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Isolation and screening of mucus-associated bacteria of the gastropod, *Drupa margariticola* for antagonistic activity

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Abstract

The mucus-associated bacteria of the gastropod, *Drupa margariticola* were screened for their ability to inhibit the human and fish pathogens. Out of the two hundred and eighty five bacterial strains isolated, 23% (65) were found to be pigmented, 71% (202) were identified as Gram-negative. A higher percentage of non-pigmented (77%) and Gram-negative (71%) strains were observed in the present investigation. 16% (46) of the isolates was found to have antagonistic activity against both human and fish pathogens tested and 63% of the Gram-negative strains (29) were found to be antibiotic producers. Antagonistic activity was found to be exhibited by pigmented strains too. A higher degree of inhibition was conferred by 3 of the isolates (D_{15}, D_{130} and D_{237}) against both human and fish pathogens. These strains exhibited full or complete degree of inhibition against *Escherichia coli*.

Key words: Gastropod, *Drupa margariticola* mucus-associated bacteria and antagonistic activity.

Introduction

Bacterial infections were considered won in the late 1960's but now antimicrobial resistance threatens to turn back. Resistance is spreading rapidly particularly where antibiotics are heavily used (Lech, 2004). The erroneous use of antibiotics both for therapeutic and aquaculture purposes has resulted in the advent of multiple drug resistance of the pathogenic bacterial strains. Hence the need of the hour is the search for novel antibiotics with lesser side effects. The ultimate source- the ocean, is a unique resource that provides a diverse of natural products primarily from bacteria and cyanobacteria and invertebrates such as corals, sponges, tunicates, bryozoans and molluscs. Many marine chemicals often possess quite novel structures which lead to pronounced biological activity and novel pharmacology (Lei and Zhou, (2001). A number of discovery efforts have yielded several bioactive metabolites which have been successfully developed by the pharmaceutical industry (Kong *et al*, 1994; Faulkner, 2001; Rosenfeld and Zobell, 1947; Fenical and Jensen; 1993 and Fenical, 1993). The study of marine bacteria has also led to the realization that microorganism from specific symbiotic relationship with marine organisms which may be responsible for the production of some bio-active compounds (Kobayashi and Ishibashi, (1993). The marine microorganisms have had a major impact on the development of medical science and these bacteria form highly specific symbiotic relationships with marine plants and animals, (Fenical, 1993). There are a number of works reporting the occurrence of bacteria associated with marine fauna, in particular, the gastropods (Kharlamenko *et al.* (2001), Distel, (1998), Belkin, *et al.* (1986), Stein *et al.* (1988) and Windoffer and Giere, (1997). Recent studies have shown that these antibacterial compounds are not only inhibiting the human pathogens but also fish pathogens (Strahl *et al.*, 2002).

Drupa margariticola is one of the commonest gastropods inhabiting the reefs and may be seen adhering the corals rocks in large numbers. Bacteria associated to the mucus of this organism are being less explored as potential sources of antagonistic compounds. This paper describes the isolation of the bacteria associated with the mucus of *D. margariticola* and testing the isolated strains for their ability to inhibit the growth of selected human and fish pathogens *in vitro*.

Materials and methods

Live *D. margariticola* (Stenoglossa: Muricidae) were collected from the intertidal region of the Tuticorin port area (Lat. 8°45'N and Long. 78°13'E). The animals were immediately transported to the laboratory and placed in the aquaria containing natural sediment and sea water. The animals were washed twice in sterile seawater and the

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shells were removed aseptically. The mucus was then collected from the animals in sterile glass tubes. Approximately 1 mL of the mucus was serially diluted and plated on two different media: 1. Zobell Marine 2216E Agar (ZMA) and 2. Cetrimide agar (supplemented with 3.5 and 1.75% Sodium chloride) using pour plating method. The plates were then incubated at room temperature for 3-4 days. Morphologically different colonies were selected randomly. The number of Gram positive and negative colonies as well as pigmented and non-pigmented colonies was noted. Axenic cultures were obtained by streaking and re-streaking on ZMA plates and subsequently stored as ZMA stab cultures at 4°C.

Screening of the isolates for antimicrobial activity

The mucus-associated isolates were applied as single streaks on pre-poured Yeast Peptone Extract (YPE) (75% sea water) agar plates and incubated at 28°C for 48 hrs. The human pathogens (*Bacillus subtilis*, *B. cereus*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *S. aureus* and *Shigella dysentriae*) were obtained from Christian Medical College, Vellore. The fish pathogens (*Proteus mirabilis*, *Serratia marcescens*, *Aeromonas formicans*, *A. hydrophila*, *Vibrio harveyii*, *V. vulnificus*, *V.campbelli* and *V. logei*) were collected from Fisheries College, Tuticorin. The test strains were grown on YPE (75% sea water) plates for three or more transfers before using them in the screening assays. All isolates were tested for the production of antibacterial metabolites using the cross streaking method (Strahl *et al.*, 2002). All the test strains were applied as single streaks perpendicular to the marine bacteria without touching it. After cross streaking with test bacteria, the plates were incubated for an additional 24 h at 28°C and observed for the inhibition of test bacterial growth. A zone inhibition is defined as an area on the test streak of reduced growth or lack of growth compared with the control plates (streaked with non-producer strains), partial inhibition as significantly reduced over 3-9 mm (P) but not completely inhibited and lack of growth over 10-19mm of the streak was defined as moderate inhibition (M). If the test organisms did not grow, it was scored as complete inhibition (C) which was about 20-25 mm.

Results

Isolation of mucus-associated bacteria

Two hundred and eighty five bacterial strains were isolated from the mucus of *D. margariticola* (232 colonies from Zobell marine agar and 48 colonies from Cetrimide agar were isolated). Out of 285 bacterial strains isolated, 23% (65) were found to be pigmented, 71% (202) were identified as Gram-negative and 29% as Gram-positive strains (Figs.1 & 2). The pigmented bacterial colonies were observed to be yellow, red, brown, orange and black in colour. A higher percentage of non-pigmented (77%) and Gram-negative (71%) strains were observed in the present investigation.

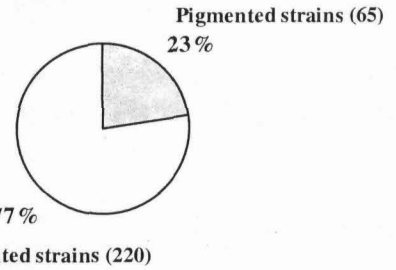

Fig 1. Percentage of pigmented and non-pigmented strains isolated from the mucus of *D. margariticola*

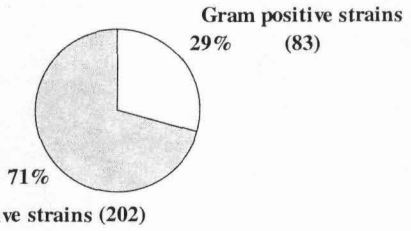

Fig 2. Percentage of Gram positive and Gram-negative strains isolated from the mucus of *D. margariticola*

Antibacterial activity of the marine bacteria

Out of the total bacterial strains (285), 16% (46) of the isolates were found to have antagonistic activity against both human and fish pathogens tested (Fig.3). 70% of the non-pigmented strains (32) and 63% of the Gram-negative strains (29) were found to be antagonistic (Figs. 4 & 5). Results of the screening of the isolates against human and fish pathogens are presented in Figures. 6 and 7 respectively. A higher degree of inhibition was conferred by 3 of the isolates (D_{15}, D_{130} and D_{237}) as shown in Figures 8, 9 and 10 against both human and fish pathogens. The strains D_{130} and D_{237} exhibited full or complete degree of inhibition against *Escherichia coli*. Strain D_{15} was able to elicit a moderate antagonism to seven of the human and fish pathogens tested. It was observed that all antagonistic strains were able to inhibit at least five of the pathogens tested.

Discussion

In the present study the non-pigmented strains (77%) were higher in numbers than the pigmented ones (23%).

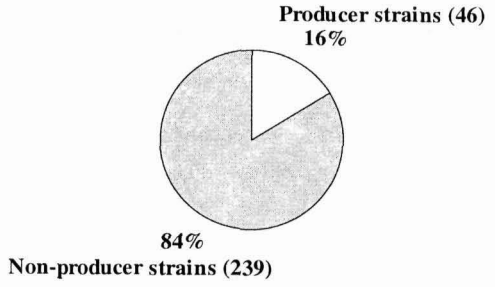

Fig 3. Percentage of producer and non- producer strains isolated from the mucus of *D. margaritícola*

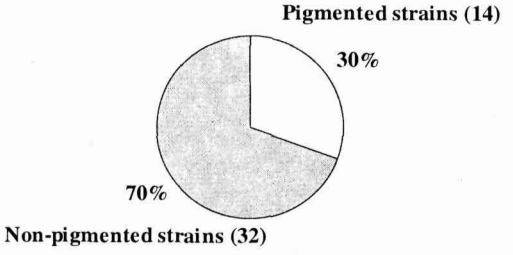

Fig 4. Percentage of pigmented and non- pigmented strains showing activity

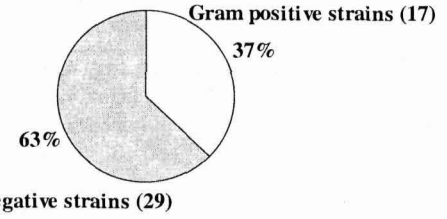

Fig 5. Percentage of Gram-positive and Gram –negative strains showing activity

This observation is on par with the findings of Jeyasekaran *et al.*(2002), who have reported that pigmented bacterial flora was lower by about 2-3 log counts than the total culturable bacterial flora observed in the marine samples from seawater, sediments, sea plants and bivalves. The finding that the percentage of Gram-positive strains was found to be lower (29) than the Gram-negative strains (71) agrees with one of the earlier works which reports that the bacteria present in seawater are mainly Gram-

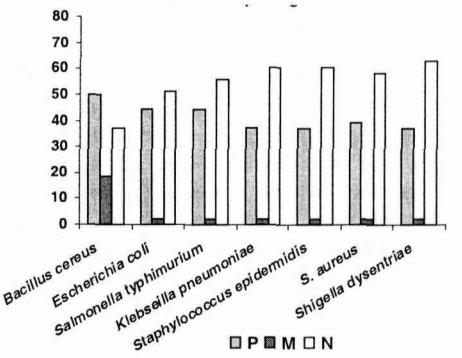

Fig. 6. Percentage of producer strains showing partial, moderate and nil inhibition against human bacterial pathogens

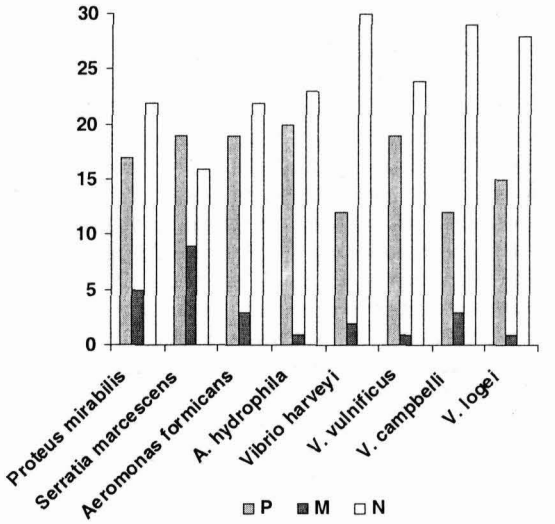

Fig. 7. Percentage of producer strains showing partial, moderate and nil inhibition against fish bacterial pathogens

negative rods (Fenical, 1993). Another study revealed that the bacterial strains isolated from various regimens of the marine environ showed that 82.8% were Gram-negative (Strahl *et al.*, 2002). There are a number of reports pertaining to the study of mucus-associated marine bacteria (Di Salvo, 1971., Ducklow and Mitchell, 1979., Rublee *et al.*, 1980; Paul *et al.*, 1986 and Coffroth,1990). A smaller percentage (16%) of the bacterial isolates was antagonistic compound producers. This agreed with the report of Nair and Simidu (1987) who stated that 8.8% out of the 45 epiphytic bacteria isolated from different marine samples displayed antistaphylococcus activity. Of the 16% (46) of the producer strains isolated in the

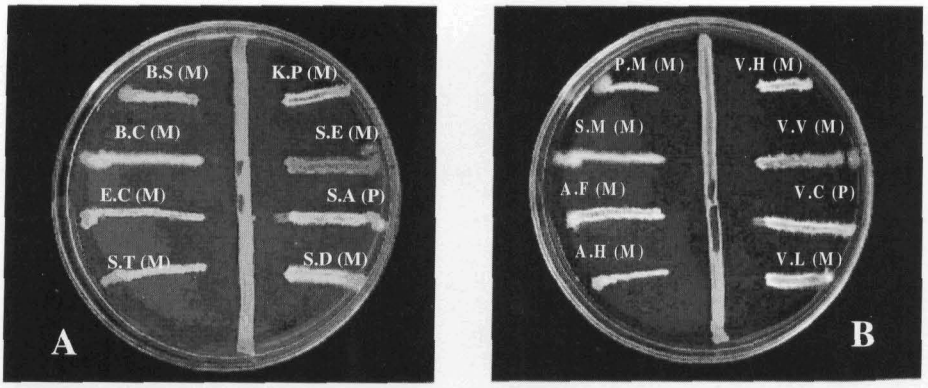

Fig. 8. Strain No. 15 antagonistic to (a) human and (b) fish pathogens

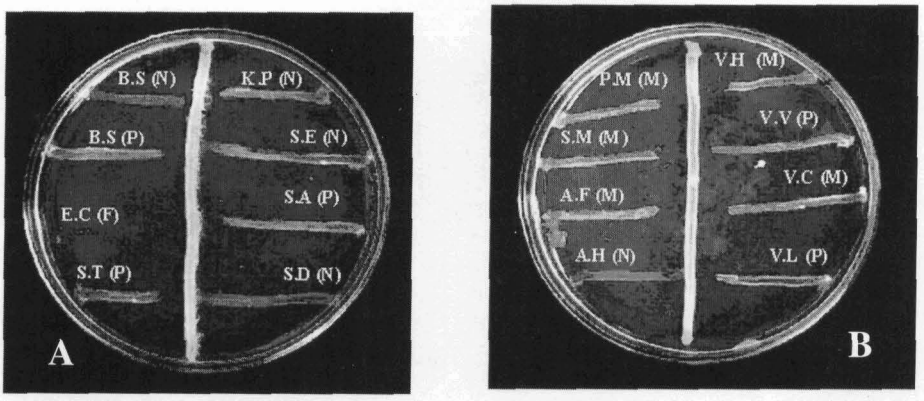

Fig. 9. Strain No : 130 antagonistic to (a) human and (b) fish pathogens

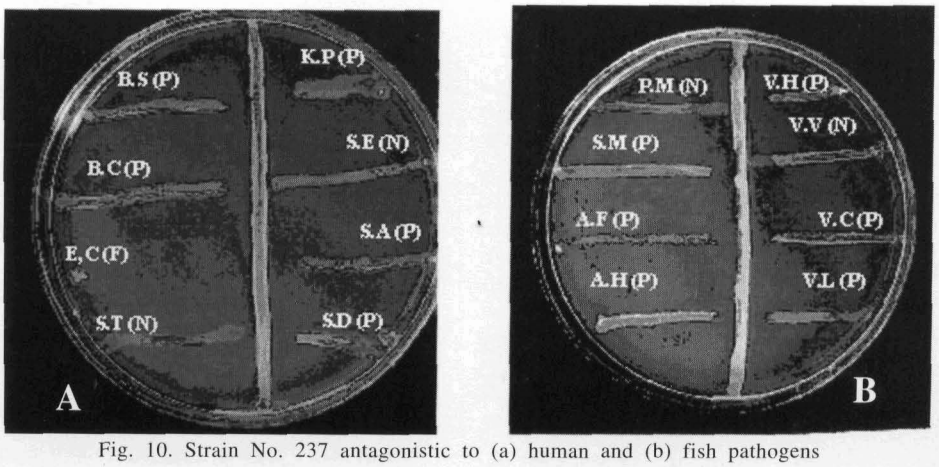

Fig. 10. Strain No. 237 antagonistic to (a) human and (b) fish pathogens

(B.S- *Bacillus subtilis*, B.C - *B. cereus*, E.C - *Escherichia coli*, S. T - *Salmonella typhimurium*, K. P- *Klebsiella pneumoniae*, S. E - *Staphylococcus epidermidis*, S. A- *S. aureus*, S. D - *Shigella dysentriae* P. M - *Proteus mirabilis*, S. M - *Serratia marcescens*, A. F - *Aeromonas formicans*, A. H - *A. hydrophila*, V. H - *Vibrio harveyii*, V. V - *V. vulnificus*, V. C. - *V. campbelli*, V. L- *V. logei*)

present study, 70% (32) were non-pigmented which contradicts the findings of Rosenfeld and Zobell (1947) who reported that most of the antibiotic-producing marine bacteria are pigmented. However, a smaller percentage of antagonistic compound producers (30%) were found to be pigmented which may be due to the reason that pigments have been associated with antibacterial activity as is the case for cyanobacteria (Lemos *et al.*, 1985). Pigmented bacteria are also known to be potential antibiotic producers as reported by Shiba and Taga (1980). One of the findings of the present study is that Gram-negative strains (29) showed comparatively higher antagonism against the test strains than the Gram-positive ones (17) which deviate from the works of Fenical (1993) who reported that most of the Gram-negative bacteria from the marine samples have chemically proven to be unproductive. In the present study, among the producer strains, D_{15}, D_{130}, D_{237} were able to inhibit a minimum of 11 of the pathogens tested with (at least a partial inhibition) zones of 3-9 mm. Similar zones of inhibition by marine antagonistic bacteria were reported by earlier workers (Rosenfeld and Zobell (1947), Patil *et al.* (2001) and Chelossi *et al.* (2004).

Marine microbes have a higher possibility of yielding natural products with unprecedented and interesting bioactivity. The antagonistic marine bacteria isolated from the mucus of *D. margariticola* may produce antibacterial compounds with novel structures which can be explored to generate pronounced biological activity in the future.

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