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Phenylalanine Stimulation of Gramicidin S Formation[1]

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Bacillus brevis produces the antibiotic gramicidin S, a cyclic peptide comprised of two pentapeptide units of the sequence D-Phe-L-Pro-L-Val-L-Orn-L-Leu. Synthesis of this antibiotic is carried out by two enzymes, GS synthetases I and II. By supplementing a defined minimal medium [glycerol, NH_4^+, tris(hydroxymethyl)aminomethane, salts] with each of the constituent amino acids (in the L form), we found that only L-phenylalanine had a marked stimulatory effect on gramicidin S production. This effect was not caused by an increase in growth, by an induction of GS synthetases I or II, or by a stabilization of these enzymes. L-Phenylalanine apparently stimulates production via its role as a precursor of the D-phenylalanine moiety of the gramicidin S molecule.

Gramicidin S (GS) is a peptide antibiotic. It has a cyclic structure consisting of two repeating pentapeptide units arranged in the sequence:

D-Phe-L-Pro-L-Val-L-Orn-L-Leu

L-Leu-L-Orn-L-Val-L-Pro-D-Phe

Two of these amino acids, D-phenylalanine and L-ornithine, do not occur in proteins. Certain strains of *Bacillus brevis* produce this antibiotic after the exponential phase of growth (2).

Many amino acids have been examined for their effect on GS formation. Leucine, valine, proline, ornithine, phenylalanine (7), glycine, tyrosine, methionine, and aspartic acid (6) have all been reported to stimulate GS production. These effects could reflect the organism's particular growth requirements, since *B. brevis* has a complex pattern of nitrogen nutrition—preferring amino N to ammonium N (E. Vandamme and A. L. Demain, Dev. Ind. Microbiol., in press). The difficulty involved in accepting the above stimulatory effects as basic is pointed out by the finding that both valine (under certain conditions) and leucine have also been reported to inhibit GS formation (6). Phenylalanine, a component amino acid of GS, shows the most dramatic stimulatory effect (1, 6). Moreover, the observations that fluorophenylalanine and β-phenyl-β-alanine inhibit GS formation but not growth (4, 5) emphasize the importance of phenylalanine. Three possibilities explain these results equally well, i.e., a phenylalanine requirement for either (i) GS synthetase formation, (ii) stabilization, or (iii) antibiotic formation. To decide between these alternatives, we undertook this study.

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MATERIALS AND METHODS

Cultures. *B. brevis* ATCC 9999 was obtained from the American Type Culture Collection and maintained at 4 C as a spore suspension. The GS bioassay strain, *B. subtilis* ATCC 6051, was obtained from P. Masurekar and maintained at 4 C as a spore suspension.

Chemicals. Nutrient broth, yeast extract, and vitamin-free Casamino Acids were obtained from Difco Laboratories (Detroit, Mich.). Triethanolamine-hydrochloride, L-ornithine, and D-phenylalanine were obtained from Schwarz/Mann (Orangeburg, N.Y.). We purchased other amino acids from General Biochemicals (Chagrin Falls, Ohio) and obtained sodium [^{32}P]pyrophosphate from New England Nuclear (Boston, Mass.). Sigma Chemicals (St. Louis, Mo.) was the source of dithiothreitol, chloramphenicol, adenosine triphosphate (disodium salt), and tris(hydroxymethyl)aminomethane (Sigma 7-9). We obtained lysozyme (egg white) from Worthington Biochemicals (Freehold, N.J.). T. Wakazawa of Meiji Seika Kaisha, Ltd. (Tokyo, Japan) generously provided the gramicidin S standard.

Media. The seed medium consisted of defined minimal medium G2T, supplemented with 0.005% vitamin-free Casamino Acids and 0.0002% yeast extract.

The defined production medium G2T (3) contained the following components per liter of distilled water: 25 g of glycerol, 12 g of $(NH_4)_2SO_4$, 6.5 g of K_2HPO_4, 1.7 g of KH_2PO_4, 203 mg of $MgCl_2 \cdot 6H_2O$, 103 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of $MnCl_2 \cdot 4H_2O$, 0.27 mg of $FeCl_3 \cdot 6H_2O$, and 12 g of tris(hydroxymethyl)-aminomethane. The phosphates were autoclaved

separately as a 10-fold concentrated solution. The Mg, Ca, and Mn salts were combined and stored as a 1,000-fold concentrate. The $FeCl_3$ was also stored as a 1,000-fold concentrate. The medium was adjusted to pH 7.5 before autoclaving.

Methods. Spores were prepared as described by Matteo et al. (3).

The inoculum was prepared by introducing 1 drop of spore suspension into 50 ml of seed medium in a 500-ml baffled flask. Germination, outgrowth, and exponential vegetative growth took place during 16 h of shaking at 37 C.

GS production in G2T medium was carried out as previously described (3).

In determining the amount of GS formation, we used *B. subtilis* ATCC 6051 in the agar diffusion assay. This method (3) involved extraction of whole broth with an equal volume of ethanol-0.02 N HCl (9:1) for 3 h prior to assay.

Dry cell weight and cell protein content were determined, cell-free extracts were prepared, and GS synthetases I and II were assayed as described by Matteo et al. (3).

RESULTS

In our earlier studies on the development of defined media (3), we devised medium G2T, which contains glycerol, ammonium sulfate, tris(hydroxymethyl)aminomethane buffer, and mineral salts. After supplementing medium G2T with the five GS-constituent amino acids (L forms), we observed a stimulation of GS production (Table 1). Each of the amino acids (0.1% concentration) was then tested singly in growing cultures for the stimulatory effect. The addition of L-phenylalanine caused a two- to threefold increase in the GS level, but no stimulation was found upon addition of L-ornithine, L-proline, L-valine, or L-leucine. No effect on growth was observed after supplementation with phenylalanine (Fig. 1). Another experiment in which phenylalanine was eliminated from the mix of the constituent amino acids resulted in GS productivity at the same level as in the G2T control. Thus, supplementing the medium with L-phenylalanine specifically stimulated antibiotic synthesis. The phenylalanine isomer that occurs in GS is the D form. Although capable of increasing GS production, D-phenylalanine inhibits the rate of growth and requires a longer fermentation to produce its effect.

We next determined the optimum level of phenylalanine for stimulation of GS synthesis. By adding 0.1% of each of the other four constituent amino acids to the G2T medium, we could insure against substrate limitation during GS synthesis. The results presented in Table 2 show the optimum phenylalanine level to be 0.1%. Supplementation with 0.5% phenylalanine resulted in a marked decrease in antibiotic production as compared to the 0.1% level. To determine whether this decrease was related to substrate limitations for the other four constituent amino acids, an additional experiment

TABLE 1. *Effect of constituent amino acids of the GS molecule on GS production*[a]

Amino acid added[b]:					Specific productivity[c] in expt:				
Phe	Leu	Orn	Pro	Val	1	2	3	4	5
−	−	−	−	−	0.04	0.04	0.05	0.05	
+	+	+	+	+	0.07	0.10			0.10
+	−	−	−	−		0.12	0.09		
−	+	−	−	−			0.03		
−	−	+	−	−				0.03	
−	−	−	+	−				0.04	
−	−	−	−	+				0.04	
−	+	+	+	+					0.05

[a] Basal medium was G2T.
[b] Added as 0.1% L form.
[c] Expressed as milligrams of GS/milligram of dry cell weight.

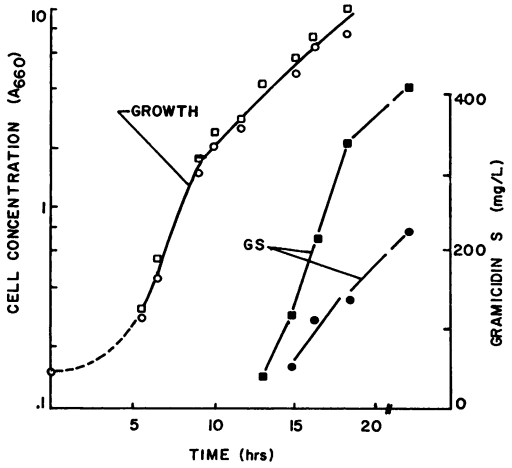

FIG. 1. *Production of GS in defined G2T medium (circles) and in G2T supplemented with 0.1% L-phenylalanine (squares). One gram of dry cell weight per liter has an absorbance (A_{660}) of 2.5.*

TABLE 2. *Effect of L-phenylalanine on GS production in the presence of 0.1% of the other constituent amino acids*[a]

L-Phenylalanine (%)	Maximum DCW[b] (g/liter)	Maximum GS (g/liter)	Specific GS (g/g of DCW)
0	6.9	0.28	0.04
0.001	6.8	0.26	0.04
0.01	6.9	0.29	0.04
0.1	6.9	0.66	0.10
0.5	6.9	0.37	0.05

[a] Basal medium was G2T plus 0.1% each of L-proline, L-leucine, L-ornithine, and L-valine.
[b] DCW, Dry cell weight.

was done in which all five GS-constituent amino acids were present at the 0.5% level. The results (Table 3) confirm that the optimum phenylalanine concentration is 0.1%, regardless of the levels of the other four GS-constituent amino acids.

We have found that, of all the natural amino acids, only phenylalanine specifically stimulates GS synthesis. A mixture of five amino acids (glutamine, histidine, arginine, methionine, and proline) markedly stimulated growth but not GS formation (3). When this enriched medium (G2T5) was supplemented with phenylalanine, GS formation was markedly increased. Again, the optimum concentration was 0.1% (Vandamme and Demain, in press).

To determine whether the phenylalanine effect was due to induction or stabilization of GS synthetases, we followed enzyme synthesis in medium G2T and in G2T supplemented with GS-constituent amino acids. Typical results (Fig. 2) indicate that, despite its marked effect on GS production, 0.1% phenylalanine alone does not increase enzyme specific activity nor does it stabilize synthetase activity (when compared to the control). Supplementation individually with each of the four remaining amino acids resulted in peak specific activities equal to or lower than the control. The simultaneous addition of all five constituent amino acids resulted in peak specific activities below the control. These results are summarized in Table 4, which shows the peak specific activities normalized with respect to the control peak values. Thus, none of the GS-constituent amino acids, alone or in combination, increase GS synthetase specific activities in G2T medium.

TABLE 3. *Effect of L-phenylalanine on GS production in the presence of 0.5% of the other constituent amino acids*[a]

L-Phenylalanine (%)	Maximum DCW[b] (g/liter)	Maximum GS (g/liter)	Specific GS (g/g of DCW)
0	6.2	0.11	0.018
0.02	6.3	0.28	0.044
0.1	7.2	0.38	0.054
0.5	6.5	0.23	0.035

[a] Basal medium was G2T plus 0.5% each of L-proline, L-leucine, L-ornithine, and L-valine.

[b] DCW, Dry cell weight.

DISCUSSION

Of all the natural amino acids, only phenylalanine, a constituent of the GS molecule, specifically stimulates GS synthesis. It does this without inducing or stabilizing GS synthetases. The most probable role of phenylalanine in stimulating antibiotic formation is that of a limiting precursor of the GS molecule.

Future studies should help to determine the effect of phenylalanine supplementation on the intracellular phenylalanine pool. If the phenyl-

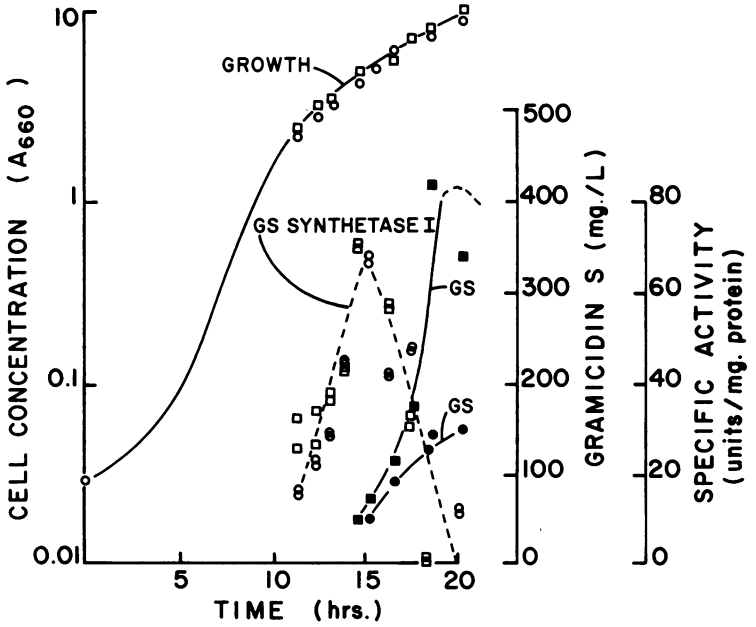

FIG. 2. *Production of GS synthetase I and GS in defined G2T medium (circles) and in G2T supplemented with 0.1% L-phenylalanine (squares). One gram of dry cell weight per liter has an absorbance (A_{660}) of 2.5.*

TABLE 4. *Effect of GS-constituent amino acids on GS synthetase formation*[a]

Amino acid added[b]:					GS synthetase I[c,d] in expt:				GS synthetase II[d,e] in expt:	
Phe	Leu	Orn	Pro	Val	1	2	3	4	2	4
−	−	−	−	−	1.00	1.00	1.00	1.00	1.00	1.00
+	+	+	+	+	0.27	0.40			0.45	
+	−	−	−	−		1.17	0.86		1.17	
−	+	−	−	−			0.44			
−	−	+	−	−				0.48		0.85
−	−	−	+	−				0.69		
−	−	−	−	+				0.56		1.14

[a] Basal medium was G2T.

[b] Added as 0.1% L form.

[c] The specific activities for the control in experiments 1, 2, 3, and 4 were 26.6, 60.8, 18.0, and 69.1 units/mg, respectively.

[d] Expressed as units of synthetase/milligram of dry cell weight.

[e] The specific activities for the control in experiments 2 and 4 were 53.7 and 37.5 units/mg, respectively.

alanine pool size affects the degree of GS synthesis, we would expect higher pool levels to occur after supplementation with the amino acid.

The observed effect of exogenous phenylalanine suggests that a mutant strain that overproduces phenylalanine would produce more GS than the parent strain would in the absence of phenylalanine supplementation. Such mutants could be important for large-scale production of the antibiotic.

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