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Enterobacter cancerogenus produces short chain *N*-3-oxo-acylhomoserine lactones quorum sensing molecules

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Abstract

Enterobacter cancerogenus strain M004 genome size is 5.67 Mb. Here, its *luxI* homologue, designated as *ecnI* (633 bp) which was cloned and overexpressed. Its AHL synthesis activity was verified using the high-resolution liquid chromatography-mass spectrometry analysis revealed the production of *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) and *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C8-HSL). The cloning and characterization of *luxI* homologue of *E. cancerogenus* strain M004 was firstly reported here.

Introduction

Bacteria are always considered to live as asocial and reclusive life forms but recently researches revealed that bacteria are able to communicate via chemical signaling system for genes regulation (Miller and Bassler, 2010). Specifically, they release, detect and respond to the accumulation of signaling molecules which are called autoinducers (Williams, 2007). The autoinducers are essential in coordinating gene expression with synchronization of activities in the entire bacterial community. Bacterial communication, commonly known as “quorum sensing

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27 (QS)", refers to the process where bacterial communication is achieved when the concentration
28 of signaling molecules reach its threshold level which is coordinated with its population density
29 (Fuqua, Winans and Greenberg, 1994a; Fuqua, Winans and Greenberg, 1996). QS provides a
30 platform where bacteria are able to reap benefits that would be unattainable to them as
31 individuals. Most Gram-negative bacteria mostly produce signaling molecules belonging to the
32 autoinducer-1 type, namely *N*-acyl homoserine lactones (AHLs) and Gram-positive bacteria use
33 oligopeptide autoinducers (Bandara et al., 2012). To date, the only shared QS mechanism for
34 both Gram-positive and Gram-negative bacteria involves the autoinducer-2 production by LuxS
35 (Xavier and Bassler, 2003).

36 AHLs produced by Gram-negative bacteria received the utmost attention where intensive studies
37 had been carried out. The simple signal response mechanism described by Engerbrecht and
38 Silverman had paved a pathway into deeper research of QS in Gram-negative bacteria (Lupp and
39 Ruby, 2005). Characteristics of AHLs are influenced by the degree of saturation, the length of
40 fatty acid side chains (ranging from 4-18 carbons) and the presence of hydroxyl-, oxo- or no
41 substituents at the C3 position (Chan et al., 2014). Although there will be variation in AHLs
42 characteristics, the lactone ring in AHLs is highly conserved within bacteria (Chan et al., 2014).
43 The communication system employs the usage of an HSL autoinducer whose synthesis is
44 dependent on a *luxI* homologue. When the AHL concentration is in threshold level, it will
45 detected and bind to a cognate transcriptional activator, *luxR* homologue that allow the
46 downstream expression leading to appropriate output (Ng and Bassler, 2009; Williams, 2007).

47 It occurred that the basic LuxI/R communication circuit would be highly conserved across
48 bacteria, but additional complexity is shown to be added with the use of multiple AHLs and
49 LuxR proteins that can act either parallel or in series (LaSarre and Federle, 2013). This can be
50 seen in the expression of phytopathogen, *Ralstonia solanacearum* LuxI/LuxR like autoinduction
51 system (SolI/SolR) to be regulated by PhcA and also RpoS (Guidot et al., 2014). Other than that,
52 the opportunistic pathogen *Pseudomonas aeruginosa* employs two pairs of LuxI/R (LasI/R,
53 RhlI/R) and function in tandem to control the virulence factors production (Pesci et al., 1997). In
54 fact, the backbone of QS would be the LuxI/R signal-response circuit but the level of regulation
55 is layered depending on the nature of bacteria. QS has been regarded a promising platform in
56 regulating various bacterial physiological activities such as biofilm (Solano et al., 2014),

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57 expression of virulence factors (Rutherford and Bassler, 2012), and swarming (Daniels et al.,
58 2004). Hence, by targeting its communication circuit would be a novel approaches either in
59 interfering its virulence or to enhance them in biotechnological purposes (LaSarre and Federle,
60 2013).

61 The genus *Enterobacter* was first proposed by Hormaeche and Edwards back in 1960s.
62 *Enterobacter* spp. are in the family of Enterobacteriaceae and are facultatively anaerobic Gram-
63 negative bacilli (Hormaeche and Edwards, 1958; Hormaeche and Edwards, 1960). They are
64 motile with peritrichous flagella and have class 1 fimbriae. *Enterobacter* spp. are commonly
65 found in soil and water. Its host range varies from plants, humans and to animals (Chen et al.,
66 2010). However, *E. cloacae* and *E. aerogenes* are responsible for the majority *Enterobacter*
67 infections, 65-75% and 15-25%, respectively (Chen et al., 2010; Keller et al., 1998).
68 *Enterobacter* spp. are commonly found in intensive care units and are responsible for 8.6 % of
69 nosocomial infections according to the US Centers for Disease Control and Prevention (CDC)
70 (Custovic et al., 2014). *Enterobacter* spp. are also involved in a considerable proportion of
71 reported bacteremia cases; in one pediatric hospital, The most common causal agent of
72 bacteremia was reported to be *Enterobacter* spp. accounting for 14 % of cases, while in adults
73 *Enterobacter* spp. are responsible for 1.5-6% of bacteremia cases (Kollef et al., 2011). Hence,
74 the roles of QS system in soil-borne or plant-associated *Enterobacter* sp., which have been only
75 rarely explored, are a valuable information.

76 Our group has been exploring the aquatic environment for bacterial communities in Malaysian
77 rainforest tropical waterfall (Tan et al., 2014). Recently, we isolated *Enterobacter* sp. strain
78 M004 (which was further identified as *Enterobacter cancerogenus*) from waterfall and its
79 genome was fully sequenced by Illumina platform and deposited in GenBank. This aquatic
80 isolate was found to secrete 3-oxo-C6-HSL and 3-oxo-C8-HSL. In the present study, we
81 analyzed its genome assembly for gene predictions and annotations. The annotated genome
82 enabled us into further investigating the putative homologues of AHL synthase, designated as
83 EcnI. The *ecnI* gene was cloned and overexpressed in *Escherichia coli* and the purified protein
84 was characterized. Mass spectrometry confirmed the productions of AHLs were directed by
85 recombinant EcnI protein.

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86 *E. cancerogenus* is one of the species of *Enterobacter* that has been reported as a pathogenic
87 microorganism (Garazzino et al., 2005; Abott and Janda, 1997). Ironically, this species has also
88 been reported to be stimulants for plant growth (Jha et al., 2012). Whether the quorum sensing
89 signaling plays a role in pathogenicity or plant association is unknown, but its presence could
90 again reflect the importance of QS in situations associated with bacterial populations thriving in
91 a limited space such as in the case of bacterial cancers or in bacteria employing an endophytic
92 lifestyle. Rezzonico and colleagues stated that QS might be the player behind dual role
93 functioning of *E. cancerogenus* and therefore the availability of whole-genome could pave the
94 path in functional study of QS in M004 in the future. To further top with this, verification of
95 synthases activity could allow us to understand that it is responsible for the AHL production
96 besides paving a way in study of the regulatory role of AHLs on virulence and unknown genetic
97 traits of this bacterium (How et al., 2015). QS as the global regulator is significantly important
98 for targeting in antimicrobial strategies to attenuate bacterial virulence and to harvest its ability
99 in biotechnological potential. Hence, this facilitates the study of QS mechanism in environmental
100 strain M004 where it could be applied in understanding clinical isolates.

101

102 **Materials and Methods**

103 **Isolation and Culturing Conditions of Bacterial Strains**

104 *Enterobacter cancerogenus* strain M004 was isolated from the freshwater source of tropical
105 rainforest waterfall known as Sungai Tua Fall (Tan et al., 2014). Bacteria were grown
106 aerobically on Trypticase Soy (TS) medium (in grams per litre: tryptone, 10; soytone extract, 5;
107 NaCl, 5; Bacto agar, 15) with incubation of 24 h at 28 ºC. Pure colonies were obtained with
108 repeated subcultures. E. coli BL21 (DE3) pLysS (Novagen, Germany) was used to propagate the
109 recombinant plasmids and to overexpress the fusion proteins. The *E. coli* strain was grown in
110 Luria Bertani (LB) medium (in grams per litre: tryptone, 10; yeast extract, 5, NaCl, 5) at 37 ºC
111 overnight (24 h). The transformed cells were grown in LB medium supplemented with two types
112 of antitiobiotics, 30 µg/ml kanamycin (Sigma, St. Louis, MO, USA) and 34 µl/ml of
113 chloramphenicol (Sigma, St. Louis, MO, USA). All bacteria isolated were stored frozen at -70 ºC.

114

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115 **DNA extractions, Library Preparation and Sequencing**

116 Genomic DNA was extracted using the MasterPure™ DNA Purification Kit (Epicentre Inc.,
117 Madison, WI, USA) (Lau, Yin and Chan, 2014). The genome of strain M004 was sequenced
118 using a Illumina MiSeq personal sequencer platform (Illumina Inc., CA).

119 **Genome Assembly and Annotation**

120 The strain M004 sequence was *de novo* assembled using the CLC Genomics Workbench 5.1
121 (CLC Bio, Denmark). The gene prediction was then performed with prokaryote gene prediction
122 algorithm by using Prodigal (version 2.60) (Hyatt et al., 2010) while the rRNA and tRNA were
123 predicted with RNAmmer (Lagesen et al., 2007) and tRNAscan SE (v.1.21) (Lowe and Eddy,
124 1997) respectively. Subsequently, the M004 sequence was annotated with Rapid Annotation
125 using Subsystem Technology (RAST) (Aziz et al., 2008) and Prokaryotic Genome Annotation
126 Pipeline (PGAP) by NCBI. The AHL synthase gene was searched out and the sequence
127 alignment was done using GenBank BLASTP program followed by phylogenetic analysis using
128 the Molecular Evolutionary Genetics Analysis (MEGA) version 5.0 (Tamura and Nei, 1993;
129 Tamura et al., 2011). The additional gene prediction analysis and functional annotation were
130 performed within IMG-ER platform. The visualization of genome linking with its various
131 functions was done by BASys (Van Domselaar et al., 2005).

132

133 **Construction of Recombinant EcnI Expression Plasmids**

134 The *ecnI* gene was synthesized by Genscript (Piscataway, NJ, USA) and ligated to expression
135 vector pET-28a(+). Two cloning site which are NdeI and BamHI were selected. In addition, *N*-
136 terminal His-tag was added into the recombinant sequence. This produced the recombinant
137 plasmid designated as pET28a-*ecnI* which allows us to have further downstream analysis. Within
138 14 days of providing the sequence information of gene of interest, 4 µg of the recombinant
139 plasmid were delivered. The plasmid was to transform into the host, *E. coli* BL21(DE3)pLysS.

140

141 **Plasmid Transformation**

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142 *E. coli* were used as competent cells for transformation which were grown as follow: A fresg 50
143 mL growth medium (LB broth) was inoculated with 1 mL of an overnight LB broth culture that
144 was grown to an optical density at 600nm of 0.4. The cells were chilled, collected by
145 centrifugation and resuspended with 25 mL of ice-chilled 0.1M $CaCl_2$. Cells suspension was put
146 on ice for 30 min. The cell pellet was re-centrifuged and resuspended in 4 mL of chilled 68 mM
147 $CaCl_2$ with glycerol added and kept in -70 ºC.

148 Plasmid delivered by Genscript was resuspended with 20 µL of sterilized water. For
149 transformation, 1 µL of the intact plasmid was added to 0.2 mL volume of competent cells (ice-
150 chilled) and incubated for 30 min. The competent cells were then subjected to a heat shock at 42
151 ºC for 50 s to enable DNA uptake as previously described (How et al., 2015). It was then
152 transferred directly into ice bath for 2 min and 700 µL of LB broth was added for resuspension
153 of the competent cells and incubated with shaking at 37 ºC for 90 min in order for full expression
154 of antibiotic resistance before plating on selective media. Transformants grew on selective agar
155 were confirmed by colony PCR.

156

157 **Colony PCR**

158 Colony PCR for *Taq* DNA polymerase with standard *Taq* Buffer was conducted according to the
159 protocol by New England Biolabs Inc. and PCR master mix was prepared. A single colony of
160 transformant was picked and mixed with the PCR reaction tube with 25 µL reaction mix. The
161 thermal cycling programs for colony PCR consisted of initial denaturation of 3 min at 94 ºC, 28
162 cycles of denaturation (94 ºC for 30 s), annealing (58 ºC for 40 s) and extension (72 ºC for 40 s).
163 The final extension was initiated for 5 min at 72 ºC. The forward and reverse primers used were
164 generated by Integrated DNA Technologies (IDT) Inc. and obtain within sequence of gene of
165 interest. The sequences of primers were obtained and confirmed using Primer3 (Koressaar and
166 Remm, 2007), The Sequence Manipulation Suite (Stothard, 2000) and OligoAnalyzer by IDT
167 (Owczarzy et al., 2008).

168

169 **Preliminary Verification of Transformant**

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170 The functionality of AHL synthase *ecnI* gene through the transformed *E. coli* BL21(DE3)pLysS
171 was screened for AHL production by cross streaking the bacterial isolates close to the bacterial
172 biosensors, *Chromobacterium violaceum* CV026 colony on a LB agar plate (24 h at 28 °C). The
173 selected colony was cultured routinely on LB medium. BL 21(DE3)pLysS also transformed with
174 pET-28a plasmid acted as a control. *E. carotovora* PNP22 and *E. carotovora* GS101 served as
175 negative and positive controls respectively. (**b**) AHL screening of strain M004 with *E. coli*
176 [pSB401]. *E. carotovora* PNP22 and *E. carotovora* GS101 served as negative and positive
177 controls, respectively.

178

179 **AHL extractions and AHL profiling**

180 Overnight culture (1 mL) was inoculated in 50 mL fresh LB medium supplemented with selected
181 antibiotics were grown at 37 ºC (up till OD_{600nm} of 0.4-0.5). Next, isopropyl-D-
182 thiogalactopyranoside (IPTG, Sigma, St. Louis, MO, USA) was added (at final concentration of
183 1.0 mM) to induce the expression of *ecnI* gene in recombinant *E. coli*. The induced culture was
184 grown for 8 h at 25 ºC with shaking. *E. coli* harboring pET28a plasmid was used as the negative
185 control. The induced *E. coli* BL21 cells (harboring pET28a-*ecnI*) was cultured in LB broth
186 buffered to pH 5.5 with 50 mM of 3-(*N*-morpholino)propanesulfonic acid (MOPS) in an
187 incubator shaker (200 rpm; 28 °C; 18 h) (Lau et al., 2014). The spent supernatant was extracted
188 twice with equal volume of acidified (0.1% v/v glacial acetic acid) ethyl acetate as described
189 previously (Ortori et al., 2011). The organic solvent was dried in fume hood and the dried
190 extracts were resuspended in 1 mL of acidified ethyl acetate and completely dried. Next, 200 µL
191 of acetonitrile (HPLC grade) was added and the mixture vortexed to dissolve the extracts. The
192 mixture was then centrifuged at 12,000 rpm for 5 min to remove any insoluble residue. The
193 dissolved sample (an aliquot of 75 µL) was withdrawn and placed in sample vials for mass
194 spectrometry analysis.

195 For the AHL profiling, an Agilent 1290 Infinity LC system (Agilent Technologies Inc., Santa
196 Clara, CA, USA) was used as the LC delivery system. The column used was the HPLC column
197 C18 column (Agilent Technologies Inc., Santa Clara, CA, USA); 2.1 mm × 50 mm, 1.8 µm
198 particle size where the injection volume was set to 2 µL. LC analysis was carried out at 37 °C

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199 column temperature with an elution of 15 min at constant flow rate of 0.3 mL/min. For the
200 mobile phase A and B, 0.1% v/v formic acid in HPLC grade MilliQ water and 0.1 % v/v formic
201 acid in acetonitrile (ACN) were used respectively. A set of gradient profiles for the HPLC
202 condition was set up as follows at (time: mobile phase A: mobile phase B): 0 min: 80:20, 7 min:
203 50:50, 12 min: 20:80, and 14 min: 80:20.

204 Next, the high resolution tandem LC-MS/MS was performed with the Agilent 6490 Triple
205 Quadrupole LC/MS system (Agilent Technologies Inc., Santa Clara, CA, USA). The
206 electrospray ionization (ESI) positive mode was used as an ion source. The probe capillary
207 voltage was set at 3 kV, nebulizer pressure at 20 psi, sheath gas at 11 mL/h, desolvation
208 temperature at 200 °C, collision energy at 5 eV and fragmentation at 380 eV. The product ion
209 *m/z* value was set at 102 which indicates the $[M+H]^+$ ion of the core lactone ring moiety and the
210 *m/z* value range of the precursor ion was set from 150 to 400. The mass spectra with respective
211 retention time were compared comparison within extracted ion and synthetic AHL compounds
212 by Agilent Mass Hunter software.

213 **Results**

214 *E. cancerogenus* strain M004 was selected for genome sequencing based on its phylogenetic
215 position and its 16S rRNA gene sequences similarity to other members of genus *Enterobacter*.
216 The genome sequence was deposited in GenBank under the accession number of
217 JRUP00000000.1 and deposited in the Genomes On-Line Database (GOLD) with ID assigned as
218 Gp0108990. The genome size of *E. cancerogenus* strain M004 is 5,670,247 bp and 53.75% G+C
219 content.

220 *Enterobacter* sp. M004 was first isolated by our group with its identification by 16S rRNA gene
221 sequencing that clustered within *Enterobacter* genus. Furthermore, due to the availability of
222 genome data from MiSeq sequencing, we searched out the 16S rRNA gene for further
223 identification. The EzTaxon database (Kim et al., 2012) was used as the preliminary 16S rRNA
224 gene sequence-based identification whereby the result indicated that the strain M004 was most
225 closely related to *E. cancerogenus* LMG 2693(T) (GenBank accession = Z96078.1; sequence
226 similarity: 99.12%). The phylogenetic analysis was then performed with the 16S rRNA gene
227 sequences of strain M004 and its related species (Figure 1). The sequences were aligned with the

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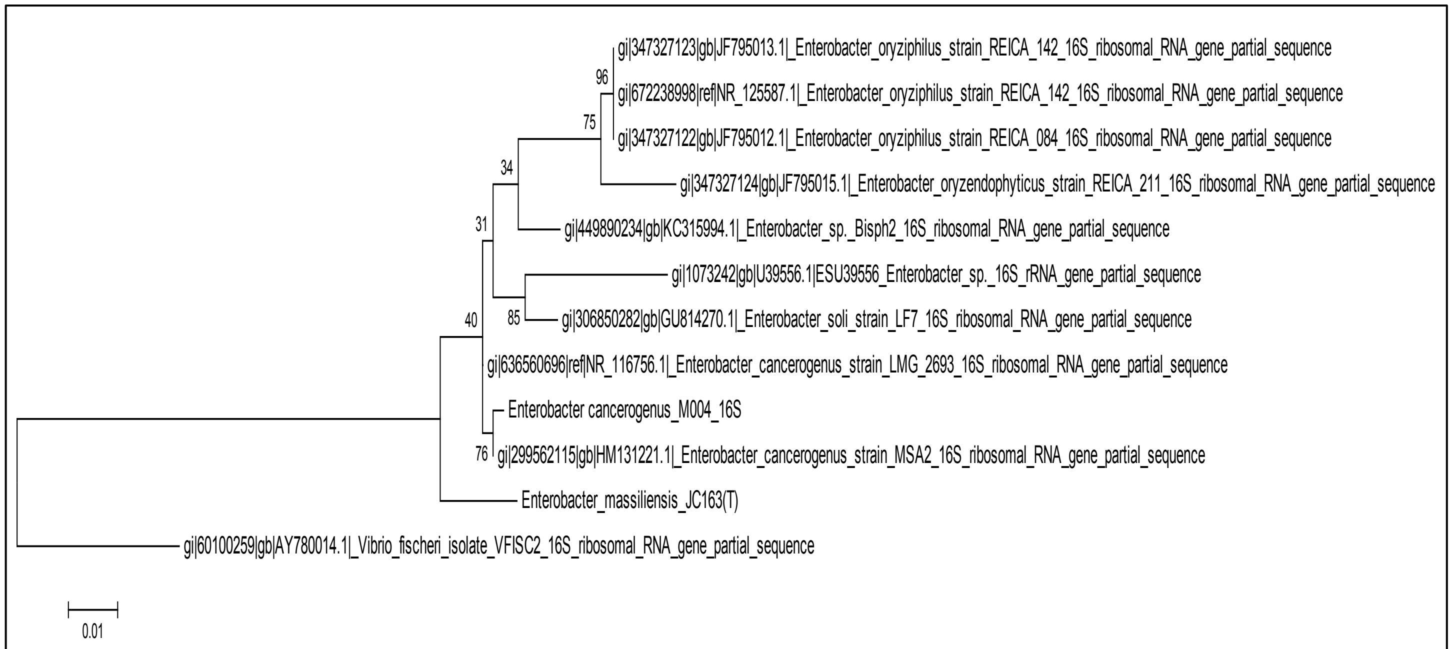
228 phylogenetic tree built using maximum-likelihood (ML) methods implanted in MEGA version 5
229 (Tamura and Nei, 1993; Tamura et al., 2011).

230



231

232 **Figure 1.** The evolutionary history was inferred by using the Maximum Likelihood method
233 based on the Tamura-Nei model.

234

235 From RAST, a 633 bp *luxI* homologue hereafter named as *ecnI*, was found in this genome which
236 is located at contig 7. This finding is parallel with the data generated by NCBI PGAP and this
237 gene shares a 77% base pair similarity with *esaI* of *Pantoea stewartii*. Analysis of the amino acid
238 sequence of *ecnI* using InterPro Scan identified the presence of an acyl-coA-*N*-acyltransferase,
239 which is the structural domain of *N*-acyl homoserine lactone synthase further confirmed us into
240 synthesizing this gene. On the other hand, the phylogenetic tree constructed based on the amino
241 acid sequence alignment illustrated that EcnI was clustered closely within other LuxI homologue
242 of *Enterobacter* and other genus that are closely related (Figure 2).

243 Adjacent to *ecnI* is a sequence encoding a QS transcriptional activator, a cognate receptor which
244 also known as *luxR* homologue (*ecnR*). The coding region was found to be sharing 83%
245 similarity with *esaR* of *P. stewartii*. Two essential domains, the antoinducer-binding domain and
246 C-terminal effector were found in protein analysis of *ecnR*.

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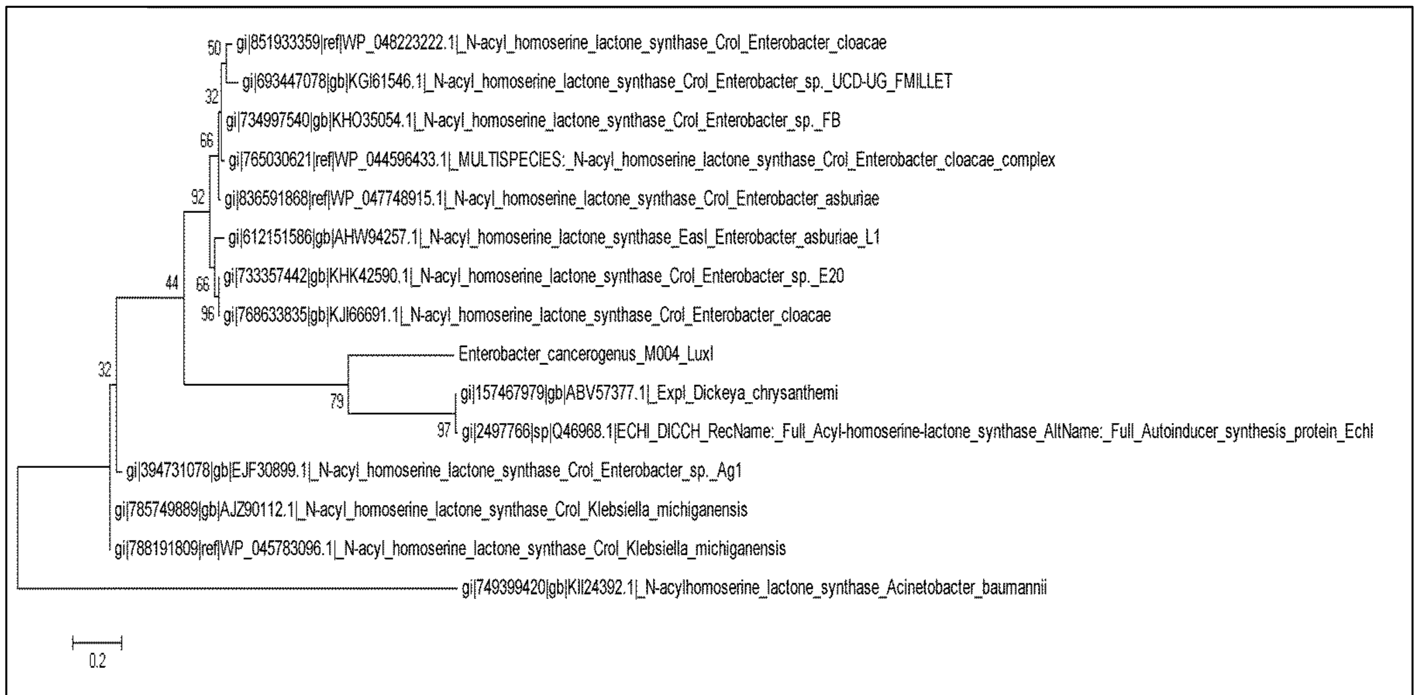

251 **Figure 2**. Phylogenetic analysis of *E. cancerogenus* M004 *ecnI* gene. The tree was constructed
252 based on the similar LuxI homologue protein sequences by Neighbor-Joining with bootstraps
253 value of 1,000 replicates.

254

255 The availability of *ecnI* sequence of strain M004 allows us to further synthesize the gene and
256 transformed into *E. coli* BL21(DE3)pLysS. Colony PCR was done to verify the presence of *ecnI*

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257 into the *E. coli*. Next, preliminary screening using CV026 biosensors showed the purple
258 pigmentation which indicates the production of AHL molecules by transformed *E. coli* (Figure 3).

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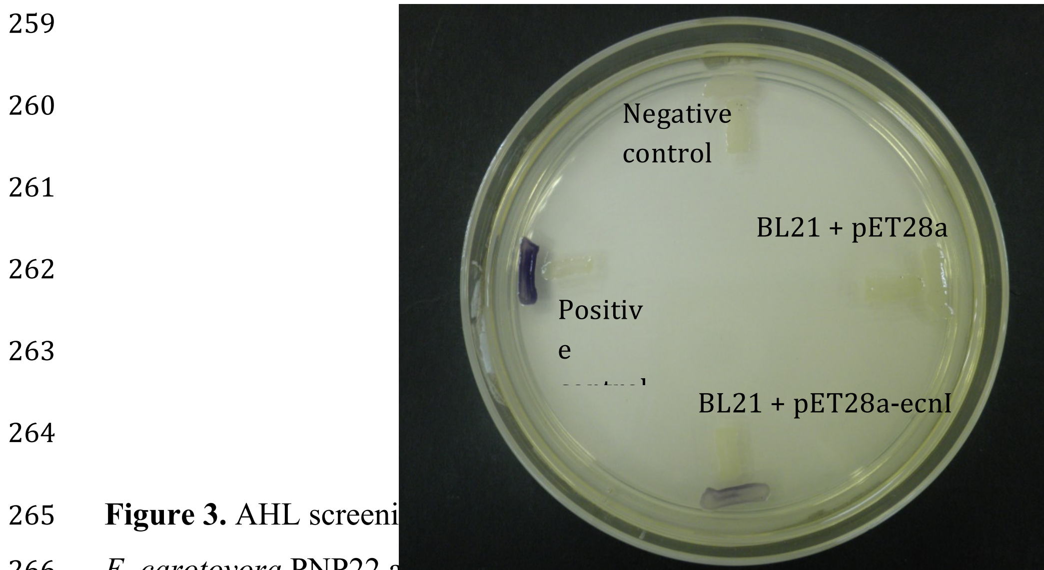

265 **Figure 3.** AHL screeni[...] n pET28a with CV026.
266 *E. carotovora* PNP22 a[...] sitive controls
267 respectively.

268

269 The extracted AHL from the spent culture supernatant of the IPTG-induced *E. coli* BL21
270 harboring pET-28a-*ecnI* was analyzed and identified via Agilent 6490 Triple-Quad LC-MS/MS
271 system. Two AHLs reported previously was confirmed to be produced by *ecnI* through analysis
272 of high-resolution mass spectrometry (Figure 4). The AHL produced were *N*-(3-oxo-hexanoyl)-
273 L-homoserine lactone (3-oxo-C6-HSL) and *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-
274 C8-HSL). The mass spectra were indistinguishable to the responding synthetic compounds at
275 their specific retention times. Both AHLs were not found in negative control.

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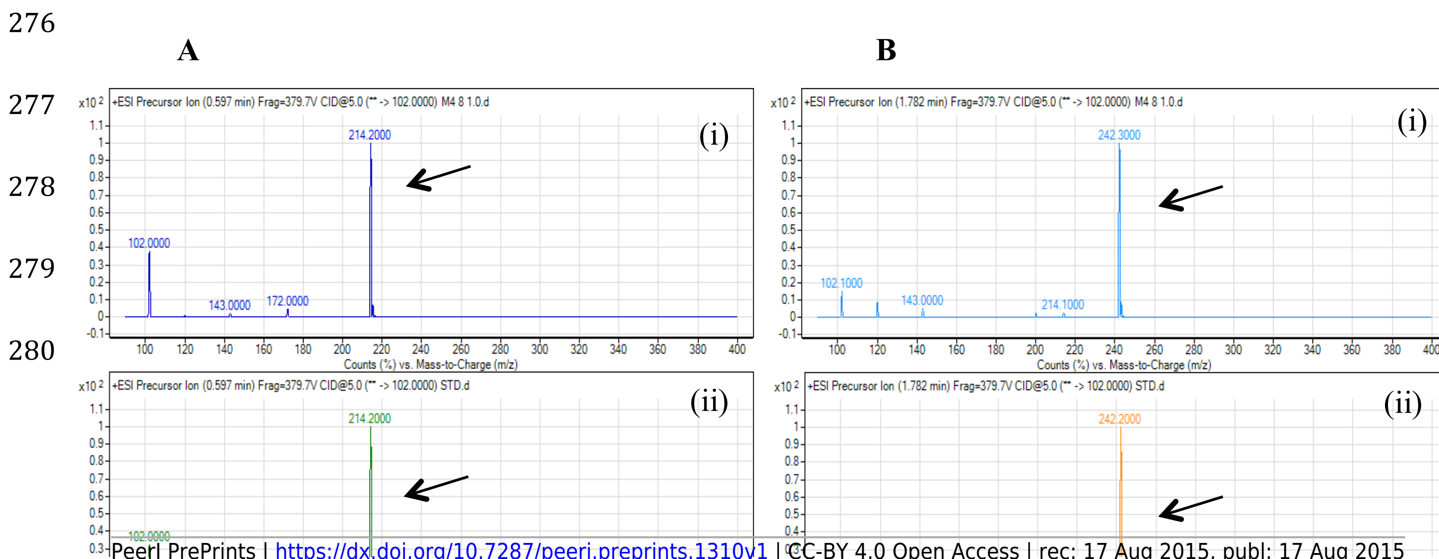

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288 **Figure 4.** Mass spectrometry (MS) analyses of the extract of spent culture supernatant of
289 induced *E. coli* BL21 cells. (A) 3-oxo-C6-HSL at *m/z* 214.2000 (B) 3-oxo-C8-HSL at *m/z*
290 242.3000 (i) Mass spectra of *E. coli* BL21 harboring pET28a-*ecnI* (ii) Mass spectra of synthetic
291 AHL (iii) Mass spectra of *E. coli* BL21 harboring pET28a alone (negative control).

292

293 **Discussion**

294 *Enterobacter* spp. could range from clinical isolates, food spoilage agent and to plant pathogens.
295 *E. cancerogenus*, a bacterium that was reported to have the ability in achieving dual role in the
296 nature as human pathogen while becoming a plant stimulant (Abbott and Janda, 1997; Rezzonico
297 et al., 2012). To date, whether pathogenicity or plant association is linked to quorum sensing
298 remains unknown, but it could be a platform to allow bacterial population in thriving with
299 limited spaces either becoming a bacterial cancers or endure into endophytic lifestyle. Therefore,
300 the isolation carried out as previously reported allows us into expanding our understanding
301 towards it genetic makeup and further searching out the genes encoded for communication
302 function.

303 The whole genome sequencing provides appreciative information towards study of genetic basis
304 of QS in *E. cancerogenus* M004 where the finding of *ecnI/R* in the genome lies parallel with the
305 common feature of *luxI/R* homologues. The InterPro scan confirmed their identity through the
306 analysis of amino acids sequence by looking into their domains. The *E. cancerogenus* M004 was

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307 reported previously to be producing 3-oxo-C6-HSL and 3-oxo-C8-HSL. The *ecnI/R* pair was
308 found to be most similar with *ecaI/R* of *P. stewartii* which is responsible with production of 3-
309 oxo-C6 HSL but production for 3-oxo-C8 HSL has no documentation.

310 In this work, the gene for putative AHL synthase from *E. cancerogenus* M004 (designated as
311 *ecnI*) has been successfully cloned and characterized. When *E. coli* harboring the pet28a-*ecnI*
312 and induced by IPTG for 8 h, its spent supernatant was profiled using LC-MS/MS confirmed the
313 profiles of both 3-oxo-C6 HSL and 3-oxo-C8 HSL suggesting that EcnI is indeed the AHL
314 synthase of *E. cancerogenus* M004. This result is in parallel with the findings by our group
315 previously which obtained the same AHL profile in strain M004 (Tan et al., 2014).

316 The information of bacterial QS related genes demonstrate initial step in elucidating the role and
317 molecular mechanism of the signaling system portrayed by the bacterium. More study should be
318 conducted in order to relate the communication with the dual roles of *E. cancerogenus* to further
319 understand their niche in the aquatic environment.

320

321 **Conclusion**

322 This study allows further investigation of the relationship between QS and how it regulates the
323 physiological activities of *E. cancerogenus* strain M004.

324

325 **Acknowledgement**

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329

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