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Sequence Analysis of the GntII (Subsidiary) System for Gluconate Metabolism Reveals a Novel Pathway for L-Idonic Acid Catabolism in *Escherichia coli*

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The presence of two systems in *Escherichia coli* for gluconate transport and phosphorylation is puzzling. The main system, GntI, is well characterized, while the subsidiary system, GntII, is poorly understood. Genomic sequence analysis of the region known to contain genes of the GntII system led to a hypothesis which was tested biochemically and confirmed: the GntII system encodes a pathway for catabolism of L-idonic acid in which D-gluconate is an intermediate. The genes have been named accordingly: the *idnK* gene, encoding a thermosensitive gluconate kinase, is monocistronic and transcribed divergently from the *idnD-idnO-idnT-idnR* operon, which encodes L-idonate 5-dehydrogenase, 5-keto-D-gluconate 5-reductase, an L-idonate transporter, and an L-idonate regulatory protein, respectively. The metabolic sequence is as follows: IdnT allows uptake of L-idonate; IdnD catalyzes a reversible oxidation of L-idonate to form 5-ketogluconate; IdnO catalyzes a reversible reduction of 5-ketogluconate to form D-gluconate; IdnK catalyzes an ATP-dependent phosphorylation of D-gluconate to form 6-phosphogluconate, which is metabolized further via the Entner-Doudoroff pathway; and IdnR appears to act as a positive regulator of the IdnR regulon, with L-idonate or 5-ketogluconate serving as the true inducer of the pathway. The L-idonate 5-dehydrogenase and 5-keto-D-gluconate 5-reductase reactions were characterized both chemically and biochemically by using crude cell extracts, and it was firmly established that these two enzymes allow for the redox-coupled interconversion of L-idonate and D-gluconate via the intermediate 5-ketogluconate. *E. coli* K-12 strains are able to utilize L-idonate as the sole carbon and energy source, and as predicted, the ability of *idnD*, *idnK*, *idnR*, and *edd* mutants to grow on L-idonate is altered.

In *Escherichia coli*, the Entner-Doudoroff (ED) pathway serves as a metabolic "funnel" receiving intermediates formed by catabolism of several sugar acids (17). Hexuronic acids undergo rearrangement in the inducible Ashwell pathways (1) to form 2-keto-3-deoxygluconate, which is then phosphorylated to produce 2-keto-3-deoxy-6-phosphogluconate (KDPG). KDPG is cleaved by KDPG aldolase, encoded by *eda*, providing for entry of carbon into glycolysis. The other enzyme of the ED pathway is 6-phosphogluconate dehydratase, encoded by *edd*, which is induced only for catabolism of gluconate and also forms KDPG, the key intermediate of the ED pathway (7). Long considered to be of more significance than is readily obvious (9), the finding that *eda* and *edd eda* double mutants are unable to colonize the mouse large intestine underscores the possible ecological importance of ED metabolism (32). The implication from these colonization studies is that colonic mucus, which contains several sugar acids, may serve as an important source of nutrients for *E. coli* in the gut.

Also participating in gluconate catabolism are several gluconate transporters and two gluconate kinases which appear, based upon their regulation, to comprise two distinct systems (2, 13). The GntI (main) system consists of *gntT*, *gntU*, and *gntK*, which code for high- and low-affinity gluconate transporters and a thermoresistant gluconate kinase, respectively (23–25, 33). Expression of the GntR regulon, that is, GntI together with the *edd-eda* operon, is negatively controlled by the *gntR* gene product. The GntII (subsidiary) system is comprised of a thermosensitive gluconate kinase and a gluconate transporter which function for gluconate catabolism in the absence of the GntI system (2, 11, 13, 22). It appears that the subsidiary gluconate transporter, which has an apparent K_m for gluconate of 60 μM (23), is encoded by a gene (*idnT*) which is adjacent to the gene encoding the thermosensitive gluconokinase (*idnK*) at 96.8 min.

The DNA sequence of the GntII system genes, located at 4492 kb on the genome, was revealed by the *E. coli* Genome Project (5, 6). If the GntII system had evolved as a subsidiary pathway for gluconate catabolism, one would expect it to contain only a gluconate transporter and gluconate kinase. However, in addition to the divergent *idnK* and *idnT* genes, this region also encodes two "dehydrogenase-like" enzymes. The similarity of *idnO* to *gno* of *Gluconobacter oxydans*, which encodes D-gluconate:NADP 5-oxidoreductase (GNO) (15), led to the testing of ketogluconates as enzyme substrates for the two newly identified dehydrogenases. A process of deductive reasoning and biochemical experiments led to the conclusion that the GntII system in fact comprises a novel metabolic pathway for catabolism of L-idonic acid, in which gluconate is a key intermediate. Accordingly, the genes involved in L-idonate metabolism have been given the designation *idn* (see Table 1 for gene nomenclature).

(Part of this work has been presented previously [3].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains and plasmids used for this study are listed in Table 2. *E. coli* strains were routinely grown at 37°C in Luria broth (LB) (19) or M63 minimal medium (28) with or without added carbohydrate (0.15%). Cell growth was measured spectrophotometrically with a

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TABLE 1. Gene and enzyme nomenclature[a]

Gene designation		Gene product	% Identity of protein[b]
Previous	New (accession no.)		
gntV	*idnK* (P39208)	D-Gluconate kinase	45 (GntK[c])
yjgV	*idnD* (P39346)	L-Idonate 5-dehydrogenase	30.6 (sheep DHSO[d])
yjgU	*idnO* (P39345)	5-Keto-D-gluconate 5-reductase	56 (GNO[e])
gntW	*idnT* (P39344)	L-Idonate transporter	61 (GntT[f])
yjgS	*idnR* (P39343)	L-Idonate regulator	46 (GntR[g])

[a] All accession numbers are Swiss-Prot database accession numbers.
[b] Percent identity of the amino acid sequence of the Idn protein to that of the protein shown in parentheses.
[c] *E. coli* gluconate kinase encoded by *gntK* (P46859).
[d] Sheep sorbitol dehydrogenase encoded by *sorD* (P07846).
[e] *G. oxydans* gluconate:NADP 5-oxidoreductase encoded by *gno* (P50199).
[f] *E. coli* gluconate transporter encoded by *gntT* (P39835).
[g] *E. coli* gluconate regulator encoded by *gntR* (P46860).

Spectronic 601 spectrometer (Milton Roy Co.). Ampicillin (50 mg/liter), tetracycline (10 mg/liter), and kanamycin (25 mg/liter) were included in growth media where appropriate.

Construction of mutants. To construct the *idnR* mutant, *E. coli* NP250, two DNA fragments were generated by PCR from *E. coli* W1485 chromosomal DNA. First, a 4.2-kb *Bam*HI-*Kpn*I fragment, using PCR primers 5′GCGGATCCGCG TAGCGATATCCTGTAAA3′ and 5′GCGGTACCCTTATGAGCTGCGTAA GCTG3′, was cloned into pUC19 to produce pNP204. Next, a 2.7-kb *Hin*dIII-*Bam*HI fragment, using PCR primers 5′GCAAGCTTGGAGCAAAATCTTCC AGCCG3′ and 5′GCGGATCCTAGAATCCGTCACCTCTGAG3′, was ligated into pNP204, generating a subclone of the *idn* genes containing a 230-bp deletion within the *idnR* gene. Then a Kan[r] gene cassette was cloned into the *Bam*HI site within the *idnR′* gene. The resulting plasmid was digested with *Kpn*I and *Pvu*II, and the fragment was purified by electroelution from an agarose gel and transformed by electroporation into *E. coli* DPB271. Kan[r] transformants were analyzed by PCR. The *idnR* mutation was then transduced into the wild-type background of *E. coli* W1485 by using the phage P1*vir* (21). The *idnD* and *gntRKU* mutants were generated in a similar way. For the *gntRKU* mutant, *E. coli* NP202, a 1.3-kb *Stu*I-*Bgl*II fragment on pTC221 (33) was replaced by the Kan[r] gene fragment by deleting the entire *gntK* gene and portions of the *gntU* and *gntR* genes. For the *idnD* mutant, *E. coli* CB350, the Kan[r] gene was cloned into *Eco*RI site of the *idnD* gene on pNP204. Last, the *edd* mutant, *E. coli* BM129, was constructed by using a Tc[r] insertion in the *Nco*I site of pTC199 (8).

Plasmids. DNA restriction digestion, ligation, transformation, and other DNA manipulations were carried out by standard methods (28). Construction of pNP204 was described above. The *idnO* gene was subcloned from pNP204 as a 1.6-kb *Eco*RI-*Nru*I fragment cloned into the *Eco*RI and *Sma*I sites of pUC18 to construct pCB95. The *idnD* gene was subcloned from pNP204 as a 1.7-kb *Hin*dIII-*Ssp*I fragment cloned into the *Hin*dIII and *Sma*I sites of pUC19 to construct pCB96. The *idnT* gene was subcloned from pNP204 as a 1.6-kb *Ssp*I-*Ssp*I fragment cloned into the *Sma*I site of pUC18 to construct pCB98. The *idnT* gene was PCR amplified from pCB98 by using primers immediately adjacent to the *idnT* structural gene (5′GCGAATTCGCTGCTTTTCTGGCACTA3′ and 5′GCGGA TCCGCATAACTTCTCCCAACGTC3′), and the PCR fragment was cloned into the *Eco*RI and *Bam*HI sites of pQE30 to construct pCU102. All plasmid constructions were confirmed by DNA sequence analysis (29).

Enzyme assays. Cells were harvested in mid-logarithmic phase, washed three times with 100 mM Tris-HCl buffer (pH 7.0), and then resuspended in 500 μl of the same buffer to a final A_{550} of 1.0. Cell suspensions were lysed by sonic oscillation for 30 s (three bursts of 10 s each, with 60 s on ice between bursts) by using a Fisher Sonic Dismembrator model 300. Crude extracts were centrifuged (13,000 × *g* for 20 min at 4°C) in order to minimize nonspecific NAD(P)H oxidase activity. 5-Ketogluconate (5KG) reduction was assayed by mixing 50 μl of cell extract with 950 μl of assay buffer containing 100 mM Tris-HCl (pH 6.5), 150 μM NADPH or NADH, and 300 mM potassium 5KG. Similarly, L-idonate and D-gluconate oxidations were assayed in a buffer containing 100 mM Tris-HCl (pH 8.0), 500 μM NAD, and 300 mM sodium D-gluconate or sodium L-idonate. Other enzyme substrates were added to final concentrations of 300 mM in the same assay buffers. All enzyme reactions were conducted at 25°C and monitored spectrophotometrically at 340 nm by using a Lambda-12 UV-visible light spectrometer (Perkin-Elmer). Protein concentrations were determined by the method of Lowry et al. (18).

Idonate uptake. *E. coli* M15(pCU102) was used to measure gluconate uptake. Expression of *idnT* was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to a culture growing in mid-logarithmic phase on LB containing 0.4% glucose, which leads to catabolite repression of the native gluconate transporters (25, 33). Uptake experiments were started by mixing 100 μl of sodium [6-^{14}C]gluconate (200 μM, 5.6 μCi/μmol) and 100 μl of the cell suspension which had been preincubated for 3 min with 2 mM competing sugar, as described previously (33). Assays were conducted in triplicate. Radioactivity was measured with a Packard Tri-Carb 2100TR liquid scintillation counter.

Purification of L-idonate. A large-scale reaction mixture containing 100 mM Tris-HCl (pH 7.0), 50 mM potassium 5KG, 100 mM NADH, and 2.5 ml of crude cell extract (described above) was mixed continuously in an Oak-Ridge tube at room temperature for 4 h. Protein was denatured by being boiled for 5 min and was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was filtered by using a 0.2-μm-pore-size Acrodisc filter (Gelman Sciences).

Low-pressure liquid chromatography (LPLC) was carried out at room temperature using a BioLogic system (Bio-Rad, Hercules, Calif.) with a formate-based AGX1 anion-exchange resin (Econo column [1 by 5 cm] equipped with a flow adapter; Bio-Rad). The sample was applied to the column, washed with 1 mM formic acid, and eluted with a linear gradient of 1 to 200 mM formic acid. Fractions were collected (Bio-Rad model 2110) and analyzed by high-pressure liquid chromatography (HPLC).

Thin-layer chromatography (TLC) of the appropriate LPLC fractions applied to glass plates (10 by 20 cm) coated with silica gel was accomplished essentially as described previously (10). Control samples were detected by spraying with 1% $AgNO_3$ in acetone, followed by air drying, fixation with 0.5 N NaOH in methanol, and baking at 100°C for 3 to 5 min. The R_f value of the sample was calculated for the control TLC plate, and the sample was scraped off of an identical plate (without detection) and then eluted from the silica gel with 4 volumes of double-distilled H_2O.

HPLC was performed by using a DX-500 system (Dionex, Sunnyvale, Calif.) equipped with a GP40 microbore gradient pump, an ED40 electrochemical detector, a Dell Optiplex XL 590 computer running PeakNet 4.11A software, and a 2-mm-diameter PA-10 anion-exchange column. Isocratic chromatography with 450 mM NaOH allowed excellent separation of the various hexonates analyzed in this study.

NMR analysis. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz on a Bruker DMX-500 instrument at room temperature. Samples were dissolved in D_2O and referenced to DOH Δ = 4.78 ppm.

Chemicals and enzymes. Restriction enzymes and DNA-modifying enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The T7 Sequenase version 2.0 kit was acquired from Amersham Life Science (Arlington Heights, Ill.). Radioactive sodium [6-^{14}C]gluconate was purchased from American Radiolabeled Chemicals (St. Louis, Mo.). TLC plates and biochemicals were obtained from Sigma Chemical Corp. (St. Louis, Mo.). The sodium L-idonate was a generous gift from Robert Lazurus (Genentech).

RESULTS

Homology searches and organization of the GntII (*idn*) genes. The arrangement of the *idn* genes is shown in Fig. 1 (6). The

TABLE 2. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype, phenotype, or characteristic	Source or reference
Strains		
W1485	K-12 wild type	CGSC[a]
DH5α	*lacZ*ΔM15 *recA*	BRL[b]
NP202	Δ(*gntR gntK gntU*)	This study
NP250	Δ*idnR*	This study
CB350	Δ*idnD recD*	This study
TUG287	F$^-$ *gntK gntV his trp*	33
DPB271	*recD*	24
P90C	*ara* Δ(*pro-lac*) *thi*	30
BM129	Δ(*edd*)	This study
M15	*recA*$^+$ *uvr*$^+$ F$^-$ *mlt gal ara lac* (pREP4)	Qiagen, Inc.
Plasmids		
pCB95	*idnO*	This study
pCB96	*idnD*	This study
pCB98	*idnT*	This study
pCU102	*idnT* in pQE30 (*idnT* controlled expression)	This study
pNP204	*idnK idnD idnO idnT idnR′*	
pUC18	*bla lacZ′*[c]	This study
pUC19	*bla lacZ′*	This study
pBR322	Tc[r] *bla*	
pQE30	Expression vector; His_6 affinity tag	Qiagen, Inc.

[a] CGSC, *E. coli* Genetic Stock Collection.
[b] BRL, Bethesda Research Laboratories.
[c] Incomplete genes.

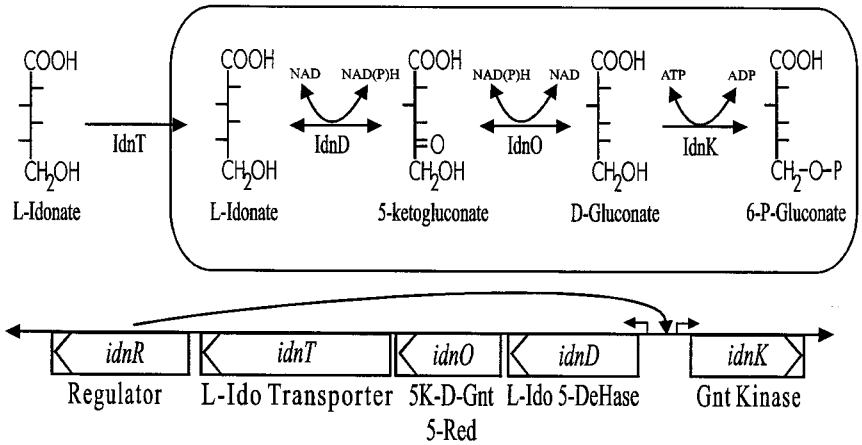

FIG. 1. Enzymes and genes of L-idonate catabolism. Abbreviations: 6-P-Gluconate, 6-phosphogluconate; L-Ido, L-idonate; 5K-D-Gnt 5-Red, 5-keto-D-gluconate 5-reductase; L-Ido 5-DeHase, L-idonate 5-dehydrogenase; Gnt, gluconate.

biochemical evidence presented below proves that these genes encode the enzymes of a pathway for L-idonate catabolism, and hence the genes have been given the designation *idn* (Table 1). Each of the peptide sequences deduced from the *idn* structural genes was used as a query against the peptide sequence database (BLASTP), and the data are summarized in Table 1. IdnK is 45% identical to GntK (33); IdnD is 31% identical to mammalian sorbitol dehydrogenase (14); IdnO is 56% identical to Gno from *G. oxydans* (15); IdnT is 61% identical to GntT (25), a match which was the highest among a total of seven gluconate permease orthologs in *E. coli* (23); and the helix-turn-helix protein IdnR is 46% identical to GntR, strongly suggesting that IdnR is involved in regulation of *idnK* and the *idnDOTR* operon.

Biochemical analysis of IdnO and IdnD. The general approach used to determine the biochemical functions of the Idn proteins involved subcloning of the corresponding gene, overproduction of the gene product, and assay of the enzyme activity in crude cell extracts. The individual gene fragments were designed to contain the structural gene only, including the ribosome binding site. The similarity of IdnO to GNO from *G. oxydans* (15) led to the testing of 2-ketogluconate and 5KG as substrates for a crude extract prepared from *E. coli* DH5α (pCB95), which was constructed to specifically overexpress IdnO. The biochemical data are summarized in Table 3. IdnO is able to reduce 5KG with either NADH or NADPH as cofactor; no other compounds which were tested could be reduced by IdnO. The reduction of 5KG by IdnO is reversible, as evidenced by oxidation of D-gluconate (and, to a 10-fold-lesser extent, 6-phosphogluconate) using NADP as a cofactor; no other sugar acids are oxidized, nor does NAD serve as a cofactor. The reactions catalyzed by IdnO were confirmed by HPLC analysis: D-gluconate is formed by reduction of 5KG (Fig. 2, chromatogram A), and 5KG is formed by oxidation of D-gluconate (data not shown). In the crude extract, IdnO shows an apparent K_m of 2 mM for gluconate and an apparent K_m of 0.5 mM for 5KG. While these K_m values are high, the kinetic data clearly indicate that the IdnO-catalyzed reaction is saturatable. Thus, IdnO encodes a specific 5-keto-D-gluconate 5-reductase. Since the D-gluconate formed by reduction of 5KG would be phosphorylated by IdnK to form 6-phosphogluconate, which would be metabolized via the ED pathway, it seemed logical that 5KG is an intermediate of the pathway leading to 6-phosphogluconate. This consideration led to the hypothesis that IdnD is involved in 5KG formation, functioning upstream in the pathway. Since IdnD showed the highest similarity to sorbitol dehydrogenase (L-iditol:2-dehydrogenase), it was anticipated that IdnD would catalyze the oxidation of 5KG at carbon number two to form 2,5-diketogluconate. Surprisingly, 5KG is not oxidized by a crude extract of *E. coli* DH5α (pCB96), which specifically overexpresses IdnD, but rather IdnD catalyzes the reduction of 5KG, using either NADH or NADPH as a cofactor. Furthermore, the reduction of 5KG is highly specific, as IdnD fails to reduce D-glucose, D-galactonate, D-galacturonate, D-glucuronate, D-sorbose, D-sorbitol, and 2-ketogluconate; nor does IdnD oxidize any of these sugars. At the time, the identity of the product formed by reduction of 5KG by IdnD was not clear.

TABLE 3. Biochemical characterization of IdnO and IdnD activities[a]

Substrate[b]	Sp act (nmol/min/mg)[c]								Approx. K_m[d]	
	IdnO				IdnD				IdnO	IdnD
	NAD	NADP	NADH	NADPH	NAD	NADP	NADH	NADPH		
D-Gluconate	107.0	1.6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	2 mM	
D-Gluconate-6-phosphate	10.2	0.4	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		
5KG	<0.01	<0.01	4,000.0	3,340.0	<0.01	<0.01	48.0	36.0	500 μM	1 mM
L-Idonate	<0.01	<0.01	<0.01	<0.01	17.4	<0.01	<0.01	<0.01		25 mM

[a] *E. coli* DH5α cells harboring plasmid pCB95 (IdnO) or pCB96 (IdnD) were induced by the addition of 1 mM IPTG for 2 h.
[b] Substrate concentrations of 300 mM.
[c] Lower than detectable activity was measured with D-glucose, D-galactonate, D-galacturonate, D-glucuronate, D-sorbose, D-sorbitol, and 2-ketogluconate.
[d] Approximate K_m values were determined with crude cell extracts.

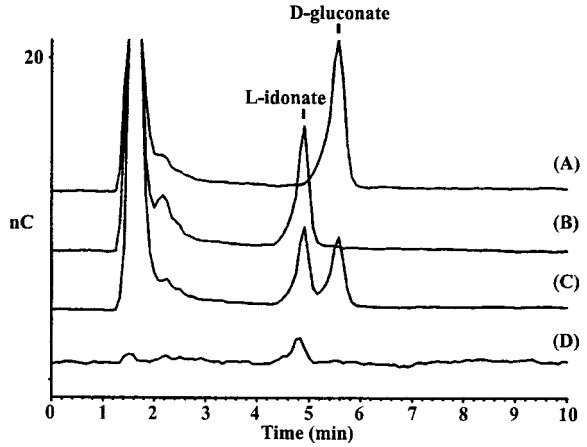

FIG. 2. HPLC analysis of substrate conversion. Conversion from 5KG to D-gluconate by 5-keto-D-gluconate 5-reductase (A), from 5KG to L-idonate by L-idonate 5-dehydrogenase (B), and from 5KG to both L-idonate and D-gluconate by 5-keto-D-gluconate 5-reductase and L-idonate 5-dehydrogenase (C) are shown. Authentic L-idonate standard (D) is also shown.

Production, purification, and analysis of the IdnD substrate. Conditions were optimized, as described in Materials and Methods, for synthesis of the 5KG reduction product by using the crude cell extract of *E. coli* DH5α(pCB96) containing IdnD. HPLC analysis showed that the resulting reaction contained an unidentified compound (Fig. 2, chromatogram B). Furthermore, this unidentified compound is distinct from D-gluconate yet possesses similar chemical properties, as indicated by the similar retention time on the anion-exchange column. An authentic standard of L-idonate was obtained and shown to cochromatograph with the unidentified compound (Fig. 2, chromatogram D). Thus, the results of HPLC analysis strongly suggested that IdnD catalyzes the reduction of 5KG to form L-idonate. The reaction mixture also contains significant amounts of 5KG (data not shown). Therefore, it was necessary to purify the L-idonate from the reaction mixture in order to prove its identity by NMR analysis. The putative L-idonate formed by IdnD was purified by anion-exchange chromatography and preparative TLC. This preparation was analyzed by proton NMR, and the putative L-idonate was shown to have the same spectrum as the authentic L-idonate standard, a spectrum which is significantly different from that of D-gluconate (Fig. 3). These data confirm that the product formed by reduction of 5KG by IdnD is indeed L-idonate. Furthermore, IdnD is able to specifically oxidize L-idonate with NAD as a cofactor to form 5KG (Table 3). Thus, IdnD is a novel enzyme, L-idonate 5-dehydrogenase; IdnD and IdnO catalyze consecutive metabolic steps which allow for conversion of L-idonate to D-gluconate, with 5-KG as an intermediate. The pathway for L-idonate catabolism is shown in Fig. 1.

Physiology of L-idonate growth, transport, and induction. As predicted, the presence of the GntII pathway in *E. coli* allows growth on L-idonate as the sole source of carbon and energy (Fig. 4). The generation time of wild-type *E. coli* W1485 on minimal medium containing L-idonate is approximately 1.4 h, compared to generation times of approximately 1.0 h on gluconate and glucose. Another prediction for growth on L-idonate is that the ED pathway is used for metabolism of the D-gluconate formed from L-idonate catabolism, and indeed an *edd* mutant grows more slowly than the wild type on L-idonate (Fig. 4), as it also does on gluconate, which is known to be metabolized in *edd* mutants via the pentose phosphate pathway (36). The reason for the extended lag phase of the *edd* mutant on L-idonate, but not on D-gluconate, is not understood at present. Furthermore, *E. coli* TUG287, a *gntK idnK* double mutant, and *E. coli* CB350, an *idnD* deletion strain, are both unable to grow on minimal medium containing L-idonate, highlighting the essential nature of the *idnK* and *idnD* genes for L-idonate catabolism (data not shown). As expected, *E. coli* NP202, a *gntRKU* deletion strain with the wild-type *idn* genes, grows well on L-idonate. Interestingly, *E. coli* NP202 is also able to grow on D-gluconate and at the same rate as the wild type, strongly suggesting that D-gluconate is able to induce *idnK* under certain conditions. Accordingly, growth of *E. coli* NP202 on rich medium containing gluconate results in a fourfold induction of gluconate kinase activity (data not shown).

Presumably, L-idonate is transported into the cell by IdnT, which was recently proven to function as a D-gluconate transporter with an apparent K_m of 60 μM for gluconate (23). Since it is not possible to confirm directly whether IdnT transports L-idonate, because radioactive L-idonate is not available, competition of unlabeled L-idonate for uptake of radioactive gluconate was measured instead. The data shown in Table 4 indicate that a 20-fold excess of unlabeled L-idonate is able to inhibit uptake of radioactive D-gluconate to about the same extent as the control value determined for a 20-fold excess of unlabeled D-gluconate. L-Iduronic acid and D-glucose did not inhibit gluconate transport, while 5-keto-D-gluconic acid had a modest effect. D-Glyceric acid, which has the same anomeric configuration as L-idonate, also had an effect. These data indicate that IdnT functions for the transport of both L-idonate and D-gluconate.

Another prediction is that L-idonate or an intermediate of its catabolism serves to induce the enzymes of the L-idonate pathway. Conditions which lead to induction of IdnO and IdnK in *E. coli* W1485 were tested (Table 5). In rich medium, there is a 15-fold induction of IdnO by L-idonate, an 80-fold induction of IdnO by 5KG, and a 4-fold induction of IdnK by 5KG. Sim-

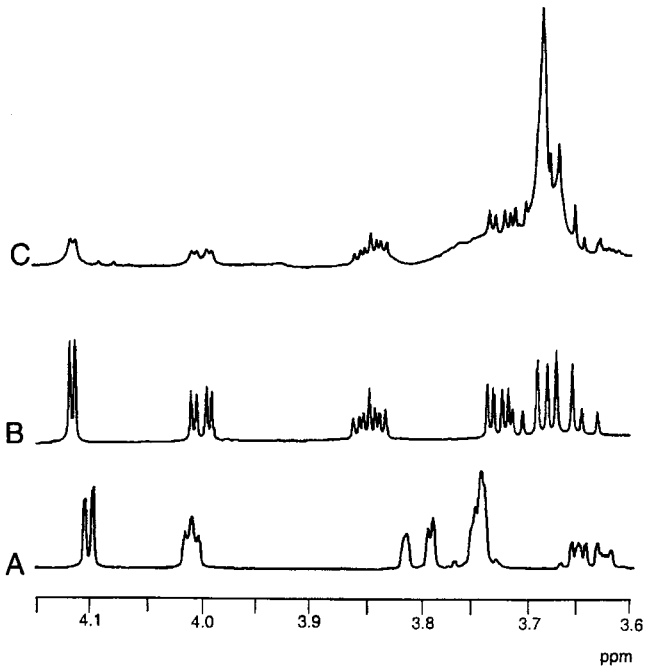

FIG. 3. Partial ^1H NMR spectra of sodium D-gluconate (A), sodium L-idonate (B), and an unknown compound (C) isolated from reaction of L-idonate 5-dehydrogenase with 5KG and NADH, indicating that the unknown compound is L-idonate. Spectra were recorded at 500 MHz in D_2O.

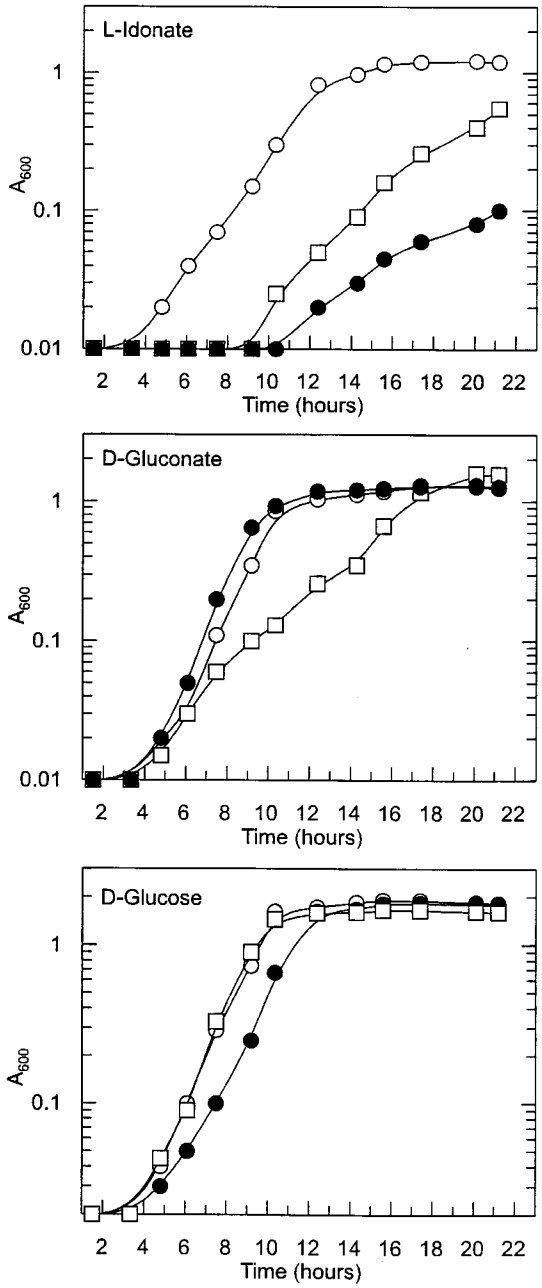

FIG. 4. Growth of *E. coli* on L-idonate, D-gluconate, and D-glucose. *E. coli* W1485 (open circles), *E. coli* BM129 (*edd* mutant; open squares), and *E. coli* NP250 (*idnR* mutant; closed circles) grown on M63 minimal medium containing L-idonate, D-gluconate, and D-glucose are shown.

ilarly, there is a 15-fold induction of IdnO on minimal medium containing L-idonate and a 20-fold induction of IdnO on minimal medium containing 5KG. These data suggest that 5KG rather than L-idonate is the true inducer. However, 5KG is a very poor growth substrate; *E. coli* W1485 has a generation time of more than 7 h when growing on minimal medium containing 5KG (not shown). Perhaps the reason for the slow growth on 5KG is poor uptake, as suggested by its modest inhibition of gluconate transport via IdnT as described above. Since induction of IdnO by L-idonate or 5KG is reduced twofold by growth on glucose, it seems likely that the *idn* genes are

TABLE 4. Inhibition of gluconate transport (IdnT) by alternative substrates

Competing sugar[a]	Rate (nmol/min/mg)	% of wild-type rate
None (control)	2.13	100
D-Gluconic acid	0.30	14
L-Idonic acid	0.42	14
D-Glucose	3.58	168
5-Keto-D-gluconic acid	1.71	80
D-Glyceric acid	1.40	66
L-Iduronic acid	2.09	98

[a] Cells preincubated in 2 mM competing sugar prior to uptake of 200 μM [^{14}C]gluconate.

catabolite repressed (Table 5). Last, an *idnR* deletion mutant, *E. coli* NP250, is unable to grow on L-idonate but grows normally on glucose and gluconate (Fig. 4). This result suggests that IdnR is a positive regulator of the *idn* regulon.

DISCUSSION

The experiments described in this paper outline a general strategy for deducing the biochemical functions of unidentified gene products based upon clues from genomic sequences. Proof for the physiological operation of a presumptive metabolic pathway can be provided by biochemical analysis of the pathway enzymes, chemical analysis of the pathway intermediates, and growth experiments involving mutants with lesions in specific steps of the pathway. This strategy was specifically applied to the GntII system of *E. coli*, which has been shown in this study to code for a previously unknown pathway for catabolism of and growth on L-idonate.

For many years, the physiology and regulation of the subsidiary gluconate pathway (GntII system) were mysteries. Genetic evidence for the presence of two systems for gluconate transport and phosphorylation was first provided by Bächi and Kornberg (2). Gluconate-fermenting pseudorevertants of *E. coli* HfrG6ΔMD2, a GntI deletion mutant (*bioH-asd*) which cannot grow on gluconate, were selected after extended incubation on minimal medium containing gluconate (11–13, 22). The pseudorevertant strains were found to have induced a thermosensitive gluconate kinase and an alternative gluconate transporter when growing on gluconate. A secondary mutation of *idnK* (*gntV*) eliminated the subsidiary gluconate kinase activity as well as the ability of the pseudorevertants to grow on gluconate (11, 13). A mutation affecting the subsidiary gluconate transporter was designated *gntS* (2), but it was never

TABLE 5. Induction of IdnO and GntK activities in *E. coli* W1485 cells[a]

Carbohydrate (0.2%)	IdnO sp act[b]		IdnK sp act,[b] LB
	LB	M63[c]	
None	10.0	NT[d]	<0.01
D-Glucose	16.0	6.2	NT
D-Gluconate	12.5	8.0	1.3
5KG	825.0	135.0	5.7
5KG + D-glucose	NT	78.0	NT
L-Idonate	158.0	99.0	NT
L-Idonate + D-glucose	NT	69.3	NT

[a] Cells all harvested during log phase.
[b] Expressed as nanomoles per minute per milligram of total cell protein.
[c] M63 minimal medium.
[d] NT, not tested.

proven whether the *gntS* locus is a structural or regulatory gene, although it has been suggested that the *gntS* product positively controls expression of *idnK* (*gntV*) (2, 11, 13). Recent evidence apparently confirms the location of *gntS* upstream of *fbp* in the 95-min region and further supports the conclusion that *gntS* is a regulatory locus (12). However, examination of the genomic sequence in the 95-min region does not provide any further insights into the nature of *gntS*. It is now believed that the subsidiary gluconate transporter is encoded by *idnT* (*gntW*), which is adjacent to the proven location of *idnK* at 96.8 min (12, 23). The nature and role of *gntS* remain obscure.

It was never established during the previous studies how the GntII system is regulated. The GntII system was thought to have evolved as a subsidiary pathway for gluconate catabolism (12). If so, the GntII system would need contain only a gluconate transporter and gluconate kinase. However, analysis of the genomic sequence containing the *idnK* and *idnT* genes indicated that this region also contains two genes that encode "dehydrogenase-like" enzymes, *idnD* and *idnO*, which are part of an operon with *idnT* and *idnR*, and have been suggested to also be constituents of the GntII system (35). The similarity of *idnO* to *gno* of *G. oxydans*, which encodes GNO (15), led us to overexpress, and thereby prove, that IdnO can interconvert 5KG and D-gluconate. Therefore, IdnO is a 5-keto-D-gluconate 5-reductase. The other dehydrogenase, IdnD, can reduce 5KG but cannot oxidize it. Therefore, IdnD is an L-idonate 5-dehydrogenase. Furthermore, the activities of both IdnD and IdnO are highly specific. The product formed by the IdnD-dependent reduction of 5KG was purified and proven by NMR analysis to be L-idonic acid. Thus, it was shown that L-idonate is converted via the two consecutive oxidation and reduction steps to D-gluconate, which is in turn phosphorylated by IdnK to form 6-phosphogluconate, an intermediate of central carbon metabolism.

The in vivo sequence of L-idonate catabolism was confirmed by the growth properties of specific pathway mutants: wild-type *E. coli* is able to grow very well on L-idonate, an *idnD* deletion mutant is unable to grow on L-idonate, an *idnK* mutant (also defective in *gntK*) is unable to grow on L-idonate, and an *edd* mutation significantly slows growth on L-idonate to the same extent as growth on D-gluconate is affected (the pentose phosphate pathway plays a backup role for 6-phosphogluconate metabolism in *edd* mutants [36]). L-Idonate is the transport substrate for IdnT, as indicated by strong competitive inhibition of D-gluconate uptake. In summary, these data prove that L-idonate is the true substrate of the GntII system and is catabolized via a pathway involving D-gluconate as an intermediate (Fig. 1).

The natural occurrence of L-idonate is apparently limited to its involvement as an intermediate in catabolism of 2,5-diketogluconate by *Erwinia* sp. (34) and *G. oxydans* (27), as well as tartaric acid formation from ascorbic acid in grapes (20, 26). Interestingly, L-idonate 5-dehydrogenase is considered to be an undesirable activity in recombinant bacteria specifically engineered to produce 2-keto-L-gulonate, a precursor of ascorbic acid biosynthesis (16). In addition to *E. coli*, the only other organism reported to grow on L-idonate is *Erwinia* sp. strain ATCC 39140 (34), and it will be interesting to find out whether other microorganisms can grow on L-idonate. The L-idonate catabolic pathway may not be unique to *E. coli* and *Erwinia* sp. strain ATCC 39140, since enzymes of ketogluconate metabolism are also present in several bacteria, including *G. oxydans* (15, 27), *Chromobacterium* (4), and *Corynebacterium* sp. (31). However, there are currently no orthologs of *E. coli idnD* present in the databases.

Genomic analysis indicates that the *idnK* gene is monocistronic and is transcribed divergently from an operon containing the *idnD-idnO-idnT-idnR* genes (6). It stands to reason that a molecular genetic analysis of the 217 bp of intervening sequence between the *idnK* and *idnD* genes will be very interesting. The fact that the GntII system encodes a pathway for catabolism of L-idonate leads to several predictions concerning regulation of the *idn* genes. First, L-idonate (or the intermediate, 5KG) should be the inducer for the pathway. This was confirmed biochemically by induction of IdnO and IdnK activities in cells grown on L-idonate. Second, the ED pathway should be induced for efficient catabolism of the 6-phosphogluconate formed from L-idonate. Third, the *idnR* product should regulate the *idn* regulon. The fact that an *idnR* deletion mutant cannot grow on L-idonate suggests that IdnR positively regulates the *idn* regulon. Last, since gluconate is an intermediate of the L-idonate pathway, there is likely to be cross talk with the *gnt* regulon (GntI). The inducer generated by the L-idonate pathway should not induce the GntI system, which is unnecessary for L-idonate catabolism, but a signal is still necessary to induce the ED pathway for growth on L-idonate; gluconate is most likely this inducer. This suggests an additional role for the *idnR* product: repression of the *gntKU* and *gntT* genes when growing on L-idonate. The presence of a highly conserved GntR binding site within the *idnK-idnD* intragenic region supports the hypothesis of cross talk from the *gnt* regulon (24, 25).

The results presented in this paper answer many of the longstanding questions concerning the GntII system (2, 13, 22, 37). It is now clear that the genes of the GntII system encode a pathway for catabolism of L-idonate in which D-gluconate is an intermediate. There are certain conditions under which D-gluconate can induce the L-idonate (GntII) pathway, but the natural inducer of the *idn* genes is apparently L-idonate. With the new understanding provided by the current study, mechanisms for induction of the GntII system by D-gluconate can now be proposed. As mentioned above, the GntI deletion strain *E. coli* HfrG6ΔMD2 is able to grow on gluconate by apparently acquiring a mutation which renders the subsidiary gluconate transporter and gluconate kinase inducible by D-gluconate. In stark contrast, it was shown that *E. coli* NP202, a *gntK* deletion mutant, is able to grow on D-gluconate. The absence of the gluconate-inducible gluconate kinase (GntK) in *E. coli* NP202 would result in intracellular accumulation of D-gluconate when cells are first exposed to D-gluconate, since this strain carries a wild-type copy of *gntT*. Under these conditions, the inducer of *idnK* could be formed from D-gluconate by reversal of the IdnO- and/or IdnD-catalyzed reactions to generate 5KG or L-idonate. Alternatively, it could be that D-gluconate is itself the inducer of the *idn* genes but that IdnR requires a higher concentration of D-gluconate for induction than does GntR. It has been previously noted that mutation of *gntR* does not affect regulation of the GntII genes (12), nor does gluconate induce *idnK* in the wild-type strain, but it remains possible that the *gntR* mutation plays a role in allowing induction of *idnK* in the particular case of *E. coli* NP202, which contains a *gntRKU* deletion. Nevertheless, the best explanation for induction of the *idn* genes by D-gluconate in *E. coli* NP202 involves formation of the inducer of the *idn* regulon. As for the pseudorevertants of *E. coli* HfrG6ΔMD2, it seems quite possible that the *gntS* locus is actually a mutation of *idnR* which leads to induction of the *idn* genes by altering the normal regulatory properties of IdnR.

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REFERENCES

1. **Ashwell, G.** 1962. Enzymes of glucuronic and galacturonic acid metabolism in bacteria. Methods Enzymol. **5:**190–208.
2. **Bächi, B., and H. L. Kornberg.** 1975. Genes involved in the uptake and catabolism of gluconate by *Escherichia coli*. J. Gen. Microbiol. **90:**321–335.
3. **Bausch, C., N. Peekhaus, T. Blais, and T. Conway.** 1997. Characterization of the gluconate subsidiary system (GntII) in *Escherichia coli*, abstr. K-75, p. 354. *In* Abstracts of the 97th General Meeting of the American Society for Microbiology 1997. American Society for Microbiology, Washington, D.C.
4. **Bernaerts, M., and J. DeLey.** 1971. 2,5-Diketogluconate formation by *Chromobacterium*. Antonie Leeuwenhoek **37:**185–195.
5. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
6. **Burland, V., G. Plunkett III, H. J. Sofia, D. L. Daniels, and F. R. Blattner.** 1995. Analysis of the *Escherichia coli* genome VI: DNA sequence of the region from 92.8 through 100 minutes. Nucleic Acids Res. **23:**2105–2119.
7. **Conway, T.** 1992. The Entner-Doudoroff pathway: history, physiology, and molecular biology. FEMS Microbiol. Rev. **103:**1–27.
8. **Egan, S., R. Fliege, S. Tong, A. Shibata, R. E. Wolf, Jr., and T. Conway.** 1992. Molecular characterization of the Entner-Doudoroff pathway in *Escherichia coli*: sequence analysis and localization of promoters for the *edd-eda* operon. J. Bacteriol. **174:**4638–4646.
9. **Fraenkel, D. G.** 1996. Glycolysis, p. 189–198. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
10. **Fried, B.** 1982. Carbohydrates, p. 255–256. *In* B. F. A. J. Sherma (ed.), Thin-layer chromatography: techniques and applications. M. Dekker, New York, N.Y.
11. **Isturiz, T., J. Vitelli-Flores, and J. Mardeni.** 1979. El metabolismo del gluconato en *E. coli*. Estudio de una mutante delecionada en la region *bioH-asd* del mapa cromosomico. Acta Cient. Venez. **30:**391–395.
12. **Isturiz, T., and J. Celaya.** 1997. The metabolism of gluconate in *Escherichia coli*. The subsidiary system and the nature of the *gntS* gene. J. Basic Microbiol. **37:**105–114.
13. **Istúriz, T., E. Palmero, and J. Vitelli-Flores.** 1986. Mutations affecting gluconate catabolism in Escherichia coli. Genetic mapping of the locus for the thermosensitive gluconokinase. J. Gen. Microbiol. **132:**3209–3212.
14. **Jeffery, J., E. Cederlund, and H. Jornvall.** 1984. Sorbitol dehydrogenase. The primary structure of the sheep-liver enzyme. Eur. J. Biochem. **140:**7–16.
15. **Klasen, R., S. Bringer-Meyer, and H. Sahm.** 1995. Biochemical characterization and sequence analysis of the gluconate:5-oxidoreductase gene from *Gluconobacter oxydans*. J. Bacteriol. **177:**2637–2643.
16. **Lazarus, R. A., R. K. Stafford, J. L. Seymour, M. S. Dennis, M. G. Lazarus, E. J. L. Hughes, H. I. Miller, C. B. Marks, and S. Anderson.** 1990. Presented at the 6th International Symposium on Genetics of Industrial Microorganisms, Strasbourg, France.
17. **Lin, E. C. C.** 1996. Sugars, polyols, and carboxylates, p. 307–342. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
18. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and F. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
19. **Luria, S. E., and M. Delbruck.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28:**491–511.
20. **Malipiero, V., H. P. Ruffner, and D. M. Rast.** 1987. Ascorbic to tartaric acid conversion in grapevines. J. Plant Physiol. **129:**33–40.
21. **Miller, J. H.** 1992. Preparation and use of P1vir lysates, p. 268–272. *In* A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Nagel De Zwaig, R., N. Zwaig, T. Isturiz, and R. S. Sanchez.** 1973. Mutations affecting gluconate metabolism in *Escherichia coli*. J. Bacteriol. **114:**463–468.
23. **Peekhaus, N., S. Tong, J. R. Reizer, M. Saier, E. Murray, and T. Conway.** 1997. Characterization of a novel transporter family that includes multiple *Escherichia coli* gluconate transporters and their homologues. FEMS Microbiol. Lett. **147:**233–238.
24. **Peekhaus, N., and T. Conway.** 1998. Positive and negative transcriptional regulation of the *Escherichia coli* gluconate regulon gene *gntT* by GntR and the cyclic AMP (cAMP)-cAMP receptor protein complex. J. Bacteriol. **180:** 1777–1785.
25. **Porco, A., N. Peekhaus, C. Bausch, S. Tong, and T. Isturiz.** 1997. Molecular genetic characterization of the *Escherichia coli gntT* gene of GntI, the main system for gluconate metabolism. J. Bacteriol. **179:**1584–1590.
26. **Saito, K., and Z. Kasai.** 1982. Conversion of L-ascorbic acid to L-idonic acid, L-idono-gammalactone, and 2-keto-L-idonic acid in slices of immature grapes. Plant Cell Physiol. **23:**499–507.
27. **Saito, Y., Y. Ishii, H. Hayashi, Y. Imao, T. Akashi, K. Yoshikawa, Y. Noguchi, S. Soeda, M. Yoshida, M. Niwa, J. Hosoda, and K. Shimomura.** 1997. Cloning of genes coding for L-sorbose and L-sorbosone dehydrogenases from *Gluconobacter oxydans* and microbial production of 2-keto-L-gulonate, a precursor of L-ascorbic acid, in a recombinant *G. oxydans* strain. Appl. Environ. Microbiol. **63:**454–460.
28. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy lac-based cloning vectors for protein and operon fusions. Gene **53:** 85–96.
31. **Sonoyama, T., B. Kageyama, S. Yagi, and K. Mitsushima.** 1987. Biochemical aspects of 2-keto-L-gulonate accumulation from 2,5-diketo-D-gluconate by *Corynebacterium* sp. and its mutants. Agric. Biol. Chem. **51:**3039–3047.
32. **Sweeney, N. J., D. C. Laux, and P. S. Cohen.** 1996. *Escherichia coli* F-18 and *E. coli* K-12 *eda* mutants do not colonize the streptomycin-treated mouse large intestine. Infect. Immun. **64:**3504–3511.
33. **Tong, S., A. Porco, T. Isturiz, and T. Conway.** 1996. Cloning and molecular genetic characterization of the *Escherichia coli gntR*, *gntK*, and *gntU* genes of GntI, the main system for gluconate metabolism. J. Bacteriol. **178:**3260–3269.
34. **Truesdell, S. J., J. C. Sims, P. A. Boerman, J. L. Seymour, and R. A. Lazarus.** 1991. Pathways for metabolism of keto-aldonic acids in an *Erwinia* sp. J. Bacteriol. **173:**6651–6656.
35. **Yamada, M., T. Kawai, and H. Izu.** 1996. Analysis of the *Escherichia coli gntT* and *gntU* genes and comparison of the products with their homologues. Eur. J. Biochem. **60:**1548–1550.
36. **Zablotny, R., and D. G. Fraenkel.** 1967. Glucose and gluconate metabolism in a mutant of *Escherichia coli* lacking gluconate-6-phosphate dehydrase. J. Bacteriol. **93:**1579–1581.
37. **Zwaig, N., R. Nagel de Zwaig, T. Isturiz, and M. Wecksler.** 1973. Regulatory mutation affecting the gluconate system in *Escherichia coli*. J. Bacteriol. **114:** 469–473.