Studies on the "Salvage" Synthesis of Ribonucleosides and their 5'-Phosphates by Microorganisms

Part II. "Salvage" Synthesis of Purine Ribosides by 8-Aza-guanine Resistant Mutants Derived from *Bacillus subtilis*

By Osamu KANAMITSU

Technical Research Laboratory, Asahi Chemical Industry Co., Ltd.,
27-36 Nakadai-3-chome, Itabashi-ku, Tokyo

Received February 18, 1970

As a part of studies on "salvage" synthesis of ribonucleosides and ribonucleotides by microorganisms,¹⁾ ability of "salvage" synthesis of *Bacillus subtilis* mutants resistant to analogues of purine bases was studied. It was found that some mutants resistant to 8-aza-guanine had ability to produce inosine and guanosine from hypoxanthine, guanine, and adenine. Recently, Midorikawa *et al.*²⁾ reported that a mutant No. 322 derived from *B. subtilis* which was deficient in GMP-reductase was able to

produce guanine and guanosine from hypoxanthine, and deficiency of GMP-reductase was an essential factor for this ability. On the contrary, we isolated several 8-aza-guanine resistant mutants of *B. subtilis* having GMP-reductase. These mutants produced inosine and guanosine from hypoxanthine almost stoichiometrically without production of guanine. In this paper, results obtained by these mutants on "salvage" synthesis of inosine and guanosine from several purine bases will be

FIG. 1. Derivation of the Mutants of *B. subtilis*.

Abbreviations. *trp*: tryptophan, *A*: adenine, *aa*: amino acid, *8-AG*: 8-aza-guanine, *Smr*: streptomycin resistant, and *8-AX*: 8-aza-xanthine.

1) O. Kanamitsu and T. Akimoto, *Agr. Biol. Chem.*, **32**, 830 (1968).

2) Y. Midorikawa, G. Akiya, A. Kuninaka and H. Yoshino, *Proc. 18th Symposium of Association of Amino Acid and Nucleic Acid*, p. 36 (1969).

described.

The mutants used were derived from *B. subtilis* Marburg strain, and resistant to 8-azaguanine. The process of derivation of these mutants was shown in Fig. 1. The mutants resistant to a purine base analogue were selected from colonies grown on the plate containing a purine base analogue at 30°C. For transfer of a genetic marker, such as adenine requirement, the technique of genetic transformation with DNA was utilized.

Media and cultivation for production of purine ribosides were as follows. Seed culture medium (medium S) contained (g/liter) KH_2PO_4 1, K_2HPO_4 2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.005, NH_4Cl 10, glucose 50, polypepton 5, casamino acid 2, yeast extract 5, meat extract 5, L-tryptophan 0.25, and pH was adjusted to 7.0 before autoclaving. Thirty ml of medium S were distributed in a 500 ml Erlenmeyer flask. Main culture medium (medium M) contained (g/liter) KH_2PO_4 1, K_2HPO_4 2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.01, NH_4Cl 20, glucose 100, yeast extract 15, casamino acid 2, L-tryptophan 0.25, purine base as indicated, and pH was adjusted to 7.0 before autoclaving. Thirty ml of medium M were distributed in a 500 ml square-shouldered flask. They were autoclaved for 6 min at 118°C. Nine hundred mg of sterilized CaCO_3 were

TABLE I. CHARACTER OF THE REPRESENTATIVE MUTANTS

Strain	Character	Addition of hypoxanthine ^{a)}	Production of	
			HR	GR
HR-25	<i>trp</i> ⁻ , <i>A</i> ⁻ , <i>aa</i> ⁻	—	###	±
		+	###	±
FA-1	<i>trp</i> ⁻ , <i>A</i> [±] , <i>aa</i> ⁻	—	±~+	±
		+	±	±
FA-1-12	<i>trp</i> ⁻ , <i>A</i> [±] , <i>aa</i> ⁻ , <i>8-AG</i> ^r	—	±~+	±
		+	±	+
No. 37	<i>trp</i> ⁻ , <i>A</i> ⁻ , <i>aa</i> ⁻ , <i>8-AG</i> ^r	—	+	+
		+	+	+
No. 37-103	<i>trp</i> ⁻ , <i>A</i> ⁻ , <i>aa</i> ⁻ , (<i>A</i> + <i>8-AG</i>) ^r	—	±	+
		+	±	±
No. 37-23	<i>trp</i> ⁻ , <i>A</i> ⁻ , <i>aa</i> ⁻ , <i>8-AG</i> ^r , (<i>A</i> + <i>8-AX</i>) ^r	—	±	+
		+	±	+

^{a)} Concentration of hypoxanthine added was 22 mM.

Abbreviations. *A*: adenine, HR: inosine, GR: guanosine, *8-AG*: 8-aza-guanine, *8-AX*: 8-aza-xanthine, *trp*: tryptophan, and *aa*: amino acid.

Designation of the amount of the products. ±: < 3 mM, +: 3~10 mM, ±: around 18 mM, ###: around 37 mM, and ###: around 56 mM.

added to medium M after autoclaving. One and a half ml of seed culture which had been cultivated for 16 hr at 32°C with shaking were inoculated to medium M. Cultivation was carried out at 32°C for 72 or 96 hr with shaking.

Determination and quantitative estimation

TABLE II. CONVERSION OF ADENINE, HYPOXANTHINE, ADENOSINE AND INOSINE BY FA-1-12

Substrate	Products				
	Adenine	Hypoxanthine	Adenosine	Inosine	Guanosine
None	t	±	t	+	±
Adenine	(###)	+	±	±	±
Hypoxanthine	t	(+)	t	±	+
Adenosine	±	±	(±)	±	+
Inosine	t	±	t	(###)	±

Substrate concentration was 40 mM. In these experiments, Polypepton (15 g/liter) was substituted for yeast extract (15 g/liter) and casamino acid (2 g/liter) in medium M. Conversion of inosine and adenosine was determined after 48 hr cultivation at 32°C with shaking. Designation of the amount of the products is the same as in Table I, and t: trace amount, if present. The amount in the bracket is that of substrate remaining.

of the products were carried out by measuring ultraviolet absorption of the eluates from paper chromatogram. Solvents used for paper chromatography were as follows; *n*-butanol: acetic acid: water (4:1:1) for separation of hypoxanthine and inosine or guanosine; methylisobutylketone: acetic acid: water (8:5:2) for separation of adenine, adenosine, hypoxanthine, and inosine or guanosine; *n*-propanol: conc. ammonia water: water (20:12:3) for separation of guanosine and xanthosine from inosine; and ethanol: 1M ammonium acetate (7:3) for separation of xanthosine from other substances.

The results obtained by the representative mutants were shown in Table I. These mutants resistant to 8-aza-guanine produced not only inosine and guanosine from hypoxanthine, but also guanosine from inosine or adenosine, and apparently they did not produce hypoxanthine, adenine or guanine. They produced guanosine from guanine, too. Studies on the conversion of added substances by FA-1-12 were summarized in Table II. Growth of FA-1-12 was severely inhibited by adenine, but the cells could deaminate adenine gradually. Hypoxanthine produced from adenine was then converted to inosine and guanosine. There was a mutant (FA-1-9) whose growth was not inhibited by adenine.

It is thought that production of inosine from

hypoxanthine might be due to weak activity of ribosidase and strong activity of "salvage" synthesis, and production of guanosine from hypoxanthine might be further due to 8-aza-guanine resistance. As for the cause of 8-aza-guanine resistance, IMP-dehydrogenase and/or XMP-aminase might be changed to some state, such as a state in which feedback inhibition or repression is less severely affected. 8-Aza-guanine resistant transformants derived from wild type strain or adenine requiring mutant (producing hypoxanthine but not inosine) were unable to produce inosine and guanosine from hypoxanthine.

Production of inosine by these mutants was larger in amount than that of guanosine. The mechanism of this phenomenon was not clear whether it depended on reductive formation of IMP from GMP once produced or depended on inhibition of production of GMP via XMP from IMP. (It was thought that inosine and guanosine were produced from IMP and GMP respectively.) These problems should be studied hereafter.

Acknowledgements. The author wishes to express his thank to Director Dr. R. Wakasa for giving a chance to study and report on this subject. Thanks are also due to Director of the Foodstuff Plant, Mr. G. Goto, Dr. T. Ohsawa, Dr. S. Ohuchi, Dr. R. Kodaira and Dr. I. Takeda for their interest and encouragement during the course of this work.