

## **Chapter Ten**

History's Windings in a Flask: Microbial Experiments into Evolutionary Contingency

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September 1862 was a dark time for the United States of America. The Civil War to bring the breakaway Confederacy back into the Union was well into its second bloody year, and Confederate general Robert E. Lee had invaded Maryland on September 4. The dispirited Union army could not manage to find Lee's army, and the US seemed assured further humiliation at a time when elections were looming. Worse, the British and French governments were considering formally recognizing and aiding the Confederacy. Victory for the Confederacy looked to be in sight (McPherson 1988).

So things stood on the morning of September 13, 1862, as the US Army sluggishly marched into Frederick, Maryland. During a stop from marching, an Indiana corporal took the opportunity to rest under a tree at the edge of a field. As he lay down, the corporal happened to see an envelope in the tall grass nearby. In this envelope, he found a piece of paper entitled "Special Order 191" that detailed Lee's marching orders (Sears 1983; McPherson 1988).

The copy of Special Order 191 soon came to Major General George B. McClellan, commanding general of the Union army. With Lee's plans in hand, McClellan was able to bring his opponent to pitched battle at Antietam on September 17. The bloody battle forced Lee to retreat back into Virginia. Morale improved in the north, and supporters of the war maintained their power in Congress after the fall elections (McPherson 1988). Antietam also allowed President Lincoln to issue the Emancipation Proclamation, making the war a crusade to end slavery. The war's new moral character effectively ended the chance for foreign intervention (Sears 1983; McPherson 1988). The Confederacy would never again be close to victory, and Antietam is now recognized as the key turning point of the war. Remarkably, this momentous event occurred because of the happenstances of a dropped envelope, where a tired man happened to rest, and where he happened to look while doing so.

Of all the ways the world could be, one of these the world is. The world is as it is because history occurred as it did, with all the many coincidences, happenstances, and freak events that played out in the tangled interplay of chance and necessity that history always involves. The range of possible futures that may result from a given present must always collapse down into a single actual outcome that in turn determines the range of possible later futures. The future is therefore dependent upon the particular causal chain of innumerable, interacting and often small antecedent factors leading up to it (Beatty and Carrera 2011; Desjardins this volume). This is to say that history is path dependent and subject to contingency; the property of historical sequences that makes history matter.

Biological evolution is also subject to a profound tension between chance and necessity. Evolutionary outcomes are determined by a complex interplay of stochastic and deterministic processes (Monod 1971; Mayr 1988). Natural selection systematically adapts organisms to the environmental conditions they encounter, but it must act on heritable variation introduced stochastically by gene flow, recombination, and, ultimately, mutation. Moreover, genetic drift can cause random loss of even the most beneficial variation that may arise. Finally, mutations with similar adaptive value in a given environment can differ greatly in their correlated effects on adaptation to other environments, as well as their effects on a variety of traits (pleiotropy) and their interactions with other mutations (epistasis), altering the genomic and organismal context in which future evolution takes place (Gould and Lewontin 1979; Jacob 1977). Consequently, what mutations occur, and the order in which they occur can profoundly impact evolutionary trajectories, evolvability, and correlated fitness in environments that may be later encountered (Lenski et al. 1991; Mani and Clarke 1990; V. S. Cooper and Lenski 2000; Weinreich, Watson, and Chao 2005). Seemingly trivial differences between lineages can even determine survival and extinction. Moreover, chaotic interactions between geological, astronomical, and climatological processes and the biosphere can cause rapid and capricious environmental changes that trigger mass extinctions in which only those lineages fortuitously pre-adapted to the new conditions survive (Lewontin 1966; Jablonski 1986; Gould 2002). Evolution is clearly a process that

takes place in lineages shaped by unique evolutionary histories billions of years long that have occurred within the Earth's singular history. It is therefore a fundamentally historical phenomenon that, just like human history, is subject to path dependence that arises from its core processes and the broader planetary context in which it occurs. It is a process that takes place in lineages shaped by unique evolutionary histories billions of years long that have occurred within the Earth's singular history. Evolution is a fundamentally historical phenomenon.

How important is the historical nature of evolution? Evolutionary historicity has been recognized since Darwin, but this question received little attention for much of evolutionary biology's history. However, Stephen Jay Gould began to highlight its importance in the 1980's.<sup>1</sup> Gould's answer to the question, most forcefully in his 1989 book, *Wonderful Life*, was a resounding "Very!" Gould suggested that evolutionary outcomes are fundamentally subject to contingency. As in human history, Gould asserted, the quirks and happenstances of the complex causal chains of evolutionary history play a critical role in determining what evolutionary outcomes result, and so small changes along the way could lead to very different outcomes (Gould 1989; Gould 2002). He famously suggested that replaying the "tape of life" from various points in the distant past would each time result in a very different biological world (1989). Gould argued that this contingency renders evolution inherently unpredictable, and therefore explicable and understandable only in retrospect using narrative, actual sequence explanations (Gould 1985b; Gould 1989; Gould 2002; Beatty 1993; Beatty 2006b; Blaser 1999; Sterelny and Griffiths 1999; Desjardins 2011).

Others have strongly disagreed with Gould. Many have suggested that widespread evolutionary

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1 Gould was actually part of a broader trend of increased interest in contingency and historicity that developed during the second half of the twentieth century, the causes of which have yet to be fully explored. This interest can be seen in popular culture, where contingency and counterfactual history came to be a mainstay of TV and film, and the science fiction sub-genre of alternate history experienced strong growth. In academia, counterfactual analysis came to be an accepted methodological tool in a variety of fields, including philosophy (Lange 2005), sociology (Harding 2003; S. L. Morgan and Winship 2007), and economics (Cowan and Foray 2002; Cartwright 2007). Increased appreciation for historical contingency and counterfactual methods also developed in both professional and popular historiography (McPherson 1988; Ferguson 1997; Bulhof 1999).

convergence indicates that contingency's scope is highly limited (Conway Morris 2003; Conway Morris 2010; Parker et al. 2013). Simon Conway Morris has argued that organisms can occupy only a limited number of possible niches that present biological challenges to which biological and physical constraints provide a limited set of solutions. Natural selection then deterministically finds these solutions (Conway Morris 2003). Consequently, were one to replay the tape of life many times, very similar outcomes would be observed (Conway Morris 2003; Conway Morris 2010; Vermeij 2006). Therefore, while contingency might provide some indeterminacy, evolution is broadly repeatable, predictable, and regular enough to be potentially described by the sorts of history-insensitive robust process explanations found in physics and chemistry (Sterelny and Griffiths 1999).

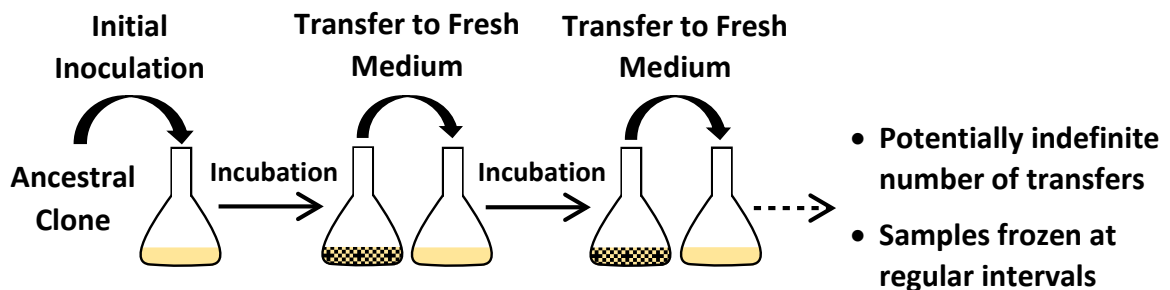
The contingency debate was hampered in its early stages by a lack of focused empirical research, in large part because evolutionary contingency is a tricky phenomenon to study. However, evolutionary contingency should have empirically evaluable effects. First, contingency should reduce or preclude evolutionary repeatability (Gould 1989; Gould 2002). This prediction is drawn from what Beatty has identified as Gould's "unpredictability" notion of contingency (Beatty 2006b; Beatty and Desjardins 2009; Beatty and Carrera 2011). Beatty also identified a second, "causal dependence", notion of contingency in Gould's writings that describes the historical path dependence of evolutionary outcomes (Beatty and Desjardins 2009; Beatty and Carrera 2011; Desjardins this volume). This notion predicts that at least some evolutionary outcomes are highly sensitive to history (Gould 2002; Beatty 2006b). Finally, an outcome contingent upon a particular prior historical path should be delayed compared to an outcome driven principally by selection (Foote 1998; Dick et al. 2009).

In recent decades, researchers have begun to evaluate evolutionary contingency at a variety of levels. These studies have included the examination of the timing and phylogenetic distribution of evolutionary innovations (Vermeij 2006), the repeatability of *Anolis* lizard ecomorph evolution on Caribbean islands (Losos et al. 1998; Losos 2010), and the effects of history on the evolution of Southeast Asian fanged frogs (Emerson 2001) and snake diets (de Queiroz and Rodríguez

2006). Some of the most intriguing work on contingency has been done using microbial evolution experiments. In this paper, I will focus on these microbial experiments, and the implications their findings hold for the issue of evolutionary contingency.

### <A> Experimental Evolution with Microbes

Experimental evolution with microbes involves maintaining populations of microorganisms, typically bacteria, yeast, or viruses, under laboratory conditions to examine evolutionary processes as they occur (Bennett and Hughes 2009; Kawecki et al. 2012). In a typical serial transfer microbial evolution experiment, a population is founded from an ancestral clone and grown in a nutrient medium under controlled conditions (figure 10.1). Following a defined incubation time, a fraction of the culture is transferred to fresh medium, and this pattern is then continued, potentially indefinitely. Experimental evolution goes back to Rev. Henry Dallinger who conducted experiments into the evolution of microbial thermotolerance in the 1880's (Dallinger 1887). Despite this early start, experimental evolution with microorganisms did not become a significant approach to studying evolution until the 1980s. In the decades since, microbial evolution experiments have proven to be a powerful way to examine a variety of difficult evolutionary issues, including historical contingency (Elena and Lenski 2003; Kacar this volume; Kawecki et al. 2012).

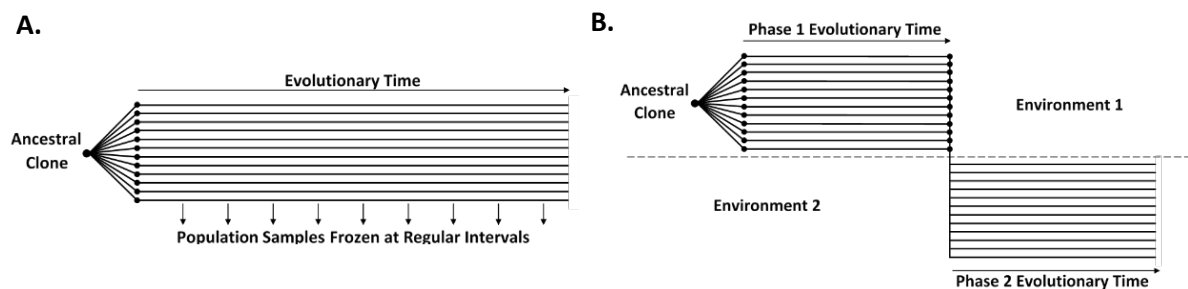


**Figure 10.1: Basic serial transfer regime used in microbial evolution experiments.**

Much of the power of microbial evolution experiments comes from the simple fact that microbes have many characteristics that make them are excellent organisms for evolution research. Many microbes have rapid generation times, some as short as 20 minutes, so hundreds to tens of thousands of generations of evolution can be studied in the course of experiments that take only weeks or years. Microbes typically remain viable after freezing, meaning that samples of evolving microbial populations may be frozen indefinitely in “fossil records” from which ancestral and evolved forms may be revived for later study. Microbial cultures can also reach extremely high population sizes despite small volumes, which ensures rich supplies of variation from mutation during experiments. Moreover, because microbes primarily reproduce by cloning, genetically identical replicate populations can be founded, which permits rigorous statistical analysis of variation that arises between populations exposed to the same conditions. Fitness assays also allow direct determination of changes in relative reproductive fitness by comparison of the growth rate of an evolved population or clone to that of its ancestor (Lenski et al. 1991). Remarkable levels of control can also be maintained, so that differences in population size, mutation supply, biotic and abiotic environmental factors, and even evolutionary history can be manipulated and examined (Levin and Lenski 1985; de Visser et al. 1999; Bohannan and Lenski 2000; Burch and Chao 1999; Burch and Chao 2000; Elena et al. 2001; Fukami et al. 2007; Meyer and Kassen 2007; Kacar this volume). Finally, modern advances in DNA sequencing and genetic engineering enable researchers to identify evolved genetic changes, and to then manipulate and directly link them to changes in fitness and phenotype (Hegreiness and Kishony 2007; Barrick et al. 2009; Barrick and Lenski 2009; Barrick and Lenski 2013; Blount et al. 2012).

Evolution experiments with microorganisms can be used to evaluate evolutionary contingency in ways that are impossible with other approaches. After all, this method permits one to come as close to replaying the “tape of life” as is foreseeably possible, albeit on a much smaller scale than Gould envisioned. Microbial evolution studies into evolutionary contingency may be divided into two general categories, which may be called “Parallel Replay” experiments, or “PREs”, and “Historical Difference”

experiments, or “HDEs” (figure 10.2). PREs involve founding identical replicate populations and evolving them under identical conditions. This set-up permits examination of the range of parallelism and divergence in evolutionary trajectories and outcomes that emerge from the effects of contingency inherent to the core evolutionary processes, as it involves what are essentially simultaneous replays of evolution from a single evolutionary point. By contrast, HDEs involve examining the impact of different evolutionary histories on subsequent evolution. In both, the frozen fossil record and the analytic tools available often make it possible to identify the genetic and ecological interactions upon which later evolutionary outcomes may be contingent. In the following, I will examine experiments and findings in each category. Space limitations preclude a thorough review of all the relevant experiments, so I will instead focus on one major experiment from each category, explicate its findings, and compare them to those of other experiments.



**Figure 10.2: Contingency Experiment Structures**

In a Parallel Reply Experiment (A), multiple, genetically-identical replicate populations are founded from a single ancestral clone and then evolved under the same conditions. At regular intervals, samples of each population are frozen for later analysis and comparison to examine evolutionary parallelism and divergence. This design is open-ended, and may be carried on indefinitely. In a typical two-step Historical Difference Experiment (B), phase one evolution consists of a PRE carried out in a given environmental condition to allow the parallel populations to accrue different evolutionary histories. Clones derived from the evolved populations are used to found new populations that are then evolved under a new environmental condition in phase 2. Typically, analysis and comparison in an HDE is done of clones used to found the second phase populations, and then of populations or clones after the completion of phase two.

## <A> Parallel Replay Experiments

If evolution is highly contingent in the unpredictability sense, then one might expect evolutionary outcomes to be fundamentally un-repeatable even from the same starting point. Parallel replay microbial evolution experiments are the simplest way to test this prediction. In these experiments, replicate populations are founded from the same genotype, and then are evolved under identical conditions (figure 10.2A). PREs therefore involve replaying the same tape of life from the same evolutionary point multiple times simultaneously (Lenski et al. 1991; Desjardins this volume). The only differences that should arise between the populations in these experiments will be those due to the stochastic influx of variation via mutation, and the action of drift and selection upon that variation.

The longest-running and best studied PRE is Richard Lenski's *E. coli* Long-Term Evolution Experiment (LTEE). The LTEE began on February 24, 1988 with the founding of twelve populations from a single clone of *E. coli* B (Lenski et al. 1991; Lenski 2004). Save for a neutral genetic marker that allows six populations to grow on arabinose (Ara<sup>+</sup>), all were initially identical. The founding strain is strictly asexual and lacks any intrinsic capacity for horizontal gene transfer (Jeong et al. 2009; Studier et al. 2009). The populations are grown at a stable 37°C with 120 rpm aeration in DM25, a carbon-limited minimal medium supplemented with 25 µg/mL glucose (Davis and Mingioli 1950). Each day, 1% of each population is transferred to fresh medium, after which each grows 100-fold, or ~6.7 generations (Lenski et al. 1991). This serial transfer regime produces a "seasonality" in which a short feast of abundant glucose after transfer is followed by famine until the next transfer (Vasi, Travisano, and Lenski 1994; Lenski 2004). Each population has evolved for more than 60,000 generations since the experiment began, and population samples have been frozen every 500 generations throughout, providing an extensive fossil record that represents a rich resource for research (Lenski 2004).

Remarkable evolutionary parallelism has been observed in the LTEE. The fitness of all twelve



populations rose rapidly and then decelerated without plateauing (Lenski and Travisano 1994; Lenski et al. 1991; Lenski 2004; Wisser, Ribeck, and Lenski 2013). All twelve have evolved faster growth rates, shorter lag phases, increased average cell size, and lower population sizes (Lenski and Travisano 1994; Vasi, Travisano, and Lenski 1994; Lenski 2004; Philippe et al. 2009). All have lost some or all of their capacity to grow on a variety of substrates other than glucose (Travisano, Vasi, and Lenski 1995; V. S. Cooper and Lenski 2000; T. F. Cooper, Rozen, and Lenski 2003). The populations have also evolved parallel changes in gene expression and regulation (T. F. Cooper, Rozen, and Lenski 2003; Philippe et al. 2007; Crozat et al. 2010; Crozat et al. 2011), protein profiles (Pelosi et al. 2006), epistatic interactions with the CRP regulon (T. F. Cooper et al. 2008), and resistance to phages T6\* and lambda (Meyer et al. 2010). Ten populations have evolved parallel changes in DNA supercoiling (Crozat et al. 2005; Crozat et al. 2010; Crozat et al. 2011). In numerous instances, the same genes have been mutated in multiple populations (V. S. Cooper et al. 2001; T. F. Cooper, Rozen, and Lenski 2003; Crozat et al. 2005; Crozat et al. 2010; Pelosi et al. 2006; Woods et al. 2006; Barrick et al. 2009; Philippe et al. 2009). Most remarkably, IS150-mediated deletions caused complete loss of capacity to grow on maltose across all populations (T. F. Cooper, Rozen, and Lenski 2003). Such parallelism is a hallmark of selection, and, indeed, a number of the parallel mutations have been demonstrated to confer increased fitness under the conditions of the experiment (V. S. Cooper et al. 2001; T. F. Cooper, Rozen, and Lenski 2003; Barrick et al. 2009; Philippe et al. 2009).

The populations have also diverged. Despite overall similarity, the populations' actual fitness trajectories show significant and persistent differences (Lenski et al. 1991; Lenski and Travisano 1994; Wisser, Ribeck, and Lenski 2013). Moreover, fitness trajectories in other environments have varied considerably (Travisano, Vasi, and Lenski 1995). Few beneficial mutations have fixed in all populations (Stanek, Cooper, and Lenski 2009; Blount et al. 2012). Indeed, each population has accumulated unique sets of mutations, including gross changes such as IS insertions, inversions, and deletions, as well as SNPs (Papadopoulos et al. 1999; Barrick et al. 2009). Six populations have

evolved high mutation rates due to substitutions in their mutation repair pathways (Sniegowski et al. 2000; Barrick and Lenski 2009; Barrick et al. 2009; Blount et al. 2012). Even those genes mutated in parallel across the populations typically differ in the location and type of mutation involved (V. S. Cooper et al. 2001; T. F. Cooper, Rozen, and Lenski 2003; Crozat et al. 2005; Pelosi et al. 2006; Woods et al. 2006). Moreover, while all populations have experienced the decay of some metabolic capacities, the capacities impacted, as well the extent of the decay, and their genetic bases have varied (V. S. Cooper and Lenski 2000; Ostrowski, Woods, and Lenski 2008).

The pattern of extensive parallelism with some divergence observed during the LTEE is typical of other PREs. Convergence to similar levels of fitness in the experimental environment (Bull et al. 1997; Fong, Joyce, and Palsson 2005; Le Gac et al. 2013; Riley et al. 2001; Treves, Manning, and Adams 1998), though not in other environments (Melnik and Kassen 2011), is remarkably common across experiments (Korona et al. 1994; Dettman et al. 2012; Kawecki et al. 2012). As in the LTEE, variation in other environments among evolved populations is common (Melnik and Kassen 2011), suggesting that parallel adaptation can be accomplished via different genetic routes. Indeed, while parallelism is seen at the level of adaptive genetic changes, it is not generally pervasive, though it is sometimes seen even at the level of specific nucleotide changes in organisms with very simple genomes (C. J. Brown, Todd, and Rosenzweig 1998; Herring et al. 2006; Bollback and Huelsenbeck 2009; Betancourt 2009; Wichman and Brown 2010). This preponderance of gross convergence in PREs strongly suggests that there are often multiple available genetic paths to similar phenotypic and adaptive states. Such multiple genetic paths mean that phenotypic similarities can mask significant genetic differences that may carry significant consequences for subsequent evolution (Bedhomme, Lafforgue, and Elena 2013). Two LTEE populations, designated Ara-2 and Ara-3, have diverged profoundly from the other ten, demonstrating that seemingly incidental genetic differences can significantly impact evolutionary outcomes.

The Ara-2 population evolved a unique ecology that has supported a long-term polymorphism.

Two monophyletic cell lineages that arose before generation 6,000 have co-existed for more than 50,000 generations. The coexistence between these two lineages, referred to as “S” and “L” for their respective small and large colony morphologies, has been maintained by negative frequency-dependent selection based on their ecological differences (Rozen and Lenski 2000). L cells are more fit than S cells in the abundant glucose environment encountered immediately after transfer. However, L cells experience higher mortality after glucose exhaustion, when S cells are more fit due to cross-feeding on substances released by lysed L cells (Rozen, Schneider, and Lenski 2005; Rozen et al. 2009). New evolution occasionally allows L to encroach on the S niche, after which S has invariably evolved to counter the encroachment. The result is a dynamic, fluctuating relationship driven by recurrent rounds of evolution that maintains diversity and is perhaps leading to incipient speciation (Rozen, Schneider, and Lenski 2005; Cohan and Perry 2007; Rozen et al. 2009; Le Gac et al. 2012). The case of Ara-2 suggests that, just as potentially important genetic differences can be masked by the superficially convergent adaptive states of evolving populations, so too can differences in population structure and ecology that should be taken into account when evaluating convergence and divergence in PREs.

The divergence of Ara-3 is even more striking. A cell lineage in Ara-3 evolved the capacity to exploit an open ecological opportunity provided by the large amount of citrate added to DM25 as a chelator to facilitate the bacteria’s acquisition of iron in the medium (Cox et al. 1970; Frost and Rosenberg 1973; Hussein, Hantke, and Braun 1981). The concentration of citrate is far higher than is necessary for this role due to DM having been developed for a particular set of experiments conducted before citrate’s biological role in *E. coli* medium was known (Davis 1949; Davis and Mingioli 1950; Blount forthcoming). The citrate is a potential carbon and energy source, but *E. coli* is unable to transport citrate into the cell during aerobic growth, preventing it from being used as a food source despite having a complete TCA cycle, and the ability to ferment citrate anaerobically (Lara and Stokes 1952; Lütgens and Gottschalk 1980; Pos, Dimroth, and Bott 1998). This Cit<sup>-</sup> phenotype is a very stable diagnostic characteristic of *E. coli* as a species, and spontaneous aerobic citrate using (Cit<sup>+</sup>)

mutants are extraordinarily rare (B. G. Hall 1982; Scheutz and Strockbine 2005).

After 33,000 generations, the Ara-3 population became several-fold larger, as Cit<sup>+</sup> variants rose to dominance in the population. These variants had evolved approximately 2,000 generations earlier due to a 2933 bp duplication that contains part of the *cit* operon regulating citrate fermentation (Pos, Dimroth, and Bott 1998; Blount et al. 2012). The duplication placed the anaerobically-expressed *citT* gene, which encodes a citrate-succinate antiporter, under the control of a promoter that normally regulates an aerobically-expressed gene, *rnk*. The new *rnk-citT* regulatory module supports weak aerobic expression of the CitT transporter, which provides marginal access to the citrate resource (Blount et al. 2012). The Cit<sup>+</sup> variants remained at low frequency until refining mutations arose, including further amplification of the duplicated segment increased dosage of the *rnk-citT* module, changes in expression of the DctA succinate transporter, and changes in carbon flow through central metabolism, all of which improved growth on citrate, producing a stronger citrate-using phenotype referred to as Cit<sup>++</sup> (Blount et al. 2012; Quandt et al. 2013; Quandt et al. under review). The Cit<sup>+</sup> variants did not sweep to fixation when they rose to numerical dominance. Instead, a small Cit<sup>-</sup> subpopulation persisted through at least generation 40,000 by evolving to cross-feed on succinate and other substances released into the medium by the Cit<sup>+</sup> cells (Blount, Borland, and Lenski 2008; Blount et al. 2012; C. B. Turner et al. under review). This long-term co-existence, along with the fact that Cit<sup>+</sup> exceeds the accepted range of variation for *E. coli*, suggests that the Cit<sup>+</sup> lineage may be an incipient species.

The Cit<sup>+</sup> trait has been experimentally demonstrated to be historically contingent. Contingent traits require multiple, non-uniquely beneficial mutations before manifestation (Blount, Borland, and Lenski 2008). Cumulative selection cannot directly facilitate the accumulation of these necessary mutations, which must instead occur as chance product of a population's evolutionary history. Contingent traits should be rare, as the necessary antecedent history is unlikely, and delayed with respect to the presentation of the ecological opportunity or environmental challenge to which they

provide access or adaptation (Foote 1998). The evolution of Cit<sup>+</sup> was therefore hypothesized to have been multi-step and contingent upon the prior accumulation of one or more mutations that produced a “potentiating” genetic background in which the rate of mutation to Cit<sup>+</sup> was much higher than in the ancestor. Consistent with this hypothesis, a series of experiments in which evolution was “replayed” from clonal genotypes isolated from various time points in Ara-3’s fossil record showed that re-evolution of the Cit<sup>+</sup> trait was much more likely to occur in replays started from later generation clones (Blount, Borland, and Lenski 2008). Fluctuation tests later showed that the ancestor’s Cit<sup>+</sup> mutation rate is immeasurably small, with an *upper bound* of  $\sim 3.6 \times 10^{-13}$  per cell per generation, while later clones have a measurable rate with a point estimate of  $\sim 6.6 \times 10^{-13}$ . The ancestral and potentiated clones have the same background mutation rate, so potentiation is not attributable to general hypermutability. Although the potentiated rate is still orders of magnitude lower than a typical mutation rate, the increase was sufficient to make the Cit<sup>+</sup> function mutationally reachable (Blount, Borland, and Lenski 2008).

The genetic basis of potentiation has not yet been determined, but suggestive details about potentiation have been uncovered. A population phylogeny based on fossil genome sequences shows that Ara-3 was diverse over much of its pre-Cit<sup>+</sup> history. Three clades, C1, C2, and C3, arose between 10,000 and 20,000 generations and then co-existed until some point after Cit<sup>+</sup> became dominant. Ecological divergence between the clades may explain this co-existence, though pre-Cit<sup>+</sup> ecology has not yet been investigated. C1 diverged before 15,000 generations, and then C2 and C3 diverged from each other before 20,000 generations. The Cit<sup>+</sup> lineage later arose from C3. Clones from all three clades yielded Cit<sup>+</sup> mutants during the replay experiments, but C3 is significantly overrepresented among them. These findings suggest that potentiation involved at least two mutations, the first of which occurred prior to C1’s divergence, and a second that occurred in C3 (Blount et al. 2012).

In principle, Cit<sup>+</sup> evolution might have been potentiated by either physical promotion of the *cit* duplication, or by functional epistatic interactions that made the *rnk-citT* module effective when it

occurred, likely by improving citrate metabolism (Blount, Borland, and Lenski 2008; Blount et al. 2012).<sup>2</sup>  $Cit^-$  clones transformed with a high copy number plasmid containing a complete *rnk-citT* module display a  $Cit^+$  phenotype. However, C3 transformants show much stronger and consistent  $Cit^+$  phenotypes than do those from the other two clades. The second potentiating mutation therefore appears to have worked by functional epistasis. Moreover, with one exception, mutations responsible for the  $Cit^+$  phenotypes of the  $Cit^+$  mutants isolated during the replay experiments are all different, though all involve *citT*. Many of the mutations involved the capture of other promoters, which is difficult to explain by physical promotion (Blount et al. 2012). It thus appears that the first potentiating mutation was also functionally epistatic, and the variety of promoters that can be coopted for *CitT* expression argues that potentiation was at the level of improved citrate metabolism. These findings pose the interesting possibility that the potentiating mutations were originally adaptive to the pre- $Cit^+$  ecological conditions of Ara-3 (Quandt et al. 2013). A similar interplay between ecology, coevolution, and epistatic genetic changes has been implicated in the contingent evolution of the capacity of phage lambda to infect through an alternate host cell surface receptor (Meyer et al. 2012).

### <A> Historical Difference Experiments

The causal dependence aspect of contingency holds that a lineage's prior evolutionary, ecological, and environmental history should leave an indelible mark in its genome that can alter and constrain its evolutionary potential (Gould 2002; Beatty 2006b). Historical difference experiments examine this aspect of contingency (Beatty 2006b). While PREs examine populations evolving from the same genotypic and historical point, HDEs examine populations evolving from different points. HDEs typically involve variations on a two-step design (figure 10.2B). Initially identical populations

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2        Contra Desjardins (this volume), both hypotheses implicate history, and differ only in whether the importance of history was in rendering possible the final necessary mutation itself (physical promotion) or the final mutation's effect (functional epistasis). Either way, historical sequences that included the prior occurrence of the potentiating mutation or mutations and the events and processes that maintained them in the population were necessary for the  $Cit^+$  function to evolve.

are first founded and evolved for some length of time under a given condition or conditions (Travisano et al. 1995; Collins, Sültemeyer, and Bell 2006). In the second step, new sets of populations are founded from those evolved in the first step, evolved under one or more new conditions, and then various traits compared. The first step therefore serves to generate different histories for the test organisms, while the second tests the evolutionary impacts of those historical differences. In some experiments, different strains or organisms with long histories outside the lab are used, which circumvents the need for the first step (F. B.-G. Moore and Woods 2006). In another experiment populations founded with clones isolated from the same population that had evolved different competing alleles were used to examine the evolutionary consequences of these alleles (Woods et al. 2011). The experiment described by Kacar (this volume) can also be seen as a form of HDE, albeit with the differences in history between phase two genotypes being limited to a single locus. In all cases, the central prediction from contingency is that differences accrued during prior histories will have detectable consequences during the second phase of evolution (Travisano et al. 1995).

The HDE design was originally developed by Travisano et al. (1995) to evaluate the roles of adaptation, chance, and history in evolution. In one experiment, a single clone was isolated from each of the LTEE populations discussed above after 2,000 generations of evolution on glucose. Each derived clone was used to found new populations that were then evolved for 1,000 generations in maltose-limited medium. The founding clones had similar fitness on glucose, but varied significantly in both cell size and fitness on maltose (Travisano et al. 1995; Travisano, Vasi, and Lenski 1995). These two traits were again assessed after the maltose evolution phase. Adaptation was expected to cause the populations to evolve by approximately the same magnitude in the same direction, while the persistence of significant trait differences between the clones noted before maltose evolution would suggest the lingering effects of history, and chance would be indicated by variation in mean trait value for populations founded from the same founding clones. A nested ANOVA was used to determine the relative contributions of the three factors to the observed trait evolution. All populations evolved

similar fitness on maltose after 1,000 generations, which was overwhelmingly attributable to adaptation. While prior history did contribute significantly to the final fitness, its effect was largely swamped by adaptation, and chance showed no significant effect. By contrast, adaptation, chance, and history were all found to have significantly contributed to the final cell size. These findings led to the conclusion that adaptation can largely overcome history's effects for traits that are subject to strong selection, but that history's mark persists far more in those traits not under selection.

A number of subsequent HDEs have found similar patterns (Pérez-Zaballos et al. 2005; Collins, Sültemeyer, and Bell 2006; Bennett and Lenski 2007; Saxer, Doebeli, and Travisano 2010; Bedhomme, Lafforgue, and Elena 2013). Deviations from this pattern have also been observed (Bollback and Huelsenbeck 2009; Flores-Moya et al. 2012). Perhaps most significantly, populations that were founded from natural isolates of *E. coli* and evolved for 2,000 generations in a novel environment reached significantly different final fitness values and they did so at significantly different tempos (F. B.-G. Moore and Woods 2006). The isolates had diverged far more during prior history than those used in other studies, which suggests that, as Travisano et al. noted, longer prior histories might produce deeper effects than they had observed.

The above suggestion points to certain difficulties inherent in the HDE design. The HDE approach is designed around the idea that the prior history provided by the first phase of evolution may alter evolutionary potential that may be detected in the second phase. This design is predicated on the assumptions that the first phase of evolution will provide sufficient history to allow sufficient resolution of history's effect, that significant divergence between populations observed during the second phases may be attributed to the different histories arise from the first phase, and that the second phase will be long enough to adequately examine the effects of history. While performing both phases of HDE evolution in the lab provides control, it may preclude accruing sufficient history in the first phase to produce detectable effects. It is possible that the findings suggesting that history is generally swamped by adaptation are artifacts of this tradeoff. Using natural isolates can provide sufficient



history to yield better resolution, but that history is unknown. Of course, as ongoing PREs like the LTEE that may be used as the first evolution phase in HDEs accrue more history, this tradeoff may be ameliorated in the course of time.

Another problem is that the second phase itself constitutes a sort of PRE, so it is always possible that divergent evolution during it may reflect different histories of the populations within the second phase of evolution rather than any differences stemming from the first phase. (It is conceivable, for instance, that adaptation to the first phase condition would not involve any mutations that would impact final fitness or subsequent fitness in the second phase condition.) It is therefore problematic that few HDEs in the literature include control populations that are evolved only under the second phase condition, which would permit disentanglement of differences arising from the two sources of history. Finally, consideration should be given as to whether or not the second evolution phase in an HDE is long enough to detect the impact of divergent first phase history. For example, while a prior history of evolution at high CO<sub>2</sub> levels was not found to significantly impact fitness evolution in *Chlamydomonas* populations, these populations did not return to normal CO<sub>2</sub> uptake characteristics following back adaptation to ambient CO<sub>2</sub> levels (Collins, Sültemeyer, and Bell 2006). Similarly, while prior evolution of the tobacco etch potyvirus to different hosts did not prevent later and equivalent phenotypic adaptation to a common host, substantial historical effects on genotype were detected (Bedhomme, Lafforgue, and Elena 2013). Both of these findings suggest that prior history can have subtle effects that may impact evolution over longer time spans. These methodological shortcomings of currently documented HDEs should be considered in the design and performance of future experiments.

## **<A> Conclusions and Implications**

The question of the scope of historical contingency's impact on evolution is one with major implications for how evolution should be approached and explained (Beatty 1993; Sterelny and

Griffiths 1999). A growing body of microbial evolution experiments explicitly designed to examine historical contingency have begun to shed much needed empirical light on this question. However, the empirical examination of evolutionary contingency is still at a relatively early stage, and it would be premature to declare that current findings have actually resolved the question. Indeed, it remains to be seen how the findings from microbial evolution experiments into contingency may apply to the broader biological world and its greater complexity and opportunities for iteration and the interaction between lineages. Nonetheless, these experimental studies have improved our understanding of contingency's role in evolution, and point to considerations for future research. The following are the five points that I think are the most important going forward.

**1. The role of contingency in evolution is constrained.** PREs have shown that initially identical populations maintained in the same environment evolve remarkably in parallel along highly similar fitness trajectories (Lenski 2004; Kawecki et al. 2012; Stern 2013). This parallelism partially extends to the genetic level, where similar adaptive mutations often accrue across multiple populations. Similarly, HDEs have generally shown that short periods of lab evolution in one environment do not impede adaptation to another environment (Travisano et al. 1995). Natural selection therefore often seems to be capable of deterministically driving broadly similar evolutionary outcomes in spite of the historicity imparted by the core processes of evolution or the effects of prior evolution.

**2. Parallelism at one level can mask divergence at another.** Parallel evolution to highly similar or identical fitness is commonly seen in evolution experiments (Lenski 2004; Kawecki et al. 2012). However, similarities in fitness can conceal important differences between evolving populations. Parallelism at one level of assessment does not necessarily imply parallelism at other levels. Variation in both genotype and unused functions among populations with similar fitness is commonly observed in both PREs and HDEs. More dramatically, LTEE population Ara-2 evolved a complex ecology that maintained a balanced polymorphism. At the level of fitness, Ara-2 was evolving

in parallel with non-polymorphic populations, and yet it did so by following a different adaptive path (Wiser, Ribeck, and Lenski 2013). These observations highlight the difficulties inherent in defining evolutionary convergence and parallelism when there are multiple levels of analytic granularity (Currie 2012). Experiments that involve assessment of parallelism and divergence would benefit from better and more extensive means of doing so. Future research will be strengthened by the development of standard measures for quantifying the parallelism and divergence identifiable in PREs and HDEs at multiple levels, including fitness, phenotype, genotype, and evolved ecology.

**3. The role of historical contingency intrinsic to the evolutionary process can be understood in terms of evolvability.** Evolvability has come to be a topic of great interest in evolutionary biology in recent years. In general, evolvability refers to the capacity of a genotype to produce new heritable variation by mutation, though some more restrictive definitions focus on the capacity to produce adaptive variation (Pigliucci 2008a). Though it has received little notice, there is clearly a deep connection between evolvability and historical contingency arising from core evolutionary processes, as both deal with evolutionary potential.

In the absence of gene flow, natural selection can only act on the variation that arises by mutation of the genomes extant within a population. However, not all variation is equally reachable from all genotypes. From any given genotype, there is a range of possible variants that can arise at frequencies determined by the number of mutations necessary to reach them and the rates at which those mutations occur. Prior history constructs the genotype and impacts future evolution by determining that from which variation arises, and thereby what variation can reasonably arise. Prior history can also alter background mutation rates, which is most obvious in the case of mutator genotypes. In so doing, history can have two possible effects. The first is potentiation, as was demonstrated in the case of  $Cit^+$  evolution in the LTEE. In the case of a variant that requires multiple mutations, history can potentiate that variant's evolution by coincidental accumulation of one or more

of the necessary mutations, thereby increasing the frequency at which the variant arises. Such a variant may be of no consequence evolutionarily, but it might confer a rare refinement of an existing trait. It might also confer a novel trait that can, like  $Cit^+$ , cause lineages to chance upon previously inaccessible novel functions that can grant access to wildly different evolutionary paths. The second effect is depotentialization, in which history reduces the likelihood of evolving a given variant. The historical accumulation of mutations that must either be reverted or compensated for in order for a variant to occur is one mechanism by which depotentialization may occur. Depotentialization is therefore related to functional loss due to mutation accumulation (V. S. Cooper and Lenski 2000). Once a mutation disables a gene required for a given function, for instance, the re-evolution of that function would be de-potentialized by the accumulation of further disruptive mutations. (Deletion, of course, may completely eliminate the possibility of re-evolution, unless there another gene or pathway that might be coopted for restored function.) Epistatic interactions have also been implicated in depotentialization. Woods et al. (2011) showed that one of two competing alleles in an LTEE population went extinct despite a significantly higher fitness benefit because its epistatic effects eliminated the fitness benefit and depotentialized the selection of a potential later mutation. Potentialization and de-potentialization therefore contribute to the changing capacity of a population to generate variation that can alter the potential for adaptation in a range of environments. This is to say that potentialization and depotentialization alter evolvability, and evolvability may therefore be a valuable way to understand and approach evolutionary contingency (Lenski, Barrick, and Ofria 2006; Colegrave and Collins 2008; Pigliucci 2008a). A synthesis between the concepts of evolvability and contingency would no doubt be helpful to future empirical and theoretical work on evolutionary contingency.

**4. History is a progressive factor in evolution.** Building on the above, as history accumulates, the effects of potentialization and de-potentialization should increase and vary, altering the evolutionary potential of the lineage to follow various trajectories. History's impacts on evolution should therefore

be progressive, leaving deeper marks over time. As Desjardins has observed, historicity comes in degrees (this volume). Moreover, history's impacts may be subject to threshold effects such that, in which avenues for future evolution may be qualitatively closed or opened by the accumulation of interacting mutations in a manner unpredictable from their subsets. In other words, history may not matter until it does matter. This is all the more so when it is considered that contingency can have impacts on multiple levels of the evolutionary process (Erwin this volume). It is therefore difficult to come to valid conclusions about contingency's potential impact on evolution from short-term experiments, and this issue should be kept in mind in future experiments.

**5. Evolutionary contingency researchers need to collaborate more broadly.** Empirical research into evolutionary contingency would benefit greatly by improved interdisciplinarity. For instance, greater collaboration between researchers using the microbial experimental evolution approach I have discussed here, paleontologists, and those who study contingency in macro-organisms is certainly called for. Systems biologists could help to develop a better and more theoretically-grounded understanding of how the complex interactions within evolving organisms impact and are impacted by contingency arising from the core evolutionary processes. Collaboration with molecular biologists and geneticists would similarly lead to better understanding of how particular molecular events and mutational processes factor into evolutionary contingency. Similarly, these researchers could help greatly in better defining convergence, and examining how divergence at the smallest level can impact later evolution.

This interdisciplinary approach to understanding contingency should not be limited to scientific fields. Historical contingency is a complex, multifaceted concept that is difficult to fully grasp and define. Gould's writings on evolutionary contingency display some of the confusion this complexity engenders. Gould never offered a single, technical definition of evolutionary contingency, and he, apparently unknowingly, articulated at least two, very different notions of contingency (Gould 1989;

Gould 2002; Beatty 2006b; Beatty and Carrera 2011; D. D. Turner 2011). Most experimental work on contingency is based on individual readings of Gould's writings, leading to a situation in which different researchers have designed experiments based on different notions of contingency without necessarily making this clear. This semantic discord has produced understandable confusion that has made it difficult to meaningfully synthesize the various interesting and illuminating experimental findings about evolutionary contingency. I propose that these conceptual difficulties could best be overcome and the study of contingency advanced by collaboration between evolutionary biologists and philosophers of science. The complexity and difficulty inherent to historical contingency makes it the sort of conceptual tangle philosophers excel at analyzing and parsing (Pigliucci 2008b). Indeed, historical contingency is a philosophically-rich area that has been the subject of much recent work (Beatty 1993; Beatty 2006b; Beatty and Desjardins 2009; Beatty and Carrera 2011; Currie 2012; Desjardins 2011; Desjardins this volume; Sterelny and Griffiths 1999; D. D. Turner 2011). These philosophers have thought deeply about the issues contingency researchers seek to address, and might help to unpack ideas of evolutionary contingency, delineate subsidiary issues within the concept, develop rigorous definitions, and trace interesting conceptual implications while also perhaps helping to guide and structure productive interdisciplinary collaborations. This conceptual work could then be used to develop more precise empirical questions and design better experiments, the results of which could then be integrated into a more coherent and unified understanding of evolutionary contingency. Indeed, a full understanding of evolutionary contingency may be contingent on such a collaboration.

### **<A> Acknowledgments**

I would like to thank my mentor Richard Lenski and lab manager Neerja Hajela for years of support and guidance, and Justin Meyer, Caroline Turner, Erik Quandt, John Beatty, Robert Pennock, David Bryson, Sabrina Mueller-Spitz, and Betül Kaçar for discussions that were helpful in the

development of the ideas behind this paper. I thank Richard Lenski and two anonymous reviewers for their suggestions on this paper. I acknowledge funding from a John Templeton Foundation Foundational Questions in Evolutionary Biology grant (FQEB #RFP-12-13), the BEACON Center for the Study of Evolution in Action (NSF Cooperative Agreement DBI-0939454), and the U.S. National Science Foundation, which has funded the LTEE (NSF; DEB-1019989).

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