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Efficient Utilization and Operation of the Gluconate-Inducible System of the Promoter of the *Bacillus subtilis gnt* Operon in *Escherichia coli*

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A DNA fragment containing the promoter of the *Bacillus subtilis* gluconate (*gnt*) operon and its first gene (*gntR*) was cloned into *Escherichia coli*. *E. coli* recognized this promoter efficiently and precisely. Moreover, the gluconate-inducible system of this operon operated even in *E. coli*.

The gluconate (*gnt*) operon of *Bacillus subtilis* is involved in gluconate catabolism in this organism (7). The *gnt* operon consists of four *gnt* genes (6) (Fig. 1). The second and third genes (*gntK* and *gntP*) encode gluconate kinase and gluconate permease, respectively; these enzymes are known to be responsible for gluconate catabolism. Recently, the first gene (*gntR*) was shown to encode a negative regulator for the *gnt* operon (5), whereas the function of the last gene (*gntZ*) remains unknown. The *gnt* operon is transcribed from the *gnt* promoter upstream of the *gntR* gene as a polycistronic mRNA (4, 6). mRNA synthesis is induced by gluconate, and this induction is repressed by glucose.

It was expected that the inducible system of the *gnt* operon might also operate in *Escherichia coli* if a fragment carrying the *gnt* promoter and the *gntR* gene were cloned into this organism, because the *gnt* promoter sequences (TTGCAT for the "−35" region and TATCAT for the "−10" region) (4) seemed to be recognized by the major *E. coli* RNA polymerase; it was also expected that an active *gntR*-encoded protein might be synthesized in *E. coli*. We chose such an *Eco*RI fragment (2.0 kilobases), cloned it into *B. subtilis* by using a promoter-probe shuttle vector (pLS353) for *B. subtilis* and *E. coli* (Fig. 1), and then transformed *E. coli* with the plasmid constructed. (Plasmid pLS353 was constructed at the Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan, and provided by H. Hirochika.) The 2.0-kilobase fragment electrophoretically isolated from an *Eco*RI digest of plasmid pgnt21 (5) was ligated with plasmid pLS353 which had been linearized with *Eco*RI. The ligated DNA was transferred to a competent culture of *B. subtilis* 61656 (*Δigf hisA1 leuA8 metB5 trpC2*) (3), and Km^r transformants were obtained. These transformants were screened for Em^r on Penassay broth (Difco Laboratories) containing 20 μg of erythromycin per ml. The Km^r and Em^r transformants obtained were analyzed for plasmids by agarose gel electrophoresis, and the plasmid carrying the properly oriented insert, whose structure is shown in Fig. 1, was designated as pgnt41. Ca^{2+}-treated *E. coli* HB101 (F^- *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-) was then transformed with pgnt41, and Ap^r transformants were obtained. These transformants also exhibited Km^r and Em^r (500 μg of erythromycin per ml in Luria-Bertani medium containing 0.1% glucose). One of the transformants was used in the following experiments after a single colony had been isolated.

If the *gnt* promoter were efficiently utilized and an active *gntR* protein were synthesized by strain HB101 bearing pgnt41, we thought it would be possible to detect proteins whose syntheses were under the control of this promoter by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) of extracts of cells exposed to gluconate. Strain HB101 bearing pgnt41 or pLS353 was grown in TA medium (TGA medium [9] without glycerol) containing erythromycin (100 μg/ml), kanamycin, and ampicillin with and without gluconate and with gluconate and glucose. The cells were harvested at the mid-logarithmic phase, and extracts of them were subjected to SDS-PAGE. We clearly detected a 29-kilodalton (kDa) protein when strain HB101 bearing pgnt41 was grown with gluconate and with gluconate and glucose (Fig. 2A, lanes 4 and 6) but not when it was grown without gluconate (lane 5) (this protein was found to represent approximately 5% of the total protein by densitometric measurements). However, strain HB101 bearing pLS353 did not produce this protein in any case (lanes 1, 2, and 3). The induction of the 29-kDa protein continued for up to 90 min after the addition of gluconate or of gluconate and glucose to the culture of pgnt41-bearing strain HB101 grown to the early logarithmic phase, and this induction was not repressed by the simultaneous addition of glucose (Fig. 2B). We recently demonstrated by SDS-PAGE that the *gntR* protein is synthesized as a 29-kDa protein in *B. subtilis* (5). Therefore, we assumed that the 29-kDa protein induced in strain HB101 bearing pgnt41 was the *gntR* protein. Analysis of the 29-kDa protein purified from this transformant indicated that it was the *gntR* protein and that it was still physiologically active (Y. Miwa and Y. Fujita, unpublished results). A negative regulator of the *gnt* operon, *gntR* protein, was undetectable in extracts of cells bearing pgnt41 by SDS-PAGE unless gluconate was added to the medium (Fig. 2). However, we postulate that an undetectable amount of the *gntR* protein made even in the absence of gluconate is sufficient to repress the *gnt* promoter; upon induction, there is a burst of its synthesis which eventually leads to renewed repression as the gluconate is used up.

To confirm the efficient utilization of the *gnt* promoter in *E. coli*, we analyzed a transcript of strain HB101 bearing pgnt41 by S1 nuclease mapping by the method of Berk and Sharp (2) as modified by us (4). We extracted the total RNAs from pgnt41-bearing strain HB101 grown with and without gluconate and with gluconate and glucose by the method of Aiba et al. (1) and from *B. subtilis* 60015 (Gnt^+ *trpC2 metC7*) grown with gluconate as described previously (6). The

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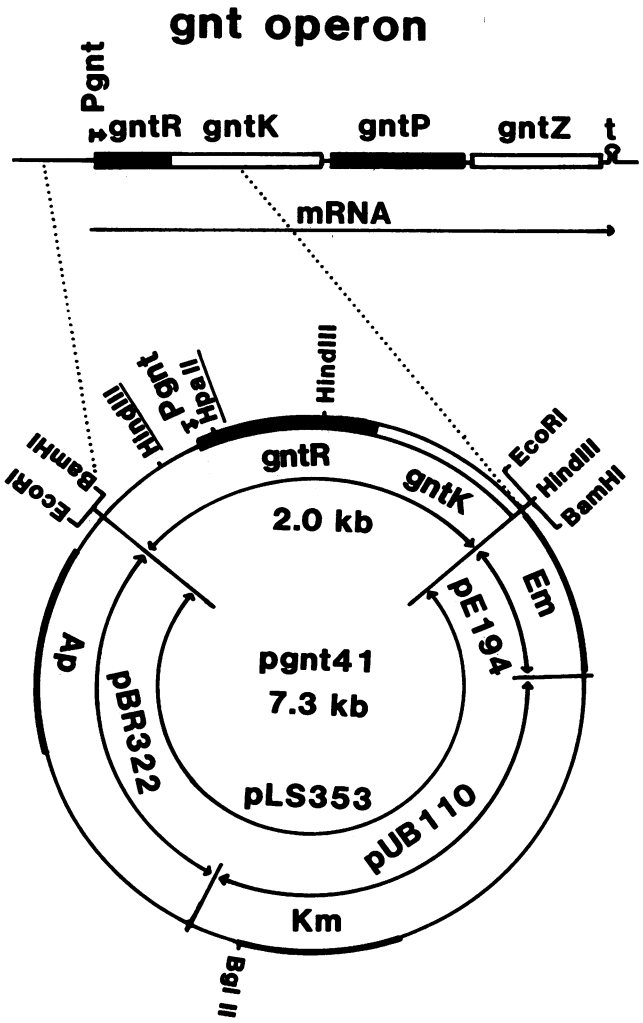
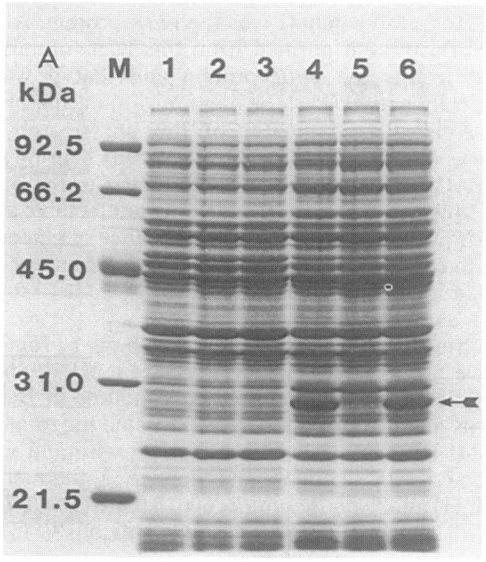
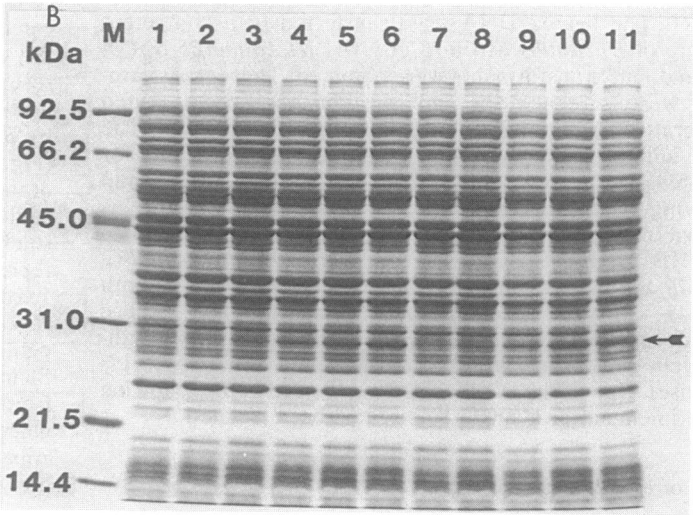


FIG. 1. Structures of the *B. subtilis gnt* operon and plasmid pgnt41. The *gnt* operon consists of four *gnt* genes. Pgnt and t represent the *gnt* promoter and terminator, respectively. mRNA (5.1 kilobases [kb]) is transcribed from the *gnt* promoter up to the terminator. The structure of plasmid pgnt41 is shown along with its partial restriction enzyme map; this plasmid possesses a 2.0-kilobase insert at the *Eco*RI site of the promoter-probe shuttle vector (pLS353) for *B. subtilis* and *E. coli*. (The underlined *Hin*dIII and *Hpa*II sites were used for the preparation of a probe used in S1 nuclease mapping experiments [see Fig. 3].) Plasmid pLS353 is composed of a promoterless erythromycin resistance gene (*ermC*) (8) of *Staphylococcus aureus* plasmid pE194 and deletion derivatives of plasmids pUB110 and pBR322. Em, Km, and Ap represent erythromycin resistance (Em^r), kanamycin resistance (Km^r), and ampicillin resistance (Ap^r) genes, respectively.

extracted RNAs were hybridized with a probe (291 base pairs) of a *Hin*dIII-*Hpa*II fragment (−201 to +91; +1 is the transcription initiation base [the locations of the *Hin*dIII and *Hpa*II sites are indicated in Fig. 1]) which had been 5′ ^{32}P labeled at the *Hpa*II site. The DNA-RNA hybrids were treated with S1 nuclease and then analyzed on a DNA sequencing gel (8%). When the probe (0.2 pmol) was hybridized with RNAs (10 μg each) extracted from *E. coli* cells grown with gluconate and with gluconate and glucose, we clearly observed a major protected fragment of 91 bases (Fig. 3, lanes 1 and 2), indicating that transcription starts from the same base as in *B. subtilis* (lane 4). However, we could not detect this fragment when the RNA extracted from pgnt41-bearing strain HB101 grown without gluconate was hybridized with the probe (lane 3). Heavy bands of the protected fragment were obtained (lanes 5 and 6) when the probe was hybridized with the same amounts (100 μg each) of RNAs from pgnt41-bearing cells grown with gluconate and with gluconate and glucose as were used for the RNA from *B. subtilis* 60015 (lane 4), suggesting that at least 10 times more of the transcript was induced by gluconate in the *E. coli* transformant than in strain 60015 or that mRNA extraction from *E. coli* was more efficient than that from *B. subtilis*.

From the results presented here, we came to the following conclusions. (i) An *E. coli* RNA polymerase efficiently utilized the *gnt* promoter from the same base as in *B. subtilis* when the cells were exposed to gluconate, which acted as an inducer, even in *E. coli*. The inducible system of the *gnt* operon probably depends only on the *gntR* protein and a particular molecule (gluconate or a catabolite derived from





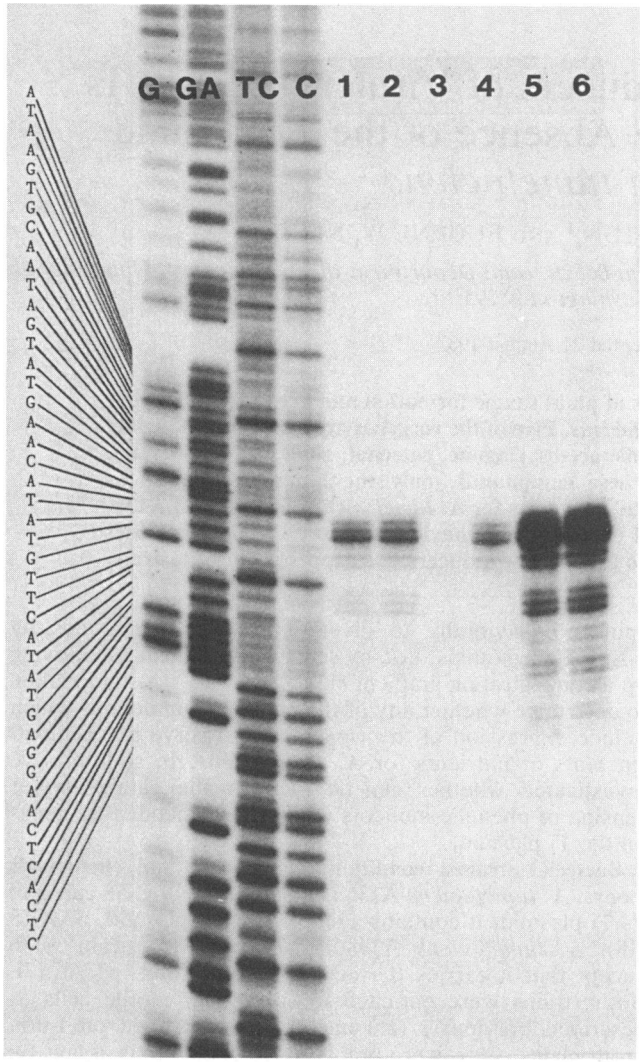


FIG. 3. S1 nuclease analysis of transcripts of strain HB101 bearing pgnt41. Total RNAs were extracted from pgnt41-bearing strain HB101 cells grown with gluconate (lanes 1 and 5), with gluconate and glucose (lanes 2 and 6), and with no addition (lane 3) and from *B. subtilis* 60015 cells grown with gluconate (lane 4). (To remove the contaminating plasmid pgnt41 from the RNAs extracted from strain HB101 cells bearing pgnt41, we treated the RNAs further with DNase I which had been treated with proteinase K in the presence of calcium as described by Tullis and Rubin [12].) The RNAs (10 μg each [lanes 1, 2, and 3] or 100 μg each [lanes 4, 5, and 6]) were hybridized with the probe. Base-specific chemical cleavages (11) of the same labeled fragments are shown in lanes G, GA, TC, and C, representing guanine, guanine-plus-adenine, thymine-plus-cytosine, and cytosine reactions, respectively. The sequence complementary to positions −23 through +23 is indicated.

gluconate). *E. coli* can produce an active *gntR* protein and regulate the concentration of the molecule, so the inducible system of the *gnt* operon can operate properly. However, we cannot exclude at present the possibility that another regulatory protein, possibly encoded in the *E. coli* chromosome, regulates this system. (ii) The *gnt* promoter was efficiently utilized even when glucose was simultaneously added with gluconate to the medium. This promoter seems to function in *E. coli* without the need for a positive regulator, such as the adenosine 3′,5′-monophosphate receptor protein, for efficient utilization. (iii) The fact that the inducible system of the *gnt* operon can operate in *E. coli* implies that this system might be useful for the construction of an expression vector for not only *B. subtilis* but also *E. coli*, in which the *gnt* promoter can be induced by such a commercially inexpensive compound as gluconate.

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FIG. 2. (A) Detection of a 29-kDa protein by SDS-PAGE of the total protein from strain HB101 bearing plasmid pgnt41. The harvested cells were washed, suspended in Laemmli sample buffer (10), and boiled for 2 min. The resulting extracts (50 μg per lane) were subjected to SDS-PAGE on a 10% gel (10). Lanes 1, 2, and 3 contain extracts from pLS353-bearing cells grown with (lane 1) and without (lane 2) gluconate and with gluconate and glucose (lane 3). Lanes 4, 5, and 6 contain extracts from pgnt41-bearing cells grown with (lane 4) and without (lane 5) gluconate and with gluconate and glucose (lane 6). Lane M contains standard proteins used as molecular weight markers. The arrow indicates the 29-kDa protein. (B) Induction of synthesis of the 29-kDa protein by gluconate in strain HB101 bearing pgnt41. The cells were harvested at 15 min (lanes 2 and 7), 30 min (lanes 3 and 8), 60 min (lanes 4 and 9), 90 min (lanes 5 and 10), and 120 min (lanes 6 and 11) after the addition of 10 mM gluconate (lanes 2 to 6) and 10 mM gluconate and 10 mM glucose (lanes 7 to 11), and extracts of them were analyzed by SDS-PAGE. Lanes 1 and M contain extracts of the cells before the addition of gluconate and standard proteins used as molecular weight markers, respectively. The arrow indicates the 29-kDa protein.