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Cloning and Molecular Genetic Characterization of the *Escherichia coli gntR*, *gntK*, and *gntU* Genes of GntI, the Main System for Gluconate Metabolism

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Three genes involved in gluconate metabolism, *gntR*, *gntK*, and *gntU*, which code for a regulatory protein, a gluconate kinase, and a gluconate transporter, respectively, were cloned from *Escherichia coli* K-12 on the basis of their known locations on the genomic restriction map. The gene order is *gntU*, *gntK*, and *gntR*, which are immediately adjacent to *asd* at 77.0 min, and all three genes are transcribed in the counterclockwise direction. The *gntR* product is 331 amino acids long, with a helix-turn-helix motif typical of a regulatory protein. The *gntK* gene encodes a 175-amino-acid polypeptide that has an ATP-binding motif similar to those found in other sugar kinases. While GntK does not show significant sequence similarity to any known sugar kinases, it is 45% identical to a second putative gluconate kinase from *E. coli*, *gntV*. The 445-amino-acid sequence encoded by *gntU* has a secondary structure typical of membrane-spanning transport proteins and is 37% identical to the *gntP* product from *Bacillus subtilis*. Kinetic analysis of GntU indicates an apparent K_m for gluconate of 212 μM, indicating that this is a low-affinity transporter. Studies demonstrate that the *gntR* gene is monocistronic, while the *gntU* and *gntK* genes, which are separated by only 3 bp, form an operon. Expression of *gntR* is essentially constitutive, while expression of *gntKU* is induced by gluconate and is subject to fourfold glucose catabolite repression. These results confirm that *gntK* and *gntU*, together with another gluconate transport gene, *gntT*, constitute the GntI system for gluconate utilization, under control of the *gntR* gene product, which is also responsible for induction of the *edd* and *eda* genes of the Entner-Doudoroff pathway.

Escherichia coli is found in the large intestines of vertebrates, usually as a minority member of the normal flora, and is capable of utilizing a wide range of carbohydrates (40). Sugars are normally catabolized via the Embden-Meyerhof-Parnas glycolytic pathway and the pentose phosphate pathway, which are the two central and constitutive routes of intermediary carbohydrate metabolism in *E. coli* (17). A third central route, the Entner-Doudoroff pathway, was discovered in 1952 in *Pseudomonas saccharophila* (12) and was later shown to be present in *E. coli* (13, 45).

The Entner-Doudoroff pathway, as it operates in *E. coli*, is specifically induced by gluconate and allows its entry into central glycolytic metabolism (for a review, see reference 6). Early genetic studies of gluconate metabolism revealed some of the genetic loci involved in gluconate transport and gluconate phosphorylation, as well as the key enzymes of the Entner-Doudoroff pathway (1, 9, 14, 24, 32, 46). There are two systems for gluconate transport and phosphorylation in *E. coli* (1, 24). GntI, the main system, contains *gntT*, *gntU*, and *gntK*, which code for high- and low-affinity gluconate transporters and a thermoresistant gluconokinase, respectively. The genes *gntT*, *gntU*, and *gntK* are located in the *bioH-asd* region of the chromosome, at 77.0 min on the *E. coli* genome (37). Induction by gluconate of the genes of the GntI system, together with the Entner-Doudoroff genes *edd* and *eda* (encoding 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase), is negatively regulated by the *gntR* product, also mapped at 77.0 min. GntII, the subsidiary system, contains *gntS* and *gntV*, located in the 96-min region, which are believed to encode a second high-affinity gluconate transporter and a thermosensitive gluconokinase, respectively (1, 29). Traditional genetic approaches have not fully explained the mechanism by which the GntII system is induced, nor have they explained why *E. coli* needs more than one gluconate transporter and gluconokinase.

Molecular genetic approaches are being used to provide additional insights into the gene expression and physiology of gluconate metabolism in *E. coli*. Previous work in this laboratory on the molecular genetics of the Entner-Doudoroff genes showed that *edd* and *eda* are cotranscribed from a gluconate-inducible promoter that appears to be devoid of any additional regulation, such as catabolite repression (11, 16). In this paper, we report on the molecular characterization of the *gntR*, *gntK*, and *gntU* genes from *E. coli*. On the basis of known locations of several *gnt* genes on the digitized *E. coli* genomic restriction map (37), specific restriction fragments containing the genes of interest were cloned from the Kohara library (25). The identities of these clones were confirmed by genetic complementation of appropriate *E. coli* mutants and by biochemical assaying, as well as by DNA sequence analysis. Expression of the *gnt* genes under various growth conditions was investigated by Northern (RNA) blot analysis, and mRNA end mapping was used to provide information about the promoter regions of these genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains, plasmids, and phages used in this study are listed in Table 1. The wild-type *E. coli* K-12 strain, *E. coli* W1485, was used for the isolation of total cellular RNA. *E. coli* DH5α was used for propagating plasmids. *E. coli* TUG287 F^-, *E. coli*

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TABLE 1. Plasmids, phages, and strains used

Strain, plasmid, or phage	Relevant genotype	Source
E. coli		
DH5α	*lacZ* ΔM15 *recA*	BRL[a]
DH5α F′	F$^+$ *lacZ* ΔM15 *recA*	BRL
W1485	K-12 wild type	Laboratory stock
HfrG6ΔMD2	*hfrG* Δ(*bioH-gntT-malA-glpRGED-glgPACXB-asd-gntUKR*) *his edd*[c]	T. Isturiz (from M. Schwartz)
TUG287	F$^-$ *gntK gntV his trp*	T. Isturiz
M6	*hfrC gntT gntU gntR*	T. Isturiz
Plasmids		
pBluescript II	*bla lacI lacZ* f1 origin	Stratagene
pTC220	3.98-kb *Bam*HI fragment (*gntR gntK gntU*)	This study
pTC220A	*gntR* probe	This study
pTC220B	*gntK* probe	This study
pTC220C	*gntU* probe	This study
pTC221	3.98-kb DNA *Bam*HI fragment in opposite orientation with respect to the *lac* promoter	This study
pTC222	*gntR* and *gntK* promoter deleted on pTC220	This study
pTC223	Frameshift mutation in *gntK* on pTC220	This study
Phages		
M13K07		Laboratory stock
λ615		Kohara library (25)

[a] BRL, Bethesda Research Laboratories.

HfrG6ΔMD2, and *E. coli* M6 were used to test genetic complementation of lesions in *gntK*, *gntR*, and *gntU*, respectively. The phagemid vector pBluescript II was used for all subclones. Phage M13K07 was used as helper phage for propagating single-stranded DNA from vector pBluescript II in *E. coli* DH5α F′.

All *E. coli* strains were routinely grown at 37°C in Luria broth (27) with or without added carbohydrate (0.4%), and growth was monitored by measuring the turbidity at 550 nm with a Spectronic 601 (Milton Roy Co.) spectrophotometer. Cultures in mid-logarithmic phase were harvested at an A_{550} of 0.4, and stationary-phase cultures were harvested approximately 3 h after inflection of the growth curve at an A_{550} of 1.8. Aerobic growth was achieved by gyratory shaking at 250 rpm, and anaerobic growth was accomplished with oxygen-free medium in sealed bottles. Phenotypes of *E. coli* strains were monitored on MacConkey indicator medium (28), bromothymol blue (BTB) medium (4), or M63 minimal medium (42) with appropriate supplements as required. Ampicillin (50 mg/liter) was included in the medium to select for cells harboring ampicillin-resistant plasmids. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 mg/liter) was used to identify recombinant plasmids with DNA insertions that inactivated β-galactosidase activity in *E. coli* DH5α.

DNA manipulations and transformation. Plasmid DNA was isolated from *E. coli* with an alkaline-sodium dodecyl sulfate (SDS) cell lysis miniprep protocol (38). For large-scale preparations, a further purification by equilibrium centrifugation in CsCl gradients was performed (38). DNA restriction digests, ligations, transformations, and other DNA manipulations were carried out by using standard methods (38), as specified by the manufacturers.

Cloning of the *gnt* genes. A 3.98-kb *Bam*HI restriction fragment, predicted to contain genes involved in gluconate utilization, was isolated from Kohara library clone no. 615 and was cloned into the *Bam*HI site of pBluescriptII SK$^-$ in both orientations to construct pTC220 and pTC221. The presence of *gntK* and *gntU* on these plasmids was confirmed by genetic complementation of *E. coli* Hfr6GΔMD2 (multiply defective in gluconate transport, phosphorylation, and gluconate regulation), which restored growth of the mutant on gluconate minimal medium. Inducibility of *edd* by gluconate in *E. coli* Hfr6GΔMD2(pTC220) was also restored, indicating the presence of *gntR*. pTC222 was prepared by digestion of pTC221 with *Bcl*I and *Hin*dIII and then by formation of blunt ends with the Klenow fragment of DNA polymerase and ligation, resulting in a clone lacking *gntR* and the first 8 codons of *gntK*. A frameshift was created in *gntK* by digestion of pTC221 with *Bcl*I and then by formation of blunt ends with the Klenow fragment of DNA polymerase and ligation, resulting in pTC223.

Enzyme assays. Cells were washed twice in 20 ml of 100 mM potassium phosphate buffer (pH 7) and were resuspended in the same buffer to a final A_{550} of 1.0. A 2-ml volume of this cell suspension was pelleted by centrifugation and resuspended in 500 μl of assay buffer, consisting of 20 mM morpholineethanesulfonic acid (MES) (pH 6.5), containing 30 mM KCl and 5 mM $MnCl_2$. Cells were disrupted by sonic oscillation for 30 s (three bursts of 10 s each, with 60 s between bursts) by using a Fisher Sonic Dismembrator model 300. After centrifugation (13,000 × *g* for 10 min at 4°C), the supernatant was used as a cell extract. The protein concentration was determined by the method described by Lowry et al. (26), with bovine serum albumin as the standard. 6-Phosphogluconate dehydratase activity (Edd) was measured spectrophotometrically as described elsewhere (45). GntK activity was assayed photometrically at 25°C essentially as described by Pouyssegur and Stoeber (35). A 50-μl volume of cell extract was added to 950 μl of assay solution containing 50 μmol of Tris-HCl buffer (pH 8.0), 3 μmol of $MgCl_2$, 0.25 μmol of $NADP^+$, 10 μmol of sodium gluconate as a substrate, and 25 μg of crystalline 6-phosphogluconate dehydrogenase as the coupling enzyme. The appearance of NADPH was monitored spectrophotometrically at 340 nm. The values were converted to specific activities, assuming a molar extinction coefficient of 6.2×10^3 for NADPH, and were expressed as nanomoles of NADPH produced per minute per milligram of total soluble cell protein.

Gluconate uptake. Cells used in uptake experiments were harvested from logarithmic cultures by centrifugation at 10,000 × *g* for 5 min at 4°C. After being washed with 0.05 M potassium phosphate buffer (pH 6.8) containing 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter, the cells were resuspended in the same buffer to an A_{550} of 5.0 and were prewarmed at 30°C for 20 min. Chloramphenicol (50 μg/ml) was added to prevent de novo protein synthesis. The uptake reaction was started by the addition of 15 μl of sodium [6-^{14}C]gluconate (3.4 Ci/mol) to 50 μl of cell suspension in order to achieve a predetermined final concentration of isotope (1 to 800 μM with a constant specific activity of 5.6 μCi/μmol). After 5 s (5-s uptake times used for single-time point kinetics reflect initial velocities, which were found to be linear for the first 20 s or longer), uptake was terminated by quenching with 5 ml of ice-cold 250 mM gluconate in 0.05 M potassium phosphate buffer maintained at a temperature of less than −3°C on a salt-ice mixture (31). The cells were filtered immediately with suction through membrane filters (0.45-μm pore size) and were washed again with 5 ml of the same buffer. The filters were then dissolved in 5 ml of scintillant in scintillation vials. Assays were conducted in triplicate. The whole process from quenching to the last step was done within 10 s. Radioactivity was measured with an LKB 1219 Rackbeta liquid scintillation counter. The data were analyzed by a least-squares fit to the hyperbola as described previously (31).

Substrate analog inhibition. For determination of the substrate specificity of gluconate transport, 50 μl of cell suspension was preincubated with competing sugar at a concentration of 2 mmol at 25°C for 3 min, and then uptake of 50 μM sodium [6-^{14}C]gluconate was assayed in the presence of a 40-fold excess of competing, unlabeled sugar. The process of filtering, washing, and counting of radioactivity was the same as that described above. The results are expressed as a fraction of the rate of [^{14}C]gluconate uptake by the control.

DNA sequencing and analysis. The 3.98-kb *Bam*HI fragment of pTC220 and pTC221, containing the same 3.98-kb *Bam*HI insert in the opposite orientation, was sequenced in both directions by the dideoxy chain termination method described by Sanger et al. (39) with the Sequenase version 2 DNA sequencing kit. Nested deletions were created with the Erase-a-base kit in accordance with the manufacturer's instructions. Single-stranded DNA from an appropriate deletion series of subclones was obtained with M13K07 helper phage according to a previously described method (38). Sequencing primer was either the T7 promoter primer or the T3 promoter primer. The sequence of the region containing the *gntK* start codon was obtained by PCR amplification (with the upstream primer, 5′-GCGGATCTATTTAAGCC, and the downstream primer, 5′-GCG CATCAACTTCATGCC) and subcloning of the blunt-end fragments into pUC18. These fragments were sequenced by the double-stranded (collapsed) plasmid method (7). DNA sequences were analyzed by using the University of Wisconsin Genetics Computing Group (UWGCG) package (10). The protein and DNA databases used for homology searches were the GenBank databases (TFASTA program [UWGCG]).

Probe construction and labeling. Antisense RNA probes were used for Northern blot hybridization. The *gntR* probe (pTC220A) was prepared from a deletion subclone of pTC220 covering positions 311 (*Sma*I site) to 977 (deletion endpoint adjacent to the T3 promoter) of the sequence (see Fig. 1). The *gntK* probe (pTC220B) was prepared from a deletion subclone of pTC221 which had been digested with *Pst*I and religated, covering positions 1390 (deletion subclone endpoint) to 1927 (*Pst*I site adjacent to T7 promoter) of the sequence. The *gntU* probe (pTC220C) was prepared from a deletion subclone of pTC221 which had been digested with *Pst*I and religated, covering positions 2431 (deletion subclone endpoint) to 3081 (*Pst*I site adjacent to the T7 promoter) of the sequence. The plasmids were linearized with appropriate restriction enzymes and then by synthesis of digoxigenin-dUTP-labeled RNAs with T3 RNA polymerase (*gntR* probe) or T7 RNA polymerase (*gntK* and *gntU* probes). The amount of digoxigenin-labeled RNA probe was estimated by direct detection of the labeled RNA probe with anti-digoxigenin-alkaline phosphatase.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from *E. coli* W1485 grown to either mid-log phase (optical density at 550 nm of 0.4) or stationary phase (optical density at 550 nm of 1.7) by the hot phenol method (7). Five milliliters of culture was pipetted into 2.5 ml of lysis buffer containing 3% SDS, 300 mM NaAC, and 30 mM EDTA at 100°C in a boiling water bath until cell lysis occurred. This was followed by two extractions with

phenol (pH 4.0) at 60°C. After a third extraction at 60°C with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1), the aqueous phase was precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water. Contaminating DNA was removed by digestion with RNase-free DNase in nick translation buffer. The concentration of RNA was determined spectrophotometrically. RNA samples were stored at −70°C.

RNA samples (5 μg) were electrophoresed on a 1.5% agarose-formaldehyde denaturing gel in MOPS buffer (20 mM 3-[*N*-morpholine]propanesulfonic acid, 5 mM sodium acetate, 2 mM EDTA [pH 7.0]). After the gel was washed twice for 5 min in deionized water, RNA was transferred overnight with 20× standard SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to a GeneScreen Plus membrane with an upward capillary system. The nucleic acids were UV cross-linked to the membrane.

After prehybridization at 42°C for 4 h, hybridization was carried out overnight at 65°C in 5× SSC–0.1% *N*-sodium lauryl sarcosinate–0.02% SDS–1% blocking agent–probe at a concentration of 20 ng/ml. The blot was washed twice with 2× SSC–0.1% SDS at room temperature for 5 min and then twice with 0.5× SSC–0.1% SDS at 65°C for 15 min. The digoxigenin-labeled probe was detected with the kit and was visualized by exposure to Kodak XAR5 film at room temperature.

Transcript end mapping. The 5′ termini of the *gntR* and *gntKU* transcripts were determined by primer extension analysis (7). An oligonucleotide (5′-GGC ATGAAGTTGATGCGC) complementary to the mRNA sequence from bases 81 to 98 downstream of the *gntK* start codon (TTG) and an oligonucleotide (5′-GCGACGGAAACCTGCTCCGG) complementary to the mRNA sequence from bases 81 to 100 downstream of the *gntR* start codon (ATG) were labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP (>5,000 Ci mmol^{-1}) as described elsewhere (38). A 20-μg amount of total RNA and 0.1 pmol (~200,000 cpm) of 5′-end-labeled oligonucleotide primer were heated at 90°C for 4 min and then hybridized at 42°C for 5 h in 10 μl of 9 mM Tris-HCl (pH 8.3)–0.35 mM EDTA (7). Reverse transcription with 30 U of SuperScript II RNase H$^-$ reverse transcriptase was carried out at 42°C for 30 min in 25 μl of a mixture containing 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 4 mM dithiothreitol, and 1 mM each deoxyribonucleotide. RNA was then degraded with DNase-free RNase A (100 μg/ml, 1 h, 37°C). A sequencing reaction with the same primer and pTC220 as the template was run in parallel as a reference for determining the endpoint of the extension product.

S1 nuclease protection experiments were as described previously (7). A 600-bp restriction fragment which begins at the *Pvu*II site 417 bases upstream of the *gntK* TTG start codon and ends at the *Pvu*I site 183 bases downstream of the *gntK* TTG start codon was end labeled with T4 DNA polymerase and [γ-^{32}P]dTTP (>5,000 Ci mmol^{-1}) (38). This radiolabeled probe (200,000 cpm) was mixed with 20 μg of total RNA in a volume of 50 μl of 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–0.4 M NaCl–1 mM EDTA (pH 8.0). The mixture was overlaid with heavy mineral oil, heated at 90°C for 10 min, hybridized at room temperature overnight, and then digested with 50 or 100 U of S1 nuclease for 30 min at 30°C. After ethanol precipitation, samples were electrophoresed adjacent to a sequence ladder in a sequencing gel.

Chemicals and enzymes. Restriction enzymes, DNA-modifying enzymes, and reverse transcriptase used throughout this study were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The Sequenase kit was ordered from U.S. Biochemical Corp. (Cleveland, Ohio). The Erase-a-base kit was purchased from Promega Corp. (Madison, Wis.). The RNA labeling and detection kit was purchased from Boehringer Mannheim Corp. (Indianapolis, Ind.). Radioactive compounds were purchased from New England Nuclear Corp. (Wilmington, Del.). Biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Cloning and expression of *gntR*, *gntK*, and *gntU*. From the previously determined map locations of *gntK*, *gntU*, and *gntR* on the digitized *E. coli* genomic restriction map (37), subclones of appropriate restriction fragments from Kohara library clone no. 615 (25) were chosen to transform *E. coli* Hfr6GΔMD2, which has a *bioH-asd* deletion and cannot normally grow on gluconate minimal medium because of defects in gluconate transport and phosphorylation. Transformants were selected for their restored ability to grow on minimal gluconate medium. The pTC220 clone, which contains a 3.98-kb *Bam*HI fragment, complemented *E. coli* Hfr6GΔMD2, indicating the presence of both *gntK* and *gntU*. The gluconate-positive phenotype was confirmed by using gluconate BTB and gluconate-MacConkey plates. The pTC220 clone was also able to rescue growth on minimal gluconate medium of *E. coli* TUG287, which is deficient in gluconokinase, and *E. coli* M6, which is deficient in gluconate transport capacity.

TABLE 2. Gluconokinase activity and [^{14}C]gluconate uptake activities

E. coli strain	Gluconokinase activity (SD)[a]	Gluconate uptake (SD)[b]
W1485	65.47 (1.39)	4.31 (0.10)
M6		0.22 (0.003)
M6(pTC220)		6.21 (0.04)
M6(pTC222)		0.19 (0.015)
M6(pTC223)		5.02 (0.16)
TUG287	0.88 (0.04)	
TUG287(pTC220)	67.25 (3.66)	
TUG287(pTC222)	0.35 (0.11)	
TUG287(pTC223)	6.21 (2.55)	

[a] Nanomoles per minute per milligram of total soluble protein.
[b] Uptake of 100 μM [^{14}C]gluconate (nanomoles per minute per milligram of total cell protein).

Further proof that the 3.98-kb *Bam*HI fragment in pTC220 does indeed code for both gluconokinase activity and gluconate transport activity was obtained by biochemical enzyme assaying and [^{14}C]gluconate uptake assaying, respectively (Table 2). Extracts of *E. coli* TUG287 showed no detectable gluconokinase activity. *E. coli* W1485 was used as a positive control. *E. coli* TUG287(pTC220) grown on gluconate expressed gluconokinase activity at a level similar to that of the wild type. Gluconokinase activity remained after incubation at 30°C for 3 h, indicating that GntK is indeed the thermoresistant gluconokinase first described by Hung et al. (23). Uptake of 10^{-4} M [^{14}C]gluconate by *E. coli* M6(pTC220) was higher than that of the *E. coli* W1485 control, confirming the biochemical function of GntU as a gluconate transporter.

The presence of the *gntR* gene on pTC220 was confirmed by analyzing the inducibility of the *edd* gene as indicated by 6-phosphogluconate dehydratase activity (Edd). The *bioH-asd* deletion mutant *E. coli* Hfr6GΔMD2, which is *gntR* negative, constitutively expresses *edd*. Inducibility of *edd* by gluconate in *E. coli* Hfr6GΔMD2 was restored by the presence of the pTC220 clone, suggesting that the *gntR* gene was also cloned in pTC220.

Sequence analysis of the *gntR-gntK-gntU* cluster. The 3.98-kb *Bam*HI fragments in pTC220 and pTC221 have different orientations with respect to the *lac* promoter. A series of overlapping, nested deletion subclones from each plasmid was prepared. The complete nucleotide sequence of the 3.98-kb *Bam*HI fragment was determined in both directions by using 28 nested deletion subclones (Fig. 1). A FRAME search with the UWGCG programs revealed three open reading frames within the sequence.

(i) gntR. The first open reading frame (996 bp) begins with an ATG start codon at bp 370 and ends with a TAA stop codon at bp 1365. A Shine-Dalgarno sequence, AGGA, occurs two nucleotides upstream of the start site (41). The downstream flanking region of the gene contains an apparent rho-independent terminator, beginning at position 1444, consisting of a palindromic sequence followed by a thymidine-rich region, which can form a stem-loop structure with 13 of 15 paired bases (44). The deduced 36-kDa, 331-amino-acid-long polypeptide appears to be a regulatory protein containing a helix-turn-helix motif (MOTIF program [UWGCG]), which is typical of a negative regulator. When the GntR peptide was used as a query sequence (TFASTA program [UWGCG]), several matches to negative regulatory proteins were found. A comparison of GntR with CcpA, the catabolite control protein from *Bacillus subtilis* (22), shows that 30.3% of the residues are

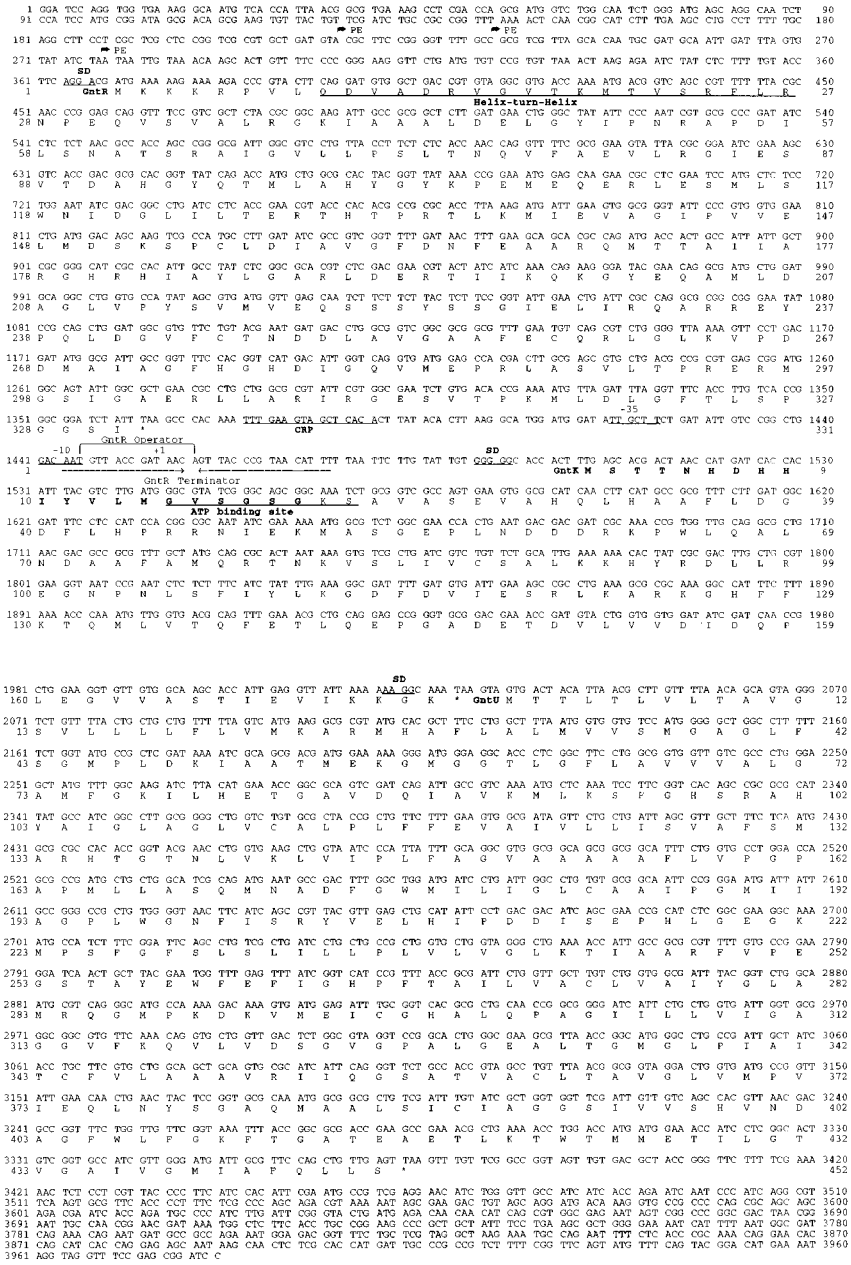

FIG. 1. Nucleotide sequences of *gntR*, *gntK*, and *gntU* genes, and the derived amino acid sequences of the corresponding polypeptides. Possible ribosome-binding sites, putative −10 and −35 elements, a putative CAP site, a helix-turn-helix motif, and ATP-binding site are underlined. The potential transcription terminator is indicated by inverted arrows. Boldfacing indicates residues that were verified by N-terminal peptide sequencing of the purified GntK protein. The possible transcriptional initiation sites for *gntR* and *gntK*, as determined by primer-extension and S1 mapping, are indicated by arrows.

identical and that 47% are chemically conserved, and a comparison of GntR with LacI from *E. coli* (15) shows that 24.4% of the residues are identical and that 44.4% are chemically conserved (data not shown).

(ii) *gntK*. The second open reading frame (528 bp), starting with a rare TTG start codon at position 1504 and ending with a TAA stop codon at position 2031, encodes a 20-kDa, 175-amino-acid polypeptide that has an ATP-binding motif similar to those found in other sugar kinases (MOTIF program [UWGCG]) and is believed to be *gntK*, the gene for glucokinase. Although there are several potential ATG start codons around the beginning of *gntK*, none are in the correct reading frame. The absence of an in-frame ATG start codon was confirmed by sequencing of the *gntK* regulatory regions of other *E. coli* strains, including *E. coli* B, *E. coli* C600, and *E. coli* K-12 (data not shown). Since GntK appeared to start with a very rare TTG (leucine) start codon, and because this was the first gluconokinase to be sequenced, the enzyme was purified and subjected to N-terminal sequence analysis. The first 20 amino acids of purified GntK precisely matched the deduced amino acid sequence derived from the *gntK* DNA sequence, beginning with the second codon (serine), indicating that the N-terminal formyl-methionine is removed in the mature protein (Fig. 1). The *gntK* gene is preceded by a string of G's, but no

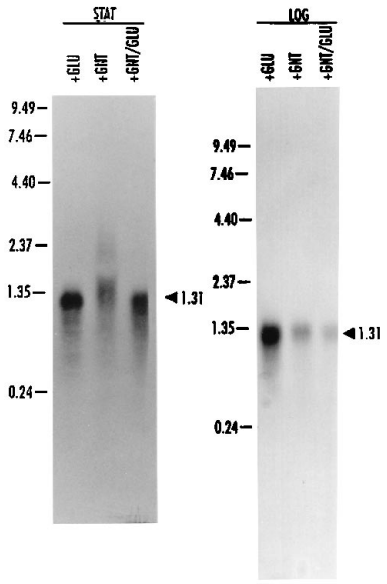

FIG. 2. Northern blot analysis of the *E. coli gntR* transcripts with a *gntR*-specific hybridization probe. RNA samples (5 μg) from *E. coli* W1485 cells grown on different carbon sources and different growth phases were loaded as follows: GLU, Luria broth (LB)-glucose-grown culture; GNT, LB-gluconate-grown culture; GLU/GNT, LB-glucose plus gluconate-grown culture; STAT, stationary phase; LOG, log phase. Size markers in kilobases are provided to the sides of the gels.

obvious Shine-Dalgarno sequence (41) was found in the region immediately upstream of the rare start codon. No potential terminator sequences were identified downstream of the *gntK* gene (44), and a third open reading frame was identified just 3 bp downstream of the *gntK* stop codon. The deduced GntK peptide sequence does not show identity to any known sugar kinase except GntV (43), which is another gluconokinase located at 96.8 min (adjacent to *leuX*) on the *E. coli* chromosome (GenBank accession numbers M96355 and U14003). Comparison of the GntK and GntV peptide sequences of *E. coli* showed the two to be 45.4% identical and 65.6% similar (data not shown).

(iii) *gntU*. The third open reading frame (1,341 bp) starts at position 2034 with a GTG start codon and ends at position 3375 with a TAA stop codon. A potential ribosome-binding site, AAGG, is located within the upstream *gntK* gene and is spaced 10 nucleotides from the start codon, which is located just 3 bp downstream of the *gntK* stop codon. A computer search of the sequence downstream of the *gntU* gene failed to reveal any canonical transcriptional terminator-like sequences, although a thymidine-rich region is present. Comparison of the deduced amino acid sequence of the third open reading frame with that in the GenBank data base (TFASTA program [UW GCG]) showed high similarity to GntP (gluconate permease) from *B. subtilis* (36), suggesting that the third open reading frame encodes a gluconate transporter. The two putative transporters are 37% identical, and 66.6% of the amino acid residues are chemically conserved (data not shown). A hydrophobic moment plot (not shown) indicated that GntU contains numerous membrane-spanning domains and a large hydrophilic loop in the middle, a secondary structure which is representative of a large family of transport proteins (21). It was also noticed that the N and C termini of GntU are unusually hydrophobic, making it unclear whether GntU contains 12 or 14 membrane-spanning domains.

Organization and transcription of the *gntR-gntK-gntU* cluster. This study confirms the gene order *gntU*, *gntK*, and *gntR* in the *bioH-asd* region of the *E. coli* genome (9). All three genes are transcribed in a counterclockwise direction and are immediately adjacent to *asd*, which is located clockwise from *asd* at 77.0 min on the *E. coli* genomic restriction map (37). The *gntU* and *gntK* genes are separated by only 3 bp. The *gntK* start codon lies 138 bp from the *gntR* stop codon.

The transcriptional organization of *gntU*, *gntK*, and *gntR* was examined by Northern blot analysis. Total RNA isolated from *E. coli* W1485 grown under various conditions was hybridized separately with *gntR*-, *gntK*-, and *gntU*-specific probes. The *gntR*-specific probe hybridized to a single 1.31-kb mRNA species, which is of sufficient length to encode only *gntR*, in RNA prepared from cells grown under all growth conditions tested, indicating constitutive expression (Fig. 2). In log-phase cells (but not stationary-phase cells) grown in the presence of gluconate, the amount of *gntR* mRNA was significantly less than the amount detected from cells grown without gluconate. Two *gntK*-specific transcripts of 2.39 and 0.65 kb were present in log-phase cells (but not in stationary-phase cells) grown on gluconate and on gluconate plus glucose (either aerobically or anaerobically), but not in the absence of gluconate, indicating transcriptional regulation by gluconate (Fig. 3). Cells grown on gluconate plus glucose contained approximately one-fourth the level of *gntK* transcripts compared with cells grown on gluconate alone, suggesting that *gntK* transcription is subject to catabolite repression. The 2.39-kb mRNA transcript is of sufficient length to encode both *gntK* and *gntU*, suggesting that the two genes are cotranscribed. The 0.65-kb transcript is of sufficient size to contain the entire coding region of *gntK*. The *gntU*-specific probe also revealed a 2.39-kb mRNA in log-phase cells grown on gluconate and on gluconate plus glucose (also at one-fourth the level of gluconate only), confirming that the 2.39-kb mRNA encodes both genes (Fig. 4). The *gntU*-specific hybridization probe also revealed a minor transcript of

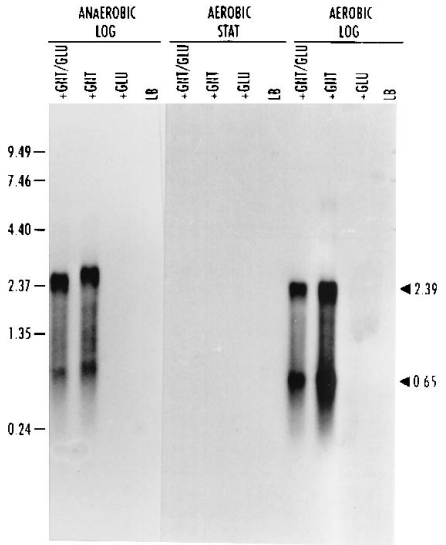

FIG. 3. Northern blot analysis of the *E. coli gntK* transcripts with a *gntK*-specific hybridization probe. RNA samples (5 μg) from *E. coli* W1485 cells grown on different carbon sources, under different growth conditions (aerobic or anaerobic) and different growth phases, were loaded as follows: GLU, culture grown on LB-glucose; GNT, culture grown on LB-gluconate; GNT/GLU, culture grown on LB-glucose plus gluconate; LB, culture grown on LB; STAT, stationary phase; LOG, log phase. Size markers in kilobases are provided to the sides of the blots.

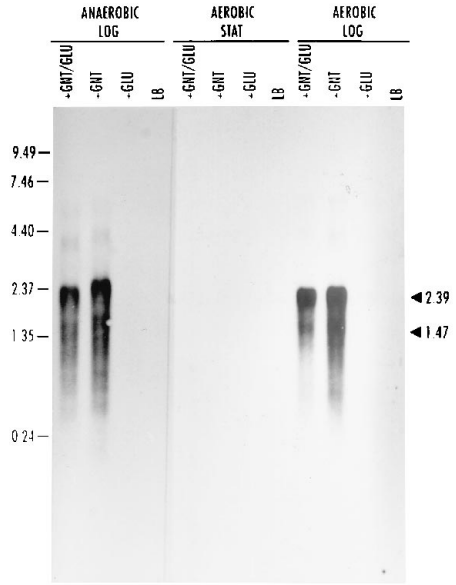

FIG. 4. Northern blot analysis of the *E. coli gntU* transcripts with a *gntU*-specific hybridization probe. RNA samples (5 μg) from *E. coli* W1485 cells grown on different carbon sources under different growth condition (aerobic or anaerobic) and different growth phases were loaded as follows: GLU, culture grown on LB-glucose; GNT, culture grown on LB-gluconate; GNT/GLU, culture grown on LB-glucose plus gluconate; LB, culture grown on LB; STAT, stationary phase; LOG, log phase. Size markers in kilobases are provided to the sides of the gels.

1.47 kb, which is of sufficient length to encode *gntU*. None of the transcripts detected with the *gntU* probe were observed in stationary-phase cultures.

To test whether *gntU* is transcribed from the *gntK* promoter only or also from a promoter within the *gntK* gene (giving rise to the 1.47-kb transcript), two clones were constructed: pTC222, containing a deletion of the *gntK* promoter and translational initiation regions, and pTC223, containing a frameshift within *gntK*. The data in Table 2 show that no gluconokinase activity (GntK) was present in *E. coli* TUG287(pTC222) grown on gluconate, while only a very low level of gluconokinase activity was present in *E. coli* TUG287(pTC223). Gluconate uptake activity (GntU) was abolished by deletion of the *gntK* promoter in *E. coli* M6(pTC222); however, gluconate uptake activity was retained in *E. coli* M6(pTC222), indicating that a frameshift within *gntK* had no effect on *gntU* expression. These data support the conclusion that *gntU* is transcribed from the promoter located upstream of *gntK* and that the *gntK*-specific 0.65-kb and *gntU*-specific 1.47-kb transcripts arise from processing of the 2.39-kb polycistronic mRNA. These data also indicate that translation of *gntU* is not obligately coupled to *gntK* translation.

Mapping of the *gntR* and *gntK-gntU* promoter regions. Total RNA isolated from wild-type *E. coli* W1485 grown on gluconate was used to map the 5′ ends of the *gntK-gntU* and *gntR* mRNAs by primer extension and by S1 nuclease protection analysis. The transcript maps of *gntR* were complex, in that multiple 5′ ends were revealed by primer extension at positions −92, −136, and −154 with respect to the ATG start codon (Fig. 5). Examination of the DNA sequence upstream from the *gntR* start codon in the region of the mapped 5′ end at −92 revealed a possible σ^{70} promoter with 4 of 6 bases conserved in both the −10 and −35 regions, separated by a 17-nucleotide spacing (44).

A single transcriptional start site for *gntK* was mapped by primer extension (and confirmed by S1 nuclease protection) at a guanine 51 bp upstream from the *gntK* start codon (Fig. 6). The putative −10 and −35 promoter regions of the *gntK* gene each share four of six positions with the canonical σ^{70} promoter (44). Interestingly, the *gntK* transcriptional start site partially overlaps the putative *gntR* terminator in a way that would not result in a stem-loop structure in the *gntKU* transcript. An excellent CAP-binding site (19) is centered at bp −70 with respect to the transcriptional start site of the *gntK* gene, explaining the phenomenon of glucose catabolite repression observed in Northern blot analysis. An apparent operator site, TGTTACCGATAACA, overlaps the *gntK* transcriptional start site. It is interesting to speculate that this is the element responsible for binding GntR and repression of *gntKU* expression. The sequence of this putative *gnt* operator is quite similar to that of the *lac* operator (5) and the *ccpA* box (20), which bind repressors with significant similarity to GntR. Primer extension and S1 nuclease protection analysis of *gntU* failed to reveal any 5′ mRNA ends within the upstream *gntK* gene, again confirming the polycistronic nature of *gntK* and *gntU* (data not shown). It is of more than passing interest that a plasmid-borne deletion subclone of *gntK* with an endpoint immediately downstream of the putative CAP-binding site at −62 with respect to the *gntK* transcriptional initiation site was not expressed, even though the gene was in the correct orientation to be transcribed from the plasmid-borne *lac* promoter. These

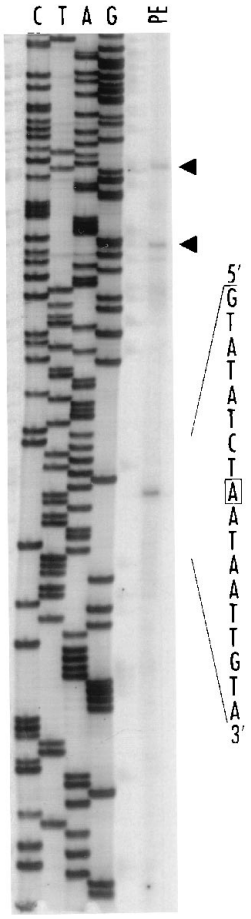

FIG. 5. Transcript mapping of the 5′ ends of *gntR* mRNA. Lanes: PE, primer extension; G, A, T, and C, the *gntR* sequence ladder (generated with the oligonucleotide used for extension).

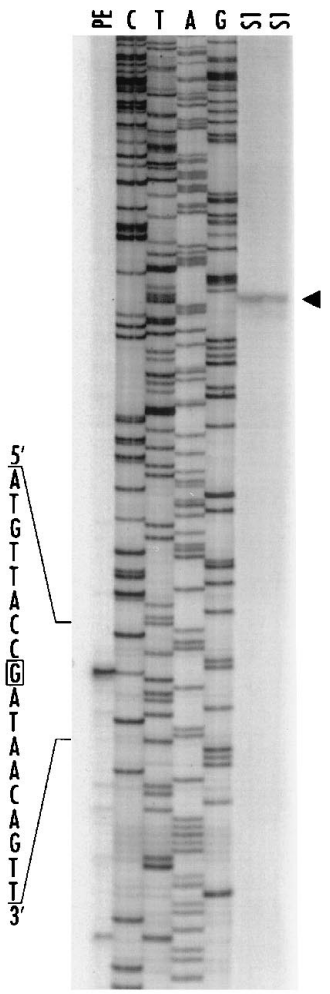

FIG. 6. Transcript mapping of the 5′ end of the *gntK* mRNA. Lanes: PE, primer extension; S1, S1 nuclease protection with 50 or 100 U of S1 nuclease; G, A, T, and C, the *gntR* sequence ladder (generated with the oligonucleotide used for extension).

results might indicate efficient termination by the *gntR* transcriptional terminator together with a requirement for the CAP-binding site (to provide a functional *gntK* promoter), which was deleted in this construction. Alternatively, the sequences immediately upstream of *gntK* might somehow be required for translation of this gene with its poor ribosome-binding site and exceedingly rare start codon. A different deletion subclone with an endpoint at −282 with respect to the *gntK* transcriptional initiation site was expressed.

Kinetic studies and substrate specificity of GntU. The initial rates of gluconate uptake by *E. coli* M6(pTC220) were measured over a range of 1 to 800 μM gluconate and showed single-phase Michaelis-Menten-type saturation kinetics, indicating only one kinetically distinguishable gluconate uptake system (Fig. 7). *E. coli* M6(pTC220) cells had an apparent K_m for gluconate of 212 μM and a V_{max} for gluconate uptake of 69 nmol/min/mg of protein, indicating that GntU is indeed the low-affinity gluconate transporter reported previously (14, 29). This K_m value is approximately 2-fold higher than previous measurements for a $GntT^-$ $GntU^+$ strain; however, it should be noted that these measurements were made in a different genetic background and did not involve a cloned transporter. In addition, the kinetics of gluconate uptake by wild-type *E. coli* W1485 grown on gluconate to log phase were measured and showed an overall apparent K_m for gluconate of 32 μM (Fig. 8). An Eadie-Hofstee plot of these gluconate uptake data for the wild-type cells showed a biphasic curve, which could indicate the simultaneous activity of two gluconate transporters, each with a different gluconate affinity (Fig. 8). The substrate specificity of GntU was analyzed by studying the ability of a number of unlabeled sugars to compete with gluconate uptake. The inclusion of a fourfold excess of several unlabeled competing sugars, including glucuronic acid, did not significantly decrease the rate at which *E. coli* M6(pTC220) took up 50 μM [^{14}C]gluconate, indicating that the GntU uptake system is highly specific for gluconate (Table 3).

DISCUSSION

The reasons for undertaking this molecular characterization of the *gntR-gntK-gntU* region of the *E. coli* genome were severalfold. First, the recent revelation that *E. coli* can utilize the Entner-Doudoroff pathway for oxidative glucose metabolism (16), as well as the widespread distribution of this pathway in microorganisms (6), suggests that the Entner-Doudoroff pathway is probably of far greater importance than is generally recognized (17). Second, the initial steps of gluconate metabolism in *E. coli* are complex, with two distinctly regulated groups of genes, which are located in different regions of the genome, being involved in gluconate transport and phosphorylation. Molecular genetic studies of the *gnt* genes should reveal how, at the physiological level, the GntI and GntII systems are utilized during metabolism of gluconate by *E. coli*. Third, despite extensive genetic and biochemical study, none of the *E. coli* genes involved in gluconate transport and phosphorylation have been subjected to molecular study.

The previously determined genetic map locations of genes involved in gluconate metabolism made cloning them straightforward (3, 9, 37). Genetic complementation, biochemical assays, and DNA sequence analysis of a 3.98-kb *Bam*HI fragment cloned from a member of the Kohara (25) library of *E. coli* firmly established the identity of three open reading frames encoding a transcriptional repressor (*gntR*), a gluconokinase (*gntK*), and a low-affinity gluconate transporter (*gntU*). The cloned 3.98-kb *Bam*HI DNA fragment allowed a *bio-asd* (GntI) deletion mutant to grow on gluconate and to regulate expression of the gluconate-responsive genes, including the genes of the Entner-Doudoroff pathway (*edd* and *eda*). The present study confirms that the *gntU-gntK-gntR* genes are immediately adjacent to *asd* at 77.0 min (in the clockwise direction) in that order.

The *E. coli gntRKU* cluster is organized somewhat differently from the analogous *gnt* operon of *B. subtilis*, which consists of four genes encoding a transcriptional repressor, gluconate kinase, gluconate permease, and 6-phosphogluconate dehydrogenase (36). In *B. subtilis*, the *gntK*, *gntP*, and *gntZ* genes are induced 50- to 100-fold by gluconate, while the *B. subtilis gntR* gene product increases only 4-fold after addition of inducer, indicating the presence of an unknown mechanism for avoiding the wasteful synthesis of repressor, which we have also observed in *E. coli*. It is interesting to note that comparison of the deduced amino acid sequences of the *E. coli gntK* and *gntR* genes with sequences of the analogous genes from *B. subtilis* failed to reveal any significant similarity.

The monocistronic *gntR* mRNA was present under all growth conditions tested, although the level of the *gntR*-specific transcript is significantly lower in log-phase cells grown on gluconate. Northern analysis clearly indicated that the *gntK* and

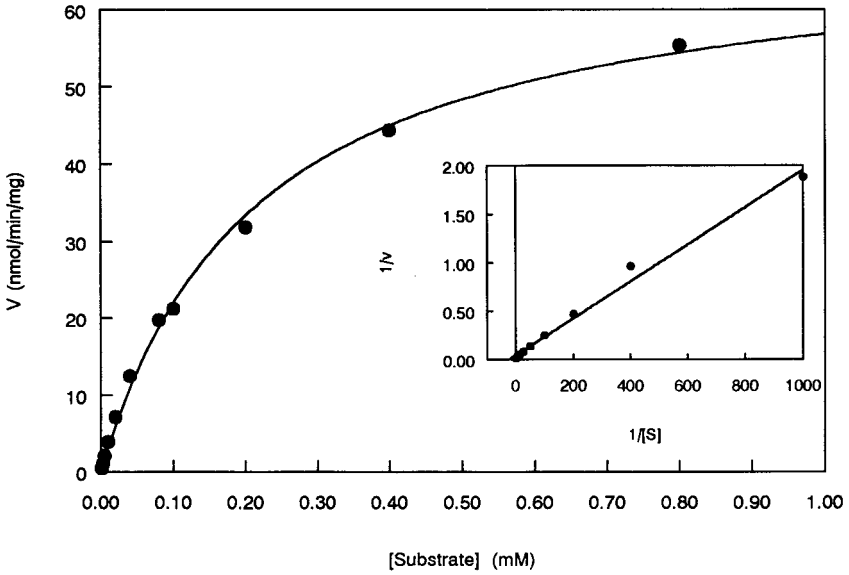

FIG. 7. GntU transport kinetics measured in *E. coli* M6(pTC220); plot of initial velocity (V) of [^{14}C]gluconate transport versus substrate concentration (inset depicts a Lineweaver-Burk plot).

gntU genes form an operon. Deletion of the *gntK* promoter eliminated expression of both *gntK* and *gntU*, confirming that the two genes are transcribed from a common promoter. Furthermore, a frameshift mutation within *gntK* did not affect *gntU* expression, indicating that while the ribosome-binding site for *gntU* is located within the upstream *gntK* gene, translation of *gntU* is not tightly coupled with *gntK* translation. The region immediately upstream of the *gntK-gntU* transcriptional start site contains −10 and −35 regions typical of a σ^{70} promoter. The presence of a CAP-binding site located at −70 with respect to the transcriptional initiation site explains the observed glucose catabolite repression effect on transcription as well as the lack of strong homology to the canonical σ^{70} promoter, which is common for CAP-activated promoters (8). A putative *gnt* operator site that overlaps the *gntK* transcriptional start site was identified by its similarity to the *lac* operator (5) and the *ccpA* box (20). Thus, the *gntK* promoter appears to be the primary locus of gluconate-dependent induction of *gntK* and *gntU*, involving the *gntR* product.

The redundancy of gluconate kinases and transporters has made it difficult to evaluate which activities are important for growth of wild-type *E. coli* on gluconate. It has long been thought that the high-affinity gluconate transporter encoded by *gntT* plays a primary role in gluconate metabolism, as does *gntK*, which encodes the primary gluconokinase (2, 33). Previous reports indicate that the high-affinity (GntT) gluconate transporter is induced for growth on gluconate while the low-affinity transporter (GntU) is not (14, 33, 34). One previous study showed that a *gntT* mutant lacking high-affinity transport but expressing the low-affinity transporter *gntU* required 7 h for

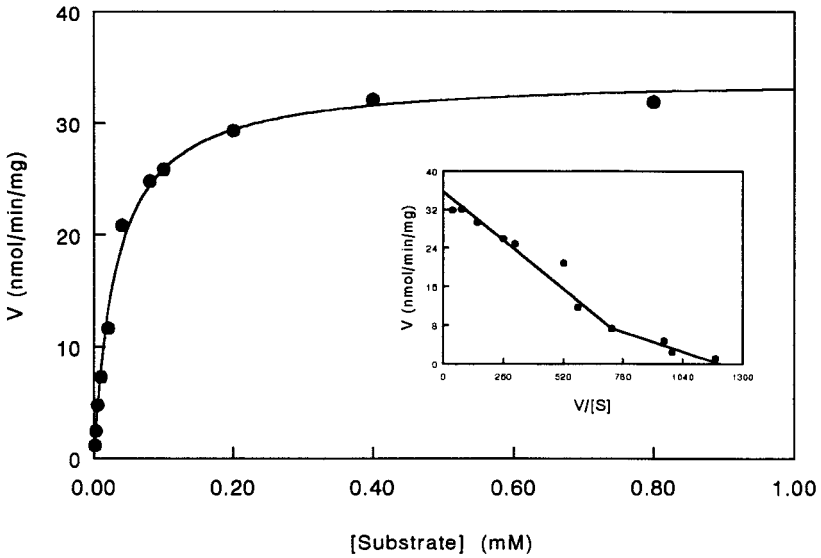

FIG. 8. Gluconate transport kinetics measured in *E. coli* W1485; plot of initial velocity (V) of [^{14}C]gluconate transport versus substrate concentration (inset depicts an Eadie-Hofstee plot).

TABLE 3. Inhibition of gluconate transport (GntU) by alternative substrates

Competing sugar[a]	Rate (nmol/min/mg [SD])	% Wild type
Control	2.10 (0.146)	100
Gluconate	0.76 (0.032)	36
Glucuronate	2.04 (0.276)	97
Maltose	2.57 (0.641)	122
Lactose	2.52 (0.154)	120
Sucrose	1.98 (0.002)	94
Mannitol	2.23 (0.099)	106
Fructose	2.19 (0.094)	104
Galactose	4.00 (0.756)	190
Glucose	2.13 (0.023)	101

[a] Cells were preincubated in 2 mM competing sugar prior to uptake of 50 μM [^{14}C]gluconate.

one cell doubling to occur when it was transferred to aerobic growth conditions with gluconate as the sole carbon and energy source, suggesting that GntU is an inefficient transport system in wild-type *E. coli* (14). In a similar study, it was found that *gntU* was progressively induced in a *gntT*::*lacZ* fusion strain (*gntT* mutant) while growing on minimal gluconate medium (40% slower than its *gntT*$^+$ parent strain), indicating that *gntU* is only expressed in the absence of GntT activity (33). It is difficult to reconcile these experimental results with those of the present study, which clearly show that *gntK* and *gntU* are cotranscribed from a common, gluconate-inducible promoter. Northern analysis of log-phase *E. coli* W1485 cells clearly indicates that *gntU* is expressed when the cells are grown on gluconate. Furthermore, the apparent K_m for gluconate in wild-type *E. coli* W1485 cells grown on gluconate is 32 μM, which is an intermediate value between the apparent K_m found for GntU (212 μM) and the previously reported K_m value of 10 μM for GntT (34). This could indicate the presence of two gluconate transporters in log-phase, wild-type *E. coli* growing on gluconate, which is further supported by a biphasic Eadie-Hofstee plot. Unfortunately, we still do not understand how *gntK* and *gntU* can appear to be differentially expressed in some strains, nor is it clear why *E. coli* possesses more than one gluconate transporter.

The modest catabolite repression of *gntKU* transcription (4-fold), by virtue of a CAP-binding site at the *gntK* promoter, confirms measurements of gluconokinase and gluconate transport made by others (2). It is quite interesting that *gntK* and *gntU* are subject to glucose catabolite repression, considering that gluconate is deemed to be more catabolite repressing than glucose (30). Nevertheless, the 4-fold-lower level of *gntKU* expression is still sufficient for cometabolism of glucose and gluconate (2, 13). It is probably important that the *edd* and *eda* genes of the Entner-Doudoroff pathway are not catabolite repressed by glucose (11).

Previous studies indicated that GntU might be less active during anaerobiosis than under aerobic conditions (46); however, Northern analysis of *gntKU* transcription did not support the possibility of gene regulation in response to oxygen tension. It could be that anaerobic conditions affect the transport function of GntU rather than the level of gene expression. It is probably significant that both the *gntK* and *gntU* reading frames begin with rare start codons and poorly conserved ribosome-binding sites; measurements of transport and gluconokinase activities in fully induced wild-type *E. coli* cells growing on rich medium containing gluconate are fairly low (Table 2) in comparison with the activities of most of the enzymes of the Embden-Meyerhof-Parnas, pentose phosphate, and Entner-Doudoroff pathways (18). A lower level of expression of GntK and GntU is consistent with a need to maintain low intracellular concentrations of 6-phosphogluconate, a metabolite which is known to inhibit growth when conditions allow its accumulation (45). The unusual translational initiation signals of *gntK* warrant further study.

The similarity of GntR to other repressor proteins, together with previous genetic studies indicating that gluconate utilization by the GntI system of *E. coli* is negatively regulated by the *gntR* product in response to induction by gluconate, argues strongly in favor of the conclusion that GntR fulfills a regulatory role similar to that of LacI for the *lac* operon (5). The developing model for gluconate induction suggests that gluconate binds to GntR, displaces it from operator sites at gluconate-regulated promoters, and allows transcription to occur. Interestingly, only a modestly conserved *gnt* operator site can be identified by visual inspection of the *edd-eda* promoter regions (43), although an obvious operator is present in the *gntK* promoter region. In addition to negative control, positive control is involved in expression of *gntK* and *gntU*, as indicated by the presence of a CAP protein binding site. Previous physiological studies have indicated that the high-affinity gluconate transporter gene, *gntT*, is regulated in a fashion similar to that for *gntKU* (33). Therefore, the molecular genetic studies presented in the present paper fully support the conclusion that the *gntK*, *gntU*, and *gntT* genes constitute the GntI system for gluconate utilization, under control of the *gntR* gene product, which is also responsible for induction of the *edd* and *eda* genes of the Entner-Doudoroff pathway.

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