The Characterization and Cloning of a Gluconate (*gnt*) Operon of *Bacillus subtilis*

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The enzymes involved in gluconate utilization in *Bacillus subtilis* seemed to be gluconate permease and gluconate kinase. Several mutants unable to grow on gluconate were isolated. The mutations they harboured (*gnt*) were clustered between *iol-6* and *fdp-74* on the *B. subtilis* chromosome (a tentative map order of *gnt-10*, *gnt-4*, *gnt-26*, *gnt-23* and *gnt-9* was obtained). The *gnt-10* mutation seemed to be located within the structural gene of the kinase, and the *gnt-23* and *gnt-26* mutations seemed to be within that of the permease. An *EcoRI* fragment (4.5 MDal) containing an intact gluconate (*gnt*) operon consisting of these two structural genes was cloned in phage $\phi 105$ by prophage transformation and was mapped physically. The physical location of the mutations coincided with their order on the genetic map. The *HindIII*-A fragment (2.4 MDal), which corrects all the *gnt* mutations, was subcloned in plasmid pC194. The fragment contained the structural genes for the gluconate permease and kinase, but not the regulatory region of the gluconate operon.

INTRODUCTION

After entering the *Bacillus subtilis* cell, gluconate is phosphorylated to gluconate 6-phosphate, which, because *B. subtilis* lacks the Entner–Doudoroff pathway, can only be metabolized through the pentose cycle. Therefore, only two enzymes may be needed for the utilization of gluconate as a carbon source; one is gluconate permease, which transports gluconate into the cell, and the other is gluconate kinase, which phosphorylates gluconate. Both enzymes are induced in response to gluconate and their induction is repressed in the presence of rapidly metabolizable carbohydrates such as glucose (Dowds *et al.*, 1978; Nihashi & Fujita, 1984). We have tentatively reported the cloning of the genes for gluconate utilization in a temperate phage, $\rho 11$ (Fujita *et al.*, 1983). In this paper, we communicate the genetic mapping and characterization of several *gnt* mutations that render the cell unable to grow on gluconate and we also report the recloning of an *EcoRI* fragment cloned in $\rho 11$ in another temperate phage, $\phi 105$. This fragment encodes an intact gluconate operon. To our knowledge, this work is the first example of the use of a *B. subtilis* host–vector system to clone an intact catabolic operon of this species.

METHODS

Bacteria, phage and plasmids. Strain 60015 (*trpC2 metC7*) is our standard strain. Among *Gnt*[−] mutants, the isolation of strain 61656 (*Nigf hisA1 leuA8 metB5 trpC2*), YF127 (*gnt-4 trpC2 metC7*) and YF029 (*gnt-9 purA16 leuA8 metB5 hisA3*) has already been reported (Fujita & Freese, 1981; Fujita & Fujita, 1983). Strain YF158 (*gnt-4 recE4 trpC2*) was constructed from YF127 by congression using the DNA of strain 1A423 (*leuA8 thr-5 argA15 recE4 r[−] m[−]*), which was obtained from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus, USA. Strain YF160 (*gnt-10 trpC2 metC7*) was isolated from strain 60015 that had been treated with

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ethylmethane sulphonate, according to the method previously described (Fujita & Fujita, 1983). Strains YF161 (*gnt-23 trpC2 metC7*) and YF162 (*gnt-26 trpC2 metC7*) were isolated as follows. After strain 60015 had been treated with ethylmethane sulphonate, cells were grown in a liquid minimal medium (S6) (Fujita & Fujita, 1983) containing glucose as sole carbon source and were plated on a solid minimal medium (N) (Fujita & Fujita, 1983) containing gluconate (0.1%) and glucose (0.01%) as carbon sources. Among approximately 20000 colonies screened, 1500 tiny colonies were picked and tested for growth on glucose and gluconate. Only two colonies, YF161 and YF162, showed a Gnt⁻ phenotype. The temperate phage, ϕ 105, was provided by F. Kawamura (Institute of Applied Microbiology, Tokyo University, Japan). Plasmids pUB110 and pC194 were obtained from Y. Sadaie (National Institute of Genetics, Mishima, Japan) and the *Bacillus* Genetic Stock Center, respectively.

Enzyme assays. Gluconate kinase (EC 2.7.1.12) was assayed as described previously (Nihashi & Fujita, 1984). The enzyme was induced by exposure of cells to 10 mM-gluconate for 2 h.

Gluconate permease was assayed essentially by the method described by Dowds *et al.* (1978). When cells had grown at 37 °C to an OD₆₀₀ of 1.0 in S6 medium containing 0.5% Casamino acids (Difco), 3 ml of the culture were harvested and washed in S6 medium plus chloramphenicol (100 µg ml⁻¹). Washed cells were suspended in 2 ml of the same medium plus chloramphenicol (100 µg ml⁻¹) and used for the permease assay as an uninduced sample. In order to induce the permease, the above culture was diluted four times with the same growth medium and the cells allowed to grow to an OD₆₀₀ of 1.0 for approximately 2 h in the presence of 10 mM-gluconate. Samples (3 ml) of the culture were harvested, washed and resuspended as described above. The suspension (2 ml) was used as an induced sample. The assay tube held, at 37 °C, 0.7 ml S6 medium plus chloramphenicol (100 µg ml⁻¹) containing D-[U-¹⁴C]gluconate (0.05 µCi, 1.9 kBq; 5.6 mCi µmol⁻¹, 210 MBq µmol⁻¹) (Amersham) and 93 µM-gluconate. Incorporation was initiated by the addition of 0.6 ml of the cell suspension to the assay tube. After incubation at 37 °C for 30 s, the suspension was filtered on a premoistened membrane (25 mm diameter, 0.45 µm pore size) (Toyo Roshi Co.). The filter pad was immediately washed three times with 5 ml S6 medium plus chloramphenicol (100 µg ml⁻¹). The pad was dried and counted in 10 ml scintillant (ACSII; Amersham).

Transduction and transformation. PBS1-mediated transduction and DNA-mediated transformation were done as described by Iijima *et al.* (1977) and Shibata & Saito (1973), respectively. Specialized transduction by phages derived from ϕ 105 was done as described by Iijima *et al.* (1980). For selection of Gnt⁺ recombinants, N medium containing 25 mM-gluconate and enriched with 0.002% yeast extract (Difco) was used.

Transformation of Gnt⁻ strains by restriction enzyme fragments was done as follows. The *gnt*⁺ fragment (1 µg) was digested with *Bgl*II, *Mlu*I, *Pst*I, *Sma*I, *Stu*I and *Hind*III. The digested samples were electrophoresed through a 1% gel of low melting point agarose (BRL) and then the bands stained with ethidium bromide (0.5 µg ml⁻¹ in H₂O) were excised. After melting the gels (65 °C, 30 min), a portion (10 µl) of the gel containing each fragment was used for transformation.

Preparation of DNA. *B. subtilis* chromosomal DNA was purified as described by Saito & Miura (1963). Phage particles (ϕ 11 and ϕ 105) and their DNA were prepared by methods essentially similar to those described by Iijima *et al.* (1980). After phages had been induced with mitomycin C, phage particles were sedimented in the presence of polyethylene glycol (Yamamoto *et al.*, 1970) and further purified by centrifugation through discontinuous CsCl gradients. Viral DNA was prepared from purified phage particles by phenol treatment. Plasmid pC194 was prepared as described by Gryczan *et al.* (1978).

Restriction nuclease reactions and agarose gel electrophoresis. Manufacturer's specifications were used for restriction enzyme digestions of plasmid and phage DNAs. Agarose gel electrophoresis was done with a horizontal submerged gel apparatus in Löening's buffer (Löening, 1968).

RESULTS

Mapping and characterization of gnt mutations

Mapping of the *gnt-4* mutation by PBS1-mediated transduction has been reported by Fujita & Fujita (1983). The map order from *sacA* to *purA* is as follows: *sacA*, *thiC*, *hsrE*, *iol-6*, *gnt-4*, *fdp-74*, *hsrB*, *ts199* and *purA*. The Δ *igf* deletion covers loci from *iol-6* to *hsrB*. In this study, the map locations of several other *gnt* mutations were determined. The *gnt-9* mutation was also located within the Δ *igf* deletion because DNA from strain 61656 (Δ *igf*) could not transform strain YF029 (*gnt-9*) to Gnt⁺. PBS1 transduction crosses revealed a map order of *iol-6*, *gnt-9* and *fdp-74*. When strain YF029 (*gnt-9 purA16*) was transduced with PBS1 propagated in strain YF127 (*gnt-4*) and then Pur⁺ recombinants were selected, two Gnt⁺ colonies were found among 161 Pur⁺ recombinants. These results suggested that *gnt-9* is very close to *gnt-4*, perhaps located between *gnt-4* and *fdp-74*. All of the *gnt* mutations in our collection, including those that had been newly isolated, *gnt-10*, *gnt-23* and *gnt-26*, were mapped by two-factor transformation crosses between Gnt⁻ strains measuring recombination indexes (Fig. 1). The crosses suggested a map order of *gnt-10*, *gnt-4*, *gnt-26*, *gnt-23* and *gnt-9*.

Fig. 1. Map of the *gnt* locus deduced from transformation crosses. In the crosses, YF141 (*gnt-4 trpC2 metB5 hisA1*) and YF029 (*gnt-9 purA16 leuA8 metB5 hisA3*) were used as recipients and strains YF160, YF161, YF162, YF127 and YF164 (*gnt-9 purA16 leuA8 metB5*) as donors. Distances are expressed as recombination indexes (Chasin & Magasanik, 1968). The ratio of *gnt*⁺ to *his*⁺ (YF141 as recipient) or *leu*⁺ (YF029 as recipient) transformants was a measure of the relative frequency of transformation to gluconate utilization.

Table 1. Induction of gluconate permease and gluconate kinase in *gnt* mutants

Gluconate kinase and permease were induced and assayed as described in Methods. Values are means of duplicate assays. Data are from a representative experiment using a complete set of the *gnt* mutants. Similar independent experiments were repeated at least twice.

Strain	Gluconate permease*		Gluconate kinase†	
	Not induced	Induced	Not induced	Induced
60015 (<i>gnt</i> ⁺)	1.14	12.39	0.4	25.4
61656 (<i>Δigf</i>)	0.15	0.15	<0.1	<0.1
YF029 (<i>gnt-9</i>)	0.09	0.15	<0.1	3.3
YF127 (<i>gnt-4</i>)	34.45	26.14	<0.1	<0.1
YF160 (<i>gnt-10</i>)	2.56	12.82	<0.1	3.9
YF161 (<i>gnt-23</i>)	0.06	0.48	0.1	19.0
YF162 (<i>gnt-26</i>)	0.37	5.25	0.1	35.1

* nmol min⁻¹ per unit OD₆₀₀.

† nmol min⁻¹ mg⁻¹.

Since the enzymes involved in gluconate metabolism are supposed to be gluconate kinase and permease, the inducibility of these two enzymes in the *Gnt*⁻ mutants was investigated (Table 1). As expected, strain 61656 (*Δigf*) could induce neither the kinase nor the permease; the *Δigf* mutation is a large deletion covering the *gnt* locus. Strain YF029 (*gnt-9*) could not induce the two enzymes as normal. Since the *gnt-9* mutation was considered to be a point mutation, this strain seemed to be impaired in a regulatory function required for the induction of both enzymes. Strain YF160 (*gnt-10*) was impaired only in the induction of the kinase, whereas strains YF161 (*gnt-23*) and YF162 (*gnt-26*) were impaired only in the induction of the permease. The former mutation seemed to be located within the structural gene of the kinase and the latter mutations were likely to be within that of the permease. Strain YF127 (*gnt-4*) could not induce the kinase at all, but unexpectedly this strain synthesized the permease constitutively.

Cloning of the *gnt*⁺ fragment in the temperate phage ϕ 105

An *EcoRI* *gnt*⁺ fragment from *B. subtilis* Marburg 168 (*trpC2*) DNA containing the genes for gluconate utilization has been cloned in a temperate phage, ρ 11, by prophage transformation

Table 2. Enzyme induction and repression by glucose in the Δigf strain lysogenic for $\phi 105gnt^+$

Strain	Gluconate permease*			Gluconate kinase†		
	Not induced	Induced	Induced (+glucose)‡	Not induced	Induced	Induced (+glucose)‡
60015 (gnt^+)	0.71	10.00	0.63	0.1	22.8	0.6
61656 (Δigf) ($\phi 105gnt^+$)	0.61	8.66	0.56	0.9	28.5	0.1

* nmol min⁻¹ per unit OD₆₀₀.† nmol min⁻¹ mg⁻¹.

‡ Glucose (10 mM) was added to the medium to repress enzyme induction.

using strain 61656 as recipient (Fujita *et al.*, 1983). Moreover, Fujita *et al.* (1983) tentatively suggested that the gnt^+ fragment contains the structural gene of the gluconate kinase and its regulatory region. However, it was very difficult to isolate the gnt^+ fragment of the recombinant phage by agarose gel electrophoresis because *EcoRI* digestion of $\rho 11$ DNA produces more than 25 fragments (Mizukami *et al.*, 1980). To isolate and analyse the gnt^+ fragment, it was recloned in another temperate phage, $\phi 105$, that is relatively small, and convenient for isolation of this fragment (Iijima *et al.*, 1980). DNA (5 μ g) of phage $\rho 11hisA^+gnt^+E$ (a recombinant $\rho 11$ possessing the gnt^+ fragment) and DNA (10 μ g) of phage $\phi 105$ were each completely digested with *EcoRI*. The digestion products were mixed and ligated using T4 DNA ligase and then used to transform strain 61656 (Δigf), lysogenic for $\phi 105$, to Gnt^+ . All of the 250 Gnt^+ transformants obtained were considered to result from the integration of the gnt^+ fragment at the site of the $\phi 105$ prophage genome, because of the absence of homology between this fragment and the chromosome of the Δigf strain. Five transformants were treated with mitomycin C and at the same time infected with wild-type $\phi 105$. All the lysates obtained exhibited the ability to transduce strain 61656 to Gnt^+ . One of the Gnt^+ transductants was used for further experiments after single colony isolation. This transductant produced, upon induction, a gnt^+ specialized transducing phage ($\phi 105gnt^+$) in the absence of helper phage $\phi 105$, but the resulting phage required helper for transduction of a Gnt^- recipient to Gnt^+ . $\phi 105gnt^+$ could transduce not only the Δigf mutant but also strains carrying all the gnt mutations ($gnt-4$, $gnt-9$, $gnt-10$, $gnt-23$ and $gnt-26$) to Gnt^+ .

The inducibility of the gluconate kinase and permease in a $\phi 105gnt^+$ lysogen of strain 61656 (Δigf) was compared with that in our standard strain 60015 (Table 2). Strain 61656 ($\phi 105gnt^+$) induced the two enzymes at normal levels upon addition of gluconate to medium, and induction was repressed by the addition of glucose. The results clearly indicated that the structural genes of the gluconate kinase and permease and regulatory regions for their expression had been cloned in $\phi 105$.

DNA from $\phi 105$ and $\phi 105gnt^+$ was digested with *EcoRI* and analysed by agarose gel electrophoresis (Fig. 2a). $\phi 105$ DNA produced nine fragments (from A to I) whereas $\phi 105gnt^+$ DNA produced a new fragment of 4.5 MDal (the gnt^+ fragment) and three of the *EcoRI* fragments of $\phi 105$ DNA (E, G and I) were missing. [The *EcoRI* cleavage map of $\phi 105gnt^+$ DNA is shown with that of $\phi 105$ in Fig. 2(b).] It was ascertained that the gnt^+ fragment was sited between fragments B and D as follows. $\phi 105$ DNA does not contain any *BglII* cleavage sites whereas $\phi 105gnt^+$ DNA had one *BglII* cleavage site in the gnt^+ fragment. After digestion of $\phi 105gnt^+$ DNA with *BglII*, the smaller fragment was isolated by agarose gel electrophoresis. *EcoRI* digestion of this fragment produced the *EcoRI*-D fragment, and the *BglII*-B fragment indicated in Fig. 3(a).

Construction of the restriction enzyme map of the gnt^+ fragment and physical localization of the gnt mutations

The restriction enzyme map of the gnt^+ fragment was constructed by digestion with *BglII*, *HindIII*, *MluI*, *PstI*, *PvuII*, *SmaI* and *StuI* (Fig. 3a). Since the gnt^+ fragment had the ability to

Fig. 2. (a) Agarose gel electrophoresis of *Eco*RI-digested samples of $\phi 105$ and $\phi 105gnt^+$ DNA. The DNA (2 μ g) was digested with *Eco*RI and electrophoresed in a 0.8% agarose gel. (b) *Eco*RI cleavage maps of $\phi 105$ and $\phi 105gnt^+$ DNA, with sizes of fragments expressed as MDal. The map of $\phi 105$ DNA has recently been corrected (Anaguchi *et al.*, 1984; Lampel *et al.*, 1984; Bugaichuk *et al.*, 1984). The cloned gnt^+ fragment (4.5 MDal) is indicated as a bold line.

transform all the Gnt^- mutants except the Δigf deletion mutant to Gnt^+ , we attempted to localize physically each gnt mutation on a particular restriction enzyme fragment by assaying fragments for their ability to transform the respective gnt mutant to Gnt^+ (Table 3). The *Bgl*II-A, *Mlu*I-A, *Pst*I-A, *Sma*I-A, *Stu*I-A and *Hind*III-A fragments transformed the $gnt-4$ mutant to the wild-type, while the *Bgl*II-A, *Mlu*I-A, *Pst*I-A, *Sma*I-B, *Stu*I-B and *Hind*III-A fragments transformed the $gnt-9$ mutant to Gnt^+ . The $gnt-10$ mutant was transformed with the *Bgl*II-B, *Mlu*I-B, *Pst*I-B, *Sma*I-A, *Stu*I-A and *Hind*III-A fragments, whereas the $gnt-26$ mutant was transformed with the *Bgl*II-A, *Mlu*I-A, *Pst*I-A, *Sma*I-A, *Stu*I-B and *Hind*III-A fragments. The $gnt-23$ mutant was transformed with *Bgl*II-A, *Mlu*I-A, *Pst*I-A and *Hind*III-A fragments. The reversion frequency of the $gnt-23$ mutation was extraordinarily high so that we could not conclude with confidence which of the *Stu*I and *Sma*I fragments exhibited the transforming activity. From these results, each gnt mutation was localized on the respective enzyme fragment (Fig. 3b). (The $gnt-23$ mutation could be localized approximately between the *Mlu*I site and the right-hand *Hind*III site.)

Fig. 3. (a) Restriction enzyme map of the *gnt*⁺ fragment. Fragments generated by *BglII*, *HindIII*, *MluI*, *PstI*, *PvuII*, *SmaI* and *StuI* digestion were alphabetically named; their sizes are expressed as MDal. (b) Localization of *gnt* mutations. This was deduced from the results of transformation experiments using fragments shown in (a) (Table 3). Transformation experiments were independently repeated at least twice using each recipient and various sets of restriction enzyme fragments; all the results obtained supported the locations shown.

Table 3. *Gnt*⁺ transforming activities of restriction enzyme fragments of the *gnt*⁺ fragment

The transformation was done as described in Methods. Independent transformation experiments were done for each recipient. Values are means of duplicate assays.

Donor fragment	No. of <i>Gnt</i> ⁺ colonies per 0.1 ml competent culture				
	Recipient:				
	YF127 <i>gnt-4</i>	YF029 <i>gnt-9</i>	YF160 <i>gnt-10</i>	YF161 <i>gnt-23</i>	YF162 <i>gnt-26</i>
<i>BglII</i> -A	600	1300	25	810	1100
<i>BglII</i> -B	1	95	380	186	76
<i>MluI</i> -A	65	550	37	450	660
<i>MluI</i> -B	8	128	900	210	110
<i>PstI</i> -A	442	1170	44	280	300
<i>PstI</i> -B	2	83	440	175	57
<i>SmaI</i> -A	1000	83	4000	230	180
<i>SmaI</i> -B	7	216	18	190	91
<i>StuI</i> -A	192	95	2800	220	56
<i>StuI</i> -B	1	212	38	250	174
<i>HindIII</i> -A	630	340	1500	770	910
No addition	0	80	8	193	95

Table 4. Induction of gluconate permease and gluconate kinase in Δigf strains bearing plasmids or $\phi 105$ derivatives containing the *HindIII*-A fragment

Plasmid or phage	Gluconate permease*		Gluconate kinase†	
	Not induced	Induced	Not induced	Induced
pCG1	5.36	4.02	28.6	15.9
pCG8	16.08	9.15	73.6	49.5
$\phi 105gnt^+H2$	2.76	2.85	16.2	11.0
$\phi 105gnt^+H4$	3.71	3.05	25.0	20.2

* nmol min⁻¹ per unit OD₆₀₀.† nmol min⁻¹ mg⁻¹.*Cloning of the HindIII-A fragment with pC194 and $\phi 105$ and its expression*

To investigate the effect of high copy number on the regulation of the expression of the cloned genes, we attempted to clone the *gnt*⁺ fragment in plasmid pUB110; these experiments failed, perhaps because the fragment was too large to be cloned in this plasmid. As an alternative approach we decided to clone the *HindIII*-A fragment (2.4 MDal) in another plasmid, pC194, because this fragment was smaller, but nevertheless was able to correct all the *gnt* mutations in transformation experiments. The *HindIII*-A fragment (2 μ g) was isolated electrophoretically from a *HindIII*-digested sample of the *gnt*⁺ fragment, and was ligated with pC194 (2 μ g) that had been digested with *HindIII*. The ligated DNA was used to obtain Gnt⁺ transformants of strain YF158 (*gnt-4 recE4 trpC2*). Out of 187 Gnt⁺ colonies obtained, ten colonies were used for plasmid extraction and analysis. Two kinds of plasmid (represented by pCG1 and pCG8) were found in which the *HindIII*-A fragment was cloned in opposite orientations. Strain 61656 (Δigf) transformed with each plasmid was used for further experiments. The inducibility of the gluconate permease and kinase in strain 61656 (Δigf) harbouring pCG1 or pCG8 [61656(pCG1 or pCG8)] was investigated. The two enzymes were synthesized constitutively in both 61656(pCG1) and 61656(pCG8) (Table 4). Specific activities of the enzymes synthesized in 61656(pCG8) were 2–3 times higher than those in 61656(pCG1).

The constitutive synthesis of the gluconate permease and kinase might possibly be attributed to the presence of multiple copies of the *HindIII*-A fragment containing pCG1 or pCG8. To test this possibility, the *HindIII*-A fragment was cloned in the genome of $\phi 105$, which can be integrated as only one copy in the host chromosome, and the expression of the genes in this fragment was investigated. The *HindIII*-A fragment was cloned by prophage transformation using 61656 (Δigf) as recipient and using similar procedures to those described above. From two Gnt⁺ transducing phages obtained ($\phi 105gnt^+H2$ and $\phi 105gnt^+H4$), DNA was prepared, digested with restriction enzymes and analysed by agarose gel electrophoresis. The electrophoretic patterns of the *EcoRI*- and the *EcoRI* + *HindIII*-digested samples of $\phi 105gnt^+H2$ were different from those of $\phi 105gnt^+H4$, but the doubly digested DNA samples gave a band of 2.4 MDal that corresponded to the *HindIII*-A fragment (data not shown). The results indicated that $\phi 105gnt^+H2$ and $H4$ possess the *HindIII*-A fragment cloned in different *HindIII* sites of $\phi 105$. Table 4 shows that strain 61656 lysogenic for either $\phi 105gnt^+H2$ or $H4$ synthesized the gluconate kinase and permease constitutively. The results imply that the constitutive synthesis of the two enzymes in strains carrying pCG1 or pCG8 was not due to the high copy number of the fragment when cloned in pC194, but rather that a regulatory function required for the induction of the enzymes was lacking in the fragment.

DISCUSSION

All of the mutations affecting the gluconate permease and kinase and the regulation of their biosynthesis were found to be clustered. On the basis of two factor crosses between Gnt⁻ mutants (Fig. 1), we distinguished on the map the *gnt-10* mutation and the *gnt-23* and *gnt-26* mutations in the structural genes of the kinase and the permease, respectively. The existence of

the *gnt-9* mutation affecting both the permease and the kinase, and the fact that the Δ *igf* strain lysogenic for ϕ 105*gnt*⁺H2 or H4 constitutively synthesized the two enzymes, suggested that the two structural genes belong to a single transcription unit, a gluconate (*gnt*) operon. However, we have not excluded the possibility that there are two separate transcription units controlled by the same regulators. The presence of the *gnt-4* mutation affecting the kinase and causing the constitutive synthesis of the permease is not completely understood. However, at present, we prefer one of the possible explanations, i.e. that the gluconate kinase protein itself may be involved in the regulation of the gluconate operon, so that the impaired gluconate kinase produced in the *gnt-4* mutant might cause the constitutive synthesis of the permease.

We previously reported the cloning of the structural gene of the gluconate kinase and regulatory regions for its expression in a phage, ρ 11 (Fujita *et al.*, 1983). In this investigation, an *Eco*RI *gnt*⁺ fragment generated from DNA of the specialized *gnt*⁺ transducing phage ρ 11 was recloned in another temperate phage, ϕ 105. The fact that the induction of the gluconate permease and kinase in the Δ *igf* strain lysogenic for ϕ 105*gnt*⁺ was indistinguishable from that in the wild-type strain (Table 2), and that ϕ 105*gnt*⁺ was able to transduce the Δ *igf* deletion as well as all the *gnt* mutants to *Gnt*⁺, strongly indicated that an intact gluconate operon had been cloned in ϕ 105. The *Hind*III-A fragment as well as the *gnt*⁺ fragment (Table 3) was able to transform all the *gnt* mutants to the wild-type. However, the Δ *igf* strain lysogenic for ϕ 105 bearing this fragment synthesized the gluconate permease and kinase constitutively. This suggests that the *Hind*III-A fragment probably contains the structural genes of the two enzymes, but does not contain any of the regulatory region of the gluconate operon.

The map order of *gnt-10*, *gnt-4*, *gnt-26*, *gnt-23* and *gnt-9* (Fig. 1) is considered to be tentative because it is based on data from two-factor crosses. However, it was not possible to carry out three-factor transformation crosses because at present there is no known easily selectable marker that is linked to the *gnt* mutations. Moreover, these mutations were too close to be ordered by PBS1 transduction crosses. All of the *gnt* mutations were successfully assigned to restriction enzyme fragments generated from the *gnt*⁺ fragment (Fig. 3*b*). Although the map order of the *gnt* mutations is tentative, it is likely to be reliable because it is reasonably consistent with the physical map. The *gnt*⁺ fragment does not complement the *iol-6* and *fdp-74* mutations (Fujita *et al.*, 1983), but it did complement all the *gnt* mutations. From these results and those obtained from the genetic crosses, the following order from *iol-6* to *fdp-74* was deduced: *iol-6*, *gnt-10*, *gnt-4*, *gnt-26*, *gnt-23*, *gnt-9* and *fdp-74*.

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