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Rapid Evolution of Citrate Utilization by *Escherichia coli* by Direct Selection Requires *citT* and *dctA*

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ABSTRACT

The isolation of aerobic citrate-utilizing *Escherichia coli* (Cit$^+$) in long-term evolution experiments (LTEE) has been termed a rare, innovative, presumptive speciation event. We hypothesized that direct selection would rapidly yield the same class of *E. coli* Cit$^+$ mutants and follow the same genetic trajectory: potentiation, actualization, and refinement. This hypothesis was tested with wild-type *E. coli* strain B and with K-12 and three K-12 derivatives: an *E. coli* Δ*rpoS*::*kan* mutant (impaired for stationary-phase survival), an *E. coli* Δ*citT*::*kan* mutant (deleted for the anaerobic citrate/succinate antiporter), and an *E. coli* Δ*dctA*::*kan* mutant (deleted for the aerobic succinate transporter). *E. coli* underwent adaptation to aerobic citrate metabolism that was readily and repeatedly achieved using minimal medium supplemented with citrate (M9C), M9C with 0.005% glycerol, or M9C with 0.0025% glucose. Forty-six independent *E. coli* Cit$^+$ mutants were isolated from all *E. coli* derivatives except the *E. coli* Δ*citT*::*kan* mutant. Potentiation/actualization mutations occurred within as few as 12 generations, and refinement mutations occurred within 100 generations. Citrate utilization was confirmed using Simmons, Christensen, and LeMaster Richards citrate media and quantified by mass spectrometry. *E. coli* Cit$^+$ mutants grew in clumps and in long incompletely divided chains, a phenotype that was reversible in rich media. Genomic DNA sequencing of four *E. coli* Cit$^+$ mutants revealed the required sequence of mutational events leading to a refined Cit$^+$ mutant. These events showed amplified *citT* and *dctA* loci followed by DNA rearrangements consistent with promoter capture events for *citT*. These mutations were equivalent to the amplification and promoter capture CitT-activating mutations identified in the LTEE.

IMPORTANCE

***E. coli* cannot use citrate aerobically. Long-term evolution experiments (LTEE) performed by Blount et al. (Z. D. Blount, J. E. Barrick, C. J. Davidson, and R. E. Lenski, Nature 489:513–518, 2012, http://dx.doi.org/10.1038/nature11514) found a single aerobic, citrate-utilizing *E. coli* strain after 33,000 generations (15 years). This was interpreted as a speciation event. Here we show why it probably was not a speciation event. Using similar media, 46 independent citrate-utilizing mutants were isolated in as few as 12 to 100 generations. Genomic DNA sequencing revealed an amplification of the *citT* and *dctA* loci and DNA rearrangements to capture a promoter to express CitT, aerobically. These are members of the same class of mutations identified by the LTEE. We conclude that the rarity of the LTEE mutant was an artifact of the experimental conditions and not a unique evolutionary event. No new genetic information (novel gene function) evolved.**

How genetic information evolves to generate new phenotypes/species is a central issue in biology. Long-term evolution experiments (LTEE) using microorganisms have been initiated by several groups, in part to empirically observe this phenomenon (1). LTEE using bacteria, bacteriophage, or yeast have distinct advantages that include high population numbers, rapid generation times, and the opportunity to freeze intermittent populations (frozen fossils) to track mutations over time. Coupled with whole-genome sequencing, evolutionary changes can be genetically characterized to identify a mutation(s) required for a specific phenotypic change and frozen intermediates can be revived to replay and confirm the events. The most famous and meticulously documented LTEE are those, initiated in 1988, in Richard Lenski's laboratory (2). Twelve parallel cultures of *Escherichia coli* REL606 (an *E. coli* B strain) have been growing aerobically in minimal salts medium with low glucose concentrations (0.0025%) for 27 years. Cultures are transferred daily into fresh medium. Frozen samples are preserved for each culture every 500 generations, providing a tremendous resource to study long-term bacterial adaptation under controlled conditions.

To date, the Lenski LTEE cultures exceed 63,000 generations, equivalent to over 1 million years of human evolution. Importantly, these experiments negate Gould's theory of contingency, i.e., the idea that if evolution were replayed, different outcomes would arise (3). All 12 cultures are undergoing surprisingly similar genetic trajectories, with one exception. After 15 years (33,000 generations), 1 of the 12 cultures increased in turbidity by utilizing citrate aerobically (Cit$^+$) (4). The minimal medium in these experiments contains 1.7 mM citrate as a chelating agent. It is well known that wild-type *E. coli* cannot use citrate as a carbon source, aerobically, because it lacks a citrate transporter, but can use citrate under anaerobic conditions via expression of the CitT citrate/succinate antiporter (5–9). Genetic analysis of Lenski's Cit$^+$

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strain showed amplification of the *citT* locus, coupled with a promoter capture event of the upstream constitutive *rnk* promoter, allowing oxic CitT expression. Replay experiments from frozen fossils have shown a recurring common theme: amplification of *citT* followed by a promoter capture of a neighboring gene or an acquisition of an insertion element upstream of *citT*, either of which allows aerobic expression. These mutations account, in part, for the novel Cit^+ phenotype. A point mutation in *dctA* (dicarboxylic acid transporter for succinate, fumarate, and malate) at position −20 from the *dctA* translational start is also a necessary mutation for efficient citrate utilization, as it allows recapture of succinate exported during citrate uptake (10). Importantly, adaptation to aerobic citrate use follows three evolutionary stages as defined by Lenski: potentiation, actualization, and refinement (10, 11). Potentiation involves mutations that may not have a discernible phenotype but that prepare the cell for future mutations leading to phenotypic change. Actualization involves a subsequent mutation(s) that generates the phenotype. Refinement is the further optimization of the phenotype once established in a population. The potentiating changes in the Cit^+ phenotype are under active investigation by Lenski and others (12) and are not yet fully documented, but the *citT* promoter capture represents actualization and the *dctA* mutation represents refinement. Some authors assert that this evolved *E. coli* Cit^+ strain represents an exceedingly rare, innovative gain-of-function mutation and argue for recognition of this *E. coli* variant as a newly evolved species (3, 13). This argument is made, in part, because citrate utilization is a key diagnostic to differentiate *E. coli* from other coliform species.

In this study, we challenged the interpretation that aerobic utilization of citrate by *E. coli* is necessarily a rare or innovative mutation warranting classification as an example of speciation. We hypothesized that the isolation of such mutants should be relatively easy, would follow the same genetic trajectory as that identified in the LTEE, and would utilize information present on the chromosome and not involve evolution of new information (novel gene function). As such, we predicted that the extremely long time required for *E. coli* to evolve to Cit^+ status was due to the LTEE conditions and not to potentiating genetic events requiring 33,000 generations. To test our hypothesis, direct selection was used. Our rationale was based on the serendipitous outcome of Hall's 1982 experiments designed to isolate *E. coli* K-12 cells able to use phenyl-arabinose (14). After 14 days of aeration in minimal medium with phenyl-arabinose, cell numbers increased. However, the cells were growing on the citrate chelator in the medium, like the Cit^+ strain from Lenski's LTEE, not the intended phenyl-arabinose substrate. Hall determined that at least two mutations are required for citrate utilization and, by transduction, mapped the mutation to the citrate operon cluster of the *E. coli* chromosome. He also speculated that these mutations activated the anaerobic citrate transporter. The adaptation was not documented by DNA analysis because genomic sequencing capabilities were not then available, nor is it clear that the Cit^+ phenotype had undergone refinement. In retrospect, Hall's experiment can be interpreted as representing unintended "direct selection" for Cit^+ mutants. The Lenski LTEE can be interpreted as representing an unintended genetic "screen" for Cit^+ mutants. Direct selection is defined as selection occurring under conditions in which only the desired mutant can grow, while a genetic screen is defined as a screen occurring under conditions in which both the desired mutant and its parent can grow. We speculated that the difference in selective conditions could account for the days that it took Hall versus the years that it took Lenski to acquire the Cit^+ phenotype.

To test our hypothesis, we did direct selection experiments in batch culture and modified direct selection experiments with weekly serial transfers. We used wild-type *E. coli* K-12, three mutant derivatives of *E. coli* K-12 (an *E. coli* Δ*rpoS*::*kan* mutant [with the σ^{38} stress response protein deleted and stationary-phase survival impaired], an *E. coli* Δ*citT*::*kan* mutant [with an anaerobic citrate/succinate antiporter deleted], and an *E. coli* Δ*dctA*::*kan* mutant [with a dicarboxylic acid transporter deleted]) as controls, and two *E. coli* B strains (B and REL606). Three medium variants were used to isolate Cit^+ mutants: (i) M9 minimal medium with citrate (6.8 mM) as the sole carbon source (M9C), (ii) M9C amended with a low concentration of glycerol (0.005%) (M9CG50) containing either 6.8 mM or 1.7 mM citrate (M9LC50), and (iii) M9C amended with a low concentration of glucose (0.0025%) (M9C25) containing either 6.8 mM or 1.7 mM citrate (M9LC25). The last medium, M9LC25, was equivalent to that used in the Lenski LTEE (2). Batch and serial transfer selection cultures were incubated aerobically, and growth was measured by turbidity and plate counts. Citrate utilization was detected using differential media and quantified by mass spectrometry (MS) of medium filtrates during various stages of growth. Cit^+ mutants were phenotypically characterized by growth dynamics and microscopy and were genetically analyzed by transduction and by genomic and regional DNA sequencing. These experiments also provided the opportunity to compare the evolutionary trajectories of direct selection and a long-term genetic screen.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. In order to use the *E. coli* K-12 Keio mutant collection that provides kanamycin (Kn)-marked mutations in every nonessential gene, systematic analyses of citrate utilization were done with this strain. The *rpoS* mutant, *dctA* mutant, and *citT* mutant control strains had *E. coli* K-12 kanamycin-marked mutations that allowed transduction experiments (see below). *E. coli* REL606 is the strain used in Lenski's LTEE. Stock cultures were maintained on Luria-Bertani (LB) agar and grown in LB broth (Difco, Detroit, MI). Directly selective citrate medium consisted of M9 mineral salts amended with 6.8 mM citrate and 50 μM thiamine (M9C). This 0.2% citrate concentration (6.8 mM versus M9 chelator concentration of 1.7 mM) is standard for a sole carbon source. It also has the added advantage of supporting more *E. coli* cell division than chelating concentrations such that initial mutants would be visually more apparent during selection. In addition, citrate at 6.8 mM or higher is used in diagnostic media to differentiate *E. coli* from citrate-utilizing coliforms. The modified directly selective citrate media consisted of M9C amended with either 0.005% glycerol (M9CG50) or 0.0025% glucose (M9C25). To reproduce the LTEE conditions, these media were also used with the lower (1.7 mM) citrate concentration (M9LC25 or M9LCG50, respectively). Colonies were isolated on LeMaster Richards minimal media supplemented with 6.8 mM citrate (15) and solidified with 1.5% Nobel agar (LRC), Simmons citrate (Becton Dickinson, Franklin Lakes, NJ), or Christensen citrate agars (Sigma, St. Louis, MO). The last two media are selective differential media used to diagnostically detect citrate utilization in enteric bacteria. In Simmons citrate agar, citrate (6.8 mM or 0.2%) is the sole carbon source and citrate-metabolizing organisms cleave citrate to oxaloacetate and acetate. Oxaloacetate dehydrogenase then converts oxaloacetate to pyruvate and CO_2, and the CO_2 is converted to sodium carbonate, resulting in an alkaline pH shift and conversion of the pH indicator, bromthymol blue, from green (neutral) to blue (alkaline). Wild-type *E. coli* does not grow on Simmons citrate agar or does so min-

TABLE 1 *E. coli* strains used in this study

Strain or phage	Genotype or parent strain and description[a]	Source
Strains		
MG1655	*rph-1* (wt)	E. Top, University of Idaho collection
JW0604-1	F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) Δ*citT750*::*kan* λ$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Keio collection, Yale University
JW3496-1	F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*dctA783*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Keio collection, Yale University
JW5437-1	F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*rpoS746*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Keio collection, Yale University
B	(wt) F$^-$	Coli Genetic Stock Center, Yale University
REL606	F$^-$ *tsx-467*(Am) *araA230 lon rpsL227* (Str^r) *hsdR* [*mal*$^+$]$_{K\text{-}12}$(λ^S)	Coli Genetic Stock Center, Yale University
Cit$^+$ mutants		
Mutants obtained by direct selection in M9C		
DV159	MG1655 (wt), Cit$^+$ at 30 days	
DV160	MG1655 (wt), Cit$^+$ at <40 days	This study
DV133T	MG1655 (wt), phage transduction from DV133	
SO191	JW3496-1 (Δ*dctA*), Cit$^+$ at 134 days	This study
DV130	JW5437-1 (Δ*rpoS*), Cit$^+$ at 43 days	
DV133	JW5437-1 (Δ*rpoS*), refined from DV130 via several cycles on SC agar	This study
DV162	JW5437-1 (Δ*rpoS*), Cit$^+$ at <40 days	This study
DV268	JW5437-1 (Δ*rpoS*), Cit$^+$ at <40 days	This study
Mutants obtained by modified direct selection in M9C25 (glucose supported)		
DV247	MG1655 (wt), Cit$^+$ at 31 days	This study
DV290	MG1655 (wt), Cit$^+$ at 35 days	This study
DV291	MG1655 (wt), Cit$^+$ at 35 days	This study
DV309	MG1655 (wt), Cit$^+$ at 42 days	This study
DV312	MG1655 (wt), Cit$^+$ at 42 days	This study
DV313	MG1655 (wt), Cit$^+$ at 42 days	This study
DV314	MG1655 (wt), Cit$^+$ at 42 days	This study
DV315	MG1655 (wt), Cit$^+$ at 42 days	This study
DV409	MG1655 (wt), Cit$^+$ at 77 days	This study
DV344	JW5437-1 (Δ*rpoS*), Cit$^+$ at 68 days	This study
Mutants obtained by modified direct selection in M9CG50 (glycerol supported)		
DV179	MG1655 (wt), Cit$^+$ at 28 days	This study
DV215	MG1655 (wt), Cit$^+$ at 21 days	This study
DV172	JW5437-1 (Δ*rpoS*), Cit$^+$ at 19 days	This study
DV216	JW5437-1 (Δ*rpoS*), Cit$^+$ at 21 days	This study
DV351	JW5437-1 (Δ*rpoS*), Cit$^+$ at 35 days and refined from papillated colony	This study
Mutants obtained by modified direct selection in M9LC25 (glucose supported, 1.7 mM citrate [low citrate])		
DV564	MG1655 (wt), Cit$^+$ at 40 days	This study
DV596	MG1655 (wt), Cit$^+$ at 56 days	This study
DV600	MG1655 (wt), Cit$^+$ at 56 days	This study
DV592	MG1655 (wt), Cit$^+$ at 63 days	This study
DV593	MG1655 (wt), Cit$^+$ at 63 days	This study
DV594	MG1655 (wt), Cit$^+$ at 63 days	This study
DV595	MG1655 (wt), Cit$^+$ at 63 days	This study
DV599	MG1655 (wt), Cit$^+$ at 63 days	This study
DV601	MG1655 (wt), Cit$^+$ at 63 days	This study
DV602	MG1655 (wt), Cit$^+$ at 63 days	This study
DV603	MG1655 (wt), Cit$^+$ at 63 days	This study
DV604	REL606, Cit$^+$ at 63 days	This study

(Continued on following page)

TABLE 1 (Continued)

Strain or phage	Genotype or parent strain and description	Source
Mutants obtained by modified direct selection in M9LCG50 (glycerol supported, 1.7 mM citrate [low citrate])		
DV545	MG1655 (wt), Cit^+ at 35 days	This study
DV546	MG1655 (wt), Cit^+ at 35 days	This study
DV571	MG1655 (wt), Cit^+ at 40 days	This study
DV572	MG1655 (wt), Cit^+ at 41 days	This study
DV605	B (wt), Cit^+ at 63 days	This study
DV608	B (wt), Cit^+ at 63 days	This study
DV609	B (wt), Cit^+ at 63 days	This study
DV610	B (wt), Cit^+ at 63 days	This study
DV611	REL606, Cit^+ at 63 days	This study
DV613	REL606, Cit^+ at 63 days	This study
DV614	REL606, Cit^+ at 63 days	This study
DV615	REL606, Cit^+ at 63 days	This study
DV616	REL606, Cit^+ at 63 days	This study
Phage P1(*vir*)	Nonlysogenic transducing phage	P. Hartzell

[a] wt, wild type; SC agar, Simmons citrate agar.

imally, producing pinpoint yellow colonies (acidic) after 1 week of incubation. Christensen citrate agar (0.3% citrate) also contains a low concentration of glucose (0.01%) and the pH indicator phenol red. *E. coli* (citrate negative) grows on the limited-glucose agar to produce visible colonies in 18 to 24 h, but the colonies are colorless to slightly yellow (acidic). Citrate-positive organisms likewise first grow on the glucose and then convert to citrate metabolism, producing cerise (alkaline) colonies by the same reaction pathway as that described for Simmons citrate agar. All bacteria were stored at −80°C in LB or M9C containing 5% glycerol. Mutants were verified to be *E. coli* by comparing levels of growth on sorbitol MacConkey agar (Difco, Detroit, MI) with 4-methyl-umbelliferyl-β-D-glucuronide (SMAC-MUG) (16) or on eosin methylene blue (EMB) agar (Difco). Bacterial strains harboring kanamycin resistance (Kn^r) were grown where noted with 50 μg/ml kanamycin (Sigma, St. Louis, MO).

Selection for *E. coli* Cit^+ mutants. Three protocols were used to select *E. coli* Cit^+ mutants from four *E. coli* K-12 strains (the wild-type, Δ*rpoS*::*kan*, Δ*citT*::*kan*, and Δ*dctA*::*kn* strains) and two *E. coli* B strains (B and REL606). In the first method, each *E. coli* K-12 strain was grown overnight at 37°C with aeration in 5 ml of LB broth, after which cells were concentrated by centrifugation, and cell pellets were washed twice with M9 salts. Cells were resuspended in 50 ml of M9C broth in 250-ml Erlenmeyer flasks to give a final concentration of 5.0×10^7 to 7.0×10^7 CFU/ml and incubated at 37°C with aeration at 160 rpm. Growth was visually monitored daily for increased turbidity and measured by absorption at an optical density at 600 (OD_{600}). An aliquot of cells was removed weekly from each flask, quantified by triplicate plate counts on LB agar, and cryopreserved. During extended incubations, flasks were rehydrated with sterile distilled water weekly to compensate for evaporation.

The second two methods consisted of modified direct selections using 50 ml of M9C in 250-ml Erlenmeyer flasks, one amended with 0.005% glycerol (M9CG50) and the other amended with 0.0025% glucose (M9C25). Both carbon concentrations supported approximately six generations of growth (OD_{600} = 0.03). Flasks were incubated at 37°C with constant shaking for 1 week and then diluted (1:100) into fresh media. This cycle was repeated until the culture turbidity at OD_{600} increased to >0.6. Aliquots were cryopreserved weekly. These experiments were also conducted with 1.7 mM citrate to determine if the concentration of citrate affected mutant selection. These low-citrate media are designated M9LC25 (glucose) and M9LCG50 (glycerol).

In all variations of Cit^+ mutant selection, once the absorbance reached an OD_{600} of >0.07 (1.7 mM citrate-containing media) or >0.6 (6.8 mM citrate-containing media), an aliquot of the culture was diluted (1:100) into 10 ml of M9C medium and incubated with aeration until absorbance (OD_{600}, >0.07 or >0.6) was again observed. Individual colonies were isolated by streak dilution on LRC agar or diluted and plated on Simmons and Christensen citrate agars. Large colonies arising after 2 to 3 days of incubation on LRC agar were likewise tested on Simmons and Christensen citrate agars. Blue colonies on Simmons citrate agar (representing strong citrate use) were repurified by 12 h of growth in LB broth and plated on fresh Simmons citrate agar. Large isolated colonies were grown in M9C broth for cryopreservation. All Cit^+ mutants were verified to be *E. coli* by patching isolated colonies sequentially onto SMAC-MUG agar, Simmons citrate agar, and Christensen citrate agar.

P1(*vir*) bacteriophage transduction experiments. Transduction experiments were conducted with P1(*vir*) bacteriophage as described by Lennox (17) at a multiplicity of infection (MOI) of 0.1:1. Donor cell lysates were preserved at 4°C using chloroform. Phage titers were quantified by plaque counts done in triplicate by 10-fold dilutions in 4 ml of LB soft agar (0.4%) seeded with 100 μl of *E. coli* K-12 wild-type cells and overlaid on LB agar plates or by dropping 10 μl of 10-fold dilutions on LB soft agar overlays seeded with 100 μl of *E. coli* K-12 cells.

Mass spectrometry of culture filtrates for metabolite identification. To verify citrate utilization, putative Cit^+ isolates were inoculated into 5 ml of M9C broth. Cultures were incubated until exponential phase and were diluted (1:100) into 50 ml of M9C in 250-ml shake flasks. Growth was monitored in 6-h intervals by determining turbidity at OD_{600}. Additionally, 1-ml aliquots were centrifuged for each time point to remove cells and subjected to filter sterilization using 0.2-μm-pore-size filters, and subsets of these samples, correlating to the various stages of growth curves, were analyzed by mass spectrometry. The concentrations of citrate, succinate, acetate, fumarate, malate, glutamine, and indole were measured using reverse-phase chromatographic separation combined with multiple reaction monitoring methods for all of the analytes except acetate, where a single ion reaction monitoring method was used. The liquid chromatographic separation was done using a Waters Acquity ultraperformance liquid chromatography (UPLC) instrument equipped with a Waters Acquity UPLC BEH C_{18} column with 1.7-μm-particle-size packing. The column was 2.1 mm in diameter by 50 mm in length and was maintained at 28°C. A binary solvent was used where solvent A was 99.9% water with 0.1% formic acid and solvent B was 99.9% acetonitrile with 0.1% formic acid. The solvent flow was 100 μl/min throughout the course of the separation. The solvent-mixing program began with the ratio of 100% solvent A/0% solvent B. The concentration of solvent B was increased linearly to 3% over 3 min following injection. The gradient rate

TABLE 2 Mass spectrometry settings for precursor masses, product ion masses, cone voltages, collision voltages, and dwell times[a]

Analyte	Precursor *m/z*	Product *m/z*	Cone V	Collision V	Dwell time (s)
Acetate	58.8	SIR	28	NA	0.11
Lactate 1	88.74	42.87	14	10	0.11
Lactate 2	88.74	44.88	14	10	0.11
Fumarate 1	114.72	26.96	26	10	0.11
Fumarate 2	114.72	70.93	26	8	0.11
Succinate 1	116.87	72.94	26	10	0.11
Succinate 2	116.87	98.32	26	11	0.11
Malate 1	132.86	132.86	70.28	30	0.11
Malate 2	132.86	132.86	70.28	30	0.11
Glutamine 1	144.91	83.97	36	14	0.11
Glutamine 2	114.91	109	36	12	0.11
Citrate 1	190.87	190.87	86.9	28	0.11
Citrate 2	190.87	190.87	110.95	28	0.11
Indole 1	117.83	117.83	64.88	48	0.745
Indole 2	117.83	117.83	90.95	48	0.745

[a] SIR, single ion reaction; NA, not applicable.

was then increased, and the concentration of solvent B reached 80% at 14 min. The ratio of solvent A to solvent B was held constant for 1 min, the concentration of solvent A was returned to 100% over the next 2.5 min, and the column was allowed to equilibrate at 100% solvent A for 2.5 min before the next injection. A 10-μl injection volume was used for all standards and samples. The column eluent flow was passed through the flow cell of an Acquity photodiode array detector and then to the electrospray ionization (ESI) sprayer of a Waters Xevo TQ MS triple-quadrupole mass spectrometer. The mass spectrometry methods were programmed to acquire data in negative ion mode for all of the analytes except indole, where the data were collected in positive ion mode. The negative ion mode capillary voltage was held at 2.5 kV, and the positive ion mode capillary voltage was held at 3.4 kV. The desolvation gas flow was 400 liters/h, the desolvation temperature was 300°C, and the source temperature was 150°C. The organic acids eluted first, and data were acquired in the first 5 min of the chromatographic run. At 5 min, the method switched to positive ion mode and the data for indole were acquired within the next 10 min. The settings for precursor masses, product ion masses, cone voltages, collision voltages, and dwell times are listed in Table 2. All standards and samples were analyzed in triplicate. The data were integrated and analyzed using TargetLynx quantitation software from Waters Corp. An external calibration was used, and the standard data were fitted to quadratic curves. The samples, except the "media only" control, were brought to a 1% concentration of formic acid by dilution of 99 μl of clarified culture media with 1 μl of formic acid prior to analysis. The "media-only" sample was diluted 10-fold and brought to a 1% concentration of formic acid before analysis.

Whole-genome sequencing. Chromosomal DNA was prepared from *E. coli* Cit$^+$ mutants designated DV130, DV133, DV133T, and DV159 (Table 1) using a GenElute bacterial genomic DNA kit (Sigma, St. Louis, MO) or a bacterial genomic DNA extraction protocol (18). Genomic DNA samples (15 μg) were sequenced using the Pacific Biosciences RS II sequencing platform at the Washington State University genomics core laboratory or the Icahn School of Medicine at Mount Sinai (NY) genomics core sequencing facilities. The sequences were deposited in the National Center for Biotechnology Information Sequence Read Archive. Sequences were assembled *de novo* via HGAP3 (PacBio) and compared to the sequences of the genome of *E. coli* K-12 MG1655.

Microscopic analysis. Digital micrographs of cells were taken at ×400 magnification using a Nikon Microphot FXA phase microscope fitted with a Photometrics Cool Snap cf camera. Bacterial colonies were photographed using a Nikon dissecting microscope at ×20 magnification with the same camera.

Nucleotide sequence accession numbers. The GenBank accession numbers for the DV133, DV159, DV133T, and DV130 sequences determined in this work are CP014348, LRDF00000000, LRDE00000000, and LRDD00000000, respectively.

RESULTS

Multiple independent *E. coli* K-12 Cit$^+$ phenotypes were isolated by direct selection using M9C. Direct selection in M9C broth containing 0.2% citrate as the sole carbon source was performed. This experiment is analogous to that performed by Hall (14) in which *E. coli* was subjected to starvation conditions. Six independent *E. coli* Cit$^+$ isolates were recovered (Table 1 and Fig. 1). Experimental conditions consisted of four flasks inoculated with *E. coli* wild-type cells or *E. coli* Δ*rpoS*::*kan*, *E. coli* Δ*citT*::*kan*, or *E. coli* Δ*dctA*::*kan* mutant cells. Bacterial growth was visually monitored daily for increased turbidity and quantified weekly by plate counts on LB agar, and aliquots were cryopreserved weekly. Two Cit$^+$ mutants arose from the *E. coli* wild-type strain, three Cit$^+$ mutants arose from the *E. coli* Δ*rpoS*::*kan* mutant strain, and one Cit$^+$ mutant arose from the *E. coli* Δ*dctA*::*kan* mutant strain. Representative growth curves from two experiments are shown in

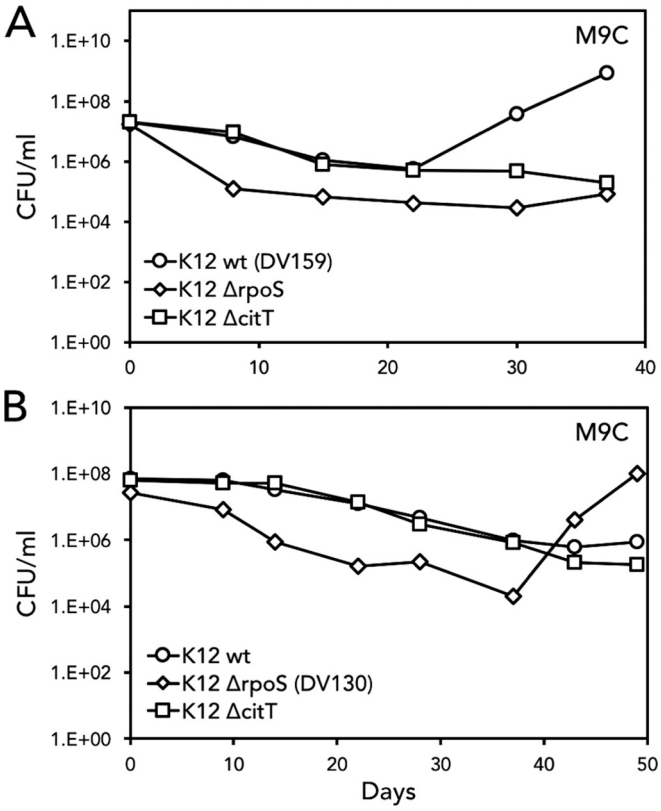

FIG 1 Direct selection of *E. coli* in minimal M9C yielded Cit$^+$ mutants for both *E. coli* wild-type and *E. coli* Δ*rpoS*::*kan* strains but not for the *E. coli* Δ*citT*::*kan* mutant strain. (A) Results of two separate representative experiments are shown. The *E. coli* wild-type (wt), *E. coli* Δ*rpoS*::*kan*, and *E. coli* Δ*citT*::*kan* strains were inoculated into individual 250-ml flasks containing 50 ml of M9 minimal medium with citrate as the sole carbon source. *E. coli* wild-type and *E. coli* Δ*citT*::*kan* levels decreased by 1 log until after day 23, when the wild-type strain (designated DV159) reinitiated exponential growth. (B) Results of a parallel experiment are shown. The level of the *E. coli* Δ*rpoS*::*kan* strain decreased by almost 2 log until after day 37, when it reinitiated exponential growth. This strain was designated DV130.

Fig. 1. During weeks of declining bacterial numbers, subpopulations presumably underwent potentiating mutations until the Cit$^+$ phenotype was actualized as evidenced by exponential growth. All Cit$^+$ *E. coli* strains were recovered within 40 days of the beginning of the selection incubations, with one exception, the *E. coli* Δ*dctA*::*kan* mutant, which arose after 134 days (Table 1). Recovery of *E. coli* Cit$^+$ cells did not occur among any of the *E. coli* Δ*citT*::*kan* mutant strains (Table 1).

Because of the potential for contamination in the extended incubation of these cultures, all Cit$^+$ isolates were meticulously confirmed to be *E. coli* by scoring 100 colonies from LB agar plates for specific *E. coli* phenotypes on SMAC-MUG agar for the *E. coli* wild-type strains and on SMAC-MUG Kn agar for the *E. coli* Δ*rpoS*::*kan* strain. All hundred colonies at each time point were sorbitol and MUG positive (fluoresced under UV light), reactions were confirmatory for *E. coli*, and the *E. coli* Δ*rpoS*::*kan* strain was positive for kanamycin resistance (data not shown). This confirmed that these cells matched the phenotype of the starting culture. Kanamycin was not used in the medium during the Cit$^+$ selection experiments. *E. coli* Cit$^+$ cells from direct selection flasks were plated on LRC agar, and a single large colony from each experiment was used for further characterization.

Multiple independent *E. coli* K-12, *E. coli* B, and *E. coli* REL606 Cit$^+$ phenotypes were isolated using modified direct selection in M9C medium supplemented with glycerol or glucose. The direct selection of Cit$^+$ mutants described above was repeated with the four *E. coli* K-12 test strains using M9C medium supplemented with either 0.005% glycerol or 0.0025% glucose, equivalent to the low-level carbon concentrations used in the LTEE by Lenski (2). The rationale for these experiments was that of providing a limiting usable carbon source to support approximately six generations of growth and thereby increasing the likelihood of mutations among replicating cells, in contrast to the limited cell division in the "starvation" direct selection described above. Glycerol was used to determine if catabolite repression or growth rate affected selection of Cit$^+$ mutants. Fourteen *E. coli* Cit$^+$ isolates (Table 1) were recovered from these experiments: 5 of 6 from glycerol-containing flasks with *E. coli* K-12 wild-type or *E. coli* Δ*rpoS*::*kan* cells and 9 of 9 from glucose-containing flasks with *E. coli* K-12 wild-type cells. After inoculation (9×10^5 to 7×10^6 CFU/ml), the turbidity of the cultures rose to an OD_{600} of 0.02 to 0.03 overnight, or the equivalent of approximately six generations based on plate count (data not shown). These cultures were maintained for 7 days in stationary phase, after which cells were inoculated into fresh media at a 1:100 dilution, similarly to Lenski's LTEE but with deliberately extended time spent in stationary phase to enrich for the desired Cit$^+$ phenotype. Results of representative experiments are shown in Fig. 2A and B. Following weekly transfers into fresh media, subpopulations presumably underwent potentiating mutations until the *E. coli* Cit$^+$ phenotype was actualized as evidenced by exponential growth of *E. coli* wild-type and *E. coli* Δ*rpoS*::*kan* cells. All but one *E. coli* Cit$^+$ strain were recovered within two to five weekly transfers, representing 12 to 30 generations. In general, adaptation to the Cit$^+$ phenotype was slower among cells in M9C supplemented with glucose than among cells in M9C supplemented with glycerol (the first mutants were obtained after the fifth versus the second transfer) (Table 1). Within 63 days, recovery of *E. coli* Cit$^+$ cells did not occur among any of the flasks containing the *E. coli* Δ*citT*::*kan* mutant or the *E. coli* Δ*dctA*::*kan* mutant. We concluded that direct selection of *E. coli* K-12 Cit$^+$ cells readily occurred when M9C medium was supplemented with low levels of glycerol or glucose, similarly to direct selection in M9C medium. Furthermore, lack of citrate-dependent growth in either the *E. coli* Δ*citT*::*kan* mutant or *E. coli* Δ*dctA*::*kan* mutant experiments showed the both of these genes were necessary for adaptation to citrate utilization within this time frame.

To show that the isolation of potentiated/actualized Cit$^+$ mutants was strain and citrate concentration independent, experiments were done using *E. coli* K-12, *E. coli* B, and *E. coli* REL606 in media with 1.7 mM citrate. Over the 9-week course, 15 of 16 *E. coli* K-12-containing flasks, 4 of 12 *E. coli* B-containing flasks, and 6 of 12 *E. coli* REL606-containing flasks gave rise to Cit$^+$ mutants (Table 1). Results of representative experiments are shown in Fig. 2C and D. As expected, reducing the citrate level by one-fourth reduced the maximum OD_{600} of *E. coli* K-12 by the same factor. Although *E. coli* B and *E. coli* REL606 followed the same pattern, the maximum OD_{600} attained was 0.08. We did not carry the *E. coli* B or *E. coli* REL606 experiments to refinement because both strains are defective in growth on succinate (data not shown), which also explains the lower level of growth. However, we predict that refined mutants would be obtained with longer selections, as shown with Cit$^+$ strain SO191, derived from the phenotypically equivalent *E. coli K-12* Δ*dctA*::*kan* mutant after 4 months. We concluded from these experiments that isolation of Cit$^+$ mutants was rapidly and readily attained regardless of the citrate concentration and strain lineage.

All independently isolated *E. coli* Cit$^+$ phenotypes had long lag phases before reinitiation of growth in M9C. Cit$^+$ cells from all three selection protocols displayed a long lag phase when transferred (1:100 dilution) from turbid primary selection media into fresh M9C broth. The length of this lag phase ranged from 4 to 7 days. Additionally, when cells were plated on Simmons citrate agar, small yellow colonies appeared only after 2 to 3 days. Colonies plated on Christensen citrate agar showed a weak, delayed cerise reaction after 24 to 36 h compared to the unselected *E. coli* strains, which all appeared as white colonies. Cells from secondary turbid M9C broth cultures were streak purified on LRC agar and consistently resulted in several larger colonies amid a background of pinpoint colonies after 2 to 3 days of incubation. These large-colony *E. coli* Cit$^+$ derivatives displayed more-rapid growth in M9C broth, and this shorter (24- to 36-h) lag phase was stable on subsequent transfers into fresh medium (see Fig. 4). Importantly, when these large-colony *E. coli* Cit$^+$ isolates were grown on Simmons citrate agar, blue colonies, indicative of strong citrate metabolism, appeared within 36 to 48 h. When the isolates were grown on Christensen citrate agar, a strong positive cerise reaction was observed within 12 h. Collectively, these strong positive reactions on citrate differential media and shortened lag phases indicated that refinement of the *E. coli* Cit$^+$ phenotype had occurred.

The long lag phase before reinitiation of growth of primary *E. coli* Cit$^+$ isolates could be shortened to 12 to 18 h by using filter-sterilized M9C spent media from turbid *E. coli* Cit$^+$ cultures, readjusted for M9 salts and citrate. Serial dilutions of spent media showed a growth-promoting dose-dependent response (data not shown). This suggested that quorum sensing could be involved. Although autoinducer 2 (AI-2) was detected in these spent medium supernates, as assayed with *Vibrio harveyi* test strains (19, 20), addition of purified AI-2 to these cultures had no growth

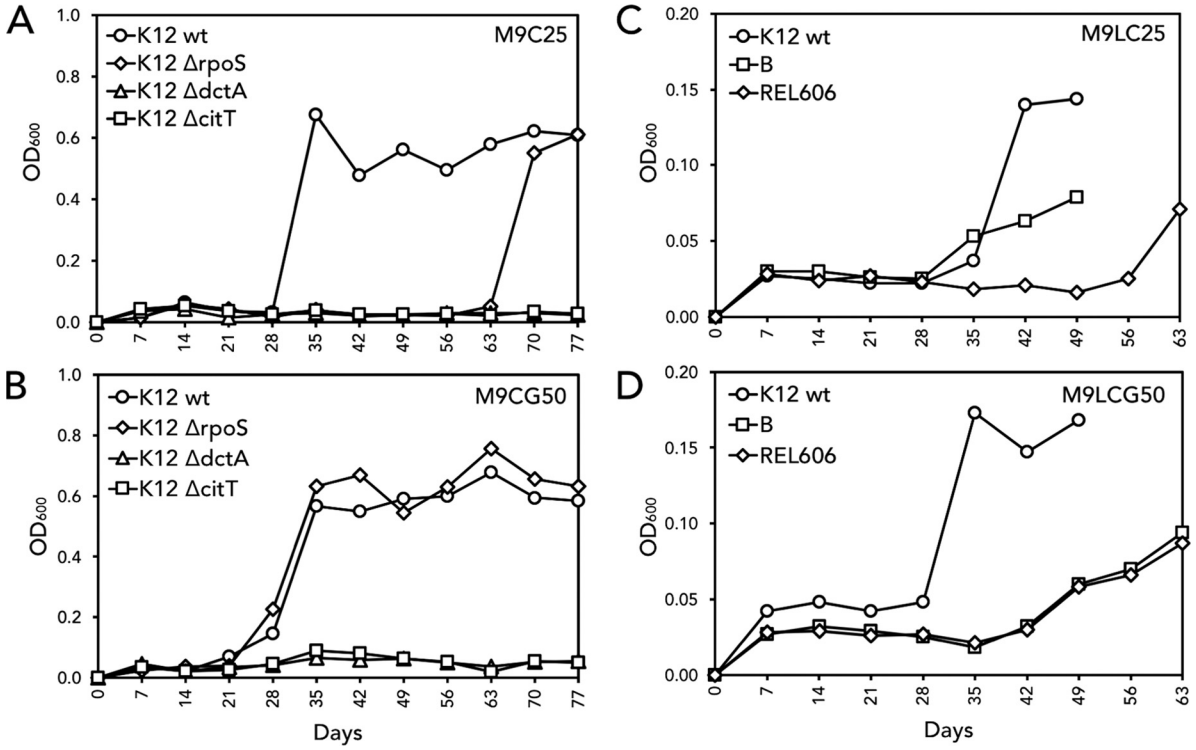

FIG 2 Modified direct selection of *E. coli* yielded Cit$^+$ mutants independently of strain and citrate concentration. Mutants arose for the *E. coli* K-12, *E. coli* K-12 Δ*rpoS*::*kan*, *E. coli* B, and *E. coli* REL606 strains but not for the *E. coli* Δ*citT*::*kan* strain or the *E. coli* Δ*dctA*::*kan* strain. Results of four separate representative experiments are shown. Each flask was inoculated with ~5.0 × 10^5 CFU/ml, and every 7 days, cultures were diluted 1:100 into fresh medium. OD_{600} levels were measured before transfer. (A and B) The *E. coli* K-12 wild-type, *E. coli* Δ*rpoS*::*kan*, *E. coli* Δ*dctA*::*kan*, and *E. coli* Δ*citT*::*kan* strains were inoculated into individual 250-ml flasks containing 50 ml of M9 minimal medium, 6.8 mM citrate, and either 0.0025% glucose (M9C25) or 0.005% glycerol (M9CG50). This level of glucose or glycerol supported six generations of growth. (A) In M9C25, Cit$^+$ mutants arose for the *E. coli* K-12 and *E. coli* Δ*rpoS*::*kan* strains after the forth and ninth transfers, respectively. (B) In M9CG50, Cit$^+$ mutants arose for the *E. coli* K-12 and *E. coli* Δ*rpoS*::*kan* strains after the third transfer. Citrate-supported growth was not recovered for either the *E. coli* Δ*dctA*::*kan* strain or the *E. coli* Δ*citT*::*kan* strain. (C and D) Similarly, the *E. coli* K-12 wild-type, *E. coli* B, and *E. coli* REL606 strains were inoculated into M9 minimal medium containing 1.7 mM citrate and either 0.0025% glucose (M9LC25) or 0.005% glycerol (M9LCG50). Cit$^+$ mutants arose in *E. coli* K-12 after five or four transfers, in *E. coli* B after four or five transfers, and in *E. coli* REL606 after eight and five transfers (panels C and D, respectively).

promoting effect and we did not pursue this further (data not shown).

Surprisingly, Cit$^+$ isolates were easily obtained using the *E. coli* Δ*rpoS*::*kan* strain. To show that lack of RpoS or of another mutation(s) present in this strain (Table 1) was not a contributing factor for citrate utilization, we made a P1(*vir*) bacteriophage lysate based on the strain DV133 isolate. This lysate was used to transduce *E. coli* K-12 wild-type cells at an MOI of 0.1:1. Transduced cells were diluted in 50 ml M9C broth and incubated at 37°C with aeration. Because citrate binds free calcium and therefore prevents P1(*vir*) phage infection (21), concern for secondary infection of transductants by transducing or lytic phage in this mixture was alleviated. After 4 days, the transduced culture displayed turbid growth with an OD_{600} of 0.7 and a sample from this flask was streaked directly onto Simmons citrate agar. Blue colonies appeared after 2 days. These colonies were sorbitol, MUG, and lactose positive and Kn^s, indicating that these transductants were not contaminated by the DV133 donor (lactose negative on EMB agar and Kn^r). This *E. coli* K-12 wild-type Cit$^+$ transductant was designated strain DV133T (Table 1).

We also identified a method of isolating refined *E. coli* Cit$^+$ cells on solid media. Actualized *E. coli* Cit$^+$ cells (long lag phase, pinpoint yellow colonies on Simmons citrate agar) were incubated with maintained humidity for several weeks. During this extended incubation period, small blue papillae arose within these yellow colonies (Fig. 3). Using a dissecting scope for viewing colonies, these papillae were picked and streaked onto fresh Simmons citrate agar. Large blue colonies appeared in 2 to 3 days and were separated from background yellow pinpoint colonies. Different papillae within the same colony or from different colonies represented independent transitions from actualized Cit$^+$ cells to the refined stage of citrate metabolism. This transition was not observed with unpotentiated *E. coli* K-12 wild-type or *E. coli* Δ*rpoS*::*kan* mutant cultures (data not shown). Using this technique, additional *E. coli* Cit$^+$ refined phenotypes were isolated.

***E. coli* Cit$^+$ phenotype growth correlated with citrate depletion in M9C broth.** Growth curve determinations were conducted with two Cit$^+$ refined isolates, DV133 and DV159. Filtered supernates obtained from these growth curve experiments were subjected to mass spectrometry at timed intervals to detect citrate and other metabolite concentrations. As shown in Fig. 4, bacterial growth correlated with depletion of citrate in the culture medium. Low levels of succinate were detected throughout these growth experiments, and acetate levels peaked in late exponential phase and dropped to undetectable levels in the stationary phases (data not shown). We did not detect any of the other organic acids

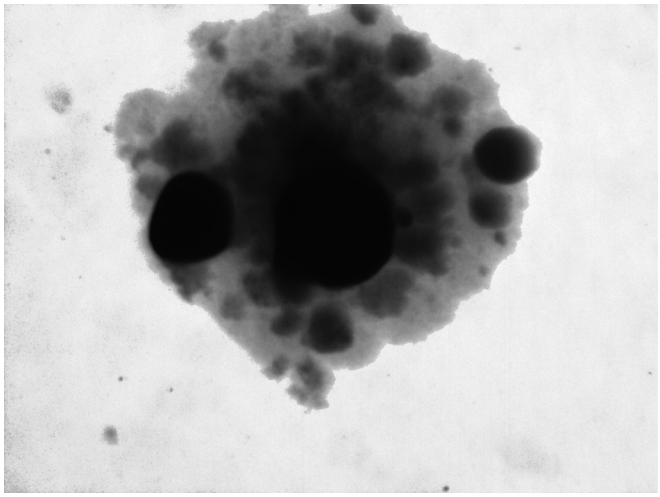

FIG 3 Extended incubation of actualized *E. coli* Cit$^+$ bacteria on Simmons citrate agar yielded refined *E. coli* Cit$^+$ papillae. Primary direct selection M9C broth that displayed growth was plated on Simmons citrate agar. Small yellow colonies appeared after 3 to 4 days of incubation, and small blue papillae (irregular darkened regions) appeared as shown after 10 to 20 days. Each blue papilla represents an independent mutation to the refinement stage.

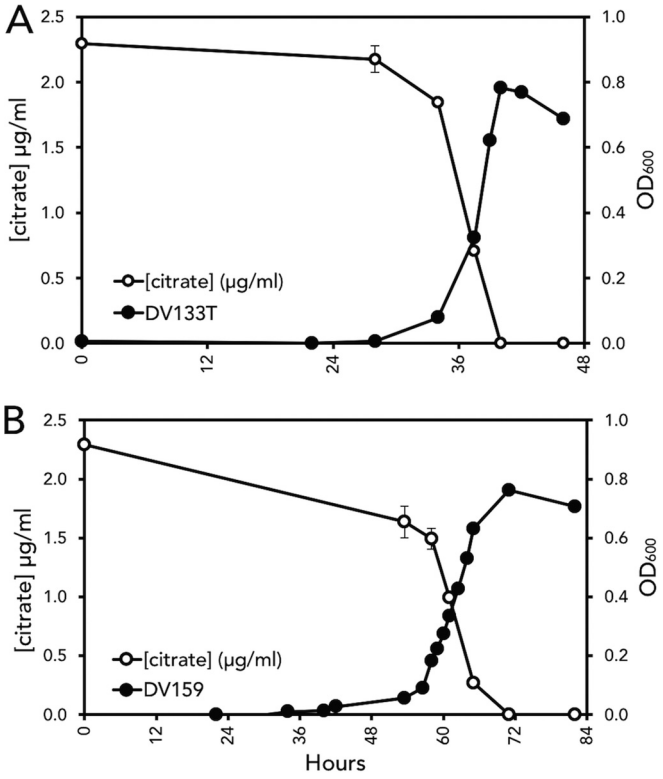

FIG 4 Mass spectrometry showed that citrate depletion correlated with *E. coli* growth. Two *E. coli* Cit$^+$ mutants derived from *E. coli* wild-type isolates, DV133T (A) and DV159 (B), were inoculated into M9C minimal medium, and the OD_{600} was measured every 6 h. Triplicate medium samples at each time point were subjected to filter sterilization, and a subset of samples corresponding to time zero and the late lag and early-, mid-, and late-exponential and stationary phases were analyzed by mass spectrometry for citric acid. Bars represent ± standard errors (S.E.).

tested. Although Kovac's reagent added to stationary-phase culture samples gave a weak positive indole reaction, we did not detect this analyte by mass spectrometry.

***E. coli* Cit$^+$ phenotypes from M9C broth were clumped or incompletely divided, and colonies derived directly from the broth were not clonal.** Microscopic examination of *E. coli* Cit$^+$ cells from M9C medium showed predominantly elongated predivisional cells, often in clumps or "rafts," estimated to contain 20 to 100 cells (Fig. 5A and C). Also, due to the deposition of these clumped cells, large blue colonies on Simmons citrate agar from this broth were not clonal (data not shown). However, this clumping phenotype was reversible when cells were grown in LB broth (Fig. 5B and D). Therefore, to obtain stable, clonal, large blue colonies on Simmons citrate agar, presumptive Cit$^+$ isolates required cycling growth through LB broth to mid-exponential phase, to reverse clumping and ensure clonal isolation. We found this purification step to be essential in the preparation of DNA for genomic sequencing.

Genetic and genomic DNA analyses of *E. coli* Cit$^+$ phenotypes showed amplification of *citT* followed by promoter capture and *dctA* amplification. Genomic DNA was prepared from four *E. coli* Cit$^+$ isolates, DV130, DV133, DV133T, and DV159 (Table 1), and was sequenced using PacBio RS II sequencing technology. *E. coli* DV159 is an independent wild-type Cit$^+$ isolate from an M9C direct selection. The other three strains represent staged isolates with respect to Cit$^+$ refinement and were used to identify genetic changes at each stage. *E. coli* DV130 (Δ*rpoS*::*kan* background) displayed weak Cit$^+$ activity evidenced by a long lag phase when placed in M9C and yellow colonies when grown on Simmons citrate agar. *E. coli* DV133 was derived from strain DV130 as a large-colony isolate from LRC agar. This refined Cit$^+$ strain had a shorter lag phase in M9C than its DV130 parental strain and yielded blue colonies on Simmons citrate agar. Strain DV133T is the *E. coli* K-12 wild type that was transduced with P1(*vir*) grown on strain DV133 and selected for Cit$^+$ in M9C broth. Like strain DV133, the DV133T transductant showed a strong Cit$^+$ phenotype upon its initial isolation, indicating that the essential mutation for strong Cit$^+$ utilization had been transduced. We reasoned that the genomic sequence of this strain in the wild-type background would facilitate identification of the mutation(s).

The read depth profile of the genome of strain DV130 showed 4-fold coverage of *citT* and 2-fold coverage of *dctA* above the normalized coverage of 100× for the rest of the chromosome, suggesting that these regions had undergone amplification (Fig. 6A). However, duplications in these regions were polished out by the software in the final assembly process. Analysis of individual "subreads" for *citT* showed tandem duplications (data not shown). The compiled genomic DNA sequence also showed a single-base-pair deletion in *dctA*, 26 nucleotides (nt) from the start of translation, 5 nt from the mutation identified by Quandt et al. (10) required for the refined Cit$^+$ phenotype (data not shown). The read depth profile of strain DV133 likewise shows increased read depths for both *citT* and *dctA*, both at 2-fold coverage, suggesting a partial reduction in *citT* amplification compared to that seen with the DV130 parental strain (Fig. 6A). Importantly, the DNA sequence of *citT* shows that *insI1*, an IS30 transposase, had been inserted 5′ to the *citT* gene, a presumptive promoter capture (Fig. 6B). This insertion was not present in any of the DV130 subreads,

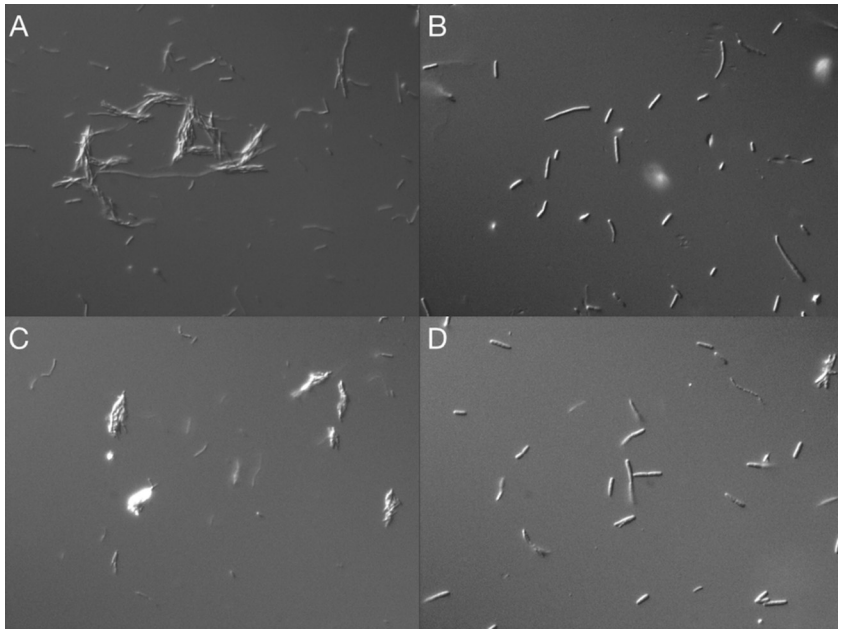

FIG 5 *E. coli* Cit$^+$ mutants grew in clumps in M9C broth but not in LB broth. Results of phase-contrast microscopy comparing levels of growth of *E. coli* Cit$^+$ mutants DV133 (A and B) and DV159 (C and D) in M9 (A and C) C and LB broth (B and D) are shown. Cells in M9C minimal medium were clumped, whereas growth in LB broth was dominated by single and predivisional cells. The pictures are representative of 10 microscopic fields; magnification, ×400.

indicating that this insertion event correlated with the switch between the potentiated Cit$^+$ weak phenotype and the strong or refined Cit$^+$ phenotype in strain DV133. This same *insl1* is present in DV133T (Fig. 6B), indicating that this region was transduced into the *E. coli* wild-type strain and confirming its role in the strong or refined Cit$^+$ phenotype. DV133T also shows an amplified *dctA* region of 140 kb generated by a recombination between *rhsA* and *rhsB*, genes that share homology and that bracket *dctA* at 79 min on the *E. coli* chromosome (Fig. 6C). There were no *dctA* point mutations in the gene or regulatory sequence.

To further verify that both *citT* and *dctA* had undergone amplification, we transduced DV133T with P1(*vir*) grown on strains JW0604-1 (Δ*citT*::*kan* mutant) and JW3496 (Δ*dctA*::*kan* mutant). DV133T cells transduced with phage grown on strain JW0604-1 were plated on Christensen citrate Kn agar and were scored for Cit$^+$. We found that 6 of 20 colonies were Kn^r and Cit$^+$. DV133T cells transduced with phage grown on JW3496 showed that 3 of 10 colonies were Kn^r and Cit$^+$. These results are compatible only with a gene duplication for both *citT* and *dctA* (22, 23).

We concluded that potentiation or actualization of Cit$^+$ first requires amplification of *citT* as shown in strain DV130. This would potentiate access to citrate by a gene dosage effect. Refinement requires promoter capture that leads to higher aerobic expression of *citT*, as shown for DV130-derivative strains DV133 and DV133T and by amplification of *dctA* by recombination at a well-documented "hot spot" for chromosome duplications (24, 25).

Sequence analysis showed that strain DV159 with *citT* had undergone a duplication and a deletion to generate a fusion of *citT* with the neighboring *uspG* gene (Fig. 6B). The *uspG* gene is stress induced and expressed at extremely high levels during carbon starvation (26, 27). Sequence analysis also showed a similar amplification of a large 140-kb region of the chromosome containing *dctA* consistent with recombination between *rhsA* and *rhsB*, as determined in strain DV133T (Fig. 6C). No point mutation(s) in *dctA* or its regulatory region was detected in DV159. The genomic rearrangement identified in strain DV159 is analogous to that of the original Cit$^+$ strain identified in the LTEE (11).

DISCUSSION

The most important finding of this work was that *E. coli* underwent rapid adaptation to aerobic citrate metabolism that was readily and repeatedly achieved using direct or modified direct selections. The genetic trajectory of this adaptation and the classes of mutations identified followed the same patterns of genetic events characterized in LTEE and centered on *citT* and *dctA* expression. Importantly, potentiated and actualized *E. coli* Cit$^+$ strains were obtained in as few as 12 generations and refined phenotypes in fewer than 100 generations. Phenotypic and genetic analyses of these *E. coli* Cit$^+$ strains provided insight into the mechanism for the adaptations and suggested why the LTEE took 33,000 generations to reach this phenotype. Also, this study provided a unique opportunity to compare the results of a direct selection with those of a long-term genetic screen. Finally, because this adaptation did not generate any new genetic information and required expanded expression of only two existing transporters (*citT* and *dctA*), generation of *E. coli* Cit$^+$ phenotypes in our estimation does not warrant consideration as a speciation event. In fact, mutations in these two loci are sufficient for the LTEE Cit$^+$ phenotype (10).

We obtained 46 independent *E. coli* Cit$^+$ phenotypes that followed a similar staged trajectory from potentiation and actualization to refinement as defined by Lenski (10, 11). The emerging *E. coli* Cit$^+$ strains, in analogy to the LTEE mutants, displayed long lag phases and weak utilization of citrate. Potentiation, in the LTEE, is undescribed due to the 33,000 generations of mutations

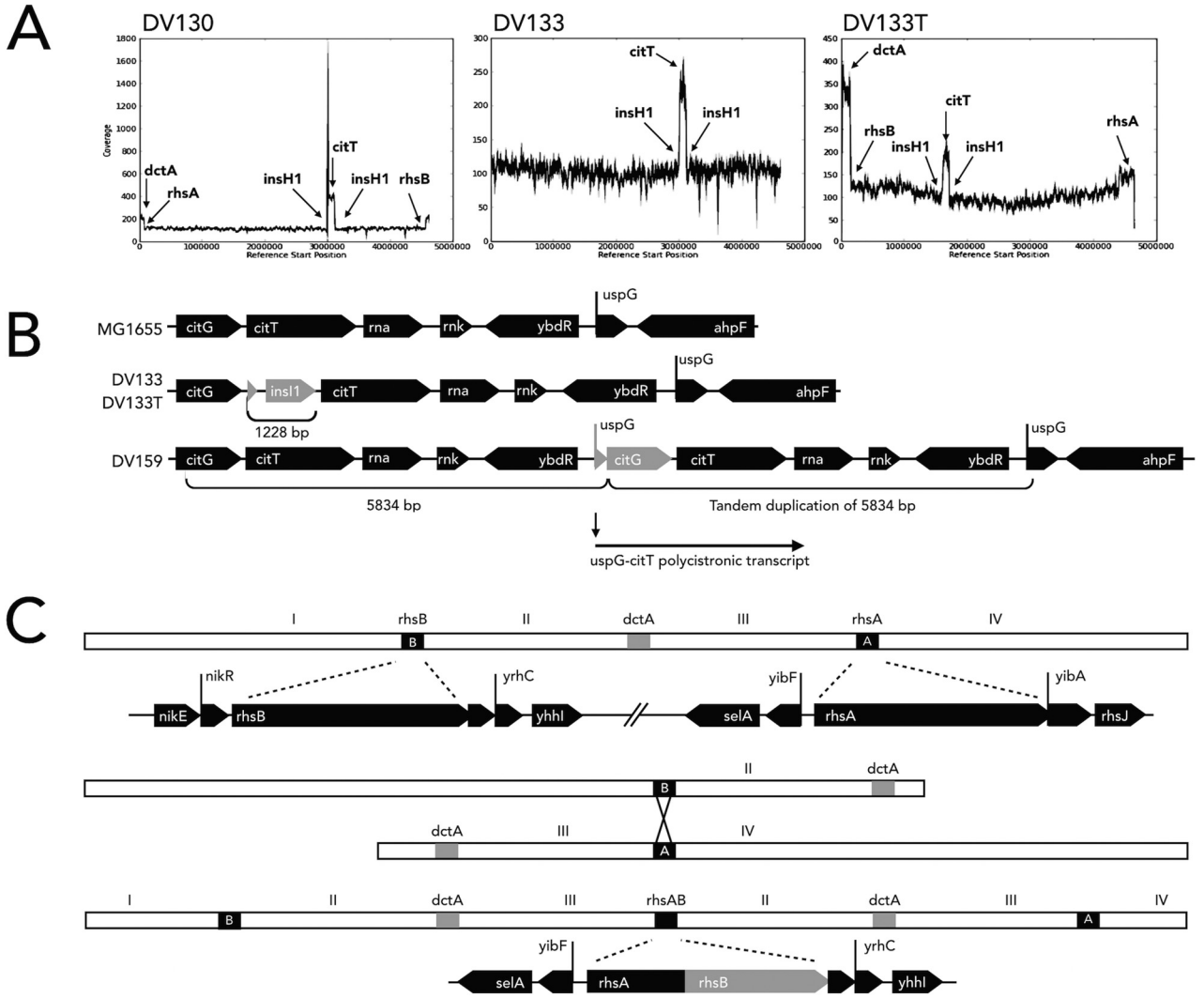

FIG 6 Genomic DNA sequence analysis of *E. coli* Cit$^+$ phenotypes showed gene amplifications associated with *dctA* and *citT* regions and promoter captures. (A) The genomic read depth profiles of *E. coli* Cit$^+$ isolates DV130, DV133, and DV133T. The weakly Cit$^+$ phenotype of DV130 shows 4-fold-increased coverage of the *citT* region and 2-fold-increased coverage of *dctA* compared to the ~100× coverage of the rest of the chromosome. (B) Strain DV133 was a strong Cit$^+$ phenotype derived from DV130. It showed a 2-fold reduction in *citT* coverage compared to the parental DV130 strain and the insertion of *insl1* 5′ into *citT*. When the *E. coli* K-12 wild-type strain was transduced with a lysate made using DV133, the same *insl1-citT* fusion was identified, confirming that this promoter capture was responsible for the transduced strong Cit$^+$ phenotype. The genomic sequence of Cit$^+$ strain DV159 showed a *citT* duplication and deletion event that fused the promoter of *uspG* to *citG*, another promoter capture event. All read depth profiles show 2-fold-increased coverage for the *dctA* region compared to the ~100× coverage for the chromosome. (C) Sequence analysis of DV133, DV133T, and DV159 showed recombination between *rhsA* and *rhsB* based on the differences in the positions of flanking genes *yibF* and *yrhC*. The genomic organization of the *E. coli* wild-type chromosome is represented by the top panel. The recombination of sister chromosomes to generate a large 140-kb duplication of this region is shown in the center panel. The gene map determined for both DV133 and DV159 is shown in the bottom panel with the *dctA* duplication.

that need to be analyzed. Because our *E. coli* Cit$^+$ cells were recovered after a minimum of 12 generations, potentiation requires only *citT* amplification, as determined for strain DV130. Increased expression of the CitT transporter allowed minimal but sufficient access to citrate and a low level of cell division. Gene amplifications are the most common mutations identified (28) and set the stage for subsequent promoter capture recombinatorial events. Thus, Cit$^+$ cells amplified for *citT*, such as the weakly Cit$^+$ DV130 cells, transitioned by *citT* promoter capture and *dctA* amplification to DV133, the strongly Cit$^+$ derivative. This combination was confirmed to be the only one required for changes by transducing this mutation into the *E. coli* K-12 wild-type strain to create DV133T.

A simple model emerges that explains the Cit$^+$ mutants derived either by direct selection or by LTEE. Cells are potentiated by *citT* amplification (>4×) and actualized by subsequent chromosome remodeling to capture a promoter that allows aerobic *citT* expression. The number of copies of *citT* required for growth before this capture is high but afterwards is decreased (2×) to reduce the fitness cost of maintaining numerous *citT* gene copies. Finally, Cit$^+$ refinement results when the DctA transporter is amplified (2×) to import succinate. The same mechanism, gene amplification, promoter capture, and subsequent reduction in gene copy number, was identified by Roth to explain the recovery of cryptic β-galactosidase expression by *E. coli* in Cairn's famous directed-mutagenesis ex-

periments (29, 30). This mechanism also explains similar adaptation events in *Salmonella* (31, 32).

We do not, as yet, fully understand the dynamics of delayed onset of growth by Cit$^+$ isolates after initial selection. Because spent media from *E. coli* Cit$^+$ isolates overrode this lag phase in a dose-dependent manner, quorum sensing may play a subtle role in this process. However, a more likely explanation is the loss of C4 dicarboxylates (succinate) that accompanies citrate uptake by CitT. Potentiated *E. coli* cells would be growing on the equivalent of a two-carbon substrate. Delayed growth may reflect a needed threshold level of succinate in the medium before citrate can be efficiently metabolized. Also, since *E. coli* K-12 can grow on succinate, this molecule may support emergence of Cit$^+$-dependent Cit$^-$-competing phenotypes in the population as they scavenge this metabolite produced from Cit$^+$ cells. Cit$^+$ cells grow in M9C as large rafts of cell complexes. We speculate that these rafts include succinate-scavenging Cit$^-$ cells because blue colonies isolated from M9C broth on Simmons citrate agar yielded various phenotypes when repurified. For this reason, we found it necessary to cycle the initial Cit$^+$ isolates through LB broth, to disperse the rafts and recover clonal isolates. In summary, it appeared that loss of succinate is a growth-limiting step at this adaptive stage.

Refinement of the *E. coli* Cit$^+$ phenotypes in our experiments required the *dctA* C4-dicarboxylate (succinate) transporter. In all experiments, the *E. coli* Δ*dctA*::*kan* strains did not produce a Cit$^+$ phenotype, with one exception, strain SO191. All genomic sequences of refined *E. coli* Cit$^+$ isolates had amplification of *dctA* or a mutation in its regulatory region. In *E. coli*, expression of *dctA* is inhibited by glucose (catabolite repression) and normally does not occur until stationary phase (33). DctA regulates its own expression, and the deletion mutation, identified in a DV130 population, centers between the −10 promoter sequence and the translational ATG start site. This may derepress its expression, as seen with the *dctA* point mutation identified in the LTEE (10). Expression of *dctA* is also dependent on the two-component regulatory DcuSR system (34, 35). DctA directly interacts with DcuSR and forms a functional tripartite transporter/sensor that localizes at the cell poles and predivision sites (36). DctA polar localization does not occur in the absence of *dcuS* expression or in a *dcuS* deletion. *E. coli* possess three additional succinate transporters, DcuA, DcuB, and DcuC, which are also under DcuSR regulation (37, 38). These three transporters, like *citT*, are expressed under anaerobic conditions. We predict that recapture of succinate may occur via activation of any of these anaerobic succinate transporters and that mutants with mutations in their regulatory sequences provide three more potential routes to refinement of a Cit$^+$ phenotype. Such mutants may represent a subset among our uncharacterized Cit$^+$ isolates such as strain SO191. The latter strain was the only *E. coli* Δ*dctA*::*kan* mutant that became Cit$^+$, which, remarkably, occurred after 134 days in stationary phase. The *E. coli* K-12 Δ*dctA*::*kan* mutant cannot utilize succinate under aerobic conditions. Preliminary characterization of the Cit$^+$ SO191 strain showed that it retained Kn^r and the ability to grow on succinate, consistent with aerobic expression of another dicarboxylic acid transporter.

One unanticipated result was the repeated successful isolation of the *E. coli* Δ*rpoS*::*kan* Cit$^+$ mutant strain. This strain was incorporated into our experiments as an intended negative control. We assumed that the RpoS stress response would be required for stress-induced mutations in starving cells. RpoS-dependent stress-induced point mutations and gene amplification occur by repair of double-stranded breaks and double-stranded ends. The former occurs as a consequence of the RpoS-controlled error-prone polymerase DinB causing nucleotide insertions, deletions, and base substitutions, and the latter occurs as a consequence of double-stranded end-initiated replication (39). RpoS-mediated stress response point mutations in chromosomes of starving *E. coli* cells have been reported to account for half of the base substitutions and frameshift mutations; thus, RpoS is considered to be essential for adaptation under starvation conditions (40). However, our results show that only gene amplification and recombination were required for generation of Cit$^+$ mutants.

Our results explain why the LTEE led to production of a single Cit$^+$ isolate. By design, the LTEE are open-ended experiments to track the evolution of 12 parallel *E. coli* cultures under controlled conditions. Aerobic citrate utilization was not a specified aim. The highly delayed evolution (33,000 generations) of Cit$^+$ strains understandably gives the impression that this was a rare, innovative evolutionary event. Our studies highlight why it was not. The LTEE design and the use of *E. coli* REL606 account for the delayed emergence of a Cit$^+$ isolate. First, potentiated and actualized cells were routinely diluted away by the LTEE daily 1:100 dilution transfers. Cells with an amplified *citT* gene had only a brief advantage in the use of citrate once glucose was expended and were likely lost with each daily dilution/transfer into fresh glucose-containing media due to the fitness cost of the maintenance of multiple gene copies. This dynamic was previously described for *Salmonella* gene amplifications (41). Gene amplification likely came to the steady state and did not reach a level that permitted the next step, the rarer promoter fusion event. Second, if actualized Cit$^+$ cells did arise, they would have a long lag phase and would have difficulty outcompeting the well-adapted *E. coli* cells specialized for growth in the LTEE low-glucose concentrations. Our results showing more-rapid recovery of Cit$^+$ mutants in glycerol than in glucose support the idea that slower cell division reduces the fitness cost of gene amplifications. Again, daily transfers to fresh glucose specifically select against slowly dividing mutants. Third, refined Cit$^+$ mutants depend on the presence of DctA to recapture succinate. Normal expression of this transporter is repressed by glucose (catabolite repression), shows autorepression, and requires DcuRS, all conditions reached in stationary phase. Thus, access to succinate occurred only briefly between LTEE transfers. Fourth, the *E. coli* strain used, REL606, has a *dcuS* five-base-pair deletion that prevents *dctA* expression (10, 12, 42). This strain is defective in the very pathway required for competitive citrate utilization. This defect also explains why our short-term direct selection yielded actualized and not refined *E. coli* REL606 Cit$^+$ cells. Thus, before citrate can be metabolized efficiently by this strain, an extra mutation to suppress this defect is required. Nonetheless, we predict that Cit$^+$ mutations, even in REL606, might have occurred sooner and repeatedly in the LTEE protocol if the *dcuS* in this strain had been repaired, because then, only gene amplifications (*citT* and *dctA*) would be required, as we found to be the case.

The following three results support this reasoning about the *dcuS* defect: (i) we rapidly isolated Cit$^+$ mutants in *E. coli* REL606 and another *E. coli* B strain; (ii) our characterized *E. coli* K-12 Cit$^+$ mutants required only *dctA* duplication, a more common process than the *dctA*-specific point mutation required to suppress a *dcuS* defect; (iii) we isolated a Cit$^+$ mutation in the *E. coli* K-12 Δ*dctA*::*kan* mutant, a strain phenotypically equivalent to the REL606 strain with a *dcuS* mutation (DctA negative), in 134 days. The

pathway to the Cit$^+$ phenotype with a defect in *dctA* expression does not require 33,000 generations of potentiating mutations. Interestingly, the time required for the LTEE Cit$^+$ mutation to occur is predictable. The frequency of the occurrence of both amplification/promoter capture (*citT*) and a point mutation (*dctA*) in the same cell under nonselective conditions is on the order of 1 in 10^{14}. This is almost exactly the number of cells Lenski screened to find Cit$^+$ and the frequency Hall predicted for his Cit$^+$ mutation (3, 14).

Our experiments also presented a unique opportunity to compare the results of a direct selection to the results of a long-term genetic screen. It is proposed that strong (direct) selection might drive an evolving population to a "quick fix rendering a better solution less accessible" (43). Our results suggest the converse is true. The *E. coli* Cit$^+$ genomes sequenced from our direct selection experiments are equivalent to those corresponding to the amplification and promoter capture mutations identified in the LTEE (11). The requirement for *dctA* activation is also common to the two experiments. We conclude there is no significant difference between these experiments with respect to the *E. coli* solutions to aerobic citrate use. Isolation of a Cit$^+$ mutant in a *dctA* deletion strain is indicative that direct selection led to an additional solution that has, as of now, not been identified in the LTEE. Because Cit$^+$ *E. coli* REL606 has been identified in only 1 of the 12 parallel cultures of the LTEE, it may well be that a weak selection inhibits access to a "citrate-use solution." Frozen fossils from the 12 LTEE cultures could be used to test this possibility. They could be revived and the experiments replayed from any generational landmark with longer incubations extended into deeper stationary phase, the selection conditions described by us, to determine if the pathway to citrate use has been preserved or lost among all 12 *E. coli* cultures.

In summary, *E. coli* can rapidly mutate to a Cit$^+$ phenotype in a relatively short time if subjected to direct selection. This indicates that the 33,000 generations to potentiate the evolutionary resources for the Cit$^+$ phenotype do not reflect a direct requirement but merely experimental conditions. As such, Cit$^+$ mutants exemplify the adaptation capability of microorganisms but, as of yet, the LTEE has not substantiated evolution in the broader sense by generation of new genetic information, i.e., a gene with a new function. Interestingly, our findings parallel the conclusions from bacterial starvation studies by Zinser and Kolter (44) in which *E. coli* adaptations were dominated by changes in the regulation of preexisting gene activities rather than by the generation of new gene activities, *de novo*. The LTEE isolation of Cit$^+$ mutants has become a textbook example of the power of long-term evolution to generate new species. But, based on our results, *E. coli* arrives at the same solution to access citrate in days versus years, as originally shown by Hall (14). In either case, genes involved in the process maintain their same function but show expanded expression by deregulation. Because of this, we argue that this is not speciation any more than is the case with any other regulatory mutant of *E. coli*. A more accurate, albeit controversial, interpretation of the LTEE is that *E. coli*'s capacity to evolve is more limited than currently assumed.

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