

Hypermutable Impedes Cooperation in Pathogenic Bacteria

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Summary

When the supply of beneficial mutations limits adaptation, bacterial mutator alleles can reach high frequencies by hitchhiking with advantageous mutations [1–9]. However, when populations are well adapted to their environments, the increased rate of deleterious mutations makes hypermutability selectively disadvantageous [6, 7, 10, 11]. Here, we consider a further cost of hypermutability: its potential to break down cooperation (group-beneficial behavior that is costly to the individual [12–14]). This probably occurs for three reasons. First, an increased rate at which ‘cheating’ genotypes are generated; second, an increased probability of producing efficient cheats; and third, a decrease in relatedness [15–18] (not addressed in the present study). We used *Pseudomonas aeruginosa*’s production of extracellular iron-scavenging molecules, siderophores, to determine if cheating evolved more readily in mutator populations. Siderophore production is costly to individual bacteria but benefits all nearby cells. Siderophore-deficient cheats therefore have a selective advantage within populations [18]. We observed the de novo evolution and subsequent increase in frequency of siderophore cheats within both wild-type and mutator populations for 200 generations. Cheats appeared and increased in frequency more rapidly in mutator populations. The presence of cheats was costly to the group, as shown by a negative correlation between cheat frequency and population density.

Results

In order to test the hypothesis that social cheats appear more rapidly in mutator populations, we followed the evolution of siderophore cheats in populations of wild-type and mutator (PAOΔ*mutS*) strains of the opportunistic pathogen *P. aeruginosa* in iron-limited medium for approximately 200 bacterial generations. The primary siderophore of *P. aeruginosa* is the yellow-green pigment pyoverdinin [19, 20], allowing pyoverdinin-negative cheat colonies to be readily distinguished from wild-types by their lack of yellow-green pigmentation [18].

In all populations, pyoverdinin-deficient mutants (cheats) arose from initially isogenic populations of siderophore producers (cooperators), and the proportion of cheats showed a net increase over time, (Figure 1). However,

the frequency of cheats was significantly greater in mutator compared with wild-type populations at the first measured time point, transfer 5, demonstrating that mutators spontaneously generate cheats more rapidly ($T(5) = 15.28$, $p < 0.0005$). The data also suggest that cheats generated from mutator populations have a greater within-population selective advantage than those generated from wild-type populations. First, the rate of increase in cheat frequency (once cheats were present in all populations, from transfer 10) was greater in mutator populations ($T(5) = 6.72$, $p < 0.001$). This difference in rates of change of cheat frequencies results from a decline in cheat frequencies from transfers 15 or 20 in four out of six wild-type populations, unlike mutator populations where cheats almost reached fixation by transfer 30 in all but one population. This decline in cheat frequencies in the wild-type populations may be due to wild-type producers having a greater probability of generating mutations that increase fitness relative to their respective cheats, as a simple result of the much larger relative size of the producer subpopulation in the wild-type microcosms. The maximum cheat frequency achieved over the period of evolution was also found to be higher in the mutator populations (mean 98.15% compared with a mean of 65.5% across the wild-type populations; $T(9) = 3.11$, $p < 0.05$). Second, the mutator populations showed much less between-population variation in cheat frequency than did the wild-type lineages, suggesting that cheats arising in mutator populations were all approaching relatively high-fitness cheating strategies (Figure 2; F-test on mean variance over all time points: $F(5,5) = 10.44$, $p < 0.05$).

Consistent with the above data, total siderophore production per cell decreased over time in all populations, and this decrease was more rapid in the mutator populations ($T(9) = 3.81$, $p < 0.005$). Interestingly, siderophore production per cell of the producer subpopulations also decreased over time (Figure 3), and again this decrease was more rapid in the mutator populations ($T(9) = 3.59$, $p < 0.005$). This could be interpreted as evidence for either the presence of partial cheats that have acquired mutations for downregulation of pyoverdinin or the fact that pyoverdinin positively regulates its own production [21]. Thus, as cheats increase in frequency and each cell receives less iron-carrying pyoverdinin, pyoverdinin synthesis may be downregulated in the producer cells.

Interestingly, there was an initial increase in siderophore production in both strains between time points 0 and 5 (wild-type: $T(5) = 2.94$, $p < 0.02$; mutator: $T(5) = 7.02$, $p < 0.0005$). This increase was more pronounced in the mutator populations ($T(9) = -2.81$, $p < 0.02$). This is probably due to an initial, facultative upregulation of siderophore production in response to iron limitation in the new growth medium, only for this acclimation effect to be damped down after time point 5 as cheats swamp the population.

Population density is expected to decrease with decreasing siderophore production as a result of iron

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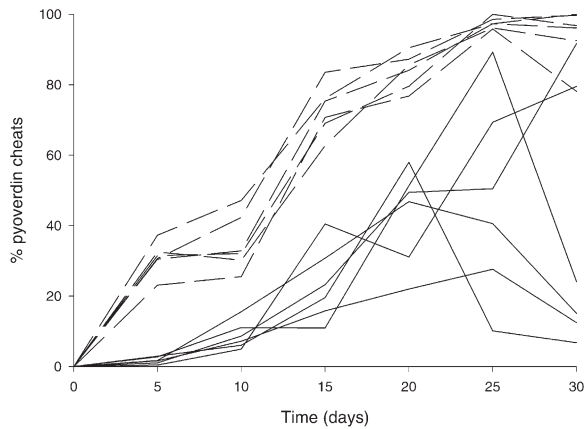


Figure 1. Cheat Frequency over Time in Evolving Wild-Type and Mutator Populations

Solid lines indicate wild-type populations; dashed lines indicate mutator populations.

limitation. Consistent with this view, population density showed a net decrease over time in all populations (Figure 4; all slopes of density against time for time points 0–30 were negative; sign test: $p < 0.0003$), and 10 out of 12 populations showed a negative relationship between cheat frequency and density (sign test: $p < 0.02$). The average density was lower in the mutator populations ($T(9) = 3.86$, $p < 0.005$), although there was no significant difference between the rate of the observed decline in wild-type and mutator populations ($T(9) = 1.20$, $p = 0.26$).

Discussion and Conclusions

In this study, we demonstrate that mutants with reduced pyoverdinin production (siderophore cheats) emerge from initially isogenic population of *P. aeruginosa* and subsequently increase in frequency over a 200-generation experiment. More importantly, we have shown that siderophore cheats were generated and spread through populations more rapidly in populations of bacteria

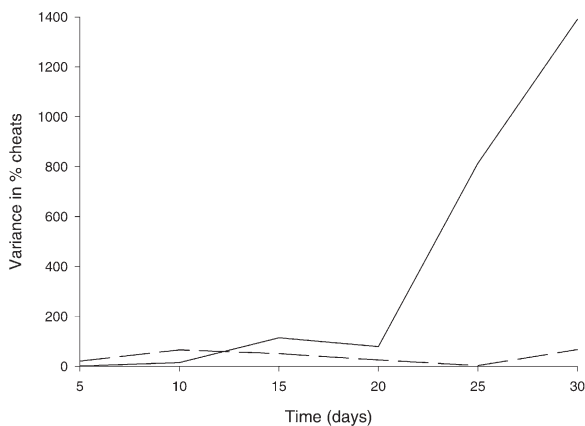


Figure 2. Variance in Cheat Frequency over Time in Wild-Type and Mutator Populations

Solid lines indicate wild-type populations; dashed lines indicate mutator populations.

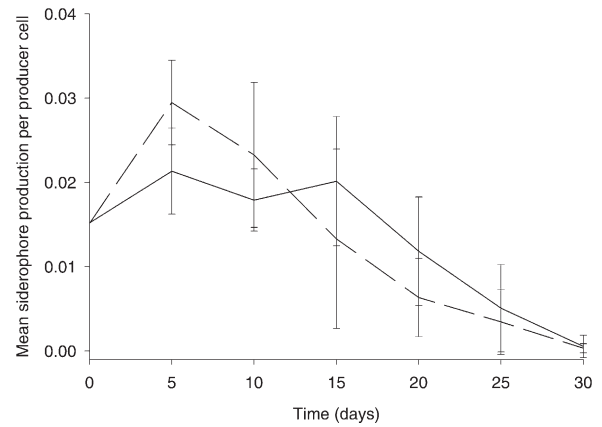


Figure 3. Mean Per Capita Siderophore Production by Producer Subpopulations in Wild-Type and Mutator Populations

Solid lines indicate wild-type populations; dashed lines indicate mutator populations. Error bars show ± 1 standard deviation.

with elevated mutation rates. Given that siderophore cheats reduced the density of bacterial populations in this and a previous study [18], mutator genotypes are likely to be selected against in environments where cooperation is strongly favored, most notably when competition occurs between populations [18].

Pyoverdinin is not the only siderophore of *P. aeruginosa* [20]. It is therefore possible that lack of green pigmentation alone is not a sufficient indicator of a siderophore cheat. To address this possibility, we also assayed both total siderophore production (by using a quantitative, chemical assay) and bacterial population density. We found that the proportion of pyoverdinin cheats positively correlated with total iron-chelating activity of media in which bacterial populations had been grown and negatively correlated with bacterial density, demonstrating that white colonies are indeed siderophore cheats and also revealing a decrease in the levels of siderophore produced by cooperator cells.

Pyoverdinin production is normally a facultative trait, with iron availability determining whether it is switched on as well as the level of production. It is therefore pos-

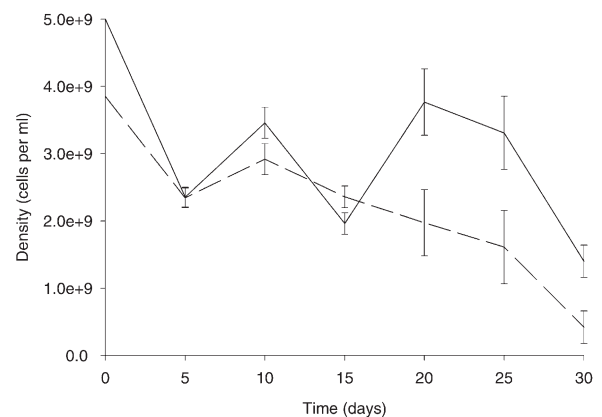


Figure 4. Population Density in Wild-Type and Mutator Populations

Solid lines indicate wild-type populations; dashed lines indicate mutator populations. Error bars show ± 1 standard deviation.

sible that our cheats are the result of non-genetic acclimation to iron limitation, rather than mutational changes. This possibility, however, seems unlikely. First, all previous work suggests siderophore production increases, rather than decreases, in response to iron limitation [20]. Second, previous work has shown that transferring one *P. aeruginosa* colony, rather than whole populations of bacteria, of competing siderophore producers or non-producers can result in the fixation of siderophore producers [18]. Such fixation would not occur if siderophore production were being facultatively decreased.

The more rapid appearance of cheats in mutator populations can simply be explained by an elevated mutation rate. However, the greater rate of sustained increase and lower between-population variance in mutator populations' cheat frequencies also suggest that mutator cheats are relatively more successful than wild-type cheats. This is consistent with an increased ability of mutator lineages to respond to selection in a novel environment [3, 4, 6–9, 22]. The greater selective advantage (relative to pyoverdinin producers) of mutator cheats compared with wild-type cheats is presumably the result of the wider range of possible mutations, including cheating mutations with lower pleiotropic fitness costs and compensatory mutations that could ameliorate any costs that do exist.

A further mechanism that may result in higher levels of cheats in mutator populations is the within-population relatedness reduction that results from elevated mutation rates. We did not investigate this mechanism here because under our experimental conditions, competition was entirely within populations. Such “local competition” or “soft selection” always favors the evolution of cheats regardless of relatedness [18, 23]. We are currently carrying out experiments to investigate how competition, relatedness, and environmental novelty interact in their effects on the success of mutator genotypes in mixed mutator/wild-type populations. Specifically, we predict that mutators will be favored under conditions of low relatedness, local competition, and environmental novelty as a result, in the first two cases, of their increased capacity to generate cheats and in the third case their greater probability of generating mutations beneficial in novel environments (independent of social interactions).

The study of bacterial cooperation may have practical implications given that many cooperative behaviors in pathogenic microorganisms are linked with virulence. The efficient exploitation of an infected host by a pathogen often relies on the cooperative production of metabolic public goods [19, 23, 24]. The formation of multicellular biofilms [25, 26] is a further example of cooperative behavior. As such, cheating mutants of pathogenic bacteria that rely on cooperative traits for efficient host exploitation show reduced virulence [19, 23, 24]. The long-term evolution of pathogen virulence can also depend on the extent to which cooperative interactions between coinfecting strains or genotypes are favored [23, 27–29].

The impact of mutators on cooperative interactions may help to explain the observed distributions of *P. aeruginosa* mutator genotypes. The frequency of mutators tends to be higher in clinical isolates of bacteria than

in conspecific environmental populations [30], reaching frequencies of up to 20% in chronic *P. aeruginosa* infections in the lungs of cystic fibrosis patients [31]. It has been suggested that this is because in vivo populations face greater fluctuations in selection pressures, as a result of the host immune system and chemotherapy [5, 7, 8, 31–33]. We suggest that, in addition, cooperation is less likely to be favored in clinical infections. Clinical *P. aeruginosa* infections, although initially clonal [34], are often long lived, resulting in considerable genetic diversification—and hence lower relatedness—over time. Furthermore, because transmission from *P. aeruginosa* infections is very low, competition will be largely local. Although we know little about the natural ecology of *P. aeruginosa* in soil and water, local patches are likely to go extinct and be recolonised by single clones from neighboring patches relatively quickly. As such, within-population relatedness will be higher and competition between groups will be greater than in clinical infections. Future characterization of clinical *P. aeruginosa* isolates with regard to cooperative traits should help to shed some light on the possibilities outlined above.

Experimental Procedures

Strains

The methionine auxotroph *P. aeruginosa* PAO6049 [19, 35] was used as the wild-type, and strain PAO Δ mutS [32], which has a deletion of the mismatch repair gene *mutS*, was used as the mutator. This strain has a spontaneous mutation rate more than two orders of magnitude higher [32] than that of strain ATC 15692 (PAO1), from which both PAO Δ mutS and PAO6049 are derived.

Growth Conditions

Six glass universals containing 6 ml Casamino acids medium (CAA: 5 g Casamino acids, 1.18 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, per liter) were inoculated with c. 10^6 overnight culture cells of PAO6049, and six with the same density of PAO Δ mutS. Populations were subjected to iron-limited conditions by the addition of 70 μ g/ml human apotransferrin (Sigma), a natural iron chelator, to each tube. Sodium bicarbonate was added to each tube to a final concentration of 20 mM, necessary for iron chelator activity [19]. Tubes were incubated for 24 hr (approximately seven generations) at 37°C on an orbital shaker. Cultures were then homogenized with a vortex mixer, and 6 μ l of culture was transferred to a new microcosm. This evolution continued for 30 days.

Every fifth day, the density, fitness, frequency of pyoverdinin-negative cheats, and total siderophore production of each culture was measured as outlined below. The founding populations were also assayed for density and total siderophore production.

Given the speed with which cheat frequency initially rose in our mutator populations, it is plausible that these populations were inadvertently pre-seeded with cheats that arose in the overnight culture used to inoculate them. Such a situation would mean that our populations were not true replicates. If this were the case, then we might expect all six populations to evolve toward the same fitness peak and so display significantly less variance in population fitness than the wild-type populations. We calculated the variance in between-population fitness for each strain at each of the measured time points. There was no significant difference in the variances between wild-type and mutator populations (T-test, $p = 0.343$), suggesting that the mutator populations are not evolving in parallel, but rather toward different peaks, and so represent true replicates.

Assays

(1) Aliquots of diluted culture were spread onto two King's medium B (KB) agar plates, and the number of co-operator (green-yellow) and cheat (completely white) colonies was scored after 19 hr of

growth. Dilutions were such that 100–200 colonies were present on each plate scored because siderophore production is affected by cell density.

(2) An aliquot of culture was centrifuged to pellet the cells, and the supernatant (containing siderophores) was stored at -20°C . The siderophore content of these supernatants was later determined via the chrome azurol S (CAS) method described by Schwyn and Neilands [36], with the modification that one volume of double-distilled water was added to the assay solution. A measure of mean siderophore production per cell in the i th microcosm is given by $[1 - (A_i/A_{\text{ref}})]/[\ln(\text{Density}_i)]$, where A = absorbance at 630 nm of the assay mixture. (All chemicals are from Sigma.)

(3) Whole-population fitness was measured by competing a sample of cells from each population against an equal number of cells of an ancestral strain oppositely marked for ability to grow on methionine-deficient media (M9 minimal). Relative fitnesses were calculated as described in [37].

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References

1. Sniegowski, P.D., Gerrish, P.J., and Lenski, R.E. (1997). Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* 387, 703–705.
2. Sniegowski, P.D., Gerrish, P.J., Johnson, T., and Shaver, A. (2000). The evolution of mutation rates: separating causes from consequences. *Bioessays* 22, 1057–1066.
3. Taddei, F., Radman, M., Maynard-Smith, J., Toupance, B., Gouyon, P.H., and Godelle, B. (1997). Role of mutator alleles in adaptive evolution. *Nature* 387, 700–702.
4. Tenaillon, O., Toupance, B., Le Nagard, H., Taddei, F., and Godelle, B. (1999). Mutators, Population Size, Adaptive Landscape and the Adaptation of Asexual Populations of Bacteria. *Genetics* 152, 485–493.
5. Rainey, B.P., and Moxon, E.R. (2000). Microbiology. When being hyper keeps you fit. *Science* 288, 1186–1187.
6. Giraud, A., Matic, I., Tenaillon, O., Clara, A., Radman, M., Fons, M., and Taddei, F. (2001). Costs and benefits of high mutation rates: Adaptive evolution of bacteria in the mouse gut. *Science* 291, 2606–2608.
7. de Visser, J.A. (2002). The fate of microbial mutators. *Microbiol.* 148, 1247–1252.
8. Schaaff, F., Reipert, A., and Bierbaum, G. (2002). An elevated mutation frequency favors development of vancomycin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 46, 3540–3548.
9. Nilsson, A.I., Kugelberg, E., Berg, O.G., and Andersson, D.I. (2004). Experimental adaptation of *Salmonella typhimurium* to mice. *Genetics* 168, 1119–1130.
10. Trobner, W., and Piechocki, R. (1984). Selection against hypermutability in *Escherichia coli* during long term evolution. *Mol. Gen. Genet.* 198, 177–178.
11. Funchain, P., Yeung, A., Stewart, J.L., Lin, R., Slupska, M.M., and Miller, J.H. (2000). The consequences of growth of a mutator strain of *Escherichia coli* as measured by loss of function among multiple gene targets and loss of fitness. *Genetics* 154, 959–970.
12. Sachs, J.L., Mueller, U.G., Wilcox, T.P., and Bull, J.J. (2004). The evolution of cooperation. *Q. Rev. Biol.* 79, 135–160.
13. Velicer, G.J. (2003). Social strife in the microbial world. *Trends Microbiol.* 11, 330–337.
14. West Eberhard, M.J. (1975). The evolution of social behavior by kin selection. *Q. Rev. Biol.* 50, 1–33.
15. Frank, S.A. (1994). Kin selection and virulence in the evolution of protocells and parasites. *Proc Biol Sci* 258, 153–161.
16. Frank, S.A. (1998). *Foundations of Social Evolution* (Princeton, NJ: Princeton University Press).
17. Hamilton, W.D. (1964). The genetical evolution of social behaviour I & II. *J. Theor. Biol.* 7, 1–52.
18. Griffin, A.S., West, S.A., and Buckling, A. (2004). Cooperation and competition in pathogenic bacteria. *Nature* 430, 1024–1027.
19. Meyer, J.M., Neely, A., Stintzi, A., Georges, C., and Holder, I.A. (1996). Pyoverdinin is essential for virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* 64, 518–523.
20. Ratledge, C., and Dover, L.G. (2000). Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* 54, 881–941.
21. Lamont, I.L., Beare, P.A., Ochsner, U., Vasil, A.I., and Vasil, M.L. (2002). Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 99, 7072–7077.
22. Rainey, P.B., Buckling, A., Kassen, R., and Travisano, M. (2000). The emergence and maintenance of diversity: Insights from experimental bacterial populations. *Trends Ecol. Evol.* 15, 243–247.
23. West, S.A., and Buckling, A. (2003). Cooperation, virulence and siderophore production in bacterial parasites. *Proc. R. Soc. Lond. B. Biol. Sci.* 270, 37–44.
24. O'Loughlin, E.V., and Robins-Browne, R.M. (2001). Effect of Shiga toxin and Shiga-like toxins on eukaryotic cells. *Microbes Infect.* 3, 493–507.
25. Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999). Bacterial biofilms: A common cause of persistent infections. *Science* 284, 1318–1322.
26. Drenkard, E., and Ausubel, F.M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 416, 740–743.
27. Frank, S.A. (1996). Models of parasite virulence. *Q. Rev. Biol.* 71, 37–78.
28. Turner, P.E., and Chao, L. (1999). Prisoner's dilemma in an RNA virus. *Nature* 398, 441–443.
29. Brown, S.P., Hochberg, M.E., and Grenfell, B.T. (2002). Does multiple infection select for raised virulence? *Trends Microbiol.* 10, 401–405.
30. LeClerc, J.E., Li, B., Payne, W.L., and Cebula, T.A. (1996). High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. *Science* 274, 1208–1211.
31. Oliver, A., Canton, R., Campo, P., Baquero, F., and Blazquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288, 1251–1254.
32. Oliver, A., Levin, B.R., Juan, C., Baquero, F., and Blazquez, J. (2004). Hypermutation and the Preexistence of antibiotic-resistant *Pseudomonas aeruginosa* mutants: Implications for susceptibility testing and treatment of chronic infections. *Antimicrob. Agents Chemother.* 48, 4226–4233.
33. Giraud, A., Matic, I., Radman, M., Fons, M., and Taddei, F. (2002). Mutator bacteria as a risk factor in treatment of infectious diseases. *Antimicrob. Agents Chemother.* 46, 863–865.
34. Finnan, S., Morrissey, J.P., O'Gara, F., and Boyd, E.F. (2004). Genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and the hospital environment. *J. Clin. Microbiol.* 42, 5783–5792.
35. Rella, M., Mercenier, A., and Haas, D. (1985). Transposon insertion mutagenesis of *Pseudomonas aeruginosa* with a Tn5 derivative: application to physical mapping of the arc gene cluster. *Gene* 33, 293–303.
36. Schwyn, B., and Neilands, J.B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56.
37. Lenski, R.E., Rose, M.R., Simpson, S.C., and Tadler, S.C. (1991). Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am. Nat.* 138, 1315–1341.