

## Genetic Analysis of a Pleiotropic Deletion Mutation ( $\Delta igf$ ) in *Bacillus subtilis*

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A  $\Delta igf$  mutation of *Bacillus subtilis* (formerly called *fdpA1*) is a large deletion causing pleiotropic defects. The mapping of the  $\Delta igf$  deletion by phage PBS1 transduction revealed the following map order: *sacA*, *thiC*, *hsrE*,  $\Delta igf$ , *ts199*, *purA*. To analyze the pleiotropic nature of the  $\Delta igf$  mutation, mutants affected in each property of the pleiotropic mutation were isolated, and the mutations were mapped. *iol* and *gnt* mutants could not grow on inositol and gluconate, respectively, and *fdp* mutants were affected only in fructose-bisphosphatase. The map order from *sacA* to *purA* was as follows: *sacA*, *thiC*, *hsrE*, *iol-6*, *gnt-4*, *fdp-74*, *hsrB*, *ts199*, *purA*. The  $\Delta igf$  deletion covered loci from *iol-6* to *hsrB*.

Fructose-bisphosphatase (EC 3.1.3.11) of bacilli has unique catalytic and physical properties which are clearly distinct from those of the enzyme from other sources, such as mammals, plants, and *Escherichia coli* (4). Interesting findings have been that the inhibition of *Bacillus subtilis* fructose-bisphosphatase by AMP can be counteracted by an increased concentration of phosphoenolpyruvate during gluconeogenesis and that its activity is under stringent control.

To investigate the physiological role of this unique enzyme, Fujita and Freese isolated a *B. subtilis* mutant unable to produce fructose-bisphosphatase and investigated its properties (5). It was surprising to find that this mutant could grow at a high rate and could sporulate well in medium containing only gluconeogenic carbon sources such as glycerol or L-malate, since it had been generally believed that fructose-bisphosphatase was the only enzyme which could supply fructose 6-phosphate (fructose 6-P). The finding clearly indicates that *B. subtilis* should be able to bypass this enzyme reaction. Furthermore, this mutation (*fdpA1*) has the pleiotropic consequence that mutants carrying it cannot grow on *myo*-inositol or D-gluconate and lack the inducibility of inositol dehydrogenase (EC 1.1.1.18) and gluconate kinase (EC 2.7.1.12), which are the first enzymes to metabolize these carbon sources. Fujita and Iijima (6) have reported in a preliminary communication that the *fdpA1* mutation is probably a large deletion which is located between *sacA* and *purA* (map order, *sacA*, *thiC*, *fdpA1*, *ts199*, *purA*) and implied that the pleiotropic nature may be attributed to the large deletion including genes involved in inositol and gluconate metabolism and in fructose-bisphosphatase activity.

This paper presents further genetic analysis of the *fdpA1* mutation, which is now called  $\Delta igf$  because of the deletion causing the inability to grow on inositol or gluconate and the lack of fructose-bisphosphatase. Mutants affecting each of the pleiotropic properties of  $\Delta igf$  strains were isolated. Their mutations were mapped and found to be located inside the deletion.

### MATERIALS AND METHODS

**Bacterial strains.** The *B. subtilis* strains used in this study are listed in Table 1.

**Transduction and transformation.** Transduction by PBS1 phage was performed according to a standard method modified by Iijima et al. (8). Transformation was performed according to a standard method modified by Shibata and Saito (11). For selection of adenine- or amino acid-independent transductants or transformants, the minimal glucose medium of Anagnostopoulos and Spizizen (2) was used. The salts of that medium, without citrate and D-glucose, are called N. N medium containing 25 mM gluconate as a carbon source was used for the selection of *gnt*<sup>+</sup> transductants or transformants. For the selection of *iol*<sup>+</sup> or  $\Delta igf$ <sup>+</sup> transductants or transformants, N medium plus 25 mM inositol (enriched with 0.002% yeast extract [Daigoeyokagaku Co., Ltd., Osaka]) was used, and for that of *fdp*<sup>+</sup> transformants of a strain (*fdp bfd*) unable to ferment gluconeogenic carbon sources, N medium plus 0.5% Casamino Acids, 0.1% Na<sub>3</sub> citrate, 0.05 mM MnCl<sub>2</sub>, and 50  $\mu$ g of tryptophan per ml was used. The transductants or transformants were tested for growth on gluconate or inositol by using N medium plus 25 mM gluconate or inositol. Amino acids and adenine were supplemented at a concentration of 50  $\mu$ g/ml to the minimal medium.

**Test of host-specific restriction activities.** Restriction activities of strains were examined by cross-streaking the colonies of transformants or transductants with appropriately modified  $\phi 105C$ , as described previously (9, 12).

TABLE 1. Origin and characteristics of *B. subtilis* strains used

Strain	Genotype <sup>a</sup>	Source (or reference)
60015	<i>trpC2 metC7</i>	Fujita and Freese (5)
61656	$\Delta$ <i>igf</i> (formerly <i>fdpA1</i> ) <i>hisA1 leuA8 metB5 trpC2</i>	Fujita and Freese (5)
61668	<i>iol-6 trpC2 metC7</i>	60015 mutagenized with EMS <sup>b</sup>
61774	$\Delta$ <i>igf bfd-1 hisA1 leuA8 metB5 trpC2</i>	61656 mutagenized with EMS
62085	<i>bfd-1 glp<sup>c</sup> hisA1 leuA8 metB5 trpC2</i>	Tf <sup>d</sup> of 61774 by 60015
QB885	<i>sacA321 thiC5 purA16</i>	R. Dedonder via H. Saito
QB944 (= Kit1)	<i>purA16 cysA14 trpC2</i>	R. Dedonder via H. Saito
ISMRBE17	<i>hsrB<sup>+</sup> hsrE<sup>+</sup> hsrR<sup>+</sup> hsrM<sup>+</sup> purB6 leuA8 metB5</i>	T. Ando
YF026	<i>sacA321 ts199 purA16</i>	H. Yoshikawa via Y. Sadaie
YF029 (= LMAH)	<i>purA16 gnt-9 leuA8 metB5 hisA3</i>	H. Yoshikawa via H. Saito
YF030	<i>sacA321 <math>\Delta</math>igf</i>	Td <sup>e</sup> of YF026 by 61774
YF062	<i>bfd-1 fdp-74 glp hisA1 leuA8 metB5 trpC2</i>	Tf of ISMRBE17 by 61668
YF081	<i>fdp-74 hisA1 leuA8 metB5 trpC2</i>	Tf of 61656 by YF062
YF086	$\Delta$ <i>igf purA16 leuA8 trpC2</i>	Tf of 61656 by QB944
YF100	$\Delta$ <i>igf hsrE<sup>+</sup></i>	Td of YF030 by ISMRBE17
YF125	<i>iol-6 hsrB<sup>+</sup> hsrE<sup>+</sup> hsrR<sup>+</sup> hsrM<sup>+</sup> purB6 metB5</i>	Tf of ISMRBE17 by 61668
YF126	<i>fdp-74 purA16</i>	Td of QB885 by YF062
YF127	<i>gnt-4 trpC2 metC7</i>	60015 mutagenized with EMS
YF130	<i>iol-6 fdp-74 hisA1 metB5 trpC2</i>	Tf of YF081 by 61668
YF149	<i>fdp-74 gnt-4 hisA1 metB5 trpC2</i>	Tf of YF081 by YF127

<sup>a</sup> The nomenclature used to designate the various genetic loci is that described in reference 7, except for the abbreviations newly introduced to denote loci involved in the metabolism of inositol (*iol*) and gluconate (*gnt*) and in the activity of fructose-bisphosphatase (*fdp*) and its bypass (*bfd*) (see text for details).

<sup>b</sup> EMS, Ethyl methane sulfonate.

<sup>c</sup> *glp* was spontaneously introduced during isolation of strain 62085.

<sup>d</sup> Tf, Transformation of the first strain by DNA of the second.

<sup>e</sup> Td, Transduction of the first strain by PBS1 phage isolated from the second.

#### Rapid screening of fructose-bisphosphatase-deficient strains.

Since *fdp* strains do not show any defect in their growth properties due to the bypass of fructose-bisphosphatase, the strains tested previously had been assayed for fructose-bisphosphatase activity in their lysates. To avoid this laborious assay, we developed a rapid method using toluol-treated cells to screen *fdp* strains among transformants or transductants (this was based on the method of fructose-bisphosphatase assay described by Fujita and Freese [4]). Strains to be tested were streaked on tryptose blood agar base (Difco Laboratories) plates containing 10 mM glucose (about 30 strains per plate). After incubation at 37°C overnight, the grown cells of each strain were suspended by a platinum loop in 0.5 ml of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH adjusted to 8.0 with KOH), 5 mM MnCl<sub>2</sub>, 5 mM phosphoenolpyruvate, and 100 mM NaCl. Cold toluol (0.15 ml) was added to each cooled suspension, and the mixture was vigorously shaken for 30 s at 4°C. The suspension (0.35 ml) was mixed with 0.2 ml of 0.27 M Tris-chloride (pH 8.0)–5.5 mM NADP<sup>+</sup>–2.7 mM fructose-bisphosphate–9 U of glucose 6-P dehydrogenase (Sigma Chemical Co.) per ml–9 U of phosphoglucosomerase (Sigma) per ml–0.11 mg of phenazinemethosulfate per ml–2.7 mg of nitrobluetetrazolium per ml and was incubated for 3 h at 22°C in the dark. Toluol-treated cells having fructose-bisphosphatase could reduce tetrazolium, to give a blue precipitate, through sequential reactions converting fructose 6-P which was produced by fructose-bisphosphatase reaction.

**Isolation of mutants unable to grow on inositol or gluconate.** Our standard strain (strain 60015) was grown to an optical density at 600 nm of 0.5 in a

synthetic salt mixture (S<sub>6</sub>) (5) containing 25 mM glucose and 50 µg each of tryptophan and methionine per ml. An amount of cells corresponding to 10 optical density units (600 nm) was centrifuged (3,000 × g, 10 min) and resuspended in 10 ml of the same synthetic medium. Ethyl methane sulfonate (0.25 ml) was added to the cell suspension (0.2 M final concentration), which was then shaken at 37°C for 1.5 h. The cells were centrifuged, washed twice in the above-described medium containing 25 mM inositol or gluconate instead of glucose, and resuspended in 30 ml of the synthetic medium containing inositol or gluconate. The culture was shaken at 37°C for 3 h, penicillin G (Meijiiseika Co., Ltd., Tokyo) was added to give a concentration of 100 U/ml, and the culture was further incubated overnight. The cells were washed in N medium and resuspended in 5 ml of N medium plus 25% glycerol. The suspension was kept at –20°C and used for the isolation of mutants.

For the isolation of mutants unable to grow on inositol, a portion of the appropriately diluted suspension was spread on an indicator plate (1% polypeptone [Daigoeiyokagaku], 1% beef extract [Kyokuto Seiyakukogyo Co., Ltd., Tokyo], 0.5% NaCl, 1.5% agar, 50 µg of 2,3,5-triphenyltetrazolium chloride per ml, 25 mM inositol; pH adjusted to 7.2 with NaOH). Inositol-fermenting colonies on the plate become white because bacteria fermenting inositol produce acid and lower the pH where the biological reduction of tetrazolium is inhibited, whereas colonies unable to ferment inositol turn deep red (10). Deep-red colonies were tested for growth on N medium plus supplemented amino acids containing 25 mM glucose or inositol, and the colonies unable to grow only on inositol were

selected. In the case of isolation of mutants unable to grow on gluconate, the indicator plate did not work well. Hence, a portion of the diluted cell suspension was directly spread on N medium plus amino acids containing glucose. Colonies that appeared were tested for growth on gluconate.

**Preparation of cell extract and fructose-bisphosphatase assay.** Cells were grown at 37°C in Penassay broth (Difco) plus 10 mM glucose to an optical density of 1 at 600 nm. The harvested cells were washed and broken by lysozyme treatment as described previously (5).

Fructose-bisphosphatase was assayed as described previously (4, 5). The fructose 6-P produced was stoichiometrically converted to NADPH by sequential reactions of phosphoglucosomerase and glucose 6-P dehydrogenase (added to the reaction mixture); NADPH formation was followed by measuring the increase of the absorbance at 340 nm.

## RESULTS AND DISCUSSION

### Properties and mapping of the $\Delta igf$ mutation.

The  $\Delta igf$  (formerly called *fdpA1*) mutant has a pleiotropic nature which results not only in the absence of fructose-bisphosphatase but also in the inability to grow on inositol or gluconate as the sole carbon source (5). Attempts to isolate revertants with respect to any of these properties have failed. The fact that transformants able to grow on inositol are also cured of the other pleiotropic defects indicates that the  $\Delta igf$  mutation is a single mutation or is composed of several linked mutations. The transformation frequency of the  $\Delta igf$  marker seems to be extremely dependent on the size of DNA because only carefully prepared DNA has transforming activity. Furthermore, when DNA is irradiated with UV light, its transforming activity for  $\Delta igf^+$  is 2,500 times more sensitive than that for amino acid independence (*hisA1*, *metB5*, and *leuA8* markers [point mutations] were compared). Thus, a larger portion of intact DNA is necessary for transformation of the  $\Delta igf$  marker than for that of other markers which are point mutations. These results (6) suggested that the  $\Delta igf$  mutation may be a large deletion causing the pleiotropic defects.

The activity of PBS1 phage for transduction of  $\Delta igf$  was not as sensitive to UV irradiation as was transforming activity. PBS1 transducing particles were irradiated with UV light, and their residual transducing activities for  $\Delta igf^+$  and amino acid independence (*hisA1*, *metB5*, and *leuA8*) were examined with strain 61656 as the recipient. If the relative inactivation rate is taken as 1 for amino acid dependence markers, then that for the  $\Delta igf$  marker would be 2.5. This difference in UV sensitivity probably originated in the large deletion of the  $\Delta igf$  mutation, as discussed for arsenate-sensitive mutations by Adams and Oishi (1).

The mapping of the  $\Delta igf$  mutation was performed by PBS1 transduction. The tentative

map order, i.e., *sacA*, *thiC*,  $\Delta igf$  (*fdpA1*), *ts199*, *purA*, has been communicated (6). Recently, Ikawa et al. (9) reported that among the genes for site-specific restriction endonuclease of *B. subtilis* which had been inserted into a recipient chromosome through transformation, *hsrE* and *hsrB* loci encoding endo R · *Bsu12311* and endo R · *Bsu12471*, respectively, were located between *sacA* and *purA* in the order *sacA*, *hsrE*, *hsrB*, *purA*. To order  $\Delta igf$ , *hsrE*, and *hsrB*, two multiple-factor transduction crosses (crosses a and b) (Table 2) were carried out. Primary selection was for transductants able to grow in the absence of adenine. We could not use *hsrB* and *hsrE* as primary selective markers because the cells that survived on a plate where  $\phi 105C$  was spread did not always have the host-specific restriction activities. A PBS1 transduction using an *Hsr*<sup>+</sup> strain as the recipient did not work because of restriction. In cross b, all of the *hsrB*<sup>+</sup> transductants lost the  $\Delta igf$  marker, so either *hsrB* and  $\Delta igf$  are very close to each other or *hsrB* is located inside the  $\Delta igf$  deletion. The map order *hsrE*,  $\Delta igf$  (or *hsrB*), *purA* was most consistent with the data of these crosses (Table 2).

**Isolation of mutants deficient in each of the pleiotropic properties of the  $\Delta igf$  strain.** The pleiotropic nature of the  $\Delta igf$  strain may be attributed to a large deletion affecting the genes

TABLE 2. Multiple-factor PBS1 transduction crosses to order *hsrE*,  $\Delta igf$ , *hsrB*, and *purA*<sup>a</sup>

A. Cross a, donor strain YF100 ( <i>hsrE</i> <sup>+</sup> $\Delta igf$ ) and recipient strain YF029 ( <i>purA</i> )				
Phenotype <sup>b</sup>				
HsrE	$\Delta igf^c$	Pur	No. of recombinants	
0	0	1	67	
0	1	1	78	
1	1	1	78	
1	0	1	1	
			224 total	
B. Cross b, donor strain ISMRBE17 ( <i>hsrE</i> <sup>+</sup> <i>hsrB</i> <sup>+</sup> ) and recipient strain YF086 ( $\Delta igf$ <i>purA</i> )				
Phenotype				
HsrE	HsrB	$\Delta igf$	Pur	No. of recombinants
0	0	0	1	40
0	1	1	1	23
1	1	1	1	42
				105 total

<sup>a</sup> Phage PBS1 transduction was performed in a standard assay as described in the text. In these crosses, *purA* was a selective marker. Implied order: *hsrE*,  $\Delta igf$  (or *hsrB*), *purA*.

<sup>b</sup> One and zero indicate donor and recipient phenotypes, respectively.

<sup>c</sup> Inability to grow on inositol was used as the determinant of the  $\Delta igf$  marker.

of fructose-bisphosphatase and of enzymes involved in gluconate and inositol metabolism. To test this hypothesis, we isolated mutants which are defective in each of the pleiotropic properties and examined whether these mutations are located in the region of the deletion.

At first, we isolated mutants which could grow on gluconate but not on inositol and had the normal level of fructose-bisphosphatase activity. Fourteen mutants of this kind were isolated from our standard strain (strain 60015) which had been treated with ethyl methane sulfonate. The gene symbol *iol* was given to these mutants. Strain 61668 (*iol-6*) was used to genetically analyze the  $\Delta igf$  deletion.

Only one mutant in which only utilization of gluconate as carbon source was affected was isolated from strain 60015. A genetic marker used for the mutant unable to grow on gluconate was given the gene symbol *gnt* in agreement with the symbol used for such mutations in *E. coli* (3). In the course of mapping experiments, we unexpectedly found that strain YF029 (= strain LMAH) had another *gnt* mutation that was called *gnt-9*. The mutant isolated, strain YF127 (*gnt-4*), was used for genetic analysis of the deletion mutation.

Mutants lacking only fructose-bisphosphatase were isolated (details will be published elsewhere), for which the gene symbol *fdp* was used in agreement with the symbol for such mutations in *E. coli* (3). Since *B. subtilis* can bypass fructose-bisphosphatase (5), an *fdp* mutant can grow on a gluconeogenic carbon source such as glycerol or malate unless a bypass mutation (which was given the gene symbol *bfd*) is introduced in addition to an *fdp* mutation. Thus, double mutants having *fdp* and *bfd* were first isolated from a mutagenized *bfd* strain by screening mutants unable to grow on a gluconeogenic carbon source. When these mutants were assayed for fructose-bisphosphatase, only two strains were found to be deficient in it. An *fdp* mutation was purified from one of them, strain YF062 (*bfd-1 fdp-74*), by PBS1 transduction and congression. The *fdp-74* strains obtained could grow on any carbon source, including L-malate, glycerol, gluconate, and inositol. (Hence, the *fdp* marker by itself could not be used for selection.) The *fdp-74* mutation was further analyzed genetically.

**Presence of *iol-6*, *gnt-4*, *fdp-74*, and *hsrB* mutations inside the  $\Delta igf$  deletion.** On the assumption that the  $\Delta igf$  strain was deleted for genes of fructose-bisphosphatase and enzymes involved in inositol and gluconate metabolism, we investigated whether DNA of the  $\Delta igf$  strains could transform *iol-6*, *gnt-4*, and *fdp-74* strains to the wild type. *iol-6* and *gnt-4* mutants (strains 61668 and YF127) were not transformed to the wild

type with DNA of strain 61656 ( $\Delta igf$ ), whereas they were transformed with DNA of strain ISMRBE17 ( $\Delta igf^+$ ). The results suggested that *iol-6* and *gnt-4* mutations are located inside the deletion. As discussed above, the *fdp* mutation by itself cannot be used as selective marker unless both the DNA donor and the recipient have *bfd* mutations. Thus, we used *bfd* recipients and donors to select transformants directly for growth on Casamino Acids as a gluconeogenic carbon source. Strain YF062 (*fdp-74 bfd-1*) was not transformed to grow on Casamino Acids with DNA of strain 61774 ( $\Delta igf bfd-1$ ), whereas it was transformed with DNA of strain 62085 ( $\Delta igf^+ bfd-1$ ). Furthermore, strain 61656 ( $\Delta igf bfd^+$ ) was transformed with DNA of strain YF062 (*fdp-74 bfd-1*) to obtain transformants able to grow on inositol as a carbon source. (*Iol*<sup>+</sup> transformants lacking fructose-bisphosphatase might be obtained because recipient strain 61656 contains a fructose-bisphosphatase bypass.) All of the transformants (40 assayed) were deficient in fructose-bisphosphatase, which indicated that DNA fragments recombined to cure the  $\Delta igf$  deletion should contain the *fdp-74* mutation. From these results, we concluded that the *fdp-74* mutation is also located inside the deletion.

*hsrB* could not be separated from  $\Delta igf$  in the PBS1 transduction cross (Table 2). To examine the possibility that *hsrB* is located inside the  $\Delta igf$  deletion, a transformation using strain 61656 ( $\Delta igf$ ) as the recipient and strain ISMRBE17 (*hsrB*<sup>+</sup> *hsrE*<sup>+</sup>) as the donor was performed to obtain  $\Delta igf^+$  transformants able to grow on

TABLE 3. Three-factor transduction crosses to order *iol-6*, *gnt-4*, *fdp-74*, and *purA*<sup>a</sup>

A. Cross c, donor strain YF130 ( <i>fdp-74 iol-6</i> ) and recipient strain YF029 ( <i>purA</i> )			
Phenotype <sup>b</sup>			No. of recombinants
<i>iol</i>	<i>Fdp</i>	<i>Pur</i>	
0	0	1	50
0	1	1	33
1	1	1	65
			148 total
B. Cross d, donor strain YF130 ( <i>fdp-74 iol-6</i> ) and recipient strain YF127 ( <i>gnt-4</i> )			
Phenotype			No. of recombinants
<i>iol</i>	<i>Gnt</i>	<i>Fdp</i>	
0	1	0	2
1	1	0	10
0	1	1	26
1	1	1	60
			98 total

<sup>a</sup> *purA*<sup>+</sup> (cross c) and *gnt-4*<sup>+</sup> (cross d) transductants were primarily selected. Implied order: *iol-6*, *gnt-4*, *fdp-74*, *purA*.

<sup>b</sup> One and zero indicate donor and recipient phenotypes, respectively.

TABLE 4. Multiple-factor transduction crosses to further order *hsrB* and *hsrE*<sup>a</sup>

A. Cross e, donor strain ISMRBE17 ( <i>hsrB</i> <sup>+</sup> <i>hsrE</i> <sup>+</sup> ) and recipient strain YF029 ( <i>purA</i> )				
Phenotype <sup>b</sup>				No. of recombinants
HsrE	HsrB	Pur		
0	0	1		92
0	1	1		48
1	1	1		23
				163 total

B. Cross f, donor strain YF125 (*iol-6 hsrB*<sup>+</sup> *hsrE*<sup>+</sup>) and recipient strain YF029 (*purA*)

Phenotype				
HsrE	Iol	HsrB	Pur	No. of recombinants
0	0	0	1	64
0	0	1	1	4
0	1	1	1	9
1	1	1	1	22
				99 total

C. Cross g, donor strain ISMRBE17 (*hsrB*<sup>+</sup> *hsrE*<sup>+</sup>) and recipient strain YF127 (*gnt-4*)

Phenotype				No. of recombinants
HsrE	Gnt	HsrB		
0	1	0		18
1	1	0		3
0	1	1		68
1	1	1		29
				118 total

D. Cross h, donor strain ISMRBE17 (*hsrB*<sup>+</sup> *hsrE*<sup>+</sup>) and recipient strain YF126 (*fdp-74 purA*)

Phenotype				
HsrE	Fdp	HsrE	Pur	No. of recombinants
0	0	0	1	77
0	1	1	1	41
1	1	1	1	16
				134 total

<sup>a</sup> *purA*<sup>+</sup> (crosses e, f, and h) and *gnt-4*<sup>+</sup> (cross g) transductants were primarily selected. Implied order: *hsrE*, *iol-6*, *gnt-4*, *fdp-74* (or *hsrB*), *purA*.

<sup>b</sup> One and zero indicate donor and recipient phenotypes, respectively.

inositol, which were then tested for *hsrB* and *hsrE* markers. All of the transformants (200 tested) were *hsrB*<sup>+</sup>, whereas only 21 transformants (of 200) were *hsrE*<sup>+</sup>. The fact that not only the transduction cross but also the transformation cross did not separate *hsrB* and  $\Delta igf$  strongly suggested that *hsrB* is in the deleted region.

**Fine mapping of the deleted region of the  $\Delta igf$  mutation.** It was shown that *iol-6*, *gnt-4*, *fdp-74*, and *hsrB* were within the  $\Delta igf$  deletion. To further analyze the deleted region, *iol-6*, *gnt-4*, and *fdp-74* were mapped by transduction (Table 3). The results of three-factor cross c revealed the order *iol-6*, *fdp-74*, *purA*. The map order *iol-6*, *gnt-4*, *fdp-74* was established by cross d. We

TABLE 5. Multiple-factor transformation cross to order *gnt-4*, *fdp-74*, and *hsrB*<sup>a</sup>

Phenotype <sup>b</sup>				No. of recombinants
HsrE	Gnt	Fdp	HsrB	
0	1	0	0	179
0	1	1	0	9
0	1	1	1	12

<sup>a</sup> Transformation was carried out in a standard assay as described in the text (DNA donor, strain ISMRBE17 [*hsrB*<sup>+</sup> *hsrE*<sup>+</sup>]; recipient, strain YF149 [*fdp-74 gnt-4*]). In this transformation, the probability of the congression of the unlinked markers was less than 1% (2 *hisA1*<sup>+</sup> and 1 *trpC2*<sup>+</sup> cells of 200 *gnt-4*<sup>+</sup> transformants analyzed).

<sup>b</sup> One and zero indicate donor and recipient phenotypes, respectively.

could not use the *iol-6* mutation as a primary selective marker because many revertants appeared on selection plates enriched with yeast extract with an *iol-6* strain as the recipient.

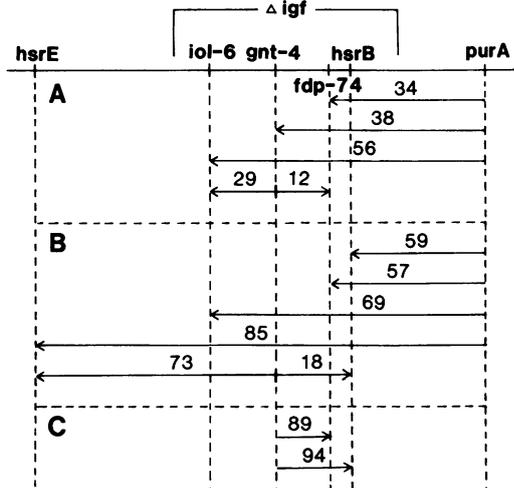


FIG. 1. Genetic map of loci within the  $\Delta igf$  deletion on the *B. subtilis* 168 chromosome by phage PBS1 transduction and DNA transformation. The recombination values were calculated as 100 minus percent cotransduction (or cotransformation). The values in panels A and B were calculated from the crosses shown in Tables 3 and 4, respectively. The variation in values was less than 10% when several independent experiments were compared. Because the values between *hsrE* (or *hsrB*) and *purA* that were obtained from the results in Table 2 decreased due to the presence of the  $\Delta igf$  deletion, these results were not included for calculation of the values in panel B. The values between the corresponding markers shown in panel B are significantly larger than those in panel A. This phenomenon was often observed in transduction crosses with *Hsr*<sup>+</sup> strains as donors. The values in panel C were calculated from the data in Table 5. Arrows point from the selected to the unselected marker in crosses. The  $\Delta igf$  mutation deleted loci of *iol-6*, *gnt-4*, *fdp-74*, and *hsrB*.

These crosses established the order *iol-6*, *gnt-4*, *fdp-74*, *purA*.

To locate *hsrB* and *hsrE*, further multiple-factor crosses were carried out (Table 4). The map orders *hsrE*, *hsrB*, *purA*; *hsrE*, *iol-6*, *hsrB*, *purA*; and *hsrE*, *gnt-4*, *hsrB* were obtained from crosses e, f, and g, respectively. Cross h revealed the order *hsrE*, *fdp-74* (or *hsrB*), *purA* (the *fdp-74* and *hsrB* markers were too close to each other to be separated). The transduction crosses established the order *hsrE*, *iol-6*, *gnt-4*, *fdp-74* (or *hsrB*), *purA*.

We could not order *fdp-74* and *hsrB*, which were located between *gnt-4* and *purA*. The transformation cross shown in Table 5 (because *fdp-74*, *hsrB*, and *hsrE* were inadequate as primary selective markers, *gnt-4* was used as a selective marker) revealed the order *gnt-4*, *fdp-74*, *hsrB*. Linkage between *hsrE* and *gnt-4* was not observed in this cross.

Figure 1 summarizes the locations of *hsrE*, *iol-6*, *gnt-4*, *fdp-74*, *hsrB*, and *purA*. To our knowledge, this paper is the first to describe a large deletion of *B. subtilis* in which various loci were analyzed. Primitive mapping experiments revealed that all of the *iol* and *gnt* mutations were located near *iol-6* and *gnt-4*, respectively (Fujita and Fujita, unpublished data). These results apparently indicate that genes involved in inositol and gluconate metabolism form two separate gene clusters near *iol-6* and *gnt-4*, respectively.

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