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Vibrio ruber (S2A1), a Marine Bacterium that Exhibits Significant Antimicrobial Activity

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ABSTRACT

A potential antimicrobial-producing marine bacterium, designated as S2A1, was isolated from a seagrass collected in Setiu Lagoon, Terengganu. S2A1 was a Gram negative rod that was motile by means of a polar flagellum. Phenotypic and genotypic characterisation indicated that strain S2A1 represented a species in the genus *Vibrio*. The antimicrobial activities of S2A1 against a number of test microorganisms showed a broad antimicrobial spectrum property with inhibition towards 25 out of 29 test microorganisms. The antimicrobial compound(s) of S2A1 was more effective against Gram-positive bacteria with 100% inhibition, compared to yeast (88.8%) and Gram-negative bacteria (75.0%) tested. High activity scores were observed when using whole cells compared to cell free extract.

Keywords: antimicrobial activity, Gram positive bacterium, *Vibrio ruber*

INTRODUCTION

The ability of marine bacteria to produce antimicrobial substance was documented 58 years ago (Rosenfeld and ZoBell, 1947). A few years later, Burkholder *et al*., (1966) identified and characterized the first antibiotic from a marine bacterium. In the years that followed these early discoveries, the number of antibiotics reported from marine bacteria has grown tremendously (Fenical, 1993; Bernan *et al*., 1997; Sponga *et al*., 1999; Jensen and Fenical, 2000; Wagner-Dobler *et al*., 2002; Behal, 2003). Urauchimycin A and B, which were isolated from *Streptomyces* sp. found in association with unidentified sponge (Imamura *et al*., 1993) were examples of novel antibiotic that had been reported from marine bacteria. Trischman *et al*., (1994) isolated a species of *Streptomyces* from surface of a jelly fish, which produced two new bicyclic peptides compounds with novel backbones (salinamides A and B). Novel cyclic decapeptide antibiotics, loloatins A-D from *Bacillus* sp. that were isolated from a marine worm (Gerard *et al.* 1999), and phenazine antibiotics termed pelagiomicins from *Pelagiobacter variabilis*, that was isolated from a seaweed (Imamura *et al*., 1997) were a few other examples of novel marine antibiotics that had been reported.

Most of the marine antibiotics were produced by Actinomycetes (*Streptomyces* sp.). This is not surprising because most of the existing antibiotics (from terrestrial bacteria) are also from the group Actinomycetales (Williams and Wellington, 1984; Fenical, 1993). Besides Actinomycetes, marine bacteria mainly *Pseudomonas* sp. (Burkholder *et al*., 1966; Wratten *et al*., 1977; Needham *et al*., 1994; Jayatilake *et al*., 1996; Debitus *et al*., 1998; Ponce *et al*., 1999; Isnansetyo *et al*., 2003; Mitova *et. al*., 2003), *Alteromonas* sp. (Gauthier, 1976; Gauthier and Flatau, 1976; Barja *et al*., 1989; Gil-Turnes *et al*., 1989; Long *et al*., 2003) and *Bacillus* sp. (Imada *et al*., 1998; Gerard *et al*., 1999; Barsby *et al*., 2001) had also been extensively studied for antimicrobial production. These bacteria were either isolated directly from seawater, sediment, surface associated or symbiotic bacteria.

Despite the vast potential offered by the marine ecosystem, limited studies have been carried out in Malaysia. Vickineswary *et al*. (1997) were among the first local researchers who reported antifungal activities from actinomycetes that were isolated from a mangrove area. Besides that, antibiotic productions were also reported from a few local marine isolates, for examples, *Bacillus pulvifaciens* (Rafat, 2002) and *Pseudomonas nautica* (Lim *et al*., 2002). In the present paper, we report the potential of a Gram negative marine bacterium, isolate S2A1, as an antimicrobial compound(s) producer. This communication also report the characterization, antimicrobial spectrum as well as the cellular distribution of the antimicrobial compound(s).

MATERIALS AND METHODS

Bacterial strains and culture conditions

The marine pigmented strain studied designated as S2A1 was isolated from the surface of a seagrass collected from Setiu Lagoon, Terengganu (east coast of Peninsular Malaysia). The bacterium was maintained in ZoBell agar slant which consisted of Bacto-peptone (5 g/l), yeast extract (1 g/l), $FePO_4$ (0.1 g/l) and bacteriological agar (12 g/l) in filtered seawater with a final pH of 7.8). Sub-culturing was carried out every 7-8 days to keep the culture viable. The red pigment produced by the isolate grown aerobically on ZoBell agar at room temperature (30 ± 2°C) was then extracted out with acetone/methanol (7:2)

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solvent system. The clear supernatant obtained after centrifugation was determined for its absorption spectrum using a VARIAN-CARY-50 Probe UV-VIS spectrophotometer, from the wave length of 300-800 nm.

The bacterium was initially identified by its colony and cell morphology after 48 hours of incubation as well as performing a Gram staining. S2A1 cells were placed onto a formvar film covered with copper grid and negatively stained with 2% methylamine tungstate (Dykstra, 1992). Cells were then observed under transmission electron microscopy (PHILIPS TEM C12, Netherlands). Phenotypic characteristics of the strain S2A1 were determined according to the identification scheme for Gram negative marine Eubacteria as proposed by Baumann and Baumann (1981). Further identification was done by using a rapid diagnostic kit (BIOLOG MICROLOG3 4.20), and 16S rRNA sequencing was conducted by the Genomic Research Lab, Shimadzu Seisakusho Co. Ltd., Kyoto, Japan.

Screening for antimicrobial activities

Preliminary screening for the production of antimicrobial compound(s) was conducted by the method of Gauthier, (1976) against 29 test microorganisms (*Acinetobacter anitratus, Burkholderia pseudomallei, Citrobacter freundii, Enterobacter aerogenes* (E114), *Escherichia coli* 0157 (E91), *Klebsiella pneumoniae, Morganella morganii, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens, Yersinia enterocolitica* (ATCC 9610), *Bacillus subtilis, Bacillus licheniformis,* multiple-drug-resistant *Staphylococcus aureus*, methycillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591), *Staphylococcus epidermidis, Staphylococcus lugdumensis, Staphylococcus saprophyticus, Streptococcus faecalis, Candida albicans, Candida famata, Candida tropicalis, Candida parapsilosis, Cryptococcus laurentii, Cryptococcus neoformans, Rhodotorula rubra, Rhodotorula minuta* and *Torulopsis glabata*) obtained from the Institute for Medical Research, Kuala Lumpur and the University Hospital, Health Campus Kubang Kerian, Kelantan. For a primary screening, S2A1 cell paste was deposited onto Trypticase Soy Agar (TSA) plates seeded with a 24 hour old test organisms. Antimicrobial activity was detected with the formation of a clear zone around S2A1 cell paste that indicated the inhibition of growth. The diameters of the inhibition zones were measured and then categorized in their respective scores, as suggested by Buck *et al.*, (1963). Scored + equivalent to 1-3 mm), ++ (>3-7 mm), +++ (>7-12 mm) and ++++ (>12.0 mm)).

Cellular distribution of antimicrobial compound(s)

Methycillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591 was selected as test microorganism for this test. The cellular distribution of the antimicrobial compound(s) was determined based on the method described by Barja *et al.* (1989). S2A1 was cultured in ZoBell medium at room temperature (28 ± 2°C) with agitation at 150 rpm (SK 600, Jeio-Tech, Korea) for 72 hours. Whole cells and supernatant were separated by centrifugation (Eppendorf, Germany) at 8,000 g for 15 min. To determine whether the antimicrobial compound(s) was excreted extracellularly, about 0.1 ml of supernatant was pipetted into wells hollowed out on TSA agar plate which had been seeded with MRSA. In order to determine whether the antimicrobial compound(s) was cell bound, a loopful of the whole cells were deposited on the seeded TSA agar. The remaining cell pellets were washed with 0.85% saline for 30 minutes and re-centrifuged at 8,000 g for 15 minutes. The washed cells were subjected to osmotic shock by suspending it in distilled water (1:50, w/v) for 1 hour followed by sonication (Bransonic, USA). After centrifuged at 8,000 g for another 15 min., 0.1 ml of the supernatant was pipetted into wells hollowed out on the seeded agar to determine possible activity of intracellular compounds.

RESULTS

Description and identification of the bacterium

S2A1 was a Gram negative bacteria with curved rods in shaped (approx. 1.6-2.4 µm long and 0.6-0.9 µm wide) that was motile by means of a single, polar flagellum as revealed by transmission electron microscopy (Figure 1). On solid agar medium it produced a bright red circular colony with an entire margin, slightly raised and non luminescent. Optimal growth occured at 25-30°C and pH 7.0 when using media prepared with fresh seawater. Growth occured between 20°- 40°C but not at 4° or 45°C. The strain was halophilic and unable to grow in the absence of NaCl.

Acid and gas were produced from fermentation of glucose. Other carbohydrates such as cellubiose, galactose, lactose, mannose, sucrose were also fermented. Methyl red, urease, oxidase and catalase reactions were negative and O/F was oxidative. However indole was not produced.

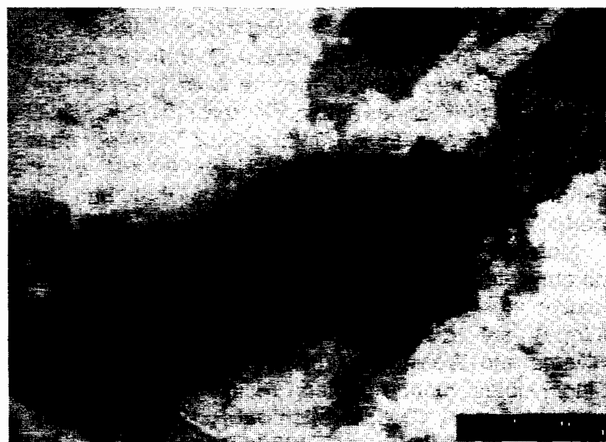

Figure 1: EM micrograph of S2A1, showing a curved rod with a polar flagellum. Bar, 0.5 µm.

S2A1 reduced nitrate to nitrite and was resistant to both 10 µg and 150 µg of the vibriostatic agent O/0129. These data supported the inclusion of strain S2A1 in the genus *Vibrio* of the family Vibrionaceae. Additional phenotypic characteristics of S2A1 are given in Table 1. Meanwhile acetone/methanol extracts of the red pigment produced by S2A1 showed a maximal absorption at the wavelength of 545 nm.

Table 1: Biological and physiological characteristics of S2A1

Characteristics	Observation	Characteristics	Observation
Colony morphology	3-4 mm, round, raised, convex, smooth	Production of:	
Cell morphology	Curved short, single rods non-sporebearing	Indole	-
Motility	+	Hydrogen sulfide	-
Relative growth	Rapid		
Metabolism	Aerobic	Utilisation of:	
		Adonitol	-
Spore formation	-	Arabinose	+
Gram stain	-	Cellubiose	-
Acid fast	-	Dextrin	+
		Dulcitol	-
Oxidative/Fermentative(O/F) test	Oxidative	Glycerol	-
Citrate utilization	-	Inositol	-
Nitrate reduction	+	Lactose	-
Methyl Red test	-	Mannose	+
Voges Proskaeur test	-	Salicin	+
Urease	-	Sucrose	+
Oxidase	-	Sorbitol	-
Catalase	-	Starch	+
0/129	Resistant		
10 µg	Resistant		
150 µg			
Temperature range for growth (°C)	15-45		
pH range for growth	4-9		
Requirement for seawater	+		
Growth in distilled water media	-		

+ -positive reaction
- -negative reaction

A rapid analysis using BIOLOG failed to yield a conclusive result on S2A1 identification, providing only a weak match (similarity index = 0.33) to *Sphingomonas parapaucimobilis*. Since the database used in the BIOLOG system was developed based on clinical rather than environmental isolates, this probably resulted in the poor match. The isolate was then further identified with 16S rRNA sequencing.

An almost complete 16S rRNA sequence (approx. 95%) of isolate S2A1 was determined. Comparative analysis confirmed the affiliation of the newly isolated strain to the genus *Vibrio* with the highest level of similarities (1425/1437@99%) to *Vibrio ruber*. As shown in Figure 2, isolate S2A1 formed a cluster with *V. gazogenes* strain ATCC 29988T and *V. ruber* (AF 42458.1) within the radiation of the *Vibrio* species. Although the similarity was high, it is believed that *V. ruber* (S2A1) and *V. ruber* (AF 42458.1) could be differentiated at the species level.

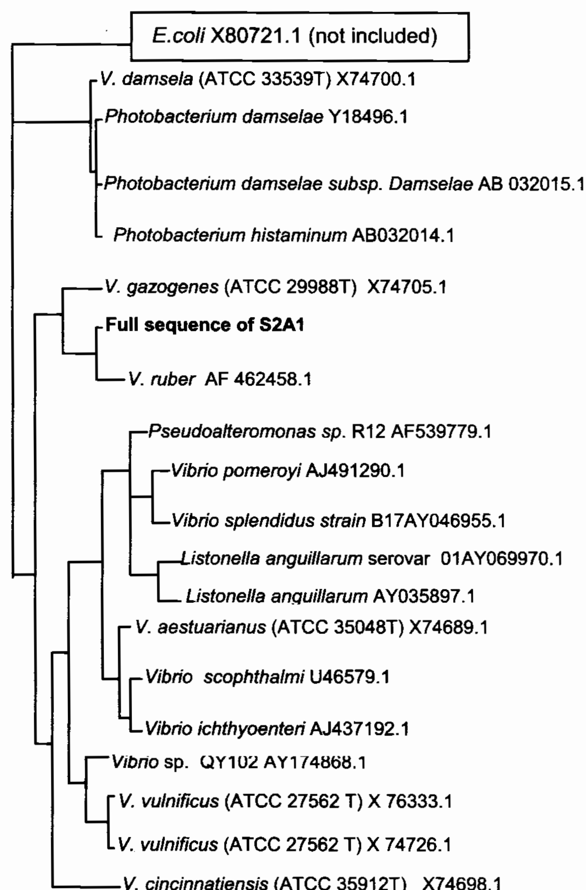

Figure 2: Unrooted phylogenetic tree derived from neighbour-joining analysis of the 16S rRNA sequences of strain S2A1 and other related species.

Antimicrobial activity of *V. ruber* S2A1

The antimicrobial activities of the strains studied are presented in Table 2. *V. ruber* (S2A1) exhibited a broad inhibitory spectrum with activities against 25 out of 29 test microorganisms of both related and non-related taxonomical groups. As shown in the same table, *V. ruber* (S2A1) was more active against Gram positive bacteria, in which 100% of the Gram positives bacteria tested were inhibited compared to 88.8% of the yeasts and 75.0% Gram negative bacteria, respectively. The highest activity score of ++++ was observed against *P. aeruginosa, B. subtilis, B. licheniformis* and *S. lugdumensis.* On the other hand, no activity was observed against *S. typhimurium, S. marcescens, M. morganii* and *R. rubra.* Interestingly, S2A1 also inhibits MRSA, which is one of the common causes of nosocomial infection.

Table 2: Antimicrobial activities spectrum exhibited by S2A1

Test Microorganisms	Activity score (inhibition zone diameter, mm)	Test microorganisms	Activity score (inhibition zone diameter, mm)
Gram negative pathogens		Yeast pathogens	
E. coli 0157 (E91)	++ (4.05 ± 1.87)	*C. tropicalis*	++ (4.03 ± 1.22)
S. typhimurium	-	*C. parapsilosis*	++ (6.77 ± 2.13)
K. pneumoniae	++ (6.81 ± 2.56)	*C. laurentii*	+++ (8.79 ± 2.96)
P. aeruginosa	++++ (21.08 ± 1.44)	*C. famata*	+++ (10.57 ± 3.50)
P. vulgaris	++ (5.05 ± 2.11)	*C. neoformans*	+++ (9.77 ± 2.77)
E. aerogenes	++ (3.98 ± 2.31)	*R. rubra*	-
C. freundii	+++ (10.52 ± 2.02)	*T. glabata*	+++ (9.08 ± 2.01)
B. pseudomallei	++ (6.01 ± 1.55)	*R. minuta*	++ (6.25 ± 1.44)
S. marcescens	-	*C. albicans*	+++ (10.27 ± 2.10)
M. morganii	-		
Y. enterocolitica	++ (5.25 ± 1.33)		
(ATCC 9610)	++ (6.28 ± 2.0)		
Gram positive pathogens			
B. subtilis	++++ (18.51 ±3.22)		
B. licheniformis	++++ (21.17 ± 4.11)		
Multiple resistant S. aureus	++ (6.26 ± 12)		
Methycillin resistant S. aureus (ATCC 33591)	++ (5.72±1.21)		
S. epidermidis S42)	+++ (11.55 ± 2.09)		
S. lugdumensis	++++ (20.51 ± 1.32)		
S. saprophyticus	+++ (12.34 ± 3.34)		
S. faecalis (S256)	++ (3.65 ± 2.23)		

- no zone of inhibition formed
+ 1.0-3.00 mm
++ >3.0-7.0 mm
+++ >7.0-12.0 mm
++++ >12.0 mm

Cellular distribution of antimicrobial compound(s)

The cellular distribution of antimicrobial compound(s) of S2A1 is presented in Table 3. The results showed that a large fraction of activity remained associated with the bacterial cells, with inhibition zones of 5.72 ± 1.21 mm. Although some of the inhibitory compound(s) were excreted into the culture medium, the activity was slightly less (4.84 ± 0.95 mm) compared when using the whole cells (5.72 ± 1.21 mm). The lowest activity (1.04 ± 0.27 mm) was observed when using the intracellular extract.

Table 3: Cellular distribution of the antimicrobial compound(s) of S2A1

Source	Antimicrobial activity (inhibition zone, mm)
Extracelullar	+ + (4.84 ± 0.95)
Intracellular	+ (1.04 ± 0.27)
Whole cells	++ (5.72 ± 1.21)

+ 1.0-3.00 mm
+ >3.0-7.0 mm
MRSA was used as test microorganism

DISCUSSION

Identification of S2A1

In the present study a red pigmented Gram negative marine bacterium, *V. ruber* (S2A1) was identified as a potential producer of antimicrobial compound(s). Association of pigmented bacteria and antimicrobial activity had been demonstrated long ago. Shiba and Taga (1980) reported high percentage of pigmented isolates with antimicrobial activity from the heterotrophic marine bacteria attached to the surfaces of the seaweed.

Meanwhile, Lemos *et al.*, (1985) in their study observed that 100% of the antibiotic-producing bacterial strains attached to the surfaces of five green and brown algal species studied were pigmented. Similarly, Nair and Simidu (1987) in their study on the distribution and significance of heterotrophic marine bacteria with antimicrobial activity found that 30% of the antibiotic-producing bacteria from seawater, sediment and phytoplankton or zooplankton from Sagami, Suruga and Tokyo Bay as well as corals and sponges from Taiwan waters were pigmented. Among the pigmented marine bacteria reported as producers of antimicrobial compound(s) were violet and red pigmented *Alteromonas* (Gauthier and Flatau, 1976; Ballester *et al.*, 1977; Barja *et al.*, 1989), brown pigmented *Pseudomonads* (Ponce *et al.*, 1999), yellow pigmented *Micrococcus* (Ponce *et al.*, 1998) and *A. citrea* (Gauthier, 1977).

S2A1 was identified as *V. ruber* by 16S rRNA sequencing. The isolation of *V. ruber* from marine environment was reported only recently by Shieh *et. al.*, (2003). They reported the isolation of a red heterotrophic marine bacterium, strain *V. ruber* sp. nov. @VRI^T from seawater samples around the coastal waters of Keelung, Taiwan and phenotypic and chemotaxonomic characterization showed that VRI^T strain ^{w}as a novel species in the genus *Vibrio*. However, antimicrobial activity tests was not performed. Therefore, this finding was the first ever reported on the isolation of *V. ruber* from Malaysia, and in fact the first reports on the antimicrobial activity of *V. ruber,* even though similar activity had been reported from other species in the *Vibrio* species (Dogett *et al.*, 1968; Jalal *et al.*, 1989; Oclarit *et al.*, 1994; Castro *et al.*, 2002).

Cellular distribution of antibiotic compound(s) in *V. ruber* (S2A1)

In this study, *V. ruber* (S2A1) was found capable of inhibiting the growth of test microorganisms by all the three types of its extracts or fractions (intracellular, extracellular and membrane bound) tested. The growth inhibition was highest when using whole cell compared to intracellular and extracellular extracts or fractions. Rosenfeld and ZoBell, (1947) also reported inhibitory compound(s) that were closely associated with bacterial cells rather than diffusing into the aqueous environment, and the similar observation was also reported by Lemos *et al.*, (1985). Barja *et al.*, (1989) also observed that in a marine *Alteromonas*, although the antibiotic compound(s) were detected in culture medium, most of the activity remains bound to the bacterial cells. However, there were also marine bacteria that produced a few types of antibiotic compounds with different cellular distribution in the same organism.

Cheng *et al.*, (1970) and Gould *et al.*, (1975) suggested that the extracellular protein in Gram negative bacteria always remains bound to the cells in the periplasmic space after excretion. The location of the inhibitory compound(s) that linked to the cells of S2A1 might indicate their possible ecological roles in the marine environment where the compound(s) could be released into the environment slowly and continuously, preventing the colonization of the adjacent space by competitors.

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