Production of Guanosine by Psicofuranine and Decoyinine Resistant Mutants of *Bacillus subtilis*

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Growth of *Bacillus subtilis* AG169 that produced large amounts of xanthosine and guanosine was inhibited by psicofuranine. When AG169 was mutated to resistance against psicofuranine, a mutant, GP-1, which yielded more guanosine was obtained. Psicofuranine did not inhibit growth of GP-1 any more. The guanosine 5'-monophosphate (GMP) synthetase activities were then assayed. In GP-1, the specific activity decreased about half, the complete loss of repression by GMP was found, and the inhibition by GMP was slightly loosed, when compared with those of AG169.

Furthermore, as growth of GP-1 was strongly inhibited by decoyinine, decoyinine resistant mutants were derived from GP-1. Of these mutants, two strains, MG-1 and MG-4, were resistant to decoyinine completely and showed the exclusive accumulation of guanosine in high yields, *i.e.* 16.0 and 15.5 g of guanosine per liter with weight yields of 20.0 and 19.4% of consumed sugar, respectively. GMP synthetase activity of MG-1 increased remarkably in comparison with that of GP-1 or AG169, and the inhibitions by GMP, psicofuranine and decoyinine were completely released in MG-1. Namely, the psicofuranine and decoyinine resistances seemed to cause mainly variations of GMP synthetase, and as results, the conversion of xanthosine 5'-monophosphate (XMP) to GMP proceeded more smoothly, and a larger amount of guanosine was accumulated.

Many studies on microbial production of guanosine so far have been performed, but mutants with high productivity of guanosine have never been obtained.^{1~5)} We have also already reported that we derived methionine sulfoxide resistant mutants from an inosine-producing strain of *Bacillus subtilis*, and obtained a mutant AG169 that did not excrete inosine but produced considerable amounts of xanthosine and guanosine.⁶⁾ But this mutant could not accumulate a sufficient amount of guanosine to be contented. In order to obtain mutants which accumulate further increased amounts of guanosine, it is necessary to derive mutants that convert XMP to GMP more smoothly, *i.e.* are released from regulation of GMP synthetase. We then tried derivation of psicofuranine and decoyinine resistant mutants from AG169.

Psicofuranine (angustmycin C) and decoyinine (angustmycin A) have been found as antibiotics that *Streptomyces hygroscopicus* produces.^{7~9)} And they are structural analogs

of adenosine.^{10,11)} They inhibit not only ribosephosphate pyrophosphokinase (EC 2.7.6.1) reaction,¹²⁾ but also mainly GMP synthetase reaction, and the mode of action is regarded as non-competitive inhibition of GMP synthetase.^{13~20)}

At first, we obtained a psicofuranine resistant mutant that produced more guanosine and less xanthosine than the parent AG169. Next, we obtained decoyinine resistant mutants from the psicofuranine resistant mutant. One of them produced a larger amount of guanosine exclusively. The present paper deals with derivation of improved guanosine-producing mutants and clarification of some of their properties, especially correlation between psicofuranine or decoyinine resistance and GMP synthetase activity.

MATERIALS AND METHODS

Bacterial strains. Psicofuranine or decoyinine resistant mutants were derived from a xanthosine- and

guanosine-producing strain of *B. subtilis*, AG169. AG169 is a histidine auxotroph, and is genetically lacking GMP reductase (EC 1.6.6.8) and succinadenosine 5'-monophosphate (succino-AMP) synthetase (EC 6.3.4.4), and is resistant to methionine sulfoxide.⁽⁶⁾

Chemicals. Psicofuranine and decoyinine were obtained from the Upjohn Company.

Culture media. Medium 1 contained 10 g of peptone, 10 g of yeast extract, 5 g of NaCl, 5 g of glucose and distilled water, in a total volume of 1 liter. The pH was adjusted to 7.0 with NaOH. Medium 2 contained 20 g of glucose, 5 g of NH_4Cl , 4 g of KH_2PO_4 , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.5 g of sodium citrate, 1 g of L-glutamic acid, 0.5 g of L-histidine, 0.1 g of adenine and distilled water, in a total volume of 1 liter. The pH was adjusted to 7.0 with KOH. Medium 3 contained 80 g of glucose, 15 g of NH_4NO_3 , 0.2 g of KH_2PO_4 , 0.4 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 g of DL-methionine, 10 g of L-glutamic acid, 0.3 g of L-histidine, 40 ml of Ajieki (soybean hydrolysate), 1.4 g of ribonucleic acid (RNA), 15 g of CaCO_3 and distilled water, in a total volume of 1 liter. The pH was adjusted to 7.5 with KOH. Calcium carbonate was sterilized separately.

Derivation of mutants. Cells (1×10^6 to 5×10^9) from the logarithmic growth phase in medium 1 were harvested, washed with 50 mM KH_2PO_4 - K_2HPO_4 buffer (pH 7.0) and exposed to 500 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) per ml in the same phosphate buffer for 50 min in an ice bath. The NG-treated cells were washed with the same phosphate buffer, diluted and spread on medium 2 agar plates supplemented with psicofuranine or decoyinine. After incubation for 2 to 5 days at 34°C, colonies appeared on the plates were picked up. In derivation of spontaneous mutants, the method was the same as described above except that NG-treatment was omitted.

Fermentation tests. Cells that grew overnight on medium 1 agar plates were inoculated into 20 ml of medium 3 in a 500 ml-flask. After cultivation for 72 hr at 34°C with shaking, 10 μl of culture broth was spotted on filter paper (Toyo Roshi No. 51). After separation by paperchromatography, using isopropanol-ammonia-water (7:2:1) as a solvent, inosine, xanthosine and guanosine were extracted from the paper with 10 ml of 0.1 N HCl for 5 hr at room temperature and were estimated spectrophotometrically.

Growth inhibition tests. Bacterial cells grown overnight in 3 ml of medium 2 in a test tube were washed with 50 mM KH_2PO_4 - K_2HPO_4 buffer (pH 7.0) and

about 10^6 cells were inoculated into 3 ml of medium 2 supplemented with psicofuranine or decoyinine. Growth was measured by absorbance at 540 nm after culturing for 24 hr at 34°C.

Enzyme preparation. Cells that had grown overnight on medium 1 agar plates were transferred to 20 ml of the inoculum medium, the composition being the same as medium 3 except that CaCO_3 was omitted. In special cases, purine bases (adenine or guanine) were used instead of RNA. After cultivation for 16 to 20 hr at 34°C, cells were harvested from culture broth by centrifugation, washed twice with a 0.85% NaCl solution containing 0.1 M ethylenediaminetetraacetic acid and three times with chilled acetone, and were dried in a vacuum.

Enzyme activity assay. GMP synthetase (EC 6.3.5.2) activity was assayed by the method of Moyed and Magasanik.⁽²¹⁾ The reaction mixture contained 3 μmol of 5'-XMP, 20 μmol of L-glutamine, 2 μmol of adenosine 5'-triphosphate (ATP), 24 μmol of MgCl_2 , 120 μmol of Tris-HCl buffer (pH 8.0), and the enzyme in a total volume of 0.5 ml. The reaction was carried out at 37°C for 30 min with gentle shaking and stopped by the addition of 3 ml of 3% perchloric acid. The amount of GMP formed was determined by measuring absorbance at 290 nm spectrophotometrically. In one case, the reaction was stopped by boiling for 3 min, and the amount of glutamic acid formed was determined by measuring absorbance at 340 nm with the use of glutamic acid dehydrogenase (EC 1.4.1.3) and nicotinamide adenine dinucleotide. Specific activity of enzyme was expressed as nanomoles of GMP or glutamic acid formed per minute per milligram of dried cells.

RESULTS

Growth inhibition of strain AG169 by psicofuranine and decoyinine, and recovery of the inhibition by guanine and xanthine

Growth of AG169 on medium 2 was inhibited remarkably by additions of 0.5 and 1.0 mg of psicofuranine or decoyinine per ml. The growth inhibition was reversed completely by 0.1 and 0.5 mg of guanine per ml, but not by 0.5 mg of xanthine per ml. The result is presented in Table I.

Derivation of psicofuranine or decoyinine resistant mutants from strain AG169 and their guanosine productivity

Mutants resistant to 0.5 and 1.0 mg of psico-

TABLE I. GROWTH INHIBITION OF STRAIN AG169 BY PSICOFURANINE AND DECOYININE AND RECOVERY OF THE INHIBITION BY GUANINE AND XANTHINE

Inhibitors (mg/ml)	Growth response (%)			
	None	Guanine (mg/ml)		Xanthine (mg/ml)
		0.1	0.5	
None	100	115	105	130
Psicofuranine	0.5	10	95	15
	1.0	5	91	10
Decoyinine	0.5	5	103	8
	1.0	2	100	2

furanine or decoyinine per ml were derived from AG169. Mutants improved in guanosine productivity were obtained from these psicofuranine resistant mutants with a frequency of 2% (4 of 200 colonies), but were not obtained from decoyinine resistant mutants at all (0 of 200 colonies). The parent AG169 accumulated 8.0 g of guanosine and 6.0 g of xanthosine per liter at an optimal RNA concentration. The best psicofuranine resistant mutant GP-1 accumulated 10.6 g of guanosine and 3.4 g of xanthosine per liter (Table III). When growth inhibition of GP-1 by psicofuranine was examined, GP-1 was not inhibited by 0.5 to 2.0 mg of psicofuranine per ml at all (Fig. 1).

FIG. 1. Growth Inhibition of Psicofuranine Resistant Mutant GP-1 and the Parent AG169 by Psicofuranine. ○—○, GP-1; ●—●, AG169.

Growth inhibition of strain GP-1 by decoyinine, and recovery of the inhibition by guanine and xanthine

Furthermore, growth inhibition of the psicofuranine resistant mutant GP-1 by decoyinine was examined. Growth of GP-1 on medium 2

was inhibited strongly by the additions of 0.5, 1.0 and 2.0 mg of decoyinine per ml. The growth inhibition was reversed completely by 0.5 mg of guanine per ml, but not by 0.5 mg of xanthine per ml (Table II). This result was the same as the case of AG169 (Table I).

TABLE II. GROWTH INHIBITION OF STRAIN GP-1 BY DECOYININE AND RECOVERY OF THE INHIBITION BY GUANINE AND XANTHINE

Inhibitor (mg/ml)	Growth response (%)		
	None	Guanine (mg/ml)	Xanthine (mg/ml)
		0.5	0.5
None	100	100	100
Decoyinine	0.1	70	100
	0.5	0	105
	1.0	0	110
	2.0	0	100

Derivation of decoyinine resistant mutants from strain GP-1 and their guanosine productivity

Mutants resistant to 1.0 mg of decoyinine per ml were derived from GP-1. Decoyinine resistant mutants appeared spontaneously at the frequency of 0.5 to 1.0 colony per 10^6 cells of GP-1. In the accumulation patterns of purine nucleosides, 22 of 56 strains produced guanosine exclusively, 30 of 56 strains produced guanosine and xanthosine like the parent, 2 of 56 strains produced guanosine and inosine, and 2 of 56 strains produced xanthosine only. Here, five mutants were selected as those that produced more guanosine than the parent. The best mutant MG-1 accumulated 16.0 g of guanosine per liter in a weight yield of 20.0% of consumed sugar. Another mutant MG-4 accumulated 15.5 g of guanosine per liter in a weight yield of 19.4% of consumed sugar. They did not accumulate inosine and xanthosine. The result is shown in Table III.

Growth inhibition of GP-1, MG-1 and MG-41 by decoyinine was examined. MG-1 was resistant to 0.5, 1.0 and 2.0 mg of decoyinine per ml. MG-41 that still produced xanthosine was more slightly resistant than the parent GP-1 at 0.5 mg of decoyinine per ml, but was sensitive to 1.0 and 2.0 mg of decoyinine per ml like the parent (Fig. 2). The

TABLE III. GUANOSINE PRODUCTIVITY OF DECOYININE RESISTANT MUTANTS DERIVED FROM GP-1

The resistance to decoyinine was investigated as described in growth inhibition tests. It was not investigated beyond 2.0 mg of decoyinine per ml.

Strain	Degree of decoyinine resistance (mg/ml)	Productivity (g/liter)			
		Ino-sine	Xantho-sine	Guanosine	Total purine nucleoside
GP-1	—	0	3.4	10.6	14.0
MG-1	2.0	0	0	16.0	16.0
MG-4	2.0	0	0	15.5	15.5
MG-7	1.0	0	1.2	12.2	13.4
MG-36	0.5	0	2.1	12.2	13.1
MG-41	0.5	0	2.5	11.8	14.3

FIG. 2. Growth Response of Decoyinine Resistant Mutants and the Parent to the Effect of Decoyinine. ○—○, MG-1 (mutant); ▲—▲, MG-41 (mutant); ●—●, GP-1 (parent).

variation of accumulation from xanthosine to guanosine responded to the degree of decoyinine resistance (Table III).

GMP synthetase activity and its inhibition by GMP in strains AG169, GP-1 and MG-1

As it is known that psicofuranine and decoyinine inhibit non-competitively GMP synthetase activity, the enzyme of psicofuranine and decoyinine resistant mutants was investi-

TABLE IV. GMP SYNTHETASE ACTIVITY AND ITS INHIBITION BY GMP IN STRAINS AG169, GP-1 AND MG-1

Strain	Specific activity of GMP synthetase (nmol/min per mg of dried cells)		
	No addition	0.6 mM GMP	4 mM GMP
AG169 (parent)	0.563	0.560	0.450
GP-1 (psicofuranine ^r)	0.270	0.260	0.240
MG-1 (psicofuranine ^r , decoyinine ^r)	0.756	0.755	0.762

gated. As shown in Table IV, specific activity of GMP synthetase of the psicofuranine resistant mutant GP-1 decreased half of that of the parent AG169. In the psicofuranine and decoyinine resistant mutant MG-1, the specific activity increased remarkably when compared with those of AG169 and GP-1. And the mutants GP-1 and MG-1 did not respond to GMP in the same manner as the parent AG169.

Inhibition of GMP synthetase activity by psicofuranine, decoyinine and adenosine in strains AG169, GP-1 and MG-1

Psicofuranine and decoyinine are analogs of adenosine. Here, inhibition of GMP synthetase activity by psicofuranine, decoyinine and adenosine was investigated. GMP synthetase activity of the parent AG169 was inhibited strongly by 2.2 and 4.4 mM psicofuranine, decoyinine and adenosine. In the psicofuranine resistant mutant GP-1, the inhibition by psicofuranine and adenosine decreased considerably, but the inhibition by decoyinine did not vary. In the psicofuranine and decoyinine resistant mutant MG-1, the inhibition was lost

TABLE V. INHIBITION OF GMP SYNTHETASE ACTIVITY BY PSICOFURANINE, DECOYININE AND ADENOSINE IN STRAINS AG169, GP-1 AND MG-1

Strain	Relative activity of GMP synthetase (%)						
	None	Psicofuranine		Decoyinine		Adenosine	
		2.2 mM	4.4 mM	2.2 mM	4.4 mM	2.2 mM	4.4 mM
AG169	100	60	45	50	40	30	20
GP-1	100	80	80	40	40	80	40
MG-1	100	100	100	100	100	100	100

completely. The result is presented in Table V.

GMP synthetase activity of strains AG169, GP-1 and MG-1 under the condition of adding guanine to culture medium

GMP synthetase activity was assayed under the condition of culturing in the medium, the composition being the same as medium 3 except that CaCO_3 was omitted and purine bases instead of RNA were used. In the parent AG169, the slight decrease of enzyme activity was observed by adding 500 mg of guanine per liter to the above culture medium. In GP-1 and MG-1, the enzyme repression by GMP was removed completely because the decrease of enzyme activity by adding 3.3 mM guanine to the culture medium was not found (Table VI).

DISCUSSION

We have tried improvement of mutants which produce guanosine in a high yield.^{5,6)} It will become a very useful method of our purpose to derive psicofuranine or decoyinine resistant mutants from a xanthosine- and guanosine-producing strain AG169, for it is thought that accumulation of a large amount of xanthosine depends on insufficiency of de-regulation of GMP synthetase. Therefore, growth inhibition of AG169 by psicofuranine or decoyinine, and its recovery by guanine or xanthine were examined. The growth was markedly inhibited by 0.5 and 1.0 mg of psicofuranine or decoyinine per ml (Table I). The growth inhibition was reversed completely by 0.1 and 0.5 mg of guanine per ml, but not by 0.5 mg of xanthine per ml (Table I). This result implies that psicofuranine or decoyinine does not disturb ribosephosphate pyrophosphokinase, but GMP synthetase. When psicofuranine or decoyinine resistant mutants were derived from AG169, improved guanosine producers were obtained with a frequency of 2% in psicofuranine resistant mutants. The best mutant GP-1 accumulated 10.6 g of guanosine and 3.4 g of xanthosine per liter

(Table III). On the other hand, any improved guanosine producers were not found in the decoyinine resistant mutants of AG169. The reason is probably that anyone of the mutants was not resistant to decoyinine completely. Growth of GP-1 was not prevented by 0.5 to 2.0 mg of psicofuranine per ml at all (Fig. 1), but was still inhibited completely by 0.5 to 2.0 mg of decoyinine per ml (Table III). From these facts, the effect of decoyinine on GMP synthetase may be slightly different from that of psicofuranine. When further mutation to decoyinine from GP-1 was performed spontaneously, decoyinine resistant mutants appeared at the ratio of 0.5 to 1.0 colony per 10^5 cells of GP-1. The best mutant MG-1 did not accumulate inosine and xanthosine, but accumulated 16.0 g of guanosine per liter in a weight yield of 20% of consumed sugar (Table III). Growth of MG-1 was not inhibited completely by 0.5 to 2.0 mg of decoyinine per ml (Fig. 2). Incomplete decoyinine resistant mutant MG-41 could not convert all of xanthosine to guanosine (Table III, Fig. 2). Thus, our purpose of obtaining a mutant which accumulates guanosine exclusively with high productivity was achieved by the psicofuranine and decoyinine resistant mutant MG-1.

The correlation between psicofuranine or decoyinine resistance and GMP synthetase activity was investigated in three strains of AG169, GP-1 and MG-1. Although the specific activity of GMP synthetase of GP-1 reduced approximately 50% when compared with that of AG169, that of MG-1 increased 35% of that of AG169 (Table IV). Inhibition of GMP synthetase activity by 4 mM GMP was observed at the degree of approximately 20 and 10% in AG169 and GP-1, respectively, but was not found in MG-1 at all (Table IV). Inhibition by psicofuranine or decoyinine in AG169 was found at the degree of about 40~60%. Inhibition by psicofuranine in GP-1 was lost considerably when compared with that of AG169, but inhibition by decoyinine was similar to that of AG169. In MG-1, the inhibitions were not observed at all (Table V). Furthermore, repression of GMP synthetase

by GMP (3.3 mM as guanine in the culture medium) was not found in GP-1 and MG-1. In the parent AG169, it was slightly recognized (Table VI).

TABLE VI. GMP SYNTHETASE ACTIVITY OF STRAINS AG169, GP-1 AND MG-1 UNDER THE CONDITION OF ADDING GUANINE TO CULTURE MEDIUM

The cell cultures were carried out by using adenine or guanine instead of RNA in medium 3.

Strain	Specific activity of GMP synthetase (nmol/min per mg of dried cells)	
	Adenine 50 mg/liter	Adenine 50 mg/liter + Guanine 500 mg/liter (3.3mM)
AG169	0.535	0.505
GP-1	0.330	0.345
MG-1	0.680	0.693

From above data, we could not conclude why GP-1 or MG-1 accumulated a larger amount of guanosine than AG169. However, in GP-1, the slight loss of inhibition or repression by GMP might cause further conversion from XMP to GMP. In MG-1, the loss of inhibition by GMP and increase of specific activity might cause smoothly conversion to GMP without pooling XMP intracellularly. Or the loss of sensitivity to psicofuranine and decoyinine in GMP synthetase might be related to the guanosine production in mechanism different from deregulation of GMP synthetase by GMP.

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