Pantoea sp. strain A4, a new plant-associated bacterium exhibiting quorum sensing activity

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Quorum sensing (QS) is a method of bacterial cell-to-cell communication to coordinate adaptive behaviour. *N*-Acyl homoserine lactone (AHL) is the most studied signalling molecule among proteobacteria. In the present study, we characterize QS activity in *Pantoea* sp. A4, a new *Rafflesia*-associated bacterium. Results show the production of purple violacein by reporter bioassay *Chromobacterium violaceum* CV026 and bioluminescence activation in *Escherichia coli* [pSB401], indicating AHL production. Using liquid chromatography-mass spectrometry, *Pantoea* sp. was found to excrete four types of AHL, namely *N*-hexanoylhomoserine lactone, 3-oxo-*N*-hexanoylhomoserine lactone, *N*-octanoylhomoserine lactone and *N*-dodecanoylhomoserine lactone. To our knowledge, there is no previous documentation of *Rafflesia*-associated bacterium that produces long chain AHL.

Keywords: *N*-acyl-homoserine lactone, *Pantoea* sp., quorum sensing, *Rafflesia*.

THE ability of a bacterium to adapt successfully in a particular environment largely depends on its ability to respond to numerous environmental factors, including temperature, pH, nutrient availability and the presence of other bacteria. It has been widely accepted that bacteria communicate with each other using small, diffusible signal molecules commonly termed autoinducers. This cell-to-cell communication mechanism or quorum sensing (QS) mediates a myriad of physiological activities among proteobacteria such as biofilm formation, conjugation and swarming motility[1,2].

The QS bacteria release autoinducers synthesized by *N*-acyl homoserine lactone (AHL) synthase (LuxI protein family) at specific stages of growth or in response to changes in the environment. At some threshold concentration, the ligand will then bind to its cognate LuxR-type protein family to form a complex. This binding allows LuxR to have a different functional conformation capable of binding DNA and subsequently activating target genes for virulence[3,4]. By far, the most widely studied QS signalling molecule in the last decade is AHL[5]. The well-studied *Vibrio fischeri* is an exemplary model for QS among Gram-negative proteobacteria. The LuxI autoinducer synthase protein from *V. fischeri* was found to produce AHL known as *N*-3-oxo-hexanoyl-L-homoserine lactone[5]. Other well-known bacterial autoinducer signalling molecules include cyclic thiolactone, hydroxyl-palmitic acid methyl ester (PAME), furanosylborate and methyl dodecenoic acid.

Extensive studies have shown that many plant pathogens such as *Agrobacterium tumefaciens* and *Pantoea stewartii* regulate their virulence by synthesizing AHL[6]. In particular, QS-regulated traits among phytopathogens include the production of siderophores, pigments, extracellular polysaccharides and degradative enzymes. These phenotypes facilitate the colonization of host surfaces and hence the pathogenicity[7]. For example, Zhang *et al.*[8] demonstrated the importance of AHL for the conjugal transfer of Ti plasmid that governs the pathogenic potential of *A. tumefaciens*. On the other hand, *P. stewartii*, the etiological agent of Stewart's wilt of sweet corn, regulates the synthesis of capsular exopolysaccharide in the presence of AHL[9].

Members of the genus *Pantoea* are widely distributed in numerous ecological niches. *Pantoea* species are primarily associated with plants, either as epiphytes or as pathogens, and some species such as *P. agglomerans* are opportunistic human pathogens. Nevertheless, the role of autoinducer AHL and its mechanism of action in *Pantoea* species remain scarce. In the present study, we report the isolation of AHL from *Pantoea* sp. strain A4 isolated from *Rafflesia* flower in the Malaysian tropical forest. Till now, only endophytic fungi have been isolated from the genus *Rafflesia*. To our knowledge, there is no previous documentation on the isolation of a bacterium from the world's largest flower. With the identified AHL profile, this facilitates the characterization of *luxI* homologue in strain A4 and a further understanding of the QS mechanism in this bacterial isolate.

Pantoea sp. A4 was isolated from the decaying flower bud of *Rafflesia cantleyi* from the tropical rainforest in Ulu Geroh, Perak, Malaysia[10]. Other bacteria used in the study included bioluminescent-based QS biosensor, *Escherichia coli* [pSB401][11] and *Escherichia coli* [pSB1142], violacein-producing AHL reporter strain, and *Chromobacterium violaceum* CV026. All bacteria were routinely maintained in LB medium (Merck, Germany). If necessary, bacteriological agar (1.5% w/v) was added to make a solidified agar. *Pantoea* sp. A4 and *C. violaceum* CV026 were grown at 28°C, whereas *E. coli* was grown at 37°C.

Bacterial 16S rDNA gene sequences of closely related *Pantoea* sp. were obtained from the NCBI database. Phylogenetic and molecular analyses were performed with MEGA version 6.0 using neighbour-joining[12] strategy as described by Chan *et al.*[13]. Bootstrap analyses of the tree were performed with 1000 re-samplings of the datasets.

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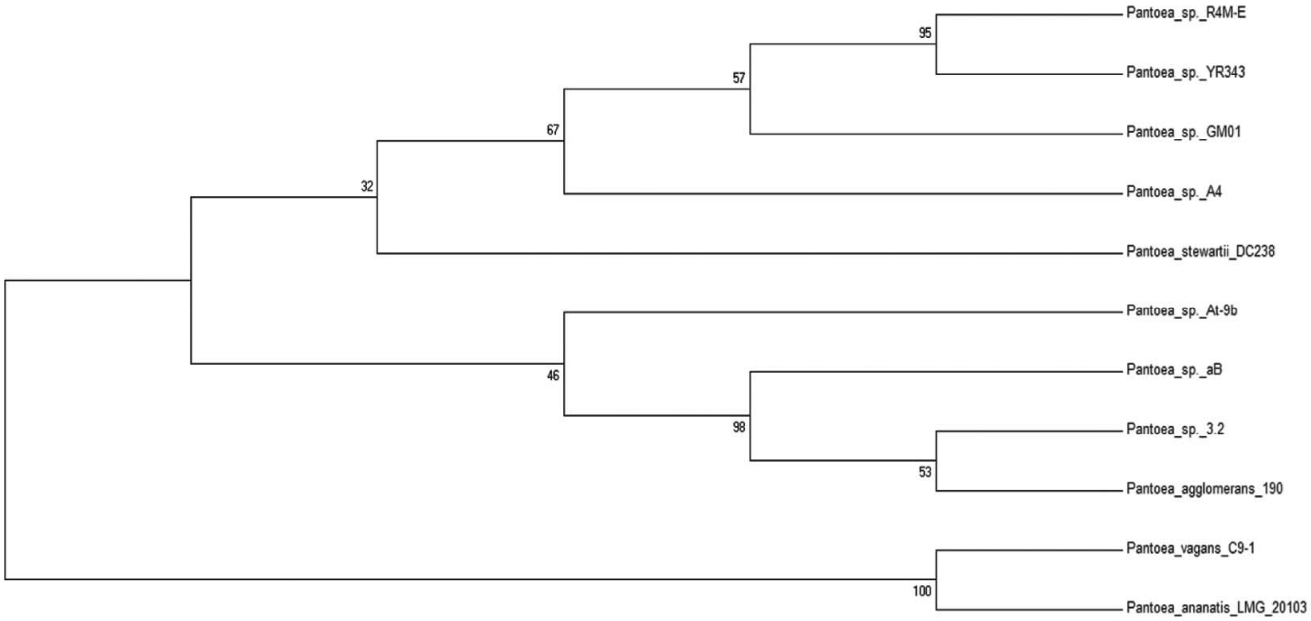

Figure 1. Phylogenetic tree generated using neighbour-joining algorithm showing the phylogenetic position of *Pantoea* sp. strain A4 using 16s rDNA sequences. The numbers at the nodes indicate the bootstrap values as percentage of 1000 replications. GenBank accession numbers (in parenthesis): *Pantoea* sp. R4M-E (GQ478260.1), *Pantoea* sp. 3.2 (GCA_000731025.1), *P. vagans* C9-1 (NC_014562), *P. agglomerans* 190 (JNGC-00000000.1), *Pantoea* sp. At-9b (NC_014837.1), *P. ananatis* LMG 20103 (NC_013956.2), *P. stewartii* subsp. Stewartii DC283 (AHIE01000042), *Pantoea* sp. aB (AEDL01000029.1), *Pantoea* sp. GM01 (NZ_AKIU00000000.1) and *Pantoea* sp. YR343 (NZ_AKIT00000000.1).

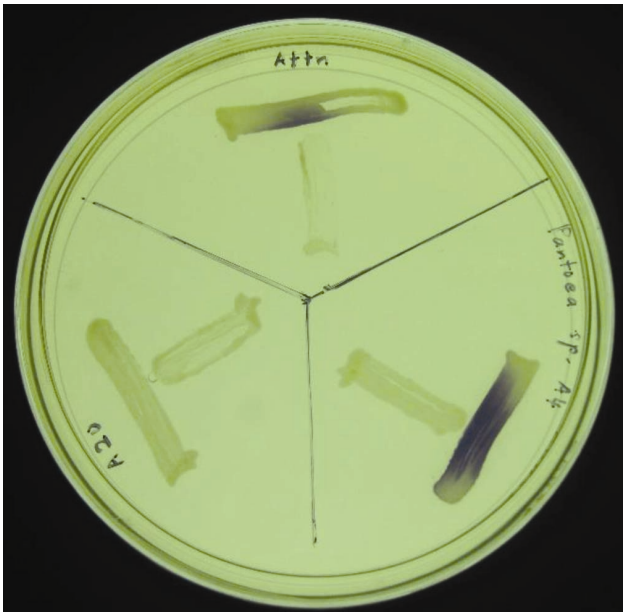

Figure 2. Detection of AHL production using cross-streaking bioassay of *Pantoea* sp. strain A4 with AHL biosensor, *C. violaceum* CV026. *E. carotovora* GS101 and *E. carotovora* PNP22 were used as positive and negative controls respectively. The production of short-chain AHLs is shown by the formation of purple pigmentation.

To detect bioluminescence from *Pantoea* sp. A4, cross-streak bioassay was perfomed as described previously[14]. Both *C. violaceum* CV026 and *E. coli* [pSB401] are short-chain AHL biosensors, whereas *E. coli* [pSB1142] is a long-chain AHL reporter strain. In the *lux*-based plasmid-bearing AHL biosensor *E. coli* [pSB401], it harbours *V. fischeri luxR* gene and the *Photorhabdus luminescens luxCDABE* casette has been fused to *V. fischeri luxI* promoter. Activation of LuxR by exogenous short-chain AHL ranging from 4 to 8 carbons, leads to the expression of *luxCDABE* and subsequently the emission of light. The production of violacein (purple zone) from *C. violaceum* CV026 bioassay was examined after overnight incubation at 28°C. The expression of bioluminescence of both reporter strains, *E. coli* [pSB401] and *E. coli* [pSB1142] was observed using ChemiDocTM MP Imaging System (Bio-Rad, Hercules, CA, USA) after overnight incubation at 37°C. *Erwinia carotovora* GS101 and *E. carotovora* PNP22 were used as positive and negative controls respectively, for pSB401 bioassay[15]. Meanwhile, *P. aeruginosa* PAO1 and *E. coli* DH5α were used as positive and negative control respectively, in pSB1142 bioassay.

An overnight bacterial culture was inoculated into 100 ml of LB broth buffered to pH 6.5 using 3-(*N*-morpholino)propanesulphonic acid (MOPS, 50 mM) and incubated in a shaking incubator (220 rpm) at 28°C. The culture was grown until it reached OD600 of 1.0 and the spent culture supernatant was extracted thrice with equal volume of acidified (0.1% v/v acetic acid) ethyl acetate[16]. The organic layer was collected and evaporated to dryness. The AHL extracts were centrifuged at 14,000 rpm

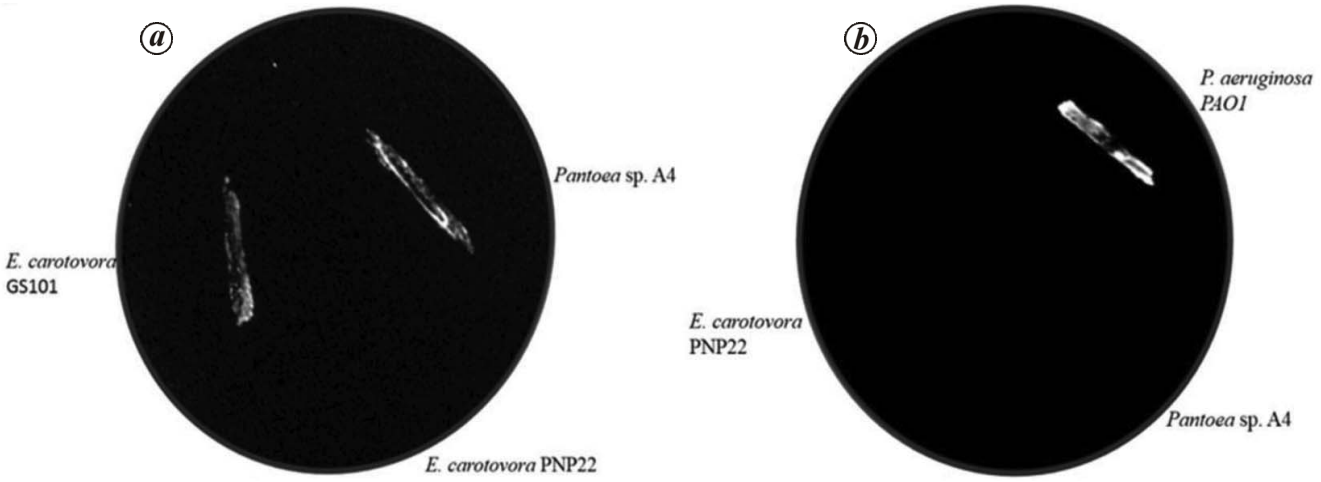

Figure 3. Cross-streaking bioassay using AHL biosensors. ***a***, *Pantoea* sp. strain A4 activated the production of bioluminescence by *Escherichia coli* [pSB401], indicating the presence of short-chain AHLs. *E. carotovora* GS101 and *E. carotovora* PNP22 were used as positive and negative control respectively. ***b***, *Pantoea* sp. A4 failed to induce the expression of bioluminescence by *E. coli* [pSB1142], suggesting the absence of long-chain AHL. *P. aeruginosa* PAO1 and *E. coli* DH5α were used as positive and negative controls respectively.

for 10 min to remove any insoluble residue before they were dissolved in acetonitrile and stored at –20°C.

The AHL profile of the culture supernatant of *Pantoea* sp. A4 was performed by suitable modification of a previously reported method[17]. For LC-MS/MS analysis, Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA) was used with an Agilent ZORBAX Rapid Resolution High Definition SB-C18 Threaded Column (2.1 mm × 50 mm, 1.8 µm particle size). The sample was injected at 2 µl and analysis was carried out using a flow rate of 0.3 ml/min at 37°C. The mobile phases *A* and *B* used were 0.1% v/v formic acid in water and 0.1% v/v formic acid in acetonitrile respectively. The gradient profile used was set at *A* : *B* 80 : 20 at 0 min, 50 : 50 at 7 min, 20 : 80 at 12 min and 80 : 20 at 14 min. Agilent 6490 Triple Quadrupole LC/MS system (Agilent Technologies Inc., USA) was employed for MS detection in ESI-positive mode. Other LC-MS parameters were set as follows: probe capillary voltage at 3 kV, sheath gas at 11 ml/h, desolvation temperature at 250°C, and nebulizer pressure at 20 psi. Nitrogen was used as the collision gas in the collisionally induced dissociation mode for MS/MS analysis, with collision energy set at 5 and 9 eV. The Agilent MassHunter software was used for MS data analysis. Known amounts of synthetic AHLs (Sigma, USA) were also loaded as standards and analysis was based on the retention index[16].

Overnight culture of *Pantoea* sp. A4 was harvested at 1000 *g*. The cell pellet was resuspended in 0.1% phosphate buffer and fixed overnight using 5% glutaraldehyde followed by post-fixing using 1% osmium tetroxide prepared in phosphate buffer for 2 h. Following this, dehydration process of post-fixed bacteria cells was performed using ethanol in gradually increasing concentration. The dehydrated post-fixed bacteria cells were then fixed again using hexamethyldisilazane (HMDS) and air-dried in a desiccator overnight at room temperature. The dried cells were then mounted onto SEM specimen stub and coated with a mixture of gold and palladium. Finally, the specimen was viewed using TM3030 Table Top Scanning Electron Microscopy (Hitachi, Japan).

A phylogenetic tree was constructed based on 16s rDNA nucleotide sequences from ten closely related *Pantoea* species obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/) (Figure 1). It was found that strain A4 was clustered closely with a few *Pantoea* species, namely *Pantoea* sp. GM01, *Pantoea* sp. strain YR343 and *Pantoea* sp. R4M-E, even though the percentage similarity was not high. On the other hand, *P. vagans* strain C9-1 and *P. ananatis* LMG20103 were distantly related to strain A4.

Bioluminescent sensors based on LuxRI regulatory system have been used widely in the identification and analysis of QS in Gram-negative bacteria. Bacterial luciferases are non-destructive, real-time reporters of gene expression to study cell-dependent AHL production for a given organism[18]. In the present study, three different AHL biosensor strains were used to determine QS activity in strain A4, namely *C. violaceum* CV026, *E. coli* JM109 [pSB401] and *E. coli* [pSB1142]. Strain A4 was shown to trigger the production of purple violacein pigment by *C. violaceum* CV026 (Figure 2). CV026 was reported to respond mainly to exogenous source of short-chained AHLs, ranging from 4 to 8 carbons in length[15]. This reporter harbours a defective *luxI* but a functional LuxR receptor protein (CviR) that causes the production of violacein pigment. The positive control *E. carotovora*

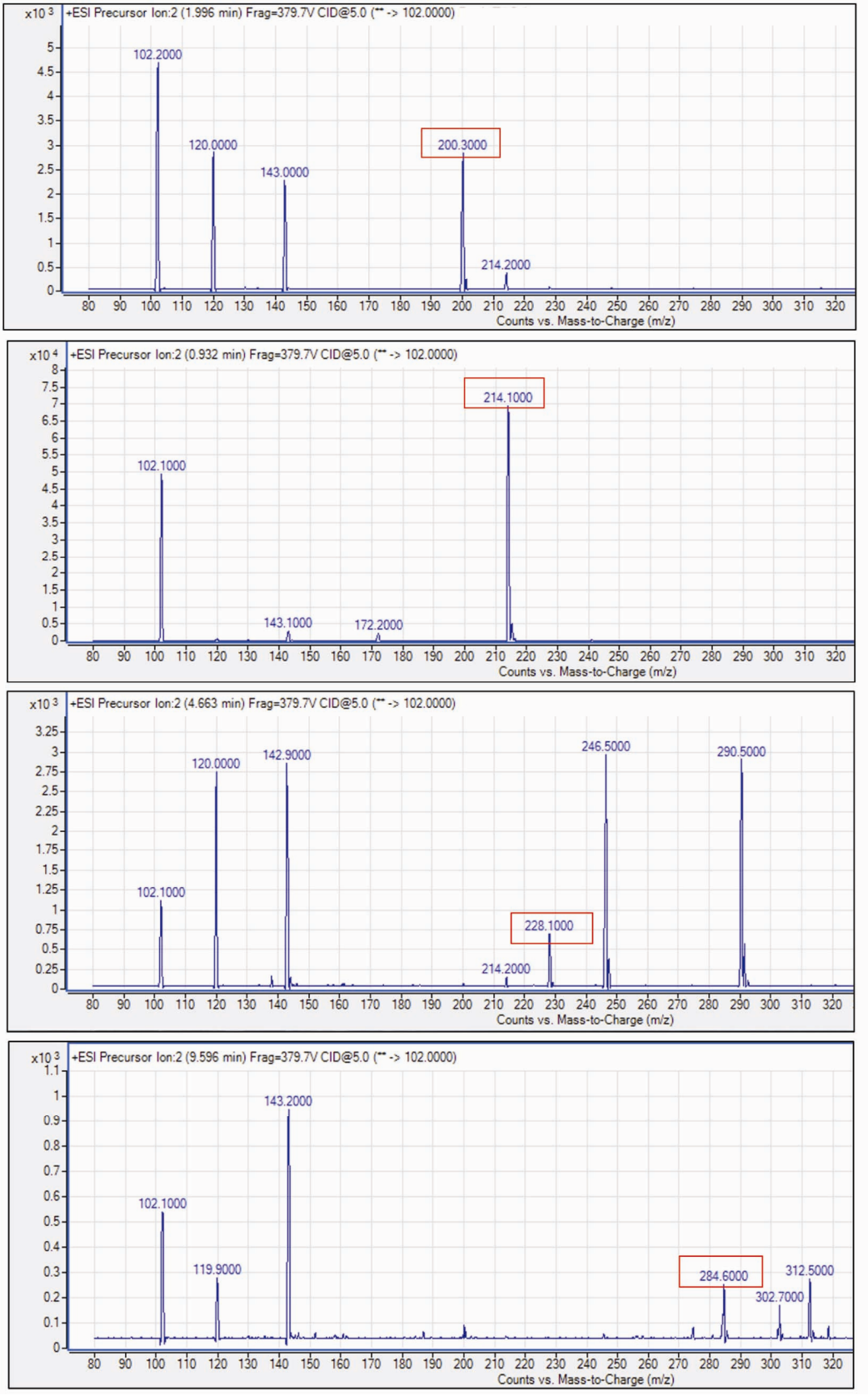

Figure 4. Mass spectra showing AHL profile of the spent supernatant of *Pantoea* sp. A4 using LC-MS/MS. The bacterium produced (***a***) C6-HSL (*m/z* 200.3000), (***b***) 3-oxo-C6-HSL (*m/z* 214.1000), (***c***) C8-HSL (*m/z* 228.1000) and (***d***) C12-HSL (*m/z* 284.6000). Peaks showing the respective AHL are boxed. The fragment ion at *m/z* 102 is a characteristic of the homoserine lactone moiety, showing the presence of AHL.

Figure 5. Scanning electron photomicrograph of *Pantoea* sp. A4. The single bacteria are rod-shaped (magnification = 25,000 ×, bar = 3.0 µm).

GS101 used in this study produces 3-oxo-hexanoylhomoserine lactone which could activate the purple pigmentation in CV026 (ref. 19).

Likewise, strain A4 was also able to stimulate bioluminesence emission by *E. coli* [pSB 401] (Figure 3 *a*). However, bioluminesence was not observed when strain A4 was cross-streaked with *E. coli* [pSB1142] (Figure 3 *b*). This suggests that A4 is a short-chain AHL producer. *E. coli* [pSB401] harbours *V. fischeri luxR* and the promoter region of *luxI* fused to *luxCDABE* from *P. luminescens*. In the presence of exogenous 3-oxo-C6-HSL or closely related short-chain AHLs which bind to LuxR, the LuxR–AHL complex is formed. This complex binds to the LuxI promoter and activates the transcription of *luxCDABE*, resulting in the emission of light. Likewise, *E. coli* JM109 [pSB1142] carries *lasR* and promoter of *lasI* from *P. aeruginosa* PAO1 fused to *luxCDABE* of *P. luminescens.* This biosensor detects AHL with acyl side chain length greater than 10 carbons[11].

Due to their hydrophobic nature, AHLs are readily partitioned from cell-free supernatant into organic solvents such as ethyl acetate. The organic solvent was acidified to prevent lactonolysis of AHLs. The culture supernatant of *Pantoea* sp. A4 was subjected to high-resolution tandem liquid chromatography quadrupole mass spectrometry (LC-MS/MS) analysis to identify its AHL profile. A list of synthetic AHLs used in this study is provided in the supplementary Table S1 (see online). From the MS analysis of the spent culture supernatant, *Pantoea* sp. A4 was shown to synthesize *N*-hexanoyl-homoserine lactone (C6-HSL, *m/z* 200.3000), *N*-(3-oxo-hexanoyl)-homoserine lactone (3-oxo-C6-HSL, *m/z* 214.1000), *N*-octanoyl-L-homoserine lactone (C8-HSL, *m/z* 228.1000) and *N*-dodecanoyl-homoserine lactone (C12-HSL, *m/z* 284.6000). In the mass spectra shown in Figure 4, the molecular mass of *m/z* 102 refers to the presence of lactone moiety to indicate the presence of AHL. The mass spectra for the four AHLs are similar to those of the corresponding synthetic standards analysed under the same parameters (Supplementary Table S2; see online). The *m/z* values obtained from the culture supernatant were very close to the theoretical value of the synthetic standards. Overall, both C6-HSL and oxo-C6-HSL were produced in higher amounts than C8-HSL or C12-HSL.

Pantoea sp. A4 is a light yellow-coloured Gram-negative bacterium. After being subjected to dehydration and fixation, when viewed under SEM, the bacteria are rod-shaped with absence of flagellum. They show a regular morphological form with size of approximately 0.2–0.5 µm in diameter and 1.0–2.5 µm long (Figure 5).

In nature, bacteria often exist in association with plants and their rhizosphere in mixed communities. In mixed populations, many biological functions of microbes and plants are likely to be determined by inter- and intraspecies signalling molecules. The QS circuit clearly has been shown to mediate a diverse array of gene expressions, some of which are involved in interaction between microbes and plant hosts. Numerous studies have shown the significant roles of plant–microbe interactions in the health and productivity of plants[20].

In the present study, *Pantoea* sp. A4 was shown to be phylogenetically related to *Pantoea* sp. strain GM01, strain YR343 and strain R4M-E. Both *Pantoea* sp. GM01 and *Pantoea* sp. strain YR343 were isolated from the rhizosphere of *Populus deltoides* (Eastern Cottonwood) in North Carolina[21], while *Pantoea* sp. R4M-E is another rhizosphere bacterium found in acid soils in the northeast of Argentina[22]. This study has also exemplified QS activity in *Pantoea* sp. A4. This strain was shown to synthesize even-numbered AHLs, namely C6-HSL, OC6-HSL, C8-HSL and C12-HSL. To our knowledge, there are no previous reports of bacteria known to be isolated from the *Rafflesia* flower, which exhibits QS activity.

LC-MS/MS has provided evidence for the presence of long-chain AHL, C12-HSL from the culture supernatant of *Pantoea* sp. A4, which was not observed from the bioreporter assay in Figure 3. This could be possibly due to the low concentration of the secreted C12-HSL that was under the detection limit of reporter strain *E. coli* [pSB 1142]. With the use of synthetic standards, LC-MS/MS is a more reliable and sensitive quantitative method for the detection of AHL in an unbiased manner, particularly in low abundance[23]. Nevertheless, biosensors are still instrumental in screening for the presence of AHLs as this method is relatively simple and produces fast results. Studies have shown that there is an inherent detection bias in many commonly used reporter strains. Thus, multiple bioreported assays need to be used for complete coverage of the AHL profile[6]. On the other hand, it is worth mentioning here that low concentration of AHLs detected in the spent culture supernatant may not reflect

the *in vivo* concentration. At the cellular level, it is possible that a low concentration of AHL could be in fact considered high if it is confined in cellular compartment. However, such hypothesis is yet to be validated and there is no concrete evidence of this so far.

Most *Pantoea* species are found to synthesize short chain AHL, mainly OC6-HSL, but strain A4 is the first bacterium found to produce long-chain AHL. Such a finding may possibly indicate a different language code used for interaction among bacteria of related species[24]. It could also play a role in preventing eavesdropping by other bacteria, hence offering a biological advantage for strain A4 to establish its niche with *Rafflesia*. However, it is not yet known if *Pantoea* sp. A4 exhibits symbiotic, pathogenic and commensal relationship with *Rafflesia*. With the ability of *Pantoea* sp. A4 to secrete both short and long-chain AHLs, it is postulated that this bacterium harbours two different LuxI homologues. Efforts are now being focused on investigating the role of these two homologues of *Pantoea* sp. and its molecular mechanism in establishing its niche with *Rafflesia.*

1. Williams, P., Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiol.*, 2007, **153**, 3923–3938.
2. Miller, M. B. and Bassler, B. L., Quorum sensing in bacteria. *Annu. Rev. Microbiol.*, 2001, **55**, 165–199.
3. Williams, P., Winzer, K., Chan, W. C. and Camara, M., Look who's talking: communication and quorum sensing in the bacterial world. *Philos. Trans. R. Soc. London, Ser. B. Biol. Sci*., 2007, **362**, 1119–1134.
4. Chan, K. G. *et al*., Quorum sensing in *Aeromonas* species isolated from patients in Malaysia. *Curr. Microbiol.*, 2011, **62**, 167–172.
5. Eberhard, A. *et al.*, Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochem.*, 1981, **20**, 2444–2449.
6. Cha, C., Gao, P., Chen, Y.-C., Shaw, P. D. and Farrand, S. K., Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. *Mol. Plant–Microbe Interact.,* 1998, **11**, 1119–1129.
7. Von Bodman, S. B., Bauer, W. D. and Coplin, D. L., Quorum sensing in plant-pathogenic bacteria. *Annu. Rev. Phytopathol*., 2003, **41**, 455–482.
8. Zhang, L., Murphy, P. J., Kerr, A. and Tate, M. E., *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. *Nature*, 1993, **362**, 446-448.
9. Beck von Bodman, S. and Farrand, S. K., Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. *J. Bacteriol*., 1995, **177**, 5000–5008.
10. Hong, K.-W. *et al*., Draft genome sequence of *Pantoea* sp. strain A4, a *Rafflesia*-associated bacterium that produces *N*-acyl-homoserine lactones as quorum-sensing molecules. *J. Bacteriol*., 2012, **194**, 6610.
11. Winson, M. *et al*., Construction and analysis of luxCDABE-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol. Lett.,* 1998, **163**, 185–192.
12. Tamura, K. *et al*., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*., 2011, **28**, 2731–2739.
13. Chan, K. G., Yin, W. F., Sam, C. K. and Koh, C. L., A novel medium for the isolation of *N*-acylhomoserine lactone-degrading bacteria. *J. Ind. Microbiol. Biotechnol*., 2009, **36**, 247–251.
14. Lau, Y., Sulaiman, J., Chen, J., Yin, W.-F. and Chan, K.-G., Quorum sensing activity of *Enterobacter asburiae* isolated from lettuce leaves. *Sensors*, 2013, **13**, 14189–14199.
15. McClean, K. *et al*., Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology*, 1997, **143**, 3703–3711.
16. Yin, W. F., Purmal, K., Chin, S., Chan, X. Y. and Chan, K. G., Long chain *N*-acyl homoserine lactone production by *Enterobacter* sp. isolated from human tongue surfaces. *Sensors* (*Basel*), 2012, **12**, 14307–14314.
17. Wong, C. S. *et al.*, Coexistence of quorum-quenching and quorum-sensing in tropical marine *Pseudomonas aeruginosa* strain MW3A. *World J. Microbiol. Biotechnol.*, 2012, **28**, 453–461.
18. Winson, M. K. *et al.*, Engineering the *luxCDABE* genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn5 constructs. *FEMS Microbiol. Lett.*, 1998, **163**, 193–202.
19. McGowan, S. *et al.*, Carbapenem antibiotic production in *Erwinia carotovora* is regulated by CarR, a homologue of the LuxR transcriptional activator. *Microbiol.*, 1995, **141**, 541–550.
20. Loh, J., Pierson, E. A., Pierson III, L. S., Stacey, G. and Chatterjee, A., Quorum sensing in plant-associated bacteria. *Curr. Opin. Plant Biol*., 2002, **5**, 285–290.
21. Pelletier, D. A. *et al*., Twenty-one genome sequences from *Pseudomonas* species and 19 genome sequences from diverse bacteria isolated from the rhizosphere and endosphere of *Populus deltoides*. *J. Bacteriol.*, 2012, **194**, 5991–5993.
22. Collavino, M., Sansberro, P., Mroginski, L. and Aguilar, O. M., Comparison of *in vitro* solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. *Biol. Fertil. Soils*, 2010, **46**, 727–738.
23. Gould, T. A., Herman, J., Krank, J., Murphy, R. C. and Churchill, M. E. A., Specificity of acyl-homoserine lactone synthases examined by mass spectrometry. *J. Bacteriol.*, 2006, **188**, 773–783.
24. Lazdunski, A. M., Ventre, I. and Sturgis, J. N., Regulatory circuits and communication in Gram-negative bacteria. *Nature Rev. Microbial*., 2004, **2**, 581–592.

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