A Novel Phage Genome Integrated into a Plasmid in *Bacillus thuringiensis* Strain AF101

By KOHZO KANDA*, YEPIN TAN† AND KEIO AIZAWA

Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan

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Bacillus thuringiensis strain AF101 possesses a single plasmid (pAF101) with a molecular size of 42 MDa (69 kb). During plasmid curing experiments in strain AF101, we found that a phage (J7W-1) was induced by ethidium bromide treatment. Moreover, the phage genome (48 kb) hybridized only with pAF101 on a Southern blot of the DNA of a cleared lysate prepared from strain AF101. Comparison of the restriction patterns of pAF101 and J7W-1 phage DNA revealed that pAF101 contains not only the entire phage DNA but also a plasmid-specific DNA region. These results indicate that the J7W-1 genome has been stably integrated into pAF101 in strain AF101. Integration of the J7W-1 genome into a plasmid was also observed after phage infection of the type strain of *B. thuringiensis* subsp. *israelensis*.

INTRODUCTION

Bacillus thuringiensis is well known for the production of parasporal inclusions (crystals) toxic to lepidopterous and dipterous insects, and it has become one of the most important non-chemical agents for the control of insect pests. The selection and breeding of effective strains of this bacterium is important to develop its utilization potential as a microbial insecticide (Aizawa, 1971). Among these effective strains, *B. thuringiensis* strain AF101 (H-serotype 4a:4b) has been selected as a strain with low toxicity to the silkworm, *Bombyx mori*, but highly toxic to the common cabbageworm, *Pieris rapae crucivora*, the fall webworm, *Hyphantria cunea*, and the diamondback moth, *Plutella xylostella* (Aizawa *et al.*, 1975). This strain is suitable for use as an insecticide in sericultural countries, particularly in Asia.

Recently, molecular biological approaches have been adopted in order to improve strains of *B. thuringiensis*. These have particularly concentrated on the crystal gene, which has been revealed to be plasmid-located, by plasmid curing (González *et al.*, 1981; Lereclus *et al.*, 1982), plasmid transfer (González *et al.*, 1983; Klier *et al.*, 1983; Battisti *et al.*, 1985), and gene cloning (summarized by Aronson *et al.*, 1986). Various strains of *B. thuringiensis* harbour a large array of plasmids; however, the genetic information located on these plasmids is not known except for the presence of the crystal gene and possibly genes associated with phage-like particles (Tam & Fitz-James, 1986).

In the present study, we investigated the biological function of a plasmid (pAF101) which is associated with crystal production in *B. thuringiensis* strain AF101 and describe the presence of a prophage in pAF101.

METHODS

Strains. *Bacillus thuringiensis* strain AF101 (H-serotype 4a:4b) reported by Aizawa *et al.* (1975) and type strains of *B. thuringiensis* used in this study were obtained from culture collections maintained in this institute.

Media. BHI broth contained 37 g brain-heart infusion (Difco) l⁻¹ (pH 7.2). LB broth was prepared as described by Maniatis *et al.* (1982). For solid medium, 15 g agar (Difco) l⁻¹ was added to LB broth. Soft agar for the assay of phage consisted of LB broth containing 0.7% agar.

† Present address: Department of Virology and Molecular Biology, Wuhan University, Wuhan, China.

Extraction and purification of plasmid. The extraction of the plasmid from *B. thuringiensis* strain AF101 was done according to the method of Battisti *et al.* (1985), except that proteinase K (Merck) was used instead of lysozyme. Plasmid in cleared lysate was purified by CsCl/ethidium bromide centrifugation (Maniatis *et al.*, 1982). The electrophoresis of DNA was done on a 0.7% agarose gel in TAE buffer (0.04 M-Tris/acetate, 2 mM-EDTA, pH 8.0) at 50 V for 120 min at room temperature.

Induction of phage. A cell suspension cultivated at 27 °C for 16 h in LB broth was inoculated (1:100) into fresh LB broth containing 0.5–5 µM-ethidium bromide, and cultured at 27 °C with continuous shaking. Cell growth was examined by measuring the optical density (660 nm) of the cell culture at 1 h intervals. The phage suspension was prepared from the cell lysate by centrifugation at 20000 *g* for 1 h at 4 °C, after removing cell debris by centrifugation at 10000 *g* for 20 min at 4 °C. Phage particles were purified by 10–40% sucrose density-gradient centrifugation using a Spinco SW25-1 rotor at 23000 r.p.m. for 1 h at 4 °C. Phage DNA was extracted by phenol treatment after incubation of the phage particles with 0.2% SDS at 37 °C for 30 min.

Phage infection. The infectivity of the phage was examined by a spot test. Cells were cultivated in LB broth at 27 °C until the mid-exponential phase and inoculated into a top layer of 3 ml 0.7% soft LB agar on an LB agar plate. Samples (50 µl) of phage suspension (approximately 7×10^6 p.f.u. ml⁻¹) were spotted on the surface and the lytic response of cells to the phage was scored after incubation of the plate at 27 °C for 24 h.

Southern blotting and hybridization. Plasmid and chromosomal DNAs in cleared lysates were separated on 0.7% agarose gels and transferred to nitrocellulose membrane (Sartorius) by the Southern blotting technique (Maniatis *et al.*, 1982). Peroxidase-labelled *Hind*III fragments of phage J7W-1 DNA were used as a hybridization probe. The enzyme-labelled DNA was prepared by using the Labezyme-POD set (Wako Pure Chemical Industries) and DNA-DNA hybridization was done according to the procedure recommended by the supplier.

Restriction enzyme analysis of plasmid and phage DNA. Plasmid and phage DNAs purified by CsCl/ethidium bromide centrifugation were digested with restriction endonucleases (Nippon Gene). The reaction conditions were as recommended by the supplier. Digests were analysed by 0.7% agarose gel electrophoresis.

RESULTS

Plasmid content of strain AF101

The cleared lysate was examined to detect a plasmid array in strain AF101 by agarose gel electrophoresis. This strain harboured only one type of plasmid, which we termed pAF101. The size of pAF101 was estimated to be 42 MDa (69 kb) by comparison with plasmids whose sizes were already known in the type strain of *B. thuringiensis* subsp. *thuringiensis* (González & Carlton, 1980).

To investigate the biological function of this plasmid, curing of pAF101 from strain AF101 was attempted by treatment with SDS and ethidium bromide. After cultivation of cells for 8 h at 27 °C in LB broth containing 0.002% SDS, one plasmid-free strain (TS-1) was selected as an acrySTALLIFEROUS strain. Strain TS-1 was confirmed as H-serotype 4a:4b, the same as the parental strain AF101, by flagellar agglutination.

Phage induction by ethidium bromide

In plasmid curing experiments, it was observed that cell lysis was induced in strain AF101 during culture in LB broth containing 0.5 µM and 5 µM-ethidium bromide (Fig. 1). Growth was completely inhibited in the presence of 50 µM-ethidium bromide. Since the cell lysis was considered to be caused by induction with ethidium bromide of a temperate phage or bacteriocin, the cell lysate was examined by electron microscopy. Phage particles (Fig. 2) were detected in the cell lysate and were found to form a single band upon sucrose density-gradient centrifugation of the lysate. Thus, it was concluded that the cell lysis during culture with ethidium bromide occurred upon the induction of one kind of temperate phage, which we termed J7W-1.

On the other hand, the phage was not induced in the plasmid-free strain TS-1 at a concentration of 1 µM-ethidium bromide. This result suggests that the induction of J7W-1 by ethidium bromide in strain AF101 depends on the presence of pAF101.

Host range and properties of J7W-1

The host range of J7W-1 was examined using 26 type strains of *B. thuringiensis* by spotting the phage suspension onto fresh lawns of each strain in soft LB agar. Two type strains (subsp. *sotto*

Fig. 1

Fig. 2

Fig. 1. Effect of ethidium bromide on the growth of strain AF101. The optical density of cell cultures in LB broth containing 0 μM (●), 0.5 μM (□), 5 μM (◇) and 50 μM (▽) ethidium bromide was measured at 660 nm.

Fig. 2. Election micrograph of phage J7W-1 detected in a cell lysate obtained by ethidium bromide treatment of strain AF101. The sample is stained with 1% (w/v) uranyl acetate. Bar, 200 nm.

and subsp. *israelensis*) showed a lytic response to infection by J7W-1. The sensitivity of these strains to J7W-1 was reduced if stationary-phase cells were used to prepare the top layers (data not shown). The other 24 type strains tested (subsp. *thuringiensis*, subsp. *finitimus*, subsp. *alesti*, subsp. *kurstaki*, subsp. *dendrolimus*, subsp. *galleriae*, subsp. *canadensis*, subsp. *entomocidus*, subsp. *subtoxius*, subsp. *aizawai*, subsp. *morrisoni*, subsp. *ostrinae*, subsp. *tolworthi*, subsp. *caucasicus*, subsp. *darmstadiensis*, subsp. *toumanoffii*, subsp. *kyushuensis*, subsp. *thompsoni*, subsp. *pakistani*, subsp. *dakota*, subsp. *indiana*, subsp. *tohokuensis*, subsp. *kumamotoensis*, and subsp. *tochigiensis*) showed no lytic response.

Phage J7W-1 has a head diameter of 65 nm and a tail length of 290 nm and is different morphologically from other temperate phages hitherto reported in *B. thuringiensis* (Colasito & Rogoff, 1969; Iwahana, 1976).

The molecular sizes of fragments produced by digestion of the J7W-1 genome with *EcoRI*, *HindIII*, *BglII* and *PstI* gave values of 48.7, 46.6, 48.8 and 47.9 kb, respectively, for the total size of the phage genome. The molecular size of the J7W-1 genome was estimated to be 48.0 kb, as the mean of these four values.

Location of phage genome

As the induction of J7W-1 appeared to be associated with pAF101, the presence of regions in the chromosome and/or the plasmid homologous to phage DNA was examined by Southern hybridization of cleared lysate against a J7W-1 DNA probe. J7W-1 DNA was able to hybridize to pAF101, but not to the chromosome (Fig. 3). Moreover, comparison of their restriction digests showed that pAF101 contained not only all the J7W-1 DNA fragments but also

Fig. 3. Location of the J7W-1 genome in strain AF101. DNAs in a cleared lysate of strain AF101 were separated by agarose gel electrophoresis (A). A Southern blot of these DNAs was probed with *Hind*III-digested J7W-1 DNA (B). The positions of pAF101 and chromosomal DNA (chr.) are indicated.

Fig. 4. Restriction patterns of pAF101 (A) in comparison with J7W-1 DNA (B). The leftmost two lanes are intact pAF101 and J7W-1 DNA. White arrowheads show DNA fragments corresponding to phage DNA. The size markers (in kb) on the right indicate the *Hind*III fragments of phage λ DNA.

additional plasmid-specific DNA fragments (Fig. 4). These results strongly suggest that the J7W-1 genome is integrated into pAF101.

The location of the prophage was also investigated in J7W-1 lysogens of the type strain of *B. thuringiensis* subsp. *israelensis*. Lysogenized strains were isolated from surviving colonies on an agar plate after infection by J7W-1, and these strains were tested to see whether they produced J7W-1 upon ethidium bromide induction. In all lysogens of subsp. *israelensis*, a newly acquired plasmid was observed (Fig. 5) and, as expected, it hybridized with J7W-1 genomic DNA (Fig. 6).

Fig. 5. Agarose gel electrophoresis of DNAs in cleared lysates prepared from the type strain of *B. thuringiensis* subsp. *israelensis* (A), lysogens (B-E) and intact J7W-1 DNA (F). The white arrowhead indicates chromosomal DNA.

Fig. 6. Location of the J7W-1 genome in a lysogenic strain of *B. thuringiensis* subsp. *israelensis*. (a) Agarose gel electrophoresis of DNAs in the cleared lysate prepared from subsp. *israelensis* (A) and a J7W-1 lysogen (B). (b) Southern blot of the same gel probed with *Hind*III-digested J7W-1 DNA. The white arrowhead indicates the new plasmid homologous to J7W-1 DNA in the lysogenic strain.

DISCUSSION

We have found that plasmid pAF101 in *B. thuringiensis* strain AF101 contains a novel temperate phage genome.

It has been reported that the crystal gene is located on plasmids in most subspecies of *B. thuringiensis*. Strain AF101 appears to possess a single species of plasmid, as is the case for the type strain of subsp. *sotto*, whose crystal gene has been cloned from a plasmid (Shibano *et al.*, 1985). The present study has shown that a plasmid-cured derivative of strain AF101 lost the ability to produce crystals. This suggests that pAF101 may be closely associated with crystal production (although the possibility that a second, undetected, plasmid is involved cannot be excluded). The location of any crystal gene in pAF101 remains to be resolved.

Ethidium bromide caused phage induction in strain AF101. Phage induction by ethidium bromide has not, to our knowledge, been previously reported. Phage induction in *B. thuringiensis*, has been reported to occur as a result of UV irradiation and treatment with

hydrogen peroxide or mitomycin C in strains of subsp. *galleriae* (Colasito & Rogoff, 1969), by UV irradiation, mitomycin C and aflatoxin B₁ treatment in a strain of subsp. *tolworthi* (Affray & Boutibonnes, 1985), and by co-cultivation of crystalliferous strain T84-A1 and acrySTALLIFEROUS strain S-16 both belonging to H-serotype 4a:4b (Iwahana, 1976). It appears that treatment with ethidium bromide can induce the phage from the lysogenic strain since it binds to DNA and inhibits DNA synthesis *in vivo*, in a similar manner to mitomycin C (Bouanchaud *et al.*, 1968). The novel phage, J7W-1, revealed to be inducible by ethidium bromide in this study, has a relatively narrow host range, i.e. the type strains of subsp. *sotto* and subsp. *israelensis*. It is noteworthy that both strains which are susceptible to infection with J7W-1 possess the same heat-stable somatic antigen (Mike, 1981).

In the lysogenic state, the genomes of most temperate phages are integrated into the host chromosome, while some, such as the prophages of P1 (Ikeda & Tomizawa, 1968) and N15 (Ravin & Shulga, 1970), exist as autonomous plasmids in the host cell. The present study demonstrates that the J7W-1 genome is integrated into plasmid pAF101 in strain AF101. This strain appears to have a single plasmid; no other satellite DNA molecules, including free J7W-1 genome, were detected. This indicates that J7W-1 prophage persists in strain AF101 as an integral part of pAF101; further study on the integration site of J7W-1 in pAF101 is in progress. Furthermore, J7W-1 prophage was present in a plasmid form in J7W-1 lysogens of subsp. *israelensis*, since a new plasmid, which hybridized with the J7W-1 genome, was detected in agarose gel electrophoresis. It is not yet clear whether the prophage is integrated into a pre-existing plasmid in these circumstances, or whether it forms an independent plasmid.

According to the evidence presented here, we suggest that phage J7W-1 could be a novel type of phage which shows a plasmid-dependent prophage state during lysogeny in *B. thuringiensis*.

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