MICROBIOLOGÍA
Acta Científica Venezolana 58 (1): 21-28, 2007

THE METABOLISM OF GLUCONATE IN *Escherichia coli.* PHYSIOLOGICAL EVIDENCES OF A REGULATORY EFFECT OF IdnR ON THE EXPRESSION OF THE gntR REGULON OPERONS

Ramírez[1], Alvaro H., Rosales, Ikerne; Porco[2], Antonietta; Díaz, Juan C., Istúriz, Tomás

Instituto de Biología Experimental, Centro de Biología Celular, Laboratorio de Fisiología y Genética de Microorganismos, Facultad de Ciencias, Universidad Central de Venezuela, Apartado Postal 47557, Caracas 1041-A, Venezuela

Recibido:13-03-2006

ABSTRACT. Uptake and phosphorylation initiate the catabolism of gluconate in *E. coli*. Such activities conform two systems, GntI and GntII, encoded by two sets of genes differently located on the *E. coli* chromosome and under different regulation. *gnt*T, *gnt*U and *gnt*K (minute 76) encode for high and low affinity gluconate transports and for a thermoresistant gluconokinase respectively, that conform GntI; the mentioned genes and those of the *edd-eda* operon (minute 41) are negatively regulated by the *gnt*R gene product conforming the gntR regulon. *idn*T and *gntV* (minute 96), encode for another gluconate transport and a thermosensitive gluconokinase, conforming GntII. These genes are presumably positively controlled by IdnR. IdnT also functions as a permease for idonate; the corresponding gene is included in the *idn*DOTR operon, responsible for idonate metabolism, in which gluconate is an intermediary. Here we report a regulatory action of IdnR on the operons of the gntR regulon; *i.e.*, *gnt*T, *gnt*KU and *edd-eda*. The expression of these operons, was diminished in a *gnt*R mutant complemented with a clone of *idn*R and also in *E. coli* mutants in which the *idn*DOTR operon is expressed in a gluconate dependent inducibility. This is the first report of a regulatory effect of IdnR on *edd-eda* operon expression. **Key words**: gluconate, regulon gntR

EL METABOLISMO DEL GLUCONATO EN *Escherichia coli*. EVIDENCIAS FISIOLOGICAS DE UN EFECTO REGULATORIO DE IdnR SOBRE LA EXPRESION DE LOS OPERONES DEL REGULON gntR

RESUMEN. El transporte y la fosforilación inician el catabolismo del gluconato en *E. coli.* Estas actividades conforman dos sistemas, GntI y GntII, codificados por dos grupos de genes, diferentemente regulados y ubicados en distintos sitios del cromosoma. Los genes *gnt*T, *gnt*U y *gnt*K (minuto 76), codifican distintas proteínas para transportes de alta y baja afinidad para gluconato y una gluconoquinasa termoresistente respectivamente, que forman el sistema GntI; los genes respectivos junto con los del operón *edd-eda* (minuto 41), son regulados negativamente por el producto de *gnt*R (minuto76) constituyendo el regulón gntR. Los genes *Idn*T y *gntV* (minuto 96), codifican otra permeasa para gluconato y una gluconoquinasa termosensible que forman GntII. IdnT funciona también como permeasa para idonato; el gen correspondiente es parte del operón *idn*DOTR, regulado positivamente por IdnR y responsable del metabolismo del idonato en el que gluconato es un intermediario. Se reporta un efecto regulatorio de IdnR sobre los operones del regulón gntR; *i.e.*, *gnt*T, *gnt*KU and *edd-eda*. La expresión de estos operones resultó disminuida en una mutante *gnt*R complementada con un clon de *idn*R y también en mutantes de *E. coli* en la que la expresión del operón *idn*DOTR se induce en presencia de gluconato. Este es el primer reporte de la acción regulatoria de IdnR sobre la expresión del operón *edd-eda.* **Palabras claves:** gluconato, regulon gntR.

INTRODUCTION

In *E. coli*, gluconate catabolism occurs via Pentose Phosphate pathway (PP), but also and mainly by the Entner-Doudoroff (ED) pathway. The latter is an inducible pathway, as those of gluconate specific phosphorylation and transport activities, when cells are grown in gluconate containing media [6,9,11,17]. Gluconate transport and phosphorylation activities, which are known as initial gluconate metabolism, occur through duplicate activities which form two systems [1,12]. These systems, GntI and GntII, are coded by two distinctly regulated sets of genes located in different regions of the bacterial chromosome [8,23]. The *bio*H-*asd* region of the chromosome, between minutes 76.3-77.1, contains genes that code for a repressor (*gnt*R), high and low affinity gluconate specific transporters, (*gnt*T and *gnt*U, respectively) and a thermoresistant gluconokinase (*gnt*K). The last three activities constitute the GntI system, considered as the main system for the initial metabolism of the substrate. The expressions of the corresponding genes, as well as those of the ED pathway (*edd-eda* operon) are negatively controlled by GntR; this regulatory network constitutes the gntR regulon. The *cys*Q-*val*S region, between minutes 95.3-96.8, contains *idn*T and *gntV*, which are genes that code for activities associated with the GntII system, *i.e.*, an alternative gluconate high affinity transport and a thermosensitive gluconokinase, respectively. IdnT funtions as a permease for gluconate and idonate; the corresponding gene *idn*T, is included in the *idn*DOTR operon, responsible of the idonate metabolism, which is divergently transcribed to *gntV* (Fig. 1).

Since the presence of two systems (GntI and GntII) was established [1,12], an important question to be addressed has been concerned with the coordination of expression between these two systems during the substrate utilization. The genetics, physiology and particular control circuits ascribed to the GntI system have been established [6,9,17,26], and later confirmed [8,9,25]; however, advances on the posed question demanded similar knowledge on the subsidiary system, aspect significantly advanced during the present and past two decades. A regulatory locus at the minute

Actual address:
[1] Centro de Microbiología y Virología Celular; laboratorio de Biología de Virus, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela.
[2] Departamento de Biología Celular, Universidad Simón Bolívar, Apartado Postal 89000, Caracas, Venezuela.

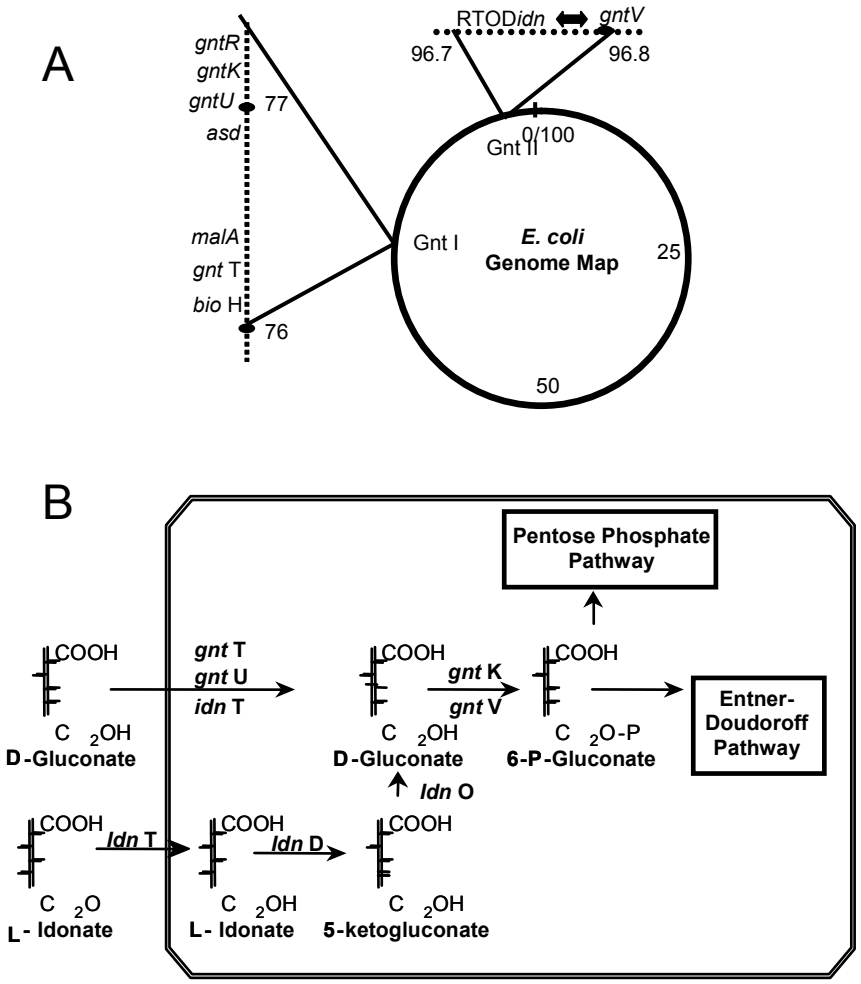

Figure 1. A: Genetic map of *E. coli* K12 showing locations of operons involved in initial gluconate and idonate metabolisms. B: Initial metabolisms of these sugars acid in *E. coli* K12.

95.3 of the genetic map, that controls positively *gntV* and also modulates, in a positive form, the expression of GntI has been reported [13,15]. Likewise, it was demonstrated that IdnR, the positive regulator of the *idn*DOTR operon [4], involved in the metabolism of gluconate and idonate, controls negatively the expression of the GntI operons [24]. The assumption that such effect is consequence of a significant homology between *idn*R and *gnt*R led us to wonder about a possible action of IdnR on the expression of the *edd-eda* operon which, similarly to the responsible operons of the GntI system, contains sites for GntR binding.

Our laboratory possesses several *E. coli* mutants that show constitutive expression of the gntR regulon. The mutations associated to these strains alter *gnt*R, whose product negatively controls the expression of *gnt*KU, *gnt*T and *edd-eda* operons, making it unable to function as a repressor of the mentioned operons [26]. In this same order of ideas, we have *E. coli bio*H-*asd* deletion mutants that have allowed the selection of derivative strains in which the activities that conform the subsidiary system GntII, are expressed in an inducible form. These mutants constitute special genetic and physiological systems to investigate the regulatory action of alternative genetic elements on the expression of genes and operons that form the gntR regulon. In the present paper we report the results obtained with *E. coli* mutants M18 [26] and C177 [12], which indicate such regulatory action of IdnR. In the former strain, carrying an ambar *gnt*R mutation, the activities of this regulon were diminished when transformed with an *idn*R clone; these same activities were also altered in a derivative of the latter strain, in which the *idn*DOTR operon is expressed in a gluconate dependent inducibility. The results suggest, additionally, that IdnR and GntR might act in concert to modulate the expression of the operons involved in the first steps of the gluconate metabolism in *E. coli.*

MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages

The strains, plasmids and bacteriophages used in this study are listed in Table I. The genetic markers are those listed by Berlyn *et al* .[5].

Media

E. coli strains were cultivated in Luria-Bertani broth (LB), mineral medium MM [22] or Casein hydrolysate (CAA; MM supplemented with 1% casein hydrolysate). LB plates, gluconate bromthymol blue indicator plates (BTB-gluconate plates)[2] and tripheniltetrazolium chloride-gluconate plates (TTZ plates)[16], were also used. When indicated, MM and

Table I. Bacterial strains, plasmids and bacteriophage.

Strain	Genotypes	Sources
DH5αMCR™	F⁻ *mcrA* Δ(*mcr-hsd*RMS-*mcr*BC) Ø80*lac*ZDM15 (*Lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1	Invitrogen
M1	Wild Type HfrC	Ref. 26
M18	Wild Type HfrC, *gnt*R1.8	Ref. 26
W3110	Wild Type F⁻	Lab. stock
W1485	K-12 Wild Type(λ⁻ *rph1*)	CGSC
HfrG6	Hfr *his*	M. Schwartz
HfrG6ΔMD2	HfrG Δ(*bio*H-*gnt*T-*mal*A-*glp*D-*asd*-*gnt*UKR) *his*	M. Schwartz
C177	HfrG Δ(*bio*H-*gnt*T-*mal*A-*glp*D-*asd*-*gnt*UKR) *his gnt*177	Ref. 12
C184	HfrG6, *his*, *gnt*177	This study
Plasmid		
pUC18	Cloning vector, *lac*Zα, Ap	Invitrogen
pUC19	Cloning vector, *lac*Zα, Ap	Invitrogen
pIdnR	pUC19 containing 1 kb *idn*R *Bam*HI fragment generated by PCR	This study
Bacteriophage		
P1		Lab. stock

CAA were supplemented with 5 µg ml^{-1} of thiamine hydrochloride, 20 µg ml^{-1} of L-amino acids as required, 500 µg ml^{-1} DL-α-ε--diaminopimelic acid (or 40 µg ml^{-1} in the cases where rich media were used, *i.e.*, LB, BTB and TTZ) and the carbon source at 2 g l^{-1} or 4 g l^{-1}, was used. If necessary, ampicillin (80 µg ml^{-1}) was added to select cells harbouring ampicillin-resistant plasmids.

Growth conditions

Cells were routinely grown aerobically at 37 °C, in volumes of 10 ml for growth curves and 20 ml for enzyme assays in 125 ml flasks fitted with side arms, on a gyratory water bath (model G776, New Brunswick) at about 200 cycles min^{-1}. In each case, the growth was monitored by reading the optical density in a Klett colorimeter with a N° 42 filter.

Assay of [U-^{14}C]gluconate uptake

Gluconate uptake activity was measured according to Porco *et al.* [20]. The specific rates of gluconate uptake are expressed as pmol taken up by 10^7 cells min^{-1}.

Preparation of crude extracts

Cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl 10 mM $MgCl_2$ (pH 7.6) and disrupted by 30s sonication pulses (2 pulses) in a Braun Sonic 2000 (12T probe, 45 wattage level) with 30s cooling periods between them. Cell debris was, in each case, removed by centrifugation at 27000xg for 15 min.

Enzyme assays

Gluconokinase, and 6-phosphogluconate dehydratase activities were assayed according to Fraenkel and Horecker [10]. The Gluconoquinase heat inactivations were performed according to Istúriz *et al.* [14]. Activities are reported as nmol min^{-1} (mg protein)$^{-1}$.

Phages and generalized transductions

A stock of phage P1 kept in our lab was used. The preparation of P1 lysates and generalized transductions were done according to Miller [16].

DNA manipulations, transformations and sequencing

Conventional and standard recombinant DNA techniques [21] were applied. Nucleotide automated sequencing with a Big Dye terminator Kit were made in a Perkin-Elmer sequencer ABI PRISM™ 377, at the Centro de Secuenciación y Análisis de Ácidos Nucleicos - Instituto Venezolano de Investigaciones Científicas (CeSAAN-IVIC).

Cloning of idnR gene

The *idn*R gene was cloned by inserting a PCR fragment, once amplified from *E. coli* M1 with a primer set consisting of PAH3 (5'CGGAATTTCCTAAAGCGTGTTGCCGTGATAAATC-3') containing an *Eco*RI site, and PAH4 (5'CGGGATCCGACGTTGGGAGAAGTTATGCG-3') containing a *Bam*HI site, into the *Eco*RI/*Bam*HI site of pUC19 cloning vector. PCR amplification was performed following Colony PCR protocol, *i.e.,* by scraping two or three colonies from agar plates, thoroughly washing the cells five times with sterile-deionized water and resuspended the final pellet in 100 µl of sterile-deionised water. PCR amplifications (30 cycles) consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min and extension at 72°C for 1 min, using sequence-specific primers and *Taq* DNA polimerase (Invitrogen- Life technologies), in a MJ Research P150 minicycler.

Probe construction and labelling

Antisense RNA probes were used for Northern blot hybridization. The sequence of the region containing the *idn*D start codon was obtained by PCR amplification with the restriction primers IdnDPstI (5'-AACTGCAGTTAGAAAACAAGCTGGACTTTTG-3') and IdnDBamHI (5'-CGGGATCCATGCAAGTGAAAACACAGTCCT-3'). The 1.021-kb *Bam*HI/*Pst*I PCR fragment was cloned into pBluescriptII SK+ (Stratagene). The sequence of the region containing the *gnt*V start codon was obtained by PCR

amplification with the restriction primers IdnK-BamHI (5'-CGGGATCCAACAACGCAGGACTGTG-3') and IdnK-KpnI (5'-GGGGTACCCTCTTTCGCACATTATTCTG-3'). The 0.788-kb *Bam*HI/*kpn*I PCR fragment was cloned into pBluescriptII SK+. The plasmids were linearized with respective sets of restriction enzymes mentioned above and then by synthesis of [α-^{32}P]-UTP-labeled RNAs with T3 RNA polimerase.

RNA isolation and Northern blot analysis

Total cellular RNA isolated from *E. coli* and different mutants strains grown to mid-log phase (optical density at 550 nm of 0.4) were obtained by the hot phenol method; likewise, probe hybridization to the membrane-bound RNA and stripping from the membranes were both made according to TONG *et al.* [23].

Chemicals

Sodium [U-^{14}C]gluconate, specific activity 5.6 mCi (0.21 GBq) nmol^{-1} was obtained from Amersham. D-gluconic acid (potassium salt), pyrimidine nucleotides, sugars, aminoacids and most other chemicals were purchased from Sigma. Media were from L-Himedia Lab. DNA enzymes were purchased from Promega and Invitrogen.

RESULTS

Molecular nature of gntR mutation in E. coli M18

In the *E. coli* mutant M18, the operons that conform the GntR regulon are expressed in a constitutive form. Early interpretations and classical mapping of the involved mutation, led to the conclusion that it was one of regulatory nature, altering *gnt*R [26]. Due to our interest in using this mutant as recipient of a pidnR plasmid containing the wild type *idn*R allele in transformation experiments, we proceeded to confirm its gluconate phenotype in molecular terms. The cloning and sequencing of *gnt*R alleles from *E. coli* M18, and its parental wild type *E. coli* M1, was accomplished and a comparative analysis between them and other *gnt*R reported alleles [23], (GenBank, EG12630, g1789846) was made. A point mutation originating a C *to* T transition at position 841 was identified (Fig. 2); it creates an Opal stop codon (UGA) which should generate a shorter product with 52 aminoacids less, as compared with its wild type allele or reported *gnt*R sequence (GenBank AE000420). Thus, the product generated must not function as the negative regulator of the regulon *gnt*R, explaining the constitutive expression of *gnt*T, *gnt*KU and *edd-eda* operons.

```
gntR GenBank            811 attgccggtttccacggtcatgacattggtcaggtgagt 850
                                I  A  G  F  H  G  H  D  I  G  Q  V  M
gntR Tong et al. 1996   811 attgccggtttccacggtcatgacattggtcaggtgagt 850
                                I  A  G  F  H  G  H  D  I  G  Q  V  M
gntR E. coli M1         811 attgccggtttccacggtcatgacattggtcaggtgagt 850
                                I  A  G  F  H  G  H  D  I  G  Q  V  M
gntR E. coli M18        811 attgccggtttccacggtcatgacattggttag       843
                                I  A  G  F  H  G  H  D  I  G  *
```

Figure 2. Comparison of mutants and wild type *E. coli gnt*R sequences.

Effect of IdnR on the E. coli gntR regulon operons

The transformations of the *E. coli* mutant M18 and the *E. coli* wild type W3110 with the plasmid pidnR carrying the wild type *idn*R allele on pUC19 vector, produced no fermentative gluconate phenotypes on TTZ and BTB-gluconate plates, suggesting that a significant and negative effect of IdnR might be altering the expression of one or more operons of the gntR regulon (Table II). Such effect did not avoid the utilization of gluconate by the transformed cells, since them remained able to grow on mineral plates containing gluconate as sole carbon and energy source.

Table II. Gluconate phenotypes on plates of *E. coli* strains

Strain	Genotype	Solid Culture Media				
		LB	LB+Amp	TTZ	BTB	MM + Gto
W3110	*HfrC* Prototrofic	+	-	W	Y	+
W3110 -19	*HfrC* Prototrofic pUC19	+	+	W	Y	+
W3110-IdnR	*HfrC P*rototrofic pAHIdnR (*)	+	+	R	W	+
M18	*gnt*R1.8	+	-	W	Y	+
M18-19	*gnt*R1.8, pUC19	+	+	W	Y	+
M18- IdnR	*gnt*R1.8 pAHIdnR (*)	+	+	R	W	+

Y (Yellow) indicates fermenting phenotype on BTB plates. W (White) designates fermenting phenotype on TTZ plates but nonfermenting on BTB plates. R (Red) points out no fermentation on TTZ plates. (+), growth; (-), non-growth; LB, Luria Bertani Broth; Amp, Ampicilline (100 μg ml^{-1}); (*), *idn*R cloned in pUC19 vector; MM + Gto, Mineral agar plates suplemented with 0.2 % gluconate plus any required nutrient.

In order to support the above results, specific activities of gluconate uptake, gluconokinase and 6-phosphogluconate dehydratase, were performed in cells extracts from *E.* coli M1, M18, M1-pUC19 and M18pidnR (Table III). While the assayed activities were expressed in inducible form in the *E. coli* wild type M1 used as a control, they were constitutive in the *E. coli* M18 and M18pUC19; *i.e.*, expressed regardless of presence of gluconate in the culture medium. Interestingly, the *E. coli* M18pidnR mutant, which contains the wild type *idn*R cloned gene, displayed these activities severely altered if compared with those exhibited by *E. coli* M18pUC19 cultivated in identical conditions (Table III). These results explained the nonfermentative gluconate phenotypes and suggested that *idn*R exerts a negative control on the expression of operons that form the gntR regulon. Noteworthy, this effect was counteracted by gluconate and not by 5-Keto-D-gluconato (5KG, Table III), necessary element for the *idn* operon expression [3].

Alternative experiments were made in order to evaluate if the effects detected in pidnR transformed cells might have been the result of the regulator over expression promoted by plasmid copy number. Thus, specific activities of gluconate uptake and 6-phosphogluconate dehydratase were performed in cellular extracts from *E. coli* mutant M18 cultured in CAA medium supplemented with gluconate or 5KG. The levels of both activities in cells cultivated in CAA-gluconate containing media resulted lower than those previously registered in extracts from cells

Table III. Activities of gluconate catabolism in *E. coli* strains

Strain	Carbon Source	[U-^{14}C] Gluconate uptake	Gluconoquinase (*)		6-Phosphogluconate dehydratase
M1	Succinate	2	1	(0 %)	10
	Gluconate	34	180	(18 %)	126
M18	Succinate	75	657	(20 %)	121
M18pUC19	Succinate	62	662	(21 %)	101
M18pidnR	Succinate	11	23	(0 %)	ND
	Gluconate	26	102	(20 %)	37
	5-keto-D-gluconate	7	37	(51%)	28
M18	Gluconate	NI	308	(10%)	46
	5-keto-D-gluconate	NI	159	(60%)	7

Cells were cultured on casaminoacids medium supplemented with succinate at 0.4% and collected during the exponential phase; then grown on the same medium with 0.4% of indicated carbon source, up to 120 UK. ND, no detected. NI, not investigated. For units, see Material and Methods. (*) Percentages in parenthesis indicate gluconokinase lability (lost of activity after extract incubation at 30 ºC for 3 h).

cultivated in CAA-succinate containing media, and even lower in extracts from cells cultured in CAA-5KG media (Tabla III). Likewise, while the gluconokinase expressed in presence of gluconate was the thermoresistant form (10% heat inactivated), the one detected in presence of 5KG was the thermosensitive one (60% heat inactivated), indicating not only the expression of the *idn* operon and consequently the *idn*R gene, but also that the expression of the gluconokinase thermoresistant was significantly compromised in this condition; also, and noteworthy, the specific activity of the 6-phosphogluconate dehydratase, was severely altered [7 nmol of 6-phosphogluconate min^{-1} (mg protein)$^{-1}$] when compared with the levels detected in succinate and gluconate containing media [121 and 47 nmol of 6-phosphogluconate min^{-1} (mg protein)$^{-1}$, repectively]. Together these results confirmed a negative regulatory effect of IdnR on the expression of the gntR regulon; they were of central importance because gave evidence for the first time of an effect of this regulator on the expression of *edd* and consequently on the *eda* gene as well.

A suitable mutant to study the effect of IdnR on the edd-eda operon

The results obtained and described before, constituted base for the study and analysis of the effect of the *idn* operon expression - through IdnR - on the expression on the *edd-eda* operon in *E. coli.* In this respect, we considered that the *E. coli* mutant C177, isolated and characterized early [12], was suitable to reach this goal. This mutant and its parental *E. coli* HfrG6ΔMD2 lack genes which code for GntI system, due to a deletion at the *bio*H-*asd* region (min. 76), that includes *gnt*R. In consequence, both express the *edd-eda* operon constitutively [26]; however, while the parental mutant does not grow on mineral medium supplemented with gluconate at concentrations routinely used (0.2-0.4%), *E. coli* C177 has a mutation at the minute 96, that enables it to utilize gluconate (0.2%) at expenses of the subsidiary system GntII, expressed in an inducible substrate-dependent form. Because these activities are involved in the idonate metabolism and the inducer of the *idn*DOTR operon is idonate or 5KG the use of induced wild type cells by this compound or 5KG/gluconate induced *E. coli* C177, seemed suitable systems to investigate the action of IdnR on the operon *edd-eda*.

The experimental approximation described above assumed, that in *E. coli* C177 cultivated in gluconate containing media the *idn* operon and *gntV* are expressed in inducible form. In order to demonstrate this hypothesis, gene transcripts were identified by Northern blot analysis in strains *E. coli* C177, *E. coli* HfrG6ΔMD2 and *E. coli* W1485 as control, each of them cultivated in LB medium alone or supplemented with gluconate or 5KG. Northern blots were performed with probes [^{23}, 0.01-0.1µg; 10^5-10^6 cpm] generated from fragments cloned in pBluescript SK+ corresponding to the coding region of *idn*D and *gntV* genes.

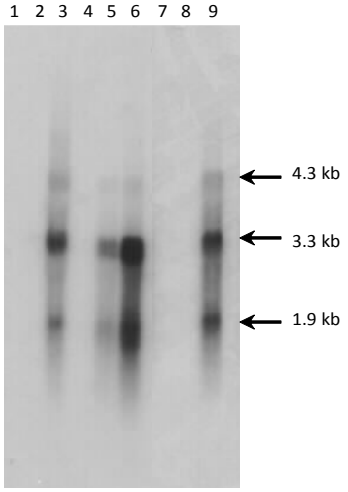

Figure 3. Northern blot analysis of the *idn*D gene. Total RNA samples (5 µg per lane) were prepared from *E. coli* strains cultivated on LB medium, LB plus 0.4% gluconate (LB+gnt), and LB plus 5-keto-D-gluconate 0,4% (LB+5KG). RNA from *E. coli* HfrG6ΔMD2, *E. coli* C177 and *E. coli* W1485 grown on LB (lanes 1, 4 and 7), on LB+gnt (lanes 2,5 and 8) and on LB+5KG (lanes 3, 6 and 9)

The hybridization with the *idn*D probe (Fig. 3), showed three bands that correspond to transcripts *idn*DO (1.9 kb) *idn*DOT (3.3 kb) and *idn*DOTR (4.3 kb) from cells cultivated in 5KG; such bands were also displayed with transcripts of *E. coli* C177 grown in gluconate containing media. The hybridization with the *gntV* probe displayed one band identifying the *gntV* transcript in each strain grown in presence of 5KG containing media. This band is also present in strains *E. coli* W1485 and *E. coli* C177 cultivated in presence of gluconate but not in *E. coli* HfrG6ΔMD2. No hybridization was observed with transcripts from cells grown in LB (data not shown).

Together, the above results indicated that in the *E. coli* C177, the *idn* operon is expressed in an inducible form when cultured in gluconate or 5KG containing media; since in both cases the *idn*R gene has to be expressed, then its action on the *edd-eda* operon, if it occurs, could be established.

Effect of IdnR on the edd-eda operon

Once established the utility of *E. coli* C177 for the studies related with the *idn*R regulator effect, this strain and its parental strains *E. coli* HfrG6ΔMD2 and HfrG6 were studied and their gluconate metabolism related activities assayed. In this sense, these strains were grown in CAA medium supplemented with fructose or gluconate (Table IV). While activities of [C-14]gluconate uptake and gluconokinase were expressed in an inducible form in *E. coli* strains HfrG6 and C177, they were not detected, as expected, in *E. coli* HfrG6"MD2; however, the activities in *E. coli* C177 were lower [55 vs 81 pmol [U-^{14}C] gluconate and 73 vs 173 nmol gluconate min^{-1} (mg protein)$^{-1}$] than those expressed in *E. coli* HfrG6 used as control. Since the characteristics of the gluconokinase expressed in each case account for which system is induced, then could be assumed that GntI was induced in the control strain (12% heat inactivated) and the subsidiary system (GntII) in *E. coli* C177 (76% heat inactivated) when both of them were grown in gluconate containing medium. Respect to the specific activities of 6-phosphogluconate dehydratase, these were expressed constitutively in both, *E. coli* C177 and its parental *E. coli* HfrG6ΔMD2, but resulted severely altered in *E. coli* C177 cultivated in presence of gluconate [Table IV; 25 vs. 135 nmol of 6-phosphogluconate min^{-1} (mg protein)$^{-1}$, respectively].

Effect of IdnR on the gntR regulon operons in presence of GntR

The fact that *E. coli* C177 expresses the *idn* operon once cultivated in gluconate containing media, allowed designing an strategy to analyse the effect of IdnR on the expression of the operons of the gntR regulon in presence of GntR. It might suggest some kind of interaction between these two regulators to modulate and to control the initial steps in *E. coli* gluconate metabolism. In order to do this, the *bio*H-*gnt*T-*mal*A-*gly-asd-gnt*UK-*gnt*R region was restored in *E. coli* C177 by transducing it to Mal$^+$, Asd$^+$ with phage P1 cultivated in *E. coli* HfrG6 and a representative transductant was designated as *E. coli* C184. These three strains gave yellow fermentative colonies on BTB-gluconate plates; however when cultivated in mineral medium with gluconate, the levels of [U-^{14}C]gluconate uptake, gluconokinase and 6-phosphogluconate dehydratase were lower in *E. coli* C184 than in *E. coli* HfrG6 (Table IV), supporting the suggested negative regulatory effect of IdnR on the expression of the gntR regulon, even in presence of GntR.

DISCUSSION

The results reported here, demonstrate a regulatory and negative effect of IdnR on the expression of the gntR regulon which, at the same time that confirm similar findings on the responsible operons of the GntI system [24], involves (as it was suggested here), a novel effect on the *edd-eda* operon.

Table IV. Activities of gluconate catabolism in *E. coli* strains.

Strain	Carbon Source	[U-^{14}C] Gluconate uptake	Gluconoquinase (*)		6-Phosphogluconate dehydratase
	Fructose	2	ND		10
HfrG6	Gluconate	82	132	(12 %)	126
	5-keto-D-gluconate	120	119	(80 %)	NI
	Fructose	ND	ND		146
HfrG6ΔMD2	Gluconate	ND	ND		135
	5-keto-D-gluconate	64	NI		NI
	Fructose	1	ND		99
C177	Gluconate	55	73	(76 %)	25
	5-keto-D-gluconate	118	81	(60 %)	NI
	Fructose	1	3		8
C184	Gluconate	61	71	(9 %)	44
	5-keto-D-gluconate	NI	NI		NI

Cells were cultured on CAA medium supplemented with succinate at 0.4% and collected during the exponential phase, then grown on the same medium with 0.4% of indicated carbon source, up to 120 UK. ND, no detected. NI, not investigated. For units, see Material and Methods. (*) Percentages in parenthesis indicate gluconokinase lability (percentage lost after 30 ºC preincubation for 3 h).

To have suitable *E. coli* mutants, allowed us to investigate the regulatory effect of IdnR in absence of GntR (*E. coli* C177), as well as in presence of this regulator altered (*E. coli* M18), or wild type (*E. coli* C184). While the first of the three named strains was suitable to demonstrate specifically the effect on the *edd-eda* operon, the latter two strains allowed to do it on the gntR regulon.

IdnR modulates negatively the gntR regulon expression

Based on the regulatory function ascribed to the *E. coli idn*R allele, the negative gluconate fermentative phenotype displayed by colonies of *E. coli* M18, once transformed with the pidnR plasmid, suggested an alteration in the utilization of the substrate that could be attributed to an IdnR negative action. Such effect altered the activities coded by the operons of the gntR regulon since it was confirmed by assays of respective specific activities; all of them showed diminished levels in extracts from *E. coli* M18pidnR (Table IV) as compared with those registered in extracts from cells transformed with the vector plasmid used as control. The enrichment in thermosensitive gluconokinase (51% inactivation) of the extract from cells cultivated in presence of 5KG allowed associating this activity with the induction of the *idn* operon. In fact, it was in this experimental condition, regardless the cells were or not transformed with the hybrid plasmid, that the regulatory effect of IdnR was more evident.

GntR might modulate the expression of gntV

It was of interest to notice in extracts of *E coli* M18 (Table IV) cultivated in succinate, some percentage (20%) of GntV activity, similar to those registered in wild type cells cultivated in conditions of gluconate promoted induction. This result, reiteratively observed in several of our *gnt*R mutants, is contrary to the accepted idea that GntR does not exert any action on the expression of the GntII system. The current knowledge [4,24] about a GntR-binding-site like sequence into the divergent region between the *idn* operon and *gntV*, gives base to suggest that GntR might be involved in the modulation of the GntII expression.

E. coli C177 metabolizes gluconate through GntII

The experimental evidence with the Northern blot showed that *E. coli* C177, contrary to its parental *E. coli* HfrG6ΔMD2 metabolizes gluconate via GntII; *i.e.,* expresses in inducible form the *idn* operon and the *gntV* gene. Because *idn*R was expressed under conditions where the *idn* operon was induced, this experimental evidence made feasible not only to demonstrate specifically the regulatory effect of IdnR on the *edd-eda* operon in absence of GntR, but also in the derivative *E. coli* C184 where it was expected, due to its genotype *gnt*R$^+$*gnt*177, that both alleles, *gnt*R and *idn*R, were simultaneously expressed when cultivated in gluconate containing medium. Indeed, the experimentation with *E. coli* C184 could be revealing a relation between the two regulators that might be part of the strategy in *E. coli* to utilize differential and efficiently the systems GntI and GntII. In this respect, it is important to point out that early studies on continuous cultures [6] indicated, that the wild type *E. coli* growing at very low dilution rates in gluconate limited mineral medium, metabolizes the substrate through GntII by expressing *gnt*V. In these conditions, which approximate the scarcity of substrate in natural bacterial environments, low basal levels of Edd are kept, so presumptively the metabolism of gluconate occurs via PP with an associate energy saving.

ACKNOWLEDGEMENTS

This work was supported by FONACIT, Grant N^0 S1-2001000704 and CDCH of Universidad Central de Venezuela, Grant N^0 P 03.00.6308.2006.

REFERENCES

1. **Bächi, B., Kornberg, H. L.** Genes involved in the uptake and catabolism of gluconate by *Escherichia coli. J. Gen. Microbiol.,* **90**: 321-335, 1975.
2. **Baker, H. V., Wolf, R. E**. Growth rate-dependent regulation of 6-phosphogluconate dehydrogenase level in *Escherichia coli* K-12: β-galactosidase expression in *gnd-lac* operon fusion strains. *J. Bacteriol.*, **153**: 771-781, 1983.
3. **Bausch, C., Peekhaus, N., Utz, C., Blais, T., Murray, E., Lowry, T., Conway, T.** Sequence analysis of the GntII (Subsidiary) system for gluconate metabolism reveals a novel pathway for l-Idonic acid catabolism in *Escherichia coli J. Bacteriol.*, **180**: 3704-3710, 1998.
4. **Bausch, C., Ramsey, M., Conway, T**. Transcriptional organization and regulation of the L-idonic acid pathway (GntII system) in *Escherichia coli. J. Bacteriol.*, **186**: 1388-1397, 2004.
5. **Berlyn, M. K. B., Brooks, L., Rudd, K. E., Singer, M. Linkage map of *Escherichia coli* K-12. Edition 9. In: Neidhardt, F. C., Curtis III, R., Ingraham, J.L., Lin, E. C., Brooks L. K., Magasanik, B., Reznikoff, W. S., Riley, M., Shaechter, M., Umbarger, E.** *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd.ed. American Society for Microbiology, Washington D. C., 1715-1902, 1996.
6. **Coello, N., Istúriz, T.** The metabolism of gluconate in *Escherichia coli*. A study in continuous culture. *J. Basic. Microbiol.,* **32**: 309-315, 1992.
7. **Cohen S. S.** Gluconokinase and the oxidative path of glucose-6-phosphate utilization. *J. Biol. Chem.*, **189**: 617-628, 1951.
8. **De Rekarte, U., Cortés, M., Porco, A., Niño, G., Istúriz, T.** Mutations affecting gluconate catabolism in *Escherichia coli*. Genetic mapping of *loci* for the low affinity transport and the thermoresistant gluconokinase. *J. Basic Microbiol.*, **34**: 363-370, 1994.
9. **Eisenberg, R., Dobrogosz, W.J.** Gluconate metabolism in *E. coli. J. Bacteriol.,* **93**: 941-947, 1967.
10. **Fraenkel, D. G., Horecker, B. L**. Pathways of D-glucose metabolism in *Salmonella typhimurium. J. Biol. Chem.*, **239**: 2765-2771, 1964.
11. **Fraenkel, D. G., Levisohn, S. R.** Glucose and gluconate metabolism in an *Escherichia coli* mutant lacking phosphoglucose isomerase. *J. Bacteriol.*, **93**: 1571-1578, 1967.
12. **Istúriz, T., Vitelli-Flores, J., Mardeni, J.** El metabolismo del gluconato en *E. coli*. Estudio de una mutante delecionada en la región *bio*H-*asd* del mapa cromosómico. *Acta Cient. Vlana.,* **30**: 391-395, 1979.

13. **Istúriz, T., Celaya, J**. The metabolism of gluconate in *E. coli*. The subsidiary system and the nature of the *gnt*S gene. *J. Basic. Microbiol.,* **37**: 105-114, 1997.
14. **Istúriz, T., Palmero, E., Vitelli-Flores, J.** Mutations affecting gluconate metabolism in *Escherichia coli*. Genetic mapping of the *locus* for the thermosensitive gluconokinase. *J. Gen. Microbiol.*, **132**: 3209-3219, 1986.
15. **Istúriz, T., Díaz-Benjumea, R., Rodríguez, N., Porco, A**. Involvement of *gnt*S in the control of GntI, the main system for gluconate metabolism in *Escherichia coli. J. Basic Microbiol.,* **41**: 75-83, 2001.
16. **Miller, J. H.** Experiments in Molecular Genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. 1972.
17. **Nagel De Zwaig, R., Zwaig, N., Istúriz, T., Sánchez, E. S**. Mutations affecting gluconate metabolism in *Escherichia coli. J. Bacteriol.*, **114**: 463-468, 1973.
18. **Peekhaus, N., Tong, S., Reizer, J., Saier Jr., M. H., Murray, E., Conway, T.** Characterization of a novel transporter family that includes multiple *Escherichia coli* gluconate transporters and their homologies. *FEMS Microbiol. Lett.*, **147**: 233-238, 1997.
19. **Porco, A., Peekhaus, N., Bausch, C., Tong, S., Istúriz, T., Conway, T.** Molecular genetic characterization of the *Escherichia coli gnt*T gene of GntI, the main system for gluconate metabolism. *J. Bacteriol.*, **179**: 1584-1590 1997.
20. **Porco, A., Alonso, G., Istúriz, T**. The gluconate high affinity transport of GntI in *Escherichia coli* involves a multicomponent complex system. *J. Basic Microbiol.,* **38**: 395-404, 1998.
21. **Sambrook, J., Fritsch, E., Maniatis, T.** Molecular cloning: a laboratory manual, 2nd. ed. Cold Sring Harbor Laboratory Press, Cold Spring Harbor, New York. 1989.
22. **Tanaka, S. S., Lerner, A., Lin, E. C. C.** Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. *J. Bacteriol.*, **93**: 642-648 1967.
23. **Tong, S., Porco, A., Istúriz, T., Conway, T.** Cloning and molecular genetic characterization of the *Escherichia coli gnt*R, *gnt*K, and *gnt*U genes of GntI, the main system for gluconate metabolism. *J. Bacteriol.*, **178**: 3260-3269, 1996.
24. **Tsunedomi, R., Izu, H., Kawai, T., Matsushita, K., Ferenci, T., Yamada, M.** The activator of GntII genes for gluconate metabolism, GntH, exerts negative control of GntR-regulated GntI genes in *Escherichia coli. J. Bacteriol.*, **185**: 1783-1795, 2003.
25. **Yamada, M., Kawai, T., Izu, H.**. Analysis of the *Escherichia coli gnt*T and *gnt*U genes and comparison of the products with their homologues. *Biosci. Biotech. Biochem.*, **60**: 1548-1550, 1996.
26. **Zwaig, N., Nagel de Zwaig, R., Istúriz, T., Wecksler, M.** Regulatory mutations affecting the gluconate system in *Escherichia coli. J. Bacteriol.,* **114**: 469-473, 1973.

Correspondencia: Tomás Istúriz, Instituto de Biología Experimental, Centro de Biología Celular, Laboratorio de Fisiología y Genética de Microorganismos, Facultad de Ciencias, Universidad Central de Venezuela, Apartado Postal 47557, Caracas 1041-A, Venezuela.
Correo electrónico: toisturiz@hotmail.com;
tomas.isturiz@ciens.ucv.ve