

The gluconate operon *gnt* of *Bacillus subtilis* encodes its own transcriptional negative regulator

(*gntR* protein/insertional mutagenesis/*gntR1* mutation/sugar acid catabolism)

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ABSTRACT The gluconate (*gnt*) operon of *Bacillus subtilis* consists of four *gnt* genes; the second and third genes code for gluconate kinase (gluconokinase, EC 2.7.1.12) and gluconate permease, respectively. A fragment carrying the promoter of this operon (*gnt* promoter) and the first gene (*gntR*) was subcloned into a promoter probe vector (pPL603B). Repression of the expression of *cat-86* gene, encoded in the vector portion of a constructed plasmid (pgnt21), that is under the control of the *gnt* promoter was removed by gluconate. The results of deletion analysis and of insertional inactivation of the *gntR* gene cloned in pgnt21 suggested that the product of the *gntR* gene, actually synthesized as a 29-kDa protein *in vivo*, is involved in repression of the *gnt* promoter. A 4-base-pair insertional mutation within the *gntR* gene constructed *in vitro* was introduced into the *B. subtilis* chromosomal *gnt* operon by use of linkage of the 4 base pairs to *gntK10* in transformation. The introduced mutation *gntR1* caused the constitutive expression of the gluconate kinase and gluconate permease genes. S1 nuclease analysis indicated that the mRNA of this operon is synthesized in the *gntR1* strain and amounts of mRNA are not changed very much by gluconate, which acts as an inducer in the wild-type gene. These results strongly indicate that the *gntR* gene codes for a transcriptional negative regulator for the *gnt* operon.

Various microorganisms are able to grow on gluconate as the sole carbon source. After entering a cell, gluconate is phosphorylated to gluconate-6-P, which is then catabolized through the pentose cycle or Entner-Doudoroff pathway. *Bacillus subtilis* does not possess the latter pathway, so only two enzymes, gluconate permease and gluconate kinase (gluconokinase, EC 2.7.1.12), seem to be specifically involved in gluconate catabolism in this organism. Both enzymes are induced by gluconate, and their induction is repressed by carbon sources that are rapidly metabolized such as glucose (1, 2).

We have characterized and cloned the gluconate (*gnt*) operon of *B. subtilis*, which includes the structural genes for gluconate permease and gluconate kinase (3). Recently, we reported the entire nucleotide sequence of the *gnt* operon (4). Analysis of the operon sequence revealed the presence of four *gnt* genes, which were designated from the 5' end as *gntR*, *gntK*, *gntP*, and *gntZ* (see Fig. 1A). The *gntK*- and *gntP*-encoded proteins were identified as gluconate kinase and gluconate permease, respectively. But we had no idea of the functions of the *gntR*- and *gntZ*-encoded proteins. Transcription of the *gnt* operon starts from the *gnt* promoter 40 base pairs (bp) upstream of the *gntR* gene and terminates 45 bp downstream of the *gntZ* gene, resulting in a polycistronic mRNA (*gnt* mRNA), the product of the four *gnt* genes (4, 7). This operon appears to be regulated mainly at the transcrip-

tion level because *gnt* mRNA synthesis is induced by gluconate and is repressed by glucose (4, 7).

Induction of the *gnt* operon by gluconate seems to be negatively controlled through a regulator of this operon, as inferred from the fact that the *gnt* promoter in a multicopy state in a cell can titrate the negative regulator to cause high expression of the *gnt* operon even in the absence of gluconate in the medium (7). However, we did not know the chromosomal location of the structural gene for this negative regulator. In this paper we describe the surprising finding that the first gene of the *gnt* operon (*gntR*) is the structural gene for the transcriptional negative regulator; thus, the *gnt* operon of *B. subtilis* encodes its own negative regulator.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids. *B. subtilis* strain 60015 (*trpC2 metC7*) is our standard strain. Strain 1A423 (*leuA8 thr-5 argA15 recE4 r⁻ m⁻*) was obtained from the *Bacillus* Genetic Stock Center (Ohio State University, Columbus, OH). Strain 61656 (Δ *igf trpC2 metB5 leuA8 hisA1*) with a deletion of the entire *gnt* operon was isolated and genetically characterized as described previously (6, 9). The isolation and properties of strain YF160 [*gntK10* (formerly *gnt-10*) *trpC2 metC7*] affecting the gluconate kinase gene were described previously (3). A ϕ 105 derivative (ϕ 105*gnt*⁺) containing an *EcoRI* fragment (7.0 kb) that carries the entire *gnt* operon was constructed by prophage transformation as described previously (3). Promoter probe vector pPL603B was a gift from D. Rothstein. Plasmids pgnt21 and pgnt23 were obtained in the course of a search for promoters within the *Bgl* II B fragment, using pPL603B as described previously (7). Strains YF170 (Δ *igf trpC2 metB5 hisA1*), lysogenized with ϕ 105*gntK10* or ϕ 105*gntR1*, and YF176 (*gntR1 trpC2 metC7*) were constructed as described below. Strains YF169 (*trpC2 recE4*) and YF177 (*gntR1 trpC2 recE4*) were constructed from strains 60015 and YF176, respectively, by conjugation with the DNA of strain 1A423.

Preparation of DNA and Transformation. Plasmids were prepared as described by Gryczan *et al.* (10). Viral DNA was prepared by phenol treatment from phage particles purified as described previously (3).

Transformation using a competent culture of *B. subtilis* was done by the method of Shibata and Saito (11). Protoplast transformation with plasmid DNA was done as described by Chang and Cohen (12).

Construction of Deletion and Insertion Derivatives of Plasmid pgnt21. For construction of pgnt21dC1, pgnt21 (0.5 μ g) was digested with *Cla* I and ligated. pgnt21dAC2 and pgnt21iA1 were constructed by double digestion with *Asp* 718 and *Cla* I, and digestion with *Asp* 718, respectively, fill-in with the Klenow fragment of DNA polymerase I, and subsequent ligation. pgnt21dP8 was constructed as follows:

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Abbreviation: CAT, chloramphenicol acetyltransferase.
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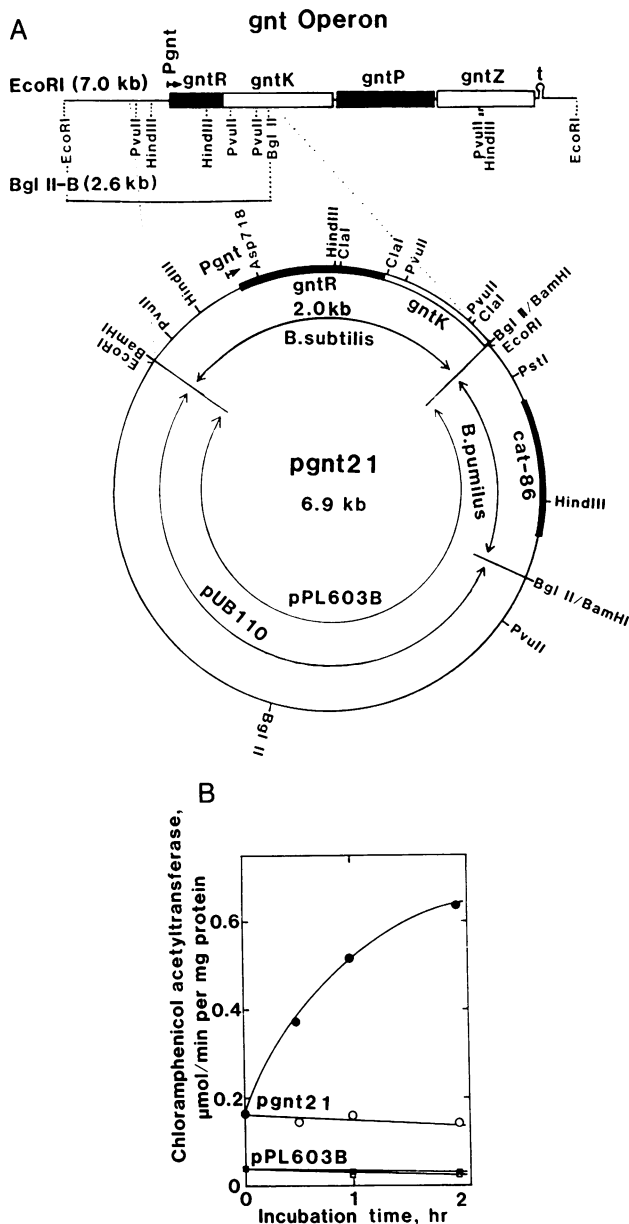


FIG. 1. (A) Structures of the *B. subtilis* *gnt* operon and plasmid *pgnt21*. The *gnt* operon consisting of four *gnt* genes is shown together with a partial restriction enzyme map of the *EcoRI* fragment of 7.0 kilobases (kb). *Pgnt* and *t* represent the *gnt* promoter and terminator. The *Bgl* II B fragment that was used for the promoter search and S1 nuclease mapping is also shown. The structure of plasmid *pgnt21* is shown together with its partial restriction enzyme map; this plasmid possesses a 2.0-kb insert at the *Bam*HI site of the promoter probe vector (pPL603B), constructed from a promoterless CAT gene of *Bacillus pumilus* (*cat-86*) and plasmid pUB110 (5). The insert carries the *gnt* promoter, the *gntR* gene, and the 5' portion of the *gntK* gene. (B) Synthesis of CAT in strain 1A423 bearing plasmid *pgnt21*. Cells bearing *pgnt21* (●, ○) or pPL603B (■, □) were grown at 37°C to $OD_{600} = 0.25$ in S6 medium (6) containing 0.5% Casamino Acids (Difco) as the sole carbon source, 50 μg each of the required amino acids per ml, kanamycin (5 μg/ml), and chloramphenicol (10 μg/ml). (The concentrations of these supplements and gluconate in S6 medium were the same throughout the experiments.) After addition of either 10 mM gluconate (●, ■) or 10 mM each of gluconate and glucose (○, □) to a culture, the cells were further incubated. Cells (10 OD_{600} units) harvested at the indicated times (0 time; no addition) were used for preparation of cell extracts as described previously (7) (one OD_{600} unit was defined as the amount of cells in 1 ml of culture that exhibited an OD_{600} nm of 1.0). Chloramphenicol acetyltransferase (CAT) was spectrophotometrically assayed as described by Shaw (8).

pgnt21 was partially digested with *Pvu* II, and the partially digested fragments were electrophoresed in low melting point agarose (1%, Bethesda Research Laboratories). After being stained with ethidium bromide, the band of a fragment shorter than the linearized plasmid was carefully excised; after the excised gel had been melted at 68°C for 30 min and treated with phenol twice, the fragment was recovered by ethanol precipitation and ligated. Each ligated fragment was transferred to protoplasts of strain 1A423 according to the method of Chang and Cohen (12). Chloramphenicol-resistant transformants were selected on a regeneration plate of DM3 (12) containing chloramphenicol (20 μg/ml) and then subjected to plasmid analysis.

Enzymes. Restriction enzymes not mentioned below, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan). *Asp* 718, *Cla* I, and calf intestine alkaline phosphatase were from Boehringer Mannheim. *Hind*III, S1 nuclease, and proteinase K were from Nippon Gene (Toyama, Japan), Sankyo (Tokyo), and Merck, respectively. All enzymes were used as recommended by the suppliers.

RESULTS

Elevated Expression of the Unrepressed *gnt* Promoter Within a Subcloned Fragment (2.0 kb). We searched for a functional promoter within the *Bgl* II B fragment (2.6 kb) from the *EcoRI* fragment (7.0 kb) that encodes the entire *gnt* operon (Fig. 1A) using promoter probe vector pPL603B containing the promoterless *cat-86* gene (5). The *Bgl* II B fragment was partially digested with *Sau*3AI to obtain a collection of sequences, and the resulting fragments were cloned into the *Bam*HI site of pPL603B. Among the 14 chloramphenicol-resistant transformants of strain 1A423 isolated, only one exhibited slightly increased chloramphenicol resistance in the presence of gluconate. Fig. 1B shows that the synthesis of CAT, which is the product of the *cat-86* gene under the control of the *gnt* promoter cloned in this transformant, escaped repression upon the addition of gluconate to the medium (the average "derepression" ratio for five experiments was 5-fold), and the derepression was under catabolite repression control (CAT synthesis in strain 1A423 bearing pPL603B was negligible). This 5-fold derepression was clearly distinguishable from those of the other 13 chloramphenicol-resistant transformants; their CAT synthesis was already highly derepressed in the absence of gluconate and was derepressed 1.1- to 1.6-fold upon gluconate addition, a typical example being strain 1A423 bearing plasmid *pgnt23* (ref. 7; Table 1).

The plasmid harbored in this transformant was designated as *pgnt21*. Its insert was analyzed by agarose and PAGE after its excision using two flanking *EcoRI* sites. As shown in Fig. 1A, this insert (2.0 kb), the longest among the 12 inserts examined, was found to carry the *gnt* promoter, the entire *gntR* gene, and nearly half of the *gntK* gene (up to the *Bgl* II site), and to be properly oriented for expression of the *cat-86* gene.

Determination of the Function of the *gntR* Product by Deletion Analysis and Insertional Inactivation. Among the inserts examined, only the 2.0-kb insert contained the *gntR* gene as well as the *gnt* promoter. Therefore, we postulated that the *gntR* protein might be involved in repression of the *gnt* promoter. To test this hypothesis, we constructed from plasmid *pgnt21* various derivative plasmids with deletions and an insert that impaired the *gntR* gene, and then we investigated CAT synthesis in strain 1A423 bearing each resultant plasmid.

Three deletion plasmids (*pgnt21dC1*, -*dAC2*, and -*dP8*) were constructed; the deletions in *pgnt21dC1* and -*dAC2* removed portions of the *gntR* gene, but the deletion in

Table 1. CAT synthesis under the control of the *gnt* promoter

Strain (plasmid)	CAT, $\mu\text{mol}/\text{min}$ per mg protein		Derepression ratio*
	- Gluco- nate	+ Gluco- nate	
1A423 (pgnt21)	0.15	0.71	4.7
1A423 (pgnt23)	1.10	1.75	1.6
1A423 (pgnt21dC1)	0.79	0.90	1.1
1A423 (pgnt21dAC2)	0.77	1.08	1.4
1A423 (pgnt21dP8)	0.12	0.68	5.7
1A423 (pgnt21iA1)	0.98	1.11	1.1
1A423 (pPL603B)	0.04	0.03	0.8

The cells were grown to $\text{OD}_{600} = 0.25$ at 37°C in S6 medium containing Casamino Acids, the required amino acids, chloramphenicol, and kanamycin. After a portion of the cells had been taken as an uninduced sample, the remaining cells were exposed to gluconate in the same medium for 2 hr with shaking. Cells (10 OD_{600} units) were lysed and then assayed as described previously (7).

*Induced enzyme activity (+ gluconate) was divided by uninduced (- gluconate) activity.

pgnt21dP8 left this gene intact (Fig. 2A). Table 1 shows that CAT synthesis in strain 1A423 bearing pgnt21dC1 and -dAC2 was high in the absence of gluconate and was derepressed only 1.1- and 1.4-fold upon gluconate addition, respectively. In contrast, synthesis of the enzyme in strain 1A423 bearing pgnt21dP8 was derepressed 5.7-fold upon gluconate addition as observed for strain 1A423 bearing pgnt21. Plasmid pgnt21iA1, with a -GTAC- insertion at a unique site of *Asp* 718 (an isoschizomer of *Kpn* I) within the *gntR* gene (Fig. 2A), was constructed through fill-in of linearized pgnt21 with the Klenow fragment of DNA polymerase I and then subsequent ligation with T4 DNA ligase. This insertion introduced a frame-shift in the *gntR* coding frame, resulting in a polypeptide of 85 amino acids instead of the *gntR* protein of 243 amino

acids (4). CAT synthesis in strain 1A423 bearing pgnt21iA1 was high in the absence of gluconate and further derepressed only 1.1-fold upon gluconate addition (Table 1). The fact that CAT synthesis under the control of the *gnt* promoter is highly derepressed in the absence of gluconate if the *gntR* gene is inactivated by deletion or insertion implies that the *gntR* protein might be a negative regulator for the *gnt* promoter.

Identification of the Product of the *gntR* Gene as a 29-kDa Protein. We obtained no evidence that the *gntR* protein [$M_r = 28,277$, deduced from its sequence (4)] was actually synthesized *in vivo*. We tried to detect this protein by NaDodSO₄/PAGE of extracts of cells bearing pgnt21 and pgnt21dP8 in which the *gntR* gene was intact.

Strain 61656 transformed with each plasmid containing an insert carrying the *gnt* promoter synthesized approximately 4 times more CAT than did strain 1A423 bearing the same plasmid when the cells were grown with gluconate (data not shown). We have no explanation at present for this observation. Total protein extracted from strain 61656 bearing each plasmid was analyzed by NaDodSO₄/PAGE (Fig. 2B). Fortunately, we could identify not only a 27-kDa protein postulated as being CAT, consisting of 220 amino acids (14), but also a 29-kDa protein as discrete bands in the electrophoretic patterns (Fig. 2B). Only extracts from cells bearing pgnt21 and pgnt21dP8 contained the 29-kDa protein; the other extracts did not contain this protein (Fig. 2B). Therefore, we concluded that the *gntR* protein was actually synthesized as a 29-kDa protein *in vivo*.

Construction of a Constitutive Mutation Expressing the *gntK* and *gntP* Genes (*gntR1*). We previously isolated a gluconate kinase⁻ mutant, the mutation (*gntK10*, formerly *gnt-10*) from which was located between the second left *Hind*III and *Bgl*II sites of the *Eco*RI fragment (7.0 kb) (see Fig. 1A) (3), indicating that the 2.0-kb insert in plasmid pgnt21 can correct the *gntK10* mutation by transformation. Plasmid pgnt21iA1 carries the -GTAC- insertion in the *gntR* gene within this

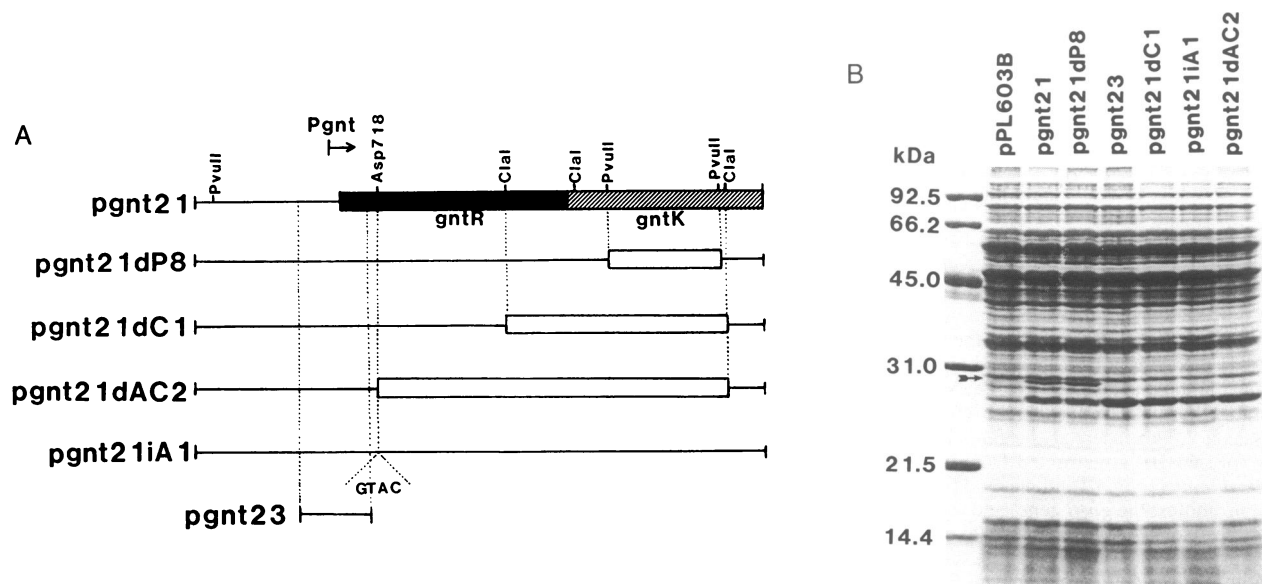


FIG. 2. (A) Construction of deletion and insertion derivatives of plasmid pgnt21. The 2.0-kb insert subcloned in pgnt21 is shown together with the frames of the *gnt* genes and the restriction enzyme sites used for plasmid construction. Open bars represent deletions. Plasmid pgnt21iA1 contains an insertion of 4 bp, -GTAC-, at the *Asp* 718 site of pgnt21. The proper insertion was confirmed by DNA sequencing. The insert (223 bp) in plasmid pgnt23 is also shown. (B) NaDodSO₄/PAGE of total protein from strain 61656 bearing the constructed plasmids. Strain 61656 bearing each plasmid was grown at 37°C to $\text{OD}_{600} = 1$ in S6 medium containing Casamino Acids, kanamycin, chloramphenicol, the required amino acids, and gluconate. Cells (9 OD_{600} units) were harvested, washed in 100 mM potassium phosphate buffer, pH 7/1 mM MgCl₂, and then suspended in 500 μl of 30 mM Tris-HCl, pH 6.6/0.1 mM phenylmethylsulfonyl fluoride. After the addition of lysozyme and DNase I (Sigma) to final concentrations of 200 $\mu\text{g}/\text{ml}$ and 10 $\mu\text{g}/\text{ml}$, respectively, the cells were lysed at 37°C for 15 min and then centrifuged at $15,000 \times g$ for 5 min. Total protein in 15 μl of the supernatant was applied to the NaDodSO₄/polyacrylamide gel (12%) and electrophoresed according to the method of Laemmli (13). An arrow and an arrowhead indicate the 29-kDa protein and CAT, respectively. The left lane contains standard molecular mass markers.

insert, which was likely linked to the *gntK10* mutation in transformation. If the *gntR* protein were actually a negative regulator for the *gnt* operon, it seemed possible to introduce the -GTAC- insertion into this operon in the *B. subtilis* chromosome and in $\phi 105gnt^+$ by transforming mutant *gntK10* strains with the DNA of pgnt21iA1 and selecting Gnt⁺ transformants that showed constitutive synthesis of the gluconate kinase and permease.

At first, we constructed strain YF170 ($\Delta igf leu^+$), lysogenized with $\phi 105gntK10$ carrying the *gntK10* in the cloned operon, by congression with the DNA of strain YF160 (*gntK10 leu⁺*) using as the recipient strain, 61656 ($\Delta igf leuA8$) lysogenized with $\phi 105gnt^+$. Among 1000 Leu⁺ transformants, only two clones were found to be immune against the $\phi 105$ wild type and to exhibit a Gnt⁻ phenotype. We examined the syntheses of gluconate kinase and permease in these two transformants and found that in only one was the permease normally induced, being lacking in the kinase; this is the phenotype of the *gntK10* strain. Then, we transformed both this transformant, strain YF170 lysogenized with $\phi 105gntK10$, and strain YF160 (*gntK10*) with the DNA of plasmid pgnt21iA1. We randomly chose 10 Gnt⁺ transformants from each of the two respective recipients, examined them as to the synthesis of the gluconate kinase and permease, and found that eight and nine transformants, respectively, synthesized constitutively gluconate kinase and gluconate permease. The resultant constitutive mutation expressing the *gntK* and *gntP* genes was designated as *gntR1*. Table 2 shows that the constructed *gntR1* strains synthesized constitutively the gluconate kinase and permease, whereas the corresponding isogenic *gnt⁺* strains normally induced both enzymes only upon the addition of gluconate to the medium.

The proper insertion of -GTAC- into the *Asp 718* site in the *gntR* gene within the *gnt* operon in $\phi 105gnt^+$ was confirmed by sequencing a fragment containing the corresponding region from the DNA of $\phi 105gntR1$ (data not shown). Furthermore, the constitutive expression of the *gntK* and *gntP* genes in strain YF177 (*gntR1 recE4*) became inducible by gluconate if this strain was transformed with pgnt21, whereas the expression remained constitutive if it were transformed with pgnt21iA1 (Table 2), indicating that this

Table 2. Constitutive expression of the *gntK* and *gntP* genes in *gntR1* strains

Strain (phage or plasmid)*	Gluconate kinase, nmol/min per mg protein		Gluconate permease, nmol/min per OD	
	- Gluco- nate	+ Gluco- nate	- Gluco- nate	+ Gluco- nate
61656($\phi 105gnt^+$)	<0.1	22.9	0.5	13.3
61656($\phi 105gntR1$)	58.2	33.1	18.2	17.1
60015	<0.1	19.2	1.0	17.7
YF176; <i>gntR1</i>	40.5	30.2	14.4	13.7
YF169	<0.1	19.2	0.8	14.1
YF177; <i>gntR1</i>	46.6	42.6	14.3	14.0
YF177(pgnt21)	<0.1	18.8	0.3	10.0
YF177(pgnt21iA1)	46.2	37.6	17.1	12.1

The cells were grown at 37°C to OD₆₀₀ = 1 in S6 medium containing Casamino Acids and the required amino acids, with or without gluconate (in the case of growth of strain YF177 bearing pgnt21 or pgnt21iA1—kanamycin and chloramphenicol were also added to the medium). The preparation of an extract of cells of 9 OD₆₀₀ units and spectrophotometric assays of gluconate kinase were done as described previously (2). Gluconate permease was assayed by radioactivity measurement of D-[U-¹⁴C] gluconate (Amersham) incorporated into whole cells as described previously (3).

*Specialized transduction with $\phi 105$ derivatives was done as described previously (3). Strains 60015 and YF176, and strains YF169 and YF177, are isogenic pairs.

constitutive *gntR1* mutation in the host chromosome could be complemented by the intact *gntR* gene in plasmid pgnt21. From these results, we concluded that the *gntR* protein was a negative regulator for expression of the *gntK* and *gntP* genes.

The *gntR1* Mutation Uncouples the Synthesis of the *gnt* mRNA from Repressive Control. We reported previously that the *gnt* operon transcribed from the *gnt* promoter as a polycistronic mRNA was probably regulated at the transcriptional level (4, 7). The results of deletion analysis and insertional inactivation of the *gntR* gene suggested that this gene coded for a transcriptional negative regulator for the *gnt* promoter because expression of the *cat-86* gene encoded in vector pPL603B depended on the promoter activity in a fragment inserted upstream of this gene (5). To confirm that the *gntR* protein is a transcriptional regulator for the *gnt* operon, we examined by S1 nuclease mapping whether or not strain YF176 carrying the *gntR1* mutation synthesized the *gnt* mRNA constitutively starting from the *gnt* promoter (Fig. 3).

We extracted total RNAs from isogenic strains 60015 and YF176 (*gntR1*) grown with and without gluconate. The extracted RNAs (100 μ g each) were hybridized with probes (0.2 pmol each), either a 2.6-kb *EcoRI*-*Bgl* II fragment (= the

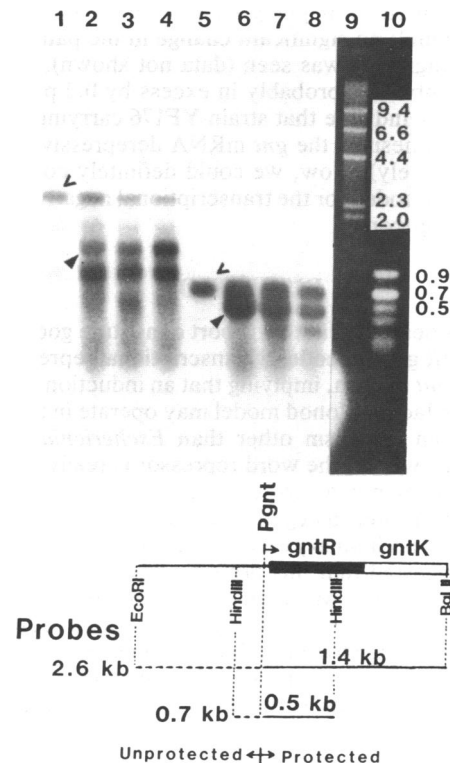


FIG. 3. Derepressive synthesis of the *gnt* mRNA in a *gntR1* strain. (Lower) Strategy used for S1 nuclease mapping to analyze *gnt* mRNA. The mapping was done by the modified method of Berk and Sharp (15) as described (4). Solid and dashed lines for the probes of 2.6 and 0.7 kb represent protected (1.4 and 0.5 kb) and unprotected regions, respectively. (Upper) Total RNA was extracted from cells grown in S6 medium containing Casamino Acids and the required amino acids, with or without gluconate. After hybridization of the *EcoRI*-*Bgl* II 2.6-kb (lanes 1-4) and *HindIII* 0.7-kb (lanes 5-8) probes ³²P-labeled at both 5' ends with the RNAs from strains 60015 (lanes 1, 2, 5, and 6) and YF176 (lanes 3, 4, 7, and 8) grown without gluconate (lanes 1, 3, 5, and 7) and with gluconate (lanes 2, 4, 6, and 8), the hybrids were digested at 37°C for 20 min with S1 nuclease (6 Sankyo units/ml) and then analyzed by alkaline agarose gel (1%) electrophoresis. The probes and protected fragments are indicated by < and \leftarrow, respectively. Lanes 9 and 10 contain molecular mass markers (in kb) of *HindIII*-digested λ DNA and *Alu* I-digested pBR322, respectively (photographed before autoradiography).

Bgl II B fragment) 5' labeled at both ends (Fig. 3, lanes 1–4) or a 0.7-kb *Hind*III fragment 5' labeled at both ends (lanes 5–8), treated with S1 nuclease and then analyzed by alkaline agarose gel electrophoresis. When the 2.4-kb probe was hybridized with the RNAs from strain 60015 grown with gluconate and from strain YF176 grown with and without gluconate (lanes 2–4), we clearly observed a protected fragment of 1.4 kb (arrowhead). In contrast, when the probe was hybridized with the RNA from strain 60015 grown without gluconate, we could not detect a protected fragment of this size (lane 1). As shown previously (4), this 1.4-kb fragment resulted from protection with the *gnt* mRNA transcribed from the *gnt* promoter that was induced by gluconate in strain 60015, and the other protected fragments might result from protection, such as that with partially degraded *gnt* mRNA. [The orientation of the 1.4-kb fragment was confirmed by further mapping that indicated proper protection of the 2.6-kb labeled fragment that had been secondarily digested with either *Hind*III or *Pvu* II before hybridization (data not shown).] As expected, on agarose gel electrophoresis a protected fragment of 0.5 kb from the 0.7-kb probe was only seen when the probe was hybridized with the RNAs from strain 60015 grown with gluconate and from strain YF176 grown with and without gluconate (lanes 6–8). On preliminary mapping using twice the amount of each probe (0.4 pmol) no significant change in the patterns of the protected fragments was seen (data not shown), suggesting that each probe was probably in excess by 0.2 pmol. These results clearly indicate that strain YF176 carrying the *gntRI* mutation synthesized the *gnt* mRNA derepressively (probably constitutively). Now, we could definitely conclude that the *gntR* gene coded for the transcriptional negative regulator for the *gnt* operon.

DISCUSSION

The results described in this report constitute good evidence that the *gntR* gene encodes a transcriptional repressor for the *B. subtilis gnt* operon, implying that an induction mechanism that fits the Jacob–Monod model may operate in an inducible operon of an organism other than *Escherichia coli* or its relatives. However, the word repressor is really too specific for the phenomenon as presently understood. Therefore, we call the *gntR* protein a negative regulator at present, although the results of preliminary experiments using the purified *gntR* protein suggested that this protein was actually a transcriptional repressor (Y. Miwa and Y.F., unpublished data).

The *B. subtilis gnt* operon is transcribed from the *gnt* promoter 40 bp upstream of the *gntR* gene as a polycistronic mRNA (4). Therefore, it is interesting that the first gene (*gntR*) for this operon is the structural gene for a transcrip-

tional negative regulator, because a cell does not need this protein after the operon has been induced. Why an apparent excess of a regulator should be synthesized after induction is not clearly understood. One explanation is that the *gntR* protein might have another function in gluconate catabolism besides being a transcriptional regulator; this protein might have its own function, or it could be a subunit of another *gnt* protein. This kind of dual function has been observed for a number of proteins (especially regulatory proteins), including a transcriptional regulator for a catabolic operon of *Salmonella typhimurium*, *put* (16). However, this explanation is unlikely because the *gntRI* strains could use gluconate as a carbon source, suggesting that the *gntR* protein is not essential for gluconate catabolism in this organism. Whether or not the location of the regulator within the *gnt* operon is unique remains to be determined, because no other catabolic system in Gram-positive bacteria has been investigated at the molecular level.

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