SEROLOGICAL PROPERTIES OF THE MOSQUITOCIDAL PROTEIN OF *BACILLUS THURINGIENSIS* AND THE MORPHOLOGY OF ITS PARASPORAL CRYSTAL

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Introduction

Bacillus thuringiensis has been known as an insect pathogen and numerous strains have been isolated from diseased insects in many countries. They are classified into some 20 subspecies based on the flagellar antigen (H-antigen) and biochemical tests[4,5]. Most subspecies normally produce a spore and a bipyramidal crystal in the cell at the end of the logarithmic phase of growth. Several subspecies, which have mosquitocidal activity, produce irregular and/or cuboidal crystals[6,10]. The crystals have been purified by isopycnic centrifugation in a NaBr density gradient[1,18].

YAMAMOTO and MCLAUGHLIN[18] have isolated two proteins of Mr 135,000 and Mr 65,000 from the crystals of mosquitocidal HD-1 strain (subsp. *kurstaki*) after an exposure of its crystals to 2-mercaptoethanol at pH 10. The 135 Kdal protein, which was the major component of the crystal and was toxic to several lepidopterous species, showed no toxicity to mosquito larvae. The 65 Kdal protein, which differed entirely from the 135 Kdal protein on the basis of biochemical tests, showed a toxicity to mosquito larvae as high as its toxicity to larvae of lepidopterous species. Furthermore, IIZUKA and YAMAMOTO[11] suggested that the mosquitocidal 65 Kdal protein crystalize in a cuboidal form.

The irregularly shaped crystals of *B. thuringiensis* subsp. *israelensis* (ONR-60 A), which was isolated by GOLDBERG and MARGALIT[7] from soil

samples taken at a mosquito larval breeding site, has showed very high toxicity to mosquito larvae[17]. Mosquitocidal toxin of subsp. *israelensis* have been identified as 28 Kdal protein[17].

In the present study, we tested the crystal proteins from 42 strains of different *B. thuringiensis* serotypes by immunoelectrophoresis, and their crystal shapes were examined with a scanning electron microscope (SEM).

Materials and Methods

Bacterial strains

Strains and sources of *B. thuringiensis* used in this study were listed in the Table 1.

Immunoelectrophoresis

We used rocket-immunoelectrophoresis which has been described by LAURELL[12]. P-1 (135 Kdal protein) and P-2 (65 Kdal protein) were isolated from the lyophilyzed crystal of subsp. *kurstaki* HD-1 according to the method of YAMAMOTO and MCLAUGHLIN[18] and used as antigens. Three antisera directed against HD-1, against P-2 protein, and against *israelensis* crystal were made in rabbit. The antigens were dialyzed in 5 mM Tris-HCl (pH 8) at 4°C and inoculated into rabbit's shoulder with Freund's adjuvants. Details of the immunization procedures were as described by CLARK and FREEMAN[2] and by YAMAMOTO and TANADA[19].

For immunoelectrophoresis, a loopful of spores and crystals which were incubated at 30°C, for 72 hr, was suspended in 30 μl of 2% 2-mercaptoethanol whose pH was adjusted to 10 with NaOH. After 30 min incubation on ice, 10 μl of Tris-HCl (pH 7) were added. The sample (4 μl) was electrophoresed on 1.2% agarose gel plate ($10 \times 10 \times 0.1$ cm) containing 125–250 μl antiserum. After electrophoresis at 100 V for 3 hr, the gel was washed in 0.8% NaCl–10 mM Tris-HCl (pH 8) for 16 hr at 37°C and stained with 0.25% Coomassie blue R-250 in 7% acetic acid and 30% methanol.

SEM

Bacteria were cultured on nutrient agar (Difco) at 30°C until almost all cells lysed (about 72 hr). The crystals and spores (about 100 mg wet weight) were washed in 10 ml of 50 mM Tris-HCl (pH 8) followed by water by repeated centrifugation at 10,000 rpm for 10 min. The final precipitate was resuspended in 1 ml of water, and 20 μl of the suspension were air-dried on an alminum disk. After the sample was coated with carbon and gold, it was observed and photographed with a SEM (JEOL, JSM-S1).

TABLE 1. Sources of the strains in *Bacillus thuringiensis*

Serotype H	Subspecies epithet	Source
1	*thuringiensis* Berliner	H. DE BARJAC, France
1	*thuringiensis* (BA-068)	E. REEVES, USA
2	*finitimus*	A. M. HEIMPEL, USA
3a	*alesti*	H. DE BARJAC, France
3a, 3b	*kurstaki* (HD-1)	H. DULMAGE, USA
3a, 3b	*kurstaki* (MC)	S. T. AMONKAR, India
4a, 4b	*sotto*	Insect Pathology Lab., Beltsville, USA
4a, 4b	*dendrolimus*	(id.)
4a, 4c	*kenyae*	H. DE BARJAC, France
5a, 5b	*galleriae*	(id.)
5a, 5c	*canadensis*	(id.)
6	*subtoxicus*	(id.)
6	*entomocidus*	Insect Pathology Lab., Beltsville, USA
7	*aizawai*	K. AIZAWA, Japan
7	*aizawai* (*juroi*)	ATCC (#21281) Rockville, USA
7	*aizawai* (HU)	Induced by T. IIZUKA from *aizawai*.
7	*aizawai* (B106)	Shionogi Co. Ltd., Japan
8a, 8b	*morrisoni*	H. DE BARJAC, France
8a, 8c	*ostriniae* (HD-501)	H. DULMAGE, USA
9	*tolworthi*	H. DE BARJAC, France
10	*darmstadiensis*	(id).
10	*darmstadiensis* (73-E-10-2)	K. AIZAWA, Japan
10	*darmstadiensis* (73-E-10-16)	(id.)
10	*darmstadiensis* (73-E-37-14)	(id.)
11a, 11b	*toumanoffi*	H. DE BARJAC, France
11a, 11c	*kyushuensis*	K. AIZAWA, Japan
12	*thompsoni*	H. DE BARJAC, France
13	*pakistani* (HD-395)	H. DULMAGE, USA
14	*israelensis* (ONR-60A)	L. GOLDBERG, USA
14	*israelensis* (922903)	L. A. BULLA
14	*israelensis* (922906)	(id.)
14	*israelensis* (922910)	(id.)
14	*israelensis* (922917)	(id.)
14	*israelensis* (922918)	(id.)

Table 1. Continued

Serotype H	Subspecies epithet	Source
14	*israelensis* (922920)	(id.)
15	*dakota* (HD-511)	H. DULMAGE, USA
16	*indiana* (HD-516)	(id.)
17	*tohokuensis*	K. AIZAWA, Japan
18	*kumamotoensis*	(id.)
19	*tochigiensis*	(id.)
	yunnanensis	(id.)
	wuhanensis	H. DULMAGE, USA

Results and Discussion

The crystals of *B. thuringiensis* strains listed in Table 1, were examined by immunoelectrophoresis using anti-*kurstaki*-serum and the results were shown in Fig. 1 and Fig. 2. The HD-1 crystal showed two peaks, one with 135 Kdal protein and the other 65 Kdal protein[11]. The peak running from the left (negative electrode) to the right (positive electrode) on the gel was of 135 Kdal protein, and the peak running to the opposite direction was of 65 Kdal protein. It was recognized that four strains, subspp. *kurstaki* HD-1, *kurstaki* MC, *kenyae* and *tolworthi* produced the peak on the negative electrode side. This indicates that crystals of subspp. *kenyae* and *tolworthi* contain a protein similar to, if not the same as, the 65 Kdal mosquitocidal toxin of HD-1.

Anti-P-2-serum was also used to check crystals of the strains listed in Table 1 (Fig. 3 and Fig. 4). It confirmed the observation with anti-*kurstaki*-serum that subspp. *kurstaki* HD-1, *kurstaki* MC, *kenyae* and *tolworthi* contains the 65 Kdal P-2 protein.

Anti-*israelensis*-serum precipitated proteins of subspp. *israelensis* and *aizawai* HU when the crystal proteins from the strains listed in Table 1 were examined by immunoelectrophoresis (Fig. 5 and Fig. 6).

Previously, crystals of many strains in *B. thuringiensis* have already been photographed by SEM[10]. In the present study, we observed morphological details of the crystals from strains which are reportedly mosquitocidal. They are subspp. *kurstaki* HD-1, *kenyae*, *galleriae*, *entomocidus*, *aizawai* and *tolworthi*[8] ; subsp. *israelensis* ONR-60 A[3] ; subsp. *kyushuensis*[14]; and subspp. *darmstadiensis* 73-E-10-2 and 73-E-10-16[15]. (Fig. 7, Fig. 8 and Fig. 9). In addition to these mosquitocidal strains, the crystal of subsp. *morrisoni* was photographed under SEM and the electron micrograph re-

vealed two crystal forms.

The shape of crystals of subspp. *kurstaki* HD-1, *kenyae*, and *tolworthi*, which were shown to produce P-2 toxin by immunoelectrophoresis, appeared to have a cuboidal body some of which were partly embedded in the bipyramidal crystal matrix as previously reported by SHARPE and BAKER[16]. The crystals of subsp. *galleriae* also contained cuboidal ones, but 65 Kdal protein did not appear on the agarose gel (Fig. 1, 2, and 3). Therefore, we re-examined subsp. *galleriae* by immunoelectrophoresis using a high concentration of antiserum (Fig. 10). As a result, the crystal protein of subsp. *galleriae* was faintly recognized by an increased volume of anti-P-2-serum, but it appeared to be different from the reaction on the proteins of subspp. *kurstaki* HD-1, *kurstaki* MC, *kenyae* and *tolworthi*. It was suggested that a crystal protein of subsp. *galleriae* had some limited antigenic sites similar to the P-2 protein of HD-1.

There were no differences in the shape of crystals among subspp. *israelensis* ONR-60 A, *israelensis* 922910 and *aizawai* HU whose proteins were precipitated with anti-*israelensis*-serum. However, crystals of subsp. *darmstadiensis* 73-E-10-2 and 73-E-10-16 which have been reported to have irregular shape by IIZUKA *et al.*[9], did not react with anti-*israelensis*-serum.

Shapes of the crystals produced by the mosquitocidal strains of *B. thuringiensis*[3,8,14,15], include cuboidal form as seen in *kurstaki* HD-1 type crystals and irregular form in subsp. *israelensis* type crystals.

We stock four strains of subsp. *aizawai*, the original *aizawai* from Dr. K. AIZAWA, *aizawai* (*juroi*) from ATCC, USA, *aizawai* HU selected by T. IIZUKA, and *aizawai* B106 (sporeless) from Shionogi Co., Ltd.. Electron micrography showed significant differences in crystal morphology among these strains. We are very concerned about the differences and propose further studies on the plasmid patterns and the insecticidal activity of these strains.

Summary

In the present study, we have demonstrated that there are three serologically groups in a number of mosquitocidal *B. thuringiensis* δ-endotoxins. They are: group 1 including those produced by subspp. *kurstaki* HD-1. *kenyae* and *tolworthi* which were precipitated with anti-*kurstaki* HD-1-serum; group 2, subspp. *israelensis* and *aizawai* HU which reacted with anti-*israelensis*-serum; group 3, subspp. *galleriae*, *entomocidus* and *darmstadiensis* which showed no crossreaction with these two antisera. Morphology, of the crystals appeared to be different among these groups when they were examined by electron microscopy.

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18. YAMAMOTO, T. and MCLAUGHLIN, R. E.: Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhynchus*. Biochem. Biophys. Res. Commun. **103**: 414–421. 1981
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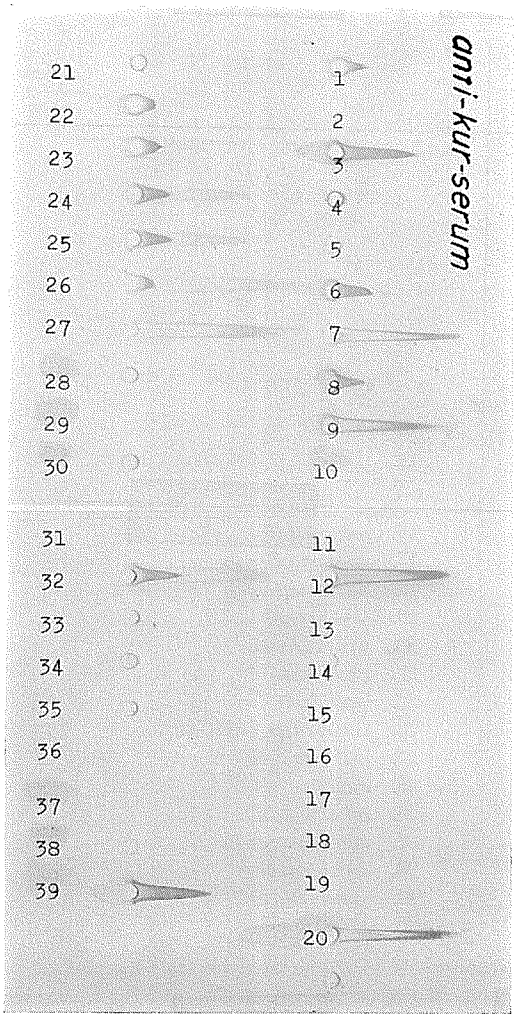
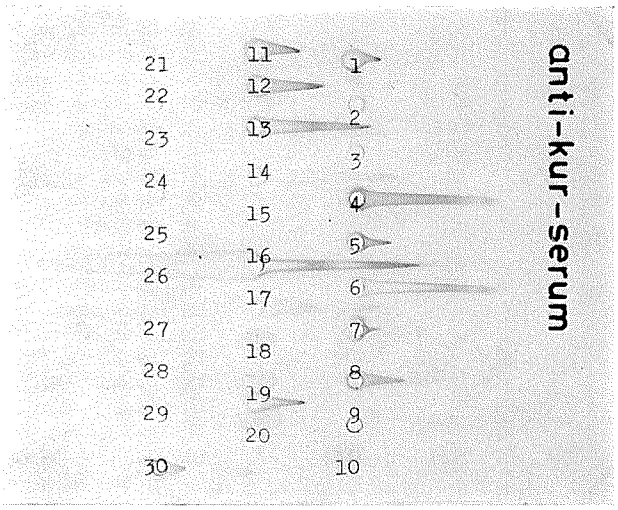


1. *thuringiensis* Berliner
2. *thuringiensis* BA-068
3. *kurstaki* HD-1
4. *finitimus*
5. *alesti*
6. *kurstaki* MC
7. *sotto*
8. *dendrolimus*
9. *kenyae*
10. *galleriae*
11. *toumanoffi*
12. *kyushuensis*
13. *thompsoni*
14. *israelensis* ONR-60A
15. *tohokuensis*
16. *kumamotoensis*
17. *tochigiensis*
18. *yunnanensis*
19. *tolworthi*
20. *aizawa* HU
21. *canadensis*
22. *subtoxicus*
23. *entomocidus*
24. *aizawai*
25. *aizawai* (*juroi*)
26. *morrisoni*
27. *darmstadiensis*
28. *darmstadiensis* 73-E-10-2
29. *darmstadiensis* 73-E-10-16
30. *darmstadiensis* 73-E-37-14
31. *aizawai* B106
32. *israelensis* 922903
33. *israelensis* 922906
34. *israelensis* 922910
35. *israelensis* 922917
36. *israelensis* 922918
37. *israelensis* 922920
38. *kurstaki* HD-1
39. *israelensis* ONR-60A

Fig. 1. Crossed immunoelectrophoresis of dissociated crystals of a variety of *B. thuringiensis* strains against anti-*kurstaki* HD-1 serum.

The sample (4 μl each) was electrophoresed on an agarose gel plate at 100 V for 3 hr at 4°C. The gel, which was made from 10 ml of 1.2% low electroendosmos agarose and contained 250 μl antiserum and 100 mM Tris-acetate (pH 8.2), was cast on a 10×10 cm glass plate. After the electrophoresis, the gel plate was washed in 0.8% NaCl buffered with 10 mM Tris-HCl (pH 8) for 16 hr at 37°C to remove unreacted serum component, dried in air, and stained with 0.25% Coomassie blue R-250. The positive electrode was on the right.



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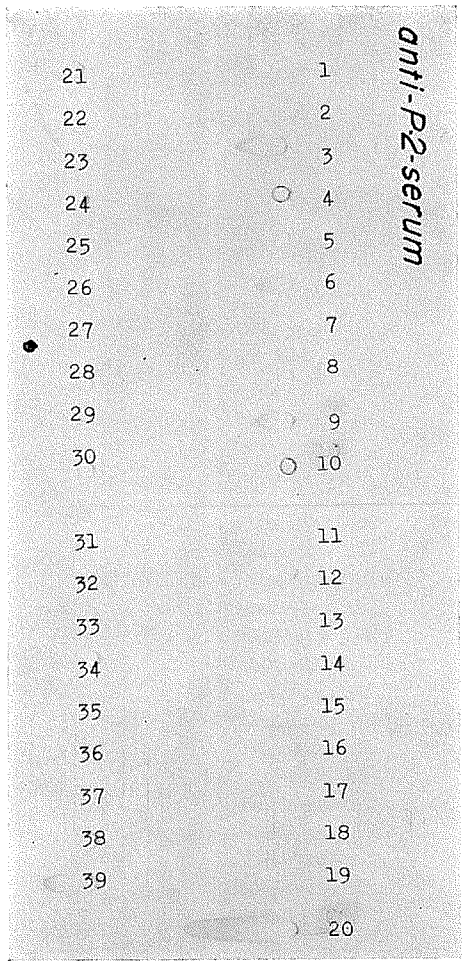
3. *alesti*
4. *kurstaki* HD-1
5. *kurstaki* MC
6. *sotto*
7. *dendrolimus*
8. *kenyae*
9. *galleriae*
10. *canadensis*
11. *subtoxicus*
12. *entomocidus*
13. *aizawai* (*juroi*)
14. *morrisoni*
15. *ostriniae*
16. *tolworthi*
17. *darmstadiensis*
18. *darmstadiensis* 73-E-10-2
19. *toumanoffi*
20. *kyushuensis*
21. *thompsoni*
22. *pakistani*
23. *israelensis* ONR-60A
24. *indiana*
25. *dakota*
26. *tohokuensis*
27. *kumamotoensis*
28. *tochigiensis*
29. *yunnanensis*
30. *wuhanensis*

Fig. 2. Crossed immunoelectrophoresis of dissociated crystals against anti-*kurstaki* HD-1-serum in the additional strains to Fig. 1.



1. *thuringensis* Berliner
2. *thuringiensis* BA-068
3. *kurstaki* HD-1
4. *finitimus*
5. *alesti*
6. *kurstaki* MC
7. *sotto*
8. *dendrolimus*
9. *kenyae*
10. *galleriae*
11. *toumanoffi*
12. *kyushuensis*
13. *thompsoni*
14. *israelensis* ONR-60A
15. *tohokuensis*
16. *kumamotoensis*
17. *tochigiensis*
18. *yunnanensis*
19. *tolworthi*
20. *aizawai* HU
21. *canadensis*
22. *subtoxicus*
23. *entomocidus*
24. *aizawai*
25. *aizawai* (*juroi*)
26. *morrisoni*
27. *darmstadiensis*
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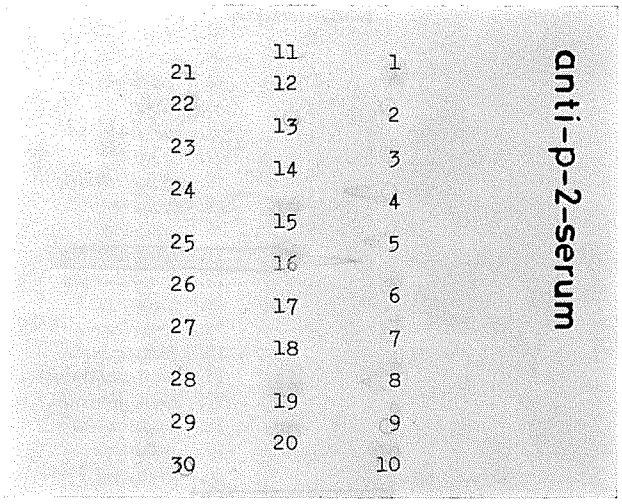
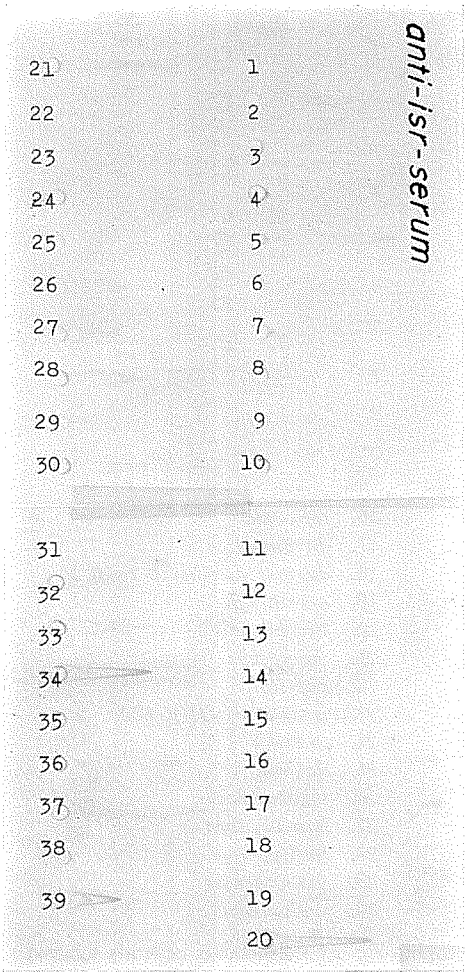
32. *israelensis* 922903
33. *israelensis* 922906
34. *israelensis* 922910
35. *israelensis* 922917
36. *israelensis* 922918
37. *israelensis* 922920
38. *kurstaki* HD-1
39. *israelensis* ONR-60A

Fig. 3. Crossed immunoelectrophoresis of dissociated crystals against anti-P-2-serum in the strains of *B. thuringiensis*.



1. *thuringiensis* Berliner
2. *finitimus*
3. *alesti*
4. *kurstaki* HD-1
5. *kurstaki* MC
6. *sotto*
7. *dendrolimus*
8. *kenyae*
9. *galleriae*
10. *canadensis*
11. *subtoxicus*
12. *entomocidus*
13. *aizawai* (*juroi*)
14. *morrisoni*
15. *ostriniae*
16. *tolworthi*
17. *darmstadiensis*
18. *darmstadiensis* 73-E-10-2
19. *toumanoffi*
20. *kyushuensis*
21. *thompsoni*
22. *pakistani*
23. *israelensis* ONR-60A
24. *indiana*
25. *dakota*
26. *tohokuensis*
27. *kumamotoensis*
28. *tochigiensis*
29. *yunnanensis*
30. *wuhanensis*

Fig. 4. Crossed immunoelectrophoresis of dissociated crystals against anti-P-2-serum in the additional strains to Fig. 3.



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2. *thuringiensis* BA-068
3. *kurstaki* HD-1
4. *finitimus*
5. *alesti*
6. *kurstaki* MC
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8. *dendrolimus*
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20. *aizawai* HU
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28. *darmstadiensis* 73-E-10-2
29. *darmstadiensis* 73-E-10-16
30. *darmstadiensis* 73-E-37-14
31. *aizawai* B106
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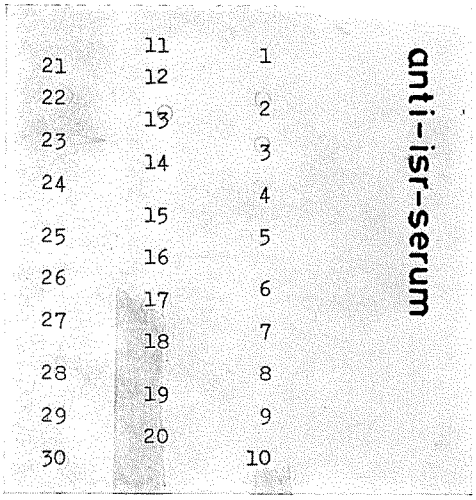
33. *israelensis* 922906
34. *israelensis* 922910
35. *israelensis* 922917
36. *israelensis* 922918
37. *israelensis* 922920
38. *kurstaki* HD-1
39. *israelensis* ONR-60A

Fig. 5. Crossed immunoelectrophoresis of dissociated crystals against anti-*israelensis*-serum in the strains of *B. thuringiensis*.



1. *thuringiensis* Berliner
2. *finitimus*
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4. *kurstaki* HD-1
5. *kurstaki* MC
6. *sotto*
7. *dendrolimus*
8. *kenyae*
9. *galleriae*
10. *canadensis*
11. *subtoxicus*
12. *entomocidus*
13. *aizawai* (*juroi*)
14. *morrisoni*
15. *ostriniae*
16. *tolworthi*
17. *darmstadiensis*
18. *darmstadiensis* 73-E-10-2
19. *toumanoffi*
20. *kyushuensis*
21. *thompsoni*
22. *pakistani*
23. *israelensis* ONR-60A
24. *indiana*
25. *dakota*
26. *tohokuensis*
27. *kumamotoensis*
28. *tochigiensis*
29. *yunnanensis*
30. *wuhanensis*

Fig. 6. Crossed immunoelectrophoresis of dissociated crystals against anti-*israelensis*-serum in the additional strains to Fig. 5.

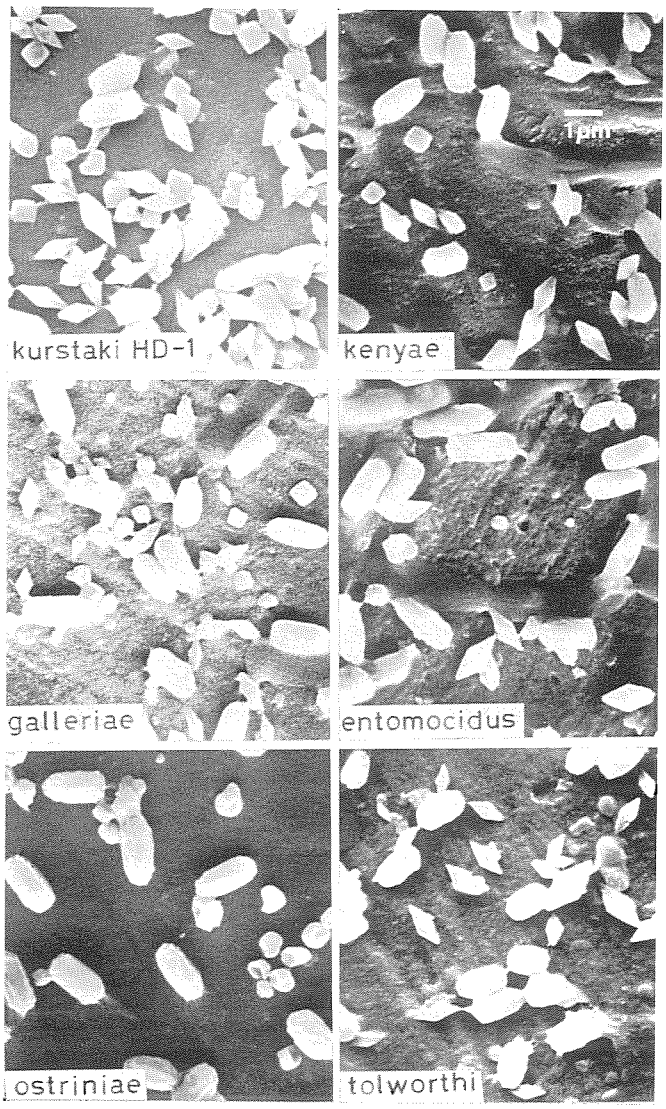

Fig. 7. Electron micrographs of crystals of *B. thuringiensis* subspecies associating which were reported to have mosquitocidal activity by HALL *et al.*[8].

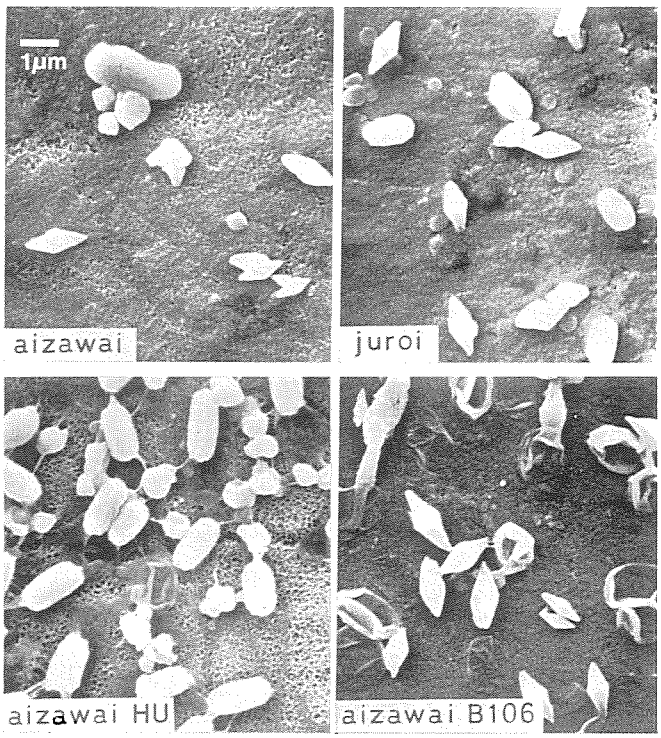

Fig. 8. Electron micrographs of crystals of *B. thuringiensis* subsp. *aizawai*.

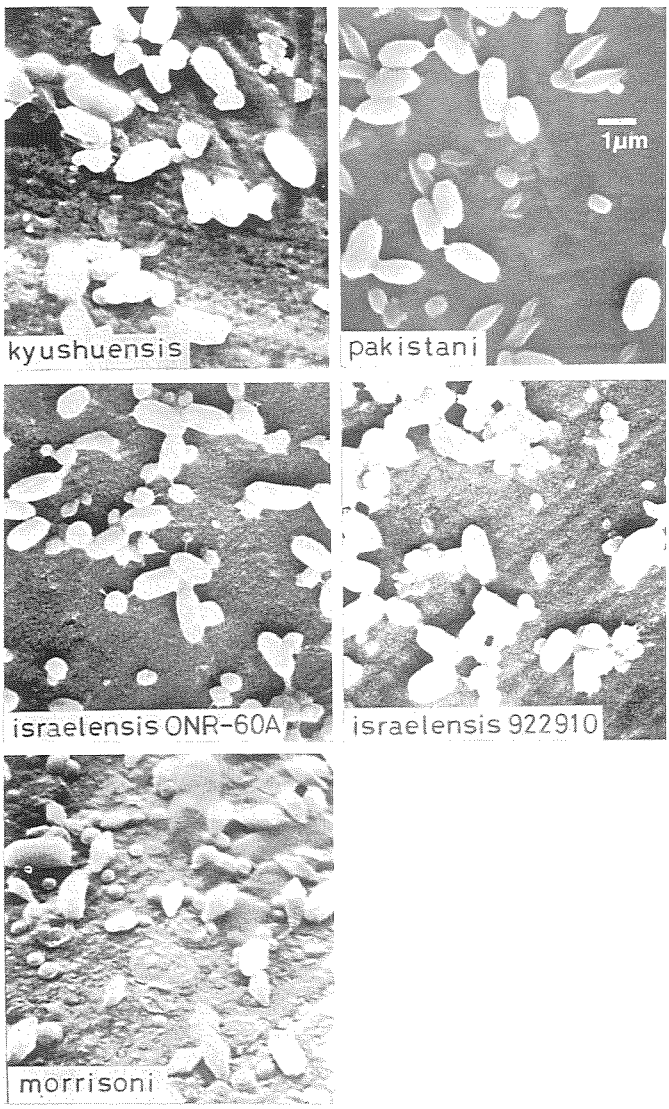

Fig. 9. Electron micrographs of crystals of *B. thuringiensis* subspecies whose mosquitocidal activity were reported by HALL *et al.*[8], by DE BARJAC[3], and by OHBA and AIZAWA[14]. The micrographs of subsp. *morrisoni* crystals appeared to be similar to that of subsp. *pakistani* crystals.

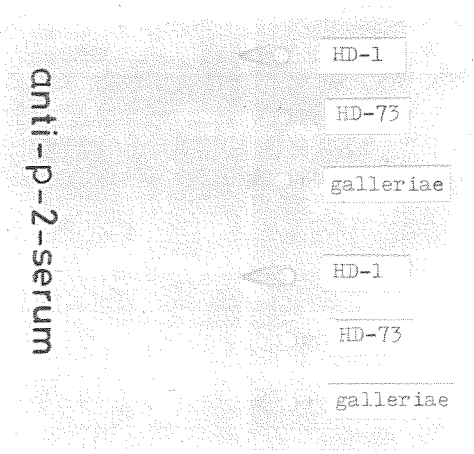

Fig. 10. Crossed immunoelectrophoresis of dissociated crystals against anti-P-2-serum in the subsp. *galleriae*. An increased volume fo anti-serum (450 μl) was added to the agarose gel.