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Biological control of the zebra mussel *Dreissena polymorpha* and the snail *Biomphalaria glabrata*, using Gramicidin S and D and molluscicidal strains of *Bacillus*

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Applications of Final Whole Culture (FWC) or primary powder material from strains of each of four *Bacillus* species (*B. alvei, B. brevis, B. circulans, B. laterosporus*) used singly, as well as the antibiotics Gramicidin S (GS) and Gramicidin D (GD) used singly, were found to be molluscicidal against several life cycle stages of the zebra mussel *Dreissena polymorpha*. Combinations of the bacterial material with either GS or GD were either additive (for GS) or antagonistic (for GD). The smaller the life cycle stage of the animal, the more sensitive it was to either the bacterial or antibiotic agent. The veliger stage was particularly sensitive to each agent, with the molluscicidal effect being more rapid in the veliger (5 h) than in the adult (6 days). The molluscicidal effects of these agents (at 1–100 μg ml^{-1}) against the veliger stages of the zebra mussel were comparable to the activity of *B. thuringiensis* and *B. sphaericus* against their target organisms. These agents used singly were also active against small adult *Biomphalaria glabrata*, the snail vector of schistosomiasis (eg at tenths of μg ml^{-1} of GS).

Keywords: biological control; veliger stage; zebra mussel (*Dreissena polymorpha*); molluscicidal strains of *Bacillus*; Gramicidin S; Gramicidin D; *Biomphalaria glabrata*; schistosomiasis

Introduction

The zebra mussel (*Dreissena polymorpha*), a mussel native to Europe's Black and Caspian Seas, was accidentally introduced into North American waters in the mid-1980s [4]. Since the discovery of the zebra mussel in Lake St Clair in June of 1988, its number and distribution have increased throughout every waterway east of the Mississippi, as far south as the deep waters surrounding New Orleans.

The female mussel is prolific, producing up to one million eggs during several spawns per year. The larval stage is planktonic and is readily dispersed through water currents [2,15]. The 'invasive' stage of the zebra mussel is the planktonic juvenile or veliger stage. If it were possible to impact this stage, then the adults would be interdicted, equivalent in many ways to attacking the more susceptible mosquito larva than the adult mosquito (the latter does the public health damage). The adult zebra mussel is capable of attaching to any hard surface and accumulating in the hundreds to hundreds of thousands per square meter of surface. When this aggregation of adult mussels starts to block water-intake of industrial plants, thousands of dollars of expenditure are necessary to remove them. Existing controls use chemical, physical and manual removal methods [2]. Biocontrol methods as an adjunct to these would be preferred for rational ecological as well as Integrated Pest Management reasons. Until recently no such biological agents were known for molluscicidal control [5,9,10].

In the middle to late 1980s, we found strains of four species of *Bacillus (B. alvei, B. brevis, B. circulans, B. laterosporus)* to be molluscicidal against *Biomphalaria glabrata*, a major vector of the tropical disease schistosomiasis [14]. The present studies are an extension of studies of the molluscicidal activity against *B. glabrata* [14].

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Materials and methods

Maintenance and production of the bacteria

The maintenance and production of all of the bacterial material (Final Whole Culture (FWC), or primary powders) were done according to the procedures for *B. brevis* of Singer *et al* [14]. The bacterial concentrations for each of the strains for the FWCs and primary powders are shown in the footnotes to Table 1. The same batch of each primary powder preparation was used for each experiment involving the powders, while fresh FWCs were used for each experiment involving FWCs.

Maintenance and bioassay for the snail, Biomphalaria glabrata

Maintenance and bioassay of the snail *Biomphalaria glabrata* were done according to the procedures of Singer *et al* [14].

Maintenance and bioassay for the adult and veliger stage zebra mussel, Dreissena polymorpha

The rearing and bioassay procedures for both the adult and the veliger stages of zebra mussel (*Dreissena polymorpha*), were carried out according to the procedures for *D. polymorpha* of Stoeckel and Garton [16]. The zebra mussels were obtained from the Illinois and Mississippi rivers.

The life cycle stages of *D. polymorpha*, as described by Claudi and Mackie [2], consist of a fertilized egg stage, followed by a veliger stage, a post-veliger stage, a settling

Table 1 Summary of the biological activity (log LC_{50})$^{-1}$ after 6 days of various molluscicidal strains of *Bacillus vs* adult life cycle stages of the zebra mussel *Dreissena polymorpha*

Agent	Adult					
	No. of trials	(>20 mm)	No. of trials	(8–12 mm)	No. of trials	(2–5 mm)
Bacillus alvei 2771-FWC (Log LC_{50})$^{-1}$	2	NA	2	$\overline{X}$ = 2.48 (0)	10	$\overline{X}$ = 2.97 (0.34)
Bacillus alvei III3DT1A-FWC (Log LC_{50})$^{-1}$	2	NA	2	$\overline{X}$ = 2.18 (0.15)	0	–
Bacillus circulans 42G1-FWC (Log LC_{50})$^{-1}$	2	NA	2	$\overline{X}$ = 1.97 (0.14)	0	–
Bacillus circulans 42G1-Primary powder (Log LC_{50})$^{-1}$	1	NA	0	–	0	–
Bacillus laterosporus 1647 Primary powder (Log LC_{50})$^{-1}$	1	NA	0	–	0	–
Bacillus brevis SS86-4 FWC (Log LC_{50})$^{-1}$	1	NA	0	–	12	$\overline{X}$ = 1.90 (0.25)

FWC = Final Whole Culture; NA = no activity; – = not done; ( ) = standard deviation; $\overline{X}$ = average; LC_{50} = dilution of FWC, primary powder, or antibiotic giving 50% death of test zebra mussel *Dreissena polymorpha*; since the values are a dilution of the FWC this requires the designation ( )$^{-1}$; we use a log value of the LC_{50}. As a consequence one should be careful when comparing the values of the FWCs (which are dilutions of the FWC) with (for example) the direct weight values of a chemical used such as Gramicidin S or D. The average Total Viable Count (TVC) for the above bacterial preparations in CPU ml^{-1} FWC or CPU mg^{-1} powder are as follows: *Bacillus alvei* 2771-FWC, 2.48×10^{-8}; *B. alvei* III3DT1A-FWC, 3.01×10^{-8}; *B. brevis* SS86-4 FWC, 6.9×10^{-8}; *B. circulans* 42G1-FWC, 4.03×10^{-8}; *B. circulans* 42G1-primary powder, 8.72×10^{-10}; *B. laterosporus* 1647-primary powder, 1.96×10^{-11}.

stage and an adult stage. The veliger stage animals can be divided into a young veliger (or pre D-stage veliger) and a straight-hinged or 'D' stage veliger. The attached adult stage begins at about a 1–3 mm shell size and can grow to 40 mm in size. From the D-stage to the adult stage the animal is microscopic, growing from slightly less than 100 μm to the 1-mm beginning adult stage. The pre D-stage animals used were 40–50 μm in size, and the D-stage animals were about 80–100 μm.

For the adult mussel bioassay of the agents used singly, we used four dilutions (1/30, 1/50, 1/100, 1/300) of the test material (FWC, or primary powder) in 15-cm glass specimen dishes with an air bubbler. Three zebra mussels were used, 3–5 mm long, when available. Due to a shortage of these very small animals, 8–12 mm animals were often used as indicated in the specific experimental protocol (Table 1). The test material was incubated at 18°C and surviving adult mussels were counted at days 3 and 6. Dead adult zebra mussels were identified as mussels that were gaping (open wide enough to indicate partial or complete atrophy of the mussel tissue) [16].

The veliger bioassays of the agents used singly were done according to the methods of Stoeckel and Garton [16] where decimal-diluted material (FWC, primary powder, antibiotic, etc) was added to 24-well micro-well plates (*ca* 3 ml well^{-1}) to which had been previously added D-stage veligers. The veligers were obtained and prepared according to the methods of Stoeckel and Garton [16]. The micro-well plates were incubated at 18°C and examined under an inverted microscope at 0, 3, and 5 h for surviving D-stage veligers. Dead D-stage veligers were not motile and usually fell apart. Zero hour was just prior to the addition of the diluted bacterial material.

Combination experiments

Combinations of each of the *Bacillus* strains with either Gramicidin S or D were tested against adult mussels (8–12 mm). The concentrations of each of the agents were the LC_{50} concentrations (as determined in the single agent experiments) and fractions of the LC_{50} concentrations (1/2, 1/4, and 1/10 LC_{50} concentrations, with 0 indicating no addition of the first and/or second agent).

Statistical analysis

The LC_{50} calculations (the dilution of the bacterial material or concentration of the antibiotic that killed 50% of the test adult or veliger zebra mussels or snails), as well as the R^2 values, were obtained by plotting the linear regression of mortality after a 6-day incubation (or in the case of the veliger bioassays, 3 or 5-h incubation) of the adult zebra mussel (or snail) *vs* the dilution of the material being tested. The R^2 value indicates the reliability of the LC_{50} values. Only data with R^2 values >0.50 were used.

For the combination experiments (molluscicidal bacterial strain and gramicidin S or D), statistical analysis was performed on SAS version 5 (SAS Institute, Cary, NC, USA)

Table 2 Summary of the biological activity (log LC_{50})$^{-1}$ after 5 h of various molluscicidal strains of *Bacillus vs* the veliger life cycle stages of the zebra mussel *Dreissena polymorpha*

Agent	Veliger			
	No. of trials	pre D-stage	No. of trials	D-stage
Bacillus alvei 2771-FWC (Log LC_{50})$^{-1}$	0	–	6	$\overline{X} = 4.72$ (0.33)
Bacillus alvei III3DT1A-FWC (Log LC_{50})$^{-1}$	0	–	6	$\overline{X} = 5.32$ (0.41)
Bacillus circulans 42G1-FWC (Log LC_{50})$^{-1}$	0	–	6	$\overline{X} = 7.02$ (1.19)
Bacillus circulans 42G1-Primary powder (Log LC_{50})$^{-1}$	1	$\overline{X} = 8.35$	0	–
Bacillus laterosporus 1647 Primary powder (Log LC_{50})$^{-1}$	1	$\overline{X} = 8.34$	0	–
Bacillus brevis SS86-4 FWC (Log LC_{50})$^{-1}$	0	–	0	–

See Table 1 for definitions and for the average Total Viable Count (TVC) for the above bacterial preparations.

using Scheffe's multiple comparison test. Scheffe's test was used because it is the most conservative and guards against type 1 error, ie rejecting a null hypothesis which is true. In terms of the null hypothesis for our combination experiments, there was no interaction between antibiotics (Gramicidin S, D) and *Bacillus* culture material. Data from each point were analyzed as a proportion using ArcSine transformation as called for in the statistical analysis program.

Results

Agents used singly

FWC (and/or primary powders) of strain(s) from each of four *Bacillus* species were tested individually against both adult (Table 1) and veliger (Table 2) stages of the zebra mussel (*D. polymorpha*), as well as against the adult snails (3–5 mm) *B. glabrata* (Table 3), and were molluscicidal against many of these targets. Several of the mussel life cycle target animals were not available in sufficient numbers to be able to compare molluscicidal activity at all of these stages. The veliger stages of the mussel were more sensitive to the FWC preparations than were the adult stages. The *B. circulans* FWC was the most active against the D-stage when compared to both *B. alvei* FWCs tested.

Table 3 Summary of the biological activity (log LC_{50})$^{-1}$ after 6 days of various molluscicidal strains of *Bacillus vs* the adult stage of the snail *Biomphalaria glabrata*

Agent	Adult (2–5 mm)	
	No. of trials	(Log LC_{50})$^{-1}$
Bacillus alvei 2771-FWC	13	$\overline{X} = 2.80$ (0.13)
Bacillus alvei III3DT1A-FWC	2	$\overline{X} = 2.59$ (0.05)
Bacillus circulans 42G1-FWC	2	$\overline{X} = 2.71$ (0)
Bacillus brevis SS86-4, FWC	11	$\overline{X} = 2.78$ (0.18)

See Table 1 for definitions and for the average Total Viable Count (TVC) for the above bacterial preparations.

The single powder preparations of *B. circulans* and *B. laterosporus* were as active against the pre-D-stage veligers as were the aforementioned FWCs against the D-stage veligers. The one case (12 trials) where the powder preparations were examined against small adult mussels (2–5 mm) the activity appeared to be low, with average (log LC_{50})$^{-1}$ values of just 1.90. This would be equivalent to a dilution of the powder of 1/79.5, whereas the *B. cereus* FWC against the D-stage veliger had high average (log LC_{50})$^{-1}$ values of 7.02 equivalent to dilutions of the FWC of 1.04×10^{-7}. The activity of the above preparations against the snail adults (Table 3) were equivalent to the activity of these preparations against the small adult mussels.

Gramicidin S and Gramicidin D were also tested against adult and veliger stages of the zebra mussel (*D. polymorpha*), as well as against adult snails (*B. glabrata*) (Table 4), and were found to be molluscicidal against many of these targets. In this case (Table 4), the smaller the number the higher the activity, whereas with the FWC preparation, the larger the number (being a dilution) the higher the molluscicidal activity. The antibiotic Gramicidin S (GS) was more active than Gramicidin D (GD) when used against the small adult (8–12 mm) mussels or against the small adult snails (3–5 mm) (Table 4). Since we have no values for GD used against D-stage veligers we cannot compare its activity to that of GS. We can say however that GS used against the D-stage veliger was quite active at an LC_{50} value of 0.21 μg GS ml^{-1}. In addition GS appears to be 1000 times more active than GD, against the small adult snails (2–5 mm), with GS having an LC_{50} value of 0.195 μg GS ml^{-1}.

Combined agents

We examined the molluscicidal effect of combinations of Gramicidin S or D and FWCs from three *Bacillus* strains, *B. alvei* 2771 (six trials using GS, four using GD), *B. alvei* III3DT1A (three trials using GS, four using GD), *B. circulans* 42G1A (two trials using GS, four using GD) against adult (8–12 mm) mussels. In these studies we expect the interactions to be within a spectrum running broadly from

Table 4 Summary of the biological activity (LC_{50}) of Gramicidin S and Gramicidin D *vs* various life cycle stages of the zebra mussel *Dreissena polymorpha* and the adult stages of the snail *Biomphalaria glabrata*

Agent	Adult (zebra mussel *Dreissena polymorpha*)					
	No. of trials	(>20 mm)	No. of trials	(8–12 mm)	No. of trials	(2–5 mm)
Gramicidin S	2		4		0	
LC_{50} (in $\mu g\,ml^{-1}$)		NA		$\overline{X} = 1.65$ (0.78)		–
Gramicidin D	2		1		0	
LC_{50} (in $\mu g\,ml^{-1}$)		NA		5.01		–

Agent	Veliger (zebra mussel *Dreissena polymorpha*)			
	No. of trials	pre D-stage	No. of trials	D-stage
Gramicidin S	0			
LC_{50} (in $\mu g\,ml^{-1}$)		–	2	$\overline{X} = 0.21$ (0.15)
Gramicidin D	0			
LC_{50} (in $\mu g\,ml^{-1}$)		–	0	–

Agent	Adult snail *Biomphalaria glabrata* (2–5 mm)	
	No. of trials	
Gramicidin S	2	
LC_{50} (in $\mu g\,ml^{-1}$)		$\overline{X} = 0.195$ (0.45)
Gramicidin D	6	
LC_{50} (in $\mu g\,ml^{-1}$)		$\overline{X} = 199.5$ (0.22)

FWC = Final Whole Culture; NA = no activity; – = not done; ( ) = standard deviation; $\overline{X}$ = average; LC_{50} = dilution of FWC, primary powder, or antibiotic giving 50% death of test zebra mussel *Dreissena polymorpha* (or the snail *Biomphalaria glabrata*).

Table 5 Example of combination test of *Bacillus alvei* 2771 FWCs and Gramicidin S

Antibiotic LC_{50} concentration	Dilution of LC_{50} concentrations				
	0	1	1/2	1/4	1/10
0	3*	2	3	3	–
1	1	0	0	0	–
1/2	3	1	1	2	–
1/4	3	0	3	3	–
1/10	–	–	–	–	3
Control	3				

∗ = Number of survivors after 6-day bioassay.
Three zebra mussels (8–12 mm) used per point.
– = Combinations that were not used in the assay.
LC_{50} concentration = that concentration (dose) previously established which results in killing half of the test animals. The amounts 1, 1/2, 1/4, 1/10 are fractions of the LC_{50} dose. 0 = no addition.

Table 6 Example of combination test of *Bacillus alvei* 2771 FWCs and Gramicidin D

Antibiotic LC_{50} concentration	Final whole culture LC_{50} concentrations				
	0	1	1/2	1/4	1/10
0	3*	2	3	3	–
1	2	2	2	3	–
1/2	3	2	3	3	–
1/4	3	3	3	3	–
1/10	–	–	–	–	3
Control	3				

∗ = Number of survivors after 6-day bioassay.
Three zebra mussels (8–12 mm) used per point.
– = Combinations that were not used in the assay.
LC_{50} concentration = that concentration (dose) previously established which results in killing half of the test animals. The amounts 1, 1/2, 1/4, 1/10 are fractions of the LC_{50} dose. 0 = no addition.

'antagonistic', to 'additive', to 'synergistic'. Rather than show data for all of the 23 combinations, we have shown one example of a combination of *B. alvei* 2771 used in combination with GS (Table 5), and one example of a combination of *B. alvei* 2771 used in combination with GD (Table 6) to illustrate the experimental design. The results of all 23 experiments were analyzed statistically. Scheffe's multiple comparison test was done on the data from all 23 combination experiments using the statistical analysis program, SAS. An example of the evaluation of LC_{50} combinations of *Bacillus alvei* 2771 FWC and Gramicidin S or Gramicidin D is shown in Table 7.

For combinations of Gramicidin S and *B. alvei* 2771 (Table 7), no statistically significant interaction was found, indicating that their effects were neither synergistic nor antagonistic but additive. There was no interaction of one agent with the other.

For combinations of Gramicidin D and *B. alvei* 2771

Table 7 Example of evaluation of LC_{50} combinations of *Bacillus alvei* 2771 FWC and Gramicidin S or Gramicidin D

Treatment	No. of trials	R^2	F stat	Probability	Assessment
B. alvei 2771	6	0.668	31.68	0.0001	Treatment statistically significant at 0.05 level
Gramicidin S alone	6	0.668	18.91	0.0001	Treatment statistically significant at 0.05 level
Gramicidin S + *B. alvei* 2771	6	0.668	1.02	0.4309	Not significant at 0.05 level, indicating no interaction between treatments
B. alvei 2771	4	0.636	9.88	0.0001	Treatment statistically significant at 0.05 level
Gramicidin D alone	4	0.636	8.58	0.0001	Treatment statistically significant at 0.05 level
Gramicidin D + *B. alvei* 2771	4	0.636	2.29	0.0315	Treatment statistically significant at 0.05 level indicating interaction

F stat = F statistic; R^2 = see text; LC_{50} = see Table 1.

(Table 7), statistically significant differences were found at the 0.05 level, indicating interaction between the two treatments. Since inspection of the data showed the molluscicidal activity of the combination of the agents to be less than the activity of either agent when used alone, we took this to mean that the combination was antagonistic rather than synergistic.

The results for combinations of either of the two antibiotics with the other two FWCs were very close to the above results with *B. alvei* 2771 and each of the two antibiotics.

Discussion

Application of bacterial FWC, or primary powder, of several strains from each of four *Bacillus* species, used singly, as well as the antibiotics Gramicidin S and Gramicidin D used singly, were molluscicidal against zebra mussels (*Dreissena polymorpha*) of several life cycle stages. These agents were also active against the snail *Biomphalaria glabrata*. Combinations of the bacterial FWC plus either Gramicidin S or D used against adult mussels (8–12 mm) were at the most additive (Gramicidin S) or antagonistic (Gramicidin D).

It was noted that the smaller the animal, the more sensitive it was to either the bacterial or antibiotic agent, with the veliger stage being particularly sensitive to each agent. In addition, the molluscicidal effect took only 5 h in the veliger, compared to 6 days for the adult.

As a result of these studies there are now available, for further exploitation, several tested molluscicidal bacterial agents active against the zebra mussel. As indicated elsewhere [14], the molluscicidal activity of these strains of *Bacillus* is the result of a molluscicidal toxin that appears to be a new and different agent from those that have been encountered before, and undoubtedly, it has a different set of biologically sensitive sites. The molluscicidal effects of these agents against the veligers (1–100 $\mu g\ ml^{-1}$) is comparable to the activity of *B. thuringiensis* and *B. sphaericus* against their target organisms [7,8,11–14].

The four species that showed molluscicidal activity, *B. alvei, B. brevis, B. circulans* and *B. laterosporus*, are part of morphological group II *Bacillus* [3,6], a designation no longer in general use but convenient in the initial description and identification of freshly isolated strains.

The sensitivity of the veliger stage of the zebra mussel to Gramicidin S and the snail was particularly striking and deserves a more detailed examination. We have found (not reported here) that Gramicidin S was also active against mosquito larvae (*Culex quinquefasciatus*), whereas in the same experiments Bacitracin, another polypeptide antibiotic, was not active against either the snail or *Culex* larvae.

The importance of the combination experiments is not only in determining whether the combined effects of the biological and biologically-derived chemicals are antagonistic, additive, or synergistic, but that several agents can be combined with apparently different sites of biological activity. This should be significant in attempting to preclude the rise of resistance if and when the agents see field use.

One major difference between the snail tested and the zebra mussel is that the snail does not have a sensitive veliger stage. Native invertebrates that do not have a veliger stage would not be as readily affected by or susceptible to these agents when used in concentrations of 1 $\mu g\ ml^{-1}$ or less. The latter gives hope of being able to selectively affect [1] the zebra mussel but not non-target-organisms.

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