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CHARACTERIZATION OF PYRIMIDINE BIOSYNTHESIS IN *ACINETOBACTER CALCOACETICUS* USING WILD TYPE AND MUTANT STRAINS

DISSERTATION

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Pyrimidine nucleotide biosynthesis was studied in *Acinetobacter calcoaceticus* ADP-1. Pyrimidine auxotrophic mutants were isolated and characterized for this purpose. One such Pyr$^-$ mutant, strain ADP-1-218 was chosen for further study. This pyrimidine auxotroph did not grow in minimal medium unless supplemented with a pyrimidine source. Uracil or uridine, but not cytidine, or cytosine, satisfied the pyrimidine requirement of strain ADP-1-218. The block in the pyrimidine biosynthetic pathway was in the third enzyme, namely dihydroorotase, which is encoded by the *pyrC* gene.

Repression and derepression studies were carried out on wild type and mutant strains. When wild type cells, grown in minimal succinate medium, were compared to the uracil grown cells, there was a 2.5 to 3.0-fold increase in aspartate transcarbamoylase (ATCase) specific activity over that found for the uracil grown cells. The mutant strain, starved for uracil for three hours, gave a 15-fold increase in ATCase specific activity when compared to the same strain

grown in uracil without starvation. Thus, *Acinetobacter* pyrimidine biosynthesis like that of *E. coli*, and unlike that of *Pseudomonas*, is regulated at the transcriptional level.

Pyrimidine nucleoside triphosphates were extracted from wild type and mutant strains with 6% TCA and quantified using HPLC. When the *pyrC* was starved for three hours for uracil the UTP and CTP pools were depleted to near zero within minutes. The UTP and CTP pools were restored to their near normal levels by subsequent growth in uracil supplemented minimal medium.

ATCase was partially purified from the derepressed mutant ADP-1-218. The enzyme was found to be like that of *Pseudomonas* with a M.W. of 480,000 Daltons, as seen in a 6% acrylamide acitivity gel. Cytidine and cytosine deaminase were missing in both wild type and mutant *Acinetobacter*. This is in keeping with the findings that cytidine and cytosine did not satisfy the pyrimidine requirement.

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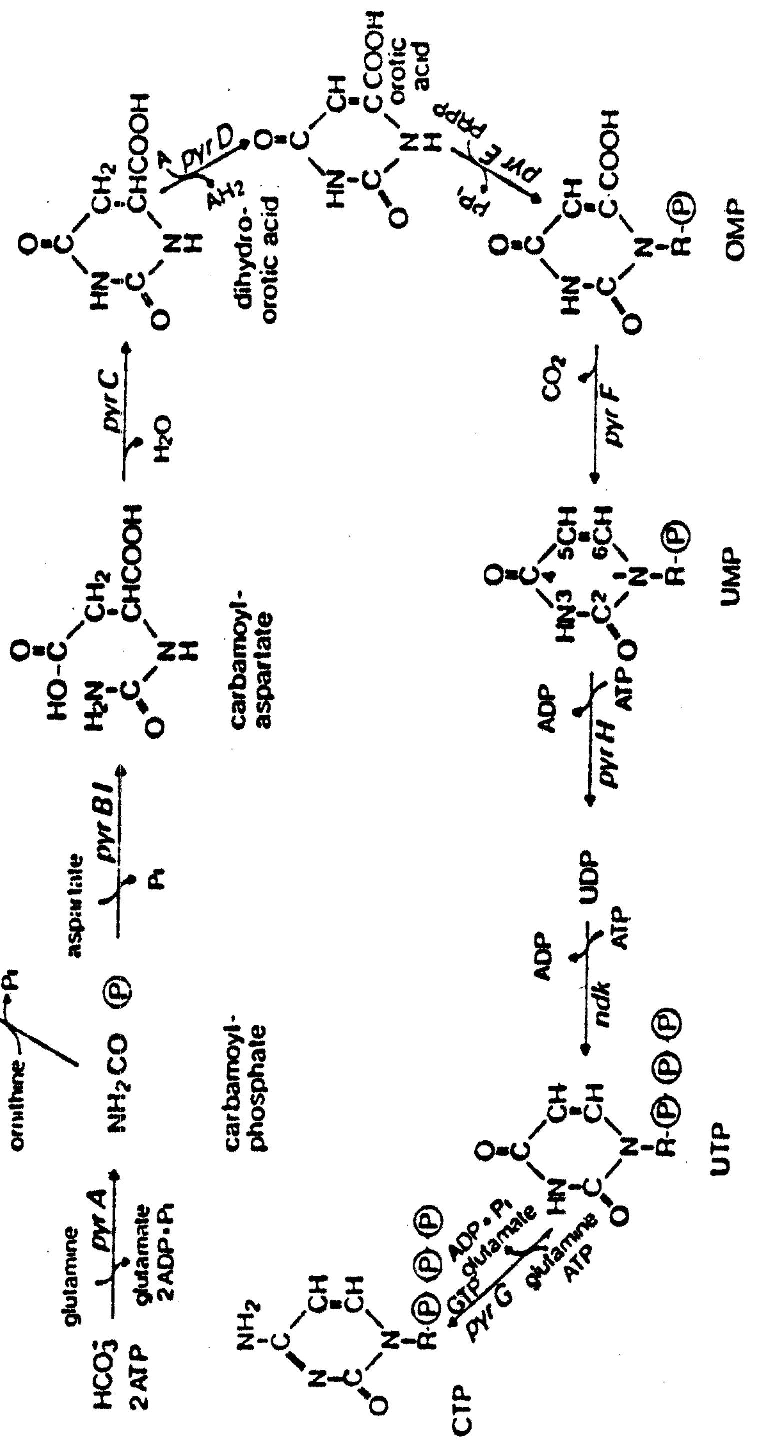
INTRODUCTION

The biosynthesis of pyrimidine nucleotides provides a system in which the biochemistry and genetics of metabolic pathways can be investigated to provide insight into a variety of metabolic processes. Most of the interest in pyrimidine metabolism has focused on the central pathway of pyrimidine biosynthesis de novo (Figure 1), which provides precursors for the formation of ribonucleotides (UTP and CTP) and deoxyribonucleotides (dCTP and dTTP) as well as other important biological molecules (O'Donovan & Neuhard, 1970; Neuhard, 1985; Neuhard & Nygaard, 1987). Most of the research performed to date has been with the pathway in *Escherichia coli* and *Salmonella typhimurium.* The pyrimidine biosynthetic pathway in *E. coli* is subjected to strict regulation. Regulation occurs at the level of enzyme synthesis or expression (repression or attenuation) and at the level of enzyme activity (allosteric regulation). Regulation of the pathway results in the economical and efficient use of precursors, intermediates and products. For example, the pathway must share metabolites with various other pathways, such as the arginine biosynthetic pathway. Also, the pathway must be regulated to rapidly adjust to changes in the metabolic state of the cell. In the case of *E. coli* or *S. typhimurium*, the organism must still produce

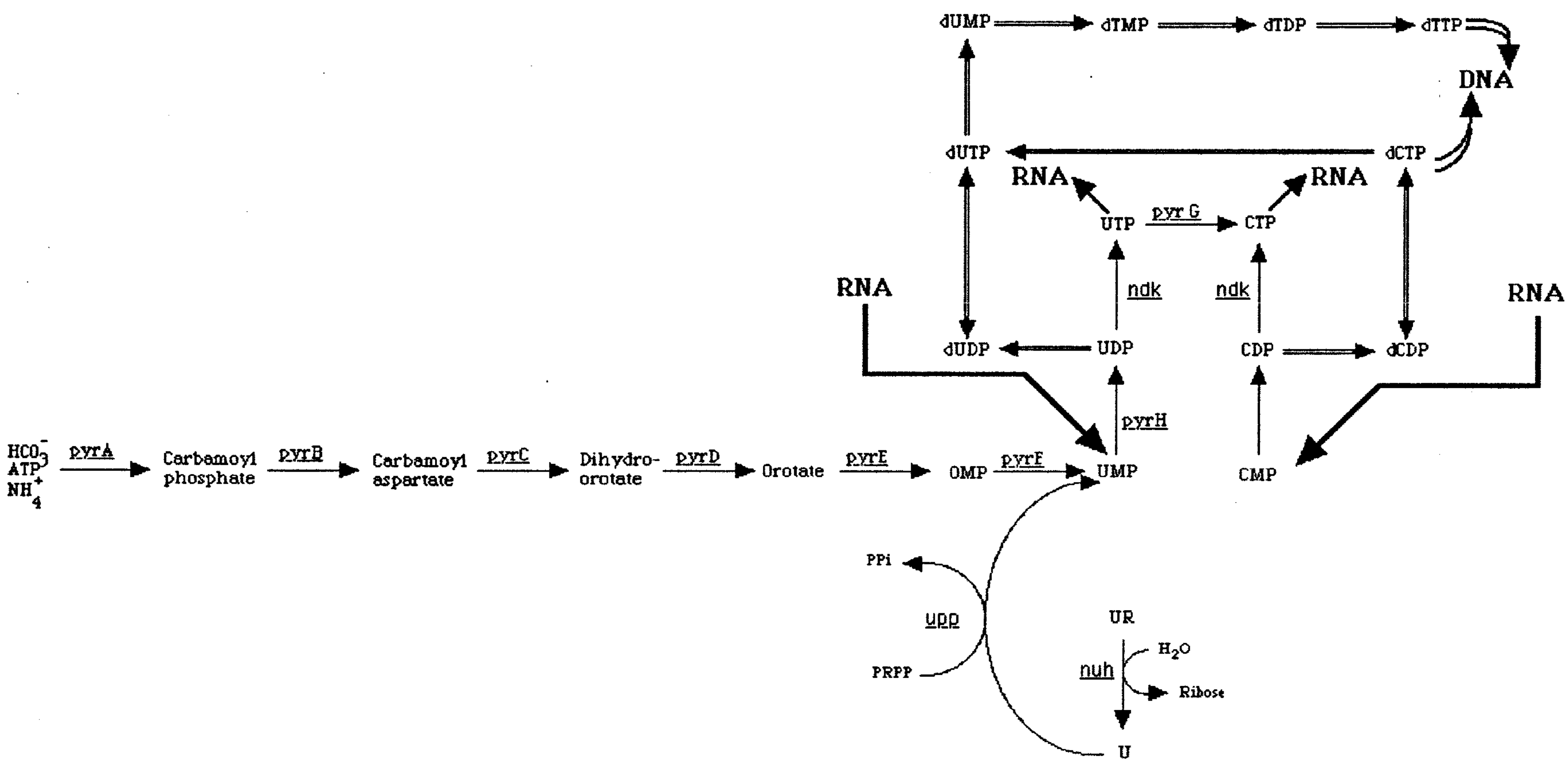
Figure 1. The synthesis of UTP and CTP de novo in bacteria. The individual enzymes are identified by their gene symbols: *argI*, ornithine carbamoyltransferase; *ndk*, nucleoside diphosphokinase; *pyrA (carAB)*, carbamoylphosphate synthetase; *pyrBI*, aspartate dehydrogenase; *pyrC*, dihydroorotase; *pyrD*, dihydroorotate transcarbamoylase; pyrE, orotate phosphoribosyltransferase; *pyrF*, orotidine 5'-phosphate decarboxylase; *pyrG*, CTP synthetase; *pyrH*, UMP kinase.


HCO$_3^-$
2 ATP
glutamine
pyr A
glutamate
2 ADP + Pi
NH$_2$CO Ⓟ
carbamoyl-phosphate
ornithine
arg I
Pi
citrulline
arginine
aspartate
pyr BI
Pi
carbamoyl-aspartate
H$_2$O
pyr C
dihydro-orotic acid
A
AH$_2$
pyr D
orotic acid
PRPP
pyr E
PPi
OMP
CO$_2$
pyr F
UMP
ADP
ATP
pyr H
UDP
ADP
ATP
ndk
UTP
ADP + Pi
glutamate
GTP
pyr G
glutamine
ATP
CTP

carbamoylphosphate for arginine biosynthesis, even when pyrimidine end products are made available from exogenously fed uracil. And since certain bacteria, such as the pseudomonads, possess the arginine deaminase pathway of arginine utilization, they must channel their endogenous pools of carbamoylphosphate efficiently between pyrimidine biosynthesis (*via* aspartate) and adenosine nucleotide maintenance (*via* carbamate kinase) (Stalon et al., 1987). Because pyrimidine nucleotides are required for RNA and DNA synthesis in fixed molar ratios, the proper balance of pyrimidine end products must be maintained differently in divergent microorganisms. Two major regulatory mechanisms include the control of enzyme activities by allosteric regulation and the control over the expression of the various pyrimidine genes. Both mechanisms respond to the levels of metabolites and to a lesser effect, intermediates in the pathway. Most of what is now known has come from studies with *E. coli*, *S. typhimurium* (Kelln et al., 1975) and other enterics (Wales & Wild 1991), though considerable insight has been gained from investigations of other bacteria such as *Bacillus subtilis* (Paulus et al., 1982; Lerner & Switzer, 1986). Available evidence indicates that the methods employed and mechanisms used for regulation are quite different among the pseudomonads (Isaac & Holloway, 1968; Chu & West, 1990) and in *Pseudomonas putida* in particular (Condon et al., 1976).

The biosynthesis of pyrimidines (Figure 2) begins with the formation of carbamoylphosphate from bicarbonate, the amide group of glutamine and ATP in a reaction catalyzed by carbamoylphosphate synthetase (CPSase, product of the *carAB* genes in *E. coli*). Carbamoylphosphate is also an intermediate in arginine biosynthesis so the enzyme CPSase actually participates in two biosynthetic pathways. The second step in the pathway and the first unique step, the committed step, is catalyzed by aspartate transcarbamoylase (ATCase encoded by *pyrBI*). ATCase condenses carbamoylphosphate with the amino group of aspartate and forms carbamoyl aspartate and inorganic phosphate. It is subject to regulation at the level of enzyme activity (Yates & Pardee, 1956) and enzyme synthesis (Beckwith et al., 1962). At the next step in the pyrimidine pathway, dihydroorotate is formed by cyclization and removal of water. This reaction is catalyzed by dihydroorotase (DHOase, product of the *pyrC* gene). Dihydroorotate is converted to orotate by dihydroorotate dehydrogenase (DHOdehase, *pyrD*) in the succeeding step. The fifth step involves the condensation of orotate with phosphoribosylpyrophosphate (PRPP) to yield the first pyrimidine ribonucleotide, orotidine-5′-monophosphate (OMP), with the elimination of pyrophosphate. This reaction is catalyzed by orotate phosphoribosyltransferase (OPRTase, product of *pyrE*). The OMP is then decarboxylated by OMP decarboxylase

Figure 2. The pyrimidine nucleotide biosynthesis de novo and salvage pathway in *Acinetobacter calcoaceticus*. The abbreviations of compounds are: U, uracil; UR, uridine; CR, cytidine; OMP, orotidine-5'-monophosphate; UMP, uridine-5'-monophosphate; UDP, uridine-5'-monophosphate; UDP, uridine-5'-diphosphate; UTP, uridine-5'-triphosphate; CTP, cytidine-5'-triphosphate. The gene designations are as follows: *pyrA*, carbamoylphosphate synthetase (CPSase, EC6.3.5.5); *pyrB*, aspartate transcarbamoylase (ATCase, EC2.1.3.2); *pyrC*, dihydroorotatase (DHOase, EC3.5.2.3); *pyrD*, dihydroorotate dehydrogenase (DHOdehase, EC1.3.3.1); *pyrE*, OMP phosphoribosyltransferase (OPRTase, EC2.4.2.10); *pyrF*, OMP decarboxylase (OMPdecase, EC4.1.1.23); *pyrH*, UMP kinase (EC2.7.4.4); *ndk*, nucleoside diphosphokinase (EC2.7.4.6); and *pyrG*, CTP synthetase (EC6.3.4.2).


HCO_3^-
ATP
NH_4^+
pyrA
Carbamoyl phosphate
pyrB
Carbamoyl aspartate
pyrC
Dihydro-orotate
pyrD
Orotate
pyrE
OMP
pyrF
UMP
pyrH
UDP
ndk
UTP
pyr G
CTP
ndk
CDP
CMP
dUDP
dCDP
dUTP
dCTP
dUMP
dTMP
dTDP
dTTP
DNA
RNA
RNA
RNA
RNA
PPi
upp
PRPP
UR
nuh
H_2O
Ribose
U

(OMPdecase, *pyrF*) to generate uridine-5′-monophosphate (UMP). The last steps in the generation of UTP involve the phosphorylation of UMP to UDP by the highly specific UMP kinase (encoded by *pyrH*), followed by the phosphorylation of UDP to UTP by a reaction catalyzed by the non-specific nucleoside diphosphokinase (*udk*). Finally, UTP is aminated to CTP by CTP synthetase (product of *pyrG*).

The pyrimidine ribonucleotide biosynthetic pathway of *E. coli* is perhaps the metabolic pathway that has been most extensively analyzed with respect to its regulation. Allosteric control of enzymatic activity of three steps in the pathway has been observed for the enzymes CPSase (Abdelal & Ingraham, 1969), ATCase (Yates & Pardee, 1956), and CTP synthetase (Long & Pardee, 1967). In *E. coli* ATCase is inhibited by CTP, the ultimate ribonucleotide end product of the pathway, while being activated by ATP in order to balance pyrimidine biosynthesis with purine biosynthesis. Furthermore, ATCase exhibits enzymatic control by responding to either substrate, aspartate or carbamoylphosphate, with positive homotropic activation. In other words, ATCase exhibits sigmoidal kinetics with response to either substrate. This cooperativity allows the enzyme to be regulated to a high degree over a relatively small range of substrate concentrations (Kantrowitz & Lipscomb, 1988; Kantrowitz et al., 1980a; Kantrowitz et al., 1980b for reviews). Thus ATCase, the enzyme catalyzing the first unique step in pyrimidine biosynthesis, is able to alter its catalytic efficiency at physiological concentrations of enzyme in response to very subtle changes in the level of a variety of changing metabolites in the cell.

The pyrimidine biosynthetic pathway is also regulated at the level of enzyme activity at two other steps in the pathway. CPSase from enteric bacteria is subjected to feedback inhibition and activation by pyrimidine and purine nucleotides (Abdelal & Ingraham, 1969; Abdelal & Ingraham, 1975). The enzyme is inhibited by UMP and activated by purine nucleotides (IMP, GMP, AMP). Ornithine is an allosteric activator of CPSase such that when arginine is limiting, ornithine concentrations rise to alleviate any UMP inhibition and activate CPSase to provide carbamoylphosphate for arginine biosynthesis. Other mechanisms to regulate the pathway operate at the level of CTP synthase. The activity of enzyme requires the activator GTP; and CTP inhibits CTP synthetase when CTP levels are high, while CTP may activate the enzyme at low concentrations (Long & Pardee, 1967).

In addition to exhibiting overall pathway control at the level of the enzyme activity of the various steps of the pathway, *E. coli* regulates the level of expression of all the genes in the de novo pyrimidine pathway. While the genes for the enzymes of pyrimidine biosynthesis are not linked, all are repressed during growth in media containing either exogenously supplied cytosine or uracil compounds.

Thus, the concentration of the enzymes in *E. coli* may fluctuate dramatically under conditions of repression or derepression in order to effect stable, balanced nucleotide pools. Again, using *E. coli* ATCase as an example, it has been shown that the *pyrBI* genes (encoding ATCase) can be controlled over a very wide range. ATCase can be derepressed up to 400-fold (Kelln et al., 1975). Indeed, using recombinant DNA technology, *E. coli* cells can be genetically manipulated to produce more than 60 per cent of their total cellular protein as ATCase (Nowlan & Kantrowitz, 1985). The regulatory region of the *pyrBI* operon from *E. coli* has been sequenced and exhibits a rho-independent terminator region (attenuator), as well as a region of dyad symmetry corresponding to a possible repressor binding site (Roof et al., 1982; Turnbough et al., 1983). Regulation is achieved by premature termination of the transcript in response to high intracellular levels of UTP. Two other genes in the pathway, *pyrE* and *pyrF*, (Poulsen et al., 1983; Poulsen et al., 1984; Jensen et al., 1986) have similar control regions. The promoter upstream regions for *pyrC* and *pyrD* have also been sequenced (Larsen & Jensen, 1985; Neuhard et al., 1986), but do not contain an attenuator like *pyrB*. Instead, they seem to be controlled by the intracellular levels of CTP, perhaps by a common regulatory protein that binds a cytidine nucleotide. The *carAB* gene in *E. coli* (*pyrA* gene in *S. typhimurium*) is regulated in a manner that allows it to be used for two separate pathways. The gene is subjected to cumulative repression in *E. coli*. The *argR* repressor bound to arginine represses the gene by binding to an operator (Arg box) next to the promoter (Gigot et al., 1980; Pierard et al., 1976). A second promoter, 70 bases upstream, is regulated by pyrimidines (Piette et al., 1984). Thus, pyrimidine biosynthesis in enteric bacteria is subjected to genetic regulation as well as enzyme regulation.

In order to obtain pyrimidines from other than the de novo pathway, *E. coli* has the capacity to utilize pyrimidine compounds scavenged from the environment and reutilized from cellular metabolism and the breakdown of messenger RNA (Neuhard, 1983; Neuhard and Nygaard, 1987). The salvage pathways for the reutilization of pyrimidine compounds are shown in Figure 2. These pathways allow cells to utilize pyrimidine bases, nucleosides and mononucleotides. The pyrimidine bases cytosine (C) and uracil (U) and pyrimidine nucleosides uridine (UR) and cytidine (CR) are channeled into UMP before conversion to cytidine nucleotides. Cytosine is deaminated to uracil which is then converted to UMP (Figure 1). Cytidine is deaminated to uridine which may be phosphorylated to UMP or more likely broken down to uracil before conversion to UMP. Likewise, exogenous uridine may be converted directly to UMP, but is mostly (75 per cent) taken through the two step conversion to UMP

(Neuhard, 1983).

The synthesis of pyrimidine nucleotides de novo does not involve nitrogenous bases or nucleosides as intermediates, however, the ability of different Pyr$^-$ mutants to use pyrimidine bases and nucleosides for growth confirms that pyrimidine salvage pathways are of widespread occurrence as shown in Figure 2. These salvage pathways are capable of converting pyrimidine bases and nucleosides into pyrimidine nucleotides. As pointed out by Neuhard (1983), the salvage pathways have three physiological functions: 1) to scavenge exogenous preformed pyrimidine bases and nucleosides for nucleotide biosynthesis, 2) to make the pentose portion of nucleosides and the amino group of cytosine available as sources of carbon, energy, and nitrogen, 3) to rephosphorylate pyrimidine bases and nucleosides produced endogenously by turnover of nucleotides from mRNA.

Other than conversion and physiological functions as mentioned the salvage pathways are also important because, 1) the extensive use of radioactively labelled pyrimidines for the study of nucleic acid synthesis *in vivo* requires a detailed knowledge of pyrimidine salvage pathways in order to select the most appropriate radioactive precursor; and 2) the salvage pathways are required to define the sensitivity of an organism to pyrimidine analogs which must be converted to their nucleotide form to be toxic. This is so because the analogs are typically metabolized by the same enzymes as are the natural pyrimidine bases and nucleosides (O'Donovan & Neuhard 1970). Thus, the pyrimidine salvage system includes the transport of the pyrimidine base or nucleoside into the cell plus its subsequent phosphorylation to the nucleotide level.

Properties of "Acinetobacter calcoaceticus"

The name *Acinetobacter* was initially used in 1954, but until recent years there has been much confusion concerning the classification of these organisms (Brisou & Prevot, 1954). The *Acinetobacter* genus contains aerobic, Gram-negative, non-motile, non-pigmented coccobacilli, which can be isolated from most environments including water, soil, sewage and within the hospital environment. Because they lack sufficient distinguished characteristics, the strains now known to be acinetobacters, were originally claimed to be members of 15 different genera, including *Moraxella*, *Achromobacter*, *Diplococcus* and *Mima* (Juni, 1978). *Acinetobacter calcoaceticus,* a common soil microorganism, was used throughout this study. This Gram-negative organism possesses a number of genetic and metabolic properties which strongly indicate its potential value towards a better understanding of the gene regulation.

Project Objectives

The major objective of this dissertation is to understand the regulation of gene expression in the pyrimidine biosynthetic pathway of *A. calcoaceticus* which is a versatile scavenger in nature.

The second objective is to study some properties of a partially purified aspartate transcarbamoylase (ATCase) from *A. calcoaceticus*. However, the specific aims and objectives accomplished by meeting the following:

1. To isolate a series of pyrimidine requiring mutants for use in studies of gene expression and nucleotide pool levels.
2. To characterize the regulation of gene expression for enzymes of the pyrimidine biosynthetic pathway of *A. calcoaceticus*. This is done by assaying the enzymes of pyrimidine metabolism under different growth conditions to ascertain if the enzymes are repressed.
3. To measure the levels of nucleotide pools under different growth conditions and to correlate changes in nucleotide pools with changes in the levels of enzymes expressed.
4. To characterize the salvage pathways by feeding wild type and mutant strains various pyrimidine bases and nucleosides, as well as base and nucleoside analogs, in order to determine the pathways for uptake and salvage of pyrimidines.
5. Comparison of the *A. calcoaceticus* ATCase with

those of *E. coli*, *P. putida*, and *P. aeruginosa*.

History and Overview of High Performance Liquid Chromatography (HPLC)

Since much of the research performed in this dissertation made use of HPLC it seems appropriate to describe the technique briefly from a historical perspective. Although certain investigations in the second part of the last century may be termed as the precursors of "chromatography", it has been established that the technique was first developed by M. S. Tswett, a Russian botanist, for the separation and isolation of plant pigments. The first description of the method was included in a paper presented in 1903 and then, a detailed report on the method and its application was published by him in 1906. Tswett also coined the name chromatography from the Greek: *chromos*, color and *graphein*, to write, referring to the bands of different colored substances which he separated on the powdered chalk columns. Tswett immediately emphasized, however, that the technique was not restricted to the separation of colored substances in plants but that it had general relevance.

In 1930-31 the method was revitalized in the laboratories of Kuhn and Lederer at Heidelberg, Germany. Within a few years, liquid column chromatography became a universally used technique without which the significant achievements in the chemistry of complex natural organic substances and in biochemistry would have been impossible. In the 20 years following the rediscovery of chromatography, the technique has been further expanded and individual variants developed. A particularly important milestone was the development of gas-liquid partition chromatography by Martin and James in 1952. This technique found immediate applications and the widespread interest in the technique initiated basic research on the theory of chromatography and in the development of standardized instrumentation in gas chromatography. This, in turn, cross-fertilized liquid chromatography by generalizing the theory and applying it specifically to this variant. This occurred in the 1960's resulting in a new explosion - the development of modern liquid chromatography now called high performance liquid chromatography (HPLC), a development still continuing today.

Liquid chromatography is one of the versions of chromatography, the most widely used analytical technique. Chromatographic processes, in general, can be defined as follows:

Chromatography is essentially a physical method of separation in which the components to be separated are distributed between two phases; one of them a stationary bed, while the other moves percolating through this bed. The chromatographic process occurs as a result of repeated sorption-desorption acts during the movement of the sample components along the stationary bed, and the separation is due to differences in the distribution coefficients of the individual sample components.

"Stationary bed" is used as a general term to denote any of the different forms in which the stationary phase may be used: for instance, it may be packed in a column or spread as a layer. The mobile phase may be gaseous or liquid.

In gas chromatography, the mobile phase is a gas while in liquid chromatography, it is a liquid. Liquid chromatography can be carried out in various systems depending on the physical form of the stationary phase. In thin-layer and paper chromatography, it is spread as a layer (plane chromatography), while in column chromatography, it is packed in a "column", which is a relatively narrow tube.

There are many ways to further divide liquid column chromatography. When this division is based on the nature of the stationary phase and the separation process, four modes can be specified as; adsorption, partition, size exclusion, and ion-exchange chromatography.

In ion-exchange chromatography, which is the method that has been used in this research, the stationary bed has an ionically charged surface of opposite charge to the sample. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger will be its attraction to the ionic surface and thus, the longer it will take to be eluted. The mobile phase is an aqueous buffer, where pH and polarity are used to control elution time from the column, (Yost, et al., 1980).

MATERIALS AND METHODS

Chemicals and Reagents

Pyrimidine bases, nucleotides, pyrimidine analogs, nucleosides, trichloroacetic acid (TCA), tri-n-octylamine, D-cycloserine, L-aspartic acid, dilithium carbamoylphosphate, carbamoylaspartate, L-dihydroorotic acid, orotic acid, 5′- phosphoribosyl-1-pyrophosphate, orotidine-5′- monophosphate, bromophenol blue and antipyrine were purchased from Sigma Chemical Company (St. Louis, Missouri); monobasic ammonium phosphate was from Mallinckrodt Inc. (Paris, Kentucky); and 1,1,2-trichlorol,2,2-trifluoroethane (Freon) was from Eastman-Kodak Company (Rochester, New York). Agar was purchased from Difco (Detroit, Michigan). Acrylamide, ammonium persulfate, TEMED (N,N,N′,N′- tetraethylmethylene diamine), and N,N′-methylene-bis-acrylamide were from BioRad laboratory (Richmond, California). All other chemicals were of analytical grade and were purchased from Fisher Scientific Company (Fair Lawn, New Jersey). Double distilled deionized water was used for all experiments with the exception of HPLC procedures in which Milli-Q water (Millipore Corporation, Bedford, Massachusetts) was used.

Bacterial Strains

Acinetobacter calcoaceticus strains were derived from strain BD413 (Juni, 1972; Juni and Janik, 1969) which is designated as ADP1. Strains ADP1 (wild-type) and ADP6 (*pcaA*$^-$) were obtained from Ms. Hayfa Al-Khatib and Dr. Mark Shanley who had received the strains from Professor L. Nicholas Ornston, Department of Biology, Yale University (New Haven, Connecticut).

Growth Media and Cultures

Bacterial cells were grown in liquid minimal medium (Cohen-Bazire, Sistrom, & Stanier, 1957) containing the following chemical composition in grams per liter of deionized water: Na_2HPO_4, 7.1, KH_2PO_4, 6.8, $(NH_4)_2SO_4$, nitrilotriacetic acid, $MgSO_4$, 28.9, $CaCl_2$, 6.67, $(NH_4)_6Mo_7O24.4H_2O$, 18.5, $ZnSO_4.\ 7H_2O$, $FeSO_4.\ 7H_2O$, $MnSO_4.H_2O$, $CuSO_4.\ 5H_2O$, $Co(NO_3)_2.\ 6H_2O$, $Na_2B_4O_7.\ 10H_2O$, Hutner's Metals '44' was used with succinate as the carbon and energy source. Pyr$^-$ mutants were supplemented with uracil at 20 µg/ml. These were periodically checked for purity on basal minimal agar plates. Gram stain tests and other appropriate morphological observations were made. All experiments were started by streaking the organism on basal minimal agar. Cells of *A. calcoaceticus* from a single, isolated colony were inoculated into basal minimal liquid medium. The cultures were incubated on a rotatory shaker at 37°C and agitated at 90 revolutions per minute. All *A. calcoaceticus* strains were grown at 37°C. Overnight cultures were transferred to a fresh basal medium and grown to about 100 Klett units (KU). Growth was measured by turbidity with a Photoelectric Klett Summerson Colorimeter (Klett Manufacturing Co., New York, N.Y.) using a green filter #54 and recorded as Klett units (KU=10^7/ml). The logarithm of the KUs was plotted against time to measure growth rate.

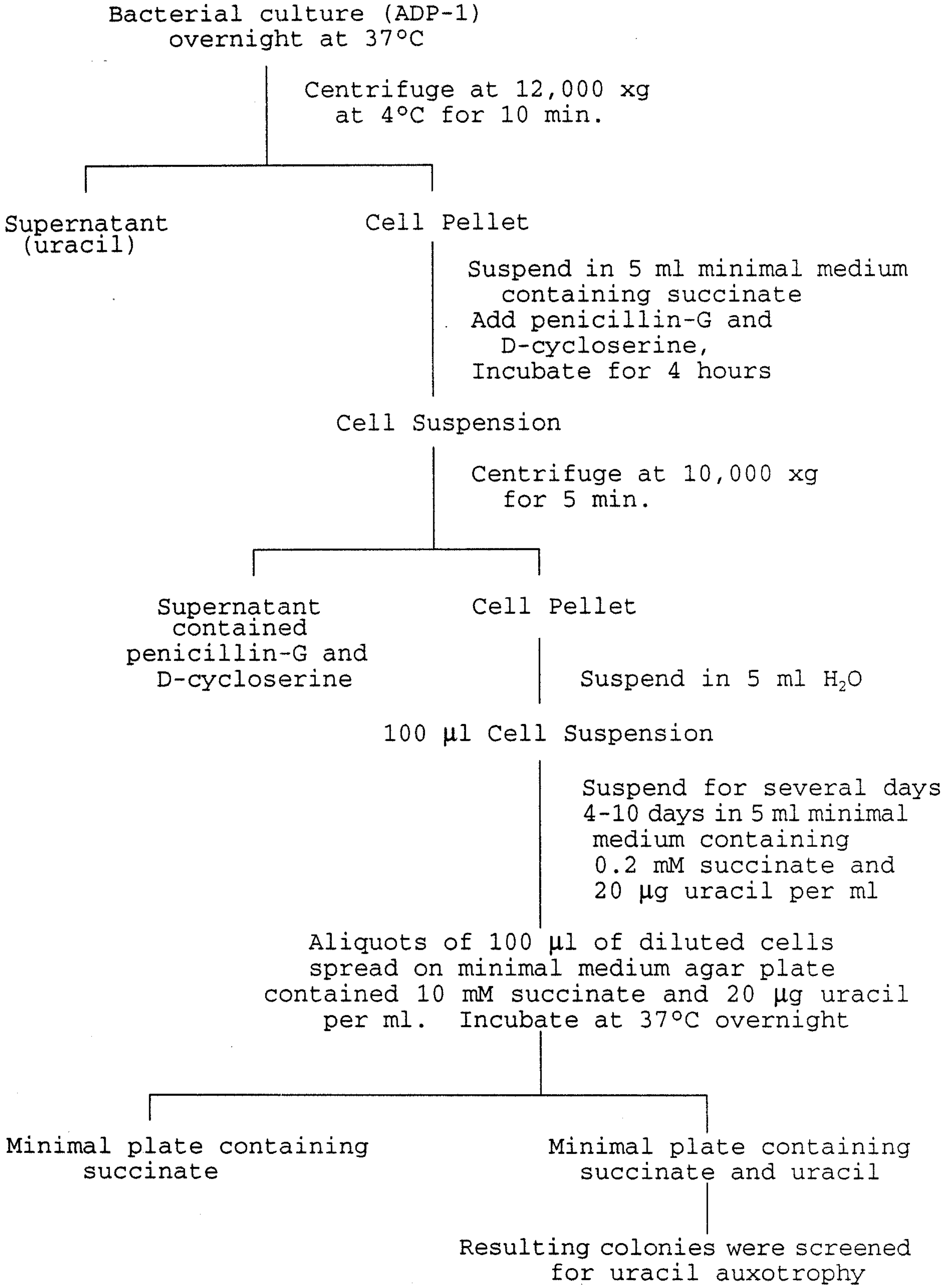
Determination of Step Blocked in Pyr$^-$ mutant

The metabolic block in pyrimidine biosynthetic pathway was examined by nutritional studies and enzyme assays. Liquid minimal medium and solid agar medium, supplemented with the intermediates of the pyrimidine pathway, as well as uracil, were used to grow the *A. calcoaceticus* mutants. Mutants which grew on a particular intermediate but not on those preceding it in the pathway indicated that the metabolic block involved the enzyme producing that particular intermediate. The *pyrB* and *pyrC* mutants were thus identified.

Isolation of Mutant Strains

A. calcoaceticus strain ADP-1, was utilized as the wild type strain in this research (Ornston et al., 1969). For isolation of uracil auxotrophic strains in *A. calcoaceticus*, spontaneous mutagenesis and penicillinG/D-cycloserine counter-selection were used (Figure 3) (Ornston et al., 1969). Several independent liquid cultures of *A.*

Figure 3. Schematic diagram representing the procedure for the isolation of pyrimidine mutants from *A. calcoaceticus* ADP-1 strain.


Bacterial culture (ADP-1) overnight at 37°C
Centrifuge at 12,000 xg at 4°C for 10 min.
Supernatant (uracil)
Cell Pellet
Suspend in 5 ml minimal medium containing succinate
Add penicillin-G and D-cycloserine,
Incubate for 4 hours
Cell Suspension
Centrifuge at 10,000 xg for 5 min.
Supernatant contained penicillin-G and D-cycloserine
Cell Pellet
Suspend in 5 ml H_2O
100 µl Cell Suspension
Suspend for several days 4-10 days in 5 ml minimal medium containing 0.2 mM succinate and 20 µg uracil per ml
Aliquots of 100 µl of diluted cells spread on minimal medium agar plate contained 10 mM succinate and 20 µg uracil per ml. Incubate at 37°C overnight
Minimal plate containing succinate
Minimal plate containing succinate and uracil
Resulting colonies were screened for uracil auxotrophy

calcoaceticus ADP-1 were grown in 5 ml amounts of minimal medium containing 0.2 mM succinate and 20 µg uracil per ml and shaken at 200 rpm, at 37°C overnight. In the morning, succinate was added to a final concentration of 5 mM and the cultures were shaken for an additional 30 minutes. The cells were harvested, washed and resuspended in minimal medium containing 5 mM succinate. Next the cells were incubated with shaking for one hour at 37°C. D-cycloserine (0.05 mg per ml) and penicillinG (5 x 10^3 units per ml) were added to kill the growing cells. After an additional 4 hours shaking, cells were harvested, washed, and resuspended with sterilized water. A 100 µl portion of the culture was transferred to fresh minimal medium containing succinate (0.2 mM) and uracil (20 µg per ml) and grown overnight. Cells were subjected to a new cycle daily for several days. After several days with the previously described treatment (at least four days and up to ten days), washed cells were diluted 10^{-6}, 10^{-7}, and 10^{-8} from the distilled water suspensions. Samples of 100 µl of the diluted cells were spread onto minimal medium agar plates that contained 10 mM succinate and 20 µg uracil per ml, and incubated at 37°C overnight. The resulting colonies were screened on solid media for uracil auxotrophy.

Extraction of Nucleotides

Volumes of 50 ml of bacterial cultures were harvested at about 100 KU, centrifuged at 4°C at 12,000 x g for 5 minutes. After decanting the supernatant, nucleotides were extracted from the cell pellet according to the scheme shown in Figure 4. One ml of ice-cold 6% (w/v) trichloroacetic acid (TCA) was added to the cell pellet, which was then thoroughly mixed for 2 minutes in the vortex mixer. The mixture was allowed to stand at 4°C for 30 minutes before centrifuging at 12,000 x g for 15 minutes. The clear supernatant was then neutralized with ice cold Freon-amine (Khym, 1975) solution (1.06 ml of 0.7 M tri-n-octylamine in 5 ml of Freon 113). The sample was then mixed on the vortex mixer for 2 minutes and allowed to separate for 15 minutes at 4°C. The top, aqueous layer, which contained the nucleotides, was removed, filtered through a 0.45 µm ACRO LC13 filter (Gelman Sciences, Ann Arbor, Michigan) and frozen at -20°C until ready for analysis.

Preparation of Cell-Free Extracts

The activity levels of enzymes of the pyrimidine biosynthetic pathway was determined in the wild type and in mutants of *A. calcoaceticus* (Figure 5). Typically, one liter of cell culture was grown to a cell density of 100 Klett units, as measured in a Klett-Summerson colorimeter equipped with a No. 54 filter. Under sterile conditions cell cultures were divided evenly into two parts each of 500 ml. After centrifugation, the cell pellets were washed and resuspended with minimal medium to remove any remaining traces of uracil. For the pyrimidine starvation

Figure 4. Schematic diagram representing the extraction procedure for ribonucleotides from *A. calcoaceticus* cultures.

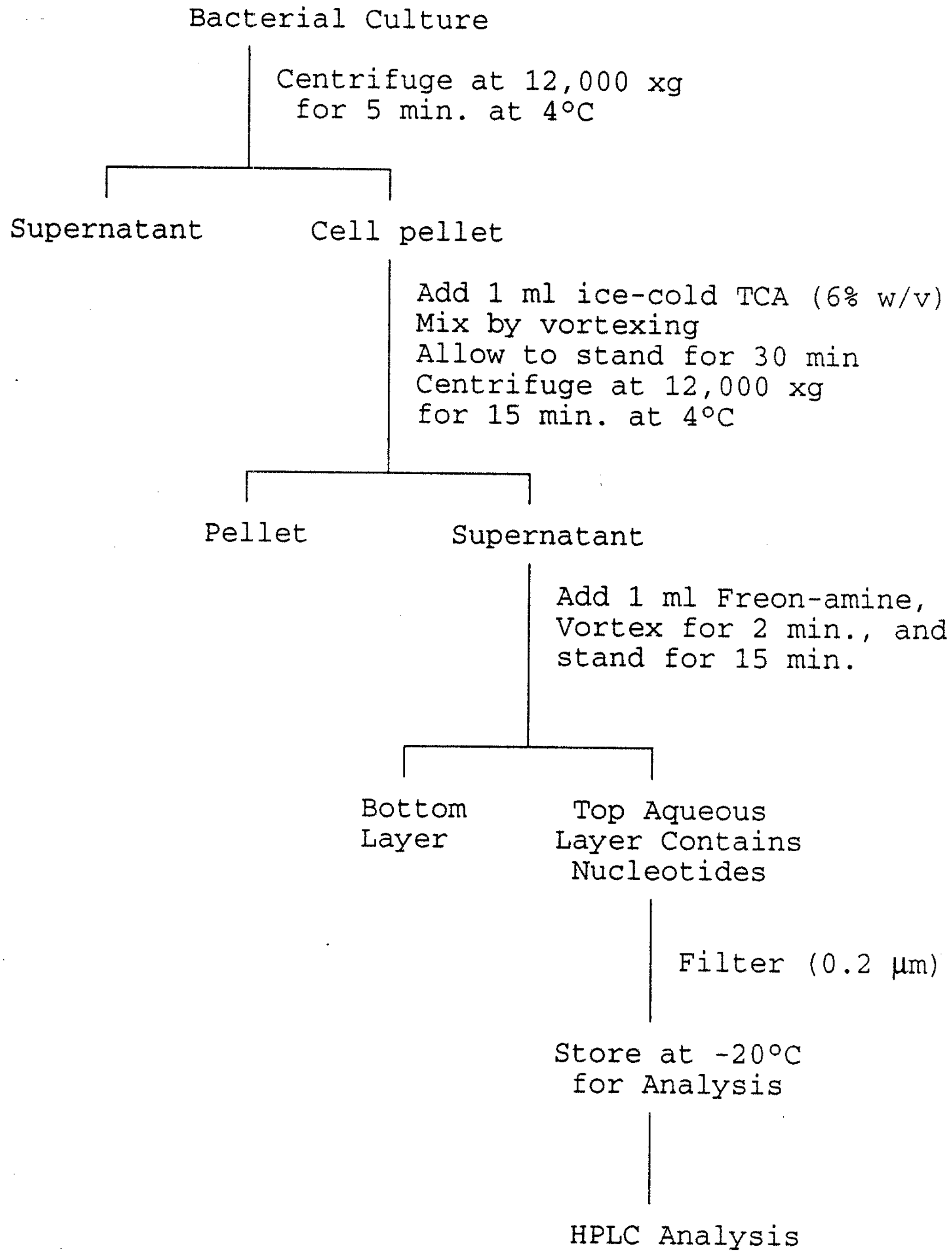
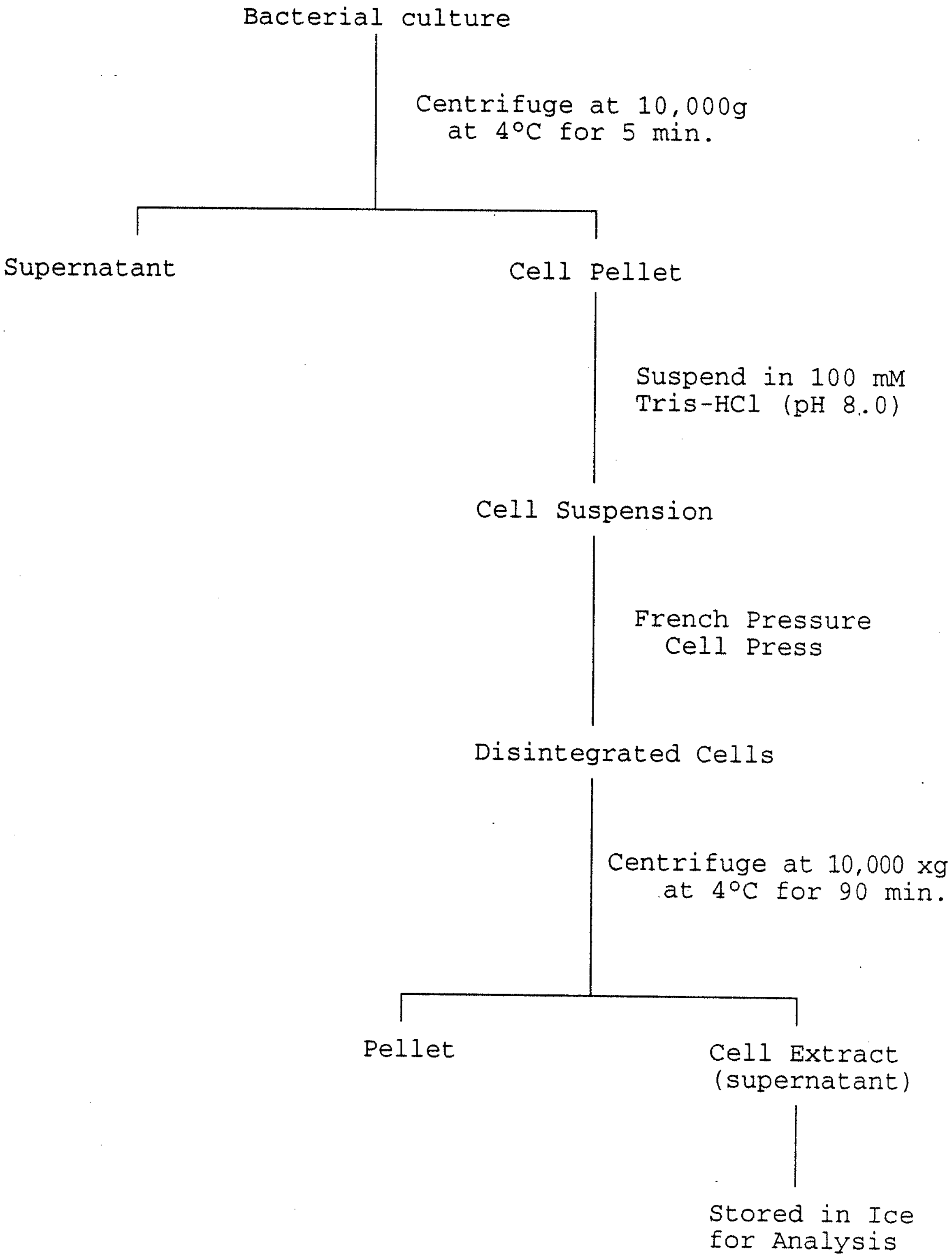

Bacterial Culture
Centrifuge at 12,000 xg
for 5 min. at 4°C
Supernatant
Cell pellet
Add 1 ml ice-cold TCA (6% w/v)
Mix by vortexing
Allow to stand for 30 min
Centrifuge at 12,000 xg
for 15 min. at 4°C
Pellet
Supernatant
Add 1 ml Freon-amine,
Vortex for 2 min., and
stand for 15 min.
Bottom
Layer
Top Aqueous
Layer Contains
Nucleotides
Filter (0.2 µm)
Store at -20°C
for Analysis
HPLC Analysis

Figure 5. Schematic diagram representing the preparation of bacterial cell-free extract.


Bacterial culture
Centrifuge at 10,000g at 4°C for 5 min.
Supernatant
Cell Pellet
Suspend in 100 mM Tris-HCl (pH 8.0)
Cell Suspension
French Pressure Cell Press
Disintegrated Cells
Centrifuge at 10,000 xg at 4°C for 90 min.
Pellet
Cell Extract (supernatant)
Stored in Ice for Analysis

experiments, one of the cell pellets was transferred to one liter of fresh minimal medium containing succinate only. The cells were collected, washed, and resuspended after 5 hours of starvation. The cell pellets were resuspended with 20 mM Tris-HCl buffer, (pH 8.6) containing 1 mM ß-mercaptoethanol (Condon, et al., 1976), and 0.02 mM zinc acetate. The cell extracts were kept on ice or chilled to 0-4°C. The suspension was passed through a French Press (SLM Aminco Corp.) to disrupt the cells by explosive decompression. The resulting cell free extract was centrifuged at 10,000 xg for 5 minutes at 4°C and 0.4 ml of the supernatant was withdrawn immediately for the assay of dihydroorotate dehydrogenase (Kelln, et al., 1975). The remaining extract was centrifuged at 10,000 xg for 1.5 hours at 4°C. The supernatants were removed and assayed immediately or were stored frozen at -20°C until further treatment.

Chromatographic Apparatus

The HPLC equipment is composed of two Waters Model 510 pumps, a Model 680 automated gradient controller, a U6K injector, a Model 740 data module (Waters Associates, Milford, Massachusetts) a SpectroMonitor Model 5000 photodiode array detector (LDC Analytical, Rivera Beach, Florida) and a Dionex AS4A column (Dionex Corp, Sunnydale, California). The entire chromatographic system except the column was stored in HPLC grade methanol when not in use, and the AS4A column was stored separately in 0.1 M NaOH. After priming the pumps, the system was flushed with distilled water at the flow rate of 4 ml per minutes for 15 minutes. The AS4A column was then connected to the system. The flow rate was decreased to 1.5 ml per minutes and kept constant for 30 minutes. Pump A was then flushed with buffer A, filtered 5 mM monobasic ammonium phosphate ($NH_4H_2PO_4$), pH 3.5; Pump B was flushed separately with buffer B, filtered 500 mM monobasic ammonium phosphate with 500 mM potassium chloride (KCl), pH 4.0. Nucleotides were detected by monitoring the AS4A ion-exchange column effluent at 254 nm with a sensitivity fixed at 0.05 absorbance unit full scale (AUFS).

Chromatographic Conditions

The entire chromatographic system excluding the column was stored in 50:50 (v/v) filtered HPLC grade methanol and filtered, double distilled water (2x) when not in use. After priming the pumps, the system was flushed with 50 ml of methanol:water mixture at 3 ml per minute. Next, the system was thoroughly washed with distilled water with the initial flow rate at 3 ml per minute. After 10 minutes, the flow rate was increased to 4 ml per minute. When the back pressure of the column dropped to 850 pounds per square inch, the methanol:water mixture was completely washed from the system. Pump A was flushed with starting buffer (filtered ultra pure, 7 mM monobasic ammonium phosphate, pH

3.8) followed by pump B which was flushed separately with final buffer (filtered, 250 mM monobasic ammonium phosphate containing 500 mM potassium chloride, pH 4.5). The LC spectrophotometer was set at 254 nm and 0.05 AUFS, and the recorder and automated gradient controller were turned on. An initial program with a linear slope (curve profile #6) of low concentration buffer was run for 10 minutes. A 10-minute reverse gradient of high concentration buffer was run, followed by a 10-minute rest with low concentration buffer.

Identification and Quantitation of Nucleotides

Nucleotide samples from *A. calcoaceticus* cells prepared as previously described were injected onto the AS4A column. Nucleotides were eluted according to the gradient program as mentioned previously. The flow rate was maintained at 1.5 ml per minutes (with exception for enzyme assay in salvage pathway at 1.00 ml). Analysis was performed at ambient temperature. Peaks were recorded on the Model 740 data module. The column was regenerated by washing with buffer A for 15 min. The sample peaks were identified by comparing their retention time with those of appropriate standards and by injecting known internal standards.

Calculations

The concentration of all nucleotides in samples was calculated by the following formula and expressed as μmol per (gram dry weight)$^{-1}$

$$\frac{Sa}{St} \times C \times \frac{V}{Vi} \times \frac{1}{DW}$$

Where Sa is the peak height of sample, St is the peak height of standard, C is the weight of compound in the standard divided by molecular weight of compound, V is the total volume of extract, Vi is the volume of extract injected, and Dw is the dry weight of the cells (Dutta and O'Donovan, 1987). Dry weight determinations were performed by filtering 25 ml of culture through a 0.2 µm filter that had been previously tared, drying the cells on the filter by vacuum desiccation, and weighing the filter to determine the weight of the cells extracted.

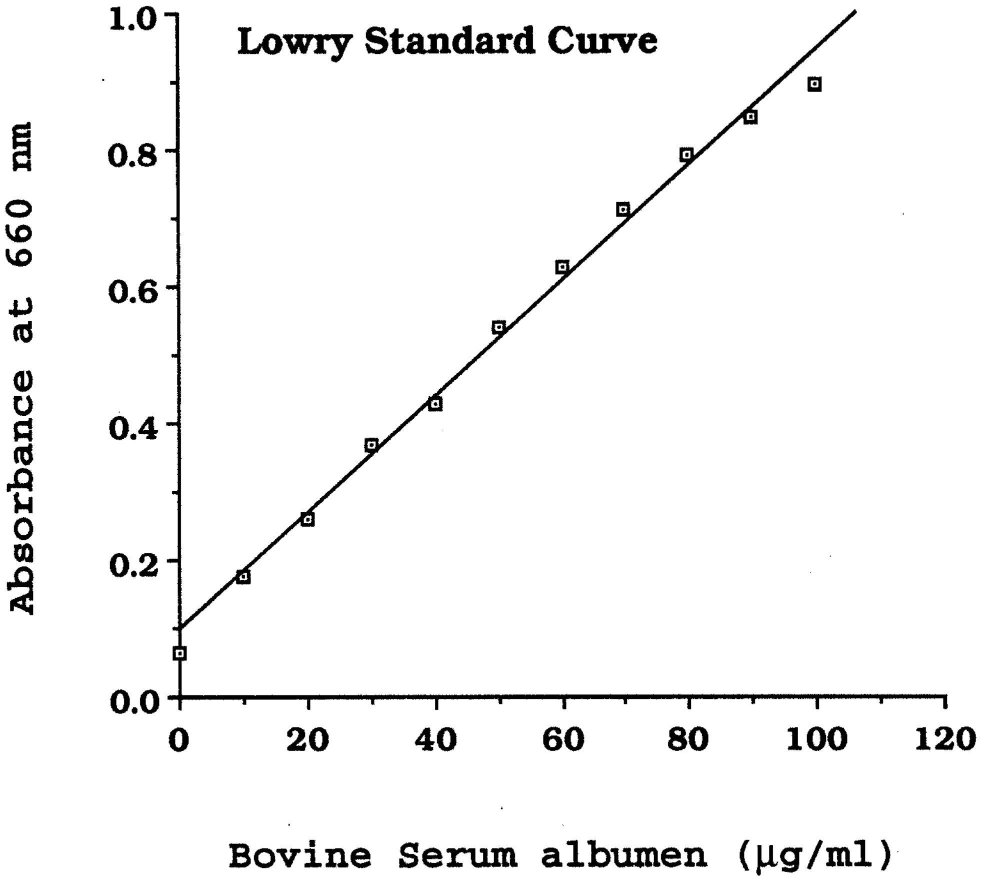
Protein Determination

All enzyme assays were performed at 30°C. This temperature was chosen due to instability of carbamoylphosphate at higher temperatures.

Specific activity of the enzymes was determined by detecting the changes in absorbance which were caused by product formation (or substrate utilization) only when that change was proportional to the reaction time and the amount of cell extract employed in reactions. Protein concentration of various cell-free extracts was determined by the Lowry method using bovine serum albumin (BSA) as a standard (Lowry et al., 1951). Reagents: Alkaline copper reagent, (mixed 0.5 ml of 2% Na/K and 0.5 ml of 1% $CuSO_4 \cdot 5 H_2O$ before adding 49 ml of 2% $NaCO_3$ in 0.1 N NaOH); Folin reagent, (diluted 1:1 with water for 1N Folin reagent); 0.1% bovine serum albumin (0.9 ml double distilled H_2O plus 0.1 ml 1% of 1 mg per ml BSA). To construct the standard curve for Lowry assay, standard tubes were set up containing from 0-100 μg BSA in 10 μg increments. To assay the samples, dilutions were made to a total volume of 200 μl. For assay, 0.8 ml of alkaline copper reagent was added to standards of 0-100 μg BSA and allowed to stand for 10 min. Next 0.1 ml of 1 N Folin reagent was added to all of the tubes and allowed to stand for 30 minutes at room temperature. The absorbance was read at 660 nm on a Beckman DU-40 spectrophotometer model against a water blank. The standard curve for the Lowry assay was prepared by plotting the absorbance at 660 nm against the concentration of BSA (Figure 6). The specific activity is expressed as nmol min^{-1} mg $protein^{-1}$.

Aspartate transcarbamoylase (ATCase) was assayed, according to the method of Adair and Jones (1972). The reaction mixture contained 100 mM Tris-HC1 buffer, (pH8.6), 10 mM aspartate, 1 μl cell-free extract, water, and 5 mM dilithium carbamoylphosphate and, if required, 1 mM ATP, 1 mM CTP, or 1 mM UTP to a total volume of 2 ml (Adair & Jones, 1972). The mixture, without carbamoylphosphate, was

Figure 6. Plot of the absorbance at 660 nm in the Lowry protein assay versus micrograms of bovine serum albumin per ml.


Lowry Standard Curve
Absorbance at 660 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Bovine Serum albumen (μg/ml)

pre-incubated at 30°C for 5 minutes, and the reaction was started by the addition of carbamoylphosphate that was made fresh and kept on ice. At 10, 20, and 30 minute intervals, 0.5 ml of sample was withdrawn and added to a tube that contained 0.5 ml water. One ml of the color mix (Prescott & Jones, 1969) was added to stop the reaction. After vigorous mixing the tubes were capped with marbles and placed in 60°C water bath under room light for 110 minutes. The absorbance of each assay tube at 466 nm was recorded. The enzyme activity was obtained by using an experimental absorption coefficient which was calculated from the slope of a standard curve, using carbamoylaspartate as the standard.

Enzyme Assays

The presence of enzymes in salvage pathway was checked in accordance with the method of Dutta et al. (1990). Twenty µl bacterial cell extract were mixed with 900 µl volumes of 1 mM stock solutions of the substrates (cytidine, uridine, or cytosine). The assay mixes contained in 1.5-ml microcentrifuge tubes were incubated at 37°C in a water bath. The reactions were terminated after 30 minutes by filtering the assay mixtures through 0.2 µm ACRO LC13 filters (Gelman, Ann Arbor, Michigan) into tubes stored on ice. Ten µl volumes of these reaction samples were injected onto an IBM C_{18} column (250 mm x 4.5 mm I.D.; particle size 5 µm; supplied by I.I.I. Supplies Co., Wallington, CT) for detection of substrates and products by HPLC, as described previously (Dutta et al. 1990).

Activity Gels

Non-denaturing 6% polyacrylamide gel electrophoresis (PAGE) was used to check for the presence of ATCase in samples of *A. calcoaceticus* cell-free extracts in the final stages of purification. The stock solution of acrylamide contained 30% (w/v) acrylamide, and 0.8% (w/v) N, N′-bis-methylene acrylamide in distilled water, 1.5 M Tris-HCl, (pH 8.8), 10% ammonium persulphate, and 0.5% (w/v) bromophenol blue. The sample buffer contained distilled water (4.7 ml), 0.5 M Tris-HCl, (4.0 ml; pH 6.8), glycerol (4.0 ml). Electrode buffer, (pH 7.0), contained 6.0 g Trizma base and 28.8 g glycine per liter of distilled water.

The 6% separating polyacrylamide gel solution contained distilled water (18.0 ml), 1.5 M Tris-HCl, pH 8.8 (7.50 ml), acrylamide bis-acrylamide (30:0.8) (7.5 ml), 10% ammonium persulfate (0.2 ml), TEMED (10 µl) with a total volume of 30.0 ml. The separating gel was made using gel solution sufficient for two 20 cm x 20 cm x 0.75 mm slab gels. The solution was aspirated for 15 minutes while in the ice container and TEMED was added with gentle swirling to avoid trapping air bubbles. Next, 18.0-20.0 ml of the separating gel was injected into the casting apparatus. After the insertion of the well-forming comb the gel was allowed to polymerize for 1-2 hours. The comb was removed from the gel, the wells washed once with electrode buffer and the core unit was placed into the tank. One liter of electrode buffer was added to the upper and lower tanks.

Samples of the cell-free extract (27-30 µl) were mixed with appropriate amount of tracking dye containing glycerol and bromophenol blue before being loaded onto a 6% non-denaturing polyacrylamide gel in a single Tris-glycine buffer, pH 7.0 (Foltermann et al., 1981). The samples were run at 90-100 volts for 18-20 hours.

Activity Stain

Gels were specifically stained for ATCase activity by a procedure developed by Bothwell (1975; Ph.D. disseration, University of California, Berkeley), and modified by K. Kedzie, (1987; Ph.D. dissertation, Texas A & M University, College Station) who used lead nitrate rather than imidazole. Following electrophoresis, the gel was dismantled and placed in a Pyrex dish, with one of the glass plates used as a support. The gel was then equilibrated with 250 ml ice cold 50 mM Tris-HCl, pH 7.0, (1.513 g Tris per 250 ml deionized H_2O) for 20 min. Five ml of 1.0 M aspartate and 10 ml of 0.1 M carbamoylphosphate (0.78 g of aspartate per 5 ml deionized H_2O and 0.153 g of carbamoylphosphate per 10 ml deionized H_2O, respectively) were then added. The gel was then incubated for 10 minutes on ice, and then washed three times using ice cold deionized H_2O. Enzymatic release of orthophosphate trapped in the gel was precipitated by the addition of 3 mM lead nitrate in ice cold 50 mM histidine, pH 7.0 (0.25 g of lead nitrate per 250 ml in 50 mM histidine pH 8.5, 2.62 g histidine per 250 ml deionized H_2O). After 15 min, lead nitrate was removed using three changes of ice cold deionized H_2O. The gel was stored overnight at 4°C in deionized H_2O prior to analysis.

RESULTS

In this study, pyrimidine biosynthesis in *Acinetobacter calcoaceticus* strain ADP-1 was partially characterized. First, a Pyr^- mutant of *A. calcoaceticus* was isolated by penicillinG and D-cycloserine counter-selection according to the method of Ornston et al., (1969). This mutant strain was designated *A. calcoaceticus* ADP1-218 by Haifa Al-Khatib.

In order to measure repression/derepression of ATCase, the Pyr^- mutant was grown in the presence and absence of pyrimidines. When starved for pyrimidines there was a 15-fold derepression observed for ATCase (Table I). The levels of UTP and CTP decreased to near zero while the intracellular concentrations of ATP and GTP were markedly increased.

Growth curves for *A. calcoaceticus*, wild type (Figure 7) and its Pyr^- mutant are shown in Figure 8. The wild type strain grows well on succinate minimal (SM) medium with or without supplement while the Pyr^- auxotroph requires uracil or uridine for growth. No other pyrimidine is able to satisfy the pyrimidine auxotrophy (Table II). Typically, when uridine was added to the medium, a much slower growth rate was observed than that observed when uracil was the pyrimidine added with turbidity reaching only 10-20 Klett units. No growth was observed when cytosine or cytidine was

Table I. ATCase specific activity from wild type and a Pyr strain of *A. calcoaceticus* in the presence and absence of uracil.

Strains	Specific Activity
ADP-1 Wild type strain + succinate	68
ADP-1 Wild type strain + uracil	22
ADP-1-218 Mutant strain + uracil	39
ADP-1-218 Mutant strain (starved)	583

Growth conditions and enzyme assays were as described in Materials and Methods. The specific activity of aspartate transcarbamoylase is given in nmol per min per (mg protein). Each specific activity measurement represents the average of two experiments.

Figure 7. Growth curves for *A. calcoaceticus* ADP-1 wild type strain in succinate minimal (SM) medium at 37°C with and without pyrimidines.

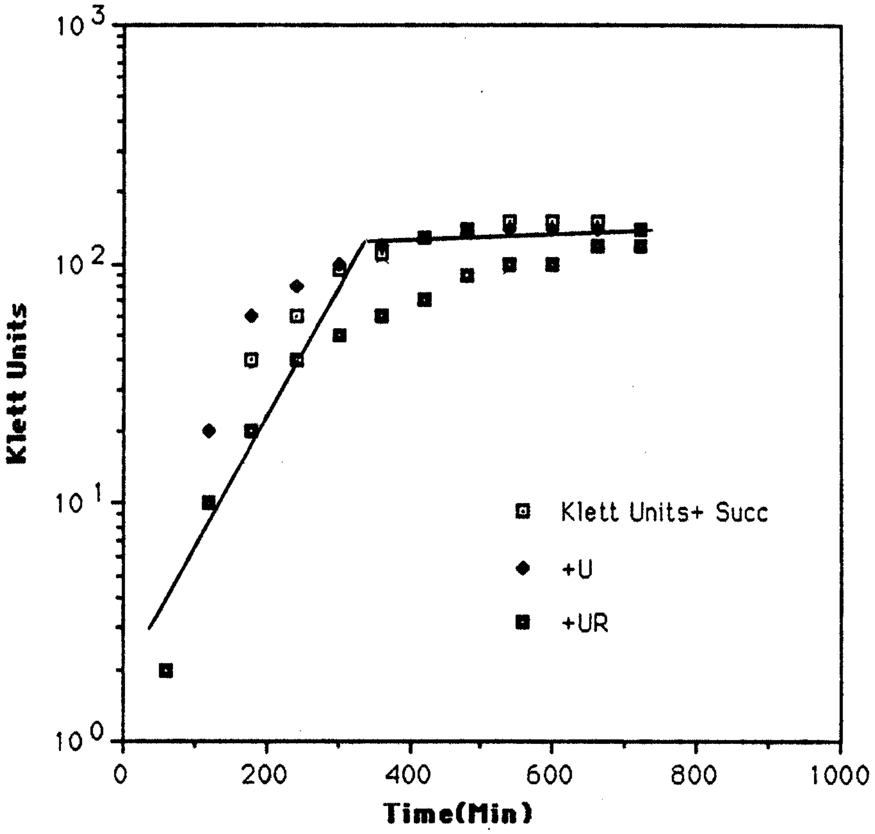

10 3
10 2
10 1
10 0
Klett Units
0
200
400
600
800
1000
Time(Min)
Klett Units+ Succ
+U
+UR

Figure 8. Growth curve for *A. calcoaceticus* ADP-1-218 mutant strain in the succinate minimal (SM) medium at 37°C with and without pyrimidines. When the cultures reached 100 KU they were starved for pyrimidines for three hours.

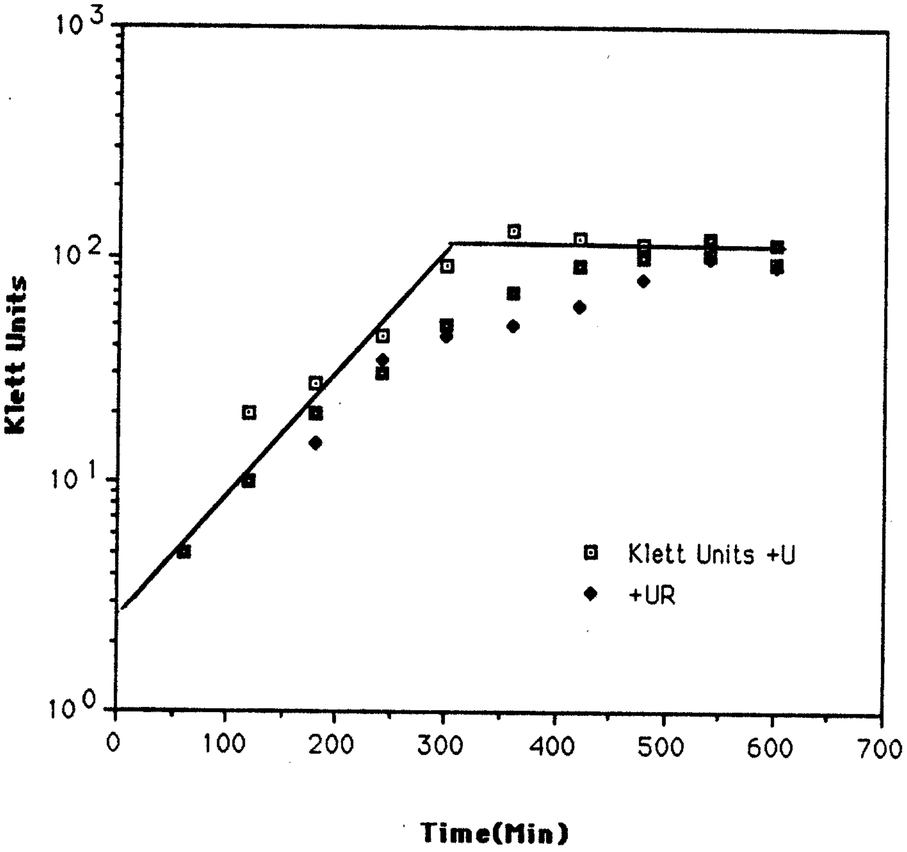

10³
10²
10¹
10⁰
Klett Units
0
100
200
300
400
500
600
700
Time(Min)
Klett Units +U
+UR

Table II. Compounds which satisfy the pyrimidine requirement for the Pyr mutant of *A. calcoaceticus*.

Addition to SM	Growth
None	-
Cytosine	-
Cytidine	-
Uracil	+
Uridine	+

+ indicates growth.
- indicates no growth.

used to satisfy pyrimidine auxotrophy. On assay, the Pyr⁻ auxotroph was found to be cytosine deaminase (*cod*) and also cytidine deaminase (*cdd*) deficient. Also cytidine deaminase was absent.

Growth curves for the *A. calcoaceticus* wild type ADP-1 strain are shown in Figure 7 where the log of the Klett Units (KU) is plotted against time. As can be seen from Figure 7, the ADP-1 strain grows equally well on succinate minimal medium and on all supplements.

Growth curves for the *A. calcoaceticus pyrC* strain are shown in Figure 8. A previous calculation indicated that the amount of exogenous pyrimidine base (5 μg/ml) or nucleoside that was needed for the auxotroph to reach 100 KU. This is seen in Figure 8 for uracil which grow exponentially to about 100 KU before all the pyrimidine nutrient was used up. After three hours of such starvation, samples for derepression measurements were taken. No growth was observed on cytosine, and little or no growth on cytidine.

All cells were harvested at 100 KU prior to derepression experiments. The mutant was assayed using the wild type strain as the control. All cultures were grown with and without uracil.

In order to measure derepression level of Pyr⁻ mutant, the enzyme ATCase was assayed. The Pyr⁻ mutant was inoculated into a Klett flask containing SM with 50 μg uracil per ml added. The strain grew to approximately 100 KU before growth was arrested. The cells were spun down, washed free of uracil and resuspended in SM without uracil and derepression of the pyrimidine enzyme started. In order to obtain the maximal derepression, the cells were starved for three hours. The cells were then centrifuged, broken in a French-press and assayed for ATCase.

The metabolic block in pyrimidine biosynthetic pathway was examined by nutritional studies and enzyme assays. The Pyr$^-$ mutant was grown on minimal liquid and agar media, supplemented with the intermediates of the pyrimidine pathway as well as uracil (50 µg/ml). Mutants which grew on a particular intermediate but not on those preceding it in the pathway indicated that the metabolic block involved the enyzme producing that particular intermediate. The metabolic block was determined by growing the cells on SM plates to which crystals of intermediates of the pyrimidine pathway were added (Table III). In this result, it was suggested that there was a block at the step where carbamoylaspartate is converted to dihydroorotate. This enzymatic step is known to be carried out by dihydroorotase (DHOase), which is encoded by the gene *pyrC* in *E. coli*. It was suggested that the Pyr$^-$ mutation was a leaky *pyrC*, because its cells produced small colonies on plates of minimal medium.

In order to characterize the regulation of gene

Table III. Determination of the metabolic block in Pyr$^-$ mutant of *A. calcoaceticus*.

Addition to SM	Growth	
	Pyr-mutant	Wild-type
None	-	+
Uracil	+	+
Carbamoylaspartate	-	+
Dihydroorotate	+	+
Orotate	+	+

+ indicates growth.
- indicates no growth.

expression of the enzymes of pyrimidine biosynthetic and salvage pathways of *A. calcoaceticus*, bacterial cell-free extracts were incubated with individual pyrimidine compounds for 30 minutes and then injected into a reversed-phase column on the HPLC. By assaying for ATCase under different growth conditions the extent of derepression can be assessed.

The presence of either uridine phosphorylase (*udp*) or nucleoside hydrolase (*nuh*) in a cell-free extract can be detected by the disappearance of cytidine or uridine and the concomitant production of cytosine or uracil. Uracil and cytosine concentrations in the range between 1 to 1000 μM were measured to produce a standard curve used in quantitation. Improved sensitivity for cytosine was observed when the integrator peak width value was adjusted between 0.1 - 0.5. Figure 9, shows a chromatogram along with its corresponding isogram, at 0.25 mM mixture of aqueous standard solution of uracil, cytosine, uridine, and cytidine. When the reaction mix of uridine and fresh undialyzed cell extract was injected, both uridine and uracil peaks resulted as shown in Figure 10 (A). However, either uridine phosphorylase or nucleoside hydrolase could have been present in the crude extract. Thus, the cell extract was dialyzed overnight against two changes of 100 mM Tris-HCl buffer (pH 8.0) to remove all phosphates and other small molecules. By means of this treatment, it appeared

Figure 9. Chromatogram (A) and isogram (B) of 0.25 mM aqueous standard solution of uridine, cytidine, uracil and cytosine. Numbers on individual peaks are retention times in min.

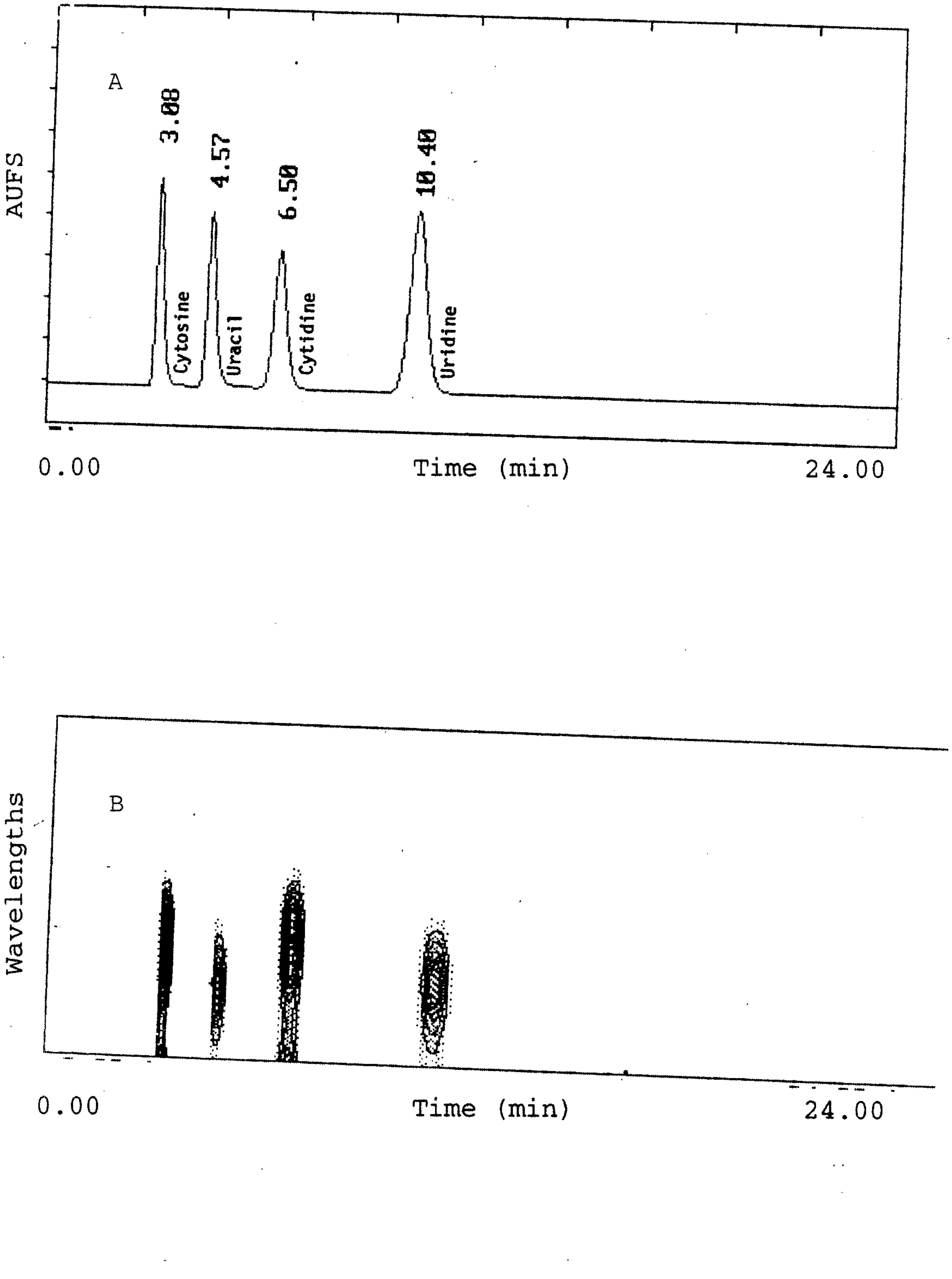
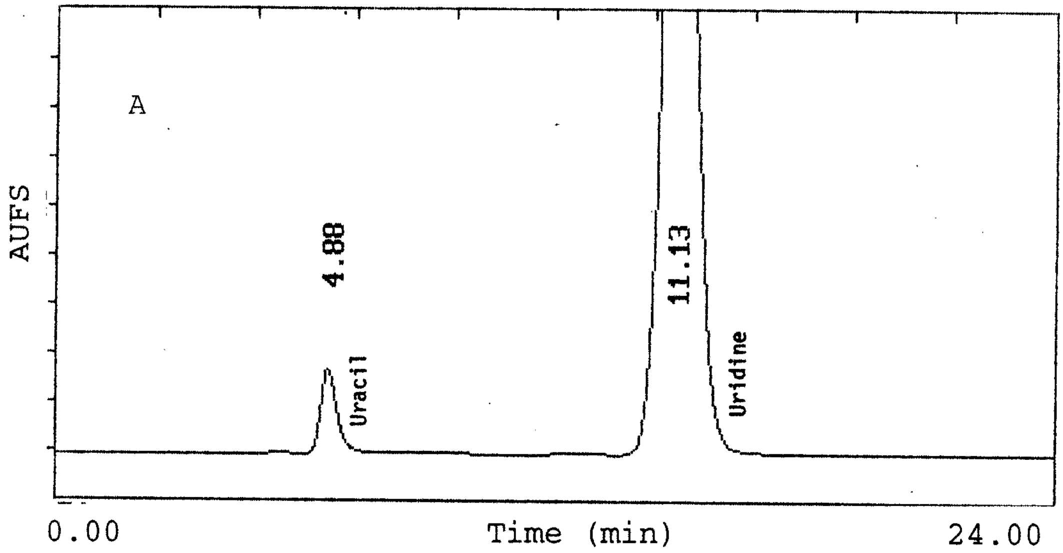
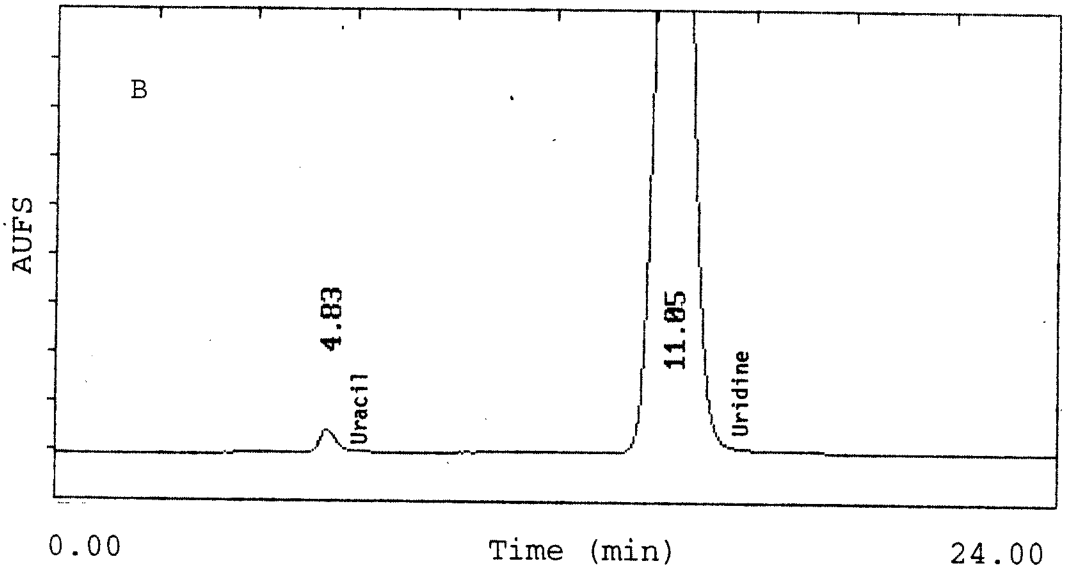

A
AUFS
3.08
4.57
6.50
10.40
Cytosine
Uracil
Cytidine
Uridine
0.00
Time (min)
24.00
B
Wavelengths
0.00
Time (min)
24.00

Figure 10. Chromatograms of pyrimidine bases and nucleosides at 254nm after incubation of 1 mM uridine with (A) undialyzed *A. calcoaceticus* cell extract and (B) dialyzed *A. calcoaceticus* wild-type ADP-1 cell extract.


A
AUFS
4.88
Uracil
11.13
Uridine
0.00
Time (min)
24.00


B
AUFS
4.83
Uracil
11.05
Uridine
0.00
Time (min)
24.00

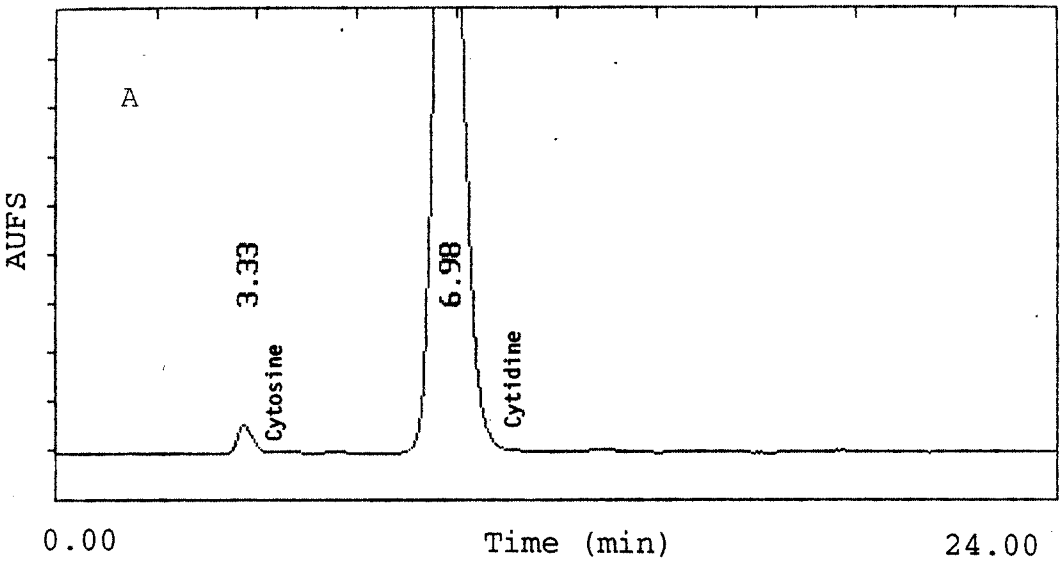
that uridine phosphorylase was not present. As Figure 10 (B) shown, uridine incubated with the dialyzed extract of strain ADP-1 still produced a uracil peak. This suggested that uridine was broken down to uracil by a nucleoside hydrolase. When cytidine was similarly incubated with the undialyzed cell extract, a large cytidine peak and a very small cytosine peak (less than 0.5% of cytidine peak) was seen. When the cell extract was dialyzed prior to incubation with cytidine a large peak but no cytosine peak appeared shown in Figure 11 (B).

The Lowry protein assay Figure 6 was used to estimate the protein content of cell-free extracts. The protein content was typically about 100 µg per ml in a cell-free extract. The carbamoylaspartate standard curve is shown in Figure 12.

An ATCase polyacrylamide gel assay was modified from an earlier procedure (Bothwell 1975) and K. Kedzie, (1987 Ph.D. dissertation, Texas A & M University College Station, Texas). A typical activity stained gel is shown in Figure 13. The ATCase of *A. calcoaceticus* was identified by comparing the band to that formed by the purified *E. coli* ATCase (Figure 13) and partially purified *Pseudomonas putida* ATCase (Figure 14). The molecular weight of the *A. calcoaceticus* enzyme was about 480,000, very similar to the mw of the *Pseudomonas* ATCase.

Quantitation of nucleotide pools in pyrimidine-

Figure 11. Chromatogram of pyrimidine bases and nucleosides at 254nm after incubation of 1 mM cytidine with (A) undialyzed *A. calcoaceticus* cell extract (B) dialyzed *A. calcoaceticus* wild-type ADP-1 cell extract.


A
AUFS
3.33
Cytosine
6.98
Cytidine
0.00
Time (min)
24.00

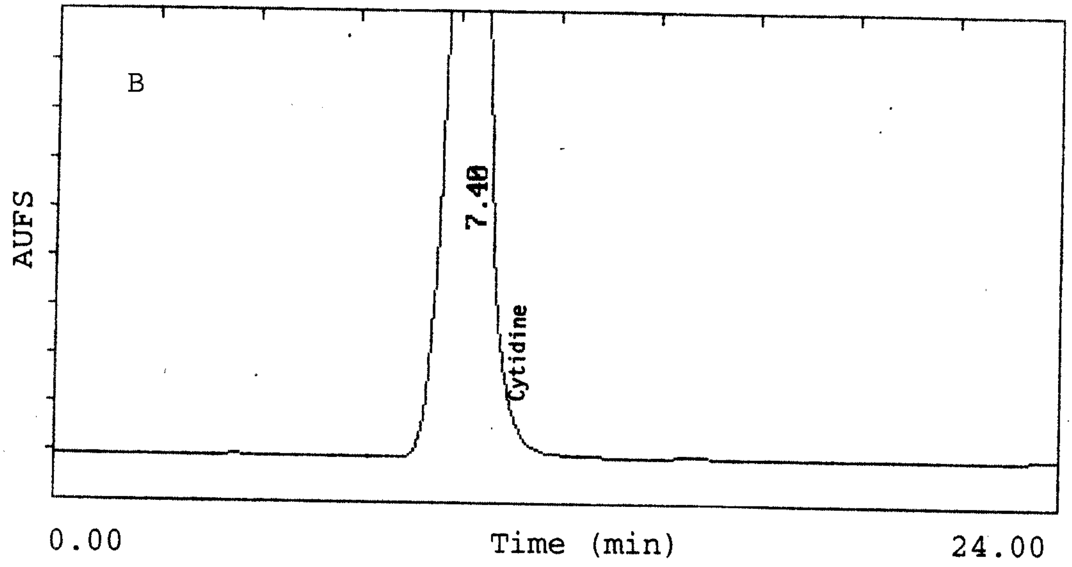

B
AUFS
7.40
Cytidine
0.00
Time (min)
24.00

Figure 12. Carbamoylaspartate standard curve. The absorbance at 466nm was plotted against the concentration of carbamoylaspartate (nM).

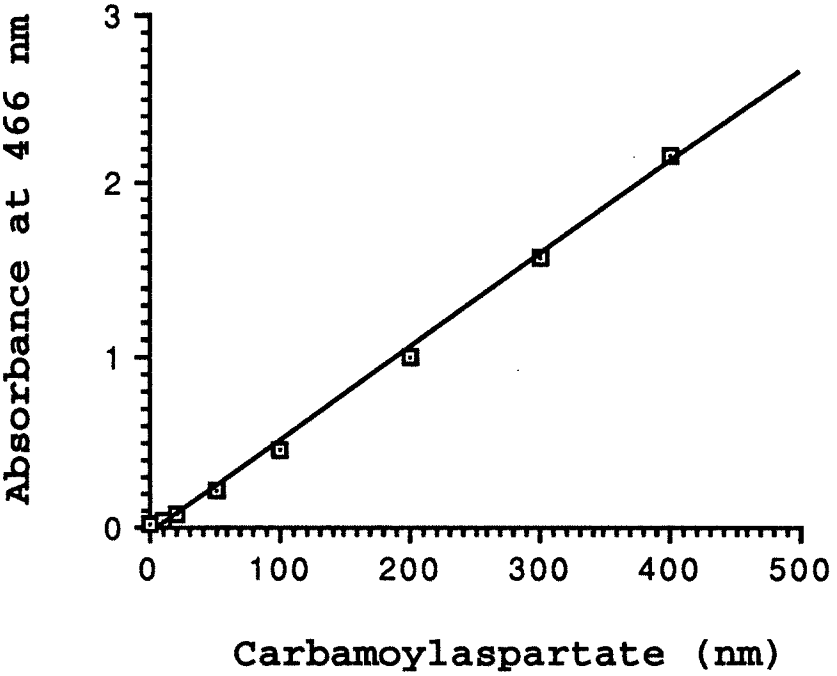

Absorbance at 466 nm
3
2
1
0
0
100
200
300
400
500
Carbamoylaspartate (nm)

Figure 13. 6% polyacrylmide ATCase activity gel. The lanes are as follows:

1. *E. coli* ATCase
2. *A. calcoaceticus* mutant ADP-1-218 -uracil (starved)
3. *A. calcoaceticus* wild type ADP-1
4. *A. calcoaceticus* wild type ADP-1 +uracil
5. *A. calcoaceticus* mutant ADP-1-218 +uracil

1 2 3 4 5

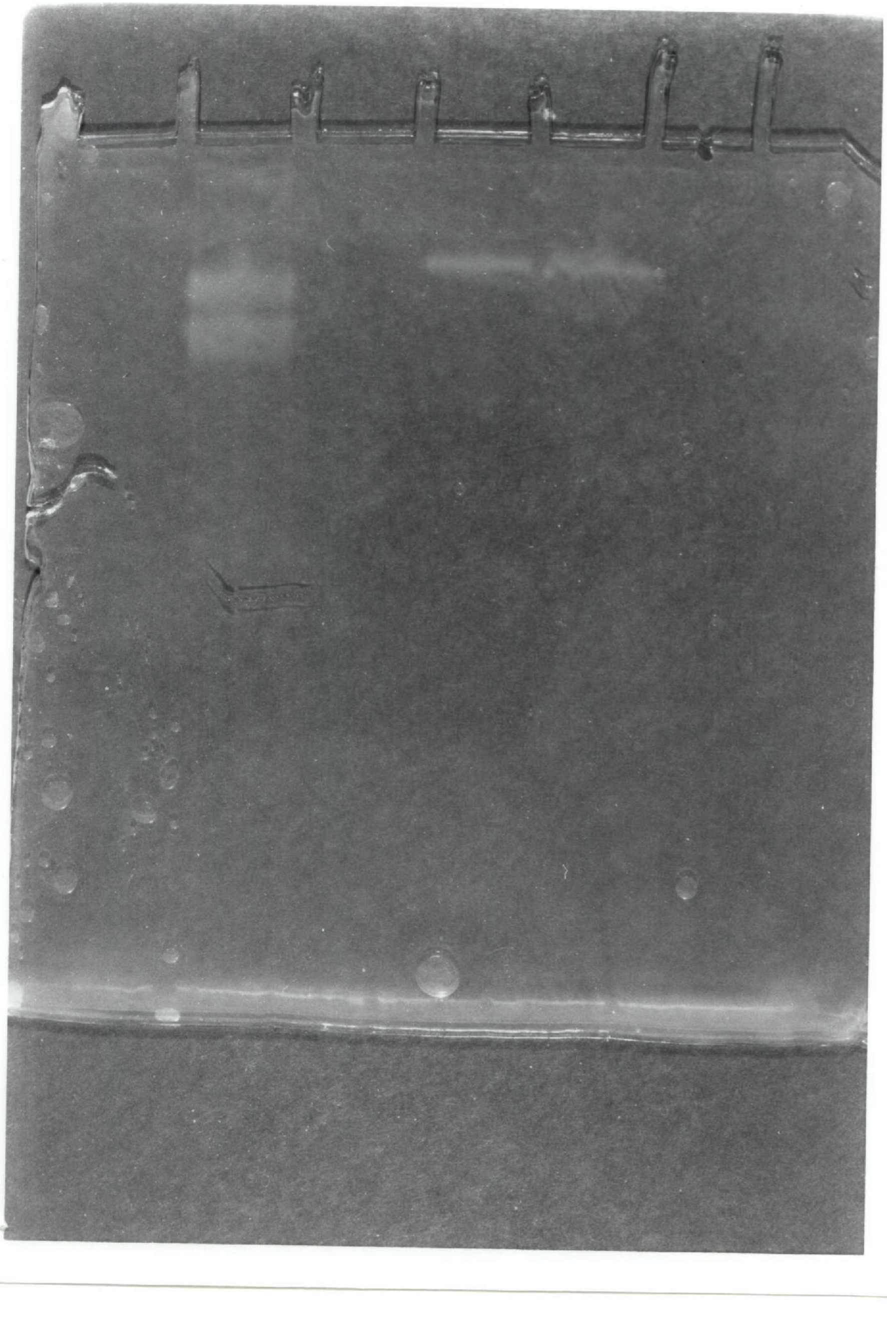
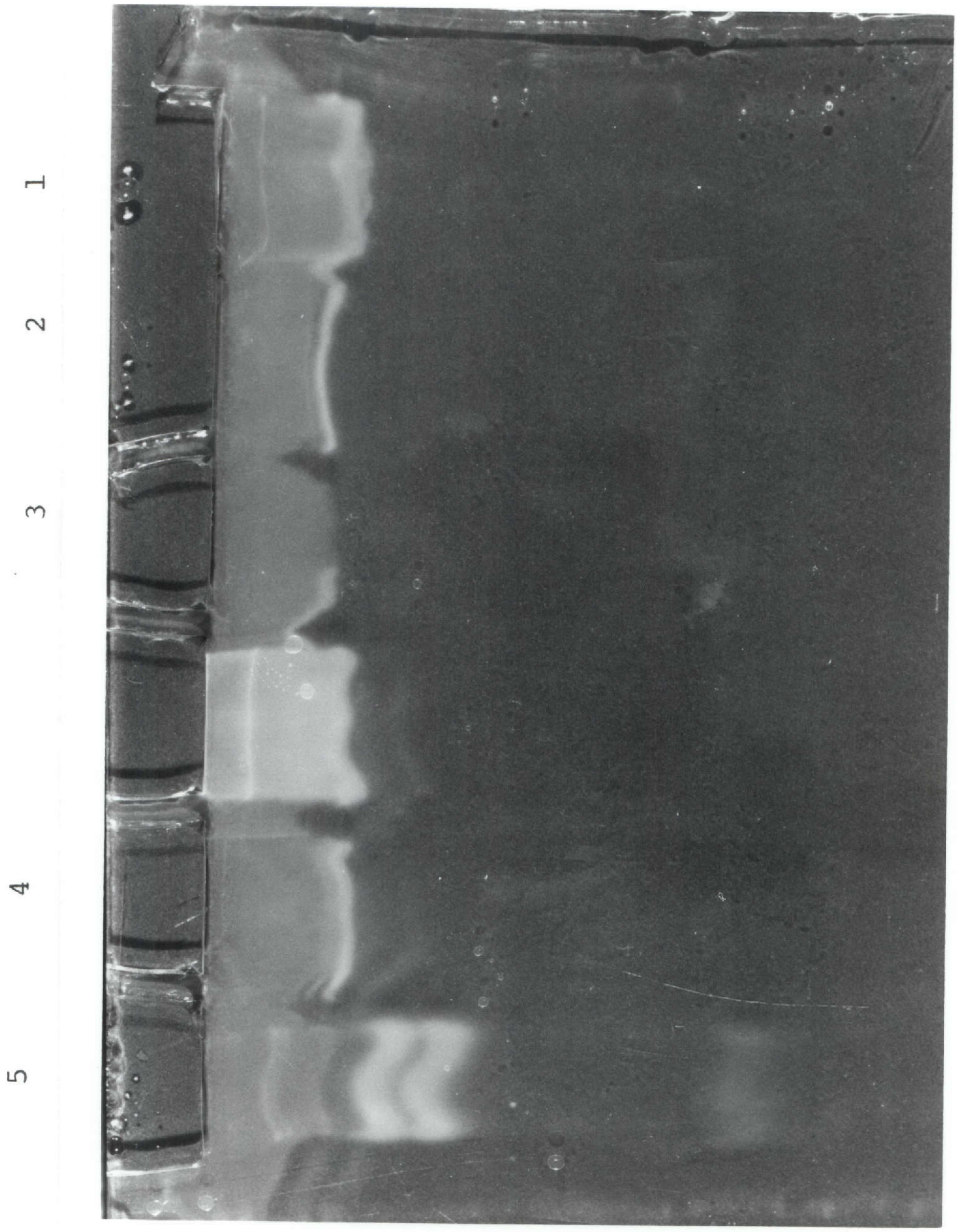


Figure 14. 6% polyacrylmide ATCase activity gel. The lanes are as follows:

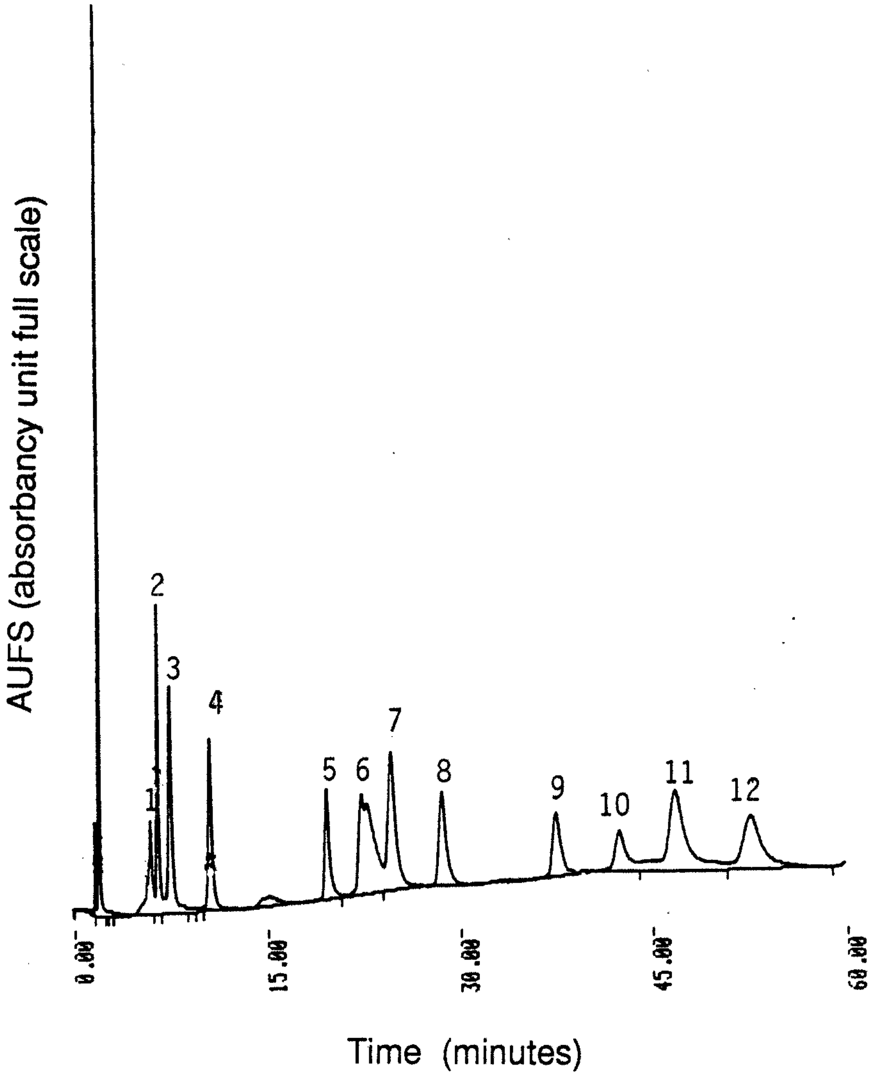
1. *P. putida* PRS2000 partially purified.
2. *P. fluorescens* ATCC-13525.
3. *P. putida* PRS2000 partial purified.
4. *A. calcoaceticus* wild type ADP-1.
5. *E. coli* EK-1104 PEK2.


1
2
3
4
5

sufficient and pyrimidine starved cells of *A. calcoaceticus* was one of the objectives of this research. It was important to ascertain if the nucleoside mono-, di-, and triphosphates could be altered in *A. calcoaceticus* strains. To answer this question, wild type *A. calcoaceticus* ADP-1, and the *A. calcoaceticus* ADP-1-218 *pyrC* were employed. Strains were grown in succinate minimal medium in the presence and absence of uracil, and uridine. All *A. calcoaceticus* cultures were grown under identical conditions on a rotary shaker water bath at 37°C. Bases and nucleosides were added at the rate of 50 µg/ml and pyrimidine auxotrophs were starved for pyrimidines for three hours at which time samples were taken. Typically, an experiment was carried out as follows: 100 to 200 ml of succinate minimal medium were inoculated with the culture to be tested in the presence (at 50 µg/ml) or absence of appropriate pyrimidines. The cells were grown to a density of 100 Klett units (10^9/ml). Fifty ml samples of culture were then harvested and treated with 6%(w/v) trichloroacetic acid to extract the nucleotides as described in Materials and Methods. Nucleotides were quantitated by HPLC.

Ribonucloeside mono-, di-, and triphosphates were measured for each of the growth conditions described in Figures 7, and 8. Profiles of the 12 ribonucleoside standards, UMP, CMP, AMP, GMP, UDP, CDP, ADP, GDP, UTP, CTP, ATP and GTP are shown in Figure 15. Since growth is

Figure 15. Chromatogram of the 12 ribonucleotide standards. The numbers refer to: 1 CMP, 2 AMP, 3 UMP, 4 GMP, 5 UDP, 6 CDP, 7 ADP, 8 GDP, 9 UTP, 10 CTP, 11 ATP, and 12 GTP. Column, radial compression Partisil 10-SAX cartridge; temperature ambient; detector sensitivity, 0.05 AUFS. Absorbance recorded at 254 nm.


AUFS (absorbancy unit full scale)
1
2
3
4
5
6
7
8
9
10
11
12
0.00
15.00
30.00
45.00
60.00
Time (minutes)

reflected best by the concentration of the triphosphates, in this dissertation only the nucleoside triphosphate levels are shown.

Table IV shows the nucleoside triphosphates for the different conditions of growth of the wild type *A. calcoaceticus* strain ADP-1. As can be seen from Table IV the overall levels of the triphosphates in *A. calcoaceticus* are similar to those reported earlier for *Salmonella typhimurium* by Kelln *et al.*, (1975). When uracil or uridine was added to the growth cultures, the UTP level increased about two-fold from 1.67 μmol/gram dry weight to 2.20 μmol (gram dry weight)$^{-1}$ while the CTP concentration increased from 0.57 to 0.90.

Table V gives the results of the nucleoside triphosphates found for the *A. calcoaceticus pyrC* mutant. It can be noted from Table V that when the *pyrC* mutant is starved for pyrimidines, the UTP and CTP levels decreased to near zero very rapidly. The possible interconversions of the nucleobases and nucleosides are presented in the Discussion.

Table IV. Nucleotide triphosphate in µmol/gram dry weight) from *A. calcoaceticus* ADP-1 wild type strain.

	Additions to SM Medium	
Nucleotide Triphosphate	None	+ Uracil
UTP	1.67	2.20
CTP	0.57	0.90
ATP	4.80	4.90
GTP	2.90	2.50

Supplements of uracil were added to succinate minimal (SM) medium at 20 µg/ml.

Table V. Nucleoside triphosphate in µmol per gram dry weight from *A. calcoaceticus* ADP-1-218 *pyrC* mutant.

	Additions to SM Medium		
Nucleoside Triphosphate	+ Uracil	+ Uridine	- Uracil
UTP	2.36	2.40	0.63
CTP	1.47	1.50	0.24
ATP	4.60	4.50	14.72
GTP	2.30	2.50	13.84

Supplements of uracil or uridine, were added to succinate minimal (SM) medium at 20 µg/ml and starvation for pyrimidines was for 3 hours.

DISCUSSION

Pyrimidine metabolism was investigated in wild-type and mutant *Acinetobacter calcoaceticus*. A Pyr$^-$ mutant was isolated for this purpose. The *pyrC* mutant was blocked in the conversion of carbamoylaspartate to dihydroorotate and required pyrimidines for growth on minimal medium. The levels of nucleotide mono-, di-, and triphosphates were measured in the wild-type and in its mutant. By starving the Pyr$^-$ mutant for pyrimidines thereby decreasing uridine and cytidine nucleotides, it was possible to derepress the synthesis of ATCase about 15-fold.

One goal of this investigation was to partially purify ATCase from *A. calcoaceticus*. It was therefore expedient to isolate a Pyr$^-$ mutant for derepression purposes. By starving this Pyr$^-$ mutant for pyrimidines it was possible to increase the synthesis of ATCase 15-fold. The mutant was found to be in the *pyrC* gene as evidenced by cross-feeding experiments (Table III). The pyrimidine requirement could be satisfied by supplying exogenous uracil or uridine, but not by cytidine or cytosine (Table II, Figure 7 and 8).

In bacterial metabolism the typical pyrimidine salvage enzymes are, uridine phosphorylase, nucleoside hydrolase, cytidine deaminase, cytosine deaminase, uridine kinase, and uracil phosphoribosyltransferase. Table VI shows the

Table VI. Distribution of pyrimidine salvage enzymes in *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa* and *Escherichia coli*.

Pyrimidine salvage Enzyme*	*A. calcoaceticus*	*P. aeruginosa*	*E. coli*
Udp	-	-	+
Nuh	+	+	-
Cdd	-	-	+
Cod	-	+	+
Udk	-	-	+
Upp	+	+	+

*The enzymes are identified by abbreviations as: Udp, uridine phosphorylase; Nuh, nucleoside hydrolase; Cdd, cytidine deaminase; Cod, cytosine deaminase; Udk, uridine kinase; Upp, uracil phosphoribosyltransferase.

enzymes operative in pyrimidine salvage pathways of *A. calcoaceticus* and for comparison, the pyrimidine salvage enzymes of *E. coli* and *P. aeruginosa*. Based on the information from this study and other studies, the pyrimidine salvage pathways of *A. calcoaceticus* can be constructed as shown in Figure 16. In comparison, the pyrimidine salvage pathways of *P. aeruginosa*, and *E. coli* are shown in Figures 17 and 18. As is evident from Table VI and Figures 16-18, the complement of enzymes in pyrimidine salvage pathway in *A. calcoaceticus* is different from that of *P. aeruginosa* and *E. coli* in the following aspects: (1) *A. calcoaceticus* differs from *E. coli* in lacking cytosine deaminase and cytidine deaminase. (2) Though it seems that *A. calcoaceticus* like *E. coli* contains uridine phosphorylase, it is probable that *A. calcoaceticus* instead contains a nucleoside hydrolase (*nuh*) that is specific for uridine (Table VI). (3) *A. calcoaceticus* does not contain cytidine deaminase and thereby is unable to metabolize cytidine. (4) The one major difference between *A. calcoaceticus* and other genera studied so far in our laboratory is its lack of cytosine deaminase (*cod*) and cytidine deaminase. Thus cytosine is not converted to uracil and accordingly, *A. calcoaceticus* Pyr$^-$ mutants are unable to use cytosine or cytidine for their pyrimidine requirement. This is in keeping with the work of ϕvrebϕ and Kleppe (1973). A similar finding was reported for *Bacillus*

Figure 16. Pyrimidine salvage pathways in *A. calcoaceticus*. Enzyme names and gene designations are: uracil phosphoribosyltransferase (*upp*); nucleoside hydrolase (*nuh*).

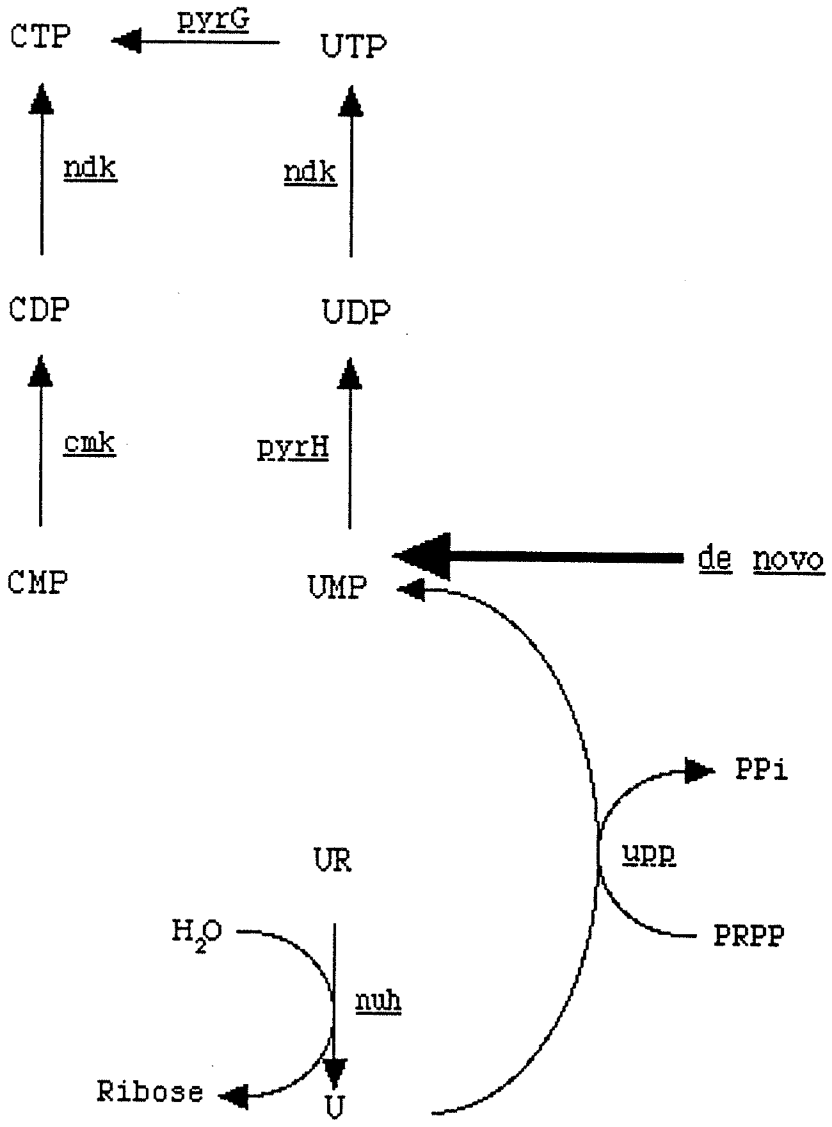

CTP
pyrG
UTP
ndk
ndk
CDP
UDP
cmk
pyrH
CMP
UMP
de novo
PPi
upp
PRPP
UR
H_2O
nuh
Ribose
U

Figure 17. Pyrimidine salvage pathways in *P. aeruginosa*. Enzyme names and gene designations are: cytosine deaminase (*cod*); uracil phosphoribosyltransferase (*upp*); nucleoside hydrolase (*nuh*); CMP or UMP phosphatases (CMP → CR; UMP → UR).

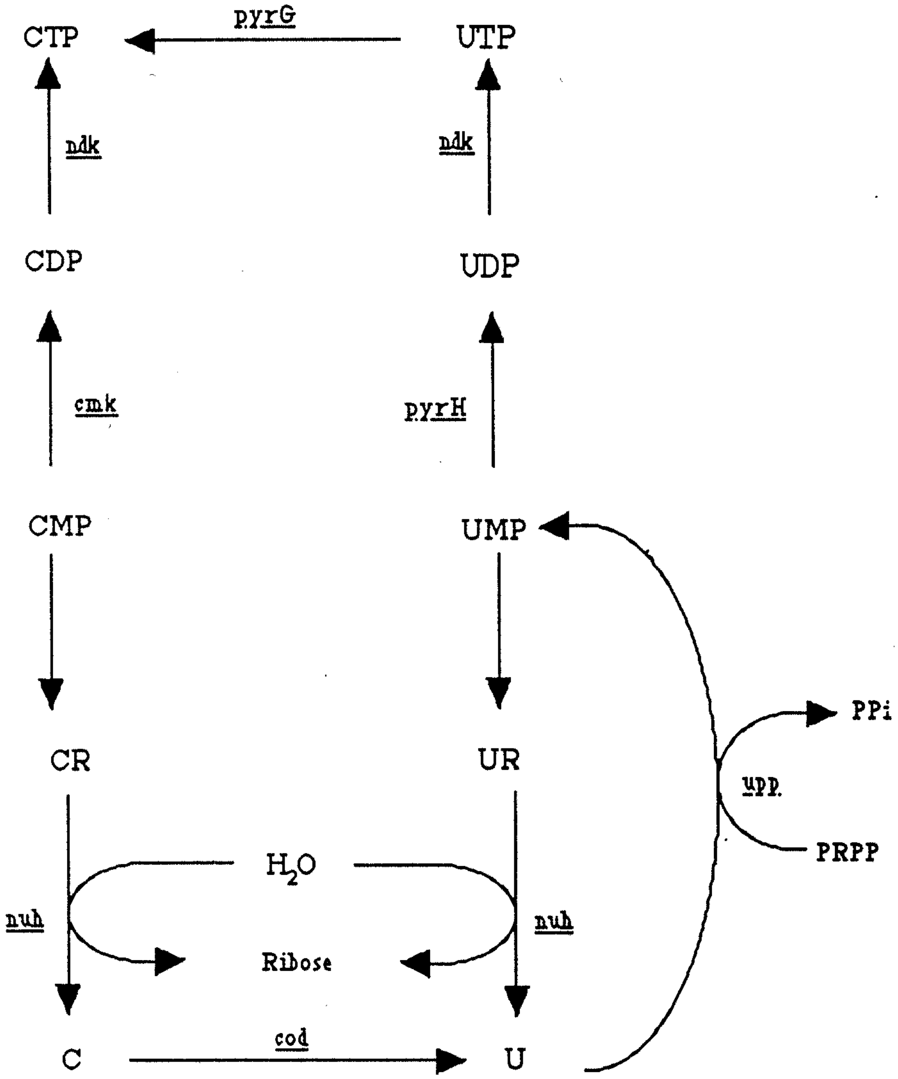
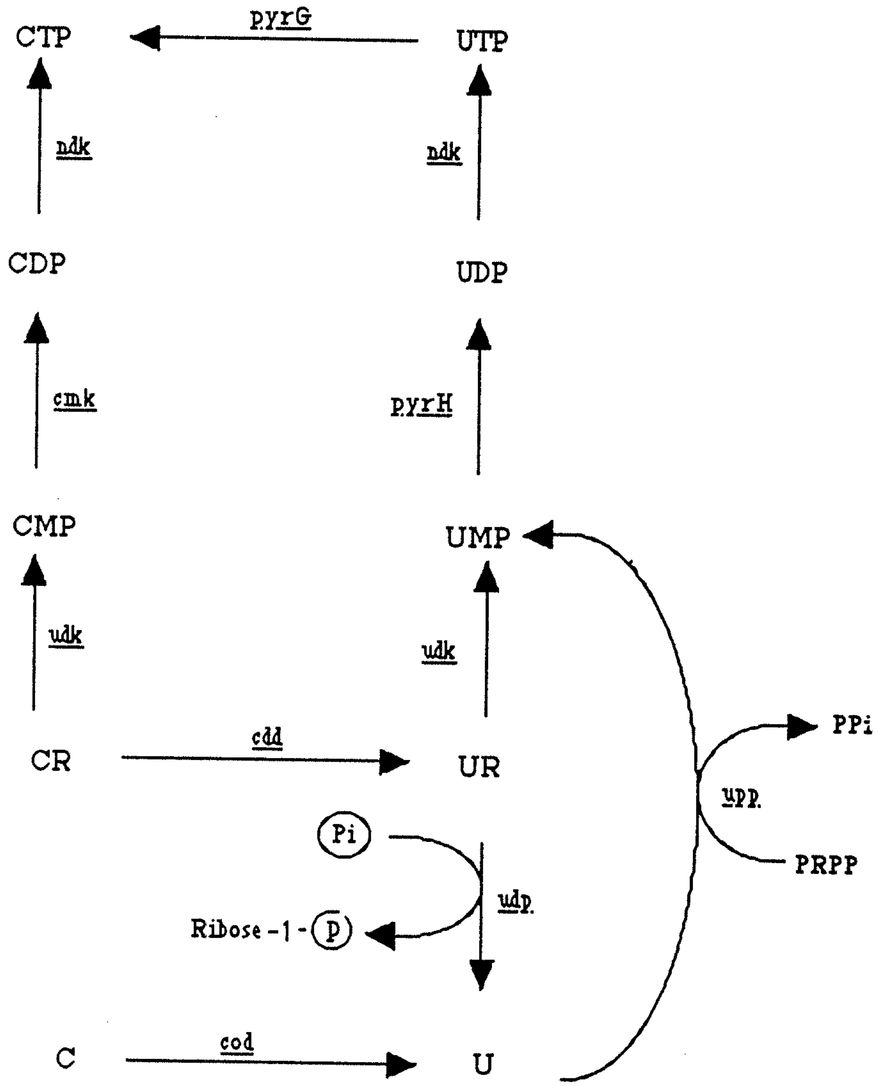

CTP
pyrG
UTP
ndk
ndk
CDP
UDP
cmk
pyrH
CMP
UMP
CR
UR
PPi
upp
PRPP
H2O
nuh
nuh
Ribose
C
cod
U

Figure 18. Pyrimidine salvage pathways in *E. coli*. Enzyme names and gene designations are: cytosine deaminase (*cod*); cytidine deaminase (*cdd*); uridine (cytidine) kinase (*udk*); uracil phosphoribosyltransferase (*upp*); uridine phosphorylase (*udp*).


CTP
pyrG
UTP
ndk
ndk
CDP
UDP
cmk
pyrH
CMP
UMP
udk
udk
CR
cdd
UR
PPi
upp
Pi
PRPP
udp
Ribose-1-P
C
cod
U

subtilis (Rebello and O'Donovan, unpublished observations).

In summary, the expression of the pyrimidine biosynthetic enzymes in *A. calcoaceticus*, is under transcriptional control with expression of ATCase being inversely proportional to the UTP concentration. The ATCase of *A. calcoaceticus* has a mw very similar to the *Pseudomonas* enzyme and differs significantly from the *E. coli* ATCase. Nucleoside triphosphate levels were comparable to those found in *S. typhimurium* (Kelln *et al.*, 1975) with starvation for uracil in a Pyr$^-$ mutant depleting the UTP pool to near zero. Concomitant to this decrease, the ATP and GTP levels increased significantly. A similar finding was observed by Dutta et al. (1992).

Thus, Pyr$^-$ mutants of *Acinetobacter* can be satisfied only by uracil and uridine. Indeed, as pointed out by ϕvrebϕ and Kleppe some Pyr$^-$ strains fail to grow on uridine making uracil phosphoribosyl transferase (*upp*) their only salvage enzyme. This might be in keeping with the unique finding that *A. calcoaceticus* is so efficient for DNA uptake for transformation. Availability of transforming DNA that became incorporated into the chromosome could make the need for the pyrimidine salvage enzymes redundant, leading to their subsequent loss. Similar findings have been reported for the purine salvage enzymes (ϕvrebϕ and Kleppe).

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