Progress in Biological Sciences
Vol. 7, Number 2, Autumn/Winter 2017/183-189 - DOI: 10.22059/pbs.2020.258364.1303

Interspecies interactions of halophilic and halotolerant actinomycetes: An example from a salt

Ensieh Salehghamari*, Fatemeh Taheri, Marzieh Hosseini, Maryam Sardabi, Seyed Alireza Etesami, Gholam Heydar Hasani, Zahra Eshrati

Department of Cell and Molecular Biology, Faculty of Biological Science, University of Kharazmi, Karaj, Iran

Received: May 20, 2019; Accepted: October 29, 2019

ABSTRACT

Interspecies interaction of actinomycetes will express new gene clusters and may therefore affect the pigmentation, sporulation and production of secondary metabolites. Actinomycetes strains were isolated from Howze Soltan Salt Lake. Binary actinomycete interaction assay was conducted to evaluate its effect on colony morphology and antibiotic production. The molecular identification of the induced strains was performed. A total of 18% of isolates induced antibiotic production of 22% of the other strains against methicillin resistance *Staphylococcus aureus* (MRSA) and 44 % of them inhibited that of 31 % of antibiotic production in other actinomycete strains. The extract of the selected strains had an inhibitory effect on the pathogen growth. Based on molecular identification, the selected isolates, called act 32, shared 98% similarity with *Streptomyces peucetius.* It is expected that by screening of actinomycetes from untouched environments and co-culture method, new metabolites can be found to treat antibiotic-resistant infectious diseases.

Keywords: Binary assay; Dual culture; Howze Soltan Salt Lake; MRSA; Secondary metabolites

Introduction

Natural products play today an important role in the discovery and development of new drugs, including antitumors, antimicrobials and anti-hypertensives (1, 2). However, there are problems with the industrial production of natural products in microorganisms. Genomic studies on certain groups of bacteria and fungi have shown that several secondary metabolite pathways are not expressed in the laboratory under standard growth conditions (3-4). Therefore, it is assumed that some potential antibiotic producing gene clusters exist in the actinomycetes genome (5). One method for more access to this potential of cultivable microorganisms is mix-culturing them as the presence of neighboring microbes can lead to the synthesis of new secondary metabolites. Today's research suggests that co-culturing of microbes can lead to induction of unknown pathways to produce bio-active materials. One report stated that in the binary culture of 76 strains of *Streptomyces*, 34% of strains induced antibiotic production in 13% of them (6).

* **Corresponding author:** esaleh@khu.ac.ir

Actinomycetes are a group of gram-positive and filamentous bacteria and have long been a great source of useful therapeutic products. These bacteria produce half of the antibiotics used to treat diseases (7, 8). Recent studies on genome sequencing and transcriptomics have shown that these bacteria also have a large reservoir of genes that are likely to produce new secreted small molecules. Still, many of these gene clusters have not been expressed under routine culture conditions (9).

In this report, we tried to do binary interspecies interaction assays in order to extract new secondary metabolites and investigate the relationships between actinomycetes. Since methicillin resistance in *Staphylococcus aureus* (MRSA) is one of the most challenging pathogens in infections and there is no research on interspecies interactions of halophilic and halotlerant actiomycetes around Howze Soltan Lake, we intend to describe the effect of dual culture on induction/inhibition of anti-MRSA metabolite production, as well as morphological changes, pigment production and sporulation of these strains.

Materials and methods

Study area and sampling

The study area was Howze Soltan Salt Lake located approximately 100 km east of Qom City in Iran (34° 30' N, 51° 52' E). Sampling was done in winter 2017. Four samples were collected from two different parts from sediments in 5 cm of rhizosphere depth and far from the roots of plants around the lake. The samples were collected in sterile screw cap bottles and were brought to the laboratory for isolation of actinomycete strains. One gram of each sample was suspended in 2 ml of distilled water and shaked for 20 minutes. The pH was measured after the sediment settled down.

Pretreatments of samples and isolation of actinomycete strains

One gram of sediment samples was heated at 100°C for 60 min. An aliquot of 100 µl of the pretreated 10^{-2}, 10^{-3}, and 10^{-4} dilutions was spread over the surface of the starch casein agar plates, including (g/l) 10 starch, 3 casein and 15 Agar containing two different NaCl concentrations (0 and 5%). Also, these plates were supplemented with nalidixic acid (50 µg/ml) and cycloheximide (100 µg/ml) to inhibit fast-growing Gram-negative bacteria and fungal growth, respectively. After 1 to 4 weeks, the colonies were picked up and re-cultured in a new plate to obtain pure colonies. Salt tolerance experiments were performed on the ISP2 medium (pH 8.2). The isolates that could have been optimally grown in media containing between 3% and 15% (w/v) salt were considered as moderately halophilic bacteria (10).

The isolates were cultured on plates for short-term storage, and long-term strain maintenance was set up in medium supplemented with 30% glycerol at −20°C.

Binary Actinomycete Interaction Assays

To test the effect of 32 actinomycete dual culture on colony morphology and metabolite production such as pigmentation or antibiotic synthesis, glycerol casein agar plates were used. One µl of spore stock of a strain was cultured 0.5 cm away from another strain, and so each of strains alone served as as a control (9). This experiment was repeated three times. Plates incubated at 29°C for 5 days until developmental progress was visible in control colonies. Plates were examined every day to observe any changes in colony morphology or pigment production. In addition, the effect of dual culture of isolated actinomycetes on antibacterial metabolite production was tested against MRSA. An overnight culture of MRSA was added to 1% LB agar (~45°C) to prepare 1% v/v mix, which were poured over the interaction assay plates. The plates were incubated overnight at 37°C and checked for altered zones of inhibition.

Extraction of dual culture plate and anti-MRSA metabolite test

The selected induced strain along with the inducing strain was cultured in a plate in a triangular shape alongside each other to do induction. Plate was incubated at 28°C for 5 days. The medium containing bacteria was mashed and mixed with ethyl acetate for 1 hour and tested for antimicrobial activity by the agar well diffusion method (11). MRSA was spread on Müller-Hinton agar plate and a 6 mm diameter well was prepared. The well was filled with 100 µl of the extract and the plate was incubated at 37°C for 48 h.

Morphological study and molecular identification

Actinomycetes colonies were characterized on the basis of colony color and morphology and gram staining by light microscopy according to International Streptomyces Project (ISP).

For molecular identification, the induced strain was grown for 5 days at 28°C with agitation in 100 ml flasks containing 10 ml of ISP-2 medium. Biomass was harvested by centrifugation at 4000 rpm for 25 min and washed twice with 0.9% sodium chloride. About 200 mg of pellet was used for DNA extraction as described by Kieser et al. (12). The *16S rDNA* was amplified using PCR with pfu DNA polymerase and primers 9F (5´ AAGAGTTTGATCATGGCTCAG 3´) and 1542R (5´ AGGAGGTGATCCAACCGCA 3´). The reaction mix (25 µl) included 0.4 mm of each primer and 4% DMSO. The reaction was started with an initial denaturation at 95°C for 300 sec followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 54.5°C for 30 sec and extension at 72°C for 90 sec, with a final extension at 72°C for 300 sec. The PCR products were analyzed by agarose gel electrophoresis and submitted for purification and sequencing to Macrogen Inc. (Seoul, Korea).

The identification of phylogenetic neighbors and the calculation of pairwise *16S rDNA* sequence similarities were achieved using the ez taxon server (http://www.ezbiocloud.net/eztaxon). Sequences were aligned using CLUSTAL X software (version 2.0, Conway Institute, USA), and a phylogenetic tree was constructed by the Neighbor Joining (NJ) method using MEGA software (version 6.0, Biodesign Institute, USA). The bootstrap was calculated from 1,000 replicates to assess the clade reliabilities.

Results

Isolation of actinomycetes

The measured pH in all samples was 8. A total of 32 actinomycetes were isolated from saline samples. 78% of strains belonged to rhizospheric sample. The halophilic test showed that 81% of isolated actinomycetes were halophiles.

Actinomycetes inhibited pigmentation, sporulation and growth

Dual cultures were observed for any colony morphology, growth and pigmentation production changes. Several actinomycetes triggered inhibition of pigmentation and sporulation. A total of 13% of them inhibited soluble pigmentation production of 9% of the others (Fig. 1) and 15% of them inhibited sporulation of 20% of the others (Fig. 2).

Figure 1. The pigmentation inhibition of some strains triggered by dual culture with other actinomycetes strains. The strains have been cultured individually as a control and binary in order to observe the inhibition of pigment production.

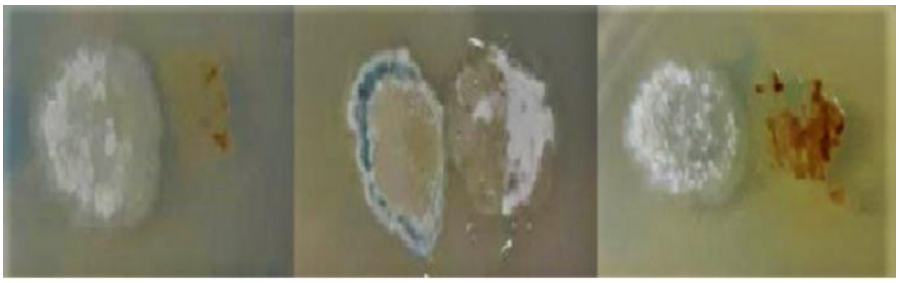

Figure 2. The sporulation inhibition of some strains triggered by dual culture with other actinomycetes strains.

Also actinomycete interaction caused phenotypic responses ranging from no stimulation development to partial colony formation. The negative control showed good growth of all the tested isolates. 35% of strains had inhibitory effects on growth of 48% of actionmycetes (Fig. 3).

Actinomycetes induced/inhibited antibacterial metabolite production

Given that regulation of actinomycetes development have been linked by the production of natural products, it was assumed that the induced changes in the strain phenotype may be associated with a change in the production of secondary products. Antibiotic production also may be induced or inhibited by an interaction. A total of 18% of isolates induced zone of clearance of 22% of the other strains which did not have any zone of inhibition against MRSA before induction (Fig. 4A). Two pairs of strains were selected for anti-MRSA metabolite extraction. Also 44% of isolated actionmycetes inhibited partially or completely the clearance zone of 31% of anti-MRSA producing strains (Fig. 5).

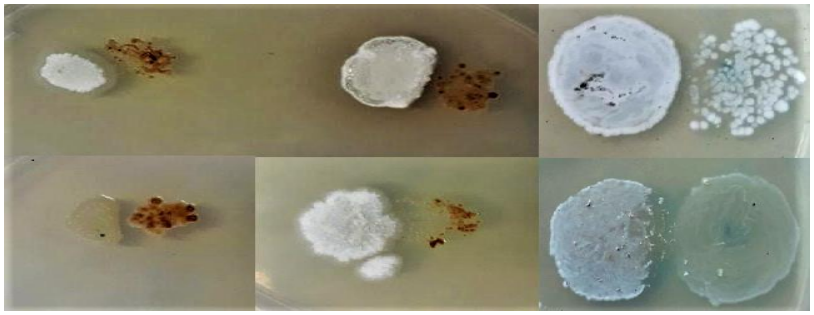

Figure 3. The growth inhibition of some strains triggered by dual culture with other actinomycetes strains.

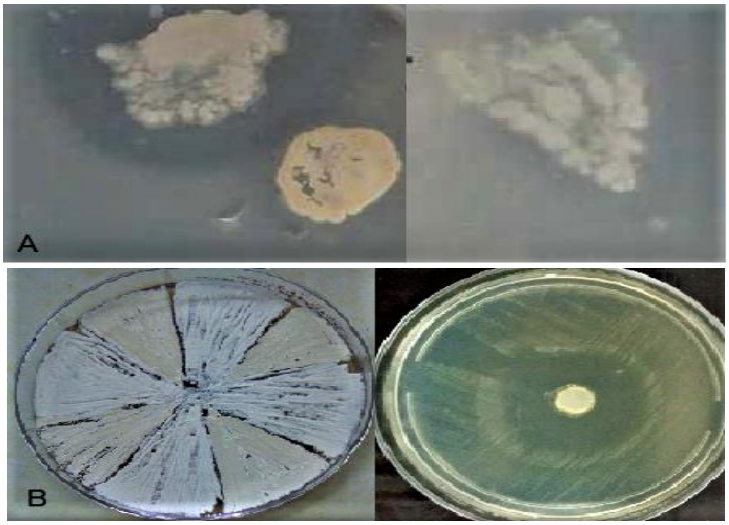

Figure 4. The anti-MRSA antibiotic induction of the strain act 32 triggered by dual culture with act 23. The strain act 32 also has been cultured individually as a control (A). Binary culture of strains act 23 and act 32 and the ethyl acetate extraction effect of the plate against MRSA with agar well diffusion method (B).

Figure 5. The anti-MRSA inhibition of some strains triggered by dual culture with other actinomycetes strains. The inhibited strain has been cultured individually as a control and the binary culture was performed to observe the inhibition of anti-MRSA production.

Induced anti-MRSA metabolite extraction

The plate of candidate interactive actinomycetes was extracted and tested with well diffusion agar method (Fig. 4B). The clearance zone of extracted anti-MRSA metabolite induced in act 23: act 32 plate (41 mm) is shown in Figure 4B. Act 32 represents the induced strain.

Bacterial identification and phylogenetic analysis

All the strains were studied for culture characteristics based on colonial morphology, ability to form aerial hyphae and substrate mycelia. Most of the isolates were slow growing, aerobic, chalky or glabrous, folded and with substrate and aerial mycelia of different colors, viz. pink, yellow, white, brown and orange with an earthy odor.

16S rDNA gene sequence of the isolate act 32 shared the highest level of sequence similarity (98%) with *Streptomyces peucetius*. DNA sequence was deposited in GenBank under accession number: MK376964. Phylogenetic trees based on the *16S rDNA* sequences of the strain act 32 using Neighbor Joining (NJ) method is shown in Figure 6.

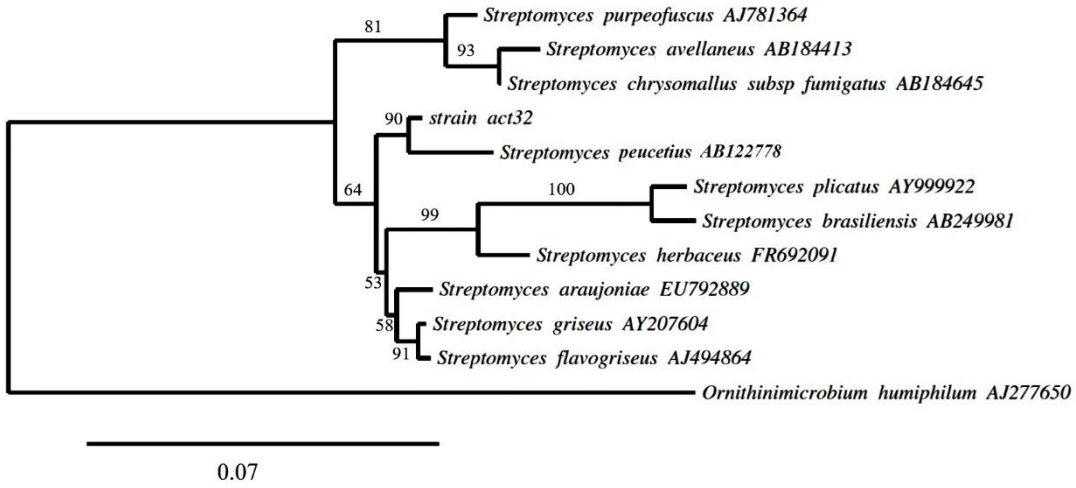

Figure 6. Phylogenetic tree based on *16S rDNA* sequences using Neighbor Joining algorithm, showing the position of isolate act 32 and its closely related species of the genus *Streptomyces*. Bootstrap values are indicated at branch-points. *Ornithimicrobium humiphilum* AJ277650 was used as the outgroup. Bar= 7% sequence divergence.

Discussion

Since bacteria in their local ecosystem are interacting with other living organisms such as animals, plants, fungi, algae and other bacteria, the expression of some of their genes is regulated for adapting to such conditions. This means that some metabolites are likely to be synthesized solely in a synergistic or antagonistic relationship (13). Therefore, since microorganism metabolite production is mostly checked in the laboratory under cotrolled conditions, the metabolites of certain genes which are crucial under stress conditions have not yet been discovered. New methods such as dual cultures or multi-species cultivation have opened up a new route to these metabolites.

The current report attempts to illustrate some of these interspecies interactions among actinomycetes that produce changes in pigment production, colony morphology, sporulation, and the production of secondary metabolites. In this report, for the first time, according to a planned schedule, halophilic and halotolerant actinomycetes were isolated from the Howze Sultan Salt Lake and their interspecies relationships were investigated. It was shown here that the greatest effects of actinomycetes co-culture were growth and sporulation inhibition as well as changes in colony morphology. About half of the strains did not grow or slightly grew under the influence of the adjacent strain, indicating the prevalence of an antagonistic relationship between actinomycete species. Another report also shows that many inter-relationships lead to a lack of growth or sporulation (14).

We also show that the relationship between actionmycetes leads to the induction or inhibition of antibacterial metabolites, and the inhibition of anti-MRSA inhibition zone was more than induction of a new one.

Therefore, this experiment also confirms the prevalence of antagonistic and inhibitory relationships among actinomycetes. In this report, for the first time, anti-MRSA metabolite produced based on interspecies relationships was extracted from *Streptomyces peucetius.*

Several other reports have shown that cross-species relationships result in induction or inhibition of antibacterial metabolites (9). This kind of inter-relationships has also been reported in some fungi (15). Studies have also been conducted on the relationship between kingdoms and the discovery of new metabolites. Pettit et al. (2009) indicated that a new metabolite called levorin was produced by a bacterium in a symbiotic relationship between *Actinomyces levoris* and *Candida tropicalis* (16).

According to our investigation, it is expected that by screening actinomycetes from untouched environments and co-culture method, new metabolites can be discovered to treat antibiotic-resistant infectious diseases, which is among the current challenges in medical treatments.

Acknowledgment

The authors gratefully acknowledge the university of Kharazmi for supporting this study. We also thank Dr. Hamid Reza Jafari for helping us to collect samples and providing some research equipment.

References

1. Banerjee, T., Duhadaway, J.B., Gaspari, P., Sutanto-Ward, E., Munn, D.H., Mellor, A.L., Malachowski, W.P., Prendergast, G.C. and Muller, A.J. (2008) A key in vivo antitumor mechanism of action of natural product-based brassinins is inhibition of indoleamine 2, 3-dioxygenase. *Oncogene*, **27**, 2851.
2. Newman, D.J. and Cragg, G.M. (2007) Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod*., **70**, 461-477.
3. Bode, H.B., Bethe, B., Höfs, R. and Zeeck, A. (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. *Chem. Bio. Chem*., **3**, 619-627.
4. Udwary, D.W., Zeigler, L., Asolkar, R.N., Singan, V., Lapidus, A., Fenical, W. and Moore, B. S. (2007) Genome sequencing reveals complex secondary metabolome in the marine actinomycete Salinispora tropica. *Proc. Natl. Acad. Sci*., **104**, 10376-10381.
5. Knight, V., Sanglier, J.J., DiTullio, D., Braccili, S., Bonner, P., Waters, J. and Zhang, L. (2003) Diversifying microbial natural products for drug discovery. *Appl. Microbiol. Biotechnol*., **62**, 446-458.
6. Ueda, K., Kawai, S., Ogawa, H.O., Kiyama, A., Kubota, T., Kawanobe, H. and Beppu, T. (2000) Wide distribution of interspecific stimulatory events on antibiotic production and sporulation among *Streptomyces* species. *J. Antibiot*., **53**, 979-982.
7. Hopwood, D.A. (2007) *Streptomyces* in nature and medicine: the antibiotic makers. Oxford University Press. New York. 272 p.
8. Patil, H.J., Srivastava, A.K., Kumar, S., Chaudhari, B.L., Arora, D.K. (2010) Selective isolation, evaluation and characterization of antagonistic actinomycetes against *Rhizoctonia solani. World. J. Microb. Biot*., **26**, 2163-2170.
9. Seyedsayamdost, M.R., Traxler, M.F., Clardy, J., Kolter, R. (2012) Old meets new: using interspecies interactions to detect secondary metabolite production in actinomycetes. *Method Enzymol*., **517**, 89-109.
10. Kushner, H., and Kamekura, M. (1988) Physiology of halophilic eubacteria. In: Rodrfguez-Valera, F. (ed.), Halophilic bacteria, vol. 1. CRC Press, Bocca Raton. Pp. 109-140.
11. Saadoun, I., and Muhana, A. (2008) optimal production conditions, extraction, partial purification and characterization of inhibitory compound(s) produced by *Streptomyces* Ds-104 isolate against multi-drug resistant *Candida albicans*. *Curr. Trends Biotechnol. Pharm*., **2**, 402-420.

12. Keiser, T., Bibb, M.J., Buttner, M.J., Charter, K.F., Hopwood, D.A. (2000) Practical *streptomyces* genetics. The John Innes Foundation. Norwich.
13. Mearns-Spragg, A., Bregu, M., Boyd, K.G., Burgess, J.G. (1998) Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Lett. Appl. Microbiol*., **27**, 142-146.
14. Mukherjee, D. (2013) The colorful lives of Actinomycetes: Exploring soil and marine actinomycetes, their secondary metabolites and the influence interspecies interactions. MBL Microbial Diversity Course, University of Michigan.
15. Degenkolb, T., Heinze, S., Schlegel, B., Strobel, G., Gräfe, U. (2002) Formation of new lipoaminopeptides, acremostatins A, B, and C, by co-cultivation of *Acremonium* sp. Tbp-5 and *Mycogone rosea* DSM 12973. *Biosci. Biotech. Bioch*., **66**, 883-886.
16. Pettit, R.K. (2009) Mixed fermentation for natural product drug discovery. *Appl. Microbiol. Biot*., **83**, 19-25.

Editorial Note

Volume 7, issue 2 of Progress in Biological Sciences was initialy scheduled to be published in December 31, 2017. However, some administrative changes leaded to a major delay in processing of the manuscripts. This issue is actually published in May 1, 2020. Editor-in-chief apologizes deeply for any inconvenience caused especially to the authors.