Enzymatic Production of Ribavirin from Pyrimidine Nucleosides by *Enterobacter aerogenes* AJ 11125

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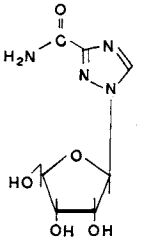
Microorganisms that produce ribavirin(1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; virazole®) directly from pyrimidine nucleosides and TCA (1,2,4-triazole-3-carboxamide) were screened from our stock cultures. Of the 400 strains tested, 16 were isolated as ribavirin-producers from uridine or cytidine. In particular, *Enterobacter aerogenes* AJ 11125, *Bacillus brevis* AJ 1282 and *Sarcina lutea* AJ 1212 were found to possess potent activities of ribavirin production from them. In the presence of intact cells of *Enterobacter aerogenes* AJ 11125, which was selected as the best strain, 110.2 mM and 67.6 mM ribavirin were produced from uridine and cytidine, respectively, on 96 hr reaction at 60°C. In addition, this strain could also produce ribavirin from guanosine, but could not produce it from orotidine, which is also a pyrimidine nucleoside.

Ribavirin, which was synthesized by Witkowski *et al.* in 1972,[1] is a broad spectrum virustatic agent and is known to be active toward various kinds of viruses *in vitro* (Fig. 1).[2~4] The antiviral mechanism of ribavirin was considered to be the inhibition of the virus-specific mRNA capping enzymes, guanyl transferase and N^7-methyl transferase or inosine monophosphate dehydrogenase.[4,5] Chemical synthetic methods for ribavirin have already been developed,[1,6] but they have a defect in that an unnecessary anomer arises as a by-product during the coupling reaction. Ochiai *et al.*[7] reported ribavirin production by a fermentation method, but the ribavirin productivity was very low.



Ribavirin

FIG. 1. The Structure of Ribavirin.

Recently, Utagawa *et al.*[8] reported the enzymatic synthesis of ribavirin through a two-step reaction from inosine *via* ribose-1-phosphate (R-1-P), an intermediate in ribavirin formation, by purine nucleoside phosphorylase (PNPase) of *Enterobacter aerogenes* AJ 11125 without the by-production of the unnecessary anomer. But ribavirin could not be produced directly from inosine and TCA because of the lower affinity of TCA for PNPase than that of hypoxanthine formed as the result of the phosphorolysis of inosine. So the isolation of R-1-P was indispensable.

This paper describes the isolation of ribavirin-producing microorganisms and the production of ribavirin directly from pyrimidine nucleosides by intact cells of *E. aerogenes* AJ 11125, which was selected as the best strain.

MATERIALS AND METHODS

Chemicals. TCA was kindly provided by Utagawa.[8] The other chemicals used were commercially available and of analytical grade.

Microorganisms. Microorganisms from stock cultures kept in our laboratory were subjected to screening for

ribavirin producers, and *Enterobacter aerogenes* AJ 11125, selected as the best strain, was used.

Preparation of intact cells. A loopful of cells of a microorganism subcultured on a bouillon agar slant was inoculated into 5 ml of medium containing 1% meat extract, 1% polypepton, 0.5% yeast extract and 0.5% NaCl in tap water, in a test tube, adjusted to pH 7.0 with 6N KOH. After aerobic incubation at 30°C for 24 hr, 1 ml of the cultured broth was transferred to 50 ml of the same medium as above in a 500-ml flask, followed by aerobic incubation at 30°C for 16 hr. The cultivated cells were then harvested by centrifugation and rinsed with 0.05 M potassium phosphate buffer (pH 7.0).

Reaction conditions. The standard conditions were as follows; a reaction mixture containing 300 mM potassium phosphate buffer (pH 7.0), 100 mM uridine or cytidine, 100 mM TCA and 50 mg/ml of intact cells, as described above, in a 5 ml test tube with a rubber plug, was incubated at 60°C for 24 hr with standing.

Analytical methods. Substrates and products were determined as described in the previous paper.[8]

TABLE I. SCREENING OF RIBAVIRIN-PRODUCING MICROORGANISMS FROM STOCK CULTURES

The reaction mixture consisted of 100 mM uridine or cytidine, 100 mM TCA, 300 mM potassium phosphate buffer (pH 7.0) and 50 mg/ml, on a wet weight basis, of rinsed cells in a total volume of 5 ml. The reaction was carried out at 60°C for 24 hr.

Strains	Ribavirin formed (mM)	
	Uridine	Cytidine
Micrococcus luteus AJ 1003	8.5	4.0
Sarcina lutea AJ 1212	24.8	13.4
Bacillus subtilis AJ 1235	18.5	1.7
Bacillus brevis AJ 1282	25.5	22.2
Corynebacterium michiganense AJ 1392	1.2	0
Arthrobacter oxydans AJ 1424	3.9	0
Arthrobacter tumescens AJ 1425	11.1	7.2
Cellulomonas flavigera AJ 1566	5.1	2.7
Erwinia herbicola AJ 2189	5.9	5.1
Escherichia coli AJ 2597	5.2	4.8
Citrobacter freundii AJ 2619	3.7	3.7
Enterobacter cloacae AJ 2662	3.1	2.4
Serratia marcescens AJ 2689	2.9	2.5
Aeromonas salmonicida AJ 2926	7.4	6.6
Salmonella schottmuelleri AJ 2930	3.0	3.1
Enterobacter aerogenes AJ 11125	38.2	23.0

RESULTS

Screening of ribavirin-producing microorganisms from stock cultures

A total of 400 strains, selected from stock cultures in our laboratory, were tested as to their ribavirin-producing ability from pyrimidine nucleosides (uridine and cytidine) and TCA.

As shown in Table I, *Enterobacter aerogenes* AJ 11125, *Sarcina lutea* AJ 1212 and *Bacillus brevis* AJ 1282 could produce ribavirin, in high yields, among the 16 strains which showed

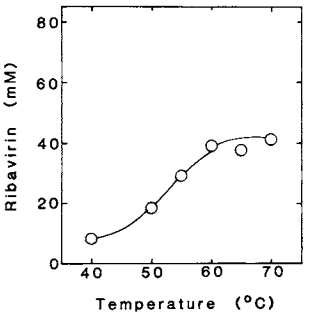


FIG. 2. Effect of Temperature on Ribavirin Formation.

The reaction mixtures, containing 100 mM uridine, 100 mM TCA, 300 mM potassium phosphate buffer (pH 7.0) and 50 mg/ml, on a wet weight basis, of rinsed cells, were incubated at various temperatures for 24 hr.

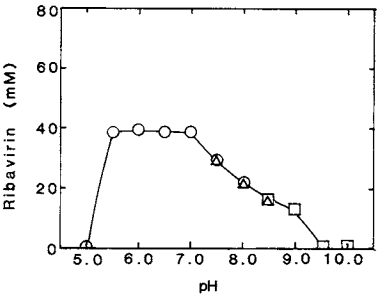


FIG. 3. Effect of pH on Ribavirin Formation.

The reaction mixtures, containing 100 mM uridine, 100 mM TCA, 50 mg/ml, on a wet weight basis, of rinsed cells 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, (□), 50 mM glycine-NaOH buffer + 300 mM K_2HPO_4.

ribavirin-producing ability. On the other hand, *Bacillus subtilis* AJ 1235, *Corynebacterium michiganense* AJ 1392 *Arthrobacter oxydans* AJ 1424 could produce ribavirin more efficiently from uridine than from cytidine. In the other strains, the productivities of ribavirin from uridine and cytidine were shown to be relatively the same.

The best strain, *Enterobacter aerogenes* AJ 11125, could produce 38.2 mM and 23.0 mM ribavirin from 100 mM uridine and 100 mM cytidine, respectively, and so was selected and used for further experiments.

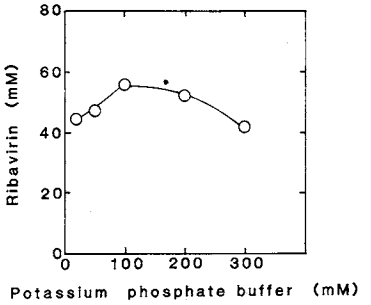


FIG. 4. Effect of the Phosphate Concentration on Ribavirin Production.

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

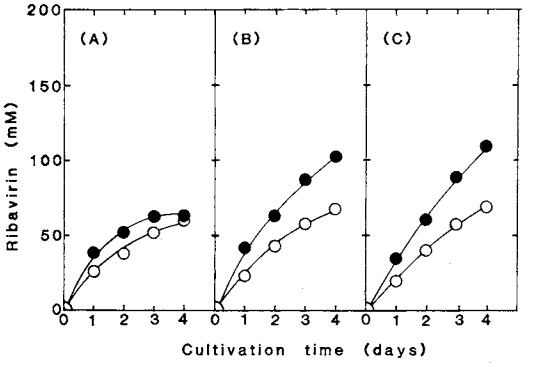


FIG. 5. Time Course of Ribavirin Production from Uridine.

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

Optimal reaction conditions

The optimal reaction conditions for ribavirin production by *E. aerogenes* AJ 11125 were determined. As shown in Fig. 2, the optimal temperature was over 60°C and ribavirin production activity was observed even at 70°C. The optimal pH was 5.5～7.0 at 60°C, as shown in Fig. 3. The optimal phosphate concentration for ribavirin production was 100 mM (Fig. 4), but the change in the activity level was only slight in the phosphate concentration range tested.

Time course of ribavirin formation

Figures 5 and 6 show the time courses of ribavirin production from uridine and cytidine, respectively, in the presence of intact cells of *E. aerogenes* AJ 11125. The amounts of ribavirin formed increased with the reaction time, and also increased in proportion to the amount of added intact cells. In contrast, the initial rate of ribavirin formation remained constant regardless of an increase in uridine or cytidine. Under the conditions with intact cells (100 mg/ml on a wet weight basis), the

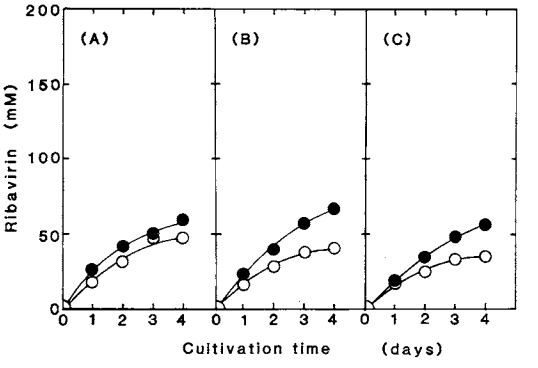


FIG. 6. Time Course of Ribavirin Production from Cytidine.

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

TABLE II. PRODUCTION OF RIBAVIRIN FROM VARIOUS NUCLEOSIDES

The reaction mixture consisted of 100 mM each nucleoside, 100 mM TCA, 300 mM potassium phosphate buffer (pH 7.0) and 50 mg/ml, on a wet weight basis, of rinsed cells in a total volume of 5 ml. The reaction was carried out at 60°C for 24 hr with standing.

Nucleosides	Ribavirin formed (mM)
Inosine	1.6
Adenosine	4.0
Guanosine	12.6
Xanthosine	7.5
AICAR*	8.2
Uridine	38.2
Cytidine	23.0
Orotidine	0

* AICAR, 4-amino-5-imidazole-3-carboxylic acid riboside.

amounts of ribavirin formed in the presence of 100 mM, 200 mM and 300 mM uridine were 61.3 mM, 103.2 mM and 110.2 mM, respectively, and those in the presence of 100 mM, 200 mM and 300 mM cytidine were 62.1 mM, 67.6 mM and 57.9 mM, respectively.

Production of ribavirin from various nucleosides

Table II shows the production of ribavirin from various nucleosides in the presence of intact cells of AJ 11125. As shown in Table II, pyrimidine nucleosides were better substrates than purine nucleosides for ribavirin production by AJ 11125. Uridine was selected as the best substrate, but ribavirin was not produced from orotidine, also a pyrimidine nucleoside. Among the purine nucleosides tested, guanosine was a better substrate as to ribavirin production than the other purine nucleosides.

DISCUSSION

Utagawa *et al.* reported the enzymatic synthesis of ribavirin through a two-step reaction from inosine by PNPase of *Enterobacter aerogenes* AJ 11125.[8] The system consisted of the following steps; 1) R-1-P production from inosine by PNPase, 2) isolation of R-1-P from the reaction mixture, and 3) ribavirin production from R-1-P and TCA by PNPase. In this system, hypoxanthine, a product of inosine phosphorolysis (1st reaction), competes with TCA in the reaction of the formation of ribavirin from R-1-P and TCA (2nd reaction), and ribavirin could not be produced directly from inosine and TCA.

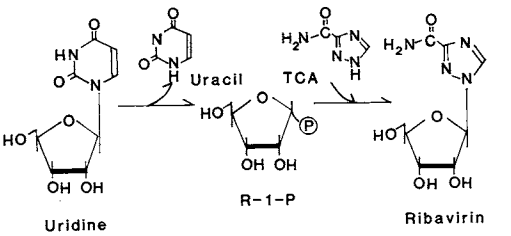


FIG. 7. The Biosynthesis of Ribavirin from Uridine.

On the basis of these observations, we considered that the direct production of ribavirin might be possible from pyrimidine nucleosides if a microorganism having both pyrimidine nucleoside phosphorylase (PyNPase) and PNPase was used. Because the enzyme catalyzing the phosphorolysis of pyrimidine nucleosides was different from the enzyme catalyzing ribavirin formation from R-1-P and TCA. Pyrimidine bases formed on the phosphorolysis of pyrimidine nucleosides were not recognized as substrates by the latter enzyme. So the ribavirin-forming reaction from R-1-P and TCA was not inhibited by pyrimidine bases. In fact, *Enterobacter aerogenes* AJ 11125, having both PyNPase and PNPase, produced ribavirin in a high yield directly from TCA and pyrimidine nucleosides such as uridine or cytidine. A proposed pathway for the ribavirin production from uridine and TCA in *Enterobacter aerogenes* AJ 11125 is shown in Fig. 7.

In addition, *Bacillus brevis* AJ 1282 and *Sarcina lutea* AJ 1212, also had potent ribavirin production activities. In the previous paper,[9] we reported the enzymatic production of ribavirin from R-1-P and TCA by various microorganisms, and the reaction of the formation of ribavirin by PNPase of AJ 11125 seemed to be the rate-limiting step for the ribavirin production. In fact, the optimal pH

for the ribavirin production by AJ 11125 was around 6, which was the optimal pH for the synthesis of ribavirin from R-1-P and TCA. On the contrary, the optimal pHs for the ribavirin production by AJ 1282 and AJ 1212 were both 7.5~8.0, which was the optimal pH for the phosphorolysis of pyrimidine nucleosides. In the latter microorganisms, it is considered that the phosphorolysis of pyrimidine nucleosides might be the rate-limiting step for the ribavirin production.

Utagawa *et al.* investigated the properties of uridine phosphorylase (URase) of AJ 11125.[10] The URase of AJ 11125 had a rather narrow substrate specificity, utilizing uridine and thymidine but not for cytidine. In contrast, it was found that ribavirin was produced directly from not only uridine but also cytidine by intact cells of AJ 11125 in this study. Judging from these results, AJ 11125 might have two types of phosphorylase, PyNPase and URase, and can produce ribavirin directly from uridine and TCA by means of either URase or PyNPase or a combination of the two enzymes in parallel with the PNPase reaction. It will be important to identify the enzymes concerned in the ribavirin production from pyrimidine nucleosides in AJ 11125.

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