Nucleic Acids Research

Identification and nucleotide sequence of the promoter region of the *Bacillus subtilis* gluconate operon

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ABSTRACT
     The nucleotide sequence (742 bp) of the promoter region of
the Bacillus subtilis gluconate (gnt) operon is presented.
Nuclease S1 mapping revealed the start point of the transcription
and suggested that the expression of this operon is probably
regulated at the transcriptional level.   The sequences of the
-35 and -10 regions suggested that RNA polymerase possessing
sigma-43 may recognize this structure.   The 223 bp fragment
containing 100 bp upstream from the transcription start site
actually exhibited a promoter activity when cloned in a promoter
probe vector of pPL603B.   This promoter activity was highly
derepressed and although still under catabolite repression.   The
fragment on a high copy plasmid could titrate a regulator of the
gnt operon so that the expression of the operon on the host
chromosome also became derepressed.

INTRODUCTION

     The lactose operon of Escherichia coli has been a model

system to investigate operon structure, gene function and

regulation of gene expression.   From such studies a detailed

understanding of the regulation of the catabolic operon has

emerged (1).   However, in Bacillus subtilis or other gram-

positive bacteria, no catabolic transcriptional system using

negative or positive regulators has been investigated.   The only

regulation system described is an anabolic trp operon of B.

subtilis whose promoter structure was recently defined (2).

Since B. subtilis has been the most studied gram-positive

bacterium, it would be of great interest to determine how a

catabolic operon is regulated and to compare the mechanism of

gene regulation with the corresponding mechanism of E. coli.   To

investigate the mechanism of catabolite repression of this

organism would be of special interest because the Bacillus

species has neither detectable cAMP (3) nor adenyl cyclase (4) so

that catabolite repression is unable to be explained by the cAMP receptor protein-cAMP regulatory complex that has been well documented in enteric bacteria (5).

Among several catabolic systems of B. subtilis that have been investigated genetically and biochemically (6), our efforts have been focussed on the gluconate utilization system. After entering the B. subtilis cells, gluconate is phosphorylated to gluconate-6-P that is then catabolized through the pentose cycle. Therefore, only two enzymes, gluconate permease and kinase, are specifically involved in the gluconate catabolism. Both enzymes are induced in response to gluconate and their induction is under catabolite repression (7,8). We have characterized and cloned the gluconate (gnt) operon containing the genes of the gluconate permease and kinase (9,10). The entire nucleotide sequence of the operon (5.5 kb) has been determined (Y. Fujita, T. Fujita, Y. Aratani and Y. Miwa, unpublished). In this paper, we report the nucleotide sequence of the promoter region of the gnt operon of B. subtilis. The restriction enzyme fragment containing 100 bp upstream from the transcription start site exhibited a promoter activity in vivo and is postulated to possess intact interacting sites with both a gnt repressor and a regulatory factor involved in catabolite repression.

MATERIALS AND METHODS
Bacterial strains, phage and plasmid
B. subtilis strain 60015 (trpC2 metC7) is our standard strain. Strain 1A423 [leuA15 thr-5 argA15 recE4 r(-) m(-)] was obtained from the Bacillus Genetic Stock Center. A ϕ105 derivative (ϕ105gnt$^+$) containing an EcoRI fragment (7.0 kb) encoding the gnt operon was constructed as described previously (10). A promoter probe vector (pPL603B) was kindly supplied by D. Rothstein.
Preparation of DNA and transformation
Viral DNA was prepared by phenol treatment from phage particles purified as described previously (10). Plasmid pPL603B was prepared as described by Gryczan et al. (11).

Transformation using the competent culture of B. subtilis was performed by the method of Shibata and Saito (12).

DNA sequence studies

The nucleotide sequence of the gnt operon was determined using the end-label method of Maxam and Gilbert (13) followed by personal computer analysis of the sequence data using the program devised by Mitsui Knowledge Industry (Tokyo, Japan).

RNA preparation and nuclease S1 mapping

Strain 60015 was grown to an optical density at 600 nm (OD_{600}) of 1.0 in the S6 medium (14) containing 0.5% Casamino Acids that was supplemented with tryptophan (50 µg/ml) and methionine (50 µg/ml), with or without 10 mM gluconate and with 10 mM each of gluconate and glucose. The cell cultures (80 ml each) were harvested and their RNA was extracted by the hot phenol method (15).

A modified Berk-Sharp procedure (16) was used to analyze RNAs. RNA samples (200 µg) were mixed with approximately 20 ng of the 291 bp HindIII-HpaII fragment which had been 5´-end labeled at the HpaII site. The mixture was precipitated with ethanol, evaporated to dryness, and dissolved in 25 µl of 40 mM 1,4-piperazinediethanesulfonic acid, pH 6.4, 400 mM NaCl, 1 mM EDTA and 80% deionized formamide (v/v). After incubation at $80^{O}C$ for 15 min, the mixture was annealed for 2 h at $37^{O}C$; then 250 µl of 4.5 mM zinc sulfate, 280 mM NaCl, 20 µg/ml denatured calf thymus DNA, and 6 units/ml nuclease S1 (one Sankyo´s unit is defined as the amount causing 1.0 increase in OD_{260}) were added and the mixture was incubated at 37° for the indicated times. The reaction was terminated by the addition of 275 µl of phenol saturated with 50 mM Tris-Cl, pH 8 and 1 mM Na-EDTA, pH 8. The RNA-DNA hybrids from the aqueous layer to which E. coli tRNA (20 µg) had been added were recovered by ethanol precipitation. The nuclease S1-resistant DNA was analyzed on a DNA sequencing gel (13).

Preparation of cell extracts and enzyme assays

Chloramphenicol acetyltransferase (CAT) was assayed essentially by the method described by Goldfarb et al. (17). The cells (10 OD_{600} units) harvested were washed in 5 ml of 1 M NaCl and 50 mM Tris-Cl, pH 8. The cell pellet was suspended in 0.5 ml of 50 mM Tris-Cl, pH 8, 0.1 mM phenylmethylsulfonyl fluoride and 50 µM dithiothreitol. After addition of lysozyme

(a final concentration of 500 µg/ml), the suspension was incubated at 37°C for 15 min. The crude extract was briefly sonicated to completely break unlysed cells. The cell lysate was centrifuged for 20 min at 27,000 x g and the supernatant was assayed as described by Shaw (18).

Kanamycin nucleotidyltransferase was assayed according to the method described by Sadaie et al. (19). The cells (15 OD_{600} units) harvested were washed in 9 ml of 50 mM Tris-Cl, pH 7. The cell pellet was suspended in 0.5 ml of the same buffer. After addition of lysozyme (200 µg/ml), the suspension was incubated at 37°C for 15 min. The crude extract was briefly sonicated and centrifuged for 20 min at 27,000 x g. Kanamycin nucleotidyltransferase activity in the supernatant was determined as described by Sadaie et al. (19).

The preparation of cell extract and the spectrophotometrical assay method of the gluconate kinase was described previously (8). The gluconate permease was determined as described previously (10).

Enzymes

Restriction enzymes not listed below, T4 polynucleotide kinase and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan). HindIII and RsaI, nuclease S1, and calf intestine alkaline phosphatase were from Nippon Gene (Toyama, Japan), Sankyo (Tokyo, Japan), and Boehringer Mannheim GmbH, respectively. All enzymes were used as recommended by the supplier.

RESULTS

Nucleotide sequence of gnt promoter region

The intact gnt operon of Bacillus subtilis has been cloned in a B. subtilis temperate phage of $\emptyset$105 using prophage transformation by use of EcoRI sites (10). The constructed phage, $\emptyset$105gnt[+], contains a new 7.0 kb EcoRl fragment (= a gnt[+] fragment). The HindIII digestion of the gnt[+] fragment produces four fragments (A to D) as aligned in Fig. 1. The HindIII-A fragment (3.7 kb) subcloned in plasmid pC194 and sequenced contains the complete structural genes of the gluconate kinase and permease (Y. Fujita, T. Fujita and Y. Aratani, unpublished).
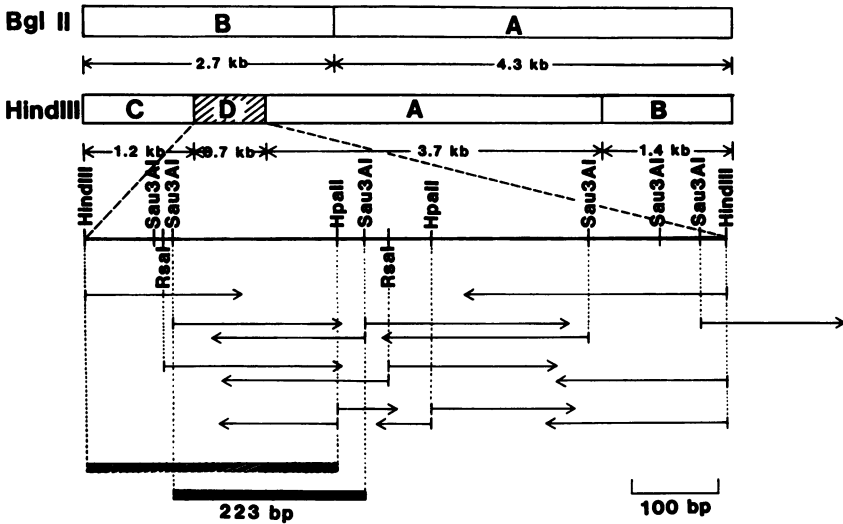
Fig. 1: Restriction endonuclease map and sequencing strategy of the HindIII-D fragment. The BglII and HindIII cleavage maps of the gnt+ fragment (7.0 kb) indicates the location of the HindIII-D fragment. The restriction enzyme map of the HindIII-D fragment was constructed by use of Sau3AI, RsaI and HpaII sites. Horizontal arrows represent the areas sequenced. The cross-hatched line between the HindIII and HpaII sites is the 291 bp fragment used for nuclease S1 mapping. The 223 bp Sau3AI fragment is represented by a thick line.

In spite of careful examination of the sequence, we could not find any promoter-like structure in the upstream region of the two structural genes.

　　To search the promoter, we continued to sequence the HindIII-D fragment that is the 5′-neighbor of the HindIII-A. Fig. 1 presents a detailed restriction map of the HindIII-D fragment and the strategy used in sequence analysis of the fragment. The nucleotide sequence was determined by the chemical modification method (13). The entire nucleotide sequence of the HindIII-D fragment (742 bp) is shown in Fig. 2. When open reading frames in the fragment (i.e., DNA sequences which when translated in a particular reading frame lack termination codons in that phase) were searched by computer, the longest frame that is another frame preceding the gluconate kinase and permease genes was identified in the coding strand of

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                                                                  -148
         AGCTTTCCT TAAAAGAAT CAGGTGCAA TGATGATTT TCATCCAAA TCGCCTCTT

                          Sau3AI          RsaI    Sau3AI-94
         TCAAAACAT GATTCACTT AACCTATTG ATCTCCAAT GTACCATAA TTGATCTGG

                                "AT-rich"                         -40
         AAATACATA CCATGCAAT ATGGTAAAA ATTTAAATA AAAATTAGA AATGAAAGT

  "-35" -35                       "-10" -10      +1              +15
         GTTTGCATA AAAGAAATA TTCACGTTA TCATACTTG TATACAAGT ATACTCCTT

  HO-UC UUUCCU  CCACU AG--                        mRNA                +67
         GAGTGAGGA AGGTGAGTGT  ATGCTA GACTCCAAA GACCTGTTG TATCCCGCA
                                      MetLeu AspSerLys AspLeuLeu TyrProAla
                      P28
                          HpaII                                      +121
         AAATGGCTC TCAAAAGCG TCAACCGGA GTTCGTGTC GCATACGAG CTGAGAATG
         LysTrpLeu SerLysAla SerThrGly ValArgVal AlaTyrGlu LeuArgMet

   Sau3AI                         RsaI                              +175
         CGGATCGTT TCAGGTCTG ATTGAAAGC GGTACCATT TTATCAGAA AATACAATC
         ArgIleVal SerGlyLeu IleGluSer GlyThrIle LeuSerGlu AsnThrIle

                                HpaII                               +229
         GCCGCCGAG TTTTCAGTA AGCCGTTCG CCGGTTCGC GAAGCGCTA AAAATACTC
         AlaAlaGlu PheSerVal SerArgSer ProValArg GluAlaLeu LysIleLeu

                                                                   +283
         GCATCCGAA AAAATCATC CGCTTAGAA CGAATGGGA GCGGTCGTA ATTGGTTTA
         AlaSerGlu LysIleIle ArgLeuGlu ArgMetGly AlaValVal IleGlyLeu

                                                                   +337
         ACTGAGAAG AAAATCGCG GAAATTTAT GATGTGCGG TTACTATTA GAAACATTT
         ThrGluLys LysIleAla GluIleTyr AspValArg LeuLeuLeu GluThrPhe

                                                        Sau3AI +391
         GTCTTTGAA CGGCTTGTC AAAATAGAC ATTGAGCCT TTAGTTAAG GATCTCAGC
         ValPheGlu ArgLeuVal LysIleAsp IleGluPro LeuValLys AspLeuSer

                                                                   +445
         AAAATTCTT GAAATGATG AAAGTCTCA ATCAAATAT GAGGATGCT GACGAATTT
         LysIleLeu GluMetMet LysValSer IleLysTyr GluAspAla AspGluPhe

                                                        Sau3AI +499
         TCATTTCAA GATGTGCTG TTCCATGAA ACGATTATC CGAGCGATT GATCATTCA
         SerPheGln AspValLeu PheHisGlu ThrIleIle ArgAlaIle AspHisSer

              Sau3AI                            +541
         TACATTCAG ATGATCTGG AACAATCTA AAACCCGTC ATGGAA
         TyrIleGln MetIleTrp AsnAsnLeu LysProVal MetGlu
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Fig. 2: Nucleotide sequence of the HindIII fragment. The coding strand of the nucleotide sequence is shown. The Sau3AI, RsaI and HpaII sites are underlined. A position of +1 is assigned as a site of transcription initiation as determined by nuclease S1 mapping. The AT-rich, -35 and -10 regions are thickly underlined. The bases (+17 to +30) show complementarity to the 3´ end of the 16 S rRNA. An ATG starting from +35 is a putative initiation codon for the P28 gene.

the two other genes. The putative translation start site could be an initiaton codon of ATG at the position of +35 (the 22nd codon of the longest frame, Fig. 2) because there was located approximately 10 bp upstream from this initiation codon, a long Shine-Dalgarno (SD) sequence (AGTGAGGAAGGTGA, +17 to +30) complementary to the 3´-end of 16S rRNA as shown in Fig. 2 [The

protein coding frame starting from the ATG (methionine) was putatively assigned as P28 gene encoding a protein whose molecular weight is 28 kDa.]. Based on the rules of Tinoco et al. (20), the calculated ΔG for the interaction between this SD sequence and 16S rRNA is -20.4 kcal/mol. Careful examination of the upstream region of the P28 gene revealed the putative -35 and -10 regions following the AT-rich region as shown in Fig. 2.

Location of the transcriptional start point of the gluconate operon

We employed the nuclease S1-mapping procedure of Berk and Sharp (16) to identify a transcriptional start site. Since the putative -35 and -10 regions were identified, a 291 bp HindIII-HpaII fragment which had been 5´-end labeled at the HpaII site (shown as a cross-hatched bar in Fig. 1) was used for hybridization. RNA preparations were isolated from strain 60015 (gnt^{+}) cells grown with or without 10 mM of gluconate and with 10 mM of gluconate and glucose. After hybridization, nuclease S1 was added to remove single-stranded nucleic acids, and the DNA-RNA hybrids were denatured and subjected to electrophoresis on DNA-sequencing gels, using as markers base-specific degradation fragments of the HindIII-HpaII fragment.

Fig. 3 shows the results of such an experiment. A specific mRNA was found only in cells grown with gluconate (lanes 1 and 2). However, this mRNA was not detected in cells both without gluconate (lanes 3 and 4) and with gluconate plus glucose (lanes 5 and 6). As expected, this mRNA (considered as mRNA for the gluconate operon) was specially synthesized from a position of +1 (adenine) of the sequence shown in Fig. 2 because only one main DNA fragment of 92 bases was observed even if the DNA-RNA hybrids were digested for different times (lanes 1 and 2).

Identification of a Sau3AI fragment containing a functional promoter of the gnt operon

Parallel to the sequencing of the HindIII-D fragment, we searched out which of the fragments produced by Sau3AI digestion of the BglII-B fragment (Fig. 1) contained a functional promoter in vivo by use of a promoter probe vector of pPL603B. The plasmid pPL603B was derived from a plasmid pPL603 which was improved by placing the BamHI site between the EcoRI sites (21,
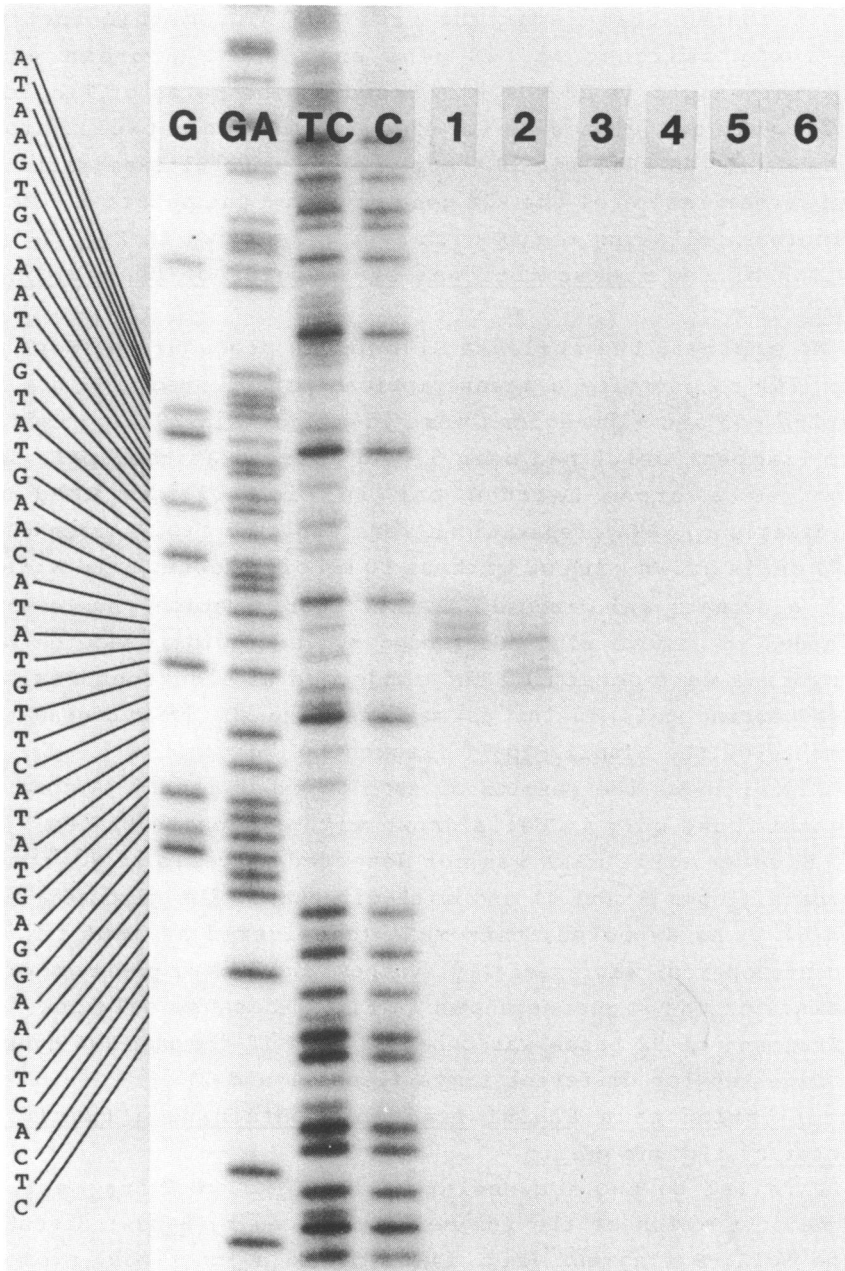
Fig. 3: Nuclease S1 analysis of transcript of the gnt operon.
The 5´-end labelled 291 bp HindIII-HpaII fragment was hybridized
with the indicated RNA samples, treated with nuclease S1 and

analyzed on a DNA sequencing gel (8%) (Details are described in
text.).   Base-specific chemical cleavages of this same labelled
fragment are shown in lanes G, GA, TC and C; each represents G,
G+A, T+C and C reaction.   RNA samples were extracted from: lanes
1 and 2, strain 60015 grown with gluconate; lanes 3 and 4, grown
without gluconate; and lanes 5 and 6, grown with gluconate and
glucose.   The RNA-DNA hybrids were treated with nuclease S1 for
5 min (lanes 1, 3 and 5) and for 15 min (lanes 2, 4 and 6).   The
sequence complementary to positions (-23 through +23) is
indicated.

D. Rothstein, personal communication).   If a Sau3AI fragment

containing a functional promoter is placed in a proper

orientation at the BamHI site of the plasmid, it allows the

expression of the downstream cat-86 gene that renders the cell

chloramphenicol-resistant (Cm^r).   The BglII-B fragment (see Fig.

1)(2 µg) containing a 5´-region of the gnt operon was partially

digested with Sau3AI because we were afraid at that time that

this enzyme might cut the gnt promoter sequence.   The partial

digest was ligated with pPL603B (1 µg) which had been digested

with BamHI.   The ligated DNA was transfered to the competent

culture of strain 1A423 (gnt^+ recE4). Cm^r transformants were

selected on plates [N medium (22) + 0.1% Na_3 citrate] containing

Cm (10 µg/ml) where Casamino Acids that do not repress the

expression of the gnt operon (14) were used as carbon source and

10 mM gluconate was supplemented.   Although 14 transformants

were obtained, we were unable to distinguish one from another in

their Cm resistancy on plates.   When one of the transformants

[strain 1A423 (pgnt23): strain 1A423 bearing a plasmid of pgnt23

(Fig. 4A)] was grown in the same but liquid medium using Casamino

Acids as carbon source without and with 10 mM gluconate, cells

exhibited their Cm resistance of 80 and 120 µg/ml, respectively.

Then, we measured the CAT activity under conditions of induction

and repression.   As shown in Fig. 4B, CAT synthesis was highly

derepressed in strain 1A423 (pgnt23) (see the activity at 0 h),

which was further induced during 2 h incubation with gluconate

but the simultaneous addition of gluconate and glucose still

repressed the synthesis of the enzyme.   The highly repressed

synthesis of the CAT was also repressed by the addition of only

glucose (10 mM) (data not shown).   On the other hand, strain

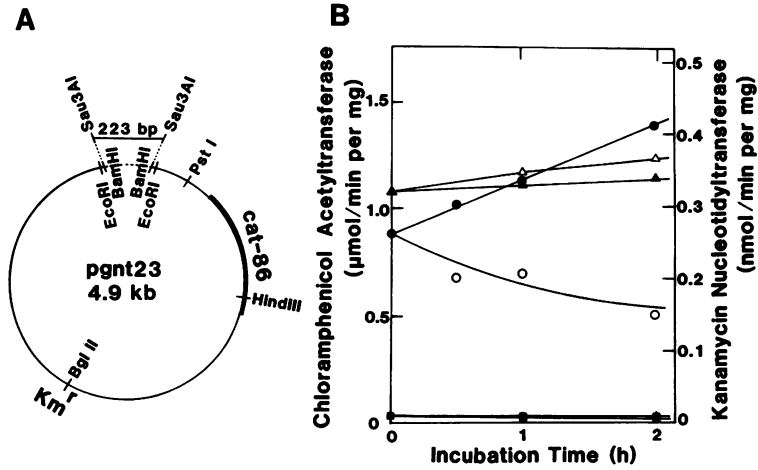1A423 (pPL603B) could not synthesize a significant level of the

A

B

Fig. 4:   A. Structure of plasmid pgnt23.   A derivative of plasmid pPL603B, plasmid pgnt23, had an insertion of the 223 bp Sau3AI fragment possessing the gnt promoter in the BamHI site of pPL603B.   This insertion allowed the expression of cat-86 gene. The BamHl site is flanked by two EcoRI sites so that the insertion is able to be excised for its analysis.   The gene encoding kanamycin nucleotidyltransferase (its location is pointed by Km^r) and the cat-86 encoding the CAT are derived from plasmid pUB110 and Bacillus pumilus, respectively (21).   B. Synthesis of the CAT and kanamycin nucleotidyltransferase in strain 1A423 bearing plasmid pgnt23.   The cells grown at 37°C overnight on tryptose blood agar base (Difco) containing 10 mM glucose and 10 µg/ml kanamycin was inoculated in 200 ml of the S6 medium containing 0.5% Casamino Acids that had been supplemented with 50 µg/ml each of arginine, threonine and leucine, 5 µg/ml kanamycin and 10 µg/ml Cm.   When the culture reached an OD_{600} of 0.25 (In the case of strain 1A423 bearing plasmid pPL603B, it took over 18 h to reach this OD), 10 OD_{600} units of the cells were harvested as uninduced sample.   The remaining culture was divided into two parts; one was incubated at 37°C with 10 mM gluconate and the other with 10 mM each of gluconate and glucose. After incubation for the indicated times, 10 OD_{600} units of the cells   were   harvested.   In   the   case   of   kanamycin nucleotidyltransferase measurements, 15 OD_{600} units of the cells were collected.   The preparation of cell extracts and enzyme assays are described in test.   CAT of strain 1A423 (pgnt23)[+ gluconate (●), + gluconate and glucose (○)] and strain 1A423(pPL603B)[+ gluconate (■), + gluconate and glucose (□)]. Kanamycin nucleotidyltransferase of strain 1A423 (pgnt23)[+ gluconate (▲), + gluconate and glucose (△)].   Uninduced samples were plotted as 0 h of the incubation time.

CAT under these conditions of cell growth (Fig. 4B).

To eliminate the possibility that the change of the CAT activity may reflect that of copy number of pgnt23 etc., due to the alteration of the medium constituent, we investigated in the same growth conditions the expression of kanamycin nucleotidyltransferase gene in pgnt23 that was originally derived from plasmid pUB110. As shown in Fig. 4B, we could not find any significant change of the enzyme activity in response to the addition of gluconate or the simultaneous addition of gluconate and glucose.

To find out which Sau3AI fragment was inserted into the BamHI site of pPL603B, the insert was excised from pgnt23 by utilizing the flanking EcoRI sites (Fig. 4A) and sequenced. The sequence of the insert revealed that a 223 bp of Sau3AI fragment (as indicated in Fig. 1) was cloned in the BamHI site of pPL603B.

Titration of gnt repressor with the 223 bp fragment

The cat-86 gene, placed under the control of the gnt promoter on a high copy plasmid, allowed a highly derepressed synthesis of the CAT. If the limited amount of a gnt regulatory protein in the cell is unable to repress the function of the promoter on a high copy plasmid despite the binding of the protein to the regulatory sequence of the promoter region, the expression of the chromosomal gnt operon as well as that of the cat-86 gene would be derepressed. To test this hypothesis, we investigated the expression of the genes of the gluconate kinase and permease on the chromosome of strain 1A423 bearing plasmid pgnt23. As shown in Fig. 5, the synthesis of the gluconate kinase and permease was highly derepressed, which were further induced during 2 h incubation with gluconate but the simultaneous addition of gluconate and glucose repressed the synthesis of the enzymes, in a similar manner as the CAT synthesis. On the other hand, strain 1A423 (pPL603B) normally induced the two enzymes and their induction is repressed by glucose (Fig. 5). It is, therefore, concluded that the titration of the gnt regulatory protein (likely gnt repressor) with the 223 bp fragment on a high copy plasmid caused the cell to synthesize at a highly
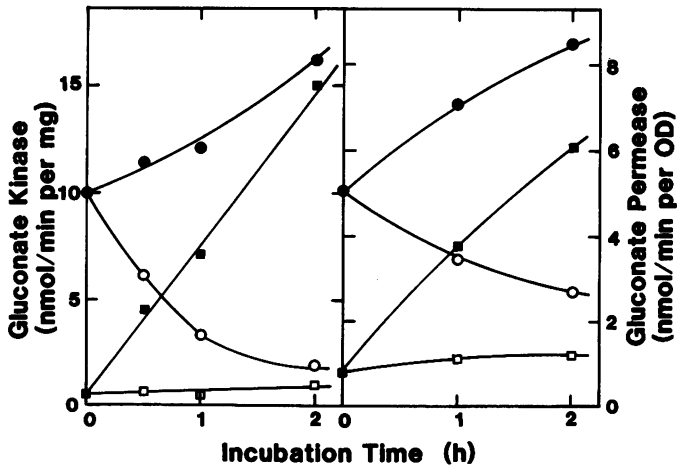
Fig. 5: Synthesis of the gluconate kinase and permease in strain 1A423 bearing plasmid pgnt23. The preparation of cell extracts and enzyme assays are described in text. Strain 1A423 (pgnt23) [+ gluconate (●), + gluconate and glucose (O)]. Strain 1A423 (pPL603B) [+ gluconate (■), + gluconate and glucose (□)].

derepressed level not only the CAT encoded in the plasmid but also the gluconate kinase and permease encoded in the host chromosome.

DISCUSSION

The entire sequence of the HindIII-A and -D fragments (4,487 bp) revealed three protein coding frames; the 5´-distal frame is designated as the P28 gene encoding a protein whose function is not known and the two following frames code for the gluconate kinase and permease (Y. Fujita, T. Fujita and Y. Aratani, unpublished results; Fig. 2). A promoter-like structure presumably utilized by B. subtilis sigma-43 (formerly sigma-55) RNA polymerase (23) was detected in the sequence of the upsteam region of the P28 gene (Fig. 2). The identification of the initiation site of the transcription by nuclease S1 mapping (Fig. 3) confirmed that this structure is actually utilized as a functional promoter in vivo. The mapping qualitatively revealed that the transcription from this site was induced upon the addition of gluconate to the medium and the induction was

repressed by glucose. Furthermore, the 223 bp fragment containing this structure on a high copy plasmid possessed a functional promoter activity and caused the Gnt^{+} cell to synthesize at a highly derepressed level the gluconate kinase and permease probably due to the titration of the gnt repressor by this fragments (Fig. 4B and 5). These results strongly indicate that this structure is a promoter of the gnt operon whose expression is regulated at the transcriptional level.

All of the promoters recognized by the sigma-43 display striking conformity in their -35 and -10 region with the corresponding consensus sequences (TTGACA and TATAAT respectively) for E. coli promoters (24). The distance between the -35 and -10 regions (17 or 18 bp) is also very well conserved. The promoter of the gnt operon that is the first sample of a catabolic promoter, shows similarity to these other described promoters, with a -35 region sequence (TTGCAT) and -10 region sequence (TATCAT) separated by a spacing of 17 bp (See Fig. 2). The -35 region is identical with that of the promoter of the Bacillus licheniformis penicillinase gene (25). If the -35 region (TTGCAT) is considered as a deletion of adenine between guanine and cytosine of the consensus sequence (TTGACA), the spacing between the -35 and -10 regions would be 18 bp. In the immediate upstream of the -35 region of the gnt promoter, a long AT-rich region comprised of 36 bp was observed (Fig. 2). Although its function is not known, it might participate directly in promoter binding or initiation as suggested in the case of the promoter of spoVG (26).

The expression of not only the chromosomal gnt operon but also the cat-86 gene under the control of the gnt promoter on the 223 bp fragment was under catabolite repression. This fact suggests that the fragment possesses an intact regulatory sequence to interact with a factor involved in the catabolite repression and that the amount of this factor is enough to regulate the promoter on a high copy plasmid. In addition, this fragment has to contain an intact sequence to interact with the gnt repressor as the 223 bp fragment on the plasmid could titrate it. These two interacting sequences are probably located at different positions within the fragment because the induction by

```
-100
   GATCTGGAAATACATACCAT G C A  IR 1
                     A T G G T A T A A
                     A
                     A
          DR         A
                     A
                     A
                     T
              T A A A T T           IR 2
              A                     T A T A +1
              A                     GC  T
              A                     T·A
              A                     T·A
              T                     C·G
   AAAGTAAAGAT                      A·T   mRNA
   G                                T A
   T                                A T
   GT TTGCAT AAAAGAAATATTCACGT TATC  ACTCCTTGAGTGAGGAAGGTGAGTGTATGCTA
      "-35"              "-10"           "SD"                         G
                                                          P28         A
                                                                      C
                                                                      T
                                                                      C
                                                                      C
   IR 3  G CTGTGCTTGAGGCCAACTGCGAAAACTCTCGGTAAAACGCCCTATGTTGTCCAGAAA
         G CATACGAGCT
           G
           A
           G
           A
           A
           T
           G
           C
           G +123
```
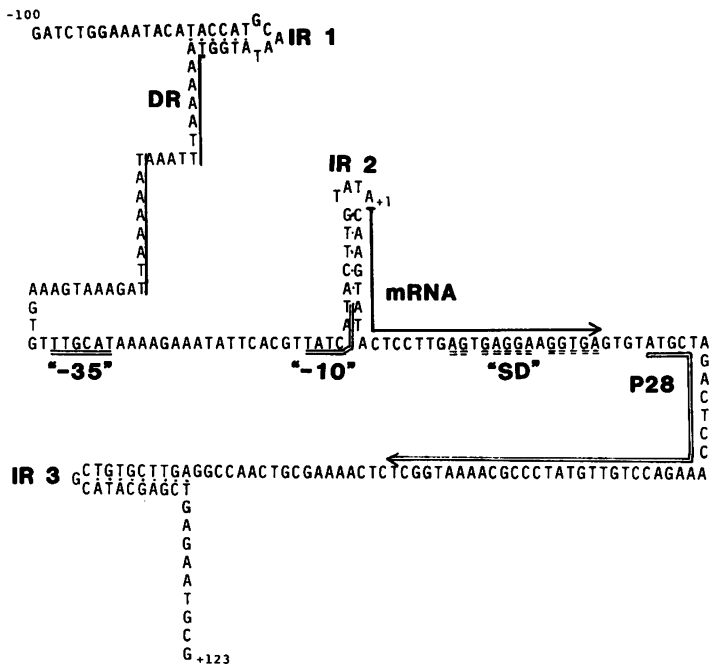
Fig. 6: Inverted repeats and a direct repeat in the 223 bp fragment. Three inverted repeats (IR 1, 2 and 3) and one direct repeat (DR) are shown. The -35, -10 and SD regions and the start points of transcription and translation are also indicated.

gluconate and its catabolite repression are likely separable as we infer from the fact that the synthesis of the gluconate kinase and permease as well as the CAT was highly derepressed but nevertheless it was under control of catabolite repression (Figs. 4B and 5).

To find out the possible location of two kinds of regulatory sequences on the 223 bp fragment, its sequence was analyzed by personal computer to search inverted and direct repeats that could be regulatory sequences. When inverted repeats were searched by the following determinants; minimum stacking length = 6 bp, maximum stacking energy ΔG=-10 kcal/mol, and maximum loopout = 20 bases), three inverted repeats (IR1, 2 and 3; ΔG = -12.6, -11.1 and -11.5 kcal/mol, respectively) were printed out as shown in Fig 6. The longest direct repeat was TAAAAATT found in the AT-rich region (Fig. 6). There are known two inverted

repeat sequences in the promoter region of <u>E. coli</u> <u>lac</u> operon (1); one is the immediate upstream of the -35 region, that interacts with the catabolite activator protein and the other is close to the initiation site of transcription, to which the <u>lac</u> reperessor binds. Since IR1 is the immediate upstream of the AT-rich region and IR2 is within the start site of transcription, they might be the possible sequences to interact with the factor involved in the catabolite repression and the <u>gnt</u> repressor.

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