

Isolation and Characterization of Pseudomonas Strain That Inhibit Growth of Indigenous and Clinical Isolate

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Citation

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Abstract

The use of microorganisms for biological purpose has become an effective alternative to control pathogens. Bacteria from marine environment are an underutilized source of novel antibiotics. A total of 100 bacteria were isolated from marine samples (Fish, Water and Crustaceans) of Baluchistan coast and screened for antagonistic interaction. Out of these 15 strains showed antibacterial activity. One strain designated as CMG1030 identified as *Pseudomonas aeruginosa* gave excellent antibacterial activity, capable of inhibiting growth of clinical isolates such as methicillin resistant *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Shigella flexnerie*, *Staphylococcus epidermidis*, as well as indigenous marine bacteria such as *Staphylococcus aureus*, *Shewanella putrefaciens*, *Vibrio alginolyticus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus subtilis*. Inhibition did not involve living cells, which has suggested that at least a substantial portion of the anti microbial activity was due to the excretion of extra cellular compound but did not give activity against *Streptococcus* (group G), *Candida albicans*, *Aspergillus niger*. Thin layer chromatography of crude extract of cells of *Pseudomonas aeruginosa* revealed one UV active fraction with biological activity. Microscopic examination of exponentially growing cells of *S.aureus* treated with crude extract of *P.aeruginosa* suggests cell lysis and some ghost cells and big cells were also observed.

INTRODUCTION

During the past two decades research on marine bacteria has highlighted the tremendous potential of these microorganisms as a source of new bioactive secondary metabolites (Jenson and Fenical, 2000 ; Ahmed et al., 2000) and there is a growing awareness of the need for development of new antimicrobial agents for the treatment of human animal and plant diseases. Marine bacteria could represent a new source of antibiotics, which are currently needed to combat emergent antibiotic resistant pathogen. Many marine heterotrophic bacteria are known to produce antibacterial substances, which inhibit or kill other bacteria. These antibacterial compounds are not only inhibitory to terrestrial bacteria but also to indigenous bacterial strains, which is of considerable ecological significance (Saz et al., 1963). It is clear that new classes of antibiotics are urgently needed. Many marine free living and sediment inhabiting marine bacteria have been shown to produce secondary metabolites that display antibacterial properties (Burgess et al., 1991 and Long and Azam, 2001) have also reported that particle attached marine bacteria are more likely to produce inhibitory compounds than free living counterparts. It has been reported that antibiotic producing marine *Pseudomonas* that produce antibiotic metabolite which are inhibitory for

both gram negative and gram positive bacteria. (Nair and Simidu, 1987)

Antibiotics have been defined as substances produced by microorganisms that in high dilution are antagonistic to the growth or life of other microorganisms (O'Grady et al., 1997). Antibiotics may be intermediates or the end product of metabolism or may be waste products or other compounds, which have antibiotic properties. There is no doubt that the discovery of antibiotics has revolutionized the world of medicine, however sixty years later humans are far from the winning battle against infectious diseases because the pathogenic strains have emerged that are virtually unresponsive to antibiotics, such multi drug resistance, arising mainly through antibiotic misuse.

The more the bacteria come in contact with antibiotics, the greater are the chances of becoming resistant. Bacteria have proved to be amazing when it comes to circumventing the action of microbial agents particular strains which are causing problems at the oment are the Vancomycin-resistant Enterococci (VRE) (Frieden et al 1993; Leclerc, 1997) and methicillin resistant *Staphylococcus aureus* (MRSA). Several strains of human pathogens have been found to

contain up to ten different genes coding for resistance (Henry, 2000). 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.

Previous studies have shown that a high percentage of marine bacteria produce antimicrobial metabolites compared with the number of planktonic isolates that produce such metabolites, however it is also observed in laboratory most isolates stop producing microbial compounds when they are continuously cultivated in shake flasks. This paper describes isolation and characterization of CMG1030 *Pseudomonas aeruginosa* producing compounds that inhibit the growth of multi resistant clinical isolates of Staph, Salmonella, Shigella etc. The antimicrobial activity has been studied using various parameters.

MATERIALS AND METHODS

BACTERIAL STRAINS USED

CMG1030 was used as producer strain and (MRSA) *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Shigella flexneri*, *Candida krusei*, *Aspergillus niger*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Vibrio alginolyticus*, *Micrococcus luteus*, *Sarcina*, *Bacillus subtilis*, *Shewanella putrefaciens*, *Proteus mirabilis* were used as test strains. All of the bacteria strains except *Vibrio alginolyticus*, *Vibrio fluvialis* were preserved stocked in trypticase soy broth (TSB medium) (Oxoid) containing 20% (vol/vol) glycerol at -70°C. *Vibrio alginolyticus*, *Vibrio fluvialis* were stocked in nutrient broth supplemented with 1.5% NaCl. The bacteria were slanted on the respective medium before using in the experiments. For MRSA in particular, the trypticase soy agar medium was supplemented with 10 µg of Oxacillin (Sigma)

SCREENING OF ISOLATES FOR INHIBITORY ACTIVITY

A total of 100 strains isolated from samples of Sindh and Baluchistan Coast were screened for production of antibacterial substance by Burkholder agar diffusion assay of Burkholder. A lawn of sensitive strain was prepared by mixing 25 ml of molten 44°C nutrient agar with 30 µL of a suspension of overnight culture of sensitive strains and it was vortexed and poured on nutrient agar plate, 10 µL of potential producer were spotted on the lawn. The plates were incubated face up for 24 at 30°C and examined for zones of inhibition (area where target isolate failed to grow). The strain (CMG1030) showing promising activities against

clinical isolates were selected for further studies.

EFFECT OF TIME ON THE PRODUCTION OF ANTIBACTERIAL METABOLITE

CMG1030 was grown in King B medium and incubated at 30°C for 72 hrs and samples were pulled out after every 6 hrs. All the samples were centrifuged and filtered using millipore filters (GyroDisc CA 0.2 µm) and activity of the filtrates were checked by agar well diffusion method.

THERMOSTABILITY OF THE ANTIBACTERIAL ACTIVITY

1.5 ml of filtrate of CMG1030 was dispensed in sterile eppendorf tubes. The tubes were incubated as follows.

- a) Room temperature for 30 minutes
- b) At 80°C for 30 minutes
- c) Autoclaved at 120°C for 20 minutes
- d) Freezer -20°C for 24 h

From each set of tubes, fractions of 100 µL were tested against MRSA for inhibitory activity.

EFFECT OF ENZYMES ON ANTIBACTERIAL METABOLITE

To find out chemical nature of the antibacterial metabolite. Filtrate of CMG1030 was treated with different enzymes (Pepsin 10 µg/mL, Protease P 10 µg/mL, Protease K 1 µg/mL, RNase 10 µg/mL) and treated samples were tested by agar well diffusion method.

PLATE ASSAY TO ESTABLISH THE BACTERIOSTATIC OR BACTERICIDAL ACTIVITY OF THE ANTIBACTERIAL METABOLITE

From inhibitory assay conducted samples with sterile cotton tips were taken from the surface of the plate where no growth of tested strain was observed the tips were surface streaked to nutrient agar plates and incubated at 30°C for 48 hrs, the plates were observed for growth or no growth.

DETERMINATION OF SIDEROPHORE

To determine whether siderophores are responsible for the antagonistic properties of CMG1030. CMG1030 was grown in nutrient agar plate supplemented with 1% ferric chloride and tested for antagonistic activity by agar well diffusion method.

ISOLATION OF ANTIBACTERIAL COMPOUND

An antibacterial compound was isolated from CMG1030

grown on king B agar plates incubated at 30°C for 5 days. Plates after incubation was cut into 1-cm square and the first extraction was carried out with 3 volume of 80% acetone with water for 2 hrs by using a rotary shaker the combined extracts were filtered through cheesecloth to remove pieces of agar other particulate matters were removed by centrifugation (9000xg for 20 minutes at 4°C the supernatant fraction was evaporated at room temperature to ensure complete solvent removal. Portioning with ethylacetate carried out a second extraction. The extracts were combined evaporated by dryness. The resulting residue was dissolved in acetone and stored at -20°C until further purified.

ANALYTICAL CHROMATOGRAPHY

Thin layer chromatography was carried out with the crude extract on silica gel (TLC Aluminum sheets silica gel 60 F254) with ethylacetate and hexane as a mobile phase (1:1). The crude extract 30µL was spotted on TLC plate and the solvent front was allowed to run for approximately 18 cm. The relative mobility of each spot was determined considering the retarding factor of each solute (RF) using an ultraviolet-light (312 - 365nm) transilluminater.

BIOASSAY OF CRUDE EXTRACTS AND ITS FRACTIONS

Crude extract and TLC elutes were tested for antibacterial activity. Test samples (100µL) were pipetted into 5mm diameter well bored into nutrient agar plates swabbed with test strain. Control plates consisted of crude extracts from un inoculated king B medium. The presence of inhibition zones around the well was recorded after incubation for 24 hrs at 30°C. Fractions from TLC plates were cut into portions. These portions were scrapped into eppendorf tubes and extracted with 100% acetone. The silica residues were removed by centrifugation and supernatant was transferred into second set of eppendorf tubes. Each fraction was concentrated by evaporating off acetone and tested for antibacterial activity by using disc diffusion method.

Incubated for 15 min at 30°C.

DETERMINATION OF MINIMAL INHIBITORY CONCENTRATION

The minimum inhibitory concentration (MICs) of crude extract was determined by the standard microdilution method described in the national committee for clinical laboratory standards using Muller Hinton broth medium (Oxoid) incubated at 37°C for 24 h.

PHASE CONTRAST MICROSCOPY

An exponential phase culture of S.aureus (A600, 0.05) was grown in Luria broth and treated with 10µg/ml of crude extract and incubated for 15 min at 30°C and slides were made in 1% agarose and observed under phase contrast microscope, particular care was taken to minimize the sample exposure to UV light, image grabbing were performed essentially as described by Edwards and Errington. (1997)

RESULTS AND DISCUSSION

Marine bacteria have been recognized as an important and untapped resource for novel bioactive compounds. A greater development of marine biotechnology will produce novel compounds that may contribute significantly towards drug development over the next decade (Kasanah & Hamann, 2004). Multidrug resistant strains such as methicillin resistant staphylococcus aureus (MRSA), Vancomycin resistant Enterococcus cause serious public health problem throughout the world. To isolate new type of antimicrobial compounds active against resistant organism's marine bacteria were isolated from Baluchistan coast (Crustaceans, Fish, and Water). 100 bacteria were isolated and tested for the production of antibacterial metabolite. A remarkable fraction 15% of the strains exhibited antagonistic properties against antibiotic resistant clinical isolates and against indigenous marine bacteria. CMG1030 identified as Pseudomonas aeruginosa showed good antimicrobial activity and was selected for further investigation. The production of inhibitory antibiotic compound by fluorescent Pseudomonas is well documented (Fravel, 1988 and Fenton, et al., 1992). CMG1030 showed excellent antibacterial activity. This strain is aerobic and failed to grow under anaerobic and acetic conditions it did not require organic growth factors, grow on acetate as the sole carbon source, was oxidase, catalase and citrate positive but indol negative and found to be resistant to several antibiotics (ampicillin, kanamycin, erythromycin, and tetracycline) and sensitive to chloramphenicol. A bioassay based on clearing zone on agar was used to determine the antibiotic activity of the metabolite produced by CMG1030 against clinical isolates and indigenous bacteria (Table 2). The antimicrobial metabolite has bactericidal activity against MRSA These results are similar to Isnansetyo et al., 2001 reported that Pseudomonas.sp isolated from marine red -alga produce anti MRSA compound having bactericidal nature.

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Figure 1

Table 1: Morphological and Biochemical Characters of CMG1030

Characteristics	Results
Gram Reaction	Negative
Cell Type	Rods (many in pairs)
Motility	Motile
Pigmentation	Green pigment
Glucose fermentation	Positive
Lactose fermentation	Negative
Sucrose fermentation	Negative
Indol	Negative
Voges-Prosekauer	Negative
Citrate Utilization	Positive
Nitrate Reduction	Negative
Urease	Negative
Catalase	Positive
Oxidase	Positive
NaCl tolerance	3%

Figure 2

Table 2: Zones of inhibition produced by whole cell, culture filtrate and crude extract of CMG1030 against clinical isolates (By agar well diffusion method)

Strains tested	Whole cell	Culture filtrate	Crude extract
MSSA	20	18	30
MRSA	21	17	32
<i>S. epidermidis</i>	18	18	33
<i>Enterococcus faecalis</i>	19	16	25
<i>Enterococcus faecium</i>	22	16	27
<i>Streptococcus group G</i>	-	-	-
<i>Cl. perfringens</i>	10	8	12
<i>Cl. sporogenes</i>	12	10	13
<i>E. coli</i>	18	16	22
<i>V. cholerae</i>	14	11	15
<i>S. typhi</i>	18	16	21
<i>Proteus morgani</i>	16	14	20
<i>Proteus mirabilis</i>	19	17	23
<i>Hafnia alvei</i>	18	15	21
<i>Klebsiella aerogens</i>	18	15	22
<i>Candida albicans</i>	-	-	-
<i>Aspergillus niger</i>	-	-	-

CMG 1030 showed varied spectra of activity inhibiting indigenous marine isolates (*Vibrio algiolyticus*, *Shewanella putrifaciens*, *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staph. epidermidis*) and antibiotic resistant clinical isolates (MRSA, *Staph. aureus*, *Staph. epidermidis*, *E. coli*). This may be due to the fact that same strain might produce different antibacterial compounds, depending on culture medium. To determine whether siderophore or antibiotics are responsible for the antagonistic properties of strain CMG1030 we tested for antibacterial activity in presence of $FeCl_3$ because this

Pseudomonas strain inhibited the growth of test strains in ferric ion rich medium, siderophore may not be involved in the antagonistic response, effect of time on production of antibacterial compound was also studied by growing it on King B medium and it was observed that degree of production of antibacterial compound increased with increasing culture age maximum zone size observed after 72 hrs of growth (Fig2).

Figure 3

Figure 1: Phase contrast images of live cells of treated with antibacterial compound, A control cells without treatment, B cells treated with Crude extract of *Pseudomonas aeruginosa*

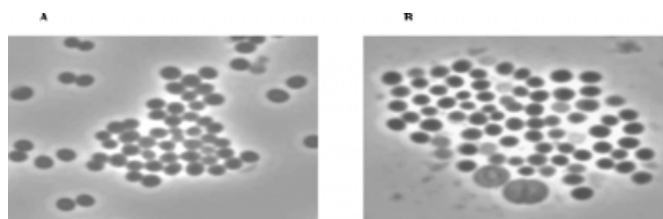
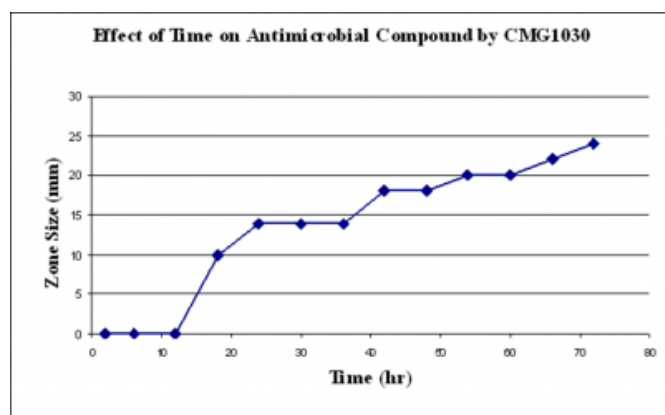


Figure 4

Figure 2: Effect of time on production of antibacterial metabolite by CMG1030



The different thermal treatments conducted with supernatants demonstrate the thermo resistance of the inhibitory metabolite. Growth inhibition zones produced were the same for all supernatants independent of the thermal treatments (Table 3) to which they were subjected these results are in agreement with the reported thermo resistant of antibiotic produced by *Pseudomonas*. Cepacia (Jayaswal 1990) and to check the effect of enzymes the supernatant was treated with different enzymes (Pepsin, Protease P, Protease K, RNase) enzymes treated supernatant was found to be resistant to enzymes indicating antibiotic metabolite is not protein in nature.

Figure 5

Table 3: Effect of different thermal treatments on the antibiotic Activity of supernatant of CMG1030

Treatments (mm)	Diameter of inhibition zone
Room temperature (30± 2°C)	22
Water bath (80±2°C)	22
Autoclaving 121±1°C)	21

Next we investigated the possibility of the production of antibiotic substances by this strain crude extract from a 5 day old culture of CMG1030 grow on King B medium were prepared by portioning with 80% acetone followed by ethylacetate these solvents were same ones used by (Jayaswal 1990) for the extraction of antifungal compounds from the strain of Pseudomonas, as (Bonsall 1997) reported these types of solvents allow extraction of metabolites such as phenazine, pyoluteorin, 2-4 diacetylphloroglucinol produces by numerous strains of Pseudomonas, extracts . The crude product appeared as dark brown oil and yield was between 100-150mg per 1000mL broth. Crude extract of CMG1030 tested positive in bioassay against all tested strains. These results indicated that the antagonistic activity was due to the production of antibacterial compound and that the compound could be extracted from growth medium by portioning with organic solvents. A thin layer chromatography of crude extract was performed and 13 bands were observed under UV transilluminater. (Table4). Bioassay of elutes from compounds separated by TLC showed one compound with activity against test strains. The MIC of isolated compound for S.aureus S. epidermidis were 75 µg/ml for Enterobacter faecalis 100 µg/ml. Log phase cells of S.aureus were treated with MIC concentration of isolated compound and incubated at 30°C for 30 min and slides were prepared and observed under phase contrast microscope, microscopic examination of crude extract treated cells of S.aureus suggest lysis of cells of S.aureus and some big and ghost cells were also observed (Fig2).

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