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In Vivo Cloning and Characterization of the *gabCTDP* Gene Cluster of *Escherichia coli* K-12

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The *gabCTDP* gene cluster, which specifies and regulates synthesis of the γ-aminobutyrate (GABA) transport carrier, of glutamate-succinic semialdehyde transaminase, and of succinic semialdehyde dehydrogenase, responsible for the uptake and metabolism of γ-aminobutyric acid in *Escherichia coli* K-12, was cloned in vivo, using the mini-Mu replicon bacteriophage Mu dI5086 as the vector. A subclone containing a 7.8-kilobase (kb) *Eco*RI-*Hin*dIII fragment complemented all of our Gab⁻ mutants. By restriction mapping, this DNA fragment was located at kb 2800.5 to 2808.5 on the physical map of the *E. coli* K-12 chromosome. A subclone containing a 1.8-kb *Eco*RI-*Sal*I fragment complemented the *gab*-repressed strain CS101A (wild-type *gabC*) but did not complement any *gab* structural gene mutants. The *gab* genes are divergently transcribed from promoters located in the vicinity of the unique *Bam*HI site. Transcription in both directions is under dual control of catabolite repression and nitrogen regulation. Using a procaryotic DNA-directed translation system, we observed three insert-coded polypeptide bands of 53 to 55, 45 to 48, and 40 to 43 kilodaltons (kDa). In vivo studies with subcloned fragments of the *gab* DNA identified the 53- to 55- and 45- to 48-kDa bands as products of the *Bam*HI-*Sal*I fragment and the 40- to 43-kDa band as the product of the *Eco*RI-*Sal*I fragment. An additional 26- to 28-kDa band was identified as the product of the *Bam*HI-*Hin*dIII fragment. Furthermore, the *Bam*HI-*Sal*I fragment was shown to specify synthesis of the two GABA enzymes, whereas synthesis of the GABA carrier was specified by the *Bam*HI-*Hin*dIII fragment. No catalytic function in addition to its regulatory role could be attributed to the *Eco*RI-*Sal*I gene product.

Laboratory strains of *Escherichia coli* K-12 (e.g., strain CS101A) are unable to utilize γ-aminobutyrate (GABA) as the sole source of nitrogen. Mutants capable of growth on GABA as the nitrogen source were isolated in this laboratory (4). Biochemical studies revealed that the mutation brought about a concomitant severalfold increase in the activities of a specific membrane-bound GABA transport carrier and two enzymes, glutamate-succinic semialdehyde transaminase (GSST) and succinic semialdehyde dehydrogenase (SSDH), catalyzing the conversion of GABA to succinate (4, 9, 20). The mutation, *gabC*, was mapped at 57.5 min on the *E. coli* K-12 chromosome. The structural genes of the GABA carrier (*gabP*), of SSDH (*gabD*), and of GSST (*gabT*) are closely linked to the regulatory locus *gabC* (13, 14). Genetic studies indicated that the *gab* genes are divergently transcribed from a promoter in the *gabD* region and that the *gabC* gene may specify a *gab* repressor (13).

Expression of the *gab* structural genes is repressed in a glucose-ammonia minimal medium and derepressed when succinate is the carbon source instead of glucose or when a poor nitrogen source (e.g., arginine, proline, or GABA itself) is used instead of ammonia (5, 23). It has been shown that glucose repression of GABA catabolism cannot be relieved by the addition of cyclic AMP and that the *gab* genes are derepressed in *cya* and *crp* mutants grown in succinate medium, conditions under which the *lac* genes are very poorly expressed (6, 21). We have further demonstrated that the activation of *gab* gene expression by nitrogen deprivation is mediated by the *gln* regulatory system (Ntr) (9, 23).

To study regulation of the *gab* system at the molecular level, it was first necessary to clone and subclone the *gab* DNA, and in particular its control region, for use in DNA sequencing and for in vivo and in vitro transcription and coupled transcription-translation studies.

This report describes the cloning and initial characterization of the *gab* gene cluster of the GABA-utilizing mutant *E. coli* K-12 CS101B.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. *E. coli* K-12 strains, plasmids, and phage used and plasmids constructed in this work are described in Table 1.

Media and growth conditions. Luria broth (LB) and MacConkey and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) indicator media were prepared as described by Miller (15). Minimal media were prepared from basal salts medium to which the appropriate carbon and nitrogen sources and other supplements were added, when required, at concentrations recommended by Miller (15). Bacteria were grown and harvested as described elsewhere (23).

Preparation of bacterial cell extracts. Extracts for use in enzyme assays were prepared as described previously (14).

Enzyme assays. GSST and SSDH activities were assayed within 2 h of extract preparation as described by Zaboura and Halpern (23). β-Galactosidase activity was measured in permeabilized cells as described by Miller (15). Galactokinase activity was determined by the following procedure. Cells from 0.5 ml of a log-phase culture (100 Klett units; no. 54 filter) were centrifuged and suspended in 20 μl of Gal buffer (50 mM Tris hydrochloride [pH 8.0], 1 mM dithiothreitol, 1 mM EDTA). A 20-μl amount of toluene was added, the suspension was vortexed, and 40 μl of lysis mix (6 mg of chloramphenicol, 2.5 mg of rifampin, 0.28 ml of 0.25 M EDTA, 0.2 ml of 14.2 M β-mercaptoethanol, 9.52 ml of H_2O) was added; the mixture was then incubated for 15 min at 32°C. A 50-μl sample of the lysed cell suspension was added

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TABLE 1. Bacterial strains, bacteriophage, and plasmids used or constructed

Strain, phage, or plasmid	Genotype or phenotype[a]	Source or reference
Strains		
CS101A	*metB*	Hfr Cavalli (1)
CS101B	*metB gabC*	14
S-5	*metB gabC gabP5*	14
M-16	*metB gabC gabD*	14
M-20	*metB gabC gabT*	14
CS101B252	*metB gabC* Δ(*srl*::Tn*10*)	13
CB454	*lacZ lacY*$^+$ *galK rpsL thi recA56*	19
UM109	*lacI*^{q} *hsdR17 mcrA*	17, 22
Phage		
Mu dI5086	Mu cts62 A^+ B^+ Km^r rep_{pMB1} *trp-lac('BAZYA)W205*	7
Plasmids		
pEG5086	pBCO::Mu dI5086 (17.2 kb)	7
pEG5086-2	Mu cts62 A^+ B^+ Km^r rep_{pMB1} *trp-lac('BAZYA)W205 gab('PDTC)CS101B* (30 kb)	Fig. 1
pEG50862-B	pEG50862 with *Bam*HI fragment removed (27 kb)	This work
pJN	Km^r Amp^r ori pBR322 (5 kb)	—[b]
pJN-1	pJN with ca. 13-kb *Eco*RI fragment from pEG5086-2 inserted (18 kb)	Fig. 3
pJN-2	As pJN-1 but *Eco*RI fragment inserted in opposite orientation (18 kb)	Fig. 3
pJN1-13	As pJN-1 but ca. 5-kb *Hin*dIII fragment including *tac* promoter deleted (13 kb)	Fig. 3
pJN1-B	As pJN-1 but ca. 9.5-kb *Bam*HI fragment including *tac* promoter and Km^r sequence deleted (8.5 kb)	Fig. 3
pJN113-B	As pJN1-13 but ca. 4-kb *Bam*HI fragment including Km^r sequence deleted (9 kb)	Fig. 3
pJN113-S	As pJN1-13 but ca. 7-kb *Sal*I fragment including Km^r sequence deleted (5.6 kb)	Fig. 3
pKK232-8	pKK, a pBR322 derivative, with a polylinker followed by a promoterless chloramphenicol resistance gene (5.1 kb)	3
pCB302a	Derived from pBR322, with promoterless genes *galK* and *lacZ* in opposite orientations and a polylinker sequence between them; Amp^r (7 kb)	18, 19
pCB302b	As pBC302a but with opposite orientation of polylinker sequence; Amp^r (7 kb)	18, 19

[a] rep, Replication; ori, origin of replication.
[b] J. J. Neitzel, Ph.D. thesis, California Institute of Technology, Pasadena, 1987.

to 50 μl of reaction mix (100 μl of 8 mM ATP, 60 μl of 32 mM NaF, 50 μl of D-[^{14}C]galactose [10 mM; 2 μCi/μmol]; 190 μl of Tris-$MgCl_2$ solution [200 mM Tris hydrochloride {pH 8.0}, 8 mM $MgCl_2$], 35 μl of lysis mix, 40 μl of H_2O) and incubated for 15 min at 32°C. A 50-μl amount of stop mix (0.1 M D-galactose, 0.01 M EDTA, 0.05 M Tris hydrochloride [pH 8.0]) was added, and the tubes were vortexed. Two samples of 40 μl from each reaction tube were spotted on DE81 Whatman filters. An additional two 40-μl samples of the complete reaction mixture were spotted (but not washed subsequently) for determination of total radioactivity. All filters except the latter two were washed three times with distilled water; all filters were dried and put in vials with 5 ml of scintillation fluid for counting of radioactivity. The results are expressed in counts per minute per sample after subtraction of the blank reading.

Transport assays. [^{14}C]GABA uptake by suspensions of nongrowing cells, in the presence of chloramphenicol, was measured as described previously (9) except that the concentrations of potassium phosphate and $MgSO_4$ were 6 and 0.1 mM, respectively; $(NH_4)_2SO_4$ was omitted from the uptake buffer.

DNA biochemistry. Restriction endonucleases and T4 ligase were obtained from New England BioLabs, Inc., U.S. Biochemical Corp., Pharmacia, and International Biotechnologies Inc. and used as specified by the manufacturers. Rapid minipreparations, large-scale plasmid DNA isolation, and other procedures were carried out as described by Maniatis et al. (12).

Transformation with plasmid DNA. The transformation procedure described by Maniatis et al. (12) was used. Usually the restriction-modification mutant strain JM109 was used in transformation to obtain better yields of transformants. In some experiments, Gab$^-$ mutants of CS101B were used directly as the primary recipients. Strain CB454 was used as the recipient when β-galactosidase and galactokinase activities were measured (18).

RESULTS

In vivo cloning. A gene library was prepared by infecting the GABA-utilizing strain CS101B with mini-Mu replicon phage Mu dI5806 (Fig. 1; see also Fig. 2 in reference 7). The heat-induced lysate of the Mu dI5806-infected CS101B was used to transduce the GABA transport-defective mutant S-5 (Table 2) to kanamycin resistance. Two thousand Km^r transductants were isolated and scored for the ability to utilize GABA as a nitrogen source. Three GABA-utilizing transductants were found; all of them yielded Mu dI5086-derived hybrid plasmids (pEG5086-1, pEG5086-2, and pEG5086-4). Transformation of mutant S-5 with pEG5086-1 or pEG5086-2 fully restored GABA transport activity (Fig. 2). pEG5086-2 was characterized by restriction mapping. It is 30 kilobases (kb) long and contains a 12.8-kb insert of bacterial DNA (Fig. 1). pEG5086-2 is rather unstable in its ability to complement Gab$^-$ mutants (Table 3).

Subcloning. Treatment of pEG5086-2 with *Bam*HI and religation resulted in the deletion of a ca. 3-kb *Bam*HI-*Bam*HI fragment. The self-religated *Bam*HI-treated pEG5086-2 plasmid lost the ability to complement mutant S-5, indicating that the *Bam*HI-*Bam*HI fragment was essential. A ca. 13-kb *Eco*RI-*Eco*RI fragment containing the essential *Bam*HI-*Bam*HI segment was cloned into the unique *Eco*RI site of plasmid pJN (Fig. 3). The pJN derived hybrid plasmids were used to transform strain S-5. Recombinant plasmids recovered from the Amp^r Km^r Gab$^+$ trans-

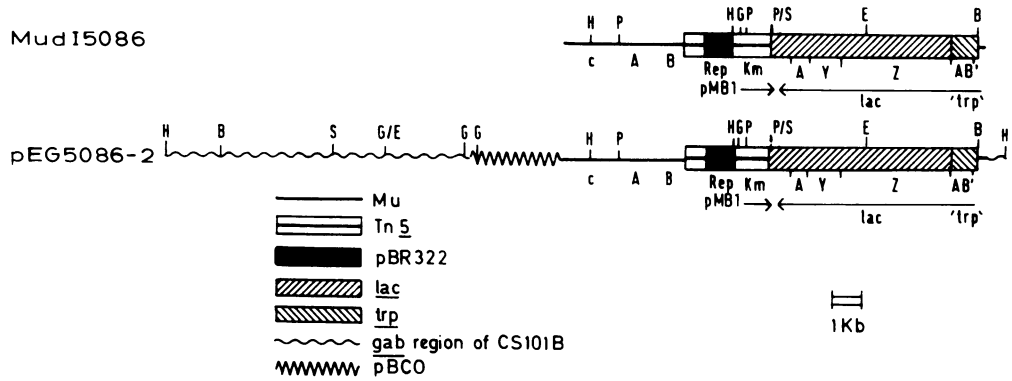

FIG. 1. Genetic and physical maps of the mini-Mu replicon phage Mu dI5086 (adapted from reference 7) and of its derivative, the *gab* DNA-containing hybrid plasmid pEG5086-2. Restriction sites: H, *Hin*dIII; P, *Pst*I; S, *Sal*I; E, *Eco*RI; G, *Bgl*II; B, *Bam*HI.

formants fell into two classes according to their *Hin*dIII restriction patterns: pJN-1, yielding 12- and 5.3-kb fragments, and pJN-2, yielding 9.5- and 7.8-kb fragments (Fig. 3). The ability of the two classes of plasmids, which integrated the *gab* DNA insert in opposite orientations, to complement all Gab$^-$ mutants (Table 4) leads to the conclusion that the ca. 13-kb insert contains its own promoter. Digestion with *Bam*HI and religation of pJN-1 yielded Amp^r Km^s plasmids that were unable to complement any of the GABA-nonutilizing mutants, in agreement with the abovementioned data showing that the *Bam*HI-*Bam*HI fragment of pEG5086-2 was essential for the complementation of mutant S-5.

*Hin*dIII digestion and religation of pJN-1 resulted in a recombinant plasmid, pJN1-13, lacking the *tac* promoter of pJN and the *Eco*RI-*Hin*dIII fragment of pEG5086-2, which contained all of the residual Mu DNA and a short sequence of the original bacterial DNA (*Bam*HI-*Hin*dIII fragment). pJN1-13 complemented all Gab$^-$ mutants (Table 4), and unlike its ancestor plasmid pEG5086-2, it was very stable. The restriction map of pJN1-13 is given in Fig. 3. The 7.8-kb *Eco*RI-*Hin*dIII bacterial insert has no internal *Eco*RI and *Hin*dIII sites and no *Pst*I, *Xba*, *Xho*, *Cla*, and *Bgl*II sites; there are unique *Bam*HI, *Sal*I, and *Pvu*II sites, two *Dra*I sites, and four *Bgl*I sites. The restriction map of this fragment is identical to that of the fragment extending from kb 2800.5 to 2808.5 on the physical map of the *E. coli* K-12 chromosome (57.5 min) (11).

Digestion of pJN1-13 with *Sal*I and religation resulted in deletion of the *Hin*dIII-*Sal*I fragment from the bacterial insert. The Amp^r Km^s 5.6-kb plasmid carried only the ca. 1.8-kb *Eco*RI-*Sal*I fragment of *gab* DNA. The pJN113-S plasmid complemented strain CS101A, enabling it to utilize GABA as a nitrogen source, but did not complement any of the other Gab$^-$ mutants (Fig. 3 and Table 4). Isolation of Amp^r Km^s transformants after *Bam*HI digestion and religation of pJN1-13 yielded a ca. 9-kb plasmid, pJN113-B, which lacked the *Hin*dIII-*Bam*HI bacterial DNA fragment. pJN113-B was unable to complement any of the Gab$^-$ mutants. The *Bam*HI-*Sal*I fragment, when introduced into plasmids pCB302a and pCB302b, complemented mutant M-20, enabling it to utilize GABA as a source of nitrogen. However, no complementation of any other Gab$^-$ mutant was observed. Fragment *Hin*dIII-*Bam*HI introduced into plasmid pCB302b was unable to complement any of the Gab$^-$ mutants (Table 4).

Analysis of *gab* DNA control sequences. *Hin*dIII-*Sal*I and *Hin*dIII-*Bam*HI *gab* DNA restriction fragments were cloned in one orientation (*Bam*HI→*Hin*dIII; *Sal*I→*Hin*dIII) in front of the promoterless chloramphenicol resistance gene of plasmid pKK232-8 (3). In both cases, transformants resistant to 50 μg of chloramphenicol per ml of LB medium were obtained; cultures transformed with the parental plasmid, with no *gab* DNA insert, showed no Cm^r colonies even at a chloramphenicol concentration as low as 5 μg/ml.

Earlier genetic experiments indicated that the *gab* gene cluster was divergently transcribed from a control region adjacent to *gabD* (13). We therefore cloned *Hin*dIII-*Sal*I, *Hin*dIII-*Bam*HI, and *Bam*HI-*Sal*I *gab* DNA fragments into a polylinker inserted in two orientations between a pair of promoterless indicator genes, *lacZ* and *galK*, according to Schneider and Beck (18, 19). Since the indicator genes are

TABLE 2. Properties of *gab* mutants[a]

Strain	Genotype	Use of GABA as nitrogen source	Sp act[b]		GABA transport activity (V_{max})[b]
			SSDH	GSST	
CS101A	*metB*	−	36	60	0.15
CS101B	*metB gabC*	+	1,264	601	1.39
S-5	*metB gabC gabP5*	−	797	608	0
M-16	*metB gabC gabD*	−	0	79	0.35
M-20	*metB gabC gabT*	−	1,080	85	1.39
CS101B252	*metB gabC* Δ(*srl*::Tn*10*)	−	651	359	0

[a] Bacteria were grown in 1% succinate–0.1% ammonia minimal medium supplemented with L-methionine (50 μg/ml). The cultures were harvested in mid-log phase (turbidity of 100 Klett units; no. 54 filter). Preparation of extracts for enzyme assays, determination of SSDH and GSST activities, and GABA transport measurements were done as described previously (9, 23).

[b] Nanomoles per milligram of protein per minute.

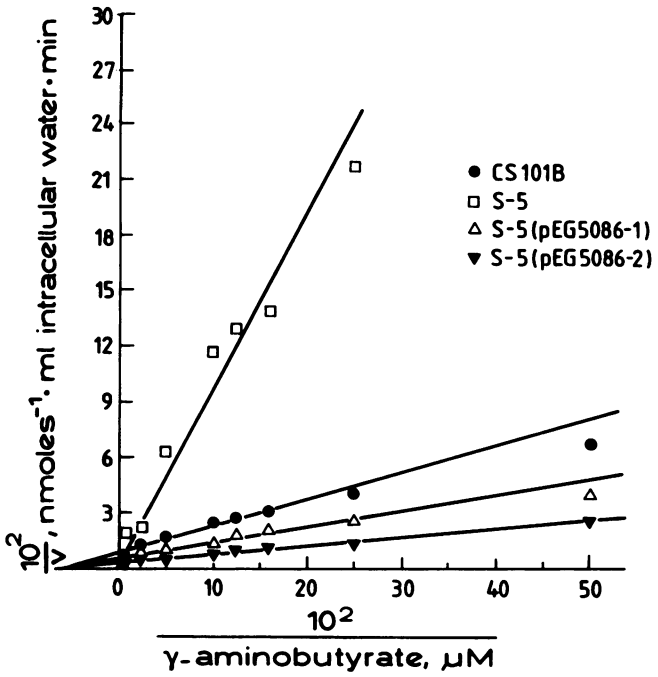

FIG. 2. Restoration of GABA uptake in GABA transport-deficient mutant S-5 transformed with hybrid plasmids carrying *gab* DNA from the GABA-utilizing mutant CS101B. The cultures were grown for three to four generations in glycerol-ammonia minimal medium. Kanamycin (50 μg/ml) was added to transformant cultures. GABA transport by cell suspensions was measured as described previously (9).

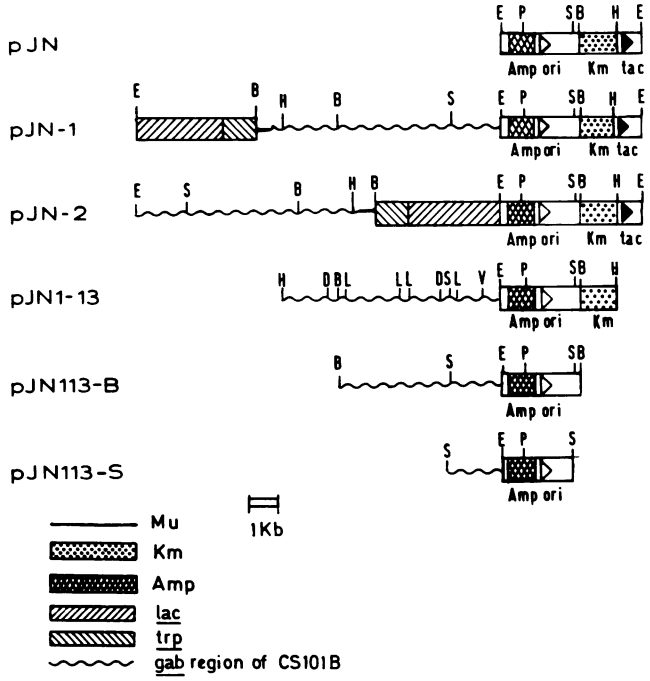

FIG. 3. Genetic and physical maps of plasmids carrying *gab* DNA from the GABA-utilizing mutant CS101B. Cleavage sites for restriction endonucleases are as in Fig. 1 except for V (*Pvu*II), L (*Bgl*I), U (*Pvu*I), and D (*Dra*I). Amp, Ampicillin; Km, kanamycin.

preceded by ribosome-binding sites and translation initiation codons but no promoter sequences, the level of expression of the genes should reflect the strength of the promoters in the *gab* DNA insert. To check whether the cloned promoters are under dual control by catabolite repression and nitrogen regulation as is the GABA catabolic pathway, strain CB454 carrying the different hybrid plasmids was grown under different conditions of catabolite repression (succinate or glucose as a carbon source), and nitrogen availability (ammonium sulfate or L-arginine as a nitrogen source) and β-galactosidase and galactokinase activities were measured. The *Bam*HI-*Hin*dIII segment had promoter activity in the *Bam*HI→*Hin*dIII direction only; the *Bam*HI-*Sal*I segment exhibited no promoter activity in either orientation, and the *Hin*dIII-*Sal*I fragment, consisting of both segments, had promoter activity in both directions (Table 5). The most plausible interpretation of these findings is that the two divergently transcribing promoters start on the *Hin*dIII side of *Bam*HI. The promoter that transcribes in the *Bam*HI→*Hin*dIII direction resides entirely within the *Bam*HI-*Hin*dIII segment, whereas the promoter that transcribes in the *Sal*I direction spans the *Bam*HI recognition site. Both promoters are subject to catabolite repression and nitrogen regulation (Ntr). To rule out the possibility that the apparent differences in promoter activities manifested by cells grown in the different media were merely due to differences in plasmid copy number, we normalized the β-galactosidase and galactokinase activities to that of the

TABLE 3. Complementation of Gab$^-$ mutants by three pEG5086-*gab* hybrid plasmids[a]

Recipient	No. of Gab$^+$ transformants/no. of Km^r transformants checked[b]			
	pEG5086	pEG5086-1	pEG5086-2	pEG5086-4
CS101A	0/16	2/5	5/8	1/11
M-16	0/19	1/6	3/5	1/12
M-20	0/17	4/13	3/5	10/23
CS101B252	0/12	10/12	10/12	5/12

[a] The Km^r transformants were streaked onto minimal medium agar plates with 0.5% glycerol as the source of carbon and 0.2% GABA as the source of nitrogen, supplemented with L-methionine (50 μg/ml).

[b] A high proportion of the recombinant plasmids lost the ability to complement Gab$^-$ mutants because of the loss of integrated bacterial DNA.

TABLE 4. Complementation of Gab$^-$ mutants by pJN and its derivatives and by pBC302 carrying *gab* DNA inserts[a]

Plasmid	*gab* DNA insert[b]	Growth				
		CS101A	M-16	M-20	S-5	CS101B252
pJN		−	−	−	−	−
pJN-1	E-H	+	+	+	+	+
pJN-2	H-E	+	+	+	+	+
pJN1-13	E-H	+	+	+	+	+
pJN113-S	E-S	+	−	−	−	−
pJN113-B	E-B	−	−	−	−	−
pBC302b *Hin*dIII-*Bam*HI		−	−	−	−	−
pBC302a *Bam*HI-*Sal*I		−	−	+	−	−
pBC302b *Bam*HI-*Sal*I		−	−	+	−	−

[a] The Amp^r transformants were streaked onto minimal medium agar plates with 0.5% glycerol as the carbon source and 0.2% GABA as the nitrogen source, supplemented with L-methionine (50 μg/ml). After 24 h of incubation at 37°C, the plates were examined for growth; + indicates growth on GABA as the nitrogen source of 10 of 10 Amp^r transformants checked.

[b] E-H, *Eco*RI-*Hin*dIII fragment; H-E, *Hin*dIII-*Eco*RI fragment; E-S, *Eco*RI-*Sal*I fragment; E-B, *Eco*RI-*Bam*HI fragment.

TABLE 5. Analysis of promoter activity in the *gab* DNA region of the *E. coli* K-12 chromosome

Plasmid[a]	Activity[b]					
	SN		GN		GA	
	β-Galactosidase	Galactokinase	β-Galactosidase	Galactokinase	β-Galactosidase	Galactokinase
pCB302a::H-B	150	0	27	0	213	85
pCB302a::H-S	28	5,299	0	2,119	92	8,242
pCB302a::B-S	0	0	0	0	0	0
pCB302b::H-B	0	2,850	0	1,032	0	2,785
pCB302b::H-S	205	90	25	0	452	350
pCB302b::B-S	0	0	0	0	0	0

[a] H-B, *Hin*dIII-*Bam*HI fragment of *gab* DNA; H-S, *Hin*dIII-*Sal*I fragment; B-S, *Bam*HI-*Sal*I fragment. The polylinkers present in the two plasmids had different restriction sites in the following order, from left to right (from *galK* to *lacZ*): pCB302a, *Sma*I-*Bam*HI-*Sal*I-*Pst*I-*Bgl*II-*Xba*I-*Hin*dIII-*Eco*RI; pCB302b, *Sma*I-*Hin*dIII-*Bgl*II-*Pst*I-*Sal*I-*Bam*HI-*Eco*RI. Strain CB454 with *lacZ* deleted and a *gal*K mutation in the chromosome was used as the bacterial host.

[b] The bacteria were grown in minimal medium with carbon and nitrogen sources as indicated: SN, 1% succinate–0.1% ammonia; GN, 0.5% glucose–0.1% ammonia; GA, 0.5% glucose–0.2% arginine. β-Galactosidase activity was measured as described by Miller (15). Galactokinase activity was measured as described in Materials and Methods. The values obtained were normalized to those of the plasmid-directed β-lactamase, the activity of which in the glucose-ammonia-grown cells was considered as 100%.

plasmid-borne β-lactamase, which does not respond to catabolite repression and nitrogen regulation and has activity directly proportional to plasmid copy number (10).

***gab* gene expression.** Plasmids pJN-1, pJN-2, and pJN1-13 were used as templates with a procaryotic DNA-directed translation kit. Three *gab*-specific proteins with molecular sizes of 53 to 55, 45 to 48, and 40 to 43 kilodaltons (kDa) were observed upon electrophoresis on sodium dodecyl sulfate-polyacrylamide (17.5%) gels (Fig. 4). Figure 5 shows an electropherogram of proteins synthesized in vivo by CS101B cultures bearing plasmids with different *gab* DNA inserts. Altogether, four *gab* DNA-specific proteins were formed, three of which were identical in size to the *gab* DNA-specific in vitro products discussed above. Two of them, the 53- to 55- and 45- to 48-kDa bands, were specified by the *Bam*HI-*Sal*I *gab* DNA fragment. The 40- to 43-kDa polypeptide was specified by the *Eco*RI-*Sal*I fragment, and the fourth polypeptide, of 27 to 28 kDa, was the product of the *Bam*HI-*Hin*dIII fragment (Fig. 5). The two products of the *Bam*HI-*Sal*I fragment were GSST and SSDH, and the product of the *Bam*HI-*Hin*dIII fragment was the GABA-specific carrier (Table 6).

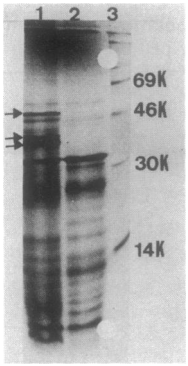

FIG. 4. Electropherogram of *gab* gene products from an in vitro *gab* DNA-directed translation system. The S-30 in vitro translation kit from Amersham Corp. was used with pJN and pJN-2 DNA as templates (see Fig. 3). The proteins were labeled with L-[^{35}S]methionine and analyzed on sodium dodecyl sulfate-polyacrylamide (17.5%) gels. Lanes: 1, pJN-2; 2, pJN; 3, protein molecular size markers. K, Kilodaltons.

DISCUSSION

We have cloned the *gab* gene cluster of the GABA-utilizing strain *E. coli* K-12 CS101B, using the in vivo cloning method of Groisman and Casadaban (7). The hybrid plasmids obtained were unstable (Table 3) because of the presence of Mu. When Mu was deleted, as in plasmid pJN1-13, stabilization of the clones was achieved. By subcloning the *gab* DNA insert, we established that the smallest fragment that still retained the ability to complement all of our *gab* mutants was the 7.8-kb *Eco*RI-*Hin*dIII bacterial insert in pJN1-13. By restriction mapping, this DNA fragment was located between kb 2800.5 and 2808.5 on the physical map of the *E. coli* K-12 chromosome (11). The regulatory *gabC* gene seems to be located in the ca. 1,800-base-pair *Eco*RI-*Sal*I fragment, since a plasmid carrying this CS101B DNA fragment alone suffices to endow strain CS101A with the ability to grow on GABA (Table 4), simultaneously enhancing the

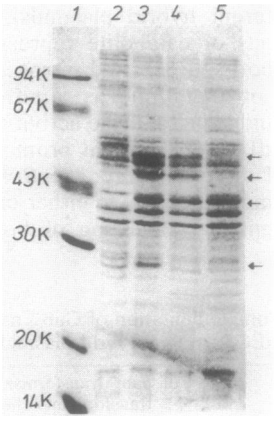

FIG. 5. Electropherogram of *gab* gene products made in vivo by cultures of CS101B bearing pJN hybrid plasmids carrying different fragments of *gab* DNA. The cultures were grown to mid-log phase (100 Klett units) in 1% succinate–0.1% ammonia minimal medium supplemented with L-methionine (50 μg/ml). A 1.5-ml culture sample was centrifuged and suspended in 120 μl of cracking buffer; 20 μl was applied to sodium dodecyl sulfate-acrylamide gels and electrophoresed as described in the legend to Fig. 4. The gels were stained with Coomassie blue. Lanes: 1, mixture of molecular size markers as indicated (K, kilodaltons); 2, pJN; 3, pJN1-13; 4, pJN113-B; 5, pJN113-S. Arrows indicate the *gab*-specific proteins.

TABLE 6. Effects of plasmids with different *gab* DNA inserts from strain CS101B on GABA transport, SSDH, and GSST activities of different *gab* mutants[a]

Mutant	Determination (nmol/mg of protein per min) with given plasmid and insert																	
	No plasmid			pCB302b, *Hin*dIII-*Bam*HI			pCB302b, *Bam*HI-*Sal*I			pJN113-B, *Eco*RI-*Bam*HI			pJN113-S, *Eco*RI-*Sal*I			pJN113, *Eco*RI-*Hin*dIII		
	Sp act		GABA transport activity (V_{max})	Sp act		GABA transport activity (V_{max})	Sp act		GABA transport activity (V_{max})	Sp act		GABA transport activity (V_{max})	Sp act		GABA transport activity (V_{max})	Sp act		GABA transport activity (V_{max})
	GSST	SSDH		GSST	SSDH		GSST	SSDH		GSST	SSDH		GSST	SSDH		GSST	SSDH	
CS101A	270	71	0.54	266	82	0.41	764	1216	0.51	2,531	5,186	0.27	327	387	3.12	1,450	1,737	>10
M-16	112	24	0.41	158	28	1.27	586	807	0.34	2,326	2,171	0.28	ND[b]	ND	0.47	1,797	ND	>10
M-20	84	507	8.10	70	484	9.94	484	1051	3.41	3,137	4,270	2.41	86	460	9.09	1,525	2,199	>10
S-5	523	389	0	426	459	1.23	430	540	0	3,605	1,171	0	395	468	0	1,459	983	>10
CS101B	572	713	4.54	410	784	9.51	636	557	6.82	3,068	4,155	2.98	371	464	5.60	1,828	4,258	>10

[a] The cultures were grown to a turbidity of 100 Klett units in minimal 1% succinate–0.1% ammonia medium supplemented with L-methionine (50 μg/ml).
[b] ND, Not determined.

activities of GABA transport, SSDH, and GSST (Table 6), but it cannot complement any of the Gab$^-$ structural gene mutants. The *Hin*dIII-*Sal*I fragment, which contains the *gab* structural genes, has divergently transcribing promoters. The promoter that transcribes in the *Hin*dIII→*Sal*I direction contains the unique *Bam*HI site, whereas the other promoter, transcribing in the opposite direction, is located within the *Bam*HI-*Hin*dIII segment. Data on GABA transport, SSDH, and GSST activities of different *gab* mutants bearing plasmids with *gab* DNA inserts of varying length (Table 6) clearly show that the 4-kb *Bam*HI-*Sal*I DNA segment codes for the two enzymes of the GABA utilization pathway, GSST and SSDH, and that the 2-kb *Bam*HI-*Hin*dIII segment contains the structural gene for the GABA carrier. It is of interest that the promoter transcribing in the *Bam*HI→*Hin*dIII direction is much stronger in the plasmid containing the *Bam*HI-*Hin*dIII fragment, which shows no promoter activity in the other direction, than in the plasmid with the *Sal*I-*Hin*dIII fragment, which shows transcription in both directions. Such competition between divergently transcribing overlapping promoters has been described previously (2).

Both promoters are under dual control of catabolite repression and of the Ntr system responding to nitrogen availability (Table 5). This dual control faithfully reflects the regulatory pattern of the GABA utilization pathway (21, 23).

Table 6 discloses two very interesting findings: (i) the presence of a plasmid carrying the *gabC*, *gabT*, and *gabD* genes but lacking the *gabP* gene (pJN113-B) resulted in much higher activities of SSDH and GSST than did the presence of a plasmid carrying the entire *gab* region (pJN-113), and (ii) the presence of plasmid pJN113-S, which carries only the mutated *gabC* gene, greatly enhanced the GABA transport activity of strain CS101A, whereas plasmid pJN113-B, which carries *gabT* and *gabD* in addition to *gabC*, actually reduced even further the low GABA transport activity of strain CS101A. Both of these phenomena may be due to competition for the Ntr activator (NRI [8, 16]) required for transcription by both diverging *gab* promoters. Titration of NRI may also explain the observation that only plasmids carrying either the *gabC* gene alone (*Eco*RI-*Sal*I) or the entire *gab* region (*Eco*RI-*Hin*dIII), but not a plasmid carrying the *Eco*RI-*Bam*HI fragment, complemented strain CS101A, enabling it to grow on GABA as the major nitrogen source.

The presence of plasmid pJN113-B, carrying the structural genes of both GSST and SSDH as well as the *gabC* allele from CS101B, resulted in a much greater increase in the synthesis of GSST and SSDH in CS101A and in mutants M-16 and M-20 than did the presence of plasmid pCB302b carrying only the *Bam*HI-*Sal*I fragment (*gabD* and *gabT*). Although we have not ruled out differences in copy number, this explanation is not very likely, since both plasmids pJN and pCB302 are derivatives of pBR322. It is possible that an NRI-binding sequence is located in the *Eco*RI-*Sal*I fragment near the *Sal*I site and that in its absence (as in pCB302b *Bam*HI-*Sal*I), the expression of *gabD* and *gabT* is not fully activated.

Finally, the presence of plasmid pJN113-S, carrying the mutated *gabC* allele from CS101B, pleiotropically enhanced the expression of all three chromosomal *gab* structural genes of the *gab*-repressed parental strain CS101A (wild-type *gabC*) but did not complement the mutated *gab* structural genes in strains M-16, M-20, and S-5 (Table 6). These findings and the fact that the *Eco*RI-*Sal*I fragment (*gabC*) codes for a protein (Fig. 5) are in agreement with earlier findings indicating that *gabC* may specify a *gab* repressor. The multiple copies of mutated *gabC* product made by the plasmid *gabC* allele possibly inactivate the *gab* repressor made by the chromosomal allele of *gabC* as a result of subunit mixing.

Using an S-30 in vitro translation system, we obtained three *gab*-specific polypeptides with molecular sizes of 53 to 55, 45 to 48, and 40 to 43 kDa. Further in vivo experiments with subcloned fragments of the *gab* DNA of CS101B showed that the larger two of these three polypeptides were the products of the GSST and SSDH structural genes located in the *Bam*HI-*Sal*I fragment, whereas the 40- to 43-kDa polypeptide was specified by the *Eco*RI-*Sal*I fragment, probably as the product of the *gabC* regulatory gene. The fourth *gab* DNA-coded protein obtained in vivo was specified by the *Bam*HI-*Hin*dIII *gab* DNA fragment and probably is the GABA carrier (*gabP*).

We are now in the process of sequencing the *gab* DNA, starting with the putative control region on both sides of the *Bam*HI restriction site.

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