JOURNAL OF BACTERIOLOGY, Jan. 1984, p. 171–178
0021-9193/84/010171-08$02.00/0

Vol. 157, No. 1

Cloning and Characterization of *gdhA*, the Structural Gene for Glutamate Dehydrogenase of *Salmonella typhimurium*

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Received 20 June 1983/Accepted 28 October 1983

Glutamic acid is synthesized in enteric bacteria by either glutamate dehydrogenase or by the coupled activities of glutamate synthase and glutamine synthetase. A hybrid plasmid containing a fragment of the *Salmonella typhimurium* chromosome cloned into pBR328 restores growth of glutamate auxotrophs of *S. typhimurium* and *Escherichia coli* strains which have mutations in the genes for glutamate dehydrogenase and glutamate synthase. A 2.2-kilobase pair region was shown by complementation analysis, enzyme activity measurements, and the maxicell protein synthesizing system to carry the entire glutamate dehydrogenase structural gene, *gdhA*. Glutamate dehydrogenase encoded by *gdhA* carried on recombinant plasmids was elevated 5- to over 100-fold in *S. typhimurium* or *E. coli* cells and was regulated in both organisms. The *gdhA* promoter was located by recombination studies and by the in vitro fusion to, and activation of, a promoter-deficient *galK* gene. Additionally, *S. typhimurium gdhA* DNA was shown to hybridize to single restriction fragments of chromosomes from other enteric bacteria and from *Saccharomyces cerevisiae*.

Glutamate dehydrogenase (GDH) (EC 1.4.1.4) in *Salmonella typhimurium*, *Escherichia coli*, and *Klebsiella aerogenes* converts α-ketoglutarate and ammonia to glutamate with the oxidation of NADPH. The enzyme from *E. coli* (31, 39) and *S. typhimurium* (10) is a 280,000-dalton hexamer with identical subunits of 47,000 daltons. Physiological and genetic studies have demonstrated that GDH catalyzes one of two possible routes for glutamate production in enteric microorganisms (37). The alternative pathway involves glutamate synthase (EC 1.4.1.13), which uses α-ketoglutarate and glutamine with the oxidation of NADPH to produce glutamate (26, 36). Either of these enzymes can function when excess ammonia is available for growth, and the loss of both activities is required to cause a glutamate requirement. However, when the ammonia concentration is growth rate limiting, the inactivation of glutamate synthase, irrespective of the functional state of GDH, is sufficient to result in glutamate auxotrophy (7, 15).

The question of why *S. typhimurium* cells growing with either excess or limiting ammonia have high GDH activities, even when it is not required to synthesize glutamate, is of particular interest (5, 14, 15, 30). The analysis of several strains with mutations in the structural gene for GDH, *gdhA*, has demonstrated that the enzyme activity is not essential as long as glutamate synthase is functional (14, 15, 30). The regulation of *ghdA* in *S. typhimurium* differs from that in *K. aerogenes*, in which growth with a limiting nitrogen source causes a decrease in GDH activity (2, 7), and from that in *E. coli*, where nitrogen limitation has no effect and only the supplementation of glucose-ammonia medium with glutamate causes a decrease in GDH activity (17, 25, 38). Thus, although similar biosynthetic routes to glutamate exist in a number of organisms, the pattern of *gdhA* regulation is unique for each.

These differences in regulation make information about the promoters and regulatory regions for *gdhA* from these organisms of special interest. To understand these, we report the construction of recombinant plasmids containing *gdhA* from *S. typhimurium* and their use to confirm the direction of *gdhA* transcription, to identify a fragment containing the *gdhA* promoter, and to develop a correspondence between the genetic and physical maps.

(A preliminary report of this work has appeared previously [Fed. Proc., **41**:2850, 1982] and was submitted by E.S.M. as part of the Ph.D. thesis to the Department of Biological Sciences, Purdue University.)

MATERIALS AND METHODS

Chemicals and enzymes. All reagents are commercially available and were obtained from Sigma Chemical Co., St. Louis, Mo.; Bio-Rad Laboratories, Richmond, Calif.; Bethesda Research Laboratories, Gaithersburg, Md.; or New England BioLabs, Beverly, Mass.

Bacterial strains and plasmids. All strains were derivatives of *S. typhimurium* LT-2 or *E. coli* K-12, unless otherwise indicated, and are listed in Table 1. pBR328 was kindly provided by F. Bolivar and pKO4 was obtained via N. Ho from M. Rosenberg. Plasmids constructed in this work are listed in Table 1. Transductions used the P22 HT105/*int* phage by the procedure of Ratzkin and Roth (28).

Media and growth conditions. The glucose-ammonia and Luria broth (LB) media were as described previously (4). Alternative nitrogen sources were added at 35 mM, and the $(NH_4)_2SO_4$ was omitted for nitrogen-limitation experiments. Antibiotics were added at 25 or 50 μg/ml.

Cultures were grown and treated as previously described (6) for enzyme assays. The glutamate dehydrogenase assay measured the rate of NADPH oxidation (15), and the specific activity was expressed as micromoles of NADPH oxidized per minute per milligram of protein. Protein was measured by the method of Lowry et al. (22).

Transformation procedures. *S. typhimurium* and *E. coli* strains were transformed by the procedure of Lederberg and Cohen (21). Although these investigators reported no differences between the transformation efficiencies of Gal^+ and Gal^- *S. typhimurium* strains, a significant difference (approximately 100-fold lower for Gal^+ strains) was observed in

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TABLE 1. Bacterial strains and plasmids used

Strain	Genotype	Source
S. typhimurium LT-2		
JB1396	*ilv-452 metA22 trpB2* H1-b(*fels-2*) *H2enx nml flaA66 strA120 xyl-404 metE551 hspLT6 hspS24 gal-496*	K. Sanderson and reference 29
JB1994	*dhuA1 hisJ5601 gal-2395* Δ(*gltB832-cod*) Δ*gdhA71*	Laboratory collection (S. A. Rosenfeld, Ph.D. thesis)
JB1995	*dhuA1 hisJ5601 gal-2395* Δ(*gltB833-cod*) Δ*gdhA71*	Laboratory collection (S. A. Rosenfeld, Ph.D. thesis)
JB2111	*dhuA1 hisJ5601 gal-2395* Δ(*gltB833-cod*) Δ*gdhA71 sr1201*::Tn*10 recA1*	P22 HT*int*-1 (28) transduction of JB1995 with TT521 as donor
JB2112	*dhuA1 hisJ5601 gal-2395* Δ(*gltB833-cod*) Δ*gdhA71 recA1*	Tetracycline-sensitive derivative of JB2111
JB2117	JB1994(pJB101)	Transformation of JB1994 with *Eco*RI ligation mixture
JB2119	JB2112(pJB101)	Transformation of JB2112 with pJB101
JB2121	JB2112(pJB102)	Transformation of JB2112 with pJB102
JB2123	JB2112(pJB103)	Transformation of JB2112 with pJB103
JB2132	TT521(pBR328)	Transformation of TT521 with pBR328
JB2134	TT521(pJB102)	Transformation of TT521 with pJB102
JB2135	TT521(pJB103)	Transformation of TT521 with pJB103
JB2138	TT521(pJB108)	Transformation of TT521 with pJB108
JB2148	*trpA49 nadB51 pncA15 gal-2398*	Gal$^-$ derivative of JF63
JF63	*trpA49 nadB51 pncA15*	J. Foster (16)
JL907	*galE hutR49*	J. L. Ingraham
SK75	*galE1797 nit-9*	S. Kustu (8)
TT521	*sr1202*::Tn*10 recA1 str*	J. Roth
E. coli		
CB123	CU1209 (pJB103)	Transformation of CU1209 with pJB103
CSR603	*uvrA6 recA1 phr-1 thr-1 thi-1 leu-6 proH2 argE3 lacY1 galK2 ara-14 mtl-1 rpsL31 tsx-33 supE44 str* Δ(*nadA-chlA*)	E. Tessman and reference 32
CU1209	*thi-1 hsdR hsdM*$^+$ *recA*	E. Harms and E. Miller
N100	*galK recA pro*	M. Rosenberg
PA340	*thi-1 thr-1 leuB6 gdh-1 hisG1 gltB31 argH1 ara-14 lacY1 gal-6 malA1 xyl-7 mtl-2 rpsL-9 tonA*1 λ^r λ *supE44*	B. Bachmann
Plasmid		
pBR328	*bla*$^+$ *tet*$^+$ *cat*$^+$	Soberon et al. (34)
pJB101	pBR328 Ω[4.91 kb:LT-2 *gdhA* 17 kb (−)]1	Chromosomal *Eco*RI fragments of JL907 inserted into *Eco*RI site of pRB328
pJB102	pBR328 Ω[4.91 kb:pJB101 *gdhA* 6.2 kb (+)]2	*Eco*RI fragment of pJB101 inserted into *Eco*RI site of pBR328
pJB103	pJB102 Δ[pBR328 4.91-1.85 kb LT-2 1.4 kb (+)]1	Removal of a *Sal*I fragment from pJB102
pJB105	pJB102 Δ[pBR328 4.91-1.25 kb LT-2 5.0 kb (+)]2	Removal of a *Hin*dIII fragment from pJB102
pJB107	pJB103 Δ[pBR328 4.81-4.91 kb LT-2 2.2 kb (+)]1	Removal of a *Pvu*II fragment from pJB103
pJB108	pBR328 Δ[4.81-4.91 kb]1 Ω[4.81,4.91 kb:pJB103 *gdhA* 2.2 kb (−)]1	*Eco*RI/*Pvu*II fragment of pJB103 inserted into *Eco*RI/*Pvu*II sites of pBR328
pJB109	pBR328 Δ[1.25-1.85 kb]2 Ω[1.25, 1.85 kb:pJB103 3.6 kb (+)]2	*Hin*dIII/*Sal*I fragment of pJB103 inserted into *Hin*dIII/*Sal*I sites of pBR328
pJB110	pBR328 Ω[4.81 kb:pJB101 *gdhA* 4.2 kb (+)]3	*Pvu*II fragment of pJB101 inserted into *Pvu*II site of pBR328
pJB111	pK04 Δ[0.00-0.31 kb]1 Ω[0.00,0.31 kb:pJB105 1.2 *gdhA'* 1.2 kb (+)]1	*Eco*RI/*Hin*dIII fragment of pJB105 inserted into *Eco*RI/*Hin*dIII sites of pK04
pK04	pBR322 derivative (*bla*$^+$ *tet*$^-$) containing a promoter-deficient *galK* gene	McKenney et al. (24)

this laboratory, and therefore Gal$^-$ derivatives were used in preference to the Gal$^+$ isogenic strain. Another transformation procedure, obtained from M. Rosenberg (personal communication), was used in later experiments and was found to be more efficient for both *E. coli* and *S. typhimurium*. This procedure differed from other transformation protocols (13, 21) only in that competent cells were prepared with a wash solution consisting of 10 mM Tris (pH 7.5) and 0.3% $MnCl_2$ and DNA was added to cells in the presence of the wash buffer plus 0.6% $CaCl_2$.

Manipulation of chromosomal and plasmid DNA. Chromosomal DNA was purified from strain JL907 by the procedures of Marmur (23) and Cosloy and Oishi (9). Plasmid DNA was prepared by the CsCl ethidium bromide buoyant density gradient procedure of Humphreys et al. (19).

Restriction endonucleases were used with buffers prepared according to the recommendations of the suppliers. DNA ligation mixtures for initial cloning were prepared by adding 30 μg of restriction endonuclease-treated chromosomal DNA from strain JL907 to 15 μg of restricted pBR328 DNA treated with bacterial alkaline phosphatase. Ligation reactions were done with excess T4 DNA ligase for 24 h at 16°C. Subclones derived from pJB101 were isolated by screening recombinant plasmid DNA in *S. typhimurium* strains JB1396 and JB2112 or in the *E. coli* strain PA340. Plasmid DNA from colonies with the appropriate phenotype

was purified and characterized by restriction analysis (3, 18). Manipulation and electrophoretic analyses of plasmids and their restriction products were performed according to described procedures (13). Plasmid DNA was stored at 4°C in buffer that was 10 mM Tris-hydrochloride (pH 7.5) and 2 mM trisodium EDTA.

In vivo labeling of proteins. For analysis of plasmid-coded polypeptides, *E. coli* CSR603 was transformed with the appropriate plasmid, and the UV sensitivity of the resulting strains was confirmed. In vivo labeling of plasmid-coded proteins was done essentially by the method of Sancar et al. (32), with the addition of 36 μCi of L-[^{35}S]methionine (>1,000 mCi/mmol; New England Nuclear Corp., Boston, Mass.) to the labeling medium. Samples were subjected to electrophoresis through 12% (wt/vol) polyacrylamide-sodium dodecyl sulfate gels as described previously (20) and analyzed by fluorography.

Mapping of *gdhA* mutations. Each strain (Table 1) having a single missense, deletion, or insertion mutation in *gdhA* was transformed separately with pJB105 or pJB109. Neither of these plasmids contains the entire *gdhA* gene, and they do not produce active GDH (see below). Isolated Ap^r transformants were scored for their ability to form Glt$^+$ recombinants by plating 0.1 ml of cells on glucose-ammonia plus ampicillin medium. The appearance of 100 to 350 colonies demonstrated the presence of the *gdhA* region on the plasmid that covered the mutation in the chromosome.

Nitrocellulose filter blot hybridization. DNA-DNA blot hybridizations were performed by the procedure of Southern (35), as modified by Davis et al. (13). The purified fragment was radioactively labeled with [α-^{32}P]dCTP (800 Ci/mmol) by using a commercially available nick-translation system (Amersham Corp., Arlington Heights, Ill.).

RESULTS

Isolation of *gdhA* on pBR328. The plasmid pBR328 (34) contains unique restriction sites in genes that confer resistance to ampicillin (Ap^r), tetracycline (Tc^r), and chloramphenicol (Cm^r). The advantage of using pBR328 as a cloning vehicle over pBR322 is the presence of unique restriction sites (*Bal*I, *Eco*RI, and *Pvu*II) in the additional antibiotic resistance gene *cat*.

S. typhimurium strains with deletion mutations in *gdhA* and *gltB* (the structural genes for GDH and glutamate synthase, respectively) were used (S. A. Rosenfeld, Ph.D. thesis, Purdue University, West Lafayette, Ind., 1981; J. Madonna, R. Fuchs, and J. Brenchley, manuscript in preparation), and one such strain, JB1994, was transformed with *S. typhimurium*-pBR328 DNA mixtures treated separately with five different restriction endonucleases. One Glt$^+$ colony, JB2117, was isolated from the DNA treated with *Eco*RI, and the purified plasmid from this strain conferred a Ap^r Tc^r Cm^s Glt$^+$ phenotype when transformed into JB1994 or the *recA* derivative, JB2112. Thus, this plasmid, pJB101, was isolated from a direct selection for glutamate prototrophs of *S. typhimurium* and contained either *gdhA* or *gltB*.

The presence of *gdhA*, and not *gltB*, on pJB101 was confirmed by examining the growth properties of plasmid-containing strains on solid media and by assaying the activities of GDH and glutamate synthase. Strains JB2117 and JB2119, which have deletions in *gdhA* and *gltB* and contain the plasmid pJB101, were grown on media containing glucose (0.4%) and either proline or arginine as growth-rate-limiting nitrogen sources. Neither strain grew on glucose-proline or glucose-arginine media. This is the result expected for a *gdhA*$^+$ *gltB* strain, suggesting the presence of a *gdhA*-containing plasmid. The presence of *gdhA* on pJB101 was confirmed by determining the level of GDH activity in strains carrying this plasmid (Table 2). These data showed a greater than 25-fold increase in the rate of NADPH oxidation by extracts prepared from pJB101-containing cells as compared to the control strain.

Complementation analysis of *pncA15* and *nit-9*. The detailed genetic analysis of the *S. typhimurium gdhA* region reported by Rosenfeld et al. (30) showed cotransducibility of *gdhA* with *pncA* and *nit-9* by phage P22HT105/*int* transduction and a clockwise order of *pncA-gdhA-nit-9* on the genetic map. Although there is a low cotransductional frequency between these markers, pJB101 was examined for its ability to complement the mutations in the *S. typhimurium* strains JB2148 (*pncA15 nadB5*) and SK75 (*nit-9*). For both strains, no transformants were obtained that showed complementation of the altered growth properties caused by *pncA15* and *nit-9*, indicating that these regions are not carried on the *gdhA*-containing DNA.

Isolation of pJB102 and partial restriction maps of the *gdhA*-containing plasmids. The *gdhA*-containing plasmid pJB101 was purified and treated with restriction endonuclease *Eco*RI and other hexanucleotide-specific enzymes. Three *Eco*RI fragments of 4.9, 6.2 and 11 kilobase pairs (kb), were generated. The 4.9-kb fragment represented the cloning vehicle, pBR328, whereas the 6.2- and 11-kb fagments were of *S. typhimurium* chromosome origin. Initial subcloning from pJB101 for the isolation of *gdhA* was done by treating pJB101 with *Eco*RI followed by ligation, transformation, and screening for complementation of Δ*gdhA71*. Using this approach, the *gdhA*$^+$ plasmid pJB102 was isolated, thereby locating *gdhA* on the 6.2-kb *Eco*RI insert.

By comparing the digestion patterns of pJB101 and pJB102, three *Ava*I, three *Pst*I, two *Bal*I, two *Sal*I, one *Hin*dIII, and one *Pvu*II sites in the 11-kb *Eco*RI fragment of pJB101 were identified. Since *gdh*A was shown to be contained entirely on the 6.2-kb *Eco*RI fragment, the precise location of these sites on the adjacent 11-kb fragment was not determined.

Unique restriction sites in the 6.2-kb *Eco*RI fragment of pJB102 were located by single and double digestions. Single sites were located for restriction endonucleases *Hin*dIII, *Pvu*II, and *Sal*I (Fig. 1), whereas no sites were found for *Ava*I, *Bal*I, or *Pst*I. Because of their distribution throughout the 6.2-kb fragment, these unique sites were used to subclone and isolate *gdhA* from pJB102.

Subcloning of *gdhA*. Subclones were constructed by digesting the parental plasmid containing the 6.2-kb *Eco*RI insert, pJB102, with one or two restriction endonucleases and ligating the products with or without the addition of pBR328 digested with the same enzyme(s). In this manner, a

TABLE 2. GDH activities for strains carrying *gdhA* plasmids[a]

Strain	GDH activity[b]	Generation time (min)
JB2112	<0.01	70
JB2132(pBR328)	0.40	70
JB2119(pJB101)	11.4	240
JB2121(pJB102)	23.4	225
JB2123(pJB103)	79.8	300

[a] The generation times and enzyme activities were determined for cells growing in glucose-ammonia media at 37°C, except for strain JB2112, to which glutamate was added as a required supplement.

[b] The activities are expressed as micromoles of NADPH oxidized per minute per milligram of protein.

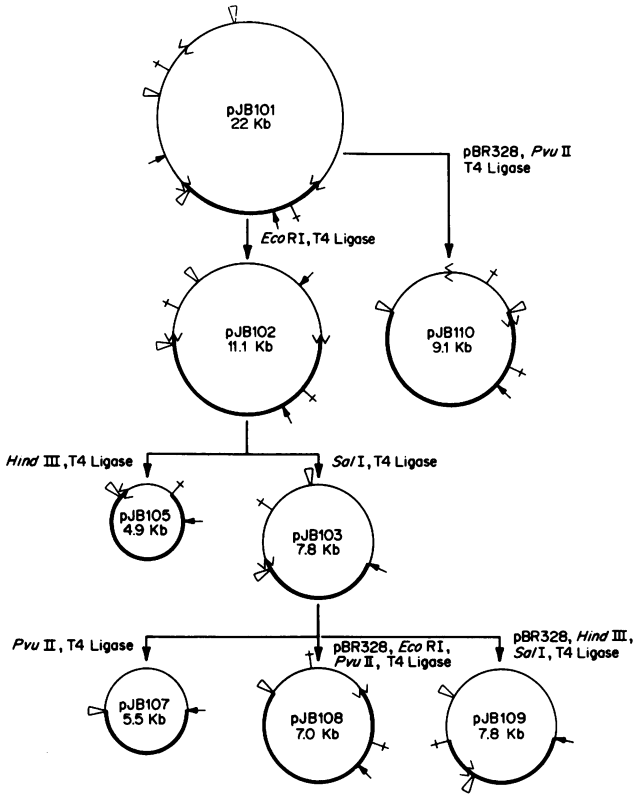

FIG. 1. pJB101 and the isolation of its derivatives. The plasmids and relative positions of the restriction sites are drawn approximately to scale. Symbols: ≷, *Eco*RI; †, *Hin*dIII; ▽, *Pvu*II; ↓ *Sal*I; bold lines, pBR328; thin lines, insert DNA.

series of plasmids was constructed (Fig. 2), and their ability to complement the Δ*gdhA71* mutation was tested. Plasmids pJB101, pJB102, pJB103, pJB108, and pJB110 complemented Δ*gdhA71* in JB2112, whereas plasmids pJB105, pJB107, and pJB109 failed to show complementation. A similar pattern of complementation was observed with these plasmids when they were transferred into the *E. coli gdh-1 gltB31* strain PA340, showing that the *S. typhimurium gdhA* gene was expressed in *E. coli* and complemented the *gdh-1* mutation. Enzyme assays of the *S. typhimurium* derivatives showed a 200-fold increase in GDH activity for a strain carrying pJB103 (Table 2). The results (Table 2) also showed that strains carrying plasmids with *gdhA* grew more slowly in glucose-ammonia medium, perhaps due to the high GDH activity. The basis for the increased GDH activity from the successive subclones (11.4 U versus 79.8 U) was not determined, but could be related specifically to *gdhA* regulation or to variation in the plasmid copy number.

The *cat* gene of pBR328 is transcribed in a clockwise direction through the *Eco*RI site and toward the *Hin*dIII site in the promoter of the *tet* gene (34). The cloned DNA inserted at the *Eco*RI site of pBR328 in pJB101 and pJB108 was in one orientation (−), whereas the insert DNA in pJB102, pJB103, and pJB110 was in the opposite orientation (+) (Fig. 1). Since *gdhA* was expressed in both orientations, the cloned gene likely contained its own promoter and was not transcribed from the adjacent *cat* promoter.

These cloning and complementation data located *gdhA* within the 2.2-kb *Eco*RI/*Pvu*II interval (Fig. 2). The GDH monomer from *S. typhimurium* has an approximate M_r of 47,000 and would therefore require 1,200 or more bases to encode approximately 390 amino acids and an adjacent regulatory region. Thus, it was inferred that the unique *Hin*dIII site between the *Eco*RI and *Pvu*II sites of pJB108 would lie within *gdhA*. The lack of complementation by pJB105 and pJB109 supported this assumption and provided two plasmids for the genetic mapping experiments described below.

Identification of plasmid-coded proteins. *E. coli* CSR603 has been used to identify proteins encoded by multicopy plasmids that escape UV damage during short periods of irradiation (32). Strain CSR603 was transformed with various *S. typhimurium gdhA*$^+$ and *gdhA* plasmids, and the polypeptides encoded by these plasmids were identified by L-[^{35}S]methionine fluorography. The *bla* gene of pBR328 encodes a major 28,000-dalton β-lactamase and the *cat* gene encodes a smaller, 22,500-dalton chloramphenicol acetyltransferase (11). These polypeptides were present (Fig. 3, lane A), but the inducible tetracycline acetyltransferase is absent, as these cells were grown without the addition of tetracycline. None of these polypeptides was made in CSR603 cells alone (lane B). Extensive synthesis of an unidentified 37,000-dalton polypeptide from pJB101 and pJB102 (lanes C and D) that was not produced by the other plasmids was detected. The coding region for this polypeptide is within the 1.4-kb *Sal*I/*Eco*RI interval of pJB102 and lies at least 2.6 kb from *gdhA* (see Fig. 2). A barely detectable polypeptide of 58,000 daltons appeared to be encoded by the 11-kb *Eco*RI fragment of pJB101. Since the presence or absence of these polypeptides had no significant affect on the level of GDH activity (see Table 3) they are likely not involved in *ghdA* expression.

The *gdhA*$^+$ plasmids (Fig. 3, lanes C, D, E, and H) directed the synthesis of a polypeptide of approximately 47,000 daltons, which was absent in the pattern produced by the noncomplementing clones (lanes F and G). These results corroborate the complementation data and show that the 47,000 M_r polypeptide corresponds to the GDH monomer.

Regulation of GDH levels. The initial characterization of the plasmids suggested that *gdhA* was expressed from its own promoter. To determine whether the expression of the plasmid gene had the same control as the chromosomal *gdhA*, cells were grown in media known to affect GDH

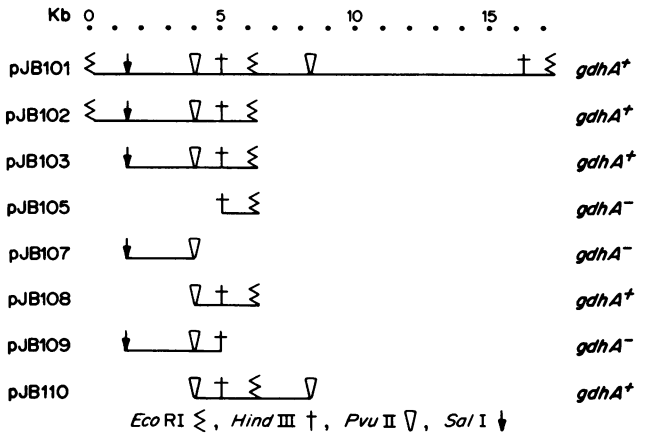

FIG. 2. Location and isolation of *gdhA*. The insert DNA present in each plasmid is aligned relative to its location in pJB101 and without regard to its orientation in the respective plasmid. The smallest derivative to complement Δ*gdhA71* is the 2.2-kb fragment in pJB108.

TABLE 3. Regulation of GDH levels

Strain	GDH activity (μmol of NADPH oxidized per min per mg of protein) in:[a]		
	Glucose-ammonia	Casamino Acids	LB
JB2132(pBR328)	0.39 (69)[b]	0.05 (64)	0.11 (38)
JB2134(pJB102)	4.32 (98)	1.41 (69)	0.58 (78)
JB2135(pJB103)	8.58 (126)	3.58 (53)	0.93 (54)
JB2138(pJB108)	4.80 (180)	0.97 (84)	ND[b]
CU1209	1.90 (74)	0.25 (57)	ND
CB123(pJB103)	29.80 (90)	4.00 (54)	ND

[a] The glucose ammonia medium was supplemented with 0.5 mM L-methionine and L-lysine for the *S. typhimurium* strains and with 4 μg of thiamine per ml for the *E. coli* strains CU1209 and CB123. The values in parentheses are the generation times (minutes).
[b] ND, Not determined.

synthesis (5). The GDH activity of *S. typhimurium* decreases in cells grown in a complex medium, such as LB, or in a medium supplemented with Casamino Acids. Specific *gdhA* control was reflected in the enzyme levels in strains carrying plasmids with *gdhA* (Table 3) and showed that each of the complementing plasmids contained important *gdhA* regulatory sequences. Regulation of *S. typhimurium gdhA* was also observed when this gene was carried in *E. coli*. Although there was some variation in the GDH levels, all strains had decreased activities in the supplemented media (Table 3).

Glutamate synthase and glutamine synthetase activities were also measured in cells grown under the growth conditions reported in Table 3, yet no differences were observed, irrespective of the presence of the *gdhA* plasmids. These activities were similar to those observed in wild-type strains, suggesting that the level of GDH has little or no effect on the expression of the structural genes for these two enzymes (data not shown).

Because the plasmid-containing strains had a slow growth rate in glucose-ammonia medium (Table 2), we examined several supplements to determine whether any restored normal growth. The addition of methionine and lysine decreased the generation time from the 225- to 300-min range (Table 2) to about 130 min. (Table 3). Although this effect is not understood, it could be related to elevated GDH activity effectively depleting the α-ketoglutarate pool and subsequently limiting synthesis of succinyl-coenzyme A for methionine and lysine formation. This is supported by the observation that the addition of succinate mimicked the effect of methionine and lysine (data not shown).

Genetic and transcriptional analysis of *gdhA*. Several strains with different *gdhA* mutations located on the *gdhA* genetic map (27) were transformed with the *gdhA* plasmids pJB105 and pJB109. Both of these plasmids were thought to carry portions of *gdhA*, since each had approximately half of the 2.2-kb *Eco*RI/*Pvu*II fragment that contains all of *gdhA*. Glt$^+$ recombinants of transformed *recA*$^+$ *gdhA* strains could be obtained if the plasmid insert DNA covered the particular *gdhA* mutation in the recipient strain. In this manner, pJB105 was shown to cover point mutations, deletion intervals, and phage Mu insertions proximal to and including *gdhA61* (Fig. 4). Conversely, pJB109 produced Glt$^+$ recombinants only in those strains with mutations found distal to *gdhA61*. Transformants of a Δ*gdhA88* strain (JB1917) produced no recombinants with either plasmid; thus, this deletion interval must span the *Hin*dIII terminal site of both inserts. A temperature-sensitive GDH is synthesized in strains having the *gdhA52* mutation, an allele in a cluster of mutations rescued by pJB109; this result supports the conclusion that the *Hin*dIII site is located in the structural gene.

The direction of *gdhA* transcription on the *S. typhimurium* chromosome is in the counterclockwise direction, from the *nit* side of *gdhA* toward *pncA* (Fig. 4) (30). Since this end of the gene was shown to include the *Eco*RI/*Hin*dIII fragment in pJB105, the N-terminus and promoter region of *gdhA* should be within this 1.2-kb interval. The direction of *gdhA* transcription on the cloned fragment was therefore deduced from the mapping experiments as being from the *Eco*RI end toward the *Hin*dIII site, with the carboxy-terminal sequences occurring proximal to the *Pvu*II site on pJB109.

Confirmation of the direction of *gdhA* transcription was obtained by cloning the 1.2-kb *Eco*RI/*Hin*dIII fragment of pJB105 into the *Eco*RI/*Hin*dIII sites of the *galK* plasmid pKO4 (24). Insertion of a promoter upstream from the galactokinase structural gene in this plasmid would activate expression of *galK* (Fig. 5).

Transformation of *E. coli* N200 (*galK*) with ligation mixtures of *Eco*RI- and *Hin*dIII-digested pK04 or with digested pK04 plus pJB105 produced only white, *gal* colonies on MacConkey agar with pK04 alone but produced 75 to 80% red, *gal*$^+$ colonies with the pK04-pJB105 mixture. Isolation and screening of several individual red colonies showed the presence of plasmids similar to pJB111 (Fig. 5) that had 3.6-kb vector and 1.2-kb insert DNA fragments. Both *E. coli* and *S. typhimurium* strains containing pJB111 are phenotypically Gal$^+$ when *galK* on the chromosome (data not shown). These data confirmed the direction of *gdhA* transcription; the 5′ end of the mRNA is initiated proximal to the *Eco*RI site.

Hybridization analysis with the 1.2-kb promoter fragment. Since *gdhA* transcription was inferred to initiate near the

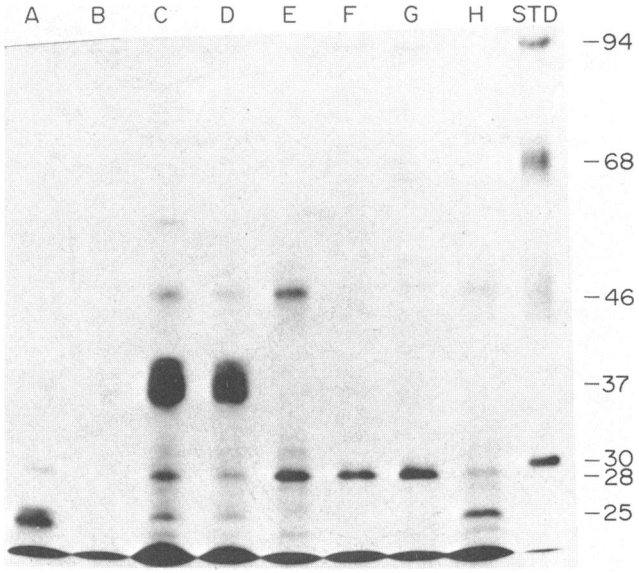

FIG. 3. Fluorograph of a sodium dodecyl sulfate-polyacrylamide gel of L-[^{35}S]methionine-labeled polypeptides from UV-irradiated cells. The strains used were the UV sensitive CSR603 or its derivatives with different plasmids. The additions to each lane are indicated by the plasmid carried by CSR603. Lane A, pBR328; B, no plasmid; C, pJB101; D, pJB102; E, pJB103; F, pJB105; G, pJB107; H, pJB108. The last lane contains ^{14}C-labeled standards at 94, 68, 46, and 30 kilodaltons.

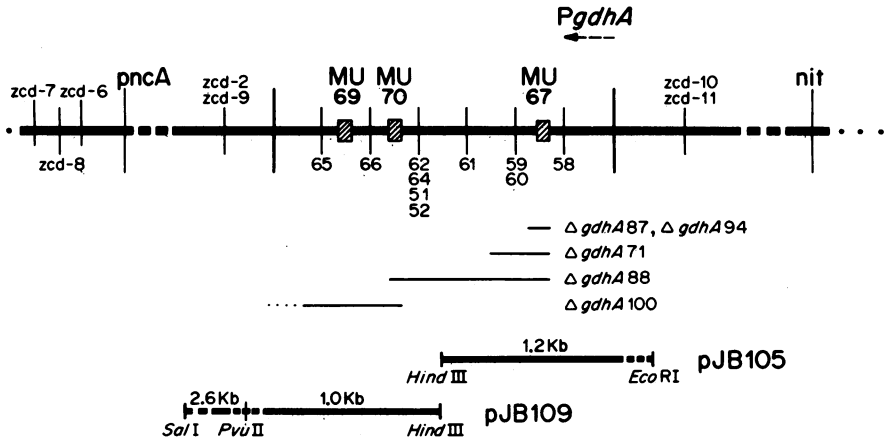

FIG. 4. Mapping of *gdhA* mutations. The *gdhA* plasmids pJB105 and pJB109 were transferred into a number of strains with previously located (30) *gdhA* mutations. Glt$^+$ recombinants were obtained between pJB105 and mutations proximal to and including *gdhA61* and between pJB109 and mutations located distal to *gdhA61*. The deletion intervals (thin lines), point mutations (numbered), phage Mu insertions, and Tn*10* insertions (*zcd* numbers) are drawn according to the genetic map. The direction of *gdhA* transcription on the chromosome is indicated by the arrow and P*gdhA*.

terminal *Eco*RI site, upstream sequences relevant to *gdhA* expression or general nitrogen metabolism could be present on the 11-kb fragment of pJB101 if these fragments are adjacent on the *S. typhimurium* chromosome.

DNA-DNA blot hybridizations were done with [α-^{32}P]dCTP-labeled 1.2-kb *Eco*RI/*Hin*dIII fragment purified from pJB111. This fragment hybridized specifically to *Pvu*II and *Eco*RI fragments of size 4.2 and 6.2 kb, respectively, from the control plasmid pJB101 (Fig. 6, lanes A and C). The 1.2-kb probe fragment also hybridized, as expected, to a 6.2-kb fragment derived from an *Eco*RI digest of *S. typhimurium* chromosomal DNA. A 4.2-kb *Pvu*II-generated chromosomal fragment did not hybridize to the probe fragment (lane B) and indicated that the 6.2 and 11-kb fragments are not adjacent on the chromosome.

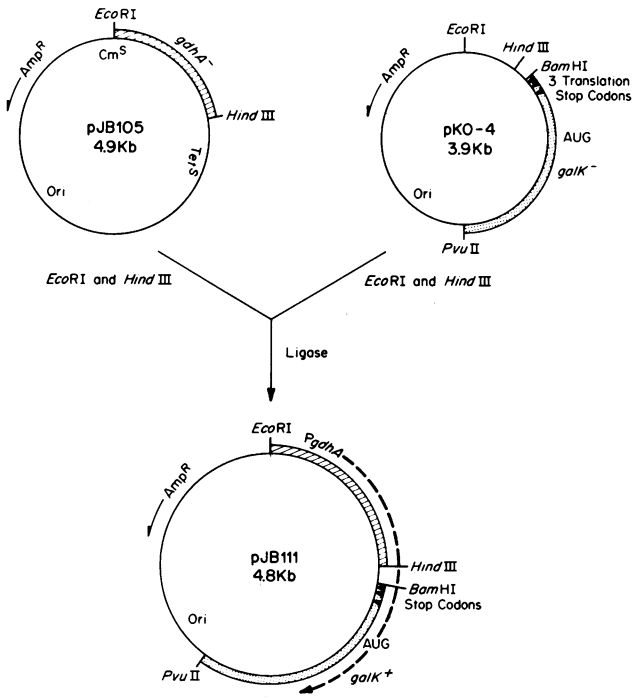

FIG. 5. Construction of a *gdhA*::*galK* fusion plasmid, pJB111. A diagrammatic representation of the promoter-deficient plasmid pKO4 and the activation of its *galK* gene by the insertion of the pJB105 1.2-kb *Eco*RI/*Hin*dIII fragment. The direction of transcription from the *gdhA* promoter (*PgdhA*) is indicated by the dashed arrow on pJB111. The *galK* gene is translated by using its own initiation codon (AUG); translational products arising from the *gdhA* initiation site are terminated at the three stop codons preceding *galK*.

To determine whether homology exists between *S. typhimurium gdhA* and other organisms, the promoter-proximal 1.2-kb fragment was used as a hybridization probe with DNA from *E. coli* K-12, *E. coli* B, *K. aerogenes*, *Serratia marsescens*, and *Saccharomyces cerevisiae* genomic DNA. All showed hybridization to the probe fragment (Fig. 6). Surprisingly, although the same amount of digested DNA was added to each lane, *S. cerevisiae* showed the greatest amount of hybridization. Thus, the cloned *S. typhimurium gdhA* fragment showed homology to DNA sequences of varied origin and suggests that these clones could facilitate the isolation or identification of *gdhA* from other organisms.

DISCUSSION

Standard recombinant DNA techniques and previously characterized glutamate auxotrophs were used to clone and partially characterize *gdhA* from *S. typhimurium*. We found transformation procedures commonly used for *E. coli* (21; above) to be nearly as effective for Gal$^-$ strains of *S. typhimurium*, which permitted the use of this microorganism for most manipulations. This was particularly important since analogous, genetically well-characterized GLT$^-$ strains of *E. coli* were not available.

Subcloning and in vivo protein labeling experiments located *gdhA* within a 2.2-kb *Eco*RI/*Pvu*II sequence interval, of which 1,200 base pairs or more would be required to code for the 47,000 M_r GDH monomer. Two plasmids, pJB105 and pJB109, contained adjacent portions of the 2.2-kb *Eco*RI/*Pvu*II fragment and were predicted to cover specific promoter-proximal or promoter-distal *gdh*A mutations. It was show (Fig. 4) that the insert DNA in pJB109 is homologous to the region on the *pncA* side of *gdhA52*, a mutation that causes the production of a temperature-sensitive GDH (14, 30). Therefore, pJB109 contains *gdhA* structural gene sequences, and the *Hin*dIII restriction site that approximately bisects

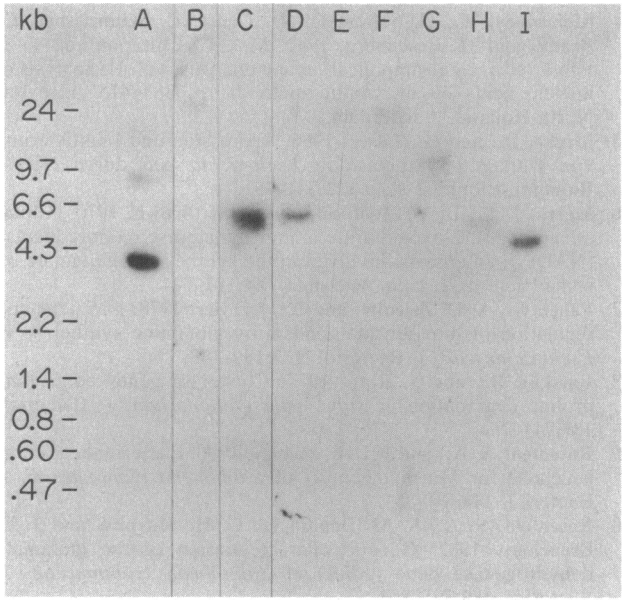

FIG. 6. Restriction digests of plasmid and chromosomal DNA hybridized to the *gdhA* promoter fragment. The probe was ^{32}P-labeled 1.2-kb *Eco*RI/*Hin*dIII promoter fragment purified from pJB111. The digested, electrophoresed, and blotted DNAs used were: (A) pJB101, *Pvu*II; (B) *S. typhimurium*, *Pvu*II; (C) pJB101, *Eco*RI; (D) *S. typhimurium*, *Eco*RI; (E) *E. coli* K-12, *Eco*RI; (F) *E. coli* B, *Eco*RI; (G) *K. aerogenes*, *Eco*RI; (H) *Serratia marsescens*, *Eco*RI; (I) *S. cerevisiae*, *Eco*RI.

the *Eco*RI/*Pvu*II fragment must be located in the *gdhA* structural gene. Since it had been shown that the direction of *gdhA* transcription is from the *nit* side of the gene toward *pncA* on the *S. typhimurium* chromosome (30; S. A. Rosenfeld, Ph.D. thesis), the *Eco*RI/*Hin*dIII fragment of pJB105 was inferred to contain the *gdhA* promoter. This plasmid contains insert DNA that is homologous only to the more promoter-proximal mutations. Although these in vivo plasmid-chromosome recombination studies were used primarily to determine the direction of transcription of the cloned gene, it is a general approach that can be used to relate a genetic map to a physical map. Further evidence for the location of the *gdhA* promoter and the direction of transcription was obtained through the construction of a *gdhA::galK* fusion plasmid (Fig. 5) that acquired the ability to synthesize galactokinase. The orientation of the *gdhA* promoter fragment in the *galK* plasmid confirmed the inferred direction of transcription derived from the in vivo recombination studies.

The observation that strains with high *gdhA* expression grew more slowly is interesting. Although the poor growth could result from many factors associated with high-level protein expression, the improvement in growth rate by the addition of methionine and lysine (or succinate) suggests that the GDH activity is deleterious. High GDH activity could produce an imbalance of reducing equivalents, since the reaction consumes NADPH, or it could decrease the availability of α-ketoglutarate for succinyl-coenzyme A synthesis. The latter possibility would be consistent with the report by Amarisingham and Davis (1) that α-ketoglutarate is needed for the induction of α-ketoglutarate dehydrogenase and with the observed partial requirement for the two amino acids, methionine and lysine, that use succinyl-coenzyme A as a substrate in their synthesis. The strains containing *gdhA* plasmids could be useful for studying the physiological effects of GDH and for devising selections for mutants with altered regulation of α-ketoglutarate dehydrogenase.

The regulation of *gdhA* expression from a number of complementing plasmids was examined, and the data showed that GDH is synthesized in various media in parallel with the pattern observed for chromosomally coded GDH (Table 3). Strains containing the *gdhA*$^+$ plasmids showed as much as a 200-fold increase in GDH levels when grown in glucose-ammonia medium compared to the control strains (Table 2). Nonetheless, as for the wild-type *S. typhimurium* strain, GDH activity was reduced in highly supplemented, Casamino Acids-containing medium. This pattern of GDH synthesis was observed with plasmids having the *gdhA* insert in both orientations relative to vector sequences.

The approximately 15-fold increase in GDH activity in *E. coli* CB123 (Table 3) was higher than anticipated based on the expression of cloned *E. coli gdhA* in *E. coli* (12, 33, 40). These reports showed only a 2.5-fold (33) or 5- to 6-fold (40) increase in plasmid-coded GDH activity in glucose-ammonia-grown cells. This is significantly lower than the activity observed with *S. typhimurium gdhA* (Table 2) and may not be attributed simply to gene dosage differences between the multicopy plasmids used. Further intergeneric physiological and genetic studies, in addition to DNA sequence analysis, should provide important insight into the mechanisms of *gdhA* control in these bacteria.

The results of the Southern blot hybridization showed that a fragment of *gdhA* hybridized to the DNA of several enteric bacteria. Thus, similarities among these genes exist and it will be important to examine the gene and control region sequences further. In addition, it appears that *Saccharomyces cerevisiae* DNA hybridized with an efficiency most comparable to the homologous *S. typhimurium* DNA, suggesting an extensive sequence homology for *gdhA* in these organisms. This not only makes further characterization of *gdhA* from these organisms of interest, but suggests that the *S. typhimurium gdhA* DNA may be a useful probe for isolating this gene from diverse organisms.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM25251 from the National Institute of General Medical Sciences, by grant PCM20882 from the National Science Foundation, and Research Career Development award GM00449 from the National Institute of General Medical Sciences to J.E.B. E.S.M. was a recipient of a National Institutes of Health predoctoral traineeship in Cellular and Molecular Biology (GM07211).

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