AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

PN-ABM-639

DATE: 10/20/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C5-141

PR

Attachment

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C5-141

BIOLOGICAL CONTROL OF MOSQUITOES BY BACILLUS SPHAERICUS

PROGRESS REPORT: AID-CDR PROJECT NO. PDC-5544-G-SS-5098-01

The goal of this project was to clone the Bacillus thuringiensis israeliensis (Bti) DNA coding for the mosquito larval toxin into Bacillus sphaericus, in order to produce an effective, broad-spectrum larvicide with greater persistence than Bti. In our reports on the first year's work, we described the successful completion of the cloning, with the production of B. sphaericus transformants much more toxic to Aedes mosquitoes than the original strain. During the first half of the second year of the project, we have completed the screening of the transformants produced in the cloning experiment, and have compared physical characteristics and flotation capability of Bti, B. sphaericus, and a successful transformant.

1. Screening:

Approximately 50 additional clones stably transformed for kanamycin (kan) resistance (the transformation system is described in our earlier reports) were screened for the presence of the Bti 28 and 65 kDa toxin proteins and for larvicidal activity against Aedes during the period covered by this report. Of these, one clone (62) was slightly more toxic to Aedes than the best of the previously tested clones (13), and a full order of magnitude more effective than the original B. sphaericus strain 2362. Clone 62 also expresses the Bti 28kDa protein well (Table 1).

The following conclusions may be drawn from the results of the screening:

a. The presence of kan (5μg/ml) after completion of growth delays sporulation and inhibits both expression of the Bti toxin proteins and larvicidal activity.

b. Expression of the Bti 28kDa protein, but not of the 65 kDa protein, correlates with larvicidal activity. It should be noted, however, that expression of both of these toxin proteins is not sufficient to determine larvicidal activity against Aedes, since several clones with strong immunological reactions to antibodies of both proteins were not more active against Aedes than the original strain 2362.

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In parallel with the screening, attempts were made to increase the plasmid copy number and thus the concentration of the toxin-coding genes by raising the kan concentration during the early stages of culture of the transformants. These attempts led to loss of toxic activity, presumably due to loss of the inserted Bti DNA from the plasmid.

2. Physical comparisons of Bti, B. sphaericus, and transformant 13:
Since B. sphaericus larvicidal activity is reportedly more persistent than that of Bti after application to mosquito breeding sites, we compared the flotation of these two strains with each other and with transformant 13. We also compared the adhesiveness to surfaces and hydrophobicity of the strains, since these physical characteristics might affect flotation under field conditions. We found that lyophilisates of sporulated cultures of B. sphaericus are both less hydrophobic and less adhesive to inert surfaces than similar preparations of Bti and that clone 13 was similar to B. sphaericus in these respects (representative results are shown in Tables 2 and 3). We also found, contrary to our expectations, that there was no great difference in flotation among the several strains (Figure 1). This suggest that the superior persistence of B. sphaericus larvicidal activity may be due to recycling within the mosquito population, rather than to any delay in sinking in water.

During the remaining months of the project, we intend to conduct further comparisons of persistence, to determine whether any of our active transformants are superior to the original Bti and B. sphaericus strains in overall larvicidal activity. To this end, we intend to compare persistence of activity against Aedes under simulated field conditions, which should provide a better indication of overall effectiveness than comparisons of flotation alone under unrealistic laboratory conditions.

Table 1: Characterization of representative clones (of approximately 100 kan-resistant transformants of B. sphaericus 2362).

Strain	*During sporulat. kan 5µg/ml	IMMUNOLOGICAL RESPONSE Bti-proteins 28kDa	65kDa	B.sph.prot 110kDa	LC_{50} µg/ml (48hrs) Aedes	Sporulat. time (hrs)
2362	–	–	–	+++	1.9	ca. 24
62	–	+++	+	+++	0.19	ca. 48
	+	++	+	+++	0.30	>48
5	–	+	–	+++	0.57	>48
	+	++	–	+++	1.16	>48
13	–	++	–	+++	0.21	>48
	+	++	–	+++	0.53	>48
96	–	–	++	+++	1.7	ca. 24
	+	–	+	+++	2.1	ca. 24

* Initial stages of culture, up to the onset of sporulation, were always in the presence of kan.

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Table 2: Adhesion to plastic of Bti, B. sphaericus, and clone 13.

Sample	OD_{600nm}			Aver.perc. decrease in O.D.	
	t_0(min)	t_{30}(min)	t_{60}(min)	t_{30}(min)	t_{60}(min)
Bti	0.500	0.350	0.320	30.0	36.0
	0.440	0.370	0.340	15.9	22.7
	0.460	0.370	0.340	19.6	26.1
				21.8±7.3	28.3±6.9
B. sphaericus	0.340	0.280	0.260	17.6	23.5
	0.340	0.310	0.290	8.8	14.7
	0.250	0.230	0.205	8.0	18.0
				11.5±5.3	18.7±4.4
Clone 13	0.250	0.255	0.220	2.0	12.0
	0.230	0.210	0.200	8.7	13.0
				3.4	12.5

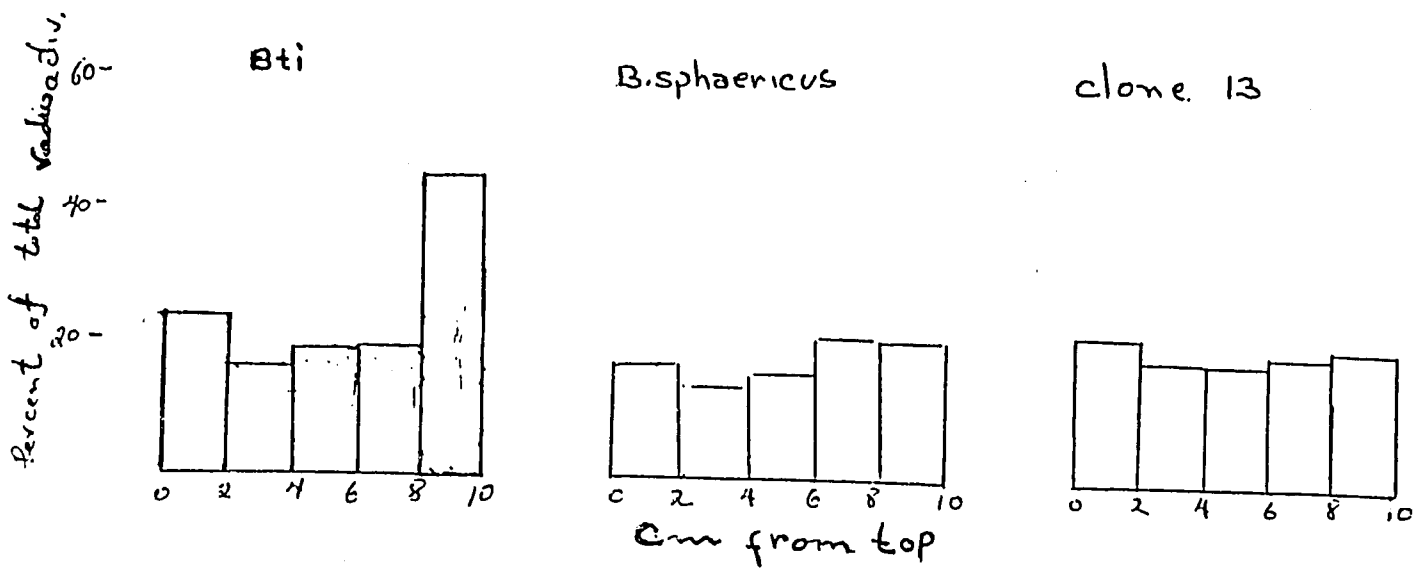
Lyophilized powders of the samples were suspended in water in the presence of 5mM $MgSO_4$. 3 ml of sample was incubated for 30 or 60 minutes at 37° C, with shaking, in a 6-5 ml polyethylene vial. The difference between the initial and final O.D. readings indicates the amount of sample adhering to the substrate.

Table 3: Hydrophobicity of suspensions of lyophilized powders of Bti, B. sphaericus, and Clone 13.

STRAIN	Hydrophobicity: Percent Decrease in OD_{434nm}	Hydrophobicity: Average percent Decrease
Expt 1: Bti	58.3	
	52.1	55.2
B. sphaericus	18.2	
	19.8	19.0
Expt 2: Bti	61.1	57.3
	55.1	
	55.7	
B. sphaericus	2.3	
	1.6	
	3.1	2.3
Clone 13	12.8	
	8.2	8.5
	4.5	

Hydrophobicity was determined by extraction into hexadecane. 0.13 ml hexadecane was aded to 4.0 ml of a suspension of O.D. about 0.5 and the sample was "vortexed" for 1 min. After 15 min. at room temperature, the OD of the aqueous phase was read and subtracted from the initial reading.

Figure 1: Percent of total radioactivity



An aqueous suspension of a lyophilized ^{14}C-labelled powder, 4 mg/ml, was added to the top of a column of water containing 1 % glycerol in 1 cm diameter dialysis tubing. After standing at room temperature for 2 hrs, the samples were frozen and sliced into 2 cm lengths. The fractions were lyophilized and the radioactivity of each fraction was determined. The radioactivity is expressed as the percent of the total radioactivity in all the fractions. Each bar represents the average of triplicate samples.