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Fermentation and toxin studies of the molluscicidal strains of *Bacillus brevis*

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SUMMARY

Strain SS86-4 was one of 40 *Bacillus brevis* strains shown to be molluscicidal to the schistosomiasis snail vector *Biomphalaria glabrata*. When grown in mB4 medium in 2-L fermentors, SS86-4 was molluscicidal only if fructose or phenylalanine was present in the medium. This is reminiscent of secondary fermentation factor effects, in this case an antioxidant effect. In vivo proteases also were capable of reducing molluscicidal activity. The molluscicidal toxin has an LC_{50} of 1 μg toxin protein ml^{-1} (approx. 1 p.p.m.) and may be described as a small proteinaceous, heat-stable, oxygen-sensitive entity associated with the particulate portion of the cell wall fraction of *B. brevis* that is formed prior to sporulation. Initial information indicates that its HPLC signature shows major peaks at 148.37 and 163.96 s and consists of two bands of approximately 5.3 kDa and 8.7 kDa on PAGE gel.

INTRODUCTION

The snail-transmitted disease schistosomiasis affects over 300 million people, mostly in developing tropical countries. Its total public health impact is surpassed only by that of malaria and tuberculosis [19]. *Biomphalaria glabrata* is the intermediate host of the disease and is therefore a primary control target. Chemicals such as copper- or tin-based molluscicides initially used to control the transmission of the disease [6,11] are no longer used due to their level of toxicity and their persistence in the environment [11]. Bacterial pesticides such as *Bacillus thuringiensis* and *Bacillus sphaericus* have been successfully used as biological control agents of insect vectors of tropical disease [9,14,15]. In a similar vein, molluscicidal strains of *Bacillus brevis* are being investigated in our laboratory and have been suggested as bacterial molluscicides [16].

To further the development of these new bacterial molluscicidal agents, this study was begun. Since there is no fermentation process for the production of molluscicidal strains of *Bacillus* such as *B. brevis*, it was our purpose to develop such a fermentation process. As molluscicidal material became available we began to characterize the toxin.

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MATERIALS AND METHODS

Bacteria

Bacteria used in this study (Table 1) were maintained on Brain–Heart Infusion agar (Difco, Detroit, MI, USA). Strains SS86-3, SS86-4, SS86-5 were derived from Ghanian culture isolates IAB-395, -416, and -418, respectively. The original Ghanian cultures were obtained courtesy of Ms Janet Ofori, Institute of Aquatic Biology, Achimote, Ghana. These cultures were previously identified by us as *Bacillus brevis* [16]. The 'WRL' cultures were originally obtained courtesy of Wellcome Research Laboratories, Beckenham, UK; the 'NRS' and 'B' cultures were originally obtained courtesy of USDA/ARS/NRRL, Peoria, USA. The *Bacillus sphaericus* culture used was *B. sphaericus* WHO/CCBC/1593.

Media and fermentation conditions

Media. Modified B4 medium (mB4) was used throughout this study. It contained (per liter) PROFLO (Procter & Gamble, Memphis, TN, USA), 10 g; fructose, 5 g; yeast extract, 2 g; vitamin solution, 1 ml; biotin solution, 1 ml; salt solution, 10 ml. Vitamin solution contained (per 100 ml) nicotinic acid, 1 g; Ca-D-pantothenic acid, 1 g; thiamine·HCl, 1 g. Biotin solution contained (per 100 ml) biotin, 100 mg. mB4 salts solution contained (per 100 ml) $MgSO_4 \cdot 7H_2O$, 8 g; $CaCl_2 \cdot H_2O$, 1 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.1 g.

TABLE 1

Molluscicidal activities found for *Bacillus brevis* strains grown in shake flasks

Culture	$(LC_{50})-1$	Culture	$(LC_{50})-1$
SS/86-3	572	SS/86-4	300
SS/86-5	1428	SS86-3/C[a]	418
SS/86-3/E	572	SS/86-3/RA	83
SS/86-4/C	83	SS/86-4/E	418
SS/86-5/C	572	SS/86-5/E	1000
SS/86-5/RA	241	NRS-136	65
NRS-380	513	B-439	159
B-444	183	B-541	300
NRS-604	241	NRS-664	300
B-691	183	NRS-1771	300
NRS-1137	300	NRS-1202	300
NRS-1203	65	NRS-1204	183
NRS-1205	65	NRS-1207	130
NRS-1206	183	NRS-1208	65
NRS-1210	65	NRS-1211	159
NRS-1373	183	NRS-1609	83
NRS-1610	130	B-1874	65
WRL-2904	572	WRL-2918	300
WRL-2934	65	WRL-2954	83
NRS-4285	65	B-4558	65

LC_{50} = Dilution of FWC, toxin, or cell fraction that kills 50% of the test snail *Biomphalaria glabrata*.

[a] /C = strain resistant to 10 μg chloramphenicol ml^{-1}; /E = strain resistant to 5 μg erythromycin ml^{-1}; /RA = strain resistant to 10 μg rifampin ml^{-1}.

Fermentation conditions. The standard inoculum buildup for the shake flasks and/or the 2-L fermentors was as previously described [18]. A BIOFLO III 2-L fermentor (New Brunswick, Edison, NJ, USA) was used for all of the experiments described with the exception of those shown in Tables 1, 2, and 4. The latter were derived from shake flask material. Harvest time for the final whole culture (FWC) was dictated by the protocol of the experiment.

Assay of molluscicidal toxicity. Maintenance of the test snail, *Biomphalaria glabrata*, and the diluent for the bioassay was deionized water containing 1 g L^{-1} of synthetic sea salts (Carolina Biological, Burlington, NC, USA). The snails were maintained in 35-L aquaria and fed lettuce on demand. For each bioassay (in 3.5 oz disposable plastic cups), four dilutions (1/30, 1/100, 1/300, 1/1000) of the test material, 3–10 snails, 3–5 mm in diameter (as available), were used. All test materials were incubated at room temperature (25 °C) and surviving snails were counted at days three and six of the treatment. Since the test snail is an albino, and one can see through the shell to see the pumping heart, it was easy to determine death. In addition, death results in a change of color of the shell (it becomes white and opaque). There is also loss of response when the snail foot is trapped with a needle. For the LC_{50} calculation (the dilution of the FWC, toxin extract, or cell component that killed 50% of the test snail population) as well as the r^2 value, data were obtained by plotting the linear regression of mortality after six days incubation of the snails vs the dilution of the material being tested. The r^2 value is obtained during the regression analysis and represents the square of the correlation coefficient giving the percent of variation in Y that can be explained by X. r^2 indicates the reliability of the LC_{50}. Only r^2 values greater than 0.70 were used. The value 0.70 is considered to be a conservative value.

Ultracentrifugation methods. Modification of a technique for localization of bacterial toxin was used [12]. FWC cells were passed through a French pressure cell (18000 p.s.i.) three times. Whole cells were removed by centrifuging at 610 × g for 10 min (done 4×). The remaining material was centrifuged at 25300 × g for 20 min, separating the cell wall material (pellet) from the membranes and cytoplasm (supernatant). The supernatant was filter-sterilized (sterilized by passage through a 0.2-μl Nalgene filter) and preserved. The pellet was layered on top of a sucrose step gradient (40, 50, 60, 70%) [12]. After centrifugation at 72000 × g for 18 h, the two resulting bands were removed, resuspended in 5 ml deionized water and the toxicity of the fractions was determined.

Toxin extraction

An alkaline extraction and a methanol extraction of whole and lysed cells were examined.

Alkaline extraction. This procedure followed the methods of Davidson [7]. Centrifuged cells from 100 ml of FWC were suspended in 25 ml of 0.05 M NaOH for 3.5 h in a Burrell wrist-action shaker. After incubation the suspension was centrifuged for 20 min at 12000 × g. The supernatant (the toxin extract) was filter-sterilized and reserved (as indicated above) and the remaining extracted cells were resuspended in 25 ml of deionized water. The toxicity of the fractions was determined.

Methanol extraction. Centrifuged FWC cells (or the same cells passed three times through a French pressure cell, as described above) were suspended in 25 ml of absolute HPLC grade methanol and incubated at room temperature for 2 h on a Burrell wrist-action shaker. The supernatant (toxin extract) and the extracted cells were treated as indicated in the alkaline extract procedure.

Treatment of toxin. As needed to preserve the toxin, the toxin was flushed for 1 min with nitrogen. Then 1 ml of a DL-dithiothreitol (Sigma, St Louis, MO, USA; 59 mg ml^{-1}) solution was added to every 6 ml of toxin to prevent oxidation.

HPLC methods

HPLC was used to analyze reference and extracted toxin samples. Initial toxin samples were examined with a Beckman (Palo Alto, CA, USA) DU 64 spectrophotometer at 200–500 nm to determine the wavelength giving maximum

absorption reference points at which investigation on the HPLC could begin. All HPLC work was completed using an ISCO model 2350 pump with a V^4 variable wavelength absorbance detector using a deuterium light source. All samples were run through a C18 reverse phase column. The solvent was absolute methanol and the flow rate was 1.0 ml min^{-1}. The recorder level was calibrated at 10 and the sensitivity level was set at 0.1 throughout the experiment. All runs on the HPLC were for 10 min and the injection volume was 0.015 ml.

SDS-polyacrylamide gel electrophoresis

The discontinuous procedure of Schagger and Von Jagow [13] was used. The polyacrylamide concentrations for the separating gel, spacer gel and stacking gel were 21.45%, 10% and 4% respectively, using a Hoefer Scientific Instruments Model SE 400 vertical slab gel unit and a 1.5-mm thick gel. Samples of the methanol extract material (where 5 ml of methanol rather than 25 ml was used) were prepared for electrophoresis by adding 0.1 ml of extract to 1.0 ml of sample buffer. The sample buffer consisted of 4 ml of 20% SDS (sodium lauryl sulfate—Sigma); 2.4 ml glycerol; 0.4 ml 2-mercaptoethanol; 2 ml of Brilliant Blue G solution; 1 m of 1 M, Tris·HCl (pH 6.8) and all were brought to a final volume of 20 ml with deionized water. The molecular weight markers were obtained from Sigma (MW-SDS-17). The staining procedure and the protocol used was that recommended by the manufacturer. The mg ml^{-1} of protein per dilution was then calculated. Means and standard deviations for the mg ml^{-1} of protein were calculated for each unknown.

Protein assay method

The concentration of protein in the toxin extracts was determined according to Bradford [5] using a Bio-Rad (Hercules, CA, USA) Protein Assay Kit. The mg ml^{-1} of protein per dilution was then calculated. Means and standard deviations for the mg ml^{-1} of protein were calculated for each unknown.

Protease assay. Protease content of each sample was determined using the Azacoll method (Calbiochem, La Jolla, CA, USA). The protocol and techniques were those recommended by the manufacturer, except initial solutions were diluted (approximately 5-fold) to allow the resulting values to fit on the generated curve.

Enzyme treatment

Three different enzymes were tested for their effects on toxin activity against both FWC and whole cell toxin. The enzymes tested were trypsin (bovine pancreas, Sigma T-8253), protease (*Streptomyces griseus*, Sigma P-5147), and lysozyme (chicken egg white, Sigma L-6876). The protocol used for these experiments was modified from the technique of Bone et al. [4]. The enzymes were tested against all samples at a concentration of 2 mg·ml^{-1} of sample. Enzymes were added to the samples and incubated for 1 h at 35 °C on an incubator shaker at 300 r.p.m. The toxicity of the samples was determined.

Heat treatment

Triplicate 3-ml samples of FWC, or toxin extract, were heat-treated at 60 and 100 °C for three separate time periods (15, 30, and 60 min). The heat-treated samples were then bioassayed.

RESULTS

The molluscicidal activity of strains of Bacillus brevis

Forty strains of *Bacillus brevis* grown in shake flasks were tested for molluscicidal activity against the target *Biomphalaria glabrata* and found to be active to varying degrees (Table 1); *B. sphaericus*/1593, the control strain, was found to be inactive. The primary powders made from Final Whole Cultures (FWC) were quite active, ranging from an LC_{50} of 5 mg L^{-1} for *B. brevis* strain SS86-4 to 300 mg L^{-1} for *B. brevis* strain 604, the species type strain. Strain SS86-4 was selected for further work (e.g. to determine optimum fermentation conditions; characterize the toxin) since it yielded the best primary powders in initial experiments.

Molluscicidal activity was not seen in the FWC when strains of *B. brevis* were grown in mB4 medium, in the absence of fructose or phenylalanine, especially in the fermentors. The molluscicidal activity was dependent on the

TABLE 2

Media additions that enhance molluscicidal activity of *Bacillus brevis*/SS86-4 grown in shake flasks*

Media addition	$(LC_{50})-1$
Sucrose	600
Melibiose	518
Inositol	400
Dextrose	600
Mannose	0
Rhamnose	200
Galactose	518
Maltose	800
Fructose	763
Inulin	692
Glycerol	1733
Ribose	150
L-arginine	1500
L-histidine	1180
L-methionine	1500
L-phenylalanine	1180

* Medium was mB4 with the listed components substituted for fructose at 1% final concentration.

LC_{50} = Dilution of FWC, toxin, or cell fraction that kills 50% of the test snail *Biomphalaria glabrata*.

inclusion of certain sugars or amino acids. If these compounds were not present, there was no activity evident in the FWC. Of the twelve sugars and four amino acids tested with strain SS86-4, all but mannose were found to result in molluscicidal activity (Table 2). The inclusion of any of the sugars, particularly fructose, resulted in several problems such as a substantial drop in pH and, as a consequence, a marked reduction in the total viable count (TVC) (Table 3). Compared to the effect of the addition of one of the 12 sugars tested, the inclusion of any of the four amino acids examined showed less adverse effects than inclusion of sugars such as fructose.

In-cycle experiments

To determine when the toxin was first produced, a series of in-cycle experiments was done in which whole culture samples were taken at various times during the fermentation (Table 3). When 0.5% fructose was used, molluscicidal activity (toxin production) appeared as early as 8 h, peaked at 12 h and declined rapidly by 24 h. All of these events took place before the initiation of sporulation, which occurred at about 24 h. The total viable count also appeared to be lowest at 12 h, as was the pH. These declines in activity, population density, and pH were not as pronounced when 0.5% phenylalanine was used as an additive. Molluscicidal activity did not appear to be reduced during sporulation, even though there appeared to be a dip in activity after 16 h.

Factors affecting the toxin during the fermentation

Two factors might account for the presence of a lower amount of toxin after 12 h of fermentation. The first was the possible susceptibility of the toxin to protease and the second was the possible susceptibility of the toxin to oxidation.

The protease content of the FWC was examined when either fructose or phenylalanine was used (Table 3); protease levels during the fermentation were high. In addition, supernatants from the fermentation (containing proteases) had the potential of inactivating extracted toxin (Table 4). The susceptibility of the toxin to oxidation was tested by

TABLE 4

Effects of DTT and in vivo protease on methanol extracted *Bacillus brevis*/SS85–4 molluscicidal toxin*

Treatment	$(LC_{50})-1$	% Reduction
Experiment A		
1) Control—no treatment (toxin)	690	
2) Toxin after four days (refrigerated)	160	77
3) Toxin treated with FWC in vivo protease at day 0, results after 4 days (refrigerated)	230	66
4) Toxin treated with DTT at day 0, results after 4 days	410	41
5) Toxin treated with DTT at day 4, results after 4 days	310	55
Experiment B		
1) mB4 FWC (growth in absence of either fructose or phenylalanine), sample taken at 8 h in-cycle	0	
2) Conditions same as B(1), + DTT	800	
Control (DTT alone)	0	

DTT = Dithiothreitol (concentration of solution, 59 mg ml^{-1}).
LC_{50} = Dilution of FWC, toxin, or cell fraction that kills 50% of the test snail *Biomphalaria glabrata*.

TABLE 3

In-cycle growth and biological activity of *Bacillus brevis*/SS86-4*

Hours	Fructose addition				Phenylalanine addition				No addition			
	TVC ($\times 10^8$)	pH	$(LC_{50})-1$	Prot.	TVC ($\times 10^8$)	pH	$(LC_{50})-1$	Prot.	TVC ($\times 10^8$)	pH	$(LC_{50})-1$	Prot.
4	3.2	7.3	NA	0.2	0.09	7.3	NA	0.2	0.2	6.9	NA	—
8	8.4	7.1	375	0.4	5.9	8.3	150	0.4	8.2	7.3	NA	—
10	—	6.6	—	—	—	8.8	—	—	—	7.3	NA	—
12	0.82	5.8	690	0.8	12.0	8.6	310	0.7	25.9	7.4	NA	—
14	—	6.8	—	—	—	8.8	—	—	—	7.6	NA	—
16	10.00	7.9	420	0.7	1.0	8.8	310	1.1	25.0	8.0	NA	—
20	2.6	8.2	420	1.0	11.0	9.1	270	1.1	24.8	8.3	NA	—
24	24.0	8.3	120	1.2	28.0	9.5	210	0.7	20.4	8.4	NA	—

* Grown in 2-L fermentors in mB4 medium with or without fructose and phenylalanine as indicated.
LC_{50} = Dilution of FWC, toxin, or cell fraction that kills 50% of the test snail *Biomphalaria glabrata*.
Prot. = Protease activity, the values indicated are absorption values, the larger the number the more enzyme active found.
NA = No activity; — = No information; TVC = Total Viable Count.

the addition of an antioxidant, dithiothreitol (DTT). The toxin could be both preserved and restored by the addition of DTT. Without DTT, the LC_{50} value of the extracted toxin after 4 days storage (refrigerated) was reduced by 77%. When DTT was added at 0 time, after 4 days the LC_{50} value of the toxin was reduced by only 41%. Adding DTT after 4 days storage reduced the LC_{50} by 55%. Furthermore, if strain SS/86-4 was grown on mB4 medium in the absence of either the fructose or phenylalanine additive, and samples taken at 8 h, no detectable toxin was produced. If, however, DTT was added to a parallel sample at 8 h (sample from the toxin obtained from growth in mB4 medium without either fructose or phenylalanine), the LC_{50} increased from 0 to 800 (Table 4).

The protein content of the toxin extract was measured. Analysis of the toxin extract showed 0.804 mg ml^{-1} of protein within the extract (Table 5). This, combined with the bioassay data, indicated an LC_{50} value of approximately 1 μg of toxin protein ml^{-1}. Controls (methanol extracts of the medium) showed no protein present. As the toxicity (LC_{50} value) declined with time (0–7 days), a corresponding decline in the amount of protein was detected.

Examination of the molluscicidal toxin

Localization of toxin. Using differential centrifugation, only the 60% sucrose fraction, noted for its cell wall fragments [12], showed molluscicidal activity (Table 6) and can therefore be considered the site of the toxin.

Toxin characteristics

Toxin extract. Three different extraction techniques were used, an alkaline extraction, a whole cell methanol extraction and a lysed cell methanol extraction. All three methods extracted toxin from the cells with only minor loss of activity. Controls (extracts of the medium itself) showed no molluscicidal activity. The whole cell methanol extraction was used for the remaining experiments since it was the easiest, least time-consuming and least complicated to perform (Table 7).

TABLE 5

Protein analysis of toxin extracts

B. brevis/SS86-4	$(LC_{50})-1$	Protein mg ml^{-1}	SD
1) FWC	800		
2) Methanol extract	800	0.804	0.123
(a) 3 days	518	0.743	0.174
(b) 7 days	175	0.180	0.109
3) Media			
(a) Methanol extract	NA	0.0	0.0

LC_{50} = Dilution of FWC, toxin, or cell fraction that kills 50% of the test snail *Biomphalaria glabrata*.
SD = Standard deviation; NA = No activity.

TABLE 6

Location of the molluscicidal toxin within the bacterial cell of *Bacillus brevis*/SS86-4

Sample	$(LC_{50})-1$
1) FWC	406
2) Supernatant	NA
3) Cytoplasmic fraction	NA
4) Cell wall fraction	400
5) Differential centrifugation of cell wall fraction	
(a) 60% band	875
(b) 40% band	NA

LC_{50} = Dilution of FWC, toxin, or cell fraction that kills 50% of the test snail *Biomphalaria glabrata*.
NA = No activity.

TABLE 7

Some characteristics of the *Bacillus brevis*/SS86-4 molluscicidal toxin

Treatment	$(LC_{50})-1$	
1) Methanol extraction		
(a) Whole cell extraction		
(i) FWC*	406	
(ii) Methanol extract	688	
(iii) Whole cells after extraction	63	
(b) Media extraction		
(i) Media extract*	NA	
(ii) Media pellet after extraction	NA	
2) Heat	FWC	Extract
(a) Untreated	406	406
(b) 60 °C, 60 min	406	406
(c) 100 °C, 60 min	313	313
3) Enzyme	FWC	Extract
1) Untreated	406	406
2) Protease	63	63
3) Trypsin	63	63
4) Lysozyme	63	406

LC_{50} = Dilution of FWC, toxin, or cell fraction that kills 50% of the test snail *Biomphalaria glabrata*.
NA = No activity; *Untreated control prior to extraction.

HPLC examination of the toxin extracts. HPLC analysis was conducted on whole cell methanol toxin extracts (Fig. 1(A)) which showed major peaks at 148.37 and 163.96 s, on a methanol extract of medium (control) (Fig. 1(B)) which showed no peaks, and on a mixed sample of 10 mg gramicidin S ml^{-1} methanol + methanol extract of medium, as a positive control (Fig. 1(C)) which showed major peaks at 129.11 and 206.53 s.

Effects of heat and enzyme treatments on toxin extracts. Toxin in both the FWC and methanol extracts was stable at 100 °C for time periods of at least 60 min (Table 7). Protease or

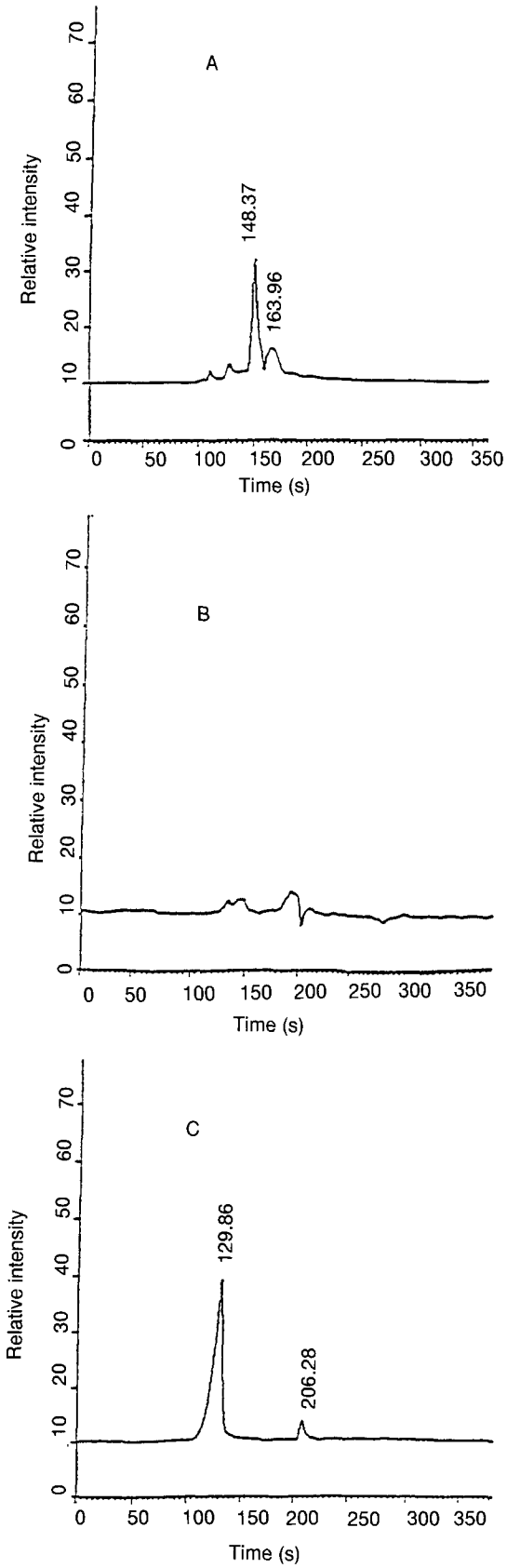

Fig. 1. HPLC analysis of whole cell methanol extracts from *Bacillus brevis*/SS86-4. (A) Whole cell methanol extract of FWC (toxin); (B) methanol extract of medium (control); (C) mixed sample of 10 mg gramicidin S ml^{-1} methanol + methanol extract of medium (positive control).

trypsin treatments were found to inhibit molluscicidal activity of both the FWC and extract samples (Table 7). Lysozyme treatment reduced molluscicidal activity in the FWC but not in the whole cell methanol toxin extract.

Molecular weight of the molluscicidal toxin. Polyacrylamide gel analysis (SDS-PAGE) of the toxin extracts showed only two faint bands (other than the known marker bands). These two bands were estimated to be 5.3 kDa and 8.7 kDa respectively.

DISCUSSION

Molluscicidal strains of *Bacillus brevis*, such as strain SS86-4, when grown in mB4 medium in 2-L fermentors, in the absence of one of several sugars such as fructose or in the absence of one of several amino acids such as phenylalanine, shows no detectable molluscicidal activity. In fact one is apt not to realize the presence of this activity. In the presence of fructose or phenylalanine or one of their congeners, we see molluscicidal activity. The molluscicidal toxin first appears in the fermentation as early as 8 h, but the amount of toxin appears to decline or disappear prior to the onset of sporulation as early as 24 h into the fermentation. In vivo proteases account for some of the degradation of the proteinaceous toxin. The toxin also appears to be sensitive to oxidation—antioxidants such as DTT can in part protect and even to a point resurrect the toxin activity. What is the role then of fructose and/or phenylalanine in the fermentation? They appear to be 'secondary factors' as classically described by Margalith [10]. Margalith describes secondary fermentation factors as those that would influence the process by steering the microbial activity toward the desired product.

One of the characteristics of a secondary factor is that it itself is not essential for growth but influences and changes the outcome of the process in other ways. One of the many examples Margalith gives is the Pasteur effect (the role of O_2 concentration on the final yeast product). In many of the examples of secondary factors that he reports, there is no information on how the factor operates (e.g. corn steep liquor, distiller's solids). In the case of the action of fructose or phenylalanine there is precedence [1,2] to hypothesize that these two factors have an antioxidant effect until they are used up. Agathos and Demain [1,2] found that gramicidin S synthetase was destroyed by oxidation during the fermentation. The addition of either fructose or phenylalanine stabilized the synthetase from oxidation. They theorized that these additives caused a low oxygen tension inside the cell (had an antioxidant effect) and that this was responsible for the synthetase preservation. In our case the additives may prevent oxidation of proteins (such as the toxin). These authors also note [1,2] that the addition of antioxidants such as DTT could preserve (and in our case, in part, also restore) activity. The presence of these two factors (fructose or phenylalanine or some of their congeners) allows our fermentation process to proceed to the point where it becomes more convenient to harvest the toxic product. The

cost of the alternative, adding DTT instead of fructose or phenylalanine, would be most prohibitive. Indeed, it is too early to tell if DTT would be able to withstand the highly aerobic conditions of the fermentor for the length of time it would be required. Reducing the aeration of the process per se did not appear to be effective either (data not shown here).

Bacillus thuringiensis and *B. sphaericus* toxins (parasporal crystals) are formed during sporulation [3,8] and the *B. brevis* toxin is formed prior to sporulation. The addition of fructose, (note the in-cycle experiments), showed good production of molluscicidal activity but the activity was likely to be destroyed later in the fermentation, when the fructose was depleted. After 12 h, the *B. brevis* population starts to increase, as does the pH, but the amount of toxin begins to decrease. This indicates to us that fructose has been completely catabolized suggesting that the culture needs to be harvested early. In the case of the addition of phenylalanine, there is less of a pH drop and unlike the case with fructose, the toxin does not appear to be destroyed once the sporulation process begins. In both cases toxin is produced before there is any evidence of spore or even prespore formation. Spores do not usually appear until after 24 h under the condition of these experiments.

Since all of the *B. brevis* strains tested showed some molluscicidal activity, it seems that the 'toxin' is rather widespread among the strains in this species. We would anticipate that only a few of the strains will provide sufficient toxin to be of much commercial interest. Future studies will determine this.

At present, the molluscicidal toxin (with an estimated LC_{50} of 1 μg toxin protein ml^{-1}, approx. 1 p.p.m.) may be described as a small proteinaceous, heat-stable, oxygen-sensitive entity associated with the particulate portion of the cell wall fraction of *B. brevis* that is formed prior to sporulation. Initial information indicates that its HPLC signature shows major peaks at 148.37 and 163.96 s and it consists of two bands of approximately 5.3 kDa and 8.7 kDa on PAGE gel. Lack of molluscicidal activity of extracts of non-inoculated mB4 medium demonstrated that the extracted toxin was a bacterial product rather than artifacts from the extraction of the medium. The molluscicidal toxin has a different HPLC signature than gramicidin S (148.37 and 163.96 s for the toxin vs 129.11 and 206.53 s for gramicidin S). Gramicidin S is toxic to the snail *Biomphalaria glabrata* and to the larvae of *Culex quinquefasciatus* whereas the *B. brevis* SS86-4 toxin is toxic against *B. glabrata* but not against *Cx quinquefasciatus* larvae (data not shown here). Bacitracin on the other hand, another polypeptide antibiotic, is not toxic for either the aforementioned snail or mosquito.

The molluscicidal toxin was found to be heat stable up to 100 °C for 60 min. This makes the *B. brevis* toxin quite different from the more heat-sensitive *B. thuringiensis* or *B. sphaericus* crystal toxins [3,8]. Protease or trypsin inhibited molluscicidal activity, indicating that the toxin is at least in part proteinaceous. The fact that lysozyme inhibited molluscicidal activity when the toxin was part of the whole cell, but had no effect when the toxic material was extracted, is most interesting and bears further examination. There are no explanations for this phenomenon at present.

The LC_{50} value of 1 μg toxin protein ml^{-1} or 1 p.p.m. would be in agreement with the activity of some of our best bacterial primary powder preparation (of several p.p.m.), indicating that the toxin is present in a rather limited amount. This is also borne out by the fact that PAGE analysis of the toxin extract shows two very faint bands of 5.3 and 8.7 kDa. The molecular weight of the *B. brevis* toxin is much smaller than either the *B. thuringiensis* or the *B. sphaericus* crystal toxins [3,8] but much larger than gramicidin S (1.06–1.34 kDa) [17].

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REFERENCES

1 Agathos, S.N. and A.L. Demain. 1983. Optimization of fermentation processes through the control of in vivo inactivation of microbial biosynthetic enzymes. In: Foundations of Biochemical Engineering (Blanch, H.W., E.T. Papoutsakis and G. Stephanopoulos, eds), pp. 53–67, American Chemical Society, Washington, DC.

2 Agathos, S.N. and A.L. Demain. 1986. Substrate amino acid-mediated stabilization of gramicidin S synthetase activity against inactivation in vivo. Enzyme Microb. Technol. 8: 465–468.

3 Baumann, P., M.A. Clark, L. Baumann and A.H. Broadwell. 1991. *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. Bacteriol. Rev. 55: 425–436.

4 Bone, L.W., K.P. Bottjer and S.S. Gill. 1986. *Trichostrongylus colubriformis*: isolation and characterization of ovicidal activity from *Bacillus thuringiensis isrealensis*. Exp. Parasitology 62: 247–253.

5 Bradford, M.M. 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72: 248–254.

6 Cheng, T.C. 1974. Molluscicides in Schistosomiasis Control. Academic Press, New York.

7 Davidson, E.W. 1983. Alkaline extraction of toxin from spores of the mosquito pathogen, *Bacillus sphaericus* strain 1593. Can. J. Microbiol. 29: 271–275.

8 Kreig, A. and H.G. Miltenburger. 1984. Bioinsecticides: 1. *Bacillus thuringiensis*. Adv. Biotechnol. Proc. 3: 273–290.

9 Luthy, P., H.R. Ebersold, J.L. Cordier and H.M. Fischer. 1985. Insecticidal metabolites of spore-forming bacilli. In: Fundamental and Applied Aspects of Bacterial Spores (Dring, G.J., D.J. Ellar and G.W. Gould, eds), pp. 475–484, Academic Press, London.

10 Margalith, P. 1964. Secondary factors in fermentation processes. Adv. Appl. Microbiol. 6: 69–90.

11 McCullogh, F.S. and K.E. Mott. 1983. The role of molluscicides in schistosomiasis control. Document WHO/SCHISTO/83.72.

12 Myers, P.S. and A.A. Youston. 1980. Localization of a

mosquito–larval toxin of *Bacillus sphaericus* 1593. Appl. Environ. Microbiol. 39: 1205–1211.

13 Schagger, H. and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166: 368–379.

14 Singer, S. 1979. Use of entomogenous bacteria against insects of public health importance. Devel. Indust. Microbiol. 20: 117–122. Society for Industrial Microbiology, Arlington, Virginia.

15 Singer, S. 1987. Current status of the microbial larvicide *Bacillus sphaericus*. In: Biotechnology Advances in Insect Pathology and Cell Culture (Maramorosch, K., ed.), pp. 133–163, Academic Press, New York.

16 Singer, S., K.A. Doherty and A.D. Stambaugh. 1988. Preliminary examination of molluscicidal strains of the genus Bacillus. 88th Annual Meeting American Society for Microbiology, Miami, Florida (Abstr., p. 76).

17 Stecher, P.G., M.J. Finkel, O.H. Siegmund and B.M. Zafranski. 1960. The Merck Index of Chemicals and Drugs, p. 497, Merck and Co. Inc., Rahway.

18 Williston, B.K. and S. Singer. 1987. Initial studies of the use of aminopeptidases for the differentiation of *Bacillus sphaericus* strains. J. Ind. Microbiol. 2: 285–292.

19 World Health Organization. 1984. Report of an informal consultation on research on the biological control of snail intermediate hosts. Document TDR/BCV-SCH/SIH/84.3, Geneva.