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ORIGINAL ARTICLE

Ecological succession in long-term experimentally evolved biofilms produces synergistic communities

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Many biofilm populations are known for their exceptional biodiversity, but the relative contributions of the forces that could produce this diversity are poorly understood. This uncertainty grows in the old, well-established communities found on many natural surfaces and in long-term, chronic infections. If the prevailing interactions among species within biofilms are positive, productivity should increase with diversity, but if they tend towards competition or antagonism, productivity should decrease. Here, we describe the parallel evolution of synergistic communities derived from a clone of *Burkholderia cenocepacia* during ~1500 generations of biofilm selection. This long-term evolution was enabled by a new experimental method that selects for daily cycles of colonization, biofilm assembly and dispersal. Each of the six replicate biofilm populations underwent a common pattern of adaptive morphological diversification, in which three ecologically distinct morphotypes arose in the same order of succession and persisted. In two focal populations, mixed communities were more productive than any monoculture and each variant benefited from the mixture. These gains in output resulted from asymmetrical cross-feeding between ecotypes and the expansion and partitioning of biofilm space that constructed new niches. Therefore, even in the absence of starting genetic variation, prolonged selection for surface colonization generates a dynamic of ecological succession that enhances productivity.
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Introduction

Microbial biofilms may be found on essentially any moist surface, including in the soil, on the exterior of larger organisms or on manmade substrates such as water supply pipes. Though seemingly simple in form and function, the carbohydrate-rich matrix that binds unicellular organisms together and affixes them to surfaces can be complex and house varying levels of species diversity. This biodiversity is not lacking for possible explanations, as residents must complete complex life histories from colonization to dispersal and must persist in highly structured environments amongst different competing populations. Neighboring cells can also produce or sequester resources that alter the growth potential of each other and change the local forces of selection, and the biofilm itself can enable variants that might be outcompeted in mass action environments to persist. The net sign of these interactions could either enhance or reduce community productivity and is typically unknown, as is the relationship between biodiversity and productivity in many communities (Cardinale *et al.*, 2007; Gross and Cardinale, 2007). Determining the nature of these interactions can contribute to our understanding of evolution in structured environments in general and of the complexity of biofilm-associated infections in particular.

Despite the basic intuition that diversity is beneficial for biological communities, the forces that generate diversity can span from antagonistic to mutualistic (Hansen *et al.*, 2007; Nadell *et al.*, 2009; Ponciano *et al.*, 2009). Antagonistic interactions could include negative effects of competition, predation (Meyer and Kassen, 2007), parasitism (Buckling and Rainey, 2002) or social cheating (Rainey and Rainey, 2003; Harrison and Buckling, 2009) that favor variation but can reduce productivity. The environmental structure of biofilms could also enable diverse populations to coexist that would not persist in mixed, mass-action environments (Rainey and Travisano, 1998; Habets *et al.*, 2006). Positive interactions among species could include cooperative production of secreted products (Brockhurst, 2007), syntrophic metabolic functions (Hansen *et al.*, 2007) or the construction of new niches for immigrants by earlier colonists (Odling-Smee *et al.*, 2003), which should increase productivity.

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In theory, the balance of these interactions could change as communities mature over longer periods of time. As examples, the complex microbial communities found in reef ecosystems (Nocker *et al.*, 2004), on river bottoms (Lyautey *et al.*, 2005) or in sewage pipes (Huang *et al.*, 2008) are likely to be many years old and reflect many of the developments in community composition associated with classical ecological succession (Odum, 1975; Connell and Slatyer, 1977). The same mechanisms that cause species turnover in the plant communities that exemplify succession are also likely to operate in microbial biofilms, with at least the same level of uncertainty about the relative role of neutral, antagonistic or facilitative processes (Connell and Slatyer, 1977).

Previous studies of experimental evolution in biofilms have occurred over relatively few generations and may not reflect the longer term dynamics occurring in natural populations. We therefore developed a novel long-term experimental model of biofilm selection requiring daily cycles of surface colonization, biofilm assembly and dispersal. Replicate populations of the bacterium *Burkholderia cenocepacia*, a common resident of the rhizosphere and a pathogen of exceptional concern to the cystic fibrosis community (Ramette *et al.*, 2005), were evolved in this model. We chose a natural isolate from the soil, strain HI2424 (LiPuma *et al.*, 2002), as the ancestral clone rather than a clinical isolate because we did not want to limit response to biofilm selection, which likely occurs during colonization of susceptible patients (Costerton, 2001). In a previous study, we found that this same strain was capable of rapid adaptation to laboratory conditions distinct from those described here, with consequences for host specificity (Ellis and Cooper, 2010). In this study, we predicted that adaptation would also be accompanied by diversification because of the structure and greater complexity of the biofilm environment.

Our initial objectives were to quantify the fitness of various evolved clones in monoculture and when assembled in the mixed community, and to identify the ecological forces favoring any variation. Bacterial species like *Burkholderia* and *Pseudomonas* that infect the lungs of cystic fibrosis patients often exhibit heritable variability in biofilm phenotypes following selection in the host lung (Singh *et al.*, 2000; Haussler *et al.*, 2003; Nguyen and Singh, 2006; Smith *et al.*, 2006), which may reflect adaptation to distinct conditions within the host. Although the contributions of each bacterial variant to the biofilm community are uncertain, some morphotypes have been associated with increased mortality (Haussler *et al.*, 2003; Chantratita *et al.*, 2007; Starkey *et al.*, 2009). Understanding how diversity influences the productivity of these infections and their consequent resilience and pathogenicity is therefore a high priority.

Following ~1500 generations of experimental evolution favoring cycles of surface colonization, biofilm construction and dispersal, we found that diversity, characterized by colony morphology, growth dynamics and region of growth in the biofilm, evolved in each of the six replicate populations. The three variants that evolved in each population appear similar to the variants in other populations, became detectable in the same order and were associated with increased cellular productivity. Further, we found that diversity was maintained by ecotypes that improve both space and resource availability in the selective environment.

Materials and methods

Experimental evolution

We devised a novel method of studying biofilm evolution by transferring biofilm-associated cells that adhered to a 7 mm plastic bead floating in a 15×180 mm test tube, which was rotating in a rollerdrum at 50 r.p.m. (Figure 1). Each day, a new bead was colonized and a new biofilm was constructed, selecting for a regular cycle of colonization and dispersal. Cells that adhered to the tube walls or remained in planktonic culture were not transferred. Six replicate biofilm populations (B populations) and six control, planktonic populations (P populations) were each founded by a single HI2424 clone and grown in 5 ml M9 minimal medium with 1 M galactose, a major constituent of mucus that promotes biofilm formation. Populations were serially transferred every 24 h for 143 days at 37 °C on a rollerdrum at 50 r.p.m. At the start of the experiment, B population transferred into fresh media produced dilutions of $\sim 1.5 \times 10^3$, but this dilution declined over time to $\sim 1.0 \times 10^3$ owing to increased

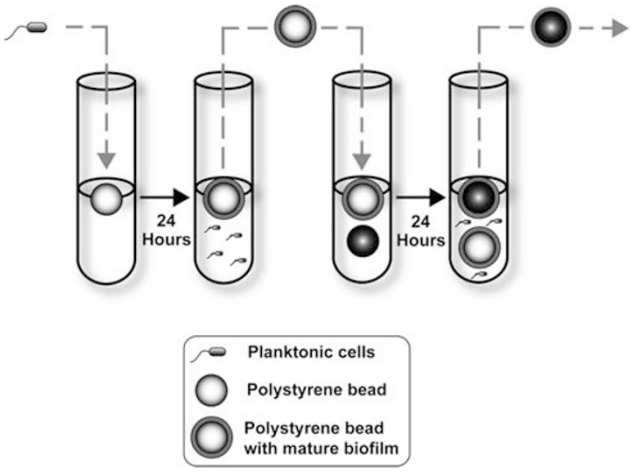


Figure 1 Model of long-term experimental evolution in biofilms. Six replicate populations founded with a single clone of *B. cenocepacia* HI2424 were propagated on 7 mm polystyrene beads, suspended in 5 ml M9 + 1 M galactose in 15×180 mm test tubes on a rollerdrum at 50 r.p.m., and were sterilely transferred to new media every 24 h. Each bead population was required to colonize a new oppositely marked bead each day. P populations were serially transferred via 1:100 dilutions of planktonic cells grown in 5 ml M9 + 1 M galactose for 24 h (not shown).

numbers of cells adhering to the bead. The number of generations is $143 \times \log_2$ (dilution), so we infer that between 1425 and 1509 generations occurred in B populations and 1000 generations occurred in P populations.

This model of biofilm selection differs from previous methods relying on static culture (Rainey and Travisano, 1998) or flow cells (Boles *et al.*, 2004) in that: (i) a new biofilm must grow on an uncolonized surface each day; (ii) the biofilm is subject to frequent shear forces in the rotating tube and (iii) samples of evolving populations can be archived and reconstituted under identical experimental conditions. In addition, our experimental model incorporates a variety of bacterial life-history components that can be separately quantified, namely dispersal, planktonic growth and surface recolonization.

Genetic manipulations

A LacZ+ mutant of *B. cenocepacia* was generated by insertion of pCELacZ (Ellis and Cooper, 2010), which inserts *lacZ* and *dhfr* in specific sites by Tn7. Transposants were competed against a *dhfr*-marked strain of HI2424 in several types of media and no fitness effect of the *lacZ* insertion was observed. Plasmids pSPR (red) and pSPY (yellow) were constructed by inserting genes encoding fluorescent proteins into pBBR1MCS, which encodes chloramphenicol resistance (Kovach *et al.*, 1995). pSPR harbors the red fluorescent protein gene from DsRedExpress (Lambertsen *et al.*, 2004) and pSPY, the *eyfp* gene (Lambertsen *et al.*, 2004), both bounded by KpnI-XmaI restriction sites that are also found in pBBR1MCS. Plasmids were introduced into evolved morphotypes by electroporation and positive selection at tryptic-soy agar + 150 μg ml^{-1} chloramphenicol.

Morphotype identification

Colony variants were scored following growth on tryptic-soy agar plates for 24 h at 37 °C and then for 48 h at room temperature. Variation in colony morphology within biofilm populations was evident following plating at generation 150; morphotype heritability was confirmed by continued isolated passage on plates and in both planktonic and biofilm environments.

Productivity assays

Biofilm productivity was quantified as colony-forming unit (CFU) per ml from beads colonized during 24 h in the selective environment, by vortexing beads in 1.5 ml phosphate-buffered saline for 1.5 min. To verify the effectiveness of vortexing for removing biofilms from beads, we examined vortexed beads under scanning electron microscopy using standard methods and found very few remaining adherent cells (at least 1000-fold reduction per unit area). Moreover, plate counts were consistent among replicates and with the optical density of the medium, which suggests that colonies were not founded by clumps, whose size should be random. Planktonic yield was measured as CFU per ml from the planktonic fraction of tubes lacking beads, without sampling wall growth. Total biomass of clones and the mixed community from population B1 was quantified with fivefold replication as follows. Cultures were grown from freezer stock in tubes containing 4 ml M9 + galactose and 1 ml Luria-Bertani broth and then subcultured into tubes containing 5 ml M9 + galactose and a 7 mm bead. The mass of each bead was measured before the experiment. Following 24 h of incubation under the selective conditions, beads were removed from each replicate, placed in separate wells of a 24-well plate and dried at 80 °C for 30 min. The mass of each bead was subsequently measured and dry weight biomass was inferred from the difference from the original uncultured bead.

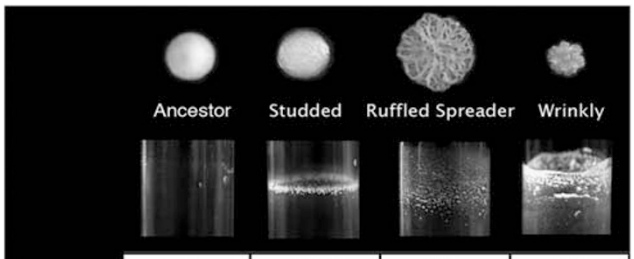
Fitness assays

Fitness was measured as $\ln(N_{t=24}/N_{t=0})$ in both environments beginning with resuspended planktonic cells or vortexed biofilms. When calculating the expected productivity based on yields of each mutant in monoculture, as in Figures 2 and 3 and Supplementary Figure S3, calculating standard confidence intervals of expected productivity was impossible as these were separate experiments. However, simulated 95% confidence intervals based on arbitrary pairings of measures from each monoculture replicate were $<0.2\%$ of the expected mean. Biofilm production was measured by crystal violet staining (O'Toole *et al.*, 1999) in 96-well plates (Corning Costar, Lowell, MA, USA) following 24 h of incubation with shaking at 37 °C and 150 r.p.m.

To examine the dynamics of attachment to the bead over the growth cycle, a single mixed culture of population B1 grown overnight under selective conditions was used to inoculate 18 fresh tubes containing beads, each grown under selective conditions. Beads from three replicate cultures were destructively sampled after 4, 8, 12, 17, 20 and 24 h, by vortexing in 1.5 ml of phosphate-buffered saline, diluting and plating on tryptic-soy (7 g l^{-1}) agar to obtain CFU per ml and morphotype frequencies at each time point.

Effects of environmental structure on biodiversity

We added an additional polystyrene bead (more structure) or removed the bead (no structure) from cultures of populations B1 and B2 grown under conditions that were otherwise identical to the selective regime. The variation in colony morphology (or biodiversity) of >200 colonies from each treatment was measured using the Shannon–Wiener index: $H = -\text{sum}(P_i \log[P_i])$, where P_i = the proportion of each given subspecies in the population.



	Ancestor	Studded	Ruffled Spreader	Wrinkly
Frequency	n.a.	88%	9%	3%
Generation detected		150	300	300
Relative fitness	1	7.15 (2.89)	8.54 (1.55)	9.49 (3.03)
Biofilm production	0.19 (0.013)	0.62 (0.054)	1.21 (0.027)	1.34 (0.040)
Planktonic growth rate	6.25 (0.26)	6.52 (0.13)	9.02 (0.010)	10.06 (0.22)

Figure 2 Adaptive diversification within *Burkholderia* biofilms. Colony morphologies (first row) and biofilm phenotypes (second row, growth on tube walls) of evolved variants following ~1500 generations of biofilm selection, their timing of detection in population B1 and their associated phenotypes. All six biofilm-evolved populations produced morphologically similar types (studded = S, ruffled spreader = R, wrinkly = W; Supplementary Figure S2) that were detected during the same intervals. Morphotypes differ in their fitness, colonization patterns, growth rates and biofilm production, when grown in monoculture. Relative fitness is colonization efficiency of evolved morphotypes relative to the ancestor. Planktonic doubling time (increasing values being disadvantageous) and biofilm production were measured using standard techniques. 95% confidence intervals of each measurement are in parentheses. NA, not applicable.

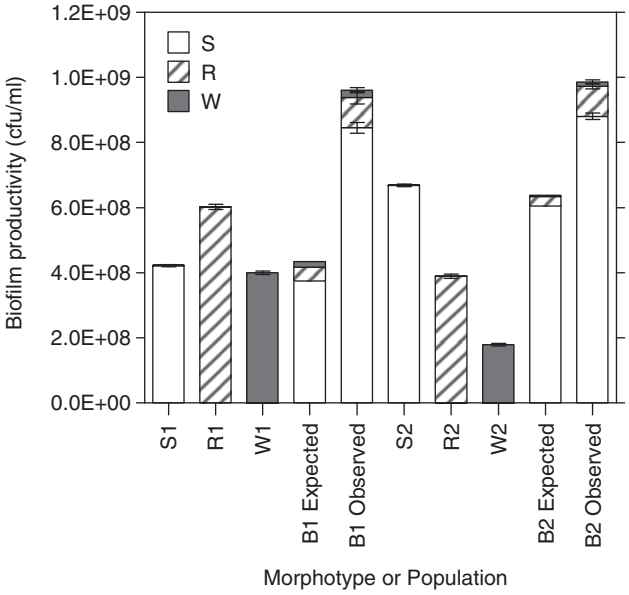


Figure 3 Productivity of S, R and W morphotypes isolated from evolved biofilm populations B1 and B2 grown in monoculture and in mixed communities. Expected productivity was calculated as the product of the proportion of each morphotype in the founding population and its yield (CFU per ml) in monoculture (Loreau and Hector, 2001). Observed productivity is the total yield of the mixed community in the experimental environment. Error bars are 95% CI based on four replicates.

Cross-feeding assays

Following 24 h of growth in the selective environment, cells were pelleted by centrifugation and the supernatant was passed through a 0.22 μm filter. Samples (200 μl) of each supernatant were added to 96-well polystyrene plates (Corning), and each morphotype was inoculated with 10^6 CFU and incubated at 37 °C for 24 h, recording optical density (OD600) following shaking every 15 min. Growth in supernatant was compared with a concurrent assay of growth in the unaltered evolution medium and expressed relative to these measures.

Microscopy

Morphotypes containing either pSPR or pSPY were grown individually on beads under standard selective conditions supplemented with 50 μg ml^{-1} chloramphenicol to maintain positive plasmid selection. Cultures were subcultured from beads into selective media containing 1 cm × 1 cm polystyrene slides. Each culture was induced with 30 μl of 10 mM isopropyl β-D-1-thiogalactopyranoside for 12 h before imaging. To label the third morphotype that was unmarked by a plasmid, slides were stained with TOPRO-3 (Invitrogen, Carlsbad, CA, USA), which stains DNA, for 1 h and destained in phosphate-buffered saline for 15 min. Slides were cleaned with ethanol on one side and placed on 75 × 25 mm glass slide (Corning). DABCO (Sigma-Aldrich, St Louis, MO, USA; 25 mg ml^{-1}) in glycerol (0.3 ml) was added to the biofilm side and a 1.5 inches glass cover slip was placed on top. Images were captured at ×200 and ×400 magnification using 2048 d.p.i. The following excitation and emission settings were used for each marker: pSPY: 514/527 nm, pSPR: 556/586 nm, TOPRO-3: 633/651 nm. Z-stack images spanned from top to bottom of biofilm. Further image analysis was conducted using Carl Zeiss Zen Light Edition 2009 software (Gottingen, Germany).

Results

Biofilm populations undergo successive adaptive diversification

As expected, biofilm production increased in all B populations but only marginally in control P populations (Supplementary Figure S1). In addition, all six B populations diversified into three classes of heritable colony morphologies (morphotypes), distinct from the ancestor, termed as smooth or studded (S), ruffled spreader (R) and wrinkly (W; population B1: Figure 2, populations B1–B6: Supplementary Figure S2). In each population, an S variant (with greater opacity, more defined colony boundaries and increased uptake of Congo red dye) was detected,

often in the majority, in the first sample at 150 generations. In the following sample at 300 generations, we detected an R variant (with rough or rugose texture and greater size), and then between 300 and 450 generations, a W variant (small, dense, highly rugose and the greatest biofilm producers). Although the morphologies of these mutant colonies varied among replicate evolved populations, they nevertheless are readily distinguishable as common types (Supplementary Figure S2) that inhabit similar regions of the test tube (Figure 1). Focusing on the clones isolated from population B1, each biofilm morphotype greatly outcompetes the ancestor in the selective environment, produces different levels of biofilm in monoculture with concomitant reduced planktonic growth rates, grows in different regions of the tube when in monoculture, and persists over nearly 5 months of transfer (Figure 2). In contrast, only one P population gave rise to a new colony type similar to S, suggesting that selection in the structured biofilm environment favors morphological and functional diversification. Indeed, eliminating structure from two of these populations at 1500 generations by removing the bead significantly reduced diversity after 24 h (Shannon–Wiener H; population B1, $t=16.30$, d.f. $=4$, $P<0.0001$; population B2: $t=385.4$, d.f. $=4$, $P<10^{-9}$). Based on the rapid decline in abundance of R and W, only S is expected to persist during prolonged transfer, and hence the stability of biofilm diversity depends on its structure.

Mixed populations are more productive because of complementary interactions

We measured effects of diversity on productivity in the selective environment by comparing the viable cell yield of two evolved mixed biofilm communities with those of their constituent morphotypes when grown alone. The cellular productivity of both mixed populations was much greater than any of their constituents in monoculture ($P<10^{-5}$ in all t-tests; Figure 3), and in both populations, the S variant attained higher yield in the mixture than in monoculture (B1: $t=47.1$, d.f. $=6$, $P<10^{-8}$; B2: $t=32.3$, d.f. $=6$, $P<10^{-8}$). This synergy did not appear to be the product of hidden diversity in the mixed sample because populations of single clones of S, R and W grown at frequencies approximating those of the mixed sample reproduced the attributes of the complete mixed community ($t=0.53$, d.f. $=8$, $P=0.61$). However, this increase in cellular productivity did not equate to increased dry weight biomass of the mixed community. Morphotypes and the mixed community varied significantly in biomass production ($F_{15,4}=17.1$, $P<0.001$) but the high-biofilm variants R and W were most productive in this assay, in the following rank order (W, R) > (S, mixed) > WT (based on Tukey–Kramer *post-hoc* tests). Given that the mixed community is >85% S, it is not surprising that its biomass is indistinguishable from that of S; further, R and W clearly produce more exopolysaccharide (Figure 2) than S.

Biodiversity may lead to increased cellular productivity of the community by two general processes: selection and complementarity (Loreau and Hector, 2001). Selection refers to 'overyielding' of the types that are most productive in monoculture, typically because they are better competitors. In contrast, complementarity reflects positive effects of biodiversity on most or all community members, either by resource partitioning or facilitation between species (Loreau and Hector, 2001). We distinguished the contributions of selection and complementarity to biofilm productivity using established methods (Price, 1970; Loreau and Hector, 2001) that test whether mixed communities depart from additive expectations of the growth of each component species, given the known starting frequencies and productivity in monoculture.

Consistent with an effect of selection, the S type remained numerically dominant in both populations. However, its proportion of the total community actually decreased slightly in both mixed populations, as the rarer R and W variants increased from their low starting frequencies, which better reflects complementarity. Although the R and W types that produce more biofilm do not grow as well as they do in monoculture, presumably because of their slower growth rates and inferiority in competing against S for nutrients (Figure 2), they are nevertheless more productive than expected when competing in mixed culture. In population B1, the productivity of S increased by 2.25-fold, R by 2.21-fold and W by 1.36-fold, resulting in 109% complementarity and −9% selection. In population B2, the productivity of S increased by 1.45-fold, R by 3.18-fold and W by 3.91-fold, resulting in 75% complementarity and 25% selection (Figure 3). Each ecotype thus benefits greatly from growth in the mixed community, causing the total populations to increase more than double in size owing to the dominant facilitative effect of complementarity.

Spatial partitioning and cross-feeding generate community synergy

We focused on two potential causes of increased productivity in the focal B1 and B2 populations: more efficient use of space by diverse types and more efficient use of available nutrients (for example, cross-feeding (Rozen *et al.*, 2009)). Partitioning of space could underlie community synergy if growth of biofilm engineers increased the total surface area for attachment and growth of all variants. Likewise, secondary metabolites could facilitate growth of other variants if they are more efficient or productive on these resources than their producers. To isolate the independent contributions of space and cross-feeding to the observed gain in productivity, we measured yields of monocultures

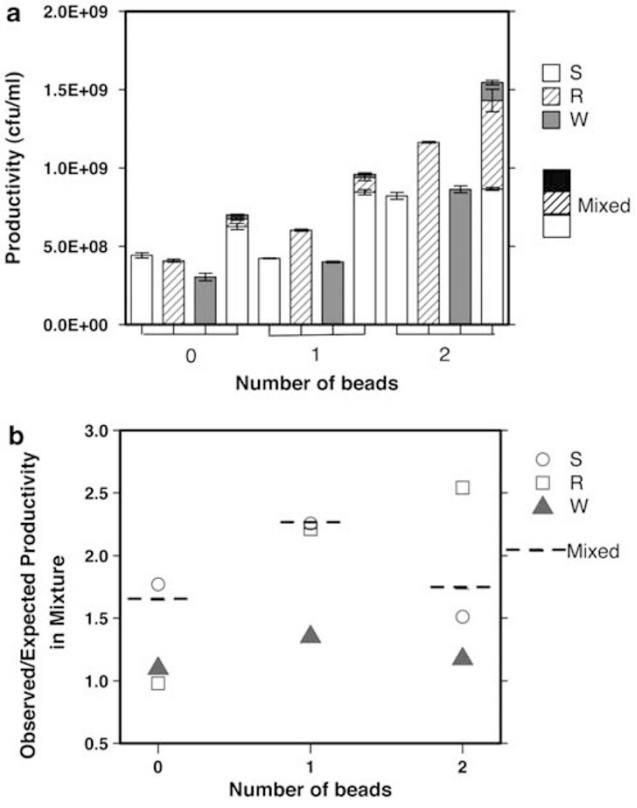


Figure 4 Contributions of cross-feeding and structure to positive effects of biodiversity. Morphotypes from population B1 were grown either in monoculture or in mixed communities in environments containing no bead (cross-feeding only), one bead (cross-feeding + structure) or two beads (cross-feeding + 2 × structure). (**a**) Productivity (CFU per ml) of monocultures and mixed communities; error bars are 95% CI based on four replicates. (**b**) Relative effect of biodiversity (observed/expected productivity) for each morphotype in the mixed communities (symbols in legend) and the overall effect on the mixed community itself (dashed line); expected yield was calculated assuming additivity (Loreau and Hector, 2001).

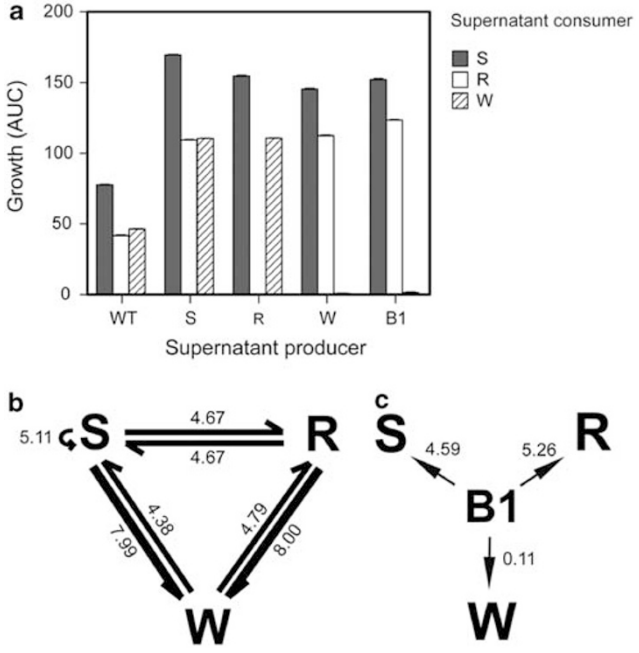


Figure 5 Absolute and relative effects of cross-feeding in pairwise interactions of supernatant producers and consumers. Isolates from population B1 and the ancestor (WT) were grown in the cell-free supernatant produced by themselves and each other type. (**a**) Growth (measured as the area under the curve of OD600 over 24 h, ± 95% CI, $n = 5$) of each morphotype in supernatants produced by fellow community morphotypes. (**b**) Cross-feeding interactions between morphotypes. Numerical values indicate fold-increase in supernatant growth relative to growth in unconditioned medium. (**c**) Effects of growth by each morphotype in the supernatant of the mixed community of population B1, calculated as in **b**.

and mixed populations in environments either lacking the bead, which eliminates structure and isolates the potential effects of cross-feeding, or containing two beads, which doubles the potential surface for adherence. In both tests, as in the long-term selection, growth on the tube walls was not sampled. Mixed populations were more productive than expected in all environments (Figure 4, Supplementary Figure S3), which demonstrates that both mechanisms operate, but S, R and W each benefit from different combinations of space and cross-feeding. Specifically, by removing the bead to emphasize cross-feeding, only S significantly benefits from growing in the mixed community ($t = 14.3$, d.f. $= 8$, $P < 0.001$), and R and W decline in frequency (Figure 4b). In contrast, by adding a bead to double the available space, the productivity of R and W increases in the mixed community (R: $t = 16.4$, d.f. $= 6$, $P < 0.001$; W: $t = 55.6$, d.f. $= 6$, $P < 0.001$) and the yield of S actually declines slightly ($t = 3.62$, d.f. $= 6$, $P = 0.011$). Mixed populations of B1 and B2 differed in their responses to varying environmental structure: in B1, the greatest advantage of diversity was found in the single-bead environment (Figure 4b), but in B2 the greatest transient advantage of diversity was found without the bead as S overgrew the mixture (Supplementary Figure S3B). These distinct responses to altered environmental structure demonstrate that the mechanisms that maintain diversity differ between populations, perhaps as a consequence of unique adaptive paths or different coevolutionary dynamics among the morphotypes.

To better quantify effects of cross-feeding, we grew isolates of S, R and W from population B1 in their own cell-free supernatant and in the supernatant of all other morphotypes plus the ancestor. Remarkably, all morphotypes grew better in supernatant than in the original medium (Supplementary Table S1), although we acknowledge that these assays were conducted under conditions different from the selection environment. However, morphotypes varied in the extent to which they improved the growth medium and these benefits were asymmetrical (Figure 5). Specifically, S grew best in its own supernatant, increasing its own productivity more than fivefold over growth in unconditioned medium (Figure 5b), but it also grew better than the other

variants in their supernatants (Figure 5a). These results demonstrate why S remains at high frequency under all conditions. In contrast, the R variant grew best in the supernatant of the mixed community, the W variant grew best in either the supernatants of S or R, and neither R nor W could grow in their own supernatants. Thus, both R and W benefit greatly from the metabolic by-products of other community members but not their own, producing a strongly interdependent food web in which only S is self-sustaining. Surprisingly, the rarest W type was essentially unable to grow in the supernatant extracted from the mixed community (Figure 5c), which may be caused by self-inhibition or by other morphotypes depleting nutrients below usable levels. For W to grow on the metabolites of S or R in a mixed community, local and structured producer–consumer interactions may be required.

Biofilm population architecture

To explore how the variants partitioned biofilm space, we imaged a mixed community of fluorescently labeled S, R and W from population B1 using confocal laser scanning microscopy (Figure 6). These images indicate that each morphotype adheres and contributes to biofilm assembly in a distinct pattern. Specifically, S cells tend to carpet the biotic surface of the biofilm (Figure 6a); R cells adhere to the plastic surface more effectively but spread rather than grow vertically (Figure 5b) and W cells produce the tallest and densest aggregates (Figure 5c). R and W variants are clearly segregated as distinct clusters across the surface, whereas S mostly inhabits a unique surface layer of the Z dimension and is more diffuse. The purple color in the assembled biofilm (Figure 6c) demonstrates a specific association between S (blue) and W (red) cells. The finding that W cells are rarely found without S may result from cross-feeding that benefits W and structural synergy that benefits S. Furthermore, the depth of the biofilm generally reaches its maximum only when all three variants are present; removing any one variant produces a biofilm with less surface coverage, height and structural resilience to physical disruption.

This biofilm architecture, in which the type that grows best (S) is found more on the biofilm exterior than on the plastic surface, led us to examine the timing of attachment of each type during the growth cycle, as the biofilm specialists R and W would be expected to attach first. Replicate cultures of the mixed population B1 were grown in the selective environment and beads were destructively sampled at regular intervals over 24 h to examine their biofilm composition. As expected, W, which sticks most strongly to the bead (and tube walls) in monoculture, is the earliest colonist and reaches a frequency of 25% between 4 and 6 h. Its frequency then begins to decline as R increases in frequency, reaching its maximum at 18 h. The density and

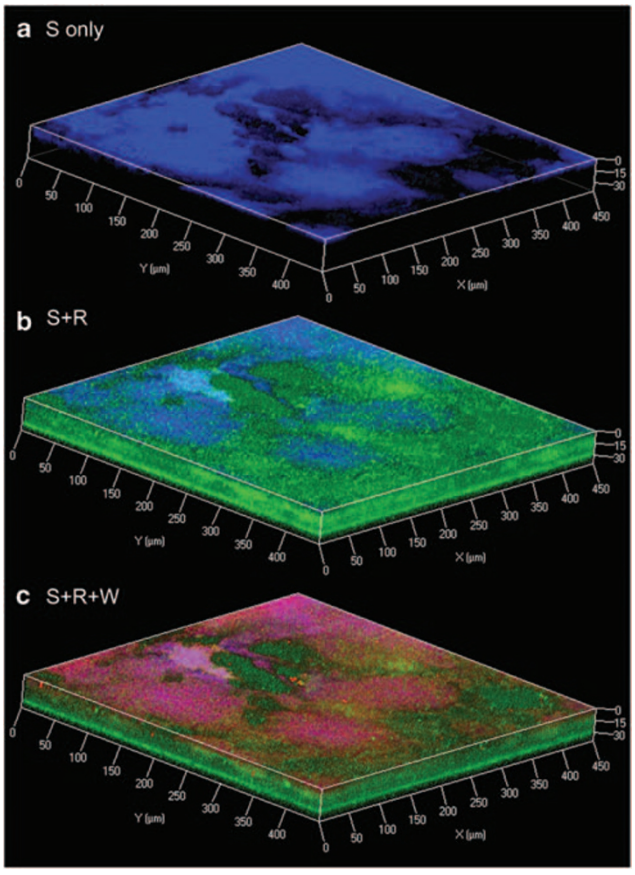


Figure 6 Confocal scanning laser microscopy of the evolved biofilm architecture. The biofilm produced by population B1 after 24 h on a polystyrene slide was rendered in three dimensions and imaged through three separate filters, a, b, and c, as follows. The entire biofilm was stained with TOPRO-3 (Invitrogen) and is projected in blue, the W morphotype carries pSPR and fluoresces red, and the R morphotype carries pSPY and is projected in green. Each morphotype inhabits a different region, and S builds clusters atop the R (indicated by yellow) and especially the W morphotype (indicated by purple).

ultimately the frequency of S rises steadily throughout the growth cycle, reaching >85% by 24 h (Figure 2). The daily assembly of the biofilm on the bead is therefore ordered in a pattern of succession that is opposite of when the ecotypes became detectable during evolution; the early evolved S attaches to the later evolved R and W, which are better surface colonists.

Discussion

Diversity is a defining trait of the biofilm lifestyle but the relative contributions of factors promoting diversity have been unclear. Biofilms are inherently structured (and slimy or sticky) environments, and this structure alone could maintain diversity that would otherwise be eliminated by competition in more uniform environments. However, the exceptional biodiversity of some microbial surface communities (>700 species may be identified in dental

plaque (Kuramitsu *et al.*, 2007) suggests that it is the interactions between species—perhaps facilitated by environmental structure—that generate biodiversity. These interactions can span from antagonistic to mutualistic, and influence community dynamics and productivity in unpredictable ways, particularly as biodiversity accumulates over longer periods of time.

To study the evolutionary consequences of prolonged biofilm selection and the ecological dynamics and forces that could contribute to biofilm diversity, we devised a novel model of biofilm selection, and studied adaptation and diversification in six B populations of *B. cenocepacia*. As a control, six replicate populations were simultaneously transferred under conditions favoring planktonic growth under the same nutrient conditions.

Not unexpectedly, we found that heritable diversity, based on colony morphology, evolved in biofilm populations but was not detected in evolved planktonic populations. However, more surprising was that novel colony types arose in a common pattern of succession in all six populations: S, then R, then W. When grown separately, these morphotypes grow in separate regions of the test tube that suggest spatial specificity of their biofilms, but when grown together, the mixed community is more productive than any one type grown alone (Figure 3). To understand the basis of this synergy, we modified the environment by increasing or decreasing the available space and thus the relative role of cross-feeding. When the plastic bead was removed, only S significantly benefited from growing in the mixed community, whereas R and W declined in number (Figure 4). In contrast, when a second bead was added, the frequency of R and W increased in the mixed community and the yield of S was unaffected. Therefore, in spite of their ability to feed on S metabolites, R and W require surface binding to persist (and benefit from increased surface area) but S can sustain itself at a lower productivity level in the absence of R and W.

Although morphotypes appeared at similar times in B populations and functioned similarly in monoculture, not every population responded equally when environmental structure was altered. In B1, the greatest advantage of diversity was found in the single-bead environment (Figure 4b), but in B2 the S morphotype grew much better than expected when the bead was removed (Supplementary Figure S3B) and the W morphotype became undetectable. These findings demonstrate that the ecological mechanisms that maintain biodiversity vary among populations as products of distinct adaptive mechanisms, forms of niche partitioning, and/or coevolution among types. Close inspection of the colony morphologies and correlated phenotypes of the S, R and W mutants from replicate populations (Supplementary Figure S2) suggests that they likely represent distinct adaptations with similar ecological consequences, but whether these different ecotypes are genetically and functionally equivalent is unknown and a subject of ongoing study.

Although this report focuses primarily on the ecological mechanisms that maintain biofilm biodiversity, we can infer how these variants initially rose to high frequency. S likely arose first because it could better exploit the selective environment than its ancestor, primarily as a better competitor for the medium but also by producing more biofilm (Figures 2 and 5). Yet during each cycle, S colonizes the new bead surface relatively late after growing best among the variants in the medium and secreting metabolites for later use. R and W may have evolved in response by exploiting the vacant bead surface and specifically consuming these metabolites (Figure 5b). Although both R and especially W remain at low frequency because of their slow growth rates (Figure 2) and because of their lesser final productivity (Figure 5a), their greatly increased biofilm production facilitate much greater attachment of S (Figures 3 and 6) and each other to the polystyrene bead. Such dynamics may therefore be viewed as one of succession enabled by niche construction (Odling-Smee *et al.*, 2003), although it is plausible that the initial invasions by biofilm specialists were driven by competition or tolerance more than facilitation (Connell and Slatyer, 1977; Day and Young, 2004).

Odum (1975) pointed out that the well-recognized phenomenon of ecological succession can be propelled by the processes of community residents themselves, including their evolved progeny, or by changing external abiotic conditions. He then defined three parameters of succession that we suggest describe the dynamics of these biofilm communities well: (1) it is orderly, reasonably directional and predictable; (2) it results from the modification of the environment by the community and (3) it maximizes productivity and stability (Odum, 1975). The parallel, sequential rise of three distinct morphotypes in each population (Figure 2, Supplementary Figure S2) fulfills the first parameter, and the increased biofilm (Figures 2 and 6) and production of novel metabolites for cross-feeding (Figure 5) fulfill the second. The third outcome of maximum productivity and stability is least certain, though we point out that these morphotypes coexisted in all replicate populations for more than 1000 generations after their origin, with obvious increases in cellular productivity and surface coverage (Figure 6). Finally, because all diversity arose from a single ancestral clone in constant abiotic conditions, these populations highlight the need to consider evolutionary processes as well as strictly ecological ones in the development of ecosystems (Odum, 1975).

Our experimental method enabled biofilm selection for 5 months but could theoretically proceed indefinitely and mimic the scale of prolonged chronic infections or more beneficial host associations. This is

also one of the first experimental evolution projects with *Burkholderia*, a functionally diverse genus that tends to associate with a wide range of hosts with varying consequences (Parke and Gurian-Sherman, 2001; Ellis and Cooper, 2010). *Burkholderia* species are found in many biofilm-associated environments and their infections are associated with biofilms, either as sources of contamination or as responses to antimicrobials. We note that the colony morphologies that evolved in each experimental population have been widely reported as associated with chronic *Burkholderia* and *Pseudomonas* infections with increased virulence and, like these experimentally evolved types, produce much greater biofilm in laboratory culture (Haussler *et al.*, 2003; Chantratita *et al.*, 2007; Starkey *et al.*, 2009) (see Chantratita *et al.*, 2007, Figure 2 in particular). A forthcoming study will compare the genetic bases of these experimental variants with those isolated from biofilm-associated infections of persons with cystic fibrosis to test whether the similar colony morphologies are caused by parallel molecular adaptations. More practically, we suggest that our model may enable manipulative experiments *in vitro* that could shed light on the patterns of succession and concomitant increased resilience of chronic biofilm-related infections (Rakhimova *et al.*, 2008).

This system also enables further mechanistic study of the evolution and maintenance of diverse communities. The relationship between diversity and productivity has remained uncertain because of the multitude of potential interactions in complex communities; for example, a positive correlation need not exist if diversification occurs by the evolution of cheats (Rainey and Rainey, 2003) or if dominant competitors arise that suppress the output of others. However, if diversification is driven by the sequential construction of new niches—here, by the likely incidental production of metabolic byproducts that enable cross-feeding and by the production of new structures for adherence by other types—a positive association between diversity and productivity becomes more likely. These findings may help explain the role of diversity not only in structured microbial communities but also in a wide range of complex natural ecosystems in which interactions for food and living space may be common. In summary, as many biofilm researchers have speculated (Stoodley *et al.*, 2002; Boles *et al.*, 2004; Brockhurst *et al.*, 2006), biofilm productivity is positively associated with the evolution of ecological diversification.

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References

Boles BR, Thoendel M, Singh PK. (2004). Self-generated diversity produces 'insurance effects' in biofilm communities. *Proc Natl Acad Sci USA* **101**: 16630–16635.

Brockhurst MA. (2007). Population bottlenecks promote cooperation in bacterial biofilms. *PLoS ONE* **2**: e634.

Brockhurst MA, Hochberg ME, Bell T, Buckling A. (2006). Character displacement promotes cooperation in bacterial biofilms. *Curr Biol* **16**: 2030–2034.

Buckling A, Rainey PB. (2002). The role of parasites in sympatric and allopatric host diversification. *Nature* **420**: 496–499.

Cardinale BJ, Wright JP, Cadotte MW, Carroll IT, Hector A, Srivastava DS *et al.* (2007). Impacts of plant diversity on biomass production increase through time because of species complementarity. *Proc Natl Acad Sci USA* **104**: 18123–18128.

Chantratita N, Wuthiekanun V, Boonbumrung K, Tiyawisutsri R, Vesaratchavest M, Limmathurotsakul D *et al.* (2007). Biological relevance of colony morphology and phenotypic switching by *Burkholderia pseudomallei*. *J Bacteriol* **189**: 807–817.

Connell JH, Slatyer RO. (1977). Mechanisms of succession in natural communities and their role in community stability and organization. *Am Nat* **111**: 1119–1144.

Costerton JW. (2001). Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends Microbiol* **9**: 50–52.

Day T, Young KA. (2004). Competitive and facilitative evolutionary diversification. *BioScience* **54**: 101–109.

Ellis CN, Cooper VS. (2010). Experimental adaptation of *Burkholderia cenocepacia* to onion medium reduces host range. *Appl Environ Microbiol* **76**: 2387–2396.

Gross K, Cardinale BJ. (2007). Does species richness drive community production or vice versa? Reconciling historical and contemporary paradigms in competitive communities. *Am Nat* **170**: 207–220.

Habets MG, Rozen DE, Hoekstra RF, de Visser JA. (2006). The effect of population structure on the adaptive radiation of microbial populations evolving in spatially structured environments. *Ecol Lett* **9**: 1041–1048.

Hansen SK, Rainey PB, Haagensen JA, Molin S. (2007). Evolution of species interactions in a biofilm community. *Nature* **445**: 533–536.

Harrison F, Buckling A. (2009). Siderophore production and biofilm formation as linked social traits. *ISME J* **3**: 632–634.

Haussler S, Lehmann C, Breselge C, Rohde M, Classen M, Tummler B *et al.* (2003). Fatal outcome of lung transplantation in cystic fibrosis patients due to small-colony variants of the *Burkholderia cepacia* complex. *Eur J Clin Microbiol Infect Dis* **22**: 249–253.

Huang LN, De Wever H, Diels L. (2008). Diverse and distinct bacterial communities induced biofilm fouling in membrane bioreactors operated under different conditions. *Environ Sci Technol* **42**: 8360–8366.

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM *et al.* (1995). 4 New derivatives of the broad-host-range cloning vector Pbbr1mcs, carrying different antibiotic-resistance cassettes. *Gene* **166**: 175–176.

Kuramitsu HK, He X, Lux R, Anderson MH, Shi W. (2007). Interspecies interactions within oral microbial communities. *Microbiol Mol Biol Rev* **71**: 653–670.

Lambertsen L, Sternberg C, Molin S. (2004). Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ Microbiol* **6**: 726–732.

LiPuma JJ, Spilker T, Coenye T, Gonzalez CF. (2002). An epidemic *Burkholderia cepacia* complex strain identified in soil. *Lancet* **359**: 2002–2003.

Loreau M, Hector A. (2001). Partitioning selection and complementarity in biodiversity experiments. *Nature* **412**: 72–76.

Lyautey E, Jackson C, Cayrou J, Rols J-L, Garabétian F. (2005). Bacterial community succession in natural river biofilm assemblages. *Microb Ecol* **50**: 589–601.

Meyer JR, Kassen R. (2007). The effects of competition and predation on diversification in a model adaptive radiation. *Nature* **446**: 432–435.

Nadell CD, Xavier JB, Foster KR. (2009). The sociobiology of biofilms. *FEMS Microbiol Rev* **33**: 206–224.

Nguyen D, Singh PK. (2006). Evolving stealth: genetic adaptation of *Pseudomonas aeruginosa* during cystic fibrosis infections. *Proc Natl Acad Sci USA* **103**: 8305–8306.

Nocker A, Lepo JE, Snyder RA. (2004). Influence of an oyster reef on development of the microbial heterotrophic community of an estuarine biofilm. *Appl Environ Microbiol* **70**: 6834–6845.

O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. (1999). Genetic approaches to study of biofilms. *Methods Enzymol* **310**: 91–109.

Odling-Smee FJ, Laland KN, Feldman MW. (2003). *Niche Construction: The Neglected Process in Evolution*, Vol. 37 Princeton University Press: Princeton, NJ.

Odum EP. (1975). *Ecology*, 2nd edn. Holt, Rinehart, and Winston: New York.

Parke JL, Gurian-Sherman D. (2001). Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. *Annu Rev Phytopathol* **39**: 225–258.

Ponciano JM, La HJ, Joyce P, Forney LJ. (2009). Evolution of diversity in spatially structured *Escherichia coli* populations. *Appl Environ Microbiol* **75**: 6047–6054.

Price GR. (1970). Selection and covariance. *Nature* **227**: 520–521.

Rainey PB, Rainey K. (2003). Evolution of cooperation and conflict in experimental bacterial populations. *Nature* **425**: 72–74.

Rainey PB, Travisano M. (1998). Adaptive radiation in a heterogeneous environment. *Nature* **394**: 69–72.

Rakhimova E, Munder A, Wiehlmann L, Bredenbruch F, Tummler B. (2008). Fitness of isogenic colony morphology variants of *Pseudomonas aeruginosa* in murine airway infection. *PLoS ONE* **3**: e1685.

Ramette A, LiPuma JJ, Tiedje JM. (2005). Species abundance and diversity of Burkholderia cepacia complex in the environment. *Appl Environ Microbiol* **71**: 1193–1201.

Rozen DE, Nadège PJ, de Visser JA, Lenski RE, Schneider D. (2009). Death and cannibalism in a seasonal environment facilitate bacterial coexistence. *Ecol Lett* **12**: 34–44.

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**: 762–764.

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA *et al.* (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* **103**: 8487–8492.

Starkey M, Hickman JH, Ma L, Zhang N, De Long S, Hinz A *et al.* (2009). *Pseudomonas aeruginosa* rugose small colony variants have adaptations likely to promote persistence in the cystic fibrosis lung. *J Bacteriol* **191**: 3492–3503; JB.00119-00109.

Stoodley P, Sauer K, Davies DG, Costerton JW. (2002). Biofilms as complex differentiated communities. *Annu Rev Microbiol* **56**: 187–209.

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