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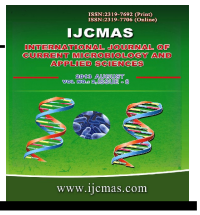

Original Research Article

Isolation of bacteria from seacoasts and screening for its antibacterial substance production

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A B S T R A C T

Ten marine bacteria were isolated and isolated for its antibacterial substance production against ten clinical isolates. The screening was carried out by different methods such as point-inoculation method, Agar-well diffusion method and screening of supernatant for antibacterial substance production. After point inoculation method and agar-well diffusion method, inhibitory activity against atleast one clinical isolate was detected for six marine isolates. The marine isolate M5 with more antibacterial activity was identified as *Lysinibacillus sphaericus* by 16S rRNA gene sequencing.

Keywords

Marine bacteria; Antibacterial substance; Pathogens.

Introduction

The oceans cover more than 70% of the earth's surface and contain about 80% of the world's plant and animal tissues. Oceans have plenty of structurally unique metabolites and other resources in the living and dead forms. About 10,000 metabolites have been isolated from different marine organisms. Among them 37% has been isolated from sponges, 21% from coelenterates, 18% from microorganisms, 9% from algae, 6% from echinoderms, 5% from tunicates, 2% from molluscs and 1% from bryozoans (Maloy Kumar *et al*., 2007).

The marine environment is a prolific resource for the isolation of less exploited microorganisms. In recent years microorganisms have become important in the study of novel microbial products exhibiting antimicrobial, antiviral, antitumor as well as anticoagulant and cardioactive properties. These active compounds may serve as model systems in the discovery of new drugs. Many organisms had developed complex adaptive and self-protecting mechanisms to survive, often associated with the production of structurally unique bioactive

compounds. Many such compounds have been extracted form various marine organisms such as bacteria, cyanobacteria, seaweeds, sponges, cnidarians, tunicates, soft corals, bryozoans, molluscs, echinoderms, fish and sea snakes. Marine bacteria produce broad-spectrum classical antibiotics and a variety of toxins such as tetradotoxins, saxitoxin, cigualoxins and brevetoxins which are useful in neuro physiological and neuro pharmacological studies. Production of antimicrobial compound seems to be a general phenomenon for most bacteria. An antimicrobial is a substance that kills or inhibits the growth of microbes such as bacteria, fungi or viruses. The discovery of antibiotics has revolutionized the world of medicine. The decreasing rate of discovery of novel drugs from established terrestrial sources has motivated the evaluation of new sources of chemically diverse objective compounds (Nathan *et al*., 2004).

Microbes are known to form a highly specific and symbiotic relationship with filter feeding organisms like sponges, algae, with their nutrient-rich and host-associated environments forming unique niche for microbial exploitation. The sponge-microbe association has attracted a number of researchers both for their diversity and secondary metabolite production which have been associated with antimicrobial, antifouling, HIV-protease inhibitory, HIV-reverse transcriptase inhibitory, immune suppressant and cytotoxic activities. Epibiotic bacteria growing on the surfaces of marine algae and other organisms live in a highly competitive environment and can produce secondary metabolites which inhibit the settlement of potential competitors such as invertebrate larvae and can antagonise other bacteria. (Mearns-Spragg *et al*., 1998). Microalgae are significant resource for bioactive metabolites, particularly cytotoxic agents with an application in cancer chemotherapy. The algal extracts with antibacterial activity can be used as antibiotics which are good for health and fails to cause side effects. (Madhan *et al*., 2005). Thus it is widely used for pharmaceutical purposes. Various strains of cyanobacteria produce intracellular and extracellular metabolites with diverse biological activities such as antifungal, antibacterial, antialgal and antiviral activity. *Streptomyces* is the largest antibiotic producing genus in the microbial world. Most *Streptomyces* and other *Actinomycetes* produce a diverse array of antibiotics including aminoglycosides, anthracyclins, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes and tetracyclins. (Nurettin *et al*., 2002). These metabolites have a wide range of applications as it possess various properties like antibacterial and antifungal activities, inhibit the growth of leukemia cell lines, and can be used to treat the immunosuppressive patients suffering from a several opportunistic pathogens. Many useful chemicals like Gliovictin have been derived from marine fungi. The oceans represent an under explored environment for microbial discovery. The marine bioactive compounds or marine natural products (MNPs) are known to be produced by many heterotrophic bacteria. These antibacterial compounds are inhibitory to terrestrial as well as indigenous bacterial strains, which is of considerable ecological significance. (Bushra Uzair *et al*., 2006).The search for new antimicrobial agent is a field of atmost importance. Marine environment provides the most effective drugs used in human therapy. The prevalence of antimicrobial resistance among key

microbial pathogen is increasing at an alarming rate worldwide. As bacteria are continuously overcoming the tools with which humans have to fight, there is a need for search for new antibiotics that affect the target. The full wealth of microbial diversity in the sea is yet to be revealed. A large fraction of marine bacteria have not been cultured yet, and novel cultivation methods need to be developed in order to culture them. Marine microorganisms have received very little attention in drug discovery mainly due to the cultural difficulty. Competition among microbes for space and nutrients in marine environment is a powerful selection pressure that endows marine microorganisms to produce marine natural products.

Today, both academic and industrial interest in marine microorganisms is on the rise. Marine microorganisms have become an important point of study in the search for novel microbial product. In the process of exploitation of marine microorganisms the following steps are to be followed: Isolation of microorganisms from marine source, screening the isolated organism for our desired characteristics, identification of organisms for biochemical or molecular methods like 16S rRNA sequencing, DNA-DNA hybridization. By considering the scope of marine bacteria and the less exploited nature of marine microorganisms, in the present study a preliminary screening was made to isolate and identify the bacteria for the production of antibacterial substances.

Materials and Methods

Sample collection

Water and wet soil samples were collected from Kollam and Kannur Seacoasts, Kerala. The four samples collected were used for the isolation of bacteria which have antibacterial activity. The samples were collected in freshly purchased polythene bags and were brought to laboratory by preventing any contamination on the way. The samples were then stored at a temperature of 4^0C until used, to minimize the metabolic activities of the microorganisms and to keep them in their exact qualitative and quantitative level of population.

Isolation of bacteria

The serial dilution spread plate method was followed to isolate bacteria from the water and soil samples. 1ml of the water sample collected was added to 9ml of sterile distilled water and was serially diluted up to 10^{-7} dilution.1g of wet soil sample was added to 100ml of sterile distilled water and serially diluted up to 10^{-7} dilution. The bacteria were isolated by spread plating 0.1ml of each of the dilution on Nutrient Agar plates. The plates were incubated at 37^0C for 24 hours to obtain colonies. The individual colonies were picked upon the basis of their macroscopic characters such as size, shape, surface appearance, texture and colour. These were purified by repeated streaking and were subcultured onto Nutrient Agar slants. The marine bacterial isolates so obtained were stored at 4^0C, for further study. The marine isolates were designated as M1, M2, M3, M4, M5, M6, M7, M8, M9, and M10.

Screening of marine isolates for their antibacterial substance production

Ten marine bacterial isolates were tested for their antibacterial activity against ten clinical isolates which included both Gram-positive and Gram-negative strains.

The clinical isolates *Bacillus subtilis, Bacillus cereus* and *Bacillus lentus* were collected from K.M.C.H College of Pharmacy, Coimbatore, *Staphylococcus aureus, Pseudomonas aeruginosa* from Kongunadu Hospital, Coimbatore, *Escherichia coli, Yersinia enterocolitica, Salmonella typhimurium, Klebsiella pneumoniae, Klebsiella aerogens* from the Department of Microbiology, Dr.N.G.P. Arts and Science College, Coimbatore.

Point inoculation method

Nutrient Agar plates were prepared. The overnight grown cultures of the clinical isolates in Nutrient broth were uniformly swabbed on the surface of the Nutrient Agar plates using sterile cotton swabs. The marine isolates from the agar slants were spotted onto nutrient agar plates seeded with actively growing cells of clinical organisms. The plates were incubated for 24 hours at 37^0C.After the incubation period the plates were observed for zone of inhibition and the results were recorded.

Agar-well diffusion method

After screening by point inoculation the antimicrobial substance production of marine isolates were tested by agar-well diffusion method. The overnight cultures of the clinical isolates in Nutrient broth were uniformly swabbed on the surface of the nutrient agar plates using sterile cotton swabs. Five wells of 6mm size were made with sterile cork borer on the seeded plates. Around 100µl of overnight grown cultures of the marine bacterial isolates in Nutrient broth, was added to each well aseptically. The plates were incubated without inverting for 24 hours at 37^0C and the zone of inhibition was noted and recorded. The marine strains showing promising activities against clinical isolates were selected for further studies.

Screening of supernatant for antibacterial substance production

Supernatant of four marine isolates having more antibacterial activity than the other isolates were tested for its antibacterial substance production. The overnight grown cultures of marine isolates grown in Nutrient broth were centrifuged at 4000 rpm for 10 minutes and supernatant was collected. The overnight cultures of the clinical isolates in Nutrient broth were uniformly swabbed on the sterile nutrient agar plates using sterile cotton swabs. Five wells of 6mm size were made with sterile cork borer on the seeded plates. Around 100µl of the supernatant collected was added to each well aseptically. The plates were incubated without inverting for 24 hours at 37^0C and the zone of inhibition was noted and recorded.

Identification of marine bacterial isolate by 16S rRNA gene sequencing

The marine isolate which showed more antibacterial activity among the organisms tested was sent to Genei, Banglore for the identification by 16S rRNA sequencing. The sequence data was aligned and analyzed for finding the closest homologs for the microbe.

Results and Discussion

Isolation of Bacteria

To isolate new type of antibacterial compounds active against resistant organisms, marine bacteria were isolated from two water samples and two soil samples of Kerala coast. The bacteria were isolated by serial dilution spread plate method. After 24 hours of incubation, isolated colonies were obtained. Ten bacteria were isolated based on their

morphological and structural characteristics (Table.1).

Screening of marine isolates for their antibacterial substance production

Screening of antimicrobial activity of ten isolates was carried out against ten clinical strains. In point inoculation method, marine isolates from agar-slants were spotted on to the Nutrient Agar. Inhibitory activity against at least one clinical isolate was detected for all the marine isolates (Table 2.)

After screening by point-inoculation method, the antibacterial substance production was tested by agar-well diffusion method. The overnight grown cultures of marine isolates in Nutrient broth were added to the wells in Nutrient Agar plates. Inhibitory activity against atleast one clinical isolate was detected for six marine isolates (Table 3). The antibacterial activity of marine isolates was compared .The majority of the strains inhibited *Pseudomonas aeruginosa.* Four marine isolates inhibited the growth of *Bacillus cereus, Klebsiella pneumoniae and Yersinia enterocolitica*. The isolate M5 presented the highest inhibition zone (45mm) against *Bacillus lentus*. *Staphylococcus aureus* and *Escherichia coli* were inhibited by two marine isolates M2 and M5 while *Salmonella typhymurium* was inhibited by the marine isolate M2 only. Those bacteria producing inhibition zone higher than 10mm against atlaest two clinical strains were selected for further characterization of their antibacterial activity. Based on this criteria, four marine bacterial isolates were selected, M2, M5, M6 and M7. To check whether the antibacterial substances produced by the marine isolates were extra cellular compounds, the supernatant of isolates grown in Nutrient broth was used. The supernatant of selected isolates M2, M5, M6 and M7 were tested for its antibacterial activity, by agar-well diffusion method. Inhibitory activity against atleast one clinical isolate was detected for the marine isolates M2 and M5 (Table 4; Figure 1).Antibacterial activity against *Bacillus lentus, Yersinia enterocolitica and Klebsiella aerogens* was detected for both M2 and M5 isolates, while no antibacterial activity against the clinical isolates was shown by the marine isolates M6 and M7. More number of clinical isolates was inhibited by the marine isolate M5.

Marine ecosystem is a rich source of various natural eco-friendly products. Marine environment provides excellent opportunity to provide compounds of rich diversity. Screening for antibacterial activity of bacteria isolated from Kollam and Kannur sea coasts were performed in this study. This study has demonstrated that the production of antibacterial substances is widespread among these bacterial strains. Of the 44 *Actinomycetes* isolated by Bhagwan *et al.*, (2007) five isolates showed antibacterial activity and were identified as *Streptomyces* species while 45.9% of the *Streptomyces* isolates from soil produced antibacterial activity (Nurettin, 2002). Bushra Uzair and Nuzhat Ahmed (2006) had isolated and characterized *Pseudomonas* strain from Sindh and Baluchistan coast.

The antimicrobial metabolite of this strain was found to have bactericidal activity against MRSA. The marine isolates obtained in the present study showed antibacterial activity against both Gram-positive and Gram-negative clinical isolates.

Table.1 Morphological characters of marine isolates

Marine Isolates	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	Optical property
M1	Circular	Entire	Raised	Small	Smooth	Shiny	Pigmented (Orange)	Opaque
M2	Irregular	Curled	Flat	Large	Rough	Dull	Non-pigmented (cream)	Opaque
M3	Circular	Entire	Flat	Pinpoint	Smooth	Shiny	Pigmented (yellow)	Opaque
M4	Circular	Entire	Raised	Small	Rough	Shiny	Non-pigmented (cream)	Opaque
M5	Circular	Curled	Raised	Moder-ate	Rough	Dull	Non-pigmented (cream)	Opaque
M6	Circular	Entire	Raised	Small	Smooth	Shiny	Non-pigmented	Transparent
M7	Irregular	Curled	Raised	Moder-ate	Smooth	Shiny	Non-pigmented (cream)	Opaque
M8	Circular	Entire	Raised	Small	Smooth	Shiny	Non-pigmented	Transparent
M9	Circular	Entire	Raised	Small	Smooth	Shiny	Non-pigmented	Transparent
M10	Circular	Entire	Flat	Pinpoint	Rough	Dull	Pigmented (yellow)	Opaque

Table.2 Screening of marine isolates for antibacterial substance production by point inoculation method

Clinical Isolates	Zone of Inhibition (mm)									
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
Bacillus subtilis	-	8	-	-	9	9	-	-	-	-
Bacillus cereus	-	6	-	7	6	9	-	10	-	-
Bacillus lentus	-	11	-	-	12	25	-	-	-	-
Yersinia enterocolitica	-	8	-	-	16	10	22	9	9	-
Pseudomonas aeruginosa	-	25	-	-	9	20	9	9	-	-
Klebsiella pnemoniae	10	8	10	-	11	11	8	-	-	-
Staphylococcus aureus	-	16	7	-	43	-	-	-	-	-
Salmonalla typhimurium	-	21	-	-	-	26	17	-	-	-
Klebsiella aeroaens	-	9	9	-	8	9	11	-	-	9
Escherichia coli	-	14	13	-	31	8	-	-	-	-

M1- M 10: Marine isolates of Bacteria

Table.3 Antibacterial substance production of marine isolates against clinical isolates by agar-well diffusion method

Clinical Isolates	Zone of Inhibition (mm)									
	M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**
Bacillus subtilis	-	11	-	-	15	13	-	-	-	-
Bacillus cereus	-	11	-	8	16	13	-	-	-	-
Bacillus lentus	-	12	-	-	45	11	-	-	-	-
Yersinia enterocolitica	-	10	-	-	31	10	9	-	-	-
Pseudomonas aeruginosa	-	19	-	-	13	16	20	8	-	-
Klebsiella pnemoniae	-	17	-	-	18	18	10	-	-	-
Staphylococcus aureus	-	13	-	-	21	-	-	-	-	-
Salmonalle typhimurium	-	25	-	-	-	-	-	-	-	-
Klebsiella aerogens	-	25	-	-	14	9	-	-	-	-
Escherichia coli	-	11	-	-	15	-	-	-	-	-

M1- M 10: Marine isolates of Bacteria

Table.4 Antibacterial activity of supernatant of selected marine isolates against clinical isolates by agar-well diffusion method

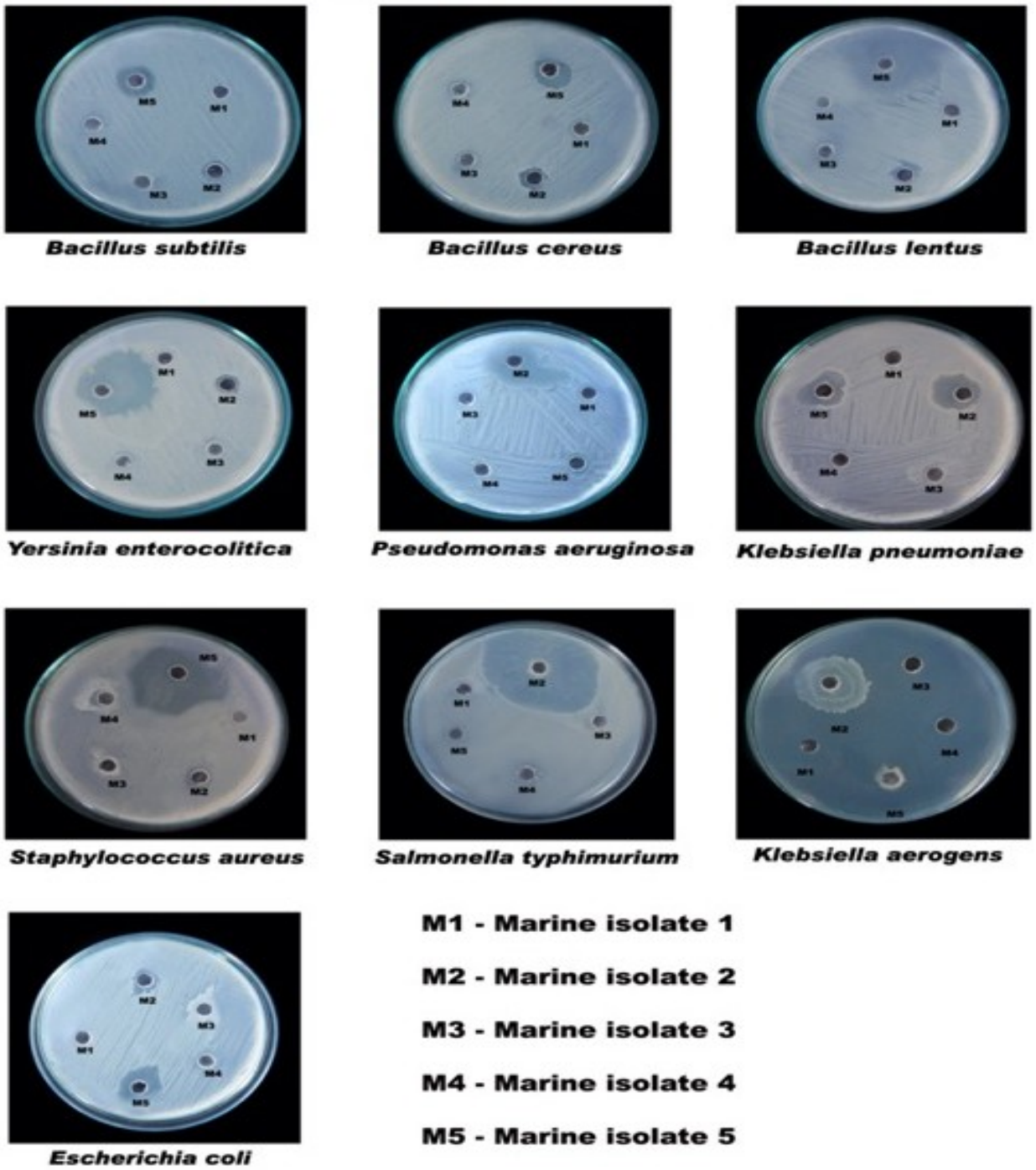
Clinical Isolates	Zone of Inhibition (mm)			
	M2	**M5**	**M6**	**M7**
Bacillus subtilis	-	10	-	-
Bacillus cereus	-	8	-	-
Bacillus lentus	8	9	-	-
Yersinia enterocolitica	-	9	-	-
Pseudomonas aeruginosa	-	10	-	-
Klebsiella pnemoniae	-	-	-	-
Staphylococcus aureus	-	12	-	-
Salmonalle typhimurium	10	-	-	-
Klebsiella aerogens	10	14	-	-
Escherichia coli	17	-	-	-

M2, M5, M6 and M7: Marine isolates of Bacteria

Marine bacteria producing a pyrrole antibiotic had been isolated and identified by Burkholde *et al.,* (1966). A new hybrid antimicrobial antibiotic, Thiomarinol, was isolated from a marine bacterium, *Alteromonas rava*. Thiomarinol showed excellent invitro antimicrobial activity against Gram-positive and Gram-negative bacteria. (Hideyuki *et al.,* 1993). Antagonistic interactions among marine pelagic bacteria were studied by Richard and Farooq (2001). Each of the 86 marine bacterial isolates were examined for their inhibition of growth of the remaining 85 isolates by agar-diffusion assay and about 53.5% of these isolates exhibited antagonistic properties against other pelagic bacteria. In the present study, remarkably large fractions, six out of ten marine isolates are showing antibacterial activity.

Among the bacteria isolated from Amazon basin 59 of 86 isolates (68.6%) displayed antimicrobial activity. Most of the strains inhibited *Bacillus cereus* and also showed activity against *Corynebacterium fimi, Escherichia coli, Staphylococcus aureus* and *Salmonella enteritidis.* (Amanda *et al.*, 2004). Around six marine isolates of the present study exhibited antibacterial activity against one or more clinical strains. The highest activity was found against *Bacillus lentus* exhibited by the isolateM5. Maximum number of isolates inhibited clinical isolates such as *Pseudomonas aeruginosa, Yersinia entercolitica* and *Bacillus cereus*.

Figure.1 Antibacterial substances production of marine bacteria against clinical isolates by agar well diffusion method



Identification of Bacteria

The isolated strain M5 showed more antibacterial activity than the other marine isolates. It was identified by 16S rRNA gene sequencing. Based on nucleotide homology and phylogenetic analysis the Microbe was detected as *Lysinibacillus sphaericus* and the nearest species was found to be *Bacillus sphaericus.*

The bacteria M5, isolated in the present study possessed more antibacterial activity and was identified as *Lysinibacillus sphaericus* by 16S rRNA gene sequencing. The information about the phylogenetic relationship of these microbe with other bacteria were found using combination of NCBI GeneBank and RDP database. The sequence description revealed that the marine isolate M5 was phylogenetically related to *Bacillus fusiformis* strain DSM 2898T, *Lysinibacillus sphaericus* strain C3-41, *Bacillus fusiformis* strain SW-B9, *Bacillus sphaericus* strain DSM 28, *Bacillus fusiformis*, *Lysinibacillus fusiformis*, *Bacillus sphaericus* strain 106 and *Bacillus macroides* strain 608. It was found to be more related to *Bacillus sphaericus* strain DSM 396.

Understanding antibiosis at the phylogenetic level may allow a more focused search for antibiotics that are active against a bacterial species or group. Such an understanding may also help to devise strategies for pathogen control in aquatic environment. Current assay for antimicrobial activity are inadequate because some antibiotic producing bacteria may require the presence of an inducer compound produced in the presence of another bacterial species. These findings have important implications for the discovery of novel antimicrobial compounds from marine bacteria and may allow the development of new methods for screening novel compounds active against multi- drug-resistant bacteria.

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