Research paper

Characterization of quorum sensing genes and N-acyl homoserine lactones in *Citrobacter amalonaticus* strain YG6

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ABSTRACT

In the phylum of Proteobacteria, quorum sensing (QS) system is widely driven by synthesis and response of N-acyl homoserine lactone (AHL) signalling molecules. AHL is synthesized by LuxI homologue and sensed by LuxR homologue. Once the AHL concentration achieves a threshold level, it triggers the regulation of target genes. In this study, QS activity of *Citrobacter amalonaticus* strain YG6 which was isolated from clams was investigated. In order to characterise *luxI/R* homologues, the genome of *C. amalonaticus* strain YG6 (4.95 Mbp in size) was sequenced using Illumina MiSeq sequencing. Through in silico analysis, a pair of canonical *luxI/R* homologues and an orphan *luxR* homologue were identified and designated as *camI*, *camR*, and *camR2*, respectively. A putative *lux* box was identified at the upstream of *camI*. The *camI* gene was cloned and overexpressed in *E. coli* BL21 (DE3) pLysS. High-resolution triple quadrupole liquid chromatography mass spectrometry (LC-MS/MS) analysis verified that the CamI is a functional AHL synthase which produced multiple AHL species, namely N-butryl-l-homoserine lactone (C4-HSL), N-hexanoyl-l-homoserine lactone (C6-HSL), N-octanoyl-l-homoserine lactone (C8-HSL), N-tetradecanoyl-l-homoserine lactone (C14-HSL) and N-hexadecanoyl-l-homoserine lactone (C16-HSL) in *C. amalonaticus* strain YG6 and *camI* gene in recombinant *E. coli* BL21(DE3)pLysS. To our best knowledge, this is the first functional study report of *camI* as well as the first report describing the production of C14-HSL by *C. amalonaticus*.

1. Introduction

The term quorum sensing (QS) was introduced by Steven Winans in 1994 (Fuqua et al., 1994; Turovskiy et al., 2007). It describes the cell-to-cell communication in the bacterial population using small diffusible chemical signalling molecules, commonly known as autoinducers, with the aim of synchronizing gene expression of the bacterial population as a whole. AHL is known to be the most widely studied type of signalling molecule in QS system. In general, AHL-type QS contains two main proteins, namely the AHL synthase and the transcriptional regulator protein, which are commonly known as the LuxI and LuxR homologues, respectively. The LuxR homologue is responsible for the synthesis of signalling molecules. In terms of interacting mechanism, the signalling molecules bind to the cognate LuxR protein homologue to form a complex which regulates the expression of targeted genes (Bassler, 1999; Miller and Bassler, 2001; Schauder and Bassler, 2001). Besides, a wide range of structural variants of the basic AHL molecules have been discovered and the signalling molecules vary in length, the degree of saturation of the acyl side chain as well as in the functional groups located at the third carbon (Decho et al., 2010).

Abbreviations: AHL, N-acyl homoserine lactone; Amino acid residues, aa; C4-HSL, N-butryl-l-homoserine lactone; C6-HSL, N-hexanoyl-l-homoserine lactone; C8-HSL, N-octanoyl-l-homoserine lactone; C14-HSL, N-tetradecanoyl-l-homoserine lactone; C16-HSL, N-hexadecanoyl-l-homoserine lactone; IPTG, Isopropyl β-D-thiogalactopyranoside; LC-MS/MS, High-resolution triple quadrupole liquid chromatography mass spectrometry; MALDI-TOF MS, Matrix-assisted laser desorption/ionization mass spectrometry; nt, Nucleotides; ORFs, Open reading frames; QS, Quorum Sensing; TCBS agar, Thiosulfate Citrate Bile Salts Sucrose agar; TSA, Tryptic Soy Agar

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This study illustrated the QS activity in \textit{Citrobacter amalonaticus}. Formerly known as \textit{Levinea amalonaticus}, this Gram-negative rod-shaped facultative anaerobe is characteristically negative in hydrogen sulfide production on triple sugar iron agar, positive for indole production, and grows in potassium cyanide (Borenstein and Schauer, 2006). A recent study has shown that QS of \textit{C. amalonaticus} isolated from the dental plaque of a patient from Malaysia and its AHL profile has been characterized (Goh et al., 2016). However, the genes that are...
responsible for the synthesis of these AHLs has yet to be verified.

In this study, the luxIR homologue, as well as an orphan luxR homologue, were identified from the draft genome of C. amalonaticus strain YG6 and designated as camIR and camR, respectively. We have cloned the camI gene into Escherichia coli BL21 (DE3)pLysS and verified that camI is responsible for the production of AHL signalling molecules using LC-MS/MS. The findings from this study provided a further understanding on the QS mechanism of C. amalonaticus, thus shedding light into its biology.

2. Materials and methods

2.1. Sampling and isolation of bacteria

Hardshell clam samples were collected from a local supermarket located in Selangor, Malaysia. The samples were placed in a clean zip lock bag, labelled and transported to the laboratory in an ice box. All the samples were processed on the same day of collection. The isolation of bacteria was performed according to the method adapted from Zarei et al. (2012). Twenty-five grams of each sample was weighed and placed into a sterile homogenizer bag containing 225 mL of alkaline peptone water. The samples were homogenized for 60 s using a stomacher (Bagmixer, New Brunswick Scientific Co., USA). The homogenate was incubated at 37 °C under aerobic condition for 18 h. After incubation and enrichment, a loopful of the enriched mixture was cultured on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (HiMedia, India) and incubated at 37 °C for 18 h. The yellow and green colour colonies on TCBS agar were selected and cultured on Tryptic Soy Agar (TSA) (HiMedia, India). The TSA plates were incubated at 37 °C under aerobic condition for 18–24 h.

2.2. Bacterial identification by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS was performed as described in Bruker direct transfer method. A thin film of a single bacterial colony was spread evenly onto an MSP 96 target polished steel BC plate, air-dried, and it was overlaid quickly with MALDI matrix (1 µL, 10 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% trifluoroacetic acid). The sample was then identified using MALDI-TOF MS (Bruker Daltonik GmbH Leipzig, Germany) along with the analysis using Bruker MALDI Biotyper Real-Time Classification (RTC) Version 3.1 and MALDI Biotyper MSP method software to generate score-oriented dendrogram.

2.3. Screening of AHL production using biosensors

C. amalonaticus strain YG6 was screened using biosensor Chromobacterium violaceum CV026, along with Pectobacterium carotovora GS101 and P. carotovora PNP22, which were used as the positive and negative controls, respectively (Chen et al., 2013; McClean et al., 1997).

2.4. Genomic DNA extraction

The genomic DNA of C. amalonaticus strain YG6 was extracted using MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), as per manufacturer protocol. The quality and quantity of DNA were assessed using Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA) and NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), respectively.

2.5. Whole genome sequencing

The Sequencing library was prepared by using Illumina Nextera DNA Sample Preparation Kit (Illumina, USA). The size distribution of the sequencing library was evaluated using High Sensitivity DNA Analysis Kit (Agilent Technologies, USA) while the concentration of the sequencing library was determined by Illumina Eco qPCR machine, as described in the KAPA Library Quantification Kits for Illumina sequencing platforms (KAPA Biosystems, Boston, MA, USA). The library was then sequenced using MiSeq Sequencer (Illumina, USA) (Chan et al., 2015).

2.6. Genome analysis

The whole genome sequence of C. amalonaticus strain YG6 was assembled using CLC Genomic Workbench and annotated using Rapid Annotations using Subsystems Technology (RAST) (Overbeek et al., 2014) and National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number LIGA00000000. The version described in this paper is version LIGA00000000.1. The rDNA sequences of strain YG6 were identified using RNaMMer 1.2 Server (Lagesen et al., 2007) and the 16S rDNA sequences were searched against NCBI database (Johnson et al., 2008), followed by the construction of phylogenetic tree using MEGA 7.0.14 with bootstrap value of 1000 and the clustering method used was maximum likelihood (Kumar et al., 2016). The phylogenetic tree was constructed using the 16S rDNA sequences of strain YG6 predicted using RNaMMer as well as other 16S rDNA sequences of other members from the genus Citrobacter.

2.7. Studies on CamI, CamR, and CamR2

The luxR homologue was identified upon annotation using RAST. Multiple LuxI and LuxR protein sequences were downloaded from NCBI followed by phylogenetic tree construction using MEGA 7.0.14, with the bootstrap value of 1000 and applying maximum-likelihood method. A similar method was used to study the conserved region of the LuxR homologue proteins. Multiple amino acid sequences were aligned using MEGA 7.0.14 and the conserved regions were illustrated using ESPript (Robert and Gouet, 2014).
Fig. 3. (A) The organization of luxR homologues and their adjacent genes in strain YG6 in comparison with the other members of Citrobacter. Autoinducer synthase genes, luxI homologues, are indicated as red arrows while the transcriptional regulators, luxR homologues, are indicated as green arrows. (B) The organization of Citrobacter orphan, luxR homologues (red arrows) and their adjacent genes. The orphan luxR homologue from strain YG6 was compared with other Citrobacter sp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.8. Cloning of camI

Recombinant camI gene expression plasmid was constructed with camI gene and overexpression vector, pET-28a(+). The resulting recombinant plasmid was designated to pET-28a(+)-camI. Subsequently, the pET-28a(+)-camI was transformed into competent E. coli BL21 (DE3)pLysS for overexpression. Chloramphenicol (34 μg/mL) and kanamycin (30 μg/mL) were supplemented to select the transformants. Afterwards, the AHL production of the desired transformant was screened using Ch. violaceum CV026 (How et al., 2015).

2.9. Identification of lux box and QS-related genes analysis

The lux box sequence was predicted and a list of lux box sequences from other well-known bacterial species was aligned using MEGA 7.0.14 and visualization of consensus sequences were generated by Genedoc software and WebLogo (Crooks et al., 2004). Bprom (www.softberry.com) was used to identify the putative promoter regions of genes adjacent to luxR and orphan luxR homologues, along with lux box predicted based on the conserved consensus sequences upstream of promoter regions.
2.10. AHL extraction and identification

The AHLs extraction method was performed as described previously (Chen et al., 2013). Briefly, strain YG6 and *E. coli* BL21 (DE3)pLysS harbouring the recombinant plasmid pET28a-caml were cultured in LB medium (50 mL) buffered with 50 mM 3-[N-morpholino] propane-sulfonic acid (MOPS) to pH 5.5 to prevent instant AHLs degradation (Yates et al., 2002). The recombinant *E. coli* was induced with 0.5 mM IPTG at OD600 for 8 h and at 25 °C. The cell-free culture supernatant was extracted twice with equal volume of acidified ethyl acetate (50 mL, 0.1% v/v glacial acetic acid) and evaporated to dryness followed by reconstitution in minimal volume of acetonitrile for subsequent analysis using LC-MS/MS. The AHL identification by LC-MS/MS was performed as described previously with slight modification (Chan et al., 2014). Precursor ion scanning mode was performed and a positive ion mode in Q3 was set to scan for m/z 102, which refers to the presence of lactone ring, hence, indicating the presence of AHLs.

3. Results

3.1. Bacterial identification

The MALDI-TOF MS showed that the identity score of strain YG6 was 2.341, that was best matched with *C. amalonaticus*. The dendrogram obtained by clustering analysis of MALDI-TOF MS spectra of *C. amalonaticus* showed that strain YG6 was clustered with *C. amalonaticus* DSM 4593T HAM (Fig. 1A). Besides, the phylogenetic analysis of the 16S rDNA genes of various members of *Citrobacter* species showed that strain YG6 was clustered with *C. amalonaticus* CECT 863T as well as *C. amalonaticus* L8A (Fig. 1B). The results of these two analyses agreed with each other, indicating that strain YG6 was *C. amalonaticus*.

3.2. Screening of AHL production in *Citrobacter amalonaticus* strain YG6

*C. amalonaticus* strain YG6 was shown to produce short-chain AHLs (ranging from C4- to C8-HSL), which induced *Ch. violaceum* CV026 to produce purple pigment violacein (Fig. 2).

3.3. luxI/R homologues identification and analysis

Upon whole genome sequencing, the filtered reads were assembled de novo into a total of 47 contigs with N50 of 330,740 bp and draft genome size of 4.95258 Mbp. The average genome coverage was 93.6× with the G + C content of 53.4%. According to RAST analysis, a total of 4510 open reading frames (ORFs) were predicted. From the annotations, a pair of *luxIR* homologues was found in contig 14 (NZ_LIGA01000014.1). The *luxI* homologue (red arrow) was located upstream of the *luxR*-type gene (green arrow) in an inward tandem arrangement, with an intergenic region of 5 bp (Fig. 3A). The *luxI* and *luxR* homologues were designated as *camI* and *camR*, respectively. A putative orphan *luxR* homologue (red arrow), located at contig 15 (NZ_LIGA01000015.1), was designated as *camR2* (Fig. 3B).

3.4. Phylogenetic and protein analysis of *CamI*, *CamR*, and *CamR2*

The phylogenetic trees of the *CamI* and *CamR* show that both proteins are closely related to the autoinducer synthases (LuxI-like protein) and transcriptional regulators (LuxR-like protein) from other *C. amalonaticus* species, respectively (Figs. 4, 5 and 6). Further, Fig. 4–6 show that *CamI*, *CamR*, and *CamR2* are evolutionally closely related to their respective homologues of *C. amalonaticus* L8A. (Pairwise alignment: *CamI* = 99% identity of amino acid residues (aa) and nucleotides (nt), *CamR* = 100% aa and 99% nt identity, *CamR2* = 100% aa and nt identity). It was noteworthy that *CamR2* was homologous to SdiA proteins from other *Citrobacter* sp. based on BLASTP analysis. It was most likely a putative SdiA homologue, sharing 84% homology with the...
Fig. 7. Multiple sequence alignment of CamR and CamR2 with LuxR homologues from other bacterial species. TraR, AfeR, CerR, CroR, LasR, RhlR, LuxR, LuxR- and orphan LuxR-like protein of *C. amalonaticus* L8A, CamR and CamR2 (GenBank ID: ABB59515.1, AAV3702.2, AAC46021.1, CBG89690.1, NP_250121.1, NP_252167.1, CAA68561.1, KEY50942.1, KEY46715.1, KOP94319.1 and KOP93373.1, respectively.) Identical residues were shown as vertically filled red bars and the conserved residues were shown as the unfilled bars. The residue numbering uses TraR in the alignment as a reference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.5. Identification of lux box

The lux boxes in luxI gene promoters are binding sites for LuxR family proteins (Egland and Greenberg, 1999). A putative lux box at position −58 to −39 relative to the translational start codon of camI was identified in this study (Fig. 8). This putative lux box was compared with lux boxes from other bacterial species, namely Vibrio fisheri (lux) (Devine et al., 1988), Pseudomonas aeruginosa (las and rhl) (Latifi et al., 1995), Ralstonia solanacearum (sol) (Flavier et al., 1997), Burkholderia cepacia (cep) (Lewenza et al., 1999), and Acidithiobacillus ferrooxidans (afe) (Rivas et al., 2005) (Fig. 9).

3.6. Cloning of camI

E. coli BL21 (DE3)pLysS harbouring pET28a(+)−camI induced C. violaceum CV026 to produce purple pigment violacein, indicating the production of short-chain AHL by the transformant.

3.7. AHL characterization

The LC-MS/MS results showed that both strain YG6 and E. coli BL21 (DE3)pLysS harbouring pET28a−camI produced the same type of AHLs, namely C4-HSL (m/z 172.0000), C6-HSL (m/z 200.0000), C8-HSL (m/z 228.0000), C14-HSL (m/z 312.0000), and C16-HSL (m/z 340.0000) (Fig. 11).

4. Discussion

Citrobacter spp. have been reported to be isolated from bivalve mollusks (Greyskov et al., 2017). However, whether C. amalonaticus is part of microflora of clams is still remain unknown. In addition to this, the previous study has shown C. amalonaticus causes enteric fever-like illness — a potentially severe systemic disease traditionally caused by Salmonella sp. (Suwansrinon et al., 2005). Interestingly, S. enterica serovar Typhimurium (S. Typhimurium) has found to be isolated from Manila clams (Rubini et al., 2018). Despite the similarity of pathology and isolation source between strain YG6 and S. Typhimurium, the correlation with QS regulation is not studied in-depth in this research.

From the whole genome analysis, a single copy of autoinducer synthase gene — camI — was identified. This finding suggested CamI was the sole AHL synthase responsible for the production of AHLs in C. amalonaticus strain YG6. The AHL profile of strain YG6 was similar to C. amalonaticus L8A (Goh et al., 2016), which were C4-HSL, C6-HSL,
C8-HSL and C16-HSL except for an additional C14-HSL that is firstly reported in this study. It is found that most members of the genus Citrobacter produce more short-chain than long-chain AHLs. For example, C. rodentium produces C4-HSL and C6-HSL (Coulthurst et al., 2007) while C. freundii produces C4-HSL, C8-HSL, C10-HSL, 3-oxo-C6-HSL and 3-oxo-C8-HSL (Huang et al., 2017; Wang et al., 2006). C4-HSL are commonly produced by C. rodentium, C. freundii and C. amalonaticus. On the other hand, C16-HSL has been documented to be synthesized by C. amalonaticus L8A (Goh et al., 2016), Paracoccus sp. (Toyofuku et al., 2017), rhizobial and roseobacterial QS system (Barth et al., 2012; Blosser-Middleton and Gray, 2001; Marketon et al., 2002; Schaefer et al., 2002; Wagner-Döbler et al., 2005), and it is neither widely reported in other bacterial species nor its QS regulation in Citrobacter spp. thus far. As mentioned in the literature review (Keller and Surette, 2006), the number of signalling molecules synthesized are inversely proportional to the cost of production in general. Moreover, the signal specificity commonly correlates with production cost. These statements might support the hypothesis that due to relatively low abundance (Fig. 10e) and production cost, (as long-chain AHL may require active efflux transport system (Pearson et al., 1999)), C16-HSL might be specific for intra-species communication of strain YG6. Besides, it was remarkable that CamI of strain YG6 synthesized five products of short-

Fig. 11. Mass spectra showing the AHL profile of C. amalonaticus strain YG6 and E. coli BL21 (DE3)pLysS:pET28a-caml. The retention time of each AHL is compared with the corresponding standard. The spectra showed that Citrobacter amalonaticus strain YG6 and E. coli BL21 (DE3)pLysS harbouring pET28a-caml produced the same type of AHLs, which are C4-HSL, C6-HSL, C8-HSL, C14-HSL, and C16-HSL. The peak m/z 102 (indicated by red arrows) refers to the lactone ring moiety of AHLs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
C8-HSL

C. amalonaticus strain YG6

(E)

Fig. 11. (continued)
and long-chain AHLs, making it a mechanistically intriguing enzyme. The rationale of CamI producing a wide range of AHLs may involve regulation of different target genes (Supplementary data). Despite the reason that non-cognate AHLs have the ability to induce excitatory or inhibitory bacterial crosstalk (Silva et al., 2017), whether multiple AHLs production contributes to competitive advantage among other AHL-producing bacteria in a natural environment is still remain unknown to the ubiquity of C. amalonaticus.

Based on the protein homology analysis, CamR shared 88% and 32% identity with LuxR homologues of C. rodentium ICC168 (CGB8969.1) and C. freundii ST2 (WP_032954343). respectively. According to Coulthurst et al. (2007), the QS system of C. rodentium is associated with the regulation of attaching and effacing pathogenesis. The secretion of AHLs by C. freundii ST2 suggests it may form microbial communities in phycosphere though no QS gene regulation is reported (Huang et al., 2017). BLASTP revealed CamR2 shared 84% homology with the SdiA of S. enterica (AAC08299). Previous study has shown SdiA from S. Typhimurium senses an extensive range of AHLs, both unmodified and modified at the C3 position of AHLS: C4-, C6-, C8-, C12-, 3-oxo-C4-, 3-oxo-C6-, 3-oxo-C8, 3-oxo-C12-homoserine lactones (de Almeida et al., 2016; Michael et al., 2001; Soares and Ahmer, 2011). In S. Typhimurium, activation of the rck operon and srg gene encodes a putative Type III secreted effecter in respond to AHLs (Soares and Ahmer, 2011). Thus, the presence of this orphan CamR2 in strain YG6 may serve as part of the mechanism for detecting the presence of other bacteria in the environment (eavesdropping). CamR and CamR2 could possibly serve as different cognate receptors for the AHLs produced by Cam of strain YG6. However, further studies on the role and mechanism of CamR and CamR2 are needed to validate this hypothesis.

From the multiple sequence alignment study, several residues of the LuxR homologues are highly conserved, namely Tyr 53, Tyr 61, Val 72, Trp 85 and Ile 110. These residues have been reported to participate in the Van der Waals interaction between LuxR homologues and AHL molecules (Zhang et al., 2002). Besides, residues Ala 38, Tyr 53, Trp 57, Trp 85 and Ile 110. These residues have been reported to participate in the hydrogen bonding between LuxR homologues and AHL molecules (Zhang et al., 2002). This in silico analysis contributes further to the prediction of the biological function of the AHL-receptors.

5. Conclusion

The autoinducer synthase (CamI) and the AHL transcriptional regulators (CamR and CamR2) of C. amalonaticus strain YG6 were identified from the bacterial genome. This study provided a functional characterization of CamI and its AHL profile. Strain YG6 was a multi-AHL producer, with the production of C4-HSL, C6-HSL, C8-HSL, C14-HSL and C16-HSL, suggesting that this bacterium might shape the microbial community in the clam. To date, this is the first report of the production of C14-HSL by C. amalonaticus.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.10.031.

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CRedit authorship contribution statement

Heng-Leong Kher: Investigation (performing most experiments), Writing- Original draft preparation, Methodology, Formal analysis, Visualization, Funding Acquisition. Thiba Krishnan: Investigation (pre-screening of the QS bacterium), Methodology. Vengadesh


Conflicts of interest

The authors declare no conflict of interest.

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