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Similarity in moth-fly specific larvicidal activity between two serologically unrelated *Bacillus thuringiensis* strains

Kazuhiko Higuchi [a], Hiroyuki Saitoh [a], Eiichi Mizuki [a,*], Michio Ohba [b]

[a] *Section of Applied Microbiology, Biotechnology and Food Research Institute, Fukuoka Industrial Technology Center, Aikawa-machi 1465-5, Kurume, Fukuoka 839-0861, Japan*

[b] *Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan*

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Abstract

Parasporal inclusions of a *Bacillus thuringiensis* isolate designated 92-KU-105-9 (H14/19) exhibited unusual larvicidal activity, specific for the moth-fly, *Telmatoscopus albipunctatus* (Diptera: Psychodidae), similar to that of a previously reported *B. thuringiensis* serovar *leesis* (H33) strain. The LC_{50} value of the purified inclusions was 4.92 μg ml^{-1} for the moth-fly larvae, while no mortality was shown in the mosquitoes *Culex pipiens molestus* and *Anopheles stephensi*, at protein concentrations up to 10 mg ml^{-1}. Morphologically, the inclusion was a homogeneous globular body surrounded by an electron-dense, thick envelope. Multilamellar inner structure was evident between envelope membrane and inclusion matrix. SDS-PAGE revealed that the inclusions consist of five proteins with molecular masses of 72, 70, 68, 56 and 30 kDa. These proteins cross-reacted with the antibodies against inclusion proteins of the serovar *leesis* strain. High homologies existed in N-terminal amino acid sequences between the three major proteins (72, 70 and 68 kDa) and the two established protein classes, Cry4A and Cry10A.

Keywords: *Bacillus thuringiensis*; Parasporal inclusion; Larvicidal activity; Moth-fly specificity; *Telmatoscopus albipunctatus*

1. Introduction

The moth-fly, *Telmatoscopus albipunctatus* (Diptera: Psychodidae), is among the medically important insects in urban environments of Japan. It causes severe nuisance problems and induces allergic reactions in humans. Also, this dipteran may carry sewage microorganisms into indoor environments since it occurs near cesspools and septic tanks. At present, there are problems in using conventional chemical insecticides for control of the psychodid flies, owing to public safety risks. Thus, *Bacillus thuringiensis*-based microbial insecticides have attracted much attention as an environmentally sound control agent for the moth-fly [1]. Previously, we found that several H serogroups of *B. thuringiensis* contained moth-fly toxic strains [2]. Of these, a strain belonging to the serovar *leesis* (H33) proved unique in having activity highly specific for this insect [3]. The objective of this study was to characterize the parasporal inclusions of another moth-fly specific isolate belonging to a different H serogroup.

* Corresponding author. Tel.: +81 (942) 306644; Fax: +81 (942) 30-7244; E-mail: emizuki@bfri01.fitc.pref.fukuoka.jp

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2. Materials and methods

2.1. Bacteria and culture condition

The strain 92-KU-105-9 used in this study was derived from a silkworm litter sample collected in Kumamoto Prefecture, Japan [4]. This strain has an H antigen sero-positive for the two reference antisera against H14 (serovar *israelensis*) and H19 (serovar *tochigiensis*) [4]. A *B. thuringiensis* serovar *leesis* strain, 88-KO-14-45 [3] was also used as a moth-fly specific reference strain. Bacteria were grown at 28°C for 5 days on nutrient agar, pH 7.6, consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), agar (2 g) and distilled water (1000 ml). Spores and parasporal inclusions were harvested by centrifugation at $10\,000 \times g$ for 20 min at 4°C.

2.2. Purification of parasporal inclusions and preparation of antiserum

Parasporal inclusions were partially purified by a biphasic separation technique [5] using dextran sulfate-polyethylene glycol. Further purification was done by sucrose density gradient centrifugation, and the purity of parasporal inclusions was finally $>99.5\%$ when monitored by phase-contrast microscopy. Antiserum against solubilized parasporal inclusion proteins of the strain 88-KO-14-45 was prepared in a Japanese white rabbit by the method described previously [3].

2.3. SDS-PAGE and immunological tests

SDS-PAGE of parasporal inclusion proteins was done by the method of Laemmli [6], using 10% separating and 4% stacking gels. After electrophoresis, the gel was stained with 0.1% Coomassie blue R250 (Sigma, St. Louis, MO). The molecular mass markers were purchased from Sigma. Immunoblot analysis and Ouchterlony double immunodiffusion tests were done as described previously [3]. Protein concentration was measured according to the method of Lowry et al. [7], using bovine serum albumin as the standard.

2.4. Electron microscopy

Bacterial cells were harvested from 2-day cultures and washed in distilled water by centrifugation. Bacteria were prefixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C overnight and postfixed in 1.33% OsO_4 in the same buffer. The sample was dehydrated in an ethanol-propylene oxide series and embedded in Epon 812 resin mixture. Ultrathin sections were made on a Reichert Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Hitachi 7200 electron microscope.

2.5. Insects and bioassay

Dipteran species used in this study were: the three mosquitoes of the family Culicidae, *Anopheles stephensi* (Beech strain), *Aedes aegypti* (Liverpool strain), and *Culex pipiens molestus*; the moth-fly, *T. albipunctatus* (Psychodidae); and the midge, *Tokunagayusurika akamusi* (Chironomidae). Two lepidopterans were also used: the silkworm, *Bombyx mori* (Bombycidae) and the diamondback moth, *Plutella xylostella* (Plutellidae). Dipteran larvae were from laboratory populations maintained at 25°C by feeding on mouse diet or dried yeast. Larvae of Lepidoptera were reared on an artificial diet. Insecticidal activity tests were done by the method of Higuchi et al. [3].

Hemolytic activity of the solubilized and protease-treated inclusion proteins was tested against human erythrocytes (blood group O) according to the method previously described [8].

2.6. N-Terminal sequencing

Parasporal inclusion proteins were separated on SDS-PAGE using 7.5% separating gel and transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membranes were stained with 0.1% Coomassie blue R250 and each protein band was excised for determination of N-terminal amino acid sequences by using an automatic sequencer Model 473A (Perkin-Elmer, Foster, CA).

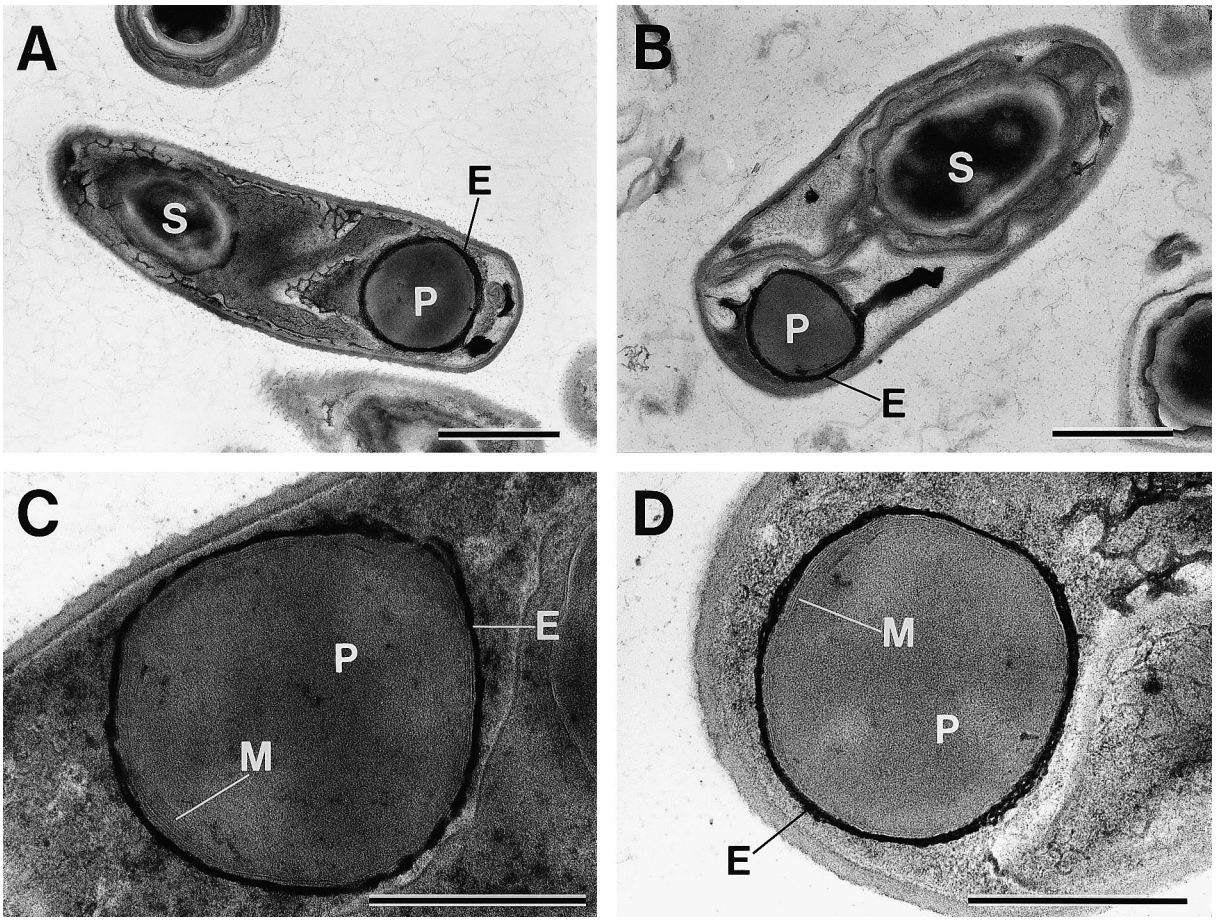

Fig. 1. Electron micrographs of parasporal inclusions from the *Bacillus thuringiensis* strain 92-KU-105-9 (A,C) and the serovar *leesis* strain 88-KO-14-45 (B,D). S, spore; P, parasporal inclusion; M, multilamellar inner layer; E, electron-dense envelope. Scale bars: A and B, 1 μm; C and D, 0.5 μm.

3. Results

3.1. Morphology of parasporal inclusion

Transmission electron microscopy revealed that the parasporal inclusions of the strain 92-KU-105-9 were globular bodies enveloped with a thick (30–80 nm) and two-layered electron-dense membrane (Fig. 1A). The size of inclusions ranged from 0.7 to 0.9 μm in diameter. Morphologically, inclusions of this strain were very similar to that of the serovar *leesis* strain 88-KO-14-45 (Fig. 1B). Inclusions of both strains also had multilamellar inner structures between the membrane and the homogeneous protein matrix (Fig. 1C,D).

3.2. Insecticidal activities

In one-dose assays, the spore/inclusion mixture of the strain 92-KU-105-9 gave no mortality to the lepidopterans, *B. mori* and *P. xylostella* and the four dipterans, *A. aegypti*, *A. stephensi*, *C. pipiens* molestus and *T. akamusi*. Table 1 shows the results of quantitative assay of the purified inclusions against dipterans, the moth-fly and the two mosquito species. There was no significant difference in the moth-fly larval LC50 values between the two strains, 92-KU-105-9 and 88-KO-14-45. No mortalities were induced in mosquito larvae by the two strains even at high protein concentrations up to 10 mg ml^{-1}.

Solubilized and proteinase-K treated inclusion

Table 1
Larvicidal activity of purified parasporal inclusions from two strains of *Bacillus thuringiensis* on the moth-fly, *Telmatoscopus albipunctatus* and the mosquitoes, *Culex pipiens molestus* and *Anopheles stephensi*

Strain	H antigen (H serovar)	LC_{50} (μg ml^{-1})[a]		
		T. albipunctatus	*C. pipiens molestus*	*A. stephensi*
92-KU-105-5	14/19	4.92 (4.19–5.77)	> 10 000	> 10 000
88-KO-14-45	33 (*leesis*)	5.87 (4.78–7.22)	> 10 000	> 10 000

[a]The values were calculated on the basis of the 24-h mortalities. The fiducial limit at the 95% level is given in parentheses.

proteins of the strain 92-KU-105-9 showed no hemolytic activity to human erythrocytes at a concentration of 1 mg ml^{-1}.

3.3. SDS-PAGE of inclusion proteins and immunological analysis

SDS-PAGE showed no differences in protein profiles of inclusions between the two strains, 92-KU-105-9 and 88-KO-14-45. Inclusions of both strains contained five proteins with molecular masses of 30, 56, 68, 70 and 72 kDa (Fig. 2A). Fig. 2B shows the immunoblot profiles of inclusion proteins of the two strains. Antibodies against 88-KO-14-45 proteins strongly reacted with the homologous antigens: the five major proteins and a protein of 60 kDa which was not visible on SDS-PAGE. The five major proteins in the strain 92-KU-105-9 were also immunoreactive for 88-KO-14-45 antiserum; however, a protein of 62 kDa was also reactive, while this protein did not appear in SDS-PAGE. In Ouchterlony tests, 88-KO-14-45 antiserum gave a single precipitin line to the homologous proteins and the heterologous 92-KU-105-9 proteins as well. A complete fusion was observed between precipitin lines formed by the two strains.

3.4. N-Terminal amino acid sequencing

The major inclusion proteins of 92-KU-105-9 and 88-KO-14-45 were examined for N-terminal amino acid sequences. As shown in Table 2f, a high similarity (91.6–100%) existed in the sequences between three (72, 70 and 68 kDa) proteins of the strain 92-KU-105-9. This was also the case observed in the strain 88-KO-14-45. Also, there was a high similarity in the sequences between the proteins, with the same molecular size, from the two strains.

4. Discussion

Previously, we reported that the mixture of spores and parasporal inclusions of the strain 92-KU-105-9 was toxic to larvae of the moth-fly [2]. It is clear from the results that the moth-fly killing activity of this strain resides in parasporal inclusions. Insecticidal activity tests showed that, like the strain 88-KO-14-45, the strain 92-KU-105-9 is not toxic against the

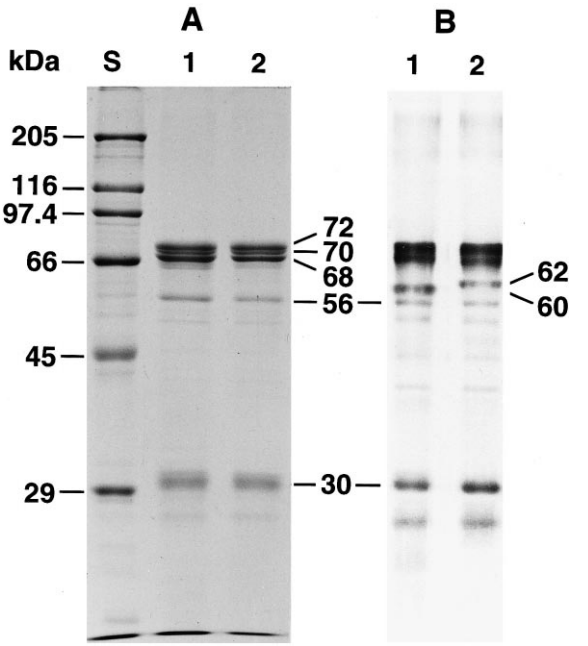

Fig. 2. SDS-PAGE and immunoblot analysis of purified parasporal inclusions. (A) Coomassie blue R-250-stained 10% SDS polyacrylamide gel. Each lane contained 5 μg of inclusion proteins. (B) Immunoblot with antiserum raised against the purified inclusions of the strain 88-KO-14-45 (*B. thuringiensis* serovar *leesis*). Each lane contained 200 ng of inclusion proteins. Lane 1, 88-KO-14-45 (serovar *leesis*); lane 2, 92-KU-105-9 (H14/19); lane S, molecular markers.

Table 2
213N-Terminal amino acid sequences of the parasporal inclusion proteins of *Bacillus thuringiensis* isolates, 92-KU-105-9 and 88-KO-14-45

Proteins (kDa)	Strains	Sequence	Source of sequence
72	92-KU-105-9	MNTYQNKNEYEILE	
	88-KO-14-45	MNTYQNKNEYE	
		MNPYQNKNEYEIF	Cry10A [12]
		MNPYQNKNEYETL	Cry4A [13]
70	92-KU-105-9	MNTYQNKNEYEIL	
	88-KO-14-45	MNTYQNKNEYEI	
		MNPYQNKNEYEIF	Cry10A [12]
		MNPYQNKNEYETL	Cry4A [13]
68	92-KU-105-9	MNPYQNXNEYEILE	
	88-KO-14-45	MNXYQNKNEYEI	
		MNPYQNKNEYEIF	Cry10A [12]
		MNPYQNKNEYETL	Cry4A [13]

dipterans other than *T. albipunctatus*. This supports our previous observation that the strain was totally non-toxic to larvae of the mosquito, *C. pipiens molestus*. The findings provide evidence that the moth-fly specific *B. thuringiensis* strains occur in serologically unrelated H serogroups.

Electron microscopic observation revealed that the parasporal inclusion of the strain 92-KU-105-9 is very similar to that of the *leesis* strain 88-KO-14-45. Inclusion of both strains were of the global shape with the homogeneous protein surrounded by thick and electron-dense envelopes. It also appeared that there is an internal multilamellar structure between envelope and matrix. In our previous study [3], this structure was not clear in the *leesis* strain 88-KO-14-45. Occurrence of the similar structure has also been reported in the strains of serovar *medellin* [9], *neoleonensis* [10] and *shandongiensis* [11].

There was no significant difference in inclusion protein profiles between the strain 92-KU-105-9 and the strain 88-KO-14-45. Also, a high similarity between the two was shown in immunoblot analysis using the antiserum against 88-KO-14-45. In addition, N-terminal amino acid sequences of the three proteins of 92-KU-105-9 had high homologies with those of 88-KO-14-45. Interestingly, as shown in Table 2, N-terminal sequences of the 92-KU-105-9 proteins resemble those of the two established classes of mosquitocidal Cry proteins, Cry10A [12] and Cry4A [13]. A high similarity in N-terminal regions was also evident between the *leesis* strain (88-KO-14-45) and the Cry10A/Cry4A proteins. However, our previous observations [3] have suggested that the 88-KO-14-45 proteins are not members of the Cry10 and Cry4 proteins. Although the moth-fly specific proteins in our strains has not been identified as yet, it is very likely that one (or more) of the three major proteins is responsible for the activity.

It has been well established that the Cry protein genes of *B. thuringiensis* are plasmid-encoded [14], and the plasmids are transmissible between strains of *B. thuringiensis* [15]. Thus, a hypothesis that follows from the fact obtained here is that the genes, encoding the moth-fly specific proteins in different H serogroups, are of the same origin and transmissible among *B. thuringiensis* natural populations. Our future studies will include identification and genetic analysis of the moth-fly specific proteins.

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