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Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance

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Variations in biofilm formation by, and antibiotic resistance of, *Pseudomonas aeruginosa* PAO1 (wild type) and the quorum-sensing-deficient mutants PDO100 (Δ*rhl*I), JP1 (Δ*las*I) and JP2 (Δ*las*IΔ*rhl*I) were studied. For PAO1, the maximum-accumulation phase of biofilm formation began immediately and a plateau phase was reached after 24 h, whereas the quorum-sensing mutants showed 36–48 h lags before entering the maximum-accumulation phase. After 72 h, the cell density of the PAO1 biofilms was *c.* 0.8–1.2 log greater than for the mutants. On a unit protein basis, total polysaccharide production was similar for PAO1 and PDO100, whereas JP1 and JP2 biofilms accumulated only *c.* 36% of the PAO1 level after 72 h. Fluorescent micrographs revealed that the PAO1 biofilms were much thicker than those of the quorum-sensing-deficient mutants. In the case of the PAO1 and PDO100 biofilms, most cells were attached to the top of the biofilm layer, whereas the bottom layer consisted predominantly of polysaccharides. The JP1 and JP2 biofilms were closely packed with cells, and little polysaccharide was visible. Cells in PAO1 biofilms were little affected by kanamycin, even at 100 mg/L, whereas those in PDO100 biofilms were susceptible to the highest concentration of kanamycin (100 mg/L) but not to lower concentrations (10 and 50 mg/L). In contrast, cells in JP1 and JP2 biofilms were susceptible to kanamycin at all three concentrations.

Introduction

The formation and persistence of biofilms can result in elevated transfer of antibiotic resistance, material deterioration and health risks.[1] The main strategies for biofilm control rely on chemical biocides or antibiotics that kill the attached microorganisms and/or remove them from the surface; however, biofilms are infamous for their resistance to antimicrobial agents. Most proposed mechanisms for the enhanced resistance to antibacterial agents observed with biofilms focus on transport limitations[2,3] and physiological adaptation.[4,5] In recent years, it has become evident that many bacteria use cell-to-cell communication systems to regulate diverse physiological processes.[6] This inter-cell communication involves a phenomenon called quorum sensing, in which bacterial cells activate specific genes in response to chemical signals released by the cells themselves into the environment. Only when a threshold concentration of the signal chemical is achieved, at high population densities, is the response triggered. Many such signal molecules are homoserine lactones (HSLs). For biofilms, cell densities may be in the region of 1.0×10^{10} cells/cm^3, or the equivalent, with the increased physical proximity of biofilm cells providing an ideal environment for inter-cell communication. These characteristics indicate that HSL-mediated gene expression in biofilms is distinctly possible and, therefore, potentially of great importance for biofilm formation and antibiotic resistance.

Although cell-to-cell communication in planktonic culture has been studied for many years, little is known about the effects of quorum sensing on biofilm formation and antibiotic resistance. Davies *et al.*[7] first demonstrated the existence of intracellular communication for *Pseudomonas aeruginosa* biofilm development. The quorum-sensing signal molecules of *P. aeruginosa* have been identified as *N*-butanoyl-L-homoserinelactone (C4-HSL) and *N*-(3-oxo-dodecanoyl)-L-homoserinelactone (3-oxo-C12-HSL), generated by the expression of *las*I and *rhl*I, respectively.[8,9] Those researchers used scanning confocal laser microscopy (SCLM) to differentiate biofilm formation by wild-type

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and Δ*las*I mutant forms of *P. aeruginosa*. They found that Δ*las*I mutant biofilms were thinner than those of the wild-type organism under the same culture conditions. It is hypothesized that the production of extracellular polysaccharide (EPS) in *P. aeruginosa* biofilms is influenced by the expression of *las*I and *rhl*I; however, the extent to which production of EPS is affected by quorum-sensing mechanisms is currently unknown. It is also proposed that EPS protects biofilm cells from interaction with antimicrobial agents. Although the role of quorum sensing in antimicrobial resistance is not yet clear, Brown & Barker[10] speculated that biofilm growth leads to an early general stress response (GSR), a major factor in bacterial antibiotic resistance. The GSR is mediated by a sigma factor, RpoS, which is regulated by quorum sensing in *P. aeruginosa*.[11] Davies *et al.*[7] also reported that *las*I mutant biofilms are more susceptible to SDS treatment than those of wild-type organisms. Since EPS absorbs and/or deactivates biocides,[12,13] it seems reasonable to speculate that biofilm antibiotic resistance could also be influenced by quorum-sensing systems.

Although evidence from SCLM demonstrated that *P. aeruginosa* biofilm formation is reduced by mutations in *las*I, expensive laboratory instruments, not typical of the general microbiological laboratory, are required to conduct this type of experiment. The aim of this paper is to demonstrate an economical approach to such study and to provide confirmation that biofilm formation and antibiotic resistance are affected by quorum-sensing deficiency.

Material and methods

Strains and media

The *P. aeruginosa* variants, wild-type PAO1, single mutants JP1 (Δ*las*I::Tn*10*, Tc^r) and PDO100 (Δ*rhl*I::Tn*501*, Hg^r) and double mutant JP2 (Δ*las*I::Tn*10*, Tc^r ; Δ*rhl*I::Tn*501*, Hg^r), were used for batch culture and biofilm continuous cultivation, for all experiments. The mutants were kindly provided by Dr Barbara H. Iglewski (Department of Microbiology and Immunology, University of Rochester, NY, USA). These strains were maintained on Luria–Bertani (LB) agar (Difco, Detroit, MI, USA) with appropriate selection and were re-streaked every 4 weeks. Trypticase Soy Broth (TSB; Difco) was used as liquid medium throughout. To maintain the cell concentrations at *c.* 10^8–10^9 cells/mL, 1/10 strength TSB was used for planktonic cultures, with 1/100 TSB used for continuous biofilm cultures.

Biofilm formation procedure

Biofilms were cultivated using drip-flow plate reactors, the detailed procedure for which has been described elsewhere.[14] They were grown on 316L stainless-steel slides (1.3 × 7.6 cm) held in parallel polycarbonate chambers, and one slide was sampled every 12 h. After sampling, the biofilm on the slide was scraped into 50 mL phosphate buffer with a sterile Teflon scrapper and homogenized using a PRO250 homogenizer (PRO Scientific Inc., Monroe, CT, USA). Homogenized biofilm suspensions were used for further analysis.

Cell density, protein and total polysaccharide assay

The homogenized biofilm samples were analysed for viable-cell and total-cell densities, total polysaccharide content and protein concentration. Viable-cell numbers were determined by serial dilution and plating on R2A agar (Difco). For total-cell numbers, suitably diluted samples were stained with 0.01% (w/v) acridine orange (Sigma, St Louis, MO, USA), and cell counts were calculated using an E600 fluorescence microscope (Nikon, Tokyo, Japan). Total polysaccharide content was determined by the phenol–sulphuric acid method,[15] with protein assessed using the total protein kit (690A, Sigma).

Fluorescent staining, cryoembedding, cryosectioning and microscopy

After 72 h of continuous culture, biofilm samples were fixed with 2.5% (v/v) formaldehyde for 45 min and then stained simultaneously with calcofluor white (75 mg/L; Sigma) and ethidium bromide (1 mg/L; Sigma) for 20 min.[16] After staining, samples were cryoembedded with Tissue-Tek OCT 4583 compound (Sakura Finetechnical Co., Tokyo, Japan), and a cryostat (Reichert-Jung Cryocut 1900, Leica Inc., Deerfield, IL, USA) was used to slice the cryo-embedded samples into 5 μm sections. Biofilms were visualized using a fluorescence microscope (E600, Nikon). Cells stained by ethidium bromide were revealed by red fluorescence detected through a Nikon G-2A filter (excitation filter, 510–560 nm; dichroic mirror, 575 nm; barrier filter, 590 nm). EPS, stained with calcofluor white, appeared as blue-white fluorescence through a UV-2A filter (excitation filter, 330–380 nm; dichroic mirror, 400 nm; barrier filter, 420 nm).

Resistance of biofilm to antibiotics

The MIC of kanamycin (Sigma) was 10 mg/L for *P. aeruginosa* PAO1 in planktonic culture and 5–7.5 mg/L for PDO100, JP1 and JP2. In biofilm resistance experiments, 1 ×, 5 × and 10 × MIC of kanamycin for PAO1 were used. After biofilms were grown for 72 h, medium containing kanamycin 10, 50 or 100 mg/L was fed to the biofilm at a steady flow rate for 2 h. The biofilm was sampled at 30 min intervals and cells were suspended in phosphate buffer by homogenization, as described above. Viable-cell and total-cell densities were determined. The surviving cell fraction was defined as the ratio of viable-cell count to total-cell count.

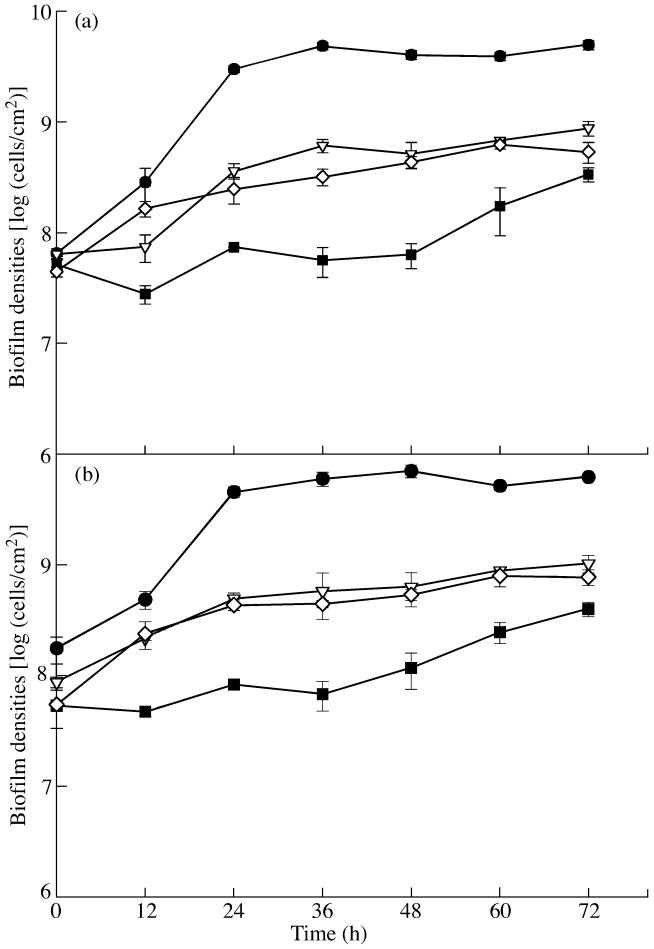

Figure 1. Net accumulation of *P. aeruginosa* PAO1 (●), PDO100 (▽), JP1 (■) and JP2 (◇), as determined by (a) viable-cell counts and (b) total-cell counts. $n = 3$; bar indicates S.E.

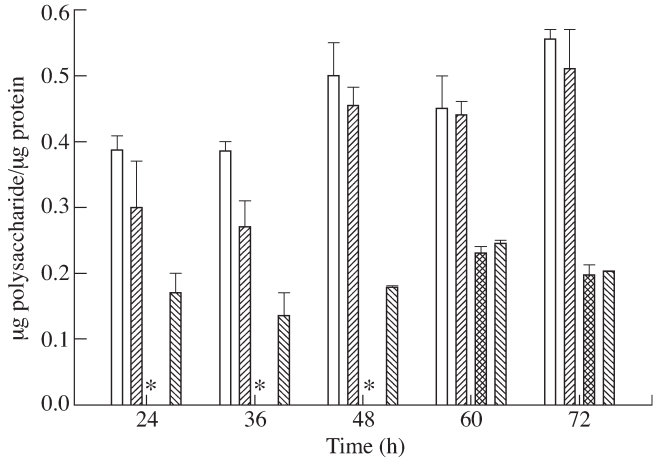

Figure 2. Total polysaccharide production relative to unit protein for *P. aeruginosa* PAO1 (□), PDO100 (▨), JP1 (▩) and JP2(▧). Asterisks indicate where JP1 was below protein detection limits. $n = 3$; bar indicates S.E.

Results

Biofilm cell density

Figure 1 depicts the net accumulations of *P. aeruginosa* wild type (PAO1) and the quorum-sensing-deficient mutants (PDO100, JP1 and JP2) in biofilms. There was no significant difference between the biofilm cell densities of the wild type and the mutants at the beginning. The wild type entered a maximum-accumulation phase immediately, reaching an accumulation plateau after 24 h, as shown by the results for both viable-cell and total-cell counts. After 72 h of accumulation, the PAO1 biofilm cell density was 9.70 ± 0.04 log cfu/cm^2 for the viable-cell count and 9.79 ± 0.02 log cell/cm^2 for the total-cell count. Cells in PDO100 and JP2 biofilms accumulated slowly for the first 36 h and then entered a stationary phase, whereas those in the JP1 biofilm showed little increase for the first 48 h. In this case, a cell density similar to those of PDO100 and JP2 was reached after 72 h. The biofilm accumulation for the wild type was more rapid than the quorum-sensing-deficient mutants and reached a steady state faster than the mutants, with a cell density 0.8–1.2 log higher than those of the mutants by the end of the experiments.

Biofilm polysaccharide production

Figure 2 presents total polysaccharide production relative to protein content for *P. aeruginosa* wild type (PAO1) and the quorum-sensing-deficient mutants (PDO100, JP1 and JP2). The polysaccharide production for PAO1 was steady for the first 36 h, and then increased over the following 36 h, with production of 0.56 ± 0.02 μg polysaccharide/μg protein after 72 h of accumulation. The polysaccharide production for PDO100 was similar to that for PAO1, increasing with time and reaching 0.51 ± 0.06 μg polysaccharide/μg protein by the end of the experiment. In comparison with PAO1 and PDO100, the polysaccharide production by JP1 and JP2 was markedly lowered. The total polysaccharide content for JP1 and JP2 biofilms did not increase significantly with time and was *c.* 36% of that of PAO1 at 72 h (0.20 ± 0.01 μg polysaccharide/μg protein).

Fluorescence micrographs

Ethidium bromide and calcofluor white were used to distinguish cells and EPS within the biofilms. Using cryo-embedding and cryosectioning techniques, the spatial distribution of cells and EPS within biofilms was observed. Figure 3 illustrates representative patterns for *P. aeruginosa* biofilm cells and EPS after 72 h of accumulation. The wild-type (PAO1) biofilms were much thicker than those of the quorum-sensing-deficient mutants (PDO100, JP1 and JP2), with PAO1 biofilm thickness ranging from 80 to 120 μm, whereas that for PDO100 was 20–40 μm and that for JP1 and JP2 was <20 μm. For PAO1 biofilms, most cells

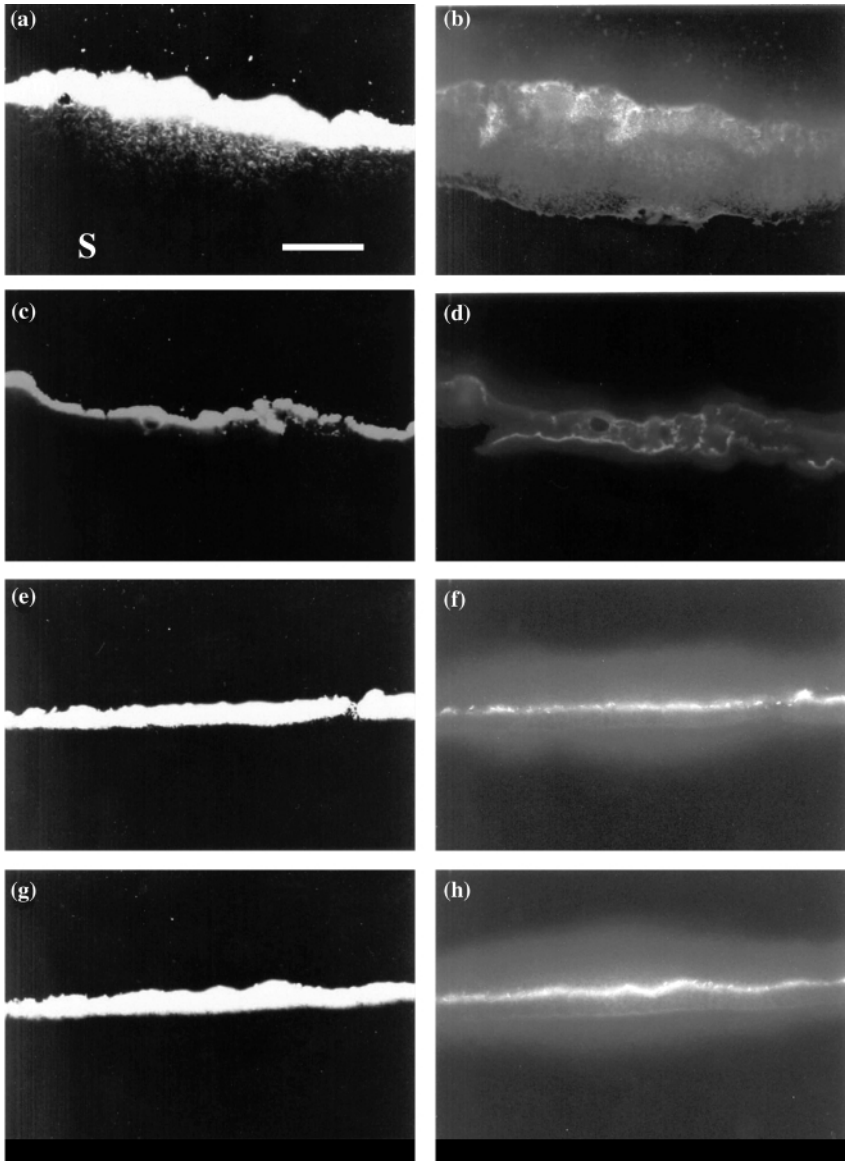

Figure 3. Epifluorescence micrographs of *P. aeruginosa* biofilm cross-sections stained by ethidium bromide (left, red under G-2A filter) and calcofluor white (right, blue-white under UV-2A filter): (a and b) PAO1; (c and d) PDO100; (e and f) JP1; (g and h) JP2. S, substratum; bar = 50 μm.

were found on the top layer, whereas EPS was found predominantly in the bottom layer (Figure 3a and b). Similar cell/EPS distribution was observed for PDO100 biofilms, although the film was less than half as thick as that of PAO1 (Figure 3c and d). The cell/EPS profiles of JP1 and JP2 biofilms were almost identical, and biofilm cells predominated in both, with little EPS visible (Figure 3e–h).

Biofilm resistance to kanamycin

Figure 4 illustrates the biofilm resistance of *P. aeruginosa* wild type (PAO1) and the quorum-sensing-deficient mutants (PDO100, JP1 and JP2) to different concentrations of kanamycin. The surviving cell fraction was used to represent the bactericidal effects and reduce the variation in cell numbers for different stainless-steel slides. For the kanamycin treatment, no obvious reduction in the surviving fraction of cells in the PAO1 biofilm was observed, with the fractions generally remaining above 0.7. Treatment with kanamycin 10 and 50 mg/L had little effect on the PDO100 biofilm; however, the surviving cell fraction fell to 0.46 after 2 h of treatment with kanamycin 100 mg/L. Compared with PAO1 and PDO100, the JP1 and JP2 biofilms were much more susceptible to kanamycin, with the surviving cell fraction for both decreasing significantly in response to all treatment, reducing to *c.* 0.2 when exposed to kanamycin 10 mg/L and to <0.1 when exposed to kanamycin 100 mg/L.

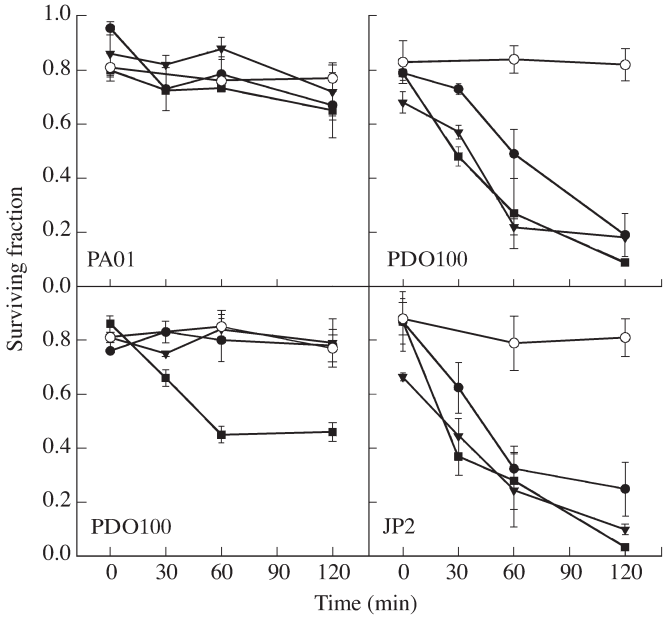

Figure 4. Surviving cell fractions of *P. aeruginosa* PAO1, PDO100, JP1 and JP2 biofilms in response to treatment with kanamycin 0 (○), 10 (●), 50 (▼) and 100 (■) mg/L. $n = 3$; bar indicates S.E.

Discussion

Biofilms consist of cells and EPS, with their accumulation a net result of planktonic cell attachment, biofilm cell growth and detachment, and EPS production. In this study, the additive effect of attachment was eliminated through the use of sterile feeding. In terms of cell density, the *P. aeruginosa* PAO1 biofilm accumulation rate was more rapid than those of the quorum-sensing-deficient mutants. This phenomenon cannot be attributed solely to the difference in growth rates, since the growth rate for PAO1 ($0.42 \pm 0.01\ h^{-1}$) is only slightly higher than for the mutants (PDO100, $0.30 \pm 0.02\ h^{-1}$; JP1, $0.30 \pm 0.01\ h^{-1}$; JP2, $0.31 \pm 0.01\ h^{-1}$). The lower biofilm cell-accumulation rates observed for the mutants also result from reduced EPS production. Without EPS protection, cells are prone to detachment from the top of the biofilm.

The cryosection photomicrograph of a PAO1 sample (Figure 3a and b) reveals that most cells occupy the top layer of the biofilm, whereas the bottom layer is mainly EPS. This finding is in agreement with the SCLM images presented by Davies *et al.*[7] and Rashid *et al.*[17] The cell/EPS distribution within biofilms of both wild-type organisms and quorum-sensing-deficient mutants (Figure 3) is consistent with the quantitative analysis of EPS (Figure 2).

Currently, the influence of quorum sensing on biofilm EPS production is unclear. Figure 2 provides an indication that EPS production is more closely related to the expression of *las*I than *rhl*I. Davies *et al.*[7] compared mature biofilms of *P. aeruginosa* PAO1, PDO100, JP1 and JP2 and determined that, after 14 days of cultivation, biofilm thicknesses of the Δ*rhl*I mutant and the wild type were similar, whereas biofilms of the Δ*las*I and Δ*las*IΔ*rhl*I variants were thinner and the cells more closely packed. Our results (Figures 2 and 3) are generally consistent with that report, except that the PDO100 biofilm was significantly thinner than the PAO1 counterpart. The difference may reflect the shorter cultivation time used in this work (3 days). Because of the influence of the *P. aeruginosa* quorum-sensing circuitry,[18] the differences in EPS production should not be attributed to the effects of a single gene. According to the quorum-sensing model proposed by Pesci *et al.*[9] for *P. aeruginosa*, both LasR-3-oxo-C12-HSL and RhlR-C4-HSL complexes will induce the expression of several genes. The LasR-3-oxo-C12-HSL complex also induces the expression of *rhl*R and, consequently, affects the formation of RhlR-C4-HSL. Although it seems reasonable to speculate from the results that EPS-associated genes are more likely to be controlled by LasR-3-oxo-C12-HSL, further investigation is required.

Little is known about the relationship between biofilm formation and the antibiotic resistance of the cells and quorum sensing. Our results reveal significant differences between the strains in antimicrobial resistance tests, as shown by the comparison between the wild-type system and the quorum-sensing-deficient mutants (Figure 4). The enhanced susceptibilities to kanamycin shown by *P. aeruginosa* JP1 and JP2 correlate with lower EPS production and thinner biofilm formation. However, the possibility of quorum sensing governing specific gene expression to modulate resistance to antibiotics of cells in biofilms should not be excluded without further investigation. Hassett *et al.*[14] have reported that quorum sensing in *P. aeruginosa* controls the expression of the catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Their results, together with ours, provide evidence that biofilms respond directly or indirectly to environmental stress via a quorum-sensing mechanism.

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