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Short communication

Pyrimidine ribonucleoside catabolism in *Pseudomonas fluorescens* biotype A

Chien-Peng Chu[1] & Thomas P. West[2]*
Department of Biological Sciences, Institute of Genetics, University of Southern Mississippi, Hattiesburg, MS 39406, USA (present address: [1] Department of Microbiology and Immunology, Temple University, Philadelphia, PA 19140, USA; [2] Station Biochemistry, South Dakota State University, Brookings, SD 57007, USA) (requests for offprints)*

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Abstract

The pyrimidine ribonucleosides uridine or cytidine were shown to serve as a source of nitrogen or carbon for the growth of *Pseudomonas fluorescens* strain A126. After incubation of either pyrimidine ribonucleoside with extracts of this strain, the resultant catabolic products were detected by thin-layer chromatography. It was found that pyrimidine ribonucleoside catabolism in this pseudomonad involved the enzymes nucleoside hydrolase and cytosine deaminase. The specific activities of both these enzymes could be influenced by the nitrogen or carbon source present in the medium.

Although pyrimidine salvage pathways in enteric bacteria have been extensively examined (O'Donovan & Neuhard 1970), few studies have been conducted regarding such pathways in *Pseudomonas* (Kelln & Warren 1974; West & Chu 1987). In the present report, we have explored the catabolism of the pyrimidine ribonucleosides uridine and cytidine by *Pseudomonas fluorescens* biotype A. Of particular interest was the effect of carbon or nitrogen source upon the levels of those pyrimidine salvage pathway enzymes that are involved in pyrimidine ribonucleoside degradation.

P. fluorescens strain A126 (Stanier et al. 1966) was grown in a minimal medium (Stanier 1947), modified as previously described (West 1989). In those experiments in which asparagine (0.2%), uracil (0.2%), cytosine (0.2%), uridine (0.2%), or cytidine (0.2%) served as the nitrogen source, $(NH_4)_2SO_4$ (0.4%) was not included in the medium. When glycerol (0.4%), succinate (0.4%), ribose (0.2%), deoxyribose (0.2%), uridine (0.2%) or cytidine (0.2%) was added as a carbon source, glucose (0.4%) and sodium citrate (0.05%) were omitted from the medium.

To learn which compounds supported the growth of the pseudomonad strain as a sole nitrogen or carbon source, approximately 10^6 washed bacterial cells were used to inoculate each respective medium (5 ml). Subsequently, these cultures were shaken (200 rpm) for 8 days at 30° C. To serve as controls minimal medium cultures, and cultures to which were added no nitrogen or carbon source, were also inoculated and shaken for 8 days at 30° C. Growth was followed turbidimetrically at 600 nm. Bacterial cell concentration (colonies/ml) was estimated from a previously determined A_{600} versus cell concentration calibration curve.

The cell-free extracts necessary for the thin-layer chromatographic and enzyme analyses were prepared using *P. fluorescens* cells grown at 30° C in 60–80 ml batch cultures provided with vigorous aeration. Cultures were harvested at a concentra-

tion of approximately 3×10^8 cells/ml and washed with 0.85% NaCl. The cells were resuspended in 1.8 ml (thin-layer chromatographic analysis) or 2.5 ml (enzyme assays) of 50 mM Tris-HCl buffer (pH 7.3) and disrupted by ultrasonic treatment at 0° C for a total of 5 min. The extract was centrifuged at 12,100 × *g* for 30 min at 4° C and the resultant cell-free supernatant fraction was used immediately.

Enzyme assays were performed at 30° C. Nucleoside hydrolase activity was measured using an assay protocol that is based upon the difference in molar extinction coefficients between uridine and uracil (5.41×10^3 $M^{-1}.cm^{-1}$) at 290 nm at pH 12 (Neuhard 1968; West & Chu 1987). A unit of nucleoside hydrolase activity is expressed as nmol uridine hydrolyzed to uracil/min. Cytosine deaminase activity was determined using a previous method in which the final cytosine concentration in the assay mix was 1 mM (West et al. 1982). A unit of cytosine deaminase activity is expressed as nmol cytosine deaminated to uracil/min. Protein was measured according to Bradford (1976) using lysozyme as the standard protein. Specific activity for both enzymes is expressed as nmol uracil formed/min per mg protein.

Ascending thin-layer chromatography at 25° C was utilized to separate the products of a uridine-containing or a cytidine-containing reaction mix. The mix (0.5 ml) consisted of 50 mM Tris-HCl buffer (pH 7.3), 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol and cell-free extract (1.05 mg protein) to which was added either 10 mM phosphate and 5 mM uridine or 0.5 mM cytidine. Each mix contained extract prepared from minimal medium-grown *P. fluorescens* cells. Both reactions were performed for 60 min at 30° C, and then were terminated with 0.50 ml absolute ethanol (0° C). The resultant residue, derived from air-evaporation of each mix, was resuspended in 0.50 ml absolute ethanol and 10 µl of the resuspension was applied to a cellulose thin-layer plate. When determining the uridine or cytidine catabolic products, 1 mg/ml solutions (prepared in absolute ethanol) of cytidine, uridine, cytosine and ribose or uridine, uracil, ribose and ribose-1-phosphate, respectively, were individually spotted (6 µl) onto the appropriate thin-layer plate to serve as standards. A solvent system composed to tertiary butanol-methyl ethyl ketone-formic acid-water (40 : 30 : 15 : 15, v/v), was used to separate the catabolic products of uridine while the cytidine catabolic products were resolved using a solvent system consisting of tertiary butanol-methyl ethyl ketone-water-formic acid (44 : 44 : 11 : 0.26, v/v) (Fink et al. 1963). The thin-layer plate was removed from the tank after the solvent front was 1 cm from the top of the plate, and was subsequently dried. The location of cytidine, uridine, cytosine or uracil on the thin-layer plates was determined by virtue of their UV fluorescence (Fink et al. 1963). Both ribose and ribose-1-phosphate were detected by their brown color after spraying the plate with an aniline phosphate reagent (Kirchner 1978). Confirmation of product identity was also provided by their observed R_f values.

To understand the catabolism of pyrimidine ribonucleosides by *P. fluorescens*, it was first necessary to determine the ability of the bacterium to utilize pyrimidine ribonucleosides, pyrimidine bases as well as ribose and deoxyribose. As is evident in Table 1, either uridine, uracil, cytidine or cytosine supported excellent growth of strain A126 as a sole nitrogen source. In contrast, while either

Table 1. Growth of *Pseudomonas fluorescens* on pyrimidines and related compounds.

Growth substrate	Bacterial cells/ml (× 10^{-6})	
	Nitrogen source[a]	Carbon source[b]
Uridine	147	46.2
Uracil	115	0.950
Cytidine	134	54.4
Cytosine	89.4	0.650
Deoxycytidine	14.8	0.100
Ribose	–	68.9
Deoxyribose	–	0.600

Cells were grown for 8 days at 30° C in liquid medium with a 0.2% (w/v) concentration of each compound. Results represent the average of 2 separate determinations that were reproducible within ± 10%. After 8 days at 30° C, control cultures of minimal medium or medium lacking a carbon and nitrogen source contained 9.5×10^7 cells/ml or 8×10^5 cells/ml, respectively.

[a] Glucose (0.4%) served as the carbon source.

[b] $(NH_4)_2SO_4$ (0.4%) served as the nitrogen source.

uridine, cytidine or ribose supported the growth of this strain as a sole source of carbon, uracil, cytosine or deoxyribose did not (Table 1). Although deoxycytidine could not support significant growth of the microorganism as either a nitrogen or carbon source, it appeared to serve as a nitrogen source (Table 1).

In enteric bacteria, the enzymes cytidine deaminase and uridine phosphorylase combine to degrade pyrimidine ribonucleosides to uracil and ribose-1-phosphate (O'Donovan & Neuhard 1970). In contrast, the pyrimidine ribonucleosides cytidine and uridine have been shown to be degraded to ribose and their respective pyrimidine bases by the enzyme nucleoside hydrolase in species of *Pseudomonas* (Sakai et al. 1968, 1976). To learn which enzymes were active in *P. fluorescens* strain A126, the products of a reaction mixture containing either uridine or cytidine were analyzed by thin-layer chromatography. Two spots were detected following thin-layer chromatography of the uridine-containing reaction mixture: one spot (R_f 0.48) was found to react with aniline phosphate as did the authentic sample of ribose (R_f 0.48) while the second spot (R_f 0.69) exhibited UV fluorescence as did the authentic uracil (R_f 0.68). Ribose-1-phosphate, which is a product of the uridine phosphorylase reaction, was not detected indicating the absence of this enzyme. After thin-layer chromatography of the cytidine-containing reaction mixture, three spots were observed. The initial spot, detected by its UV fluorescence, was found to co-migrate with the cytidine standard (R_f 0.16). The second spot (R_f 0.23), also detected by its UV fluorescence, migrated in a nearly identical manner to authentic cytosine (R_f 0.22). The third spot (R_f 0.39) reacted with aniline phosphate as did authentic ribose (R_f 0.40). Uridine, an expected product of the cytidine deaminase reaction, was not present on the chromatogram after the thin-layer separation. Therefore, strain A126 would appear to lack significant cytidine deaminase activity. Chromatographic analysis of the products would seem to indicate that nucleoside hydrolase is highly active in strain A126 extracts. This was confirmed by enzyme assay (Table 2).

Considering its role in the salvage of cytosine from cytidine degradation in bacteria (West et al. 1982), the enzyme cytosine deaminase, which can catalyze the deamination of cytosine to uracil, was also assayed in this study. As can be seen in Table 2, low cytosine deaminase activity was detected in strain A126. Under standard conditions, its deaminase activity was observed to be at least 100-fold lower than its nucleoside hydrolase activity (Table 2).

The levels of the two pyrimidine salvage enzymes, namely nucleoside hydrolase and cytosine

Table 2. Effect of nitrogen source upon nucleoside hydrolase and cytosine deaminase activities in *P. fluorescens*.

Nitrogen source	Nucleoside hydrolase (U/mg protein)	Cytosine deaminase (U/mg protein × 10)
$(NH_4)_2SO_4$	74.6	4.06
Asparagine	82.3	10.1
Uracil	8.56	6.70
Cytosine	24.2	13.8
Uridine	160	3.78
Cytidine	216	4.77

Nitrogen sources were included in the growth medium as indicated in the text. Cell-free extracts, prepared from cells harvested in exponential phase, were assayed for nucleoside hydrolase and cytosine deaminase activities as described in the text. Results represent the average of 2 separate determinations that were reproducible within ± 10%.

Table 3. Effect of carbon source upon pyrimidine salvage enzyme activities in *P. fluorescens*.

Carbon source	Nucleoside hydrolase (U/mg protein)	Cytosine deaminase (U/mg protein × 10)
Glucose	74.6	4.06
Glycerol	87.7	4.25
Succinate	35.2	5.16
Ribose	301	2.98
Uridine	560	1.23
Cytidine	726	5.59

Each carbon source was added to the growth medium at the concentration given in the text. Cells were harvested in exponential phase. The resultant cell-free extract was assayed for nucleoside hydrolase and cytosine deaminase activities as given in the text. Results represent the average of 2 separate determinations that were reproducible within ± 10%.

deaminase, were examined under various growth conditions. Initially, the effect of nitrogen source upon nucleoside hydrolase activity was examined. Substituting asparagine for $(NH_4)_2SO_4$ as a sole source of nitrogen affected nucleoside hydrolase activity only slightly, while hydrolase activity was significantly diminished by growing cells with uracil or cytosine as a sole nitrogen source (Table 2). In contrast, when uridine or cytidine served as the nitrogen source in the medium, approximately a 2-fold or 3-fold increase in nucleoside hydrolase activity was observed, respectively relative to the $(NH_4)_2SO_4$) control. This indicated that growth on its substrate uridine or cytidine does influence enzyme activity (Table 2).

Because cellular nitrogen can be provided by cytosine deamination, cytosine deaminase activity was examined in relation to the nitrogen source present in the growth medium. If asparagine, replaced $(NH_4)_2SO_4$ as the sole nitrogen source, cytosine deaminase activity was noted to increase better than 2-fold (Table 2). Repression of cytosine deaminase synthesis by ammonium ions could be a possible explanation for the low activity in minimal medium-grown cells. Cells grown on uracil or cytidine as a nitrogen source contained higher cytosine deaminase activity than those grown on minimal medium (Table 2). Dissimilarly, growth on uridine as a nitrogen source decreased deaminase activity. Relative to minimal medium-grown cells, cytosine deaminase activity in strain A126 cells was found to increase more than 3-fold if its substrate cytosine was present in the medium as a sole source of nitrogen (Table 2). This finding would seem to be in agreement with that of a prior study which reported the induction of cytosine deaminase synthesis in the fluorescent pseudomonad *Pseudomonas putida* after its growth on cytosine as a nitrogen source (Kim et al. 1987).

The nature of the carbon source also affected the levels of nucleoside hydrolase and cytosine deaminase in this strain (Table 3). When glycerol was substituted for glucose as a carbon source, no major change in hydrolase activity was seen (Table 3). A drop in nucleoside hydrolase activity was observed when succinate served as the carbon source. These data do not indicate that catabolite repression of nucleoside hydrolase was an important regulatory factor. The substitution of ribose, uridine or cytidine for glucose as a carbon source drastically altered the level of nucleoside hydrolase. Its activity was found to increase approximately 4-fold, 8-fold or 10-fold after growth on ribose, uridine or cytidine, respectively (Table 3). This could be a result of regulation of nucleoside hydrolase at the level of enzyme activity or synthesis. As might be expected, replacing glucose with glycerol as the carbon source in the growth medium had little effect on the level of cytosine deaminase in strain A126 (Table 3). If either succinate or cytidine was present as the sole carbon source, a small increase in deaminase activity was noted (Table 3). A decrease in deaminase activity was observed when ribose or uridine substituted for glucose in the minimal medium (Table 3). These findings are in accordance with the apparently greater role of cytosine deaminase in nitrogen metabolism than in carbon metabolism.

Overall, this study presents evidence that the pyrimidine salvage pathway enzymes nucleoside hydrolase and cytosine deaminase are active in *P. fluorescens* strain A126. Moreover, the levels of these enzymes in strain A126 have been found to respond directly to the nitrogen or carbon source included in the growth medium. This response probably serves as an energy-conserving mechanism that would seem to be consistent with the exceptional ability of this pseudomonad to survive harsh environmental conditions.

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