PYRIMIDINE SALVAGE ENZYMES IN MICROORGANISMS:
LABYRINTHS OF ENZYMATIC DIVERSITY

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Debrah A. Beck, B.S., M.A.
Denton, Texas
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Pyrimidine salvage pathways are essential to all cells. They provide a balance of RNA synthesis with the biosynthetic pathway in pyrimidine prototrophs and supply all the pyrimidine requirements in auxotrophs. While the pyrimidine biosynthetic pathway is found in almost all organisms and is nearly identical throughout nature, the salvage pathway often differs from species to species, with aspects of salvage seen in every organism. Thus significant taxonomic value may be ascribed to the salvage pathway.

The pyrimidine salvage pathways were studied in 55 microorganisms. Nine different salvage motifs, grouped I-IX, were identified in this study based on the presence of different combinations of the following enzymes: cytidine deaminase (Cdd), cytosine deaminase (Cod), uridine phosphorylase (Udp), uracil phosphoribosyltransferase (Upp), uridine hydrolase (Udh), nucleoside hydrolase (Nuh), uridine/cytidine kinase (Udk), 5'-nucleotidase and CMP kinase (Cmk).

*Escherichia coli*, the archetype species, was placed in Group I with nine other enteric bacteria, including *Enterococcus, Shewanella* and two vibrio species. All organisms in Group I contained the enzymes Cdd, Cod, Udp, Upp, Udk, Cmk, 5'-nucleotidase and Udh. Special significance is placed on Udh because organisms with Group I enzymes, except for Udh, were placed in Group II. This group included *Burkholderia cepacia, Haemophilus, Proteus vulgaris, Rhizobium loti* and three *Pseudomonas* species. Group III contained *Saccharomyces* and *Pseudomonas indigofera*, lacking only Udp from Group I. *Bacillus, Bacteroides* and *Micrococcus*, lacking Cod,
constituted Group IV. *Staphylococcus* was the sole occupant of Group V with no Cod or Udp but having Cdd and Udh. Group VI contained members of the fluorescent *Pseudomonas*, with Cod and Nuh, but without Cdd or Udk. The presence of Udp and Nuh provided a separate group for *Pseudomonas putida* as Group VII. *Neisseria*, *Moraxella* and *Acinetobacter* contained only one salvage enzyme, Upp as Group VIII. *Clostridium* species with Cdd, Udh and Udp comprised Group IX.

Because it lacked uridine kinase, a Upp⁻ mutant of *P. aeruginosa* was resistant to fluorouridine. When a functional Udk gene, on plasmid pDEB1, was transformed into this *Pseudomonas*, it became sensitive to fluorouridine for the first time.
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CHAPTER I

INTRODUCTION

A labyrinth is a roundabout or circuitous path with meandering twists and turns, causing the explorer to wander to and fro through a tortuous scheme of passages. Labyrinth may also refer to a long-winded, obscure or evasive speech containing circumlocution, digression or evasion (Doob, 1990). In another context, it refers to mental confusion or uncertainty as in Milton's "Paradise Lost" (1667):

Others apart sat on a hill retir'd,
In thoughts more elevate, and reason'd high
Of Providence, foreknowledge, will, and fate,
Fix'd fate, free will, foreknowledge absolute,
And found no end, in wand'ring mazes lost.

Chaos and despair may be involved in negotiating a labyrinth if no map is available to the mazewalker and especially if the possibility of death awaits beyond every turn. In one Cretan myth, King Minos had such an intricate and deadly maze constructed that all of the King's enemies sentenced to death were thrust into the maze. Within the convoluted pathways lived a half human-half bull monster, the Minotaur, which fed on the unfortunates doomed to wander the monster's lair (Doob, 1990).

A similar fate awaits the student who enrolls for a stint in the half biology-half chemistry world of Biochemistry wherein many scholars are doomed to wander through a
profusion of tangled pathways, while always carrying the fear of being unable to negotiate the maze unscathed. In the Minotaur myth, the story does have a happy ending for one Athenian youth sentenced to death in the labyrinth. He received clues to negotiating its intricacies (from the King's daughter) and was able to make good his escape.

The same holds true for negotiating the biochemical pathways involved in pyrimidine metabolism which encompasses two major pathways, the biosynthetic and salvage pathways (Fig. 1) which intersect at UMP. At first glance, the pathways appear to be a complicated maze of molecules. However, when clues to solving the convoluted pathways are taken one by one, the labyrinth becomes, not a complicated maze, but a guide by which to perpetuate life.

**Synthesis of pyrimidine nucleotides de novo.** Both UTP and CTP are synthesized via the biosynthetic pathway (Fig. 2). Carbamoylphosphate is synthesized in the following reaction by carbamoylphosphate synthetase (CPSase) which is activated by ornithine and IMP and inhibited by UMP in *E. coli*: glutamine + HCO₃⁻ + 2ATP → Carbamoylphosphate + glutamate + 2ADP + Pi. The second step in the pathway is the catalysis of carbamoylphosphate and aspartate to carbamoyl aspartate by aspartate transcarbamoylase (ATCase) with the carbamoylphosphate being used also in the synthesis of arginine in a competitive pathway. This branch point is an important regulatory site in the pathway with the ATCase being activated by ATP and inhibited by CTP in *E. coli.* Pyrimidine synthesis proceeds via the catalysis of carbamoyl aspartate (CA) to dihydroorotate (DHO) by dihydroorotase (DHOase) and from there to orotate (OA) by the oxidation of dihydroorotate by dihydroorotate dehydrogenase. To make orotidine 5'-monophosphate (OMP), phosphoribosylpyrophosphate (PRPP) donates the phosphoribosyl moiety, in a reaction catalyzed by orotate phosphoribosyltransferase. With the removal of a carboxyl group from the number six carbon in the OMP ring by OMP decarboxylase, uridine 5'-monophosphate (UMP) is formed. With the successive addition
of γ-phosphate groups from ATP to UMP to make UDP and UTP by uridine 5'-monophosphate kinase and nucleoside diphosphokinase (nudiki), respectively, one nucleotide that goes into making RNA, UTP, has been successfully completed. To generate CTP, a synthetase launches the amination of UTP in the presence of GTP and glutamine using one ATP to carry the reaction forward. The synthetase is feedback inhibited by CTP and activated by UTP (Neuhard & Nygaard, 1987).

**Synthesis of pyrimidine nucleotides by salvage enzymes.** In the salvage/recycling pathways (Fig. 3), patterns of enzymatic diversity have converged in elegant simplicity. The RNA salvage pathway consists of only two nucleosides, uridine and cytidine and two bases, uracil and cytosine and their accompanying enzymes.

The salvage pathway in Fig. 3 belongs to *E. coli*, the bacterium to which all results have been compared. In my research, I examined the pyrimidine salvage pathways of 44 prototrophs and 11 auxotrophs which were specially selected for their mutations in both the biosynthetic and salvage pathways. In addition to the pathway represented by *E. coli*, eight more different salvage pathways were discovered, each one distinct from that of *E. coli*, the prototype organism.

The pyrimidine salvage pathway in *E. coli* in this work was studied *in vitro* using High Performance Liquid Chromatography (HPLC) exploiting the unique ability of the machine to recognize and register products of enzyme reactions after incubation of cell extracts with pure substrates of pyrimidine nucleosides and bases.

**RNA precursors: Uracil.** In some organisms uracil has two pathways to make UMP. The primary way and the shorter, is the catalysis of uracil to UMP by the enzyme, uracil phosphoribosyltransferase (Upp), utilizing a molecule of PRPP (Andersen *et al.*, 1992). The other pathway takes advantage of uridine phosphorylase (Udp) in a high background of ribose-1-phosphate to make uridine. Uridine is then converted to UMP by uridine kinase (Udk) with GTP donating its γ-phosphate (Neuhard & Nygaard, 1987).
Control of Upp lies in the concentration of ppGpp, UMP, UDP and UTP all of which inhibit the Upp while GTP activates it (Rasmussen et al., 1986).

Cytosine. Cytosine deaminase (CodA) catalyzes the deamination of cytosine to uracil in an irreversible hydrolytic reaction (West et al., 1982). Upon starving the cells for pyrimidines and nitrogen, the enzyme is derepressed but is repressed by purines (Chargaff & Kream, 1948; West et al., 1982).

Uridine. Uridine is a substrate for four different enzymes: uridine phosphorylase (Udp), uridine kinase (Udk), uridine hydrolase (Udh) and the broadly specific nucleoside hydrolase (Nuh). All four reactions have not been found in one organism exclusively, but E. coli does have the first three (this work, 1995).

As mentioned above, uridine is converted to UMP by a uridine kinase (Udk) with GTP the phosphate donor. This uridine kinase is also active for cytidine. Uridine is also converted phosphorolytically to uracil by uridine phosphorylase.

As was discovered in this research, uridine can be a substrate, in many organisms, for both nucleoside hydrolase (Nuh) and uridine hydrolase (Udh). This hydrolytic reaction is irreversible and proceeds from uridine to uracil by Udh. The phosphorylase requires induction in E. coli and Salmonella and is accomplished by cytidine in the former and by cytidine and uridine in the latter.

Cytidine. There is a three-pronged fork in the salvage pathway when cytidine is concerned. In the enteric bacteria, cytidine is phosphorylated to CMP by uridine kinase. However, when cytidine is available most of it is converted to uridine by cytidine deaminase which catalyzes the formation of uridine by deaminating cytidine hydrolytically (Ashley & Bartlett, 1984).

Cytidine has also been shown to be a substrate for Nuh in Pseudomonas (Terada et al., 1967; this work) to produce cytosine.
Existence of the pyrimidine salvage pathway in any organism is of primary importance so that pyrimidine auxotrophs and organisms which have no biosynthetic pathway may be supplied exogenous pyrimidines. Organisms having a mutation in the pyrimidine biosynthetic pathway could not survive without a source of pyrimidines. The salvage pathway is the means by which preformed pyrimidine bases and nucleosides may be fed to pyrimidine auxotrophs and thus ensure their survival.

Not only is the salvage pathway used to feed pyrimidine auxotrophs, more importantly, it is the mRNA recycling plant in the cell. Other, smaller, contributors to the nucleotide recycling pool include the products of tRNA processing (Cudny & Deutscher, 1980), of DNA ligations (Neuhard, 1983) and of turnover of nucleotide coenzymes such as NAD$^+$ (Tritz, 1987).

**mRNA degradation:** To be able to reuse the rich source of pyrimidines from the degradation of mRNA to 5'-mononucleotides, UMP and CMP, is a boon to the cell in several ways. The phosphorylated compounds in the cell are toxic if allowed to accumulate, and thus must be processed quickly for the well-being of the cell (O'Donovan, 1978). If a block occurs in the degradation of mRNA, the cell is poisoned by its own nucleotides (O'Donovan & Shanley, 1995).

In a bacterial cell approximately 20% of the dry weight in that cell is RNA of all types but up to 60% of the RNA made is actually mRNA. Since mRNA does not accumulate in the cell and comprises over half the total concentration of RNA, it is evident that it is rapidly degraded (O'Donovan & Shanley, 1995).

The fact that mRNA is so unstable and has a short half-life ensures an ever ready source of pyrimidines and purines for synthesis of a cell's DNA and RNA. Typically, rates of mRNA degradation are between two and four minutes in *E. coli*, but RNA has been degraded in as little as 20-30 seconds and as slowly as 50 minutes (Pedersen *et al.*, 1978; Nilsson *et al.*, 1984; Donovan & Kushner, 1986; Emory & Belasco, 1990;
Baumeister et al., 1991). To explain the rapid turnover of mRNA, Graves and colleagues (1987) suggested that a ribonuclease may be associated with the ribosome itself, with the accompanying ribonuclease degrading the mRNA upon completion of its task as messenger (the most primal level of "shooting the messenger"!) or else the mRNA actually activates a nuclease upon completion of its role in the ribosome (Graves et al., 1987). Another explanation by Graves is that in the mRNA's journey within the ribosome complex, the mRNA's secondary structure may be altered which in turn exposes the mRNA's nuclease recognition sites to ribonucleases lurking about the ribosome.

Another cause of rapid degradation of mRNA is the occurrence of premature nonsense mutations in DNA and thus also in its message (Peltz & Jacobson, 1993). By inserting a series of six amber mutations at strategic points along the mRNA in the *Saccharomyces* gene, *PKG1*, it was determined that the mRNA with a nonsense mutation allowing translation of 55% or less of the protein, was degraded up to 12 times faster than those mRNAs containing a stop codon in the last 33% of the mRNA. This decreased the half-life of the mRNA only four-fold (Peltz & Jacobson, 1993). Such ruthless pruning of the degenerate mRNA ensures that little energy is wasted in translating a truncated protein when there is no hope of making a useful protein. The DNA and RNA are degraded to smaller pieces enzymatically (CMP, UMP, CDP and UDP, cytidine, uridine, cytosine and uracil) and thus become substrates so that the salvage pathway can use them again. A vast amount of cellular energy is expended just to maintain mRNA until it delivers its message to the ribosome and the protein is made (Ehretsmann et al., 1992).

The breakdown of mRNA has been studied most extensively in *E. coli* in which the genes responsible for encoding the enzymes are *rnb* and *pnp*. The proteins produced from these two genes are ribonuclease II (RNase II) and polynucleotide phosphorylase (PNPase) respectively (Donovan & Kushner, 1986). When both genes were knocked out in *E. coli*, no mRNA degradation occurred and the cells died. When only one gene was
knocked out, leaving the second gene to function on its own, that enzyme (whether phosphorylase or hydrolase) can fully substitute for its partner in degradation (Donovan & Kushner, 1986).

Preliminary work by Boyer and associates (Chaney & Boyer, 1972 and Duffy et al., 1972) showed that while E. coli's method of degrading its RNA was hydrolytic (to 5'-monophosphates), the method used predominantly by Bacillus subtilis was phosphorolytic (to 5'-diphosphates). Later work on E. coli revealed that 90% of its mRNA was, indeed, broken down hydrolytically using RNase II. The enteric bacteria do not, however, rely totally on one enzyme to degrade all their mRNA. They also have a functional, but less active PNPase, which degrades the remaining 10% to diphosphates. Both the RNase II and the PNPase are processive enzymes, that function in the 3' → 5' direction and have been classified as exoribonucleases.

Subsequent work by Deutscher & Reuven, (1991) on B. subtilis confirmed that its mRNA was broken down phosphorolytically to the 5'-diphosphate level with RNase II being absent entirely. However, B. subtilis had a unique PNPase, distinct from its E. coli counterpart which cleaved mRNA to 5'-diphosphates. Thus, Bacillus contained a dual degradation system, just as in E. coli. Its second degradation enzyme is a ribonuclease which acts hydrolytically and is much less active than the phosphorylase (Deutscher & Reuven, 1991).

Salvage enzymes in E. coli. In this study, pyrimidine salvage pathways with their similarities and differences have been grouped for convenience. The combinations of the different enzymes of the pyrimidine salvage pathways may be used as an aid in putting together a bacterial taxonomic jigsaw puzzle. In my research I have discovered nine different recycling schemes entitled, Groups I-IX, for pyrimidine salvage.

I have found that the salvage pathways may differ among different genera and sometimes even between species as in Pseudomonas. Nevertheless, E. coli has been used
as the comparative organism in all cases and has been chosen as the prototype species in
Group I of the nine groups. Its salvage pathway consists of cytidine deaminase (Cdd),
uridine phosphorylase (Udp), uridine hydrolase (Udh), cytosine deaminase (Cod), uracil
phosphoribosyltransferase (Upp), uridine kinase (Udk), cytidine 5'-monophosphate kinase
(Cmk) and nucleotide monophosphate glycosylase (Nmg).

The reactions carried out by the salvage enzymes may be seen below (equations 1-9):

Cdd catalyzes the following hydrolytic reaction:

\[
\text{Equation 1} \quad \text{Cytidine} + H_2O \xrightarrow{\text{Cdd}} \text{Uridine} + \text{NH}_3 \quad (\text{O'Donovan & Neuhard, 1970}).
\]

\[E. coli\] also has a uridine phosphorylase (Udp):

\[
\text{Equation 2} \quad \text{Uridine} + \text{Pi} \xleftarrow{\text{Udp}} \text{Uracil} + \text{Ribose-1-phosphate} \quad (\text{O'Donovan & Neuhard, 1970}).
\]

An additional enzyme, uridine hydrolase, was found in \[E. coli\] which catalyzed the
conversion of uridine to uracil:

\[
\text{Equation 3} \quad \text{Uridine} + H_2O \xrightarrow{\text{Udh}} \text{Uracil} + \text{Ribose} \quad (\text{Sakai et al., 1976, this work}).
\]

The fourth salvage enzyme in \[E. coli\] is the cytosine deaminase (Cod):

\[
\text{Equation 4} \quad \text{Cytosine} + H_2O \xrightarrow{\text{Cod}} \text{Uracil} + \text{NH}_3 \quad (\text{O'Donovan & Neuhard, 1970}).
\]

\[
\text{Equation 5} \quad \text{Uracil} + \text{PRPP} \xrightarrow{\text{Upp}} \text{UMP} + \text{Pi}.
\]

The Upp in Equation 5 is found in \[E. coli\] and all other organisms.

\[
\text{Equation 6} \quad \text{Uridine} + \text{GTP} \xrightarrow{\text{Udk}} \text{UMP} + \text{GDP}.
\]

The reactions in Equation 6 and 7 are found in most organisms.

\[
\text{Equation 7} \quad \text{CMP} + \text{ATP} \xrightarrow{\text{Cmk}} \text{CDP} + \text{ADP}.
\]

\[
\text{Equation 8} \quad \text{CMP} + H_2O \xrightarrow{\text{Nmg}} \text{Cytosine} + \text{Ribose-5-phosphate}.
\]
(Equation 9) Cytidine (Uridine) + H₂O $\rightarrow^{n^{\text{nuh}}} \text{Cytosine (Uracil)}}$ + Ribose.

In my work, I assayed Cdd, Udp, Cod, Nuh and Udh for all organisms.

There are four types of phosphorylases found in the salvage pathways of various organisms: (1) purine nucleoside phosphorylase: these enzymes use both purine ribonucleosides and purine deoxyribonucleosides as substrates but substrate specificities vary (Parks & Agarwal, 1972; Scocca, 1978); (2) thymidine phosphorylases which are specific for deoxyuridine and deoxythymidine; cytidine is not used as a substrate (Hammer-Jespersen, 1983); (3) pyrimidine nucleoside phosphorylases are active for thymidine, uridine and pyrimidine analogs but not for cytidine or deoxycytidine. Activity for deoxyribonucleosides is lower than for the ribonucleosides (Hammer-Jespersen, 1983); and (4) uridine phosphorylase which is specific for uridine and uracil.

The hydrolases are grouped according to their specificities: (1) (ribo)nucleoside hydrolases which use all four nucleosides, both purine and pyrimidine as substrates, (Takagi & Horecker, 1957; Hammer-Jespersen, 1983); (2) purine ribonucleoside hydrolases are specific for purines, only (Hammer-Jespersen, 1983); (3) purine deoxyribonucleoside hydrolases (Koszalka & Krenitsky, 1979) are specific for purine deoxyribonucleosides and (4) uridine hydrolases are specific for uridine and 5-methyluridine (Magni et al., 1975; Magni et al., 1976).

In cells with both a phosphorylase and a hydrolase, two assays were performed. The second assay I developed to prove conclusively if the enzyme were a phosphorylase or a hydrolase. In the first assay, incubation of cell extract containing the phosphorylase and the substrate, uridine, was done at physiological temperature for a specified time. The products of the reactions were then injected onto a reverse phase (HPLC) column and a peak corresponding to an enzyme product was observed.
The second assay was done by incubating cell extract with uracil and ribose-1-phosphate as substrates and measuring the peak of uridine which appears only if a phosphorylase is present:

\[ \text{Uracil} + \text{Ribose-1-phosphate} \xrightarrow{\text{udp}} \text{Uridine} + \text{Pi}. \]

If no peak of uridine formed, provided that the cells degraded uridine, then the enzyme was a hydrolase since the nucleoside hydrolase and uridine hydrolase reactions are irreversible. The nucleoside hydrolase catalyzes the reactions from cytidine to cytosine and from uridine to uracil while the uridine hydrolase uses only uridine as a substrate:

\[ \text{Cytidine} + \text{H}_2\text{O} \rightarrow_{\text{nuk}} \text{Cytosine} + \text{Ribose}, \]

and/or

\[ \text{Uridine} + \text{H}_2\text{O} \rightarrow_{\text{nuk}} \text{Uracil} + \text{Ribose}. \]

The enzyme not represented in \textit{E. coli} is the (ribo)nucleoside hydrolase, henceforth known as nucleoside hydrolase. The bacterium does, however, contain a uridine hydrolase. This may be tested in appropriate mutant strains (see Results).

As a control, the assay may be done after dialysis which removes inorganic phosphate (Pi) from the cell extract. Since Pi is needed by uridine phosphorylase, and not the hydrolase, to catalyze the phosphorolytic reaction from uridine to uracil, no uracil is seen if a phosphorylase is present. Hydrolase does not require Pi, is unaffected by the dialysis and uracil is produced. Thus, hydrolase or phosphorylase activity may be confirmed by dialysis as well. In some instances both a hydrolase and phosphorylase have been reported (Sakai \textit{et al.}, 1976, this work).
Cytidine deaminase catalyzes the hydrolysis of cytidine to uridine in a non-reversible reaction releasing ammonia as a byproduct:

\[
\text{Cytidine} + H_2O \rightarrow \text{Uridine} + \text{NH}_3.
\]

Because all organisms do not employ the same salvage enzymes it is possible to use the different enzyme combinations for taxonomic differentiation when taken together with other data. Recently, *Burkholderia cepacia* was reclassified from the genus, *Pseudomonas*, to the genus, *Burkholderia*, on the basis of rRNA-DNA homology (Palleroni *et al.*, 1973; De Vos & De Ley, 1983; De Vos *et al.*, 1985; De Vos *et al.*, 1989; Willems *et al.*, 1989; Willems *et al.*, 1990; Willems *et al.*, 1992) and other relevant characteristics. In these studies I found *Burkholderia cepacia* salvage enzymes to be different from those of the fluorescent *Pseudomonas* species.

**Pyrimidine analogs.** A pyrimidine analog is defined as a pyrimidine base or nucleoside with an additional functional group added to a member of the ring. For example, 5'-fluorouracil has a fluoro group attached at the fifth carbon in the pyrimidine ring. It is recognized as uracil by the regular salvage and biosynthetic enzymes and is phosphorylated in the pathway, causing death to the cell:

\[
\text{FU} \rightarrow \text{FUMP} \rightarrow \text{FUDP} \rightarrow \text{FUTP} \rightarrow \text{RNA} \rightarrow \text{Death.}
\]

In any population of organisms there are a few which are naturally resistant to fluoro-analogs. When 5'-fluorouracil is administered to the cells, fluorouracil-resistant organisms survive which are naturally devoid of uracil phosphoribosyltransferase.

Four general principles exist regarding incorporation of analogs: (a) to be toxic to the cell, the analog must be converted to the nucleotide level; (b) the analog may be toxic if
it inhibits a step (or steps) in the pathway or if it is incorporated into RNA (5'-FUTP); (c) analog toxicity may be reversed by addition of natural bases and nucleosides; (d) two classes of analog-resistant mutants may be found: those which have natural mutations in the gene encoding the enzyme that catalyzes the reaction involving the analog (e.g. naturally occurring 5'-fluorouracil resistance due to the existence of a mutation in uracil phosphoribosyltransferase in the population) and those which are pyrimidine overproducers. The latter class produce so much extra pyrimidines that they compete with the analog for incorporation into RNA (i.e. the cell does naturally, what is stated in (c) above) (O'Donovan & Neuhard, 1970). An additional way that organisms may be resistant to analogs is if they do not take them into the cell at all (lacking permeases) (O'Donovan & Neuhard, 1970).

5'-Fluoro- analogs. Those organisms which lack uracil phosphoribosyltransferase are 5'-fluorouracil resistant since they are unable to catalyze the conversion of 5'-fluorouracil to 5'-fluoroUMP. Organisms which possess uridine kinase are still 5'-fluorouridine sensitive since the 5'-fluorouridine is catalyzed to 5'-fluoroUMP by uridine kinase which can then incorporate the fluoro-compounds into RNA. These cells are also resistant to 5'-fluorocytidine since uridine kinase is also used in the catalysis of cytidine and accordingly, 5'-fluorocytidine as well.

Organisms that are resistant to 5'-fluorocytosine while remaining sensitive to 5'-fluorouracil, lack cytosine deaminase.

Exposure to 5'-fluorocytosine forces the selection for organisms devoid of cytosine deaminase while administration of 5'-fluorodeoxycytidine in the presence of uracil selects for mutations in cytidine deaminase assuming that the organism possesses cytidine deaminase (O'Donovan & Neuhard, 1970). Through the use of analogs, genetic studies can be done to determine which steps in the pathway are present.
Analogs used as antimicrobial drugs. Another reason for which the analogs and the salvage pathway are important is that analogs may be used as antitumor drugs in human beings (Bruckner & Creasey, 1974); 5′-fluorocytosine has been successfully administered to patients with candidiasis with no severe side effects (Eilard et al., 1974) the drug azidothymidine (AZT) has been used in the treatment of Acquired Immune Deficiency Syndrome. Cryptococcus neoformans -related meningitis has been successfully treated synergistically with 5′-fluorocytosine and amphotericin B (Hamilton & Elliott, 1975) which in high doses, alone, is neurotoxic (Utz et al., 1964 and Douglas & Healy, 1969).

While not an analog, uridine has been used as an anxiolytic "drug" in treating neurological disorders in human beings (Connolly, 1994).

Pyrimidine auxotrophs. Even though the two divergent biosynthetic and recycling pathways have the same destination, the choice of pathways does not really exist in any organism, for in the majority of cases, the "choice" to use the salvage pathway over the biosynthetic pathway is not really an option, but a necessity. Many organisms can use cytidine, cytosine, uridine or uracil as preformed pyrimidines to feed pyrimidine auxotrophs which thus can survive mutations in either the biosynthetic or salvage pathways. In Mycoplasma, for example, there are no biosynthetic pyrimidine and purine pathways. As a result, the organism depends solely on its salvage enzymes for nucleotides with which to make DNA and RNA.

As can be seen in Fig. 2, the biosynthetic pyrimidine pathway utilizes no nucleosides or bases as intermediates in the synthesis of nucleotides for RNA and DNA. One or all four of the nucleosides and bases may be used exclusively by pyrimidine auxotrophs to satisfy the pyrimidine requirement. Thus, many organisms are able to use cytidine, cytosine, uridine or uracil as precursors to DNA and RNA whenever there is a mutation in the biosynthetic pathway (Moore & Boylen, 1955). The ability to use nucleosides and bases allows an auxotroph to avoid the lethal circumstance of being unable
to supply nucleotides for RNA and DNA synthesis. This ingenious safeguard has evolved in the majority of organisms to ensure that their genetic material remains intact and that reproduction of the DNA and ultimately of the organism itself continues. In fact, among all the organisms studied, not one was devoid of all salvage enzymes! However, organisms exist which live successfully without the biosynthetic pathway and rely solely on the salvage pathway for supplements of pyrimidine nucleosides and bases to fulfill nucleic acid requirements.

The profound need for a salvage pathway is exhibited in eukaryotes by *Homo sapiens* when there is a mutation in orotate phosphoribosyltransferase which catalyzes the conversion of orotate to orotidine 5'-monophosphate (OMP) by UMP synthase. This is a bifunctional enzyme in mammals which also functions as OMP decarboxylase: Orotate $\rightarrow$ OMP $\rightarrow$ UMP. Human beings devoid of the orotate phosphoribosyltransferase activity of UMP synthase have a genetic disease called orotic aciduria, the result of orotic acid buildup due to the inability of the organism to process orotate. The symptoms include large concentrations of orotic acid in the urine, severe anemia and retardation of growth (Voet & Voet, 1990). Upon administration of cytidine and/or uridine to affected patients, the salvage enzymes cytidine deaminase and uridine kinase, go into action furnishing vital pyrimidines needed for normal growth and development of the organism. An additional benefit of being able to feed uridine is that one is able to swell the UTP pools (inhibits CPSase) and CTP pools (inhibits ATCase) and to shut down the biosynthetic pathway altogether, thus alleviating the orotic acid buildup.

Pyrimidine auxotrophy is not the normal condition in most organisms, yet there are some organisms which are known to have no biosynthetic pathway and rely solely on the salvage pathway for replenishing the pyrimidine nucleotide supply. Two such organisms are *Bdellovibrio bacteriovorus* (Rosson & Rittenberg, 1981) and *Chlamydia trachomatis* (McClarty & Qin, 1993).
Transport of nucleosides and bases into the cell. While nucleosides and bases are freely transported into the cell as needed for DNA and RNA synthesis, nucleotides are not transported into the cell by the majority of bacteria since charged molecules are not easily transported (Neuhard & Nygaard, 1987). Nevertheless, some parasitic genera, such as Chlamydia (with no biosynthetic pathway) have developed mechanisms for uptake of host ribonucleotides from which deoxyribonucleotides are synthesized (McClarty & Qin, 1993). However, in E. coli, the well known enteric bacterium, uraA, the gene responsible for synthesis of uracil permease genes is linked but separate from the upp gene encoding uracil phosphoribosyltransferase (Munch-Petersen & Mygind, 1983). In yeast species uracil permease is a plasma membrane protein (Silve et al., 1991). The gene responsible for uptake of cytosine (codB) is closely linked to the structural gene, codA, but is separate from it (deHaan et al., 1972) although the two may be in an operon.

Nucleosides are actively transported into the cell by two systems in E. coli: the G system and the C system. The G system is nonspecific and transports both pyrimidine and purine nucleosides. However, the C system specifically transports pyrimidines and adenosine. Organisms which are devoid of both systems cannot grow on nucleosides as a source of carbon and energy but retain the ability to use the ribonucleosides as pyrimidine and purine sources (Munch-Petersen & Mygind, 1983).

Degradation of the pyrimidine ring. Pyrimidine nucleotide precursors not only provide material for making DNA and RNA, but the pyrimidine ring may also be utilized as a carbon and energy source and as a source of nitrogen. However, the ring must be broken in order for the atoms to be freed for use in their more fundamental forms.

In the organisms containing cytosine and cytidine deaminases, the ammonia byproducts are available to satisfy the organism's nitrogen requirement (Vogels & van der Drift, 1976).
The pyrimidine ring may also be broken down to be used as a carbon and energy source in some organisms through its oxidative and reductive degradation (Vogels & van der Drift, 1976). The reductive pathway is of primary importance and is seen below:

Cytosine $\rightarrow$ Uracil + NH$_3$ $\rightarrow$ Dihydrouracil $\rightarrow$ N-carbamoyl-β-alanine $\rightarrow$ β-alanine.

The β-alanine is used further in the production of coenzyme A: β-alanine + pantoate $\rightarrow$ pantothenate $\rightarrow$ coenzyme A (West et al., 1985).

The oxidative breakdown of cytosine, uracil, thymine and orotate is utilized by some pseudomonads for carbon and/or nitrogen:

\[
\text{Cytosine} \quad \downarrow \\
\text{Orotate} \rightarrow \text{Uracil + NH$_3$} \rightarrow \text{Barbiturate} \rightarrow \text{Urea + Malonate} \quad \text{(Hayashi & Kornberg, 1952; Vogels & van der Drift, 1976).}
\]

The oxidative catalysis of uracil to barbiturate is accomplished by uracil dehydrogenase with the conversion of barbiturate to urea and malonate by barbiturase.

Negotiation of the pyrimidine salvage pathways is not so much like wandering aimlessly in a labyrinth, as it is a series of intersecting roads. Some roads are blocked, but alternative routes or detours are usually provided to ensure the survival of the species. I have learned of at least nine such pathways which ensure a species' longevity. A tour of the pathways leads one to begin to understand "Providence, foreknowledge, will, and fate..." but not to wander in mazes lost (Milton, 1962).
Fig. 1. Pyrimidine pathways in microorganisms.
Fig. 2. Pyrimidine biosynthetic and salvage pathways in Escherichia coli.
Fig. 3. Pyrimidine salvage pathway in *Escherichia coli*. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. uridine hydrolase (Udh), 4. cytosine deaminase (Cod), 5. Cytidine deaminase (Cdd), 6. uridine kinase (Udk), 7. 5'-nucleotidase, 8. CMP glycosylase, 9. CMP kinase (Cmk).
CHAPTER II

METHODS

In his Il Saggiatore, Galileo Galilei, (1623) stated:

Philosophy is written in this grand book - I mean the universe - which stands continually open to our gaze, but it cannot be understood unless one first learns to comprehend the language and interpret the characters in which it is written... without which it is humanly impossible to understand a single word of it; without these, one is wandering about in a dark labyrinth.

Galileo was speaking of mathematics as the language of the universe, but DNA and RNA are the written language of life, with purines and pyrimidines being the alphabet which forms the words. For many years, scientists were "wandering about in a dark labyrinth" of ignorance regarding the double helix. DNA is the blueprint of life on earth and RNA molecules carefully execute and build from that blueprint the molecules of metabolism which perpetuate the life processes. Both pyrimidines and purines are produced biosynthetically in most organisms but the processes which actually keep an organism functioning effectively and efficiently involve the faithful reproduction of molecules of DNA and RNA and the reuse and recycling of degraded RNA. This recycling process is one which all cells have developed and have utilized over millennia in order to survive, with little assistance - or interference - from Homo sapiens until the present century. When man-made mutagens accomplish in an instant what nature has evolved
through all of time, *H. sapiens* plays god to all other organisms. Even though man continues to develop new and better mutagens, organisms continue to develop means of survival. One such means which is the recycling and reutilization or salvaging of RNA degradation products to make RNA and DNA once again and to continue that process until death occurs. Since some organisms utilize a different salvage pathway from those of other organisms, I have investigated the salvage enzymes in 55 organisms. Their comparative biochemistry comprise this dissertation.

There are many methods by which pyrimidines and their attendant enzymes are studied... by spectrophotometric assays of enzyme products, by identifying intermediates in mutants blocked in specific steps, by molecular biology and its repertoire of numerous techniques for manipulation of both DNA and RNA, by thin layer chromatography and radioactive studies, but in this study, the techniques used to delineate the salvage enzymes were those of high performance liquid chromatography (HPLC). HPLC is a method of separating molecules into individual components using two phases. One phase is a stationary bed which is the packing found inside the stainless steel column and the other is a mobile phase which is moved along the stationary bed and through the column using high pressure to force the sample through. Separation of the substrates from products in the enzyme assays based upon the difference in affinity of the two for the silica bedding (in a reverse phase column). A hydrophobic surface was created on the inside of the reverse phase column atop the silica bedding by coating the silica with organochlorosilane. In reversed phase chromatography, the stationary bed is non-polar while the mobile phase is a polar liquid such as water or alcohol. The more nonpolar the sample, the longer it is retained on the column (Yost, *et al.*, 1980).

Preparation of the mobile phase: a 5 mM solution of the ammonium phosphate buffer was prepared by dissolving 0.575 g monobasic ammonium phosphate in 1000 ml HPLC grade H$_2$O. Dilute HCl was added to the buffer until the pH read 3.5. The solution
was then filtered through a 0.45 μm Gelman filter using a vacuum pump attached to a flask. After filtration, the solution was degassed by aspiration while being stirred for one hour. The mobile phase was then ready to be pumped through the HPLC system. Approximately 30 ml, at one ml per minute, were pumped through the HPLC before any assays were done in order to equilibrate the column and the buffer.

The HPLC equipment utilized for assay of the salvage enzyme activity was a Waters 510 pump, Waters Model U6K Universal Liquid Chromatography Injector, a SpectroMonitor 5000 Photodiode Array detector, a Waters Model 740 Data Module, and reverse phase columns by Beckman and Rainer: Ultrasphere ODS 5μm, 4.6 mm ID x 25 cm reverse phase column and Waters NovaPak™ C18 Reverse Phase Column. The mobile phase was 50 mM ammonium phosphate monobasic (Mallinckrodt, HPLC grade), pH 3.5 (HCl) using HPLC grade, filtered water at 10 megohms/cm (Millipore, Milli-Q™ Water System).

The separation process was carried out as follows: the sample was injected into the mobile phase stream through the U6K injector of the HPLC instrument using a Hamilton syringe. As the sample of nucleosides and/or bases passed through the column in the polar mobile phase (ammonium phosphate buffer) each of the nucleosides and bases was retained on the silica bed according to its polarity. The sample components (cytidine, uridine, cytosine and uracil) are eluted from the stationary phase at different times (retention time in minutes) thus forming peaks at different times on the chromatogram and recorded by the data module or computer. In the present research, cytosine eluted first, then uracil followed by cytidine and uridine.

Standards of each nucleoside and base were injected onto the column so that appropriate retention times might be noted for the unknown products in each assay of salvage enzymes. Chromatograms showing retention times of standard pyrimidines are seen in Figs. 4-6.
In order to determine the retention time of a substance, standards were maintained at 1mM throughout, and 30 μl of each were injected onto the HPLC each time. Fig. 4 shows the chromatogram of a cytosine standard with the retention time at 2.6 minutes. Fig. 5 is the chromatogram of the standard sample of uracil which has the retention time of 3.6 minutes. The cytidine standard in Fig. 6 was eluted at 4.5 minutes and the uridine in Fig. 6 came off at 7.1 minutes. The ribose-1-phosphate used in the uridine phosphorylase assay was retained on the column (Fig. 7). A composite of standards of the two nucleosides and the two bases used as substrates and/or products is shown in Fig. 7 with cytosine at 2.6 minutes, uracil at 3.5 minutes, cytidine at 4.4 minutes and uridine at 6.8 minutes.

As a column ages, the retention times may vary and the peaks may develop shoulders and broaden toward the end of the run. Therefore, standards were run as retention times drifted. The standards may show somewhat different retention times, but peak integrity was retained.

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains as well as other members of the Enterobacteriaceae were grown in *E. coli* minimal medium with 0.2% (w/v) glucose as carbon source (Miller, 1972). Cytidine, cytosine and uridine were added at 100 μg/ml for induction of the salvage enzymes. The *E. coli* minimal medium contained K$_2$HPO$_4$ (10.5 g); KH$_2$PO$_4$ (4.5g); (NH$_4$)$_2$SO$_4$ (1.0 g); Na$_2$citrate (0.5 g) dissolved sequentially in 988 ml distilled deionized H$_2$O. After being autoclaved and cooled, sterile solutions of 0.2% glucose (final concentration), 1 mM MgSO$_4$ and thiamine (10 μg/ml, filter sterilized) were added to the minimal medium (Miller, 1972).

*E. coli* was also grown on Luria-Bertani (LB) broth which consists of Bacto tryptone (10.0 g); Bacto yeast extract (5.0 g); NaCl (10.0 g) dissolved in 1000 ml distilled H$_2$O (Sambrook *et al.*, 1989).
The *Pseudomonas* and *Pseudomonas*-like organisms (*Brevundimonas*, *Comamonas*, *Burkholderia*, *Stenotrophomonas*, *Shewanella*) were grown on *Pseudomonas* minimal medium using 0.2% succinate as a carbon source. The *Pseudomonas* minimal medium was made up as follows: 0.5 M Na$_2$HPO$_4$ (25 ml); 0.5 M KH$_2$PO$_4$ (25 ml); 10% (NH$_4$)$_2$SO$_4$ (10 ml); concentrated base (10 ml; recipe follows) which are added to distilled deionized H$_2$O (930 ml) in a two liter Erlenmeyer flask (Ornston & Stanier, 1966).

To make one liter of concentrated base for *Pseudomonas* minimal medium: (Note: divergence from this protocol results in inability to get the components to go into solution) To 600 ml of H$_2$O, nitrilotriacetic acid (20.0 g) was added which should be dissolved completely before addition of any other compounds. The solution was neutralized with KOH (14.6 g). Additional chemicals were added and dissolved completely before adding the next compound in the order given: MgSO$_4$ (anhydrous); CaCl$_2$ *7 H$_2$O (6.67 g); (NH$_4$)$_6$Mo$_7$O$_{24}$ *7 H$_2$O (18.5 g); FeSO$_4$ *7 H$_2$O (198 mg); and 100 ml "Metals 44" (protocol follows). Before adding additional H$_2$O to make a liter, the pH of the solution was adjusted to 6.8 before bringing the volume to 1000 ml. Typically, a precipitate formed but this went into solution upon prolonged stirring; the solution changed from a straw color to a bright yellow. This concentrated base may be stored at 4°C for one year (it may also be stored in toluene, but storage in the cold, alone, is sufficient) (Ornston & Stanier, 1966).

**Hutner's Metals 44 protocol:** Into 800 ml distilled deionized H$_2$O the following compounds were dissolved (in order): EDTA, free acid (2.5 g); ZnSO$_4$ *7 H$_2$O (10.95 g); FeSO$_4$ *7 H$_2$O (5.0 g); MnSO$_4$ *H$_2$O (1.54 g); CuSO$_4$ *5 H$_2$O (392 mg); CuSO$_4$, anhydrous (251 mg); Co(NO$_3$)$_2$ *6 H$_2$O (250 mg) or Na$_2$B$_4$O$_7$ *10 H$_2$O (177.0 mg). In order to prevent precipitation, a few drops of H$_2$SO$_4$ were added. Additional H$_2$O was added to bring the solution up to one liter. The "Metals 44" was a lime green color and
may be stored at 4°C indefinitely (care should be taken to watch for fungal growth even in the cold) (Ornston & Stanier, 1966).

*Micrococcus luteus* was grown in tryptic soy broth (Difco). The *Vibrio* species were grown in tryptic soy broth plus 3% NaCl. *Saccharomyces cerevisiae* was grown in nutrient broth (Difco) plus one per cent sucrose. *Bacillus* were grown in Luria Bertani broth (LB) and tryptic soy broth plus cytidine, uridine and cytosine for induction.

All *Rhizobium* strains were grown in yeast mannitol broth plus cytidine, uridine and cytosine. Strains of *Staphylococcus* and lactic acid bacteria were grown in LB and tryptic soy broth to which cytidine, uridine and cytosine were added as potential inducers. *Haemophilus* were harvested from chocolate plates purchased from Remel. *Neisseria* were grown on blood agar plates (Remel, Lenexa, KA 66215). *Clostridium* and *Bacteroides* were grown in fluid thioglycollate medium (Anaerobe Systems, San Jose, CA) in an anaerobe jar.

All strains grown on plates of solid agar were harvested using sterile cotton swabs and transferred to 15 ml plastic conical tubes containing HPLC breaking buffer (50 mM Tris-HCl, enzyme grade; pH 7.0 and modified with MgCl₂; Dutta *et al.*, 1990).

All cultures (100 ml amounts) were grown to 100 Klett Units (10⁹ cells/ml) where 1 Klett Unit (KU) equals 10⁷ cells/ml (measured with a Klett-Summerson photoelectric colorimeter, using a green filter No. 54). Cells were pelleted in 50 ml plastic conical tubes at 3000 x g for 10 minutes at 2°C and the supernatant discarded.

**Disrupting cells.** All cells were washed in HPLC breaking buffer, spun down and the supernatant was again discarded. An additional two milliliters of HPLC breaking buffer were added to the tube containing the cell pellets respectively and the cells were resuspended in the buffer. In order to break the cells, equal quantities of 5 μm glass beads (Sigma) were added to each 50 ml conical tube and mixed on a vortex mixer at high speed.
for up to 15 minutes depending on the durability of the cell. Gram negative cells were vortex mixed for two to three minutes. Gram positive cells were vortex mixed up to 15 minutes. *Staphylococcus aureus* cells were broken using 2.5 mg lysostaphin per ml. The *S. aureus* cells and lysostaphin were mixed together and incubated at 37°C for 30 minutes.

When glass beads were used to break cells, the beads and cell debris were allowed to settle to the bottom of the 50 ml conical tube after which the supernatant was transferred to 1.5 ml microcentrifuge tubes and spun down in a microcentrifuge for 5 minutes at 10,000 x g. The remaining supernatant containing the salvage enzymes was removed from the debris using a 3cc B-D® Syringe and a monoject® hypodermic needle. 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 two liters of a 50 mM Tris buffer placed on a stirrer at 4°C. Dialysis of the cell extract removed phosphates and other ions required for catalysis of salvage enzyme reactions. Both the filtered supernatant and the filtered, *dialyzed* supernatant were frozen at -20°C until used in enzyme assays.

**Enzyme assays using HPLC.** Isolation and identification of pyrimidine salvage intermediates were done by HPLC. The following enzyme assays were performed.

(a) **Uridine phosphorylase - forward reaction:** Uridine + Pi $\underset{\text{nth}}{\rightarrow}$ Uracil + Ribose-1-phosphate. The result of the uridine phosphorylase assay was observed by incubation of 20 μl filtered, dialyzed crude cell extract in HPLC breaking buffer with 80 μl of uridine as substrate (1mM). The assay mix was placed in a 0.6 ml microcentrifuge tube, labelled and incubated for 10 minutes at 37°C. The reaction tube containing the enzyme assay mixture was placed on ice until injected on the HPLC. A 30 μl sample of the enzyme reaction mix (UR + dialyzed, cell extract) was injected onto a reverse phase column. Isocratic elution was utilized at a flow rate of 1.0 ml/minutes. Compounds were monitored via UV spectrum at 261 nm.
The resulting peaks of uridine (substrate) and uracil (product) corresponded to uridine and uracil standards (1mM) which were injected onto the HPLC column prior to injection of experimental samples (Figs. 5-9). Thus, pyrimidine nucleoside and base products catalyzed by salvage enzymes were seen at specific retention times on the chromatogram and indicated the presence of uridine phosphorylase when a product peak of uracil was not formed.

(b) Uridine phosphorylase - reverse reaction, Uracil + Ribose-1-phosphate $\xrightarrow{\text{urid}}$ Uridine + Pi. To prove conclusively the presence of the phosphorylase, the following assay was developed for this study: Into a 0.6 ml microcentrifuge tube were placed, 80 µl uracil (1mM substrate), 20 µl filtered, dialyzed crude cell extract and 20 µl of ribose-1-phosphate (2.5 mg/ml) (ribose source) and incubated at 37°C for 10 minutes as above. A 30 µl sample of this enzyme mix was injected onto the HPLC. Formation of a peak of uridine (product) from uracil (substrate) indicated presence of a uridine phosphorylase phosphorylase.

(c) Uridine hydrolase, Uridine + H$_2$O $\xrightarrow{\text{urid}}$ Uracil + Ribose. Incubation of 20 µl dialyzed cell extract and 80 µl uridine (1mM substrate) were incubated for 10 minutes at 37°C. The enzyme reaction was stopped by placing the reaction mix on ice until ready for injection. To observe the products of the enzyme assay, a 30 µl sample was injected onto the column and elution of a peak of uracil from uridine indicated the presence of uridine hydrolase.

(d) Cytidine deaminase, Cytidine + H$_2$O $\xrightarrow{\text{Cdd}}$ UR + NH$_3$. The cytidine deaminase assay was performed using one assay. By incubating undialyzed cell extract (in HPLC breaking buffer) (20 µl) and 80 µl of the 1mM substrate, cytidine (CR) at 37°C for 10 minutes, the assay of Cdd was completed. Again, to observe product formation a 30 µl sample of the reaction mix was injected onto the HPLC and analysis of
peak formation correlated with catalysis of $\text{Cyt} \rightarrow^\text{Cdd} \text{UR}$ (Dutta et al., 1990) if cytidine deaminase (Cdd) were present.

(e) Cytosine deaminase, Cytosine $+ \text{H}_2\text{O} \rightarrow^\text{Cdd} \text{Uracil} + \text{NH}_3$. This deamination assay was performed by incubating 20 µl undialyzed cell extract (in HPLC breaking buffer) with 80 µl of the cytosine substrate (1mM). The reaction was stopped, as before, by placing the reaction tubes on ice. Again, a 30 µl sample was injected onto the HPLC and observation of a uracil peak (product) indicated the presence of cytosine deaminase ($\text{C} \rightarrow^\text{Cdd} \text{U}$).

(f) Nucleoside hydrolase, Cytidine $+ \text{H}_2\text{O} \rightarrow^\text{nuk} \text{Cytosine} + \text{Ribose}$ and Uridine $+ \text{H}_2\text{O} \rightarrow^\text{nuk} \text{Uracil} + \text{Ribose}$. The nucleoside hydrolase uses both cytidine and uridine as substrates. Therefore two separate assays are done for this enzyme. When 20 µl of dialyzed cell extract (in HPLC breaking buffer) are incubated with 80 µl cytidine, a peak of cytosine was observed if nucleoside hydrolase were present. A second 20 µl sample of dialyzed cell extract (in HPLC breaking buffer) was incubated with 80 µl uridine. The formation of a peak of uracil upon injection of a 30 µl sample onto the HPLC indicated that the hydrolase was present.

(g) Controls. Both the filtered cell extract and the filtered, dialyzed cell extract were assayed for nucleosides and bases by incubation of 20 µl cell extract in 80 µl of tris buffer (HPLC breaking buffer) for 10 minutes at 37°C.

Pyrimidine standards. In order to determine the retention times of the pyrimidine nucleosides and bases, 1 mM pyrimidine standards were injected into the reverse phase HPLC column. Each pyrimidine standard came off the column at a different time (Figs. 4-6). Fig. 4 shows cytosine retained at 2.684 min and uracil at 3.648 min; the cytidine peak was at 4.591 min (Fig. 5) and uridine at 7.118 min. In the ribose-1-phosphate standard, there is no peak (Fig. 6), indicating that it was retained on the column
within the nine minute time period of the HPLC assay. The chromatogram in Fig. 6(b) shows four pyrimidine standards all in the same chromatogram, showing that no one nucleoside or base has the same retention time as another, and that the peak of each is distinguishable from its neighboring peak. On different reverse phase HPLC columns, even of the same brand, the retention times may vary somewhat. However, the nucleosides and bases were still retained in the same order (cytosine, uracil, cytidine and uridine) in each assay.

Regeneration of reverse phase column: When the reverse phase columns began to age the peaks on the chromatograms began to broaden, and solutes from the mobile phase collected within the column and at its entrance (usually after approximately 300 assays were run). The reverse phase columns were regenerated using the following procedure: the column was removed from the HPLC and reattached in the reverse direction (i.e. the column outlet end was reattached to the inlet tubing leaving the column inlet without attached tubing) so that solvent could run through the column and into a beaker, not through the photodiode array detector. Ten column volumes of water (approximated 40 ml) were pumped through the column at a rate of two ml HPLC grade H$_2$O per minute. The water was collected in a beaker under the column. Next, five column volumes of each of the following HPLC grade solvents were pumped through the open column: methanol, isopropanol and hexane. Then the process was reversed and five more column volumes of isopropanol, methanol and water were passed through the column. The column was removed and replaced in its normal position so that the flow would be as indicated in the direction of the arrow printed on the side of the stainless steel casing (Johns, 1989). Both the inlet and outlet lines leading to and from the column were reattached and 100 ml of mobile phase (ammonium phosphate buffer) were passed through the column before any assays were undertaken. This procedure added approximately 100-150 assays to the life of the column.
Cloning and expression of uridine kinase gene from *E. coli* in *Pseudomonas aeruginosa*. Because *Pseudomonas* was known to be devoid of the enzyme, uridine kinase (Udk), and was resistant to 5-fluorouridine, a functional Udk from *E. coli* was transformed into *Pseudomonas aeruginosa*. In order to do this, the gene for Udk (*udk*) from *E. coli* was cloned and transformed into *Pseudomonas aeruginosa*. This was achieved as follows:

The strains used included *Pseudomonas aeruginosa* PAO1 which was grown in *Pseudomonas* minimal medium, *E. coli* SΦ5114 (*leu, thr, cdd, upp, udk, cod*) and *E. coli* SΦ5123 pEUK20 (plasmid containing uridine kinase) which were grown in *E. coli* minimal medium plus 0.2% casamino acids to satisfy the leucine and threonine requirements. Plasmid pEUK20 is a pUC19 derivative with a 780 bp *EcoRI/HindIII* fragment containing the *E. coli udk* with its own promoter. The *HindIII* site included the *udk* stop codon. Also in the *HindIII* portion was the 29 bp *EcoRI/HindIII* fragment of pBR322. All bacteria were grown aerobically at 37°C.

The *E. coli* DH5α pUCP19 was grown in *E. coli* minimal medium and 0.2% glucose as a carbon source, plus 100 μg ampicillin per ml. The vector plasmid, pUCP19, (Fig. 7) into which the *udk* gene was cloned contained both *E. coli* and *Pseudomonas* origins of replication and a gene for ampicillin resistance (and thus carbenicillin resistance as well).

The pUCP19, was a derivative of pUC19 (Schweizer, 1991). Its approximate 4.5 kb size was due to a stabilizing fragment inserted into the *NarI* site at nucleotide number 235 (Fig. 7). It retained the multiple cloning site containing the *HindIII* and *EcoRI* restriction sites.

*E. coli* SΦ5114 containing pDEB1 was grown in *E. coli* minimal medium plus 100 μg ampicillin per ml to ensure that the plasmid pDEB was maintained.
The *P. aeruginosa* PAO1 containing pDEB1 was grown on *Pseudomonas* minimal plates with succinate as a carbon source, and with 500 µg carbenicillin per ml to ensure plasmid maintenance.

**Bulk plasmid preparation.** Plasmid DNA was prepared in bulk from *E. coli Sophageal* with pEUK20 (carrying the functional *udk* gene) and *E. coli DH5α* containing pUCP19 (vector plasmid) by the method of Sambrook *et al.*, (1989) with modifications according to R. C. Benjamin and M. S. Shanley respectively. Broth tubes containing 5 ml LB broth were inoculated with the organisms containing the plasmids to be bulked. Appropriate antibiotics were added to the tubes to maintain the plasmids. For both *E. coli* cultures grown above, the 5 ml tubes each contained 100 µg ampicillin per ml. The tubes of *E. coli Sophageal* with pEUK20 and *E. coli DH5α* with pUCP19 were grown overnight at 37°C on a shaker and used to inoculate one liter flasks of LB broth. The plasmids which were to be used for the bulk preparation were both high copy number plasmids and were not amplified with chloramphenicol.

The one liter flasks were grown overnight in a 37°C shaker/incubator. The cells were then harvested by pouring and balancing 500 ml of each culture in 500 ml centrifuge bottles and were spun down using a previously cooled (4°C) GS3 rotor at 10,810 x g for 15 minutes. The supernatant was poured off and the pellets resuspended in 20 ml cold 0.15 M NaCl in Oak Ridge centrifuge tubes (additional 0.15M NaCl was added to tubes in order to balance them.) The tubes were placed in a cold (4°C) SA600 rotor and centrifugation was at 5211 x g for 5 minutes.

The following steps were done in Dr. Kunz's cold room. The pellets were resuspended in 10 ml of 50 mM Tris, 25% sucrose, pH 8.0 using a spatula to break the pellet loose and by vortex mixing them so that no clumps remained.
Into each tube was added two ml lysozyme (5 mg/ml mixed in H₂O) and mixed by inversion. (The lysozyme helped to break the cell walls.) The cell-lysozyme mix was left on ice in the cold room for five minutes.

In order to chelate Ca²⁺ and Mg²⁺ in the mixtures, four ml of cold 0.25M Na₂EDTA (pH 8.0) were added to each tube, mixed by inversion and left on ice in the cold room for five additional minutes. After addition of five ml cold NaCl and mixing gently by inversion, the E. coli chromosome was made insoluble in the mixture. Violent agitation was avoided in this step as breakage of DNA might have occurred had it been vortex mixed.

Two ml of 10% SDS were then added to disrupt the cell membranes and mixed gently by inversion of the tubes. (The SDS was not kept cold as cooling caused the sodium dodecyl sulfate to come out of solution.) The two tubes were then left in the cold room for two hours.

After the two hour period, the two tubes were placed in a SA600 rotor and centrifuged at 39,410 x g for 60 minutes. After being spun down, the supernatants were poured into a graduated cylinder and one volume of isopropanol was added to each 8 ml sample. Each mixture was poured into a GSA centrifuge tube and placed in a dry ice-ethanol (95%) bath for 20 minutes until frozen solid. The contents of each tube were then thawed in a tray of tap water and centrifuged in a GSA rotor at 10,410 x g for 20 minutes. The pellets were resuspended in 10 ml of 10mM Tris, 1mM EDTA, pH 8.0 (TE) and placed in the cold room. A magnetic stir bar was added to each centrifuge tube, the tubes placed on a stir plate in a cold box and allowed to stir slowly for two hours. DNAse-free RNAse was added while the mixtures were stirring (20 μg/ml; 20 μl of 10 mg/ml stock RNAse solution was added per 10 ml of plasmid mixture).
In order to clarify the presence of the plasmids, samples of each were run on a 1% agarose gel (1.0 g high melt agarose dissolved in 100ml Tris Acetate EDTA buffer: (TAE) 50x: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M Ethylenediamine tetraacetic acid (EDTA) (pH 8.0). The DNA was stained with ethidium bromide (2 ml of a 10 mg/ml solution were added to the molten agarose before pouring the gel). Since bands for each of the plasmids were noted upon inspection with a UV lamp, the bulking protocol proceeded. In order to remove undissolved material from the mixtures, the remainder were spun down in Oak Ridge tubes using an SA600 rotor at 14,470 x g for 10 minutes.

To provide a gradient for separation of the chromosomal DNA and plasmid DNA, 5.3 g CsCl per 5 ml of sample was added to each Ti1270 ultracentrifuge tube (per liter of culture grown) using a syringe and a 19 gauge needle. The tubes were filled to the shoulder, leaving enough room to add ethidium bromide. The tubes were balanced to a weight within 0.01g of each other and the ethidium bromide added to the neck with a Pasteur pipette (10 mg/ml per eight ml solution). The tubes were crimp-closed using Ultracrimp™ caps, the lights were extinguished and the tubes inverted to mix the ethidium bromide throughout the DNA-CsCl mixture. This step was done in the dark to prevent cleavage of ethidium bromide bound DNA by light.

The ultracentrifuge caps were placed atop the Ti1270 tubes and the tubes placed opposite each other in the ultracentrifuge. The DNA was spun in a Sorvall OTD75B ultracentrifuge in a T-1270 rotor (not chilled) for 40 hours at 118,700 x g.

After centrifugation, the rotor was removed and placed on the rotor stand in order to transport the tubes to the plasmid extraction room. The process of extracting the plasmid band from the tube was done in the dark. In order to see the two separate plasmid and chromosomal DNA bands, a handheld UV lamp was placed close to the tube (secured in a ringstand and clamp apparatus). A beaker was placed beneath the tube to collect the ethidium bromide-cesium chloride overflow from the tube. Two 20 gauge syringe needles
per tube were required. One needle was used to introduce an air hole in the sloping shoulder of the tube. The other needle was attached to a syringe and inserted just above the lower, plasmid band and used to suction off the plasmid DNA. The DNA saturated with ethidium bromide was washed and extracted from the ethidium bromide using butanol saturated with water. The butanol extraction was performed until the DNA supernatant was no longer pink. The CsCl was removed from the mixture by dialyzing the samples in three liters of 1 M tris buffer, pH 7.0.

After dialysis, the concentration of DNA in each tube was determined spectrophotometrically at 260 nm using a quartz cuvette (to equal 50 μg/ml). The concentration of the pEUK20 plasmid was 930 ng/μl and for the pUCP19 the concentration was 1010 ng/μl. In order to check for plasmid degradation, the pEUK20 was electrophoresed through a 1.0% agarose gel in 1x TAE buffer and it was determined that the DNA had not been degraded. Subsequently, a restriction digest of the pEUK20 plasmid containing the uridine kinase gene (udk) was performed. The plasmid was a pUC18 derivative with an approximately 780 bp EcoRI/HindIII fragment containing the E. coli udk with its own promoter. The HindIII site included the udk stop codon. Also in the HindIII end was a 29 bp EcoRI/HindIII fragment of pBR322 (Fig.4). This EcoRI site was selected to cut out the udk gene.

To determine how much DNA to add to the restriction digest to have 20 μg DNA for the digest, the concentration of DNA is divided into the 20 μg. For the donor plasmid
\[
\frac{20\mu g}{930\mu g/\mu l} = 21\mu l
\]
the amount to add to the restriction digest was 21 μl. Other components of the restriction digest included 6 μl EcoRI buffer (10x); 32 μl H₂O; and 1 μl EcoRI enzyme. The restriction digest mix was incubated 1.5 hours at 37°C in a microcentrifuge tube.
Upon completion of the restriction digest, the digested pEUK20 DNA was run out on a 1% agarose gel. The gel was designed with one small well into which was placed the molecular weight marker, a *Hind*III digest of lambda DNA (United States Biochemical, Cleveland, OH) (20 µl DNA and 4 µl 5x loading buffer) and one very large well which held the 60 µl of restriction cut pEUK20 DNA (60 µl restriction cut pEUK20 and 12 µl loading buffer). The gel was run at 80V for 1.5 hours. When viewed under UV, the *Hind*III cut lambda exhibited bands of molecular weight 23,130; 9,416; 5,582; 4,361; 2,322; 2,027, 564 and 125. The pEUK20 had two bands of approximate molecular weights 780 and 3,800. The band with MW 780 corresponded in size to the *udk* fragment which was excised from the pEUK20.

Since the *udk* was ready for insertion into the vector, the vector itself had to be digested so that the *udk* could be ligated into the vector. This was accomplished by determining the amount of DNA to be used in the restriction digest:

\[
\frac{20\mu g}{1010\mu g / \mu l} = 0.019 = 19 \mu l \text{ (the amount of DNA added)}.
\]

The restriction enzyme digest mix included 19 µl pUCP19 DNA; 6 µl EcoRI buffer; 34 µl H₂O; 1 µl *EcoRI* restriction endonuclease (added last). To keep the digested vector from rejoining its DNA, the digested DNA was treated with bacterial alkaline phosphatase in the following manner. Into the 60 µl restriction mixture of pUCP19 vector DNA was added two µl bacterial alkaline phosphatase (BAP) and incubated for three hours in a 65°C water bath. The tube was removed from the water bath, spun down (10 sec) in a microcentrifuge to collect the condensation on the walls of the tube and 50 µl TE buffer were added (TE: 10 mM Trizma base, 1 mM EDTA, pH 8.0 with HCl). Several phenol washes were done as follows: 100 ml water-saturated phenol (contained 0.1% 8-hydroxyquinoline for color) was added to the pUCP19 digest and vortex mixed. The tube was centrifuged for three minutes at 10,000 x g. The lower phenol layer was removed and discarded using a 100 µl micropipettor. The
remaining TE/pUCP19 DNA digest was transferred to a sterile 1.5 ml microcentrifuge tube and an additional 100 μl water-saturated phenol were added. The tube was once again vortex mixed, centrifuged for 3 minutes at 10,000 x g and the phenol layer removed as before. Diethyl ether (100 μl) was added to the tube and vortexed. The tube was centrifuged for 30 sec and the ether layer (top layer) was removed under a hood, using a drawn out 9" Pasteur pipette. The tube was left open under the hood to allow the remainder of the ether to evaporate.

After the DNA was cleaned, 3 M sodium acetate (0.1 x volume) was added and the mixture mixed well. Then three times the volume of ice cold 100% ethanol was added to precipitate the DNA and mixed. It was then placed in the -80°C freezer for 30 minutes. After thawing, it was spun down in a microcentrifuge in a cold box at 10,000 x g for 15 minutes leaving the DNA in a pellet in the bottom of the tube. The supernatant was discarded using a drawn out 9" Pasteur pipette and the DNA was washed using ice cold 70% ethanol. It was mixed gently by inversion, centrifuged at 10,000 x g for 5 minutes and the supernatant discarded. The DNA was placed in the Speed Vac vacuum centrifuge (Savant instruments) for five minutes, 50 μl distilled deionized water were added to dissolve the DNA by vortex mixing it and it was incubated at 65°C for 10 minutes after which it was mixed by vortex once again. It was frozen at -20°C, thawed five minutes in the 65°C water bath, vortexed and stored at -20°C.

Meanwhile, the 780 bp udk insert in the gel run previously was prepared for electroelution. A small piece of the agarose gel containing the 780 bp band was cut from the agarose gel by placing the gel on a UV light box and carefully cutting with a new razor blade (gloves and eye goggles were worn to protect from ethidium bromide and UV respectively). The fragment of agarose gel containing the 780 bp EcoRI fragment was inserted into a six inch portion of dialysis tubing which had been rinsed well in water and clamped at one end. A stainless steel spatula was used to aid in guiding the DNA-laden
agarose into the tubing which contained 500 μl 0.25x TAE buffer. The open end of the tubing was clamped shut. The dialysis bag was then placed horizontally in a gel box containing enough TAE buffer (0.25x) to cover the dialysis tubing. A large black rubber stopper was placed atop the tubing to keep it from floating to the top. The gel was electrophoresed overnight (approximately 10 hours) at 25V. In order to loosen the DNA from the walls of the dialysis tubing, the poles of the electrophoresis apparatus were reversed and current applied for 15 sec at 100V. A siliconized Pasteur pipette was used to draw out the TAE buffer containing the DNA from the dialysis tubing. Care was taken to exclude any agarose in the transfer of the TAE to the microcentrifuge tube, but a quick spin (3 minutes) was done to ensure that any extraneous agarose did not contaminate the TAE. A phenol extraction was done (the same procedure as in the bacterial alkaline phosphatase (BAPping) protocol above, except that 80 μl of additional buffer were added the phenol extraction in order to get enough volume to successfully pull off. The cells were stored at -20°C until needed.

In order to ligate the udk into the pUCP19 vector, concentrations of the DNA from each preparation were taken. The 780 bp udk fragment was calculated as follows:

\[0.090 \text{ OD} \times 10 \text{ (dilution factor)} \times 50 \mu l \text{ total volume} = 45 \mu g/ml\]

with the pUCP19 vector DNA calculated as follows:

\[0.052 \text{ OD} \times 10 \text{ (dilution factor)} \times 50 \mu l \text{ total} = 26 \mu g/ml \text{ or } 1300 \text{ ng/50} \mu l.\]

The ligation mixture included 4.0 μl vector DNA; 2.0 μl udk DNA; 3.0 μl 20 mM ATP; 3.0 μl 20 mM dithiothreitol; 3.0 μl 10x buffer; 1.0 μl DNA ligase; 14 μl distilled deionized H₂O. It was ligated at 25°C overnight and named pDEB1 (Fig.7).

**Preparation of competent cells.** The preparation of the competent cells was achieved by inoculation of 100 ml LB broth with a 5 ml culture of *E. coli* DH5α which had been grown overnight (Sambrook *et al.*, 1989). The 100 ml of *E. coli* were grown to mid-log phase and harvested by centrifugation for five minutes at 2000 x g at 4°C. After the
supernatant was discarded, the cells were gently resuspended in cold (4°C) TFB1 buffer (30 ml/100 ml culture) and incubated on ice for 90 minutes (TFB1: 100 mM RbCl, 50 mM MnCl₂, 30 mM Kacetate, 10 mM CaCl₂, 15% glycerol, pH 5.8, filter sterilize). After being incubated on ice, the cells were spun down (5 minutes at 833 x g at 4°C) and the supernatant was discarded. The pellet was kept on ice. The cells were resuspended carefully in ice cold TFB2 buffer (4 ml/100 ml culture) (TFB2: 10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂, 15% glycerol, pH 8.0; autoclaved). The cells were gently resuspended by shaking (were not vortex mixed). Aliquots of 500 ml were placed in sterile microfuge tubes and frozen at -80°C until needed for transformation.

**Transformation of E. coli DH5α with pDEBl.** The transformation of E. coli was after the technique of Sambrook et al., (1989). Thirty |μl of the newly ligated pDEBl were placed into a sterile 1.5 ml microcentrifuge tube. After thawing the competent E. coli cells on ice they were gently resuspended and 125 ml were added to the pDEBl in the microcentrifuge tube. The transformation mixture was incubated in a 42°C water bath for two minutes after which 500 |μl of PSI broth were added to grow up the cells (PSI: 5 ml LB; 20 μl MgSO₄ (1 M); 15 μl KCl (1 M). The microfuge tube was placed in an empty 10 ml glass test tube which acted as a holder in the incubator-shaker and shaken for 90 minutes at 37°C.

While the E. coli containing the newly transformed pDEBl were growing up, plates were prepared to conduct the β-galactosidase assay on the newly grown cells. The plates were LB agar plates with 100 μg ampicillin per ml. Ten μl isopropyl thiogalactoside (IPTG) were placed in the center of the LB/Ampicillin plates above. Immediately after, 50 ml of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; the chromagen) were placed in the same spot as the IPTG and the two substances were spread over the surface of the plates. Fifty |μl of the newly transformed E. coli containing pDEBl were spread on the surface of the β-galactosidase assay plates. The plates were incubated at 37°C.
After 36 hours of growth, 24 white colonies were "picked" and each one transferred to a 5 ml tube of LB respectively. The organisms were grown for 10 hours at 37°C while shaking. A rapid plasmid preparation was done on each tube of cells according to the protocol of Zhou et al., (1990).

To prove that the vector had, indeed, taken up the udk DNA, the newly ligated pDEB1 was digested with EcoRI which resulted in a 780 bp fragment being cut out of the pDEB1 when run out on a 1% agarose gel. Tubes 1, 3, 5, 10, 11, 14 and 19 contained plasmids with the udk insert.

**Determination of udk DNA insert orientation.** The restriction enzyme HindIII (New England Biolabs) was chosen to test the orientation of the inserted udk fragment in pUCP19 from tubes 5, 10, 11, 14 and 19. After digestion with HindIII, the "correctly orientated plasmid had an 829 bp HindIII fragment excised from the multiple cloning site in the pUCP19 vector portion of pDEB1 as the DNA was run out on a 1% agarose gel and the appropriate sized bands were seen stained with ethidium bromide using a UV lamp. The DNA from the original "clone" tubes were found in tubes 5, 10, 11 and 14. This was further proof that the udk fragment had been inserted into the vector.

Another rapid plasmid preparation was done using the clones in tubes 5, 10, 11 and 14 after each was grown up in respective tubes of LB supplemented with 100 μg ampicillin per ml. Upon completion of the rapid prep and again checking each clone for the insert by digesting each one with HindIII, the samples were electrophoresed and 829 bp bands resulted after running the DNA at 80V for one hour. Clone 10 was chosen (since it had the largest band of 829 bp) as the one most likely to contain the pDEB1 plasmid and was bulked by the method previously described. The concentration of plasmid DNA (pDEB1) was 1.6 mg/ml.

**Preparation of recipient cells for transformation.** A culture of *P. aeruginosa* PAO1 grown in *Pseudomonas* minimal medium with succinate as a carbon and
energy source, was plated on *Pseudomonas* minimal medium agar plates. A crystal of 5-fluorouracil was placed in the center of each plate and the plates incubated at 37°C. After two days a zone of killing was seen on the plate but resistant (FU *, upp*) colonies were seen growing within the zone of killing adjacent to the crystal of fluorouracil. These resistant organisms were not mutagenized by the 5-fluorouracil but were naturally occurring resistant organisms in the population.

One of those small resistant colonies was chosen and grown in five ml of *Pseudomonas* minimal medium and replated on a *Pseudomonas* minimal medium plate. A small crystal of fluorouracil was placed in the center of the plate, the plate incubated at 37°C. After two days, no zone of killing was observed, indicating that the PAO1 strain which had been selected for fluorouracil resistance had been successfully chosen. The fluorouracil resistant pseudomonad was grown in LB (5 ml) at 37°C overnight.

To determine if *P. aeruginosa* PAO1 (wild type) was naturally resistant to 5-fluorouridine, the PAO1 upp* cells selected previously were grown in *Pseudomonas* minimal medium and spread on a *Pseudomonas* minimal plate. A crystal of 5-fluorouridine was placed in the center and incubated at 37°C for two days. The pseudomonad grew to the crystal of FUR which indicated that the *Pseudomonas* had no uridine kinase.

The organism used as the control was *E. coli* SΦ 5114 (upp*, udk*, cdd*). It was grown in a five ml culture of *E. coli* minimal medium (glucose as carbon and energy source) at 37°C overnight. The organism was tested to make certain that its mutations were true by plating it on *E. coli* glucose minimal medium and subjecting it to 5-fluorouracil and 5-fluorouridine treatment on two separate minimal plates. Since no zones of killing were found, and the organisms grew up to the crystals of FU and FUR, the mutations in upp and udk were still present.

The *P. aeruginosa* PAO1 was transformed using the Bagdasarian method (Bagdasarian & Timmis, 1982) using rubidium chloride and magnesium chloride.
Transformation of the *E. coli* SF5114 *udk*<sup>−</sup>, *upp*<sup>−</sup> was by the method of Rod Kelln (personal communication).

**Complementation of *Pseudomonas aeruginosa* PAO1 by *E. coli* uridine kinase:** Aliquots were plated on selective agar plates containing ampicillin for the *E. coli* and carbenicillin for the *P. aeruginosa*. Growth was checked after one and two days.

The FU<sup>+</sup> *Udk*<sup>−</sup> *E. coli* SF5114 transformed with pDEB1 (*Udk*<sup>+</sup>, *amp*<sup>+</sup>) were plated on *E. coli* glucose minimal medium plus ampicillin (100 μg Amp/ml). The *P. aeruginosa* PAO1 *Upp*<sup>−</sup>pDEB1 was plated on *Pseudomonas* minimal medium plus carbenicillin (500 μg Car/ml). A crystal of 5-fluorouridine was placed in the center of each plate. Both organisms exhibited a zone of killing on the pyrimidine analog plates and successfully took up the uridine kinase gene as well as the antibiotic resistance markers. The experiment was also done using 5'-fluorocytidine. A crystal of 5'-fluorocytidine was placed in the center of a lawn of *P. aeruginosa* containing pDEB1 and on a lawn of *E. coli* containing pDEB1. On the *E. coli* pDEB1 plate there was a zone of killing but on the *P. aeruginosa* pDEB1 plate the organisms grew up to the crystal. In the *E. coli* the uridine kinase had activity for both cytidine and uridine. In the *Pseudomonas*, cytidine and thus its analogs, did not get into the cell. This would explain how the pseudomonad could be sensitive to 5'-fluorouridine but not to the 5'-fluorocytidine.

**Controls.** Aliquots of pDEB1 DNA were plated on *Pseudomonas* minimal medium containing 500 μg carbenicillin per ml and on *E. coli* minimal medium containing 100 μl ampicillin per ml. No growth occurred.

*E. coli* SF5114 and *P. aeruginosa* PAO1 were plated on the selective media above. The *E. coli* did not grow on the ampicillin plates nor did the *P. aeruginosa* grow on the carbenicillin plates. The *P. aeruginosa* grew on the ampicillin plates.
Recovery of pDEB1 on agarose gel. Both *E. coli* pDEB1 and *P. aeruginosa* pDEB1 were grown up in 5 ml cultures of LB plus ampicillin and carbenicillin respectively and rapid preps were performed. The plasmid DNA was digested with *Hind*III and run out on a 1% agarose gel at 80V for one hour. Fragments in the size of 829 bp were recovered in the *E. coli* pDEB1 lanes but none was recovered in the *P. aeruginosa* pDEB1 lanes.
Fig. 4. HPLC chromatogram standards. (a) Cytosine standard peak at 2.6 min retention time; (b) Uracil standard peak at 3.6 min retention time.
Fig. 5. HPLC chromatogram standards. (a) Cytidine standard peak at 4.5 min retention time; (b) Uridine standard peak at 7.1 min retention time.
Fig. 6. HPLC chromatogram standards. (a) Ribose-1-phosphate standard with no peaks; (a) standards of cytosine (2.6 min), uracil (3.5 min), cytidine (4.4 min) and uridine (6.8 min).
Table 1. Bacterial stains and plasmids.

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<td><strong>Mutant Strains</strong></td>
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<td><em>Escherichia coli</em> DH5α</td>
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<td>Above 30°C ura</td>
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<td><em>Salmonella typhimurium</em> KP1130</td>
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**Plasmids**

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<td>pUCP18</td>
<td>pBR322-derived vector with <em>Pseudomonas</em> and <em>E. coli</em> oriV5, AmpR</td>
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<td>pEUK20</td>
<td>pBR322 &amp; pUC18 derivative bearing Tdk</td>
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<td>pDEB1</td>
<td>pUCP18 &amp; pEUK20 derivative bearing Tdk</td>
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Fig. 7. Construction of plasmid pDEB1.
CHAPTER III

RESULTS

In a radio speech in 1939, Sir Winston Churchill told the British people that "I cannot forecast to you the action of Russia. It is a riddle wrapped in a mystery inside an enigma." (Churchill, 1939). Bacteria could well be described, for example, in those same terms, for just when one discovered that bacteria were sensitive to a particular antibiotic, they may have changed! Next day they were found to be resistant to that same antibiotic. Because antibiotic resistance is often carried on plasmids and because plasmids are freely exchanged between different bacteria, the use of antibiotic resistance as a stable characteristic is unreliable. Indeed, the use of any classification that is plasmid-mediated as a stable taxonomic marker is unwise. Yet, we actually exploit this natural ability of bacteria when we use antibiotic resistance markers to determine if a gene has been cloned successfully in the laboratory. Bacteria have had to use this ability to resist antibiotics in nature for millions of years and those that survived have incorporated these resistance genes into their DNA. Taxonomists look for similar traits in organisms just to classify and organize them. Actually, differences can be just as helpful in organizing bacteria into more precise groupings such as families.

I, too, am looking for pieces in the bacterial jigsaw puzzle. I believe that it is possible to use the pyrimidine salvage enzymes to fill in a few more pieces of the jigsaw, while the nine salvage pathways I have discovered may not tell the entire story of bacteria, it may be a method of discerning some of the "mystery inside an enigma" called "bacteria."

The presence or absence of the pyrimidine salvage enzymes is known to vary in different organisms. Those enzymes that show greatest variance from organism to
organism, namely, cytosine deaminase, cytidine deaminase, uridine phosphorylase, uridine hydrolase and nucleoside hydrolase were assayed in 44 prototrophs and also in 11 mutants as well. A typical assay with cytosine, uracil, cytidine and uridine peaks would show retention times of 2.6, 3.5, 4.4 and 6.8 minutes respectively. Except for mutant strains which lacked these enzymes, uracil phosphoribosyltransferase and uridine kinase enzymes were presumed to be present based on data from the literature. These enzymes were not assayed in this work.

Of the approximately 2200 HPLC chromatographic assays performed, 290 may be seen in Figs. 9-153. For each set of chromatograms, two controls were run. One was on the cell extract, prepared as described in Methods, which was filtered through a 0.45 μm syringe filter. This was to show if any of the four substrates used in all assays were present in the cell extract after filtration. Filtration removed cell debris but not the pyrimidines uracil, uridine, cytosine or cytidine. The second control was on the dialyzed cell extract which should have removed all pyrimidines from the cell extract. Typically, cell extracts contained varying amounts of pyrimidines, especially uracil, as seen in most of the controls. However, there should be no pyrimidines in the cell extracts after dialysis. This was generally the case. Occasionally, minute traces of compounds were seen as peaks with retention times close to or coincident with those of the four pyrimidines assayed. Such peaks may be the authentic pyrimidines that escaped removal by the dialysis or may represent non-pyrimidine compounds with retention times and absorptivity near those of the pyrimidines. The appearance of each peak was treated on an individual basis.

**Group I results:** The salvage enzyme combination used to place organisms in Group I is seen in Fig. 8. The salvage enzymes, cytidine deaminase (Fig. 8, reaction 5), cytosine deaminase (Fig. 8, reaction 4), uridine phosphorylase (Fig. 8, reaction 2) and uridine hydrolase (Fig. 8, reaction 3) were active in 14 of the 44 species of organisms
examined. The actual chromatograms of the salvage enzymes assayed have been included. Group I chromatograms may be found in Figs. 9-52.

In the ATCC strain 25922 of *E. coli*, the prototype organism, the dialyzed cell extract (Fig. 9) contained no pyrimidines on the HPLC chromatogram while the filtered cell extract (Fig. 9) contained a minute amount of uracil with a peak at a retention time of 4.1 minutes.

Fig. 10 shows the cytidine deaminase and cytosine deaminase assays. In Fig. 10, the presence of cytidine deaminase in *E. coli* was determined by formation of a peak of uridine product at a retention time of 9.3 minutes upon addition of cytidine substrate.

In Fig. 10 is shown the results of the cytosine deaminase assay. At a retention time of 3.0 minutes, a peak of the cytosine substrate not yet converted to uracil is seen. The product of the assay, uracil, appeared as a peak at a retention time of 4.3 minutes. Fig. 11 denotes the presence of both uridine hydrolase (Udh) and uridine phosphorylase (Udp). Fig. 11(a) shows the results of incubating the dialyzed cell extract with uridine. The characteristic uracil peak appeared at a retention time of 4.0 minutes which indicated that a hydrolase was present in the assay performed before injection into the column. In addition to the uridine hydrolase, another enzyme was found in Group I which degraded uridine. That enzyme was uridine phosphorylase which also catalyzed the reaction of uracil (at a retention time of 4.0 minutes) and ribose-1-phosphate to produce uridine seen at a retention time of 8.3 minutes in Fig. 11(b).

Figs. 12-14 represent assays of salvage enzymes in the K12 strain of *E. coli*. The salvage enzymes in the K12 strain were identical to those found in the ATCC strain above. In Fig. 12(a), the dialyzed cell extract had less than 3% uracil while the filtered cell extract in Fig. 12(b) had only traces of uracil at retention times of 3.9 minutes and uridine at 8.6 minutes. The product of the cytidine deaminase assay (Fig. 13), uridine, gave a massive peak at a retention time of 8.3 minutes (the uracil peak, a product of the uridine

...
phosphorylase and uridine hydrolase reactions can also be seen in Fig. 13 as well). During
the assay period, the cytidine was depleted and all converted to uridine. However, its
presence was confirmed when additional cytidine was added to the assay mix and incubated
for a shorter time after which a peak of cytidine appeared at a retention time of 5.4 minutes
with uridine at 8.3 minutes (data not shown). The combined action of cytidine deaminase
and uridine phosphorylase or uridine hydrolase produced uridine first and then uracil.

The product of the cytosine deaminase assay, uracil, was noted at a retention time
of 3.9 minutes in Fig. 13(b). In Fig. 14, the dialyzed cell extract containing the presumed
uridine hydrolase was incubated with uridine and produced a peak of uracil with the
characteristic retention time of 4.1 minutes. The dialyzed cell extract also contained a
uridine phosphorylase. Fig. 14(b) shows the assay for the uridine phosphorylase which
produced a large peak of uridine (at a retention time of 8.6 minutes) from uracil (4.1
minutes). One other strain of *E. coli*, TB2, was tested for pyrimidine salvage enzymes
giving three in all in which no differences were found among the salvage enzymes (data not
shown).

The presence of the enzyme nucleotide glycosylase is frequently inferred in
literature surveys but rarely are assays proving its presence shown. In the following assay,
the presence of a nucleotide glycosylase is proven in a strain where uridine/cytidine kinase
was shown to be present. Figs. 15-16 are chromatograms of the *E. coli* mutant, SΦ5110
(Table 1) which has genetic blocks in the enzymes, cytidine deaminase and cytosine
deaminase. In the control chromatograms in Fig. 15 no pyrimidines are shown in the
diallyzed cell extract and only small amounts of cytosine, uracil and cytidine in the
undialyzed extract. When the dialyzed cell extract was used instead of the undialyzed one,
the cytosine peak was not present. This proved the presence of nucleotide glycosylase. If
a nucleoside hydrolase had been present in this strain, cytosine would have appeared
whenever cytidine was used as substrate. Thus the substrate flow from Cytidine (CR) →
CMP → Cytosine (C) → Uracil (U) is seen. This eliminated the nucleoside hydrolase from consideration.

When the undialyzed extract was incubated with cytidine, peaks of cytosine and uracil emerged. Since there was no cytidine to uridine conversion due to the lack of cytidine deaminase in the mutant the only way for cytosine to be formed would be as follows: CR → CMP → C.

Other organisms with Group I salvage enzymes are *Citrobacter freundii* (Figs. 17-19); *Enterobacter cloacae* (Figs. 20-22); *Hafnia alvei* (Figs. 23-25); *Klebsiella pneumoniae* (Figs. 26-28); *Proteus mirabilis* (Figs. 29-31); *Salmonella typhimurium* (Figs. 32-34); *Serratia odorifera* (Fig. 35-37); *Yersinia enterocolitica* (Figs. 38-40); *Shewanella putrefaciens* (Figs. 41-43); *Vibrio alginolyticus* (Figs. 44-46); *V. parahaemolyticus* (Figs. 47-49); and *Enterococcus faecalis* (Figs. 50-52).

The Group I salvage enzymes are Cod, Cdd, Udp, Udh and (Upp, Udk).

**Group II results:** The presence of uridine hydrolase distinguishes Group I and III organisms from Group II members (Fig. 53). Accordingly, a separate Group II has been set aside for those organisms containing uridine phosphorylase but lacking uridine hydrolase. The enzymes cytidine deaminase, cytosine deaminase and uridine phosphorylase were found in eight of the 44 species examined. *Burkholderia cepacia* was the archetype organism of Group II. In Fig. 54, which shows the two control assays, the dialyzed cell extract had no background in Fig. 54(a) and the undialyzed cell extract in Fig. 54(b) had a small peak of uracil. Both the dialyzed and undialyzed cell extracts were compared with the four subsequent assays. In the cytidine deaminase assay, (Fig. 55(b) the substrate peak, cytidine was at 5.06 minutes with the product, uridine, at 7.56 min. Fig. 55(b) shows the cytosine deaminase assay with the substrate (cytosine) at 2.8 minutes and the product (uracil) at 3.77 min. Since the cell extract in both assays in Fig. 55 was undialyzed, the area of the uracil peak found in the filtered cell extract was subtracted from
that of the uracil peak found in Fig. 54(b) to determine that uracil was, indeed, hydrolyzed from cytosine. The uracil produced from the cytosine deaminase hydrolysis was well above the background found in the undialyzed cell extract.

An assay for both uridine phosphorylase and uridine hydrolase (Fig. 56), shows that the *B. cepacia* has uridine phosphorylase only. Since all small molecules including phosphates were removed from the cell extract by the dialysis, the uridine phosphorylase was inactive and no product was produced. Were the hydrolase present, a peak of uracil would have formed since no phosphates are necessary for the hydrolase reaction to occur. That no uracil peak appeared (Fig. 56(a) from the dialyzed extract with uridine as substrate suggested that a phosphorylase present. To confirm the presence of phosphorylase, a special assay devised for this work was done using uracil, ribose-1-phosphate and dialyzed cell extract. As in Fig. 56(b), when a peak of uridine formed, the enzyme uridine phosphorylase must be present since the phosphorylase reaction is reversible while the hydrolase reaction is not. There was no activity with cytidine to cytosine, again suggesting that nucleoside hydrolase was absent.

Other members containing Group II salvage enzymes are: *Haemophilus influenzae* (Figs. 57-59); *Mycobacterium smegmatis* (Figs. 60-62); *Proteus vulgaris* (Figs. 63-65); *Pseudomonas mendocina* (Figs. 66-68); *Pseudomonas pseudoalcaligenes* (Figs. 69-71); *Pseudomonas stutzeri* (Figs. 72-74); and *Rhizobium loti* (Figs. 75-77).

The five Group II salvage enzymes are Cdd, Cod, Udp, (Upp, Udk).

**Group III results:** The Group III salvage scheme (Fig. 78) is distinguished from that of Groups I and II by the fact that it contained cytidine deaminase, cytosine deaminase and uridine hydrolase (but no uridine phosphorylase). All were found in the two species examined. The eukaryotic organism, *Saccharomyces cerevisiae* (Figs. 79-81) and the prokaryote *Pseudomonas indigofera* (Figs. 82-84) have the Group III salvage enzymes Cod, Cdd, Udh, and (Upp, Udk).
Group IV results: The Group IV salvage seen in Fig. 85, is most like Group II except that it has no cytosine deaminase. Three species of bacteria of those examined were found to contain cytidine deaminase and uridine phosphorylase. The bacteria which had these two salvage enzymes included *Bacillus megaterium* (Figs. 86-88); *Bacteroides fragilis* (Figs. 89-91); and *Micrococcus luteus* (Figs. 92-94).

The Group IV salvage enzymes are Cdd, Udp and (Upp, Udk).

Group V results: Only one bacterium, namely *Staphylococcus aureus*, was found to contain the salvage scheme in Group V and shown in Fig. 95. The assays are shown in Figs. 96-98. The control assays for the dialyzed cell extract (Fig. 96) and the undialyzed cell extract (Fig. 96) were performed so that comparisons of actual assays could be made against the pyrimidines in the cell extracts. There was a small amount of uracil and cytidine in the undialyzed sample. The cytidine deaminase assay (Fig. 97) produced a uridine product from cytidine. The uracil seen in the cytosine deaminase assay (Fig. 97) was not sufficient to register over the amount of uracil in the background; in other words, no cytosine deaminase was present. In the uridine hydrolase assay (Fig. 98), a very large peak of uracil was seen as a product of the substrate uridine even in dialyzed cell extracts, proving that the enzyme present was a hydrolase. In Fig. 98(b), is proof that a uridine phosphorylase was not present since a peak of uridine did not emerge when uracil and ribose-1-phosphate were incubated with undialyzed cell extracts.

The Group V salvage enzymes are Cdd, Udh, (Upp, Udk).

Group VI results: Thirteen of 44 organisms assayed contained nucleoside hydrolase and cytosine deaminase only, which constitutes a divergence from the other organisms seen in the previous Groups I-V (Fig. 99). Group VI, composed primarily of *Pseudomonas* and former *Pseudomonas* species, contained the salvage enzymes, nucleoside hydrolase which catalyzes cytidine to cytosine and uridine to uracil irreversibly.
This group also contained cytosine deaminase but no cytidine deaminase or uridine phosphorylase. *Pseudomonas* species are also devoid of uridine kinase.

*Pseudomonas aeruginosa* was the prototype organism of Group VI. Neither the dialyzed cell extract (Fig. 100) nor the undialyzed cell extract (Fig. 100) from *P. aeruginosa* PAO1 contained any nucleosides or bases which would interfere with accurate readings of subsequent chromatographic assays. In Fig. 101(a), the substrate, cytidine, was incubated with undialyzed cell extract and a peak of cytosine product was formed, indicating that a nucleoside hydrolase was present. Since a peak of uridine did not form, no cytidine deaminase was present. The organism did, however, have cytosine deaminase, which can be seen by the production of uracil from cytosine (Fig. 102). In Fig. 102(a), the second half of the nucleoside hydrolase assay may be seen. The broadly specific hydrolase, even with dialyzed cell extract, converted the uridine substrate to uracil since no phosphates are required for the hydrolase function. Further proof of the existence of the hydrolase (Fig. 102) was seen when uracil and ribose-1-phosphate were incubated with the cell extract and gave no uridine peak. The hydrolase does not work in the reverse direction: uracil $\rightarrow$ uridine or, cytosine $\rightarrow$ cytidine.

The organisms which have the Group VI salvage enzymes include strains of *Pseudomonas aeruginosa* in addition to strain PAO1. These are strain ATCC 10145 (Figs. 103-105) and ATCC 27853 (Figs. 106-108). Other organisms with the same salvage enzymes include *P. aureofaciens* (Figs. 109-111); *Brevundimonas diminuta* (Figs. 112-114); *P. fluorescens* (Figs. 115-117); *Alcaligenes faecalis* (Figs. 118-120); *Comamonas acidovorans* (Figs. 121-123); *C. testosteroni* (Figs. 124-126); *Rhizobium leguminosarum* Biovar *trifolii* (Figs. 127-129); *R. meliloti* (Figs. 130-132); and *Stenotrophomonas maltophilia* (Figs. 133-135).

The Group VI salvage enzymes include Cod, Nuh and (Upp).
Group VII Results: The presence of uridine phosphorylase in addition to nucleoside hydrolase causes *P. putida* to be placed in a separate Group VII (Fig. 136) to the other pseudomonads. *P. putida* (Figs. 137-139) has the same salvage enzymes as the previous group of pseudomonads except that it contains an additional uridine degrading enzyme, uridine phosphorylase, not present in other members of Group VI. *P. putida* contains nucleoside phosphorylase (Fig. 138), cytosine deaminase (Fig. 138) and uridine phosphorylase (Fig. 139) but no cytidine deaminase (Fig. 139).

Group VII salvage enzymes included Cod, Nuh, Udp and (Upp).

Results for Group VIII: Four bacterial species were found to lack cytidine deaminase, cytosine deaminase, uridine hydrolase or nucleoside hydrolase and uridine phosphorylase (Fig. 140). They include *Acinetobacter calcoaceticus* (Figs. 141-142); *Moraxella catarrhalis* (Figs. 143-145); *Neisseria meningitidis* (Figs. 146-147); and *Neisseria subflava* (Figs. 158-149). All four contain uracil phosphoribosyltransferase but no uridine kinase.

The only Group VIII salvage enzyme was Upp.

Group IX Results: The presence of an additional uridine degrading enzyme, uridine hydrolase, was found in the Group IX enzymes, which included uridine phosphorylase and cytidine deaminase (Fig. 150). *Clostridium perfringens* is the only member of Group IX (Figs. 151-153).

Group IX salvage enzymes are Cdd, Udp, Udh and (Upp, Udk).

These nine groups are treated in more detail in the Discussion.

Transformation of *Pseudomonas aeruginosa* with *E. coli* udk gene.

As a genus, *Pseudomonas* is devoid of nucleoside kinases including uridine/cytidine kinase. As a consequence of this, *Pseudomonas aeruginosa* is resistant to all 5-fluoroanalogs except 5-fluorouracil. However, it is not known whether resistance to 5-fluorouridine is due solely to the lack of uridine kinase or due to the lack of nucleoside
permease. To answer this question, a functional uridine kinase gene from *E. coli* was cloned into plasmid pDEB1 and transferred into a *P. aeruginosa* Upp<sup>-</sup> strain which had been selected by its resistance to 5-fluorouracil. An *E. coli* strain with similar mutant blocks as *P. aeruginosa*, namely *upp*, *cdd* and *udk* was used as a control into which the *udk* gene was also transferred as described in the Methods. Prior to the transformation, both the *E. coli* *udk upp* and the *P. aeruginosa* *udk upp* were resistant to 5-fluorouridine. After transforming each strain with pDEB1, both the *P. aeruginosa* and the *E. coli* strains, became sensitive to 5-fluorouridine indicating that a functional uridine kinase was present in both organisms. When fluorocytidine was used, only the *E. coli* strain was sensitive, the *P. aeruginosa* strain being completely resistant.
Fig. 8. Pyrimidine salvage pathway in Group I. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. uridine hydrolase (Udh), 4. cytosine deaminase (Cod), 5. cytidine deaminase (Cdd), 6. uridine kinase (Udk), 7. 5'-nucleotidase, 8. CMP glycosylase, 9. CMP kinase (Cmk).
Fig. 9. HPLC chromatograms of *Escherichia coli* ATCC 25922 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 10. HPLC chromatograms of *Escherichia coli* ATCC 25922 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 11. HPLC chromatograms of *Escherichia coli* ATCC 25922 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 12. HPLC chromatograms of *Escherichia coli* K12 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 13. HPLC chromatograms of *Escherichia coli* K12 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 14. HPLC chromatograms of *Escherichia coli* K12 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 15. HPLC chromatograms of Escherichia coli Φ5110 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 16. HPLC chromatograms of *Escherichia coli* SF5110 nucleotide glycosylase assay. (a) Assay using undialyzed cell extract and cytidine substrate. (b) Assay using dialyzed cell extract and cytidine indicating presence of nucleotide glycosylase.
Fig. 17. HPLC chromatograms of *Citrobacter freundii* ATCC 8090 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 18 HPLC chromatograms of *Citrobacter freundii* ATCC 8090 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 19. HPLC chromatograms of *Citrobacter freundii* ATCC 8090 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 20. HPLC chromatograms of *Enterobacter cloacae* ATCC 13047 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 21. HPLC chromatograms of *Enterobacter cloacae* ATCC 13047 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 22. HPLC chromatograms of *Enterobacter cloacae* ATCC 13047 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 23. HPLC chromatograms of *Hafnia alvei* ATCC 13337 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 24. HPLC chromatograms of *Hafnia alvei* ATCC 13337 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 25. HPLC chromatograms of *Hafnia alvei* ATCC 13337 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 26. HPLC chromatograms of Klebsiella pneumoniae ATCC 13883 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 27. HPLC chromatograms of *Klebsiella pneumoniae* ATCC 13883 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 28. HPLC chromatograms of *Klebsiella pneumoniae* ATCC 13883 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 29. HPLC chromatograms of *Proteus mirabilis* ATCC 7002 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 30. HPLC chromatograms of *Proteus mirabilis* ATCC 7002 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 31. HPLC chromatograms of *Proteus mirabilis* ATCC 7002 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 32. HPLC chromatograms of *Salmonella typhimurium* LT2 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 33. HPLC chromatograms of *Salmonella typhimurium* LT2 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 34. HPLC chromatograms of *Salmonella typhimurium* LT2 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 35. HPLC chromatograms of *Serratia odorifera* ATCC 33077 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 36. HPLC chromatograms of *Serratia odorifera* ATCC 33077 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 37. HPLC chromatograms of *Serratia odorifera* ATCC 33077 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 38. HPLC chromatograms of *Yersinia enterocolitica* ATCC 23715 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 39. HPLC chromatograms of *Yersinia enterocolitica* ATCC 23715 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 40. HPLC chromatograms of *Yersinia enterocolitica* ATCC 23715 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 41. HPLC chromatograms of *Shewanella putrefaciens* ATCC 8071 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 42. HPLC chromatograms of *Shewanella putrefaciens* ATCC 8071 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 43. HPLC chromatograms of *Shewanella putrefaciens* ATCC 8071 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 44. HPLC chromatograms of *Vibrio alginolyticus* ATCC 17749 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 45. HPLC chromatograms of *Vibrio alginolyticus* ATCC 17749 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 46. HPLC chromatograms of *Vibrio alginolyticus* ATCC 17749 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 47. HPLC chromatograms of *Vibrio parahaemolyticus* ATCC 17802 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 48. HPLC chromatograms of *Vibrio parahaemolyticus* ATCC 17802 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 49. HPLC chromatograms of *Vibrio parahaemolyticus* ATCC 17802 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 50. HPLC chromatograms of *Enterococcus faecalis* ATCC 29212 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 5.1. HPLC chromatograms of *Enterococcus faecalis* ATCC 29212 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 52. HPLC chromatograms of *Enterococcus faecalis* ATCC 29212 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 53. Pyrimidine salvage pathway in Group II. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. cytosine deaminase (Cod), 4. cytidine deaminase (Cdd), 5. uridine kinase (Udk), 6. 5'-nucleotidase, 7. CMP glycosylase, 8. CMP kinase (Cmk)
Fig. 54. HPLC chromatograms of *Burkholderia cepacia* ATCC 25416 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 55. HPLC chromatograms of *Burkholderia cepacia* ATCC 25416 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 56. HPLC chromatograms of *Burkholderia cepacia* ATCC 25416 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 57. HPLC chromatograms of *Haemophilus influenzae* ATCC 13337 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 58. HPLC chromatograms of *Haemophilus influenzae* ATCC 13337 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 59. HPLC chromatograms of *Haemophilus influenzae* ATCC 13337 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 60. HPLC chromatograms of *Mycobacterium smegmatis* controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 61. HPLC chromatograms of *Mycobacterium smegmatis* cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 62. HPLC chromatograms of *Mycobacterium smegmatis* uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
**Fig. 63.** HPLC chromatograms of *Proteus vulgaris* ATCC 13315 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 64. HPLC chromatograms of *Proteus vulgaris* ATCC 13315 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 65. HPLC chromatograms of *Proteus vulgaris* ATCC 13315 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 66. HPLC chromatograms of *Pseudomonas mendocina* ATCC 10541 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 67. HPLC chromatograms of *Pseudomonas mendocina* ATCC 10541 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 68. HPLC chromatograms of *Pseudomonas mendocina* ATCC 10541 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 69. HPLC chromatograms of *Pseudomonas pseudoalcaligenes* ATCC 17440 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 70. HPLC chromatograms of *Pseudomonas pseudoalcaligenes* ATCC 17440
cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay.
(b) Positive cytosine deaminase assay.
Fig. 71. HPLC chromatograms of *Pseudomonas pseudoalcaligenes* ATCC 17440 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 72. HPLC chromatograms of *Pseudomonas stutzeri* ATCC 11607 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 73. HPLC chromatograms of Pseudomonas stutzeri ATCC 11607 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 74. HPLC chromatograms of *Pseudomonas stutzeri* ATCC 11607 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 75. HPLC chromatograms of *Rhizobium loti* ATCC 33669 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 76. HPLC chromatograms of *Rhizobium loti* ATCC 33669 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 77. HPLC chromatograms of *Rhizobium loti* ATCC 33669 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 78. Pyrimidine salvage pathway in Group III. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. cytosine deaminase (Cod), 4. cytidine deaminase (Cdd), 5. uridine kinase (Udk), 6. 5'-nucleotidase, 7. CMP glycosylase, 8. CMP kinase (Cmk).
Fig. 79. HPLC chromatograms of *Saccharomyces cerevisiae* controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 80. HPLC chromatograms of *Saccharomyces cerevisiae* cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 81. HPLC chromatograms of *Saccharomyces cerevisiae* uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 82. HPLC chromatograms of *Pseudomonas indigofera* ATCC 19706 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 83. HPLC chromatograms of *Pseudomonas indigofera* ATCC 19706 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 84. HPLC chromatograms of *Pseudomonas indigofera* ATCC 19706 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 85. Pyrimidine salvage pathway in Group IV. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. cytidine deaminase (Cdd), 4. uridine kinase (Udk), 5. 5'-nucleotidase, 6. CMP kinase (Cmk)
Fig. 86. HPLC chromatograms of *Bacillus megaterium* controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 87. HPLC chromatograms of *Bacillus megaterium* cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Negative cytosine deaminase assay.
Fig. 88. HPLC chromatograms of *Bacillus megaterium* uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 89. HPLC chromatograms of Bacteroides fragilis ATCC 25285 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 90. HPLC chromatograms of *Bacteroides fragilis* ATCC 25285 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Negative cytosine deaminase assay.
Fig. 91. HPLC chromatograms of *Bacteroides fragilis* ATCC 25285 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 92. HPLC chromatograms of *Micrococcus luteus* ATCC 49732 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 93. HPLC chromatograms of *Micrococcus luteus* ATCC 49732 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Negative cytosine deaminase assay.
Fig. 94. HPLC chromatograms of Micrococcus luteus ATCC 49732 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 95. Pyrimidine salvage pathway in Group V. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. cytidine deaminase (Cdd), 4. uridine kinase (Udk), 5. 5'-nucleotidase, 6. CMP kinase (Cmk)
Fig. 96. HPLC chromatograms of *Staphylococcus aureus* ATCC 29213 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 97. HPLC chromatograms of *Staphylococcus aureus* ATCC 29213 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Negative cytosine deaminase assay.
Fig. 98. HPLC chromatograms of *Staphylococcus aureus* ATCC 29213 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Negative uridine phosphorylase assay
**RNA**  
\[ \text{CTP} \leftrightarrow \text{UTP} \]

**mRNA**  
\[ \text{CDP} \]

**UDP**  
\[ \text{mRNA} \]

**CMP**  
\[ \text{CR} \]

**UMP**  
\[ \text{UR} \]

**C**  
\[ \text{U} \]

\[ \text{H}_2\text{O} \]

**Ribose-5-\( ^\text{P} \)**

1. Uracil phosphoribosyltransferase (Upp)
2. Uridine hydrolase (Udh)
3. Cytosine deaminase (Cod)
4. 5'-nucleotidase
5. CMP glycosylase
6. CMP kinase (Cmk)

*Fig. 99.* Pyrimidine salvage pathway in Group VI. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. cytosine deaminase (Cod), 4. 5'-nucleotidase, 5. CMP glycosylase, 6. CMP kinase (Cmk)
Fig. 100. HPLC chromatograms of *Pseudomonas aeruginosa* PAO1 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 101. HPLC chromatograms of *Pseudomonas aeruginosa* PAO1 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 102. HPLC chromatograms of *Pseudomonas aeruginosa* PAO1 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 103. HPLC chromatograms of *Pseudomonas aeruginosa* ATCC 10145 controls.

(a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 104. HPLC chromatograms of *Pseudomonas aeruginosa* ATCC 10145 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 105. HPLC chromatograms of *Pseudomonas aeruginosa* ATCC 10145 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 106. HPLC chromatograms of *Pseudomonas aeruginosa* ATCC 27853 controls.

(a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 107. HPLC chromatograms of *Pseudomonas aeruginosa* ATCC 27853 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 108. HPLC chromatograms of *Pseudomonas aeruginosa* ATCC 27853 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 109. HPLC chromatograms of *Pseudomonas aureofaciens* ATCC 13985 controls.

(a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 110. HPLC chromatograms of *Pseudomonas aureofaciens* ATCC 13985 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. III. HPLC chromatograms of *Pseudomonas aureofaciens* ATCC 13985 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 112. HPLC chromatograms of *Brevundimonas diminuta* ATCC 11568 controls.

(a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 113. HPLC chromatograms of *Brevundimonas diminuta* ATCC 11568 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 114. HPLC chromatograms of *Brevundimonas diminuta* ATCC 11568 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 115. HPLC chromatograms of *Pseudomonas fluorescens* CW1013 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 116. HPLC chromatograms of *Pseudomonas fluorescens* CW1013 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 117. HPLC chromatograms of *Pseudomonas fluorescens* CW1013 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 118. HPLC chromatograms of *Alcaligenes faecalis* ATCC 8750 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 119. HPLC chromatograms of *Alcaligenes faecalis* ATCC 8750 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 120. HPLC chromatograms of *Alcaligenes faecalis* ATCC 8750 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 121. HPLC chromatograms of *Comamonas acidovorans* ATCC 15668 controls.

(a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 122. HPLC chromatograms of *Comamonas acidovorans* ATCC 15668 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 123. HPLC chromatograms of *Comamonas acidovorans* ATCC 15668 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 124. HPLC chromatograms of *Comamonas testosteroni* ATCC 11996 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 125. HPLC chromatograms of *Comamonas testosteroni* ATCC 11996 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 126. HPLC chromatograms of *Comamonas testosteroni* ATCC 11996 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 127. HPLC chromatograms of *Rhizobium leguminosarum* Biovar *trifolii* ATCC 14479 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 128. HPLC chromatograms of *Rhizobium leguminosarum* Biovar *trifolii* ATCC 14479 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 129. HPLC chromatograms of *Rhizobium leguminosarum* Biovar *trifolii* ATCC 14479 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 130. HPLC chromatograms of *Rhizobium meliloti* ATCC-19930 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 131. HPLC chromatograms of *Rhizobium meliloti* ATCC-19930 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 132. HPLC chromatograms of *Rhizobium meliloti* ATCC-19930 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 133. HPLC chromatograms of *Stenotrophomonas maltophilia* ATCC 13637 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 134. HPLC chromatograms of *Stenotrophomonas maltophilia* ATCC 13637 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 135. HPLC chromatograms of *Stenotrophomonas maltophilia* ATCC 13637 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 136. Pyrimidine salvage pathway in Group VII. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. uridine hydrolase (Udh), 4. cytosine deaminase (Cod), 5. 5'-nucleotidase, 6. CMP glycosylase, 7. CMP kinase (Cmk)
Fig. 137. HPLC chromatograms of *Pseudomonas putida* PRS2000 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 138. HPLC chromatograms of *Pseudomonas putida* PRS2000 nucleoside hydrolase and cytosine deaminase assays. (a) Positive nucleoside hydrolase assay and negative cytidine deaminase assay. (b) Positive cytosine deaminase assay.
Fig. 139. HPLC chromatograms of *Pseudomonas putida* PRS2000 uridine hydrolase and uridine phosphorylase assays. (a) Positive nucleoside hydrolase assay. (b) Positive uridine phosphorylase assay.
Fig. 140. Pyrimidine salvage pathway in Group VIII. Enzymes are 1. uracil phosphoribosyltransferase (Upp) and 2. CMP kinase (Cmk)
Fig. 141. HPLC chromatograms of *Acinetobacter baumannii* ATCC 19606 cytosine deaminase assay and control assay. (a) Negative cytosine deaminase assay. (b) Undialyzed cell extract.
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Fig. 144. HPLC chromatograms of *Moraxella catarrhalis* ATCC 25296 cytidine deaminase and cytosine deaminase assays. (a) Cytidine deaminase assay. (b) Cytosine deaminase assay.
Fig. 145. HPLC chromatograms of *Moraxella catarrhalis* ATCC 25296 19606 uridine hydrolase and uridine phosphorylase assays. (a) Negative uridine hydrolase assay. (b) Negative uridine phosphorylase assay.
Fig. 146. HPLC chromatograms of Neisseria meningitidis ATCC 13077 cytosine deaminase assay and control assay. (a) Negative cytosine deaminase assay. (b) Undialyzed cell extract.
Fig. 147. HPLC chromatograms of *Neisseria meningitidis* ATCC 13077 uridine hydrolase and cytidine deaminase assays. (a) Negative uridine hydrolase assay and negative uridine phosphorylase assay. (b) Negative cytidine deaminase assay.
**Fig. 148.** HPLC chromatograms of *Neisseria subflava* ATCC 49275 cytosine deaminase assay and control assay. (a) Negative cytosine deaminase assay. (b) Undialyzed cell extract.
**Fig. 149.** HPLC chromatograms of *Neisseria subflava* ATCC 49275 uridine hydrolase and cytidine deaminase assays. (a) Negative uridine hydrolase assay and negative uridine phosphorylase assay. (b) Negative cytidine deaminase assay.
Fig. 150. Pyrimidine salvage pathway in Group IX. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. uridine hydrolase (Udh), 4. cytidine deaminase (Cdd), 5. uridine kinase (Udk), 6. 5'-nucleotidase, 7. CMP kinase (Cmk)
Fig. 151. HPLC chromatograms of *Clostridium perfringens* ATCC 13124 controls. (a) Dialyzed cell extract. (b) Undialyzed cell extract.
Fig. 152. HPLC chromatograms of *Clostridium perfringens* ATCC 13124 cytidine deaminase and cytosine deaminase assays. (a) Positive cytidine deaminase assay. (b) Negative cytosine deaminase assay.
Fig. 153. HPLC chromatograms of *Clostridium perfringens* ATCC 13124 uridine hydrolase and uridine phosphorylase assays. (a) Positive uridine hydrolase assay. (b) Positive uridine phosphorylase assay.
When is a labyrinth not a labyrinth? When the correct turns within it are no longer a puzzle... then the maze of pathways simply becomes a highway, a well-traveled road. For the Minotaur within the Cretan labyrinth, his lair was never a maze, but a way to entrap food. It was also his home. To the microorganism, and other organisms as well, the pyrimidine salvage pathway is not really a conundrum. It is just a way to make DNA and RNA. The organism does not care if it has one enzyme or 20 to make its salvage pathway... what does matter is that the enzymes can be used as guideposts by "outsiders" who wonder at the complexity of even one-celled organisms. If it is possible to block a pathway and to kill the monster within before it kills us, whether Minotaur or microorganism, then it is important to know the beast within the maze.

In this work, I studied the pyrimidine salvage enzyme activities using HPLC in cell extracts of 55 microorganisms, 44 of which were prototrophs with the remainder being mutant strains of both *E. coli* and *Salmonella typhimurium*. Each mutant organism contained a complete block in a particular step of the pyrimidine salvage pathway and was important in establishing baseline standards for the HPLC assays. All assays were analyses of the enzymes *in vitro* with the presence or absence of the selected enzymes being judged against the standards. In a recent study on mollicutes, McElwain and colleagues (1988) stated that enzyme reactions using crude cell extracts are "to be viewed with great reservation... [since] the assays may not be sufficiently sensitive... to detect activity because they lack necessary... cofactors or are inappropriately incubated...". It is
further postulated that enzyme activity may also be lost due to extraction methods or by the fact that some reactions may have to compete with stronger enzyme reactions. I believe that I have alleviated many if not all of these negative statements regarding the use of enzyme assays *in vitro*. The HPLC assay is very sensitive and measures nanomolar concentrations of nucleosides and bases in the cell extract (Dutta *et al.*, 1990). The results of all my assays are to be seen in Table 2 and are meant to show conclusively that an enzyme is present or absent in the cell. No absolute quantitative measurements are represented by the chromatograms though peak height or areas under the curve are indicated on each chromatogram. Results were mostly in accord with the findings of others where relevant, but not all organisms had been previously examined.

For clarity, the different enzyme pathways have been arbitrarily divided into nine different salvage pathway schemes based on the combination of salvage enzymes found in the organisms of each group. Table 2 summarizes the results of my research by group.

Pyrimidine recycling (salvage) pathways are ubiquitous in prokaryotes. The salvage enzymes and their activities are generally consistent for *bona fide* members of a genus and thus may be used as a taxonomic marker. Salvage schemes are particularly useful for phylogenetically recalcitrant organisms that straddle generic lines. Recently, *Burkholderia cepacia* was reclassified from the genus *Pseudomonas*. Yabuuchi *et al.*, (1992) proposed that the new genus, *Burkholderia*, be established for *Pseudomonas cepacia* and other members of the RNA homology group II.

Oyaizu and Kamagata (1983) grouped the diverse pseudomonads on the basis of the quinone system and cellular fatty acids, as did Stead (1992). Palleroni and colleagues (1973) established a different basis for distinguishing the members of the genus, *Pseudomonas*. They divided the genus into five groups based on ribosomal DNA (rDNA) homology. rDNA is more conserved than the rest of the genomic DNA. The RNA homology groups developed by Palleroni *et al.*, (1973) based on 16S rRNA and DNA-
DNA homology among species (Dams et al., 1983; Li et al., 1993) are listed below and include certain organisms in my research:

rRNA homology group I:

*Pseudomonas aeruginosa*

*Pseudomonas fluorescens*

*Pseudomonas putida*

*Pseudomonas stutzeri*

*Pseudomonas mendocina*

*Pseudomonas pseudoalcaligenes*

rRNA homology group II:

*Pseudomonas* (now *Burkholderia*) *cepacia*

rRNA homology group III:

*Pseudomonas* (now *Comamonas*) *acidovorans*

*Pseudomonas* (now *Comamonas*) *testosteroni*

rRNA homology group IV:

*Pseudomonas* (now *Brevundimonas*) *diminuta*

rRNA homology group V:

*Pseudomonas* (now *Stenotrophomonas*) *maltophilia*

Whereas Palleroni was able to use five rRNA homology groups for the *Pseudomonas* organisms, I have chosen to place the 44 organisms I studied into nine groups based on small but highly selective differences in the pathways.
As can be seen from Table 2, Group I includes nine members of the family Enterobacteriaceae. Also included are *Shewanella putrefaciens* (a former *Pseudomonas*), two Vibrionaceae family species which are closely related to the enterics, and *Enterococcus faecalis*.

The finding of *Shewanella putrefaciens* (formerly *Pseudomonas putrefaciens*) among Group I members was unexpected. Clearly, its repertoire of enzymes, including cytidine deaminase (Cdd), cytosine deaminase (Cod), uridine phosphorylase (Udp), uridine hydrolase (Udh), uracil phosphoribosyltransferase (Upp), and uridine/cytidine kinase (Udk) differs significantly from those of the true *Pseudomonas* such as *P. aeruginosa* and other members as classified in rRNA homology group I (Palleroni *et al.*, 1973; Johnson & Palleroni, 1989). These are to be found in my scheme under Group VI. The inclusion of the two vibrio species was not surprising because of their close familial relationship to the Enterobacteriaceae.

Because it shares many characteristics of its co-inhabitants in the large intestine, *Enterococcus faecalis* is seen to behave as a *bona fide* enteric bacterium with regard to its pyrimidine salvage enzymes. These characteristics include the following: (a) it is a facultative anaerobe; (b) it has a fermentative metabolism (sucrose, lactose, glycerol, sorbitol, mannitol); (c) it grows between 10°C-45°C; (d) it is a member of the normal intestinal flora in human beings (Holt, *et al.*, 1994); and (e) it contains all Group I salvage enzymes, Cdd, Cod, Udp, Udh, Upp, and Udk.

Its inclusion strengthens the premise that salvage enzymes can be used as pertinent taxonomic markers particularly with regard to ecological niches.

As can be seen from Table 2, Group II is very similar to Group I except that it lacks the enzyme uridine hydrolase (Udh). At first, I placed members of Group I and Group II in the same large group since only subtle differences existed between them. However, after careful consideration, due to the unique nature of the uridine hydrolase, I made two
separate groups as is now shown. Group II contains eight diverse members. These include *Burkholderia cepacia*, *Haemophilus influenzae*, *Mycobacterium smegmatis*, *Proteus vulgaris*, three *Pseudomonas* species and *Rhizobium loti*. Each contains the salvage enzymes Cdd, Cod, Udp, Upp and Udk. At first glance the finding of *Burkholderia cepacia* and three other rRNA homology group I members in Group II should be surprising. Despite the avowed catabolic versatility of *Burkholderia cepacia* and the three other pseudomonads (Stanier, *et al*., 1966), I found that they lack the salvage enzyme scheme seen for *Pseudomonas aeruginosa* and other "true" members. Many former *Pseudomonas* species have been renamed in the past five years (Yabuuchi, *et al*., 1992) with the ultimate plan being to retain only three species, namely, *P. aeruginosa*, *P. putida* and *P. fluorescens* as true *Pseudomonas* members. I concur based on some of my research.

*Proteus vulgaris* is the only enteric bacterium examined that differed from the Group I enterics. It is placed in Group II because it lacks uridine hydrolase (Udh). This placement is vindicated by the finding that *Proteus vulgaris* also contains tryptophanase and produces acid in maltose and sucrose fermentations while *Proteus mirabilis* lacks tryptophanase and does not produce acid (Holt *et al*., 1994). It is clearly different from *Proteus mirabilis*.

One very different member of this group, *Rhizobium loti*, is a pleomorphic Gram negative rod that fixes nitrogen in symbiosis with leguminous plants. This is a contrast to the salvage enzymes from two other *Rhizobium* species, *R. leguminosarum* and *R. meliloti* which I have shown to contain Group VI enzymes.

I found that salvage Group III had two organisms, (Table 2) one the eukaryotic microorganism, *Saccharomyces cerevisiae* and a prokaryote, *Pseudomonas indigofera*. Group III is similar to Group I in that the Group III salvage lacks a uridine phosphorylase, but has all the other Group I salvage enzymes. Jund & Lacloute (1970) discovered the
yeast had Cdd, Cod and Udh. The rRNA homology of *P. indigofera* has not been examined and thus it has not been assigned to one of the rRNA homology groups. The presence of Udk was first described by Kern (1990) in *Saccharomyces*.

Among the Gram positive organisms containing the salvage enzymes Cdd, Udk, Udp (known as Pdp) but no Cod, and constitute Group IV are members of the aerobic endospore-forming rods, *Bacillus megaterium*. Other members with Cdd and Udp activity (but no Cod or Udh) are the Gram positive coccus, *Micrococcus luteus* and a microaerophilic Gram negative rod, *Bacteroides fragilis*. *B. subtilis* salvage enzymes have been characterized by Rima & Takahashi, (1977) who discovered Cdd; the Udk was found by Fucik, *et al.*, (1974). The Pdp (Udp activity) in *B. subtilis* was found by Rumyantseva, *et al.*, (1979) and the absence of Cod was substantiated by Waleh & Ingraham, (1976).

In addition to the prokaryotes listed, the eukaryotic organism, *Homo sapiens* also has Group IV salvage.

*Staphylococcus aureus*, the Gram positive coccus, is the only member thus far which has the fifth group of salvage enzymes, Cdd, Udh, Udk and Upp. Like organisms in the previous four groups, *S. aureus* contains a Cdd but differs in that it has only a Udh.

Group VI as mentioned previously houses many pseudomonads, both true *Pseudomonas* species and also former pseudomonads. *Comamonas acidovorans*, *C. testosteroni*, *Brevundimonas diminuta*, *Alcaligenes faecalis* and *Stenotrophomonas maltophilia* are included. These organisms have Cod, Nuh and Upp, but no Cdd or Udp or Udk. Other organisms with Group VI enzymes are *Rhizobium leguminosarum* and *R. meliloti*.

The organisms, *Acinetobacter baumannii*, *Neisseria meningitidis*, *N. subflava* and *Moraxella catarrhalis* (formerly *Neisseria*) use only one salvage enzyme, uracil phosphoribosyltransferase (Upp). These organisms constitute Group VIII. Perhaps the
fact that these organisms are able to utilize natural transformation in taking up DNA may have some bearing on their lack of salvage enzymes. Similar results were obtained by Jyssum (1971) for *Neisseria meningitidis* (Jyssum & Jyssum, 1979) and also for *Moraxella* and *Acinetobacter* (Jyssum & Øvrø, 1974; Jyssum & Bøvre, 1974).

The last group, number IX has but one organism, the obligate anaerobic Gram positive rod, *Clostridium perfringens*. This organism combines the enzymes from Group IV and V salvage, possessing Cdd, Udh, Udp, Upp and Udk enzymes. Other *Clostridium* species are likely to find inclusion in Group IX.

The nine groups chosen by me are not meant to be all inclusive. The literature contains many partial studies on individual enzymes but there are few reports of salvage *in toto* for any organisms.

One comprehensive study by Hammer and her coworkers (Martinussen et al., 1994; Martinussen & Hammer, 1995) concerned *Lactococcus lactis* and other members of the lactic acid bacteria. They found that *L. lactis* had Cdd, Udk, Pdp (Udp activity, but has activity for both uridine and thymidine) and Upp. Kalckar (1951) and Uerkvitz (1971) found that some lactobacilli require purine and pyrimidine bases for growth. *Lactobacillus delbruckii, Lactobacillus helveticus, Lactobacillus leichmanii* and *Lactobacillus acidophilus* (Durham & Ives, 1971) cannot metabolize deoxyribonucleosides (contain no nucleoside phosphorylases or hydrolases, DeoA). Another lactic acid bacterium, *Lactobacillus casei* contains Udp and thymidine phosphorylase (Tpp, DeoA) (Avraham, *et al.*, 1988). In 1951, Kalckar showed that it had no growth requirement for deoxyribonucleosides, was devoid of trans-N deoxyribosylases and Nuh, but contained Udp.

*L. pentosus* contained separate hydrolase enzymes specific for pyrimidine nucleosides or purine nucleosides (Nuh) (Lampen & Wang, 1952).

An interesting difference in the lactobacilli and other bacteria is that instead of the usual ribonucleoside diphosphate reductase which catalyzes the synthesis of all four
deoxyribonucleoside diphosphates from their corresponding ribonucleoside diphosphates (Stubbe & Kozarich, 1980; Stubbe & Ackles, 1980), the lactobacilli have ribonucleoside triphosphate reductase (Thelander & Reichard, 1979; Babior, 1975). The ribonucleoside triphosphate reductase (cobalamine dependent) catalyzes the synthesis of deoxyribonucleoside triphosphates from their corresponding ribosomal triphosphates.

In another lactic acid bacterium, Streptococcus hemolyticus was discovered a nucleoside phosphorylase (Udp) but no nucleoside hydrolase (Lees & Jago, 1977; Taketo & Taketo, 1974).

Because of the diverse nature of the lactic acid bacteria a complete study of their salvage enzymes would be beneficial.

In research by Auling & Moss (1984; Auling, et al., 1982) on the coryneform bacterium, Brevibacterium ammoniagenes, they found that the organism had no Cod or Cdd but does have Upp, Udh and Udp. This would place Brevibacterium in a tenth salvage group.

Other pyrimidine salvage work has been done by other laboratories. In human beings, Camiener & Smith (1965) found that Cdd activity was highest in the liver after testing for the salvage enzyme activity in liver, kidney, heart and muscle. Cdd has also been found in human leukocytes (Silber, 1967; Teng, 1975) and in white blood cells (granulocytes) but little or no activity for Cdd was found in red blood cells (RBCs) (Chabner et al., 1974). Cdd has also been found in human lymphocytes (Abell & Marchand, 1973) and in spleen tissue (Malathi & Silber, 1971).

Staub (1994) states that three tissue sources are remarkable in their use of salvage in the human body: (1) monocytes or macrophages; (2) polymorphonuclear leucocytes; and (3) the central nervous system. The pyrimidines are scavenged mainly as nucleosides whereas the purines are predominantly salvaged as free bases.
There is indirect evidence that uridine phosphorylase is present in human beings, as well as uracil phosphoribosyltransferase from the use of 5-fluoropyrimidine analogs and their conversion to fluoronucleotides. Pinedo and Peters (1988) describe the metabolism of 5'-fluorouracil as having two pathways to FUMP: $5FU + \text{PRPP} \rightarrow 5FUMP + \text{PPi}$, with Upp responsible for the conversion of FU to FUMP as in bacteria. In the second pathway, the reaction proceeds in two steps which utilize uridine phosphorylase and uridine kinase as seen in the following reaction:

$$5FU \xrightarrow{5FU} 5\text{FUR} \xrightarrow{5\text{FUMP}} 5\text{FUMP}.$$ The 5-fluoropyrimidines are metabolized by the normal cellular enzymes which handle the natural pyrimidines. These fluoropyrimidines, though highly toxic, have been used for some time as a defense against certain cancers (breast, colorectal, head and neck). Their toxicity can be limited to a certain extent by feeding uridine intravenously upon and after administration of the 5'-fluorouracil as an antitumor drug (van Groeningen et al., 1989). That these pyrimidine analog drugs are more toxic to tumors is because the tumor cells exhibit a higher metabolic activity than normal cells and thus incorporate the toxic pyrimidines at a faster rate. This has been described as a pyrimidine metabolic imbalance in cancer cells by Weber (1993). Weber compared the activity of pyrimidine biosynthetic and salvage enzymes of normal liver cells with those of rapidly growing hepatoma cells. Some of his findings shown below were dramatic for selected enzymes:
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Normal liver</th>
<th>Hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Activity</td>
<td>% normal liver</td>
</tr>
<tr>
<td>Ribonucleotide reductase</td>
<td>23</td>
<td>18,348</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>56</td>
<td>5,806</td>
</tr>
<tr>
<td>dTMP kinase</td>
<td>420</td>
<td>7,000</td>
</tr>
<tr>
<td>Uracil phosphoribosyltransferase</td>
<td>19,000</td>
<td>760</td>
</tr>
<tr>
<td>Uridine phosphorylase</td>
<td>164,000</td>
<td>671</td>
</tr>
<tr>
<td>Aspartate carbamoyl-transferase (ATCase)</td>
<td>448,000</td>
<td>706</td>
</tr>
</tbody>
</table>

Accordingly, toxic fluoro- analogs are taken up more quickly by the tumor cells - making it a race between the cancer and normal cells for the uptake of the toxic pyrimidines and their incorporation into RNA and DNA. Sometimes, the tumor cells "win" and are killed before the fluoro- compounds become too toxic for the patient.

Another type of analog is 6-azathymidine which inhibits thymidine kinase: (TdR → TdR → dTMP) in humans. The 6-azathymidine is used in conjunction with methotrexate drugs (impair thymidylate synthase: dUMP → dTMP) to achieve synergistic cytotoxicity in cancer cells by blocking both the biosynthetic pathway (ThyA) and the salvage pathway (TK) to the cancer cells (Weber, 1993).

As mentioned above, the toxicity of the 5-fluorouracil (FU) may be allayed by the administration of uridine concurrently with the FU treatment. Because of the high activity of uridine kinase in humans, the uridine competes with the FU for incorporation into RNA and relieves some of the toxic effects of the analog. The side effect of excessive doses of uridine is a high fever. Uridine has also been used as an anxiolytic drug (Connolly, 1994) in the treatment of depression.
The importance of the salvage pathway in human beings can also be seen when mutations have occurred in the biosynthetic and degradative pathways. When a mutation occurs in the bifunctional UMP synthase, catalyzing OA → OMP → UMP, orotic aciduria results. Orotic aciduria is the result of orotic acid buildup due to the inability of the organism to process orotate. The symptoms result in large concentrations of orotic acid in the urine, severe anemia and retardation of growth (Jones, 1980). Upon administration of cytidine and/or uridine to patients with this hereditary disorder, the human salvage enzymes, cytidine deaminase and uridine kinase are induced to produce the vital pyrimidine nucleotides for normal growth and development of the organism. An additional benefit of being able to feed uridine to alleviate the blocked biosynthetic pathway is that uridine nucleotides feedback inhibit the first step in the pyrimidine biosynthetic pathway. This stops the production of orotic acid and alleviates excess orotic acid in the urine, one symptom of the disease (Voet & Voet, 1990).

The condition of orotic aciduria in humans is analogous to a deficiency of uridine-5'-monophosphate synthase (DUMPS) in the Holstein cow where it is seen also as a heterozygous condition. Robinson and his colleagues at the University of Illinois have studied this genetic disorder in a herd of Holsteins that contain carriers of the disease. The homozygous recessive state is not found in full-term calves, since homozygotes are spontaneously aborted at 60 days of gestation. However, heterozygous cows experience none of the adverse symptoms of humans, but the condition is manifested by increased level of orotate in milk and urine, longer gestation periods and greater milk production (Robinson et al., 1983; Robinson et al., 1984; Harden & Robinson, 1987).

In one study by Zaharevitz et al., (1992) on the contributions of the pyrimidine de novo and salvage pathways in mouse tissues and tumors in vivo showed that the pathway de novo contributes the major portion of the uracil nucleotide pool in the mouse intestine, while salvage makes the larger contribution in the kidney, with salvage and de novo
synthesis contributing equally to the pools in the liver. In that same study of pyrimidine pools in tumors, (L1210 leukemia, P388 leukemia, B15 melanoma and Nettesheim lung carcinoma) the biosynthetic pathway was found to contribute five times more pyrimidine precursors than that of the salvage pathway. This is important when appropriate drugs are planned for use against tumors.

Although the pyrimidine salvage pathway is but a small part of an organism's metabolic activity, it is an essential part. No organism has been found which has no salvage enzymes. On the contrary, organisms exist which have no biosynthetic pyrimidine pathway. One of these is Chlamydia psittaci an obligate intracellular parasite (McClarty & Qin, 1993; Hatch, 1976).

Another organism without a pyrimidine biosynthetic pathway is Mycoplasma mycoides (Mitchell & Finch, 1977, 1979), a small Gram negative bacterium without a cell wall, known as a mollicute. The mollicutes, bacteria that are enclosed in a plasma membrane without a cell wall are divided into two groups. The first group contains facultative anaerobes and microaerophiles including the genera Mycoplasma, Acholeplasma, Spiroplasma and Ureaplasma. The second group of mollicutes contains obligate anaerobes including the genera Anaeroplasms and Asteroleplasina (Holt et al., 1994).

Upp was found in all mollicutes tested (Finch & Mitchell, 1992). Udp was found in all except Spiroplasma, which, like Mycoplasma, lacks the pyrimidine biosynthetic pathway (Pollack, et al., 1989). Udk was found in most Mycoplasma species as well as in Anaeroplasma intermedium and Spirolasma citri (Finch & Mitchell, 1992).

Studies of the mollicutes are very important because these organisms are implicated in so many human infections including Acquired Immune Deficiency Syndrome (AIDS), pneumonia, septicemia after renal transplantation, trauma, in brain abscesses and
osteomyelitis lesions (Taylor-Robinson, 1995). They are important, too, because these organisms escape killing by many antibiotics because of their lack of a cell wall.

Experiments with the mollicutes are also important because they bring new insights concerning the phylogeny and rapid evolution of organisms since there is a lack of conserved sequences in their 5S ribosomal RNA (McElwain et al., 1988). It is thought that the absence of dUTPase in all mollicutes (except Mycoplasma) may contribute to the mollicutes' ability to evolve so quickly (Williams & Pollack, 1988).

Recycling of RNA nucleotides is also an integral part of the salvage design. In most prokaryotes, RNA is broken down to CMP and UMP but in Bacillus, a polynucleotide phosphorylase (PNPase) rather than the more ubiquitous hydrolytic ribonuclease II (RNaseII) predominates in the breakdown of mRNA. In E. coli, 90% of its mRNA is degraded by the RNaseII (O'Donovan & Shanley, 1995). Whatever the degrading enzyme, its value cannot be underestimated in the cell's survival and in the regulation of gene expression.

As seen above, pyrimidine salvage is another method of obtaining phylogenetic answers. There are no organisms with which human beings come in closer contact than bacteria. Elucidation of the pyrimidine salvage pathways of these bacteria and our own, provides a more complete understanding of the microorganisms with which we share life. The groupings of enzymes may change as more is learned about each organism's pathway. Certain errors due to failure to induce an inducible enzyme, low enzyme concentration and even interfering enzymes may have occurred and will be corrected just as I have done in a few cases. Further analysis of these pyrimidine salvage enzymes' kinetics, size determination, constitution of subunits and sequence analysis is warranted and may be the object of subsequent research.

In some organisms their salvage pathways do, indeed, resemble a labyrinth, a complicated maze that ensures existence in a hostile microbial world. Other organisms
have hardly a beaten track to call a salvage pathway. Nonetheless, all organisms have
some semblance of a salvage and recycling pathway. They cannot live without one. I,
myself, have chosen a lesser known path of pyrimidine salvage:

"...I shall be telling this with a sigh

Somewhere ages and ages hence:

Two roads diverged in a wood, and I-

I took the one less traveled by,

And that has made all the difference."

(Frost, 1916)
Table 2. Summary of pyrimidine salvage enzyme assays.

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CHAPTER V

REFERENCES


Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K., & De Ley, J. (1992). Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax as Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax*


