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Quorum-sensing activity and related virulence factor expression in clinically pathogenic isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Respiratory isolates of *Pseudomonas aeruginosa* were collected from 58 critically-ill patients with ventilator-associated pneumonia. Expression of elastase and pyocyanin was assessed semi-quantitatively, while quorum-sensing activity was assessed by quantifying the levels of the autoinducers *N*-3-oxododecanoyl-L-homoserine lactone (C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL). Correlations were sought between quorum-sensing activity and the expression of these two virulence factors, and all results were compared to those obtained with the laboratory reference strains PA103, a strain defective in quorum-sensing, and PAO1, a functional quorum-sensing strain. More than two-thirds of clinically pathogenic isolates had increased levels of elastase and/or pyocyanin, and high quorum-sensing activity, as assessed by autoinducer levels. However, a strong correlation between quorum-sensing activity and virulence factor production was revealed only for elastase and not for pyocyanin (C12-HSL/elastase, $r = 0.7$, p 2×10^{-9}; C4-HSL/elastase, $r = 0.7$, p 2×10^{-9}). These data suggest that the pathogenicity of *P. aeruginosa* isolates from critically-ill patients with ventilator-associated pneumonia is caused, at least in part, by an increase in elastase production regulated by quorum-sensing, while increased pyocyanin production in these isolates may be regulated predominantly by mechanisms other than quorum-sensing.

Keywords Elastase, pathogenicity, *Pseudomonas aeruginosa*, pyocyanin, quorum-sensing, virulence factors

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INTRODUCTION

Pseudomonas aeruginosa, an opportunistic human pathogen, is the most common Gram-negative bacterium causing nosocomial infections such as ventilator-associated pneumonia (VAP). Furthermore, *P. aeruginosa* is characterised by a high degree of clinical pathogenicity, with an attributable mortality that reaches 38% in patients with VAP [1,2]. This high pathogenicity is associated with a wide range of mechanisms and virulence factors [3]. The synthesis and secretion of a number of virulence factors are controlled by quorum-sensing (QS). QS is a bacterium-to-bacterium signalling system that allows the bacteria to sense their environment and coordinate the expression of various genes within the entire bacterial population [4–6].

P. aeruginosa possesses two distinct, but interacting, well-characterised QS systems, *las* and *rhl*. These systems are hierarchically superimposed, with the *las* system controlling the *rhl* system [7]. Each of these systems comprises a homoserine lactone synthase (LasI or RhlI, respectively), a molecule termed an autoinducer (*N*-3-oxododecanoyl-L-homoserine lactone or C12-HSL, and *N*-butanoyl-L-homoserine lactone or C4-HSL, respectively) that is freely diffusible across bacterial membranes [8], and a transcriptional activator (LasR and RhlR, respectively) that regulates gene transcription [9,10]. During the bacterial growth

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phase, the autoinducer levels (C12-HSL and C4-HSL) accumulate to finally reach a critical threshold concentration at which the autoinducer binds to its cognate receptor, which is a transcriptional activator (LasR or RhlR, respectively), leading to the induction or repression of target genes.

Among up-regulated target genes are those leading to the secretion of extracellular virulence factors. The *las* system controls the expression of virulence factors such as LasA protease, LasB protease (elastase), alkaline protease, exotoxin A and LasI, while the *rhl* system controls the expression of rhamnolipid, LasA protease, LasB protease, RhlI and pyocyanin. This regulation of virulence factor expression by QS suggests that QS might have a major role in *P. aeruginosa* pathogenicity, and virulence studies in animals with laboratory reference strains have suggested that the QS system is crucial in the pathogenesis of *P. aeruginosa*-induced pneumonia [11–13]. However, laboratory reference strains may have lost important pathophysiological characteristics, and their study might therefore lead to inaccurate or clinically irrelevant correlations [14]. Furthermore, the role of QS in clinical isolates from typical human lung infections caused by *P. aeruginosa* has not been documented.

A number of studies have focused on QS-deficient clinical isolates and investigated their genotypic, as opposed to phenotypic, characteristics [15–18], while others have focused on isolates from cystic fibrosis (CF) patients. In clinical isolates from CF patients, *P. aeruginosa lasR* transcription correlated with the transcription of *lasA*, *lasB* and *toxA*, suggesting that the *las* system controls virulence gene expression during the course of this specific infection [19]. Autoinducers have also been detected in sputum from these patients [20–22], but no correlation between autoinducer levels and virulence factor expression was revealed [20]. Relatively modest correlations in transcript levels among *lasR*, *lasB* and *aprA* were found in non-CF isolates from different sources (e.g., pneumonia, water) [23], but this correlation was observed in only 50% of the isolates. Phenotypic characterisation of respiratory *P. aeruginosa* isolates has only been performed in a single study [24], which revealed that three of nine isolates colonising mechanically-ventilated patients were defective in the production of both autoinducers and extracellular virulence factors. However, the number of non-clonal isolates studied was too small to make a correlation between autoinducers and virulence factors. Furthermore, this study concerned only colonised patients.

The aim of the present study was to examine the hypothesis that clinically pathogenic *P. aeruginosa* isolates from critically-ill patients with VAP would have increased QS activity and a resulting increased expression of elastase and pyocyanin, which are virulence factors considered to be QS-regulated. The results obtained were compared to those obtained with two reference laboratory strains, PA103, a *lasR* mutant strain, and PAO1, a QS wild-type strain with high levels of autoinducer and elastase production [25].

MATERIALS AND METHODS

Clinical isolates

P. aeruginosa clinical isolates were grown from frozen cultures isolated originally from routine tracheal aspirate samples of patients with VAP who were hospitalised in intensive care units (ICUs) between February 2003 and December 2004. VAP was defined as a lower respiratory tract infection that developed after mechanical ventilation for ≥3 days. Clinical, biological or radiological criteria for pneumoniae were: new lung opacity on two chest radiographs at >12-h intervals plus two of the following findings: presence of either fever (temperature ≥38.2°C) or hypothermia (temperature <36°C), a white blood cell count of >10 000 cells/mm^3 or <4000 cells/mm^3, and purulent endotracheal aspirate. Quantitative microbiological criteria were ≥10^4 CFU/mL from bronchoalveolar lavage fluids, ≥10^6 CFU/mL from endotracheal aspirates, or ≥10^3 CFU/mL from protected specimen brush cultures. Therapeutic criteria were the initiation or modification of antibiotic therapy. The clinical isolates were collected by the Pyopneumagen group of 12 French university hospitals in Angers, Caen, Lille, Paris, Roubaix and Tourcoing. The study was approved by the institutional review boards of the participating institutions. Samples were obtained after informed consent of the patients or their legal representative. Exclusion criteria were CF, neutropenia and previous lung colonisation by *P. aeruginosa*. Two standard laboratory reference strains were used as controls: *P. aeruginosa* PA103 (ATCC 29260), which is a *lasR* mutant, and the wild-type strain PAO1 (ATCC 39018).

Genotypic characterisation of the isolates

The clonality of the *P. aeruginosa* isolates was investigated using standard pulsed-field gel electrophoresis (PFGE) procedures. Following overnight digestion of genomic DNA at 37°C with *Dra*I (New England Biolabs, Beverly, MA, USA), electrophoresis was performed in agarose 1% w/v gels using a CHEF-DR III System (BioRad, Hercules, CA, USA). The criteria of Tenover *et al.* [26] were used to distinguish clonal isolates from non-clonal isolates. Unrelated isolates were initially defined as those with differences in at least three PFGE bands.

Determination of autoinducer concentrations

Culture supernatants were extracted with ethyl acetate, and autoinducer concentrations were determined in bioassays using *P. aeruginosa* PAO-JP2 (pECP61.5) for C4-HSL [27] and *Escherichia coli* MG4 (pPCS.1) for C12-HSL [28]. In brief, extracts were added to cultures of these bacteria, which harboured a plasmid with a transcriptional fusion of a QS transcriptional activator and the β-galactosidase reporter gene *lacZ*, so that exogenous autoinducer resulted in induction of β-galactosidase. Thus, β-galactosidase activity, measured as described by Miller [29], was proportional to the autoinducer concentration. Standard curves were constructed using known quantities of synthetic C12-HSL or C4-HSL (Aurora Biosciences, Coralville, IA, USA). Reporter strain cultures were grown for 14 h at 37°C with shaking in Medium A (K_2HPO_4 10.5 g/L, KH_2PO_4 4.5 g/L, $(NH_4)_2SO_4$ 1 g/L, Na_3-citrate.$2H_2O$ 0.5 g/L, yeast extract 0.5 g/L) [30], supplemented with ampicillin 100 mg/L, glucose 0.4% w/v and 1 mM $MgSO_4$, and were then subcultured in the same medium. Autoinducers were added to the culture at an OD_{660} of 0.3, after which the cells were incubated until an OD_{660} of 0.7 was reached. The culture was then resuspended in the same volume of Medium A supplemented with yeast extract 0.05% w/v. The β-galactosidase activity was assayed using 2-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich, Lyon, France) as described by Miller [29].

Elastase assays

The level of LasB protease (elastase) activity in the supernatant fraction of isolates was determined spectrophotometrically at OD_{600} using an elastin Congo red assay as described previously [27]. Absorption caused by pigments produced by *P. aeruginosa* was corrected by subtracting the absorbance at OD_{495} of control samples incubated in the absence of elastin Congo red. Values were expressed as the ratio $(OD_{495}/OD_{600}) \times 100$. All assays were performed in triplicate.

Pyocyanin assays

The pyocyanin assay was based on the absorbance of pyocyanin at 520 nm in an acidic solution as described previously [31]. Values were expressed as the ratio $(OD_{520}/OD_{600}) \times 100$. All assays were performed in triplicate.

Statistical analyses

Statistical analyses were performed using Pearson's correlation coefficient or simple linear regression. A logarithmic transformation was first applied to C4-HSL, C12-HSL, pyocyanin and elastase to respect the normality assumption. All statistical analyses were performed using R software v.1.9.1 (http://www.r-project.org) [32].

RESULTS

Collection of clinical isolates

Between February 2003 and December 2004, 58 *P. aeruginosa* isolates were collected from 58 patients. To avoid the collection of duplicate isolates associated with hospital outbreaks, each isolate was typed by PFGE. Overall, 96% of the isolates were non-clonal (data not shown); four epidemiologically-related profiles were shared by isolates from four patients from two hospitals. Replicate isolates were excluded from the study, leaving 56 genotypically distinct isolates, collected from 56 patients, for further analysis.

Levels of elastase and pyocyanin

In-vitro levels of elastase and pyocyanin produced by the 56 non-clonal isolates were compared with those produced by the *lasR* mutant strain (PA103) [31] and a QS wild-type strain (PAO1). Seventeen (30.3%) isolates produced less elastase than the *lasR* mutant strain PA103 [33], two (3.6%) isolates produced higher levels of elastase than PAO1, and 37 (66.1%) isolates produced levels of elastase between those of PA103 and PAO1 (Fig. 1a). The pyocyanin assays revealed that 34 (60.7%) of the isolates produced more pyocyanin than PAO1, one (1.8%) isolate produced less pyocyanin than PA103, and 21 (37.5%) isolates produced levels between those of PA01 and PA103 (Fig.1b). Overall, these results suggest that *P. aeruginosa* isolates associated with VAP had, on average, lower elastase activity than PAO1, but higher pyocyanin production than PAO1.

Autoinducer levels

To assess the QS activity in each isolate, autoinducer concentrations were determined and compared with those of the *lasR* mutant and wild-type reference strains. At an OD_{600} of 2.0 (late exponential phase), levels of C12-HSL ranged from 1.01×10^{-8} mol/L to 1.65×10^{-4} mol/L, and levels of C4-HSL ranged from 1.49×10^{-5} mol/L to 6.28×10^{-3} mol/L (Fig. 2). Most isolates produced levels of C12-HSL and C4-HSL between those of PA103 and PAO1 (69.6% and 62.5% of the isolates, respectively). Only one isolate produced more C12-HSL than PAO1, and none produced levels of C4-HSL equivalent to those of PAO1. The levels of C12-HSL and C4-HSL were lower than those of PA103 for 16 (29%) and 21 (37.5%) isolates, respectively (Fig. 2). These results mirrored those for elastase production, suggesting that increased levels of

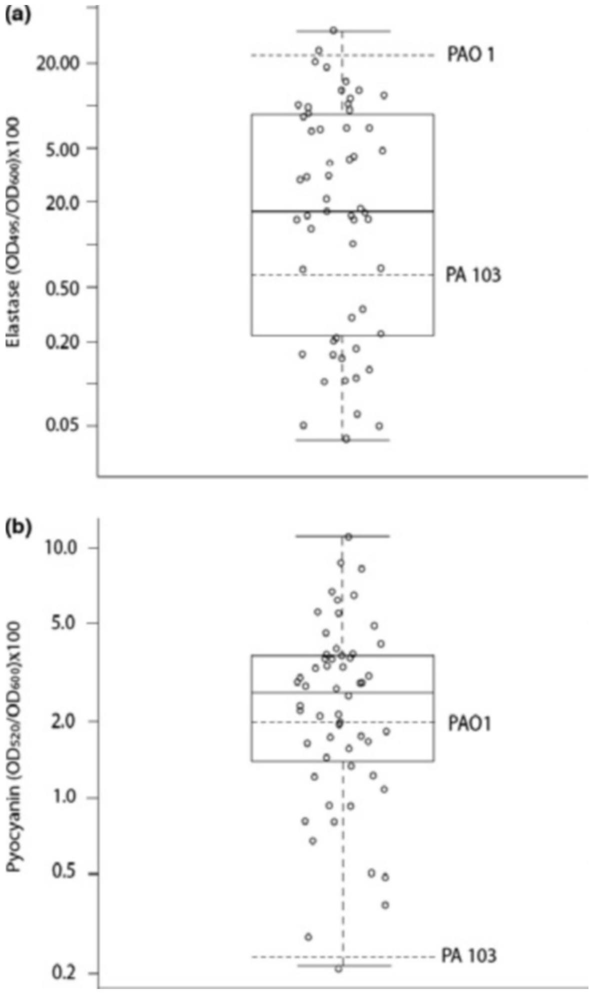

Fig. 1. Box-plot of the production levels of quorum-sensing-related virulence factors in culture supernatants from 56 non-clonal clinically pathogenic isolates of *Pseudomonas aeruginosa* and comparison with laboratory reference strains PAO1 and PA103. Logarithmic scales are used. Dotted lines show the relative levels of production of reference strains PAO1 and PA103. (a) Elastase production as assessed by elastin Congo red assay, expressed as OD_{495}/OD_{600}. (b) Pyocyanin production as assessed by absorbance in an acidic solution expressed as OD_{520}/OD_{600}.

elastase in clinically pathogenic strains may be caused by QS. However, the results were not completely superimposable on those for pyocyanin production, suggesting that QS via *las* and *rhl* might not be the only system regulating the increased levels of pyocyanin production. A correlation analysis of these two QS components revealed that there was a strong correlation between C4-HSL and C12-HSL levels ($r = 0.59$, p 1.3×10^{-6}), which supports the known interaction between these two QS systems [7,33].

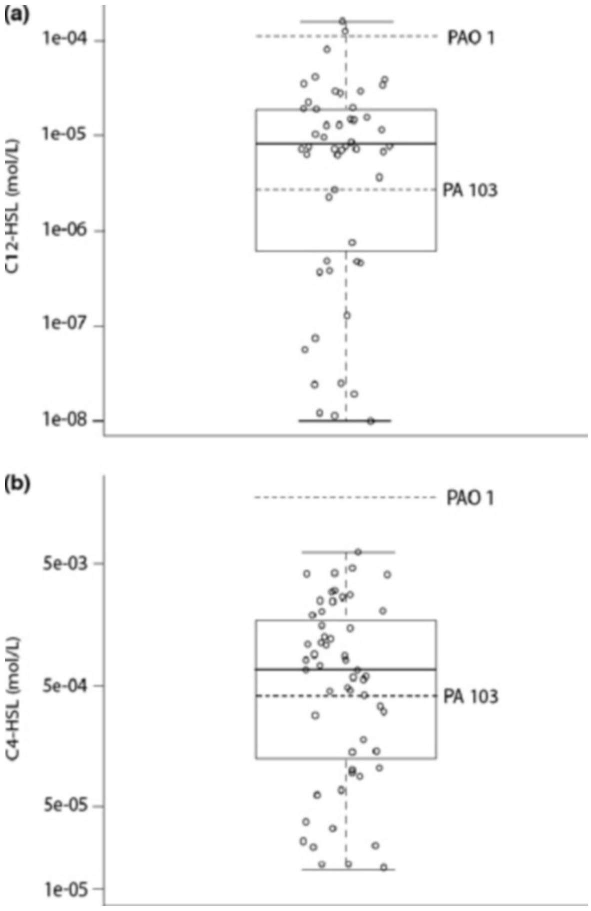

Fig. 2. Production of autoinducers (a) *N*-3-oxododecanoyl-L-homoserine lactone (C12-HSL) and (b) *N*-butanoyl-L-homoserine lactone (C4-HSL) in culture supernatants from 56 non-clonal clinically pathogenic isolates of *Pseudomonas aeruginosa* and comparison with laboratory reference strains PAO1 and PA103. Results are expressed in μmol/L on logarithmic scales.

Relationships between QS activity and virulence factor levels

The pathogenicity of the clinical isolates might be caused by increased production of QS-related virulence factors and increased QS activity. A strong correlation ($r = 0.7$; p 2×10^{-9}) was revealed between levels of elastase and *las* system autoinducer (C12-HSL) in the 56 clinical isolates. There was also a correlation ($r = 0.7$; p 2.2×10^{-9}) between levels of elastase and *rhl* system autoinducer (C4-HSL) in the 56 isolates. These correlations suggested that the *rhl* QS system might be at least as important as the *las* system in regulating elastase production.

In contrast, there was only a poor correlation ($r = 0.3$; p 0.02) between pyocyanin and C4-HSL levels, and no significant correlation with C12-HSL levels. This indicates that only the *rhl* QS system may play a role in increased pyocyanin production in these isolates. However, since there was, at most, only a poor correlation between HSL levels and increased pyocyanin production, it is likely that other mechanisms were predominantly responsible for regulating pyocyanin production in these isolates.

DISCUSSION

The present study confirmed the hypothesis that most clinically pathogenic *P. aeruginosa* strains associated with VAP in critically-ill patients have increased expression of two virulence factors, elastase and pyocyanin, which are considered to be regulated by QS, and that, concomitantly, most strains have increased QS activity. The fact that such results were observed in two-thirds of the isolates studied suggests that increased QS activity and QS-regulated virulence factor production may play a role in the clinical pathogenicity of *P. aeruginosa*.

Elastase, a metalloproteinase secreted by a type II secretion system, has been shown to destroy respiratory epithelium tight junctions, leading to increased permeability disorders, increased interleukin-8 levels and a decreased host immune response [34,35]. Therefore, it is not surprising that most (two-thirds) of the clinically pathogenic isolates in the present study should have increased levels of production of such a virulence factor. The finding of a strong correlation between QS activity and elastase levels is also consistent with previous studies concerning the regulation of elastase production by QS in laboratory strains [10,36,37].

The remaining one-third of isolates (also pathogenic) had levels of elastase, C12-HSL and C4-HSL that were lower than those of PA103, the *lasR* mutant strain. This agrees with the results of a previous study, which revealed that 33% of *P. aeruginosa* isolates colonising intubated patients were defective in the production of both autoinducers and extracellular virulence factors [24]. Such defective isolates often have mutational defects in both *lasR* and *rhlR* [23,24], but the sequences of these genes were not determined in the present study. Although QS and elastase-deficient strains were found among these clinically pathogenic isolates, this does not contradict the theory that QS-regulated virulence may play a role in *P. aeruginosa* pathogenicity, but simply emphasises the fact that pathogenicity may be regulated by a wide range of mechanisms [3].

The levels of pyocyanin in these clinically pathogenic isolates were lower than those in PA103 for only one pyocyanin-deficient isolate, with most (60.7%) isolates having pyocyanin levels that were higher than those of the QS wild-type strain PAO1. The high levels of pyocyanin production in these isolates from patients with VAP suggest that pyocyanin may play a major role in acute pneumonia, in agreement with the results of studies using animal models, which revealed an important role for pyocyanin in oxidative-stress-related tissue damage [38,39]. However, unlike elastase, the high levels of pyocyanin were not well-correlated with high levels of C4-HSL, C12-HSL or elastase, which suggests that increased production of pyocyanin was not regulated via QS in these isolates, although pyocyanin is considered to be regulated by QS. Indeed, pyocyanin expression is under RhlI control [37], which may explain the poor correlation between pyocyanin and C4-HSL levels. Since the *las* system positively regulates expression of both *rhlR* and *rhlI* [7,40], a correlation between C12-HSL and pyocyanin levels was also sought, but was not detected. This result indicates that, of the two QS systems *las* and *rhl*, probably only *rhl* regulates pyocyanin expression. Although most previous studies have suggested the existence of a quasi-exclusive, direct or indirect, QS-regulated control mechanism for the two differentially regulated phenazine biosynthetic operons involved in pyocyanin production [41–43], it seems that other regulatory mechanisms could be responsible for the increased production of pyocyanin in these isolates [44,45].

Most previous studies have investigated laboratory reference strains and, with the exception of research concerning QS in strains infecting CF patients, the only studies that have focused on clinically relevant strains have concerned eye infections [15] or lung colonisation, but not infection, in critically-ill patients [24]. The present study is the first to characterise QS-related virulence by a phenotypic analysis of both virulence factor and HSL production in clinically pathogenic isolates from patients with VAP. The fact that *P. aeruginosa* is responsible for severe nosocomial pneumonia in mechanically-ventilated patients [1,46,47] supports the choice of clinical isolates from ICU patients with VAP as clinically pathogenic strains. However, a limitation of the present study is that the results were obtained in in-vitro conditions, which might vary considerably from conditions *in vivo*, where the microenvironment in which *P. aeruginosa* grows may modify the phenotypic characteristics of strains.

In conclusion, the results obtained in the present study suggest that a QS-related increase in virulence factors, e.g., elastase, may play a role in the pathogenicity of *P. aeruginosa* strains causing VAP in ICU patients. Investigations of this role could yield new therapeutic strategies, and the results should therefore be confirmed in animal models and future prospective clinical studies. Increased pyocyanin production may also play a role in clinically pathogenic strains, although regulation of pyocyanin production may be independent of the *las* and *rhl* QS systems. Other possible non-QS mechanisms regulating pyocyanin production deserve investigation as an entirely new field of research concerning *P. aeruginosa.*

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