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

III. ENZYME ACTIVITIES OF THE MUTANTS

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As reported previously (1), the five mutants of *Bacillus subtilis* K requiring D-glutamic acid for growth are classified into three groups. Mutants 1a and 2a grow on media supplemented with either L-alanine, D-alanine, D-aspartic acid or D-glutamic acid, mutants 3a and 4a on media with either L-aspartic acid, L-glutamic acid, D-aspartic acid or D-glutamic acid, and mutant 3d on media with either D-alanine, D-aspartic acid or D-glutamic acid. These dependencies were thought to be caused by the lack of enzymes involved in the biosynthesis of these amino acids. It is not unusual in microorganisms that a strain turns out incapable of producing an enzyme as a result of gene mutation (2). On this basis, several enzymes that were considered important for the biosynthesis of D- and L-glutamic acid were investigated.

MATERIALS AND METHODS

Strains: A wild-type strain and the four mutants, 2a, 3a, 4a, 3d were employed.

Media: Medium A consists of components of minimal medium (1), 0.1% nutrient broth (v/v) and 200 μ g/ml of D,L-glutamic acid. B is a medium lacking nutrient broth of medium A. Medium A is used for mutants 2a, 3a and 4a, and medium B for 3d.

Preparation of cell extract: Mutants, 2a, 3a and 4a were cultivated in 100 ml of medium A at 30° for 15–17 hours, and mutant 3d in medium B for 17–20 hours. These cultivations were carried out on a shake machine. Cells at the late logarithmic phase were harvested by centrifugation and washed several times with 0.1 M phosphate buffer (pH 7.2). The washed cells were suspended heavily in 0.01 M phosphate buffer (pH 7.2, ca. 50 mg wet cells per ml) and treated by a 10 kc sonic oscillator (Tōyō Rikō, Type 50-2) for 20 minutes. The resulting disrupted cells were centrifuged at 10,000 $\times$ g for 15 minutes, and the supernatant thus obtained was dialysed against 0.01 M phosphate buffer (pH 7.4) for 20 hours at 5°. This preparation is to be called "dialysed extract."

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Measurement of enzyme activities (1) *Racemases* — To measure the activities of enzymes (racemases) that catalyse the reaction between L-alanine and D-alanine (3) and between L-glutamic acid and D-glutamic acid (4, 5), 0.4 ml dialysed extract, 40 μ g pyridoxal phosphate and 100 μ mole L-alanine or D-glutamic acid were dissolved in 1 ml of 0.1 M phosphate buffer (pH 8.0). The mixture was incubated at 37° for 40 minutes, and D-alanine and L-glutamic acid formed were measured by the method to be described in a later section. The specific activity is defined as “amounts of D-alanine or L-glutamic acid formed (μ mole)/mg protein/hour.”

(2) *Transaminases* (6) — Transaminase activities were measured as follows. 0.4 ml dialysed extract, 40 μ g pyridoxal phosphate, and each 100 μ mole D-glutamic acid and pyruvic acid or each 100 μ mole L-alanine and α -ketoglutaric acid were mixed in 1 ml of 0.1 M phosphate buffer (pH 8.0), and D-alanine or L-glutamic acid formed after 40 minutes at 37° was measured. The specific activity is defined as “amounts of D-alanine or L-glutamic acid formed (μ mole)/mg protein/hour.”

(3) *Dehydrogenases* (7–9) — To measure the activities of L-alanine-, L-glutamic acid- or L-aspartic acid-dehydrogenases, a reaction mixture consisting of 0.4 ml dialysed extract, 10 μ mole L-alanine (or L-glutamic acid, or L-aspartic acid), 0.5 μ mole DPN (or TPN) dissolved in 3 ml sodium pyrophosphate buffer (pH 8.3) was prepared. The mixture was placed at room temperature (23°–25°), and optical density was measured at intervals using a Shimadzu spectrophotometer. Reaction after the addition of each substrate was recorded. The specific activity is defined as “change in absorption values at 340 m μ /mg protein/minute.”

Determination of L-glutamic acid and D-alanine: To stop reactions, trichloroacetic acid (TCA) was added in a concentration of 5% (w/v). The mixture was centrifuged, and then the supernatant was treated with ether to remove the TCA. L-Glutamic acid or D-alanine in the sample was assayed manometrically by using L-glutamic acid decarboxylase prepared from *Escherichia coli* for the determination of L-glutamic acid (10), and D-amino acid oxidase prepared from sheep kidney for D-alanine (11). Protein was determined by the method of Lowry et al (12) using bovine serum albumin as the standard.

Chemicals: D-, L- and DL-amino acids used in this work were supplied from Ajinomoto Co. Inc., DPN and TPN from Sigma Chemical Co., and pyridoxal phosphate, pyruvic acid and α -ketoglutaric acid from Nutritional Biochemical Cooperation.

RESULTS

Alanine and glutamic acid racemase activities

It is considered that racemases are essential catalyzers for the synthesis of D-amino acids (6). To know whether the wild-type strain and the mutants might possess the racemases or not, their cell free extracts were reacted

with D-(or L-) alanine or D-(or L-) glutamic acid in the presence of pyridoxal phosphate. The result was that the crude extract from the wild-type strain could convert D-alanine to L-alanine and *vice versa*. Meanwhile, no conversion of D-(L-)glutamic acid to L-(D-)glutamic acid was brought about by the extract (figure 1). Moreover, the latter reaction was not found even when the extract was prepared from the cells at different growth phases or under conditions where reaction mixtures of different pH values were employed. Therefore, it is concluded that alanine racemase plays an important role in the biosynthesis of D-amino acids, but glutamic acid racemase does not.

Table 1 shows racemase activities of the wild-type strain and the four mutants. Although glutamic acid racemase activity was not found in the mutants as in the case of wild-type strain, alanine racemase activities were detected in all the strains tested. Among them, the activities in mutants 3a and 4a were higher than in the wild-type, and the activity in mutant 3d was equivalent to 10% of that in the wild-type. Since the addition of crude extract of mutant 3d to the extract of the wild-type did not reduce the enzyme activity, it is assumed that the low alanine racemase activity in mutant

Fig. 1. Alanine and glutamic acid racemase activities in a wild-type strain.

The reaction mixture consists of 1 ml undialysed extract, 500 μmole alanine or glutamic acid (D or L), 100 μg pyridoxal phosphate and 0.02 M phosphate buffer (pH 8.0). The final volume is 5 ml. Substrates: ● D-alanine, ○ L-alanine, △ D-glutamic acid, ▲ L-glutamic acid.

Table 1. Alanine and glutamic acid racemase activities.

Strains	Specific activity*	
	Alanine racemase	Glutamic acid racemase
2a	72.0	0
3a	92.5	0
4a	149.5	0
3d	4.9	0
Wild strain K	55.0	0
K+3d**	60.1	—

* See text.

** K+3d indicates a mixture of dialysed extract of K and crude extract of 3d (1:1).

3d is not due to the co-existence of inhibitory substances. This supposition is supported by fact that the mutant 3d regains the normal alanine racemase activity when it reverts to prototrophs in spontaneous mutation. A similar phenomenon was observed, as shown in Table 2, even in revertants of mutants 2a, 3a and 4a.

Table 2. Alanine and glutamic acid racemase activities in revertants.

Strains**	Specific activity*	
	Alanine racemase	Glutamic acid racemase
2a-R4	60.2	0
3a-R1	71.5	0
4a-R1	50.3	0
3d-R119	66.0	0
Wild strain K	55.0	0

* See text.

** 2a-R4 is a revertant of 2a which was isolated from a plate containing minimal medium and 100 μ g/ml L-glutamic acid. 3a-R1 and 4a-R1 were isolated from a plate containing minimal medium and 100 μ g/ml DL-alanine, and 3d-R119 from a plate containing complete medium.

Alanine-glutamic acid transaminase activities

The results obtained in the preceding experiments suggest that the negative growth of mutant 3d on L-amino acids and the positive response on D-amino acids might be due to the low activity of alanine racemase. But, since it was not the case with other mutants, an attempt was made to examine their transaminase activities (Table 3). As a result of experiments aiming at measuring the transamination between glutamic acid and alanine, it was found that mutant 4a possessed a low activity of transaminase, about 25% of that in the wild-type strain, and mutant 3a, which is similar to 4a in its response pattern, possessed an activity almost equal to that of the wild-type. It was also shown that L-alanine-L-glutamic acid transaminase activity in these strains is, in general, lower than D-alanine-D-glutamic acid trans-

Table 3. Alanine-glutamic acid transaminase activities.

Strains	Specific activity*	
	D-ala-D-glu	L-ala-L-glu
2a	21.4	1.17
3a	29.9	4.55
4a	4.87	4.72
3d	23.8	0.35
Wild strain K	19.7	2.31

* See text.

aminase activity of the same strains. The difference was greatest in mutant 3d.

Alanine-, Glutamic acid-, Aspartic acid-dehydrogenase activities

The dehydrogenases were studied by using DPN, TPN, or a mixture of them as the hydrogen acceptor. As the data in Table 4 show, L-alanine-

Table 4. Alanine-, glutamic acid- and aspartic acid-dehydrogenase activities*.

Strains	Specific activity** ($10^3 \Delta E_{340}/\text{min/mg protein}$)		
	AIDH (DPN)	GIDH (DPN, TPN)	AsDH (DPN, TPN)
2a	13.2	0	0
3a	29.0	0	0
4a	35.0	0	0
3d	42.3	0	0
Wild strain K	104	0	0

* The abbreviations used are: AIDH, L-alanine-dehydrogenase; GIDH, L-glutamic acid-dehydrogenase; AsDH, L-aspartic acid-dehydrogenase.

** See text.

dehydrogenase was found in all the mutants tested as well as in the wild-type strain, while L-aspartic acid- and L-glutamic acid-dehydrogenases were not. The results coincide with the data reported by BRAUNSTEIN (13). Although alanine-dehydrogenase activities of the mutants were, generally speaking, lower than that in the wild-type, the activity in mutant 2a was exceptionally low. This finding supports the following view though not conclusively. If D-amino acids are produced via L-alanine as it is widely believed, a defect in alanine-dehydrogenase production would disturb the synthesis of these amino acids, and as a result, the requirement of L-alanine, D-alanine, D-aspartic acid, and D-glutamic acid for growth would appear. Because mutant 2a really depends on these amino acids, this hypothesis would be allowed only when the synthesis of L-aspartic acid and L-glutamic acid from any sources other than L-alanine is evidenced. To test this possibility, products from fumaric acid formed by the extract of mutant 2a was examined by paper chromatogram, and, as it was expected, the presence of two L-amino acids was ascertained.

DISCUSSION

It is by no means easy to determine the correlation between growth velocity and enzyme activity. A strain of low enzyme activity does not always grow slowly unless the activity is below the limiting degree, and what is important, the limiting degree is different from strain to strain. The second difficulty is that the enzyme activity determined with cell free extract does not represent the actual activity in the intact cell. Unfortunately, these

problems have not yet been solved completely in microbiology notwithstanding its importance in dealing with experimental results.

Nevertheless, it is allowed in the field of biochemical genetics to discuss the gene-enzyme relationship by assaying enzyme activity of a mutant. In general, a mutant having a defect in a certain gene locus can not produce an enzyme or can it produce an enzyme with low activity (14). It may be fair, therefore, to suppose that the D-glutamic acid-requiring mutants reported in the previous paper (1) might have a defect in their enzyme system. As seen below, the results in this paper support the view although it is still uncertain whether the defect (the low activity) is below the limiting activity that allows the growth.

Among the four mutants studied, the defect in mutant 3d is most obvious. In the mutant, the alanine racemase activity was about 10% of that in the wild-type strain. If D-amino acids are to be produced via L-alanine and D-alanine as it is widely accepted, the mutant should require D-alanine, D-aspartic acid, and D-glutamic acid. This expectation agrees with the response pattern of mutant 3d. It appears that D-alanine is converted to D-glutamic acid by transamination, and the reaction proceeds towards D-glutamic acid in living cells, because the amino acid is essential for the formation of cell wall (15). It is assumed too that D-aspartic acid is converted to D-glutamic acid under a similar mechanism.

As described in the last part of the preceding chapter, the site of the genetic block in mutant 2a seems to be between pyruvate and L-alanine. The requirement of L-alanine, D-alanine, D-aspartic acid and D-glutamic acid is the result of this genetic block. It is supposed too that the requirement of D-aspartic acid and D-glutamic acid by mutant 4a is caused by the low activity of D-alanine-D-glutamic acid transaminase. But, as a matter of fact, the strain requires L-aspartic acid and L-glutamic acid in addition to the D-amino acids. It is necessary therefore to explain this growth response pattern to prove simultaneous occurrence of depression and induction, that is, the pathway for the synthesis of L-glutamic acid and L-aspartic acid must be closed and the pathway for the synthesis of D-glutamic acid from L-glutamic acid or L-aspartic acid must be opened. These reactions were examined carefully, but all attempts ended in failure.

As mutant 3a, a spontaneous mutant of 3d, requires L-glutamic acid, L-aspartic acid, D-glutamic acid and D-aspartic acid just like mutant 4a in spite of its higher activity of D-alanine-D-glutamic acid transaminase, the correlation between enzyme activity and requirement is not so simple as to allow us to draw a ready conclusion. The authors' favorite opinion is that these enzymes are related to each other in their formation, and the requirement is the combined effects of genetic damage and the interaction between two or more enzyme forming systems. BRAUNSTEIN's finding (13) that a *Am* mutant of *B. subtilis* turned out to be a producer of L-glutamic acid-dehydrogenase in compensation of L-alanine-dehydrogenase is very interesting in this respect.

SUMMARY

To look for the site of genetic block in the mutants of *Bacillus subtilis* K requiring D-glutamic acid, several enzymes that were considered important for the biosynthesis of D- and L-glutamic acid were investigated. In mutant 3d, alanine racemase activity was about one tenth of that in the wild-type strain. It appears therefore that the block site in this mutant is between L-alanine and D-alanine. The mutant requires D-alanine, D-aspartic acid, or D-glutamic acid for growth.

L-Alanine dehydrogenase activity was exceptionally low in mutant 2a. Because the mutant is able to synthesize L-aspartic acid and L-glutamic acid from fumarate too, the requirement of L-alanine, D-alanine, D-aspartic acid, or D-glutamic acid may be due to the slow reaction between pyruvate and L-alanine.

Mutant 4a possesses a low activity of D-alanine-D-glutamic acid transaminase and responds to L-aspartic acid, L-glutamic acid, D-aspartic acid, or D-glutamic acid. The dependency on D-aspartic acid or D-glutamic acid is reasonable if these D-amino acids are produced from D-alanine by transamination, while the dependency on L-aspartic acid or L-glutamic acid remains unexplained. The correlation between a genetic damage and an enzyme formation was discussed in this connection.

REFERENCES

- (1) H. MOMOSE: *This Journal*, **7**, 359 (1961).
- (2) C. W. BEADLE: *J. Biol. Chem.*, **156**, 683 (1945).
- (3) W. A. WOOD, and J. C. GUNSALUS: *J. Biol. Chem.*, **190**, 403 (1951).
- (4) P. AYENGAR, and E. ROBERT: *J. Biol. Chem.*, **197**, 453 (1952).
- (5) S. A. NARROD, and W. A. WOOD: *Arch. Biochem. Biophys.*, **35**, 462 (1952).
- (6) C. B. THORNE, C. G. GOMEZ, and R. D. HOUSEWRIGHT: *J. Bact.*, **69**, 357 (1955).
- (7) J. M. WIAME, and A. PIERARD: *Nature*, **176**, 1073 (1955).
- (8) G. FALCONE, G. SALVATORE, and I. COVELLI: *Biochim. Biophys. Acta*, **36**, 390 (1959).
- (9) A. PIERARD, and J. M. WIAME: *Biochim. Biophys. Acta*, **37**, 490 (1960).
- (10) W. W. UMBREIT, R. H. BURRIS, and J. F. STAUFFER: "*Manometric Techniques*" 207 (1957).
- (11) H. A. KREBS: *Biochem. J.*, **29**, 1620 (1935).
- (12) O. H. LOWRY, N. J. ROSENBROUGH, and R. J. RANDALL: *J. Biol. Chem.*, **193**, 265 (1951).
- (13) M. M. HONG, S. S. SHEN, and A. E. BRAUNSTEIN: *Biochim. Biophys. Acta*, **36**, 288 (1959).
- (14) D. M. BONNER, C. YANOFSKY, and C. W. H. PARTRIDGE: *Proc. Nat. Acad. Sci. U. S.*, **38**, 25 (1951).
- (15) H. MOMOSE: *This Journal*, **7**, 365 (1961).

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