A SIMPLE SCREENING METHOD FOR INSECTICIDAL SUBSTANCES FROM ACTINOMYCETES

BERNARD FABRE, ELISE ARMAU, GILLES ETIENNE[†], FRÉDÉRIC LEGENDRE and GÉRARD TIRABY*,[†]

Laboratoire de Recherches Cayla, Centre commercial de Gros, Avenue de Larrieu, 31094 Toulouse Cédex, France
[†]Laboratoire de Microbiologie et Génétique appliquées du C.N.R.S, 118 route de Narbonne, Université Paul Sabatier, 31062 Toulouse Cédex, France

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A simple and selective assay system was developed in the search for new insecticidal substances from *Actinomycetales* strains propagated on solid culture media. The strains were first tested for their ability to produce antimicrobial compounds. Only strains displaying weak or no activity were retained and screened in the insecticidal bioassay. Microbial solid cultures were given as food to larvae and to adults of *Musca domestica* to detect insecticide producers. A second phase, after extraction of the active compounds, consisted of an evaluation of the insecticidal potency and a primarily biological identification of the products synthesized by the selected strain.

Of 6,280 actinomycete strains which were screened, 47 were active but only 30 of these were finally chosen in the second phase of screening. All these strains, except one, produced known metabolites such as piericidins, avermectins or valinomycin. The one strain, CL307-24, and its insecticide products appeared novel and will be the topic of further study.

For many years, pest control has been primarily based on the utilization of compounds originating from chemical synthesis. However, certain insect control problems, such as resistance, toxicity, persistence, which require new and safer pesticides, have led to the development of natural products and their semisynthetic derivatives. This approach was particularly developed for products from higher plants such as the pyrethroides[1]; however, microbial metabolites, which have been studied primarily for their antimicrobial activities, can also be used for pest control. Some antibiotics have been found to possess insecticidal properties. They include inhibitors of respiration (antimycin A, patulin, piericidins) and protein synthesis (cycloheximide and tenuazonic acid) and membrane active-agents (dextruxin, beauvericin and some polyene macrolide antibiotics)[2]. Other microbial products have been studied specifically for their insecticidal activities, *e.g.* nikkomycins[3], prasinons[4], milbemycins[5], and a few of them have been commercialized as anti-parasitic compounds; avermectins[6], tetranactin[7]. All of these compounds are secondary metabolites produced by *Actinomycetales* strains. These bacteria may produce yet undiscovered bioactive substances, provided a new screening assay could be applied to their fermentation products[8].

We devised an assay system able to rapidly detect and characterize insecticidal activities from bacterial strains. This report concerns the development of this assay system and its application to a large number of *Actinomycetales* strains propagated on solid culture.

Materials and Methods

Microorganisms, Media and Culture Conditions

All microorganisms screened in this program were isolated clones belonging to the *Actinomycetales*

order and originated from the laboratory collection at Cayla. This collection consisted of *Streptomyces* (70%), *Micromonospora* (20%), and rare *Actinomycetales* (10%). The strains were propagated on GAPY medium[9], inoculated with a loopful on agar plates and incubated at 27°C for 7 to 10 days.

Collection strains consisted of *Streptomyces coeruleorubidus* ATCC 31276, *Streptoverticillium* sp. ATCC 15003, *Streptomyces rimofaciens* ATCC 21066, *Streptomyces pactum* NRRL 2939, *Streptomyces avermitilis* NRRL 8165, *Streptomyces tendae* ATCC 31160, *Streptomyces prasinus* NCIB 10719.

Detection of Antimicrobial Metabolites

Strains were tested by the agar piece method[10]. The indicator microorganisms and their media were the following: *Bacillus subtilis* ATCC 6633 and *Escherichia coli* HB101, Antibiotique No. 2 medium (Bio-Mérieux, France); *Trichoderma reesei* QM9414, Potato dextrose agar (Difco); *Saccharomyces cerevisiae* FL200, glucose 2%, Neopeptone (Difco) 1%, yeast extract (Difco) 1%, agar 1.5%.

Detection of Insecticide Producers

Tests were carried out on the last larval instar and adults of *Musca domestica*. Half the agar content of a 55-mm diameter Petri dish (7.5 ml GAPY medium) on the surface of which the bacteria had developed was placed in a large 90-mm diameter Petri dish with 10 larvae. The other half (7.5 ml) was ground in 2 ml of distilled water with an Ultra-turax and introduced in a large Petri dish with 10 pupae just before the pupae hatched into flies. Controls consisted of non-inoculated GAPY medium placed at 20°C and given as food to 10 larvae or 10 pupae as described above. A typical control group of 10 larvae in this insecticide screening test would consume the major part of the media by the second or the 3rd day and 10 of 10 larvae would pupate by the 4th day. On days 8 and 9 most pupae would hatch into flies. We could expect about one half of the adult flies to die by day 12 and almost all by day 14. The criteria used for describing a specimen as active were; the death of at least 6 of the 10 larvae after 1 week, or at least 6 of 10 pupae not hatching, or the early death of adult flies, or a combination of these. Positive controls consisted either of actinomycete strains from various collections, producers of known insecticidal compounds, or purified powders of commercialized insecticides added at different concentrations to the GAPY medium just prior to pouring plates. These preparations were given as food to insects as described above.

Secondary Phase Screening

Preparation of Crude Extracts: Whole agar culture media from the large Petri dishes of the selected strains were extracted twice with 30 ml of MeOH. After centrifugation (3,200 × *g*, 15 minutes), the methanolic solution was recovered and dried under vacuum. The residue was then dissolved in 3 ml of distilled water. One third of this solution was extracted with 1 ml of chloroform in an Eppendorf tube and centrifuged a few seconds at 12,000 × *g* (Apelex Z230 M, Apelex, France). Aqueous and organic phases were separated and the chloroform phase was dried under vacuum. Part of the chloroform residues were dissolved in DMSO to a final concentration of 100 mg/ml. An acetone solution was also prepared at a concentration of 40 mg/ml.

Insecticide Tests:

(a) Injection tests; 1 μl of both DMSO and aqueous solutions from each strain was separately injected under the cuticle of 10 larvae with a microsyringe. For each test, controls with DMSO or H_2O were carried out. The larval mortality was recorded 72 hours after the injection.

(b) Ingestion tests; 500 μl of aqueous solution and acetone preparation diluted at 1/20 in distilled water (50 μl in 950 μl) were spotted into separated Petri dishes and 10 adult flies were allowed to drink *ad libitum*. The aqueous extracts were directly used. The mortality count was made 72 hours after the test.

(c) Topical application; 5 μl of acetone preparations diluted 1/2 with acetone were applied with a microsyringe on the cuticle of 10 larvae and on the thorax of adults previously anaesthetized with diethyl ether vapor. Aqueous extracts, 1 μl, were applied in the same manner. The larvae experienced insecticidal action either by mortality by day 3 or by the non-hatching of the pupae into flies. Activity on adult flies was evaluated by mortality by day 3. Commercialized insecticides solubilized in the same solvents as the crude extracts were included in the assay as controls.

Analytical Methods

TLC: Crude extracts prepared from avermectin- or piericidin-producing strains were spotted on a precoated silica gel thin-layer plate (Silica gel 60 F_{254}, Merck, France) developed with an appropriate solvent system (piericidins: Chloroform - MeOH, 90:10; avermectins: Chloroform - ethyl acetate - MeOH - methylene chloride, 9:9:1:2) and observed under UV light.

HPLC: The system developed for the analysis of the different compounds employed a 5-μm Beckman Ultrasphere C18 analytical reverse phase column (Beckman Instruments, France) and a flow rate of 1 ml per minute. A Gilson pump Model 303, a manometric module Model 802C (Gilson Medical Electronics S.A., France) and a Rheodyne injector Model 7125 (Rheodyne Inc. Cotati, CA, U.S.A.) were used. The column effluents were monitored by a Beckman 156 Refractive index refractor. Samples of 10 μl, containing 500 to 1,000 μg of each compound, dissolved in MeOH were injected. The different solvent systems were: MeOH - H_2O (83:17); MeOH - H_2O (85:15) and MeOH - H_2O - trifluoroacetic acid (93:7:5 mM) for piericidins, avermectins and valinomycin, respectively.

Purification of Valinomycin

The organic extract of a *Streptomyces* strain characterized early in this study as a producer of valinomycin was applied to a column of silica gel (E. Merck, France) packed with chloroform. The column was eluted stepwise with a mixed solvent of chloroform - MeOH (100:0, 99:1, 97:3, 95:5, 90:10 and 0:100).

Identification of Valinomycin-producing Strains: The contents of Petri dishes of all actinomycete strains presumed to produce valinomycin according to the response of a biological test were extracted with chloroform. The chloroform residue was dissolved in 1 ml of MeOH and purified through a C18 cartridge Sep-Pack (Waters Ass., Milford, MA, U.S.A.). The MeOH was removed under vacuum and the extract was analyzed by comparing its HPLC chromatographic profile with that of a co-injection of purified valinomycin.

Insecticides

Diflubenzuron, DDT, lindane, parathion, deltametrine were gifts of M. MAUCHAMPS, Inra Versailles, France. Nikkomycins X and Z were from Bayer, Rfa; monensin and cycloheximide from Sigma; antimycin A from Ayerst.

Results

The screening program comprised two phases. In the first, the strains were tested for their ability to produce antimicrobial compounds and only ones displaying weak or non-activity were retained and screened in the bioassay to detect insecticide producers. This greatly reduced the number of microorganisms to be tested in the secondary screening and avoided the detection of toxic and known antimicrobial compounds possessing insecticidal activity. The second phase consisted of an evaluation of the insecticidal potency and a primarily biological identification of the products synthesized by the selected strain.

Insecticide Bioassay Evaluation, Primary Phase Screening

Sensitivity of the bioassay was evaluated using nine known insecticidal drugs included in GAPY plates (Table 1) and cultures of seven collection strains (Table 2). Drugs like deltametrine, lindane, parathion and DDT displayed a paralysis of the nervous system followed by the death of the insects. Deltametrine was the most effective showing activity at a concentration as low as 3 μg/ml for larvae and 1 μg/ml for adults. With diflubenzuron, no larval mortality was observed even at high concentrations; however, hatching into flies was inhibited at 3 μg/ml. Except for *S. tendae*, which under our conditions was inactive, all the control strains produced insecticidal activity.

Tests for Insecticidal Potency Measurement, Secondary Phase Screening

Five insecticidal drugs and four collection strains were tested in the secondary phase of the screening procedure. After extraction of the solid culture of collection strains, the organic and aqueous extracts were tested on larvae by injection and topical application and on the adults by ingestion and topical application (Tables 3 and 4).

In the case of insecticide producers, all active fractions were recovered from chloroform residues, in agreement with the solubility characteristics of the synthesized compounds.

Only diflubenzuron had no activity on the adults and did not induced the death of the larvae by injection. Most of the drugs were more active on the larvae by injection than by contact. In general, it was more difficult to compare the different activities on the adults. The toxicity by topical application was exclusive whereas by ingestion the flies were also in contact with the active material and a poisoning resembling that of a topical application might occur.

The crude extract of *S. avermitilis* was the most active on the adults and on the larvae by ingestion, but was 1,000-fold less active by topical application. For the crude extract of *S. pactum*, the levels of toxicity by injection and by topical

Table 1. Sensitivity of bioassay to known insecticides from chemical synthesis or microbial origin.

	Activity[a] on larvae (μg/ml)	Activity[a] on adults (μg/ml)
Diflubenzuron	3[b]	>100
DDT	>100	3
Lindane	10	3
Parathion	10	1
Deltametrine	3	1
Antimycin A	>100	10
Monensin	100	30
Cycloheximide	30	>100
Nikkomycins X and Z	100	>100

[a] Concentration of the product included in the consumed medium necessary to kill 60% of the larvae or to shorten the life of adult flies by a factor of two.

[b] Concentration needed to inhibit emergence as adults.

Table 2. Sensitivity of bioassay to collection strains producing insecticidal compounds.

Collection strains	Insecticide products	Activity on larvae	Activity on adults
Streptoverticillium sp. ATCC 15003	Bleomycins	+	+
Streptomyces coeruleorubidus ATCC 31276	Anthracyclines	+	−
S. rimofaciens ATCC 21066	Destomycins	+	+
S. pactum NRRL 2939	Piericidins	+	+
S. avermitilis NRRL 8165	Avermectins	+	+
S. tendae ATCC 31160	Nikkomycins	−	−
S. prasinus NCIB 10719	Prasinons	+	−

+: Active (as defined in Materials and Methods), −: not active.

Table 3. Sensitivity of tests performed in the secondary screening to synthetic chemical insecticides.

	Activity on larvae		Activity on adults	
	Injection[a]	Topical application[a]	Ingestion[b]	Topical application[a]
Diflubenzuron	>100	100	>2	>100
DDT	10	>100	2	1
Lindane	1	10	0.2	1
Parathion	0.1	1	0.02	1
Deltametrine	0.001	0.01	0.02	0.1

[a] μg per insect required to kill 100% of the larvae or 100% of the adults by 72 hours.

[b] Concentration in mg/ml of offered suspension necessary to kill 100% of the adults flies by 72 hours.

Table 4. Activity of the known insecticide producers in the secondary screening procedure.

	Activity on larvae		Activity on adults	
	Injection[a]	Topical application[a]	Ingestion[b]	Topical application[a]
Streptomyces avermitilis	0.1	100	2	1
S. pactum	1	10	2	100
S. tendae	>100	>100	>2	>100
S. prasinus	10	>100	2	100

[a] μg per insect required to kill 100% of the larvae or 100% of the adults by 72 hours.
[b] Concentration in mg/ml of offered suspension necessary to kill 100% of the adult flies by 72 hours.

Table 5. Performance of primary phase screening.

Parameter	Total	Adults	Larvae	Larvae+adults
Number of positive strains	47	6	4	37
% of 6,280 strains screened	0.75	0.1	0.06	0.59

Table 6. Activity of selected strains in the secondary screening procedure.

Number of strains	Activity on larvae		Activity on adults	
	Injection[a]	Topical application[a]	Ingestion[b]	Topical application[a]
Type A: 6	1~10	10~100	0.2~2	10~100
Type B: 1	0.1	100	2	1
Type C: 22	1~10	>100	0.2~2	1~100
Type D: 1	10	>100	0.2	>100

[a] μg per insect required to kill 100% of the larvae or 100% of the adults by 72 hours.
[b] Concentration in mg/ml of offered suspension necessary to kill 100% of the adult flies by 72 hours.

application differed by a factor of 10. The crude extract of *S. prasinus* was active except by topical application. The nikkomycins produced by *S. tendae* were not detected in the test.

Insecticide Screening Performance

Primary Phase Screening

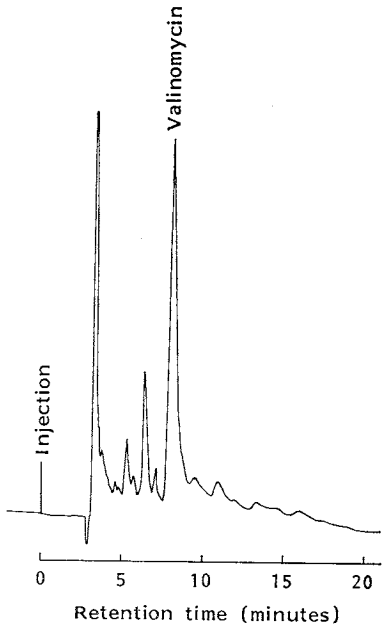
6,280 actinomycete strains were tested for their ability to produce antimicrobial compounds. Of these, 2,560 inactive or weakly active on any panel indicator strain (40.7%) were selected for the insecticide screening, 47 of which (0.75%) were scored as positive and retained for secondary screening (Table 5). Most of these exerted harmful effects on both larvae and adult flies.

Secondary Phase Screening

To enhance the screening performance for the second step, we retained only the strains which showed a level of activity comparable to those of reference insecticide producers. Thus 17 strains of insufficient activity were discarded: 6 of them active only on adults, 4 strains active only on larvae and 7 strains active on both adults and larvae.

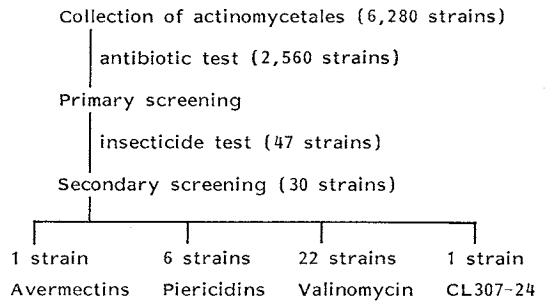
The 30 actinomycete strains finally selected are listed in Table 6 arranged by the nature of activity. Groups A and B strains showed insecticidal effects similar to those of *S. pactum*[11] and *S. avermitilis*[6], respectively. Most strains belonging to type C differed from type A by the absence of harmful effects by topical application on larvae.

Fig. 1. A typical HPLC profile of a crude extract of a type C strain.



Column: Beckman Ultrasphere C18 (4.6×250 mm), mobile phase: MeOH - H_2O - TFA (93:7:5 mM), flow rate: 1 ml/minute, detector: refractive index.

Scheme 1. Procedure and results of screening.



Only one strain is representative of type D whose main characteristic was high activity upon ingestion to adult houseflies.

Identification of Products

The strains were grown on GAPY medium. After extraction the active metabolites of types A and B strains were identified as piericidins[11] and avermectins[6] respectively by classical TLC and HPLC analysis. For the identification of its insecticidal compounds, one of the first encountered type C strains was chosen and grown on GAPY medium. After extraction and purification, the active product was analyzed by spectrometric methods (UV, electron impact mass spectrum (EI-MS)) and identified as valinomycin[12]. The powder was subsequently used as a standard. The 21 other strains were routinely analyzed as described in Materials and Methods. All of these strains produced valinomycin. A typical HPLC profile of a crude extract of these strains is depicted in Fig. 1. One strain co-produced the macrotetrolide antibiotics as did the strain of *Streptomyces tsusimaensis* reported by NISHIMURA *et al.*[18]. The insecticide produced by the type D strain was not identified as a known compound. Its analysis will be published in detail elsewhere. The results of this screening are summarized in Scheme 1.

Discussion

Actinomycetales are well known for their ability to produce a wide variety of secondary metabolites. The search for novel metabolites with specific anti-parasitic activity developed in the last 10 years resulting in the discovery of products such as avermectins and tetranactin which are now commercialized.

Different strategies could be employed to develop a profitable insecticide screening program from microbial metabolites. For instance, insecticidal properties can be screened from various antimicrobial substances. This was the approach in a related field where bialaphos[14], originally described as an antifungal compound was developed as a potent herbicide. An alternative approach would consist of the search for novel metabolites from particular microbial strains isolated from soil samples collected in unusual niches around the world. We choose the latter approach because Cayla Laboratories hold a collection of such microorganisms constituted during different screenings for antimicrobial, herbicidal or insecticidal substances. The wide diversity of this particular collection as a source of

biological compounds enhances the probability of discovering new insecticides. For this purpose, we developed a rapid and reliable screening procedure for insecticide producers among the strains from the collection. The screening was applied only to actinomycete isolates devoid of any antimicrobial activity on solid production medium. The selective insecticide bioassay was based on the detection of mortality among larvae or flies fed *ad libitum* by the whole agar medium containing active compounds which diffused from mycelia of candidate producer strains.

A single agar plate per strain was convenient for the primary screen. This technique allowed easy manipulation and required a minimum of materials, time and staff. The GAPY agar medium was used for growth, maintenance of the strains, production and detection of microbial metabolites by the agar piece method and as bait in the insecticide test. The early elimination of the strains producing antimicrobial agents reduced the investigation to 40% of the initial number of strains and avoided detection of known insecticidal antibiotics like antimycin A[2], cycloheximide[2] and streptothricin[15]. Many other toxic substances *e.g.* antitumor compounds, were excluded because of their antimicrobial activities.

Other compounds such as valinomycin possess an antimicrobial activity which is not detectable under our conditions. This molecule does not diffuse into the agar medium because of its ionophorous character. Some novel insecticide compounds might be co-produced with antimicrobial substances by a strain that we eliminated in our test. Moreover, the GAPY medium might not be the appropriate production medium for some strains. However, the advantages of our technique as a rapid and quantitative screen overrode these limitations.

According to the sensitivity test evaluated with known insecticides (Table 1), the level of detection was about 3 to 10 μg/ml. Wild type strains selected in this study produced about 10 to 50 μg of active compound per ml of GAPY medium. Therefore, this insecticide screening allowed the detection of products either 5-fold less active than the known insecticides or produced at a 5-fold lower level. The reliability of the primary screening was confirmed by the positive response of known producer strains, with the exception of *S. tendae*. Nikkomycins, weakly active in bioassay at 100 μg/ml, are inhibitors of chitin synthetase and disturb the ecdysis of the larvae which is associated with synthesis and degradation of chitin, the main component of insect cuticle. In our insecticide tests, the nymphal ecdysis was the only target for this kind of product. Nevertheless, diflubenzuron (benzoylphenylurea)[16], which does not inhibit chitin synthetase but rather affects chitin metabolism[17] was harmful to the nymphal instar in our test. Therefore, if the bioassay was not sensitive enough to detect exclusively the inhibitors of chitin synthetase, it was able to reveal the compounds acting on other steps of chitin metabolism.

The secondary phase screening eliminated 17 insufficiently active strains and retained only those active on both larvae and adults. Thus, the primary test on the adults appeared to be too sensitive for our purposes and could be abandoned. In addition, this second step gave indications of the type of activity and the solubility of the insecticide. A major part of synthetic insecticides are freely soluble in organic solvents, such as chlorinated hydrocarbons, organophosphorus carbamate, benzoylphenylurea and pyrethroids. Some insecticidal antibiotics such as streptothricin[15], C80-30 A, B and C[18], destomycins[19] and MYC 8005[20] are soluble in water. Destomycins were detected in our test. In the group of insecticides from microbial metabolites without antimicrobial activity, only nikkomycins and allosamidin[21] are soluble in water. A screening directed at products soluble in the aqueous phase would enhance the probability of finding new compounds.

The discovery of several strains producing piericidins and valinomycin emphasizes the natural occurrence of these microorganisms. On the contrary, only one strain was found with a phenotype similar to *S. avermitilis* producing the same pattern as avermectins. Strain CL307-24 and its insecticide product appeared novel. The results of its study will be published in detail elsewhere.

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