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Glyceraldehyde 3-Phosphate Dehydrogenase Mutants of *Escherichia coli*

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We describe glyceraldehyde 3-phosphate dehydrogenase mutants of *Escherichia coli*. The gene (*gap*) is at approximately 34 min, with the transductional order *gap-fadD-eda*. One *gap* mutant is temperature sensitive and has a heat-labile enzyme. Another is amber.

Until recently, there were no bacterial mutants blocked in glycolysis after glyceraldehyde 3-phosphate. But in 1972 Freese et al. (9) reported a *Bacillus subtilis* mutant lacking phosphoglycerate kinase, obtained as an isolate unable to grow on glucose unless supplemented with citrate or malate. We have used a similar rationale with *Escherichia coli* and report here mutants lacking glyceraldehyde 3-phosphate dehydrogenase. During this work, Irani and Maitra (12) also reported *E. coli* mutants lacking this enzyme, and, although our selection techniques differed, we may have the same mutants and will therefore not discuss the selection or phenotype in great detail. We do, however, report a genetic map position (*gap*) quite different from theirs and show, with a temperature-sensitive mutant, that it is likely the structural gene. An amber mutant has also been found.

MATERIALS AND METHODS

Media and organisms. Mutants lacking glyceraldehyde 3-phosphate dehydrogenase were isolated from each of the following strains of *E. coli*: K10 (HfrC *tonA22 lambda*, ref. 1); JC411 (F⁻ *metB leu his argG lac malA xyl mtl gal str sup-59*, ref.1); and DF230, obtained from NP37 (HfrC *tonA22* λ^+ *pheS*^{ts}, ref. 5) as a spontaneous revertant to growth on malate at 30 C (the malate lesion of NP37 was not known). The mutants are listed in Table 1.

The strains used for mapping include KL96 (Hfr *thi rel*, ref. 14); DF1651 (F⁻ *edd galK his pyrD pps str tyrA*, ref. 19); and DF250 (F⁻ *eda-1 fadD88 str*), obtained in a mating of strains DF71 (HfrH *eda-1*, ref. 6) and K63 (F⁻ *fadD88 his str*, ref. 20) with *his*$^+$ selection and screening of the other markers according to growth on glucuronate and oleate.

Media were as described (8), with carbon sources 4 mg/ml unless otherwise specified. Fatty acid plates for testing *fadD* contained sodium oleate (1 mg/ml) and 1% Brij-35 (18). Matings and transductions were according to Miller (17), except that malate minimal medium was supplemented with glycerol and 0.1% Casamino Acids was used instead of broth in experiments with *gap* mutants. Recombinants were purified on selective plates before scoring of unselected markers.

Assays. Most assays are described or given reference in the footnotes to Table 1. Others are as follows. For initial screening of glyceraldehyde 3-phosphate dehydrogenase mutants we used the reductive-dephosphorylation assay described by D'Alessio and Josse (3). A modification of the oxidative-arsenolysis assay used by those same authors was used in the heat lability test. A sample of extract containing approximately 90 μg of protein was added to 1 ml of 0.05 M sodium pyrophosphate (pH 8.5) containing 3 mM 2-mercaptoethanol, placed 5 min in a water bath at the desired temperature, chilled in ice, and returned to 25 C; then, a 0.92-ml volume was assayed for remaining activity by addition of 0.25 μmol of nicotinamide adenine dinucleotide, 5 μmol of $Na_2HAsO_4 \cdot 7H_2O$, 0.4 μg of triose phosphate isomerase (from rabbit muscle), and 0.5 μmol of dihydroxyacetone phosphate, and the change in absorbance was recorded as usual. Other assays of glyceraldehyde 3-phosphate dehydrogenase are cited in the text.

Selection of mutants. Logarithmic-phase broth cultures of *E. coli* strains K10 and JC411 were mutagenized with ethyl methane sulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine according to Miller (17). Survivors were allowed to grow out overnight at 37 C in minimal medium containing malate supplemented with glycerol (12.5 mM). Samples of these cultures were then treated with penicillin (17) in the presence of malate alone and plated on glycerol-supplemented malate media. After 3 days at 37 C colonies were transferred to plates containing glucose, glycerol, glycerate, malate, and glycerol-supplemented malate. Mutant strains that grew only on the last of these media were purified, and extracts were prepared from 25-ml cultures to determine their glyceraldehyde 3-phosphate dehydrogenase activities. The "directed" mutagenesis of strain DF230 [*pheS*^{ts}] entailed plating appropiate dilutions of nitrosoguanidine-mutagenized cultures on glycerol-supplemented malate plates and incubating for 6 h at 30 C before shifting the plates to the nonpermissive

TABLE 1. *Specific activities of some enzymes in extracts of nine gap mutants and their parents*

Strain[a]	Enzyme (sp act; U/mg of protein)[b]								
	gap	*pgk*	*pgm*	*eno*	*zwf*	*tkt*	*fda*	*pgi*	*pfk*
K10	0.72	2.21	2.13	0.93	0.21	0.25	0.10	0.68	0.64
DF220 (*gap-1*)	<0.02	2.79	2.19	1.16	0.19	0.25	0.14	0.88	1.08
DF221 (*gap-2*)	<0.02	2.38	2.20	1.07	0.22	0.29	0.15	0.87	0.84
JC411	0.45	1.54	1.57	0.83	0.25	0.17	0.09	—	—
DF225 (*gap-3*)	<0.02	1.84	1.17	0.95	0.22	0.20	0.12	—	—
DF230	0.43	2.04	1.68	0.73	0.22	0.19	0.09	—	—
DF231 (*gap-4*)	<0.02	2.01	2.54	1.41	0.20	0.12	0.15	—	—
DF232 (*gap-5*)	<0.02	2.27	1.84	1.06	0.19	0.23	0.12	—	—
DF233 (*gap-6*)	<0.02	2.61	2.17	1.08	0.19	0.23	0.13	—	—
DF234 (*gap-7*)	0.15	2.34	1.61	0.77	0.19	0.19	0.15	—	—
DF235 (*gap-8*)	<0.02	1.91	1.92	0.88	0.21	0.19	0.15	—	—
DF236 (*gap-9*)	<0.02	1.87	1.51	0.73	0.17	0.13	0.11	—	—

[a] DF220 and DF221 are, respectively, ethyl methane sulfonate- and nitrosoguanidine-induced *gap* derivatives of K10. DF225 was obtaind by nitrosoguanidine treatment of JC411. The remaining six *gap* strains were derived from DF230 (*pheS*) by directed mutagenesis.

[b] Cell-free extracts were prepared from 250-ml cultures, stationary phase, grown at 30 C in malate (25 mM) minimal medium supplemented with glycerol (12.5 mM) and 0.1% Casamino Acids. The cells were collected by centrifugation, washed once with 0.9% NaCl, and resuspended (ca. 10^{11} cells/ml) in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8), 0.01 M $MgCl_2$, and 1 mM dithiothreitol. After sonication (1 min/ml, −4 C; Biosonik II ultrasonicator, Bronwill Scientific Co.), the crude extracts were centrifuged (35,000 rpm, 2 h; Spinco 50Ti rotor) to remove nicotinamide adenine dinucleotide, reduced form, oxidase. Assay of glyceraldehyde 3-phosphate dehydrogenase (*gap*) was modified from Duggleby and Dennis (4) and contained (per 1 ml) 135 μmol of tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.8), 0.2 μmol of ethylenediaminetetraacetic acid, 3.3 μmol of cysteine-hydrochloride, 0.2 μmol of $MgCl_2$, 2.0 μmol of nicotinamide adenine dinucleotide, 10 μmol of K_2HPO_4, 0.5 μmol of adenosine 5′-diphosphate, 4 μg of phosphoglycerate kinase (from yeast, Boehringer Co.), 0.4 μg of triose phosphate isomerase (from rabbit muscle, Boehringer Co.), and 0.5 to 5 μl of extract. The reaction was initiated with 0.5 μmol of dihydroxyacetone phosphate, and increase in absorbance at 25 C was followed with a Gilford model 240 spectrophotometer. Under these conditions reaction rates were proportional to the amount of extract for at least 45 s. Other assays were phosphoglycerate kinase (*pgk*, ref. 3), phosphoglycerate mutase (*pgm*, ref. 10), enolase (*eno*, ref. 10), glucose 6-phosphate dehydrogenase (*zwf*, ref. 7), transketolase (*tkt*, assay 2a of ref. 13), fructose diphosphate aldolase (*fda*, ref. 16), phosphoglucose isomerase (*pgi*, ref. 7), and phosphofructokinase (*pfk*, ref. 8). Specific activities are units (micromole of product formed per minute) per milligram of protein. Protein was measured according to Lowry et al. (15) using bovine serum albumin corrected for moisture content as standard.

temperature, 42 C (2). Colonies appearing at this temperature were screened for the mutant phenotype as described above.

Chemicals. Dihydroxyacetone phosphate was obtained as the dimethylketal di-monocyclohexylamine salt from Sigma Chemical Co., St. Louis, Mo.; 65% D-glyceric acid was from Aldrich Chemical Co., Milwaukee, Wis.; $NaH_2{}^{32}PO_4$ was purchased from New England Nuclear Corp., Boston, Mass., and β-*O*-methyl-galactoside was a gift of that company. Enzymes used in the various assays were from Boehringer Mannheim Corp., New York, N.Y.

RESULTS AND DISCUSSION

Isolation of mutants. Mutants were selected (see Materials and Methods) that grew as patches on minimal medium containing malate and glycerol, but not on malate, or several other single carbon sources, alone. Extracts of three independent strains (DF220, DF221, and DF225) proved to have less than 5% of parental levels of the glyceraldehyde 3-phosphate dehydrogenase oxidative phosphorolysis activity in vitro (see Table 1). Similar results were obtained with other assays, including the oxidative-arsenolysis of glyceraldehyde 3-phosphate, the adenosine 5′-triphosphate-dependent reduction of 3-phosphoglycerate, and the $^{32}P_i$–adenosine 5′-triphosphate–γ–^{32}P exchange reactions described by D'Alessio and Jossi (3). Mixed wild-type and mutant extracts gave additive activities.

Growth. Growth on a variety of media was tested using plates spread with approximately 50 colony-forming units. The growth phenotype of the three mutants was similar to that reported by Irani and Maitra (12). None of the following single carbon sources could support growth: glucose, glycerol, gluconate, glucuronate, fructose, galactose, ribose, glycerate, lactate, acetate, succinate, and malate. Growth occurred when malate was supplemented with glycerol (12.5 mM), ribose (12.5 mM) or β-*O*-

methyl galactoside (1 mM). α-Glycerol phosphate and various carbohydrates, such as glucose, galactose, maltose, and fructose, on the other hand, could not substitute for glycerol and even inhibited to varying degrees the growth of the mutants on glycerol-supplemented malate. Varying the concentration of the glycerol supplement between 0.1 and 100 mM did not appreciably affect the doubling times of the mutants, although the lower amounts limited yield in liquid culture. Both succinate and Casamino Acids (0.4% Difco) were found to substitute well for malate, whereas glycerate and lactate served poorly.

Mapping. Preliminary crosses suggested that the mutant locus (to be called *gap*) was in the *his* region. Using strain DF225 (*gap-3 his*) as recipient in an interrupted mating with strain KL96 (an Hfr with origin just clockwise to *his* which injects *his* early), recombinants which could grow on glucose (i.e., *gap*$^+$) appeared within 5 min after *his*$^+$. Likewise, an F-duction with the same recipient, using an F'*his* strain (KLF48/KL159; see ref. 23), gave approximately equal numbers of presumed merodiploids by selection for *his*$^+$ (on glycerol-supplemented malate plates) or for *gap*$^+$ (selected on glucose plates with histidine). These results placed *gap* at approximately 32 to 35 min.

gap was found to co-transduce with markers in the 34-min region; phage P1vir grown on strain DF250 (*eda-1, fadD88*) was used to transduce strains DF220 (*gap-1*), DF221 (*gap-2*), and DF225 (*gap-3*) to growth on glucose. Scoring of the unselected markers (Table 2) best accords with the gene order *eda-fadD-gap*. The presence of parental levels of glyceraldehyde 3-phosphate dehydrogenase activity in several of the transductants was confirmed by in vitro assay. The similar co-transduction frequencies for the three mutants and the failure of a P1vir lysate prepared on any one of these strains to transduce the others to growth on glucose suggest close linkage of the three independent mutations.

The mutants selected by Irani and Maitra (12) lacked either glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, or enolase. In "preliminary" conjugation experiments, they placed the loci for these mutations between the origin of strain KL16 (ca. 55 min) and *tyrA* (ca. 50 min) and suggested the abbreviation *gad* for the first of these genes. Our assignment is different (ca. 34 min), and we use the abbreviation *gap* (since *gad* has been used for a different enzyme [22]). We show below that *gap* is probably the structural gene for glyceraldehyde 3-phosphate dehydrogenase, and it would be of some interest if another (regulatory?) locus lies elsewhere. We do confirm (below) their assignment of the gene for enolase (*eno*).

TABLE 2. *Transduction of gap*[a]

Recipient	No. of *gap*$^+$ recombinants screened	Recombinant classes (%)			
		fad$^+$ *eda*$^+$	*fad* *eda*$^+$	*fad* *eda*	*fad*$^+$ *eda*
DF220 (*gap-1*)	337	66.2	29.0	4.7	0
DF221 (*gap-2*)	351	58.4	37.0	4.5	0
DF225 (*gap-3*)	340	67.6	28.8	3.5	0

[a] The donor was DF250 (*eda-1, fadDi8*), and selection was on glucose. *fad* was scored on oleate plates, and *eda* was scored on glucuronate plates.

"Directed mutagenesis" of DF230. The mapping of *gap* placed it slightly clockwise to a known cluster at 33 min, which includes the gene for phenylalanyl-transfer ribonucleic acid synthetase (2), and, indeed, the *pheS* marker of strain NP37 was found to co-transduce approximately 10% with the *gap* allele in DF225 (selecting on glucose at 30 C and scoring for *pheS* at 42 C). Since nitrosoguanidine-induced reversion has been used as a technique for selection of linked mutations (11), we treated a culture of strain DF230 (*pheS*) with nitrosoguanidine and selected revertants to growth at 42 C (on glycerol-supplemented malate plates; see Materials and Methods). Of approximately 1,600 colonies screened, 11 had the *gap* mutant phenotype, and six of these were found to lack glyceraldehyde 3-phosphate dehydrogenase in vitro. Five strains, DF231,-232,-233,-235, and -236, appeared identical to the original three mutants with respect to their phenotypes and co-transduction frequencies with *eda-1*. The sixth strain, DF235, had a temperature-sensitive phenotype, being able to grow on a variety of carbon sources at 30 C but only on glycerol-supplemented malate at 42 C. Extracts of a culture of strain DF234 grown at 42 C on this media possessed less than 5% of parental glyceraldehyde 3-phosphate dehydrogenase activity in vitro, whereas a culture grown at 30 C had approximately 30% of the parental activity (Table 1). When samples of extracts from cultures grown at 30 C were pretreated by heating in a water bath (see Materials and Methods) a marked difference in enzyme thermolability was observed; incubation for 5 min at 50 C destroyed over 95% of the activity present in extracts of DF234, whereas less than 30% of the activity in extracts of the parent, DF230, was lost. To show that this strain indeed carried a single mutation at *gap*, and thus that *gap* is probably the structural gene for glyceral-

dehyde 3-phosphate dehydrogenase, the mutation was transferred to another strain. Since strain DF230 and its derivatives were not able to support adequately the growth of P1vir, the presumed *gap*(ts) mutation of DF234 was first introduced by mating into DF1651 (*pps*) by selection on lactate at 30 C and screening recombinants for the temperature-sensitive *gap* phenotype. One such recombinant, DF237, whose *gap*(ts) genotype was confirmed by in vitro thermolability studies, was used to grow P1vir, which was then used to transduce strain DF71 (*eda-1*) to growth on galacturonate at 30 C. The co-tranduction frequency (5 out of 150) of *gap* (ts), as assessed by screening transductants for growth on glucose at 42 C and in vitro thermolability, closely corresponded to the frequencies characteristic of the other mutants studied.

Reversion studies. Fifty independent and spontaneous revertants of strains DF220, -221, and -225 to growth on glucose at 30 C were selected and yielded a broad spectrum of phenotypes for aerobic growth on glucose, glycerol, glycerate, and malate at 30 and 42 C. In general, from 30 to 60% of the revertants had growth properties typical of their respective parents and on assay had regained normal levels of glyceraldehyde 3-phosphate dehydrogenase activity. Most of the other revertants grew poorly or not at all on glycerate and malate, espically at 42 C, and several of these were shown to have regained only 10 to 20% of the parental levels of enzyme activity. This latter class of revertants might correspond to the type reported by Irani and Maitra (12).

Three of the 50 independent revertants of strain DF221 (*gap-2*) to growth on glucose at 30 C allowed the suppression of the amber mutation present in the T4 strain H36, but not of a T4 ochre mutation. The levels of glyceraldehyde 3-phosphate dehydrogenase activity in these revertant strains were 10 to 15% of wild type. Thus, *gap-2* is probably an amber mutation. However, attempts to directly introduce known suppressors into DF221 using P1vir grown on CA5013 (*supD*) or CA161 (*supE*) by selection on glucose and screening of purified transductants for suppression of T4 strain H36 were unsuccessful; presumably, either *gap*$^+$ transductants grew faster than suppressed mutants, or these suppressors were not effective on *gap-2*.

Other enzymes. Crude extracts of the *gap* mutants were screened for several other enzymes of central intermediary metabolism; there were no marked effects on any of the activities surveyed (Table 1).

Enolase mutants. As mentioned above, Irani and Maitra also found mutants lacking phosphoglycerate kinase or enolase (12). One of our mutants, strain DF260, an isolate from the nitrosoguanidine mutagenesis of strain DF230 (*pheS*), was found to contain apparently normal levels of glyceraldehyde 3-phosphate dehydrogenase but to lack enolase (ca. 10% parental level). The enolase locus (*eno*) was found (12) to be co-transducible with *lysA* (ca. 55 min), and we have confirmed that position for the *eno* mutation of strain DF260 (*eno*). We used strain DF260 as recipient in a transduction with strain AT713 (*argA8 cysC39 lysA10*, ref. 21), selecting *eno*$^+$ on glucose supplemented with the amino acids and scoring. Three hundred and four transductants gave the following recombinant classes (percentage): *arg*$^+$ *cys lys*$^+$, 42.1; *arg*$^+$ *cys*$^+$ *lys*$^+$, 33.2; *arg cys lys*$^+$, 12.8; *arg cys*$^+$ *lys*$^+$, 9.2; *arg cys*$^+$ *lys*, 2.0; *arg*$^+$ *cys*$^+$ *lys*, 0.3; *arg cys lys*, 0.3; and *arg*$^+$ *cys lys*, 0.0. These results suggest the gene order *lysA-argA-eno-cysC*.

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LITERATURE CITED

1. Bachmann, B. J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
2. Böck, A., and F. C. Neidhardt. 1967. Genetic mapping of phenylalanyl-sRNA synthetase in *Escherichia coli*. Science **15**:78–79.
3. D'Alessio, G., and J. Josse. 1971. Glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, and phosphoglyceromutase of *Escherichia coli*. J. Biol. Chem. **246**:4319–4325.
4. Duggleby, R. G., and D. T. Dennis. 1974. Nicotinamide adenine dinucleotide-specific glyceraldehyde 3-phosphate dehydrogenase from *Pisum sativum*. J. Biol. Chem. **249**:167–174.
5. Eidlic, L., and F. C. Neidhardt. 1965. Protein and nucleic acid synthesis in mutants of *Escherichia coli* with temperature-sensitive aminoacyl ribonucleic acid synthetases. J. Bacteriol. **89**:706–711.
6. Fradkin, J. E., and D. G. Fraenkel. 1971. 2-Keto-3-deoxygluconate 6-phosphate aldolase mutants of *Escherichia coli*. J. Bacteriol. **108**:1277–1283.
7. Fraenkel, D. G., D. Kotlarz, and H. Buc. 1973. Two fructose 6-phosphate kinase activities in *Escherichia coli*. J. Biol. Chem. **248**:4865–4866.
8. Fraenkel, D. G., and S. R. Levisohn. 1967. Glucose and gluconate metabolism in an *Escherichia coli* mutant lacking phosphoglucose isomerase. J. Bacteriol. **93**:1571–1578.
9. Freese, E., Y. K. Oh, E. B. Freese, M. D. Diesterhaft, and C. Prasad. 1972. Suppression of sporulation of *Bacillus subtilis*, p. 212–221. *In* H. O. Halvorson, R. Hanson and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
10. Grisolia, S. 1962. Phosphoglyceric acid mutase, p.

236–242. *In* S. P. Colowick and N. D. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
11. Guerola, N., J. L. Ingraham, and E. Cerda-Olmedo. 1971. Induction of closely-linked multiple mutations by nitrosoguanidine. Nature (London) **230**:122–125.
12. Irani, M., and P. K. Maitra. 1974. Isolation and characterization of *Escherichia coli* mutants defective in enzymes of glycolysis. Biochem. Biophys. Res. Commun. **56**:127–133.
13. Josephson, B. L., and D. G. Fraenkel. 1969. Transketolase mutants of *Escherichia coli*. J. Bacteriol. **100**:1289–1295.
14. Low, K. B. 1972. *Escherichia coli* K-12 F-prime factors, old and new. Bacteriol. Rev. **36**:587–607.
15. 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.
16. Maitra, P. K., and Z. Lobo. 1971. A kinetic study of glycolytic enzyme synthesis in yeast. J. Biol. Chem. **246**:475–488.
17. Miller, J. H. 1972. Experiments in molecular biology. Cold Spring Harbor Laboratory, New York.
18. Overath, P., G. Pauli, and H. V. Schairer. 1969. Fatty acid degradation in *Escherichia coli*. Eur. J. Biochem. **7**:559–574.
19. Peyru, G., and D. G. Fraenkel. 1968. Genetic mapping of loci for glucose 6-phosphate dehydrogenase, gluconate 6-phosphate dehydrogenase, and gluconate 6-phosphate dehydrase in *Escherichia coli*. J. Bacteriol. **95**:1272–1278.
20. Pouyssegur, J. M. 1971. Localization génétique de mutations 2-céto-3-désoxy-6-P-gluconate aldolase negatives chez *E. coli* K-12. Mol. Gen. Genet. **113**:31–42.
21. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of *Escherichia coli*. Bacteriol. Rev. **31**:332–353.
22. Taylor, A. L., and C. D. Trotter. 1972. Linkage map of *Escherichia coli* strain K-12. Bacteriol. Rev. **36**: 504–524.
23. Vinopal, R. T., and D. G. Fraenkel. 1975. *pfkB* and *pfkC* loci of *Escherichia coli*. J. Bacteriol. **122**:1153–1161.