JOURNAL OF BACTERIOLOGY, Jan. 2006, p. 409–423 Vol. 188, No. 2
0021-9193/06/$08.00+0 doi:10.1128/JB.188.2.409–423.2006

Characterization and Use of Catabolite-Repressed Promoters from Gluconate Genes in *Corynebacterium glutamicum*†

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Received 2 September 2005/Accepted 22 October 2005

The genes involved in gluconate catabolism (*gntP* and *gntK*) in *Corynebacterium glutamicum* are scattered in the chromosome, and no regulatory genes are apparently associated with them, in contrast with the organization of the *gnt* operon in *Escherichia coli* and *Bacillus subtilis*. In *C. glutamicum*, *gntP* and *gntK* are essential genes when gluconate is the only carbon and energy source. Both genes contain upstream regulatory regions consisting of a typical promoter and a hypothetical cyclic AMP (cAMP) receptor protein (CRP) binding region but lack the expected consensus operator region for binding of the GntR repressor protein. Expression analysis by Northern blotting showed monocistronic transcripts for both genes. The expression of *gntP* and *gntK* is not induced by gluconate, and the *gnt* genes are subject to catabolite repression by sugars, such as glucose, fructose, and sucrose, as was detected by quantitative reverse transcription-PCR (qRT-PCR). Specific analysis of the DNA promoter sequences (P*gntK* and P*gntP*) was performed using bifunctional promoter probe vectors containing *mel* (involved in melanin production) or e*gfp2* (encoding a green fluorescent protein derivative) as the reporter gene. Using this approach, we obtained results parallel to those from qRT-PCR. An applied example of in vivo gene expression modulation of the *divIVA* gene in *C. glutamicum* is shown, corroborating the possible use of the *gnt* promoters to control gene expression. *glxR* (which encodes GlxR, the hypothetical CRP protein) was subcloned from the *C. glutamicum* chromosomal DNA and overexpressed in corynebacteria; we found that the level of *gnt* expression was slightly decreased compared to that of the control strains. The purified GlxR protein was used in gel shift mobility assays, and a specific interaction of GlxR with sequences present on P*gntP* and P*gntK* fragments was detected only in the presence of cAMP.

Corynebacterium glutamicum is a nonpathogenic gram-positive soil bacterium of considerable interest to biotechnological industries for its role in amino acid and nucleotide production (46); in addition, *C. glutamicum* presents an emerging role as a nonpathogenic model for the study of related bacteria with clinical relevance, such as pathogenic corynebacteria (23) or mycobacteria (16). The genera *Corynebacterium* and *Mycobacterium* are included in the suborder *Corynebacterineae*, which belongs to the mycolata, a broad and diverse group of mycolic acid-containing actinomycetes (52). *C. glutamicum* is able to grow on a variety of carbohydrates and organic acids (such as acetate, lactate, and gluconate) used as single and combined sources of carbon and energy; gluconate is an important carbon source for many microorganisms in their natural environments and is required by *Escherichia coli* to colonize the mouse large intestine (71). In general, only two specific enzymes are required for gluconate catabolism; extracellular gluconate is incorporated into bacterial cells by a specific gluconate permease (GntP) and phosphorylated to 6-phosphogluconate by a gluconate kinase (GntK) (31). Thus, 6-phosphogluconate can be channeled either through the pentose phosphate pathway or through the Entner-Doudoroff pathway, which is present only in certain microorganisms (11, 59).

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† Supplemental material for this article may be found at http://jb.asm.org/.

In *E. coli*, GntI is considered to be the main system for gluconate metabolism which comprises (i) an operon with three components, *gntR-gntK-gntU*, coding for a repressor protein, gluconate kinase, and gluconate permease, respectively (32) and (ii) two additional genes for gluconate permeases (*gntP* and *gntT*) scattered throughout the *E. coli* chromosome (39, 60). In several *Bacillus* species, *gnt* genes are clustered and form the *gntRKPZ* operon, encoding the repressor, gluconate kinase, gluconate permease, and 6-phosphogluconate dehydrogenase, respectively (19, 63, 77). Both systems are transcriptionally regulated, i.e., induced by gluconate and repressed by glucose (9, 58, 60). In *E. coli*, another subsidiary gluconate system (GntII) has been previously described (30), but it is now considered the catabolic pathway for L-idonate where D-gluconate is an intermediary (4); the *idn* idonate operon (the former GntII) is repressed by a regulatory protein (IdnR) and globally repressed by the carbon source (5).

Carbon catabolite repression (CCR) is an environment-sensing mechanism used by bacteria for establishing priorities in carbon metabolism. In gram-negative bacteria, the foremost component of transcriptional control is cyclic AMP (cAMP) receptor protein (CRP), which acts as a global transcriptional activator in the presence of cAMP (8). The CCR mechanism in low-G+C-content gram-positive bacteria is different and is not based on the presence of a typical CRP protein able to bind or respond to cAMP (65). In this group of bacteria, three elements are crucial to CCR (68): CcpA (carbon catabolite protein), HPr (transcriptional regulator), and CRE (*c*atabolite-*r*esponsive *e*lement). The location of the CRE site within a given promoter appears to determine whether CcpA functions

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or description[a]	Reference or source[b]
Strains		
E. coli S17-1	Mobilizing donor strain, *pro recA*, which has a RP4 derivative integrated into the chromosome	66
E. coli DH5α	r_K^- m_K^-; used for general cloning	27
E. coli JM109 (DE3)	Strain used to express the *glxR* gene after induction by IPTG	76
C. glutamicum ATCC 13032	Wild-type strain	ATCC
C. glutamicum RES167	Restriction-deficient *C. glutamicum* 13032 derivative used as the host for recombinant plasmids in conjugation or transformation experiments	75
C. glutamicum TRA-8	Gluconate permease mutant obtained by unspecific integration of a mobilizable suicide vector into *C. glutamicum*	47
C. glutamicum GNTP	Gluconate permease mutant obtained by specific integration of pKP1 into the *C. glutamicum gntP* gene	This work
C. glutamicum GNTK	Gluconate kinase mutant obtained by specific integration of pKMM3 into the *C. glutamicum gntK* gene	This work
Plasmids		
pUC18/19	*E. coli* vectors containing *lacZ*, *bla*	Fermentas
pBluescript KS/SK	*E. coli* vectors containing *bla*, *lacZ*, *orif1*	Stratagene
pULMV00	pUC18 derivative containing the *gntP* gene from *C. glutamicum* in a 4.1-kb BamHI fragment	This work
pULMV05	pUC18 derivative containing the *gntP* gene in a 2.7-kb BamHI-KpnI fragment	This work; Fig. 1A
pKSK11	pBS SK derivative containing the *gntK* gene from *C. glutamicum* in a 740-bp BamHI-HindIII fragment amplified by PCR	This work; Fig. 1B
pGEM-TEasy	Vector used to clone PCR-amplified products using *Taq* enzyme	Promega Co., Madison, WI
pGEM-TK	pGEM derivative plasmid containing the *gntK* PCR-amplified fragment from the RACE assay	This work
pGEM-TP	pGEM derivative plasmid containing the *gntP* PCR-amplified fragment from the RACE assay	This work
pK18mob	Mobilizable plasmid containing an *E. coli* origin of replication and *kan*	67
pKP1	pK18mob derivative containing an internal fragment of *gntP* (600 bp) obtained by PstI+BglII digestion of plasmid pULMV05	This work; Fig. 1A
pKMM3	pK18mob derivative containing an internal fragment of *gntK* (200 bp)	This work; Fig. 1B
pECM2	Mobilizable *E. coli-C. glutamicum* bifunctional plasmid containing the *kan* and *cat* genes and *oriT*	33
pEM2	pECM2 derivative lacking the unique EcoRI site and the *cat* gene	2
pECP	pECM2 derivative carrying the complete *gntP* gene from *C. glutamicum* and the *cat* gene	This work; Fig. 1A
pECK	pECM2 derivative carrying the complete *gntK* gene from *C. glutamicum* and the *cat* gene	This work; Fig. 1B
pXEGFP2	pIJ2925 derivative containing the *egfp2* gene under the xylanase promoter P*xysA* and flanked by transcriptional terminators T1 and T2	J. M. Fernández-Ábalos (unpublished)
pKEGFP2	pXEGFP2 derivative containing the *egfp2* gene under the P*kan* promoter from pXHis1-NPro instead of P*xysA*	This work; Fig. 1C
pXHis1-NPro	pIJ2925 derivative containing the Δ*xysA*-His_6 P*kan* and flanked by transcriptional terminators T1 and T2	1
pNPro	pXHis1-NPro derivative lacking the Δ*xysA*-His_6 afforded by XhoI-NotI digestion, Klenow treatment, and ligation	This work
pEGFP	pEM2 derivative containing *egfp2* under the control of P*kan* and flanked by transcriptional terminators T1 and T2	This work; Fig. 1C
pEGNC	pEGFP derivative vector containing the promoterless *egfp2* gene	This work; Fig. 1C
pJMFA24	*E. coli* promoter probe vector containing the promoterless *kan* as a reporter	J. M. Fernández-Ábalos (unpublished)
pJMF-MP	pJMFA24 derivative containing the promoter region of *gntP* amplified by PCR as a 270-bp BamHI-NdeI fragment	This work
pJMF-MK	pJMFA24 derivative containing the promoter region of *gntK* amplified by PCR as a 250-bp EcoRI-NdeI fragment	This work
pEMel-1	Mobilizable *E. coli*-corynebacteria promoter probe vector containing the *melC1* operon as a reporter under the control of P*kan*	2
pEMel-2	pEMel-1 derivative lacking the P*kan* fragment	2
pEMel-P	pEMel-1 derivative containing the *melC1* operon under the control of the 270-bp P*gntP* (*PgntP-melC1*)	This work
pEMel-K	pEMel-1 derivative containing the *melC1* operon under the control of the 250-bp P*gntK* (*PgntK-melC1*)	This work
pEGFP-MP	pEGFP derivative containing *egfp2* under the control of the 270-bp P*gntP* (*PgntP-egfp2*)	This work; Fig. 1C
pEGFP-MK	pEGFP derivative containing *egfp2* under the control of the 250-bp P*gntK* (*PgntK-egfp2*)	This work; Fig. 1C

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant genotype or description[a]	Reference or source[b]
pKCRP	pK18mob derivative containing a 300-bp PCR-amplified internal fragment of *C. glutamicun glxR*	This work
pNProCRP	pNPro derivative plasmid containing the *glxR* gene under P*kan* and flanked by transcriptional terminators T1 and T2	This work
pEMCRP	pEM2 derivative containing the *glxR* gene under P*kan* (P*kan-glxR*)	This work
pET-28a	Vector used for construction of fused proteins containing a His_6 tail at the N end	Novagen
pETCRP	pET-28a derivative containing the *glxR* gene from *C. glutamicum*	This work
pEAG2	pECM2 derivative containing *divIVA-egfp2* under the control of *divIVA* promoter (P*div*)	61
pEPDG	pEGFP-MP derivative containing the *divIVA-egfp2* cassette under the control of P*gntP*	This work
pEKDG	pEGFP-MK derivative containing the *divIVA-egfp2* cassette under the control of P*gntK*	This work

[a] *kan*, *bla*, *cat*, and *egfp2* are genes for kanamycin, ampicillin, and chloramphenicol resistance and for green fluorescent protein, respectively; *oriT* is the transfer origin for mobilization.
[b] ATCC, American Type Culture Collection.

as an activator when CRE is located upstream of the promoter or as a repressor when CRE is overlapping or downstream of the promoter (48).

Many global and specific transcriptional regulators involved in gene expression have been described for bacteria. In *C. glutamicum*, only the following six transcriptional regulators have been deeply analyzed during the last couple of years: the negative transcriptional regulators McbR (64), RamB (24), AcnR (41), and HspR (18); the transcriptional activator ClgR (17, 18); and GlxR, which is involved in modulating expression of *aceB* (codes for malate synthase). GlxR shares common features with the CRP from *E. coli*, such as a 27% identity in amino acid sequence and the presence of a cAMP-binding motif where cAMP is the modulating agent involved in gene expression; heterologous *glxR* can complement *E. coli* CRP mutants (37). In most cases, inactivation of the aforementioned regulators leads to a different expression pattern of the target gene, except in the case of GlxR, where *glxR* mutants have not been obtained. In addition, several *C. glutamicum* genes are transcriptionally regulated by a dual mechanism: GlxR and RamB regulate *aceB* (24, 37), and the aconitase gene *acn* is regulated by AcnR plus at least one more system (41).

Gene-modulated systems are powerful tools for studying gene function and for the validation of drug targets in bacteria (14). In *E. coli* and *Bacillus*, there are useful inducible and repressible systems based on P*lac*, P*tac*, or phage promoters (15) to assess the effect of the expression or depletion of cloned gene products; it is also possible to modulate the expression of the associated genes using regulated promoters based on the arabinose operon from *E. coli* (26, 50) or the xylose operon from *Bacillus subtilis* (38). Unfortunately, there are not efficient systems available to modulate gene expression in corynebacteria, and systems based on P*lac* (lactose) or P*bad* (arabinose) from *E. coli* or P*xyl* (xylose) from *Bacillus* do not work proficiently in corynebacteria, due mainly to the inability to metabolize those carbon sources (6, 62). Owing to the huge biotechnological importance of *C. glutamicum*, its genome has been sequenced by different groups and is now available (29, 34). Our interest in gluconate metabolism in *C. glutamicum* arose from results obtained when we were studying the nonspecific integration of suicide plasmids in the *gntP* gene of *C. glutamicum* (47) and continues presently because the regulatory properties of the *gnt* promoters may be a way to control expression of cell division genes in corynebacteria (61, 62).

In this work, we analyzed the genes involved in gluconate metabolism with special emphasis on their promoters. Herein, we provide evidence of the absence of a *gntR*-like gene in the genome of *C. glutamicum* and suggest that the expression of the *gntK* and *gntP* genes is governed by a global regulatory mechanism responsive to the carbon source. In addition, an applied example based on *divIVA* gene expression using the modulated *gnt* promoters is shown.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown on Luria-Bertani complex medium (27) or VB minimal medium (74) at 37°C. Corynebacterial strains were grown at 30°C in Trypticase soy broth from Oxoid (TSB complex medium without glucose), TSA (TSB supplemented with 2% agar), and minimal medium (MM) for corynebacteria lacking a carbon source (35) and supplemented with either 1% glucose (MMG), 1% gluconate (MMGn), 1% acetate (MMA), 1% fructose (MMF), or 1% sucrose (MMS). Where specified, TSA, TSB, or MM was supplemented with different quantities (up to 4%) of sugars and/or organic acids. When required, the following supplements were added to the culture media: kanamycin (50 μg/ml for *E. coli*; 25 μg/ml for corynebacteria), ampicillin (100 μg/ml), and chloramphenicol (100 μg/ml for *E. coli*; 10 μg/ml for corynebacteria). For melanin production in corynebacteria, TSA was supplemented with tyrosine (8.2 mM) and cupric sulfate (2 mM) added after sterilization. All cultures were grown in Erlenmeyer flasks at 30°C (for *Corynebacterium* strains) or 37°C (for *E. coli*) at 250 rpm. Corynebacterial cells were harvested at an optical density at 600 nm (OD_{600}) of 1.5 for conjugation assays and for RNA isolation.

DNA/RNA manipulations and analyses. Plasmid DNA isolation, restriction endonuclease digestion, agarose gel electrophoresis, Southern blot analysis, and molecular cloning were performed by using standard procedures (36). PCR products in this work were generated by *Pfu* (for expression) and *Taq* DNA polymerase (Stratagene) using the *C. glutamicum* ATCC 13032 genome as the template and primers listed in Table 2. *E. coli* cells were transformed by the method of Hanahan (27). Mobilization of plasmids from *E. coli* to coryneform strains was accomplished as described previously (47).

DNA labeling and hybridization for Southern blotting was performed using a digoxigenin (DIG) High Prime kit from Roche (Mannheim, Germany) according to the manufacturer's specifications. DNA labeling and detection for gel-retarding analysis was performed using a second-generation DIG gel shift kit from

TABLE 2. Primers used in this study[a]

Oligonucleotide	Target gene[b]	Nucleotide sequence (5′–3′)[c]
gnt1	P*gntP*	CGGGATCCCGGCGGGTTAGATGAAGGGATTTT
gnt2	P*gntP*	GGAATTCCATATGGGGTGATCCTTCGTGAAAATTTG
gnt3	*gntK*	CGCGGATCCAAACATACAGTCCCCGTGATG
gnt4	*gntK*	CCCAAGCTTTCGATTGTTAGATGTGAGGAAAAG
gnt5	P*gntK*	CGGAATTCCAGGAAGTATCCGCTCCACG
gnt6	P*gntK*	GGAATTCCATATGACGACAATATGTAAGCCTTC
gnt7	$gntP_2$	TCCGCCGGCCTCCTA
gnt8	$gntP_{1,2}$	TTCGCCGAGGAATCCTAGAA
gnt9	$gntK_2$	TGGCTGCGGCAAATCC
gnt10	$gntK_{1,2}$	ATTCCGAGCTCCGCTGC
gnt11	raceK_1	TCGCGGAGCCACTTGCCA
gnt12	raceK_2	GCCGTGGAGGTGGACGAA
gnt13	raceP_1	GCGACCGAGGCGAGTGTT
gnt14	raceP_2	CACCGAGTGCGAAAGCTGC
crp1	*crp*	GGAATTCACCTCCGGCAAAGTGAAGC
crp2	*crp*	GGAATTCGCGGCCTGGGACGTC
crp3	*crp*	GGAATTCCATATGGAAGGTGTACAGGAG
crp4	*crp*	GGAATTCCATATGTTATCGAGCGCGAC
div1	*divIVA*	GGAATTCCATATGCCGTTGACTCCAG
div2	*divIVA*	GGAATTCCATATGCTCACCAGATGGC

[a] The sequences of the primers were derived from the EMBL nucleotide sequence database. The accession number for the genome of *C. glutamicum* is NC003450.
[b] Primers used for the first step of RT-PCR (cDNA synthesis) are indicated by a subscript 1; primers used for qRT-PCR analysis are indicated by a subscript 2.
[c] Underlined sequences indicate the presence of restriction enzyme targets.

Roche (Mannheim, Germany). PCR-amplified DNA was always sequenced when it was used for expression.

RNA for Northern blotting, reverse transcription-PCR (RT-PCR), quantitative RT-PCR (qRT-PCR), and rapid amplification of cDNA ends (RACE) analysis was isolated from *C. glutamicum* strains grown in TSB or in MM containing different metabolizable carbon sources (acetate, gluconate, glucose, fructose, and sucrose) using an RNeasy commercial kit (QIAGEN); for Northern blots, 15-μg portions of total cellular RNA were denatured by incubation for 10 min at 65°C in formaldehyde-MOPS (morpholinepropanesulfonic acid) gel-loading buffer, electrophoresed through a 1.5% agarose gel containing formaldehyde-MOPS buffer, and then transferred to nylon membranes (Hybond; Amersham). Filters were hybridized with a 600-bp PstI-BglII internal fragment of the *gntP* gene (Fig. 1A) or with the whole *gntK* gene (740 bp) (Table 2; Fig. 1B) obtained by PCR amplification using gnt3/gnt4 probes. Probes were labeled by nick translation (Amersham) using [α-^{32}P]dCTP (6,000 Ci/mmol; Amersham) and purified by using a Magic-Clean system (Promega). Filter hybridization and film development were accomplished with conventional protocols (36).

For RT-PCR, 1 μg of the isolated RNA was used as the template to generate single-strand cDNA from the *gntP* and *gntK* genes with avian myeloblastosis virus reverse transcriptase (first-strand cDNA synthesis kit for RT-PCR; Roche) and primers gnt8 and gnt10, respectively (Table 2; Fig. 1A and B). Primers were designed using the Primer Express software v2.0 (Applied Biosystems) and checked for PCR amplification using *C. glutamicum* genomic DNA as the template. RNA samples were also tested for contamination with genomic DNA.

For qRT-PCR, 1/20 of each RT-PCR product was used as the template for DNA amplification, using specific primer pairs for each gene (gnt7/gnt8 for *gntP* and gnt9/gnt10 for *gntK* [Table 2; Fig. 1A and B]), 12.5 μl of master mix reagent (Applied Biosystems), and up to 25 μl of water. Reactions were performed with an ABI Prism 7000 sequence detection system (Applied Biosystems), and the results were processed using specific software (ABI Prism 7000 SDS software). The results obtained were referred to as the C_T (cycle threshold) values. In all cases, the oligonucleotides used in qRT-PCR were designed to have similar melting temperatures (59°C) and to amplify DNA fragments of similar lengths (around 50 nucleotides).

For identification of the transcriptional start site of *gnt* genes, a 5′-to-3′ RACE kit from Roche (Mannheim, Germany) was used. For the assay, 3 μg of RNA was used as the template for specific *gntK* cDNA production using primer gnt11, and the PCR was performed using the primer pair gnt12/poliT (the latter from the kit); for *gntP*, cDNA was generated using primer gnt13 and the primer pair gnt14/poliT for the further PCR (Table 2; Fig. 1A and B). The purified PCR products were isolated and cloned into pGEM-TEasy vector, yielding recombinant plasmids pGEM-TK and pGEM-TP (Table 1); these were further sequenced (five of them), and their corresponding transcriptional start sites were determined.

Comparisons of DNA or protein sequences were carried out online (http://www.ebi.ac.uk) using FASTA (57), BLAST (3), and CLUSTAL W (10). Phylogenetic analyses were performed using MEGA3 (molecular evolutionary genetics analysis software) (42) with the neighbor-joining method; this information is shown in the supplemental material.

Construction of recombinant plasmids. *gntP* was identified in a 4.1-kb sequenced fragment (EMBL databank accession number, AJ296014) obtained from a genomic library of *C. glutamicum* ATCC 13032 (28) by use of an internal fragment of the *gntP* gene as a probe (47); the 4.1-kb BamHI fragment was subcloned in pUC18, yielding plasmid pULMV00 (Table 1). A 2.7-kb BamHI-KpnI internal fragment of pULMV00 containing the *gntP* gene was subcloned into pUC18, yielding the recombinant plasmid pULMV05 (Fig. 1A; Table 1).

gntK was amplified from the *C. glutamicum* chromosome with gnt3/4 primers, which contained restriction sites for BamHI and HindIII at their 5′ ends (Table 2; Fig. 1B). The amplified 740-bp DNA fragment was isolated, digested, and ligated to the BamHI-HindIII-digested pBluescript SK, yielding plasmid pKSK11 (Fig. 1B).

Internal fragments of *gntP* (600 bp) and *gntK* (200 bp) were obtained from plasmids pULMV05 (PstI plus BglII digested) and pKSK11 (HaeIII digested and purified the 200-bp fragment), respectively, and ligated to the mobilizable plasmid pK18mob, yielding plasmids pKP1 for *gntP* and pKMM3 for *gntK* (Table 1; Fig. 1A and B). Both plasmids are mobilizable and were used to obtain the kanamycin-resistant mutant strains *C. glutamicum* GNTP and *C. glutamicum* GNTK (Table 1).

To complement *C. glutamicum* GNTP, the whole *gntP* gene was isolated as a 2.7-kb BamHI-KpnI (blunt-ended) fragment from plasmid pULMV05 and ligated to pECM2 (BamHI-SmaI digested) (Table 1), yielding plasmid pECP (Table 1; Fig. 1A). To complement *C. glutamicum* GNTK, *gntK* was isolated from plasmid pKSK11 as a 740-bp BamHI-HindIII fragment (the latter target was blunt ended) and cloned into pECM2 (BamHI-SmaI digested), making plasmid pECK (Table 1; Fig. 1B); pECP and pECK were mobilized from *E. coli* to *C. glutamicum*, and transconjugants were selected by chloramphenicol resistance.

A promoter probe vector to quantify promoter strength in *C. glutamicum* was constructed using the *egfp2* gene (69) as the reporter as follows. An EcoRI-NdeI fragment containing the xylanase promoter (P*xysA*) from pXEGFP2 (Table 1) was replaced by the 0.9-kb *kan* promoter (P*kan*) from Tn*5* present in plasmid pXHis1-NPro (1), yielding plasmid pKEGFP2 (Table 1). A BglII fragment from pKEGFP2 containing the P*kan-egfp2* gene was subcloned into the bifunctional mobilizable plasmid pEM2, yielding plasmid pEGFP (Fig. 1C). Any promoter with EcoRI-NdeI ends can be introduced into pEGFP by replacement of the EcoRI-NdeI fragment (P*kan*). Plasmid pEGNC (Table 1), a P*kan*-less pEGFP derivative plasmid obtained by EcoRI-NdeI digestion and blunted by Klenow treatment and ligation was always used as a negative control for fluorescence. *C. glutamicum* strains containing pEGFP derivatives were grown in liquid media

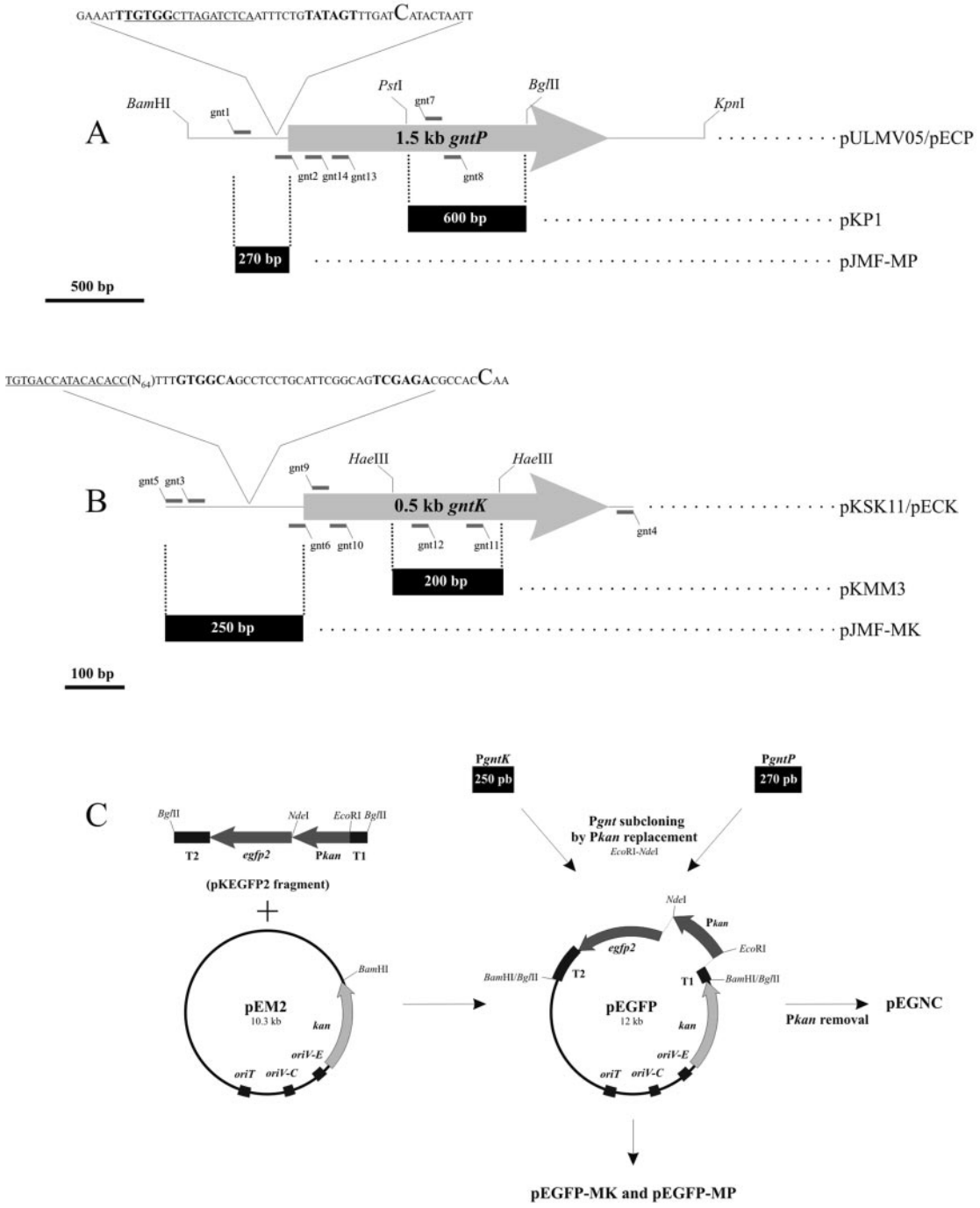

FIG. 1. Schematic representation of the *gntP* (A) and *gntK* (B) genes involved in gluconate catabolism in *C. glutamicum* and construction of plasmid pEGFP and derivatives (C). In panels A and B, arrows represent ORFs; black boxes indicate internal fragments used in gene disruption assays and promoter fragments obtained by PCR amplification using specific primers. The names of the plasmids are indicated to the right. The upper sequences show the transcription start point of genes (large C), the −10 and −35 promoter boxes (bold), and the putative CRP-binding sites (underlined). Primers used for PCR (Table 2) are also indicated. (C) Plasmid pEGFP was obtained by ligation of pEM2 BamHI digested to the BglII fragment (from pKEGFP2), which contains the gene for green fluorescent protein (*egfp2*). pEGFP-MP and -MK were obtained by pEGFP replacement of the original P*kan* by the P*gntP* and P*gntK* fragments subcloned from pJMF-MP and -MK, respectively (Table 1). pEGNC was obtained by removal of the EcoRI-NdeI fragment (P*kan*-less) from pEGFP.

(OD_{600} = 0.5); samples were then loaded into 96-well microtiter plates, and their fluorescence levels were measured on a Synergy HT multidetection microplate reader fluorimeter (Bio-Tek Instruments, Inc.).

To detect the presence of functional promoters immediately upstream from the *gntP* and *gntK* genes, DNA fragments were amplified by PCR using genomic DNA from *C. glutamicum* and the primer pairs gnt1/gnt2 for P*gntP* and gnt5/gnt6 for P*gntK* (Table 2; Fig. 1A and B). The P*gntP*-amplified fragment (270 bp) was BamHI-NdeI digested and ligated to the *E. coli* promoter probe vector pJMFA24 (Table 1), yielding plasmid pJMF-MP (Fig. 1A). Similarly, the P*gntK*-amplified fragment (250 bp) was EcoRI-NdeI digested and ligated to pJMFA24,

affording plasmid pJMF-MK (Fig. 1B). P*gntP* and P*gntK* were isolated from plasmids pJMF-MP and pJMF-MK, respectively, by EcoRI-NdeI digestion, and DNA fragments were further subcloned into the bifunctional *E. coli-C. glutamicum* promoter probe plasmids pEMel-1 and pEGFP (Table 1), generating plasmids pEMel-P, pEMel-K, pEGFP-MP, and pEGFP-MK (Table 1; Fig. 1C).

For the cloning of the *glxR* gene from *C. glutamicum*, a 684-bp fragment corresponding to the full open reading frame (ORF) was PCR amplified using the primer pair crp3/crp4 (Table 2), digested with NdeI, and subcloned into the NdeI-digested plasmids pNPro and pET-28a (Table 1), yielding plasmids pNProCRP and pETCRP, respectively (see below). pNProCRP was BglII digested, and the 1.5-kb fragment containing the *glxR* gene under the kanamycin promoter (P*kan-glxR*) was subcloned into the bifunctional mobilizable plasmid pEM2 (BamHI digested; Fig. 1C), making plasmid pEMCRP (Table 1), which was further transferred to *C. glutamicum*.

An internal fragment from *glxR* (300 bp) was PCR amplified using the primer pair crp1/crp2 (Table 2), and the purified fragment was digested with EcoRI and further subcloned into pK18mob, resulting in plasmid pKCRP (Table 1). Plasmid pKCRP was transferred from *E. coli* S17-1 to *C. glutamicum* by conjugation to disrupt the chromosomal copy of *glxR*.

The presence of a single NdeI site in plasmids pEGFP-MP and pEGFP-MK allowed us to study the expression of any *C. glutamicum* gene as a translational fusion with EGFP2 (as an NdeI-ended fragment) under the control of P*gntP* or P*gntK*. To isolate the *divIVA* gene as an NdeI fragment, a 1.1-kb PCR product corresponding to the full ORF without a stop codon was obtained from the chromosomal DNA of *C. glutamicum* ATCC 13032 using the primer pair div1/div2 (Table 2), digested with NdeI, and cloned into the NdeI-digested plasmids pEGFP-MP and pEGFP-MK (Table 1; Fig. 1C), yielding plasmids pEPDG and pEKDG (Table 1), which contained the *divIVA-egfp2* translational fusion under the control of P*gntP* and P*gntK*, respectively.

Purification of His-tagged GlxR protein in *E. coli*. Plasmid pETCRP carries the coding sequence of GlxR with an amino-terminal six-His tag label. Purification of the His-tagged protein from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced (1 mM) *E. coli* JM109 (DE3)(pETCRP) cells grown at 37°C in LB medium supplemented with 100 μg/ml ampicillin was accomplished according to the standard procedures of the manufacturer (Novagen). When indicated, the six-histidine tag was removed by treatment with thrombin (Novagen) after purification.

EMSA. Three DNA probes obtained from *C. glutamicum* were used for the electrophoretic mobility gel shift assays (EMSA); two of the probes correspond to the 270-bp P*gntP* and 250-bp P*gntK* fragments, and the third was a 250-bp fragment (P*arsB1*) corresponding to the promoter region of the arsenic detoxification operon from *C. glutamicum* (53), which was used as a negative control due to the absence of hypothetical CRP-binding sequences. Probes were nonradioactively labeled by use of a DIG High Prime kit from Roche (Mannheim, Germany), and samples were further diluted and dot blot analyzed to optimize the quantity of DNA used in the assay. The binding reaction mixture contained the labeled DNA fragments (around 10 ng), the purified GlxR protein (from 0.1 to 5 μg), 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, 0.05 μg of poly(dI-dC), and cAMP (0.05 to 0.6 mM) when indicated; 10 μl of the reaction mixture was incubated at 25°C for 30 min and further analyzed on 6% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels in the presence or absence of cAMP. After electrophoresis, gels were blotted to nylon membrane (Hybond) using an electroblotting device (Bio-Rad). For detection, the chemiluminescent substrate CSPD (Amersham) was used and further exposed with X-ray films. Specific binding was confirmed by adding to the reaction mix the same unlabeled DNA probe (100 to 1,000 times).

Microscopic techniques. Fluorescence microscopy of *C. glutamicum* cells was performed according to protocols described previously (61).

RESULTS

Molecular analysis of the *gnt* genes from *C. glutamicum* ATCC 13032. Several years ago, we described the characterization of a transconjugant (*C. glutamicum* TRA-8) obtained by stable integration of a suicide conjugative plasmid in the *gntP* gene from *C. glutamicum* (47). This gene (EMBL databank accession number, AJ296014) was cloned in plasmid pULMV05 (Fig. 1A), and sequence analysis revealed the presence of a 463-amino-acid ORF with significant homology to bacterial gluconate permeases. Once the complete genome sequence of *C. glutamicum* (NC003450) became available (29), a second gene involved in gluconate metabolism (*gntK*) was amplified from the *C. glutamicum* chromosome by PCR; the expected 740-bp PCR DNA fragment was obtained and cloned into pBluescript SK, affording plasmid pKSK11 (Fig. 1B). In both cases, the sequences surrounding *gntP* and *gntK* were analyzed in order to identify any other gene that might be involved in gluconate catabolism (especially for the *gntR* gene, encoding the gluconate repressor), although the search was unsuccessful.

Examination of the DNA sequences located upstream from *gntP* and *gntK* revealed the presence of several putative −10 and −35 RNA polymerase-binding sites for *C. glutamicum*, and therefore it was necessary to characterize the transcription start points (TSPs) for both genes. PCR-amplified fragments were subcloned into pGEM-TEasy by use of a 5′-to-3′ RACE kit, and the resulting plasmids, pGEM-TP and pGEM-TK, were sequenced, leading to the identification of TSPs for *gntP* and *gntK* (Fig. 1A and B); in both cases, the TSP was a cytosine. The *C. glutamicum gntP* and *gntK* −10 boxes are much more conserved than the −35 boxes, compared with the consensus −10 and −35 hexamers described for corynebacteria (TAnaaT and ttGcca, respectively [lowercase letters indicate less conserved nucleotides]) (55, 56); in both cases, the −10 and −35 boxes were separated by 18 nucleotides (Fig. 1A and B). In addition, two putative CRP-binding sites (CBS) were located upstream from *gntP* (5′-TGTGG-N_6-TCTCA-3′) and *gntK* (5′-TGTGA-N_6-ACACC-3′) (Fig. 1A and B). Both boxes matched the consensus CRP-binding site box (5′-TGTGA-N_6-ACACT-3′) described previously by de Crombrugghe et al. (12). No putative consensus binding sequences for GntR (5′-ATGTTA-N_4-TAA CAT-3′) or IdnR (5′-ATGTTA-N_4-TAACGT-3′) from *E. coli* (5, 60) or for GntR interaction (the dyad symmetry sequence ATACTTGTA) from *B. subtilis* (22) were found upstream from the *gntP* or *gntK* genes. The absence of consensus GntR binding sites upstream from the *gnt* genes and the failure to find a *gntR* homolog on the chromosome of *C. glutamicum* might suggest a nonconventional mechanism for regulation of the *gnt* genes in this bacterium.

Gene disruption and complementation of the *gnt* genes. To confirm the participation of *gntP* and *gntK* in gluconate catabolism in *C. glutamicum*, we performed gene disruption experiments using conjugative suicide plasmids containing internal fragments of both *gnt* genes. Suicide plasmids pKP1 and pKMM3 (Fig. 1A and B) were transferred by conjugation from *E. coli* to *C. glutamicum* RES167, and in both cases kanamycin-resistant transconjugants were obtained, suggesting that the disruption of both genes is not lethal. As expected, the transconjugants were able to grow in MMG but not in MMGn (containing gluconate instead of glucose). Transconjugants disrupted either in *gntP* or in *gntK* were named *C. glutamicum* GNTP and *C. glutamicum* GNTK, respectively. These mutants were able to grow in MMGn when plasmids containing the whole *gntP* or *gntK* genes were introduced by conjugation into *C. glutamicum* GNTP or *C. glutamicum* GNTK (pECP or pECK, respectively; Fig. 1A and B). Therefore, it is reasonable to point out that the products of the *gntP* and *gntK* genes seem to be required for gluconate catabolism in *C. glutamicum*.

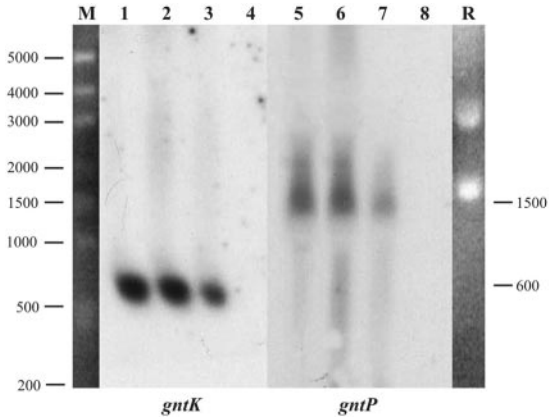

FIG. 2. Northern blot analysis of the *gntK* and *gntP* transcripts from *C. glutamicum*. Total RNA was isolated from cultures grown on MMA (1% acetate) (lanes 1 and 5), on MMGn (1% gluconate) (lanes 2 and 6), on MMG (1% glucose) (lanes 3 and 7), and on TSB (lanes 4 and 8); 15 μg of RNA was loaded per lane. Estimated sizes of the *gntP* and *gntK* transcripts (in bases) are shown at the right and were determined using a high-range RNA ladder (M) and rRNAs from *C. glutamicum* (R). Filters were hybridized with a 600-bp PstI-BglII internal fragment of the *gntP* gene or with the whole *gntK* gene (740 bp) obtained by PCR.

Expression analysis of the *gnt* genes. Northern analyses were done using RNA obtained from *C. glutamicum* RES167 grown in minimal media containing 1% acetate (MMA), 1% gluconate (MMGn), or 1% glucose (MMG) or in TSB. The probes used for Northern blotting were the internal PstI-BglII fragment from *gntP* (600 bp) and the whole *gntK* gene (740 bp) isolated from pKSK11 (Fig. 1A and B). Figure 2 shows (i) that *gntP* and *gntK* are expressed as monocistronic transcripts of 1.5 and 0.6 kb, respectively; (ii) that equivalent expression was observed in samples from MMA and MMGn; and (iii) that little expression (MMG) or no apparent expression (TSB) for either *gnt* gene indicates the importance of the carbon source in *gnt* gene expression.

To precisely quantify the expression levels of *gntP* and *gntK* under different culture conditions, qRT-PCR was performed with mRNA isolated from *C. glutamicum* grown in MM supplemented with different carbon sources and concentrations (acetate, gluconate, glucose, fructose, and sucrose). mRNAs from different cultures were used to obtain specific cDNA for *gntP* and *gntK*, using primers gnt8 and gnt10, respectively, and further amplified using the primer pairs gnt7/gnt8 and gnt9/gnt10, respectively (Fig. 1A and B). Table 3 summarizes the qRT-PCR data as follows. (i) The expression level of the *gntK* gene is always higher than that of *gntP* in the same culture conditions (indicated by lower C_T values). (ii) The estimated numbers of specific mRNA molecules for *gntK* or *gntP* in cultures containing gluconate or acetate (at 1% or 4%) were equivalent, suggesting a noninducible *gnt* gene expression in presence of gluconate. (iii) For different nutritional conditions, the scale of expression of *gnt* genes was as follows (from high to low): acetate/gluconate, glucose, fructose, and sucrose. (iv) The expression of the *gnt* genes was dependent not only on the catabolized carbon source but also on its concentration in the culture media. (v) The expression levels of the *gnt* genes in media supplemented with gluconate plus glucose or gluconate plus fructose were slightly lower than the expression levels in glucose or fructose medium lacking gluconate.

Homologous and heterologous analysis of the promoter activity from the *gnt* genes. Promoter regions of *gntP* (P*gntP*; 270 bp) and *gntK* (P*gntK*; 250 bp) were PCR amplified and cloned into the *E. coli* promoter probe vector pJMFA24, which contains the promoterless *kan* gene as the reporter, yielding plasmids pJMF-MP and pJMF-MK (Fig. 1A and B). *E. coli* strains containing plasmids pJMF-MP and pJMF-MK were resistant to up to 50 μg/ml of kanamycin. Therefore, the P*gntP* and

TABLE 3. qRT-PCR analysis of *gnt* gene expression from *C. glutamicum* grown in supplemented MM for corynebacteria[a]

MM supplemented with:	C_T for[b]:		Ratio for[c]:	
	gntK	*gntP*	*gntK*	*gntP*
Gluconate 1%[d]	16.64 ± 0.06	17.38 ± 0.07	1 ± 0.04	1 ± 0.05
Gluconate 4%	18.81 ± 0.04	19.61 ± 0.03	4.5 ± 0.12	4.69 ± 0.10
Acetate 1%	16.70 ± 0.04	17.59 ± 0.04	1.04 ± 0.03	1.15 ± 0.04
Acetate 4%	18.85 ± 0.03	19.68 ± 0.02	4.62 ± 0.10	4.92 ± 0.07
Glucose 1%[d]	20.42 ± 0.05	22.44 ± 0.02	13.73 ± 0.49	33.35 ± 0.47
Glucose 4%	22.28 ± 0.05	23.43 ± 0.04	49.86 ± 1.76	66.25 ± 1.87
Fructose 1%	22.35 ± 0.04	23.68 ± 0.03	52.34 ± 1.47	78.79 ± 1.65
Fructose 4%	23.69 ± 0.03	24.58 ± 0.02	132.51 ± 2.78	147.03 ± 2.05
Sucrose 1%	22.43 ± 0.04	23.75 ± 0.03	55.33 ± 1.55	82.71 ± 1.73
Sucrose 4%	23.75 ± 0.03	24.64 ± 0.02	138.14 ± 2.90	153.27 ± 2.14
Gluconate 1% + glucose 1%	20.71 ± 0.04	22.67 ± 0.03	16.79 ± 0.47	39.12 ± 0.82
Gluconate 1% + fructose 1%	22.40 ± 0.03	23.76 ± 0.03	54.19 ± 1.14	83.28 ± 1.75
Fructose 2% + glucose 2%	23.71 ± 0.02	24.60 ± 0.02	134.36 ± 1.88	149.08 ± 2.08
Gluconate 1% + pEMCRP	17.30 ± 0.04	18.22 ± 0.03	1.58 ± 0.04	1.79 ± 0.04
Glucose 1% + pEMCRP	20.89 ± 0.05	22.82 ± 0.04	19.02 ± 0.67	43.41 ± 1.22

[a] These values correspond to the mean of three independent experiments (standard deviation values are also indicated).

[b] C_T is defined as the cycle at which fluorescence is determined to be statistically significantly above background and is inversely proportional to the log of the initial copy number; this value was calculated automatically by the ABI Prism 7000 SDS software.

[c] The relative number of specific mRNA molecules for each gene in the different conditions was calculated assuming that at the threshold point, the number of amplified DNA molecules (N) in one or another growth condition would be the same and proportional to the initial number of RNA molecules (n) according to the following equation: $N = n \times 2^{C_T}$. Ratio indicates the repression ratio estimated by dividing the amount of mRNA molecules obtained in the presence of 1% gluconate (the highest level of expression) by the amount of mRNA molecules in each condition tested.

[d] The same C_T values were obtained for *C. glutamicum* [pEM2] when it was grown at the indicated conditions.

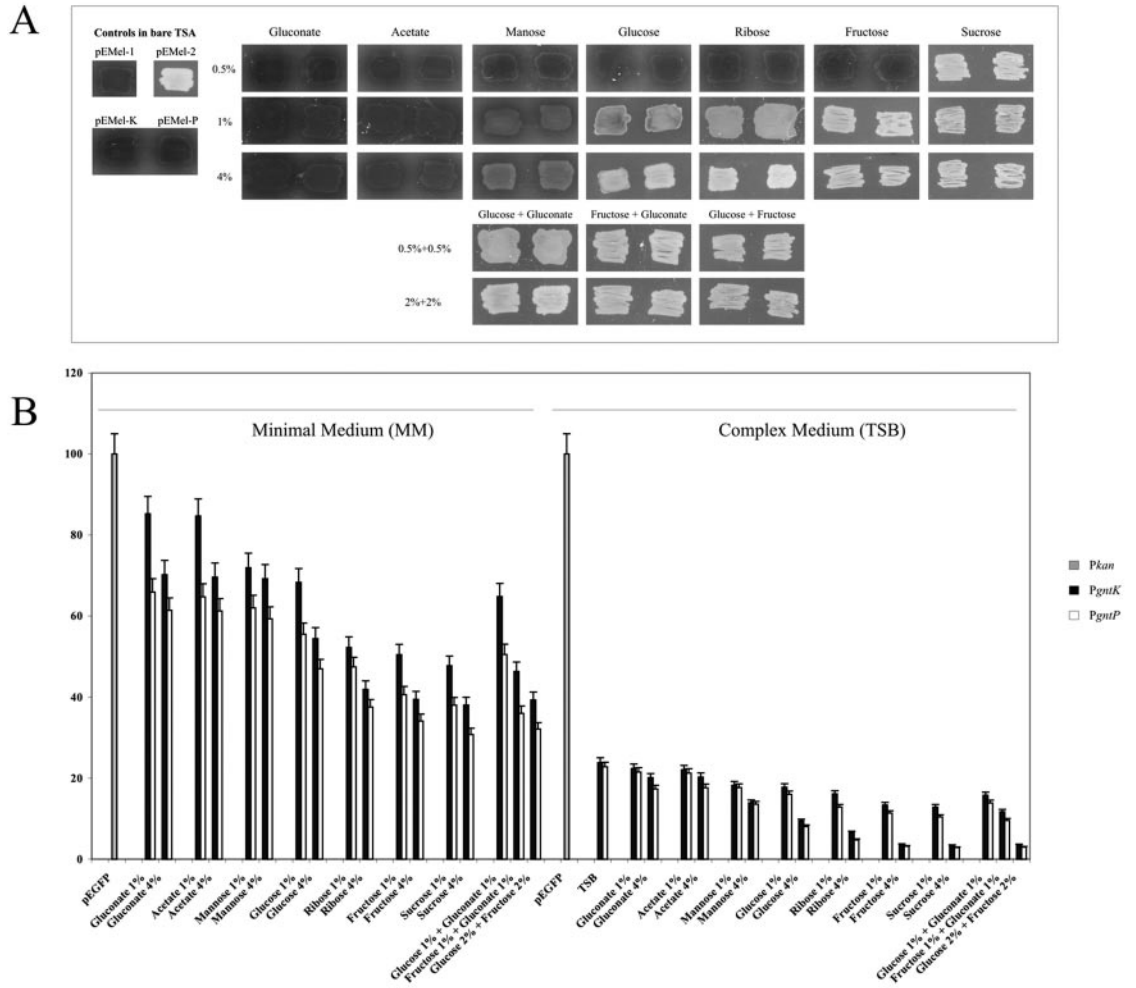

FIG. 3. Quantification of promoter strength using the *melC1* operon and the *egfp2* gene as reporters. (A) *C. glutamicum* strains containing plasmids pEMel-K and pEMel-P were grown on TSA (supplemented with tyrosine and copper sulfate) in the presence of different carbon sources and concentrations, as indicated. *C. glutamicum* strains containing plasmids pEMel-1 and pEMel-2 (Table 1) were used as positive and negative controls for melanin production, respectively; in all cases, the patch pairs correspond to *C. glutamicum*(pEMel-K) (left patches) and *C. glutamicum*(pEMel-P) (right patches). (B) *C. glutamicum* strains containing plasmids pEGFP-MK (black bars) and pEGFP-MP (white bars) were grown in MM or TSB in the presence of increasing concentrations of different carbon sources, and the fluorescence of EGFP2 was measured. *C. glutamicum* strains containing plasmid pEGFP and pEGNC (Fig. 1C) were used as positive and negative controls, respectively. The intrinsic fluorescence level from *C. glutamicum*(pEGNC) was subtracted from all the values obtained. In each case, the fluorescence level of each sample was divided by the OD_{600} of the sample. The values are the means of four independent experiments; standard deviations are indicated on the bar tops.

P*gntK* promoters from *C. glutamicum* are recognized by the *E. coli* RNA polymerase, like many corynebacterial promoters (55, 56). P*gntP* and P*gntK* were also cloned into the bifunctional promoter probe vectors pEMel-1 and pEGFP (Table 1; Fig. 1C) to confirm their promoter activities in corynebacteria. *C. glutamicum* transconjugants containing plasmids pEMel-P and pEMel-K (Table 1) were grown separately in TSA medium supplemented with different carbon sources and with tyrosine and cupper sulfate for the identification of a dark color in the strain when the *mel* operon is expressed. As can be seen in Fig. 3A, similar expression levels of *mel* under the control of P*gntP* or P*gntK* were phenotypically observed in the presence of the organic acids gluconate, acetate, and lactate (the latter not shown), regardless of the concentrations used. The strongest repressor effect was caused by sucrose, followed in order of strength of effect by fructose, ribose, glucose, and mannose. In the presence of two carbon sources (combining gluconate, fructose, and glucose), the repressor effect seems to be stronger than that in the presence of individual sugars; alone, these results support those obtained by qRT-PCR analysis but include additional sugars (ribose and mannose). Minor differences were seen, but they could be attributed to the fact that the growth media used in the experiments differed (MM for qRT-PCR and TSA for *mel* expression).

To monitor the promoter activity of P*gntP* and P*gntK* in the presence of different carbon sources using an alternate method, pEGFP derivative plasmids pEGFP-MP and pEGFP-MK were used (Fig. 1C). *C. glutamicum*(pEGFP-MP) and *C. glutamicum*(pEGFP-MK) were grown in liquid MM or TSB in the presence of different carbon sources at various concentrations.

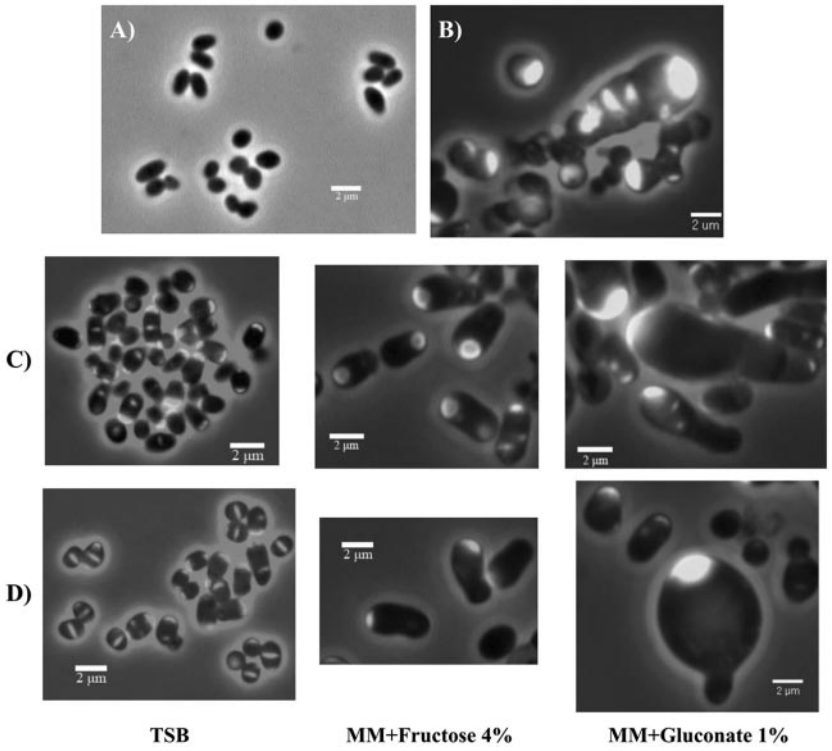

FIG. 4. Expression of the essential morphogen *divIVA* under the control of P*gntP* and P*gntK.* (A) Phase contrast of *C. glutamicum* ATCC 13032. (B to D) Overlays combining phase contrast and fluorescence microscopy of the following strains: *C. glutamicum*(pEAG2) (P*divIVA-divIVA-egfp2*) grown in TSB (B); *C. glutamicum*(pEKDG) (P*gntK-divIVA-egfp2*) cultured under the indicated conditions (C); *C. glutamicum*(pEPDG) (P*gntP-divIVA-egfp2*) cultured under the indicated conditions (D). Bars, 2 μm.

Plasmid pEGFP, which contains a strong and constitutive promoter (P*kan*) not repressed by these carbon sources, was used as a control; the fluorescence value obtained from *C. glutamicum*(pEGFP) cultured on MM or TSB was set at 100%, and the other data were normalized to this value. The results in Fig. 3B indicate the following. (i) The level of fluorescence from *C. glutamicum*(pEGFP-MK) (P*gntK*) was always higher than that from *C. glutamicum*(pEGFP-MP) (P*gntP*), independent of the carbon source or growth medium (MM or TSB). (ii) The repressor effect was always 50 to 60% higher in complex medium (TSB) than in defined medium (MM). (iii) Plasmids containing P*gntK* and P*gntP* were repressed to the same extent by mannose, glucose, ribose, fructose, and sucrose, with the last exerting the greatest catabolic repression. (iv) The repressor effect was dependent on the concentration of the carbon source (increasing at higher concentrations). (v) The repression effect seems to increase slightly when gluconate and an additional repressor sugar are present in the media.

***PgntP* or P*gntK* can be used to modulate the expression of essential cell division genes.** The *divIVA* gene from *Brevibacterium lactofermentum* (presently known as *C. glutamicum* [44]) is located downstream from the cell division cluster (*dcw*), and its product (DivIVA) has a possible function at the growing cell poles of *C. glutamicum* (61). Overexpression of the translational fusion *divIVA-egfp2* (from plasmid pEAG2) in *C. glutamicum* led to an altered morphology: the cells were rounded, larger, and swollen and tended to grow in a polar fashion, with the DivIVA-EGFP2 product located mainly at the cell poles. It has been suggested that DivIVA participates in the maintenance of cell morphology in *C. glutamicum* (61).

We constructed plasmids pEPDG and pEKDG (Table 1), in which P*gntP* and P*gntK* controlled the expression of the *divIVA-egfp2* translational fusion. The plasmids were transferred by conjugation to *C. glutamicum* ATCC 13032 for comparison with our previous results (61). Transconjugants containing either plasmid were grown in TSB or MM containing different sugars, and the expression levels relative to the morphological changes were compared to that of *divIVA-egfp2* under its own promoter (plasmid pEAG2; Table 1). *C. glutamicum*(pEAG2) displayed the previously described altered morphology (Fig. 4) independently of the medium used for growth (TSB or MM). However, when *C. glutamicum*(pEPDG) and *C. glutamicum*(pEKDG) were grown in TSB, their morphologies were similar to that of the wild type, suggesting low levels of expression of DivIVA-EGFP2 under the control of P*gntP* or P*gntK* (Fig. 4). This is supported by the low level of apical fluorescence detected from *C. glutamicum*(pEPDG) or *C. glutamicum*(pEKDG).

C. glutamicum(pEPDG) and *C. glutamicum*(pEKDG) were also grown in MM in the presence of gluconate (1%) or fructose (4%) to control the expression of *divIVA-egfp2* under the control of P*gntP* or P*gntK*. As can be observed in Fig. 4, larger and swollen cells were observed in the presence of gluconate, but the cell size and the fluorescence level were not affected as much by the presence of fructose. It can also be observed that the fluorescence of the DivIVA-EGFP2 fusion under P*gntK*

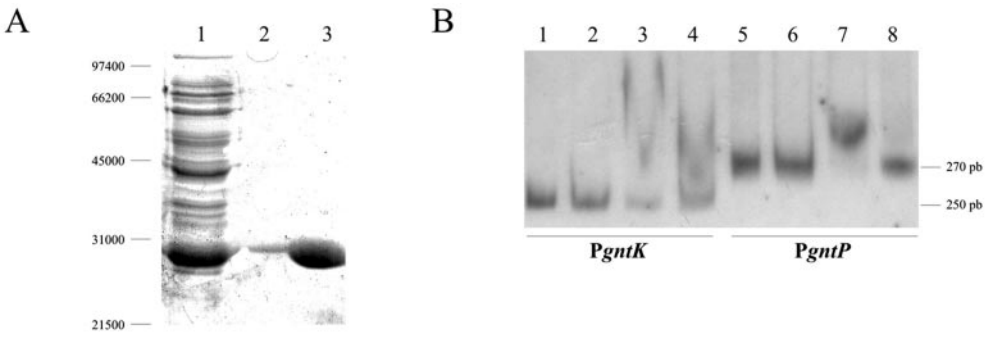

FIG. 5. GlxR protein purification and EMSA using P*gntK* and P*gntP* (A) SDS-PAGE of GlxR. Cell extract of the induced *E. coli*(pNProCRP) (lane 1); elution fraction containing the diluted GlxR protein (lane 2); elution fraction containing concentrated GlxR (lane 3). Molecular mass markers are indicated to the left (Da). (B) Gel-retarding analysis of the following: samples containing only the DNA probe in the reaction mixture (lanes 1 and 5); samples containing the corresponding DNA probe and 0.1 μg of the purified GlxR protein (lanes 2 and 6); samples containing the DNA probe, 0.1 μg of GlxR, and 0.2 mM of cAMP (lanes 3 and 7); samples with the same components as those in lanes 3 and 7 but supplemented with 500 times the amount of the corresponding unlabeled probe (lanes 4 and 8). The sizes of the P*pgntK* and P*gntP* probes are indicated.

was stronger than that under P*gntP*, confirming our results regarding promoter strength (see above).

Cloning and expression of the *glxR* gene. As indicated above, no typical binding domains for GntR interaction were found in the promoter region of *gntP* or *gntK*; however, consensus sequences corresponding to the CBS described for catabolite repression in gram-negative bacteria were found upstream from *gntP* and *gntK*. We then performed a search for a hypothetical *crp* gene on the genome sequences of *C. glutamicum*, *C. efficiens*, and *C. diphtheriae* (GenBank accession numbers BX927147, BA4035, and BX248353, respectively). We were able to identify a sequence encoding a common 227-amino-acid CRP protein, previously described as the glyoxylate regulator GlxR (37). This protein showed significant homology with putative transcriptional regulators from *Mycobacterium tuberculosis* and *Streptomyces coelicolor* and with CRP regulators from *E. coli* and *Vibrio cholerae*. To test the possible participation of the GlxR protein on the regulation of the *gnt* genes, the *C. glutamicum crp1* gene (also called the *glxR* gene herein) was PCR amplified and subcloned into pNPro (pNProCRP), and the BglII cassette was cloned into the bifunctional *E. coli*-corynebacteria expression vector pEM2 (Fig. 1C), yielding the plasmid pEMCRP, which contains the *glxR* gene under the *kan* promoter (P*kan-glxR*). With the *glxR* gene present on a multicopy plasmid, *C. glutamicum*(pEMCRP) showed delayed growth, in agreement with the results previously published by Kim et al. (37). This strain was grown in MMG (1%) or MMGn (1%), and the expression of *gntP* and *gntK* was evaluated by qRT-PCR; the transcription levels of the *gnt* genes in *C. glutamicum*(pEMCRP) were slightly lower than those of *C. glutamicum*(pEM2) and *C. glutamicum* without plasmid, which were used as C_T control values (Table 3).

The suicide plasmid pKCRP, which contains a 300-bp internal fragment from *glxR* (Table 1), was repeatedly transferred from *E. coli* to *C. glutamicum* by conjugation, but kanamycin-resistant transconjugants were never obtained; this result might indicate that *glxR* is essential for the viability of *C. glutamicum*, as was described previously (37).

Purification of the GlxR protein and binding analysis to the *gnt* promoter regions. A His-tagged GlxR protein was purified from *E. coli*(pETCRP) and analyzed by SDS-PAGE (Fig. 5A); its molecular mass (27 kDa) coincides with the expected size deduced from the gene sequence.

To determine if purified protein (His-tagged GlxR) binds to the *gnt* promoter regions, P*gntP* and P*gntK* were used as probes in gel shift mobility assays. We used the promoter region of the arsenic detoxification operon P*arsB1* (53) as the negative control. Starting from the protocol described by Kim et al. (37), we optimized the reaction conditions for each probe and obtained gel-retarding bands only for probes P*gntP* and P*gntK*. The presence of retarding bands was directly dependent on the presence of cAMP in the reaction mixture (Fig. 5B); bands were detectable when the cAMP values were between 0.05 and 0.6 mM (and above). No retarding bands were observed in the absence of cAMP or His-tagged GlxR (Fig. 5B). Treating His-tagged GlxR with thrombin to remove the His tag had no effect on binding (data not shown). When the P*arsB1* probe was used, no retardation was observed (data not shown). In addition, the binding specificity of GlxR to P*gntP* or P*gntK* was confirmed by adding increasing amounts of unlabeled probe (Fig. 5B). No differences were found on the levels of retarding bands from the gel shift assay when cAMP was present or absent in the SDS-PAGE and/or in the electrophoresis buffer.

DISCUSSION

C. glutamicum can metabolize numerous carbon sources, including glucose, fructose, sucrose, ribose, mannose, lactate, acetate, and gluconate. In general, gluconate is incorporated into bacterial cells by a gluconate permease (GntP) and is then converted to 6-phosphogluconate by a gluconate kinase (GntK); 6-phosphogluconate is channeled through the pentose phosphate pathway and through the Entner-Doudoroff pathway, if present (59). Owing to the presence of *edd* and the absence of *eda*, which encode the Entner-Doudoroff enzymes 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate-aldolase, respectively, in the genome of *C. glutamicum* (as it was deduced from the database), it may be assumed that gluconate/6-phosphogluconate is metabolized through the pentose phosphate pathway. To our knowledge, no studies

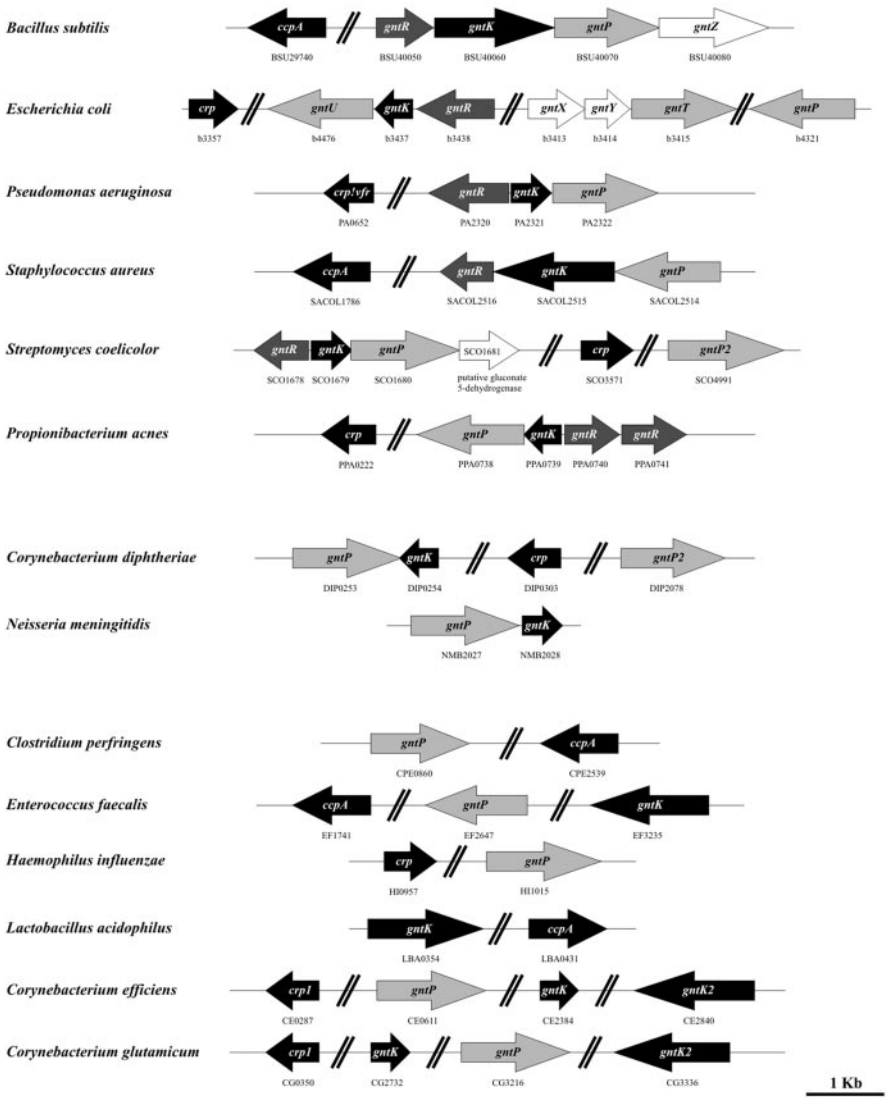

FIG. 6. Schematic organization of the *gnt* genes/operons in various bacteria. All of the nucleotide sequences were taken from the EBI (*E*uropean *B*ioinformatics *I*nstitute, Hixton Hall, United Kingdom) database; the *gnt* gene locations for *E. coli* and *Bacillus* have been published previously (21, 60, 72). Genes and their respective products are as follows: *gntR*, gluconate repressor; *gntK*, gluconate kinase; *gntP*, gluconate permease; *gntU*, low-affinity gluconate permease; *gntT*, high-affinity gluconate permease; *gntZ*, 6-phosphogluconate dehydrogenase; *gntX*, putative periplasmic gluconate-binding protein; *gntY*, putative membrane-bound protein; *crp*, cAMP receptor protein; *ccpA*, carbon catabolite protein.

specifically addressing gluconate metabolism in corynebacteria have been conducted, except for those regarding the use of gluconate as a secondary carbon source in nongrowing cells of *C. glutamicum* (7, 43).

Microorganisms able to metabolize gluconate can contain a typical *gnt* operon which includes genes for the repressor protein (*gntR*), for gluconate permease (*gntP* or the equivalent), and for gluconate kinase (*gntK*). This organization is present in the *E. coli* GntI system (72) and in *B. subtilis* (20), *Pseudomonas aeruginosa* (accession number, NC002516), *Staphylococcus aureus* (NC002952), *Streptomyces coelicolor* A3 (2) (NC003888), and *Propionibacterium acnes* (NC006085), as shown in Fig. 6. A *gnt* operon structure lacking *gntR* is present on the genomes of *Neisseria meningitidis* (NC003116) and *Corynebacterium diphtheriae* (NC002935). The most frequent situation is the presence of one or two genes of the *gnt* system scattered on the chromosome, as occurs in *Clostridium perfringens* (NC003366), *Enterococcus faecalis* (NC004668), *Haemophilus influenzae* (NC007146), *Lactobacillus acidophilus* (NC006814), *Corynebacterium efficiens*, and *C. glutamicum* (Fig. 6).

A second hypothetical gluconate kinase gene (*gntK2*) was located in a recent sequence analysis of the *C. glutamicum* and *C. efficiens* genomes (Fig. 6); comparative analysis of the ORFs showed that the *gntK2* gene is larger than *gntK* (494 and 156 amino acids, respectively) and also showed certain similarities to the *gntK* gene from *Bacillus* (513 amino acids); moreover, phylogenetic analysis of bacterial GntK performed with the MEGA3 program displays different associations for *gntK* and *gntK2* (see Fig. S1 in the supplemental material); the former is well positioned close to the actinobacteria, whereas *gntK2* (ab-

sent in *C. diphtheriae*) is related to the low-G+C-content gram-positive bacteria. In addition, the evidence obtained from this work suggests that *gntK2*, if functional, is not involved in gluconate catabolism in *C. glutamicum*, since *C. glutamicum* GNTK (a mutant with *gntK* disrupted) was unable to grow in MM containing gluconate. A similar analysis was performed with *gntP* (see Fig. S2 in the supplemental material) and *gntR* (see Fig. S3 in the supplemental material) and showed a high level of conservation (likeness) in the sequences of both genes among the analyzed bacteria (even though in this analysis no specific *gntR* was found in *C. glutamicum* or within the *Corynebacterineae* group).

Northern blotting analysis showed that *gntP* and *gntK* are transcribed in *C. glutamicum* as single monocistronic units of 1.5 and 0.6 kb, respectively. The expression of the *gntK* and *gntP* genes is not induced by gluconate, since equivalent levels of expression were obtained when acetate was the only carbon source present in the media (Fig. 2; Table 3). An important repressor effect was observed in the presence of several assimilable carbon sources, such as glucose, fructose, and sucrose (Table 3). The repression caused by sucrose and fructose is the strongest and highest when the sugar concentration is increased (Table 3). In all cases, the basal level of expression of the *gntK* gene was higher than that in *gntP* (Fig. 2; Table 3). The repressor effect of glucose or fructose did not revert when gluconate was added to the culture; on the contrary, a slight increase of *gnt* gene repression was continuously observed by qRT-PCR (Table 3) and by *mel* (Fig. 3A) and *gfp* (Fig. 3B) expression analysis. A slight repressor effect was also observed when cells were grown in MMGn containing 4% gluconate rather than 1% (Table 3 and Fig. 3B).

Parallel gene expression results were obtained when *C. glutamicum* strains containing bifunctional promoter probe vectors with *mel* and *egfp2* under the control of *gnt* promoters (P*gntP* and P*gntK*) were assayed; in addition to the results described above, we observed repression of the reporter genes when ribose or mannose was present (Fig. 3A and B). Strains containing plasmids with the fully expressed *mel* operon are easily visualized by its typical dark color. The assay was performed in complex medium (TSA) (Fig. 3A), since phenotypical differences when cells were grown in MM were not appreciated due to the high level of expression of the *mel* operon in these culture conditions. In addition, to evaluate the fluorescence levels from cultures containing pEGFP derivatives grown at different conditions, we applied a fast and confident method in which the promoter fusion results were correlated to those obtained by qRT-PCR without the tedious process of mRNA isolation. The data differences seen in these two procedures could be due to the presence of several copies of the promoter probe vector in the cells, which contrasts with the quantification by qRT-PCR of single-copy gene expression.

Several putative regulators with partial similarity to CcpA were found in *Streptomyces* (73) and *Corynebacterium* (54). However, *C. glutamicum* does not have genetic information for HPr kinase/phosphatase, the central enzyme of CCR in low-GC-content gram-positive bacteria (54); therefore, due to the presence of typical CBS upstream from *gntP* and *gntK*, we decided to look for CRP-type regulators. We searched the *C. glutamicum* genome for a putative *crp* gene using the *crp* homologues of *Mycobacterium* and *Streptomyces* and phylogenetic

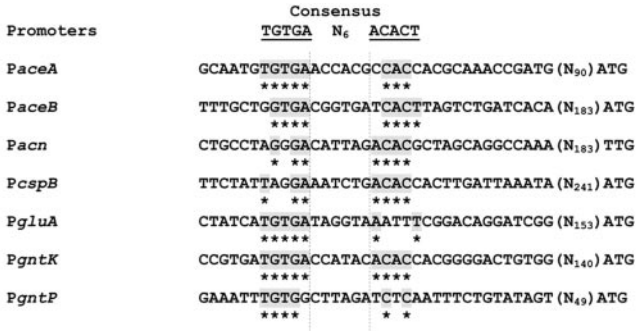

FIG. 7. Hypothetical presence of CBS consensus motifs at the promoter region of *C. glutamicum* catabolite-regulated genes. Promoter sequences and their corresponding products (or function) are as follows: P*aceA*, isocitrate lyase; P*aceB*, malate synthase; P*acn*, aconitase; P*csp*, surface-layer protein; P*glu*A, glutamate uptake; P*gntK*: gluconate kinase; P*gntP*: gluconate permease. Asterisks and gray shading indicate conserved nucleotides with the consensus sequence described for catabolite repression. (N_x) indicates the distance (in nucleotides) corresponding to the gap.

analysis (see Fig. S4 in the supplementary material). We found that the *crp1* gene (CG0350; Fig. 6) was exactly the same as *glxR* as described by Kim et al. (37) for the strain *C. glutamicum* ATCC 13059. These authors mentioned the heterologous complementation of *E. coli* CRP mutants by GlxR protein and suggested a possible interaction of GlxR with other promoters (37). *glxR* seems to be essential for cell viability, in agreement with the results previously mentioned (37) and in contrast to all transcriptional regulators described so far for *C. glutamicum*, where the corresponding encoded genes were not essential (24, 41, 64). We indicate the presence (based on database gene alignment) of the *crp* and *ccpA* genes located on the chromosome of the above-mentioned bacteria (Fig. 6).

Due to the presence of typical CBS upstream from *gntP* and *gntK*, we looked for such sites around the promoter region of *C. glutamicum* genes shown to be regulated by carbon sources, such as *aceA* and *aceB* (37), *acn* (41), *gluA* (40), and *cspB* (70). The *aceA* and *aceB* genes coding for isocitrate lyase and malate synthase, respectively, are transcriptionally regulated by the RamB repressor; the effect of RamB was evaluated in media containing glucose (24). In addition, GlxR has been described as a carbon catabolite transcriptional regulator involved in *aceB* gene expression (37), although an equivalent mechanism is mentioned for *aceA*. The expression of *acn*, which codes for aconitase, is modulated by the transcriptional repressor AcnR (41). However, in Δ*acnR* mutants of *C. glutamicum*, an increase in aconitase activity (1.5- to 2.3-fold) in cultures grown on acetate, citrate, or propionate compared to the activity of cultures grown on glucose was observed. Therefore, an additional carbon-dependent regulatory mechanism has been suggested (41). *gluA* is the first gene from the *gluABCD* operon involved in glutamate uptake in *C. glutamicum* (40); a repressor effect on glutamate uptake was established when cells were grown in the presence of different carbon sources (such as glucose or fructose) and compared to cells grown on glutamate as the sole carbon source (54). In the case of *cspB* (coding for the S-layer protein PS2), the catabolite repressor effect was measured by analyzing the amount of PS2 present in cultures grown in the presence of lactose or glucose or by measuring the β-galactosidase activity from *C. glutamicum* strains

containing the *cspB* promoter fused to *lacZ* (P*cspB-lacZ*) grown in media containing lactate or glucose. Both methods showed a repressive effect of glucose (70). The CBS consensus sequence, TGTGA-N_6-ACACT, matched with the analyzed carbon catabolite-regulated promoters from *C. glutamicum* (Fig. 7). The physical position of CBS on P*gntP* and P*gntK* could be a determining factor for the modulation of gene expression, as with CRE sequences (48). In the case of P*gntP*, the hypothetical CBS is partially included in the −35 box, and therefore its inherent promoter activity could be lower and could strengthen the repressor effect of the sugars (Fig. 1A; Table 3). The contrary may be true for P*gntK*, where the CBS is located upstream of the −35 box (Fig. 1B; Table 3). Therefore, based on our results for the expression of the *gnt* genes (Fig. 2 and Table 3) and the amount of EGFP2 produced from *gnt* promoters (Fig. 3B), we can conclude that *gntP* is expressed in *C. glutamicum* at lower levels and is more sensitive to catabolite repression than *gntK*.

EMSA using purified GlxR from *C. glutamicum* and P*gntP* and P*gntK* showed a specific DNA-GlxR interaction; therefore, the *gnt* genes would be subject to carbon catabolite regulation, with the binding of GlxR to the promoters mediated by the intermediate regulator, cAMP. In *C. glutamicum*, high levels of cAMP are present when cells are grown on glucose medium, while low levels are present when cells are grown on acetate (37, 45). The repressor effect observed in *C. glutamicum* grown in media containing sucrose or fructose was high compared with that seen with other sugars (ribose, glucose, or mannose) or organic acids (acetate, lactate, or gluconate); this could be due to the specific or unspecific mechanism of sugar uptake (25, 49) and/or to the generation of certain glycolytic intermediates. These intermediates could be involved directly or indirectly with cAMP production in catabolite repression. We also observed a higher repressor effect when *C. glutamicum* was grown in complex medium (TSB) than when it was grown in defined medium (MM) (Fig. 2; Fig. 3A and B). In *B. subtilis*, the inositol dehydrogenase gene (*idn*) is repressed by the presence of carbohydrates (51); analogous results, described by Deutscher et al. (13) for a *B. subtilis* mutant, showed that the inositol dehydrogenase activity was almost completely repressed when the strain was grown in rich medium but only partially repressed when the grown was in MM (23-fold and 4-fold, respectively). We do not know the possible mechanism involved in the increased repressor effect of TSA/TSB medium, but equivalent results were obtained when Luria-Bertani complex medium (lacking glucose) was used. Perhaps the presence of very little sugar in the complex medium would be responsible for a certain repressor effect, but it is more probable that additional unknown factors are involved.

In this work, we describe the fact that *gntP* and *gntK*, which are involved in gluconate metabolism in *C. glutamicum*, are subject to carbon catabolite regulation mediated by cAMP and GlxR; expression of the *glxR* gene does not seem to be the limiting factor, as its overexpression [in *C. glutamicum* (pEMCRP)] decreased the expression of the *gnt* genes slightly (Table 3). However, the presence of additional regulatory mechanisms involved in *gnt* gene expression is possible. The promoters of the gluconate system in corynebacteria display a weak to strong repression as a function of the media (complex or minimal) and of the presence of certain sugars (mannose, glucose, ribose, fructose, and sucrose). The effect of catabolic repression is higher when the repressor agents, sugars, are present in large quantities.

The availability of regulated promoters in corynebacteria will allow us to control the expression of essential genes, particularly those studied in basic and medical research. An example of the regulation of an essential gene from *C. glutamicum* which is involved in cell division is reported in this work (Fig. 4); the overexpression of *divIVA* from *C. glutamicum* leads to an aberrant cell morphology. We were able to modify the cell morphology from large and irregular to normal by use of vectors containing the *divIVA-egfp2* gene fusion product under the control of the *gnt* promoters and specific nutritional conditions.

ACKNOWLEDGMENTS

We thank Brett Geissler for the critical revision of the manuscript.

M. Letek, N. Valbuena, E. Ordóñez, and A. Ramos were recipients of fellowships from the Junta de Castilla y León (E.O. and A.R.) and Ministerio de Educación y Ciencia (M.L. and N.V.). This work was supported by grants from the Junta de Castilla y León (LE 24/01), Diputación de León (2003FMC), Universidad de León (ULE-2001-03), and Ministerio de Ciencia y Tecnología (BIO 2002-03223).

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