SOME MUTANTS OF BACILLUS BREVIS DEFICIENT IN GRAMICIDIN S FORMATION

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SUMMARY. Twenty gramicidin-less mutant strains of B. brevis were isolated by N-methyl-N'-nitro-N-nitrosoguanidine treatment. These mutants have been classified so far into 3 groups. The first group lacked a phenylalanine activating and racemizing enzyme. The second group did not have a proline, valine, ornithine and leucine activating enzyme complex. The third group was deficient in both the enzyme fractions. Enzymes obtained from the first and second group mutants were complementary for gramicidin S formation.

To elucidate the mechanism of gramicidin S formation, it is of particular interest to investigate the enzyme system for gramicidin S formation in the mutants of B. brevis which are not able to form gramicidin S. Kurahashi et al.(1) briefly reported on such mutants which were obtained by ultraviolet irradiation. We obtained 20 gramicidin-less mutants of B. brevis by N-methyl-N'-nitro-N-nitrosoguanidine(NTG) treatment. This report concerns the characteristics of an enzyme system of some gramicidin-less mutants obtained.

MATERIALS AND METHODS

NTG treatment on a wild strain of B. brevis was carried out mainly according to the method described by Adelberg et al.(2). A three-hour culture of wild strain was incubated with 1000 μg

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per ml of NTG in nutrient broth for 1 hour at 37 C and after washing out the NTG with a minimum medium containing glutamate and yeast extract by centrifugation the mutagenized cells were cultured in nutrient broth at 37 C for 12 hours. First screening was performed on nutrient agar plate. Of 6300 mutagenized colonies isolated, 20 strains which did not show any inhibitory zone against Staphylococcus aureus were selected as gramicidin-less mutants. After bacteriological purification on each mutant, all these mutants were identified as B. brevis by morphological characteristics and quantitative agglutination tests using antiserum of rabbit against wild strain of B. brevis. In the second screening, the alcoholic extracts of all these mutant cells did not inhibit the growth of Staphylococcus aureus in a serial dilution method, and furthermore, any gramicidin S was not detected on paper chromatography of these extracts.

The cell-free enzyme system responsible for gramicidin S formation was prepared as previouly described (3) with slight modification. The ammonium sulfate fraction was applied to a Sephadex G-200 column (1.5 X45 cm) and eluted with 0.02 M Tris-HCl buffer, pH 7.6, containing 0.25 mM EDTA, 0.01 M $MgCl_2$ and 1.0 mM β-mercaptoethanol according to the method described by Tomino et al.(4). Flow rate was 5 ml per hour and 1.5 ml aliquots were collected.

Assay of incorporation of L-^{14}C-phenylalanine into gramicidin S and D-phenylalanyl-L-prolyl diketopiperazine was performed as described previously(5). After incubation at 37 C for 1 hour, the reaction mixture was extracted with 3 ml of ethanol containing 300 μg of carrier gramicidin S and centrifuged. The precipitate was again extracted with 3 ml of ethanol. The combined ext-

ract was poured into a Dowex-50 column (1 X 3 cm) prewashed with ethanol. The column was eluted with 100 ml of ethanol, 100 ml of distilled water, 100 ml of 4 M ammoniacal water and 100 ml of 4 M ammonia in 50 % ethanol, succesively. The ethanol extract was evaporated to dryness and the residue was dissolved in 2 ml of 50 % ethanol. Radioactivity of the 1 ml aliquot was determined by Nuclear Chicago gas-flow counter for diketopiperazine formed. The 4 M ammoniacal ethanol fraction was treated as described above for gramicidin S. Radioactive substances were tested by paper chromatography developed with a butanol-acetic acid-water system and identified as the diketopiperazine and gramicidin S, respectively, by a radioactive paper chromatography scanner.

The assay method of amino acid dependent $^{32}PP_i$-ATP exchange reaction was the same as previously described(5).

The protein concentration was determined by the method of Kalcker(6).

RESULTS AND DISCUSSION

NTG treatment gave several different types of gramicidin-less mutants of B. brevis which could not synthesize gramicidin S in both the growing cells and the cell-free system. Three groups of mutant strains have been established so far at enzymatic level. The first group (Strains BI-7, C-1 and E-1) lacked a D-phenylalanine activating and/or racemizing enzyme (Fig. 2). The second group (Strains BI-5 and C-2) did not have a L-proline, L-valine, L-ornithine and L-leucine activating enzyme complex (Fig.3). The third group (Strains BI-1, BII-4 and C-4) did not contain either fraction (Fig. 4). Figure 1 shows the pattern of a wild strain as a comparison. As shown in these Figures, all strains had strong valine and leucine dependent $^{32}PP_i$-ATP exchange activities in the second protein peak, but these were due to amino acyl-tRNA

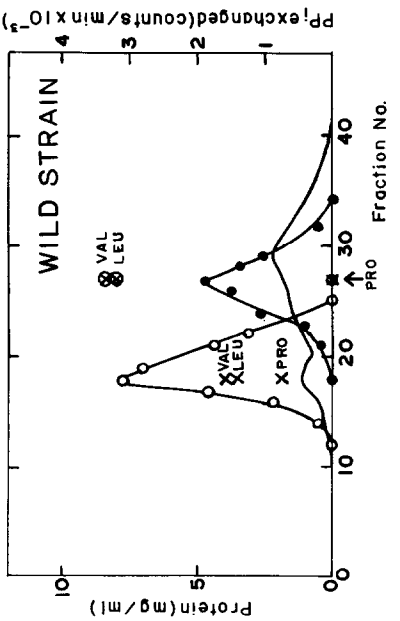

Fig. 1.

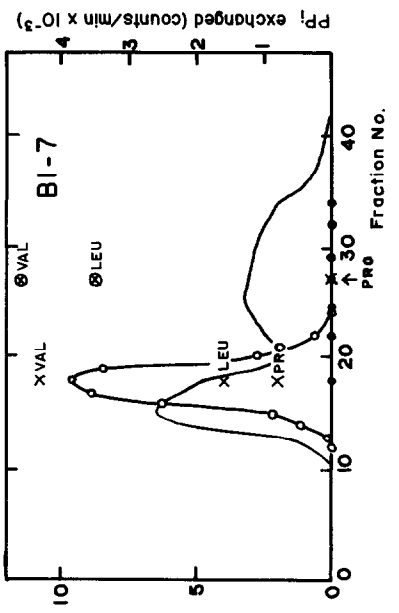

Fig. 2.

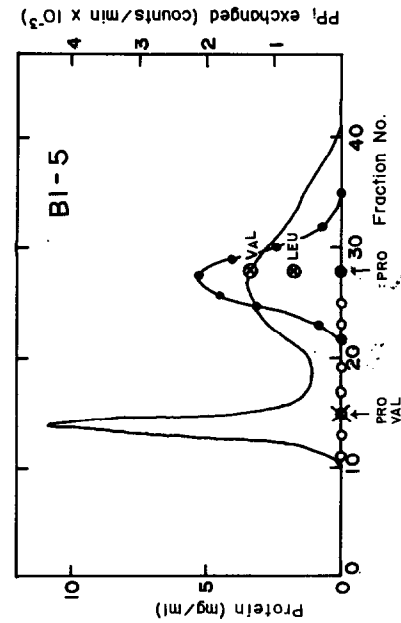

Fig. 3.

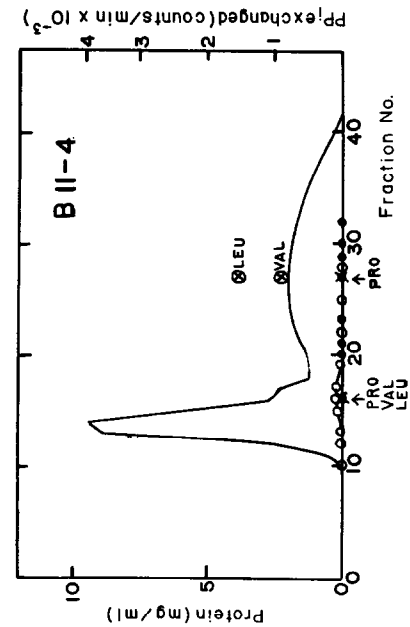

Fig. 4.

synthetases and had no relation to gramicidin formation. For formation of gramicidin S and D-phenylalanyl-L-prolyl diketopiperazine, ammonium sulfate fraction of each mutant was used after dialysis against a 0.02 M Tris-HCl buffer, pH 7.6, containing 1.0 mM β-mercaptoethanol for 16 hours at 3 C.

TABLE I

Enzyme Source	L-^{14}C-Phenylalanine incorporated into	
	Gramicidin S (counts/min.)	D-Phenylalanyl-L-prolyl Diketopiperazine (counts/min.)
Wild strain	3,898	7,486
BI-5	57	241
BI-7	56	212
BII-4	74	329
BI-5 + BI-7	6,540	5,414
BII-4 + BI-5	71	319
BII-4 + BI-7	69	308

The assay was carried out as described in the text. Six mg of protein (ammonium sulfate fraction dialyzed against a 0.02 M Tris-HCl buffer, pH 7.6, containing 1.0 mM β-mercaptoethanol) were used as enzymes.

Figures 1, 2, 3 and 4. The elution patterns of ammonium sulfate fractions obtained from the wild and mutant strains of B. brevis from Sephadex G-200 column. 100 mg to 120 mg of protein was applied to a column. For the condition of elution and the determination of amino acid dependent $^{32}PP_i$-ATP exchange reaction see the text. —— protein concentration; O——O L-ornithine activating activity; ●——● D-phenylalanine activating activity; X amino acid activating activities in the enzyme complex and ⊗ amino acyl-tRNA synthetases activities in the second protein peak.

Table I shows that each enzyme of any gramicidin-less mutant used alone can not synthesize either the diketopiperazine or gramicidin S. Whereas the combination of the enzymes from mutant BI-5 which lacked an activating enzyme complex, and mutant BI-7 deficient in a phenylalanine activating enzyme, results in the synthesis of both the diketopiperazine and gramicidin S.

It is evident from these results that the enzymes obtained from mutants BI-5 and BI-7 were complementary for gramicidin S formation, as expected from the results of gel-filtration experiments.

Other types of gramicidin-less mutants are now being investigated in this laboratory.

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