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Modulation of Antibacterial Activity of Actinomycetes by Co-culture with Pathogenic Bacteria

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Abstract

In this study, we investigated the effect of pathogenic bacteria on the production of antibiotics by actinomycetes. We used four strains of actinomycetes-ANAM-5, ANAM-39, AIAH-10 and ANTS-1, which were isolated from the soils of Sundrabans, Bangladesh. All the strains were cultured in absence or presence of either alive or heat-killed human pathogens (*Staphylococcus aureus* and *E. coli*). The antibacterial activity of cultured cell-free supernatant fluid was analyzed by disc diffusion assay against the inducer strains. Three out of the four marine actinomycetes tested showed enhanced antibacterial activity against *Staphylococcus aureus* and *E. coli*. ANAM-5 and AIAH-10 showed enhanced antibacterial activity when co-cultured with *Staphylococcus aureus* whereas ANAM-5 and ANAM-39 showed enhanced antibacterial activity when co-cultured with *E. coli*. The highest enhancement of antibacterial activity was exhibited by the strain ANAM-5 against *Staphylococcus aureus* (from 9 mm to 18 mm inhibition zone). The study has important ecological and biotechnological implications in case of microbial competition in the natural environment and may become helpful to discover novel classes of antibiotics with high specificity and huge production.

Key words: Co-culture, actinomycetes, antibacterial activity, cross signaling.

Introduction

There is an immediate need to discover and develop new antibiotics because of the steady rise of old antibiotic resistant bacteria which is an imminent and urgent threat to individual patients and public health (Xinxuan *et al.,* 2010). For this, special attention was focused on the microbes since decades for resolving the problem of antibiotic resistance, as they are capable of producing a wide array of antimicrobial compounds (Berdy, 2005; Wise, 2008). Among such microorganisms, actinomycetes are of special interest, since they are prolific producers of antibiotics and other industrially useful secondary metabolites (Bredholt *et al.,* 2008; Takahashi and Omura, 2003). Compared with terrestrial organisms, the secondary metabolites produced by marine organisms have stronger bioactivities, and more novel & unique structures owing to the complex living circumstance and diversity of species (Carte, 1996; Schwartsmann *et al.,* 2001). Although microbial metabolites remain the most pivotal source of novel antibiotics, new approaches are required to improve the efficiency of the discovery process (Thumar *et al.,* 2010).

Microbial competition is thought to be the selective force that promotes biosynthesis of bioactive natural products. Interactions, such as symbiosis, competition and allelopathy are central feature of microbial communities (Bassler and Losick, 2006; Hibbing *et al.,* 2010). Antibiotic production of microorganisms is an adaptive defense mechanism, which is enhanced by the presence of competing organisms (Patterson and Boils, 1997). Many silent biosynthetic genes are only activated under specific conditions for the enhance production of antibiotics

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(Scherlach and Hertweck, 2009). Thus, new cultivation approach, particularly strategies similar to the natural habitats in which microorganisms defending themselves against competitive bacteria can be used to enhance the production of secondary metabolites (Kanagasabhapathy and Nagata, 2007). So, co-culture of microorganisms can be used as an alternative way to screen production antimicrobial drugs. The present study was carried out to evaluate the effect of co-culture of actinomycetes with pathogenic bacteria (*Staphylococcus aureus and E. coli*) on antibiotic production.

Materials and Methods

Isolation of actinomycetes: Soil samples were collected from the different location of mangrove forest Sundrabans, Bangladesh. The collected soil samples were dried in a hot air oven at 60-65^{o}C for about three hours to reduce the number of bacteria in the soil other than actinomycetes and soil suspensions were made using sterilized distilled water. Starch-casein-nitrate agar medium (Starch 1.0 g, Casein 0.4 g, Potassium Nitrate 0.5 g, Potassium Monohydrogen Phosphate 0.2 g, Magnesium Phosphate 0.1g, Calcium Carbonate 0.1 g and Agar 15 g per liter) was used as medium for isolation of actinomycetes. Incubation at 30^{o}C for seven days results in the formation of actinomycetes colonies that were recognized by their characteristic appearance (Sarker *et al.,* 2012; Haque *et al.,* 2012). The purified actinomycetes were preserved on yeast-extract-glucose-agar slants at 4°C for further works.

Growth and culture conditions: Yeast-extract glucose broth media (Yeast extract 0.25g/100ml, Glucose 0.5g/100ml) was used to culture actinomycetes strains (ANAM-5, ANAM-39, AIAH-10 and ANTS-1). Pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli* were cultured separately in nutrient broth medium (P^{H} 7.2) which contained (per liter) bacto peptone (Difco) 5 g, NaCl 5 g, bacto yeast extract (Difco) 1.5 g and beef extract 1.5 g. Actinomycetes strains were grown separately in a culture test tube containing 20 ml of medium. Inducer strains such as *Staphylococcus aureus* and *E. coli* were grown separately overnight and 20 µl culture of inducer strains either alive or heat-killed cells (121^{o}C, 15 min) was inoculated after 12 h of culturing actinomycetes strains (20 ml) for the enhancement of antibacterial activity. The experiment was performed by culturing the actinomycetes strains in absence or presence of alive or heat-killed *Staphylococcus aureus* or *E. coli*. All the culture tubes were then shaken on a rotary shaker (220 rpm) at 31^{o}C for 5 days. After 5 days, the culture filtrate was separated by centrifugation at 4000 rpm for 10 minutes at 10^{o}C and clear supernatant was separated.

Screening of antibacterial activity: Antibacterial activity of the extracts was performed in triplicate by the paper disc diffusion technique in agar-plated petri dishes using *Staphylococcus aureus* and *E. coli* as test organisms (Bauer *et al.,* 1966). These test organisms were available in the Pharmaceutical Microbiology Research Laboratory, Department of Pharmacy, Rajshahi University, Bangladesh. Clear supernatant (30 µl) was used to saturate a sterilized Whatman filter paper disc (6 mm), allowed to dry at room temperature and placed onto nutrient agar plates inoculated with the test organisms. All the plates were incubated at 37˚C for 24 h. The antibacterial activity was then evaluated by measuring the diameter of zone of inhibition (ZOI) around each paper disc.

Results and Discussion

In our experiment, we have investigated the effect of antibiotic production by marine actinomycetes namely ANAM-5, ANAM-39, AIAH-10 and ANTS-1 in the presence of pathogenic bacteria such as *Staphylococcus aureus* and *E. coli*. The results showed that exposure to cells of both alive and heat-killed *Staphylococcus aureus* and *E. coli* enhanced the antibacterial activity in most of the cases by marine actinomycetes. In some instances, there was no effect on antibacterial activity while in other cases it caused inhibition of activity.

Effect of alive and heat-killed cells of Staphylococcus aureus on antibacterial activity by ANAM-5, ANAM-39, AIAH-10 and ANTS-1: We have observed that the antibiotic production by ANAM-5 was triggered in the presence of alive *Staphylococcus aureus*, whereas the production was inhibited for others (Table 1).

The zone of inhibition of cell-free supernatant (30 µl) of 5 days old culture of ANAM-5 was only 9 mm as tested by disc diffusion assay against *Staphylococcus aureus*. Whereas the ZOI of cell-free supernatant (30 µl) of co-culture of ANAM-5 and alive *Staphylococcus aureus* was 18 mm against *Staphylococcus aureus*. It is clear that the

ZOI of supernatant of co-culture increased about two fold against *Staphylococcus aureus* compared to control. In contrast, the ZOI of supernatant of co-culture with heat-killed cells of *S. aureus* did not show any effect on antibacterial activity against same organism. In case of AIAH-10 we have seen an opposite findings that the production of antibiotic by this bacterium was not influenced in the presence of live cells of *Staphylococcus aureus* but induced by dead cells. The ZOI of cell free supernatant (30 µl) of 5 days old culture of AIAH-10 was 13.3 mm, whereas in presence of alive *Staphylococcus aureus* cells the zone reduced to 12 mm. However, the heat-killed cells of *Staphylococcus aureus* triggered antibacterial production by AIAH-10 and the ZOI was increased to 19.7 mm against *Staphylococcus aureus*. However, both the alive and dead cells of *Staphylococcus aureus* could not induce the antimicrobial activity by ANAM-39 and ANTS-1, rather the antibacterial production was inhibited significantly in case of ANTS-1. The ZOI of cell-free supernatant of ANTS-1 (monoculture) was 20.7 mm, which reduced to 13.3 mm and 13 mm for alive and heat-killed cells treated co-culture respectively (Table 1).

Table 1. Effect on induction of antibiotic production by actinomycetes exposed to alive and heat-killed cells of *Staphylococcus aureus* (inducer strain).

Actinomycetes strains	Zone of inhibition (in mm) against *Staphylococcus aureus*		
	Monoculture (Control)	Co-culture of actinomycetes and alive cells of *Staphylococcus aureus*	Co-culture of actinomycetes and heat-killed cells of *Staphylococcus aureus*
ANAM-5	9 ± 1	18 ± 0.0	8 ± 1
ANAM-39	9.7 ± 1.5	8.3 ± 0.6	8 ± 1
AIAH-10	13.3 ± 0.6	12 ± 1	19.7 ± 0.6
ANTS-1	20.7 ± 1.5	13.3 ± 2.08	13 ± 1

Data represent as means ± standard deviations of values from triplicate experiments.

Table 2. Effect on induction of antibiotic production by actinomycetes exposed to alive and heat-killed cells of *E. coli* (inducer strain).

Actinomycetes strains	Zone of inhibition (in mm) against *E. coli*		
	Monoculture (Control)	Co-culture of actinomycetes and alive cells of *E. coli*	Co-culture of actinomycetes and heat-killed cells of *E. coli*
ANAM-5	9.3 ± 0.6	14.3 ± 0.6	7 ± 1
ANAM-39	9.7 ± 1.5	8.3 ± 0.6	13.3 ± 0.6
AIAH-10	10.7 ± 0.6	11.3 ± 1.5	9 ± 1
ANTS-1	22 ± 1	14.7 ± 1.5	16 ± 1

Data represent as means ± standard deviations of values from triplicate experiments.

Effect of live and heat-killed cells of E. coli on antibacterial activity by ANAM-5, ANAM-39, AIAH-10 and ANTS-1: The presence of alive *E. coli* cells stimulated antimicrobial activity by ANAM-5. The zone of inhibition (ZOI) of cell-free supernatant (30 µl) of 5 days old culture of ANAM-5 was only 9.3 mm as tested by disc diffusion assay against *E. coli*. Whereas the ZOI of cell-free supernatant (30 µl) of co-culture ofANAM-5 and alive *E. coli* was 14.3 mm. It is clear that the ZOI of supernatant of co-culture increased significantly against *E. coli* compared to control.

In case of ANAM-39, we found an opposite results that antibacterial activity was enhanced due to the exposure of heat-killed *E. coli* cells, whereas activity was inhibited by alive cells. The ZOI of cell free supernatant (30 µl) of 5 days old culture of ANAM-39 was 9.7 mm, whereas in presence of alive *E. coli* cells the zone reduced to 8.3 mm. However, the heat-killed cells of *E. coli* triggered antibacterial production by ANAM-39 and the ZOI was increased to 13.3 mm against *E. coli*. In case of ANTS-1, antibacterial activity was not enhanced following exposure of alive and dead cells of *E. coli*;

rather the antibacterial production was inhibited significantly. The ZOI of cell-free supernatant of ANTS-1 (monoculture) was 22 mm, which decreased to 14.7 mm & 16 mm for alive and heat-killed cells treatment respectively (Table 2).

Cross-species induction and enhancement of antibacterial activity was first studied in *Vibrio harveyi* (Bassler *et al.,* 1997). Induction of antibacterial activity by epibiotic bacteria following exposure to terrestrial bacteria has also been reported (Means-Spragg *et al.,* 1998). These inductions of antibacterial activity might be due to the response of chemical signals, so called quorum sensing membrane permeable molecules received from the potential competitive strain, which leads to an antagonistic reaction (Burgess *et al.,* 1999). In our experiment, pathogenic bacteria both alive and heat-killed (inducer strains) were directly introduced into the culture medium of actinomycetes during growth and co-cultivated for certain periods. This mixed cultivation of marine actinomycetes with pathogenic bacteria may allow the signal transduction between them, which is the possible reason of the stimulation or inhibition of antibacterial activity of marine actinomycetes. Another reason is that competition for nutrition and space among bacteria leads to the induction of antibacterial activity of marine actinomycetes. It was also suggested that the enhancement of antimicrobial compounds production by bacteria might be due to deter or kill a potential competitors (Means-Spragg *et al.,* 1998). Induction and enhancement does not occur in all actinomycetes and differs depending on the pathogen used. We have also observed that actinomycetes strains such as ANAM-5 and AIAH-10 responded to *Staphylococcus aureus* whereas strain ANAM-39 responded favourably to cultures of *E. coli*. A reason for this phenomenon might be due to the variation of specificity of signal transduction process of the heterogeneous group of actinomycetes.

Figure 1. Plate showing enhanced antibacterial activity by the marine actinomycetes in the presence of pathogenic bacteria against target strains. *Staphylococcus aureus* and *E. coli* were used for inducing the antimicrobial activity of ANAM-5, ANAM-39 & AIAH-10. In all these plates, C = control, L = alive cells of inducer strain & K = heat-killed cells of inducer strain.

Conclusion

In our experiment, we found that both alive and dead cells of human pathogens such as *Staphylococcus aureus* and *E. coli* induced antibacterial activity of marine actinomycetes of Bangladesh. These findings have important implications for the discovery of potent antibiotics from marine bacteria and may allow as an alternative method for screening antibacterial compounds from microbes.

Declaration of Interests

The authors declare that they have no conflict of interests.

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