Evidence for posttranscriptional regulation of synthesis of the *Bacillus subtilis* Gnt repressor

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Received 12 March 1990; revised version received 3 May 1990

Transcription of the *Bacillus subtilis gnt* operon results in a polycistronic mRNA that encodes from the 5′ end the Gnt repressor, gluconate kinase and permease. The RNA is drastically induced through inactivation of the Gnt repressor by gluconate. The results of deletion analysis of the *gnt* promoter region upstream of the repressor gene indicated that no other promoter except the *gnt* promoter was present for expression of the gluconate kinase gene. In contrast to the synthesis of gluconate kinase and permease, which was markedly induced by gluconate, the results of a radioimmunoassay for the Gnt repressor indicated that synthesis of the Gnt repressor from the induced mRNA was posttranscriptionally repressed.

Gluconate operon; Transcription, Promoter region; Gene expression regulation, *Bacillus subtilis*

1. INTRODUCTION

After entering the *Bacillus subtilis* cell with the aid of gluconate permease, gluconate is phosphorylated to gluconate 6-phosphate by gluconate kinase, and is then catabolized through the pentose cycle. The *gnt* operon, specifically involved in this gluconate catabolism, consists of four *gnt* genes: (from the 5′ end) *gntR, gntK, gntP* and *gntZ* [1]. The *gntR, gntK* and *gntP* genes encode the Gnt repressor, gluconate kinase and permease, respectively [1–3], while the function of the *gntZ* gene remains unknown. The *gnt* operon is transcribed from the *gnt* promoter upstream of the *gntR* gene to the *gnt* terminator downstream of the *gntZ* gene as a polycistronic mRNA of *gnt* [1,4]; no other promoter is likely to be present in the regions upstream of the *gntK* and *gntP* genes [1]. This transcription is negatively regulated through the interaction of the Gnt repressor with the *gnt* operator, located at the transcription initiation site; a marked induction of transcription is mediated through inactivation of the Gnt repressor by the inducer gluconate [2,3,5].

In the absence of gluconate, the *B. subtilis gnt* operon is autorepressed by the Gnt repressor because it encodes its own repressor [2,3]. This autorepression allows the synthesis of basal levels of gluconate kinase and permease, which are essential for normal induction of the *gnt* operon [6]. Upon induction of the *gnt* operon, the Gnt repressor is inactivated by added gluconate and the cell likely does not require the Gnt repressor any longer. However, induction of *gnt* mRNA synthesis from the *gnt* promoter upstream of the *gntR* gene would lead to the synthesis of unnecessary Gnt repressor as well as gluconate kinase and permease, unless the synthesis of the Gnt repressor is posttranscriptionally repressed. To explore whether repressor synthesis from the increased *gnt* mRNA upon the addition of gluconate might be posttranscriptionally regulated, we first demonstrated by deletion analysis of the *gnt* promoter region that no other promoter except the *gnt* promoter is present in the region upstream of the *gntK* gene. Then we developed a radioimmunoassay for the Gnt repressor protein involving antiserum raised against the purified Gnt repressor protein. With this assay we examined the absolute levels of the Gnt repressor, as well as the activities of gluconate kinase and permease in *B. subtilis* cells grown with and without gluconate.

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Abbreviations: SDS, sodium dodecyl sulfate; OD, optical density; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1. *Materials*

B. subtilis strain 60015 (*trpC2 metC7*) is our standard strain. Strain 61656 (*Δigf trpC2 metB5 leuA8 hisA1*) was described previously [7,8]. Strain DB204 (*trpC2 lys-1 phe-1 nprR2 nprE2 aprA3 ispA1* chloramphenicol-resistant) is a triple mutant deficient in intracellular serine protease and extracellular alkaline and neutral protease [5].

DNA restriction and modification enzymes were from Takara Shuzo (Kyoto, Japan).

2.2. *Deletion analysis of the promoter region of the* gnt *operon*

To construct plasmid pgnt37, the *Rsa*I-*Kpn*I fragment carrying nucleotides −109 to +153 (the transcription initiation nucleotide is

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00145793/90/$3.50

numbered +1) was cloned into plasmid pWP19 [5] which had been digested with *Sma*I and *Kpn*I. The resulting plasmid pgnt36 was digested with *Bam*HI and *Kpn*I, and the fragment carrying the *gnt* promoter was recloned into the *Bam*HI and *Kpn*I sites of plasmid pgnt34 whose construction was previously described [5].

Deletion derivatives of plasmid pgnt37 were constructed as follows: plasmid pgnt37 was cleaved with *Bam*HI, digested with BAL 31 nuclease-S (1.0 unit, 30°C, 6 min), treated with the Klenow fragment of DNA polymerase I, and ligated with *Bam*HI linker (8mer; Takara). After digestion with *Kpn*I and *Bam*HI, 100–240 bp fragments were electrophoretically isolated and ligated with pgnt34 which had been digested with *Bam*HI and *Kpn*I. Plasmids containing appropriate sizes of deletion were selected and the end points of all the deletions were determined by sequencing.

For subtilisin assay, strain DB204 bearing each deletion derivative was grown in Schaeffer's medium [9] with or without 10 mM gluconate as described previously [5]. Subtilisin was assayed by the method of Millet using Hide Powder Azure (Sigma) [10] with modifications as described previously [5].

2.3. *Radioimmunoassay for the Gnt repressor*

The Gnt repressor was purified as described previously [3] from an overproducing *Escherichia coli* strain, HB101, harboring a plasmid (pgnt41) carrying *gntR*. In the case of the most purified preparation (Fraction V), the purity was estimated to be 99% by SDS-polyacrylamide gel analysis. This preparation was used to generate antiserum in a New Zealand White rabbit as described previously [7]. Radioiodination of the purified Gnt repressor (10 μg) was performed with Iodogen [11] (Pierce) and $Na^{125}I$ (18.5 MBq (0.5 mCi); Amersham), as described previously [3].

B. subtilis cell extracts were prepared by the method of Gunsalus et al. [12], with modifications, from exponentially growing cells (OD_{600} = 0.5) in S6 medium [7] containing 0.5% Casamino Acids (Difco) with or without 10 mM gluconate. Cells (100 OD_{600} units) were washed in PBS (150 mM NaCl, 10 mM $NaPO_4$ (pH 7.4)) solution and suspended in 0.5 ml of PBS containing 0.5% bacitracin (Sigma). Cell extracts were prepared by sonication and centrifugation (20000 × *g* for 20 min at 4°C). Gluconate (5 mM) was routinely added before cell disruption to insure that the Gnt repressor remained free of cellular DNA. Under these conditions, 8 mg of protein was extracted from 100 OD_{600} units of cells (2×10^{10}).

Radioimmunoassaying for the Gnt repressor protein was performed by the method of Gunsalus et al. [12] with modifications. Each assay tube contained 100 μl of ^{125}I-labeled Gnt repressor (9×10^3 cpm), the Gnt repressor as a standard or a sample in 300 μl, and 100 μl of the anti-Gnt repressor antiserum which had been diluted 1/5000 with PBS containing 0.2% normal rabbit serum (all other reagents were prepared in PBS containing 0.5% bacitracin). The sample tubes were briefly mixed and then incubated for 24 h at 4°C. To each sample was added 100 μl of a suspension of fixed *Staphylococcus aureus* cells (Cowan I strain) (10% wet weight/volume in 20 mM KPO_4 (pH 7.4), 0.15 M NaCl and 0.05% NaN_3), which was prepared as described by Mori et al. [13]. After gentle shaking for 1 h at room temperature, the tubes were centrifuged at 2000 × *g* for 5 min at room temperature, and then the supernatants were carefully aspirated off. The pellets were suspended in 1 ml of 10 mM Tris-Cl (pH 7.4), 2 mM Na-EDTA (pH 7.4), 0.1% Triton X-100 and 0.1% SDS. After recentrifugation, the supernatants were thoroughly aspirated off before determining the radioactivity in the pellets, at 60% counting efficiency (Autowell counter, Hitachi Medico RMA-1W-2ch).

3. RESULTS AND DISCUSSION

S1 nuclease analysis and an extensive search of the *Bacillus subtilis gnt* operon for its promoters revealed one promoter upstream of the *gntR* gene (the *gnt* promoter) as well as the two tandem internal promoters upstream of the *gntZ* gene [1,4]. The *gntR*, *gntK* and *gntP* genes might be transcribed only as the polycistronic *gnt* mRNA from the *gnt* promoter which is induced by gluconate. To directly demonstrate that no other promoter except the *gnt* promoter is present in the region upstream of the *gntK* gene, we performed deletion analysis of a fragment carrying the upstream portion of the *gnt* operon which had been cloned into the promoter probe vector pWP19, which carries the *aprA* reporter gene encoding subtilisin.

Plasmid pgnt37 carries the 1.1 kb fragment possessing sequences of the *gnt* operon extending from −109 to +888, which includes the *gnt* promoter, the *gntR* gene, and the N-terminal portion of the *gntK* gene (Fig. 1). The end points of all the deletions from nucleotide −109 are also shown in Fig. 1. Table I shows the activities of subtilisin in the medium when strain DB204 bearing each deletion derivative was grown with or without gluconate. Strain DB204, bearing plasmid pgnt37dM50, synthesized a considerable level of subtilisin without addition of gluconate, and gluconate further induced subtilisin by gluconate just as with pgnt37, demonstrating that a deletion up to nucleotide −49 (pgnt37dM50) did not affect expression of the *aprA* gene. Strain DB204 bearing pgnt37dM34 carrying a deletion destroying the '−35' region of the *gnt* promoter diminished expression of the *aprA* gene down to the level expressed by plasmid pWP19. Further deletions up to nucleotide +26 (pgnt37dM19, pgnt37dM10 and pgnt37dP27) also completely destroyed the promoter activity, except that a deletion to −19 resulted in a slight increase of the promoter activity due to unknown reasons. These results clearly indicate that no other promoter except the *gnt* promoter drives for expression of the *gntK* gene.

To determine whether repressor synthesis is post-transcriptionally regulated, we developed a radioimmunoassay for the repressor protein. The anti-Gnt repression antiserum was used at a final dilution of 1/25000, which typically bound 20–30% of the ^{125}I-labeled Gnt repressor. Increasing amounts of the purified Gnt repressor gave the displacement curve shown in Fig. 2. The midpoint was approximately 3 ng of Gnt repressor per tube. Over the useful range of the assay (1–10 ng of Gnt repressor per tube), the coefficient of variation within the assay was less than 7%.

Levels of the Gnt repressor were readily measurable in 1–10-μl samples of cell extracts prepared from strain 60015 which had been grown with or without gluconate. Furthermore, these samples gave displacement curves that were almost parallel to that of the purified Gnt repressor (Fig. 2). Strain 61656, carrying a large deletion (Δigf) covering the *gnt* operon [8,14], is unable to synthesize any Gnt repressor protein. As shown in Fig. 2, a cell extract of strain 61656 contained very low displacing activity. This activity was subtracted from the displacing activities in cell extracts of

strain 60015 upon calculation of the intracellular Gnt repressor levels (Table II).

To determine whether expression of the *gntR* gene is posttranscriptionally regulated in contrast to that of the *gntK* and *gntP* genes, we compared the absolute intracellular levels of the Gnt repressor, determined in the radioimmunoassay, with the specific activities of gluconate kinase and permease in strain 60015 cells

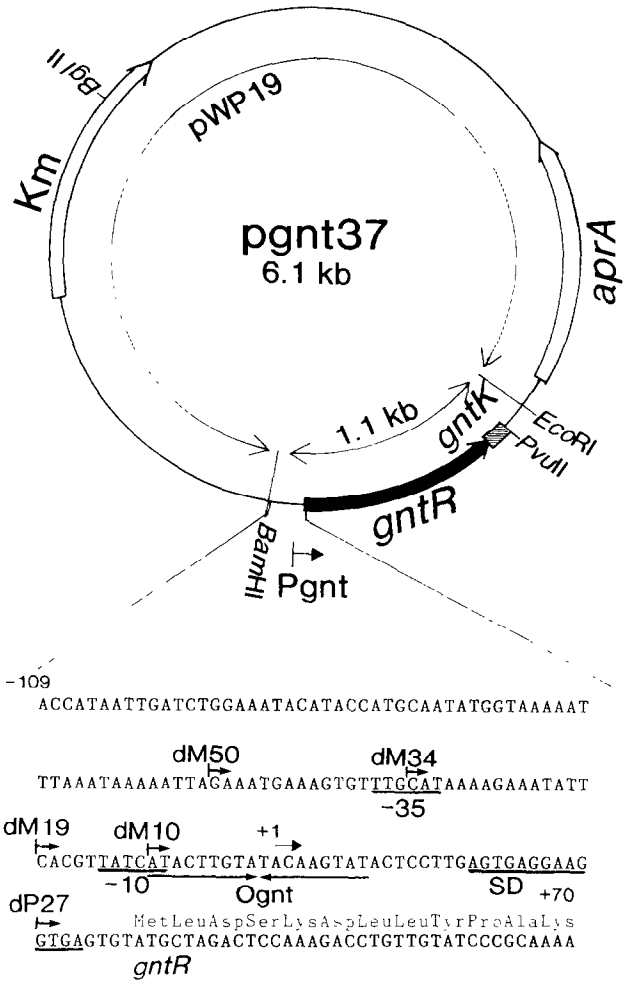

Fig. 1. Structures of plasmid pgnt37 and its deletion derivatives. Plasmid pWP19 is a plasmid pUB110 derivative containing a promoterless subtilisin gene (*aprA*) preceded by a plasmid pUC19 polylinker [5]. The 1.1 kb fragment carries the upstream region of the *gnt* operon (−109 to +888; the transcription initiation nucleotide is numbered +1) in which the *gnt* promoter (Pgnt), the entire *gntR* gene, and the N-terminal portion of the *gntK* gene are included. Km denotes the gene encoded in pUB110 for kanamycin nucleotidyltransferase. At the bottom, the nucleotide sequence surrounding the transcription initiation site of the *gnt* operon is shown. Deletion derivatives of plasmid pgnt37 (plasmids pgnt37dM50, pgnt37dM34, pgnt37dM19, pgnt37dM10, and pgnt37dP27) carry deletions from the nucleotide of −109 to −49, −33, −18, −9 and +26, respectively, whose end points are indicated by dM50, dM34, dM19, dM10 and dp27. The region showing dyad symmetry which is indicated by convergent arrows is the *gnt* operator (Ognt). The '−35' and '−10' regions of the *gnt* promoter and the Shine-Dalgarno (SD) sequence of the *gntR* gene are also shown.

Table I

Expression of the *aprA* gene encoded in plasmid pgnt37 and its deletion derivatives

Plasmid in DB204	Gluconate addition	Subtilisin activity (units/OD_{600} per ml × 10^3)
pWP19	minus	6
	plus	4
pgnt37	minus	54
	plus	638
pgnt37dM50	minus	41
	plus	634
pgnt37dM34	minus	5
	plus	4
pgnt37dM19	minus	16
	plus	17
pgnt37dM10	minus	5
	plus	3
pgnt37dP27	minus	6
	plus	5

Shown are the activities of subtilisin which was synthesized in *B. subtilis* strain DB204 bearing each deletion derivative of plasmid pgnt37. Cells were grown with or without gluconate and subtilisin activity in the medium was measured as described in section 2. One unit of subtilisin activity was defined as the amount of enzyme that produced soluble dye giving an A_{595} of 1 in 1 ml

which had been exposed or not exposed to gluconate (Table II). (Gluconate kinase and permease were assayed as described previously [7,14].) An extract of cells grown without gluconate contained 12 ng of Gnt repressor per mg of protein, whereas it contained specific activities of gluconate kinase and permease of 0.2 and 0.3 nmol/min/mg of protein, respectively. This amount of Gnt repressor, which corresponds to approximately 48 molecules of Gnt repressor per cell (Table II), might be enough to repress expression of the

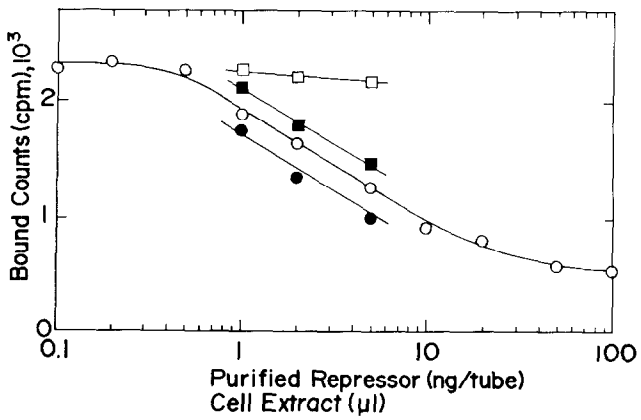

Fig. 2. Displacement of ^{125}I-labeled Gnt repressor from anti-Gnt repressor by purified Gnt repressor and cell extracts of *B. subtilis* strain 60015 grown with (16.4 mg/ml) and without (16.2 mg/ml) gluconate, and strain 61656 grown without gluconate (18.2 mg/ml). Pure Gnt repressor is expressed as nanograms per tube (○). The *B. subtilis* cell extracts, expressed as μl of extract, were: strain 60015 grown with (●) and without (■) gluconate, and strain 61656 grown without gluconate (□).

Table II

Levels of Gnt proteins in *B. subtilis* strain 60015 cells exposed and unexposed to gluconate

Exposure to gluconate	Gnt protein		
	GntR	GntK	GntP
	Gnt repressor (ng/mg) (fold)	Gluconate kinase (nmol/min/mg) (fold)	Gluconate permease (nmol/min/OD_{600}) (fold)
Grown without	12 (1)	0.2 (1)	0.3 (1)
Grown with	43 (4)	19.2 (96)	18.3 (61)
Exposed for 1 h	27 (2)	15 1 (76)	12.8 (43)
Exposed for 2 h	29 (2)	17.2 (86)	15.4 (51)

Shown are levels of Gnt proteins in cells exposed and unexposed to 10 mM gluconate. Cells were grown and the *gnt* repressor was determined as described in section 2. 1 ng of Gnt repressor per 1 mg of extracted protein corresponds to approximately 4 molecules of Gnt repressor (dimer [3]) per cell, assuming that the Gnt repressor protein was completely recovered from the cells under the extraction conditions described in section 2. Cells grown under the same conditions as those for the repressor determination were used to directly assay gluconate permease and to prepare a cell extract for the gluconate kinase assay as described previously [7,14] The assays for the gluconate kinase and permease are described in section 2. The induction levels (fold) when the levels of Gnt proteins in cells grown without gluconate are expressed as 1

gnt operon, even though 9 molecules of Gnt repressor are bound to one *gnt* operator [3]. During growth with gluconate, the Gnt repressor was induced only 4-fold in contrast to the 96- and 61-fold induction of gluconate kinase and permease, respectively. During 1- and 2-h exposure to gluconate, the Gnt repressor was induced only 2-fold, but gluconate kinase and permease were induced 76- and 43-fold on 1 h exposure, and 86- and 51-fold on 2 h exposure, respectively. These results suggest that upon over 30-fold induction of *gnt* mRNA synthesis by gluconate [1,4,6], only expression of the *gntR* gene is posttranscriptionally regulated, in contrast to that of the *gntK* and *gntP* genes.

The possibility that induction of the gluconate kinase and permease by gluconate might be attributed to a direct stimulation of their activities by gluconate is unlikely, because the induction of the *gnt* mRNA by gluconate [1,4,6] is similar to that of the gluconate kinase and permease enzyme activities. Also, addition of gluconate to crude extracts which had been prepared from cells grown without gluconate did not stimulate the activity of the gluconate kinase (unpublished observation by Y. Fujita).

One explanation for the molecular mechanism underlying the posttranslational repression induced by gluconate could be that the *gnt* mRNA is degraded specifically from the 5′ end in the presence of gluconate, which in turn prevents the translation of the *gntR* gene. Another possibility is that only translation of the *gntR* gene is repressed in the presence of gluconate, without degradation of the *gnt* mRNA. Thus, *B. subtilis* cells growing with gluconate would be able to save energy which would otherwise be used to synthesize unnecessary Gnt repressor protein from the increased *gnt* mRNA. However, we cannot exclude the explanation that the Gnt repressor is specifically unstable only in cells exposed to gluconate, in contrast to the gluconate kinase and permease, so that unnecessary Gnt repressor protein rapidly turns over.

Further experiments to determine which of these explanations is the case may reveal a novel regulation mechanism of gene expression.

Acknowledgements: A part of this work was performed at the Department of Biochemistry, Hamamatsu University School of Medicine. We wish to thank M. Fujita for his encouragement to complete this work. We are also grateful to Y Shibata for his help in deletion analysis of the *gnt* promoter region. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan

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