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ISOLATION AND CHARACTERIZATION OF *RHEINHEIMERA* SP. FROM BLACK TIGER SHRIMP *PENAEUS MONODON*

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Abstract: Two bacterial strains designated C9 and C11 were isolated and characterized from black tiger shrimp *Penaeus monodon* maintained in laboratory conditions. The shrimp exhibited tiny white spots along with clinical signs of bacterial infection, probably, due to suboptimal water quality and resulting stress. Absence of white spot syndrome virus was confirmed by PCR. Bacterial isolates obtained from the haemolymph, hepatopancreas and gills were predominated by two types of strains. Strains C9 and C11 were Gram negative, aerobic, nonpathogenic, pigmented, rod shaped and motile. C9 was highly pigmented whereas C11 was less pigmented. Optimum growth temperature for C9 and C11 was 25^0C. Lower salinity was more suitable for growth and pigmentproduction. On the basis of the 16SrRNA gene sequence, the strains C9 and C11 were allotted the accession numbers KR135116 and KR135117, respectively. Strains C9and C11 exhibited no differences in their 16S rRNAsequences. They were shown to belong to the class Gammaproteobacteria, being most closely related to *Rheinheimera aquimaris* with 87% bootstrap support. The DNA G+C content of the two strains C9 (53.36mol%) and C11 (53.14mol%) are the highest reported so far among *Rheinmeinhera* spp. Molecular data demonstrated that both the strains showed 96% bootstrap support witheach other butdifference in their phenotypic characteristics strongly support that they belong to different biotypes.
Keywords: *Rheinheimera,* gammaproteobacteria, *Penaeus monodon,* brackish water.

Introduction

Various types of stressors can predispose a cultured animal to disease, particularly when held in confined conditions. Stressful condition leads to a decrease in diversity of the microbial community and an increase in the numbers of functionally specific microbial groups (Dean-Ross and Mills 1989). These pathogens are generally harmless but they have the ability to change unpredictably to abiotic and biotic factors (Aujoulat *et al* 2012). Conditions such as poor water quality, temperature changes, nutritional deficiencies and over stocking lead to the emergence of opportunistic pathogens, which might become harmful to the cultured animals (Sousa *et al* 1999; Adanir and Turutoglu 2007).

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In the present study, apparently healthy tiger shrimp *Penaeus monodon* were brought from a hatchery, tested and confirmed free of the presence of the commonly occurring viruses, viz; MBV, HPV, IHHNV and WSSV and maintained in the lab for acclimatization and further studies. Small white spots developed on the carapace of the shrimp. They also had typical signs of bacterial infection such as cut antenna, slight reddish discolouration and blackish scar formation on carapace. In the present study, we report the isolation and characterization of two strains of the bacteria in the genus *Rheinheimera*.

Till date, 15 species of the genus *Rheinheimera* have been reported. It was first described by (Brettar *et al* 2002) when they characterized the blue coloured isolates from different depth stations in the central Baltic Sea and proposed the name *Rheinheimera baltica*. Subsequently, *R. pacifica* (Romanenko *et al* 2003), *R. perlucida* (Brettar *et al* 2006), *R. aquimaris* (Yoon *et al* 2007), *R. chironomi* (Halpern *et al* 2007), *R.texasensis* (Merchant *et al* 2007), *R. soli* (Ryu *et al* 2008), *R. tangshanensis* (Zhang *et al* 2008), *R.aquatica* (Chen *et al* 2010b), *R.nanhaiensis* (Li *et al* 2011), *R.longhuensis* (Liu *et al* 2012), *R.tilapia* (Chen *et al* 2013), *R.hassiensis* and *R.muenzenbergensis* (Suarez *et al* 2014) and *R. tuosuensis* have been described (Zhong *et al* 2014). The different species of this genus of bacteria were isolated from seawater, freshwater, chironomid egg mass, soil, alkaline lake, marine sediments, rice roots, fresh water culture pond, rhizosphere of salt tolerant plant species and from saline lake. In this study, we report the isolation and characterization of two *Rheinheimera* strains isolated from cultured black tiger shrimp *Penaeus monodon* during August 2014 in Kerala, India. This is the first report of the presence of *Rheinheimera* sp. in brackish water black tiger shrimp *Penaeus monodon*.

Materials and Methods

The black tiger shrimp *Peneaus monodon* were collected from a reputed hatchery in Kerala. The shrimps were of uniform size, feeding normally with no visible signs of disease. They were kept in a well aerated tank at 6-7 gL^{-1} salinity for acclimatization and subsequent use for further studies. After 10-12 days, antenna cut was observed in some of the shrimps and they developed pinkish to slight reddish discolouration. Some of the shrimps showed tiny white spots on the body surface, with a few showing blackish scar formations on the carapace typical of host defense response (**Fig.1**). They were tested by PCR for the detection of white spot syndrome virus (WSSV) using the IQ 2000 WSSV detection kit.

Fig. 1: Bacterial infection in *P.monodon*

Enumeration of total bacterial population:

The total bacterial densities were enumerated using spread plate method. Shrimps were surface disinfected and one gram tissue samples of gill, muscle, hepatopancreas and 100µl of haemolymph were taken aseptically and processed separately. The tissue samples were homogenized in 9ml of sterile 0.85% saline using sterile mortar and pestle. 0.1ml of each dilution was spread plated onto nutrient agar plates (Himedia) in duplicates and incubated for 24 hour at ambient temperature for enumeration of total heterotrophic bacteria. The haemolymph sample was directly plated on nutrient agar plates without further dilution. Out of the different morphotypes obtained, brownish to black colour colonies that predominated were selected and characterized in the present study. The isolates were subcultured on nutrient agar and Zobell's marine agar medium (Himedia). Single colonies were transferred at least three times to ensure purity, which was verified by colony morphology and pigmentation.

Physiological and Biochemical characterization:

Growth and pigment production at 0, 25, 37 and 42^0C and salt tolerance at 0% (without NaCl supplementation), 0.4, 0.8, 1.2, 1.6 and 2% (w/v NaCl) were tested in nutrient agar and Zobell's marine agar. Biochemical characteristics such as Grams reaction, cytochrome oxidase and catalase were determined. Indole, Methyl red, Voges-Proskaur and citrate utilization, nitrate reduction test were performed. Reaction to different aminoacids such as arginine, ornithine and lysine were also tested. Motility was observed using motility test medium. Furthermore, production of hydrogen sulphide, carbohydrate production test, hydrolysis of starch and urea, antibiotic sensitivity against 18 antibiotics were tested by Kirby Bauer disc diffusion method on Muller Hinton Agar plates (Bauer *et al* 1966). The antibiotics used for the test included Azithromycin (15mcg/disc), Amikacin (30mcg/disc), Ampicillin (10mcg/disc), Cefepime (30mcg/disc), Ciprofloxacin (5mcg/disc), Chloramphenicol (30mcg/disc), Carbenicillin (100mcg/disc), Erythromycin (15mcg/disc), Gentamycin

(10mcg/disc), Kanamycin (30 mcg/disc), Methicillin (5 mcg/disc), Nalidixic acid (30mcg/disc), Penicillin (2 units/disc), PolymyxinB (50 units/disc), Streptomycin (10mcg/disc), Tetracycline (30mcg/disc), Trimethoprim (5 mcg/disc) and Vancomycin (30mcg/disc).

Biofilm assay:

Pure culture of the two strains C9 and C11 were inoculated to sterile glass petriplates containing nutrient broth. Two glass plates which were not inoculated with bacterial strain served as control. The inoculated and control plates were incubated at 25 and 37^0C for 24 h. After incubation, the broth was aseptically removed from the plate for biofilm assay using the crystal violet binding method (Stepanovic *et al* 2004). Plates were washed 3 times with 5ml sterile distilled water to remove the nonattached bacteria and the remaining broth from the plate. The adhered bacteria were stained with 0.1% solution of crystal violet for 15 min and then the excess stain washed off under running tap water. The plates were turned upside down and allowed to dry for a few hours before observing the biofilm formation.

WSSV detection:

DNA extraction was done from 20-30 mg shrimp sample using DTAB-CTAB extraction procedure as described in the IQ 2000 kit (GeneReach Biotechnology Corp., Taiwan). PCR reaction was carried out in a thermal cycler (Biorad) using WSSV specific primers provided in the kit, following the reaction conditions mentioned in the kit. PCR reaction products were separated on 1% agarose gel and observed in a gel documentation system for DNA visualisation.

Bacterial DNA extraction and PCR amplification of 16S rRNA:

Following biochemical characterization upto genus level, the cultures were grown overnight in LB broth at 28^0C. DNA extraction from bacterial samples was carried out using the protocol of Sambrook *et al* 1989. The broth was centrifuged and the pellet resuspended in 567µl of TE buffer with 24µl lysozyme (10mg/ml). 30µl 10% SDS and 3µl of 20mg/ml proteinase K were added to it, mixed well and incubated at 37^0C for 1 h.100µl of 5M NaCl was then added to adjust the NaCl concentration of the DNA solution. 80µl of 10% CTAB was then added slowly and mixed thoroughly before incubation at 65^0C for 10 min. Following two extractions with an approximately equal volume of chloroform/isoamyl alcohol and two extractions with an equal volume of phenol/chloroform/isoamyl alcohol, the DNA was precipitated with 2 volumes of absolute ethanol and washed with cold 70% ethanol. The dried DNA pellet was dissolved in TE buffer, pH 8.0.To identify the strains,

amplification of the 16S rRNA was carried out using primers (Weisburg *et al* 1991) by PCR in a Biorad thermal cycler. Forward primer with sequence AGAGTTTGATCCTGGCTCAG and reverse primer with sequence TACGGCTACCTTGTTACGACTT were used.For PCR, reaction mix (50µl) consisted of 2µl of genomic DNA, 5µl of 10X Taq buffer (10mM Tris-HCl, pH – 9.0., 1.5mMMgCl_2, 50mMKCl, 0.01% gelatin), 4µl of dNTP mix (200µM), 10pmols of each of forward and reverse primers and 1.5 U of Taq polymerase and 34.5µl of distilled water. The reaction condition used was as follows; initial denaturation at 95^0C for 5 min followed by 30 cycles of 95^0C for 1 min, 60^0C for 1 min, 72^0C for 1.5 min and a final extension at 72^0C for 5 min. The purity and size of PCR products were verified by electrophoresis in 1.5% agarose gel with 1X TAE buffer at 80V and visualized in gel doc imaging system.

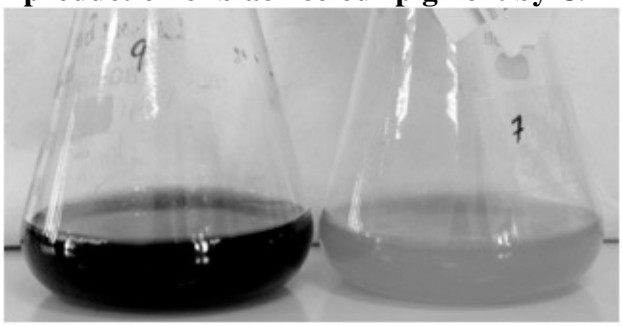
Sequencing and determination of phylogenetic relationships:

The amplified DNA fragments were sequenced with an automated ABI 3100 Genetic analyzer using fluorescent label dye terminators, by M/s Eurofins, Bangalore. Sequencing and assembling of the 16S rRNA gene were carried out as described by (Lane 1991). The resultant 16S rRNA gene sequence of two strains C9 and C11 were compared with available gene sequences from GenBank using the BLAST program and submitted to the GenBank. The sequences were aligned by the multiple alignment package CLUSTAL W. Neighbour joining tree were obtained using MEGA software version 6 (Tamura *et al* 2013).

Results

PCR test carried out to detect WSSV on shrimps that had small white spots on carapace yielded negative result. On bacterial isolation it was found that black to brownish coloured colonies predominated in the nutrient agar plates. The colonies were circular, smooth, regular, shining, convex and opaque. The isolated strains were aerobic, motile, gram negative, oxidase and catalase positive rod shaped bacterium. On nutrient agar, the colony colour ranged from brown to black. However, on ZMA plates they appeared dark creamish initially. After 24h. incubation the strains released more amounts of pigment into the medium. Two types of colonies C9 and C11, different in their pigment production, were observed. After 24 h, C9 was highly pigmented with dark brownish to blackish on ZMA as well as on NA, while C11 showed light black colour on NA and light brown colouration on ZMA (**Fig. 2**). It was noticed that when the incubation time was increased upto 4-5 days the complete medium in the plate turned black. The pigmentation started from the centre of the colony, spread and later covered the entire medium.

Fig. 2: Nutrient broth inoculated with C9 (left) and C11 (right). Note the intense production of black colour pigment by C9



C 9 was weakly positive for arginine and ornithine dihydrolase and completely negative for lysine; whereas C11 was positive for arginine, weakly positive for ornithine and completely negative for lysine. Out of the 21 sugars tested, C9 was able to utilize maltose and glucose; while C11 could utilise only glucose. Both the strains gave negative reaction to all the other sugars. C9 reduced nitrate to nitrite and hydrolysed urea and starch. However, there was no difference in the reaction to IMVIC test, with both the strains testing negative for Indole, methyl red, Voges Proskaur and citrate utilization tests.C11, on the other hand, was nitrate negative, not able to produce urease and amylase (**Table 1**).

Table. 1: Biochemical characterization of KR135116 and KR135117

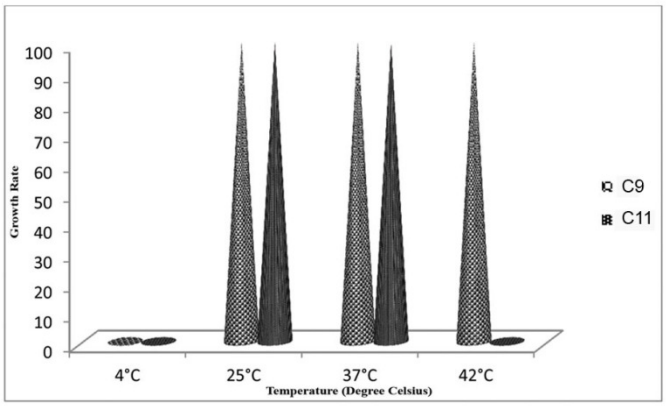
Characteristics	**KR135116**	**KR135117**
Pigmentation	Dark black	Brownish
Gram reaction	Positive rod	Positive rod
Motility	+	+
Catalase	+	+
Oxidase	+	+
Indole	-	-
Methyl red	-	-
VogesProskaur	-	-
Citrate utilisation	-	+
TSI	-	-
Arginine	w+	+
Lysine	-	-
Ornithine	w+	w+
Growth at (^{0}C)		
4	-	-
25	+	+
37	+	+
42	+	-
Growth in NaCl (%)		
0	+	+
1	+	+
2	+	+

3	-	-
6	-	-
Hydrolysis of		
Starch	+	-
Urea	+	-
Nitrate reduction	+	-
Utilisation of		
Glucose	+	-
Arabinose	-	-
Lactose	-	-
Maltose	+	-
Mannose	-	-
Mannitol	-	-
Melibiose	-	-
Raffinose	-	-
Rhamnose	-	-
Cellobiose	-	-
Galactose	-	-
Xylose	-	-
Inositol	-	-
Sorbitol	-	-
Adonitol	-	-
Fructose	-	-
Salicin	-	-
Sucrose	-	-
Dulcitol	-	-
Inulin	-	-
Susceptability to		
Azithromycin	S	S
Amikacin	S	S
Ampicillin	S	S
Cefepime	S	S
Ciprofloxacin	S	S
Chloramphenicol	S	S
Carbenicillin	Resistant	S
Erythromycin	S	S
Gentamycin	S	S
Kanamycin	S	S
Methicillin	Resistant	Resistant
Nalidixic acid	S	S
Penicillin	Resistant	Resistant
Polymyxin B	S	S
Strptomycin	S	S
Tetracyclin	S	S
Trimethoprim	S	S
Vancomycin	S	S

S – sensitive, w+ weakly positive, - negative, + positive

The bacterial strain C9 grew well and produced black colour on nutrient agar and Zobell marine agar incubated at 25^0C, 37^0C and 42^0C. In the case of C11 strain too, growth was observed only at 25^0Cand 37^0C. Optimum growth for both the strains was at 25^0C. At 37^0C, both the strains C9 and C11 took 48h for complete growth with the production pigment in nutrient broth. They did not grow at low temperature (4^0C). However, at 42^0C, growth was observed only in C9 and not in C11 (**Fig. 3**).

Fig. 3: Graphical representation of growth by C9 and C11 at different temperatures



Maximum growth and pigment production of C9 strain was observed when the medium was supplemented with 0.8% NaCl. It grew well at lower salinities (0.4%) and even without NaCl supplementation (0%). Growth and pigment production was considerably less when NaCl supplementation was increased to 1.2%. There was no growth at 1.6% and 2% NaCl supplementation. On the other hand, growth of C11 was, in general, very much less at all concentrations of salt tested in the present study. It grew only at a narrow range of salt concentration (0.4-0.8% salt supplementation) **(Fig. 4a and b**).

Fig. 4a: Graphical representation of pigment production by C9 and C11 at different NaCl concentrations

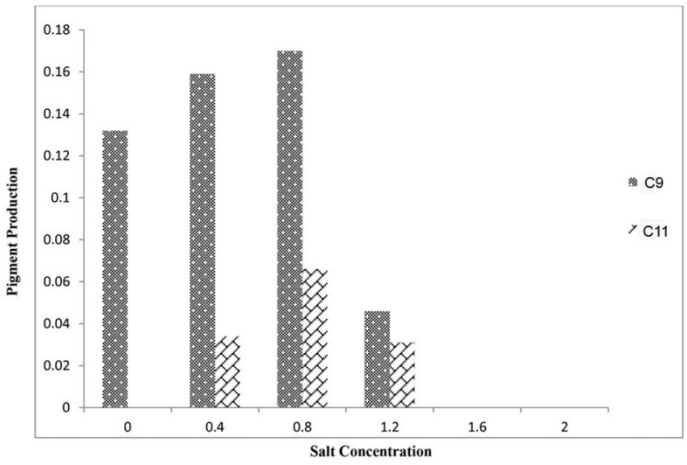
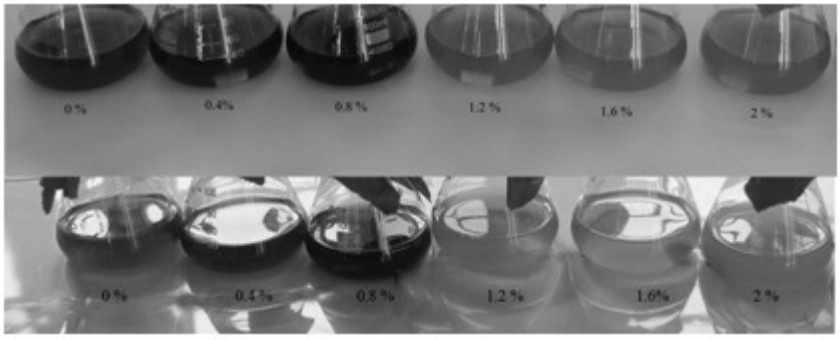
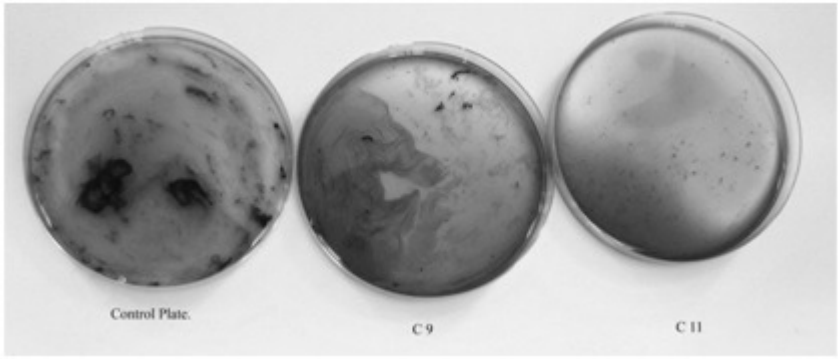


Fig. 4b: Production of pigment by C9 (top) and C11 (bottom) on nutrient broth medium supplemented with different NaCl Concentration (from left - 0, 0.4, 0.8, 1.2, 1.6 and 2%)



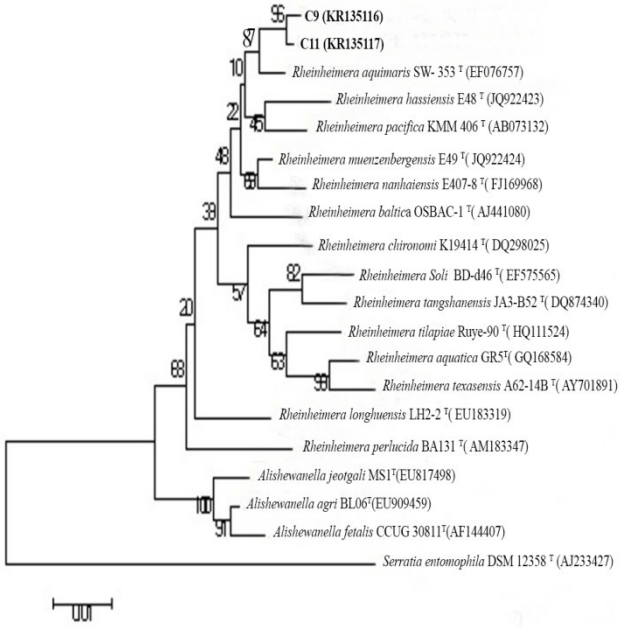
C9 showed resistance to carbenicillin, methicillin and penicillin but was sensitive to other antibiotics tested. C11 showed resistance against methicillin and penicillin but not carbenicillin. Both the strains isolated in the present study did not produce biofilm at 25 and 37^0C. However, a slight biofilm like formation that was not adhesive was noticed with C9 at 25^0C (**Fig. 5**).

Fig. 5: Biofilm assay - control (right) C 9 (middle) and C 11(left)



Phylogenetic relationships among strains C9and C11 with some related taxa are shown in **Fig.6**. The 16S rRNA gene sequence analysis revealed that strains C9 and C11 are most closely affiliated phylogenetically to the genus *Rheinheimera* and were allotted the accession numbers KR135116 and KR135117 respectively. The neighbor – joining phylogenetic tree showed that strains were closely related with *R.aquimaris* with 87% boot strap support. G+C content of strain C9 was 53.36 mol% and C11 was 53.14mol%.

Fig. 6: Phylogenic affiliations ofstrains C9 and C11 andmembers of some related species based on 16S rRNAgene sequences. The branching pattern was generated by the neighbour joining method



Discussion

Colony morphology and biochemical characterization of C9 and C11 were almost similar to other *Rheinheimera* species reported previously. Production of deep brown to black pigmentation was the striking feature observed with these isolates.

Pigmentation is a common feature of bacteria. Usually when environmental conditions become stressful most bacteria are known to produce pigments. However in both the isolates, growth rate of the bacteria and pigment production were positively correlated. Temperature and salinity were found to be influencing factors in pigment production. Nothing is known about the chemistry, production mechanism and ecological role of the pigment. Production of a blue pigment, glaukothalin by marine *Rheinheimera* sp. was reported in 2009 (Grossart *et al* 2009). They too observed that salinity was one of the factors that affected pigment production. They suggested that availability of soluble organic matter affects pigment production of the strains and demonstrated that addition of arginine, an N-rich amino acid, greatly increased the production of glaukothalin. Pigment production has been reported in three other *Rheinheimera* species. *R. tilapiae, R.aquimaris* and *R. aquatica* produced yellow, yellowish white and greenish yellow pigmentation, respectively. *Rheinheimera* genus has been isolated from both marine and freshwater origin. *R.baltica*, *R.pacifica*, *R.perlucida*, *R.aquimaris* were isolated from marine origin, (Brettar *et al* 2002; Romanenko *et al* 2003;

Brettar *et al* 2006 and Yoon *et al* 2007) whereas *R.tilapiae* and *R.texasensis* were isolated from freshwater (Chen *et al* 2013 and Merchant *et al* 2007).

Optimum temperature for growth for C9 and C11 was 25^0C. The highest temperature for growth among *Rheinheinmera* spp. has been reported for *R.chironomi* isolated from egg mass. This species could grow even at a very low temperature of 4^0C (Halpern *et al* 2007). In the present study, strain C9 grew even at a temperature increased upto 42^0C with pigment production. The optimum growth temperature for other *Rheinheimera* species (*R.tuosuensis*, *R.longhuensis*, *R.perlucida*, *R.aquimaris*, *R.baltica* and *R.chironomi*) showed a range between 20-25^0C, 26-34^0C, 20-30^0C 30-37^0C, 20-25^0C and 4-40^0C respectively (Zhong *et al* 2014; Liu *et al* 2012; Brettar *et al* 2006; Yoon *et al* 2007; Brettar *et al* 2002 and Halpern *et al* 2007).

In the present study, the tiger shrimps were maintained at a salinity of 6-7 gL^{-1} in the lab. Therefore, we expected the bacterial strains to grow in media with low salt concentrations. The study of NaCl requirement of the strains confirmed that these strains grew well at low salt concentrations (0%, 0.4% and 0.8% supplementation in nutrient broth). It may be noted that nutrient agar contains 0.8% NaCl. The growth rate and pigment production was decreased with increasing salt concentration. Optimum NaCl concentration of C9 was 0-0.8% whereas the optimum range for C11 was 0.4-0.8%. Tolerance to salinity of different *Rheinheinmera* sp. can be linked to the habitat from where it had been isolated. For instance, the isolates from marine or estuarine habitats had higher tolerance to salinities as demonstrated for *R. pacifica* (0-8%), (Romanenko *et al* 2003) *R.tuosensis* (3-4%) (Zhong *et al* 2014) and *R.baltica* (1-3%) (Brettar *et al* 2002). It has been reported that *R.baltica* strains do not require NaCl for growth. The freshwater isolates *R.tilapiae* and *R.texasensis* didnot require NaCl supplementation for their growth and did not grow at NaCl concentrations greater than 1% (w/v) (Chen *et al* 2013 and Merchant *et al* 2007).

Biofilms contribute a protected mode of growth for microbes that allows survival in a hostile environment. Most of the pathogenic bacteria are able to produce biofilm. Biofilm shows a characteristically higher degree of resistance to antimicrobial agents (Costerton *et al* 1999). In the present study, both the strains did not produce characteristic biofilm, suggesting that the two strains may be non-pathogenic.

Phylogenetic analysis based on 16s rRNA sequences confirmed that the strains C9 and C11 belong to *Rheinheimera* species, yet differed from other recognized members of the same species through differences in phenotypic properties. The G+C content of C9 (KR135116)

and C11(KR135117) is the highest reported among *Rheinheimera* spp.. *R. aquimaris* which is phylogenetically most closely related has a G+C content of 50.1-50.5 mol% (Yoon *et al* 2007). The G+C content of other *Rheinheimera*spp ranges between 48.2-51.0mol%. (Romanenko *et al* 2003; Yoon *et al* 2007; Chen *et al* 2010b; Chen *et al* 2013 and Zhong *et al* 2014). The highest G+C content yet reported for *Rheinheimera* species was 51.9% for *R. aquatic* (Chen *et al* 2010b). In the present study, DNA G+C content of C9 is 53.36 mol% and C11 is 53.14 mol% respectively. Strain C9 and C11 showed 96% bootstrap value, with each other indicating that they belong to the same genomic species, as suggested by (Wayne *et al* 1987). Even though they were genetically highly similar, they showed some intraspecific dissimilarity in their phenotypic expression. These findings strongly support that KR135116 and KR135117 may be different biotypes or biovars.

Acknowledgements

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