Effects of PslG on the Surface Movement of *Pseudomonas aeruginosa*

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ABSTRACT PslG attracted a lot of attention recently due to its great potential abilities in inhibiting biofilms of *Pseudomonas aeruginosa*. However, how PslG affects biofilm development still remains largely unexplored. Here, we focused on the surface motility of bacterial cells, which is critical for biofilm development. We studied the effects of PslG on bacterial surface movement in early biofilm development at a single-cell resolution by using a high-throughput bacterial tracking technique. The results showed that compared with no exogenous PslG addition, when PslG was added to the medium, bacterial surface movement was significantly (4 to 5 times) faster and proceeded in a more random way with no clear preferred direction. A further study revealed that the fraction of walking mode increased when PslG was added, which then resulted in an elevated average speed. The differences of motility due to PslG addition led to a clear distinction in patterns of bacterial surface movement and retarded microcolony formation greatly. Our results provide insight into developing new PslG-based biofilm control techniques.

IMPORTANCE Biofilms of *Pseudomonas aeruginosa* are a major cause for hospital-acquired infections. They are notoriously difficult to eradicate and pose serious health hazards to human society. So, finding new ways to control biofilms is urgently needed. Recent work on PslG showed that PslG might be a good candidate for inhibiting/disassembling biofilms of *Pseudomonas aeruginosa* through Psl-based regulation. However, to fully explore PslG functions in biofilm control, a better understanding of PslG-Psl interactions is needed. Toward this end, we examined the effects of PslG on the surface movement of *Pseudomonas aeruginosa* in this work. The significance of our work is in greatly enhancing our understanding of the inhibiting mechanism of PslG on biofilms by providing a detailed picture of bacterial surface movement at a single-cell level, which will allow a full understanding of PslG abilities in biofilm control and thus present potential applications in biomedical fields.

KEYWORDS *Pseudomonas aeruginosa*, biofilm, Psl, PslG, bacterial movement

Biofilms of *Pseudomonas aeruginosa* are a major cause of hospital-acquired infections (1, 2). Compared with planktonic cells, bacteria in biofilms can tolerate a high dosage of antibiotics (3–5) and exhibit strong antibiotic resistance and enhanced capabilities for escaping from immune systems (6, 7). Biofilms are notoriously difficult to eradicate and pose serious health hazards to human society. Therefore, biofilm formation of *P. aeruginosa* has been a fast-growing research area in the last 2 decades.

A typical process of biofilm formation starts with the initial attachment of bacterial

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cells on surfaces, continues with microcolony formation and maturation, and finally ends with dispersion of free cells from matured biofilms to start the development cycle of a “next generation” (8). The regulation of bacterial motility is critical for biofilm formation. *P. aeruginosa* has two types of motility appendages: a flagellum and type IV pili (TFP). The flagellum helps cells to overcome the repulsion forces of surfaces and attach on them (9). After attaching, cells move on surfaces by TFP-driven twitching motility (10–13). A previous study showed two types of twitching mode, flat “crawling” and upright “walking,” which were suggested to be different surface exploration strategies (9). Bacterial motility can greatly affect the structure of biofilms. It has been shown that biofilms formed by $\Delta pilA$ mutants had irregular and protruding structures while wild-type (WT) organisms formed a flat carpetlike biofilm under the same growth conditions (14).

In biofilms, bacteria are embedded in a polymeric substance matrix. Exopolysaccharides secreted by bacterial cells are the main components of the matrix. They form the skeleton of a biofilm and maintain biofilm integrity. *P. aeruginosa* cells secrete mainly three types of polysaccharides: Psl, Pel, and alginate. Psl is a neutral polysaccharide consisting of a pentasaccharide repeat containing D-mannose, L-rhamnose, and D-glucose, and 11 polysaccharide synthesis locus (*psl*) genes, *pslACDEFGHIJKL*, are required for Psl production (15). For *P. aeruginosa* strain PAO1, Psl plays a key role in surface attachment and biofilm development, which has been shown to act as a “molecular glue” to promote cell-surface and cell-cell adhesions, especially at early stages of biofilm development (16–19).

Besides the passive role of Psl as a molecular glue, recent work showed an active role for Psl in the regulation of bacterial motility and of a second messenger, c-di-GMP (20–22). Irie et al. showed that secreted Psl could act as a signal to stimulate two guanylate cyclases, SiaD and SadC, to increase the production of c-di-GMP and then promote the biofilm formation of *P. aeruginosa* (21, 23). Eliminating Psl, on the other hand, could reduce the intracellular c-di-GMP level and weaken the tolerance of bacteria to antimicrobial drugs (24). Using a bacterial tracking technique, which enables a detailed study at a single-cell resolution, Zhao et al. found that Psl trails left by cells affected the motion of cells that came across those trails later on, and bacterial surface movement followed a Psl-based power law distribution, which led to microcolony formation (25). A fiber-like Psl matrix has also been observed in biofilms, and evidence suggested that the pattern was formed by TFP-driven twitching motility (16). In *Myxococcus xanthus*, it has been shown that extracellular polysaccharides on surfaces could bind to TFP and trigger pilus retraction (26). All those results demonstrate the important roles of exopolysaccharides, particularly that of Psl in biofilm development. Therefore, finding ways to reduce the production and/or interfere with the surface distribution of Psl becomes a promising strategy to control biofilm formation.

Recent work on PslG showed that PslG might be a good candidate for biofilm disassembly through Psl-based regulation. PslG is a protein encoded by the *pslG* gene of *Pseudomonas aeruginosa*. It is a glycosyl hydrolase. PslG was first considered to be necessary for Psl synthesis, as deletion of the *pslG* gene led to a loss of Psl production (27). But later work showed that deletion of *pslG* in the earlier work might have affected the gene expression downstream of *pslG* and thus resulted in the loss of Psl production, and the absence of PslG itself did not affect Psl production. In contrast, overexpression of PslG could affect Psl and biofilm formation (28). Yu et al. showed that PslG could disassemble a matured biofilm by degrading the Psl matrix within minutes at a nanomolar concentration, indicating its great potential in combating biofilms (29). However, how exactly PslG is involved in regulating bacterial behavior is largely unexplored. Particularly, how PslG affects bacterial motion is still unclear.

In this work, we studied the effects of PslG on bacterial surface movement in early biofilm development at a single-cell resolution by using a high-throughput bacterial tracking technique (25, 30). We showed that after adding PslG exogenously to the medium, bacteria moved significantly (4 to 5 times) faster on the surface and in a more random way with no clear preferred direction. A further study showed that the fraction

of walking mode increased, which contributed significantly to the observed increase in the measured average speed. The differences of motility due to PslG addition resulted in a clear distinction in patterns of bacterial surface movement. With PslG addition, the trajectory was rough and the efficiency of surface coverage increased. The results also showed that biofilm development was greatly affected. It took bacteria roughly two times longer to form microcolonies with PslG addition than without. This work revealed the effects of PslG on bacterial surface motion and may help to get a full evaluation of PslG abilities in inhibiting/disassembling biofilms.

RESULTS

With PslG addition, the fraction of walking mode during cell movement increased, which on average resulted in a faster surface motion of bacteria. *P. aeruginosa* cells use TFP to move on surfaces in a way called twitching, in which a pilus first extends out and attaches to a surface spot, and then retraction of the pilus will pull the cell body forward. During twitching, the pulling force of TFP and the adhesive force between the attached surface and the cell body, which are coated with sticky exopolysaccharides, mainly determine how a cell moves (31). PslG is expected to affect bacterial motility by degrading Psl and thus changing the cell-surface adhesive interaction. To test this hypothesis quantitatively, bacterial surface movements with and without exogenously added PslG were recorded by time-lapse imaging under a microscope. Then, a thorough image analysis was performed. The results showed that the speed of bacterial movement on average increased significantly when 50 nM PslG was added into the medium (Fig. 1A). For wild-type (WT) bacteria, the cell speed increased from $2.2 \pm 0.2 \mu\text{m}/\text{min}$ to $9.8 \pm 1.5 \mu\text{m}/\text{min}$. The distribution of speed was broadened with PslG addition, probably due to an increased diffusive behavior that is not Psl associated. In order to show clearly the role of the PslG-Psl interaction, we also measured cell speeds for the Psl-overproducing (Psl⁺⁺) $\Delta P_{psl}/P_{BAD}\text{-psl}$ strain (32) with 1% arabinose addition and for the Psl⁻ $\Delta ps/BCD$ strain. The $\Delta P_{psl}/P_{BAD}\text{-psl}$ strain is an arabinose-inducible strain, and with 1% arabinose addition in the medium, Psl is overproduced. The $\Delta ps/BCD$ strain is a Psl knockout strain and did not produce Psl. The speed of Psl⁺⁺ cells increased from $1.7 \pm 0.3 \mu\text{m}/\text{min}$ when no PslG was added to $10.6 \pm 0.6 \mu\text{m}/\text{min}$ when PslG was added. As a control experiment, for Psl⁻ cells, there was no significant difference in cell speed with and without PslG addition, confirming that PslG affects cell motility through the PslG-Psl interaction. For $\Delta P_{psl}/P_{BAD}\text{-psl}$ cells under 0% arabinose addition, their speed still showed an increase from $5.0 \pm 1.2 \mu\text{m}/\text{min}$ to $10.4 \pm 1.2 \mu\text{m}/\text{min}$ when PslG was added (see Fig. S1 in the supplemental material), which might be due to the possible leakage of Psl production (19). Interestingly, after adding PslG, both WT and Psl⁺⁺ cells moved even faster than those Psl⁻ cells.

There are two types of modes for twitching: crawling and walking. In order to understand how PslG addition induced a faster bacterial surface motion, the effect of PslG on the two twitching modes was further investigated. We classified cells into three groups based on their twitching mode, i.e., crawling group, walking group, and crawling-walking group for cells that crawl only, walk only, and both crawl and walk, respectively, during the whole observation time. We found that with PslG addition, for the WT, the fraction of cells in the crawling-walking group increased significantly from 32% to 70%, and the fraction of cells in walking group also increased, but slightly, from 8% to 12%. In contrast, the fraction of cells in the crawling group was reduced by a large amount, from 59% to 18%. Similar trends were observed for Psl⁺⁺ cells when PslG was exogenously added, with an increase of fraction from 22% to 57% in the crawling-walking group and from 13% to 22% in the walking group and a decrease of fraction in the crawling group from 65% to 21% (Fig. 1B). These results clearly showed that the fraction of walking mode during cell twitching increased significantly when PslG was added exogenously.

With PslG addition, the cell speeds of all three groups showed an ~2.5-fold increase, in which the cell speed of the crawling-walking group increased from $4.1 \mu\text{m}/\text{min}$ to

FIG 1 Effects of PslG addition on the surface motility of bacteria. (A) Cell speeds with and without exogenous PslG addition for both WT and Psl⁺⁺ bacteria. (B) Fractions of cells that crawl only, walk only, or both crawl and walk. (C) Residence times of cells with and without PslG addition for both WT and Psl⁺⁺ bacteria. The data shown in this figure were collected for time periods of 12 h, 17 h, 13 h, and 20 h after inoculation for WT, WT + PslG, Psl⁺⁺, and Psl⁺⁺ + PslG bacteria, respectively. For all results with PslG addition, 50 nM PslG was used. The Psl⁺⁺ label refers to $\Delta P_{psl}/P_{BAD-psl}$ cells with 1% arabinose. Statistical significance was measured using one-way analysis of variance (ANOVA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All data are the averaged results for three repeats, and more than 1,000 tracked cells were examined for each condition.

10.1 $\mu\text{m}/\text{min}$ (see Fig. S2 in the supplemental material), while the cell speed of the crawling group increased from 1.5 $\mu\text{m}/\text{min}$ to 3.3 $\mu\text{m}/\text{min}$ and the cell speed of the walking group increased from 3.6 $\mu\text{m}/\text{min}$ to 7.4 $\mu\text{m}/\text{min}$. However, the observed 4- to 5-fold increase in the average speed when PslG was added cannot be attributed solely to the increase in the magnitude of speed of the three groups. Considering that walking is typically faster than crawling, the enlarged fraction of the walking mode would also contribute significantly to the increased average speed. In other words, that cells moved faster is not just because their speeds of walking and/or crawling increased *per se* but also because they walked more frequently.

Interestingly, a new type of surface motion, which we called “cartwheel,” was observed for cells in the crawling-walking group during their crawling-walking conversions. A representative cartwheel motion includes a sequence of a cell standing up on one pole, lying down on the surface, and then standing up again on the other pole (see Fig. S3 in the supplemental material). The speed of cartwheel was faster than that of crawling or walking, and as a result, the crawling-walking group has the highest average speed among three groups.

The residence time of cells is another quantity closely related to cell-surface adhesion and cell motility. It is defined as the time during which a cell stays on the surface. In experiments, the residence time was measured as the time difference between when a cell first attached to the surface and when it detached from the surface. For a cell that stayed until dividing, the end time point would be when it divided into two separate daughter cells. With PslG addition, the average residence time of WT cells was reduced by about one-half, from 24 min to 11 min. Similarly, for Psl⁺⁺ cells, the average residence time was reduced from 28 min to 9 min (Fig. 1C).

These results are consistent with the picture that by adding PslG, Psl gets degraded, which then weakens the adhesion between cell bodies and the substrate surface, so it is easy for cells to twitch in walking mode, as TFP can easily pull cell bodies upward to be upright due to reduced adhesion. This induces an enlarged fraction of walking mode, which then increases the cell speed on average and displays a superdiffusive behavior. In addition, bacterial detachment is enhanced due to the weakened cell-surface adhesion, as well as the enlarged fraction of walking mode, since upright configuration has been suggested to be related to bacterial detachment (33). A higher detachment probability thus results in a reduced residence time.

With PslG addition, bacteria moved on a surface in a more random way with no clear preferred direction and generated more-curly trajectories. Using a metric common for evaluating the diffusive behavior of micrometer-sized objects, we calculated the mean square displacements (MSDs) of cells. The slope of MSD in a log-log plot reflects the shapes of cell trajectories. The results showed that without exogenously added PslG, WT cells moved superdiffusively with an MSD slope of 1.4, and with added PslG, cells moved with an MSD slope of 1.3. For Psl⁺⁺ cells, the MSD slope was reduced from 1.3 in the case of no PslG addition to 1.2 in the case of PslG addition (see Fig. S4 in the supplemental material). These results implied that after addition of PslG into the medium, cells moved in a more random way.

Graphically we can see this trend by looking at the distribution of the moving directions of cells. Figure 2 shows representative results. Without adding PslG exogenously, the distribution of moving directions for both WT and Psl⁺⁺ cells showed a clear preferred direction (Fig. 2A), and this agreed with the observed trajectories that typically were less confined and extended more along some specific direction in space (Fig. 2C). However, with PslG addition, the distribution of moving direction became less anisotropic and did not show a clear preferred direction (Fig. 2B). The trajectories under this condition were typically confined and curled (Fig. 2D), similar to Psl⁻ bacterial trajectories (see Fig. S5 in the supplemental material). The curled wavy parts of trajectories were often where cells stood up and walked, in agreement with observations reported earlier (9, 11).

The observed less-directional motion is understandable when considering that more cells displayed walking mode. When cells crawl, they lie down and the contact area between the cell body and the substrate surface would be larger than the contact area when cells walk, in which only one cell pole contacts with the substrate surface. Since the cell-surface binding interaction (in which Psl-mediated interaction plays a major role) is proportional to the contact area, it is easier for standing cells to turn to a quite different direction in the next motion than lying-down cells. Therefore, the higher fraction of walking mode would increase the probability of large-angle deviation from the previous motion and thus result in curled trajectories with no preferred direction. The walking mode in *P. aeruginosa* has a role similar to that of the “tumbling” state in *Escherichia coli* (34).

With PslG addition, bacterial visits on a surface were uniform and the efficiency of surface coverage increased. Psl has been shown to act as a signal to regulate bacterial surface movement and to induce a power law distribution of bacterial visits at early stages of biofilm formation (25). We would expect that the surface exploration pattern of bacteria will be changed due to the degradation of Psl by adding PslG. In order to illustrate how the pattern is changed, we measured the distribution of bacterial visits and its evolution on a surface. With PslG addition, bacteria moved faster and in

FIG 2 Effects of PslG addition on the direction of bacterial surface motion. (A) Representative probability distributions of moving angle, $p(\theta)$, for a WT cell (red) and a Psl⁺⁺ cell (green) without exogenous PslG addition. θ is defined as the angle of a cell's displacement vector between two consecutive frames relative to a horizontal reference axis. (B) Representative $p(\theta)$ for a WT cell (red) and a Psl⁺⁺ cell (green) with PslG addition. (C and D) Representative trajectories of $\Delta P_{psl}/P_{BAD}$ -psl cells under 1% arabinose without (C) and with (D) PslG addition.

a more random way; hence, the efficiency of surface coverage by bacterial visits was enhanced, as shown in Fig. 3A. For the surface coverage maps shown in Fig. 3A, red indicates the surface area that has been visited or contaminated, while black indicates a "fresh" surface area. Compared with the condition under which no PslG was added exogenously, with PslG addition the surface coverage was enhanced from 38% to 86% for WT and from 14% to 56% for Psl⁺⁺ cells for a total of 50,000 bacterial visits (Fig. 3A). The efficiencies of surface coverage for both WT and Psl⁺⁺ cells under the condition of PslG addition were at the same level as that for Psl⁻ cells, which had a surface coverage of $\sim 72\%$ for a total of 50,000 visits (see Fig. S6 in the supplemental material).

To measure the distribution of bacterial visits, a bacterial visit frequency map was constructed by a bacterial tracking technique (Fig. 3B) and a histogram analysis was performed (Fig. 3C and D). Without exogenous PslG, cells followed a Psl-based rich-get-richer mechanism, which resulted in a distribution of bacterial visits that could be approximated by a power law with an exponent of -2.5 ± 0.1 for WT cells and an exponent of -1.8 ± 0.1 for Psl⁺⁺ cells, consistent with the results reported in earlier work (25). With PslG addition, the distribution of bacterial visits shifted to a power law with an exponent of -3.8 ± 0.07 for WT and an exponent of -3.3 ± 0.03 for Psl⁺⁺ cells. A power law distribution with a more negative exponent indicates that the distribution is more uniform. So, the results implied that PslG addition induced a more uniform bacterial visit distribution on the surface by degrading Psl and hence reducing the guidance role of Psl in bacterial movement.

PslG addition retarded microcolony formation greatly. Formation of a microcolony, a cluster of tens of cells, is the first step in communal biofilm organization. To quantitatively evaluate the biofilm-inhibiting effect of PslG, we measured the microcolony formation time, defined as the time taken from inoculation to the observation of the first microcolony (defined as clusters of 30 cells in this work) appearing in the

FIG 3 Efficiency of surface coverage and bacterial visit distribution. (A) Surface coverage maps at a total of 7,500 (corresponding to incubation times of 1.7 h, 2.2 h, 3.3 h, and 3.4 h for WT, WT + PslG, Psl⁺⁺, and Psl⁺⁺ + PslG cells, respectively) and 50,000 (corresponding to incubation times of 3.8 h, 10.4 h, 10.7 h, and 15.8 h for WT, WT + PslG, Psl⁺⁺, and Psl⁺⁺ + PslG cells, respectively) bacterial visits for both WT and Psl⁺⁺ cells with and without PslG addition. Red indicates the surface area that has been visited or contaminated, while black indicates a “fresh” surface area. Bacteria in the current frame are shown in blue. The surface coverages are 2.7%, 9.2%, 1.8%, and 9.2% at 7,500 visits and 37.5%, 85.7%, 13.5%, and 55.4% at 50,000 visits for WT, WT with PslG, Psl⁺⁺, and Psl⁺⁺ with PslG cells, respectively. (B) Bright-field images and visit frequency maps at a total of ~200,000 visits (corresponding to incubation times of 5.6 h, 16.3 h, 13.9 h, and 19.4 h for WT, WT + PslG, Psl⁺⁺, and Psl⁺⁺ + PslG cells, respectively), when microcolonies began to form. (C) The graph on the left displays the visit frequency distributions for WT cells without (red) and with (magenta) PslG addition, and the one on the right shows the visit frequency distributions for Psl⁺⁺ cells without (olive) and with (green) PslG addition. Bars, 10 μm. Lines in panel C are power law fittings.

chosen field of view (Fig. 4A). PslG was added to culture medium at inoculation. We found that the microcolony formation time was 2 to 3 times longer with PslG addition than without. For WT, when there was no exogenous PslG addition, the measured microcolony formation time was 8.5 ± 0.6 h. However, after adding PslG, the number of bacteria in the chosen field of view was still relatively small in the first 8 h, and a microcolony did not form until 21.1 ± 2.1 h after inoculation. For Psl⁺⁺ cells to form a microcolony, without exogenous PslG it took 13.5 ± 1.2 h, whereas with PslG addition, it took about 27.0 ± 7.1 h (Fig. 4B).

FIG 4 Measurement of the time required for microcolony formation. (A) An example of a microcolony formed by WT cells cultured in a flow cell for about 9 h in the case of no PslG addition (marked in red) and for about 18 h in the case of PslG addition (marked in magenta). (B) Time required to form the first microcolony (defined as clusters of 30 cells in this work) that appeared in the chosen field of view for both WT and Psl⁺⁺ cells with and without PslG addition. Statistical significance was measured using one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bars, 10 μm .

DISCUSSION

It has been shown that PslG can degrade Psl and hence inhibit biofilm formation (28, 29). In this work, we provided a detailed picture at the single-cell level to understand the mechanism of this discovery. Our results clearly revealed that by adding PslG in the medium to degrade Psl, both bacterial motility and cell-surface adhesion were altered due to the loss of Psl regulation on these processes, which then resulted in the inhibition of biofilm formation by retarding the appearance of microcolonies with a substantial time delay.

One role of Psl is to enhance cell-surface adhesion. If Psl is degraded by PslG, then cell-surface adhesion will be weakened, and this will lead to a higher fraction of walking mode as observed in the experiment for both WT and Psl⁺⁺ cells with 50 nM PslG addition. This is also consistent with the observation of a higher fraction of standing cells in Psl-deficient mutants. For one cell to stand up by pilus retraction, it has to break up the adhesion between the cell body and the substrate surface, in which Psl is a major binding agent. The adhesion force between a *P. aeruginosa* cell and a titanium surface has been measured to be ~ 2 nN (35). Although the exact value of the adhesion force will vary with cell types and properties of substrate surfaces (35), it is reasonable to estimate the adhesive force between a *P. aeruginosa* cell and the glass surface to be in the order of nanonewtons. A single pilus can generate a force of ~ 100 pN (36), and a cooperative effort of several pili can produce a force of up to ~ 1 nN (37). Moreover, it has been observed that the maximum-force events by cooperation of multiple pili appeared rarely and low-force events typically involving a single pilus were the majority. Thus, without reducing the cell-surface adhesion, multiple pili (with a single pilus force of ~ 100 pN) are likely needed to pull cooperatively in order to generate enough force (~ 1 nN) to let cells stand up. This agrees with the fact that under typical conditions, WT cells in crawling mode are observed more frequently than those in walking mode (9). When PslG is added, degrading of Psl weakens the cell-surface adhesion, and fewer pili or even a single pilus can generate enough force to break up the binding to let cells stand up.

The walking mode on average has a greater speed than crawling (33). It has been suggested that the walking mode is efficient for local exploration, which is typically

superdiffusive, while crawling is better for directional long-distance exploration, which can be both superdiffusive and subdiffusive (9, 33). If cells walk more often, it will result in a superdiffusive motion with an increased speed. Under our conditions, cells moved 4 to 5 times faster, with more walking mode induced by PslG addition both for WT and for Psl⁺⁺ organisms.

The residence times for both WT and Psl⁺⁺ decreased when PslG was added, indicating a higher detaching probability. Considering the degradation of Psl, the results are, not surprisingly, similar to those for Psl-deficient mutants. This is also consistent with the observation of an enlarged fraction of walking mode when PslG was added, since the standing-up configuration has been suggested to be closely related to detachment (33).

Besides the glue function, Psl could also act as a signal for bacterial surface exploration and c-di-GMP regulation (21, 25). When cells move on a surface, by degrading Psl with PslG, they lose the guidance of Psl. Since there is no accumulation of intact Psl, there is no Psl hot spot to attract cells and hence no Psl-based positive feedback, so cells move in a random way that finally leads to a more uniform distribution of bacterial visits, as shown by a more negative power law exponent. Thus, an enlarged fraction of walking mode, a reduced residence time, and a more uniform distribution of bacterial visits altogether contributed to the inhibition of biofilm development.

Interestingly, we found that after PslG was added, both WT and Psl⁺⁺ cells moved even faster than Psl⁻ cells, and they also took a longer time to form microcolonies than Psl⁻ cells did. These results suggest that PslG might have an effect on other extracellular polymeric substances besides Psl, which has also been implied in earlier work (29). However, these results could be also affected by possible lubricating effects of degraded Psl. Further work is needed to test those hypotheses. Furthermore, keeping the flow containing PslG for a long enough time under our conditions, bacteria eventually formed microcolonies. This seems to suggest that although Psl is one of the key polysaccharides for the biofilm development (38), when Psl gets degraded, bacteria may mobilize other metabolic regulatory mechanisms to enhance the role of other extracellular polysaccharides in biofilms. In fact, it has been reported that in an extended cultivation of *psl* mutants of *P. aeruginosa*, mutations with upregulated expression of Pel were obtained and the overproduction of Pel was able to partially compensate for the loss of Psl (38). It would be intriguing to identify enzymes for other exopolysaccharides and combine them to enhance the inhibition and disassembling effect on biofilms.

Other substances that can be used for biofilm control have been reported (39–42). Rhamnolipids at low concentrations in the presence of caprylic acid have shown promising activities for inhibition/disruption of *P. aeruginosa* biofilms (39), and nitroxide molecules can be used to induce biofilm dispersion by preventing bacterial swarming (43). However, compared with those substances, PslG has several unique properties. It has been shown to have little impact on the physiology of bacteria within biofilms regarding gene expression (29). PslG is also not toxic to human epithelial cells and immune cells (32). More importantly, only a nanomolar amount of PslG is needed for an effective inhibition and disassembling behavior of biofilms to be observed (29). All those results support that PslG is a promising candidate for controlling biofilms in the clinic. Our results will thus allow a full understanding of PslG abilities and help to develop new PslG-based methods for biofilm control in areas of medical devices like implants and catheters.

In summary, by using a state-of-the-art bacterial tracking technique, we have revealed the effects of PslG on bacterial motility, which is important for biofilm development. WT and Psl⁺⁺ cells in the presence of 50 nM PslG behaved very similarly to Psl⁻ cells, which confirmed the fact that PslG targets Psl. Our results indicate that it is promising to use PslG to degrade Psl, which can alter cell-surface adhesion and bacterial surface exploration and then interrupt the dynamics of biofilm formation at early stages to prevent biofilm development.

MATERIALS AND METHODS

Bacterial strains and media. We used *P. aeruginosa* wild-type (WT) strain PAO1 and its isogenic $\Delta ps/BCD$ mutant and the Psl-inducible $\Delta P_{psl}/P_{BAD-psl}$ strain in this study (32). The $\Delta ps/BCD$ mutant could not produce Psl; the $\Delta P_{psl}/P_{BAD-psl}$ strain can use arabinose to regulate Psl production. For the $\Delta P_{psl}/P_{BAD-psl}$ mutant, 0% and 1% amounts of arabinose (Sigma-Aldrich) were added into the medium to control the production of Psl.

Strains were grown on LB agar (LB, 10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) plates at 37°C for 12 h. An inoculum was prepared by growing strains in test tubes containing FAB medium [2 g liter⁻¹ (NH₄)₂SO₄, 9 g liter⁻¹ Na₂HPO₄·7H₂O, 3 g liter⁻¹ KH₂PO₄, 3 g liter⁻¹ NaCl, 93 mg liter⁻¹ MgCl₂, 14 mg liter⁻¹ CaCl₂·2H₂O, 1 ml liter⁻¹ trace metals solution (200 mg liter⁻¹ CaSO₄·2H₂O, 200 mg liter⁻¹ FeSO₄·7H₂O, 20 mg liter⁻¹ MnSO₄·H₂O, 20 mg liter⁻¹ CuSO₄·5H₂O, 20 mg liter⁻¹ ZnSO₄·7H₂O, 10 mg liter⁻¹ CoSO₄·7H₂O, 10 mg liter⁻¹ Na₂MoO₄·2H₂O, 5 mg liter⁻¹ H₃BO₃)] (44) with 30 mM glutamate (Sigma-Aldrich), shaking at 220 rpm and 37°C for about 10 h to an optical density at 600 nm (OD₆₀₀) of ~0.5. Cultures were then diluted to an OD₆₀₀ of ~0.01 in FAB medium with 0.6 mM glutamate, which was used for injection into a flow chamber. For all biofilm inhibition experiments, PslG was supplied into FAB at 50 nM final concentration. A 50 nM concentration of PslG has been shown to degrade biofilm sufficiently (29). Growth of *P. aeruginosa* with and without PslG was measured, and the results showed that PslG had no effect on bacterial growth (see Fig. S7 in the supplemental material). A fully automated inverted microscope was used to observe bacterial movements on a glass surface.

Protein purification. The DNA sequence encoding PslG (residues 31 to the end) was amplified from the genomic DNA of *P. aeruginosa* PAO1 and cloned into pGLo1, a derivative of pET15b with a PPase cleavage site. The protein was expressed in *E. coli* BL21(DE3) cells induced with 0.12 mM isopropyl β -D-thiogalactopyranoside at 16°C overnight. For protein purification, bacterial cells were harvested by centrifugation for 15 min at 5,200 $\times g$, resuspended in binding buffer (25 mM Tris-HCl [pH 8.0], 200 mM NaCl), and lysed by sonication. The lysate was centrifuged at 28,000 $\times g$ for 50 min, and the supernatant was loaded onto Ni-chelating Sepharose (GE Healthcare). After washing with binding buffer, the resin with bound protein was incubated with 0.12 mg/ml PPase overnight in binding buffer at 4°C to remove the His tag. Subsequently, the protein was eluted with binding buffer. The eluate was then loaded onto an ion-exchange column (source 15Q; GE Healthcare) and eluted using a 120-ml linear gradient of 0 to 1 M NaCl in 25 mM Tris-HCl buffer (pH 8.0). The protein was further purified by using a Superdex 200 (GE Healthcare) column with 10 mM Tris-HCl (pH 8.0)–100 mM NaCl as eluate buffer.

Flow cell assembly and sterilization and washing of the system. Flow cells made of polycarbonate were purchased from the Department of Systems Biology, Technical University of Denmark. Each flow cell has three identical rectangle channels (40 mm by 4 mm by 1 mm) (30) and was assembled by attaching a cover glass as the substratum as previously described (45). The assembled flow cell was connected to a syringe through a 0.22- μ m filter (Millipore Millex) using silicon tubing. Then, the whole system was sterilized overnight with 3% H₂O₂ at 3 ml/h using a syringe pump (Harvard Apparatus). After sterilization, autoclaved, deionized water was used to wash the whole system overnight (25). Before inoculation of bacteria into the flow cell, the system was flushed for 5 min at a flow rate of 30 ml/h by FAB medium with 0.6 mM glutamate using a syringe pump (Harvard Apparatus). Then, the medium flow was stopped and 1 ml of a diluted bacterial culture (OD₆₀₀ of ~0.01) was injected directly into the channel of the flow cell using a 1-ml syringe equipped with a needle. A 5-min incubation period was allowed after inoculation to let cells attach to the surface, which was then followed by a medium flow with a large flow rate of 30 ml/h for 5 min to wash out floating cells. After that, the flow rate was set to 3 ml/h, and image recording was started. In this work, the flow cell experiments were conducted at 30°C.

Data acquisition. Images were captured using an electron-multiplying charge-coupled-device (EMCCD) camera (Andor iXon) on a Leica DMI8 microscope equipped with the Zero Drift autofocus system. The image size is 66.49 μ m by 66.49 μ m (1,024 by 1,024 pixels). The bright-field images were recorded with a 100 $\times$ oil objective (plus a $\times 2$ magnifier) every 3 s for total recording times of about 12 h for runs without PslG and 24 h for runs with PslG. Each data set, which had 10,000 to 30,000 frames, can accommodate up to 1,000,000 bacterial images. There are at least three replicates for each condition.

Image analysis. Images were processed and analyzed in the same way as described in reference 25. Simply speaking, 16-bit grayscale images were first converted to binary images for the detection of bacteria with a standard image processing algorithm. Geometry information of cells such as center position, size, and aspect ratio, etc., were then collected. Bacterial trajectories were obtained by connecting cell positions in all frames of a time series, from which bacterial motion can be measured and analyzed.

The crawling and walking states of bacteria were classified by the aspect ratio of projected cell images (length/width). Cells were considered to be in crawling status if they had an aspect ratio of ≥ 2 , and cells were considered to be in walking status if they had an aspect ratio of < 2 . The mean square displacement (MSD) of cells was calculated as $\Delta r^2(\tau) = [r(t + \tau) - r(t)]^2$, where $r(t)$ is the position vector of a cell at time t , and τ represents the time interval (33). Surface coverage and bacterial visit frequency analysis were done in the same way as in a previous work (25). Simply speaking, the surface coverage was generated from the bacterial trajectories using the area of each tracked cell. The visit frequency distributions were generated at full data resolution; for the visit maps, the center of the cell was used to mark the trail and each of these marks was spread over a square patch eight pixels (~0.5 μ m) wide, in accordance with bacterium width.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00219-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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We declare that we have no competing interests.

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