doi:10.1111/j.1558-5646.2009.00708.x

COEVOLUTION BETWEEN COOPERATORS AND CHEATS IN A MICROBIAL SYSTEM

Quan-Guo Zhang,[1,2,3] Angus Buckling,[1,4] Richard J. Ellis,[2,5,6] and H. Charles J. Godfray[1,7]

[1]*Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK*

[2]*NERC Centre for Population Biology, Imperial College London, Silwood Park Campus, Ascot, Berkshire SL5 7PY, UK*

[3]*E-mail: quan-guo.zhang@zoo.ox.ac.uk*

[4]*E-mail: angus.buckling@zoo.ox.ac.uk*

[5]*E-mail: r.ellis@vla.defra.gsi.gov.uk*

[7]*E-mail: charles.godfray@zoo.ox.ac.uk*

Received September 25, 2008
Accepted March 24, 2009

In many circumstances organisms invest in cooperative activities to increase their mutual fitness but are susceptible to cheats that obtain the benefits of cooperation without investment. Natural selection may favor cooperators that resist cheats, and cheats that avoid such resistance; in theory the coevolutionary interaction may be sustained and dynamic. Here, we report evidence of antagonistic coevolution between cooperators and cheats involved in biofilm formation by *Pseudomonas fluorescens* bacteria. Two distinct phenotypes occur in static culture tubes: one that can form a biofilm at the air–broth interface and thus obtain improved access to oxygen, and one that colonizes the broth phase but which can also invade, and weaken, the biofilm produced by the other type. Over serial passage, biofilm producers (considered here as cooperators) evolve to become better at resisting invasion, and biofilm nonproducers (cheats) evolve to be more efficient invaders. Each type has higher performance (resistance in the case of cooperators and biofilm invasion for cheats) in competition with isolates of the other type from their past compared to that from their future, indicating a dynamic coevolutionary interaction. Such coevolution may have important consequences for the maintenance of cooperation.

KEY WORDS: **Biofilm, microcosm, *Pseudomonas fluorescens*, smooth morph (SM), wrinkly spreader (WS).**

In many situations individual organisms can cooperate to increase their mutual fitness. But cooperation may not be evolutionarily stable if one individual continues to enjoy a group benefit when cheating, that is when it ceases to engage in the cooperative behavior with its attendant costs. How cooperation that is resistant to cheating evolves and is maintained has been a major preoccupation of evolutionary biology for over 40 years (Hamilton 1964; Maynard Smith 1964; Maynard Smith and Szathmary 1995), with kin selection (Hamilton 1964) and reciprocal altruism (Trivers 1971) now considered the most important processes increasing the frequency of cooperation (Sachs et al. 2004; Lehmann and Keller 2006; Nowak 2006; West et al. 2006; Rankin et al. 2007; West et al. 2007b). However, natural selection acting on cooperation and cheating may not lead to a simple static resolution. A dynamic equilibrium may occur with reciprocal selection leading to new cheating behaviors followed by the emergence of new strategies among cooperators to resist cheats, akin to arms races in the evolution of predators and prey or hosts and parasites (van Valen 1973; Chao et al. 1977; Anderson and May 1982; Hamilton et al. 1990; Thompson 1998; Abrams 2000; Buckling and Rainey 2002; Brockhurst et al. 2003; Morgan et al. 2005). Alternatively, cycles may occur in the frequency of cooperative behavior, for example when the benefits and costs of cooperating are frequency dependent, and when there is a time lag that prevents the frequency

[6]Current address: Molecular Pathogenesis and Genetics, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, KT15 3NB, UK

of cooperators and/or cheaters from responding instantaneously to changes in relative fitness (Riolo et al. 2001; van Baalen and Jansen 2003). Demonstrating dynamic equilibria or cyclic cooperation is challenging in large multicellular eukaryotes that are the subjects of most research in social evolution because of the requirements for long-term, replicated data.

Microbial systems are increasingly being used to explore questions in social evolution (Rainey and Rainey 2003; Kerr et al. 2006; MacLean and Gudelj 2006; West et al. 2006; Diggle et al. 2007b; Ross-Gillespie et al. 2007; Sandoz et al. 2007). Partly this is because their short generation time and small sizes allow long-term, replicated data on experimental evolution to be collected relatively easily, but also because recent research has revealed unexpected complexity in the social interactions of microbes (Crespi 2001; West et al. 2007a). Many bacteria cooperatively release substances into the external environment that enable them to break down potential food sources (Greig and Travisano 2004; Sandoz et al. 2007), render toxic substances harmless (Ellis et al. 2007), scavenge for limiting nutrients such as iron (West and Buckling 2003; Griffin et al. 2004), increase mobility over surfaces (Velicer and Yu 2003; Daniels et al. 2004), or in the case of pathogenic species overcome host defenses (Meyer et al. 1996; Brown 1999; Harrison et al. 2006). Interactions between individuals may even involve complex signaling systems (quorum sensing) that allow the assessment of local population density, where production of the signal molecules is a cooperative behavior (Diggle et al. 2007a; Williams et al. 2007). In all these cases mutant bacteria that do not pay the costs of excreting the extracellular compounds may still reap the benefits and thus increase in frequency when rare. Perhaps the most celebrated examples of microbial cooperation occur in the slime mold *Dictyostelium discoideum* (Strassmann et al. 2000; Fortunato et al. 2003) and the "slime bacterium" *Myxococcus xanthus* (Velicer et al. 2000; Fiegna et al. 2006), which form multicellular fruiting bodies containing dispersive spores. Individuals that do not enter the spore body sacrifice themselves for the benefit of the group, and in mixed lineages conflict occurs over which cells contribute to spore formation.

Bacterial cells are often found in nature as biofilms, bound together by an extracellular matrix composed of material, typically polysaccharides, secreted by individual cells (Davies et al. 1998; Kolter and Greenberg 2006). They may be composed predominantly of a single type of bacteria, or involve more than one species (Tolker-Nielsen and Molin 2000; Palmer et al. 2001; Kolenbrander et al. 2002). Biofilms have numerous functions including preventing cells being washed away in moving media, protection from predators and viruses, and in pathogenic organisms escape from host immune defenses or antibiotics (Govan and Deretic 1996; Tolker-Nielsen and Molin 2000; Drenkard and Ausubel 2002; Matz and Kjelleberg 2005; Harrison et al. 2007; Meyer and Kassen 2007). Although in some cases biofilm formation may be a simple emergent property of the actions of numerous cells acting independently, there is also evidence for the involvement of quorum sensing and more active behaviors such as cell death that releases extracellular DNA that has a structural function in biofilms (Davies et al. 1998; Whitchurch et al. 2002). However, the production of extracellular polysaccharides is costly and cells that fail to produce such compounds may still be able to attach to, and benefit from, biofilms (Rainey and Rainey 2003; Brockhurst et al. 2007; Xavier and Foster 2007).

Pseudomonas fluorescens, which belongs to the Gamma Proteobacteria, has become widely used in studies of microbial ecology and evolution (Rainey and Travisano 1998; Hodgson et al. 2002; Rainey and Rainey 2003; Kassen et al. 2004; MacLean 2005; Brockhurst et al. 2006; Venail et al. 2008). When grown in static tubes of growth medium initially isogenic cultures differentiate into a variety of phenotypes that can be distinguished by heritable colony morphologies. The different phenotypes fall into three classes that occupy different spatial niches in the culture tubes (Rainey and Travisano 1998; Buckling et al. 2000; Kassen et al. 2000). "Smooth morphs" (SM) resemble the ancestral type and inhabit the liquid phase. "Wrinkly spreaders" (WS) form a biofilm at the air–liquid interface by overproducing cellulose-like polymers. Finally, rather rare "fuzzy spreaders" (FS) colonize the bottom of the tubes (and are not considered further in this article). The growth rate of SM is higher than WS in exponential phase, suggesting that the production of the polymer that makes up the biofilm is costly (Rainey and Rainey 2003; MacLean et al. 2004). However, it is thought that the biofilm provides a group benefit to WS cell by enhancing their access to oxygen (Rainey and Rainey 2003). This cooperative behavior of WS can be exploited by SM phenotypes, which invade the biofilms, gaining the benefit of oxygen access without paying the cost of high polymer production. The WS cells suffer from these cheats as a biofilm carrying SM cells is constitutively weaker than a pure-WS one and has a higher probability of collapsing and sinking into the relatively anoxic culture medium (Rainey and Rainey 2003; Brockhurst et al. 2006, 2007).

In this study, we explore the intraspecific coevolution of cheating by SM and resistance by WS clones. Our major question is whether there is a dynamic turnover of genotypes within these two phenotypic classes in which new cheating variants of SM are countered by new resistance mutants of WS and vice versa. We make use of the fact that bacteria can be frozen and stored for future study, and investigate the cooperator–cheat interactions using a methodology previously developed to explore the coevolutionary interactions between bacteria and phage (Buckling and Rainey 2002; Brockhurst et al. 2003). Serial cultures of the bacterium are propagated and the performance of each type (cooperator or cheat) is estimated when competed against isolates of the other type from past, contemporary, or future passages.

Dynamic turnover is indicated when current cooperator or cheat phenotypes perform better against the alternative phenotype from the past, and worse against those from the future.

Methods

ORGANISMS

The experiments were carried out using *P. fluorescens* SBW25, which was originally isolated from sugar beet roots (Rainey and Bailey 1996), and its modified variant, SBW25EeZY6KX (Bailey et al. 1995). The latter contains two constitutively expressed marker gene cassettes: one consisting of genes encoding kanamycin resistance and catechol 2,3-dioxygenase and the other consisting of *lacZY* genes enabling the utilization of lactose.

EXPERIMENTAL POPULATIONS

The interactions between SM and WS phenotypes from six independently evolving populations were studied. In each, cultures of SBW25EeZY6KX were propagated in 30 mL universal containers with loose lids (referred to below as microcosms) containing 6 mL of King's Medium B (KB). Initially, 10^8 cells from an isogenic culture were added to each microcosm, which was statically incubated at 28°C. Sixty microliters (1%) of each culture was transferred to fresh media every seven days for five transfers. At each transfer every microcosm was vortexed and a sample was stored at −80°C in 50% glycerol. In addition, samples of each culture were grown on KB agar plate; one colony of the numerically most dominant SM and one colony of the most dominant WS (determined on the basis of colony morphology) were isolated, propagated, and stored at −80°C.

THE EFFECT OF SM ON THE QUALITY OF BIOFILMS

We tested whether the presence of SM decreased the strength of the biofilms formed by the WS phenotypes. The most dominant SM and WS phenotypes were isolated from each of the six selection lines at transfers 1 and 5. For each combination of line and transfer, 36 replicate static microcosms were set up, 12 inoculated with SM, 12 with WS, and 12 with both. Each microcosm was initiated with 10^8 cells per phenotype and maintained for seven days. Biofilms appeared at the air–liquid interface in pure WS and mixed cultures, and also in pure SM cultures from transfer 2 onwards although these were visibly much weaker. The fraction of microcosms where the biofilms collapsed within seven days was recorded. We used the probability of collapse as a measure of biofilm quality, with lower values indicating higher quality. The proportional data were compared using paired-sample *t*-tests after arcsine transformation.

Obtaining a direct measure of the number of SM cells in pure SM biofilms and in biofilms composed of both types of cell would clearly demonstrate whether the SM phenotype benefits from integrating itself in WS biofilms. However, pure SM and often mixed biofilms were so fragile that it was impossible to collect them intact and hence estimate cell density.

MEASUREMENT OF SM CHEATING ABILITY AND WS RESISTANCE

To determine whether SM phenotypes evolved so that they were better able to integrate themselves into a biofilm, and similarly WS morphs evolved so that they were better able to resist the invasion by SM, each trait was measured in bacteria collected at different times when competed against a standard reference isolate of the other type. To obtain the latter, *P. fluorescens* SBW25 was cultured in a static microcosm for seven days and the numerically most dominant SM and WS phenotypes were isolated to be used as the reference isolates.

To test SM invasion ability, 10^8 cells of SM from each evolved line at each transfer and the standard WS were inoculated into a microcosm and cultured for two days. A sample of the biofilm was collected using a wire loop and vortexed in M9 salt solution (Na_2HPO_4, $6gL^{-1}$; KH_2PO_4, $3gL^{-1}$ NH_4Cl, $1gL^{-1}$; NaCl, $0.5gL^{-1}$), which was then diluted and plated onto KB agar. The proportion of SM cells in the biofilm was recorded as an estimate of SM cheating ability (Rainey and Rainey 2003; Brockhurst et al. 2006). The same experimental design was used to test the ability of different isolates of WS to resist invasion by the standard SM isolate. Each SM or WS was assayed three times, and the average values were used in analyses. The data were analyzed by mixed-model analysis of covariance (ANCOVA) with transfer number as a continuous explanatory variable and selection line as a random factor. The proportions of SM cells were arcsine-transformed before analysis.

MEASUREMENT OF COEVOLUTION BETWEEN SM AND WS

Coevolution was studied by comparing the performance of SM or WS phenotypes when competed against isolates of the other type from (1) one transfer in the past, (2) the contemporary transfer, and (3) one transfer in the future. The proportion of SM cells in biofilms of WS from the different transfers was determined as described above. The numerically most dominant WS and SM collected at each transfer were tested, with three replicates for each. If a single SM isolate performs better against WS isolates from the past compared with the future (shown as a negative slope when the fraction of SM in the biofilm is plotted against WS transfer number), this indicates the evolution of the WS phenotype to resist cheating. Similarly, if a single WS isolate performs better against SM isolates from the past compared with the future (a positive slope when the fraction of SM in the biofilm is plotted against SM transfer number), this shows that the SM morph is evolving an improved ability to invade biofilms. If both patterns

occur this indicates coevolution. The slopes of these two types of relationships were determined by ANCOVA, with transfer number as a continuous explanatory variable and selection line as a random factor.

Results

The colony morphology of the most dominant SM or WS phenotypes on agar plates appeared constant over time. Initially, pure cultures of the SM phenotype could not form a biofilm. SM isolated at transfer 2 formed very thin biofilms whereas the biofilms formed by SM isolates from transfer 3 onwards appeared as thick as normal WS biofilms; however, they were less robust and easily broken, whereas WS biofilms could often be lifted intact using a wire loop. We assessed the robustness of biofilms formed by a single phenotype or a mixture of both phenotypes (the proportion of biofilms that collapsed within seven days) at transfers 1 and 5. The biofilms produced in mixed cultures of SM and WS bacteria had a higher probability of collapsing than biofilms of pure WS cultures (Fig. 1). This was observed for both bacteria isolated at transfer 1 ($t = 2.72$, df $= 5$, $P = 0.042$) and those at transfer 5 ($t = 3.36$, df $= 5$, $P = 0.020$). There was no significant difference in the fraction of collapsed biofilms between transfer 1 and 5 for either the pure WS ($t = 1.01$, df $= 5$, $P = 0.359$) or mixed cultures ($t = 1.39$, df $= 5$, $P = 0.223$). Pure SM microcosms did not produce biofilms at transfer 1 whereas there was no significant difference at transfer 5 in the probability of collapse of pure SM and mixed biofilms ($t = 1.18$, df $= 5$, $P = 0.292$).

The ability of SM cells to invade biofilms formed by the standard reference WS isolate increased over time (Fig. 2; ANCOVA, $F_{1,23} = 9.56$, $P = 0.005$). This significant result was largely due to differences between SM isolated at transfer 1 and those at later

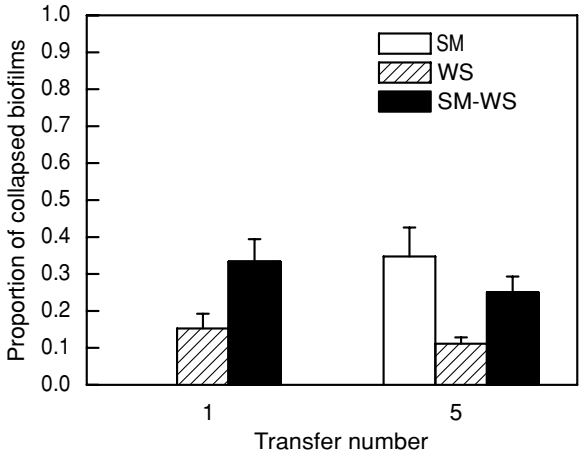

Figure 1. The probability of collapse of biofilms composed of different combinations of bacterial phenotypes isolated at transfer 1 or 5. Note that SM from transfer 1 did not form observable biofilms. Graph bars show mean ± SE ($N = 6$).

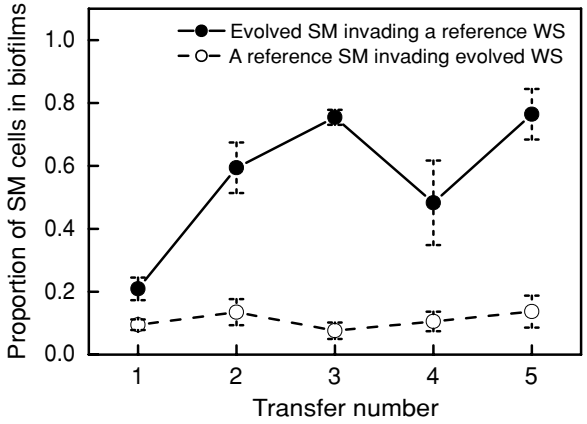

Figure 2. The ability of SM isolated at different transfers to invade biofilms of a reference WS isolate and that of a reference isolate of SM to invade biofilms of WS isolated at different times. The proportion of SM cells (mean ± SE; $N = 6$) in mixed biofilms is plotted.

transfers, and there was no significant increase in integration with transfer number beyond 1 ($F_{1,17} = 0.63$, $P = 0.439$). The proportion of SM cells in the biofilms could be surprisingly high: 70–80% for transfer 3 and 5. In contrast, the ability of the reference SM strain to invade biofilms of WS isolated from different transfers was low (∼10%) and did not change with time (Fig. 2; $F_{1,23} = 0.24$, $P = 0.631$).

If coevolution occurs we expect that WS or SM isolates will perform better when competed against isolates of the alternative phenotype from their past compared with their future. We thus expect positive and negative slopes when the fraction of SM cells in the biofilms is plotted against the transfer number from which the SM and WS competitors, respectively, were isolated. The results confirm this pattern. Consider first the simple sign of the slope of the 10 different relationships (five in Fig. 3A and five in Fig. 3B), nine of the 10 slopes are in the direction predicted if coevolution has occurred (sign test, $P < 0.01$). For the assays of the WS phenotypes in competition with past, current, and future SM all slopes are positive: four slopes are statistically significant ($P < 0.05$) and the remaining one (for WS from transfer 4) is marginally significant ($P = 0.076$; Fig. 3A). For the assays of the SM phenotype in competition with the three types of WS, four of the five slopes are negative with three cases being statistically significant ($P < 0.05$); one slope (for SM from transfer 5) is nonsignificantly positive ($P > 0.50$; Fig. 3B).

Given that SM phenotypes evolved to be able to produce their own weak biofilms, it is possible that the coevolution observed between SM and WS (Fig. 3) might represent an increase in the capacity of both SM and WS to form biofilms rather than reciprocal interactions involving exploitation and resistance. This possibility could be ruled out by demonstrating that the fitness of pure WS and pure SM biofilms does not change over time.

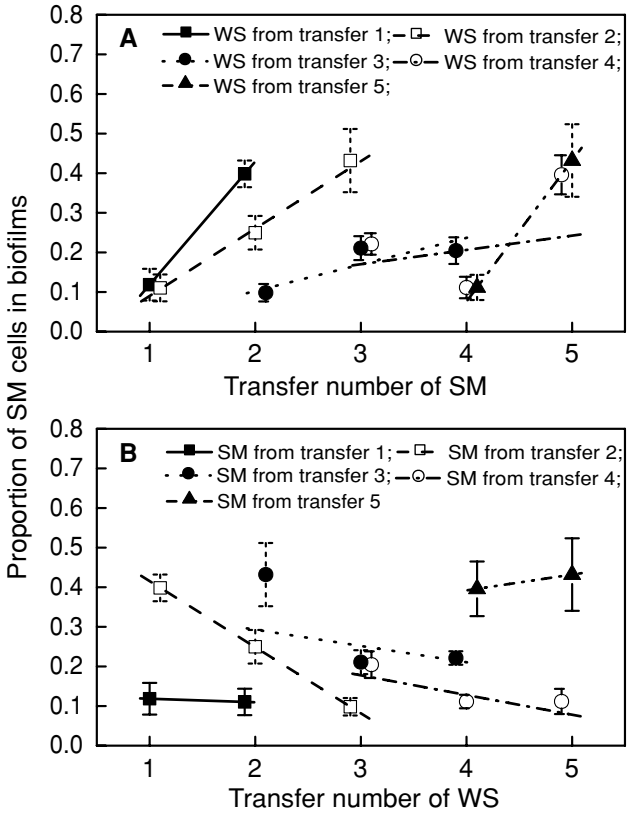

Figure 3. The evolution of SM invasion ability and WS resistance. Each set of lines in (A) show (from left to right) the ability of SM from one transfer in the past, from the contemporary transfer, and from one transfer in the future to invade the biofilm of the contemporary WS. In (B) each set of lines show (from left to right) the ability of contemporary SM to invade the biofilms formed by WS from one transfer in the past, the contemporary transfer, and from one transfer in the future. Each datapoint shows the mean (±SE), across six selection lines, of competition between the most dominant SM and WS isolates.

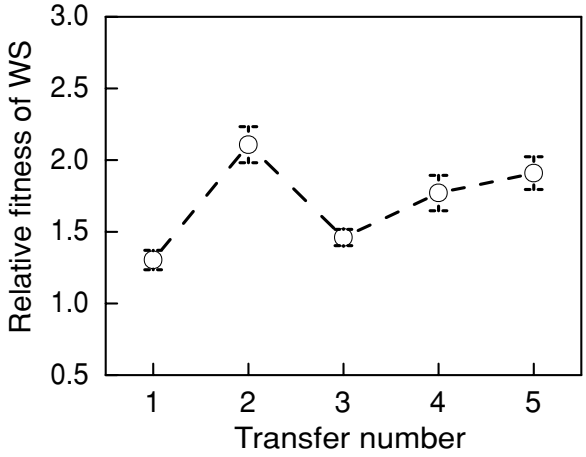

Figure 4. The relative fitness of WS phenotypes extracted at different transfers when competed against the standard WS isolate. The relative fitness was calculated as the ratio of the estimated Malthusian parameters (m) of the test and standard WS isolates ($m = \ln(N_f/N_0)$, where N_0 is the starting density and N_f the final density).

Unfortunately, this was not possible for pure SM biofilms that were too flimsy to extract entire. However, competing WS phenotypes isolated from different transfers against the standard WS showed no significant increase in fitness over time (Fig. 4). There was a tendency for fitness to increase ($F_{1,23} = 3.27$, $P = 0.083$) but this was due to isolates from transfer 1 having comparatively low fitness; over transfers 2–5 the relative fitness showed no consistent change ($F_{1,17} = 0.18$, $P = 0.675$).

Discussion

Previous work has shown that biofilm formation by WS phenotypes increases their fitness by allowing them access to a more aerobic environment, although the production of cellulose-like polymer is also costly for the cells (Rainey and Rainey 2003; MacLean et al. 2004). It is also suggested that SM cells either do not contribute or contribute less to biofilm production, and thus their presence in WS biofilms makes the structure less robust and more likely to collapse into the broth (Rainey and Rainey 2003; Brockhurst et al. 2006, 2007), a finding we confirm here. These observations and experiments suggest that the WS and SM phenotypes of *P. fluorescens* can be considered cooperators and cheats, respectively.

The chief object of the present study was to determine whether there was dynamic coevolution between cheat and cooperator phenotypes in which cheats evolve improved means of integrating themselves in the biofilms, which are counteracted by cooperators evolving improved methods of prevention. Were this to happen, we predicted that each phenotype would be more successful in competition with the other phenotype from its immediate past compared to its immediate future (with current phenotype intermediate). We can compare this prediction with that from another model of how WS and SM types might evolve, that is, SM and WS are niche specialists in the broth phase and the air–broth interface, respectively, with the two phenotypes evolving to be more dissimilar in a process of character displacement (Brown and Willson 1956; Connell 1980; Schluter 1988, 1993, 2000; Grant and Grant 2006; Steiner et al. 2007). Under the character displacement hypothesis competition between SM and WS is predicted to become less severe over time; this prediction receives no support as we find that the negative effects of SM and WS on each other increased over time. Our results thus strongly support the cooperator/cheat coevolution hypothesis over character displacement.

In the assays involving the reference isolates of the two phenotypes, the standard SM was unable to invade the biofilms of any of the WS isolates sampled at the five transfer points. SM

from transfer 1 could not invade the standard WS, but SM from transfers 2–5 could, to roughly the same extent. The results suggest that single or multiple key innovations or improvements have to arise in SMs before they can establish themselves in biofilms, and that it takes approximately two transfers (~12–15 generations) for this to happen. The result that biofilms with evolved SM and standard WS contain very high percentages of the former phenotype (40–80%; Fig. 2) compared to those observed in the coevolution experiments (usually lower than 40%; Fig. 3) suggests that counter-adaptations to limit SM invasion did evolve in WS bacteria.

Over the course of the experiment the SM phenotypes evolved to be able to produce their own weak biofilms, probably through the external secretion of at least some biofilm-forming polymer. An increase in fitness over time of SM relative to WS might therefore have resulted from an increased ability of SM to form biofilms per se, rather than increased exploitation of WS biofilms. This, however, seems unlikely given that previous studies have demonstrated that SM do exploit WS biofilms (Rainey and Rainey 2003; Brockhurst et al. 2006, 2007), and the evidence from this study that WS evolved an increased ability to prevent SM from invading biofilms after transfer 2 (see Figs. 3 and 4). Furthermore, mixed SM–WS biofilms at transfer 1 were not different in strength from those at transfer 5, yet transfer 5 SM isolates were much fitter than transfer 1 SM isolates when measured against the standard WS. Together, these results strongly indicate that the increase in SM fitness is largely due to a better ability to exploit WS rather than an improved ability to form biofilms in their own right. We suspect that the weak biofilm-forming ability of late-transfer SM is simply a byproduct of improved cheating performance. What we do not know is whether this evolved polymer production is "purely selfish" and harms the WS phenotypes by increasing the number of SM cells in the biofilm, or whether by producing some polymer the SM cells help stabilize the biofilm to benefit the WS cells. Simulations have also shown that polymer production may confer a direct fitness benefit by reducing the access of competing neighbors to oxygen (Xavier and Foster 2007).

It is possible that the increased ability of the WS phenotype to resist SM was not directly selected but instead a byproduct of competition among WS phenotypes to use better the broth–surface niche. We think this is unlikely because WS phenotypes did not show consistently improved performance over time when competed against the standard WS clone (Fig. 4). Selection for improved biofilm formation may occur in systems such as this (Xavier and Foster 2007) and it would be interesting to know if there are trade-offs between biofilm formation and the ability to resist SM morphs.

The evolution of WS resistance to invasion by SM raises further interesting conceptual issues. Just as biofilm formation can be exploited by cheats (the SM phenotype) then so potentially can resistance to SM (Rankin et al. 2007): for example a WS cell that did not invest into the resistance mechanism would benefit from the presence of the other WS cells that did. The highly spatial structure of biofilms provides a simple and plausible explanation for the success of resistant WS. First, SM invasion is likely to be reduced most strongly in the immediate locality of resistant WS. Second, the invasion of nonresistant WS will be minimized because neighboring cells of resistant WS are likely to be more genetically similar than the average WS cell in the biofilm (i.e., have higher relatedness; Hamilton 1964). Resistant WS are therefore likely to reach higher local densities, and competition across the whole biofilm at each transfer will ensure the increase of the resistant WS population.

A better understanding of the genetic basis of the cheating and resistance behaviors in this system will help test our interpretation of these results. However, this goal still seems some way off. Currently, we know little about the mechanistic basis of the ability of SM to invade biofilms, and the ability of WS to resist invasion. Both traits are likely to be continuous rather than discrete, and their evolution probably involves changes in the quantitative production of different substances determined by differential gene expression. Existing studies on biofilm formation and colony morphology (Spiers et al. 2002; Goymer et al. 2006; Bantinaki et al. 2007) suggest that the relationship between genotype and phenotype may be quite complex within the two morphs and hence difficult to untangle. There is some evidence, however, that WS cells produce allelochemicals that suppress the growth of SM cells near the biofilm (Rainey and Travisano 1998; Day and Young 2004; Rainey 2005). However, although genetic dissection of these traits may require considerable effort, the system is likely to be far more tractable than understanding the mechanistic genetics of equivalent traits in multicellular eukaryotes.

Although the biological outcomes are often not in question, there is currently much controversy about how best to interpret cooperative behaviors—whether as kin selection or a form of group selection (Wilson and Wilson 2007; West et al. 2008). Indeed, the evolution of cooperation in this system (Rainey and Rainey 2003) has been used as an example in which a group selection perspective might be more fruitful (Wilson and Wilson 2007, and see the recent commentary by Kohn 2008). In our system, WS cells within a biofilm are closely related with daughter cells literally stuck together and we find evolution to be most naturally explained by kin selection theory (Griffin et al. 2004; Sachs et al. 2004; Kerr et al. 2006; Lehmann and Keller 2006; West et al. 2006, 2007b) although alternative interdemic group selection formulations are equally valid. Although not applicable to the evolutionary changes we observe with our experimental design, it is possible that selection operating at the level of the biofilm, involving the differential death and reproduction of biofilm units

with the latter acting as replicators sensu Dawkins (1982), might lead to the evolution of cooperative behavior beyond that predicted by kin selection in biofilm-forming microorganisms in natural environments. However, the conditions under which this occurs may be limited as biofilms typically originate from single cells. It is important, though, to distinguish this type of group selection from that which can also be explained by kin selection.

Biofilm formation is very common in natural microbial communities, and we would expect the conflicts observed in our artificial system also to occur in more natural systems, although the interactions there are likely to be more complex, probably also involving interspecific cooperation and cheating. An example of this type of complexity has been found in the medically important *Pseudomonas aeruginosa*. There is evidence that cells in biofilms produce new variants at a greater rate than those in liquid medium (associated with *recA* activity) and that this diversity is adaptive in creating a biofilm that can resist different challenges (Boles et al. 2004). It would be interesting to explore whether the generation of genetic diversity also produces noncooperative variants, or whether a function or byproduct of enhanced diversity is better control of cheating genotypes (Brockhurst et al. 2006; Jansen and van Baalen 2006).

The concept and possibility of coevolutionary cycles is well established in victim–exploiter interactions in which two participants are different species (van Valen 1973; Chao et al. 1977; Anderson and May 1982; Hamilton et al. 1990; Thompson 1998; Sasaki and Godfray 1999; Abrams 2000; Buckling and Rainey 2002; Brockhurst et al. 2003; Morgan et al. 2005; Decaestecker et al. 2007; Raffel et al. 2008). Theoretical studies have also explored equivalent cyclic dynamics in cooperator–cheat systems that are a type of intraspecific victim–exploiter system (e.g., Riolo et al. 2001; van Baalen and Jansen 2003; Jansen and van Baalen 2006). However, to date experimental evidence for such cycles has been scarce, in large part because of the logistic difficulty of performing long-term studies of cooperative behavior. We believe work with microorganisms is valuable both in its own right and because it can reveal the presence of patterns that will guide research in less experimentally tractable multicellular eukaryote systems.

ACKNOWLEDGMENTS

We thank M. Travisano and three anonymous reviewers for their helpful comments. This work was funded by UK's Natural Environment Research Council (NERC) through the NERC Centre for Population Biology at Imperial College London, and by the Leverhulme Trust.

LITERATURE CITED

Abrams, P. A. 2000. The evolution of predator-prey interactions: theory and evidence. Annu. Rev. Ecol. Syst. 31:79–105.

Anderson, R. M., and R. May. 1982. Coevolution of hosts and parasites. Parasitology 85:411–426.

Bailey, M. J., A. K. Lilley, I. P. Thompson, P. B. Rainey, and R. J. Ellis. 1995. Site directed chromosomal marking of a fluorescent pseudomonad isolated from the phytosphere of sugar beet: stability and potential for marker gene transfer. Mol. Ecol. 4:755–763.

Bantinaki, E., R. Kassen, C. G. Knight, Z. Robinson, A. J. Spiers, and P. B. Rainey. 2007. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. III. mutational origins of wrinkly spreader diversity. Genetics 176:441–453.

Boles, B. R., M. Thoendel, and P. K. Singh. 2004. Self-generated diversity produces "insurance effects" in biofilm communities. Proc. Natl. Acad. Sci. USA 101:16630–16635.

Brockhurst, M. A., A. D. Morgan, P. B. Rainey, and A. Buckling. 2003. Population mixing accelerates coevolution. Ecol. Lett. 6:975–979.

Brockhurst, M. A., M. E. Hochberg, T. Bell, and A. Buckling. 2006. Character displacement promotes cooperation in bacterial biofilms. Curr. Biol. 16:2030–2034.

Brockhurst, M. A., A. Buckling, and A. Gardner. 2007. Cooperation peaks at intermediate disturbance. Curr. Biol. 17:761–765.

Brown, S. P. 1999. Cooperation and conflict in host-manipulating parasites. Proc. R. Soc. Lond. B 266:1899–1904.

Brown, W. L., and E. O. Willson. 1956. Character displacement. Syst. Zool. 5:49–64.

Buckling, A., and K. Rainey. 2002. Antagonistic coevolution between a bacterium and a bacteriophage. Proc. R. Soc. Lond. B 269:931–936.

Buckling, A., R. Kassen, G. Bell, and P. B. Rainey. 2000. Disturbance and diversity in experimental microcosms. Nature 408:961–964.

Chao, L., B. R. Levin, and F. M. Stewart. 1977. Complex community in a simple habitat: experimental study with bacteria and phage. Ecology 58:369–378.

Connell, J. H. 1980. Diversity and the coevolution of competitors, or the ghost of competition past. Oikos 35:131–138.

Crespi, B. J. 2001. The evolution of social behavior in microorganisms. Trends Ecol. Evol. 16:178–183.

Daniels, R., J. Vanderleyden, and J. Michiels. 2004. Quorum sensing and swarming migration in bacteria. FEMS Microb. Rev. 28:261–289.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298.

Dawkins, R. 1982. The extended phenotype. Oxford Univ. Press, Oxford.

Day, T., and K. A. Young. 2004. Competitive and facilitative evolutionary diversityfication. BioScience 54:101–109.

Decaestecker, E., S. Gaba, J. A. M. Raeymaekers, R. Stoks, L. Van Kerckhoven, D. Ebert, and L. De Meester. 2007. Host-parasite 'Red Queen' dynamics archived in pond sediment. Nature 450:870–873.

Diggle, S. P., A. Gardner, S. A. West, and A. S. Griffin. 2007a. Evolutionary theory of bacterial quorum sensing: when is a signal not a signal? Philos. Trans. R. Soc. Lond. B 362:1241–1249.

Diggle, S. P., A. S. Griffin, G. S. Campbell, and S. A. West. 2007b. Cooperation and conflict in quorum-sensing bacterial populations. Nature 450:411–414.

Drenkard, E., and F. M. Ausubel. 2002. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature 416:740–743.

Ellis, R. J., A. K. Lilley, S. J. Lacey, D. Murrell, and H. C. J. Godfray. 2007. Frequency-dependent advantages of plasmid carriage by Pseudomonas in homogeneous and spatially structured environments. ISME J 1:92–95.

Fiegna, F., Y. T. N. Yu, S. V. Kadam, and G. J. Velicer. 2006. Evolution of an obligate social cheater to a superior cooperator. Nature 441:310–314.

Fortunato, A., D. C. Queller, and J. E. Strassmann. 2003. A linear dominance hierarchy among clones in chimeras of the social amoeba *Dictyostelium discoideum*. J. Evol. Biol. 16:438–445.

Govan, J. R., and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. 60:539–574.

Goymer, P., S. G. Kahn, J. G. Malone, S. M. Gehrig, A. J. Spiers, and P. B. Rainey. 2006. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. II. role of the GGDEF regulator WspR in evolution and development of the wrinkly spreader phenotype. Genetics 173:515–526.

Grant, P. R., and B. R. Grant. 2006. Evolution of character displacement in Darwin's finches. Science 313:224–226.

Greig, D., and M. Travisano. 2004. The Prisoner's Dilemma and polymorphism in yeast SUC genes. Proc. R. Soc. Lond. B 271:S25–S26.

Griffin, A. S., S. A. West, and A. Buckling. 2004. Cooperation and competition in pathogenic bacteria. Nature 430:1024–1027.

Hamilton, W. D. 1964. The genetical evolution of social behaviour. I and II. J. Theor. Biol. 7:1–52.

Hamilton, W. D., R. Axelrod, and R. Tanese. 1990. Sexual reproduction as an adaptation to resist parasites (a review). Proc. Natl. Acad. Sci. USA 87:3566–3573.

Harrison, F., L. Browning, M. Vos, and A. Buckling. 2006. Cooperation and virulence in acute *Pseudomonas aeruginosa* infections. BMC Biol. 4:21.

Harrison, J. J., H. Ceri, and R. J. Turner. 2007. Multimetal resistance and tolerance in microbial biofilms. Nat. Rev. Micro. 5:928–938.

Hodgson, D. J., P. B. Rainey, and A. Buckling. 2002. Mechanisms linking diversity, productivity and invasibility in experimental bacterial community. Proc. R. Soc. Lond. B 269:2277–2283.

Jansen, V. A. A., and M. van Baalen. 2006. Altruism through beard chromodynamics. Nature 440:663–666.

Kassen, R., A. Buckling, G. Bell, and P. B. Rainey. 2000. Diversity peaks at intermediate productivity in a laboratory microcosm. Nature 406:508–512.

Kassen, R., M. Llewellyn, and P. B. Rainey. 2004. Ecological constraints on diversification in a model adaptive radiation. Nature 431:984–988.

Kerr, B., C. Neuhauser, B. J. M. Bohannan, and A. M. Dean. 2006. Local migration promotes competitive restraint in a host-pathogen 'tragedy of the commons.' Nature 442:75–78.

Kohn, M. 2008. The needs of the many. Nature 456: 296–299.

Kolenbrander, P. E., R. N. Andersen, D. S. Blehert, P. G. Egland, J. S. Foster, and R. J. Palmer. 2002. Communication among oral bacteria. Microbiol. Mol. Biol. Rev. 66:486–505.

Kolter, R., and E. P. Greenberg. 2006. The superficial life of microbes. Nature 441:300–302.

Lehmann, L., and L. Keller. 2006. The evolution of cooperation and altruism—a general framework and a classification of models. J. Evol. Biol. 19:1365–1376.

MacLean, R. 2005. Adaptive radiation in microbial microcosms. J. Evol. Biol. 18:1376–1386.

MacLean, R. C., and I. Gudelj. 2006. Resource competition and social conflict in experimental populations of yeast. Nature 441:498–501.

MacLean, R. C., G. Bell, and P. B. Rainey. 2004. The evolution of a pleiotropic fitness tradeoff in *Pseudomonas fluorescens*. Proc. Natl. Acad. Sci. USA 101:8072–8077.

Matz, C., and S. Kjelleberg. 2005. Off the hook—how bacteria survive protozoan grazing. Trends Microb. 13:302–307.

Maynard Smith, J. 1964. Group selection and kin selection. Nature 201:1145–1147.

Maynard Smith, J., and E. Szathmary. 1995. The major transitions in evolution. W.H. Freeman, Oxford.

Meyer, J. M., A. Neely, A. Stintzi, C. Georges, and I. A. Holder. 1996. Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. Infect. Immun. 64:518–523.

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

Morgan, A. D., S. G. Gandon, and A. Buckling. 2005. The effect of migration on local adaptation in a coevolving host-parasite system. Nature 437:253–256.

Nowak, M. A. 2006. Five rules for the evolution of cooperation. Science 314:1560–1563.

Palmer, R. J., K. Kazmerzak, M. C. Hansen, and P. E. Kolenbrander. 2001. Mutualism versus independence: strategies of mixed-species oral biofilms in vitro using saliva as the sole nutrient source. Infect. Immun. 69:5794–5804.

Raffel, T. R., L. B. Martin, and J. R. Rohr. 2008. Parasites as predators: unifying natural enemy ecology. Trends Ecol. Evol. 23:610–618.

Rainey, P. B. 2005. Bacteria evolve and function within communities: observations from experimental *Pseudomonas* populations. Pp. 83–102 *in* M. McFail-Ngai, B. Henderson, and N. Ruby, eds. The influence of cooperative bacteria on animal host biology. Cambridge Univ. Press, Cambridge.

Rainey, P. B., and M. J. Bailey. 1996. Physical and genetic map of the *Pseudomonas fluorescens* SBW25 chromosome. Mol. Microb. 19:521–533.

Rainey, P. B., and K. Rainey. 2003. Evolution of cooperation and conflict in experimental bacterial populations. Nature 425:72–74.

Rainey, P. B., and M. Travisano. 1998. Adaptive radiation in a heterogeneous environment. Nature 394:69–72.

Rankin, D. J., K. Bargum, and H. Kokko. 2007. The tragedy of the commons in evolutionary biology. Trends Ecol. Evol. 22:643–651.

Riolo, R. L., M. D. Cohen, and R. Axelrod. 2001. Evolution of cooperation without reciprocity. Nature 414:441–443.

Ross-Gillespie, A., A. Gardner, S. A. West, and A. S. Griffin. 2007. Frequency dependence and cooperation: theory and a test with bacteria. Am Nat 170:331–342.

Sachs, J. L., U. G. Mueller, T. P. Wilcox, and J. J. Bull. 2004. The evolution of cooperation. Q. Rev. Biol. 79:135–160.

Sandoz, K. M., S. M. Mitzimberg, and M. Schuster. 2007. Social cheating in *Pseudomonas aeruginosa* quorum sensing. Proc. Natl. Acad. Sci. U SA 104:15876–15881.

Sasaki, A., and H. C. J. Godfray. 1999. A model for the coevolution of resistance and virulence in coupled host-parasitoid interactions. Proc. R. Soc Lond. B 266:455–463.

Schluter, D. 1988. Character displacement and the adaptive divergence of finches on islands and continents. Am. Nat. 131:799–824.

———. 1993. Adaptive radiation in sticklebacks: size, shape, and habitat use efficiency. Ecology 74:699–709.

———. 2000. The ecology of adaptive radiation. Oxford Univ. Press, Oxford.

Spiers, A. J., S. G. Kahn, J. Bohannon, M. Travisano, and P. B. Rainey. 2002. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. genetic and phenotypic bases of wrinkly spreader fitness. Genetics 161:33–46.

Steiner, C. F., C. E. Caceres, and S. D. P. Smith. 2007. Resurrecting the ghost of competition past with dormant zooplankton eggs. Am. Nat. 169:416–422.

Strassmann, J. E., Y. Zhu, and D. C. Queller. 2000. Altruism and social cheating in the social amoeba *Dictyostelium discoideum*. Nature 408:965–967.

Thompson, J. N. 1998. Rapid evolution as an ecological process. Trends Ecol. Evol. 13:329–332.

Tolker-Nielsen, T., and S. Molin. 2000. Spatial organization of microbial biofilm communities. Microb. Ecol. 40:75–84.

Trivers, R. L. 1971. The evolution of reciprocal altruism. Q. Rev. Biol. 46:35–57.

van Baalen, M., and V. A. A. Jansen. 2003. Common language or Tower of Babel? On the evolutionary dynamics of signals and their meanings. Proc. R. Soc. Lond. B 270:69–76.

van Valen, L. 1973. A new evolutionary law. Evol. Theor. 1:1–30.

Velicer, G. J., and Y. T. N. Yu. 2003. Evolution of novel cooperative swarming in the bacterium *Myxococcus xanthus*. Nature 425:75–78.

Velicer, G. J., L. Kroos, and R. E. Lenski. 2000. Developmental cheating in the social bacterium *Myxococcus xanthus*. Nature 404:598–601.

Venail, P. A., R. C. MacLean, T. Bouvier, M. A. Brockhurst, M. E. Hochberg, and N. Mouquet. 2008. Diversity and productivity peak at intermediate dispersal rate in evolving metacommunities. Nature 452:210–214.

West, S. A., and A. Buckling. 2003. Cooperation, virulence and siderophore production in bacterial parasites. Proc. R. Soc. Lond. B 270:37–44.

West, S. A., A. S. Griffin, A. Gardner, and S. P. Diggle. 2006. Social evolution theory for microorganisms. Nature Rev. Microb. 4:597–607.

West, S. A., S. P. Diggle, A. Buckling, A. Gardner, and A. S. Griffin. 2007a. The social lives of microbes. Ann. Rev. Ecol. Evol. Syst. 38:53–77.

West, S. A., A. S. Griffin, and A. Gardner. 2007b. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. J. Evol. Biol. 20:415–432.

West, S. A., A. S. Griffin, and A. Gardner. 2008. Social semantics: how useful has group selection been? J. Evol. Biol. 21: 374–385.

Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick. 2002. Extracellular DNA required for bacterial biofilm formation. Science 295:1487.

Williams, P., K. Winzer, W. C. Chan, and M. Camara. 2007. Look who's talking: communication and quorum sensing in the bacterial world. Philos. Trans. R. Soc. B 362:1119–1134.

Wilson, D. S., and E. O. Wilson. 2007. Rethinking the theoretical foundation of sociobiology. Q. Rev. Biol. 82:327–348.

Xavier, J. B., and K. R. Foster. 2007. Cooperation and conflict in microbial biofilms. Proc. Natl. Acad. Sci. US A 104:876–881.

Associate Editor: M. Travisano