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Phenotypic and Molecular Characterisation of Rhamnolipids in Clinical, Animal and Environmental Potential Mutant *LasR* of *P. aeruginosa*

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ABSTRACT

Background: *Rhamnolipids (RL) are natural glycolipids produced by Pseudomonas aeruginosa. Despite their use in the agricultural, environmental, bioremediation, petroleum, pharmaceutical and food industries, these rhamnolipid biosurfactants have not been characterised in clinical, animal or environmental strains.*

Objective: *The aim of this study was to characterise the rhamnolipids produced by P. aeruginosa strains of diverse origin.*

Methodology: *A total of 97 P. aeruginosa strains of clinical (30), environmental (32) and animal (35) origin were identified using bacteriological methods and PCR followed by sequencing. The sensitive, semi-quantitative blue agar test and detection of the RhlI and RhlR genes by PCR were used to characterise the phenotypic and molecular production of rhamnolipids, respectively. Potential LasR mutants were identified from iridescent and autolytic metallic colonies on the same agar.*

Results: *In order of decreasing importance, P. aeruginosa strains of clinical (83.3%), environmental (78.1%) and animal (62.9%) origin produced different levels of rhamnolipids. Rhamnolipid-producing strains showed patches of autolysis (63.9%), iridescence (51.4%) and autolytic iridescence (41.7%). The RhlI synthase and the RhlR regulator were mainly detected in environmental strains with an identical prevalence of 66.4%. The prevalence of RhlI (47.2%) and RhlR (47.2%) detected was also identical for clinical and animal strains.*

Conclusions: *This study shows the need to study the factors controlling rhamnolipid production in P. aeruginosa.*

Keywords
Rhamnolipids, *P. aeruginosa*, *Rhl* gene, Biosurfactants.

Introduction

Surfactant is a main lipid component of cleaning detergents. As its name suggests, surfactant stimulates activity on the surface to be cleaned to help trap dirt and remove it from the surface [1]. Surfactants are surface-active agents synthesised chemically or biologically called biosurfactants [2].

Biosurfactants are microbial secondary metabolites produced in a medium containing one or more carbon sources [3,4]. They are classified according to the biochemical nature of the surfactant produced by the micro-organism [5]. There are five main classes of biosurfactants: glycolipids, lipopeptides, phospholipids, liposaccharides and neutral lipids [4,6]. The most widely studied glycolipids are rhamnolipids (RL) [7,8]. These rhamnolipid-type biosurfactants can reduce surface tension, stabilise emulsions and promote foam formation, and are generally non-toxic, non-hazardous and biodegradable [3,9].

Rhamnolipids (RLs) are glycolipid biosurfactants composed of rhamnose linked to highly effective β-hydroxy fatty acids, which are produced by various bacterial species, notably of the genus *Pseudomonas* and *Burkholderia* [4,9]. The biosynthesis of rhamnolipids involves 3 enzymes encoded by the *RhlA*, *RhlB* and *RhlC* genes [10,11]. *Pseudomonas aeruginosa* produces around 25 rhamnolipids, distinguished either by the length of the chain or the degree of saturation of the fatty acid [12]. Rhamnolipids are mainly studied in *P. aeruginosa* strains because of the involvement in surface 'swarming' motility, biofilm development and the virulence of this bacterium [13].

The diversity of these biosurfactants makes them an interesting group of molecules for application in many fields [14]. They are used in public health, agriculture, agri-food, waste management, environmental pollution control and the degradation of hydrocarbons in soil [2,15]. Despite their low toxicity, higher biodegradability, selectivity and high specific activity at extreme temperatures, pH and salinity, rhamnolipid-type biosurfactants have not been characterised in *P. aeruginosa* strains [3,12].

The aim of this study was to characterise the rhamnolipids produced by *P. aeruginosa* strains of clinical, environmental and animal origin.

Material and Methods

Isolation and identification of *P. aeruginosa*

Ninety seven (97) isolates of *P. aeruginosa* were obtained from clinical samples (30) (blood, stools, pus and urine), environment (32) samples (animal products market stalls, animal products cutting instruments, animal products market floors) and animal products (35) (fish and beef). Strains of *P. aeruginosa* were isolated and identified with the basis of standard microbiological techniques. The molecular identification and sequencing of *P. aeruginosa* strains were carried out using the 16S gene as described in our previous works [16].

Phenotypic Detection of Rhamnolipid

The production of rhamnolipids (RL) was assessed in the different strains using a sensitive, semi-quantitative test on blue agar as described in the method of Siegmund and Wagner [17]. This blue agar is composed of $N_{a2}HPO_4$ (0.9 g/L); KH_2PO_4 (2.3 g/L); $CaCl_2.2H_2O$ (0.1 g/L); $MgSO_4.7.H_20$ (0.4 g/L); $NaNO_3$ (2 g/L); Tryptone (1 g/L), 2 mL/L mineral salts (trace elements) supplemented with 0.2 g/L hexadecyltrimethylammonium bromide (CTAB) (Sigma Chemicals Co), 0.015 g/L methylene blue, 20g/L substrate (Glycerol or mannitol) and 13 g/L agar. Trace elements preserved at 4°C consisted of $FeSO_4,7H_20$ (2 g/L); $MnSO_4.H_2O$ (1.5 g/L); $(NH_4)_6M_{O7}O_{24}.4H_2O$ (0.6g/L). The ingredients were added one after the other and the whole was homogenised, with a pH maintained at 7 after preparation.

A volume of 5 µL of 24-hour bacterial culture, taken from TSB broth, was spread onto blue agar plates and incubated at 37°C for 24 hours. After 24 hours of incubation, the plates were incubated again at 30°C for 3 to 4 days to develop the rhamnolipid (RL) production zones. Finally, the plates were incubated at 4°C for 24 hours to increase the contrast of the zones against the background. The production of rhamnolipid biosurfactants in *P. aeruginosa* strains is indicated by the formation of a dark blue halo around the colonies.

Phenotypic detection of *lasR* mutant of rhamnolipid-producing strain

After prolonged growth of purified colonies on Siegmund and Wagner blue agar [17], potential *LasR* mutants were identified from iridescent and autolytic metallic colonies. The metallic, shiny colony iridescent appearance reflects an accumulation of 4-hydroxy-2-heptylquinoline.

Molecular Characterization of Rhamnolipid

Extraction and purification of the genomic DNA of *P. aeruginosa*

Clinical, animal and environnemental strain of *Pseudomonas aeruginosa* were harvested from an overnight broth culture. Genomic DNA was extracted and purified according to the method described by Amutha and Kokila [18]. After extraction, DNA was diluted and stored at -20°C to serve as a DNA template for polymerase chain reactions (PCR).

Reaction Mixture

The 25 µL reaction mixture consisted of 14 µL of sterile Milli-Q water (milli-Q ™, Millipore Corporation, Foster City, CA, USA); 5 µL of 10 X concentration loading buffer ; 1.5 µL of $Mgcl_2$, 25 mM (Promega Corporation, Madison, WI, USA) ; 0.5 µL of d'NTPs, 10 mM ; 0.5 µL of each primer, 10 mM (TranS, AP111 5U, Macau City, China) ; 0.5 µL of BSA, 20 mg/mL and 0.5 µL of Easy Tag® DNA polymerase with a final concentration of 1.5 U (TranS, AP111 5U, Macau City, China) and 2 µL of the DNA matrix.

Sterile Milli-Q water and the reference strain *P. aeruginosa* PA14 were used as negative control and positive control, respectively, for each PCR reaction run.

Amplification of *RhlI and RhlR* genes

Amplification of the *RhlI and RhlR* gene was performed according to the method described by Al-Kilabi et al. [19] using primers F: 5'-TTCATCCTCCTTTAGTCTTCCC-3′ and R: 5′-TTCCAGCGATTCAGAGAGC-3′) for *RhlI;*
F: 5'-TGCATTTTATCGATCAGGGC-3′ and R: 5′-CACTTCCTTTTCCAGGACG-3′) for *RhlR*. The amplification program included an initial denaturation of 5 min at 95°C followed by 33 cycles of denaturation (95°C for 30 s), annealing (65°C for 60 s) and extension (72°C for 90 s), with a single final extension of 5 min at 72°C. The samples were stored at 4 °C until the Thermocycler was stopped.

Statistical Analysis

Data were entered with SPSS Statistics 20.0 data processing software (IBM Corporation, SPSS Inc, Chicago, USA) and transferred to Excel. Data were entered with Data Gen 5 v. 2.04™. The data were analyzed by t-test at a statistical level of $\alpha < 0.05$ (Excel, MS office).

Results

Percentage of rhamnolipid production in *P. aeruginosa*

The production of rhamnolipids is higher in clinical (83.3%) and environmental (78.1%) strains and lower in animal strains (62.9%) (Figure 1). There is no significant difference between the rate of rhamnolipid production in clinical and environmental strains.

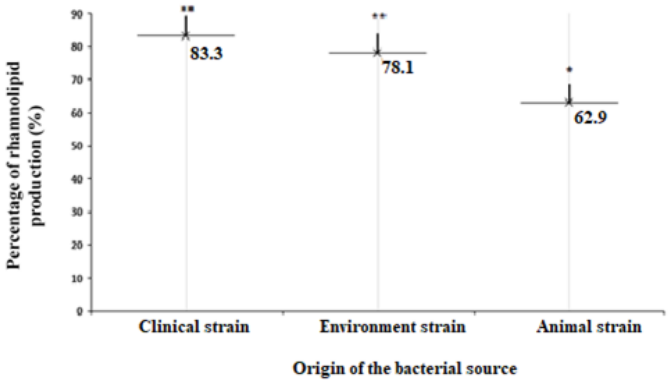

Figure 1: Production de rhamnolipides chez *Pseudomonas aeruginosa.*

LasR Mutant Potential and Rhamnolipid Production

Rhamnolipid-producing *P. aeruginosa* strains presented plaques of autolysis and iridescence, phenotypes characteristic of potential *LasR* mutants (Figure 2). These phenotypes ranging from 64.0% to 84.0% were mainly expressed in environmental strains. Of all the strains studied, 74.2% were rhamnolipid producers. These results also indicated that 63.9% and 51.4% of the rhamnolipid-producing strains presented autolysis and iridescence plaques, respectively (Table 1). Only 41.7% of these rhamnolipid-producing strains were iridescent autolytic (Table 1).

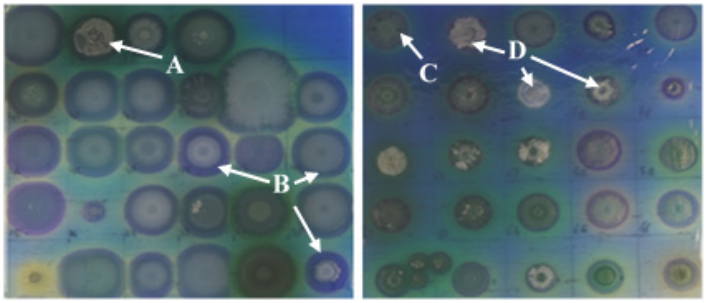

Figure 2: Rhamnolipid produced by potential *LasR* mutant of *P. aeruginosa* strains:
A: Autolytic iridescent rhamnolipid-producing strains; B: Rhamnolipid-producing strains C: Autolytic rhamnolipid-producing strains; D: Iridescent rhamnolipid-producing strains Rhamnolipid production on blue agar (MSM-SW) after 24 hours growth at 37°C, 3-4 days at 30°C and one night at 4°C, rhamnolipid production is detected by the formation of a dark blue halo around the colonies.

Molecular Determinants Involved in Rhamnolipid Production

The electrophoretic profile indicates that some strains of *P. aeruginosa* harbor the gens *Rhl* which encodes the production of rhamnolipids (Figure 3). The *Rhl* gene was mainly detected in environmental strains with a prevalence of 66.4% for both the *RhlI* synthase and the *RhlR* regulator (Figure 4). The prevalence of the *RhlI* (47.2%) and *RhlR* (47.2%) genes detected in the clinical and animal strains was identical (Figure 4).

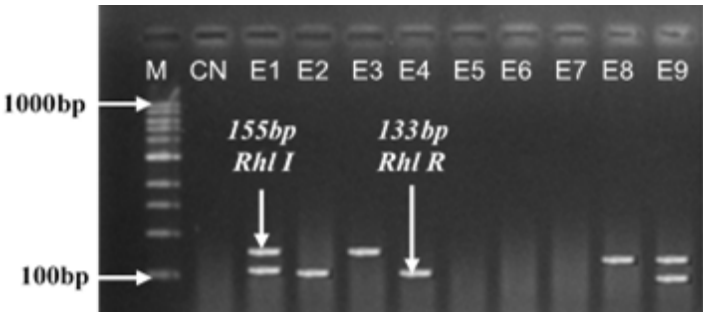

Figure 3: Electrophoretic profile of genes involved in rhamnolipid production.

Table 1: Characteristic phenotypes of potential *LasR* mutant and rhamnolipid.

Phenotypic characteristics	***Clinical strain* (N =30)**	***Environnemnt strain* (N =32)**	***Animal strain* (N =35)**	***Total* (N =97)**
Rhamnolipid	25 (83.3)	25 (78.1)	22 (62.9)	72 (74.2)
Autolysis (autolytic)	13 (52.0)	21 (84.0)	12 (54.5)	46 (63.9)
Iridescence (iridescent)	10 (40.0)	18 (72.0)	9 (40.9)	37 (51.4)
Iridescent autolytic	06 (24.0)	16 (64.0)	8 (36.4)	30 (41.7)

Ligne 1 et 9: Présence de gène RhlI et *RhlR chez P. aeruginosa; ligne 3 et 8: présence de gène RhlI chez P. aeruginosa; ligne 2 et 4: présence de gène RhlR CN: control négatif; M: Marqueur de poids moléculaire; Rhl: Gene codant pour la production de rhmnolipd, RhlI* (synthase d'une Homosérine lactone (C4-HSL)); *Rhl R* (régulateur).

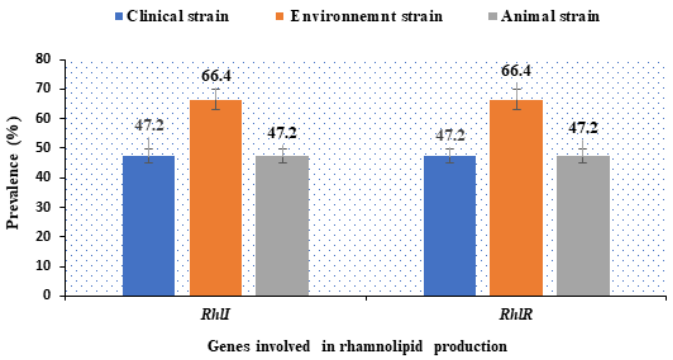

Figure 4: Molecular determinants involved in the production of rhamnolipids.
Rhl: Gene coding for the production of rhmnolipd, *RhlI* (synthase of a homoserine lactone (C4-HSL)); *RhlR* (regulator).

Discussion

Rhamnolipids are remarkably promising biodegradable biosurfactants for use in agriculture, bioremediation, the petroleum, pharmaceutical, agrifood and environmental industries [9,20]. A better understanding of gene regulation and environmental triggers could contribute to an increase in the yield of industrial rhamnolipid production.

In this work, we described the genetic determinants at the origin of rhamnolipid production in *P. aeruginosa* and their implication for both pathogenicity and biotechnological research. Of all the *P. aeruginosa* strains studied, 74.2% were phenotypically rhamnolipid-producing. The results showed that rhamnolipid biosynthesis was higher in clinical and environmental strains but lower in animal strains. There was no significant difference between the yield of rhamnolipid production in clinical and environmental strains. This biosynthesis of rhamnolipids by the strains studied could be explained by several environmental stimuli such as cell density, stress and nutritional deficiency [21,22].

All the phenotypes observed could be explained by the presence of genes involved in the synthesis of rhamnolipids. These genes could regulate the production of these rhamnolipids by the *lasI-lasR* system via *RhlI-RhlR* [23]. Molecular characterisation of the strains studied showed that the *Rhl* gene was mainly detected in environmental strains, with a prevalence of 66.4% for both the *RhlI* synthase and the *RhlR* regulator. These detected genetic determinants could explain the phenotypes observed in the different strains [11]. These rhamnolipid-type biosurfactants can promote colonisation of animal products and human cells by isolated *P. aeruginosa* strains [20]. Consequently, the rhamnolipids produced by clinical, environmental and animal strains could be involved in changing the hydrophobicity of the cell surface, in the solubilisation of the PQS (*Pseudomonas* quinolone signal), in swarming mobility and in biofilm architecture.

Furthermore, given their important ecological role due to their low toxicity and biodegradability, these rhamnolipids detected may be useful molecules in medicine and agriculture [2,15]. These rhamnolipids could form part of alternative strategies for reducing or replacing pesticides in agriculture [13].

In addition, better production, separation and purification of these rhamnolipids in industrial quantities may enable them to be applied in other fields, such as cosmetics [2,24]. In this study, rhamnolipid-producing strains of *P. aeruginosa* also showed patches of autolysis and iridescence, phenotypes characteristic of potential *LasR* mutants. These phenotypes ranged from 64.0% to 84.0% and were mainly detected in environmental strains.

These results show that the phenotypes observed could contribute to the pathogenicity of the *P. aeruginosa* strains studied, particularly in environmental strains. Indeed, certain *LasR* mutants are traditionally associated with virulence, antimicrobial resistance, protease secretion and biofilm formation. These results are in line with those of Li et al., [12], who reported in their study that biofilm production was significantly associated with the presence of *LasR* mutants. Several other studies, have shown that the existence of *LasR* mutants associated with the presence of *Rhl* genes denotes the importance of these determinants in the virulence of *P. aeruginosa* strains [25]**.** The present work has discussed the production of rhamnolipid biosurfactants as a promising and functional alternative for various biotechnological applications [4]. These rhamnolipids are being studied not only as virulence factors in *P. aeruginosa* but also as promising compounds for industrial processes.

Conclusion

This study highlighted the phenotypic and molecular production of rhamnolipids in *P. aeruginosa* of various origins. A wide variety of *P. aeruginosa* produce potent rhamnolipids, which vary according to their origin. The genetic basis of rhamnolipid production in *P. aeruginosa* was determined. The *Rhl* gene was mainly detected in environmental strains. A better understanding of the regulatory mechanisms of the genes involved in the production of rhamnolipids can help optimise their industrial yield. On the other hand, mastery of these determinants and environmental triggers can also help to control the involvement of these genes in the virulence of strains.

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