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Degradation of pyrimidine ribonucleosides by *Pseudomonas aeruginosa*

Thomas P. West[1,2]

[1] *Olson Biochemistry Laboratories, Department of Chemistry and Biochemistry, South Dakota Dakota State University, Brookings, SD 57007, USA (present address);* [2] *Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS 39406, USA*

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Abstract

Pyrimidine ribonucleoside degradation in the human pathogen *Pseudomonas aeruginosa* ATCC 15692 was investigated. Either uracil, cytosine, 5-methylcytosine, thymine, uridine or cytidine supported *P. aeruginosa* growth as a nitrogen source when glucose served as the carbon source. Using thin-layer chromatographic analysis, the enzymes nucleoside hydrolase and cytosine deaminase were shown to be active in ATCC 15692. Compared to $(NH_4)_2SO_4$-grown cells, nucleoside hydrolase activity in ATCC 15692 approximately doubled after growth on 5-methylcytosine as a nitrogen source while its cytosine deaminase activity increased several-fold after growth on the pyrimidine bases and ribonucleosides examined as nitrogen sources. Regulation at the level of protein synthesis by 5-methylcytosine was indicated for nucleoside hydrolase and cytosine deaminase in *P. aeruginosa*.

Introduction

The microorganism *Pseudomonas aeruginosa* is an opportunistic human pathogen whose clinical significance has been established with regard to hospital infections (Gilardi 1991). Although the genus *Pseudomonas* has undergone taxonomic reassignment (De Vos & De Ley 1983; Gilardi 1991), *P. aeruginosa* has remained in rRNA homology group I. Only recently have studies concerning pyrimidine ribonucleoside degradation in other related *Pseudomonas* species assigned to rRNA homology group I been conducted (Chu & West 1990; West 1991). Prior work has explored pyrimidine utilization and the presence of pyrimidine salvage pathway enzyme activities in *Pseudomonas fluorescens* (Chu & West 1990), *Pseudomonas pseudoalcaligenes* (West 1991) and *Pseudomonas alcaligenes* (West 1991). Pyrimidine ribonucleoside degradation in *P. aeruginosa* has not been fully examined with respect to the enzymes involved despite pyrimidine salvage pathways being critical to the reutilization of pyrimidines (O'Donovan & Neuhard 1970). A comparison of the mode of pyrimidine ribonucleoside degradation operating in the other rRNA homology group I members with *P. aeruginosa* has not been undertaken and could prove valuable from a taxonomic perspective. In this investigation, pyrimidine ribonucleoside utilization as well as the effect of nitrogen source on enzyme activities involved in pyrimidine ribonucleoside degradation were examined in *P. aeruginosa* ATCC 15692.

Materials and methods

In this study, *P. aeruginosa* PAO1 ATCC 15692 (Holloway 1955) and *Escherichia coli* K-12 wildtype strain EMG2 (CGSC # 4401) (Bachmann 1972) were used and grown in a minimal medium that has been previously described (West 1989). Glucose, ribose or succinate (0.4%) was utilized as the carbon source and $(NH_4)_2SO_4$ (0.4%) served as the nitrogen source in the medium. The final concentration of each pyrimidine tested as a nitrogen or carbon source tested in the medium was 0.2%.

Each nitrogen or carbon source was tested for its ability to support *P. aeruginosa* growth in liquid medium cultures (5 ml) that were inoculated with about 10^7

washed cells. Subsequently, all cultures were shaken for 72 h at 37° C. Growth was determined spectrophotometrically at 600 nm and was considered to occur if the absorbance was ≥ 0.1 (1×10^8 cells/ml). Bacterial cell concentration (cells/ml) of each culture was estimated using an A_{600} versus cell concentration calibration curve.

Batch cultures (60 ml) of *P. aeruginosa* were grown shaking in 250 ml flasks at 37° C and harvested when a concentration of about 5×10^8 cells/ml was achieved. When investigating whether the increase in the pyrimidine salvage enzyme activities after growth on 5-methylcytosine required protein synthesis, ATCC 15692 was grown in 120 ml glucose minimal medium containing 0.4% $(NH_4)_2SO_4$ as the nitrogen source until mid-exponential phase. The cells were collected, washed and resuspended into a glucose minimal medium containing 5-methylcytosine as the nitrogen source. This culture was divided into two cultures of equal volume and 0.1 mg/ml chloramphenicol was added to only one of these cultures. The two cultures were grown at 37° C for 4 h and then harvested. Harvested cells were washed with 0.85% NaCl and then were resuspended in 5 ml of 50 mM Tris-HCl buffer (pH 7.30). The cells were ultrasonically disrupted in an ice bath using 30 s bursts for a total of 5 min where sufficient time was provided for each extract in ice to maintain its temperature at 4° C. The extract was centrifuged at 12 100 × g for 30 min at 4° C. The cell-free extract was dialyzed overnight at 4° C against 500 ml of sonication buffer.

Enzyme activities were assayed at 37° C. Nucleoside hydrolase, cytosine deaminase, cytidine deaminase and uridine phosphorylase activities were assayed using prior procedures (Beck et al. 1972; West 1994). Protein was measured according to Bradford (1976) where lysozyme served as the standard. Specific activity for each enzyme was expressed as nmol product formed/min per mg protein.

The cytidine-containing reaction mix or uridine-containing reaction mix (with 10 mM phosphate added only to the latter mix) was prepared as previously described (West 1994). The catabolic products were separated using ascending thin-layer chromatography. Extract (1.30 mg protein) from *P. aeruginosa* cells grown on glucose minimal medium with $(NH_4)_2SO_4$ as the nitrogen source was added to each mix. After incubating the mixes for 60 min at 37° C, each reaction was halted with ice-cold absolute ethanol (0.50 ml). Following centrifugation to remove precipitate, the supernatant was air-evaporated and the resultant residue was resuspended in absolute ethanol (0.50 ml). Resuspended residue (10 μl) and standard solutions (6 μl) were spotted onto cellulose thin-layer plates. For separation of cytidine catabolic products, a solvent system (solvent 1) composed of tertiary butanol-methyl ethyl ketone-water-formic acid (44:44:11:0.26, v/v) was utilized (Fink et al. 1963; Chu & West 1990). Uridine catabolic products were separated using a solvent system (solvent 2) consisting of tertiary butanol-methyl ethyl ketone-water-formic acid (40:30:15:15, v/v) (Fink et al. 1963; Chu & West 1990). On each thin-layer plate, cytidine, cytosine, uridine and uracil were detected using UV fluorescence while ribose and ribose-1-phosphate were identified by their brown color after treatment with an aniline phosphate reagent (Kirchner 1978; Chu & West 1990). Observed R_f values were used to identify products.

Table 1. Growth of *P. aeruginosa* on pyrimidine ribonucleosides and related compounds

Growth substrate	Bacterial cells/ml ($\times 10^8$)	
	Nitrogen source[a]	Carbon source[b]
Uracil	7.44 (0.06)	0.00 (0)
Cytosine	6.99 (0.07)	0.00 (0)
5-Methylcytosine	4.43 (0.04)	0.04 (0)
Thymine	6.04 (0.06)	0.00 (0)
Uridine	5.18 (0.06)	0.42 (0.04)
Cytidine	5.55 (0.08)	0.70 (0.06)
Ribose	–	2.15 (0.05)

Liquid medium cultures were grown for 72 h at 37° C. Results represent the mean of 3 separate determinations. The number in parentheses indicates the standard deviation. After 72 h at 37° C, control cultures of glucose/$(NH_4)_2SO_4$ minimal medium, medium lacking a carbon source or medium lacking a nitrogen source contained 7.19×10^8 cells/ml, 4.00×10^6 cells/ml, or 4.00×10^6 cells/ml, respectively. [a] Glucose (0.4%) served as the carbon source. [b] $(NH_4)_2SO_4$ (0.4%) served as the nitrogen source.

Results and discussion

Utilization of pyrimidine bases and ribonucleosides by *P. aeruginosa* as sole nitrogen or carbon sources to sustain its growth was examined (Table 1). Uracil, cytosine, 5-methylcytosine, thymine, uridine or cytidine were able to support the growth of ATCC 15692 as a sole nitrogen source (Table 1). Although the pyrimidines were unable to support the growth of this pseudomonad as sole carbon sources, ribose was capable of supporting its growth (Table 1). Thus, *P. aeruginosa* resembled other pseudomonads in that pyrimidine

Table 2. Thin layer chromatographic identification of cytidine and uridine catabolic products

Compound	R_f values		UV	Aniline
	Solvent 1	Solvent 2	fluorescence	phosphate
Cytidine	0.17	–	+	-
Cytosine	0.21	–	+	-
Uridine	0.49	0.53	+	-
Uracil	0.61	0.66	+	-
Ribose	0.41	0.48	-	+
Ribose-1-phosphate	–	0.09	-	+
Product 1	0.17	–	+	-
Product 2	0.23	–	+	-
Product 3	0.40	–	-	+
Product 4	0.62	–	+	-
Product 5	–	0.47	-	+
Product 6	-	0.53	+	-
Product 7	–	0.67	+	-

Extracts were prepared from cells grown on medium containing 0.4% glucose and 0.4% ammonium sulfate. Reaction mixtures containing cytidine or uridine were incubated for 60 min at 37° C. +, positive reaction. Cytidine and uridine catabolic products were separated using solvent 1 and solvent 2, respectively.

Table 3. Effect of nitrogen and carbon source upon nucleoside hydrolase and cytosine deaminase activities in *P. aeruginosa*

Nitrogen source	Carbon source	Nucleoside hydrolase (mU/mg protein)	Cytosine deaminase (mU/mg protein)
$(NH_4)_2SO_4$	Succinate	20.3 (0.7)	9.1 (0.2)
$(NH_4)_2SO_4$	Ribose	38.7 (0.7)	14.5 (0.3)
$(NH_4)_2SO_4$	Glucose	36.6 (0.3)	4.4 (0.3)
Uracil	Glucose	34.2 (0.4)	84.8 (2.9)
Cytosine	Glucose	51.7 (0.7)	23.4 (0.6)
5-Methylcytosine	Glucose	73.9 (0.8)	91.6 (1.7)
Thymine	Glucose	38.8 (1.2)	69.9 (0.8)
Uridine	Glucose	63.4 (1.1)	11.6 (0.8)
Cytidine	Glucose	63.2 (0.6)	35.9 (1.4)

Nitrogen and carbon sources were included in the culture medium as stated in the text. Nucleoside hydrolase and cytosine deaminase activities were assayed at 37° C in the cell-free extracts derived from 3 different cultures as indicated in the text (mU = nmol/min). Results are the mean of 3 separate determinations where the number in parentheses indicates the standard deviation.

bases and ribonucleosides served as excellent nitrogen sources for growth (Chu & West 1990; West 1991).

It was also of interest to identify which enzymes were involved in pyrimidine ribonucleoside metabolism in *P. aeruginosa*. In other pseudomonads, the enzymes nucleoside hydrolase and cytosine deaminase were active in such metabolism (Terada et al. 1967; Sakai et al. 1976; Chu & West 1990; West 1991). The former enzyme catalyzes the hydrolytic degradation of pyrimidine and purine ribonucleosides to their respective bases and ribose (Terada et al. 1967) while the latter enzyme catalyzes the deamination of cytosine or 5-methylcytosine to uracil or thymine, respectively, in pseudomonads (Sakai et al. 1975). This contrasts the combination of the enzymes cytidine deaminase and uridine phosphorylase that are active in cytidine catabolism in enteric bacteria (O'Donovan & Neuhard 1970; Sakai et al. 1976). Thin-layer chromatographic analyses of cytidine and uridine catabolic products were undertaken in attempt to resolve which enzymes were involved in *P. aeruginosa* ribonucleoside metabolism. Initially, the cytidine catabolic products were analyzed (Table 2) and 4 cytidine catabolic products were detected after incubation with cell extract (products 1–4). The spots on the plate were identified as cytidine (product 1), cytosine (product 2), ribose (product 3) and uracil (product 4). With the detection of cytosine plus ribose but no uridine (a product of cytidine deaminase), nucleoside hydrolase was likely active in ATCC 15692 cells. The presence of uracil on the plate probably resulted from cytosine deamination by cytosine deaminase.

Analysis of the uridine catabolic products using cell extract (Table 2) revealed 3 catabolic products including ribose (product 5), uridine (product 6) and uracil (product 7). The detection of only ribose and no ribose-1-phosphate (uridine phosphorylase reaction product) again indicated hydrolase activity. Overall, it appeared that nucleoside hydrolase and cytosine deaminase were active in *P. aeruginosa* cells and this was confirmed by enzyme assay (Table 3). Although nucleoside hydrolase and cytosine deaminase were active, the absence of cytidine deaminase and uridine phosphorylase activities in *P. aeruginosa* cannot be concluded unless proven by assay. Cytidine deaminase and uridine phosphorylase were assayed in extracts prepared from glucose minimal medium-grown cells of *P. aeruginosa* and of the enteric *E. coli* K12 wildtype strain (this strain is known to contain both enzyme activities) (O'Donovan & Neuhard 1970). While cytidine deaminase or uridine phosphorylase activity could not be detected in ATCC 15692 cells, cytidine deaminase or uridine phosphorylase activity in the *E. coli* cells was 59.8 (2.7) nmol deoxyuridine/min per mg protein or 41.1 (2.0)

nmol uracil/min per mg protein (standard deviation in parentheses), respectively. The presence of nucleoside hydrolase and cytosine deaminase in *P. aeruginosa* ATCC 15692 indicated that its mode of pyrimidine ribonucleoside catabolism was analogous to that found in other species of *Pseudomonas* (Sakai et al. 1976; Chu & West 1990; West 1991).

The effect of growth medium upon nucleoside hydrolase and cytosine deaminase activities in *P. aeruginosa* was investigated. Initially, the effect of carbon source on these enzyme activities was studied. As seen in Table 3, hydrolase activity in glucose-grown (generation time 97 min) or ribose-grown cells (generation time 154 min) was nearly double compared to succinate-grown cells (generation time 116 min). In contrast, cytosine deaminase activity in ribose- or succinate-grown cells was more than double its activity in glucose-grown cells (Table 3).

Next, the effect of nitrogen source upon nucleoside hydrolase and cytosine deaminase activities in ATCC 15692 glucose-grown cells was determined. Growth on uracil (generation time 222 min) or thymine (generation time 354 min) as a nitrogen source had little effect on hydrolase activity (Table 3). Relative to $(NH_4)_2SO_4$-grown cells, its activity did increase after growth of ATCC 15692 on cytosine (generation time 189 min), uridine (generation time 648 min), cytidine (generation time 157 min) or 5-methylcytosine (generation time 790 min) with its activity being highest in 5-methylcytosine-grown cells (Table 3). In *P. fluorescens*, nucleoside hydrolase activity also doubled after growth on the nitrogen source uridine or cytidine (Chu & West 1990). All of the nitrogen sources examined elevated cytosine deaminase activity by more than two-fold compared to $(NH_4)_2SO_4$ (Table 3). The largest increases in cytosine deaminase activity were observed (Table 3) after growth on 5-methylcytosine (21-fold), uracil (19-fold) or thymine (16-fold). Correspondingly, growth of *P. fluorescens* or *P. pseudoalcaligenes* on cytosine or uracil as a nitrogen source increased cytosine deaminase activity (Chu & West 1990; West 1991). Interestingly, *P. aeruginosa* growth on uracil has been shown to induce its pyrimidine base catabolic enzyme activities (Kim & West 1991). The rise in cytosine deaminase activity after *P. aeruginosa* growth on uracil suggests that this enzyme may have a role in pyrimidine base catabolism.

Possible regulation of nucleoside hydrolase and cytosine deaminase syntheses were investigated. Nucleoside hydrolase or cytosine deaminase activity was 28.1 (0.2) nmol uridine/min per mg protein or 4.6 (0.3) nmol uracil/min per mg protein (standard deviation in parentheses), respectively, in extracts prepared from *P. aeruginosa* cells initially grown in a minimal medium with $(NH_4)_2SO_4$ and then grown for 4 h in a minimal medium containing 5-methylcytosine plus the prokaryotic protein synthesis inhibitor chloramphenicol. These activities were similar to those observed in $(NH_4)_2SO_4$-grown cells (Table 3). In the absence of chloramphenicol, hydrolase activity rose approximately 1.7-fold to 48.1 (2.2) nmol uridine formed/min per mg protein while deaminase activity rose about 6.3-fold to 29.1 (0.3) nmol uracil/min per mg protein (standard deviation in parentheses) compared to the chloramphenicol-treated cells. The increases in both activities appeared to be dependent upon ongoing protein synthesis and thus, regulation for these enzymes was concluded to exist at the level of protein synthesis in *P. aeruginosa*.

In summary, *P. aeruginosa* ATCC 15692 utilized pyrimidine ribonucleosides for growth by virtue of its pyrimidine salvage pathway enzymes which were both regulated by 5-methylcytosine. Taxonomically, *P. aeruginosa* and other *Pseudomonas* species of the rRNA homology group I appeared to be closely related if their mode of pyrimidine ribonucleoside catabolism was used as a criterion.

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