JOURNAL OF BACTERIOLOGY, Mar. 1971, p. 1224-1226
Vol. 105, No. 3

Printed in U.S.A.

Nalidixic Acid-Resistant Mutants of *Escherichia coli* Deficient in Isocitrate Dehydrogenase

ROBERT B. HELLING AND JOHN S. KUKORA
Department of Botany, University of Michigan, Ann Arbor, Michigan 48104

Received for publication 29 December 1970

icd Mutants of *Escherichia coli* K-12, selected for their resistance to nalidixic acid, are deficient in isocitrate dehydrogenase.

Among mutants of *Escherichia coli* selected for resistance to low levels of nalidixic acid (Nal), a reversible inhibitor of deoxyribonucleic acid (DNA) synthesis (6), many have simultaneously acquired a new growth factor requirement (9). These auxotrophs fall into two groups. Mutants of the first group specifically require adenine as the result of loss of adenylosuccinate lyase activity (due to mutation of the *purB* gene; 9). Mutants of the other group of Nal-resistant (Nal^r) auxotrophs require Casamino Acids for growth. In this communication we present evidence identifying the enzymatic lesion in the most frequent class of amino acid-requiring mutants.

AB259 (HfrH, *thi*) and KL98 (Hfr, λ-lysogenic), derivatives of *E. coli* K-12, were obtained from A. Taylor and B. Low. From these strains Nal^r mutants were selected for growth on tryptone-agar containing 10 μg of Nal per ml. Generally, from 10 to 80% of the Nal^r auxotrophs obtained responded well to both L-glutamate and to L-proline in auxanograms (with glucose as energy source), and somewhat more poorly to 2-oxoglutarate and to L-glutamine. (Other Casamino Acid-requiring Nal^r auxotrophs which do not respond to glutamate were also obtained, but we have not identified the enzymatic lesion in any of these mutants. Reduced sulfur-containing compounds such as methionine can substitute for the Casamino Acid requirement of some of the mutants.) Most mutant cultures contained a high frequency of revertants. Simultaneously with reversion to prototrophy, the cells reacquired sensitivity to Nal. Thus both drug resistance and the requirement for Casamino Acids appear to result from the same mutation.

Although the response to glutamate (and to proline) was obvious in an auxanogram, the mutant cells grew slowly when streaked to minimal glucose plus glutamate, poorly (not at all in some cases) on succinate plus glutamate, and no growth was observed on acetate plus glutamate, or on medium not supplemented with glutamate. The poor growth may be, in part, the result of poor glutamate uptake and metabolism in *E. coli* K-12 as suggested from studies on the use of glutamate as sole carbon source (8). Growth was somewhat better on glucose or succinate supplemented with proline rather than glutamate, but in no case did a mutant grow with acetate as energy source. The wild type grew faster than the Nal^r auxotrophs in nutrient broth (24-min doubling time for KL98 versus 60 min for KL98 *icd-5*).

These results suggested that the mutant cells were deficient in synthesis of glutamate, probably because of deficiency in 2-oxoglutarate, an intermediate in the tricarboxylic acid cycle and the immediate precursor of glutamate (Fig. 1).

To test this possibility, the ability of mutant and wild-type cells to respire a variety of tricarboxylic acid cycle-related compounds was examined using nutrient broth-grown cells and standard respirometric procedures (16). Although mutant and wild type respired 2-oxoglutarate equally well, glucose, pyruvate, succinate, and malate were respired less well in the mutant than in the parent strain. The mutant failed to respire acetate (even after incubation in minimal medium containing 0.4% acetate plus 40 μg of L-glutamate per ml for 3.5 hr) and only a small amount of oxaloacetate (OAA) was respired. These results showed that the cause of respiratory deficiency in the mutant lies somewhere before 2-oxoglutarate (which was respired well), but after OAA and acetate. Thus the mutants could be deficient in citrate synthase, aconitase, or isocitrate dehydrogenase, each of which is required to respire acetate and OAA and to synthesize 2-oxoglutarate. [The small amount of OAA which was respired could result from a contaminant in the OAA (e.g., pyruvate resulting from spontaneous decarboxylation), or from conversion of OAA to pyruvate via either malic enzyme, or phosphoenolpyruvate carboxykinase and pyruvate kinase.]

The activity of the three enzymes was meas-

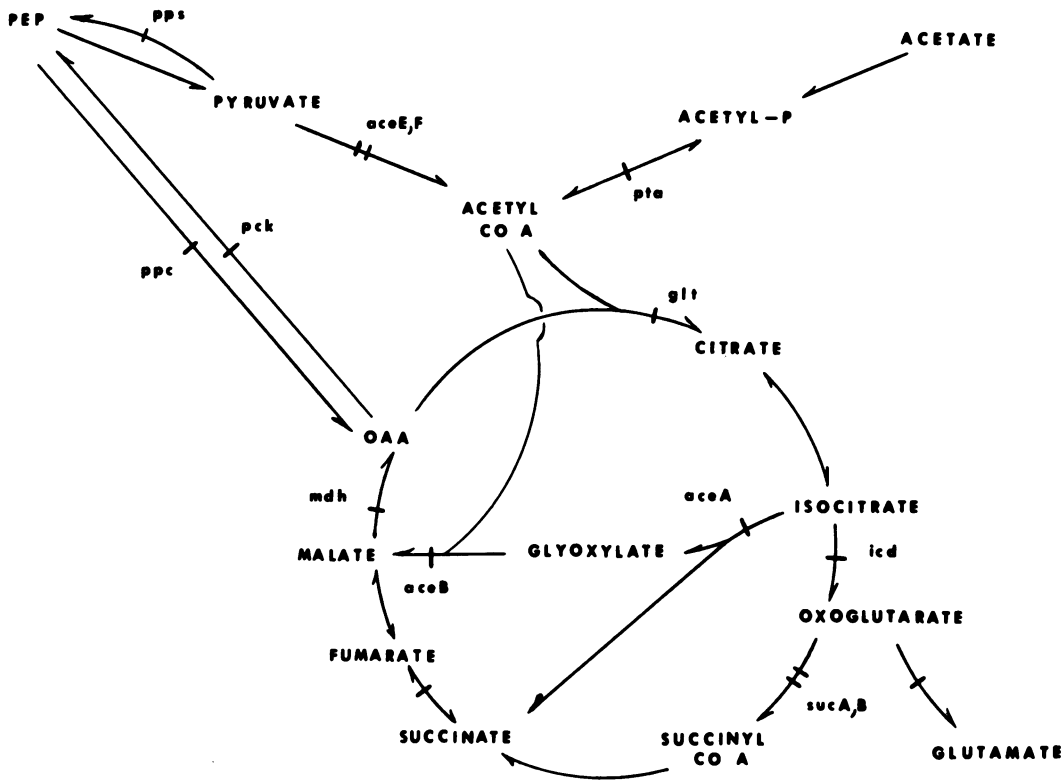

FIG. 1. *Metabolic pathways showing mutant blocks affecting tricarboxylic acid cycle and tricarboxylic acid cycle-related enzymes. Enzymes or enzyme complexes blocked in mutants are: pps (phosphopyruvate synthase, 15), aceE and aceF (pyruvate dehydrogenase, 15), pta (phosphate acetyltransferase, 12), gltA (citrate synthase, 15), aceA (isocitrate lyase, 15), aceB (malate synthase A, 15), icd (isocitrate dehydrogenase [NADP]), sucA and sucB (oxoglutarate dehydrogenase, 15), mdh (malate dehydrogenase, 2), ppc (phosphopyruvate carboxylase, 15), pck (phosphoenolpyruvate carboxykinase, 13). Mutants deficient in succinate dehydrogenase (2) and glutamate dehydrogenase (17) were reported but no mutant designation was assigned.*

ured. The mutants tested had somewhat higher levels of citrate synthase and aconitase than the wild type, but completely lacked isocitrate dehydrogenase (Table 1). Mixing experiments showed that the absence of enzyme activity in the mutant did not result from an inhibitor capable of inhibiting enzyme from wild-type cells. Similar results were obtained with each of five other phenotypically similar Nal^r mutants isolated from two different derivatives of *E. coli* K-12. We designate these mutants *icd* mutants, *icd* standing for isocitrate dehydrogenase.

The mutants showed normal glutamate dehydrogenase activity (Table 1). Citrate lyase, which converts citrate to OAA and acetate, was present at the same uninduced level in both mutant and wild type. This enzyme is known to be induced in *E. coli* by citrate but only during anaerobic growth (7).

During growth on acetate or fatty acids (which form acetyl coenzyme A), isocitrate can be metabolized by an alternative pathway making use of the two inducible enzymes of the glyoxylate cycle, isocitrate lyase, and malate synthase A. This pathway allows the cell to meet the requirement for OAA resulting from growth and from loss of tricarboxylic acid cycle intermediates in biosynthesis. Isocitrate lyase was assayed, and the enzyme level was shown to be much lower in the mutant than in the wild type when grown on nutrient broth (Table 1). However, the enzyme was induced in the mutant when the cells were incubated in minimal acetate plus glutamate medium (Table 1). Presumably, in the mutant both the repression of isocitrate lyase synthesis and the inability to respire acetate via the glyoxylate cycle are the result of high levels of phosphoenolpyruvate (PEP) when grown on nutrient broth. PEP has been shown to act as repressor and also to inhibit isocitrate lyase activity (10).

TABLE 1. *Enzymatic activities in wild-type and mutant strains*[a]

Enzyme	Strain			
	KL98	KL98 *icd-5*	AB259	AB259 *icd-3*
Aconitase (EC 4.2.1.3)	290	414		
Citrate synthase (EC 4.1.3.7)	103	311	87	105
Isocitrate dehydrogenase (EC 1.1.1.42)	552	<4	304	<5
Citrate lyase (EC 4.1.3.6) ...	1.1	1.6	1.3	0.98
Isocitrate lyase (EC 4.1.3.1).	61	4.8		
Isocitrate lyase (acetate)[b] ...	69	37		
Glutamate dehydrogenase (EC 1.4.1.4)[c]	135	132		

[a] Nanomoles of substrate transformed per minute per milligram of protein. Overnight cultures in nutrient broth were washed in 1 mM ethylenediaminetetraacetic acid (*p*H 7.4), resuspended to 1.5×10^9 cells per ml in the assay buffer, and sonically treated with 75% intensity (four 15-sec pulses) at 4 C, by using a Biosonic II ultrasonic probe with needle tip. After centrifuging 30 min at 5,000 × *g*, the supernatant solution was used for enzyme assay. Protein was determined by the Folin phenol procedure (11). Activity of all enzymes was measured spectrophotometrically at room temperature (about 25 C) by using freshly prepared extracts. Citrate synthase was assayed according to the DTNB method of Srere (14) by using 0.1 M tris(hydroxymethyl)aminomethane buffer, *p*H 7.5. Aconitase was measured by following the enzyme-dependent disappearance of *cis*-aconitate at 240 nm (5). Enzyme was measured in a 3-ml cuvette without activation and without NaCl. Other enzymes were assayed as described elsewhere: glutamate dehydrogenase (17), citrate lyase (3), isocitrate lyase (4), isocitrate dehydrogenase (1).

[b] Nutrient broth-grown cells were washed and shaken in minimal medium containing 0.4% acetate plus 40 μg L-glutamate per ml for 3.5 hr before harvesting.

[c] Nutrient broth-grown cells were washed and shaken in minimal medium containing 0.2% D-glucose for 3.5 hr before the cells were harvested, and extracts were prepared as described.

The cause of drug resistance is obscure and may be an indirect effect of accumulation of intermediates before the block. There is no obvious structural similarity of Nal and any of the immediate precursors of 2-oxoglutarate.

This work was supported by National Science Foundation grant GB-5737 and by Institutional Research grant IN-40J to the University of Michigan from the American Cancer Society. J. K. held a summer fellowship funded by American Cancer Society Institutional Research grant IN-40J.

We thank A. Taylor and B. Low for strains, and R. Davis, H. Ikuma, and E. Juni for their criticisms.

LITERATURE CITED

1. Cribbs, R., and E. Englesberg. 1964. L-Arabinose negative mutants of the L-ribulokinase structural gene affecting the levels of L-arabinose isomerase in *Escherichia coli*. Genetics **49**:95-108.
2. Courtright, J. B., and U. Henning. 1970. Malate dehydrogenase mutants in *Escherichia coli*. J. Bacteriol. **102**:722-728.
3. Dagley, S. 1969. Citrate lyase, p. 160-163. *In* J. M. Lowenstein (ed.), Methods in enzymology, vol. 13. Academic Press Inc., New York.
4. Dixon, G. H., and H. L. Kornberg. 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72**: 3P.
5. Fansler, B., and J. M. Lowenstein. 1969. Aconitase from pig heart, p. 26-30. *In* J. M. Lowenstein (ed.), Methods in enzymology, vol. 13. Academic Press Inc., New York.
6. Goss, W. A., W. H. Dietz, and T. M. Cook. 1965. Mechanism of action of nalidixic acid on *Escherichia coli*. II. Inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. **89**:1068-1074.
7. Gunsalus, I. C. 1958. Organic acid aldolases: microbial C_2 formation reactions. Proc. 4th Int. Congr. Biochem. Vienna **13**:226-250.
8. Halpern, Y. S., and M. Lupo. 1965. Glutamate transport in wild-type and mutant strains of *Escherichia coli*. J. Bacteriol. **90**:1288-1295.
9. Helling, R. B., and B. S. Adams. 1970. Nalidixic acid-resistant auxotrophs of *Escherichia coli*. J. Bacteriol. **104**: 1027-1029.
10. Kornberg, H. L. 1966. The role and control of the glyoxylate cycle in *Escherichia coli*. Biochem. J. **99**:1-11.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
12. Morgan, M. J., and H. L. Kornberg. 1969. Regulation of sugar accumulation by *Escherichia coli*. FEBS Let. **3**:53-56.
13. Sanderson, K. 1970. Current linkage map of *Salmonella typhimurium*. Bacteriol. Rev. **34**:176-193.
14. Srere, P. A. 1969. Citrate synthase, p. 3-11. *In* J. M. Lowenstein (ed.), Methods in enzymology, vol. 13. Academic Press Inc., New York.
15. Taylor, A. L. 1970. Current linkage map of *Escherichia coli*. Bacteriol. Rev. **34**:155-175.
16. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric techniques, 4th ed. Burgess, Minneapolis.
17. Vender, J., and H. V. Rickenberg. 1964. Ammonia metabolism in a mutant of *Escherichia coli* lacking glutamate dehydrogenase. Biochim. Biophys. Acta **90**:218-220.