PARADOXICAL EFFECT OF ARGININE ON GRAMICIDIN S FORMATION BY *BACILLUS BREVIS*

Sir:

Previous studies[1,2] from this laboratory revealed that six amino acids have a positive effect on growth and/or production of gramicidin S (GS) by *Bacillus brevis* ATCC 9999. Phenylalanine acts as a limiting precursor of GS[3] whereas a mixture of the other five amino acids (proline, histidine, arginine, methionine and glutamine) stimulates growth.

In the present work, we attempted to assess the relative importance of the individual members of the growth-stimulatory mixture and found arginine to have major but paradoxical effects on GS biosynthesis.

Materials and Methods

Culture

B. brevis ATCC 9999 was maintained as a spore suspension (8×10^8 spores per ml). The GS bioassay strain, *Bacillus subtilis* ATCC 6051, was also maintained at 4°C as a spore suspension.

Medium and growth conditions

Chemically-defined medium (F3/6)[2] was used for both germination and fermentation. F3/6 medium contained per liter of distilled water: 10 g of D-fructose, 6.5 g of K_2HPO_4, 1.7 g of KH_2PO_4, 203 mg of $MgCl_2\cdot6H_2O$, 103 mg of $CaCl_2\cdot2H_2O$, 10 mg of $MnCl_2\cdot4H_2O$, 0.27 mg of $FeCl_3\cdot6H_2O$, 1.5 g of L-proline, 1.3 g of L-histidine, 1.3 g of L-glutamine, 0.5 g of L-methionine, 3 g of L-arginine and 1 g of L-phenylalanine. Fructose and phosphates were autoclaved separately and the pH was adjusted to 7.4 with 1 N HCl before autoclaving. Modifications of the F3/6 medium are described in the text.

For germination, one drop of spore suspension of *B. brevis* was introduced into 25 ml of medium in a 250-ml Erlenmeyer flask and the flasks were shaken on the rotary shaker (220 rpm, 2 inch diameter orbit) at 37°C for 16 hours. The germinated culture was used to seed fermentation flasks as follows: 1 ml of germinated culture was added to 25 ml of medium in a 250 ml flask or in larger scale fermentations, 10 ml of the germination culture into 300 ml medium in a 2,800 ml FERNBACH flask. The fermentation flasks were incubated on the rotary shaker at 220 rev./min. and 37°C. For growth and GS determinations, a small amount of whole broth was removed from the flasks at indicated times.

Analytical methods

GS assays were as described previously.[1] Growth was measured with a KLETT SUMMERSON colorimeter using a red filter.

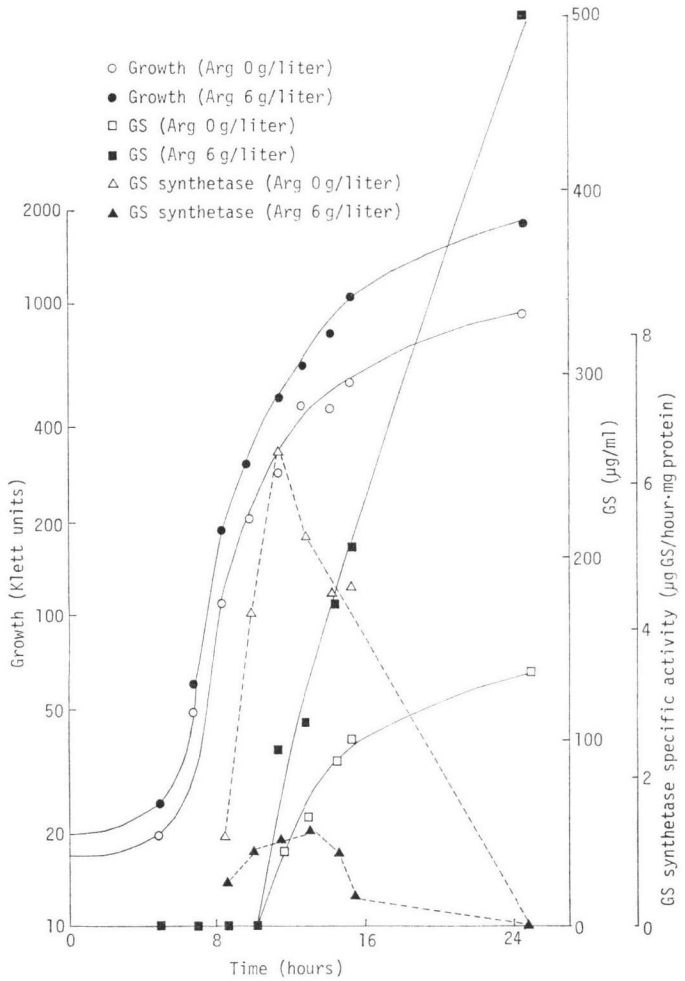
In assaying the activity of the GS synthetase complex, cells were harvested by centrifugation in the cold at $12,000\times g$ for 10 minutes and pellets were stored at −20°C. The protein determination (biuret method), the preparation of crude cell-free extracts, and the assay procedure

Table 1. Effect of arginine in various growth media.

Expt.	Amino acids in growth medium[a]						Maximum growth (Klett units)	Maximum GS (μg/ml)	Maximum specific GS (μg/ml/Klett unit)
	Pro	Gln	His	Met	Phe	Arg (g/liter)			
1	−	+	+	+	+	0	1,500	60	0.040
	−	+	+	+	+	3	2,000	192	0.096
2	−	+	+	+	+	0	1,100	46	0.042
	−	+	+	+	+	3	1,700	133	0.078
3	−	−	−	+	+	3	1,060	98	0.092
	−	−	−	+	+	4	1,180	193	0.160
4	−	+	+	+	+	0	920	138	0.150
	−	+	+	+	+	1.5	1,280	240	0.190
	−	+	+	+	+	6.0	1,880	500	0.270

[a] The concentrations of Pro, Gln, His, Met, and Phe in all experiments where they were included were 1.5, 1.3, 1.3, 0.5 and 1.0 g/liter respectively.

Fig. 1. Effects of L-arginine (6g/liter) on growth, GS formation, and GS synthetase formation by *Bacillus brevis* ATCC 9999.



using L-(^{14}C) ornithine for the activity of GS synthetase complex were carried out as described[4] except for the following procedures in the enzyme assay. After the enzyme reaction was stopped in an ice bath, 20 μl of the reaction mixture was applied to a filter paper square (2×2 cm, SCHLEICHER and SCHUELL grade 595). The papers were dried under an infrared lamp and transferred into a large beaker containing 10 ml of cold 6% trichloroacetic acid (TCA) per paper square. The contents were mixed for 30 minutes in the cold gently with a magnetic stirrer to remove TCA soluble materials from the papers. The washing procedure was carried out for a total of 3 washings and the papers were dried under an infrared lamp. The filter papers with the washed precipitates were transferred to scintillation vials and counted.

Results

Experiments were carried out in which individual amino acids were omitted from medium F3/6 and the effects on growth and GS formation were examined. Omission of proline or glutamine appeared to have no effect. Omission of histidine decreased growth and GS formation to a limited extent. As expected, omission of methionine precluded growth since it is the sole source of sulfur in F3/6 medium. The decrease in production upon elimination of phenylalanine was expected.[3] Arginine was the most interest-

ing since its omission led to decreased growth and to even more dramatic decrease in GS production. Experiments were next carried out in various media modifications as shown in Table 1. In experiments 1 and 2, the dramatic effect of arginine omission is shown in a medium lacking proline. In experiment 3 conducted in a simpler medium, increasing the arginine concentration from the usual 3 to 4 g/liter showed a marked positive effect on production. In experiment 4, a concentration dependent effect of arginine was observed.

In the next series of experiments, GS synthetase production was examined when proline, glutamine, histidine and arginine were individually removed. In the case of the first three amino acids, omission led to a moderate drop in specific activity of GS synthetase. However in the case of arginine elimination, a major increase in specific activity was observed despite the fact that growth and GS synthesis were decreased (Fig. 1).

This apparent repression of soluble GS synthetase activity was totally unexpected. To determine whether arginine also inhibited activity, it was added to the GS synthetase assay at 37 g/liter (0.2 M). Activity was inhibited but only to a minor extent, *i.e.* about 10%. It is doubtful then that the lowering of soluble GS synthetase activity by growth in arginine could be due to inhibition of enzyme activity. Further experimentation will be necessary to explain how a nutrient could repress an antibiotic synthetase yet increase specific production of the antibiotic.

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