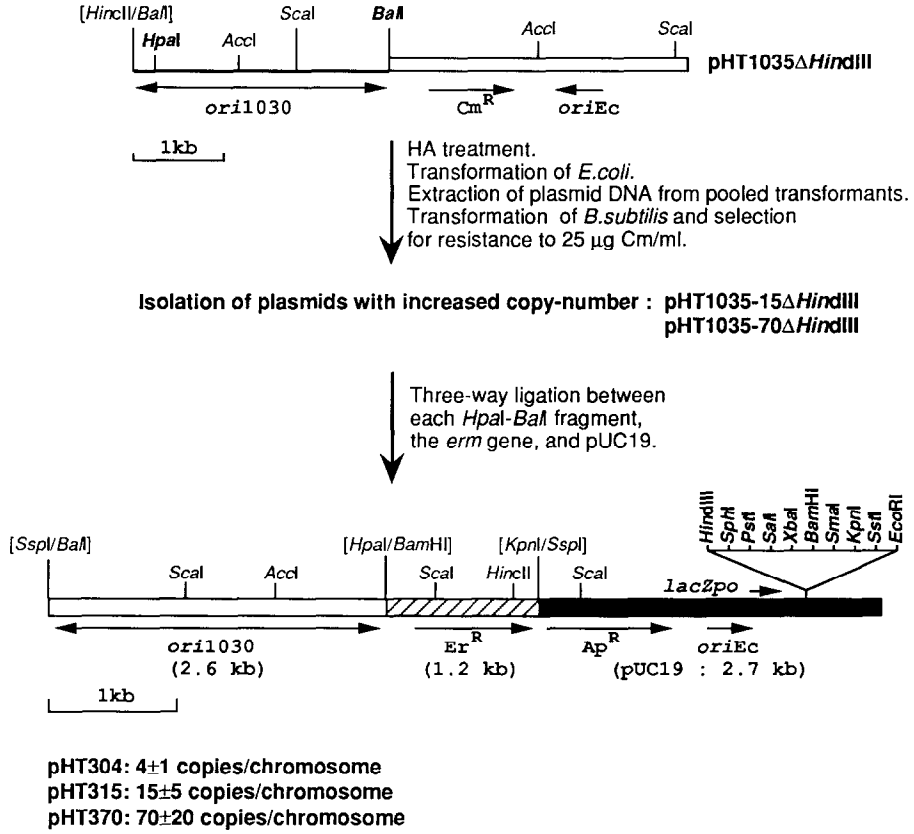

Fig. 1. Construction of shuttle vectors with various copy-numbers. The *Hin*dIII site of pHT1035 was removed by *Hin*dIII cleavage, filling-in the ends, and ligation. In vitro mutagenesis of pHT1035*ΔHin*dIII was carried out by HA treatment as previously described (Humphreys et al., 1976). A 20 µg of treated DNA was used to transform *E. coli* strain JM83. Approx. 10000 clones resistant to Cm (5 µg/ml) were pooled and plasmid DNA was extracted. Subsequently, competent *B. subtilis* cells were transformed with about 10 µg of plasmid DNA and plated on LB agar containing 25 µg of Cm per ml. From about 100 Cm^R clones obtained, 14 were tested for their plasmid content. Agarose gel electrophoresis of the DNA preparations indicated that nine of them harbored plasmids with a higher copy-number than the parental plasmid pHT1035*ΔHin*dIII (result not shown). Two clones harboring plasmids with intermediate (pHT1035-15*ΔHin*dIII) and high-copy-number (pHT1035-70*ΔHin*dIII) were selected. The 2.6-kb *Hpa*I-*Bal*I DNA fragment carrying the replication and stability regions of pHT1030 was isolated and purified separately from each of pHT1035*ΔHin*dIII, pHT1035-15*ΔHin*dIII and pHT1035-70*ΔHin*dIII. A 1.2-kb *Kpn*I-*Bam*HI fragment carrying the constitutive *erm* gene from Tn*1545* (Trieu-Cuot et al., 1990) was isolated as a *Asp*718-*Bam*HI fragment and the ends made blunt with PolIk. Each *Hpa*I-*Bal*I fragment was cloned at the *Ssp*I site of pUC19 with the fragment containing the *erm* gene in a three-way ligation. The three ligation mixtures were separately used to transform the *E. coli* strain JM83, and the recombinant clones were selected on LB agar plates containing Ap (100 µg/ml) and Er (150 µg/ml). The shuttle vectors, pHT304, pHT315 and pHT370, in which the different DNA fragments are cloned at the same place in the same orientation were identified by restriction mapping. The sites destroyed at the blunt junctions are represented in brackets. Arrows above Cm^R, Er^R, Ap^R and in front of *lacZpo* indicate the direction of transcription of the *cat*, *erm*, *bla* and *lacZ* genes, respectively. Arrow above *ori*Ec (*ori* from pBR322) indicates the direction of replication of pBR322. The plasmid copy-number in *B. thuringiensis* is indicated for each vector. The double arrow above *ori*1030 (*ori* from pHT1030) indicates that the orientation of the replication of pHT1030 is not known. The unique restriction sites of the plasmids are shown in bold type.

the *cat* gene of pC194, and the *ori* of pBR322 (Lereclus et al., 1988). The unique *Hin*dIII site of pHT1035 was removed (Fig. 1) in order to avoid any duplication of the restriction sites of pUC19 (Yanisch-Perron et al., 1985) used for the construction of the shuttle vectors. The modification at the *Hin*dIII site does not impede the replication and stability functions of the plasmid, since pHT1035*ΔHin*dIII was able to transform *B. subtilis* strain 168 (Anagnostopoulos and Spizizen, 1961) to Cm^R (10 μg/ml), and 90% of cells contained the plasmid after about 25 generations in nonselective LB medium. Thus, the frequency of generating a plasmid-free cell in *B. subtilis* is about 4×10^{-3}.

(2) Selection of high-copy-number derivatives of pHT1035Δ Hin*dIII*

The pHT1035*ΔHin*dIII plasmid DNA was treated with HA (Fig. 1) and was then used to transform *E. coli* cells in order to recover convenient plasmid DNA molecules (multimeric forms) for transforming *B. subtilis*. Plasmids with an increased copy-number were then selected by plating the transformed *B. subtilis* cells onto LB agar medium containing a concentration of Cm (25 μg/ml) preventing the growth of the cells harboring the low-copy-number pHT1035*ΔHin*dIII (4 ± 1 copies per equivalent chromosome).

Two plasmids with an increased copy-number were selected for further study (Fig. 1). Their copy-numbers were determined by densitometric analysis of gel electrophoretograms using the parameters described by Projan et al. (1983). The plasmids were designated pHT1035-15*Δ* *Hin*dIII and pHT1035-70*ΔHin*dIII and have 15 ± 5 and 70 ± 20 copies per equivalent chromosome, respectively. They are segregationally stable since 100% of cells contained the plasmid after about 25 generations in nonselective LB medium (the frequency of generating a plasmid-free cell was found to be less than 4×10^{-4}).

(3) Construction of the shuttle vectors

The replication and stability regions of pHT1030 were shown to be entirely included in the 2.6-kb *Hpa*I-*Bal*I fragment carried by pHT1035 (Fig. 1; D.L., unpublished results). Consequently, this DNA fragment was isolated from each of the plasmids, pHT1035*ΔHin*dIII, pHT1035-15*ΔHin*dIII and pHT1035-70*ΔHin*dIII and separately used to construct pHT304, pHT315 and pHT370, respectively (Fig. 1).

These plasmids can be used in the same way as pUC19 for cloning experiments in *E. coli* since the fragments were inserted at the *Ssp*I site interrupting neither the polylinker cloning sites nor the *bla* gene. No site of the pUC19 polylinker is duplicated.

These shuttle vectors were used to transform the *B. thuringiensis* strain *kurstaki* HD1 Cry^-B by the electroporation procedure as previously described (Lereclus et al., 1989a). The recombinant clones were selected on LB agar plates containing Er (25 μg/ml). Densitometric analysis of total DNA preparations extracted from these clones showed that the three shuttle vectors had a copy-number in *B. thuringiensis* similar to that of the corresponding parental plasmids in *B. subtilis* (Fig. 1).

(b) Relationship between δ-endotoxin production and plasmid copy-number

A 3-kb *Hin*dIII restriction fragment, carrying a *cryIIIA gene isolated from the coleopteran-specific B. thuringiensis* strain LM79 (J. Chaufaux, M. Marchal and D.L., unpublished data), was subcloned at the *Hin*dIII site of each of the vectors pHT304, pHT315 and pIIT370. The resulting three plasmids (pHT304Ω*cryIIIA*, pHT315Ω*cryIIIA* and pHT370Ω*cryIIIA*), carrying the *cryIIIA* gene in the same orientation, were introduced into the *B. thuringiensis* strain *kurstaki* HD1 Cry^-B by electroporation (about 5×10^2 transformants were obtained per μg of each plasmid DNA).

The recombinant clones were grown for two days at

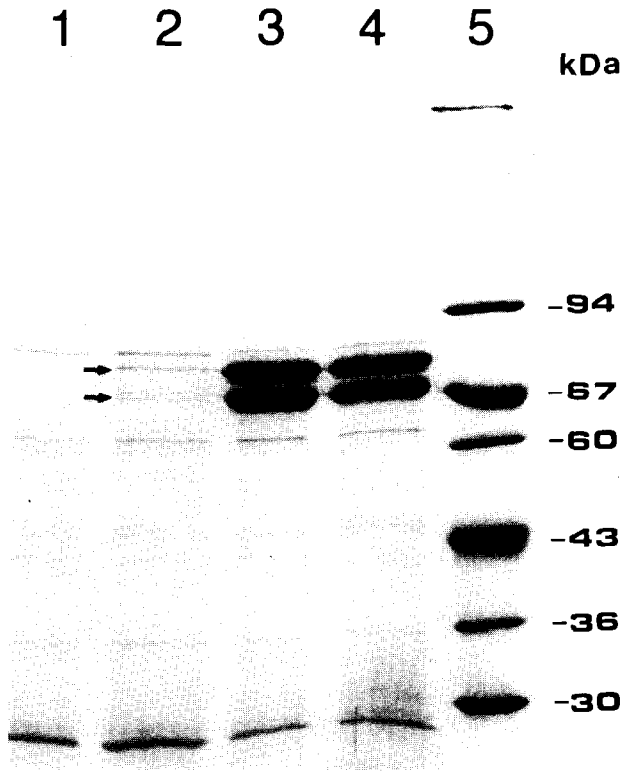

Fig. 2. Protein analysis of *B. thuringiensis* transformants expressing the *cryIIIA* gene. Spore-crystal preparations were obtained and analysed by 0.1% SDS–10% PAGE and Coomassie blue staining as previously described (Lereclus et al., 1989a). An identical volume of sample (20 μl) was loaded in each well. Lanes **1–4**, strain *kurstaki* HD1 Cry^-B carrying pHT315; pHT304Ω*cryIIIA*; pHT315Ω*cryIIIA* and pHT370Ω*cryIIIA*, respectively. Lane **5**, molecular weight markers. Arrows indicate the crystal components of 73 and 67 kDa.

30°C in HCT medium (Lecadet et al., 1980) in the presence or absence of 25 μg of Er per ml. Spore-crystal preparations harvested from the cultures were examined by phase-contrast microscopy and by SDS–PAGE (Fig. 2). The *kurstaki* HD1 Cry⁻B recombinants harboring pHT304Ω*cryIIIA* produced no detectable parasporal inclusions, whereas the cells harboring either pHT315Ω*cryIIIA* or pHT370Ω*cryIIIA* produced flat rhomboid crystals characteristic of the strains active against coleopteran larvae (Herrnstadt et al., 1986). The major components of these crystals were two polypeptides of about 73 kDa and 67 kDa (Fig. 2). This is in agreement with previous results which indicated that the *cryIIIA* gene from the *B. thuringiensis* strain *tenebrionis* produced 73-kDa and 65-kDa peptides (Sekar et al., 1987). The 65-kDa component might result from proteolytic cleavage of the 73-kDa peptide. Small amounts of these polypeptides were found in the spore preparations recovered from the bacteria harboring pHT304Ω*cryIIIA* (Fig. 2, lane 2).

The results shown in Fig. 2 correlate with the quantity of proteins contained in the spore and spore-crystal preparations, as estimated by the procedure of Lowry et al. (1951). In comparison with bacteria carrying the vector alone, no significant difference of protein production was detected with bacteria carrying pHT304Ω*cryIIIA*, both produced 0.11 ± 0.01 mg of protein per ml. However, the bacteria harboring pHT315Ω*cryIIIA* or pHT370Ω*cryIIIA* produced 0.29 ± 0.03 mg of protein per ml. Therefore about 0.18 mg CryIIIA protein is produced by these recombinant clones during sporulation. The important difference in toxin production obtained with pHT304Ω*cryIIIA* and pHT315Ω*cryIIIA* strongly suggests that the level of gene expression is not exclusively related to the vector copy-number, but can result from additional factors. The existence of a repressor can be hypothesized as an additional factor, only detectable when the *cry* gene is present at a low-copy-number in the cell. The repressor being titrated and then inefficient when the *cryIIIA* gene is cloned in a high-copy-number plasmid.

In native *B. thuringiensis* strains, the *cry* genes are located on large low-copy-number plasmids; however, several copies of *cry* genes are generally found in a given strain (cf. Lereclus et al., 1989b, for review). This is the case for the strain LM79 which contains at least two copies of the *cryIIIA* gene (D.L., unpublished results). This natural situation is therefore in agreement with our results suggesting a relation (although indirect) between *cry*-gene expression and *cry*-gene copy-number.

There was no significant difference between the amounts of δ-endotoxin produced by the cells harboring pHT315Ω*cryIIIA* and those harboring pHT370Ω*cryIIIA*, thus indicating that above fifteen copies of plasmid per chromosome, factors other than *cry*-gene copy-number limit the synthesis of crystal protein.

It is worth noting that the production of δ-endotoxins in the recombinant strains is not affected by the presence or absence of Er, suggesting that plasmids are stably maintained in the host cells. Indeed, after about 15 generations of growth followed by sporulation in HCT medium without antibiotic, all of the spores tested contained the plasmid. One hundred colonies started from spores were tested for each recombinant strain. The results showed that plasmid-less segregants arise at an inferior frequency of 7×10^{-4} per generation.

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