ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1994, p. 1314–1319
0066-4804/94/$04.00+0

Vol. 38, No. 6

Characterization and Biological Activity against *Naegleria fowleri* of Amoebicins Produced by *Bacillus licheniformis* D-13

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Received 27 October 1993/Returned for modification 13 January 1994/Accepted 16 March 1994

The strain *Bacillus licheniformis* D-13 produces three hydrophobic peptides (amoebicins d13-A, d13-B, and d13-C) that elicit antiamoebic activity against human-pathogenic and nonpathogenic species of *Naegleria* and have a broad spectrum of antibacterial activity. The three amoebicins have the same amino acid composition (three Asp, two Glu, two Val, and nine Leu residues) and molecular weight (1,870). Amoebicin d13-B causes lysis of amoebae through disorganization of the cell membrane. It also induces permeability to ^{86}Rb and membrane disruption in asolectin vesicles.

Free-living amoebae of the genera *Naegleria* and *Acanthamoeba* are able to cause diseases in humans and other animals. Normally, these amoebae live as phagotrophs in moist environments, ponds, rivers, streams, and lakes, where they feed on bacteria (6, 15, 20, 26, 27). However, as opportunists they may produce serious infections of the eye and central nervous system. The biology, ecology, and pathology of opportunistically pathogenic, free-living amoebae have been reviewed recently in detail (12). *Naegleria fowleri* is responsible for a rapidly fatal infection involving the central nervous system called primary amebic meningoencephalitis (4, 13). Infection often occurs in healthy young people who have a recent history of swimming in fresh water. The most widely used antibiotic against this disease is amphotericin B (19, 23). Other compounds have been reported to have antiamoebic activity in vitro (8, 19, 22, 24, 25), but their efficacy in vivo has not been proved. The reduced number of chemotherapeutic agents available for treatment of this disease and their limited efficacy and toxic side effects are the main concerns in the search of new antiamoebic substances.

A screening for the production of antiamoebic substances by bacteria isolated from soil, mud, and water samples was carried out in our laboratory. Three strains of *Bacillus licheniformis* isolated from soil (named A12, D-13, and M-4) showed a marked amoebolytic activity against human-pathogenic amoebae (5). Activity from strain M-4 was best produced during sporulation in media buffered at pH 5.8. Partially purified preparations from this strain obtained by ammonium sulfate precipitation and gel filtration showed strong amoebolytic activity and a narrow antibacterial spectrum (5). Moreover, two amoebolytic substances (amoebicins A12-A and A12-B) were purified from strain A12. They were hydrophobic peptides with a low molecular weight (1,430) containing the amino acids Asp, Glu, Ser, Pro, and Tyr. Both peptides were active against pathogenic amoebae and fungi (9). The purification and biological activity of new antiamoebic peptides produced by strain D-13 are reported in this paper.

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MATERIALS AND METHODS

Microorganisms and culture conditions. The producer strain *B. licheniformis* D-13 was isolated from a soil sample (5). The amoebae used in this study (see Table 3) and their growth conditions are described elsewhere (9). For the assays of biological activity, amoebae were grown axenically in Cline medium (14). Peritoneal and splenic cells obtained from BALB/c mice as described previously (18) were grown in RPMI 1640 (Sigma) supplemented with 10% bovine fetal serum (RPMI-BFS) at 37°C under a 5% CO_2 atmosphere.

Activity assays. Samples were assayed for antiamoebic activity as described elsewhere (9), in 6.4-mm-diameter flat-bottomed microtiter wells (96-well microtiter plates; Corning Glass Works, Corning, N.Y.) containing 6×10^3 amoebae in a final volume of 200 μl. The cytological effects were visualized under an inverted light microscope (Carl Zeiss, Jena, Germany). The number of intact amoebae was determined with a hemocytometer.

Purified amoebicins were tested on murine cells by a colorimetric assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] as described elsewhere (16). Cells were incubated with amoebicins in 96-well microtiter plates at 4×10^3 cells per well. At desired intervals, 50 μg of MTT in phosphate-buffered saline was added to each well and the cells were incubated for 4 h before the results were read on a Whittaker microplate reader 2001 as described previously (16).

The antibacterial activity of amoebicins was tested as described previously (9). One arbitrary unit (AU) was defined as the highest dilution of the sample to cause lysis of ca. 50% of the amoebae or to produce a visible halo of inhibition on a bacterial lawn. The titer, in AU per milliliter, was calculated from the inverse of this dilution.

Production of amoebicidal activity. The production of amoebicins by *B. licheniformis* D-13 was tested in brain heart infusion (BHI) (BBL, Cockeysville, Md.) or tryptic soy broth (BBL) dissolved in 75 mM sodium phosphate buffer, pH 7.2, as described previously (5), and the antiamoebic activity of cell-free supernatants was determined.

Purification of antiamoebic activity. One liter of culture supernatant of the producer strain was mixed with 500 ml of methanol and passed through a column (1 by 15 cm) packed with solid-phase extraction bulk packing (Supelclean LC-8) (Supelco Inc., Bellefonte, Pa.). The retained material was washed with 100 ml of 35% methanol in water and eluted with

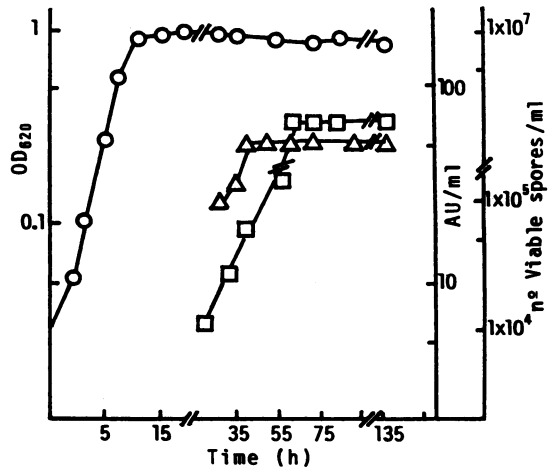

FIG. 1. Production of antiamoebic activity by *B. licheniformis* D-13 incubated at 28°C under agitation in buffered BHI broth. The optical density at 620 nm (OD_{620}) (○), the number of spores (△), and the titer of antiamoebic activity against *N. fowleri* HB-1 (□) are represented.

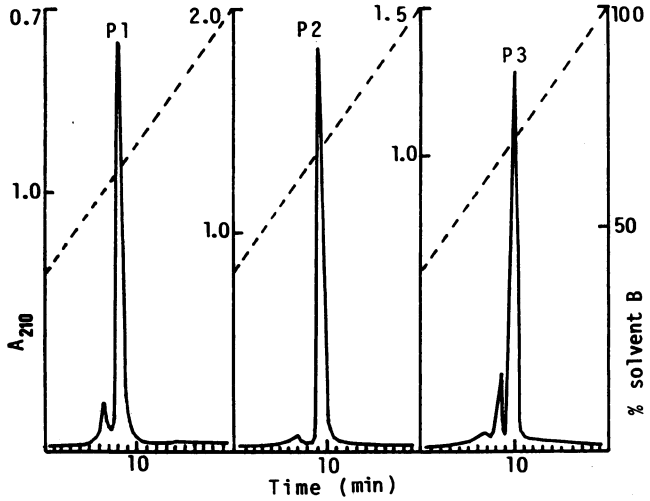

FIG. 2. Purification of amoebicins from *B. licheniformis* D-13 on an Ultrapore reversed-phase solid chromatography column. The elution conditions are described in Materials and Methods. The solvent concentration gradient (– – –) and the recorded A_{210} of the column effluent (——) are represented. P1, P2, and P3 refer to amoebicins d13-A, d13-B, and d13-C, respectively.

50 ml of methanol-acetonitrile (1:1, vol/vol). The eluate was lyophilized and redissolved in 50 ml of methanol-water (1:2, vol/vol). Portions (10 ml) of this material were loaded on a Spherisorb C-6 column (1 by 25 cm) (Phase Separations, Inc., Norwalk, Conn.) in 10 mM trifluoroacetic acid (TFA) and separated with two combined linear gradients (0 to 20% over 5 min and 20 to 100% over 30 min) of isopropyl alcohol-acetonitrile (2:1) in 4 mM TFA at a flow rate of 2 ml/min. The eluate was collected in different fractions, which were lyophilized and redissolved in 30% methanol before they were tested for antiamoebic activity. The active fractions were loaded separately on an Ultrapore reversed phase solid chromatography column (0.4 by 7.5 cm) (Altex, Berkeley, Calif.) equilibrated in 10 mM TFA. The column was eluted with a 40 to 100% linear gradient of isopropyl alcohol-acetonitrile (2:1) in 4 mM TFA over 20 min at a flow rate of 1 ml/min.

The molecular weights of the amoebicins were estimated by gel filtration. Aliquots (20 μl) were chromatographed on a glass column (3 by 500 mm) (Omni International Inc., Waterbury, Conn.) packed with Fractogel TSK HW-50 (S) (Merck, Darmstadt, Germany) in 75% methanol. The column was operated with a Pharmacia-LKB high-pressure liquid chromatography (HPLC) system (Pharmacia, Barcelona, Spain) at a flow rate of 9 ml/h. Aprotinin (6.5 kDa), bacitracin (1.4 kDa), polymyxin B (1.2 kDa), valinomycin (1.1 kDa), and erythromycin (0.73 kDa) were used as markers.

Physicochemical characterization. Active samples were tested for sensitivity to extremes of heat and pH or to inactivation by the enzymes trypsin, pronase, proteinase K, β-glucuronidase, and lipase as described elsewhere (9).

The sample protein concentration was determined by the method of Bradford (2), and the presence of phosphorus and neutral sugars was quantitated by methods described elsewhere (1, 7). The optical absorbance spectrum of the purified amoebicins was recorded in a UV/Vis Beckman DU-70 spectrophotometer. Amino acid composition was determined as described previously (10), and the protein content was correlated with the recorded absorbance in order to calculate the extinction coefficients. The N-terminal amino acid sequence was determined by Edman degradation with an Applied Bio-Systems 470A protein sequencer. Fatty acid analysis was carried out by a previously described procedure (9, 21).

Fluorescence microscopy of treated amoebae. Aliquots (50 μl) of exponentially growing cultures of *N. fowleri* HB-1 were deposited on slides and incubated at 37°C for 30 min to allow the cells to adhere to the glass. The supernatant was removed, and 100 μl of a solution containing 1 μg of 4′,6-diamidino-2-phenylindole (DAPI) (Pierce, Rockford, Ill.) per ml in McIlvaine buffer (82 mM citric acid monohydrate–36 mM Na_2HPO_4, pH 7.2) plus 5 mM $MgCl_2$ was deposited on the preparations. After 30 min of incubation in the dark, the preparations were washed with McIlvaine buffer and covered with a solution of 50% glycerol in McIlvaine buffer containing different concentrations of amoebicin. Preparations were observed at regular intervals under a Dialux 22/22EB microscope equipped for epifluorescence (Leitz, Wetzlar, Germany).

Preparation of asolectin vesicles. L-α-Phosphatidylcholine from soy beans (type IV-S; Sigma) was homogenized (6 mg) in 2 ml of 20 mM sodium phosphate buffer (pH 7.2) by sonication. ^{86}Rb (2.7 mCi/mg) was added to a final concentration of 1 μCi/ml, and the mixture was sonicated for 1 min. The

TABLE 1. Purification of the different amoebicins produced by *B. licheniformis* D-13[a]

Fraction	Total protein (μg)	Total activity (AU)	Sp act (AU/μg of protein)
Crude supernatant	335,000	65,000	0.19
Bulk LC-8	66,000	40,000	0.60
Spherisorb C-6			
Fraction 1	5,950	7,500	1.26
Fraction 2	6,250	9,500	1.52
Fraction 3	4,700	7,000	1.48
Ultrapore			
Amoebicin d13-A	2,600	4,750	1.82
Amoebicin d13-B	3,250	6,000	1.84
Amoebicin d13-C	2,350	4,250	1.80

[a] One liter of culture supernatant was fractionated as described in Materials and Methods. Activity against *N. fowleri* HB-1 (= ATCC 30174) is expressed in arbitrary units.

TABLE 2. Amino acid compositions of amoebicins from *B. licheniformis* D-13

Amino acid	No. of amino acids in the indicated amoebicin[a]		
	d13-A	d13-B	d13-C
Asp	3 ± 0.10	3 ± 0.08	3 ± 0.09
Glu	3 ± 0.08	3 ± 0.12	3 ± 0.11
Val	2 ± 0.07	2 ± 0.06	2 ± 0.08
Leu	9 ± 0.12	9 ± 0.15	9 ± 0.13

[a] Values represent means of three determinations ± standard deviations.

suspension was frozen in liquid nitrogen, thawed in a water bath at 45°C, and sonicated briefly (15 s). The vesicle suspension was chromatographed on a column (0.5 by 10 cm) packed with Sephadex G-25 to remove the untrapped radioactive material. The vesicles were eluted with 20 mM sodium phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min and used immediately. The vesicle suspension was incubated at room temperature with purified amoebicin d13-B (1 μg/ml). At the desired intervals, portions (100 μl) of the vesicle suspension were filtered through Millipore GSTF02500 filters, the filters were washed with 2 ml of 100 mM lithium chloride, and the radioactivity retained in the filters was measured.

Electron microscopy observation of amoebae and liposomes treated with amoebicin. Aliquots of exponentially growing cells of *N. fowleri* HB-1 were treated with amoebicin d13-B (2 AU/ml) and observed under a Zeiss DSM 950 scanning electron microscope by a previously described procedure (5).

Samples of liquid vesicles obtained by sonication as described above were treated with amoebicin d13-B and deposited on Formvar-coated copper grids. The preparations were washed with 20 mM sodium phosphate buffer (pH 7.2) and stained with 2% uranyl acetate before they were observed under a Zeiss 902 transmission electron microscope.

RESULTS

Bacterial growth and amoebicin production. The highest yields of antiamoebic activity were obtained when the producer strain was grown in BHI broth buffered with 75 mM sodium phosphate, pH 7.2. Production of activity in this medium was not affected by incubation temperatures of 28 or 37°C, but it was favored (1.5-fold) by agitation.

Production of antiamoebic activity occurred during late stationary phase, regardless of the growth conditions tested. The relationship between amoebicin production and sporulation was investigated in cultures grown in buffered BHI broth at 28°C under agitation. Amoebicidal activity was detected in culture supernatants at the onset of sporulation, and its production reached a plateau of 65 AU/ml at 60 h (Fig. 1). For practical reasons, the cultures were collected at 72 h of incubation and the cells were removed by centrifugation.

Purification. Antiamoebic activity from the spent medium of *B. licheniformis* D-13 was retained by solid-phase extraction LC-8 bulk packing at 33% methanol while the pigmented material from the broth was washed off. Activity in the partially purified concentrate was further separated from most of the contaminants by reversed-phase chromatography on a Spherisorb C-6 column. Three peaks of A_{210} (but none at 280 nm) were detected at the position where amoebicidal activity eluted (at a 60% concentration of solvent B). When the fractions corresponding to the three peaks were chromatographed separately on a Ultrapore reversed-phase column, they eluted at slightly different retention times (Fig. 2). The highly purified preparations obtained after this step showed a specific activity of 1.8 AU/μg of protein against *N. fowleri* HB-1. We call these fractions amoebicins d13-A, d13-B, and d13-C. The purified substances were soluble in polar solvents (such as methanol) at concentrations above 30% but were insoluble in water. The yields of the different purification steps and the specific activities of the different fractions against *N. fowleri* HB-1 are

TABLE 3. Spectrum of inhibition of the purified amoebicins from *B. licheniformis* D-13[a]

Organism	AU/μg of protein		
	d13-A	d13-B	d13-C
Acanthamoeba sp. strain Gr-1	<0.02	<0.02	<0.02
N. fowleri			
S-3 (= ATCC 30809)	1.84	1.86	1.82
HB-1 (= ATCC 30174)	1.82	1.84	1.80
Naegleria lovaniensis Aq/9/1/45D	1.78	1.80	1.75
Naegleria gruberi CCAP 1516/1e	1.80	1.82	1.80
Alcaligenes facecalis	2.75	2.75	2.75
B. licheniformis			
M-4	11.00	11.00	11.00
A12	11.00	1.00	11.00
Bacillus megaterium	22.00	22.00	22.00
Corynebacterium glutamicum CECT 78	11.00	11.00	11.00
Enterococcus faecalis			
S-13	5.50	5.50	5.50
S-14	5.50	5.50	5.50
S-48	2.75	2.75	2.75
S-86	5.50	5.50	5.50
Micrococcus luteus	5.50	5.50	5.50
Mycobacterium phlei	11.00	11.00	11.00
Pseudomonas reptilovora N5	5.50	5.50	5.50
Other bacteria[b]	<0.02	<0.02	<0.02

[a] Serial dilutions of samples were assayed as described in Materials and Methods.

[b] The following bacteria were resistant to amoebicins: *B. licheniformis* D-13 (the producer strain), *Escherichia coli* U-9, a *Proteus* sp., *Salmonella typhimurium* LT2, and *Staphylococcus aureus* ATCC 8.

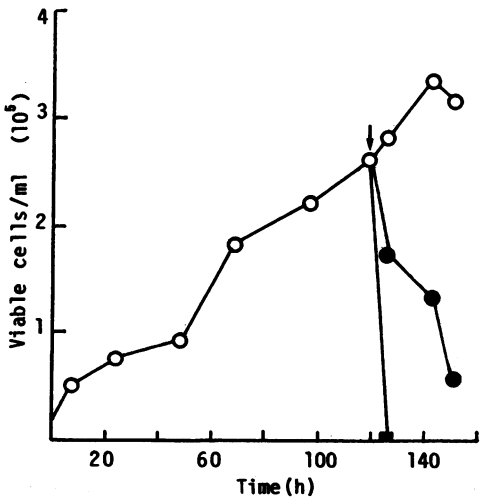

FIG. 3. Effect of amoebicin d13-B on the number of viable cells of *N. fowleri* HB-1. The assay was carried out in Cline medium, and the numbers of intact amoebae in controls (○) and in cultures treated with 1 (●) or 2 (■) AU of amoebicin per ml were determined with a hemocytometer. The average values of triplicate counts are represented. The arrow indicates amoebicin addition.

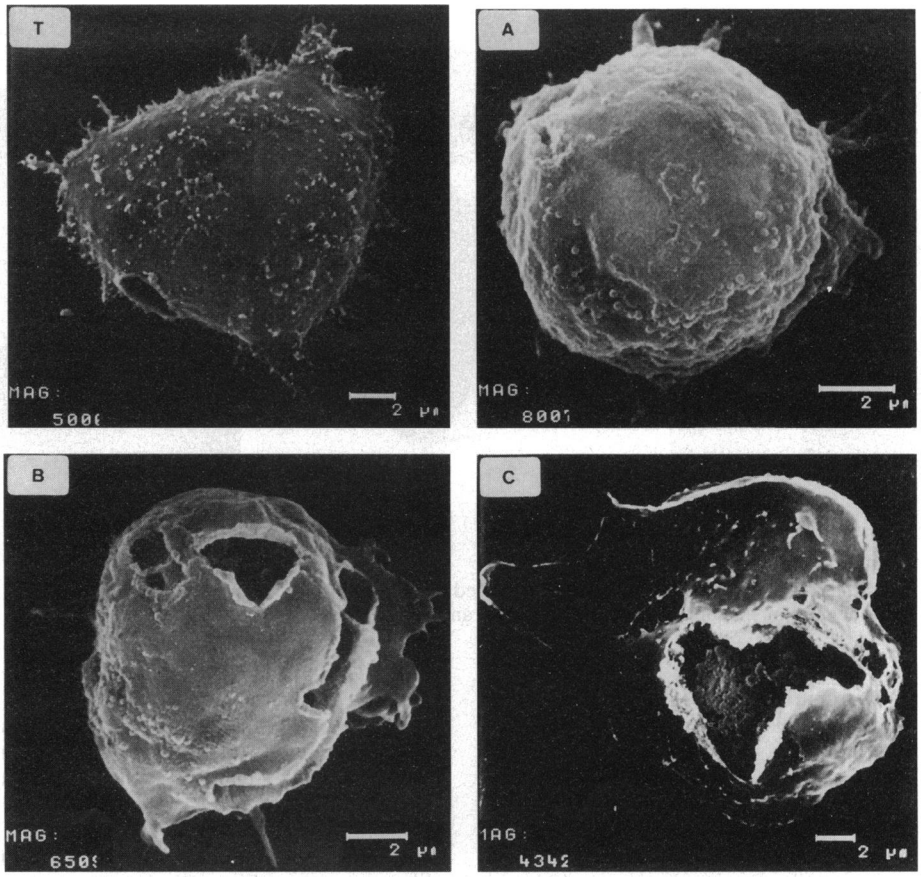

FIG. 4. Scanning electron micrographs of the test strain *N. fowleri* HB-1 (T) and of cells treated for 10 (A), 20 (B), or 40 (C) min with purified amoebicin d13-B (2 AU/ml).

listed in Table 1. The amount of amoebicin d13-B recovered was slightly larger than those of the other two. The percentage of activity found in the purified fractions was 23% of the initial activity.

Physicochemical characterization. The three amoebicins were stable in a pH range from 2.5 to 9.5, and they retained 100% of the activity after being heated at 100°C for 30 min and also after being stored at −20°C for 6 months. Since purified amoebicins were not soluble in aqueous buffers, a mixture of partially purified amoebicins in 20 mM Tris-HCl (pH 7.2) was tested for sensitivity to various enzymes. The mixture retained 100% of its activity after being treated for 1 h with proteases (trypsin, pronase, and proteinase K), lipase, or β-glucuronidase.

The purified amoebicins did not contain any phosphorus, fatty acids, or neutral sugars. The three fractions had identical amino acid compositions: Asp, Glu, Val, and Leu (Table 2). The minimum molecular weight estimated from their amino acid contents was 1,870. The three amoebicins eluted at the same volume on a Fractogel TSK HW-50 (S) column, and this corresponded to a molecular weight of 1,700. Amoebicins showed a maximum light A_{210}, but none above 240 nm. The E_{210} extinction coefficients calculated from the protein content and the UV absorbance (in [milligrams per milliliter]$^{-1}$ × centimeter^{-1}) were 6.69×10^{-3}, 6.82×10^{-3}, and 6.58×10^{-3} for amoebicins d13-A, d13-B, and d13-C, respectively. No amino acid residues were detected after the N-terminal sequence of amoebicin d13-B, suggesting that this peptide is cyclic or blocked at its amino terminus.

Spectrum of inhibition and biological activity. Purified amoebicins were active against several bacteria, most of them gram-positive species (Table 3). Both pathogenic and nonpathogenic species of *Naegleria* were susceptible, while *Acanthamoeba* sp. strain Gr-1 was resistant. The specific activity of amoebicin d13-B against *N. fowleri* HB-1 was 1.86 AU/μg of protein in our assay (0.55 μg in 200 μl).

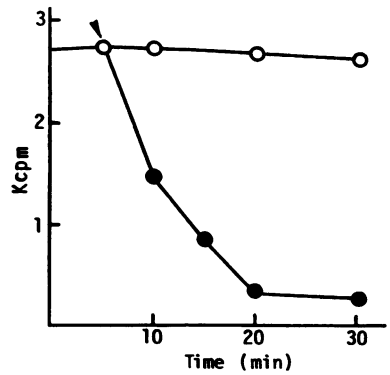

FIG. 5. Effect of amoebicin d13-B on liposomes loaded with ^{86}Rb. The radioactivity retained by untreated liposomes (○) and by liposomes treated with 1 μg of purified amoebicin d13-B per ml (●) was measured at regular intervals. The average values obtained from three determinations are represented. The arrowhead indicates the time of d13-B addition.

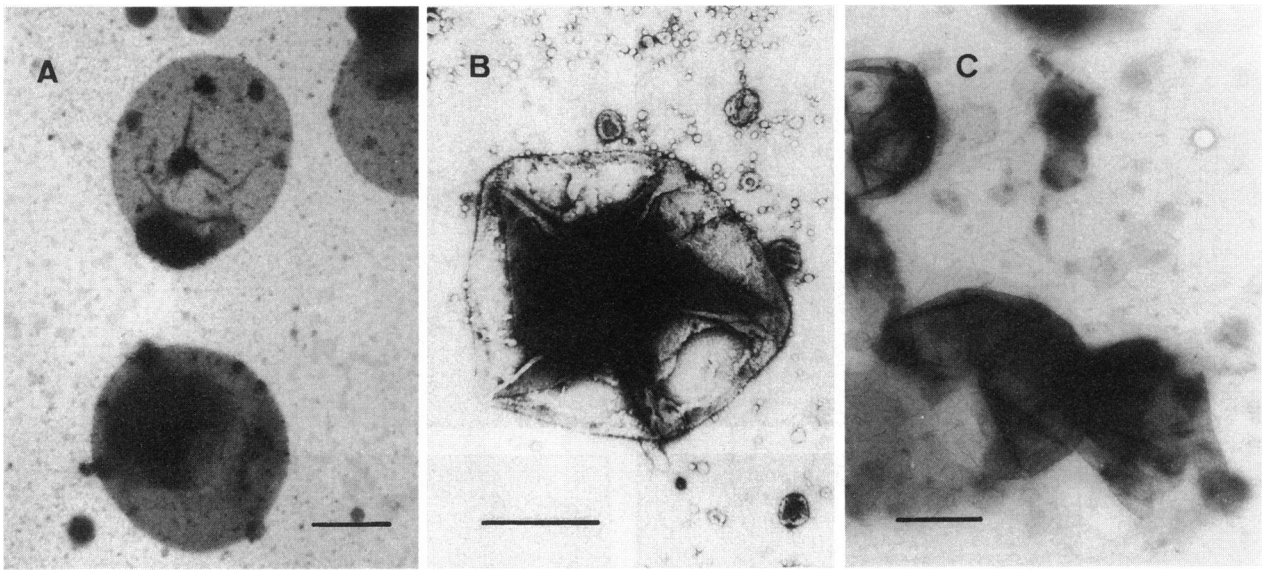

FIG. 6. Electron micrographs of asolectin vesicles (liposomes) stained with uranyl acetate as described in Materials and Methods. (A) Controls. (B and C) Liposomes treated with amoebicin d13-B (1 μg/ml) for 15 and 30 min, respectively. Bars, 0.1 μm.

A cytotoxicity assay was carried out with peritoneal and splenic cells of BALB/c mice. Cells were incubated in triplicate for 3 or 12 h with different amounts of amoebicins (0.2 to 2 μg in 200 μl) before MTT was added. A 3-h period of incubation with 2 μg of amoebicin reduced cell viability of peritoneal and splenic cells by 8 and 10%, respectively, while no effect was observed at lower amoebicin concentrations. The numbers of viable peritoneal and splenic cells after incubation for 12 h with 2 μg of amoebicin were 72 and 65% of controls, respectively, and 85 or 81% for cells incubated with 0.55 μg. We did not test higher amoebicin concentrations as they interfered with the assay.

Addition of purified amoebicins to exponentially growing trophozoites of *N. fowleri* HB-1 caused a progressive decrease in the number of cells that was proportional to the amount of amoebicin added. The effect of amoebicin d13-B on the number of cells is shown in Fig. 3. Changes in the morphology of treated amoebae were easy to observe under an inverted light microscope. First, the cells became round with a dense granular cytoplasm, and substantial lysis of the cell population occurred soon after. Past 10 h of incubation, the cultures contained abundant cell debris, but no intact amoebae were found. Amoebae stained with DAPI were observed under a fluorescence microscope. Treatment with amoebicin d13-B caused lysis of the cell membrane and loss of the cytoplasmic content, while the nuclei remained intact and stained with the fluorescent dye. Some nuclei surrounded by membrane ghosts could also be observed.

Preparations of amoebae treated with amoebicin d13-B for different periods of time were observed under a scanning electron microscope (Fig. 4). Amoebae turned into round cells after 10 min of treatment (Fig. 4A). Visible damage to the cell membrane could be observed after 20 min of incubation (Fig. 4B). After 40 min, the preparations contained severely damaged amoebae whose cytoplasmic content had burst out because of chaotic disorganization of the cell membrane (Fig. 4C).

Liposomes loaded with ^{86}Rb were treated with amoebicin d13-B (1 μg/ml) for various periods (Fig. 5). A rapid efflux of ^{86}Rb was observed following addition of this amoebicin. Treated liposomes lost 90% of trapped ^{86}Rb after 15 min of incubation. It was not necessary to generate an artificial membrane potential across the liposome membrane for amoebicin d13-B to be active on the membrane.

Liposomes treated with amoebicin d13-B were observed by transmission electron microscopy (Fig. 6). A short treatment with amoebicin induced the formation of peculiar cloverleaf-like structures (Fig. 6B). Further treatment with amoebicin caused lysis of the liposomes (Fig. 6C).

DISCUSSION

The production of antibiotics with antiamoebic activity by strains of *B. licheniformis* isolated from natural habitats may be important for various reasons. First, the severity of the disease caused by human-pathogenic amoebae is a stimulus for the trial of new antibiotics. Second, there may be ecological implications since producers of amoebicins may share the same habitats as amoebae. Finally, amoebicins may constitute a new class of antibiotics from *Bacillus* spp.

The three substances with antiamoebic activity purified from culture supernatants of *B. licheniformis* D-13 are hydrophobic peptides with identical molecular weights and amino acid compositions. The similarity in the chemical and physical characteristics of the three fractions isolated suggests that they may be the same substance. However, slight differences must exist to account for their different retention times on HPLC.

The production of various substances with antiamoebic activity by different strains of *B. licheniformis* has common features: (i) production occurs during sporulation; (ii) the substances produced by strains A12 and D-13 consist of two or three related peptides with low molecular weights; (iii) both types of amoebicins lack a free amino terminus, suggesting that they may be cyclic peptides; and (iv) the amoebicins are very resistant to extremes of pH, heat, and proteolytic enzymes. Although both types of amoebicins contain the amino acid residues Asp and Glu, the rest of the residues are different, as are the spectra of UV light absorbance. The amino acid compositions of amoebicins from *B. licheniformis* D-13 are also

different from those of other antibiotics from *B. licheniformis* (3, 11, 17).

The antiamoebic spectrum of amoebicins from *B. licheniformis* D-13 is restricted to species of *Naegleria*, while *Acanthamoeba* sp. strain Gr-1 is resistant. This strain is also resistant to purified amoebicins from *B. licheniformis* A12 (9) and to crude extracts from *B. licheniformis* M-4 (5). Amoebicins from strain D-13 are also active against several bacteria, the most susceptible strains belonging to the genera *Bacillus*, *Corynebacterium*, *Enterococcus*, and *Mycobacterium*. These strains are at least twice as susceptible as amoebae.

Preliminary cytotoxicity assays indicate that murine cells are affected by amoebicins. Nevertheless, they are much less sensitive than amoebae since the amount of amoebicin required to partially reduce the number of viable cells during a 12-h incubation period was fourfold higher than that required to reduce the number of intact amoebae by 50% in 1 h. It may be possible to chemically modify the structure of amoebicins in order to modulate their biological activity and selectively minimize undesirable side effects.

The type of damage caused by amoebicins on amoebae and liposomes clearly indicates that the primary site of action is the cell membrane. Furthermore, localized membrane damage can be observed on preparations of amoebicin-treated amoebae or liposomes examined under an electron microscope. It is tempting to suggest that amoebicins from strain D-13 are therefore cytolytic pore-forming peptides.

ACKNOWLEDGMENTS

We thank Tecnical Services of the University of Granada for gas spectrometry-mass spectrometry and electron microscopy.

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