REAL-TIME OBSERVATION OF *Pseudomonas aeruginosa* BIOFILM FORMATION USING CONFOCAL MICROSCOPY SYSTEM WITH A FLOW CELL TECHNIQUE

PENGAMATAN LANGSUNG PEMBENTUKAN BIOFILM *PSEUDOMONAS AERUGINOSA* MENGGUNAKAN SYSTEM MIKROSKOP KONFOKAL DENGAN TEKNIK FLOW CELL

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ABSTRACT

Pseudomonas merupakan salah satu genus bakteri yang memiliki manfaat di bidang pertanian. Bakteri tersebut diketahui termasuk mikroba yang menghasilkan sekret berupa polimer ekstraseluler yang berperan dalam pembentukan biofilm. Penelitian ini bertujuan untuk mengetahui pembentukan biofilm pada *Pseudomonas aeruginosa* dengan memanfaatkan sistem mikroskop confocal yang terintegrasi dengan teknik flow cell. Pembentukan biofilm pada *Pseudomonas aeruginosa* diatur oleh 3 gen yaitu *algD*, *pelA* and *pslA*. Hasil menunjukkan bahwa teknik flow cell dengan mikroskop konfokal dapat digunakan untuk melihat secara langsung pembentukan biofilm. Berdasarkan hasil pengamatan mikroskop confocal diketahui pembentukan biofilm strain a*lgD*, *pslA* and PAO1 (*wild type*) masih rendah.

Keywords : biofilm, Pseudomonas aeruginosa, mikroskop confocal, teknik flow cell

INTRODUCTION

Bacterial life includes the steps in which cells are associated and form a biofilm on a surface. The formation of these surface communities and their inherent resistance to antimicrobial agents are the cause of many persistent and chronic infections (Costerton *et al*., 1994). Microscopic analysis indicated that biofilm formation occurs in a sequential process of (i) transport of microbes to a surface, (ii) initial fixation, (iii) micro-colony formation and (iv) biofilm maturation (Tolker-Nielsen *et al*., 2000; Sauer *et al*., 2002). The biofilms formed by *Pseudomonas aeruginosa*

under favorable conditions were found to be heterogeneous with fungal forms where glucose was used as a carbon source (Davies *et al*., 1998), and flat, uniform and dense where citrate was used as a carbon source (Heydorn *et al*., 2002). Suggesting that the development of *P. aeruginosa* biofilm is dependent on the carbon source

Flagella are necessary for the formation of biofilms (Klausen *et al*., 2003), and likely to contribute to persistent colonization. TFP (type IV pili) allows bacteria to move on solid surfaces (Mattick, 2002), attach to epithelial cells, and contribute to the formation of biofilms (Kang *et al*, 1997, Mattick, 2002, O'Toole and Kolter, 1998).

The microorganisms involved in denitrification are facultative heterotrophic and are usually abundant in sewage; examples are *Pseudomonas aeruginosa*. In a study was reported that *P.aeruginosa* have Polyhidroxy alkanoat (PHA) synthases of class I and II as a catalyst to make biodegradable plastic from palm oil mill effluent (Jaeger *et al*. 1995 ; Verlinden *et al*. 2007 in Sudesh, 2013). *P. aeruginosa* uses flagella and TFP to swarm across semi-solid surfaces. Swarming occurs on semi-solid surfaces (0.5-0.7% agar) when a specific carbon source and nitrogen sources are provided (Kohler *et al*., 2000; Rashid & Kornberg, 2000). Nevertheless, it has been shown that strains without TFP can also swarmer but that their biofilm has a different morphology from the wild-type strain (Kohler *et al*., 2000).

Therefore, it is important to know how *P.aeruginosa* is able to form biofilm through direct observation using confocal microscope integrated with flow cell technique. The results of this study are useful as information in the treatment of

liquid waste that utilizes *P. aeruginosa* as a degradation agent.

MATERIALS AND METHODS

The bacterial strains, culture conditions, and growth of the biofilm

All the strains used come from the Lorient (LBCM), France : the wild-type strain of *Pseudomonas aeruginosa* PAO1, as well as several strains mutated on several loci (*pelA*, *pslA* and *algD*), these genes being responsible for the production of Exopolysaccharides. The bacteria were grown in LB (*Luria Broth*) medium.

Motility tests

Swimming, swarming and twiching were studied with agar containing 0.3%, 0.6% and 1.5% agar. These agar plates were inoculated with pre-culture bacteria using a sterile cone. For swimming and swarming, 5 µL of bacterial suspension was deposited on the agar, while for twitching, the agar was stabbed with a cone previously plunged into preculture.

The swarming and swarming agars were incubated at 37 ° C for 24 h and 48 h respectively. Regarding twitching, it were also incubated at 37 ° for 120 hours. After this time, the agar was removed and a violet crystal staining was performed to reveal the bacteria spread out in the bottom of the plate. The tests were carried out in duplicate. For each motility, the diameter was measured. The results obtained with the mutants were expressed as a ratio relative to PAO1.

Microplate adhesion test (plate 1)

In the adhesion test, a microplate was used, as well as a spectrophotometer to measure OD_{590} obtained. One hundred µL of bacterial culture of OD equal to 1 was placed in the wells (5 wells / strain). After one hour of adhesion, the wells were rinsed with physiological water and then crystal violet was set for 15 minutes

to color the adhered bacteria. The dye was then removed and the wells were again rinsed several times with physiological water to remove excess crystal violet. After these rinses, the remaining dye (having colored the bacteria) was solubilized with absolute ethanol. The wells containing the same strain were pooled in a spectrophotometric tank to read OD at 590 nm.

Formation of biofilm on microplate (Plate 2)

A second plate was designed to study the formation of biofilm. It had undergone the same treatments as the first plate until the stage preceding the coloring. Instead of the violet crystal, LB medium had been added so that the bacteria had a nutrient source to form a biofilm. After 24 hours of incubation at 37 ° C, this LB was removed, the wells rinsed and then a violet crystal staining was also carried out as for the adhesion test. The OD at 590 nm was read after dissolving the dye with absolute ethanol.

For both tests, the volume in the tanks was increased by addition of absolute ethanol so that the spectrophotometer could read the values. A dilution was therefore carried out. The results used in this study are the values read multiplied by the dilution factor.

Real-time observation of the formation of biofilm by "flow cell" technique

PAO1 and the mutants *pelA* and *algD* were grown at room temperature in three flow cell channels. This system coupled with a bubble trap for biofilm observation was invented by Caldwell and then modified by adding computer image processing. The flow cell system was assembled and prepared as described previously (Heydorn *et al*., 2002 ; Hentzer *et al*., 2001). The substrate is composed of a microscope slide. The

bacterial culture for inoculation of the flow cells was prepared as follows**.**

Only one colony of each strain was used to inoculate test tubes containing low-salt LB broth. The specimens were then incubated overnight in a shaker at 37 ° C. The cultures were diluted to an OD 600 of 0.1 to 0.9% NaCl and used for inoculation. The flow chambers were inoculated with duplicate strains by injecting 350 µL of the inoculum prepared with a 0.5 ml syringe (28G1 / 2; 0.336313 mm). After inoculation, the flow cells were left at room temperature for 1 h without flux to allow attachment of the cells to the substrate. The media flow was resumed at a constante rate of 3 ml H-1 using a Marlow 205S peristaltic pump conforming to a flow rate of 0.2 mms-1 in the flow cells.

Data analysis

The data were obtained in a massive growth zone, the amount of bacteria that develops and the value of the spectrographs (turbidity). Then the data obtained from each formula were analyzed using an ANOVA followed by the Tukey test at the significance level of 95% using the SPSS version 22. The resulting images, tables and graphs were depicted descriptively.

RESULTS AND DISCUSION

In this study, the effect of suppression of the a*lgD, pslA* and *pelA* genes in the *P. aeruginosa* PAO1 strain was studied on adhesion, motility as well as the ability to form a biofilm. For the observation of the biofilm, two methods were used: the microplate assay with crystal violet staining, and the observation by confocal microscopy using fluorochromes.

Adhesion

The microplate test was to evaluate the attachment of bacteria on the microplate surface and the amount of biofilm. This test was intended to determine the ability of *P. aeruginosa* adheres to the surface of the object. Results were illustrated in Figure 1. The adhesion was observed by light microscopy under white light after one hour of adhesion without flux. Figure 2 showed the results obtained. The results showed that there was no significant difference between mutants *pel*A, *psl*A and PAO1. Figure 1 also showed that the absorbance of *algD* was higher than *pelA*, *pslA* and PAO1. According to Gauthier *et al*. (1989) Klausen, et.al (2003). Adhesion, which corresponds to reversible adsorption of cells. This step involves only physical processes (electrostatic interaction, electrodynamics) and depends on the nature of the support and its preconditioning (presence of corrosion tubers, for example). The bacteria are fixed only reversibly to the support ; They easily detach under the action of hydrodynamic constraints imposed by the environment. This phase is generally a specific and of short duration (5 to 10 hours). This is why Figure 2 showed that the adhesion was not significant after one hour for the three strains used. Indeed, the secretion of exopolymers by the microorganisms allows them to consolidate their adhesion to the support, forming around the bacterium, an envelope, called glycocalix. Contamination in ethanol might cause the absorbance value of PAO1 to be negative.

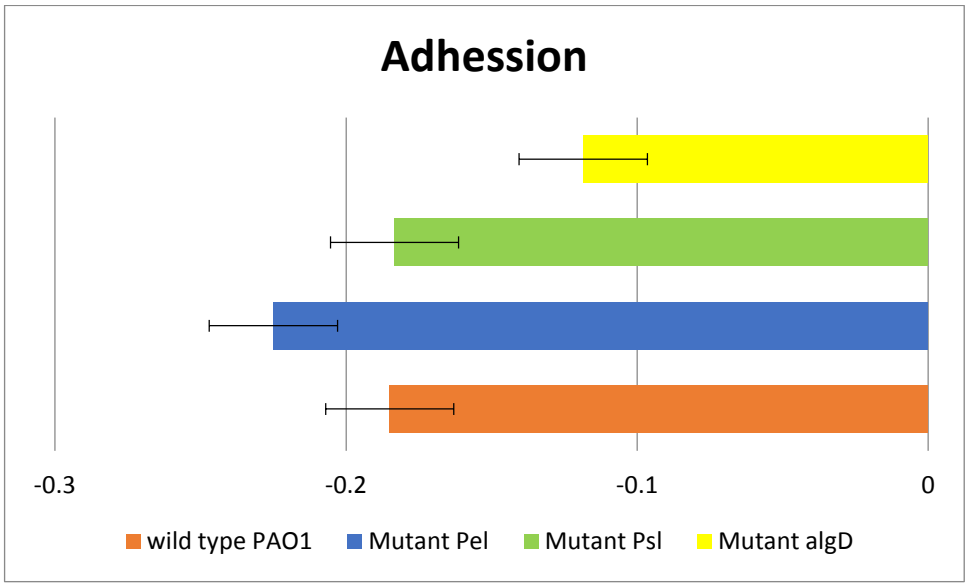


Figure 1. Adhesion results (mean absorbance value).

Figure 2. A. Mutant *AlgD*; B. Wild-type Strain (PAO1) and C. Mutant *pslA*

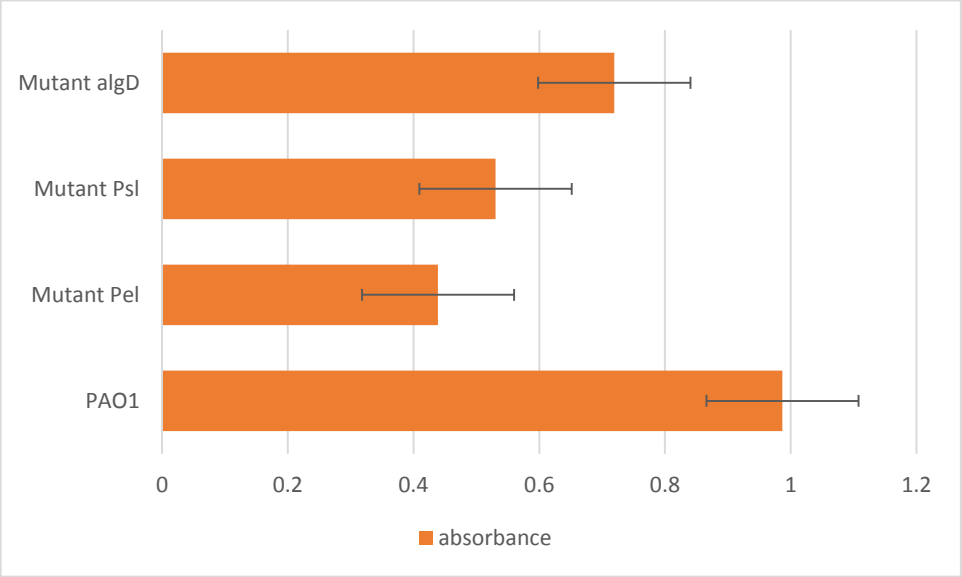
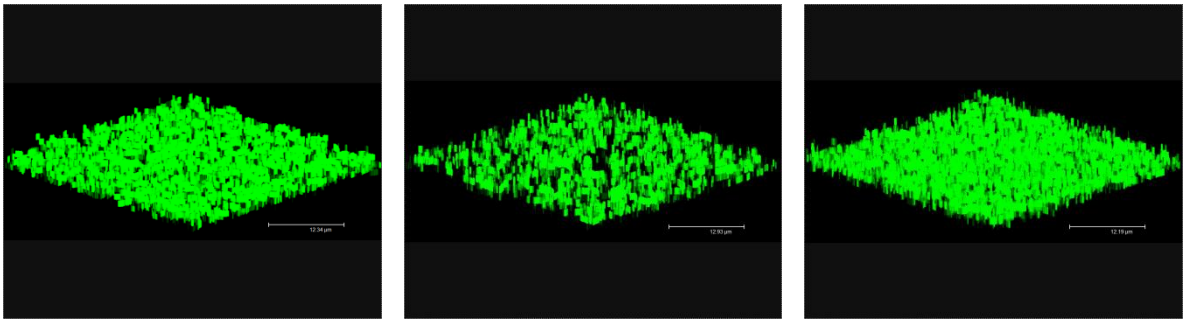


Figure 3. Biofilm results (mean absorbance value)



A B C

Figure 4. A. *algD* mutant ; B. Wild-type Strain and C. pslA Mutant. Genetic degradation occurred on PAO1 led to a decrease of biofilm formation.

Biofilm Formation

On the other hand, the microplate tests was also conducted to determine the amount of biofilm formed. The results of this microplate test are shown in Figure 3. The analysis of the statistical data described that the treatment showed a significant difference with wild-type strain. In this case, the PAO1 bacterial strain gave the highest absorbance value compared to the others. *P. aeruginosa* PAO1 tend to have the ability to form a biofilm that was higher than other types. It is clear that *pelA*, *alg*D, and *pslA* gene

plays a role in the formation of biofilm, the absence of these genes lead to lower biofilms formation.

The role of Exopolysaccharide (EPS) are providing food for the biofilm, involved in the defense mechanisms of the host, contributing the aggregation and adhesion of surfaces, to survive under conditions where the cells planktonic are not able to survive. EPS contains the main content of polysaccharides, amino acids, proteins and fats. The flow cell experiments were carried out to see if the genes studied had a role in the formation and architecture of biofilm in *P. aeruginosa* PAO1 on glass plate. This type of experimental device has been used in several studies to analyze the architecture of *P. aeruginosa* biofilms (Hentzer *et al*., 2001; Davey *et al*., 2003).

One of the main differences between the two systems (flow cell and microplate) is the availability of nutrients. The microplate model provides static conditions in which the nutrients are exhausted during the exponential phase (Steinberger *et al*., 2002), while the flow cell model offers flow conditions, where the environment is renewed and replenishment occurs. Nutrients and the removal of metabolites were produced by bacteria. The combination of two biofilms growth models is a strategy often used to obtain additional information on the various structural and functional aspects of biofilms (Mah *et al*., 2003, Werner *et al*., 2004, Waite *et al*., 2005) . Both models seem to give the same results for mutants *ps1*A, *pelA* and PAO1. The two mutants formed less biofilm than the wild-type PAO1 strain. The images obtained by confocal microscopy show poorly structured flow cell biofilms, the biofilms formed were really very thin (Figure 4). No significant differences were observed. This leads us to conclude that the ability of the mutants to form the biofilm is not enough for an

hour. Moreover, the absence of genes (*pelA*, *pslA*, *algD*) leads to lower the formation of biofilms. It encode proteins to synthesize the communication signals between cells and initiate the formation of the polysaccharide (Stathopoulos, 2000).

***Motility of** P.Aeruginosa* **PAO1, *pelA, algD, and pslA*.**

Three types of motility (swimming, swarming and twiching) were studied (Figure 5). The diameter measured was that of the displacement observed for each strain. According to the statistical test carried out for swimming, there was no significant difference was observed between *psl*A and *pel*A. *alg*D mutants had the higher motility of swimming test. The adhesion test on a microplate showed that the *algD* gene is not necessary in the first step of initiation of the biofilm. Wozniak, *et al*., (2003) observed that alginate was not a significant element of EPS in the biofilm formation of PA14 and PAO1. The first gene of this operon, *algD*, encodes the GDP dehydrogenase mannose, an enzyme essential for the synthesis of alginate. The product of the *algD* gene catalyses the first step involved in the synthesis of alginate and is not known to be involved in another cellular process (Wozniak, *et al*., 2003).

The swarming test results showed that the *pelA*, *pslA* and *algD* strains had a higher motility than the wild-type strain (PAO1). Swarming allows displacement on semi-solid surface. The mutants showed a strong swarming over the wild-type strain. Nevertheless, there is no difference among the mutants. Mutants therefore arrived more at swarmer, which may be due to the increase in their production of biosurfactants, such as rhamnolipids. They can reduce the surface tension of the surrounding liquid. Rhamnolipids are heat-resistant glycolipid biosurfactants. It is composed

of rhamnose bound to β-hydroxylated fatty acids. *P. aeruginosa* produces 25 rhamnolipids which are different either by the length of the chain or by the degree of saturation of the fatty acid (Deziel *et al*., 1999). In addition, rhamnolipids play a role in the change in hydrophobicity of the cell surface (Al-Tahhan *et al*., 2000), solubilization of PQS (Pseudomonas quinolone signal), and swarming motility (Deziel *et al*., 2003).

This test aimed at observing the motility on a solid surface. Based on statistical test for twitching, there was no significant difference between the mutant *alg*D and the wild-type strain. But, significanly different with mutants *pel*A and pslA. At this stage, the bacteria secrete EPS matrix to form a biofilm. Although the mutants do not have the EPS gene as PAO1, they still can grow to form biofilms. This suggests that there has been a genetic change in the mutants so that the biofilms were formed. Alternatively, genetic degradation occurred so that the ability to form biofilms in PAO1 decreased. This can be proved by searching under the confocal microscope (Figure 4).

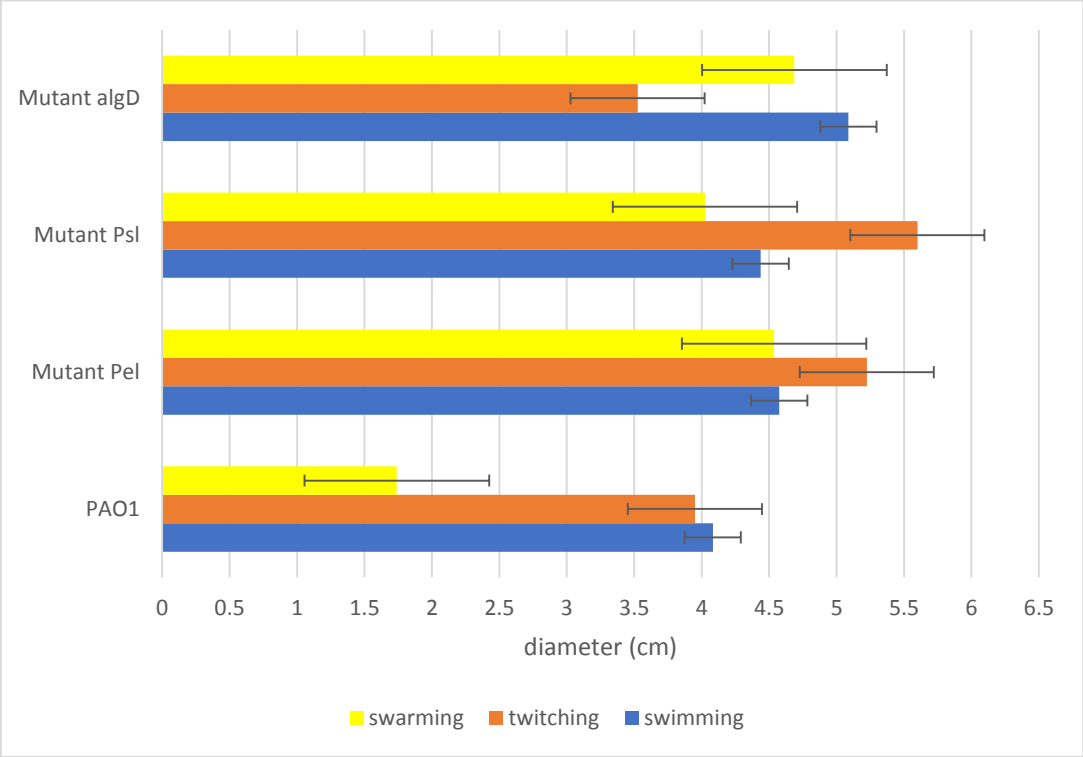


Figure 5. Motility of *P.aeruginose* PAO1, *pel*A, *psl*A, and *alg*D

CONCLUSION

Based on our result, the ps1A, pelA and AlgD genes play a role in biofilm formation. The absence of these genes leads to the formation of biofilms either low. It could be concluded also that confocal microscopy system with a flow cell technique can be used for real time observation of *Pseudomonas aeruginosa* Biofilm Formation.

REFERENCES

Al-Tahhan, R. A., Sandrin, T. R., Bodour, A. A. & Maier, R. M. (2000).Rhamnolipid-induced removal of lipopolysaccharide from Pseudomonas aeruginosa: effect on cell surface properties and interaction with hydrophobic substrates. Appl Environ Microbiol66, 3262–3268.

Costerton, J. W., Lewandowski, Z., DeBeer, D., Caldwell, D., Korber, D. & James, G. (1994). Biofilms, the customized microniche. J Bacteriol 176, 2137–2142.

Davey, M. E., Caiazza, N. C. & O'Toole, G. A.(2003). Rhamnolipid surfactant production affects biofilm architecture in Pseudomonas aeruginosaPAO1. J Bacteriol185, 1027-1036.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280, 295–298.

Déziel, E., Lepine, F., Dennie, D., Boismenu, D., Mamer, O.A. & Villemur, R. (1999).Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by Pseudomonas aeruginosa strain 57RP grown on mannitol or naphthalene. Biochim Biophys Acta1440, 244–252.

Déziel, E., Lepine, F., Milot, S. & Villemur, R. (2003).RhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology149, 2005– 2013.

Drenkard, E. (2003). Antimicrobial resistance of Pseudomonas aeruginosa biofilms. Microbes Infect 5, 1213–1219.

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H. & Prince, A. 1998. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. Infect Immun 66, 43–51.

Gauthier, Y. et Isoard P., 1989, L'adhésion des bactéries sur les surfaces, Industries alimentaires et agricoles, 106, (1-2), p. 31-33.

Hentzer, M., Teitzel, G.M., Balzer, G. J., Heydorn, A., Molin, S., Givskov, M. & Parsek, M. R. 2001.Alginate overproductionaffects Pseudomonas aeruginosabiofilm structure and function.J Bacteriol183, 5395–5401.

Heydorn, A., Ersboll, B., Kato, J., Hentzer, M., Parsek, M. R., Tolker- Nielsen, T., Givskov, M. & Molin, S. 2002. Statistical analysis of Pseudomonas aeruginosa biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. Appl Environ Microbiol 68, 2008–2017.

Kang, P. J., Hauser, A. R., Apodaca, G., Fleiszig, S. M., Wiener- Kronish, J., Mostov, K. & Engel, J. N. 1997. Identification of *Pseudomonas aeruginosa* genes required for epithelial cell injury. Mol Microbiol 24, 1249–1262.

Klausen, M, A. Heydorn, P.Ragas, L. Lambertsen, A. Aaes-Jørgensen, S. Molin and T.Tolker-Nielsen, 2003. Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. *Molecular Microbiology* (2003) 48(6), 1511–1524.

Kohler, T., Curty, L. K., Barja, F., van Delden, C. & Pechere, J. C. (2000). Swarming of *Pseudomonas aeruginosa* is dependent on cell-tocell signaling and requires flagella and pili. J Bacteriol 182, 5990–5996.

Mah TF, Pitts B, Pellock B, Walker GC, Stewart P.S, *et al*. (2003) A genetic basis for Pseudomonas aeruginosabiofilm antibiotic resistance. Nature 426: 306–310.

Mattick, J. S. (2002). Type IV pili and twitching motility. Annu Rev Microbiol 56, 289–314.

McDougald, D., Klebensberger, J., Tolker-Nielsen, T., Webb, J. S., Conibear, T., Rice, S. A., Kirov, S. M., Matz, C. & Kjelleberg, S. (2008). Pseudomonas aeruginosa: a model for biofilm formation. In Pseudomonas. Model Organism, Pathogen, Cell Factory, pp. 215–253. Edited by B. H. A. Rehm. Weinheim: Wiley-VCH Verlag.

O'Toole, G. A. & Kolter, R. (1998). Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol 30, 295–304.

Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J.W. & Davies, D. G. (2002). Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. J Bacteriol 184, 1140–1154.

Sauer, K., Cullen, M. C., Rickard, A. H., Zeef, L. A., Davies, D. G. & Gilbert, P. (2004). Characterization of nutrient-induced dispersion in Pseudomonas aeruginosa PAO1 biofilm. J Bacteriol 186, 7312–7326.

Semmler, A., C. B. Whitchurch, and J. S. Mattick. 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. Microbiology 145:2863–2873.

Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407:762–764.

Skerker, J.M., and Berg, H.C. (2001) Direct observation of extension and retraction of type IV pili. *Proc Natl Acad Sci USA* 98: 6901–6904.

Stathopoulos Christos, Hendrixson David R. Thanassi David G., Hultgreen Scott J.,2000, Secretion of Virulence determinants by the general secretory pathway in Gram – negative pathogens : an evolving story, Microbes and infection,2, p 1061 – 1072

Steinberger, R. E., Allen, A. R., Hansa, H. G. & Holden, P. A. (2002). Elongation correlates with nutrient deprivation in Pseudomonas aeruginosa unsaturated biofilms. Microb Ecol43, 416–423. Stewart, P.S., Peyton, B.M., Drury, W.J., and Murga, R. (1993) Quantitative observations of heterogeneities in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* 59: 327–329.

Stoodley, P., Wilson, S., Hall-Stoodley, L., Boyle, J. D., Lappin-Scott, H. M. & Costerton, J. W. (2001). Growth and detachment of cell clusters from mature mixed-species biofilms. Appl Environ Microbiol 67, 5608–5613.

Tolker-Nielsen, T., Brinch, U.C., Ragas, P.C., Andersen, J.B., Jacobsen, C.S., and Molin, S. (2000) Development and dynamics of *Pseudomonas* sp. biofilms. *J Bacteriol* **182:** 6482–6489.

Waite, R. D., A. Papakonstantinopoulou, E. Littler, and M. A. Curtis.2005. Transcriptome analysis of Pseudomonas aeruginosa growth: comparison of gene expression in planktonic cultures and developing and mature biofilms. J. Bacteriol.187:6571–6576.

Wehmhoner, D.,Haussler, S., Tummler, B., Jansch, L., Bredenbruch, F., Wehland, J. & Steinmetz, I. (2003). Inter- and intraclonal diversity of the Pseudomonas aeruginosa proteome manifests within the secretome. J Bacteriol 185, 5807–5814.

Werner, E., Roe, F., Bugnicourt, A., Franklin, M. J., Heydorn, A., Molin, S., Pitts, B. & Stewart, P. S. (2004).Stratified growth in Pseudomonas aeruginosabiofilms.Appl Environ Microbiol70, 6188–6196

Wozniak, Daniel J., Timna J. O. Wyckoff, Melissa Starkey, Rebecca Keyser, Parastoo Azadi, George A. O'Toole, and Matthew R. Parsek, 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 Pseudomonas aeruginosa biofilms. PNAS 13(100). 7907–7912.