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ANTIBACTERIAL ACTIVITY OF A MARINE BACTERIUM AGAINST PATHOGENIC AND ENVIRONMENTAL ISOLATES OF *VIBRIO* SPECIES

Bushra Uzair, Nuzhat Ahmed and Farzana Kousar
Center for Molecular Genetics, University of Karachi, Karachi-75270, Pakistan
e-mail: shanibushra@yahoo.com

ABSTRACT: The marine environment covers three quarters of the surface of the planet is estimated to be home to more than 80% of life and yet it remains largely unexplored. The rich diversity of marine flora and fauna and its adaptation to the harsh marine environment coupled with new developments in biotechnology, has opened up a new exciting vista for extraction of bioactive products of use in medicine. In this study inhibitory activity of a marine bacterium isolated from gut of ribbonfish was studied against pathogenic and environmental isolates of *Vibrio* species. This strain was identified as *Pseudomonas stutzeri* and it was found active against *V. harveyi* (luminescent bacteria), *V. cholerae*, *V. alginolyticus*, *V. damseal*, *V. fluvialis*. The antibacterial substance produced by *Psaeudomonas stutzeri* was soluble in organic solvent and closely bound to external surface of bacterial cells. Reduction of the absorbance of the *V.cholerae* cell suspension was observed when log phase cells of *V.cholerae* were treated with MIC and 4xMIC concentration of crude extract of *Pseudomonas stutzeri.*

KEY WORDS: Antibacterial; marine bacteria; agar well diffusion method; extraction.

INTRODUCTION

The sea is an immense and practically unexploited source of new potentially useful biologically active compounds. Marine microorganisms comprise a comparatively untapped reservoir of commercially valuable compounds with antibacterial, antiviral and anticancer properties Colwell and Hill (1992). Marine microorganisms have become an important tool of study in the search for novel products. Today both academic and industrial interest in marine microorganisms is on the rise, the results of these efforts have led to several significant discoveries of compounds of medical importance Paul (1992). Marine microorganisms are particularly attractive because they fit in with the traditional pharmaceutical 'model' of a natural product drug source moreover supply to bulk amounts of a microbial derived drug can be addressed by large scale fermentation of bio active marine microorganisms. Fluorescent *Pseudomonads* have been used as biocontrol agents in several rhizosphere studies Sullivan *et al* (1992) where their inhibitory activity has been attributed to a number of factors, such as the production of antibiotics Mazzola, *et al* (1992) and Shanahan *et al* (1992), hydrogen cyanide Voisard *et al* (1989), or iron-chelating siderophores Kloepper *et al* (1980). *Pseudomonads* constitute a large part of the microflora of the gills, skin, and intestinal tracts of live fish Cahill (1990) and are only rarely reported as pathogens of fish Hatai, *et al* (1988). As with their terrestrial counterparts, aquatic *Pseudomonads* are often antagonistic against other microorganisms Gram (1993) including fish-pathogenic bacteria Smith *et al* (1993).

Changes in global climate have raised concerns about the emergence and resurgence of infectious diseases. *Vibrio cholerae* is a reemerging pathogen that proliferates and is transported on marine particles Colwell (1996). The *Vibrio* genus contains motile, Gram-negative bacteria that are obligate aerobes. *Vibrio* has a recognizable curved shape and single polar flagella. Although *Vibrio* species are non-invasive pathogens, they cause some of the most serious cases of diarrhea and thousands of people die from infection annually. Pathogenic biotypes of *V. cholerae* are annually responsible for more than 300,000 cases of cholera, 6,500 of which are lethal, in over 50 countries worldwide. (World Health Organization 2000) The ecology of *V. cholerae* is intimately coupled with its attachment to particles, and these interactions are considered important in its transmission from aqueous environments to humans Colwell *et al* (2003). The association of *V. cholerae* with both phytoplankton Huq *et al* (1990) and zooplankton Kaper *et al* (1979) is well documented. *V. cholerae* can reach such high abundance (10^4 to 10^5) on larger particles that a single particle is sufficient to provide an infectious dose to humans Colwell (1996). The present study was undertaken to determine the antibacterial potential of marine *Pseudomonas stutzeri* isolated from fish to evaluate its antivibrio activity and to assess the potential of the aquatic *Pseudomonas* sp for the production of secondary metabolites with antibiotic activity.

MATERIALS AND METHODS

Bacterial strains:

Clinical isolates were collected from various medical centers of Pakistan. Marine *Vibrio* strains were isolated from fish and crustaceans samples. Identification of each bacterial culture was done by conventional methods; gram-negative rods were identified with API20E and 20NE system. All *Vibrio* species strains were stocked in alkaline peptone water (ASW medium). *Pseudomonas* strains were stocked in brain heart infusion broth containing 20% glycerol. The bacteria were slanted on the respective media before being used in the experiments.

Anti *Vibrio* activity of *Pseudomonas stuzeri*:

In vitro antibacterial activity of *Pseudomonas stutzeri* was tested against *V. harveyi* (luminiscent bacteria), *V. cholerae*, *V. alginolytticua*, *V. damseal*, *V. fluvialis* by agar well diffusion method. Different strains of *Peudomonas* sp were grown in brain heart infusion broth and incubated at 37°C for 48 h. After 48 h cell free neutralized supernatants were obtained by centrifugation (10,000rpm for 30 min at 4°C). The cells were discarded and pH of the supernatant was adjusted to 7.0 with 1 N NaOH. Lawn of *Vibrio* sp was prepared on blood agar plates and wells of (5mm diameter) were cut and 100µl of supernatant of *Pseudomonas* sp were poured into the wells and plates were incubated at 37°C for12 to 48 h and examined for zone of inhibition (area where target isolate failed to grow).

Effect of enzymes on antibacterial metabolite:

To find out chemical nature of the antibacterial metabolite. Filtrate of *Pseudomonas stutzeri* was treated with different enzymes (Pepsin10µg/mL, ProteaseP10µg/mL,

ProteaseK1μg/mL, RNase10μg/mL) and treated samples were tested by agar well diffusion method.

Preparation of crude extract:

A seed culture was prepared and inoculated onto King B agar medium plates and the plates were incubated at 30°C for 5 days. The culture was first extracted with 3 volumes of 80% acetone with water and then with ethyl acetate from the agar surface at room temperature. The combined ethyl acetate extract was evaporated under vacuum to yield the crude ethyl acetate extract (2.67g).

Determination of Minimal Inhibitory Concentration:

The minimal inhibitory concentration of crude extract was determined by disc diffusion method of Bauer *et al* (1966). Sterile discs containing different concentrations of crude extract (varying from 0.01 to 100 μg/disc) were prepared. The prepared discs were placed on the surface of MH agar plate seeded with test culture and incubated at 37°C for 24 hours. The results were noted on the basis of presence or absence the zone of inhibition. The MIC was determined as the lowest concentration of the crude extract showing zone of inhibition.

Bacteriolytic assay:

The seed culture of *V. cholerae* in alkaline peptone water (ASW) were washed twice with sterile distilled water containing 0.9% NaCl and the absorbance was adjusted to 0.05 at 600nm.The bacterial cell suspension was divided into aliquots of 5 ml each placed in sterile test tube and exposed to crude extract at MIC and 4xMIC concentration, untreated bacterial suspension were used as the negative control. These test tubes were incubated at 30° C by shaking at 120 rpm the absorbance was measured at 0, 60, 120,180, and 240, 300,60 min

Result and Discussion:

Antibacterial metabolite producing strain was isolated from gut of ribbonfish caught from Baluchistan coast of Pakistan. This strain was identified as *Pseudomonas stutzeri*, which produced green pigment on King B agar medium. Loss in pigmentation was observed when grown on seawater-based medium. It was capable of growth up to 4% sodium chloride (NaCl) and it failed to grow on medium without NaCl. This bacterium was negative for luminescence, amylase, gelatinase, catalase, urease, indol production and positive for oxidase and citrate utilization. These characters placed this bacterium under the genus *Pseudomonas*. As seen in table 1 all the pathogenic and environmental isolates of *Vibrio*. sp were sensitive to this strain with varying degree of inhibition. Antibacterial activity among marine bacteria is a well-known phenomenon and demonstrated in a number of studies (Rosenfeld and Zobell 1947; Anderson *et al* 1974;Lemos *et al* 1985; Dapazo *et al* 1988; Mc Carthy *et al* 1994 and Alim Isnansetyo *et al* 2003). The cell free supernatant and crude extract display inhibitory active against *V.harveyi* (luminescent bacteria) (Fig. 1), *V. cholerae*, *V. alginolytticua*, *V. damseal*, *V. fluvialis*. No loss in antibacterial activity was observed on treatment with proteolytic enzyme, (Pepsin10μg/mL, ProteaseP10μg/mL, ProteaseK1μg/mL, RNase10μg/mL) by agar well diffusion method. This indicated that the antibacterial substance might not be

proteinaceous in nature. The antibacterial substance produced by *Pseudomonas stuzeri* was recovered from the cells and the King B agar medium inoculated with *Pseudomonas stuzeri* after 5 days of incubation using 80% acetone and ethyl acetate. Burkholder *et al* 1966 observed better antibiotic production by a marine bacterium on solid medium. Since the antibacterial substance could be recovered from both the cells and King B agar medium, it can be inferred that the antibacterial substance may be bound to the outer cell surface and secreted into the solid medium. This corroborates with earlier observations (Rosenfeld and Zobell 1947; Lemos *et al* 1985) reported that the inhibitory compounds remains closely bound to the cells. The production of antibiotic substance by *Pseudomonas stutzeri* was observed to be growth associated phenomenon in an earlier study and probably released as a secondary metabolite into the surrounding environment. According to Lemos *et al* (1985), if the antibiotic remains bound to cells, they can be excreted slowly and continually to the environment preventing colonization of the adjacent space by competitors. A rapid release of the antibiotic substance by antibiotic producing bacteria probably would not provide them any competitive advantage because the seawater would immediately wash it away. The observation of the present study further indicated that the antibacterial compound of *Pseudomonas stutzeri* could be an organic compound or polysaccharide as other antibacterial substances recovered from marine bacteria using ethyl acetate have turned out to be a a pyrole (Burkholder *et al* 1966) or quinolinol (Wratten *et al* 1977) or tyrosol and isatin(Gil-Turnes and Finical 1992)or a polysaccharide(Anderson *et al* 1974). Reduction of the absorbance of the *V.cholerae* cell suspension was observed when log phase cells of *V.cholerae* were treated with MIC and 4xMIC concentration of crude extract of *Pseudomonas stutzeri* (Fig 2). These results indicated that crude extract lyse *Vibrio cholerae.* The MIC of crude extract for *V.cholerae and V.damsel* were 5-7 µg/ml for *V.harveyi and V.alginolyticus* was 6-8µg/ml.

Table 1. Zones of inhibition produced by culture filtrate of *Pseudomonas stutzeri* against *Vibrio* species (By agar well diffusion method).

Strains tested	No of strains tested	Size of zone of inhibition (mm)
V. harveyi	3	18
V. cholerae	5	16
V. alginoilticus	6	15
V. damseal	5	18
V. fluvialis	4	20
V. homini	5	16

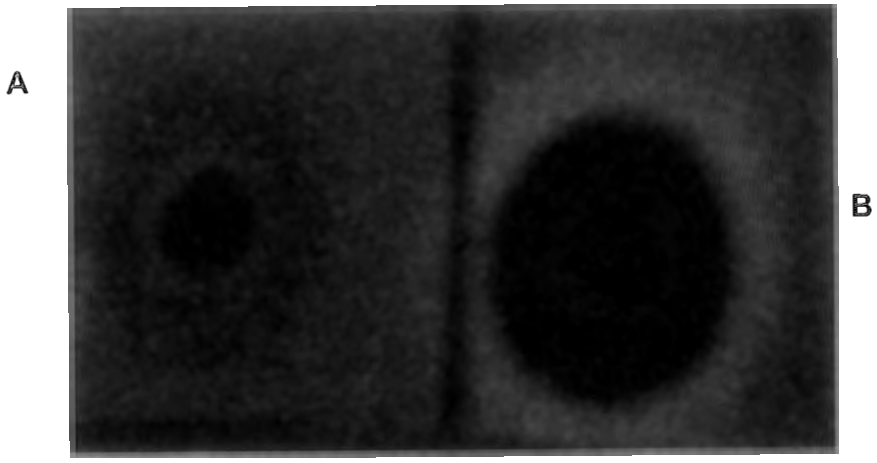

Fig. 1. Bioassay plate showing antibacterial activity of culture filtrate of *Pseudomonas stutzeri* against *Vibrio harveyi by agar well diffusion method* A: No zone of inhibition B, Zone of inhibition.

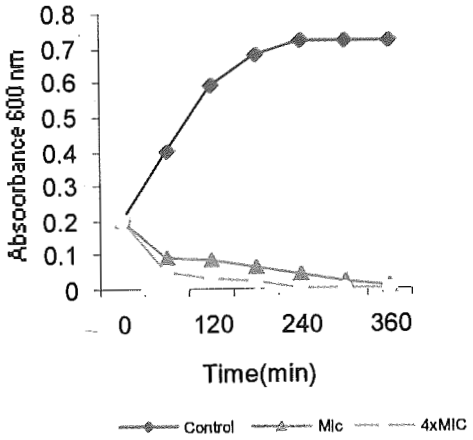

Fig. 2. Bacteriolytic activity of crude extract of *Pseudomonas stuzeri* against *Vibrio cholerae* at MIC and 4xMIC.

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REFERENCES

Anderson, R. J., M. S. Wolfe and D.J. Faulkner, 1974. Autotoxic antibiotic production by a marine chromobacterium. Marine Biology, 27: 281-285.

Bauer A. W., W. Kirby, M. M. Sherrisc, M. Turck, 1966. Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology*, 45: 493-6.

Burkholder, P. R., R. M. P.Fisher, F.P. Leitz. 1996. Production of a pyrrole antibiotic by a marine bacterium. *Applied Microbiology*, 14: 649-653

Colwell, R.R. 1996. Global climate and infectious disease: the cholera paradigm. *Science* 274: 2025-2031.

Colwell, R. R., A. Huq, M. S. Islam, K.M.A. Aziz, M. Yunus, N.H. Khan, A. Mahmud, R. B. Sack, G. B. Nair, J. Chakraborty, D. A. Sack, and E. Russek-Cohen, 2003. Reduction of cholera in Bangladeshi villages by simple filtration. *Proc. Natl. Acad. Sci.* USA 100: 1051-1055.

Colwell, R. R. and R. T. Hill. 1992. Microbial Diversity In: Diversity of oceanic life: An evaluation review, pp100-106, Peterson, M.N.A (ed). The center for strategic and international studies, Washington. D.C

Dopazo, C. P., M. L. Lemos, C. Lodeiros, J. Bolinches, J. Lbarja and A. E. Toranzo 1988. Inhibitory activity of antibiotic producing marine bacteria against fish pathogens. *Journal Applied Bacteriology*, 65: 97-101.

Gilturn and W. Fenical, 1992. Embryos of *Homarus americanus* are protected by epibiotic bacteria. *Biological Bulletin,* 182: 105-108.

Gram, L. 1993. Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas* strains isolated from spoiled and fresh fish. *Applied Environment Microbiology,* 59: 2197-2203

Hatai, K., and L. G. Willoughby. 1988. *Saprolegnia parasitica* from rainbow trout inhibited by the bacterium *Pseudomonas fluorescens. Bulletin of European Associations of Fish Pathologists,* 8: 27-29

Huq, A., R. R. Colwell, R. Rahman, A. Ali, M. A. Chowdhury, S. Parveen, D. A. Sack, and E. Russek-Cohen. 1990. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. *Applied Environment Microbiology,* 56: 2370-2373.

Isnansetyo, A. and Y. Kamei. 2003. A bactericidal antibiotic produced by a new marine bacterium, *Pseudomonas phenolica* sp.nov.O-BC30 T, against methicillin – resistant *Staphylococcus aureus.* Antimicrob. Agents Chemother. 47: 480-488.

Kaper, J., H. Lockman, R. R. Colwell and S. W. Joseph. 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. *Applied Environment Microbiology,* 37: 91-103.

Kloepper, J. W., J. Leong, M. Teintze and M. N. Schroth. 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature. 286: 885-886.

Lemos, M. L., A. E. Toranzo and J. L. Barja, 1985. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. *Microbiological ecology*, 11: 149-163.

Mazzola, M., R. J. Cook, L. Thomashow, D. M. Weller and L.S. Pierson, III. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent *Pseudomonads* in soil habitats. *Applied Environment Microbiology,* 58: 2616-2624.

McCarthy, S.A., A.R. M.Johnson and D. Kakimoto, 1994. Characterization of an antibiotic produced by *Alteromonas luteoviolacea* Gauthier 1882,85 isolated from Kinko Bay, Japan. *Journal Applied Bacteriology,* 77: 426-432.

Pascual, M., M. J. Bouma and A. P. Dobson. 2002. Cholera and climate: revisiting the quantitative evidence. *Microbes Infection*, 4: 237-245.

Paul V.J (ed) 1992. Ecological roles of marine natural products. Ithaca comstock pp-245.

Rosenfeld, W. D. and C. E. Zobell. 1947. Antibiotic production by marine microoganisms. *Journal Applied Bacteriology,* 154: 393-398.

Shanahan, P., D.J. O'Sullivan, P. Simpson, J.D. Glennon and F. O'Gara, 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent *pseudomonad* and investigation of physiological parameters influencing its production. *Applied Environment Microbiology,* 58: 353-358

Smith, D.C., M. Simon, A.L. Alldredge and F. Azam, 1992. Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. *Nature* 359: 139-142.

Voisard, C., C. Keel, D. Haas and G. Defago, 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J. 8: 351-358.

World Health Organization, 2000. Cholera. Wkly. *Epidemiological Review*, 76: 1.

Wratten, S.J., M.S. Wolfe, R.J. Anderson and D.J Faulkner, 1997. Antibiotic metabolites from marine *Pseudomonas. Antimicrobial agents Chemother.* 11: 411-414.

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