JOURNAL OF BACTERIOLOGY, Feb. 1985, p. 563–566
0021-9193/85/020563-04$02.00/0

Vol. 161, No. 2

Repressor for the *sn*-Glycerol-3-Phosphate Regulon of *Escherichia coli* K-12: Cloning of the *glpR* Gene and Identification of Its Product

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Received 10 September 1984/Accepted 14 November 1984

The *glpR* gene encoding the repressor for the *glp* regulon of *Escherichia coli* was cloned from a library of *Hin*dIII DNA fragments established in bacteriophage lambda. Phages harboring *glpR* were isolated by selection for *sn*-glycerol-3-phosphate dehydrogenase function encoded by *glpD*, which is adjacent to *glpR* on the *E. coli* linkage map. Restriction endonuclease analysis and recloning of DNA fragments localized *glpR* to a 3-kilobase-pair *Eco*RI-*Sal*I segment of DNA. Strains exhibiting constitutive expression of the *glp* operons were strongly repressed after introduction of multicopy plasmids containing the *glpR* gene. Analysis of proteins labeled in minicells harboring either *glpR*$^+$ recombinant plasmids or a *glpR*::Tn*5* derivative showed that the *glpR* gene product is a protein with an apparent molecular weight of 33,000.

The catabolism of *sn*-glycerol-3-phosphate (glycerol-P) is mediated by the components of the *glp* regulon of *Escherichia coli* (10). The *glp* genes are organized in different operons which map in three separate regions on the chromosome (1). The *glpK-glpF* operon encoding cytoplasmic glycerol kinase and a membrane diffusion facilitator for glycerol maps near 88 min; the *glpT-glpQ* operon encoding the membrane-associated glycerol-P permease and periplasmic glycerophosphodiester glycerophosphodiesterase (8) and the *glpA-glpB* operon (7) encoding anaerobic glycerol-P dehydrogenase map near 49 min; the *glpD* operon encoding aerobic glycerol-P dehydrogenase maps near 75 min. Each operon is negatively regulated by a single repressor encoded by *glpR*, which maps adjacent to *glpD*. The inducer for the system is glycerol-P (5).

The *glp* operons exhibit differential sensitivity to the *glp* repressor, with the *glpD* expression ca. 10-fold more sensitive to repressor than the other operons (10). The *glp* operons are also regulated by the cyclic AMP-catabolite gene activator protein system, and expression of the dehydrogenase genes (*glpA-glpB* and *glpD*) is controlled by anaerobiosis (7).

Little is known about the mechanism for the differential repression of the *glp* operator regions by the *glpR* product. Studies aimed at elucidation of factors influencing repressor-operator interactions would be facilitated if purified repressor protein and operator DNA were available. All of the *glp* regulatory regions have been cloned (4, 9, 18), but the repressor has not been identified. Cloning of the *glpR* gene and identification of its gene product are reported here.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains used are listed in Table 1. The *glpK-lacZ* protein fusion was isolated by insertion of λ p*lac*Mu1 (3) into GD1 (*zih-730*::Tn*10*), selecting for Lac$^+$. A P1 lysate grown on the pooled Lac$^+$ colonies (500 transductants) was used to transduce TS100(λ), selecting for tetracycline (Tc) resistance (Tc^r). A Lac$^+$ transductant (GD2) exhibiting no glycerol kinase activity (16) was chosen for further studies. Strains SH301 and SH302 were obtained by lysogenization of MC4100 and TS100 with λ p*glpT668* (12).

Growth media. Media were prepared according to Miller (14) and Davis et al. (6). If needed, antibiotics were added at the following concentrations: ampicillin (Ap), 50 to 100 μg/ml; kanamycin (Km), 25 μg/ml; and tetracycline, 10 μg/ml.

Screening of the *glpR* phenotype. Plasmids to be tested for the *glpR* phenotype were introduced into SH304 (*glpR glpT-lacZ*). *glpR*$^+$ transformants of SH304 were only weakly Lac$^+$ on MacConkey-lactose indicator plates. SH304(pBR322)was strongly Lac$^+$ on these plates.

TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Origin (reference)
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 thiA1 deoC1 ptsF25 flbB5301*	(19)
TS100	MC4100 *glpR2*	(9)
ECL371	MC4100 Δ*glpD102 sdh-9*	(7)
SH250	TL45 *phoR1 recA1 srl*::Tn*10*	(12)
GD1	MC4100 *glpR2 zih-730*::Tn*10*	This study
GD2	MC4100 *glpR2 zih-730*::Tn*10* (λ *pglpK-lacZ*)	This study
SH300	MC4100 Δ*glpD102 sdh*$^+$	P1(MC4100) → ECL371[a]
SH301	MC4100 (λ *pglpT668*)	This study
SH302	MC4100 *glpR2* (λ *pglpT668*)	This study
SH304	MC4100 *glpR2* (λ *pglpT668*) *recA1 srl*::Tn*10*	P1(SH250) → SH302[b]
SH305	MC4100 Δ*glpD102 recA1 srl*::Tn*10*	P1(SH250) → SH300[b]
8	HfrC *glpD3 glpR2 phoA8 relA1 fhuA22* (λ)	(5)
DS410T	F$^-$ *minA minB ara lacY malA mtl xyl rpsL thi fhuA azi gyrA* Δ(*glpT-glpA*)*593*	(9)
LE392	F$^-$ *hsdR514 supE44 supF58 lacY1 galK2 galT2 metB1 trpR55*	(13)
NM303	*mgl recA1 lacZ lacY*$^+$ (λ)	N. Müller

[a] Selection was for Sdh$^+$ on minimal medium A (14) agar plates containing 0.4% sodium succinate.
[b] Selection was for Tc^r.

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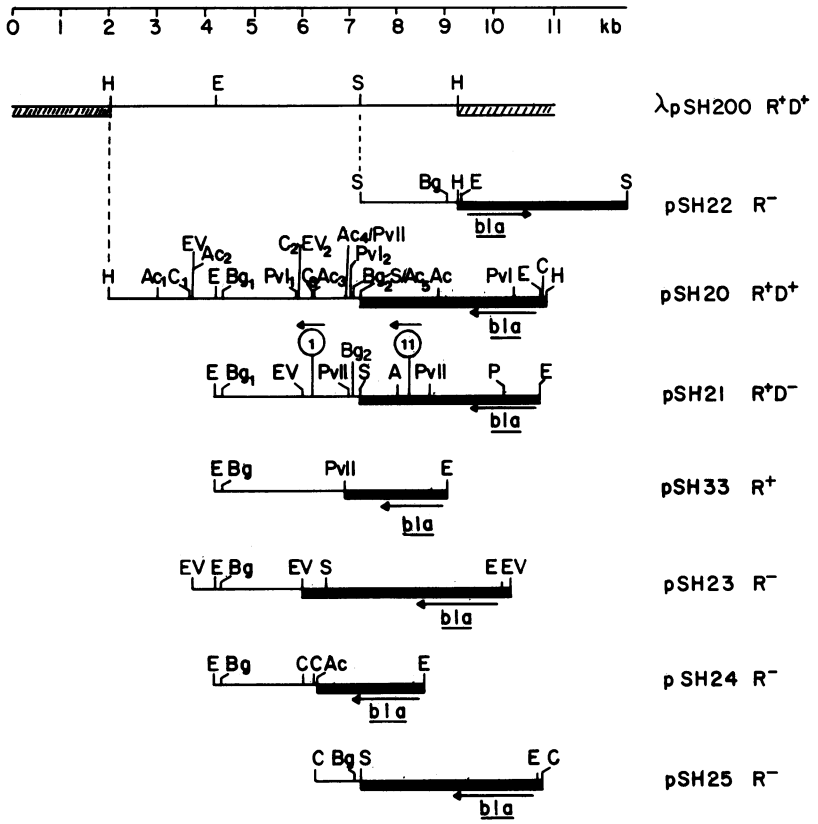

FIG. 1. Restriction endonuclease maps of recombinant phages and plasmids. The restriction endonucleases used were *Acc*I (Ac), *Ava*I (A), *Bgl*II (Bg), *Cla*I (C), *Eco*RI (E), *Eco*RV (EV), *Hin*dIII (H), *Pvu*I (PvI), *Pvu*II (PvII), and *Sal*I (S). Restriction sites occurring more than once in the chromosomal inserts are designated by numbers. Plasmids pSH20 and pSH21 do not contain *Bam*HI and *Xho*I sites. The *bla* gene encodes the β-lactamase of pBR322. The *glpR* phenotype conferred by the phage and plasmids is given alongside. The numerals 1 and 11 in pSH21 indicate the locations of the Tn*5*-1 and Tn*5*-11 insertion sites. The orientation of the Tn*5* insertions with respect to the direction of transcription of the kanamycin resistance gene (17) is indicated by an arrow. No functional *Eco*RV or *Acc*I restriction sites were regenerated during the constructions of pSH23 and pSH24, respectively.

DNA manipulations. Plasmid and phage λ DNA were prepared as described previously (20). Restriction enzyme digestions, electrophoresis of restriction fragments, and ligations with T4 DNA ligase were performed as previously described (20).

Isolation of plasmids containing Tn*5* insertions in *glpR*. To identify plasmids with a Tn*5* insertion in *glpR*, strain NM303(pSH21) was mutagenized with λ *rex*::Tn*5* as described previously (20). Plasmid DNA preparations from 12 independent kanamycin-resistant cultures were used to transform strain SH304 (*glpR glpT-lac*) to Ap^r Km^r on MacConkey-lactose agar. One colony exhibiting a strong Lac^+ phenotype was identified. It contained a Tn*5* insertion in the cloned *glpR* gene (Tn*5*-1; Fig. 1). A Tn*5* insertion in the vector DNA (Tn*5*-11; Fig. 1) was also saved to serve as a control.

Analysis of plasmid-encoded proteins in minicells. Minicells were purified from 200 ml of an overnight culture of the minicell-producing strain DS410T grown in LB medium (6) containing the appropriate antibiotics. Proteins were labeled with [^{35}S]methionine during a 60-min incubation at 37°C in minimal medium A (14) containing 0.4% glucose as described by Schweizer and Boos (21).

Analytical techniques. *glpT*-dependent glycerol-P transport was measured as described by Larson et al. (9). Aerobic glycerol-P dehydrogenase activity in cell-free extracts was assayed by the method of Weiner and Heppel (22), and β-galactosidase activity was assayed as described by Miller (14). Cell-free extracts were prepared from cells grown overnight in 5 ml of the indicated growth medium. Harvested cells were washed once in 5 ml of 80 mM Tris-hydrochloride (pH 7.3) and suspended in 1 ml of the same buffer. After sonication (100 s with intermittent cooling), cell debris was removed by centrifugation at 35,000 × *g* for 20 min at 4°C. Protein concentrations were determined by the method of Lowry et al. (11).

RESULTS AND DISCUSSION

Identification of *glpD*$^+$*glpR*$^+$ transducing λ phages. It was anticipated that the *glpR* gene may be found on a restriction fragment containing *glpD* because these genes are 99% linked in P1 transduction (5). Therefore, phages carrying *glpD*$^+$ were selected by lytic complementation of a *glpD* strain with glycerol as carbon source (6). Strain ECL371 (Δ*glpD102*) was used as a recipient for the λgt7 *Eco*RI (6) and λNM762 *Hin*dIII (15) libraries. *glpD*$^+$ transducing phages were detected in the λNM762 *Hin*dIII library but not in the λgt7 *Eco*RI library. Phages from two single plaques were purified on strain LE392, and the presence of the *glpD* gene was verified by reinfection of strain ECL371 as described above. One of these phages was saved and designated λpSH200. To determine whether λpSH200 carried the *glpR* gene, strain 8 (*glpR2 glpD3*) was lysogenized with λpSH200. Table 2 shows that both *glpD*-encoded glycerol-P

dehydrogenase and *glpT*-encoded glycerol-P transport activity were repressed in this lysogenic strain. Both activities were induced by the addition of glycerol-P to the growth medium. The basal *glpT* activity in strain 8(λpSH200) was probably high because a significant portion of the cells of the overnight culture had lost the $glpR^+$ transducing phage. We determined titers of up to 10^7 PFU of λpSH200 per ml in the supernatant of overnight cultures grown in Casamino Acids. Therefore, a significant number of cells expressed the *glp* system constitutively, and the observed induction ratio was lower than expected. *glpD* expression was more sensitive to repression by the *glpR* protein than *glpT* expression, a finding which is in agreement with previously reported results (5). These results indicate that λpSH200 carries the *glpR* gene.

Subcloning of *glpR* from λpSH200. To facilitate the subcloning of the *glpR* gene, we constructed a restriction endonuclease cleavage map of the cloned chromosomal DNA fragment. λpSH200 carried a single 7.4-kilobase (kb) *Hin*dIII fragment between the λ arms (11.2 and 23.1 kb [11]). This *Hin*dIII fragment contained a single *Eco*RI site and a single *Sal*I site, but no *Bam*HI site (Fig. 1). Cleavage of the 7.4-kb *Hin*dIII fragment with *Sal*I produced fragments of 2.0 and 5.4 kb. Both fragments were subcloned into pBR322 (2), yielding plasmids pSH22 and pSH20 (Fig. 1). The *glpR* gene resided on the 5.4-kb *Hin*dIII-*Sal*I fragment (pSH20). Subcloning of the small *Eco*RI-*Sal*I fragment of λpSH200 into pBR322 (pSH21; Fig.1) localized the *glpR* gene to this 3-kb fragment of DNA. The recombinant plasmid pSH21 harboring $glpR^+$ effectively repressed expression of *glpD*, *glpT*, and *glpK* (Table 3). All three activities were only partially inducible by the addition of glycerol-P to the growth medium.

Localization of the *glpR* gene on pSH21. To further localize the *glpR* gene, transposon Tn*5* derivatives of pSH21 were isolated. Insertion Tn*5*-1 inactivated *glpR*, as indicated by constitutive expression of *glpD* and *glpT* in strain SH304 (*glpR2*) harboring pSH21::Tn*5*-1 (Table 3). This insertion mapped ca. 0.2 kb from the *Eco*RV-2 site (Fig. 1). Insertion Tn*5*-11 mapped in the pBR322 region of pSH21. The plasmid harboring this insertion remained $glpR^+$ and therefore effectively repressed expression of *glpD* and *glpT* (Table 3). Subcloning DNA fragments and isolation of deletion derivatives of pSH21 allowed more precise determination of the location of *glpR*. The 2.2-kb *Eco*RV fragment of pSH20 was cloned into the single *Eco*RI site of pBR322, yielding pSH23 (Fig. 1). This plasmid did not harbor an intact *glpR* gene. Plasmid pSH24 is an *Acc*I deletion derivative of pSH21 and is *glpR*. Manipulations at the *Acc*I-3 site inactivated the *glpR* gene, indicating that the *glpR* gene is located in the proxim-

TABLE 2. Effect of λpSH200 ($glpR^+$ $glpD^+$) on aerobic glycerol-P dehydrogenase (*glpD*) and glycerol-P active transport (*glpT*) activities in strain 8 (*glpR2 glpD3*)

Strain	Addition[a]	*glpD* activity[b]	*glpT* activity[c]
8	None	1	50.0
8	Glycerol-P	2	50.0
8(λpSH200)	None	1	10.7
8(λpSH200)	Glycerol-P	24	28.3

[a] Strains were grown overnight in minimal medium A containing 1% vitamin assay Casamino Acids (Difco Laboratories) as the sole carbon source. Where indicated, 10 mM glycerol-P was present initially.
[b] Expressed as nanomoles of glycerol-P oxidized per minute per milligram.
[c] Expressed as picomoles of glycerol-P taken up in 1 min by 10^9 cells.

TABLE 3. Effect of pSH21 and Tn*5* insertion derivatives on expression of *glpD*, *glpT*, and *glpK*

Plasmid (genotype)	Addition[a]	Glycerol-P dehydrogenase[b]	*glpT-lacZ* activity[c]	*glpK-lacZ* activity[d]
pBR322	None	50	5.0	2.7
pBR322	Glycerol-P	26	2.8	3.2
pSH21 ($glpR^+$)	None	1	0.08	0.07
pSH21 ($glpR^+$)	Glycerol-P	2	2.70	0.81
pSH21::Tn*5*-1 (*glpR*::Tn*5*)	None	35	5.0	ND[e]
pSH21::Tn*5*-1 (*glpR*::Tn*5*)	Glycerol-P	21	3.9	ND
pSH21::Tn*5*-11 (pBR322::Tn*5*)	None	2	0.07	ND
pSH21::Tn*5*-11 (pBR322::Tn*5*)	Glycerol-P	2	0.84	ND

[a] Additions were as described in Table 2, footnote *a*.
[b] Expressed as nanomoles of glycerol-P oxidized per minute per milligram of protein in cell-free extracts of strain SH304 (*glpR2*).
[c] Expressed as micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein in cell-free extracts of strain SH304 (*glpR2 glpT-lacZ* [transcriptional fusion]).
[d] Expressed as micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein in toluenized cells of strain GD2 (*glpR2 glpK-lacZ* [protein fusion]).
[e] ND, Not determined.

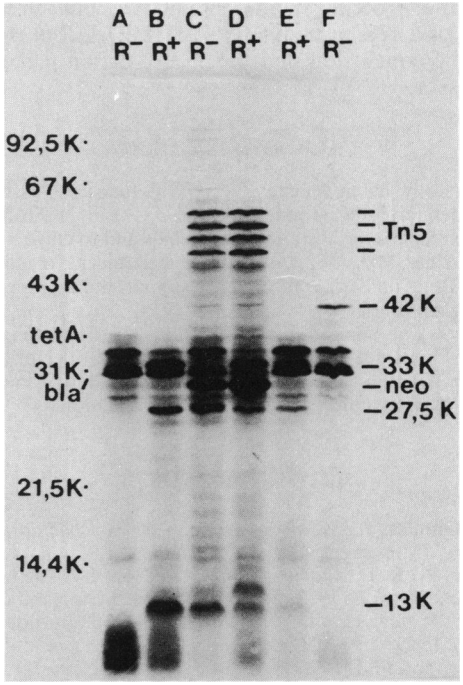

FIG. 2. Expression of plasmid-encoded proteins in minicells. Minicells were labeled with [^{35}S]methionine and separated on a 15% sodium dodecyl sulfate polyacrylamide gel (9). The gel was dried and subjected to fluorography. Lanes A through F: DS410T containing the plamids pBR322, pSH21, pSH21::Tn*5*-1, pSH21::Tn*5*-11, pSH20, and pSH22, respectively. The positions of plasmid-encoded proteins other than resistance proteins are marked, followed by their molecular masses in kilodaltons. The positions of Tn*5*-encoded proteins are also indicated. bla, β-Lactamase; tetA, tetracycline resistance protein; neo, neomycin phosphotransferase. The positions of marker proteins with their molecular masses in kilodaltons are shown. These included phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme.

ity of the *Acc*I-3 site of pSH21 or spans this restriction site. In contrast, pSH33, a *Pvu*II deletion derivative of pSH21, carried a functional *glpR* gene. Therefore, the *glpR* gene is located to the left of the single *Pvu*II site in the region of the *Eco*RV-2 and *Acc*I-3 sites of the chromosomal DNA carried by pSH21 (Fig. 1).

The exact location of the *glpD* gene on the chromosomal DNA fragment carried by λpSH200 was not determined in the studies described in this paper. pSH20 conferred a $glpD^+$ phenotype upon strain SH305 (Δ*glpD102*), whereas pSH21 did not. Therefore, we conclude that the *glpD* structural gene is at least partially located within the *Hin*dIII-*Eco*RI fragment of the chromosomal DNA carried by λpSH200 and pSH20.

Identification of the *glpR* gene product. Plasmid-encoded proteins were labeled in minicells and detected by fluorography after separation on sodium dodecyl sulfate-polyacrylamide gels (Fig. 2). Comparison of the proteins encoded by $glpR^+$, *glpR* plasmids, and two Tn*5*-mutagenized derivatives of pSH21 indicated that a protein with an apparent molecular weight of 33,000 (33K protein) is the *glpR* gene product. Inactivation of this protein by the Tn*5*-1 insertion (Fig. 2, lane C) was accompanied by loss of *glpR* function (Table 3). Besides the *glpR* gene product, two other 13K and 27.5K proteins were encoded by the 3.0-kb *Eco*RI-*Sal*I DNA fragment carried by pSH20 and pSH21 (Fig. 2, lanes B and E). Neither of these proteins was affected by the Tn*5*-1 insertion (Fig. 2, lanes C and D). Plasmid pSH22 encoded none of the proteins mentioned above, but directed the synthesis of a 42K protein (Fig. 2, lane E). All of the results support the conclusion that the 33,000-dalton protein is the *glp* repressor.

ACKNOWLEDGMENTS

We gratefully acknowledge the skillful technical assistance of Anke Middendorf. We are indebted to E.C.C. Lin, N. Müller, and J. Tommassen for bacterial strains and phages and to Gaye Sweet who constructed the *glpK-lacZ* fusion and measured its regulation. We also thank RoxAnn Karkhoff for moral support during preparation of this manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the North Atlantic Treaty Organization. H.S. is a recipient of a Feodor-Lynen fellowship from the Alexander von Humboldt Foundation.

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