Contribution of the production of quorum sensing to some phenotypic characteristics of *Pseudomonas aeruginosa* clinical strains

C. Nagant, M. Seil, A. Nachtergael, S. Dulanto and J. P. Dehaye

Laboratoire de Chimie biologique et médicale et de Microbiologie pharmaceutique, Faculté de Pharmacie, Université libre de Bruxelles, Brussels, Belgium

The contribution of quorum sensing in some phenotypic and pathogenic characteristics of *Pseudomonas aeruginosa* was studied. The production of acylhomoserine lactones (AHL) by planktonic cultures of eight clinical and reference strains of *P. aeruginosa* was evaluated using two biosensors. The adhesion of the bacteria on a surface (Biofilm Ring Test \( \text{BFRT} \)), their capacity to develop a biofilm (crystal violet staining method, \( \text{CVSM} \)), their sensitivity to tobramycin and their secretion of proteases or of rhamnolipids were also measured. The production and the release of AHL widely varied among the eight strains. An analysis of the extracts by TLC showed that 3-oxo-C\(_8\)-HSL, 3-oxo-C\(_{10}\)-HSL and 3-oxo-C\(_{12}\)-HSL were released by the five strains producing the highest amount of C\(_n\)≥6-HSL. The genes *las* and *lasR* involved in the synthesis and response to 3-oxo-C\(_{12}\)-HSL were detected in the genomes of all strains. Two clinical strains had deletions in the *lasR* gene leading to truncation of the protein. One subpopulation of the PAO1 strain had a major deletion (98 bp) of the *lasR* gene. Strains with significant mutations of *lasR* secreted the lowest amount of AHL, probably due to deficiencies in the self-induction and amplification of the synthesis of the lactone. These strains formed a biofilm with low biomass. C\(_4\)-HSL production also differed among the strains and was correlated with rhamnolipid production and biofilm formation. Whereas the production of AHL varied among *P. aeruginosa* strains, few correlations were observed with their phenotypic properties except with their ability to form a biofilm.

**INTRODUCTION**

*Pseudomonas aeruginosa* is an opportunistic pathogen for plants, invertebrates and vertebrates and is frequently associated with nosocomial infections (Fridkin et al., 1999). Critically ill patients in intensive care units or immunocompromised patients with chemotherapy-induced neutropenia, diabetes, AIDS, severe burns or skin abrasion are more susceptible to infections (Pizzo, 1999). There is also a very close association between *P. aeruginosa* and cystic fibrosis (CF) (Hauser et al., 2011). Once inside the organism, *P. aeruginosa* colonizes a surface by forming a biofilm (Mulcahy et al., 2008). Bacteria encapsulated in the biofilm have an increased resistance to host defences (Leid, 2009) or to antibiotherapy (Mah et al., 2003). It is clearly established that when bacteria move from a planktonic way of life to a biofilm mode of growth, they acquire some properties reminiscent of social behaviour (Parsek & Greenberg, 2005). Within microcolonies, the bacteria seem to detect the number of adjacent cells by a mechanism called quorum sensing (QS), a name coined by Fuqua et al. (1994). Each cell synthesizes and releases inducers in the surrounding medium inducers. When the cellular density reaches a critical threshold, these inducers trigger paracrine stimulation of neighbouring cells. They bind to transcription factors that regulate the expression of enzymes responsible for their own synthesis, creating a positive feedback loop or regulating the expression of proteins involved in diverse responses, such as antibiotic resistance or biofilm development. Two major mechanisms based on acylated homoserine lactones (AHL) have been described in *P. aeruginosa* (Ruimy & Andremont, 2004). Las and Rhl are the two communication systems using N-3-oxododecanoyl homoserine lactone (3-oxo-C\(_{12}\)-HSL) and N-butanoyl homoserine lactone (C\(_4\)-HSL), respectively. AHL synthesis and secretion are variable among clinical strains of *P. aeruginosa* (Geisenberger et al., 2000). We previously reported that eight mucoid and nonmucoid strains of *P. aeruginosa* have distinct phenotypic characteristics. The...
purpose of this work was to study whether this phenotypic heterogeneity could be correlated with differential production of AHL by the various clinical and reference strains.

**METHODS**

**Bacterial strains.** The *P. aeruginosa* CGMCC 1.860 strain was kindly provided by Dr J. Zhong (East China University of Science and Technology, Shanghai, China), and the *A. tumefaciens* NTL4(pZLR4) strain was provided by Professor S. Farrand (University of Illinois, Urbana-Champaign, USA). The two strains have been genetically modified to be used as bioassay strains for C4-HSL (Yong & Zhong, 2009) and C6≥5-HSL (Cha et al., 1998), respectively. The *P. aeruginosa* CGMCC 1.860 strain was grown overnight at 28°C in the presence of 100 mg/ml ampicillin and 250 mg/tetracycline. Cultures of *A. tumefaciens* NTL4(pZLR4) were grown overnight at 28°C in Autodissolved media (AB) and incubated under constant shaking supplemented with 30 mg gentamicin.

Clinical isolates from sputum of CF patients at the Erasmus Hospital in Brussels (*P. aeruginosa* PYO1 and PYO2 strains), respiratory tract clinical isolates from CF patients at the University Hospital of Ghent (*P. aeruginosa* MC75-450457, MC99-450467 and MC93-450507) and the reference strains *P. aeruginosa* PAO1 (ATCC15692), ATCC9027 and ATCC15442 were tested. Four of these eight strains had a mucoid phenotype (PYO2, MC75-450457, MC99-450467 and MC93-450507). The strains were stored at -20°C in glycerol. Before their use, the strains were spread onto Mueller–Hinton solid medium and incubated at 37°C for 24 h. After culturing, the strains were identified by applying the API 20 NE system (bioMe´rieux). The strains were grown overnight at 28°C in glycerol. Before their use, the strains were spread onto Mueller–Hinton solid medium and incubated at 37°C for 24 h. After culturing, the strains were identified by applying the API 20 NE system (bioMe´rieux). The bacteria were plated not more than three times onto Mueller–Hinton solid medium. Colonies were then transferred into sterile brain heart infusion medium (BHI) and incubated under constant shaking (150 r.p.m.) at 37°C in an orbital shaking incubator (Gallenkamp Orbital Incubator, Sanyo). After overnight incubation, the culture was adjusted to a final optical density of 600 nm (OD600) of 1.00 ± 0.05 by adding sterile BHI medium. The initial bacterial suspension (IBS) was obtained by a 250-fold dilution of the bacterial suspension. The same IBS was prepared for the Biofilm Ring Test (BFRT) and the crystal violet staining method (CVSM).

**Assay for the detection of C4-HSL produced by *P. aeruginosa.*** The secretion and synthesis of C4-HSL by the various *P. aeruginosa* strains were studied according to the methods described by Yong & Zhong (2009) using the bioassay strain *P. aeruginosa* CGMCC 1.860. This strain is unable to produce C4-HSL because it lacks *rhlI.* It overexpresses *rhlR*, rendering it very sensitive to exogenous C4-HSL. In response to RhlR activation, this environmental strain forms biofilms. The adhesion of the bacteria on an abiotic surface synthesis a blue pigment that is easily detected. Briefly, the clinical and reference strains of *P. aeruginosa* were incubated overnight at 37°C in LB medium. The next day, the OD600 was adjusted to 1.00 ± 0.05. Next, 5 ml bacterial culture samples were centrifuged at 3000 r.p.m. for 10 min. The supernatants were extracted three times with 5 ml ethyl acetate. The organic extracts were pooled, and the water remaining in the extracts was removed with magnesium sulfate. After filtration, the extracts were evaporated to dryness. The residues were dissolved in 1 ml HPLC-grade acetonitrile, 500 μl extract samples were dried under a stream of nitrogen and the residue was incubated in the presence of 1 ml bioassay strain previously diluted with PB to an OD600 of 0.05. The bacterial suspension and the HSL extracts were vigorously mixed and incubated for 24 h at 30°C under constant shaking. The cultures were then centrifuged at 3000 r.p.m. for 10 min, and the supernatants were extracted twice with 0.5 ml chloroform. After evaporation of the chloroform, the residues were dissolved in 500 μl methanol, and then methanolic solution samples (150 μl) were transferred to the wells of a 96-well UV star plate (Greiner Bio-One), and the absorbance of the blue pigment was measured at 299 nm with a plate reader.

**Microplate assay for the detection of C6≥5-HSL produced by *P. aeruginosa.*** After overnight culture, the cellular suspensions of the various strains were adjusted to a final OD600 of 1.00 ± 0.05. After centrifugation of the bacterial suspensions, 700 μl supernatant was vigorously mixed with the same amount of ethyl acetate. The upper layer (300 μl) was evaporated, and the residue was dissolved in 10 μl 10 mM HCl solution (30-fold concentration of C6≥5-HSL). *A. tumefaciens* NTL4(pZLR4) was inoculated in a 96-microwell plate to a final volume of 100 μl per well, and 2 μl extracted C6≥5-HSL was added to each well. A blank (without ethyl acetate extract) was run in parallel. The plate was incubated for 18 h at 28°C to promote lacZ reporter gene expression. Then, 20 μl from each well was transferred into a new 96-microwell plate containing 80 μl Z-buffer (60 mM NaHPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4 and 50 mM β-mercaptoethanol). Next, 25 μl 1 mg/ml 4-methylumbelliferyl β-D-galactopyranoside (MUG) solution in DMSO was added to the wells, and the fluorescence generated by the hydrolysis of MUG by β-D-galactosidase was measured for 30 min with a plate reader at excitation and emission wavelengths of 360 and 460 nm, respectively.

**TLC analysis of C6≥5-HSL produced by *P. aeruginosa.*** TLC analysis was performed as described by Shaw et al. (1997). The *P. aeruginosa* strains were grown to stationary phase in 5 ml AB medium supplemented with 0.4% glucose. After centrifugation, the supernatant was extracted twice with equal volumes of ethyl acetate. The organic phases were pooled, dried over magnesium sulfate, filtered and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl ethyl acetate containing 0.1% acetic acid. The AHL present in the acidified extract was separated by chromatography on a reverse-phase plate with a solvent system of methanol/water (60/40, v/v). The bioassay strain was prepared in AB medium containing 0.4% mannitol, 0.7% agar and 60 mg X-Gal l−1. After development, the chromatographic plate was transferred in a Pexiglas jig and overlaid with a thin pellicle (~3 mm) of the soft bioreporter strain/agar mixture. The agar was allowed to dry, and the plate was incubated for 24 h at 28°C. During this incubation, blue spots appeared where AHL had migrated. A digital image of the plate was analysed with the ChemiDoc XRS+ system using Image Lab 3.0 software. The Rs of the spots were compared with those of known standards (Shaw et al., 1997).

**Study of the adhesion of the various strains and their ability to develop a biofilm.** The adhesion of the bacteria on an abiotic surface was performed using the BFRT, and the biomass of the biofilm formed by the bacteria adherent to the bottom of microtitre plate wells was assayed using the CVSM as previously described (Nagant et al., 2010a).

**Sensitivity of the various strains to tobramycin.** The eight *P. aeruginosa* strains were incubated for 24 h at 37°C in the wells of a 96-microwell plate (Nunc MicroWell Plates; Nalge Nunc) as previously described (Moskowitz et al., 2004; Nagant et al., 2010b). The plates were covered with lids presenting 96 pegs (Nunc TSP, Transferable Solid Phase Screening System; Nalge Nunc) that were immersed in the wells and on which the biofilm could build up. After this incubation, the pegs of the lid were washed, the cover was transferred to a new plate and the pegs were plunged into wells filled with fresh medium in the absence or in the presence of 4 μg tobramycin ml−1. Young biofilms were exposed to the drugs for 24 h before the bacteria were quantified to determine the number of c.f.u. on the pegs.
Assay of protease activity by the various strains. *P. aeruginosa* strains were incubated in 10 ml mineral salt (MS) medium containing 40 mM K2HPO4, 15 mM KH2PO4, 1 mM MgSO4, 7.5 mM (NH4)2SO4, 0.4% (w/v) glycerol (pH 7.0) and 2% (v/v) skimmed milk at 37 °C for 48 h. After this incubation, bacterial suspension OD600 was adjusted to 0.60 ± 0.0. The bacterial suspensions were centrifuged at 10 000 r.p.m. for 15 min at room temperature. Next, 500 µl supernatant was incubated with 100 µl 30 mg ml⁻¹ azocasein solution (Sigma). A blank assay was run in parallel with only MS medium and azocasein. The reaction mixtures were incubated at 37 °C for 3 h. At the end of the incubation, the reaction was stopped with 100 µl 20% (w/v) trichloroacetic acid, and the samples were kept at room temperature for 10 min. The mixture was centrifuged at 10 000 r.p.m. for 10 min. Supernatant absorbance was measured at 405 nm using a plate reader.

Assay of rhamnolipid production by the various strains. The amounts of rhamnolipids (3-deoxy-hexose) produced by the eight strains of *P. aeruginosa* were estimated by measuring rhamnose with a colorimetric test (Wilhelm *et al.*, 2007). The cells were adjusted to an initial OD580 of 0.05 ± 0.01 and grown for 48 h in 20 ml proteose-peptone/glucose/ammonium salts (PPGAS) medium at 30 °C and 120 r.p.m. to stimulate rhamnolipid production. The bacterial suspension was centrifuged, and 300 µl supernatant was extracted twice with 600 µl diethylether. The extracts were pooled and evaporated to dryness. The extracts were then dissolved with 100 µl distilled water and mixed with 100 µl 1.6% orcinol and 800 µl 60% sulfuric acid. After incubation at 80 °C under constant shaking at 175 r.p.m. for 30 min, the absorbance was measured at 421 nm using a plate reader. The assay was done in triplicate, and the results were compared with a standard curve established with known concentrations of rhamnose.

Detection and sequencing of selected genes contributing to the quorum sensing signalling pathways. The genomic DNA was released with lysis buffer and extracted with the GenElute bacterial genomic DNA kit (Sigma) according to the manufacturer’s instructions. Oligonucleotide primers designed by Schaber *et al.* (2004) (Eurogentech) were used to detect the *lasI*, *lasR*, *rhlI*, and *rhlR* genes (Table 1). The PCR was performed in a 50 µl final volume containing a half volume of Master Mix 2 × (Fermentas), 2 µl genomic DNA and 200 pmol of each primer in water. The sequences were amplified using a Bio-Rad iCycler with the following protocol used for PCR: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 120 s (*lasI* and *lasR*) or denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 120 s (*rhlI* and *rhlR*). Thirty-four cycles were performed. The products were next cleaned using the DNA Clean-Concentrator-5 kit (ZymoResearch) and sequenced by Eurofins DNA (Ebersberg). The comparison of the four genes with the published genomes of various *P. aeruginosa* strains was performed with a multigenic blast search. The reference strain with the best score was used as the reference, and each gene was then compared with the sequence found in the genome of the selected reference strain. The analysis was performed using the sequences obtained with both primers (forward and reverse). The comparison between the sequence of the protein of the reference strain and of the tested strain was performed using the SIM alignment tool (ExpASy).

**RESULTS AND DISCUSSION**

Study of the production of C₄-HSL and Cₙ≥₆-HSL

In the first experiment, C₄-HSL production by eight *P. aeruginosa* strains was measured. As shown in Fig. 1(a), three mucoid strains (PY02, MC75-450457 and MC099-450507) and one nonmucoid strain (PA01) produced the highest amounts of C₄-HSL. The four other strains (PY01, MC099-450467, ATCC15442 and ATCC9027) produced comparable, intermediate amounts of C₄-HSL. There was no obvious difference between mucoid and nonmucoid strains. A similar experiment was performed using the *A. tumefaciens* NTL4(pZLR4) reporter strain to study Cₙ≥₆-HSL synthesis and release (Fig. 1b). This strain expresses TraR, a transcription factor that, unlike LasR or RhlR, is rather unspecific and responds to several AHLs with an acyl group longer than four carbons (Cha *et al.*, 1998). The expression of *lacZ* by the bioreporter strain secondary to the interaction between TraR and Cₙ≥₆-HSL varied with the extracts from the eight strains (PY02 > MC75-45057

**Table 1. Oligonucleotide primers used to amplify and sequence rhlI, rhlR, lasI and lasR genes**

Four QS genes were amplified by PCR and sequenced. The primers used to amplify the sequences were designed according to Schaber *et al.* (2004). The PCR protocol is described in Methods.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
<th>Primers</th>
<th>Position in the genome</th>
<th>Size of the amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhlI</td>
<td>PA3476</td>
<td>5'-ACGGGTGACGCACCTCACAC-3'</td>
<td>3889126—3889144</td>
<td>626 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5'-CTTTGTCACTGATGATTGCT-3'</td>
<td>3889730—3889751</td>
<td></td>
</tr>
<tr>
<td>rhlR</td>
<td>PA3477</td>
<td>5'-GTCTCATGAGGACCCAGC-3'</td>
<td>3889922—3889940</td>
<td>731 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5'-CAATGAGGAATGAGGAGG-3'</td>
<td>3890633—3890652</td>
<td></td>
</tr>
<tr>
<td>lasI</td>
<td>PA1432</td>
<td>5'-ATGATCGTGAACATTGTCGCGC-3'</td>
<td>1559254—1555275</td>
<td>607 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5'-GTATGAAACGGCAGTGCT-3'</td>
<td>1559842—1559860</td>
<td></td>
</tr>
<tr>
<td>lasR</td>
<td>PA1430</td>
<td>5'-ATGCCCTTGTGAGCAGGTT-3'</td>
<td>1558171—1558189</td>
<td>726 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5'-GCAAGATCAGAGTATAAGACCCA-3'</td>
<td>1555871—1555896</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
(a) Production of C$_2$-HSL

(b) Production of C$_{10}$-HSL

(c) BFRT

(d) CVSM

(e) Production of rhamnolipids

(f) Protease activity

(g) Sensitivity to tobramycin

<table>
<thead>
<tr>
<th>Strain</th>
<th>BFRT</th>
<th>CVSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYO2/MC075MC093MC099</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC0999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC15442</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PYO1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC98927</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration of rhamnose (µg ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
</tr>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>4.0</td>
</tr>
<tr>
<td>6.0</td>
</tr>
<tr>
<td>8.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensitivity to tobramycin (Drop of log c.f.u. ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
</tr>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>4.0</td>
</tr>
<tr>
<td>6.0</td>
</tr>
<tr>
<td>8.0</td>
</tr>
</tbody>
</table>
Considering that spontaneous mutations of quorum sensing genes, especially lasR, are frequent (Sandoz et al., 2007), the lasI, rhlI, rhlR and lasR genes were sequenced after PCR amplification. For each strain, a BLAST search was performed with a combination of the sequences of the four genes. As expected, the best scores for the four genes of the reference PAO1 strain were obtained with the following strains: PAO1 (4384)>LESB58 (4323)>M18 (4312). This order was different with all the other strains (LESB58>M18>PAO1) except for PYO1 (LESB58>PAO1>M18). It should be mentioned that the scores obtained by comparing the eight tested strains with the LESB58, PAO1 and M18 strains were never very different (<2%). The sequences of the four genes of all the strains (except PAO1) were compared with the genome of the LESB58 strain, a hypervirulent strain first isolated in 1996 from patients with CF (Cheng et al., 1996).

Only silent point mutations were found in lasI, rhlI and rhlR. LasR was the only gene with mutations affecting the structure of the protein. This is consistent with reports that clinical strains of P. aeruginosa isolated from patients with CF show a high rate of mutations of the lasR gene: after colonization of CF lung, 60% of the isolates carried a lasR mutation (Smith et al., 2006). The MC099-450467 and PY01 strains had the most significant mutations of lasR. In the MC099-450467 strain, the G584A mutation introduced a premature STOP codon at position 195, which truncated the C-terminal domain of the protein (residues 195–237). In the PY01 strain, a 2 bp deletion provoked a frameshift of the reading frame, which resulted in major changes in the primary structure of the protein (modification of the translation product after R122). These major alterations of the LasR protein might account for the low level of AHL produced by the PY01 and MC099-450467 strains. Three strains had missense mutations in the alpha helix involved in the binding of LasR to DNA (Bottomley et al., 2007). One of these mutations (V208M in the MC093-450507 strain) affected a conserved residue, but the role of this valine has not been defined (Bottomley et al., 2007). Its substitution by another hydrophobic amino acid should not greatly affect its activity. The two other mutations concerned the M212; M212T for ATCC9027 and M212V for ATCC15442. This amino acid belongs to an alpha helix interacting with DNA (Bottomley et al., 2007). It is substituted by either valine, another hydrophobic acid (ATCC15442) or by threonine, a polar residue (ATCC9027). This mutation might account for the low level of AHL observed for this strain.

Two lasR genes were amplified in the reference strain PAO1. One of these genes was identical to the published sequence of the lasR gene of this strain. A subpopulation of PAO1 had a major deletion that covered most of the fragment. This experiment was repeated with another PAO1 strain provided by Dr O. Vandeputte (Vandeputte et al., 2011). Only one lasR amplicon could be observed with this strain, confirming that a mutation had occurred in our PAO1 strain during conservation. Spontaneous mutations of PAO1 occurring after several passages have been described by Klockgether et al. (2010). Sandoz et al. (2007) suggested that these mutations would arise under conditions in which nutrients are in full supply and QS signals are dispensable. According to these authors, a QS-proficient strain and a QS-deficient cheater strain might coexist in a heterogeneous population.

In a next step, TLC was performed to separate the AHL produced by the strains, and the spots were detected with an overlay of the A. tumefaciens NTL4(pZLR4) bioreporter strain. The migration of AHL produced by P. aeruginosa was compared with that of the standards (Table 2). In agreement with Cha et al. (1998) the bioreporter strain detected AHL with acyl chains from 6 to 12 carbon atoms. No spot was observed with three strains (MC099-450467, PY01 and ATCC9027). Three spots were detected in the culture medium of the five other strains producing AHL (PY02>MC75-45057>ATCC15442>MC093-450507>ATCC PAO1). These spots migrated with Rfs compatible with 3-oxo-C9-HSL, 3-oxo-C10-HSL and 3-oxo-C12-HSL (Table 2). These three AHLs are synthesized by LasI. It is widely accepted that the two quorum-sensing systems of P. aeruginosa are mostly responsive to C4-HSL (RhlR) and to C10- and 3-oxo-C12-HSL (LasR). The role of 3-oxo-C9-HSL or 3-oxo-C10-HSL remains elusive. It was recently reported that the sensitivity of P. aeruginosa to 3-oxo-C9- or 3-oxo-C10-HSL was regulated by the MexAB-OprM efflux pump (Minagawa et al., 2012). This pump mediates the efflux of long-chain 3-oxo-AHL. Mutations of this protein might contribute to the intracellular accumulation of 3-oxo-C10-HSL at concentrations sufficient to activate LasR. The 3-oxo-C9-HSL that diffuses out of bacteria might interact
The culture media of the eight strains were extracted with ethyl acetate and 4 μl extracts were spotted on a reverse-phase plate. The plate was developed with a solution of methanol/water, and the AHLs were revealed with an overlay of soft agar containing the bioreporter strain and X-Gal. The migration of various AHLs was measured using Image Lab 3.0 software with the ChemiDoc XRS+ system. The results presented in the Table are the Rfs of the different spots and are expressed in cm. They are the means of two to three experiments. Columns 2 and 3 represent AHL standards that were used for comparison. N.M. Not measured. U.D., Undetectable.

with transcriptional proteins of other Gram-negative bacteria and contribute to the development of multispecies biofilms (Riedel et al., 2001).

**Phenotypic characteristics**

**Adhesion and formation of a biofilm by the various strains.** The adhesion of the various strains on 96-microwell plate bottoms was measured with the BFRT (Fig. 1c). This test estimates the migration of magnetic beads and their ability to agglomerate at the centre of the well during exposure to a magnet. This is reflected by a high (>12) biofilm index (BFI). The formation of a biofilm in the wells interferes with the motility of the beads, and the BFI decreases down to values around 2. As shown in Fig. 1(c), the immobilization of the beads varied among the strains (ATCC PAO1=ATCC9027>ATCC15442 >MC093-450507>MC75-450457=PYO1=PYO2=MC099-450467). Three nonmucoid strains had very low BFs consistent with complete biofilm formation within 1 h. The MC093-450507 strain was the only mucoid strain that could immobilize the magnetic beads (BFI significantly lower than control, 6.26 ± 0.33, n=6). The CVSM provides a good estimate of the amount of biofilm formed over longer times. After 24 h, a mucoid strain (MC099-450467) and two nonmucoid strains (PYO1 and ATCC9027) formed very little biofilm (OD540<0.5). The mucoid MC75-450457 strain formed an intermediate biofilm (0.5<OD540<1.0). Two mucoid strains (PYO2 and MC093-450507) and two nonmucoid strains (ATCC PAO1 and ATCC15442) formed considerable biofilm (OD540>1.5) (Fig. 1d).

**Production of rhamnolipids by the various strains.** Rhamnolipid production was estimated with rhamnose assays (Fig. 1e). Four strains were strong producers of rhamnose (MC093-450507>ATCC PAO1>MC75-450457>PYO2). The PYO1 strain produced a small but significant amount of the sugar. The three remaining strains (ATCC9027, ATCC15442 and MC099-450467) did not produce any significant levels.

**Protease secretion by the various strains.** The activity of proteases secreted by *P. aeruginosa* was tested using azo-casein as a substrate. As shown in Fig. 1(f), two mucoid strains (MC75-450457 and MC093-450507) had the highest activity among the eight strains. The two other mucoid strains (PYO2 and MC099-450467) and the non-mucoid PYO1 strain had no activity (MC75-450457>MC093-450507 =ATCC PAO1>ATCC9027=ATCC15442>>PYO2>MC099-450467=PYO1).

**Sensitivity of the various strains to tobramycin.** Tobramycin is an aminoglycoside especially active against *P. aeruginosa*. The antibiotic was tested at a 4 μg ml⁻¹ concentration. The four non-mucoid strains were very sensitive to the drug (PYO1>ATCC PAO1>ATCC15442>ATCC9027). The c.f.u. decreases ranged from 3.48 log c.f.u. ml⁻¹ to 6.14 log c.f.u. ml⁻¹. The four mucoid strains were much less sensitive to the drug. The number of c.f.u. ml⁻¹ only decreased by 1.95 log for the MC75-450457 strain, the mucoid strain that was the most sensitive to tobramycin. The MC093-450507 strain was fully resistant to the aminoglycoside (Fig. 1g).

**Correlation between AHL production and phenotypic characteristics**

There was no correlation between the production of C₆-HSL and the adhesion of bacteria (BFRT), biofilm production (CVSM), sensitivity to tobramycin, protease secretion or rhamnolipid production. C₄-HSL production was correlated with rhamnolipid production (Fig. 2a). This correlation is secondary to the regulation of the rhlAB operon by the RhlR-C₄-HSL complex (Ochsner & Reiser, 1995). The production of C₄-HSL also correlated with biofilm formation (CVSM) (Fig. 2b) but not with the initial

![Table 2. TLC of the HSL produced by *P. aeruginosa*](image-url)
adhesion of the bacteria on an abiotic surface, sensitivity to tobramycin or the secretion of proteases. The presence of AHL in the sputum of patients with CF infected with P. aeruginosa was first noticed by Singh et al. (2000). These results were partly confirmed by Middleton et al. (2002), who detected and assayed AHL with acyl groups from 4 to 12 carbons in sputum from some CF patients. Erickson et al. (2002) reported that P. aeruginosa synthesized 3-oxo-C12-HSL within the lungs of CF patients. Based on these results, it was suggested that the formation of a biofilm by P. aeruginosa and hence its pathogenicity were correlated with its ability to release AHL (Høiby et al., 2010).

CONCLUSION

Our results confirm the variability of AHL production among clinical strains in laboratory growth conditions. Four strains secreted high amounts of C4-HSL, and some of the tested strains also produced AHL that could bind to TraR, the transcription factor expressed by A. tumefaciens. The strains that did not produce AHL had significant mutations in lasR. This work also illustrates the lack of a relationship between production of C4-HSL by a strain and its capacity to produce rhamnolipids, to adhere and to develop a biofilm on an abiotic surface or its sensitivity to tobramycin within a biofilm. Our results also show some positive correlation between C4-HSL production, the synthesis of rhamnolipids and the propensity of a strain to develop a biofilm. These results suggest that assaying HSL production by a planktonic culture has some predictive value regarding the propensity of a strain to form a biofilm.

ACKNOWLEDGEMENTS

This work was supported by the Fonds National de la Recherche Scientifique (F.N.R.S) (grant number 3.4577.10) and by an institutional grant. C. N. is a research fellow sponsored by the F.N.R.S. The authors have no conflicts of interest to declare.

REFERENCES


Geisenberger, O., Givskov, M., Riedel, K., Høiby, N., Tümmler, B. & Eberl, L. (2000). Production of N-acetyl-l-homoserine lactones by Bruxelles for the reference strain ATCC PAO1. The authors are also very grateful to Dr S. K. Farrand (University of Illinois at Urbana-Champaign, USA) for the A. tumefaciens NTL4 reporter strain, to Dr J. J. Zhong (East China University of Science and Technology, Shanghai, China) for the P. aeruginosa CGMCC 1.860 strain, to Ms M. Faes for her technical help with TLC and to Ms N. Payrat for her help in the study of HSL. The authors have no conflicts of interest to declare.


