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A multivariate approach to correlate bacterial surface properties to biofilm formation by lipopolysaccharide mutants of *Pseudomonas aeruginosa*

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ABSTRACT

Bacterial biofilms are involved in various medical infections and for this reason it is of great importance to better understand the process of biofilm formation in order to eradicate or mitigate it. It is a very complex process and a large range of variables have been suggested to influence biofilm formation. However, their internal importance is still not well understood. In the present study, a range of surface properties of *Pseudomonas aeruginosa* lipopolysaccharide mutants were studied in relation to biofilm formation measured in different kinds of multi-well plates and growth conditions in order to better understand the complexity of biofilm formation. Multivariate analysis was used to simultaneously evaluate the role of a range of physiochemical parameters under different conditions. Our results suggest the presence of serum inhibited biofilm formation due to changes in twitching motility. From the multivariate analysis it was observed that the most important parameters, positively correlated to biofilm formation on two types of plates, were high hydrophobicity, near neutral zeta potential and motility. Negative correlation was observed with cell aggregation, as well as formation of outer membrane vesicles and exopolysaccharides. This work shows that the complexity of biofilm formation can be better understood using a multivariate approach that can interpret and rank the importance of different factors being present simultaneously under several different environmental conditions, enabling a better understanding of this complex process.

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1. Introduction

The ability of bacteria to adhere to natural and artificial materials is a first step in biofilm formation, is central for bacterial survival, and has great clinical and environmental relevance [1–3]. Initial adhesion is dependent on a large number of parameters [4,5]. Although surface properties like hydrophobicity and cell charges have been used to explain bacterial adhesion to surfaces, it is not clear how these properties co-influence adhesion under different conditions and how important their effect is in comparison to other surface properties [5,6]. One approach that can help to disentangle a large number of variables and interpret their individual correlation to biofilm formation is multivariate analysis [7] which is used, in this study, for a detailed investigation of bacterial biofilm formation.

Both specific and non-specific interactions mediate bacterial adhesion. Non-specific interactions include electrostatic charges, hydrophobicity and van der Waal's forces. These properties are influenced by the bacterial cell surface composition and medium constituents [5]. Specific interactions can occur between an abiotic surface and proteins, sugars and lipids in cell membrane [8,9] and is often related to specific receptors. Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria, and it plays an important role for interactions with abiotic surfaces, as well as for cell integrity and immunogenic responses [10]. The LPS structure of the cell wall has influence on biofilm formation [11,12] and enhanced biofilm has been observed in deep rough LPS mutants of *Escherichia coli* [11]. LPS have three distinct domains: lipid A, core oligosaccharides and O-antigen [18] (Fig. 1). In general, LPS phenotype is described as smooth, rough and deep

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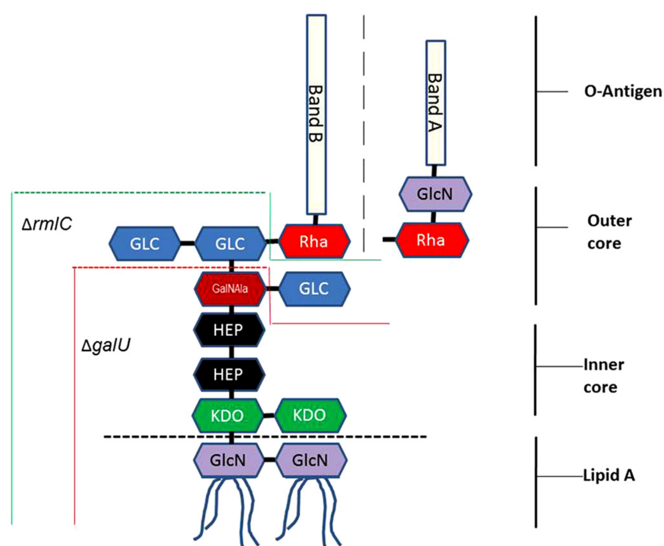

Fig. 1. Schematic representation of lipopolysaccharide (LPS) of *P. aeruginosa* (wild type strain PAO1) with O-antigen (A- and B-band), outer and inner core [18]. The truncation of LPS for mutants without O-antigen (*ΔgalU* and *ΔrmlC*) is marked by red and green lines. The other LPS mutants have more than one chemotype of LPS including O-antigen with either A-band or B-band as described in Table S1. PAO1, *Δgmd*, *ΔwbpA* and *ΔwapB* mutants have, apart from A- and/or B-band, uncapped LPS with two sugar residues (Rha and GLC) more than *ΔrmlC* does. *ΔwapB* has an additional uncapped LPS similar to ΔrmlC but with only one additional Rha. The sugar abbreviations are as follows: GlcN = 3-(acetyl amino)-3-deoxy-D-glucose), KDO = 3-deoxy-D-manno-oct-2-ulosonic acid, HEP = heptose, GalNAla = 2-(2-L-alanyl)-2 deoxy-D-galactosamine, Rha = rhamnose GLC = glucose. (For interpretation of the references to color in this legend, the reader is referred to the web version of the article.)

rough. Cell phenotype where O-antigen caps the core is referred to as "smooth" LPS phenotype, while strains lacking O-antigen are termed "rough" LPS [18], and if the outer core is missing "deep rough". Furthermore, motility of bacteria (swimming, swarming and twitching caused by appendages like flagella and pili) and production of surface exopolysaccharides (EPS) has been reported to influence biofilm formation [13–17].

Pseudomonas aeruginosa is a common soil bacterium [19], and a well-established model organism for studying bacterial biofilm formation. Furthermore, *P. aeruginosa* is of clinical relevance e.g. for infections related to medical devices such as contact lenses and endotracheal tubes. It is also a bacterium that is commonly found in natural and artificial environments [19]. In a previous study, we showed that LPS mutants of *P. aeruginosa* with different hydrophobicity and negative zeta potential colonized surfaces differently [1], which urged us to further investigate the influence of bacterial surface properties on bacterial adhesion and biofilm formation. In this work we try to understand how bacterial cell surface properties relate to biofilm formation and if these properties and processes change under different environmental conditions such as composition of growth medium and serum content. LPS structures of the mutants used in the present study [18] are shown in Fig. 1. The mutations resulting in these different LPS structures, affect the genes involved in biosynthesis of LPS monomers (*gmd*, *galU*, *rmlC*), glycosyl transferase (*wapB*) and *algD* homologue (*wbpA*) which is essential for biosynthesis of GDP-D-mannose [20]. The resulting LPS mutants differ in phenotype of O-antigen (A- or B-band) that "caps" the core LPS structure. The strains often have both capped and uncapped LPS in the outer membrane. The mutant *ΔwbpA* has A-band but no B-band [21], *ΔrmlC* and *ΔgalU* have neither A- nor B-band and both have different forms of a truncated outer core in LPS [10,22], *ΔwapB* has both A- and B-band similar to wild strain PAO1, coexisting with some truncated uncapped LPS [23], *Δgmd* lack the A-band [24]. The strains used in this study differ from LPS mutant used in previously published studies, as different genes with different functional roles were mutated. The reason for using the selected LPS mutants were that they differed to a large extent in surface physicochemical properties, while they were not expected to show large differences in other bacterial properties. In this work, we investigate how variations in amount of biofilm formed by LPS mutants was correlated to a combination of several factors: cell wall composition (with respect to peptidoglycan, protein, sugars and lipids), hydrophobicity, cell charges (as reflected in Zeta potential) and aggregation of bacterial cells, type of polystyrene culture plate, motility (swimming, swarming and twitching), secretion of exopolysaccharides, and release of outer membrane vesicles (OMVs).

To be able to simultaneously interpret all the above mentioned parameters we used multivariate analysis. Previously multivariate analysis has been used to investigate the variance of surface physiochemical properties between a large range of bacterial species with the conclusion that 70% of the variability between strains could be explained by four components that were roughly described as presence of protein and phosphorous, negative charge, hydrophobic properties and iso-electric point of the bacterium [25,26]. Thus, previous work indicates that these factors could play a large role in governing biofilm formation and result in differences between strain biofilm phenotype. In this article we will start by discussing the individual parameters for the whole strain collection of *P. aeruginosa* and their correlation to biofilm formation. Thereafter, we will move on to discuss the information obtained from the multivariate analysis of the entire dataset.

2. Materials and methods

2.1. Strains, media, multiwell plates and LPS gel profile

Strains used in this study are shown in Table S1 [10,14,20–24] and Fig. 1. The LPS gel profile was confirmed using a previously described method (Fig. S1) [27]. All strains were cultured in isosensitest media (Oxoid LTD, Hampshire, England) [1] or AGSY media [28]. Since LPS mutants were constructed by gene replacement that confers resistance to gentamycin, this antibiotic was added where required. Effect of serum on biofilm formation was studied by adding 10% sterile filtered fetal bovine serum (Sigma Life Science) in respective media. Three kinds of 96-well multiwell plates were used – standard plates (96-well polystyrene microtiter plate, Corning 3595, New York, NY), cell grade™ (96-well BRAND*plates*® Cell Grade™) [29], inert grade™ (96-well BRAND*plates*® Inert Grade™) [30]. Composition and hydrophobicity of these multi-well plates are shown in Table S2.

2.2. Biofilm formation and surface characterization measurements

More details of each experimental assay can be found in supplementary information. Biofilm formation was measured quantitatively by the crystal violet method as described previously [31]. Outer membrane fractionation was done using the N-lauryl sarcosyl method [32]. Selected protein bands were identified by MALDI-MS by Alphalyse Denmark. OMVs were isolated as reported previously with brief modification outlined in the supplement [33]. The amounts of OMVs were quantified by the Bradford method on the basis of protein content. Surface exopolysaccharides were isolated as mentioned previously with brief modifications outlined in the supplement [34]. The amount of polysaccharide was estimated by the phenol sulfuric acid method [35]. Surface composition analyses of bacteria, grown overnight (24 h) on agar plates

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