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Cell Lysis of *Bacillus subtilis* Caused by Intracellular Accumulation of Glucose-1-Phosphate

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Mutants deficient in both glucose-6-phosphate dehydrogenase and phosphoglucose isomerase lysed 4 to 5 h after growth in nutrient medium containing glucose, or after prolonged incubation if the medium contained galactose. The lysis could be prevented by the addition of any other rapidly metabolizable carbon source such as fructose, glucosamine, or glycerol. The glucose-induced lysis was also abolished by introduction of a third mutation lacking phosphoglucose mutase activity but not by a third mutation lacking uridine diphosphate-glucose pyrophosphorylase or teichoic acid glucosyl transferase activity. Galactose-induced lysis was prevented only if the additional mutation abolished the uridine diphosphate-glucose pyrophosphorylase activity. The results showed that lysis was caused by the intracellular accumulation of glucose-1-phosphate, which in turn inhibited at least one of the two enzymes that convert glucosamine-6-phosphate to *N*-acetyl glucosamine-6-phosphate.

Glucose (Glc) is not only a good carbon source for the growth of *Bacillus subtilis*, but it also affects several physiological processes. For example, it suppresses sporulation (12, 14, 15, 16, 21, 36), initiates germination (23, 24, 34, 43), induces Glc-phosphoenolpyruvate phosphotransferase (14), and represses isocitrate dehydrogenase (10, 42) and the citrate transport system (44). To determine which metabolites of Glc cause some of these physiological effects, we have isolated mutants that are deficient in one or more of the enzymes of Glc-6-phosphate metabolism. With one of these mutants, it was shown earlier in this laboratory that Glc-6-P accumulation suppresses sporulation (15). In the course of these examinations, we observed that certain mutants lysed when they were grown in the presence of Glc, whereas others did not. It will be shown below that the lysis is caused by the intracellular accumulation of Glc-1-phosphate (Glc-1-P), which in turn inhibits the conversion of glucosamine-6-P into mucopeptides.

MATERIALS AND METHODS

Bacteria and phages. All bacterial strains (Table 1) were derived from the transformable strain 60015, which requires L-tryptophan and L-methionine for growth (see Results for nomenclature). The isolation of mutants carrying additional mutations lacking phosphoglucose isomerase [P-Glc-isomerase, (i) strain 61158] and phosphoglucomutase [P-Glc-mutase, (m) strain 61370] activities was described earlier (15). The mutant lacking uridine diphosphate (UDP)-glucose pyrophosphorylase activity [UDP-Glc-pyrophosphorylase, (p) strain 61150] was derived as a spontaneous phage SP8-resistant mutant of strain 60015. A (t) mutant (61145) lacking teichoic acid glucosyl transferase (TAG-transferase) was kindly given to us by Frank Young.

Whereas the (m) strain 61370 had been obtained by transforming the P-Glc-mutase deficiency from 61154 back into 60015, the (i) strain 61158 was a direct mutant of strain 60015 without genetic purification. After strain 61158 had been used to produce several double and triple mutants, it was found to lack the ability to grow on fructose as sole carbon source. This separate mutation, which was also found in the strains derived from 61158, was eliminated by transformation with deoxyribonucleic acid (DNA) of the standard strain (60015) and selection for growth on fructose. The experiments were then repeated for several of the derived strains to ensure that the conclusions derived in this paper were not influenced by the additional mutation (Table 1). The results with the purified mutants will be given where relevant.

The (di) mutant 61324, deficient in both P-Glc-isomerase and Glc-6-P dehydrogenase, was derived from the (i) mutant 61158, as described elsewhere (Lin and Prasad, manuscript in preparation), by isolation of colonies unable to grow on a substrate of N plus Glc. The fructose-related deficiency also contained in the mutant was transformed out, producing strain 61375. The activities of different enzymes in the

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TABLE 1. *Abbreviations and nomenclature of mutants*

Strain	Genotype[a]	Abbreviated enzyme defect[b]	Derived by	Parent	Information donor (if any) or obtained from	References
60015	*metC trpC*	(+)	Ultraviolet light	168-strain	Nester (SB26)	
61158	*fru metC trpC pgi*	(i)	Spontaneous	60015		15
61372	*metC pgi trpC*	(i)	Transformation	61158	60015	
61154	*glpK gtaC metC trpC*	(m)	Spontaneous	60015		15
61370	*gtaC metC trpC*	(m)	Transformation	61154	60015	
61150	*gtaD metC trpC*	(p)	Spontaneous	60015		
61145	*gtaA trpC*	(t)	Ultraviolet light	168-strain	Young (BY12)	47
61324	*fru gpd metC pgi trpC*	(di)	Ethyl methane sulfonate	61158		
61375	*gpd metC pgi trpC*	(di)	Transformation	61324	60015	
61371	*fru gpd metC gtaC trpC*	(dm)	Transformation	61364	60015	
61364	*fru gpd metC gtaC pgi trpC*	(dim)	Transformation	61324	61154	
61445	*fru gpd gtaD metC pgi trpC*	(dip)	Transformation	61324	61150	
61368	*fru gpd gtaA metC pgi trpC*	(dit)	Transformation	61324	61145	

[a] The genotype nomenclature agrees with that used by Young and Wilson (48). Additional abbreviations: *fru*, unable to grow on fructose; *glpK*, lacks glycerokinase; *gpd*, deficient in Glc-6-P-dehydrogenase; *gtaD*, lacks UDP-Glc-pyrophosphorylase (gene may be identical to that of *gtaB*); *pgi*, deficient in P-Glc-isomerase.

[b] The abbrevations of enzyme defects refer to: (d), Glc-6-P-dehydrogenase; (i), P-Glc-isomerase; (m), P-Glc mutase; (P), UDP-Glc pyrophosphorylase; (t), teichoic acid glucosyl transferase.

standard strain 60015 and the (d) and (di) mutants are compared in Table 2.

The (dim) strain 61364 was isolated by transforming the P-Glc-mutase-minus property of (m) strain 61370 into the (di) strain 61324; the bacteria were plated on TBAB (see below) and later overlaid with soft agar containing 10^8 SP8 phage. Phage-resistant colonies were purified, tested for their growth on agar containing N and Glc, and examined for their enzymes (Table 3). The (dip) strain 61445 was similarly derived from the (di) strain by transforming it with DNA of the (p) mutant 61150. The (dit) mutant 61368 was derived by transforming the (di) mutant 61375 with DNA of the (t) strain 61145. Colonies resistant to phage SP8 were isolated and assayed for the presence of Glc-6-P dehydrogenase and P-Glc-isomerase. Without DNA addition, no phage-resistant colonies were found.

All mutants were preserved in phosphate magnesium buffer plus 25% glycerol at −70 C for long-term storage and at −30 C for daily use.

Phage SP8 was obtained from W. R Romig and kept inside spores of strain 60015. A purified protease-free phage preparation was kindly provided by N. L. Richards of this laboratory.

Media. TBAB plates contained 33 g of tryptose blood agar base (Difco) per liter. Soft agar contained 1% tryptone (Difco) and 0.7% agar. The phosphate-buffered (pH 6.5) nutrient sporulation medium, described previously (11), was supplemented with 25 μg of L-tryptophan per ml and 10 μg of L-methionine per ml (NSMP medium).

Phosphate magnesium buffer contained (per liter): K_2HPO_4, 14 g; KH_2PO_4, 6 g; and $MgSO_4 \cdot 7\ H_2O$, 0.25 g. Minimal salts medium N consisted of phosphate magnesium buffer supplemented with 2.0 mg of ammonium sulfate, 10^{-6} M $FeCl_3$, 10 μg of L-methionine, per ml, and 25 μg of L-tryptophan per ml; for growth experiments, 5 mg of a carbon source (adjusted to pH 7.0 by KOH or HCl where necessary) per ml were added. Transformation media have been described by Anagnostopoulos et al. (1).

Growth conditions. Bacteria, grown overnight at 37 C on TBAB plates, were inoculated into NSMP medium (plus 5 mg of any desired carbon source per ml) at an initial absorbancy for a 1-cm path at 600 nm (A_{600}) of 0.1 to 0.2 (except where stated otherwise) and shaken on a reciprocal water bath-shaker at 37 C and 120 strokes per min. The bacterial culture occupied 10% or less of the volume of the culture flask. For removal of samples, the shaker was stopped for less than 10 s. The A_{600} was measured in a Gilford spectrophotometer after the culture was diluted in phosphate magnesium buffer to an A_{600} of less than 0.8.

Transformation. DNA was prepared by the method of Massie et al. (32), and transformation experiments were carried out by the method of Anagnostopoulos et al. (1).

Chemical assays. Protein was determined by the method of Lowry et al. (30), with bovine serum albumin fraction V (Armour Pharmaceutical Co., Kankakee, Ill.) as the standard. The Glc-6-P concentration was measured in both the bacterial pool and the extracellular medium as follows. A 2-ml volume of culture was filtered through a membrane filter (pore size 0.45 μm, diameter 25 mm; Gelman Instrument Co., Ann Arbor, Mich.) and washed with 10 ml of phosphate-magnesium buffer at room temperature. The filter was immediately extracted with 2 ml of

boiling water for 5 min, and the debris was removed by centrifugation. The filtrate was also incubated for 5 min in boiling water. The assay mixture (1.0 ml) contained 50 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) adjusted with KOH to pH 8.0, 10 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), and 50 to 200 μliters of sample. The A_{340} was measured, 1.5 to 3.0 IU of Glc-6-P dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) was added, and the increase in absorbance was recorded. The concentration of Glc-6-P was calculated by using the molar extinction coefficient of NADPH, 6,220 (22). The addition of phosphate buffer, equivalent to the amount contained in 200 μliters of NSMP, did not inhibit the reaction.

Intracellular Glc-1-P was assayed as follows. A 30-ml volume of the culture (of A_{600} = 0.8 to 1, see Table 4) was filtered through a membrane filter (0.45-μm pore size; Millipore Corp.), and the cells were washed with 10 ml of phosphate magnesium buffer. The filter was transferred to a tube containing 4 ml of ice-cold water, 200 μliters of 5 M perchloric acid was added, and the tube was kept on ice for 20 min with occasional shaking; 600 μliters of saturated $KHCO_3$ was slowly added. After 10 min of incubation on ice, the tube was centrifuged and the supernatant was assayed for both Glc-6-P and Glc-1-P, as described above, except that 1.5 to 3.0 IU of P-Glc-mutase was also added for the Glc-1-P determination.

Enzyme assays. Bacteria were grown in NSMP to an A_{600} of 1.0 to 1.2; enzyme production was arrested by the addition of 100 μg of chloramphenicol per ml; and the cells were harvested by centrifugation and washed with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 8.0) containing 100 μg of chloramphenicol per ml. For assays of P-fructokinase, Glc-6-P dehydrogenase, P-Glc-isomerase, P-Glc-mutase, 6-P-gluconate dehydrogenase, transaldolase, and transketolase, the cells were suspended in this buffer at an A_{600} of 40 and lysed by lysozyme (100 μg/ml) treatment for 30 min at 37 C. For the assay of fructose-6-P amidotransferase and UDP-Glc-pyrophosphorylase, the cells were passed through a small French pressure cell (no. 4-3399, American Instrument Co., Inc., Silver Spring, Md.). To stabilize the UDP-Glc-pyrophosphorylase activity, the cells were first suspended, before being broken, in a buffer containing 10 mM imidazole-hydrochloride (pH 7.0), 10 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid (EDTA pH 7.0), and 10% (vol/vol) glycerol. The cell extracts were centrifuged for 20 min at 35,000 × *g*, and the supernatants (crude extract) were removed carefully and kept on ice until they were assayed on the same day.

All enzymes coupled to the reduction of NADP were assayed at 25 C by recording the change of A_{340}. For Glc-6-P dehydrogenase the reaction mixture contained: 50 mM K-TES (pH 8.0), 0.3 mM $NADP^+$, 3 mM $MgCl_2$, 5 mM Glc-6-P, and 10 to 50 μliters of cell extract (30 to 150 μg of protein). For P-Glc-isomerase the reaction mixture contained the same as above, but instead of Glc-6-P it contained 2 mM fructose-6-P and 1.5 to 3.0 IU of Glc-6-P dehydrogenase. Phosphoglucomutase was measured by the method of Joshi and Handler (26); the reaction mixture contained 100 mM glycine-NaOH buffer (pH 9.0), 20 mM L-cysteine (freshly neutralized), 0.3 mM $NADP^+$, 2 mM $MgCl_2$, 0.1 mM Glc-1,6-diphosphate, 1.5 to 3.0 IU of Glc-6-P dehydrogenase, 4 mM Glc-1-P, and 100 μliters of crude extract. (A 10-min preincubation period at 25 C before the addition of Glc-1-P was essential.) At pH 7.6, at which the mammalian enzyme is usually assayed, the specific activity was five to ten times lower than at pH 9.0.

The activity of UDP-glucose pyrophosphorylase was assayed in both directions. For the reverse reaction, the assay mixture (1.0 ml) contained 50 mM glycine-NaOH (pH 8.5), 0.5 mM 2-mercaptoethanol, 2.0 mM $MgCl_2$, 0.2 mM $NADP^+$, 0.4 mM UDP-glucose, 10 mM sodium pyrophosphate, 1.5 to 3.0 IU each of Glc-6-P dehydrogenase and P-Glc-mutase, and 50 μliters of crude extract. The reaction was initiated by addition of pyrophosphate, and the increase of A_{340} was recorded. For the forward reaction the assay mixture (100 μliters) contained 50 mM glycine-NaOH (pH 8.5), 10 mM $MgCl_2$, 2 mM uridine triphosphate, 0.015 mM [U-^{14}C]Glc-1-P (10 μCi/μmol), and 10 μliters of crude extract. The reaction was run for 15 min at 37 C in a glass tube (10 by 75 mm) and stopped by immersing the tube in boiling water for 2 min. Control tubes were prepared in the same way and were boiled at zero time. After chilling the tubes for 5 min in ice, 45 μliters of 1 M Tris-chloride (pH 8.0) and 5 μliters of alkaline phosphatase (2 to 3 U, Worthington Biochemical Corp., Freehold, N.J.) were added. The tube was incubated for 40 min at 37 C and then centrifuged at 3,000 × *g* at 25 C. The supernatant was collected and stored in ice. For chromatography, diethylaminoethyl (DEAE)-cellulose (DE-81; 2 by 20 cm) paper strips with graphite pencil lines drawn at 7, 11, and 17 cm from one end of the strip were purchased from H. Reeve Angel & Co., Inc., Clifton, N.J. A 100-μliter volume of the alkaline phosphatase-treated reaction mixture was added to the paper strips within the area marked by the lines at 7 and 11 cm. The strips were dried at room temperature and then developed by descending chromatography with glass-distilled water until the water reached the 17-cm mark. Under these conditions, UDP-Glc stayed at the origin, whereas Glc (derived from unreacted Glc-1-P by the action of alkaline phosphatase) moved with the solvent front. After air-drying, the portion between 7 and 11 cm was placed in a scintillation vial with 10 ml of scintillation fluid (14) and counted.

Fructose-6-P amidotransferase was assayed by the method of Ghosh and Roseman (19) by using an incubation period of 30 min at 37 C.

Conversion of glucosamine-6-phosphate to N-acetyl glucosamine-1-phosphate. Bacteria were grown in a medium containing NSMP plus 2 mg of Glc per ml to an A_{600} of 0.9 to 1.0, harvested, washed and suspended in 50 mM Tris-chloride (pH 8.0) to a final A_{600} of 33, and passed through a small French pressure cell. Unbroken cells were removed by centrifugation at 1,900 × *g* for 6 min, and the crude extract containing cell debris was dialyzed for 4 to 5 h at 4 C against 1,000-times the volume of the mixture containing 10 mM Tris-chloride (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM 2-mercaptoethanol. The

assay mixture (500 μliters) for *N*-acetyl-glucosamine-1-phosphate formation contained 100 mM Tris-chloride (pH 8.0, 1 mM D-glucosamine-6-P, 1 mM acetyl coenzyme A, 2 mM $MgCl_2$, 8 mM 2-mercaptoethanol, 0.02 mM Glc-1,6-diphosphate, and 100 μliters of the dialyzed extract. After incubation for 30 min at 37 C in a glass tube (10 by 75 mm), the reaction was stopped by the addition of 25 μliters of 1.5 N HCl. The tube was immersed for 10 min in boiling water (which removes phosphates from *N*-acetyl-glucosamine-1-P) and chilled in ice, and then 25 μliters of 40% (wt/vol) trichloroacetic acid was added. The mixture was spun for 8 min at 3,000 × *g*, and the amount of acetylated amino sugar in the supernatant was determined by the method of Bates and Pasternak (3).

Measurement of antibiotic sensitivity. A 100-ml volume of the (di) mutant was grown in NSMP or in NSMP plus 5 mg of Glc per ml. At different times, samples containing a total of 5 to 6 A_{600} units of cells were washed by centrifugation with NSMP at room temperature, resuspended in 10 ml of NSMP (at 37 C), and transferred to 125-ml flasks which contained 1 μg of staphcillin per ml or 0.5 μg of vancomycin per ml. The flasks were shaken in a water bath-shaker at 37 C, and the A_{600} was followed.

Preparation of cell wall and measurement of its autolysis. Cells were grown in NSMP or in NSMP plus 5 mg of Glc per ml and harvested at an A_{600} of 0.8 to 0.9. Cell walls were prepared by the method of Young et al. (49). Their autolysis was measured at 38 C as described by Young (46). The assay mixture (1.0 ml) contained 25 mM $NaHCO_3$ buffer (pH 9.6), 100 mM KCl, and enough cell wall suspension to give an initial A_{600} of 0.8 to 1.0. The reduction of A_{600} was recorded at 38 C.

Measurement of ATP synthesis and the incorporation of [^{14}C]thymine into the trichloroacetic acid-precipitable fraction. Adenosine triphosphate (ATP) was measured as described earlier (28). The incorporation of [^{14}C]thymine into the trichloroacetic acid-precipitable fraction was measured as described by Rana and Halvorson (35).

Microscope observations. For phase-contrast microscopy at 2,000-fold magnification, a portion of the culture was mixed with an equal volume of a solution containing 20% Ficoll and 2% carboxymethyl cellulose, and the mixture was applied to a slide.

Chemicals. All chemicals were reagent quality. All biochemicals and enzymes were products of either Sigma Chemical Co. or Boehringer Mannheim. Vancomycin was a gift from Eli Lilly & Co., Indianapolis, Ind.

RESULTS

Nomenclature and enzyme activities of mutants. The isolation of the different mutants used in this paper (Table 1) is described above. To allow an easy description of single, double, and triple mutants, the deficiencies in enzyme activities relevant to this paper will be abbreviated by (d), (i), (m), (p), and (t), as defined in Table 1 and illustrated in Fig. 1. The standard (+) genotype will not be mentioned.

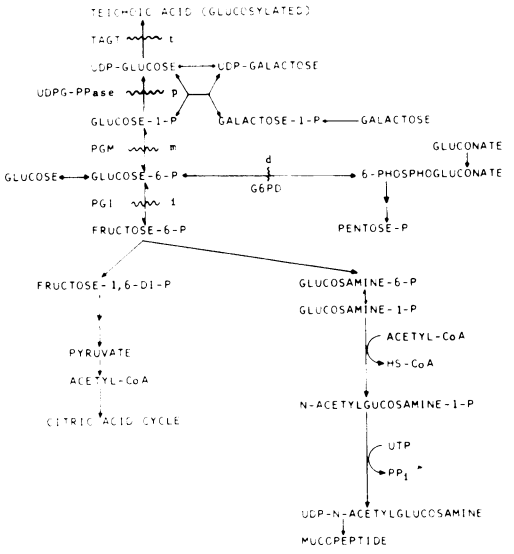

FIG. 1. *Location of mutations on the biochemical chart. Abbreviations: G6PD, Glc-6-P-dehydrogenase [mutation (d)]; PGI, P-Glc-isomerase [mutation (i)]; PGM, P-Glc-mutase [mutation (m)]; UDPG-PPase, UDP-Glc-pyrophosphorylase [mutation (p)]; TAGT, teichoic acid glucosyl transferase [mutation (t)].*

Tables 2 and 3 show the activities of different enzymes for the standard strain (60015) and for different mutants. Whereas in the appropriate mutants containing (m), (p), or (i) mutations the P-Glc-isomerase, P-Glc-mutase, and UDP-Glc-pyrophosphorylase activities were reduced more than 50-fold compared to the strains in which these activities were normal, the Glc-6-P dehydrogenase activitty of the (d) mutation was reduced only fivefold if 5 mM Glc-6-P was used as substrate. The properties of normal and mutant Glc-6-P dehydrogenase were therefore compared with crude enzyme extracts. At 5 mM Glc-6-P, both enzyme activities exhibited the same pH dependence: in 50 mM Tis-chloride buffer the pH optimum was 8.0; in 50 mM K-TES buffer the activity continued to increase up to pH 9.0; in 50 mM K-phosphate buffer the activity was reduced by 30% or more for all pH values between 5.5 and 7.5. But the K_m values of the two enzymes for Glc-6-P observed in K-TES buffer (pH 8) differed significantly: the K_m of the Glc-6-P dehydrogenase mutant enzyme was 35 mM, 175 times higher than the K_m of 0.2 mM observed for the standard enzyme.

Glucose-induced spheroplast formation and cell lysis of (di) mutants. The addition of Glc to NSMP medium increased the final A_{600} to which the standard strain or the (i), (m), or (p) strains grew. In contrast, (di) strains grew to a lower A_{600} in the presence of Glc than in its

absence and then lysed (Fig. 2). After the maximal A_{600} had been reached, the titer of colony-forming units decreased more rapidly than the A_{600}, indicating that many cells retained their ability to scatter light longer than their ability to grow after plating (Fig. 3). In fact, many cells showed protuberances or spheroplast formation, as could be seen when they were mixed with Ficoll and carboxymethylcellulose (used to protect them from lysis and to reduce motility) and inspected under the phase-contrast microscope (Fig. 3). No such effects were observed for the standard strain or the (i) or (m) mutants.

The spheroplast formation and cell lysis indicated that the (di) mutant grown in NSMP plus Glc experienced some alteration in its cell wall. This was also demonstrated by the higher susceptibility of the cells to staphcillin or vancomycin, both of which are known to inhibit cell wall synthesis (4, 40). Figure 4 shows an immediate onset of cell lysis when the (di) mutant was grown in NSMP plus Glc to an A_{600} of 0.65 and then one of the antibiotics was added. In contrast, when the strain was grown in NSMP alone, growth continued for about 30 min before lysis began. The susceptibility to the antibiotics increased during growth in NSMP plus Glc from almost no effect within the first 2 h after inoculation to a maximal effect shortly before

TABLE 2. *Enzyme activities of strains 61158 and 61324*

Name of enzyme	Classification no.	Sp act[a] (nmols/min/mg of protein)				
		Standard 60015	Strains (i)		Strains (di)	
			61158	61372 (purified)[b]	61324	61375 (purified)
Glucose-6-phosphate dehydrogenase	EC 1.1.1.49	120	120	108	20	30
6-Phosphogluconate dehydrogenase	EC 1.1.1.43	80	65	ND[c]	75	ND
UDP-glucose pyrophosphorylase	EC 2.7.7.9	7.2	ND	ND	5.8	ND
Phosphoglucosemutase	EC 2.7.5.1	26	22	22	25	28
Phosphoglucose isomerase	EC 5.3.1.9	600	5	2	8	2
Transaldolase	EC 2.2.1.2	25	26	ND	26	ND
Transketolase	EC 2.2.1.1	20	16	ND	16	ND
Phosphofructokinase	EC 2.7.1.11	60	43	ND	43	ND

[a] Extracts of cells grown in NSMP were assayed as described in Materials and Methods.
[b] Purified refers to the genetic removal of a fructose deficiency described in Materials and Methods.
[c] ND, Not determined.

TABLE 3. *Specific activities of different enzymes in mutants defective in glucose-6-phosphate metabolism*

Strain	Abbreviated enzyme defect[a]	Sp act[b] (nmol/min/mg of protein)				
		G6P-dehydrogenase (G6PD)	Phospho-Glc isomerase	Phospho-Glc mutase	UDP-Glc pyrophosphorylase	
					Reverse	Forward
60015		120	544	27	7.8	3.4
61158	(i)	94	5	20	ND	ND
61372	(i)	108	2	22	ND	ND
61370	(m)	95	398	<0.1	ND	ND
61150	(p)	105	402	16	<0.5	<0.01
61324	(di)	19	7	25	ND	ND
61375	(di)	30	2	28	5.8	3.8
61371	(dm)	22	605	<0.1	ND	ND
61364	(dim)	16	11	<0.1	ND	ND
61445	(dip)	20	10	18	<0.5	<0.01
61368	(dit)	26	2	28	ND	ND

[a] See footnote to Table 1 for details.
[b] Cells were grown in NSMP, and extracts were prepared and assayed as described in Materials and Methods.

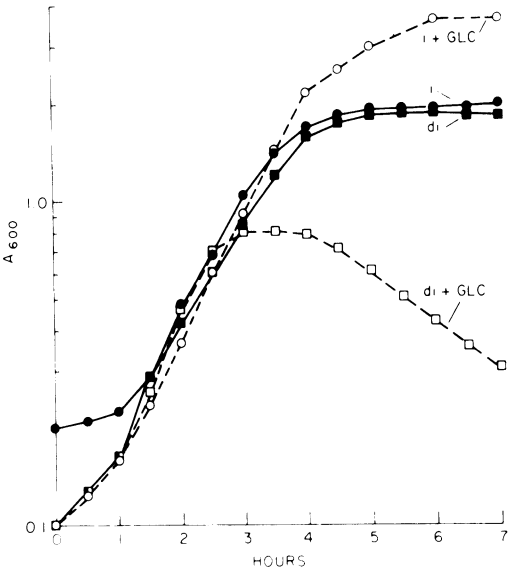

FIG. 2. *Effect of the addition of glucose to NSMP medium on the growth of different mutants. Symbols: closed, full lines = NSMP alone; open, dashed lines = NSMP plus 5 mg of Glc per ml. Strains: 61158 (i) = (O), 61324 (di) (□). Growth proceeded to a higher A_{600} with Glc than without, and no lysis was observed, also for strains 60015 (standard), 61370 (m), and 61371 (dm).*

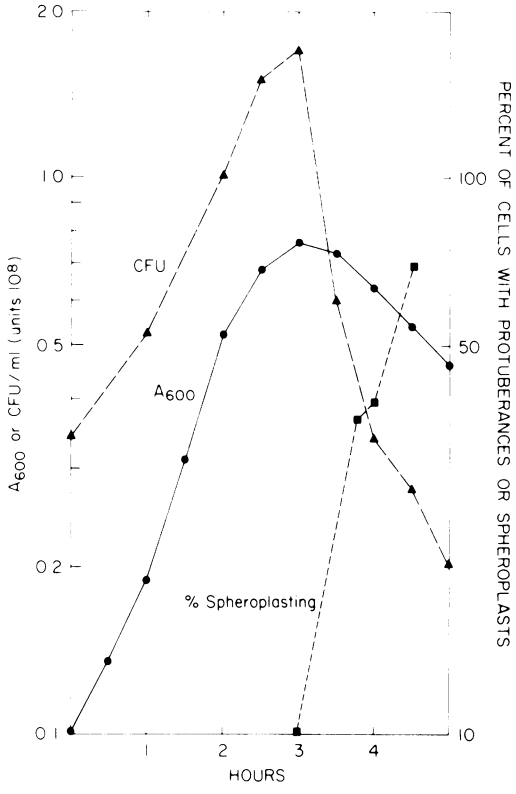

FIG. 3. *Effect of glucose on cell population and spheroplast formation in strain 61324 (di). Symbols: ▲, colony-forming units (CFU/ml); ●, A_{600}; and ■, percentage of cells spheroplasting.*

the time at which the culture would start to lyse by itself. No such increased susceptibility was observed when the standard strain was grown in NSMP plus Glc and then exposed to the antibiotics.

The addition of Glc in NSMP did not show any significant effect on the rate of DNA synthesis or the ATP level in the (di) mutant. The Glc-induced lysis does not appear to be caused by the induction of defective phages, because the culture lysed only slowly and the (di) supernatant was unable to kill *B. subtilis* W23 cells.

Evidence that lysis is caused by intracellular accumulation of Glc-1-phosphate. The initiation of lysis required a certain time of growth in the presence of Glc, irrespective of the initial cell titer. When the (di) mutant was pregrown in NSMP and then inoculated at different initial A_{600} values into NSMP plus Glc, lysis always started 3 to 4 h after the inoculation, irrespective of the final A_{600} of the culture (Fig. 5).

This indicated that lysis resulted from some intracellular Glc metabolite that started to accumulate after the cells had been transferred to the Glc-containing medium. Lysis, caused by accumulation of this compound, occurred in the

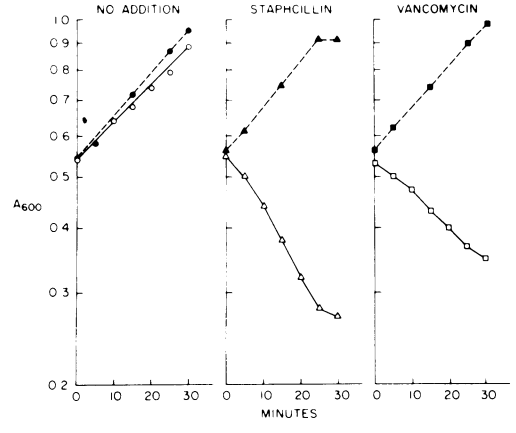

FIG. 4. *Antibiotic susceptibility of strain 61324 (di) grown in NSMP and NSMP plus 0.5% Glc. Symbols: NSMP-grown cells (closed symbols), NSMP plus Glc-grown cells (open symbols). No antibiotic (circles), 1 μg of staphcillin per ml (triangles), and 0.5 μg of vancomycin per ml (rectangle).*

(di) but not the single mutants, apparently because the latter could still metabolize Glc-6-P via glycolysis or the pentose shunt. The accumulation of Glc-6-P was therefore measured in different strains growing in NSMP plus 5 mg of Glc per ml (Fig. 6). The (di) mutant accumulated at the time of cell lysis only slightly more Glc-6-P inside the cells than the (i) mutant, but it accumulated a much higher concentration of Glc-6-P in the medium than the standard and the (i) strain. To determine the effect of extracellular Glc-6-P on cell lysis, the (di) mutant was grown in NSMP containing 5 mg of Glc-6-P per ml. No lysis was observed at any time. Growth in the presence of 5 mg of 2-deoxy-Glc or α-methylglucoside per ml, Glc analogues that are phosphorylated in the 6-position but not further metabolized (14), also did not cause lysis of the (di) mutant. Consequently, neither intra- nor extracellular Glc-6-P seem to be responsible for lysis.

Since Glc-6-P was excluded as the lysis-inducing compound, accumulation of glucose-1-P (Glc-1-P) or some sugar nucleotide should be the cause of lysis. In fact, the (dim) mutant, in which Glc-6-P cannot be metabolized into Glc-1-P, etc., did not lyse in NSMP plus Glc; its final A_{600} was about the same with and without Glc (Fig. 7). In spite of the absence of lysis, the (dim) mutant accumulated about the same amount of Glc-6-P inside cells as the (di) mutant (Fig. 6). To identify the lysis-inducing compound, two additional triple mutants, (dip) and (dit), were used that are blocked in one of the next two enzymes of the path leading to the glucosylation of teichoic acid (Fig. 1). Both mutants produced in NSMP plus Glc the same lysis as the (di) mutant (Fig. 7). This showed that neither UDP-Glc accumulation nor the glucosylation of teichoic acid were responsible for the lysis, but that lysis was caused by Glc-1-P. Since extracellular Glc-1-P (5 mg/ml) did not lyse the (dim) mutant, its intracellular accumulation apparently was essential.

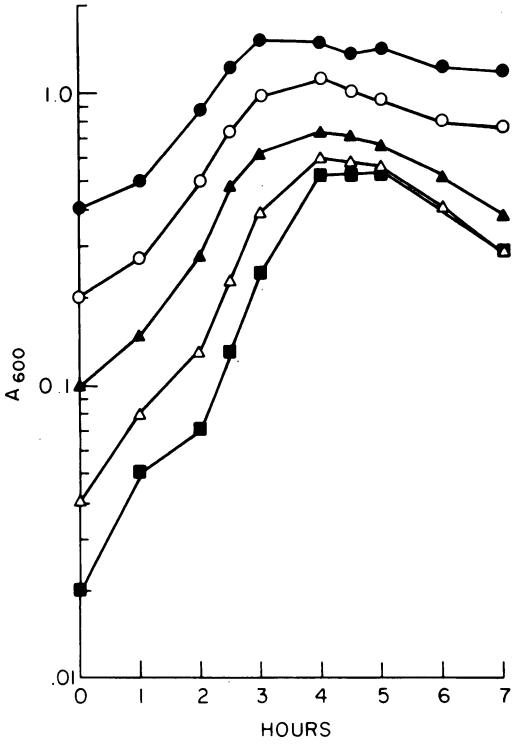

FIG. 5. *Dependence of the maximal cell titer (A_{600}) of strain 61324 (di) on the size of the initial inoculum. Medium: NSMP plus 5 mg of Glc per ml.*

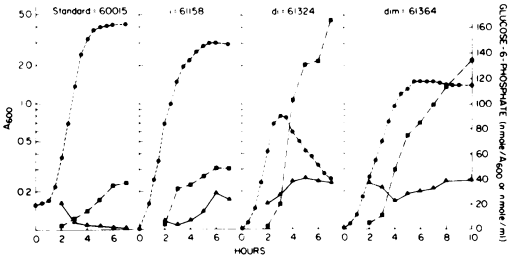

FIG. 6. *Growth and Glc-6-phosphate accumulation in standard and mutant strains grown in NSMP plus 0.5% Glc. Symbols: ●, A_{600}; ▲, Glc-6-P inside cells (in nanomoles/A_{600}); ■, Glc-6-P in the medium (nanomoles per milliliter).*

This conclusion was supported by the lysis-inducing effect of galactose (Gal), a compound that can be used by *B. subtilis* as a sole carbon source only very slowly (doubling time 5 h). Gal addition (5 mg/ml) to NSMP did not significantly increase the final A_{600} of the standard strain. Nevertheless, its presence caused, after prolonged incubation, lysis in the (m), (di), (dim), and (dit) mutants, but not in the standard strain nor the (p) or (dip) mutants (Fig. 7). Gal apparently caused the accumulation of a lysis-inducing compound; since the last two mutants did not lyse, UDP-Glc-pyrophosphorylase, which converts UDP-Glc to Glc-1-P, is necessary for this accumulation from Gal.

The intracellular accumulation of Glc-1-P was directly measured in different strains grown in NSMP plus 5 mg of Glc per ml. The amount of intracellular Glc-1-P was always much smaller than that of Glc-6-P (Table 4). However, the (dim) mutant contained less than $1/5$ of the Glc-1-P present in the (di) or the (dip) mutants, whereas the Glc-6-P concentration was high in all three strains. The absence of lysis for the (dim) mutant can therefore be

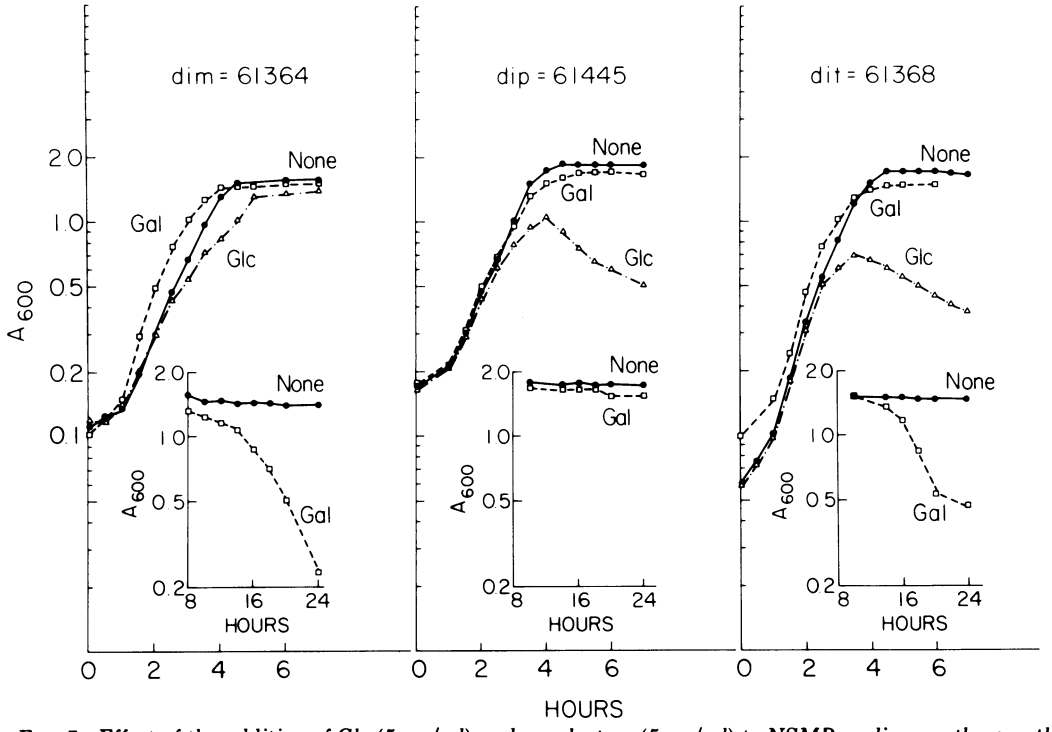

FIG. 7. *Effect of the addition of Glc (5 mg/ml) and D-galactose (5 mg/ml) to NSMP medium on the growth and lysis of strains 61364 (dim), 61445 (dip), and 61368 (dit).*

TABLE 4. *Intracellular accumulation of glucose-phosphates in various mutants*[a]

Mutant	Abbreviated enzyme defect[b]	A_{600}	nmol/A_{600} of culture	
			Glc-6-P	Glc-1-P
61324	(di)	0.873	21.4	5.6[c]
61364	(dim)	1.024	37.8	0.3
61445	(dip)	0.859	29.7	4.9

[a] Cells were grown in NSMP plus 5 mg of Glc per ml to the indicated A_{600}, collected on a Millipore filter, and extracted, and sugar-phosphates were assayed as described in Materials and Methods.

[b] See footnote to Table 1 for details.

[c] When mutant (61324) was grown in NSMP plus Glc plus either fructose, glucosamine, gluconate, glycerol, malate, or mannose (5 mg/ml) to A_{600} = 0.8 to 1, the intracellular concentration of Glc-1-P ranged from 1.5 to 3.1 nmol/A_{600} (and Glc-6-P ranged from 26 to 34 nmol/A_{600}).

ascribed to the absence of Glc-1-P accumulation. Since one A_{600} unit corresponds to about 1 μliter of cells, the intracellular concentration of Glc-1-P in the (di) and (dip) mutants was, at the time measured in Table 4, about 5 mM.

Indications that lysis is caused by inhibition of the muramic acid pathway. The lysis of the (di) mutant in NSMP plus Glc could be prevented by the addition of (5 mg/ml) any rapidly-metabolizable carbon source (except Glc) such as fructose, gluconate, glucosamine, glycerol, malate, or mannose (Fig. 8). These compounds also prevented the formation of spheroplasts. The cells grew to a higher A_{600} (3 to 4) because they could metabolize these carbon sources to compounds required for growth. In such cells, the concentration of Glc-1-P was reduced by a factor of 2 to 3 (Table 4), presumably because the compound could be partially utilized for glucosylation of teichoic acid. The absence of cell lysis under these conditions could therefore be due to both the reduction in the Glc-1-P concentration, reducing the extent of inhibition, and to the increase in the concentration of glucosamine derivatives, which can be produced from the above carbon sources and can compete with the inhibitory Glc-1-P by mass action. Which of these factors plays the more important role depends on the K_m and K_i values of the inhibited enzyme. Slowly metabolizable compounds, e.g., citrate, succinate, or L-α-glycerophosphate, which support growth as sole carbon source only after a long lag period and at a low rate, did not prevent lysis.

All preceding results suggested that lysis resulted from the inhibition of some enzyme in

the glucosamine pathway. The first enzyme in this pathway, L-glutamine-D-fructose-6-phosphate amidotransferase (EC 2.6.1.16), which in crude cell extracts showed a K_m for fructose-6-P of 11 mM, was not sensitive to inhibition by up to 10 mM of Glc-1-P (or Glc-6-P or UDP-Glc) (Table 5). Therefore, the effect of Glc-1-P on the subsequent reactions was tested by measuring the formation of *N*-acetyl-glucosamine-1-P from glucosamine-6-P and acetyl coenzyme A, which is catalyzed by two enzymes (3) (Fig. 1). Glc-1-P strongly inhibited this reaction, whereas Glc-6-P or other compounds tested had no significant effect (UDP-Glc slightly stimulated the reaction) (Table 6).

Comparison of cell wall autolysis and glucose-induced lysis. While this work was in progress, Forsberg et al. (9) published information indicating that a lesion in phosphoglucomutase reduces autolysis of *B. licheniformis* cell wall. This phenomenon is not related to our glucose-induced lysis of *B. subtilis*, as concluded from the data presented in Table 7, in which the autolysis of cell walls isolated from different mutants grown in NSMP or in NSMP plus Glc is measured. (i) After growth in NSMP plus Glc, cell walls of the standard strain autolyzed as well as those of the (di) mutant, whereas our glucose-induced lysis was observed only for the (di) mutant. (ii) Autolysis of Glc-grown cultures was reduced in both the (dim) and the (dip) mutants, whereas our glucose-induced lysis was observed only in the (dip) mutant. (iii) Both cell wall autolysis and glucose-induced lysis were observed in the (dit) mutant, which shows that they did not require the glucosylation of teichoic acid.

Consequently, autolysis seems to require the production of UDP-Glc (or possibly some galactose derivative), in contrast to our lysis phenomenon which depends upon the accumulation of Glc-1-P.

TABLE 5. *Effect of different compounds on the activity of L-glutamine-D-fructose-6-phosphate amidotransferase*

Addition to reaction mixture[a]	Concn (mM)	nmol of glucosamine-phosphate formed per min per mg of protein[b]
None		17.2
UDP-glucose	10.0	16.9
	5.0	16.8
	1.0	17.5
Glucose-1-phosphate	10.0	17.4
	5.0	17.3
	1.0	17.7
Glucose-6-phosphate	10.0	13.8

[a] The reaction mixture (250 μliters) contained: K-phosphate buffer, pH 7.4 (40 mM); fructose-6-P (20 mM); L-glutamine (12 mM); 2 mercaptoethanol (4 mM); and crude cell extract of strain 60015 (200 μg of protein).

[b] No inhibition was also observed for UDP-*N*-acetyl glucosamine (0.1 mM), thymidine diphosphate-glucose (5.0 mM), adenosine diphosphate-glucose (5.0 mM) and guanosine diphosphate-glucose (2.0 mM).

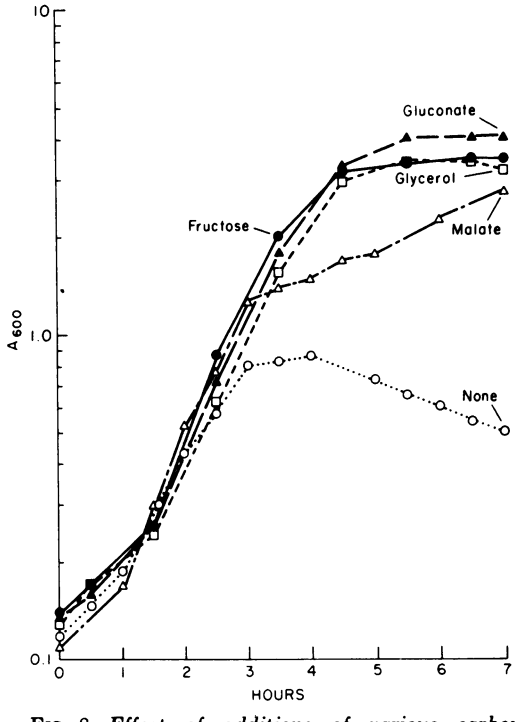

FIG. 8. *Effect of additions of various carbon sources (5 mg/ml) to NSMP plus Glc (5 mg/ml) medium on the lysis of the (di) strain 61324. Symbols: ○, no addition; △, potassium malate; □, glycerol; ●, fructose; and ▲, potassium gluconate. Lysis was also prevented by glucosamine (1 mg/ml) but not by citrate, succinate, or α-glycerophosphate.*

DISCUSSION

The mutations (i, m, p, or t) employed in this paper (Fig. 1) exhibited no or very low specific activities for the respective enzymes. The Glc-6-P dehydrogenase deficiency of the (d) mutation was only partial, the K_m being 175 times higher than that of the standard enzyme; in excess substrate, the enzyme activity in crude extracts was about ⅛ of the standard strain. Variants of Glc-6-P dehydrogenase exhibiting a deficiency in enzyme activity due to altered pH optima, heat stability, K_m for

TABLE 6. *Inhibition of the conversion of D-glucosamine-6-phosphate to N-acetyl D-glucosamine-1-phosphate*

Addition to reaction mixture[a]	Concn (mM)	Sp act[b]	Inhibition[c] (%)
None		52.4	
Glucose-1-phosphate	1.0	22.7	56.7
	2.0	12.9	75.4
	3.0	7.4	85.9
	4.0	5.5	94.5
Glucose-6-phosphate	10.0	47.8	8.8
UDP-glucose	10.0	54.8	−4.6
UDP-galactose	10.0	52.8	−0.7
UDP-*N*-acetyl glucosamine	1.0	51.5	1.7

[a] The reaction mixture (500 μliters) contained: Tris-chloride buffer, pH 8.0 (100 mM); D-glucosamine-6-phosphate (1 mM); acetyl coenzyme A (1 mM); $MgCl_2$ (2 mM); β-mercaptoethanol (8 mM); glucose-1,6-diphosphate (0.02 mM); and crude extract of strain 61364 grown in NSMP plus 2 mg of Glc per ml (1,060 μg of protein).

[b] Nanomoles of acetylated sugar formed per milligram of protein per 30 min.

[c] No significant inhibition was observed for the following uridine, guanosine, cytosine, and adenosine mono-, di, or triphosphates compounds (10 mM): UTP, UDP, UMP, GTP, GDP, GMP, CTP, CDP, CMP, ATP, ADP and AMP.

TABLE 7. *Autolysis of cell wall of different mutants grown in NSMP and NSMP plus 5 mg of Glc per ml*

Strain	Abbreviated enzyme defect[a]	% Reduction of A_{600} in 15 min of walls of cells grown in:	
		NSMP	NSMP + Glc
60015	Standard	40	44
61324	(di)	14	34
61364	(dim)	12	15
61445	(dip)	11	15
61368	(dit)	25	32

[a] See footnote to Table 1 for details. Cell walls were isolated as described in Materials and Methods and suspended in 25 mM bicarbonate buffer (pH 9.6) and 100 mM KCl. Autolysis was measured at 38 C by the decrease of A_{600} from an initial value of 0.8 to 1.

Glc-6-P and $NADP^+$, electrophoretic mobility, and chromatographic behavior on DEAE-Sephadex are not uncommon (5, 27, 31). The Glc-6-P dehydrogenase defect in *B. subtilis* made it impossible for the double mutant (di) to grow on Glc as sole carbon source, whereas the single P-Glc-isomerase (i) mutants could grow, although at a slower rate (15), presumably by utilizing Glc-6-P via the pentose shunt.

When the mutants (di), (dip), or (dit) were grown in NSMP plus Glc, they began to lyse after 3 to 4 h, whereas no such lysis was observed for the standard strain, single (i), (m), or (p) mutants, or the (dim) mutant. This and other experimental results show that the lysis is caused by the accumulation of Glc-1-P. The restoration of growth by other carbon sources and the inhibition of *N*-acetyl glucosamine-1-P synthesis by Glc-1-P indicate further that lysis results from the inhibition of muramic acid production. This conclusion agrees with the appearance of spheroplasts and with the increased sensitivity of (di) cells toward antibiotics that inhibit cell wall synthesis. At present we do not know whether Glc-1-P inhibits the phosphoglucosamine mutase or the glucosamine-1-P acetyltransferase.

The accumulation of phosphorylated sugar intermediates has frequently been observed to cause toxic effects in bacteria (2, 6–8, 13, 17, 18, 25, 29, 33, 38, 41, 45), insects (39), HeLa cells (20), and humans (37). The effect of Gal on mutants of *Escherichia coli* has been studied in particular detail (18, 29, 41, 45). It was found that mutants lacking Gal-1-P uridyl transferase, UDP-galactose-4′-epimerse, or UDP-glucose pyrophosphorylase lyse when they are grown in a nutrient medium containing Gal. Since Gal transport, galactokinase, or Gal-1-P uridyl transferase mutants did not exhibit this lysis, it was assumed that lysis results from the accumulation of some Gal nucleotide. This phenomenon is similar to the one reported here, except that in *B. subtilis* lysis is caused by Glc-1-P. In *B. subtilis* Gal is slowly metabolized, so that in (m) or (dim) mutants Glc-1-P can accumulate and cause lysis only after prolonged incubation. In (dip) mutants, Gal does not cause any lysis, presumably because the UDP-Glc-pyrophosphorylase is necessary to accumulate enough Glc-1-P from Gal, whereas Gal-1-P uridyl transferase and the other Gal enzymes alone are not sufficient to cause this accumulation. The Gal system of *E. coli* is inducible by Gal and repressible by Glc. Since the above authors found that the addition of Glc prevented the lysis by Gal, they concluded that this rescue function of Glc resulted from the repression of the Gal metabolism. Our findings would suggest that Glc could in addition provide the precursor for the glucosamine path and thereby circumvent the lytic effect of any compound that might have accumulated. In our mutants Glc could not be metabolized to glucosamine derivatives, because the necessary enzymes (P-Glc-isomerase or Glc-6-P dehydrogen-

ase) were missing. However, the addition of any other compound that could be metabolized to glucosamine effectively prevented the lysis.

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