STUDIES ON MUTANTS OF *BACILLUS SUBTILIS* REQUIRING D-GLUTAMIC ACID

II. UNBALANCED GROWTH DEATH OBSERVED IN MUTANT 3d

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It is known that unbalanced synthesis of cell wall and cytoplasm brings a bacterium to death (unbalanced growth death). For example, a mutant of *Escherichia coli* incapable of synthesizing diaminopimelic acid will die of itself provided that the acid is not supplied (1, 2). The phenomenon is understood to be an indication that the synthesis of cytoplasm proceeds independently of that of cell wall in which diaminopimelic acid is an essential component, and, owing to the lack of balance, the cells perish. Because D-glutamic acid is known to be a component of cell wall in a certain kind of bacteria, a similar situation is conceivable. To test this possibility, a mutant of *Bacillus subtilis* requiring D-glutamic acid for growth was placed in a medium devoid of D-glutamic acid.

MATERIALS

Strains: A wild-type strain and the following two mutants of *B. subtilis* K were used. 3d is a mutant which is unable to synthesize D-glutamic acid, D-aspartic acid, and D-alanine. This inability is due to the lack of an enzyme, alanine racemase (see the following paper). 3a is a mutant which is incapable of synthesizing both D- and L-glutamic acid.

Media: MM medium and DG medium were described in the preceding paper (3). M-N is a modified minimal medium containing no nitrogen sources (ammonium chloride and ammonium nitrate).

METHODS AND RESULTS

1. *Growth of 3d in DG, MM, and M-N media*

Although growth of mutant 3d in DG and MM media had been expressed with nephelometric values, it was attempted in this experiment to produce growth curves by counting viable cells. It was done so because the nephelometric values do not always reflect real viable cell numbers. To count

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Fig. 1. Growth curves of strain 3d

MM: minimal medium, DG: MM plus 100 μ g/ml D-glutamic acid, M-N: MM minus nitrogen source.

2. Growth of 3a in DG and MM media

Similarly, mutant 3a was cultivated in DG and MM media. In contrast to mutant 3d which showed residual growth in MM medium, the viable cell numbers did not change significantly throughout the whole course of incubation. Results are shown in Figure 2.

3. Presence of D-glutamic acid in cell wall

In order to test whether or not D-glutamic acid would be a component of the cell wall of *B. subtilis* K, the following experiments were carried out. Vegetative cells were harvested from a culture of wild-type strain and ground in a mortar with fine glass beads. Residual intact cells were spun out at $1,000\times g$ for 15 minutes, and cell walls in the supernatant were collected at a higher speed ($10,000\times g$, 15 minutes). The cell walls thus obtained were

viable cells, aliquots were withdrawn periodically from the cultures on a seesaw-type shake machine, and plated onto DG agar medium after appropriate dilution. Cultivation was carried out at 37° .

As the curves in Figure 1 indicate, the viable cells in DG medium increased continuously with time lapse, while the cells in MM medium increased for a while and decreased rapidly after a lapse of seven hours. The latter type of growth curve will be referred to as "residual growth curve." It is because growth was determined nephelometrically that no residual growth was observed in Figure 1 of the preceding paper (3). When the mutant was cultivated in M-N medium, the viable cells increased but only to a negligible degree.

Fig. 2. Growth curves of strain 3a.

MM: minimal medium
DG: MM plus 100 μ g/ml D-glutamic acid

washed twice with 1 M NaCl and distilled water. The yield was 0.1 g in dry weight per 16 g wet cells.

50 mg of the dry material in 5 ml of 3N hydrochloric acid was sealed in a small tube, and heated at 110° for 20 hours. The hydrolysate was dried up *in vacuo* and humins that appeared after the addition of water filtered off. This procedure was repeated several times and finally the content was filled up to 1 ml with water. Amino acids in this solution were separated by the chromatographic technique using a mixture of solvents (butanol 4, acetic acid 1, and water 2). Glutamic acid (D or L) was extracted with water from the corresponding area on the paper.

L-Glutamic acid was assayed manometrically using glutamic acid decarboxylase prepared from *E. coli* (4), while D-glutamic acid was assayed biologically depending on the dosage-growth curve given in Figure 2 of the preceding paper (3). Total glutamic acid was assayed by the ninhydrin method (5). As a matter of fact, the extract mentioned above did support the growth of 3d, the mutant which is able to grow only in the presence of D-glutamic acid (Figure 3). When assayed biologically, 1.65 mg D-glutamic acid was found in 100 mg dried cell walls, amounting to 81.3% of the total glutamic acid contained in the material (Table 1). The remainder is L-form. Thus, the presence of D-glutamic acid in the cell wall of *B. subtilis* K was evidenced.

Fig. 3. Growth response of mutant 3d to glutamic acid fraction of cell walls and protoplasts.

MM: minimal medium, DG: MM+10 µg/ml D-glutamic acid, C-G: MM+glutamic acid fraction from cell walls, P-G: MM+glutamic acid fraction from protoplasts (C-G and P-G contain ca. 4 µg/ml glutamic acid respectively.)

Table 1. Ratio of D- and L-glutamic acid to total glutamic acid in protoplasts and cell walls

	Amount of glutamic acid in 100 mg of each fraction (mg)			
	Protoplasts 2.50		Cell walls 2.03	
Total glutamic acid				
D-Glutamic acid*	—	0.3	—	1.65
L-Glutamic acid**	2.44	—	0.29	—

* assayed biologically.

** assayed manometrically (see text).

4. *Absence of D-glutamic acid in cytoplasm*

Protoplasts were prepared to estimate the amount of D-glutamic acid in cytoplasm of the bacillus. First, 300 mg washed cells were suspended in 1 ml of the solution consisting of 20% sucrose and 0.05 M phosphate buffer (pH 7.0), and then treated with 300 μ g lysozyme for three hours at 37°. The precipitate after centrifugation at $10,000 \times g$ for 5 minutes was washed twice with 0.5 M KCl and dried. 1.5 g dried protoplast was obtained from 14 g wet cells.

The material was hydrolyzed by the method described above, and D- and L-glutamic acid were assayed. The glutamic acid fraction prepared from this material did not support the growth of 3d (Figure 3). As a result of the assay, it was found that the fraction consisted of about 98% L-glutamic acid and 2% D-glutamic acid. Because the content of D-glutamic acid in the material is too small to be considered a component of protoplast (cytoplasm and cytoplasmic membrane), it is supposed that the biological significance of D-glutamic acid in cytoplasm, if any, is negligible when compared with that in the cell wall.

DISCUSSION

As has been expected in the introduction, the cells of mutant 3d perished when they were placed in a medium devoid of D-glutamic acid. The residual growth that lasted for seven hours might be due to the surplus D-glutamic acid brought about by inoculum. At the expense of it, cell walls would have been reproduced for a while. Meanwhile, when the surplus D-glutamic acid was used up, there would be no supply of D-glutamic acid, and the cell wall formation would stop. Even at this time, synthesis of cytoplasm would proceed normally, because the strain was capable of synthesizing L-amino acids from raw materials. Thus, the synthesis of cytoplasm and cell wall would lose balance. The growth curve of mutant 3d in M-N medium supports the above mentioned view (Figure 1). In this medium, the synthesis of cytoplasm as well as that of cell wall would not have taken place because of the lack of nitrogen sources. On account of this balanced deficiency, no significant residual growth resulted. The absence of residual growth in the culture of mutant 3a in MM medium (Figure 2) is considered to be due to the same reason. The mutant can not synthesize both D- and L-glutamic acid.

D-Glutamic acid was found mainly in cell wall fraction and in a negligible amount in cytoplasm fraction. This result is in agreement with the data so far published (6). It is concluded, therefore, that the death of mutant 3d in MM medium is a similar phenomenon to that found in a diaminopimelic acid requiring mutant of *E. coli* (1, 2). In the latter case, diaminopimelic acid is known to be a component of cell wall of the bacterium.

SUMMARY

When mutant 3d is incubated in a medium devoid of D-glutamic acid, the number of viable cells increases for a while and after the lapse of seven hours begins to decrease abruptly. Because D-glutamic acid is a major component of cell wall and a minor component of cytoplasm, and because the strain cannot synthesize D-glutamic acid although it can synthesize L-glutamic acid and other L-amino acids, it is concluded that the phenomenon mentioned above is caused by the unbalanced synthesis between cell wall and cytoplasm.

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