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# Rapid Evolution of Culture-Impaired Bacteria during Adaptation to Biofilm Growth

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## SUMMARY

Biofilm growth increases the fitness of bacteria in harsh conditions. However, bacteria from clinical and environmental biofilms can exhibit impaired growth in culture, even when the species involved are readily culturable and permissive conditions are used. Here, we show that culture-impaired variants of *Pseudomonas aeruginosa* arise rapidly and become abundant in laboratory biofilms. The culture-impaired phenotype is caused by mutations that alter the outer-membrane lipopolysaccharide structure. Within biofilms, the lipopolysaccharide mutations markedly increase bacterial fitness. However, outside the protected biofilm environment, the mutations sensitize the variants to killing by a self-produced antimicrobial agent. Thus, a biofilm-mediated adaptation produces a stark fitness trade-off that compromises bacterial survival in culture. Trade-offs like this could limit the ability of bacteria to transition between biofilm growth and the free-living state and produce bacterial populations that escape detection by culture-based sampling.

## INTRODUCTION

Biofilms are matrix-encased bacterial aggregates that are ubiquitous in nature and in chronic human infections. When grown in biofilms, bacteria develop high-level resistance to many types of stress, including antibiotic treatment, oxidant and desiccation stress, as well as resistance to predation (Costerton et al., 1999; Hall-Stoodley et al., 2004). These phenotypes are thought to enhance bacterial survival in harsh conditions.

Paradoxically, recent observations in medical, environmental, and laboratory settings indicate that periods of biofilm growth can impair the ability of bacteria to grow in laboratory culture (Alam et al., 2007; Ehrlich et al., 2002; Hall-Stoodley et al.,

2006; Martin et al., 2010; Shen et al., 2010; Zandri et al., 2012). For example, environmental *Vibrio cholerae* in aquatic biofilms, infecting *Haemophilus influenzae* in middle-ear biofilms, and Staphylococcal species forming biofilms on human catheters become impaired in culture growth, or nonculturable (Alam et al., 2007; Ehrlich et al., 2002; Hall-Stoodley et al., 2006; Zandri et al., 2012). That a culture-resistant phenotype arises in these settings is surprising because the species involved could thrive in culture prior to passaging through biofilms and because culture conditions are designed to be optimal for growth.

Poor culture growth has important consequences, as culture is the primary method for detecting and identifying environmental and pathogenic bacteria. In addition, culture impairment makes functional studies of bacteria and biotechnology applications extremely difficult. Mechanisms that could impair culture growth of biofilm bacteria are poorly understood.

One possibility is that biofilm growth induces a state akin to the "viable-but-nonculturable" phenotype, which may be a consequence of exposure to sublethal stress (Oliver, 2010; Post et al., 1995).

In addition to phenotypic mechanisms, theory predicts that culturable bacteria could become culture-impaired through genetic trade-offs. Trade-offs evolve when genetic adaptations to one set of conditions reduce fitness in others (Futuyma and Moreno, 1988). In one mechanism that generates trade-offs, the same mutation produces the fitness cost and benefit. This is likely due to limits on how much a particular function can be improved for one environment, before its ability to operate in other conditions deteriorates (Jaenicke, 1991; Russell, 2000). In another mechanism producing trade-offs, fitness costs and benefits are caused by different mutations. In this case, the beneficial mutations are selected for, whereas the detrimental alleles randomly accrue (Elena and Lenski, 2003; Kassen, 2002).

Trade-offs that differentially affect biofilm and culture fitness seem possible because selective pressures within and outside of biofilms differ markedly. For example, biofilm bacteria are closely aggregated and thus are subject to strong gradients of nutrients, oxygen, and wastes (Stewart and Franklin, 2008). Biofilms must also sustain adherence functions and matrix
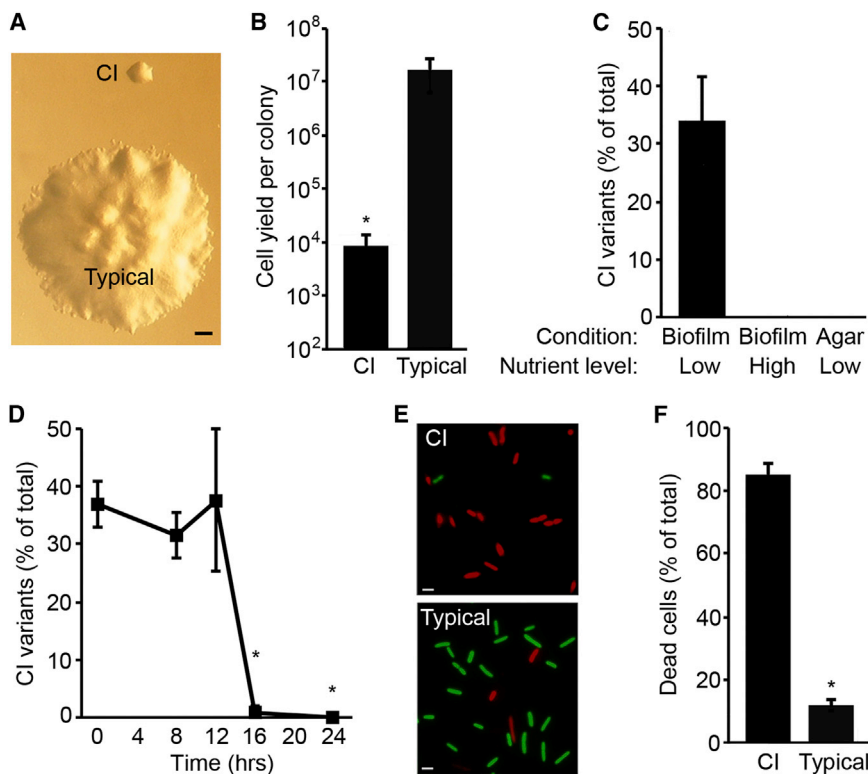
**Figure 1. Culture-Impaired *P. aeruginosa* Variants Evolve during Biofilm Growth**

(A) Morphology of a CI-variant and typical colony after overnight incubation on standard agar. The scale bar represents 100 μm.

(B) Cell yield (number of cells that produce colonies) from 1-day-old CI-variant and typical colonies. The error bars in (B)–(D) and (F) represent SD. *p = 0.01.

(C) Abundance of CI variants in 3-day-old biofilms supplied low (0.03% tryptic soy broth) and high (3% tryptic soy broth) strength media and in bacterial populations passaged on agar containing 0.03% tryptic soy broth so that cells grew for ~32 generations.

(D) Viability of CI variants in shaken-batch cultures inoculated with $10^3$ bacteria from 3-day-old biofilms. The abundance of CI variants was monitored by plating samples from broth cultures on agar. *p = $3 \times 10^{-4}$.

(E) Micrograph of LIVE/DEAD viability staining of cells from CI-variant and typical colonies. The scale bar represents 1 μm.

(F) Relative abundance of dead cells in CI-variant and typical colonies after overnight incubation. *p = $3.9 \times 10^{-9}$.

production, which are not needed in culture. In addition, work with several species indicates that biofilm growth rapidly generates genetic variation (Allegrucci and Sauer, 2007; Boles et al., 2004; Hansen et al., 2007b; Koh et al., 2007; Savage et al., 2013; Starkey et al., 2009; Traverse et al., 2013; Waite et al., 2001; Yarwood et al., 2007). This may increase the probability that mutations producing trade-offs occur (Elena and Lenski, 2003; Futuyma and Moreno, 1988).

In the course of other experiments, we observed that biofilm growth promoted the evolution of variants that are severely impaired in culture. Surprisingly, these variants evolved in a *Pseudomonas aeruginosa* strain that had been acclimated to laboratory culture for decades and is also a model strain for biofilm studies. We refer to these variants as "culture-impaired" as they exhibited markedly compromised survival in culture but were not strictly unculturable. Because opportunities to understand mechanisms impairing culture fitness are rare, we studied the conditions that generate the variants and the molecular basis of the culture-resistant phenotype. We also explored whether this phenotype was due to a genetic trade-off between biofilm and culture fitness and investigated the evolutionary mechanism that produced it.

## RESULTS AND DISCUSSION

### *P. aeruginosa* Biofilms Produce Variants that Spontaneously Die in Culture

In a previous study, we observed that, when biofilm-grown *P. aeruginosa* was plated on standard laboratory agar, several colony morphologies were apparent (Boles et al., 2004).

This study utilized a biofilm reactor (Huang et al., 1998) in which medium is continuously dripped onto a bacterial growth surface. This system models biofilms of relatively large biomass (cell yields of ~$10^9$ bacteria on a 9 cm$^2$ surface), which form under low shear conditions at an air-surface interface. Such conditions may be present in certain natural environments, in industrial equipment, and in the oral cavity (Goeres et al., 2009). In addition, biofilms grown in this system generate bacterial variants similar to those that evolve during the biofilm infections of cystic fibrosis (Barth and Pitt, 1995; Boles et al., 2004; Ernst et al., 2003; Starkey et al., 2009; Wei et al., 2011).

The most abundant morphotype from these biofilms exhibited barely perceptible growth (Figure 1A). These variants (called culture-*impaired* or "CI" variants) produced colonies that were one-tenth the diameter and contained 1,000-fold fewer viable cells than the bacteria from which they evolved (Figures 1A and 1B). Despite their poor performance in culture, CI variants were strongly selected for in biofilms. They increased from 0% to ~35% of the population in just 3 days (Figure 1C) and were the most prevalent morphotype to emerge (Boles et al., 2004).

The stunted phenotype of CI variants was heritable (Figure S1A), indicating genetic changes had occurred, and was observed in shaken (Figure 1D) and static (Figure S1B) broth cultures, anaerobic conditions (Figure S1C), and when various media were used (Figures S1C and S1D). We measured cell viability after 1 day of colony growth and found that, whereas typical colonies contained a low proportion of dead cells (12% on average), variant colonies contained predominantly dead cells (an average of 85%; Figures 1E and 1F). If sustained, these high death rates could explain the 1,000-fold lower cell yields of CI-variant colonies and could cause the variants to die out in culture.
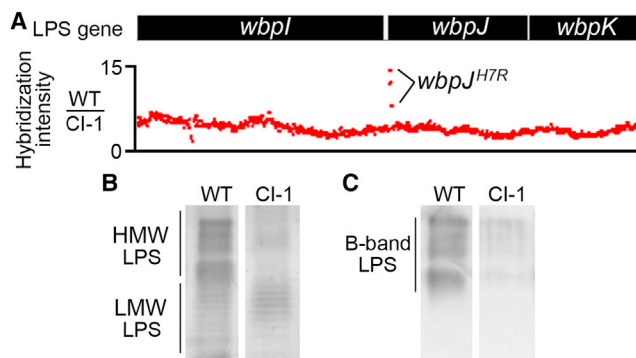
**A** LPS gene



**Figure 2. A CI Variant Has a B-Band LPS Mutation**
(A) Hybridization of DNA from a CI variant (CI-1) to a *P. aeruginosa* whole-genome tiling array identified a *wbpJ*$^{H7R}$ mutation. Genes in the LPS biosynthetic operon are indicated with black boxes. The red points on the graph indicate ratios of the hybridization intensity of genomic DNA from wild-type *P. aeruginosa* (WT) versus a CI variant (CI-1) to probes with WT gene sequences. A positive ratio indicates poor hybridization of CI-1 DNA relative to the WT due to a mutation in the CI variant.
(B and C) SDS-PAGE silver staining (B) and western immunoblotting analysis (C) of LPS from wild-type and CI-1 variant cells. HMW, high molecular weight; LMW, low molecular weight.

The biofilms in which CI variants evolve and the cultures in which they spontaneously die differ in two major conditions: biofilm growth and the nutrient level supplied. Biofilm reactors typically use dilute growth media to model natural environments and because nutrient limitation is thought to promote biofilm growth (Goeres et al., 2009; James et al., 1995). Notably, CI variants did not evolve at measurable rates during *P. aeruginosa* growth on agar containing dilute medium or in biofilms grown with rich medium (Figure 1C). Thus, the combination of biofilms and a relatively low nutrient supply was required.

**Culture-Impaired Variants Harbor B-Band LPS Gene Mutations**
We used a genomic tiling array to identify mutations acquired by a CI variant. This work identified nonsynonymous mutations in the *mexT* transcriptional regulator and in *wbpJ* (Figure 2A), a gene mediating biosynthesis of a polysaccharide that decorates some lipopolysaccharides (LPS). We thought the *mexT* mutation was not likely to be responsible for the culture-resistant phenotype, because this gene is dispensable for growth in the laboratory (Maseda et al., 2000).

In contrast, two established mutant libraries (that involve culturing steps) failed to isolate *wbpJ* mutants (Jacobs et al., 2003; Lewenza et al., 2005), suggesting that *wbpJ* may be critical in culture. WbpJ is a putative glycosyltransferase implicated in the biosynthesis of the long polysaccharide chains known as B-band O-antigen, and below, we refer to LPS-harboring B-band O-antigen as B-band LPS (Burrows et al., 1996). Consistent with this function, analysis of LPS by silver staining and immunoblotting showed that this CI variant (Figures 2B, 2C, and S1E) and seven others from independent experiments (see Figures 4D, S2F, and S4A) produced substantially less B-band LPS than the parental bacteria. In contrast, A-band LPS (which

carries a different O-antigen) was made by CI-variant bacteria (Figures S1F, S2F, S4A, and S4B below).

**B-Band LPS Gene Mutations Sensitize Bacteria to a Self-Produced Antibiotic**
How could a B-band LPS biosynthetic gene mutation impair culture growth? In addition to structural functions, B-band LPS confers resistance to a self-produced antimicrobial agent intended to target other organisms (Köhler et al., 2010). B-band LPS on the bacterial cell envelope masks the receptor for R2 pyocin, an antimicrobial that resembles a bacteriophage and kills bacteria by permeabilizing membranes (Köhler et al., 2010). These observations led us to hypothesize that CI variants die in culture because B-band LPS deficiency sensitizes bacteria to killing by self-produced R2 pyocin. This hypothesis makes several predictions.

First, *P. aeruginosa* should produce sufficient R2 pyocin in laboratory culture to kill sensitive cells. As shown in Figure 3A, wild-type *P. aeruginosa* grown in broth and agar cultures produced enough extracellular R2 pyocin to kill a susceptible *P. aeruginosa* indicator strain.

Second, CI variants should be R2-pyocin sensitive. As shown in Figure 3B, R2 pyocin prevented growth of CI variants (but not other variant types), whereas control preparations from a Δ*R2* strain did not. Also consistent with this prediction, when biofilm-grown cells were incubated in broth culture, CI variants precipitously lost viability at the time that R2 pyocin levels increased (Figure 3C).

A third prediction is that preventing R2-pyocin synthesis should eliminate the culture-resistant phenotype of R2-sensitive variants. To test this, we grew biofilms using *P. aeruginosa* in which R2-pyocin genes were inactivated, cultured them on standard agar, and examined ~500 colonies from each biofilm. No colonies with the culture-resistant phenotype were identified in five independent biofilm experiments (Figure 3D).

Notably, the Δ*R2* strain did produce R2-sensitive variants at the same relative abundance as the wild-type in biofilms (Figure 3E). However, because these sensitive variants did not produce R2 pyocin, they formed colonies with typical size (Figure 3F), levels of cell death (Figures 3G and S2A), and cell yield (Figure S2B) when grown on agar. They also retained viability in broth culture (Figure 3C). These experiments indicate that CI variants die in culture because they evolved LPS mutations that compromise their resistance to self-produced R2 pyocin.

**Several B-Band LPS Gene Mutations Can Produce the Culture-Impaired Phenotype**
The rapidity with which the variants become abundant led us to hypothesize that the B-band LPS mutations mediating the CI phenotype also caused the variant's strong biofilm fitness advantage. We explored this hypothesis in two ways.

First, we reasoned that mutations in a variety of B-band biosynthetic genes should be found in R2 pyocin-sensitive variants emerging from biofilms. To test this, we sequenced the genomes of four variants that had evolved in independent biofilm experiments. All contained mutations altering conserved residues of B-band biosynthetic proteins (Figures 4A and S2C–S2E), all were B-band LPS deficient (Figures S2F and S2G),
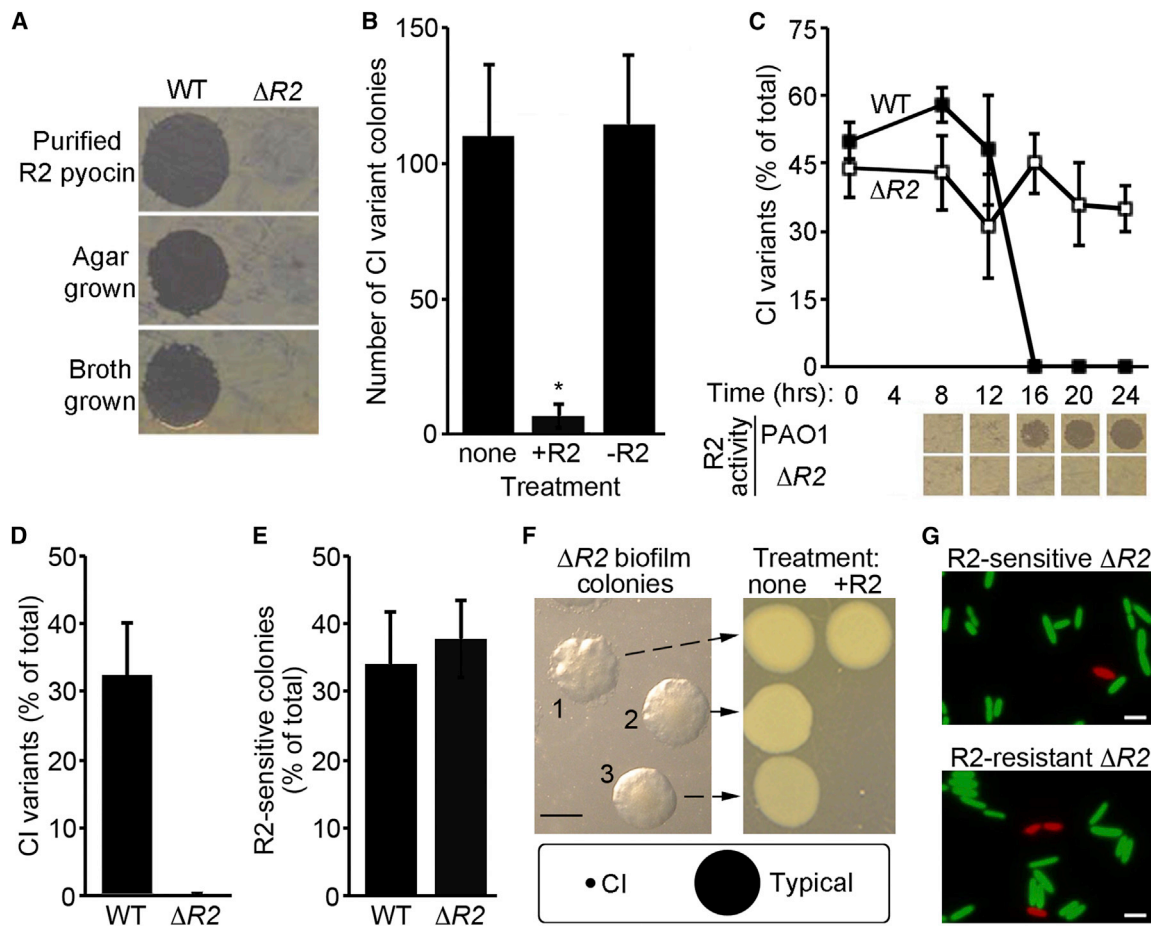
**Figure 3. B-Band LPS Deficiency Sensitizes Bacteria to Self-Produced R2-Pyocin and Impairs Culture Growth**

(A) R2-pyocin activity in cultures of wild-type and Δ*R2* bacteria grown on agar and in broth. Areas of clearing represent growth inhibition of an indicator strain and indicate R2-pyocin activity.

(B) Number of CI-variant colonies on untreated agar (none), agar containing purified R2-pyocin (+R2), and agar containing a pyocin preparation from Δ*R2* cells (−R2). The error bars in (B) and (C)–(E) represent SD. *p = 2.6 × 10$^{-5}$.

(C) Performance of R2-pyocin-sensitive variants generated by wild-type (filled squares) and Δ*R2* (open squares) biofilms in shaken planktonic batch cultures. R2-pyocin activity in cultures was monitored by plating samples of filtered culture supernatants onto a lawn of bacteria of an R2-pyocin-sensitive indicator strain. Clearing of the indicator strain is indicative of R2-pyocin activity.

(D) Relative abundance of CI-variant colonies produced by cells from 3-day-old wild-type and Δ*R2* biofilms; 500 colonies were examined from each of five independent biofilm experiments.

(E) Relative abundance of R2-pyocin-sensitive bacteria generated by wild-type and Δ*R2* biofilms.

(F) Colonies from Δ*R2* biofilms (left panel) were tested for R2-pyocin sensitivity by exposure to purified R2-pyocin (right panel). Colonies 2 and 3 are R2-pyocin-sensitive variants. Note that these R2-pyocin-sensitive colonies are the same size as the R2-pyocin-resistant colony (colony 1) and larger than CI variants from wild-type biofilms. Representative sizes of typical and CI-variant colonies are shown below. The scale bar represents 500 μm.

(G) Micrograph of LIVE/DEAD viability staining of cells from Δ*R2* biofilm colonies that are sensitive and resistant to R2-pyocin. The scale bar represents 1 μm. Note that this experiment also indicates that, in the absence of R2-pyocin production, B-band LPS loss had no independent effect on LIVE/DEAD staining.

and all exhibited R2-pyocin sensitivity (Figure S2H). Furthermore, genetic complementation reversed these phenotypes (Figure S2G and S2H).

Second, we engineered two of the identified mutations into *P. aeruginosa* and competed them in biofilms against their isogenic parent. We performed these experiments in the Δ*R2* background, as this eliminates the culture-impaired phenotype and enables fitness to be evaluated in isolation. Cells harboring the *wbpJ*$^{H44Y}$ or *wbpA*$^{V41F}$ mutations showed a strong fitness advantage, increasing from 1% to over 50% of the biofilm pop-

ulation after only 3 days (Figures 4B and S3A). Genetic complementation that restored B-band LPS eliminated this competitive advantage (Figures 4B and S3A). Of note, the fitness of B-band LPS-deficient cells in biofilms was not strictly dependent on their frequency or the presence of other phenotypes in the population (Figures S3B and S3C). However, their fitness advantage in biofilms required low nutrient conditions (Figure S3D).

These experiments indicate that B-band LPS mutations produce both the strong fitness advantage observed in biofilms and the lethal phenotype in culture. Thus, the impaired culture
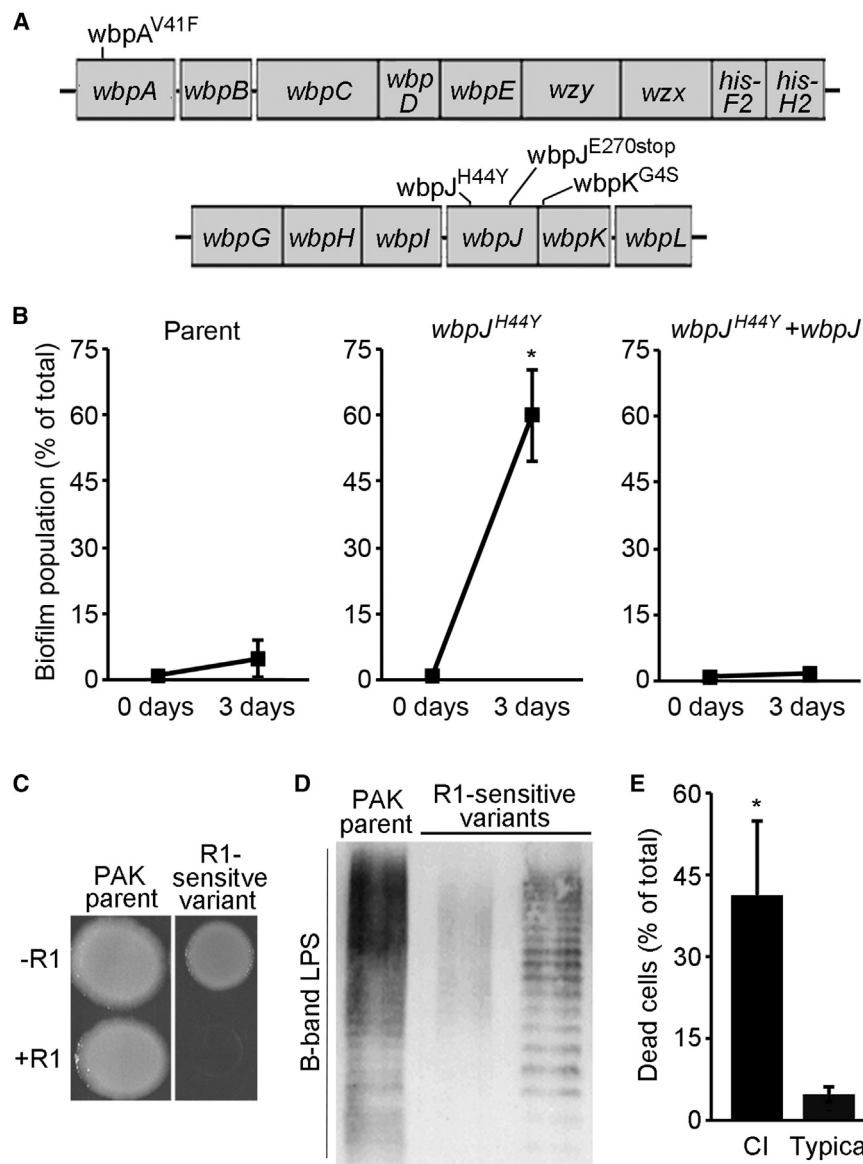
**Figure 4. B-Band LPS Mutations Increase Bacterial Fitness in Biofilms and Arise from Several *P. aeruginosa* Strains**

(A) Location of B-band LPS mutations identified by whole-genome sequencing of independently evolved variants. All mutations affect genes known to mediate B-band LPS synthesis.

(B) The biofilm fitness of the indicated strains was tested by cocultivation with parental Δ*R2* bacteria. Test strains were inoculated at a starting ratio of 1%. The error bars represent SD. *p = 4.6 × 10$^{-3}$.

(C) R1-pyocin sensitivity of typical and variant colonies from PAK Δ*R1* biofilms.

(D) Western immunoblot analysis of LPS isolated from parental PAK Δ*R1* cells (the strain used to initiate biofilm growth) and biofilm-evolved R1-pyocin-sensitive variants shows reduced B-band LPS production by the variants.

(E) Abundance of dead cells in CI variant and typical colonies from wild-type PAK biofilms as measured by LIVE/DEAD viability staining. The error bars represent SD. *p = 2.8 × 10$^{-5}$.

Arguing against an energy conservation mechanism, we found that CI variants produce increased amounts of A-band LPS (Figures S1F, S2F, S4A, and S4B), which could reduce energy savings from B-band loss. Furthermore, inactivating B-band LPS biosynthesis did not produce a growth advantage in shaken liquid cultures, provided that R2 pyocin genes are also inactivated (Figure 3C). It is possible that the energy conservation advantage is only manifested in biofilms. This could occur because aggregated growth produces nutrient gradients that starve some biofilm cells or because of the costs of biofilm-specific functions like matrix production. An alternative possibility is that B-band loss produces a fitness advantage by enhancing the cells' ability to aggregate or adhere within biofilms, as has been observed in other systems (Hansen et al., 2007a; Spiers and Rainey, 2005).

**Other Strains Generate Culture-Impaired Variants by a Similar Mechanism**

Environmental *P. aeruginosa* strains produce a variety of R-pyocins and different B-band LPS types (Köhler et al., 2010), and the trade-off between culture and biofilm fitness could depend on a specific R-pyocin-LPS interaction. To investigate this, we studied five additional *P. aeruginosa* strains expressing different LPS and R-pyocin types.

We found that biofilm growth by the PAK and NIH K strains promoted the evolution of variants that were sensitive to their own R-pyocins and deficient in B-band LPS (Figures 4C, 4D, and S4A–S4C). Furthermore, variants of both strains

growth phenotype develops through the evolutionary mechanism known as antagonistic pleiotropy, in which a single genetic adaptation produces an advantage in one environment but a fitness cost in another.

Identifying how B-band loss produces a fitness advantage in biofilms will require additional work. We considered the possibility that cells forego B-band production to conserve energy or limited resources. Consistent with this idea, we found that CI variants produce less PSL exopolysaccharide (Figure S3E), which is a biofilm matrix component (Ma et al., 2012). Also consistent, increasing the nutrient concentration of the biofilm medium eliminated the selective advantage conferred by the *wbpJ* mutation in competitions (Figure S3D) and markedly reduced the number of CI variants emerging from wild-type *P. aeruginosa* biofilms (Figure 1C).

exhibited compromised survival in culture (Figures 4E and S4D). Thus, strains that produce different pyocin and LPS types generate culture-impaired variants by the same general mechanism.

## Conclusions

Impaired culture growth can be an inherent property of bacterial species, be caused by unsuitable culture conditions, or be a consequence of phenotypic changes in otherwise culturable cells. Our experiments illustrate another mechanism, mediated by a fitness trade-off produced by genetic adaptation to biofilm growth conditions. The rapidity with which this trade-off evolved was remarkable, as approximately 35% of the biofilm population was culture impaired after only 3 days. The fitness consequences of the trade-off were also significant. Because of enhanced biofilm performance, small numbers of CI-variant cells quickly dominated biofilm populations. However, in culture, the variants produced three orders of magnitude fewer cells and exhibited death rates that would lead to extinction if sustained. This trade-off suggests that, in addition to its well-known effect of enhancing bacterial stress resistance, biofilm growth can have unappreciated negative consequences for bacteria.

That *P. aeruginosa* rapidly evolved such a stark trade-off with opposing effects on fitness inside and outside of biofilms is surprising, as its large genome is highly enriched in regulatory elements and it is a model organism for both culture growth and biofilm formation (Costerton et al., 1999; Stover et al., 2000). Despite this, regulatory mechanisms were inadequate to mediate the conflicting demands of biofilm and culture growth. The parental *P. aeruginosa* cells were highly fit in culture but needed a genetic adaptation to enhance biofilm fitness. Likewise, the CI variants exhibited high performance in biofilms but could not mitigate the lethal effect of biofilm-enhancing adaptations in culture. Less-versatile organisms could be even more prone to antagonistic fitness trade-offs that restrict their ability to live outside their native environments.

A key question for future research is whether biofilm growth in natural settings promotes genetic fitness trade-offs that impair bacterial survival outside the protected biofilm environment. Whereas additional work will be required, observations suggest that trade-off mechanisms similar to the one we identified may operate. For example, *P. aeruginosa* causing cystic fibrosis infections (in which biofilms are implicated) evolve mutations inactivating B-band LPS biosynthesis at high frequencies, just as occurred in our laboratory model (Davis et al., 2013; Lam et al., 1989; Smith et al., 2006; Spencer et al., 2003; Warren et al., 2011). Natural biofilms also promote the evolution of adaptations like motility loss and exopolysaccharide overproduction, which could compromise the fitness of cells leaving biofilms without directly affecting bacterial viability (Smith et al., 2006; Starkey et al., 2009; Folkesson et al., 2012). Together, these findings suggest that the transition between biofilm growth and the free-living state can be costly for bacteria. Our work also raises the possibility that biofilms in natural environments and human infections produce sizable culture-resistant subpopulations that thrive in situ but fail to be detected by culture-based sampling.

## EXPERIMENTAL PROCEDURES

### Strains, Plasmids, and Growth Conditions

Strains and plasmids are available upon request, and details on their construction are in the Supplemental Experimental Procedures. Bacteria were grown at 37°C in 3% tryptic soy broth (TSB) or on 3% tryptic soy agar (TSA) unless otherwise noted. Biofilm cultivation, harvesting, and plating were done as previously described (Boles et al., 2004). Frequency of CI- and R-pyocin-sensitive variants was determined by scoring the morphology or R-pyocin sensitivity of ~500 colonies, respectively (see below).

### Growth Assays

The cell yield (cells capable of forming a colony) of colonies on agar was determined by plating serial dilutions of individual colonies suspended in PBS. Colony suspensions were also used to determine R-pyocin sensitivity of colonies (see below). An unpaired Student's t test was performed to evaluate significance. For growth assays in broth, $10^2$–$10^3$ biofilm cells were used to inoculate cultures containing TSB. Paired Student's t tests were used to assess significance relative to time zero.

### Evaluation of Levels of Cell Death within Colonies

Biofilm-derived cells and their $\Delta R$-pyocin derivatives were plated on nitrocellulose membranes laid upon TSA. After overnight incubation, five CI-variant colonies on membranes were individually resuspended in 25 $\mu$l 0.85% NaCl. R-pyocin-sensitive variants were also identified by R-pyocin sensitivity assays (see below). The frequency of viable cells within the suspension was determined by using the LIVE/DEAD cell viability assay kit (Invitrogen). Approximately 600–1,000 cells were scored per colony, and statistical significance was assessed using an unpaired Student's t test.

### LPS Purification and Analysis

LPS was purified and analyzed by SDS-PAGE silver staining and western immunoblotting as previously described (Burrows et al., 1996; Lau et al., 2009). The monoclonal antibodies used, MF15-4 and MF83-1, are specific against serotype O5 (PAO1) and O6 (PAK) B-band LPS, respectively (Lam et al., 1987).

### R-Pyocin Purification and Assays

Preparations of R-pyocin, and R-pyocin activity assays were performed as previously described (Williams et al., 2008). To detect R-pyocin activity, supernatants from broth cultures or from resuspended colonies (~100 colonies per 1 ml) were filtered and spotted onto a lawn of the indicator strain.

The R2-pyocin sensitivity of CI variants was tested by plating equal numbers of cells on agar on which was spread 150 $\mu$l of a one-tenth dilution of R-pyocin preparation from PAO1 (+R2) or an otherwise isogenic $\Delta R2$ strain (−R2). CI variants from six independent biofilms were tested, and statistical significance relative to the untreated control was assessed with a paired Student's t test. R-pyocin sensitivity of colonies from $\Delta R2$ biofilms was assayed by suspending cells in PBS with purified R-pyocin and then spotting this mixture onto standard agar. Growth of cells after overnight incubation was indicative of resistance; no growth was indicative of sensitivity.

### Mapping Polymorphisms and Genome Sequencing

DNA polymorphisms were mapped by using a high-density, whole-genome tiling array of the PAO1 genome that was designed by Nimblegen (Albert et al., 2005). DNAs from wild-type and a CI-1 variant (CI-1) were fragmented and hybridized on a single microarray to quantify hybridization intensities for each probe. Each wild-type (WT) probe value was divided by their counterpart in the CI-variant data set to generate the WT/CI-1 data set. Probes spanning mutations in the CI genome display lower hybridization intensities than in the WT data set, causing the ratio of probe values at a given locus in the WT/CI-1 data set to be positive. Fourteen putative polymorphic loci in the CI-1-variant genome were identified, and Sanger sequencing verified that two of these loci had bona fide mutations.

For whole-genome sequencing, DNA was extracted and purified from PAO1, $\Delta R2$, and four R-pyocin-sensitive (RPS) variants derived from $\Delta R2$ biofilms (RPS-1–RPS-4). DNA samples were sequenced using Illumina Genome

Analyzer technology (Illumina) and compared to the PAO1 reference genome (Stover et al., 2000). Mutations were confirmed using Sanger sequencing.

## Biofilm Fitness Tests

To measure biofilm fitness, B-band-deficient strains were labeled with a tetracycline resistance marker and competed against an unlabeled $\Delta R2$ parent strain by using the indicated ratios of the two strains to initiate biofilm growth. After 72 hr, biofilms were plated on standard agar and the resulting colonies were tested for resistance to 225 $\mu$M tetracycline to determine the relative abundance of competing strains in biofilms. Four independent experiments were performed for each competition. A paired Student's t test was used to assess statistical significance.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article at http://dx.doi.org/10.1016/j.celrep.2013.12.019.

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