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Isolation and characterization of quorum-sensing signalling molecules in *Pseudomonas aeruginosa* isolates recovered from nosocomial infections

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Pseudomonas aeruginosa is one of the most common pathogens in nosocomial infections. Many studies have documented the role of quorum-sensing (QS) systems in antibiotic tolerance of *P. aeruginosa*. *N*-acyl homoserine lactones (AHLs) serve as QS signalling molecules and can be a target for modulating bacterial pathogenicity. In this study, nosocomial isolates of *P. aeruginosa* were characterized for the presence of different types of QS signalling molecules. AHLs were solvent extracted and quantified by determination of β-galactosidase activity using the *Escherichia coli* MG4 reporter strain. Further characterization was performed by analytical thin layer chromatography coupled with detection using the *Agrobacterium tumefaciens* A136 biosensor strain. All *P. aeruginosa* isolates produced AHLs, but there were differences in the quantity and nature of AHLs. We identified AHLs belonging to C4-homoserine lactone (HSL), C6-HSL, C8-HSL, C10-HSL and C12-HSL. AHL profiling of *P. aeruginosa* isolates showed differences in the amounts and types of AHLs, suggesting differences in the virulence factors and the potential for infection. Our results may be investigated further using animal model systems.

Key words: *Pseudomonas aeruginosa*; *N*-acyl homoserine lactones; nosocomial infection; β-galactosidase; quorum-sensing molecules.

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Pseudomonas aeruginosa is a gram-negative organism commonly found in soil, water, skin flora and most indoor environments. It thrives on medical equipment, including catheters, causing cross-infections in hospitals and clinics (1). *P. aeruginosa* is an opportunistic pathogen, especially in immunosuppressed patients and in those with cystic fibrosis, malignancy or trauma (2).

Many virulence factors in *P. aeruginosa* are regulated by quorum-sensing (QS) molecules, mainly *N*-acyl homoserine lactones (AHLs). These small diffusible molecules can be detected by surrounding organisms (3), and transcriptional regulators are activated when

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the concentration of AHLs reaches a threshold level. This mechanism of communication enables bacteria to act as a community and coordinate regulation of gene expression, including virulence genes. This coordination gives the bacteria an advantage over host defences and thus plays a role in pathogenesis (4, 5), which has been confirmed in a number of different animal models (6). In this study, we have analysed QS signals in nosocomial isolates of *P. aeruginosa* by using a bacterial biosensor able to detect exogenous AHLs (7).

MATERIALS AND METHODS

Bacterial strains

A total of 31 non-repetitive isolates of *P. aeruginosa* were recovered from patients with nosocomial infections at King Khaleed Hospital, King Saud University, Riyadh, Saudi Arabia between August 2011 and March 2012. The strains were obtained from invasive and non-invasive sites, including wounds, blood, sputum, throat swabs and urine. To identify and quantify AHLs, the *Escherichia coli* MG4 pKDT17 reporter strain (Sigma Chemical Co., Bangalore, India) was grown on nutrient agar containing 100 μg/mL ampicillin. *E. coli MG4 pKDT17* contains a *lasB'-lacZ* detection system and a *lasR* gene under the control of the *lac* operon promoter. This reporter strain produces β-galactosidase in response to a wide range of AHLs, including 3-hydroxy-, 3-oxo- and unsubstituted AHLs with side chain lengths of 8–14 carbons. AHL-biosensing *Agrobacterium tumefaciens* A136 (ATCC accession number BAA-2240) was grown on Minimal A medium containing 30 μg/mL gentamicin, and was used for analytical thin layer chromatography (TLC). *A. tumefaciens* A136 biosensor does not produce AHLs, but contains a plasmid harbouring the traR promoter, and *traG*::*lacZ* transcriptional fusion protein traG::lacZ is activated in the presence of exogenous AHL, resulting in the appearance of a blue colour by β-galactosidase activity. Most biosensors respond to a limited number of AHLs because of the specificity of their R protein (7).

Extraction of AHLs

Cultures were grown overnight in Luria-Bertani (LB) broth (HiMedia, Mumbai, India), and supernatants were extracted twice with equal volumes of acidified ethyl acetate. Pooled extracts were dried over anhydrous magnesium sulphate and then dried by evaporation using a rotator evaporator. Residues were resuspended in 100 μL of HPLC-grade ethyl acetate.

Analytical TLC

To characterize the different AHLs, TLC was carried out as described previously (8). Briefly, 10 μL of sample was applied to the silica gel TLC plates ($C18_{RP}$ Silica gel plates; Merck, Darmstadt, Germany). The chromatograms were developed using a mixture of chloroform and methanol (9:1 v/v). Once the solvent had migrated to within 2 cm of the top, the plates were air dried. The plates were then overlaid with a thin film of 1% agar containing 65 μg/mL X-gal (9683801L; Promega BioSciences, San Luis Obispo, CA, USA) and seeded with the biosensor strain, *A. tumefaciens* A136. All experiments were performed in duplicate. AHLs were identified by comparing the retention factor (Rf) of synthetic standard AHLs (Sigma Chemical Co.) and test AHL spots. The Rf of the spots from the isolates was calculated and compared with that of the standards. Acyl homoserine lactone (HSL) standards [mixture of C4 (BHL), C6 (HHL) and C8 (OOHL)] (lane 1) were submitted to TLC and visualized with the biosensor as done for acyl HSLs produced by isolates of *P. aeruginosa* (lanes 2-6).

AHL quantification by β-galactosidase assay

The quantity of AHLs was measured by determining the β-galactosidase activity with o-nitrophenyl-D-galactopyranoside substrate (ONPG; Sigma Chemical Co.) as described earlier (9). Briefly, each strain was grown in 2 mL LB broth overnight at 37 °C and 5 mL LB broth was inoculated and incubated at 37 °C until the exponential growth phase. Dilutions of 0.5 mL cell suspensions with 0.5 mL Minimal A medium were prepared in triplicate and two controls were prepared with 1.0 mL Minimal A medium. To permeabilize the cells, one drop of 0.1% SDS and two drops of chloroform were added using a Pasteur pipette. The tubes were placed in a 30 °C water bath and allowed to equilibrate for about 2 min. Then, 0.2 mL ONPG (final concentration of 4 mg/mL in 0.1 M phosphate buffer pH 7.0.) was added to each tube, followed by vortexing to initiate the reaction. When a yellow colour developed, 0.5 mL of 1 M Na_2CO_3 was added to stop the reaction. The absorbance for each tube was determined within 1 h and used to calculate the activity of β-galactosidase in Miller units (9). For each strain extract, the values presented are means of three independent experiments.

RESULTS

With the biosensor strain, *A. tumefaciens* A136 and the agar overlay technique purple spots were found in the position of the C4 and

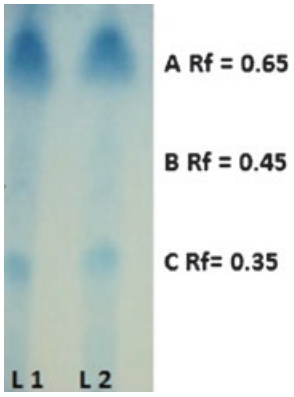

Fig. 1. Retention factor of an isolate (lane 1) and the control (lane 2).

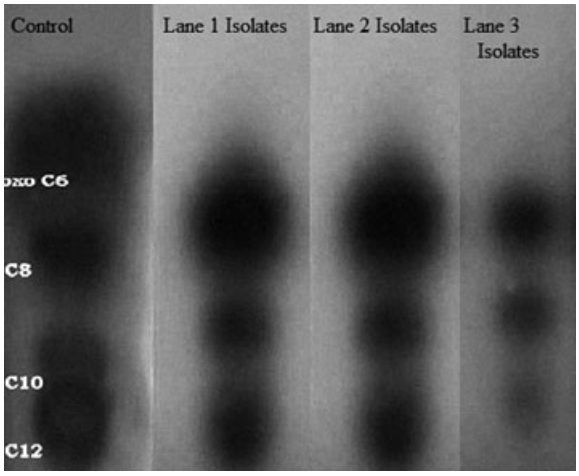

Fig. 2. Different types of *N*-acyl homoserine lactones based on retention factor.

C6-HSL standards (Fig. 1). All *P. aeruginosa* isolates in our study showed production of AHLs and most of the isolates showed production of different types of AHLs on the basis of relative Rfs (Fig. 2). Twenty-seven of the 37 isolates (87%) were positive for C8-HSL, 26 (83%) for C6-HSL, 18 (58%) for C12-HSL, 17 (54%) for C10-HSL and only 10 (32%) for oxo-C6-HSL.

DISCUSSION

In this study, we used the *A. tumefaciens* A136 biosensor strain to screen, identify and quantify AHLs in nosocomial isolates of *P. aeruginosa* from King Khaleed Hospital, Riyadh, Saudi Arabia. By using the *A. tumefaciens* biosensor strain, all *P. aeruginosa* isolates in our study showed production of AHLs, which was further confirmed by the blue colouration of the *E. coli* MG4 reporter strain. The types of AHL produced by *P. aeruginosa* in our study have not been reported previously. Therefore, we applied TLC to extracts and developed the plates with the biosensor.

A previous study has indicated that *Chromobacterium violaceum* CV026 cannot detect AHLs with longer acyl side chains (10), and therefore a broad range biosensor, *A. tumefaciens* A136, was employed in our study. Besides high sensitivity to low levels of AHLs with long acyl side chains, the strain can detect most AHLs, including C8–C12-HSLs and oxo-C6-HSL (7, 10). By using only one biosensor, we found more than two different types of AHL in most isolates. The presence of at least two types of AHLs, such as C12-HSL, C6-HSL, oxo-C6-HSL and C4-HSL, has been detected previously with the *A. tumefaciens* A136 biosensor strain. Previous reports have also shown the production of different types of AHLs, such as C8-HSL, C10-HSL and C12-HSL, in clinical isolates (7). Our quantitative results based on induced β-galactosidase activity showed that isolates from the same source produced different amounts of AHLs in addition to various types of QS molecules. This result suggests notable strain differences in terms of AHL production.

In conclusion, the methods applied here were useful to detect production of AHLs in pure cultures of nosocomial *P. aeruginosa* isolates, and showed the presence of more than one type of AHL in most strains. Because of the key role in QS, the production of AHLs might be a target for detection in clinical samples.

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