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Extracellular GroEL Promotes adherence of *ΔrhlA* mutant to inert surfaces in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is particularly notorious producing robust biofilm. Bacterial biofilms are three-dimensional systems, attached to inert or living surfaces that are surrounded by an extracellular matrix consisting of; extracellular DNA, proteins, exopolysaccharides, and secondary metabolites (Guilbaud *et al*., 2017).

There are evidences indicating that a biofilm matrix forms micro-environments in which cells are optimally organized to resist stress and use available nutrients. For example, eDNA acts as a biofilm scaffold by binding with other biomolecules, such as peptides, enzymes, proteins, and polysaccharides (Withchurch *et al*., 2002; Das *et al*., 2016). Zhang *et al*., (2015) pointed out that the polysaccharides provide mechanical stability, mediate bacterial adhesion to surfaces, and form a cohesive, three-dimensional network that connects and immobilizes biofilm cells. However, the complex network leading to complete biofilm formation is not well understood. Alayande *et al*., (2018) reported that the quorum sensing system molecules and unknown secreted proteins involve *P. aeruginosa* biofilms.

Biofilm formation is considered as a survival strategy against environmental stresses such as; nutrient deficiency, pH inconsistency, UV damage, metal toxicity, and hosts of immunological responses during infection (Kumar *et al*., 2017). Rhamnolipid (rhl) is a secondary metabolite produced by *P. aeruginosa*, and it alters cell–cell and cell–surface interactions. It is a well-wetting agent for initial bacterial attachment as well as detachment (Kim *et al*., 2015).

In this study, we investigated the physiological response of inorganic (Pi-PO4) deficiency; which has an increased effect on rhamnolipid production in *P. aeruginosa*, with respect to virulence and biofilm-related proteins. The proteins were obtained through extracellular and cytoplasmic proteomic analysis using non-rhamnolipid produced *P. aeruginosa ΔrhlA* strains (constructed for this study by deleting the *rhlA* gene from wild type PAO1, Nottingham Collection strain using the method of alleic exchange), and mono and di-rhamnolipid produced strain PAO1 wild type (Nottingham Collection strain), grown in the populously rhl-producing medium, the proteose-peptone ammonium salts (PPGAS).

Mass spectrometry data collected from extracellular proteomic; showed the proteins were largely involved in bacterial attachment and biofilm

formation, as well as virulence factors. Between these proteins, GroEL was down-regulated in *P. aeruginosa ΔrhlA* mutant vs the PAO1 wild type strain. GroEL was the unique cytoplasmic protein detected in extracellular space. Indeed, it has already been observed on membrane-associated periplasmic, and cytoplasmic spaces (Blatch and Edkins, 2015). Our report indicated that this protein is abundantly located in the cytoplasm based on the cytoplasmic proteomic result using the strain *ΔrhlApVLT31groEL*. The GroEL protein was over-expressed in the *P. aeruginosa ΔrhlA* strains, the groEL operon from the existing plasmid pVLT31groEL (Institute of Molecular Enzyme Technology, Research Center in Julich, IMET culture collection), and a control empty vector, pVLT31 (IMET culture collection). They were introduced chemically into the *ΔrhlA* strain, and *groEL* expression was induced under standard İzopropil-β-D-tiyogalaktopiranozit-IPTG conditions vs *ΔrhlApVLT31* (empty vector). GroEL protein overexpression was verified by the extracellular and cytoplasmic proteomics by the Two Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) (Fig. 1). The extracellular and cytoplasmic protein samples were prepared from the same cultures, which were optimally induced at the same initial concentration of bacteria for 24 h pre-cultures. The main cultures were incubated for an extra 24 h in rhamnolipid production conditions (PPGAS medium). The extracellular and cytoplasmic protein extracts were prepared with *ΔrhlApVLT31groEL* and *ΔrhlApVLT31* (empty vector) strains (Hirose *et al*., 2000). Protein quantification was carefully measured using the 2D Quant Kit (GE Healthcare). The first dimension was performed using IPG strips, covering a pH range of 3-11. Further separation of the proteins according to size was determined using 12% SDS-PAGE (Fig. 2). After Colloidal Coomassie Blue staining of the gels, the images were analyzed using the 2D software Decodon analysis system. To identify the proteins in the modified Δ*rhlA* strain; the corresponding bands were eluted from the gel, tryptic digests were performed, and MALDITOF-MS was performed using an Ultraflex III TOF / TOF mass spectrometer (Bruker Daltonics, Bremen). To compare the measured mass pattern with the theoretical proteome of *P. aeruginosa* PAO1, Protein Prospector software was used. In both gels, these proteins were identified as a 57 kDa GroEL protein. According to the results; the cytoplasmic GroEL was 6-fold more than extracellular GroEL, indicating that almost 20% of GroEL was released from the cell into extracellular space. The presence of GroEL in the extracellular space, whether it is the result of cell disruption or not, should be examined. To eliminate autolysis; β-lactamase enzyme activity located in the periplasm was measured, and LipH an inner membrane protein in the LifS class in *P. aeruginosa*, was detected immulogically. β-lactamase activity from cell extracts and supernatants were assayed spectrophotometrically; by measuring the hydrolysis of 150μM of chromogenic substrate from the group of cephalosporin, which is hydrolyzed by β-lactamases in 50 mM sodium phosphate buffer (pH 7.2) at 30°C for 15 min., using a Spectrophotometer (Thermo Genesis) at wavelength of 405 nm. The kinetic parameters were determined by using SpfX program. The supernatants of *ΔrhlApVLT31* (e.v), *ΔrhlApVLT31groEL*, wild type PAO1, and medium control were negative for extracellular β-lactamase activity, whereas, the PAO1 cell-disrupted sample was positive. LipH was immunologically detected by Western blot using a LipH antibody. For this purpose, the expression cultures were diluted to an optical density (OD) of 0.10 at 580 nm, and expression was induced in a culture with an OD of 0.3- 0.5 at 580 nm, by the addition of IPTG (0.4 mM final concentration). Cultures were incubated at 30°C for 16 h, and total cell extracts were prepared. Immunological detection of LipA and LipH by SDS-PAGE and Western blot was performed. Immunoblot analysis showed that samples prepared from the supernatant and from the cell extract were negative and positive for LipH

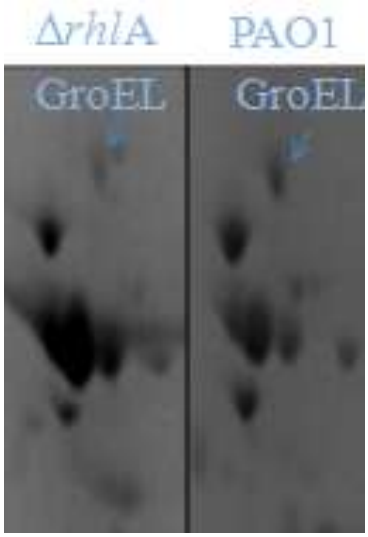

Fig. 1: Extracellular proteome from *ΔrhlA* and PAO1. The image shows the extracellular proteins on 2D-Gels. The arrows indicate GroEL protein in *ΔrhlA* and PAO1. The culture supernatants of different strains from three independent experiments were adjusted so that they corresponded to the same OD_{580} nm.

Fig. 2: Cytoplasmic proteome from *ΔrhlA* and PAO1. The image shows the supervision of the cytoplasmic proteins on 2D-Gels. The arrows indicate GroEL protein in Δ*rhl*ApVLT31 (empty vector-orange) and Δ*rhl*ApVLT31*gro*EL. The culture supernatants of different strains from three independent experiments were adjusted so that they corresponded to the same OD_{580} nm. Using the 2D software Delta2D (Decodon) were superimposed on each other to be compared.

protein, respectively. Results of the β-lactamase assay and Western immunoblot analysis showed that cytoplasmic β-lactamase enzyme or inner membrane protein LipH were not found in the supernatant of *ΔrhlApVLT31* (e.v), *ΔrhlApVLT31groEL* or wild type PAO1. These two autolysis control tests showed that cytoplasmic and inner membrane proteins present in the sample supernatants cannot result from autolysis. Moreover, because the *ΔrhlA* strain has no rhamnolipid production to function as a bio-detergent and causes cell lysis, the extracellular GroEL's possible function needs to be determined. GroEL protein has been shown previously to facilitate the membrane proteins' folding process, and participates in the bacterial attachment to surfaces or tissues (Jeffery, 2018).

Considering the data obtained for extracellular proteins; down-regulated in Δ*rhl*A strain, played a role in attachment and biofilm formation. GroEL overexpression proteomic data directed to the extracellular GroEL, contributed to the attachment of *ΔrhlA* strain to the inert surface (Fig. 3). This hypothesis was proved by the crystal violet attachment assay. All cultures were prepared as previously described, and diluted to an OD of 0.1. 100 µl aliquots of the bacterial strain were pipetted into the wells of a 24-well PVC plate. The plate was incubated for eight hours (initial attachment) at

Fig. 3: Adherence assay from over-expressed GroEL in *P. aeruginosa ΔrhlA* (*ΔrhlApVLT31groEL*) and empty vector control (*ΔrhlApVLT31*). The adherence assay for 8 h showed 3 replicates of the CV staining for *ΔrhlApVLT31groEL* and *ΔrhlApVLT31*.

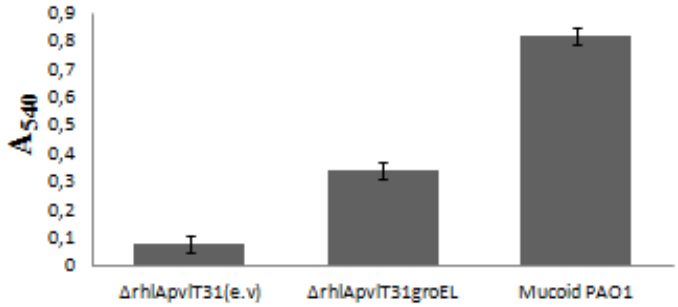

Fig. 4: Adherence assay measurement of CV at OD_{540}. The graphic shows the measurement of the CV at OD_{540}. Three independent experiments were conducted and replicated for the measurement of each strain. Mucoid PAO1 were used as a control known as strong biofilm producer strain (IMET Culture Collection).

37°C. The attached bacteria were stained with 0.1% crystal violet (CV). The amount of CV stain was quantified by dissolving the stain in 200 µl of ethanol (80%), and then CV concentration was determined by measuring the OD at 540 nm of the dissolved CV dye (Grafik). Results indicated that *ΔrhlApVLT31groEL* adhered to the surface at least five-times more than *ΔrhlApVLT31* (empty vector) (Fig. 4).

Although the mechanism by which groEL released from the cell was unclear; since it did not carry a signal peptide, it can be assumed that the protein was released by a vacuolar membrane, or secreted by type III or IV secretion machinery, or by a simple system such as an ABC transporter in the extracellular space (Tsugawa *et al*., 2007).

In this study, we have identified a non-classical secreted protein with a new surface attachment function in *P. aeruginosa*.

Two hypotheses can be proposed for GroEL's contribution to attachment or biofilm formation. First, the reactivity of the proteins or cells was altered by GroEL, to promote the attachment of *P. aeruginosa*. In accordance with our findings, GroEL overexpression led to up-regulation of the flagellar cap FliD and flagellin type B protein, which were responsible for mucin adhesion and the initial attachment to the surfaces, respectively (Campodonico *et al*., 2010; Haiko and Westerlund-Wikstrom, 2013). The second was relevant for altering the surface charge by rhamnolipid, to prepare the surfaces for initial attachment. GroEL overexpression obviously promotes

bacterial attachment to the inert surfaces, which is especially important for developing a medical device depending on *P. aeruginosa* infections.

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