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Development of a Mechanism of Action-Based Screen for Anthelmintic Microbial Metabolites with Avermectinlike Activity and Isolation of Milbemycin-Producing *Streptomyces* Strains

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A high-volume screen for anthelmintic microbial metabolites with an avermectinlike mode of action was developed. The primary screen used the free-living nematode *Caenorhabditis elegans* in a whole-organism assay. The specificity for avermectinlike compounds resides in the secondary screen, which takes advantage of the chloride channel-opening properties of the avermectins. By using standard microelectrode techniques, membrane conductance changes following exposure to extracts of microbial cultures were measured in the walking leg stretcher muscle fibers of the lined shore crab *Pachygrapsus crassipes*. The avermectins and related milbemycins give a characteristic response of rapid loss of membrane resistance coupled with a slight hyperpolarization of the membrane. This is partially (near 50%) reversible with the chloride channel blocker picrotoxinin. Four morphologically similar cultures that produced avermectinlike activities were identified by this screen. Isolation of the active components from one of these cultures (strain UC 8984) followed by nuclear magnetic resonance spectroscopy resulted in the identification of milbemycins α_1 and α_3. These metabolites are members of a large family of milbemycins produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus* NRRL 5739. Systematic studies revealed that strain UC 8984 is also a *S. hygroscopicus* strain, but which is taxonomically distinct from NRRL 5739.

The avermectins are a series of highly potent anthelmintic and insecticidal compounds of the macrolide structural class produced by the soil bacterium *Streptomyces avermitilis* (2). Since the discovery of the avermectins, members of the structurally and functionally similar milbemycin family have been isolated from *Streptomyces hygroscopicus* subsp. *aureolacrimosus* (19), *Streptomyces cyaneogriseus* subsp. *noncyanogenus* (3), *Streptomyces* sp. strain E225 (10), and *Streptomyces thermoarchaensis* (21a).

These compounds owe their efficacy primarily to their unique mode of action, whereby they are able to stimulate the conductance of chloride ions across muscle membranes in arthropods and nematodes (12, 22). The γ-aminobutyric acid (GABA)-associated chloride channel blocker picrotoxinin has been found to reverse some of the effects of avermectin (8). Many of these mode of action studies have been carried out in crustaceans (8, 22), because the physiology of the nervous systems of selected crustaceans is well characterized and amenable to study by electrophysiological techniques.

We took advantage of the ionophoric properties of the avermectins to devise a screen for microbial products which possess the same mode of action. By using standard microelectrode techniques, microbial culture extracts can be assayed for specific avermectinlike effects on membrane electrical properties of stretcher muscle fibers from the lined shore crab *Pachygrapsus crassipes*. This report describes the development of the assay, its implementation in a high-volume screening regimen, and the identification of a group of milbemycin-producing streptomycetes.

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MATERIALS AND METHODS

Microbial strains. *S. avermitilis* UC 8346 (ATCC 31,267 [2]) produces avermectin and was used as a positive control for the development of the screening protocol. It was obtained from the American Type Culture Collection (Rockville, Md.). *Streptomyces lividans* TK 64 was provided by D. A. Hopwood (John Innes Institute, Norwich, United Kingdom). *S. hygroscopicus* subsp. *aureolacrimosus* NRRL 5739 (19) produces a complex of milbemycins and was obtained from the Agricultural Research Center Collection (Northern Regional Research Laboratory [NRRL], Peoria, Ill.). Strain UC 8984 was isolated from a soil sample collected in Indiana.

Culturing conditions. For all agar-grown cultures Bennett agar (5) was used, and the cultures were incubated at 28°C. Liquid fermentations for the production of active metabolites were grown by using a seed stage medium (20 g of lactose per liter, 15 g of distiller's solubles per liter, 5 g of Ardamine PH yeast extract per liter [medium pH 7.0]). Seed cultures were scaled up to provide an inoculum for a 250-liter batch fermentation by using Production Medium B, which was described previously for the production of avermectin (2).

***Caenorhabditis elegans* assay.** Determination of activity against the free-living nematode *C. elegans* was performed by previously published procedures (17). Briefly, 20 to 30 *C. elegans* (at various stages of development) were added to 2 ml of a solution containing 1.8 ml of M-9 buffer (17), 0.2 ml of an overnight culture of *Escherichia coli*, 100 U of nystatin per ml, and 100 μg of chloramphenicol per ml (both antibiotics were from Sigma Chemical Co., St. Louis, Mo.).

Extracts (see below) in dimethyl sulfoxide (DMSO) were added, and the mixtures were covered and incubated in the dark for 7 days. Each sample was run in duplicate. Readings were made under a dissecting microscope and assigned a numerical score based on the number of surviving nematodes: 0, >50 worms; 1, 21 to 50 worms; 2, 11 to 20 worms; 3, 6 to 10 worms; 4, 1 to 5 worms; 5, 0 worms. Scores from duplicate samples were averaged to yield the final score.

Primary screening protocol. The screening of soil isolates was done by inoculating agar plates with individually isolated cultures in grids of 23 cultures on 100-mm-diameter Petri dishes. The cultures in these plates were allowed to grow at 28°C for 5 days and were then replica plated. The replicas were grown for an additional 5 days, while the master plates were saved. The replicas were then extracted as described below, and the extracts were assayed for nematocidal activity against *C. elegans*. Each of the 23 colonies from master plates whose replicates contained activity were then used to inoculate individual 100-mm-diameter plates. After 5 days, an agar plug (4 mm in diameter) of each culture was removed and extracted as described below. These extracts were reassayed against *C. elegans*. For active cultures, the remainder of the agar plates (used to supply the plugs) were extracted. These extracts served as the samples for the shore crab muscle resistance assay.

Shore crab muscle resistance assay. The effects of ivermectin (22,23-dihydroavermectin B_1) and agar plate extracts on chloride channel activity were assessed by the shore crab muscle resistance assay. This assay has been described previously (20) as a means for detecting avermectin-induced chloride conductance and is a modification of the technique used by Fritz et al. (8) to study the actions of ivermectin on lobster muscle fibers. The assay measures changes in membrane resistance (R_m) and membrane potential (E_m) in isolated leg muscle fibers of the shore crab in response to drug treatment followed by replacement with the chloride channel blocker picrotoxinin. Briefly, extract samples (5 μl of 100 to 120 μl of total extract from each agar plate) or ivermectin standard solutions were diluted in 100 μl of DMSO and were then further diluted 1:100 in shore crab Ringer solution (SCRS [20]) immediately prior to testing. R_m and E_m were recorded from four to six muscle fibers in each of two to three legs at time points 10 and 1 min prior to and 1, 5, and 15 min following replacement of normal SCRS with 5 ml of SCRS containing 1% sample in DMSO. Following the 15-min exposure, sample-containing SCRS was replaced with SCRS containing 10 μM picrotoxinin (exchanged four times), and recordings were obtained at 1, 5, and 10 min thereafter. The reversibility of extract effects was tested by recording R_m and E_m 5 min after replacing picrotoxinin-containing SCRS with normal SCRS (exchanged four times). R_m and E_m values recorded from individual muscle fibers within a leg were standardized to the 0-min value to obtain percent changes in R_m or millivolt changes in E_m. The datum points in Fig. 1 to 3 and Table 1 represent the mean ± 1 standard error for the values obtained from two to three separate preparations.

Agar culture extraction procedures. Whole agar plate cultures (grids of colonies or single isolate streak plates) were sliced into sections, placed in a 125-ml Erlenmeyer flask, and chopped into small pieces by using a weighing spatula. Acetone with 1% 1 N HCl (15 ml) was added to cover all agar pieces and mixed well. The flasks were capped with Parafilm and allowed to stand at room temperature for 2 h. Chloroform (10 ml) was added, and the covered flasks were swirled lightly and allowed to stand for an additional 30 min. During this time, the agar pieces floated to the top of the flask and the chloroform settled to the bottom. The chloroform was then pipetted from the bottom of the flask and filtered through a glass microfiber filter (Whatman GF/A) into scintillation vials. The extracts were dried and then resuspended in 200 μl of DMSO for assay against *C. elegans*.

Individual agar plugs were extracted in a single step. Plugs were placed in 1.5-ml microcentrifuge tubes, and 1 ml of acidified acetone was added. After the tubes were left to stand at room temperature for 2 h, the plugs were removed with forceps and the acetone was decanted into scintillation vials for drying. The dried residues were resuspended in 100 μl of DMSO prior to dosing *C. elegans*.

HPLC metabolite profile. Residues from the concentration of culture extracts were dissolved in methanol and studied by high-pressure liquid chromatography (HPLC). This HPLC metabolite profile method was adapted from a similar method that is useful in avermectin analysis (13). It employed a Brownlee 5-μm RP-18 reversed-phase column and guard cartridge (22 and 3 cm, respectively) maintained at 60°C. The programmed elution sequence began at 82% methanol in water pumped at 1 ml/min and then was increased linearly to 4 ml/min between 10 and 15 min. The flow rate remained at 4 ml/min for an additional 20 min before a 100% methanol flush and reequilibration to the beginning solvent conditions. A Varian 5500 instrument was used with a 10-μl injection loop. The column effluent was monitored at 240 nm, and data were recorded by using a Varian 4270 integrator.

Correlation of anthelmintic activity with TLC and HPLC metabolite profiles. The extract from an entire agar plate culture was divided into a 10% portion for an HPLC metabolite profile and *C. elegans* assay and a 90% portion for application to thin-layer chromatography (TLC). The concentrated extract (250 μl in methylene chloride) was applied as follows: 120 μl was streaked at the origin of each of two silica gel GF panels (5 by 20 cm; Analtech, Inc.); a single (5-μl) spot was applied to a second pair of identical panels. The analytical panels were developed in the same solvent chambers as the streaked, correlation panels were. The solvent systems were methylene chloride-methanol (19:1) and hexane-isopropyl alcohol (17:3). The developed, analytical panels were studied first under 254-nm UV light, the quenching was marked, and then the panels were visualized with acidic spray and heat charring. The developed, streaked panels were divided into R_f sections, and the silica gel of each section was scraped off and eluted with 2 ml of methylene chloride-acetone-methanol (1:1:1). Eluates from each R_f section were evaporated under a nitrogen stream, and the residue was dissolved in 100 μl of DMSO. A portion of this solution was assayed with *C. elegans*, and another portion was studied by the HPLC metabolite profile method.

Taxonomic studies of UC 8984. The aerial mycelia of the cultures grown on Bennett agar and ISP media 2, 3, and 4 (Difco) for 14 days at 28°C were examined directly under a light microscope. Spore surfaces were examined by scanning electron microscopy by the methods of Dietz and Mathews (7).

Physiological characterizations of cultures grown on various media (5) were carried out by the methods of Shirling and Gottlieb (15). Observations were made after 14 days of incubation at 28°C. Colors were assigned to the aerial mass, reverse, and pigments. The growth temperature range was determined on Bennett agar.

To prepare whole-cell hydrolysates, a modified procedure

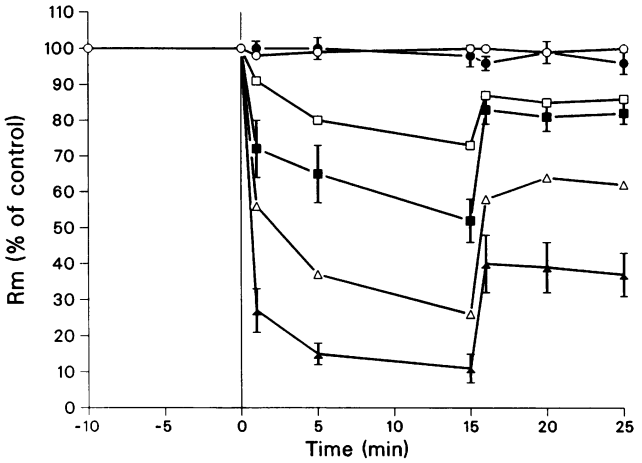

FIG. 1. Concentration- and time-dependent effects of ivermectin on shore crab muscle membrane resistance. Results are the mean ± 1 standard error of the percent change in R_m recorded in three separate preparations. After 15 min, the SCRS medium containing ivermectin was replaced with SCRS containing 10 μM picrotoxinin. The R_m values recorded following a 15-min exposure to ≥0.01 μM ivermectin were significantly lower than pretreatment (0-min) values ($P \leq 0.05$). Lower concentrations of ivermectin and 0.1% DMSO had no effect. Increases in R_m following picrotoxinin addition were significant by the 20-min time point ($P \leq 0.05$). ○, 0.1% DMSO; ●, 0.003 μM ivermectin; □, 0.01 μM ivermectin; ■, 0.03 μM ivermectin; △, 0.1 μM ivermectin; ▲, 1.0 μM ivermectin.

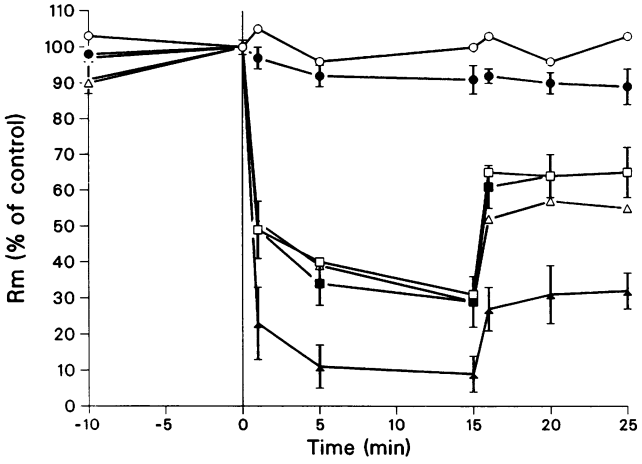

FIG. 3. Effects of extracts of four soil isolate agar plate cultures on shore crab muscle membrane resistance. Cultures were extracted and extracts were assayed as described in the text. The R_m values recorded following a 15-min exposure to all four extracts were significantly lower than pretreatment (0 min) values ($P \leq 0.05$). After 15 min, the SCRS medium containing culture extracts was replaced with SCRS containing 10 μM picrotoxinin. The increases in R_m values following replacement of each active extract with picrotoxinin were significant by the 20-min time point ($P \leq 0.05$). ○, 1% DMSO; ●, uninoculated agar; □, UC 8984; ▲, UC 8985; △, UC 8986; ■, UC 8987.

of Becker et al. (1) was used. The cultures were grown in ISP medium 1 (Difco) in shake flasks at 28°C for 72 h. After incubation, the cells were collected by filtration, washed twice with deionized water, and air-dried at room temperature. Dried cells (10 mg) were hydrolyzed in 1 ml of 6 N HCl in culture tubes (16 by 125 mm) for 20 min by autoclaving them at 121°C. After cooling, 1 ml of chloroform was added to the tube and the solution was mixed on a vortex mixer (Fisher Scientific). The upper layer was pipetted off and filtered through Whatman No. 1 paper into a 10-ml beaker. The filtrate was washed and subjected to TLC as described by Staneck and Roberts (18).

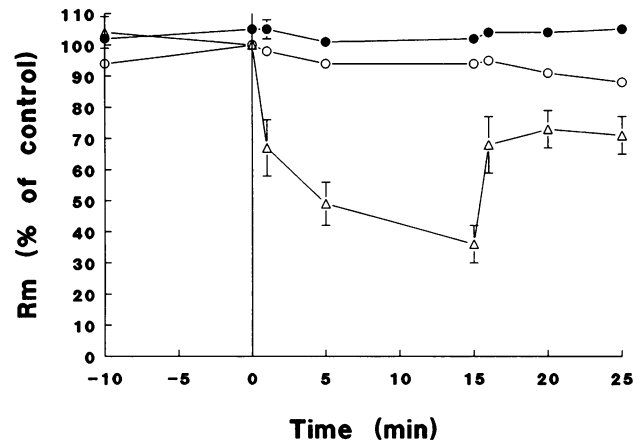

FIG. 2. Effects of agar plate culture extracts of *S. avermitilis* UC 8346 and *S. lividans* TK 64 on shore crab muscle membrane resistance. Cultures were extracted and extracts were assayed as described in the text. The R_m values recorded following a 15-min exposure to the *S. avermitilis* extract were significantly lower than pretreatment (0-min) values ($P \leq 0.05$). Extracts of *S. lividans* or uninoculated agar did not affect the R_m. After 15 min, the SCRS medium containing culture extracts was replaced with SCRS containing 10 μM picrotoxinin. The increase in R_m following replacement of the *S. avermitilis* extract with picrotoxinin was significant by the 20-min time point ($P \leq 0.05$). ○, uninoculated agar; ●, TK 64; △, UC 8346.

Isolation and identification of anthelmintic metabolites from UC 8984. The mycelial cake of a 250-liter fermentation of UC 8984 was partially defatted by washing it with two 40-liter portions of hexane and was then extracted with two 80-liter portions of acetone. The acetone extract was stripped to 13 liters of aqueous slurry, which was extracted with three 8-liter portions of methylene chloride. Solvent removal gave 138 g of a very crude, thick oil. Small batches of this were first defatted by equilibration in a solvent mixture of hexane-acetonitrile (1:1) at about 20 g/liter. The acetonitrile layers were concentrated to dryness, and the residue was triturated three times with heptane (about 50 ml/g), which removed

TABLE 1. Effects of agar plate extracts on the E_m of shore crab muscle fibers

Culture extracted[a]	E_m (mV)[b]
S. lividans TK 64	−0.2 ± 0.3
S. avermitilis	
UC 8346	−6.1 ± 0.7[c]
UC 8984	−3.7 ± 0.4[c]
UC 8985	−2.0 ± 0.6
UC 8986	−4.1 ± 0.8[c]
UC 8987	−1.7 ± 0.6

[a] Extracts were prepared as described in the text.
[b] Expressed as the mean ± 1 standard error within-fiber change of E_m between 0 min (before treatment) and 15 min (after treatment) recordings.
[c] The change in E_m at 15 min was significantly greater than that recorded following exposure to SCRS containing 1% DMSO ($P \leq 0.05$).

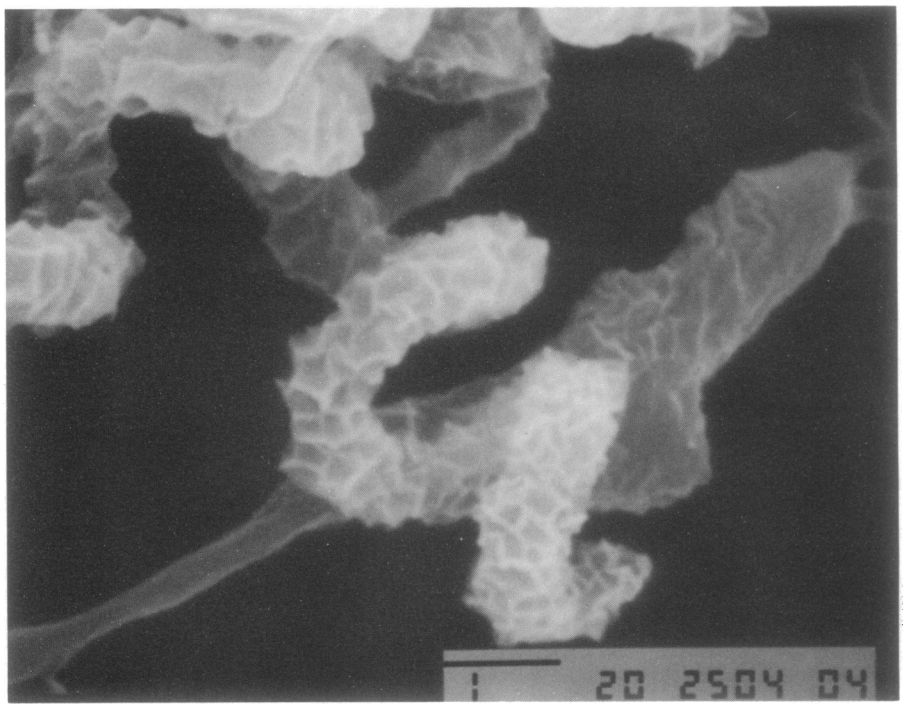

FIG. 4. Scanning electron micrograph of UC 8984, showing spiral sporophores with rugose surfaces. Magnification, ×15,000; bar, 1 μm.

much of the lipid fraction from the 13.7 g of residue remaining. This material was subjected to countercurrent distribution (200 tubes, 10 ml of hexane-acetonitrile per phase). Final chromatography of 0.5 g of active material was done over a Lobar size B LiChroprep Si 60 column (E. Merck) eluted with 0 to 5% methanol in chloroform. Active fractions from this column were studied by ^{13}C nuclear magnetic resonance.

Reagents. Picrotoxinin was obtained from Sigma. Ivermectin was deformulated from the commercial product Ivomec. The Ivomec paste was diluted with a minimal amount of water and extracted three times with one-half the original volume of methylene chloride. The organic phase was washed twice with a one-third volume of water and was then dried over $MgSO_4$. Following filtration and evaporation, the oily crude product was purified on a silica gel column by using ethyl ether elution.

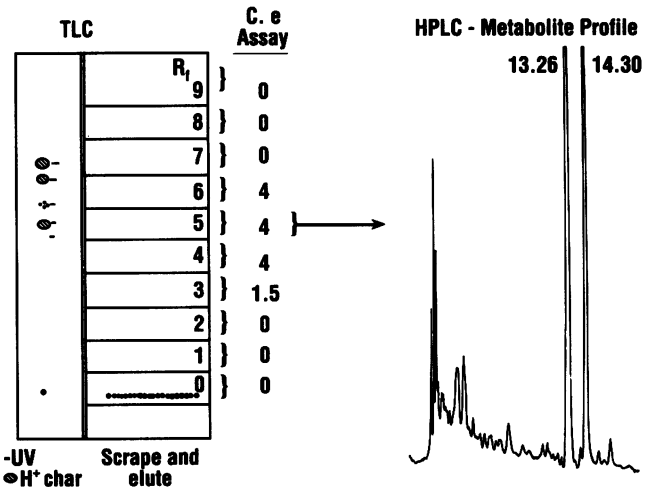

FIG. 5. Correlation of metabolite activity of UC 8984 by TLC and HPLC analyses. A concentrated extract of UC 8984 was applied to the TLC plate as described in the text. The solvent system was hexane-isopropyl alcohol (17:3). Elution, *C. elegans* (C. e) assay, and HPLC metabolite profile procedures are described in the text. H^+ char, visualized with acid and heat charring.

RESULTS

Effects of known avermectin-producing and nonproducing cultures on shore crab muscle membrane resistance. Figure 1 shows the response of shore crab muscle fibers to different concentrations of ivermectin. The response was concentration dependent and consisted of a rapid decline in the R_m

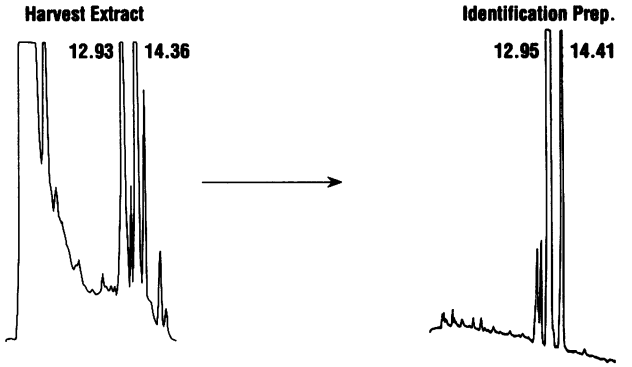

FIG. 6. Purification of active components from a 250-liter fermentation of UC 8984. The HPLC metabolite profile method and UC 8984 process procedure were carried out as described in the text.

TABLE 2. Culture characteristics of *S. hygroscopicus* subsp. *aureolacrimosus* NRRL 5739 and UC 8984[a]

Medium	Parameter[b]	NRRL 5739	UC 8984
Bennett agar	G	Good	Good
	AM	Gray (abundant)	Gray, black patches (moderate)
	R	Brownish gray	Brownish gray
	SP	None	Yellow
Czapek sucrose	G	Good	Moderate
	AM	Gray (moderate)	Light gray (light)
	R	Light gray	Pink gray
	SP	None	Pink
Maltose-tryptone	G	Good	Good
	AM	Light gray (abundant)	Light gray (moderate)
	R	Light grayish brown	Light brownish gray
	SP	None	Light yellow
ISP medium 2	G	Good	Moderate
	AM	Gray	Gray
	R	Brown	Brown
	SP	None	Light yellow
ISP medium 3	G	Good	Good
	AM	Gray (abundant)	Gray (moderate)
	R	Brownish gray	Olive gray
	SP	None	Yellow
ISP medium 4	G	Good	Good
	AM	Gray (abundant)	Whitish gray (moderate)
	R	Brownish gray	Brownish gray
	SP	None	None
ISP medium 5	G	Good	Good
	AM	Gray	Light gray (light)
	R	Light gray	Light brown
	SP	None	None
ISP medium 6	G	Good	Good
	AM	No aerial mass	No aerial mass
	R	Yellowish brown	Yellowish brown
	SP	Light brown	None
ISP medium 7	G	Good	Good
	AM	Gray (abundant)	Gray (moderate)
	R	Light yellowish brown	Brown
	SP	None	Brown
ISP medium 8	G	Good	No growth
	NR	+	

[a] All readings were taken after 14 days of growth at 28°C.
[b] G, growth; AM, aerial mass color; R, reverse color; SP, soluble pigment color; NR, nitrate reduction (+, positive).

over the first minute following drug administration; this was sustained throughout the 15-min period. When ivermectin was replaced with 10 μM picrotoxinin, the R_m recovered approximately 50% of its value that was lost upon treatment with ivermectin. The extract of the *S. avermitilis* UC 8346 agar plate culture showed a qualitatively equivalent response approximately equal to that of 0.1 μM ivermectin, while extracts of the *S. lividans* TK 64 and uninoculated agar showed no response (Fig. 2). The addition of a known amount of ivermectin to the *S. avermitilis* extract was followed by quantitative detection of that amount in the assay (data not shown). Thus, the assay can detect submicromolar ivermectin equivalents of avermectin in extracts of agar-grown cultures. The response was not affected by background readings in nonproducing cultures or the agar medium and was not attenuated by any extract constituents (within this dosage range).

Detection of avermectinlike activity in extracts of four soil isolates. After the screening of approximately 12,000 isolated cultures, 4 cultures were discovered which gave similar results in the shore crab muscle resistance assay. Extracts from these cultures produced conductance changes in shore crab muscle which were identical to those recorded upon exposure to extracts containing avermectin (Fig. 3). That is, under the conditions described above, these extracts produced (i) rapid and profound reductions of R_m which were (ii) sustained over time (i.e., nondesensitizing) and (iii) accompanied by a 2- to 4-mV hyperpolarization of E_m (Table 1). The R_m effects were (iv) partially (40 to 60%) antagonized by the chloride channel blocker picrotoxinin but (v) were irreversible, because the R_m recovered by picrotoxinin was lost following replacement with normal SCRS (data not shown). The decline in R_m (Fig. 3) and the concomitant hyperpolarization that was recorded (Table 1), together with the antagonism of these responses by picrotoxinin (Fig. 3), suggest that the active component(s) in these extracts stimulates chloride conductance in shore crab muscle. The responses exhibited no tendency to desensitize, which suggests that the chloride channel itself, rather than the GABA receptor per se, is the target. The GABA receptor in this preparation does desensitize upon exposure to GABA or muscimol (20). On the basis of comparative data from studies with known quantities of ivermectin (Fig. 1), the levels of activity observed in the isolate extracts is approximately equal to that of 0.03 to 1.0 μM ivermectin.

Taxonomic studies of UC 8984. Upon analysis of the four active isolates, it quickly became evident that they were all closely related. They were all isolated from Indiana soils, and on Bennett agar, they all produced a soluble yellow pigment which diffused throughout the agar and accumulated in droplets of exudate on the colony surface. Gray spore masses revealed coiled spore chains. Also, HPLC analysis of the agar plate extracts produced quite similar patterns. Detailed taxonomic studies were thus carried out only on the culture selected for large-scale fermentations (UC 8984). This culture was compared with the milbemycin-producing isolate *S. hygroscopicus* subsp. *aureolacrimosus* NRRL 5739.

The mature sporophores of UC 8984 formed spirals in dense clusters (Fig. 4). The spore surfaces were rugose. Vegetative hyphae showed no evidence of fragmentation. These characteristics were identical to those of NRRL 5739. Morphological and cultural characteristics pertinent to the differentiation between NRRL 5739 and UC 8984 are given in Table 2. Neither strain produced a melanoid pigment in ISP medium 1 (Difco). The color of the aerial mass produced by both cultures belonged to the gray series of Tresner and Backus (21).

Both cultures utilized a spectrum of sugars (glucose, sucrose, xylose, inositol, mannitol, fructose, and rhamnose). NRRL 5739 utilized arabinose in addition, but not raffinose. UC 8984 utilized raffinose well, but not arabinose. This culture produced a soluble yellow pigment on inositol and fructose. Neither culture utilized cellulose.

NRRL 5739 grew in the temperature range of 18 to 55°C

TABLE 3. ^{13}C chemical shifts of UC 8984 metabolites[a]

Carbon	UC 8984 metabolites	Milbemycin D[b]
1	173.6	173.6
2	45.6	45.8
3	118.1	118.2
4	137.7	137.8
5	67.5	67.4
6	79.2	79.3
7	80.2	80.3
8	139.5	139.6
9	120.1	120.4
10	123.4	123.5
11	142.7	142.8
12	36.5	36.0
13	48.5	48.6
14	136.9	136.9
15	120.9	121.0
16	34.6	34.7
17	67.7	67.8
18	35.5	36.7
19	68.7	68.7
20	41.2	41.4
21	97.5	97.5
22	35.6	35.8
23	27.7	28.1
24	35.9	31.6
25	71.3	78.4
26	19.9	19.9
27	68.4	68.5
28	22.3	22.3
29	15.4	15.5
30	17.8	17.4
31	19.4 25.5 (weak)	28.4
32	10.2 (weak)	14.2
33		21.0

[a] The spectrum was obtained in $CDCl_3$; δ values are in parts per million downfield from tetramethylsilane on a Bruker 300-MHz instrument.
[b] Data were obtained from references 3 and 14.

(light growth at 55°C). UC 8984 was much more restricted in its growth temperature range, growing at 24 and 37°C (no growth at 18 or 45°C). Both cultures contained LL-diaminopimelic acid; no *meso* isomer was detected.

The macroscopic, microscopic, and whole-cell hydrolysate properties of UC 8984 clearly belong to the genus *Streptomyces*. The spore chain morphology and rugose spore surface place the strain in the species *S. hygroscopicus* (6, 16). However, the growth temperature range, pigmentation, and carbon utilization pattern indicate that UC 8984 and *S. hygroscopicus* subsp. *aureolcarimosus* NRRL 5739 are different strains.

Identification of anthelmintic compounds produced by UC 8984. Agar plate extracts were fractionated on TLC streak plates, and the fractions were tested for their activity against *C. elegans*. Correlation of anthelmintic activity with TLC zones and HPLC peaks defined certain metabolites for isolation and quantification (Fig. 5). Only peaks present in the anthelmintic fractions and absent from inactive fractions were selected. By using these criteria, 13-min and 14-min peaks were eligible metabolites (Fig. 5). These peaks served as internal markers for the purification processes. The enrichment for these metabolites during the 250-liter fermentation purification process leading to the material analyzed by ^{13}C nuclear magnetic resonance is shown in Fig. 6. Table 3 shows the resonances found for the sample for which the

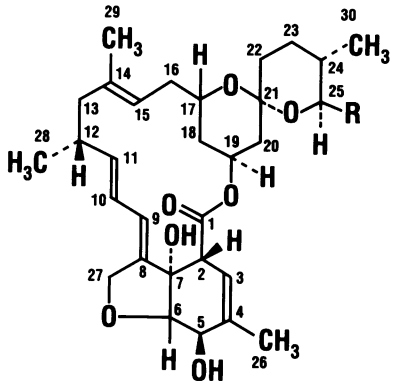

FIG. 7. Milbemycin structures. R for milbemycin α_1, CH_3; R for milbemycin α_3, CH_2CH_3; R for milbemycin D, $CH(CH_3)_2$.

results are shown in Fig. 6 compared with those reported for milbemycin D. The only significant differences were for carbons 31, 32, and 33. Interpretations of these results identify the major anthelmintic UC 8984 metabolite as milbemycin α_1(13-min peak), with a minor amount of milbemycin α_3(14-min peak). These structures are shown in Fig. 7.

DISCUSSION

The discovery of the avermectins and milbemycins has allowed the investigation of a new target site for anthelmintic compounds and insecticides. The primary site and mechanism of avermectin action on nematodes have not been determined, although considerable evidence implicates the chloride channel on axial muscle (11, 12). In crustaceans, the effects of avermectin are localized at the chloride channels in muscle (22), some of which are associated with GABA receptors. GABA is the major inhibitory neurotransmitter at muscle synapses in nematodes (4) and crustaceans (9). Activation of the GABA receptor hyperpolarizes muscle membranes in these organisms, resulting in sustained paralysis. In gastrointestinal nematodes, paralysis of the axial musculature is all that would be required for the parasite to be eliminated from the host.

The avermectins were discovered by testing fermentation preparations directly in nematode-infected mice (2). Milbemycin-producing cultures have been singled out by using whole-nematode in vitro screens (3). Neither of these methods gives any information about the mode of action of active compounds, and in vitro screens routinely pick up a wide variety of generally toxic microbial metabolites. By measuring changes in membrane resistance in response to the addition of experimental samples and known chloride channel blockers, the shore crab muscle resistance assay can reveal in vitro effects of the samples on chloride conductance. The avermectins were shown to give a response in this assay which is particular to this class of compounds: rapid membrane hyperpolarization and loss of membrane resistance which is nondesensitizing and only partially (near 50%) reversible upon the addition of picrotoxinin.

By incorporating the shore crab muscle resistance assay into an in vitro screening regimen following an assay for nematocidal activity, we were able to identify four cultures within a 4-month period which gave the avermectinlike response. These soil isolates proved to be similar in appearance and metabolite profiles. The use of fractionated, correlative TLC greatly expedited the identification of the

active components from the culture UC 8984. Following purification, these were determined by ^{13}C nuclear magnetic resonance to be milbemycins α_1 and α_3.

These two milbemycins are members of a large milbemycin family produced by *S. hygroscopicus* subsp. *aureolacrimosus* NRRL 5739 (19). Taxonomic studies showed that UC 8984 is also a strain of *S. hygroscopicus*, although comparison of growth temperature range, carbon source utilization, and physical appearance indicates that they are distinct strains. *Streptomyces* sp. strain E225 produces a different family of milbemycins and is similar to *S. hygroscopicus* subsp. *aureolacrimosus* in carbon source utilization and some physiological parameters (10). The report describing *Streptomyces* sp. strain E225 (10) notes that, by using whole-organism in vitro screening, metabolites of the milbemycin α series were detected from other isolates. We also routinely find cultures from a variety of sources which have the same milbemycin α metabolite profiles and morphological features as UC 8984. These isolates likely represent a series of closely related strains that have milbemycin production and physical appearance in common.

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