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Artificial selection of microbial ecosystems for 3-chloroaniline biodegradation

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Summary

We present a method for selecting entire microbial ecosystems for bioremediation and other practical purposes. A population of ecosystems is established in the laboratory, each ecosystem is measured for a desired property (in our case, degradation of the environmental pollutant 3-chloroaniline), and the best ecosystems are used as 'parents' to inoculate a new generation of 'offspring' ecosystems. Over many generations of variation and selection, the ecosystems become increasingly well adapted to produce the desired property. The procedure is similar to standard artificial selection experiments except that whole ecosystems, rather than single individuals, are the units of selection. The procedure can also be understood in terms of complex system theory as a way of searching a vast combinatorial space (many thousands of microbial species and many thousands of genes within species) for combinations that are especially good at producing the desired property. Ecosystem-level selection can be performed without any specific knowledge of the species that comprise the ecosystems and can select ensembles of species that would be difficult to discover with more reductionistic methods. Once a 'designer ecosystem' has been created by ecosystem-level selection, reductionistic methods can be used to identify the component species and to discover how they interact to produce the desired effect.

Introduction

The pollution of soil and water by industrial chemicals is a pervasive problem afflicting the modern world. One of the more promising approaches to ameliorating environmental contamination has been the development of microbial agents capable of degrading toxic or recalcitrant substances in treatment facilities or *in situ* (Timmis *et al.*, 1994; Atlas and Bartha, 1998). A great deal of research has been devoted to finding the organisms, usually bacteria, that are capable of altering or degrading such pollutants to environmentally tolerable forms. Bacteria are particularly suitable for biodegradation applications because of the wide variety of carbon sources or electron acceptors used by various taxa. Bacteria also naturally inhabit substrates, such as soil, that would be difficult or expensive to decontaminate by chemical or physical means (Timmis *et al.*, 1994; Atlas and Bartha, 1998).

The traditional approach of microbiology has been to isolate individual strains of bacteria with the desirable biodegradative abilities (Hegeman, 1985). Because of problems in seeding environmental spills with exogenous, isolated degraders, another approach has been to enrich toxic sites in order to step up the metabolic rates of indigenous consortia of degrading organisms. These methods rely on Beijerinck's assumption that 'everything is everywhere, the environment selects' (Van Iterson *et al.*, 1983), which, if not literally true, entails a 'luck-of-the-draw' approach to finding effective degraders. In this paper, we describe a third approach that involves selecting whole microbial ecosystems for desired properties. So far, we have used the method to evolve soil ecosystems that promote or inhibit plant growth (Swenson *et al.*, 2000), aquatic ecosystems that raise or lower the pH of their environment (Swenson *et al.*, 2000) and aquatic ecosystems that degrade the toxic industrial compound 3-chloroaniline (this paper).

Before describing the ecosystem selection procedure, it will be helpful to review existing methods for facilitating microbial biodegradation in more detail.

Current methods of biodegradation

A sampling of the recent literature provides examples of the range of current approaches to biodegradation. Researchers have identified strains of bacteria that have the ability to break down or alter environmental pollutants such as petroleum products, polychlorinated biphenyls (PCBs) (Wagner-Döbler *et al.*, 1998; Hrywna *et al.*, 1999), other halogenated aromatics (Sharak Genthner *et al.*, 1997; Zeyer *et al.*, 1985), toluene (Hubert *et al.*, 1999) and nitrogen-containing heterocyclic aromatic analogues of polycyclic aromatic hydrocarbons (Pfaller *et al.*, 1999). Organisms capable of such activity are often isolated by a

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screening process in which the target substance is provided as the carbon source or electron acceptor for the growth of a generalized inoculum (Hegeman, 1985; Maymó-Gatell *et al*., 1997; Van Agteren *et al*., 1998). The strain or strains that are capable of survival in the presence of the xenobiotic are then isolated in pure culture (Hegeman, 1985; Zeyer *et al*., 1985; Peters *et al*., 1997; Flashner *et al*., 1999; Hubert *et al*., 1999; Pfaller *et al*., 1999).

Molecular techniques can be used to monitor indigenous or introduced degraders or to engineer recombinant organisms, endowing them with the genes coding for enzymes required by particular steps in a given catabolic pathway (Timmis *et al*., 1994). Because many of the genes coding for catabolic enzymes can be found on transposons or plasmids, Peters *et al*. (1997) were able to track the horizontal *in situ* transfer of a plasmid-borne operon coding for phenol metabolism. By introducing specific dehalogenation genes into a bacterial strain capable of co-metabolizing PCBs, Hrywna *et al*. (1999) demonstrated an improvement in the ability of the recipient strain to grow upon and metabolize much higher concentrations of substrate. Novel developments along these lines include the modification of degrading bacteria to fluoresce when actively breaking down a target substance, providing an *in situ* indicator of degradative progress (Ripp *et al*., 2000). Flashner *et al*. (1999) used a 'directed evolution' technique, involving repeated cycles of screening intentionally mutated gene libraries, to increase the reactivity of enzymes that facilitate biodegradation.

Enriching the natural environment to accelerate the metabolic activities of indigenous degraders is often preferable to seeding with exogenous organisms (Atlas and Bartha, 1998). This approach can take advantage of organisms that have already adapted to catabolize the pollutant substance. One technique has been the enrichment of natural systems with a structural analogue of the target contaminant, inducing the expression of enzymes that can co-metabolize the target (You and Bartha, 1982; Wagner-Döbler *et al*., 1998). Another method has involved the addition of exogenous nutrients to affected systems, thereby stimulating the activity of indigenous degraders (Piehler *et al*., 1999).

One advantage of boosting the activity of indigenous organisms is that single strains of bacteria are often insufficient to degrade certain substances – complete catabolism may require consortia or communities composed of two or more taxa (Wolfaardt *et al*., 1994; Caldwell *et al*., 1997; Sharak Genthner *et al*., 1997; Wagner-Döbler *et al*., 1998; Hubert *et al*., 1999; Piehler *et al*., 1999). Wolfaardt *et al*. (1994) demonstrated that consortia of bacteria and of bacteria plus an alga were capable of degrading diclofop methyl, whereas none of the isolates from the consortia could do so alone. O'Neill *et al*. (1998) described perchloroethylene and tetrachloroethene (TCE) degradation by microbial mats containing multiple prokaryote species, including aerobes and anaerobes. In a recent paper on the isolation of a bacterium that breaks down TCE, the authors noted that the focal bacterial strain required by-products from other bacterial strains in order to convert TCE to less harmful ethene (Maymó-Gatell *et al*., 1997). Such cases have been described as examples of 'syntrophy' (Hamilton, 1984), in which multiple species are effectively participating in a kind of metabolic co-operation (Van Agteren *et al*., 1998). Syntrophic communities can be cultured in the laboratory using continuous culture techniques that essentially select for those consortia capable of metabolizing a chosen pollutant (Hegeman, 1985).

Artificial ecosystem selection

Figure 1 illustrates how we used the general concept of ecosystem selection in our 3-chloroaniline biodegradation experiment. 3-Chloroaniline is mildly toxic and has mutagenic properties (Van Agteren *et al*., 1998). It is a ubiquitous by-product of the industrial synthesis of several common substances, as well as a break-down product of some explosives and herbicides (Zeyer *et al*., 1985).

The experiment began with 120 test tubes containing a sterile tryptic soy broth medium and a controlled

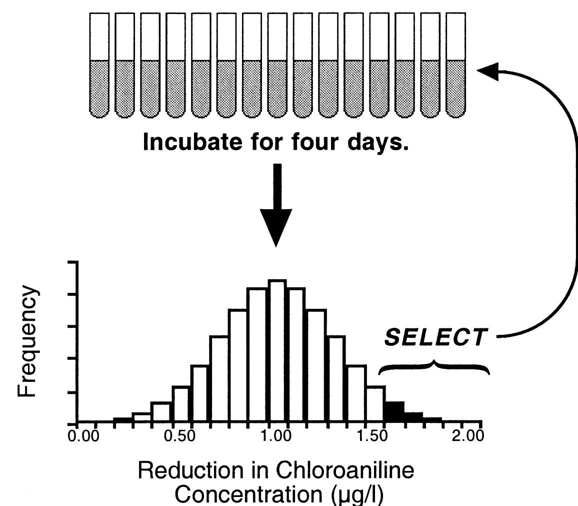

Fig. 1. The ecosystem selection procedure used in our experiment. Within each treatment, we inoculated 15 test tubes containing a dilute nutrient broth and 12.2 $\mu g\ l^{-1}$ 3-chloroaniline with a sample of a naturally occurring bacterial community. We then incubated the test tubes for 4 days and assessed their ability to degrade the xenobiotic. The three test tubes from the high end of the ecosystem-level phenotypic distribution (idealized in the figure) were selected to inoculate a new set of test tubes. The selection cycle was repeated for 30 generations. Control lines followed the same procedure, with the exception that randomly selected test tubes were used to inoculate subsequent generations.

concentration of 3-chloroaniline (see *Experimental procedures* for details). The test tubes were divided into four selection lines and four non-selection lines with 15 test tubes per line. Each test tube was inoculated with planktonic microorganisms in 1 ml of unsterilized pond water from a single source. After a 4 day incubation period, each test tube was measured for the amount of 3-chloroaniline degraded. Within each selection line, the three test tubes with the highest degradative ability were mixed and used to inoculate a new 'generation' of 15 test tubes. Within each non-selection line, three test tubes were chosen at random (without regard to their degradative ability) and used to inoculate a new generation in a similar fashion. The experiment was continued for 30 4 day ecosystem generations.

It should be obvious that our procedure is a standard artificial selection experiment, except that we are selecting whole ecosystems on the basis of their properties rather than single individuals. An ecosystem is defined as a system formed by the interaction of a community of organisms with their environment. Because the phenotypic trait that forms the basis of selection is a property of the physical environment (3-chloroaniline concentration), we are selecting at the ecosystem level in every sense of the word. Nevertheless, if the ecosystems exhibit phenotypic variation and if the 'offspring' ecosystems resemble their 'parents', the ecosystems in the selected lines should become progressively better at degrading 3-chloroaniline as the generations proceed. We might know little about the species, genes and their interactions with each other and their physical environment that we have selected but, despite our ignorance at the mechanistic level, we would have created 'designer ecosystems' that are exceptionally good at the task they were selected to perform. It is worth noting that artificial selection experiments at the individual level also produce phenotypes without knowledge of the underlying mechanisms (e.g. egg production in hens without knowledge of the genetic and physiological causes). Although mechanistic understanding is always desirable, it is a strength of artificial selection experiments that mechanistic understanding is not required.

On closer examination, it may seem puzzling that the vital ingredients of variation and heritability exist at the ecosystem level. Why should the test tubes vary when they are initially physically identical and are inoculated with so many millions of organisms from the same source? Even if differences are observed, why should they remain stable in the face of selection processes taking place within each test tube? Problems such as these have made higher level selection a controversial topic among evolutionary biologists (for reviews, see Sober and Wilson, 1998; Swenson *et al.*, 2000). However, complex systems theory can help to explain how variation and heritability can exist at the ecosystem level.

A complex system interpretation

Complex systems are often highly sensitive to initial conditions. Arbitrarily small differences between replicate systems do not remain small but act as a seed for the development of larger differences over time. This has been called 'the butterfly effect' for weather systems; a butterfly flapping its wings over Brazil can be the seed that alters weather patterns in Europe a month later (Lorenz, 1993).

The test tubes in our experiment are complex biological systems that initially differ only slightly (by sampling error) in their species and genetic composition. We have found that they exhibit the butterfly effect, just like the weather. Over the course of the 4 day incubation period, they become very different in their species composition (and probably their genetic composition within species), with consequences for 3-chloroaniline degradation. In short, variation among ecosystems ceases to be a puzzle when the ecosystems are recognized as complex systems.

Of course, if the ecosystems always exhibited sensitive dependence on initial conditions, they would not have the stability that is required for 'offspring' ecosystems to resemble their 'parents'; there would be variation, but no heritability. Fortunately, there are combinations of species and genes that are locally stable. Ecosystems that degrade 3-chloroaniline and exhibit local stability will faithfully transmit their properties to the next generation; their properties will be heritable.

Computer scientists have developed methods for solving problems that have an astronomical number of possible solutions, such as the travelling salesman problem, which seeks to minimize the length of a path that connects a large number of points (Mitchell, 1998; Fogel, 1999). These methods often emulate an evolutionary process in which a number of solutions are compared, and the best are selected, from which a number of new solutions are generated by mutation or recombination. The solutions that evolve by this procedure are not always the very best, but they are far better than the average solution. Our experiment can be seen as a physical realization of these computer algorithms, in which the problem is to find an ecosystem that performs a given function, and there are an astronomical number of combinations of species and genes within species from which to find a solution. The experiment explores the parameter space by creating a population of ecosystems and selecting the best from which a new population of ecosystems are formed. The final product of selection may not be the best ecosystem for performing the function, but it is likely to be far better than average. Now that we have described the experiment from both the evolutionary and complex system perspectives, we can proceed to the results.

Results

Figure 2 shows the degradative ability of the four selected and four non-selected lines over the course of 30 ecosystem generations (an ecosystem generation is the 4 day incubation period and therefore includes many microbial generations). For every ecosystem generation, the amount of degradation that occurred in sterile medium over the same period was used as a baseline to define the zero value on the *y*-axis. The first point to notice is that the eight lines diverged widely in their degradative abilities within a few ecosystem generations. Even without being selected, some lines were relatively good and other lines were relatively poor at degrading 3-chloroaniline. The divergence among lines in degradative ability can be explained only by a corresponding divergence in the species and/or genetic composition of the lines, despite the fact that they started out nearly identical. Thus, the butterfly effect operated at the level of the eight replicate lines.

Within each line, sensitive dependence operated at a smaller scale to create differences among the test tubes during each ecosystem generation. We measured evolution within each line by comparing the average degradative ability for five early generations (4–8) with five late generations (25–30, excluding generation 27, in which we experienced measurement problems; Table 1). Figure 2 and Table 1 show that three of the four selected lines (E1, E2 and E3) responded to selection, significantly improving their degradative ability over the course of the 30 ecosystem generations. The fourth line (E4) did not respond to selection and, in fact, appears to have retarded the degradation of 3-chloroaniline, compared with the degradation that takes place in sterile medium (the zero line on the *y*-axis). The regression lines in Fig. 2 indicate the clear upward trend for E1–E3 and the downward trend for E4.

In the four non-selected lines, the parent ecosystems were chosen at random without respect to their degradative

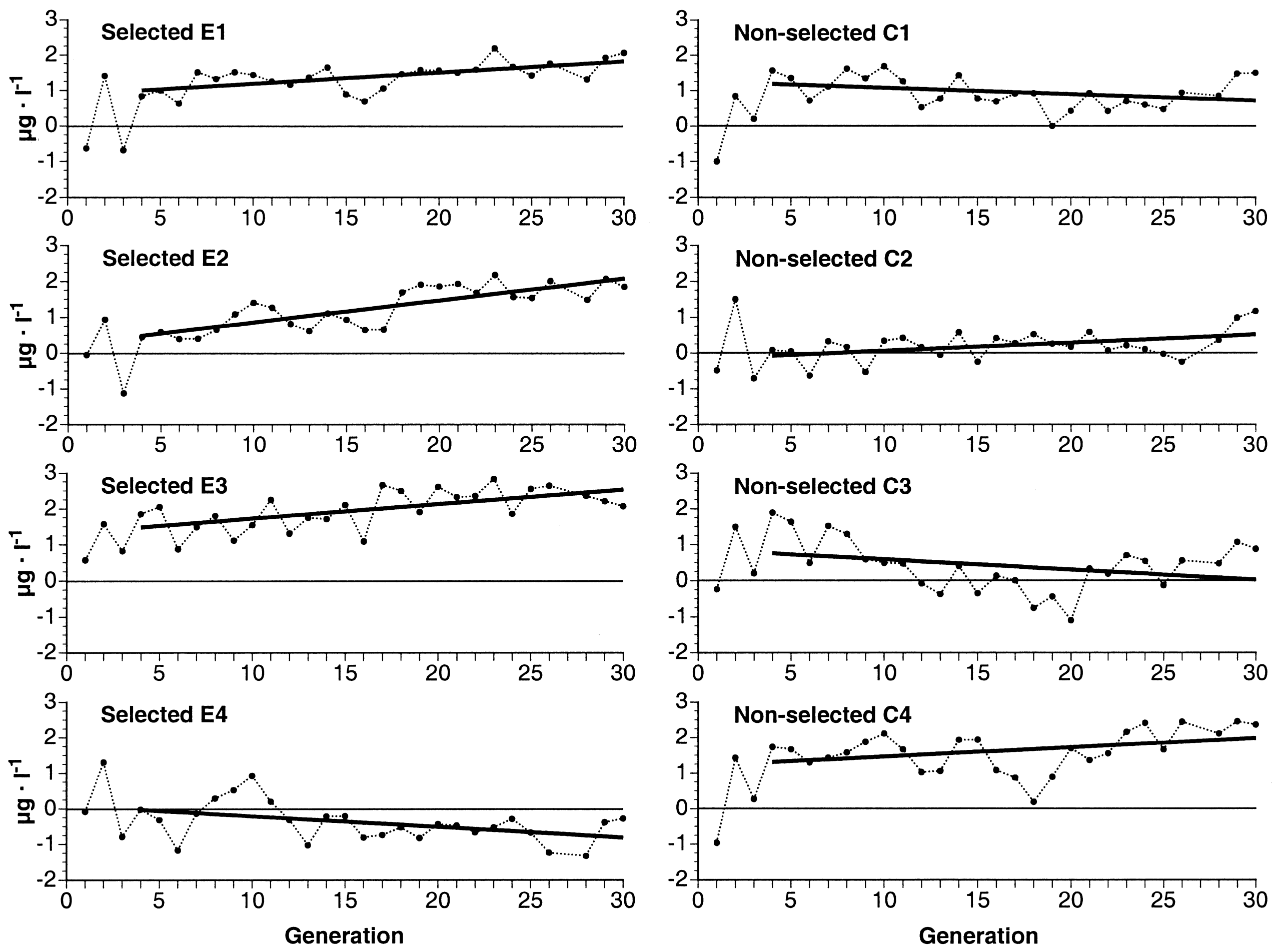

Fig. 2. Change in concentration of 3-chloroaniline in the medium of test tube microcosms as a result of ecosystem-level selection. Absorbance of light at 550 nm wavelength was measured and converted into concentration of 3-chloroaniline ($\mu g\ l^{-1}$) based on an overall regression of sterile samples of known concentration run with each generation of the experiment. Each point represents the mean of 15 replicate microcosms. Superimposed trend lines represent the least squares linear best fit of mean values from generations 4 to 30 (omitting the 'experimental noise' of the first three generations).

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Table 1. Individual ANOVAs for each replicate line, comparing chloroaniline reduction in five 'early' generations (4–8) with that in the same line in five 'late' generations (25–30; excluding generation 27, in which we encountered measurement problems).

Source	Sum of squares	DF	Mean square	F-ratio	*P*
E1	19.422	56	0.347	1.804	0.006**
Error	17.692	92	0.192		
E2	26.560	64	0.415	3.256	0.000***
Error	10.833	85	0.127		
E3	18.042	57	0.317	1.505	0.040*
Error	19.351	92	0.210		
E4	15.152	59	0.257	1.039	0.429
Error	22.242	90	0.247		
C1	10.477	56	0.187	0.645	0.961
Error	26.690	92	0.290		
C2	17.879	61	0.293	1.322	0.115
Error	19.514	88	0.222		
C3	21.968	62	0.354	2.005	0.001**
Error	15.200	86	0.177		
C4	16.581	56	0.296	1.331	0.112
Error	20.250	91	0.223		

'E' refers to experimental lines (four replicates), 'C' to randomly selected control lines (four replicates). $n = 150$ (two sets of five pooled generations, 15 microcosms per line per generation), $\alpha = 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

ability. Although these lines varied widely in their ability to degrade chloroaniline, none of them systematically increased in ability over the course of the 30 generations. One line (C3) showed a significant decrease (Table 1), whereas another (C4) was particularly good at breaking down chloroaniline from the beginning of the experiment and maintained its ability throughout the experiment. Thus, the systematic increase that occurred in three of the four selected lines can be attributed to ecosystem-level selection. A repeated measures analysis of variance (ANOVA) indicates a significant selection–time interaction ($F_{28,2772} = 10.062$, $P < 0.001$; cf. Fig. 3), illustrating the overall efficacy of selection, even including the line (E4) that did not respond to selection. The fact that the (between subjects) effect of selection on the ability to break down chloroaniline was not significant ($F_{1,99} = 2.441$; $P = 0.121$) results from the great heterogeneity that developed among lines noted earlier (Fig. 2). It is interesting that the selected lines only marginally exceeded the degradative ability that one of the non-selected lines achieved without selection.

Discussion

The experiment reported here, combined with our earlier experiments that produced fourfold differences in plant growth and 25-fold differences in hydrogen ion concentration, demonstrate that microbial ecosystems possess the heritable variation that allows their properties to be moulded by artificial selection procedures. The theoretical significance of these findings is discussed in Swenson *et al.* (2000); briefly, they help to resolve the controversial subject of multilevel selection and to integrate complex systems theory with evolutionary theory. In this paper, we are stressing the practical significance of the research as a method of evolving whole ecosystems to perform useful functions such as bioremediation. The method is simple, can proceed without detailed knowledge of the organisms and their interactions, and can select successful combinations of organisms (and genes within organisms) that would be difficult or impossible to discover otherwise.

Artificial ecosystem selection departs from traditional microbiological research in its ignorance of the specific organisms and mechanistic interactions that are being selected. It is a strength of the approach that 'designer ecosystems' can be developed to perform practical functions without the time-consuming research required for detailed mechanistic understanding. However, mechanistic understanding is also highly desirable. The best research programme would begin with artificial selection to discover combinations of species and genes that function well together with respect to a given task, followed by more traditional microbiological research to identify the organisms and their interactions. We have conducted some preliminary research along these lines (unpublished data). It is clear from microscopic examination, isolation cultures and the visual appearance of the test tubes that different multispecies communities evolved in each line, including cyanobacteria as well as spirochaetes, bacilli and cocci of various sizes and degrees of motility. Preliminary high-performance liquid chromatography (HPLC) time-course analysis of two lines (E1 and C3) revealed that they were modifying their chemical environments in different ways. Mechanistic knowledge will be more useful for ecosystems known to be functionally adaptive than for ecosystems that are merely average (and possibly greatly below average) with respect to the practical goals that we are trying to be achieve.

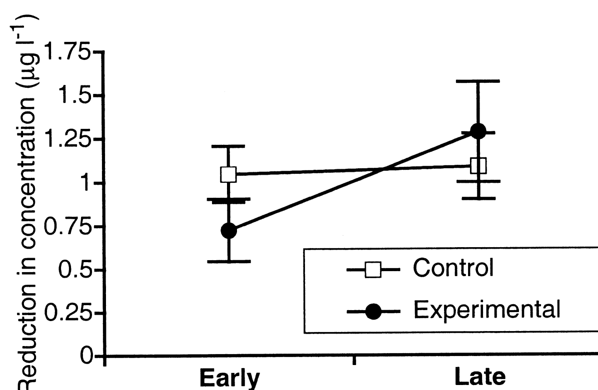

Fig. 3. Overall change in ability to degrade 3-chloroaniline over time, selected (experimental) versus non-selected (control) treatments. Means (± SE) of four experimental and four control lines in five 'early' generations (4–8) and five 'late' generations (25, 26 and 28–30).

An ecosystem selection experiment requires a number of decisions concerning the size of the experimental unit, the specific medium, the duration of the ecosystem generation, the size of inoculum that connects one generation to another, whether to mix the selected ecosystems (similar to sexual reproduction) or to keep them separate (similar to asexual reproduction), and so on. It is highly unlikely that the experiment reported here represents the optimal conditions for ecosystem-level selection. For example, variation occurred at the level of single test tubes, but also at the level of the eight replicate lines. If we repeated the experiment on a larger spatial and temporal scale, selecting the best line to initiate a new set of lines, we might have produced an even greater response to selection. Alternatively, ecosystem selection might work better at a much smaller scale, such as the biofilms that form on the surface of glass or resin beads. The technology exists to bind molecules (e.g. 3-chloroaniline) to beads in such a way that the beads undergo a colour change as the result of degradation of the bound substance (Lam *et al*., 1991; 1995; Warren *et al*., 1992; Liang *et al*., 1996; Shah *et al*., 1997). Thousands of beads could be screened for the degradative ability of their surface biofilms, which could then be used to inoculate a new generation of beads. More research is required to fine tune the methods of ecosystem selection, but our experiments provide a promising start.

The consequences of complexity add an exciting and unpredictable dimension to ecosystem selection experiments. If microbial ecosystems are as vulnerable to the butterfly effect as the weather, then variation can be created at almost any scale. Even very large units that start out virtually identical will eventually vary in ways that are likely to create correlated variation in the selected trait. On the other hand, the exact nature of the variation may be difficult to predict. It is possible and even likely that, if we repeated our experiment without changing any of the methods, we would select different suites of organisms and genes that degrade 3-chloroaniline via different mechanistic pathways (a result that has been observed in artificial selection experiments at the individual level; Cohan, 1984). The same complex interactions that create variation also include regions of local stability, which can have both positive and negative effects on ecosystem selection. For example, one of the four selected lines was stable with respect to the property of retarding degradation, despite selection of the test tubes within the line that were best with respect to degradation, generation after generation. Stability in this case resulted in the absence of heritability. On the other hand, one of the non-selected lines happened by chance to be stable with respect to the property of enhancing degradation. When ecosystem selection 'discovers' a combination of species and genes that is locally stable and well adapted with respect to the selected trait, that combination prevails over less stable and less well-adapted combinations, which appears in the experiment as heritable variation and a response to selection.

The fact that 'designer ecosystems' can be stable in addition to well adapted with respect to the selected trait is important from the practical standpoint, because it means that they can remain stable after selection has been relaxed. For example, once selected, a designer ecosystem could be cultured in large batches to be applied in the field without losing its efficacious properties.

Ever since Darwin, individual organisms have been regarded as a malleable clay sculpted by natural selection. Moths can be black or white, the beaks of finches can be thin or stout, and guppies can be colourful or drab depending on differences in survival and reproduction coupled with heritable variation. When people do the selecting, dogs can be large or small, chickens can be naked or sport fantastic plumage, and cows can become milk or meat producers. Ecosystems have almost never been regarded as malleable in the same way, such that a single handful of soil can promote or inhibit plant growth or a single cup of pond water can preserve or degrade a toxic compound, all depending on differences in survival and reproduction coupled with heritable variation. We do not know whether natural selection occurs at the ecosystem level, but artificial selection provides a powerful tool for moulding the properties of domesticated ecosystems for practical purposes.

Experimental procedures

We collected and combined initial samples of naturally occurring planktonic bacterial communities from Harpur Pond on the Binghamton University Nature Preserve. A total of 120 test tubes were prepared with 29 ml each of a liquid growth medium consisting of equal volumes of sterile distilled water and general purpose tryptic soy broth. 3-Chloroaniline was added to the medium to a final concentration of 12.2 $\mu g\ l^{-1}$. Fifteen test tubes were randomly assigned to each of four replicate selected experimental lines and four replicate non-selected control lines, and 1.0 ml of the well-mixed natural sample was added to each test tube. In addition, 15 test tubes containing 30 ml each of the sterilized water–broth–chloroaniline medium without bacterial inocula was used to provide a baseline measurement of the abiotic degradation of chloroaniline. All the test tubes were covered and incubated without mixing in an environmental chamber under a 16 h light/8 h dark cycle at 22°C and 18°C respectively. Cultures were grown for 4 days, after which the concentration of chloroaniline remaining in the medium was measured.

The method for measuring the concentration of chloroaniline was adapted from Snell and Snell (1967). The liquid medium in each test tube was thoroughly mixed, and

≈ 1.5 ml was poured into a 2 ml centrifuge tube. These were centrifuged at 13 000 r.p.m. for 3 min, after which 20 μl of the supernatant was pipetted into one well of a 96-well tissue culture plate. Aliquots of 20 μl of 1:5 (v/v) HCl and 20 μl of 0.25% sodium nitrite were then added to each sample. The samples were allowed 15 min for the chloroaniline to diazotize. Samples of 20 μl of 2.5% ammonium sulphamate and 20 μl of naphthylethylenediamine were added, the solution was diluted to 200 μl, and 20 min was allowed for the reaction to reach completion. Initial measurements indicated that 20 min was sufficient time to obtain a stable absorbance reading. Absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) spectrophotometric reader at a wavelength of 550 nm. Absorbance values were subtracted from the mean absorbance of the 15 sterile 'blank' samples to calculate the amount of chloroaniline degraded by the bacterial communities.

Within each of the four experimental groups, we selected the three test tubes showing the greatest reduction in concentration of chloroaniline. The contents were combined, and 1 ml of the mixture was used to inoculate each of a new set of 15 test tubes containing 29 ml of fresh, sterile growth medium including 12.2 $\mu g\ l^{-1}$ 3-chloroaniline. Three test tubes were selected randomly from each of the four control groups, their contents were mixed, and the mixture was used to inoculate 15 new test tubes. We prepared a set of 15 fresh 'blank' test tubes and placed the entire set of 135 test tube microcosms in the growth chamber for a 4 day growth period as before. This growth–assay–selection cycle was repeated for a total of 30 generations.

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References

Atlas, R.M., and Bartha, R. (1998) *Microbial Ecology: Fundamentals and Applications.* Menlo Park, CA: Benjamin/Cummings.

Caldwell, D.E., Wolfaardt, G.M., Korber, D.R., and Lawrence, J.R. (1997) Cultivation of microbial consortia and communities. In *Manual of Environmental Microbiology.* Hurst, C.J., (eds). Washington, DC: American Society for Microbiology Press.

Cohan, F.M. (1984) Can uniform selection retard random genetic divergence between isolated conspecific populations? *Evolution* **38**: 495–504.

Flashner, Y., Mendelson, I., Ordentlich, A., Grosfield, H., Leitner, M., Balan, A., *et al.* (1999) Directed evolution of a bacterial pesticides-degrading enzyme. In *Novel Approaches for Bioremediation of Organic Pollution.* Fass, R., Flashner, Y., and Reuveny, S. (eds). New York: Kluwer Academic/Plenum Publishers.

Fogel, L.J. (1999) *Intelligence Through Simulated Evolution: Forty Years of Evolutionary Programming.* New York. Wiley & Sons.

Hamilton, W.A. (1984) Energy sources for microbial growth: an overview. In *Aspects of Microbial Metabolism and Ecology.* Codd, G.A. (ed.). New York: Academic Press.

Hegeman, G. (1985) The mineralization of organic materials under aerobic conditions. In *Bacteria in Nature*, **Vol. 1**. Bacterial Activities in Perspective. Leadbetter, E.R., and Poindexter, J.S. (eds). New York: Plenum Press.

Hrywna, Y., Tsoi, T.V., Maltseva, O.V., Quensen, J.F., III, and Tiedje, J.M. (1999) Construction and characterization of two recombinant bacteria that grow on *ortho-* and *para*-substituted chlorobiphenyls. *Appl Environ Microbiol* **65**: 2163–2169.

Hubert, C., Shen, Y., and Voordouw, G. (1999) Composition of toluene-degrading microbial communities from soil at different concentrations of toluene. *Appl Environ Microbiol* **65**: 3064–3070.

Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.J., Kazmierski, W.M., and Knapp, R.J. (1991) A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* **354**: 82–84.

Lam, K.S., Wade, S., Abdul-Latif, F., and Lebl, M. (1995) Application of a dual color detection scheme in the screening of a random combinatorial peptide library. *J Immunol Methods* **180**: 219–223.

Liang, R., Yan, L., Loebach, J., Ge, M., Uozumi, Y., Sekanina, K., *et al.* (1996) Parallel synthesis and screening of a solid phase carbohydrate library. *Science* **274**: 1520–1522.

Lorenz, E.N. (1993) Predictability: does the flap of a butterfly's wings in Brazil set off a tornado in Texas? Appendix 1. In *The Essence of Chaos.* Lorenz, E.N. (ed.). Seattle: University of Washington Press.

Maymó-Gatell, X., Chien, Y.-T., Gossett, J.M., and Zinder, S.H. (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* **276**: 1568–1571.

Mitchell, M. (1998) *An Introduction to Genetic Algorithms.* Cambridge, MA: MIT Press.

O'Neill, W., Nzengung, V., and Noakes, J. (1998) Biodegradation of tetrachloroethylene and trichloroethylene using mixed species microbial mats. In *Bioremediation and Phytoremediation: Chlorinated and Recalcitrant Compounds.* Wickramanayake, G.B., and Hinchee, R.E. (eds). Columbus, OH: Battelle Press.

Peters, M., Heinaru, E., Talpsep, E., Wand, H., Stottmeister, U., Heinaru, A., *et al.* (1997) Acquisition of a deliberately introduced phenol degradation operon, *pheBA*, by different indigenous *Pseudomonas* species. *Appl Environ Microbiol* **63**: 4899–4906.

Pfaller, S.L., Sutton, S.D., and Kinkle, B.K. (1999) *Sphingomonas* sp. strain Lep1: an aerobic degrader of 4 methylquinoline. *Can J Microbiol* **45**: 623–626.

Piehler, M.F., Swistak, J.G., Pinckney, J.L., and Paerl, H.W. (1999) Stimulation of diesel fuel biodegradation by indigenous nitrogen fixing bacterial consortia. *Microb Ecol* **38**: 69–78.

Ripp, S., Nivens, D.E., Ahn, Y., Werner, C., Jarrell, J.I.V., Easter, J.P., *et al.* (2000) Controlled field release of a bioluminescent genetically engineered microorganism for bioremediation process monitoring and control. *Environ Sci Technol* **34**: 846–853.

Shah, A., Rahman, S.S., De Biasi, V., and Camilleri, P. (1997) Development of colorimetric method for the detection of amines bound to solid support. *Anal Commun* **34**: 325–328.

Sharak Genthner, B.R., Townsend, G.T., and Blattmann, B.O. (1997) Reduction of 3-chlorobenzoate, 3-bromobenzoate, and benzoate to corresponding alcohols by *Desulfomicrobium escambiense*, isolated from a 3-chlorobenzoate dechlorinating coculture. *Appl Environ Microbiol* **63**: 4698–4703.

Snell, F.D., and Snell, C.T. (1967) *Colorimetric Methods of Analysis: Including Photometric Methods*, **Vol. IVA**. Princeton: Van Nostrand Company.

Sober, E., and Wilson, D.S. (1998) *Unto Others: the Evolution of Altruism.* Harvard: Harvard University Press.

Swenson, W., Arendt, J., and Wilson, D.S. (2000) Artificial ecosystem selection. *Proc Natl Acad Sci USA* **97**: 9110–9114.

Timmis, K.N., Steffan, R.J., and Unterman, R. (1994) Designing microorganisms for the treatment of toxic wastes. *Annu Rev Microbiol* **48**: 525–557.

Van Agteren, M.H., Keuning, S., and Janssen, D.B. (1998) *Handbook on Biodegradation and Biological Treatment of Hazardous Organic Compounds.* Boston: Kluwer Academic Publishers.

Van Iterson, G., Jr, L.E.Den Dooren de Jong, , and Kluyer, J.J. (1983) *Martinus Wilem Beijerinck: His Life and Work.* Madison, WI: Science Technical.

Wagner-Döbler, I., Bennasar, A., Vancanneyt, M., Strömpl, C., Brümmer, I., Eichner, C., *et al.* (1998) Microcosm enrichment of biphenyl degrading microbial communities from soils and sediments. *Appl Environ Microbiol* **64**: 3014–3022.

Warren, T.M., Williams, V., and Fletcher, M. (1992) Influence of solid surface, adhesive ability, and inoculum size on bacterial colonization in microcosm studies. *Appl Environ Microbiol* **58**: 2954–2959.

Wolfaardt, G.M., Lawrence, J.R., Robarts, R.D., and Caldwell, D.E. (1994) The role of interactions, sessile growth, and nutrient amendments on the degradative efficiency of a microbial consortium. *Can J Microbiol* **40**: 331–340.

You, I.-S., and Bartha, R. (1982) Stimulation of 3,4-dichloroaniline mineralization by aniline. *Appl Environ Microbiol* **44**: 678–681.

Zeyer, J., Wasserfallen, A., and Timmis, K.N. (1985) Microbial mineralization of ring-substituted anilines through an *ortho*-cleavage pathway. *Appl Environ Microbiol* **50**: 447–453.