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ISOLATION OF NEW ENTOMOPATHOGENIC STRAINS OF BACILLUS THURINGIENSIS AND BACILLUS SPHAERICUS

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ABSTRACT

During 1984 and 1985, a survey was undertaken to isolate new strains of *Bacillus thuringiensis(B.t.),* pathogenic to mosquitoes, from soil samples collected in Israel. The isolation protocol involved suspension of the sample in buffered saline, vortexing to release bacteria adsorbed onto the soil particles and heat shocking the suspension to kill vegetative cells and non-spore-formers prior to plating a sample on LB solid medium The heat treatment removed a large number of the background contaminents. Selection of the pathogenic strains was achieved through assay of a 48 hour liquid culture of isolates *against Culexpipiens* and *Aedes aegypti* larvae, rather than through use of selective media. The process was good for the isolation of mosquito pathogenic strains of both *B.t.* and *B. sphaericus.* Three isolates, s-2-6, s-2-13 and 37. K 30, serotyped as *B.t.* var *israelensis/aizawi,* were highly toxic to A. *aegypti* larvae. Isolate s-2-6, with a calculated toxicity of 17 800 ITU/mg, was the most toxic strain recovered.

Work i s currenlly underway to isolate B.t strains from soil samples taken from different ecological and geographic zones in Kenya. The protocol adopted is more specific for the isolation of *B.t.*. It involves the use of a liquid medium incorporating 0.25M Na acetate. Use of Na acetate at this concentration supresses the germination of any *B.t.* spores in the soil sample added to the medium, but facilitates the germination of other soil Bacilli over the 4 to 5 hours shaken incubation period. Heat treatment of a sample taken from the soil culture of 80'C for 4 minutes kills the developing vegetative cells and non-spore-forming bacteria. Plating a sample of the heat treated suspension on enriched NA then allows *B.t.* spores to germinate wilh many of the background species removed. The isolates are then screened for activity against Lepidopteran pest species and mosquitoes.

This method has so far produced 10 crystal forming isolates. These exhibit varying levels of pathogenicity to the pest species used in the preliminary assays conducted so far.

Bacillus thuringiensis (Berliner) *(B.t)* has shown great potential for development and use in a number of pest management programmes. It is desirable that strains continue to become available for possible development for a number of reasons. These include discovery of strains of increased potency, active against the indigenous pest species in the country of isolation. Strains with a wider host range could reduce the number of treatments applied to a crop for pest control purposes. Strains may be isolated showing activity to different insect orders, e.g. *B.t.* var. *tenebrionis* (Krieg et al., 1983) which is active against certain Coleoptera. *B.t.* var *israelens'ts* (Goldberg and Margalit, 1977) and *B.t.* var *morrisoni* (PG-14) (Padua et al., 1984) which show high and specific pathogenicity to mosquito larvae, differing from previously isolated strains which were active against Lepidoptera. Strains may be found which show better fermentation or storage characteristics. The elucidation of strains more amenable to genetic manipulation is also an important consideration with the advances made in molecular biology. Furthermore, restrictions are in force on the introduction of exotic

microorganisms for pest control purposes. India, for example, requires that only indigenous species of *B.t.* be used for insect control. The situation can be alleviated, as in India, where *B.t.* strains are isolated from local material.

Bacillus sphaericus (Neide) is also an excellent entomopathogenic species for mosquito control. Unfortunately its development was overshadowed by the discovery of *B.t.i.* However, with the isolation of strains showing increased levels of pathogenicity (Lacey and Singer, 1982; Weiser, 1984) interest was renewed in this bacterium. It is now a valuable candidate organism for large scale mosquito control programmes. It is therefore, also desirable that additional strains of *B. sphaericus* possessing high mosquito larvae toxicity and other advantageous traits become available for possible field use.

MATERIALS AND METHODS

1. Isolation of mosquito-active *Bacillus* spp. in Israel

During 1984 and 1985, a survey was undertaken to isolate bacteria, pathogenic to mosquitoes, from soil samples collected and processed in Israel. The protocol of isolation adopted was simple and relatively straightforward (Brownbridge and Margalit, 1986). Briefly, the method used was as follows:

Mud or soil samples were suspended in tubes containing sterile buffered saline solution and vigorously agitated using a vortex to release bacteria adsorbed onto the soil particles. The tubes were heated at 78°C for 12 minutes to reduce the unwanted micro-organisms, i.e. non-spore-formers and vegetative cells. A sample of the heat treated suspension was then spread evenly over the surface of solid medium. The plates were incubated at 30°C and examined for bacterial colonies after 48 and 72 hours.

The isolated growth of the colonies facilitated their removal from the plates by means of an inoculation loop when colonies were selected and subcultured for evaluation in a screening assay.

Mosquito pathogenic strains were selected by assay of 48 hour liquid cultures against *Culex pipiens* and *Aedes aegypti* larvae. This method was employed in preference to the use of selective media as it enabled both *B.t.* and *B. sphaericus*, as well as other potentially mosquito pathogenic strains of bacteria, to be recovered using only one procedure.

Isolates showing pathogenicity to the mosquito larvae were cultured in a peptone-glucose-salts medium and assayed over a range of dilutions. From these tests, the most toxic isolates were chosen.

2. Isolation of *B.t.* strains in Kenya

Work is currently underway to isolate *B.t.* strains from soil samples taken from different ecological and geographical zones in Kenya. The protocol adopted thus incorporates the use of defined media to reduce other bacteria from the processed material. The methodology is based on that of Travers et al. (1987), and involves the use of a liquid medium containing 0.25m Na acetate, which selects against other *Bacillus* spp. in the sample material.

Initially, a sample of the soil is placed in 100 ml. flasks containing PBS and a number of micro elements. The flasks are incubated at 30°C, 300 rpm for 24 hours. The treatment is designed to release bacteria from the soil and promote spore formation in the *Bacillus* spp.

A 1 ml. sample of the suspension is then transferred to 100 ml. flasks containing 10ml of L broth buffered with 0.25m Na. acetate. The flasks are incubated at 30°C, 300 rpm for 4 hours. Use of Na acetate at this concentration supresses the germination of any *B.t.* spores contained in the sample, but facilitates the germination of other soil bacilli. Heat treatment at 80°C for 4 minutes kills the vegetative cells and non-spore-forming bacteria. The heat-treated suspension is then plated on enriched nutrient agar which allows *B.t.* spores to germinate, with the majority of the unwanted background bacteria removed. The isolates thus obtained are subcultured and examined microscopi-

cally for the presence of crystals. The crystals formers are then screened for activity against Lepidopteran pest species, e.g. *Chilo parellus*, and mosquito larvae.

RESULTS

1. Israel isolates

Over 130 soil samples from 80 diverse mosquito breeding habitats were processed and several hundred sporeforming bacteria were isolated and assayed. Nine *B.t.* strains, toxic to mosquito larvae, were recovered. These were identified, using the serum agglutination test devised by de Barjac and Bonnefoi (1962), as *B.t. israelensis/aizawi*, *B.t. israelensis*, and *B.t. entomocidus* (Brownbridge and Margalit, 1987).

Three isolates, s–2–6, s–2–13 and 37. K 30, identified as *B.t.* subsp. *israelensis/aizawi*, were highly toxic to *A. aegypti* larvae. Isolate s–2–6, with a calculated toxicity of 17 800 ITU/mg, was the most toxic strain recovered (Table 1) (Brownbridge and Margalit, 1987).

TABLE 1
Mosquito pathogenic strains of *B.t.* isolated from soil and mud samples collected in Israel

Isolate code	*B.t.* serovar	ITU/mg vs. *A. aegypti* L4 larvae[1]
IPS–82[2]	israelensis	15000
1884[3]	israelensis	7000
S–2–6	israelensis/aizawai	17800
37.k 30	israelensis/aizawai	15600
S–2–13	israelensis/aizawai	13800
36.k 2297	israelensis	7100
33.14	israelensis	6100
35.28	israelensis	4700
35.k 36	israelensis	3500
S–2–4	entomocidus	250
S–2–5	entomocidus	140

[1]ITU values calculated by comparison with the IPS–82 powder. All assays performed on lyophilized powder preparations against early fourth stage *A. aegypti* larvae.
[2]International Standard Reference Preparation of *B.t.i.* obtained from the Institut Pasteur, Paris, with an arbitrarily assigned value of 15000 ITU/mg vs L4 *A. aegypti* larvae.
[3]Reference strain of *B.t.i.* obtained from the Institut Pasteur cultured and preserved in our laboratory under identical conditions of media and regime as other *B.t.* types isolated in this survey.

Bacteria of typical *B. sphaericus* appearance were the most common types isolated from material collected in the survey (Brownbridge and Margalit, 1987). Nineteen isolates were sufficiently toxic to be retained for bioassay against *C. pipiens* larvae. The isolates were classified according to their reaction to a series of lytic bacteriophages (Yousten, personal communication).

The phage testing enabled the *B. sphaericus* strains to be placed in either Phage Group 3 or Group 4 (Yousten, 1984). The most toxic strains recovered were all in Phage 3. Two strains, coded 2615

TABLE 2
Bacillus sphaericus strains isolated from soil/mud samples collected in Israel (only the most active isolates listed)

WHO/CCBC accession number	Phage group	ITU/mg US L4 *C. pipiens larvae*[1]
RB–80[2]	3	1000
2362[3]	3	910
2613	3	1100
2615	3	1500
2619	3	1300
2631	3	1450
2626	4	130
2630	4	350

[1]ITU values calculated by comparison with the RB–80 Standard. Assays performed on acetone powders vs early fourth stage *C. pipiens* larvae.
[2]International Standard Reference Preparation produced from *B. sphaericus* strain 1593–4 and obtained from the Institut Pasteur. Assigned on arbitrary value of 1000 ITU/mg vs. *C. pipiens* larvae.
[3]*B. sphaericus* reference strain obtained from Dr. A.A. Yousten and produced in our laboratory under the same regime as all other isolates tested.

and 2631, with approximately 1500 ITU/mg were the most potent isolates recovered (Table 2). They were approximately 50% more active than the RB–80 *B. sphaericus* reference preparation.

There was also an interesting geographical distribution of the material from which the different phage groups were isolated. Bacteria belonging to Phase Group 3 were recovered from material collected in the Central Negev region of Israel. Material taken from sources close to the Dead Sea produced isolates belonging to Group 4, indicating that the environment may exert an influence upon the occurrence of different entomopathogenic strains (Brownbridge and Margalit, 1987).

The protocol adopted in Kenya has so far produced eight crystal forming isolates pathogenic to one or more of the pest species used in the preliminary screening assays (Table 3). These and others

TABLE 3
B.t. isolates from soils collected in Kenya

Isolate Code	Pest species	
	C. partellus	*A. aegypti*
MF–1–1	+	–
MF–1–3	+	–
MF–1–4	+	–
MF–2B–3	+	+/–
MF–3A–1	+	–
MF–4B–1	+	–
MF–4B–2	+	–
MF–9–3	+	–

+ = pathogenic
– = non-pathogenic
+/– = slightly pathogenic

that will become available, are now the subject of further quantitive evaluation. These experiments are designed to elucidate the most toxic strains for use in future field experiments.

DISCUSSION

Entomopathogenic *Bacillus* strains were recovered in both surveys from soil samples collected in a variety of ecological and environmental locations. In this respect, this study differs from the usual isolation of entomopathogenic bacteria, where the micro-organisims are obtained from diseased or dead insects.

This study serves to illustrate the value of soils as a source of insect pathogenic bacteria, where *B.t.* and *B. sphaericus* spores are known to survive for long periods (Hertlein et al., 1979; Petras and Casida, 1985). Soils thus appear to have excellent potential for further exploitation, and if such isolation schemes are adopted in several countries, then it seems likely that indigenous strains of entomopathogenic bacteria would become available for development and use against a variety of pest species.

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