Enzymatic Production of Ribavirin from Purine Nucleosides by *Brevibacterium acetylicum* ATCC 954

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Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Virazole®), which is known as a potent antiviral agent, was synthesized directly from purine nucleosides and 1,2,4-triazole-3-carboxamide (TCA) by a newly selected bacterium, *Brevibacterium acetylicum* ATCC 954. Among the purine nucleosides tested, guanosine and xanthosine were selected as the best substrates as to ribavirin production. The highest amount of ribavirin produced was 229 mM from 300 mM guanosine and 300 mM TCA on 96 hr reaction at 60°C. In this system involving ATCC 954, ribavirin was effectively even produced at a limited concentration of phosphate (about 10 mM). ATCC 954 could also produce ribavirin directly from inosine and TCA in a high yield, but could hardly produce it directly from adenosine and TCA. In contrast, a bacterium belonging to the genus, *Bacillus*, could produce ribarivin directly from adenosine and TCA in a high yield.

It is well known that enzymes catalyzing the transglycosylation of ribonucleosides and deoxyribonucleosides are widely distributed in mammalian cells and bacteria.[1~20] Several nucleosides and their analogs have been synthesized through the application of these reactions.[21~24] Recently, Utagawa *et al.* reported the nucleoside-*N*-arabinosyl group transfer reaction.[25,26] They also reported ribavirin formation through the following three-step process involving two enzymatic reactions; (1) the formation of ribose-1-phosphate (R-1-P) through the enzymatic phosphorolysis of inosine catalyzed by purine nucleoside phosphorylase (PNPase) of *Enterobacter aerogenes* AJ 11125, (2) the isolation of R-1-P from the reaction mixture, and (3) the formation of ribavirin from the isolated R-1-P and TCA, also catalyzed by the PNPase of *E. aerogenes* AJ 11125.[27] The authors have widely screened microorganisms producing ribavirin from pyrimidine nucleosides and TCA, and selected *E. aerogenes* AJ 11125[28] and *Erwinia carotovora* AJ 2992[29] as representative strains. Both strains could produce ribavirin directly from pyrimidine nucleosides and TCA in high yields, but could hardly produce it from purine nucleosides and TCA. On the basis of these results, microorganisms producing ribavirin directly from purine nucleosides and TCA were screened to construct a more efficient ribavirin-producing system without the R-1-P isolation step.

This paper describes screening as to the ribavirin production from purine nucleosides and TCA, and investigation of the reaction conditions for ribavirin production from guanosine and TCA by a newly selected bacterium, *Brevibacterium acetylicum* ATCC 954.

MATERIALS AND METHODS

Chemicals. Guanosine was the product of Ajinomoto Co., Inc. TCA was kindly provided by Dr. T. Utagawa of our laboratory. The other chemicals used were commercially available and of analytical grade.

Microorganisms. Microorganisms from stock cultures kept in our laboratory were used. The best strain, *Brevibacterium acetylicum* ATCC 954, was used.

Screening methods. The medium and the cultivation method used for the screening were the same as described in the previous paper.[29] The reaction mixture for the screening, containing 50 mM guanosine or adenosine, 50 mM TCA, 300 mM potassium phosphate buffer(pH 7.0) and rinsed cells, 50 mg/ml on a wet weight basis, in a total volume of 5 ml in a test tube, was incubated at 60°C for 3 hr and 24 hr.

Media and culture conditions. All the procedures used were the same as described in the previous paper.[29]

Reaction with intact cells. The basal reaction mixture consisted of 100 mM guanosine, 100 mM TCA, 50 mg/ml of cells (wet weight basis) and 300 mM potassium phosphate buffer (pH 7.0). The reaction was carried out at 60°C for 24 hr with standing.

Identification of the reaction product. The reaction product were identified by comparison of the infra-red, NMR and mass spectra with those of authentic ribavirin. The NMR and mass spectra were obtained in D_2O with a Nihondenshi type JNM-GX400 and a Nihondenshi type DX-300, respectively. The infra-red spectrum was obtained with a Bio-Rad Laboratories type FTS-15E.

Analytical methods. Ribavirin formed in the reaction mixture was separated by silica gel thin layer chromatography with a solvent system of *n*-butanol–iso-propanol–water–NH_4OH = 10 : 70 : 20 : 2, by volume. Ribavirin was qualitatively detected and also quantitatively determined by the same methods as described in the previous paper.[29]

RESULTS

Screening of ribavirin-producing microorganisms from stock cultures

A total of 425 strains belonging to 39 genera, selected from stock cultures in our laboratories, were tested as to their ribavirin-producing ability, from guanosine and TCA, or adenosine and TCA.

As shown in Table I, 45 strains produced ribavirin directly from guanosine and TCA, and 50 strains directly from adenosine and TCA, respectively. The strains showing high ribavirin production ability are listed in Tables II and III. Through the screening, *Brevibaterium acetylicum* ATCC 954 was selected as the best strain producing ribavirin directly from guanosine and TCA.

TABLE I. DISTRIBUTION OF RIBAVIRIN-PRODUCING BACTERIA

The reaction mixtures, containing 50 mM guanosine or adenosine, 50 mM TCA, 300 mM potassium phosphate buffer (pH 7.0) and cells, 50 mg/ml on a wet weight basis, were incubated at 60°C for 3 hr.

Genus	Tested	Ribavirin-producing bacteria	
		Guanosine	Adenosine
Achromobacter	34	2	1
Acinetobacter	2	0	0
Aeromonas	2	1	0
Agrobacterium	5	0	0
Alcaligenes	13	0	0
Arthrobacter	20	1	0
Bacillus	28	5	10
Beijerinckia	1	0	0
Brevibacterium	29	5	3
Cellulomonas	4	4	0
Citrobacter	6	0	0
Corynebacterium	28	4	0
Escherichia	32	4	7
Enterobacter	4	1	1
Erwinia	49	3	18
Flavobacterium	14	1	0
Hafnia	1	0	0
Jensenia	1	0	0
Klebsiella	4	0	0
Kluyvera	5	2	2
Kurtia	1	0	0
Microbacterium	3	0	0
Micrococcus	15	0	0
Mycoplana	4	0	0
Nocardia	19	0	0
Planococcus	2	0	0
Protaminobacter	3	0	0
Proteus	11	0	0
Propionibacterium	1	0	0
Pseudomonas	30	2	0
Rhizobium	5	0	0
Salmonella	4	4	0
Sarcina	9	0	0
Serratia	14	2	5
Sporosarcina	1	0	0
Staphylococcus	1	0	0
Streptomyces	11	3	0
Vibrio	4	1	2
Xanthomonas	5	0	1

Optimal reaction conditions for ribavirin production from guanosine and TCA by Brevibacterium acetylicum ATCC 954

The optimal pH was around 7.0 (Fig. 1). The optimal temperature was 70°C in the

TABLE II. SCREENING OF RIBAVIRIN-PRODUCING BACTERIA, FROM GUANOSINE AND TCA

The reaction mixtures, containing 50 mM guanosine, 50 mM TCA, 300 mM potassium phosphate buffer (pH 7.0) and cells, 50 mg/ml on a wet weight basis, were incubated at 60°C for 24 hr.

Strains	Ribavirin (mM)
Achromobacter winogratski AJ 2427	2.9
Aeromonas salmonicida AJ 2926	2.1
Arthrobacter nucleogenes AJ 1769	12.7
Bacillus cereus AJ 1269	30.4
Bacillus brevis AJ 1282	13.9
Brevibacterium acetylicum AJ 1442 (ATCC 954)	37.0
Brevibacterium testaceum AJ 1464	4.0
Brevibacterium flavum AJ 1485	2.0
Brevibacterium linens AJ 1520	5.7
Brevibacterium imperiale AJ 1526	10.4
Cellulomonas flavigera AJ 1569	2.0
Citrobacter freundii AJ 2619	2.6
Citrobacter intermedius AJ 13544	13.6
Escherichia coli AJ 3937	4.4
Escherichia intermedia AJ 2607	3.1
Erwinia carotovora AJ 2992	4.4
Flavobacterium sulfreum AJ 2456	3.2
Kluyvera citrophila AJ 2626	3.2
Salmonella typhimurium AJ 2635	1.9
Salmonella scottmuelleri AJ 2930	1.2
Serratia marcescens AJ 2951	3.5
Streptomyces rimosus AJ 9042	5.3
Streptomyces tanashiensis AJ 9082	8.9
Streptomyces humifer AJ 9138	15.8
Vibrio metschnikovii AJ 2806	6.8

TABLE III. SCREENING OF RIBAVIRIN-PRODUCING BACTERIA, FROM ADENOSINE AND TCA

The reaction mixtures, containing 100 mM adenosine, 100 mM TCA, 300 mM potassium phosphate buffer (pH 7.0) and cells, 50 mg/ml on a wet weight basis, were incubated at 60°C for 24 hr.

Strains	Ribavirin (mM)
Bacillus subtilis AJ 1314	7.1
Bacillus cereus AJ 1265	5.5
Bacillus megaterium AJ 3284	16.5
Bacillus circulans AJ 1285	6.5
Bacillus firms AJ 1287	9.9
Bacillus sphericus AJ 1309	0.9
Bacillus licheniformis AJ 1352	2.1
Bacillus pulvifaciens AJ 1353	10.0
Brevibacterium acetylicum AJ 1442 (ATCC 954)	8.6
Brevibacterium pusillum AJ 1462	0.9
Escherichia coli AJ 2599	2.3
Escherichia freundii AJ 2608	11.3
Erwinia carotovora AJ 2753	2.5
Serratia marcescens AJ 2704	3.2
Vibrio tyrogenes AJ 2805	8.0

initial period of the reaction, but was around 65°C after 24 hr reaction (Fig. 2). The optimal phosphate concentration for ribavirin production was around 10 mM (Fig. 3).

Time course of ribavirin production from guanosine and TCA

The time courses of ribavirin production in the presence of various concentrations of guanosine, TCA and cells of ATCC 954 are shown in Fig. 4. Only a little difference was observed between 50 mg/ml and 100 mg/ml of cells (wet weight basis) as to ribavirin production. The amounts of ribavirin produced were 82 mM, 162 mM and 229 mM in the presence of 100 mM, 200 mM and 300 mM guanosine and TCA, respectively, on 96 hr reaction at 60°C with

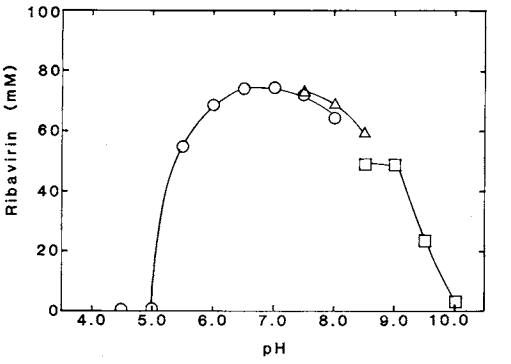

FIG. 1. Effect of the pH on Ribavirin Production.

The reaction mixtures, containing 100 mM guanosine, 100 mM TCA, cells, 50 mg/ml on a wet weight basis, and the indicated buffer were incubated at 60°C for 24 hr. (○) 300 mM potassium phosphate buffer; (△) 50 mM Tris-HCl buffer + 300 mM K_2HPO_4; and (□) 50 mM Glycine-NaOH buffer + 300 mM K_2HPO_4.

100 mg/ml of cells (wet weight basis) of ATCC 954.

Ribavirin production from various nucleosides by ribavirin-producing bacteria

The ribavirin production from various nucleosides by ribavirin-producing bacteria in-

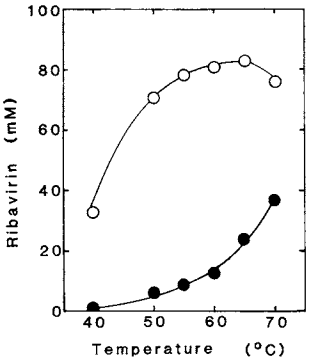

FIG. 2. Effect of the Temperature on Ribavirin Production.

The reaction mixtures, containing 100 mM guanosine, 100 mM TCA, 300 mM potassium phosphate buffer (pH 7.0) and cells, 50 mg/ml on a wet weight basis, were incubated at the indicated temperatures for 3 hr and 24 hr. (●) 3 hr reaction and (○) 24 hr reaction.

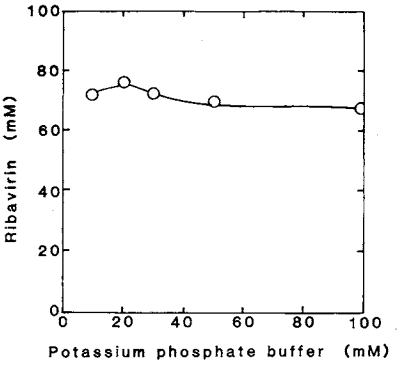

FIG. 3. Effect of the Phosphate Concentration.

The reaction mixtures, containing 100 mM guanosine, 100 mM TCA, cells, 50 mg/ml on a wet weight basis, and the indicated concentrations of potassium phosphate buffer (pH 7.0) were incubated at 60°C for 24 hr.

cluding ATCC 954 is shown in Table IV.

Brevibacterium acetylicum ATCC 954, selected as the best producer of ribavirin, *Bacillus brevis* AJ 1282 and *Bacillus cereus* AJ 1269 more efficiently produced ribavirin from TCA and purine nucleosides, such as guanosine, xanthosine and inosine, than from TCA and pyrimidine nucleosides, such as uridine and cytidine. But only *Bacillus megaterium* AJ 3284 could not produce ribavirin from TCA and pyrimidine nucleosides such as uridine and cytidine.

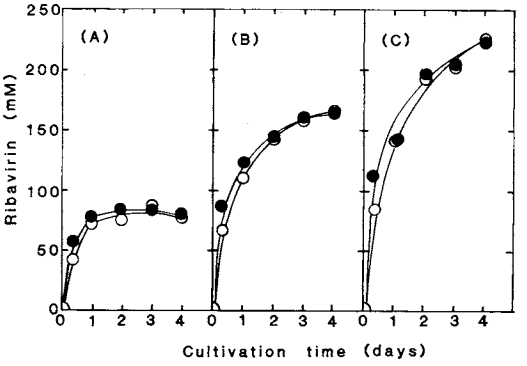

FIG. 4. Time Course of Ribavirin Production from Guanosine and TCA by ATCC 954.

The reaction mixtures, containing the indicated concentrations of guanosine, TCA and cells, and 300 mM potassium phosphate buffer (pH 7.0) were incubated at 60°C. (A) 100 mM guanosine and 100 mM TCA, (B) 200 mM guanosine and 200 mM TCA and (C) 300 mM guanosine and 300 mM TCA, as substrates, were used.
(○) 50 mg/ml and (●) 100 mg/ml cells (wet weight basis).

In contrast, *Enterobacter aerogenes* AJ 11125 and *Erwinia carotovora* AJ 2992 more efficiently produced ribavirin from TCA and pyrimidine nucleosides than from TCA and purine nucleosides. *Erwinia carotovora* AJ 2992 only produced it from TCA and orotidine.

Time course of ribavirin production from inosine and TCA

Utagawa *et al.* reported that ribavirin production directly from inosine by *Enterobacter aerogenes* AJ 11125 was rather difficult because of the lower affinity of TCA for PNPase than that of hypoxanthine, and the isolation of R-1-P from the reaction mixture was indispensable.[27] In contrast, *Brevibacterium acetylicum* ATCC 954 could produce ribavirin directly from inosine in a high yield (Table IV). The time course of ribavirin production from inosine and TCA with cells of ATCC 954 was investigated (Fig. 5). The amounts of ribavirin produced increased with the reaction time, 56 mM, 68 mM and 95 mM ribavirin being obtained in the presence of 100 mM, 200 mM and 300 mM inosine and TCA, respectively, on 96 hr reaction at 60°C with 100 mg/ml cells (wet

TABLE IV. RIBAVIRIN PRODUCTION FROM VARIOUS NUCLEOSIDES BY ATCC 954 AND OTHER MICROORGANISMS

The reaction mixtures, containing 100 mM nucleosides, as indicated in the table, 100 mM TCA, 300 mM potassium phosphate buffer (pH 7.0) and cells, 50 mg/ml on a wet weight basis, were incubated at 60°C for 24 hr.

Strains	Ribavirin (mM)							
	HxR	GR	AR	XR	AICAR	UR	CR	OR
Brevibacterium acetylicum (ATCC 954)	14.3	74.0	8.6	68.6	2.2	11.0	0.9	0
Bacillus brevis (AJ 1282)	0.9	45.9	6.5	65.9	4.0	25.5	22.2	0
Bacillus cereus (AJ 1269)	4.2	63.0	29.0	54.4	3.7	2.4	1.7	0
Bacillus megaterium (AJ 3284)	2.0	23.6	20.2	31.5	1.7	0	0	0
Enterobacter aerogenes (AJ 11125)	1.6	12.6	4.0	7.5	8.2	38.2	23.0	0
Erwinia carotovora (AJ 2992)	2.2	7.3	4.4	6.0	6.7	24.3	17.1	39.8

HxR, inosine; GR, guanosine; AR, adenosine; XR, xanthosine; AICAR, 5-amino-4-imidazolecarboxamide riboside; UR, uridine; CR, cytidine; OR, orotidine.

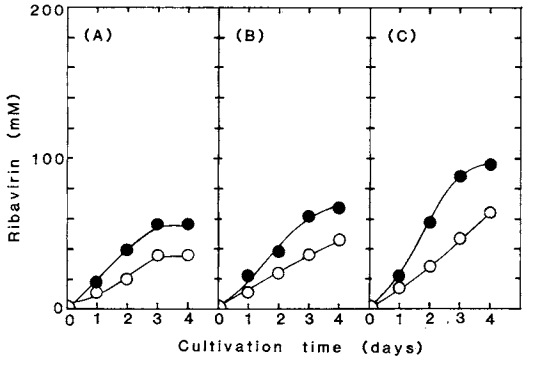

FIG. 5. Time Course of Ribavirin Production from Inosine and TCA by ATCC 954.

The reaction mixtures, containing the indicated concentrations of inosine, TCA and cells, and 300 mM potassium phosphate buffer (pH 7.0) were incubated at 60°C.
(A) 100 mM inosine and 100 mM TCA, (B) 200 mM inosine and 200 mM TCA and (C) 300 mM inosine and 300 mM TCA, as substrates, were used.
(○) 50 mg/ml; (●) 100 mg/ml cells (wet weight basis).

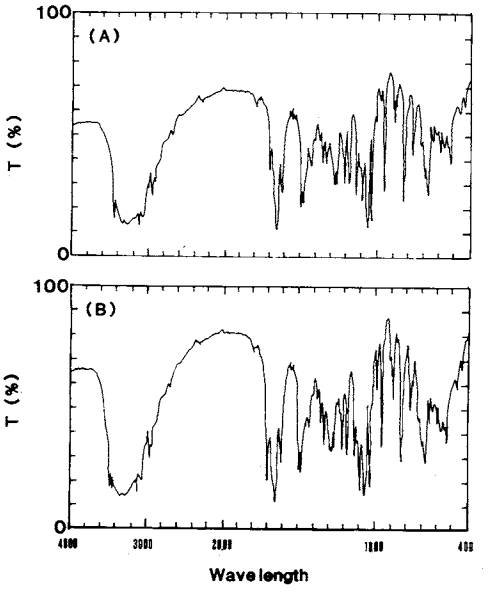

FIG. 6. The Infra-red Spectrum of the Isolate from the Reaction Mixture.

(A) authentic ribavirin and (B) ribavirin produced by ATCC 954.

weight basis) of ATCC 954.

Identification of the reaction product

The ribavirin produced was isolated from the reaction mixture by chromatographies on Diaion SK-1B (H^+ form) and SP-207 (Mitsubishikasei, Japan). The molecular weight was determined to be 244 from the Fab-mass spectrum. The NMR and infra-red spectra

of the isolate also coincided with those of authentic ribavirin. The infra-red spectrum of the isolate from the reaction mixture is shown in Fig. 6. The results of elementary analysis of the isolate were as follows, Found: C, 39.27; H, 4.86; N, 22.82. (Calcd. for $C_8H_{12}O_5N_4$: C, 39.34; H, 4.95; N, 22.94%).

DISCUSSION

Through the screening, *Brevibacterium acetylicum* ATCC 954 was selected as the best strain as to ribavirin production from guanosine and TCA. ATCC 954 also produced ribavirin from xanthosine and TCA in a high yield. The reaction seemed to consist of the following two successive reactions catalyzed by PNPase; 1) R-1-P production from guanosine or xanthosine and phosphate, and 2) ribavirin production from R-1-P and TCA. The high ribavirin productivity seemed to be due to the following reasons. As the three-dimensional structures of guanosine and xanthosine were shown to resemble that of ribavirin, as judged on X-ray crystallography,[30] guanine and xanthine, formed through the phosphorolysis of the corresponding nucleosides, were considered to inhibit the latter reaction by competing with TCA. But guanine and xanthine showed lower solubilities in the reaction mixture than TCA, a base moiety of ribavirin. So, TCA easily reacted with R-1-P without inhibition by guanine or xanthine.

It is known that hypoxanthine, a base moiety of inosine, is a competitive inhibitor with TCA for PNPase as to ribavirin production.[27] But ATCC 954 showed good ribavirin production from inosine and TCA. This may be due to the difference in affinity for the PNPase of ATCC 954 between hypoxanthine and TCA. ATCC 954 hardly produced ribavirin from adenosine and TCA. In contrast, *Bacillus cereus* AJ 1269 and *Bacillus megaterium* AJ 3284 could produce ribavirin from adenosine and TCA, and AJ 1269 also produced ribavirin from guanosine and TCA, or xanthosine and TCA, but could hardly produce it from inosine.

Lewis and Glantz[31] reported that the PNPase derived from rabbit liver acted on xanthosine as a extremely poor substrate and could not phosphorolyze adenosine at all. But ATCC 954 could produce ribavirin from xanthosine and TCA in a high yield, and AJ 1269 and AJ 3284, which belong to the genus, *Bacillus*, could produce ribavirin from adenosine and TCA. Judging from these results, purine nucleoside-phosphorolysing enzymes in microorganisms might be enzymes with wide substrate specificities for various nucleosides, or the enzymes from AJ 1269 and AJ 3284 might be specific enzymes catalyzing adenosine phosphorolysis. All the microorganisms did not produce ribavirin from nucleotides, such as guanosine-5′-monophosphate, under the basal reaction conditions.

In the system involving ATCC 954, the ribavirin-producing reaction could proceed well even at a low concentration of phosphate. This suggests that phosphate might be recycled efficiently in this reaction system.

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