Volume 84(3). Published September, 01, 2021
www.jnsciences.org
E-ISSN 2286-5314

Phospholipid Alterations in *Pseudomonas* swarmer cells from inoculums at different stages of growth

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Abstract –: Depending on the age of the inoculums for swarming, *Pseudomonas aeruginosa* undergoes a multicellular behavior called swarming which seems to be closely related to the age of the inoculums and therefore to the fatty acid profile of the membrane of swarmer cells.
- This study was conducted to investigate the role of fatty acids in regulation of swarming motility in *Ps.aeruginosa*.
- We identified the fatty acid composition in swarmer cells of *Ps. aeruginosa*, from inoculums at different stages of growth. Preculture of four inoculums were stopped at different times corresponding to the point of log, exponential, early stationary and late stationary phases. Then, these inoculums were used for swarming assay. In parallel, we analyzed the membrane composition of swarmer cells, scarped from the edge of the swarm zone, to investigate the possible role of fatty acids in regulation of swarming in *Ps. aeruginosa.*
- Here, we report that it is possible that endogenously fatty acids can be released from phospholipids of the membrane and could act as intracellular signals to regulate swarming in *Ps. aeruginosa*.
- Together, these data suggest an hypothetic correlation between physical state of the membrane lipid bilayer and swarming phenotype of *Ps. aeruginosa*.

Keywords: Phospholipids, Malondialdehyde, *Ps. aeruginosa*, swarming, growth

1. Introduction

The gram-negative bacterium *Ps. aeruginosa* is adept at coordinating individual cells to participate in several surface-associated behaviors (Daniel 2010). This ability benefits this microbe, presumably through improving access to nutrients, in Biofilms organization (Aloui et al. 2010). Given the extent of problems caused by biofilms, there has been a significant effort to develop new anti-biofilm strategies via the comprehension of several mechanisms implicated in biofilm formation, like swarming motility (Daniel 2010).

Therefore, *Ps. aeruginosa* is able of well-known types of motilities like swimming and swarming. Swarming is a quick and coordinated translocation of a bacterial population across a semisolid surface (Daniel 2010). When cells are inoculated at the surface of swarm plates, regular colonies are formed initially. Afterward, cells at the rim of the colonies undergo differentiation to form elongated, multinucleate and hyper-flagellated swarm cells after activation of flagella production (Shively and Benson 1967).

In the other hand, the bacterial membrane phospholipids have been reported to be noticeably affected by several growth conditions like pH, growth temperature, carbon source, salinity and the growth phase (Syakti 2006). However, no studies have reported change in membrane composition, in swarmer bacteria. Concerning the growth phase, several changes in bacterial lipid composition have been reported to occur during the transition from the exponential growth phase to the stationary growth phase. Cardiolipin or diphosphatidyl glycerol has been observed to accumulate during the stationary phase of growth in *Clostridium butyricum* (Shively and Benson 1967), *Staphylococcus aureus* (Houtsmuller and Van Deenan 1965), and *Thiobacillus thiooxidans* (Shively and Benson 1967). The disappearance of phosphatidyl glycerol (PG) has been noted in *E. coli* (Syakti 2006).

Many studies have shown that membrane composition is strongly regulated to offset adaptation process probably enables vital membrane functions to continue (Diefenbach et al.1992). In spite of these

environmental changes, microorganisms adjust their fatty acid composition to keep most favorable permeability of cytoplasmic membranes (Shively and Benson 1967). Microorganisms are obliged to adapt the physical state of their membranes to environmental conditions but they have a limited range of possibilities for regulating the stability of the lipid bilayer. An effective way for this is to modify the ratio of saturated (SFA) to unsaturated fatty acids (UFA) of the phospholipids (Diefenbach et al.1992). Isomers of the unsaturated fatty acids found in *Ps. aeruginosa* (C16:1ω7 and C18:1ω9) are representatives of the aerobic pathway of fatty acid biosynthesis. In this biosynthetic pathway, bacteria employ the multi-component membrane desaturases enzymes (Lai et al.1998). Bacteria utilizing desaturases enzymes have a synthetase that produces only SFA. The only way for these bacteria to produce UFA, most commonly palmitoleic (C16:1ω7) and oleic acids (C18:1ω9), is by the action of the desaturase enzyme (Houtsmuller and Van Deenan 1965). So, after synthesis of the saturated acyl chains and integration into phospholipids, the fatty acid composition of phospholipids change significantly in this bacterium (Mâalej et al 2013). Therefore, the proportion of saturated to unsaturated fatty acids in the membrane can be modified in non-growing cells. In most of the previous works, phospholipid fatty acid of bacterial membrane was obtained from the exponential growth phase (Kloula Ben Ghorbal et al 2013; Mâalej et al 2013; Khefacha et al 2014). However, there is a lack of information about the modification of fatty acid composition during stationary growth phase (Syakti 2006).

Here, we report the kinetics of several such changes in *Ps. aeruginosa* swarmer cells, from inoculums at different stages of growth. The amounts of the three mean classes of phospholipids and of fatty acids in total lipids and in each of the phospholipid classes has been also measured in the membrane of swarmer cells. This research on mechanisms of biofilm resistance will help to develop strategies to prevent and combat *Pseudomonas* biofilms.

This study aimed at assessing the fatty acid composition of *Pseudomonas* bacteria recovered from inoculums at different stages of growth, to understand the role of membrane fatty acids composition in regulation of swarming and also in biofilm formation, a solicited process in *Pseudomonas* infections.

2. Materials and Methods

2.1. Bacterial strain and growth conditions

For preculture preparation, *Ps. aeruginosa* PAO1 was grown overnight in Tryptocasein Soja Broth (TSB) at 37° C. One percent of the overnight preculture was then added to Tryptocasein Soja Broth (TSB) and grown until point of log, exponential, early and late stationary phases of growth. Inoculums were normalized to undergo swarming assay with the same cell density.

2.2. Swarming behavior assay

Swarming migration distance assay was performed as described by Liaw (2003). Media used for assay consisted of 0.6% (wt/vol) Biolife –agar with 30g/liter Biolife TSB, to which 5 g/liter glucose was added. Swarm plates were typically allowed to dry at room temperature overnight before being used. The inoculums used for swarming were liquid cultures (10 µl) grown until point of log, exponential, early and late stationary phases of growth. Inoculums, each with the same initial population density were inoculated centrally onto the surface of dried TSB swarming agar. Swarming plates were then incubated at 37° C overnight (18 h) and the swarming zone was photographed. The swarming migration distance that cells moved from the point of inoculation was assayed by measuring the swarm fronts of the bacterial cells, after 18 hours. Then, swarmer cells at the edge of the swarm surface were scraped and centrifuged. The pellets were washed twice with NaCl (0.9 %). Then, the pellets were used for total lipids extraction.

2.3. Extraction of total lipids

The lipid extraction procedure was as previously described by Bligh & Dyer (1959). Briefly, the extraction was conducted in a monophasic system (Dichloromethane/methanol; 3:1 v/v). After centrifugation, the chloroform layer obtained was removed. Chloroform layer was then evaporated in a rotary evaporator at 40° C. The lipid residue was immediately dissolved in a small volume of hexane and stored under a nitrogen atmosphere until chromatographic analyses were carried out (Bligh and Dyer 1959).

2.4. Thin-layer chromatography (TLC)

The extracted lipids were separated on TLC silica gel plates. The plates were impregnated and developed in methanol shortly before sample application and then activated by heat treatment at 110° C for 30 minutes. A one-dimensional ascending development was in the following solvent system: chloroform- acetone-methanol-glacial acetic acid-water (50:20:10:10:5, v:v:v:v:v) (Tremoliers and Lepage 1971). Phospholipids were visualized with iodine vapors and their R_f were calculated and compared with known standards. The corresponding bands were marked, scraped off the plates and then eluted for fatty acids analysis (Kloula et al. 2013).

2.5. Fatty acids analysis

Methylation of the FA was achieved according to the method described by Cecchi et al. (1985). The membrane FA composition was determined on a GC system (Agilent Technologies 6890NR model [Network GC System]) equipped with a flame ionization detector (FID), an electronic pressure control (EPC) injector. A polyethylene glycol fused silica capillary column (Innowax, 30 m × 0.25 mm × 0.25 µm film thickness) purchased from Agilent (Wilmington, Delaware, USA) was used. The column was operated at 150° C for 1 min, the temperature was raised by 15° C/min^{-1} to 210° C for 5 minutes and then raised by 5° C/min^{-1} to 250° C and maintained until the end of analysis (25 min). N_2 was used as the carrier gas at a flow rate of 150 KPascal and H_2 at a flow rate of 25 ml/min. Peak areas were quantified using chromatography software (Agilent Technologies ChemStation Family data analysis). The FAMEs were first identified by comparing their retention times with those of known standards and results were expressed as relative percentages of each FA (Cecchi et al. 1985).

2.6. Measurement of lipid peroxidation

Swarmer cells, collected from the edge of the swarm zone, were harvested. The pellets were weighted and then suspended in 300 µl of Tris EDTA (TE) buffer. Lipid peroxide was extracted by addition of 150 µl of 0.5% Thiobarbituric acid (TBA) and 150 µl of 2% trichloracteic acid (TCA) followed by incubation at 95°C for 5 min. After cooling to room temperature, MDA was extracted with equal volume of chloroform, and the concentration of MDA, formed by reacting with TBA to give red species in the aqueous phase, had a maximum of absorbance at 535 nm. MDA concentration was determined by measuring optical density at 535 nm with a path length of 1 cm with a spectrophotometer (Spectro UVS- 2700 Dual BEAM LABOMED, INC) and then expressed as nmol of MDA produced per mg of protein (Heath and Packer 1986).

2.7. Statistical analysis

The mean values and the standard deviation were calculated from the data obtained with three separate experiments. These data were carried out using Student's t-test. Statistical significance was set at < 0.05.

3. Results and Discussion

3.1. Motility behavior of *Pseudomonas* from inoculums at different stages of growth

For swarming behavior study, cells of the inoculums were normalized to see differences in swarming behavior depending on the growth phase of the inoculum. As shown in figure (1), surface migration at the colony periphery of *Ps. aeruginosa* was generally en masse with rafts of cells that migrated away from the colony forefront. This migration was preceded by a shiny mucoid layer which presumably consists of exopolysaccharide, alginate, biosurfactant and rhamnolipid (Figure 1). These rafts are more sizeable for inoculums at the early and late stationary growth phase (Figure 1.C, D) with an important colony diameter of the swarm surface.

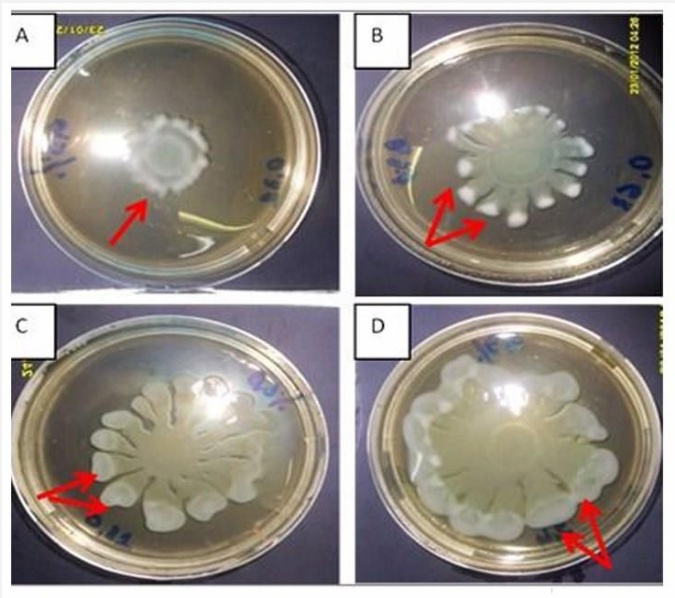

Fig 1 Photographs of Swarming zones of *P.aeruginosa*, from inoculums at different stages of growth

Swarming zones were measured 18h after inoculating 0.6% TSA plates; zone diameters (means ± standard deviations; n = 3) are listed for each optical density

(A) inoculum O.D = 0.5, (B) inoculum O.D = 0.6, (C) inoculum O.D = 1 and (D) inoculum O.D = 3 are shown. The arrows indicate cell proliferation in the edge of the swarm zone

Figure (2) shows that colony diameter is considerable for PAO1 inoculums in end log phase (2.27 cm) and in early stationary phase of growth (2.49 cm). This finding suggests that older swarmer (inoculums at stationary growth phase) cells are trying to find a new niche with more propitious nutriment in the edge of the Petri dishes with an important aptitude of swarming. Swarmer cells were scraped from the edge of the swarm surface to be analyzed by TLC and GC.

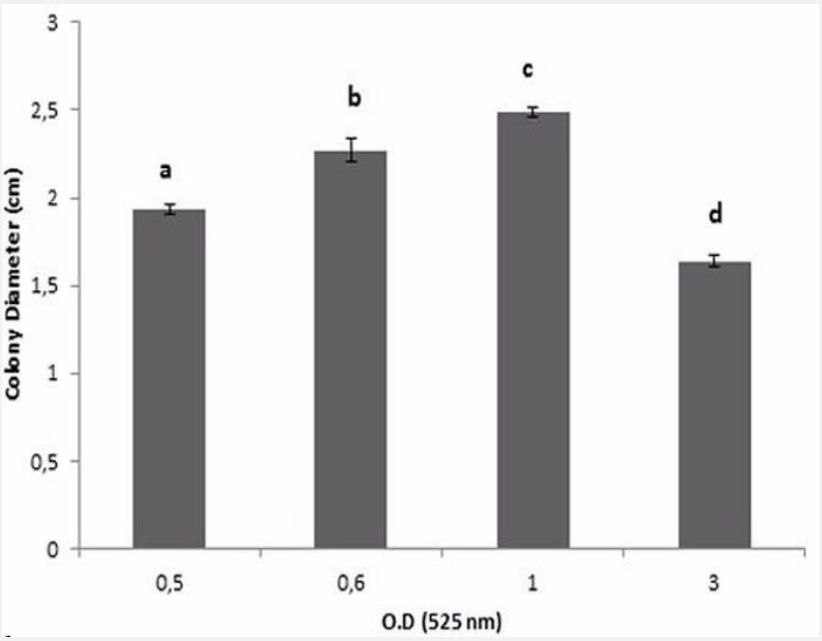

Fig 2 Diameter variation of swarming zones of *P.aeruginosa*, from inoculums at different stages of growth
The results are the means of three determinations ($P < 0.05$, student's test). Different letters mean significant difference ($P < 0.05$) for each growth phase. Same letters means no significant difference. Vertical bars represent the standard deviation from the mean.

3.2. Amounts of PE, PG and Cd differ in swarmer cells, according to the inoculums age

Thin-layer chromatograms (TLC) of lipid extracts of *Ps. aeruginosa* swarmer cells yielded three mean spots. The spots were identified by their behavior in one thin-layer solvent systems. Spot 1 is Cardiolipin which is diphosphatidyl glycerol (Cd), spot 2 is Phosphatidyl Glycerol (PG) and spot 3 is Phosphatidyl Ethanolamine (PE).

Same phospholipids species were observed for swarmer cells, independently of the inoculums age. Moreover, Phosphatidylethanolamine (PE) and cardiolipine (Cd) showed significant ($P < 0.05$) different distribution patterns in swarmer cells, according to the inoculums age (Figure 3). For inoculums in stationary phase of growth, PE rate was significantly ($P < 0.05$) enhanced up to twofold, in swarmer cells. However, since we have not significant lowering of the rate of Phosphatidyl Glycerol (PG), we can't speak about the conversion of PG to Cd, in swarmer cells. In parallel, the corresponding phospholipids bands were extracted and chromatographed to find out fatty acids profile.

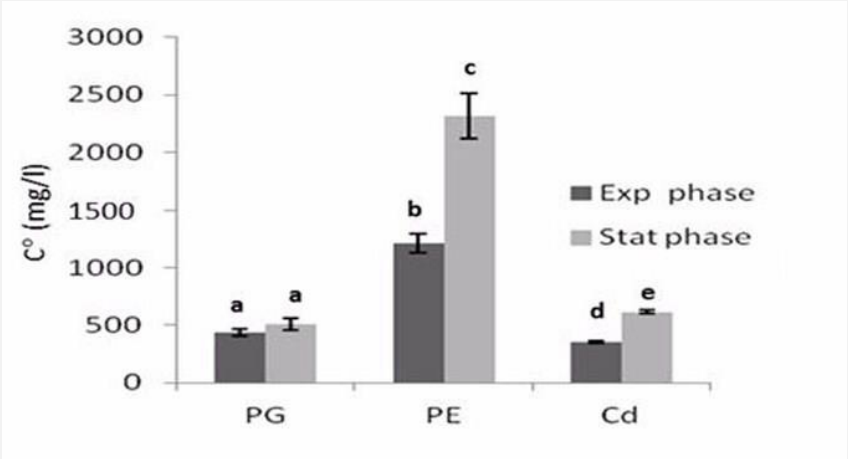

Fig 3 Changes in the relative amounts of certain phospholipids.

PG, Phosphatidyl Glycerol; PE, Phosphatidyl Ethanolamine and CL, Cardiolipine in Pseudomonas swamer cells, according to the inoculum's growth phase
The results are the means of three determinations ($P < 0.05$, student's test). Different letters mean significant difference ($P < 0.05$) for each phospholipid. Same letters means no significant difference.

3.3. Fatty acid profiles differ in PE, PG and Cd of swarmer cells, according to the inoculums age

As shown in figure (4), FA composition of phospholipids in *Ps. aeruginosa* swarmer cells was highly modified, depending on the growth phase of the inoculums cells. This change is marked by a significant ($P < 0.05$) increase of the ratio UFA/SFA, which means an increase in UFA proportion for PE and PG. This change is counteracted by a general decrease in SFAs in swarmer cells from inoculums at exponential phase of growth (O.D =0.6). In another hand, a decrease of the ratio UFA/SFA was seen, for Cd, especially when inoculums are in stationary growth phase.

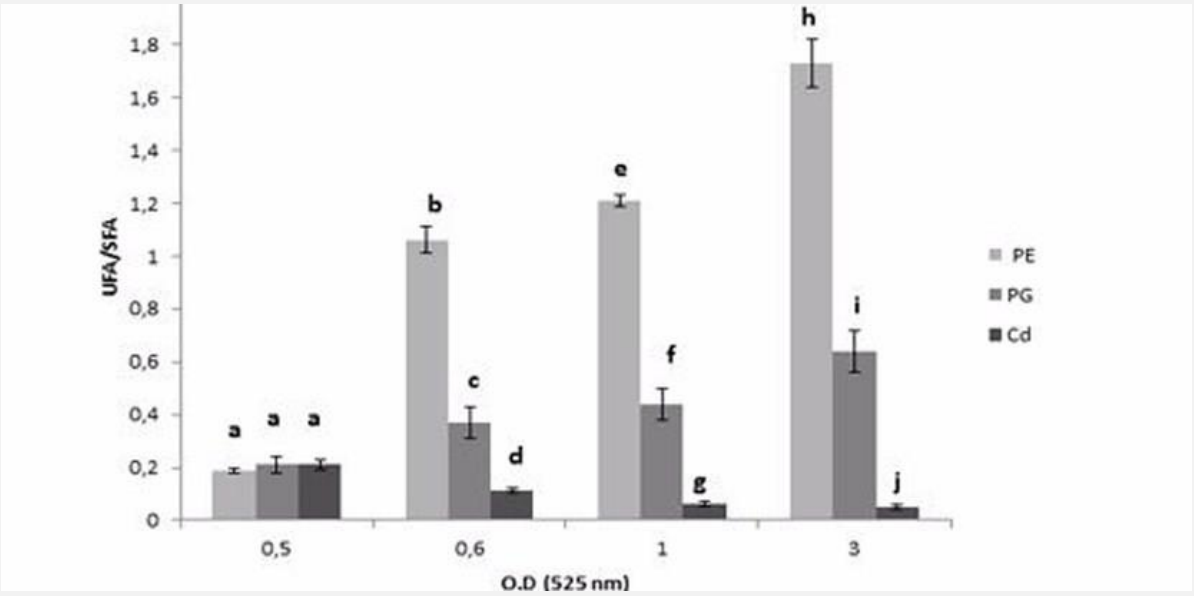

Fig 4 Course of the degree of unsaturation of fatty acids for PE, PG and Cd in *Pseudomonas* swarmer cells, depending on the growth phase of the inoculum
The results are the means of three determinations ($P < 0.05$, student's test). Different letters mean significant difference ($P < 0.05$). Same letters means no significant difference.

Accordingly, if inoculums cells are in stationary phase of growth, fatty acid composition of swarmer cells is characterized by a significant increase in the degree of unsaturation, respectively for PE and PG balanced by an increase in the degree of saturation for Cd.
This result show that the degree of unsaturation of membrane lipids is apparently not frozen after cessation of growth, for inoculums at stationary growth phase. The results also have shown that the cells are still able to modify their fatty acids composition which is in perfect accord with the aerobic way described above (Diefenbach et al. 1992). This way is independent of lipid synthesis and thereby of growth of the cells (Bligh and Dyer 1959).
For inoculums in stationary growth phase, since existing lipids, the palmitic acid (C16:0) and the stearic acid (C18:0) are used as the substrate, lipid synthesis is not required and proportions of these SFAs are significantly diminishing as they are converted into their analogous UFAs, palmitoleic (C16:1ω7) and oleic acid (C18:1ω9). Therefore, the UFAs/SFAs index increased progressively, in swarmer cells and was 1.5-fold higher when inoculums are at the end of the log phase (from 1.27 to 1.85).
As the aerobic fatty acid biosynthetic pathway is a post-synthesis modification process, in growing (exponential growth phase) or non-growing cells (stationary phase growth), the proportion of SFA to UFA in the membrane can be modified but not with the same speed. The experiments of this study with *Ps. aeruginosa* PAO1 swarmer cells have shown that the degree of desaturation of membrane lipids is apparently slow down after cessation of growth in the cells
of the inoculum. Nevertheless, the results revealed that the cells can modify their fatty acids, as this way is independent of lipid synthesis and thereby of the cells growth.
For non-growing cells of the inoculum (stationary phase growth), the desaturation of UFAs seems to be an appropriate and energetically practical way to modify membrane fluidity compared with a mechanism based

on de-novo synthesis of fatty acids (Diefenbach et al. 1992). In the other hand, the slight increase in the relative content of cellular PUFAs (from 0.64% to 1.48%) shows that their biosynthesis takes place mainly under stressful conditions, during the phase of stationary cellular activity. It is also believed that an increase in PUFAs represents a need for greater insaturation during bacterial growth (Russell and Fukunaga 1990).

3.4. Fatty acid profiles differ in TL of swarmer cells, according to the inoculums age

Then, we followed the degree of unsaturation by measuring the ratio of Unsaturated FA to Saturated FA in total lipids (TL), in C16 family and in C18 family. All of these results were reported in figure 5.

Results showed that, for inoculums at stationary growth phase, swarmer cells are characterized by a significant increase ($P < 0.05$) in the degree of unsaturation in C18 family. Therefore, UFA/SFA ratio is enhanced, in swarmer cells, from 5.47 to 45.22. However, for (C16) family UFA/SFA ratio did not vary. As a result, the relative ratio UFA/SFA for (C18) family gave distinct values for each physiological state, in swarmer cells, according to the age of the inoculum.

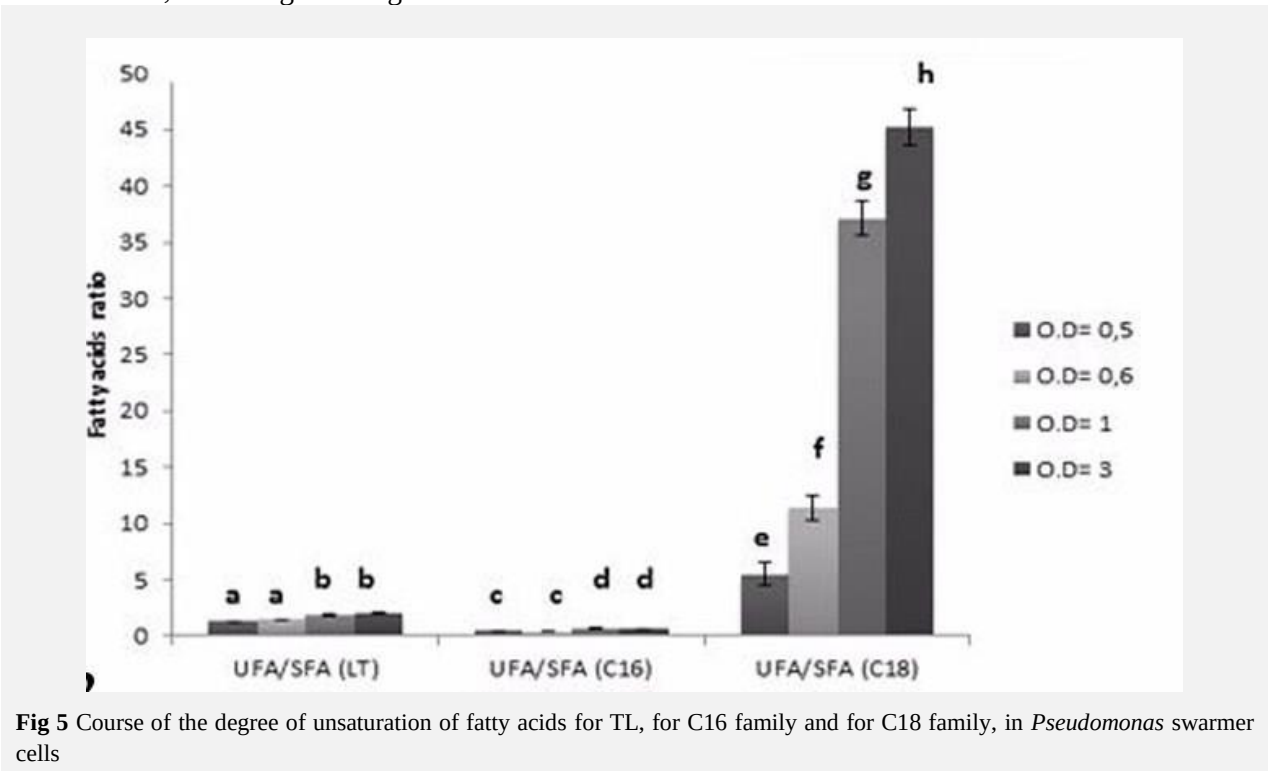

Fig 5 Course of the degree of unsaturation of fatty acids for TL, for C16 family and for C18 family, in *Pseudomonas* swarmer cells

The results are the means of three determinations ($P < 0.05$, student's test). Different letters mean significant difference ($P < 0.05$) for each fatty acids family. Same letters means no significant difference.

So, because the cellular fatty acid profile is closely related to the swarming phenotype and possibly linked to the age of the inoculums, Lai and collaborators (1998) propose a preserved mechanism of swarming in Gram-negative bacteria through the control of membrane stability (Lai et al. 1998). Otherwise, fatty acids can function as intercellular communication signals (Liaw et al. 2004) and so, in *Ps. aeruginosa*, fatty acids also affect swarming through this mechanism. We found that the relative abundance ratio of Unsaturated Fatty Acids to Saturated Fatty Acids which is about twofold higher in swarmer cells when inoculums are in stationary growth phase (1.98) than in early log phase (1.27), led to good discrimination between the different physiological states.

Consecutively, correlation between fatty acid composition and swarming phenotype was investigated in this study. Moreover, as it was confirmed that inhibition of swarming by SFA was unlikely to be due to its inhibitory effect on cell growth, SFAs may act as signals to regulate swarming (Mallej et al. 2013) .The presence of saturated or unsaturated fatty acids strongly affects swarming in *S.marcescens*, *P.mirabilis* and *Ps.aeruginosa.* Moreover, it was reported for *Sinorhizobium meliloti* (Soto et al. 2002) and *Xanthomonas campestris* that fatty acids or their derivatives have been shown to be implicated in regulation of swarming differentiation (Daniel 2010). According to our results, when inoculums are in stationary growth phase, SFAs are diminishing and are converted into their analogous UFAs and so this modified composition is suitable to a greater swarm surface. All of these data reveal that fatty acids could act as intracellular signals to control swarming and expose a new mechanism through which fatty acid exerted its regulatory effect (Shinitzky and Barenholz 1974).

3.5. Lipid peroxidation in swarmer cells

To identify the possible interaction between hydroxyl radicals overproduced during growth and cellular membrane phospholipid content, TBA test was performed to measure lipid peroxidation. Our results indicate that lipid peroxidation in *Ps. aeruginosa* swarmer cells was significantly ($P < 0.05$) induced at measurable levels, from 7.8 for young inoculums to 55.25 nmol of MDA/mg of protein, for more old inoculums. In the other hand, as shown in Figure (6), polyunsaturated fatty acids (PUFA) percentage is insignificantly ($P > 0.05$) increased, in swarmer cells, from 0.4%, for inoculums at the end of exponential growth phase, to 0.49%, when inoculums are in early
stationary growth phase. This may suppose a possible conversion of PUFA into malondialdehyde. However, for inoculums at the end of the stationary growth phase, lipid peroxidation decreased significantly ($P < 0.05$), in swarmer cells, until 19.72 nmol of MDA /mg of protein and PUFA percentage augmented significantly ($P < 0.05$) (up to 2.58%).

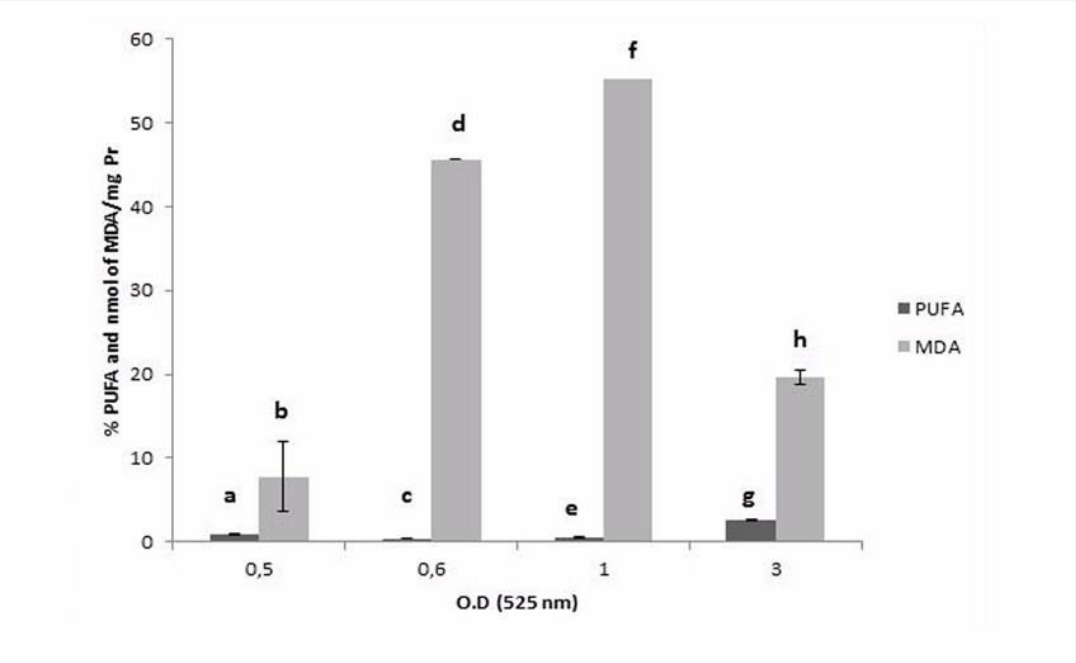

Fig 6 Correlation between Polyunsaturated Fatty acids (PUFA) content (%) and Malondialdehyde (nmol of MDA/ mg Protein) production in swarmer cells, from inoculums at different stages of growth

The results are the means of three determinations ($P < 0.05$, student's test). Different letters mean significant difference ($P < 0.05$) between PUFA and MDA content for each growth phase. Same letters means no significant difference.

MDA, a major oxidation product of peroxidized polyunsaturated fatty acids has been used to determine the degree of lipid peroxidation and as a biological marker of oxidative stress (Mâalej et al. 2013; Khefacha et al. 2014). The normal level of MDA, in swarmer cells, might be due to the enhanced activities of antioxidant enzymes which can scavenge excess lipid peroxidaton (Meng et al. 2009). For inoculums at end log and early stationary growth phase, PUFAs amount are diminishing. This finding can be explained by a significant increase in reactive oxidative species in *Pseudomonas* swarmer cells which is in concordance with the decrease in PUFA content dosage. We propose that peroxidation of PUFA is highly enhanced by membrane free radical reactions in swarmer cells from old inoculums, thereby causing widespread lipid peroxidation.

4. Conclusion

As a conclusion, we have a well-known result supposing that cellular fatty acid profile is strongly correlated to the swarming phenotype and most likely linked to the age of the inoculums .In line with this proposition, this change in membrane composition was found to be closely related to swarming phenotypes in *P .mirabilis*. Here, with *Ps. aeruginosa* PAO1 membrane desaturation was markedly seen especially for C18 family and consecutively membrane composition was adjusted which in turn determines important colony diameter of swarmer cells and more developed swarming phenotypes.

In this respect, it is important to elucidate the molecular mechanisms underlying fatty acid regulation of swarmer bacteria in the order of developing new anti-biofilm strategies via the comprehension of several mechanisms implicated in biofilm formation, like swarming motility.

Acknowledgments

The authors would like to thank Dr. Ines Mehri at the department of biology, faculty of sciences, University of Tunis el Manar, Tunisia, for practical provision.

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