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Micron Scale Spatial Measurement of the O_2 Gradient Surrounding a Bacterial Biofilm in Real Time

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ABSTRACT Bacteria alter their local chemical environment through both consumption and the production of a variety of molecules, ultimately shaping the local ecology. Molecular oxygen (O_2) is a key metabolite that affects the physiology and behavior of virtually all bacteria, and its consumption often results in O_2 gradients within sessile bacterial communities (biofilms). O_2 plays a critical role in several bacterial phenotypes, including antibiotic tolerance; however, our understanding of O_2 levels within and surrounding biofilms has been hampered by the difficulties in measuring O_2 levels in real-time for extended durations and at the micron scale. Here, we developed electrochemical methodology based on scanning electrochemical microscopy to quantify the O_2 gradients present above a *Pseudomonas aeruginosa* biofilm. These results reveal that a biofilm produces a hypoxic zone that extends hundreds of microns from the biofilm surface within minutes and that the biofilm consumes O_2 at a maximum rate. Treating the biofilm with levels of the antibiotic ciprofloxacin that kill 99% of the bacteria did not affect the O_2 gradient, indicating that the biofilm is highly resilient to antimicrobial treatment in regard to O_2 consumption.

IMPORTANCE O_2 is a fundamental environmental metabolite that affects all life on earth. While toxic to many microbes and obligately required by others, those that have appropriate physiological responses survive and can even benefit from various levels of O_2, particularly in biofilm communities. Although most studies have focused on measuring O_2 within biofilms, little is known about O_2 gradients surrounding biofilms. Here, we developed electrochemical methodology based on scanning electrochemical microscopy to measure the O_2 gradients surrounding biofilms in real time on the micron scale. Our results reveal that *P. aeruginosa* biofilms produce a hypoxic zone that can extend hundreds of microns from the biofilm surface and that this gradient remains even after the addition of antibiotic concentrations that eradicated 99% of viable cells. Our results provide a high resolution of the O_2 gradients produced by *P. aeruginosa* biofilms and reveal sustained O_2 consumption in the presence of antibiotics.

KEYWORDS biofilm, oxygen, antibiotics, electrochemistry, *Pseudomonas aeruginosa*, antibiotic resistance

Molecular oxygen (O_2) is one of the most important molecules dictating bacterial lifestyle and behavior. For organisms capable of tolerating O_2, it can provide a means to remove excess electrons formed during metabolism. While general fundamentals of O_2 consumption are well established, the role of O_2 is complex in bacterial communities, including those associated with human infection, since O_2 levels vary tremendously based on the infection site and the host response (1–3). In addition,

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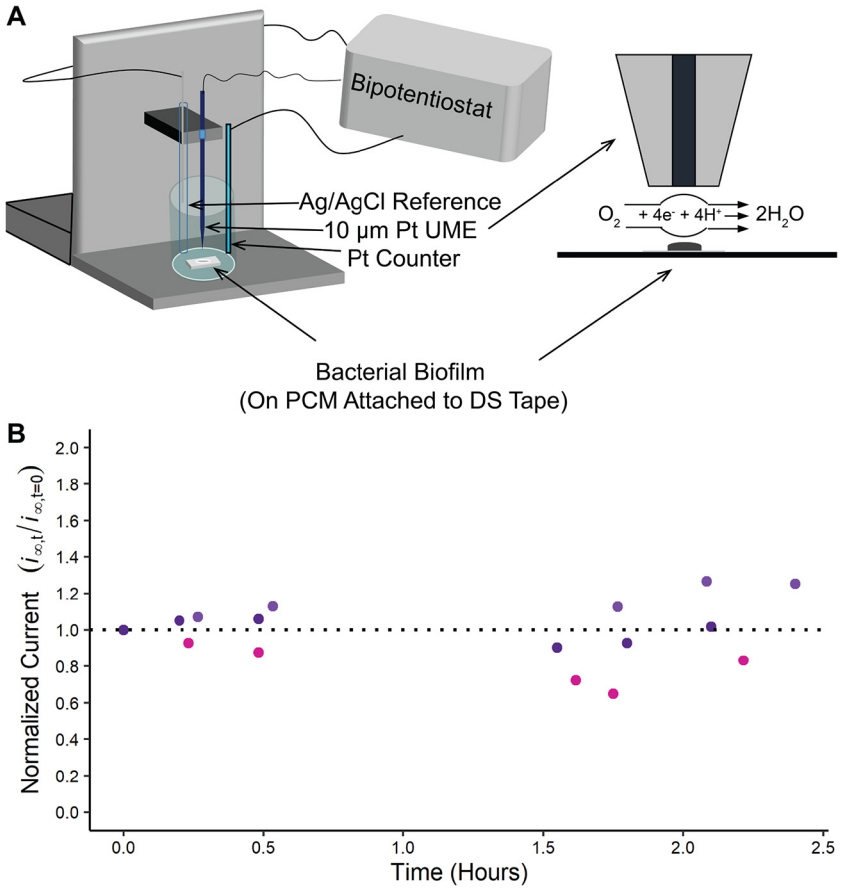

FIG 1 Experimental system and SECM detection of the O_2 gradient surrounding a *P. aeruginosa* biofilm. (A) Schematic of SECM setup for measurement of O_2 gradient surrounding a *P. aeruginosa* biofilm (left), including a closeup of the SECM cell and O_2 reduction reaction at the UME tip (right). (B) The platinized UME continuously monitors bulk O_2 levels through measurement of tip current over several hours without loss of sensitivity. The *y* axis (ordinate) is the ratio of the tip current at each time point divided by the tip current at time zero. Each color represent biological replicates. PCM, polycarbonate membrane; DS, double-sided; UME, ultramicroelectrode.

bacteria in many infections grow as sessile communities called biofilms (4), and the three-dimensional structure of these communities can affect O_2 levels throughout the biofilm.

Previous work has shown that O_2 gradients within biofilms affect their biology (5, 6). This has prompted an examination of O_2 levels within and surrounding biofilms. In particular, stagnant biofilms rapidly deplete O_2 and waste material buildup occurs as a result of mass transport limitation at the surface of biofilms (7). Although it is clear that O_2 levels are decreased within the biofilm, the levels immediately adjacent to the biofilm surface have not been thoroughly investigated in static biofilms, in part due to the difficulties in robustly measuring O_2 with high spatial precision (5, 8–13). To address this gap in knowledge, we developed a system to spatially measure O_2 levels above a microbial biofilm in real time at the micron scale. We chose the facultative anaerobe *Pseudomonas aeruginosa* strain PA14 for these studies since this opportunistic pathogen preferentially utilizes aerobic respiration (14), and its physiology and behavior are highly influenced by O_2 availability (14, 15).

A significant challenge that was overcame is the inherent difficulty with continuously measuring O_2 over extended time periods. To address this challenge, we developed a system using electrochemical methods to measure O_2 in real-time with micron-scale spatial resolution (Fig. 1A). O_2 can be detected electrochemically through a four-electron reduction on a platinum ultramicroelectrode (UME) (Fig. 1A) (16). How-

ever, platinum UMEs readily deactivate which leads to long wait times for O_2 current stabilization and sub-nA current (see Fig. S1 in the supplemental material). To address this challenge, we optimized a platinization protocol that coats the UME surface with platinum particles that actively reduce O_2 while avoiding severe changes in the geometry of the UME surface (see Fig. S2). Importantly, our platinum UMEs had a higher electroactive area and could continuously monitor O_2 levels over several hours without loss of sensitivity (Fig. 1B). This is especially important because the current measured in bulk was approximated to be 205 μM; since current measured is directly proportional to O_2 concentration, stability ensures accurate O_2 measurement despite each platinized UME used only once per experiment and having slightly variable degrees of platinization or size after polishing.

We next sought to measure O_2 levels surrounding a *P. aeruginosa* biofilm using scanning electrochemical microscopy (SECM). The *P. aeruginosa* strain chosen for this work (*fliC9*::MrT7) (17) has an inactivated flagellar motor protein, rendering the strain unable to leave the biofilm via swimming motility. Biofilms of the *P. aeruginosa fliC* mutant were formed on polycarbonate membranes as previously described for electrochemical studies (18). Membrane biofilms were grown for 8 h on Todd Hewitt broth (THB) agar, yielding an ~3-mm-diameter nascent biofilm containing $\sim 4 \times 10^7$ bacteria (see Fig. S3). These biofilms contain fewer cells than those used in previous studies (5, 8) focused on O_2 consumption to better mimic biofilms observed in human infections. After formation, the membrane containing the biofilm was removed from the agar plate and attached to the bottom of a glass vial using double-sided tape and covered with ~5 ml of morpholinepropanesulfonic acid (MOPS)-glucose minimal medium.

To measure O_2 levels above the biofilm, a 10-μm-diameter platinized UME was approached to 40 μm above the biofilm surface using ferrocenyl methyl trimethylammonium ($FcMTMA^+$; the toxicity and stability are assessed in Text S1 in the supplemental material) as the redox mediator (Fig. S4 and S5) using SECM (19). The UME tip was then poised at −0.5 V (O_2 reduction potential), with a wait time of 5 min; afterward, the UME was retracted at 6 μm/s while continually measuring O_2 until bulk O_2 levels were detected, ~1,400 μm above the biofilm surface (the O_2 gradient calculations are detailed in Text S1). The O_2 levels above the biofilm resembled a sigmoidal curve with no O_2 detectable until ~200 μm above the biofilm (Fig. 2A; see also Fig. S6). Assuming a 10-pA minimal background current, the detection limit of the UME is ~1 μM.

For comparison, we created an O_2 gradient without a biofilm using a platinum electrode the same size as the biofilm as the SECM substrate. The 3-mm platinum electrode was held at three potentials (0.1, 0, and −0.5 V versus Ag/AgCl) for 5 min, and then the O_2 gradient was measured as described for the biofilm (detailed in Text S1). The biofilm O_2 gradient was similar to the −0.5 V poised electrode gradient, which is the potential at which O_2 reduction is mass transport limited at the surface of the electrode. The biofilm O_2 gradient was distinct from the other potentials at which O_2 was being consumed at a submaximal rate (i.e., limited in part by kinetics and not predominantly by mass transport). Using Comsol Multiphysics to digitally simulate O_2 consumption (Fig. 2B; see also Fig. S7), we approximated the flux of O_2 at the surface of the biofilm to be 8.2×10^{-7} mol/cm²/s (detailed in Text S1). Assuming each cell has a dimension of 1.5 μm × 0.8 μm, 9.8×10^{-15} mol/s O_2 or 5.9×10^9 molecules of O_2 per second were consumed by each bacterium. Collectively, these results reveal that *P. aeruginosa* biofilms produce a hypoxic zone that can extend hundreds of microns from the biofilm surface within minutes, and the biofilm consumes O_2 at a maximum rate.

To assess the effect of antibiotic treatment on biofilm O_2 consumption, we treated our biofilms with 400 times the MIC of the antibiotic ciprofloxacin (40 μg/ml) and then measured the O_2 gradient above the biofilm. We first confirmed that ciprofloxacin does not interfere with the electrochemical signal for O_2 quantification (Fig. S8; see also Text S1). Ciprofloxacin treatment of the biofilm was performed by initially adding 20 μg/ml ciprofloxacin and measuring the O_2 response 1.5 h after submersion in MOPS-glucose (Fig. 2C). After we observed no immediate change in signal, we treated the biofilm with another 20 μg/ml. After addition of the second dose of ciprofloxacin, the O_2 gradient

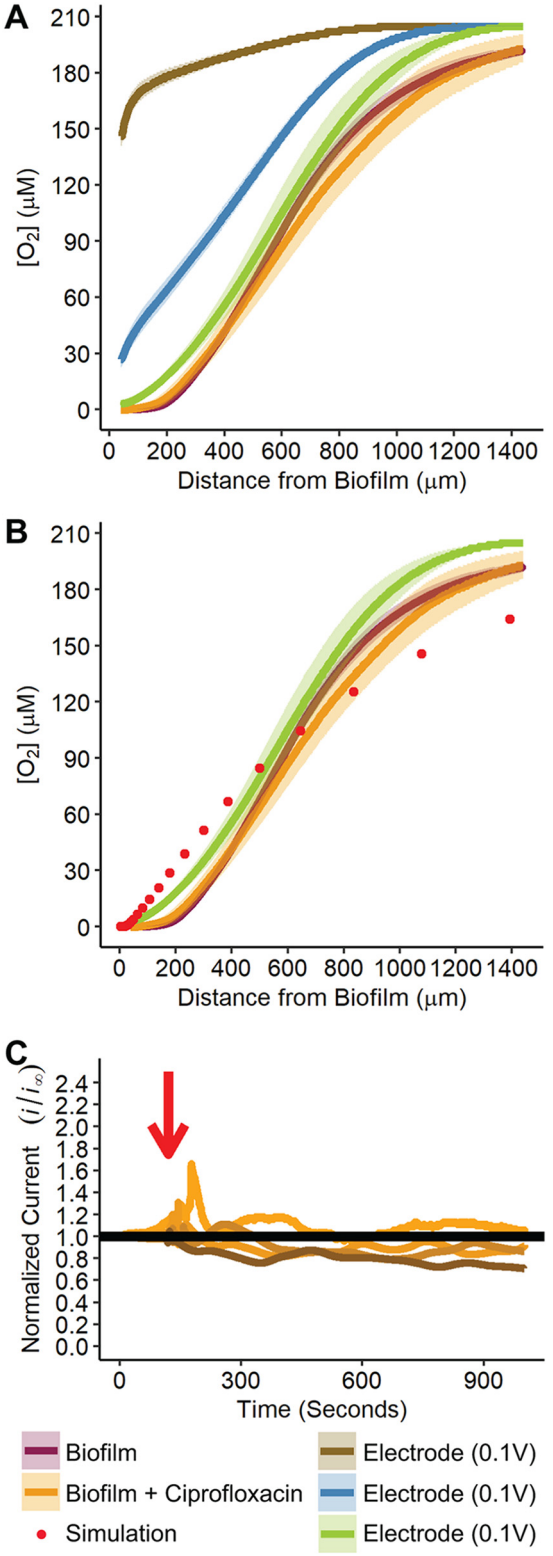

FIG 2 *P. aeruginosa* rapidly produce O_2 gradients that are resilient to antibiotic treatment. (A) O_2 gradients above the surface of *P. aeruginosa* biofilms, *P. aeruginosa* biofilms treated with ciprofloxacin, and for reference a 3-mm platinum electrode poised at 0, 0.1, and −0.5 V versus Ag/AgCl (different electrode potentials correspond to various O_2 consumption rates). $n = 4$ biological replicates for Electrode 0.1 V, Electrode 0 V, Electrode −0.5 V, and Biofilm + Ciprofloxacin, and $n = 16$ biological replicates for biofilm. For all O_2 gradients, shading represents one standard deviation from the mean (solid line). (B) Digital simulation (red circles) to estimate O_2 consumption rates of the biofilm. The model was solved by Comsol Multiphysics (5.3a; COMSOL, Inc., Burlington, MA) using the electrochemical

(Continued on next page)

was measured for 50 min with no observable change in the O_2 gradient, a total of 1 h and 35 min after ciprofloxacin was first added. Despite the fact that addition of ciprofloxacin reduced the number of viable bacteria in the biofilm by 100-fold to $\sim 2.4 \times 10^5$ bacteria, there was no change in the O_2 gradient or O_2 consumption rates (Fig. 2A and B).

While prior work has primarily measured bulk O_2 at the biofilm/air interface, we show that, in contrast, at a stagnant biofilm/liquid interface a hypoxic region forms several hundred microns above the biofilm surface. Containing only $\sim 4 \times 10^7$ bacteria, our biofilms consumed O_2 at maximum rates and continued to do so despite 99% killing by ciprofloxacin. These data corroborate similar findings that bulk respiratory activity and carbon consumption persists despite antibiotic exposure (20, 21). Given this high O_2 consumption rate and the observation that biofilms of this size exist in human implant/catheter infections (22), we propose that biofilms are capable of rapidly depleting local O_2 in chronic infections even during antibiotic challenge. Ultimately, the experimental system developed in this work provides a valuable framework for studying biofilm O_2 consumption.

MATERIALS AND METHODS

Instrumentation. Initial electrochemistry experiments were performed using a BioLogic SECM (model M470). Biofilm experiments measuring O_2 gradients by scanning distances were done using a CHI model 920D scanning electrochemical microscope (CH Instruments). For all experiments, a three-electrode setup was used. This consisted of a 10-μm-diameter platinum UME (working electrode), Ag|AgCl|Saturated KCl (reference electrode to which all potentials are referred to in all experiments), and platinum wire (counter electrode). An in-depth protocol for UME fabrication may be found elsewhere (19).

Ultramicroelectrode fabrication and SECM cell setup. An in-depth protocol for UME fabrication may be found elsewhere (19). Briefly, platinum (99.9% purity) wire, 10-μm diameter, temper: hard (Goodfellow Metals, Cambridge, United Kingdom; product PT005107) was used for the preparation of the SECM UME tip. The metal wire was heat sealed with a heating coil under vacuum in a glass capillary. The tip was sharpened to an RG of $\sim$10, where RG is the ratio of the glass diameter to wire diameter. Prior to electrochemical experiments, UMEs were sonicated in a water bath for 30 s. Platinization significantly alters the surface and UMEs were seldom repolished and replatinized for reuse.

Bacterial strain culture and preparation. *P. aeruginosa* (PA14) *fliC9*::MrT7 mutant was obtained from a PA14 nonredundant transposon insertion mutant set (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi) (17). Biofilms were grown in THB agar for 8 h at 37°C, at which point an $\sim$3-mm-diameter biofilm formed before transfer to the SECM cell. All SECM experiments were performed using MOPS minimal media (23) containing 20 mM glucose. CFU were enumerated at the end of experimentation by removing media above the biofilm, substantially vortexing the biofilm off the polycarbonate membrane, and plating on THB agar plates overnight at 37°C.

Platinizing UMEs. Handmade 10 μm platinum UMEs (as described above) were sonicated in water, acetone, and water. A modified protocol for platinizing UMEs was used that may be found elsewhere (24) with an adjusted recipe for the platinization solution containing 0.250 ml of H_2PtCl_6 and 0.4 mg of $Pb(NO_3)_2$ up to a final volume of 7.36 ml in 1$\times$ phosphate-buffered saline (pH 7.4). Geometric and electroactive effects on the UME surface resulting from platinization were measured to confirm the stability and reproducibility of platinization. An in-depth review of platinizing electrodes can be found elsewhere (25).

Measuring O_2. PA14 tn::*fliC* biofilms were grown as described above. After 8 h growth, the polycarbonate membrane was removed and attached to the bottom of a custom glass vial using double sided tape. UMEs were cycled in platinizing solution (same as above) from 0.2 V to -0.3 V versus Ag/AgCl at 100 mV/s until the maximum limiting current increased $\sim 1.2\times$ for $FcMTMA^+$ oxidation (the synthesis is detailed in Text S1). After platinization and ensuring proper geometric area of the UMEs, 1 mM $FcMTMA^+$ was added to MOPS-glucose media, and approximately 5 ml was added to the vial containing the biofilm. A three-electrode setup using a platinum wire counter, and Ag/AgCl reference electrodes were connected. Platinized UMEs were precisely positioned with micron-scale accuracy using SECM. SECM

FIG 2 Legend (Continued)
analysis module in two-dimensional axial symmetry using stationary conditions with a parametric sweep of the "d" or distance between UME tip and substrate (Fig. S5, and detailed in Text S1). (C) Changes in O_2 concentration 600 μm above a biofilm measured as a response to ciprofloxacin treatment. At 120 s, the first dose of 20 μg/ml ciprofloxacin was added (designated by red arrow). Each line represents a biological replicate. The *y* axis (ordinate) is the ratio of the tip current at each time point divided by current measured before ciprofloxacin addition (i.e., a value of 1 indicates no change in current after ciprofloxacin addition). There were changes immediately after ciprofloxacin addition (peaks at red arrow), likely a result of the mixing caused by addition of ciprofloxacin to the growth media above the biofilm. Importantly, the current quickly stabilized.

positions UMEs at defined distances from the biofilm surface using an electroactive mediator while observing tip current changes as a function of distance. For this work, we chose the electroactive mediator $FcMTMA^+$ since it is neither consumed by nor is toxic to *P. aeruginosa*. UMEs were poised at 0.5 V to oxidize $FcMTMA^+$, approached within ~40 μm above the surface (within the hindered diffusion region corresponding to a decrease in signal to ~95% limiting current), and then poised at −0.5 V and retracted at 6 μm/s to measure O_2 gradients. For antibiotic chronoamperometry curves, UMEs were positioned first approximately 600 μm for the first addition of ciprofloxacin or control, approximately 1 h and 30 min elapsed after MOPS-glucose was added over the biofilm. Ciprofloxacin was added slowly during this time at ~7.5 mm above the biofilm, and the stage was attached to the vial containing the biofilm was rotated 10 times in a circular motion immediately after addition. The UME was then approached approximately 300 μm above the biofilm for the second addition of ciprofloxacin, approximately 2 h elapsed after MOPS-glucose was added over the biofilm. Ciprofloxacin or control was then added quickly at ~7.5 mm above the biofilm. For both additions, a 2-min window was given before antibiotics were added to the media and current was measured for a minimum of 1,000 s in total. O_2 concentration gradients were immediately measured in triplicate during each biological replicate after the second addition of antibiotics to determine the O_2 consumption rates.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, PDF file, 0.2 MB.

FIG S1, PDF file, 0.1 MB.

FIG S2, PDF file, 0.1 MB.

FIG S3, PDF file, 0.04 MB.

FIG S4, PDF file, 0.1 MB.

FIG S5, PDF file, 0.3 MB.

FIG S6, PDF file, 0.2 MB.

FIG S7, PDF file, 0.03 MB.

FIG S8, PDF file, 0.2 MB.

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REFERENCES

1. Mettraux G, A Gusberti F, Graf H. 1984. Oxygen tension (pO2) in untreated human periodontal pockets. J Periodontol 55:516–521. https://doi.org/10.1902/jop.1984.55.9.516.
2. Hopf HW, Hunt TK, West JM, Blomquist P, Goodson WH, Jensen JA, Jonsson K, Paty PB, Rabkin JM, Upton RA, von Smitten K, Whitney JD. 1997. Wound tissue oxygen tension predicts the risk of wound infection in surgical patients. Arch Surg 132:997–1004. https://doi.org/10.1001/archsurg.1997.01430330063010.
3. Kalani M, Brismar K, Fagrell B, Ostergren J, Jörneskog G. 1999. Transcutaneous oxygen tension and toe blood pressure as predictors for outcome of diabetic foot ulcers. Diabetes Care 22:147–151. https://doi.org/10.2337/diacare.22.1.147.
4. James GA, Swogger E, Wolcott R, Pulcini E. d, Secor P, Sestrich J, Costerton JW, Stewart PS. 2008. Biofilms in chronic wounds. Wound Repair Regen 16:37–44. https://doi.org/10.1111/j.1524-475X.2007.00321.x.
5. Walters MC, III, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 47:317–323. https://doi.org/10.1128/aac.47.1.317-323.2003.
6. Wessel AK, Arshad TA, Fitzpatrick M, Connell JL, Bonnecaze RT, Shear JB, Whiteley M. 2014. Oxygen limitation within a bacterial aggregate. mBio 5:e00992. https://doi.org/10.1128/mBio.00992-14.
7. Stewart PS. 2012. Mini-review: convection around biofilms. Biofouling 28:187–198. https://doi.org/10.1080/08927014.2012.662641.
8. Dietrich LEP, Okegbe C, Price-Whelan A, Sakhtah H, Hunter RC, Newman DK. 2013. Bacterial community morphogenesis is intimately linked to the intracellular redox state. J Bacteriol 195:1371–1380. https://doi.org/10.1128/JB.02273-12.
9. Ahimou F, Semmens MJ, Haugstad G, Novak PJ. 2007. Effect of protein, polysaccharide, and oxygen concentration profiles on biofilm cohesiveness. Appl Environ Microbiol 73:2905–2910. https://doi.org/10.1128/AEM.02420-06.
10. Kühl M, Jørgensen BB. 1992. Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. Appl Environ Microbiol 58:1164–1174. https://doi.org/10.1128/AEM.58.4.1164-1174.1992.
11. de Beer D, Stoodley P, Roe F, Lewandowski Z. 1994. Effects of biofilm structures on oxygen distribution and mass transport. Biotechnol Bioeng 43:1131–1138. https://doi.org/10.1002/bit.260431118.
12. Rasmussen K, Lewandowski Z. 1998. Microelectrode measurements of local mass transport rates in heterogeneous biofilms. Biotechnol Bioeng 59:302–309. https://doi.org/10.1002/(SICI)1097-0290(19980805)59:3<302::AID-BIT6>3.0.CO;2-F.
13. Staal M, Borisov SM, Rickelt LF, Klimant I, Kühl M. 2011. Ultrabright planar optodes for luminescence life-time based microscopic imaging of O_2 dynamics in biofilms. J Microbiol Methods 85:67–74. https://doi.org/10.1016/j.mimet.2011.01.021.
14. Alvarez-Ortega C, Harwood CS. 2007. Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. Mol Microbiol 65:153–165. https://doi.org/10.1111/j.1365-2958.2007.05772.x.

15. Schertzer JW, Brown SA, Whiteley M. 2010. Oxygen levels rapidly modulate *Pseudomonas aeruginosa* social behaviours via substrate limitation of PqsH. Mol Microbiol 77:1527–1538. https://doi.org/10.1111/j.1365-2958.2010.07303.x.
16. Sánchez-Sánchez CM, Bard AJ. 2009. Hydrogen peroxide production in the oxygen reduction reaction at different electrocatalysts as quantified by scanning electrochemical microscopy. Anal Chem 81:8094–8100. https://doi.org/10.1021/ac901291v.
17. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM. 2006. An ordered, nonreundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc Natl Acad Sci U S A 103:2833–2838. https://doi.org/10.1073/pnas.0511100103.
18. Liu X, Ramsey MM, Chen X, Koley D, Whiteley M, Bard AJ. 2011. Real-time mapping of a hydrogen peroxide concentration profile across a polymicrobial bacterial biofilm using scanning electrochemical microscopy. Proc Natl Acad Sci U S A 108:2668–2673. https://doi.org/10.1073/pnas.1018391108.
19. Bard AJ, Mirkin MV. 2012. Scanning electrochemical microscopy, 2nd ed. Taylor & Francis, Abingdon-on-Thames, UK.
20. Stewart PS, White B, Boegli L, Hamerly T, Williamson KS, Franklin MJ, Bothner B, James GA, Fisher S, Vital-Lopez FG, Wallqvist A. 2019. Conceptual model of biofilm antibiotic tolerance that integrates phenomena of diffusion, metabolism, gene expression, and physiology. J Bacteriol 201:e00307-19. https://doi.org/10.1128/JB.00307-19.
21. Simkins JW, Stewart PS, Codd SL, Seymour JD. 2019. Non-invasive imaging of oxygen concentration in a complex *in vitro* biofilm infection model using (19) F MRI: persistence of an oxygen sink despite prolonged antibiotic therapy. Magn Reson Med 82:2248–2256. https://doi.org/10.1002/mrm.27888.
22. Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, Jensen PØ, Høiby N. 2013. The *in vivo* biofilm. Trends Microbiol 21:466–474. https://doi.org/10.1016/j.tim.2013.06.002.
23. Palmer KL, Mashburn LM, Singh PK, Whiteley M. 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. J Bacteriol 187:5267–5277. https://doi.org/10.1128/JB.187.15.5267-5277.2005.
24. Li Y, Sella C, Lemaître F, Guille Collignon M, Thouin L, Amatore C. 2013. Highly sensitive platinum-black coated platinum electrodes for electrochemical detection of hydrogen peroxide and nitrite in microchannel. Electroanalysis 25:895–902. https://doi.org/10.1002/elan.201200456.
25. Feltham AM, Spiro M. 1971. Platinized platinum electrodes. Chem Rev 71:177–193. https://doi.org/10.1021/cr60270a002.