DETECTION OF SINGLE-STRANDED COHESIVE ENDS IN THE GENOME OF *BACILLUS THURINGIENSIS* TEMPERATE PHAGE KK-88

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Summary. – Cohesive ends (*cos* sites) were detected in the genome of temperate KK-88 phage in *Bacillus thuringiensis* after analysis of the phage DNA generated by the 3'-5' exonuclease activity of Klenow fragment of DNA polymerase I. In addition, unlike the 5'-protruding ends of coliphage lambda genome, the ends of KK-88 phage genome were found to be 3'-protruding. The restriction map of the phage genome was also constructed on the basis of position of the *cos* fragments.

Key words: Bacillus thuringiensis; temperate phage KK-88; 3'-protruding cohesive ends; restriction map

Bacillus thuringiensis is a Gram-positive soil bacterium that is known to produce an insecticidal crystalliferous toxin (delta-endotoxin) thought to play an important role in the microbial control of pest insects. Since various strains of *B. thuringiensis* are found to be naturally associated with temperate phages, it has been suggested that temperate phages could serve as potential transduction vehicles in studies aimed at characterizing this bacterium genetically (Lecadet *et al.*, 1980; Landen *et al.*, 1981; Heierson *et al.*, 1983; Barsomian *et al.*, 1984; Walter and Aronson, 1991).

We have previously described the association of *B. thuringiensis* strain AF101 with two temperate phages, KK-88 and J7W-1. Although both phages can lysogenize the same bacterial cell, they can be induced selectively using UV irradiation and ethidium bromide treatment, respectively (Kanda and Aizawa, 1989). Furthermore, there exist characteristic differences in the nature of lysogenic state of the two temperate phages. This is especially true for J7W-1 phage which integrates its genome into a plasmid of host bacterium (Kanda *et al.*, 1989, 1998).

Studies in this laboratory were focused on how these phage genomes are organized. It is hoped that such studies will lead to the use of these phages as valuable molecular tools for the genetic analysis of *B. thuringiensis*. In the present study we have determined the cohesive ends (*cos* site) and constructed the restriction map of the genome of the UV inducible KK-88 phage.

Temperate KK-88 phage was induced from *B. thuringiensis* strain AF101 according to Kanda *et al.* (1989). The phage particles were purified by isopycnic centrifugation in CsCl density gradient at 55,000 rpm for 4 hrs at 20°C using a HITACHI RP100NT rotor. DNA was then extracted from the phage particles (density 1.46 g/ml) with 0.1% w/v sodium dodecyl sulfate (SDS) for 30 mins at 37°C, followed by phenol/chroloform extraction and ethanol precipitation. Probe DNA for use in Southern blot hybridization experiments was labelled with digoxygenin-dUTP according to the method recommended by the manufacturer (Boehringer Mannheim). Restriction enzymes and other enzymes used in this study were purchased from Boehringer Mannheim.

In experiments designed to detect the cohesive ends of the KK-88 phage genome, *Xba*I digests of the phage DNA were heat-treated at 65°C for 15 mins in order to dissociate any ends joined by hydrogen bonds. The *Xba*I digests without heat treatment served as controls. The restriction patterns of the unheated *Xba*I digests showed the presence of a 4.4 kbp fragment, however, a decrease in its amount was observed with the concomitant appearance of two new smaller fragments of 0.5 kbp and 3.9 kbp in

Abbreviations: dNTP = deoxynucleoside triphosphate; SDS = sodium dodecyl sulfate

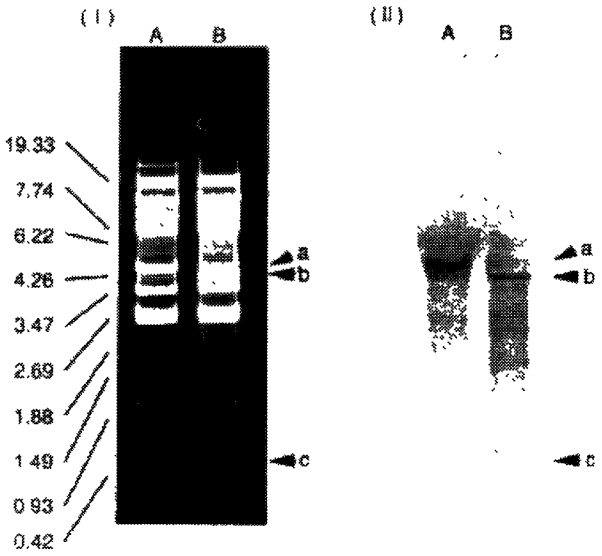

Fig. 1
Detection of the KK-88 genome *cos* site
I: Agarose gel electrophoresis. KK-88 phage DNA *Xba*I digests before (lane A) and after heat treatment (lane B). Size markers (kbp) shown on the left. II: Southern blot hybridization of the same gel probed with the 4.4 kbp *Xba*I fragment Black arrowheads indicate the 4.4 kbp (a), 3.9 kbp (b), and 0.5 kbp (C) fragments.

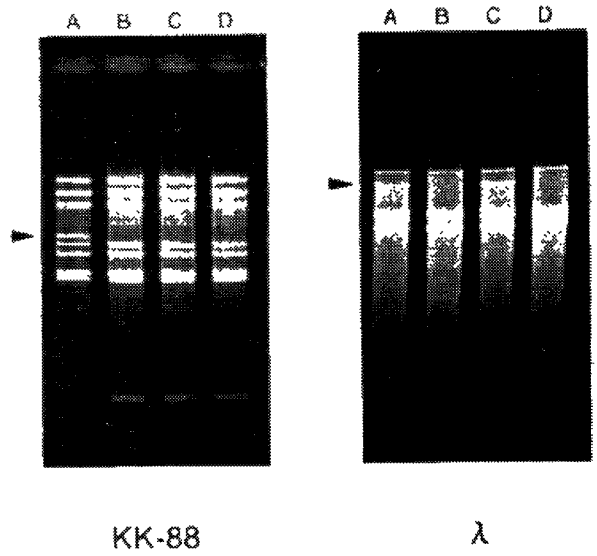

Fig. 2
The ability of *cos* site-containing fragments of *Xba*I KK-88 phage DNA digest and *Bam*HI lambda phage DNA digest to reanneal after enzymatic treatments
No enzymatic treatment (lanes A). Mung bean nuclease treatment (lanes B). Klenow fragment treatment without dNTPs (lanes C) Klenow fragment treatment with dNTPs (lanes D).
Black arrowheads indicatethe fragments containing reannealed *cos* site

the heat-treated *Xba*I digests (Fig. 1, I). Furthermore, both the 0.5 kbp and 3.9 kbp fragments were found to hybridize with the 4.4 kbp fragment (Fig. 1, II). These results suggest that the KK-88 phage genome is linear with cohesive termini on the 0.5 kbp and 3.9 kbp *Xba*I fragments, usually observed as the end-joined 4.4 kbp DNA on the agarose gel.

The structure of the termini of KK-88 phage genome was further characterized by Mung bean nuclease and the Klenow fragment of DNA polymerase I. Mung bean nuclease is specific for single-stranded DNA. The Klenow fragment can have either exonuclease activity from 3'-OH terminus or 5' to 3' polymerase activity depending on the template DNA in the presence of deoxynucleoside triposphates (dNTPs). Intact phage DNA was first passed through two heat-cool cycles (65°C for 15 mins and 0°C for 15 mins) to separate the cohesive ends before a short incubation (no longer than for 5 mins) at 37°Cwith the appropriate enzyme. After heating at 65°C for 10 mins to terminate the enzymatic reactions, the DNA was extracted with phenol/chloroform and ethanol-precipitated. The enzyme-treated phage DNAs were further digested with the appropriate restriction enzyme (*Xba*I for KK-88 and *Bam*HI for lambda phages). The samples were again heated at 65°C for 30 mins to separate the ends and then incubated at 25°C for at least 1 hr to allow for reannealing.

The results of these experiments are summarized in Fig. 2. The coliphage lambda, known to have 5'-protruding cohesive ends of 12 bp in length (Wu and Taylar, 1971), was used as control. When the *Xba*I digest of linearized KK-88 phage DNA was treated with Mung bean nuclease, the ability of the 0.5 kbp and 3.9 kbp fragments to reanneal and form the end-joined 4.4 kbp fragment was abolished (lanes A and B). Thus, it was again confirmed that the phage genome possesses single-stranded cohesive ends in the 4.4 kbp *Xba*I fragment.

The polarity of protruding ends of the phage genome was also analyzed by the ability of the generated ends to reanneal after a treatment with Klenow fragment in the absence or presence of dNTPs. It was found that the ends of KK-88 phage genome were degraded by this treatment in the absence of dNTPs, in contrast to those of coliphage lambda genome (lanes A and C). When the phage DNA was treated with the Klenow fragment in the presence of dNTPs, as predicted, the synthesis of chains complementary to the single-stranded ends resulted in the inability of the ends to reanneal. Furthermore, the single-stranded ends of KK-88 phage DNA were also unable to reanneal after this treatment (lanes A and D).

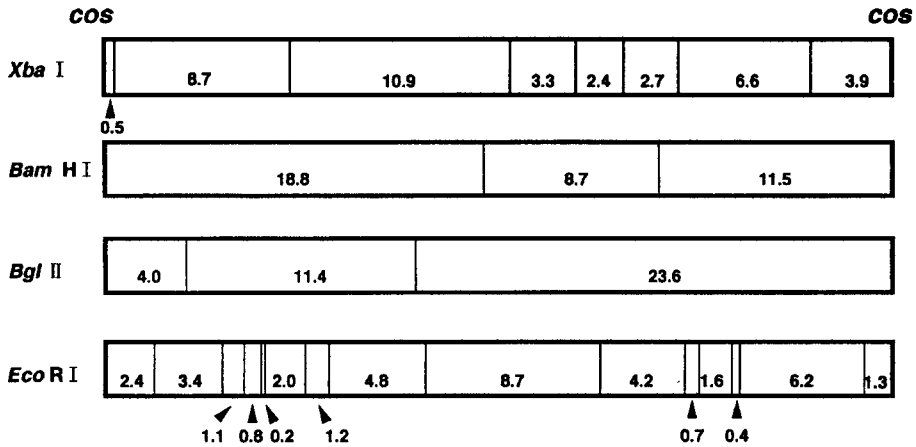

Fig. 3
The restriction map of the 39.0 kbp KK-88 phage genome

Therefore, using KK-88 phage DNA as substrate, the Klenow fragment acted only as an exonuclease even in the presence of dNTPs (as in case of the treatment without dNTPs). We thus conclude that unlike the 5'-polarity of the lambda phage genome, the KK-88 phage genome possesses 3'-protruding single-strand cohesive ends. Our data are compatible with the findings that temperate phages in genus *Bacillus*, e.g. temperate phage ϕ 105 of *B. subtilis,* also possess 3'-protruding ends (Ellis and Dean, 1985).

Having determined which *Xba*I restriction fragments of KK-88 phage DNA (0.5 kbp and 3.9 kbp) contained the *cos* site, we attempted to construct a restriction map of the genome. Starting with the fact that the *Xba*I fragments containing the *cos* site had to be situated on both ends of the map, the sequence and relative positions of the other *Xba*I fragments were determined using partial and/or double restriction enzyme digestion with *Xba*I, *Bam*HI, *Bgl*II and *Eco*RI. The relative positions on the map of the restriction sites generated by these four enzymes were confirmed after hybridization of the digests with each other (Fig. 3). Based on the mean values of the size of the restriction digests generated by *Xba*I, *Bam*HI, *Bgl*II and *Eco*RI, we estimated the size of the genome at 39.0 kbp.

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