Screening of molecular markers of quorum sensing in *Pseudomonas aeruginosa* strains isolated from clinical infections

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Abstract

Pseudomonas (Ps.) aeruginosa is an increasingly prevalent opportunistic human pathogen responsible for severe nosocomial infections, life-threatening infections in immunocompromised persons, and chronic infections in cystic fibrosis patients. The capacity of Ps. aeruginosa to produce different type of infections, often overwhelming infections is due to an arsenal of virulence factors. Many extracellular virulence factors secreted by Ps. aeruginosa have been shown to be controlled by a complex regulatory circuit involving quorum sensing (QS) (cell-to-cell signaling) systems that allow the bacteria to produce these factors in a coordinated, cell-density dependent manner (3). The purpose of this study was to characterize the molecular markers of quorum-sensing in Pseudomonas aeruginosa strains isolated from different clinical specimens. Our results demonstrates that all the analyzed Ps. aeruginosa strains have functional quorum-sensing systems, that shows the involvement of these cell-to-cell signaling systems in control of coordinated expression of virulence determinants in Ps. aeruginosa strains isolated from clinical infections. Knowledge of how quorum sensing systems of Ps. aeruginosa operate during infection may help us to find a new approach to the treatment of chronic Ps. aeruginosa infections.

Keywords: virulence, molecular markers, *N*-acylhomoserine lactones, quorum-sensing

Introduction

Ps. aeruginosa is the most common Gram-negative bacterium found in nosocomial and life threatening infections of immunocompromised patients. Patients with cystic fibrosis are especially disposed to *Ps. aeruginosa* infections, and for these persons the bacterium is responsible for high rates of morbidity and mortality. The capacity of *Ps. aeruginosa* to produce different type of infections, often overwhelming infections is due to an arsenal of virulence factors. Some of them are cell-associated (flagellum, pilus, nonpilus adhesins, alginate/biofilm, lipopolysaccharide) and the other are extracellular virulence factors (proteases, such as elastase, Staphylolytic protease and alkaline protease, hemolysins, pigments, i.e. pyocyanin and pyoverdine, exotoxin A and exoenzyme S) [3].

Many extracellular virulence factors secreted by *Ps. aeruginosa* have been shown to be controlled by a complex regulatory circuit involving quorum sensing (QS) systems/cell-to-cell signaling systems that allow the bacteria to produce these factors in a coordinated, cell-density dependent manner [7, 8]. These QS systems exert their action by small diffusible signal molecules called *N*-acylhomoserine lactones (AHLs) and beside production of virulence factors, coordinate different activities including bioluminescence, plasmid conjugation, metabolic processes dependent on cellular density, competence induction and sporulation. QS influences the ability to form surface-associated, structured and cooperative consortia referred to as biofilms, that plays an important role in bacterial pathogenesis and is a common cause of persistent infections. Bacteria from biofilm are resistant to disinfectants, antibiotics and the action of host immune defenses [4, 11].

Ps. aeruginosa possesses at least two well-defined, interrelated QS systems, las and *rhl*, that control the production of different virulence factors, motility and biofilm formation. Each QS system consists of two components, the autoinducer synthases (*LasI* and *RhlI*, respectively) and their cognate transcriptional regulators (*LasR* and *RhlR*, respectively). *LasI* is the synthase for the autoinducer N-(3-oxododecanoyl) homoserine lactone ($3OC_{12}$-HSL), while *RhlI* synthesizes the autoinducer N-butyryl homoserine lactone (C_4-HSL). The signal molecules interact with his cognate transcriptional regulator to induce the expression of target genes. At high

cell density, $3OC_{12}$-HSL and C_4-HSL reach critical levels and activate their regulators, which in turn enhance the transcription of different virulence genes [5]. Therefore, functional quorum sensing requires transcription of the autoinducer synthase (*lasI, rhlI*) and response regulator (*lasR, rhlR*) and production of autoinducers. The *las* system has been shown to modulate expression of *lasI* itself, *lasB* (elastase), *lasA* (Staphylolytic protease), *apr* (alkaline protease), the *xcp* secretion pathway, twitching motility and *rhlR.* The *rhl* system modulates expression of *rhlI* itself, *rhlAB* (rhamnolipid biosynthesis), *lasB*, twitching motility and *rpoS.* These two QS systems are hierarchically linked, it has also been shown that the *las* QS system controls the *rhl* QS system at both the transcriptional and posttranslational levels [1]. The discovery of this link between the systems indicated that they were arranged in a hierarchy where the *las* QS system is dominant over the *rhl* QS system [7].

Material and methods

i) Bacterial strains

In this study there were analyzed ten *Pseudomonas (Ps.) aeruginosa* strains selected from a strains pool isolated between 2005-2007 from patients hospitalized in Fundeni Hospital, Bucharest. These strains were isolated from different clinical (tracheo-bronchic secretions, wound secretion and sputum) specimens and were identified by help of conventional and API microtests and VITEK I automatic system. The phenotypic characterization for the presence of enzymatic virulence hallmarks (table 1) was established by cultivating the strains in specialized media for enzymes activity detection.

ii) Methods

PCR analysis of the QS genes.

PCR assays were used for the detection of QS genetic support, i.e. *lasI, lasR, rhlI* and *rhlR* encoding for the two QS *las* and *rhl* in *Ps. aeruginosa* strains analyzed.

One colony of each strain was inoculated into 5 ml of BHI (Broth Heart Infusion) and grown overnight at 37°C with shaking. DNA extraction was performed by using Wizard DNA Genomic Purification kit (Promega, USA) according to the manufacturer′s recommendations.

For PCR analysis of the quorum-sensing genes chromosomal DNA was extracted from the ten clinical isolates and utilized as templates in PCR experiments. One set of oligonucleotide primers for gene amplicons were diagnosed by Primer Express (Applied Biosystems) corresponding to different regions within the *lasI, lasR, rhlI,* and *rhlR* genes and synthesized (table 2) [9]. PCR was performed by adding 1 µl of chromosomal template DNA and 31.7 µl of water to 50 µl of a PCR mixture that includes 5 U of GoTaq Flexi DNA polymerase, 25 mM MgCl2, 10 mM (total) deoxynucleoside triphosphates, in 0.5-ml PCR tubes. A negative control (pure water) was included. Thermal cycling was performed in a Thermo Cycler (My Research - Biorad). For gene amplicons PCR conditions for the amplification step were: 35 cycles of PCR, with 1 cycle consisting of denaturation (30 seconds at 94 °C), annealing (30 seconds at 50 °C), and extension (2 minutes at 72 °C) and 1 elongation cycle of 72 °C for 5 minutes. The lengths of the PCR products were estimated by comparison with the 100 bp DNA ladder molecular size markers (Promega) (Fig. 1, 2).

Results and discussion

The aim of this study was to determine the presence of QS genes in *Ps. aeruginosa* analyzed. Since the QS systems control the production of different virulence factors, it is possible that the loss of one or both systems severely compromises the ability of *Ps. aeruginosa* to cause infections in humans.

The expression of soluble, enzymatic factors (haemolytic activities, lecithinase, lipase, caseinase, amylase, DN-ase) in *Ps. aeruginosa* strains was assayed by spot plate method using specific media, as previously described [2].

Table 1. Soluble virulence factors expressed by the analyzed *Ps. aeruginosa* strains.

No.	*Ps. aeruginosa strain*	Source of isolation	Lecithinase	Lipase	Amylase	DN-ase	Caseinase	Hemolysins
1	101	Tracheo-bronchic	□	++	++	-	□□	-

		secretion						
2	1558	Tracheo-bronchic secretion	-	-	+	-	□	-
3	111	Wound secretion	-	+	+	-	++	++
4	1443	Wound secretion	+	-	++	-	+++	++
5	1093	Tracheo-bronchic secretion	++	-	++	-	+++	
6	1561	Wound secretion	++	+	+	-	+++	++
7	20	Sputum	+	+	++	-	++	++
8	1442	Tracheo-bronchic secretion	++	++	+□	_	++	++
9	84	Tracheo-bronchic secretion	++	+	-	-	++	-
10	1562	Wound secretion	-	+	-	-	++	+

The presence of QS systems *las* and *rhl* in *Ps. aeruginosa* strains studied was confirmed by the size of the amplified products with specific primers for *lasI* and *lasR* genes, encoding the QS system *las,* and for *rhlI* and *rhl* R genes, encoding the QS system *rhl*.

Table 2. The primers utilized in PCR experiments for amplicons of the QS genes.

Gene	Forward primer	Reverse primer
lasI	5'-ATGATCGTACAAATTGGTCGGC-3'	5'-GTCATGAAACCGCCAGTCG-3'
lasR	5'-ATGGCCTTGGTTGACGGTT-3'	5'GCAAGATCAGAGAGTAATAAGACCCA-3'
rhlI	5'-CTTGGTCATGATCGAATTGCTC-3'	5'-ACGGCTGACGACCTCACAC-3'
rhlR	5'-CAATGAGGAATGACGGAGGC-3'	5'-GCTTCAGATGAGGCCCAGC-3'

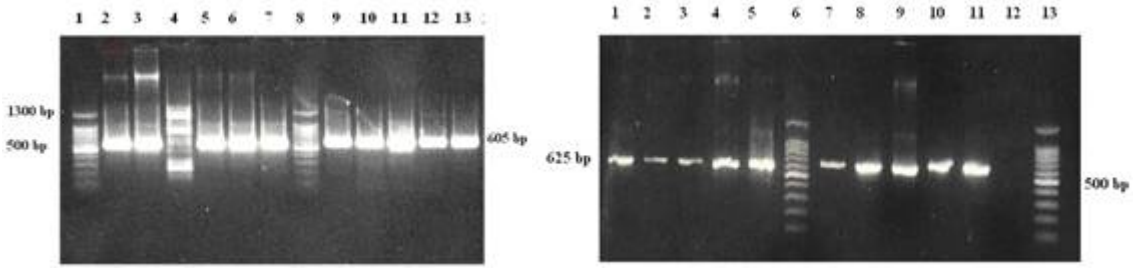

Figure 1. Ethidium bromide-stained 1.5% agarose gel showing the amplified products of *lasI* and *rhlI* genes. Lines 1 - DNA ladder 100bp, 2 – *Ps. aeruginosa* 101, 3 – *Ps. aeruginosa* 1558, 4 - DNA ladder 100bp, 5 - *Ps. aeruginosa* 111, 6 - *Ps. aeruginosa* 1443, 7 - *Ps. aeruginosa* 1093, 8 - DNA ladder 100bp, 9 - *Ps. aeruginosa* 1561, 10 - *Ps. aeruginosa* 20, 11 - *Ps. aeruginosa* 1442, 12 - *Ps. aeruginosa* 84, 13 - *Ps. aeruginosa* 1562.

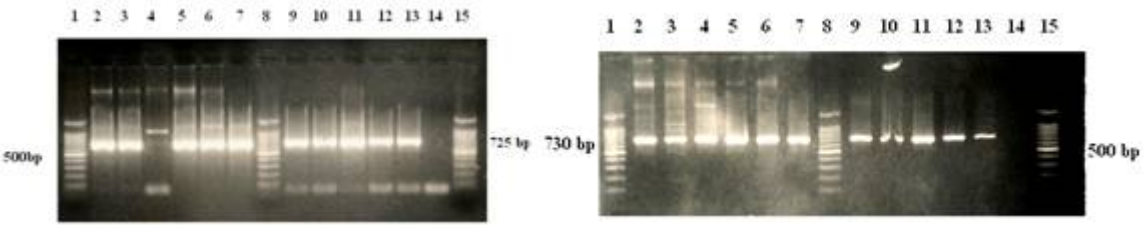

Figure 2. Ethidium bromide-stained 1.5% agarose gel showing the amplified products of *lasR* and *rhlR* genes. Lines 1 - DNA ladder 100bp, 2 – *Ps. aeruginosa* 101, 3 – *Ps. aeruginosa* 1558, 4 - DNA ladder 100bp, 5 - *Ps. aeruginosa* 111, 6 - *Ps. aeruginosa* 1443, 7 - *Ps. aeruginosa* 1093, 8 - DNA ladder 100bp, 9 - *Ps. aeruginosa* 1561, 10 - *Ps. aeruginosa* 20, 11 - *Ps. aeruginosa* 1442, 12 - *Ps. aeruginosa* 84, 13 - *Ps. aeruginosa* 1562, 14 – pure water, 15 - DNA ladder 100bp.

The results obtained shows that these strains possesses some of these soluble, enzymatic factors (lecithinase, lipase, amylase, caseinase and haemolysins) that plays important roles during infections and their production is coordinately regulated by QS systems *las* and *rhl*.

Multiple studies have demonstrated the contribution of QS to the pathogenesis of *Ps. aeruginosa*. The success of any pathogen in the colonization of a sensitive host and the development of an infectious process is depending on its ability to sense its environment and to modulate the expression of the genes encoding factors required for the colonization of a new habitat. In human *Ps. aeruginosa* infections implicating the bacterial adherence and biofilm development on medical devices, as well as its resistance to biocides are all regulated by QS mechanisms. The QS systems might enable *Ps. aeruginosa* to overcome host defense mechanisms. Isolated production of extracellular virulence factors by a small number of bacteria would probably lead to an efficient host response neutralizing these compounds. However, the coordinated expression of virulence genes by an entire bacterial population once a certain density has been reached might allow *Ps. aeruginosa* to secrete extracellular factors only when they can be produced at high enough levels to overcome host defenses. These factors could alter the precarious balance between host defenses and production of bacterial toxins, leading to invasion of blood vessels, dissemination, systemic inflammatory-response syndrome, and finally death. Even appropriate antibiotic therapies are often unable to stop this course; therefore, the process must be blocked early, before virulence gene expression can be coordinated.

The signaling pathway is a target for design of small-molecule inhibitors because *Ps. aeruginosa* require quorum sensing to produce virulence factors in response to association with a human host [10]. Knowledge of the molecular mechanisms underlying these signaling systems and their control of virulence, biofilm formation, and pathogenicity may help us to find a new approach to the treatment of chronic *Ps. aeruginosa* infections [6].

Our results demonstrates that all the *Ps. aeruginosa* strains analysed have functional quorum-sensing systems, that shows the involvement of these cell-to-cell signaling systems in control of coordinated expression of virulence determinants at *Ps. aeruginosa* strains isolated from clinical infections.

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