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Isolation of a Mutant of *Escherichia coli* with a Temperature-sensitive Fructose-1,6-Diphosphate Aldolase Activity

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ABSTRACT

BÖCK, AUGUST (Purdue University, Lafayette, Ind.), AND FREDERICK C. NEIDHARDT. Isolation of a mutant of *Escherichia coli* with a temperature-sensitive fructose-1,6-diphosphate aldolase activity. J. Bacteriol. **92**:464–469. 1966.—A mutant of *Escherichia coli* was isolated which was able to grow in rich medium at 30 C but not at 40 C. Upon exposure to 40 C, the cells immediately stopped ribonucleic acid (RNA) and deoxyribonucleic acid synthesis, but protein synthesis continued at a diminished rate for a short time. Addition of chloramphenicol did not release RNA synthesis from inhibition at 40 C. Synthesis of β-galactosidase could be induced at high temperature despite the presence of glucose in the medium, indicating a lesion in glucose catabolism. Of many catabolic enzymes tested in cell-free extracts, only fructose-1,6-diphosphate aldolase activity appeared to be altered in the mutant cells. No activity was demonstrable in extracts of mutant cells grown at either 30 or 40 C, but determination of glucose-oxidation patterns revealed that the enzyme is probably active in vivo at 30 C. Temperature-resistant secondary mutants were found to have partially or fully restored aldolase activity, and temperature-resistant recombinants had normal aldolase activity, indicating that the growth pattern and the altered aldolase had a common genetic basis. Linkage data permitted the assignment of an approximate map location for the mutated aldolase gene.

During the search for mutants of *Escherichia coli* having a temperature-sensitive lesion in the synthesis of macromolecules, a number of isolates were obtained in which after a shift to 40 C ribonucleic acid (RNA) and deoxyribonucleic acid synthesis stopped immediately, and the synthesis of protein continued, at a diminished rate, for a short time (7). This pattern of macromolecule synthesis by a strain with relaxed control over RNA synthesis growing in a nutritionally rich medium suggests a lesion in RNA synthesis—either in a nucleoside or nucleotide kinase or in the polymerizing step itself. The desirability of strains with such lesions led us to select one of these isolates, called h8, for detailed biochemical analysis.

The inability of this mutant to grow at 40 C has somewhat unexpectedly proved to be caused by an altered fructose-1,6-diphosphate aldolase activity. The affected enzyme cannot be demonstrated in extracts prepared from mutant cells at any temperature, as is the case for certain other temperature-sensitive mutants (e.g., 2). Nevertheless, there is evidence that aldolase is active in vivo in mutant h8 at the permissive temperature.

Absence of aldolase activity in the mutant at 40 C confers several interesting properties on these cells, including an extreme sensitivity to minute amounts of many hexoses.

Identification of mutant h8 as a temperature-sensitive aldolase mutant is described in this paper. The companion paper deals with the physiological consequences of aldolase deficiency.

MATERIALS AND METHODS

Organisms. The principal experiments were performed with *E. coli* K-10, a B_1-requiring wild strain with relaxed control over RNA synthesis, and with mutant h8, a temperature-sensitive derivative of K-10. In addition, *E. coli* strains AB1117 no. 1 and AB2297

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(obtained from E. Adelberg of Yale University) were employed in the genetic crosses.

Growth of cultures. Except in the mutant isolation procedures, which required a special medium described below, the following two media were used: (i) a minimal medium consisting of basal salts solution P (4), 0.2% $(NH_4)_2SO_4$, 0.4% carbon source, and, when required, 5 μg/ml of thiamine; and (ii) an enriched medium containing 10% tryptone, 5% yeast extract, and 0.2% glucose. Liquid cultures were grown aerobically on a rotary-action shaker at 30 or 40 C. Growth was measured by optical density in a Zeiss spectrophotometer (model PMQ II) at 420 mμ.

Isolation of mutant h8. The wild strain, K-10, was grown overnight in the mutant-isolation medium described by Eidlic and Neidhardt (2). A 5-ml portion of the culture was adjusted to *p*H 7.0 and placed in a 250-ml flask with 100 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 30 min (ca. 90% killing). The mutagen was diluted out by adding 45 ml of rich medium, and the culture was incubated at 30 C for 5 hr to allow expression of the mutants. An appropriate dilution was plated on rich plates and incubated for 24 hr at 35.5 C. From these plates the smallest colonies were picked, streaked on plates, and incubated at 30 C. Separate colonies from these plates were suspended in 1 ml of saline, and a loopful of each suspension was put on two plates; one was incubated at 30 C, and the other at 40 C. Cells giving rise to colonies only at the lower temperature were studied further; one such mutant is h8. [By this variation of the original method (7), the use of penicillin at the high temperature can be avoided, and mutants can be isolated for which 40 C is a lethal, not just a bacteriostatic, temperature.]

Chemical assays. Protein and RNA were measured by colorimetric assays as described previously (2).

Incorporation studies. To estimate protein and nucleic acid synthesis by isotope incorporation, cultures were grown in minimal medium containing either C^{14}-labeled L-arginine (100 μg per ml; ca. 500 counts per min per μg) or C^{14}-labeled uracil (40 μg per ml; ca. 500 counts per min per μg). The cells from culture samples were collected on membrane filters and washed thoroughly with ice-cold minimal medium lacking a carbon source. Radioactivity was measured in a thin end-window, gas-flow counter.

Enzyme assays. β-Galactosidase was assayed in toluene-treated cells by measuring the hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG) according to the directions of Herzenberg (5). One unit of enzyme was defined as that which hydrolyzes 1 mμmole of ONPG per min.

Enzyme extracts for measurement of fructose-1,6-diphosphate aldolase were prepared by suspending medium-washed cells in 0.05 M tris(hydroxymethyl)-aminomethane-HCl (*p*H 7.8) and treating them with a Branson sonic oscillator. The extracts were centrifuged at 20,000 $\times$ *g* for 15 min, and the supernatant fluids were dialyzed against buffer overnight. Aldolase activity was measured in two different ways. In the first, the formation of triose hydrazone was measured by the method of Sibley and Lehninger (8) as modified by Dounce et al. (1). The second assay employed a coupled enzymatic test in which the rate of formation of glyceraldehyde-3-phosphate was measured by a nicotinamide adenine dinucleotide (NAD)-coupled oxidation by glyceraldehyde-3-phosphate dehydrogenase. For the latter assay, the reaction mixture consisted of 3×10^{-4} M NAD; 1.67×10^{-2} M fructose-1,6-diphosphate (*p*H 7.4); 2.67×10^{-2} M glycine; 1.73×10^{-2} M $Na_2HAsO_4 \cdot 7H_2O$; 0.2 mg of crystalline glyceraldehyde-3-phosphate dehydrogenase; and 100 to 300 μg (protein) of the dialyzed crude extract. Increase in optical density at 340 mμ was followed in a Gilford spectrophotometer at 25 C.

All enzyme assays were performed under conditions where the activity measured was proportional to the amount of protein added. All of the enzymes (except the mutant aldolase) were stable under the conditions employed.

Glucose metabolism. C6-C1 ratios of glucose oxidation were measured by suspending washed glucose-grown cells to an optical density (420 mμ) of 1.0 in diluted (1:5) minimal medium lacking $(NH_4)_2SO_4$ and a carbon source. Portions (10-ml) were placed in bubbling tubes connected with CO_2-adsorption columns containing 10% KOH, and were aerated for 30 min. Glucose-*1-C*^{14} or glucose-*6-C*^{14} (100 μg/ml; 0.5 μc/ml) were then added. After incubation at an appropriate temperature, the reaction was terminated by adding trichloroacetic acid to a final concentration of 5%. The aeration was continued for 5 min. The radioactivity in trichloroacetic acid-insoluble material, in CO_2, and in the medium was then determined with the aid of a Nuclear Chicago thin end-window gas-flow counter.

Genetic crosses. Matings were performed between AB1117 no. 1, K-10, or AB2297 (Hfr strains) and h8, after the latter strain (which is also an Hfr) was converted into an F^- phenocopy. The procedures employed have been fully described (2).

RESULTS AND DISCUSSION

E. coli mutant h8 was chosen for analysis on the basis of its response to a temperature shift from 30 to 40 C. When a culture of h8 growing in glucose minimal medium was shifted from 30 to 40 C, there was a slow synthesis of protein (measured as L-arginine incorporation), amounting to a 22% net increase over a 2-hr period (Table 1). During the same period, there was a slight decrease in the amount of nucleic acid in the cells (measured as C^{14}-labeled uracil). In contrast, the same temperature shift merely accelerated the balanced growth of the parental strain, K-10. Identical responses occurred in enriched medium. A similar experiment (not shown), in which the C^{14}-labeled compounds were added to unlabeled cells at the time of the temperature shift, indicated that there was no great destruction of protein that might have masked synthesis at 40 C, and that the only uracil incorporation was into a small amount of rapidly labeled nucleic acid (presumably the messenger

TABLE 1. *Increase in optical density and incorporation of C^{14}-labeled uracil and C^{14}-labeled arginine by normal cells and by temperature-sensitive mutant cells (h8) after a shift to 40 C*

Organism	Time after shift to 40 C	Optical density[a]	Isotope incorporated[a]	
			C^{14} (as L-arginine)	C^{14} (as uracil)
	min			
K-10 (wild)	0	1.00	1.00	1.00
	30	1.88	2.13	1.83
	60	3.35	3.53	3.02
	90	4.80	4.87	4.48
h8 (mutant)	0	1.00	1.00	1.00
	60	1.06	1.16	0.91
	120	1.10	1.22	0.87
	180	1.16	1.22	0 88

[a] All values are normalized to the value at zero-time. The absolute values for strain K-10 at zero-time were: optical density, 0.80; C^{14}-arginine, 6,350 counts/min; C^{14}-uracil, 1,850 counts/min. For strain h8, the values were, respectively: 0.39, 2,200, and 872.

RNA fraction, since some protein synthesis was occurring).

In cells with relaxed control of RNA synthesis, inhibition of protein synthesis usually fails to block RNA synthesis. The behavior of strain h8 at 40 C, therefore, suggested either a lesion in RNA synthesis or some drastic abnormality in energy metabolism, but not a primary lesion in protein synthesis. This suggestion was strengthened by the finding that chloramphenicol failed to permit RNA synthesis in h8 cells growing in minimal medium when the temperature was increased to 40 C.

Measurements of several enzymes involved in RNA synthesis or in nucleotide metabolism (phosphoribosyl adenosine triphosphate pyrophosphorylase, uridine triphosphate aminase, uridine kinase, and RNA polymerase) failed to reveal any differences between h8 and K-10 cells, so an experiment was devised to test the possibility that h8 possessed a temperature-sensitive step in an indispensable catabolic process. During biosynthetically restricted growth, the presence of a utilizable carbon and energy source usually severely inhibits the induced synthesis of new catabolic enzymes. This phenomenon, catabolite repression, can be used as a diagnostic tool to help decide whether inhibited cells are primarily restricted in a biosynthetic step or in a catabolic step.

To measure catabolite repression in strain h8, a culture growing in glucose minimal medium at 30 C was supplemented with 10^{-3} M methyl-β-D-thiogalactoside (TMG), and 1 hr later the cells were harvested and assayed for β-galactosidase activity. These cells had a specific activity of less than 1.0, compared to a value of ca. 200 when growing in lactose minimal medium at the same temperature. A second culture was shifted to 36 C at the time of the TMG addition. Growth continued at this partially restrictive temperature, but at only 50% of the rate at 30 C; nevertheless, considerable β-galactosidase (about 5 to 10% of the value when growing in lactose medium at the same temperature) was synthesized in 1 hr. This failure of catabolite repression during growth inhibition suggests that glucose catabolism in h8 is abnormal at high temperature.

To discover the site of the postulated lesion in glucose catabolism, the growth of h8 was tested on different sources of carbon and energy. Growth on glucose, fructose, and gluconate was normal at 30 C, but was completely inhibited at 40 C; glycerol and succinate, on the other hand, supported near-normal growth at both temperatures (Fig. 1). Since glucose cannot, and glycerol can, support growth at 40 C, attention was concentrated on the early reactions in glucose catabolism via the Embden-Meyerhof and the hexose monophosphate pathways.

Cell-free extracts were made from strain h8 and K-10 and surveyed for a number of catabolic enzyme activities: hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphofructose isomerase, phosphofructokinase, and fructose-1,6-diphosphate aldolase. All of these enzyme activities except the last one were approximately the same in mutant and parental extracts. As shown in Table 2, h8 extracts contained less than 2% of the fructose-1,6-diphosphate aldolase activity of K-10 extracts in both of the assay systems employed. Mixtures of h8 and K-10 extracts exhibited an aldolase specific activity consistent with a simple dilution of the K-10 activity by inert h8 protein.

Even when extracts were made from h8 cells growing at 30 C, no aldolase activity could be demonstrated. Other instances are known in which mutations rendering an enzyme sensitive to high temperature appear to decrease the enzyme's in vitro stability so much that activity cannot be demonstrated in vitro even at temperatures where it can be demonstrated in vivo (e.g., 2). To discover whether h8 cells have a functional fructose-1,6-diphosphate aldolase at 30 C, the pattern of glucose metabolism by resting suspensions of these cells incubated at 30 and 40 C was investigated.

The data in Fig. 2 show the fate of carbons 1

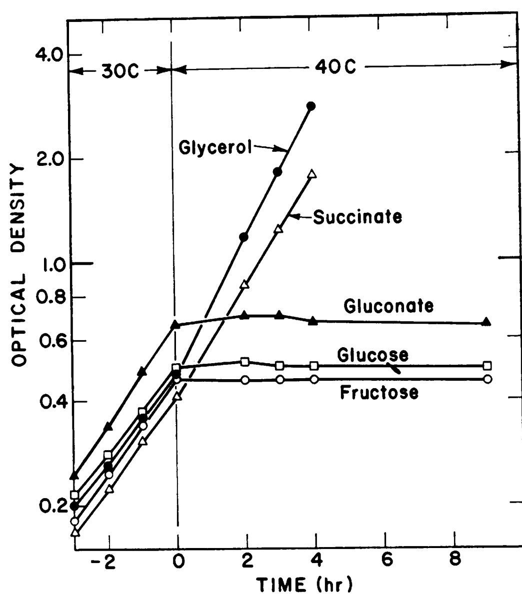

FIG. 1. *Growth of mutant h8 at 30 C and at 40 C in minimal medium containing various carbon sources. The optical density of the cultures at 420 mμ is plotted on a logarithmic scale as a function of time. The carbon sources were all employed at 0.4% final concentration. The temperature was rapidly changed from 30 to 40 C at zero-time.*

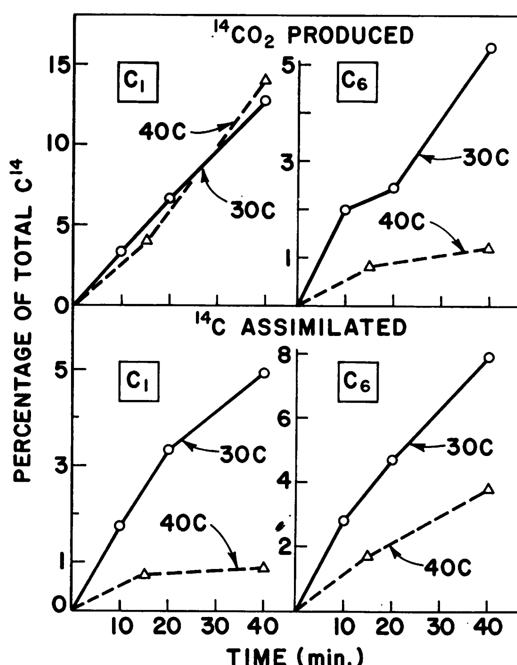

FIG. 2. *Fate of C^{14}-labeled carbon during the metabolism of glucose-1-C^{14} and glucose-6-C^{14} by mutant h8 at 30 and at 40 C. The radioactivity of the indicated fractions is expressed as a percentage of the total radioactivity (counts per minute) of the glucose in each tube (1 mg; 500,000 counts per min), and is plotted as a function of time.*

TABLE 2. *Aldolase activity of extracts prepared from normal cells and from temperature-sensitive cells (h8)*

Source of extract	Temperature of growth[a]	Aldolase specific activity	
		Colorimetric assay[b]	NAD-coupled assay[c]
	C		
K-10 (wild)	30	172	3.60
	40	145	4.56
h8 (mutant)	30	4	<0.05
	40	3	<0.05
K-10 + h8 (1:1 mixture)	40	74	2.40

[a] Wild cells were grown at the temperatures indicated. Mutant cells were grown at 30 C and, where indicated, exposed to 40 C for 1 hr.

[b] The values are expressed as Klett units per 0.1 mg of protein per 30 min.

[c] The values are expressed as micromoles of fructose-1,6-diphosphate cleaved per hour per milligram of protein.

and 6 from differentially C^{14}-labeled glucose at the two temperatures. The rate of CO_2 production from C1 was almost the same at both 30 and 40 C. In contrast, the CO_2 yield from C6 was decreased 5- to 10-fold by raising the temperature from 30 to 40 C, indicating a marked reduction in the operation of the Embden-Meyerhof glycolytic pathway. That the temperature shift did not result in a similar decrease in CO_2 production from C1 could be a result of two factors: (i) raising the temperature might increase the activity of the enzymes of the hexose monophosphate pathway, and (ii) blocking glycolysis might provide a greater supply of glucose-6-phosphate for the hexose monophosphate pathway. The pattern of C^{14} assimilated into trichloroacetic acid-insoluble cell material is consistent with a reduction in the Embden-Meyerhof pathway. Except for some slight incorporation within the first 15 min, C1-carbon fails to be assimilated at 40 C, indicating that glucose is metabolized at this temperature exclusively via routes that convert C1 into CO_2. Carbon is assimilated from C6 at approximately half the rate at 40 C as at 30 C.

If raising the temperature from 30 to 40 C only interrupts glucose catabolism via the Embden-Meyerhof pathway by destroying aldolase activity, one should perhaps expect some con-

tinued slow growth at 40 C as a result of glucose catabolism via the hexose monophosphate route. Wang et al. (10) estimated that the latter route can account for 28% of glucose catabolism in *E. coli*. Indeed, an appreciable amount of glucose was oxidized by h8 at 40 C via the pentose cycle (Fig. 2), but no growth occurred. Experiments dealing with this problem are presented in the accompanying paper.

The pattern of glucose metabolism in vivo is consistent with the conclusion that the temperature-sensitive growth response of mutant h8 is a consequence of the possession of a temperature-sensitive aldolase. Genetic evidence supports this conclusion. Temperature-resistant secondary mutants were isolated by plating cells of strain h8 on rich medium at 40 C. Eight of these "revertants" were examined for aldolase activity. Four of the eight had regained a wild-type level of aldolase activity; of the remainder, one had 5%, two had 20%, and one had 80% of the full wild-type activity. These results are strong evidence that aldolase activity and the temperature-sensitive growth of h8 have a common genetic basis. The results of two genetic crosses lead to the same conclusion (Table 3). Recombinants were obtained by mating two different Hfr strains with F$^-$ phenocopies of strain h8. The selected factors were the temperature resistance of the Hfr cells and the *ade*$^+$ character of the F$^-$ cells. In both experiments, recombinants were obtained with high frequency; of a total of 30 recombinants from which extracts were made to test for aldolase, all possessed wild-type levels of this enzyme activity.

TABLE 3. *Genetic relationship between temperature sensitivity (ts) and aldolase activity in mutant h8*

Hfr parent	F$^-$ parent	No. of *ts*$^+$ *ade*$^+$ recombinants per 100 Hfr cells	No. of recombinants with normal aldolase/no. tested
AB1117 no. 1 (*ts*$^+$ *ade*$^-$)	h8 (*ts*$^-$ *ade*$^+$)	0.7	15/15
AB2297 (*ts*$^+$ *ade*$^-$)	h8 (*ts*$^-$ *ade*$^+$)	2.1	15/15

Genetic mapping of enzymes of intermediary metabolism (excluding those which initiate degradation of various carbon compounds) has not progressed far, chiefly because of the scarcity of appropriate mutants. Recently, however, mutants deficient in fructose-1,6-diphosphatase activity have been identified (3) and genetically located (11), approximately at minute 84 on the *E. coli* chromosome as described by Taylor and Thoman (9). The functional proximity of this enzyme to fructose-1,6-diphosphate aldolase prompted us to determine the approximate map location of the mutant h8 lesion. Accordingly, matings were performed as shown in Table 4. The linkage data obtained in these matings are inconsistent with a map location near that of fructose-1,6-diphosphatase, and suggest a location near minute 60 on the *E. coli* chromosome.

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TABLE 4. *Approximate map position of ald*$^+$ *(ts) from linkage analysis with nonselected markers in uninterrupted matings*[a]

Hfr	F$^-$	Selected marker from Hfr	Linkage (%) of selected with nonselected markers					Approx. map location of *ts*$^+$ (min)[b]
			str	*ser/gly*	*xyl*	*iv*	*ts*	
K-10 (*try*$^-$ *str-s* *ts*$^+$)	h8 (*ser/gly*$^-$ *str-r* *ts*$^-$)	*ts*$^+$	56.2	88.5	—	—	—	58.1
K-10 (*try*$^-$ *str-s* *ts*$^+$)	h8 (*ser/gly*$^-$ *str-r* *ts*$^-$)	*ser/gly*$^+$	26.2	—	—	—	69.0	59.4
AB1117 no. 1 (*xyl*$^-$ *pro*$^-$ *ade*$^-$ *str-s* *ts*$^+$)	h8 (*str-r* *ts*$^-$)	*ts*$^+$	80.0	—	60.0	—	—	60.5
AB2297 (*xyl*$^-$ *iv*$^-$ *ade*$^-$ *str-s* *ts*$^+$)	h8 (*str-r* *ts*$^-$)	*ts*$^+$	77.5	—	37.0	27.0	—	61.5

[a] From these four matings, the frequencies of individual recombinant classes consistently indicated that the order of the markers studies is: *ser/gly, ts, str, xyl, iv*. For example, the second mating yielded the following frequencies of recombinants: *ser/gly*$^+$ *ts*$^+$ *str-r*, 46%; *ser/gly*$^+$ *ts*$^+$ *str-s*, 23%; *ser/gly*$^+$ *ts*$^-$ *str-r*, 27.8%; *ser/gly*$^+$ *ts*$^-$ *str-s*, 3.2%.

[b] Numbers show approximate location of *ts*$^+$ on the chromosome map given by Taylor and Thoman (9). The linkage data were corrected for double crossovers. Mating experiments were performed at 30 C.

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