Bioactive Compounds with DNA-Binding and Antibiotic Properties from Marine Sediment Microorganisms

HAIDE MAY ALOOT
FRANCO TEVES
LYDIA BAJO
CARMELITA HANSEL

Abstract

The marine environment has recently become the focus of intensive investigation in the global search for new drugs. Aside from marine invertebrates and algae, a number of microorganisms have been shown to synthesize compounds with a variety of specific bioactivities. As a continuing research program in our laboratory, we targeted on screening of sediment microorganisms obtained from an estuarine environment near the Layawan River in Oroquieta City, Misamis Occidental. Using our own modification of a novel screening protocol employing a combination of biomolecular (DNA-binding)-chemical (one and two-dimensional thin layer chromatography) techniques, we were able to identify several actinomycetes (*Nocardia* sp., *Actinomyces* sp., and *Streptomyces* sp.), non-filamentous bacteria (*Listeria* sp., *Corynebacterium* sp., and *Bacillus* sp.), and fungi (*Aspergillus* sp., *Rhizopus* sp. and *Penicillium* sp.) that produce putative DNA-binding antibiotics. The assay was analogous to gel retardation assays but done on preparative TLC plates rather than on agarose gel in electrophoretic systems. Further, we also introduced a two-level antibiotic production screening to target only putative antibiotics with DNA-binding properties. Our modification has the potential for shortening the screening process for new

HAIDE MAY ALOOT, graduate student, M.S. Biology Program, MSU-IIT. FRANCO G. TEVES, Professor, MSU-IIT, Department of Biological Sciences, College of Science & Mathematics, OVCRE, Director of Research, LYDIA BAJO, Professor, Department of Chemistry, College of Science and Mathematics, MSU-IIT, CARMELITA HANSEL, Professor, Department of Biological Sciences, College of Natural Sciences & Mathematics, MSU Main Campus, Marawi City.

potent drugs based on DNA-binding properties of microbial metabolites. DNA-binding is a definite indicator of gene regulatory function exemplified by peptides which are anti-tumor antibiotics. Therefore, the most significant result of our study is the demonstration of production of similar bioactive metabolites by our microbial isolates using a modified biomolecular-chemical screening procedure. This will certainly have a profound impact on the local pharmaceutical industry in the near future.

Introduction

One of the global health problems today is the emergence of multiple drug resistant strains of microorganisms. This has in part caused the race for the discovery of new drugs. In addition, the increased incidence of cancer of various types also necessitates the search for alternative anti-neoplastic natural compounds. About 60 to 80% of drugs such as antibiotics, anti-tumor, anti-cancer, and anti-malarials have been produced from natural products and/or their derivatives (Fransworth, 1994; Cragg *et al.*, 1997; Maier, 1999; Harvey, 2000). The chemical novelty associated with natural products is higher than any other source (Harvey, 2000). The rigorous search for natural products with unique chemistry and biological activity has led to the investigation of many terrestrial and aquatic organisms including plants, animals and microorganisms. Such investigations have revealed that the marine environment is indeed a very prolific source of natural product with diverse structural types than the terrestrial environment (Fuoco *et al.*, 1997). As for microbial sources, marine strains are considered more complex than their terrestrial counterparts. However, exploration of the marine environment as source of natural products has been affected by the problem of inaccessibility. Thus, much focus has been directed on marine sediments that harbor microorganisms that can survive in a less saline environment. These microorganisms play an important role in the degradation of coarse particulate organic matter in sediments (Holguin *et al.*, 2001; Fenchel *et al.*, 1998), thereby providing nutrients for other sediment microorganisms. The vital role of microorganisms in sediments makes them an interesting source for the investigation of natural products.

Discovery of novel compounds does not depend solely on the source but also with the screening strategy and the techniques used in compound collection. New strategies and technologies have been employed in order to determine novel compounds that are different from those already obtained. Biological and chemical screenings are not uncommon in the search for biologically important compounds (Maier *et al.*, 1999). One of the classical screening techniques is the *in*

vitro test for inhibitory effect of a compound (Wilson,2002). Nonetheless, investigators have established certain protocols for faster and more efficient screening. One of those is the integration of whole cell and mechanism-based assay for creating and developing anti-fungal drugs (Fuoco *et al.*, 1997). Recently, a new screening strategy has been formulated known as biomolecular-chemical screening in which DNA-binding capacity of pure compound or crude extract from microorganisms was established using a convenient chemical technique, the thin layer chromatography or TLC (Maier *et al.*, 1999). This screening method combines the chemical screening strategy with binding studies of biological relevance. This is a very powerful tool in exploring the natural sources of biologically active compounds. Developing further this technique to target antibiotics with DNA-binding properties is a task that could allow for the isolation of DNA-binding antibiotics, a class of bioactive substances where anti-tumor antibiotics belong.

Objectives of the Study

The main objective of this study is the isolation of bioactive compounds from marine sediment microorganisms with antibiotic and DNA-binding properties using our modification of the novel biomolecular-chemical screening previously described by Maier *et al.* (1999). This significantly allows for the selection of potential antibiotic anti-tumor active microbial metabolites. Secondary to this objective is the partial survey of the sediment microbial communities found near the mouth of the Layawan River, Oroquieta City, of which no data are available to date.

Scope and Limitation

This study is limited to the isolation of active metabolites with antibiotic and DNA-binding properties from either aerobic or facultatively anaerobic microorganisms in marine sediments obtained near the mouth of the Layawan River, Oroquieta City. Microorganisms targeted are those belonging to the actinomycete group, heterotrophic bacteria and fungi that are able to grow in modified conventional culture media. Antibiosis is established using *Staphylococcus aureus, Bacillus subtilis, Proteus vulgaris, Escherichia coli, Pseudomonas aeruginosa, Saccharomyces cerevisiae,* and *Aspergillus niger.* Analyses were conducted in the Molecular Genetics and Microbial Technology Laboratory, Department of Biological Sciences, MSU-IIT.

Materials and Methods

Collection of Sediment Samples and Microbial Isolation

Sediments were collected near the mouth of the Layawan River, Oroquieta City, in the month of September, 2003 during low tide. The samples were kept in sterile one-liter beakers covered with black plastic and stored at 4°C (Sponga *et al.*, 1999) prior to processing in the laboratory.

Actinomycetes

Three ten-gram portions of sediment were prepared from each sediment sample and placed in sterile 250 ml beakers. The beakers were covered with black plastic. The samples were oven-dried for 4 days at 28°C to reduce the number of vegetative cells of fast-growing actinomycetes (Williams *et al.*, 1972; Sponga *et al.*, 1999; El-Tarabily, 2002). Dried sediment samples were diluted in filtered sterilized seawater and serially diluted. Plating was done using modified glycerol arginine vitamin agar prepared using filtered seawater to which was added 50 μg/ml nystatin to inhibit fungal growth. Plates were covered with black plastic and incubated at 28°C for seven days. Re-streaking for purification was performed using the same media and conditions with final incubation of two weeks with daily inspection for colonial growth.

Non-filamentous Heterotrophic Bacteria

Dilution of sediment samples and serial dilutions were prepared following the protocols described by Harley and Precott (1996), Benson (1998) and Sponga *et al.* (1999). Samples were plated on marine agar medium with nystatin as anti-fungal agent. The plates were wrapped in black plastic and incubated for 7 days at 28°C with daily inspection for growth.

Fungi

The method described by Benson (1998) was modified as used in the sample preparation for the isolation of sediment fungi. Plating was done using potato dextrose agar prepared with filtered seawater and with 50μg/ml each of streptomycin and erythromycin (ASM, 1999). Purification of colonies was done following the methods described by Koneman *et al.* (1978), Soman *et al.* (1999)

and Sponga *et al.* (1999) with plates covered with black plastic and incubated for seven days at 22°C.

Antibiotic Production Assay

A two-level antibiotic production assay was performed on all isolates using the test organisms previously mentioned using either nutrient agar or potato dextrose agar. Initial screening was done by cross-streaking technique described by Kreig *et al.* (1981), Benson (1998) and Sponga *et al.* (1999). A second screening was done using the disc diffusion assay as decribed by Raymundo *et al.* (1991) and Benson (1998).

Characterization and Identification of Microbial Isolates

Isolates were characterized and identified up to the genus level using the scheme described by Raymundo *et al.* (1991), Enriquez *et al.* (1995) and Benson (1998).

Extraction of Metabolites

The isolates were first massively grown in appropriate agar media, which were then diced using sterile spoon and wire mesh and placed in sterile one-liter beakers. A two-fold volume of ethyl acetate was then added and allowed to stand for 24 hours. The resulting eluates were first filtered using sterile gauze pads followed by filter paper filtration with Whatman #2 filter paper. The volume of the filtrates was measured, after which the samples were kept in sealed sterile glass vials to avoid evaporation. Concentration of the samples was done using a rotary evaporator, followed by reconstitution of the weighed dry crude extract in a measured amount of ethyl acetate and storage in sealed sterile glass vials with refrigeration. Another disc diffusion assay was conducted to determine if the antibiotic properties were retained in the cell extracts.

Biomolecular-Chemical Screening of Extracts

Preparation of Salmon Sperm DNA

Salmon sperm DNA purchased from Sigma (USA) was diluted with Tris-EDTA (TE) buffer to obtain a stock concentration of 2mg/ml and stored at 4°C in the refrigerator.

One-dimensional Thin Layer Chromatography

Several solvent systems were first tested to determine which solvents would give good separation of crude extract components. Samples were spotted on preparative TLC plates and developed in improvised chromatography tanks. Detection of spots was done by means of UV extinction at 254 nm and Rf values determined. Spot location and solvent front were marked with pencil at the back of the TLC plates.

Two-dimensional Thin layer Chromatography

Reference and test TLC plates were first run in one dimension using appropriate solvent systems as above. In the second dimension, salmon sperm DNA suspension previously was spotted above the spot locations in the test plates but not in the reference plates. Both plates were then allowed to develop. Spots were visualized by UV extinction. Rf values were calculated for specific spots and Rf ratios (Rf2/Rf1) were determined, with Rf1 referring to the Rf value of spots in the absence of DNA (reference) and Rf2 as the Rf value of spots in the presence of DNA (test). Ratios less than 1.0 would indicate DNA-binding.

Results and Discussion

Sediment Microorganisms and Antibiotic Production

A total of 46 actinomycete isolates were obtained in which five antibiotic producers were selected for the biomolecular-chemical screening (Table 1 and Figure 1).

Table 1. Antibiotic-producing actinomycetes obtained from sediment.

ISOLATES	SOURCE	
	Sampling Area	Dilution
GAVA 16a (*Nocardia* sp.)	A_3	10^{-1}
GAVA 29c (*Actinomyces* sp.)	A_3	Stock suspension
GAVA 34 (*Nocardia* sp.)	A_3	Stock suspension
GAVA 50b (*Nocardia* sp.)	A_2	Stock suspension
MA 47 (*Streptomyces* sp.)	A_2	10^{-5}

Most of the test organisms were inhibited except for *P. aeruginosa* and *B. subtilis* which were not inhibited by Gava 34. Antibiotic production by actinomycetes is anticipated as a defense mechanism to protect its food source during the transition from growth of vegetative mycelium to aerial mycelium (Chater and Merrick, 1979; Miguelez *et al.*, 2000; Challis and Hapwood, 2003). Of the 75 heterotrophic bacteria obtained, five antibiotic producers were chosen for the biomolecular-chemical screening (Table 2). It would be interesting to find out what the chemical nature is of these antibiotics, because most of the bacterial and fungal antibiotics that are known are produced by terrestrial species.

As shown in Table 2, three non-filamentous bacterial species produced antibiotics based on the cross-streaking studies similar to Figure 1. One thing unusual is that these three genera have member species that are pathogenic to man. It is still not known if these are only transient sediment inhabitants, or are actually part of the microbial loop. More light can be shed once the chemical nature of the antibiotics they produce is resolved.

Table 2. Antibiotic-producing non-filamentous heterotrophic bacteria isolated from sediment.

ISOLATES	SOURCE	
	Sampling Area	Dilution
MA 44p (*Listeria* sp.)	A_2	10^{-5}
MA 52bp (*Listeria* sp.)	A_2	10^{-7}
MA 61p (*Corynebacterium* sp.)	A_3	10^{-3}
MA 64g (*Bacillus* sp.)	A_3	10^{-3}
MA 72g (*Listeria* sp.)	A_3	10^{-5}

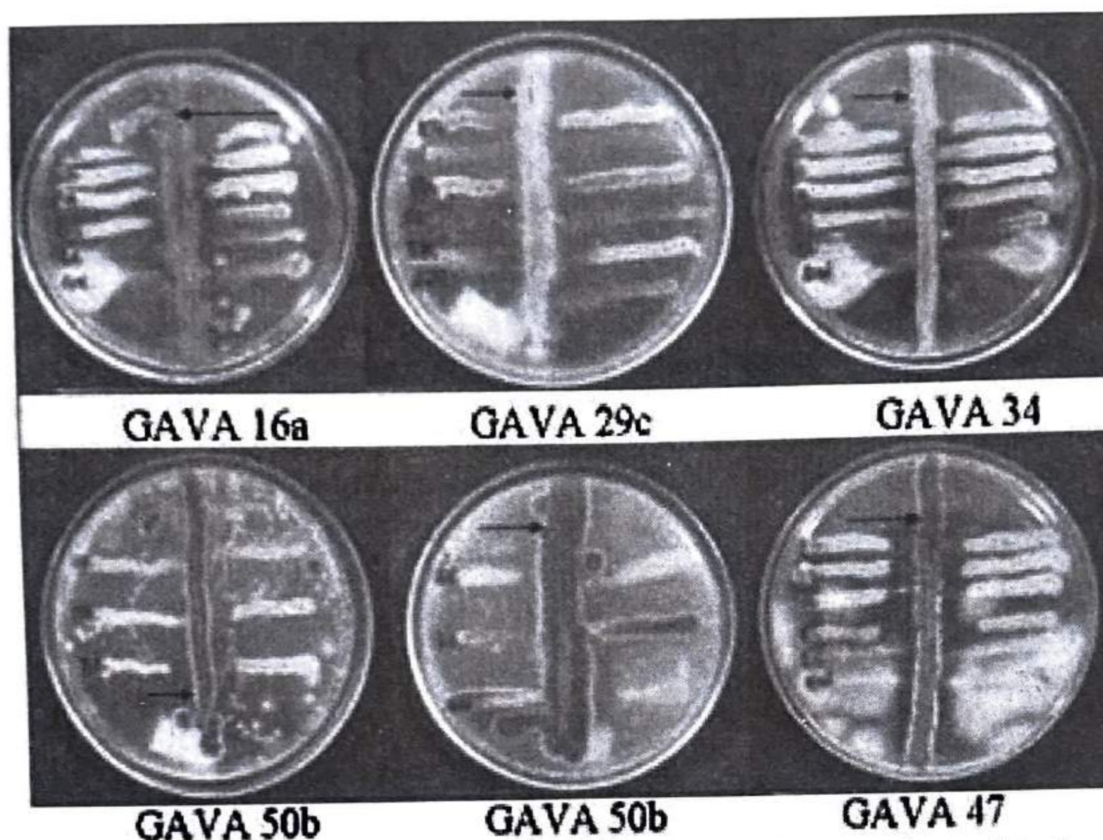

Figure 1. Cross-streaking results in the first-level antibiotic production screening. Vertical streaks with arrows represent the actinomycete isolates. Horizontal streaks represent the different test organisms used in the assay.

Twenty-six fungal isolates were obtained from the marine sediment samples. Five antibiotic producers were selected for the biomolecular-chemical screening (Table 3).

Table 3. Antibiotic-producing fungi isolated from sediment.

ISOLATES	SOURCE	
	Sampling Area	**Dilution**
PDA 2 (*Mucor* sp.)	A_1	10^{-1}
PDA 12 (*Aspergillus* sp.)	A_2	10^{-3}
PDA 28 (*Aspergillus* sp.)	A_2	Stock suspension
PDA 29 (*Rhizopus* sp.)	A_1	Stock suspension
PDA 30a (*Penicillium* sp.)	A_3	Stock suspension

Production of antibiotics is not limited to the actinomycetes, but also includes other differentiating microorganisms such as fungi and myxobacteria (Shi and Zusman, 1993). Fungal antibiotics are well known, among which are the beta-lactams as well as some peptide antibiotics. The four fungal genera represented by the isolates, namely, *Mucor* sp., *Aspergillus* sp., *Rhizopus* sp., and *Penicillium* sp., are common in the terrestrial ecosystem. Again, the chemical nature of the antibiotics they produce will help to shed light on the role of these fungi in the marine sediment. Figure 2 shows an example of the disc diffusion method for determining antibiotic production, in this case, that of actinomycete sediment isolates. This is the second-level antibiotic production assay conducted to verify results from the cross-streaking method (first-level antibiotic production assay).

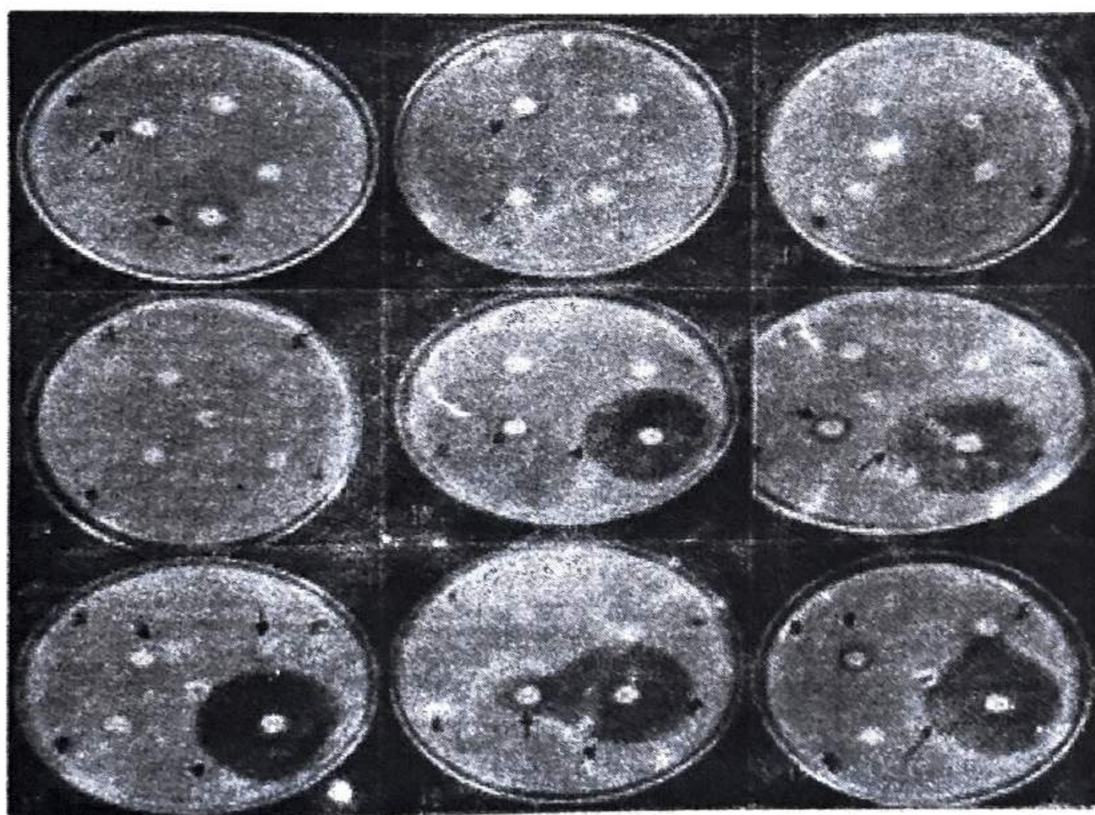

Figure 2. Set up for the second-level antibiotic production assay using the disc diffusion method.

Biomolecular-Chemical Screening

The ultimate goal of isolating DNA-binding antibiotics from sediment microorganisms depended on the thin layer chromatography experiments in one-dimensional and two-dimensional formats. Table 4 shows the Rf ratios for the different separated spots from the crude cell extracts. These spots represented individual chemical components which were then evaluated for their DNA-binding properties. As shown in the table, there is a range of Rf values which indicate potential DNA-interactions (values less than 1.0). For practical reasons, all values less than 1.0 indicate DNA-binding, but for a more stringent screening, if values at the 0.9 levels are excluded, four bioactive components would still pass the biomolecular-chemical screening as DNA-binding antibiotics (Rf ratios 0.600, 0.810, 0.850, and 0.894). This is very significant considering the fact that the potent anti-tumor drugs available in the market possess both antibiotic and DNA-binding properties. In addition, this is the first study conducted in the Philippines utilizing a targeted biomolecular-chemical screening assay for marine sediment microorganisms.

In a study by Puvvada *et al.* (1993), DNA-binding affinity of pyrrolo[2,1-c][1,4] benzodiazepine (PBD), an anti-tumor antibiotic, was quantitatively determined through inhibition of restriction endonuclease *Bam*HI. Inhibition of *Bam*HI activity controlled endonuclease cleavage. Anti-tumor antibiotics are known as non-covalent DNA-binding drugs that interact with DNA in a variety of different ways including intercalation, DNA strand breakage, and inhibition with the enzyme topoisomerase II but they lack the specificity of the antimicrobial antibiotics and thus produce significant toxicity. Another example was dactinomycin that inhibits DNA-directed RNA synthesis at low concentrations and inhibits DNA synthesis at higher concentrations. The dactinomycin binds to double-stranded DNA due to the presence of guanine, permitting RNA chain initiation but blocking chain elongation. This blockage is responsible for the cytotoxic effect of the antibiotic (pharmacology.unmc.edu/cancer/antibio.htm). Our results are, therefore, very significant in the light of these available information on previously studied anti-tumor antibiotics.

Table 4. Rf ratios of spots after 1D and 2D TLC of the cell extracts from selected isolates.

ISOLATE	*SOLVENT*	1-D		2-D		RF2/RR1 RATIO
	RATIO	w/ DNA	w/o DNA	w/ DNA	w/o DNA	
MA 44p	Ethylacetate:.5M Ammonium acetate	0.42	0.50	**	**	-
		0.69	0.69	0.71	0.78	**0.910**
		0.96	0.96	**	0.83	-
MA 52bp	Butanol:Chloroform	0.93	0.90	0.93	0.91	1.020
		0.97	0.98	0.81	0.78	1.040
MA 61p	Butanol:Chloroform	0.47	*	0.72	*	-
		0.67	0.79	0.92	**	-
		0.90	0.875	0.92	0.925	**0.990**
		0.97	0.94	0.94	0.825	1.140
MA 64g	Butanol:Chloroform	0.89	0.89	0.93	0.89	1.040
		0.96	0.97	0.83	0.92	**0.900**
MA 72g	Butanol:Chloroform	0.88	0.88	0.88	0.92	**0.960**
		0.97	0.97	0.86	0.81	1.060
PDA 2	Ethylacetate:.5M Ammonium acetate	*	0.56	*	0.56	-
		0.74	0.76	**	**	-
		0.96	0.92	0.90	0.64	1.410
PDA 12	Butanol:Chloroform	0.28	0.20	0.17	0.21	**0.810**
		0.80	0.76	0.73	0.65	1.120
		0.96	0.96	0.86	0.78	1.100
PDA 28	Methanol:1M ammmonium acetate	*	0.23	**	0.45	-
		0.64	0.63	0.575	0.45	1.280
		0.68	0.73	0.64	0.675	**0.950**
		0.79	0.79	0.74	0.87	**0.850**
		0.92	0.89	0.89	0.92	**0.970**
PDA 29	Ethylacetate:.5M Ammonium acetate	0.37	0.36	**	0.34	-
		0.96	0.94	0.81	0.72	1.130
PDA 30a	Butanol:Chloroform	0.20	0.21	0.12	0.20	**0.600**
		0.43	0.50	**	**	-
		0.97	0.96	0.74	0.80	**0.925**
GAVA 16a	Butanol:Chloroform	0.52	*	**	*	-

		0.80	0.67	0.87	0.91	**0.960**
		0.96	0.90	0.79	0.81	**0.980**
GAVA 29c	Ethylacetate:.5M Ammonium acetate	0.35	*	**	*	-
		0.61	0.45	0.52	0.57	**0.910**
		0.78	0.77	0.81	0.86	**0.940**
		0.96	*	**	*	-
GAVA 34	Butanol:Chloroform	0.83	0.85	0.87	0.79	1.100
		0.97	0.97	0.90	0.82	1.098
GAVA 50b	Butanol:Chloroform	0.43	*	**	*	-
		0.85	0.9	0.83	0.77	1.080
		0.95	0.97	0.82	0.87	**0.940**
MA 47	Butanol:Chloroform	0.43	0.50	**	**	-
		0.83	0.87	0.93	0.93	1.000
		0.97	0.98	0.61	0.69	**0.880**

Legend: *- no separation ** - lost after 2-D TLC
Highlighted values - with DNA affinity

Figure 3. DNA-binding affinity of GAVA 50b extract in two-dimensional thin layer chromatography. [Arrows indicate locations of molecules in the second dimension both in the measuring (with DNA) and reference (without DNA) plates].

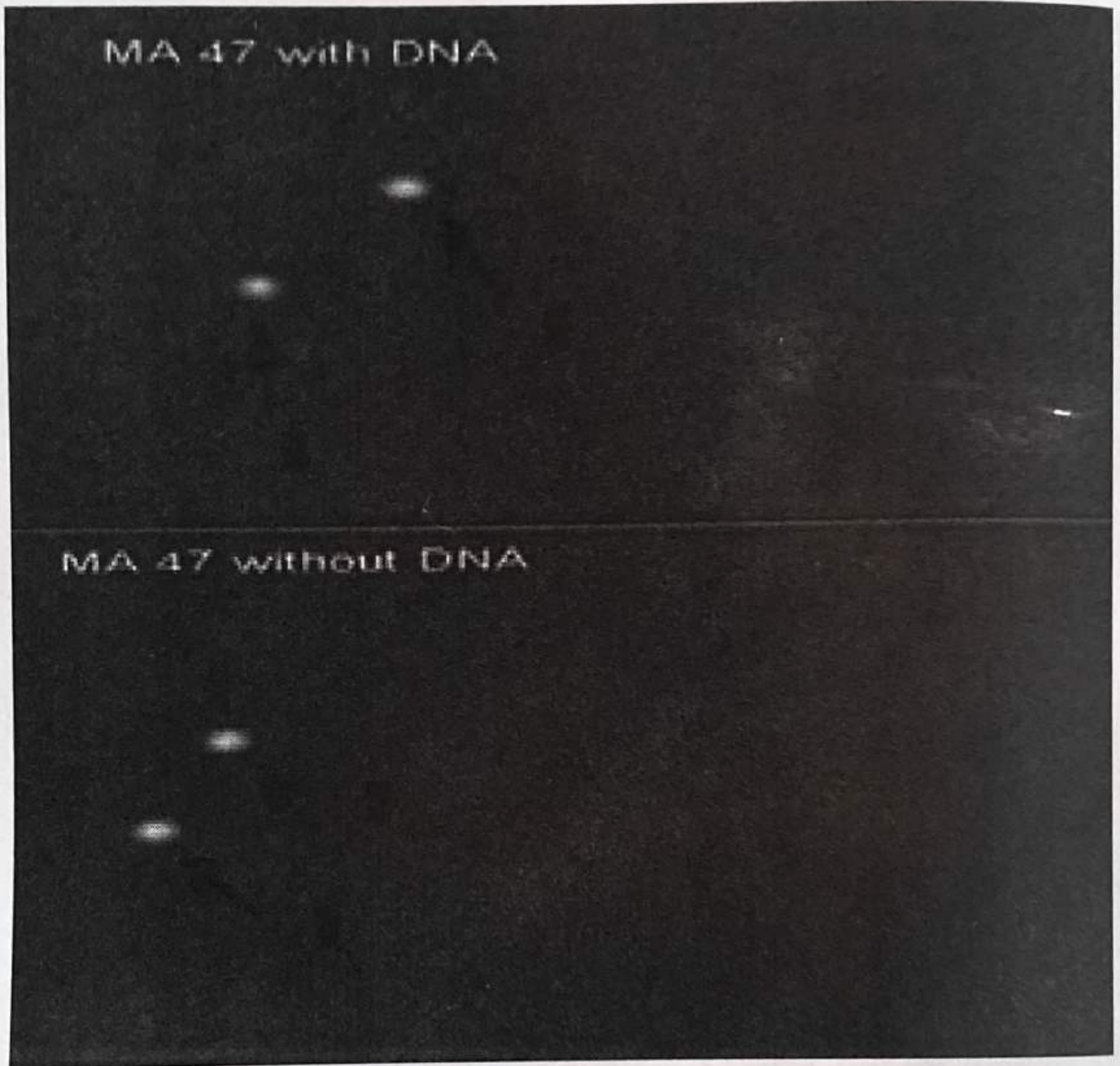

Figure 4. DNA-binding affinity of MA 47 extract in Two-dimensional Thin Layer Chromatography. [Arrows indicate locations of molecules in the second dimension both in the measuring (with DNA) and reference (without DNA) plates].

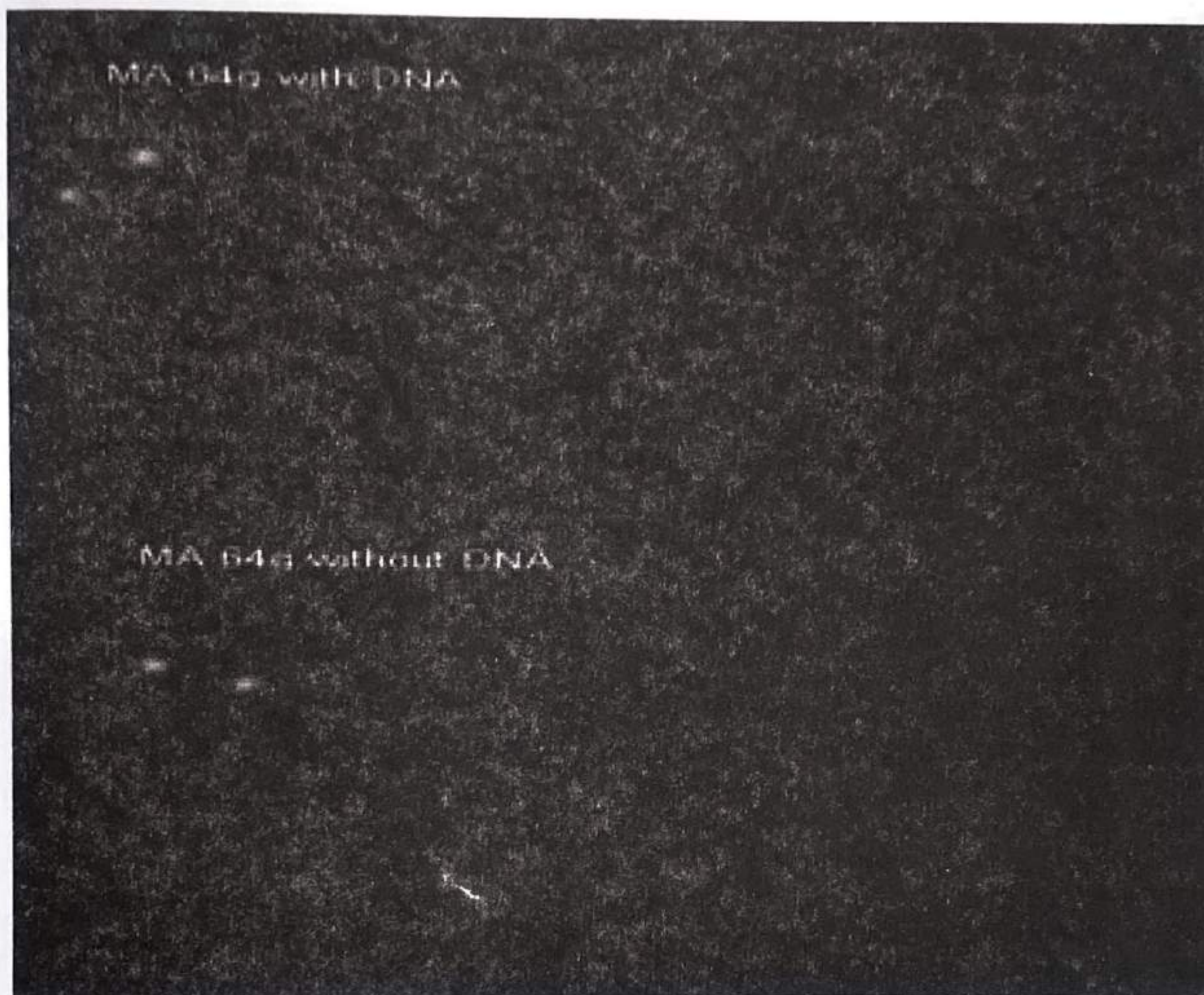

Figure 5. DNA-binding affinity of MA 64a extract in Two-dimensional Thin Layer Chromatography. [Arrows indicate locations of molecules in the second dimension both in the measuring (with DNA) and reference (without DNA) plates].

In Figures 3, 4 and 5 are shown actual results of the 2D-TLC/DNA-binding assay. The novelty and efficiency of this technique are demonstrated by the fact that as TLC provides a chemical "fingerprint" of the individual components of the crude cell extracts, DNA-binding simultaneously screens components which are biologically active at the molecular level. In addition to this format developed by Maier *et al.* (1999), since we have introduced a two-level antibiotic production assay as a modification, we are able to directly screen for antibiotics with DNA-binding properties. Compounds that bind DNA are most

certainly able to regulate gene expression so that in terms of pharmacologic action, these are the most potent. Antibiotics that are able to bind DNA belong to a class of biologically active compounds where anti-tumor antibiotics are members. We are able therefore to show actual selective cytotoxic effects of our compounds to tumor cells in our sequel studies.

Summary and Conclusion

Sediment microorganisms belonging to the actinomycete group (GAVA 16a, 29c, 34, 50b, and MA 47), non-filamentous bacteria (MA 44p, 52bp, 61p, 64g, and 72g), and fungi (PDA 2, 12,, 28, 29, and 30a) have been isolated from marine sediments near the mouth of the Layawan River, Oroquieta City, Misamis Occidental. GAVA 16a, 34, and 50b were identified as belonging to the genus *Nocardia*, whereas GAVA 29c was identified as an *Actinomyces* sp. MA 47 belong to the genus *Streptomyces*. Three of the bacterial isolates belong to the genus *Listeria* (MA 44p, 52bp, and 72g), one to the genus *Corynebacterium* (MA 61), and one to the genus *Bacillus* (MA 64g). Two fungal isolates belong to the genus *Aspergillus* (PDA 12 and 28), one to the genus *Mucor* (PDA 2), one *Rhizopus* sp. (PDA 29), and one *Penicillium* sp. (PDA 30a).

Cell extracts of these organisms have been shown to inhibit the growth of test microorganisms in cross-streaking and disc diffusion set ups. Several components of the cell extracts, which have been separated using 1D and 2D thin layer chromatography exhibited DNA-binding properties. Rf ratios (Table 4) of spots or components showing potential DNA-binding ranged from 0.600 to 0.990, although only four may be considered as having clear DNA-binding capabilities, if more stringent criteria are applied which would exclude all Rf ratios at the 0.9 level.

Results of our study clearly show that targeted biomolecular-chemical screening zeroed in on DNA-binding antibiotics can be a powerful tool for drug discovery of active compounds with immense economic and pharmaceutical values.

Recommendations

The results we have obtained are promising thus far and we recommend the complete chemical elucidation of the specific DNA-binding antibiotics and testing for their selective cytotoxicity to tumor cells for possible application in humans. It is also recommended that identification of the specific DNA sequences, motifs, or modules to which the above compounds bind, as well as specific genes possibly regulated, be undertaken

Literature Cited

BENSON, H.J. 1998. Microbiological applications: laboratory manual in general microbiology. 7th ed. New York: McGraw-Hill Co., Inc.

CHALLIS, G. L. and D. A. HOPWOOD. 2003. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. PNAS. 100 (2): 4555 14561(www.pnas.org/content/vol100/suppl_2/).

CRAGG, G.M. *et al.* 1997. Natural products in drug discovery and development. J. Nat. Prod. 60:52-60.

ENRIQUEZ, G.L., L.S. SANIEL, R.R. MATIAS and G.L. GARIBAY. 1995. Laboratory manual in general microbiology. Manila: UP Press.

EL-TARABILY, K.A. 2002. Total microbial activity and microbial composition of a mangrove sediment are reduced by oil pollution at a site in the Arabian Gulf. Can. J. Microbiol. 48:176-182.

FRANSWORTH, N.R. 1994. Ethnobotany and the search for new drugs. New York: John Wiley and Sons, Inc.

FUOCO, D.M., B.D. TUCKER, R.L. WOOD, N.L. RAUERT, T.K. MILLER, A.E. WRIGHT, P.J. MCCARTHY, S, POMPONI and C.P. SELITRENIKOFF. 1997. Creating and developing an anti-fungal drug discovery program. Biotechnology Perspective. 47(6):280-285.

KONEMAN, E.W., G.D. ROBERTS and S.E. WRIGHT. 1978. Practical laboratory mycology. 2nd ed. New York: The Williams and Wilkins Co.

KREIG, N.R. 1981. Manual of methods for general bacteriology. Washington, D.C.: American Society for Microbiology Press.

MAIER, A., C. MAUL, M. ZERLIN, I. SATTLER, S. GRABLEY, and R. THIERICKE. 1999. Biomolecular-chemical screening approach for the discovery of biologically active secondary metabolites I. Screening, strategy and validation. J. Antibiot. 52(11): 945-951.

MAIER, A., C. MAUL, M. ZERLIN, I. SATTLER, S. GRABLEY and R. THIERICKE. 1999. Biomolecular-chemical screening approach for the discovery of biologically active secondary metabolites II. Application studies with pure metabolites. J. Antibiot. 52(11): 952-959.

PUVVADA, M.S., J.A. HARTLEY, T.C. JENKINS and D.E. THURSTON. 1993. A quantitative assay to measure the relative DNA-binding affinity of pyrrolo [2,1-c][1,4] benzodiazepine (PBD) antitumor antibiotics based on the inhibition of restriction endonuclease *Bam*HI. Nucleic Acids Research 21(16): 3671-3675.

PRESCOTT, L.M., J.P. HARLEY and D.A. KLEIN. 1993. Microbiology 2nd ed. New York: Wm.C. Brown Publishers.

RAYMUNDO, A.K., A.F. ZAMORA, and I.F. DALMACIO. 1991. Manual on microbiological techniques. Manila: Technology and Livelihood Center.

SPONGA, F., L. CAVALETTI, A. LAZZARINI, A. BORGHI, I. CICILIATO, D. LOSI and F. MARINELLI. 1999. Biodiversity and potentials of marine-derived microorganisms. Journal of Biotechnology. 70:65-69.

SHI, W. and D.R. ZUSMAN 1993. Fatal attraction. Nature 366: 414-415.

SOMAN, A.G., J.B. GLOER and D.T. WICKLOW. Antifungal and antibacterial metabolites from a sclerotium-colonizing isolate of *Mortierella vinacea*. J. Nat. Prod. 62:386-388.

TOUCHSTONE, J.C. 1992. Practice of thin layer chromatography 3rd ed. New York: John Wiley and Sons, Inc.

YOSHIKAWA, K., T. TAKADERA, K. ADACHI, M. NISHIJIMA and H. SANO. 1997. Korormicin: a novel antibiotic specifically active against marine gram-negative bacteria produced by a marine bacterium. J. Antibiot. 50(11): 949-953.