Selection for Citrate Synthase Deficiency in *icd* Mutants of *Escherichia coli*

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Cultures of isocitrate dehydrogenase-deficient *(icd)* mutants were overgrown by double mutants *(icd glt)* lacking citrate synthase activity also. The *icd* mutants grew more slowly than wild-type cells or the double mutants because they accumulated an inhibitory metabolite (possibly citrate). Intracellular citrate levels were several hundred-fold higher in *icd* cells than in wild-type or *icd glt* cells. Final growth yields of the wild type and the *icd* mutant on limiting glucose were equivalent and greater than the growth yield of *icd glt* double mutants. The *icd* gene mapped between 60 and 74 min. *icd* mutants were resistant to nalidixic acid, but *glt* and *icd glt* mutants and wild-type cells were sensitive, indicating that resistance results from accumulation of isocitrate, citrate, or a derivative of these compounds.

A large proportion of mutants of *Escherichia coli* resistant to low levels of nalidixic acid (Nal), an inhibitor of deoxyribonucleic acid (DNA) synthesis, require L-glutamate or L-proline for growth and are *icd* mutants. The growth factor requirement results from the lack of isocitrate dehydrogenase (ICD) activity (6, 7). Although several physically distinct forms of ICD have been identified in *E. coli* (11), the growth factor requirement and the lack of any enzyme activity in extracts of such mutants suggest that the different isoenzymes are controlled by the same gene and may contain identical polypeptides. Similar mutants have been isolated from *Salmonella typhimurium* by using the same procedure (1).

These mutants were found to grow slowly relative to wild-type cells both in enriched media (nutrient broth) and in defined media (minimal glucose plus glutamate; 7). However, faster-growing variants have been found to appear frequently in *icd* mutant cultures. By identifying the additional enzymatic deficiency in these secondary mutants and comparing the growth rates and growth yields of the single and double mutants, we conclude that the poor growth of *icd* mutants results from the accumulation of an inhibitory intermediate.

The appearance of the secondary mutants has also enabled us to examine the basis of Nal resistance in *icd* mutants. Why lack of ICD activity should cause Nal resistance is not immediately obvious, but all possible explanations can be categorized as follows. (i) A *cis* effect of the *icd* gene on expression of a second gene responsible for resistance. In this case, the loss of ICD activity would be a fortuitous consequence of selection for an altered phenotype of the second gene. (ii) A direct role of the ICD protein (or of the ICD messenger ribonucleic acid) in producing drug resistance or sensitivity. For example, the enzyme could act directly to activate the drug or, alternatively, it might interact with a membrane complex in which DNA synthesis occurs. (iii) Lack of a normal product of ICD activity. Such a product might be 2-oxoglutarate or reduced nicotinamide adenine dinucleotide phosphate. (iv) Accumulation of an intermediate compound producing Nal resistance. Presumably this would be isocitrate, aconitate, citrate, or a derivative of one of these.

Analysis of the secondary mutants with faster growth rates that appear in cultures of *icd* strains has enabled us to rule out the first three hypotheses for Nal resistance, thereby showing the *icd* mutants accumulate a compound that results in Nal resistance.

MATERIALS AND METHODS

Bacterial strains. Mutants were isolated from *E. coli* K-12 strains AB259 (HfrH *thi*) and W620 (*thi-1 pyrD36 gltA6 galK30 str-129*; 6). *glt* cells are deficient in citrate synthase activity and therefore require glutamate or proline. They have growth rates similar to *icd glt* mutants. A *glt+* derivative of strain W620 was constructed by transduction of *glt+* from AB259. *icd* mutants were isolated as described (7). *icd glt* mutants arose spontaneously in the *icd* strains and were identified as faster-growing colonies on tryptone agar or on glucose minimal medium plus glutamate. *glt* strains were constructed by transduction with phage P1*bt* as described else-

where in this paper. Strain K264 [HfrH *thi* Δ *(gal-bio) uvrB*] was obtained from D. Friedman. Other males used in conjugation were obtained from the *E. coli* Genetic Stock Center.

Media. The growth media have been described elsewhere (5). Glucose (0.2%) was used as carbon and energy source in minimal medium. L-Amino acids were added to 40 µg/ml as necessary. Thiamine was added to all media to 1 µg/ml. Resistance to Nal was determined on tryptone agar containing 10 µg of Nal per ml.

Enzyme studies. Nutrient broth cultures were grown overnight at 37 C for enzyme assays. The cells were sonically treated, and the crude extracts were used for measuring the enzyme activity as described (7). At the end of each experiment, the purity of the strains was tested by streaking the samples on tryptone agar with and without Nal (10 µg/ml) and on minimal glucose medium with and without glutamate.

To stabilize isocitrate dehydrogenase, DL-isocitrate was added to the cell suspension to 3.3 mg/ml before sonic oscillation. ICD activity was determined immediately after extraction. Citrate synthase activity was determined in samples that had been frozen and stored at −20 C overnight. The enzyme reactions were followed in a recording spectrophotometer at room temperature (approximately 25 C).

Fluorometric assays. Endogenous levels of citrate and isocitrate were measured fluorometrically as described by Williamson and Corkey (13). Cultures were grown in minimal glucose medium containing Casamino Acids (0.1%), thiamine, and L-glutamate (20 µg/ml) and harvested during exponential growth or after overnight growth (stationary phase). The cells from 100-ml samples were centrifuged and resuspended in 4 ml of water. Two 2-ml samples were removed, and 0.5 ml of 5% perchloric acid was added to each. The samples were allowed to stand at room temperature for 2 min. Then each was centrifuged 5 min at 5,100 × *g*, and the supernatant solution was retained. A 0.2-ml amount of *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES, 1 M, pH 6.5) was added to each sample. K_2CO_3 (1 M) was added dropwise with agitation until the pH was adjusted to be about 6.5. The samples were centrifuged once more, and each supernatant solution was retained for citrate and isocitrate measurement. Values determined on duplicate samples agreed within 4%. Control experiments showed that known amounts of citrate added to a cell pellet before extraction were recovered quantitatively in the final extract.

Growth studies. Overnight cultures in glucose minimal medium supplemented with Casamino Acids (0.1%) and thiamine were centrifuged, washed twice in 0.85% NaCl, resuspended in a similar medium without Casamino Acids to an absorbance of approximately 0.3 at 420 nm, and shaken at 37 C. After 1 h, 5-ml samples were added to 20 ml of glucose minimal medium plus amino acids at the desired concentration, and shaking was continued at 37 C. Samples were periodically removed to measure the absorbance at 420 nm, diluting in the

same medium as required to give an absorbance below 1.0.

Genetic analysis. Approximate positions of gene loci were determined as described by Low and Wood (10). Transduction with phage P1*bt* was done as described elsewhere (5), except that during phage adsorption the concentration of calcium chloride was increased to 0.25 M. After 3 days of growth at 37 C, the transductants were transferred to an identical medium and, after growth, scored for unselected markers by replica plating from the secondary plates.

RESULTS

Secondary mutants. As reported earlier (7), *icd glt*+ mutants grew slowly in nutrient broth relative to the wild type. The mutants also grew relatively slowly on all other media tested. The mutants grew on a minimal glucose medium supplemented with 0.1% Casamino Acids at approximately 75% the rate of the wild type, but achieved the same final growth yield (Fig. 1, Table 1).

By using auxanograms with glucose as energy source and with or without glutamate and proline, an attempt was made to find other supplements specifically stimulating growth of the mutant. On solid media, L-arginine consistently improved the growth response in the presence of glutamate or proline, presumably by sparing the glutamate otherwise needed for arginine synthesis. No effect of arginine on growth rate in liquid culture was apparent, however, in the presence or absence of glutamate or proline. No other supplement tried (amino acids, vitamins, and bases) specifically stimulated growth of *icd* mutants.

Minimal glucose plates streaked with several *icd* mutants were placed in sealed anaerobic chambers containing GasPaks (BBL) and incubated at 37 C. The mutants still exhibited a requirement for glutamate or proline, demonstrating that a single ICD serves to produce glutamate during both aerobic and anaerobic growth.

Although the original *icd* mutants reverted to prototrophy, occasional nutrient broth-grown cultures were found to contain no revertants. Cells from these cultures grew as rapidly as the wild type on complex media or on glucose plus Casamino Acids, although the growth yield was somewhat less (Fig. 1). The strains still exhibited a requirement for glutamate or proline and could not be transduced to prototrophy. These results suggested that the faster-growing mutant strains contained two (or more) mutations, each conferring a glutamate requirement for growth.
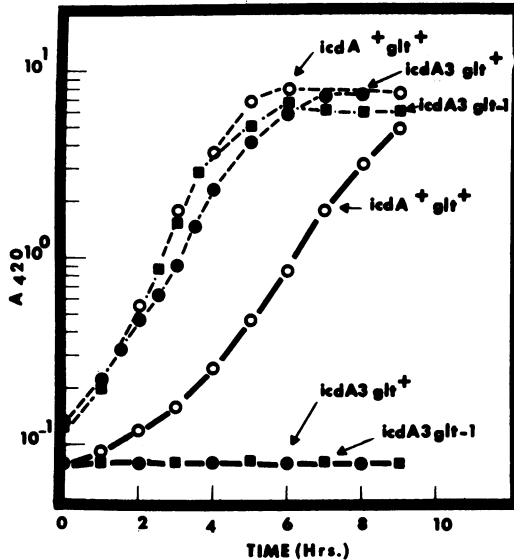
Identification of the second mutation. The

FIG. 1. *Growth of wild-type and mutant strains in glucose minimal medium and in glucose minimal medium supplemented with Casamino Acids. Solid lines represent growth in unsupplemented medium, and dashed lines represent growth in medium supplemented with Casamino Acids (0.1%).*

TABLE 1. *Growth rates of wild-type and mutant strains[a]*

Genotype	Doubling time (min) on medium supplemented with:				
	None	CAA	Gluta-mate	Proline	Proline, glutamate
icd+ glt+	60	32	60	60	58
icdA3 glt+		45	192	80	72
icdA3 glt-1		35	96	63	62

[a] Minimal medium contained 0.2% glucose as energy source and was supplemented as indicated with 0.1% Casamino Acids (CAA), 40 µg of glutamate per ml, or 40 µg of proline per ml. Strains include AB259 and mutant derivatives of AB259.

poor growth of *icd* mutants might result from accumulation of an inhibitory intermediate or from the drain on potential energy by formation of intermediate compounds such as citrate, aconitate, or isocitrate, which are not further metabolized. In either case, relief should come from blocking citrate synthase activity. Consistent with this expectation, each of five faster-growing derivatives of *icd* mutants lacked citrate synthase activity as well as isocitrate dehydrogenase activity (Table 2), but still retained the activity of other tricarboxylic acid cycle enzymes including aconitase (data to be reported elsewhere).

gltA mutants (lacking citrate synthase activity) have been mapped at 16 min and are co-transducible with the *gal* locus (8). If our secondary mutants are altered in the *gltA* gene, these mutations should also lie at approximately 16 min. We demonstrated that this was indeed the case as follows. Phage P1 stocks grown on each of the strains lacking both ICD and citrate synthase activities were used separately to transduce a *gal* strain (*glt+* W620) to *gal+*. With phage from each double-mutant strain, a mutation giving a glutamate requirement was cotransduced with *gal+* (Table 3). One such transductant resulting from each of four double-mutant donor strains was examined and found to have isocitrate dehydrogenase activity but to lack citrate synthase activity (Table 2).

The *gltA* gene is not jointly cotransducible with *gal+* and *bio+* although *gal+* and *bio+* have a cotransduction frequency of 1% (8). *bio* is far to the opposite side of *gal* from *glt,* and the inability to cotransduce all the three genes together presumably reflects limitation on the amount of DNA that can be packaged within P1. Neither *gltA6* (a bona fide *gltA* mutation) nor *glt-1* could be cotransduced with *gal+* to a recipient containing a genetic deletion extending from *gal* to beyond *bio* (strain K264). These results show that the *glt-1* mutation lies to the left of *gal,* as does the established *gltA6* mutation.

Phage grown on each of four single *glt* mutants were used to transduce W620 *(gal gltA)* to *gal+* and *glt+*. In each case, *glt+* transductants appeared at a frequency between 0.04 and 4% that of *gal+* transductants from the same cross. Such close linkage is consistent with the location of mutations within a single gene. We conclude that secondary mutants appearing in cultures initiated with *icd* cells have the genotype *icd glt.*

The *icd* gene is not closely linked to the *glt* gene because each of the *gal+* transductants lacking citrate synthase activity retained ICD activity. Similarly, the glutamate requirement imposed by *icd* mutations was not cotransducible with *gal+* (less than 0.5% cotransduction using phage grown on *icd* single-mutant strains).

By mating, we were able to determine the approximate location of the *icd* gene. Strains HfrH (origin 87 min, transfers clockwise), KL16 (origin 56 min, transfers counterclockwise), and KL25 (origin 74 min, transfers clockwise) transferred the *icd+* gene very late. On the other hand, AB313 (origin 74 min, transfers counterclockwise) and AB312 (origin 60 min, transfers clockwise) transferred the *icd+* gene within 15 min, showing that *icd* lies between 60 and 74 min. Similar results were obtained with

six recipients derived from two different K-12 strains, each containing an independently arising *icd* mutation.

Growth. We compared the growth behavior of wild-type parent cells (AB259), an *icdA3* derivative, and an *icdA3 glt-1* secondary mutant appearing as a faster-growing colony on tryptone agar seeded with *icdA3* mutants. The growth rate of the *icd glt* double mutant was faster than that of the *icd* single mutant on both complex and defined media (Fig. 1 and 2, Table 1). As shown in Fig. 2 and 3 and Table 1, the double mutant grew much faster on glucose than did the *icd* single mutant, but achieved the same final density when limited by glutamate. On glucose supplemented with proline, both *icd glt⁺* and *icd glt* cells showed a lag before growth began (not shown). However, once adapted to proline, growth was faster than on glucose plus glutamate (Fig. 2, Table 1). The lag presumably reflected the time required for synthesis of the transport system and/or catabolic enzymes required to convert proline to glutamate.

When the medium was supplemented with both proline and glutamate, the *icd* and *icd glt* cells each showed the faster growth rate characteristic of growth utilizing proline, but they lacked the growth lag observed in medium supplemented with proline alone (Table 1).

The growth rates on glucose with limiting glutamate or proline are shown for each strain in Fig. 2. The amino acid concentration had no obvious effect on the growth rate of wild-type cells. The growth rate of *icd* and *icd glt* cells increased as the glutamate concentration was increased, but did not achieve the rate of the wild type. At all concentrations, the growth rate of the double mutant was greater than that of the single. The growth response to proline was consistently greater than to glutamate, and on proline the growth rate of the double mutant was almost equal to that of the wild type. The growth yields on limiting glutamate and proline were essentially equivalent for each of the mutant strains (Fig. 3). Growth yields of the mutants did not increase at glutamate concentrations in excess of 70 μg/ml. This might result from induction at high glutamate concentrations of an enzyme system catabolizing glutamate.

The viable counts of the two mutants and of wild-type cells were equivalent when plated on tryptone agar and on glucose salts medium supplemented with Casamino Acids, glutamate, or proline.

The reduced growth rate of *icd* cells might result from the inability to utilize the energy source with maximal efficiency because citrate and citrate derivatives formed cannot be further metabolized. If so, the growth yield of the *icd* mutant on limiting glucose should be less than that of the wild type and of the double mutant. Contrary to this expectation, the growth yields of the wild type and the *icd* mutant did not differ significantly from each other,

TABLE 2. *Enzyme activities in wild-type and mutant strains*

Enzyme	Enzyme activity[a]				
	AB259 *icd⁺ glt⁺*	AB259 *icdA3 glt⁺*	AB259 *icdA3 glt-1*	W620 *icd⁺ glt⁺*	W620[b] *icd⁺ glt-1*
Citrate synthase (EC 4.1.3.7)	123	272	<2	75	<2
Isocitrate dehydrogenase (EC 1.1.1.42)	696	<1	<1	479	643

[a] Expressed as nanomoles of substrate transformed per minute per milligram of protein.

[b] *icd⁺ glt-1* W620 was constructed by transduction of *glt-1 gal⁺* from *icdA3 glt-1 gal⁺* AB259 to *glt⁺* W620.

TABLE 3. *Frequency of cotransduction of glt and gal*

Donor markers[a]	Recipient markers[b]	Selected marker	Unselected donor marker	Cotransduction frequency
icd⁺ glt⁺ gal⁺	*icd⁺ gltA6 gal*	*gal⁺*	*glt⁺*	0.45 (42/94)
icd⁺ glt⁺ gal⁺	*icd⁺ gltA6 gal*	*glt⁺*	*gal⁺*	0.18 (33/178)
icdA3 glt-1 gal⁺	*icd⁺glt⁺ gal*	*gal⁺*	*glt*	0.16 (24/151)
icd-102 glt-2 gal⁺	*icd⁺glt⁺ gal*	*gal⁺*	*glt*	0.29 (41/140)
icd-103 glt-3 gal⁺	*icd⁺glt⁺ gal*	*gal⁺*	*glt*	0.15 (27/184)
icd-104 glt-4 gal⁺	*icd⁺glt⁺ gal*	*gal⁺*	*glt*	0.09 (20/211)

[a] Donor strains are derivatives of AB259.
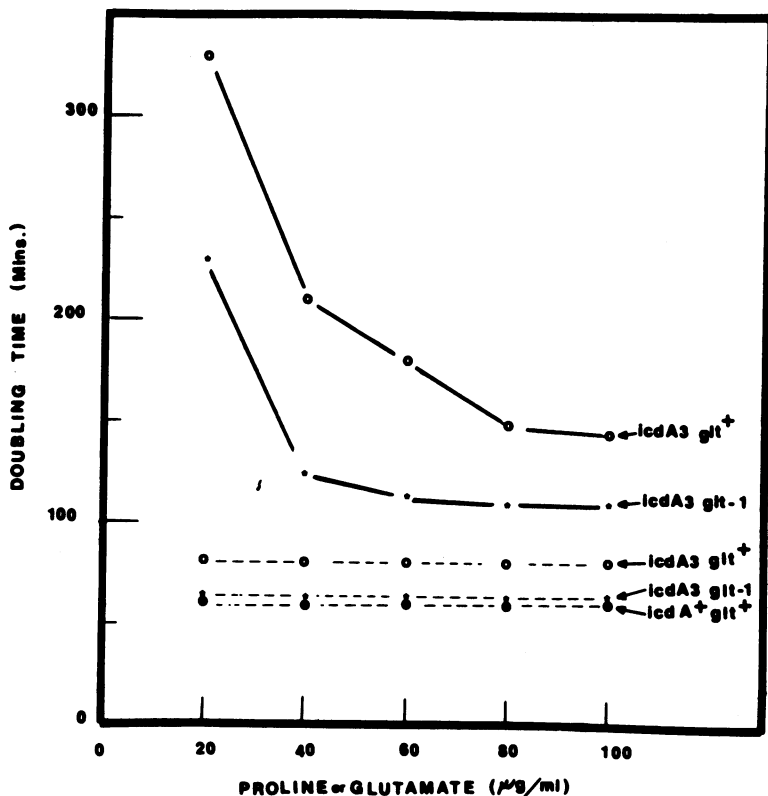
[b] Recipient strain is W620 or W620 *glt⁺*.

FIG. 2. *Growth rates of wild-type and mutant strains as a function of proline or glutamate concentration. Dashed lines indicate growth on proline; continuous lines indicate growth on glutamate. Growth rate of the wild type was not affected detectably by the presence of glutamate or proline at these concentrations.*

but the growth yield of the double mutant was substantially less (Fig. 4). These results show that the reduced growth rate of *icd* cells is not the result of decreased efficiency of energy utilization, but rather must result from accumulation of an inhibitory intermediate (because *icd* cells blocked in synthesis of citrate by a *glt* mutation grow faster).

Citrate and isocitrate accumulation. Our previous results suggested that citrate and isocitrate accumulated in *icd glt⁺* cells but not in *icd glt* cells. To test this possibility directly, we measured the intracellular levels of these intermediates. The single mutants accumulated several hundred times as much citrate and isocitrate as the wild type and (as expected) no detectable citrate or isocitrate was present in *icd glt* cells (Table 4).

Resistance to Nal. The availability of strains of the four genotypes *icd⁺ glt⁺*, *icd glt⁺*, *icd⁺ glt*, and *icd glt* afforded us an opportunity to test each of the hypotheses enumerated previously. The Nal resistance of each strain was tested (Fig. 5). The results are clear cut—*icd*

glt⁺ strains are Nal resistant relative to strains of the other three genotypes. If resistance were due to lack of an end product of ICD activity (hypothesis iii), *icd⁺ glt* and *icd glt* would be Nal resistant. If resistance resulted from a *cis* effect on a neighboring gene (hypothesis i) or from a direct effect of the *icd* gene product (hypothesis ii), *icd⁺ glt* strains should be Nal sensitive but *icd glt* strains should be Nal resistant. Since none of these possibilities is realized, hypothesis iv must be correct; namely, Nal resistance results from accumulation of a metabolic intermediate.

DISCUSSION

Both poor growth rate and the resistance to nalidixic acid characteristic of mutants deficient in ICD probably stem from the accumulation of an inhibitory product. Because these effects are not observed in cells additionally lacking citrate synthase activity, the responsible compound(s) is presumably citrate, isocitrate, aconitate, or a derivative of one of these. Consistent with this interpretation, we showed

by direct measurement that large amounts of citrate and isocitrate accumulate within *icd* cells.

The equivalence of the growth yields of the wild type and the *icd* mutant and the poorer yields of cells blocked in citrate synthase as well *(icd glt)* indicate that the glyoxylate cycle must be functional in the single mutants. In support of this conclusion, further detailed studies of acetate metabolism in *icd* cells show that isocitrate lyase (the first enzyme in the glyoxylate cycle) is inducible in such cells and that they fully utilize the acetate produced from glucose (Lakshmi and Helling, in preparation).

The target of the growth-inhibitory compound is unknown. It does not appear to be at the level of specific transport of the required

amino acid, since the ratio of growth rates of *icd glt* to *icd glt⁺* cells is nearly constant over a range of glutamate concentrations, and the growth rate of *icd glt⁺* cells is independent of proline concentrations at concentrations of 20 µg/ml and greater (Fig. 2). It could be at the level of transport of other materials required by the cell. We have observed no difference in rates of uptake of α-methylglucoside by wild-type and *icd* cells (Adams and Helling, unpublished data).

Why accumulation of citrate (or a derivative) causes Nal resistance is unknown. Under our conditions, the principal effect of Nal is to block DNA synthesis (2). There is no obvious structural similarity between Nal and any tricarboxylic acid cycle-related compound with the ex-
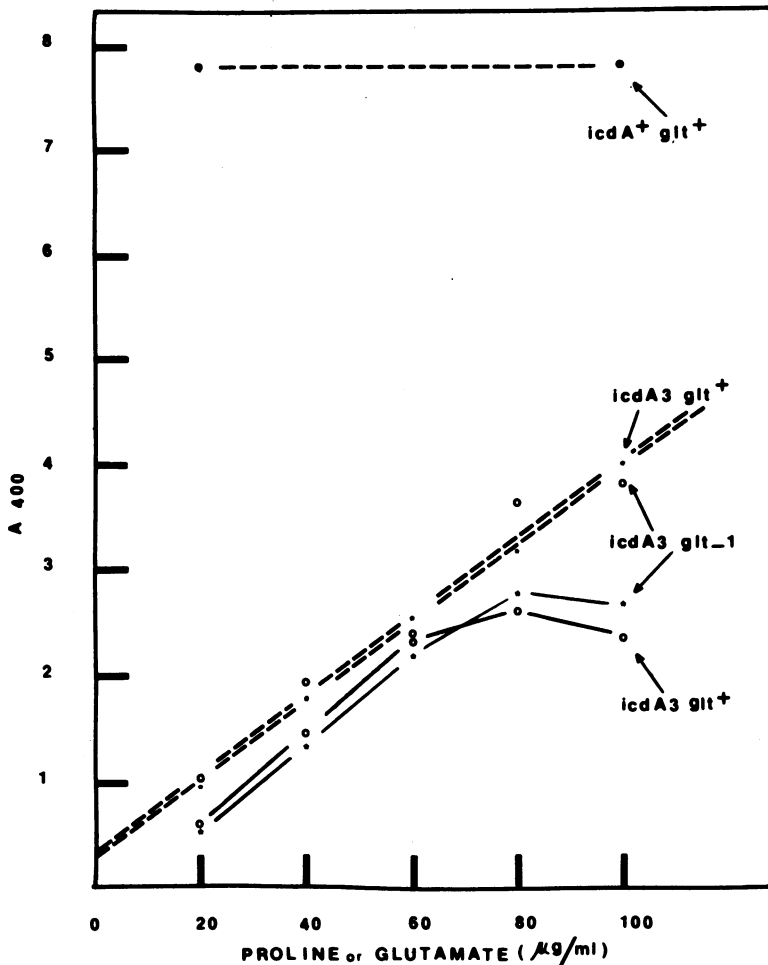

FIG. 3. *Growth yields of wild-type and mutant strains as a function of proline or glutamate concentration. Dashed lines indicate growth on proline and were fitted by the method of least squares. Continuous lines indicate growth on glutamate and were fitted by eye. Growth yield of the wild type was not affected detectably by the presence of glutamate or proline at these concentrations.*
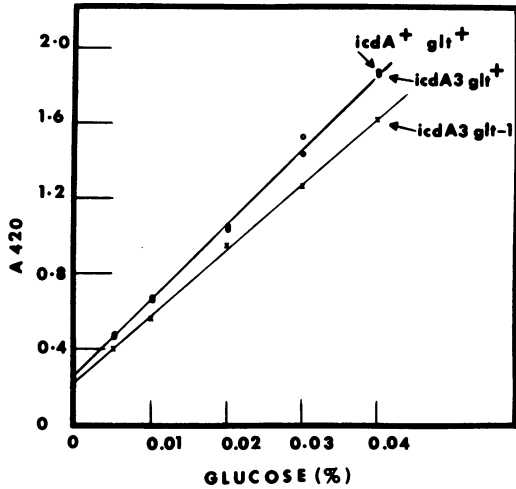
FIG. 4. *Growth yields of wild-type and mutant strains in glucose-limited medium. L-Glutamate and L-proline were each added to 40 µg/ml. Lines were fitted by the method of least squares.*

TABLE 4. *Intracellular citrate and isocitrate levels*

Strain	Citrate[a]		Isocitrate[a]	
	Exponential	Stationary	Exponential	Stationary
icd⁺ glt⁺	0.9	0.1	<0.25	<0.25
icdA3 glt⁺	290	152	30.5	16.6
icdA3 glt-1	<0.1	<0.1	<0.25	<0.25

[a] Nanomoles × 10⁻¹⁰ of citrate or isocitrate per cell. Figures represent the average of duplicate samples. Cells of the three strains had equal volumes as determined from absorbance measurments and viable counts.

FIG. 5. *Colony-forming ability on tryptone agar plus nalidixic acid. Single colonies were picked into 1 ml of tryptone broth and grown overnight at 37 C. Appropriate dilutions were spread on the surface of tryptone agar containing Nal at the concentration indicated. Responses of AB259 icd⁺ glt⁺ (O), AB259 icdA3 glt⁺ (●), AB259 icdA3 glt-1 (■), and W620 icd⁺ glt-1 (▲) strains are shown.*

ception of oxalacetate. Oxalacetate accumulated in *icd* mutants might competitively block the interaction of Nal and its target. We have no evidence to support this hypothesis. Malate, succinate, and oxalacetate do not alter the sensitivity of wild-type or *icd* cells to the drug. Drug resistance could result from inhibition of drug transport or drug activation. Earlier experiments suggested that Nal was transported by the phosphotransferase system because a *ctr* mutant was Nal resistant (6). However, we have tested other *ctr* mutants deficient either in enzyme I or in the Hpr protein and found that they remained Nal sensitive. Thus, the phosphotransferase system is not necessary for Nal uptake. The Nal resistance of the original *ctr* strain probably resulted from an unrelated but closely linked mutation.

The genes for glutamate dehydrogenase, succinate dehydrogenase, and two components of 2-oxoglutarate dehydrogenase had previously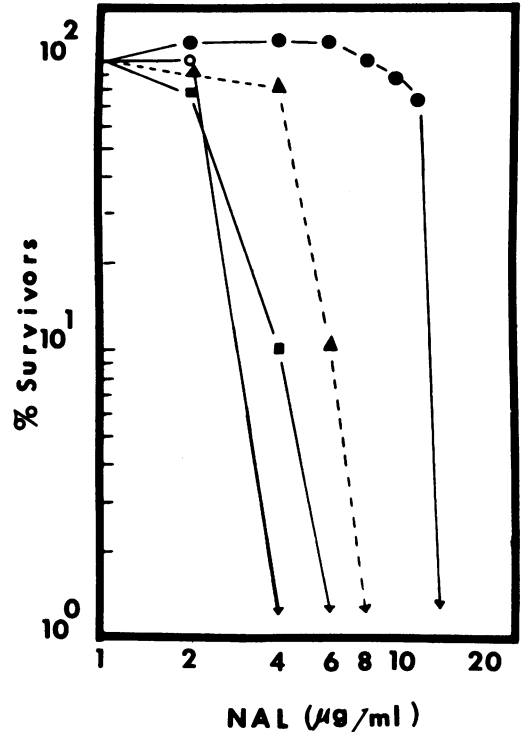 been shown to cluster very closely at 16 min on the standard map (12), raising the possibility that all tricarboxylic acid cycle genes might be adjacent, presumably for control purposes (9). However, the genes for malate dehydrogenase (4), lipoate dehydrogenase (3), and now isocitrate dehydrogenase have been shown to lie elsewhere, ruling out the hypothesis of a common control over all tricarboxylic acid cycle genes through gene clustering.

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