

Cloning, Expression, and Purification of Glutamine Synthetase from *Clostridium acetobutylicum*

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A glutamine synthetase (GS) gene, *glnA*, from the gram-positive obligate anaerobe *Clostridium acetobutylicum* was cloned on recombinant plasmid pHZ200 and enabled *Escherichia coli* *glnA* deletion mutants to utilize $(\text{NH}_4)_2\text{SO}_4$ as a sole source of nitrogen. The cloned *C. acetobutylicum* gene was expressed from a regulatory region contained within the cloned DNA fragment. *glnA* expression was subject to nitrogen regulation in *E. coli*. This cloned *glnA* DNA did not enable an *E. coli* *glnA ntrB ntrC* deletion mutant to utilize arginine or low levels of glutamine as sole nitrogen sources, and failed to activate histidase activity in this strain which contained the *Klebsiella aerogenes hut* operon. The GS produced by pHZ200 was purified and had an apparent subunit molecular weight of approximately 59,000. There was no DNA or protein homology between the cloned *C. acetobutylicum* *glnA* gene and GS and the corresponding gene and GS from *E. coli*. The *C. acetobutylicum* GS was inhibited by Mg^{2+} in the γ -glutamyl transferase assay, but there was no evidence that the GS was adenylylated.

Clostridium acetobutylicum is a gram-positive obligate anaerobe which sporulates and produces acetone and butanol from a variety of carbohydrate substrates. Nitrogen levels have been shown to be important for solventogenesis and endospore formation (18). In the nitrogen metabolism of bacteria, glutamine synthetase (GS) (EC 6.3.1.2) plays a central role as it catalyzes one of the main reactions by which ammonia is assimilated (39): $\text{L-glutamate} + \text{NH}_4^+ + \text{ATP} \rightarrow \text{L-glutamine} + \text{ADP} + \text{P}_i$.

The regulation of transcription of the structural gene for GS, *glnA*, has been extensively studied in the gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*. In these bacteria transcription of *glnA* is regulated by the products of the *ntrB* (*glnL*) and *ntrC* (*glnG*) genes, which are linked to the *glnA* gene, as well as by the product of the unlinked *ntrA* (*glnF*) gene (11, 16, 20, 23, 27). However, there is no evidence for regulatory *ntr* genes in the gram-positive aerobic sporulating bacterium *Bacillus subtilis* (9, 12, 20), and GS seems to play a role in regulating its own synthesis (6, 30).

Because nitrogen metabolism and its regulation are important for solvent production and sporulation in *C. acetobutylicum*, we cloned, purified, and investigated the regulation of GS as part of a research program aimed at elucidating the molecular biology of this potentially important industrial bacterium.

MATERIALS AND METHODS

Plasmids, bacteria, and growth conditions. The plasmids and bacterial strains used in this study are listed in Table 1. pEcoR251, a gift from M. Zabeau, Plant Genetic Systems, Ghent, Belgium, is a positive selection vector containing the *E. coli* *EcoRI* gene under the control of the lambda rightward promoter, the ampicillin resistance gene, and the pBR322 origin of replication. It was derived from the pCL plasmids described by Zabeau and Stanley (41). The *EcoRI* gene product, which is expressed at high levels by the lambda promoter on pEcoR251, is lethal unless insertional inacti-

vated or regulated by plasmid pC₁₈₅₇ which contains a temperature-sensitive lambda repressor gene (28). The *EcoRI* gene has a single *Bgl*II cloning site. *C. acetobutylicum* P262 was grown from heat-shocked spores to mid-

TABLE 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant genotype	Reference or source
<i>E. coli</i>		
SF1001	(<i>glnA-ntrC</i>)2000	30
	<i>glnE1::Tn5</i>	
HB101	<i>leuB6 trpE38 metE70</i>	21
	<i>recA13 supE44</i>	
YMC10	Wild type	2
YMC11	(<i>glnA-ntrC</i>)2000	2
ET8051	(<i>rha-glnA</i>) <i>hutC_k</i> ^a	27
<i>C. acetobutylicum</i>		14
P262		
Plasmids		
pEcoR251	<i>E. coli</i> -positive selection vector	M. Zabeau, Plant Genetic Systems, Ghent, Belgium
pHZ200	pEcoR251 derivative containing the <i>C. acetobutylicum</i> <i>glnA</i> gene	This study
pHZ200-2	pHZ200 derivative containing the <i>Bgl</i> II DNA fragment in the reverse orientation	This study
pHZ201-203	Deletion derivatives of pHZ200	This study
pHZ212	Internal <i>EcoRI</i> fragment from pHZ200 cloned into the <i>EcoRI</i> site of pBR325	This study
p804	pBR322 derivative containing an <i>E. coli</i> <i>glnA</i> gene	38

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^a *hutC_k*, High basal level of histidase.

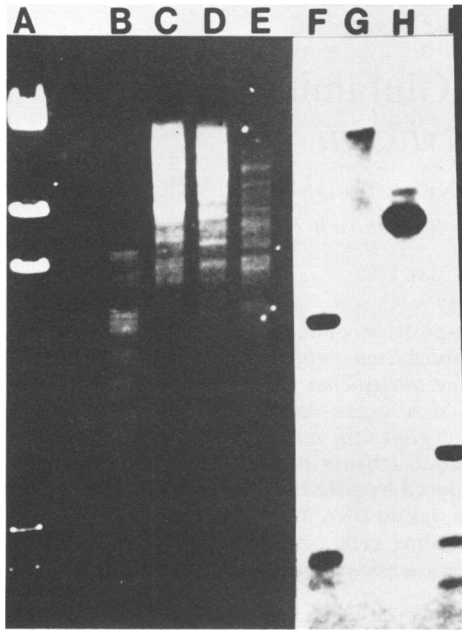


FIG. 1. Hybridization of ^{32}P -labeled pHZ200 with *C. acetobutylicum* DNA. Lane A, Lambda DNA digested with *Hind*III; lanes B through E, ethidium bromide-stained gel containing various restriction enzyme digestion products of *C. acetobutylicum* DNA (lane B, *Hind*III; lane C, *Pst*I; lane D, *Bgl*II; lane E, *Eco*RI); lanes F through H, autoradiogram of lanes B through E hybridized with ^{32}P -labeled pHZ200.

log phase in buffered *Clostridium* basal medium (25). The *E. coli* strains were grown in Luria medium (24) and glucose minimal medium (24), with either $(\text{NH}_4)_2\text{SO}_4$ (1 g/liter) or 20 mM glutamine as the sole nitrogen source.

Preparation of DNA. Plasmid DNA was prepared by the alkali-lysis method of Ish-Horowicz and Burke (13). Total cellular DNA from *C. acetobutylicum* P262 was prepared by a modification of the method of Marmur (22). *C. acetobutylicum* cultures (1 liter) were harvested at an optical density of 600 nm of 0.5 to 0.6 units, and protoplasts were produced from the cells as described by Allcock et al. (1). Sodium dodecyl sulfate (SDS) was added to the protoplasts to give a final concentration of 1%, and the lysed culture was extracted twice with TE (10 mM Tris hydrochloride [pH 8.0], 1 mM Na_2EDTA)-saturated phenol and twice with water-saturated ether. The lysate was then exhaustively dialyzed against TE at 4°C.

Construction of a *C. acetobutylicum* genomic library and the isolation of the *C. acetobutylicum glnA* gene. *C. acetobutylicum* DNA was partially digested with *Sau*3A and fractionated on a sucrose density gradient. Fragments of 4 to 7 kilobase pairs (kbp) in size were ligated with *Bgl*II-digested pEcoR251 and used to transform *E. coli* HB101 competent cells. Ampicillin-resistant transformants were pooled in lots of 500 each. Plasmid DNA was extracted from each of these pools and used to transform *E. coli* ET8051. Selection for *glnA* gene expression was carried out on minimal agar containing $(\text{NH}_4)_2\text{SO}_4$ (1 g/liter).

DNA hybridization. Total cellular DNA from *C. acetobutylicum* was digested with a variety of restriction enzymes, resolved by electrophoresis on 0.7% agarose Tris acetate gels, and transferred to nitrocellulose paper (BA85;

Schleicher & Schuell, Inc., Keene, N.H.) by the method of Smith and Summers (32). Probes were prepared by nick translation with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ as described by Rigby et al. (29).

Enzyme assays. Assays for GS activity were done by the γ -glutamyl transferase (γ -GT) assay described by Shapiro and Stadtman (31) and the biosynthetic reaction described by Bender et al. (3). Histidase activity was assayed by the method of Smith et al. (33). β -Lactamase was assayed by the microiodometric assay described by Sykes and Nordström (36). Protein was determined by the method of Lowry et al. (19).

In vitro transcription-translation. Plasmid DNA or gel-purified DNA fragments (7) were transcribed and translated in an *E. coli* DNA-directed translation system (N.380) supplied by Amersham, England and used according to the instructions of the manufacturer.

Purification of GS. The purification of the *C. acetobutylicum* GS encoded by pHZ200 was achieved by a modification of the method of Streicher and Tyler (35). Washed cells from a late-exponential-phase culture (1 liter) of ET8051 (pHZ200) were suspended in 10 ml of imidazole (IM) buffer (20 mM [pH 7.5], containing 10 mM MgCl_2 , 2 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). The preparation was sonicated, and the cellular debris was removed by centrifugation at $16,000 \times g$ for 45 min. The supernatant was subjected to two cycles of centrifugation at $100,000 \times g$ for 60 and 120 min. The final pellet (P1) was suspended in 10 ml of IM buffer and centrifuged at $100,000 \times g$ for an additional 120 min. The resultant pellet (P2) was suspended in 1 ml of IM buffer. The P2 fraction was heated at 55°C for 10 min and then cooled in an ice bath. Denatured proteins were removed by centrifugation at $12,000 \times g$ for 15 min to yield the supernatant fraction S2. The S2 fraction was passed through a Sephadex G-100 column, and GS was eluted in the void volume (E1). The E1 fraction was chromatographed on a Sephadex G-200 column with IM buffer as the column buffer, yielding fraction C1. The cloned *C. acetobutylicum* GS was also purified by the method of Thomas and Durbin (37).

Preparation of antibodies. Rabbits were immunized by weekly subcutaneous injections of 100 μg of the partially purified protein (fraction C1), together with 500 μl of Freund adjuvant. Serum was obtained after 5 weeks and preadsorbed with an *E. coli* ET8051 cell extract.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the methods of Laemmli (17) and O'Farrell (26). Proteins from crude extracts were used at 45 μg per lane, while 15 μg of purified sample was used per lane. Radioactively labeled proteins were loaded at 20,000 cpm per lane. Molecular weight markers labeled with ^{35}S by use of the ^{35}S sulfur labeling reagent (Amersham) were used with radioactive samples.

Western blotting. Polypeptides resolved by SDS-PAGE were electroblotted onto nitrocellulose by the procedure outlined in the Genescreen Instruction Manual (NEF-972; New England Nuclear Corp., Boston, Mass.). Detection of the antigen was carried out by the procedure outlined in the Genescreen manual.

Electron microscopy. Purified GS was fixed with 3% (wt/vol) glutaraldehyde, stained with 2% (wt/vol) uranyl acetate, and observed with an electron microscope (EM109; Zeiss).

SVP digestion. Snake venom phosphodiesterase (SVP) digestion was carried out on the partially purified enzyme (fraction C1) as described by Shapiro and Stadtman (31),

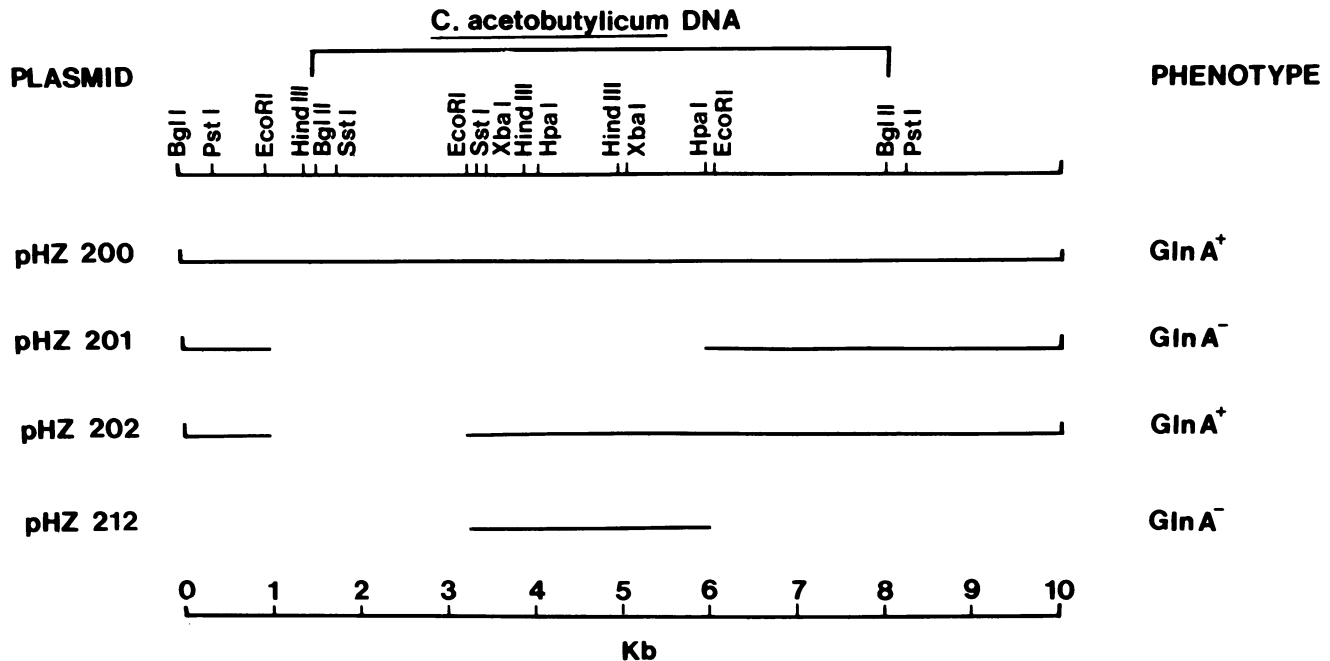


FIG. 2. Restriction and deletion map of pHZ200.

using purified adenylylated *Vibrio alginolyticus* GS as a positive control (5).

RESULTS

Cloning of the *C. acetobutylicum glnA* gene in *E. coli*. A library of *C. acetobutylicum* P262 DNA was established in *E. coli* HB101 by insertional inactivation of the *EcoRI* gene of pEcoR251. Plasmid DNA prepared from pools of clones containing *C. acetobutylicum* DNA was used to transform the *E. coli* ET8051 *glnA*⁻ *nrB*⁻ *nrC*⁻ deletion strain. Four transformants from one pool complemented the *glnA*⁻ phenotype in *E. coli* ET8051. Plasmid DNA was prepared from these colonies and was used to retransform *E. coli* ET8051 and YMC11. All the plasmids isolated from the *glnA*⁺ Ap^r transformants had inserts of the same size and the same *PstI* digestion profile.

One plasmid was chosen for further study. This plasmid, designated pHZ200, was capable of directing the synthesis of a GS enzyme which was active in both the γ -GT and the glutamine biosynthetic assays. *E. coli glnA*⁻ strains containing this plasmid grew in minimal medium containing 1 g of (NH₄)₂SO₄ per liter at the same rate as *E. coli* YMC10, which is the wild type with respect to the *glnA* gene (generation time of 120 min). Under nitrogen-limiting conditions (15 mM glutamate as sole nitrogen source), ET8051(pHZ200) had a doubling time of 190 min compared with 325 min for YMC10.

DNA homology. The origin of the 6.5-kbp insert in pHZ200 was determined by Southern blotting and DNA hybridization with ³²P-labeled pHZ200 (Fig. 1). A strong positive signal was obtained with various fragments in restriction digests of *C. acetobutylicum* DNA. A single high-molecular-weight band was obtained in the case of *PstI* and *BglII* digests (Fig. 1, lanes G and H), while multiple fragments, indicative of internal recognition sites, were apparent in *HindIII* and *EcoRI* digests (Fig. 1, lanes F and I). Even when the hybridization was done under conditions of low stringency (<20%), no DNA homology was detected in DNA

hybridization experiments between the insert contained in pHZ200 and the insert in plasmid p804, which contained the *E. coli glnA* gene.

Restriction endonuclease mapping of the cloned *C. acetobutylicum* DNA fragment. The restriction endonuclease map of pHZ200 was obtained by complete single or double digestions with a variety of restriction endonucleases (Fig. 2). The localization of the *glnA* gene was determined by the isolation and characterization of pHZ200 deletion plasmids (Fig. 2). Analysis of the deletion plasmids pHZ201, pHZ202, and pHZ212 indicates that the *C. acetobutylicum glnA* gene is located in a central position in the 6.5-kbp insert of *C. acetobutylicum* DNA in pHZ200.

Control of expression of the cloned *glnA* gene. The 6.5-kbp *C. acetobutylicum* DNA insert contained in pHZ200 was recloned in pEcoR251 in the opposite orientation (pHZ200-2), and the orientation was confirmed by restriction mapping. *E. coli* ET8051(pHZ200) and ET8051(pHZ200-2) exhibited the same levels of γ -GT activity under all growth conditions.

pHZ202 was an *EcoRI* deletion plasmid which still exhibited GS activity. In pEcoR251 the rightward lambda promoter controlling the expression of the *EcoRI* gene was situated on an *EcoRI-HindIII* fragment. This *EcoRI-HindIII* region was deleted in pHZ202, and it is concluded that the expression of the *C. acetobutylicum glnA* gene is not controlled by the lambda rightward promoter of pEcoR251.

Purification of plasmid-encoded GS. The pHZ200-encoded GS was purified 51-fold by the method of Streicher and Tyler (35) and by Sephadex G-100 and G-200 column chromatography (Table 2). SDS-PAGE of the purified GS (C1 fraction) revealed the presence of a major and a minor polypeptide with apparent molecular weights of approximately 59,000 and 61,000, respectively (Fig. 3, lane F). These two polypeptides were also obtained after purification by the method of Thomas and Durbin (37).

Antibodies raised against the C1 fraction were used in Western blots to challenge proteins from crude extracts of *E.*

TABLE 2. Purification of the cloned *C. acetobutylicum* GS from *E. coli* ET8051(pHZ200)

Fraction ^a	Vol (ml)	Protein (mg)	GS activity (μmol of γ-glutamyl hydroxamate/min)	Sp act (μmol of γ-glutamyl hydroxamate/mg of protein per min)	Purification (fold)
Crude extract	10	11,460	86,300	7.53	1
S1	20	11,344	83,950	7.40	1
P1	10	2,608	37,970	14.56	2
P2	1	1,522	25,890	17.01	2.3
S2	1	147	4,315	29.30	4
E1	0.7	14.4	870	60.25	8
C1	0.5	0.2	78	388.00	51

^a GS activity was determined by the γ-GT assay. The details of the purification procedure and the fractions S1, P1, P2, S2, E1, and C1 are described in the text.

coli ET8051, and *E. coli* ET8051(pHZ200) resolved by SDS-PAGE and transferred to nitrocellulose (Fig. 4). The antiserum reacted with two polypeptides with apparent molecular weights of approximately 59,000 and 61,000 in extracts of *E. coli* ET8051(pHZ200) and one polypeptide with a molecular weight of approximately 61,000 in extracts of *E. coli* ET8051. When crude extracts of *E. coli glnA*⁺ HB101 cells were used, the antiserum only reacted with a 61,000-molecular-weight polypeptide. Western blot analysis with the antiserum made against the purified GS produced by *E. coli* ET8051(pHZ200) against *C. acetobutylicum* cell extracts revealed a single band of homology with an apparent molecular weight of approximately 59,000.

SVP treatment failed to produce any detectable change in the mobility of either of the two polypeptides observed in the C1 fraction.

Electron microscopy of the C1 fraction after negative staining showed the presence of molecules with a central hole and hexagonal shape, which are features characteristic of the dodecamer assembly of the GS subunits from other bacteria (10) (Fig. 5).

In vitro transcription-translation. pHZ200 and pHZ202, which conferred the *glnA* phenotype in *E. coli glnA*⁻ dele-

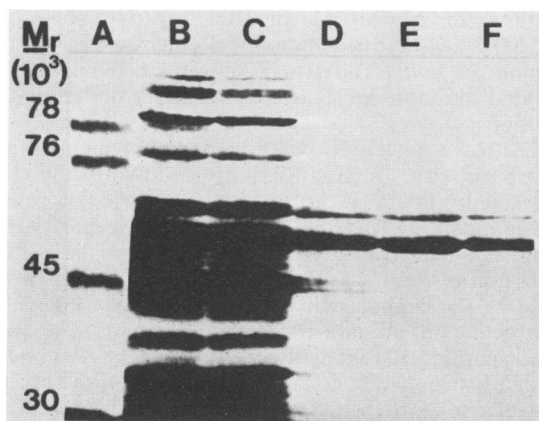


FIG. 3. SDS-PAGE of cell extracts and purified, cloned *C. acetobutylicum* GS from *E. coli* ET8051(pHZ200). The details of the purification procedures and the fractions S1, P1, P2, and C1 are described in the text. The lanes contained molecular weight markers (lane A), crude extract, 45 μg (lane B); S1 fraction, 45 μg (lane C); P1 fraction, 30 μg (lane D); P2 fraction, 15 μg (lane E); C1 fraction, 15 μg (lane F).

tion strains, directed the synthesis in vitro of a major polypeptide with an apparent molecular weight of 59,000 (Fig. 6, lanes D and E). The 59,000-molecular-weight polypeptide was not produced by pEcoR251, pBR325, or pHZ212 (Fig. 6, lanes C, H, and F), which is a deletion derivative of pHZ200 (subcloned in pBR325), and did not complement the *glnA*⁻ genotype. The internal *EcoRI* fragment of pHZ200 on its own did not direct the synthesis of a polypeptide (Fig. 6, lane G), whereas the same fragment cloned into pHZ212 produced at least two polypeptides with molecular weights below 45,000 (Fig. 6, lane F). It is suggested that the internal *EcoRI* fragment did not contain a regulatory region and that the polypeptides obtained with pHZ212 were due to readthrough from plasmid regulatory regions located on the pBR325 DNA. The *BglIII* fragment containing the 6.5-kbp *C. acetobutylicum* insert from pHZ200 was purified by gel electrophoresis and electroelution onto a DEAE membrane, and directed the synthesis in vitro of a polypeptide with an apparent molecular weight of approximately 59,000 (Fig. 6, lane I).

Enzyme activities. GS was assayed in *E. coli* ET8051 (pHZ200) and *C. acetobutylicum* with either cell extracts or whole cells permeabilized by 0.1 mg of hexadecyltrimethylammonium bromide (CTAB) per ml. Treatment of crude extracts with CTAB had no detectable effect on GS levels. Co²⁺, Cd²⁺, Hg²⁺, Na⁺, and Ca²⁺ were tested for their ability to substitute for Mn²⁺ in the γ-GT assay. Although none of these cations inhibited the γ-GT activity assayed in the presence of Mn²⁺, they were unable to substitute for Mn²⁺ in the reaction. Mg²⁺, however, showed a marked effect on Mn²⁺-supported γ-GT activity, irrespective of the levels of available nitrogen. In wild-type *E. coli* strains the velocity of the reaction is maximal in the presence of 3 mM Mg²⁺ (31, 35), but in *E. coli* ET8051(pHZ200), the velocity of the reaction was maximal in the absence of Mg²⁺ (Fig. 7). The GS was very sensitive to Mg²⁺, and 75% inhibition of activity was obtained when 10 mM Mg²⁺ was added. SVP

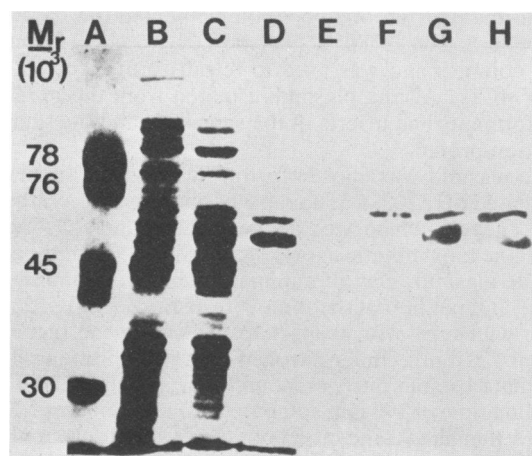


FIG. 4. SDS-PAGE and immunoblot of crude extracts and purified GS samples from various *E. coli* strains. The SDS-PAGE-fractionated proteins were transferred to nitrocellulose paper and challenged with antiserum prepared from purified samples of GS (C1 fraction). Lanes A through D, SDS-PAGE gel of Coomassie blue-stained extracts. The lanes contained molecular weight markers (lane A); ET8051, crude extract, 45 μg (lane B); ET8051 (pHZ200), crude extract, 45 μg (lane C); and C1 protein, 15 μg (lane D). Lanes E through H, immunoblot of gels depicted in lanes A through D, respectively.

treatment did not alter this Mg^{2+} sensitivity. Significant levels of biosynthetic activity were also observed for GS from the plasmid in *E. coli* in the presence of either Mg^{2+} or Mn^{2+} . Transformation of pHZ200 into *E. coli* SF1001, an adenyltransferase mutant which is incapable of adenylating GS molecules, did not alter the Mg^{2+} sensitivity of the plasmid-encoded GS.

The effect of nitrogen levels on the expression of the cloned *C. acetobutylicum* GS activity in *E. coli* was determined by comparing GS activity in cells grown under nitrogen-excess or nitrogen-limiting conditions. GS activity in the *E. coli* YMC10 wild type was repressed by excess glutamine (Table 3). Although the cloned *C. acetobutylicum glnA* gene in *E. coli* produced high levels of GS activity in excess nitrogen, GS activity was increased approximately fivefold when cells were shifted to limiting nitrogen conditions, and extremely high levels of GS activity were obtained. These high levels of GS activity were not due to changes in plasmid copy number because there was <5% variation in the β -lactamase (a plasmid-encoded gene product) levels after growth in the nitrogen-rich and -limiting media.

In *E. coli* and *S. typhimurium* the *ntxB* and *ntxC* genes, which are closely linked to the *glnA* gene, regulate high-affinity arginine and glutamine transport systems and the activation of the *K. pneumoniae hut* operon carried by *E. coli* ET8051 (16, 23, 27, 38). The ability of the *E. coli* YMC10, ET8051, and ET8051(pHZ200) strains to grow on minimal media containing arginine or low levels of glutamine was determined (Table 3). The wild-type YMC10 strain grew on these media, but ET8051 and ET8051(pHZ200) were unable to do so.

The *K. pneumoniae hut* operons carried by *E. coli* ET8051 and YMC10 contain a *hutC* mutation (38), so that the basal level of histidase was rather high in the absence of the normal inducer histidine (Table 3). However, results of

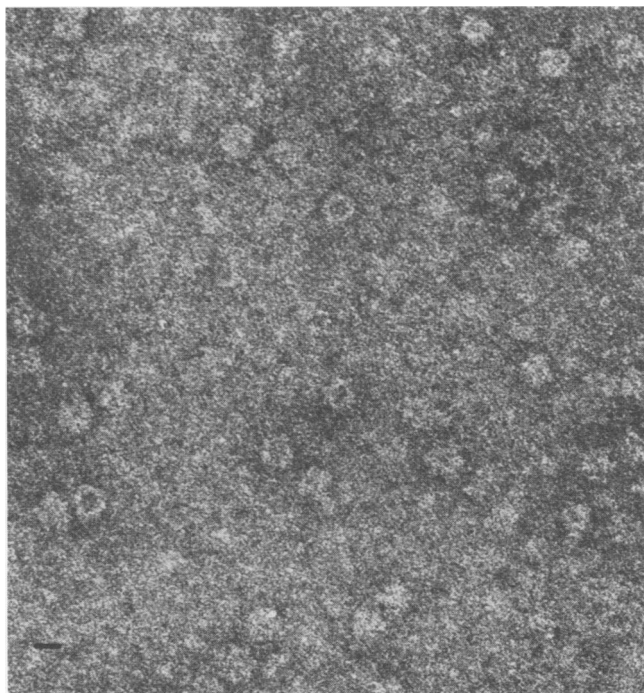


FIG. 5. Electron micrograph of purified *C. acetobutylicum* GS obtained from *E. coli* ET8051(pHZ200). Samples from fraction C1 were examined. Magnification, $\times 420,000$. Bar, $0.001 \mu m$.

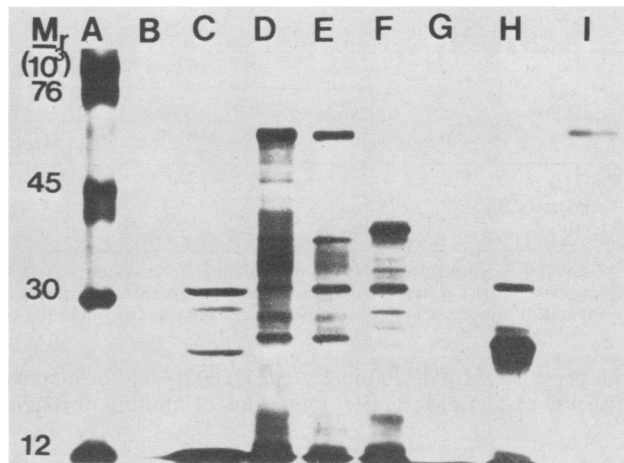


FIG. 6. Plasmid-encoded polypeptides produced in vitro with an *E. coli* cell-free transcription-translation system. Lane A, ^{35}S -labeled molecular weight markers; lane B, zero DNA control; lane C, pEcoR251; lane D, pHZ200; lane E, pHZ202; lane F, pHZ212; lane G, internal *EcoRI* fragment of pHZ200; lane H, pBR325; lane I, purified *BglII* fragment from pHZ200 containing the 6.5-kb *C. acetobutylicum* insert.

experiments in which *E. coli* ET8051 was used show that this relatively high level of histidase activity is not affected by growth in limiting or excess nitrogen, whereas the *E. coli* YMC10 wild type showed an approximately 3.5-fold increase in histidase activity by growth in limiting nitrogen.

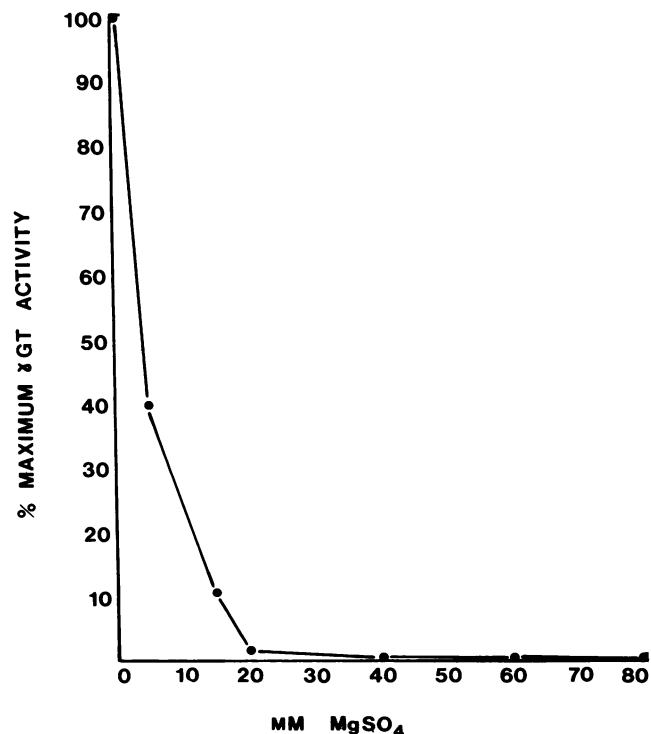


FIG. 7. Inhibition of cloned *C. acetobutylicum* GS in *E. coli* ET8051(pHZ200) by Mg^{2+} . Mg^{2+} was added at various concentrations to extracts of *E. coli* ET8051(pHZ200), and the effect of the cation on γ -GT activity was assayed after 15 min at $37^\circ C$.

TABLE 3. Expression of GS and histidase activity and growth on $(\text{NH}_4)_2\text{SO}_4$, arginine, and low levels of glutamine as sole sources of nitrogen of the *E. coli* wild type and *glnA ntrB ntrC* deletion strains with and without pHZ200

Strain	Growth on ^a :			Enzyme activity on the following growth media ^b :			
	20 mM NH_4^+	0.5 mM glutamine	0.5 mM glutamine-10 mM arginine	0.15 mM glutamine		15 mM glutamine	
				Histidase	GS	Histidase	GS
ET8051	-	-	-	0.23	0	0.22	0
ET8051(pHZ200)	+	-	-	0.22	49.20	0.22	9.81
YMC10	+	+	+	0.78	1.05	0.20	0.51

^a Growth was scored after 5 days of incubation at 37°C. All of the strains grew well on 15 mM glutamine.

^b Histidase activity is expressed as micromoles of urocanate formed per milligram of protein per minute. GS activity is expressed as micromoles of γ -glutamyl hydroxamate formed per milligram of protein per minute. Growth media contained 15 mM glutamate in addition to the glutamine concentrations indicated.

The presence of pHZ200 in *E. coli* ET8051 did not increase the level of histidase under conditions of limiting nitrogen.

DISCUSSION

A. 6.5-kbp DNA fragment was cloned from the gram-positive obligate anaerobe *C. acetobutylicum* P262 and shown to complement the *glnA* lesion in *E. coli* ET8051. Although there was no detectable DNA or protein homology between the cloned *C. acetobutylicum glnA* gene and GS, and the *E. coli glnA* gene and GS, respectively, the cloned gene and gene product functioned very efficiently in *E. coli* and enabled a *glnA*⁻ deletion strain to grow approximately 1.7-fold faster than a wild-type *E. coli* strain under nitrogen-limiting conditions. A lack of DNA and protein homology between a cloned *B. subtilis glnA* gene and GS and the corresponding *E. coli* gene and enzyme has also been reported (12).

Two different purification procedures resulted in partially purified fractions with high specific activities, but the preparations contained two polypeptides with apparent molecular weights of approximately 61,000 and 59,000. Western blotting indicated that the 61,000-molecular-weight polypeptide was an *E. coli* ET8051 host-encoded contaminating product which copurified with the 59,000-molecular-weight polypeptide in the two purification procedures. Western blotting with the GS antiserum and the *C. acetobutylicum* cell extracts indicated that the cloned gene product is the GS from *C. acetobutylicum*.

In vitro transcription and translation studies with plasmid DNA containing functional *C. acetobutylicum glnA* genes and portions of the purified 6.5-kb *C. acetobutylicum* DNA insert indicated that the 59,000-molecular-weight polypeptide was the *C. acetobutylicum* GS subunit. The 59,000-molecular-weight polypeptide falls within the size range reported for other GS subunits (4, 5, 37, 40). Assuming that the enzyme is a typical dodecamer, an assumption which is substantiated by the electron micrographs which show molecules with a central hole and hexagonal shape, the particle mass for the undissociated GS would be approximately 708,000.

The conclusion that the *C. acetobutylicum* GS is not regulated by an adenylation-deadenylation system is based on the following evidence. (i) GS from the cloned gene in *E. coli* and from *C. acetobutylicum* was very sensitive to Mg^{2+} under all growth conditions. (ii) SVP treatment did not affect this Mg^{2+} sensitivity or alter the mobility of the purified GS in SDS-PAGE. (iii) The Mg^{2+} sensitivity was observed in adenylyltransferase mutants which were unable to adenylylate GS. (iv) Significant levels of biosynthetic activity were observed in the presence of Mg^{2+} and Mn^{2+} . Kleiner (15) reported that the GS of *Clostridium pas-*

teurianum is not regulated by adenylation. The absence of an adenylation system has also been reported for the *Anabaena* GS cloned in *E. coli* (8) and for the *B. subtilis* GS cloned both in *E. coli* and *B. subtilis* (30).

The cloned *C. acetobutylicum glnA* gene was expressed at the same level of activity after insertion in both orientations in pEcoR251. The conclusion that the cloned *glnA* gene is expressed from a *C. acetobutylicum* regulatory region in *E. coli* is supported by the in vitro transcription-translation studies with the purified 6.5-kbp *C. acetobutylicum* DNA insert. This insert produced a polypeptide with an apparent molecular weight of approximately 59,000 in the *E. coli*-derived in vitro system. Deletion of the lambda rightward promoter did not affect the expression of the cloned *glnA* gene. Whether or not the *glnA* promoter that functions in *E. coli* also functions as such in *C. acetobutylicum* is not known and remains to be determined.

The *C. acetobutylicum glnA* gene in *E. coli* produced relatively high levels of GS activity in excess nitrogen. Nevertheless, the *C. acetobutylicum glnA* gene was subject to nitrogen regulation in *E. coli*, as under conditions of limiting nitrogen there was an approximately fivefold increase in GS activity and very high levels of GS activity were produced. The increase in GS activity under nitrogen-limiting conditions was not due to a change in copy number, as another plasmid-encoded enzyme, β -lactamase, did not show the same variation in activity.

The cloned *C. acetobutylicum glnA* DNA fragment was unable to complement certain nitrogen-regulatory gene functions in *E. coli ntrB* and *ntrC* deletion strains. pHZ200 did not activate histidase production or allow growth on arginine or low concentrations of glutamine in *E. coli glnA ntrB ntrC* deletion strains.

Although *C. acetobutylicum* is a gram-positive obligate anaerobe with an average G+C content of 29% (34), the *glnA* gene is the third *C. acetobutylicum* gene which has been shown to be expressed from its own promoter in gram-negative *E. coli* strains (G+C content, 51%). Recently, we reported that a cellulase gene and a cellobiase gene are expressed from a *C. acetobutylicum* promoter in *E. coli* (42). The expression of *C. acetobutylicum* genes in *E. coli* augurs well for future genetic manipulation experiments with this industrial bacterium.

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