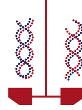

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Assessment of change in biofilm architecture by nutrient concentration using a multichannel microdevice flow system

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A new multichannel microdevice flow system with stainless steel flow chamber was used for architecture visualization, development monitoring and structural quantification of GFP-labeled *Pseudomonas aeruginosa* PAO1 live biofilms. Direct in situ investigations using confocal laser scanning microscopy (CLSM) at 72 h revealed structural pattern differences as a result of nutrient concentration gradients. When grown in LB medium, round, dispersed cellular aggregates were formed whereas in 1/3-diluted LB medium, biofilms were mostly flat and compact. However, COMSTAT analyses showed no considerable differences in biomass and thickness between the two LB concentrations. Characterization of time-dependent development of biofilms grown in 1/3-diluted LB medium showed full maturation of colonies by 120 h reaching maximum biomass at 17.1 μm^3/μm^2 and average thickness at 44.4 μm. Consequent thinning and formation of openings through interior in colonies occurred by 168 h. These results suggest that the new system tested allowed a fast and thick biofilm development on the surface of the stainless steel flow chamber. These findings may provide better estimates of biofilm activity and systematic evaluation of the effects of different parameters on biofilm morphology and development in industrial and biomedical systems.

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[**Key words:** Biofilm architecture; Flow system; Multichannel microdevice; Confocal laser scanning microscopy; *Pseudomonas aeruginosa*]

Biofilms are sessile microbial communities embedded in a self-produced extracellular polymeric matrix (1). They are reported to exhibit a high degree of resistance to antimicrobial agents, host immune system, and environmental stresses (2–4). Medically important biofilms are responsible for many chronic, nosocomial, and device-related infections (3,5). In industries, biofilms cause biofouling, biocorrosion, product contamination, and act as pathogen reservoirs that result in considerable loss of productivity (2,3). Hence, many of the biofilm studies target their prevention, control, and removal from adhesion sites (6,7). Although several devices and techniques such as flow cells, capillary tubes, glass cover slides, and microtiter plate-based assays are available for generation and study of in vitro biofilms (7,8), there are disadvantages in their uses. Some of their respective limitations include incompatibility with large-scale screening, fastidious biofilm growth protocols, high costs, limited capacities for high-resolution structural biofilm characterization, and time-consuming ex-situ preparation that may disrupt native biofilm structures (8). Biofilm growth and development on glass substratum of conventional flow cells may not also accurately represent biofilms in most medical or industrial environments (8).

Due to a complex interaction between external and internal processes, biofilm architecture or pattern formation is heterogenous and unique among different biofilms (9). Motility patterns in *Pseudomonas aeruginosa* biofilms have been shown to be dependent on nutritional conditions. With glucose as carbon source, the attached *P. aeruginosa* differentiate initially into non-motile and motile subpopulations that eventually form mushroom-shaped multicellular structures (10,11). The non-motile subpopulation with repressed twitching motility becomes the stalk of the mushroom, forming a platform for the motile twitching subpopulation to ascend and form the mushroom cap. In contrast, citrate-grown biofilms are in hypermotile state during the initial phase of biofilm formation and thus do not form stalks but form flat, homogeneous biofilms (10,12). Under denitrifying conditions, *P. aeruginosa* PAO1 form mostly filamentous cells in three-dimensional mesh-like structures with channels or spaces in between large macrocolonies (13).

To address the challenge of developing a portable, reusable device whose surface material resembles most biofilm environments and at the same time allow non-destructive, high-resolution characterization of live biofilm structures and development, we designed a new "multichannel microdevice flow system" with a nine-channel stainless steel flow chamber. Combined with the use of confocal laser scanning microscopy (CLSM) and COMSTAT (14), our protocol and design allowed rapid, multiple, parallel screening and analysis of three-dimensional structures and

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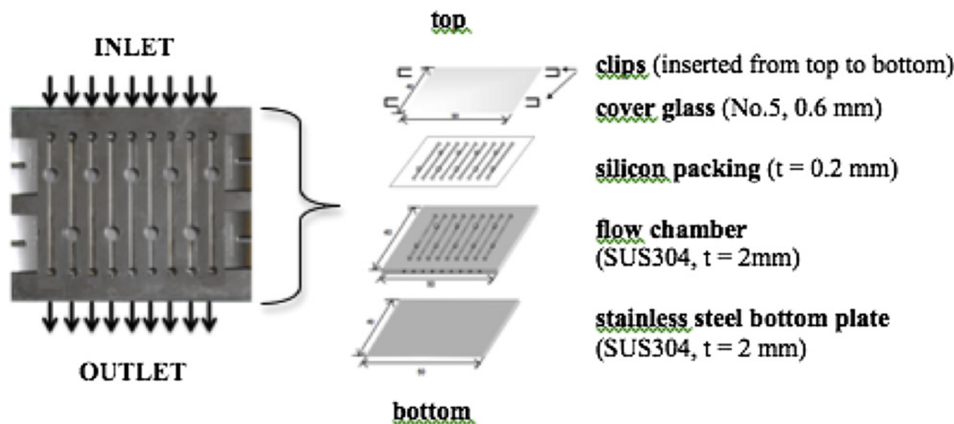

FIG. 1. The assembled set-up (left) and individual components (right) of the multichannel microdevice. The stainless steel flow chamber, with 9 parallel channels and stainless steel bottom plate, are covered on top with transparent silicon packing and cover glass for imaging with any type of microscope. Each channel in the flow chamber, which is 30 mm long, 1 mm wide, and 1 mm deep, has an inlet and outlet on opposite ends where culture medium is passed. The whole assembly is secured by clips. Size: 40 × 50 × 4 mm.

development of *P. aeruginosa* PAO1 biofilms. Differences in biofilm architecture of *P. aeruginosa* PAO1 induced by nutrient rich and diluted growth media were determined. Considering that the multichannel microdevice simulate surface environments in food processing plants, medical devices, and engineered systems where biofilms grow and cause contaminations and infections (15–17), we propose here its suitability for medical and industrial biofilm experiments targeted for growing, monitoring, and eradicating biofilms.

MATERIALS AND METHODS

Bacterial strain and culture conditions *P. aeruginosa* strain PAO1 containing the GFP plasmid pMRP9-1 (18) was used in this study. Overnight cultures of *P. aeruginosa* PAO1 were grown in Luria-Bertani (LB) medium supplemented with 300 μg/ml carbenicillin, with 13 h shaking at 28°C. A 50-μl volume of overnight culture was transferred to 5 ml fresh LB medium with carbenicillin and subjected to 4 h shaking at 28°C. A 1-ml sample of this culture with a final optical density at 600 nm of 0.6 was added to 9 ml of LB medium for inoculation in the multichannel microdevice. For flow experiments, biofilms were supplemented with LB and 1/3-diluted LB media (Bacto tryptone 3.3 g/l, Bacto yeast extract 1.6 g/l, and NaCl 5 g/l).

Multichannel microdevice flow system *P. aeruginosa* PAO1 was grown on a multichannel microdevice (developed in this laboratory and Kyowa Fine Tech. Co., Ltd., Japan) at 28°C. The components of the microdevice, as shown in Fig. 1, include a central stainless steel flow chamber consisting of 9 parallel channels, each channel measuring 30 mm long × 1 mm wide × 1 mm deep. Each channel has an inlet and outlet with a diameter of 1 mm. The hole area in each channel, with a depth of 2 mm, is intended for diffusion experiments and is not used in the current study.

Biofilm cultivation The structural formation of *P. aeruginosa* PAO1 biofilm in the microdevice supplied with different concentrations of LB medium was investigated. Three experiments were conducted and in each experiment, two channels were used for flow of LB medium and two channels for flow of 1/3-diluted LB medium.

The microdevice was soaked in deionized water and autoclaved at 121°C for 15 min. A 1-ml volume of inoculum was injected into each of the four channels and left for 1 h to allow attachment of *P. aeruginosa* PAO1 to the stainless steel substratum. Medium was supplied with the multichannel pump (IPC8, ISMATEC, Germany) via Tygon tubings at a flow rate of 11 ml/h. The effluents were collected in 1 l flasks. Fig. 2 shows the multichannel microdevice flow system.

To characterize time-dependent biofilm development, three independent experiments were performed with the same protocol described above. *P. aeruginosa* PAO1 was grown in two channels continuously pumped with 1/3-diluted LB medium for 7 days.

Confocal laser scanning microscopy (CLSM) and image acquisition To assess the structure and development of *P. aeruginosa* PAO1 biofilms grown in the microdevice, microscopic observations and image acquisitions were done using the Olympus CLSM FV 1000D (Olympus, Tokyo, Japan). Images were obtained using 20x/0.75NA. Three-dimensional images and optical z-sections were generated using the Olympus LCSM FV 1000D software. For microscopic observations of biofilms grown in different concentrations of LB medium, 7 image stacks were acquired from each of the four channels at 72 h in each experiment and collected at 1-μm intervals in z-section. A total of 42 image stacks were analyzed for biofilms supplemented with LB medium as well as for biofilms supplemented with 1/3-diluted LB medium. For characterization of time-dependent biofilm development in the microdevice, 7 image stacks were acquired from each of the two channels at days 1, 5 and 7 (24, 120 and 168 h after inoculation).

Image analysis by COMSTAT CLSM-captured images were analyzed quantitatively using COMSTAT software (14) written on the Matlab platform (The MathWorks, Inc., MA, USA). Images collected as z-stacks by 3D reconstruction consisted of a series of images with 1-μm intervals in z-section from the

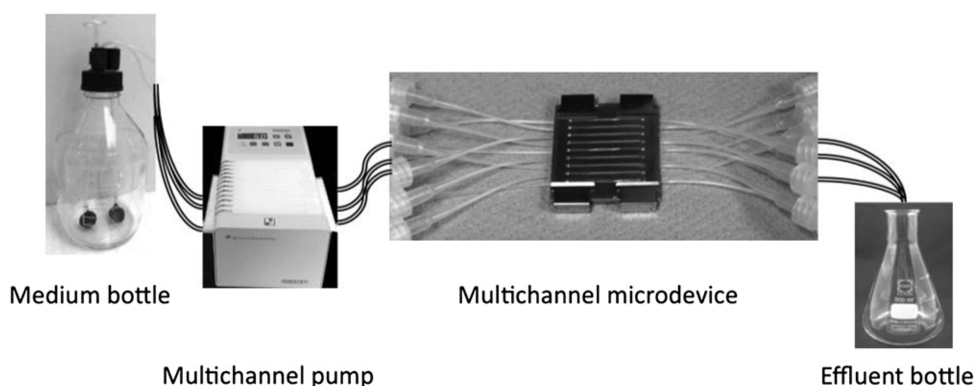

FIG. 2. Schematic representation of the multichannel microdevice flow system. Liquid medium in reservoir bottle with bubble trap is pumped using a multichannel pump into the microdevice. The effluent is collected in a flask. Influent and effluent Tygon tubings and series connectors are used in this once-through, continuous culture system.

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