Characterization of *Pseudomonas aeruginosa* fatty acid profiles in biofilms and batch planktonic cultures

Jerry Chao, Gideon M. Wolfaardt, and Michael T. Arts

Abstract: The fatty acid composition of *Pseudomonas aeruginosa* PAO1 was compared between biofilm and batch planktonic cultures. Strain PAO1 biofilms were able to maintain a consistent fatty acid profile for up to 6 days, whereas strain PAO1 batch planktonic cultures showed a gradual loss of *cis*-monounsaturated fatty acids over 4 days. Biofilms exhibited a greater proportion of hydroxy fatty acids but a lower proportion of both cyclopropane fatty acids and saturated fatty acids (SAFAs). SAFAs with ≥16 carbons, in particular, decreased in biofilms when compared with that in batch planktonic cultures. A reduced proportion of SAFAs and a decline in overall fatty acid chain length indicate more fluidic biophysical properties for cell membranes of *P. aeruginosa* in biofilms. Separating the biofilms into 2 partitions and comparing their fatty acid compositions revealed additional trends that were not observed in the whole biofilm: the shear-nonremovable layer consistently showed greater proportions of hydroxy fatty acid than the bulk liquid + shear-removable portion of the biofilm. The shear-nonremovable portion demonstrated a relatively immediate decline in the proportion of monounsaturated fatty acids between days 2 and 4; which was offset by an increase in the proportion of cyclopropane fatty acids, specifically 19:0cyc(11,12). Simultaneously, the shear-removable portion of the biofilm showed an increase in the proportion of *trans*-monounsaturated fatty acids and cyclopropane fatty acids.

Key words: biofilms, batch planktonic culture, *Pseudomonas aeruginosa*, bacteria, fatty acids, physiological heterogeneity.

Résumé : Une comparaison de la composition en acides gras de *Pseudomonas aeruginosa* PAO1 maintenu en culture planctonique en lot ou en biofilm a été réalisée. Les biofilms de PAO1 maintenaient un profil en acides gras stable pendant 6 jours, alors que les cultures planctoniques en lot de PAO1 perdaient graduellement des acides gras *cis*-monoinsaturés au cours des quatre premiers jours. Les biofilms contenaient davantage d'acides gras hydroxylés et une proportion plus faible d'acides gras cyclopropaniques et d'acides gras saturés. La quantité d'acides gras saturés de 16 carbones ou plus diminuait dans les biofilms comparativement aux cultures planctoniques en lot. La proportion réduite d'acides gras saturés et la diminution de la longueur de la chaine d'acides gras en général indique que les membranes cellulaires de *P. aeruginosa* en biofilm sont plus fluides. La séparation des biofilms en deux phases et la comparaison de leur composition en acides gras a révélé d'autres tendances qui n'étaient pas observées dans les biofilms entiers : la couche non détachable par cisaillement possédait systématiquement des concentrations plus élevées d'acides gras hydroxylés comparativement à la portion détachable par cisaillement ou la phase liquide. La proportion d'acides gras monoinsaturés de la portion non détachable par cisaillement déclinait presqu'immédiatement entre les jours 2 et 4, ce qui était compensé par une augmentation de la proportion d'acides gras cyclopropaniques, spécifiquement le 19 :0 cyc(11,12). Simultanément, la proportion d'acides gras *trans*-monoinsaturés et d'acides gras cyclopropaniques augmentait dans la portion du biofilm détachable par cisaillement.

Mots-clés : biofilms, culture planctonique en lot, *Pseudomonas aeruginosa*, bactéries, acides gras, hétérogénéité physiologique.

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Introduction

As early as the 1970s, total cellular fatty acid analysis has been proposed as a possible method for rapidly identifying bacterial species in laboratory cultures (Moss and Dees 1975; Moss 1981). This approach allows researchers to identify bacterial species that would be difficult to distinguish through classic biochemical tests (Walker et al. 1993; Whittaker et al. 2005). Numerous studies have since documented the fatty acid profiles of various bacterial species (Johns and

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Perry 1977; Nichols 2003; Whittaker et al. 2007). While fatty acid profiling has been used to identify bacteria and characterize metabolic functions, specific details on how bacterial fatty acids relate to the physiological status of biofilms are relatively scarce.

The majority (70%–90%) of total cellular fatty acids reside in the acyl constituents of cell membrane phospholipids, whereas neutral lipids are relatively minor constituents of the total lipids in bacteria (Lennarz 1966; Nichols and Mancuso Nichols 2008). The fatty acid composition of cell membrane affects their biophysical properties (i.e., fluidity and flexibility) and, consequently, the efficiency of many membrane-related functions (Zhang and Rock 2008). Thus, the biochemical competency of membranes is one of the most important factors regulating cellular processes in prokaryotic organisms. In addition, the enzymatic activity of aerobic and anaerobic fatty acid desaturation mechanisms during synthesis, along with postsynthesis modifications on existing unsaturated phospholipid fatty acid (PLFA), can alter the membrane properties to improve viability of the bacterial cells (Garwin et al. 1980; de Mendoza and Cronan 1983; Fulco 1983; Thompson 1992). For example, bacterial cell membrane fatty acid composition shifts in response to changes in environmental conditions, such as temperature (Nichols 2003; Zhu et al. 2005; Zhang and Rock 2008), pH (Giotis et al. 2007), salinity (Komaratat and Kates 1975), hydrostatic pressure (DeLong and Yayanos 1985), and organic solvent exposure (Mrozik et al. 2004; Nielsen et al. 2005).

Considerable effort has been devoted to understanding the structure and function of biofilms, from both cellular and ecological perspectives. Biofilms can be defined as communities of microorganisms that have accumulated at interfaces and where multicellular behavior allows for specialization and cooperation between individual cells (Denkhaus et al. 2007). Biofilm communities differ from planktonic bacterial cultures not only in terms of their metabolic activity but they also display stage-specific phenotypes during development (Sauer et al. 2002) and considerable spatial heterogeneity of physiological condition (Bester et al. 2005; Stewart and Franklin 2008). Previous studies on differentiation of metabolic activity in biofilms mostly involved microscopy combined with reporter probes and (or) genes that targeted various indicators of physiological activity in individual cells. More recently, selective removal of the shear-susceptible layers of biofilms, combined with CO_2 analysis, has provided a direct measure of metabolic activity while overcoming some of the inherent disadvantages of microscope-based techniques (Bester et al. 2010).

Cell attachment during biofilm formation has been associated with the genetically controlled expression of cell surface adhesion molecules (Goller et al. 2006; Ma et al. 2006). However, it has been suggested that cell adhesion can also be affected by the biophysical properties of cell membranes, which in turn, are controlled largely by fatty acid composition (Aricha et al. 2004). Given that the cell membrane is a vital barrier that strongly influences bacterial physiology, it is reasonable to expect that cellular fatty acid composition also plays a central role in the complex regulation of biofilm–planktonic transitions. Comparing biofilm and planktonic batch culture fatty acid profiles should therefore provide an indication of their physiological condition and further emphasize the heterogeneous nature of bacterial physiology.

In this study, fatty acid profiles of biofilms versus batch planktonic cultures of the model bacteria *Pseudomonas aeruginosa* were compared. To further assess biofilm structural organization, we examined the fatty acid composition of 2 distinct zones of the biofilm: (1) the upper active-phase containing the shear-removable zone, and (2) the tightly bound shear-nonremovable, surface-associated zone.

Material and methods

Bacterial strain selection

Pseudomonas aeruginosa PAO1 was chosen as the primary model organism for all experiments because of its well-known capacity for biofilm formation and its abundance in a wide range of environments. All stock cultures were stored at –80 °C in 1.5 mL Eppendorf microcentrifuge tubes with 40% glycerol. To preserve strain consistency, inocula used for all experiments came from overnight cultures (10% tryptic soy broth at 37 °C) derived from these cryogenically frozen stock cultures (70 μL).

Batch planktonic cultures

Batch planktonic cultures were grown in 100 mL of sterile 10% trypic soy broth in 250 mL Erlenmeyer flasks. The cultures were inoculated with 70 μL of *P. aeruginosa*, grown at room temperature (25 ± 2 °C), and shaken at 250 r/min. Aliquots (50 mL) of mature batch cultures were transferred into a 50 mL conical centrifuge tube and centrifuged for 20 min at 3100*g*. The resulting pellet of cells was aseptically transferred into a 2.0 mL cryogenic vial and stored (–80 °C) until fatty acid extraction.

Biofilm cultures

Biofilms were cultivated in 50-cm-long silicon tube reactors (VWR No. 60985-736; ~9.5 mm i.d., volume = ~35 mL/reactor). A Watson–Marlow 205S peristaltic was used to deliver 1% tryptic soy broth at the rate of 15 mL/h to the reactor at room temperature. The flow through system was inoculated with 1 mL of overnight *P. aeruginosa* culture by direct injection through the silicon tubing with a sterile syringe. The flow was turned off during inoculation and kept off for 1 h to allow for some cell attachment. As described by Sauer et al. (2002), total biofilm biomass was harvested by physical agitation and squeezing; but without purging the bulk liquid from the tubing. Biofilm matrix released into the bulk liquid was then poured into 50 mL conical centrifuge tubes and centrifuged for 20 min at 3100*g*. The cell pellets were transferred to 2 mL cryogenic vials and stored at –80 °C until fatty acid extraction.

Partitioning of biofilms

The shear-removable biofilm layer at the biofilm–liquid interface was removed, as described by Bester et al. (2010); in essence, this method involves the introduction of an air bubble into the flow chambers by temporarily disconnecting the silicon tube from the growth medium reservoir upstream of the peristaltic pump. The loosely attached biofilm biomass (shear-removable portion of the biofilm) was subse-

quently collected at the effluent end of the growth chamber into 50 mL conical centrifuge tubes. To collect the surface-associated layer (i.e., the shear-nonremovable portion), the medium reservoir was reconnected to the growth chamber and then harvested by using the total biofilm collection method described above. Both partitions were centrifuged and stored at –80 °C.

Bacterial fatty acid analysis

All samples were freeze-dried for 1 day at –62 °C under vacuum prior to fatty acid extraction, as described by Steger et al. (2003) and Hoffmann et al. (2008). The fatty acid analysis method was adapted from MIDI Inc. (Newark, Delaware, USA) with minor modifications: (1) temperature was changed from 100 °C to 80 °C in the saponification reaction, (2) phase separation used 1.50 mL of hexane – methyl *tert*-butyl ether instead of 1.25 mL, and (3) an additional extraction was carried out with 1.0 mL of hexane. In brief, ~5 to 10 mg of freeze-dried bacterial sample was used in each extraction. The method began with saponification using 1 mL of sodium hydroxide (3.75 mol/L) in 1:1 methanol–water at 80 °C for 30 min, followed by transesterification (methylation) with 2 mL of 10% hydrochloric acid in methanol at 80 °C for 10 min, then phase separation with 1.50 mL of 1:1 hexane – methyl *tert*-butyl ether, a wash with 3 mL of 0.3 mol/L sodium hydroxide, followed by an extra extraction with 1 mL of hexane.

The resulting whole-cell fatty acid methyl esters (FAMEs) were analyzed with a Varian CP-3800 gas chromatograph with a flame ionization detector. A 5% phenylmethyl silicone fused silica capillary column (Agilent HP-Ultra 2 19091B-102; 25 m × 0.20 mm i.d. × 0.33 μm film thickness) was used to separate the FAMEs. Oven temperature programming was as follows: start at 50 °C and hold for 0.5 min, increase to 170 °C at the rate of 35 °C/min, increase to 225 °C at 4 °C/min, and then ramp to 310 °C at 60 °C/min and hold for 1.15 min. Other gas chromatography parameters included 0.5 μL injection volume, 1.0 split ratio, and 1.0 mL/min constant flow of helium (carrier gas). Bacterial acid methyl ester standard (Sigma No. 47080-U) with the added 21:0 (Sigma No. H3265) reference standard was used for identification and quantification. Additional peaks were identified using the following individual FAME standards from Matreya LLC: 3-hydroxydecanoate (No. 1728), 3-hydroxyhexadecanoate (No. 1740), methylhexadecanoate (No. 1614), *cis*-11-octadecenoic acid (No. 1266), *trans*-11-octadecenoic acid (No. 1262), and *cis*-9,10-methyleneoctadecanoic acid (No. 1822). A 4-point linear calibration curve was constructed with the bacterial acid methyl ester + 21:0 standard mix to quantify the peaks for each sample. All gas chromatography data were analyzed with Varian Galaxie™ software (version 1.9.3).

Statistical analysis

Fatty acid data are reported as mol% of total fatty acid; this allows for direct comparisons independent of the amount of biomass in the sample. All statistical analyses were performed with SigmaStat software for Windows (version 3.5) using 95% confidence intervals. Pair-wise comparisons were performed using 2-sample *t* tests. Comparisons with multiple treatment groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test if the ANOVA indicated a significant result. Proportional data were arcsine square-root transformed prior to applying statistical tests to satisfy the condition of normality.

Results

Fatty acid profiles of *P. aeruginosa*

From the fatty acid profiles of a 2-day-old batch planktonic *P. aeruginosa* shown in Table 1, monounsaturated fatty acids (MUFAs) and saturated fatty acids (SAFAs) represented the greatest proportion of total fatty acids: ~43.60 and 31.60 mol% of total fatty acid, respectively. Among these, *cis* 18:1n-7 (~31.00%) and 16:0 (~26.70%) were the 2 most abundant fatty acid. Cyclopropane and hydroxy fatty acids (~9.60 and 15.20 mol% of total fatty acid), typical for gram-negative bacteria, were also observed in *P. aeruginosa*.

Fatty acid profile of *P. aeruginosa* batch planktonic culture over time

The batch culture is a closed system where growth typically ceases as a result of the accumulation of metabolic waste products or when a limiting nutrient is depleted. To determine the appropriate age of planktonic cultures for comparison with biofilm cultures, it was necessary to observe how the planktonic bacterial fatty acid profile changed over an extended period of the growth cycle.

No significant differences were observed in the concentration of SAFAs of planktonic *P. aeruginosa* cultures up to day 4. While total hydroxy fatty acids and cyclopropane fatty acids showed an increasing trend and MUFAs showed a decreasing trend, none of the changes in these fatty acid classes were significant (Table 1). Further isomer-specific comparison revealed that *cis*-MUFA, in particular, decreased with the age of the culture (ANOVA, $p = 0.04$). *Trans*-MUFAs showed an increasing trend, but it was not significant (ANOVA, $p = 0.17$). However, the combination of *trans*-MUFA and cyclopropane fatty acid showed a significant increase over the 4 day period (ANOVA, $p < 0.001$; Tukey's HSD, $p \leq 0.02$).

Increases in the ratio of cyclopropane fatty acids relative to their respective MUFA precursors were also observed in *P. aeruginosa*; these included 19:0cyc(11,12) from *cis* 18:1n-7, and 17:0cyc(9,10) from *cis* 16:1n-7 (Table 2).

Pseudomonas aeruginosa biofilm fatty acid profile over time

The fatty acid profiles of 2-, 4-, and 6-day-old *P. aeruginosa* biofilms cultured in the continuous flow system were compared (Table 1). No significant difference for any of the major fatty acid classes was observed for biofilm culture up to day 6 (ANOVA, $p > 0.05$). The proportion of *cis*- to *trans*-MUFA, as well as the proportion of other fatty acids did not show any discernable differences.

Comparing fatty acid profiles of *P. aeruginosa* biofilms and planktonic cells

Because we demonstrated that total biofilm fatty acid profile was not influenced by age, the assumption was made

Table 1. *Pseudomonas aeruginosa* fatty acid profile in 1-, 2-, 3-, and 4-day-old planktonic culture with 2-, 4-, and 6-day-old total biofilm culture (upper + lower biofilm).

	Planktonic cultures (n = 6)				Total biofilm cultures (n = 6)		
Fatty acid	1 day	2 days	3 days	4 days	2 days	4 days	6 days
11:0	—	—	—	—	—	—	—
2-OH-10:0	—	—	—	—	—	—	—
3-OH-10:0	5.32±0.39	4.53±0.44	5.02±0.67	4.67±0.25	6.90±0.22	6.52±0.33	7.52±0.40
12:0	2.56±0.16	2.71±0.26	3.27±0.30	3.48±0.55	2.24±0.08	2.14±0.10	2.83±0.38
13:0	—	—	—	—	—	—	—
2-OH-12:0	6.72±0.23	7.09±0.31	7.56±0.36	8.41±1.22	7.13±0.22	6.88±0.26	6.53±0.22
3-OH-12:0	3.27±0.17	3.60±0.18	3.77±0.30	4.18±0.36	3.22±0.18	3.18±0.14	3.56±0.26
14:0	0.62±0.03	0.64±0.03	0.73±0.13	0.67±0.08	0.98±0.24	1.10±0.20	0.75±0.04
i-15:0	—	—	—	—	0.69±0.31	—	3.36±0.82
a-15:0	—	—	—	—	0.29±0.15	—	1.37±0.32
15:0	0.39±0.06	0.43±0.08	0.38±0.07	0.47±0.09	0.69±0.31	0.71±0.07	0.53±0.04
2-OH-14:0	—	—	—	—	—	—	—
3-OH-14:0	—	—	0.01±0.01	—	0.24±0.15	0.51±0.18	0.31±0.06
i-16:0	0.05±0.02	—	0.35±0.22	0.02±0.02	0.28±0.13	1.88±0.63	1.10±0.25
cis 16:1n-7	10.85±1.77	9.35±1.78	7.14±1.07	5.47±0.57	12.62±0.75	11.84±0.42	11.71±0.56
trans 16:1n-7	1.66±1.22	1.96±1.22	3.15±1.72	3.26±1.94	2.47±0.79	2.24±0.62	2.30±0.40
16:0	25.53±1.73	26.71±2.26	25.48±1.98	25.92±1.60	20.81±0.88	20.59±0.66	19.32±0.25
i-17:0	—	—	0.01±0.01	0.02±0.01	0.25±0.11	0.84±0.55	1.22±0.27
a-17:0	—	—	—	—	0.05±0.03	0.70±0.25	0.40±0.09
17:0cyc(9,10)	1.93±0.62	3.11±0.75	2.98±0.87	4.08±1.39	1.27±0.13	1.37±0.27	1.28±0.13
17:0	0.26±0.05	0.30±0.06	0.16±0.07	0.26±0.05	0.21±0.05	0.27±0.02	0.22±0.02
2-OH-16:0	—	—	—	—	—	—	—
3-OH-16:0	—	—	—	—	—	—	—
i-18:0	—	—	—	—	—	—	—
18:2n-6	—	—	—	0.01±0.01	—	—	—
cis 18:1n-9	0.21±0.09	0.31±0.05	0.15±0.10	0.17±0.11	0.06±0.04	—	—
cis 18:1n-7	34.27±1.73	31.00±2.45	26.58±2.63	23.31±3.14	36.34±0.59	35.61±0.70	34.19±0.81
trans 18:1n-9	1.00±1.00	—	1.71±1.71	—	—	—	—
trans 18:1n-7	0.12±0.08	3.15±1.46	0.99±0.89	3.77±2.37	0.73±0.16	0.65±0.05	1.40±0.59
18:0	0.72±0.13	0.83±0.15	1.13±0.28	0.77±0.11	0.38±0.09	0.50±0.05	0.52±0.10
i-19:0	—	—	—	—	—	—	—
a-19:0	—	—	—	—	—	—	—
19:0cyc(9,10)	—	—	—	—	—	—	—
19:0cyc(11,12)	4.51±1.81	7.00±2.34	9.44±3.38	11.07±3.36	2.13±0.26	2.48±0.38	2.23±0.25
19:0	—	—	—	—	—	—	—
20:0	—	—	—	—	—	—	—
ΣHydroxy fatty acid	15.31±0.60	15.22±0.23	16.37±0.54	17.26±1.67	17.51±0.23	17.09±0.46	17.78±0.50
ΣBranched fatty acid	—	—	0.36±0.22	0.04±0.03	1.72±0.87	3.42±1.24	4.92±1.67
ΣIso	—	—	0.36±0.22	0.04±0.03	1.21±0.55	2.72±1.05	3.78±1.28
ΣAnteiso	—	—	—	—	0.34±0.18	0.70±0.25	1.14±0.40
ΣCyclic fatty acid	6.44±2.40	9.59±3.08	12.42±4.18	15.14±4.68	3.41±0.39	3.85±0.65	3.51±0.37
ΣSAFA	30.07±2.08	31.62±2.78	31.13±2.39	31.56±2.41	25.32±1.03	25.31±0.73	24.06±0.58
ΣMUFA	48.11±4.30	43.57±5.71	39.72±6.41	35.99±7.71	52.22±0.88	50.33±0.53	49.60±1.26
Σ*cis*	45.34±3.36	40.56±4.13	33.88±3.43	28.95±3.57	49.02±1.23	47.45±0.95	45.90±1.27
Σ*trans*	2.78±2.19	3.01±1.98	5.85±3.43	7.03±4.31	3.20±0.86	2.88±0.63	3.70±0.98

Note: Values are shown in unit of mol% of total fatty acid ± standard error. MUFA, monounsaturated fatty acid; SAFA, saturated fatty acid.

that sampling biofilms on any of the 3 collection dates (days 2, 4, or 6) for comparison with 2-day-old batch planktonic culture would have the same outcome. However, we elected to use the 6-day-old biofilm culture because of the increased biomass available for fatty acid analyses.

There was a difference in SAFAs between planktonic cells and biofilms (31.6–24.1 mol%), but this difference was borderline significant (t test, p = 0.05). Further comparison among the individual SAFAs of *P. aeruginosa* revealed that only SAFAs ≥16 carbons long showed a decrease, while other SAFAs were slightly greater in the biofilm (Table 3). The most abundant SAFA (palmitic acid, 16:0) declined by ~27.7% in biofilms compared with planktonic cells (t test, p = 0.01). Although only a low amount of stearic acid (18:0) was found in *P. aeruginosa*, biofilms had ~37.6% less 18:0 than planktonic cells (t test, p = 0.04).

Total cyclopropane fatty acid also showed a decreasing trend in the biofilm compared to planktonic cells, but the

Table 2. Ratio (± standard error) of postsynthesis-derived fatty acids (cyclopropane and *trans*-MUFA) to *cis*-MUFA in batch planktonic *Pseudomonas aeruginosa* cultures.

	Culture age			
Ratio of fatty acid	1 day	2 days	3 days	4 days
19:0cyc(11,12) to *cis* 18:1n-7	0.15±0.09	0.26±0.10	0.44±0.17	0.60±0.19
17:0cyc(9,10) to *cis* 16:1n-7	0.24±0.09	0.41±0.15	0.55±0.21	0.89±0.31
Cyclopropane fatty acid + *trans*-MUFA to *cis*-MUFA	0.22±0.06	0.35±0.08	0.60±0.13	0.82±0.10

Note: MUFA, monounsaturated fatty acid.

Table 3. Mol% of total fatty acid (± standard error) and percent change of detectable saturated fatty acids (SAFAs), cyclopropane fatty acids, and hydroxyl fatty acids between *Pseudomonas aeruginosa* batch planktonic and biofilm cultures.

Fatty acid	Planktonic (n = 6)	Biofilm (n = 9)	% Change
SAFA			
12:0	2.71±0.26	2.83±0.38	4.5
14:0	0.64±0.03	0.75±0.04	17.1
15:0	0.43±0.08	0.53±0.04	24.3
16:0*	26.71±2.26	19.32±0.25	–27.7
17:0	0.30±0.06	0.22±0.02	–27.0
18:0*	0.83±0.15	0.52±0.10	–37.6
Cyclopropane fatty acid			
17:0cyc(9,10)	3.11±0.75	1.28±0.13	–58.8
19:0cyc(11,12)*	7.00±2.34	2.23±0.25	–68.2
Hydroxy fatty acid			
3-OH-10:0*	4.53±0.44	7.52±0.40	65.9
2-OH-12:0	7.09±0.31	6.53±0.22	–8.0
3-OH-12:0	3.60±0.18	3.56±0.26	–1.0

Note: Asterisk (*) indicates fatty acids with significantly different proportions at $p \leq 0.05$ between the 2 culture types.

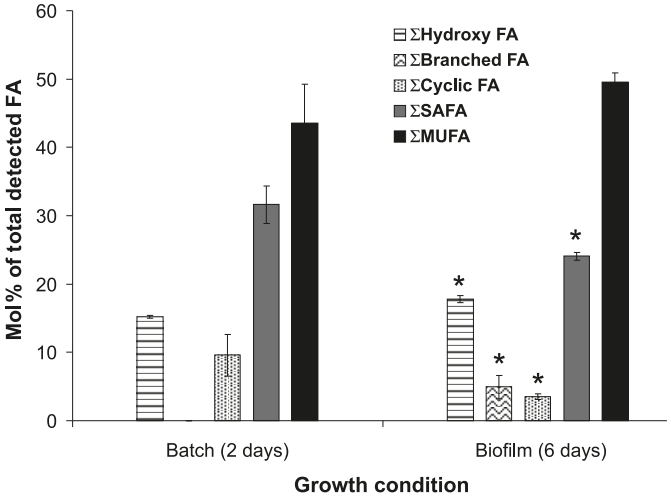


Fig. 1. Comparison of major fatty acid (FA) classes between 2-day-old *Pseudomonas aeruginosa* batch planktonic cells and 6-day-old *P. aeruginosa* biofilm cultures. Asterisks above bars (*) indicate a significant difference ($p \leq 0.05$) from the batch culture. Error bars express standard errors. MUFA, monounsaturated fatty acid; SAFA, saturated fatty acid.

difference in the total proportions was not significant. Among the 2 fatty acids that make up the total cyclopropane fatty acid in *P. aeruginosa*, only 19:0cyc(11,12) showed a significant decline (~68.2% less; t test, $p = 0.03$) in biofilms (Table 3).

Of the different fatty acid classes, hydroxy fatty acids demonstrated a significant increase in abundance in the biofilms than in planktonic cells (15.2 to 17.8 mol%, t test, $p = 0.003$) (Fig. 1). Further, among the 3 hydroxy fatty acids, 3-OH-10:0 (4.5 to 7.5 mol%) contributed most significantly to the observed increase in total hydroxy fatty acid (t test, $p < 0.001$) (Table 3).

Another observation was the presence of branched fatty acids in the gram-negative *P. aeruginosa* biofilms. This is interesting because branched fatty acids are usually considered to be associated with gram-positive bacteria (O'leary 1962; Kaneda 1991). As much as 4.9 mol% of branched chain fatty acids was observed in the biofilm; an amount that is comparable to that of cyclopropane fatty acids (~3.5 mol%). Among the branched-fatty acids found in *P. aeruginosa* biofilms, i-15:0 accounted for ~45.2% of total branched fatty acids; others included a-15:0 (~18.8%), i-16:0 (~15.0%), i-17:0 (~16.5%), and a-17:0 (~3.8%).

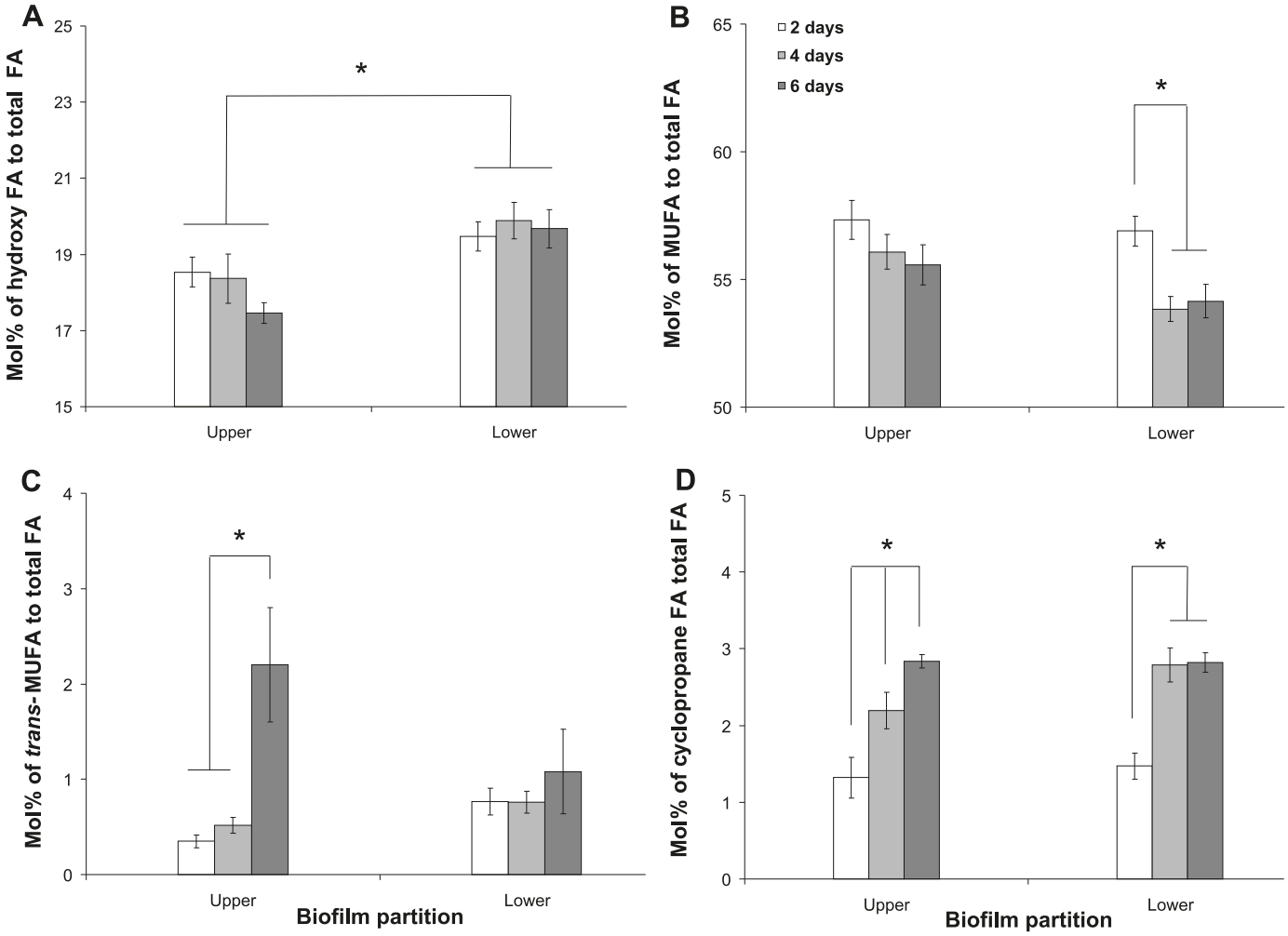
Fatty acid profile of shear-removable and shear-nonremovable *P. aeruginosa* biofilm partitions over time

To obtain sufficient biomass for fatty acid analysis, it was only practical for us to partition the biofilm culture into 2 phases: (1) the upper layer that includes both shear-removable attached cells and planktonic cells in the bulk liquid and (2) the lower layer was dominated by surface-attached cells that could be removed only by physical disruption (herein referred to as the shear-nonremovable layer).

A comparison of the major fatty acid classes revealed that there was a significant difference in the proportion of hydroxy fatty acids between the 2 partitions (Fig. 2A). The shear-nonremovable layer had a greater proportion of hydroxy fatty acid than the active upper phase for all 3 time periods (ANOVA, $p < 0.001$; Tukey's HSD, $p < 0.05$). The proportion of hydroxy fatty acids in the upper phase also appeared to decrease over time, but the difference was not significant. Among the 3 detectable hydroxy fatty acids in *P. aeruginosa* (Table 4), 3-OH-10:0 contributed most significantly to the differences in total hydroxy fatty acid.

The proportion of MUFAs gradually declined in the upper layer of the biofilm (Fig. 2B). MUFAs in the lower partition declined between days 2 and 4, then remained stable between days 4 and 6 (Tukey's HSD; $p = 0.004$ and 0.009, days 2 to 4 and days 2 to 6, respectively). Further, isomer-

Fig. 2. Mol% of (A) hydroxy fatty acid (FA), (B) monounsaturated fatty acid (MUFA), (C) *trans*-MUFA, and (D) cyclopropane fatty acid between the shear-removable region and the shear-nonremovable region of 2-, 4-, and 6-day-old *Pseudomonas aeruginosa* biofilm cultures. Asterisks (*) indicate significant difference ($p < 0.05$). Error bars express standard errors.



specific comparison revealed that the proportion of *cis*-MUFAs had a similar pattern as total MUFA over time for both partitions. The proportion of *trans*-MUFAs showed a drastic increase on day 6 in the upper partition but remained the same in the lower partition (Fig. 2C).

A significant difference in the proportion of cyclopropane fatty acids as a function of biofilm age was observed (ANOVA, $p < 0.001$) (Fig. 2D). The proportion of cyclopropane fatty acids increased over time for both partitions. However, the increase in cyclopropane fatty acids appeared to have stabilized by day 4 for the lower shear-nonremovable partition, as no significant change was observed between days 4 and 6.

No significant differences were observed in the proportions of total SAFA either as a function of time or between the 2 layers (ANOVA, $p > 0.05$).

Discussion

Fatty acid profiles of *P. aeruginosa* biofilms remained more stable than batch planktonic cultures over time

There were a total of 16 fatty acids consistently identified in *P. aeruginosa* biofilms throughout our experiments, which is in broad agreement with previously reported findings (Dubois-Brissonnet et al. 2000; Mrozik et al. 2004). Gram-negative bacteria have been shown to respond to limiting nutrients by converting existing MUFAs to cyclopropane fatty acids in membrane phospholipids (Piotrowska-Seget and Mrozik 2003; Kim et al. 2005). Increases in the proportion of *trans*-MUFAs + cyclopropane fatty acids to the *cis*-MUFAs over time suggests that the batch culture experienced resource deprivation, i.e., MUFAs underwent postsynthesis modifications (Denich et al. 2003). The fact that the increase in *trans*-MUFAs was not significant on its own indicates that the cells experienced gradual deprivation up to day 4. For example, Härtig et al. (2005) demonstrated in various *Pseudomonas putida* strains that *cis–trans* isomerization only occurs in cells subjected to abrupt disturbance. *cis* to *trans* isomerization had also been shown to contribute to *P. putida*'s rapid short-term response to withstand toluene stress and its ability to grow at temperatures above the optimal 37 °C (Junker and Ramos 1999). Because *trans*-MUFAs have a higher phase-transition temperature, phospholipids containing *trans*-MUFAs have physical properties that re-

Table 4. *Pseudomonas aeruginosa* fatty acid profile in shear-removable layer (upper sheared) and shear-nonremovable layer over 2, 4, and 6 days.

	Upper sheared (n = 3)			Lower attached (n = 3)		
Fatty acid	2 days	4 days	6 days	2 days	4 days	6 days
11:0	—	—	—	—	—	—
2-OH-10:0	—	—	—	—	—	—
3-OH-10:0	7.69±0.09	7.15±0.39	7.39±0.08	8.19±0.17	8.16±0.31	8.23±0.41
12:0	2.11±0.14	2.07±0.14	2.92±0.32	2.08±0.13	2.13±0.04	2.29±0.14
13:0	—	—	—	—	—	—
2-OH-12:0	7.33±0.08	7.16±0.20	6.53±0.17	7.34±0.18	7.44±0.06	7.53±0.34
3-OH-12:0	3.52±0.36	4.06±0.09	3.55±0.29	3.95±0.13	4.30±0.16	3.92±0.20
14:0	0.77±0.14	0.65±0.09	0.68±0.12	0.53±0.02	0.70±0.17	0.65±0.12
i-15:0	—	0.17±0.17	—	—	—	—
a-15:0	—	—	—	—	—	—
15:0	0.40±0.02	0.42±0.07	0.48±0.06	0.43±0.01	0.58±0.15	0.53±0.10
2-OH-14:0	—	—	—	—	—	—
3-OH-14:0	—	—	—	—	—	—
i-16:0	—	0.27±0.27	—	0.01±0.01	—	—
cis 16:1n-7	17.52±0.61	15.88±0.37	14.37±0.21	15.88±0.20	14.50±0.37	14.21±0.52
trans 16:1n-7	0.25±0.04	0.35±0.06	1.54±0.43	0.52±0.10	0.51±0.08	0.72±0.29
16:0	18.73±0.66	19.20±0.13	19.36±0.05	18.54±0.23	19.48±0.33	19.31±0.32
i-17:0	—	—	—	—	—	—
a-17:0	—	—	—	—	—	—
17:0cyc(9,10)	0.59±0.11	0.86±0.10	1.03±0.02	0.66±0.08	1.17±0.06	1.08±0.06
17:0	0.17±0.02	0.15±0.01	0.16±0.01	0.17±0.01	0.20±0.04	0.19±0.02
2-OH-16:0	—	—	—	—	—	—
3-OH-16:0	—	—	—	—	—	—
i-18:0	—	—	—	—	—	—
18:2n-6	—	—	—	—	—	—
cis 18:1n-9	0.37±0.10	0.26±0.06	0.18±0.05	0.25±0.04	0.33±0.09	0.29±0.04
cis 18:1n-7	39.09±0.28	39.43±0.70	38.82±1.19	40.00±0.49	38.25±0.29	38.57±0.63
trans 18:1n-9	—	—	—	—	—	—
trans 18:1n-7	0.10±0.03	0.17±0.02	0.66±0.17	0.24±0.04	0.25±0.03	0.36±0.15
18:0	0.60±0.19	0.43±0.04	0.37±0.02	0.36±0.01	0.36±0.02	0.39±0.01
i-19:0	0.05±0.05	—	—	0.03±0.03	—	—
a-19:0	—	—	—	—	—	—
19:0cyc(9,10)	—	—	—	—	—	—
19:0cyc(11,12)	0.73±0.16	1.33±0.15	1.80±0.08	0.81±0.10	1.62±0.17	1.74±0.15
19:0	—	—	—	—	—	—
20:0	—	—	—	—	—	—
ΣHydroxy fatty acid	18.54±0.39	18.37±0.65	17.47±0.27	19.48±0.39	19.90±0.48	19.68±0.50
ΣBranched fatty acid	0.05±0.05	0.44±0.44	0.17±0.17	0.04±0.04	—	—
ΣIso	0.05±0.05	0.44±0.44	—	0.04±0.04	—	—
ΣAnteiso	—	—	0.17±0.17	—	—	—
ΣCyclic fatty acid	1.32±0.27	2.19±0.24	2.84±0.08	1.47±0.17	2.79±0.22	2.82±0.13
ΣSAFA	22.77±0.87	22.92±0.35	23.95±0.46	22.11±0.28	23.47±0.63	23.35±0.30
ΣMUFA	57.33±0.76	56.08±0.68	55.58±0.79	56.90±0.59	53.85±0.49	54.15±0.65
Σ*cis*	56.98±0.79	55.56±0.76	53.38±1.16	56.13±0.47	53.08±0.50	53.07±0.50
Σ*trans*	0.35±0.07	0.52±0.08	2.20±0.60	0.77±0.14	0.76±0.11	1.08±0.44

Note: Values are in units of mol% of total fatty acid (± standard error). MUFA, monounsaturated fatty acid; SAFA, saturated fatty acid.

semble those containing SAFAs, i.e., increased rigidity and decreased permeability of the cell membrane (Zhang and Rock 2008).

Based on how the proportion of MUFAs and cyclopropane fatty acids in the batch planktonic *P. aeruginosa* changed, it was reasonable to suspect that the fatty acid constituents were continually compromised over the 4 day period. Thus, to strike a balance between capturing a representative fatty acid profile with the need to obtain sufficient biomass for fatty acid analysis, 2-day-old batch planktonic cultures were selected for comparison with biofilm cultures.

The production of CO_2 had been used to differentiate between various populations within the biofilm community (Bester et al. 2010). Biofilm-derived planktonic cells and shear-removable biofilm layers have been shown to be metabolically less active than exponentially growing batch planktonic cells but more active than the base layer within the

biofilm (Bester et al. 2010). Here, we were interested in determining whether a biofilm community's total fatty acid composition changes as it progress through its growth and development. We found that the fatty acid profile of biofilms reached a comparatively stable state by day 2 and stayed that way over the 6 day period. Bester et al. (2009) demonstrated that *P. aeruginosa* biofilm has the potential to reach steady state within 40 h in terms of planktonic cell yield. It thus appears that despite spatial variation within biofilms, these structures maintain a steady-state over time in terms of form (fatty acid in this study; biofilm thickness and structure as shown in other studies) and function provided that environmental parameters, such as nutrient level and flow rate, remain constant.

SAFA profile indicated less rigid membrane structure in *P. aeruginosa* biofilms

The observed decline in SAFA proportions and the overall decrease in fatty acid chain length of *P. aeruginosa* biofilms imply that their cell membrane properties were more consistent with a less rigid membrane structure. This was rather unexpected, since previous findings in surface-associated biofilm on glass surfaces (Gianotti et al. 2008) and deep subsurface sediment biofilms (Tunlid et al. 1989) both showed a greater proportion of SAFA; a membrane attribute that is thought to provide structural integrity to bacteria in the biofilm.

An example of a decrease in the overall fatty acid chain length and degree of saturation in the bacterial cell membrane has been shown in *Rhodococcus erythropolis*, resulting from exposure to terpenes (de Carvalho and da Fonseca 2007). These modifications in *R. erythropolis*' fatty acids decreased cell hydrophobicity, and this was believed to participate in cell dispersion in this species. Although *Candida parapsilosis* biofilms demonstrated a more flexible membrane property than planktonic cells, it was apparently the result of increased expression of ergosterol (equivalent of cholesterol in animal cells) rather than a function of fatty acid composition (Rossignol et al. 2009).

It is possible that our experimental system designed to cultivate biofilms (i.e., silicon tubes that allow notable diffusion of oxygen from the outside to the base of the biofilm), together with a constant supply of a labile nutrient, results in constant biofilm turnover. In contrast, the higher degree of oxygen depletion that probably occurs in the deeper layers of biofilms grown on gas-impermeable substrates (e.g., glass, metal) would result in a more stratified metabolism and cell viability; information that may be of great relevance to industrial biofilm bioreactor design.

Pseudomonas aeruginosa biofilms maintained a consistent physiological condition in term of fatty acid composition

Cyclopropane fatty acid derives from the addition of a single carbon unit; *S*-adenosyl-L-methionine to existing *cis*-MUFA in the PLFA (Buist and MacLean 1981). Both cyclopropane and *trans*-MUFA derive from postsynthesis modification of *cis*-MUFA and are regarded as mechanisms for bacteria to maintain optimal membrane flexibility and improve membrane PLFA stability, all the while regulating the penetration of undesirable molecules during the stationary phase of batch cultures (Smith et al. 2000; Piotrowska-Seget and Mrozik 2003; Kim et al. 2005; Whittaker et al. 2005).

Compared with the batch planktonic culture, the lower proportion of cyclopropane fatty acids observed in our biofilms and the lack of significant change in the abundance of *trans*-MUFA, suggest that *P. aeruginosa* in a biofilm community was able to maintain a consistent physiology in terms of its fatty acid composition. The bulk of the biofilm remained physiologically active and did not reach a condition similar to the stationary phase of a planktonic culture; a state that is probably maintained in biofilms as long as the external environment does not drastically change. This result therefore cautions against the generalized use of terms such as "young" or "mature" biofilms based on biofilm age without also making specific reference(s) to their physiological condition.

Branched-chain fatty acids in *P. aeruginosa* biofilms

Branched-chain fatty acids in gram-positive bacterial phospholipids have an influence on membrane physiology that is similar to MUFA in gram-negative bacteria (Silbert et al. 1973; Giotis et al. 2007). Although branched-chain fatty acids are rarely found and little is known about their function in gram-negative bacteria, they have been reported in the literature. Moss and Dees (1975) studied the fatty acid composition of various *Pseudomonas* spp. and found that iso-branched fatty acids, specifically i-15:0, accounted for 30%–60% of total fatty acid in *Pseudomonas maltophilia*. Mrozik et al. (2004) also found branched-chain fatty acids in *Pseudomonas stutzer* (0.8%) and *Pseudomonas vescularis* (7.5%); these branched-chain fatty acids, especially iso-branched, increased when the cultures were exposed to naphthalene. A study by Inoue et al. (2008) found that exposure to anteiso-branched a-15:0 repressed the flagella-driven motility of *P. aeruginosa*, with a consequent 31% repression in biofilm formation.

We observed a more prominent presence of branched-chain fatty acids in *P. aeruginosa* biofilms compared with batch planktonic cells. It is possible that branched-chain fatty acids have other roles in biofilm physiology that are yet to be discovered. We suggest that branched-chain fatty acids may repress biofilm formation in *P. aeruginosa* and perhaps participate in the cell release phase of biofilm development. For example, Davies and Marques (2009) have demonstrated the ability of short-chain fatty acid to act as cell-to-cell communication molecules in bacteria and fungi. *cis*-2-Decenoic acid (*cis* 10:1n-8) in nano-molar concentration was able to induce dispersion of biofilm microcolonies for many bacteria species, including *P. aeruginosa*.

Physiological heterogeneity of biofilm cultures

Since the biofilm cultures in this study were originally sampled as a composite, our observed fatty acid profiles constitutes an 'average' of the biofilm as a whole, which may have been dominated by the loosely bound surface-associated cells in the boundary layers. Hence, we performed a further investigation on the fatty acid profile of 2 different partitions within biofilm cultures.

Heterogeneity in biofilm physiology is reflected in the chemical gradient present within biofilms (Stewart and Franklin 2008). The absence of complete exchange with the

environment creates isolated microniches which provide conditions whereby localized populations are subject to physiological adaptation and variation in genetic expression. The physiological heterogeneity in biofilms can be described as active aerobic, active anaerobic, nonactive and (or) dormant but viable and dead cells (Bester et al. 2010). In contrast, well-mixed batch planktonic cultures are generally considered to have homogeneous physiological activities (Stewart and Franklin 2008).

The upper shear-removable layer can contain more than half of the biofilm cells, but the most active cells, in terms of protein synthesis (Werner et al. 2004), DNA replication and respiration (Rani et al. 2007) typically reside just at the biofilm–liquid interface under continuous flow conditions; whereas cells in bulk liquid contributes relatively little to the total activity (only <4%) (Kroukamp and Wolfaardt 2009; Bester et al. 2010).

Biofilm shear-nonremovable layer showed stable and greater proportion of hydroxy fatty acid

A greater proportion of hydroxy fatty acids was observed in the lower shear-nonremovable layer than the upper active layer in our biofilms. Hydroxy fatty acids are an integral part of the lipopolysaccharides in gram-negative bacteria. It is a structural component in the lipid-A structure of the lipopolysaccharide that anchors the polysaccharide to the exterior of the outer cellular membrane. Härtig et al. (2005) found that the proportion of hydroxy fatty acids was slightly lower during the stationary phase of the microbial growth cycle, but the reason for the response was unclear. However, *P. aeruginosa* has the ability to modify its lipopolysaccharide structure (Buchanan et al. 2009). For example, environmental *P. aeruginosa* strains have five 10-carbon fatty acid chains and one 12-carbon fatty acid chain on the lipid-A structure, whereas *P. aeruginosa* isolates from cystic fibrosis patients contained 16-carbon fatty acid chains, and ~48% of isolates from severely ill patients produced 7 fatty acid chains from lipid-A. Addition and (or) elongation of fatty acid chains changes the isoelectric properties of lipid-A and are associated with an increased resistance to specific classes of antibiotics (i.e., cationic antimicrobial peptides). Thus, we speculate that the greater concentrations of hydroxy fatty acid that we observed may potentially be an additional factor that can be correlated to a biofilm's antimicrobial resistance capacity; particularly, for cells deeper within the biofilm matrix.

Rhamnolipid in the extracellular polymeric substance (Soberón-Chávez et al. 2005) and the energy reserve molecule polyhydroxyalkanoic acid (Rojas-Rosas et al. 2007) of *P. aeruginosa* biofilms are alternative sources of 3-hydroxy fatty acid. Large quantities of rhamnolipid and polyhydroxyalkanoic acid can be found in bacteria when nutrients are limited or when a specific essential nutrient is depleted, thereby, halting cell division (Sutherland 1982; Manca et al. 1996; Ayub et al. 2006). Cells in the shear-nonremovable layer may be experiencing an accumulation of polyhydroxyalkanoic acid and (or) rhamnolipid because of limited nutrient exposure that is typically observed with cells located within the biofilm matrix.

Biofilm shear-nonremovable layer showed more rapid loss of MUFA

Since *cis*-MUFA accounted for the majority of the total MUFA in *P. aeruginosa*, it was reasonable for *cis*-MUFA to respond the same as total MUFA for both partitions over time. The proportion of MUFA showed a steep decline in the lower layer that coincided with a sharp increase of cyclopropane fatty acids between days 2 and 4; the proportion of total cyclopropane fatty acid then remained consistent up to day 6. This perhaps is an indication of the ability of the shear-nonremovable portion of biofilms to reach a stable state between days 2 and 4 in terms of fatty acid composition. Our observation showed a limiting range in the proportion of cyclopropane fatty acid necessary for the substrate-associated *P. aeruginosa* to sustain a nonactive and (or) dormant, but viable, state.

Since a relatively small proportion of the biomass is composed of active cells in the upper shear-removable layer (Kroukamp and Wolfaardt 2009; Bester et al. 2010), continual stratification of biofilms over time likely explains the gradual decline of *cis*-MUFA, as opposed to the steep decline observed in the lower shear-nonremovable layer between days 2 and 4. The proportion of total cyclopropane fatty acid in the upper layer (between days 4 and 6) reached a stable state later than the lower layer. In addition, an increase in the proportion of *trans*-MUFA was only observed in the upper layer between days 4 and 6. Since *cis–trans* isomerization has been suggested to be a rapid short-term response in *P. putida* membrane homeostasis (Junker and Ramos 1999; Härtig et al. 2005), the presence of increased *trans*-MUFA in our experiment might be associated with cells in the intermediate layers beneath the active cells beginning to experience nutrient stress. To confirm this speculation, it will be necessary to use a more refined separation method to distinguish the active layer from the rest of the shear-removable biomass.

Conclusions

Through the use of fatty acid analysis to assess the physiological condition of bacteria, we were able to observe the gradual conversion of *cis*-MUFA to *trans*-MUFA and cyclopropane fatty acid in batch planktonic cultures of *P. aeruginosa*. In contrast, the fatty acid profiles of biofilm cultures of *P. aeruginosa* remained relatively consistent. Our results suggest that *P. aeruginosa* planktonic stationary-phase cultures were more stressed than biofilm cultures in terms of nutrient availability (i.e., the fatty acids associated with stress were elevated in the planktonic cultures). Simultaneously, biofilms displayed a relatively less rigid membrane biophysical structure than batch planktonic cells, as evidenced by a lowered proportion of SAFA and an overall decrease in the fatty acid chain length. It is possible that the gas permeability of the silicon tubes used in our experiments permitted sufficient oxygen to penetrate deep into the biofilms, thus reducing the stratification effect. This is an area that deserves further investigation for potential application in biofilm reactors.

There was a consistently greater concentration of hydroxy fatty acid in the shear-nonremovable region than in the active shear-removable region of *P. aeruginosa* biofilm over

time. The shear-nonremovable region also showed a relatively more abrupt decrease in MUFAs that may primarily be attributed to their conversion into cyclopropane fatty acids between days 2 and 4. However, the shear-removable region showed evidence of *cis–trans* conversion along with a gradual increase in the proportion of cyclopropane fatty acids, suggesting that the cells within the shear-removable layer were exposed to a more diverse range of physiological conditions.

Our fatty acid analyses highlighted the physiological heterogeneity that exists between biofilms and planktonic cells and also between 2 different regions within the biofilm. We therefore suggest fatty acid analyses is a useful tool to further investigate the unique physiology, community structure, and development of biofilms.

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