

# Heavy-Metal Tolerance and Antibiotic Susceptibility of Red Pigmented Bacteria Isolated from Marine Environment

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## ABSTRACT

This study was undertaken to determine heavy metal resistance and antibiotic susceptibility of three non-pathogenic red pigmented bacteria namely WPRA3, SM11-3j and SC-G18, isolated from marine environments of Malaysia. The bacteria isolates were identified by 16S rRNA sequencing and by biochemical and morphological tests. The 16S rRNA gene sequences of all isolates showed  $\geq 96\%$  similarity to *Serratia* spp. Antibiotic susceptibility test of isolates was assayed according to the Kirby-Bauer disc diffusion method. All isolates were highly resistant to beta-lactam antibiotics, but were susceptible to quinolone antibiotics. Minimum inhibitory concentration (MIC) of nine heavy metals ( $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$ ) against the bacteria isolates were determined via the plate-dilution method. The isolates exhibited resistance to  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$  and  $\text{Zn}^{2+}$ . Isolates WPRA3 and SM11-3j showed higher multiple tolerances to heavy metals. The results obtained indicate that bacteria from marine environments of Malaysia present interesting metabolic activities, which should be studied and explored for potential biotechnological applications.

**Keywords:** Antibiotic Susceptibility; Heavy Metal Resistance; *Serratia* sp; Marine Bacteria

## 1. Introduction

The genus *Serratia* are gram negative bacteria, classified in the large family of Entrobacteriaceae of the class Gammaproteobacteria. *Serratia* species are widespread in the environment, occurring in soil, water, plants, vertebrates and humans. *S. marcescens* has been identified as the cause of many hospital epidemics [1]. The greatest impediment to the successful treatment of bacterial diseases is antibiotic resistance that is widespread and occurs in numerous bacterial genera [2]. Winshell and Neu [3] divided *Serratia marcescens* strains into three groups on the basis of antimicrobial resistance and pigment production. Group I, comprising non-pigmented strains, are resistant to most antibiotics. Group II, which are non-pigmented, and group III, which are pigmented, are susceptible to many antimicrobial agents. In general the pigmented strains are more susceptible to antibiotics.

Pollution of the marine environment by heavy metals has been recognized as one of the important problems that emerged from industrial, sewage treatment discharges and anti-fouling paints and natural sources include weathering of rocks, leaching of soils and volcanic

eruptions [4,5]. Four classes of heavy metal can be differentiated in seawater depending on their concentration [6]. Class I, common elements with concentrations between 100 nM and 1  $\mu\text{M}$  (Fe, Zn, Mo), class II, elements with concentrations between 10 nM and 100 nM (Ni, Cu, As, V, Mn, Sn, U), class III, rare elements (Co, Ce, Ag, Sb) and finally class IV, elements just below the 1 nM level (Cd, Cr, W, Ga, Zr, Th, Hg, Pb) [7]. Sn, Ce, Ga, Zr and Th have no biological influence because of the low solubility of the tri- or tetravalent cations [6]. Fe, Mo and Mn are important trace elements with low toxicity and Zn, Ni, Cu, V, Co, W and Cr are toxic elements with high to moderate importance as trace elements; As, Ag, Sb, Cd, Hg, Pb and U have limited beneficial function, but have to be considered as toxins [7]. In this study, we evaluated the heavy metal resistance and antibiotic susceptibility of three red pigmented *Serratia* sp. isolates designated WPRA3, SM11-3j and SC-G18.

## 2. Experimental

### 2.1. Bacterial Strain

Red pigmented bacteria designated as WPRA3, SM11-3j and SC-G18 were isolated from seawater of Port Dickson,

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mussel from Sungai Merbok (Kedah) and sea cucumber from Tinggi Island of Malaysia, respectively. Isolates were routinely grown in Marine Agar (MA; Difco). Identification of isolates was done by 16S rRNA sequence analysis. PCR amplification of the 16S rRNA gene was performed using two oligonucleotide primers, forward 5'-CTCCTACGGGAGGCAGCAG-3' and reverse 5'-WATTACCGCGGCKGCTG-3'. The PCR programme was set using thermal minicycler (MJ Research, USA) as follows: initial denaturation was carried out for 5 min at 94°C. It was followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1.5 min with a further 10 min extension at 72°C, using UniversAll™ tissue PCR kit. PCR product was purified using QIAquick PCR Purification Kit (QIAGEN, Germany) according to manufacturer's instructions. Nucleotide sequences were analyzed by using BioEdit Sequence Alignment Editor. The 16S rRNA gene sequences of the isolates were compared with available 16S rRNA gene sequences in GenBank databases using the BLASTn search facility (<http://www.ncbi.nlm.nih.gov>). Morphological and biochemical tests were determined according to Bergey's Manual using an isolated colony of bacteria from MA.

## 2.2. Antibiotic Susceptibility Assay

Antibiotic susceptibility test of the isolated bacteria was assayed according to the Kirby-Bauer disc diffusion method given by Bauer *et al.* [8]. Briefly, isolated bacteria were cultured in marine broth (MB; Difco) at 28°C for 24 hours. Sterile swabs were immersed in the microbial suspensions ( $10^8$  cells/mL) and applied to Muller Hinton Agar (MHA; Difco) plates immediately. Standard antibiotics (Oxoid) used were penicillin (10 U), ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (10 µg, 30 µg), nalidixic acid (30 µg), streptomycin (10 µg), gentamicin (10 µg) and kanamycin (30 µg) were placed on the agar in the MHA plates. The plates were incubated at 28°C for 24 hours. The result of inhibition zones of the isolates were interpreted as sensitive (S), intermediate (I) and resistant (R) according to the references to the standard provided by BD BBL™ Sensi-Disc™ Zone Interpretation Set (Oxoid). All of the experiments were performed in triplicate.

## 2.3. Heavy Metal Resistance

Isolated bacteria were tested to determine the minimal inhibitory concentrations (MICs) of the metals by the plate-dilution method as described by Malik and Jaiswal [9]. The metals  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  were used as  $\text{NiSO}_4$ ,  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{Cr}(\text{NO}_2)_3 \cdot 9\text{H}_2\text{O}$ ,  $\text{ZnSO}_4$ ,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ ,  $\text{Pb}(\text{NO}_3)_2$ ,  $\text{HgCl}_2$ ,  $\text{CdSO}_4$  and  $\text{CuSO}_4$  respectively. Stocks of the metal salts

were prepared in deionised water and sterilized by filter membrane (0.2 µm) and stored at 4°C. The heavy metal solutions were added to tryptone soy agar (TSA; Difco) in various concentrations ranging from 50 to 800 µg/mL. The plates were incubated at 28°C for 48 hours. The concentration of the metal at which the isolate failed to grow on the plate was considered as the MIC. Colour changes of the isolate were also observed.

## 3. Results and Discussion

### 3.1. Bacterial Strains

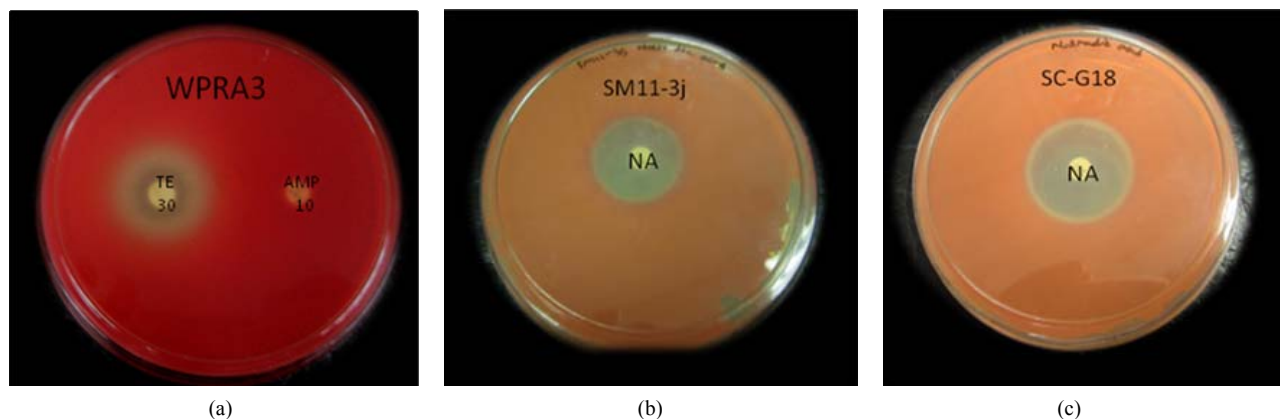
Isolated bacteria were Gram-negative, facultative anaerobic, red pigmented and short rod-shaped bacteria. They were positive for catalase, the citrate utilization test,  $\beta$ -galactosidase, lysine decarboxylase and ornithine decarboxylase. Oxidase and indole tests were negative. Isolate WPRA3 was negative for the Voges Proskauer test, while SM11-3j and SC-G18 isolates were positive. Comparative 16S rRNA gene sequence analysis showed that all three isolates WPRA3, SM11-3j and SC-G18 shared  $\geq 96\%$  similarities with *Serratia* sp.

### 3.2. Antibiotic Susceptibility

This assay revealed that isolates WPRA3, SC-G18 and SM11-3j were highly resistant to penicillin, ampicillin and tetracycline. Isolated bacteria showed high sensitivity to nalidixic acid and they were also susceptible to streptomycin, kanamycin and gentamicin. All isolate were susceptible to chloramphenicol. Antibiotic resistance of the isolate WPRA3, to tetracycline and ampicillin is shown in **Figure 1(a)**, while antibiotic susceptibility of the isolates SM11-3j and SC-G18 to nalidixic acid is shown in **Figures 1(b)** and **(c)**. The antibiotic susceptibility of the isolates WPRA3, SM11-3J and SC-G18 were similar to other non-pathogenic *Serratia* species such as *S. marcescens* subsp. sakuensis JCM 11315 [10], but they were more resistant to antibiotics than *S. nematodiphila* DZ0503SBS1 [11], although in comparison with pathogenic *Serratia* species such as *S. marcescens* strain 08 [12] and *S. marcescens* [13] they were more susceptible to antibiotics. **Table 1** shows the antibiotic susceptibility of isolates WPRA3, SM11-3J and SC-G18 in comparison with other *Serratia* species.

### 3.3. Heavy Metal Resistance

Isolate WPRA3 exhibited the highest heavy metal resistance as compared to isolates SM11-3j and SC-G18. The heavy metal resistance patterns of isolates WPRA3, SM11-3j and SC-G18 were  $\text{Mn, Pb} > \text{Zn} > \text{Cr} > \text{Ni} > \text{Co}$ ,  $\text{Mn} > \text{Cr} > \text{Zn} > \text{Ni}$ ,  $\text{Co}$  and  $\text{Zn} > \text{Ni}$ ,  $\text{Cr} > \text{Co}$  respectively (**Table 2**). Isolates WPRA3, SM11-3j and SC-G18 were unable to grow in metal-agar media at any concentration



**Figure 1.** Antibiotic resistance of isolate WPR3 to tetracycline (TE) and ampicillin (AMP) (a), antibiotic resistance of isolates SM11-3j (b) and SC-G18 (c) to nalidixic acid (NA).

**Table 1.** Antibiotic susceptibility of isolated bacteria in comparison with other *Serratia* species.

Antibiotics	A <sup>1</sup>	B <sup>2</sup>	C <sup>3</sup>	D <sup>4</sup>	E <sup>5</sup>	F <sup>6</sup>	G <sup>7</sup>
Penicillin (10 µg)	R	R	R	S	R	R	R
Ampicilin (10 µg)	R	R	R	S	R	R	R
Tetracycline (30 µg)	R	R	R	S	R	R	R
Gentamicin (10 µg)	S	S	S	S	S	R	R
Chloramphenicol (10 µg, 30 µg)	S	S	S	S	S	S	R
Kanamycin (30 µg)	S	S	S	S	S	R	R
Nalidixic acid (30 µg)	S	S	S	NA	NA	R	S
Streptomycin (10 µg)	S	S	S	S	S	R	R

A<sup>1</sup> = *Serratia* sp. strain WPR3 (JX020764); B<sup>2</sup> = *Serratia* sp. strain SM11-3j; C<sup>3</sup> = *Serratia* sp. strain SC-G18; D<sup>4</sup> = *S. nematophilila* DZ0503SBS1 [12]; E<sup>5</sup> = *S. marcescens* subsp. *sakuensis* JCM 11315T [11]; F<sup>6</sup> = *S. marcescens* strain 08 [13]; G<sup>7</sup> = *S. marcescens* [14]; R = resistance; S = susceptible; NA = not available.

**Table 2.** Minimum inhibitory concentrations of heavy metal for isolated bacteria.

Salt	MIC (ppm)		
	WPR3	SM11-3j	SC-G18
NiSO <sub>4</sub>	450 - 500	100	200
CoSO <sub>4</sub> ·7H <sub>2</sub> O	400 - 450	100	100
Cr(NO <sub>2</sub> ) <sub>3</sub> ·9H <sub>2</sub> O	600	300	200
MnSO <sub>4</sub> ·H <sub>2</sub> O	750 - 800	700	-
Pb(NO <sub>3</sub> ) <sub>2</sub>	750	-	-
CuSO <sub>4</sub>	-	-	-
HgCl <sub>2</sub>	-	-	-
ZnSO <sub>4</sub>	700 - 750	200	500
CdSO <sub>4</sub>	-	-	-

-No growth.

of copper, mercury and cadmium, may because of high toxicity of these metals [7]. Meanwhile, Isolates WPR3 and SM11-3j showed the highest resistance to Mn, may due to the lowest toxicity of Mn amongst other elements

[7]. In the presence of Ni, Pb and Co, isolate WPR3 did not change its colony color and shape, while the color of the colony changed from red to pink in the presence of Zn. Isolate WPR3 was unable to produce color in the presence of Mn and Cr. Color of colonies of isolates SM11-3j and SC-G18 were also changed from red to pink in the presence of Zn. Some *Serratia* species have also been reported as heavy metals tolerant, such as; *Serratia* sp. K1RP-49 could tolerate Pb, whereas it could not grow in the medium supplemented with Cd, Cr, Cu, Ni and Zn [14]. *S. marcescens* Strain C-1 was reported as Cobalt and Nickel resistant. The MIC values for strain C-1 were 30 mM Co (II) and 20 mM Ni (II) [15]. *Serratia* (PL18), isolated from a pitch lake located in Trinidad, was able to tolerate Pb (4 mM), Ni (5 mM), V (9 mM), Se (9 mM) and Zinc sulfate (9 mM) [16]. Moreover, some studies have evaluated the use of *S. marcescens* for the degradation of Mo [17,18] and Cr [18,19].

#### 4. Conclusion

Biochemical, morphological and molecular characteris-

tics showed that the red pigmented isolates belonged to the genus *Serratia*. The *Serratia* sp. isolate WPRA3 (JX020764) and *Serratia* sp. isolate SM11-3j have broad ranges of multi heavy metal (Ni, Co, Cr, Zn, Mn and Pb both in broth and agar medium) and antibiotic (penicillin, ampicilline and tetracycline) resistance. Thus these isolates could potentially be used in the treatment of Industrial effluents. They could also serve as models for studies on heavy metal and antibiotic resistance in marine bacteria.

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