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Molecular Analysis of the *glpFKX* Regions of *Escherichia coli* and *Shigella flexneri*

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We have identified a new gene, *glpX*, belonging to the *glp* regulon of *Escherichia coli*, located directly downstream of the *glpK* gene. The transcription of *glpX* is inducible with glycerol and *sn*-glycerol-3-phosphate and is constitutive in a *glpR* mutant. *glpX* is the third gene in the *glpFKX* operon. The function of GlpX remains unknown. GlpX has an apparent molecular weight of 40,000 on sodium dodecyl sulfate-polyacrylamide gels. In addition to determining the *E. coli glpX* sequence, we also sequenced the corresponding *glpFKX* region originating from *Shigella flexneri*, which after transfer into *E. coli* was instrumental in elucidating the function of *glpF* in glycerol transport (D. P. Richey and E. C. C. Lin, J. Bacteriol. 112:784–790, 1972). Sequencing of the *glpFKX* region of this hybrid strain revealed an amber mutation instead of the tryptophan 215 codon in *glpF*. The most striking difference between the *E. coli* and *S. flexneri* DNA was found directly behind *glpK*, where two repetitive (REP) sequences were present in *S. flexneri*, but not in the *E. coli* sequence. The presence or absence of these REP sequences had no effect on transport or on growth on glycerol. Not including the REP sequence-containing region, only 1.1% of a total of 2,167 bp sequenced was different in the two sequences. Comparison of the sequence with those in the EMBL data library revealed a 99% identity between the last third of *glpX* and the first part of a gene called *mvrA*. We show that the cloned *mvrA* gene (M. Morimyo, J. Bacteriol. 170:2136–2142, 1988) originated from the 88-min region of the *Escherichia coli* chromosome and not, as reported, from the 7-min region and that the gene product identified as MvrA is in fact encoded by a gene distal to *glpX*.

Glycerol can enter the *Escherichia coli* cytoplasm by passive or facilitated diffusion. After transport into the cytoplasm, glycerol is phosphorylated by glycerol kinase, encoded by *glpK*, and thus trapped inside the cell as *sn*-glycerol-3-phosphate (G3P) (13). The existence of a transport system, encoded by *glpF*, facilitating the passage of glycerol across the cytoplasmic membrane was demonstrated by Sanno et al. (45). Sweet et al. (50) cloned *glpF*, the gene encoding the glycerol facilitator, showed that it complemented a chromosomal glycerol transport mutation, and identified its product as a membrane protein with an apparent molecular weight of 25,000. *glpF* is the promoter proximal gene in an operon with *glpK* (50), located at 88 min on the *E. coli* chromosome (3). Richey and Lin (41) observed that wild-type *Shigella flexneri* M4243 was glycerol transport negative although glycerol kinase positive and attributed this to a natural mutation in *glpF*. This phenotype was transferred by P1 transduction into an *E. coli glpK* mutant, selecting for growth on glycerol. The resulting glycerol transport-negative strain Lin282 (41) was used as a negative control in investigations of the properties of the glycerol facilitator in vivo (15). Both *S. flexneri* and the *E. coli* strain with the *glpFK* region from *S. flexneri*, referred to here as a hybrid strain, were shown to have a growth disadvantage at low glycerol concentrations (41).

The *glpFK* operon belongs to the *glp* regulon, whose products participate in uptake and metabolism of glycerol, G3P, and glycerophosphodiesters. The phosphodiesterase, GlpQ, hydrolyzes glycerophosphodiesters (the deacylated products of phospholipids) to G3P and alcohol. G3P is subsequently transported into the cytoplasm by the G3P permease, GlpT. Internal G3P is converted to dihydroxyacetone phosphate by either the aerobic (GlpD) or the anaerobic (GlpACB) dehydrogenase; dihydroxyacetone phosphate is then further metabolized in the glycolytic pathway. The *glp* genes and operons are negatively controlled by the GlpR repressor (reviewed in reference 23).

Although the metabolism of glycerol is well understood, some unanswered questions remain. Two genes, *glpE* and *glpG*, were shown to belong to the *glp* regulon and to encode 13- and 26-kDa proteins whose functions are unknown (47). Mutants defective in *glpF* exhibit a decrease in the passive permeability of the membrane, which manifests itself in the increase of resistance to ethanol or tetracycline, and a decrease in the passive diffusion of *o*-nitrophenyl-β-galactoside (53). Here we report another *glp* regulon gene, the most distal gene in the *glpFK* operon. Because the function of the gene product (deduced molecular weight, 35,769) is as yet unknown, we named the gene *glpX*.

MATERIALS AND METHODS

Bacterial strain construction. Table 1 lists the bacterial strains used in this study. P1 transductions, with P1*vir*, were performed according to the method of Miller (31). VT57 was constructed by using a P1 lysate of Lin282 (*S. flexneri* M4243 *glpF* in *E. coli*) and strain GD202 (*glpK*) as recipients, selecting for growth on glycerol and screening for glycerol transport negativity. VT56 and VT55 were constructed by transducing the *trp*114::Tn*10* of VT1 and VT3 (VT3 with *S. flexneri glpF*) to Trp$^+$ with a P1 lysate from T1GP (*cls*). The transductants were tested for resistance to 3,4-dihydroxybutyl-1-phosphonate, conferred by the *cls* mutation (16). To construct VT185, VT3 was transduced to Trp$^+$ with a P1 lysate from T10GP (*cls*$^+$).

Growth. For genetic constructions or DNA preparation, bacterial strains were grown in rich medium (LB) (31). For

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TABLE 1. Bacterial strains

Strain	Description	Construction,[a] reference, or source
BW7622	Hfr KL96 *trpB114*::Tn*10* *thi*-1 *relA1*	55
GD95	MC4100 *glpF*(Am) (*S. flexneri*) *cdh4*::Tn*10*	P1 MW1104 →VT57
HB290	MC4100 *minB rpsL mgl*	14
LE392	*supF supE44 lacY metB tonA galK trgR hsdR*	12
Lin 282	HfrC Δ*phoA glpF*(Am) (*S. flexneri* M4243)	41
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL50 relA*1 *deoC ptsF25 rbsR thi flbB5301*	7
MW1104	Hfr *relA1 metA7 spoT1 cdh*4::Tn*10*	5
Ra2	Hfr *mal-28 sfa-4 supE42*	24
RJ70	MC4100 *glpF*::Tn*10*	R. Jin
TG1	MC4100 Δ(*lac-pro*) *supE thi hsdD5*/F' *traD36 proA*⁺*B*⁺ *lacI*ᵠ *LacZ*ΔM15	6
T1GP	F⁻ *cls met ilv lacI supE44 supF*(?)	39
T10GP	F⁻ *cls*⁺ *met ilv lacl supE*44 *supF*(?)	39
TS100	MC4100 *glpR*	T. Silhavy
VT1	MC4100 *trpB114*::Tn*10*	P1BW7622→MC4100
VT3	MC4100 *glpF*(Am) (*S. flexneri*) *trpB114*::Tn*10*	P1 BW7622→VT57
VT4	Ra2 *glpF*(Am) (*S. flexneri*) *supE cdh-4*::Tn*10*	P1 GD95→Ra2
VT55	MC4100 *cls glpF*(Am) (*S. flexneri*)	This study
VT56	MC4100 *cls*	This study
VT57	MC4100 *glpF*(Am) (*S. flexneri*)	This study
VT132	MC4100 *cls glpF*::Tn*10*	P1 RJ70→VT56
VT183	LE392 *glpF*::Tn*10*	P1 RJ70→LE392
VT185	MC4100 *supF glpF*(Am) (*S. flexneri*)	This study

[a] The donor and recipient in constructions by P1 transduction are indicated.

transport and β-galactosidase assays, strains were grown in minimal medium A (MMA) (31) supplemented with 0.4% Casamino Acids (Difco). When necessary, chloramphenicol or spectinomycin was used at a concentration of 15 or 50 μg/ml, respectively, in MMA or at twice these concentrations in rich medium (antibiotics from Sigma). Cells were routinely grown at 37°C overnight. Growth media were obtained from Difco.

Plasmid construction. Plasmids used in this study are listed in Table 2. DNA methods were those of Maniatis et al. (27) and Silhavy et al. (48). pVT13 contains the 6.0-kb *Bam*HI-*Pvu*II fragment of pGD31 (Fig. 1), cutting in the first third of *glpK* and in the *cdh* gene (see Fig. 1), cloned into pHSG575 (digested with *Bam*HI-*Sma*I). pVT20 and pVT28 were constructed by digesting pDG31 (*E. coli* DNA) and chromosomal DNA from VT57 (hybrid strain) with *Pst*I and ligating fragments of approximately 5 kb into pHSG575 (Fig. 1). The ligation mixture was transformed into VT183, selecting for Cmʳ and screening for complementation of the GlpK⁻ phenotype (growth on glycerol). pVT21 and pVT29 contain the same inserts in the same vector as pVT20 and pVT28, respectively, but in opposite orientation. Subcloning of the 2.9-kb *Hin*dIII fragment into pHSG575 yielded pVT15, which complemented the GlpK⁻ phenotype. pVT54 was derived from pVT21 after digestion with *Hin*cII and religation of the 5.1-kb fragment. This resulted in deletion of *glpF*, *glpK*, and part of the *glpX* gene (see Fig. 5A). pVT60 contains the 1-kb *Hin*dIII-*Pst*I fragment of pVT54 ligated into *Hin*dIII-*Pst*I-digested vector pHSG575. pVT59 was constructed by digesting pVT54 with *Sac*I and filling the 3' sticky ends by using T4 DNA polymerase prior to ligation. Digestion of pVT54 with *Sma*I and *Nru*I followed by religation yielded pVT65, in which a portion of *glpX*, contained on the small *Sma*I-*Nru*I fragment, was deleted. The *glpX*::*lacZ* protein fusion in pVT46 was constructed by digestion of pVT21 with *Fsp*I, cutting 430 bp after the start of the *glpX*

TABLE 2. Plasmids

Plasmid	Description	Reference
pBR322	Apʳ Tcʳ	4
pGD31	pBR322 Apʳ *glpFKX cdh tpi sbp orf1 orf2*	This study
pHP45	Omega element Spcʳ Smʳ Apʳ	40
pHSG575	pSC101 Cmʳ *lacZ'*	51
pNM482	pMC1403 Apʳ *lacZ/Y*	32
pVT13	pHSG575 Cmʳ *glpX orf1 orf2 tpi*	This study
pVT15	pHSG575 Cmʳ *glpKX*	This study
pVT20/21	pHSG575 Cmʳ *glpFKX orf1 orf2*	This study
pVT28/29	pHSG575 Cmʳ *glpF*(Am) *glpKX orf1 orf2* (*S. flexneri*)	This study
pVT46	pHSG575 Cmʳ *glpFK glpX*::*lacZ*	This study
pVT47	pHSG575 Cmʳ *glpF*(Am) *glpK glpX*::*lacZ* (*S. flexneri*)	This study
pVT54	pHSG575 Cmʳ *orf1 orf2*	This study
pVT59	pHSG575 Cmʳ *orf1*	This study
pVT60	pHSG575 Cmʳ *orf2*	This study
pVT65	pHSG575 Cmʳ *orf1 orf2*	This study
pVT70	pHSG575 Cmʳ Spcʳ *glpF glpX orf1 orf2 glpK*::Ω (*Eco*RV)	This study

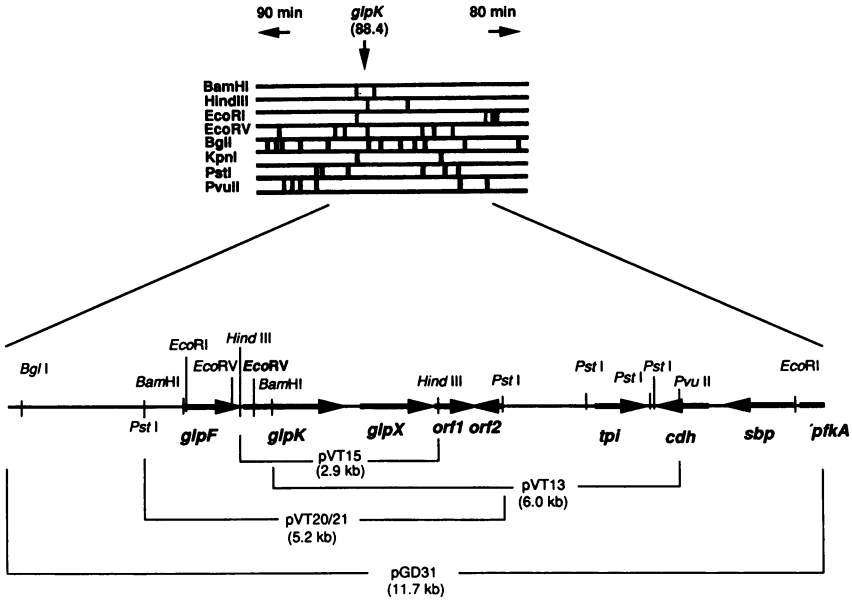

FIG. 1. Restriction map of the *glpFKX* region of the *E. coli* chromosome. Inserts of constructed plasmids, subcloned into the vector pHSG575, are indicated. External restriction sites are taken from the work of Kohara et al. (19). The direction of transcription of the *glpFKX* operon is towards *tpi* or counterclockwise on the *E. coli* chromosome, opposite of the direction suggested in the eighth edition of the linkage map (3).

gene (see Fig. 3), and ligation with the 3.2-kb *Sma*I-*Dra*I fragment from pNM482, containing the *lacZ* gene. Construction of pVT47 was the same as that for pVT46, starting with pVT29, containing the *S. flexneri glpFK* region. Continuous reading frames were created. The *glpX*::*lacZ* fusions were expressed from the *glpFK* operon promoter. The 1,853-bp *Fsp*I fragment containing DNA downstream of *glpX* was deleted. pVT70 contains the *Sma*I-digested polar interposon Ω (from pNM482 [40]) inserted into the *Eco*RV site in *glpK* (in pVT21); the 289 bp between the two adjacent *Eco*RV sites were deleted (Fig. 1) and the *Hin*dIII site between the *Eco*RV sites was lost.

Cloning strategy for sequencing. The 1.4- and 1.6-kb *Bgl*I fragments of pVT20 and -28 were digested with *Eco*RII and *Alu*I, with the intention of identifying smaller fragments with different electrophoretic mobilities. The 0.6-kb *Alu*I and 0.5-kb *Eco*RII fragments (Fig. 2) were cloned in both directions into the *Hin*cII site from M13mp19 (for the *Eco*RII fragment, after filling the 3′ ends with T4 DNA polymerase) and were sequenced by using the M13 primer. An open reading frame of 225 amino acids was found. The rest of the *glpX* gene was sequenced with the help of synthetic primers (positions shown in Fig. 2). The *Bam*HI-*Hin*dIII fragment from pVT20 (2.4 kb), starting within the *glpK* gene (Fig. 2), was cloned into M13mp18 and -19 (30, 58) and then used as template DNA. The specific primers were 17-mers (Microsynth) and were used as follows: 150 nmol was resuspended in 100 ml of H_2O, and 1 ml of a 1:1,000 dilution was used for each annealing. Two open reading frames downstream of *glpX*, *orf*1 and *orf*2, were partially sequenced. The *Hin*dIII-*Pst*I fragment containing *orf*1 and *orf*2 (Fig. 1) was cloned into M13mp18 and -19 and sequenced in only one direction by using the M13 primer. For sequencing *glpF*, the 0.8-kb *Bam*HI-*Eco*RV fragments of pVT20 and pVT28 (Fig. 1) were used. Fragments were cloned into M13mp18 and -19 and then transformed into TG1. Single-stranded DNA was prepared as described by Silhavy et al. (48). DNA sequencing was done according to the dideoxy method of Sanger et al. (44) using the description of United States Biochemical Corp. (54). α-^{35}S-dATP (0.5 μl; 5 μCi, 10 μM; New England

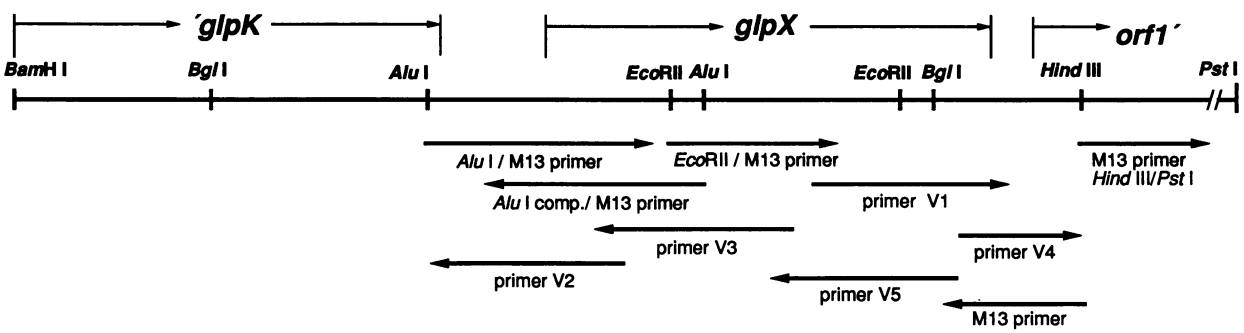

FIG. 2. Sequencing strategy for *glpX*. DNA templates and primers are indicated. The exact locations of the synthetic primers V1 to V5 are shown in Fig. 3. Only the two important *Alu*I and *Eco*RII sites are shown. *Alu*I comp., complementary strand.

Nuclear) was included in each reaction. Unless otherwise indicated, the M13 primer from the United States Biochemical kit was used. Sequences were compared with those in the EMBL data library (9). The Mail-FASTA service is based on the search algorithm, which is implemented in the FASTA program of Pearson and Lipman (36). Promoter homology scores were calculated according to the method of Mulligan et al. (34).

Minicells. Minicells from 200 ml of LB overnight cultures of strain HB290 carrying the indicated plasmids were prepared according to the methods of Maegher et al. (26) and Russel and Model (43). Aliquots of the prepared minicells at an optical density at 600 nm of 0.5 were labeled with 10 μCi of [^{35}S]methionine (New England Nuclear) for 5 min. The chase was done with 0.3 mM methionine for 10 min. After being washed, the cells were resuspended in 25 μl of sample buffer and incubated for 60 min at 37°C (GlpF aggregates at higher temperatures). Samples were loaded onto sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels. After electrophoresis, the gel was stained with Coomassie brilliant blue (Serva) for visualization of the protein standard and later autoradiographed overnight. Gels, buffers, and staining and destaining solutions were prepared as described by Laemmli (21).

Nucleotide sequence accession numbers. The sequence of *glpX* from *E. coli* as shown in Fig. 3 was submitted to the EMBL data library and assigned accession number Z11767. The sequence of the *glpX* region from *S. flexneri*, also including the repetitive (REP) sequences and the beginning of *orf1*, has accession number Z11766. The sequence of the *S. flexneri glpF* gene, as shown in Fig. 7, has accession number Z11768.

RESULTS

***glpX* sequence.** Figure 1 shows the endonuclease restriction map of the relevant region around the *glpFK* operon of *E. coli*. We sequenced DNA beginning at the 3′ end of *glpK* and continuing into *orf1*, after cloning *Alu*I, *Eco*RII, and *Bam*HI-*Hin*dIII fragments into M13. The sequencing strategy as well as the positions of the primers used is shown in Fig. 2. The obtained sequence is shown in Fig. 3. After the TAA stop codon of the *glpK* gene at position 17, we observed an open reading frame starting with ATG at position 25 and extending to the stop codon TGA at position 1162. The *glpX* gene starts with the ATG at position 154, as is clear from comparison of the intergenic region between *glpK* and *glpX* with the corresponding sequence from *S. flexneri*, which we also determined (see below).

The DNA sequence of *glpK* and *glpX* from *S. flexneri* is nearly identical to that from *E. coli*, except in the intergenic region between *glpK* and *glpX*. In *S. flexneri*, two REP sequences (49) (Fig. 4A), which are only partially present in the *E. coli* sequence (Fig. 4B; boldface letters at positions 23 to 66 in Fig. 3), were found. The start of *glpX* in both the *E. coli* and *S. flexneri* sequences is identical and is preceded by a typical ribosomal binding site (SD in Fig. 3), concurring in 6 nucleotides with the consensus sequence (11). Consistent with this assignment of the *glpX* start is the observation that the gene product is identical in size whether encoded by *E. coli glpX* or by *S. flexneri glpX* (lanes labelled pVT20 and pVT28, respectively; Fig. 5B). The *glpX* gene consists of 1,011 nucleotides encoding a polypeptide chain of 337 amino acids with the calculated molecular weight of 35,769. The *glpX* stop codon was followed by a typical rho-independent transcription terminator (42) with a stem length of 9 nucleotides, a 4-base loop, and a tail of 7 Ts (boldface letters underlined in Fig. 3).

Distal to *glpX* we recognized another open reading frame, *orf1*, beginning with ATG at position 1261 and continuing to the end of the sequenced DNA. This sequence encodes the amino-terminal portion of ORF1, a 28-kDa protein that was identified by the minicell technique (see below) (Fig. 5B).

Expression of *glpX* in minicells and induction by G3P. To learn whether the *glpX* gene belonged to the *glp* regulon, *glpX* was cleaved with *Fsp*I (Fig. 3) and fused to *lacZ*, yielding pVT46. pVT47 was constructed similarly by using DNA from *S. flexneri*. The *glpFK* operon promoter was present on these plasmids. Analysis of plasmid-encoded proteins in minicells (Fig. 6) proved that *lacZ* was fused to the gene coding for the GlpX protein. From the *glpX* sequence (Fig. 3), we had learned that *glpX* stopped 210 bp in front of the *Hin*dIII site used for constructing pVT15 (Fig. 1). Therefore, the only protein besides GlpK and GlpF encoded by pVT15 and pVT20 had to be the GlpX protein (Fig. 6). This protein band with a size of approximately 40 kDa disappeared in pVT46 and pVT47. Instead, a band with the apparent molecular weight of 130,000 was expressed from these two plasmids carrying *glpX*::*lacZ* fusions. The size agreed with the expected hybrid protein size of 116 kDa from β-galactosidase plus 14.9 kDa from the truncated GlpX protein. The existence of the fusion protein confirmed the reading frame of *glpX* (Fig. 3). The ORF1 protein is also indicated in Fig. 6.

β-Galactosidase assays (Table 3) showed that *glpX* expression from pVT46 could be induced fivefold with glycerol or G3P and was constitutively expressed in *glpR* strains. The constitutive level of expression in the *glpR* mutant was considerably higher than that in glycerol-induced wild-type strains. Schweizer et al. (46) also observed lower levels of expression of *glpD*::*lacZ* and *glpT*::*lacZ* fusions induced with G3P in a wild-type strain than in a *glpR* mutant. In pVT47 (DNA from *S. flexneri*), both the maximal expression and the induction of *glpX*::*lacZ* were less than those of the *E. coli* fusion (pVT46). As shown in Fig. 7, *glpF* from *S. flexneri* contains an amber mutation that is slightly polar on the expression of distal genes. This is consistent with the observation by Kim and Corwin (18) that glycerol kinase, encoded by *glpK*, was expressed in *S. flexneri* at half the level of that in *E. coli*. Suppression of *glpF*(Am) in a *supF* mutant (VT185, Table 3) resulted in *glpX* expression like that in *E. coli*, demonstrating that *glpX* belongs to the *glpFKX* operon.

Even though it is clear that *glpX* is expressed from the *glpF* promoter and thus part of the operon, we cannot exclude the possibility of an additional separate promoter for *glpX*. The *glpX* gene was still expressed in minicells carrying plasmid pVT13 with an intact *glpX* gene but lacking the operon promoter, *glpF*, and part of *glpK*. Similarly, introduction of an omega interposon (40) into the *Eco*RV site of *glpK* (pVT70) did not prevent the expression of *glpX* in minicells (data not shown).

The function of GlpX remains unknown. The hydrophobicity plot according to Kyte and Doolittle (20) showed a protein with no extended hydrophobic or hydrophilic regions. Thus, the GlpX protein is probably not a membrane protein. GlpX does not appear to have a signal sequence. Glycerol transport measurements in wild-type strains or *glpF* mutants transformed with pVT46 (*glpF*$^+$*K*$^+$*glpX*::*lacZ*) or pVT20 (*glpF*$^+$*K*$^+$*X*$^+$) showed a slight increase in the rate of uptake when strains transformed with pVT46 were compared with those transformed with pVT20 (data not shown).

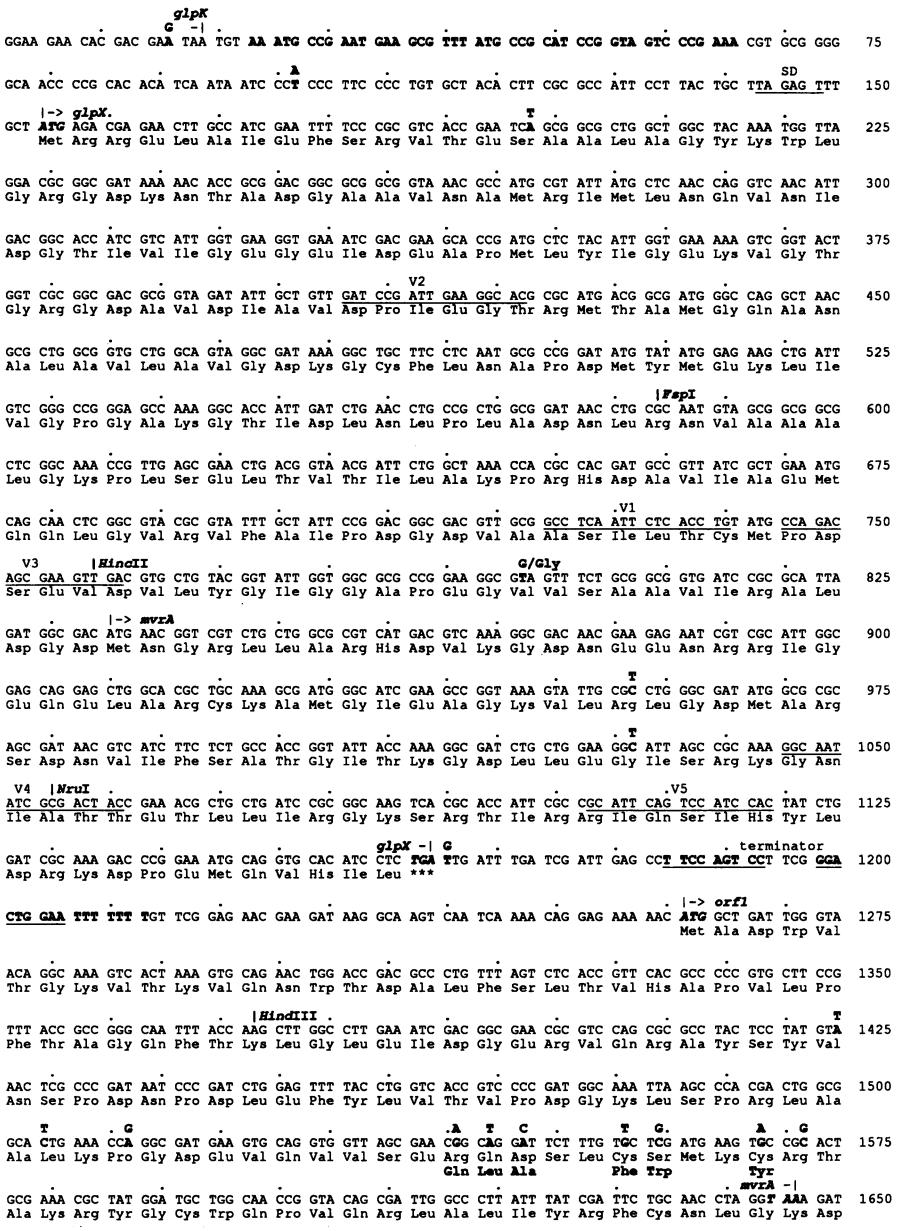

FIG. 3. Sequence of *glpX* and downstream region. The primers used (V1 to V5) are indicated. The possible Shine-Dalgarno sequence of *glpX* is underlined. The start and stop codons of the reported *mvrA* gene (33) are indicated. The TAA stop codon of *mvrA* (position 1644) is out of frame with the *orf1* sequence. Important restriction sites are indicated. The *Fsp*I site is identical to the fusion joint of the *glpX*::*lacZ* fusion of pVT46 and pVT47. The incomplete open reading frame (*orf1*) sequence is shown. Indicated in boldface are the nucleotides that are different in the *S. flexneri* sequence.

Potential clues for the function of GlpX came from comparison of its amino acid sequence with those in the EMBL library. The highest homology found was 44% identity in 95 amino acids to a protein of unknown function, the product of an open reading frame located in a cluster of genes encoding glycolytic enzymes (1). The homology was between the entire unknown open reading frame, *urf*1 (95 amino acids long), and the last third of the GlpX sequence. In addition, an identity of 68% in 31 amino acids with fructose-1,6-biphosphatase from *Synechococcus leopoliensis* (28) was found. No specific functions for the 31-amino-acid stretch of this enzyme or for the Urf1 protein have been identified (36a).

GlpX also shows some homology to various ATPases but not to the conserved nucleotide-binding fold.

Part of the *glpX* nucleotide sequence is similar to that of *mvrA*. In a comparison of the *glpX* nucleotide and protein sequences with those of the EMBL gene bank library, a sequence with high homology (96% identity in 410 nucleotides and 99% in 107 amino acids) was found. This sequence belonged to an *E. coli* gene, *mvrA*, whose product was described as conferring resistance to methyl viologen (MV), an active oxygen radical propagator (33). Morimyo had isolated mutants sensitive to MV in the presence of oxygen and had mapped the *mvrA* mutation by Hfr crosses at 7 min on the *E. coli* chromosome. The *mvrA* gene had been cloned

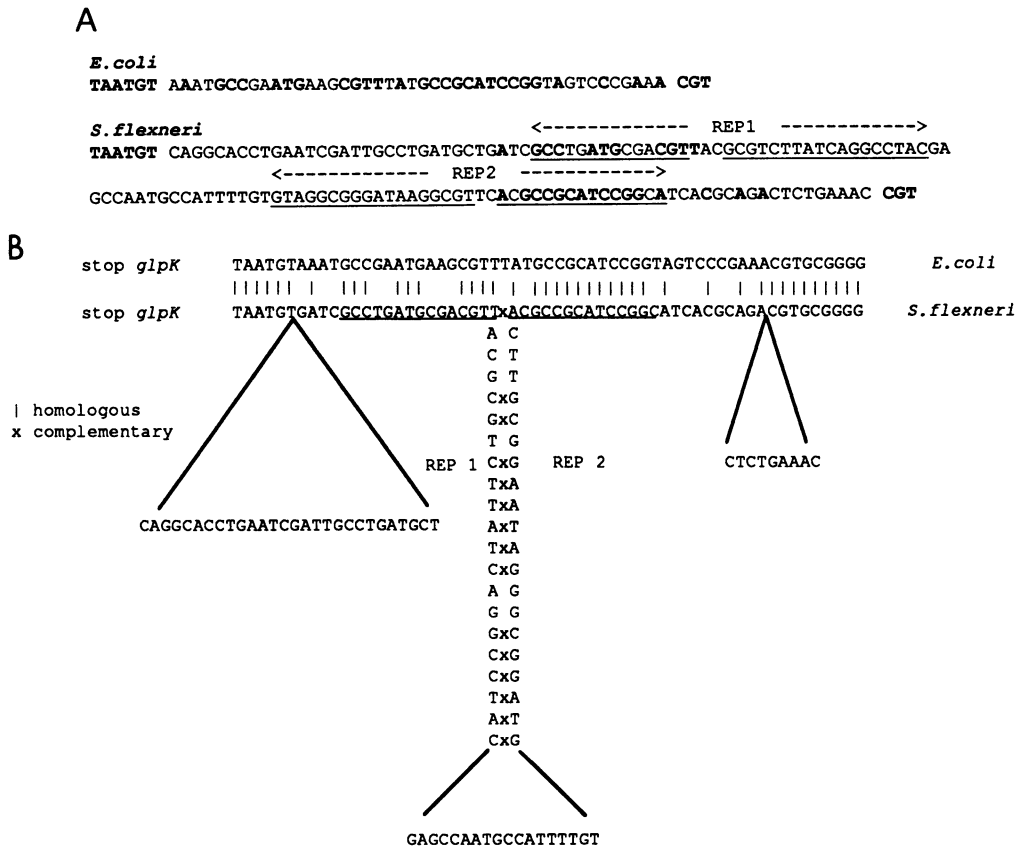

FIG. 4. Difference between the *S. flexneri* and *E. coli glpK* and *glpX* intergenic regions. (A) Both sequences begin with the TAA stop codon of the *glpK* gene and end with CGT, 84 bp prior to the ATG start codon of *glpX*. Before and after these positions, both sequences are nearly identical. REP refers to the extragenic palindromic sequences as defined by Stern et al. (49). (B) The two sequences are aligned to show that the *E. coli* sequence perhaps previously included the REP sequences present in the *S. flexneri* sequence but that they were lost by deletion. This model is analogous to the proposal of Levinson and Gutman (22).

from an *E. coli Pst*I gene bank by screening for MV resistance. The restriction map of the cloned 5.2-kb *Pst*I fragment, which complemented the MV sensitivity (33), was identical to that of our pVT20 (Fig. 1). Comparison of these sites with the map described by Kohara et al. (19) showed no similarity in the 6- to 8-min region, but they could be easily identified at 88 min, where the *glpFKX* operon is located (Fig. 1). Therefore, either Morimyo's mapping of the *mvrA* mutation was incorrect or the cloned DNA fragment from the 88-min region complements the mutation in the 7-min region, without being identical with the *mvrA* gene.

The MvrA protein is encoded on the DNA downstream of *glpX*. Comparison of the *glpX* and *mvrA* sequences revealed some differences. The published *mvrA* gene sequence started at position 835 in the sequence shown in Fig. 3, 682 bp downstream of the *glpX* start codon. The reading frame of *mrvA* was the same as that of *glpX*, but the *glpX* stop codon did not appear in the published *mvrA* sequence (33). Instead, 5 bp in front of the stop, an extra C appeared in the *mvrA* sequence, prolonging the reading frame to the end of the reported 807-bp *mvrA* gene. Comparing the *mvrA* sequence to that of *glpX*, we found that in the published *mvrA* sequence 2 nucleotides were missing, 1 was extra, and 6 nucleotides were different from those in the sequence shown in Fig. 3. The postulated promoter for *mvrA* (33) had a score of only 18% (34). Experiments were performed to test Morimyo's conclusions. The product of the *mvrA* gene had been described as a 29.7-kDa protein. This was reminiscent of the 28-kDa protein (ORF1) expressed from pVT20 in minicells (Fig. 5B and 6). This protein and also a 16-kDa one (ORF2) were encoded by DNA located between the *Hin*dIII and *Pst*I sites of pVT20 (Fig. 5A). Therefore, both were missing in pVT15. Starting from pVT21 (carrying the same insert as pVT20), we subcloned the *Hin*cII-*Pst*I fragment (Fig. 5A) with which Morimyo (33) presumably had worked (pMV1-4-2). Starting with the resulting plasmid, pVT54, three further plasmids were constructed (pVT59/60/65, as described in Materials Methods; Fig. 5A).

Plasmid-encoded proteins were examined in minicells and are shown in Fig. 5B. pVT54 encoded both proteins ORF1 and ORF2. *glpX* had been cleaved by *Hin*cII, accompanied by the disappearance of the 40-kDa band. The pVT54 protein profile was the same as that of Morimyo's plasmid pMV1-4-2, although he did not mention the 16-kDa protein (ORF2), which could be seen at the bottom of his SDS-polyacrylamide gel (see Fig. 7 in reference 33). pVT60 (*Hin*dIII-*Pst*I) did not show the 28-kDa protein (ORF1), indicating that the DNA between the *Hin*cII and *Hin*dIII sites was required for expression of the *orf*1 gene. In pVT59, the 16-kDa protein disappeared, indicating that *Sac*I had cut within the *orf2* gene. From pVT60 and pVT59, we learned that *orf1* was the gene following *glpX*. Finally, pVT65 proved that the *mvrA* gene product was not encoded as described by Morimyo. In his localization (33) of the *mvrA* gene, the *Nru*I site should

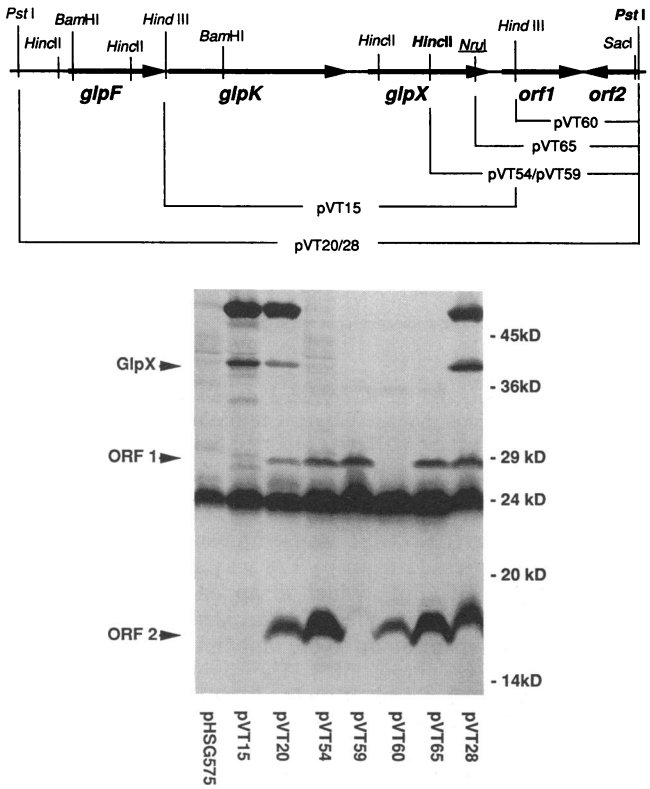

FIG. 5. Gene products encoded distal to *glpK*. (A) Restriction map of the chromosomal inserts of pVT20/21 (5.2 kb), pVT54, pVT59, pVT60, and pVT65. In pVT59, the *Sac*I site from pVT54 is lost. (B) Proteins expressed from the indicated plasmids were labeled with [^{35}S]methionine and separated by SDS-polyacrylamide gel electrophoresis. The proteins GlpX, ORF1, and ORF2 are indicated; molecular sizes (in kilodaltons) are indicated on the right.

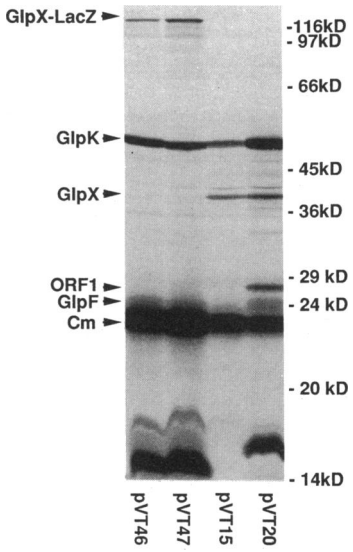

FIG. 6. GlpX-LacZ hybrid protein. Proteins were expressed in minicells containing the plasmids pVT46, pVT47, pVT20, and pVT15 (Fig. 1). The GlpX-LacZ fusion protein, GlpX, GlpK (56 kDa [37]), GlpF (25 kDa [50]), ORF1, and chloramphenicol acetyltransferase (Cm) from the vector pHSG575 are indicated. Molecular sizes (in kilodaltons) are indicated on the right.

cut the gene encoding the 28-kDa protein into two pieces (Fig. 3). This was not the case, since the 28-kDa protein was still synthesized.

The order of the three open reading frames downstream of *glpK* has now been established as *glpX*, *orf1*, and *orf2* (40, 28, and 16 kDa, respectively). The 29-kDa protein, seen by Morimyo on SDS-polyacrylamide gels, is ORF1. Delimitation of the *mvrA* gene by Tn*1000* insertion mutagenesis followed by localization via restriction analysis (33) apparently led to incorrect conclusions.

Does ORF1 confer MV resistance? Because the *mvrA* gene had been cloned by Morimyo (33) via complementation in MV-sensitive mutants, we wanted to test whether this was the function of ORF1. Therefore, we examined the ability of different strains carrying various plasmids to grow in LB supplemented with 0.5 to 4 mM MV. No difference was observed for wild-type strains with or without plasmids pVT54 and pVT20 (data not shown). Growth always stopped in the presence of more than 3 mM MV. Because pVT54 carries the same insert as Morimyo's plasmid pMV1-4-2, it should also confer resistance to MV. Morimyo reported the isolation of an MV-sensitive mutant that could be complemented with plasmid pMV1-4-2. Since this mutant is not available (33a), the effect of ORF1 on MV resistance could not be tested. The resistance might be detectable only on the basis of complementation, and the cloned gene might be incapable of increasing the normal MV tolerance of a wild-type strain. Comparison of the ORF1 partial nucleotide and amino acid sequences with those in the EMBL data bank (9) showed no significant homology to other proteins.

The *glpF* sequence of *S. flexneri*. We had observed that the *glpF* mutation from *S. flexneri* caused, in addition to a glycerol transport-negative phenotype, an alteration in the general permeability properties of the *E. coli* membrane (53). It was therefore of interest whether the *glpFKX* region of *S. flexneri* would exhibit major differences in comparison to the *E. coli* sequence. Southern blot analysis (data not shown) demonstrated that the only difference between the chromosomal DNA from wild-type *E. coli* and the *E. coli-S. flexneri* hybrid strain was in a *Bgl*I fragment containing the end of *glpK* plus distal DNA, which was 0.2 kb longer in the hybrid strain. We cloned the *S. flexneri* DNA from the chromosome of the hybrid strain into a low-copy-number vector. We

TABLE 3. Induction of *glpX*::*lacZ*

Strain	Plasmid	β-Galactosidase activity[a]		Induction factor[b]
		Uninduced	Induced[c]	
MC4100	pTV46	0.9	4.4	5
MC4100	pVT47	1.0	2.0	2
TS100 (*glpR*)	pVT46	11	ND[d]	12
TS100 (*glpR*)	pVT47	3.2	ND	3.2
VT185 (*supF*)	pVT47	1.1	5.5	5
T1GP (*cls supF*?)[e]	pVT47	0.8	5.2	6.5
T10GP (*cls*$^+$ *supF*?)[e]	pTV47	0.7	3.9	5.6

[a] The β-galactosidase activity (in nanomoles per minute × milligrams of protein) was measured in permeabilized cells from overnight cultures, as described by Miller (31).

[b] Cells in the logarithmic phase of growth exhibited the same factors of induction but at levels three- to fourfold higher than those given.

[c] Glycerol (5 mM) or G3P was added as inducer.

[d] ND, not determined.

[e] These strains were described as *supE*, not *supF* (39).

FIG. 7. The *glpF* sequence from *S. flexneri* contains an amber mutation. The *S. flexneri* and the *E. coli glpF* were sequenced up to the *Eco*RV site. Shown is the sequence of *S. flexneri*. Indicated in boldface are the nucleotides that are different in the two sequences. The sequence distal to the *Eco*RV site is taken from the *E. coli glpF* sequence (35). The A in position 518 was reported to be a T.

subcloned and sequenced the DNA distal to *glpK*, the region containing the difference between *S. flexneri* and *E. coli*. Since we expected the relevant alteration of the mutant to be distal to *glpK*, we first focused our attention on this region. *Alu*I cuts 128 nucleotides before the *glpK* stop codon (37). The *S. flexneri* and *E. coli* '*glpK* sequences in this area were identical, with one exception: in *S. flexneri*, the last nucleotide before the stop codon was G, not A, without changing the C-terminal glutamic acid. The *glpK* stop codons were followed by the 3 identical nucleotides, TGT, and then by completely different sequences (Fig. 4). After 44 nucleotides in *E. coli* and 138 in *S. flexneri*, the DNA sequences were again identical starting with CGT; 416 nucleotides with only two differences followed. This common sequence contained *glpX*. Thus, the region of nonhomology between *S. flexneri* and *E. coli* was limited to a segment directly distal to *glpK*, which was 94 nucleotides longer in *S. flexneri*. Comparison of the *S. flexneri* DNA sequence with those of the EMBL library revealed that it contained two REP sequences. These extragenic palindromic sequences (approximately 35 bp) are highly conserved inverted repeats with hundreds of copies in the *E. coli* chromosome (8). These sequences are also present in *Shigella sonnei* and *Shigella boydii* (10). As can be seen in Fig. 4, the two REP sequences have opposite orientation. REP1 agrees with the consensus sequence (49, 57), and REP2 differs in 1 nucleotide. Cloning the REP sequences behind the *E. coli glpFK* operon revealed that the glycerol transport-negative phenotype as well as the general membrane permeability properties of the hybrid strain (53) were not due to the difference in the DNA distal to *glpK* but were present in the *S. flexneri glpF* DNA. We therefore sequenced the *S. flexneri glpF* gene.

First, the *E. coli glpF* sequence was compared with those of Muramatsu and Mizuno (35) and Weissenborn et al. (56). Ours agreed with the latter, differing in one nucleotide from the former. Nucleotide 518 is an A, not a T, translating into aspartate instead of valine. The sequence from *S. flexneri* (Fig. 7) differed in four nucleotides from the *E. coli* sequence: the two differences at positions 84 and 312 did not change the amino acid sequence, whereas the one at 730 yielded serine instead of glycine. The fourth was an amber mutation at nucleotide 659 in the *S. flexneri glpF* gene.

In pulse-chase labeling experiments with minicells, the truncated GlpF protein of *S. flexneri* was detectable. It can be seen in Fig. 8 that *E. coli* GlpF, encoded by pVT20, was stable during the 20-min chase. When programmed by pVT28 (*S. flexneri*), no GlpF was synthesized even at 0-min chase time. Expressed from pVT28 was a faint band at 20 kDa, too small for GlpF, which disappeared after 20 min. This must be the truncated *S. flexneri glpF*(Am) gene product, the polypeptide up to the amber stop codon (calculated molecular weight, 22,565). This polypeptide was less stable than GlpF and not active in glycerol transport.

Suppression of the amber mutation in *glpF*. We had previously observed that introduction of a *cls* mutation (reduced cardiolipin synthesis) into the hybrid strains restored glycerol transport (53). The source of the *cls* mutation was strain T1GP, derived from PA3092, which was described as *supE* (38). On the *E. coli* linkage map (3), *supE* is located at 15 min while *supF* is located at 27 min, near *cls*. Possibly, strain PA3092, used as the *cls* donor, carried, instead of *supE* or in addition to *supE*, a *supF* mutation that was cotransduced with *cls*. Introducing an authentic *supF* mutation from a *cls*$^+$ strain into a strain with the *S. flexneri glpF* also yielded a glycerol transport-positive phenotype (VT185; Table 4).

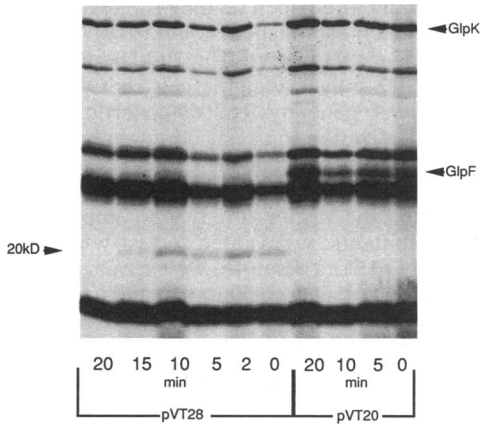

FIG. 8. The *glpF* from *S. flexneri* produces a truncated protein in minicells. Minicells programmed with pVT20 [*E. coli glpF*$^+$) and pVT28 (*S. flexneri glpF*(Am)] were pulse-labeled for 2 min with [^{35}S]methionine and chased with unlabeled methionine for the indicated times. The reactions were stopped with 12% trichloroacetic acid. The amounts of minicells were always the same, but difficulties in resuspending the trichloroacetic acid pellet led to differences in the amounts of protein applied to the gel.

TABLE 4. Suppression of glycerol uptake in a *glpF*(Am) strain by *supE* and *supF*

Strain[a]	Relevant genotype	Glycerol uptake[b]
MC4100[c]	Wild type	70
VT57	*glpF*(Am) (*S. flexneri*)	0.3
VT55	*glpF*(Am) (*S. flexneri*) *cls supF*	40
VT185	*glpF*(Am) (*S. flexneri*) *cls*$^+$ *supF*	42
VT4	*glpF*(Am) (*S. flexneri*) *supE*	0.3
VT132	*glpF* (*E. coli*) *cls supF*	0.2
MC4100[c] pVT28	plasmid *glpF*(Am) (*S. flexneri*)	28
Ra2 (*supE*)[c] pVT28	plasmid *glpF*(Am) (*S. flexneri*)	40

[a] Strains were grown overnight in MMA with 0.4% Casamino Acids. Strains without plasmids were induced with 5 mM G3P.

[b] Glycerol (in picomoles) taken up per 10^9 cells at a substrate concentration of 0.1 μM.

[c] The strains are chromosomally *glpF*$^+$.

Also, when the original *cls* strain, T1GP, was transformed with a plasmid containing a *glpX-lacZ* fusion distal to the *glpF*(Am) from *S. flexneri* under its own promoter control, suppression of β-galactosidase activity was observed, whereas the same plasmid in a *supF*$^+$ strain exhibited polar effects on the expression of β-galactosidase activity (Table 3).

In a *supE* background, the transport ability was not regained (VT4; Table 4) and only small amounts of GlpF were made (data not shown).

Thus, it became clear that the previously observed supression of glycerol transport in the *glpF* mutation from *S. flexneri* was not due to a *cls* mutation but to a *supF* mutation located next to *cls*.

DISCUSSION

We have identified *glpX*, a new *glp* gene, and determined its DNA sequence (1,011 bp, 337 amino acids). *glpX* is located directly downstream of the *glpFK* operon, at 88 min on the *E. coli* chromosome. Expression of *glpX*, measured as β-galactosidase activity of a *glpX*::*lacZ* gene fusion present on a low-copy-number plasmid, was induced fivefold by glycerol or G3P and 12-fold in a *glpR* mutant. GlpX has an apparent molecular weight of 40,000 on SDS-polyacrylamide gels. A rho-independent terminator is located after the *glpX* stop codon. *glpX* is the third gene in the *glpFK* operon. The *S. flexneri glpF* amber mutation was shown to reduce *glpX* expression by a factor of three (Table 3). In a suppressor mutant (*supF*), the plasmid-encoded *S. flexneri glpX* expression increased to wild-type levels (Table 3). This demonstrated that *glpX* is part of a *glpFKX* operon. Surprisingly, the insertion of a polar omega interposon (40) in *glpK* did not abolish expression of *glpX* in minicells. Therefore, it is plausible that *glpX* is preceded by an additional promoter that is active under certain conditions.

The function of GlpX is not yet known. It is possible that some of the chromosomal Tn*5* insertions isolated by Lupski et al. (25) were located in *glpX*. They described mutants with reduced growth on glycerol despite wild-type glycerol kinase activity and no glycerol transport defect. The phenotype was complemented by plasmids containing *glpK* plus downstream DNA (37) that would include *glpX*. Therefore, GlpX may not be required for growth on glycerol but may increase its utilization, although the established *glp*-mediated glycerol metabolic pathway does not offer an explanation. From this pathway there is no need for an additional enzyme, and therefore, *glpX* mutants are not expected to have a Glp$^-$ phenotype. Yet, an alternative pathway for glycerol dissimilation in the *E. coli* mutant has been reported: the oxidation of glycerol to dihydroxyacetone by the NAD$^+$-linked glycerol dehydrogenase (2), which was reported to be weakly expressed in the wild-type strain. No genetic characterization was described. The enzyme was partially purified from a wild-type strain by Asnis and Brodie (2) and later from a *glpK glpD glpR* mutant (29) that had regained the ability to grow on glycerol (52). Kelley and Dekker (17) showed that the D-1-amino-2-propanol:NAD$^+$ oxireductase from wild-type *E. coli* was identical to the glycerol dehydrogenase from the mutant. Glycerol dehydrogenase had an apparent molecular weight of 39,000 (52), similar to the 40-kDa size observed for GlpX. It will be necessary to construct a mutant that expresses GlpX in the absence of glycerol kinase in order to test for the role of this protein in glycerol metabolism.

We found three open reading frames downstream of the *glpK* gene, in the order *glpX*, *orf1*, and *orf2*. ORF1, not GlpX, is identical to the MvrA protein as reported by Morimyo (33). He isolated mutants sensitive to MV and characterized an *mvrA* mutation. Plasmid pMV1-4-2, constructed by Morimyo, complemented the MV^r phenotype. This plasmid contains the *glpFKX orf1 orf2* region described in this paper. Although the *mvrA* mutation was mapped at 7 min on the *E. coli* chromosome, the complementing DNA fragment originated from the 88-min region.

Comparing the *glpFKX* sequence of *E. coli* with that from *S. flexneri* revealed a high degree of identity. This homology was interrupted in the region between *glpK* and *glpX*. Two REP sequences (8, 49) were found in the hybrid strain, while in *E. coli* only small remnants of this sequence were observed (Fig. 4). The presence of these REP sequences had no apparent effect on glycerol transport activity or growth on glycerol. This was clear from results of experiments in which the plasmid-borne *glpF*(Am) was replaced by the wild-type *E. coli glpF* gene.

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