LETTERS

Evolution of species interactions in a biofilm community

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Biofilms are spatially structured communities of microbes whose function is dependent on a complex web of symbiotic interactions[1,2]. Localized interactions within these assemblages are predicted to affect the coexistence of the component species[3–5], community structure[6] and function[7–10], but there have been few explicit empirical analyses of the evolution of interactions[11]. Here we show, with the use of a two-species community, that selection in a spatially structured environment leads to the evolution of an exploitative interaction. Simple mutations in the genome of one species caused it to adapt to the presence of the other, forming an intimate and specialized association. The derived community was more stable and more productive than the ancestral community. Our results show that evolution in a spatially structured environment can stabilize interactions between species, provoke marked changes in their symbiotic nature and affect community function.

Life is sustained through the activities of microorganisms[8–10], most of which function within biofilm communities composed—in some instances—of hundreds of metabolically diverse species[12–14]. Rules governing biofilm assembly, function and evolution have been largely unexplored[15,16], but as with communities of macroorganisms, local interactions between component organisms in spatially structured environments are likely to be of central importance[11,17–20]. Here we use a community composed of two bacterial species to explore experimentally how spatial structure allows local species interactions to evolve and affect community function.

Acinetobacter sp. (strain C6) and *Pseudomonas putida* (strain KT2440) are two unrelated soil-inhabiting bacteria[21]. When propagated in an environment with benzyl alcohol as the sole carbon source, the persistence of *P. putida* is dependent on the presence of *Acinetobacter*. *Acinetobacter* partitions the primary carbon source into benzoate, a substrate that is partly excreted by *Acinetobacter* and can be metabolized by *P. putida*[22]. The relationship between *Acinetobacter* and *P. putida* thus approximates that of a host (*Acinetobacter*) and a commensal (*P. putida*) and it is expected to be frequency dependent—although *P. putida* is not expected to persist below a threshold concentration of benzyl alcohol[23]. We tested this prediction by determining the ability of each species to invade, when rare, a population dominated by the other species, and did so first in a homogeneous (spatially unstructured) chemostat environment and second in a spatially structured environment: a glass surface within a biofilm flow chamber.

Acinetobacter increased in frequency from rare with a relative fitness significantly greater than zero at all benzyl alcohol concentrations tested (see Supplementary Methods). In the chemostat environment *P. putida* coexisted with *Acinetobacter*, but only at benzyl alcohol concentrations above about 430 μM (Fig. 1). When cocultivated on a glass surface, *P. putida* and *Acinetobacter* coexisted in a stable frequency-dependent manner at benzyl alcohol concentrations as low as about 130 μM. Spatial structure thus extended the range of resource concentrations over which the two species coexist. This is most readily explained by higher local concentrations of benzoate in the biofilm flow chambers, but spatial clustering may also facilitate coexistence by minimizing potential competitive interactions, such as those likely to arise from competition for oxygen.

The structure of this two-species community was investigated by examining the glass surface of a 24-h-old biofilm flow chamber by microscopy. This revealed a mutually exclusive association, with

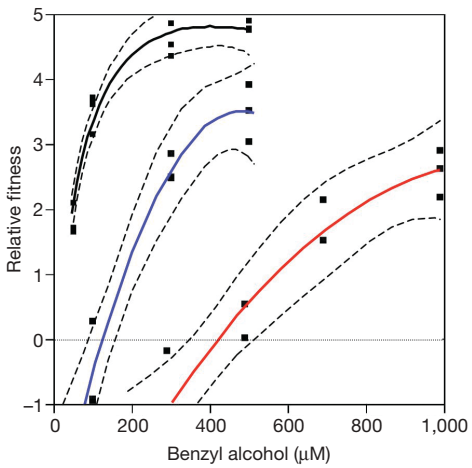

Figure 1 | Coexistence of *P. putida* KT2440 and *Acinetobacter* sp. C6 in structured (biofilm flow chamber) and unstructured (chemostat) environments with benzyl alcohol as the sole carbon source. Fitness measures were determined over 24 h at different concentrations of benzyl alcohol and are the difference between the malthusian parameter of the initially rare species (*P. putida*) and that of the common species (*Acinetobacter*): a fitness of zero indicates equal competitive ability[28]. Lines are second-order polynomials plus 95% confidence curves fitted to the data (a minimum of three replicate measurements were determined at a minimum of three different concentrations of benzyl alcohol in each experiment). The red line denotes the fitness of ancestral *P. putida* in chemostat culture, the blue line the fitness of *P. putida* in biofilm flow chambers, and the black line the fitness of the derived rough variant of *P. putida* in biofilm flow chambers. The threshold concentration of benzyl alcohol above which *P. putida* and *Acinetobacter* coexist is indicated at the point at which each curve crosses the reference line. A curve for the fitness of the derived rough variant of *P. putida* mutant in chemostat culture is not shown, but in this environment this genotype was unable to increase in frequency against *Acinetobacter* populations at benzyl alcohol concentrations lower than about 950 μM (at 1 mM benzyl alcohol the fitness of derived *P. putida* was 0.69 (95% confidence interval 0.165–1.215)).

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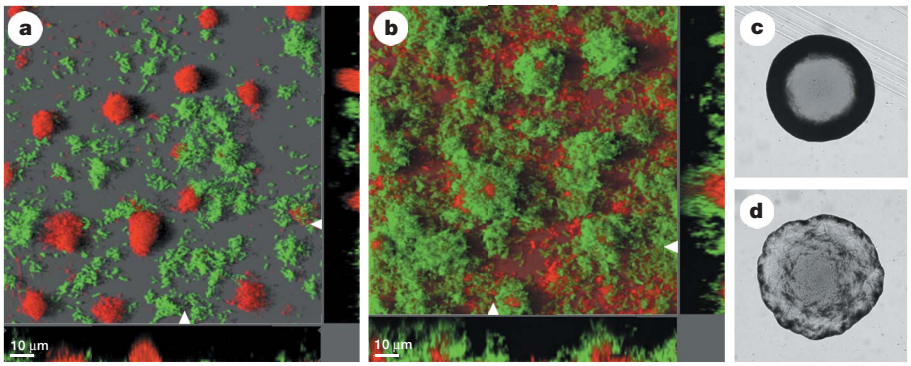

Figure 2 | Characteristic biofilm and colony phenotypes of the ancestral and the derived rough variant of *P. putida*. Flow-chamber biofilms were propagated for 24 h with 500 μM benzyl alcohol as the sole carbon source. **a**, **b**, Confocal scanning laser microscope (CSLM) micrographs were obtained of mixed biofilms containing *Acinetobacter* sp. C6 (red) and ancestral *P. putida* (green) (**a**) and *Acinetobacter* sp. C6 (red) and a rough variant of *P. putida* (green) (**b**). The main panels show simulated fluorescence projection images (SFP): the outer horizontal and vertical panels show a cross-section through the *x*–*z* dimension at the positions marked by the arrows. The images shown are representative of eight images taken from each of two independent flow chambers. **c**, The smooth colony morphology of the ancestral *P. putida*. **d**, The rough colony morphology of a biofilm-derived genotype.

discrete colonies of *Acinetobacter* surrounded by loose assemblages of *P. putida* (Fig. 2a). After propagation for five days the association changed. At separate focal points across the surface of replicate glass plates, *P. putida* grew in intimate contact with *Acinetobacter*. Within a further five days *Acinetobacter* colonies had been overgrown by a mantle of *P. putida*.

To determine the cause of this altered community structure, cells were harvested from 16 independent ten-day mixed-species flow chambers and spread on agar plates. Careful observation showed that all agar plates harboured a common rough-colony variant of *P. putida* that was phenotypically distinct from the ancestral type (Fig. 2c, d). The phenotype of the variant was heritable, and when inoculated together with ancestral *Acinetobacter* into fresh biofilm flow chambers the structure of the resulting community was radically altered: instead of a mutually exclusive association the two species grew in close contact; *P. putida* formed a mantle over the top of *Acinetobacter* colonies (Fig. 2b). This structure was achieved within 24 h and was fully reminiscent of that observed previously in the five-day flow chambers.

Niche specialist genotypes can evolve when populations experience selection in spatially structured environments[19]. The rough variant of *P. putida* might reflect a specific adaptive response to the physical environment afforded by the biofilm flow chamber, to the biological environment afforded by *Acinetobacter*, or to a combination of both of these. To discriminate between these possibilities, we inoculated replicate flow chambers with *P. putida* and maintained them for ten days in the absence of *Acinetobacter*. Bacteria were harvested from the surface of glass plates from biofilm flow chambers and cultured on agar plates. No rough variants of *P. putida* were observed in any of five independent biofilm chambers, and *P. putida* taken from ten-day flow chambers did not grow as a mantle over the top of *Acinetobacter* colonies. Further, no rough variants were detected after propagation of *P. putida* in chemostat culture with *Acinetobacter*.

To examine further the evolutionary significance of the rough *P. putida* variant, its competitive fitness relative to the ancestral genotype was determined in the presence of *Acinetobacter* in both biofilm flow chambers and chemostat culture. The fitness of the rough variant was dependent on environment (by analysis of variance (ANOVA), $F_{1,4} = 1050.94$; $P < 0.0001$) and significantly greater than the wild type in biofilm flow chambers. Enhanced fitness in the biofilm flow chamber was traded against a significantly reduced fitness in chemostat culture (Fig. 3).

In a separate but parallel experiment the fitness of derived *P. putida* was determined relative to the ancestral genotype in the absence of *Acinetobacter* (*P. putida* is unable to grow on benzyl alcohol in the absence of *Acinetobacter*; benzoate was therefore supplied as the sole carbon source). ANOVA showed that the fitness of the derived rough *P. putida* genotype was not affected by the structure of the environment ($F_{1,4} = 0.03$; $P = 0.87$) and did not differ significantly from the ancestral genotype (Fig. 3).

Together these data indicate that neither spatial structure nor the presence of *Acinetobacter* was sufficient to promote the evolution of the rough variant types: both were necessary, but the adaptive response was specific to *Acinetobacter*.

Suspecting that adaptive evolution on the part of *P. putida* might have stabilized the interaction further, we examined the effect of benzyl alcohol concentration on the coexistence of *Acinetobacter* and the derived rough *P. putida* genotype. In spatially structured flow chambers the two species coexisted stably at the lowest concentration (50 μM) of benzyl alcohol tested (Fig. 1)—a significant improvement in community stability compared with that of the ancestral community. Consistent with the evolution of a more specialized relationship was our observation that coexistence of the derived community was severely compromised in the chemostat environment: the benzyl alcohol concentration required for coexistence exceeded 950 μM (see legend to Fig. 1).

Stabilization of the interaction between *Acinetobacter* and *P. putida* is likely to have implications not only for community structure but also for the symbiotic relationship and function of the community. One way of assessing this impact is by the analysis of productivity (biomass) of the individual species grown alone and in combination (Fig. 4). After three days of propagation in flow chambers with benzyl alcohol as the sole carbon source, the productivity of the derived community was significantly greater than that of both the ancestral community and *Acinetobacter* alone (ANOVA: $F_{2,6} = 106.57$; $P < 0.0001$). This effect was attributable to enhanced productivity of *P. putida* and occurred despite a deleterious effect of *P. putida* on *Acinetobacter*—the species on which *P. putida* is reliant. Selection in a spatially structured environment thus changed the relationship between the two species and tipped the balance towards a more exploitative interaction, but overall community function was enhanced.

Last, in an attempt to understand the genetic and physiological causes of the newly emerged symbiosis, the mutation(s) responsible for the mantle-like growth of *P. putida* were sought (see Supplementary Methods). Two independent mutations were identified in *wapH* (PP4943)—a gene involved in lipopolysaccharide (LPS) biosynthesis (see Supplementary Table 1 and Supplementary Fig. 1). In *P. aeruginosa* the homologous *wapH* gene is necessary for the assembly of outer core LPS[24].

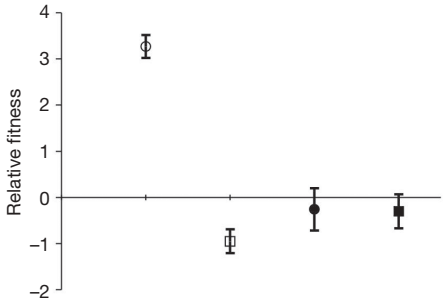

Figure 3 | Competitive fitness of the rough variant of *P. putida*. Fitness was determined relative to ancestral *P. putida* (initial ratio 1:1) in the presence (open symbols) and absence (filled symbols) of *Acinetobacter* in both spatially structured (circles) and unstructured (squares) environments. *P. putida* is unable to grow on benzyl alcohol in the absence of *Acinetobacter*; the fitness of derived *P. putida* in the absence of *Acinetobacter* was therefore determined in environments containing 200 μM benzoate as the sole carbon source. Data are means and 95% confidence intervals; fitness values with confidence intervals that span zero are not significantly different from that of the ancestral genotype. See the text for ANOVA.

To determine the evolutionary significance of the *wapH* mutation, a non-polar mutation was made in *wapH* from the ancestral strain (see Supplementary Methods). This mutant (SNZ83) was phenotypically indistinguishable from the spontaneously arising rough mutants. The fitness of the mutant was determined by competition with the isogenic wild-type strain (SM1243) in biofilm flow chambers in the presence of *Acinetobacter*. Its fitness relative to the wild type was 3.4 (95% confidence interval 3.214–3.639) and was indistinguishable from that of the spontaneously occurring rough mutant analysed previously (Fig. 3). Mutations within *wapH* are therefore sufficient to generate the rough *P. putida* variant.

The physiological basis of the interaction is complex: *P. putida* is dependent on *Acinetobacter* for benzoate, but the two species also require oxygen. When the concentration of oxygen falls below a threshold, wild-type *P. putida* detaches and leaves the biofilm, resulting in uncolonized zones surrounding each *Acinetobacter* colony (Fig. 2a). Physiological changes in derived *P. putida* (it becomes sticky) cause it to associate with *Acinetobacter* (but not the glass surface), such that the oxygen-dependent detachment programme is overridden. This leads to the intimate association observed in Fig. 2b and is the cause of enhanced fitness in the structured environment in the presence, but not the absence, of *Acinetobacter* (Fig. 3). The close proximity of derived *P. putida* to *Acinetobacter* means that it acquires more benzoate, but this has a detrimental effect on *Acinetobacter* (Fig. 4), most probably due to intensified competition for oxygen (S.K.H., J.A.J.H., M. Gjermansen, T. Martini Jørgensen, T. Tolker-Nielsen and S.M., unpublished observations).

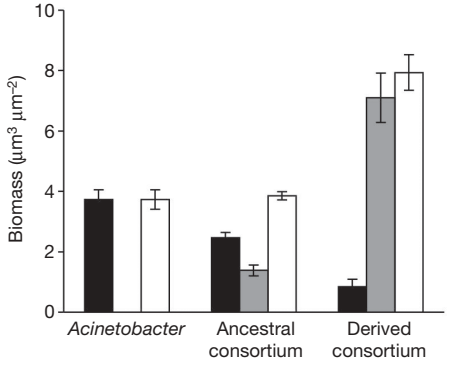

Figure 4 | Productivity of ancestral and derived communities. Biomass was determined after three days by CSLM imaging and COMSTAT analysis. Black bars represent *Acinetobacter*, light grey bars represent *P. putida*, and white bars represent total biomass. Data are means and 95% confidence intervals from three replicate biofilm flow chambers. The negative effect of ancestral *P. putida* on *Acinetobacter* is a consequence of competition for oxygen.

Biofilm communities might lack the grandeur of a tropical rainforest, but not their complexity, or their significance in terms of ecosystem function[8–10]. Central to the structure, composition and function of any community is a complex web of interactions[11]. Although ecologists often consider such interactions to be hard-wired, they are capable of rapid evolutionary change[25,26]. Here we have witnessed the first step in the evolution of an interaction between two species with marked effects on community function. The simplicity of our experimental design enables an explicit statement of causality. Spatial structure was the key environmental factor: it allowed the establishment of a structured community (Fig. 2a) and provided the selective conditions necessary for the interaction between *Acinetobacter* and *P. putida* to evolve. Within a short duration the community structure changed: the abundance of each type was altered, and the community became more stable and more productive. Interactions are the key to understanding biological communities: the real challenge is to understand how interactions evolve[11].

METHODS

Strains and growth conditions. *Acinetobacter* sp. strain C6 (streptomycin-resistant derivative) was isolated from a toluene gas reactor[21]. The strain was grown at 30 °C in Luria–Bertani (LB) agar with streptomycin (100 μg ml^{-1}) for plate enumerations. The soil bacterium *Pseudomonas putida* KT2440 (ref. 27) (devoid of plasmid pWW0) and derivatives were grown at 30 °C in minimal AB agar supplemented with 40 mM sodium citrate for plate enumerations. Mixed-species biofilm and chemostat populations were propagated on FAB minimal medium (AB minimal medium with 10 μM Fe-EDTA replacing $FeCl_3$) containing benzyl alcohol as specified. Single-species biofilm and chemostat populations of *P. putida* were propagated with FAB medium containing 200 μM sodium benzoate.

Fitness. Population densities (N_i) were determined at times $t = 0$ and at $t = T$. The malthusian parameter[28] (m_i), which is the average rate of increase, was calculated for both competitors: $m_i = \ln[N_i(T)/N_i(0)]/T$. Relative fitness is expressed here as the selection rate constant: $r_{ij} = m_i - m_j$, resulting in a fitness of zero when competing organisms are equally fit.

Biofilm experiments. Biofilms were grown at 24 °C in three-channel flow chambers with individual channel dimensions of $1 \times 4 \times 40$ mm^3. The continuous-flow system was assembled and prepared as described[29], with an additional overnight wash with sterile milliQ-water. After inoculation the medium flow was stopped for 1 h and resumed at a rate of 3.3 ml h^{-1} (see Supplementary Methods).

Populations were prepared for inoculation into flow chambers by first growing strains overnight in shaken broth culture. The population density of each type was adjusted as necessary and introduced into the chamber. Initial ($t = 0$) and final ($t = 24$ h) population densities were determined either by colony counts on agar plate culture or by measurement of biomass. In chambers containing both *Acinetobacter* and *P. putida*, the density of each type was derived from measures of biomass by fluorescence imaging: 12 images from each biofilm were recorded at random along the flow channel with a Zeiss LSM510 Confocal Scanning Laser Microscope equipped with a 40×/1.3 numerical aperture (n.a.) Plan-Neofluar oil objective plus filter sets for monitoring green fluorescent protein (GFP; green, *P. putida*) and Syto62 (red, *Acinetobacter*) (see Supplementary Methods). Images were analysed by the COMSTAT program[30], calculating the biomass volume (μm^3 μm^{-2}) of each strain. Biofilm images for illustration were recorded with a 63×/1.4 n.a. Plan-APOChromat objective and processed with the IMARIS software package (Bitplane AG). As this is a destructive process, measures at $t = 0$ were obtained from paired control chambers. In chambers without *Acinetobacter*, population densities were obtained by counting colonies arising on agar plates after appropriate dilution. Biofilms were propagated with 500 μM benzyl alcohol unless otherwise stated. To check for the presence of rough variants of *P. putida* in biofilm flow chambers propagated in the absence of *Acinetobacter*, a minimum of 500 colonies were examined from each of five independent biofilm chambers.

Chemostat experiments. Chemostat vessels (300 ml running volume, dilution rate 0.6 h^{-1}) were operated with a cell density of (1–5) × 10^7 ml^{-1} depending on the carbon source concentration. Before inoculation, cells from overnight cultures were acclimated for about 6 h in batch culture. Chemostat cultures were grown at 24 °C with aeration. Initial ($t = 0$) and final ($t = 24$ h) population ratios

were determined by plate enumeration. To check for the evolution of the derived rough variant of *P. putida* in chemostat culture a minimum of 1,000 colonies were sampled from each of three replicate chemostats.
Genetic analyses. See Supplementary Methods.

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1. Davey, M. E. & O'Toole, G. A. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64,** 847–867 (2000).
2. Tyson, G. W. *et al.* Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428,** 37–43 (2004).
3. Kerr, B., Riley, M. A., Feldman, M. W. & Bohannan, B. J. M. Local dispersal promotes biodiversity in a real-life game of rock–paper–scissors. *Nature* **418,** 171–174 (2002).
4. Hassell, M. P., Comins, H. N. & May, R. M. Species coexistence and self-organizing spatial dynamics. *Nature* **370,** 290–292 (1994).
5. Durrett, R. & Levin, S. The importance of being discrete (and spatial). *Theor. Popul. Biol.* **46,** 363–394 (1994).
6. Tilman, D. Niche tradeoffs, neutrality, and community structure: a stochastic theory of resource competition, invasion, and community assembly. *Proc. Natl Acad. Sci. USA* **101,** 10854–10861 (2004).
7. Loreau, M. *et al.* Ecology—Biodiversity and ecosystem functioning: Current knowledge and future challenges. *Science* **294,** 804–808 (2001).
8. Newman, D. K. & Banfield, J. F. Geomicrobiology: how molecular-scale interactions underpin biogeochemical systems. *Science* **296,** 1071–1077 (2002).
9. Battin, T. J., Kaplan, L. A., Denis Newbold, J. & Hansen, C. M. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature* **426,** 439–442 (2003).
10. Labrenz, M. *et al.* Formation of sphalerite (ZnS) deposits in natural biofilms of sulfate-reducing bacteria. *Science* **290,** 1744–1747 (2000).
11. Thompson, J. N. *The Coevolutionary Process* (Univ. of Chicago Press, Chicago, IL, 1994).
12. Ley, R. E. *et al.* Unexpected diversity and complexity of the Guerrero Negro hypersaline microbial mat. *Appl. Environ. Microbiol.* **72,** 3685–3695 (2006).
13. Kroes, I., Lepp, P. W. & Relman, D. A. Bacterial diversity within the human subgingival crevice. *Proc. Natl Acad. Sci. USA* **96,** 14547–14552 (1999).
14. Ram, R. J. *et al.* Community proteomics of a natural microbial biofilm. *Science* **308,** 1915–1920 (2005).
15. Boles, B. R., Thoendel, M. & Singh, P. K. Self-generated diversity produces 'insurance effects' in biofilm communities. *Proc. Natl Acad. Sci. USA* **101,** 16630–16635 (2004).
16. An, D., Danhorn, T., Fuqua, C. & Parsek, M. R. Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. *Proc. Natl Acad. Sci. USA* **103,** 3828–3833 (2006).
17. Montoya, J. M., Pimm, S. L. & Sole, R. V. Ecological networks and their fragility. *Nature* **442,** 259–264 (2006).
18. Tilman, D. & Kareiva, P. (eds) *Spatial Ecology* (Princeton Univ. Press, Princeton, NJ, 1997).
19. Rainey, P. B. & Travisano, M. Adaptive radiation in a heterogeneous environment. *Nature* **394,** 69–72 (1998).
20. Forde, S. E., Thompson, J. N. & Bohannan, B. J. Adaptation varies through space and time in a coevolving host–parasitoid interaction. *Nature* **431,** 841–844 (2004).
21. Moller, S., Pedersen, A. R., Poulsen, L. K., Arvin, E. & Molin, S. Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative in situ hybridization and scanning confocal laser microscopy. *Appl. Environ. Microbiol.* **62,** 4632–4640 (1996).
22. Christensen, B. B., Haagensen, J. A., Heydorn, A. & Molin, S. Metabolic commensalism and competition in a two-species microbial consortium. *Appl. Environ. Microbiol.* **68,** 2495–2502 (2002).
23. Turner, P. E., Souza, V. & Lenski, R. E. Tests of ecological mechanisms promoting the stable coexistence of two bacterial genotoypes. *Ecology* **77,** 2119–2129 (1996).
24. Lam, J. S., Matewish, M. & Poon, K. K. H. in *Pseudomonas* Vol. 3 (ed. Ramos, J. I.) 3–53 (Kluwer/Plenum, New York, 2004).
25. Urban, M. C. & Skelly, D. K. Evolving metacommunities: toward an evolutionary perspective on metacommunities. *Ecology* **87,** 1616–1626 (2006).
26. Thompson, J. N. & Cunningham, B. M. Geographic structure and dynamics of coevolutionary selection. *Nature* **417,** 735–738 (2002).
27. Franklin, F. C., Bagdasarian, M., Bagdasarian, M. M. & Timmis, K. N. Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. *Proc. Natl Acad. Sci. USA* **78,** 7458–7462 (1981).
28. Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am. Nat.* **138,** 1315–1341 (1991).
29. Christensen, B. B. *et al.* Molecular tools for study of biofilm physiology. *Methods Enzymol.* **310,** 20–42 (1999).
30. Heydorn, A. *et al.* Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146,** 2395–2407 (2000).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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