EXPERIMENTAL EVOLUTION AND ECOLOGICAL CONSEQUENCES: NEW NICHES AND CHANGING STOICHIOMETRY

By

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ABSTRACT

EXPERIMENTAL EVOLUTION AND ECOLOGICAL CONSEQUENCES:
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Evolutionary change can alter the ecological conditions in which organisms live and continue to evolve. My dissertation research used experimental evolution to study two aspects of evolutionary change with ecological consequences: the generation of new ecological niches and evolution of the elemental composition of biomass. I worked with the long-term evolution experiment (LTEE), which is an ongoing experiment in which *E. coli* have evolved under laboratory conditions for more than 60,000 generations. The LTEE began with extremely simple ecological conditions. Twelve populations were founded from a single bacterial genotype and growth was limited by glucose availability.

In Chapter 1, I focused on a population within the LTEE in which some of the bacteria evolved the ability to consume a novel resource, citrate. Citrate was present in the growth media throughout the experiment, but *E. coli* is normally unable to consume it under aerobic conditions. The citrate consumers (Cit^+) coexisted with a clade of bacteria which were unable to consume citrate (Cit^-). Specialization on glucose, the standard carbon source in the LTEE, was insufficient to explain the frequency-dependent coexistence of Cit^- with Cit^+. Instead Cit^- evolved to cross-feed on molecules released by Cit^+. The evolutionary innovation of citrate consumption led to a more complex ecosystem in which two co-existing ecotypes made use of five different carbon sources.

After 10,000 generations of coexistence, Cit^- went extinct from the population (Chapter 2). I conducted replay experiments, re-evolving for 500 generations 20 replicate

populations from prior to extinction. Cit^- was retained in all populations, indicating that the extinction was not deterministic. Furthermore, when I added small numbers of Cit^- to the population after extinction, Cit^- was able to reinvade. It therefore appears that the Cit^- extinction was not due to exclusion by Cit^+, but rather to unknown laboratory variation.

Chapter 3 shifts focus to studying evolutionary changes in stoichiometry, the ratio of different elements within organisms' biomass. Variation in stoichiometry between organisms has important ecological consequences, but the evolutionary origin of that variation had not previously been studied experimentally. Growth in the LTEE is carbon limited and nitrogen and phosphorus are abundant. Additionally, daily transfer to fresh media selects for increased growth rate, which other research has suggested correlates to higher phosphorus content. Consistent with our predictions based on this environment, clones isolated after 50,000 generations of evolution had significantly higher nitrogen and phosphorus content than ancestral clones. There was no change in the proportion of carbon in biomass, but the total amount of carbon retained in biomass increased, indicating that the bacteria also evolved higher carbon use efficiency.

To test whether the increases in nitrogen and phosphorus observed in the LTEE were a result of carbon limitation or were side effects of other selective factors in the experiment, I evolved clones from the LTEE for 1000 generations under nitrogen rather than carbon limitation (Chapter 4). The stoichiometry of the bacteria did change over the course of 1000 generations, indicating that evolution of stoichiometry can occur over relatively short time frames. Unexpectedly however, the evolved bacteria had higher nitrogen and phosphorus content. It appears that the bacteria were initially poor at incorporating nitrogen into biomass, but evolved improved nitrogen uptake.

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They say, “The grass is always greener on the other side of the fence.” That doesn’t apply to my graduate school experience in the Lenski lab at MSU. I can’t imagine a better place for me to have ended up. I am exceedingly grateful to the people who have made my PhD experience so rewarding.

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CHAPTER 1:

EVOLUTION AND COEXISTENCE IN RESPONSE TO A KEY INNOVATION IN A LONG-TERM EVOLUTION EXPERIMENT WITH *ESCHERICHIA COLI*

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Evolution does not produce novelties from scratch. It works on what already exists, either transforming a system to give it new functions or combining several systems to produce a more elaborate one.

–Francois Jacob

Abstract

Evolution of a novel function can greatly alter the effects of an organism on its environment. These environmental changes can, in turn, affect the further evolution of that organism and any coexisting organisms. We examine these effects and feedbacks following evolution of a novel function in the long-term evolution experiment (LTEE) with *Escherichia coli*. A characteristic feature of *E. coli* is its inability to consume citrate aerobically. However, that ability evolved in one of the LTEE populations. In this population, citrate-utilizing bacteria (Cit^+) coexisted stably with another clade of bacteria that lacked the capacity to utilize citrate (Cit^-). This coexistence was shaped by the evolution of a cross-feeding relationship in which Cit^+ cells released the dicarboxylic acids succinate, fumarate, and malate into the medium, and Cit^- cells evolved improved growth on these carbon sources, as did the Cit^+ cells. Thus, the evolution of citrate

consumption led to a flask-based ecosystem that went from a single limiting resource, glucose, to one with five resources either shared or partitioned between two coexisting clades. Our findings show how evolutionary novelties can change environmental conditions, thereby facilitating diversity and altering both the structure of an ecosystem and the evolutionary trajectories of coexisting organisms.

Introduction

Although ecology and evolutionary biology are often treated as distinct disciplines, scientists as far back as Darwin have recognized that the two are closely intertwined (Darwin 1881; Dawkins 1982; Lewontin 2001). Recent research on the interplay between ecological and evolutionary processes (Matthews et al. 2011; Schoener 2011) has emphasized that the two can interact even over short time scales. Evolutionary innovations—and in particular the emergence of qualitatively new functions—have the potential to alter ecological conditions both for the organisms that evolved the innovation and for coexisting organisms. Perhaps the most dramatic example of this interplay in the history of life on Earth was the evolution of oxygenic photosynthesis. This new way of obtaining energy from light was extraordinarily successful, but it produced oxygen as a by-product. Over time, oxygen accumulated in the atmosphere, where it acted as a toxin to most extant life (Knoll 2003; Sessions et al. 2009). Many organisms were likely driven extinct, but others evolved mechanisms to tolerate and use oxygen; thus, the oxygenated world created new ecological niches requiring aerobic respiration and perhaps favoring multicellularity (Schirrmeister et al. 2013).

The origin of a novel function is, by its nature, a rare event. Examples such as the evolution of oxygenic photosynthesis can only be studied using the fossil and geological records, making it difficult to resolve the order of events and examine the consequences of the innovation. The evolution of a new function can lead to major changes in an ecosystem, as some lineages may be driven extinct while others evolve into new niches. Ideally, one would like to be able to study the ecological and evolutionary consequences of an evolutionary innovation by directly comparing the properties of living organisms from before and after the innovation and measuring their relative fitness by competing the ancestral and derived forms in the environments where they evolved. In fact, the evolution of a novel function in a laboratory evolution experiment gives us the opportunity to do just that.

The long-term evolution experiment with *Escherichia coli* (LTEE), in which this novel function arose, is an ongoing experiment in which 12 populations founded from a common ancestor have been evolving in identical environments for more than 25 years. The initial ecology of the LTEE was deliberately made very simple, with the bacteria growing on a single carbon source, glucose, in a carbon-limited medium (Lenski et al. 1991). The culture medium also contained a large quantity of a potential second carbon source, citrate, but this resource was not available for *E. coli* metabolism. In fact, the inability to grow aerobically on citrate is one of the diagnostic features of *E. coli* (Koser et al. 1924). However, after more than 30,000 generations, one of the populations, called Ara-3, evolved the ability to use the citrate (Blount et al. 2008). After 33,000 generations, the citrate-consuming bacteria (Cit^+) became numerically dominant in the population, and the total population size increased several fold. Following this large increase in numbers,

much of the existing diversity in the population was eliminated, but a clade that could not consume citrate (Cit$^-$) persisted and exhibited negative frequency-dependent coexistence with the Cit$^+$ cells (Blount et al. 2008; Blount et al. 2012). The clade of Cit$^-$ cells that persisted and the clade in which the Cit$^+$ phenotype arose had diverged about 10,000 generations before the origin of the Cit$^+$ function. All of the Cit$^-$ cells isolated after the Cit$^+$ type became numerically dominant came from this same clade (Blount et al. 2012).

We set out to study the ecological consequences of the evolution of citrate consumption and how it affected the further evolution of both the Cit$^+$ and Cit$^-$ lineages. In particular, we focused on studying the mechanism by which the Cit$^-$ clade coexisted with the Cit$^+$ clade. At least two distinct mechanisms could underlie this coexistence. First, a tradeoff between growth on glucose and citrate might allow the Cit$^-$ lineage to persist as a specialist on glucose. Indeed, a comparison of the growth curves of Cit$^+$ and Cit$^-$ cells showed that the Cit$^+$ cells had a longer lag time when growing under the same conditions as used in the LTEE (Blount et al. 2008). Second, Cit$^-$ cells might cross-feed on one or more carbon compounds released by the Cit$^+$ cells. We define cross-feeding as the consumption by one group of organisms of products produced by another group of organisms. The two mechanisms are not mutually exclusive and could act together to enable the coexistence of the Cit$^+$ and Cit$^-$ lineages.

The emergence of cross-feeding interactions has been observed repeatedly in evolution experiments with *E. coli* (Rosenzweig et al. 1994; Treves et al. 1998), and it is predicted to occur by theory under certain conditions (Doebeli 2002). Cross-feeding occurs when, in the process of growing on a primary resource, one bacterial ecotype secretes a byproduct that is consumed by another ecotype. Cross-feeding is favored when

bacteria can grow more quickly by not fully degrading their primary resource, but instead secrete incompletely digested byproducts, opening the opportunity for the evolution of specialists on the byproducts. Cross-feeding has evolved in at least one other population of the LTEE, in which two lineages have stably coexisted for tens of thousands of generations (Rozen and Lenski 2000; Le Gac et al. 2012); in another population, a negative frequency-dependent interaction arose, probably also involving cross-feeding, that led to transient coexistence for several thousand generations (Maddamsetti et al. 2015; Ribeck and Lenski 2015). However, cross-feeding interactions may be less common in the LTEE than in some other evolution experiments because the relatively low glucose input and resulting low bacterial density limit the accumulation of secondary metabolites (Rozen and Lenski 2000). Moreover, mathematical modeling shows that cross-feeding will be less favored under a serial-transfer regime like that of the LTEE, where population densities are low much of the time, than in continuous culture, where populations are always near their maximum density (Doebeli 2002).

One potentially important set of molecules for cross-feeding in the Cit^+/Cit^- system includes the C_4-dicarboxylic acids succinate, fumarate, and malate. The Cit^+ phenotype is based on the aerobic expression of the gene encoding the CitT transporter protein (Blount et al. 2012). CitT is a generalized di- and tricarboxylic acid antiporter that can import citrate, succinate, fumarate, and malate in exchange for export of any of these molecules (Pos et al. 1998). Thus, any net import of citrate into the cell requires that the CitT transporter pump succinate, fumarate, or malate out of the cell, making these C_4-dicarboxylates likely molecules for cross-feeding.

Here we show that a key innovation in a long-term experiment with *E. coli*, the evolution of aerobic citrate metabolism, altered the ecological conditions experienced by the bacteria, influencing the subsequent evolution of both the lineage that evolved the new function as well as another surviving lineage that did not. In particular, we demonstrate that Cit^+ cells modified the environment by releasing additional carbon sources—the C_4-dicarboxylates—into the medium. The coexisting Cit^- bacteria evolved to cross-feed on those resources, adapting to the modified environment but resulting in a cost to their fitness when growing on glucose in the absence of the Cit^+ cells. The Cit^+ bacteria also evolved improved growth on the additional resources, thus competing with the Cit^- lineage for both the glucose and C_4-dicarboxylates.

Methods

Long-term evolution experiment with E. coli

The long-term evolution experiment (LTEE) consists of 12 evolving populations of *E. coli* B, which are serially propagated in 10 mL of Davis minimal medium with 25 mg/L glucose (DM25). All populations were initiated from two founder clones that differ by a neutral mutation. Cultures are grown in a 37°C shaking incubator under well-mixed, aerobic conditions. Growth in DM25 is carbon-limited with a final population density of ~5 x 10^7 cells/mL in the absence of citrate consumption. Growth of Cit^+ cells remains carbon-limited (Fig. 1.1) with a population density of ~2 x 10^8 cells/mL. Each population is diluted 1:100 into fresh medium daily, allowing regrowth of the population for ~6.6 generations per day. Every 500 generations, samples of each population are frozen

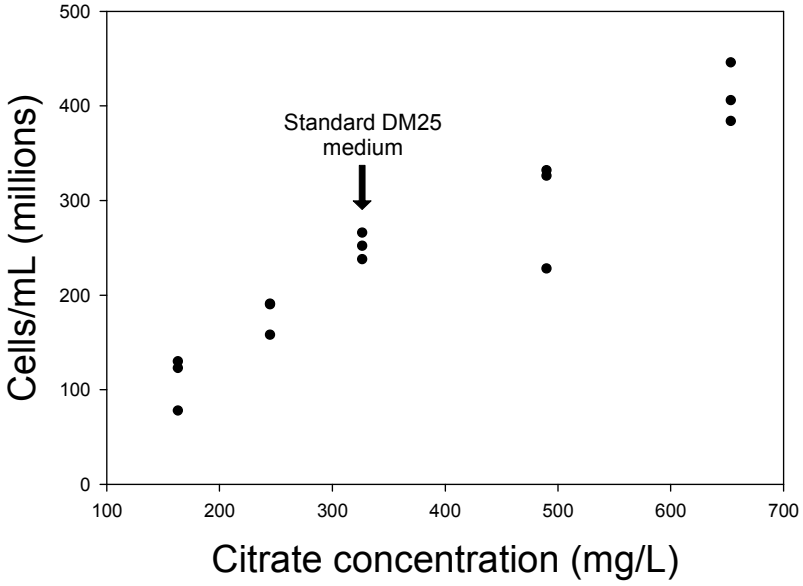

Figure 1.1: Density of a 40,000-generation Cit+ clone when grown in DM25 medium with varying citrate concentrations. Each data point represents a separate replicate culture. The continued increase in population density with increased carbon availability shows that the Cit+ population density is carbon-limited. Cell densities are based on colony-forming units.

at -80°C. These frozen organisms are viable and can be revived for later experimentation. More details on the methods used in the LTEE are given in Lenski et al. (1991).

Strains used and conditioning procedure

Other than the ancestral clone, REL606, all clones were isolated from the LTEE population, designated Ara-3, in which the ability to consume citrate evolved. Unless otherwise specified, the Cit^- clones used in this study were from the Cit^- clade (called clade 2 in Blount et al. 2012) that persisted after the evolution of citrate consumption. Clones from the Cit^+ clade vary in their ability to grow on citrate. All clones from the Cit^+ clade (called clade 3 in Blount et al. 2012) used in this study that were isolated at 32,000 generations or later have a Cit^+ phenotype; however, clones from that clade that were isolated at earlier time points have a Cit^- phenotype. Members of the Cit^- and Cit^+ clades were identified by mutations unique to each lineage, as described in Blount et al. (2012). Table 1.1 lists all of the strains used in this study.

Prior to initiating each experiment, we revived samples of frozen isolates by first growing them in Luria-Bertani broth (LB). We then conditioned them to the environment of the LTEE by growing them for 2 days in DM25, with transfers into fresh medium every 24 h.

Resource assays

We hypothesized that the Cit^+ cells released C_4-dicarboylates into the culture medium, as a consequence of the antiporter mode of action of the CitT protein. To test this hypothesis, we employed gas chromatography–mass spectrometry (GCMS) to measure

Generation	ID Number	Description
0	REL606	Founding clone of Ara-3
30,000	ZDB357	Cit^- clone
30,000	CBT1	Ara^+ mutant of ZDB357
31,000	ZDB374	Cit^- clone
31,000	ZDB200	Cit^- clone (sequencing only)
32,000	ZDB188	Cit^- clone
32,500	ZDB158	Cit^- clone (sequencing only)
33,000	CZB193	Cit^- clone (sequencing only)
33,000	CZB194	Cit^- clone (growth curves)
33,000	CZB195	Cit^- clone (fitness measurements)
33,000	CZB207	Ara^+ mutant of CZB195
34,000	ZDB86	Cit^- clone
34,000	CBT2	Ara^+ mutant of ZDB86
34,000	ZDB87	Cit^- clone (sequencing only)
34,000	ZDB88	Cit^- clone (sequencing only)
35,000	ZDB92	Cit^- clone
36,000	ZDB99	Cit^- clone
37,000	ZDB104	Cit^- clone
38,000	ZDB111	Cit^- clone
39,000	ZDB576	Cit^- clone
40,000	REL10988	Cit^- clone
41,000	ZDB597	Cit^- clone
42,000	ZDB600	Cit^- clone
43,000	ZDB606	Cit^- clone
30,000	ZDB17	Cit^- clone from Cit^+ clade
31,000	ZDB23	Cit^- clone from Cit^+ clade
32,000	ZDB172	Cit^+ clone
33,000	CZB154	Cit^+ clone
34,000	ZDB83	Cit^+ clone
35,000	ZDB89	Cit^+ clone
36,000	ZDB96	Cit^+ clone
37,000	ZDB101	Cit^+ clone
38,000	ZDB107	Cit^+ clone
39,000	ZDB575	Cit^+ clone
40,000	REL10979	Cit^+ clone
40,000	CBT3	λ-sensitive mutant of REL10979
41,000	CBT4	Cit^+ clone
42,000	CBT7	Cit^+ clone
43,000	CBT10	Cit^+ clone

Table 1.1: List of the bacteria used in Chapter 1. Unless otherwise noted, all of the Cit^- clones from generations 30,000 to 43,000 belong to the Cit^- clade which persisted after the Cit^+ lineage became numerically dominant (Blount et al. 2012).

the concentrations of succinate, fumarate, and malate in the medium over the course of a 24-h transfer cycle. We measured these C_4-dicarboxylate concentrations in cultures of the following strains: REL606, the founding strain of the Ara-3 population; a 40,000-generation clone from the Cit^- clade; and eight clones from the Cit^+ clade sampled every 2,000 generations from 30,000 to 44,000 generations. Of the clones from the Cit^+ clade, the 30,000-generation clone had a Cit^- phenotype, the 32,000-generation clone exhibited weak growth on citrate, and the remaining clones exhibited strong growth on citrate. After reviving and conditioning the clones as described above, we transferred the cultures to fresh DM25 medium. At 0, 2, 4, 6, 8, 12 and 24 h after transfer, 300 mL of each culture was collected using a sterile syringe, immediately filter-sterilized by passing the culture medium through a 0.25-µm filter, and frozen at -80°C for later analysis.

We measured the succinate, fumarate, and malate concentrations in these samples using an Agilent 5975 GCMS system at the Michigan State University Research Technology Support Facility. Because DM25 medium contains a high concentration of phosphate that obscured all other molecules in full-scan mode, we could not conduct a broad chemical survey. We instead used selective ion measurement to isolate the spectral signatures specific to succinate, fumarate, and malate. Because of this limitation, we cannot eliminate the possibility that Cit^+ cells also released other molecules into the culture medium.

We also measured the concentration of citrate in the medium to compare the timing of citrate consumption to the timing of succinate, fumarate, and malate release. We measured citrate consumption by the 30,000-, 32,000-, 34,000- and 40,000-generation Cit^+ clones with five-fold replication. We collected filtered samples of culture

medium at 0, 1, 2, 3, 4, 6, 9, and 24 h, following the same protocol as above. We then measured the concentration of citrate in these samples using a Megazyme citric acid assay kit.

Growth using C_4-dicarboxylates as carbon sources

To assess changes in the ability of clones from the Cit^- and Cit^+ clades to grow on succinate, fumarate, and malate, we analyzed growth curves for Cit^- and Cit^+ clones isolated every 1,000 generations from 30,000 to 43,000 generations. This range includes clones from three periods of interest: (i) prior to the origin of the Cit^+ phenotype (30,000 to 31,000 generations; (ii) when Cit^+ cells were present, but very rare (32,000 to 33,000 generations); and (iii) after the several-fold population expansion of the Cit^+ lineage (34,000 to 43,000 generations). The growth trajectories of each clone were assessed for three replicate cultures in media with a single alternative carbon source in the place of glucose. In addition to the C_4-dicarboxylates succinate, fumarate, and malate, we also measured the growth of the Cit^- clones on acetate, a common substrate for cross-feeding in *E. coli* (Rosenzweig et al. 1994; Treves et al. 1998).

We used glucose as a carbon source during conditioning so that all cultures would have a similar initial density. For the Cit^- clones, growth curves were conducted in DM medium. However, growth curves of the Cit^+ clones were conducted in M9 minimal salts medium (Sambrook and Russell 2001) because DM medium contains citrate, which would confound the assays. As a result, the growth curves for the Cit^+ and Cit^- clones are not directly comparable; instead, comparisons of the growth curves provide information on the changes that evolved within each clade over time. The concentrations of the

carbon sources used were as follows: 30.5 mg-succinate/L, 39.5 mg-fumarate/L, 45.7 mg-malate/L, and 34.4 mg-sodium acetate/L. We chose these concentrations because they give stationary-phase population densities similar to those in DM25. Growth curves were generated in 96-well microplates by measuring optical density (OD) at 420 nm every 10 min for 24 h (or 48 h, where noted) using a VersaMax automated plate reader (Molecular Devices).

We analyzed two characteristics of the growth curves: the final OD value at 24 h, and the time required to reach stationary phase. We determined time to stationary phase by visually inspecting each log-transformed growth curve and identifying the time point when a population switched from exponential growth to either a stable or declining OD. These visual assessments were performed twice, in a blind fashion, and the results were extremely consistent between repetitions.

Fitness assays

To determine how evolutionary changes in the Cit^- lineage affected the fitness of Cit^- cells in the presence and absence of Cit^+ cells, we conducted a series of competition experiments between a reference 30,000-generation Cit^- clone and Cit^- clones sampled every 1,000 generations from 30,000 to 40,000 generations. All Cit^- clones used in these assays were from the Cit^- clade. All of the Cit^- clones competed against the reference clone, in both the presence and absence of a mutant derived from a Cit^+ clone from 40,000 generations, with three replicates of each competition. Competitions ran for one standard 24-h cycle, and fitness values were calculated as the ratio of realized population growth rates during the competition assay (Lenski et al. 1991). The Cit^- clones used

were the same as those for the growth measurements above, with the exception of 33,000-generations, for which we inadvertently used a different clone, CZB195, instead of CZB194. However, we confirmed in separate measurements that CZB195, like CZB194 was unable to grow on the C_4-dicarboxylates.

The reference clone, called CBT1, was an arabinose-consuming (Ara^+) mutant of a Cit^- clone isolated from the 30,000-generation population (Table 1.1). Arabinose consumption is a neutral marker under the conditions of the LTEE (Lenski et al. 1991) that allows the differentiation of bacteria by their colony color when grown on tetrazolium arabinose (TA) agar plates (Levin et al. 1977; Lenski et al. 1991). To isolate CBT1, we grew the 30,000-generation Cit^- clone in 10 mL of DM25 medium, then centrifuged the culture and plated the concentrated cells onto a minimal arabinose agar plate. After incubation, a single colony from the plate was streaked twice on minimal arabinose agar plates. A colony from the second plate was grown in LB, and a sample of that culture was frozen. We then confirmed that CBT1 was selectively neutral compared to its parent clone by conducting a seven-day competition between the two clones under the same conditions as the LTEE.

To conduct competitions between Cit^- clones in the presence of Cit^+ cells, we needed a way to prevent the Cit^+ bacteria from growing on the TA plates. To this end, the competitions were conducted using a λ-sensitive mutant, called CBT3, of a Cit^+ clone isolated from the population at 40,000 generations. Although the founding clone of the LTEE was sensitive to bacteriophage λ, resistant mutants were favored and went to fixation in population Ara-3 by 10,000 generations (Meyer et al. 2010). Resistant strains exhibit reduced expression of LamB, a maltose transport protein that is also the

adsorption site for λ (Pelosi et al. 2006; Meyer et al. 2010). To isolate the λ-sensitive mutant, CBT3, we selected for a maltose-consuming mutant following the procedure described above for isolating an Ara^+ mutant, substituting minimal maltose for minimal arabinose agar plates. We confirmed that CBT3 was sensitive to phage λ by plating onto TA plates with and without λ. The fitness of the Cit^+ λ-sensitive mutant was similar to that of its λ-resistant parent in one-day competition assays (relative fitness 0.98 ± 0.06, mean ± 95% confidence interval, n = 10), and the Cit^+ population size was unchanged.

For all competitions between Cit^- clones that were performed in the presence of Cit^+ cells, the cultures were plated together with phage λ to prevent the growth of Cit^+ colonies. However, a few Cit^+ colonies were able to grow on these plates, presumably due to mutations to λ-resistance or incomplete coverage of λ on the plates. To correct for this problem, we transferred a sample of all Ara^- colonies onto Christensen's citrate agar, an indicator medium for the Cit^+ phenotype (Reddy et al. 2007). Any colonies that showed a positive reaction on Christensen's agar were excluded from the counts used to measure the relative fitness of the Cit^- competitors.

Based on the outcome of these experiments, we decided to compete Cit^- clones isolated at 33,000 and 34,000 generations directly against one another. We measured the relative fitness of the 33,000- and 34,000-generation clones with ten-fold replication in both the presence and absence of Cit^+, following the same procedure as above. Half of the replicates had the 33,000-generation clone competing against an Ara^+ mutant of the 34,000-generation clone (named CBT2, isolated as described above), and half had the 34,000-generation clone competing against an Ara^+ mutant of the 33,000-generation clone (called CZB207).

Genomic analysis

The genomes of seven clones from the Cit$^-$ clade were sequenced by Blount et al. (2012). We inspected the genome sequences to look for candidate mutations that might have affected the evolution of C_4-dicarboxylate consumption. Three clones—ZDB357, ZDB200, and ZDB158 from generations 30,000 to 32,500 (Table 1.1)—could not grow on the C_4-dicarboxylates. The other four clones—ZDB87, ZDB99, ZDB111, and REL10988 from generations 36,000 to 40,000 (Table 1.1)—exhibited strong growth on the C_4-dicarboxylates. We used the breseq 0.23 software (Deatherage and Barrick 2014) to generate a list of mutations that were present in the C_4-dicarboxylate-consuming clones, but absent in the clones that lacked the ability to grow on C_4-dicarboxylates.

Genetic manipulation

We constructed an isogenic derivative of the ancestral clone REL606 that carried the *dcuS* allele found in a 34,000-generation Cit$^-$ clone called ZDB86. To do so, we used the pKOV plasmid and the methods in Link et al. (Link et al. 1997). We performed Sanger sequencing to confirm that the evolved *dcuS* allele was present in the resulting construct, ZDB1052.

Results

Resource assays

Consistent with our expectation based on the antiporter mechanism used for citrate uptake, we found that Cit$^+$ clones secrete succinate, fumarate, and malate into the culture medium. The concentrations of these C_4-dicarboxylates remained low throughout the 24-h transfer cycle in cultures of the ancestral strain (REL606), the 40,000-generation Cit$^-$ clone, and a 30,000-generation clone from the clade that later gave rise to the Cit$^+$ lineage (Fig. 1.2). By contrast, both succinate and fumarate concentrations were elevated in all cultures of all of the Cit$^+$ clones during at least part of the 24-h cycle (Fig. 1.2A-B). Malate concentrations were also noticeably elevated in all except one (from generation 36,000) of the Cit$^+$ clones that we analyzed (Fig. 1.2C).

The peak succinate levels in medium with Cit$^+$ clones from 34,000 generations and later occurred at 4 or 6 h after inoculation and ranged from 0.9 to 2.1 mg/L (Fig. 1.2A). Succinate concentrations then declined to below detectable levels by 8 h. The culture medium of the 32,000-generation Cit$^+$ clone, which grows weakly on citrate, showed a very different pattern, with a peak succinate concentration of 2.1 mg/L at 12 h and 1.3 mg/L remaining at 24 h.

Fumarate and malate exhibited similar patterns to succinate, with peak fumarate concentrations ranging from 0.6 to 1.1 mg/L and peak malate concentrations from 3.2 to 11.0 mg/L (Fig. 1.2B-C). (The 36,000-generation Cit$^+$ clone was atypical, however, in that it did not produce any measurable amount of malate.) As with succinate, the 32,000-generation, weakly Cit$^+$ clone released fumarate and malate, but did not fully consume

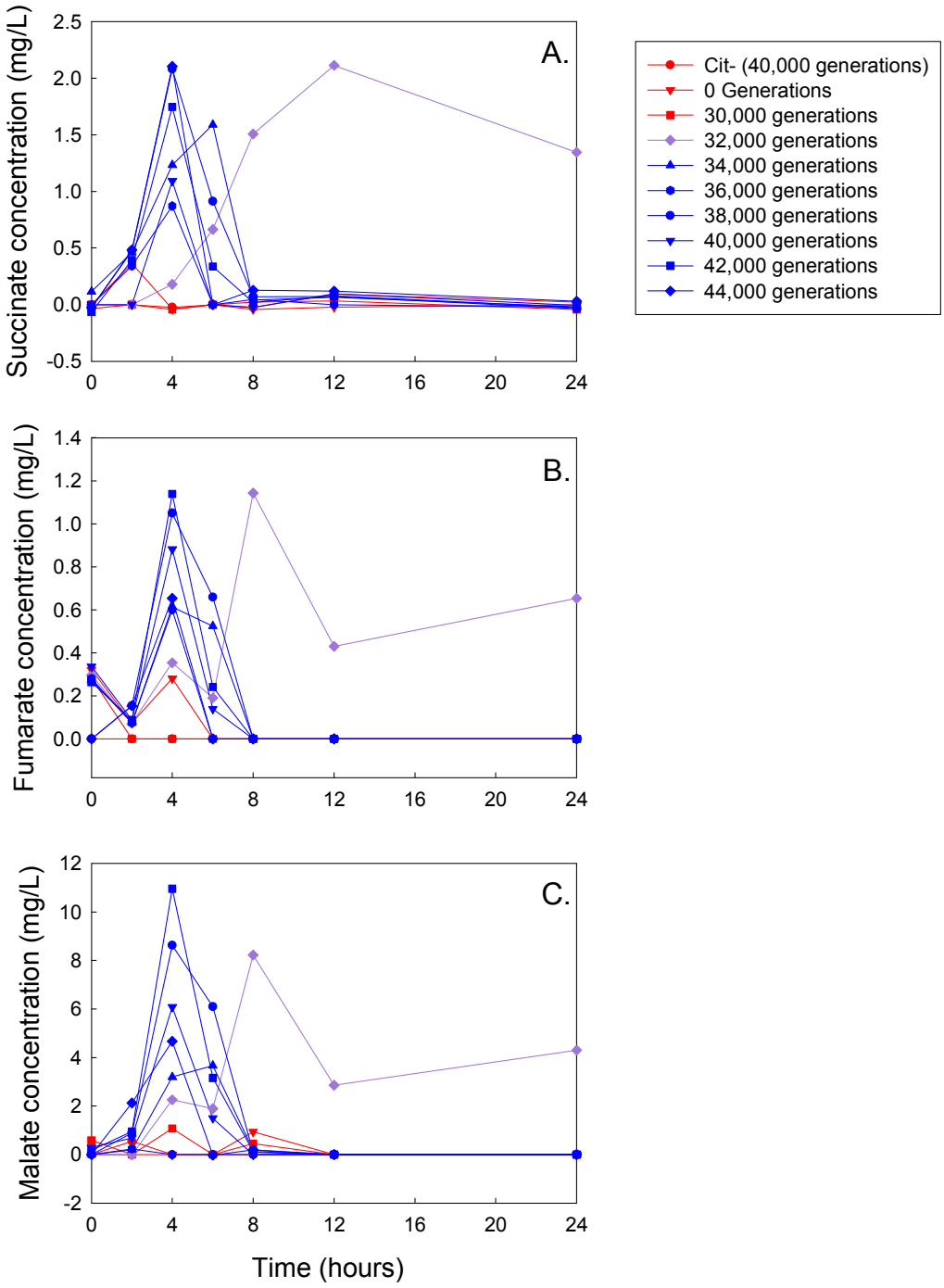

Figure 1.1: Concentration of succinate (A.), malate (B.) and fumarate (C.) in filtered medium sampled throughout a 24-hour growth period. Red points indicate clones with a Cit– phenotype, purple indicates a weakly Cit+ clone, and blue indicates strongly Cit+ clones.

them from the medium. None of the C_4-dicarboxylates showed any obvious trend, upward or downward, in peak concentration over evolutionary time.

Fig. 1.3 shows the concentration of citrate in the culture medium during the growth of several Cit^+ clones. The earliest Cit^+ clone from generation 32,000 drew down citrate only slightly, if at all, over the full 24-h transfer cycle. By contrast, both Cit^+ clones that we tested from generations 34,000 and 40,000 rapidly removed citrate in the period from 4 to 6 h, which coincides with the period when the C_4-dicarboxylates reached their highest levels (Fig. 1.2). By 9 h, both these clones drew down the citrate concentration to below the detection limit.

Growth using C_4-dicarboxylates as carbon sources

Clones from the Cit^- clade isolated before the demographic shift (30,000–33,000 generations) did not exhibit a measurable increase in OD even after 24 h in glucose-free medium supplemented with any of the three C_4-dicarboxylates (Fig. 1.4A-C). However, all Cit^- clones from 34,000 generations and later showed improved growth on succinate, fumarate, and malate with OD values significantly greater than zero after 24 h (Fig. 1.4D-F). These later clones also reached stationary phase in fewer than 24 h, and the time they took to reach stationary phase declined between 34,000 and 43,000 generations (Fig. 1.4D-F).

Clones from the Cit^+ clade also showed negligible growth on C_4-dicarboxylates through 32,000 generations (Fig. 1.5A-C). Beginning at 33,000 generations, Cit^+ clones had significantly improved growth on succinate, fumarate, and malate as sole carbon sources (Fig. 1.5A-C). The 32,000-generation clone had a weak Cit^+ phenotype, but it

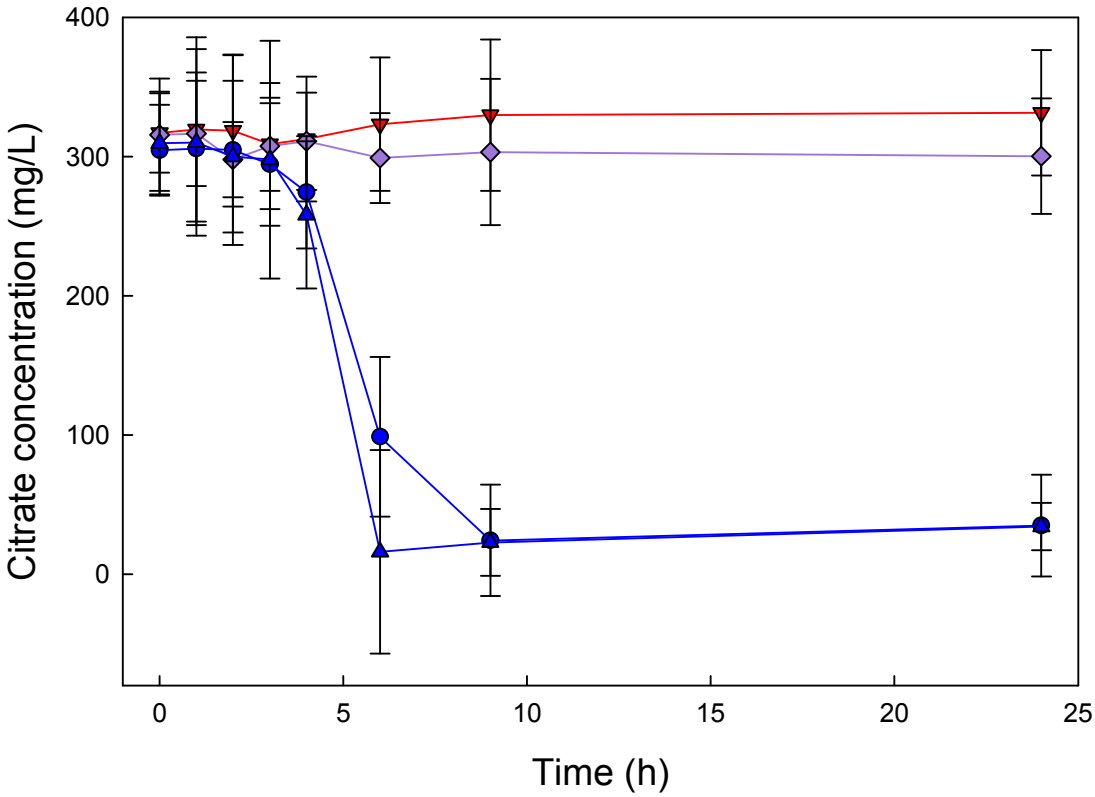

Figure 1.3: Citrate concentration in filtered medium sampled throughout a 24-hour growth period. Red points indicate clones with a Cit^- phenotype, purple indicates a weakly Cit^+ clone, and blue indicates strongly Cit^+ clones.

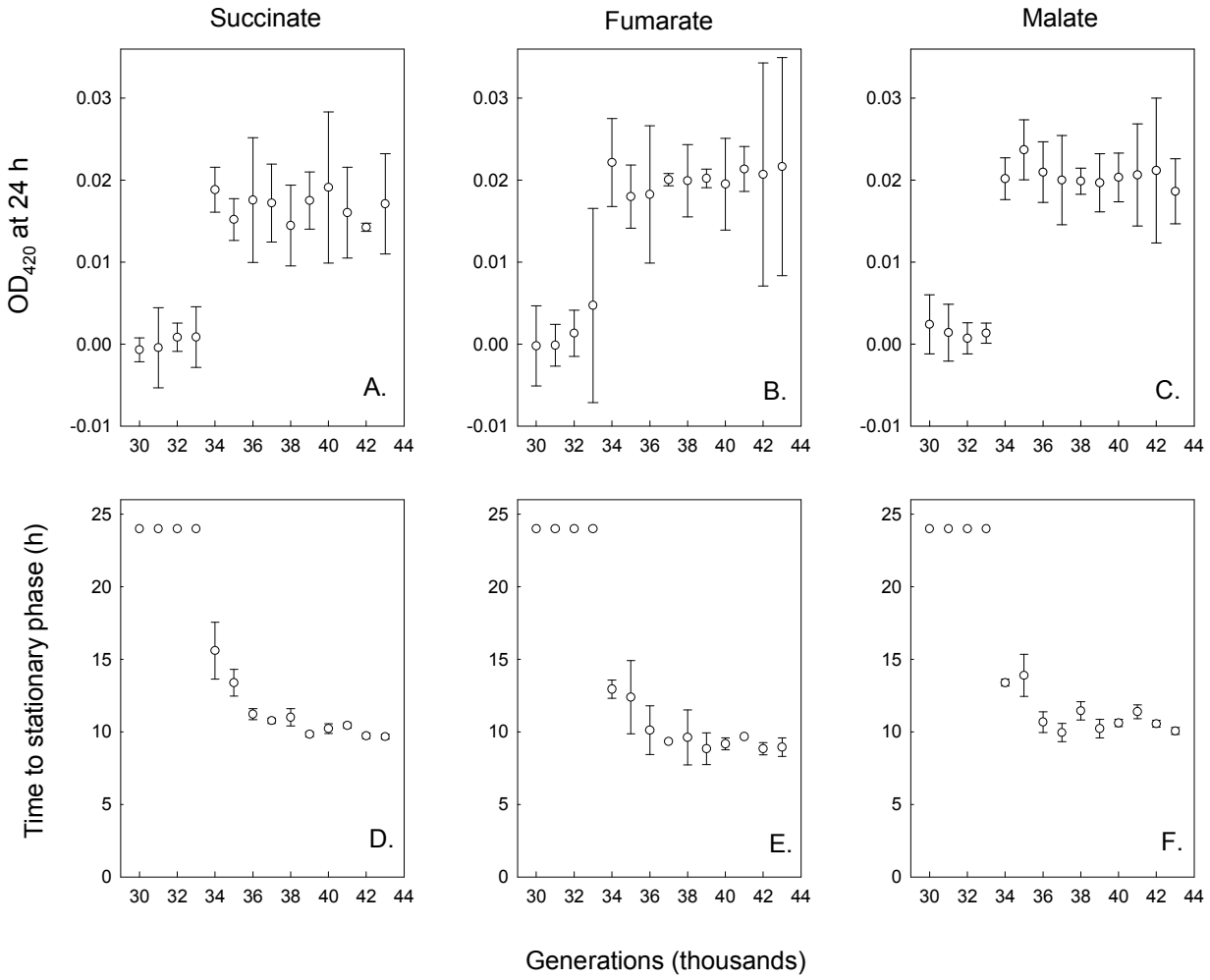

Figure 1.4: Final OD at 420 nm and time to stationary phase of clones from the Cit$^-$ clade grown in DM medium, with 30.5 mg /L succinate, 39.5 mg/L fumarate, or 45.7 mg/L malate. All data shown are means with 95% confidence intervals.

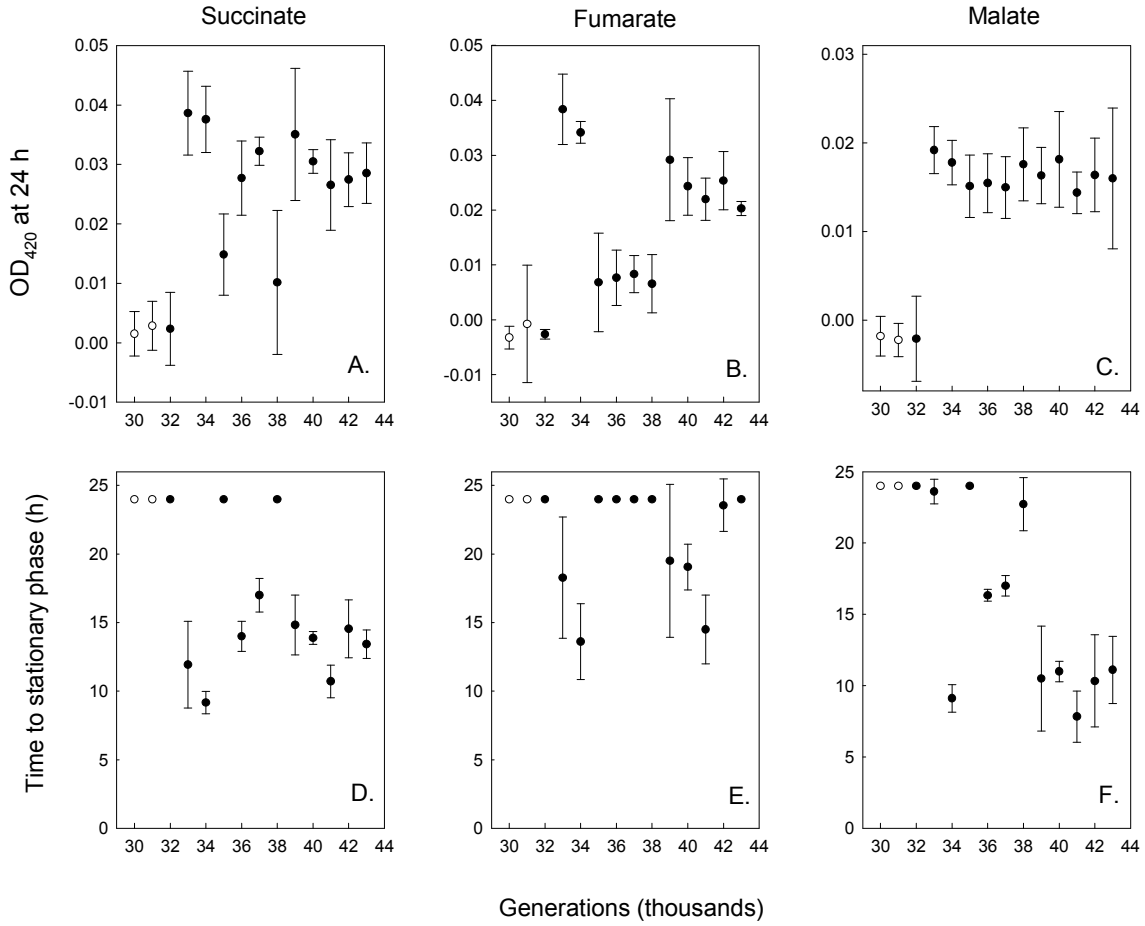

Figure 1.5: Final OD at 420 nm and time to stationary phase of clones from the Cit^+ clade grown in M9 medium, with 30.5 mg /L succinate, 39.5 mg/L fumarate, or 45.7 mg/L malate. Closed circles indicate clones with a Cit^- phenotype and open circles indicate clones with a Cit^+ phenotype. All data shown are means with 95% confidence intervals.

showed no growth on succinate, fumarate, or malate, indicating that the ability to grow on these carbon sources was not simply a pleiotropic effect of the same mutations that allowed growth on citrate. Unlike the clones from the Cit$^-$ clade, Cit$^+$ clones did not show consistent improvement across the generations in their growth in succinate medium. For example, the Cit$^+$ clones from generations 35,000, 38,000 and 46,000 had lower final OD values than other late-generation Cit$^+$ clones and did not reach stationary phase within 24 h when growing on succinate (Fig. 1.5D). Growth of the Cit$^+$ clones on fumarate and malate was similarly variable (Fig. 1.5E-F), but the particular clones with slower growth or lower final density differed across the three substrates.

In contrast to the pattern of improved growth on the C_4-dicarboxylates succinate, fumarate, and malate after the demographic expansion of the Cit$^+$ lineage, clones from the Cit$^-$ clade showed reduced growth on acetate, both in terms of final OD values (Fig. 1.6A) and time to stationary phase (Fig. 1.6B). Thus, acetate cross-feeding is unlikely to contribute to the coexistence of the Cit$^-$ and Cit$^+$ lineages. Moreover, the reduced growth on acetate indicates that the improved growth of Cit$^-$ clones on C_4-dicarboxylates is specific to those molecules, rather than a general improvement in either growth or the ability of Cit$^-$ cells to shift from growing on glucose to other carbon resources.

Fitness assays

If the Cit$^-$ lineage has evolved to feed on byproducts secreted by the Cit$^+$ lineage, then it should show evidence of evolving higher fitness, relative to its Cit$^-$ predecessors, in the presence of Cit$^+$ cells than in their absence. We tested this prediction by competing clones from the Cit$^-$ clade isolated at generations 30,000 and 32,000, and then every

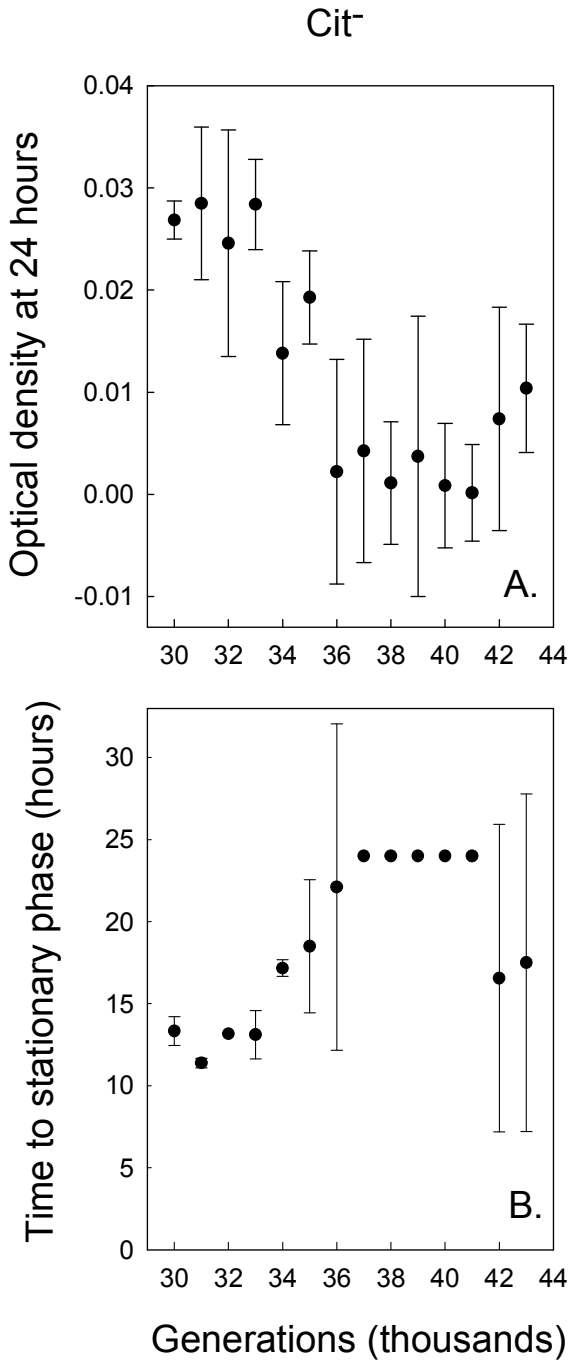

Figure 1.6: Final OD at 420 nm and time to stationary phase of clones from the Cit$^-$ clade growing in DM medium with 30.5 mg/L acetate. All data shown are means with 95% confidence intervals.

1,000 generations from 33,000 to 43,000 generations, against a reference Cit^- clone from 30,000 generations, both in the presence and absence of a common Cit^+ clone that could be excluded when counting the two Cit^- competitors (see Methods). In the presence of Cit^+ cells, the fitness of the Cit^- clones trended higher in later generations, with mean fitness values relative to the 30,000-generation reference significantly greater than unity for 9 of the 11 time points tested from generations 33,000 to 43,000 (Fig. 1.7). However, in the absence of Cit^+ cells, the fitness of the Cit^- clones actually declined over time (Fig. 1.7). These results not only support the cross-feeding hypothesis, but they also suggest that the adaptation of the Cit^- lineage to the cross-feeding niche imposed a tradeoff with respect to growth in its previous glucose-only niche.

Although the overall trend in these data is consistent with our expectations, it is surprising that the increase in the fitness of the Cit^- clones, when assayed in the presence of Cit^+ cells, seems to have begun by 33,000 generations (Fig. 1.7), whereas we did not see any evidence of their growth on C_4-dicarboxylates until generation 34,000 and later (Fig. 1.4). If the differences in fitness of the Cit^- cells in the presence and absence of Cit^+ cells were caused by the evolution of the capacity for growth on the C_4-dicarboxylates, then we would expect the 34,000-generation C_4-dicarboxylate-consuming Cit^- clone to be more fit than the 33,000-generation Cit^- clone in the presence of Cit^+ cells and, perhaps, less fit in the absence of Cit^+ cells. This discrepancy might support a more complicated scenario than that envisioned in our hypothesis, or it might indicate limited statistical resolution given only three competition assays performed for each combination of clone and treatment. To gain better resolution of changes in fitness over this critical period, we competed the Cit^- clones from 33,000 and 34,000-generations against one another, using

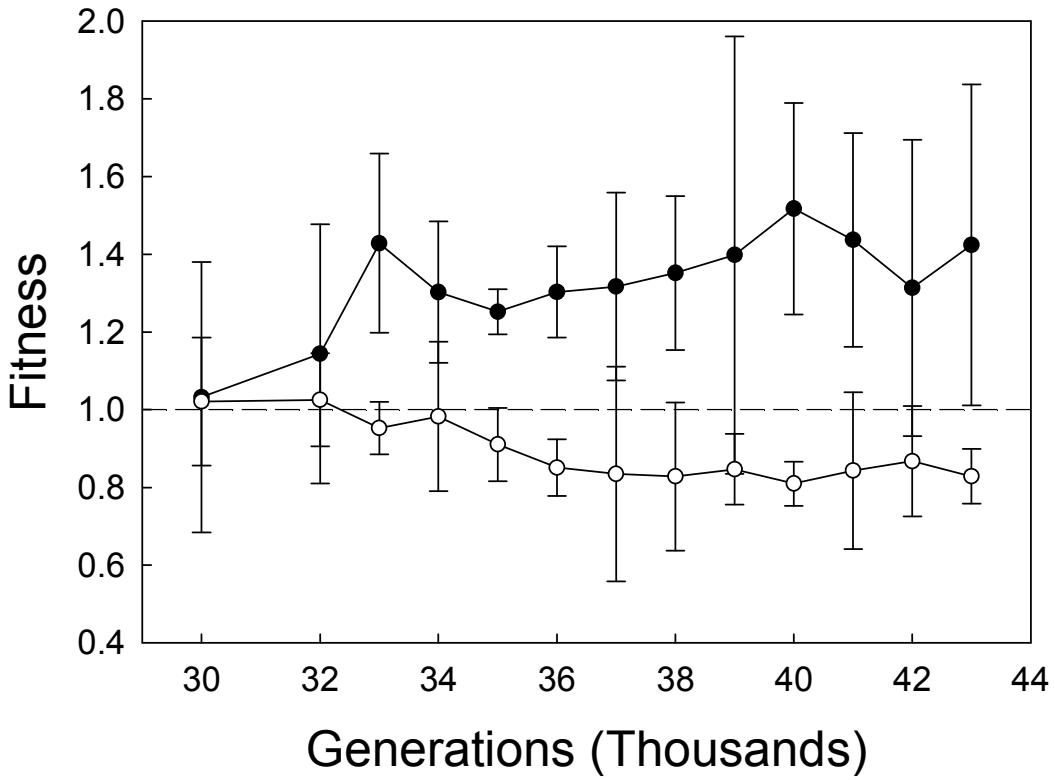

Figure 1.7: Fitness of clones from the Cit$^-$ clade relative to a 30,000-generation clone from the Cit$^-$ clade. Competitions in the presence of Cit$^+$ cells (filled circles) were conducted between the two Cit$^-$ competitors when they were grown together with a 40,000-generation Cit$^+$ clone. Competitions without Cit$^+$ cells (open circles) were performed with only the two Cit$^-$ competitors. All data shown are means with 95% confidence intervals.

a neutral genetic marker to distinguish them (see Methods and Table 1.1). Consistent with our predictions, the later C_4-dicarboxylate-consuming clone had a fitness of 1.14 ± 0.04 (mean ± 95% confidence interval, n = 10) relative to the earlier clone that could not use those substrates, when they competed in the presence of Cit^+ cells. Moreover, in the absence of the Cit^+ cells, the 34,000-generation Cit^- clone had a fitness of only 0.94 ± 0.02 relative to the 33,000-generation Cit^- clone, providing further support for the hypothesis that adaptation of the Cit^- cells to growth on C_4-dicarboxylates imposed a tradeoff with respect to their performance in their original glucose-only niche.

Genomic analysis

We compared the sequenced genomes of seven Cit^- clones that differed in their capacity for growth on C_4-dicarboxylates, generating a list of 19 distinguishing mutations that might underlie that phenotype (Table 1.2). We performed Sanger sequencing to determine the presence or absence of many of these mutations in six additional Cit^- clones: CZB193, CZB194, and CZB195, which did not grow on C_4-dicarboxylates; and ZDB86, ZDB88, and ZDB92, which did grow on those substrates. These additional data narrowed the list of candidates to nine mutations (Table 1.2). Of all these candidates, the 5-bp deletion in the *dcuS* gene is the only one that has clear relevance to the C_4-dicarboxylate growth phenotype. The DcuS protein is a C_4-dicarboxylate sensor that induces expression of the *dctA* gene, which in turn encodes DctA, a dicarboxylic-acid transporter protein (Janausch et al. 2004). The founding strain of the LTEE had a 5-bp insertion in *dcuS* (Jeong et al. 2009), which eliminated the DcuS function and thereby

Position	Mutation	Annotation	Gene	Description
1,140,245	A→T	N38Y (AAT→TAT)	*rimJ* →	ribosomal-protein-S5-alanine N-acetyltransferase
1,761,565	G→A	E441K (GAG→AAG)	*ydiD* →	hypothetical protein
1,782,849	G→A	A167V (GCC→GTC)	*ydiY* ←	hypothetical protein
2,191,174	**IS*150* (+) +3 bp**	**coding (411-413/999 nt)**	***mglB*** ←	**methyl-galactoside transporter subunit**
2,507,912	A→C	T452P (ACC→CCC)	*narQ* →	sensory histidine kinase in two-component regulatory system with NarP (NarL)
2,784,696	**Δ1,396 bp**		***[ygcR]–[ygcS]***	***[ygcR], [ygcS]***
2,899,574	**IS*150* (+) +3 bp**	**coding (419-421/1626 nt)**	***yqeB*** ←	**conserved protein with NAD(P)-binding Rossman fold**
2,918,435	A→C	N439H (AAC→CAC)	*ygfU* →	predicted transporter
2,983,794	**C→T**	**D259D (GAC→GAT)**	***yggW*** →	**coproporphyrinogen III oxidase**
3,046,412	G→A	intergenic (-409/+77)	*yghJ* ← / ← *yghK*	predicted inner membrane lipoprotein/glycolate transporter
3,141,566	C→T	L242L (CTC→CTT)	*ygjE* →	predicted tartrate:succinate antiporter
3,288,053	**C→G**	**V70L (GTC→CTC)**	***arcB*** ←	**hybrid sensory histidine kinase in two-component regulatory system with ArcA**
3,407,922	**C→A**	**G197G (GGG→GGT)**	***kefB*** ←	**glutathione-regulated potassium-efflux system protein**
3,570,410	**A→C**	**intergenic (-1/-390)**	***yhiO*** ← / → ***uspA***	**universal stress protein UspB/universal stress global response regulator**
3,798,623	IS*1* (-) +9 bp	coding (444-452/924 nt)	*yicL* →	predicted inner membrane protein
4,164,385	G→A	R1177H (CGC→CAC)	*rpoB* →	DNA-directed RNA polymerase subunit beta
4,313,510	**C→T**	**A265A (GCG→GCA)**	***yjdB*** ←	**predicted metal dependent hydrolase**
4,329,504	**Δ5 bp**	**pseudogene (875-879/912 nt)**	***dcuS*** ←	**sensor of fumarate two-component regulatory system; b4125_2**
4,414,736	A→G	intergenic (+182/-127)	*fklB* → / → *cycA*	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase)/D-alanine/D-serine/glycine transporter

Table 1.2: List of mutations present in all four sequenced C_4-dicarboxylate-consuming Cit^- clones, but absent from all three Cit^- clones that cannot grow on the C_4-dicarboxylates. The mutations shown in bold font are the reduced set of candidates for conferring growth on C_4-dicarboxylates that were not eliminated after performing additional Sanger sequencing of the relevant genes in other clones. Arrows in the fourth column indicate the direction of transcription of the mutated gene.

prevented expression of the *dctA* gene and its encoded dicarboxylic-acid transporter (Yoon et al. 2012; Quandt et al. 2014).

Genetic manipulation

We tested whether the deletion in *dcuS* was, in fact, a gain-of-function mutation that conferred on the Cit^- lineage the ability to grow on the C_4-dicarboxylates that were secreted by the Cit^+ lineage. To do so, we constructed ZDB1052, an isogenic derivative of the ancestor REL606 except with the *dcuS* allele from a 34,000-generation Cit^- clone called ZDB86. We then measured the growth over 48 h of REL606, ZDB86, and ZDB1052 on succinate, fumarate, and malate.

ZDB1052 had improved growth on succinate, fumarate, and malate compared to REL606 (Fig. 1.8A-C). The densities achieved by ZDB1052 on these substrates after 48 h were comparable to those reached by ZDB86, the earliest Cit^- clone in our study to exhibit the C_4-dicarboxylate growth phenotype and the source of the evolved *dcuS* allele. However, ZDB1052 grew more slowly on the C_4-dicarboxylates than did ZDB86. ZDB1052 also grew more slowly on glucose, and reached a slightly lower final density on that substrate, than did REL606, its counterpart with the ancestral *dcuS* allele (Fig. 1.8D). This result provides direct evidence that adaptation to growth on the C_4-dicarboxylates caused a tradeoff in terms of growth on glucose, the sole carbon source that was available to the ancestral bacteria. ZDB86 does not show any indication of that tradeoff (Fig. 1.8A-D), but that is not surprising because it contains many mutations that were beneficial for growth on glucose, which had fixed before citrate utilization evolved and C_4-dicarboxylates appeared.

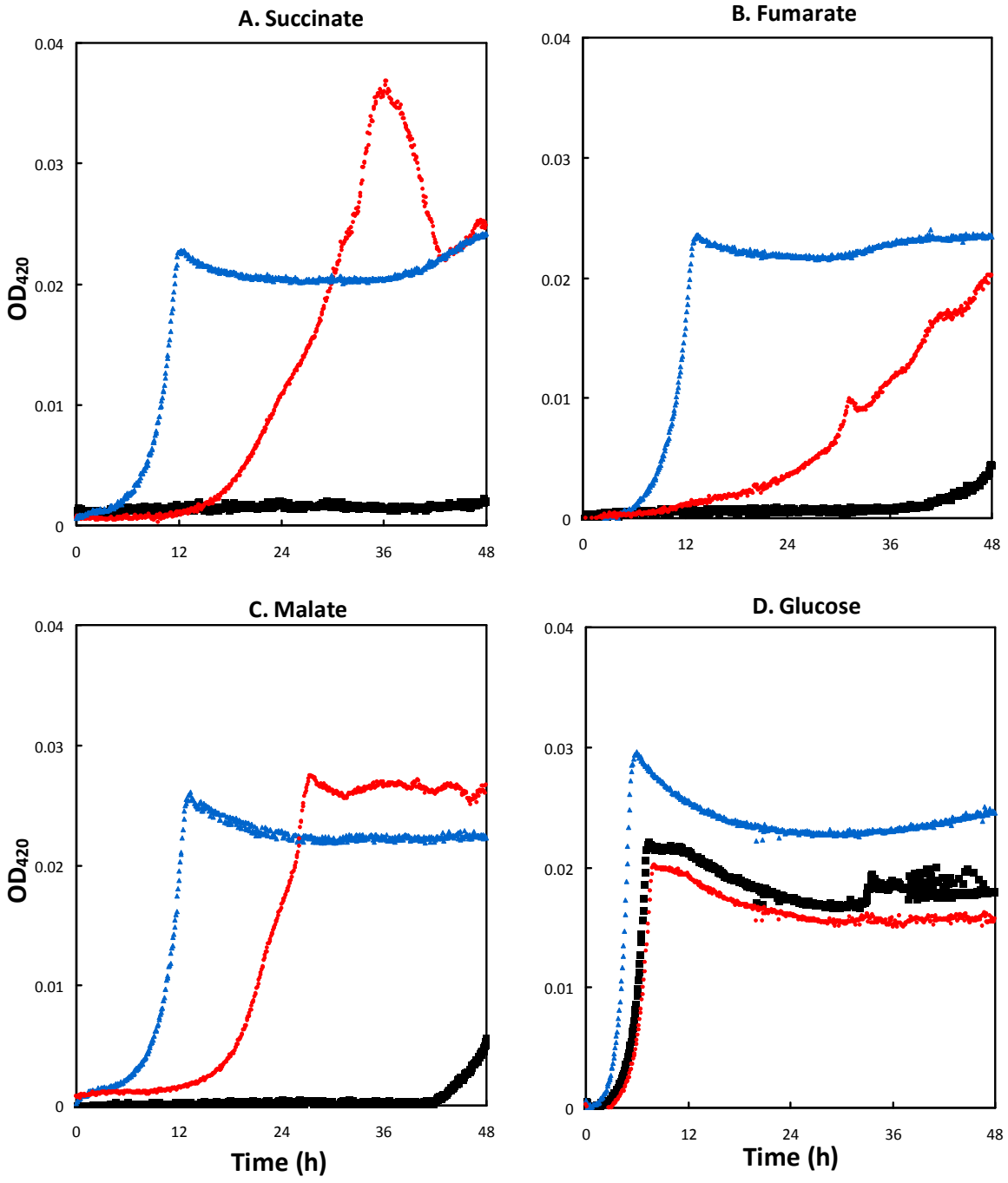

Figure 1.8. Growth curves showing OD at 420 nm of the ancestral strain (REL606, black squares), a 34,000-generation Cit$^-$ clone (ZDB86, blue triangles), and a modified ancestral strain with the evolved *dcuS* allele (ZDB1052, red circles) grown in DM medium supplemented with (A) 30.5 mg/L succinate, (B) 39.5 mg/L fumarate, (C) 45.7 mg/L malate, or (D) 25 mg/L glucose.

The ancestral strain, REL606, did not grow appreciably on C_4-dicarboxylates during the 24-h interval between transfers of the LTEE, but it did show slight growth between 36 and 48 h on two of these compounds (Fig. 1.8A-C). This pattern suggests that growth on C_4-dicarboxylates has a long lag and is slow, but not absent, in the ancestral genotype. Alternatively, this growth could have resulted from a mutant with the ability to grow on C_4-dicarboxylates. We did not try to distinguish these alternatives because the purpose of collecting these data was to test whether the evolved *dcuS* allele led to improved growth on C_4-dicarboxylates, which it clearly did.

Discussion

The ability to grow aerobically on citrate evolved in only one of the twelve LTEE populations (Blount et al. 2008), even after 60,000 generations and billions of spontaneous mutations in each population (Lenski 2004). This evolutionary innovation provided access to a previously unavailable resource, and it caused dramatic ecological changes in the flask-based ecosystem where it arose. Owing to the abundant citrate in the medium, the total population experienced a several-fold increase in density when the Cit^+ cells became dominant in the population. However, the Cit^+ lineage did not eliminate all of the non-citrate consumers from the population. Instead, two ecotypes, one Cit^+ and one Cit^-, coexisted for thousands of generations in a frequency-dependent manner. We sought to understand the ecological dimensions of this prolonged coexistence. Based on several lines of evidence, we discovered that the Cit^- lineage was able to persist, at least in part,

by evolving a cross-feeding relationship with the Cit$^+$ lineage, one in which three C_4-dicarboxylates were the secreted metabolites.

We ruled out the possibility that the Cit$^-$ lineage coexisted with the Cit$^+$ lineage by improvements in their growth on the exogenously supplied glucose. Although Cit$^-$ cells initially had an advantage over Cit$^+$ cells in competition for glucose (Blount et al. 2008), improved growth on glucose did not sustain the Cit$^-$ lineage. In fact, the fitness of the Cit$^-$ lineage in the standard glucose-limited medium, when measured in the absence of a Cit$^+$ population, actually declined over evolutionary time (Fig. 1.7).

However, the fitness of clones from the Cit$^-$ clade increased over evolutionary time when they competed against their predecessor in the presence of Cit$^+$ cells (Fig. 1.7). This finding supports our hypothesis that the Cit$^-$ lineage adapted to changes in the environment caused by the activity of Cit$^+$ cells. In particular, Cit$^+$ cells secreted the C_4-dicarboxylates succinate, fumarate, and malate into the culture medium, whereas Cit$^-$ cells (including predecessors from the clade that generated the Cit$^+$ lineage) did not (Fig. 1.2). The earliest Cit$^+$ clone that we tested showed only weak growth on citrate (Blount et al. 2012), but it, too, released C_4-dicarboxylates into the medium. However, it did not fully draw them back down during the course of the 24-h transfer cycle, whereas Cit$^+$ clones from later generations both released these C_4-dicarboxylates and then drew them down below detectable levels. Although the observed concentrations of the endogenously produced succinate, fumarate, and malate were low relative to the exogenously added concentrations of glucose and citrate, the total amounts of C_4-dicarboxylate secreted and available to support growth would probably be much higher if they could be integrated over the course of an entire cycle. In fact, the citrate transporter protein CitT transports

one molecule out of the cell for each molecule that it transports into the cell (Pos et al. 1998). While some of these antiporter events may have involved the exchange of one citrate molecule for another, the Cit^+ populations (except in their earliest form) eventually consumed all of the citrate that was available each day (Fig. 1.3). It follows logically, then, that the total quantity of C_4-dicarboxylate molecules released into the medium was equal to the number of citrate molecules consumed, implying substantial opportunity for growth on C_4-dicarboxylates and, consequently, strong selection to improve growth on those resources. C_4-dicarboxylate concentrations were generally highest from about 4 to 6 h after inoculation, which coincided with the period when citrate was being drawn down from the medium (Fig. 1.3), corroborating the biochemical evidence (Pos et al. 1998) that the release of C_4-dicarboxylates is simultaneous to citrate uptake.

In addition to showing that the Cit^+ population released C_4-dicarboxylates into the medium, and that the Cit^- population was becoming more fit in the presence (but not the absence) of the Cit^+ population, we also tested explicitly whether the Cit^- lineage evolved to exploit those C_4-dicarboxylates. Indeed, we found that C_4-dicarboxylate consumption by the Cit^- lineage evolved in close temporal proximity to the rise of the Cit^+ population to numerical dominance between 33,000 and 33,500 generations. Clones isolated from the Cit^- lineage through 33,000 generations showed no measurable growth on succinate, fumarate, or malate, but clones from 34,000 generations and later grew well on all three C_4-dicarboxylates (Fig. 1.4). Moreover, the time for populations to reach stationary phase, which is a function of both the length of the lag phase and the subsequent growth rate, declined in later generations, indicating continued adaptation of the Cit^- population to growth on C_4-dicarboxylates. This improvement was not a generalized improvement in

fitness because the growth of the Cit$^-$ clones on both glucose and acetate declined over this period.

The Cit$^+$ lineage also evolved improved growth on C_4-dicarboxylates, although that improvement was somewhat more sporadic than the improvements seen in the Cit$^-$ lineage (Fig. 1.5). The earliest clone able to grow on citrate, from generation 32,000, did not show any detectable growth on C_4-dicarboxylates, indicating that improved growth on these compounds was not simply a pleiotropic effect of the ability to consume citrate. The greater variability of growth on C_4-dicarboxylates in the Cit$^+$ lineage suggests that selection to use them was either weaker or less effective in this lineage than in the Cit$^-$ lineage. That variability may reflect the fact that Cit$^+$ cells can also metabolize citrate, a more abundant and energetically valuable resource than any of the C_4-dicarboxylates, so that mutations that improve growth on citrate might be favored even if they reduce growth on one or more of these byproducts.

In both the Cit$^+$ and Cit$^-$ lineages, the mechanism underlying the improved growth on C_4-dicarboxylates appears to be increased expression of the DctA transporter protein. DctA is a proton motive force-driven transporter that *E. coli* requires for transport of C_4-dicarboxylates during aerobic metabolism (Davies et al. 1999). The ancestral strain of the LTEE had a 5-bp frameshift mutation in the *dcuS* gene, which encodes a regulator that promotes expression of DctA (Jeong et al. 2009; Quandt et al. 2014). In the Cit$^+$ lineage, previous work showed that a mutation in the promoter region of the *dctA* gene caused increased DctA expression and restored the ability to grow on C_4-dicarboxylates (Quandt et al. 2014). Similarly, in the Cit$^-$ lineage, we have showed that a 5-bp deletion in *dcuS* that restored its reading frame enabled growth on C_4-dicarbolyates (Fig. 1.8).

The consumption by Cit^- cells of the C_4-dicarboxylates released by Cit^+ cells is an example of cross-feeding, a phenomenon that has been observed in several previous microbial experiments (Rosenzweig et al. 1994; Treves et al. 1998). Cross-feeding typically occurs when one organism evolves to grow faster by partially degrading a primary resource, leaving a secondary resource for another organism to consume. In this canonical scenario, incomplete degradation of the primary resource is adaptive because it leads to faster growth on the primary resource (Doebeli 2002; Pfeiffer and Bonhoeffer 2004). However, the evolution of cross-feeding between the Cit^+ and Cit^- lineages follows a somewhat different pattern. The release of C_4-dicarboxylates by the Cit^+ cells does not itself appear to be beneficial to them. Indeed, while the Cit^- lineage evolved increased growth on the C_4-dicarboxylates, so too did the Cit^+ lineage. Thus, unlike the typical cross-feeding relationship, the two ecotypes have evolved increased, not decreased, overlap in their resource usage. Instead of being an adaptive trait, cross-feeding in this case appears to reflect evolutionary 'bricolage' (Jacob 1977), whereby evolution tinkers with existing components to solve new problems.

The Cit^+ lineage studied here evolved the ability to consume citrate via multiple mutations including, in particular, a complex rearrangement that allowed aerobic expression of an existing CitT transporter (Blount et al. 2012). Most *E. coli* cells can express the CitT protein only under anaerobic conditions, where cells ferment citrate into succinate (but only when they also have another growth substrate in addition to citrate). Under anaerobic conditions, the use of a transporter that exchanges citrate for succinate has a clear benefit because succinate is an end product of fermentation. However, in the Cit^+ lineage, the CitT transporter is expressed under carbon-limited, aerobic conditions

where succinate and other C_4-dicarboxylates provide a useful source of energy and carbon. Many other bacterial species use proton or sodium gradients for citrate transport (Boorsma et al. 1996; Bott 1997). Furthermore, in the only other reported case where mutations gave rise to aerobic consumption of citrate by *E. coli* (Hall 1982), the citrate importation appears to have been powered by the proton motive force (Reynolds and Silver 1983), although the genetic and mechanistic bases are unknown in that case. If the Cit^+ ecotype in the LTEE had instead evolved to use a proton or sodium gradient for citrate uptake, then it would not have exported C_4-dicarboxylates. A hypothetical Cit^+ mutant that employed a proton or sodium gradient would probably be favored over the evolved Cit^+ bacteria that release valuable molecules that are often lost to competitors. Instead, as a consequence of evolutionary tinkering, or bricolage, the Cit^+ lineage first evolved to express the previously unexpressed CitT antiporter under aerobic conditions (Blount et al. 2012) and then to reabsorb the exported C_4-dicarboxylates via the proton motive force by using the previously unexpressed DctA transporter (Quandt et al. 2014). While this solution seems not to have been the most elegant or efficient, it nonetheless proved highly successful for the Cit^+ clade.

It is also interesting to consider that the coexistence of the Cit^+ and Cit^- ecotypes might depend on this mode of evolutionary tinkering. That is, if the Cit^+ ecotype did not release C_4-dicarboxylates, then the Cit^- lineage would not have been able to evolve to make better use of those resources. In theory, a glucose-specialist ecotype could coexist by out-competing the Cit^+ bacteria for the exogenously supplied glucose, but in practice this coexistence seems unlikely because other LTEE populations, which had evolved for 50,000 generations as glucose specialists, could not invade Cit^+ populations when

introduced at low frequency (Wiser et al. unpublished data). Thus, glucose specialization does not appear to be sufficient to sustain long-term stable coexistence.

As evolution proceeds, it is possible that refinement of citrate transport by the Cit^+ bacteria will erode the cross-feeding niche, perhaps even leading to the extinction of the Cit^- ecotype. For example, the Cit^+ bacteria might evolve to preferentially export less energetically valuable molecules (*e.g.*, malate rather than succinate) in exchange for citrate. Or the Cit^+ lineage might eventually eliminate the Cit^- bacteria by evolving better uptake of the C_4-dicarboxylates, particularly given its larger population and much higher mutation rate (Blount et al. 2012) than the Cit^- clade. It is even possible that a transport protein that uses proton motive force to import citrate could eventually evolve in the Cit^+ lineage. For now, though, the evolution of citrate importation via the CitT antiporter has altered the Ara-3 population of the LTEE from its simple ancestral ecosystem with one available source of carbon to a more complex system with at least five carbon sources that together support the coexistence of two distinct ecotypes.

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CHAPTER 2:

REPLAYING EVOLUTION TO TEST THE CAUSE OF EXTINCTION OF ONE ECOTYPE IN AN EXPERIMENTALLY EVOLVED POPULATION

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Abstract

In a long-term evolution experiment with *Escherichia coli*, bacteria in one of twelve populations evolved the ability to consume citrate, a previously unexploited resource in a glucose-limited medium. This innovation led to the frequency-dependent coexistence of citrate-consuming (Cit^+) and non-consuming (Cit^-) ecotypes, with Cit^- bacteria persisting on the exogenously supplied glucose as well as other carbon molecules released by the Cit^+ bacteria. After more than 10,000 generations of coexistence, however, the Cit^- lineage went extinct; cells with the Cit^- phenotype dropped to levels below detection, and the Cit^- clade could not be detected by molecular assays based on its unique genotype. We hypothesized that this extinction event was a deterministic outcome of evolutionary change within the population, specifically the appearance of a more-fit Cit^+ ecotype that competitively excluded the Cit^- ecotype. We tested this hypothesis by re-evolving the population from one frozen sample taken just prior to the extinction and from another sample taken several thousand generations earlier, in each case for 500 generations and with 20-fold replication. To our surprise, the Cit^- type did not go extinct in any of these replays, and Cit^- cells also persisted in a single replicate that was propagated for 3,000 generations. Even more unexpectedly, we showed that the Cit^-

ecotype could reinvade the Cit^+ population after its extinction. Taken together, these results indicate that the extinction of the Cit^- ecotype was not a deterministic outcome driven by competitive exclusion by the Cit^+ ecotype. The extinction also cannot be explained by demographic stochasticity, as the population size of the Cit^- ecotype should have been many thousands of cells even during the daily transfer events. Instead, we infer that the extinction must have been caused by a rare chance event in which some aspect of the experimental conditions was inadvertently perturbed.

Introduction

In studying patterns of extinction over the course of evolutionary history, a key question is the extent to which extinctions are random or deterministic events (Jablonski 1986; Yedid et al. 2012). Very small populations can go extinct as a consequence of demographic stochasticity (Lande 1993; Melbourne and Hastings 2008; Wootton and Pfister 2013), that is, random fluctuations in population size that reflect the discrete nature of birth and death processes in finite populations. In the case of sexually reproducing organisms, inbreeding depression can also contribute to the extinction of small populations (Saccheri et al. 1998; O'Grady et al. 2006). Other causes of extinction, such as asteroid impacts and anthropogenic changes, may also be random (at least insofar as they are exogenous to the affected organisms), yet they can eliminate large populations and even extinguish entire clades (Alvarez et al. 1980; Jablonski 1986). In other cases, though, extinctions might be more or less deterministic in the following sense: if the extinctions reflect processes that are endogenous to the organisms and the communities in

which they are embedded, then the extinctions would be predictable and repeatable if the same system could be observed multiple times. For example, one lineage may be fated to extinction because it cannot evolve and adapt as well as its competitors or other biological enemies (Bengtsson and Milbrink 1995; Woods et al. 2011). These different causes of extinction may also have different consequences for how an ecosystem recovers from extinction (Solan et al. 2004; Yedid et al. 2009).

Unfortunately, it is difficult to identify definitively the causes of most extinctions in geological history. Even for contemporary extinctions, one cannot generally manipulate and test possible causes of extinction by taking the extinct organisms and reintroducing them into the same ecosystems. However, as we will show, when an extinction occurs during a controlled laboratory experiment with microbes, it is possible, at least in principle, to perform replay experiments to test certain causes of extinction directly and determine whether the extinction was a random or deterministic event.

Here we examine possible causes of an extinction event in a long-term evolution experiment with *E. coli* (LTEE). Because *E. coli* can be revived from frozen stocks (Lenski et al. 1991), the LTEE has a living fossil record with which a population's evolution can be replayed, starting from one or more frozen samples, under conditions that are as close as possible to those of the original evolutionary event (Blount et al. 2008; Woods et al. 2011). In the event of a lineage's extinction, these samples allow the population to be revived from a point prior to the extinction and re-evolved in many parallel replicates to determine the frequency with which that lineage's extinction happens again. Microbes have the additional advantages of short generation times and

large population sizes, allowing observation of substantial evolutionary change over tractable time spans (Lenski et al. 1991; Kawecki et al. 2012).

The LTEE consists of 12 independently evolving populations, each of which was founded from one of two *E. coli* strains that differ by a selectively neutral marker (Lenski et al. 1991). Growth in the LTEE is carbon-limited, with glucose as the primary carbon source. The 12 populations had been evolving for more than 50,000 generations at the time of the experiments reported here. In one of the 12 populations (called Ara-3), the novel ability to grow aerobically on citrate evolved after more than 30,000 generations (Blount et al. 2008). The inability to grow aerobically on citrate is one of the identifying traits of *E. coli* (Koser 1924), so the abundant citrate present in the LTEE culture medium was previously unavailable to the bacteria. The population size increased several-fold after citrate consumption evolved (Blount et al. 2008), and the evolution of that new ability substantially altered the ecological conditions of the population (Chapter 1).

The citrate-consuming lineage (Cit^+) did not fix in the population but instead coexisted with a clade that could not grow on citrate (Cit^-). These two clades coexisted in a negative frequency-dependent fashion for more than 10,000 generations. The initial phase of coexistence involved a tradeoff, whereby the Cit^+ cells grew more slowly on glucose than their Cit^- counterparts during part of the daily growth cycle (Blount et al. 2008). In particular, the Cit^+ cells exhibited a longer lag phase prior to commencing growth after the daily transfers into fresh medium. Following the emergence of the Cit^+ lineage, each clade continued to evolve, though with a different suite of mutations accumulating in each (Blount et al. 2012). Notably, the Cit^+ clade evolved a higher mutation rate and accumulated many more mutations than did the Cit^- clade (Blount et al.

2012). The Cit^- cells adapted to the presence of the Cit^+ lineage, at least in part, by evolving the ability to consume additional energy-containing carbon molecules released by the Cit^+ bacteria (Chapter 1). The physiological mechanism for the evolution of citrate consumption in the Cit^+ lineage was a mutation causing aerobic expression of the CitT transporter protein (Blount et al. 2012). CitT is an antiporter that allows the Cit^+ bacteria to import citrate in exchange for various C_4-dicarboxylic acids including succinate, malate, and fumarate (Pos et al. 1998). The Cit^- ecotype evolved improved growth on these C_4-dicarboxylic acids, which enabled the coexistence of the Cit^- and Cit^+ ecotypes (Chapter 1).

Beginning at 44,000 generations, however, we were unable to detect Cit^- cells in the population. Here we document that the Cit^- ecotype went extinct in the population. Prior to that time, the Cit^- lineage was present at frequencies of one or more percent of the total population, which had increased to $>10^8$ cells per mL (and $>10^9$ cells in the 10-mL of medium in the flask) at the end of the daily growth cycle. Even after the 100-fold daily dilution, a lineage that comprised 1% of the total population would include many thousands of cells. Therefore, the extinction of the Cit^- ecotype cannot be explained by demographic stochasticity (i.e., as a statistical fluctuation associated with a simple birth-death process). We therefore hypothesized that the extinction was caused by the on-going evolutionary adaptation of the Cit^+ lineage. Given the larger population size and higher mutation rate of the Cit^+ lineage, such an outcome would not be unexpected. There are multiple ecophysiological scenarios whereby the Cit^+ lineage could have evolved to drive the Cit^- ecotype extinct. If the Cit^+ bacteria had evolved to export fewer or less valuable C_4-dicaboxylate molecules, then this would have reduced the carbon and energy available

to the Cit$^-$ cells. Similarly, if the Cit$^+$ bacteria, or a subpopulation within the Cit$^+$ clade, had evolved improved ability to compete for either the glucose or the C_4-dicaboxylates, then that shift also could have driven the Cit$^-$ ecotype extinct.

Beneficial mutations in the LTEE require hundreds, if not thousands, of generations to achieve fixation, with progressively longer periods required in later generations as the opportunity for further fitness improvement declines (Wiser et al. 2013). Therefore, if the hypothesis that the on-going adaptation of the Cit$^+$ lineage drove the Cit$^-$ lineage extinct was correct, then we would expect the responsible Cit$^+$ mutants to have been well-established numerically (even if still a minority genotype) long before the extinction played out; and we would therefore also expect the extinction to be highly reproducible if we replayed evolution from the sample taken within 500 generations before the extinction of the Cit$^-$ ecotype.

We tested whether the extinction of the Cit$^-$ lineage was deterministic, in the sense of being repeatable going forward from the 43,500-generation sample, by (i) reviving that population sample along with an earlier one from generation 40,000; (ii) propagating 20 replicate populations derived from each of those samples for 500 generations; and (iii) determining whether the Cit$^-$ ecotype was present after each of the 40 total replays. Based on the considerations above, we predicted that the Cit$^-$ type would persist in most or all of the replay populations started from the 40,000-generation sample, whereas the Cit$^-$ lineage would go extinct in most or all of the replays started with the 43,500-generation sample. We also tested whether a Cit$^-$ clone could invade and thereby re-establish its frequency-dependent coexistence with Cit$^+$ clones and populations from after the extinction event.

Methods

Long-term evolution experiment with E. coli

The LTEE is an ongoing experiment in which 12 independent populations of *E. coli* are evolving in a glucose-limited Davis-Mingioli minimal medium, called DM25. Six of the populations were founded from REL606, a clone that cannot grow on arabinose (Ara^-) and six were founded from REL607, a clone that differs from REL606 by a point mutation that confers the ability to grow on arabinose (Ara^+). DM25 does not contain arabinose, and REL606 and REL607 have equal fitness in the LTEE environment. The focal population in this chapter, Ara-3, was founded from REL606 and is Ara^-. In addition to having 25 ml/L glucose, DM25 contains 326.6 mg/L citrate, an additional carbon source that *E. coli* cannot consume under aerobic conditions. However, a lineage in Ara-3 evolved the ability to consume citrate after more than 30,000 generations (Blount et al. 2008). The Cit^+ lineage coexisted with a Cit^- lineage for at least 10,000 generations. Cit^+ and Cit^- clones used in our experiments were identified as described in Blount et al. (2012). More details on the LTEE methods are described in Lenski et al. (1991).

Assays for presence of the Cit^- phenotype and genotype

We tested for the extinction of the Cit^- lineage using two methods. First, we measured the frequency of cells with the Cit^- phenotype in both DM25, the standard culture medium of the LTEE, and DM25 without citrate. We used DM25 without citrate

in addition to the standard culture medium because the proportion of Cit$^-$ cells is higher when populations are grown in medium without citrate, thus increasing the probability of detecting them. We knew from prior experiments that Cit$^-$ cells were present in the population through at least 43,000 generations (Chapter 1), and so we revived frozen population samples at 1,000 generation intervals from 40,000 to 46,000 generations as well as populations from 43,500 and 50,000 generations (listed in Table 2.1). Revived samples were grown overnight in Luria-Bertani medium (LB), then diluted 10,000-fold into DM25 or DM25 without citrate and grown for 24 h. The cultures were then diluted 100-fold into the same two media and grown again for 24 h. We diluted and plated samples from each culture onto tetrazolium arabinose (TA) indicator agar plates (Levin et al. 1977; Lenski et al. 1991). From the TA plates, we tested 64 colonies from each culture for growth on minimal citrate plates (Blount et al. 2008). For any colony that did not grow on minimal citrate, we confirmed the Cit$^-$ phenotype using Christensen's agar (Reddy et al. 2007), which produces a color reaction in response to growth on citrate.

We tested for the presence of the Cit$^-$ genotype in populations from the same time points using the polymerase chain reaction (PCR) to amplify a segment of the genome known to have been deleted in the Cit$^+$ clade (Blount et al. 2012). In addition to serving as a confirmation of the results from the phenotypic assay, the PCR assay also tested the possibilities that (i) the Cit$^-$ phenotype was still present in the population, but had lost the ability to form colonies, and (ii) the Cit$^-$ clade was still present but had evolved the ability to consume citrate.

Generation	ID Number	Description
40,000	REL10969	Ara-3 population
41,000	REL11013	Ara-3 population
42,000	REL11039	Ara-3 population
43,000	REL11079	Ara-3 population
43,500	REL11091	Ara-3 population
44,000	REL11103	Ara-3 population
45,000	REL11151	Ara-3 population
46,000	REL11190	Ara-3 population
50,000	REL11354	Ara-3 population
40,000	REL10979	Cit^+ clone
42,000	CBT7	Cit^+ clone
43,000	CBT10	Cit^+ clone
44,000	CBT13	Cit^+ clone
45,000	REL11161	Cit^+ clone
50,000	REL11364	Cit^+ clone
43,000	ZDB605	Cit^- clone
43,000	CBT28	Ara^+ mutant of ZDB605

Table 2.1: List of the bacteria used in Chapter 2.

Replays of the extinction event

We hypothesized that ongoing adaptation of the Cit^+ lineage caused the extinction of the Cit^- lineage. If this were the case, then the Cit^+ genotypes that drove the Cit^- lineage extinct should already have been present in the population sample collected and frozen within the 500 generations prior to extinction, given the time required for new beneficial mutations to fix in an LTEE population. To test whether adaptation of the Cit^+ lineage had, in fact, caused the Cit^- extinction, we conducted evolutionary replay experiments with the samples from 40,000 and 43,500 generations of population Ara-3. Based on our hypothesis, we expected Cit^- to go extinct in many or all of the replay populations founded from the 43,500-generation sample, but no or few extinctions in replay populations founded from the 40,000-generation sample. We maintained 20 replicate populations started from each sample for 500 generations under the same conditions as the LTEE. Samples from these populations were frozen every 100 generations. We then tested for the presence of the Cit^- lineage in the final, 500-generation samples by the PCR assay.

To test for Cit^- extinction over a longer time period, we also propagated one population revived from the 43,000-generation sample of population Ara-3 for 2,500 generations. Samples of this population were frozen every 500 generations. We tested for the presence of the Cit^- lineage by the PCR assay at each 500-generation interval.

Invasion experiments

If our hypothesis that ongoing adaptation of the Cit$^+$ lineage caused the extinction of the Cit$^-$ lineage were correct, then Cit$^-$ cells should not be able to reinvade the Cit$^+$ lineage after the extinction. We considered two possible cases of adaptation of the Cit$^+$ lineage. In one, the trait that allowed the Cit$^+$ lineage to exclude the Cit$^-$ lineage spread to fixation or near fixation in the Cit$^+$ clade. In that case, we would expect that a Cit$^-$ invader could not reinvade cultures of either individual Cit$^+$ clones or entire Cit$^+$ populations from after the extinction event. Alternatively, the Cit$^+$ trait that led to exclusion of Cit$^-$ might have spread only into a subpopulation of the Cit$^+$ clade, perhaps because the fitness benefit of the trait was frequency dependent or perhaps because its benefit was small and therefore slow to spread. If this were the case, then we would expect that a Cit$^-$ invader could reinvade cultures of at least some Cit$^+$ clones, but not Cit$^+$ populations from after the extinction event.

To test whether the extinction of the Cit$^-$ lineage was caused by changes in the Cit$^+$ lineage that eliminated the Cit$^-$ ecotype's advantage when rare, we tracked whether a Cit$^-$ clone could become established when introduced at low and high frequencies to cultures of Cit$^+$ bacteria after the extinction event. To monitor the abundance of the Cit$^-$ clone in the population, we used a selectively neutral Ara$^+$ mutant of a Cit$^-$ clone from 43,000 generations as the invading strain. Because the Ara-3 population is Ara$^-$, the Cit$^+$ bacteria cannot grow on citrate-free minimal arabinose plates (same recipe as minimal citrate, but with 4 g/L L-arabinose in place of citrate), allowing us to track the population size of the initially rare Cit$^-$ clone.

The invading Cit$^-$ clone was added to three replicate cultures of Cit$^+$ clones from 40,000, 42,000, 43,000, 44,000, 45,000 and 50,000 generations at initial densities of ~3 x 10^3 (low) and ~3 x 10^8 cells/mL (high). We included time points before the extinction as controls, because we expected that the Cit$^-$ clone could invade those Cit$^+$ clones. In addition, we added the same Cit$^-$ strain to whole-population samples from generations 44,000, 45,000, and 50,000. We did not include whole-population samples from time points before the extinction, because the Cit$^-$ cells already present in those samples would confound the assay. In all cases, the clones and populations were revived from frozen stocks and acclimated in DM25 for two days prior to the start of the experiment, as described for the experiments above. Each day for 7 days, we transferred the cultures via 1:100 dilutions and maintained them under the same conditions as the LTEE. Each day we measured the Cit$^-$ population size by plating on citrate-free minimal arabinose agar. All reported cell densities are based on colony-forming units.

Results

Assays for presence of the Cit$^-$ genotype and phenotype

The PCR assay for the presence of the Cit$^-$ genotype produced clear bands at all time points up to and including 43,500 generations (Fig 2.1). At 44,000 generations and beyond, we did not observe any amplification. Examination of phenotypes also indicated that the Cit$^-$ lineage went extinct between 43,500 and 44,000 generations. Cit$^-$ cells were present through 43,500 generations in all cultures, but they were not detected at any later time point. Prior to the 44,000-generation sample, on average 4 of the tested 64 colonies

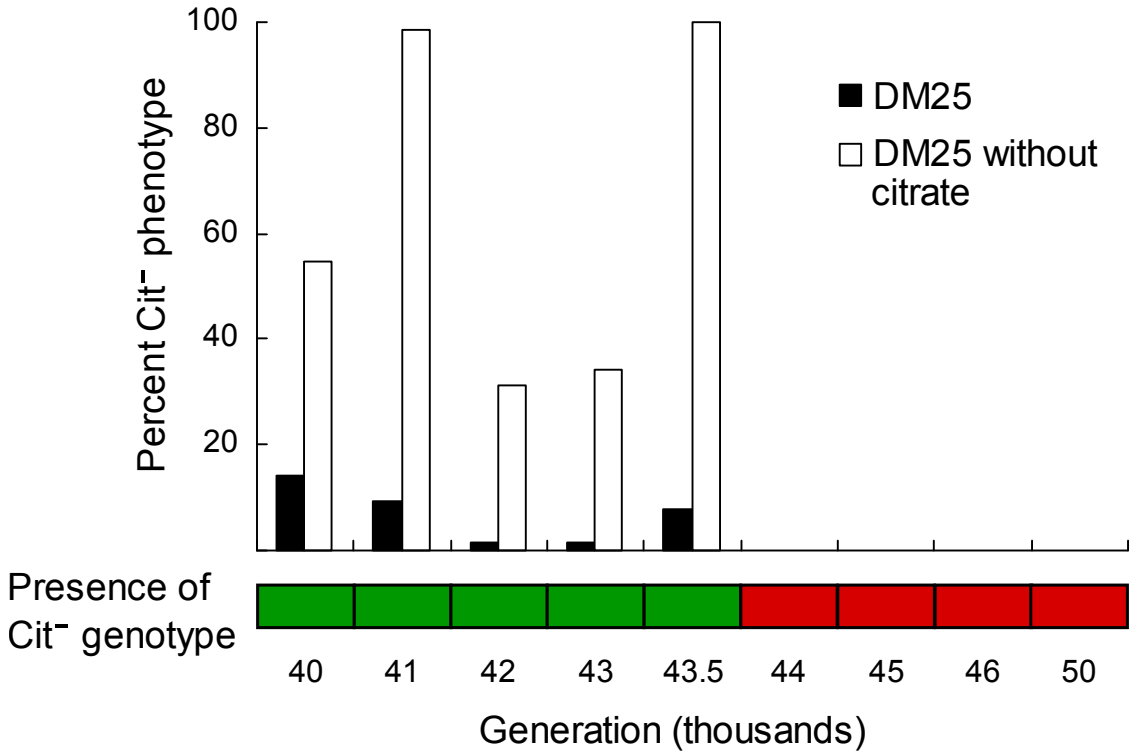

Figure 2.1: Recovery of Cit⁻ bacteria by generation from the Ara-3 population of the LTEE. The bar graph shows the percentage of clones with a Cit⁻ phenotype recovered in two media, DM25 and DM25 without citrate. The lower grid indicates the presence (green) or absence (red) of the Cit⁻ genotype, as determined by a PCR assay. Taken together, these data show that the Cit⁻ lineage went extinct between 43,500 and 44,000 generations.

(~6%) had the Cit$^-$ phenotype when cultures were grown in DM25 medium (Fig 2.1). Note that this proportion does not necessarily represent the proportion of Cit$^-$ bacteria in the population because plating efficiency (the proportion of cells that form colonies) might vary between lineages and over time. We also tested the Cit$^+$/Cit$^-$ phenotype of colonies produced by cells from cultures that were grown in DM25 without citrate, a medium that selects for Cit$^-$ bacteria in the population. In DM25 without citrate, 41 of 64 tested colonies (~64%) had the Cit$^-$ phenotype, on average, prior to the extinction. There was no indication of a downward trend in the proportion of Cit$^-$ bacteria in the generations before the extinction.

Replays of the extinction event

Twenty replicate populations founded from the 43,500-generation population sample evolved for 500 generations under the standard LTEE conditions. PCR assays showed that the Cit$^-$ clade persisted in all 20 cases through the end of the experiment. The Cit$^-$ lineage also persisted in all 20 of the control populations founded from the 40,000-generation population sample as well as in the population founded from the 43,000-generation population sample that evolved for 2,500 generations.

Invasion experiments

When a 43,000-generation Cit$^-$ strain was introduced at a low density (~3 x 10^3 cells per mL) to a culture containing a single Cit$^+$ genotype from several time points before and after the extinction, the population of the Cit$^-$ clone increased by about 1000-fold, reaching an average density of ~4 x 10^6 cells per mL after 7 days (Fig 2.2A). When

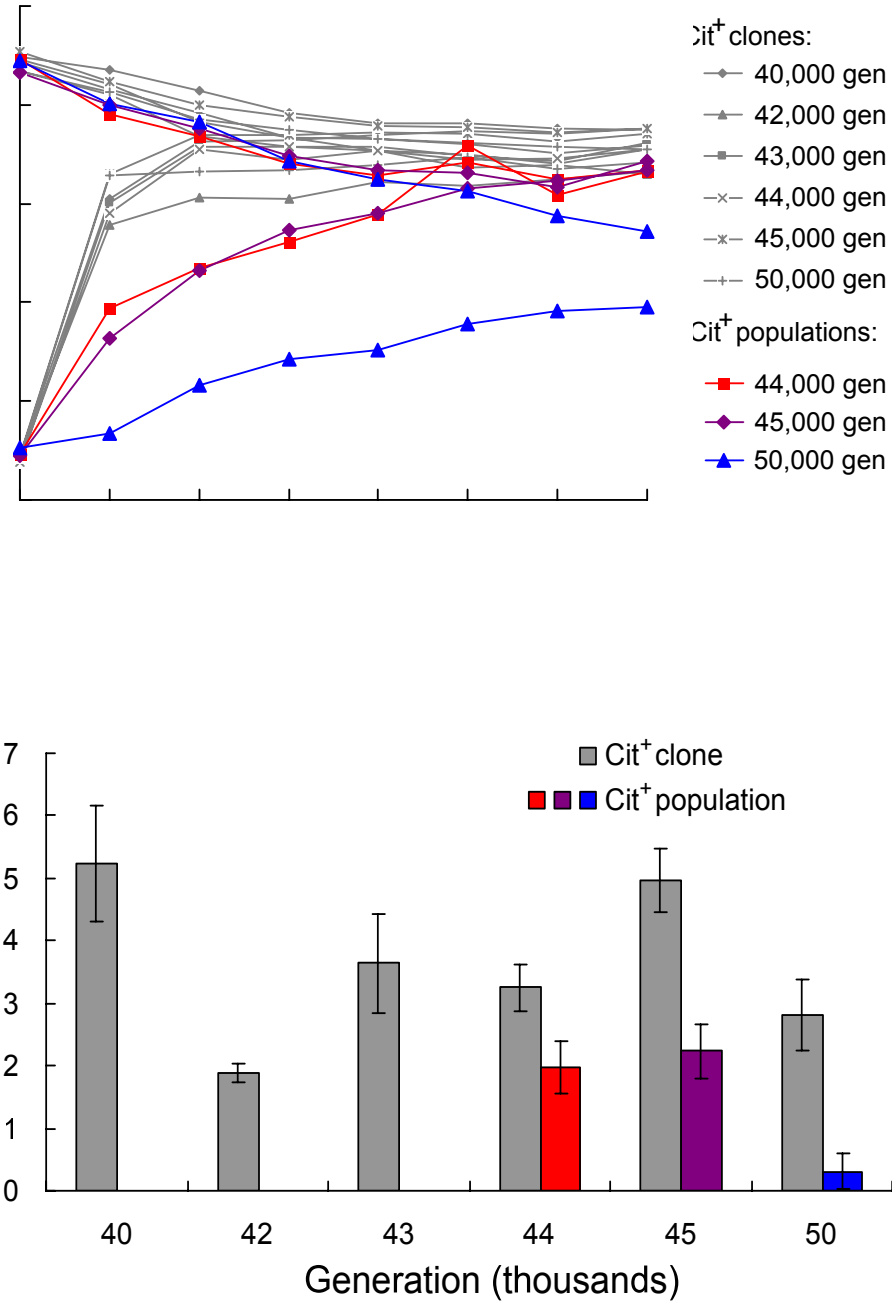

Figure 2.2: A Cit$^-$ clone can reinvade the Ara-3 population after the extinction event. A. Trajectory of the mean population density of the Cit$^-$ clone over 7 days, starting from both high and low initial densities and introduced into Cit$^+$ clones or populations as indicated in the legend at right. Error bars are omitted for clarity. B. Mean density of the Cit$^-$ clone on day 7 of the invasion experiments, including both high and low initial density treatments. The generation of the resident Cit$^+$ clone (gray bars) or population (colored bars) is shown along the x-axis. Error bars are 95% confidence intervals.

the same Cit$^-$ clone was added at a high density (~3 x 10^7 cells per mL), the Cit$^-$ population declined to the same average population size of ~4 x 10^6 cells per mL on day 7. These results indicate that Cit$^-$ and Cit$^+$ clones can coexist owing to a negative frequency-dependent interaction, regardless of whether the Cit$^+$ clone was isolated before or after the extinction of the Cit$^-$ lineage.

We also observed invasions and coexistence mediated by negative frequency-dependent selection when the same Cit$^-$ clone was added to cultures containing the complete mixture of Cit$^+$ genotypes present in the population at 44,000, 45,000, and 50,000 generations. When using the 44,000- and 45,000-generation populations, the final density of the Cit$^-$ clone was within the general range observed when it invaded the Cit$^+$ clones, although its initial rate of increase when rare was noticeably slower (Fig 2.2A). The equilibrium density of the Cit$^-$ clone was also slightly lower (Fig 2.2B) when it invaded the entire population than when it invaded a clone from the same generation. When added to the 50,000-generation population—more than 6,000 generations after the extinction of the Cit$^-$ lineage—the 43,000-generation Cit$^-$ clone could still invade the Cit$^+$ population (Fig 2.2A). However, the final Cit$^-$ population size was an order of magnitude lower (3 x 10^5 cells per mL, mean of high and low initial density treatments) than the final density of Cit$^-$ cells in any other treatment (Fig 2.2B).

Discussion

Three lines of evidence indicate that the Cit$^-$ ecotype, which had coexisted with the Cit$^+$ ecotype for more than 10,000 generations, went extinct between 43,500 and

44,000 generations. First, no clones with the Cit$^-$ phenotype were isolated from 44,000 generations or later (Fig 2.1), indicating that the Cit$^-$ phenotype was absent from (or at least much rarer in) the population. Second, PCR assays also failed to amplify a Cit$^-$ lineage-specific locus from population samples collected after 43,500 generations. Third, if the Cit$^-$ ecotype remained in the population, then the Cit$^-$ ecological niche would be occupied and the Cit$^-$ bacteria would be at a frequency-dependent equilibrium with Cit$^+$. In this case, a Cit$^-$ strain from an earlier generation, when added at low density, should not be able to invade the population. In contrast to this prediction, however, a Cit$^-$ clone from 43,000 generations reinvaded the Ara-3 populations at multiple time points tested after the Cit$^-$ extinction, including even 6,000 generations later (Fig 2.2).

The ability of a Cit$^-$ clone to reinvade the population long after the Cit$^-$ ecotype went extinct also implied that, contrary to our predictions, the Cit$^-$ extinction was not caused by the Cit$^+$ bacteria evolving to exclude the Cit$^-$ ecotype from the population. Our evolution replay experiments also supported this result. The Cit$^-$ lineage persisted for 500 generations in all 20 replicate populations founded from the last population sample prior to the Cit$^-$ extinction, as well as in 20 replicate populations started from an earlier point. Moreover, in an additional population that was founded from the 43,000-generation population, the Cit$^-$ lineage has continued to coexist with the Cit$^+$ ecotype through at least generation 45,500, which is more than 1,500 generations after the Cit$^-$ lineage went extinct. The Cit$^-$ extinction in Ara-3 therefore appears to be a rare occurrence, and not a repeatable outcome of competitive exclusion by Cit$^+$.

As explained in the Introduction, we expected that, if the extinction of the Cit$^-$ lineage had been driven by on-going adaptive evolution of the Cit$^+$ lineage, then that

adaptation would be captured in the population sample collected immediately prior to extinction. However, our results also eliminate the possibility that the extinction of the Cit^- lineage was caused by some rare, highly beneficial mutation that arose in the Cit^+ lineage at some point after the prior population sample and spread quickly through the population. The presence of such a mutation in the Cit^+ population would have prevented the reinvasion of the population by Cit^- cells in subsequent generations.

Thus, the cause of the Cit^- extinction remains unknown. We have rejected our hypothesis that evolutionary changes in the Cit^+ bacteria drove the extinction. Moreover, a purely stochastic extinction of the Cit^- lineage is also extremely unlikely. The LTEE is propagated by 1:100 daily transfers, and the total number of Cit^- cells in the culture prior to transfer (i.e., after 24 h) was consistently measured at $>10^7$ prior to their extinction. Therefore, the probability that no Cit^- cells would be present in the population after a transfer, given that each cell has a 99% chance of not being transferred, is less than $0.99^{10,000,000} \approx 10^{-43,700}$. Even if some perturbation were to cause a moderate decline in the Cit^- population size, its density should then have tended to increase back towards its equilibrium owing to the negative frequency-dependent relationship between the Cit^+ and Cit^- ecotypes (Blount et al. 2008; Turner et al. *In review*), making a stochastic extinction even more unlikely.

In populations large enough that demographic stochasticity is unlikely to cause extinction, the most likely causes of random extinction are environmental stochasticity and random catastrophes (Lande 1993). Environmental stochasticity refers to changes in population size caused by environmental variation over time, which should be minimal in the context of a controlled laboratory experiment like the LTEE. Furthermore, the large

size of the Cit$^-$ population should have made it robust to environmental stochasticity caused by the typical fluctuations that simply cannot be avoided in any experimental setting. We propose, therefore, that the most likely cause of the Cit$^-$ extinction was a "random catastrophe", that is a large, rare event that reduced the size of the Ara-3 population as a whole or, at least, the Cit$^-$ lineage within it. We do not know the nature of this random catastrophe, but it could have been some unusual variation in laboratory conditions, such as soap residue in a flask or problems with the deionized water used to make the culture medium. Although the Cit$^-$ population was too large for purely stochastic extinction from demographic fluctuations, its population size was substantially smaller than that of the Cit$^+$ population, making the Cit$^-$ lineage more vulnerable to such occurrences (Spiller et al. 1998; Schoener et al. 2001).

In addition to demonstrating that the extinction of the Cit$^-$ population was not a deterministic event, we also found evidence that there has been ecological diversification in the Cit$^+$ clade. The equilibrium Cit$^-$ population density was significantly lower when it was grown with the whole Cit$^+$ population than when it was grown with a single Cit$^+$ clone at each time point tested. This result suggests that some Cit$^+$ genotypes in the population were better at competing with the Cit$^-$ invader for shared resources than were the particular Cit$^+$ clones tested individually at those time points. The effect of this diversity was particularly pronounced at 50,000 generations, where the equilibrium population size of the Cit$^-$ cells when grown together with the Cit$^+$ population was nearly an order of magnitude lower than in earlier generations (Fig 2.2). This decline in the reinvasion ability of the Cit$^-$ clone indicates that, by 50,000 generations, a subpopulation of Cit$^+$ bacteria had evolved improvements that exploited or otherwise impacted the niche

previously occupied by the Cit$^-$ bacteria. Thus, rather than the Cit$^-$ lineage having been driven extinct by the Cit$^+$ bacteria evolving into its niche, it instead appears that the extinction of the Cit$^-$ lineage opened a niche that a Cit$^+$ subpopulation has begun to exploit.

The occurrence of this extinction during an evolution experiment allowed us to test the hypothesis that the extinction was caused by the evolution of other organisms, something that would not be possible to test in natural systems. Having found that the extinction of the Cit$^-$ lineage was likely caused by a rare environmental perturbation—a random catastrophe, as it were—we can take advantage of another feature of experimental evolution. In particular, we can now study, going forward, the consequences of that extinction event by following two 'alternative histories' of evolution, one with and one without the extinction. To that end, we are continuing to propagate the population that was founded from a sample taken before the Cit$^-$ extinction and evolved for 2,500 generations. We have designated it Ara-7, and it is the thirteenth population in the LTEE, although it is well behind the other populations in terms of its total elapsed generations owing to the fact that it was only re-started from the 43,000-generation sample of Ara-3 when that population was at generation 56,500. In the future, Ara-3 and Ara-7 together will allow us to compare the outcomes in these two populations, which differ in the occurrence of the extinction of the Cit$^-$ bacteria. Will the continued presence of the Cit$^-$ cells in Ara-7 prevent members of the Cit$^+$ clade from diversifying and occupying the Cit$^-$ niche, as the Cit$^+$ clade appears to be doing in Ara-3? Will the Cit$^+$ bacteria in Ara-3 eventually evolve to occupy fully the Cit$^-$ niche, such that pre-extinction members of the Cit$^-$ clade can no longer re-invade the Cit$^+$ population?

Might a new clade with a Cit^- phenotype emerge from within the Cit^+ clade in Ara-3? All this remains to be seen.

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CHAPTER 3:

EVOLUTION OF ORGANISMAL STOICHIOMETRY IN A 50,000-GENERATION EXPERIMENT WITH *ESCHERICHIA COLI*

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Abstract

Organismal stoichiometry refers to the relative proportion of chemical elements in the biomass of organisms, and it can have important effects on ecological interactions from population to ecosystem scales. Although stoichiometry has been studied extensively from an ecological perspective, little is known about rates and directions of evolutionary changes in elemental composition in response to nutrient limitation. We measured carbon, nitrogen, and phosphorus content of *Escherichia coli* evolved under controlled carbon-limited conditions for 50,000 generations. The bacteria evolved higher relative nitrogen and phosphorus content, consistent with selection for increased use of the more abundant elements. Total carbon assimilated also increased, indicating more efficient use of the limiting element. Altogether, our study shows that stoichiometry evolved over a relatively short time-period, and that it did so in a predictable direction given the carbon-limiting environment.

Introduction

At the coarsest level, organisms consist of a mixture of elements in varying proportions. Common patterns in the ratios of elements are seen across life on Earth: all

life requires large quantities of carbon, hydrogen, oxygen, nitrogen and phosphorus, and all life requires more carbon than nitrogen and more nitrogen than phosphorus. Because the cycling of carbon, nitrogen and phosphorus in ecosystems is driven in large part by biological processes, these elements tend to be the main focus of ecological stoichiometry (Sterner and Elser 2002). While all life shares broadly similar elemental profiles, the ratios vary substantially both inter- and intra-specifically (Vanni et al. 2002; Klausmeier et al. 2004; Bertram et al. 2008; Zimmerman et al. 2014). Over several decades, the field of ecological stoichiometry has established that stoichiometric variation has important ecological consequences. However, much less is known about the evolutionary origins of variation in organismal stoichiometry. It is not known whether organismal stoichiometry is a labile trait that can evolve fairly quickly when conditions change or if it is more constrained. Addressing this lack of knowledge is important because evolved changes in organismal stoichiometry might alter ecosystem processes (Elser 2006a) as well as shape responses to anthropogenic changes (Munday et al. 2013). In this paper, we present evidence from a laboratory experiment that changes in stoichiometry can evolve over an observable timescale and in predictable directions.

Variation in organismal stoichiometry affects many different levels of biological organization. At the physiological level, the growth-rate hypothesis predicts a correlation between the growth rate of individual organisms and their phosphorus content, driven by the greater need for phosphorus-rich ribosomes in rapidly growing organisms (Sterner and Elser 2002; Elser et al. 2003). Many groups of organisms exhibit this correlation, including zooplankton (Acharya et al. 2004), insects (Elser et al. 2006; González et al. 2014), phytoplankton (Hillebrand et al. 2013), and heterotrophic bacteria (Makino et al.

2003; Makino and Cotner 2004; Chrzanowski and Grover 2008). Differences in stoichiometry among organisms can also affect community and ecosystem processes such as predator-prey interactions (Meunier et al. 2012), competitive interactions (Gurung et al. 1999), and nutrient cycling (Elser et al. 1998; Vanni et al. 2002).

The relative availability of elements in the environment plays an important role in organismal stoichiometry. As a particular element becomes increasingly scarce, the proportion of that element in biomass generally declines, although the degree of physiological plasticity varies among organisms and across elements (Sterner and Elser 2002). However, the effect of elemental abundance on the evolution of organismal stoichiometry is not well established, however. All else being equal, one would expect selection for efficient nutrient use or "sparing", such that the proportion of a scarce element in biomass would decrease over evolutionary time because selection favors organisms that require less of that element. However, the proportion of a scarce element in biomass might instead increase over evolutionary time. The adaptive value of that element for performing important functions could outweigh selection for nutrient sparing. Also, the evolution of improved mechanisms to acquire a scarce element could alleviate the effects of its scarcity. Because organisms often exhibit plasticity in response to nutrient scarcity, the improved uptake of a scarce element could lead to a higher proportion of that element in an organism's biomass (Bragg and Wagner 2007).

Current evidence in support of the evolution of nutrient sparing is mixed. Several comparative studies of the elemental composition of proteins support the nutrient-sparing hypothesis. For example, in both *Escherichia coli* and yeast, proteins involved in the acquisition of nitrogen, phosphorus and sulfur each have a lower content of the element

they acquire, suggesting that selection acted to reduce the nutrient content of proteins expressed under nutrient scarcity (Baudouin-Cornu et al. 2001). Terrestrial plants, whose growth is frequently limited by nitrogen, exhibit evidence of nitrogen sparing in their genomes and proteomes (Elser 2006b; Acquisti et al. 2009; but see Günther et al. 2013). Additionally, a recent study of a *Daphnia pulicaria* population, based on rearing individuals from an egg bank that had been deposited over many centuries in lake sediments, found that relaxation of phosphorus limitation in the lake led to reduced phosphorus-use efficiency in the *Daphnia* (Frisch et al. 2014). However, contrary to the predictions of the nutrient-sparing hypothesis, Bragg and Wagner (2007) found that proteins whose expression decreased in yeast experimentally evolved under carbon limitation were disproportionately carbon poor compared to the rest of the proteome. They suggested that the evolution of improved uptake of carbon might have alleviated the degree of carbon limitation, thereby allowing greater expression of carbon-rich proteins. The experiments analyzed by Bragg and Wagner (2007) ran for only a few hundred generations, so their results might reflect a short-term process whereas nutrient sparing might dominate over the long term.

The potential for evolved changes in stoichiometry has important implications for understanding how organisms will respond to anthropogenic change. The phytoplankton of the world's oceans provide a critical ecosystem service by taking up atmospheric carbon dioxide. Half of all carbon removal from the atmosphere is performed by oceanic phytoplankton (Field et al. 1998), and some of this carbon is then exported to the ocean sediments, which act as a long-term carbon sink (Longhurst and Harrison 1989; Richardson and Jackson 2007). In order to make predictions about future climate change,

it is therefore important to understand how phytoplankton growth will be affected by changing environmental conditions. A number of models (Quere et al. 2005; Follows et al. 2007) make such predictions based on our knowledge of phytoplankton ecology, but none of them incorporate evolutionary changes (Munday et al. 2013). Increases in sea-surface temperatures have already led to declines in phytoplankton biomass, associated with more severe nutrient limitation (Boyce et al. 2010). One process that might mitigate the effect of declining nutrient availability is evolution of changes in the stoichiometric ratios of elements in phytoplankton biomass. Evolution of stoichiometry could also affect the consequences of anthropogenic increases in nutrient availability in other ecosystems, such as those caused by runoff from agricultural systems into aquatic environments.

Many questions need to be answered before we can predict how stoichiometry might evolve in response to global change. Over what time spans can it evolve and at what rate? Does selection generally favor the reduced use of scarce resources? Is the proportional use of some elements more evolutionarily flexible than others? We can begin to address these questions using experimental evolution, in which organisms are allowed to evolve under controlled conditions in the laboratory. Microbes are valuable subjects for experimental evolution because they can evolve quickly due to their large population sizes and short generation times (Lenski et al. 1991). An additional benefit is that many microbes can be frozen throughout the course of the experiment and later revived for analysis. This feature allows ancestral and evolved organisms to be compared directly, under the same conditions in which they evolved.

To examine the potential for evolutionary change in organismal stoichiometry, we compared the carbon, nitrogen and phosphorus content of biomass in ancestral and

evolved *E. coli* cells from a 50,000-generation laboratory evolution experiment (Lenski et al. 1991; Wiser et al. 2013). We predicted that the evolved bacteria would have lower carbon content, relative to nitrogen and phosphorus, compared to the ancestral strain for two reasons. First, the evolving bacteria have been maintained under carbon limitation in a medium that contains high concentrations of nitrogen and phosphorus (Lenski et al. 1991). All else being equal, we expected that such conditions should favor selection for reduced use of carbon in biomass, as predicted by the nutrient-sparing hypothesis. Similarly, the high concentrations of nitrogen and phosphorus should release the bacteria from any prior selection for reduced use of those elements. Second, the bacteria undergo daily batch transfer to fresh medium, a condition that selects for faster growth rates (Vasi et al. 1994). According to the growth-rate hypothesis (Sterner and Elser 2002), this regime should also select for an increase in the relative proportion of phosphorus in the bacterial biomass.

Methods

Long-term evolution experiment

The long-term evolution experiment (LTEE) was founded with *E. coli* B strain REL606 (Lenski et al. 1991; Jeong et al. 2009). Six of the 12 populations started directly from REL606; The other six populations began with a mutant clone, REL607, that differed by a neutral marker. The populations have been maintained in Davis-Mingioli minimal medium supplemented with 25 mg glucose and 2 mg thiamine per liter (DM25). The bacteria can use both glucose and thiamine as a carbon sources, but not citrate

(another component of DM25 that the ancestral strain and most evolved populations cannot use). Given the glucose and thiamine in DM25, the molar ratios of C:N:P available to the bacteria are 1:17:50. For the one population, designated Ara-3, that evolved the ability to consume citrate (Blount et al. 2008; Blount et al. 2012), the available C:N:P ratios are 1.1:1:3. Each population is serially propagated by a 1:100 dilution into fresh medium each day; it then grows 100-fold until the available carbon is depleted, and it thus goes through ~6.6 generations per day. Samples of each population are frozen every 500 generations. Portions of these frozen cultures can be revived, allowing direct comparison of ancestral and evolved bacteria. Lenski et al. (1991) provide more details about the methods used in the LTEE.

Sample collection for stoichiometric analyses

We used a paired sampling design in which a clone isolated from each of the 12 populations at 50,000 generations was paired with its ancestral clone, either REL606 or REL607. Each clone was revived from a frozen stock and conditioned for one day in Luria-Bertani medium. After an additional day of conditioning in DM25, each clone was transferred, via a 1:100 dilution, to 500 mL of DM25. After 24 h, each culture was then split into two 250-mL aliquots, each of which was filtered through a pre-combusted glass-fiber filter. In the case of population Ara-3, which evolved the ability to consume citrate, we filtered only 125 mL due to the increased density of the population. Each filter was rinsed by filtering an additional 250 mL of 8.5% sterile saline solution made with distilled deionized water in order to remove dissolved nutrients present in the medium from the filter. The dry mass of each filter was measured before and after filtering. For

each culture, one filter was analyzed for phosphorus content and the other for both carbon and nitrogen content.

We performed sample collection on four different dates with six cultures (three pairs of ancestral and evolved clones) filtered on each day. Pairs of ancestral and evolved clones were assigned to sampling dates randomly. On each day, 250 mL of sterile DM25 was filtered through each of two filters and processed identically to all other samples. These filters served as blanks in subsequent analyses.

To confirm that glass-fiber filters effectively collected both evolved and ancestral bacteria, we compared the number of colony-forming units in filtered and unfiltered medium. The filters retained more than 99% of both ancestral and evolved bacteria.

Elemental analysis

Elemental content was measured by the Nutrient Analytical Services Laboratory at the University of Maryland Center for Environmental Science. Phosphorus mass was determined via extraction to dissolved phosphate followed by molybdenum blue colorimetric analysis (Aspila et al. 1976) using an Aquakem 250 photometric analyzer. Carbon and nitrogen content were measured by elemental analysis (U.S. EPA 1997) using an Exeter Analytical Model CE-440 Elemental Analyzer.

Nucleic-acid content

The total nucleic-acid content of ancestral and evolved clones in stationary phase was measured after 24 h in DM25. The bacteria were revived from frozen stocks and

conditioned as described above. Cells were then lysed by heating, and their nucleic-acid concentration was measured in duplicate using the Quant-iT RiboGreen assay kit.

Data analyses

All values for biomass, carbon, nitrogen and phosphorus were corrected for background levels by subtracting the corresponding value measured from a blank filter on the same day. Background levels were always small (< 15%) relative to the measured value. Biomass was calculated as the change in dry mass of the filter before and after filtering. The percentages of carbon, nitrogen and phosphorus were calculated as the mass of each element divided by the total biomass. All nutrient ratios were calculated as molar ratios.

Two-tailed paired *t*-tests were used to test for differences between evolved and ancestral biomass, nucleic-acid content, percentages of C, N and P, and molar C:N, C:P and N:P ratios. Statistical analyses were conducted using JMP 9.0 software.

Results

Carbon, nitrogen and phosphorus content

We observed substantial differences in the ratios of carbon, nitrogen and phosphorus between the ancestral and evolved clones (Fig. 3.1). Among the evolved clones, however, one had distinctly different C:N and C:P ratios compared to the other eleven, and that one was from the population that evolved the ability to consume the citrate present in the medium. As explained in the Methods, this population subsequently

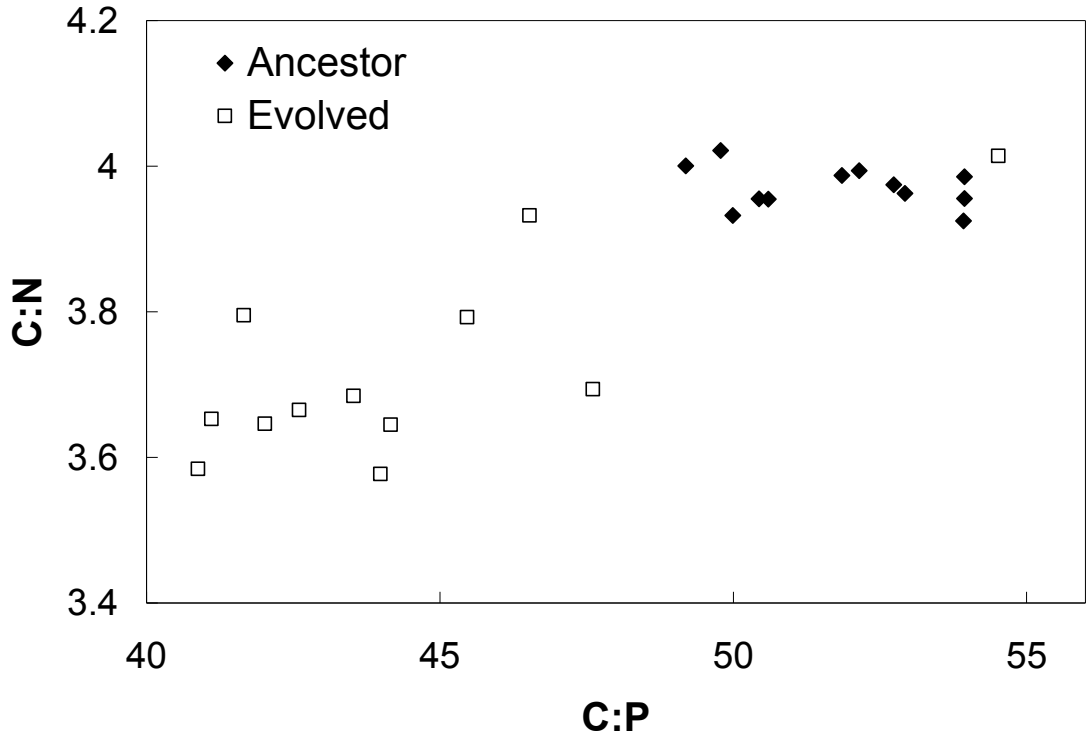

Figure 3.1: The molar C:N and C:P ratios of the ancestral (filled diamonds) and 50,000-generation evolved (open squares) clones from the long-term evolution experiment with *E. coli*. The evolved clone at the far upper right is from the population that evolved the ability to consume citrate, and it was excluded from the statistical analysis owing to the much higher carbon availability that it experienced.

experienced conditions with much higher carbon availability than the other populations. The sample from this population reached a biomass concentration of 108.7 mg/L at 24 h, whereas the samples from the other populations had biomass concentrations between 10 and 18 mg/L. The citrate-consuming clone also had the highest measured C:N and C:P ratios of any of the evolved clones, with ratios as high as or higher than any samples of the ancestral clones. Given the distinctive growing conditions experienced by this clone, we excluded it from our statistical analyses. (Note, however, that inclusion of the citrate consuming clone does not affect whether the comparisons reported below are statistically significant at the 0.05 level, with two exceptions: the N:P ratio would no longer differ between the ancestral and evolved clones, while their nucleic-acid content per biomass would differ.)

The average molar C:N and C:P ratios both decreased significantly in the evolved clones compared to their ancestors (Fig. 3.1, paired, two-tailed *t*-tests, C:N $P < .001$, C:P $P < .001$). Also, the average molar N:P ratio declined from 13.1 to 11.8 ($P < .001$). These changes in C:N and C:P ratios were driven by increases in the percentages of both nitrogen and phosphorus in the evolved clones. The percentage of biomass comprised of nitrogen atoms increased from 11.1% to 12.0% ($P = .012$), and that of phosphorus increased from 1.9% to 2.3% ($P < .001$). The percentage of carbon did not significantly change (Fig. 3.2A, $P = .888$).

The significant increases in the percentages of both nitrogen and phosphorus in the evolved clones' biomass implies that the proportion of one or more other elements must have declined. One possibility is a decline in the percentage of some unmeasured element, such as hydrogen, oxygen, sulfur or potassium. The other possibility is that the

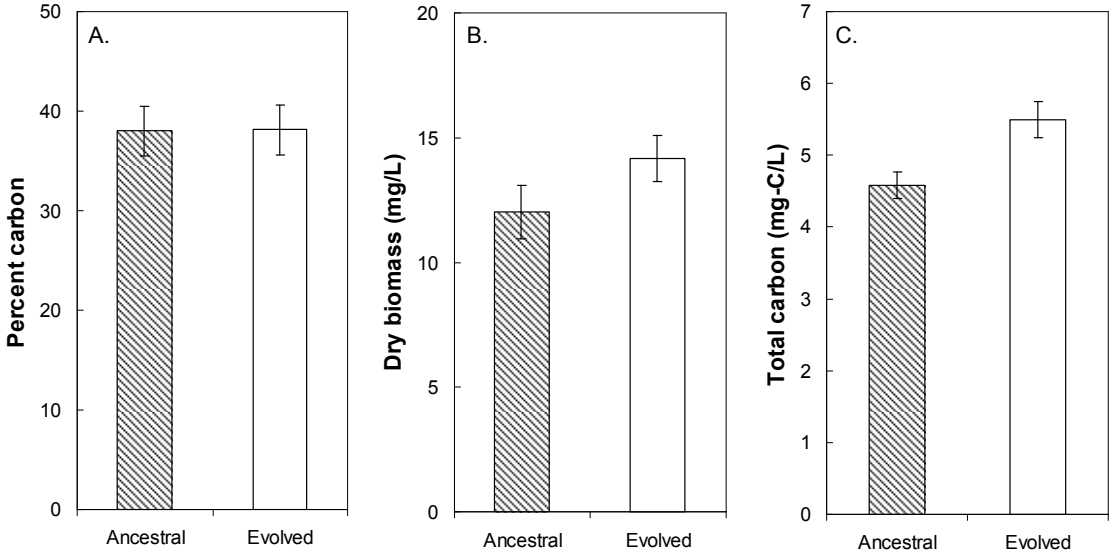

Figure 3.2: A. The percent carbon in cellular biomass did not differ between ancestral and evolved clones ($P = 0.888$). B. Total dry biomass was significantly higher in the evolved clones than in the ancestral clones ($P < 0.001$). C. The total carbon retained in biomass was also significantly higher in the evolved than the ancestral clones ($P < 0.001$). All data shown are means with 95% confidence intervals.

carbon content declined slightly, but that the decline was not detected. An evolved carbon content of 36.7% would be sufficient to offset the combined 1.3% increase in the nitrogen and phosphorus content; that level is well within the 95% confidence interval of 35.6 to 40.6% for the carbon content of the evolved bacteria.

Biomass and carbon retention

The total bacterial biomass concentration increased by 17.7%, from 12.3 to 14.5 mg/L, in the evolved bacteria (Fig. 3.2B, $P < 0.001$). Although the percentage of carbon in the biomass did not change significantly, the total amount of carbon retained increased by 17.8%, from 386 to 455 µmol C/L (Fig. 3.2C, $P < .001$), thus mirroring the increase in total biomass.

Nucleic-acid content

The nucleic-acid content per culture volume increased 23.6%, from 344 to 425 µg/mL ($P < .001$), in the evolved bacteria. However, most of this change reflected the increase in overall biomass. The proportion of nucleic acid in the biomass averaged 28.3 µg-nucleic acid/mg-biomass in the ancestral clones and 29.6 µg-nucleic acid/mg-biomass in the evolved clones. This difference was not significant ($P = .116$), although the trend was in the predicted direction. Given that nucleic acids are about 8.7% P (Sterner and Elser 2002), then the change in nucleic-acid content could account for at most a 0.1 ug-p/mg-biomass increase in phosphorus content, which constitutes only ~3% of the observed 3.6 ug-p/mg-biomass increase in phosphorus content per biomass.

Discussion

Our results demonstrate that organismal stoichiometry can evolve quickly, at least in microorganisms, with changes observed over an experimental time span, albeit a long one that has run for decades (Fig. 3.1). To our knowledge, this is the first experimental observation of evolutionary changes in the overall stoichiometry of organisms, rather than inferred from observational evidence. The average C:P ratio decreased by 14% and the average C:N ratio declined by 6% during the evolution experiment. These changes were evolved, heritable changes that occurred in addition to any plastic physiological response. Previous work has shown that *E. coli* cells also exhibit a plastic response to variation in nutrient supply, with their C:P ratio decreasing ~25% in response to declines in the C:P supply ratio, while the C:N supply ratio was held constant (Makino et al. 2003, C:N did not vary). Overall, these results indicate that evolutionary changes, even over an experimental time scale, can add to plastic physiological responses and further alter organismal stoichiometry. Moreover, these evolutionary changes can be of comparable magnitude to the plastic responses.

As predicted by the nutrient-sparing hypothesis, we observed significant declines in both the C:N and C:P ratios in biomass. These changes are consistent with selection for decreased carbon use relative to the use of nitrogen and phosphorus. However, we cannot distinguish the direct effect of selection due to carbon limitation from the indirect effects of selection favoring other traits in the evolution experiment. For example, some portion of the stoichiometric changes that we observed could be correlated responses to selection

for larger cell size (Mongold and Lenski 1996), faster growth rate (Vasi et al. 1994), or other traits.

The uniquely high C:N and C:P ratios of the clone from the citrate-consuming lineage, which had access to 10 times as much carbon as any other population (Blount et al. 2008), provides further evidence that the declines in the C:N and C:P ratios in the other populations were beneficial specifically under the very low C:N and C:P supply ratios of the LTEE. However, the higher relative carbon content of the citrate-consuming clone is not necessarily an evolutionary response; its higher carbon content might also be, in whole or in part, a plastic physiological response to the higher carbon availability that it experiences in the experimental medium of the LTEE.

The proportion of cellular phosphorus contained in nucleic acids was much lower in our study than in previous experiments with *E. coli* (Makino et al. 2003). Our measurements were made during stationary phase, when carbon has been exhausted, rather than during exponential growth. Both DNA and RNA levels are closely tied to growth rate in bacteria, because the number of genome copies and transcription rate are higher in growing cells (Schaechter et al. 1958; Akerlund et al. 1995). Therefore, it is not surprising that nucleic-acid content would be quite low during stationary phase. The low nucleic-acid content, together with the lack of any significant change in the nucleic-acid content per biomass, indicates that the overall increase in the percentage of phosphorus in evolved cells reflects changes in cellular components other than nucleic acids. Based on the growth-rate hypothesis, we expected to see higher nucleic-acid content in the evolved cells because they exhibit a shorter lag time and faster growth rate upon exiting stationary

phase (Vasi et al. 1994). However, our results are not a direct test of the growth-rate hypothesis, which focuses on growing organisms, rather than ones in stationary phase.

Given the carbon-limited conditions of the LTEE, one might reasonably expect that the strongest selection would be to reduce the proportion of carbon in the bacterial biomass. Therefore, it is notable that increases in nitrogen and phosphorus content drove the changes in the C:N and C:P ratios, while there was no significant change in the percentage of carbon in the biomass. Taken at face value, this finding suggests that the proportion of carbon in biomass may be less evolutionarily flexible than the proportions of nitrogen and phosphorus. Alternatively, changes in carbon content may be more difficult to detect because carbon makes up a much larger portion of biomass.

Although the proportion of carbon in biomass remained constant, the total amount of carbon that accumulated in the biomass increased significantly in the evolved bacteria (Fig. 3.2B). This increase was associated with an increase in the total biomass (Fig. 3.2C). The increase in carbon retention was not caused by an increase in the amount of carbon consumed because all of the bacteria (with the exception of the citrate consumer that was excluded from analysis) consumed the same 25 mg/L of glucose. Therefore, to retain more carbon in biomass, the bacteria must have released less carbon, either as carbon dioxide or byproducts excreted into the medium (and not then recycled for growth). Our results parallel those of an experiment in which yeast were evolved under carbon limitation for 350 generations (Goddard and Bradford 2003). The evolved yeast retained more carbon in biomass, but the proportion of carbon in biomass remained unchanged. It may be that carbon-use efficiency is more evolutionarily flexible than the proportion of carbon in biomass, at least in some organisms and contexts.

In this regard, it is also interesting that the LTEE provides no evidence of a tradeoff between growth rate and carbon-use efficiency. Instead, the evolved bacteria have increased in both their growth rate (Vasi et al. 1994; Novak et al. 2006) and carbon-use efficiency (this study; see also Vasi et al. 1994; Lenski and Mongold 2000; Novak et al. 2006 for indirect evidence based on optical density and total biovolume of cells, rather than on biomass). A tradeoff between growth rate and yield is one of the most well-established patterns in life history traits (Pfeiffer et al. 2001; MacLean 2008; Bachmann et al. 2013; Meyer et al. 2015). Under the unstructured (i.e., well mixed) conditions of the LTEE, selection for increased growth rate is strong and direct, whereas there is no direct selection for increased yield (Vasi et al. 1994); for example, a mutant that grows faster, despite using resources less efficiently, would still have a competitive advantage in the LTEE because the reduced resources would affect all competitors equally. Thus, it is surprising to observe an increase in biomass. It appears, therefore, that the ancestor was sufficiently maladapted to the conditions of the LTEE that both growth rate and yield could increase (Novak et al. 2006).

The LTEE is an ongoing experiment, and the trend of decreasing C:N and C:P ratios might well continue over longer time spans. The most likely mechanisms for the changes in stoichiometry observed thus far are changes in the abundance of proteins and other broad classes of cellular components that differ in their elemental content. Over longer timescales, selection for nutrient sparing might also change the composition of individual proteins or other specific components. We focus on proteins in the discussion below because we can easily calculate the effect of individual mutations on the elemental content of proteins. However, similar logic applies for other cellular components.

Comparative studies have found that selection can affect the amino-acid content of proteins in organisms, reducing their use of the most limiting elements (Baudouin-Cornu et al. 2001; Elser 2006b). However, the fitness benefit of any mutation changing a single amino acid is likely to be extremely small. Following the approach of Bragg and Wagner (2009), and given the effective population size for the LTEE of 3.3×10^7 (Lenski et al. 1991), we calculate that mutations that save a single carbon atom would be visible to selection, such that $s > 1/(2N_e)$ (Kimura 1983), in proteins with more than ~450 copies per cell. Individual amino acid changes, specifically Trp to Gly, can save as many as 9 carbon atoms per protein molecule (Bragg and Wagner 2009). A mutation that saved 9 carbon atoms per protein molecule could be selected for in proteins with ~50 or more copies per cell. Of course, such a mutation would require a very long time to achieve fixation. First, the mutation would have to escape drift loss while it was still rare; a successful escape would require many millions of mutational events, given that the probability of escaping drift loss is only ~4s (Gerrish and Lenski 1998). Also, in the asexual regime of the LTEE, large-effect mutations are expected to drive out smaller effect mutations due to clonal interference (Gerrish and Lenski 1998). For example, consider a mutation that saves 9 carbon atoms per molecule in an abundant protein with 10,000 copies per cell. Even after escaping drift and without competition from other mutations, this mutation would require on the order of 10^6 generations (~400 years) to approach fixation in the population.

Our results demonstrate the potential for experimental studies to shed new light on evolutionary changes in stoichiometry, since measurable inherited changes can occur over experimentally tractable time scales. The relatively rapid evolutionary changes in

stoichiometry also raise the possibility that large-scale changes in nutrient availability in Earth's ecosystems, for example those caused by climate change or increased fertilizer use, could drive evolutionary changes in organismal stoichiometry. Shifts in organismal stoichiometry could, in turn, alter the effects of anthropogenic change on ecological interactions and ecosystem processes. Evolution of stoichiometry might be usefully incorporated into eco-evolutionary models, such as those that forecast the response of global phytoplankton communities to climate change (Follows et al. 2007; Munday et al. 2013). In order to successfully model the evolution of stoichiometry, however, we need to know much more about how stoichiometry responds to selection, and experimental evolution offers a valuable tool for acquiring that knowledge.

Acknowledgments

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APPENDIX

APPENDIX

Trajectory of changes in stoichiometry in a single population during 60,000 generations of evolution

Authors: Caroline B. Turner, Brooke Sommerfeld, and Richard E. Lenski

Following the methods described above in the main body of Chapter 3, we also measured the biomass (Fig. 3.3), carbon to nitrogen (Fig. 3.4) and carbon to phosphorus (Fig. 3.5) ratios of clones from multiple time points in a single population of the LTEE. The focal population, designated Ara-1, has been used in a number of previous studies which examined a single population in detail. We measured the stoichiometry of two clones isolated every 500 generations for the first 2,000 generations, every 5,000 generations up to 40,000 generations, and at 50,000 and 60,000 generations. We excluded data from the two 40,000 generation clones due to suspected contamination.

The C:N and C:P ratios declined over time, consistent with the results in the main body of Chapter 3. The decrease appears to have been steepest in the earliest generations, but, at least in the case of C:N, appears to have continued at a slower rate in later generations. In contrast, biomass increased sharply between 500 and 1,000 generations, but had no clear directional trend in later generations.

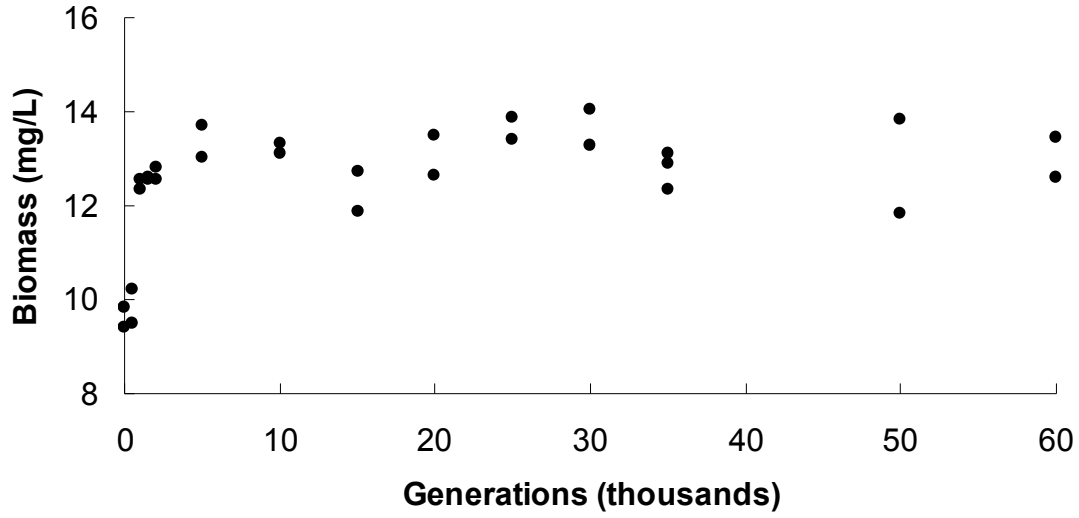

Figure 3.3: Biomass of clones isolated from population Ara-1 through evolutionary time.

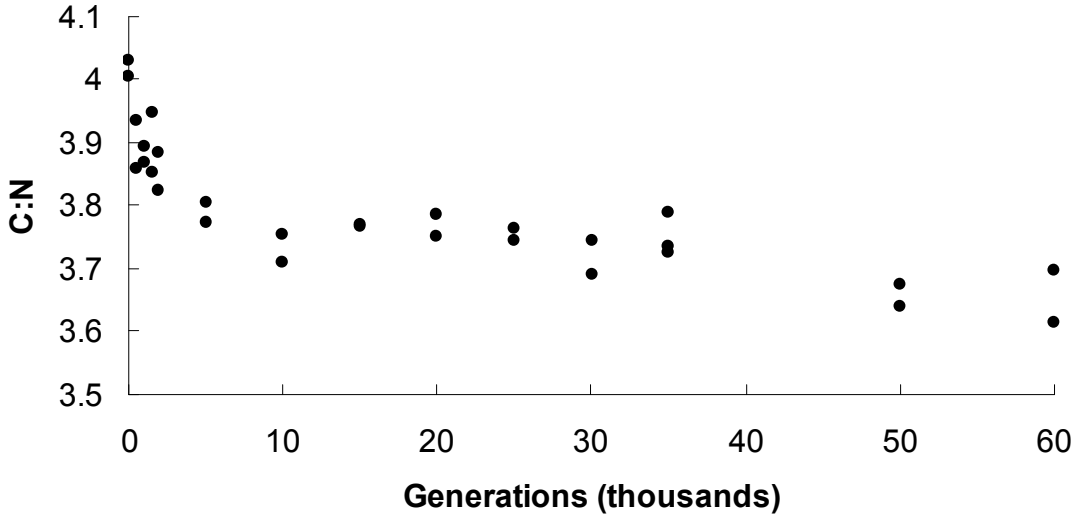

Figure 3.4: Carbon to nitrogen ratio (molar) of clones isolated from population Ara-1 through evolutionary time.

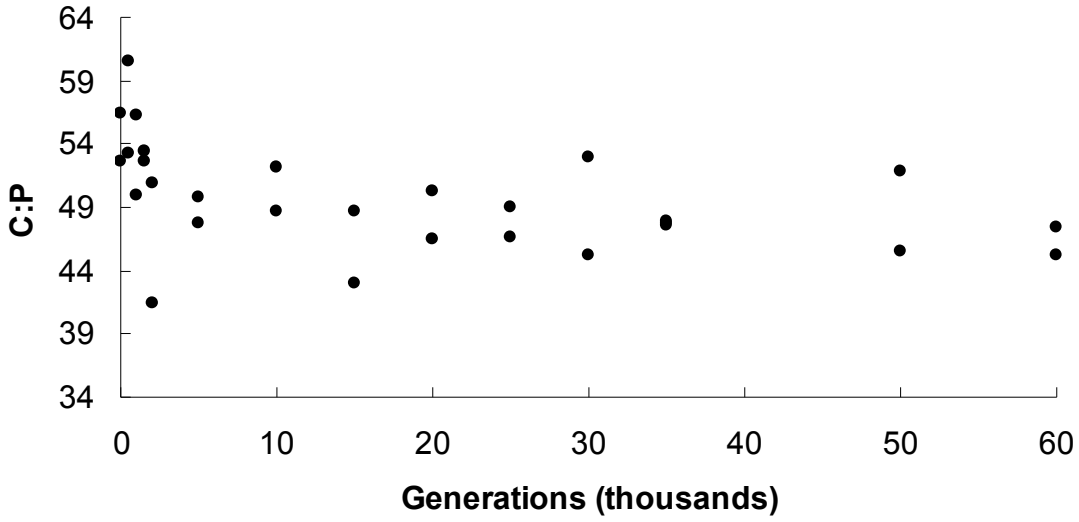

Figure 3.5: Carbon to phoshorus ratio (molar) of clones isolated from population Ara-1 through evolutionary time.

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LITERATURE CITED

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CHAPTER 4:

EXPERIMENTAL EVOLUTION OF ORGANISMAL STOICHIOMETRY IN *ESCHERICHIA COLI* PROPAGATED UNDER NITROGEN LIMITATION

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Abstract

While variation in elemental composition is known to have important ecological consequences, much less is known about how stoichiometry changes over evolutionary time. In Chapter 3, we demonstrated that *Escherichia coli* evolved lower C:N and C:P ratios and increased carbon-use efficiency after 50,000 generations of experimental evolution under carbon limited, high nitrogen and phosphorus conditions. However, that experiment did not differentiate between adaptation to carbon limitation and adaptation to other aspects of the evolutionary environment. To isolate the effect of nutrient limitation, we further evolved clones from the original 50,000-generation experiment under either carbon-limited or nitrogen-limited conditions for 1,000 generations. Contrary to our expectations, the *E. coli* evolved under nitrogen limitation had a higher percentage of nitrogen in their biomass than the ancestral strains. This change appears to be due to an increase in the amount of nitrogen incorporated into biomass. Initially, the nitrogen limited *E. coli* incorporated only 60% of the nitrogen in the culture medium into biomass. This improved slightly to 64% in the evolved *E. coli*, with the additional nitrogen allocated towards an increase in the proportion of nitrogen in biomass, rather than

towards increased total biomass. Our results show that stoichiometry can evolve over relatively short time scales, but that organisms do not necessarily evolve decreased use of scarce elements. Instead, in both this experiment and in the long-term evolution experiment, we observed increases in the proportion of the limiting nutrient that was incorporated into biomass, indicating that selection for improvements in nutrient uptake and retention may be stronger than selection for nutrient sparing.

Introduction

Biological organisms are made up of chemical elements in varying proportions. The proportion of different elements, the stoichiometry of organisms, both affects and is affected by their environment. The stoichiometry of phytoplankton helps shape the nutrient profile of the oceans (Redfield 1958; Weber and Deutsch 2010), the rate of carbon sequestration (Hessen et al. 2004), and the nature of ecological interactions (Gurung et al. 1999). At the same time, organismal stoichiometry can also respond to environmental changes. In particular, the relative amount of elements such as carbon, nitrogen, and phosphorus supplied to organisms can affect the ratios of those elements in organismal biomass (Sterner and Elser 2002). The stoichiometry of these organisms, in turn, can feed back and affect their environment.

Organismal stoichiometry can respond to changes in elemental availability through two mechanisms, which operate over different timespans. Over one or a few generations, organisms can exhibit a plastic response, while evolutionary changes in stoichiometry can occur over many generations. The plastic response of organisms has

been studied more extensively. Generally, the proportion of a limiting element in organismal biomass decreases as that element becomes scarcer, although the degree of variability varies among taxa (Sterner and Elser 2002). The plasticity of stoichiometry could be due to regulatory responses of organisms in response to limitation, such as the nitrogen regulatory protein C regulon in *Escherichia coli* (Zimmer et al. 2000). Plasticity could also be a direct response to scarcity in which rates of synthesis of cellular components are slowed when a particular element becomes scarce.

The evolutionary response of organisms to changes in elemental availability is harder to study than the plastic response because it occurs over longer times. As a result, the rate, magnitude and direction of evolutionary changes in stoichiometry in response to elemental limitation is not well established. All else being equal, one would expect that organisms which make less use of a scarce resource will produce more offspring than organisms which require more of that resource and will therefore have a selective advantage. This logic leads to the 'nutrient-sparing hypothesis', that evolutionary response to scarcity parallels the physiological response and leads to reduced use of scarce elements.

To date, evidence for this hypothesis is primarily based on comparative genomics and proteomics, either by comparison of the stoichiometry of the genomes or proteomes of organisms which differ in their history of nutrient limitation or by comparison of the stoichiometry of proteins which are expressed under different nutrient limiting conditions. The evidence from these studies generally indicates that organisms can evolve decreased use of limiting elements. For example, sulfur assimilatory proteins in *E. coli* and yeast have lower sulfur content than the proteome as a whole, and the same pattern

holds for carbon and nitrogen assimilatory enzymes (Baudouin-Cornu et al. 2001). Plant genomes and proteomes also show evidence of selection for nutrient sparing under nitrogen limitation, based on comparisons with animals and with agricultural plants, both of which are less likely to experience nitrogen limitation (Elser 2006; Acquisti et al. 2009). However, given the widely different evolutionary histories of these groups, it is difficult to eliminate the possibility that differences in organismal stoichiometry are driven by factors other than nutrient availability (Günther et al. 2013).

To overcome these limitations, it is valuable to be able to compare the stoichiometry of evolved and ancestral organisms which have evolved under known conditions. For organisms evolving in the wild, this is possible only in a few unusual cases. Because *Daphnia* zooplankton produce eggs which remain viable in layers of lake sediment, offspring can be raised from the young and compared. *Daphnia* grown from the egg bank of a lake which had experienced 120 years of elevated phosphorus levels, exhibited declines in phosphorus use efficiency, but no change in the phosphorus content of their biomass (Frisch et al. 2014). An alternative approach is to study the evolution of organisms experimentally. Microbes, with their small size, large populations and short generation times, allow for evolution to studied over tractable time scales under controlled laboratory conditions (Lenski et al. 1991; Kawecki et al. 2012). A further advantage is that many microbes can be frozen and later regrown, allowing for simultaneous comparison of ancestral and evolved strains.

We are aware of only one previous study that used experimental evolution to evaluate the nutrient-sparing hypothesis. That study compared proteins which differed in their expression between yeast strains which had evolved for 200-500 generations under

carbon limitation and their ancestors, but did not measure the stoichiometry of overall biomass (Bragg and Wagner 2007). In contrast to the predictions of the nutrient-sparing hypothesis, proteins with higher expression in evolved yeast than in ancestral yeast were more carbon-rich than the proteome as a whole. Bragg and Wagner suggest that evolution of improved carbon uptake may have alleviated the degree of carbon limitation, allowing expression of more carbon rich molecules. Increased use of a limiting element could also evolve for other reasons. For example, the adaptive benefit of increased use of a scarce element could outweigh any benefit of nutrient sparing.

In Chapter 3, we found that *E. coli* that evolved under carbon limitation with abundant nitrogen and phosphorus in the laboratory for 50,000 generations had lower C:N and C:P than their ancestors. This result was consistent with the nutrient-sparing hypothesis. However, the bacteria were adapting not just to carbon limitation, but also to other aspects of their environment. We could not eliminate the possibility that the increased nitrogen and phosphorus content of the evolved clones was an epistatic effect of adaptation to batch culture, minimal medium, or the particular temperature, pH, etc. of the long-term evolution experiment (LTEE).

To address this limitation of our previous study, we conducted a controlled experiment in which we evolved a set of clones from the LTEE under both carbon and nitrogen limited conditions. Based on the nutrient-sparing hypothesis, we predicted that clones from populations evolved under nitrogen-limited conditions would have lower relative nitrogen content than their ancestors. For the carbon-limited populations, our previous experiments indicate that higher nitrogen content should evolve under carbon limitation. However, because many of the founding clones in this experiment were

already adapted to carbon limitation we thought it just as likely that the stoichiometry of bacteria would not exhibit further change. Contrary to our predictions, we found that the bacteria evolved under nitrogen limitation had higher nitrogen content than their ancestors.

Methods

Evolution experiment

We evolved 24 populations, each founded from a clone from the LTEE, for 1000 generations. We established four populations from each of six founding clones- two replicate populations evolving under carbon limitation and two replicate populations evolving under nitrogen limitation (Table 4.1). The 12 carbon-limited populations were grown in Davis minimal medium with 25 mg/L glucose and 1000 mg/L ammonium sulfate, under the same conditions as the LTEE (Lenski et al. 1991). Conditions for the 12 nitrogen-limited populations differed only in the amount of carbon and nitrogen added to medium. Nitrogen limited medium contained 250 mg/L glucose and 20 mg/L ammonium sulfate. We confirmed that growth in this medium was limited by nitrogen availability by varying the concentration of ammonium sulfate in the medium and confirming that higher concentrations of nitrogen resulted in higher population density. The concentration of nitrogen was chosen to produce roughly similar population densities as under carbon limitation. We chose to study nitrogen rather than phosphorus limitation because DM25 is phosphorus buffered and reducing the phosphorus concentration would have required the addition of a different buffer, which would have created an additional difference

Medium	Population	0-generation clone	1000-generation clone
Carbon limited	A	REL606	CBTn288
Carbon limited	B	REL606	CBTn300
Carbon limited	C	REL11330 (Ara-1)	CBTn290
Carbon limited	D	REL11330 (Ara-1)	CBTn302
Carbon limited	E	REL11333 (Ara-2)	CBTn292
Carbon limited	F	REL11333 (Ara-2)	CBTn304
Carbon limited	G	REL11336 (Ara-4)	CBTn294
Carbon limited	H	REL11336 (Ara-4)	CBTn306
Carbon limited	I	REL11339 (Ara-5)	CBTn296
Carbon limited	J	REL11339 (Ara-5)	CBTn308
Carbon limited	K	REL11389 (Ara-6)	CBTn298
Carbon limited	L	REL11389 (Ara-6)	CBTn310
Nitrogen limited	M	REL606	CBTn312
Nitrogen limited	N	REL606	CBTn276
Nitrogen limited	O	REL11330 (Ara-1)	CBTn266
Nitrogen limited	P	REL11330 (Ara-1)	CBTn278
Nitrogen limited	Q	REL11333 (Ara-2)	CBTn268
Nitrogen limited	R	REL11333 (Ara-2)	CBTn280
Nitrogen limited	S	REL11336 (Ara-4)	CBTn270
Nitrogen limited	T	REL11336 (Ara-4)	CBTn282
Nitrogen limited	U	REL11339 (Ara-5)	CBTn272
Nitrogen limited	V	REL11339 (Ara-5)	CBTn284
Nitrogen limited	W	REL11389 (Ara-6)	CBTn274
Nitrogen limited	X	REL11389 (Ara-6)	CBTn286

Table 4.1: List of the ancestral and evolved clones for each population.

between this experiment and the LTEE. Cultures were transferred daily via 1:100 dilution and population samples were frozen every 100 generations.

The founding clones used in the experiment consisted of clones isolated from the 50,000 generation samples from 5 of the LTEE populations and also the original founding strain of those populations, REL606 (Table 4.1). The LTEE consists of 12 populations, 6 of which were founded from REL606, which lacks the ability to consume arabinose. The 50,000-generation clones we used constituted clones from all Ara^- populations of the LTEE, with the exception of the population, designated Ara-3, in which the ability to consume citrate evolved. Our previous work (Chapter 3) showed that the Ara-3 population, which had access to much more carbon than the other populations, had substantially different stoichiometry than the other evolved clones. We chose to use clones from 50,000 generations because these populations had evolved under carbon limitation for a long period of time, and previous work (Chapter 3) showed that these clones had higher nitrogen and phosphorus content than their ancestors. We expected that these clones would have the greatest likelihood of showing decreases in nitrogen content over the course of a 1,000 generation experiment.

We collected samples of sterile nitrogen-limited medium and of the deionized water with which the medium was made each time we prepared media during the evolution experiment. The dissolved nitrogen content of these samples was measured using a Shimadzu TOC carbon analyzer equipped with a module for chemiluminescence detection of nitrogen. We collected a total of eight samples, however one sample of medium became visibly contaminated and was excluded from our results.

Following the evolution experiment, we isolated clones from the 1,000 generation population samples by plating a portion of the population on tetrazolium arabinose (TA) agar plates (Levin et al. 1977; Lenski et al. 1991) and streaking a colony from those plates onto a second TA plate. From the second plate we inoculated one colony into Luria-Bertani medium (LB), incubated the culture overnight and then froze a sample of the culture with glycerol at -80° C.

Fitness

We measured the fitness of all ancestral and evolved clones relative to the clone REL607, an arabinose-consuming mutant of REL606. Arabinose consumption is a neutral marker under the conditions of the LTEE (Lenski et al. 1991). In measuring fitness as well as all other assays in this study, we used a paired sampling design, in which we compared each evolved clone with its ancestor. In all cases we measured traits in the environment in which the derived clone had evolved. To condition the cells to the growth conditions, samples of frozen clones were first grown in LB, then transferred via 1:10,000 dilution into either nitrogen- or carbon-limited medium. Other than varying the media, conditions were the same as in the LTEE. Following conditioning, equal volumes of REL607 and either and evolved or ancestral clone were added to fresh growth medium. The population density of each competitor was measured by plating onto TA plates before and after 24 h of growth. TA plates allow differentiation of Ara^+ and Ara^- competitors by colony color. We calculated the fitness of the competitor relative to REL607 as described by Lenski et al (1991).

Measurement of stoichiometry

We measured the carbon, nitrogen and phosphorus content of the ancestral and evolved clones when grown under the conditions in which they evolved. After reviving and acclimating the clones to the growth conditions, as described above, we transferred 5 mL of each culture into 500 mL of nitrogen- or carbon-limited medium and incubated the cultures for 24 hours. We then divided each culture in half and vacuum-filtered each 250 mL aliquot using a pre-combusted glass-fiber filter. We next rinsed the medium from the filters by filtering an additional 250 mL of saline solution made up with Milli-Q purified water. One filter was used for phosphorus measurements and the other for nitrogen and phosphorus measurements. Biomass was measured as the difference in weight of dry filters before and after filtering.

We filtered bacteria on eight sampling dates with 3 pairs of ancestral and evolved clones sampled on each date. On any given sampling date we filtered cultures grown in only one type of media and alternated between nitrogen- and carbon-limited media between dates. Each pair of ancestral and evolved clones was randomly assigned a sampling date. On each sampling date, we also processed filter blanks by following the same procedure as above, but with sterile medium.

Carbon, nitrogen and phosphorus content was measured by the Nutrient Analytical Services Laboratory at the University of Maryland Center for Environmental Science. Phosphorus content was measured via extraction to dissolved phosphate followed by molybdenum blue colorimetric analysis (Aspila et al. 1976) using an Aqaukem 250 photometric analyzer. Carbon and nitrogen were determined by elemental

analysis (U.S. Environmental Protection Agency 1997) using an Exeter Analytical Model CE-440 Elemental Analyzer.

Carbon usage

To evaluate how carbon use evolved under nitrogen-limited conditions, we measured the concentration of glucose remaining in the media at the end of a 24 h growth period. After reviving, conditioning and growth, we filter sterilized and froze a sample of the culture medium. We later thawed these samples and measured glucose concentration using a colorimetric glucose assay kit (Cayman Chemical) and a VersaMax automated plate reader (Molecular Devices). Glucose concentration was measured for three replicate cultures of paired ancestral and evolved clones. We did not measure the concentration of glucose remaining under carbon limitation because we knew from prior tests that, under those conditions, glucose levels are always below detection by 24 h.

Statistical analysis

We tested for evolutionary changes between ancestral and derived clones using paired, two tailed *t*-tests. All statistical analysis was conducted using JMP 9.0 software. In assays where measurements were made in triplicate, we used the mean of the three measurements in our statistical analysis.

Results

Fitness improved under nitrogen, but not carbon, limitation

We measured fitness relative to a common competitor to assess whether fitness improved during evolution under nitrogen- and carbon-limited conditions. All competitions were conducted against the reference clone REL607, which differs from REL606 by a neutral mutation which confers the ability to grow using arabinose. As expected, the populations founded from the REL606 ancestor of the LTEE had an initial relative fitness near one under both carbon and nitrogen limitation. Under carbon limitation, the relative fitness of the ancestral clones which had evolved for 50,000-generations in the LTEE was consistently greater than one. The fitness of the founding clones under nitrogen limitation varied, with some 50,000-generation clones exhibiting higher fitness than REL606 and others having lower fitness. It appears that adaptation to carbon limitation during the LTEE does not consistently result in either a trade-off with or a synergistic effect on fitness under nitrogen limitation.

There was no significant change in fitness between ancestral and evolved clones in the carbon-limited populations (Fig. 4.1A, paired, two-tailed *t*-test $P = 0.3469$). However, fitness of the nitrogen-limitation evolved clones was significantly higher than their ancestors, indicating that substantial adaptation occurred in the nitrogen-limited populations, but not the carbon-limited populations (Fig. 4.1B, $P < 0.001$). We were unable to measure the fitness under nitrogen limitation for populations 3 and 9, which were founded from the 50,000-generation Ara-2 clone. That clone and its descendents did not consistently produce colonies on TA agar after growing under nitrogen limitation.

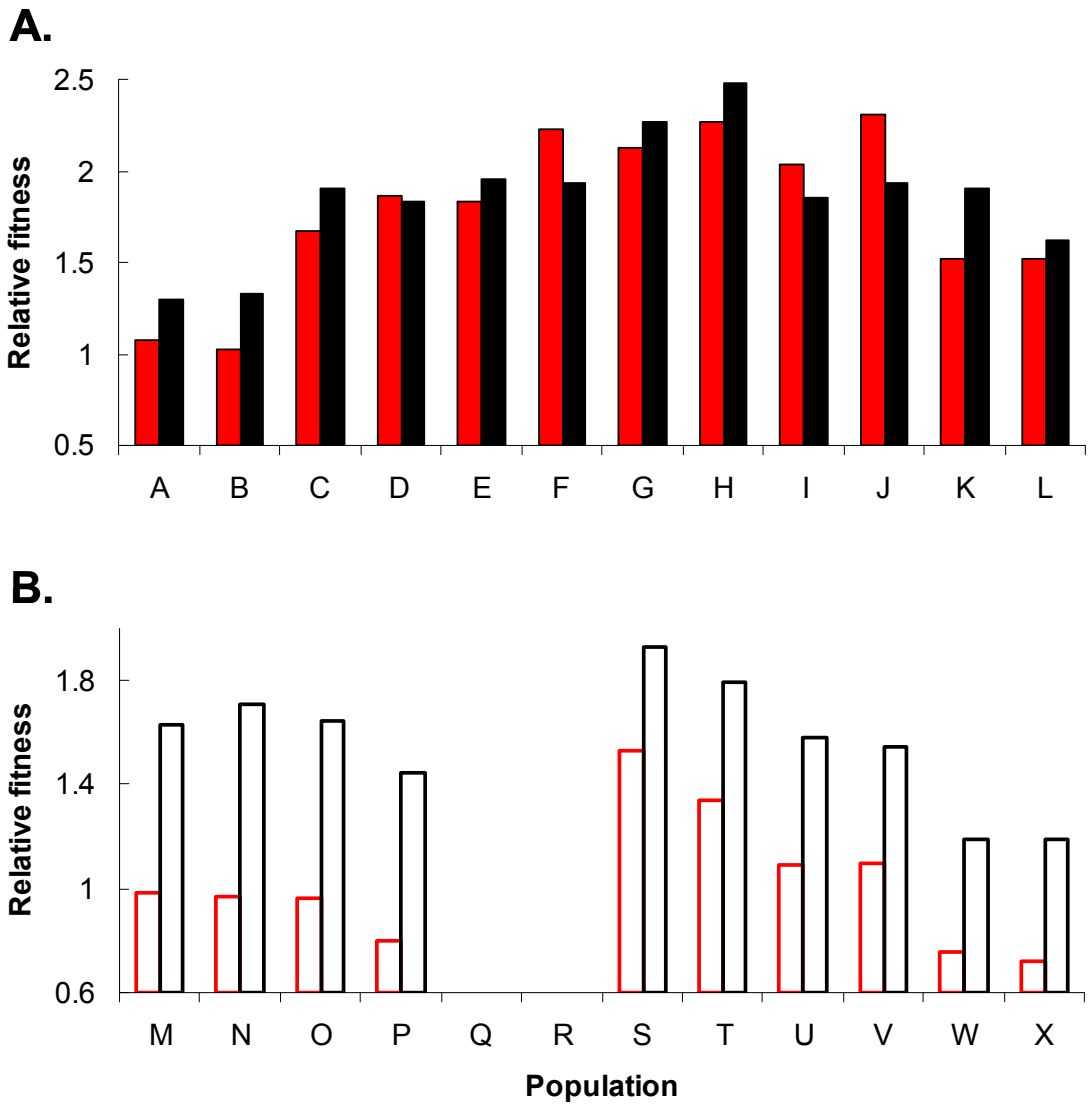

Figure 4.1: The fitness of ancestral (red) and evolved (black) clones relative to a common competitor under A. carbon limitation and B. nitrogen limitation. All data are means of three replicate competitions. Data is absent for populations 3 and 9 under nitrogen limitation due to low plating efficiency of those clones when grown under nitrogen-limited conditions.

However, the patterns in the growth of the common competitor, which we were able to measure, are consistent with improvement in fitness between the founding and evolved clones. The common competitor underwent more generations of growth when grown in the presence of the ancestral clone than when grown in the presence of the evolved clone from those populations.

Biomass

The mean dry biomass in cultures of ancestral and evolved carbon-limited clones was 12 and 13 mg/L, respectively. The mean biomass in cultures of ancestral and evolved nitrogen-limited clones was 36 and 33 mg/L, respectively. There was no significant difference between ancestral and evolved clones under either carbon ($P = 0.88$) or nitrogen ($P = 0.18$) limitation.

Stoichiometry

Our measurements of stoichiometry contained several data points which were distinctly different from other measurements. Under nitrogen limitation there were two measurements with substantially higher C:N and C:P than the others (Figs. 4.2 and 4.3B). These measurements were both of the ancestral clone isolated from population of the LTEE designated Ara-5. This clone also exhibited high biomass and appears to have accumulated excess carbon when grown under nitrogen limitation. Additionally, amongst the carbon limited clones (Fig. 4.3A), one pair of ancestral and evolved clones had a substantially lower C:N ratio than the others. Because of these data points, we analyzed all changes in elemental content using Wilcoxon Signed Rank tests, a non-parametric

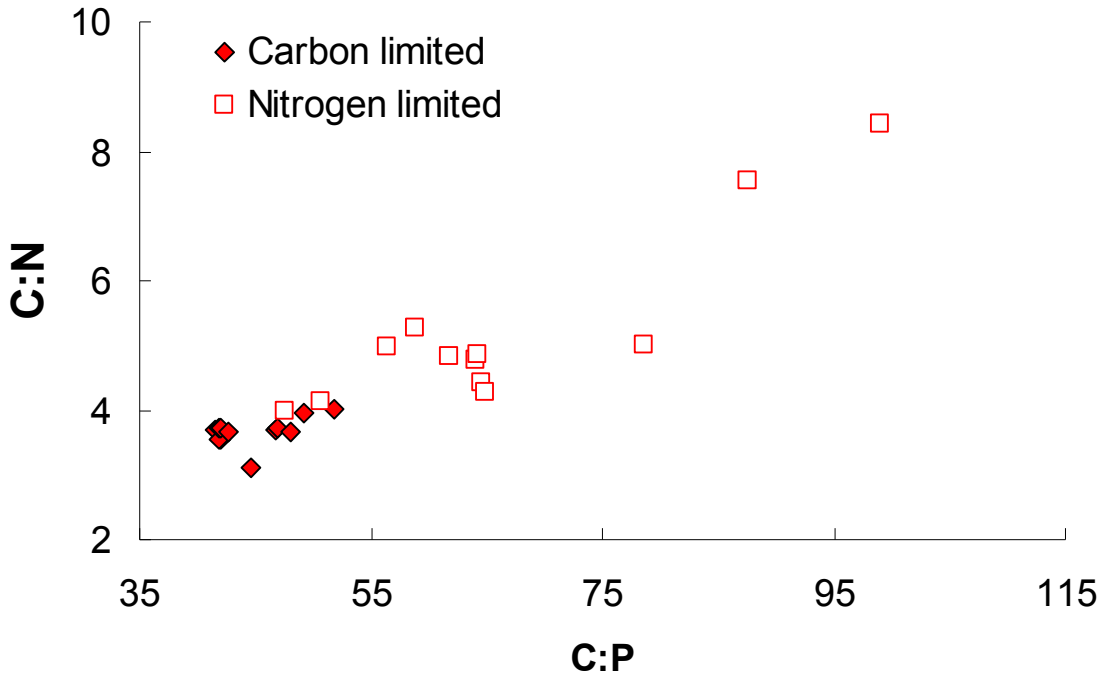

Figure 4.2: The C:N and C:P ratios of the ancestral clones when grown under carbon-limited (filled diamond) or nitrogen-limited (open square) conditions. Each data point represents a single measurement.

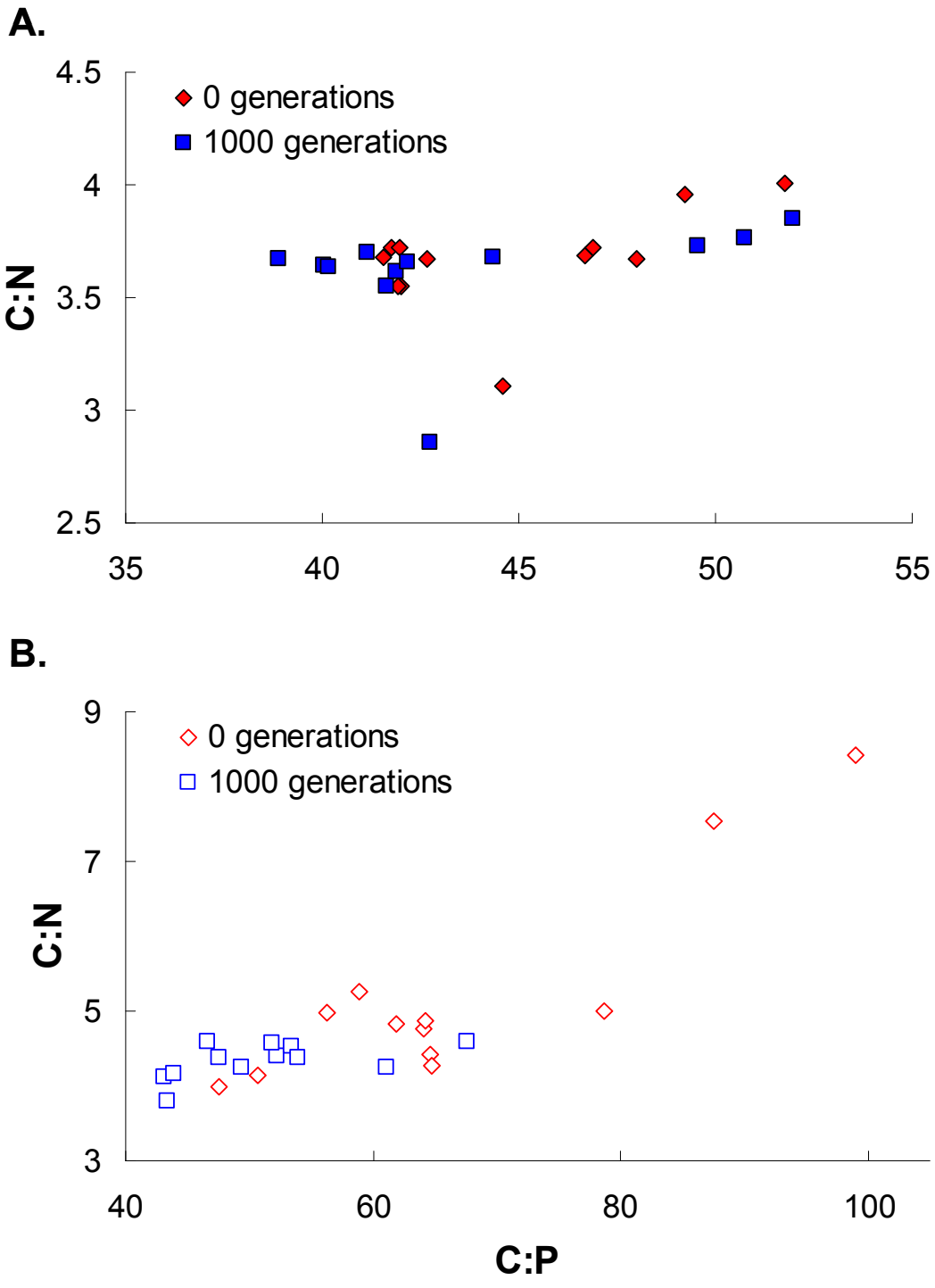

Figure 4.3: The C:N and C:P ratios of ancestral (red diamonds) and evolved (black squares) clones. Panel A. shows clones under carbon limitation; panel B shows clones under nitrogen limitation. Data points for the ancestral clones are the same as those shown in Fig. 4.2.

parametric comparison, in addition to *t*-tests. The significance of results using the Wilcoxon Rank Sum test differed only in the case of C:N ratios of ancestral and evolved clones. Unless otherwise specified, the *P* values listed below are for *t*-test comparisons.

We examined the stoichiometric plasticity of the bacteria in our experiment by testing whether the carbon, nitrogen, and phosphorus content of the ancestral clones differed when grown under carbon limitation and nitrogen limitation. The ancestral clones had higher molar C:N ($P = 0.003$) and C:P ($P < 0.001$) ratios when grown under nitrogen limitation than when grown under carbon limitation (Fig. 4.2). This is consistent with the expected pattern of reduced use of limiting elements. There was no difference in N:P ($P = 0.239$) between carbon and nitrogen limitation.

Under carbon limitation, there was no overall difference in the percentages of carbon ($P = 0.572$), nitrogen ($P = 0.827$) or phosphorus ($P = 0.989$) or in C:P ($P = 0.323$) or N:P ($P = 0.760$) ratios between the ancestral and evolved clones (Fig. 4.3A). The C:N ratio was significantly lower in evolved clones than ancestral clones according to the Wilcoxon Signed Rank test ($P = 0.016$), but did not meet the $P < 0.05$ significance threshold according to the *t*-test ($P = 0.058$).

In contrast to our predictions, we found no evidence for evolution of decreased nitrogen use under nitrogen limitation. In fact, C:N content of the evolved bacteria may even have been lower than that of the ancestors (Wilcoxon $P = 0.027$, *t*-test $P = 0.064$) and the percentage of nitrogen in biomass increased significantly ($P = 0.040$). Interestingly, the phosphorus content also increased, both in terms of C:P ratio ($P = 0.016$) and percentage of phosphorus in biomass ($P = 0.009$). We did not observe a significant change in N:P ($P = 0.052$) or percent carbon ($P = 0.269$).

Carbon usage

There was no significant change in the amount of glucose consumed between ancestral and evolved clones under nitrogen limitation (Fig. 4.4, $P = 0.589$). Across all clones, the average glucose consumption was 197 mg-glucose/L or 79 mg-C/L. Given that an average of 13.5 mg-C/L was incorporated into biomass, this implies that only ~17% of carbon consumed was used to produce biomass. In contrast, under carbon limitation, where all 25 mg-glucose/L was consumed and an average of 5.1 mg-C/L was incorporated into biomass, more than 50% of carbon consumed went into biomass.

Discussion

We evolved replicate populations under both nitrogen and carbon limitation to test how varying nutrient availability affects the evolution of stoichiometry. The populations evolved under nitrogen limitation exhibited substantial adaptation to these new conditions over the course of 1,000 generations (Fig. 4.1B). In the course of this evolution, changes in stoichiometry also evolved (Fig. 4.3B). Our previous results (Chapter 3) demonstrated that stoichiometry evolved during 50,000 generations, but these experiments demonstrate that stoichiometry can evolve over much shorter timespans as well. This potential for rapid evolution of stoichiometry due to *de novo* mutations implies that evolutionary changes in stoichiometry in response to changing nutrient availability could be an important process in natural systems as well.

Unlike the nitrogen-limited clones, ancestral and evolved clones from carbon-limited populations did not improve in fitness (Fig. 4.1). This was consistent with our

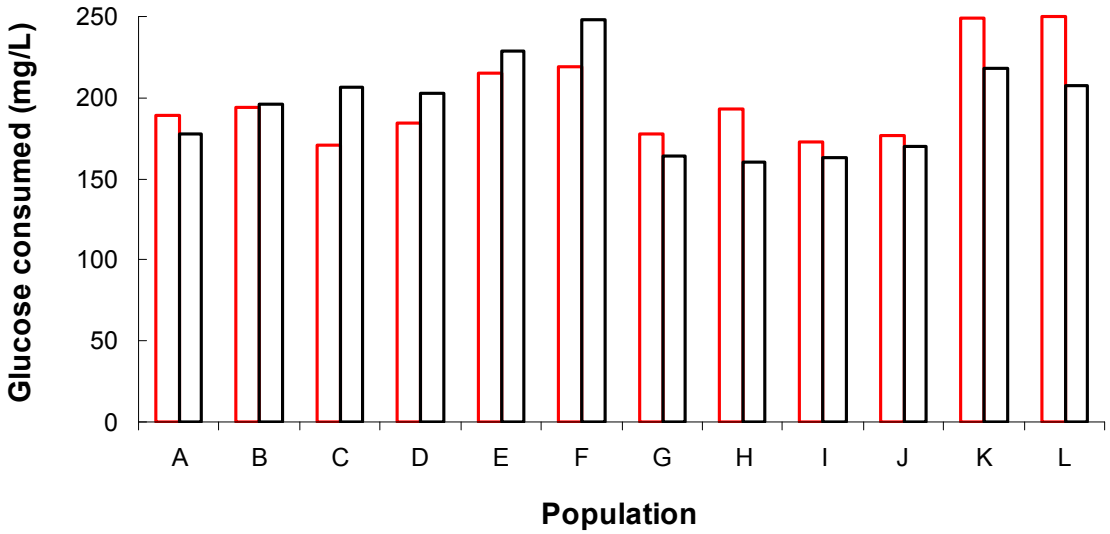

Figure 4.4: Glucose consumption in ancestral (red) and evolved (black) clones during 24 h of growth under nitrogen limitation. All data shown are means of three replicate assays.

expectations, given that the founding clones of most of the populations had already evolved for 50,000 generations under identical conditions. While fitness is still improving in the LTEE populations at 50,000 generations, the rate of change has slowed to the point that the change in fitness over 1,000 generations would be difficult to detect (Wiser et al. 2013). However, there was a trend toward lower C:N ratios in evolved clones (Fig. 4.3B), though the statistical significance of the trend was borderline. The decrease in C:N ratio is consistent with the decrease in C:N over 50,000 generations in the LTEE. No other aspects of elemental composition differed between ancestral and evolved clones under nitrogen limitation.

Based on the nutrient-sparing hypothesis we predicted that the bacteria evolved under nitrogen limitation would have a lower proportion of nitrogen in their biomass. However, if anything, the evolved clones had higher nitrogen content than their ancestors. The percentage of nitrogen in biomass increased and the C:N ratio declined, though the statistical significance of the decline in C:N was borderline. The evolution of increased nitrogen content is not consistent with the nutrient sparing hypothesis, but it is consistent with a previous study of experimental adaptation of yeast to carbon limitation. In that study, proteins which had increased expression in evolved relative to ancestral yeast were more carbon-rich than the proteome as a whole (Bragg and Wagner 2007). Bragg and Wagner suggested that the yeast may have evolved improved carbon uptake and that this improvement alleviated the degree of carbon limitation, allowing the expression of carbon-rich proteins. Supporting this interpretation, we found that the clones evolved under nitrogen limitation exhibited improved nitrogen uptake. When growing under nitrogen-limited conditions, the ancestral clones incorporated only 60% of

the available nitrogen into biomass (Fig. 4.5). However, nitrogen incorporation into biomass improved to 64% in the evolved clones. This is still a low efficiency and there may be potential for substantial further evolutionary improvement.

Evolution of improved uptake does not preclude the possibility that selection for nutrient sparing can occur under some circumstances. However, it may be that when mutations which improve uptake are available, they provide a greater fitness benefit than mutations that result in decreased use of scarce elements. Because the organisms in our experiment reproduce entirely asexually, mutations of larger fitness benefit will outcompete smaller-benefit mutations through clonal interference (Gerrish and Lenski 1998). It is also possible that under the serial-transfer conditions used in this experiment and the LTEE higher nitrogen content is favored, regardless of the supply ratio of elements. The serial transfer regime favors cells which can grow quickly after being transferred to new medium (Vasi et al. 1994). It could be that cells with higher nitrogen content prior to transfer are able to grow more quickly following transfer.

Given the improved nitrogen uptake in the clones evolved under nitrogen limitation, there are two ways that the clones could have allocated that additional nitrogen. Additional nitrogen could have been used to produce additional biomass, keeping the proportion of nitrogen in the biomass the same. Alternatively, additional nitrogen could have been allocated to increase the proportion of nitrogen in biomass, keeping biomass constant. A combination of these two allocation patterns is also possible. However, given that we observed no increase in biomass in evolved clones (and if anything a downward trend), and an increase in the percentage of nitrogen in biomass, it appears that the bacteria exhibited the latter pattern. This could suggest that higher

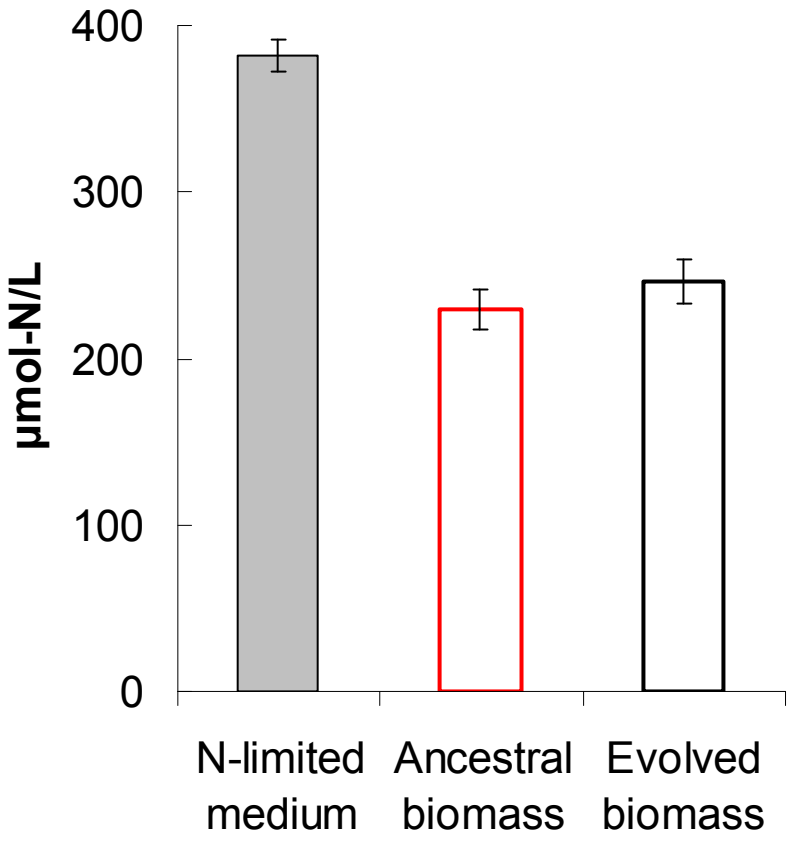

Figure 4.5: Total dissolved nitrogen in the nitrogen-limited medium compared to the nitrogen accumulated in the biomass of the ancestral and evolved clones. Total dissolved nitrogen is the mean of 7 samples of sterile medium collected throughout the evolution experiment. Each value of nitrogen accumulated in biomass is the mean of 12 clones. Error bars are 95% confidence intervals.

nitrogen content was more beneficial to the bacteria than increased yield, however we cannot distinguish whether the allocation of nitrogen was selected for under the conditions of the experiment or if the allocation was simply an intrinsic response.

Interestingly, evolution under nitrogen limitation, but not carbon limitation, also resulted in changes in phosphorus content (Fig. 4.3B), even though phosphorus availability was the same in both treatments. The phosphorus content of the evolved clones was higher than that of ancestral clones, both in terms of C:P ratio and the percentage of phosphorus in biomass. The ancestral clones exhibited a plastic response to nitrogen limitation in which clones grown under nitrogen limitation had a higher C:P ratio than the same clones grown under carbon limitation (Fig. 4.2). A likely explanation for this is that nitrogen availability affected the proportion of cellular components rich in both nitrogen and phosphorus compared to carbon. Alternatively, lack of nitrogen could have hindered the cells' ability to take up phosphorus. In either case, the improved uptake of nitrogen would allow phosphorus content to increase also.

Although there was no difference in the amount of glucose consumed in cultures of ancestral and evolved clones under nitrogen limitation, glucose consumption was notably high across all of the nitrogen-limited clones. Under carbon-limited conditions, about 50% of the carbon consumed was incorporated in biomass, with the rest released either as carbon dioxide of as exudates into the medium. Under nitrogen-limited conditions, only ~17% of carbon consumed was incorporated into biomass. The low proportion of carbon incorporated into biomass is could be due to the bacteria continuing to respire carbon, even after the population had stopped growing due to nitrogen limitation.

Evolutionary changes in stoichiometry have the potential for important consequences as human induced changes shaped the relative availability of elements. Our results indicate that stoichiometry can evolve even over as short a span as 1000 generations. However, the direction of stoichiometric change and whether and under what circumstances organisms will evolve reduced use of limiting elements remains unclear. In order to incorporate changes in stoichiometry into models of response to climate change (Follows et al. 2007; Munday et al. 2013), we will need to improve our ability to predict how stoichiometry will evolve.

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