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Mapping and Cloning of *gldA*, the Structural Gene of the *Escherichia coli* Glycerol Dehydrogenase

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***gldA*, the structural gene for the NAD$^+$-dependent glycerol dehydrogenase, was mapped at 89.2 min on the *Escherichia coli* linkage map, cotransducible with, but not adjacent to, the *glpFKX* operon encoding the proteins for the uptake and phosphorylation of glycerol. *gldA* was cloned, and its position on the physical map of *E. coli* was determined. The expression of *gldA* was induced by hydroxyacetone under stationary-phase growth conditions.**

In *Escherichia coli* the products of the *glp* regulon are responsible for salvaging the glycerol moiety of degradation products of phospholipids and triglycerides (21). All genes of the *glp* system are genetically regulated by the repressor protein, GlpR (9, 28), with internal *sn*-glycerol-3-phosphate (G3P) being the inducer (8). G3P uptake is catalyzed by the G3P permease GlpT, a cytoplasmic membrane protein encoded by *glpT* (7, 19). A transport system facilitating the passage of glycerol, particularly at a low concentration, through the *E. coli* inner membrane exists (27): the glycerol facilitator, encoded by *glpF* (30). Glycerol is phosphorylated by glycerol kinase and trapped as G3P in the cell (8). Glycerol kinase activity is increased in vivo by the presence of the glycerol facilitator. Therefore, effective glycerol phosphorylation relies on the interaction between the facilitator (encoded by *glpF*) and the kinase (encoded by *glpK*) (35). In cellular extracts the K_ms of glycerol kinase were 1.3 μM for glycerol (8, 11), 0.5 mM for dihydroxyacetone (DHA) (11), and 3 mM for L-glyceraldehyde (9), indicating that the primary role of glycerol kinase is the utilization of glycerol. The next step in the catabolism of G3P is the conversion to dihydroxyacetone phosphate (DHAP), catalyzed by two *glp* gene-encoded G3P dehydrogenases (G3P DH): the aerobic G3P DH, GlpD, which uses molecular oxygen or nitrate as an electron acceptor (17), and the anaerobic G3P DH, GlpABC (6, 15), with fumarate or nitrate as an electron acceptor. The resulting DHAP enters then the glycolytic pathway. Even though glycerol metabolism seems to be well established (reviewed in reference 22), the functions of the three proteins encoded by *glpE* and *glpG* (20) and *glpX* (34) remain unknown.

Martin et al. (23) described a mutant isolated as a glycerol plus pseudorevertant from a *glpD glpR glpK* mutant. This mutant used an enzyme other than glycerol kinase for the first step of glycerol dissimilation. The enzyme was identified as an NAD$^+$-linked glycerol dehydrogenase (GLDH) catalyzing the oxidation of glycerol to DHA. The enzyme was purified from this mutant strain (33) and shown to have an apparent molecular weight of 39,000. The GLDH was later shown to be identical to the purified D-1-amino-2-propanol oxireductase from wild-type *E. coli* (14).

Although *glpK* mutants are unable to grow on glycerol, GLDH must exist even in wild-type *E. coli* since it was purified from a wild-type strain (1). The derepression of GLDH alone cannot be sufficient to explain growth on glycerol of a *glpK* mutant. To enter glycolysis, DHA first has to be phosphorylated. In *E. coli* no DHA kinase has been found as yet. However, a DHA-specific enzyme II of the phosphotransferase system has been proposed (13). Consequently, DHA, produced internally, would be excreted into the medium and subsequently recaptured by the DHA-specific enzyme II delivering DHAP into the cytoplasm, where it could enter glycolysis.

The GLDH enzyme described here should not be mistaken for an enzyme found in *Klebsiella pneumoniae* that formally catalyzes the same reaction, the NAD$^+$-dependent oxidation of glycerol to DHA. This enzyme is part of a pathway for the utilization of glycerol under anaerobic conditions, a pathway which does not exist in *E. coli* (29, 32).

The *gldA* gene is cotransducible with *glpFKX*. We learned from M. Varga and E. C. C. Lin (34a) that the gene encoding the GLDH (now called *gldA*) was cotransducible with *glpK*, positioned at 88.6 min on the *E. coli* chromosome. This suggested to us that *glpX* (34), the gene of unknown function, distal to *glpK*, might encode the GLDH. To determine the cotransduction frequency of the mutation causing the defect in GLDH to *glpK* and the order of neighboring genes, a P1 lysate of RJ70 (*glpF*::Tn*10*, polar on *glpK*) was transduced into Lin424 by selecting for tetracycline resistance and screening for growth on glycerol minimal medium. Strain Lin424 lacks glycerol kinase and overproduces GLDH (23), most likely because of a promoter mutation in *gldA* (*gldA*$^{++}$). Thirty-one percent of the tetracycline-resistant transductants were no longer able to grow on glycerol. A second transduction with a P1 lysate of Lin424 was done with strain VT199 (*met*::Tn*10* *glpK*) by selecting for Met$^+$ and screening for growth on glycerol minimal medium. Sixty percent of the Met$^+$ transductants could still grow on glycerol, indicating the presence of *gldA*$^{++}$. Three factor crosses were done with VT197 and VT198. A P1 lysate of VT197 (*glpK*::*lacZ cdh4*::Tn*10*) was used to transduce Lin424 by selecting tetracycline resistance and screening for growth on glycerol and the formation of blue colonies on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-containing minimal maltose plates. All colonies that were white (and thus contained the *glpK* region of Lin424) could grow on glycerol. A P1 lysate of strain Lin424 was used to transduce VT198 (*glpK*::*lacZ met*::Tn*10*) by selecting for Met$^+$. Sixty-five percent of the Met$^+$ transductants could grow on glycerol, indicating the presence of *gldA*$^{++}$. Of the colonies

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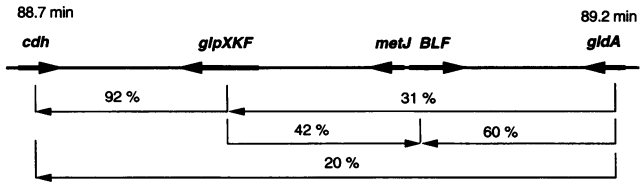

FIG. 1. Position of *gldA* on the *E. coli* linkage map as determined by P1 transduction. The cotransduction frequencies are expressed in the percentage of screened transductants in each cross. Arrows indicate the selected marker in each cross. The arrows in the upper lane indicate the direction of transcription of the different operons.

that were white on minimal maltose plates containing X-Gal, 52% of them could grow on glycerol. These results showed that *gldA* was not identical to *glpX*, and it allowed us to order the genes in the following way: *cdh glpFKX metJBLF gldA* (Fig. 1).

Cloning of the *gldA* gene. Different strains (Table 1) were transformed with a gene bank, constructed by ligating partially *Sau*3A-digested *E. coli* chromosomal fragments (6 to 20 kb) isolated from strain ECL116 (5) into the *Bam*HI site of pBR322. The recipients were strains RCD3 and VT200, both of which were *glpD glpK* double mutants and therefore unable to grow on glycerol as the sole source of carbon. Ampicillin-resistant transformants were selected and screened for growth on glycerol minimal plates. Since the recipients lacked the aerobic G3P DH, clones containing *glpK* would not be obtained by this selection. Two different plasmids were found to bring about growth on glycerol: pVT86 and pVT87. Digestion with different restriction enzymes showed that these two plasmids contained overlapping fragments with lengths of about 8.7 and 10 kb. Digestion with *Bgl*I (Fig. 2A, lanes 1 and 2) showed that both plasmids matched in two fragments (the 5.3-kb fragment belongs to the insert, and the 2.3-kb fragment belongs to pBR322). When digested with *Hin*dIII (Fig. 2A, lanes 6 and 7) both plasmids contained a 1.3-kb fragment. In GLDH tests, extracts of strain RCD3 after retransformation with pVT86 and pVT87 showed 70-fold-higher GLDH activity than did extracts of strain RCD3.

Localization of the *gldA* gene on the physical map of *E. coli*. The analysis of the DNA fragments allowed their identification on the physical map of *E. coli* (16, 26). It revealed the location of the *gldA* region at 89.2 min, at a distance of about 20 kb from the *glpK* gene in a clockwise direction (Fig. 2B). As part of the analysis of the *E. coli* genome (89.2 to 92.8 min), the sequence of an unknown open reading frame near *katG*, named f380 (composed of 380 amino acids), has recently been determined and shown to exhibit homologies to a GLDH from *Bacillus stearothermophilus* (2). Partial sequence analysis of our clone containing *gldA* showed identity with the sequence reported by Blattner et al. (2), as shown in Fig. 2B.

Identification of the *gldA* gene product in minicells. We cloned the *Eco*RI fragment of pVT86 into the low-copy-number plasmid pHGS576 (31), resulting in pVT88. It contained the 2.6-kb *Eco*RI fragment of pVT86 (Fig. 2A, lanes 3 and 4), consisting of a 2.2-kb fragment of the insert and 377 bp from pBR322, the distance between the *Bam*HI and the *Eco*RI sites in pBR322. The reason for cloning *gldA* into a low-copy-number vector was that in overnight cultures of strains containing pVT86, lysed cells could be seen, indicating growth inhibitory conditions although the culture grew to a high optical density. Extracts of strain RCD3 carrying pVT88 showed high activity in GLDH assays (Table 2). Figure 3 shows the proteins encoded on plasmids pVT86, pVT87, and pVT88 after labeling with [^{35}S]methionine in minicells. Three proteins (approximately 39, 23, and 75 kDa) were synthesized when pVT87 was used as a template. With pVT86 as a template, the 39-kDa and the 23-kDa proteins were also synthesized, while the 75-kDa protein of pVT87 was truncated to 70 kDa. pVT88 encoded, in addition to chloramphenicol acyltransferase, only the 39-kDa protein. Since the position of the chloramphenicol acetytransferlase is identical to that of the 23-kDa protein, it is unclear whether this protein is still encoded by pVT88. Purified GLDH was shown to have an apparent molecular weight of 39,000 in sodium dodecyl sulfate (SDS)-polyacrylamide gels (33). Since pVT88 encoded the 39-kDa protein, since cellular extracts of strains carrying this plasmid exhibited high GLDH activity (Table 2), and since the analysis of the genes surrounding *gldA* (Fig. 2B) excluded the presence of the gene encoding

TABLE 1. Bacterial strains

Strain	Description	Construction[a] reference or source
DLT242	MC4100 *gldA*::Tn*10* Δ(*glpFKX*)[b]	D. Latour
GD31	MC4100 ϕ(*glpK*::*lacZ*)hyb	P1 GD32→MC4100
GD32	MC4100 ϕ(*glpK*::*lacZ*)hyb *glpR*	30
GD202	MC4100 *glpK*	30
GD237	MC4100 *met*::Tn*10* *glpR*	P1 of Tn*10* pool→MC4100 *glpR*
GD251	MC4100 Δ(*glpD-malT*)	Excision of λ(*glpD-lacZ*)
ECL116	F$^-$ *endA hsdR* Δ(*argF-lac*)*U169 thi*	5
HB290	MC4100 *minB rpsL mgl*	12
VT197	MC4100 ϕ(*glpK*::*lacZ*)hyb *cdh*4::Tn*10*	P1 MW1104→GD31
VT198	MC4100 ϕ(*glpK*::*lacZ*)hyb *met*::Tn*10*	P1 GD237→GD31
VT199	MC4100 *glpK met*::Tn*10*	P1 GD237→GD202
VT200	MC4100 Δ(*glpD-malT*) ϕ(*glpK*::*lacZ*)hyb	P1 GD237→GD251 P1→the above transductant, selecting Met$^+$
RCD3	Lin424 ϕ(*glpK*::*lacZ*)hyb (*gldA* of MC4100)	P1 GD237→Lin424 P1 GD32→the above transductant
Lin424	*glpK* Δ(*glpDR*) *gldA*$^{++}$	23
MC4100	F$^-$ *araD*139 Δ(*argF-lac*)*U169 rpsL50 deoC ptsF25 rbsR relA1 thi fblB5301*	4
MW1104	Hfr *relA1 metA7 spoT1 cdh4*::Tn*10*	3
RJ70	MC4100 *glpF*::Tn*10*	R. Jin

[a] The donor and recipient in constructions by P1 transduction are indicated.

[b] Hybridizing restriction fragments obtained from DNA of DLT242 with a probe from the *glpFKX* region revealed that it carried a deletion beginning at the middle of the *glpF* gene, taking out the entire *glpK* gene, and ending at the 3′ end of *glpX*.

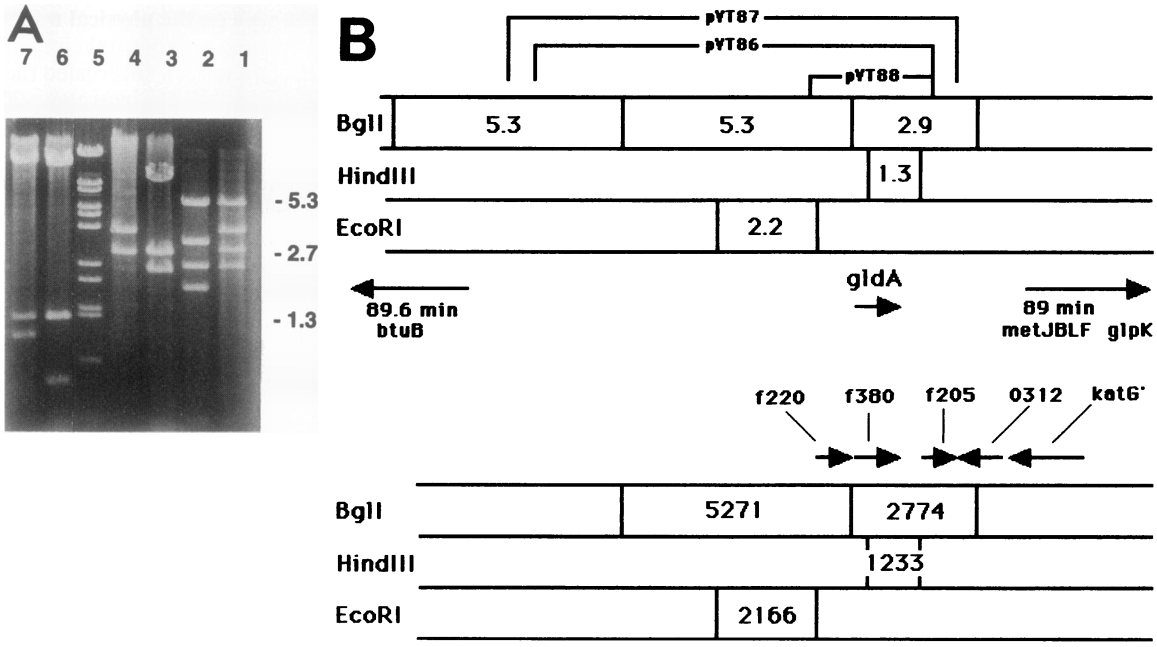

FIG. 2. Restriction map of the 89.2-min region of the *E. coli* chromosome. (A) Restriction analysis of pVT86, pVT87, and pVT88. Lane 5 shows the size standard of λ DNA digested with *Bst*EII. The lengths (in kilobases) of relevant restriction fragments are indicated on the right. Lanes: 1 and 2, pVT87 and pVT86, respectively, DNA digested with *Bgl*I; 3 and 4, fragments of pVT86 and pVT88, respectively, after digestion with *Eco*RI; 6 and 7, pVT86 and pVT87, respectively, digested with *Hin*dIII. (B) (Top) Restriction fragments shown in panel A placed in the physical map of *E. coli* (16, 26) around *gldA*. The inserts of pVT86, pVT87, and pVT88 are indicated. The distances (in kilobases) between the restriction sites are indicated. (Bottom) Detailed view of the same area, according to sequence analysis of the *E. coli* chromosome from 89.2 to 92.8 min by Blattner et al. (2). The numbers refer to the number of nucleotides in the sequence. The numbers attached to the arrows indicate open reading frames; f380 is identical to *gldA*.

the 23-kDa protein on pVT88, it was clear that the 39-kDa protein band represented GLDH and that the corresponding gene was carried by all three plasmids.

Characterization of the GLDH activity. GLDH assays at pH 9.3 with extracts of different strains under different growth conditions were done. The results are shown in Tables 2 and 3. GLDH activity in MC4100 cells was not induced with G3P, but the addition of 50 mM hydroxyacetone to the low-phosphate growth medium and growth to stationary phase yielded a

TABLE 2. Induction and repression of GLDH activity

Strain	Growth condition[a]	GLDH activity[b]
MC4100	No additions	18.2
	+ 10 mM G3P	17.0
	+ 10 mM glucose	2.0
	+ 50 mM hydroxyacetone	970
Lin424	No additions	1,280
RCD3	No additions	15
RCD3/pVT88	No additions	4,170
DLT242	No additions	0.03

[a] Overnight cultures (20 ml of low-phosphate G+L medium [10] containing 1 mM KH_2PO_4 and 0.5% Casamino Acids) were used. The cells were washed in cold 100 mM potassium phosphate (pH 7.0) and resuspended in 1.5 ml of the same buffer. Cells were lysed by disruption in a French pressure cell (Aminco) at 18,000 psi. The debris and remaining cells were removed in an Eppendorf centrifuge. The GLDH activity was measured at room temperature by the linear increase at A_{340} produced by the addition of 100 mM glycerol to a reaction mixture containing cell extract, 30 mM ammonium sulfate, 100 mM potassium carbonate buffer (pH 9.3), and 1.2 mM NAD^+ (33).

[b] Activities are given in micromoles of NAD^+ reduced per minute per milligram of total protein.

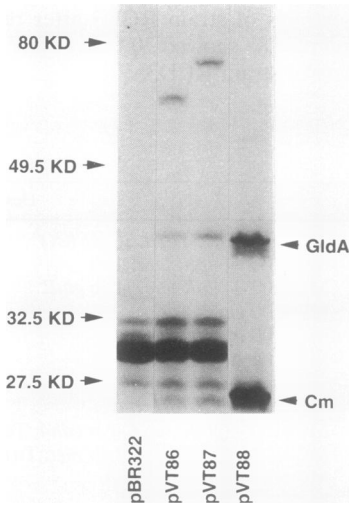

FIG. 3. GldA protein as expressed in minicells. The GldA protein and chloramphenicol acetyltransferase (Cm) are indicated on the right; molecular sizes (in kilodaltons [KD]) are indicated on the left. Minicells from 200 ml of LB overnight cultures of strain HB290 carrying the indicated plasmids were prepared according to published methods (24). Samples containing 0.1 mg of cell protein of the prepared minicells were labeled with 10 μCi of [^{35}S]methionine (New England Nuclear) for 15 min. After they were washed, the cells were resuspended in 25 μl of sample buffer and incubated for 60 min at 37°C. Samples were then loaded onto SDS–10% polyacrylamide gels (18).

TABLE 3. Regulation of the GLDH activity in different strains bearing pVT88

Strain	Growth condition[a]	GLDH activity
DLT242 (*gldA*)	Stationary or logarithmic ± hydroxyacetone	0.03
DLT242/pVT88	Stationary	12,034
	Logarithmic	5,650
	Logarithmic + hydroxyacetone	7,460
RCD3 (*gldA*$^+$ of MC4100)	Stationary	19.0
	Logarithmic	4.6
	Logarithmic + hydroxyacetone	9.0
RCD3/pVT88	Stationary	4,280
	Logarithmic	700
	Logarithmic + hydroxyacetone	963

[a] Overnight cultures were diluted 1:100 and grown for 5 h (logarithmic) or overnight (stationary) in G+L medium–1 mM P_i–0.5% Casamino Acids. Induction with 50 mM hydroxyacetone was done for 2 h before harvesting. Otherwise, the preparation of the extracts and the enzymatic assay were done as described in footnote *a* of Table 2.

[b] Activities are given in micromoles of NAD^+ reduced per minute per milligram of total protein.

TABLE 4. K_m and V_{max} of GLDH for glycerol assayed in different strains and at different pHs

Strain	Growth condition	K_m (mM)	V_{max}[a]
MC4100[b]	Stationary	38	23.6
	Stationary + hydroxyacetone	36	1,035
DLT242/pVT88[b]	Stationary	4.1	9,900
	Logarithmic	3.6	3,750
	Logarithmic + hydroxyacetone	3.8	5,520
RCD3/pVT88[c]	Stationary, pH 10	2.4	2,630
	Stationary, pH 9.3	4.0	3,210
	Stationary, pH 8.0	13	633
	Stationary, pH 7.0	17	328
Lin424[c]	Stationary, pH 9.3	3.8	1,650
	Stationary, pH 7.0	46	495

[a] V_{max} is given in micromoles of NAD^+ reduced per minute per milligram of protein.

[b] Assays with extracts from cultures grown in G+L medium–1 mM P_i–0.5% Casamino Acids were used; when indicated, induction was done with 50 mM hydroxyacetone. Otherwise, the preparation of the extracts and the enzymatic assay were done as described in footnote *a* of Table 2, with the exception that various glycerol concentrations were used for the determination of K_m.

[c] Extracts from overnight cultures (MMA [25]–0.5% Casamino Acids) were used.

50-fold increase in activity, nearly reaching the level observed in extracts of the constitutive strain, Lin424. To observe induction with hydroxyacetone, the use of low-phosphate medium was necessary. When cultures grown in minimal medium A (MMA) (25) (high phosphate) were supplemented with hydroxyacetone, they were lysed after overnight growth. The reason appears to be the elevated production of methylglyoxal from DHA (8, 13). Growth in MMA in the presence of glucose repressed the GLDH activity about 10-fold. This repression could be seen only when cultures were grown in MMA but not with G+L medium (the medium of Garen and Levinthal [10]) plus 1 mM P_i. The effect of hydroxyacetone on the induction of GLDH activity was also tested in strains bearing pVT88. Since strains carrying this plasmid showed lysis when cultures were grown overnight in the presence of hydroxyacetone, even in low-phosphate medium, the induction by hydroxyacetone was done with logarithmically growing cells for 2 h. Induction was at most 1.5-fold (Table 3). In the absence of hydroxyacetone, the expression of GLDH in stationary phase was routinely higher than during logarithmic growth. Thus, full expression of GLDH is achieved only by the combination of hydroxyacetone and stationary-phase growth conditions. High expression of *gldA* in the constitutive strain Lin424 was independent of hydroxyacetone or stationary-growth conditions. Interestingly, the expression of *gldA* from pVT88 was dependent on the strain background. It was considerably higher in DLT242 than in RCD3 (Tables 2 and 3).

Determination of the kinetic constants of GLDH with glycerol as a substrate. GLDH assays at pH 9.3 with cell extracts of the *gldA*$^+$ strain MC4100, strain Lin424 (expressing *gldA*$^+$ at high levels), strain RCD3/pVT88, and strain DLT242/pVT88 (overnight and logarithmic cultures) were done with glycerol concentrations ranging from 1 to 100 mM. Lineweaver-Burk plots led to a K_m of 38 mM and a V_{max} of 23.6 μmol of NAD^+ reduced per min per mg of total protein for GLDH from MC4100. Induction with hydroxyacetone did not affect the K_m but led to a 44-fold-higher V_{max} (Table 4). Our standard assay at pH 9.3 revealed that the enzyme of the overproducing strain Lin424 and the activity of the plasmid-encoded GLDH enzyme exhibited a 10-fold-lower K_m than did the enzyme from strain MC4100 (Table 4). When the enzyme assay was done at different pHs, again the difference between the enzymes from strains MC4100 and Lin424 as well as the plasmid-encoded enzyme was apparent. Whereas no enzymatic activity could be observed at pH 7 for the extract of strain MC4100 (data not shown), the enzyme of strain Lin424 as well as the plasmid-encoded enzyme still showed considerable activity. Apparently, aside from the fact that glycerol cannot induce *gldA*, the altered affinity for glycerol and the altered pH dependency of the enzyme from MC4100 must be responsible for the inability of *glpK* derivatives of MC4100 to grow on glycerol at pH 7, even at a 300 mM concentration, despite the presence of hydroxyacetone as the inducer of GLDH.

Nucleotide sequence accession number. The sequence of f380 has been entered in the EMBL data base as ECOUW89 under accession no. U00006 (2).

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