Diversity of *Bacillus thuringiensis* environmental isolates showing larvicidal activity specific for mosquitoes

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(Received 5 April 1993; revised 24 June 1993; accepted 6 July 1993)

Seven mosquito-specific strains of *Bacillus thuringiensis* isolated in Japan were examined for their flagellar (H) antigenicities and the properties of parasporal inclusion proteins. They were assigned to six H serovars: serovar *fukuokaensis* (H 3ade); serovar *canadensis* (H 5ac); serovar *darmstadiensis* (H 10); serovar *kyushuensis* (H 11ac); serovar *shandongiensis* (H 22); and an undescribed serovar belonging to H serotype 20. Purified parasporal inclusions exhibited moderate mosquito larvicidal activities with LC₅₀ values ranging from 1 µg ml⁻¹ to 10 µg ml⁻¹. The inclusions of these strains consisted of highly heterogeneous multiple protein components, and five distinct patterns were evident in their SDS-PAGE profiles. Antibodies against *kyushuensis* inclusion proteins were reactive with all strains to varying degrees, while *israelensis* antibodies gave only relatively weak reactions with the seven strains. Similarity in antibody-binding profiles was associated with similarity in SDS-PAGE profiles. In a given strain, different antisera gave altered immunoblot profiles. Haemolytic activity was shown by solubilized parasporal inclusion proteins of five of the seven strains.

Introduction

Goldberg & Margalit (1977) isolated a *Bacillus* strain possessing a high larvicidal activity, specific for mosquitoes, from the soil of a mosquito-breeding site in Israel. On the basis of this strain, de Barjac (1978) established the *Bacillus thuringiensis* serovar *israelensis* (H antigen 14). Thereafter, many workers have reported the occurrence of highly mosquitocidal *B. thuringiensis* strains, belonging to serovar *israelensis* (Balaraman *et al.*, 1981; Zhang *et al.*, 1984; Shim *et al.*, 1990; Abdel-Hameed *et al.*, 1990), serovar *morrisoni* (Padua *et al.*, 1984), and serovar *medellin* (Ordaz *et al.*, 1992), in various regions of the world. However, it is generally accepted that the mosquito-specific *B. thuringiensis*, with moderate to low toxicities, is also disseminated in natural environments. In fact, our surveys on the geographical distribution of this bacterium in Japan have found many strains with moderate toxicity levels (Ohba & Aizawa, 1979, 1986a, 1990; Padua *et al.*, 1980; T. Ishii & M. Ohba, unpublished results). Properties of highly toxic strains have been investigated extensively because of their possible use for microbial control of medically important dipterous pests (de Barjac & Sutherland,

1990). In contrast, only a few studies have focused on the group with moderate to low toxicities.

In this paper, we describe the comparative characterization of moderately toxic strains isolated in Japan. Our study shows that the populations of mosquito-specific *B. thuringiensis* in Japan are highly heterogeneous in flagellar antigenic structure and in the composition of parasporal inclusions.

Methods

Bacteria. The seven Japanese strains of *B. thuringiensis* used in this study are listed in Table 1. Of these, three reference strains were from the culture collection of this institute. Four other strains were newly isolated in this study from soils in Japan according to the method of Ohba & Aizawa (1986b). The type strains of *B. thuringiensis* serovar *israelensis* and serovar *sotto* (H 4ab) were also used as a mosquitocidal reference and a Lepidoptera-specific control, respectively. To prepare sporulated cultures, bacteria were grown on nutrient agar, pH 7.8, at 27 °C for 4 d. Formation of spores and parasporal inclusions was monitored by phase-contrast microscopy.

Qualitative and quantitative toxicity tests. Four *B. thuringiensis* strains, newly isolated in this study, were examined for their qualitative larvicidal activity against three species of Lepidoptera and two species of Diptera. To test the toxicity against the silkworm, *Bombyx mori*, ten third- to fourth-instar larvae were fed at 25 °C for 3 d on a mulberry leaf smeared with 0.2 ml spore/parasporal inclusion mixture (approximately 10⁸ spores ml⁻¹). For the tests with the fall webworm, *Hyphantria cunea*, and the smaller tea tortrix, *Adoxophyes orana*, five to ten larvae (fourth or fifth instar) were fed for 7 d on a bacteria-

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Table 1. Japanese strains of *B. thuringiensis* used in this study

<i>B. thuringiensis</i> strain	Flagellar (H) antigen	H serovar	Source or reference
Type strain of serovar <i>kyushuensis</i>	11ac	<i>kyushuensis</i>	Ohba & Aizawa (1979)
84-I-1-13	3ade	<i>fukuokaensis</i>	Ohba & Aizawa (1990)
73-E-10-2	10	<i>darmstadiensis</i>	Padua <i>et al.</i> (1980)
88-KG-2-21	10	<i>darmstadiensis</i>	This study, soil, Kagawa Pref.
89-T-5-9	5ac	<i>canadensis</i>	This study, soil, Tokyo
89-T-34-14	20*	—	This study, soil, Tokyo
89-ST-1-25	22	<i>shandongensis</i>	This study, soil, Saitama Pref.

* Not seroreactive for 20b and 20c factor sera.

contaminated artificial diet (0.3–0.5 ml bacterial suspension per 10 g diet). The type strain of serovar *sotto* was also tested in the same manner as a Lepidoptera-toxic control. Toxicity tests with the mosquitoes, *Aedes aegypti* and *Culex pipiens pallens*, were done by introducing 10 to 20 larvae (3–4-d-old) into a test tube containing 10 ml bacterial suspension (approximately 10^7 spores ml⁻¹). Mosquito larvae were kept at 25 °C and the mortality was examined daily for 2 d.

Quantitative toxicity tests were performed with purified parasporal inclusions. Twofold serial dilutions were prepared in distilled water. For the assay with *B. mori*, 20 second-instar larvae were fed at 25 °C for 3 d on an artificial diet (2.5 g) contaminated with 0.1 ml of each dilution. Assays were done in duplicate. The test with *A. aegypti* was done by introducing 30 second-instar larvae into 15 ml of each dilution. Larvae were kept unfed at 25 °C and the 24 h mortality was scored. Assays were done in triplicate and the LC₅₀ values were determined by probit analysis (Finney, 1952).

H serotyping. Reference H antisera to the type strains of the known *B. thuringiensis* H antigenic serovars were prepared according to the method of Ohba & Aizawa (1978). For H serotyping of the strains, actively motile bacteria were selected by passing through Craigie's tubes at 37 °C for 24 h. Slide agglutination was performed by mixing one drop of 3–4-h-old flagellated broth culture of the bacteria on a glass slide with one drop of 20–50-fold dilution of H antiserum. Specific H agglutination was determined 3–5 min after mixing.

Purification and solubilization of parasporal inclusions. Sporulated cultures were harvested by centrifugation at 10000 g for 20 min at 4 °C, and the pellet was washed three or four times by centrifugation in 1 M-NaCl at 4 °C. Parasporal inclusions were partially purified by a biphasic separation technique (Goodman *et al.*, 1967) using dextran sulphate/polyethylene glycol. The lower phase, containing parasporal inclusions, was collected and washed three times in distilled water by centrifugation. Further purification was done by NaBr density-gradient centrifugation according to the method of Held *et al.* (1990). The isolated inclusions were washed three times with distilled water and their purity was monitored by phase-contrast microscopy. Purified parasporal inclusions were suspended in distilled water and stored at –20 °C until needed.

Parasporal inclusions were solubilized by treatment at 37 °C for 60 min in 50 mM-Na₂CO₃ (pH 10.0)/10 mM-dithiothreitol (DTT)/1 mM-EDTA. Insoluble materials were pelleted by centrifugation at 10000 g for 10 min at 4 °C, and the resulting supernatant was referred to as solubilized parasporal inclusions.

Protease treatment of solubilized parasporal inclusions. Solubilized inclusion proteins (1 mg ml⁻¹) were treated with proteinase K (10 µg ml⁻¹; Sigma) at 37 °C for 30 min. The reaction was stopped by addition of 1 mM-PMSF.

Protein determination. Protein concentrations were determined by the Bio-Rad Protein Assay Kit with bovine IgG as the standard.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (1970), using 10% separating and 4% stacking gels. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie blue R-250 (Sigma). The molecular masses of proteins were determined by using protein standards obtained from Sigma.

Production of inclusion protein antibodies and immunoblotting. Antisera were raised in rabbits against parasporal inclusion proteins of the type strains of *B. thuringiensis* serovar *israelensis* and serovar *kyushuensis*. Rabbits were immunized by three subcutaneous injections, at weekly intervals, of the solubilized parasporal inclusions, mixed with an equal volume of Freund's complete adjuvant (Difco). One week later, these were followed by intravenous booster injections of adjuvant-free solubilized inclusion proteins. The rabbits were bled one week after the last intravenous injection. The total amount of inclusion proteins injected was 2 mg and 9 mg for serovar *kyushuensis* and serovar *israelensis*, respectively. IgG fractions were purified with ImmunoPure IgG Purification Kit (Pierce) according to manufacturer's instructions.

For immunoblot analysis (Towbin *et al.*, 1979), the separated proteins were transferred electrophoretically from an SDS-polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane using an ATTO Holizblot (ATTO) according to the manufacturer's instructions. The transferred proteins were probed first with a 1:2000 dilution of primary antibodies (IgG fractions: 1 mg ml⁻¹) followed by a 1:2000 dilution of goat anti-rabbit IgG-conjugated alkaline phosphatase (TAGO). Visualization was performed with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl toluidine (Wako Pure Chemical) as the colour substrate.

Haemolytic activity. Human (blood group AB) erythrocytes were washed twice by centrifugation at 800 g with 20 mM-Tris-buffered saline, pH 8.0. They were then resuspended to a concentration of 2% (v/v) in Tris-buffered saline, and twofold serial dilutions of solubilized inclusion preparations were made in the same solution. The dilution (100 µl) was mixed with an equal volume of 2% erythrocyte suspension in a well of a round-bottomed 96-well microtitre plate (Sanko). After incubation for 18 h at 27 °C, the plate was centrifuged at 800 g for 10 min, and the supernatant was examined for the amount of haemoglobin released. Haemolytic activity was expressed as units per mg protein; one unit was defined as the activity required to increase the absorbance at 540 nm by 0.1 after 18 h incubation at 27 °C. Each assay was repeated three times.

Results

H serotyping and insecticidal activity

H antigenicities of the three reference strains and the four new soil isolates were analysed by slide agglutination tests with the H antisera against the known serovars of *B.*

Table 2. Mosquito larvicidal activity of purified parasporal inclusions from eight *B. thuringiensis* strains

Strain	LC ₅₀ (µg ml ⁻¹) for second-instar larvae of <i>Aedes aegypti</i> *
<i>israelensis</i> type strain	0.010 (0.009–0.013)
<i>kyushuensis</i> type strain	3.69 (3.04–4.52)
84-I-1-13	1.73 (1.21–2.28)
73-E-10-2	1.48 (1.28–1.71)
88-KG-2-21	1.15 (0.96–1.31)
89-T-5-9	1.02 (0.73–1.30)
89-T-34-14	10.34 (9.07–11.95)
89-ST-1-25	2.64 (2.20–3.14)

* The fiducial limit at the 95% level is given in parentheses.

Fig. 1. Comparative SDS-PAGE of parasporal inclusion proteins from mosquito-specific *B. thuringiensis* strains. Lanes: 1, the type strain of *B. thuringiensis* serovar *israelensis*; 2, the type strain of serovar *kyushuensis*; 3 and 4, strains of serovar *darmstadiensis*, 73-E-10-2 and 88-KG-2-21; 5, strain 89-T-34-14 (undescribed serovar belonging to serotype 20); 6, strain 89-T-5-9 (serovar *canadensis*); 7, strain 89-ST-1-25 (serovar *shandongiensis*); 8, strain 84-I-1-13 (serovar *fukuokaensis*); S, protein standards. Each lane contained 10 µg inclusion proteins. The gel was stained with Coomassie brilliant blue.

thuringiensis. As shown in Table 1, six strains were assigned to the five known serovars of *B. thuringiensis*. One isolate, designated 89-T-34-14, was seropositive to H antiserum against serovar *yunnanensis* (H antigen 20ab). However, it did not react with factor sera against 20b and 20c H antigenic subfactors.

Qualitative oral toxicity tests revealed that spore/parasporal inclusion mixtures of the four new soil isolates were all toxic to mosquito larvae; they produced 90–100% mortality in *A. aegypti* and *C. pipiens pallens* in 2 d. In contrast, there was no mortality in three species of Lepidoptera. The type strain of serovar *sotto* exhibited toxicity to lepidopterous larvae.

Mosquito larval LC₅₀ values of the purified inclusions were determined. Inclusions of the seven Japanese strains had LC₅₀ values ranging from 1 µg ml⁻¹ to 10 µg ml⁻¹ (Table 2). They were 100–1000 times less toxic than *israelensis* inclusions. No mortality was induced in

second-instar *B. mori* larvae by these mosquitocidal strains, even at a high inclusion concentration of 1 mg ml⁻¹.

SDS-PAGE analysis of parasporal inclusion proteins

Purified parasporal inclusions of the type strain of serovar *israelensis* and the seven Japanese strains were examined for their protein components by SDS-PAGE (Fig. 1). Parasporal inclusions of the strains consisted of highly heterogeneous multiple protein components, with molecular masses ranging from 20–29 kDa to 130–140 kDa. The major protein profile of serovar *israelensis* was substantially different from those of the seven Japanese strains, among which five distinct patterns were observed in their SDS-PAGE profiles. The strain of serovar *shandongiensis* (89-ST-1-25) had a protein profile similar to that of the type strain of serovar *kyushuensis*. Similarity in the major protein profiles was also evident between the two strains of serovar *darmstadiensis*, 73-E-10-2 and 88-KG-2-21, although a minor difference was observed. Inclusions of the three other strains had different protein profiles. Of several major proteins, the 130–140 kDa proteins were commonly detected in the type strain of *israelensis* and the six Japanese strains, with only strain 84-I-1-13 (serovar *fukuokaensis*) lacking the protein of this class. Proteins of the 25–29 kDa class were present in all strains tested; the 20 kDa proteins were detected in the two *darmstadiensis* strains and the strain belonging to serotype 20.

Immunoblot analysis

As shown in Fig. 2(b), antibodies against inclusion proteins of the serovar *kyushuensis* strain reacted strongly, not only with homologous proteins (25 kDa, 60–70 kDa and 150 kDa), but with all major proteins (25 kDa, 60–70 kDa and 150 kDa) of the strain 89-ST-1-25 belonging to serovar *shandongiensis*. Immunoblot profiles of these two strains were very similar to each other. Further, *kyushuensis* antibodies showed immuno-reactivity to inclusion proteins of the other mosquitocidal strains. In particular, inclusion proteins of the two *darmstadiensis* strains, 73-E-10-2 and 88-KG-2-21, and the strain 89-T-34-14 (serotype 20), strongly cross-reacted; however, immunoblot profiles of the two *darmstadiensis* strains were quite different from that of strain 89-T-34-14. The two other strains, serovar *canadensis* and serovar *fukuokaensis*, reacted weakly with *kyushuensis* antibodies.

The antibodies of serovar *israelensis* reacted strongly with the homologous antigens and moderately with 14–15 kDa inclusion proteins of the *kyushuensis* type strain and the strain 89-ST-1-25 of serovar *shandongiensis*.

Fig. 2. Immunoblots of parasporal inclusion proteins from the mosquito-specific strains of *B. thuringiensis*. The proteins were probed with antibodies raised against solubilized inclusion proteins of (a) *B. thuringiensis* serovar *israelensis* and (b) the type strain of serovar *kyushuensis*. Each lane contained 100 ng inclusion proteins. Lanes: 1, the type strain of serovar *israelensis*; 2, the type strain of serovar *kyushuensis*; 3 and 4, strains of serovar *darmstadiensis*, 73-E-10-2 and 88-KG-2-21; 5, strain 89-T-34-14 (undescribed serovar belonging to serotype 20); 6, strain 89-T-5-9 (serovar *canadensis*); 7, strain 89-ST-1-25 (serovar *shandongiensis*); 8, strain 84-I-1-13 (serovar *fukuokaensis*). Pointers correspond to the positions (kDa) of protein standards in SDS-PAGE.

(Fig. 2a). Other heterologous strains exhibited only weak cross-reactions with *israelensis* antibodies. Binding of the antibodies to standard proteins was not observed in this study. In addition, binding of non-immune antibodies to parasporal inclusion proteins was not evident.

Haemolytic activity

Solubilized parasporal inclusions of the seven Japanese strains and the type strain of serovar *israelensis* were examined for their haemolytic activity against human erythrocytes. As shown in Table 3, most strains showed haemolytic activity. However, it was not detected in two strains, 89-T-5-9 (serovar *canadensis*) and 89-T-34-14 (serotype 20), even at a high protein concentration of

Table 3. Haemolytic activity of solubilized parasporal inclusions against human erythrocytes

Strain	Haemolytic activity (units per mg protein)*	
	Solubilized parasporal inclusions†	Solubilized and protease-treated parasporal inclusions‡
Reference strains		
<i>israelensis</i>	160	320
<i>kyushuensis</i>	10	80
73-E-10-2	2.5	40
84-I-1-13	2.5	0
Soil isolates		
88-KG-2-21	5	20
89-T-34-14	0	0
89-T-5-9	0	0
89-ST-1-25	10	40

* One unit was defined as the activity required to increase the absorbance at 540 nm by 0.1 after 18 h incubation at 27 °C.

† The solubilization was performed with 50 mM-Na₂CO₃/10 mM-DTT/1 mM-EDTA.

‡ Solubilized parasporal inclusions were treated with proteinase K at a final concentration of 10 µg ml⁻¹.

1 mg ml⁻¹. Among the positive strains, serovar *israelensis* had the highest level of haemolytic activity, while the other strains had low activities. Proteinase K treatment of solubilized inclusions of all but one of the positive strains resulted in a marked increase in their haemolytic activities; the activity of the *fukuokaensis* strain (84-I-1-13) was destroyed by this treatment. Solubilized inclusions of the two strains, 89-T-5-9 (serovar *canadensis*) and 89-T-34-14 (serotype 20), did not show any haemolytic activity even after treatment with proteinase K.

Discussion

B. thuringiensis strains exhibiting mosquito-specific toxicity are widely found in soils and insect-breeding environments in Japan (Ohba & Aizawa, 1979, 1986b; Padua *et al.*, 1980; T. Ishii & M. Ohba, unpublished results). They have been assigned to serovars *kyushuensis* (Ohba & Aizawa, 1979, 1986a), *darmstadiensis* (Padua *et al.*, 1980), *fukuokaensis* (Ohba & Aizawa, 1990), *amagiensis* and an undescribed serovar (T. Ishii & M. Ohba, unpublished results). In the present study, three more serovars were added to the mosquito-specific *B. thuringiensis* flora in Japan, indicating that the bacterial populations of this pathotype are highly heterogeneous in H antigenic structures. Wang *et al.* (1986) and Pietrantonio & Gill (1992) reported that the type strain of the serovar *shandongiensis* has no insecticidal activity against the larvae of Diptera and Lepidoptera. In addition, mosquito-toxic strains are presently unknown in the serovar *canadensis*. Thus, this

report is the first to show the occurrence of mosquitocidal strains in these two serovars of *B. thuringiensis*. The occurrence of two or more pathotypes in a given serogroup has been demonstrated in serovars *darmstadiensis* (Padua *et al.*, 1980), *morrisoni* (Padua *et al.*, 1984; Krieg *et al.*, 1987) and *fukuokaensis* (Ohba & Aizawa, 1990). These findings, together with the present results, show the discrepancy between H antigenicity and insecticidal properties, although a general correlation is evident between these two phenotypes.

The seven Japanese strains were similar in that they produced spherical to irregular-shaped parasporal inclusions. We detected five distinct patterns in SDS-PAGE profiles of these mosquito-specific parasporal inclusions, although some similarity was also observed between the patterns. Protein profiles for the type strain of *kyushuensis* and the two reference strains, 73-E-10-2 and 84-I-1-13, were consistent with those observed by previous investigators (Earp *et al.*, 1987; Drobniowski & Ellar, 1989; Held *et al.*, 1990; Yu *et al.*, 1991; Ishii & Ohba, 1992). Parasporal inclusions of most strains contained proteins with high molecular masses of 130–140 kDa. At present, little information is available on the actual mosquitocidal toxins in these strains. However, it is likely that the 130–140 kDa proteins are primarily responsible for mosquitocidal activity, because the proteins of this class are the protoxins in most insecticidal strains of *B. thuringiensis*, including serovar *israelensis* (Höfte & Whiteley, 1989).

Interestingly, parasporal inclusions of the serovar *fukuokaensis* strain (84-I-1-13) lacked the protein of this class. Yu *et al.* (1991) suggested that the proteins of 72–90 kDa are the protoxin in this strain. Kim *et al.* (1984) isolated a 67 kDa larvicidal protein from parasporal inclusions of the strain 73-E-10-2. However, it is likely that this protein is a proteolysis product of the higher-molecular-mass protoxin.

There was a high similarity in protein profiles between the strain of serovar *shandongiensis* (89-ST-1-25) and the type strain of *kyushuensis*. A high similarity between the two was also shown in immunoblot analysis with *kyushuensis* antibodies. In another study of ours (T. Ishii & M. Ohba, unpublished results), *kyushuensis*-type parasporal inclusions were found in several soil isolates belonging to serovar *amagiensis* and an undescribed serovar. Our results suggest that the *kyushuensis*-type inclusion is widely disseminated in multiple serovars of *B. thuringiensis*.

It has been demonstrated that the parasporal inclusions of the existing mosquito-specific *B. thuringiensis* strains are immunologically related to varying degrees (Earp *et al.*, 1987; Padua *et al.*, 1988; Held *et al.*, 1990; Cheung & Kim, 1990; Yu *et al.*, 1991; Ishii & Ohba, 1992). In this study, we showed that mosquito-specific

strains tested shared common inclusion protein antigens with both type strains of *kyushuensis* and *israelensis* to various extents. It is noteworthy that the two antibodies gave different immunoblot profiles to a given strain. Interestingly, the 14–15 kDa proteins of *kyushuensis* and *shandongiensis* strains showed substantial immunoreactivity to *israelensis* antibodies, while no immunobinding of *kyushuensis* antibodies occurred. At present, no explanation is available for this contradictory result.

It is well accepted that the 25–29 kDa proteins of mosquito-specific *B. thuringiensis* strains are cytolytic, upon proteolytic digestion, for vertebrate and insect cells (Thomas & Ellar, 1983; Gill & Hornung, 1987; Yu *et al.*, 1991; Knowles *et al.*, 1992). In the present study, we detected proteins of this size in all strains tested. This is of interest because human erythrocyte lytic activity was not associated with two strains, 89-T-5-9 and 89-T-34-14, even after treatment with protease. However, more research with various cell types is needed, since previous workers have demonstrated that the activity of the *B. thuringiensis* cytolytic delta-endotoxin varies considerably, depending on the source of erythrocytes (Drobniowski & Ellar, 1989). Several investigators have made the interesting observation that the 25 kDa cytolytic proteins of *israelensis* and of strain PG-14 (serovar *morrisoni*) are of low toxicity to mosquitoes, but strongly enhance the mosquitocidal-activity of the other co-existing inclusion proteins (Wu & Chang, 1985; Yu *et al.*, 1987). Whether and how the 25–29 kDa proteins of our strains participate in killing mosquito larvae will be the subject of future work.

We thank Mr K. Miyamoto for assistance in testing the insecticidal activity of *Bacillus thuringiensis* strains against Lepidoptera. We also thank Dr T. Kwarabata for critically reading the manuscript.

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