

Influence of the Hydrodynamic Environment on Quorum Sensing in *Pseudomonas aeruginosa* Biofilms^{∇†}

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We provide experimental and modeling evidence that the hydrodynamic environment can impact quorum sensing (QS) in a *Pseudomonas aeruginosa* biofilm. The amount of biofilm biomass required for full QS induction of the population increased as the flow rate increased.

Quorum sensing (QS), or intercellular signaling, is used by a wide range of bacterial species to coordinate gene expression in a population (8, 26). One of the best-studied QS mechanisms is the acyl homoserine-lactone (AHL) system used by many different gram-negative bacterial species (7, 8). QS has been characterized primarily in planktonic batch culture, where its onset corresponds to a specific population density at which an inducing concentration of signal occurs. This has promoted the description of QS as a cell density-dependent signaling mechanism.

In the environment, bacteria rarely encounter conditions similar to a well-mixed planktonic culture. Unlike the steady signal accumulation in batch cultures, signal levels in environmental systems might be affected by signal diffusion between bacteria and the surrounding environment. Therefore, an interesting question is this: where can QS-relevant concentrations of signal be found in the environment? One potential answer is in surface-associated communities called biofilms. Microorganisms in the natural environment often live in biofilms, where they grow in close proximity to each other (5, 15). McLean et al. demonstrated that biofilms present on rocks submerged in flowing aquatic systems produce measurable amounts of AHLs (14), supporting the idea that physiologically relevant concentrations of signal might be present in some environmental biofilm systems.

Several physical, biological, and chemical factors have the potential to influence QS in biofilm systems (12). The hydro-

dynamic environment is one such factor with the potential to influence QS in several ways. First, signal produced in the biofilm will diffuse into the bulk fluid and will be washed away (i.e., advected). Thus, not all of the signal will remain in the local environment due to mass transfer effects. Second, mass transfer also can produce nutrient gradients, and the nutritional environment has been shown to influence QS (1, 6). For example, iron availability can modulate QS in *Pseudomonas aeruginosa* (1, 6), while other components of the nutritional environment can delay the onset of QS (28). Third, the hydrodynamic environment can affect biofilm density and thickness (2, 18, 25), which in turn can affect signal gradients.

The common soil and aquatic bacterium *P. aeruginosa* is a paradigm organism for the study of AHL-based QS and biofilms. In this study, we examined the relationship between hydrodynamic environment and QS induction in a developing *P. aeruginosa* biofilm. We hypothesized that as fluid flow rate in a biofilm system increased, the amount of biofilm biomass required for full QS induction of the population also would increase.

We used predictive modeling to gauge how flow conditions might affect the amount of biomass required for full QS induction of the population in a simple laboratory biofilm. This model focuses primarily on the effect of signal washout from the system. Several parameters were included in the model (see Table S1 in the supplemental material), including estimated per-cell signal production rate, biofilm biomass accumulation, Fickian diffusion of signal in the biofilm, and assumed geometry of a flat, two-dimensional biofilm. Based on past studies, we assumed the signal concentration required for full *lasB* induction to be 23 nM 3-oxo-dodecanoyl homoserine-lactone (3, 4, 17). We used a Stokes flow model for the fluid flow over the biofilm, which induced a diffusion boundary layer. The only nutrient gradient accounted for was dissolved oxygen, which was used as a measure of metabolic activity for estimating signal production at different depths in the biofilm. In our model, as flow rate increased, the predicted amount of biofilm biomass (CFU per attachment surface area) required

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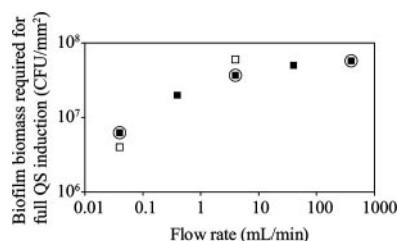


FIG. 1. Comparison of the amounts of biofilm biomass required for full QS induction of the population from model simulations (■) and from tube biofilm reactor experiments (□). The three modeled data points that were tested experimentally are circled.

for full QS induction of the population increased as well (Fig. 1).

To experimentally examine our hypothesis, we used *P. aeruginosa* strains harboring fluorescence-based transcriptional reporters for the well-studied QS-regulated gene *lasB* (see Table S2 in the supplemental material) (9, 10, 17). These reporters were characterized initially in planktonic batch culture, and green fluorescent protein (GFP) fluorescence was monitored as a function of culture density. The chromosomal reporter strain (*P. aeruginosa* PAO1 *lasB::gfp*) was found to induce rapidly between mid- and late logarithmic phase (see Fig. S1 in the supplemental material), and the plasmid reporter strain (*P. aeruginosa* PAO1 *plasB::gfp*) showed similar results (data not shown). As expected, the reporters failed to fluoresce in their associated QS-deficient (*lasI rhII*) strains (data not shown). All of these observations are consistent with previously published *lasB* expression data. Using an AHL bioassay (16) on a planktonic batch culture (Luria-Bertani [LB] medium at 23°C), the *plasB::gfp* reporter strain was induced maximally at a 3-oxo-dodecanoyl homoserine-lactone concentration of 17 nM (data not shown). For the rest of this study, QS was said to be fully induced in a reporter biofilm once the biofilm reached 100% of the maximum normalized fluorescence (i.e., relative fluorescence units divided by optical density at 600 nm) of the reporter strain in a planktonic batch culture.

Three modeled data points (Fig. 1), corresponding to flow rates of 0.04 ml/min, 4.0 ml/min, and 380 ml/min, were chosen for analysis in laboratory biofilms. These flow rates correspond to velocities of 0.15 mm/s, 15 mm/s, and 1,400 mm/s, respectively, and to Reynold's numbers of 0.3, 30, and 3,000, respectively. For the two lower flow rates, biofilms were cultured in a once-through silicone tube biofilm reactor similar to those described previously (13, 20, 22) (Fig. 2A), where the flow was driven by a peristaltic pump. For the highest flow rate, to avoid the large volumes of growth medium necessary to run the reactor in a once-through format, biofilms were cultured in a recycle reactor similar to that described previously (23) (Fig. 2B). In the recycle reactor, fresh medium was pumped via a peristaltic pump from an influent reservoir to a mixing chamber, where it was mixed with recycled medium. To achieve the high flow rate (380 ml/min), a gear pump was used to pump the medium in the recycle loop. The hydraulic residence time in the system was approximately 35 min, which minimized planktonic accumulation. For both types of reactors, biofilms of *P. aeruginosa* PAO1 *lasB::gfp* were cultured at room temperature in LB medium on sections of 7.6-cm silicone tubing with a

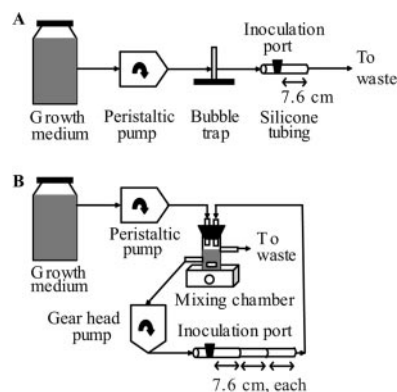


FIG. 2. Schematics depicting tube biofilm reactors. (A) Once-through reactor for flow in the laminar regime. (B) Recycle reactor for flow in the transitional regime between laminar and turbulent.

2.4-mm inner diameter. At various time points, the biofilm was harvested by centrifugation and was resuspended in $1 \times$ phosphate-buffered saline by several cycles of sonication and vortexing; the absence of cell clumps in the suspension was verified by microscopy. Total biofilm biomass was determined by plate counts for each harvested sample. Biofilms grown at the two lower flow rates showed similar biofilm accumulation kinetics (Fig. 3), in which a steady-state level of biofilm biomass ($\sim 5 \times 10^7$ CFU/mm²) was reached after approximately 100 h. Biofilms grown at the highest flow rate showed a lower steady-state level of biofilm biomass ($\sim 5 \times 10^6$ CFU/mm²), which was reached after approximately 170 h. Scanning electron microscopy was performed on the biofilms grown at the two lower flow rates. The biofilms appeared to be fairly flat and uniform, forming a confluent mat on the tube surface (see Fig. S2 in the supplemental material); thus, the experimental biofilms formed in the tube biofilm reactors were similar in structure to the modeled two-dimensional biofilms.

Expression of *lasB* in the biofilm population was monitored over time using fluorimetry (Fig. 4) and microscopy (data not shown). At the lowest flow rate (0.04 ml/min), the amount of biofilm biomass required for full QS induction of the population was estimated to be 4×10^6 CFU/mm² (Fig. 4A). At the intermediate flow rate (4.0 ml/min), the amount of biofilm

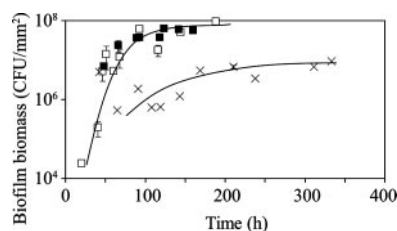


FIG. 3. Accumulation of biofilm biomass in the tube biofilm reactor. Flow in the laminar regime, 0.04 ml/min (□) and 4.0 ml/min (■); flow in the transitional regime between laminar and turbulent, 380 ml/min (×). Lines on the plot show the trend of the data. For simplicity, error bars representing the standard deviations are shown only for the data points that are based on the averages of at least two separate harvested biofilms. The data points without error bars are based on results from a single harvested biofilm. The biofilm biomass for each harvested biofilm was measured by triplicate plate counts.

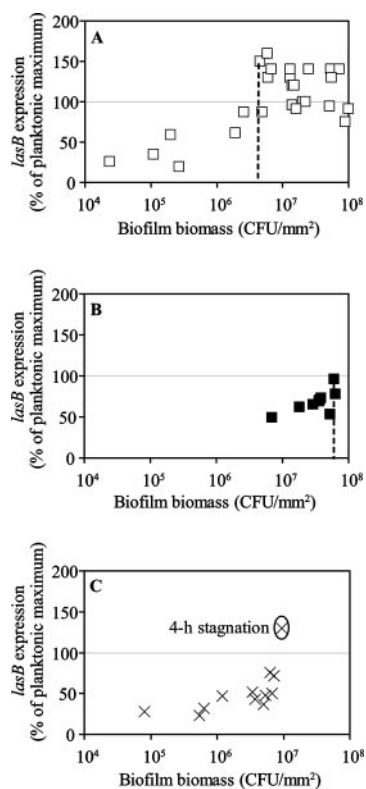


FIG. 4. QS induction in the tube biofilm reactor. Flow rates were (A) 0.04 ml/min, (B) 4.0 ml/min, and (C) 380 ml/min (with and without stagnation). The vertical dashed line indicates the biofilm biomass at which QS was fully induced (i.e., *lasB* expression at 100% of planktonic maximum).

biomass required for full QS induction was estimated to be 6×10^7 CFU/mm² (Fig. 4B). The highest flow rate was achieved using the recycle reactor. Signal accumulation in the recycled medium was monitored and found to be below the detection limit of the AHL bioassay (data not shown). Full QS induction of the biofilm population grown at the highest flow rate was never observed, even after 200 h of reactor operation, although an upper limit of 80% induction of the population was seen (Fig. 4C). However, QS was fully induced in this population after the flow was stagnated for 4 h (Fig. 4C).

Our data and model suggest that signal washout might explain why QS fails to fully induce at the highest flow rate, although we cannot rule out mass transfer effects on nutrient gradients. These data also suggest that QS may not be fully operative at high flow rates and thus not play a significant role in biofilm formation. Supporting this point, Purevdorj et al. demonstrated that *P. aeruginosa* wild-type and QS mutant strains formed nearly identical biofilms under high flow conditions (19).

At the two lower flow rates, which are in the laminar flow region, our experimental values for QS induction were very close to the values predicted by the model (Fig. 1). At the highest flow rate, which is in the transitional regime between laminar and turbulent, full QS induction of the population was not seen experimentally; this was consistent with the model, since the maximum biofilm biomass in the flowing experimen-

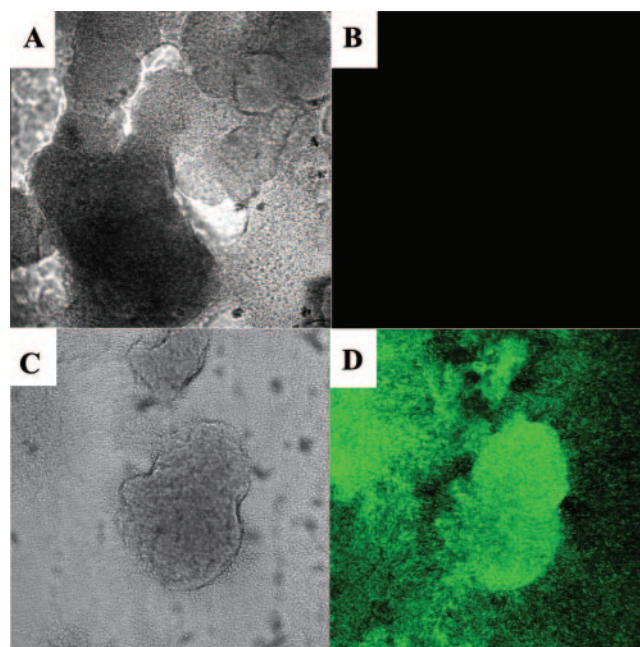


FIG. 5. QS induction in the flow cell biofilm reactor. Transmitted light and epifluorescent maximum projections of the confocal scanning laser microscopy stacks are shown for a biofilm grown in the flow cell at 1 ml/min. (A) Transmitted light image and (B) corresponding epifluorescent image showing low *lasB* expression in the biofilm under flowing conditions. (C) Transmitted light image and (D) corresponding epifluorescent image showing full *lasB* expression after a 6-h stagnation period.

tal system (7×10^6 CFU/mm² [Fig. 4C]) never reached the level predicted by the model to be necessary for full QS induction (6×10^7 CFU/mm² [Fig. 1]).

To demonstrate some of the same principles in a different format, we next examined *lasB* expression in a flow cell biofilm reactor (24). This system was inoculated with *P. aeruginosa* PAO1 *lasB::gfp* and operated with LB medium at room temperature. This system is very similar to that shown in Fig. 2A, except that the biofilm was grown in a 1-mm- by 1-mm-square glass capillary (Friedrick & Dimmock, Millville, NJ), with flow rates of 0.005 ml/min, 0.1 ml/min, and 1.0 ml/min. These flow rates correspond to velocities of 0.08 mm/s, 1.7 mm/s, and 17 mm/s, respectively, and to Reynold's numbers of 0.08, 1.7, and 17, respectively. The advantage of the flow cell biofilm reactor is that it can be interrogated by direct microscopy. Biofilm biomass and structural characteristics were measured using the image analysis software COMSTAT (11) (see Fig. S3 in the supplemental material). In this system, the biofilm was much more structured and heterogeneous than were the tube biofilms (Fig. 5). At earlier points in biofilm development, *lasB* was not expressed in the flow cell system although a significant amount of biofilm biomass was present (Fig. 5A and B). To determine if the mass transfer environment might be responsible, flow was stopped, and much of the population was observed to be expressing *lasB* within 6 h (Fig. 5C and D). Nearly uniform levels of expression were seen throughout the system, also demonstrating that oxygen is not limiting for proper GFP folding. To complement this experiment, another biofilm was grown to a point of development similar to that shown in Fig.

5, and inducing concentrations (10 μ M) of the two primary AHLs, 3-oxo-dodecanoyl homoserine-lactone and butyryl homoserine-lactone, were supplied exogenously. The entire population was seen to rapidly induce within 10 min (data not shown). Taken together, the tube biofilm reactor data and the flow cell data suggest that mass transfer influences QS induction in a developing biofilm.

Environmentally and clinically relevant biofilms are found in hydrodynamic environments that range from turbulent flow (e.g., biofilms on a rock in a river) to little or no flow (e.g., biofilms in the airways of people suffering from cystic fibrosis). In this report, we provide evidence that the hydrodynamic environment can impact QS induction in biofilms. Our study focused on a single, well-known QS-regulated gene. Other researchers have shown that there are multiple classes of QS-regulated genes (21, 27), and our future studies will examine if representatives of these other classes of QS-regulated genes also are subject to the effects of the hydrodynamic environment in biofilms. Another limitation of our study is that our simple model does not account for nutritional parameters known to influence QS. As the effect of the nutritional environment on QS becomes better understood, we will attempt to incorporate these nutritional parameters into our model and test the model experimentally. As researchers attempt to understand and control QS in environmentally and clinically relevant contexts, consideration of factors such as the hydrodynamic environment will become increasingly important in developing an accurate model of these complex situations.

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