Antibacterial activity of marine bacteria from Arabian Sea of Pakistan

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Citation

N Ahmed, B Uzair, S Ayaz, V Ahmed. *Antibacterial activity of marine bacteria from Arabian Sea of Pakistan*. The Internet Journal of Microbiology. 2007 Volume 4 Number 2.

Abstract

Objective: In this study the potency of free-living and animal and plant associated marine bacteria to produce antimicrobial substances has been studied in 150 strains isolated from different samples of Arabian Sea of Pakistan coast.

Method: Antibacterial activity of isolated bacterial strains was checked by cross streak and agar well diffusion method. Crude extract were prepared from antibacterial metabolite producing strains using organic solvents.

Results: A total of six strains showed antibacterial activity against Aeromonas punctata, Kokuria marina, Rothia marina, Vibrio cholerae, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Staphylococcus aureus, methicillin resistant Staphylococcus aureus and Proteus vulgaris. Production of antibacterial metabolites varied within 24 to 72 h, in marine broth 2216. A total of 6 crude ethyl acetate extracts of antibacterial substance were prepared from the antibacterial metabolite producing strains grown on marine agar 2216 and screened for their antibacterial activity against test bacterial strains. Out of 6 extracts 3 extracts showed activity against marine and clinical isolates. Antibacterial metabolite producing strains were identified by 16S rRNA gene sequence analysis. Active bacterial strains belonged mainly to genera of Pseudomonas, Bacillus and Bravebacterium.

Conclusion: The recovery of strains with antimicrobial activity suggests that marine environment may represent an ecological niche which harbors a largely uncharacterized microbial diversity and a yet unexploited potential in the search for new secondary metabolites.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years. An impressive number of modern drugs have been isolated from microorganisms, mainly based on their use in traditional medicine. In the past century, however, an increasing role has been played by microorganisms in the production of antibiotics and other drugs (Fenical, 1993).

The importance of terrestrial bacteria and fungi as sources of valuable bioactive metabolites is very well established for more than half a century. As a result, over 120 of the most important medicines (penicillins, cyclosporin A, adriamycine, etc.) in use today are obtained from terrestrial microorganisms (Alanis, 2005).

For more than two decades, there has been an ongoing quest to discover new drugs from the sea (Anand et al., 2006). Most efforts have been directed towards chemical studies of marine invertebrates (Chin et al., 2006). Although these studies have indeed proven that marine invertebrates are an important source of new biomedical leads, a fact well demonstrated by the number of compounds currently in clinical trials, it has proven notoriously difficult to obtain adequate, reliable supplies of these compounds from nature. Because of these problems, a new avenue of study focusing on marine microorganisms has been gaining considerable attention (Faulkner, 2002). At first sight thus, the expectable enormous biodiversity of marine microorganisms might have been the reason for the interest in their study (McCarthy et al., 2004). Although marine microorganisms are not well defined taxonomically, preliminary studies indicate that the wealth of microbial diversity in the world's oceans, make this a promising frontier for the discovery of new medicines (Blunt et al., 2004).

Marine bacteria are most generally defined by their

requirements of seawater, or more specifically sodium for growth. In the case of marine fungi, which in general do not display specific ion requirements, obligate marine species are generally considered to be those that grow and sporulate exclusively in a marine habitat. Although such definitions can prove useful, they tend to select for a subset of the microorganisms that can be isolated from any one environment. This problem is compounded in the case of near - shore or estuarine samples where a large percentage of the resident microbes are adapted to varying degrees of marine exposure. For the purpose of microbial drug discovery, it seems only logical to study all microbes that can be isolated from the marine environment. Based on the species studied, most of the new compounds reported from marine microorganisms were obtained from species that can, in principle, be isolated from both land and sea. Although these facultative marine species are clearly a good source of novel metabolites, their ecological roles and degrees of adaptation to the marine environment is largely unknown (Bush, 2004).

Screening of marine bacteria isolated from the surface of marine algae and invertebrates has shown that a high percentage produce antimicrobial metabolites (Bergess et al., 1999). The first antibiotic from marine bacterium was identified and characterized in 1966 (Burkholder et al., 1966). In addition, bacteria in biofilms formed on the surface of marine organisms have been documented to contain a high proportion of antibiotic producing bacteria than some other marine environment (Lemos et al., 1985; Anand et al., 2006). Marine epiphytic bacteria, associated with nutrient rich algal surfaces and invertebrates, have also been shown to produce antibacterial secondary metabolites, which inhibit the settlement of potential competitors (Bernan et al., 1997).

A number of surface associated marine bacteria have also been found to produce antibiotics (Holmstrom and Kjelleberg 1999; Hans et al., 2004). A Bacillus sp isolated from a marine worm in Papua New Guinea produced a novel cyclic decapeptide antibiotic, loloatin B, which inhibit growth of MRSA (methicillin resistant Staphylococcus aureus) and VRE (Vancomycin resistant Enterococcus) (Gerard et al., 1999). The marine bacterium Alteromonas rava was found to produce a new antibiotic thiomarinol (Shiozawa et al., 1993). Antibiotics from marine microorganisms have been reported, including loloatins from Bacillus. Agrochelin and sesbanimides from Agrobacterium (Acebal et al., 1999), pelagiomicins from Pelagiobacter variabilis (Imamura 1997), pyrones from Pseudomonas (Singh et al., 2003).

The present study was undertaken to screen potential antibacterial metabolite producing strains isolated from marine samples of Arabian Sea of Pakistan.

MATERIALS AND METHODS

BACTERIAL CULTURES

The strains used in this work were isolated from marine environments of Arabian Sea of Pakistan. Indicator microorganisms for the characterization of antimicrobial activity are listed in Table 1. Bacteria were maintained as stock cultures at -20ºC in marine broth (Difco, Detroit, USA) supplemented with 20 % glycerol. Strains were propagated twice before use in experiments.

ISOLATION OF BACTERIAL STRAINS FROM MARINE SAMPLES

Different samples of fish crustaceans, sponges, seaweeds, water, sediment, stones were collected from Arabian Sea of Pakistan and analyzed for the isolation of morphologically different bacterial strains in marine broth 2216. Selected bacterial strains were identified by 16S rRNA gene sequence analysis.

SCREENING OF ANTIBIOTIC ACTIVITY BY CROSS STREAK METHOD

Isolated bacterial strains were analyzed for their inhibitory activity. A fresh colony of potential antibacterial metabolite producing strain was picked with the help of sterile sharp ended tooth pick and inoculated as a streak across the surface of Brain Heart Infusion agar. Inoculated Heart Infusion agar plates were incubated at 30°C for 12-48 h. Keeping the plate inverted a 9-cm2 Whatman's filter (3M) paper was placed onto the lid and it was impregnated with 0.3ml of chloroform, plate was kept closed for 30 min and filter paper was removed. Suspected sensitive strains were cross-streaked to the producing strain using a blunt ended toothpick. Plates were incubated at 30°C for12 to 48 h and observed for zone of inhibition at each side of the plate.

SCREENING OF ANTIBIOTIC ACTIVITY BY AGAR WELL DIFFUSION METHOD

Antibacterial activity was assayed in duplicate by agar well diffusion method using methicillin resistant S. aureus, S. saprophyticus, E. coli, P. vulgaris, Rothia marina, Bacillus leichniformis, and Aeromonas punctata, as test organisms. Wells of 5 mm diameter were punched in Brain Heart Infusion agar plates seeded with test organisms. Overnight

culture (20µl) of indicator bacterial strains was added in the wells. Plates were then incubated overnight at 37°C for 24 hours. Where upon inhibitory activity was detected as a zone of clearing in the turbid agar around the wells containing antibacterial activity (positive samples). The diameter of the clearing zones was measured in (mm) to obtain a semi quantitative determination of the concentration of the antibacterial compound.

HEMOLYTIC ACTIVITY

Hemolytic activity was also determined by agar diffusion technique (Monteiro, 2002). One micro liter of antibacterial metabolite producing strain suspensions were placed on the surface of plates containing blood agar medium and incubated at 30ºC and 37ºC. After 72 h of incubation, the diameters of hemolysis zones were measured in (mm), and the results were expressed as hemolytic activity (mm).

DETERMINATION OF SIDEROPHORES

To determine whether siderophores are responsible for the antagonistic properties of antibacterial metabolite producing strains, the strains were grown in nutrient agar supplemented with 1% ferric chloride and tested for antagonistic activity by agar well diffusion method.

EFFECT OF TIME ON THE PRODUCTION OF ANTIBACTERIAL METABOLITE

Antibacterial metabolite producing strains were grown in Brain Heart Infusion Broth and incubated at 30°C for 72 hrs. Samples were pulled out after every 6 hr and were analyzed for antibacterial activity by agar well diffusion method.

PREPARATION OF CRUDE EXTRACT

Seed cultures of antibacterial metabolite producing strains were prepared by growing antibacterial metabolite producing strains in Brain Heart Infusion broth. Seed cultures of antibacterial metabolite producing strains were inoculated on Brain Heart Infusion agar. The inoculated plates were incubated at 30ºC for 5 days. After incubation the agar was removed from the plates and cut into1-cm square agar pieces and extracted with 80%. The extracts were filtered through cheese cloth to remove pieces of agar while other particulate matter was removed by centrifugation (9,000xg for 15 min at 4°C). NaCl (5 g) was dissolved in 100ml of the aqueous concentrate, which then was extracted three times with ethyl acetate. The combine ethyl acetate extract was evaporated under vacuum to yield the crude ethyl acetate extract.

RESULTS AND DISCUSSION

The search for novel antibiotics has gained urgency because of increased occurrence of multi-drug resistant human pathogens. Many clinically relevant microbes have developed resistance resulting not only from the exposure to sub lethal concentrations of antibiotics in hospital environment but also in animal farms where antibiotics are used as growth enhancers (Witte, 1999).

Marine bacteria have been recognized as an important and untapped resource for novel bioactive compounds. The chemical compounds of marine microorganisms are less well known than those of their terrestrial counterparts However, in the last decade several bioactive compounds have been isolated from marine bacteria and are new resources for the development of medically useful compounds (Donia and Haman 2003; Anand et al., 2006). Antibacterial activity among marine bacteria is a well-known phenomenon and has been demonstrated in a number of studies (Isnansetyo et al., 2003; Uzair et al., 2006c).

The aim of the present study was to isolate marine bacteria from Arabian Sea of Pakistan coast producing antibacterial compounds. Different samples were collected from Arabian Sea of Pakistan coast such as crustaceans (crabs, shrimps, bivalve), different kinds of fish, water samples (surface and deep sea), sponges, sea weeds, stones, rocks and plants. Bacterial strains were isolated from these samples using marine medium 2216 using aged sea water. A total of 150 different bacterial strains were isolated, purified and preserved. All of the isolated bacterial strains were tested for their ability to produce antibacterial activity by cross streak and agar well diffusion method. Out of the 150 bacterial strains six bacterial strains showed antibacterial activity against clinical and marine bacteria (Figure 1 & 2). These antibacterial metabolite producing strains were identified by 16S rRNA gene sequence analysis as Bacillus subtilis, Bacillus lecheniformis, Pseudomonas aeruginosa, Pseudomonas putida, and Brevibacterium frigoritolerans. The antibacterial spectrum of all the strains showing inhibitory activity is presented in (Table 1). Among the antibacterial metabolite producing strains Pseudomonas aeruginosa showed activity against both Gram-negative and Gram-positive bacteria. Antibacterial activity of marine Pseudomonas sp has been reported earlier (Uzair et al., 2006c). Pseudomonas aeruginosa showed good activity against Gram-positive bacteria including methicillin resistant Staphylococci. Whereas B. leichniformis only inhibited growth of Gram -positive bacteria (Table1)

In this study all of the six antibacterial metabolite-producing strains were isolated from attached sources. High recovery of strains with antimicrobial activity suggested that marine attached sources are a rich source of novel microorganisms with potential pharmacological relevant bioactivity. Lemos et al., (1985) demonstrated that the production of compounds by microbes that are found on the surfaces of organisms would be extremely advantageous as many of these microbes are less difficult to culture.

Screening procedures gave some indication about the nature of compound involved in antibacterial activity of all the six strains, which gave positive results. In several tests with culture filtrates of all the six strains, the antibacterial substance involved in the inhibition was detected in cell free culture filtrate of Pseudomonas aeruginosa, Pseudomonas putida and Bacillus subtilis but culture filtrate of Bacillus lechinofrmis showed weak antibacterial activity whereas culture filtrates of Brevebacterium friotolerense and uncultured bacterium did not show any activity. In recent years, fluorescent Pseudomonads have drawn attention worldwide because of the production of secondary metabolites such as siderophores, antibiotics, volatile compounds HCN, enzymes and phytohormones (Isnansetyo and Kamei, 2003).

During this study the best antibacterial metabolite producing strains Pseudomonas aeruginosa and Pseudomonas putida showed varied spectra of activity, inhibiting indigenous marine isolates (V. algiolyticus, Sh. putrifacians, E. coli, B. subtilis, S. aureus, S. epidermidis) and antibiotic resistant clinical isolates (MRSA, S. aureus, S. epidermidis, E. coli).

Addition of $FeCl_3$ to the growth medium showed no influence on the antibacterial activity of all the antibacterial metabolite producing bacterial strains. The strain inhibited the growth of bacteria in ferric ion rich medium, indicating siderophore might not be involved in the antagonistic response.

The effect of time on the production of antibacterial compound was studied by growing all the antibacterial metabolite producing strains on marine broth 2216 and it was observed that degree of production of antibacterial compound increased with increasing culture age, maximum zone size was observed after 72 hrs of growth (Figure 3). The different thermal treatments conducted with supernatants of Pseudomonas putida, P aeruginosa and B. subtilis demonstrated the thermo resistance property of the inhibitory metabolite. Growth inhibition zones produced were the same for all supernatants independent of the thermal treatments to which they were subjected. These results are in agreement with the reported thermo resistance activity of antibiotic produced by Pseudomonas cepacia (Jayaswal, 1990).

Antibiotic substances from antibacterial metabolite producing strains were extracted using organic solvents. Crude extract was prepared from a 5 day old culture of antibacterial metabolite producing strains grown in brain heart infusion agar by extracting the antibacterial substances from growth medium with 80% acetone and ethyl acetate. Crude extract of P. aeruginosa, P. putida and B. subtilis tested positive in bioassay against the test strains. While crude extract of Brevibacterium frigotolernse, uncultured bacterium and B. leciniformis did not show any activity. The antibacterial compound was only detected at high cell densities, and this indicated that the production was probably controlled in a quorum-dependent manner. Indeed, quorum sensing has been shown to control the production of antibacterial compounds in several bacteria.

Figure 1

Figure 1: Antibacterial activity of marine bacteria against

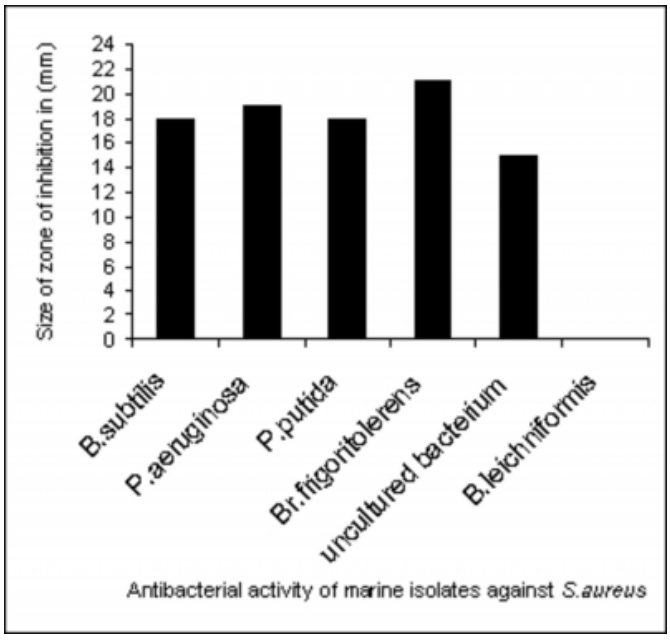

Figure 2

Figure 2: Bioassay plate showing antibacterial activity of marine bacteria by agar well diffusion method A against B against

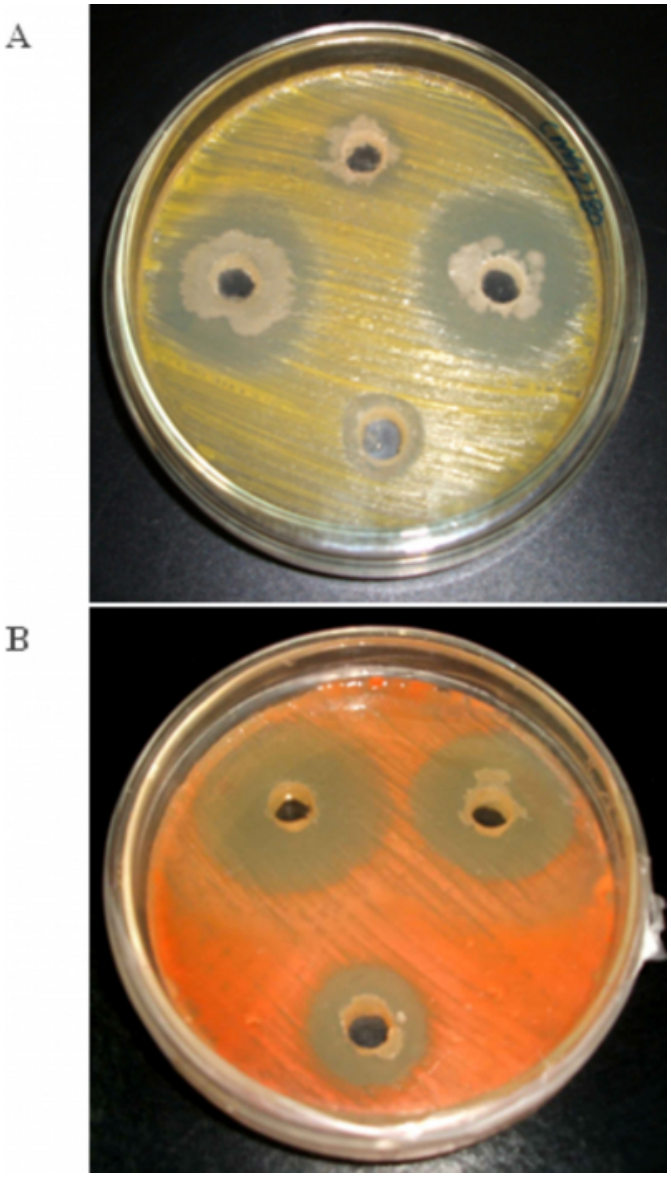

Figure 3

Figure 3: Effect of time on the production of antibacterial metabolite by marine bacterial strains

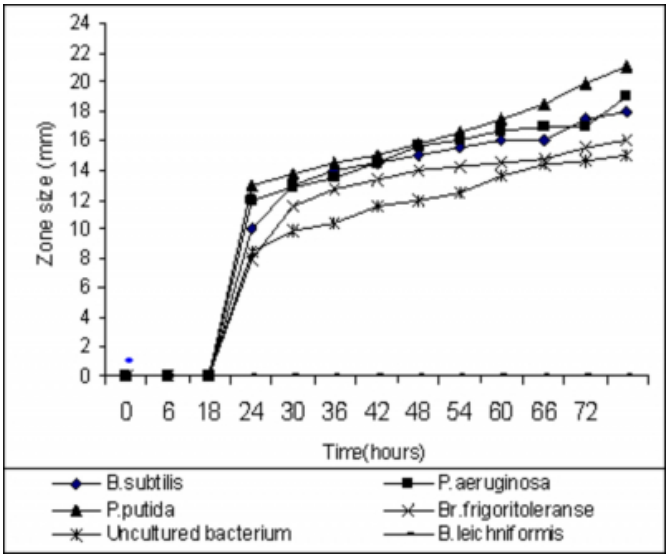

Figure 4

Table 1: Antimicrobial activity of marine isolates of Arabian sea of Pakistan coast against clinical and marine isolates following agar well diffusion method

Producer strains	Indicator strains(zones of inhibition in mm)									
	M1	M2	M3	M4	M5	C1	C2	C3	C4	C5
B. leichniformis	-	17	14	-	-	-	-	14	-	-
B. subtilis	15	-	18	21	16	15	-	18	-	-
P. aeruginosa	17	15	18	17	17	18	16	17	16	18
P. putida	15	-	15	14	18	16	14	15	16	17
Br. frigoritoleranse	-	-	20	19	17	-	-	-	-	-
Uncultured bacterium	-	17	15	14	17	-	20	21	-	-

Key; - no activity; M1 (*Aermonas punctata*); M2 (*Kokoris marina*); M3 (*Rothia sp*) M4(*Vibrio. sp*);M5(*S.aureus*);C1(*S.epidermidis*);C2(*E.coli*);C3(*S.aureus*); C4(methicillin resistant *S.aureus*);C5(*Poteus vulgaris*)

Figure 5

Table 2: Hemolytic activity of antibacterial metabolite producing isolates at different temperatures after 72 hours of incubation. Hemolytic activity (mm) is expressed as diameter of hemolysis zone

Antibacterial metabolite producing strains	Temperature 30°C	Temperature 37°C
B. leichniformis	13	10
B. subtilis	9	9
P. aeruginosa	-	-
P. putida	-	-
Br. frigoritoleranse	9	11
Uncultured Bacterium	-	-

Key; -ve no activity

CONCLUSION

In conclusion the results obtained during this study indicated that the antagonistic activity was due to the production of antibacterial compound in case of Pseudomonas aeruginosa, Pseudomonas putida and B. subtilis and the compound could be extracted from growth medium by extraction with organic solvents whereas crude extract of antibacterial activity producing strain Bacillus lecheniformis, Brevibacterium frigoritolerans did not show any activity. This investigation reveals the importance of marine bacteria in the evaluation of antibacterial activity.

ACKNOWLEDGMENT

We thank Higher Education Commission of Pakistan for generous support of this research by providing research grant under National Research programme for Universities

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