Biosynthesis of Tyrocidines By Extracts of Two Strains of *Bacillus brevis*[1] (35048)

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A number of strains of *Bacillus brevis* produce a variety of antibiotic polypeptides belonging to two major families: the linear gramicidins and the cyclic tyrocidines. These polypeptides are produced by a non-ribosomal, soluble enzyme system, and the mechanisms by which they are synthesized have attached considerable attention as models for the production of other bacterial peptides.

The structures of the four tyrocidines can be represented as follows (1–4):

Tyrocidine A
- Phe - D-Phe - Asn - Gln - Tyr - Val - Gln - Leu - D-Phe - Pro -

Tyrocidine B
- Trp - D-Phe - Asn - Gln - Tyr - Val - Gln - Leu - D-Phe - Pro -

Tyrocidine C
- Trp - D-Trp - Asn - Gln - Tyr - Val - Gln - Leu - D-Phe - Pro -

Tyrocidine D
- Trp - D-Trp - Asn - Gln - Trp - Val - Gln - Leu - D-Phe - Pro -

The terminal amino acids in each sequence are linked to each other to form a cyclic structure. All amino acids are in L-configuration except for those designated "D". The amino acids which vary from one tyrocidine to another are underlined.

Mach and Tatum (5) reported that the distribution of tyrocidines produced by intact cells of strain ATCC 10068 of *B. brevis* could be altered by changing the relative concentration of tryptophan and phenylalanine in the medium in which the cells were suspended. Since the composition of the peptide produced reflected the composition of the medium, it appeared that a single enzyme system, with a low degree of specificity for the aromatic amino acids concerned, was responsible for the synthesis of all four of the tyrocidines produced by this organism. Note that the substitution of tryptophan for phenylalanine and tyrosine appears to occur in a regular sequence, however, since only the isomers given above have been reported to be present. This suggests that the synthetic complex must control the substitution process so that the second phenylalanine is replaced only after the first, and the tyrosine is replaced only when both phenylalanines have been substituted.

Studies on the tyrocidine synthetic system from a closely related strain of *B. brevis*, ATCC 8185, which produces an identical spectrum of antibiotic peptides, indicated that the system from this organism was not greatly influenced by the amino acid composition of the medium (6). Because of this discrepancy, and because of the great interest of a system for peptide synthesis which is capable of such a flexible and specific response to changes in substrate concentrations, it appeared to be worthwhile to prepare cell-free extracts from the ATCC 10068 strain of *B. brevis* in order to demonstrate that the observations were not actually due to the induction of new enzymes, or other artifacts introduced by the use of living cells.

Methods. B. brevis ATCC 10068 was grown in a New Brunswick Shaker-incubator at 37°. The medium used was the asparagine–glycerol minimal medium of Mach *et al.* (7) supplemented with 0.3% yeast extract and 2% glucose.

[1] This research was supported by a grant from the National Institutes of Health (GM 13312).

The preparation of cell-free extracts was modified from Bhagavan *et al.* (8) to compensate for the more gradual release of the synthetic system from this strain.

All procedures were carried out at 4°. Several variations of the method for distintegrating the cells were tried, and the following appeared to give the best results. The cells were washed twice with 0.025 *M* magnesium acetate and once with 0.025 *M* magnesium acetate in 0.01 *M* mercaptoethanol (solution A). The cells were then frozen at −20° for 1 hr before disruption. They were thawed, suspended in 1.5 vol of solution A, and sonicated at 60W and 20 kc for 10 min. Five periods of sonication, each 2 min long were used, and the suspension was chilled in ice between periods to control its temperature. After sonication it was homogenized for 6 min in Potter–Elvehjem homogenizer. Unbroken cells and debris were removed by centrifugation at 30,000*g* for 45 min, and the supernatant phase was centrifuged again at 105,000*g* for 1 hr to remove ribosomes.

Protein content was determined by biuret method (9). The incubation mixture had the following ingredients in each tube unless otherwise specified (3–5 mg of protein), 20 μmoles of ATP, 10 μmoles phosphoenolpyruvate, 10 μg pyruvate kinase (Calbiochem), 25 μmoles magnesium acetate, 10 μmoles mercaptoethanol, 200 μmoles Tris–HCl (pH 7.4), 0.8 μCi ^{14}C-ornithine (Nutritional Biochemicals, sp act 11 mCi/mmole), and 1.0 μmole of each of the 19 or 20 other ^{12}C-amino acids. Incubation was for 5 hr at 37°. ^{14}C-Ornithine was chosen as labeled amino acid in the reaction mixture since this amino acid does not occur in the gramicidins, the other type of polypeptide antibiotics synthesized by the organism. The peptides were isolated as previously described (8).

The four tyrocidines were separated from each other on 1 × 150-cm column of Sephadex G-25 (Pharmacia Fine Chemicals) by the procedure of Ruttenberg and Mach (4). Four milligrams of commercial tyrocidine (Mann Research Laboratories) were added to each sample as carrier before chromatography. The absorbancies of each fraction were determined at 280 mμ on a Beckman DU spectrophotometer, and aliquots were assayed for radioactivity.

TABLE I. The Incorporation of Ornithine-^{14}C into the Tyrocidines in the Presence of Varying Amounts of Phenylalanine and Tryptophan by Extracts of the Two Strains of *B. brevis*.

Ratio Phe/Trp	Tyrocidine	Total cpm in peptide ATCC 8185	Total cpm in peptide ATCC 10068
1/1	A	20	1220
	B	30	2630
	C	30	1770
	D	2760	3810
10/1	A	590	4680
	B	540	2020
	C	550	1890
	D	2690	750
1/10	A	530	820
	B	530	2020
	C	550	1790
	D	2520	5980

Results. The preparation of the cell-free extracts and the incubation conditions required for maximum peptide synthesis were generally the same as those reported for similar systems from other strains of this bacterium. There appeared to be only two significant differences: (1) The synthetic system was appreciably more difficult to separate from the cells so that more prolonged sonication was required, and (2) magnesium was readily lost from the extracts on dialysis, while in those from strain ATCC 8185 it appeared to be bound sufficiently tightly that a requirement for this ion was difficult to demonstrate.

When peptide-synthesizing extracts from each of the strains of *B. brevis* were incubated with 1 μmole of each amino acid found in the tyrocidines, the distribution of peptides produced by the two systems was markedly different. As shown in Table I, extracts from ATCC 8185 produce tyrocidine D almost exclusively, while extracts from ATCC 10068 not only produce this peptide but the other three as well, and in only slightly smaller quantities. Increasing the quantities of either phenylalanine or tryptophan to 10 μmoles in the incubation mixture appeared to stimulate synthesis of tyrocidines

A through C by extracts of ATCC 8185 slightly, but without affecting the marked bias towards tyrocidine D production. On the other hand, extracts of ATCC 10068 were strongly responsive to the change in amino acid concentration. This system produces 6 moles of tyrocidine A for every mole of tyrocidine D when phenylalanine was present at high levels, but the ratio was reversed when tryptophan was the more abundant amino acid (Table I).

In order to confirm the identification of each fraction obtained from the column, peptides labeled with either tryptophan-^{14}C or tyrosine-^{14}C (both from New England Nuclear Corporation) were prepared. The fraction identified as tyrocidine A contains no tryptophan, and was significantly labeled by this amino acid. Similarly, tyrocidine D contains no tyrosine, and the fraction thought to include this peptide was unlabeled by tyrosine.

Discussion. The present study confirms the observations of Mach and Tatum (5) with *B. brevis* ATCC 10068 and shows that even in a cell-free extract lacking ribosomes in which no protein synthesis can occur, the distribution of the tyrocidines produced is altered by changes in the concentrations of tryptophan or phenylalanine. Thus, enzyme induction cannot be responsible for this shift.

It is interesting that extracts of the closely related bacterium, *B. brevis* ATCC 8185, should behave so differently in producing mainly tyrocidine D under all conditions. This organism normally synthesizes the same complex of tyrocidines as ATCC 10068 as well as the mixture of open-chain antibiotic peptides called the gramicidins which are also common to the two strains. The preparation of cell-free extracts which will synthesize all four tyrocidines from *B. brevis* 10068 should make possible more detailed studies of the mechanism of synthesis of these antibiotic peptides. Lipmann and his colleagues have conducted such studies (10, 11) on the similar gramicidin S system, but the controlled variability of this tyrocidine-producing complex from strain ATCC 10068 should make its study unusually interesting.

It seems possible that the loss or inactivation of some component of the active complex, such as a tyrosine-activating enzyme, during preparation of the 8185 extracts may account for this difference in activities. If so, this difference in the stability of the complex is another major point of difference between the two strains.

Summary. Two different strains of the bacterium, *Bacillus brevis,* each of which produces the antibiotic, cyclic, decapeptides called tyrocidines have been studied. The four tyrocidines differ only in the position occupied by three of their four aromatic amino acid residues. Two of these positions may be occupied by either phenylalanine or tryptophan and the third by tyrosine or tryptophan, with the substitutions occurring in a definite sequence. (See structures given in the introductory paragraphs). A report that these four peptides are produced *in vivo,* by a single enzyme system with a low specificity for the aromatic amino acids, was confirmed with cell-free extracts in which the possibility of protein synthesis could be excluded. A second strain of the bacterium yielded extracts which did not show how this lack of specificity.

We express our appreciation to Miss Ruth Ann Noble for her technical assistance in this work.

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Received June 22, 1970. P.S.E.B.M., 1970, Vol. 135.