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Physical and Genetic Characterization of the Glucitol Operon in *Escherichia coli*

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The glucitol (*gut*) operon has been identified in the colony bank of Clark and Carbon (A. Sancar and W. D. Rupp, Proc. Natl. Acad. Sci. USA 76:3144–3148, 1979). We subcloned the *gut* operon by using pACYC184, pACYC177, and pBR322. The operon, which is encoded in a 3.3-kilobase nucleotide fragment, consists of the *gutC*, *gutA*, *gutB*, and *gutD* genes. The repressor of the *gut* operon seemed to be encoded in the region downstream from the operon. The gene products of the *gut* operon were identified by using maxicells. The apparent molecular weights of the glucitol-specific enzyme II (product of the *gutA* gene), enzyme III (product of the *gutB* gene), and glucitol-6-phosphate dehydrogenase (product of the *gutD* gene) were about 46,000, 13,500, and 27,000, respectively, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

There are three hexitol transport systems in *Escherichia coli* specific for D-glucitol, D-mannitol, and D-galactitol. These transport systems are known to function via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (10, 11, 21). These three systems have been the subject of intensive study, and their characteristics have in part provided the basis for conclusions about the evolution of the PTS as a whole (12, 13, 21, 23). Earlier work (10, 11) suggested that all three hexitol transport systems consist of the same constituents, a sugar-specific enzyme II and the general PTS constituents enzyme I (EC 2.7.3.9) and HPr (23). These early studies were confirmed by more recent experiments on the mannitol PTS. Homogenous mannitol-specific enzyme II (enzyme II^{Mtl}) was reconstituted in an artificial phospholipid membrane and shown to catalyze the concomitant transport and phosphorylation of its sugar substrate (14). The mannitol operon has been cloned and proved to consist of the *mtlA* gene, which encodes enzyme II^{Mtl}, as well as the *mtlD* gene, which encodes mannitol-1-phosphate dehydrogenase (7).

More recently, it was found that the glucitol PTS includes an additional protein, the glucitol-specific enzyme III (enzyme III^{Gut}) (5, 26). This observation as well as genetic mapping studies led to the conclusion that the gene order to the *gut* operon is *gutC-gutA-gutB-gutD* (12, 13, 26, 27). The *gut* operon was mapped at 58 min on the *E. coli* chromosome (10, 17). Additionally, hybrid ColE1 plasmids carrying the *gut* operon were identified in a colony bank of Clarke and Carbon (3, 25).

Recent studies on the glucitol PTS (5; F. C. Grenier and M. H. Saier, Jr., unpublished results) and the mannitol PTS (6, 9, 14) revealed that the properties of the glucitol-enzyme II-III pair are strikingly similar to those of the mannitol enzyme II. The results appeared to lead to the hypothesis that these two systems have a common evolutionary origin (21, 23). However, because the purified glucitol (sorbitol)-6-phosphate (EC 1.1.1.140) and mannitol-1-phosphate dehydrogenases were found to be very different in structure and enzymatic properties (20) and because the glucitol operon had not been subjected to the approach of the molecular geneticist, the evolutionary relationships of the two operons to each other remained uncertain.

In the present work, we have subcloned the *gut* operon, which encodes the structural genes *gutA*, *gutB*, and *gutD*, and identified the products of these genes in maxicells. The previously suggested gene order (26) has been confirmed, and a restriction map of the operon has been determined. This work represents the first phase of the molecular genetic characterization of the glucitol operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* 236sr (Hfr *leuB6 thi-1 lacZ4 mtlA9 rpsL8 supE44 srl recA*) (8) and JM103 [Δ(*lac pro*) *thi rpsL supE endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q}*ZΔM15*] (18) were used as host strains for cloning the *gut* operon. *Salmonella typhimurium* LJ530 (*mtlA61 gutC151 gutB152*) and LJ531 (*mtlA61 gutC151 gutA153*) (26) were used in complementation assays. pLC18-42, which carries the *gut* operon, was from the Clarke and Carbon colony bank (3, 25). CSR603 (24) was used for maxicell experiments. pACYC184, pACYC177 (2), pBR322 (1), and pUC18 (18) were used as cloning vectors.

DNA manipulations. Restriction endonuclease cleavage, ligation, transformation, DNA isolation, and polyacrylamide gel electrophoresis (PAGE) were performed as described (16). Bal 31 deletion analysis was carried out according to instructions provided by the suppliers of the enzymes. After digestion of the plasmid DNA (8 μg) with a restriction endonuclease, the material was treated with phenol, precipitated with ethanol, and dried. The DNA was suspended in a reaction mixture (50 μl) containing 20 mM Tris hydrochloride (pH 8.0), 12 mM $MgCl_2$, 12 mM $CaCl_2$, 200 mM NaCl, and 1 mM EDTA. The reaction was initiated by the addition of Bal 31 endonuclease (1 U) at 37°C. At intervals of 5 min, 5 μl of the mixture was removed, brought to 65°C for 10 min, and kept at −20°C before the next step. The extent of the deletion was monitored by agarose gel electrophoresis with about half of each sample. Frameshift mutations were induced essentially by the method described previously (28).

Growth media and selection conditions. Transformants were selected on EMB plates (Difco Laboratories) containing 0.5% glucitol and ampicillin (50 μg/ml), kanamycin (25 μg/ml), or chloramphenicol (20 μg/ml). For enzyme assays, cells were grown in LB medium (19) containing 0.5% glucitol and the antibiotics mentioned above in the same concentrations as used in the solid media.

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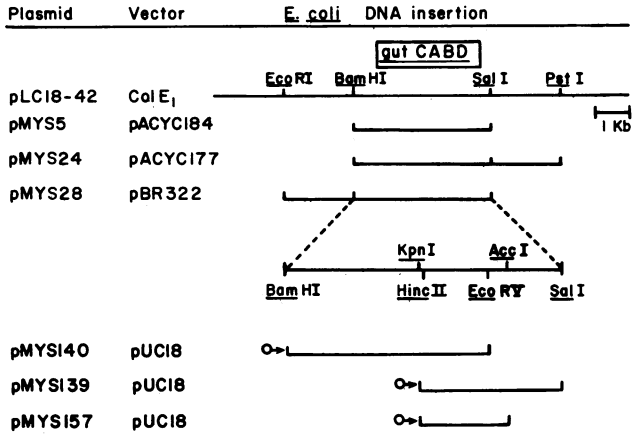

FIG. 1. Subcloning of the *gut* operon. The *gut* operon in pLC18-42 (3, 25) was cloned between the *Bam*HI and *Sal*I sites of pACYC184, the *Bam*HI and *Pst*I sites of pACYC177, and the *Eco*RI and *Sal*I sites of pBR322. Left columns present the resultant plasmids and vectors used. Diagrams to the right represent the tentative locations of the *gutCABD* operon and illustrate insertion fragments (solid lines) of *E. coli* chromosomal DNA in the vectors. The middle diagram is a restriction map of the 4-kb fragment between the *Bam*HI and *Sal*I sites. The *Sal*I site is also digested by *Acc*I and *Hinc*II. The bottom diagrams represent further subcloning of the 4-kb *Bam*HI-*Sal*I fragment. pMYS140 and pMYS139 have insertions of the *Bam*HI-*Eco*RV and *Kpn*I-*Sal*I fragments from pMYS24 between the *Bam*HI and *Hinc*II sites and between the *Kpn*I and *Sal*I sites of pUC18, respectively. pMYS157 is a pMYS139 derivative in which the *Acc*I-*Sal*I fragment was deleted.

Enzyme assays. Cell cultures (250 ml) were grown to the late log phase and then harvested, washed, and suspended in 5 ml of buffer (10 mM Tris hydrochloride, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5). The cells were broken by passage through a French pressure cell (10,000 lb/in^2), and unbroken cells were removed by centrifugation. The crude extracts were used for assay.

Phosphoenolpyruvate-dependent glucitol phophorylation activity was assayed as described previously (5). Phosphorylation was determined in a total volume of 0.2 ml containing 50 mM Tris hydrochloride (pH 8.0), 12.5 mM $MgCl_2$, 25 mM KF, 2.5 mM dithiothreitol, 5 mM phosphoenolpyruvate, and 0.01 mM [^{14}C]glucitol (5 μCi/μmol). A high-speed supernatant which contained excess quantities of enzyme I and HPr was supplied to the assay mixture as described previously (22). For the complementation assays, extracts from *S. typhimurium* LJ530 and LJ531 were used as sources of enzyme II^{Gut} and enzyme III^{Gut}, respectively. Glucitol-6-phosphate dehydrogenase assays were performed as described previously (20). Protein was determined by the method of Lowry et al. (15), with bovine serum albumin as the protein standard.

Identification of plasmid-encoded proteins in maxicells. Maxicell experiments were performed by the method of Sancar et al. (24) with *E. coli* CSR603 harboring the plasmid of interest. Plasmid-encoded proteins were labeled with a ^{14}C-labeled protein hydrolysate (15 μCi/ml) in Hershey medium containing 0.5% glucitol (instead of glucose) and 1 mM cyclic AMP.

Enzymes and radionucleotides. Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were obtained from Bethesda Research Laboratories. ^{14}C-protein hydrolysate (specific activity, 57 mCi/milliatom carbon) was a product of Amersham Corp.

RESULTS

Cloning of the *gut* operon encoding the enzyme II^{Gut}, enzyme III^{gut}, and glucitol-6-phosphate dehydrogenase. The *gut* operon has been reported to be included in pLC18-42 (3, 25). With the combinations *Bam*HI and *Sal*I, *Bam*HI and *Pst*I, and *Eco*RI and *Sal*I, pLC18-42 was digested and ligated with vectors pACYC177, pACYC184, and pBR322, respectively, which had been digested with the same combinations of enzymes. The ligated materials were introduced into strain 236sr, which contained *gut* and *mtl* mutations as genetic markers. Among the transformants obtained on EMB plates containing glucitol and the appropriate antibiotics, colonies capable of fermentation were screened. The sizes of the insertion fragments in pMYS5, pMYS24, and pMYS28 (Fig. 1) were estimated to be about 4, 6, and 6 kilobases (kb), respectively, by agarose gel electrophoresis. The restriction sites of the *Acc*I, *Hinc*II, and *Kpn*I endonucleases in pMYS5 were identified in the corresponding positions on plasmids pMYS24 and pMYS28 (Fig. 1). From these results, it is clear that the *gut* operon was located in the 4-kb region between the *Bam*HI and *Sal*I sites.

Phosphoenolpyruvate-dependent phosphotransferase and glucitol-6-phosphate dehydrogenase activities. Cells harboring individual recombinant plasmids carrying the *gut* operon expressed higher activities than cells harboring only the vector in both the PTS and dehydrogenase assays (Table 1). The cells harboring pMYS24 showed inducible expression of both enzyme activities, while the cells harboring pMYS5 and pMYS28 showed constitutive expression of both activities. pMYS5 and pMYS28 lacked the *Sal*I-*Pst*I fragment which was present in pMYS24. The latter two plasmids showed 9- to 18-fold-higher activities of both enzymes than did the former plasmid. These data suggested that the complete *gut* operon is localized between the *Bam*HI and *Sal*I sites and that the *gut*R gene, encoding a repressor of the *gut* operon, is located either in the 2-kb region between the *Sal*I and *Pst*I sites or close to the *Sal*I site. This positional assignment of the *gut*R gene relative to the *gut* operon agrees with the mapping results published by Csonka and Clark (4).

Localization of the *gut* operon. To analyze the region of the *gut* operon between the *Bam*HI and *Sal*I sites, Bal 31 deletion analysis was carried out (Fig. 2). pMYS5 was digested with *Bam*HI or *Sal*I, treated with Bal 31 exonuclease, and ligated with T4 DNA ligase. Chloramphenicol-resistant transformants were checked for their fermentation responses on EMB plates containing glucitol, and the approximate sizes of the plasmids were determined by agarose gel electrophoresis. Deletion sizes were then esti-

TABLE 1. Induction of the glucitol-specific enzymes

Strain	Glucitol (0.5%) added to LB broth	PEP-dependent[a] glucitol phosphotransferase (nmol/min per mg of protein)	Glucitol-6-phosphate dehydrogenase (μmol/min per mg of protein)
236sr(pACYC177)	+	2.2	0.01
	−	2.5	0.01
236sr(pMYS24)	+	23	0.92
	−	7.5	0.19
236sr(pMYS5)	+	210	17
	−	290	17
236sr(pMYS28)	+	210	16
	−	240	17

[a] PEP, Phosphoenolpyruvate.

mated by PAGE after digestion of the deletion plasmids with the *Taq*I, *Sau*3A, or *Alu*I endonucleases in which pMYS5 and the 4-kb fragment between *Bam*HI and *Sal*I digested with the same endonucleases were used as controls.

Cells bearing the plasmid pMYS35, which contains an internal deletion mutation, lost enzyme II^{Gut} activity but still possessed low activities of enzyme III^{Gut} and glucitol-6-phosphate dehydrogenase compared with the parental plasmid pMYS5. The activities from pMYS35 were at least 5- to 10-fold higher than those of cells bearing the vector pACYC184. These results indicate that pMYS35 lacked the promoter of the *gut* operon and at least part of the enzyme II^{Gut} structural gene. The residual activities of the III^{Gut} and glucitol-6-phosphate dehydrogenase might be due to readthrough from a promoter on the vector. Similar results were obtained with the deletion mutant pMYS20, which lacked the portion of the *gut* operon between *Bam*HI and *Kpn*I from pMYS5 (Fig. 2). In cells harboring pMYS34, no activity of enzyme II^{Gut} was detected, but low activities of III^{Gut} and the glucitol-6-phosphate dehydrogenase were present. pMYS31 and pMYS32 were capable of glucitol fermentation, but pMYS35 and pMYS34 were not (Fig. 2). Therefore, the promoter of the *gut* operon appears to be located about 0.5 to 1.0 kb to the right side of the *Bam*HI site in the 4-kb fragment. Furthermore, there seem to be no distinct promoters for the genes encoding III^{Gut} and the glucitol-6-phosphate dehydrogenase.

Deletion mutants (pMYS46 and pMYS47) generated by restriction at the *Sal*I site and lacking 0.1 to 0.2 kb of DNA exhibited a positive glucitol fermentation phenotype. We could not obtain deletion mutations between pMYS47 and pMYS45 (indicated by a bracket in Fig. 2). A possible explanation for this might be that this region includes the *gutD* gene. Deletion mutants which lack *gutD* but still possess the complete *gutA* and *gutB* genes are unstable in vivo. From these considerations, it is suggested that the gene

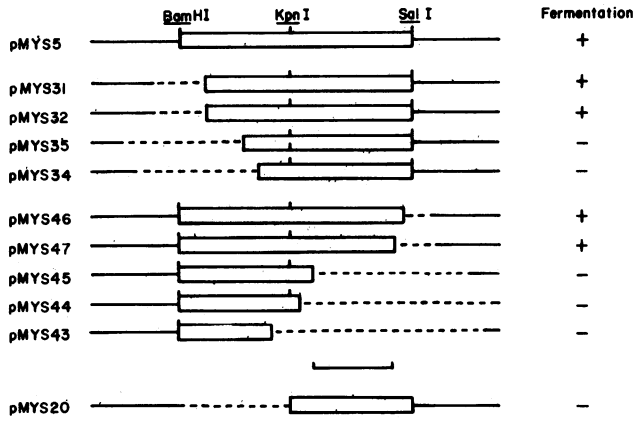

FIG. 2. Deletion mutants of pMYS5. The top diagrams show Bal 31 deletion mutations from the *Bam*HI site in pMYS5. Middle diagrams show Bal 31 deletion mutations from the *Sal*I site in pMYS5. The bottom diagram illustrates the deletion mutation between the *Bam*HI and *Kpn*I sites which resulted from digestion with *Bam*HI and *Kpn*I, treatment with T4 DNA polymerase in the presence of deoxynucleoside triphosphates, and ligation. The open boxes indicate the insertion fragments containing the *gut* operon from the *E. coli* chromosome, and the solid lines indicate parts of the vector, pACY184. The deleted regions are indicated by dotted lines. A bracket shows the region in which it seemed to be difficult to make deletions, possibly because this is the region encoding the *gutD* gene.

TABLE 2. Complementation assays of clones carrying the *gutA* and *gutB* genes and of a frameshift mutation at the *Kpn* site

Host	Plasmid	PEP-dependent[a] glucitol phosphotransferase (nmol/min per mg of protein) in extract from:		Glucitol-6-phosphate dehydrogenase (μmol/min per mg of protein)
		LJ530[b]	LJ531[c]	
JM103	pUC18	3.0	1.5	0.004
	pMYS139	870	2.0	43
	pMYS140[d]	20.0	220	0.094
	pMYS157	820	1.7	0.004
236sr	None	3.0	2.0	0.004
	pMYS170	310	3.0	14

[a] PEP, Phosphoenolpyruvate.
[b] This extract contained all enzymes essential for glucitol phosphorylation except enzyme III^{Gut}.
[c] This extract contained all enzymes essential for glucitol phosphorylation except enzyme II^{Gut}.
[d] Elevated activities of III^{Gut} and glucitol-6-phosphate dehydrogenase with pMYS140 may be due to dilution of the *gut* promoter by the cloned promoter and increased expression of the chromosomal *gut* operon.

order of the *gut* operon is *gutC-gutA-gutB-gutD* and that the operon is distal to the *Bam*HI site. This gene order is coincident with previously published transductional analyses (12, 13, 26, 27). Furthermore, the *gut* operon appears to start about 0.5 kb to the right of the *Bam*HI site and end about 0.2 kb to the left of the *Sal*I site (Fig. 1). The operon is probably encoded in a 3.3-kb fragment.

Localization of *gutA*, *gutB*, and *gutD* genes. To determine the precise localization of the *gutA*, *gutB*, and *gutD* genes, further subcloning was performed. The *Bam*HI-*Eco*RV and *Kpn*I-*Sal*I fragments from pMYS24 were inserted by ligation into the *Bam*HI-*Hin*cII site and the *Kpn*I-*Sal*I site of pUC18, respectively (Fig. 1). The recombinants were selected as white colonies on an LB plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (30 μg/ml) and ampicillin after the ligated materials were introduced into JM103. The insertions were analyzed by restriction mapping. The direction of the *lac* promoter in the recombinants proved to be the same as that of the *gut* operon. Complementation assays with *S. typhimurium* LJ530 (*gutA*$^+$ and *gutB*) and LJ531 (*gutA* and *gutB*+) revealed that pMYS140, possessing the *Bam*HI-*Eco*RV fragment, expressed enzyme II^{Gut} activity (Table 2), while pMYS139, possessing the *Kpn*I-*Sal*I fragment, expressed enzyme III^{Gut} and glucitol-6-phosphate dehydrogenase activities. The activities of the enzyme III and glucitol-6-phosphate dehydrogenase in cells harboring pMYS140 were slightly higher than in cells harboring pUC18. The explanation for this observation may be that the repressor of the *gut* operon was diluted by the many copies of the promoter, so that the chromosomal *gut* operon was partially expressed.

pMYS157 was constructed by deletion of the fragment between the *Acc*I and *Sal*I restriction sites of pMYS139. Since the *Sal*I site is also cleaved by *Acc*I, the deletion was introduced by complete digestion with *Acc*I, followed by ligation. The presence of the deletion was ensured by restriction mapping analysis. Cells harboring pMYS157 retained enzyme III activity but not glucitol-6-phosphate dehydrogenase activity (Table 2). Therefore, it is clear that the *gutB* gene is encoded in a 1.5-kb nucleotide fragment between the *Kpn*I and *Acc*I sites, while the *gutD* gene is encoded in the region around the *Acc*I site, immediately after the *gutB* gene.

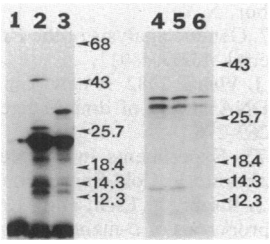

FIG. 3. Analysis of the gene products encoded by the *gut* operon. Maxicells were irradiated and labeled with ^{14}C-protein hydrolysate, and the proteins were separated on a 13% polyacrylamide gel. An autoradiogram of the gel is presented. Lanes: 1, CSR603 with no plasmid; 2, pMYS5; 3, pACYC184; 4, pMYS139; 5, pMYS157; 6, pUC18. Molecular weight markers used were bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), lysozyme (14,300), and cytochrome *c* (12,300). Sizes are indicated (in kilodaltons).

To confirm the region including the *gutA* gene, frameshift mutations were introduced at the *Kpn*I sites. pMYS5 was digested with *Kpn*I, treated wtih T4 DNA polymerase, and ligated with T4 DNA ligase. With these procedures, four nucleotides were deleted in each strand at the *Kpn*I site (28) so that the frame of one enzyme was changed when the *Kpn*I site was present in one of the coding sequences. The ligation mixture was used for the transformation of strain 236sr, nonfermentating colonies were selected, and the plasmids from the colonies were checked for the missing *Kpn*I site by digestion with *Kpn*I. One of the resultant frameshift mutants, pMYS170, was used for the PTS complementation assay (Table 2). The results clearly indicate that the cells harboring plasmid pMYS170 lacked only enzyme II^{Gut} activity. Thus, the *Kpn*I site exists in the *gutA* gene. These results confirm that the gene order of the *gut* operon is *gutC-gutA-gutB-gutD*, as described above.

Identification of gene products of the *gut* operon with maxicells. pMYS5 and its vector, pACYC184, were introduced into CSR603 (24), and the gene products of the *gut* operon were detected by sodium dodecyl sulfate-PAGE. (Fig. 3). Protein bands of molecular weights 33,000 and 24,000 in the cells harboring pACYC184 (Fig. 3, lane 3) appeared to correspond with the products of the tetracycline and chloramphenicol resistance genes, respectively. In the cells harboring pMYS5 (Fig. 3, lane 2), in which the 4-kb fragment of the *gut* operon was inserted into the tetracycline resistance gene (Fig. 1), the protein band of M_r 33,000 disappeared, and two protein bands of 46,000 and 27,000 M_r appeared. The latter two proteins were lacking in cells harboring its vector, pACYC184. The protein bands of 46,000 and 27,000 daltons had the same molecular weights as the partially purified enzyme II^{Gut} from *S. typhimurium* (F. C. Grenier and M. H. Saier, Jr., unpublished results) and homogenous glucitol-6-phosphate dehydrogenase from *E. coli* (20), respectively. The band corresponding to enzyme III^{Gut} may overlap those of the other proteins encoded on the vector.

Since enzyme III^{Gut} could not be detected in the experiment described above with cells harboring pMYS5, other subclones were used for detection of this protein. pMYS139 and pMYS157 have insertions of the 2.4-kb fragment between the *Kpn*I and *Sal*I sites and of the 1.5-kb fragment between the *Kpn*I and *Acc*I sites of the vector pUC18, respectively (Fig. 1). The proteins of M_r 30,000 and 26,900 in cells harboring pUC18 appeared to correspond to the precursor and mature forms of β-lactamase derived from the ampicillin resistance gene (Fig. 3, lane 6). In cells harboring pMYS139, two protein bands of 27,000 and 13,500 M_r were detected in addition to the bands detected in cells harboring its vector, pUC18, In cells harboring pMYS157, which lacked glucitol-6-phosphate dehydrogenase due to deletion of the 0.8-kb *Acc*I-*Sal*I fragment from pMYS139, the protein band of M_r 13,500 was detected but that of M_r 27,000 was lacking. The protein of M_r 13,500 must be enzyme III^{Gut}, since the molecular weight agreed with that of purified III^{gut} from *S. typhimurium* (5). These results confirm that the molecular weights of enzyme II^{Gut}, III^{Gut}, and glucitol-6-phosphate dehyrogenase are 46,000, 13,500, and 27,000, respectively (5, 20).

We also identified the gene products of the *gut* operon in experiments with cellular fractionation procedures, including butanol-urea extraction (22) (data not shown). In these experiments as well as in maxicell experiments, we noticed that it was most difficult to detect enzyme II^{Gut} and that the glucitol-6-phosphate dehydrogenase was more easily detected than the two PTS enzymes. Because it is not likely that the individual genes of the *gut* operon have their own promoters (as discussed above), the differences in the levels of the gene products may be due to translational or post-translational regulatory interactions.

DISCUSSION

In the work described in this communication, we subcloned the *gut* operon and determined the approximate locations of the *gutC*, *gutA*, *gutB*, and *gutD* genes in the operon. We also identified the products of these genes in experiments with maxicells. Evaluation of the sum of the molecular weights of enzyme II^{Gut} (46,000), III^{Gut} (13,500), and glucitol-6-phosphate dehydrogenase (27,000) suggests that at least 2.5 kb of DNA is required to encode these structural genes. The *gutC* gene, the presumed promoter-operator region, is likely to be very small. The total estimated length of the *gut* operon is in accord with the length (about 3.3 kb) which was estimated in the experiments with deletion mutants done to determine the location of the operon. The DNA sequence determination of the *gut* operon will be valuable in comparative studies with the *mtl* operon. Such information may provide clues to the evolutionary relationship between these two operons.

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