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Pathways of Pyrimidine Salvage in *Pseudomonas* and Former *Pseudomonas*: Detection of Recycling Enzymes Using High-Performance Liquid Chromatography

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Abstract Pyrimidine salvage pathways are vital for all bacteria in that they share in the synthesis of RNA with the biosynthetic pathway in pyrimidine prototrophs, while supplying all pyrimidine requirements in pyrimidine auxotrophs. Salvage enzymes that constitute the pyrimidine salvage pathways were studied in 13 members of *Pseudomonas* and former pseudomonads. Because it has been established that all *Pseudomonas* lack the enzyme uridine/cytidine kinase (Udk) and all contain uracil phosphoribosyl transferase (Upp), these two enzymes were not included in this experimental work. The enzymes assayed were: cytosine deaminase [Cod: cytosine + H_2O → uracil + NH_3], cytidine deaminase [Cdd: cytidine + H_2O → uridine + NH_3], uridine phosphorylase [Udp: uridine + P_i ↔ uracil + ribose – 1 - P], nucleoside hydrolase [Nuh: purine/pyrimidine nucleoside + H_2O → purine/pyrimidine base + ribose], uridine hydrolase [Udh: uridine/cytidine + H_2O → uracil/cytosine + ribose]. The assay work generated five different Pyrimidine Salvage Groups (PSG) designated PSG1 – PSG5 based on the presence or absence of the five enzymes. These enzymes were assayed using reverse phase high-performance liquid chromatography techniques routinely carried out in our laboratory. *Escherichia coli* was included as a standard, which contains all seven of the above enzymes.

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Introduction

Pseudomonads are ubiquitous soil bacteria, Gram-negative, oxidase positive, aerobic rods that are motile by one or more polar flagella. They have a strictly oxidative metabolism and display extreme versatility in their use of carbon and energy sources. The landmark paper of Stanier, Palleroni, and Doudoroff in 1966 provided a rational subdivision of the genus *Pseudomonas* into species [27]. A total of 267 strains, collected worldwide, were subjected to exhaustive study, after which 10 *Pseudomonas* species emerged including *Pseudomonas aeruginosa* (the type species), *P. putida*, *P. aureofaciens, P. fluorescens, P. acidovorans, P. multivorans*, and *P. maltophilia*. In 1973, the pseudomonads were divided into five distinct groups, designated rRNA groups I–V based on their rRNA/DNA hybridizations [22, 23]. Since 1992, many former *Pseudomonas* members have been reassigned to new genera [38]. Members of rRNA homology group I were retained as the genus *Pseudomonas sensu stricto*, whereas members of rRNA homology groups II–V have been reassigned to new genera. In this study, we have examined the pyrimidine salvage pathways in seven members of rRNA homology group I and we have also explored the salvage pathways in one member of rRNA homology groups II–V. The salvage pathways in *Escherichia coli* are included for comparison [17, 24].

Pyrimidine salvage pathways are vital for all bacterial cells in that they share the synthesis of RNA with the biosynthetic pathway in pyrimidine prototrophs, while supplying all the pyrimidine requirements in auxotrophs [18]. The salvage enzymes scavenge bases, nucleosides, and nucleotides for recycling to RNA while making available the pentose moieties of nucleosides for carbon and energy sources. The amino groups of pyrimidines are

removed by oxidative deaminations to supply nitrogen sources, and the pyrimidine ring itself is opened up both oxidatively [29] and reductively [29, 33, 36, 37] for further catabolism.

The catabolic products utilized for recycling are mostly ribonucleoside-5′-monophosphates from mRNA degradation with minor contributions from DNA ligations [17], tRNA processing [7], and the turnover of nucleotide coenzymes [28].

Whereas the biosynthetic pathway of pyrimidine ribonucleotides is virtually the same for all organisms, the salvage pathway differs among some species, making it valuable as a potential taxonomic marker. Although the biosynthetic pathway is present in all but a few bacteria that are obligate intracellular parasites [4], some aspect of the salvage pathway is present in every bacterium [2].

The pyrimidine salvage pathways for *Escherichia coli* and *Salmonella typhimurium* have been explored in meticulous detail by Neuhard and colleagues [17–19], but except for the work of West and collaborators [6, 14, 30–37], no similar detail has been reported for pyrimidine salvage pathways in the pseudomonads. Because of the ubiquity and importance of *Pseudomonas*, in particular *P. aeruginosa*, we present here the pyrimidine salvage pathways in 13 members of *Pseudomonas* and former pseudomonads.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains utilized in this study are listed in Table 1. The *Escherichia coli* strain was grown in *E. coli* minimal medium with 0.2% (wt/vol) glucose as carbon source [16]. Cytidine, cytosine, and uridine at 1 mM final concentration were added for induction of the synthesis of the salvage enzymes. The *Pseudomonas* and former *Pseudomonas* bacteria *(Brevundimonas, Comamonas, Burkholderia, Stenotrophomonas, Shewanella)* were grown in *Pseudomonas* minimal medium [21] using 0.2% succinate as a carbon source. All cultures were grown to 100 Klett Units (KU) where 1 KU equals 10^7 cells/ml (measured with a Klett-Summerson photoelectric colorimeter, with a 1-cm light-path length, using a green filter no. 54).

HPLC Equipment and Mobile Phase

The HPLC equipment utilized for assay of the salvage enzyme activity was a Waters 510 pump, Waters Model U6K Universal Liquid Chromatograph Injector, a SpectroMonitor 5000 Photodiode Array detector, a Waters Model 740 Data Module, and reverse-phase columns by Beckman and Rainen: Ultrasphere ODS5, µm, 4.6 mm ID × 25 cm reverse-phase column and Waters NovaPak™ C18 Reverse Phase Column. The mobile phase was 50 mM ammonium phosphate monobasic [Mallinckrodt, high-performance liquid chromatography (HPLC) grade], pH 3.5 (HCl) using HPLC grade, filtered water at 10 megohms /cm (Millipore, Milli-QTM Water System).

Table 1 Strains used in this research

Strains	RRNA group	Reference
Brevundimonas diminuta	IV	ATCC 11568
Burkholderia cepacia	II	ATCC 25416
Comamonas testosteroni	III	ATCC 11996
Escherichia coli	Control	K12, ATCC 25922
Pseudomonas aeruginosa	I	ATCC 10145, 27853
P. aureofaciens	I	ATCC 13985
P. fluorescens	I	CW 1013; West T. P.
P. indigofera	*	ATCC 119706
P. mendocina	I	ATCC 10541
P. pseudoalcaligenes	I	ATCC 17440
P. putida	I	PRS2000; Holloway, B. W.
P. stutzeri	I	ATCC 11607
Shewanella putrefaciens	*	ATCC 8071
Stenotrophomonas maltophilia	V	ATCC 13637

Roman numerals denote assigned rRNA homology groups [22]. Asterisks (*) denote strains not assigned to a homology group. *Escherichia coli* is used as a standard control

Disruption of Cells

Cells were pelleted in 50 ml plastic conical tubes at 3000 *g* for 10 min at 2°C and the supernatant was discarded. All cells were washed in HPLC breaking buffer (50 mM Tris-HCl, enzyme grade; pH 7.0 and modified with Mg Cl_2) [12]. The cell pellets were resuspended in the buffer with equal quantities of 5 µm glass beads (Sigma) and mixed on a vortex mixer at high speed for 2 to 3 min to disrupt the cells. The supernatant was filtered through a 0.45 µl Gelman or Whatman syringe filter. Half the cell extract was placed in dialysis tubing and dialyzed overnight in 2 L of a 50 mM Tris buffer placed on a stirrer at 4°C. This extract was subsequently utilized for the salvage enzyme assays.

HPLC Assays

For the nucleoside hydrolase (Nuh) assays, the method of Lee [15] was used. Samples of 20 µl of cell extract,

dialyzed overnight against 2 L of HPLC breaking buffer prior to assaying for nucleoside hydrolase, were added to 80 µl of 1 mM substrate (adenosine, guanosine, cytidine, or uridine) in the breaking buffer and incubated for 10 min at 37°C for *E. coil* and *P. aeruginosa* and 30°C for the other pseudomonads. The reactions were terminated after 10 min by filtering the assay mixtures through 0.2 µm filters into tubes stored on ice. A control containing 50 µl of cell extract in 450 µl HPLC breaking buffer was also prepared in this manner. To observe the products of the enzyme assays, a 30-µl sample was injected onto the reverse-phase column described above. Compounds were detected by monitoring the column effluent at 254 nm with a sensitivity set at 0.06 absorbance units full scale. Individual nucleosides and bases were identified using retention times established by monitoring known standards and their retention times on the reverse-phase column [15]. For the uridine/cytidine hydrolase (Udh) assays, the above procedure was followed except that only uridine and cytidine were used as substrates. The cytidine deaminase (Cdd), cytosine deaminase (Cod), and uridine phosphorylase (Udp) assays were performed as above except that undialyzed cell extract was used for each assay. For the Cdd assay, undialyzed cell extract was incubated with 1 mM cytidine substrate for 10 min at 30°C [12]. The Cod assay was the same as the Cdd except that cytosine was used in place of cytidine [12]. In order to distinguish between Nuh, Udh, and Udp, two assays were devised for Udp. The forward catabolic reaction (uridine + Pi → uracil + ribose – 1 - P) was performed by incubating 20 µl filtered dialyzed cell extract in the HPLC breaking buffer with 80 µl of 1 mM uridine. The reverse anabolic reaction (uracil + ribose – 1 - P → uridine + Pi) was performed to prove conclusively the presence of uridine phosphorylase. In this assay, 20 µl of filtered dialyzed cell extract, 20 µl of ribose – 1 - P (1 mM) and 80 µl uracil (1 mM) substrate were incubated for 10 min at 30°C.

Hydrolase reactions are irreversible and take place using both dialyzed or undialyzed cell extracts, whereas uridine phosphorylase reactions, which are reversible, do not occur using dialyzed extracts unless phosphate is re-introduced. Thus, one can distinguish between Udh and Udp because in the Udp assay the dialysis removes all inorganic phosphate required for the phosphorylase activity. The dialysis has no effect on hydrolase reactions.

Results

The pyrimidine salvage enzymes of all strains listed in Table 1 were assayed using reverse-phase HPLC methods. For each set of chromatograms, two controls were run. One control was performed on the cell extract to determine whether any of the organisms contained pyrimidine substrates or products in the cell extract. The second control was run on the dialyzed cell extract to see whether any pyrimidines were present in the cell extract after dialysis. These assays of the key salvage enzymes generated five distinct Pyrimidine Salvage Groups (PSG) as designated PSG1–PSG5 based on the presence or absence of the five enzymes. The enzymes were assayed as described in Materials and Methods and included cytidine deaminase (Cdd), cytosine deaminase (Cod), nucleoside hydrolase (Nuh), uridine/cytidine hydrolase (Udh), and uridine phosphorylase (Udp).

Pyrimidine Salvage Group 1 (PSG1)

This group includes *Pseudomonas aeruginosa*, *P. aureofaciens*, *P. fluorescens*, *Brevundimonas diminuta*, *Comamonas testosteroni*, and *Stenotrophomonas maltophilia*. The latter three members were formerly listed in the Pseudomonas genus but have been moved to three new genera. All members of PSG1 contained the enzymes Cod and Nuh.

Pyrimidine Salvage Group 2 (PSG2)

This group includes *P. mendocina, P. pseudoalcaligenes, P. stutzeri*, and *Burkholderia cepacia.* The latter strain was a former *Pseudomonas* species but has been placed in the new genus, *Burkholderia*. These strains showed activity for Cod, Cdd, and Udp.

Pyrimidine Salvage Group 3 (PSG3)

This group has only one member, namely, *P. indigofera*, and contains the enzymes Cod, Cdd, and Udh.

Pyrimidine Salvage Group 4 (PSG4)

P. putida is the sole member of pyrimidine salvage group four (PSG4). It contains the enzymes Cod and Nuh like members of PSG1, but unlike those members it also contains the enzyme Udp.

Pyrimidine Salvage Group 5 (PSG5)

Escherichia coli was used as the standard control. It contains the enzymes Cdd, Cod, Udp, Udh, and Nuh. It also contains the enzymes uridine/cytidine kinase, Udk, absent

in *Pseudomonas*, and uracil phosphoribosyltransferase, Upp, which is present in all strains listed in Table 1. *Shewanella putrefaciens*, formerly *Pseudomonas putrefaciens*, contained the same salvage enzymes as did *E. coli*. Both were arbitrarily placed in pyrimidine salvage group 5 (PSG5). These details are summarized in Table 2.

Discussion

Based on the results derived in this research and on data reported elsewhere [2, 6, 20, 30–37], we suggest five different salvage schemes for the five PSGs mentioned above. These are shown in Figure 1 panels PSG1 through PSG5. Pathways of pyrimidine salvage in *Pseudomonas* differ significantly from those of *E. coli* (Fig. 1, panel PSG1 *versus* panel PSG5). *Pseudomonas*, as a genus, lacks Cdd and uridine/cytidine kinase (Udk) but contains Cod and Nuh. Pyrimidine recycling pathways for *E. coli* are shown in Figure 1, panel PSG5. As can be seen from the figure, *E. coli* contains both Cdd and Cod, Udk, Udp, and Udh. When we first reported uridine hydrolase activity [2, 3] we did not find hydrolase activity with cytidine as substrate, although this has since been demonstrated in our laboratory [13]. In their seminal paper, Petersen and Moller [24] showed that *E. coli* not only contained a uridine/cytidine (pyrimidine) hydrolase, which they renamed RihA (for ribonucleoside hydrolase A), but also a nucleoside hydrolase, which they named RihC, and a third hydrolase renamed RihB, which was active with pyrimidine nucleosides. In addition to the above, *E. coli* also possessed the conventional Udp. Even when the three hydrolases were knocked out [24], the *E. coli* cells grew normally, which begs the question as to the role of these three isofunctional enzymes. This question is briefly dealt with below.

As can be seen in Table 2 and Figure 1, typically *Pseudomonas* contains a ribonucleoside hydrolase (Nuh), which degrades both purine and pyrimidine ribonucleosides but not uridine phosphorylase (Udp). An exception to this is seen for *P. putida*, where both a ribonucleoside hydrolase and a uridine phosphorylase are observed [2]. This is in agreement with the findings of Yamamoto et al. [39], who separated *P. putida* from the other pseudomonads [39]. Thus, the salvage enzymes may be used for taxonomic purposes. The finding of a ribonucleoside hydrolase and uridine phosphorylase for the same reaction is not unusual because it is seen in *E. coli* [2, 24, 25] and other enterics [2], in the coryneform bacteria [1] and again for mRNA degradation in *E. coli* and *Bacillus subtilis* [8, 9].

In a much earlier study, Boyer and coworkers [5, 11] reported that whereas *E. coli* degraded its mRNA hydrolytically to 5′ monophosphates, *Bacillus subtilis* did so phosphorolytically to diphosphates [8]. These findings were reinvestigated more recently [9, 10] where the original findings were confirmed. However, *E. coli* was found to degrade not all but 90% of its mRNA hydrolytically using RNase II (*rnb*), but in the absence of RNase II, a less active polynucleotide phosphorylase (PNPase, *pnp*) degrades the remaining 10% in phosphorolytic fashion to diphosphates. Similarly, in *B. subtilis* where there is no RNase II, a polynucleotide phosphorylase, analogous to the *E. coli* enzyme, degrades mRNA to diphosphates. A less active hydrolytic ribonuclease is also present in *B. subtilis*. The importance of mRNA degradation and its purine and pyrimidine salvage is borne out by the fact that a double mutant lacking functional *pnp* and *rnb* is lethal in *E. coli* [10].

Table 2 Pyrimidine salvage groups (PSG) in selected *Pseudomonas* species and former pseudomonads

Pyrimidine Salvage Group	Organisms	Enzymes				
		Cdd	Cod	Nuh*	Udh	Udp
PSG1	*Pseudomonas aeruginosa*	−	+	+	−	−
	Pseudomonas aureofaciens	−	+	+	−	−
	Pseudomonas fluorescens	−	+	+	−	−
	Brevundimonas diminuta	−	+	+	−	−
	Comamonas testosteroni	−	+	+	−	−
	Stenotrophomonas maltophilia	−	+	+	−	−
PSG2	*Burkholderia cepacia*	+	+	−	−	+
	Pseudomonas mendocina	+	+	−	−	+
	Pseudomonas pseudoalcaligenes	+	+	−	−	+
	Pseudomonas stutzeri	+	+	−	−	+
PSG3	*Pseudomonas indigofera*	+	+	−	+	−
PSG4	*Pseudomonas putida*	−	+	+	−	+
PSG5	*Shewanella putrefaciens*	+	+	+	+	+
	Escherichia coli	+	+	+*	+	+

E. coli is included as the standard control. Nuh* has been renamed Rih C in *E. coli* [24]. Pyrimidine salvage schemes are seen in Figure 1

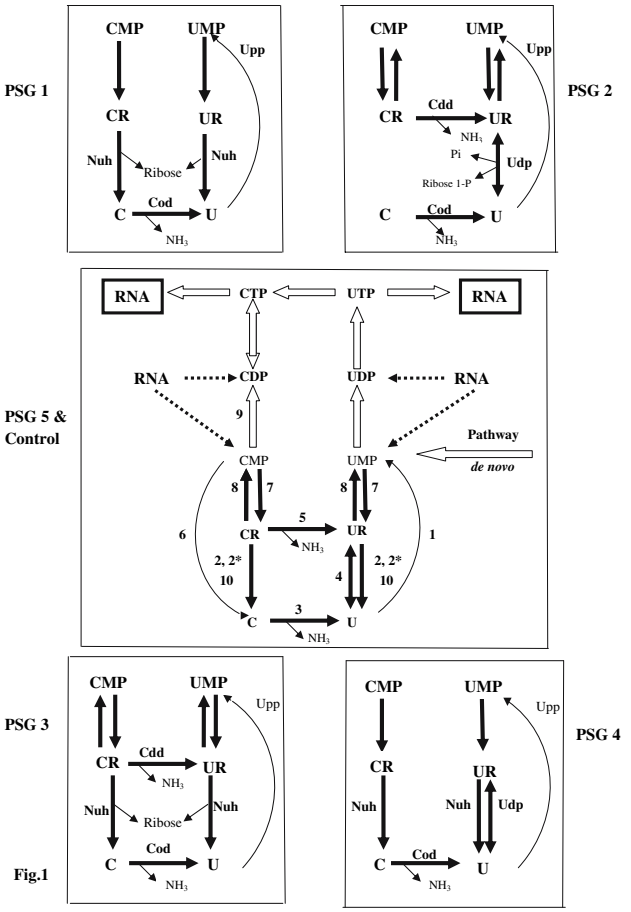

Fig. 1 Pyrimidine salvage pathways for the five groups (PSG1–PSG5) of pseudomonads and Ec control listed in Table 2. Enzymes are: 1. Uracil phosphoribosyltransferase (Upp), 2. Nonspecific ribonucleoside hydrolase (Nuh, Ps; RihA Ec), 2.* Pyrimidine specific ribonucleoside hydrolase (Rih A, Ec), 3. Cytosine deaminase (Cod), 4. Uridine phosphorylase (Udp), 5. Cytidine deaminase (Cdd), 6. CMP glycosylase, 7. Nucleotidase, 8. Uridine/Cytidine kinase (Udk), 9. CMP kinase (Cmk), 10. Pyrimidine–specific ribonucleoside hydrolase (Rih B, Ec). Ec, *Escherichia coli*; Ps, *Pseudomonas*; Cytidine 5′ monophosphate (CMP); Uridine 5′ monophosphate (UMP); Cytidine (CR); Uridine (UR)

The choice between phosphorolytic and hydrolytic degradation of mRNA and nucleosides is likely to reflect energy considerations in the two niches occupied by *E. coli* and *B. subtilis*, although neither organism operates in an all-or-nothing mode. On the one hand, the presence of a uridine hydrolase and a Udp in *E. coli* gives the organism considerable flexibility in coping with the feast or famine surges of the colon [26]. On the other hand, in an energy-poor environment, such as experienced by *P. putida*, corynebacteria [1, 2, 11], and *B. subtilis* in the soil, phosphorolytic breakdown is likely to predominate to produce diphosphates. This also preserves the energy of the phosphodiester bond. In all cases, the major degradation scheme is likely to be induced by the environmental conditions. Organisms that can adjust their hydrolase-to-phosphorylase ratio are favored in times of stress. Thus, a common feature of mRNA and nucleoside degradation is that both seem to require hydrolytic *and* phosphorolytic breakdown. This may account for the presence of both a hydrolase and a phosphorylase in several organisms.

We followed up on the prevalence of uridine/cytidine (pyrimidine) hydrolase (Udh) in other bacteria and found it widely distributed in all enteric bacteria. It was also found in *Staphylococcus aureus, Pseudomonas indigofera, Stenotrophomonas maltophilia, and Shewanella putrefaciens*, among others [2, 3, 8]. In some cases, the cleavage of the *β-N*-glycosyl linkage is carried out by one enzyme only, namely, the hydrolase, as in *Pseudomonas indigofera*. In most cases of degradation, however, there appears to be both a phosphorolytic and a hydrolytic breakdown of mRNA products, especially ribonucleosides.

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