Glycerol-deprival Autolysis in a *Bacillus subtilis* Mutant Requiring Large Amounts of Glycerol

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(Received 30 June 1977)

A mutant (strain G311) of *Bacillus subtilis* w23, which requires abnormally large amounts of glycerol, was isolated and characterized. Glycerol consumption is high in the mutant even in the presence of glucose. With limiting glycerol, growth stops in mid-exponential phase and autolysis occurs (glycerol-deprival autolysis). During this autolysis the cell wall lytic enzyme seems to be more active. Inhibitors of protein synthesis or energy supply prevented both autolysis and increase in the activity of the wall lytic enzyme. Synthesis of glycerol-catabolizing enzymes in G311 was less sensitive to catabolite repression than in the parent strain. The results suggest that G311 has a defect in the regulation of the *glp* system and that exhaustion of glycerol causes an increase in autolysis.

INTRODUCTION

Glycerol-requiring mutants which lack glycerol-catabolizing enzymes are useful tools in studies on phospholipid metabolism (Mindich, 1970). It is easy to stop phospholipid synthesis by glycerol deprivation and to specifically label lipids with radioactive glycerol in strains with both defects. In a search for glycerol auxotrophs of *Bacillus subtilis*, a mutant (designated G311) was obtained which required abnormally large amounts of glycerol and autolysed after cessation of growth due to glycerol starvation.

The pathway of glycerol dissimilation in *B. subtilis* has already been established (Mindich, 1968; Lindgren & Rutberg, 1974). Glycerol is first phosphorylated with ATP to glycerol 3-phosphate by glycerol kinase and then dehydrogenated by NAD-independent glycerol-3-phosphate dehydrogenase to dihydroxyacetone phosphate which can enter the common pathway of intermediary metabolism. The first two enzymes for glycerol dissimilation are known to be synthesized after induction with glycerol or glycerol 3-phosphate and are coded by a set of genes designated *glp*. The regulatory system of *glp* genes has been described by Lindgren & Rutberg (1976). However, the pathway of glycerol synthesis in *B. subtilis* and the enzymic defects in glycerol auxotrophs have not been identified. It is possible that G311 is defective not only in glycerol or glycerol 3-phosphate synthesis but also in the regulation of glycerol dissimilation. The present report concerns the characteristics and mechanism of glycerol-deprival autolysis, which is a novel type of bacterial autolysis, and the induction pattern of glycerol-catabolizing enzymes in strain G311.

METHODS

Bacterial strains, media and growth. *Bacillus subtilis* w23 (Stm⁺, Met⁻, Ade⁻) was used as the parent strain. Strain G311, a glycerol-requiring mutant, was isolated after treatment of strain w23 with ethyl methane-sulphonate (2%, w/v) and plating on GAA agar plates (see below) with or without glycerol (200 µg ml⁻¹). Strain B71, kindly provided by Dr L. Mindich, is a *glp* and *glyc* (= auxotrophic for glycerol; Mindich, 1970) mutant of w23. GAA medium contained 0.5% (w/v) glucose and a mixture of amino acids approximating 5 mg casein hydrolysate ml⁻¹. For liquid cultures, the basic medium contained 0.5% (w/v) of vitamin-free Casamino acids (Nissui Pharmaceutical Co., Tokyo, Japan) and 0.05% (w/v) yeast extract (Daigo Eiyo Chemical Co., Osaka, Japan). Where indicated glycerol and/or glucose, each at 0.5% final concentration, were added to induce or repress the *glp* system. Adenine sulphate (10 µg ml⁻¹) was added to all media, and agar plates always contained 50 µg streptomycin ml⁻¹. All ingredients were dissolved in an inorganic salts solution (Koga & Kusaka, 1968) and the pH was adjusted to 7.2. Bacteria were grown at 37 °C with shaking. Growth was measured in a Kotaki 5D turbidimeter (660 nm filter); 100 units corresponded to 6.0 × 10⁸ bacteria ml⁻¹.

Determination of cell wall autolysis. Bacteria were disrupted by sonication (100 W) and the sonicate was centrifuged at 1000 g for 30 min to remove intact cells. The supernatant was centrifuged again at 10000 g for 10 min and the pellet was washed three times with 50 mM-Tris/HCl buffer (pH 7.4). The whole procedure was carried out at 4 °C. The walls were suspended in 0.01 M-phosphate buffer (pH 7.5) and their autolysis was followed turbidimetrically with a Shimadzu UV200 spectrophotometer at 650 nm.

Measurement of radioactivity. Aqueous samples (1.0 ml) or dried membrane filters were mixed with 10 ml toluene/Triton X-100 (2:1, v/v) scintillation fluid (Patterson & Greene, 1965) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Preparation of cell extracts and enzyme assays. Bacteria were harvested at mid-exponential phase by centrifugation, and washed once in 0.01 M-phosphate buffer (pH 7.5). Cell extracts were prepared by grinding with quartz powder or by sonication in the same buffer. The quartz powder and cell debris were removed by low-speed centrifugation and the supernatant was immediately used for the enzyme assays. NAD-independent glycerol-3-phosphate dehydrogenase [EC 1.1.99.5; *sn*-glycerol-3-phosphate: (acceptor) oxidoreductase] was determined spectrophotometrically with 2,6-dichlorophenolindophenol as hydrogen acceptor. The reaction mixture (final volume 3 ml) contained (µmol): sodium phosphate (pH 7.5), 190; 2,6-dichlorophenolindophenol, 0.129; sodium DL-α-glycerophosphate, 10; KCN, 30; cell extract and water. The molar extinction coefficient of 2,6-dichlorophenolindophenol at 600 nm was taken as 21.5 × 10³ l mol⁻¹ cm⁻¹ at pH 7.5 (Armstrong, 1964). Glycerol kinase [EC 2.7.1.30; ATP: glycerol 3-phosphotransferase] was estimated by NAD⁺ reduction coupled with glycerol-3-phosphate dehydrogenase [EC 1.1.1.8; *sn*-glycerol-3-phosphate: NAD⁺ 2-oxidoreductase] from rabbit muscle as described by Lin *et al.* (1962). Enzyme activities were expressed as nmol 2,6-dichlorophenolindophenol or NAD⁺ reduced min⁻¹ (mg protein)⁻¹ at 25 °C. The protein concentrations of the extracts were measured according to Lowry *et al.* (1951) with bovine serum albumin as standard.

Chemicals. Crystalline rabbit muscle glycerol-3-phosphate dehydrogenase and NAD were purchased from Boehringer; adenine sulphate, 2,6-dichlorophenolindophenol, hydrazine hydrate and glycerol from Wako Pure Chemical Industries, Osaka, Japan; ATP from Daiichi Pure Chemicals Co., Tokyo, Japan; sodium DL-α-glycerophosphate from Tokyo Kasei Kogyo Co., Tokyo, Japan; [1,3-¹⁴C]glycerol (> 40 mCi mmol⁻¹) from New England Nuclear; chloramphenicol from Fujisawa Pharmaceutical Co., Osaka, Japan; streptomycin sulphate from Ban-yu Pharmaceutical Co., Tokyo, Japan.

RESULTS AND DISCUSSION

Glycerol-deprivation autolysis

Strain G311 did not grow in GAA medium without glycerol but grew normally in GAA medium containing 200 µg glycerol ml⁻¹ (Fig. 1). The parent strain w23 did not require glycerol. For spore formation in G311, the glycerol concentration required was about 10 times higher than that required for vegetative growth. In medium with limiting amounts of glycerol (80 µg ml⁻¹), the growth rate of G311 was similar to that in the presence of sufficient glycerol, but growth stopped in mid-exponential phase and 30 min later the cells began to lyse (Fig. 1). The level of growth at which autolysis was initiated depended on the amount of glycerol present in the medium (glycerol-deprivation autolysis, Fig. 2).

Fig. 1

Fig. 2

Fig. 1. Growth of *B. subtilis* G311 and B71 in GAA medium at 37 °C in the presence of glycerol at 200 (○), 80 (●) and 0 $\mu\text{g ml}^{-1}$ (□) for G311 and 20 $\mu\text{g ml}^{-1}$ (■) for B71.

Fig. 2. Growth and autolysis of *B. subtilis* G311 in the presence of limiting amounts of glycerol. Glycerol was supplied to the medium at 100 (○), 50 (●) and 25 $\mu\text{g ml}^{-1}$ (□).

Effect of inhibitors on glycerol-deprivation autolysis

Glycerol-deprivation autolysis was inhibited by chloramphenicol (100 $\mu\text{g ml}^{-1}$, Fig. 3), 2,4-dinitrophenol or NaN_3 (data not shown). These results suggest that lysis depends on protein synthesis or energy supply. Effective inhibition by chloramphenicol was achieved when the drug was added before or just at the initiation of autolysis, but when it was added to a culture after autolysis had begun, the rate of lysis was little affected (Fig. 3). Autolysis of isolated cell walls indicated that wall lytic activity increased during the 30 min after cessation of growth, i.e. walls prepared from a culture 30 min after cessation of growth autolysed faster than those prepared at the time when growth first ceased (Table 1). This increase in the rate of autolysis of isolated walls did not occur when chloramphenicol, 2,4-dinitrophenol or NaN_3 was added to the glycerol-limiting culture at the time of maximum growth (Table 2). The similarities between the increases or inhibition of autolysis in cells and walls suggests that a wall lytic enzyme is possibly involved in glycerol-deprivation autolysis.

Glycerol consumption by strain G311

The glycerol concentration required for growth of G311 was about 10 times higher than that for B71, a *glyc* and *glp* mutant of *B. subtilis* (Fig. 1). This high glycerol requirement might be due to abnormal consumption of this compound. Consumption of glycerol by G311 was estimated by following the loss of radioactivity from a culture grown in the presence of glycerol (200 $\mu\text{g ml}^{-1}$) and [$1,3\text{-}^{14}\text{C}$]glycerol (0.067 $\mu\text{Ci ml}^{-1}$). At intervals, 1 ml samples were taken and filtered through a membrane (0.45 μm pore size; Toyo Filter Paper Co., Tokyo, Japan), and the radioactivity in the bacteria and culture filtrates was measured. About 45% of the glycerol (90 $\mu\text{g ml}^{-1}$) was lost from the culture to the air and only 25% (50 $\mu\text{g ml}^{-1}$) was assimilated by the bacteria (Fig. 4). The radioactive material remaining in the medium after growth may contain products derived from glycerol. Calculations showed that more than 140 $\mu\text{g glycerol ml}^{-1}$ in the medium was consumed by G311 even in the presence of glucose which should have a repressive effect on the *glp* system. We decided, therefore, to determine the induction pattern of the glycerol dissimilation enzymes in G311.

Table 1. *Lysis of isolated cell walls*

Walls of strain G311 grown in the presence of $100\ \mu\text{g}$ glycerol ml^{-1} were isolated 30 min before (1), just at (2) and 30 min after (3) the time when maximum growth was reached. Isolated walls were incubated at 37°C and their autolysis was evaluated turbidimetrically.

Walls isolated at stage:	Rate of autolysis (% decrease in $E_{650}\ \text{min}^{-1}$)
1	1.41
2	1.65
3	2.74

Table 2. *Effect of chloramphenicol on cell wall autolysis*

Strain G311 was grown in the presence of $100\ \mu\text{g}$ glycerol ml^{-1} and chloramphenicol ($100\ \mu\text{g}\ \text{ml}^{-1}$) was added to the culture at the time of maximum growth. Walls were isolated at the indicated times after the addition of chloramphenicol and their rates of autolysis were determined.

Time of isolation (min)	Rate of autolysis (% decrease in $E_{650}\ \text{min}^{-1}$)
30	1.4
60	1.4
90	1.7
120	1.0

Fig. 3

Fig. 3. Effect of chloramphenicol on autolysis in *B. subtilis* G311 grown in the presence of $100\ \mu\text{g}$ glycerol ml^{-1} . The culture was divided into five equal portions; chloramphenicol ($100\ \mu\text{g}\ \text{ml}^{-1}$) was added to cultures 1 to 4 (○, □, △, ▽, respectively) at the times indicated by the arrows and incubation was continued. Chloramphenicol was not added to the fifth culture (●).

Fig. 4

Fig. 4. Dissimilation of glycerol in *B. subtilis* G311 grown in glucose/Casamino acids medium with $[1,3-^{14}\text{C}]$ glycerol ($0.067\ \mu\text{Ci}\ \text{ml}^{-1}$) and $200\ \mu\text{g}$ glycerol ml^{-1} . Radioactivity was measured in 1 ml samples of culture (○), culture filtrate (□) and bacteria (△). Growth (●) was measured turbidimetrically at 660 nm.

Activities of glycerol-catabolizing enzymes

Extracts of strain w23 grown with Casamino acids as sole carbon source had low activities of glycerol-3-phosphate dehydrogenase and glycerol kinase (Table 3). When glycerol was added to the culture, synthesis of both enzymes was induced and this synthesis was repressed by the addition of glucose (glucose repression or catabolite repression, Table 3). These results are similar to those of Lindgren & Rutberg (1974). Since G311 does not grow in the absence of glycerol, enzyme activities were compared in bacteria grown in the presence of limiting ($50\ \mu\text{g}\ \text{ml}^{-1}$) and excess ($5\ \text{mg}\ \text{ml}^{-1}$) glycerol. In G311, glycerol kinase activity was

Table 3. *Activities of enzymes of glycerol catabolism*

Activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. Values quoted are averages of at least two determinations.

Carbon source	Glycerol kinase activity		Glycerol-3-phosphate dehydrogenase activity	
	Strain w23	Strain G311	Strain w23	Strain G311
Casamino acids	6	—	5	—
Casamino acids + glycerol ($50 \mu\text{g ml}^{-1}$)	13	129	6	0
Casamino acids + glycerol (5 mg ml^{-1})	44	96	80	22
Casamino acids + glycerol (5 mg ml^{-1}) + glucose (5 mg ml^{-1})	0	66	4	11

slightly higher in bacteria grown in limiting glycerol, and was much higher than in the parent strain. Enzyme formation in w23 was completely repressed by glucose, but in G311 it was not. The glycerol kinase activity in G311 growing in the presence of glucose was very high (Table 3).

The formation of NAD-independent glycerol-3-phosphate dehydrogenase was inducible in both strains (Table 3). However, in the presence of glucose, enzyme formation was repressed only by 50% in G311, whereas in w23 it was decreased to 5%. The activity in the presence of glucose was much higher in G311 than in w23.

These results suggest that formation of glycerol kinase and glycerol-3-phosphate dehydrogenase, the key enzymes for glycerol catabolism in *B. subtilis*, is not effectively repressed by glucose in G311 and large amounts of glycerol would, therefore, be consumed even in the presence of glucose.

Cell wall lytic activity was involved in the lysis of G311 as described above. However, the process by which glycerol exhaustion results in autolysis is as yet unknown. Several factors which induce bacterial autolysis have been reviewed by Ogata (1976). In general, these factors seem to cause physiological disturbances in the bacteria. In many cases, there appears to be no activation nor derepressed synthesis of a lytic enzyme but rather a change in the spatial relationship of the bound wall lytic enzyme and its substrate, which results in an apparent increase of the rate of wall autolysis. It is likely that glycerol starvation in G311 induces similar effects through cessation of synthesis of membrane lipid. Although the wall of strain w23 contains ribitol teichoic acid, there is also some glycerol teichoic acid in the membrane (Coley, Duckworth & Baddiley, 1975). Cessation of synthesis of glycerol teichoic acid cannot be excluded as a possible cause of glycerol-deprival autolysis.

The fact that walls prepared from bacteria treated with inhibitors did not show an increased rate of autolysis may suggest that autolysis is the result of synthesis or derepression of a lytic enzyme. However, the preventive effect of the inhibitors on autolysis is more likely to be explained by the prevention of unbalanced synthesis of membrane material and other cellular constituents which may cause an apparent acceleration of wall lytic enzyme activity.

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