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Filamentous growth of *Pseudomonas aeruginosa*

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SUMMARY

The growth of two strains of *Pseudomonas aeruginosa* in stirred batch cultures was monitored by optical density, DNA concentration, and acridine orange direct cell count measurements. Growth of adherent bacteria in pure culture was also observed on suspended glass discs by light and scanning electron microscopy. Strain MUCOID produced significant numbers of filamentous cells in broth culture and in the adherent population, while strain PAO 381 did not produce elongated cells. Filamentous growth of MUCOID could be prevented by the addition of 5×10^{-2} M Mg^{2+}. However, the addition of 0.66 mM EDTA caused an increased proportion of the population (> 50%) of MUCOID cells to become filamentous in broth culture. The results are discussed and related to theories regarding bacterial plasticity, and filamentation of normally bacillary cells.

INTRODUCTION

Bacillary (rod-shaped) bacteria in adherent populations often grow into long filamentous cells. This phenomenon has been described in laboratory experiments by observing *Pseudomonas* sp. [13], *Klebsiella* sp., *Enterobacter* sp., *Acinetobacter* sp. [20], and *Sphaerotilus natans* [14]. The presence of filamentous bacteria in well-developed natural biofilms has also been reported (e.g., Refs. 1, 2, 17 and 21). Filamentous bacteria in biofilms have been demonstrated to influence energy-loss processes (e.g., heat transfer resistance and fluid frictional resistance) in tubular systems [13,16] and the relationship between cell morphology and an actual energy loss in tubular flow systems has been established [12,13].

Many physical and chemical agents can render bacterial cells incapable of dividing. Cell elongation can be induced in batch cultures of growing bacteria by a low dose of a growth-inhibiting agent (e.g., antibiotics, antimetabolites or disinfectants), rapid increases of growth rate, and exposure to high or low temperatures or ultraviolet light. This report describes a case in which a regularly bacillary bacterial species produced filaments in batch cultures as well as in a biofilm attached to suspended glass discs. Filamentous growth was influenced by the concentration of available magnesium ion in the media.

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MATERIALS AND METHODS

Bacteria

Two strains of *Pseudomonas aeruginosa* were grown in pure cultures in nutrient broth (NB). The cultures were in 250 ml of medium (in 500 ml Erlenmeyer flasks), constantly stirred at 22°C. The two laboratory strains of *P. aeruginosa* used were MUCOID (observed to produce filaments) and PAO 381 (filaments never observed).

Assays

Samples of the culture fluid were regularly taken over the duration of the experiments (24–35 h) and the optical density (OD), DNA concentration, and cell numbers were determined.

The optical density of each culture was measured at a wavelength of 600 nm using a Spectronic 20 spectrophotometer.

The concentration of DNA was determined with the fluorochrome compound Hoescht 33258 (Calbiochem, La Jolla, CA). Two milliliters of culture were filtered on to a 0.45 μm Millipore filter, resuspended in a phosphate saline solution (0.05 M Na_2HPO_4, 0.05 M NaH_2PO_4, 2.0 M NaCl; pH 7.4), sonicated to disrupt the cells, and then the fluorochrome was added to a final concentration of 0.1 mg of Hoescht 33258 per ml of sample. The concentration of DNA was determined in relation to *Escherichia coli* DNA (Sigma) standard solutions in an Aminco spectrophotofluorometer (excitation λ, 356 nm; emission λ, 458 nm), after the manner of Labarca and Paigen [8].

Acridine orange (AO) direct cell counts were made to determine the concentration of cells in the culture fluids. Specimens for epifluorescent microscopy were stored in 0.5% glutaraldehyde (in 0.1 M cacodylate buffer). One milliliter of the glutaraldehyde-fixed sample was filtered through a 25 mm Nucleopore filter (0.2 μm) which was then covered with 0.01% AO (in 0.1 M phosphate buffer, pH 7.5) for 2 min. The solution of AO was then pulled through the filter and the specimen was rinsed with isopropanol, using mild vacuum filtration. The stained bacteria were observed and counted under a Zeiss Standard 16 microscope. The microscopy and counting procedures were as detailed by Costerton [5]. The number of filamentous cells and the cell lengths were also determined by AO epifluorescence microscopy. Ten randomly selected areas of each stained filter (prepared as above) were selected and the cell lengths were determined using a calibrated ocular micrometer. Filamentous cells were expressed as a percentage of the total number of cells on each filter.

Modified Robbins device

The modified Robbins device, shown in Fig. 1, was developed as an artificial multiport sampling device and was described in detail by Nickel et al. [15]. The device was fitted with glass discs which could be mounted flush with the inner surface of the device so as not to disturb the flow characteristics of the culture fluid passing through the inner chamber. The glass discs to which the biofilm adhered could be removed and/or replaced aseptically.

Scanning electron microscopy

The glass discs were placed in a fixative solution consisting of 5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) for 1 h at room temperature, followed by dehydration in a series of aqueous ethanol solutions (20–100%) and alcoholic freon 113 solutions (30–100%) and then air-dried. Samples were sputter-coated with gold and examined using a Hitachi 5456 scanning electron microscope.

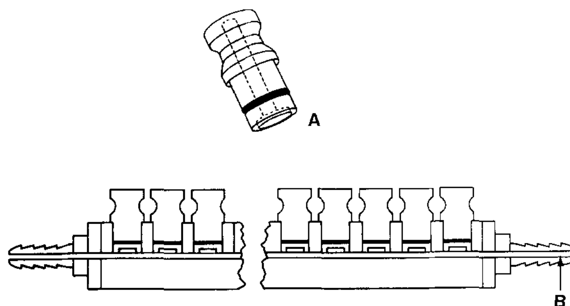

Fig. 1. Modified Robbins device showing the sample plug to which the glass discs were attached (A) and the fluid flow chamber (B).

Transmission electron microscopy

Bacteria were fixed in 5% glutaraldehyde (0.1 M, pH 7.2) for 2 h at room temperature. The material was then washed five times in the buffer, post-fixed with 2% OsO_4 in buffer, washed five more times with the buffer, and dehydrated through a series of acetone (30–100%) washes. After further dehydration in propylene oxide, the specimens were embedded in Spurr [19] low-viscosity embedding resin, sectioned, stained with uranyl acetate and lead citrate, reinforced with evaporated carbon and examined with a Hitachi 600 transmission electron microscope at an accelerating voltage of 50 kV.

Media

Nutrient broth (NB; Difco) was prepared according to package directions. NB was supplemented with Mg^{2+} (5×10^{-2} M $MgSO_4$), glucose (5 mM), and/or EDTA (0.66 mM) in certain experiments. A minimal medium (M9) was also employed, consisting of 22.4 mM Na_2HPO_4, 8.5 mM NaCl, 18.5 mM NH_4Cl and 0.2% glucose.

RESULTS

Filamentous cells were defined as those which were 6 μm or longer, twice the normal length of *P. aeruginosa* rods [4]. The filamentous cells were aseptate as evidenced in the transmission and scanning electron micrographs (Figs. 2 and 3). The MUCOID strain produced filaments under some experimental conditions whereas strain PAO 381 was never observed to do so.

The growth characteristics of the MUCOID and PAO 381 strains differed in at least one respect. The growth curves of the two strains were similar when increases in their respective changes of DNA concentrations over time were measured (Fig. 4) but differed when the increases in cell numbers were monitored (Fig. 5). The MUCOID strain showed a sharp decrease in the growth rate, measured by cell number, at about the time that filaments were seen. This apparent downshift was not noticed when the growth of the MUCOID strain was monitored by optical density. This can be explained by

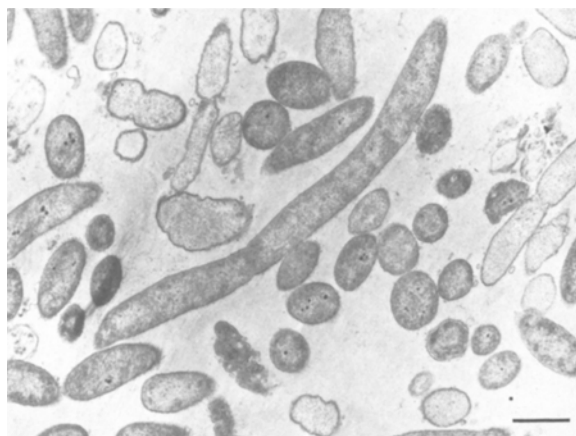

Fig. 2. Transmission electron micrograph of a thin section of *P. aeruginosa* strain MUCOID (bar = 1 μm). Note the length of the cells and the lack of septa.

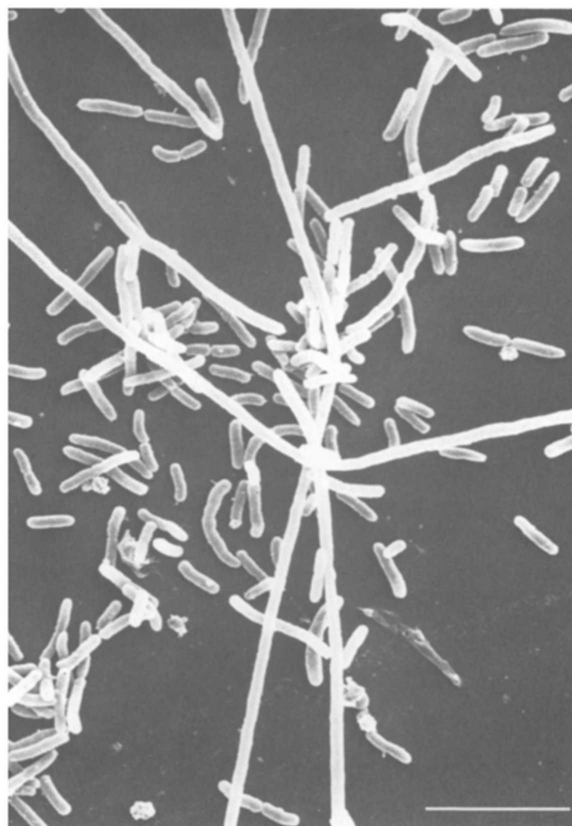

Fig. 3. Scanning electron micrograph of filaments of *P. aeruginosa* strain MUCOID attached to a glass disc (bar = 5 μm).

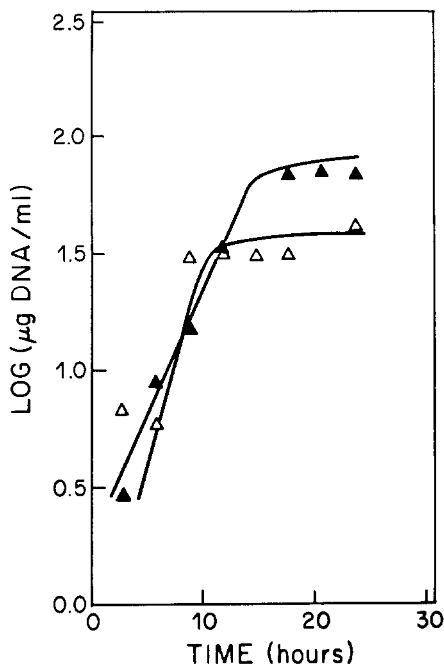

Fig. 4. Growth curves (DNA vs. time) of NB batch cultures of *P. aeruginosa* strains MUCOID (▲) and PAO 381 (△).

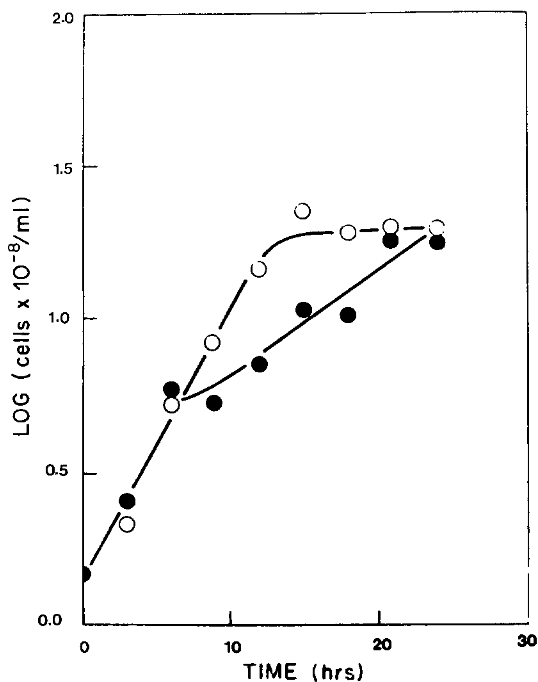

Fig. 5. Growth curves (cell number vs. time) of NB batch cultures of *P. aeruginosa* strains MUCOID (●) and PAO 381 (○).

noting that the MUCOID culture was increasing in turbidity, in part, by extending bacillary cells, as well as by increasing cell number, as opposed to dividing with the resultant increase in OD solely due to increased cell numbers. Apparently, DNA replication was continuing at a constant rate, suggesting that multiple copies of the genome were present in each filamentous cell.

When filaments were intitially observed in a broth culture, it was suggested that the bacteria were experiencing a microaerophilic environment due to the rapid growth of the culture and that this affected cell division. To preclude this possibility, air was bubbled vigorously through the culture using an Elite 800 fish tank pump (Rolf C. Hagen Corp., Mansfield, MA). This procedure had no effect on the proportion of filaments seen in the culture (data not shown) Another nutrient, glucose, was also suspected of playing a potential role in influencing filamentation. However, supplemental glucose (5 mM) added to the nutrient broth or added to a minimal medium (Fig. 6) failed to influence the percentage of filaments (Table 1).

Fig. 7 shows the growth curves which were obtained when the MUCOID strain was grown in NB and in NB supplemented with 5×10^{-2} M Mg^{2+}.

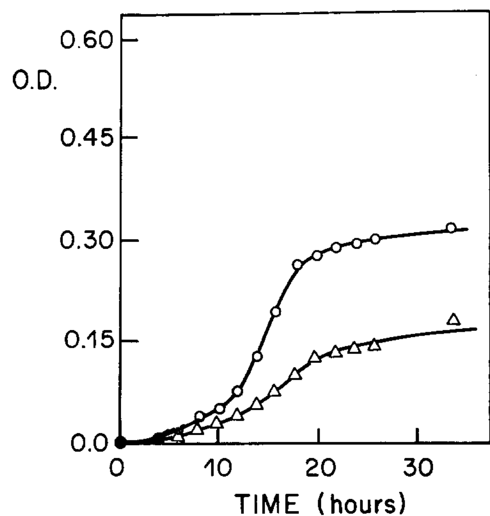

Fig. 6. Growth curves (OD vs. time) of batch cultures of *P. aeruginosa* strain MUCOID in M9 medium + glucose (○) and NB + EDTA (△).

Table 1

Filamentous growth of *P. aeruginosa* strain MUCOID in aerobic broth cultures

Time (h)	% Filamentous cells				
	NB	glucose[a]	Mg^{2+} [a]	EDTA[a]	M9 + glucose
0	0	0	0	0	0
2.5	0	0	0	0	0
6	0	0	0	0	0
8	0.1	0.1	0	0	1.1
10	1.0	1.9	0	0.3	3.1
12	1.9	3.4	0	0.4	4.4
14	2.4	2.4	0	0.4	2.4
16	2.8	2.5	0	3.9	6.3
18	2.8	2.3	0	6.0	3.9
20	1.5	1.6	0	11.9	3.8
22	0.8	1.2	0.1	36.8	3.4
24	0.2	0.2	0.1	52.6	1.4
25.5	0.2	0.2	0.1	41.5	0.1
34	0	0	0	21.2	0.7

[a] Represents supplement to nutrient broth (NB).

The changes of the OD of the two cultures are quite similar. However, Table 1 shows that the increased Mg^{2+} concentration in the culture resulted in the proportion of filaments being reduced, essentially to zero. When EDTA (0.66 mM) was added to the culture media with the resulting decrease in the growth rate, as indicated by OD (Fig. 6), the proportion of filamentous cells increased to a maximum of 52.6% of the cells (Table 1). EDTA added to NB in which strain PAO 381 was grown did not slow the growth rate as drastically, nor were there any filamentous cells observed (data not shown). From these data it was apparent that magnesium ion must, in some way, affect the growth of the MUCOID strain cells. Moreover, as shown in Table 1, filaments which were never seen in lag phase emerged and reached their greatest proportion in exponential phase, and diminished in frequency, to 0% in some cases, in stationary phase cultures.

In the biofilm studies, cultures were pumped from a continuous culture vessel through the modi-

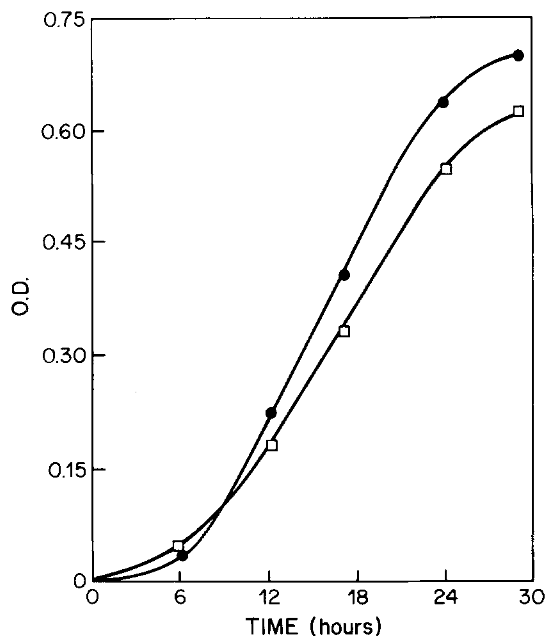

Fig. 7. Growth curves (OD vs. time) of batch cultures of *P. aeruginosa* strain MUCOID in NB (□) and NB + Mg^{2+} (●).

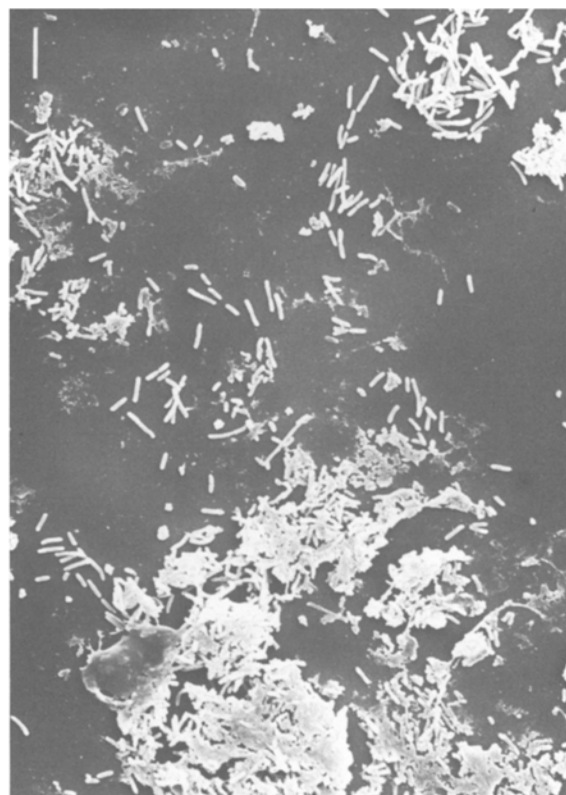

Fig. 8. Scanning electron micrograph of *P. aeruginosa* strain MUCOID attached to a glass disc. Note the condensed glycocalyx surrounding the cells. (Bar = 5 μm).

fied Robbins device at a rate of 50 ml/h, with fresh medium replenishing the culture vessel at the same rate. The biofilm which developed on the glass discs contained large numbers of filamentous bacterial cells (Fig. 3). The filaments took significantly longer to develop on the surface of the glass discs than they did in broth culture (19 h vs. < 10 h). The filaments on the glass discs were frequently associated with large amounts of glycocalyx which surrounded the adherent bacteria prior to significant numbers of filaments being observed (Fig. 8).

DISCUSSION

Filamentous growth of *P. aeruginosa* can be considered as cell growth without normal cell division. This effect can be mediated by a variety of external agents [9,17]. In this report, we present a case in which a normally bacillary organism grew filamentously under conditions of magnesium depletion. This report joins with a report by Jensen and Woolfolk [7] in demonstrating that a number of *Pseudomonas* spp. are able to grow filamentously under certain conditions. Jensen and Woolfolk demonstrated that *P. putida* isolated from caffeine and environmental isolates of *P. fluorescens* grew filamentously when oxygen became a limiting factor and that, by increasing the aeration of the culture, filamentation could be suppressed, unlike our findings with the MUCOID strain of *P. aeruginosa*. However, similar to our results, they noted that supplemental Mg^{2+} could prevent filamentation whereas added EDTA enhanced filamentation of their strains of *Pseudomonas*. Jensen and Woolfolk [7] were unable to induce filamentation of their strains of *P. aeruginosa* under any of the conditions employed.

It was of interest that filaments were absent during the lag phase (Table 1) under all conditions tested, as noted by Jensen and Woolfolk [7]. Rapid growth and low Mg^{2+} concentrations appeared to be requirements, in the systems tested, for the growth of filamentous bacteria. The requirement for active growth was apparent when it was noted that the formation of filamentous cells ceased, and the already formed filaments began to fragment (Table 1) when the bacteria ceased rapid growth and entered stationary phase. The culture then returned to a normal, bacillary population.

An idea presently receiving increased attention is that bacteria have what may be termed a 'plasticity spectrum'. This means, essentially, that there is a range of biochemical [3] and morphological [2, 9] characteristics which bacteria may exhibit, depending on their growth conditions, i.e., sessile, planktonic, continuous culture, iron-limited, etc. It has been documented that a 'lack of essential nutrients for a growing culture results in dramatic changes in general biochemistry and in envelope structure' which are characteristic of a specific nutrient [3]. The data presented in this report add evidence that cellular morphology may likewise be affected by the growth conditions.

Many environmental changes affect the response of a phenotypically plastic bacterial cell. One of these conditions may be a change in the growth rate of the bacteria – a less recognized but important factor in the phenotypic response of bacteria [3]. Brown and Williams [3] hypothesized that responses to growth rate are linked to the various mechanisms which bacteria employ to enhance their survival, depending on the environmental stresses causing changes in the growth rate.

When the MUCOID strain was growing in a flowing system and allowed to colonize the surface of glass discs in the modified Robbins device, we see that a significant proportion of the cells became filamentous. This may be explained, in part, by the fact that the surface-attached bacteria are seen to be surrounded by a mass of anionic glycocalyx (Fig. 8). The glycocalyx has been suggested to be of importance to surface-attached bacteria [6] and may serve as a method through which sessile bacteria are able to concentrate nutrients as they flow past the organisms. In this instance, the glycocalyx may be acting to trap the cationic magnesium ions, preventing them from reaching the cells as rapidly as would have been the case if the cells did not have this extracellular covering. Thus the sessile bacteria, while still rapidly growing, were experiencing a depletion of magnesium.

The growth rate, as measured by cell numbers, for the MUCOID strain batch culture demonstrated a reduction at the time that the proportion of filaments increased (Fig. 5, Table 1), an observation not seen in the DNA curve (Fig. 4), OD curve (Fig. 7), or the cell number curve for strain PAO 381 (Fig. 5), suggesting that cells of the MUCOID strain are elongating while not concomitantly slowing the production of cellular components. The reduction in the rate of increase of cell numbers occurred at the time at which we hypothesize that the Mg^{2+} level is sufficiently depleted for this particular strain to be induced to form filaments. The change from bacilli to filaments in both cases (sessile and planktonic bacteria) may be in response to an effort to increase the surface area of the cells in an attempt to sequester greater amounts of Mg^{2+}. This is particularly true of the sessile cells, which may become filamentous in order to extend into the menstruum to be exposed to potentially larger amounts of Mg^{2+}. The lack of septation in the filaments indicates that Mg^{2+} may be limiting in some enzyme system(s) involved in septation. The lack of filaments in lag and stationary phases may simply reflect that slowly growing cells do not require as much rapidly available Mg^{2+}, and therefore do not form elongated cells.

It is important to note that the plasticity theory suggests that different genomes respond differently to different environmental and growth conditions. Thus, in the case of a specific genome, MUCOID, the cells respond to a decrease in the amount of available Mg^{2+} by a resultant change in the cellular morphology. Under the same conditions, a different genome, PAO 381, of the same species does not respond in a similar manner. In natural settings, we would therefore expect the, often observed, wide range of morphologies due in part to responses of different genomes to existing environmental conditions.

The development of natural bacterial populations on submerged surfaces is usually described as a succession of genera beginning with bacillary cells that are adept at attachment and rapid growth, then proceeding to include filamentous genera. The bacteria in industrial cooling tower biofilms [11] and biofilms on engineered surfaces exposed to well-aerated natural waters are always pseudomonads. At the same time, the presence of filamentous bacteria in these biofilms is common [12,13,19]. The succession from bacillary to filamentous cells, using as inoculum a pure culture of *P. aeruginosa* in a model tubular flow system, has also been observed [12]. The present data help to establish that filamentous growth of bacillary bacteria can be demonstrated in laboratory pure culture experiments without the use of an external agent that inhibits cell division. We propose that although bacterial succession on surfaces in natural settings undoubtedly occurs, the filamentous biofilm population is partly the result of growth without division by bacteria described as bacillary under pure culture, nutrient-rich conditions. We emphasize that filamentous biofilm populations play a key role in the energy losses associated with many fouling biofilms [13]. In particular, bacterial genera that initially colonize natural and engineered surfaces in aquatic environs (e.g., *Pseudomonas*) contribute significantly to these filamentous biofilms. The observation of progression of genera from bacillary cells to filamentous cells on the surfaces of flow systems, such as heat exchangers, may not reflect solely a change of genera but also the response of certain bacterial genomes to different environmental stresses.

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