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Sequence Analysis of Putative *luxS* Gene Involved in Prodigiosin Biosynthesis from Philippine Local Strains of *Serratia marcescens*

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Abstract

Prodigiosin produced by Serratia marcescens is a bacterial metabolite that has antibiotic, immunosuppressive and anticancer properties involving the luxS gene. The putative luxS gene of the five local S. marcescens strains (B1748, B111, B112, B211, and B212) was amplified by PCR and sequenced and their nucleotide and amino acid sequences were characterized through bioinformatics. The resulting putative luxS nucleotide sequences of the five local strains are highly similar to the nucleotide sequence of two strains of S. marcescens in the EMBL database (Acc. No. EF164926.1 and Acc. No. AJ628150.1) having a maximum identity of 98%, 94%, 97%, 98%, and 100% respectively. However, the five local strains were more related to the clade of EF164926.1 than that of AJ628150.1 using neighbor-joining method of MEGA ver.5. BLASTP Homology search was done and B1748 and B212 strains showed a high degree of homology (100%) with a protein product of the luxS gene of Neisseria gonorrhoeae and Salmonella enterica correspondingly. In addition, a putative conserved domain was detected in all protein sequences from the local strains of S. marcescens. The conserved domain was in the luxS superfamily consisting of the luxS protein involved in autoinducer AI-2 synthesis and its hypothetical relatives. Results of in silico analyses used in this study confirmed presence of putative luxS gene in local strains of S. marcescens with high potential prodigiosin production needed in the manufacture of pharmacological important products. This is the first report in the Philippines on the presence of luxS gene from local isolates of S. marcescens.

Keywords: Prodigision, *luxS* gene, *Serratia marcescens*.

Introduction

Serratia marcescens is a species of gram-negative, short rod-shaped, enteric bacterium in the family Enterobacteriaceae that inhabits a wide variety of ecological niches and causes disease in plant, vertebrate and invertebrate hosts[1]. It is classified as an opportunistic pathogen that causes clinically problematic nosocomial infections because multi-drug resistance is widespread within the species[2].

However, one important characteristic feature of many *S. marcescens* strains which could be of importance to mankind in many ways is the production of cell-associated red color pigment called prodigiosin[3]. Prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) is a linear tripyrrole typical secondary metabolite, appearing only in the later stages of bacterial growth[4]. Prodigiosin may conform to the classical description of a secondary metabolite, having no essential role in the growth or survival of the cell, but instead acting as an overflow for 'waste' products from primary metabolism[5].

Prodigiosins have no defined role in the physiology of producing strains as reported by Demain[6] and Williams et al[7] but has been reported to possess numerous biological activities including antifungal, antibacterial, antiprotozoal and antimalarial properties. Prodigiosins also display immunosuppressive, proapoptotic and anticancer properties and are therefore potentially interesting candidates for drug development[8,9].

The regulation of prodigiosin production is complex, with many environmental inputs[10]. As recently shown in the study of Vendeville et al[11], prodigiosin is regulated by a proposed interspecies quorum sensing (QS) signalling system, AI-2 (LuxS) in some *Serratia* strains. Quorum sensing (QS) is a mechanism whereby bacteria can regulate gene expression in response to the population cell density via detection of a diffusible signaling molecule[12]. In *S. marcescens* 274, *luxS* synthesizes the AI-2 signal, which is required for maximum pigmentation[13]. The *luxS* mutant of Sma 274 exhibits reduced prodigiosin production, haemolysis and virulence, and *luxS* regulation of pigment production does indeed occur via an extracellular signal, most likely AI-2[13].

Recently, *Serratia marcescens*, has been a subject of research interest by the scientific community because of its developing therapeutic potential[14,15]. With this importance, this study is conducted to elucidate results of previous researches on the detection of *luxS* gene in the local strains used through PCR amplification and sequence analysis so as to apply the potential of prodigiosin in local experiments. Result of this study is the first report in the Philippines on the presence of putative *luxS* gene from local isolates of *S. marcescens*.

Material and Methods

Five (5) local strains of *Serratia marcescens* designated as B1748, B111, B112, B211, and B212 were used in this study (figure-1) and were obtained from the strains maintained in the Molecular Biology Laboratory, of the MSU-IIT Department of Biological Sciences, Iligan City, Philippines. All strains were revived from stock cultures and were grown overnight at 37ºC in 3 ml of Luria-Bertani broth which were inoculated later on a Luria agar.

Genomic DNA Extraction and PCR Amplification: Local strains of *Serratia marcescens* were used as the source of target genomic DNA for PCR amplification. Genomic DNA extraction was achieved through the use of Wizard Genomic DNA Purification Kit (Promega Corp., USA), following the manufacturer's instruction. The genomic DNA samples were run on the agarose gel electrophoresis. The gel was then stained using methylene blue to check the presence of genomic DNA.

A nested manufacturer's (GoTaq® PCR Core System I Kit) PCR protocol was followed to amplify the *luxS* gene from genomic DNA of the five local strains of *S. marcescens*. The primers FluxS (5'-GCTGGAACACCTGTTCGC-3') and RluxS (5'-ATGTAGAAACCGGTGCGG-3') were complementary to the conserved published *luxS* gene sequences of various *S. marcescens* strains downloaded from GenBank.

PCR amplification was carried out in a 100µl sample consisting of 10µl of bacterial genomic DNA extract and 90µl of reaction mixtures, which contained 34µl of sterile distilled water, 12µl of 25mM MgCl2, 20µl of 5x Green Flexibuffer, 2µl of 10mM PCR Nucleotide Mix, 10µl of upstream primer, 10µl of downstream primer and 2µl of Taq DNA Polymerase 5u/ml. The reaction mix was overlaid with 75µl of Mineral Oil to prevent evaporation during thermal cycling and was centrifuged in a refrigerated centrifuge machine for 5 seconds. The reaction was performed in a PTC-100 Programmable Thermal Controller with the parameter settings as follows: 1 cycle (predenaturation 94ºC, 5 min; annealing 55ºC, 2 min; and extension 72ºC, 3 min); 30 cycles (denaturation 94ºC, 45 sec; annealing 55ºC, 45 sec; and extension 72ºC, 3 min); 1 cycle (final extension 72ºC, 10 min); and stored at 4ºC. PCR products were resolved by electrophoresis on a 1.4% (w/v) agarose gel/1 x TAE gel and were visualized over a fluorescent lamp after 3 hours of methylene blue staining.

The resulting PCR products were sent to Macrogen Inc. (Seoul, Korea) for sequencing.

Nucleotide and Amino Acid Sequence Analysis: To determine sequence similarity or homology of the *luxS* gene, these nucleotide sequences were compared against sequences found in nucleotide database using the National Center for Biotechnology Information (NCBI) BLASTN tool available at the European Bioinformatics Institute.

The amino acid sequence of the *S. marcescens luxS* gene was deduced using the Expert Protein Analysis System (ExPASY) Translate tool. All the amino acid sequences obtained from the local strains of *S. marcescens* were submitted to the Expasy ProtParam Tool to determine the molecular weight, isoelectric point, N- terminal and amino acid composition.

The deduced protein sequences of the *S. marcescens* were compared with nonredundant protein sequence databases using the BLASTP homology tool of NCBI.

Multiple-sequence alignment was performed using the ClustalW algorithm from the European Bioinformatics Institute (EBI) toolbox.

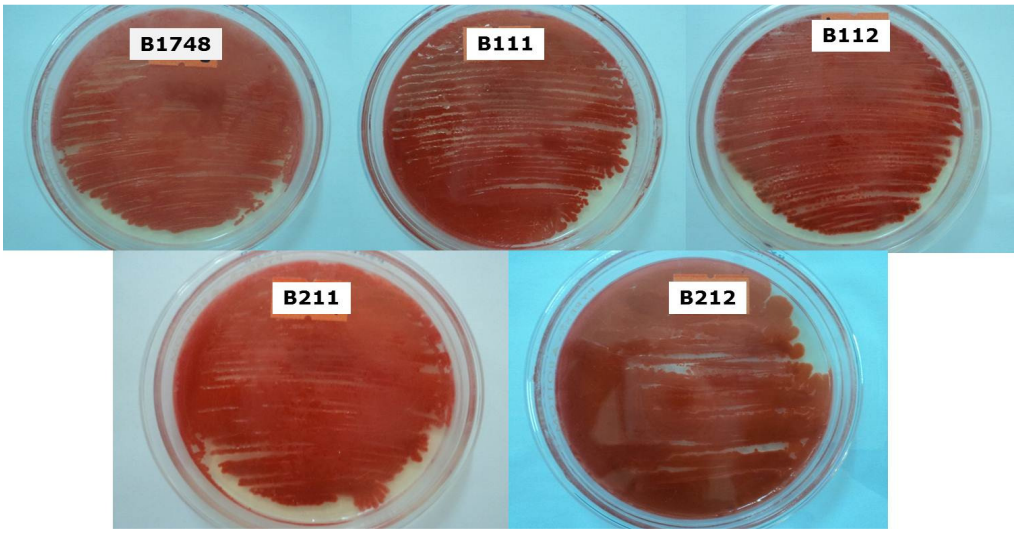

Figure – 1
Cultures of the five local strains of *S. marcescens*

To identify conserved domains, or functional units, within a protein query sequence, the CD-search tool of NCBI was used. CD-search provides annotation of domain footprints and conserved functional sites on protein sequences.

The cladogram tree was constructed using the neighbor-joining (NJ) method of the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0.

Results and Discussion

Nucleotide Sequence Analysis: The resulting nucleotide sequences as performed by the Korean Macrogen Sequencing Services are as follows: the B1748 *luxS* fragment was 678 bp long; and that of the *luxS* fragments of B111, B112, B211, B212 strains were 678 bp, 202 bp, 198 bp, 73 bp and 201 bp long respectively. All five of the nucleotide sequences of the putative *luxS* gene obtained belonged to the *luxS* sequences of *S. marcescens* based on BLASTN search results.

Table – 1
NCBI BLASTN homology search for highly similar sequences on *luxS* gene

Sample from this study	Length (bp)	Accession number	Percent Identity
B1748	678	EF164926.1 and AJ628150.1	98% (53/54)
B111	202	EF164926.1 and AJ628150.1	94% (49/52)
B112	198	EF164926.1 and AJ628150.1	97% (44/45)
B211	73	EF164926.1 and AJ628150.1	98% (55/56)
B212	201	EF164926.1 and AJ628150.1	100% (44/44)

Sequence BLASTN result (table-1) for the *luxS* fragment of B1748 strain of this study showed 98% similar to the two published *luxS* sequences of *Serratia marcescens* in NCBI EMBL-Bank database. These were the *Serratia marcescens* strain H3010 autoinducer-2 synthase (*luxS*) gene, complete cds, 66 bp (Acc. No. EF164926.1) and *Serratia marcescens luxS* gene for autoinducer-2 synthase, strain ATCC 274, 2850 bp (Acc. No. AJ628150.1). Sequences B111, B112, and B211 of this study were similar to the same sequences in the database having a maximum identity of 94% (49/52), 97% (44/45), and 98% (55/56) respectively. It should be specially noted that B212 is identical (100% or 44/44) to the two *luxS* sequences mentioned above. These results suggest that the cloned DNA fragment is most likely the partial *luxS* gene fragment of *S. marcescens* strains.

Amino Acid Sequence Analysis: Figure 2 shows the nucleotide sequence and their corresponding deduced amino acid sequence from the *luxS* fragments of the *S. marcescens* strains B1748, B111, B112, B211, and B212. It also shows that the 678 bp *luxS* fragment of *S. marcescens* B1748 strain corresponds to a 223 amino acid (deduced by the ExPASY Translate tool) polypeptide with a molecular weight (MW) of 25372.0 Daltons and isoelectric point (pI) of 9.96. The translational product of ORF1 deduced from the 202 bp nucleotide sequence of *luxS* fragment of *S. marcescens* B111 strain is a protein of 66 amino acids (aa) with a molecular weight (MW) of 7877.1 Daltons and 11.65 pI. Strains B112, has corresponding polypeptide length of 65 amino acids with a calculated molecular weight of approximately 7210.1 Da and isoelectric point of 10.28. The amplified *luxS* fragment of strains B211 and B212 contained open reading frames (ORF) that encoded a protein of 23 amino acids (calculated molecular weight of 2644.0 Da and 6.03 pI) and 66 amino acids (6944.8 Da and 7.09 pI) respectively.

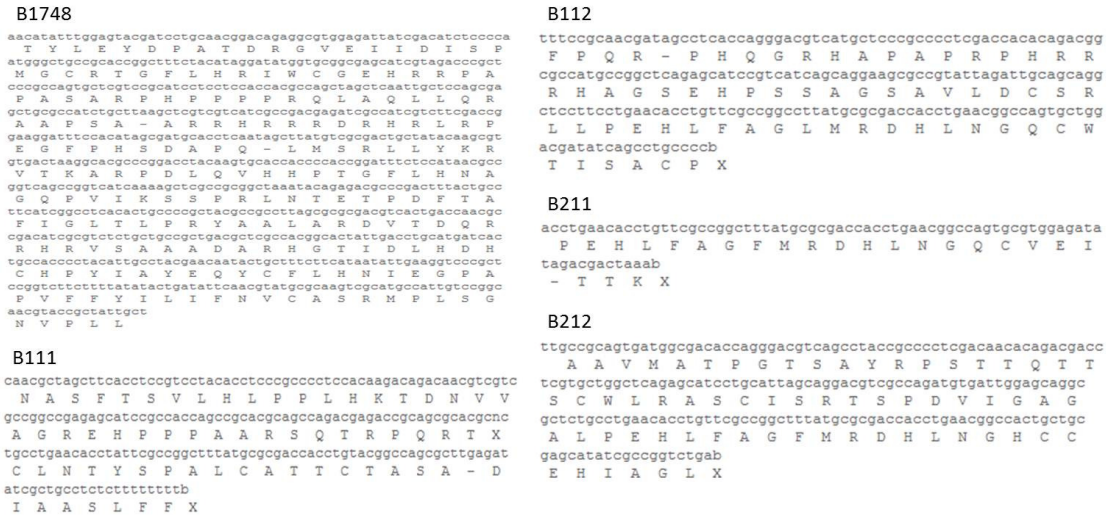

Figure – 2
Nucleotide sequences of *luxS* fragment from the local strains of *S. marcescens* and their corresponding amino acid sequences

Analysis of the nucleotide sequences of *S. marcescens* strains identified an open reading frame (ORF), where the translated amino acid sequence showed significant and conserved homology to the *luxS* from other microorganisms. Each of the putative ORFs is designated as ORFs 1, 2, and 3 in Table 2. Translated amino acid sequence were then compared with entries in the NCBI GenBank (non-redundant protein sequences) in order to find sequences related to the LuxS family of proteins in the local strains of *S. marcescens* using the BLASTP program.

Two *S. marcescens* strains (B1748 and B212) exhibited a high degree of sequence similarity at the protein level with the genes involved in prodigiosin biosynthesis in some bacterial species (table-2). BLASTP homology search of the B1748 strain showed highest sequence similarity of 100% with a protein product of the LuxS of *Neisseria gonorrhoeae* and *Ruminococcus flavefaciens*. Database searches also revealed that all three strains namely, B111, B112 and B212 strains have significant sequence similarity (73%, 93% and 100% respectively) with *Salmonella enterica* subsp. (accession no. EIC61144.1). The amino acid sequence of B112 is 93% identical to LuxS from *Neisseria meningitides* while B212 strain is 83% identical to LuxS protein sequences of *Salmonella enterica* subsp. *Enterica serovar Typhi* strain. In the protein sequences of B211, a high sequence similarity of 95% is shown to the two species of *Serratia* namely, *Serratia odorifera* (DSM 4582 strain) and *Serratia proteamaculans* (568 strain). Protein sequences of B111 strain on the other hand showed a weaker homology with the sequences of *Campylobacter showae* having a maximum identity of 70%.

Multiple Alignment of the LuxS Protein Sequences: The amino acid sequences of *luxS* gene from the local strains of *Serratia marcescens* were multiple-aligned with the LuxS protein sequences from various gram-positive and gram-negative bacteria using ClustalW2 multiple sequence alignment with default settings (figure-3). ClustalW2 analyzes the best match for the selected sequences and lines them up so that the identities, similarities and differences can be seen (European Bioinformatics Institute). An asterisk (*) indicates positions which have a single, fully conserved residue; colon (:) indicates conservation between groups of strongly similar properties; and period (.) indicates conservation between groups of weakly similar properties. These consensus symbols (asterisks, colon and periods) in the alignment are designated by the ClustalW2 program under their corresponding positions. The numerical position of the last amino acid in each row is indicated on the right. Gaps are represented by dashes.

The conserved regions (*), as well as the strong (:) and weak (.) similar properties in the LuxS proteins from the other microorganisms matched with the same regions in the LuxS amino acid sequences of the local strains of *S. marcescens*. Their corresponding physiochemical properties (small or hydrophobic include aromatic except Tyr, Acidic, Basic – H, and Hydroxyl + sulfhydryl + amine + G) of the residues are indicated in red, blue, magenta and green, respectively. They may have an important role in the catalytic activity of LuxS. Sequence alignment of 11 LuxS proteins from the local isolates and a wide range of bacterial species revealed a conserved region. It consists of the amino acid Leucine which is occupied by hydrophobic residues that are particularly well conserved across all species. On the entire alignment, seven conserved substitutions and one semi conserved substitution were exhibited. All of the substitution residues were found only in the protein sequences of *S. marcescens* B1748 strain. Identifying the type of conserved amino acids in the alignment plays a helpful role of predicting protein function and structure.

Table – 2
BLAST protein homology search of ORFs of *S. marcescens* strains

***S. marcescens* Strains**	**ORF**	**Accession**	**Homology**	**Percent Identity**
B1748	ORF 3	YP_209115.1; ABC60260.1	S-ribosylhomocysteinase [*Neisseria gonorrhoeae* FA 1090]; putative AI-2 synthase [*Ruminococcus flavefaciens*]	100% 100%
B111	ORF 1	EIC61144.1; ZP_05364224.1	S-ribosylhomocysteinase, partial [*Salmonella enterica* subsp; S-ribosylhomocysteinase LuxS [*Campylobacter showae* RM3277]	73% 70%
B112	ORF 1	EIC61144.1; YP_001598394.1	S-ribosylhomocysteinase, partial [*Salmonella enterica* subsp. *enterica serovar* Heidelberg str. 41565]; S-ribosylhomocysteinase [*Neisseria meningitidis* 053442]	93% 93%
B211	ORF 2	ZP_06637083.1; YP_001477082.1	S-ribosylhomocysteine lyase [*Serratia odorifera* DSM 4582]; S-ribosylhomocysteinase [*Serratia proteamaculans* 568]	95% 95%
B212	ORF3	EIC61144.1; ZP_03342525.1	S-ribosylhomocysteinase, partial [*Salmonella enterica* subsp. *enterica serovar* Heidelberg str. 41565]; S-ribosylhomocysteinase [*Salmonella enterica* subsp. *enterica serovar* Typhi str. 404ty]	100% 83%

Figure – 3
Multiple alignment of the LuxS protein sequences of *Serratia marcescens* strains with the luxS from *Neisseria gonorrhoeae* (acc. no. YP_209115.1), *Neisseria meningitides* (acc. no. YP_001598394.1), *Salmonella enterica* subsp. (acc. no. EIC61144.1), *Salmonella enterica* subsp (acc. no. ZP_03342525.1), *Serratia odorifera* (acc. no. ZP_06637083.1) and *Serratia proteamaculans* (acc. no. YP_001477082.1)
Legend: (*)-conserved region; (:)-conserved substitution; and (.)-semi conserved substitution

Sequencing result also shows the presence of the precursors to prodigiosin similar to the report of Williams[16] such acetate, serine, alanine, methionine and proline. Recently, the mechanism of proline incorporation into a pyrrole moiety has been shown biochemically and a pathway for synthesis of undecylprodigiosin proposed[17,18].

Conserved Domain Identification: A putative conserved domain was detected in all protein sequences of the local strains of *S. marcescens* used in this study. The conserved domain was in the LuxS superfamily consists of the LuxS protein involved in autoinducer AI-2 synthesis and its hypothetical relatives. Analysis of the functional domains among the luxS homologues may help unravel the underlying mechanism of luxS function. CD-search acknowledges that protein domain families may be very diverse and that they may contain sets of related subfamilies.

LuxS (S-ribosylhomocysteinase) is a key metabolic enzyme that catalyzes the cleavage of the thioether bond in S-ribosylhomocysteine (produced by the detoxification of S-adenosylhomocysteine) to produce homocysteine (which is recycled back to methionine) and AI-2, the precursor of type II bacterial quorum sensing molecule[19,20]. Winzer et al[21] reported that AI-2 is not a single chemical entity but is a collective term used for a group of furanone derivatives which form spontaneously from the same precursor, 4,5,-dihydroxy-2,3-pentanedione (DPD). Furanones have been implicated as signaling molecules in both bacteria and eukaryotes.

Further, Medina et al[22] cited that the function of *luxS* to generate homocysteine plays an important role in bacterial metabolism due to homocysteine's three main fates: to be remethylated to methionine, to enter the cysteine biosynthesis pathway, and to be released into the extracellular environment.

Phylogenetic Analysis: A phylogenetic analysis of the *luxS* nucleotide sequences was performed by the neighbour-joining method using MEGA. Based on the cladogram tree result (figure-4), all five local strains of *S. marcescens* used in this study appear to be evolutionarily more related to that of *Serratia marcescens* strain H3010 (Acc. No. EF164926.1) than to the *Serratia marcescens* strain ATCC 274 (Acc. No. AJ628150.1). It also shows that the two local strains of *S. marcescens*, B112 and B212, are almost identical to each other.

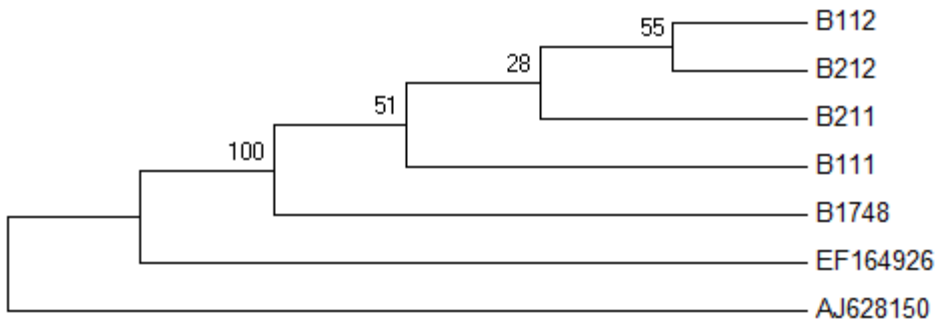

Figure – 4
Cladogram tree result by NJ (all gap-free sites) method of the MEGA software with bootstrap values

The method for the amplification and sequencing of *luxS* genes from local strains of *S. marcescens* reported in this paper may be useful for the rapid development of specific probes, and for taxonomic analysis. Future studies will include taxonomic identification by examining their morphological and biochemical properties, cloning of *luxS* genes into vectors, construction of *luxS* mutants and confirmation by gene inactivation.

Conclusion

The *in silico* analyses conducted in this study confirmed the presence of putative *luxS* gene in local strains of *S. marcescens*. Knowing its presence in local strains could boost the interest of the local scientific community towards further studies on production of prodigiosin using local strains for prospects of pharmacological applications and other possible industrial utilization.

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