Sporeless Mutants of *Bacillus thuringiensis*. II. Mutants derived from var. *thuringiensis* and var. *sotto*

In order to keep our world clean, biological methods of insect control have recently been investigated by many scientists. Among many biological control agents found, *Bacillus thuringiensis* seemed to be most useful because it is harmless to humans, domestic animals, wildlife and plants. However, since *B. thuringiensis* preparations as they appear on the market contain living spores of the organism, they possess the potential for introducing unforeseen disturbances in the ecological balance for the future. Hence, a microbial insecticide lacking viability but retaining activity is most desirable.

In the previous paper[1], we have reported that 5 sporeless mutant strains, 2 derived from *kurstaki* and 3 from *aizawai*, were selected after mutagenic treatment, and that they were characterized by a complete lack of spores and yet maintain intact insecticidal activity. Most mutant cells usually autolized at the end of cultivation and the remaining cells were easily killed by heat treatment without lowering the toxic activity. Thus, the use of these sporeless mutants was suggested as microbial producers of the insecticidal toxin in commercial preparations without bacterial viability.

Since *B. thuringiensis* has many varieties[2], each of which shows a different spectrum of insecticidal effect on different species of lepidoptera larvae, many sporeless mutants derived from the different strains can be expected. This report concerns sporeless mutants newly obtained from the different varieties.

B. thuringiensis var. *sotto* and var. *thuringiensis* were used as parent cultures. The parent strains were cultivated on GNB-broth (glucose 0.1%, polypeptone 1.0%, beef extract 0.5%, NaCl 0.2%, pH 7.0). The cell paste obtained was suspended in saline containing 0.1% N-methyl-N'-nitro-N-nitrosoguanidine[3] for 30 min at 30 °C. Following dilution, transfer onto GNB-agar plate and incubation, mutant colonies having a slightly translucent consistency were selected. The details of cultivation and selection of mutants has been reported elsewhere[1].

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[2] H. T. Dulmage, J. Invert. Path. *18*, 363 (1971).
[3] E. A. Adelberg, M. Mandel and G. Chein Ching Chen, Biochem. biophys. Res. Commun. *18*, 788 (1965).

Table I. Viable cell counts after heat treatment[a] (/ml)

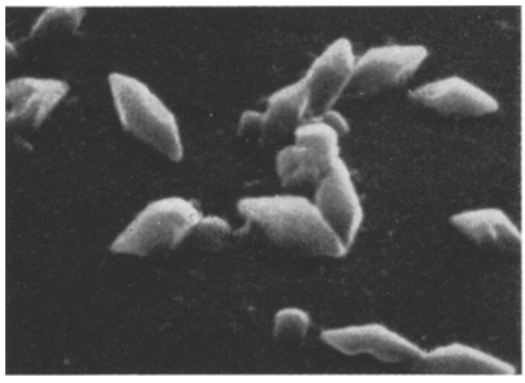
	BT var. *thuringiensis*			BT var. sotto	
Temperature (30 min)	Original strain	M 1	M 67	Original strain	S 162
Non-treated	1.10×10^9	8.80×10^6	2.19×10^7	8.30×10^8	1.34×10^7
40 °C	1.04×10^9	7.05×10^5	8.60×10^5	9.10×10^8	5.20×10^5
50 °C	1.27×10^9	1.67×10^4	211	1.06×10^9	15
60 °C	1.09×10^9	492	12	9.20×10^8	5
65 °C	1.30×10^9	337	0	9.20×10^8	0

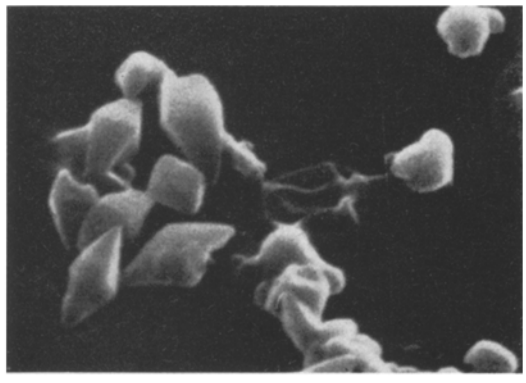
[a] All strains were cultivated on GNB-broth for 48 h at 30 °C; 1.5 ml of each culture broth was transferred into a test tube and heated at various temperatures for 30 min.

Table II. Insecticidal effects of sporeless mutants treated at 65 °C for 30 min

Name[a]	Dry wt.[b] (μg/ml of broth)	N. of crystals/ml of broth[c]	Temperature (65 °C 30 min)	No. of viable cells/ml	Insecticidal effect[d] LD_{50}[e]	
					Bombyx	*Samia*
thuringiensis						
mutant M 1	670	2.7×10^8	—	9×10^6	6.6	< 1
			65 °C	0	8.6	—
mutant M 67	700	2.0×10^8	—	2×10^7	5.4	< 1
			65 °C	223	6.2	—
Original strain	1.580	1.4×10^9	—	1×10^9	6.1	3
			65 °C	1×10^9	11.0	—
sotto						
mutant S 162	320	1.9×10^8	—	1×10^7	5.4	1
			65 °C	0	4.6	—
Original strain	490	1.0×10^8	—	8×10^8	16.0	31
			65 °C	8×10^8	14.5	—
aizawai[f] mutant I 45	970	8.5×10^8	—	8×10^6	3.4	—
			65 °C	0	3.4	—

[a] All strains were cultivated on GNB-broth for 3 ~4 days at 30 °C. [b] Dry weight was expressed the mean value of 3 repeated cultivations. [c] Crystalline bodies were counted irrespective of being inside of cells or free. [d] Bioassay was done using 40 *Bombyx* larvae (1st day of 3rd instar) and 30 *Samia* larvae (1st day of 2nd instar) for each experimental lot. Each strain was tested through 5 series of dilutions. [e] LD_{50} was calculated from the "probit" and expressed in μg/ml of test solutions employed. [f] I 45 is a sporeless mutant derived from *aizawai*[1].





Figs. 1, 2. *thuringiensis* sporeless mutant, M 1 (Figure 1) and M 67 (Figure 2). ×10000. No spores are seen. Bare crystalline bodies, sharply pointed and diamond-shaped are seen together with (Figure 2) or without (Figure 1) cell ghosts.

The genetically stable sporeless mutants were cultivated in GNB-broth for 2–4 days at 30 °C. Precipitates following the centrifugation of the culture-broth were washed several times with 0.01 *M* phosphate buffer pH 7.0 and then resuspended in H_2O. These samples were submitted for bioassay and cytological observations.

Insecticidal activity was tested with silkworm, *Bombyx mori*, reared on fresh mulberry leaves, and with Eri-silkworm, *Samia cynthia ricini*, reared on artificial diets. Methods for bioassay and electron microscopy have been reported previously[1].

Four criteria were emphasized in selection of mutant strains: 1. lack of ability to form the spore and a lack of back mutation with the repeated cultivations. 2. High degree of spontaneous autolysis at the end of cultivation, 3. having a big crystalline body and 4. retaining the strong insecticidal toxicity. Sofar, we obtained 2 mutants from *thuringiensis* and 1 from *sotto* that seemed to be highly satisfactory under all the 4 conditions.

As seen in Table I, the temperature sensitivity of the sporeless mutants was examined after the cultivation on GNB-broth for 48 h at 30 °C. Although mutant M1 was somewhat heat-resistant, the practical thermal death point of all mutants was 60 °C, the same as seen in the cases of mutants from the strains of *kurstaki* and *aizawai*[1].

A heavy leakage or autolysis occurred during the 48-h stage of mutant strains. The number of viable cells (non-treated) was much less than that for parent strains, but numbers of crystalline bodies per ml of the broth were found to be nearly the same in parents and mutant strains (see Table II). Although mutant M1 derived from *thuringiensis* was sporeless, as determined by microscopic observation and by heat-treatment of 70 °C for 30 min, after heat treatment at 65 °C, a very small number of viable cells could be found. Table II demonstrates the dry weight, number of crystals and the insecticidal activity of sporeless mutants.

After 3–4 day cultivation, all broths were repeatedly washed as mentioned above and dry weight and number of crystalline bodies were measured. Since yields of 3 day cultivation were nearly the same as those of 4 day cultivation, the average value of 3 repeated cultivations was shown in Table II. Yields of mutant strains were less than half that of the original strain under the conditions of fermentation employed.

The number of crystalline bodies was counted under the phase contrast microscope, irrespective of being inside of cells or free. It was found that mutant M1, M67 and S162 had approximately equal number of crystalline bodies, namely $2–3 \times 10^8$/ml of broth.

Insecticidal activity of sporeless mutants, with and without heat treatment of 65 °C for 30 min, was measured by the mortality over 3 days of *Bombyx* and *Samia*. LD_{50} was calculated from the 'probit' and expressed in μg/ml of test solutions employed (Table II). In general, the toxic activities of mutants M1 and M67 seemed essentially the same as that of the original *thuringiensis* strain. However, the toxicity of mutant S162 seemed to

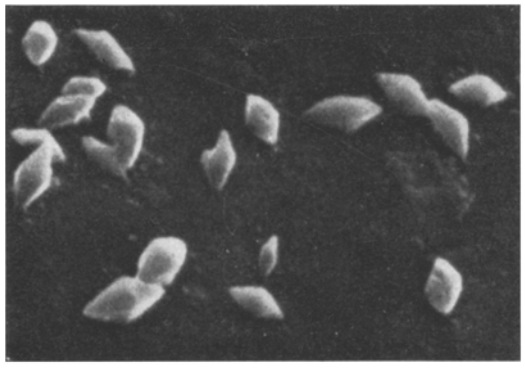


Fig. 3. *sotto* sporeless mutant, S162. ×10000. Crystalline bodies are much smaller than those of the M1 and M67.

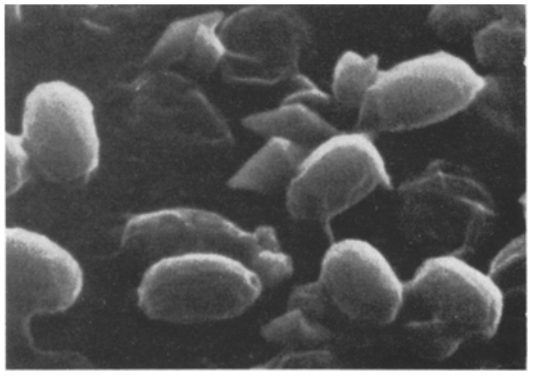


Fig. 4. *thuringiensis* wild strain. ×10000. Many ellipsoid spores and some diamond-shaped crystalline bodies are seen among the lysed cell ghosts.

be much stronger on both silkworms than that of original strain, *sotto*. No change of toxic activity was found in the all 3 mutants after the heat treatment.

Scanning electron micrographs of sporeless mutants showed only regularly shaped bipyramidal crystalline bodies laid free on a background of lysed cell envelopes which sometimes form cell ghosts or networks (Figures 1–3). In contrast, many ellipsoidal spores were observed besides octahedral crystalline bodies and cell envelopes in the original wild strains (Figure 4). In general, crystalline bodies of mutant S162 (Figure 3) were much smaller than those of the mutants M1 (Figure 1) and M67 (Figure 2), a condition which also occurs in the original *sotto* strain. All mutants had a size and shape of crystalline bodies similar to those of their parent strains.

The combination of biological inviability, and complete retention of toxic activity in preparations derived from sporeless mutants of *Bacillus thuringiensis* would provide a safer source of microbial insecticides as well as being a valuable source of starting material for the purification and characterization of the δ-endotoxin.

Summary. Three sporeless mutants of *Bacillus thuringiensis*, 2 derived from var. *thuringiensis* and 1 from var. *sotto* were selected after mutagenic treatment. They were completely lacking in ability to form spores, yet maintained intact insecticidal activity.

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[5] Daiwa Kasei, K. K. Hieda, Kosei-cho, Koga, Shiga, 520–30 (Japan).

Isatin-3-Anils as Excystment and Cysticidal Agents Against *Schizopyrenus russelli*

Generally relapses have been encountered in treated cases of chronic amoebiasis. This has been attributed to the fact that the drugs discovered so far have little or no effect on the cystic stage of amoeba.

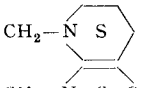
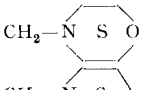
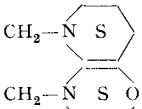
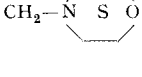
Isatins have generally been associated with antiviral activity[1]. Other biological responses exhibited by isatins include antibacterial[2], anthelmintic[3] and hypotensive actions[4]. In this communication we wish to report for the first time their cysticidal action and their ability to cause excystment. A series of isatin-3-anils(I) have been screened for their cysticidal activity and their role as excystment agents.

It is interesting to note that certain compounds of this series have shown cysticidal activity and also caused excystment simultaneously. This type of behaviour in a single substance has perhaps not been reported earlier.

Materials and methods. Isatin-3-anils(I) were obtained by condensing isatin with aromatic amines in ethanolic medium containing 2 drops of glacial acetic acid. The resulting condensed products were then subjected to Mannich reaction conditions[5].

[1] T. S. Osdene, in *Medicinal Chemistry*, 3rd ed. (Ed. A. Burger; Wiley Interscience, New York, N.Y. 1970), p. 662.
[2] R. S. Varma and W. L. Nobles, J. Pharm. Sci. *14*, 881 (1975).
[3] R. Cavier, R. Royer, R. Rips and L. Rene, Chim. Ther. *4*, 21 (1969); chem. Abstract *70*, 113686s (1969).
[4] Brit. Patent No. *1*, 240, 648 (1971); chem. abstr. 75, 118342q (1971).
[5] R. S. Varma, Polish J. Pharmac. Pharmac. in press (1975).

Isatin-3-anils (I) as excystment and cysticidal agents

Specimen No.	R	R′	Excystment (%)	Mortality (%)
1	4—OCH_3	H	50	20
2	2—OCH_3	H	28	—
3	4—CH_3	H	15	—
4	3—CH_3	H	30	40
5	4—Cl	H	nil	37
6	4—Ph	H	10	40
7	4—OCH_3	CH_2— 	nil	28
8	3—CH_3	CH_2— 	nil	20
9	4—Ph	CH_2— 	5	—
10	4—Ph	CH_2— 	10	10
11 Control (cysts + *E. coli* extract)			96	—