Gluconate as suitable potential reduction supplier in *Corynebacterium glutamicum*. Cloning and expression of *gntP* and *gntK* in *Escherichia coli*

ANTONIETTA PORCO¹, ELIDA E. GAMERO², ELENA MYLONÁS¹ and TOMÁS ISTÚRIZ²

¹ Departamento de Biología Celular, Universidad Simón Bolívar, Apartado Postal 89000, Caracas-Venezuela.

² Instituto de Biología Experimental, Facultad de Ciencias, Universidad Central de Venezuela, Apartado Postal 47557, Caracas 1041-A, Venezuela

ABSTRACT

Corynebacterium glutamicum is widely used in the industrial production of amino acids. We have found that this bacterium grows exponentially on a mineral medium supplemented with gluconate. Gluconate permease and Gluconokinase are expressed in an inducible form and, 6-phosphogluconate dehydrogenase, although constitutively expressed, shows a 3-fold higher specific level in gluconate grown cells than those grown in fructose under similar conditions. Interestingly, these activities are lower than those detected in the strain *Escherichia coli* M1-8, cultivated under similar conditions. Additionally, here we also confirmed that this bacterium lacks 6-phosphogluconate dehydratase activity. Thus, gluconate must be metabolized through the pentose phosphate pathway. Genes encoding gluconate transport and its phosphorylation were cloned from *C. glutamicum*, and expressed in suitable *E. coli* mutants. Sequence analysis revealed that the amino acid sequences obtained from these genes, denoted as *gntP* and *gntK*, were similar to those found in other bacteria. Analysis of both genes by RT-PCR suggested constitutive expression, in disagreement with the inducible character of their corresponding activities. The results suggest that gluconate might be a suitable source of reduction potential for improving the efficiency in cultures engaged in amino acids production. This is the first time that gluconate specific enzymatic activities are reported in *C. glutamicum*.

Key terms: *Corynebacterium glutamicum*, gluconate metabolism, gnt

INTRODUCTION

Corynebacterium glutamicum is an aerobic, gram-positive, non-sporulating and non-pathogenic bacillus that lives in the soil. It is a microorganism widely used in the industrial production of primary metabolites, such as L-glutamate, L-lysine and nucleotides, because of which there is ongoing interest in developing more efficient strains of corynebacterium. Research has resulted in significant advances in the biochemistry, physiology and molecular genetics of this organism, with special attention to its aminoacids biosynthetic pathways (Hermann, 2003; Jetten and Sinskey, 1995, Kaliwonski et al.,

2003). Better conditions and more efficient *C. glutamicum* strains for amino acid production are obtained through cloning and the characterization of the genes involved in their biosynthesis, as well as metabolic studies with emphasis on the carbon flux distribution between glycolysis and the pentose phosphate pathway (PPP) under particular conditions of growth (Eggeling, 1994, Kirchner and Tauch, 2003, Sahm et al., 2000, Vallino and Stephanopoulos 1993). Our interest in *C. glutamicum* is associated with the gluconate metabolism, which is one of our areas of study in *E. coli* (De Rekarte et al., 1994; Istúriz et al., 1986; Porco et al., 1998). It was known (Vallino and Stephanopoulos,

1994b) that *C. glutamicum* grows in mineral media with glucose as the sole carbon source. However, if supplemented only with gluconate, the growth is linear, produces cell lysis and the Entner-Doudoroff pathway (EDP) activities are not detected (Lee et al., 1998). The addition of glucose to the gluconate containing cultures alleviates this problem. Moreover, the specific production of L-lysine in this microorganism was enhanced when gluconate was used as a secondary carbon source with glucose, presumably by relieving the limiting factors in the lysine synthesis rate as the NADPH supply (Lee et al., 1998). Diverse metabolic flux studies have revealed a correlation between lysine production and the NADPH supplied by carbon flux through the pentose phosphate pathway (Wittmann and Heinzle, 2002, Beker et al., 2007). Recently, metabolic flux engineering has addressed the over expression of the *zwf* gene, which encodes glucose 6-phosphate dehydrogenase, resulting in increased lysine production, probably due to an overall NADPH excess (Becker et al., 2007).

In regard to the organization of the initial gluconate metabolism genes in *C. glutamicum*, a recent study identified and analyzed *gntP* and *gntK* as the genes involved, which are responsible of the gluconate transport and the gluconokinase activity respectively (Letek et al., 2006). Although the expression analysis revealed monocistronic transcripts and constitutive expression for both genes (Letek et al., 2006), a subsequent study (Frunzke et al., 2008) identified two regulators (*GntR1* and *GntR2*), which repress the expression of genes involved in gluconate metabolism (e.g. *gntK*, *gntP* and *gnd*) in the absence of the substrate.

Here we report physiological and genetic studies on the initial steps of gluconate utilization by *C. glutamicum*, i.e., substrate transport and its phosphorylation. While in *E. coli*, this acid sugar once phosphorylated is both, oxidatively decarboxylated by the 6-phosphogluconate dehydrogenase (*Gnd*), the third enzyme of the pentose phosphate pathway (PPP) and dehydrated by the 6-phosphogluconate dehydratase (*Edd*), the

first enzyme of the EDP (Fraenkel, 1996), in *C. glutamicum*, gluconate seems to feed exclusively in the PPP (Vallino and Stephanopoulos, 1994b). We have confirmed that *C. glutamicum* grows on modified mineral medium supplemented with gluconate as a sole carbon source. In this condition, while the *Edd* activity is not detected, low gluconokinase and gluconate transport activities are expressed in an inducible form. The growth of cells in mineral medium supplemented with glucuronate indicated the presence of the second enzyme of the EDP, 2-keto-3-deoxy-6-phosphogluconate aldolase (*KDPG* aldolase), which was confirmed through enzymatic assays. Using oligonucleotide primers designed from the *C. glutamicum* genome sequences reported in the GenBank (NC 003450), the gluconokinase and gluconate permease genes were amplified from this bacterium, cloned and expressed in appropriate *E. coli* mutants. RT-PCR experiments indicated that these genes are expressed constitutively in *C. glutamicum*, which is not in agreement with the inducible character of the corresponding enzymatic activities. Because the results suggest that gluconate catabolism in *C. glutamicum* is a suitable reduction potential supplier, the complementation of culture media with this substrate might be used to improve efficiency in the amino acids production state of the bacteria.

MATERIALS AND METHODS

Organisms

The strains and plasmids used in this study are listed in Table I. *E. coli* strains are K12 derivatives.

Media

Mineral medium [MM (Tanaka et al., 1967)], containing 5 $\mu\text{g ml}^{-1}$ of thiamine hydrochloride, 20 $\mu\text{g ml}^{-1}$ of L-amino acids as required and the carbon source at 2 g l^{-1} , was used. Luria broth (Lb), Lb plates and gluconate bromthymol blue indicator plates [GBTB plates (Istúriz et al., 1986)] were

also used. If necessary, ampicillin (Amp, 80 $\mu\text{l ml}^{-1}$) was added to select cells harboring ampicillin-resistant plasmids. CAA medium was MM supplemented with 10 g l^{-1} of casein hydrolysate. *C. glutamicum* was grown in a mineral *C. glutamicum* citrate medium [MCGC (von der Osten, 1989)], supplemented with DL- α - ϵ -diaminopimelic acid (500 $\mu\text{g ml}^{-1}$), L-homoserine (80 $\mu\text{g ml}^{-1}$) and carbohydrates at 5 g l^{-1} .

Growth of bacteria

The 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 G76, 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 (one Klett unit is approximately 2×10^6 cell ml^{-1}).

Preparation of crude extracts

Cells were harvested by centrifugation, re-suspended in 50 mM Tris-HCl 10 mM MgCl_2 (pH 7.6) and disrupted by 30s sonication pulses (16 and 2 pulses for *C. glutamicum* and *E. coli* respectively) in a Braun Sonic 2000 (12T probe, 45 wattage level) with cooling periods between pulses. Cell debris was removed by centrifugation at 2700xg for 15 min.

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

Sodium [U - ^{14}C]gluconate [specific activity 5.6 mCi (0.21 GBq) nmol^{-1}], obtained from Amersham, was used at $2 \times 10^{-5}\text{M}$ to measure gluconate uptake activity according to Porco et al. (1998). The specific rates of gluconate uptake are expressed as pmol incorporated by 10^7 cells min^{-1} .

Enzyme assays

The gluconokinase, gluconate 6-phosphate dehydrogenase, 6-phosphogluconate dehydratase (Edd) and KDPG aldolase (Eda) activities were assayed as previously described (Fraenkel and Horecker, 1964). KDPG for Eda assays was obtained as described (Conway et al., 1991): *E. coli* DF214 carrying the pT280 plasmid, which contains the *E. coli edd* gene, generated by PCR and inserted immediately downstream of the *lac* promoter (Egan et al., 1992), was grown overnight in Lb containing ampicillin (100 $\mu\text{g ml}^{-1}$) and IPTG (0,5 mM) at 37 °C. Cultures were centrifuged for 5 min. (3000xg) and the cells re-suspended in buffer MES- MgCl_2 [50mM 2(N-morpholino) ethanesulfonic acid, 10 mM MgCl_2] to a final A_{550} of 1. Cells from 2 ml of this suspension, once centrifuged as before, were re-suspended in 500 μl of the same buffer, and

TABLE I

Bacteria and plasmids

Strain	Relevant characteristics	Origin
<i>C. glutamicum</i> ATCC13032	Wild type	ATCC
<i>E. coli</i> DF214(pTC280)	F ⁻ <i>eda</i> -1, Δ (<i>zwf</i> - <i>edd</i>), His, <i>pgi</i> ::mu, <i>str</i> . (pTC280)	Conway, T.
<i>E. coli</i> TMC297	F ⁻ <i>gntR gntK</i> ^C <i>gntU gntT gntV</i> His ⁻ Trp ⁻ Gal ⁻	This lab
<i>E. coli</i> TGN282	F ⁻ <i>gntK gntU gntV</i> His ⁻ Trp ⁻ Gal ⁻	This lab
<i>E. coli</i> DH5 α (mcr)	F ⁻ <i>mcrA</i> Δ (<i>mrr hsdRMS mcrB</i>) Φ 80 <i>dlacZ</i> M15 Δ (<i>lacZYA</i> <i>argF</i>) U169 <i>endA1 deo R thi sup</i> E44 <i>gyrA</i> 96 <i>relA1</i>	This lab
<i>E. coli</i> M1-8	HfrC <i>gntR</i>	This lab
Plasmid		
pCR®2.1-TOPO®	<i>lacZ</i> α	Invitrogen
pTC280	<i>Ptac lacI</i> ^q <i>edd</i>	Conway, T.

disrupted by two 30s sonication pulses. The disrupted cells were centrifuged (27000xg, 15 min.) and the supernatant was added to 12.5 ml of 6-phosphogluconate 5 mM. This mixture was incubated at 37 °C, for 30 min, heat inactivated by incubation at 90 °C for 5 min., centrifuged (27000xg, 15 min.) and the supernatant was used as a substrate for KDPG aldolase assays. Activities are reported as nmol min⁻¹ (mg protein)⁻¹.

DNA isolation and manipulation

Plasmids and total DNA were isolated using standard DNA manipulation protocols (Ausubel et al., 1999; Birboim and Doly, 1982). In order to obtain the total DNA from *C. glutamicum*, cells were pretreated by re-suspension in lysis buffer containing lysozyme (15 mg ml⁻¹) and incubated in a shaking bath for 3h, at 37 °C.

PCR amplification, cloning and sequencing

For amplification of genes encoding gluconate transport and phosphorylation activities from *C. glutamicum*, primers were designed from the GenBank DNA sequences of the corresponding putative genes (accession number NC003450). Primers P1 (5'-AGCCGGATACAATCCCAATACAGC-3') and P2 (5'-CGATTTTCAGTCGGATTATCACCCG-3'), for gluconate permease gene, and primers P3, (5'-AAACTTACGCCAGGAAGTATCCGC-3') and P4, (5'-GTGTTCTTGCCATCCATTGTGCC-3') for gluconokinase gene. *Taq* polymerase from Invitrogen was used for PCR. Samples of 50 µl, were prepared according to the manufacturer's instructions. The mixture was heated at 94 °C, for 5 min, followed by 30 cycles of the following program: 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C. PCR products were analyzed by agarose (1.5%) gel electrophoresis and sequenced by using an automated ABI 377 instrument (CeSAAN, IVIC). PCR products were cloned into the pCR®2.1-TOPO vector (Promega) according to the instructions of the manufacturer. They were then used to transform the *E. coli* DH5α(mcr) strain.

RT PCR analysis

To examine the *gntK* and *gntP* gene expression, reverse transcription-PCR (RT-PCR) analysis was performed with the total RNA isolated by the Trizol reagent (GIBCO/BRL) according to the instructions of the manufacturer. The cells were pre-cultured at 37 °C, in MCGC medium with fructose and collected during the log phase. Aliquots of the pre-culture were diluted 10-fold in the same medium, and cultures (20 ml) in the presence of fructose and glucose were carried out at 37 °C, until reaching an absorbance of 0.6 at 600nm. In each case, the RT reaction was carried out with 1 µg of the respective total RNA with M-MLV Reverse Transcriptase, using random primers, following the manufacturer's instructions. PCR (35 cycles) performed with the primers P5 (5'-ACCCAGCTAACGCAGTGTC-3') and P6 (5'-CGGTTGCCTAGGAAGAACAG-3'), and primers P7 (5'-AGCAGCCGAAGGCTTACATA-3') and P8 (5'-CAACCTGGACTAGCCACCAT-3') for *gntP* and *gntK* ORFs, respectively, consisted of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The relative amounts of RT-PCR products on the gel were compared by measuring the band density after the color of the image obtained was reversed by using a model GS-700 imaging densitometer (Bio-Rad). This experiment was repeated at least twice. As controls, PCR were carried out in RNA samples without RT.

RESULTS

Gluconate catabolism in C. glutamicum

Initially, we confirmed previous results (Lee et al., 1998; Vallino and Stephanopoulos, 1994b) about the capability of *C. glutamicum* (ATCC 13032) to utilize gluconate as a sole energy and carbon source. This strain, pre-cultivated in Lb, grew aerobically at 37 °C in MCGC medium supplemented with glucose (0.5%)

or gluconate (0.5%) with generation times of 90 and 130 minutes, respectively. The lag period was about an hour in the former condition and two hours in the latter. Fructose, galactose, maltose and glucuronic acid were also used as carbon sources for *C. glutamicum* growth (data not shown).

We investigated the presence of gluconate activities in *C. glutamicum* grown in MCGC medium, supplemented with fructose, gluconate, or glucuronate. Table II shows that while the activities for transport and phosphorylation of this substrate are induced in the gluconate culture [$32 \text{ pmol} \times 10^7 \text{ cells min}^{-1}$ and $47 \text{ nmol min}^{-1}(\text{mg prot})^{-1}$, respectively], 6-phosphogluconate dehydratase is not detected in any of the conditions assayed. However, KDPG aldolase is induced when the cells are grown in the presence of glucuronate [$38 \text{ nmol min}^{-1}(\text{mg prot})^{-1}$]. Likewise, the specific activity of 6-phosphogluconate deshydrogenase, expressed in a semiconstitutive form, shows a 3-fold higher level in gluconate than in fructose. Interestingly, the transport and phosphorylation activities in *C. glutamicum* were lower than those detected in the *E. coli* strain M1-8 grown under similar experimental conditions. Although gluconate is not a good carbon source for *C. glutamicum* growth (Vallino and Stephanopoulos, 1994a), the capture and

phosphorylation of this substrate might improve bacterial amino acid production by the generation of reducing power via the Gnd enzyme.

Cloning of genes for transport and phosphorylation of gluconate in C. glutamicum

Recently, two genes, *gntK* and *gntP*, involved in gluconate catabolism of *C. glutamicum* were reported (Letek et al., 2006). Based on that information, and having at our disposal *E. coli gntK* and *gntT* mutants, we proceeded to demonstrate through cloning, complementation and enzymatic assays in the above *E. coli* suitable mutants, if the *C. glutamicum* ORFs reported by GenBank and investigated by Letek et al. (2006) certainly encode activities of transport and phosphorylation of gluconate. It is known that *C. glutamicum* genes are expressed in *E. coli* (Eikmanns, B. 1992).

Two sets of primers were prepared, which were designed from sequences reported in the GenBank as responsible ORFs for the transport (GenBank ID 1020851) and phosphorylation (GenBank ID 1020432) of gluconate. The two PCR products obtained from the *C. glutamicum* (ATCC 13032) genome were of approximately 1723 bp and 861 bp,

TABLE II

Activities of gluconate catabolism in *C. glutamicum*

Strain	Carbon source	[U- ^{14}C] gluconate uptake	Gluconokinase	6- phosphogluconate dehydrogenase	6-phosphogluconate dehydratase	KDPG aldolase
<i>E. coli</i> M1-8	Fructose	NI	388 ± 12.7	152 ± 9.1	242 ± 7.7	189 ± 12
<i>C. glutamicum</i> (ATCC 13032)	Fructose	ND	ND	94 ± 3.5	ND	ND
	gluconate	32 ± 3.5	47 ± 2.8	291 ± 11.6	ND	ND
	glucuronate	ND	ND	25 ± 2.8	ND	38 ± 2.8

Cells were cultivated in Lb and collected during the exponential phase; then grown to 120 Klett Units in MMCGC (*C. glutamicum*) and MM (*E. coli* M1-8), supplemented with the indicated carbon sources. NI, not investigated. ND, not detected. For units, see Materials and methods. The values for the activities represent means $\pm$ standard deviations from two independent experiments.

respectively. Once the sequences of both PCR products were confirmed, they were cloned in the vector pCR®2.1-TOPO to create plasmids pTAEP and pTAEK, to be used for complementation assays in *E. coli* mutants TMC297 and TGN282, which are unable to utilize gluconate because of defects in transport and phosphorylation of this substrate, respectively (De Rekarte et al., 1994).

Sequence analysis of the C. glutamicum cloned fragments

According to the genomic sequence reported by GenBank (NC 003450), the 1723 bp cloned fragment (3.109.625 bp - 3.107.902 bp) carried by the plasmid pTAEP, contains one ORF of 1392 bp, which specifies a polypeptide of 463 amino acids long (51 kD). Chromosomal sequence analysis for this ORF by the PC/GENE program identified its product as a transporter with 13 transmembrane helices. A similar analysis of the 861 bp fragment (2.631.283 bp - 2.630.422 bp) carried by the pTAEK plasmid, allowed for the identification of one 504 bp ORF, which encodes a gluconate kinase of 167 amino acids long (18 kD), with an ATP-binding-site-domain. Both ORFs are monocistronic and separated by approximately 477.5 kb in the *C. glutamicum* genome.

In agreement with previous reports (Patek et al., 2003; Letek et al., 2006), presumptive promoter regions with a -10 (TATAGT) for *gntP* and -10 (TATGAT) for the *gntK* ORF were identified. Sequences resembling -35 regions, which is not conserved in *C. glutamicum* (Patek et al., 2003), were not identified.

The deduced amino acid sequences of the ORFs from pTAEP y pTAEK resemble those of the corresponding proteins in *E. coli*. The former product has 28%, 27% and 30% identity with GntT, GntU and IdnT, respectively, and the latter has 42% identity with GntK. According to the data bank, no other *C. glutamicum* ORF has been identified as a presumptive protein encoding for a gluconate transporter or gluconate phosphorylation activity.

E. coli complementation by C. glutamicum cloned genes

In correspondence with the sequence analysis, the pTAEP clone complemented the *E. coli* mutant TMC297 on both mineral gluconate and GBTB plates, indicating that the genomic *C. glutamicum* DNA fragment carried by this clone certainly includes the *gntP* gene, specifying gluconate transport activity. On the contrary and unexpectedly, the pTAEK clone did not complement the *E. coli* mutant TGN282 on similar plates.

The failure of pTAEK to complement the *E. coli* mutant TGN282 was a deceptive presumption. We observed that transformed colonies did not arise on mineral gluconate plates. However, those that arose on GBTB plates were white (non-fermentative) and particularly smaller than *E. coli* DH5 α (mcr) transformed colonies with the same plasmid, but selected on Lb Amp-plates. This observation suggested that the failure of growth could be the result of a 6-phosphogluconate accumulation due to high levels of gluconokinase activity, when the transformed cells were selected on gluconate containing plates. The toxicity caused by the intracellular accumulation of phosphorylated compound has been reported (De Rekarte et al., 1994).

Activities of gluconate metabolism in E. coli transformed cells

In order to demonstrate the expression of the *C. glutamicum* cloned gluconate genes in *E. coli*, and also to support the above hypothesis, the specific activities for transport and phosphorylation of gluconate were estimated in transformed *E. coli* DH5 α (mcr) cells, as well as in transformed *E. coli* mutants, cultivated in a CAA medium supplemented with fructose or gluconate (Table III). In the *E. coli* mutant TMC297 carrying the pTAEP plasmid, the gluconate transport was expressed in a partially constitutive form (691 pmol x 10⁷ cells min⁻¹ in fructose vs 1194 pmol x 10⁷ cells min⁻¹ in gluconate). *E. coli* mutant TGN282, transformed with the pTAEK plasmid, did not grow in gluconate-supplemented medium. However, when

these cells were cultivated in fructose, they registered high levels of gluconate kinase activity [$2894 \text{ nmol min}^{-1}(\text{mg prot})^{-1}$]. Similar results were observed in the *E. coli* DH5 α (mcr) carrying the same plasmids; while *E. coli* DH5 α (mcr)(pTAEP) grew on gluconate containing medium and showed high levels of gluconate uptake ($425 \text{ pmol} \times 10^7 \text{ cells min}^{-1}$ cultivated in mineral fructose), *E. coli* DH5 α (mcr)(pTAEK) did not grow on gluconate supplemented medium, but showed high levels of gluconate kinase specific activity when cultivated in fructose [$1429 \text{ nmol min}^{-1}(\text{mg prot})^{-1}$], confirming that the segment carried by the plasmid certainly encodes a gluconokinase

Expression of C. glutamicum gntP and gntK genes

In order to examine the possibility of identifying the inducible character of GntP and GntK in expression studies, RT PCR

assays were made from RNA isolated from *C. glutamicum*, cultivated in gluconate or fructose as sole carbon sources. The results did not show significant differences between the two growth conditions investigated, suggesting a constitutive expression for these genes (data not shown).

DISCUSSION

The data reported here provides evidences for the presence, in *C. glutamicum*, of enzymes involved in the transport and phosphorylation of gluconate, which are codified by *gntP* and *gntK* genes, respectively. Basic and novel aspects of the gluconate catabolism in this bacterium have been investigated. The results indicate that *C. glutamicum* grows exponentially in MCGC medium with gluconate (0.5%) as the sole carbon source and, for the first time, data involving specific gluconate

TABLE III
Activities of gluconate catabolism in *E. coli* transformed cells

Strain	Carbon source	[U- ^{14}C] gluconate uptake	Gluconokinase
TMC297	Fructose	ND	NI
	Fructose - gluconate	ND	NI
TGN282	Fructose	NI	ND
	Fructose - gluconate	NI	ND
TMC297 (pCR $^{\text{®}}$ 2.1-TOPO $^{\text{®}}$)	Fructose	ND	NI
	Fructose - gluconate	ND	NI
TGN282 (pCR $^{\text{®}}$ 2.1-TOPO $^{\text{®}}$)	Fructose	NI	ND
	Fructose - gluconate	NI	ND
TMC297 (pTAET)	Fructose	691 ± 9.9	NI
	Gluconate	1194 ± 12	NI
TGN282 (pTAEK)	Fructose	NI	2894 ± 9.1
	Gluconate	NI	NG
DH5 α (mcr) (pCR $^{\text{®}}$ 2.1-TOPO $^{\text{®}}$)	Fructose	ND	ND
DH5 α (pTAET)	Fructose	425 ± 12.7	NI
	Gluconate	NI	NI
DH5 α (pTAEK)	Fructose	NI	1429 ± 13.4
	Gluconate	NI	NG

Cells were grown in Lb and collected during the exponential phase; then cultivated in CAA medium with the indicated carbon sources at 0.2%, from approximately 10 KU up to 120 KU. Transformed cells were grown in the presence of ampicillin (100 $\mu\text{g/ml}$). NI, not investigated. ND, not detected. NG, no growth. For units, see Materials and methods. The values for activities represent means $\pm$ standard deviations from two independent experiments.

activities in these conditions are reported. Gluconate, once incorporated and phosphorylated, is decarboxylated oxidatively via PPP, because this bacterium lacks Edd, but not Gnd. These characteristics, and the enhancement of Gnd specific activity in gluconate containing cultures, which is mainly addressed to generate reducing power, might facilitate the search for strategies to improve the efficiencies of *C. glutamicum* in production conditions. It is known that the yield of L-lysine by this bacterium is increased if cultivated in glucose plus gluconate (Lee et al., 1998; Coello et al., 1992). This result, as well as ours, can be explained by the coordinate and negative regulation of GntR1 and GntR2, two redundant repressors of *gntP*, *gntK* and *gnd* whose actions are interfered by gluconate (Frunzke et al., 2008). The *C. glutamicum* growth in a mineral gluconate medium results in the derepression of the mentioned genes with a significant increase in the levels of Gnd, previously expressed as constitutive basal activity. Because gluconate cometabolizes with glucose in this bacterium, the growth rate in mineral gluconate plus glucose medium not only increases (compared to the medium supplemented with either substrate {Frunzke et al., 2008}), but also participates in the generation of reduction potential, as gluconate is totally metabolized via PPP and glucose can still be partitioned at the glucose-6-phosphate level. The importance of the reducing power in the *C. glutamicum* lysine production was also observed through the over-expression of the *zwf* gene (Becker et al., 2007); so the utilization of gluconate by this bacterium with the corresponding increase of Gnd activity and reduction potential, might stimulate the optimization of culture conditions to improve production conditions without complex genetic manipulations.

Initial activities of gluconate metabolism in C. glutamicum

The specific GntP and GntK activities were expressed in an inducible form and showed lower levels than those of the *E. coli* mutant

(*gntR*) M1-8, cultivated in MCGC medium with gluconate (Table 2). The low levels of specific activities detected for GntP and GntK might explain why *C. glutamicum* shows linear growth in a basic mineral medium supplemented with gluconate as sole carbon source (Vallino and Stephanopolous, 1994b), and why this growth is improved when glucose is added or when a special mineral media (MCGC), which contains citrate, is used. Perhaps gluconate is not able to support the growth of this bacteria and an additional source of energy is required. The fact that these low levels are detected, even in cells cultivated in MCGC supplemented with gluconate, explains the difficulty of detecting gluconate transport and gluconokinase activities in extracts from cells cultivated in the same media with fructose; due probably to catabolite repression caused by the presence of this substrate (Letek et al., 2006), or the effect of regulators recently revealed (GntR1 and GntR2; Frunzke et al., 2008).

The inducible character of GntP and GntK seems to contrast with the constitutivity of their respective genes as reported by Letek et al. (2006) and observed by us on the basis of non-quantitative expression studies. In this concern, it is clear that the negative regulatory circuit uncovered by Frunzke et al. (2008, see above) certainly supports the inducibility, not only of these genes, but also the semiconstitutivity of *gnd*. Consequently, the dissimilarity between our results might be due to different sensitivities among the techniques used, since contrarily to RT-PCR, the enzyme assay registers the final product, *i.e.*, the protein; alternatively, the presence of a unknown regulatory circuit blocking the translation of messengers in conditions of non-induction, should not be discarded. It is not advantageous for the cell, in energetic terms, to synthesize GntP and GntT in absence of gluconate.

C. glutamicum gntP and *gntK* genes, cloned in the pCR[®]2.1-TOPO vector, were expressed constitutively in *E. coli* mutants lacking their own gluconate activities. Specific levels of GntP and GntK in the

transformants were particularly high, probably as a consequence of the multicopy character of the vector used, so it was not possible to infer some effects of the intracellular medium. Notably, GntK activity could be registered only in extracts of cells cultivated in MCGC plus fructose, where the formation of gluconate 6-phosphate is low and the toxicity of the cell is not compromised. Because the Gnd activity in *C. glutamicum* increases in the presence of its substrate and seems to be a signal of production conditions, it would be of interest to study this in mutants with a high capacity to form gluconate 6-phosphate from gluconate.

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REFERENCES

- AUSUBEL F, BRENT R, KINSTON R, MOORE D, SEIDMAN J, SMITH J, STRUHL K (1999) in: John Wiley & Sons, Inc. (Eds.), Short protocols in molecular biology, New York, pp. 2.12-2.14
- BECKER J, KLOPPROGGER C, HEROLD A, ZELDER O, BOLTEN CJ, WITTMANN C (2007) Metabolic flux engineering of L-lysine production in *Corynebacterium glutamicum*-over expression and modification of G6P dehydrogenase. *J Biotechnol* 132: 99-109
- BIRBOIM H, DOLY J (1982) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res* 7: 513-523
- COELLO N, PAN JG, LEBEAULT JM (1992) Physiological aspects of L-lysine production: effect of nutritional limitations on a producing strain of *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* 38: 259-262
- CONWAY T, FLIEGE R, JONES-KILPATRICK D, LIU J, BARNELL WO, EGAN S E (1991) Cloning, characterization and expression of the *Zymomonas mobilis eda* gene that encodes 2-keto-3-deoxy-6-phosphogluconate aldolase of the Entner-Doudoroff pathway. *Mol Microbiol* 5: 2901-2911
- DE REKARTE U, CORTES M, PORCO A, NIÑO G, ISTÚRIZ T (1994) 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
- EGAN S, FLIEGE R, SHIBATA A, WOLF R, CONWAY T (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
- EGGELING L (1994) Biology of L-lysine overproduction by *Corynebacterium glutamicum*. *Amino Acids* 41: 261-272
- EIKMANN S, B (1992) Identification, sequence analysis, and expression of a *Corynebacterium glutamicum* gene cluster encoding the three glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and triosephosphate isomerase. *J Bacteriol* 174: 6076-6086
- FRAENKEL D (1996) Glycolysis In: NEIDHARDT FC, CURTISS R, INGRAHAM JL, LIN EC, LOW KB, MAGASANIK B, REZNIKOFF WS, RILEY M, SHAECHTER M, UMBARGER HE (eds). *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology. 2nd ed. Washington: American Society for Microbiology DC, pp: 189-198
- FRAENKEL DG, HORECKER BL (1964) Pathways of D-glucose metabolism in *Salmonella typhimurium*. *J Biol Chem* 239: 2765-2771
- FRUNZKE J, ENGELS V, HASENBEIN S, GÄTGENS C, BOTT M (2008) Co-ordinated regulation of gluconate catabolism and glucose uptake in *Corynebacterium glutamicum* by two functionally equivalent transcriptional regulators, GntR1 and GntR2. *Mol Microbiol* 67: 305-322
- HERMANN T (2003) Industrial production of amino acids by coryneform bacteria. *J Biotechnol* 104: 155-172
- ISTÚRIZ T, PALMERO E, VITELLI-FLORES J (1986) Mutations affecting gluconate catabolism in *Escherichia coli*. Genetic mapping of the locus for the thermosensitive gluconokinase. *J Gen Microbiol* 132: 3209-3219
- JETTEN M, SINSKEY A (1995) Recent advances in the physiology and genetic of amino acid-producing bacteria. *Crit Rev Biotech* 15: 73-103
- KALIWONSKI J, BATHE B, BARTELS D, BISCHOFF N, BOTT N, BURKOVSKI A, DUSH N, EGGELING L, EIKMANN S, GAIGALA L, GOESMANN A, HARTMANN M, HUTHMACHER K, KRAMER R, LINKE B, MCHARDY AC, MEYER F, MOCKEL B, PFEFFERLE W, PUHLER A, REY DA, RUCKERT C, RUPP O, SAHM H, WENDISH VF, WIEGRABE I, TAUCH A (2003) The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derive aminoacids and vitamins. *J Biotechnol* 104: 5-25
- KIRCHNER O, TAUCH A (2003) Tools for genetic engineering in the aminoacid-producing bacterium *Corynebacterium glutamicum*. *J Biotechnol* 104: 287-299
- LETEK M, VALBUENA N, RAMOS A, ORDÓÑEZ E, GIL JA, MATEOS L (2006) Characterization and use of catabolite-repressed promoters from gluconate genes in *Corynebacterium glutamicum*. *J Bacteriol* 188: 409-423
- LEE HW, PAN JG, LEBEAULT JM (1998) Enhanced L-lysine production in threonine-limited continuous culture of *Corynebacterium glutamicum* by using gluconate as a secondary carbon source with glucose. *App Microbiol Biotech* 49: 9-15
- PATEK M, NESVERA J, GUYONVARCH A, REYES O, LEBLON G (2003) Promoters of *Corynebacterium glutamicum*. *J Biotech* 104: 311-323
- PORCO A, ALONSO G, ISTÚRIZ T (1998) The gluconate high affinity transport of GntI in *Escherichia coli* involves a multicomponent system. *J Basic Microbiol* 38: 395-404

- SAHM H, EGGELING L, DE GRAAF AA (2000) Pathway analysis and metabolic engineering in *Corynebacterium glutamicum*. *Biol Chem* 381: 899-910
- TANAKA SS, LERNER A, LIN EC (1967) Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. *J Bacteriol* 93: 642-648
- VALLINO JJ, STEPHANOPOULOS G (1993) Metabolic flux distributions in *Corynebacterium glutamicum* during growth and lysine overproduction. *Biotechnol Bioengin* 41: 633-646
- VALLINO JJ, STEPHANOPOULOS G (1994a) Carbon flux distributions at the pyruvate branch point in *Corynebacterium glutamicum* during lysine overproduction. *Biotech Prog* 10: 327-334
- VALLINO JJ, STEPHANOPOULOS G (1994b) Carbon flux distributions at the glucose 6-phosphate branch point in *Corynebacterium glutamicum* during lysine overproduction. *Biotech Prog* 10: 320-326
- VON DER OSTEN CH, GIOANNETTI C, SINSKEY AJ (1989) Design of a defined medium for growth of *Corynebacterium glutamicum* in which citrate facilitates iron uptake. *Biotech Letter* 11: 11-16
- WITTMANN C, HEINZLE E (2002) Genealogy profiling through strain improvement by using metabolic network analysis: metabolic flux genealogy of several generations of lysine-producing corynebacteria. *Appl Env Microbiol* 69: 5843-5859