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The Glutamate Uptake Regulatory Protein (Grp) of *Zymomonas mobilis* and Its Relation to the Global Regulator Lrp of *Escherichia coli*

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After being expressed in *Escherichia coli* JC5412, which is defective in glutamate transport, a *Zymomonas mobilis* gene which enabled this strain to grow on glutamate was cloned. This gene encodes a protein with 33% amino acid identity to the leucine-responsive regulatory protein (Lrp) of *E. coli*. Although overall glutamate uptake in *E. coli* was increased, the protein encoded by the cloned fragment repressed the secondary H^+/glutamate transport system GltP by interaction with the promoter region of the *gltP* gene. It also repressed the secondary, H^+-coupled glutamate uptake system of *Z. mobilis*, indicating that at least one role of this protein in *Z. mobilis* is to regulate glutamate transport. Consequently, it was designated Grp (for glutamate uptake regulatory protein). When expressed in *E. coli*, Grp repressed the secondary H^+/glutamate transport system GltP by binding to the regulatory regions of the *gltP* gene. An *lrp* mutation in *E. coli* was complemented in *trans* with respect to the positive expression regulation of *ilvIH* (coding for acetohydroxy acid synthase III) by a plasmid which carries the *grp* gene. The expression of *grp* is autoregulated, and in *Z. mobilis*, it depends on growth conditions. The putative presence of a homolog of Grp in *E. coli* is discussed.

The aerotolerant anaerobic gram-negative bacterium *Zymomonas mobilis*, which metabolizes sugars by the Entner-Doudoroff pathway (14), is of interest as an effective producer of ethanol (34). Besides studies of glucose uptake by facilitated diffusion (3, 11) and uptake of glutamine (2) and glutamate (35), not much information is available on transport processes in this bacterium. Glutamate uptake is mediated by a secondary $2H^+$/glutamate symport system (35). In *Escherichia coli*, three L-glutamate transport systems have been identified: (i) a sodium-dependent, glutamate-specific system (GltS), (ii) a proton symport system for glutamate and aspartate (GltP), and (iii) a binding protein-dependent, glutamate/aspartate transport system (8, 16, 29, 38). The *gltS* and the *gltP* genes have been cloned and sequenced (10, 23, 43, 44). Although the corresponding gene products are functional, *E. coli* K-12 and B cannot grow on glutamate as the sole source of carbon and energy. Mutants that have higher levels of glutamate uptake activities and grow on glutamate (18) have been isolated. It has been suggested that the glutamate uptake systems in *E. coli* are repressed in response to nitrogen regulation (9, 27), but information on the regulation of glutamate transport in *E. coli* and other bacteria is scarce.

In this study we identified a novel transcriptional regulatory protein in *Z. mobilis*, of which the gene (*grp*) was cloned by the complementation of an *E. coli* strain which has a low overall level of glutamate uptake activity. Grp increases the overall glutamate uptake activity in *E. coli* but negatively affects glutamate uptake in *Z. mobilis*. The deduced Grp protein exhibits significant amino acid similarity to Lrp, a global transcriptional regulator of *E. coli* (7, 31, 50). This important regulatory protein modulates, either negatively or positively, the expression of a number of operons coding for proteins of very diverse functions, like amino acid biosynthesis, amino acid degradation, transport of amino acids and oligopeptides, and pilin synthesis. These operons are defined as members of the leucine-Lrp regulon (51). The presence of leucine often modulates the activity of Lrp, either in a positive or in a negative direction. It has been suggested that leucine acts as a regulatory signal in response to the availability of amino acids and other nitrogen compounds in the environment and may thus be essential for the coordination of metabolism during shifts between feast and famine (9).

MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The *E. coli* strains were grown at 37°C with vigorous aeration in Luria broth (LB), M9 (36), M9G (M9 in which ammonium chloride was replaced by L-glutamate at a final concentration of 40 mM), or SSA minimal medium (20). If necessary, the media were supplemented with the following additions (in micrograms per milliliter): leucine, 100; isoleucine, 25; valine, 50; proline, 50; thiamine, 10; carbenicillin, 100; kanamycin, 25; tetracycline, 20; chloramphenicol, 50; and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 50. In some experiments, minimal medium containing 0.2% lactose was used. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM where indicated. The *Z. mobilis* strains were grown anaerobically overnight at 30°C in complex medium or minimal medium as described previously (6, 15).

Assays. The method of Miller (28) was used for the measurement of β-galactosidase activity, except that the buffer lacked KCl and contained 0.25 mM $MnSO_4$. Gel retardation assays similar to those described by Ricca et al. were performed (33). The 150-bp fragment containing the region upstream of the translation start codon of *gltP* and including the ribosome binding site (44) was amplified by PCR with primers PM1 and PM2 (PM1, 5′-GCGGAATTCAAGG GTTGCGCAACATACC-3′; PM2, 5′-GCGAATTCATGAATGACTTCCTCA ATG-3′) (see below, DNA manipulations). The DNA fragment was cut with *Eco*RI (the respective sites were introduced by the primers) and end labeled with [^{32}P]dATP by using the Klenow fragment of DNA polymerase (36). Cell lysates were obtained as described by Ricca et al. (33). For the binding assay, each sample contained (in a total volume of 20 μl) a ^{32}P-labeled DNA fragment, 4.5×10^4 cpm; crude extract (1 to 10 μg of protein) or purified protein; calf thymus DNA, 5 μg; and a solution containing 50 mM NaCl, 0.1 mM sodium-EDTA, 0.1

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Bacterial strains		
Z. mobilis ZM6 (ATCC 10988)	Wild type	42
E. coli		
JC5412	No growth on glutamate as the sole source of carbon and energy, *secBC*	49
CV975	F^- *ara thiD* Δ(*lac-pro*) *ilvIH*::MudI1734	32
CV1008	CV975 (*lrp-35*::Tn*10*)	32
M15	*recA*$^+$ *uvr*$^+$ F^- *mtl gal ara lac* (pREP4)	8
MC1061	*araD139* (*ara leu*)*7697 lacX74 galU galK hsdR hsdM rpsL*	52
S17-1	Mobilizing donor strain	40
Plasmids		
pSUP104	Shuttle vector (*Z. mobilis* and *E. coli*)	40
pQE-30	Expression vector, His affinity tag	41
pMC1871	*lacZ* in pBR322	39
pGBT521	*gltP* in pUC18	45
pBW1	*gltP* in pBR322	47
pKNP-42	4.2-kb fragment from *Z. mobilis* in pKK223-3	This study
pGRP521	*grp* in pGBT521	This study
pBRP52	*grp* in pBW1	This study
pMP-90	*grp-lacZ* fusion in pMC1871	This study
pSLG-90	*grp-lacZ* fusion in pSUP104	This study
pKG-7	*grp* in pKK223-3	This study
pPN-31	*grp* in pQE-30	This study
pSG-12	*grp* in pSUP104	This study
pREP4	*lacI*^{q}	Qiagen

mM dithiothreitol, 4 mM magnesium acetate, 20 mM Tris-acetate (pH 7.9), and 12.5% (vol/vol) glycerol. The samples were incubated at room temperature for 15 min and fractionated by electrophoresis in 2% agarose gels (150 V, 3 h) and Tris-borate-EDTA buffer (pH 8.1). Gels were dried at 65°C under vacuum and subjected to autoradiography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (24). To identify proteins and molecular weight protein standards, the gels were stained with Coomassie brilliant blue as described by Sambrook et al. (36).

Measurement of amino acid uptake. *E. coli* cells, grown in LB or minimal medium (M9 or SSA), were harvested by centrifugation at 5,000 × *g* for 10 min. The cell pellets were washed three times with 25 ml of buffer A (50 mM potassium phosphate, pH 6.9, and 2 mM magnesium chloride). The pellets were then resuspended in the same buffer to a final optical density at 600 nm (OD_{600}) of approximately 200 and stored on ice. The transport activity of the cells remained constant for at least 4 h of storage. Levels of uptake of the ^{14}C-labeled amino acids were assayed at 37°C and at a final OD_{600} of 2 to 5 in 50 mM potassium phosphate (pH 6.9)–2 mM magnesium chloride–20 mM NaCl–10 mM glucose. Following a 3-min incubation at 37°C under continuous aeration, uptake was started by adding ^{14}C-labeled amino acids. The uptake reaction was stopped at different times by rapid filtration through glass-fiber filters. The filters were washed once with 10 volumes of ice-cold minimal medium and dried. Radioactivity was measured by liquid scintillation counting. Amino acid uptake by *Z. mobilis* cells was measured as described for *E. coli*, except that MES buffer (100 mM morpholineethanesulfonic acid [pH 5.5], 10 mM sodium chloride, 10 mM potassium chloride, 50 mM glucose) was used, and aeration was avoided.

DNA manipulations. Mini- and large-scale preparations of plasmid DNA were obtained by the alkaline lysis method (4). Chromosomal DNA was isolated as described by Eddy et al. (13). *E. coli* cells were transformed by the rubidium chloride method (36) or by electroporation (12). The transfer of plasmids from *E. coli* to *Z. mobilis* was accomplished by conjugation with *E. coli* S17-1 (1). PCR (30) was carried out with *Taq* DNA polymerase from New England BioLabs with the recommended buffers, 1 nmol of primers, 20 ng of plasmid DNA, and 1.25 mM deoxynucleoside triphosphates in a total volume of 100 μl. Forty cycles of amplification were performed (for 1 min at 94°C, 2 min at 59°C, and 1 min at 72°C).

Cloning of the *grp* gene. The *grp* gene was cloned by the complementation of *E. coli* K-12, strain JC5412, which does not grow on glutamate as its sole source of carbon and energy (43). Chromosomal DNA of *Z. mobilis* was partially digested by *Eco*RI, *Hin*dIII, or *Pst*I and fractionated by PAGE (6% [wt/vol] polyacrylamide). Fragments of 2 to 20 kb were isolated and ligated into the linearized and dephosphorylated expression vector pKK223-3. The ligation constructs were transformed into *E. coli* JC5412 by electroporation as previously described (12). Transformants were selected on M9G plates (supplemented with carbenicillin and IPTG), and plasmids were isolated and used to retransform *E. coli* JC5412 in order to distinguish between a Glu^+ revertant and true transformants.

DNA sequencing. The 1,676-bp *Eco*RI-*Asp*HI fragment was isolated from plasmid pKNP-42 and subcloned in both orientations into pUC18. Exonuclease III from Promega (Heidelberg, Germany) was used to generate two sets of deletions by the method of Henikoff (21). A subset of these deletions was used for DNA sequence determination. Both strands of the deletion clones were sequenced by the dideoxy chain termination method (37). The Sequenase kit (Bio-Rad Laboratories) was used in sequencing double-stranded DNA. The programs FASTA and BLASTA from HUSAR 2.1 and 3.0 (DKFZ, Heidelberg, Germany) and PC-gene (Intelli Genetics) were used for computer-assisted sequence analysis.

Isolation of the Grp protein. Grp was isolated with the Qiaexpress protein expression and purification system from Qiagen (Hilden, Germany). The 495-bp fragment containing the coding region of the *grp* gene was synthesized by PCR with the primers PLO5 and PLO7 (PLO5, 5′-GCGGATCCATGATCAGAA AGCTGGATG-3′; PLO7, 5′-CGCGTCGACTTAGGCTTTGCGCTCTGCC-3′) and plasmid DNA of pKG-7 as the template. After being digested with the endonucleases *Eco*RI and *Sal*I, the fragment was cloned in vector pQE-30. The resulting plasmid, pPN-31, carried the *grp* gene fused to six histidine codons at the 5′ end, and the fusion gene was localized downstream of the inducible T5 promoter. Plasmid pPN-31 was transformed into *E. coli* M15, carrying the *lacI* gene on plasmid pREP4. The resulting *E. coli* M15 strain (pPN-31) was grown in LB medium to an OD_{600} of 0.8, after which the expression of the modified Grp protein was induced by the addition of 1 mM IPTG for 4 h. The cells were harvested by centrifugation, suspended in cold TG buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl), and disrupted by sonication. Cell debris was removed by centrifugation. The supernatant was applied to a nickel nitrilotriacetate column equilibrated with TG buffer. The column was washed with 50 mM sodium phosphate (pH 6.0)–300 mM NaCl–10% glycerol to an OD_{280} of ≤0.01. The column was eluted with a concentration gradient of 0 to 0.5 M imidazole, and 1-ml fractions were collected. Samples of these fractions were analyzed by SDS-PAGE.

Construction of plasmids. Plasmid pSG-12 was constructed by ligating the 1,676-bp *Eco*RI-*Asp*HI DNA fragment from plasmid pKNP-42 into the shuttle vector pSUP104, which had been digested with *Eco*RI and *Pst*I. Plasmids pGRP521 and pBRP52 were constructed by ligating the 1.2-kb *Hin*dIII *grp*-containing fragment of pKNP-42 into pGBT521 and pBW1, respectively. Plasmids pMP90 and pSLG90 were constructed as follows. The 870-bp *Eco*RI-*Eco*RV DNA fragment of pKG-7 was treated with the Klenow fragment of DNA polymerase and ligated into plasmid pMC1871, which had been cleaved with *Sma*I. From the resulting plasmid, pMP90, the 3.9-kb *Pst*I DNA fragment was isolated and ligated in the *Pst*I site of vector pSUP104.

Chemicals. Radiochemicals were purchased from Amersham International (Buckinghamshire, United Kingdom). The following labeled compounds were used: L-[U-^{14}C]glutamic acid, L-[U-^{14}C]glutamine, L-[U-^{14}C]aspartic acid, L-[U-^{14}C]proline, L-[U-^{14}C]leucine, and [α-^{32}P]dATP. Biochemicals and endonucleases were from Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany), or Sigma (St. Louis, Mo.).

Nucleotide sequence accession number. The sequence of *grp* has been submitted to GenBank under accession number X84019.

RESULTS

Cloning of the *grp* gene of *Z. mobilis*. In experiments to identify the genes of the glutamate uptake system from *Z. mobilis*, the *grp* gene from *Z. mobilis* was cloned by the complementation of *E. coli* JC5412 for growth on glutamate as its sole source of carbon and nitrogen (see Materials and Methods). One of the positive transformants, *E. coli* JC5412(pKNP-42), harbored plasmid pKK223-3 with an insert of 4.2 kb (Fig. 1). This plasmid, pKNP-42, conferred a Glu^+ phenotype on *E. coli* JC5412 upon retransformation. In cells of *E. coli* JC5412 (pKNP-42), the level of glutamate transport activity was more than twofold higher than that of *E. coli* JC5412 cells harboring plasmid pKK223-3 (Fig. 2). To identify the smallest fragment that allowed *E. coli* JC5412 to grow on M9G, we constructed

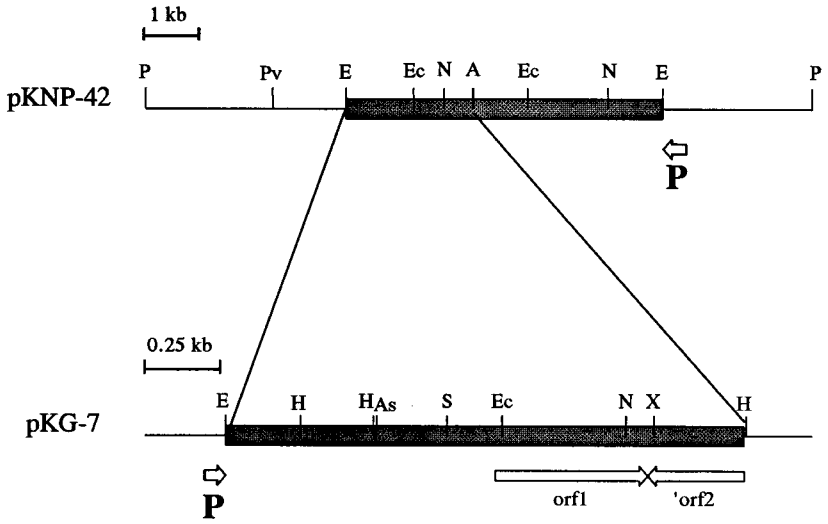

FIG. 1. Restriction map of insert DNA of pKNP-42 and of the subclone pKG-7. The orientation of the *tac* promoter in both plasmids is indicated by an arrow over the letter P. Abbreviations: A, *Asp*HI; As, *Asu*II; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII; N, *Nco*I; P, *Pvu*II; Pv, *Pvu*I; X, *Xma*III.

subclones of the 4.2-kb fragment in pKK223-3. The smallest insert which resulted in a Glu^+ phenotype for *E. coli* JC5412 led to plasmid pKG-7, which contained a 1,676-bp *Eco*RIII-*Asp*HI DNA fragment (Fig. 1). The recombinant strain *E. coli* JC5412(pKG-7) showed increased levels of glutamate transport activity similar to those observed for *E. coli* JC5412 (pKNP-42) (data not shown). In later experiments we also found that the expression of a significantly smaller *Hin*dIII-*Asp*HI fragment of 1,196 bp, which contains less than 300 bp upstream of *orf1*, led to a Glu^+ phenotype. When this fragment was cloned in plasmid pKK223-3 and transformed into *E. coli* JC5412, however, the addition of IPTG resulted in strong growth inhibition, presumably because of the proximity of the *tac* promoter, resulting in a high level of overexpression of the *orf1* gene product. Without the addition of IPTG, the same complementation described for the 1,676-bp *Eco*RI-*Asp*HI fragment was observed (data not shown). For this reason, in further experiments we used the 1,676-bp *Eco*RI-*Asp*HI fragment. This fragment was cloned in pUC vectors and sequenced as described in Materials and Methods. The complete sequence is shown in Fig. 3. Two open reading frames were identified, *orf1* and *orf2*. *orf1* is located between positions 870

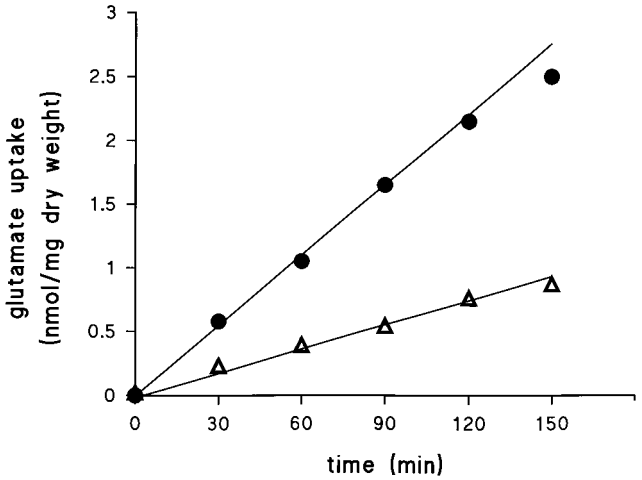

FIG. 2. Glutamate uptake in *E. coli* JC5412(pKNP-42) (filled circle) and *E. coli* JC5412(pKK223-3) (open triangle). The cells were grown in minimal medium supplemented with carbenicillin and IPTG with 40 mM L-glutamate and 20 mM glucose. Cells were diluted to a final concentration of 1.2 mg (dry weight)/ml into a buffer containing 50 mM potassium phosphate (pH 6.9), 2 mM $MgCl_2$, and 10 mM glucose. After 3 min of incubation, 50 μM labeled L-glutamate was added, and transport assays were carried out as described in Materials and Methods.

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EcoRI
GAATTCTCCAAAAGCAAAAGTAAATTTAGCGAGAAGTGTAAAAAGATAGATAACAGCTAT  60
CTGTTCTTAGCCGCTATTTAAGGCAATAAAATATATAACGAAATAAGGTTTTTTAGATAT 120
ATTTTTTTCAAAAATATTAAACAATTATATTACAAGGCGAAAAAATATTATGCTGATCTT 180
TTTAAGATAGGATAAGCGCCTTTCTCTCCAATATAAGAGCCGCCATAGTGTAAAACGAAC 240
GTCAGTACAAGCTTCTGGGTTTATCAACTCTGTTGCCAGTAAATTATCCTTCATTATACG 300
CTATGTCGGTAACAGCCTTAAAGGAATGAGGGCTGATCGGTTCTGTTCTGAGGTCGGGCA 360
AGATTGTTCTGATTTTAACCGATACATGAAATCTTAAGTTAAATTCTACAGTTGGAAATC 420
AAGGAATATGGATAGGCAAAAAACCTCCGGATTCCGGTCCTTTTGTTGGTGAAGGAAAAG 480
AAGCTTTCGAAATTGAGATAGCGTCTACCGGTGAAGTTATTCCGGTTACATCTGGCCAAA 540
CAATTGCAGATGCCTTGGAAAAAGCAGGTATCGAAACGGGTATCGCCTGTGAAGAGGGGT 600
TTGCGGGGCTTGCATGGTTGGATTGGTCTCGGGGAAAGTCGATCATCGTGACCATATCCA 660
GTCAGAAGAAGAAAAAGCCCAGAATAAAGAAATCGCTATTTGTTGTTCGGGGCGTCGTTC 720
TGCCCGTTTAGTTCTGGATCTCTAGTAATTCAATTCGACAGTGATCAGTTTTTTGTGAAG 780
GGCAGAATTTTAAAAAAAATTGCAGATTTAGATGCTTGATATTATAAATTTTTAAACAAAA 840
          RBS
AATGAAATTTTCTGGGAAAAATACCTAATTATGATCAGAAAGCTGGATGATTTTGATATC 900
                        grp->M  I  R  K  L  D  D  F  D  I
AAAATTTTAGTCCTGCTTCAGGATGATGCCACTGCGACTATGGCTGAATTATCAGAAAAA 960
 K  I  L  V  L  L  Q  D  D  A  T  A  T  M  A  E  L  S  E  K
ACCGGTCTGTCAGCGAATGCGTGTTGGCGTCGTATCCGGCTTTTAGAAGCGGATGGTGTT 102
 T  G  L  S  A  N  A  C  W  R  R  I  R  L  L  E  A  D  G  V
ATCAAAAATCGCGTCACTTTGCTTGATCCCCAGAAAATCGGGCTTGGTATTACAGTGTTC 108
 I  K  N  R  V  T  L  L  D  P  Q  K  I  G  L  G  I  T  V  F
GTCTGTATCACTGTGCGGAACATTCCCAATGATTGGTTGGATAACTTCCTGCAAATCGTC 114
 V  C  I  T  V  R  N  I  P  N  D  W  L  D  N  F  L  Q  I  V
AATGAATCGCCGGAAGTGATAGAGTTTTATCGTTTGGCAGGGGACATTGACTATCTTTTG 120
 N  E  S  P  E  V  I  E  F  Y  R  L  A  G  D  I  D  Y  L  L
AAGTTACAGGTCGCTAGTATTAGTGAATATCATCGTCTTTATAAAAAGCTGGTCAGCCGT 126
 K  L  Q  V  A  S  I  S  E  Y  H  R  L  Y  K  K  L  V  S  R
GTGAAGCTGACGGATGTAAGTGCTATTTTCTCCATGGAAGAGCTAAAACATAGCACAATT 132
 V  K  L  T  D  V  S  A  I  F  S  M  E  E  L  K  H  S  T  I
CTGCCTTTGCCAGAAACTTCAGATAAGGCAGAGCGCAAAGCCTAATATTCTTCGATTAGA 138
 L  P  L  P  E  T  S  D  K  A  E  R  K  A  *
                                    *  Y  E  E  I  L
CGGCCGTCATGTAAGGTAACGATCCTATCCATCTTGCGAGCCATAGCCATATTATGGGTC 144
R  G  D  H  L  T  V  I  R  D  M  K  R  A  M  A  M  N  H  T
GCTATGATAGCGGTAGAACCTTGGCCGCGCACCAATCTTAAAAATTCATGCAGCACAATA 150
A  I  I  A  T  S  G  Q  G  R  V  L  R  L  F  E  H  L  V  I
TCACCGGTAGCTTCATCGAGATTGCTTGTAGGTTCATCTGCTAGAACCAAGGCTGGACGA 156
D  G  T  A  E  D  L  N  S  T  P  E  D  A  L  V  L  A  P  R
TTGGCTAAAGCGCGTGCCACGGCTACCCGTTGTTGCTGACCCCCTGATAGTTGGGAAGGG 162
N  A  L  A  R  A  V  A  V  R  Q  Q  Q  G  G  S  L  Q  S  P
TAATGTTTAGACGTTCTTCCAGTTTAAAGAGTTGAGCAAAAAATGGGCGTGCTC     167
Y  H  K  L  R  E  E  L  K  L  S  N  L  L  F  H  A  H  E <-'orf2
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FIG. 3. Nucleotide sequence of the 1,676-bp *Eco*RI-*Asp*HI DNA fragment isolated from *Z. mobilis*. The amino acid sequence deduced from the DNA sequences of *orf1* and *orf2*, as well as a putative ribosome binding site (RBS, underlined), is shown.

```
                                                    50
                                          ---helix-turn-helix----
Lrp           MVDSKKRPGKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVR
Grp(ORF1)     MI.......RKLDDFDIKILVLLQDDATATMAELSEKTGLSANACWRRIR
BkdR          M........RKLDRTDIGILNSLQENARITNAELARSVNLSPTPCFNRVR
Consenus      .........*.LD..D..IL..LQ....*...EL.....LS...C..R*R

              -------                               100
Lrp           RLERQGFIQGYTALLNPHYLDASLLVFVEITLNRGAPDVFEQFNTAVQKL
Grp(ORF1)     LLEADGVIKNRVTLLDPQKIGLGITVFVCITVRNIPNDWLDNFLQIVNES
BkdR          AMEELGVIRQQVTLLSPEALGLDVNVFIHVSLEKQVEQSLHRFEEEIAER
Consensus     ..E..G.I.....LL.P..*.*.*.VF*.***..........F...*...

                                                    150
Lrp           EEIQECHLVSGDFDYLLKTRVPDMSAYRKLLGETLLRLPGVNDTRTYVVM
Grp(ORF1)     PEVIEFYRLAGDIDYLLKLQVASISEYHRLY.KKLVSRVKLTDVSAIFSM
BkdR          PEVMECYLMTGDPDYLLRVLLPSIQALERFL.DYLTRLPGVANIRSSFAL
Consensus     .E*.E.....GD.DYLL*..*.......*.....L.....*.........

Lrp           EEVKQSNRLVI........KTR 164
Grp(ORF1)     EELKHSTILPLPETSDKAERKA 164
BkdR          KQVRYKTALPLP..ANGMTLRE 161
Consensus     ...*....L.*...........
```

FIG. 4. Alignment of the deduced amino acid sequences of Grp, Lrp, and BkdR. The potential helix-turn-helix is indicated. The consensus sequence is indicated, and similar amino acids are marked by asterisks.

and 1364. *orf2* was identified on the opposite strand and encodes the C-terminal part of the ATPase subunit of a binding protein-dependent transport system. Orf1 contains 164 amino acid residues, corresponding to a molecular mass of 18,690 Da. Orf1 shows 33% amino acid identity (52% similarity) to Lrp from *E. coli* and 35% identity (54% similarity) to BkdR, a transcriptional regulator from *Pseudomonas putida* (26). The alignment of all three proteins is shown in Fig. 4. A region which fits the criteria of a helix-turn-helix motif, by which the glycine residue in position 32 corresponds to position 9 of the turn postulated by Brennan and Mathews (5), is marked. The cloned gene was named *grp* (for glutamate uptake regulatory protein) according to its function, as described below.

Complementation of the *lrp* mutation by the *grp* gene. Since Grp from *Z. mobilis* and Lrp from *E. coli* exhibit significant amino acid similarity, the possibility existed that Grp is the Lrp analog in *Z. mobilis*. To establish whether the *grp* gene of *Z. mobilis* could complement an *lrp* mutation in *E. coli*, pKG-7 was transformed into *E. coli* CV1008, which lacks significant expression of the *ilvIH* operon because of a Tn*10* insertion in *lrp*. The *lrp* mutant strain *E. coli* CV1008 and the corresponding parent strain *E. coli* CV975 both contain the *lacZ* gene fused to a part of the *ilvIH* operon, which allows *ilvIH* expression to be measured as β-galactosidase activity (32). In agreement with previously reported results (32), *E. coli* CV975, carrying the control plasmid pKK223-3, showed a high level of β-galactosidase activity. The activity was repressed more than fourfold when cells were grown in minimal M9 medium in the presence of leucine (Table 2). In the *lrp* mutant strain *E. coli* CV1008(pKK223-3), the reporter gene was expressed only at a low level, irrespective of whether leucine was present. *E. coli* CV1008(pKG-7), which carried the plasmid-encoded *grp* gene, regained the ability to grow on lactose plates and exhibited a high level of β-galactosidase activity which was essentially unaffected by leucine. Similarly, the *lrp* wild-type strain (CV975) carrying plasmid pKG-7 showed a high level of activity of the *ilvIH* β-galactosidase reporter, which was reduced only about 30% by leucine. The addition of IPTG led to only about a 25% change in the level of β-galactosidase activity, which indicates that the expression of the *grp* gene was mainly dependent on its own promoter. These results suggest that Grp is able to stimulate transcription of the *ilvIH* operon but, unlike Lrp, in a leucine-independent manner. We have tested other amino acids for their influence on *ilvIH* expression, e.g., glutamate, glutamine, aspartate, and proline, but none of these was effective. The constitutively high expression of Grp in strain *E. coli* CV975(pKG-7) indicates that either Grp activity is dominant over that of Lrp or Grp can modulate Lrp activity.

We have, in addition, investigated the effect of *grp* on the expression of the two high-affinity uptake systems for leucine, LIV-I and LS, which are known to be repressed by Lrp and in which leucine acts as an essential corepressor. In the *E. coli* wild-type strain CV975, leucine transport was strongly decreased in the presence of leucine (19). The high-affinity leucine uptake systems in mutant *E. coli* CV1008 were constitutively derepressed. Transformation of these strains with pKG-7 did not affect the leucine transport either in the presence or in the absence of leucine (data not shown). These results indicate that in contrast to that of the *ilvIH* operon, the expression of LIV-I and LS was not affected by Grp.

Effect of the *grp* gene on glutamate transport in *E. coli*. The increased glutamate transport activity in *E. coli* JC5412(pKNP-42) suggested that Grp affects the expression of at least one glutamate transport system in *E. coli*. In comparison with that in *E. coli* JC5412(pKNP-42), the effect of Grp on glutamate uptake was much more pronounced in *E. coli* CV975(pKG-7) and CV1008(pKG-7). The level of uptake activity in strain CV975(pKG-7) was sixfold higher than that of the parental strain *E. coli* CV975(pKK223-3), and leucine had only a small effect on the activity (Table 3). The positive effect of Grp on glutamate uptake was completely independent of the presence of glutamate in the growth medium (data not shown). These results indicate that Grp acts positively on glutamate transport, probably by activating the expression or by preventing repression of one of the transport systems in *E. coli*. To discover which of the three different glutamate uptake systems of *E. coli* was affected by Grp, the activity of the individual systems was modulated with specific inhibitors. In *E. coli* CV975(pKG-7),

TABLE 2. Complementation of a chromosomal *lrp* mutation by plasmid-encoded *grp* from *Z. mobilis* in different *E. coli* strains

E. coli strain	Leucine addition (0.2 mM)	Growth on lactose[a]	β-Galactosidase activity [mU/(min · mg protein)]	
			With IPTG	Without IPTG
CV975(pKK223-3)	−	++	1,170	1,095
	+	−	240	252
CV1008(pKK223-3)	−	−	30	32
	+	−	20	20
CV1008(pKG-7)	−	+	620	430
	+	+	590	395
CV975(pKG-7)	−	++	1,250	910
	+	+	810	610

[a] −, no growth; +, medium growth; ++, good growth.

TABLE 3. Effects of the inhibitors β-hydroxyaspartate and L-aspartate on glutamate uptake activity in *E. coli* CVC975

E. coli strain	Leucine addition (0.2 mM)	Glutamate uptake activity (nmol · min^{-1} · mg [dry weight]$^{-1}$) with:		
		No addition	β-Hydroxy-aspartate (6 mM)	L-Aspartate (3 mM)
CV975(pKK223-3)	+	8.40	2.80	1.05
	−	4.55	0.95	0.75
CV975(pKG-7)	+	21.80	7.50	2.50
	−	26.40	6.40	3.10

the increased transport activity was strongly inhibited by L-aspartate and by β-hydroxyaspartate (Table 3). Since the H^+-dependent glutamate/aspartate transport system (GltP) is affected by these two inhibitors (17), we investigated the effect of Grp on *gltP* expression. For this purpose, *E. coli* JC5412 was transformed separately with pGBT521 and pBW1, both of which contain *gltP* together with flanking regions of different sizes. The *E. coli* strains JC5412(pGBT521) and JC5412 (pBW-1) showed increased glutamate transport activity in comparison with that of strain JC5412 after growth on glutamate medium (Table 4). The higher glutamate uptake rate in *E. coli* JC5412(pGBT521) relative to that of *E. coli* JC5412 (pBW-1) is presumably the result of a difference in the copy numbers of the two plasmids. When cells were grown in LB medium, the glutamate uptake activity was strongly reduced, which indicated that *gltP* expression was reduced in a rich medium in the presence of glucose. In order to study the effect of Grp on *gltP* expression, the 1.2-kb *Hin*dIII DNA fragment containing the *grp* gene was cloned in the single *Hin*dIII restriction sites of plasmids pGBT521 and pBW-1. The resulting plasmids pGRP521 and pBRP52, carrying the *grp* gene in different positions and orientations with respect to the position of the *gltP* gene, were transformed into *E. coli* JC5412. In the resulting *E. coli* strains JC5412(pGRP521) and JC5412 (pBRP52), the glutamate transport activity encoded by *gltP* was strongly reduced. The same observations were made with *E. coli* JC5412(pGBT521/pSG-12), containing the *gltP* gene on plasmid pGBT521 and the *grp* gene on the compatible plasmid pSG-12. These results suggest that Grp is able to repress *gltP* expression in *trans*, probably by binding to the *gltP* promoter region. Since these experiments were carried out in the

TABLE 4. Effects of Grp on glutamate transport activity in *E. coli* strains carrying the *gltP* gene

E. coli strain	Relevant phenotype	Growth on glutamate[a]	Glutamate transport activity (nmol · min^{-1} · mg [dry weight]$^{-1}$) in:	
			LB medium	Minimal medium with glutamate
JC5412	GltP$^-$ GltS$^-$	−	0.30	0.41
JC5412(pSG-12)	Grp$^+$	+	0.30	1.05
JC5412(pGBT521)	GltP$^+$	++	1.70	9.20
JC5412(pGRP521)	GltP$^+$ Grp$^+$	−	0.84	1.20
JC5412(pGBT521/pSG-12)	GltP$^+$ Grp$^+$	−	0.85	0.95
JC5412(pBW-1)	GltP$^+$	++	0.91	4.60
JC5412(pBRP52)	GltP$^+$ Grp$^+$	−	0.62	0.90

[a] Growth in minimal medium with 40 mM glutamate as the sole source of carbon and nitrogen. −, no growth; +, medium growth; ++, good growth.

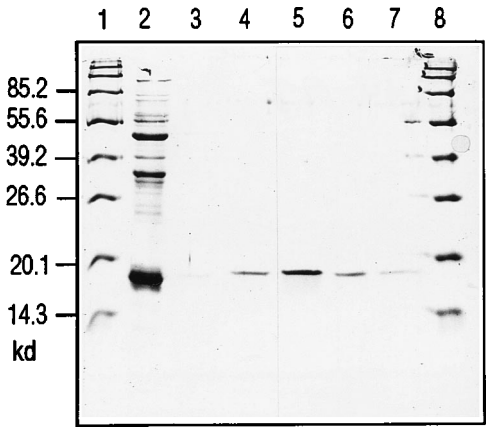

FIG. 5. SDS-PAGE of the purified, His-tagged Grp. Lanes 1 and 8, molecular weight standards; lane 2, cell extract; lanes 3 to 7, peak fractions of the nickel nitrilo-triacetate column which were eluted at 0.25 to 0.3 M imidazole.

lrp$^+$ strain JC5412, an effect of Lrp on *grp* expression could not be ruled out. Therefore, we compared *gltP* expression in *E. coli* wild-type CV975 and in the *lrp* mutant *E. coli* CV1008. Since we observed very similar levels of glutamate uptake activity, i.e., expression of the *gltP* gene, there is no indication of an Lrp effect on *gltP* expression, at least when *gltP* is within a plasmid.

Binding of the isolated Grp to the regulatory regions of *gltP*. To test whether Grp is able to repress the expression of GltP by binding to the putative regulatory region of the *gltP* gene, we purified the Grp and applied a gel retardation assay. The Grp protein was isolated by the His tag modification system. After overexpression of the His-tagged Grp protein in *E. coli* M15(pPN-31), the protein was isolated in native form with a nickel nitrilo-triacetate resin. Samples of the purified protein were fractionated by SDS-PAGE (Fig. 5). The yield of pure protein was about 6 mg from 0.5 g of cells (wet weight). The purified Grp has an apparent molecular mass of 19 to 20 kDa as determined by SDS-PAGE, which is in agreement with the theoretical molecular mass (18,690 Da) deduced from the DNA sequence of *grp*. Since regulation of *gltP* expression was observed with a fragment containing the structural gene and only 150 bp of 5′ DNA, a DNA fragment containing this upstream region was amplified by PCR and end labeled with [^{32}P]dATP. The DNA fragment containing the 150 bp upstream of the initiation codon of *gltP* was incubated with different amounts of purified Grp protein in the presence or absence of crude extract from *E. coli* JC5412. The samples were fractionated by electrophoresis on a 2% agarose gel. A fraction of the labeled fragment migrated more slowly in the presence than in the absence of purified Grp (Fig. 6). The formation of this shift band was proportional to the concentration of the Grp protein, which indicates that the retardation of the DNA fragment was limited by the amount of purified Grp protein. We tested DNA fragments of similar sizes in the neighborhood of the *grp* gene (both upstream and downstream) and did not observe the formation of a shift band upon the addition of Grp protein (not shown). Since the retardation occurred only in the presence of the crude extract, at least one additional component is required. As possible candidates for this factor we tested some amino acids (glutamate, aspartate, glutamine, and leucine) for their ability to elicit the binding of purified Grp protein to the *gltP* promoter region, but none of these amino acids had an effect (data not shown). Boiling the

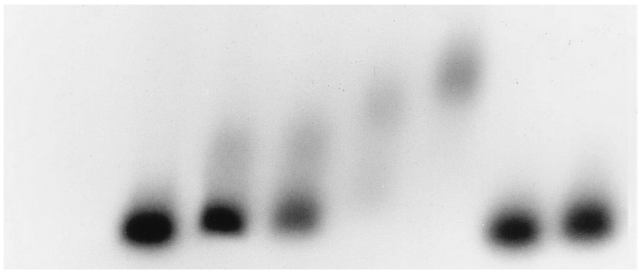

FIG. 6. Gel retardation assay of Grp binding to the regulatory regions of the *gltP* gene. Increasing amounts of purified Grp were added to a DNA fragment containing the putative regulatory regions of the *gltP* gene in the presence (+) or absence (−) of 10 μg of crude cell extract. See the text for further conditions.

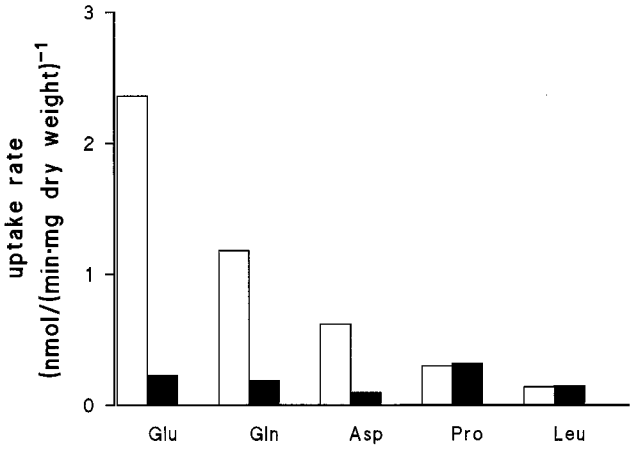

FIG. 7. Transport of glutamate (Glu), glutamine (Gln), aspartate (Asp), proline (Pro), and leucine (Leu) in *Z. mobilis* ZM6(pSUP104) (open bar) and *Z. mobilis* ZM6(pSG12) (filled bar). Strains were grown in minimal medium with 100 mM glucose and 100 mM glutamate. Uptake rates of labeled amino acids at a 100 μM concentration were measured after 5 min of preincubation with 100 mM glucose.

extract did not change its activity in the shift assay. In summary, we conclude that Grp is able to repress *gltP* expression presumably by binding to the regulatory region of the *gltP* gene and that the Grp protein needs a coeffector for this activity.

Regulation of *grp* expression. Since it is known that the expression of *lrp* from *E. coli* strongly depends on growth conditions (19), we analyzed whether the expression of *grp* is regulated both in *E. coli* and in *Z. mobilis*. For this purpose, we constructed plasmids carrying a *grp* promoter-*lacZ* transcriptional fusion. Plasmid pMP90 contains 870 bp of upstream DNA, and the first nine codons of the *grp* gene fused to the *lacZ* gene without its promoter. In this construct the expression of β-galactosidase should be under the control of the *grp* promoter. The fusion gene was isolated as a 3.9-kb *Pst*I DNA fragment and was ligated into the shuttle vector pSUP104. The resulting plasmid, pSLG-12, was transformed into the *Z. mobilis* wild-type strain ZM6. The *grp* expression was measured as a function of the growth conditions and the growth phase. In stationary growth phase the specific level of β-galactosidase activity was at least twofold higher than that of the exponential growth phase and increased about 50% when cells were grown in minimal medium instead of LB medium (Table 5). These results are in the range of the extent of regulation normally observed in *Z. mobilis* (22) and indicate that the level of *grp* expression is higher under limited than under good growth conditions. In *E. coli*, the expression of the same fusion gene was not affected by different growth conditions. Expression in *E. coli* was dependent on the copy numbers of the plasmids pMP90 (with a high copy number) and pSLG90 (with a low copy number). Furthermore, *E. coli* MC1061(pSLG90/pKG-7), which carried the *grp* gene on a second plasmid, showed a strongly decreased level of expression of the fusion gene. This result indicates that in *E. coli*, Grp represses its own synthesis probably by binding to a regulatory region upstream of the structural gene. This phenomenon is similar to the autoregulation of Lrp in *E. coli.*

Multicopy effect of the *grp* gene in *Z. mobilis*. To investigate the regulatory function of Grp in *Z. mobilis*, the uptake of some amino acids in the transconjugant strain *Z. mobilis* ZM6(pSG-12) was measured. This strain contains the wild-type *grp* allele on the shuttle vector pSG-12 (10 to 20 copies per cell) in addition to the chromosomal *grp* gene. In comparison with that of the control, *Z. mobilis* ZM6(pSUP104), the transport activity for glutamate, aspartate, and glutamine was strongly decreased, whereas the uptake rates for proline and leucine were not significantly different (Fig. 7). The glutamate uptake activity of the parent strain was inhibited more than 90% by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone and competitively inhibited by L-aspartate but not by glutamine (data not shown), the substrate of the previously characterized high-affinity glutamine transport systems in *Z. mobilis* (2). The simplest interpretation of these results is that Grp repressed both the carbonyl cyanide *m*-chlorophenylhy-

TABLE 5. Specific activity of β-galactosidase in strains containing a *grp-lacZ* fusion gene on plasmids

Strain	Relevant genotype	Sp act of β-galactosidase (mU/mg of protein) on:			
		LB medium[a]		Minimal medium	
		Exp.	Stat.	Exp.	Stat.
Z. mobilis ZM6(pSLG90)	*grp-lacZ grp*	1,080	2,120	1,450	3,100
E. coli					
MC1061(pMC1871)	*lacZ*	0	0	ND[b]	ND
MC1061(pMP90)	*grp-lacZ*	1,260	1,300	1,180	1,320
MC1061(pSLG90)	*grp-lacZ*	420	480	390	390
MC10618pSLG90/pKK223-3)	*grp-lacZ*	390	490	370	380
MC1061(pSLG90/pKG-7)	*grp-lacZ grp*	80	120	110	160

[a] Exp., cells were grown to a final OD_{600} of 0.8 (exponential growth phase); Stat., cells were grown overnight (stationary growth phase).
[b] ND, not determined.

drazone-sensitive, secondary glutamate/aspartate transport system (35) as well as the glutamine uptake system (2). We suggest that the expression of both systems is regulated by Grp. The regulatory effect of Grp on glutamate uptake thus seems to be similar to that observed in the case of Lrp in *E. coli* with respect to the high-affinity leucine transport systems LIV-I and LS, which are both repressed by Lrp (19).

DISCUSSION

In this study, a gene cloned from *Z. mobilis* was shown to confer increased glutamate uptake when expressed in an *E. coli* strain with low levels of glutamate uptake activity. The cloned gene encodes a regulatory protein with a high level of sequence similarity to the leucine-responsive regulatory protein (Lrp) from *E. coli*. The same *E. coli* strain (JC5412) and the same selection method have previously been used to clone glutamate transport genes by heterologous complementation (43, 46). Although the nature of the mutation which leads to low levels of glutamate uptake activity in *E. coli* JC5412 is not known, the observed correlation between the levels of glutamate uptake in recombinant strains of *E. coli* JC5412 and the ability to grow on this substrate indicate that transport is in fact the limitation in using glutamate as the substrate in *E. coli* JC5412.

Because some elaborated molecular methods are lacking in *Z. mobilis*, e.g., gene disruption or gene replacement, the functional aspects of Grp were studied in *E. coli* mutants deficient in Lrp. Besides having many other functions, Lrp is a positive regulator of expression of *ilvIH. ilvIH* codes for acetohydroxy acid synthase III, the key enzyme of branched chain amino acid biosynthesis. Leucine acts as an antagonist in this regulation. Consequently, in *E. coli* wild-type cells the expression of *ilvIH* was activated by Lrp and repressed when the cells were grown in the medium containing leucine (32). The expression of Grp from *Z. mobilis* in the corresponding *E. coli* strains complemented the regulation of the *ilvIH* operon. Similar to Lrp (50), the *Z. mobilis* regulatory protein autoregulates its own expression probably by binding to DNA upstream of the structural gene. Other experiments indicate that Grp has functional properties different from those of *E. coli* Lrp as follows. (i) Grp's expression regulates glutamate uptake systems, e.g., the *E. coli* GltP system and other, perhaps so-far-unknown glutamate uptake systems, but not the branched chain amino acid uptake systems, e.g., the *E. coli* LIV-I and LS systems. (ii) The effect of Grp on the expression of the *ilvIH* operon was not modulated by leucine or any other amino acid tested. Grp has properties typical of a transcriptional regulation factor, e.g., a classic helix-turn-helix motif, and the ability to interact with regulatory regions of the *E. coli gltP* gene. Since Grp negatively affects the expression of *E. coli gltP*, but since glutamate uptake activity is increased, one or more other genes encoding a glutamate uptake system(s) must be positively regulated by Grp.

Several features of the function of Grp have been elucidated in *Z. mobilis*. Grp represses the uptake systems for glutamate/aspartate and glutamine in *Z. mobilis*. Whereas the *Z. mobilis* glutamate uptake system is mechanistically similar to GltP of *E. coli* (35), glutamine uptake is presumably catalyzed by a binding protein-dependent system (2). Similar to Lrp in *E. coli* (25, 48, 50), expression of the *Z. mobilis* Grp is autoregulated and depends on growth conditions. The dependence on nutrients and supplements was not observed when Grp was expressed heterologously in *E. coli.*

Finally, a further interesting conclusion can be drawn from these results. We showed that *Z. mobilis* Grp effectively modulates several genes in *E. coli*, the regulation of which genes is not mediated by the Lrp protein. However, the expression of *E. coli* genes which are normally regulated by Lrp in a leucine-dependent manner, e.g., the *ilvIH* operon, is influenced by *Z. mobilis* Grp in a different way. These observations argue for the existence of a yet-unknown regulator protein in *E. coli* with functions similar to those of Grp. Approaches based on the structural similarities of *Z. mobilis* Grp and *E. coli* Lrp to identify this protein in *E. coli* by PCR techniques have failed so far.

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