JOURNAL OF BACTERIOLOGY, Aug. 1974, p. 431-442

Vol. 119, No. 2
Printed in U.S.A.

Glycerol Metabolism in *Bacillus subtilis*: Gene-Enzyme Relationships

VIVEKA LINDGREN AND LARS RUTBERG

Department of Bacteriology, Karolinska Institutet, S 104 01 Stockholm 60, Sweden

Received for publication 20 February 1974

Bacillus subtilis mutants unable to catabolize glycerol (Glp mutants) were isolated and mapped. The location of the mutations on the chromosome was determined by a density transfer technique and confirmed by PBS1 transduction and transformation. The different mutations were ordered relative to each other. Mutations rendering the cells glycerol auxotrophic were also mapped and found not to be linked to the Glp mutations.

Two pathways for the dissimilation of glycerol have been described in bacteria. One involves a nicotinamide adenine dinucleotide (NAD)-dependent glycerol dehydrogenase (EC 1.1.1.6) and a dihydroxyacetone kinase; this pathway is used under anaerobic conditions by *Aerobacter aerogenes* (21, 24). The other pathway involves a glycerol kinase (EC 2.7.1.30) and an NAD-independent glycerophosphate (GP) dehydrogenase (EC 1.1.99.5; hereafter referred to as GP dehydrogenase), and is used by *Bacillus subtilis* (27) and aerobically grown *Escherichia coli* (6, 13, 20). In the presence of an electron acceptor other than oxygen, *E. coli* can use either the GP dehydrogenase or a flavine-dependent GP dehydrogenase (10, 18). In extensive studies Lin and co-workers have shown that the genes for glycerol kinase, GP dehydrogenases, and also the genetic determinants for uptake of glycerol and GP constitute at least three separate operons that all are affected by the product of a single regulatory gene (6, 8, 18, 19). Mutants defective in glycerol kinase or GP dehydrogenase fail to grow with glycerol as a sole carbon source but can grow on glucose. Mutants with an absolute requirement for glycerol have also been isolated in both *B. subtilis* (28) and *E. coli* (17). In *E. coli* these mutants have been shown to lack a reduced NAD phosphate-dependent GP dehydrogenase (EC 1.1.1.94), indicating that this is the main enzyme used for producing GP in the cell when grown in the absence of glycerol or GP. The enzymatic defect in glycerol-requiring mutants of *B. subtilis* has not been identified. All phospholipids presently identified in *B. subtilis* are glycerophosphatides (3, 31). The isolation of glycerol-requiring mutants has made possible studies of the effects of arrest of net phospholipid synthesis on different cellular functions (17, 28, 29, 35). Since glycerol can be used also as a carbon and energy source in *B. subtilis*, an understanding of the regulation of phospholipid synthesis in this organism requires knowledge of the pathways for synthesis and breakdown of glycerol and glycerophosphate and the genetic organization of these pathways.

This investigation was undertaken to study the genetic organization of glycerol metabolism in *B. subtilis*. We used strain 168 and various derivatives thereof. *B. subtilis* 168 contains polyglycerophosphate (5), which strain W23 does not (2), but only 168 and not W23 is open to proper genetic analyses.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. The *glp* nomenclature was introduced by Lin and co-workers to designate in *E. coli* the genes governing the synthesis of the enzymes and permeases specifically involved in the catabolism of glycerol and L-α-GP (8). The *glp* nomenclature has also been used for *B. subtilis* (28). It should be noted, however, that in *E. coli* the *glp* denotation was not introduced until it had been conclusively shown that the different enzymes and permeases belonged to the same regulon, the specific inducer being L-α-GP (8, 14, 19). It has previously been suggested that GP (or some further metabolite) and not glycerol is the true inducer of the NAD-independent GP dehydrogenase as well as glycerol kinase also in *B. subtilis* (33). We will here present genetic data suggesting a common regulation of glycerol kinase, GP dehydrogenase, and a GP transport system in this species. We have therefore adopted the *glp* designation although it has not been clearly shown whether glycerol or GP is the true inducer of glycerol kinase and the GP transport system in *B. subtilis*.

Media. The minimal salt solution was that described by Anagnostopoulos and Spizizen (1) supple-

TABLE 1. *List of Bacillus subtilis strains*

Strain[a]	Relevant properties	Origin or derivation
W168	Wild type	J. Spizizen
168 TT	*thyA, thyB, trpC2*	J. A. Hoch
BR51	*trpC2, metC7*	J. Spizizen
BR56	*trpC2, ura-1, purB*	J. Spizizen
BR85	*trpC2, argC4*	J. Spizizen
BR95	*trpC2, pheA1, ilvC1*	J. Spizizen
BR98	*trpC2, hisB, ura-2*	J. Spizizen
BD92	*trpC2, hisA, cysB*	J. A. Hoch
GtaC	*trpC2, lys-3, metB10, gtaC*	F. E. Young
GlpD6	Lacks NAD-independent GP dehydrogenase	NTG, W168
GlpD8-2	Lacks NAD-independent GP dehydrogenase	NTG, W168
GlpD12-11	Lacks NAD-independent GP dehydrogenase	NTG, W168
GlpD13-10	Lacks NAD-independent GP dehydrogenase	NTG, W168
$GlpP_i9$-3	Pleiotropic glycerol mutant	NTG, W168
$GlpP_i12$-6	Pleiotropic glycerol mutant	NTG, W168
$GlpP_i18$	Pleiotropic glycerol mutant	NTG, W168
$GlpP_i18$-R2 and $GlpP_i18$-R9	Spontaneous *glp*$^+$ revertants of *$glpP_i18$* with constitutive NAD-independent GP dehydrogenase and uninducible glycerol kinase	$GlpP_i18$
$GlpP_i18$-R2 GlpK21	NTG-induced mutant of *$glpP_i18$-R2;* lacks glycerol kinase	$GlpP_i18$-R2
$GlpP_i18$-R1	NTG-induced revertant of *$glpP_i18$;* grows on glycerol at 45 C but not at 30 C	$GlpP_i18$
GlpK3-7	Lacks glycerol kinase	NTG, W168
GlpK11-4	Lacks glycerol kinase	NTG, W168
Glyc-1	Glycerol auxotroph	NTG, W168
Glyc-2	Glycerol auxotroph	NTG, W168
Glyc-3	Glycerol auxotroph	NTG, W168

[a] The various *glp* and *glyc* mutations were introduced into other strains by congression. This is indicated in the text as BR95 GlpD6, GtaC, GlpK21, etc.

mented with 10^{-5} M $MnCl_2$. Glucose or glycerol was added at a concentration of 5 g/liter if not otherwise indicated. When the cells were grown on glycerol as the sole source of energy and carbon, citrate was omitted from the minimal salt solution. Min-CH is minimal salt solution with glucose (5 g/liter) and casein hydrolysate (0.5 g/liter; Difco). LP medium is the low-phosphate medium described by Miki et al. (26) with sodium lactate omitted. Since GP dehydrogenase mutants do not grow on this low-phosphate medium (probably because of the presence of glycerol in the peptone), the phosphonomycin sensitivity of these mutants was assayed on low-phosphate plates with 1 g of casein hydrolysate per liter instead of 10 g of peptone per liter and with inorganic phosphate added at a final concentration of 0.18 mM. The media were solidified by addition of Noble agar (Difco), 18 g/liter. PB is antibiotic medium no. 3 (Difco), and TBAB plates were made from tryptose blood agar base (Difco).

Isolation of mutants. *B. subtilis* W168 growing exponentially in PB was treated with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; K&K Laboratories, Inc., Hollywood, Calif.) per ml for 30 min. The cells were then harvested, resuspended in fresh PB, and grown on a rotary shaker at 37 C overnight. For the selection of Glp mutants, an appropriate dilution of the mutagenized culture was spread on TBAB or min-CH plates. After 15 to 20 h of incubation at 37 C, these master plates were replicated to minimal glycerol and minimal glucose plates. Colonies which grew on minimal glucose but not on minimal glycerol were selected. Such colonies were restreaked twice on minimal glucose plates and then analyzed for the presence of inducible activities of glycerol kinase and GP dehydrogenase. Glycerol auxotrophic mutants were selected by spreading a mutagenized culture on min-CH plates supplemented with glycerol (50 μg/ml) and then replicating to minimal glucose plates with and without glycerol. Phosphonomycin-resistant mutants were selected on LP plates containing 1 mM phosphonomycin.

To avoid the selection of mutants belonging to the same clone, only one mutant of a certain phenotype was kept from each mutagenized culture. Most of the mutant alleles were subsequently introduced into an isogenic background by transforming, for example, strain BR95 with saturing concentrations of the mutant deoxyribonucleic acid (DNA) (congression).

Growth of cells and preparation of extracts for enzymatic assays. The bacteria were inoculated at a density of approximately 4×10^7 cells/ml into 1-liter Erlenmeyer flasks containing 100 ml of min-CH and required amino acids (20 mg/liter). After 3.5 h of growth on a rotary shaker at 37 C, the cells were centrifuged and resuspended in 100 ml of fresh

minimal salt solution with the same additions as above with the exception of glucose. Inducer (40 mM DL-α-GP or 20 mM glycerol) was added as indicated in the text, and the cells were grown on the rotary shaker for 1 h.

For preparation of enzyme extracts, the cells were washed once with cold 0.9% NaCl, resuspended in 2 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, pH 7.4, and sonicated while chilled in an ice bath in a 100-W ultrasonic disintegrator (Measuring & Scientific Equipment Ltd., London). The sonicated suspension was centrifuged at 17,000 × *g* for 15 min. Enzymatic activities in the supernatant were measured.

Glycerol kinase activity was always assayed immediately after preparation of the cell extract. In most cases the activity of the GP dehydrogenase was assayed at the same time. In a few cases the extracts were kept at −20 C until the next morning, when the GP dehydrogenase assay was performed. We found the loss of enzyme activity under these conditions to be less than 10% (data not shown).

In *E. coli*, L-α-GP uptake is competitively inhibited by inorganic phosphate (12). Our minimal salt solution contained inorganic phosphate at a concentration of approximately 100 mM. Obviously inorganic phosphate has an inhibitory effect on the entrance of GP in *B. subtilis* also, since strain W168 would not grow on 40 mM DL-α-GP in this high-phosphate medium but did grow in a low-phosphate (1 mM), Tris-buffered minimal salt solution (Fig. 1). Growth on GP is not dependent on hydrolysis of the phosphoric ester bond since glycerol kinase mutants that could not grow on glycerol did grow at a slow rate on GP in low-phosphate minimal salt solution (Fig. 1).

However, also in the high-phosphate medium with 40 mM DL-α-GP, enough GP was taken up to induce the glycerol kinase and GP dehydrogenase (compare in Table 2 the enzyme activities in extracts from cells grown without any inducer and with GP, respectively). Under the growth conditions used in the experiments (i.e., with high-phosphate medium), there was no measurable activity of alkaline phosphatase, whereas in the low-phosphate medium we found a variable phosphatase activity depending on the growth phase (not shown).

Thus there is a twofold advantage in using the high-phosphate medium with DL-α-GP as the inducer: the difference between inducing and noninducing conditions regarding available carbon and energy sources is minimized, and the alkaline phosphatase activity is both repressed and inhibited (C. Anagnostopoulos, Fed. Proc. **19**:48, 1960; 26).

Wild-type bacteria and our different Glp mutants grew at the same rates in high-phosphate minimal salt solution with glucose (5 g/liter) and casein hydrolysate (1 g/liter) (data not shown). When glucose was replaced by 20 mM glycerol or 40 mM DL-α-GP, growth of GP dehydrogenase mutants and also of pleiotropic $GlpP_I$ mutants was inhibited (see Results). The toxic effect of GP on GP dehydrogenase mutants has been studied previously (7, 30).

Enzyme assays. The assays of the activities of glycerol kinase and of GP dehydrogenase have been described by Lin et al. (20). In the glycerol kinase assay, the phosphorylation of glycerol is coupled to the reduction of NAD by means of NAD-dependent L-α-GP dehydrogenase from rabbit muscle. The in-

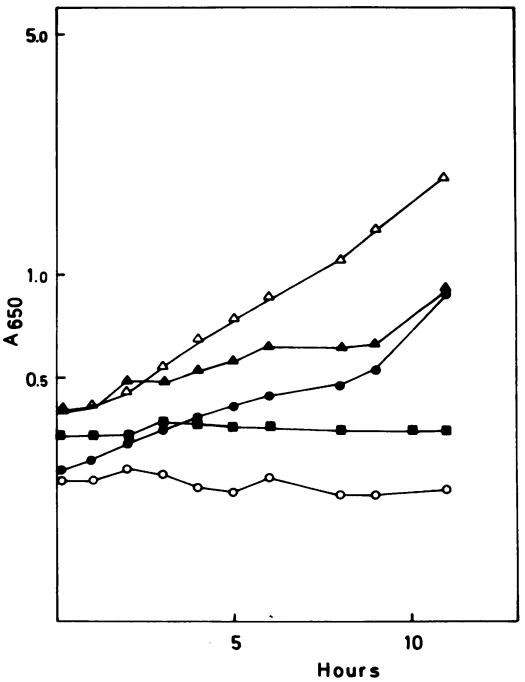

FIG. 1. *Growth of W168 and BR95 GlpK21 on glycerol (20 mM) or DL-α-GP (40 mM) as sole carbon source. W168 on glycerol in low-phosphate minimal salt solution, Δ; W168 on DL-α-GP in low phosphate minimal salt solution, ▲; W168 on DL-α-GP in high-phosphate minimal salt solution, ■; BR95 GlpK21 on glycerol in low-phosphate minimal salt solution, ○; BR95 GlpK21 on DL-α-GP in low-phosphate minimal salt solution, ●.*

TABLE 2. *Activities of glycerol-catabolizing enzymes in wild-type and glycerol kinase mutants*

Strain	Inducer	Sp act	
		Glycerol kinase	GP dehydrogenase
W168		24	2
W168	40 mM DL-α-GP	110	119
W168	20 mM glycerol	97	110
BR95		59	2
BR95	40 mM DL-α-GP	148	58
BR95	20 mM glycerol	98	70
BR95 GlpK3-7	40 mM DL-α-GP	1	43
BR95 GlpK3-7	20 mM glycerol	0	1
BR95 GlpK11-4	40 mM DL-α-GP	0	40
BR95 GlpK11-4	20 mM glycerol	0	1
BR95 GlpK21	40 mM DL-α-GP	0	102
BR95 GlpK21	20 mM glycerol	0	0

crease in absorbance at 340 nm was followed in a Beckman model DB-GT spectrophotometer using the double beam and with a W&W model 2011 recorder attached. The activity of the NAD-independent GP dehydrogenase is determined by measuring the rate of the reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide to its formazan, which has an absorption maximum at 550 nm.

NAD-dependent glycerol dehydrogenase activity was assayed according to the method of Lin and Magasanik (22), in which the reduction of NAD is followed spectrophotometrically at 340 nm. The specific activity for all enzymes is given as nanomoles of substrate converted per minute per milligram of protein. All enzyme assays were performed at 25 C.

The protein concentrations of the extracts were determined according to Lowry et al. (23).

Density transfer experiments. With some modifications, the density transfer experiments were performed as described by O'Sullivan and Sueoka (32). Spores with tritiated DNA were prepared by incubating 168 TT on TBAB plates containing 1 μCi of [*methyl*-^{3}H]thymine per ml (specific activity, 20.4 Ci/mmol) at 30 C for 7 to 10 days. The cells were then scraped off the plates, washed six times with cold, sterile, deionized water, and treated with 400 μg of lysozyme per ml for 2 h at 37 C. Sodium dodecyl sulfate was then added at a final concentration of 10 mg/ml, and incubation continued for 15 min at 60 C. Finally the spores were washed six times with cold, sterile water. The spore preparations were tested for heat resistance by incubation at 80 C for 10 min. Only preparations showing 100% survival after such treatment were used.

Germination was initiated by incubating the spores in PB at 70 C for 15 min. 5-Bromo-2'-deoxyuridine (80 μg/ml) and [^{14}C]thymidine (0.05 μCi/ml; specific activity, 62 mCi/mmol) were then added, and the spores were germinated at 37 C with aeration. Incorporation of [^{14}C]thymidine under these conditions was found to start 50 to 60 min after the addition of 5-bromo-2'-deoxyuridine and [^{14}C]thymidine. Cell samples for DNA extraction were taken at 60, 65, and 75 min. The procedure for cell lysis was the one described by O'Sullivan and Sueoka (32). Lysate (2 ml) was mixed with 1 ml of sterile 0.02 M NaCl-0.001 M ethylenediaminetetraacetic acid, pH 8.2, and 3.8 g of CsCl, sheared by pipetting up and down a few times with a 1-ml pipette, and then centrifuged at 31,000 rpm for 68 h at 4 C in a Spinco model L ultracentrifuge with a Spinco SW50 rotor. From the bottom of each tube a total of 38 fractions were collected and diluted with 1.0 ml of sterile 0.02 M NaCl-0.001 M ethylenediaminetetraacetic acid, pH 8.2. Samples were then taken from each fraction and precipitated with cold 10% trichloroacetic acid on HA membrane filters (Millipore Corp., Bedford, Mass.). The filters were washed, dried, and then put in scintillation vials with 10 ml of scintillation liquid {10 g of 2,5-diphenyloxazole; 0.125 g of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene per liter of toluene}. The radioactivity was counted in a Nuclear-Chicago Mark liquid scintillation computor model 6860.

In the DNA taken out 60 and 65 min after germination had been initiated, there was no radioactivity representing a fully heavy DNA fraction (which should have been due to premature re-initiation of replication). In the DNA sample extracted after 75 min, there may have been a minor fully heavy fraction, which, however, was very small compared with the light and hybrid fractions and which we therefore considered negligible. The percentage of the total ^{3}H counts that was found in the hybrid fraction was a measure of the extent of the first round of replication.

To determine the relative location of a *glp* mutation, 0.1 ml from the light and hybrid fractions of each DNA preparation was used to transform competent cells of the Glp mutant and of strains bearing markers with known positions on the chromosome. The result for each marker is given as the percentage of the total number of transformants that is obtained with the DNA from the hybrid fraction.

Transduction. Transducing PBS1 lysates were obtained by the method of Young et al. (36). The procedure for transduction experiments was according to Hoch (16). Before plating on selective media the cells were washed once in a sterile minimal salt solution.

Transformation. DNA was extracted essentially according to the principles developed by Marmur (25). Transformation experiments were performed as described by Anagnostopolous and Spizizen (1), except that the first period of growth was extended to 5.5 h. Maximal level of competence was obtained 90 min after the shift. At this time the cells were exposed to DNA for 30 min and then plated on selective media.

Scoring of recombinants. Glp$^+$ recombinants were selected on minimal glycerol agar plus required amino acids. They were restreaked once and then scored for unselected markers. The GtaC character was scored as resistance to phage ϕ29 as described by Young et al. (36). The scoring is made on TBAB plates with about 10^8 phages spread on each plate. As controls, the GtaC strain and the ϕ29-sensitive recipient were streaked on each plate together with the recombinants.

Chemicals. Crystalline rabbit muscle L-α-GP dehydrogenase, adenosine triphosphate, NAD, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide, phenazin methosulfate, DL-α-GP, and 5-bromo-2'-deoxyuridine were purchased from Sigma Chemical Co., St. Louis, Mo.; hydrazine from Eastman Kodak Co., Rochester, N.Y.; glycerol from BDH Chemicals Ltd., Poole, England; and [*methyl*-^{3}H]thymine and [^{14}C]thymidine from The Radiochemical Centre, Amersham, England. The disodium salt of phosphonomycin (molecular weight, 182) was a gift from Merck Sharp & Dohme International.

RESULTS

Activities of glycerol-catabolizing enzymes in B. subtilis. Extracts of strain W168 grown in the presence of glycerol or GP contained activity of glycerol kinase, GP dehydrogenase, and NAD-dependent glycerol dehydrogenase

(Tables 2 and 3). The levels of glycerol dehydrogenase, but not of the other two enzymes, were unaffected by the presence or absence of glycerol or GP. Mutants lacking GP dehydrogenase failed to grow with either glycerol or GP as sole carbon source. Mutants lacking glycerol kinase would grow on GP but not on glycerol (Fig. 1). These results are in agreement with data previously published by Mindich (27) and clearly show that the main pathway for glycerol dissimilation in *B. subtilis* is by glycerol kinase and GP dehydrogenase. The dihydroxyacetone phosphate thus formed can then enter the common intermediary metabolism.

Mutants lacking glycerol kinase; GlpK mutants. In strain W168, glycerol kinase was induced to give three- to fivefold higher activity in cell extracts when the cells were grown in the presence of glycerol or GP (Table 2). Mutants lacking glycerol kinase while still having GP dehydrogenase activity were rare among the Glp mutants isolated. Of about 100 mutants analyzed from 20 independently mutagenized cultures, only two GlpK mutants were found. These mutants, GlpK3-7 and GlpK11-4 (Table 2), grew on GP but not on glycerol.

It may be that glycerol kinase mutants are initially hard to find if they mask themselves as pleiotropic mutants, assuming that inducing levels of GP cannot be reached inside cells lacking glycerol kinase. To test this hypothesis, we isolated a kinase mutant from a strain which constitutively produces GP dehydrogenase ($GlpP_i18$-R2; see later section) and has only basal, noninducible levels of glycerol kinase. The kinase mutation, GlpK21, was then introduced into strain BR95 by congression; the derived strain, BR95 GlpK21, proved fully inducible for GP dehydrogenase, using GP as inducer. Glycerol would not induce GP dehydrogenase in this or any other strain tested which lacked glycerol kinase activity (33). Thus, as in *E. coli* (19), GP is probably the inducer for GP dehydrogenase.

Mutants lacking NAD-independent GP dehydrogenase: GlpD mutants. About 20% of all glycerol mutants isolated lacked GP dehydrogenase only. Four independently isolated GlpD mutants were chosen for further studies (Table 4). Growth of GlpD mutants is inhibited in the presence of glycerol due to accumulation of toxic amounts of GP inside the cells (30), as has also been found in *E. coli* (7). Glycerol kinase is internally induced in GlpD mutants also when these mutants are grown in a minimal medium with a defined mixture of amino acids added (Table 4).

Pleiotropic glycerol mutants: GlpP mutants. Pleiotropic mutants were initially recognized by their inability to grow on glycerol.

TABLE 4. *Activities of glycerol-catabolizing enzymes in GP dehydrogenase mutants*[a]

Strain	Inducer	Sp act	
		Glycerol kinase	GP dehydrogenase
BR95 GlpD6		105	0
BR95 GlpD6	40 mM DL-α-GP	55	1
GlpD8-2		48	0
GlpD8-2	40 mM DL-α-GP	27	2
GlpD12-11		75	0
GlpD12-11	40 mM DL-α-GP	33	0
GlpD13-10	40 mM DL-α-GP	46	1
W168		24	2
W168	40 mM DL-α-GP	110	119

[a] Values for W168 are relisted from Table 2 for comparison. In the case of BR95 GlpD6, the casein hydrolysate in the growth medium was replaced by a defined mixture of amino acids.

TABLE 3. *Activity of NAD-dependent glycerol dehydrogenase in the wild type and in two Glp mutants*[a]

Strain	Growth medium	Sp act of NAD-dependent glycerol dehydrogenase
W168	Minimal salt solution with glucose (5 g/liter) and casein hydrolysate (0.5 g/liter)	16
W168	Minimal salt solution with glycerol (3 g/liter) and casein hydrolysate (0.5 g/liter)	16
BR95 GlpD6	Minimal salt solution with glucose (5 g/liter) and casein hydrolysate (0.5 g/liter)	27
BR95 $GlpP_i18$	Minimal salt solution with glucose (5 g/liter) and casein hydrolysate (0.5 g/liter)	12

[a] Strains were inoculated at a cell density of approximately 4×10^7 cells/ml and grown on a rotary shaker at 37 C to 2×10^8 cells/ml.

These mutants have only basal or lower levels of both glycerol kinase and GP dehydrogenase also when grown in the presence of glycerol or GP (Table 5). The mutations giving pleiotropic effects can be introduced into strain BR95 by congression. Pleiotropic mutants revert to wild-type phenotype at a frequency of generally about 10^{-8}.

TABLE 5. *Activities of glycerol-catabolizing enzymes in pleiotropic Glp mutants*

Strain[a]	Inducer	Sp act	
		Glycerol kinase	GP dehydrogenase
$GlpP_I$9-3		6	1
$GlpP_I$9-3	40 mM DL-α-GP	28	4
BR95 $GlpP_I$9-3	40 mM DL-α-GP	25	1
BR95 $GlpP_I$9-3	20 mM glycerol	23	0
$GlpP_I$12-6		12	3
$GlpP_I$12-6	40 mM DL-α-GP	19	1
BR95 $GlpP_I$12-6	40 mM DL-α-GP	10	2
BR95 $GlpP_I$12-6	20 mM glycerol	11	1
$GlpP_I$18	40 mM DL-α-GP	20	4
BR95 $GlpP_I$18		23	0
BR95 $GlpP_I$18	40 mM DL-α-GP	18	2
BR95 $GlpP_I$18	20 mM glycerol	17	3
W168		27	3
W168	40 mM DL-α-GP	125	83
W168	20 mM glycerol	108	97

[a] Activities in the wild type are given in the last three lines.

The pleiotropic mutants are all resistant to the antibiotic phosphonomycin (0.2 mM), which in several bacteria can be taken into the cells by the GP transport system (15). GlpD and GlpK mutants were sensitive to phosphonomycin (Table 6). The pleiotropic mutants grew as well as wild type on glucose, mannose, and mannitol. Consequently they are not of the type described by Gay et al. (11). Growth of the pleiotropic mutants is inhibited by glycerol (20 mM) or GP (40 mM, in high-phosphate medium); thus they are probably not merely uptake mutants. It is most probable that the pleiotropic mutants, $GlpP_I$, are effected in the regulation of the glycerol-catabolizing enzymes. We have isolated revertants of $GlpP_I$18 able to grow on glycerol as sole carbon source. Out of 14 independent revertants studied, 12 had a wild-type phenotype in that they were inducible for both glycerol kinase and GP dehydrogenase. However, two revertants were isolated, $GlpP_I$18-R2 and $GlpP_I$18-R9, which had the following properties. They were still uninducible for glycerol kinase but produced GP dehydrogenase constitutively. In wild-type cells, glucose repressed the levels of activity of both GP dehydrogenase and glycerol kinase (Table 7). Revertants $GlpP_I$18-R2 and $GlpP_I$18-R9, however, gave the same level of GP dehydrogenase irrespective of the presence of inducer and/or glucose. One explanation for the properties of the revertants could be that the original *$glpP_I$18*

TABLE 6. *Phosphonomycin sensitivity of wild type and different Glp mutants*[a]

Strain	Phosphonomycin concentration (mM)						
	0	0.025	0.05	0.1	0.2	0.5	1.0
A							
W168	+	−	−	−	−	−	−
BR95	+	±	−	−	−	−	−
BR95 GlpK21	+	±	−	−	−	−	−
BR95 GlpK3-7	+	±	−	−	−	ND	ND
BR95 GlpK11-4	+	±	−	−	−	ND	ND
BR95 $GlpP_I$18	+	+	+	+	+	−	−
BR95 $GlpP_I$9-3	+	+	+	+	+	±	−
BR95 $GlpP_I$12-6	+	+	+	+	+	−	−
B							
W168	+	−	−	−	−		
BR95	+	±	±	−	−		
BR95 GlpD6	+	±	±	−	−		
BR95 GlpD8-2	+	±	−	−	−		
BR95 GlpD12-11	+	±	−	−	−		
BR95 $GlpP_I$18	+	+	+	+	+		

[a] In A, sensitivity was assayed on LP plates; in B, it was assayed on low phosphate-casein-hydrolysate plates. The strains were streaked with sterile toothpicks on the plates. Symbols: +, good growth after 20 h at 37 C; ±, poor growth; −, no growth. ND, Not done.

mutation is located in a locus, which determines a repressor (or activator), that regulates the activity of the *glpK* and the *glpD* loci, respectively. The reversions R2 and R9 could then be located in an operator-promoter region for the *glpD* locus. This model suggests that, although *glpK* and *glpD* are sensitive to the product of the same regulatory gene, they may be part of separate operons.

If *glpP$_I$18* defines the locus for a repressor affecting the activity of *glpK* and *glpD*, it should be possible to isolate constitutive and temperature-sensitive (ts) constitutive revertants of GlpP$_I$18. We have not yet found any revertants constitutive for both glycerol kinase and GP dehydrogenase. We isolated five independent ts revertants of GlpP$_I$18 (four spontaneous, one induced with NTG). These revertants grew at 45 C with glycerol as sole carbon source but poorly at 30 C on glycerol. They also did not grow on TBAB plates at 30 C for reasons unknown. At 45 C the ts revertants did not produce the Glp enzymes constitutively. The ts revertants became sensitive to phosphonomycin at 45 C.

Mapping of glycerol mutants by density transfer. The relative locations of mutations *glpP$_I$18* and *glpD6* on the *B. subtilis* chromosome were determined by the density transfer technique (32). The following reference markers were used: *purB*, *hisA*, *cysB*, *argC*, *metC*, and *ura-1*. *hisA* consistently replicated slightly ahead of *purB*, as reported by O'Sullivan and Sueoka (32) for their *his* marker. Others have mapped *hisA* distal to *purB* (4, 9). However, since the order of the markers, except *purB-hisA*, found in our experiments agreed with previously published data on the relative locations of these markers on the *B. subtilis* chromosome (9), they were used to identify the approximate locations of *glpD6* and *glpP$_I$18*. Both *glp* markers appeared in the replicated DNA fraction after *purB* and *cysB* but before *argC*, *metC*, and *ura-1* (Table 8). The *glpK* locus was not mapped by this technique.

Linkage between glpP$_I$18, glpK21, and glpD6 by transformation. To determine whether *glpK21* is located in the same region of the *B. subtilis* chromosome as *glpP$_I$18* and *glpD6*, the recombination index was determined for these markers as described by Dubnau et al. (9). The results of these experiments show that all three markers are linked by transformation (Table 9). The order of markers indicated by the experiments is *glpP$_I$18-glpK21-glpD6*.

Linkage between glpK21 and glpD6 to argC and gtaC by PBS transduction. Both *glpK21* and *glpD6* were linked to *argC* by PBS1 transduction (Table 10). The crosses were performed only in one direction, selecting for Arg$^+$ recombinants, since these recombinants are frequently lost when Arg$^+$ is used as unselected marker (36). Strain BR95 GlpP$_I$18 is little motile and could not be transduced with PBS1 for any marker tested. No linkage was found by PBS1 transduction between *glpK21* or *glpD6* and *cysB* or *hisA*, respectively. *glpK21* and *glpD6* were 100% co-transduced with *gtaC* (Table 11); *gtaC* and *argC* showed about 10% co-transduction with PBS1 (36).

Three-factor transformation crosses with Glp mutants and GtaC. All Glp mutations tested were linked to *GtaC* by transformation (Table 12). The relative order of the Glp mutations was next determined in three-factor transformation crosses involving two different Glp

TABLE 7. *Activities of glycerol-catabolizing enzymes in revertants from a pleiotropic Glp mutant*[a]

Strain	Inducer	Glucose	Sp act	
			Glycerol kinase	GP dehydrogenase
GlpP$_I$18-R2		–	25	72
GlpP$_I$18-R2	40 mM DL-α-GP	–	28	77
GlpP$_I$18-R2		+	32	103
BR95 GlpP$_I$18-R9		–	39	63
BR95 GlpP$_I$18-R9	40 mM DL-α-GP	–	31	56
BR95 GlpP$_I$18-R9		+	31	84
W168		–	32	3
W168	40 mM DL-α-GP	–	122	118
W168	40 mM DL-α-GP	+	26	4
W168		+	17	2

[a] Activities in the wild type are given in the last four lines. The procedures for growth of cells, preparation of enzyme extracts, and enzyme assays were as described in Materials and Methods, except that glucose (5 g/liter) was present in the growth medium during the induction period when indicated in the Table.

mutations and the *gtaC* marker in different combinations. Selection was always made for Glp$^+$ recombinants, and the distribution of the *gtaC* alleles among these recombinants was scored. We assume that the least frequent class of recombinants is the one requiring the largest number of crossovers. The results of these crosses are given in Table 13. The crosses are best compatible with the following marker order: *glpP$_I$18-glpK21-glpD6-gtaC*. This order agrees with that derived from recombination index and also with the two-factor transformation crosses.

Mapping of reversion GlpP$_I$18-R2. As mentioned above, GlpP$_I$18-R2 is a revertant of GlpP$_I$18; this revertant is uninducible for glycerol kinase and constitutive for GP dehydrogenase. To map the position of the R2 reversion, the following transformation cross was made. DNA was extracted from GlpP$_I$18-R2 GlpK21 and used to transform GlpD6 GtaC. GlpP$_I$18-R2 GlpK21 is an NTG-induced glycerol kinase mutant isolated from GlpP$_I$18-R2 (Table 1); it produces GP dehydrogenase constitutively and is resistant to phosphonomycin, suggesting that both the GLpP$_I$18 and the R2 mutations are retained. Glp$^+$ transformants were selected for in the cross between GlpP$_I$18-R2 Glp K21 and GlpD6 GtaC. These transformants were twice restreaked on minimal glycerol plates and then tested for the state of the *gtaC* allele and for constitutivity of GP dehydrogenase. Out of 18 recombinants tested (Table 14), seven were constitutive for GP dehydrogenase. The distribution of the *gtaC* marker was not significantly different among the constitutive recombinants (2 out of 7) compared with the recombinants which had an inducible GP dehydrogenase (4 out of 11). Since the order of the *glp* markers is *glpP$_I$18-glpK21-glpD6*, and we select primarily for a crossover between *glpK* and *glpD*, the results of the above cross are not compatible with a location of reversion R2 close to or to the left of *glpK21*. Instead, the results are best

TABLE 9. *Two-factor transformation crosses between different glp mutants*[a]

DNA donor	Recipient	Recombination index
GlpP$_I$18	BR95 GlpD6	0.39 ± 0.04
GlpD6	BR95 GlpP$_I$18	0.40 ± 0.02
BR95 GlpK21	BR95 GlpD6	0.24 ± 0.03
BR95 GlpK21	BR95 GlpP$_I$18	0.18 ± 0.04

[a] Each cross was performed with three different DNA concentrations in the range of 0.01 to 0.5 μg/ml. The recombination index was calculated as the ratio between the frequency of Glp transformants in the cross between two Glp mutants and the frequency of Glp$^+$ transformants in a cross between the same recipient and wild-type DNA. The transformant frequencies are standardized by reference to the unlinked *trpC* marker (BR95 GlpD6 and BR95 GlpP$_I$18 are Trp$^-$, and the other strains are Trp$^+$).

TABLE 10. *PBS1-mediated linkage between argC and the glpD6 and glpK21 loci*[a]

Donor	Total no. of recombinants tested	Co-transduction of the unselected *glp* marker[b]
BR95 GlpD6	260	4
BR95 GlpK21	260	6

[a] Recipient was BR85 (TrpC2 ArgC4); selected phenotype was Arg$^+$; unselected phenotype was Glp.
[b] [(Arg$^+$ Glp recombinants)/(total number of Arg$^+$ recombinants)] × 100.

TABLE 11. *PBS1-mediated linkage between the glpD6 and glpK21 loci and gtaC*[a]

Recipient	Total no. of recombinants tested	Co-transduction of the unselected marker *gtaC*[b]
BR95 GlpD6	104	100
BR95 GlpK21	80	100

[a] Donor was GtaC; selected phenotype was Glp$^+$; unselected phenotype was GtaC.
[b] [(Glp$^+$ GtaC recombinants)/(total number of Glp$^+$ recombinants)] × 100.

TABLE 8. *Distribution of markers in density transfer transformation experiments*

Percent DNA in hybrid density region[a]	Percent transformants obtained with hybrid density DNA[b]							
	hisA	*purB*	*cysB*	*glpD6*	*glpP$_I$18*	*argC*	*metC*	*ura-1*
10	16	5	4	0	0	0	0	0
15	33	9	8	2	2	0	0	0
21	46	22	25	5	10	2	0	0
26	46	43	43	26	25	23	5	1

[a] [(^{3}H counts/min in hybrid region)/(total count/min)] × 100.
[b] [(Number of transformants obtained with hybrid density DNA)/(total number of transformants)] × 100. The recipients in the transformation experiments were BD92, BR51, BR56, BR85, BR95 GlpD6 and BR95 GlpP$_I$18.

TABLE 12. *Transformation linkage between gtaC and different glp loci*[a]

Recipient	Total no. of recombinants tested	Co-transformation of the unselected marker *gtaC*[b]
BR95 GlpP$_I$12-6	100	56
BR95 GlpP$_I$18	297	61
BR95 GlpP$_I$9-3	104	68
GlpK3-7	208	66
BR95 GlpK21	312	67
BR95 GlpK11-4	104	78
BR95 GlpD6	312	77
GlpD12-11	153	79
GlpD8-2	104	86
GlpD13-10	152	86

[a] Donor was GtaC; selected phenotype in all crosses was Glp$^+$; unselected phenotype in all crosses was GtaC.

[b] [(Glp$^+$ GtaC recombinants)/(total number of Glp$^+$ recombinants)] × 100. The final DNA concentration was generally 0.002 to 0.006 μg/ml. For BR95 GlpK21 and BR95 GlpK11-4, the DNA concentration was 0.01 to 0.02 μg/ml due to reduced transformability of these strains. The transformations of BR95 GlpP$_I$18, BR95 GlpK21 and BR95 GlpD6 were carried out at the same time with the same DNA preparation. The other transformation experiments were performed with different DNA preparations from GtaC.

compatible with a location of R2 between *glpK21* and *glpD6*, although it cannot be quite excluded that R2 is located to the right of *glpD6*.

Phosphonomycin-resistant mutants. All pleiotropic Glp mutants studied were resistant also to the antibiotic phosphonomycin. In several bacteria this drug is known to irreversibly inhibit pyruvate-uridine diphospho-*N*-acetylglucosamine transferase (15), and can be taken into the cells by the same mechanism as GP. We also found that our strains of *B. subtilis* were sensitive to phosphonomycin. Ten independent resistant mutants of strain W168 were isolated. Eight of those could no longer grow on GP but did grow on glycerol as sole carbon source. The mutation simultaneously giving resistance to phosphonomycin and inability to grow on GP can be transformed into BR95 at a limiting DNA concentration. We have not yet mapped the position of these mutations on the *B. subtilis* chromosome.

Glycerol auxotrophic mutants: Glyc mutants. Three independent glycerol requiring mutants were isolated from NTG-treated W168. The enzymatic defect in these mutants has not been identified (28). All three mutations were linked to *trpC* and *hisB*, the order of markers being *glyc-trpC-hisB* (Tables 15 and 16).

TABLE 13. *Ordering of glpK, glpD and glpP by three-factor transformation crosses involving the gtaC locus*[a]

Cross[b]	Total no. of recombinants tested	Frequency of GtaC in the recombinants
D: + *glpD6 gtaC* R: *glpK21* + +	156	0.14
D: *glpK21* + *gtaC* R: + *glpD6* +	212	0.59
D: + *glpD6 gtaC* R: *glpK3-7* + +	104	0.19
D: *glpK3-7* + *gtaC* R: + *glpD6* +	156	0.62
D: + *glpK21 gtaC* R: *glpP$_I$18* + +	156	0.11
D: *glpP$_I$18* + *gtaC* R: + *glpK21* +	156	0.76
D: + *glpK3-7 gtaC* R: *glpP$_I$18* + +	155	0.12
D: *glpP$_I$18* + *gtaC* R: + *glpK3-7* +	156	0.65
D: + *glpD6 gtaC* R: glpP$_I$18 + +	304	0.03
D: *glpP$_I$18* + *gtaC* R: + *glpD6* +	312	0.55
D: + *glpK21 gtaC* R: *glpP$_I$9-3* + +	104	0.23
D: + *glpK21 gtaC* R: *glpP$_I$12-6* + +	94	0.21
D: + *glpD6 gtaC* R: *glpK11-4* + +	156	0.09
D: *glpK21* + *gtaC* R: + *glpD8-2* +	88	0.85
D: *glpK21* + *gtaC* R: + *glpD12-11* +	104	0.73
D: *glpD6* + *gtaC* R: + *glpD13-10* +	133	0.81

[a] Selected phenotype in all crosses was Glp$^+$; unselected phenotype in all crosses was GtaC.

[b] D was relevant donor genotype; R was relevant recipient genotype. *glpK*, *glpD*, *glpP*, and *gtaC* denote the mutant alleles; + indicates the corresponding wild-type allele. All donors had the genetic background of the *gtaC* strain, whereas all recipients with the exception for those in the last three crosses had the BR95 genetic background. *glpD8-2*, *glpD12-11*, and *glpD13-10* were in W168.

DISCUSSION

Our results confirm Mindich's earlier findings (27) that the main pathway for glycerol dissimilation in *B. subtilis* proceeds by way of a glycerol kinase and an NAD-independent GP dehydrogenase. We have found that, in *B. subtilis* as in *E. coli* (19), both enzymes are inducible. The basal level of glycerol kinase in uninduced cells, however, is sufficiently high to allow growth on glycerol (e.g., mutant GlpP$_I$18-R2).

TABLE 14. *Result of transformation cross between gtaC glpD6 and glpP$_I$18-R2 glpK21*[a]

No. of recombinants			
R2$^+$ GtaC$^+$	R2$^+$ GtaC	R2 GtaC$^+$	R2 GtaC
7	4	5	2

[a] Donor was GlpP$_I$18-R2 GlpK21; recipient was GtaC GlpD6; selected phenotype was Glp$^+$. R2 and GtaC denote the mutant characters (constitutive GP dehydrogenase activity and ϕ29 resistance, respectively); R2$^+$ and GtaC$^+$ denote the wild type. The final DNA concentration was 0.005 μg/ml. None of the Glp$^+$ transformants had been transformed for the unlinked lysine marker.

TABLE 15. *Transformation linkage between trpC2 and glyc*[a]

Donor	Total no. of recombinants tested	Co-transformation of unselected markers	
		glyc[b]	*hisB*$^+$[c]
glyc1	104	28	75
glyc2	104	34	72
glyc3	104	36	71

[a] Recipient was BR98 (*trpC2 hisB ura-2*); selected phenotype was Trp$^+$.

[b] [(Trp$^+$ Glyc recombinants)/(total number of Trp$^+$ recombinants)] $\times$ 100.

[c] [(Trp$^+$ HisB$^+$ recombinants)/(total number of Trp$^+$ recombinants)] $\times$ 100. The final DNA concentration was 0.002 to 0.005 μg/ml.

TABLE 16. *Three-factor transformation crosses between trpC2, hisB, and glyc3*[a]

Cross	Total no. of recombinants tested	Frequency of glyc in the recombinants
D: *glyc3* + *hisB* R: + *trpC* +	104	0.26
D: *glyc3 trpC* + R: + + *hisB*	104	0.07

[a] Selected phenotype was TrpC$^+$ HisB$^+$. D is relevant donor genotype; R is relevant recipient genotype. *trpC*, *hisB*, and *glyc3* denote the mutant alleles; + denotes the corresponding wild-type alleles. The recipient in the first cross was BR95, in the second cross a BR98 derivative. Both the donors had the BR98 genetic background.

Several mutants which specifically lack either glycerol kinase or GP dehydrogenase activity have been isolated. Definite proof that these mutations define the structural genes for respective enzymes must await the isolation of mutants with altered properties of the enzymes. However, since all four *glpD* mutations and all three *glpK* mutations analyzed mapped close to each other, respectively, and since we did not find any properties in the GlpK or GlpD mutants that could not be accounted for by their specific enzymatic defects, we assume that *glpK* and *glpD* define the structural genes for glycerol kinase and GP dehydrogenase.

In *E. coli*, transport systems for GP and for glycerol have been defined and shown to be under the same genetic control as the structural genes for glycerol kinase and GP dehydrogenase. We were not able to obtain good data on the rate of uptake of glycerol or GP (34), probably due to the cells content of poly-GP. However, indirect experiments strongly suggest that in *B. subtilis* also there is a specific uptake system for GP, which is subject to control by the same regulatory genes that regulate the activity of the *glpK* and *glpD* genes. In the presence of GP, we find that *B. subtilis* becomes phenotypically resistant to the antibiotic phosphonomycin, a finding which we interpret to indicate competition for a common transport system (15). All pleiotropic mutants (see later) studied were resistant to phosphonomycin in addition to being noninducible for glycerol kinase and GP dehydrogenase.

In *E. coli* Lin and co-workers have conclusively shown that GP is the inducer of the *glp* genes (14, 19). In *B. subtilis* also GP is the inducer for *glpD* and probably also for *glpK* (34). We find that in mutants lacking glycerol kinase activity GP but not glycerol will induce GP dehydrogenase. In mutants lacking GP dehydrogenase activity, glycerol kinase is internally induced. The internal concentration of GP is increased in these mutants (30).

In *E. coli* the genes for glycerol kinase, GP dehydrogenase, and for transport of GP map at separate locations and constitute three separate operons. These operons are subject to negative control by the product of a fourth, regulatory gene. Mutations at this regulatory locus can give noninducibility, constitutivity, or temperature-sensitive constitutivity of the *glp* genes (6). The control system for the *glp* genes in *E. coli* is thus similar to that of the classical *lac* operon.

Besides *glpK* and *glpD*, we identified in *B. subtilis* a third *glp* locus, called *glpP$_I$*. The *glpP$_I$* locus was closely linked to *glpK* and *glpD*, the order being *glpP$_I$-glpK-glpD*. Mutations at *glpP$_I$* gave pleiotropic effects; GlpP$_I$ mutants had noninducible levels of glycerol kinase and GP dehydrogenase and had increased resistance to phosphonomycin. GlpP$_I$ mutants could not grow with either glycerol or glycerophosphate as a sole carbon source; growth was actually inhibited by these substances, indicating that they

could enter $GlpP_I$ mutants. $GlpP_I$ mutants could grow on several other carbohydrates. Three types of revertants of mutant $GlpP_I$-18 were isolated. One class had a wild-type phenotype. A second class could grow on glycerol at 45 C but grew very poorly on glycerol at 32 C. These ts revertants were inducible for *glpK* and *glpD* at 45 C and produced nearly normal activities of glycerol kinase and GP dehydrogenase at this temperature in the presence of inducer. They were sensitive to phosphonomycin at 45 C. A third class of revertants, $GlpP_I$18-R2, produced GP dehydrogenase constitutively but remained noninducible for glycerol kinase and resistent to phosphonomycin. GP dehydrogenase was not glucose repressed in these revertents. The R2 reversion maps at a locus distinct from $glpP_I$, probably between *glpK* and *glpD*. The R2 reversion probably represents a new, or altered, promoter-operator region for *glpD*.

All of the above properties suggest that $glpP_I$ defines a locus involved in regulation of *glpK* and *glpD* activity. Although *glpK* and *glpD* are closely linked by transformation and may lie adjacent to each other, no decision can as yet be made whether they are part of a single or of two separate operons. To study these problems, additional pleiotropic glp mutants must be isolated and characterized.

ACKNOWLEDGMENTS

Kerstin Nilsson provided able technical assistance.

This investigation was supported by grants from the Swedish Cancer Society (73:135, 627-B73-02XA) and from Karolinska Institutets Forskningsfonder.

LITERATURE CITED

1. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741-746.
2. Armstrong, J. J., J. Baddiley, and J. G. Buchanan. 1961. Further studies on the teichoic acid from *Bacillus subtilis* walls. Biochem. J. **80**:254-261.
3. Bishop, D. G., L. Rutberg, and B. Samuelsson. 1967. The chemical composition of the cytoplasmatic membrane of *Bacillus subtilis*. Eur. J. Biochem. **2**:448-453.
4. Borenstein, S., and E. Ephrati-Elizur. 1969. Spontaneous release of DNA in sequential genetic order by *Bacillus subtilis*. J. Mol. Biol. **45**:137-152.
5. Burger, M. M., and L. Glaser. 1964. The synthesis of teichoic acids. I. Polglycerophosphate. J. Biol. Chem. **239**:3168-3177.
6. Cozzarelli, N. R., W. B. Freedberg, and E. C. C. Lin. 1968. Genetic control of the L-α-glycerophosphate system in *Escherichia coli*. J. Mol. Biol. **31**:371-387.
7. Cozzarelli, N. R., J. P. Koch, S. Hayashi, and E. C. C. Lin. 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. **90**:1325-1329.
8. Cozzarelli, N. R., and E. C. C. Lin. 1966. Chromosomal location of the structural gene for glycerol kinase in *Escherichia coli*. J. Bacteriol. **91**:1763-1766.
9. Dubnau, D., C. Goldthwaite, I. Smith, and J. Marmur. 1967. Genetic mapping of *Bacillus subtilis*. J. Mol. Biol. **36**:163-185.
10. Freedberg, W. B., and E. C. C. Lin. 1973. Three kinds of controls affecting the expression of the *glp* regulon in *Escherichia coli*. J. Bacteriol. **115**:816-823.
11. Gay, P., P. Cordier, M. Marquet, and A. Delobbe. 1973. Carbohydrate metabolism and transport in *Bacillus subtilis*. A study of ctr mutations. Molec. Gen. Genet. **121**:355-368.
12. Hayashi, S., J. P. Koch, and E. C. C. Lin. 1964. Active transport of L-α-glycerophosphate in *Escherichia coli*. J. Biol. Chem. **239**:3098-3105.
13. Hayashi, S., and E. C. C. Lin. 1965. Capture of glycerol by cells of *Escherichia coli*. Biochim. Biophys. Acta **94**:479-487.
14. Hayashi, S., and E. C. C. Lin. 1965. Product induction of glycerol kinase in *Escherichia coli*. J. Mol. Biol. **14**:515-521.
15. Hendlin, D., E. O. Stapely, M. Jackson, H. Wallick, A. K. Miller, F. J. Wolf, T. W. Miller, L. Chaiet, F. M. Kahan, E. L. Foltz, H. B. Woodruff, J. M. Mata, S. Hernandez, and S. Mochales. 1969. Phosphonomycin, a new antibiotic produced by strains of *Streptomyces*. Science **166**:122-123.
16. Hoch, J. A. 1971. Genetic analysis of pleiotropic negative sporulation mutants in *Bacillus subtilis*. J. Bacteriol. **105**:896-901.
17. Hsu, C. C., and C. F. Fox. 1970. Induction of the lactose transport system in a lipid-synthesis-defective mutant of *Escherichia coli*. J. Bacteriol. **103**:410-416.
18. Kistler, W. S., and E. C. C. Lin. 1971. Anaerobic L-α-glycerophosphate dehydrogenase of *Escherichia coli*: its genetic locus and its physiological role. J. Bacteriol. **108**:1224-1234.
19. Koch, J. P., S. Hayashi, and E. C. C. Lin. 1964. The control of dissimilation of glycerol and L-α-glycerophosphate in *Escherichia coli*. J. Biol. Chem. **239**:3106-3108.
20. Lin, E. C. C., J. P. Koch, T. M. Chused, and S. E. Jorgensen. 1962. Utilization of L-α-glycerophosphate by *Escherichia coli* without hydrolysis. Proc. Nat. Acad. Sci. U.S.A. **48**:2145-2150.
21. Lin, E. C. C., A. P. Levin, and B. Magasanik. 1960. The effect of aerobic metabolism on the inducible glycerol dehydrogenase of *Aerobacter aerogenes*. J. Biol. Chem. **235**:1824-1829.
22. Lin, E. C. C., and B. Magasanik. 1960. The activation of glycerol dehydrogenase from *Aerobacter aerogenes* by monovalent cations. J. Biol. Chem. **235**:1820-1823.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
24. Magasanik, B., M. S. Brooke, and D. Karibian. 1953. Metabolic pathways of glycerol dissimilation. A comparative study of two strains of *Aerobacter aerogenes*. J. Bacteriol. **66**:611-619.
25. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208-218.
26. Miki, T., Z. Minami, and Y. Ikeda. 1965. The genetics of alkaline phosphatase formation in *Bacillus subtilis*. Genetics **52**:1093-1100.
27. Mindich, L. 1968. Pathway for oxidative dissimilation of glycerol in *Bacillus subtilis*. J. Bacteriol. **96**:565-566.
28. Mindich, L. 1970. Membrane synthesis in *Bacillus subtilis*. I. Isolation and properties of strains bearing mutations in glycerol metabolism. J. Mol. Biol. **49**:415-432.
29. Mindich, L. 1970. Membrane synthesis in *Bacillus subtilis*. II. Integration of membrane proteins in the absence of lipid synthesis. J. Mol. Biol. **49**:433-439.
30. Oh, Y. K., E. B. Freese, and E. Freese. 1973. Abnormal

septation and inhibition of sporulation by accumulation of L-α-glycerophosphate in *Bacillus subtilis* mutants. J. Bacteriol. **113**:1034-1045.
31. Op den Kamp, J. A. F., I. Redai, and L. L. M. van Deenen. 1969. Phospholipid composition of *Bacillus subtilis*. J. Bacteriol. **99**:298-303.
32. O'Sullivan, A., and N. Sueoka. 1969. Sequential replication of the *Bacillus subtilis* chromosome. IV. Genetic mapping by density transfer experiment. J. Mol. Biol. **27**:349-368.
33. Saheb, S. A. 1972. Etude de deux mutants du métabolisme du glycérol chez *Bacillus subtilis*. Can. J. Microbiol. **18**:1315-1325.
34. Saheb, S. A. 1972. Perméation du glycérol et sporulation chez *Bacillus subtilis*. Can. J. Microbiol. **18**:1307-1313.
35. Willecke, K., and L. Mindich. 1971. Induction of citrate transport in *Bacillus subtilis* during the absence of phospholipid synthesis. J. Bacteriol. **106**:514-518.
36. Young, F. E., C. Smith, and B. E. Reilly. 1969. Chromosomal location of genes regulating resistance to bacteriophage in *Bacillus subtilis*. J. Bacteriol. **98**:1087-1097.