# Molecular Genetic Analysis of Gluconate Uptake and Catabolism in *Escherichia coli*

by

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## Abbreviations

aa, amino acid(s)
b, base
bp, base pair(s)
β-Gal, β-galactosidase
CAT, chloramphenicol acetyltransferase
CRP, cAMP receptor protein
FITC, fluorescein isothiocyanate
GntI, major gluconate uptake and catabolism system
GntII, subsidiary gluconate uptake and catabolism system
*gntK*, gene encoding gluconate kinase in GntI
GntK, gene product of *gntK*
*gntP*, gene encoding high affinity gluconate permease
GntP, gene product of *gntP*
*gntR*, gene encoding regulator of the *gnt* genes
GntR, gene product of *gntR*
*gntT*, gene encoding high affinity gluconate permease in GntI
GntT, gene product of *gntT*
*gntU*, gene encoding low affinity gluconate permease in GntI
GntU, gene product of *gntU*
*gntV*, gene encoding gluconate kinase in GntII
GntV, gene product of *gntV*
*gntW* (*gntS*), gene encoding gluconate permease in GntII
GntW, gene product of *gntW*
*gntH*, gene encoding regulator of the *gnt* genes
GntH, gene product of *gntH*
kb, kilobase pair(s) or 1000 bp
*lacZ*, gene encoding β-galactosidase
nt, nucleotide(s)
ORF, open reading frame
REP sequence, repetitive extragenic palindromic sequence

## Introduction

In *Escherichia coli*, gluconate can be provided as an exogenous carbon source, but in addition it may be produced by the direct oxidation of glucose by glucose dehydrogenase (GDH). There are at least two systems for glucose uptake and catabolism in *E. coli* (Fig. 1). One is the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Postma *et al.*, 1989) that phosphorylates glucose to glucose-6-phosphate and concomitantly imports it into the cells, which is then catabolized by the Emden-Meyerhof-Parnas (EMP) pathway (Frankel, 1987). The other is glucose oxidation system (van Schie *et al.*, 1985), in which glucose is oxidized at the periplasm by the membrane-bound GDH and the resulting gluconate is imported through the inner membrane by gluconate permeases. The imported gluconate is subsequently phosphorylated to gluconate-6-phosphate (Eisenberg & Dobrogosz, 1967), which is then catabolized by the Entner-Doudroff (ED) and pentose phosphate (PP) pathways (Conway, 1992; Eisenberg & Dobrogosz, 1967). Glucose is preferentially taken by the former system into the cells as the most convenient carbon source. Although in the presence of various sugars, the catabolic pathway of glucose predominantly functions and those of most sugars are repressed by the glucose effect (Magasanik & Neidhardt, 1970) that is assumed to be performed by PTS (Saier, 1989), *E. coli* is able to take up and catabolize gluconate with glucose, without the repression by glucose (Lin, 1996; Porco *et al.*, 1997). A few sugars including gluconate are not subject to the glucose effect. The facts emphasize that the ability to catabolize gluconate plays an important physiological role for the organism although the catabolism has not been elucidated in detail.

In general, sugar uptake and catabolism are important for bacteria to acquire carbon and energy sources, which are indispensable for their growth. Especially, when they start to proliferate from the depleted intracellular energy condition such as stationary phase or resting state, they should require the energy as a starter for import of various extracellular substrates. For acquisition of the energy, GDH may be suitable that is involved in forming membrane potentials by the glucose oxidation at the periplasm followed by electron transfer to ubiquinone and cytochrome oxidase (van Schie *et al.*, 1985). Additionally, after sugar import, the ED pathway via the glucose oxidation system may have the advantage of faster energy production than the EMP pathway via the PTS system because reaction steps in the former pathway are less than those in the latter. Therefore, it may be possible that the glucose oxidation system followed by the ED pathway contributes for cells to survive and compete with other bacteria under the nutrient-limited condition, and that the two systems for glucose uptake are assumed to be selectively used in response to the cell growth phase or environmental conditions. Although a number of biochemical and molecular genetic studies on PTS and GDH in *E.*

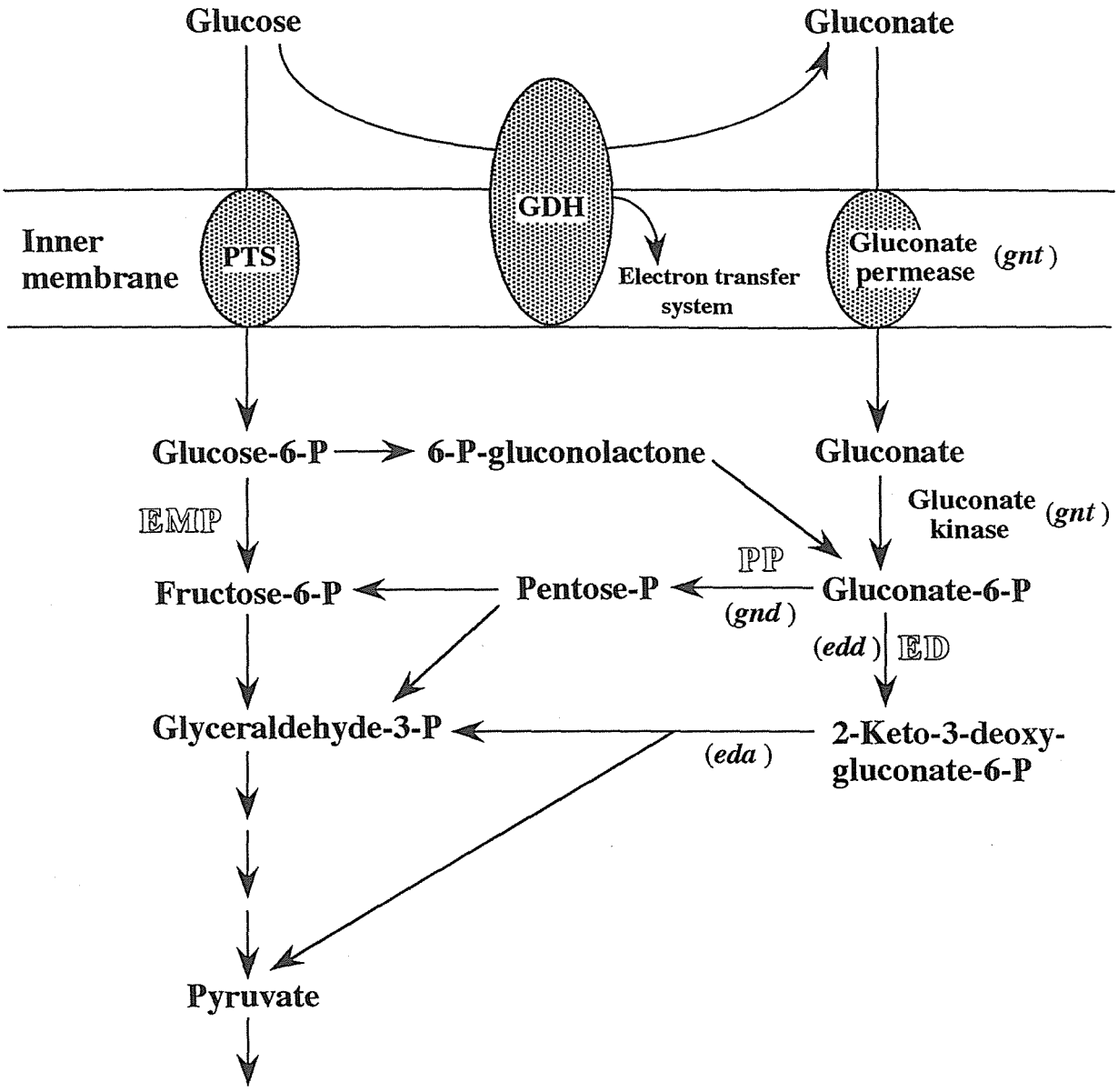


**Figure 1. Pathways of gluconate catabolism in *E. coli*.**
The first step of gluconate catabolism in *E. coli* is its entry into cells by gluconate permease and it is subsequently phosphorylated to gluconate-6-phosphate by gluconate kinase. Gluconate-6-phosphate is then catabolized by the Entner-Doudoroff (ED) and pentose phosphate (PP) pathways. The uptake and utilization system of gluconate in *E. coli* is closely related to that of glucose, which consists of two systems, the phosphotransferase system (PTS) and glucose dehydrogenase (GDH)-dependent glucose oxidation system. Glucose is phosphorylated to glucose-6-phosphate by the PTS system and catabolized by the Emden-Meyerhof-Parnas (EMP) pathway. On the other hand, glucose is oxidized at the periplasm by GDH and the resulting gluconate is imported into cells by gluconate permease.

*coli* have been performed, no study has been reported on the physiological significance of the glucose oxidation system, especially gluconate uptake and catabolism.

The initial gluconate uptake and phosphorylation can be achieved by two different systems, GntI and GntII (Istúriz *et al.*, 1986). The GntI that functions as a main system consists of high and low affinity gluconate permeases and a thermoresistant gluconate kinase encoded by the *gntT, gntU,* and *gntK* genes, respectively (Hung *et al.*, 1970; Zwaig *et al.*, 1973; Bächi & Kornberg, 1975). The GntII consists of another gluconate permease and a thermosensitive gluconate kinase encoded by the *gntW* (*gntS*) and *gntV* genes, respectively (Hung *et al.*, 1970; Zwaig *et al.*, 1973; Bächi & Kornberg, 1975). It is thought to be a subsidiary system, but little is known about it. The genes for the GntI and GntII are located around 77 and 96 min on the *E. coli* genome, respectively (Nagel de Zwaig *et al.*, 1973; Faik & Kornberg, 1973; Zwaig *et al.*, 1973; Miller, 1992). The expression of the GntI genes appears to be positively controlled by cAMP (Bächi & Kornberg, 1975). GntR has been believed to act as a repressor for the GntI genes, *edd* encoding 6-phosphogluconate dehydratase, and *eda* encoding 2-keto-3-deoxy-6-phosphogluconate aldolase (Zwaig *et al.*, 1973; Istúriz *et al.*, 1986). The latter two gene products are constituents of the ED pathway. Recently, another gene, *gntP*, encoding a high affinity gluconate permease was discovered in *E. coli*, and its expression was shown to be repressed by the presence of gluconate or glucose (Klemm *et al.*, 1996). GntP was thus hypothesized to allow entry of gluconate into cells for induction of other *gnt* genes. To elucidate the physiological significance and the mutual regulation of two glucose uptake systems in glucose utilization of *E. coli*, it may be crucial to define the molecular mechanism of the gluconate uptake and catabolism.

To do so, I have achieved several molecular genetic and biochemical studies on the *gnt* genes. In CHAPTER 1, the *gnt* genes, *gntRKU*, involved in the GntI system of *E. coli* were cloned and characterized to define their gene organization and expressional regulation. Moreover, by homology searching, their gene products were predicted and the possible GntII genes were also expected. CHAPTER 2 showed purification and characterization of the thermoresistant gluconate kinase (EC 2.7.1.12) encoded by *gntK* gene and compared its characteristics with those of the thermoresistant gluconate kinase reported by Vivas *et al* (1994). In CHAPTER 3, four homologues to GntT in *E. coli* were shown, which in addition to the GntT and GntU appear to constitute a GntP family with similar topological structures consisting of 14 membrane-spanning segments. CHAPTER 4 showed the expressional regulation of the *gntT* gene in the GntI and the transport ability and specificity of the gene product. In CHAPTER 5, the *gnt* genes, *gntV, yjgV, yjgU, gntW,* and *gntH,* involved in the GntII system were cloned and characterized. Their expressional control and mutual regulation between the GntI and GntII systems were analyzed.

# CHAPTER 1

## Gene organization and transcriptional regulation of the *gntRKU* operon involved in gluconate uptake and catabolism of *Escherichia coli*

## Summary

The *gntRKU* operon encoding part of the GntI system involved in gluconate uptake and catabolism by *Escherichia coli* was cloned and characterized. The operon was shown to encode its repressor, a thermoresistant gluconate kinase, and a low affinity gluconate permease. CAT fusion analysis revealed that the operon has a promoter for *gntR* and another for *gntKU*, and that the *gntR* gene is constitutively expressed, while that of *gntKU* is regulated positively by the cAMP-CRP complex and negatively by GntR. Read-through transcription from the *gntR* promoter into *gntK* was decreased in the presence of GntR, although GntR did not repress its own promoter. In addition, transcriptional attenuation was observed after the *gntK* gene, so *gntU* expression is reduced presumably to modulate the production of the low affinity gluconate permease according to the available concentration of gluconate.

## Results and Discussion

### Cloning of the *gnt* genes involved in gluconate uptake and catabolism of *E. coli*

As described in Materials and Methods, I screened a genomic library from *E. coli* W3110 and obtained plasmid pGNT5, which conferred a positive gluconate fermentation phenotype upon the Gnt$^-$ (*gntT*$^-$) mutant. The genomic position of the inserted DNA fragment on pGNT5 was at 77 min, where the genes of the GntI system are mapped (Hung *et al.*, 1970; Zwaig *et al.*, 1973; Bächi & Kornberg, 1975; Miller, 1992). Based on the physical map of pGNT5, a larger clone, pGNT15, was obtained from the 16A5 Kohara phage clone (Fig. 2).

Gluconate kinase activity was measured in cells harboring pGNT5 or pGNT15, which were cultured under various conditions (Table 1). In the absence of gluconate, the activity of YU120 cells harboring pGNT5 or pGNT15 was about 300- and 1.4-fold higher, respectively, than that of YU120 cells harboring the vector. The higher activity from pGNT5 in the absence of gluconate may be due to de-repression caused by the absence of *gntR* on the plasmid (all strains except Hfr G6MD2 possessed *gntR* on the chromosome). The lower activity from pGNT15 seems to be due to the presence of the *gntR* gene encoding a repressor (see below) on the plasmid. Comparable activities were obtained using Hfr G6MD2 as a host strain. The activity of CA8306 (*Δcya*) harboring these clones was higher in the presence of cAMP, suggesting that *gntK* expression is controlled by cAMP-CRP. In contrast, the activities of cells harboring pGNT5 decreased in the presence of gluconate, although those of LJ288 and CA8306 harboring the vector were high. The decrease may be due to the catabolite repression via reduction of intracellular cAMP because cAMP-free cells, CA8306 harboring pGNT5 in the absence of cAMP, showed no such decrease by the addition of gluconate.

### Nucleotide sequencing of the cloned *gnt* genes and *gntR* encoding the repressor of the GntI system

The nucleotide sequence of part of the genomic DNA fragment of pGNT15 was determined to find three open reading frames (Fig. 3). Each ORF is accompanied by a canonical ribosome recognition sequence (Shine & Dalgarno, 1974) upstream of its initiation codon. In front of both ORF1 and ORF2, possible -10 and -35 promoter sequences occur (see later), but no such sequence appears in front of ORF3. A sequence with dyad symmetry followed by a series of T residues with the potential to form a stem-loop structure was found within the beginning of ORF3. This structure resembles a ρ-independent terminator, which may attenuate transcription as described below. Additionally, there is a second sequence with dyad symmetry which may act as a ρ-independent terminator, following ORF3.

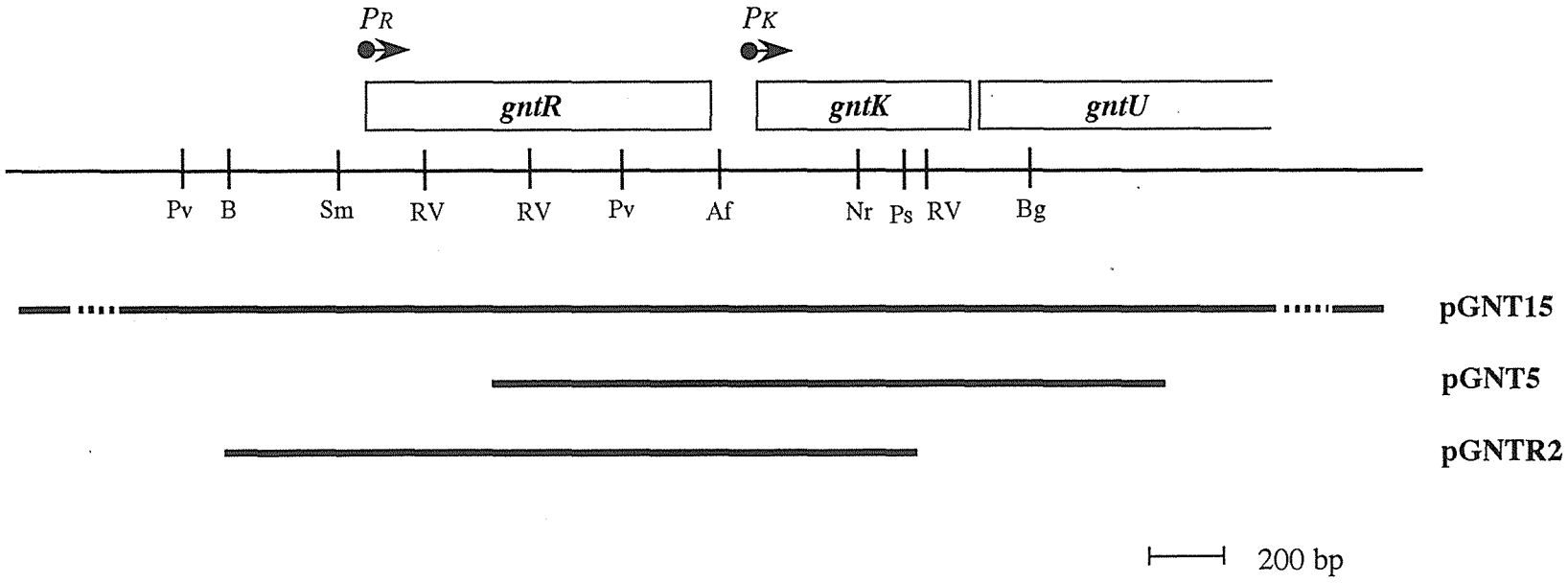


**Figure 2. Physical map of the *gntRKU* operon and *gnt* gene clones.**
The *gntRKU* genes are adjacently located at 75.5 min on the *E. coli* W3110 genome, and the *gntR, K,* and *U* genes corresponding to ORF1, ORF2, and ORF3, respectively, are represented by boxes. The transcriptional start sites and directions of the promoters in front of *gntR* (*PR*) and *gntK* (*PK*) are shown by circles and arrows, respectively. Plasmid pGNT15 with the 10-kb *Eco*RI fragment from the Kohara clone, 16A5, includes the entire *gntRKU* genes. Plasmid pGNT5 isolated from a genomic library bears a 2.0-kb fragment bearing the *gntK* gene. Plasmid pGNTR2 with the 1.9-kb *Bam*HI-*Pst*I fragment from pGNT15 bears the *gntR* gene. Pv, *Pvu*II; B, *Bam*HI; Sm, *Sma*I; RV, *Eco*RV; Af, *Afl*II; Nr, *Nru*I; Ps, *Pst*I; Bg, *Bgl*II.

**Table 1. Gluconate kinase activity of the cells harboring various *gnt* clones.**

| Strain | Gluconate kinase activity (mU/mg) [a] | | | | | |
|---|---|---|---|---|---|---|
| | +gluconate | -gluconate | +cAMP +gluconate | +cAMP -gluconate | -cAMP +gluconate | -cAMP -gluconate |
| LJ288 (pBR322) | 34 | 1.3 | - [b] | - | | |
| LJ288 (pGNT5) | 46 | 150 | - | - | | |
| LJ288 (pGNT15) | 1.9 | 1.5 | - | - | | |
| YU120 (pBR322) | 0.5 | 0.92 | - | - | | |
| YU120 (pGNT5) | 45 | 290 | - | - | | |
| YU120 (pGNT15) | 1.6 | 1.3 | - | - | | |
| Hfr G6MD2 (pBR322) | 0.26 | 0.28 | - | - | | |
| Hfr G6MD2 (pGNT5) | 550 | 1040 | - | - | | |
| Hfr G6MD2 (pGNT15) | 2.4 | 0.7 | - | - | | |
| CA8306 (pBR322) | | | 41 | 6.7 | 1.9 | 2.5 |
| CA8306 (pGNT5) | | | 450 | 900 | 40 | 32 |
| CA8306 (pGNT15) | | | 49 | 26 | 5.4 | 6.5 |

[a] Cells were incubated at 37 °C for 4 h with or without gluconate and/or cAMP. Gluconate kinase activity was measured as described in Materials and Methods. Reported values are the averages of 2-4 independent experiments performed in triplicate.

[b] Not determined.

```
        10         20         30         40         50         60
CTGGTGCTCTCGCCGGATGCGCGAGATGGTTCATTGAAAGTGCATCAGGATATGGAACTG
        70         80         90        100        110        120
TACCGCTGGGCGTTGCTGAAAGATGAGCAGTCGGTGCATCAGATTGCCGCTGAACGCCGC
       130        140        150        160        170        180
GTCTGGATCCAGGTGGTGAAAGGCAATGTCACCATTAACGGCGTGAAAGCCTCGACCAGC
       190        200        210        220        230        240
GATGGTCTGGCAATCTGGGATGAGCAGGCAATCTCCATCCATGCGGATAGCGACAGCGAA
       250        260        270        280        290        300
GTGTTACTGTTCGATCTGCCGCCGGTTTAAAACTCAACGGCATCTTTGAAGCCTGCCTTT
       310        320        330        340        350        360
TTGCAGGCTTCCTCGCTCGCTCCGGTCGCGTGCTGATGTACGCTTCCGGGGTTTTGCCGC
       370        380        390        400        410        420
GTCGTTAGCACAATGCGATGCAATTGATTTAGTGTATATCTAATAATTGTAAACAAGCAC
       430        440        450        460        470        480
TGTTTTCCCCGGGAAGGTTCTGATGTGTCCGTGTTAAACTAAGAGAATCTATCTCTTTTG
                  -35                        -10
       490        500        510        520        530        540
TACCTTCAGGACGATGAAAAAGAAAAGACCCGTACTTCAGGATGTGGCTGACCGTGTAGG
        ____ _
            M  K  K  K  R  P  V  L  Q  D  V  A  D  R  V  G
            gntR start
       550        560        570        580        590        600
CGTGACCAAAATGACGGTCAGCCGTTTTTTACGCAACCCGGAGCAGGTTTCCGTCGCTCT
 V  T  K  M  T  V  S  R  F  L  R  N  P  E  Q  V  S  V  A  L

       610        620        630        640        650        660
ACGCGGCAAGATTGCCGCGGCTCTTGATGAACTGGGCTATATTCCCAATCGTGCGCCCGA
 R  G  K  I  A  A  A  L  D  E  L  G  Y  I  P  N  R  A  P  D

       670        680        690        700        710        720
TATCCTCTCTAACGCCACCAGCCGGGCGATTGGCGTCCTGTTACCTTCTCTCACCAACCA
 I  L  S  N  A  T  S  R  A  I  G  V  L  L  P  S  L  T  N  Q

       730        740        750        760        770        780
GGTTTTCGCGGAAGTATTACGCGGAATCGAAAGCGTCACCGACGCGCACGGTTATCAGAC
 V  F  A  E  V  L  R  G  I  E  S  V  T  D  A  H  G  Y  Q  T

       790        800        810        820        830        840
CATGCTGGCGCACTACGGTTATAAACCGGAAATGGAGCAAGAACGCCTCGAATCCATGCT
 M  L  A  H  Y  G  Y  K  P  E  M  E  Q  E  R  L  E  S  M  L

       850        860        870        880        890        900
CTCCTGGAATATCGACGGCCTGATCCTCACCGAACGTACCCACACGCCGCGCACCTTAAA
 S  W  N  I  D  G  L  I  L  T  E  R  T  H  T  P  R  T  L  K

       910        920        930        940        950        960
GATGATTGAAGTGGCGGGTATTCCCGTGGTGGAACTGATGGACAGCAAGTCGCCATGCCT
 M  I  E  V  A  G  I  P  V  V  E  L  M  D  S  K  S  P  C  L

       970        980        990       1000       1010       1020
TGATATCGCCGTCGGTTTTGATAACTTTGAAGCAGCACGCCAGATGACCACTGCCATTAT
 D  I  A  V  G  F  D  N  F  E  A  A  R  Q  M  T  T  A  I  I

      1030       1040       1050       1060       1070       1080
TGCTCGCGGGCATCGCCACATTGCCTATCTCGGCGCACGTCTCGACGAACGTACTATCAT
 A  R  G  H  R  H  I  A  Y  L  G  A  R  L  D  E  R  T  I  I

      1090       1100       1110       1120       1130       1140
CAAACAGAAGGGATACGAACAGGCGATGCTGGATGCAGGCCTGGTGCCATATAGCGTGAT
 K  Q  K  G  Y  E  Q  A  M  L  D  A  G  L  V  P  Y  S  V  M
```

GGTTGAGCAATCTTCTTCTTACTCTTCCGGTATTGAACTGATTCGCCAGGCGCGGCGGGA
V E Q S S S Y S S G I E L I R Q A R R E

1210 1220 1230 1240 1250 1260
ATATCCGCAGCTGGATGGCGTGTTCTGTACGAATGATGACCTGGCGGTCGGCGCGGCGTT
Y P Q L D G V F C T N D D L A V G A A F

1270 1280 1290 1300 1310 1320
TGAATGTCAGCGTCTGGGGTTAAAAGTTCCTGACGATATGGCGATTGCCGGTTTCCACGG
E C Q R L G L K V P D D M A I A G F H G

1330 1340 1350 1360 1370 1380
TCATGACATTGGTCAGGTGATGGAGCCACGACTTGCGAGCGTGCTGACGCCGCGTGAGCG
H D I G Q V M E P R L A S V L T P R E R

1390 1400 1410 1420 1430 1440
GATGGGCAGTATTGGCGCTGAACGCCTGCTGGCGCGTATTCGTGGCGAATCTGTGACACC
M G S I G A E R L L A R I R G E S V T P

1450 1460 1470 1480 1490 1500
GAAAATGTTAGATTTAGGTTTCACCTTGTCACCGGGCGGATCTATTTAAGCCCACAAATT
K M L D L G F T L S P G G S I TER

1510 1520 1530 1540 1550 1560
TGAAGTAGCTCACACTTATACACTTAAGGCATGGATGGATATTGCTTCTGATATTGTCCG
-35

1570 1580 1590 1600 1610 1620
GCTGGACAATGTTACCGATAACAGTTACCCGTAACATTTTTAATTCTTGTATTGTGGGGG
-10

1630 1640 1650 1660 1670 1680
CACCACTTTGAGCACGACTAACCATGATCACCACATTTACGTCTTGATGGGCGTATCGGG
M S T T N H D H H I Y V L M G V S G

*gntK* start

1690 1700 1710 1720 1730 1740
CAGCGGCAAATCTGCGGTCGCCAGTGAAGTGGCGCATCAACTTCATGCCGCGTTTCTTGA
S G K S A V A S E V A H Q L H A A F L D

1750 1760 1770 1780 1790 1800
TGGCGATTTCCTCCATCCACGGCGCAATATCGAAAAAATGGCGTCTGGCGAACCACTGAA
G D F L H P R R N I E K M A S G E P L N

1810 1820 1830 1840 1850 1860
TGACGACGATCGCAAACCGTGGTTGCAGGCGCTGAACGACGCCGCGTTTGCTATGCAGCG
D D D R K P W L Q A L N D A A F A M Q R

1870 1880 1890 1900 1910 1920
CACTAATAAAGTGTCGCTGATCGTCTGTTCTGCATTGAAAAAACACTATCGCGACTTGCT
T N K V S L I V C S A L K K H Y R D L L

1930 1940 1950 1960 1970 1980
GCGTGAAGGTAATCCGAATCTCTCTTTCATCTATTTGAAAGGCGATTTTGATGTGATTGA
R E G N P N L S F I Y L K G D F D V I E

1990 2000 2010 2020 2030 2040
AAGCCGCCTGAAAGCGCGCAAAGGCCATTTCTTTAAAACCCAAATGTTGGTGACGCAGTT
S R L K A R K G H F F K T Q M L V T Q F

2050 2060 2070 2080 2090 2100
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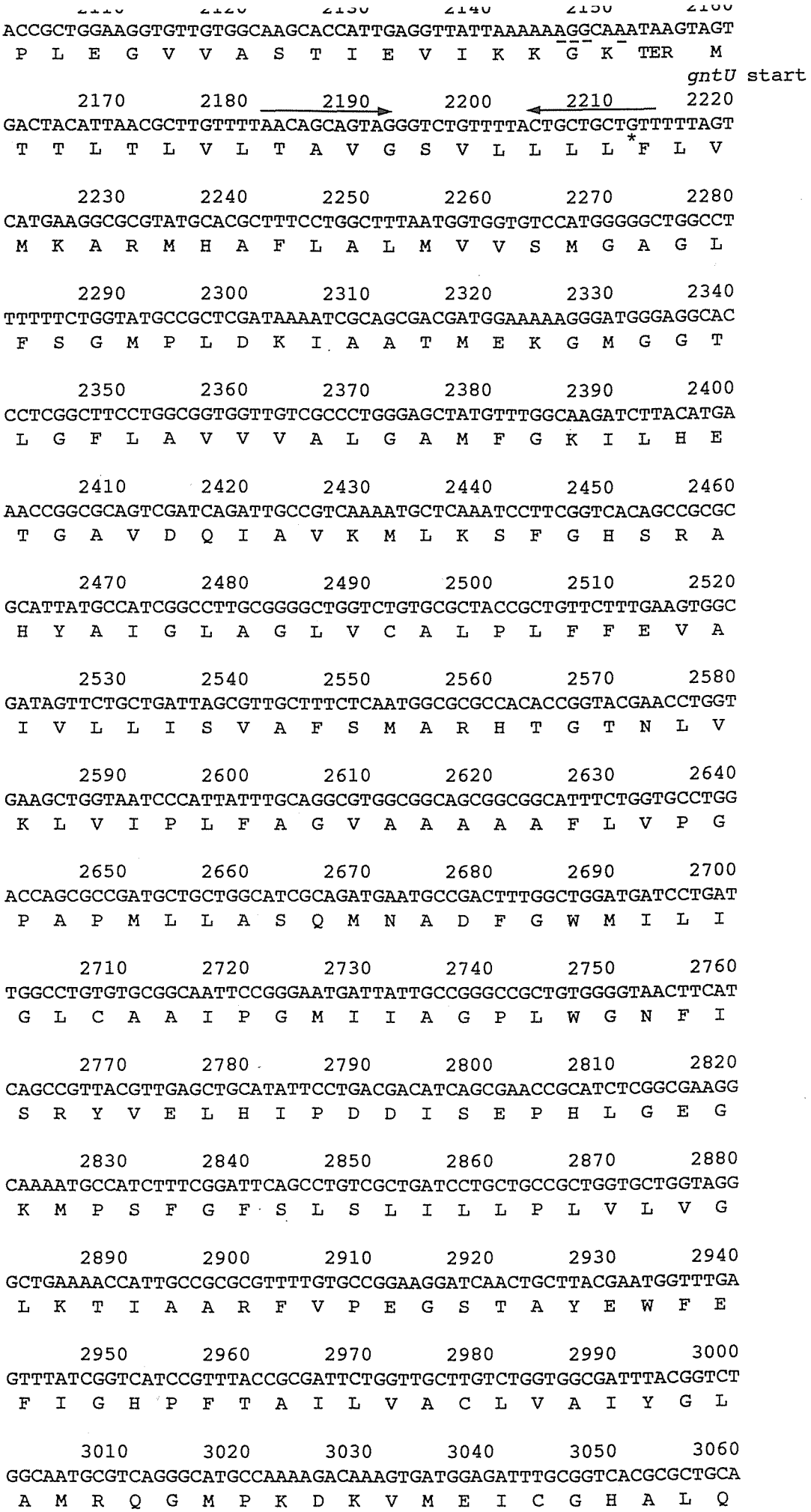
E T L Q E P G A D E T D V L V V D I D Q


ACCGCTGGAAGGTGTTGTGGCAAGCACCATTGAGGTTATTAAAAAAGGCAAATAAGTAGT
P L E G V V A S T I E V I K K G K TER M
gntU start
2170 2180 2190 2200 2210 2220
GACTACATTAACGCTTGTTTTAACAGCAGTAGGGTCTGTTTTACTGCTGCTGTTTTTAGT
T T L T L V L T A V G S V L L L L * F L V
2230 2240 2250 2260 2270 2280
CATGAAGGCGCGTATGCACGCTTTCCTGGCTTTAATGGTGGTGTCCATGGGGGCTGGCCT
M K A R M H A F L A L M V V S M G A G L
2290 2300 2310 2320 2330 2340
TTTTTCTGGTATGCCGCTCGATAAAATCGCAGCGACGATGGAAAAAGGGATGGGAGGCAC
F S G M P L D K I A A T M E K G M G G T
2350 2360 2370 2380 2390 2400
CCTCGGCTTCCTGGCGGTGGTTGTCGCCCTGGGAGCTATGTTTGGCAAGATCTTACATGA
L G F L A V V V A L G A M F G K I L H E
2410 2420 2430 2440 2450 2460
AACCGGCGCAGTCGATCAGATTGCCGTCAAAATGCTCAAATCCTTCGGTCACAGCCGCGC
T G A V D Q I A V K M L K S F G H S R A
2470 2480 2490 2500 2510 2520
GCATTATGCCATCGGCCTTGCGGGGCTGGTCTGTGCGCTACCGCTGTTCTTTGAAGTGGC
H Y A I G L A G L V C A L P L F F E V A
2530 2540 2550 2560 2570 2580
GATAGTTCTGCTGATTAGCGTTGCTTTCTCAATGGCGCGCCACACCGGTACGAACCTGGT
I V L L I S V A F S M A R H T G T N L V
2590 2600 2610 2620 2630 2640
GAAGCTGGTAATCCCATTATTTGCAGGCGTGGCGGCAGCGGCGGCATTTCTGGTGCCTGG
K L V I P L F A G V A A A A A F L V P G
2650 2660 2670 2680 2690 2700
ACCAGCGCCGATGCTGCTGGCATCGCAGATGAATGCCGACTTTGGCTGGATGATCCTGAT
P A P M L L A S Q M N A D F G W M I L I
2710 2720 2730 2740 2750 2760
TGGCCTGTGTGCGGCAATTCCGGGAATGATTATTGCCGGGCCGCTGTGGGGTAACTTCAT
G L C A A I P G M I I A G P L W G N F I
2770 2780 2790 2800 2810 2820
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S R Y V E L H I P D D I S E P H L G E G
2830 2840 2850 2860 2870 2880
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K M P S F G F S L S L I L L P L V L V G
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GCTGAAAACCATTGCCGCGCGTTTTGTGCCGGAAGGATCAACTGCTTACGAATGGTTTGA
L K T I A A R F V P E G S T A Y E W F E
2950 2960 2970 2980 2990 3000
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F I G H P F T A I L V A C L V A I Y G L
3010 3020 3030 3040 3050 3060
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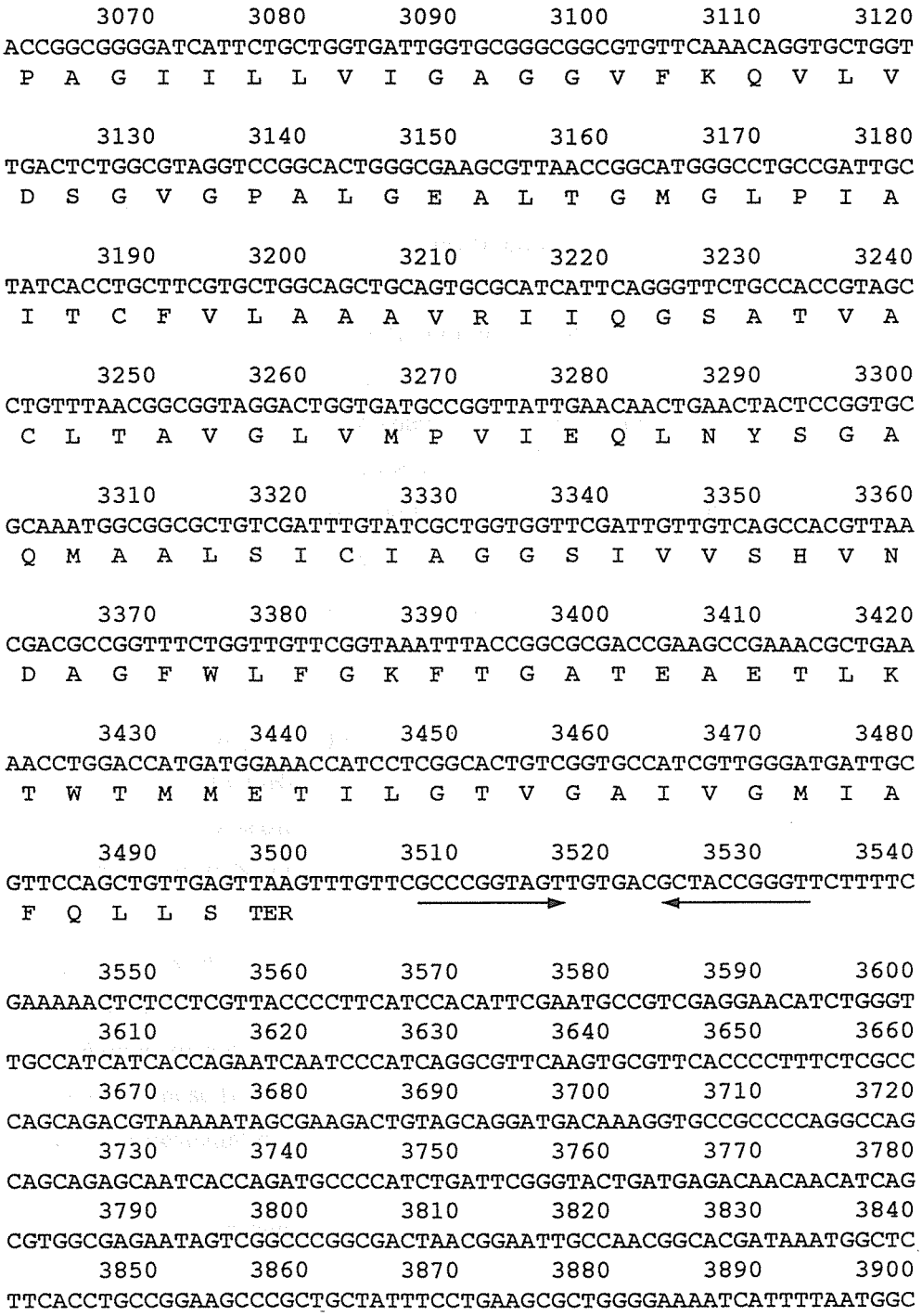


**Figure 3. Nucleotide sequence of the *gntRKU* genes and their deduced amino acid sequences.**

ORF1 (positions 494-1489), ORF2 (positions 1628-2155), and ORF3 (positions 2159-3499) correspond to the *gntR, K*, and *U* genes, respectively. The arrow with a circle (position 1575) represents the initiation and direction of the *gntK* mRNA. The *gntK* promoter and one of the possible *gntR* promoters in front of their coding regions are represented by boxes. A possible ρ-independent terminator (positions 3508-3533) downstream from *gntU* is shown by arrows. Broken underlines represent putative ribosome-recognition sequences. Putative GntR- (positions 1568-1585) and cAMP-CRP binding sequences (positions 1496-1516) are represented by bold characters and an underline, respectively. Arrows at the beginning of *gntU* show an inverted repeat sequence (positions 2182-2214), which seems to correspond to the transcriptional attenuation site after *gntK*. The asterisk indicates the mutation site (position 2212) in the sequence of pGNTUp-CAT15M. Dots represent a putative ATP-binding site in GntK. The nucleotide sequence data reported in this paper were reported in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D84362.

A homology search of the DNA and protein databases was performed using the deduced amino acid sequences of the three ORFs. ORF1 encodes a protein homologous to repressors including CytR, GalR, and LacI (Weickert & Adhya, 1992) as shown in Fig. 4. The homology and the function as described below suggest that ORF1 is the *gntR* gene encoding a repressor for the *gnt* genes in the GntI system. GntR is predicted to consist of 331 amino acid residues with a molecular mass of 36,422 Da. A comparison of the GntR primary structure with those of the repressors in the CytR family revealed that GntR also has a helix-turn-helix motif at its N-terminus, which may be responsible for DNA binding and it may also have domains related to dimerization and inducer binding at its C-terminus (Weickert & Adhya, 1992). The homology search also identified a protein, encoded by a gene (*yjgS* in SWISS-PROT) at 96 min, with 46.1% identity to GntR. I designated the gene *gntH*. The homology and the location of the gene suggest that GntH is a regulator for the *gnt* genes.

**The *gntK* gene encoding the gluconate kinase of the GntI system**

The protein encoded by ORF2 is homologous to the adenylylsulfate kinase of *E. coli* (22.3%) and it possesses a sequence similar to the ATP-binding consensus sequence (Walker *et al.*, 1982) at its N-terminal portion (Fig. 3). When pGNT5 bearing only ORF2 was introduced into YU120 or Hfr G6MD2, gluconate kinase activity remarkably increased (Table 1). Moreover, the N-terminal 10 amino acid sequence of the protein purified from YU120 harboring pGNT5 completely agreed with that deduced from the nucleotide sequence, except for the first Met, and the purified enzyme was heat stable (Izu *et al.*, 1996). These findings, as well as the genomic position, suggest that ORF2 encodes the thermoresistant gluconate kinase, GntK. GntK consists of 175 predicted amino acid residues with a molecular mass of 19,543 Da, which was consistent with that of the purified protein estimated by SDS-polyacrylamide gel electrophoresis. It is notable that the *gntK* gene bears a rare UUG initiation codon. I also found a protein with 47.2% identity to GntK, which was deduced from the nucleotide sequence at 96 min, that should be the thermosensitive gluconate kinase, GntV, of the GntII system.

**The *gntU* gene encoding the low affinity gluconate permease of the GntI system and putative constituents of the GntII system**

ORF3 starts with the GUG initiation codon only 3 nucleotides after the stop codon of *gntK*, and encodes a protein consisting of 447 amino acid residues with a molecular mass of 46,415 Da. The ORF3-encoded protein is homologous to the gluconate permease of *Bacillus subtilis* (36%) and GntP of *E. coli* (32%). YU120 harboring pGNT15 has gluconate permease activity whilst YU120 harboring the vector has none (unpublished). These and the evidence that the *gntU* gene is located near the *gntR* and *gntK* genes (Nagel

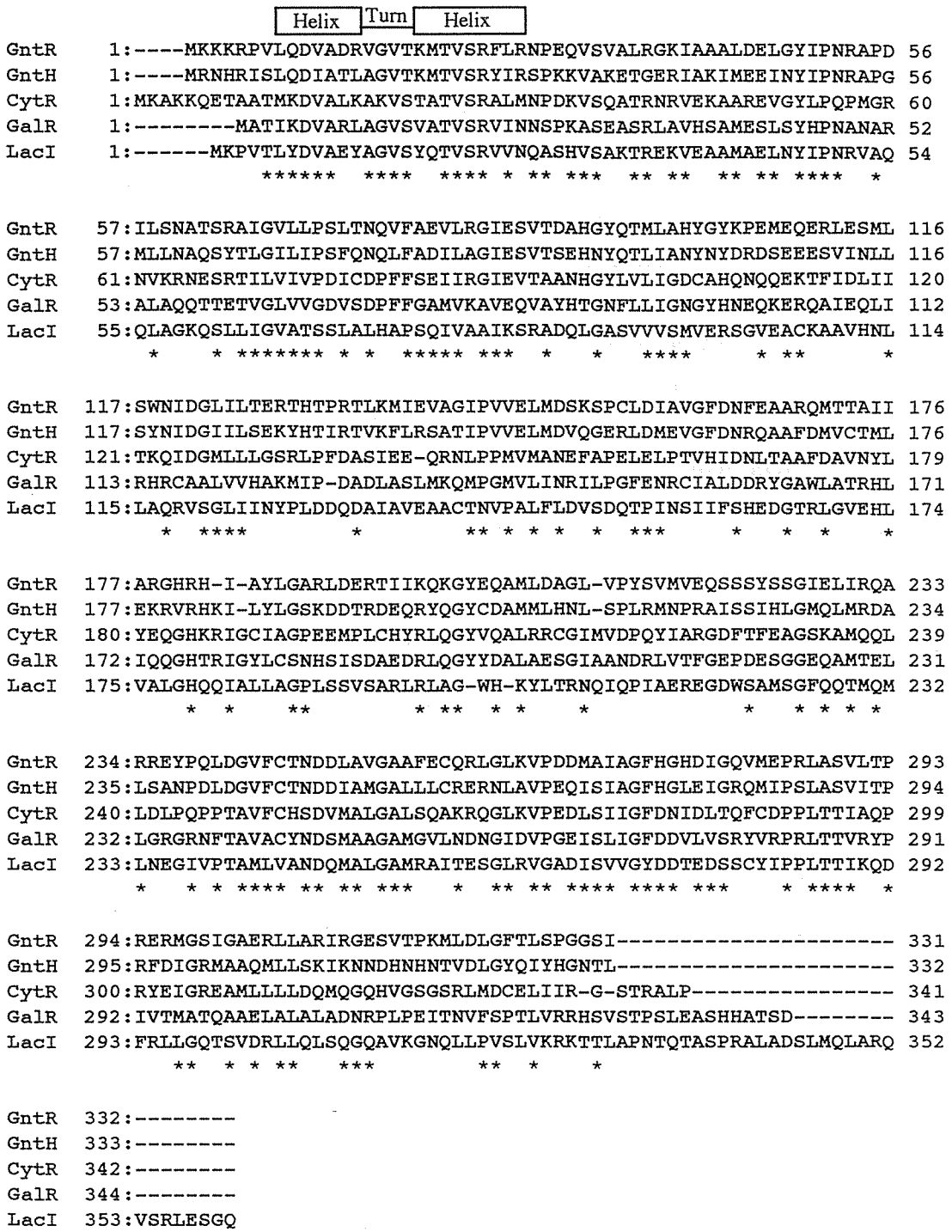


**Figure 4. Comparison of the amino acid sequence of GntR with those of CytR, GalR, and LacI.**

A database search showed that GntH is highly homologous to GntR and may be a regulator for the *gnt* genes. The same and conservatively substituted amino acid residues in more than four proteins are marked with asterisks. Putative helix-turn-helix motifs are shown near the N-termini.

de Zwaig *et al.*, 1973) suggest that ORF3 is the *gntU* gene encoding a low affinity gluconate permease.

I also found a protein, encoded by *yjgT*, with 36% identity to GntU, and its gene is located near *gntH* encoding the GntR homologue and close to *gntV* encoding the GntK homologue. Therefore, *yjgT* may be the previously reported *gntS* gene, which has been described as a gene encoding a gluconate permease of the GntII system. The nucleotide sequence in the databases reveals that *gntV*, *yjgT*, and *gntH* occur in this order, and it is likely that *yjgT*, *gntH*, and two other unknown *orf*s are co-transcribed, whereas *gntV* is transcribed in the opposite direction. Further studies are required to compare gene organization and the physiological functions of the two Gnt systems.

**Promoters of the *gntRKU* genes and expression of the *gntR* gene**

The promoters of the *gnt* genes and regulation of their expression were analyzed using operon fusions with the promoter-less *cat* gene (Fig. 5). DNA fragments including parts of the *gnt* genes were inserted between the *cat* gene and an upstream terminator, which prevents read-through transcription from promoters on the vector. The *cat* gene is thus expressed under the control of the inserted promoter. The CAT fusion plasmids were introduced into TG1 cells, and the resistance of the transformants was tested on LB plates containing 20 μg/ml chloramphenicol. All transformants except for cells harboring pGNTU-CAT8 grew on the plates, suggesting that there are at least two promoters, one in front of the *gntR* coding region and the other in front of *gntK*, and that no, or an extremely weak promoter is located in front of the *gntU* coding region.

The CAT activity of CA8306 cells harboring those fusion plasmids was measured under various conditions (Table 2). CAT activity from pGNTR-CAT11 including the 5'-coding region of the *gntR* gene and its 5'-flanking region was constant under all conditions tested. The pGNTR-CAT14 and pGNTR-CAT27, which include less of the 5'-flanking region of *gntR* than that in pGNTR-CAT11, had activities similar to that of pGNTR-CAT11. Therefore, the *gntR* gene seems to be constitutively expressed and it is not affected by cAMP or gluconate in the medium. At least one promoter for *gntR* appears to be located between positions 430 and 494 corresponding to the segment between the *Sma*I site and the initiation codon of *gntR* (Fig. 3). Within this region, there is a possible promoter sequence (TTCTGA---16 b---TAAGAG at positions 438-465). However, the CAT activity from pGNTR-CAT11 was higher than that of pGNTR-CAT14 or pGNTR-CAT27, suggesting that DNA upstream of the *Sma*I site is important for activity of the proposed promoter or other promoters are present in this region. The latter hypothesis is supported by the observation that a few weak initiation sites for the *gntR* transcript, both before and after the possible promoter sequence, were identified by primer extension mapping even when it was carried out at high temperature (data not shown). Therefore, the *gntR* gene may be transcribed by more than one weak promoter.

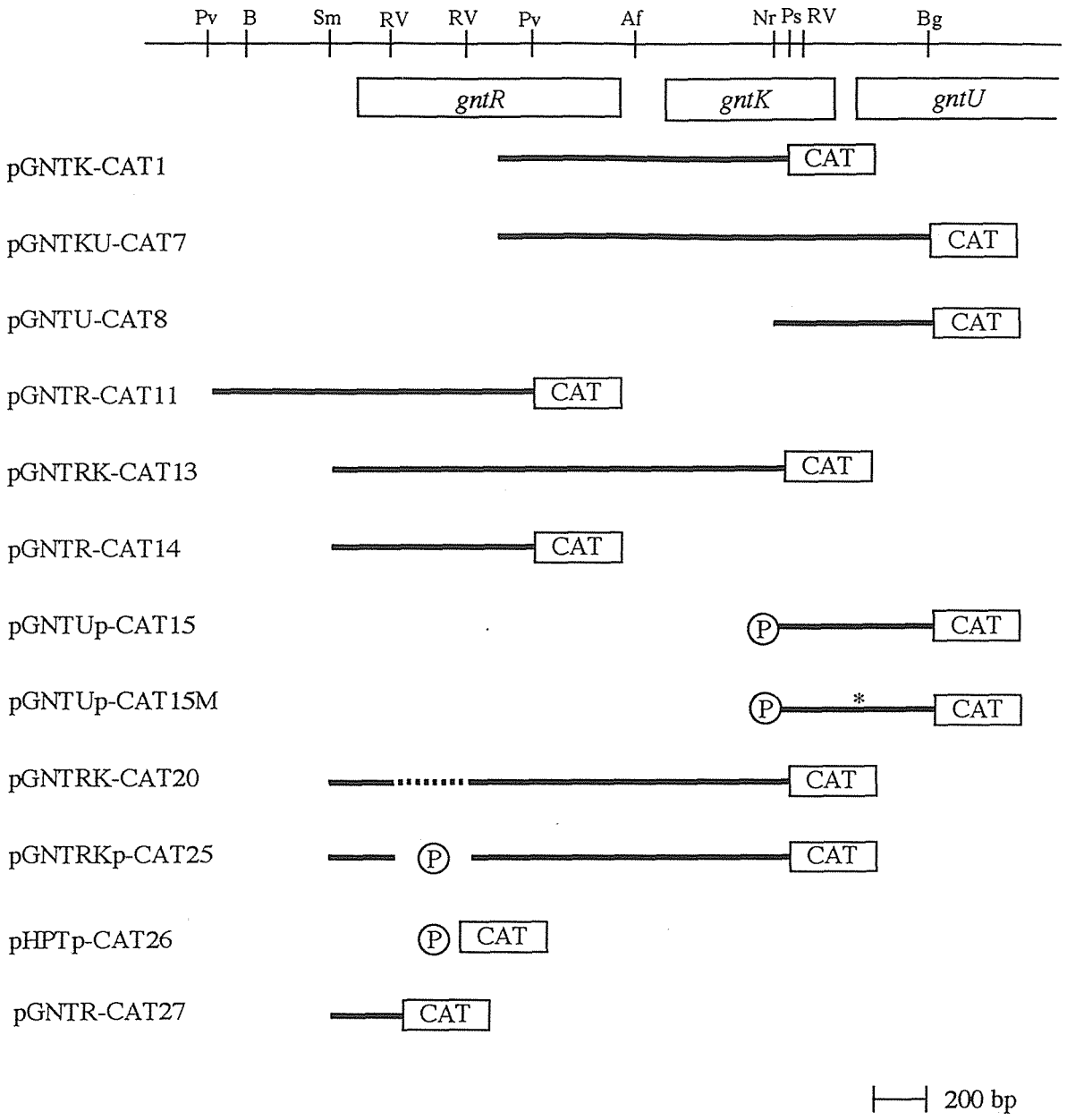


**Figure 5. CAT operon fusions constructed with the *gntRKU* genes.**
CAT operon fusions with the *gnt* genes were constructed as described in Materials and Methods. The regions inserted in the operon fusions are indicated by heavy solid lines, and the promoter-less *cat* genes are shown by boxes. A dotted line in pGNTRK-CAT20 indicates the deleted region. P in a circle indicates the additional inserted promoters: *hpt* promoter for pGNTRKp-CAT25 and pHPT-CAT26 and *orf-f*268 promoter for pGNTUp-CAT15 and pGNTUp-CAT15M. The asterisk represents the mutation site in pGNTUp-CAT15M.

**Table 2. Expressional regulation of the *gntRKU* genes.**

| Strain | CAT activity (mU/mg) [a] | | | |
|---|---|---|---|---|
| | +cAMP +gluconate | +cAMP -gluconate | -cAMP +gluconate | -cAMP -gluconate |
| CA8306 (pGNTK-CAT1) | 580 | 520 | 16 | 6.0 |
| CA8306 (pGNTKU-CAT7) | 100 | 100 | 5.8 | 1.7 |
| CA8306 (pGNTU-CAT8) | <0.002 | <0.002 | <0.002 | <0.002 |
| CA8306 (pGNTR-CAT11) | 27 | 26 | 26 | 24 |
| CA8306 (pGNTRK-CAT13) | - [b] | - | - | 2.9 [c] |
| CA8306 (pGNTR-CAT14) | 14 | 12 | 11 | 13 |
| CA8306 (pGNTRK-CAT20) | - | - | - | 20 |
| CA8306 (pGNTRKp-CAT25) | - | - | - | 39 |
| CA8306 (pHPTp-CAT26) | - | - | - | 29 |
| CA8306 (pGNTR-CAT27) | 11 | 12 | 12 | 10 |
| CA8306 (pKK232-8) | <0.002 | <0.002 | <0.002 | <0.002 |

[a] Cells were incubated at 37 °C for 4 h with or without gluconate and/or cAMP and CAT activity was measured as described in Materials and Methods. Reported values are the averages of 2-4 independent experiments performed in triplicate.

[b] Not determined.

[c] Compared with the activity from pGNTRK-CAT20, the decrease may be due to the intact *gntR* gene.

**Promoter of the *gntKU* genes and regulation of their expression**

Primer extension mapping was performed to determine the mRNA initiation site of the *gntK* gene. One band (corresponding to C at position 1575) was detected in the presence of pGNTKU-CAT7 but not in that of the vector (Fig. 6 and data not shown). The band corresponding to the chromosomal mRNA was undetectable under the conditions tested, probably because of the low RNA concentration. From the band position, pGNTKU-CAT7, TTGCTT---17 b---GACAAT at positions 1542 to 1570 is expected to be the -35 and -10 sequences of the promoter as shown in Fig. 3. Upstream of the possible promoter, there is a sequence (AA-TTTGA--T---TCA-ACT at positions 1496-1516) homologous to the cAMP-CRP consensus sequence (AA-TGTGA--T---TCA-ATA, Ebright *et al.*, 1989).

I thus examined the effect of cAMP on expression of the *gntK* gene (Table 2). The CAT activity from pGNTK-CAT1 increased remarkably in the presence of cAMP. These results and the occurrence of a putative cAMP-CRP binding site suggest that the *gntK* gene expression is positively regulated by cAMP-CRP at the transcriptional level. The putative cAMP-CRP binding sequence resembles that of canonical FNR (Scott *et al.*, 1995), but it appears that FNR is not involved in the regulation of *gntK* expression, because the gluconate kinase activity from pGNT15 in $Fnr^+$ cells grown under oxygen-limited conditions was similar to that under aerobic conditions (data not shown). In the presence of cAMP, the induction caused by adding gluconate was not significant, probably because of catabolite repression, as described above. On the other hand, the CAT activity from pGNTU-CAT8 was undetectable under all conditions tested, suggesting that *gntU* does not have its own promoter and that it is transcribed from the *gntK* promoter.

**Transcriptional attenuation within the beginning of *gntU***

The CAT activity from pGNTKU-CAT7 also depended on cAMP, like pGNTK-CAT1 but it was 3- to 6-fold lower under all conditions tested (Table 3). To examine whether or not transcription from the *gntK* promoter terminates following the *gntK* gene, Northern blot analysis was performed with RNAs from CA8306 harboring pGNTKU-CAT7 (Fig. 7). In the absence of GntR on the plasmid, 600 and 1900 nt RNA bands were detected in the presence of cAMP or cAMP and gluconate. In contrast, only weak bands corresponding to the two RNAs were seen in the presence of GntR, cAMP and gluconate, after a long exposure. The 600 and 1900 nt bands correspond to the predicted length of transcripts from the *gntK* promoter to the inverted repeat sequence near the beginning of the *gntU* coding region (Fig. 3) and to that from the *gntK* promoter to terminators *T*1 and *T*2 of ribosomal RNA downstream of the *cat* gene on the vector, respectively. The shorter band was estimated to be about 5 times more intense than that

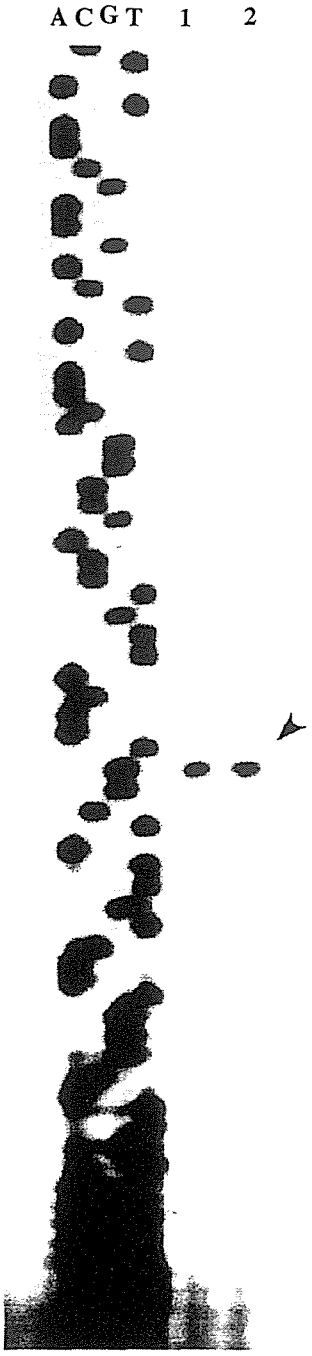


**Figure 6. Primer extension mapping of the *gntK* gene.**
Nucleotide sequencing and primer extension reactions were performed using the same FITC-labeled primer. Nucleotide sequence of the sense strand of the DNA around the mRNA initiation site is shown in lanes A, C, G, and T. Lanes 1 and 2, mRNA isolated from CA8306 cells harboring pGNTKU-CAT7, which had been grown in the presence of 0.5% gluconate and 1 mM cAMP or 1 mM cAMP, respectively.

**Table 3. Expressional regulation of the *gntKU* genes in the presence of GntR.**

| Strain | CAT activity (mU/mg)[a] | | | Gluconate kinase activity (mU/mg)[a] | |
|---|---|---|---|---|---|
| | +cAMP +gluconate | +cAMP -gluconate | -cAMP -gluconate | +cAMP +gluconate | +cAMP -gluconate |
| CA8306 (pYY2/pGNTK-CAT1) | 850 [100%] | 710 [100%] | 9.0 | - | - |
| CA8306 (pGNTR2/pGNTK-CAT1) | 210 [25%] | 81 [11%] | 2.0 | - | - |
| CA8306 (pYY2/pGNTKU-CAT7) | 140 [100%] | 280 [100%] | - [b] | 330 [100%] | 910 [100%] |
| CA8306 (pGNTR2/pGNTKU-CAT7) | 18 [13%] | 6.3 [2.3%] | - | 48 [15%] | 37 [4.1%] |
| CA8306 (pYY2/pGNTUp-CAT15) | 3.0 | - | - | - | - |
| CA8306 (pGNTR2/pGNTUp-CAT15) | 3.0 | - | - | - | - |
| CA8306 (pYY2/pGNTUp-CAT15M) | 20 | - | - | - | - |
| CA8306 (pGNTR2/pGNTUp-CAT15M) | 20 | - | - | - | - |

[a] Cells were incubated at 37 °C for 4 h with or without gluconate and/or cAMP. The control plasmid pYY2 is a derivative of pACYC177, which has a frameshift mutation at the *Pst*I site in the *bla* gene. Enzymes were assayed as described in Materials and Methods. The activity in the presence of GntR in each strain is expressed as a percentage of that in the absence of GntR and shown in brackets. Reported values are the averages of 2-4 independent experiments performed in triplicate.

[b] Not determined.

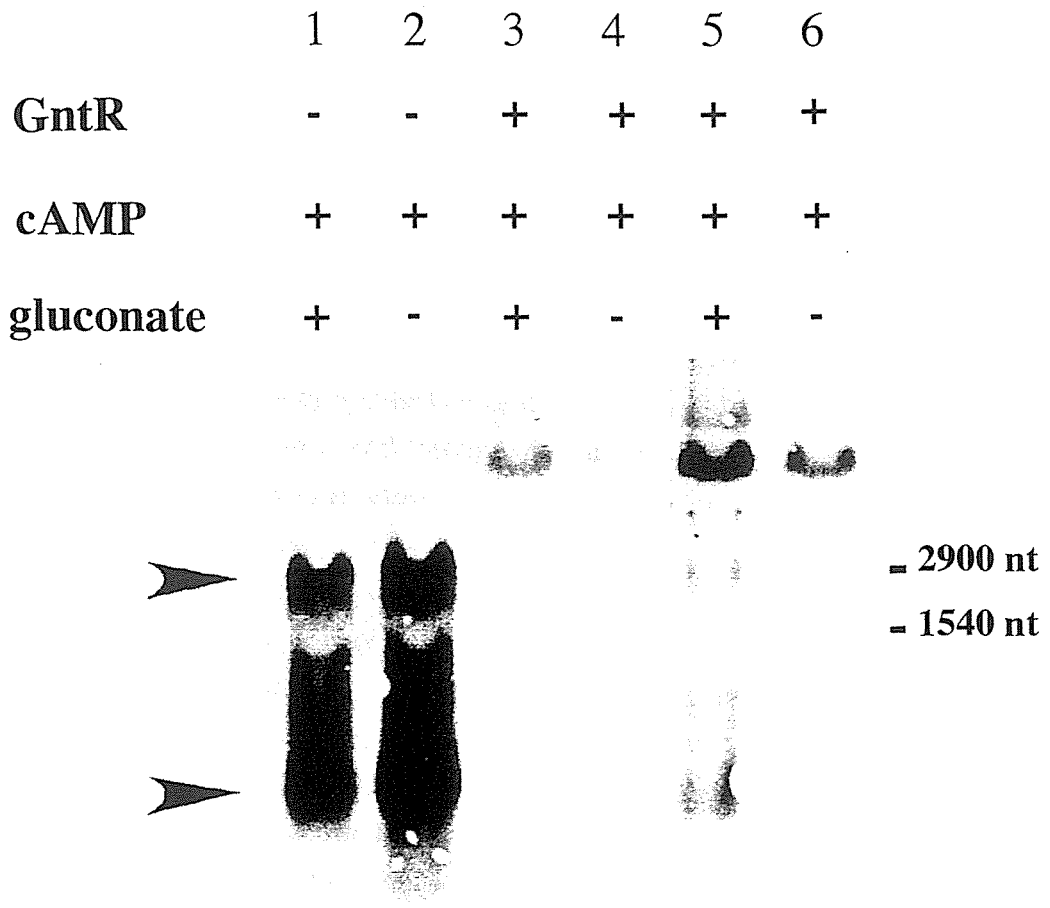


**Figure 7. Northern blot of transcriptional attenuation after the *gntK* gene.** RNAs were isolated from CA8306 cells harboring pGNTKU-CAT7 and pYY2, a control vector, which had been grown in the presence of 1 mM cAMP and 0.5% gluconate (lane 1) or 1 mM cAMP (lane 2) and from CA8306 cells harboring pGNTKU-CAT7 and pGNTR2 bearing the *gntR* gene, which had been grown in the presence of 1 mM cAMP and 0.5% gluconate (lane 3) or 1 mM cAMP (lane 4) and Northern blotted using the 190-bp *Nru*I-*Eco*RV fragment in the *gntK* gene as a probe. Lanes 5 and 6 show longer exposures of lanes 3 and 4, respectively. Two major bands are shown by arrow heads. Longer and weaker bands above the 2900 nt marker in lanes 3 and 4 may be transcripts, including the truncated *gntK* gene that started from a promoter on pGNTR2. Size markers were 23S (2900 nt) and 16S (1540 nt) rRNAs.

of the longer band under both conditions, and this ratio of the intensities is comparable with that of CAT activities between pGNTK-CAT1 and pGNTKU-CAT7.

To elucidate the mechanism by which the 600 nt RNA was produced, a mutant that increased the activity of the read-through transcription to *gntU* was isolated from pGNTUp-CAT15, in which the *Nru*I-*Bgl*II fragment including the inverted repeat after *gntK* was placed under the control of a foreign, relatively weak and constitutive promoter (Fig. 5). Cells harboring the resultant mutant, pGNTUp-CAT15M, had 6-fold higher CAT activity than those harboring pGNTUp-CAT15 (Table 3). G was mutated to A at position 2212 within the inverted repeat sequence, corresponding to the stem of the potential stem-loop structure. Although the effect of the mutation on *gntK* expression was also tested after the mutation was moved into pGNTKU-CAT7, no influence was observed (data not shown). Therefore, the G to A mutation should reduce the stability of the stem-loop structure and enhance read-through transcription. The stem-loop structure seems to be an attenuator for the *gntK* transcript, resulting in reduced expression of the *gntU* gene as shown in Fig. 7.

The presence of a smear extending downwards from about 1500 nt, suggests another explanation for the 600 nt band. It may be due to the stabilization of mRNAs by the secondary structure formed at the dyad symmetry, against 3' to 5' exonucleases following endonucleolytic attack in the *gntU* mRNA.

**Read-through transcription from the *gntR* promoter to *gntK***

Under non-induced conditions, the CAT activity from pGNTRK-CAT20 bearing both *gntR* and *gntK* promoters was nearly equal to that from pGNTR-CAT27 bearing the *gntR* promoter plus that from pGNTK-CAT1 bearing the *gntK* promoter (Table 2). The data indicate that there is no strong terminator between the end of the *gntR* and the beginning of the *gntK* coding regions and that there is read-through transcription from the *gntR* promoter to *gntK*. To test the read-through transcription, an additional promoter from the *hpt* gene (Yamada *et al.*, unpublished) was inserted into the *Eco*RV site inside *gntR* on pGNTRK-CAT20, generating pGNTRKp-CAT25. Compared with CAT activity from pGNTRK-CAT20 or its control plasmid, pHPTp-CAT26 bearing only the *hpt* promoter, that of pGNTRKp-CAT25 was increased by the *hpt* promoter. Therefore, read-through transcription can occur from *gntR* to *gntK*. The read-through transcription may be repressed by GntR, which was indicated by the difference in the CAT activities between pGNTRK-CAT13 ($gntR^+$) and pGNTRK-CAT20 ($gntR^-$). Studies using the *gntR* clone in *trans* described below also support this idea. These data in addition to those obtained from pGNTK-CAT1 and pGNTKU-CAT7 suggest that the *gntR*, *gntK*, and *gntU* genes constitute an operon, even though transcription from the *gntR* promoter is relatively weak.

Since read-through transcription from the *gntR* promoter was significant under de-repressed conditions (no *gntR* on the plasmid), it would be significant for the expression of *gntK* or *gntU* in the presence of gluconate or its derivative, which may induce the removal of GntR from the binding site but only when the intracellular cAMP concentration is low. The same situation seems to exist in the *E. coli lac* operon where *lacI* is expressed from its own promoter immediately upstream from the inducible *lacZYA* operon encoding catabolic enzyme and permease. It is notable that *lacI* mRNA terminated in the control region for the *lacZYA* genes, and that the estimated efficiency of the termination was 20% *in vitro* and 80% *in vivo* (Horowitz & Platt, 1982; Cone *et al.*, 1983). The influence of LacI on the termination, however, has not yet been tested. On the other hand, the situation would appear to be different in the *B. subtilis gnt* operon, where the *gntRKP* genes are co-transcribed from the *gntR* promoter, but no internal promoter for *gntK* is detectable (Fujita *et al.*, 1986; Fujita & Fujita, 1987).

**Effect of GntR on transcription from the *gntK* promoter**

To examine the effect of GntR on transcription from the *gntK* promoter, both pGNTR2 bearing *gntR* and plasmids bearing CAT fusions were introduced into CA8306 and the CAT activity of the transformants was measured under the conditions shown in Table 2 (Table 3). The activities from pGNTK-CAT1 and pGNTKU-CAT7 were reduced 9- and 44-fold, respectively, by GntR in the presence of cAMP. Gluconate and cAMP caused 2.5- and 3.0-fold increases, respectively. Gluconate kinase activity from pGNTKU-CAT7 similarly changed in the presence of GntR under the same conditions. The increase of gluconate kinase activity was comparable in CA8306 harboring pBR322 (Table 1) which carries the *gntRKU* gene on the chromosome. Therefore, it is likely that experiments with pGNT-CAT fusion and pGNTR plasmids reflect chromosomal phenomena. The much higher induction in LJ288 harboring pBR322 may be because the genetic background is different from that of CA8306.

Northern blotting revealed a large reduction in *gntK* transcription by GntR (Fig. 7), which appears to be comparable to that in the CAT activity from pGNTKU-CAT7. The reduction by GntR, however, was not complete (Table 3) probably because the plasmid copy number of pGNTR2, a pACYC177 derivative, is lower than that of pGNTK-CAT1 or pGNTKU-CAT7, a pBR322 derivative. It appeared that the transcription from the *gntK* promoter is negatively regulated by GntR as shown in Fig. 8. I note that an inverted repeat sequence AATGTTACCGATAACAGT, overlaps the -10 sequence of the possible *gntK* promoter (Fig. 3), which could be the binding site for the repressor.

### Effect of GntR on the *gntR* transcription and on read-through transcription from the *gntR* promoter to *gntK*

I tested the effect of GntR on the transcription from the *gntR* promoter. The CAT activities from the pGNTR-CAT11 and pGNTR-CAT27 were not repressed (Table 4), indicating that GntR is not involved in the expressional regulation of its own gene. On the other hand, the activity from pGNTRKp-CAT25 was reduced by GntR under non-induced conditions, but the activity from the control plasmid, pHPTp-CAT26, was not. The values obtained by subtracting the activities of pGNTK-CAT1 corresponding to the *gntK* promoter under the non-induced condition as shown in brackets in Table 4, suggest that GntR reduces the read-through transcription from the *hpt* promoter on pGNTRKp-CAT25 and presumably also from the *gntR* promoter. This could be due to steric hindrance from GntR binding to the putative GntR-binding site of the *gntK* operator (Fig. 8).

### Differential expression among the *gnt* genes

The results described above suggest that the *gntK* gene is the most transcribed in the presence of gluconate of the cistrons of the *gntRKU* operon. Northern blotting and CAT fusion experiments indicate that *gntK* is transcribed about 5- and 20-fold more than *gntU* and *gntR*, respectively. To further compare the expression of these genes, we examined their codon usages (Ikemura, 1981). The *gntK* gene preferentially used optimal codons for Pro, Lys, Arg, and Gly. The *gntU* and *gntR* genes used a mixture of non-optimal and optimal codons, indicating that both genes are moderately translated. The *gntK* and *gntU* genes possess the non-optimal initiation codons, UUG and GUG, respectively. Reddy *et al.* (1985) reported that the relative amounts of protein produced were 3:2:1 for AUG:GUG:UUG initiation codons. Thus, despite the fact that *gntK* has the worst translational initiation codon, the much higher transcription of that gene should ensure it is the major product. These findings suggest that the *gntK* gene is organized so that it is expressed more frequently than the *gntU* gene, and that the *gntR* gene is expressed less often than the other two genes. The predominance of *gntK*, over *gntU* expression may be because GntT is the preferred transporter for gluconate utilization. Differential expression of the *gnt* genes would contribute to the rational utilization of available gluconate.

## Materials and Methods

### Materials

Restriction enzymes, T4 DNA ligase and DNA sequencing kits were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (MA, USA). The enzyme 6-

**Table 4. Expressional regulation of the *gntR* gene in the presence of GntR.**

| Strain | CAT activity (mU/mg) [a] | | |
|---|---|---|---|
| | +cAMP +gluconate | +cAMP -gluconate | -cAMP -gluconate |
| CA8306 (pYY2/pGNTR-CAT11) | 15 | 15 | - |
| CA8306 (pGNTR2/pGNTR-CAT11) | 15 | 15 | - |
| CA8306 (pYY2/pGNTRKp-CAT25) | - [b] | - | 34 [25] [c] |
| CA8306 (pGNTR2/pGNTRKp-CAT25) | - | - | 14 [12] |
| CA8306 (pYY2/pHPTp-CAT26) | - | - | 13 |
| CA8306 (pGNTR2/pHPTp-CAT26) | - | - | 13 |
| CA8306 (pYY2/pGNTR-CAT27) | - | - | 3.0 |
| CA8306 (pGNTR2/pGNTR-CAT27) | - | - | 3.0 |

[a] Assay conditions were the same as those described in Table 4. Reported values are the averages of 2-4 independent experiments performed in triplicate.
[b] Not determined.
[c] The values in brackets were obtained by subtracting the corresponding activity from pGNTK-CAT1 in the absence of cAMP and gluconate as shown in Table 4.

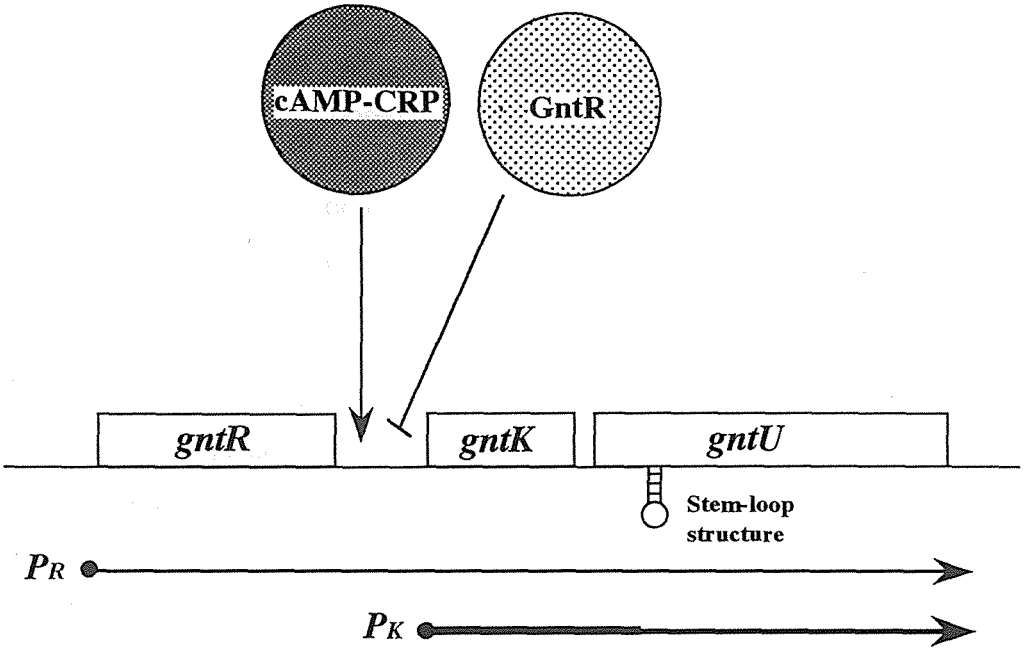


**Figure 8. Model of the regulation of the *gntRKU* gene expression in *E. coli*.** The adjacent *gntRKU* genes may be transcribed from promoters in front of *gntR* (*PR*) and *gntK* (*PK*). Their transcriptional initiation sites and directions are shown by circles and arrows, respectively. The *PK* promoter is regulated positively by cAMP-CRP complex and negatively by GntR, and the *PR* promoter is constitutively expressed. Transcription from the *PR* promoter seems to be terminated by the presence of GntR around the GntR-binding site overlapping the *PK* promoter. Transcription from the *PK* promoter seems to be attenuated at the potential stem-loop structure in front of the *gntU* gene.

phosphogluconate dehydrogenase used in the gluconate kinase assay was from Boehringer Mannheim (Mannheim, Germany). Other chemicals were of analytical grade.

**Bacterial strains and plasmids**

The bacterial strains used in this study were derivatives of *E. coli* K-12. Their relevant genotypes and plasmids are shown in Table 5.

**Isolation of a Gnt$^-$ mutant**

A Gnt$^-$ mutant strain was isolated to clone the genes involved in the *E. coli* gluconate uptake and catabolism. LJ288 was exposed to N-methyl-N′–nitro-N-nitrosoguanidine (Miller, 1992) and selected using penicillin (Saier & Roseman, 1972). The resulting cells were spread on EMB plates (Miller, 1992) containing 0.5% gluconate. A mutant strain, YU120, which showed negative gluconate fermentation on the plate, was isolated and used to clone the *gnt* genes. The mutation seemed to occur in the *gntT* gene. This is because the growth characteristics were similar to those of the *gntT*$^-$ (but not to those of *gntR*$^-$, *gntK*$^-$, or *gntU*$^-$) mutants of the GntI system. Fermentation was negative in the early, and positive in the late growth phase, and the mutation affected the activities of both gluconate kinase and permease (Nagel *et al.*, 1973, data not shown). Furthermore, fermentation became positive when a *gntT* clone was introduced into YU120 as described in CHAPTER 3 (Izu *et al.*, 1997b). The estimated reversion frequency of the mutant was $1\times10^{-6}$, suggesting that it carries a point mutation.

**DNA manipulations and sequencing**

Conventional recombinant DNA techniques were applied (Sambrook *et al.*, 1989). DNA was sequenced by dideoxy-chain termination (Sanger *et al.*, 1977) using M13 mp18 or 19 (Yanisch-Perron *et al.*, 1985) as a vector. Northern blotting proceeded as described (Sambrook *et al.*, 1989). The nucleotide sequence of the cloned genes and the deduced amino acid sequence were compared with those listed in the DNA and protein databases of GENETYX (Software Development, Tokyo, Japan).

**Cloning of the *gnt* genes**

A genomic library was constructed using *E. coli* W3110 genomic DNA (Sambrook *et al.*, 1989). The DNA (20 μg) was partially digested with *Sau*3AI, and 2.0 to 5.0-kb DNA fragments were resolved by 6% polyacrylamide gel electrophoresis. The fragments were then ligated into pBR322 which had been digested with *Bam*HI, and introduced into the Gnt$^-$ (*gntT*$^-$) strain, YU120, using an *E. coli* Pulser (Bio-Rad, California, U.S.A.). The transformants were screened on EMB plates containing 0.5% gluconate and ampicillin (100 μg/ml), and three were fermentation-positive. From these, cloned plasmid DNAs were isolated and analyzed by restriction mapping. The genomic location

**Table 5. Bacterial strains and plasmids.**

| Strain or plasmid | Genotype or description | Reference or source |
|---|---|---|
| Strains | | |
| W3110 | Wild type | Ourlaboratory stock |
| CA8306 | Hfr Hayes $Sm^S$ $B1^-$ Δ*cya* | J. Beckwith |
| LJ288 | Δ*pts* | M. H. Saier, Jr. |
| YU120 | LJ288, *gnt* | This study |
| Hfr G6MD2 | $\lambda^-$ *his323 Δ(bioH-asd)29* | M. Schwartz |
| TG1 | *supE hsdΔ5 thi Δ(lac-proAB)* F '*traD36 proAB* $lacI^q$ *lacZΔM15* | Sambrook *et al.* (1985) |
| Plasmids | | |
| pBR322 | $amp^r$ $tet^r$ | Bolivar *et al.* (1977) |
| pKK232-8 | $amp^r$ promoter-less *cat* gene | Brosius (1984) |
| pACYC177 | $amp^r$ $kan^r$ | Chang & Cohen (1978) |
| pACYC184 | $tet^r$ $cat^r$ | Chang & Cohen (1978) |
| pYY2 | frame-shift mutation of the *bla* gene on pACYC177 | Yamada *et al.* (1995) |
| pUCGCD1 | *gcd* and part of *hpt* in pUC118 *lacZ* gene | Yamada *et al.* (1993) |
| pGNT5 | *gntK* in pBR322 | This study |
| pGNT15 | *gntRKU* in pACYC184 | This study |
| pGNTR2 | pACYC177 with the *Bam*HI-*Pst*I fragment from pGNT15 | This study |
| pGNTK-CAT1 | pKK232-8 with the *Bam*HI-*Pst*I fragment from pGNT5 | This study |
| pGNTKU-CAT7 | pKK232-8 with the *Bam*HI-*Bgl*II fragment from pGNT5 | This study |
| pGNTU-CAT8 | pKK232-8 with the *Nru*I-*Bgl*II fragment from pGNT5 | This study |
| pGNTR-CAT11 | pKK232-8 with the *Pvu*II fragment of pGNT15 | This study |
| pGNTRK-CAT13 | pKK232-8 with the *Sma*I-*Pst*I fragment of pGNT15 | This study |
| pGNTR-CAT14 | pKK232-8 with the *Sma*I-*Pvu*II fragment of pGNT15 | This study |
| pGNTUp-CAT15 | pGNTU-CAT8 derivative with insertion of a constitutive promoter at the *Sma*I-*Nru*I site | This study |
| pGNTUp-CAT15M | pGNTUp-CAT15 derivative with a mutation between *gntK* and *gntU* | This study |
| pGNTRK-CAT20 | deletion of the *Eco*RV fragment of pGNTRK-CAT13 | This study |
| pGNTRKp-CAT25 | pGNTRK-CAT20 with the *Hin*cII fragment of pUCGCD1 | This study |

| | | |
|---|---|---|
| pHPTp-CAT26 | pKK232-8 with the *Hin*cII fragment of pUCGCD1 | This study |
| pGNTR-CAT27 | pKK232-8 with the *Sma*I-*Eco*RV fragment of pGNT15 | This study |

of the cloned genes was determined using an *E. coli* ordered phage library (Kohara, *et al.*, 1987) and detected using an ECL kit (Amersham, Buckinghamshire, England). The genomic location analysis and gluconate kinase assay indicated that one clone (pGNT5) included the *gntK* gene and the other two included unknown genes mapped at 76 and 88 min. Plasmid pGNT5 encoded only the gluconate kinase of the GntI. The reason why a clone containing only *gntK* with positive fermentation was isolated from the *gntT* background, might be depletion of the repressor by its binding to the *gntK* operator, thus leading to expression of the *gntKU* operon. Fermentation was consistently positive when the 250-bp *Afl*II-*Taq*I fragment (positions 1523-1773), including the *gntK* operator and part of its coding sequence was subcloned. Based on the genomic position of the fragment inserted into pGNT5, plasmid pGNT15 covering the whole *gntRKU* operon was constructed by subcloning the 10-kb *Eco*RI fragment derived from λ phage 16A5 of the Kohara library (Kohara, *et al.*, 1987) at the *Eco*RI site on pACYC184 (Fig. 2). The *gntR* gene was subcloned by inserting the 1.9-kb *Bam*HI-*Pst*I fragment of pGNT15 at the *Bam*HI-*Pst*I site on pACYC177, generating pGNTR2.

**Primer extension mapping**

The primer (GCGCCACTTCACTGGCGACCGCAGA) labeled with FITC, was synthesized by Sawady Technology (Tokyo, Japan) and used to analyze the *gntK* promoter. Total RNA was isolated using hot phenol (Aiba *et al.*, 1981), from CA8306 cells harboring pGNTKU-CAT7, which were grown at 37 °C for 4 h in the presence of 0.5% gluconate and/or 1 mM cAMP. Primer extension reactions were performed at 50 °C for 60 min in a 0.5-ml microcentrifuge tube in a final volume of 20 μl, in which 1 μg of the total RNA, 2 pmol of the FITC-labeled primer, a 200 μM concentration of dNTPs, and r*Tth* DNA polymerase (Perkin-Elmer-Cetus Instruments) were added in 30 mM Tricine (pH 8.5), 75 mM potassium acetate, 1.1 mM $Mn(OAc)_2$, and 10% glycerol. Nucleotides were sequenced using the same FITC-labeled primer and pGNTKU-CAT7 DNA as a template. Both products were analyzed on a nucleotide sequencer SQ3000 (Hitachi Electronics Engineering, Tokyo, Japan) after separation on a 6.1 M urea-6% polyacrylamide gel.

**Operon fusion constructs**

Operon fusions with the *cat* gene were constructed to examine the promoter activity of the *gnt* genes and the regulation of their expression. DNA fragments from the *gnt* genes were isolated and inserted in front of the promoter-less *cat* gene on pKK232-8 (Brosius, 1984). Plasmids pGNTK-CAT1, pGNTKU-CAT7, pGNTU-CAT8, pGNTR-CAT11, pGNTRK-CAT13, pGNTR-CAT14, and pGNTR-CAT27 have the 1.2-kb *Bam*HI-*Pst*I fragment of pGNT5, the 1.7-kb *Bam*HI-*Bgl*II fragment of pGNT5, the 0.5-kb *Nru*I-*Bgl*II fragment of pGNT5, the 1.2-kb *Pvu*II fragment of pGNT15, the 1.6-kb *Sma*I-*Pst*I

fragment of pGNT15, the 0.8-kb *Sma*I-*Pvu*II fragment of pGNT15, and the 230-b *Sma*I-*Eco*RV fragment of pGNT15, respectively. Plasmid pGNTRK-CAT20 was constructed by deleting the 0.3-kb *Eco*RV fragment from pGNTRK-CAT13. The *hpt* promoter (0.7-kb *Hin*cII fragment) from pUCGCD1 (Yamada *et al.*, unpublished) was inserted into the *Eco*RV site on pGNTRK-CAT20 and into the *Sma*I site on pKK232-8 to generate pGNTRKp-CAT25 and pHPTp-CAT26, respectively.

**Isolation of a mutant with a nucleotide substitution at the potential stem-loop structure just after the *gntK* gene**

A constitutive promoter from the *E. coli*-genomic promoter library (Talukder *et al.*, unpublished) was inserted at the *Sma*I-*Nru*I site of pGNTU-CAT8, generating pGNTUp-CAT15. The pGNTUp-CAT15 DNA was mutagenized using hydroxylamine (Manayan *et al.*, 1988). DNA (100 μl) in 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA was mixed with 200 μl of 0.6 M $NH_2OH$ in 40 mM $K_2HPO_4$ and 1 mM EDTA, then incubated overnight at 37 °C. The DNA sample was then dialyzed three times against 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and recovered by ethanol precipitation. The DNA was then introduced into CA8306 and transformants that grew on LB plates containing a high concentration (70 μg/ml) of chloramphenicol were selected, because CA8306 harboring pGNTUp-CAT15 can grow only at low concentrations (20 μg/ml) of chloramphenicol. The isolate, pGNTUp-CAT15M, carried a mutation (G to A at position 2212) in the potential stem-loop structure and had no other mutations within the *gntK* and *gntU* genes.

**Enzyme assay**

For the gluconate kinase assay, cells harboring plasmids were grown in LB medium (1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl) containing ampicillin (100 μg/ml) at 37 °C for 4 h, harvested by centrifugation, and washed twice with 0.85% NaCl. To induce the *gnt* gene expression, 0.5% gluconate was added to the medium. Crude extracts were prepared by sonic oscillation followed by centrifugation at 8,000 × *g* for 5 min to remove intact cells. Gluconate kinase activity was measured using 6-phosphogluconate dehydrogenase as described (Frankel & Horecker, 1964). For the CAT assay using *gnt-cat* operon fusions, CA8306 cells harboring each fusion plasmid were grown under different test conditions in LB medium containing ampicillin (100 μg/ml) at 37 °C for 4 h, harvested and washed twice with 0.85% NaCl. To induce expression of the fusion genes, 0.5% gluconate and/or 1 mM cAMP was added to the medium. Crude extracts were prepared as described above, and the activity of chloramphenicol acetyltransferase (CAT) was measured as described (Close & Rodriguez, 1982). Protein was determined by the Biuret method (Layne, 1957).

# CHAPTER 2

## Purification and characterization of the *Escherichia coli* thermoresistant gluconate kinase encoded by the *gntK* gene

## Summary

A thermoresistant gluconate kinase encoded by the *gntK* gene of *Escherichia coli* K-12 was purified and characterized. The *K*m values of the purified enzyme for gluconate and ATP are 42 μM and 123 μM, respectively, and the activity was not altered by the presence of pyruvate. The enzyme was shown to function as a dimer with two identical subunits of 18.4 kDa. These characteristics appear to be distinct from those of the thermoresistant gluconate kinase reported by E. I. Vivas, A. Liendo, K. Dawidowicz, and T. Istúriz (1994) *J. Basic. Microbiol.* 16, 117-122.

## Results and Discussion

### Purification of thermoresistant gluconate kinase encoded by the *gntK* gene

Purification of the thermoresistant gluconate kinase in *E. coli* was performed with crude extracts prepared from YU120 cells harboring an expression plasmid of the *gntK* gene, pGNT5 (CHAPTER 1; Izu *et al.*, 1997a). The crude extracts were passed through a DEAE-Toyopearl column followed by a phosphocellulose column (Table 6). The gluconate kinase was finally purified 62.5-fold to the crude extracts with a specific activity of 250 U/mg of protein in an overall recovery of 55%. The final material was examined and compared with other fractions by SDS-15% PAGE (Fig. 9). An 18.4-kDa band corresponding to that deduced from the nucleotide sequence of the *gntK* gene was found in more than 95% purity. To estimate the catalytic form of the gluconate kinase, the purified protein was subjected to HPLC analysis under the conditions of 50 mM potassium phosphate (pH 7.0) containing 100 mM NaCl. The estimated molecular mass was 38 kDa in contrast with 18.4 kDa estimated by SDS-PAGE. Therefore, the thermoresistant gluconate kinase is assumed to function in a dimer composed of two identical subunits.

The N-terminal 10 amino acid sequence of the purified gluconate kinase was determined. The resultant sequence, Ser-Thr-Thr-Asn-His-Asp-His-His-Ile-Tyr, completely agreed with that deduced from the nucleotide sequence of *gntK* (CHAPTER1; Izu *et al.*, 1997a) except for the first Met, which may be posttranslationally removed in cells. The coding sequence of the *gntK* gene is thus initiated with an unusual initiation codon of TTG and consists of 176 codons including a TAA stop codon according to the nucleotide sequence, and the gene product is calculated to have a molecular mass of 19.5 kDa, which is consistent with that obtained from SDS-PAGE. From database searching was found a hypothetical gene product, designated GntV, with 47% identity to GntK. Its gene is located between the *pepA* and *leuX* genes on the genome sequence. These facts suggest that the *gntV* gene may encode the thermosensitive gluconate kinase of the GntII system.

At the N-terminal portion of the enzyme, a sequence from positions 16 to 38 homologous to the ATP-binding consensus sequence, Val-X-Gly-Ser-Gly-X-Ser-$X_2$-Ala-$X_{11}$-Leu-Asp (Walker *et al.*, 1982), was found, suggesting that ATP regarding the kination reaction binds to the domain including the sequence. Such a sequence was also found at the N-terminal portion of the hypothetical GntV.

### Characterization of the *E. coli* thermoresistant gluconate kinase

To examine thermoresistancy of the purified gluconate kinase, I measured the remaining activity after the enzyme was treated as reported by Istúriz *et al.* (Istúriz *et al.*, 1986). As a result, 100% of the gluconate kinase activity was retained after 30-min

**Table 6. Purification summary of the thermoresistant gluconate kinase encoded by the *gntK* gene.**

| Step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 180 | 720 | 4.0 | 1 | 100 |
| DEAE-Toyopearl | 10.6 | 472 | 44.5 | 11.1 | 65.5 |
| Phosphocellulose | 1.57 | 393 | 250 | 62.5 | 54.6 |

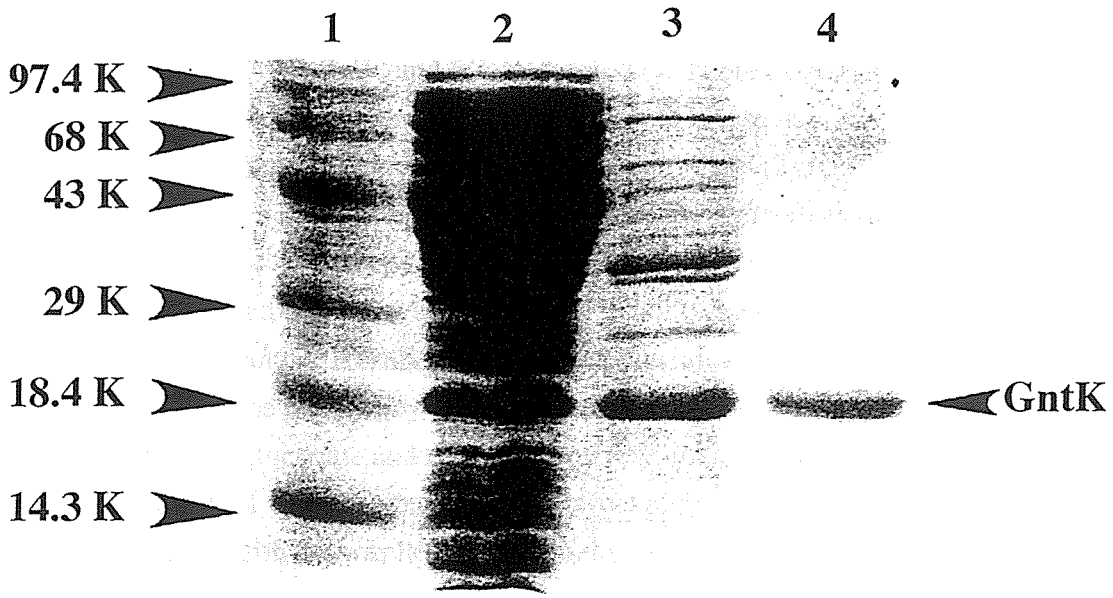


**Figure 9. SDS-PAGE of the thermoresistant gluconate kinase encoded by the *gntK* gene.**
Samples through the purification process of the thermoresistant gluconate kinase were analyzed by SDS-15% PAGE. Lanes 2-4 showed crude extracts (60 μg), the active fraction from DEAE-Toyopearl column chromatography (67 μg), and the active fraction from phosphocellulose column chromatography (12 μg). Bands corresponding to the thermoresistant gluconate kinase are indicated by an arrowhead. Molecular weights of the prestained standard markers (lane 1) are shown in a kilo scale on the left: phosphorylase *b* (97,400), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), β-lactoglobulin (18,400), and lysozyme (14,300).

incubation at 30 °C. While, under the same condition, the thermosensitive enzyme was reported to lose the activity more than 75% (Istúriz *et al.*, 1986). Additionally, its thermoresistancy was tested after 20 min or 40 min incubation at 25 °C, 30 °C, 40 °C, and 50 °C, and found that only at 50 °C, the activity was reduced to be 63% and 50% after 20 min and 40 min incubation, respectively, compared with that at 25 °C. Optimum temperature of the purified enzyme was also determined to be 45 °C; in comparison with the activity (325 U/mg) at 45 °C, the activities at 25 °C, 30 °C, 40 °C, 50 °C, 55 °C, and 60 °C were 75%, 86%, 97%, 85%, 57%, and 6%, respectively. Therefore, these results indicate that the purified enzyme is the thermoresistant gluconate kinase in *E. coli*.

The kinetic property of the purified gluconate kinase was examined (Table 7). By the assay method with pyruvate kinase and lactate dehydrogenase, the *K*m values for gluconate and ATP were estimated to be 42 μM and 123 μM, respectively, and the *V*max to be 285 U/mg. It was reported that pyruvate acts as a metabolic repressor for the GntII system (Istúriz *et al.*, 1986; Bächi & Kornberg, 1975), and that another *E. coli* thermoresistant gluconate kinase purified by Vivas *et al.* showed 3.9- and 4.4-fold increase in the *K*m values for gluconate and ATP, respectively, in the presence of 1 mM pyruvate (Vivas *et al.*, 1994). I thus also examined the effect of pyruvate on the enzyme activity by the assay method with 6-phosphogluconate dehydrogenase. When pyruvate was added in the assay mixture at the concentrations of 1 mM and 10 mM, the gluconate kinase activity was not changed compared with that in the absence of pyruvate. Therefore, the results indicate that pyruvate has no effect on the activity of the thermoresistant gluconate kinase from *gntK*.

**Comparison of GntK with a thermoresistant gluconate kinase reported recently**

Vivas *et al.* (Vivas *et al.*, 1994) also purified and characterized a thermoresistant gluconate kinase from an *E. coli* K-12 derivative strain, Ca26 (HfrH, *gntR20, gntV18, thiA, strA*) that constitutively produces the thermoresistant gluconate kinase but lacks the thermosensitive one (Istúriz *et al.*, 1986). However, the characteristics of the reported enzyme are quite different from those of GntK purified in this study as follows: first, the specific activity of the reported enzyme was 30 U/mg of protein that was about 8.3 times lower than that of the purified GntK. Second, the native molecular mass of the reported enzyme was 100 kDa with three identical subunits of approximately 29.5 kDa, which completely differs from that deduced from the *gntK* nucleotide sequence. Additionally, the native molecular mass of GntK was 38 kDa with two identical subunits of 18.4 kDa. Third, the *K*m values of the reported enzyme for gluconate and ATP were determined to be 20 μM and 45 μM, respectively, in the presence of 2 mM ATP or 10 mM gluconate, which are 2 and 3 times, respectively, lower than those of GntK even under their conditions. The *K*m value for gluconate of the purified GntK is consistent with that of

**Table 7. Characteristic comparison of the thermoresistant gluconate kinases purified from *E. coli*.**

| | GntK | Reported gluconate kinase [a] |
|---|---|---|
| Native *Mr* | 38 kDa (homodimer) | 100 kDa (homotrimer) |
| Subunit *Mr* | 18.4 kDa | 29.5 kDa |
| Thermoresistance | stable | stable |
| Optimum temperature | 45 °C | 55 °C |
| *K*m for gluconate | 42 (4.0) [b] μM | 20 μM |
| *K*m for ATP | 123 (3.5) [b] μM | 45 μM |
| Effect of pyruvate | no effect | increased *K*m for gluconate and ATP |

[a] Reported by Vivas *et al.* (1994).
[b] Experiments were performed four times and the values with standard deviation were shown.

thermoresistant gluconate kinase determined with *E. coli* crude extracts (Hung *et al.*, 1970). Finally, the apparent *K*m values of the reported enzyme for gluconate and ATP increased in the presence of 1 mM pyruvate, but pyruvate up to 10 mM showed no effect on GntK, which seems to be consistent with the previous report that pyruvate did not affect the activity of the thermoresistant gluconate kinase in the GntI system (Hung *et al.*, 1970; Bächi & Kornberg, 1975). Therefore, I conclude that GntK purified here is different from the thermoresistant gluconate kinase purified by Vivas *et al.*, and that in addition to GntK and GntV, another thermoresistant gluconate kinase may occur in *E. coli*.

## Materials and Methods

### Purification of gluconate kinase encoded by *gntK*

YU120 [Δ*pts gntT*] cells harboring pGNT5, which bears the *E. coli gntK* gene (CHAPTER 1; Izu *et al.*, 1997a), were grown in LB for 15 h, harvested, washed twice with 0.85% NaCl, and suspended with 20 mM Tris-HCl buffer (pH 7.0). The suspended cells were disrupted by passing three times through a French pressure cell press (16,000 p.s.i.), and after removing cell debris by centrifugation at 8,000 x *g* for 10 min, crude extracts were obtained. The extracts were then applied onto a DEAE-Toyopearl column equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was washed with 10 bed volumes of the same buffer. The enzyme was eluted by a gradient composed of 4 bed volumes of the same buffer and 4 bed volumes of the same buffer containing 0.3 M NaCl. Active fractions eluted at about 0.15 M NaCl were pooled and dialyzed against 20 mM Na-acetate (pH 6.0). The dialyzed material was successively applied on a phosphocellulose column equilibrated with 20 mM Na-acetate buffer (pH 6.0). The column was then washed with 10 bed volumes of the same buffer and further with 10 bed volumes of the same buffer containing 0.3 M NaCl. The enzyme was eluted by a gradient composed of 4 bed volumes of the same buffer containing 0.3 M and 0.7 M NaCl. Active fractions eluted at about 0.55 M NaCl were pooled, concentrated by ultrafiltration with a Toyo UK50 membrane filter. The resultant material was found to have a homogeneity of more than 95%, judging from SDS-15% PAGE.

### Enzyme assay

Gluconate kinase activity was measured at 25 °C by the assay system with pyruvate kinase and lactate dehydrogenase (Boehringer Mannheim, Mannheim, Germany) as described (Kayne, 1973) in 50 mM Tris-HCl (pH 7.0) containing 5 mM phosphoenolpyruvate, 65 mM KCl, 20 mM $MgSO_4$, 90 μM NADH, 3.2 mM ATP, and 1 mM sodium gluconate, or by another assay system with 6-phosphogluconate dehydrogenase (Boehringer Mannheim) (Frankel & Horecker, 1964) in 50 mM Tris-HCl

(pH 7.0) containing 20 mM $MgSO_4$, 400 μM $NADP^+$, 3.2 mM ATP, and 1 mM sodium gluconate. The former was used for the assay in enzyme purification, determining kinetic parameters or optimum temperature, and testing enzyme thermoresistancy. Whereas, the latter was used for determining the specific activity of purified enzyme and examining effect of pyruvate on the enzyme activity. *K*m values for gluconate and ATP were determined in the presence of 3.2 mM ATP or 1 mM gluconate. The kinetic constants were estimated by a program, EnzymeKinetics (Trinity Software Technical Support, NH, USA). According to Istúriz *et al.*. (Istúriz *et al.*, 1986), thermoresistancy was examined by measuring the remaining activity at 25 °C after 3 h incubation at 30 °C in 0.2 ml volumes of enzyme solution (250 μg/ml in 20 mM Tris-HCl, pH 7.0), and the activity was compared with that of the same solution without the treatment. Thermoresistancy was also examined by measuring activity at 25 °C after 20 min or 40 min incubation at 25 °C, 30 °C, 40 °C, or 50 °C in the same enzyme volume. For determining optimal temperature, assay solution only containing buffer was preincubated for 20 min at different temperatures and after addition of other components, the reaction was started by the addition of enzyme. The background activity was also obtained by performing the same procedure without gluconate. All these assays were carried out at least three times.

**N-terminal sequencing**

N-terminal sequencing was performed on an automatic gas phase sequenator, PSQ2 (Shimadzu, Kyoto, Japan). The purified gluconate kinase (100 μg) was subjected to SDS-12% PAGE and transferred to a polyvinylidene difluoride membrane. Using a single band obtained from the membrane, the N-terminal amino acid sequence was determined.

**Determination of a native molecular mass**

The purified gluconate kinase (244 μg) was applied onto a TSK gel G3000 SW column (7.5 x 60 cm). HPLC (Shimadzu) was performed with 50 mM potassium phosphate (pH 7.0) containing 100 mM NaCl as a HPLC running buffer under the conditions of pressure 63 kg/cm and flow rate 0.6 ml/min. Standard proteins were also applied onto the same column under the same condition.

**Database searching**

Database searching was carried out using GENETYX (Software Development, Tokyo, Japan).

# CHAPTER 3

## Analysis of the *Escherichia coli gntT* and *gntU* genes and comparison of the products with their homologues

## Summary

The *Escherichia coli* gluconate permease genes, *gntT* and *gntU*, were cloned and characterized. At least four homologues to GntT were found in *E. coli* by database searching. These proteins including GntT and GntU appear to have similar topological structures with 14 membrane-spanning segments, suggesting that they constitute a GntP family.

## Results and Discussion

In the gluconate uptake and catabolism of *Escherichia coli*, the GntI system is known to function predominantly at the initial step, which appears to be supported by the GntII system as a subsidiary (Istúriz *et al.*, 1986). Three genes, *gntT*, *gntU*, and *gntS*, encoding gluconate permeases in the two systems have so far been identified genetically and mapped on the genome (Istúriz *et al.*, 1986; Nagel de Zwaig *et al.*, 1973; Bächi & Kornberg, 1975). Recently, the *gntP*gene encoding another gluconate permease was also shown to be involved in gluconate uptake in *E. coli* (Klemm *et al.*, 1996).

To discover the physiological function of those permeases in gluconate metabolism, I subcloned the *gntT* gene and analyzed the *gntT* and *gntU* genes using conventional recombinant DNA techniques (Sambrook *et al.*, 1989). The *gntT* gene was reported to occur between the *bioH* and *malA* loci and near the *gntRKU* genes (Nagel de Zwaig *et al.*, 1973). Thus, from a Kohara phage clone, E3C10 (Kohara *et al.*, 1987), a mini-library was made and introduced into a strain, YU120 (CHAPTER 1; Izu *et al.*, 1997a), lacking *gntT*. A clone with the positive fermentation was then isolated on EMB (Miller, 1992) plates containing 0.5% gluconate. From the clone, a 2.0-kb fragment was subcloned into pBR322. The resultant clone, pGNTT20, showed a positive gluconate fermentation in YU120 and also in a strain, G6MD2 [$\lambda^-$, *hisA323*, *Δ(bioH-asd)29*] provided by P. Postma, which deletes the region including *gntT*. The nucleotides of the inserted DNA were then sequenced, and one ORF was found in it, suggesting that GntT consists of 437 amino acid residues with a molecular mass of 45.9 kDa. The sequence obtained here was found to match completely with that of *orf-o437* in the GenBank database, which was analyzed by the genomic sequencing project. GntT has significant sequence similarity (50%) to the *Bacillus subtilis* gluconate permease (GntP) (Fujita *et al.*, 1986) as shown in Fig. 10 and Table 8.

I have cloned the *gntRKU* operon, and characterized its gene organization and expressional regulation as described in CHAPTER 1 (Izu, *et al.*, 1997a). Here, all of the nucleotides of *gntU* were sequenced. The initiation codon for GntU with a possible ribosome recognition sequence was found after *gntK*, and the ORF with 448 codons is followed by a possible ρ-independent terminator. The nucleotide sequence of the region including *gntU* appeared in the databases during the time we sequenced its nucleotides. From sequence comparison, it was found that one base (CGAAGCGTT) at the 333th amino acid residue of GntU in our sequence was absent in the sequence of the databases. Based on our sequence, GntU was deduced to consist of 447 amino acid residues with a molecular mass of 46.4 kDa. The protein shows 36% and 35% similarity to the *B. subtilis* GntP and to GntT, respectively. The lack of one base may be from a sequencing error because the protein deduced from the databases had a much shorter C-terminus than GntT, and lower similarity.

Searching for homologues in *E. coli* to GntT was done using the SWISS-PROT database, and four proteins, DsdC, YjiB, YjgT, and YjhF, in addition to GntU were uncovered (Fig. 10). Out of the proteins, DsdC and YjiB were reported to be a D-serine permease in the database and a transporter (encoded by the *gntP* gene) with high affinity for gluconate (Klemm *et al.*, 1996) respectively, and the other two were found to be uncharacterized previously. DsdC, YjiB, and YjgT have 35%, 40%, and 61% similarity to GntT, respectively, and 36%, 37%, and 50% to the *B. subtilis* GntP, respectively (Table 8). The remaining one, YjhF, was also significantly similar to GntT, but its reported size was about 70 amino acid residues shorter than that of GntT. We thus carefully searched three different frames on the reported nucleotide sequences, finding one possible frame shift at the C-terminus, which seems to be caused by lacking one base at the site corresponding to the 354th amino acid residue of the revised YjhF. Because the C-terminal sequence of the revised protein was significantly similar to those of the homologues including GntT, it is assumed that the lacking of one base was due to failing in nucleotide sequencing. The revised YjhF has 48% and 60% similarity to GntT and the *B. subtilis* GntP, respectively. In *Haemophilus influenzae* Rd, a homologue, GntPh, to GntT was also found by database searching but has a slightly longer C-terminus than the others (Fig. 10). On the basis of the similarity to the *B. subtilis* GntP, it is suggested that those proteins, including GntT and GntU, constitute a GntP family.

To examine whether those proteins have topological structures similar to that of the *B. subtilus* GntP or not, their hydropathy plots (Kyte & Doolittle, 1982) were compared (data not shown). They show the profiles resembled to each other and have 14 hydrophobic segments, enough to span the *E. coli* cytoplasmic membrane. Therefore, it is suggested that they form similar structures in the membrane, and that YjgT and YjhF may also function as transporters. It is noteworthy that the GntP family may be unique in respect of having 14 membrane-spanning segments since a large family of transporters have 12 membrane-spanning segments (Griffith *et al.*, 1992). Therefore, the family may be classified into one subgroup of the entire transporters.

The functions and gene organization of those homologues were deduced according to the EMBL database. The *yjgT* gene is located close to the *yjgS* (*gntH*), *yjgU*, *yjgV*, and *gntV* genes at the *pepA-gntV* loci at 96.7 min. GntV and YjgS (GntH) show 47% similarity to a gluconate kinase, GntK, in the GntI system and 46% similarity to a repressor, GntR, for genes in the system, respectively. Moreover, YjgU shows 42% similarity to 2-deoxy-D-gluconate 3-dehydrogenase, KduD, in *Erwinia chrysanthemi* (Miller, 1992). Thus, they might be constituents of the GntII system, and especially, it is possible that GntV and GntH are a gluconate kinase and a regulator for the system, respectively. The *yjgT* gene might be the *gntS* (*gntW*) gene encoding a high affinity gluconate permease in the GntII system because it is located near *gntV* and *yjgT* and no ORF similar to GntT was found between *aidB* and *rpsF* near 95.3 min where *gntS* was

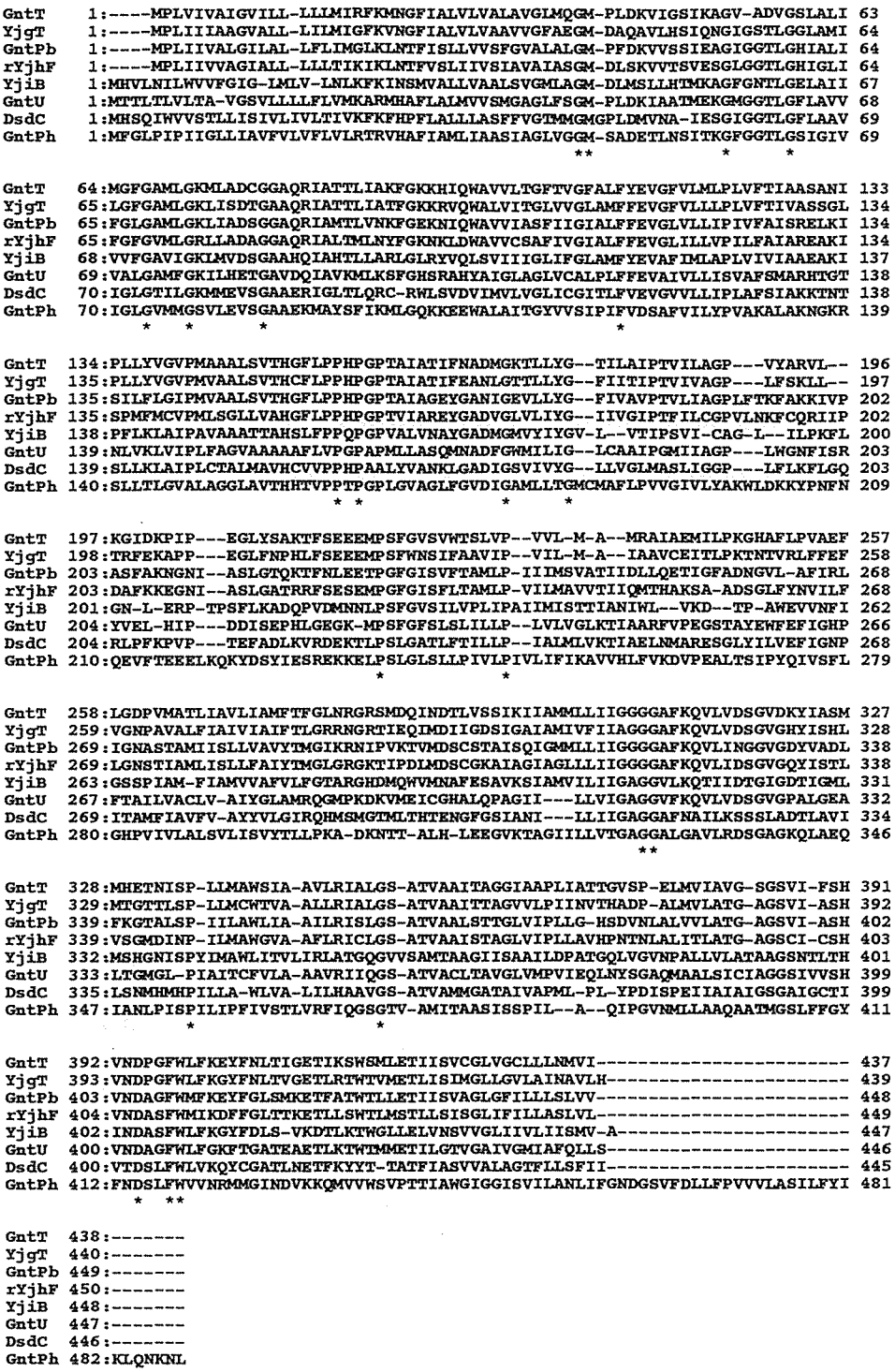


**Figure 10. Sequence alignment of gluconate permeases, GntT and GntU, with their homologues.**

Sequence alignment was done by using the MAlign program of GENETYX (Software Development, Tokyo, Japan). GntT, GntPb, and GntU are a high affinity gluconate permease in *E. coli*, a gluconate permease in *B. subtilus*) and a low affinity gluconate permease in *E. coli,* respectively. YjgT, YjiB, and DsdC were selected as homologues in *E. coli* to GntT from the databases and GntPh also selected as a homologue in *H. influenzae* Rd from the databases. rYjhF was a sequence revised from YjhF in the databases. DsdC and YjiB were reported to be D-serine permease in the databases and a high-affinity gluconate permease, respectively, and YjgT and YjhF were uncharacterized previously. Asterisks show amino acid residues conserved among the proteins.

**Table 8. Percent identities among proteins of gluconate permeases, GntT and GntU, and their homologues.**

| | YjgT [a] | GntPb | rYjhF | YjiB | GntU | DsdC | GntPh |
|---|---|---|---|---|---|---|---|
| GntT | 61 | 50 | 48 | 40 | 35 | 35 | 30 |
| YjgT | | 50 | 48 | 40 | 36 | 32 | 28 |
| GntPb | | | 60 | 37 | 36 | 36 | 28 |
| rYjhF | | | | 36 | 33 | 33 | 29 |
| YjiB | | | | | 32 | 32 | 24 |
| GntU | | | | | | 35 | 30 |
| DsdC | | | | | | | 32 |

[a] Proteins are as described in the legend to Figure 10.

originally mapped (Bächi & Kornberg, 1975). On the other hand, the *yjhF* gene occurs at 97 min, where no such gene related to sugar metabolism has been reported. Since YjhF is located the most closely to the *B. subtilis* GntP in the phylogenetic tree (data not shown), it may be another gluconate permease in *E. coli*. For identifying the physiological functions and roles of the genes discovered here, biochemical and molecular genetic experiments are required, which are necessary for understanding the gluconate uptake and catabolism of *E. coli*.

## Materials and Methods

### Materials

Restriction enzymes, T4 DNA ligase, and the DNA sequencing kit were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (MA, USA). Other chemicals were of analytical grade.

### Bacterial strains

*E. coli* K-12 strains used in this study were YU120, *Δpts*, *gntT* (Izu *et al.*, 1997a) ; JM103, *Δ(lac-pro) thi strA supE endA sbcB hspR4* F' *traD36 proAB lacI*$^{q}$ *ZΔM15* (Messing, 1983).

### Subcloning *gntT* and nucleotide sequencing

Conventional recombinant DNA techniques (Sambrook *et al.*, 1989) were used for subcloning and analyzing genes. To subclone the *gntT* gene, E3C10 phage DNA (2 μg) from the Kohara library (Kohara *et al.*, 1987) was partially digested with *Sau*3AI and ligated with pBR322 (Bolivar *et al.*, 1977) which had been digested with *Bam*HI. The ligated material was introduced into YU120, and the cells were grown on EMB plates (Miller, 1992) containing 0.5% gluconate and ampicillin (100 μg/ml).

DNA sequencing for the *gntT* and *gntU* genes was carried out by the dideoxy-chain termination method (Sanger, 1977) using M13 mp18 or 19 (Yanish-Perron *et al.*, 1985) as a vector. Southern blot analysis (Sambrook *et al.*, 1989) was performed for mapping the cloned *gntT* gene with the Kohara ordered phage library (Kohara *et al.*, 1987). DNA fragments for the sequencing and the blotting were prepared from purified plasmid DNAs after restriction enzyme digestion and polyacrylamide gel electrophoresis (Sambrook *et al.*, 1989).

### Homology searching, hydropathy analysis, and construction of phylogenetic tree

Nucleotide sequences and the deduced amino acid sequences were compared with those listed in the EMBL, GenBank, SWISS-PROT, and NBRF-PIR databases from

GENETYX (Software Development, Tokyo, Japan). Hydropathy analysis was performed by the Kyte and Doolittle method (Kyte & Doolittle, 1982). Phylogenetic tree was constructed by the UPGMA method (Sokal & Michener, 1958; Nei, 1987) in GENETYX.

# CHAPTER 4

## Characterization of the *gntT* gene encoding a high affinity gluconate permease in *Escherichia coli*

## Summary

The *gntT* gene encoding a high affinity gluconate permease of *Escherichia coli* K-12 was characterized. Primer extension and *lacZ*-operon fusion analyses revealed that *gntT* has one strong and two weak promoters, all of which are regulated positively by cAMP-CRP and negatively by GntR. The weak promoters became constitutive when separated from the upstream region including the strong promoter that overlaps a putative GntR-binding sequence. Gluconate-specific uptake activity was observed with cells harboring the *gntT* plasmid clone, which was enhanced by the presence of *gntK* encoding gluconate kinase.

## Results

### Gluconate uptake activity from the *gntT* gene

To examine the function of the cloned *gntT*, we measured gluconate uptake activity was measured using a host strain, Hfr G6MD2, which lacks *gntT* as well as *gntRKU* (Fig. 11). Cells harboring pGNTT20, bearing *gntT*, showed the significant activity compared to cells harboring the vector alone or pGNT5, bearing *gntK*. The uptake activity in pGNTT20 increased linearly till 5 min and reached the steady state thereafter. There results suggest that GntT is able to import gluconate into the cells.

The uptake activity of cells harboring pGNTTK, bearing *gntT* and *gntK*, was found to be significantly higher than that of cells harboring pGNTT20. The rate limiting step in this uptake process is thought to be the transport step not the kination for the following reasons. First, analysis with *lacZ* operon fusions revealed that *gntT* was expressed about 7-fold lower than *gntK* (data not shown). Second, the turnover rate of GntT in gluconate transport is expected to be much lower than that of GntK whose turnover in kination was calculated to be about 160/s (CHAPTER 2; Izu *et al.*, 1996).

The uptake data in pGNTTK, therefore, indicate that GntT activity is enhanced by the presence of GntK. The enhancement was observed not only at the early phase where the uptake activity increased in time-dependent manner, but also at the late steady state phase. The higher steady state values in pGNTTK may be due to increase in gluconate uptake by conversion of gluconate to gluconate-6 phosphate. In the case of pGNTT20, the steady state values were low presumably because the accumulated gluconate inside the cells repress GntT activity. On the other hand, the enhanced activity at the early phase, specially till 3 min by pGNTTK, may be due either to the conversion of uptaken gluconate to gluconate-6 phosphate, or to stimulation of transport ability of GntT by interaction with GntK.

The *K*m value of GntT for gluconate was estimated to be 13 μM in the absence of GntK and 20 μM in the presence of GntK, suggesting that GntK has a little effect on the affinity of GntT for gluconate. The values of GntT were about 10-fold lower than that of GntU (Tong *et al.*, 1996) but were nearly equivalent to that of GntP (Klemm *et al.*, 1996), suggesting that the *gntT* gene encodes a high affinity gluconate permease. The uptake activity of Hfr G6MD2 cells harboring pGNTTK was inhibited strongly by the addition of cold gluconate but not by other sugars tested (Table 9). The permease was thus specific for gluconate. The specificity of GntT was similar to that of GntU (Tong *et al.*, 1996) and was higher than that of GntP (Klemm *et al.*, 1996) with substrates tested. Thus, *E. coli* has been shown to have at least three gluconate permeases.

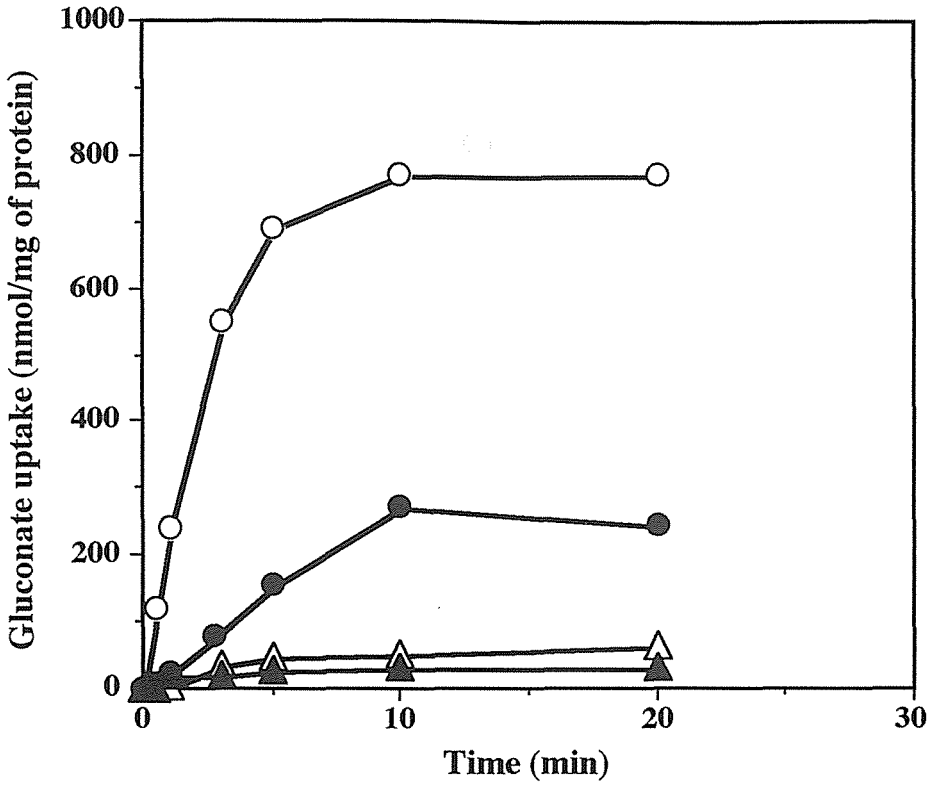


**Figure 11. Gluconate uptake activity from pGNTT20 bearing the *gntT* gene or pGNTTK bearing the *gntT* and *gntK* genes.** Gluconate uptake assay was performed as described in Materials and Methods. Hfr G6MD2 lacking the *gntRKU* and *gntT* genes was used as a host strain. Values are the averages of three assays. Cells harboring pGNTTK (open circles), pGNTT20 (closed circles), pGNT5 bearing the *gntK* gene (open triangles) or vector (pBR322; closed triangles) were subjected to the time course experiment.

**Table 9. Inhibition of gluconate uptake by alternative sugars in Hfr G6MD2 harboring pGNTTK.**

| Competitor | Activity of gluconate uptake [a] (nmol/min/mg) | % |
|---|---|---|
| Control | 1.23 (± 0.12) | 100 |
| Gluconate | 0.09 (± 0.05) | 7.6 |
| 2-Keto-gluconate | 1.29 (± 0.1) | 105 |
| Glucuronate | 1.13 (± 0.11) | 92 |
| Glucose | 1.08 (± 0.13) | 88 |
| Fructose | 1.09 (± 0.12) | 83 |
| Galactose | 1.40 (± 0.1) | 114 |
| Mannitol | 1.52 (± 0.14) | 124 |
| Sucrose | 1.22 (± 0.11) | 99 |
| Maltose | 1.19 (± 0.06) | 97 |

[a] Assays were performed more than three times. Mean uptake activities are shown with standard deviations.

**Promoter and operator analysis by operon fusion with *lacZ* and primer extension**

To examine the promoter of the *gntT* gene and the regulation of its expression, we constructed operon fusions with *lacZ* were constructed as shown in Fig. 12. These fusions were introduced into MC1000 and β-Gal activity was measured. Cells harboring pGNTT-LAC2 or pGNTT-LAC5 exhibited less activity than those harboring pGNTT-LAC4, but more than those harboring the vector alone. These results suggested that there are at least two promoters, one upstream from the *Kpn*I site, positions 240 to 245 (Fig. 13), and the other downstream from the same *Kpn*I site.

To determine the position of the promoter, I performed primer extension analysis. A strong band and two weak bands corresponding to positions 238, 280, and 283, respectively, were detected from YU230 harboring pGNTT-LAC4 (Fig. 14A). The strong band was also detected from pGNTT-LAC2, and two bands corresponding in position to the two weak bands were also detected from pGNTT-LAC5 (Fig. 14B and C). The promoter (P1) sequence for the strong band was thus predicted to be TATCAT (-10 sequence) and TTCTCA (-35 sequence), and P2 and P3 for the weak bands to be TACAAT and AATAAT (-10 sequence) and GTACCA and CAAACA (-35 sequence), respectively, as shown in Fig. 13, all of which are similar to the canonical $\sigma^{70}$-dependent promoter sequence (Miller, 1992).

The intensities of the three bands in pGNTT-LAC4 were reduced by the presence of pGNTR2 bearing the *gntR* gene (Fig. 14A), suggesting that the promoters are negatively regulated by GntR. Consistently, a putative GntR-binding sequence GATGTTACCCGTATCATT was detected from position 216 to 233 overlapping with the P1 sequence (Fig. 13), which is similar to that found at the operator region of the *gntK* gene as described in CHAPTER 1 (Tong *et al.*, 1996; Izu *et al.*, 1997a). GntR thus seems to function as a negative regulator of *gntT* by binding to the putative sequence (see below). No such sequence, however, was found around the P2 and P3 sequences. Since P2 and P3 are close to the putative GntR-binding sequence overlapping with P1, the transcription from these promoters may also be influenced by GntR binding.

The addition of cAMP increased the intensities of all three bands in the presence and absence of GntR, suggesting that cAMP-CRP acts as a positive regulator of *gntT*. A putative cAMP-CRP binding sequence (TAATATGACCAACCTCTCATA at positions 157-177) homologous to the canonical sequence (Ebright *et al.*, 1989) was detected 24 bp upstream of the -35 sequence of P1. The cAMP-CRP complex thus seems to bind to the putative sequence and to enhance *gntT* transcription (see below).

There is a sequence homologous to an REP sequence (Stern *et al.*, 1984) following the termination codon of *orf191*. Although most REP sequences are located between cistrons in various operons (Stern *et al.*, 1984), no functional relationship between ORF191 and GntT was suggested by database searching. ORF191 encodes a predicted

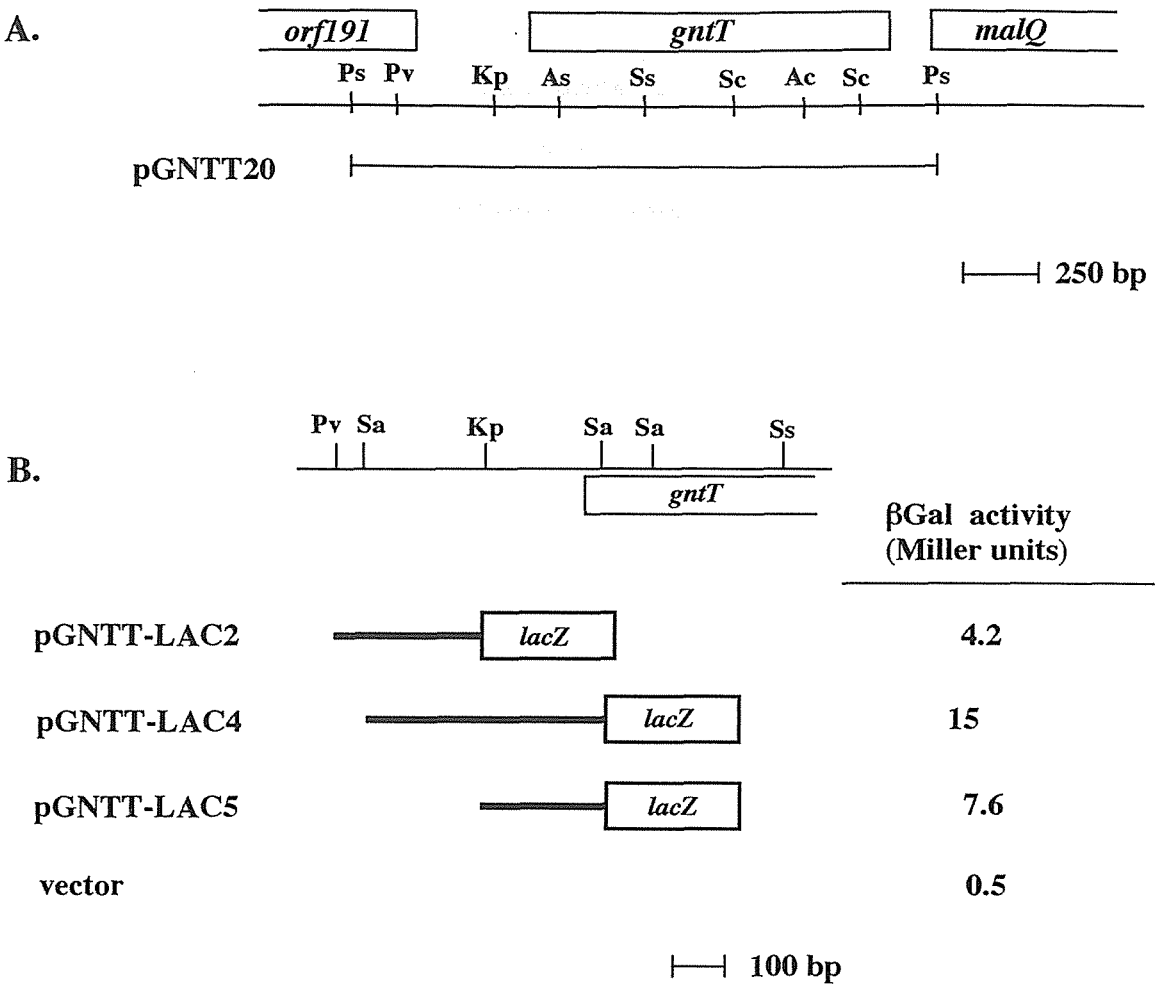


**Figure 12. Structures of the *gntT* clone, pGNTT20, and *gntT-lacZ* fusions.**
(A) The *gntT* gene is located at 76.4 min on the *E. coli* W3110 genome. Plasmid pGNTT20 has the 2.0-kb *Pst*I fragment from the Kohara clone, E3C10.
(B) *gntT-lacZ* operon fusions, pGNTT-LAC2, -LAC4, and -LAC5, were constructed as described in Materials and Methods. MC1000 cells harboring one of pGNTT-LACs and pGNTR2 bearing *gntR* were grown for 4 h and their βGal activities were measured (Miller, 1992). The regions inserted in the operon fusions are indicated by heavy solid lines, and the promoterless *lacZ* genes are shown by boxes. Ps; *Pst*I, Pv; *Pvu*II, Kp; *Kpn*I, As; *Ase*I, Ss; *Ssp*I, Sc; *Sca*I, Sa, *Sau*3AI.

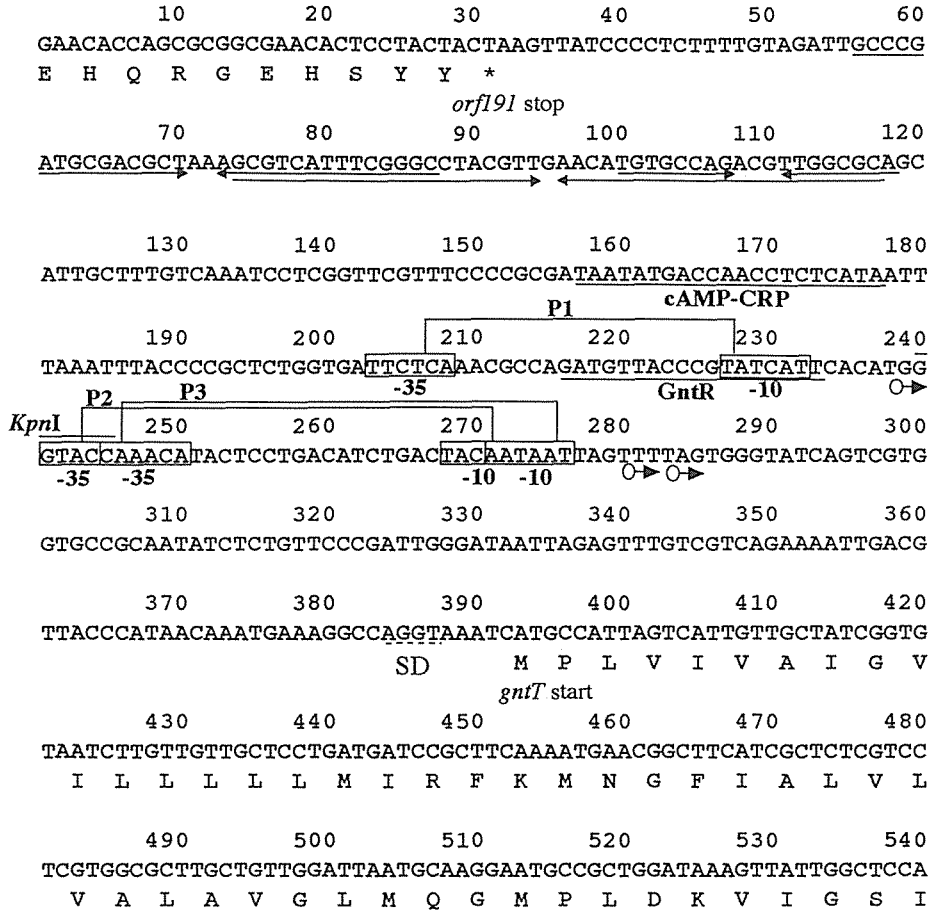


**Figure 13. Nucleotide sequence of the 5'-flanking region of the *gntT* gene.** *orf191* ends at position 33 and *gntT* starts at position 393. Arrows with a circle at positions 238, 280 and 283 represent the initiation site and direction of the *gntT* mRNA synthesis. The corresponding promoter sequences (P1, P2, and P3) are represented by boxes. The broken underline represents a putative ribosome-recognition sequence. The putative GntR-binding (positions 216-233) and cAMP-CRP binding sites (positions 157-177) are represented by underlines. The REP sequence (positions 56-118) is represented by arrows. Accession number for *orf191*, *gntT*, and *malQ* is U18997.

polypeptide of 191 aa residues with a molecular mass of 21 kDa which is expected to be a soluble protein from the results of hydropathy analysis (data not shown), and has 74% identity to a protein (Accession number: T31774) at the transformation locus in *Haemophilus influenzae*.

**Effect of GntR on *gntT* expression**

The effect of GntR on *gntT* expression was examined using the operon fusions, where pGNTR2 bearing *gntR* was added in *trans* (Table 10). Plasmid pGNTR2 or control vector pYY2 was introduced into YU230 cells harboring one of pGNTT-LACs. In the cases of pGNTT-LAC2 and -LAC4, β–Gal activities of the cells harboring pGNTR2 were about 15% of those of control cells harboring pYY2 without gluconate and cAMP. The activities were increased 2- to 3-fold by the addition of gluconate. Such reduction by GntR and increase by gluconate were also observed in the primer extension experiments in the cases of pGNTT-LAC4 and -LAC2 (Fig. 14A and B, and data not shown). Therefore, it was confirmed that GntR is a negative regulator of *gntT*. Since a putative GntR-binding sequence overlaps with the P1 sequence, the repression appears to be achieved by steric hindrance to binding of RNA polymerase to the promoter.

On the other hand, no such reduction of β–Gal activity by GntR was observed in pGNTT-LAC5 bearing P2 and P3 (Table 10). Primer extension analysis showed reduction in density of both bands for P2 and P3 in the presence of GntR in pGNTT-LAC4 (Fig. 14A), but the corresponding bands at the same position in pGNTT-LAC5 were not reduced by the presence of GntR and no other band in addition to those for P2 and P3 was detected in pGNTT-LAC5 under the induced and non-induced conditions (Fig. 14C and data not shown). Therefore, P2 and P3 are thought to become constitutive after separated from the upstream region including P1, which seems to be due to deletion of the GntR-binding sequence.

Reduction of β–Gal activity by the addition of gluconate was observed in pGNTT-LAC2/pYY2 and pGNTT-LAC4/pYY2 under the conditions without cAMP, and a similar effect of gluconate was observed by primer extension analysis. This reduction might be due to repression by catabolite(s) derived from gluconate, which should not be cAMP-dependent because the used host strain, YU230 lacks adenylate cyclase.

**Effect of cAMP on *gntT* expression**

β–Gal activities from pGNTT-LAC2 and -LAC4 in the *Δcya* background were increased 3- and 4-fold, respectively, by the addition of cAMP in the presence of GntR and gluconate (Table 10). Such activation by cAMP was also observed in primer extension experiments (Fig. 14A and B). These results are consistent with the existence of the putative cAMP-CRP binding sequence upstream from P1. Therefore, it is suggested that P1 is dependent on cAMP-CRP. In the absence of GntR and gluconate,

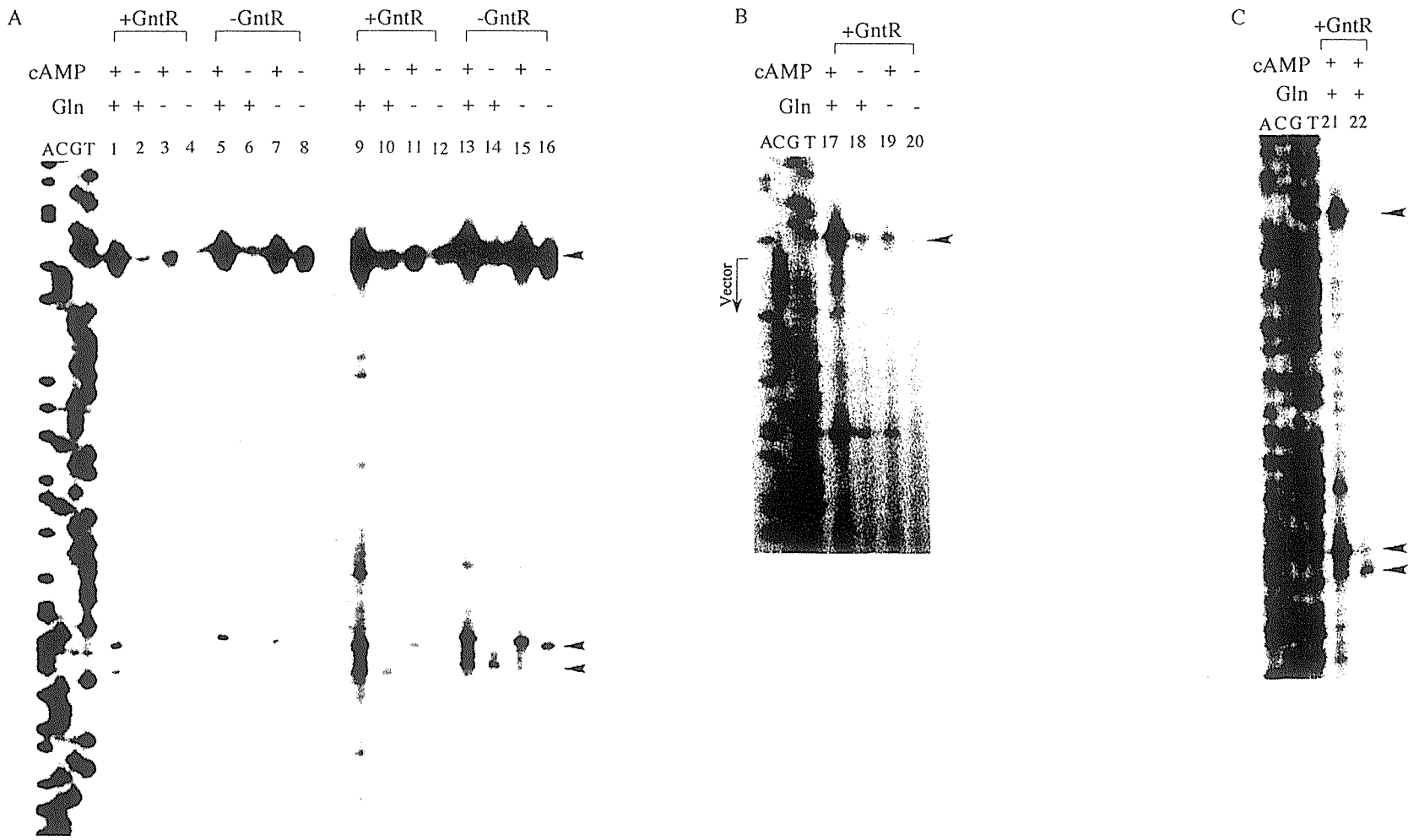


**Figure 14. Primer extension analysis of the *gntT* gene.**
Primer extension and nucleotide sequencing were performed with the same FITC-labeled primer. Nucleotide sequence of the sense strand of the DNA around the transcription start point is shown in lanes A, C, G, and T. (A) In lanes 1-4, mRNA was used from YU230 cells harboring pGNTT-LAC4 and pGNTR2, which were grown with or without 1 mM cAMP or 0.5% gluconate (Gln) as shown on the top. In lanes 5-8, mRNA was from YU230 cells harboring pGNTT-LAC4 and pYY2, a control plasmid. Lanes 9-16 were exposed for longer than lanes 1-8. (B) In lanes 17 -20, mRNA was used from YU230 cells harboring pGNTT-LAC2 and pGNTR2, which were grown with or without 1 mM cAMP or 0.5% gluconate. (C) In lanes 21 and 22, mRNA was used from YU230 cells harboring pGNTT-LAC4 and pGNTR2, and harboring pGNTT-LAC5 and pGNTR2, respectively, which were grown with 1 mM cAMP and 0.5% gluconate. Arrowheads indicate significant bands. Upper band corresponds to the transcript from the promoter, P1 shown in Fig. 13 and lower two bands correspond to the transcripts from P2 and P3.

**Table 10. Regulation of the *gntT* gene expression.**

| Strain | β–Gal activity (Miller units) [a] | | | |
|---|---|---|---|---|
| | +gluconate +cAMP | +gluconate -cAMP | -gluconate +cAMP | -gluconate -cAMP |
| YU230 (pGNTT-LAC2/ pYY2) | 36 | 8.5 | 36 | 21 |
| YU230 (pGNTT-LAC2/pGNTR2) | 27 | 9.2 | 4.2 | 3.2 |
| YU230 (pGNTT-LAC4/ pYY2) | 84 | 26 | 80 | 69 |
| YU230 (pGNTT-LAC4/pGNTR2) | 73 | 19 | 15 | 8.1 |
| YU230 (pGNTT-LAC5/pYY2) | 3.6 | 5.9 | 7.3 | 7.4 |
| YU230 (pGNTT-LAC5/pGNTR2) | 5.8 | 8.0 | 7.6 | 8.3 |

[a] Values are the averages of more than three independent experiments.

β-Gal activities from pGNTT-LAC2 and -LAC4 in the absence of cAMP were about 70% of those in the presence of cAMP, indicating that *gntT* expression is not highly dependent on cAMP in the absence of GntR, whereas *gntKU* expression is completely dependent on cAMP as described in CHAPTER 1 (Izu *et al.*, 1997a).

Primer extension analysis revealed that the intensities of the bands for P2 and P3 in pGNTT-LAC4 also increased in the presence of cAMP (Fig. 14A) but little change was observed in pGNTT-LAC5 (data not shown). β-Gal activity from pGNTT-LAC5 was not changed by the addition of cAMP (Table 10). Therefore, P2 and P3 appear to be influenced mainly by binding of cAMP-CRP to the putative site upstream from P1.

## Discussion

To elucidate the physiological function of the *gntT* gene in gluconate uptake and catabolism of *E. coli*, I analyzed the transport ability of the product and showed that it specifically takes up gluconate with high affinity into cells. When *gntT* was expressed together with *gntK* cloned on the same vector, the transport activity was significantly enhanced. It suggests that as indicated genetically, GntT is functionally related to GntK though *gntT* is located at some distance from *gntK*. The cooperation with GntK may be important for the cells specially in the presence of relatively low concentrations of gluconate.

I also addressed regulation of *gntT* expression. Experiments with primer extension and operon fusions suggested that the major promoter, P1, of the gene is repressed by GntR and the repression is relieved by the addition of gluconate, and that cAMP-CRP functions as a positive regulator for the promoter. Two weak promoters, P2 and P3, were found which were also influenced by GntR and cAMP-CRP. The promoters became constitutive after separated from the upstream region including P1, probably because of elimination of the GntR effect. Since P2 and P3 in pGNTT-LAC4 seem to have no overlapping GntR-binding sequence but to be subjected to the GntR control, it may be possible that the weak promoters avoid the GntR effect by factor(s) or event(s) like DNA bending to be a constitutive promoter, which would be required for initial gluconate uptake for induction of the *gnt* operon expression.

## Materials and Methods

### Materials

Restriction enzymes, T4 DNA ligase and a DNA sequencing kit were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (MA, USA). Sodium D-[1-

$^{14}$C] gluconate was from American Radiolabeled Chemicals Inc. (St. Louis, USA). Other chemicals were of analytical grade.

**Bacterial strains and plasmids**

The bacterial strains used were derivatives of *E. coli* K-12. Their relevant genotypes and plasmids are shown in Table 11. *Δcya*, deletion of adenylate cyclase gene, was transferred by P1 transduction (Miller, 1992) from CA8306, generating YU230.

**Construction of *gntT* clones and operon fusions**

Conventional recombinant DNA techniques were applied (Sambrook *et al.*, 1989). To confirm the constructs, nucleotide sequencing was performed by the dideoxy-chain termination method (Sanger *et al.*, 1977).

Plasmid pGNTT20 bearing *gntT* was constructed by insertion of the 2.0-kb *Pst*I fragment from the Kohara clone E3C10 into pBR322 (Fig. 12A) (Yamada *et al.*, 1996). pGNTTK bearing *gntT* and *gntK* was constructed by insertion of the 1.2-kb *Eco*RI-*Hin*dIII fragment from pGNTKU-CAT7 (CHAPTER 1; Izu *et al.*, 1997a) into the *Eco*RI-*Hin*dIII site on pGNTT20. Hfr G6MD2 cells harboring pGNTTK showed stronger fermentation than those harboring pGNTT20 on EMB plates (Miller, 1992).

Operon fusions with the *lacZ* gene were constructed to examine regulation of *gntT* expression. DNA fragments from the *gntT* gene were isolated and inserted in front of the promoterless *lacZ* gene in pCB192 (Schneider & Beck, 1986). Plasmids pGNTT-LAC2, -LAC4, and -LAC5 (Fig. 12A) have the 286-bp *Pvu*II-*Kpn*I fragment, the 454-bp *Sau*3AI fragment, and the 254-bp *Kpn*I-*Sau*3AI fragment, respectively, from pGNTT20. The *Kpn*I site of the fragments was blunt-ended by treatment with T4 DNA polymerase as described (Yamada & Nakazawa, 1984) before insertion into the vector.

**Primer extension mapping**

Primer extension was performed as described in CHAPTER 1 (Izu *et al.*, 1997a). An FITC-labeled primer, CGCCAGGGTTTTCCCAGTCACGAC, synthesized by Sawady Technology (Tokyo, Japan), was used to analyze the *gntT* promoter. Total RNA was isolated by the hot phenol method (Aiba *et al.*, 1981) from cells harboring plasmids, which were grown at 37 °C for 8 h in the presence or absence of 0.5% gluconate and/or 1 mM cAMP. Primer extension reaction was carried out at 60 °C for 60 min in 0.5-ml microcentrifuge tubes in a final volume of 20 μl containing 3 μg of total RNA, 1 pmol of FITC-labeled primer, 200 μM dNTPs and 5 units of r*Tth* DNA polymerase (Perkin-Elmer-Cetus Instruments) in 30 mM Tricine (pH 8.5), 75 mM potassium acetate, 1.1 mM $Mn(OAc)_2$ and 10% glycerol. Nucleotide sequencing reaction was carried out using the same FITC-labeled primer and pGNTT-LAC4 DNA as a template. Both samples were

**Table 11. Bacterial strains and plasmids.**

| Strain or plasmid | Genotype or description | Reference or source |
|---|---|---|
| Strains | | |
| MC1000 | *araD139 Δ(ara, leu)7697* | Casadaban & Cohen |
| | *ΔlacX74 galU galK strA* | (1980) |
| CA8306 | Hfr Hayes $Sm^S$ $B1^-$ *Δcya* | J. Beckwith |
| YU230 | MC1000, *Δcya* | This study |
| Hfr G6MD2 | $\lambda^-$ *his323 Δ(bioH-asd)29* | M. Schwartz |
| TG1 | *supE hsdΔ5 thi Δ(lac-proAB)* | Sambrook *et al.* |
| | F *'traD36 proAB* $lacI^q$ *lacZΔM15* | (1985) |
| Plasmids | | |
| pBR322 | $amp^r$ $tet^r$ | Bolivar *et al.* (1977) |
| pGNTT20 | pBR322 with the 2.0-kb *Pst*I fragment bearing *gntT* | Yamada *et al.* (1996) |
| pGNTKU-CAT7 | $amp^r$ $cm^r$ *gntK* truncated *gntU* | Izu *et al.* (1997a) |
| pGNT5 | pBR322 with the 2.0-kb fragment bearing *gntK* | Izu *et al.* (1997a) |
| pGNTTK | pGNTT20 with the 1.2-kb *Eco*RI-*Hin*dIII fragment bearing *gntK* from pGNTKU-CAT7 | This study |
| pCB192 | $amp^r$ *lacZ galK* | Schneider & Beck (1986) |
| pYY2 | frame-shift mutation of the *bla* gene on pACYC177 | Yamada *et al.* (1995) |
| pGNTR2 | pACYC177 derivative, *gntR* | Izu *et al.* (1997a) |
| pGNTT-LAC2 | pCB192 with the 286-bp *Pvu*II-*Kpn*I fragment from pGNTT20 | This study |
| pGNTT-LAC4 | pCB192 with the 454-bp *Sau*3AI fragment from pGNTT20 | This study |
| pGNTT-LAC5 | pCB192 with the 254-bp *Kpn*I-*Sau*3AI fragment from pGNTT20 | This study |

analyzed using an SQ3000 nucleotide sequencer (Hitachi Electronics Engineering, Tokyo, Japan) with a 6.1-M urea-6% polyacrylamide gel.

**Gluconate uptake assay**

Gluconate uptake assay was carried out using $^{14}$C-gluconate by a modification of the method of Saier *et al.* (1976). Cells for the uptake assay were prepared as follows. Cells grown at 37 °C for 4 h were harvested, washed with 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM $MgSO_4$, and resuspended in the same buffer (adjusted to about 3.0 at $OD_{600}$). The cells were then incubated at 30 °C for 20 min in the presence of chloramphenicol (30 μg/ml) and stored at 0 °C until use. The cells were used within 3 h. In time course experiments, 300 μl aliquots of the cell suspension were preincubated in test tubes at 30 °C for 5 min, and then the reaction was started by addition of the labeled sugar. The time course of uptake was determined by transferring 50 μl aliquots of the suspension at various times to precooled tests tube containing 1 ml of 50 mM potassium phosphate, pH 7.0, containing 250 mM gluconate. The mixtures were immediately transferred on Millipore filters (0.45 μm pore size) and filtered rapidly, and the cells were then washed three times with 3 ml of the same buffer at room temperature. In kinetic experiments, the uptake reaction was performed by the same procedure used in the time course experiments except the reaction time of 30 sec. The filters were dried and radioactivity was determined by liquid scintillation spectrometry using a toluene-based liquid scintillation fluid (Nacalai Tesque, Inc., Kyoto, Japan). In competition assays, gluconate uptake assay was performed as described above with 40 μM sodium [1-$^{14}$C] gluconate but in the presence of a 40-fold concentration of alternative sugars.

**Enzyme assay**

Cells harboring plasmids were grown in LB medium (1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl) containing ampicillin (100 μg/ml) and/or kanamycin (50 μg/ml) at 37 °C for 4 h or 8 h and subjected to β–Gal assay (Miller, 1992). To induce the *gntT* expression, 0.5% gluconate or 1 mM cAMP was added to the medium.

# CHAPTER 5

## Characterization of the *gnt* genes in GntII system and transcriptional regulation of the GntI and GntII genes involved in gluconate uptake and catabolism of *Escherichia coli*

## Summary

The *gnt* genes in GntII system that is involved in the *Escherichia coli* gluconate uptake and catabolism were cloned and characterized. By nucleotide sequence analysis and homology searching, 5 genes, *gntV, yjgV, yjgU, gntW,* and *gntH* were identified which encode a thermosensitive gluconate kinase, an alcohol dehydrogenase-like protein, a 2-deoxy-D-gluconate 3-dehydrogenase-like protein, a gluconate permease, and a regulator, respectively. The *gntV* gene appears to be monocistronic and other genes constitute the *yjgV-yjgU-gntW-gntH* operon. Primer extension and *lacZ*-operon fusion analyses indicated that there are three promoters in front of *yjgV, gntH,* and *gntV*, and that the former expression is enhanced by cAMP-CRP complex. The expression from the *gntT* and *gntK* promoters in the GntI system was repressed by GntR and GntH at the early and late growth phases and induced by the addition of gluconate, whereas the expression from the promoters in the GntII was repressed by GntR at the late growth phase and activated by GntR and GntH in the presence of gluconate at the same phase. Single-copy GntI gene-*lacZ* operon fusion analysis revealed that in the presence of gluconate, their expression increased at the early growth phase and decreased before the late growth phase. Moreover, the expression of *gntR* and *gntH* was increased significantly by GntH and slightly by GntR in the presence of gluconate at the late growth phase, indicating that GntR and GntH seem to accumulate inside cells. Basing on all these results, I discussed the system relay from GntI to GntII along with cell growth via the mutual expressional control that is directed by GntH and GntR.

## Results

### Cloning and characterization of the *gnt* genes in GntII system of *Escherichia coli*

The *gnt* genes in GntII system were reported to be located between *pepA-gntV* loci at 96.9 min on the *E. coli* genome (Istúriz *et al.*, 1986). Based on the data, I cloned these genes from a Kohara phage clone E4D8 as described in Materials and Methods. In the locus, there are five genes, *gntV, yjgV, yjgU, gntW* (*yjgT*), and *gntH* (*yjgS*) as listed in database and as expected in CHAPTERs 1 and 3 (Yamada *et al.*, 1996; Izu *et al.*, 1997a) (Fig. 15). The function of their products was deduced by homology searching with the EMBL database. GntV, GntW, and GntH show 47% similarity to a gluconate kinase, GntK, 61% to a gluconate permease, GntT, and 46% to a repressor for the *gnt* genes, GntR, respectively, which are involved in GntI system. The *yjgT* gene may be the *gntS* gene encoding a high affinity gluconate permease for the GntII system because it is located near *gntV* and no ORF similar to GntT was found between *aidB* and *rpsF* near 95.3 min where *gntS* was originally mapped (Bächi and Kornberg, 1975; Yamada *et al.*, 1996). However, Istúriz & Celaya demonstrated that *gntS* may specify a trans-acting positive regulator for expression of at least *gntV*, instead of a gluconate permease postulated previously (Istúriz & Celaya, 1997). I thus denote *yjgT* encoding a gluconate permease as *gntW* to distinguish it (Peekhaus *et al.*, 1997) from *gntS*. YjgU shows 42% similarity to 2-deoxy-D-gluconate 3-dehydrogenase, encoded by *kduD* in *Erwinia chrysanthemi*, and YjgV belongs to the zinc-containing alcohol dehydrogenase family or ribitol dehydrogenase family. However, the physiological functions of these two gene products are not clear. From promoter analysis and gene organization, which are described below in addition to these homologies, I assume that they may also constitute the GntII system, and that GntV, GntW, and GntH are a gluconate kinase, a gluconate permease, and a repressor for the genes in the gluconate utilizing system, respectively.

### Sequence characteristics of the *gnt* genes in GntII system

(i) *gntV*. The *gntV* ORF consists of 564 b with an ATG initiation codon at position 359 (Fig. 16). A possible ribosome-binding sequence, AGGA, occurs 8 b upstream of the initiation codon. A DNA sequence, GCGCCTTACCGTCGCGCACATGCTGGATGG CGTCGTTAATTTT, 35 b downstream of its stop codon may be a rho-independent terminator that is able to form a stem-loop structure followed by a series of T residues, and there is a stop codon of the other ORF (*yjgB*) 3 b downstream of the stop codon of *gntV* on the antisense strand. These facts suggest that the *gntV* gene is monocistronic. GntV is predicted to consist of 187 amino acid residues with a molecular mass of 21,004 Da, which possesses a sequence ,GVSGS GKTLIGSKV, similar to the ATP-binding consensus sequence (Walker *et al.*, 1982) at its N-terminal portion. The primer extension

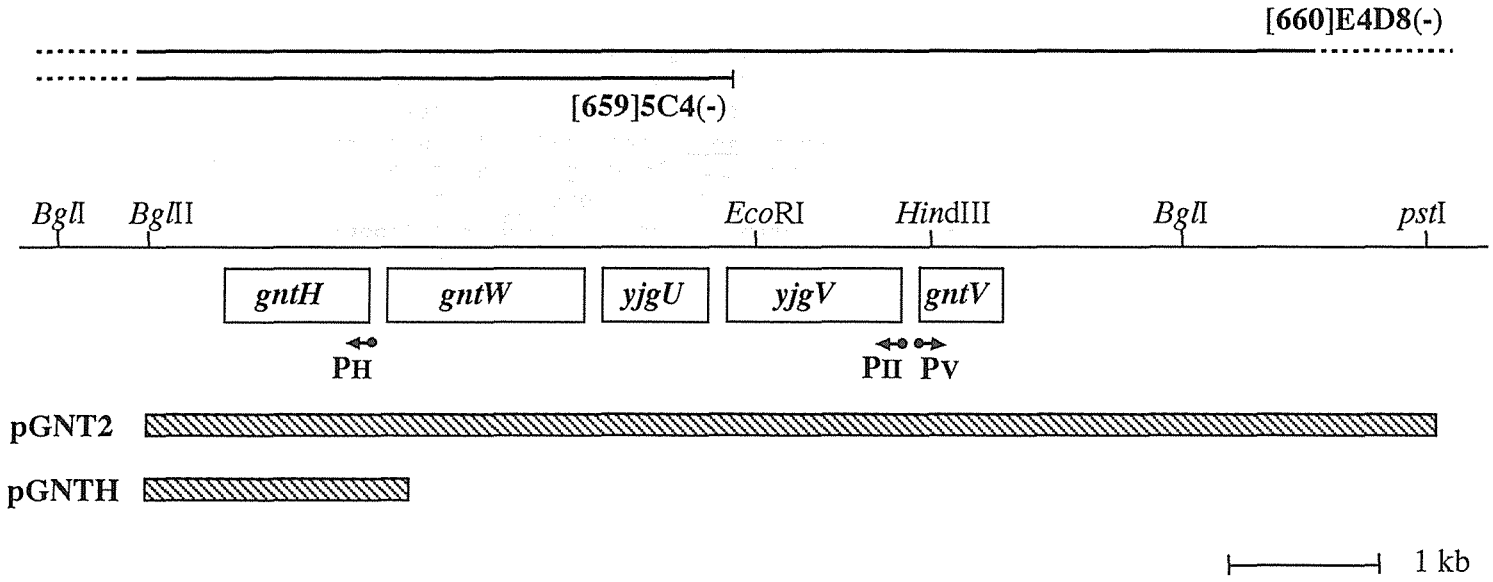


**Figure 15. Physical map of the *gntV, yjgV, yjgU, gntW*, and *gntH* genes, and *gnt* gene clones.**
The *gntV, yjgV, yjgU, gntW*, and *gntH* genes are located at 96.9 min on the *E. coli* W3110 genome and represented by boxes. Parts of Kohara clones, E4D8 and 5C4, are shown on the top. The promoters in front of *gntV* (PV), *yjgV* (PII) and *gntH* (PH) are shown by arrows with closed circles. Plasmid pGNT2 with the 8.4-kb *Bgl*II-*Pst*I fragment from E4D8 bears the *gntV, yjgV, yjgU, gntW*, and *gntH* genes. Plasmid pGNTH with the 1.7-kb *Dra*I fragment bears the *gntH* gene.

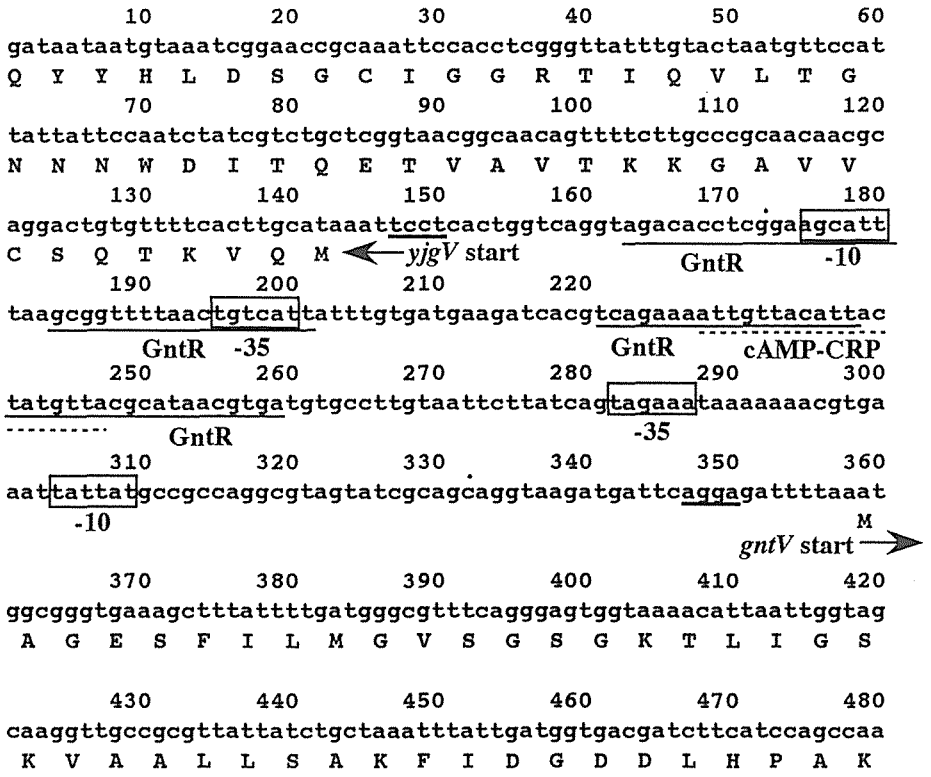


**Figure 16. Nucleotide sequence between *yjgV* and *gntV*.**
Nucleotide sequence between *yjgV* and *gntV* and parts of amino acid sequences of their products are shown. The arrows at positions 143 and 359 represent the initiation and direction of the *yjgV* and *gntV* coding sequence. Their possible promoters and SD sequences are represented by boxes and broken underlines, respectively. The initiation of mRNA of *yjgV* and *gntV* were determined by primer extension analysis, and the initiation and direction are represented by arrows at positions 172 and 332, respectively. Possible GntR-binding sequences at positions 162-180, 183-201, 220-238, and 240-258 are represented by underlines. A cAMP-CRP binding site at position 228-247 is by a broken underline.

mapping was performed for determining the mRNA initiation site of *gntV* (data not shown). As a result, one band that corresponds to C at the position 26 b upstream of the initiation codon was detected. Thus, a sequence, TATTAT---16 b---TGAAA, being at the site 49 b upstream of the initiation codon seems to be -10 and -35 sequences of the promoter.

(ii) *yjgV*. The *yjgV* ORF encodes 343 amino acid residues, from which a molecular mass of the product is estimated to be 37,146 Da. The *yjgV* gene is located with the *yjgU*, *gntW*, and *gntH* genes, and the direction of transcription of these genes are opposite to that of the *gntV* gene (Fig. 16). A possible ribosome-binding sequence, AGGA, also occurs 4 b upstream of its initiation codon. The primer extension revealed that the mRNA initiation site of *yjgV* is G at the position 29 b upstream of the initiation codon. A sequence, AATGCT---14 b---ATGACA, being at the site 32 b upstream of the initiation codon seems to be -10 and -35 sequences of the promoter. No potential terminator was found downstream of *yjgV*, so that read-through transcription appears to occur from this promoter to the following three genes. Thus, the *yjgV* gene is assumed to constitute an operon with the *yjgU*, *gntW*, and *gntH* genes.

(iii) *yjgU*. The *yjgU* ORF consisting of 765 b starts with an ATG initiation codon at the position 23 b downstream of the stop codon of the *yjgV* gene. The possible promoter of *yjgU* was not found at the proximal upstream region. A possible ribosome-binding sequence, AAGGA, was found 8 b upstream of the initiation codon. YjgU is predicted to consist of 254 amino acid residues with a molecular mass of 27,563 Da.

(iv) *gntW*. From the position 61 b downstream of the stop codon of *yjgU*, the *gntW* gene starts its ORF with an ATG initiation codon, which consists of 1,320 b, encoding a 439-amino acid peptide with a molecular mass of 46,041 Da. Between *yjgU* and *gntW*, there are two inverted repeat sequences, GCAGCGCGAAAGCGCTGCTTTT and GAA AGCGCTGCTTTT. The former is similar to the typical rho-independent terminator, and the latter may disturb the terminator formation because both sequences are overlapping. However, no possible promoter for *gntW* was found in this region. A possible ribosome-binding sequence, AAAG, occurs 3 b upstream of the initiation codon. Peekhaus *et al.* reported that GntW is a member of the GntP family and a gluconate inducible and stationary phase-specific gluconate permease, whose *K*m value for gluconate is 60 μM and *V*max is 26 nmol/min/mg of protein (Peekhaus *et al.*, 1997).

(v) *gntH*. The *gntH* ORF consisting of 999 b starts with an ATG initiation codon at the position 66 b downstream of the *gntW* gene. A possible ribosome-binding sequence, AGAA, occurs 3 b upstream of the initiation codon. The primer extension was not successful, but *gntH* seems to have its own promoter, TTGCAC---16 b---TGTGAT, corresponding to -10 and -35 sequences, at the position 48 b upstream of the initiation codon. The other ORF encoding an unknown protein starts 77 b downstream of the stop codon of *gntH*, and a possible rho-independent terminator, GTTAATTGCCCGCAC

GCCGGACACAGAACTGTTTTT, of *gntH* is overlapped with the N-terminal region of the ORF. Therefore, the read-through transcription from the *yjgV* promoter and transcription from the *gntH* promoter seem to stop at this point. An inverted repeat sequence, TCGGGGGAAGATTGAAGATTCCCCCGA, occurs between *gntW* and *gntH*, which might be a regulator binding sequence to control the read-through transcription from *gntW* to *gntH*. GntH is predicted to consist of 332 amino acid residues with a molecular mass of 37,567 Da. GntH appears to belong to the CytR family (Weickert & Adhya, 1992), one of bacterial regulator families, and is highly homologous to GntR as shown in CHAPTER 3 (Yamada *et al.*, 1996). Comparison of the GntH primary structure with those of regulators in this family revealed that GntH has a helix-turn-helix motif, LQDIATLAGVTKMTVSRYIR, at its N-terminus, which may be responsible for DNA binding, and domains related to dimerization and inducer binding at its C-terminus (Weickert & Adhya, 1992).

(vi) *The region between gntV and yjgV*. As described in CHAPTERs 1 and 4, GntR may bind to an inverted repeat sequence, AATGTTACCGATAACAGT, overlapping with the -10 sequence of the *gntK* promoter (Izu *et al.*, 1997a) and to an inverted repeat sequence, GACGTTACCCATAACAAA, in the promoter-operator region of *gntT* (Izu *et al.*, 1997b). Four sequences similar to these GntR binding sequences were found between *gntV* and *yjgV*. Two of them, CACGTTATGCGTAACATA and AATGTAA CAATTTTCTGA, overlap with a possible cAMP-CRP binding sequence, TAACATAG TAATGTAACAATT, as shown in Fig. 16. It is known that cAMP-CRP complex has a stimulating effect on the binding of the RNA polymerase to the promoter, and then the DNA appears to wrap around both proteins to form the open complex (Mollegaard *et al.*, 1993). Therefore, a protein-protein interaction between GntR or GntH and cAMP-CRP might be expected in the expressional regulation of the *gnt* genes as demonstrated in the CytR-dependent *deo* gene expression (Sogaard-Andersen & Valentin-Hansen, 1993). Whereas, the remaining two sequences, AATGACAGTTAAAACCGC and AATGCTTC CGAGGTGTCT, overlapping with the promoter sequence of the *yjgV* gene may be involved in the repression of expression of the *yjgV-yjgU-gntW-gntH* operon by preventing RNA polymerase from binding to the promoter.

## Transcriptional units of the *gnt* genes in GntII system and regulation of their expression

Transcriptional units of the genes in GntII system and regulation of their expression were analyzed by using operon fusions with the promoter-less *lacZ* gene (Fig. 17). DNA fragments including various parts of the *gnt* genes were inserted in front of the *lacZ* gene, which is expressed under the control of an inserted promoter. The *yjgU-lacZ* operon fusion could not be constructed as a recombinant exibiting a blue colony on X-gal plates, probably because *yjgU* has no own promoter. β-Gal activity of YU230 cells harboring

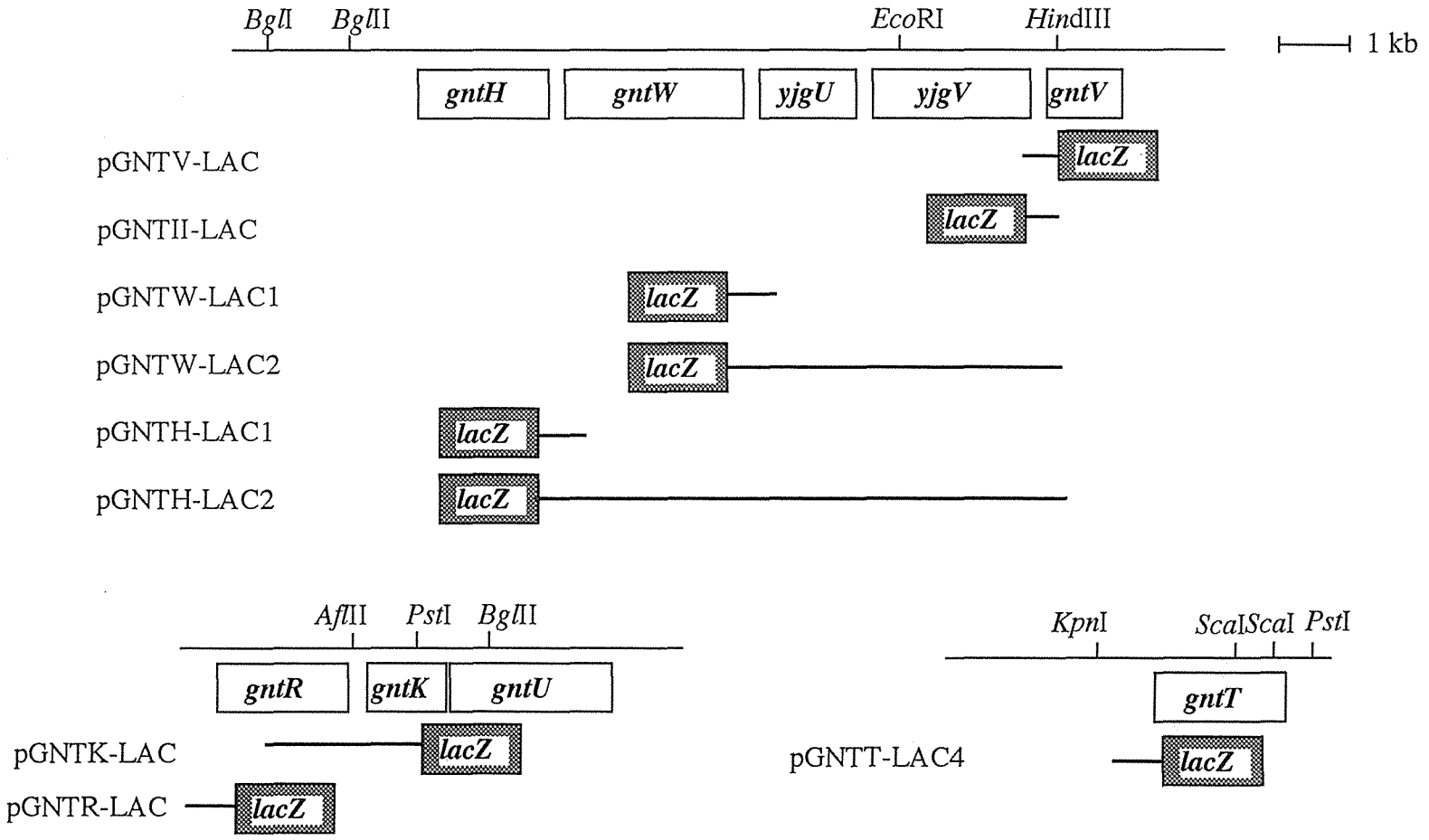


**Figure 17. Construction of *gnt-lacZ* operon fusions.**
The *gnt-lacZ* operon fusions were constructed as described in Materials and Methods. The regions inserted in the operon fusions are indicated by solid lines, and the promoter-less *lacZ* genes are shown by dotted boxes.

each fusion plasmid was then measured (Table 12). Significant β-Gal activities were detected in the cells harboring pGNTV-LAC, pGNTII-LAC, pGNTH-LAC1, or pGNTH-LAC2, and weak β-Gal activities in the cells harboring pGNTW-LAC1 or pGNTW-LAC2 compared to those of cells harboring the vector, pCB182. These results suggest that the *gntV*, *yjgV*, and *gntH* genes have their own promoters, which are also demonstrated from their nucleotide sequences and primer extension analysis as described above. The read-through transcription from the *yjgV* promoter to the *gntW* and *gntH* genes seems to occur because β-Gal activity from pGNTW-LAC2 bearing the inserted fragment from the 5'-flanking region of *yjgV* to the 5'-coding region of *gntW* was higher than that from pGNTW-LAC1 bearing the fragment from the 5'-flanking to 5'-coding regions of *yjgV*, and because the activity from pGNTH-LAC2 bearing the fragment from the 5'-flanking region of *yjgV* to the 5'-coding region of *gntH* was higher than that from pGNTH-LAC1 bearing the fragment from the 5'-flanking to 5'-coding regions of *gntH* (Table 12). However, β-Gal activity from pGNTW-LAC2 was not equal to the sum of that from pGNTII-LAC and that from pGNTW-LAC1, and the activity from pGNTH-LAC2 was also not equal to the sum of that from pGNTII-LAC, that from pGNTW-LAC1, and that from pGNTH-LAC1. The inequality may be due to the inhibition of read-through transcription from the *yjgV* promoter to *gntW* and *gntH* by the mechanism like an attenuation, which may be performed by several inverted repeats existing between *yjgU* and *gntW* and between *gntW* and *gntH* as described above. Transcription level of *gntW* was found to be very low under the conditions tested, and the *gntW* transcription appears to be mostly dependent on the read-through transcription from the *yjgV* promoter. On the other hand, *gntH* is assumed to be predominantly transcribed by its own promoter, and the read-through transcription from the *yjgV* promoter seems to be minor. These results indicate that *yjgV*, *yjgU*, *gntW*, and *gntH* constitute the operon structure.

To analyze the effect of gluconate and cAMP on the expression of *gntV* and *gntW*, β-Gal activities of cells harboring pGNTV-LAC and pGNTII-LAC were measured under the various conditions (Table 12). The activities from pGNTV-LAC and pGNTII-LAC were stimulated in the presence of cAMP. As described above, a sequence homologous to the cAMP-CRP consensus sequence was found between *gntV* and *yjgV*, which overlaps with two possible GntR-binding sequences. cAMP-CRP complex may be involved in the stimulation of expression of the *gnt* genes related to the GntII system in some extent, through the mechanism that cAMP-CRP complex may operate with GntR via protein-protein interaction rather than as a sole activator as described above and below. Under this condition, β-Gal activities from all fusion plasmids decreased in the presence of gluconate presumably via catabolite repression (Hogema *et al.*, 1997), although GntV and GntW were reported to be gluconate-inducible enzymes (Istúriz *et al.*, 1986; Peekhaus *et al.*, 1997). Under another condition where GntH or GntR was

**Table 12. Regulation of the *gntV*, *yjgV*, *gntW*, and *gntH* expression.**

| Strain | β-Gal activity (Miller units)[a] | | | |
|---|---|---|---|---|
| | +gluconate +cAMP | -gluconate +cAMP | +gluconate -cAMP | -gluconate -cAMP |
| YU230 (pGNTV-LAC/ pYY2) | 51 | 79 | 45 | 59 |
| YU230 (pGNTII-LAC/ pYY2) | 27 | 30 | 10 | 14 |
| YU230 (pGNTW-LAC1/ pYY2) | 2.0 | 3.9 | -b | - |
| YU230 (pGNTW-LAC2/ pYY2) | 5.9 | 9.3 | - | - |
| YU230 (pGNTH-LAC1/ pYY2) | 21 | 39 | - | - |
| YU230 (pGNTH-LAC2/ pYY2) | 33 | 64 | - | - |
| YU230 (pCB182/ pYY2) | 1.0 | 0.8 | | |

[a] Values are the average of more than three independent experiments. Cells were incubated at 37 °C for 4 hours with or without gluconate and/or cAMP, and β-Gal activity was measured as described in Materials and Methods.

[b] Not determined.

provided enough, the *gntV* and *yjgV* promoter activities were increased by the presence of gluconate as shown below.

**Expressional regulation of the *gnt* genes in GntI system and comparison with that of the *gnt* genes in GntII**

The effect of gluconate and cAMP on the expression of the genes in GntI system was also analyzed using operon fusions with the promoter-less *lacZ* gene (Fig. 17). β-Gal activities from both pGNTT-LAC4 and pGNTK-LAC were stimulated in the presence of cAMP (Table 13), suggesting that the *gntT* and *gntK* genes in the GntI system are regulated positively by the cAMP-CRP complex as described in CHAPTERs 1 and 4 (Izu *et al.*, 1997a; 1997b). Transcription level of the GntI genes seems to be at least three times higher than that of the GntII genes under the condition tested, which is almost consistent with the ratio of gluconate kinase activities of GntK and GntV that was reported previously (Istúriz *et al.*, 1986). However, when cells were cultivated in continuous culture with gluconate at high oxygen concentration, the specific activities of GntK and GntV were differently influenced by the culture dilution rate (Coello & Istúriz, 1992). Gluconate kinase activity from GntV was predominant at low dilution rate, that is the high cell density condition, and the activity from GntK increased with increasing dilution rate to make the low cell density condition. This suggests that the expression of *gntK* and *gntV* is differently regulated in response to bacterial cell density under aerobic condition, and their products appears to act at the early and late growth phases, respectively.

To analyze the growth phase-dependent expression of *gntT* and *gntK*, single-copy *gntT::lacZ* and *gntK::lacZ* operon fusions were constructed in the *E. coli* genome as described in Materials and Methods. β-Gal activities from the fusions were measured in the presence or absence of gluconate along with the cell growth (Fig. 18-A and B). As a result, the activities of both fusions were detected at the early growth phase and decreased rapidly before entry into the late growth phase, indicating that the *gntT* and *gntK* genes in the GntI system are expressed at the early growth phase. Porco *et al.* also reported that the *gntT* gene was expressed at the logarithmic phase (Porco *et al.*, 1997). After decreasing expression of the GntI genes, the expression of the GntII genes at the late growth phase is expected based on the following results and previous reports (Coello & Istúriz, 1992; Peekhaus *et al.*, 1997).

**Effect of GntR and GntH on transcription of the GntI genes**

To examine the effect of GntR and GntH on the transcription of the *gntT* and *gntK* genes related to GntI system, pGNTR2 bearing *gntR*, pGNTH bearing *gntH*, or the control vector, pYY2 was co-introduced with the *gntT*- or *gntK-lacZ* operon fusion into

**Table 13. Regulation of the *gntT* and *gntK* expression.**

| Strain | β-Gal activity (Miller units)[a] | | | |
|---|---|---|---|---|
| | +gluconate +cAMP | -gluconate +cAMP | +gluconate -cAMP | -gluconate -cAMP |
| YU230 (pGNTT-LAC4/ pYY2) | 84 | 80 | 26 | 69 |
| YU230 (pGNTK-LAC/ pYY2) | 150 | 100 | 40 | 61 |

[a] Values are the average of more than three independent experiments. Cells were incubated at 37 °C for 4 h with or without gluconate and/or cAMP, and β-Gal activity was measured as described in Materials and Methods.

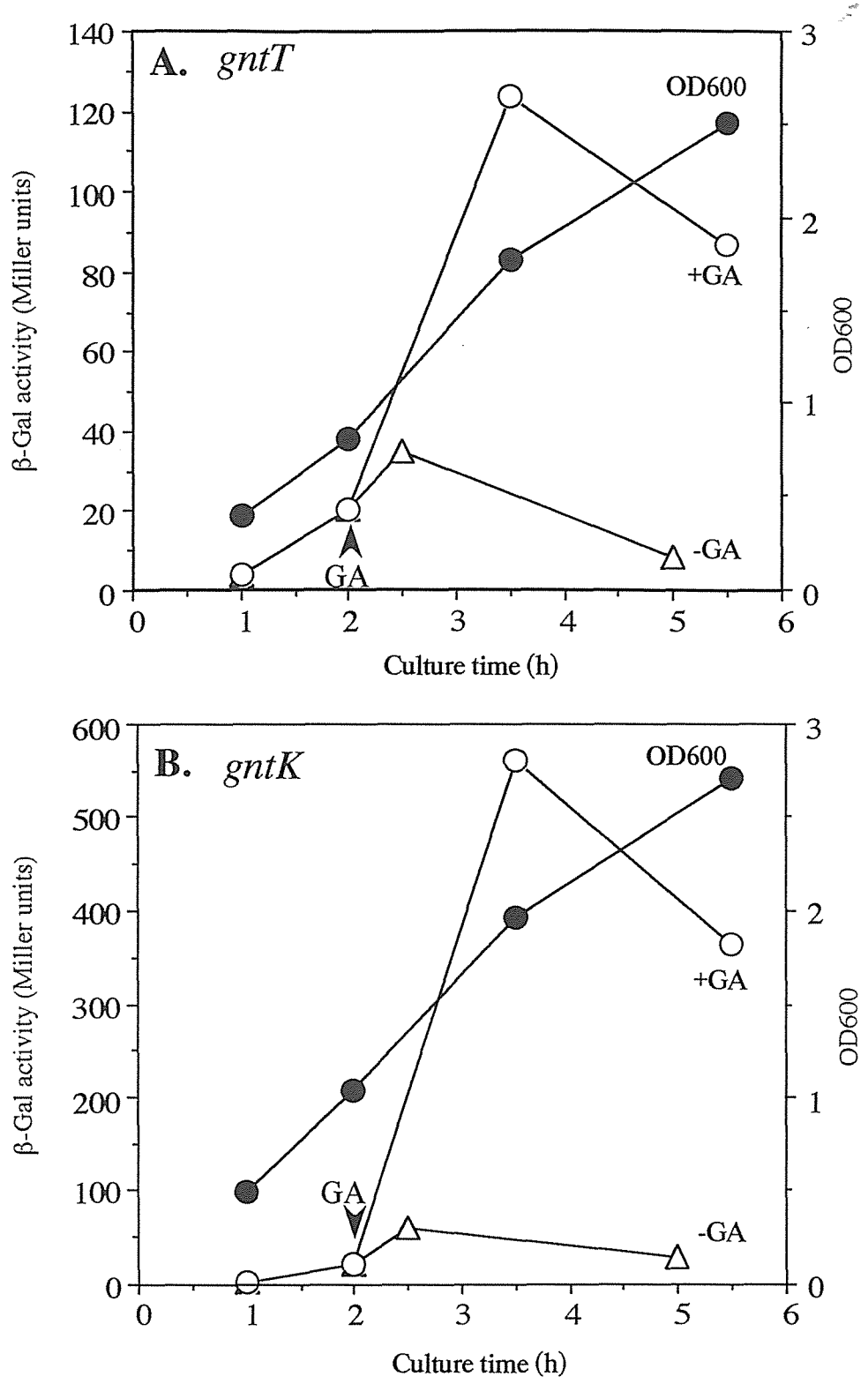


**Figure 18. Expression of the single-copy *gntT::lacZ* (A) and *gntK::lacZ* (B) operon fusions along with cell growth.**
Growth phase-dependent expression of *gntT* and *gntK* was examined at 37 °C in LB medium. After the 2-h incubation, 0.5% gluconate was added to the culture medium as indicated by arrow heads. Closed circles indicate the growth curve (OD600). Open circles and triangles indicate β-Gal activities (Miller units) from the culture with and without gluconate, respectively.

YU230, and β-Gal activities of the transformants were measured (Table 14). The activities from pGNTT-LAC4 and pGNTK-LAC were reduced 5.3- and 14.5-fold in the 4 h culture, and 14.8- and 10-fold in the 24 h culture, respectively, by the presence of GntR, and also reduced 5.0- and 4.5-fold in the 4 h culture, and 3.1- and 4.0-fold in the 24 h culture, respectively, by the presence of GntH under the condition without gluconate. These data suggest that the *gntT* and *gntK* genes in the GntI system are negatively regulated by both GntR and GntH. The repression by both factors were released by the addition of gluconate: the activities from pGNTT-LAC4 and pGNTK-LAC in the presence of GntR were increased by gluconate 1.2- and 6.3-fold in the 4 h culture, and 1.4- and 1.9-fold in the 24 h culture, respectively, and the activities in the presence of GntH were increased 2.8- and 2.5-fold in the 4 h culture, and increased 1.0- and 1.4-fold in the 24 h culture, respectively. These results suggest that GntH also functions as a repressor for the GntI genes like GntR (Izu *et al.*, 1997), and that gluconate or its derivative directs GntH to detach from the operator to induce expression of *gntT* or *gntK*. Since GntH shares 46% homology to GntR as described in CHAPTERs 1 and 3 (Yamada *et al.*, 1996; Izu *et al.*, 1997a), both repressors may share the same element for binding. GntH, however, may have lower affinity for the element than GntR because the repression ratio by GntH was lower than that of GntR in both pGNTT-LAC4 and pGNTK-LAC.

**Effect of GntR and GntH on transcription of the GntII genes**

To examine the effect of GntR and GntH on the transcription from the *gntV* and *yjgV* promoters in GntII system, β-Gal activities were measured of the cells that were co-transformed with either pGNTR2 or pGNTH, and the GntII gene-*lacZ* operon fusion plasmid, pGNTV-LAC or pGNTII-LAC (Fig. 16) under the various conditions (Table 15). The activities from both fusion plasmids decreased a little in the presence of GntR or GntH in the 4 h and 24 h cultures except for GntH in pGNTII-LAC, under the conditions without both gluconate and cAMP. When gluconate was added, although the catabolite repression was seen in the control cells harboring pYY2, the activities were increased by the presence of GntR and GntH in the 24 h culture, and the level of activities was significantly higher than that of cells harboring the control plasmid, pYY2. Similar increase by gluconate was observed in the presence of cAMP. Therefore, GntR and GntH seem to function as an activator for the *gntV* and *yjgV* promoters. Moreover, the increase ratio of the activities to those of the control cells in the 24 h culture was higher than that in the 4 h culture. The facts suggest that the accumulation of GntR or GntH at the late growth phase may cause the increase of the GntII genes expression. Interestingly, the activation by GntR or GntH was enhanced by cAMP in the case of pGNTII-LAC, but not of pGNTV-LAC, indicating that the *yjgV* promoter may be

**Table 14. Regulation of the *gntT* and *gntK* expression in the presence of GntR or GntH.**

| Strain | β-Gal activity (Miller units)[a] | | | |
|---|---|---|---|---|
| | +gluconate | | -gluconate | |
| | 4 h | 24 h | 4 h | 24 h |
| YU230 (pGNTT-LAC4/ pYY2) | 84 | 360 | 80 | 490 |
| YU230 (pGNTT-LAC4/ pGNTR2) | 73 | 260 | 15 (19%)[b] | 33 (6.7%) |
| YU230 (pGNTT-LAC4/ pGNTH) | 30 | 360 | 16 (20%) | 160 (33%) |
| YU230 (pGNTK-LAC/ pYY2) | 150 | 110 | 100 | 380 |
| YU230 (pGNTK-LAC/ pGNTR2) | 24 | 58 | 6.9 (6.9%) | 38 (10%) |
| YU230 (pGNTK-LAC/ pGNTH) | 59 | 150 | 22 (22%) | 96 (25%) |

[a] Values are the averages of more than three independent experiments. Cells were incubated at 37 °C for 4 or 24 h with cAMP and with or without gluconate, and β-Gal activity was measured as described in Materials and Methods.

[b] The activities in the presence of GntR or GntH are expressed as a percentage of that in the absence of them, which are shown in brackets.

**Table 15. Regulation of the *gntV* and *yjgV* expression in the presence of GntR or GntH.**

| Strain | β–Gal activity (Miller units) [a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | +gluconate +cAMP | | -gluconate +cAMP | | +gluconate -cAMP | | -gluconate -cAMP | |
| | 4 h | 24 h | 4 h | 24 h | 4 h | 24 h | 4 h | 24 h |
| YU230 (pGNTV-LAC/ pYY2) | 51 | 160 | 79 | 290 | 45 | 170 | 59 | 520 |
| YU230 (pGNTV-LAC/ pGNTR2) | 74 (145%)[b] | 330 (206%) | 60 | 180 | 49 (109%) | 720 (424%) | 52 | 330 |
| YU230 (pGNTV-LAC/ pGNTH) | 37 (73%) | 350 (219%) | 52 | 320 | 30 (67%) | 430 (253%) | 40 | 230 |
| YU230 (pGNTII-LAC/ pYY2) | 27 | 75 | 30 | 160 | 10 | 17 | 14 | 81 |
| YU230 (pGNTII-LAC/ pGNTR2) | 18 (67%) | 260 (347%) | 23 | 85 | 7.7 (77%) | 36 (212%) | 8.8 | 47 |
| YU230 (pGNTII-LAC/ pGNTH) | 26 (96%) | 620 (827%) | 26 | 170 | 17 (170%) | 220 (1294%) | 15 | 83 |

[a] Values are the averages of more than three independent experiments. Cells were incubated at 37 °C for 4 or 24 h with or without gluconate and/or cAMP, and βGal activity was measured as described in Materials and Methods.

[b] The activities in the presence of GntR or GntH are expressed as a percentage of that in the absence of them, which are shown in brackets.

positively regulated not only by GntR or GntH but also by cAMP-CRP complex. Thus, the gluconate metabolism in *E. coli* is subject to both positive and negative control.

**Mutational analysis of the possible GntR-binding element upstream of the *gntK* gene**

As described in CHAPTERs 1 and 4, an inverted repeat sequence AATGTTACC GATAACAGT (R1) overlapping with the -10 sequence of the *gntK* promoter (Izu *et al.*, 1997a), and a sequence GATGTTACCCGTATCATT overlapping with the promoter 1 (P1) of the *gntT* gene (Izu *et al.*, 1997b) may be the binding site of the GntR. The other possible GntR-binding sequence (R2) exists close to R1 (Fig. 19). To determine the GntR-binding element involved in regulation of the *gnt* genes, the possible GntR-binding sequences upstream of the *gntK* gene were changed by site-directed mutagenesis to produce mutant derivatives of *gntK-lacZ* operon fusions, pGNTK-LACM1, M6, and M8, containing mutations at R1, R2, and both R1 and R2, respectively. β-Gal activities of cells harboring pGNTR2, pGNTH, or control plasmid, pYY2, with one of the mutant *gntK-lacZ* fusions were then measured (Table 16). In the presence of pYY2, the activities from all mutant fusion plasmids increased compared to those of the wild type plasmid. The increase may be due to either that GntR or GntH from the chromosome was too low amount to inhibit the expression because of the reduced affinity between the factors and the mutated DNA, or that the mutations increased the expression by elevation of the promoter activity. The latter, however, may not be the case because all mutations were from TA to GT. On the other hand, β-Gal activities from pGNTK-LACM1 and M6 were repressed by GntR or GntH from plasmid, pGNTR2 or pGNTH, like the wild type pGNTK-LAC under the condition without gluconate. The activity from pGNTK-LACM1 in the presence of pGNTR2 or pGNTH was increased by the addition of gluconate and the increase ratio was larger than that of the wild type plasmid. Similar induction by gluconate was observed in the case of pGNTK-LACM6. Therefore, the binding affinity of GntR or GntH to pGNTK-LACM1 or M6 seems to be weakened in the presence of gluconate compared to the wild type pGNTK-LAC, although the factor-dependent repression was not weakened without gluconate. Whereas, β-Gal activities from pGNTK-LACM8 bearing mutations at both R1 and R2 sites were not repressed by GntR or GntH even without gluconate. These results suggest that these two GntR-binding sequences, R1 and R2, are required for the repression of the *gntK* expression and GntR and/or GntH may function as a tetramer. Porco *et al.* also indicated two operators at the regulatory regions of *gntT* and *gntV* which allow formation of a DNA loop bridged by a tetrameric GntR (Porco *et al.*, 1997), as described in the case of LacI and GalR (Choy & Adhya, 1996). These results confirmed that GntH repress the *gntK* expression and can bind to the GntR element because the repression by GntH was similarly influenced by the mutations.

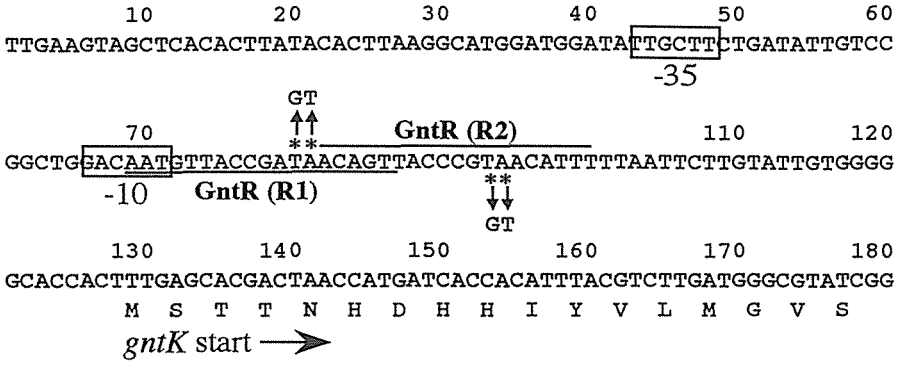


**Figure 19. Mutation sites of possible GntR-binding sequences (R1 and R2) upstream of *gntK*.**
Nucleotide sequence of the 5'-flanking region of *gntK* and the N-terminal amino acid sequence of GntK are shown, and its promoter sequence is represented by boxes. Possible GntR-binding sequences upstream of *gntK*, R1 and R2, are shown by an underline and overline, respectively. Mutation M1, M6, and M8 possess base substitutions of TA to GT in the R1 sequence, TA to GT in the R2 sequence, and TA to GT in both R1 and R2 sequences. The substitution bases are represented by asterisks.

**Table 16. Effect of the mutation of possible GntR-binding sequences upstream of *gntK* on the *gntK* expression.**

| Strain | β-Gal activity (Miller units)[a] | |
|---|---|---|
| | +gluconate | -gluconate |
| YU230 (pGNTK-LAC/ pYY2) | 150 | 100 |
| YU230 (pGNTK-LAC/ pGNTR2) | 24 | 6.9 (6.9%)[b] |
| YU230 (pGNTK-LAC/ pGNTH) | 59 | 22 (22%) |
| YU230 (pGNTK-LACM1/ pYY2) | 950 | 540 |
| YU230 (pGNTK-LACM1/ pGNTR2) | 640 | 33 (6.1%) |
| YU230 (pGNTK-LACM1/ pGNTH) | 430 | 140 (26%) |
| YU230 (pGNTK-LACM6/ pYY2) | 450 | 440 |
| YU230 (pGNTK-LACM6/ pGNTR2) | 300 | 35 (8.0%) |
| YU230 (pGNTK-LACM6/ pGNTH) | 270 | 130 (30%) |
| YU230 (pGNTK-LACM8/ pYY2) | 490 | 630 |
| YU230 (pGNTK-LACM8/ pGNTR2) | 480 | 440 (70%) |
| YU230 (pGNTK-LACM8/ pGNTH) | 760 | 650 (103%) |

[a] Values are the averages of more than three independent experiments. Cells were incubated at 37 °C for 4 or 24 h with cAMP and with or without gluconate, and β-Gal activity was measured as described in Materials and Methods.

[b] The activities in the presence of GntR or GntH are expressed as a percentage of that in the absence of them, which are shown in brackets.

**Effect of GntR and GntH on the transcription of their own genes encoding regulators for gluconate utilization genes**

Expression of the *gntR* and *gntH* genes was analyzed under the various conditions (Table 17) using operon fusions with the promoter-less *lacZ* gene, pGNTR-LAC, pGNTH-LAC1, and pGNTH-LAC2 (Fig. 16). β-Gal activity of cells harboring pGNTR-LAC and pYY2, increased in the 24 h culture without gluconate compared to that in the 4 h culture. Such increase was not observed under the conditions with gluconate, which may result from catabolite repression. The activities from pGNTR-LAC were little affected by pGNTR2 or pGNTH in the 4 h culture with or without gluconate. This is consistent with results exhibited in CHAPTER 1, in which *gntR* was constitutively expressed and GntR did not repress its own promoter at least in the 4 h culture (Izu *et al.*, 1997a). Under the conditions with gluconate, β-Gal activity from pGNTR-LAC was significantly increased by the presence of pGNTH, and slightly by pGNTR2 at 24 h compared to those by pYY2. Therefore, the *gntR* expression may gradually increase along with cell growth when gluconate is absent, and be stimulated by GntH and slightly by GntR when gluconate is present.

β-Gal activity of cells harboring pGNTH-LAC2 and pYY2 also increased in the 24 h culture without gluconate compared to that in the 4 h culture, but the activity of cells harboring pGNTH-LAC1 and pYY2 was not. Therefore, the increase of the *gntH* expression in pGNTH-LAC2 may be due to the read-through transcription from the *yjgV* promoter but not to the own promoter. β-Gal activity from pGNTH-LAC2 was also stimulated in the presence of pGNTR2 or pGNTH in the 24 h culture under the conditions with gluconate. Since no such stimulation was observed in the case of pGNTH-LAC1, the stimulation may also be due to the read-through transcription from the *yjgV* promoter. From these results and those using pGNTR-LAC, it is assumed that GntR and GntH are accumulated inside cells along with cell growth, which in turn repress the gene expression in the GntI system and activate in the GntII system. Especially, since GntH seems to strongly activate the *gntR* and *yjgV* promoters, it may function as a main regulator at the late growth phase. While, under the conditions without gluconate, the expression of the *gntR* and *gntH* genes is also stimulated at the late growth phase, which may be required for the tight repression of the *gnt* genes.

**Gel-shift analysis using purified GntR and CRP**

To determine the binding sites of GntR and cAMP-CRP, gel-shift analysis was carried out using purified GntR and CRP. The target DNA fragments were prepared by PCR followed by digestion with appropriate restriction enzymes. As for pGNTT-LAC4 and pGNTK-LAC, shifted bands were observed (lanes 2 and 3 in Fig. 20-A and B), indicating that GntR binds to the operator-promoter regions of the *gntK* and *gntT* genes. The bindings to *gntT* (lane 4 in Fig. 20-A) and to *gntK* (lanes 4 and 5 in Fig. 20-B) were

**Table 17. Regulation of the *gntR* and *gntH* expression in the presence of GntR or GntH.**

| Strain | β-Gal activity (Miller units)[a] | | | |
|---|---|---|---|---|
| | +gluconate | | -gluconate | |
| | 4 h | 24 h | 4 h | 24 h |
| YU230 (pGNTR-LAC/ pYY2) | 63 | 66 | 75 | 320 |
| YU230 (pGNTR-LAC/ pGNTR2) | 100 (159%)[b] | 130 (197%) | 110 | 390 |
| YU230 (pGNTR-LAC/ pGNTH) | 63 (100%) | 310 (470%) | 70 | 240 |
| YU230 (pGNTH-LAC2/ pYY2) | 33 | 26 | 64 | 140 |
| YU230 (pGNTH-LAC2/ pGNTR2) | 33 (100%) | 42 (162%) | 43 | 190 |
| YU230 (pGNTH-LAC2/ pGNTH) | 83 (252%) | 75 (288%) | 54 | 220 |
| YU230 (pGNTH-LAC1/ pYY2) | 21 | 35 | 39 | 52 |
| YU230 (pGNTH-LAC1/ pGNTR2) | 28 (133%) | 44 (126%) | 44 | 65 |
| YU230 (pGNTH-LAC1/ pGNTH) | 28 (133%) | 6.6 (19%) | 30 | 9.0 |

[a] Values are the averages of more than three independent experiments. Cells were incubated at 37 °C for 4 or 24 h with cAMP and with or without gluconate, and β-Gal activity was measured as described in Materials and Methods.

[b] The activities in the presence of GntR or GntH are expressed as a percentage of that in the absence of them, which are shown in brackets.

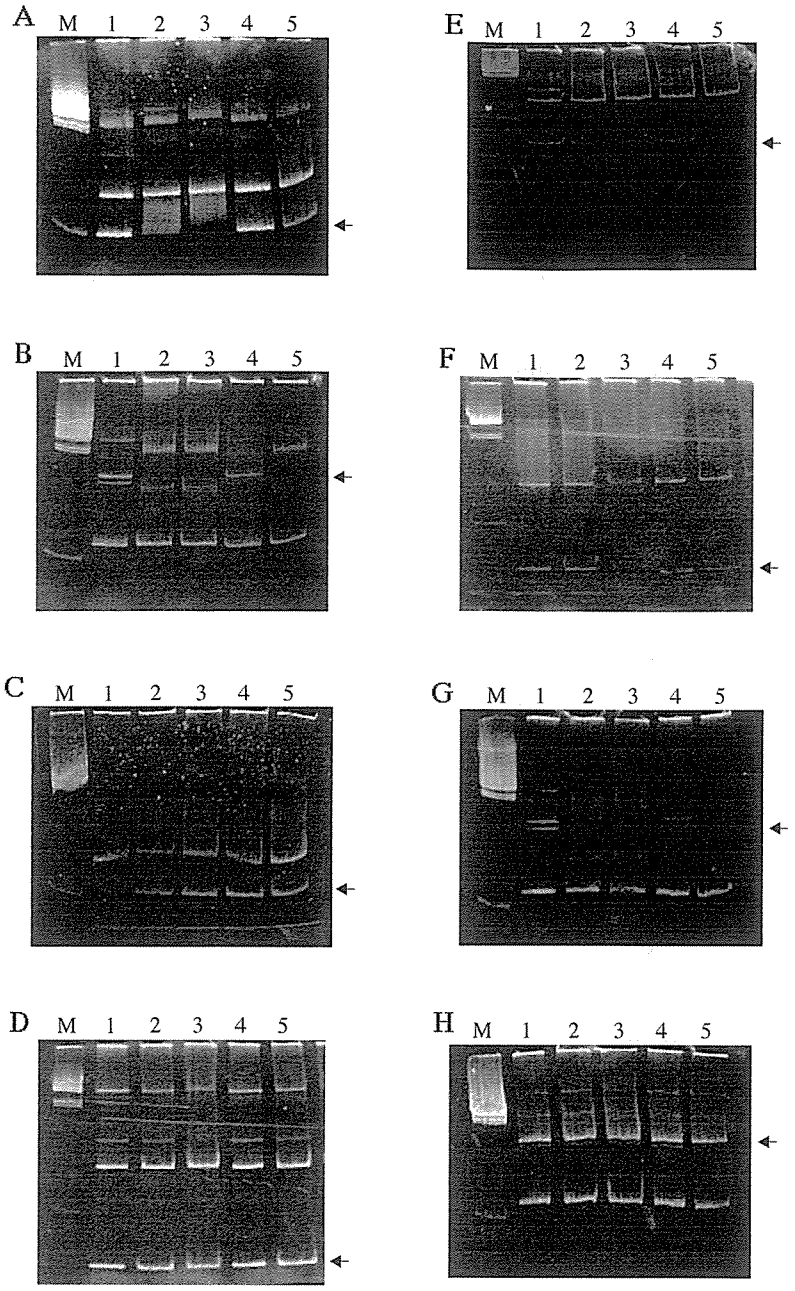


**Figure 20. Gel-shift analysis to identify GntR-binding sites in the *gnt* genes.**
Gel-shift analysis was performed as described in Materials and Methods. In PCR amplification, pGNTT-LAC4 (A and C), pGNTK-LAC (B), pGNTV-LAC (D), pGNTII-LAC (E and F), pGNTK-LACM6 (G), and pGNTK-LACM8 (H) DNAs were used as templates. The PCR products were digested with *Eco*RI (A and C), *Afl*II (B, G, and H), and *Sal*I (D, E, and F) and then incubated with purified GntR. In all panels except C and F, the incubation was performed as follows; without GntR and gluconate for lane 1, 0.5 μg of GntR for lane 2, 1.0 μg of GntR for lane 3, 0.5 μg of GntR with 30 mM gluconate for lane 4, and 1.0 μg of GntR with 30 mM gluconate for lane 5. In panels C and F, the incubation was as follows; 1.0 μg of GntR for lane 1, 1.0 μg of GntR with 5 mM gluconate for lane 2, 1.0 μg of GntR with 10 mM gluconate for lane 3, 1.0 μg of GntR with 30 mM gluconate for lane 4, and 1.0 μg of GntR with 60 mM gluconate for lane 5. pGNTT-LAC4, pGNTK-LAC, pGNTV-LAC, and pGNTII-LAC have the 5'-flanking regions of the *gntT*, *gntK*, *gntV*, and *yjgV* genes, respectively, and the fragment containing the 5'-flanking region of each *gnt* gene is indicated by an arrow. pGNTK-LACM6 and M8 have the same 5'-flanking region of the *gntK* gene as pGNTK-LAC but with substituted bases at the R2 and both R1 and R2, respectively, as shown in Fig. 19. Lanes M shows *Hin*dIII digested-λ phage DNA fragments as molecular markers.

inhibited almost completely or slightly, respectively, by the addition of gluconate, a possible inducer, indicating that the gluconate effect on the binding is different between the *gntK* and *gntT* gene operators. Thus, the effect of gluconate in the various concentrations was examined on the GntR-binding ability to *gntT* (Fig. 20-C). No shifted band was observed in pGNTT-LAC4 with 5, 10, 30, and 60 mM gluconate (lanes 2-5 in Fig. 20-C), although a shifted band was still observed in pGNTK-LAC with 30 mM gluconate when the same amount of GntR was used (lane 5 in Fig. 20-A). These results are consistent with those in Table 15, in which the *gntT* expression in the presence of GntR was derepressed easier than the *gntK* by the addition of gluconate. Therefore, it is again suggested that *gntK* is repressed by GntR stronger than *gntT*. Such differential repression seems to be reasonable because the expression of *gntT* encoding a high affinity gluconate permease may be induced at the low concentration of gluconate to uptake gluconate, which then induces other *gnt* gene expression. This also suggests that the operator of each *gnt* gene has the different affinity for GntR.

On the other hand, no shifted band was observed in the pGNTV-LAC and pGNTII-LAC under the conditions tested (Fig. 20-D and E). Since GntR and GntH appear to act as an activator of the *gntV* and *yjgV* genes in the presence of gluconate at the late growth phase, gel-shift analysis for *yjgV* was carried out at the various concentrations of gluconate. No shifted band, however, was found in pGNTII-LAC with 5, 10, 30, and 60 mM gluconate (lanes 2-5 in Fig. 20-F). Therefore, the GntR-binding to the *gntV* and *yjgV* promoters might thus require the formation of heterodimer between GntR and GntH, or the binding with cAMP-CRP complex as described above or other factors specific for the late growth phase. The gel-shift analysis with GntH seems to be necessary for further study.

Gel-shift analysis was also performed with DNA fragments from pGNTK-LACM6 and M8 containing mutations at the possible GntR-binding sequences in the *gntK* gene (Fig. 16). As a result, pGNTK-LACM6 showed the weaker shift than the wild type pGNTK-LAC (lanes 2 and 3 in Fig. 20-G; lanes 2 and 3 in Fig. 20-A), but pGNTK-LACM8 did not (lanes 2-5 in Fig. 20-H). The mutation at the R2 site seems to be insufficient to prevent GntR from binding to the fragment, and the mutations at both R1 and R2 sites be sufficient. These results were consistent with those in Table 17. The gel-shift analysis with pGNTK-LAC DNA exhibited the two shifted bands (Fig. 20-B), suggesting that the *gntK* gene has two GntR-binding sites.

Gel-shift analysis was also performed with purified CRP. As for pGNTK-LAC, pGNTT-LAC4, and pGNTV-LAC, shifted bands were observed only in the presence of both cAMP and CRP (lanes 4 and 5 in Fig. 21-A, B, and C). All *gntK*, *gntT*, and *gntV* genes have possible cAMP-CRP binding sequences as described above in this CHAPTER (Izu *et al.*, 1997a and b; Porco *et al.*, 1997). Therefore, cAMP-CRP complex may be involved in the expressional control of the *gnt* gene expression.

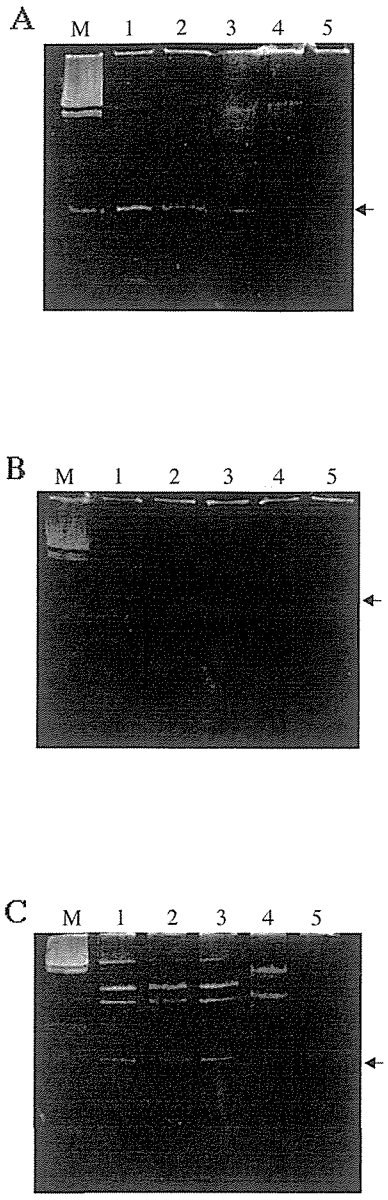


**Figure 21. Gel-shift analysis to identify cAMP-CRP binding sites in the *gnt* genes.** Gel-shift analysis was performed as described in Materials and Methods. In PCR amplification, pGNTT-LAC4 (A), pGNTK-LAC (B), and pGNTV-LAC (C) DNAs were used as templates. The PCR products were digested with *Eco*RI (A), *Pst*I (B), and *Sal*I (C) and the incubated with purified CRP. In all panels, the incubation was performed as follows; without CRP and cAMP for lane 1, 1.2 μg of CRP for lane 2, 2.4 μg of CRP for lane 3, 1.2 μg of GntR with 1 mM cAMP for lane 4, and 2.4 μg of CRP with 1 mM cAMP for lane 5. pGNTT-LAC4, pGNTK-LAC, and pGNTV-LAC have the 5'-flanking regions of the *gntT*, *gntK,* and *gntV* genes, respectively, and the fragment containing the 5'-flanking region of each *gnt* gene is indicated by an arrow. Lanes M show *Hin*dIII digested-λ phage DNA fragments as molecular markers.

## Discussion

The *gntV, gntW,* and *gntH* genes in the GntII system of *E. coli* were cloned from a phage clone E4D8 in the Kohara library based on the previous genetic studies (Istúriz *et al.,* 1986) and database searching. The former reported to encode a thermosensitive gluconate kinase appears to be monocistronic, and the latter two genes to be cistrons in an operon consisting of *yjgV, yjgU, gntW,* and *gntH* which encode an alcohol dehydrogenase-like protein, a protein homologous to 2-deoxy-D-gluconate 3-dehydrogenase, a gluconate permease homologous to GntT and GntU, and a regulator homologous to GntR, respectively. Analysis with *lac*-operon fusions and primer extension revealed that there are three promoters in front of *gntV, yjgV,* and *gntH,* in the region encompassing these genes, and *gntW* and *gntH* are predominantly transcribed from the promoters of *yjgV* and *gntH,* respectively.

The expression from the *yjgV* promoter but not from the *gntV* promoter was shown to be positively regulated by cAMP-CRP such as the *gntT* and *gntK* genes (Izu *et al.,* 1997a and b). In addition to this regulation, a mutual gene regulation between the GntI and GntII systems was found when the effect of GntR and GntH on the *gnt* genes was analyzed. The expression from the *gntT* and *gntK* promoters in the GntI system was repressed by both GntR and GntH at both the early and late growth phases, and derepressed by the addition of gluconate. This is the first evidence that GntH in the GntII system regulates the GntI genes. Although the results related to GntR just confirm the fact that they are gluconate-inducible genes, which were described in CHAPTERs 1 and 3, and also reported previously (Tong *et al.,* 1996; Izu *et al.,* 1997a and b; Porco *et al.,* 1997). The *gntT* and *gntK* genes are inducibly expressed by the addition of gluconate at the early growth phase, but the expression is then ceased at the late growth phase, which were shown with single-copy *lacZ* operon fusions in this study and also in the previous study (Porco *et al.,* 1997). The ceasing of the GntI system seems to be due to accumulation of GntR and GntH inside cells because the expression of both regulator genes gradually increase along with cell growth. On the other hand, the *gntV* and *yjgV* promoters in the GntII system are positively regulated by both GntR and GntH at the late growth phase in the presence of gluconate.

Such growth phase-dependent relay from the GntI to GntII system were reported by Coello and Istúriz in the experiments (Coello and Istúriz, 1992), where at high oxygen concentration in continuous culture, most of gluconate kinase activity was from GntK at low cell density and from GntV at high cell density. Therefore, it is likely that via the GntR and GntH-dependent regulation, cells start to uptake gluconate by the GntI system expressed inducibly, and then the expression becomes decreasing and successively the GntII system is expressed. GntR and GntH are thus important for such a relay of the *gnt* gene expression in response to cell density. GntR and GntH seem to have different

affinities for each promoter in the *gnt* genes, which were indicated by experiments with *lacZ*-fusion plasmids and gel-shift analysis. Such difference and their functions as a repressor for the GntI system and an activator for the GntII may give the diversity of the *gnt* gene expression so as to switch from the GntI to GntII system. Gluconate-free GntH or GntR would occur in addition to gluconate-bound form at the late growth phase where GntR and GntH are accumulated and the concentration of gluconate decreased. To achieve the expressional relay, the gluconate-free factors may repress the GntI system and the gluconate-bound activates the GntII system.

cAMP-CRP complex involved in the gene expressional control may provide the information about the state of sugars metabolizing inside cells. cAMP-CRP may stimulate the expression from the *yjgV* promoter in the GntII system in cooperation with inducer-bound GntR or GntH, which may be different from the case of the GntI gene repression that may be performed only by GntR or GntH. The additional regulation could be involved in the expression of the *yjgV-yjgU-gntW-gntH* operon in the GntII system like the attenuation in the GntI system that may reduce the read-through transcription from the *gntK* promoter to *gntU* and might be regulated by GntR as also indicated in CHAPTER 1 (Izu *et al.*, 1997a). Istúriz and Celaya demonstrated that *gntS* might specify a positive regulator involved in the control of at least the expression of *gntV* (Istúriz and Celaya, 1997), and it could make more diversity of regulation of the *gnt* genes.

## Materials and Methods

### Materials

Restriction enzymes, T4 DNA ligase and DNA sequencing kits were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (MA, USA). Primers and those labeled with FITC were synthesized by Sawady Technology (Tokyo, Japan). Other chemicals were of analytical grade.

### Bacterial strains and plasmids

The bacterial strains used in this study were derivatives of *E. coli* K-12. Their relevant genotypes and plasmids are shown in Table 18.

### DNA manipulations and sequencing

Conventional recombinant DNA techniques were applied (Sambrook *et al.*, 1989). Nucleotide sequencing was carried out by the dideoxy-chain termination method (Sanger *et al.*, 1977). The nucleotide sequence of the cloned genes and the deduced amino acid sequence were compared with those listed in the DNA and protein databases of GENETYX (Software Development, Tokyo, Japan).

**Table 18. Bacterial strains and plasmids.**

| Strain or plasmid | Genotype or description | Reference or source |
|---|---|---|
| Strains | | |
| MC1000 | *araD139 Δ(ara, leu)7697 ΔlacX74 galU galK strA* | Casadaban & Cohen (1980) |
| YU230 | MC1000 *Δcya* | Izu *et al.* (1997b) |
| TG1 | *supE hsdΔ5 thi Δ(lac-proAB)* F'*traD36 proAB lacI*$^q$ *lacZΔM15* | Sambrook *et al.* (1989) |
| P90C | *ara Δ(lac-pro) thi* | Miller *et al.* (1992) |
| NK7049 | *ΔlacX74 galOP308 rpsL* | Simons *et al.* (1987) |
| Plasmids | | |
| pBR322 | amp$^r$ tet$^r$ | Bolivar *et al.* (1977) |
| pACYC177 | amp$^r$ kan$^r$ | Chang & Cohen (1978) |
| pCB182 | amp$^r$ *lacZ galK* | Schneider & Beck (1986) |
| pCB192 | amp$^r$ *lacZ galK* | Schneider & Beck (1986) |
| pRS551 | amp$^r$ kan$^r$ promoter-less *lacZ* | Simons *et al.* (1987) |
| pKK232-8 | amp$^r$ promoter-less *cat* gene | Chang & Cohen (1978) |
| pGNT2 | pACYC177 with *gntV, yjgV, yjgU, gntW,* and *gntH* | This study |
| pGNT5 | pBR322 with *gntK* | Izu *et al.* (1997a) |
| pGNT15 | pACYC184 with *gntRKU* | Izu *et al.* (1997a) |
| pGNTT20 | pBR322 with *gntT* | Izu *et al.* (1997b) |
| pGNTK-CAT1 | pKK232-8 with the *Bam*HI-*Pst*I fragment from pGNT5 | Izu *et al.* (1997a) |
| pYY2 | frame-shift mutation of the *bla* gene on pACYC177 | Yamada *et al.* (1995) |
| pGNTR2 | pACYC177 with *gntR* | Izu *et al.* (1997a) |
| pGNTH | pACYC177 with *gntH* | This study |
| pGNTT-LAC4 | pCB192 with the 460-bp *Sau*3AI fragment from pGNTT20 | Izu *et al.* (1997b) |
| pGNTK-LAC | pCB192 with the 1.2-kb *Sma*I -*Hin*dIII fragment from pGNTK-CAT1 | This study |
| pGNTK-LACM1 | pGNTK-LAC derivative with a mutation at the R1 site upstream of *gntK* (M1) | This study |
| pGNTK-LACM6 | pGNTK-LAC derivative with a mutation at the R2 site upstream of *gntK* (M6) | This study |
| pGNTK-LACM8 | pGNTK-LAC derivative with mutations at the R1 and R2 sites upstream of *gntK* (M8) | This study |

| | | |
|---|---|---|
| pGNTR-LAC | pCB182 with the 480-bp promoter-operator region of *gntR* | This study |
| pGNTV-LAC | pCB182 with the 320-bp promoter-operator region of *gntV* | This study |
| pGNTII-LAC | pCB182 with the 320-bp promoter-operator region of *yjgV* | This study |
| pGNTW-LAC1 | pCB182 with the 550-bp fragment upstream of *gntW* | This study |
| pGNTW-LAC2 | pCB182 with the 2.3-kb fragment from the promoter-operator of *yjgV* to *gntW* | This study |
| pGNTH-LAC1 | pCB182 with the 570-bp fragment upstream of *gntH* | This study |
| pGNTH-LAC2 | pCB182 with the 3.7-kb fragment from the promoter-operator of *yjgV* to *gntH* | This study |
| pRSGNTT | pRS551 with the 460-bp fragment from the promoter-operator of *gntT* | Kawai (1997) |
| pRSGNTK | pRS551 with the 500-bp fragment from the promoter-operator of *gntK* | Kawai (1997) |

**Cloning of the *gnt* genes in GntII system**

Plasmid pGNT2 bearing *gntV*, *yjgV*, *yjgU*, *gntW* and *gntH* in the GntII locus was constructed by inserting the 8.4-kb *Bgl*II-*Pst*I fragment from a Kohara clone, E4D8 (Kohara *et al.*, 1987) into the *Bam*HI-*Pst*I site on pACYC177. Plasmid pGNTH bearing *gntH* was constructed by subcloning the 1.7-kb *Dra*I fragment of pGNT2 into the *Hin*cII site on pACYC177.

**Construction of *gnt-lacZ* operon fusions**

Operon fusions of the *gnt* genes with the *lacZ* gene were constructed (Fig. 17) to examine expressional regulation of the *gnt* genes. DNA fragments from the *gnt* genes were isolated and inserted in front of the promoter-less *lacZ* gene on pCB192 and pCB182 (Schneider and Beck, 1986). Plasmid pGNTK-LAC is a pCB192 derivative with the *Sma*I-*Hin*dIII fragment bearing the 5'-flanking region of *gntK* from pGNTK-CAT (Izu *et al.*, 1997a). Construction of plasmid pGNTT-LAC4 was described in CHAPTER 4 (Izu *et al.*, 1997b). Other *gnt-lacZ* fusions were constructed with PCR products as follows. Single-stranded synthetic oligonucleotide primers were designed with either *Bam*HI or *Sal*I sites to create PCR products with the *Bam*HI and *Sal*I sites at the 5'- and 3'-ends, respectively. Pairs of the upstream and downstream primers used in the PCR amplification for construction of *lacZ*-operon fusions pGNTV-LAC [1], pGNTII-LAC [2], pGNTH-LAC1 [3], pGNTH-LAC2 [4], pGNTW-LAC1 [5], pGNTW-LAC2 [6], and pGNTR-LAC [7] were 5'-GTAGGATCCTCGGTAACG GCAACAGTT-3' and 5'-GTAGTCGACACCGAACTGTTCCCGATG-3' for [1]; 5'-GTAGGATGGGTGGGTGAAACGCCCATC-3' and 5'- GTAGTCGACTCGGTAA CGGCAACAGTT-3' for [2]; 5'-ATCGGATCCACTGTTGCGGCGCTGTTG-3' and 5'-AGAGTCGACAGATACCGAGGGTATAAC-3' for [3]; 5'-GTAGGATGGGTGG GTGAAACGCCCATC-3' and 5'-AGAGTCGACAGATACCGAGGGTATAAC-3' for [4]; 5'-GATGGATCCGCGAACTGGGACGTGACA-3' and 5'-CCTGTCGACGCC GATACCATTTTGTAT-3' for [5]; 5'-GTAGGATGGGTGGGTGAAACGCCCATC-3' and 5'-CCTGTCGACGCCGATACCATTTTGTAT-3' for [6]; and 5'-GTAGGAT CCAGGTGGTGAAAG-3' and 5'-GTAGTCGACAGAGCGACGGAAACCTGC-3' for [7], respectively. The products from PCR with pGNT2, except for pGNT15 used for construction of pGNTR-LAC, as a template were subcloned into the *Bam*HI-*Sal*I site of pCB182. Subcloned *gnt-lacZ* fusions were co-transformed into YU230 with pYY2 as a control plasmid, pGNT2 bearing *gntR*, or pGNTH bearing *gntH*. Double-transformants were screened on LB plates containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml), and subjected to β-Gal assay.

**Construction of single-copy *gntT::lacZ* and *gntK::lacZ* fusions on the genome**

Single-copy *gntT::lacZ* and *gntK::lacZ* fusions on the genome were constructed according to Simons *et al.* (Simons *et al.*, 1987). The PCR fragments from the 5'-flanking region to parts of coding regions of the *gntT* and *gntK* genes were subcloned into the *Eco*RI-*Bam*HI site of pRS551 to generate pRSGNTT and pRSGNTK, respectively. To obtain the PCR fragments, pairs of the upstream and downstream primers and the templates used were 5'-ATGGAATTCCTTTTGTAGATTGCCCG-3', 5'-ATGGGATCCATTCCTTGCATTAATCC-3', and pGNTT17 (Izu *et al.*, 1997b) for the fragments of *gntT*, and 5'-ATGGAATTCGGCGAATCTGTGACACC-3', 5'-ATGG GATCCTTCGATTACCTTCACG-3', and pGNTK-LAC for those of *gntK*. *E. coli* strain P90C transformed with pRSGNTT or pRSGNTK was used as a host strain for growth of phage λRS45 (Simons *et al.*, 1987) to prepare phage lysate, according to standard methods (Silhavy *et al.*, 1984). To perform homologous recombination between λRS45 genomic DNA and pRSGNTT or pRSGNTK, *E. coli* strain NK7049 was infected with the lysate and the recombinants were screened on LB plates containing kanamycin (35 μg/ml), streptomycin (50 μg/ml), and X-Gal (0.005%). Isolated blue colonies were then subjected to β-Gal assay.

**Primer extension mapping**

Primer extension was performed as described in CHAPTER 1 (Izu *et al.*, 1997a). An FITC-labeled primer, CGCCAGGGTTTTCCCAGTCACGAC, synthesized by Sawady Technology (Tokyo, Japan), was used to analyze the *gntV*, *yjgV*, and *gntH* promoters. Total RNA was isolated by the hot phenol method (Aiba *et al.*, 1981) from cells harboring each *lacZ* operon fusion plasmid, which were grown at 37 °C for 8 h. Primer extension reaction was carried out at 50 °C for 120 min in 0.5-ml microcentrifuge tubes in a final volume of 20 μl containing 5 μg of total RNA, 1 pmol of FITC-labeled primer, 200 μM dNTPs and 5 units of r*Tth* DNA polymerase (Perkin-Elmer-Cetus Instruments) in 30 mM Tricine (pH 8.5), 75 mM potassium acetate, 1.1 mM $Mn(OAc)_2$ and 10% glycerol. Nucleotide sequencing reaction was carried out using the same FITC-labeled primer and each *lacZ* operon fusion plasmid as a template. Both samples were analyzed using an SQ3000 nucleotide sequencer (Hitachi Electronics Engineering, Tokyo, Japan) with a 6.1-M urea-6% polyacrylamide gel.

**Site-directed mutagenesis of possible GntR-binding sequences upstream of the *gntK* gene**

Site-directed mutagenesis was performed using the Mutan-Super Km kit (Takara Shuzo) based on the oligonucleotide-directed dual amber method. Original nucleotide sequences of R1 and R2 are AATGTTACCGATAACAGT and ACAGTTACCCGT

AACATT (Fig. 20), respectively. Mutation 1 (M1), Mutation 6 (M6), and Mutation 8 (M8) have the substitution mutations on R1, R2, and both R1 and R2, respectively, and their sequences are AATGTTACCGAGTACAGT (M1), ACAGTTACCCGGTACATT (M6), and AATGTTACCGAGTACAGTTACCCGGTACATT (M8), respectively. Their effect was examined by gel-shift analysis and measurement of β-Gal activity from pGNTK-LAC containing the mutations.

**GntR purification**

GntR was purified with the Glutathione S-transferase (GST) Gene Fusion System (Pharmacia Biothech). The *gntR*-GST gene fusion plasmid that produces the GntR protein fusion with GST at its N-terminus was constructed as follows. The PCR fragment bearing the whole *gntR* gene was produced by amplification with an upstream primer, ACCGAATTCATGAAAAAGAAAAGACCC, and downstream primer, ACAGTCGACTTAAATAGATCCGCCCGG, and pGNTR2 as a template, digested with *Eco*RI and *Sal*I, and then inserted into the *Eco*RI-*Sal*I site of pGEX4T-1 (Pharmacia Biothech). The GntR-GST fusion protein was expressed in TG1 cells after induction with IPTG in LB medium for 6 h, and crude extracts of the cells were prepared. The purified GntR was used for gel-shift analysis after removing of the GST portion by thrombin protease cleavage.

**Gel-shift analysis**

Specific DNA binding of GntR and CRP was tested by gel-shift analysis (Fujita & Miwa, 1989) with some modifications. The PCR products were used as a DNA fragment after appropriate restriction enzyme digestion, which were amplified with an upstream primer, 5'-GCGCGTGCAGCCCTTATT-3' corresponding to the upstream sequence of the multicloning site on pCB182 and pCB192 and downstream primer, 5'-TTCTGGTGCCGGAAACCAGGCAAAG-3' corresponding to the 5'-coding region of *lacZ*, and with *gnt-lacZ* operon fusions as templates. The PCR product from pGNTT-LAC4 was digested with *Eco*RI to produce the 800-b and 480-b fragments of which the latter bears the *gntT* promoter-operator. The PCR products from pGNTV- and GNTII-LAC were digested with *Sal*I to produce the 850-b and 350-b fragments of which the latter bears the *gntV* or *yjgV* promoter-operator. The PCR products from pGNTK-LAC, -LACM1, -LACM6, and -LACM8 were digested with *Afl*II to produce the 1.4-kb and 690-b fragments of which the former bears the *gntK* promoter-operator. In some cases, the PCR product from pGNTK-LAC was digested with *Pst*I to produce the 1.2-kb and 0.9-kb fragments of which the former bears the *gntK* promoter-operator. About 0.5 pmol of the DNA fragments were mixed with purified GntR or CRP at 30 °C for 30 min in a 30 μl mixture of 30 mM Tris-HCl (pH7.5), 0.6 mM EDTA, 0.6 mM DTT, and 30

mM KCl followed by electrophoresis at room temperature with 5% polyacrylamide gels and staining with ethidium bromide.

**Enzyme assay**

Cells harboring operon fusion plasmids with the *lacZ* gene in multi or single-copy were grown at 37 °C for 15 h in 3 ml of LB medium containing ampicillin (100 μg/ml) and/or kanamycin (50 μg/ml). The preculture was diluted 30-fold with LB medium containing the same antibiotics to further incubate for appropriate time. The cells was then subjected to β-Gal assay (Miller, 1992). To analyze the *gnt* expression, 0.5% gluconate and/or 1 mM cAMP was added to the medium.

## Conclusions

*Escherichia coli* cells import gluconate and subsequently phosphorylate it to gluconate-6-phosphate by two different systems, GntI and GntII. Most genes in both systems and the related genes had genetically been identified, and *gntH* and *gntP* were found by this study (Table 19). Here, molecular biological and biochemical studies on the *gnt* genes were performed to define their gene organization and expressional regulation. The gene organization was summarized in Fig. 22.

The GntI system consists of a high affinity gluconate permease, a low affinity gluconate permease, and a thermoresistant gluconate kinase, which are encoded by the *gntT, gntU,* and *gntK* genes, respectively. The *gntT* gene is monocistronic with one strong and two weak promoters, which is located at 76.4 min on the *E. coli* chromosome. The *gntU* and *gntK* genes constitute an operon structure with the *gntR* gene encoding a regulator, which occur at 77.1 min. The *gntRKU* operon has a promoter for *gntR* and another for *gntKU*. The GntII system consists of a high affinity gluconate permease and a thermosensitive gluconate kinase encoded by the *gntW* and *gntV* genes, respectively. Additionally, a high affinity gluconate permease and a regulator encoded by the *gntP* and *gntH* genes, respectively, are uncovered by homology searching. The *gntW* and *gntH* genes constitute an operon with the *yjgV* and *yjgU* genes encoding an alcohol dehydrogenase-like protein and a protein homologous to 2-deoxy-D-gluconate 3-dehydrogenase, respectively, and upstream of this operon, the *gntV* gene exists as a monocistronic gene with its own promoter, which is transcribed in opposite direction to the operon. All these genes are located at 96.9 min except for *gntP* that occurs at 98 min. The *yjgV-yjgU-gntW-gntH* operon has at least two promoters in front of the *yjgV* and *gntH* genes, and the read-through transcription appears to occur from the *yjgV* promoter to *yjgU, gntW,* and *gntH* genes. Thus, it is revealed that the *E. coli* gluconate utilization system possesses 4 permeases, 2 kinases, and 2 specific regulators, which may give the possibility to adapt to various growth conditions via the ingenious expressional regulation of the *gnt* genes as mentioned below.

In the process of uptake and catabolism of gluconate, the GntI system functions as a main system and had been expected to be assisted by the GntII system. The GntII, however, appears to mainly contribute at high cell density, so that the GntI acts at the early growth phase followed by the GntII at the late growth phase. Such a system relay along with cell growth is achieved by a unique gene expressional control as shown in Fig. 23. The *gntT, gntK,* and *yjgV* promoters in the GntI and GntII are positively regulated by cAMP-CRP complex. Although the intracellular cAMP concentration is known to reflect the amount of glucose available, glucose was not used in this study, so that enough amount of cAMP may present even in the early growth phase. Thus, the control by cAMP-CRP complex is not shown in Fig. 23. Under the conditions without

**Table 19. The *gnt* genes of *E. coli***

| | Gene | Map position (min) | Function of gene products |
|---|---|---|---|
| GntI | *gntT* | 76.4 | high affinity permease |
| | *gntU* | 77.1 | low affinity permease |
| | *gntK* | 77.1 | thermoresistant kinase |
| GntII | *gntW* | 96.9 | permease |
| | *gntV* | 96.9 | thermosensitive kinase |
| | *gntR* | 77.1 | regulator |
| | *gntH* | 96.9 | regulator |
| | *gntP* | 98.0 | permease |

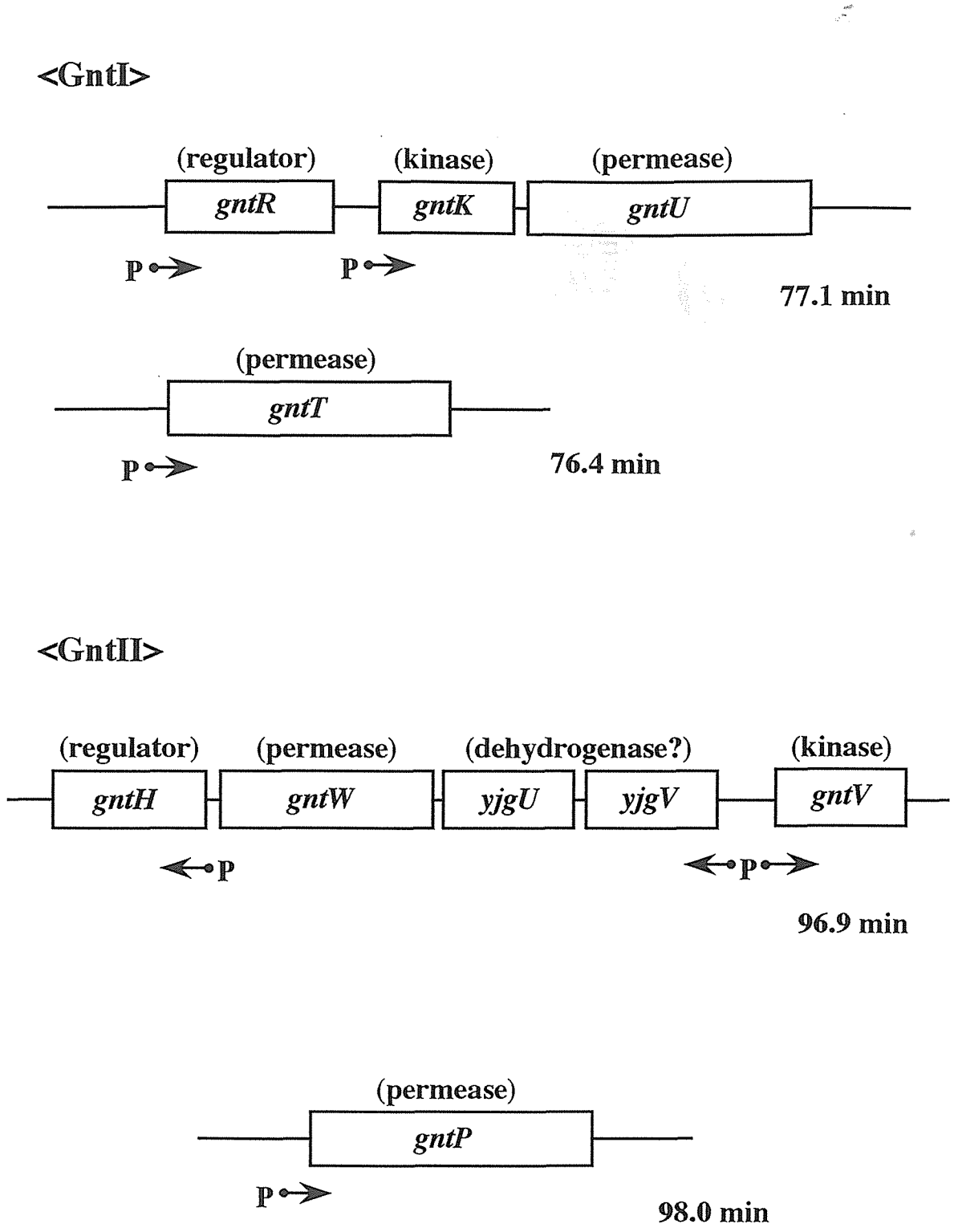


Figure 22. Organization of the *gnt* genes of *E. coli*.

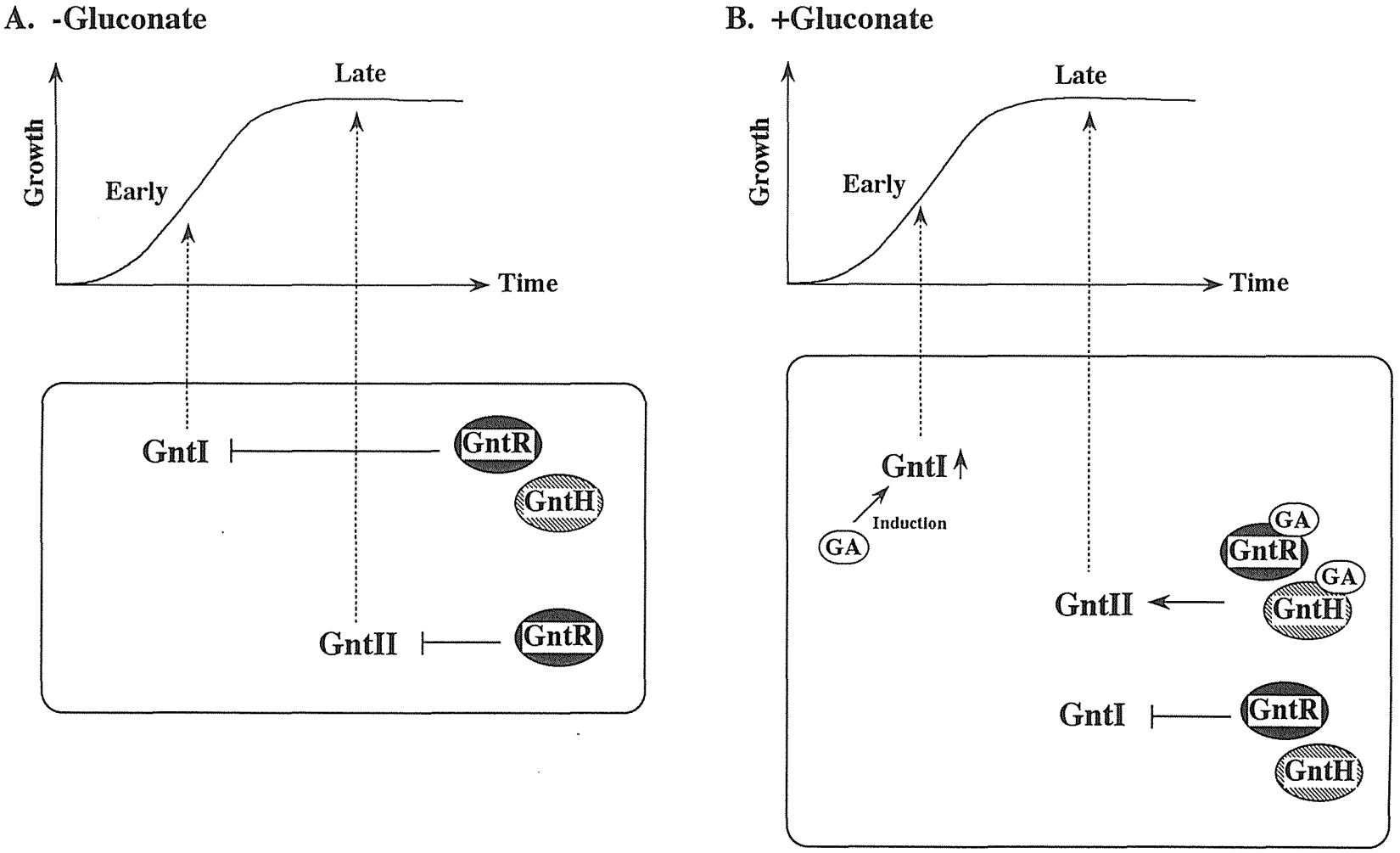


**Figure 23. Summary of expression of the *gnt* genes.**

gluconate (Fig. 23-A), both GntR and GntH repress the expression from the *gntT* and *gntK* promoters in GntI at both the early and late growth phases, and GntR represses the expression from the *gntV* and *yjgV* promoters in the GntII at the late growth phase. By the addition of gluconate (Fig. 23-B), the *gntT* and *gntK* genes in GntI are inducibly expressed at the early growth phase, but the expression is then decreased at the late growth phase. The *gntV* and *yjgV* genes are little expressed at the early growth phase and their expression is activated by both gluconate-bound GntR and GntH at the late growth phase (Fig. 23-B). Since at the late stage, gluconate concentration becomes low and both GntR and GntH are accumulated inside cells, the resultant gluconate-free GntR and GntH repress the GntI gene expression. Consequently, the GntI functioning at the early stage is switched with the GntII. GntH mainly contributes the activation of the GntII gene expression and also enhances the *gntR* expression at the late stage. Therefore, GntH appears to be important for the mutual regulation between the two systems and for a system relay from the GntI to GntII.

In this study, I have identified many genes involved in gluconate uptake and catabolism system of *E. coli* and defined the unique and complex mechanism of their expressional control. No such complexity was found for other sugars, suggesting that the gluconate system is physiologically important. GntP, a high affinity gluconate permease whose gene expression is repressed by the presence of gluconate (Klemm *et al.*, 1996), is hypothesized to import gluconate at low concentration even under the nutrient-limited condition. Gluconate is different from other sugars in respect to that *E. coli* can uptake and catabolize it with glucose by the systems insensitive to the glucose effect. This organism would predominantly utilize gluconate under certain conditions because the gluconate catabolism via Entner-Doudoroff pathway has the advantage of quickly producing energy. Acquisition of such gluconate utilization system may allow the organism to provide the ability to proliferate or survive under various conditions.

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要旨

大腸菌におけるグルコン酸取り込みおよび異化の分子遺伝学的解析

大腸菌の細胞外グルコースの利用はホスホトランスフェラーゼ系およびグルコース酸化系によって行われ、この2つの系は細胞の増殖段階や生育環境に応じて、選択的に使い分けがなされていると予想される。後者の系ではペリプラズムにおいてグルコースがグルコース脱水素酵素により酸化され、グルコン酸として細胞内に取り込まれ、その後、エントナー・ドウドロフおよびペントースリン酸経路によりさらに異化される。この系は低エネルギー状態におけるエネルギー獲得系として、また、迅速なエネルギー生産系として生理的に重要であると予想されるが、その分子機構はほとんど明らかにされていない。本研究では大腸菌のグルコン酸の取り込みと異化に関する酵素遺伝子の分子遺伝学的、生化学的解析を行ない、グルコース利用におけるグルコン酸取り込み系の生理的役割を明らかにすることを試みた。

1）*gntRKU*オペロンの遺伝子構造と発現調節

大腸菌のグルコン酸の取り込みと異化には、主として機能するGntI系と、補助的に機能するGntII系とが存在する。*gntK*と*gntU*はGntI系のリン酸化酵素と低親和性輸送体をコードし、調節タンパクをコードする*gntR*とともにゲノム上の77分にオペロンを構成することが示された。*gntRKU*は*R*と*K*の前にプロモーターをもっており、*gntR*は構成的に発現し、*gntKU*はcAMP-CRPにより正に、GntRにより負に制御されていることが明らかとなった。また、*gntKU*間で転写減衰が生じており、これがGntRによって促進されることが観察された。この機構によって、グルコン酸濃度に応じた*gntU*の発現調節が成されると推測された。

2）GntKの精製と解析

GntKの精製を行ない、酵素学的解析を行なった結果、GntKは18.4kDaの同一なサブユニットからなる二量体で機能し、グルコン酸とATPに対する*K*m値はそれぞれ42μM、120μMと見積られた。N末のアミノ酸配列の決定や分子量の測定から、*gntK*遺伝子との対応をつけた。

3）GntPファミリーの発見

大腸菌のグルコン酸取り込みに輸送体GntT, U, Wの関与が示唆されていたが、大腸菌ゲノムの塩基配列を用いてホモロジー検索を行なった結果、新たに3つの相同タンパクを見いだした。このうちの1つであるGntPのグルコン酸取り込みへの関与を予測し、合計5つのグルコン酸輸送体の存在を明らかにした。また、これらは他の糖輸送体と異なる14個の膜貫通領域を持つGntPファミリーを形成している。

4）*gntT*の発現調節とGntTの機能解析

*gntT*はGntI系の高親和性輸送体をコードし、ゲノム上の76.3分に単独で存在する。*gntT*

は1つの強いプロモーターと2つの弱いプロモーターを持ち、cAMP-CRPにより正に、GntRにより負に制御されていることが明らかとなった。GntTのグルコン酸輸送活性はGntKの共存により促進され、これらが共同で機能することが推測された。

5）*gntV, gntW, gntH*の遺伝子構造解析とGntIおよびII遺伝子の発現調節

*gntV, gntW*はそれぞれGntII系のリン酸化酵素と輸送体をコードし、ゲノム上の96.9分に位置する。*gntW*は脱水素酵素をコードすると推測される*yjgV*や*yjgU*および調節タンパクをコードする*gntH*とともに*yjgV-yjgU-gntW-gntH*オペロンを構成し、一方、*gntV*はこのオペロンと反対に向き合い単独で存在することが明らかとなった。プロモーターはそれぞれ*gntV, yjgV, gntH* の前に位置し、*gntW, gntH*は*yjgV*のプロモーターによるリードスルー転写により読まれていることがわかった。

GntI遺伝子は増殖初期および後期においてGntRやGntHによって、またGntII遺伝子は増殖後期においてGntRによって、それぞれ負の制御を受けることが明らかとなった。グルコン酸の添加によって、GntI遺伝子の発現は増殖初期に速やかに誘導され、その後、直ちに減少するが、GntII遺伝子の発現は増殖後期においてGntRやGntHによって高められることが確認された。このような巧妙な機構によって、増殖初期にGntIが発現し、引き続いてGntIIが発現して、環境に応じたグルコン酸利用を可能にしていると予想された。

細胞内への糖の取り込みは炭素源およびエネルギー源の獲得過程として生理的に重要である。大腸菌においてグルコースは利用しやすい糖であり、優先的に代謝される。しかしながら、他の糖と異なり、グルコン酸はグルコースと同時に代謝可能な珍しい糖であり、本研究により、その取り込みに複数のタンパクが関与し、巧みな制御が行われていることが示唆された。この複雑な機構はグルコン酸利用系の生理的重要性を暗示するものであるが、マウス大腸内での大腸菌コロニー形成にグルコン酸輸送系が必須であるという事実からも、多様な条件や環境での生理的役割がうかがえる。また、大腸菌が多様なグルコン酸取り込み機構および制御機構を獲得することによって、様々な環境下での生存を可能にしてきたと予想される。

## List of Publications Related to This Thesis

1. Yamada, M., Kawai, T., & Izu, H. (1996). Analysis of the *Escherichia coli gntT* and *gntU* genes and comparison of the products with their homologues. *Biosci. Biotech. Biochem.* **60**, 1548-1550.
**(CHAPTER 3)**

2. Izu, H., Adachi, O., & Yamada, M. (1996). Purification and characterization of the *Escherichia coli* thermoresistant gluconokinase encoded by the *gntK* gene. *FEBS Lett.* **394**, 14-16.
**(CHAPTER 2)**

3. Izu, H., Adachi, O., & Yamada, M. (1997). Gene organization and transcriptional regulation of the *gntRKU* operon involved in gluconate uptake and catabolism of *Escherichia coli*. *J. Mol. Biol.* **267**, 778-793.
**(CHAPTER 1)**

4. Izu, H., Kawai, T., Yamada, Y., Aoshima, H., Adachi, O., & Yamada, M. (1997). Characterization of the *gntT* gene encoding a high-affinity gluconate permease in *Escherichia coli*. *Gene,* **199**, 203-210.
**(CHAPTER 4)**

5. Aoshima, H., Yokoyama, T., Tanizaki, J., Izu, H., & Yamada, M. (1997). The sugar specificity of $Na^+$/glucose cotransporter from rat jejunum. *Biosci. Biotech. Biochem.* **61**, 979-983.

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