

1 **Discovery of *Pandoraea pnomenusa* RB38 *N*-acyl homoserine Lactone Synthase (PpnI)**
2 **and its Complete Genome Sequence Analysis**

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12

13 **Abstract:** In this study, we sequenced the genome of *P. pnomenusa* RB38 and
14 reported the finding of a pair of cognate *luxI/R* homologs which we firstly coined
15 as *ppnI*, which is found adjacent to a *luxR* homolog, *ppnR*. An additional orphan
16 *luxR* homolog, *ppnR2* was also discovered. Multiple sequence alignment revealed
17 that PpnI is a distinct cluster of AHL synthase compared to those of its nearest
18 phylogenetic neighbor, *Burkholderia* spp. When expressed heterologously and
19 analysed using high resolution tandem mass spectrometry, PpnI directs the
20 synthesis of *N*-octanoylhomoserine lactone (C8-HSL). To our knowledge, this is
21 the first documentation of the *luxI/R* homologs of the genus of *Pandoraea*.

22 **Keywords:** Quorum sensing; cell-to-cell communication; *N*-acyl homoserine lactone
23 (AHL); Miseq; PacBio; whole genome mapping (WGM); Opgen; Multi Locus
24 Sequence Typing (MLST); *ppnI*; *ppnR*

25

26 **INTRODUCTION**

27 The theory of “quorum sensing” (QS) was coined in the late nineties describing
28 bacterial cell-to-cell communication for various gene expression regulations (Bainton *et al.*
29 1992; Miller & Bassler 2001; Schauder & Bassler 2001). This communication is
30 accomplished through secretion and detection of small hormone-like chemical molecules
31 known as autoinducers which facilitate intra- and inter-species microbial communications.
32 There are different classes of autoinducers where upon reaching a threshold concentration,
33 these signaling molecules activate and stimulate a wide variety of gene expression (Davies *et*
34 *al.* 1998; Williams *et al.* 2007). The most studied QS molecules is *N*-acyl homoserine lactone
35 (AHL) which is secreted by Gram-negative proteobacteria especially in the class of alpha-,
36 beta- and gamma-proteobacteria subdivisions. AHL typically consists of a homoserine
37 lactone moiety (Williams *et al.* 2007) and an *N*-acyl side chain with various chain length (C4-
38 C18), degree of saturation at C-3 position and presence of a hydroxy-, oxo- or no substituent
39 at the C3 position (Chhabra *et al.* 2005). AHL synthase, also known as the LuxI homologs,
40 together with the AHL receptor protein known as LuxR homologs, are two typical principal
41 protein families in AHLs QS system. Briefly, in this QS system, AHLs are secreted by LuxI
42 homologs until a threshold concentration of AHL is attained before they bind to LuxR
43 homologs and subsequently activate a cascade of QS-regulated gene expression (Fuqua,
44 Parsek & Greenberg 2001; Swift *et al.* 2001; Swift *et al.* 1996).

45 *Pandoraea* was believed to be originated from the term “Pandora box” which referred
46 to the source of all evil in Greek mythology. Predominantly isolated from cystic fibrosis (CF)
47 patients, *Pandoraea* species were also recovered from other clinical specimen and soil
48 environment (Coenye *et al.* 2000; Daneshvar *et al.* 2001). Clinical manifestations of this
49 terrorizing pathogen revolved around nosocomial infections with its capability to deteriorate
50 lung function (Caraher *et al.* 2008; Costello *et al.* 2011; Stryjewski *et al.* 2003) and even
51 causes multiple organ impairment (Stryjewski *et al.* 2003). However, detailed mechanism of
52 its colonization remain unknown despite emerging clinical documentations on this respiratory
53 pathogen (Atkinson *et al.* 2006; Daneshvar *et al.* 2001; Stryjewski *et al.* 2003). To date,
54 *Pandoraea* sp. is recognized as one of the less studied CF pathogens that requires further
55 investigations particularly in its bacterial pathogenicity (Callaghan & McClean 2012). To
56 aggravate the situation, *Pandoraea* sp. was often misidentified in many clinical laboratories
57 leading to the lack of clinical documentation on its virulence potential (Hogardt *et al.* 2009).
58 On the other hand, *Pandoraea* sp. have considerable attractions in biotechnological
59 applications with various degradation abilities such as lignin degradation (Shi *et al.* 2013),

60 polychlorinated biphenyls (PCBs) biodegradation (Dhindwal *et al.* 2011), and sulphur oxidation
61 (Anandham *et al.* 2008).

62 Previously, we reported the first documentation of *P. pnomenusa* RB38 isolated from
63 a non-operating landfill site which produce C8-HSL (Ee *et al.* 2014b). As there is no report of
64 AHL synthase in the *Pandoraea* genus, we sought to identify the presence of the AHL
65 synthase in the genome of *P. pnomenusa* RB38 and further study it. We started the
66 experiment by sequencing the complete genome of *P. pnomenusa* RB38 to provide the vital
67 groundwork to understand this strain comprehensively prior to gene hunting. As QS is well-
68 known to regulate various gene expressions such as virulence factors, identification of the
69 LuxI/R homologs will be useful for further investigations on the QS-regulated gene
70 expression. To our best knowledge, this is the first documentation of the QS system in the
71 genus of *Pandoraea*.

72 **METHODS**

73 **Bacterial Strains and Culture Conditions**

74 LB medium (Scharlau, Spain) was used as the only culture media in the experiment.
75 The AHLs biosensors used in this experiment was *Chromobacterium violaceum* CV026,
76 *Escherichia coli* [pSB401] and *E. coli* [pSB 1142] while *Erwinia carotovora* GS101 and *E.*
77 *carotovora* PNP22 was used as the positive and negative control for screening of AHLs
78 production. All isolates were cultured routinely in Luria-Bertani (LB) agar or broth in 28°C
79 with exception to *Escherichia coli* [pSB401], *E. coli* [pSB 1142] and *E. coli*
80 BL21(DE3)pLysS, which were cultured aerobically at 37°C.

81 **Complete Genome Sequencing**

82 Complete genome sequencing was performed using Pacific Biosciences (PacBio) RS
83 II Single Molecule Real Time (SMRT) sequencing technology (Pacific Biosciences, Menlo
84 Park, CA) as described previously (Chan, Yin & Lim 2014; Ee *et al.* 2014c). Briefly, the
85 prepared 10-kb template library was sequenced on 4 single molecule real time (SMRT) cells
86 using P4-C2 chemistry. *De novo* assembly was performed by filtering insert reads using
87 RS_filter protocol (version 2.1.1) prior to assembly with Hierarchical Genome Assembly
88 Process (HGAP) workflow in SMRT portal (version 2.1.1). Gene prediction was conducted
89 using Prodigal version 2.60 (Hyatt *et al.* 2010).

90 **Whole Genome Mapping**

91 Whole genome mapping was performed using OpGen Argus® system (OpGen,
92 Gaithersburg, MD) according to manufacturer's instructions. Briefly, high molecular weight
93 DNA was isolated from single colony of sample strain using Argus High Molecular Weight
94 (HMW) DNA Isolation Kit. DNA quality and concentration were determined using the Argus
95 QCard kit. Single DNA molecules were then flowed through a microfluidic channel that was
96 formed by Channel Forming Device (CFD), and were immobilized on charged glass surface.
97 By using Enzyme Chooser software, BamHI was selected as the optimal restriction
98 endonuclease for *P. pnomenusa* RB38, based on the FASTA-formatted sequence generated
99 from PacBio RS II sequencing technology. The DNA molecules were digested on the glass
100 surface to maintain the fragment order, and were stained with fluorescence dye. The image of
101 DNA fragments were captured using fluorescence microscopy and fully automated image-
102 acquisition software. The single-molecule maps were assembled by overlapping DNA
103 fragment patterns to produce a whole genome map (WGM) with a minimum of 30X
104 coverage. The WGM was aligned with PacBio FASTA-formatted sequences using sequence
105 placement tool in MapSolver software (OpGen, Gaithersburg, MD).

106 **Identification of Putative *N*-acyl Homoserine Lactone Synthase and Gene Cloning**

107 The predicted open reading frames (ORF) were further annotated by comparing
108 against NCBI-NR (<ftp://ftp.ncbi.nlm.nih.gov/blast/db/>) and Uniprot databases
109 (<http://www.uniprot.org/>) to locate the AHL synthase. The putative *ppnI* sequence was then
110 sent to GenScript Inc. for gene cloning service where it was cloned in pUC57 vector
111 (GeneScript, Piscataway, NJ) prior to direct cloning into pGS-21a expression vector. The
112 resulting pGS-21a::*ppnI* plasmid was transformed into competent *E. coli* BL21(DE3)pLysS.
113 Ampicillin (100µg/ml) and chloramphenicol (34µg/ml) (CalBioChem, Merck Millipore,
114 Billerica, MA) were added for transformation selection purpose.

115 **Screening of AHL Production**

116 Preliminary screening of AHL was performed by streaking transformed *E. coli* with
117 gene of interest against *C. violaceum* CV026 biosensor prior to 37°C incubation overnight. *E.*
118 *coli* harboring only vector pGS-21a without the gene of interest was included as negative
119 control.

120 AHL extraction was performed as previously described (Ee *et al.* 2014a). Briefly,
121 planktonic culture of transformed *E. coli* with gene of interest was extracted twice with equal
122 volume of acidified ethyl acetate (0.1% glacial acetic acid) and left to complete desiccation
123 until further analysis (Ortori *et al.* 2011).

124 AHL profile was confirmed using LC-MS/MS triple quadrupole mass spectrometry
125 (Agilent 1290 Infinity LC and Agilent 6490 Triple Quadrupole LC/MS systems, Agilent
126 Technologies, Santa Clara, CA) as described previously (Ee *et al.* 2014a; Lim *et al.* 2014).
127 AHL detection was performed using precursor ion mode where the precursor ion *m/z* value
128 was scanned from 80 to 400. Agilent MassHunter software was used for data analysis.

129 **Thin Layer Chromatography**

130 Thin layer chromatography was conducted with loading of 25 μ L of extracted AHLs
131 (in 100 μ L of ACN) on activated reverse phase C18 TLC plate (TLC aluminium sheets 20cm
132 \times 20cm, RP-18 F254s, Merck, Darmstadt, Germany) (Shaw *et al.* 1997). Synthetic AHLs of
133 *N*-octanoyl-L-homoserine lactone (C8-HSL) (Sigma–Aldrich, St Louis, MO) were included as
134 positive control and the chromatography was performed in 60% methanol: 40% water volume.
135 Once completed, the TLC plate was air-dried and seeded with overnight culture of CV026
136 biosensor before it was left for overnight incubation (Chen *et al.* 2013; Lim *et al.* 2014).

137 **RESULTS AND DISCUSSION**

138 **Complete Genome Sequencing**

139 In this study, PacBio RSII SMRT sequencing technology was used as the sequencing
140 platform in which the genome of *P. pnomenus* RB38 was assembled into a single contig
141 (GenBank accession number CP007506.1). With an average coverage of 190-fold, 4755
142 ORFs were revealed in the 5.3797Mb complete genome of *P. pnomenus* RB38. By using
143 Gepard (Krumisiek, Arnold & Rattei 2007), a dot matrix analysis was performed on the
144 FASTA formatted sequence file of the genome which confirmed the circular topology of the
145 assembly (data not shown).

146 This complete genome was then validated using OpGen WGM processed with
147 BamHI. Optical mapping is commonly used as one of the laboratory techniques to provide a
148 structural scaffold for contigs orientation as well as to visually identify errors in genome

149 assemblies by using constructed whole genome restriction maps (Nagarajan, Read & Pop
150 2008). Perfect alignment of the WGM (5.146Mb) constructed with the complete genome
151 assembly of *P. pnomenusa* RB38 confirmed the accuracy of the finished genome sequence.

152 **Multilocus Sequence Typing (MLST)**

153 *Pandoraea* spp. belong to the beta-subclass of Proteobacteria with *Burkholderia* and
154 *Ralstonia* as the closest neighbor (Coenye *et al.* 2000). In clinical microbiology laboratory,
155 *Pandoraea* spp. is often misidentified as *Burkholderia cepacia* complex (Bcc) or *Ralstonia*
156 spp or initially reported as non-fermentative Gram-negative bacilli (Aravena-Román 2008;
157 Coenye *et al.* 2001). Initial annotation of *P. pnomenusa* RB38 complete genome using Rapid
158 Annotation using Subsystem Technology (Version 4.0) (<http://rast.nmpdr.org/rast.cgi>)
159 misidentified *Burkholderia* sp. CCGE1001 as the closest identity. However, isolate
160 identification performed in previous study using 16S rDNA sequencing and Matrix-assisted
161 Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)
162 identified strain RB38 as *P. pnomenusa* [24].

163 Multilocus sequence typing (MLST) is considered as the “gold standard” of accurate
164 typing and identification of bacterial species (Larsen *et al.* 2012). As there is currently no
165 MLST studies available for *Pandoraea* species, we employed the *Burkholderia* MLST
166 database as a reference and also to study the possibility of employing *Burkholderia* MLST in
167 distinguishing *Pandoraea* genus effectively. With the availability of the whole genome
168 sequence data for strain RB38, Multilocus Sequence Typing (MLST) analysis was performed
169 (**Table 1**), where seven conserved housekeeping genes (*atpD*, *gltB*, *gyrB*, *recA*, *phaC*, *lepA*
170 and *trpB*) from genomic sequences of *P. pnomenusa* RB38 were blasted against NCBI-NR
171 database for the nearest identity. As expected, all conserved housekeeping genes with
172 exception of *lepA* gene successfully distinguished *Pandoraea* sp. as the closest organism [24].

Genes	First Match			Second Match		
	Strain	Identities	Expect	Strain	Identities	Expect
ATP synthase beta chain (<i>atpD</i>)	<i>Pandoraea</i> sp. SD6-2	444/463 (96%)	0	<i>Burkholderia vietnamiensis</i> G4	425/464 (92%),	0
Glutamate synthase large	<i>Pandoraea</i> sp. SD6-2	1496/1563 (96%),	0	<i>Burkholderia terrae</i>	1308/1566 (84%)	0

subunit (<i>gltB</i>)						
DNA gyrase subunit B(<i>gyrB</i>)	<i>Pandoraea</i> sp. SD6-2	744/825 (90%),	0	<i>Burkholderia ambifaria</i> MC40-6	657/830 (79%)	0
Recombinase A (<i>recA</i>)	<i>Pandoraea</i> sp. SD6-2	338/349 (97%)	0	<i>Burkholderia rhizoxinica</i> HKI 454	305/342 (89%)	0
Acetoacetyl-CoA reductase(<i>phaC</i>)	<i>Pandoraea</i> sp. SD6-2	224/247 (91%)	e-169	<i>Burkholderia phymatum</i> STM815	173/247 (70%)	e-130
GTP binding protein (<i>lepA</i>)	Not Found	Not Found	Not Found	Not Found	Not Found	Not Found
Tryptophan synthase subunit beta (<i>trpB</i>)	<i>Pandoraea</i> sp. SD6-2	384/400 (96%)	0	<i>Ralstonia pickettii</i> 12J	335/393 (85%),	0

173 **Table 1: Multi-Locus Sequence Typing Analysis of *P. pnomenusa* RB38.** Seven
 174 housekeeping genes in *P. pnomenusa* RB38 were analyzed where six out of seven conserved
 175 genes of *Pandoraea* were successfully distinguished from *Burkholderia*. All six MLST
 176 sequences shows an expect-value of 0.0, which reflects high similarity except for acetoacetyl-
 177 CoA reductase (*phaC*) that gives the value of expect-value169 for *Pandoraea* sp. SD6-2 and
 178 expect-value130 for *Burkholderia phymatum* STM815. As shown below, *P. pnomenusa*
 179 RB38 is highly similar to *Pandoraea* sp. SD6-2 with higher identities.

180 **Identification and *in silico* Analysis of *luxI/R*-Type QS Genes**

181 We previously reported the QS activity of *P. pnomenusa* RB38 (Ee *et al.* 2014b). In
 182 this study, we identified the putative *luxI* and *luxR* homologs from the annotated genome.
 183 Firstly, a 786 bp putative *N*-acyl homoserine lactone synthase (DA70_23485) (designated as
 184 *ppnI* gene) was identified. Conserved domain analysis of the predicted proteome of this gene
 185 indicated presence of autoinducer synthase domain (PFAM signature: PF00765) which
 186 further confirmed that this gene is a genuine LuxI homolog. Further, a 702 bp putative
 187 cognate LuxR homolog (DA70_23490) (designated as *ppnR* gene) located in close proximity
 188 and in a convergent transcriptional orientation to the *ppnI* gene was also manually identified
 189 (**Figure 1**). Presence of LuxR homolog in close proximity to the LuxI homolog is commonly
 190 observed in the typical LuxI/LuxR-type QS circuit (Schaefer *et al.* 2013). In order to confirm

191 the authenticity of this putative LuxR homolog, the predicted protein sequence was scanned
 192 and was confirmed to contain the universal conserved domain organization of LuxR proteins
 193 namely: the autoinducer binding domain (PFAM03472) and C-terminal DNA-binding
 194 domain of LuxR-like proteins (cd06170) (Choi & Greenberg 1992; Fuqua, Parsek &
 195 Greenberg 2001; Hanzelka & Greenberg 1995).

196 In addition, further search in the genome also indicated presence of an additional
 197 putative *luxR* homologous gene (designated as *ppnR2*) which was not associated with a *luxI*
 198 homolog and is therefore referred in this study as a putative orphan LuxR regulator. Orphan
 199 LuxR is hypothesized to occur as a result of genes re-organizations, horizontal gene transfer
 200 or independent evolution of transcriptional regulatory circuits (Patankar & González 2009b).
 201 Various studies have reported identification of orphan LuxR in numerous bacteria and orphan
 202 LuxR was also found to interact with AHLs to regulate a variety of gene expression (Malott
 203 *et al.* 2009; Patankar & González 2009a; Subramoni & Venturi 2009)

204 Furthermore, phylogenetic analyses based on amino acid sequences performed
 205 indicated that both the PpnI/PpnR1 pair and the orphan PpnR2 are distant from LuxI or LuxR
 206 homologues of its closest phylogenetic neighbour, both *Burkholderia* and *Ralstonia* species
 207 (**Figures 1, 2, 3**). To the best of our knowledge, this is the first documentation of LuxI/R
 208 homologs of the *Pandoraea* species.

209

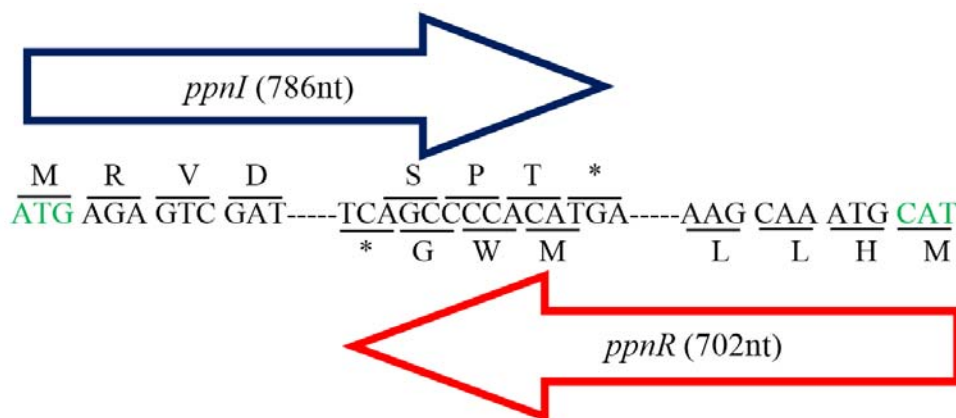
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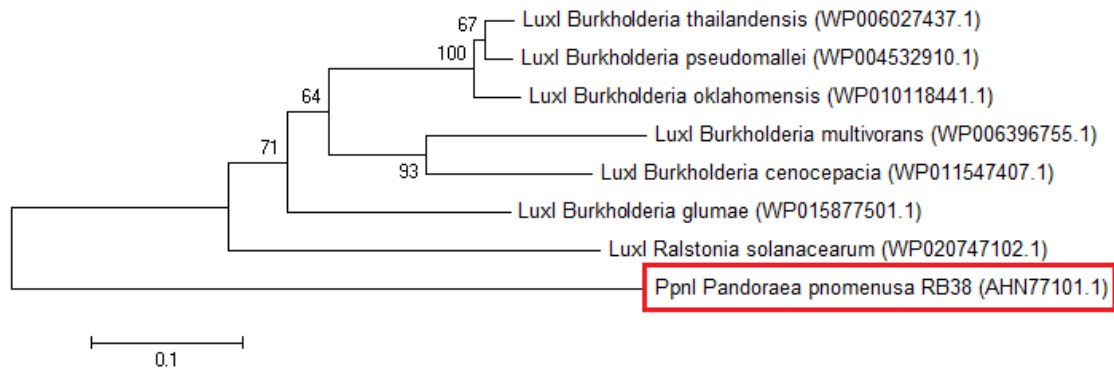
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215 **Figure 1. Gene map showing organization of *ppnR* (*luxR* homolog) and *ppnI* (*luxI***
 216 **homolog).** The direction of the arrows indicated the orientation of both genes where *ppnI* is
 217 in 5'-3' direction while *ppnR* is in 3'-5' direction. A line was used to indicate the nucleotide
 218 sequences and its respective amino acid sequence. Start codon, Methionine (M) was

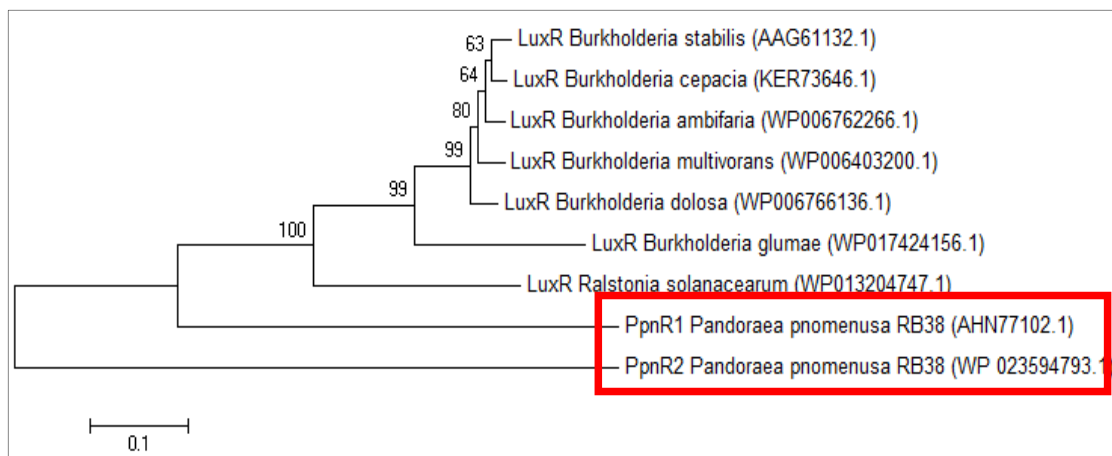
219 represented by green font; while asterisk represented stop codon (TGA). The *ppnR* and *ppnI*
220 genes sequences had been deposited in GenBank database with GenBank accession number
221 AHN77102.1 and AHN77101.1, respectively.



222

223

224 **Figure 2. Phylogenetic tree of PpnI.** Neighbor-Joining method (Saitou & Nei 1987) was
225 used in MEGA6 (Tamura *et al.* 2011) where bootstrap tests (1000 replicates) were shown
226 next to the branches (Felsenstein 1985). This analysis involved 8 amino acid sequences with
227 their GenBank accession numbers as listed: LuxI *Burkholderia thailandensis*
228 (WP006027437.1), LuxI *Burkholderia pseudomallei* (WP004532910.1), LuxI *Burkholderia*
229 *oklahomensis* (WP010118441.1), LuxI *Burkholderia multivorans* (WP006396755.1), LuxI
230 *Burkholderia cenocepacia* (WP015877501.1), LuxI *Burkholderia glumae* (WP015877501.1),
231 LuxI *Ralstonia solanacearum* (WP020747102.1) and PpnI *Pandoraea pnomenusa* RB38
232 (AHN77101.1).



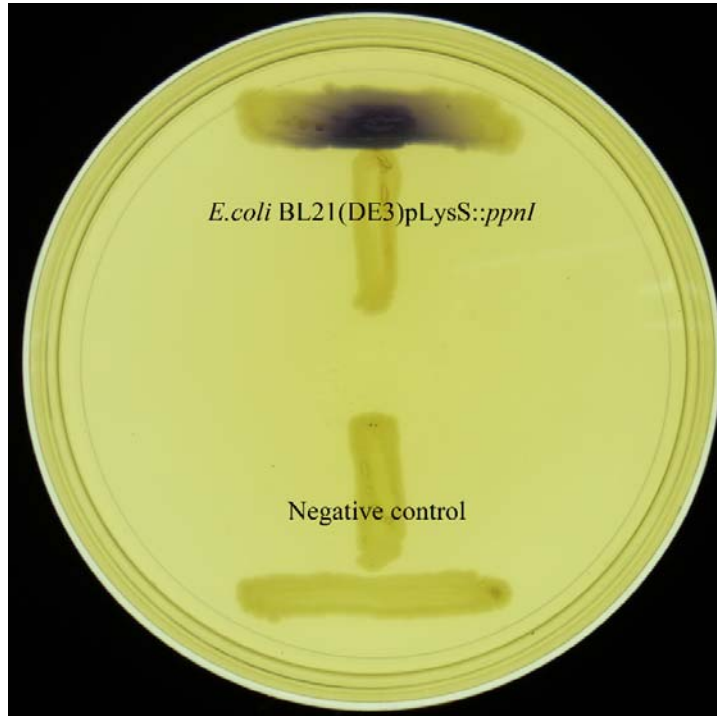
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234 **Figure 3. Phylogenetic tree of PpnR.** Neighbor-Joining method (Saitou & Nei 1987) was
235 used in MEGA5 (Tamura *et al.* 2011) where bootstrap tests (1000 replicates) were shown
236 next to the branches (Felsenstein 1985). This analysis involved 9 amino acid sequences with
237 their GenBank accession numbers as listed: LuxR *Burkholderia stabilis* (AAG61132.1),
238 LuxR *Burkholderia cepacia* (KER73646.1), LuxR *Burkholderia ambifaria*
239 (WP006762266.1), LuxR *Burkholderia multivorans* (WP006403200.1), LuxR *Burkholderia*
240 *dolosa* (WP006766136.1), LuxR *Burkholderia glumae* (WP017424156.1), LuxR *Ralstonia*
241 *solanacearum* (WP013204747.1), PpnR1 *Pandoraea pnomenusa* RB38 (AHN77102.1) and
242 PpnR2 *Pandoraea pnomenusa* RB38 (WP023594793.1).

243 **Functional Study of Putative *ppnI* Gene**

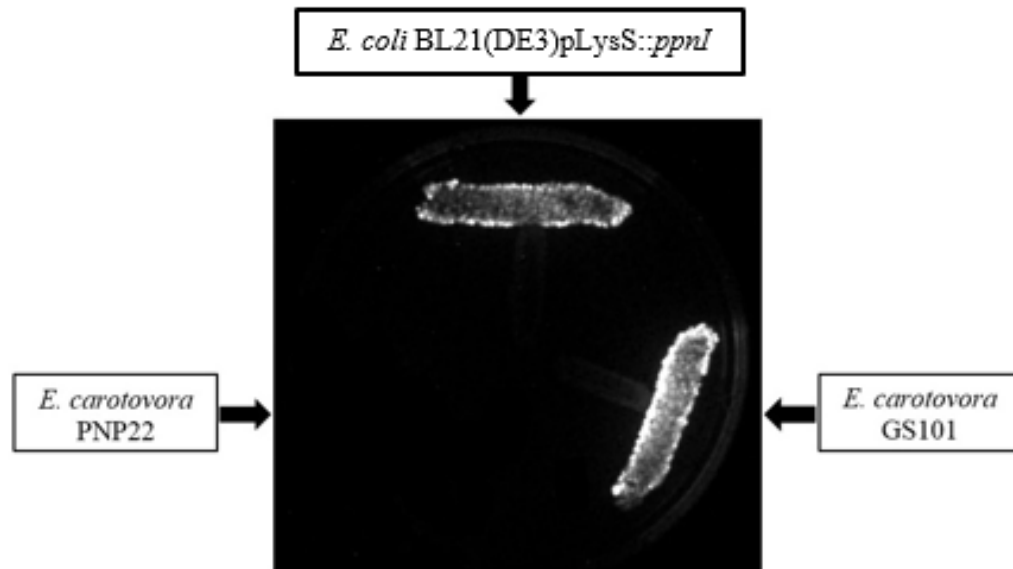
244 For functional studies, we cloned the putative *ppnI* into a pGS-21a expression vector and
245 subsequently transformed the pGS-21a::*ppnI* plasmid into competent *E. coli*
246 BL21(DE3)pLysS. AHL screening were performed using *C. violaceum* CV026 biosensor with
247 *E. coli* BL21(DE3)pLysS::*ppnI*. The result of the cross-streak bioassay demonstrated
248 activation of purple violacein secretion of *C. violaceum* CV026 (**Figure 4A**) as well as
249 chemiluminescence activity of *E. coli* [pSB 401] indicating the production of short chain
250 AHLs by the *ppnI* gene (**Figure 4B**). Besides that, formation of a sole purple violacein spot
251 on CV026 lawn which correspond to the same retention time of the synthetic C8-HSL
252 suggested that the *ppnI* is responsible for the production of C8-HSL in *P. pnomenusa* RB38
253 (**Figure 5**). The AHL profile of *ppnI* was further verified using LC/MS mass spectrometry
254 system and only C8-HSL was detected in the supernatant of transformed *E. coli* BL21
255 suggesting that *ppnI* is indeed the functional LuxI synthase of *P. pnomenusa* RB38.

256 **4(A)**



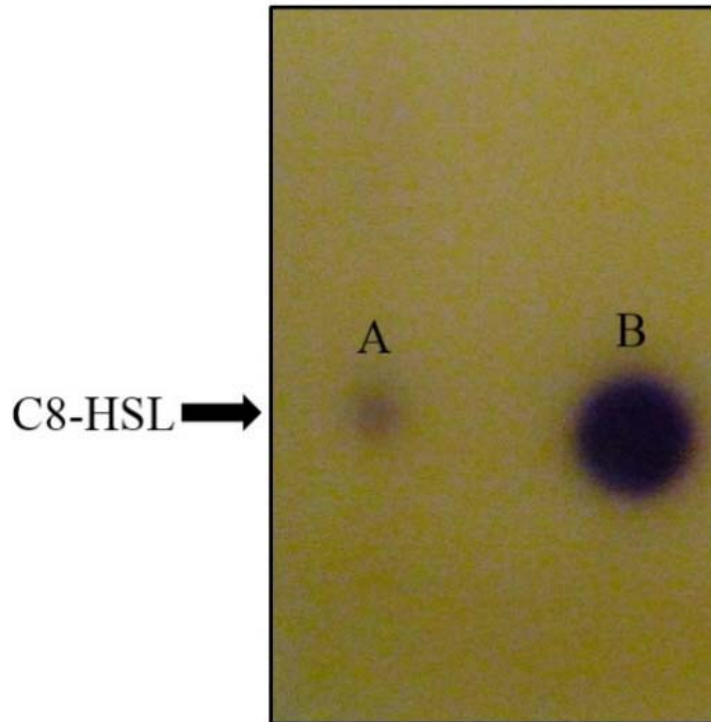
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258 **4(B)**



259

260 **Figure 4. Cross Streiking bioassay. (A) CV026 bioassay.** Purple pigmentation indicated
261 secretion of short chain AHLs from *E. coli* BL21(DE3)pLysS::ppnI. **(B) *E. coli* [pSB 401]**
262 **chemilumiscence bioassay.** Expression of chemiluminescence activity in *E. coli* [pSB
263 401] demonstrated the detection of short chain AHLs. *E. carotovora* GS101 and *E.*
264 *carotovora* PNP22 served as the positive and negative control respectively in both
265 experiment.



266

267

268 **Conclusion**

269 Here, we reported the complete genome of *P. pnomenusa* RB38 and the discovery of its AHL
270 synthase, designated as *ppnI* gene and its LuxR homolog receptor, *ppnR* gene, as well as an
271 additional orphan LuxR regulator, *ppnR2* gene. Short chain AHL, C8-HSL was detected in the
272 spent culture supernatant of *E. coli* BL21(DE3)pLysS::*ppnI* which confirmed that *ppnI* gene is
273 a functional AHL synthase.

274 **ADDITIONAL INFORMATION AND DECLARATIONS**

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281 **Author Contributions**

282 Yan-Lue Lim, Robson Ee, Kah-Yan How, Siew-Kim Lee and Wai-Fong Yin performed the
283 research, data analysis and prepared for manuscript. Kok-Gan Chan designed, supervised and
284 approved the experiments.

285 **Conflicts of Interest**

286 The authors declare no conflict of interest.

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