

Apparent Surface Associated Lag Time in Growth of Primary Biofilm Cells

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ABSTRACT

The ability of microorganisms to form biofilms has been well documented. Bacterial cells make a transition from a planktonic state to a sessile state, replicate, and subsequently populate a surface. In this study, organisms that initially colonize a “clean” surface are referred to as “primary” biofilm cells. The progeny of the first generation of sessile cells are known as “secondary” biofilm cells. This study examined the growth of planktonic, primary, and secondary biofilm cells of a green fluorescent protein producing (GFP+) *Pseudomonas aeruginosa* PA01. Biofilm experiments were performed in a parallel plate flow cell reactor with a glass substratum. Individual cells were tracked over time using a confocal scanning laser microscope (CSLM). Primary cells experience a lag in their growth that may be attributed to adapting to a sessile environment or undergoing a phenotypic change. This is referred to as a surface associated lag time. Planktonic and secondary biofilm cells both grew at a faster rate than the primary biofilm cells under the same nutrient conditions.

Introduction

Biofilms are complex communities of microorganisms pervasive in the natural environment. The current conceptual model of a biofilm portrays an ingeniously complicated multi-species entity where ecological microniches are created and occupied by specific organisms [4, 5]. Bacteria are known to attach to abiotic materials and other organisms in all manner of aqueous environments. Following attachment

of the colonizing bacteria, growth of the sessile population and induction of new organisms into the community occurs. The resulting biofilm is an intricate assortment of micro- and macroorganisms [11] that can be responsible for many types of human infections, the corrosion of metals, the reduced efficiency of industrial heat dissipation equipment, and the degradation of waste materials [6].

The first stages of biofilm development, termed “initial events,” have been thoroughly studied but are not well understood. One of the most useful results from research into initial events in biofilm formation has been a conceptual model of biofilm accumulation [10]. The processes involved in the initial events of biofilm formation have been suc-

cinctly summarized by Mueller et al. [23] and include substratum conditioning, transport of cells to the surface, adsorption of the cells to the surface, transformation of these cells from reversible to irreversible adsorption, desorption of reversibly adsorbed cells, growth of irreversibly adsorbed cells, and erosion/detachment of cells resulting from growth.

Although there is a reasonable amount of research being done on all of the above steps in biofilm formation, a great deal of current interest resides in understanding the manner in which reversibly adsorbed cells make the transition to become irreversibly adsorbed. These “parent” organisms form the basis for the ensuing biofilm. Observations on the behavior of these cells have become possible with the advent of advanced microscopy techniques, including confocal scanning laser microscopy, and improved image analysis methods. Based on these methods, it has been found that parent cells can form dense microcolonies and then disperse in a matter of minutes [24]. Upon replication, daughter cells can pack tightly near the parent cell location, spread out upon the surface, be cast back into the bulk fluid, form long chains of cells, or roll along the substratum [8, 16, 17, 26, 27]. The behavior exhibited by these cells is thought to vary according to species [17], although environmental conditions may also play a role.

Direct observation and detailed explanations of the characteristic behavior during initial attachment of bacteria, including *Pseudomonas fluorescens* have been accomplished [19]. Planktonic cells approach the surface and attach. Rotational behavior was observed for up to half an hour on the surface with rotation gradually slowing and ceasing, resulting in a longitudinal irreversible attachment. Rotational behavior was also seen with detaching or emigrating cells prior to moving to a nearby location where the sequence of rotation and attachment was repeated. Following longitudinal attachment, cell division occurred resulting in two daughter cells with a clear space of 0.2 to 0.3 μm . The standard colonization and growth pattern for *P. fluorescens* is adsorption to a surface as single cells, development of 8–16 cell microcolonies, and emigration of the daughter cells to form 8–16 cell microcolonies elsewhere. This type of approach in studying biofilms was emulated in our investigation of a surface associated lag in growth.

To be able to adequately model and understand the growth of biofilms, knowledge of differences and similarities between sessile and planktonic modes of growth is needed. For example, it is extremely convenient to use kinetic expressions developed using planktonic cells to model biofilm growth. One of the first efforts to address differences, or lack

thereof, between sessile and planktonic cells was reported by Bakke et al. [1]. It was shown that *Pseudomonas aeruginosa* behaves similarly in planktonic and biofilm cultures where diffusional resistance is negligible. This conclusion allowed the kinetic and stoichiometric parameters for *P. aeruginosa* derived from planktonic cultures to describe steady-state biofilm processes [1]. However, this straightforward application of planktonic parameters to biofilms is not always appropriate. In one case, the relationship between substrate concentration and growth rate for planktonic cells was not observed for sessile cells [20]. A detailed review of surface and planktonic growth has examined several other contradictory findings where growth of a sessile population deviated from that of a planktonic population. The conclusion was that the lack of consistency is due to a variety of experimental conditions and analytical methods [28]. Clearly, more research is needed in this area.

Inherent in both the change from reversible to irreversible adsorption and the potential disparity in growth rates observed in planktonic and biofilm cells is the concept of phenotypic change. It is now a widely accepted premise that bacteria undergo phenotypic changes when a transition from a planktonic state to a sessile state occurs. The fact that biofilm cells are markedly different from planktonic cells is not trivial, as this may help explain the increased resistance to antibiotics [6] and other morphological changes [8].

The process of becoming a sessile organism may involve a sweeping number of genetic changes that cause the sessile cell to become phenotypically distinct from its planktonic counterpart [5]. It has been demonstrated that adhesion to a surface appears to rapidly initiate the phenotypic expression of a range of genes not used by planktonic organisms [4, 5, 6, 15]. Evidence of this type of behavior was first presented by Dagostino et al. [7]. Using a lacZ reporter system, two marine bacteria were shown to express certain genes upon attachment to surfaces. These genes were not expressed when the cells were in a planktonic state [7]. In many cases, genes that are involved in EPS production are up-regulated after an attachment event [11]. Supporting research has shown that new protein synthesis is required for *P. fluorescens* to form biofilms on abiotic surfaces. The production of these unknown proteins is part of a speculated common genetic pathway used to form biofilms on a range of abiotic surfaces [25].

Specific research in this area has identified a portion of the regulated genes that are being expressed in this phenotypic change. An example is the algC gene, which is a key component in the alginate production pathway in *P. aeru-*

ginosa. Within the span of minutes after a planktonic cell adheres to a surface, this gene is expressed and eventually results in the abundance of alginate, which is a primary constituent of the EPS produced by biofilms of this organism [9]. Expression of these genes may result in a lag in growth, since cells would be directing metabolic energy into EPS, rather than into replication [11].

The reported research addresses two areas of biofilm development: the change from reversibly to irreversibly adsorbed cells and the subsequent growth of the organisms. The data arose from direct, *in situ* observation of biofilm cells in a parallel plate flow cell visualized by confocal laser scanning microscopy. The purpose was to qualitatively compare the growth characteristics of planktonic cells and the resulting primary and secondary biofilm cells. In the experiments described here, *P. aeruginosa* PA01 cells underwent this transition and there appeared to be a temporary halt in their growth and division cycle.

Materials and Methods

Terminology

In the context of this manuscript, *planktonic cells* are those cells that have never existed in an attached mode of growth. *Primary* biofilm cells are those that have made the transition from a planktonic state to a sessile state. The progeny of these organisms are referred to as *secondary* biofilm cells.

Organism

Pseudomonas aeruginosa PA01 is a motile, aerobic, Gram-negative rod. This particular strain contained a plasmid that conveyed a resistance to 150 mg/L⁻¹ of carbenicillin and encoded the production of a green fluorescent protein (GFP). The GFP was constitutively expressed, thus enabling each cell to be "self labeling." The excitation wavelength of the GFP was 488 nm and an emission maximum was at 512 nm. This organism was obtained from Dr. Mike Franklin, Department of Microbiology, Montana State University. Stock cultures of the organism were prepared using R2A agar (Difco) which contained 150 mg/L⁻¹ of carbenicillin (Sigma). The culture was harvested, placed in a 2% peptone–20% glycerol solution, and stored in 2.0-ml vials at –70°C.

Equipment

A parallel plate flow cell reactor was used for primary and secondary biofilm experiments (Fig. 1). The experimental equipment included media and waste containers, assorted tubing, chemostat reactor, parallel plate flow cell reactor, pump, inoculation vessel, bubble trap, and a batch reactor. The nutrient medium contained K₂HPO₄ (0.7 g L⁻¹), KH₂HPO₄ (0.3 g L⁻¹), (NH₄)₂SO₄ (0.1 g L⁻¹),

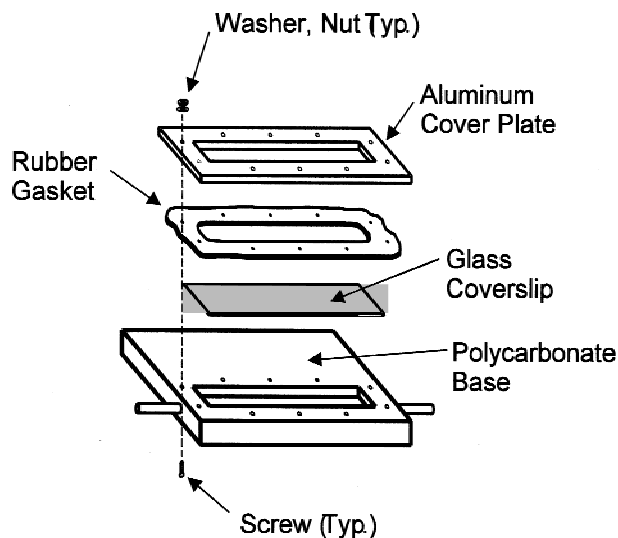


Fig. 1. Schematic diagram of a parallel plate flow cell reactor.

MgSO₄ · 7H₂O (0.01 g L⁻¹), and glucose (0.4 g L⁻¹) and was prepared and sterilized separately from the remainder of equipment. The various vessels and tubing were cleaned by washing with soap and water and sterilized by autoclaving dry for 20 min at 121°C and 20 psi. The tubing upstream from each of the reactors was replaced for each experiment.

Reactors

A chemostat was used to grow inocula for both the flow cell and batch culture experiments. The reactor was inoculated with an aliquot of the frozen stock culture and operated at room temperature with a volume of 500 ml and a 5-h residence time. The culture in the reactor was mixed using a magnetic stir bar and plate and aerated using laboratory pressurized air through bacterial air vents (Gelman Sciences). The bacteria were grown in a chemostat environment for 48 h (approximately 10 residence times) using the medium described above.

A batch reactor was utilized, also with the medium described above, for the planktonic growth experiments. Batch culture experiments were conducted for periods of 6 and 12 h. First 1.0 L of medium was sterilized in a 2.0 L Erlenmeyer flask. An inoculum was withdrawn from a 48-h chemostat culture and placed in the vessel. The batch culture was incubated at room temperature and well mixed using a magnetic stir bar and plate. Population measurements, as detailed later, were made at the beginning, middle, and end of the 6- or 12-h period via direct microscopic cell counts.

Cells growing in a sessile state were observed on the "wet side" of the coverglass in a parallel plate flow cell reactor. Prior to inoculation, the flow cell and all upstream tubing were filled with sterile medium to purge air from the system. Approximately 15 ml of inoculum were withdrawn from a 48-h chemostat culture and placed into the inoculation vessel, upstream of the flow cell. The inoculum was rapidly mixed using a magnetic stir bar and plate. A

portion of the stirred inoculum was pumped through the flow cell for approximately 1 min. After cells had been introduced into the flow cell reactor and attached to the coverglass, the inoculum flow was terminated and flow of sterile, cell-free medium commenced. The flow rate was set at 2.0 ml min^{-1} , which is laminar flow ($Re \sim 5.5$) with flow channel dimensions being $12 \text{ mm} \times 1.5 \text{ mm}$ in cross-section and 37 mm in length.

Data Collection

After inoculation of the flow cell reactor, the adhered cells were subjected to uninterrupted, cell-free nutrient flow for 10 min. Subsequently, a random field of view was chosen to be observed. The flow cell reactor was left in place on the microscope stage and not moved for the duration of the experiment. The “primary” cells in this field of view were then observed using a confocal laser scanning microscope at $1,000\times$ magnification (Leica TCS-NT) every 15 min for a period of 12 h to determine population growth. The fluorescence emitted from the GFP produced by the bacteria was used to locate individual cells. The excitation of the protein was achieved with the 488-nm laser light source. The fluorescent emission maximum at 512 nm, was registered after passing through a BF filter block (500 to 550 nm, cutoff). At each interval, the desired field of view was first checked for focus by eye with transmitted white light. Subsequently, the image was captured using the laser light source. This method greatly reduced the amount of time the sample was subjected to exposure by the laser beam, which is important in ensuring that the exposure did not influence the growth rate of the organisms. Previous work by others has shown that the use of a confocal scanning laser microscope does not impact the growth rate of cells [2]. The pump supplying nutrient flow was temporarily halted to prevent pulsing of the coverglass when an image was taken. Images of “secondary” cells were captured in the same manner; however, the flow cell reactor was subject to uninterrupted flow of medium for 24 h prior to the 12-h observation period.

Data Analysis

Various TIF format images from both batch culture/planktonic cells (cells captured on filter as described below) and flow cell experiments were captured from the confocal microscope. The TIF image files were imported into an image analysis software package, Image-Pro Plus, Version 3.00.00 (Media Cybernetics). The cells were counted by eye after the images were captured. The Image Pro software allowed for the marking of each cell as it was counted, and the software then assigned each marked object an identifying number and the x - y coordinate.

To determine planktonic population growth, direct cell counts were performed at the beginning and end of two 6-h and four 12-h periods using GFP+ cultures. Percent population increase was obtained by removing a sample (10 ml initially, 1 ml at the end of the experiment), vortexing to break up cell clusters, diluting if necessary, and filtering onto the surface of a 25 mm diameter, 0.2 micron

Table 1. Descriptive quantities and formulas^a

Value	Formula
Total number of cells, n_T	$N_0 + n_P$
Base number of cells, N_B	$N_0 - N_{0E}$
% of original cells that emigrate	$\{N_{0E}/N_0\}\%$
% of original cells that divide	$\{N_{0D}/N_0\}\%$
% of original cells that do not divide or detach	$\{N_{0R}/N_0\}\%$
% Surface accumulation increase	$\{n_P/N_0\}\%$
% Population increase	$\{n_P/N_B\}\%$
% Population detach	$\{(n_{PE} + N_{0E})/n_T\}\%$

^a N_0 , original colonizing cells; n_P , progeny cells; N_{0E} , original cells that emigrate; N_{0D} , original cells that divide; N_{PE} , progeny cells that emigrate; N_{0R} , number of original cells

pore size polycarbonate filter (Osmonics). The filters were then placed on microscope slides and the fluorescent cells were examined using the same microscope, light source, and filter settings described previously. From each filter, 12 to 15 separate fields of view were counted to determine the approximate number of cells per filter, from which the number of cells per milliliter in the batch culture was extrapolated. The average beginning and end cell concentrations and corresponding standard errors of the mean were calculated for each sample. Population increase was determined by dividing the change in cell concentration by the beginning concentration.

Determining the growth of a sessile population was more complicated. Each of the cells in the initial field of view, or image, was identified and assigned an object number using image analysis software, as described previously. The original colonizing cells, their fate, and their progeny were tracked over the course of the experiment. Cells were observed to divide, emigrate, or remain in the field of view without replicating. An emigration event is defined as a cell leaving the field of view. This is not necessarily a detachment event because many cells were observed to move under their own power to locations just outside the field of view. Four flow cell reactor experiments were conducted for primary and secondary biofilm cells. Each time, a specific field of view was observed for the duration of the experiment, and individual biofilm cells were tracked. A description of the quantities measured and the formulas used to calculate them are provided in Table 1.

Results

Three types of experiments were performed that dealt with the growth of planktonic, primary biofilm, and secondary biofilm cells. In each experiment, the purpose was to quantify the growth of each population to determine any differences between primary biofilm cells, their progeny, and their planktonic counterparts.

The cell concentrations of the batch cultures, shown in

Table 2. Planktonic growth results

Culture ID	Time (h)	Mean initial concentration (cells ml ⁻¹)	SEM ^a	Mean final concentration (cells ml ⁻¹)	SEM ^a	% population increase	Average % population increase
B1	12	1.40E+04	1.08E+03	1.08E+05	4.78E+03	671.43	
B2	12	No count	N/A	1.44E+05	6.32E+03	N/A	
B3	12	1.31E+04	5.79E+02	1.72E+05	7.23E+03	1212.98	
<i>Average</i>		<i>1.36E+04</i>	<i>8.30E+02</i>	<i>1.41E+05</i>	<i>6.11E+03</i>		
B4	12.17	1.91E+05	7.95E+03	3.13E+06	1.10E+05	1538.74	
B5	12.17	2.25E+05	7.07E+03	No count	N/A	N/A	
B6	12.17	2.30E+05	4.95E+03	1.38E+06	4.47E+04	500.00	
<i>Average</i>		<i>2.15E+05</i>	<i>6.66E+03</i>	<i>2.26E+06</i>	<i>7.74E+04</i>		<i>~12 h</i> 981%
B7	5.95	5.88E+04	1.84E+04	2.08E+05	8.63E+03	253.74	
B8	5.45	9.94E+04	8.60E+03	2.32E+05	1.24E+04	133.40	<i>~6 h</i> 194%

^a SEM, standard error of the mean

Table 2, were measured at the beginning and end of each experiment. The average increase after 12 h was 981%. The percent population increase was determined by:

% increase =

$$(\text{Change in concentration} \div \text{Initial concentration}) \times 100$$

The planktonic populations exhibited an average of nearly 1,000% increase over a 12-h period and roughly a 200% increase in 6 h. The planktonic cells that were used to inoculate the batch reactors originated in a chemostat culture with a residence time of 5 h, which corresponds to a doubling time of 3.5 h. If the cells were to continue to replicate at that rate, the observed population increases are exactly what would be expected. From this information, it is evident that the cells which were transferred from the planktonic state in the chemostat to the planktonic state of a batch culture incurred no significant lag time and continued to replicate without any hindrance at a specific growth rate of 0.2 h⁻¹.

To determine the fate of primary and secondary cells, an initial field of view was chosen and the primary cells identified. These original, individual cells were then observed for the duration of the experiment and their fate was recorded. The description of the data and the calculations have been listed previously; the results are presented in Table 3.

The two key calculations made to illustrate the growth of biofilm cells are percent population change and percent surface coverage change. Invariably, there were cells present in the original field of view that emigrated before the end of the experiment. Cells growing in the attached state

were quite active, as evidenced by many cells moving around on the substratum during the experiment, the total distance moved being in the tens of microns. As a result, a number of the original cells emigrated, leaving the chosen field of view (average of 16.6% emigrated, 76.2% remained; Table 3). Because of this mobility and the associated uncertainty about division if time intervals were excessively long, it was necessary to track individual cells on a 15-min interval to make an accurate judgment concerning their growth. It is unknown whether these emigrating cells divided during the remainder of the observation period. Therefore, the measurement of population increase is determined solely for cells whose fate is known. Surface accumulation increase is a measure of the change in total numbers of cells attached in a field of view and is affected by cell division and emigration. There was no reattachment of cells in any field of view during these experiments of GFP+ cells.

Cells that made the transition from a planktonic state to a sessile state did not behave in the same manner as the planktonic, batch culture grown cells. The primary cells that colonize the surface are the first, or parent, generation of biofilm cells. After initial colonization, there was only a 15.1% increase in surface accumulation, and a 17.4% actual population increase over 12 h (Table 3). Also, an average of only 7.2% of the primary cells divided during the first 12 h after initial colonization. Because such a great proportion of cells exhibited no growth, it was not possible to determine a representative mean division time, which is needed to estimate growth rate.

Table 3. Detailed biofilm growth results^a

PRIMARY Biofilm Culture Results															
Experiment	Time (h)	N ₀	N _{OE}	N _{OD}	N _{OR}	n _P	n _{PE}	n _T	N _B	% original emigrate	% original divide	% original remain	% surface accum.	% pop. increase	% pop. emigrate
P1	8.5	13	3	1	9	1	0	14	10	23	8	69	7.7	10.0	21.4
P2	12.5	46	4	7	35	12	7	58	42	9	15	76	26.1	28.6	19.0
P3	12	95	19	0	76	0	0	95	76	20	0	80	0.0	0.0	20.0
P4	12.5	34	5	2	27	9	5	43	29	15	6	79	26.5	31.0	23.3
Total		186	31	10	147	22	12	210	157	16	5	78	11.7	14.0	20.5
Avg. %										16.6	7.2	76.2	15.1	17.4	20.9
SECONDARY Biofilm Culture Results															
Experiment	Time (h)	N ₀	N _{OE}	N _{OD}	N _{OR}	n _P	n _{PE}	n _T	N _B	% original emigrate	% original divide	% original remain	% surface accum.	% pop. increase	% pop. emigrate
S1	11	24	11	13	0	14	14	38	13	46	54	0	58.3	107.7	65.8
S2	10	4	0	3	1	10	3	14	4	0	75	25	250.0	250.0	21.4
S3	10	12	2	10	0	17	10	29	10	17	83	0	141.7	170.0	41.4
S4	10	8	4	4	0	8	4	16	4	50	50	0	100.0	200.0	50.0
Total		48	17	30	1	49	31	97	31	35	63	2	102.1	158.1	49.5
Avg. %										28.1	65.6	6.3	137.5	181.9	44.6

^a N₀, original colonizing cells; N_{OE}, original cells that emigrate; N_{OD}, original cells that divide; N_{OR}, original cells that remain and neither divide nor emigrate; n_P, progeny cells; n_{PE}, progeny cells that emigrate; n_T, total number of cells; N_B, base number of cells

Once a primary cell replicates, the two daughter cells are known as secondary biofilm cells. These cells are different in one significant way from their parent cells. These bacteria did not undergo a change in environment; they originated in an attached state and did not partition onto the surface from the bulk or planktonic phase. This group of cells did not grow as fast as their planktonic counterparts, but did grow at a greater rate than the primary cells. In approximately a 10-h period, there was a 137.5% increase in surface accumulation and a 181.9% population increase. The mean specific growth rate was 0.1 h⁻¹.

Comparing the patterns shown by primary and secondary biofilm cells, it is evident that the secondary cells are replicating at a significantly faster rate. Only 7.2% of primary cells replicated during the 12-h observation period compared to 65.6% of secondary cells. The secondary biofilm cells are also more active than the primary cells; 76% of primary cells neither divided nor emigrated, compared with only 6% of secondary cells. In addition, twice the total population of secondary cells emigrated as compared to the primary colonizers (44.6 vs 20.9%, respectively).

Discussion

The perceived model of biofilm formation includes the attachment of planktonic (primary) cells and their physiologi-

cal transition, resulting in a lag in growth. Following the transition to a biofilm phenotype (secondary cells) growth resumes and the biofilm accumulates. Depending on the source of the colonizing cells, it is possible that the biofilm phenotype is already being expressed; this may occur if the attaching cells are recently detached biofilm organisms. Another potential phenomenon is the reversion of attached biofilm cells to the planktonic phenotype to facilitate detachment. The research reported herein was designed to address the concept that a lag phase occurs upon initial attachment of truly planktonic cells, and this lag phase is indicative of a physiological change.

The term planktonic has historically referred to cells that are suspended in solution and not attached to a surface or neighboring cells. A sessile or biofilm organism has been defined as an organism that is attached to a surface and not suspended in a liquid culture. In recent years, the distinction between the terms has come to imply not only environmental differences, but phenotypic differences as well [4, 5, 6, 11, 15]. An underlying tenet of our research is that a change in phenotype between the planktonic and biofilm states does not occur instantaneously. Accordingly, a detached biofilm cell that is suddenly entrained in the bulk fluid is an organism in a planktonic environment, but temporarily expressing a biofilm phenotype, provided that the detachment event is not a function of down-regulation of biofilm phenotype genes.

One mechanism for determining whether cells undergo a change in phenotype upon initial attachment is to compare the growth rates of cells before and immediately after attachment and at subsequent time intervals. Comparisons between the growth of planktonic and biofilm cells have been made previously [1, 8, 12, 13, 14, 20, 21, 28, 29]. The results of these studies vary, indicating that planktonic cells can grow faster, slower, or at the same rate as biofilm cells. It is probable that the variability in results stems from whether or not the colonizing cells are from a truly planktonic source or from detached biofilm cells still expressing the biofilm phenotype. For example, Bakke et al. proposed that growth rates of *Pseudomonas aeruginosa* biofilm cells and planktonic cells are the same provided the microenvironment of the cells is the same [1]. If this is true, the kinetic parameters determined for a planktonic culture could be extrapolated to thin biofilms where diffusion limitation is negligible [25]. This simplification is not universally true, as evidenced in our research: where the biofilms are only a few microns thick and diffusion limitations are not present, growth rate differed with time after attachment.

There are other cases where organisms do not exhibit a lag in replication after initial attachment [14, 16, 17]. These results would appear to contradict the findings of our work. However, inocula in these other studies likely consisted of biofilm phenotype cells rather than cells expressing a purely planktonic phenotype. For example, Lawrence and Caldwell [17] utilized a river water inoculum, which most likely contained detached biofilm cells. In another study [3] where no difference in growth rate was detected, the source of the inoculum for the system is not defined. However, reference is made to batch cultures that used glass beads as a surface for attachment for the organism used in the experiments. In both of the above cases, it is probable that detached biofilm cells expressing the biofilm phenotype were used as the inoculum; no lag time would be expected because no change in phenotype would be required.

There are also instances where chemostats were used to provide the inocula for flow cell experiments where cell behavior and growth rate were observed. In these cases, it is more likely that cells would truly be expressing a planktonic phenotype provided that the surface area to volume ratio and dilution rate of the chemostat favors planktonic over biofilm growth. In this case, any adherence and subsequent growth on a surface would necessitate a change to a biofilm phenotype. Mueller studied the behavior of sessile cells on various surfaces, with the inoculum being grown in a large chemostat [22, 23]. The flow cell reactor system used in his

studies is operated in a similar manner to the one described herein and the organism is the same species but a different strain: *Pseudomonas aeruginosa* ERC. Mueller does not specifically discuss the aspect of a lag time but the data presented indicate the occurrence of such an event. In the only other documented case where a chemostat is used, there is no noted lag phase after attachment [18], but no information is given on the manner in which the chemostat is constructed, and the dilution rate is given as 0.693 h^{-1} or a doubling time of 1 h. No information is given for the maximum specific growth rate of this organism on the carbon source used (glucose), but it is likely that at this dilution rate, a substantial biofilm population was favored. Therefore, it is possible that the inoculum for these attached cell experiments may have been detached biofilm. Regardless, these data indicate the critical need for understanding the nature of the inoculum used to generate the initial biofilm.

The results of this work indicate that when *Pseudomonas aeruginosa* PA01 cells make the transition from a planktonic to a sessile environment, there is a lag time in growth. This observation is consistent with the concept that these cells undergo a phenotypic change. The change could be substantial and the amount of time to initiate these changes significant if the number of genes being up- or down-regulated is as large as has been postulated [6]. The end product of several of these genes may be extracellular materials [9], indicating that a portion of metabolic energy is required for this task at the expense of creating new cells. Overall, the results of this research are consistent with the current conceptual model of biofilm formation where it has been postulated that there is a distinct phenotypic difference between biofilm and planktonic cells.

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