

Prodigiosin - an antibacterial red pigment produced by *Serratia marcescens* IBRL USM 84 associated with a marine sponge *Xestospongia testudinaria*

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ABSTRACT

The objectives of present study are to extract the antibiotic compound from marine isolate and to determine its *in vitro* antimicrobial activity against bacteria. A marine bacterial isolate *Serratia marcescens* IBRL USM 84 was isolated from the surface of a marine sponge *Xestospongia testudinaria*. This species of bacteria produced red pigment with antibacterial activity. The red antibacterial pigment was produced intracellularly and inhibited 13 out of 18 tested bacteria, with Gram positive was more susceptible than the Gram negative bacteria. The growth and antibacterial red pigment production profiles demonstrated the highest antibacterial red pigment production was achieved at the 48 hours of cultivation (14.08 U/ml) time in marine broth when incubated at 25 °C with 150 rpm agitation. The antibacterial red pigment was extracted, purified and confirmed as prodigiosin.

INTRODUCTION

The increasing numbers in bacterial resistance to a number of antimicrobial agents is becoming a major health problem worldwide. Besides, the increasing use and misuse of existing antibiotics in human, veterinary medicine and in agriculture has further aggravated the problem (Todar, 2004). Common species among them are methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* and *Mycobacterium tuberculosis* (Barrett and Barrett, 2003; Dancer, 2008). It is further stated that, about 70% of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment (Todar, 2004). Thus, there is an urgent need for the discovery of new classes of antimicrobial compounds to overcome existing resistance mechanisms and to effectively combat these human pathogens that can cause life-threatening infections. The importance of terrestrial microbes

including bacteria as sources of valuable secondary bioactive metabolites is very well established and over 120 of the most important antibiotics in use today are obtained from terrestrial microorganisms (Alanis, 2005). Along with the deep studies of marine natural products biosynthesis, some evidence indicates that many bioactive compounds previously found in marine animals and plants were in fact produced or metabolized by associated microorganisms (Rinehart, 2000; Osinga *et al.*, 2001). Competition among microbes for space and nutrient in marine environment is a powerful selection pressure that endows marine microorganisms to possess many natural products, pharmaceutical and industrial values (Armstrong *et al.*, 2001). Recently many reports have been published on the production of potential antimicrobial compounds from marine bacteria associated with sponges, macroalgae and invertebrates. The first antibiotic from marine bacterium was identified and characterized in 1966 (Burkholder *et al.*, 1966). Sponges are the most primitive of multi-celled animals and have existed for more than 800 million years (Radjasa *et al.*, 2007). They are known to produce secondary metabolites which play a decisive ecological role, protecting them against potential invaders, predators or other competitors (Pawlik *et al.*, 2002).

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Bacteria growing on the surface of sponges live in a highly competitive environment in which access to space and nutrients are limited (Burgess *et al.*, 1999; Slattery *et al.*, 2001). Secondary metabolites produced by sponge-associated bacteria far exceed those produced by planktonic bacteria (Lu *et al.*, 2009). Several potentially therapeutic compounds identified in sponges have striking similarities to metabolites derived from their associated microorganisms (Radjasa *et al.*, 2007; Thiel and Imhoff, 2003). Hence, sponge-associated bacteria become a highly potential source for the production of antibiotic compounds.

The aim of the present study was to extract the red pigment from culturable marine bacteria associated with a marine sponge *Xetospongia testudinaria* and to evaluate its antibacterial activity against a series of microorganisms.

MATERIALS AND METHODS

Culture maintenance

Bacterial isolate IBRL USM 84 was isolated from the surface of a local marine sponge, *X. testudinaria* which was collected from Bidong Island, Terengganu, Malaysia. This isolate was supplied by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia. The bacterial culture was maintained on the marine agar (Difco, United Kingdom) slant at 25 °C for 72 hours before storing at 4 °C for subsequent studies. The stored bacterial culture was subcultured regularly at every month to maintain viability.

Cultivation, extraction of the crude extract and cellular distribution of antibacterial compounds in the isolate IBRL USM 84 cell

Ten colonies of the isolate IBRL USM 84 were inoculated in to 250 ml Erlenmeyer flask containing 50.0 ml of marine broth medium (Difco, United Kingdom) and incubated at 25 °C with 150 rpm of agitation for 72 hours. The culture was then separated by centrifugation at 5,000 g (Sigma 42k, Sartorius, Germany) for 20 minutes at 4 °C. The supernatant was tested for extracellular antibacterial activity using disc diffusion susceptibility test.

The pellet was washed twice with 0.85 % (w/v) physiological saline for 30 minutes and recentrifuged at 5000 g for 20 minutes. The washed cell pellets were then soaked in 50.0 ml sterile distilled water for an hour at 4 °C to give osmotic shock condition to the cells. The cells were broken up by adding 10 sterile glass beads (0.4 mm diameter) and vigorously agitated using vortex (Donna and Nicholas, 1997). The suspension was then separated by centrifugation at 5,000 g for 20 minutes at 4 °C. The resultant cell suspension after cell disruption was centrifuged and the supernatant was tested for intracellular antibacterial activity. The test bacteria used in this experiment was MRSA. The results were expressed as mean value \pm standard error of the inhibition zone obtained with three replicates for each experiment.

Test microorganism

Eighteen bacterial cultures such as *Acinetobacter anitratus*, *Agrobacterium tumefaciens*, *Azotobacter* sp., *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Citrobacter freundii*, *Erwinia* sp., *Escherichia coli*, *Micrococcus* sp., Methicillin resistant *Staphylococcus aureus* (MRSA), *Proteus rettgeri*, *Salmonella parathypi*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus aureus* and *Yersinia* sp. provided by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, USM, were used as test bacteria in this study. The culture was grown on nutrient agar (Merck, Germany) and incubated at 37 °C for 24 hours before storing them at 4 °C until further use. The stored bacterial culture was subcultured regularly at every month to maintain viability.

Inoculum preparation

Inoculum was prepared by inoculating five colonies from > 24 hour old of bacterial culture into a universal bottle containing 5.0 ml of Tripcase Soy broth and incubated for 6 hours. The logarithmic phase culture was adjusted to yield a cell density of approximately 1×10^8 colony forming unit per ml (cfu/mL) as described in Clinical and Laboratory Standards institute (CLSI) standard M26-A (NCCLS 1998).

Disc diffusion susceptibility test

The assay was performed according to the CLSI standard M2-A9 (NCCLS, 1998) with slight modifications. Using a sterile cotton swab, tested bacterial inoculum of approximately 1×10^8 cfu/ml was streaked onto the surface of a Soy Tripcase agar plate. Sterile Whatman antibiotic discs (6.0 mm diameter) impregnated with 20 μ l of supernatant were then placed on the surface of the inoculated medium. Distilled water was applied as a negative control and 30 μ g/ml of chloramphenicol was used as a positive control. The plates were incubated at 37 °C for 24 hours and the diameter of the clear zone was measured. The results were expressed as mean value \pm standard error of the inhibition zone obtained with three replicates for each experiment.

Determination of antibacterial activity secreted by the isolate IBRL USM 84 against MRSA growth

The amount of antibacterial activity secreted by the isolate was measured quantitatively using the method described previously by Lorian (1991). About 0.1 ml of the > 24 hour old MRSA cell suspension ($3 - 4 \times 10^5$ cells/ml) was inoculated in 7.9 ml nutrient broth followed by the addition of 2.0 ml intracellular extraction of the isolate *Serratia marcescens* IBRL USM 84 obtained from different cultivation time. The mixture was then incubated at 37°C for 14 hours. The degree of inhibition of the test bacteria (MRSA) was determined based on the decrease in the culture turbidity measured spectrophotometrically (Spectronic Unicam, United State) at 560 nm, compared to control. The control consisted the same material as for the test culture but with no addition of the isolate *Serratia marcescens* IBRL USM 84

cultivation broth, instead it was replaced by addition of another 2.0 ml of nutrient broth. To facilitate the expression of the potency of the antibacterial activity of the compounds secreted by the isolate *Serratia marcescens* IBRL USM 84, the antibacterial activity was defined as one unit (U) of the antibacterial activity which resulted the reduction or inhibition of 1.0% on the growth of MRSA in the liquid medium which was used as the test microorganism.

Serratia marcescens IBRL USM 84 Cell growth determination

The cell growth was determined spectrophotometrically (Spectronic Unicam, Genesys 10UV, United State) at 540 nm and the actual concentration was compared to standard curve prepared prior determination.

Extraction, purification and identification of prodigiosin

The red pigment produced by the isolate *Serratia marcescens* IBRL USM 84 was extracted and analyzed by method of Cang *et al.*, (2000) and Alliohosseni *et al.*, (2008). Three milliliter of acetone was added into 1.0 ml of a thick IBRL USM 84 cell suspension and agitated at 150 rpm for 30 minutes at room temperature (30±2 °C).

The mixture was centrifuged at 5000 g for 20 minutes. The red colored supernatant was mixed with petroleum ether with the ratio of 1:2 in a separating funnel and shook vigorously until the red pigment was in the petroleum ether. The extracted red colored petroleum ether solution was evaporated using a rotary evaporator under reduced pressure until the dried red pigment obtained and was used for spectral analysis.

Fifty microgram of dried red pigment was dissolved in 10.0 ml of absolute ethanol and in order to get an acidic condition, 1.0 ml of 1.0 M HCl was added into the solution. To get an alkaline solution, 1.0 ml of 1.0 M NaOH was added and to get a neutral condition 1.0 ml of sterile distilled water was added into the solution. A spectral analysis of the pigment in acidic, alkaline and neutral solution was determined using a spectrophotometer (Spectronic Unicam, Genesys 10UV, United State) at 400-600 nm.

Statistical analysis

The significant difference of the mean data was analyzed using one way Analysis of variance (ANOVA) and Duncan test with PASW Statistics 18 version.

RESULTS

Antibacterial activity of the intracellular compounds of *Serratia marcescens* IBRL USM 84

The antibacterial activities of the test bacterial strains studied are presented in Table 1. The commercial antibiotics chloramphenicol has shown significantly ($P>0.05$) larger inhibition zone compared to the intracellular extract of *Serratia marcescens* IBRL USM 84. The red pigment intracellular extract of *Serratia marcescens* IBRL USM 84 exhibited a broad inhibitory spectrum activity against 13 out of 18 test bacteria. These results are supported by Figure 1 where it shows the present of the

antibacterial pigment in the cells. The intracellular antibacterial pigment production is higher as the incubation time increased from 48 (Fig. 1a) to 72 hours (Fig. 1b). However, it was noted that all the test bacteria were resistant to the extracellular extract treatment.

MRSA produced the largest diameter inhibition zone 22.5 cm (data not shown) in agar disc diffusion assay test and it is also a pathogenic bacteria which commonly causes of nosocomial infections was chosen for further study.

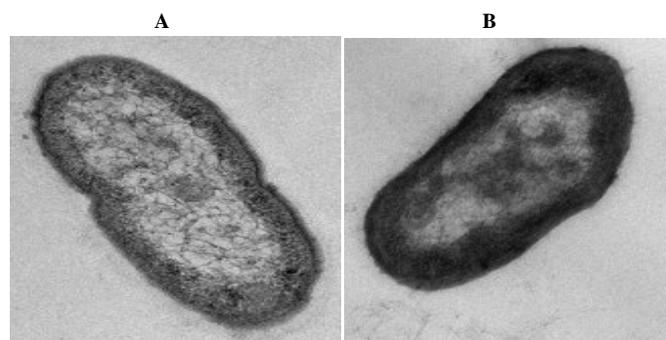


Fig. 1: TEM micrographs of the *Serratia marcescens* IBRL USM 84 cells. (A) 48 hours and (B) 72 hours of incubation at 25°C.

Table 1: Antimicrobial activity of cellular distribution of antibacterial compounds extracted from *Serratia marcescens* IBRL USM 84.

Test microorganisms	Inhibition zone (mm)		
	I	E	C
<i>Acinetobacter anitratus</i> -	+	-	++
<i>Agrobacterium tumefaciens</i> +	++	-	+++
<i>Azotobacter</i> sp.	-	-	++
<i>Bacillus licheniformis</i>	++	-	+++
<i>Bacillus cereus</i>	++	-	++
<i>Bacillus subtilis</i>	+	-	+++
<i>Bacillus thuringiensis</i>	+	-	++
<i>Citrobacter freundii</i>	-	-	++
<i>Erwinia</i> sp.	+	-	++
<i>Escherichia coli</i> -	+	-	++
<i>Micrococcus</i> sp.	+	-	++
Methicillin resistant <i>Staphylococcus aureus</i> (MRSA)	+++	-	+++
<i>Proteus rettgeri</i>	-	-	++
<i>Salmonella paratyphi</i>	-	-	++
<i>Staphylococcus epidermidis</i>	+	-	++
<i>Staphylococcus saprophyticus</i>	+	-	+++
<i>Staphylococcus aureus</i>	+	-	+++
<i>Yersinia</i> sp.	-	-	++

Note: The antimicrobial activity was determined based on the diameter of inhibition zone (mm). The results were recorded according to following scale: +++ = ≥ 15 mm, ++ = 10-14 mm, + = ≤ 9 mm, - = no inhibition zone. I = Intracellular extract of *Serratia marcescens* IBRL USM 84, E= Extracellular extract of *Serratia marcescens* IBRL USM 84, C = Chloramphenicol.

Profile of antibacterial red pigment production by *Serratia marcescens* IBRL USM 84 against MRSA

Figure 2 shows the profile of antibacterial red pigment produced by *Serratia marcescens* IBRL USM 84 in 72 hours of cultivation in marine broth medium. The antibacterial activity was detected at 8 hours (4.41 U/ml) of cultivation and increased gradually and achieved it maximal value of 14.08 U/ml at the 48 hours of cultivation. The activity was then decreasing slowly thereafter until 72 hours of cultivation. The growth of *Serratia*

marcescens IBRL USM 84 also follow the same pattern as the antibacterial activity and achieved its maximal growth of 2.10 g/L at the 48 hours of cultivation.

These results suggested that the antibacterial red pigment production is growth dependent. There was a slight increased in pH of the medium from 7.5 at the beginning of the cultivation to 9.6 at 72 hours of cultivation showing that the bacterial cells need an alkaline condition to produced the antibacterial activity significantly.

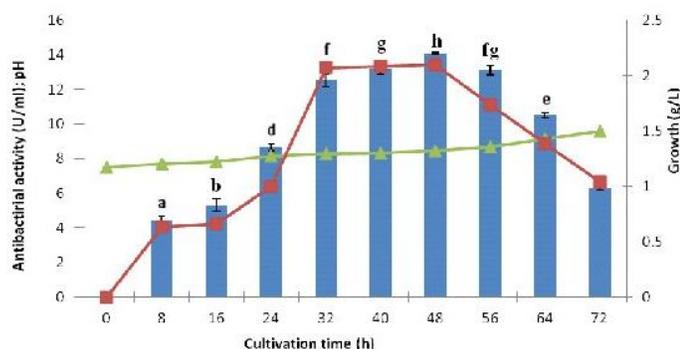


Fig. 2. Profiles of antibacterial red pigment production and cell growth of *Serratia marcescens* IBRL USM 84.

Note: ▲ pH; ■ antibacterial activity; ■ growth. (Error bars refer to standard deviation calculated from the triplicate samples. Means with different letters indicated significantly different at $p \leq 0.05$, Duncan test).

Identification of red pigment

The red pigment displayed maximum absorbance at 465 nm wavelengths under alkaline conditions and at 535 nm under acid conditions (Fig. 3).

In acidic condition, the pigment is red, symmetrical narrow band with a peak at 535 nm and a slight shoulder on the lower wavelength limb of the curve. In alkaline solution, the pigment is orange-yellow due to a broader, less intense, roughly symmetrical band centered at about 465 nm. There appears to be a definite isobestic point at about 495 nm. Maximum absorption spectrum of the prodigiosin produced by *Serratia marcescens* IBRL USM 84 was in accordance with the literature (Williams, 1973).

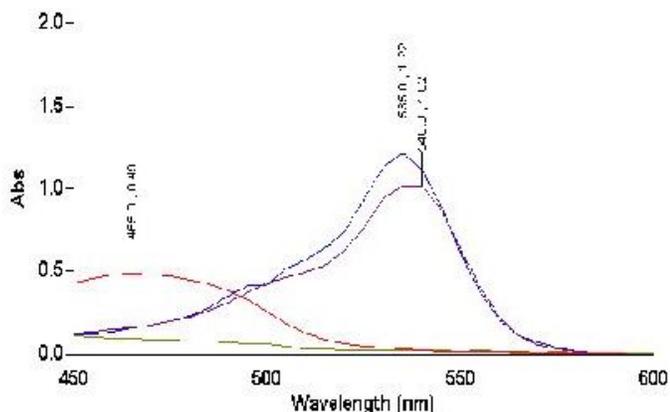


Fig. 3: Absorbance of the red pigment in acidic and alkaline solution.

Note: - - - alkaline medium (NaOH and ethanol); — acidic medium (HCl and ethanol); ··· neutral medium (water and ethanol)

DISCUSSION

The main and most plentiful pigment group in marine pigmented bacteria is carotenoid which usually appears orange, yellow or red in colour (Marit *et al.*, 2010). The pigment also exhibits antimicrobial including antibacterial activity (Gulani *et al.*, 2012).

Serratia marcescens IBRL USM 84 was isolated from the surface of a marine sponge and its red pigment which was confirmed as prodigiosin exhibited antibacterial activity. Marine sponges are reported as rich sources of natural compounds, which exhibit wide variety of biological activity (De Rosa *et al.*, 2003). Hence, many novel microorganisms with potential biological activity were isolated from marine sponges (Hentschel *et al.*, 2001; Devi *et al.*, 2010). Moreover, the numbers of bacteria associated with marine sponges are two or three times higher than seawater because of the specific surface and internal environmental niche of sponges (Friedrich *et al.*, 2001).

Marine bacterial isolates have been proven to contain active compounds, prodigiosin (red pigment), violacein (violet pigment) and pyocyanin (blue-green pigment) that are known to have active compounds such as antimicrobial activity, antiviral, antitumor, antiprotozoa, antioxidant, anticancer and much more (Kim *et al.*, 2010; Solieve *et al.*, 2011). However, red pigment has higher antimicrobial especially antibacterial activity followed by orange, yellow and green (Solieve *et al.*, 2011). Among marine bacteria that have been reported to produce red pigment with prodigiosin or antibacterial activity are *Serratia marcescens* (Solieve *et al.*, 2011), *Streptomyces* sp. (Gerber, 1975), *Pseudomonas* sp. (Gandhi *et al.*, 1976), *Pseudoalteromonas* sp. (Fehér *et al.*, 2008), *Paracoccus* sp. (Lee *et al.*, 2004) and *Vibrio* sp. (Kumar and Nair, 2007).

The production of red pigment by *Serratia marcescens* IBRL USM 84, a non-clinical strain is scientifically important because there might be a relationship or correlation between pigment production to the absence of plasmid that was also reported by Gargallo-Viola (Gargallo-Viola, 1989). Thus, pigment production by non-clinical strains of *Serratia marcescens* was of interest for further studies to determine a relationship, if any, of loss of pigment production to pathogenicity (Hardjito and Colwell, 2002).

Serratia marcescens IBRL USM 84 produced intracellular antibacterial red pigment prodigiosin which showed higher susceptibility against Gram positive than Gram negative bacteria. This may be attributed to the fact that these two groups differ in their structure of the cell wall components. The same characteristics were observed in other antimicrobial studies of plant extract against pathogenic bacteria (Darah *et al.*, 2013; Tong *et al.*, 2014). Various studies were reported to investigate the mechanism of actions involved in bacterial killing process. Among them are the interactions of antibacterial compound with the cell membrane (Hyldgaard *et al.*, 2012).

A Gram negative cell wall consists of lipopolysaccharide that can act as toxin to protect bacteria against predator (Barnett,

1992). The component of lipopolysaccharide can also makes the cell wall impermeable to lipophilic solutes (Pandey, 2004), hence gives more protection in Gram negative bacteria. The cell wall of Gram-positive bacteria has a thick layer of peptidoglycan and should be more susceptible because not an effective permeability barrier (Pandey, 2004). The pigmented marine natural products can also contribute to a variety of applications, from health, cosmetic up to the flavours of food additives, paint industry, fabric dye, and ink manufacture. Thus, the variety application of marine pigment has made it as an interesting subject of investigation in scientific knowledge.

CONCLUSION

A marine sponge associated bacterial isolate *Serratia marcescens* IBRL USM 84 was found to produce significant intracellular antibacterial red pigment which was confirmed as prodigiosin. It inhibited a series of test bacteria and Gram positive was more susceptible than Gram negative bacteria. To the best of our knowledge, this is the first report concerning the isolation of a marine sponge associated bacterial isolate, *Serratia marcescens* IBRL USM 84 which has a potential to produce prodigiosin. Thus, the further study was to optimize the intracellular antibacterial red pigment production and to characterize its properties which can contribute to a variety of applications in different field of industries.

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