In Situ Biomineralization and Particle Deposition Distinctively Mediate Biofilm Susceptibility to Chlorine

Xiaobao Li,^a David L. Chopp,^b William A. Russin,^c Paul T. Brannon,^c Matthew R. Parsek,^d Aaron I. Packman^a

Department of Civil and Environmental Engineering, Northwestern University, Evanston, Illinois, USA^a; Department of Engineering Sciences and Applied Mathematics, Northwestern University, Evanston, Illinois, USA^b; Biological Imaging Facility, Northwestern University, Evanston, Illinois, USA^c; Department of Microbiology, University of Washington, Seattle, Washington, USA^d

Microbial biofilms and mineral precipitation commonly co-occur in engineered water systems, such as cooling towers and water purification systems, and both decrease process performance. Microbial biofilms are extremely challenging to control and eradicate. We previously showed that *in situ* biomineralization and the precipitation and deposition of abiotic particles occur simultaneously in biofilms under oversaturated conditions. Both processes could potentially alter the essential properties of biofilms, including susceptibility to biocides. However, the specific interactions between mineral formation and biofilm processes remain poorly understood. Here we show that the susceptibility of biofilms to chlorination depends specifically on internal transport processes mediated by biomineralization and the accumulation of abiotic mineral deposits. Using injections of the fluorescent tracer Cy5, we show that *Pseudomonas aeruginosa* biofilms are more permeable to solutes after *in situ* calcite biomineralization and are less permeable after the deposition of abiotically precipitated calcite particles. We further show that biofilms are more susceptible to chlorine killing after biomineralization and less susceptible after particle deposition. Based on these observations, we found a strong correlation between enhanced solute transport and chlorine killing in biofilms, indicating that biomineralization and particle deposition regulate biofilm susceptibility by altering biocide penetration into the biofilm. The distinct effects of *in situ* biomineralization and particle deposition on biocide killing highlight the importance of understanding the mechanisms and patterns of biomineralization and scale formation to achieve successful biofilm control.

The development of biofilms in engineered water systems is usually detrimental. Excessive biofilm growth decreases process performance; for example, it reduces the heat exchange efficiency in cooling towers and hinders the flow through high-pressure water treatment membranes (1, 2). Biofilms in water systems are also potential reservoirs of human pathogens (2–6). Biofilms are difficult to eradicate because they are much less susceptible to antimicrobials and biocides than suspended microbial cells (7, 8). The decreased susceptibility of biofilms to biocides results from a combination of the decreased penetration of solutes into the biofilm structure, the protection provided by extracellular polymers, reduced cellular metabolism within biofilms, and the development of resistant cellular phenotypes (persisters) (9–11). Chlorine is one of the most commonly used biocides for biofilm control in engineered water systems (12, 13). Prior studies have found that the limited transport of chlorine to the interior of biofilms reduces the overall killing efficacy of chlorination (12, 14).

Biofilm biofouling is often associated with the formation of scale, which is an inorganic mineral precipitate that commonly includes calcium carbonate, sulfate, and phosphate (1, 15, 16). The co-occurrence of a biofilm and mineral formation has been widely found in reverse osmosis membrane systems, cooling towers, and water distribution pipes (17–19). Biofouling and scale fouling combined decrease the flow rate and process performance and greatly increase operational costs and energy demand (1, 20). Scale can form either abiotically as a result of chemical oversaturation or biotically through microbially catalyzed biomineralization (15). Microbial growth facilitates mineral precipitation and alters the composition and morphology of scale deposits (21, 22). Biofilms induce mineral precipitation by providing nucleation sites, concentrating cations and anions, and altering the local pH (23–25). Biofilms with mineral deposits, termed crystalline

or lithifying biofilms, present a morphology and a mechanical strength very different from those of the more common mucoid biofilms (24, 26, 27). However, the specific effects of *in situ* biomineralization and particle deposition on the susceptibility of biofilms to biocides are not understood. While mucoid biofilms have been widely studied (28), little information on the processes that regulate the development of biofilms with mixed organic and mineral matrices is available, and the interactions between biofilms and scale formation are still largely unexplored.

In our previous investigations, we used *Pseudomonas aeruginosa* as a model organism to study calcium carbonate biomineralization in biofilms (29). *P. aeruginosa* is a common pathogen in aquatic systems and building water systems, as well as an organism well studied as a model for biofilm formation (30, 31). We found that *in situ* carbonate biomineralization produced unexpected spatial patterns, in that it primarily occurred from the base of the biofilm architecture rather than at the biofilm surface. The biomineralized deposits grew over time, deformed the biofilm structure, and detached cells from the biofilm (29). Based on these

Received 10 December 2015 Accepted 29 February 2016

Accepted manuscript posted online 4 March 2016

Citation Li X, Chopp DL, Russin WA, Brannon PT, Parsek MR, Packman AI. 2016.

In situ biomineralization and particle deposition distinctively mediate biofilm susceptibility to chlorine. Appl Environ Microbiol 82:2886–2892.

doi:10.1128/AEM.03954-15.

Editor: H. Nojiri, The University of Tokyo

Address correspondence to Aaron I. Packman, a-packman@northwestern.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.03954-15>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

previous findings, we hypothesized that *in situ* biomineralization should enhance internal solute transport within biofilms and therefore increase biofilm killing by biocides. Conversely, particle deposition at the biofilm surface should reduce solute penetration into the biofilm and thus reduce biofilm killing. We tested these hypotheses by comparing the propagation of a fluorescent tracer and killing by chlorine in *P. aeruginosa* biofilms before and after *in situ* biomineralization and particle deposition.

MATERIALS AND METHODS

Experimental setup, bacterial strains, and inoculation procedure. We observed changes in solute transport and chlorine killing in *P. aeruginosa* PAO1-*gfp* biofilms before and after biomineralization and particle deposition. PAO1 biofilms were grown in flow cells following a protocol described previously (32). Prior to inoculation, the flow cell system was sterilized by autoclaving. To prepare the bacterial inoculum, a log-phase bacterial culture was diluted in 1% tryptic soy broth (TSB) to a final optical density at 600 nm (OD_{600}) of 0.01. One milliliter of the inoculum was then injected into the flow cell chamber using a syringe. The inoculated bacteria were allowed to attach to the cover glass under static (no-flow) conditions for 1 h. The flow was then resumed and a growth medium made of 1% TSB was supplied to the flow cell at room temperature (22°C) and a constant flow rate of 10 ml/h for 3 days.

Calcite biomineralization and particle deposition in biofilms. Calcium carbonate biomineralization was induced by treating 3-day-old PAO1 biofilms with a supersaturated calcium carbonate medium composed of 15 mM (each) $CaCl_2$ and $NaHCO_3$ in 1% TSB at a flow rate of 10 ml/h for 12 h. The medium has a pH of 7.6 and a calcium carbonate saturation index with a $\log\Omega$ value of 1.88 at room temperature (~22°C) under atmospheric CO_2 concentrations, yielding the biomineralization of calcium carbonate within the biofilms. We previously showed that this medium is nontoxic to PAO1 biofilms and planktonic cells (29).

In separate experiments, fine abiotic calcite particles were deposited on the surface of 3-day-old PAO1 biofilms. A calcite particle suspension was obtained by mixing 0.5 M solutions of $CaCO_3$ and $NaHCO_3$ for 30 s. The distribution of particle sizes in the suspension is shown in Fig. S1 in the supplemental material. One milliliter of the calcite particle suspension was injected into flow cells containing 3-day-old biofilms. The flow cell was inverted during injection to enable injected particles to settle onto the biofilm surface. Residual suspended particles were then rinsed out with 1% TSB for 15 min at a flow rate of 10 ml/h.

Imaging procedure. Biofilms, mineral precipitates, and injected particles were imaged by confocal microscopy (TCS SP2; Leica) with a 63× oil objective and a 488-nm argon laser. The biofilms were imaged through the visualization of constitutively expressed green fluorescent protein (GFP). Mineral deposits in the biofilms were imaged by collecting the confocal laser reflectance signal following methods described previously (29).

Biofilm permeability assay. Biofilm permeability was analyzed by visualizing the propagation of the fluorescent tracer Cy5 into the biofilm (32). Cy5 does not bind to PAO1 biofilm extracellular polymeric substances (EPS), and the injected Cy5 can be completely washed out from the biofilm (33). One milliliter of a 20- μ g/ml Cy5 solution was injected upstream of the flow cell and then pumped into the flow cell at a constant flow rate of 10 ml/h. Time series images of Cy5 penetration into individual biofilm clusters were then obtained at a frame rate of 0.16 Hz for 15 min. To directly quantify the effects of biomineralization on solute transport into and within biofilms, Cy5 transport was observed before and after biomineralization in a single field of view. In separate experiments, Cy5 transport into the biofilms was also observed before and after particle deposition.

Transport into the biofilm was quantified in terms of an effective diffusion coefficient (D_e) on the basis of the following one-dimensional diffusion model:

$$\frac{\partial C}{\partial t} = D_e \left(\frac{\partial^2 C}{\partial r^2} + \frac{1}{r} \frac{\partial C}{\partial r} \right) - \alpha C \quad (1)$$

where C is the radially averaged Cy5 concentration calculated from the fluorescence intensity measured by confocal microscopy, r is the distance from the biofilm surface, αC represents the first-order removal of Cy5 in the biofilm, and t is time. Equation 1 is in cylindrical coordinates because diffusion was analyzed in horizontal image planes representing cross sections of biofilm clusters, and the transport into the biofilm was observed to be radial at these locations. The values of D_e and α were determined by high-precision numerical solution of equation 1 to the sample experimental data at discrete values r_i . The values of D_e and α minimized the error measurement $\sum_i [C(i) - C(r_i)]^2$, where r_i is the location of the sample points, $C(r_i)$ is the numerical solution of equation 1, and $C(i)$ is the experimentally measured concentration at r_i . Equation 1 includes an effective diffusion coefficient because solute transport in biofilms can involve a combination of diffusion and advection (34). The diffusion curves stabilized near the center of the colony, suggesting the possible sequestration or degradation of Cy5 inside the biofilm. Therefore, we introduced a concentration first-order sink term ($-\alpha C$) in the diffusion model. We use this one-dimensional diffusion model here only to compare changes in effective biofilm transport properties following biomineralization and particle deposition. A more detailed assessment of transport within biofilms should include three-dimensional advection, mixing, sorption, and degradation (34). Details of the diffusion model output are shown in Table S1 in the supplemental material and are discussed in the text in the supplemental material.

Chlorine killing. To assess how biomineralization and particle deposition affected biofilm susceptibility to killing by chlorine, 3-day-old biofilms with and without biomineralization and particle deposition were treated with 100 ppm NaClO for 30 min at a flow rate of 10 ml/h. Following treatment, the biofilms were rinsed with 1% TSB at the same flow rate for 10 min to remove the residual NaClO. Live/dead staining was then used to visualize the resulting pattern of biofilm killing. A 1-ml solution containing 6.7 μ M SYTO 9 and 100 μ M propidium iodide (PI) (FilmTracer LIVE/DEAD biofilm viability kit; Life Technology) was slowly injected into the flow cell and allowed to stain the biofilm for 15 min under static conditions. The unbound stain was then rinsed with 1 ml of 1% TSB before imaging. Stained biofilms were imaged using confocal microscopy as described above.

Permeability and killing assays were also conducted with control biofilms without mineral precipitation. These biofilms were untreated or exposed to undersaturated calcium carbonate solutions composed of 20 mM $CaCl_2$ only, 20 mM $NaHCO_3$ only, and 5 mM (each) $CaCl_2$ and $NaHCO_3$. All of these experiments were performed in triplicate. Live/dead staining of the negative controls for biocide exposure, i.e., biofilms subjected to biomineralization but not chlorine treatment, was also performed to evaluate the effect of biomineralization on cell death in biofilms.

To confirm that the introduced Ca^{2+} or $CaCO_3$ did not affect the vulnerability of PAO1 cells to chlorine, we also assayed the chlorine killing of planktonic PAO1 cells in a 96-well plate. Killing was observed under the following conditions: (i) with 100 ppm NaClO, (ii) with 100 ppm NaClO and 20 mM $CaCl_2$, and (iii) with 100 ppm NaClO, 20 mM $CaCl_2$, and 20 mM $NaHCO_3$. Each well included 40 μ l of PAO1 cell culture (in TSB; log phase, $OD_{600} = 0.5$), 140 μ l of test medium, and 20 μ l of NaClO stock solution (1,000 ppm). Each well had a final chlorine concentration of 100 ppm and a final volume of 200 μ l. Negative controls for killing were also performed without NaClO. The cell cultures were incubated at 22°C with shaking (225 rpm) in a microplate reader (Synergy Mx; BioTek), and the OD_{600} of the culture was monitored for 8 h. Each condition was tested in triplicate.

Image analysis. Three-dimensional images of biofilms, mineral deposits, and chlorine killing patterns were generated from confocal image stacks using the VOLOCITY visualization software package (PerkinElmer Inc.). Cy5 diffusion curves and chlorine killing maps were generated by

FIG 1 PAO1 biofilm morphology after no treatment (a), after *in situ* calcite biomineralization (b), and after calcite particle deposition (c). Images are overlays of calcite deposits (blue) and biofilm biomass (green) (b and c). (Left) Three-dimensional opacity views; (right) orthogonal sections.

analyzing time series Cy5 propagation images and live/dead staining image stacks using the Biofilm Spatial Pattern Analysis (BioSPA) software package. We also used BioSPA to calculate the volume of live and dead biomass (defined as m_L and m_D , respectively). The overall killing efficacy (K) was then calculated as $K = m_D / (m_L + m_D)$. The depth over which chlorine killed the biofilm was also calculated from live/dead image stacks using the diffusion distance function in COMSTAT image analysis software (35).

RESULTS

Spatial patterns of calcite biomineralization and particle deposition in biofilms. Three-day-old PAO1 biofilms showed mushroom-shaped colonies surrounded by flat lawns of cells (Fig. 1a). Three-day-old biofilms exposed to an oversaturated calcium carbonate solution for 12 h biomineralized calcite inside the biofilm structure (Fig. 1b). Calcite biomineralization did not occur under undersaturated conditions (see Fig. S2 in the supplemental material). Conversely, abiotically precipitated particles deposited on the biofilm surface (Fig. 1c). Abiotically precipitated particles were substantially smaller than the biomineralized deposits formed *in situ* (Fig. 1). The diameters of the abiotic particles were primarily in the range of 4 to 6 μm (see Fig. S1 in the supplemental material), whereas the biomineral deposits produced *in situ* were larger than 20 μm (29).

Cy5 transport patterns in biofilms after biomineralization and particle deposition. We visualized the time series propagation of Cy5 into biofilm colonies to quantify the changes in solute transport patterns within the biofilm after biomineralization and particle deposition. As expected, a steep Cy5 concentration gradient from the biofilm surface to the interior developed (Fig. 2a). Cy5 penetration into the biofilm increased substantially after biomineralization, leading to a 30.2% increase in the effective dif-

FIG 2 Cy5 transport in PAO1 biofilm colonies. (Top) Planar heat maps of Cy5 intensity in and around a biofilm colony. Black lines, the edges of the biofilm colonies. Bars = 10 μm . (Bottom) Curves for Cy5 penetration into an untreated biofilm (a), a biofilm after *in situ* biomineralization (b), and a biofilm after particle deposition (c). Images were obtained after 190 s of Cy5 transport, when the concentration profiles reached steady state. The penetration profiles obtained at different times are presented in Fig. S4 in the supplemental material. D_e is the Cy5 effective diffusion coefficient calculated from the concentration-time distribution. The effective diffusion coefficient is not available (N.A.) for the particle deposition case (c) because the one-dimensional diffusion model cannot uniquely distinguish Cy5 propagation through the particle layer and the underlying biofilm on the basis of the resolution of the available data.

FIG 3 (a to c) Chlorine killing in 3-day-old PAO1 biofilms (a), 3-day-old PAO1 biofilms after *in situ* biom mineralization (b), and 3-day-old PAO1 biofilms after particle deposition (c). (Top) Planar confocal images with live/dead staining. Dead cells (stained by PI) appear in red, and live cells appear in green. Three-dimensional opacity views of the same results are shown in Fig. S5 in the supplemental material. (Bottom) Planar killing heat maps. Chlorine killing is limited to the biofilm surface in untreated biofilms (a), occurs much more deeply in biofilms after biom mineralization (b), and is significantly hindered in biofilms after particle deposition (c). (d) Killing efficacy for each treatment. Error bars are standard deviations from triplicate experiments.

fusion coefficient (Fig. 2a and b). Cy5 penetration did not change in control experiments with undersaturated calcium carbonate solutions (see Fig. S3 and Table S1 in the supplemental material), confirming that the enhanced Cy5 transport resulted specifically from biom mineralization. Conversely, particle deposition significantly hindered Cy5 penetration, because particles deposited on the biofilm surface presented a barrier to solute transport into the biofilm (Fig. 1c and 2c). Following particle deposition, the Cy5 concentrations just within the biofilm decreased significantly relative to those in untreated and biom mineralized biofilms (compare the concentration profiles in Fig. 2c [biofilms with particle deposits] with those in Fig. 2a [untreated biofilms] and b [biom mineralized biofilms]), and the concentrations in the biofilm interior continued to decrease. The Cy5 profile shows the radially averaged concentration. The Cy5 concentration observed at the biofilm surface after particle deposition is lower because particles partially block the transport of Cy5 into the biofilm and lower the average Cy5 concentration at the biofilm surface. These results show that *in situ* biom mineralization and particle deposition had opposite impacts on solute transport into and within biofilms: *in situ* biom mineralization enhanced transport, while particle deposition on the biofilm surface hindered transport.

Chlorine killing patterns in biofilms after biom mineralization and particle deposition. Internal transport limitations are recognized to be one of the main reasons for the reduced susceptibility of biofilms to antimicrobials (9), suggesting that the increased biofilm permeability caused by biom mineralization could increase the susceptibility of biofilm-resident cells to chlorine killing. We tested this hypothesis by subjecting untreated biofilms, biom mineralized biofilms, and biofilms onto which particles were deposited to continuously supplied 100 ppm NaClO for 30 min at a flow rate of 10 ml/h (see Materials and Methods). Biom mineralization significantly enhanced the chlorine killing of biofilms (Fig. 3a and b; see

Fig. S5 in the supplemental material). Chlorine treatment of untreated biofilms resulted in the killing of $57.4\% \pm 3.7\%$ of biofilm cells. Chlorine killing of biom mineralized biofilms was dramatically greater: $91.3\% \pm 9.6\%$ (Fig. 3d). Chlorine killing also occurred deeper in biom mineralized biofilms (Fig. 3a and b; see also Fig. S5 and S6 in the supplemental material), suggesting that biom mineralization enhanced the transport of the biocide to the biofilm interior. In biofilms with surface-deposited particles, the chlorine killing efficacy decreased to $25.7\% \pm 11.9\%$, and the killing was limited to a thin layer at the biofilm surface (Fig. 3c and d; see also Fig. S5 and S6 in the supplemental material). All killing results were obtained with biofilms with comparable morphologies, live biomasses, and total biovolumes (see Fig. S7 in the supplemental material). In addition, chlorine treatment did not trigger a significant detachment of the biofilm biomass (see Fig. S8 in the supplemental material). Perturbations of the solution chemistry associated with biom mineralization did not significantly affect cell killing by chlorine: dissolved calcium and carbonate only slightly decreased the killing of planktonic PAO1 cells by chlorine (see Fig. S9 in the supplemental material), while in control experiments with chlorine treatment of biofilms in undersaturated calcium carbonate medium, killing similar to that of untreated biofilms was seen (see Fig. S10 in the supplemental material). Therefore, the observed changes in biofilm killing directly resulted from calcite biom mineralization within the biofilm and abiotic particle deposition on the biofilm surface.

The observed trends in chlorine killing, which was greater in biom mineralized biofilms and lower in biofilms with abiotic particle deposits, exactly follow the trends in solute penetration into the biofilm. Indeed, the chlorine killing efficacy showed a strong positive correlation with the effective diffusion coefficient describing conservative transport into and within the biofilm (Fig. 4; $P < 0.05$). These results indicate specifically that biom mineralization en-

FIG 4 Enhanced solute transport induced by biomineralization increases biofilm killing by chlorine ($P < 0.05$). D_e and D_e^* , the effective diffusion coefficients obtained from Cy5 transport into the same biofilm colony before and after treatment. Component observations are provided in Table S1 in the supplemental material. Averaged over all replicate experiments, biomineralization increased the effective diffusion coefficient by 36.0% and the chlorine killing efficacy by 33.9%. The black dashed line shows a linear regression of all data.

hances killing by increasing the biofilm permeability and, thus, the delivery of the biocide into the interior of the biofilm.

DISCUSSION

We showed that *in situ* calcite biomineralization and the deposition of abiotically precipitated fine calcite particles distinctively impacted biofilm transport processes and susceptibility to chlorine. *In situ* biomineralization enhanced solute penetration and facilitated chlorine killing in *P. aeruginosa* biofilms. Conversely, the deposition of calcite particles onto the biofilm surface hindered solute transport and decreased chlorine killing. The latter observation is consistent with the findings of a previous study showing that the deposition of calcite and clay particles decreased the susceptibility of biofilms to biocides (36). Further, we previously observed that *in situ* biomineralization of calcite occurs throughout the biofilm, while abiotically precipitated particles deposit onto the biofilm surface (29). By combining independent analyses of biofilm morphology, solute transport, and biocide killing, we have provided here mechanistic insight into the link between the accumulation of mineral deposits and biofilm killing: mineral deposits that penetrate the biofilm increase solute transport and susceptibility to biocides and antimicrobials, while mineral deposits that largely cover the biofilm surface hinder the penetration of solutes from the bulk fluid into the biofilm and thus reduce the level of killing by antibiotic agents delivered via the bulk fluid. It is challenging to ascertain the specific role of biomineralization in increasing solute transport in biofilms, as little is known about the impacts of biomineralization on biofilm properties. Solute transport in biofilms is strongly influenced by biofilm structural properties, including internal porosity, cell density, and EPS density (37–39). The results that we have presented here show that *in situ* biomineralization produces minerals that gradually perforate the biofilm architecture (Fig. 1) (29). It is likely that biomineralization increases biofilm permeability both by introducing channels through the biofilm and by reshaping the internal biofilm structure. Biomineralization also clearly increased the het-

erogeneity of the biofilm in terms of the overall architecture and morphology (Fig. 1) (29). We also observed increased fluctuations in Cy5 transport within the biofilm after biomineralization (Fig. 2b), indicating that biomineralization increased the structural heterogeneity of the biofilm to solute transport. We previously observed that biomineralization also triggers significant cell detachment from biofilms (29), which decreases cell density. This provides a mechanism for increased solute transport because biofilm permeability is inversely related to cell density (40). Based on the combination of the new results presented here and information from the literature, we conclude that biomineralization increases biofilm permeability via a combination of increasing permeability by forming pores or channels, decreasing the local cell density, and increasing the internal heterogeneity within the biofilm. Further elucidation of these mechanisms will require detailed characterization of the biofilm fine structure with high-resolution imaging of biofilms with multiple types of microbially catalyzed biomineralization.

Such distinctive impacts of *in situ* biomineralization and particle deposition highlight the importance of understanding the spatial patterns of mineral formation and particle deposition within biofilms. Biomineralization and particle deposition are important in diverse clinical, engineered, and geological settings (24, 41, 42). These processes are expected to be especially important in the development of recalcitrant biofilms in engineered systems that favor the precipitation and deposition of mineral phases, such as water treatment systems, distribution systems, and cooling systems. In systems where particle deposition is the dominant mode of biofilm-mineral interactions, for example, in water filtration systems with high particle inputs (43), biofilms should present a higher recalcitrance because of the decreased susceptibility of particle-coated biofilms to biocides. Conversely, in systems with conditions that are near or above mineral saturation conditions, *in situ* biomineralization is expected to dominate and biofilms should be less recalcitrant and more readily controlled by biocides. However, biomineralization can also disrupt the biofilm structure and release biofilm-resident cells (29), which presents a concern because of the potential for this process to facilitate the dissemination of pathogens (44, 45). It should also be noted that *in situ* biomineralization and the deposition of abiotic particles often occur simultaneously in biofilms under oversaturated conditions (29, 46). Overall changes in key biofilm properties, such as permeability and biocide susceptibility, therefore should depend on the relative importance of these two processes, as they are regulated by the saturation state of CaCO_3 and other common scale-forming minerals, local flow conditions around the biofilms, and biofilm community metabolism. Our findings suggest that both biofilm activity and local precipitation/deposition conditions are critical to the successful control of biofilm growth and scale fouling.

Our findings have further implications for biofilm control in other contexts, particularly biofilm-based infections. Biomineralization is commonly found in catheter-associated urinary tract infections (47, 48). Recent studies have also suggested links between recalcitrant respiratory infections and the formation of pulmonary and bronchial stones (49). Limited penetration of antimicrobials into biofilms is one of the main causes for the failed antimicrobial treatment and recurrent biofilm infections (33, 50, 51). Similar to the patterns in chlorine killing that we observed here, antimicrobial killing activity is also commonly limited to the

biofilm surface (52, 53). Furthermore, biofilm killing by antimicrobials has been found to significantly increase after treatments that enhance antimicrobial penetration (54). Based on the results presented here, we expect that biomining should also considerably affect the efficacy of antimicrobial therapies for biofilm-based infections by changing the patterns of delivery of antimicrobial agents into biofilms.

More generally, internal transport limitations are recognized to be a primary mechanism of biofilm physiological heterogeneity because patterns of substrates, nutrients, and metabolic products within biofilms result from a combination of influx from the surrounding bulk fluid, microbial metabolism, and the internal redistribution of solutes (55–57). Physiological heterogeneity in biofilms therefore both regulates and responds to internal physical and chemical gradients. Here we observed that biomining and particle deposition significantly altered both the rates and patterns of solute transport in biofilms. Associated changes in basic internal biofilm transport processes have important implications, as nutrient and substrate availability regulate local and global metabolism, biofilm growth and cell differentiation, phenotype and genotype distributions, and interspecies interactions in biofilms (58–61). Our findings therefore suggest that biomining and particle deposition are important general regulators of biofilm complexity.

ACKNOWLEDGMENTS

Imaging work was performed at the Northwestern University Biological Imaging Facility, generously supported by the Northwestern University Office for Research. Confocal microscopy was performed on a Leica TCS SP2 laser scanning confocal microscope system purchased with funds from the Northwestern University Office for Research.

X.L. and A.I.P. conceived the project and designed studies. X.L. performed experimental measurements. X.L., W.A.R., P.T.B., and A.I.P. developed the imaging methods. D.L.C. developed the numerical method for diffusion analysis. X.L. and D.L.C. performed image and data analysis. M.R.P. constructed strains and informed the experimental design. X.L. and A.I.P. wrote the paper. All authors discussed the results and commented on the manuscript.

FUNDING INFORMATION

This work was supported by grant R01AI081983 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

- Baker JS, Dudley LY. 1998. Biofouling in membrane systems—a review. *Desalination* 118:81–89. [http://dx.doi.org/10.1016/S0011-9164\(98\)00091-5](http://dx.doi.org/10.1016/S0011-9164(98)00091-5).
- Mattiasandholm T, Wirtanen G. 1992. Biofilm formation in the industry—a review. *Food Rev Int* 8:573–603. <http://dx.doi.org/10.1080/87559129209540953>.
- Hugosson A, Hjorth M, Bernander S, Claesson BEB, Johansson A, Larsson H, Nolskog P, Pap J, Svensson N, Ulleryd P. 2007. A community outbreak of Legionnaires' disease from an industrial cooling tower: assessment of clinical features and diagnostic procedures. *Scand J Infect Dis* 39:217–224. <http://dx.doi.org/10.1080/00365540601001930>.
- Characklis WG, Cooksey KE. 1983. Biofilms and microbial fouling. *Adv Appl Microbiol* 29:93–138. [http://dx.doi.org/10.1016/S0065-2164\(08\)70355-1](http://dx.doi.org/10.1016/S0065-2164(08)70355-1).
- Lim AL, Bai R. 2003. Membrane fouling and cleaning in microfiltration of activated sludge wastewater. *J Membr Sci* 216:279–290. [http://dx.doi.org/10.1016/S0376-7388\(03\)00083-8](http://dx.doi.org/10.1016/S0376-7388(03)00083-8).
- Herzberg M, Elimelech M. 2007. Biofouling of reverse osmosis membranes: role of biofilm-enhanced osmotic pressure. *J Membr Sci* 295:11–20. <http://dx.doi.org/10.1016/j.memsci.2007.02.024>.
- Cloete TE, Jacobs L, Brozel VS. 1998. The chemical control of biofouling in industrial water systems. *Biodegradation* 9:23–37. <http://dx.doi.org/10.1023/A:1008216209206>.
- Cochran WL, McFeters GA, Stewart PS. 2000. Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. *J Appl Microbiol* 88:22–30.
- Mah TFC, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9:34–39. [http://dx.doi.org/10.1016/S0966-842X\(00\)01913-2](http://dx.doi.org/10.1016/S0966-842X(00)01913-2).
- Messiaen AS, Nelis H, Coenye T. 2014. Investigating the role of matrix components in protection of *Burkholderia cepacia* complex biofilms against tobramycin. *J Cystic Fibrosis* 13:56–62. <http://dx.doi.org/10.1016/j.jcf.2013.07.004>.
- Stewart PS, Franklin MJ, Williamson KS, Folsom JP, Boegli L, James GA. 2015. Contribution of stress responses to antibiotic tolerance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 59:3838–3847. <http://dx.doi.org/10.1128/AAC.00433-15>.
- De Beer D, Srinivasan R, Stewart PS. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol* 60:4339–4344.
- Behnke S, Camper AK. 2012. Chlorine dioxide disinfection of single and dual species biofilms, detached biofilm and planktonic cells. *Biofouling* 28:635–647. <http://dx.doi.org/10.1080/08927014.2012.700705>.
- Chen X, Stewart PS. 1996. Chlorine penetration into artificial biofilm is limited by a reaction-diffusion interaction. *Environ Sci Technol* 30:2078–2083. <http://dx.doi.org/10.1021/es9509184>.
- Sheikholeslami R. 1999. Composite fouling—inorganic and biological: a review. *Environ Prog* 18:113–122. <http://dx.doi.org/10.1002/ep.670180216>.
- Ognier S, Wisniewski C, Grasmick A. 2002. Characterisation and modelling of fouling in membrane bioreactors. *Desalination* 146:141–147. [http://dx.doi.org/10.1016/S0011-9164\(02\)00508-8](http://dx.doi.org/10.1016/S0011-9164(02)00508-8).
- Thompson J, Lin N, Lyster E, Arbel R, Knoell T, Gilron J, Cohen Y. 2012. RO membrane mineral scaling in the presence of a biofilm. *J Membr Sci* 415:181–191.
- Li H, Hsieh MK, Chien SH, Monnell JD, Dzombak DA, Vidic RD. 2011. Control of mineral scale deposition in cooling systems using secondary-treated municipal wastewater. *Water Res* 45:748–760. <http://dx.doi.org/10.1016/j.watres.2010.08.052>.
- Flemming HC. 2002. Biofouling in water systems—cases, causes and countermeasures. *Appl Microbiol Biotechnol* 59:629–640. <http://dx.doi.org/10.1007/s00253-002-1066-9>.
- Guo WS, Ngo HH, Li JX. 2012. A mini-review on membrane fouling. *Bioresour Technol* 122:27–34. <http://dx.doi.org/10.1016/j.biortech.2012.04.089>.
- Ben Chekroun K, Rodriguez-Navarro C, Gonzalez-Munoz MT, Arias JM, Cultrone G, Rodriguez-Gallego M. 2004. Precipitation and growth morphology of calcium carbonate induced by *Myxococcus xanthus*: implications for recognition of bacterial carbonates. *J Sediment Res* 74:868–876. <http://dx.doi.org/10.1306/050504740868>.
- Buczynski C, Chafetz HS. 1991. Habit of bacterially induced precipitates of calcium-carbonate and the influence of medium viscosity on mineralogy. *J Sediment Petrol* 61:226–233. <http://dx.doi.org/10.1306/D42676DB-2B26-11D7-8648000102C1865D>.
- Cockell CS, Tsikos H, Durante M, Parnell J. 2007. Microbe-mineral interactions in naturally radioactive beach sands from Espirito Santo, Brazil: experiments on mutagenicity. *Radiat Environ Biophys* 46:247–253. <http://dx.doi.org/10.1007/s00411-007-0100-9>.
- Dupraz C, Reid RP, Braissant O, Decho AW, Norman RS, Visscher PT. 2009. Processes of carbonate precipitation in modern microbial mats. *Earth Sci Rev* 96:141–162. <http://dx.doi.org/10.1016/j.earscirev.2008.10.005>.
- Decho AW. 2010. Overview of biopolymer-induced mineralization: what goes on in biofilms? *Ecol Eng* 36:137–144. <http://dx.doi.org/10.1016/j.ecoleng.2009.01.003>.
- Cox AJ, Hukins DWL. 1989. Morphology of mineral-deposits on encrusted urinary catheters investigated by scanning electron-microscopy. *J Urol* 142:1347–1350.
- Zhao Q, Liu Y, Wang C, Wang S, Muller-Steinhagen H. 2005. Effect of surface free energy on the adhesion of biofouling and crystalline fouling. *Chem Eng Sci* 60:4858–4865. <http://dx.doi.org/10.1016/j.ces.2005.04.006>.
- Hall-Stoodley L, Stoodley P. 2009. Evolving concepts in biofilm infections. *Cell Microbiol* 11:1034–1043. <http://dx.doi.org/10.1111/j.1462-5822.2009.01323.x>.

29. Li X, Chopp DL, Russin WA, Brannon PT, Parsek MR, Packman AI. 2015. Spatial patterns of carbonate biomineralization in biofilms. *Appl Environ Microbiol* 81:7403–7410. <http://dx.doi.org/10.1128/AEM.01585-15>.
30. Moritz MM, Flemming HC, Wingender J. 2010. Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials. *Int J Hyg Environ Health* 213:190–197. <http://dx.doi.org/10.1016/j.ijheh.2010.05.003>.
31. Wang H, Edwards M, Falkinham JO, Pruden A. 2012. Molecular survey of the occurrence of *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa*, and amoeba hosts in two chloraminated drinking water distribution systems. *Appl Environ Microbiol* 78:6285–6294. <http://dx.doi.org/10.1128/AEM.01492-12>.
32. Li X, Song JL, Culotti A, Zhang W, Chopp DL, Lu N, Packman AI. 11 March 2015. Methods for characterizing the co-development of biofilm and habitat heterogeneity. *J Vis Exp* <http://dx.doi.org/10.3791/52602>.
33. Tseng BS, Zhang W, Harrison JJ, Quach TP, Song JL, Penterman J, Singh PK, Chopp DL, Packman AI, Parsek MR. 2013. The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. *Environ Microbiol* 15:2865–2878. <http://dx.doi.org/10.1111/1462-2920.12155>.
34. Stewart PS. 1998. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. *Biotechnol Bioeng* 59:261–272. [http://dx.doi.org/10.1002/\(SICI\)1097-0290\(19980805\)59:3<261::AID-BIT1>3.0.CO;2-9](http://dx.doi.org/10.1002/(SICI)1097-0290(19980805)59:3<261::AID-BIT1>3.0.CO;2-9).
35. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, Molin S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146:2395–2407. <http://dx.doi.org/10.1099/00221287-146-10-2395>.
36. Srinivasan R, Stewart PS, Griebel T, Chen CI, Xu XM. 1995. Biofilm parameters influencing biocide efficacy. *Biotechnol Bioeng* 46:553–560. <http://dx.doi.org/10.1002/bit.260460608>.
37. Sutherland IW. 2001. The biofilm matrix—an immobilized but dynamic microbial environment. *Trends Microbiol* 9:222–227. [http://dx.doi.org/10.1016/S0966-842X\(01\)02012-1](http://dx.doi.org/10.1016/S0966-842X(01)02012-1).
38. Beyenal H, Lewandowski Z. 2002. Internal and external mass transfer in biofilms grown at various flow velocities. *Biotechnol Prog* 18:55–61. <http://dx.doi.org/10.1021/bp010129s>.
39. Vogt M, Flemming HC, Veeman WS. 2000. Diffusion in *Pseudomonas aeruginosa* biofilms: a pulsed field gradient NMR study. *J Biotechnol* 77:137–146. [http://dx.doi.org/10.1016/S0168-1656\(99\)00213-8](http://dx.doi.org/10.1016/S0168-1656(99)00213-8).
40. Stewart PS. 2003. Diffusion in biofilms. *J Bacteriol* 185:1485–1491. <http://dx.doi.org/10.1128/JB.185.5.1485-1491.2003>.
41. Battin TJ, Kaplan LA, Newbold JD, Hansen CME. 2003. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature* 426:439–442. <http://dx.doi.org/10.1038/nature02152>.
42. Donlan RM. 2001. Biofilms and device-associated infections. *Emerg Infect Dis* 7:277–281. <http://dx.doi.org/10.3201/eid0702.010226>.
43. Kang ST, Subramani A, Hoek EMV, Deshusses MA, Matsumoto MR. 2004. Direct observation of biofouling in cross-flow microfiltration: mechanisms of deposition and release. *J Membr Sci* 244:151–165. <http://dx.doi.org/10.1016/j.memsci.2004.07.011>.
44. Langmark J, Storey MV, Ashbolt NJ, Stenstrom TA. 2005. Accumulation and fate of microorganisms and microspheres in biofilms formed in a pilot-scale water distribution system. *Appl Environ Microbiol* 71:706–712. <http://dx.doi.org/10.1128/AEM.71.2.706-712.2005>.
45. Ferguson C, Husman AMD, Altavilla N, Deere D, Ashbolt N. 2003. Fate and transport of surface water pathogens in watersheds. *Crit Rev Environ Sci Technol* 33:299–361. <http://dx.doi.org/10.1080/10643380390814497>.
46. Benzerara K, Menguy N, Lopez-Garcia P, Yoon TH, Kazmierczak J, Tyliszczak T, Guyot F, Brown GE. 2006. Nanoscale detection of organic signatures in carbonate microbialites. *Proc Natl Acad Sci U S A* 103:9440–9445. <http://dx.doi.org/10.1073/pnas.0603255103>.
47. Saint S, Chenoweth CE. 2003. Biofilms and catheter-associated urinary tract infections. *Infect Dis Clin North Am* 17:411–432. [http://dx.doi.org/10.1016/S0891-5520\(03\)00011-4](http://dx.doi.org/10.1016/S0891-5520(03)00011-4).
48. Barber AE, Norton JP, Spivak AM, Mulvey MA. 2013. Urinary tract infections: current and emerging management strategies. *Clin Infect Dis* 57:719–724. <http://dx.doi.org/10.1093/cid/cit284>.
49. Kennedy MP, Noone PG, Carson J, Molina PL, Ghio A, Zariwala MA, Minnix SL, Knowles MR. 2007. Calcium stone lithoptysis in primary ciliary dyskinesia. *Respir Med* 101:76–83. <http://dx.doi.org/10.1016/j.rmed.2006.04.007>.
50. Fux CA, Costerton JW, Stewart PS, Stoodley P. 2005. Survival strategies of infectious biofilms. *Trends Microbiol* 13:34–40. <http://dx.doi.org/10.1016/j.tim.2004.11.010>.
51. de la Fuente-Nunez C, Reffuveille F, Fernandez L, Hancock REW. 2013. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Opin Microbiol* 16:580–589. <http://dx.doi.org/10.1016/j.mib.2013.06.013>.
52. Bjarnsholt T, Ciofu O, Molin S, Givskov M, Hoiby N. 2013. Applying insights from biofilm biology to drug development—can a new approach be developed? *Nat Rev Drug Discov* 12:791–808. <http://dx.doi.org/10.1038/nrd4000>.
53. Hentzer M, Givskov M. 2003. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J Clin Invest* 112:1300–1307. <http://dx.doi.org/10.1172/JCI20074>.
54. Carmen JC, Nelson JL, Beckstead BL, Runyan CM, Robison RA, Schaalje GB, Pitt WG. 2004. Ultrasonic-enhanced gentamicin transport through colony biofilms of *Pseudomonas aeruginosa* and *Escherichia coli*. *J Infect Chemother* 10:193–199. <http://dx.doi.org/10.1007/s10156-004-0319-1>.
55. Xu KD, Stewart PS, Xia F, Huang CT, McFeters GA. 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl Environ Microbiol* 64:4035–4039.
56. Stewart PS, Franklin MJ. 2008. Physiological heterogeneity in biofilms. *Nat Rev Microbiol* 6:199–210. <http://dx.doi.org/10.1038/nrmicro1838>.
57. Zhang W, Sileika TS, Chen C, Liu Y, Lee J, Packman AI. 2011. A novel planar flow cell for studies of biofilm heterogeneity and flow-biofilm interactions. *Biotechnol Bioeng* 108:2571–2582. <http://dx.doi.org/10.1002/bit.23234>.
58. O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. *Annu Rev Microbiol* 54:49–79. <http://dx.doi.org/10.1146/annurev.micro.54.1.49>.
59. Zhang W, Sileika T, Packman AI. 2013. Effects of fluid flow conditions on interactions between species in biofilms. *FEMS Microbiol Ecol* 84:344–354. <http://dx.doi.org/10.1111/1574-6941.12066>.
60. Stoodley P, Dodds I, Boyle JD, Lappin-Scott HM. 1998. Influence of hydrodynamics and nutrients on biofilm structure. *J Appl Microbiol* 85:19S–28S. <http://dx.doi.org/10.1111/j.1365-2672.1998.tb05279.x>.
61. Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56:187–209. <http://dx.doi.org/10.1146/annurev.micro.56.012302.160705>.