

## Chromosomal Mutation for Citrate Utilization by *Escherichia coli* K-12

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A mutant strain of *Escherichia coli* K-12 that utilizes citrate as a sole source of carbon and energy was isolated. Citrate utilization arose as the consequence of two mutations in genes *citA* and *citB*, which are linked to the *gal* operon. The mutant strain expresses a semiconstitutive citrate transport system, and it utilizes both citrate and isocitrate as carbon and energy sources. It is capable of utilizing *cis*- and *trans*-aconitate, but only if it is preinduced by growth on citrate.

*Escherichia coli* is unable to utilize citrate as a sole source of carbon and energy (9), and this citrate-negative phenotype forms an important basis for the classification of *E. coli* (15). A number of investigators have reported the isolation of citrate-utilizing *E. coli* from a variety of birds and mammals, but the Cit<sup>+</sup> phenotype has always been associated with the presence of Cit<sup>+</sup> plasmids (6-8). *E. coli* is not, however, totally inert toward citrate. It may utilize citrate anaerobically provided that a second substrate such as glucose or lactate is available to provide reducing power for the formation of succinate from oxaloacetate (13). The failure of *E. coli* to utilize citrate aerobically has been attributed to the absence of a citrate transport system (10), but the co-metabolism of citrate implies that *E. coli* does possess a citrate transport system that functions anaerobically (13).

Here I report the isolation of a spontaneous mutant of *E. coli* K-12 that utilizes citrate aerobically as a sole source of carbon and energy.

### MATERIALS AND METHODS

**Bacterial strains.** All strains used were *E. coli* K-12. Relevant genotypes are given in Table 1, where references giving complete genotypes are cited.

**Media.** Minimal medium A contained, per liter, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 7 g of K<sub>2</sub>HPO<sub>4</sub>, 0.42 g of sodium citrate, 0.1 g of MgSO<sub>4</sub>, 1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mg of thiamine, and 20  $\mu$ M FeCl<sub>3</sub>. Medium B was identical to medium A except that it contained no sodium citrate; it contained instead 0.5 g of NaCl per liter. L-broth was as described by Miller (14). The following was employed as a carbon source: 2 g of glucose per liter, 2.5 g of glycerol per liter, or 2.5 g of carboxylic acids per liter.

**Growth conditions.** Cultures were grown at 37°C with constant aeration.

**Genetic procedures.** Matings (conjugations) were carried out in L-broth as described by Miller (14) at a ratio of five F<sup>-</sup> cells per Hfr cell. Transductions were mediated by bacteriophage P1 *cam clr* (*ts100*) as described by Miller (14).

**Growth rates.** Growth rates were determined by monitoring the turbidity of cultures at 600 nm in a Gilford model 250 spectrophotometer. For each determination, cultures were monitored for a minimum of two mass doublings, and at least eight turbidity readings were recorded. The growth rates are given as the first-order rate constant in hours<sup>-1</sup> as determined from a least-squares fit of ln absorbance versus time. Values are given  $\pm$  the 95% confidence interval based upon a minimum of four independent determinations.

**Citrate uptake.** Exponentially growing cells were harvested by centrifugation at room temperature, washed, and suspended at 37°C in 0.1 M Tris-hydrochloride buffer (pH 7.0) containing 10 mM MgCl<sub>2</sub>. The assay was initiated by adding [1,5-<sup>14</sup>C]citrate (1 mCi mmol<sup>-1</sup> Amersham Corp.). Cultures contained 200  $\mu$ g (dry weight) of cells per ml and were shaken at 37°C throughout the assay. Samples were withdrawn at indicated intervals, collected upon HAWP filters (Millipore Corp.), and transferred immediately to scintillation vials containing ACS (Amersham) aqueous liquid scintillation cocktail. In no case was more than 5% of the citrate taken up from the medium in the course of an assay.

### RESULTS

**Isolation of a Cit<sup>+</sup> strain.** In an attempt to isolate mutants utilizing phenyl arabinoside, for reasons unrelated to this study, strain D21 was inoculated into medium A containing 10 mM phenyl-arabinoside as a carbon source. The culture was shaken at 37°C in a sealed sidearm flask, and the turbidity was monitored periodically without opening the flask. The culture absorbance at 600 nm declined slowly from 0.12 (equivalent to about 10<sup>8</sup> cells per ml) to about 0.06 over a period of 12 days. On day 14, the absorbance had risen to 0.19, but it failed to increase further. Upon subculturing into the same medium, a 1,000-fold dilution of the culture reached an absorbance of 0.13 overnight, but failed to grow further upon the addition of more phenyl arabinoside. This behavior suggested that the culture was not utilizing phenyl

TABLE 1. Bacterial strains

Strain	Relevant genotype	Source or reference
D21	<i>hfrC lacZ</i> ( $\Delta$ W4680) <i>spc</i> <i>ebgA116 ebgR7</i>	2
D2004	<i>citA</i> <sup>+</sup> <i>citB</i> <sup>+</sup> mutant of strain D21	This study
SJ7	F <sup>-</sup> <i>argG metC lacZ tolC</i> <i>ebgA</i> <sup>0</sup> <i>ebgR</i> <sup>+</sup>	4
CSH57	F <sup>-</sup> <i>his trp gal rpsL</i>	14
CA190	HfrH <i>galK</i>	B. Bachmann
CBT38	F <sup>-</sup> <i>rpsL leuB lacY dctB</i>	B. Bachmann
GS-1	F <sup>-</sup> <i>rpsL galK dctB</i> (Recombinant from CA190 $\times$ CBT38 mating)	This study

arabinoside as a carbon source. Medium A, like many *E. coli* media, contains citrate. Upon being subcultured into medium A containing no added carbon source, the culture achieved an overnight absorbance of 0.14, suggesting that citrate was indeed being utilized. The culture was streaked onto medium A agar containing 2.5 g of sodium succinate per liter, and a single colony was isolated and designated strain D2004. All further studies utilized medium B.

To be sure that strain D2004 was *E. coli* K-12 and was a mutant of strain D21, it was compared with strain D21 in a variety of tests. Strain D2004 produced indole, was methyl red positive, was Voges-Proskauer negative, and formed colonies with a green metallic sheen on eosin methylene blue agar. It was sensitive to streptomycin, ampicillin, and nalidixic acid, but resistant to spectinomycin. It was sensitive to the coliphages T4, T6, lambda, P1, and male-specific R17. In all of these respects it was identical to strain D21. Strain D21 is deleted for the *lacZ* gene, but it carries mutations which allow it to utilize lactose via its second  $\beta$ -galactosidase, *ebg* enzyme (2). The *ebgA* and *ebgR* genes are located at 66 min on the *E. coli* map, and they are linked to the *tolC* gene at 65 min (3, 4). Strains D21 and D2004 were mated with strain SJ7, and *rpsL tolC*<sup>+</sup> recombinants were selected. The mating with strain D21 showed 19% recombination between the *ebg* and *tolC* genes, and strain D2004 showed 21% recombination between those genes. By all of these criteria strain D2004 is indeed a mutant of strain D21.

**Genetic analysis.** The method of isolation and the fact that we utilize no strains carrying Cit<sup>+</sup> plasmids in this laboratory made it seem unlikely that strain D2004 acquired citrate utilization via a plasmid. I mapped the location of the *cit*<sup>+</sup> gene on the *E. coli* chromosome. There were no Cit<sup>+</sup> colonies among the *tolC*<sup>+</sup> recombinants from

the mating between strain D2004 and strain SJ7. This made it unlikely that the *cit*<sup>+</sup> mutation lay in the region from 65 min clockwise to 12 min (the origin for HfrC). Strain D2004 was therefore mated with the *his trp gal* strain CSH57 (Table 2). The results of those matings suggested that the *cit*<sup>+</sup> gene was located distal, but linked, to the *gal* operon.

To locate the *cit*<sup>+</sup> gene more precisely, phage P1 grown on strain D2004 was used to transduce strain CSH57. Of 222 *gal*<sup>+</sup> transductants, 11 (5%) were *cit*<sup>+</sup>. I was unable to select any *cit*<sup>+</sup> transductants directly, for reasons discussed below.

Several observations suggested that citrate utilization did not arise from a simple point mutation.

The mutation to *cit*<sup>+</sup> is extremely rare. Citrate is included in many classical mineral salts media used for the routine cultivation of *E. coli* (14). Many studies, particularly chemostat studies, subject large populations of cells to long-term growth under near-starvation conditions where *cit*<sup>+</sup> mutants would be strongly selected. Spontaneous single-base substitutions occur in *E. coli* at a rate of about 10<sup>-10</sup> per cell division. Thus, a steady-state population of 10<sup>10</sup> cells in a glucose-limited chemostat maintained at a specific growth rate of 0.17 h<sup>-1</sup> or less would be displaced by *cit*<sup>+</sup> mutants within 40 generations if *cit*<sup>+</sup> arose via a single-base substitution. The fact that such mutations have not been previously reported despite the extensive use of citrate in minimal media argues that *cit*<sup>+</sup> is not the result of a simple mutation.

The conjugation experiments (Table 2) showed that the fraction of *cit*<sup>+</sup> recombinants recovered when *gal*<sup>+</sup> was selected was much lower than the fraction of *gal*<sup>+</sup> recombinants recovered when *cit*<sup>+</sup> was selected. This could be attributed to the gradient of transmission resulting from spontaneous mating interruption; it would be interpreted as *cit*<sup>+</sup> being distal to *gal*<sup>+</sup> in the cross. When *cit*<sup>+</sup> was selected, there was only 20% recombination between *cit*<sup>+</sup> and *gal*<sup>+</sup>, indicating that *cit*<sup>+</sup> is less than 1 min from *gal*<sup>+</sup>.

TABLE 2. Mating between strain D2004 and strain CSH57<sup>a</sup>

Selected marker	Fraction of progeny with unselected marker:			
	<i>his</i> <sup>+</sup>	<i>trp</i> <sup>+</sup>	<i>gal</i> <sup>+</sup>	<i>cit</i> <sup>+</sup>
<i>his</i> <sup>+</sup>		ND	ND	0.003
<i>trp</i> <sup>+</sup>	ND		0.39	0.06
<i>gal</i> <sup>+</sup>	0.44	0.44		0.17
<i>cit</i> <sup>+</sup>	0.48	0.53	0.80	

<sup>a</sup> Based on 700 colonies scored for each selected marker. ND, Not determined.

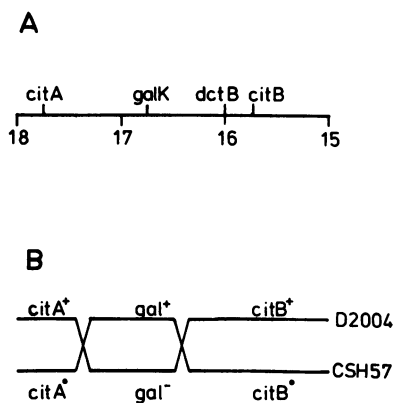


FIG. 1. Proposed genetic map of citrate genes. (A) *galK* and *dctB* loci placed according to Bachmann and Low (1); placement of *citA* and *citB* based on data in Table 2 and transduction data in the text. These locations should not be considered exact. (B) Genetic exchanges consistent with the data in Table 2.

Spontaneous interruption reduces the recovery of distal markers by no more than 5%/min under these mating conditions and therefore does not account for the reduction from 80% linkage between *cit* and *gal* when *cit*<sup>+</sup> is selected to 17% linkage when *gal*<sup>+</sup> is selected.

P1 lysates prepared on strain D2004 transduced the genes *gal*<sup>+</sup>, *argG*<sup>+</sup>, *metC*<sup>+</sup>, *his*<sup>+</sup>, and *trp*<sup>+</sup> efficiently into appropriate recipients, but they did not transduce *cit*<sup>+</sup> directly. Part of the failure to transduce *cit*<sup>+</sup> directly can be attributed to the fact that when the *cit*<sup>+</sup> strain is grown on broth (as the recipient is for transductions), the plating efficiency on citrate minimal medium is about 5% of the plating efficiency on glucose minimal medium. Plating efficiency alone, however, did not account for the failure to recover *cit*<sup>+</sup> transductants.

The observed 20% recombination between *cit* and *gal* when *cit*<sup>+</sup> was selected in conjugation experiments predicts a value of about 30% cotransduction between *cit* and *gal*, based upon the mapping function of Wu (17). The observed 6% cotransduction between *gal* and *cit* was therefore much less than expected.

These considerations suggested that the Cit<sup>+</sup> phenotype resulted from two mutations in two genes. To locate the *cit*<sup>+</sup> genes more precisely, the *galK* *dctB* strain GS-1 was constructed. GS-1 is unable to utilize citrate, galactose (*galK*), or succinate (the *dctB* gene specifies a component of the dicarboxylic acid transport system [11]). Strain GS-1 was transduced with P1 phage grown on strain D2004, and both *galK*<sup>+</sup> and *dctB*<sup>+</sup> transductants were selected. The cotransduction frequencies between *galK* and *dctB* were 28% when *galK* was selected and 23%

when *dctB* was selected. These frequencies are consistent with the distance of 0.75 min between *galK* and *dctB* (1, 16). The cotransduction frequency between *galK* and *cit*<sup>+</sup> was 5%, of which 80% were also *dctB*<sup>+</sup>. If *cit*<sup>+</sup> were a point mutation, this would be interpreted as gene order *galK*-*dctB*-*cit*, with *cit*<sup>+</sup> being about 1.5 min from *dctB*. This interpretation predicts that the cotransduction frequency between *dctB* and *cit*<sup>+</sup> would be about 25% when *dctB* is selected and that the majority of those cotransductants would be *galK*<sup>-</sup>. Contrary to those expectations, when *dctB* was selected only 3% were *cit*<sup>+</sup> cotransductants, and all 13 of those were also *galK*<sup>+</sup>. These data are not consistent with *cit*<sup>+</sup> being due to a single point mutation.

The Cit<sup>+</sup> phenotype appears to be the result of two separate point mutations separated by slightly less than the length of a transducing fragment, i.e., by about 2 min (Fig. 1). If citrate utilization requires a mutation in both *citA* and *citB* genes, then mutation to citrate utilization is certainly expected to be a very rare event. When *cit*<sup>+</sup> was selected in conjugation experiments, the *cit*<sup>+</sup> *gal*<sup>-</sup> recombinants obtained were the result of two exchanges, one between *citA* and *gal* and the other between *gal* and *citB* (Fig. 1). Previous results (3, 4; unpublished data) indicate a recombination frequency of about 40%/min; thus, placement of the *cit* genes about 1 min on either side of *gal* would be consistent with the observed 20% double exchanges leading to *cit*<sup>+</sup> *gal*<sup>-</sup> recombinants. This placement also accounts for the low recovery of *cit*<sup>+</sup> recombinants when *gal*<sup>+</sup> was selected. When *gal*<sup>+</sup> was selected, only those progeny in which no exchanges occurred in the interval between *citA* and *citB* would be *cit*<sup>+</sup>. The expected frequency of *cit*<sup>+</sup> based upon zero exchanges would be reduced by about another 5% due to spontaneous interruption over the interval between *gal* and *citB*. This arrangement is also consistent with the transduction data. *Cit*<sup>+</sup> transductants could occur only when *citA* and *citB* cotransduced; thus, the apparent cotransduction frequency of any gene located between the two *cit* genes and *cit*<sup>+</sup> would be the same and would reflect only the cotransduction frequency between *citA* and *citB*. The necessity of two gene functions for citrate utilization is not particularly surprising. Uptake of dicarboxylic acids, including succinate, requires the functioning of both the *dctA* and *dctB* genes (11, 12).

**Characteristics of strain D2004.** It is clear that citrate is an excellent carbon source for strain D2004 (Table 3). Although the *cit*<sup>+</sup> mutations did not affect growth rates on glucose or glycerol, strain D2004 grew 70% faster than its parent on succinate and 2.8 times faster on fumarate. Strain D2004 utilizes citrate, isocitrate, *cis*-acon-

TABLE 3. Growth rate comparisons<sup>a</sup>

Carbon source	Strain D21	Strain D2004
Glucose	0.548 ± 0.007	0.537 ± 0.015
Glycerol	0.427 ± 0.013	0.414 ± 0.018
Succinate	0.294 ± 0.015	0.501 ± 0.025
Malate	0.388 ± 0.013	0.433 ± 0.014
Fumarate	0.158 ± 0.013	0.444 ± 0.015
Citrate	<0.01	0.360 ± 0.011
Isocitrate	<0.01	0.084 ± 0.006
cis-Aconitate	<0.01	0.516 ± 0.033 <sup>b</sup>
trans-Aconitate	<0.01	0.477 ± 0.024 <sup>b</sup>
Tricarballylate	<0.01	<0.01

<sup>a</sup> Values are first-order growth rate constants in reciprocal hours ± 95% confidence limits.

<sup>b</sup> Growth occurred only if the cultures were pre-grown on citrate.

itate, and *trans*-aconitate (Table 3); however, *cis*-aconitate and *trans*-aconitate were utilized only if the cultures are pregrown in citrate. By standard methods (5, 6), strain D2004 would therefore be classified as positive for citrate and isocitrate, but negative for *cis*-aconitate, *trans*-aconitate, and tricarballylate. This pattern is unlike that of any other members of the family *Enterobacteriaceae* or any of the Cit<sup>+</sup> plasmids tested (5, 6).

Strain D2004 always exhibited a long growth lag (10 to 48 h) when shifted from other carbon sources to citrate. The only exception was that cultures growing on isocitrate grew without lag when shifted to citrate. To eliminate the possibility that the Cit<sup>+</sup> phenotype was unstable and that the observed lag was due to selection of a minority Cit<sup>+</sup> population within cultures grown on other carbon sources, I measured the plating efficiency of glucose-, succinate-, and citrate-

grown cultures of D2004 on citrate, succinate, and glucose minimal media. The plating efficiencies on the three minimal media were indistinguishable, and they were independent of the carbon source in the growth medium. It is concluded that the growth lag is physiological.

**Citrate uptake.** The parental strain D21 was unable to take up detectable citrate whether the cells were grown in succinate or glycerol, or upon either substrate in the presence of 2.5 g of citrate per liter. Citrate-grown cells of strain D2004 (Fig. 2) took up citrate rapidly, reaching a steady-state internal concentration within 3 min. The observed plateau in uptake was not due to exhaustion of citrate during the assay, for less than 2% of the citrate was taken up. It is possible that the actual flux across the membrane is faster than these data indicate and that citrate is actually being trapped as some intermediate. If that is the case, then the observed steady-state level reflects the internal concentration of intermediate plus citrate. The initial rate of citrate uptake in glycerol- or in succinate-grown cells was 3.1 μmol/min per g (dry weight) of cells; the initial rate in citrate-grown cells was 8.7 μmol/min per g (dry weight) (Fig. 2). These data suggest that the citrate uptake system is induced no more than two- or threefold. Therefore, it seems unlikely that the observed growth lag is

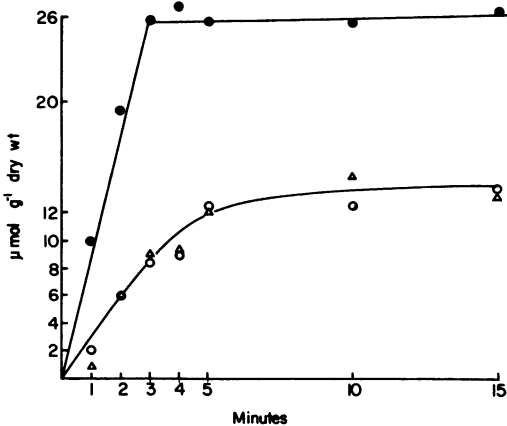


FIG. 2. Uptake of 0.4 mM citrate by strain D2004. Symbols; ●, citrate-grown cells; ○, succinate-grown cells; △, glycerol-grown cells.

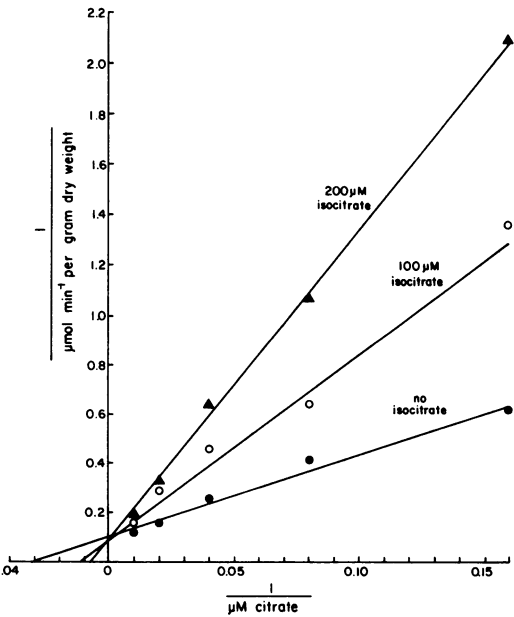


FIG. 3. Double-reciprocal plot showing effect of isocitrate concentration on uptake of [<sup>14</sup>C]citrate (8.0 mCi/mmol) by strain D2004. The straight lines are least-squares fits to the double-reciprocal data.

due to a requirement for induction of the citrate transport system.

The initial rate of citrate uptake was measured over a concentration range from 6.25 to 400  $\mu\text{M}$  citrate. A computer analysis of those initial rates yielded a  $V_{\max}$  of  $10.0 \pm 1.8 \mu\text{mol/min per g}$  (dry weight) and an apparent  $K_m$  of  $36 \pm 11 \mu\text{M}$  citrate. Isocitrate was a competitive inhibitor of citrate uptake (Fig. 3), with an apparent  $K_i$  of 65 to 70  $\mu\text{M}$  isocitrate. *cis*-Aconitate, *trans*-aconitate, and tricarballoylate also inhibited citrate uptake, but the inhibition was not examined in detail.

### DISCUSSION

The observation that the citrate transport system is semiconstitutive, but that cells lag for several hours upon being shifted to citrate, suggests that other physiological adaptations are required for *E. coli* to utilize citrate. This suggestion is supported by the observation that strain D2004 can utilize *cis*- and *trans*-aconitate only if pregrown on citrate. It seems likely that some component required for tricarboxylic acid utilization is induced by citrate and isocitrate, but not by *cis* or *trans*-aconitate. These observations do, however, support the generally held idea that transport is the major barrier to citrate utilization by *E. coli*.

The data suggest that the *citA* and *citB* genes together specify a citrate transport system. These data cannot distinguish whether *citA* and *citB* specify different structural components in the citrate transport system or whether one gene specifies a structural component and the other gene plays a strictly regulatory role. Because *cit*<sup>+</sup> *dctB*<sup>-</sup> recombinants were recovered, the hypothesis that the *dctB* gene product is involved in citrate transport can be ruled out.

The mutational activation of these genes suggests that they should be considered cryptic genes which have persisted in a silenced state since *E. coli* diverged from its Cit<sup>+</sup> ancestor. That suggestion is supported by the observation that the kinetic parameters for citrate uptake in strain D2004 are of the same order as those for succinate uptake in *E. coli* ( $K_m = 14 \mu\text{M}$  succinate;  $V_{\max} = 20 \mu\text{mol/min per g}$  [dry weight]) (11). It is unlikely that mutations conferring a specificity for citrate upon some already existing transport system would result in such a low  $K_m$  for citrate. More detailed comparisons between the citrate transport system of strain D2004, the citrate transport systems specified by Cit<sup>+</sup> plas-

mids in *E. coli*, and citrate transport systems of such citrate-utilizing organisms as *Salmonella* and *Klebsiella* should shed some light on the possibility that the citrate genes are cryptic in wild-type *E. coli*.

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