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Transcription of Sialic Acid Catabolism Genes in *Corynebacterium glutamicum* Is Subject to Catabolite Repression and Control by the Transcriptional Repressor NanR

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ABSTRACT

Corynebacterium glutamicum **metabolizes sialic acid (Neu5Ac) to fructose-6-phosphate (fructose-6P) via the consecutive activity of the sialic acid importer SiaEFGI, *N*-acetylneuraminic acid lyase (NanA), *N*-acetylmannosamine kinase (NanK), *N*-acetylmannosamine-6P epimerase (NanE), *N*-acetylglucosamine-6P deacetylase (NagA), and glucosamine-6P deaminase (NagB). Within the cluster of the three operons *nagAB*, *nanAKE*, and *siaEFGI* for Neu5Ac utilization a fourth operon is present, which comprises *cg2936*, encoding a GntR-type transcriptional regulator, here named NanR. Microarray studies and reporter gene assays showed that *nagAB*, *nanAKE*, *siaEFGI*, and *nanR* are repressed in wild-type (WT) *C. glutamicum* but highly induced in a Δ*nanR C. glutamicum* mutant. Purified NanR was found to specifically bind to the nucleotide motifs A[AC]G[CT][AC]TGATGT C[AT][TG]ATGT[AC]TA located within the *nagA-nanA* and *nanR-sialA* intergenic regions. Binding of NanR to promoter regions was abolished in the presence of the Neu5Ac metabolism intermediates GlcNAc-6P and *N*-acetylmannosamine-6-phosphate (ManNAc-6P). We observed consecutive utilization of glucose and Neu5Ac as well as fructose and Neu5Ac by WT *C. glutamicum*, whereas the deletion mutant *C. glutamicum* Δ*nanR* simultaneously consumed these sugars. Increased reporter gene activities for *nagAB*, *nanAKE*, and *nanR* were observed in cultivations of WT *C. glutamicum* with Neu5Ac as the sole substrate compared to cultivations when fructose was present. Taken together, our findings show that Neu5Ac metabolism in *C. glutamicum* is subject to catabolite repression, which involves control by the repressor NanR.**

IMPORTANCE

Neu5Ac utilization is currently regarded as a common trait of both pathogenic and commensal bacteria. Interestingly, the nonpathogenic soil bacterium *C. glutamicum* efficiently utilizes Neu5Ac as a substrate for growth. Expression of genes for Neu5Ac utilization in *C. glutamicum* is here shown to depend on the transcriptional regulator NanR, which is the first GntR-type regulator of Neu5Ac metabolism not to use Neu5Ac as effector but relies instead on the inducers GlcNAc-6P and ManNAc-6P. The identification of conserved NanR-binding sites in intergenic regions within the operons for Neu5Ac utilization in pathogenic *Corynebacterium* species indicates that the mechanism for the control of Neu5Ac catabolism in *C. glutamicum* by NanR as described in this work is probably conserved within this genus.

The Gram-positive *Corynebacterium glutamicum* is mostly known for its application in the industrial production of amino acids, mainly L-glutamate and L-lysine (1, 2), and has become a versatile cell factory for the production of various commodity products (3–5). *C. glutamicum* utilizes a large variety of sugars and organic acids as sources of carbon and energy (6, 7) and additionally has been genetically engineered for the utilization of alternative feedstocks such as starch, glycerol, xylose, glucuronic acid, and *N*-acetylglucosamine (8–12). In contrast to many other bacterial species, *C. glutamicum* prefers to use multiple carbon sources simultaneously. It simultaneously metabolizes glucose together with other carbon sources such as sucrose, fructose, maltose, gluconate, ribose, pyruvate, and acetate and thereby exhibits monophasic growth (13–18). Diauxic growth of *C. glutamicum* and consecutive utilization of the provided carbon sources have been observed so far for media that contain glutamate or ethanol in addition to glucose (6, 19–21). Recently, uptake and utilization of the sialic acid *N*-acetylneuramic acid (Neu5Ac) were reported to be inhibited in glucose-cultivated *C. glutamicum* cells (22). This finding suggests the presence of a third example of carbon catabolite repression (CCR) in *C. glutamicum*, although neither the transcription of genes for Neu5Ac utilization has been analyzed

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nor have the underlying mechanisms been identified and characterized.

C. glutamicum harbors a full set of genes for Neu5Ac catabolism, which are clustered within the genome (23). Uptake of Neu5Ac is brought about in *C. glutamicum* by the ABC transporter here named SiaEFGI, encoded by *cg2937* (*siaE*), *cg2938* (*siaF*), *cg2939* (*siaG*), and *cg2940* (*siaI*) (22). As depicted in Fig. 1, Neu5Ac is then metabolized by the consecutive action of the *N*-acetylneuraminic acid lyase NanA (encoded by *cg2931* [*nanA*]), the *N*-acetylmannosamine kinase NanK (encoded by *cg2932* [*nanK*]), the *N*-acetylmannosamine-6-phosphate epimerase NanE (encoded by *cg2935* [*nanE*]), the *N*-acetylglucosamine-6-phosphate deacetylase NagA (encoded by *cg2929* [*nagA*]), and the glucosamine-6-phosphate deaminase NagB (encoded by *cg2928* [*nagB*]) to pyruvate, acetate, ammonia, and fructose-6-phosphate (fructose-6P) (22). The enzyme NagB is also required for utilization of glucosamine as a source of carbon and nitrogen, which is taken up via the glucose specific permease EII^{Glc} of the phosphoenolpyruvate sugar phosphotransferase system (PTS) (24). For the efficient utilization of glucosamine by *C. glutamicum* increased expression of *nagB* is required, which is brought about either by ectopic expression or by a point mutation within the promoter region of the *nagAB* operon in the spontaneous mutant *C. glutamicum* M4 (24). The genes for Neu5Ac utilization are organized in *C. glutamicum* in three clustered operons, namely, *nagAB*, *nanAKE*, and *siaEFGI*. Within this cluster of operons a fourth operon is present, which comprises the open reading frame (ORF) *cg2936* for a putative GntR-type transcriptional regulator and ORF *cg2935* (*nanP*) for a putative sialidase (22).

The sialic acid Neu5Ac is the terminal moiety of glycan molecules present on the surface of eukaryotic cells (25–28). Besides serving as attachment and recognition point for various pathogens, Neu5Ac is also an important source of carbon and energy available in various host niches such as the oral cavity and the respiratory, intestinal, and urogenital tracts (29–32). Therefore, Neu5Ac metabolism and its control have been studied in detail for various, often pathogenic, bacteria such as *Escherichia coli*, *Vibrio vulnificus*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Clostridium perfringens*, and the probiotic *Bifidobacterium breve* (33–38). In *H. influenzae*, transcription of the *nan* and the *siaPT* operons for Neu5Ac utilization is repressed by SiaR and is activated in the presence of glucosamine 6-phosphate (see Table S1 in the supplemental material), which is an intermediate of Neu5Ac catabolism and acts as a coactivator of the RpiR-type regulator SiaR (37, 39–41). The RpiR-type transcriptional repressor NanR of *V. vulnificus* controls $nanT_{PSI}AR$ (for the triapartite ATP-independent transporter, Neu5Ac aldolase, and the *nan* gene repressor), *nanEK*, and the *nagA* operons (42, 43). Binding of the Neu5Ac catabolism intermediate *N*-acetylmannosamine-6-phosphate (ManNAc-6P) to *V. vulnificus* NanR mediates relocation of residues in the ligand binding site, thus alleviating the interaction between the NanR dimer and DNA and subsequently relieving the repression by NanR and inducing transcription of the *nan* operons (42) (see Table S1). Different from the RpiR-type regulators of Neu5Ac metabolism of *H. influenzae* and *V. vulnificus*, the RpiR-type regulator NanR of *S. pneumoniae* acts as a transcriptional activator of Neu5Ac catabolism genes (34). In *E. coli* and *B. breve*, transcriptional control of genes for Neu5Ac utilization is brought about by GntR-type transcriptional repressors each also named NanR, which both depend on Neu5Ac as the sole inducer (44–46) (see Table S1). Mechanisms for the control of Neu5Ac catabolism have hitherto not been studied for *C. glutamicum*.

In this communication, we show that in *C. glutamicum* the *cg2936*-encoded GntR-type transcriptional regulator, here named NanR, represses transcription of its own gene and of all operons for Neu5Ac catabolism, namely, *nagAB*, *nanAKE*, and *siaEFGI*, by binding within the respective promoter regions. Repression of Neu5Ac catabolism operons in *C. glutamicum* is shown to be responsible for the preferential utilization of glucose or fructose compared to NeuAc, and the role of NanR for this new example of CCR in *C. glutamicum* is analyzed. As opposed to the GntR-type repressors of Neu5Ac catabolism of *E. coli* and *B. breve*, Neu5Ac did not interfere with *C. glutamicum* NanR as the inducer. Instead, we show that the presence of ManNAc-6P as well as *N*-acetylglucosamine-6P (GlcNAc-6P) alleviates binding of *C. glutamicum* NanR to DNA fragments carrying NanR-binding sites. Finally, we discuss the relevance of these findings for the control of Neu5Ac metabolism in the related pathogenic species *Corynebacterium ulcerans*, *Corynebacterium diphtheriae*, and *Corynebacterium pseudotuberculosis*.

MATERIALS AND METHODS

Microorganisms, plasmids, and cultivation conditions. Strains and plasmids used in this study are listed in Table 1. The CGXII minimal medium used for cultivation of *C. glutamicum* has been described previously (47) and contained glucose, fructose, and/or Neu5Ac at concentrations indicated in Results. LB medium (48) was used as a complex medium for *C. glutamicum* and *E. coli*. When appropriate, kanamycin (50 μg ml^{-1}) and/or isopropyl-β-D-thiogalactopyranoside (IPTG; 100 μM) was added to the medium. *C. glutamicum* was grown aerobically at 30°C as 10-ml cultures in 125-ml baffled Erlenmeyer flasks, and *E. coli* was grown at 37°C as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Growth of *E. coli* and of *C. glutamicum* strains in liquid cultures was followed by measuring the optical density at 600 nm (OD_{600}).

Quantification of glucose, fructose, and Neu5Ac. For quantification of glucose, fructose, and Neu5Ac in the culture broth, aliquots of the culture were withdrawn, cells were removed by centrifugation (5 min at 13,000 × *g* and 4°C), and the supernatant was used for the determination of sugars with a Hitachi high-performance liquid chromatography (HPLC) system equipped with a refractive index detector (L2490; used for glucose and fructose quantification) and a UV detector (L2400; used for Neu5Ac quantification) and a Nucleo Sugar 810H column (Macherey & Nagel). The mobile phase was 0.01 M H_2SO_4 with a flow rate of 0.5 ml min^{-1}, the column temperature was 40°C, and fluorescence was recorded at 210 nm.

DNA preparation, manipulation, and transformation. Standard procedures were employed for chromosomal as well as plasmid DNA isolation and for molecular cloning and transformation of *E. coli*, as well as for electrophoresis (48). Isolation of plasmids and chromosomal DNA of *C. glutamicum* was performed as described previously (49). Transformation of *C. glutamicum* was performed by electroporation as described previously (50). PCR experiments were performed in a Flexcycler (Analytik Jena) with Phusion DNA polymerase (New England BioLabs) with oligonucleotides obtained from Eurofins MWG Operon and listed in Table S1 in the supplemental material. All restriction enzymes, T4 DNA ligase, and shrimp alkaline phosphatase were obtained from New England BioLabs and used according to the manufacturer's instructions.

Construction of plasmids and strains. Inactivation of the chromosomal *nanR* gene in *C. glutamicum* was performed using crossover PCR and the suicide vector pK19*mobsacB*. Flanking regions of the gene of roughly 560 bp were amplified by using the primer pairs D_nanR_P1 and D_nanR_P2 and D_nanR_P3 and D_nanR_P4. The PCR products were

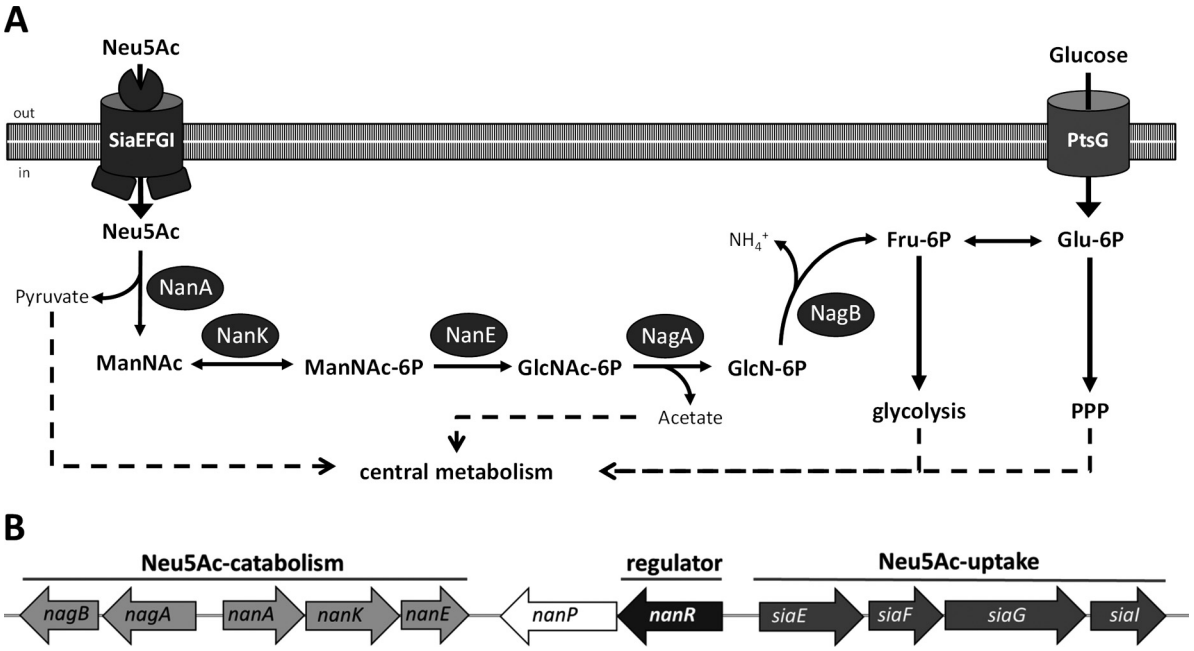

FIG 1 (A) Schematic diagram of the pathway for Neu5Ac metabolism in *C. glutamicum*. SiaEFGI, ABC transporter for Neu5Ac; NanA, *N*-acetylneuraminic acid lyase; NanK, *N*-acetylmannosamine kinase; NanE, *N*-acetylmannosamine-6P epimerase; NagA, *N*-acetylglucosamine-6P deacetylase; NagB, glucosamine-6P deaminase; PtsG, glucose-specific EII permease of the PTS. (B) Genetic organization of the genes for Neu5Ac utilization in *C. glutamicum*.

fused via the complementary artificial overhangs provided in the primers and in a PCR using both of the PCR products together with the primers D_nanR_P1 and D_nanR_P4. Using the PCR-generated 5′ XmaI and 3′ PstI restriction sites of the 1,146-bp fusion PCR product, the construct was ligated into pK19mobsacB and transformed into *E. coli*. The recombinant plasmid pK19*mobsacB*Δ*nanR* was isolated from *E. coli* and electroporated into WT *C. glutamicum*. By application of the method described in reference 51, the complete chromosomal *nanR* gene was deleted via homologous recombination (double crossover). Deletion of *nanR* was verified by PCR using the primer check_D_nanR_fwd and check_D_nanR_rev, resulting in an 848-bp fragment for the WT and a 197-bp fragment for the deletion mutant.

For IPTG-inducible overexpression in *C. glutamicum* strains, vector pEKEx2 was used. For construction of pEKEx2-*nanR*, the gene was am-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 phoA supE44 hsdR1 recA1 endA1 gyrA96 thi-1 relA1*	89
BL21(DE3)	*ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	90
C. glutamicum strains		
ATCC 13032	Wild type	American Type Culture Collection
Δ*nanR* mutant	WT *C. glutamicum* with deletion of *nanR* (*cg2936*)	This work
Plasmids		
pEKEx2	Expression vector; p*tac lacI*^{q} Km^r	91
pEKEx2-*nanR*	pEKEx2 carrying the *nanR* (*cg2936*) gene	This work
pEKEx2-*siaEFGI*	pEKEx2 carrying the genes *siaE* (*cg2937*), *siaF* (*cg2938*), *siaG* (*cg2939*), and *siaI* (*cg2940*)	This work
pK19*mobsacB*	Km^r; mobilizable *E. coli* vector for the construction of insertion and deletion mutations in *C. glutamicum* (*oriV sacB lacZ*α)	51
pK19*mobsacB*Δ*nanR*	Km^r; pK19*mobsacB* with the deletion construct for the gene *nanR* (*cg2936*)	This work
pASK_IBA3	Expression vector, *tetA* promoter, C-terminal Strep-tag II; Amp^r	IBA GmbH
pASK_IBA3_*nanR*	pASK_IBA3 carrying the *nanR* gene	This work
pEPRI	Promoter probe vector carrying the promoterless *gfp* gene; Km^r	52
pEPRI_PRnagAB_WT	pEPRI containing the *nagA* (*cg2929*) promoter fragment from *C. glutamicum* ATCC 13032	24
pEPRI_PRnagAB_M4	pEPRI containing the *nagA* (*cg2929*) promoter fragment from *C. glutamicum* M4	24
pEPRI-PR*nanA*	pEPRI containing the *nanA* (*cg2931*) promoter fragment	This work
pEPRI-PR*siaE*	pEPRI containing the *siaE* (*cg2937*) promoter fragment	This work
pEPRI-PR*nanH*	pEPRI containing the *nanH* (*cg1756*) promoter fragment	This work
pEPRI-PR*nanR*	pEPRI containing the *nanR* (*cg2936*) promoter fragment	This work

plified via PCR from genomic DNA of *C. glutamicum* ATCC 13032 using the oligonucleotide primer OE_nanR_fwd and OE_nanR_rev (see Table S2 in the supplemental material); the resulting 801-bp PCR product was then cut using BamHI and *Sbf*I and cloned into the PstI/BamHI-cut plasmid pEKEx2. For overexpression of *siaEFGI*, the genes were PCR amplified with the primers siaEFGI_fwd and siaEFGI_rev. The 5,619-bp PCR product was cloned using the PCR-generated 5′ *Sbf*I site and the 3′ blunt end into the PstI- and *Eco*IRI-cut pEKEx2, leading to pEKEx2-*siaEFGI*. The recombinant plasmids were isolated from *E. coli* DH5α, controlled by sequencing (GATC Biotech AG, Constance, Germany), and transformed into *C. glutamicum* strains.

Construction of *gfp* reporter gene fusions. In order to monitor the activity of promoters from *C. glutamicum*, transcriptional fusions with the promoterless *gfp* gene were used based on the corynebacterial promoter-probe vector pEPR1 (52). The *siaEFGI* promoter region (−207 to +45 in relation to the adenosine residue of the *siaE* ATG start codon) was amplified by PCR from genomic DNA of WT *C. glutamicum* by using primers PsiaE_for and PsiaE_rev, resulting in a 272-bp PCR product. The *nanAKE* promoter region promoter region (−179 to +29 in relation to the adenosine residue of the *nanA* ATG start codon) was amplified by PCR using primers PnanA_for and PnanA_rev, resulting in a 229-bp PCR product. The *nanR* promoter region (−278 to +59 in relation to the adenosine residue of the *nanR* ATG start codon) was amplified by PCR using primers PnanR_for and PnanR_rev, resulting in a 359-bp PCR product. The *nanH* promoter region (−136 to +179 in relation to the adenosine residue of the ATG *nanH* start codon) was amplified using primers PnanH_fwd and PnanH_rev, resulting in a 336-bp PCR product. The PCR products were digested at primer-generated restriction sites (see Table S2) and ligated into the multiple-cloning site of pEPRI in front of the *gfp* gene, resulting in the plasmids pEPRI-P*siaE*, pEPRI-P*nanA*, pEPRI-P*nanR*, and pEPRI-P*nanH*. Promoter activities of *C. glutamicum* strains carrying promoter test vectors were measured by determining the *gfp* fluorescence in relation to the OD_{600} using a Tecan M200 Infinite plate reader (Tecan, Crailsheim, Germany).

[^{14}C]Neu5Ac and [^{14}C]glucose uptake studies. *C. glutamicum* cells were grown to early exponential phase (3 h), harvested by centrifugation, washed twice with ice-cold CGXII medium, suspended to an OD_{600} of 2 with CGXII medium, and stored on ice until the measurement. Before the transport assay, cells were incubated for 3 min at 30°C; the reaction was started by addition of 1 μM to 500 μM [^{14}C]*N*-acetylneuraminic acid (specific activity, 55 mCi mmol^{-1}; American Radiolabeled Chemicals). At given time intervals (15, 30, 45, 60, and 90 s), 200-μl samples were filtered through fiberglass filters (type F; Millipore, Eschborn, Germany) and washed twice with 2.5 ml of 100 mM LiCl. The radioactivity of the samples was determined using scintillation fluid (Rotiszinth; Roth, Germany) and a scintillation counter (LS 6500; Beckmann, Krefeld, Germany). Kinetic parameters as well as standard errors were derived from nonlinear regressions according to the Michaelis-Menten equation by using Sigma Plot software. Uptake of [^{14}C]glucose was analyzed at a concentration of 100 μM as described previously (53).

NagA and NagB activity assays. Exponentially growing cells of *C. glutamicum* strains were harvested by centrifugation (10 min at 3,200 × *g* and 4°C) and washed twice with 50 mM Tris-HCl, pH 8.0. Cells were disrupted by ultrasonic treatment (UP 200S; Dr. Hielscher GmbH, Teltow, Germany) with an amplitude of 50% and a duty circle of 0.5 for 7 min. The cell suspension was centrifuged for 1 h at 4°C and 16,000 rpm. Glucosamine 6-phosphate deaminase and *N*-acetylglucosamine-6P deacetylase activities of the supernatant were determined according to the assays described by Uhde et al. (24) and Matano et al. (10), respectively.

Purification of NanR. For heterologous expression, the *nanR* gene was amplified from genomic DNA of *C. glutamicum* ATCC 13032 by PCR using the primers IBA_nanR_fwd and IBA_nanR_rev, and the resulting 792-bp PCR product was then cloned into the vector pASK-IBA3 (IBA GmbH, Göttingen, Germany) according to the supplier's manual. Expression of the *nanR-strep* gene from plasmid pASK_IBA3_*nanR* was induced by the addition of anhydrotetracycline (2 μg ml^{-1}) at an OD_{600} of 1, and cells were harvested 6 h later by centrifugation at 5,000 × *g* for 5 min. The cells were washed twice with wash buffer (100 mM Tris-HCl, pH 8.0, and 150 mm NaCl), resuspended in wash buffer, and disintegrated by ultrasonic treatment with a Branson 250 sonifier at an output control of 2.5 and a duty cycle of 25% for 1.5 min. After centrifugation at 15,000 × *g* for 20 min, the supernatant was subjected to Strep-tag purification (IBA GmbH) according to the manufacturer's protocol. For storage, glycerol was added to the purified NanR protein (final amount 10% [vol/vol]), and aliquots were then stored at −20°C until further use. Protein concentrations were determined using the Roti-Nanoquant kit (Roth) with bovine serum albumin as the standard. SDS-PAGE was performed according to the method of Laemmli (54). Loading buffer (4×) contained 8% (wt/vol) SDS, 20% (vol/vol) glycerol, 10 mM EDTA, 100 mM Tris-HCl (pH 6.8), 2% (vol/vol) β-mercaptoethanol, and 1 mg/ml of bromphenol blue. Western blot experiments for detection of the streptavidin-tagged NanR protein by using antibodies raised against Strep-tag II (IBA GmbH) were performed as described for the uptake carrier BetP (55).

EMSA. Electophoretic mobility shift assays (EMSAs) with NanR were performed as described previously (56). Briefly, various concentrations (0 to 0.3 μg) of purified NanR were mixed with 15 ng of DNA probes generated by PCR in DNA binding buffer (40 mM Tris-HCl, 10% [vol/vol] glycerol, 0.2 M KCl, 4 mM dithiothreitol [DTT] [pH 7.5]) in a total volume of 10 μl. The DNA fragments were obtained by PCR with the primers listed in Table S2 in the supplemental material using genomic DNA from *C. glutamicum* ATCC 13032 or *C. glutamicum* M4 as the template and primer combinations mentioned in the Results section and purified by gel extraction using the NucleoSpin PCR cleanup and gel extraction kit (Macherey & Nagel) according to the manufacturer′s instructions. After incubation for 20 min at 30°C, the samples were separated on 9% native polyacrylamide gels at a constant electric current of 30 mA at 4°C, stained, and photographed as described previously (56). To test for possible effectors, the protein was incubated with glucosamine-6-phosphate (50 μg), glucosamine (50 μg), Neu5Ac (up to 100 μg), GlcNAc (50 μg), GlcNAc-6P (up to 50 μg), glucose (50 μg), glucose-6-phosphate (50 μg), and ManNAc-6P (up to 50 μg) in the binding buffer for 5 min before addition of DNA fragment PnagA and incubation for another 30 min. With the exception of ManNAc-6P and GlcNAc-6P (from Carbosynth Limited), the sugars and sugar phosphates tested as effector molecules in EMSAs were purchased from Sigma-Aldrich.

Gene expression analysis. For the comparison of transcriptomes of *C. glutamicum* strains, cells growing exponentially in LB medium were harvested at an OD_{600} of 3.5. RNA purification, transcription to cDNA, fluorescent labeling, hybridization, and data analysis were performed as described previously (57–59). Only hybridization signals exceeding background noise by at least a factor of 3 were considered (GENEPIX 3.0). Normalized ratios of medians were taken to reflect relative mRNA levels. Slot blot experiments were performed as described previously (60). For hybridization, digoxigenin (DIG)-11-dUTP-labeled gene-specific antisense RNA probes against *ptsG* mRNA and the 16S rRNA were prepared from PCR products (generated with oligonucleotides listed in Table S2) carrying the T7 promoter by *in vitro* transcription (1 h at 37°C) using T7 RNA polymerase (MBI Fermentas) as described previously (61).

Computational analysis. Databank searches were carried out by using BLAST (62), protein sequences were analyzed using CLUSTAL W (63), and protein domain assignments were performed using SUPERFAMILY (64). The following NCBI-GI accession numbers for protein sequences were retrieved from the KEGG database (65): 476417323 for *B. breve* NanR, 49176329 for *E. coli* NanR, 62391489 for *C. glutamicum* NanR, 375292402 for *C. diphtheriae* NanR, 300859481 for *C. pseudotuberculosis* NanR, and 384516670 for *C. ulcerans* NanR. Discovery of motives in sets of sequences was performed using GLAM2 (66), and FIMO (67) was used for motif scanning in the *C. glutamicum* ATCC 13032 (GenBank accession number NC_006958), *C. diphtheriae* INCA402 (NC_016783), *C. ulcerans*

809 (NC_017317), and *C. pseudotuberculosis* FRC41 (NC_014329) genome sequences.

RESULTS

Utilization and uptake of the Neu5Ac are inhibited in glucose-cultivated as well as fructose-cultivated *C. glutamicum* cells. Uptake and utilization of Neu5Ac were recently reported to be inhibited in glucose-cultivated *C. glutamicum* cells (22); however, utilization of both carbon sources at the same time was hitherto not analyzed. In growth experiments in minimal medium with a mixture of 0.3% (wt/vol) glucose and 0.2% (wt/vol) Neu5Ac, consecutive utilization of the two substrates was observed for WT *C. glutamicum*. As depicted in Fig. 2A, utilization of Neu5Ac only started after 5 h of cultivation, after the initially provided glucose was completely consumed. The growth rate decreased from initially 0.35 ± 0.03 h^{-1} in the course of glucose utilization to 0.09 ± 0.02 h^{-1} when Neu5Ac was utilized. In addition, coutilization of Neu5Ac and fructose was tested. As shown in Fig. 2C, when 0.3% fructose and 0.2% Neu5Ac were used as substrates, WT *C. glutamicum* also showed consecutive utilization of these two sugars and utilization of Neu5Ac only began after 4 h of cultivation, after fructose was completely consumed. The growth rate decreased from initially 0.43 ± 0.04 h^{-1} in the course of fructose utilization to 0.09 ± 0.02 h^{-1} during Neu5Ac utilization. To analyze Neu5Ac uptake in *C. glutamicum* cells from different cultivations, transport assays with ^{14}C-labeled Neu5Ac were established. Neu5Ac uptake in Neu5Ac-cultivated cells showed simple saturation kinetics with a K_m of 10 ± 2 μM and a maximum uptake rate (V_{max}) of 9 ± 1 nmol min^{-1} mg of cells (dry mass)$^{-1}$ (see Fig. S1 in the supplemental material). To analyze the influence of precultivation on different carbon sources, transport measurements were performed at a Neu5Ac concentration of 100 μM. No uptake of ^{14}C-labeled Neu5Ac was detected in cells of WT *C. glutamicum* cultivated on glucose and fructose, whereas uptake of ^{14}C-labeled Neu5Ac proceeded at a rate of 11 ± 2 nmol min^{-1} mg of cells (dry mass)$^{-1}$ in WT *C. glutamicum* cells cultivated on Neu5Ac (Fig. 2F). To test for carbon source-dependent transcriptional regulation of the *siaEFGI* operon for the Neu5Ac transporter, a transcriptional fusion between the *siaE* promoter region and the promoterless *gfp* gene was constructed in the promoter probe vector pEPPRI and the resulting plasmid, pEPRI-PR*siaE*, was transformed into WT *C. glutamicum*. Whereas high green fluorescent protein (GFP) fluorescence was observed for WT *C. glutamicum*(pEPRI-PR*siaE*) cultivated on Neu5Ac, only residual GFP fluorescence was detected when this strain was cultivated on glucose or fructose (Fig. 3A). Taken together, these results indicate the presence of a CCR mechanism in *C. glutamicum* for the inhibition of Neu5Ac utilization in the presence of glucose and fructose.

Deletion of *cg2936* enables Neu5Ac and glucose as well as Neu5Ac and fructose coutilization in *C. glutamicum*. The genes for Neu5Ac utilization in *C. glutamicum* comprise four clustered operons, namely, *nagAB*, *nanAKE*, and *siaEFGI* (22, 23) and a fourth operon with *cg2936* encoding a putative GntR-type transcriptional regulator (Fig. 1B). Upon deletion of *cg2936*, the resulting mutant strain was able to grow without any lag phase in minimal medium with Neu5Ac as the sole source of carbon and energy (Fig. 2E). As expected (22), a lag phase of about 5 to 6 h was observed for the parental strain (Fig. 2E). Furthermore, transport assays with ^{14}C-labeled Neu5Ac showed high transport activities, 23 ± 2 nmol min^{-1} mg of cells (dry mass)$^{-1}$, 19 ± 3 nmol min^{-1} mg of cells (dry mass)$^{-1}$, and 26 ± 2 nmol min^{-1} mg of cells (dry mass)$^{-1}$, for cells of the *cg2936*-deficient strain cultivated on glucose, fructose, and Neu5Ac, respectively (Fig. 2F). Subsequently, the *siaE* promoter activity was assayed in WT *C. glutamicum* and in the *cg2936* deletion mutant using the promoter test plasmid pEPRI-PR*siaE*. Independent of the carbon source used for cultivation, higher GFP fluorescence was observed for the *cg2936* deletion mutant than for WT *C. glutamicum*(pEPRI-PR*siaE*) (Fig. 3A). Taken together, these data indicate that the *cg2936* gene encodes a transcriptional regulator for the genes for Neu5Ac transport, and we therefore refer to it as *nanR*. Accordingly, the *cg2936* mutant was designated *C. glutamicum* Δ*nanR*.

When *C. glutamicum* Δ*nanR* was cultivated in minimal medium with glucose plus Neu5Ac as sources of carbon and energy, growth proceeded at a rate of 0.45 ± 0.02 h^{-1} and the two substrates were consumed in parallel (Fig. 2B). However, in these cultivations the glucose consumption rate of the Δ*nanR* mutant was lowered to 3.6 ± 0.2 mmol of C g of cells (dry mass)$^{-1}$ · h^{-1}. For the parental strain, WT *C. glutamicum*, a glucose consumption rate of 10.3 ± 0.6 mmol of C g of cells (dry mass)$^{-1}$ · h^{-1} had been determined when the strain was cultivated on glucose as the sole substrate (15) as well as on glucose plus Neu5Ac (Fig. 2A). In addition, growth of the Δ*nanR* mutant was also slowed down in cultivations with glucose as the sole carbon source compared to that of WT *C. glutamicum* (growth rates of 0.11 ± 0.02 h^{-1} and 0.35 ± 0.03 h^{-1} were determined, respectively [see Fig. S2A in the supplemental material]). These results indicate the presence of a limitation in glucose catabolism in *C. glutamicum* Δ*nanR*. Upon plasmid-encoded expression of *nanR* in *C. glutamicum* Δ*nanR*(pEKEX2-*nanR*), growth with glucose as the sole substrate was restored to a rate of 0.27 ± 0.03 h^{-1} (see Fig. S2A). Growth of the empty-vector control strain *C. glutamicum* Δ*nanR*(pEKEX2) on glucose remained slow. Deletion of *nanR* did not negatively affect growth on the glycolytic substrates maltose, fructose, and sucrose (data not shown). The metabolic pathways for glucose, maltose, and fructose in *C. glutamicum* differ only in their initial steps until glycolytic intermediates are formed (7). For glucose utilization, just the first two steps, namely, uptake and phosphorylation to glucose-6-phosphate, are specific, which both are brought about by the *ptsG*-encoded EII^{Glc} of the PTS (68). However, Northern blot analyses and uptake experiments with ^{14}C-labeled glucose showed that neither *ptsG* transcript levels (see Fig. S2C) nor activity of the glucose uptake system (see Fig. S2B) is reduced in *C. glutamicum* Δ*nanR* compared to that in WT *C. glutamicum*. Thus, the reason for the poor growth of *C. glutamicum* Δ*nanR* in minimal medium with glucose and the decreased glucose consumption rate in cultivations of this strain with glucose plus Neu5Ac remains elusive. Although glucose utilization was slightly slower in *C. glutamicum* Δ*nanR*, we conclude that deletion of *nanR* enables coutilization of glucose and Neu5Ac, and NanR probably acts as a transcriptional repressor of the *siaEFGI* operon coding for the Neu5Ac transporter.

When the NanR-deficient strain *C. glutamicum* Δ*nanR* was cultivated in minimal medium with fructose plus Neu5Ac, growth proceeded at a rate of 0.50 ± 0.04 h^{-1} and the two carbon sources were coutilized. A fructose consumption rate of 10 ± 1 mmol of C g of cells (dry mass)$^{-1}$ · h^{-1} was determined for *C. glutamicum* Δ*nanR* in cultivations with fructose plus Neu5Ac. Nearly identical fructose consumption rates of 11 ± 1 mmol of C g of cells (dry mass)$^{-1}$ · h^{-1} and 11 ± 1 mmol of C g of cells (dry mass)$^{-1}$ · h^{-1}

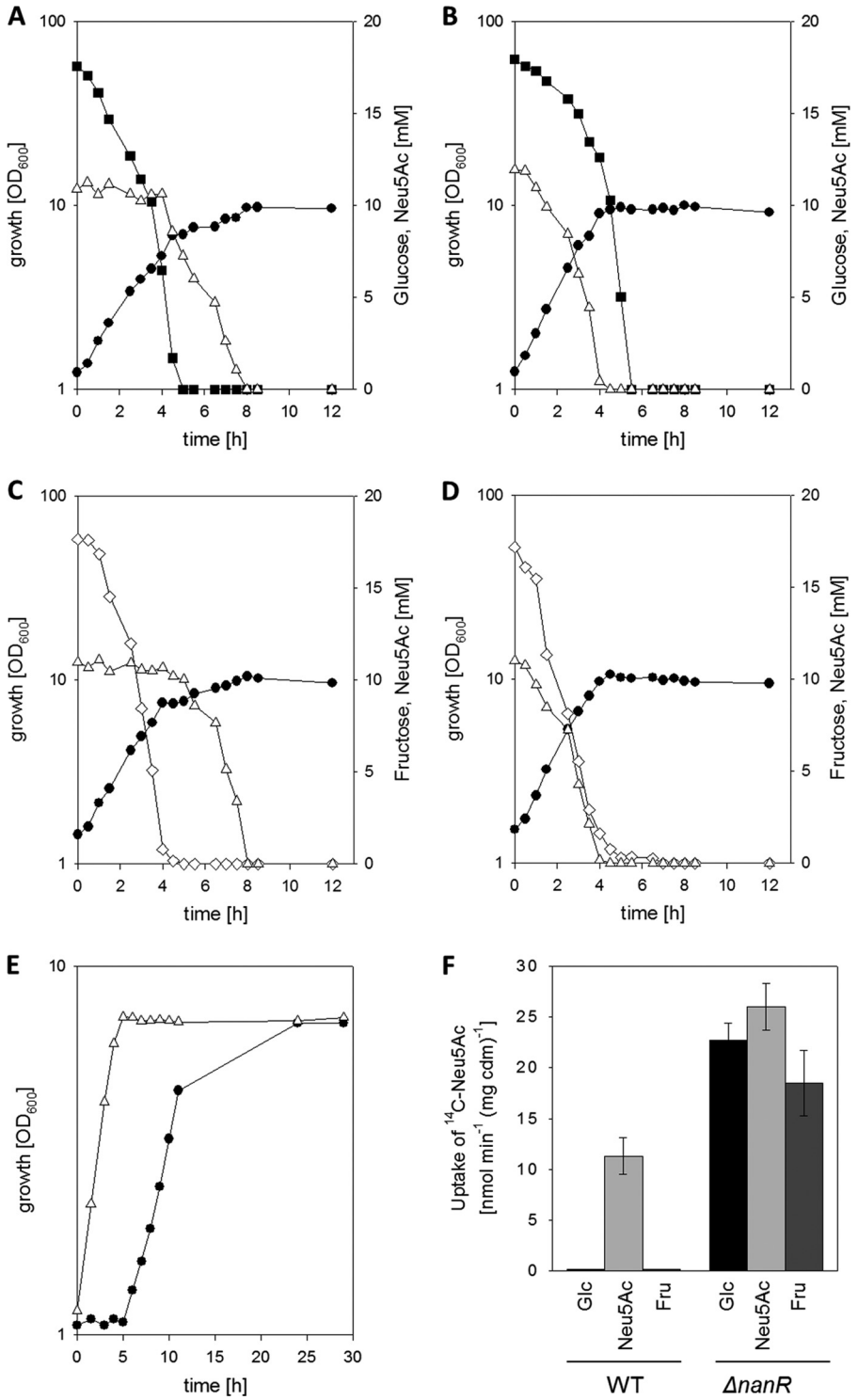

FIG 2 (A to D) Growth and substrate consumption in minimal medium with 0.3% (wt/vol) glucose plus 0.2% (wt/vol) Neu5Ac (A and B) or with 0.3% (wt/vol) fructose plus 0.2% (wt/vol) Neu5Ac (C and D) of WT *C. glutamicum* (A and C) and *C. glutamicum* Δ*nanR* (B and D). Solid circles, growth; open triangles, Neu5Ac; solid squares, glucose; open diamonds, fructose. (E) Growth of WT *C. glutamicum* (solid circles) and *C. glutamicum* Δ*nanR* (open triangles) in minimal medium with 0.2% (wt/vol) Neu5Ac. Three independent cultivations were performed; data from one representative experiment are shown. Results of all of the cultivations were comparable. (F) Rates of [^{14}C]Neu5Ac uptake of WT and Δ*nanR C. glutamicum* cells cultivated in minimal medium with different substrates. Data represent mean values and standard deviations of three independent measurements from two independent cultivations.

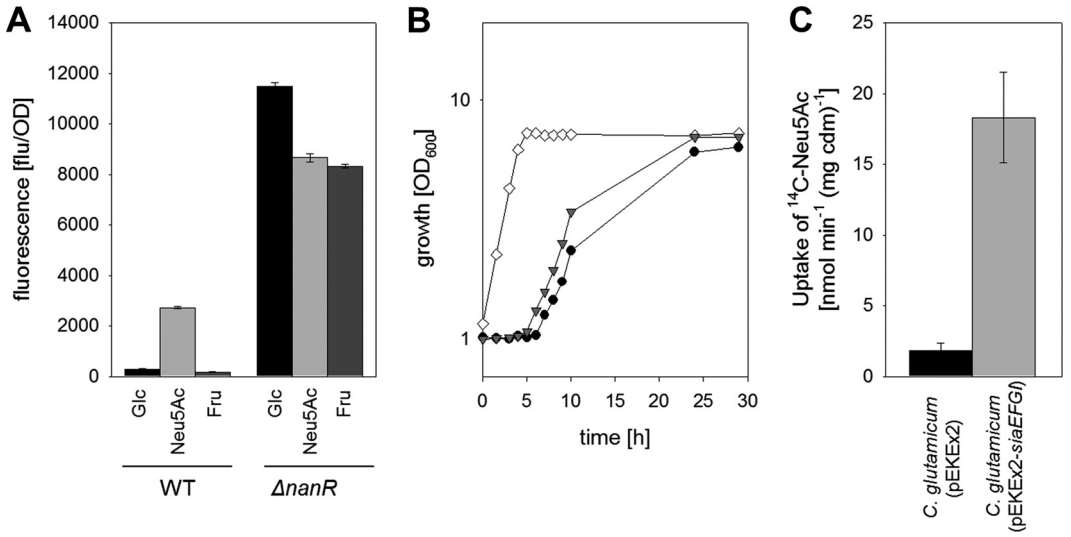

FIG 3 (A) Analyses of *siaE* promoter activity, measured as relative fluorescence of the GFP reporter upon expression of the *gfp* gene under the control of the *siaE* promoter in *C. glutamicum*(pEPRI-PR*siaE*) and *C. glutamicum* Δ*nanR*(pEPRI-PR*siaE*) cells cultivated for 8 h in minimal medium plus glucose, Neu5Ac, or fructose. Data represent mean values and standard deviations of two independent measurements each from three independent cultivations. (B) Growth of *C. glutamicum* Δ*nanR* (open diamonds), *C. glutamicum*(pEKEx2) (solid circles), and *C. glutamicum* (pEKEx2-*siaEFGI*) (gray triangles) in minimal medium with 0.2% (wt/vol) Neu5Ac. (C) Rates of [^{14}C]Neu5Ac uptake of *C. glutamicum*(pEKEx2) and *C. glutamicum*(pEKEx2-*siaEFGI*) cells cultivated in minimal medium with 0.3% (wt/vol) fructose. Data represent mean values and standard deviations of three independent measurements from two independent cultivations.

were determined for the *C. glutamicum* Δ*nanR* mutant and WT in cultivations on fructose, respectively. Thus, it can be concluded that deletion of *nanR* enables the efficient coutilization of fructose and Neu5Ac in *C. glutamicum* and does not, in contrast to the situation with glucose, negatively affect fructose metabolism.

Identification of the *C. glutamicum* NanR regulon. Transcription of the *siaEFGI* genes for Neu5Ac uptake is repressed by NanR in the presence of both glucose and fructose. Plasmid-encoded overexpression of *siaEFGI* indeed caused significantly increased Neu5Ac transport in fructose-cultivated cells of the strain *C. glutamicum*(pEKEx2-*siaEFGI*), whereas only residual Neu5Ac transport activity was measured for the empty-vector control strain *C. glutamicum*(pEKEx2) when cultivated on fructose (Fig. 3C). Despite the presence of high Neu5Ac transport activity, growth of *C. glutamicum*(pEKEx2-*siaEFGI*) on Neu5Ac after precultivation on fructose started only after a prolonged lag phase, as was also observed for *C. glutamicum*(pEKEx2) (Fig. 3B). In contrast, no lag phase was observed for the NanR-deficient mutant strain *C. glutamicum* Δ*nanR* under these conditions (Fig. 3B). This observation suggested that regulation by NanR might pertain to further genes for Neu5Ac utilization besides the Neu5Ac transporter *siaEFGI* operon. To identify the complete NanR regulon, the transcriptome of the mutant *C. glutamicum* Δ*nanR* was compared to that of the *C. glutamicum* WT by DNA microarray analyses during exponential growth in LB medium. Table S3 in the supplemental material lists genes that showed statistically significant ($P < 0.05$) expression changes by at least a factor of 4. It is noteworthy that deletion of *nanR* substantially increased expression of a small group of genes localized in proximity to *nanR* itself, i.e., 10-fold to several hundredfold. These genes, *nagA*, *nagB*, *nanA*, *nanK*, *nanE*, and, as expected, *siaE*, *siaF*, *siaG*, and *siaI*, are involved in Neu5Ac utilization and are derepressed in the absence of NanR. Moreover, expression of the propionate utilization operons *prpDBC* and *prpD2B2C2*, located elsewhere on the chromosome, increased up to 14-fold.

To further analyze transcriptional control of the *nanAKE* and *nagAB* operons as well as *nanR* itself, reporter plasmids which carry transcriptional fusions between the promoter regions and the promoterless *gfp* gene in the test vector pEPRI were constructed and transformed into WT and Δ*nanR C. glutamicum*. Analyses of the GFP fluorescence in samples of the resulting strains from cultivations on fructose showed a higher activity of all tested promoters in the NanR-deficient strain than in the WT (Fig. 4). These data confirm that NanR acts as a transcriptional repressor of the *siaEFGI*, *nanAKE*, and *nagAB* operons and additionally of its own gene, *nanR*.

Deletion of *nanR* enables efficient utilization of glucosamine. The increased expression levels of *nagA* and *nagB* in the absence of NanR are reflected by the specific activities of the encoded enzymes (see Table S4 in the supplemental material). The specific activities of NagA and NagB were about 20-fold and 40-fold higher, respectively, in *C. glutamicum* Δ*nanR* than in WT *C. glutamicum*. As increased expression of *nagB* was shown to be required for efficient utilization of glucosamine as the substrate (24), growth of *C. glutamicum* Δ*nanR* in minimal medium with glucosamine as the sole carbon source was tested. As depicted in Fig. S4A in the supplemental material, indeed good growth on glucosamine was observed for *C. glutamicum* Δ*nanR* (growth rate of 0.17 ± 0.03 h^{-1}), whereas, as expected, only slow growth, with a rate of 0.08 ± 0.02 h^{-1}, was observed for WT *C. glutamicum* under the same conditions. Ectopic expression of *nanR* in *C. glutamicum* Δ*nanR* using the plasmid pEKEx2-*nanR* resulted in slow growth of *C. glutamicum* Δ*nanR*(pEKEX2-*nanR*) on glucosamine, whereas the empty-vector control strain *C. glutamicum* Δ*nanR*(pEKEX2) grew well in the same medium (see Fig. S4B). Taken together, these results confirm that NanR also represses

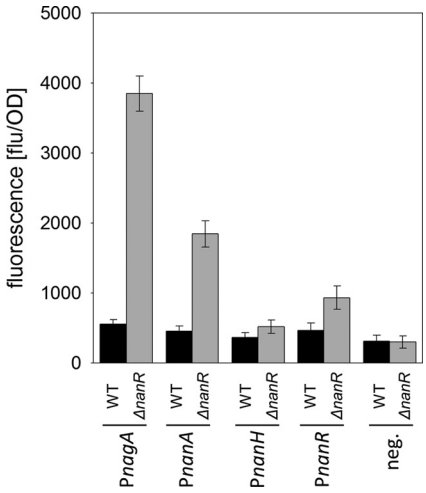

FIG 4 Analyses of *nanA*, *nagA*, *nanH*, and *nanR* promoter activities in WT and Δ*nanR C. glutamicum* strains cultivated for 8 h in minimal medium with 0.3% (wt/vol) fructose, expressed as relative fluorescence of the GFP reporter upon expression of the *gfp* gene under the control of the *nanA*, *nagA*, *nanH*, or *nanR* promoter present in the plasmid pEPRI-PR*nanA*, pEPRI-PR*nagAB*, pEPRI-PR*nanH*, or pEPRI-PR*nanH*, respectively. As a control, relative fluorescence was measured for cells of *C. glutamicum*(pEPRI) and *C. glutamicum* Δ*nanR*(pEPRI) carrying the empty vector pEPRI. Data represent mean values and standard deviations of two independent measurements each from three independent cultivations.

transcription of the *nagAB* operon and thus of glucosamine utilization. Moreover, the good growth of *C. glutamicum* Δ*nanR* on glucosamine also shows that the *ptsG*-encoded EII^{Glc} is present and active in NanR-deficient *C. glutamicum* strains, as uptake and phosphorylation of glucosamine are exclusively brought about by EII^{Glc} (24).

Binding of purified Strep-tagged NanR protein to promoter/operator regions and identification of NanR-binding sites. To test for the binding of NanR to the *siaEFGI*, *nagAB*, and *nanAKE* promoter regions, we assayed the binding of purified NanR to promoter regions *in vitro*. For this purpose, NanR was synthesized as a Strep-tagged fusion protein in *E. coli* BL21(DE3) and purified to apparent homogeneity by affinity chromatography as described in Materials and Methods. For EMSAs, different amounts of purified NanR protein were incubated with DNA fragments and separated on 10% native polyacrylamide gels.

First, binding of NanR to the *nagA* and *nanA* intergenic region was tested using the 433-bp probe PnagA covering the region 121 bp downstream of the *nanA* ATG start codon and 98 bp downstream of the *nagA* ATG start codon, which was generated by PCR using the primers nagA-for and nagA-rev and WT *C. glutamicum* DNA as the template (Fig. 5A; see also Fig. S5 in the supplemental material). As shown in Fig. 5B, NanR indeed bound to the probe PnagA, which covers the *nagA* and *nanA* intergenic region. A complete mobility shift was observed when 0.2 μg of purified NanR protein was added to the probe (Fig. 5B, lane 3), which corresponds to a 120-fold molar excess. In *C. glutamicum* M4, a T-to-C point mutation within the *nagA* promoter region leads to high, constitutive expression of the *nagAB* operon (24). The probe PnagA-M4 was generated using the primers nagA-for and nagA-rev and DNA isolated from *C. glutamicum* M4 as the template and, thus, carries the T-to-C point mutation. No shift of the probe PnagA-M4 was observed in EMSAs using 0.1 to 0.3 μg of purified NanR (Fig. 5C). Also, when concentrations up to 2 μg of purified NanR were used (corresponding to a 1,200-fold molar excess), no shift upon adding the probe PnagA-M4 was observed. These data show that the exchanged nucleotide residue within the *nanA-nagR* intergenic region is required for binding of the repressor NanR. In addition, binding of purified NanR to the probes PnagA-A and Pnag-B, which carry truncated versions of the *nagA-nanA* intergenic region, was tested (Fig. 5A). In EMSAs, binding of NanR to the probe PnagA-A was observed, whereas no binding of NanR to the probe PnagA-B lacking the region 58 bp upstream of the *nagA* start codon was detected (Fig. 5D).

Since NanR also represses transcription of *siaEFGI* and *nanR* in addition to *nagAB* and *nanAKE*, binding of purified NanR to the *siaE-nanR* intergenic region was analyzed in EMSAs using the 338-bp probe PnanR, which covers the region 30 bp upstream of the *siaE* ATG start codon and 56 bp downstream of the *nanR* ATG start codon (Fig. 6A; see also Fig. S6 in the supplemental material). A complete shift of the probe PnanR was observed when 0.2 μg of purified NanR was added (Fig. 6B), which corresponds to a 100-fold molar excess. To identify the NanR-binding site, the nucleotide sequences of the intergenic regions of *nagA-nanA* and *siaE-nanR* were compared using the program GLAM2. By this means the nucleotide sequences ACGTATGATGTCTTATGTCTA CGGA within the *nagA-nanA* intergenic region and ACGTCTGA TGTCTGATGTATATTGA within the *siaE-nanR* were found, which are identical in 20 of 25 positions. Actually, the T-to-C mutation in the *nagA* promoter of *C. glutamicum* M4 is located within this 25-bp nucleotide sequence, and moreover, the nucleotide sequence is not present in the truncated probe PnagA-B, which was not bound by NanR. When binding of NanR to truncated versions of the *siaE-nanR* intergenic regions was tested, no shifts were observed for the probes PnanR-B and PnanR-C (Fig. 6B), which lack the ACGTCTGATGTCTGATGTATATTGA nucleotide sequence.

Based on the results from the EMSAs, the preliminary consensus motif ACGT[AC]TGATGTCT[TG]ATGT[AC]TA[CT][TG] GA for binding of NanR to the promoters of *siaE*, *nanR*, *nagA*, and *nanA* was determined. This consensus motif was then used to search within the *C. glutamicum* genome for further putative NanR-binding sites using the software FIMO. By this means, a further nucleotide sequence with a *P* value below 1×10^{-6} was identified upstream of *cg1756*, which is annotated as *nanH* and putatively encodes a secreted sialidase (23). The identified nucleotide sequence AAG**CAT**GATGTCAGATGTCTAATTG overlaps the ATG start codon of *cg1756* (indicated in bold). In EMSAs with purified NanR, a shift of the probe PnanH covering the promoter region of *nanH* was observed, whereas no shift of the control probe Pcont was detected at identical NanR concentrations (Fig. 6C).

To test for NanR-dependent transcriptional control of *nanH*, the promoter probe plasmid pEPRI-P*nanH*, which carries a transcriptional fusion between the *nanH* promoter region and the promoterless *gfp* gene, was constructed. Only residual GFP fluorescence was detected for WT *C. glutamicum*(pEPRI-PnanH) cultivated on fructose. GFP fluorescence was only slightly higher, not significantly increased, in the corresponding NanR-deficient strain *C. glutamicum* Δ*nanR*(pEPRI-P*nanH*) (Fig. 4). These results of the reporter gene experiments show that *nanH* possesses a weak promoter whose activity is only slightly affected by NanR.

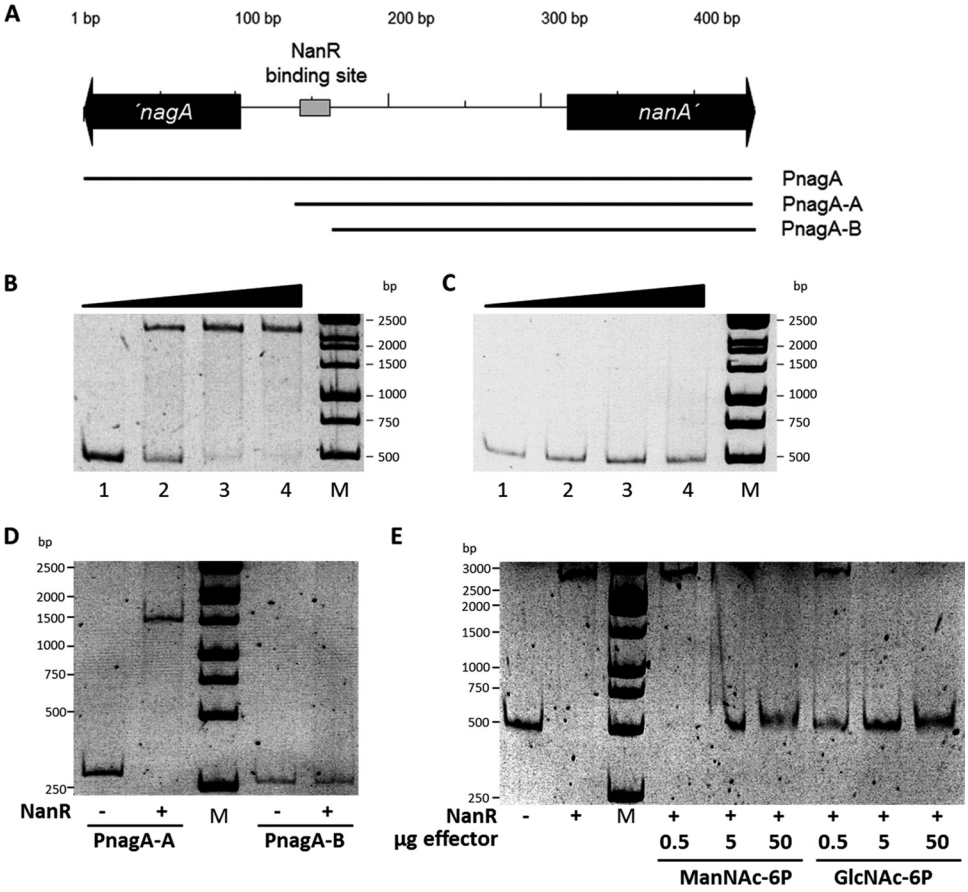

FIG 5 Electrophoretic mobility shift assays with NanR and probes covering the *nanA-nagA* intergenic region. DNA probes containing the *nagA-nanA* intergenic region were incubated with various concentrations of NanR. (A) Schematic illustration of the intergenic region of the *C. glutamicum nagA* and *nanA* genes, probes used for EMSAs, and localization of NanR-binding site. (B and C) Representative EMSAs using NanR (0, 0.1, 0.2, and 0.3 μg) with 10 ng of the probes PnagA (B) and PnagA (C). (D) EMSAs using 0.3 μg of NanR (indicated by plus signs) with 10 ng of the probes PnagA-A or PnagA-B. (E) Identification of GlcNAc-6P and ManNAc-6P as NanR effector molecules. Shown are results of EMSAs using NanR (0.2 μg; 0.7 μM) with the Probe PnagA (10 ng; 3.7 nM) and 0.5, 5, or 50 μg of GlcNAc-6P (0.17 mM, 1.66 mM, and 16.6 mM) or 0.5, 5, or 50 μg of ManNAc-6P (0.14 mM, 1.44 mM, and 14.49 mM). Lanes M, molecular size markers.

The low transcription might explain why no change of *nanH* transcript amounts was detected in microarray analyses of differential transcription in WT and Δ*nanR C. glutamicum*. These results demonstrate that NanR controls transcription of *siaEFGI*, *nagAB*, *nanAKE*, and *nanR* by binding within the promoter regions and at least binds also to the *nanH* promoter. Analyses of the promoter regions of aforementioned operons using GLAM2 led to the determination of the 21-bp consensus motif A[AC]G[CT][AC]TG ATGTC[AT][TG]ATGT[AC]TA for NanR binding to its target promoters.

GlcNAc-6P and ManNac-6P inhibit binding of NanR to its cognate promoter regions. Analyses of the NanR amino acid sequence using the program SUPERFAMILY suggested that this protein belongs to the GntR family of DNA binding proteins and possesses an N-terminal winged-helix DNA binding domain and a C-terminal GntR ligand-binding domain. Based on this C-terminal domain, NanR can be placed within the FadR branch of the large GntR family of transcriptional regulators (69). Moreover, NanR from *C. glutamicum* is highly similar to the well-characterized GntR-type (FadR subfamily) transcriptional regulators of sialic acid metabolism from *B. breve* (33% identity; E value, 5e−33) and *E. coli* (25% identity; E value, 5e−9), which both depend on Neu5Ac as the inducer (45, 46). Considering the observed induction of the genes for Neu5Ac utilization upon cultivation with Neu5Ac, the similar metabolic pathways present in *C. glutamicum*, *B. breve*, and *E. coli*, and the alignment of amino acid sequences of the NanR proteins from these three species, which demonstrates well-conserved C termini (see Fig. S7 in the supplemental material), we hypothesized that also *C. glutamicum* NanR repression might be relieved by binding of Neu5Ac. To test this hypothesis, in EMSAs with 0.3 μg of NanR, which ensures a complete shift of the probe PnagA, increasing concentrations of Neu5Ac (up to 30 mM) were added to the reaction mixture. Analysis of the gels demonstrated that NanR binding to the probe PnagA was not altered in the presence Neu5Ac (see Fig. S8C). Besides Neu5Ac, effects on NanR binding to PnagA of the metabolites GlcNAc, glucosamine, glucose-6-phosphate, glucose, GlcNAc-6P, glucosamine-6-phosphate, and ManNAc-6P were also tested in EMSAs. Only the presence of GlcNAc-6P and ManNAc-6P inhibited binding of NanR to the probe (Fig. 5E). Specifically, addition of 0.6 mM GlcNAc-6P or 2.1 mM ManNAc-6P to the reaction mixture inhibited the shift of the probe PnagA by

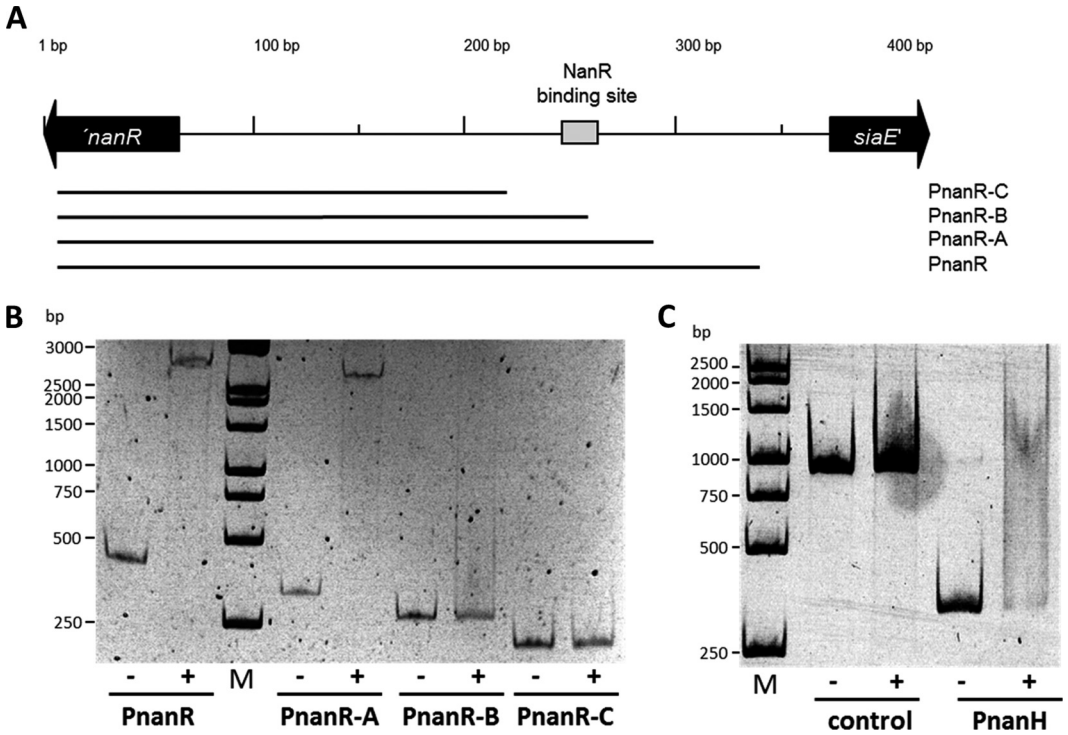

FIG 6 Electrophoretic mobility shift assays with NanR and probes covering the *nanR-siaE* intergenic region. DNA probes containing the *nanR-siaE* intergenic region were incubated with various concentrations of NanR. (A) Schematic illustration of the intergenic region of the *C. glutamicum nanA* and *siaE* genes, probes used for EMSAs, and localization of the NanR-binding site. (B) Representative EMSAs using 0.2 μg of NanR with 10 ng of each of the probes PnanR, PnanR-A, PnanR-B, and PnanR-C. (C) Representative EMSA using 0.2 μg NanR with 10 ng of the probe PnanH, which covers the *nanH* promoter. As a negative control, 10 ng of a PCR product of the *nanR* gene generated with the primers OE_nanR_fwd and OE_nanR_rev was used.

NanR in EMSAs (see Fig. S8). These results suggest that conditions leading to high concentrations of either one of the two intermediates of Neu5Ac degradation, GlcNAc-6P and ManNAc-6P, in *C. glutamicum* will induce sialic acid catabolism by inhibiting binding of the GntR-type transcriptional regulator NanR to promoters of its target genes.

Transcription of *siaEFGI*, *nagAB*, *nanAKE*, and *nanR* is subject to catabolite repression. In addition to the consecutive utilization of two carbon sources, a further characteristic of CCR is the repressed transcription of genes for the utilization of the nonpreferred substrate when both substrates are present in the culture broth. Analyses of kinetics of *siaE* promoter activities in WT *C. glutamicum*(pEPRI-PR*siaE*) showed a delayed increase of GFP fluorescence in cultivations with Neu5Ac plus fructose compared to cultivations with Neu5Ac as the sole substrate (Fig. 7A and B). In cultivations of WT *C. glutamicum*(pEPRI-PR*siaE*) with 0.2% (wt/vol) Neu5Ac plus 0.25% (wt/vol) fructose, GFP fluorescence started to increase after 6 h of cultivation, when the initially provided fructose was completely consumed (Fig. 7B). Analyses of *siaE*, *nagA*, *nanA*, and *nanR* promoter activities after 5 h of cultivation in minimal medium with 0.2% (wt/vol) Neu5Ac plus 0.25% (wt/vol) fructose or in minimal medium with 0.2% (wt/vol) Neu5Ac showed that transcription of all tested promoters was low when fructose was present in the medium (Fig. 7C). Furthermore, when glucose was added to cells of WT *C. glutamicum*(pEPRI-PRnagAB_WT) precultivated with Neu5Ac as the sole substrate, GFP fluorescence transiently deceased (see Fig. S9 in the supplemental material). This finding indicates that transcription from the *nagA* promoter stopped after glucose addition, which fits within the concept of CCR by presence of fructose or glucose. To analyze the role of NanR for CCR of Neu5Ac catabolism genes by fructose, *siaE*, *nagA*, *nanA*, and *nanR* promoter activities were analyzed in the NanR-deficient strain *C. glutamicum* Δ*nanR* after 5 h of cultivation in minimal medium with 0.2% (wt/vol) Neu5Ac plus 0.4% (wt/vol) fructose or in minimal medium with 0.2% (wt/vol) Neu5Ac. Whereas no significant reduction of *siaE* promoter activity by the additional presence of fructose in the culture broth was observed, *nagA*, *nanA*, and *nanR* promoter activities were significantly lower in cultivations of *C. glutamicum* Δ*nanR* in medium with Neu5Ac plus fructose than in cultivations with Neu5Ac as the sole substrate (Fig. 7C). These results show that despite the lack of NanR, the presence of fructose still leads to a partial repression of the transcription of the operons *nagAB*, *nanAKE*, and *nanR*.

The presence of fructose and glucose, two sugars taken up via the PTS in *C. glutamicum* (7), prohibited induction of the genes for Neu5Ac utilization. In *E. coli*, activity of the PTS inhibits activity of several other transporters for sugar uptake, e.g., the lactose permease LacY and the ABC transporter for maltose uptake $MalEFGK_2$, which leads to inducer exclusion (70, 71). To analyze the effects of the presence of glucose and fructose on Neu5Ac transport by SiaEFGI, uptake of ^{14}C-labeled Neu5Ac was analyzed in cells of *C. glutamicum*(pEKEx2-*siaEFGI*) incubated for 3 min at 30°C with 500 μM either glucose or fructose before addition of the ^{14}C-labeled Neu5Ac (cells were cultivated in LB medium). Surprisingly, uptake of ^{14}C-labeled Neu5Ac was even faster in the

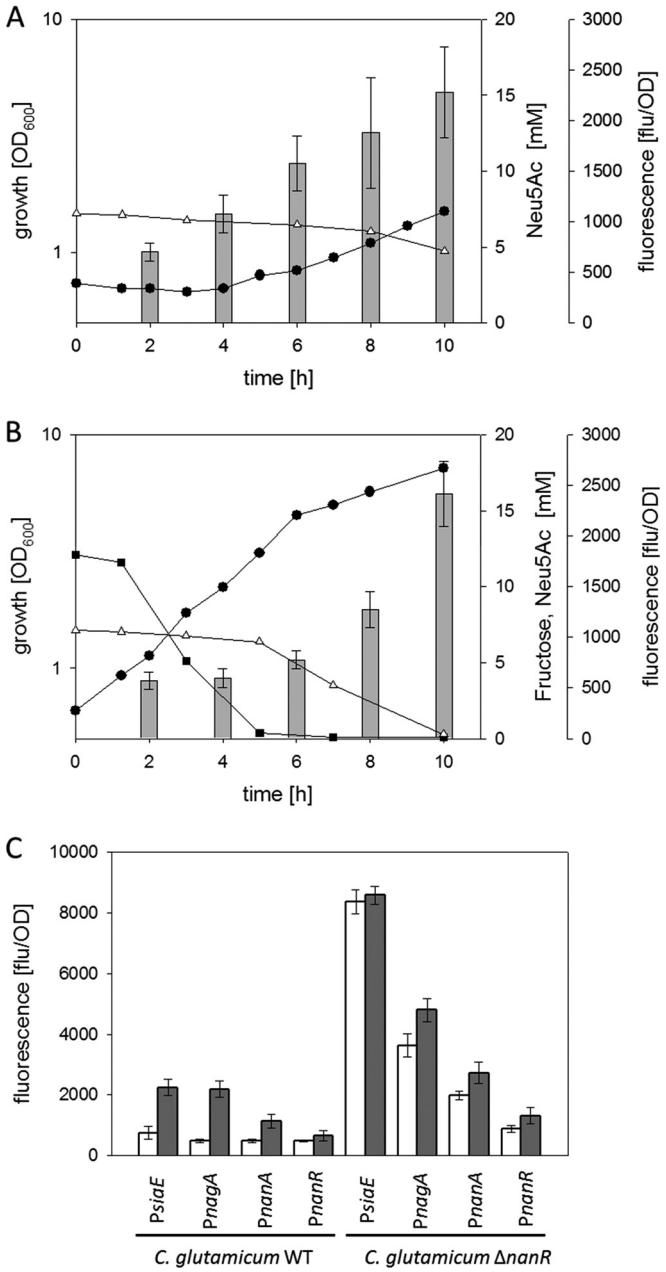

FIG 7 (A and B) Growth (black circles), substrate concentrations (white triangles, Neu5Ac; black squares, fructose), and relative fluorescence of the GFP reporter (gray bars) in cultivations of *C. glutamicum* WT (pEPRI-PR*siaE*) on minimal medium with 0.2% (wt/vol) Neu5Ac (A) or 0.2% (wt/vol) Neu5Ac plus 0.25% (wt/vol) fructose (B) as substrates. (C) Analyses of *siaE*, *nanA*, *nagA*, and *nanR* promoter activities in WT *C. glutamicum* and *C. glutamicum* Δ*nanR* strains after 5 h of cultivation in minimal medium with 0.2% (wt/vol) Neu5Ac plus 0.4% (wt/vol) fructose (white boxes) or in minimal medium with 0.2% (wt/vol) Neu5Ac (gray boxes). Three independent cultivations were performed. Growth data from one representative experiment are shown; results of all of the cultivations were comparable. GFP fluorescence data represent mean values and standard deviations of two independent measurements each from three independent cultivations.

presence of glucose (27.9 ± 3.4 nmol min^{-1} mg of cells [dry mass]$^{-1}$) or fructose (26.4 ± 2.8 nmol min^{-1} mg of cells [dry mass]$^{-1}$) than ^{14}C-labeled Neu5Ac uptake in cells preincubated for 3 min in the absence of additional sugars (18.2 ± 3.2 nmol min^{-1} mg of cells [dry mass]$^{-1}$). These results clearly show that inducer exclusion by glucose and fructose is not involved in the CCR of Neu5Ac catabolism genes in *C. glutamicum*.

Taken together, these findings indicate that besides NanR a second, hitherto-unidentified, NanR-independent regulatory mechanism is involved in the observed CCR of the genes for Neu5Ac catabolism in the presence of glucose and fructose.

DISCUSSION

In this study, we have shown that in *C. glutamicum*, the *cg2939*-encoded regulator protein NanR controls as a repressor transcription of the operons *siaEFGI*, *nagAB*, and *nanAKE* required for uptake and metabolization of Neu5Ac. In contrast to the GntR-type regulators of genes for Neu5Ac utilization from *E. coli* and *B. breve*, which both depend on the presence of Neu5Ac (45, 46) as an effector, binding of *C. glutamicum* NanR to its target promoters was relieved in the presence of 0.6 mM GlcNAc-6P and at slightly higher concentrations of ManNAc-6P. Control by intermediates of Neu5Ac degradation has also been reported for the RipR-type regulators of Neu5Ac metabolism from *V. vulnificus*, *S. pneumoniae*, *S. aureus*, and *H. influenzae*, which are activated in the presence of ManNAc-6P, ManNAc, and GlcN-6P, respectively (39, 42, 72, 73) (see Table S1 in the supplemental material). Thus, NanR of *C. glutamicum* is the first transcriptional regulator of sialic acid metabolism depending on the inducer GlcNAc-6P and the first of the GntR-type regulators independent of Neu5Ac (see Table S1). The use of GlcNAc-6P as a signal molecule for the control of Neu5Ac degradation in *C. glutamicum* is plausible, as this organism cannot utilize GlcNAc as the substrate for growth (10). In *E. coli*, for which the pathway for sialic acid utilization is regarded as an addition to the pathway for the utilization of the amino sugar GlcNAc (74), distinct regulatory circuits exist for differential control of the genes for Neu5Ac utilization and genes for GlcNAc utilization. Transcription of the genes *nagE*, *nagA*, and *nagB* for GlcNAc utilization is controlled in *E. coli* by the ROK-type transcriptional regulator NagC, which is displaced from its DNA targets by interacting with GlcNAc-6P (75). When Neu5Ac is metabolized in *E. coli*, GlcNAc-6P formed by the consecutive action of NanA, NanK, and NanE induces *nagAB* transcription via NagC and, thus, synthesis of the enzymes NagA and NagB, required for the last two steps of Neu5Ac degradation (74, 76). Similarly, GlcNAc-6P derived from Neu5Ac induces the NanR regulon in *C. glutamicum*. In the related *C. glycinophilum*, GlcNAc uptake and phosphorylation are accomplished by the *nagE*-encoded EII permease of the PTS (10). Alongside heterologous expression of *nagE* from *C. glycinophilum*, plasmid-encoded overexpression of endogenous *nagA* and *nagB* was shown to be necessary for efficient GlcNAc utilization in *C. glutamicum* (10). Moreover, plasmid-encoded overexpression of *nagE* from *C. glycinophilum* in *C. glutamicum* M4 did not lead to efficient growth on GlcNAc (10), despite the fact that this strain possesses high levels of NagA and NagB due to a point mutation within the promoter of *nagAB* and is thus able to efficiently utilize GlcN (24). This result indicates that besides the GlcNAc-6P-dependent transcriptional regulation of the operons for Neu5Ac metabolism mediated by NanR, further, hitherto-unidentified regulatory mechanisms for the control of amino sugar metabolism in *C. glutamicum* have to exist.

To accomplish the observed sequential utilization of glucose or fructose as preferred substrates together with Neu5Ac as the less-

favorable substrate, a regulatory mechanism for CCR is required to inhibit induction of the Neu5Ac utilization genes. As transcription of Neu5Ac genes was still slightly repressed by the presence of fructose and glucose in *C. glutamicum* Δ*nanR* compared to cultivations with Neu5Ac as the sole substrate, CCR is probably controlled independently of NanR. In several bacteria, complex regulatory networks for the control of consecutive utilization of carbon sources have been described, and often the PTS is involved in the underlying signal transduction pathways (70, 71). Mechanisms underlying CCR by PTS substrates often include inducer exclusion (70, 71). When Neu5Ac uptake by the ABC transporter SiaEFGI was analyzed in *C. glutamicum*(pEKEx2-*siaEFGI*), preincubation with a 50 μM concentration of either of the preferred substrates glucose and fructose did not lead to a diminished rate for Neu5Ac uptake, as would have been expected if inducer exclusion occurs. Apart from inducer exclusion, also several other regulatory mechanisms for CCR are also controlled by PTS activity. In the Gram-positive *S. pneumoniae*, transcription of *nanA* is repressed by CcpA (34), the global regulator responsible for CCR in this organism (77). The presence of preferred substrates results in HPrK-catalyzed phosphorylation of the PTS component HPr at Ser-46, which, in turn, binds to and activates CcpA, and the resulting CcpA and P-Ser46-HPr complex then binds to DNA, which leads to repression of target promoters (71, 78). Although a serine residue is indeed present at position 46 in *C. glutamicum* HPr, it is unlikely that a CcpA-like mechanism underlies the observed repression of Neu5Ac catabolism in *C. glutamicum* in the presence of glucose or fructose, as neither a gene for nor activity of HPrK was detected in *C. glutamicum* (79, 80). In the Gram-negative bacteria *V. vulnificus* and *H. influenzae*, CCR is brought about by the cyclic AMP (cAMP) receptor protein (CRP) (37, 39, 43). The CRP orthologue GlxR has been identified in *C. glutamicum* as one of the central regulators of its metabolism, which controls more than 200 genes (81–83). However, no GlxR-binding sites have been identified within the *nagA-nanA* and *nanR-siaE* intergenic regions, nor has binding of GlxR to DNA fragments of this region been observed (81, 82, 84). Thus, it is unlikely that CCR of Neu5Ac metabolism in *C. glutamicum* is mediated by GlxR. Involvement of the PTS in the control of Neu5Ac metabolism remains to be investigated; however, the growth defect of the NanR-deficient strain on the PTS substrate glucose and the positive effects on Neu5Ac uptake indicate the presence of possible regulatory interactions.

Utilization of Neu5Ac as a substrate for growth is a common trait of a large variety of microorganisms inhabiting various niches within eukaryotic hosts and has therefore been associated with pathogenicity or at least commensal lifestyles (85). Therefore, the presence of a functional pathway for Neu5Ac utilization and distinct mechanisms for its control seem rather odd in a nonpathogenic soil bacterium such as *C. glutamicum*. Consequently, this trait of *C. glutamicum* was regarded as a remnant, possibly inherited from related pathogenic species such as *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, and *Corynebacterium pseudotuberculosis* (22). These three species indeed harbor gene clusters for Neu5Ac transport and utilization highly similar to the cluster described for *C. glutamicum* (22). Moreover, genes for putative GntR-type regulators are present within these clusters, and the gene products in fact are highly similar to NanR from *C. glutamicum* (see Fig. S10 in the supplemental material). Similar to the case with *C. glutamicum*, the genes encoding the putative Neu5Ac uptake systems are located in the genomes of *C. diphtheriae* (strain INCA 402), *C. ulcerans* (strain 809), and *C. pseudotuberculosis* (strain FRC41) adjacent to the gene for the corresponding transcriptional regulator, separated by a short intergenic region (86–88). In analyses of the genome sequences using FIMO and the consensus motif for *C. glutamicum* NanR-binding sites, we were able to identify highly conserved 21-bp motifs within the intergenic regions of these related species (see Fig. S11). The conserved genomic organization and the conserved regulator binding motifs indicate that the control of Neu5Ac metabolism by NanR in *C. glutamicum* may be rather similar to that of the related pathogenic species *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* and might thus be regarded as a model for the control of Neu5Ac utilization within the genus *Corynebacterium*.

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