COMPARISON OF ASPARTATE TRANSCARBAMOYLASE AND PYRIMIDINE SALVAGE IN *SPOROSARCINA UREAE, SPOROLACTOBACILLUS INULINUS, LACTOBACILLUS FERMENTUM, AND MICROCOCCUS LUTEUS*

THESIS

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

For the Degree of

MASTER OF SCIENCE

By

Vincent N. Barron, B.S.
Denton, Texas

August, 1994

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The enzyme that catalyzes the committed step in pyrimidine biosynthesis, aspartate transcarbamoylase, has been compared in selected endospore-forming organisms and in morphologically similar control organisms.

The ATCases and pyrimidine salvage from *Sporosarcina ureae, Sporolactobacillus inulinus, Lactobacillus fermentum,* and *Micrococcus luteus* were compared to those of *Bacillus subtilis*. While the ATCases *from Sporosarcina ureae, Sporolactobacillus inulinus*, and *L. fermentum* were found to exhibit characteristics to that of *Bacillus* with respect to molecular weight and kinetics, *M. luteus* ATCase was larger at approximately 480 kDa. Furthermore, pyrimidine salvage in *Sporosarcina ureae* and *M. luteus* was identical to those of *B. subtilis*, while pyrimidine salvage of *Sporolactobacillus inulinus* and *L. fermentum* resembled that of the pseudomonads.

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INTRODUCTION

Enzyme characterization and regulation of pyrimidine nucleotides are relatively unexplored areas in all but a few key genera. The molecular genetics and enzymology of pyrimidine biosynthesis have been dutifully studied in genera such as *Escherichia, Salmonella, Bacillus, Yersinia,* and most recently *Pseudomonas*. Little is known about the biosynthesis of pyrimidine nucleotides in the endospore-forming organisms other than in *Bacillus subtilis* and *Bacillus caldolydicus* (Switzer and Quinn, 1993). Two such organisms are *Sporosarcina ureae* and *Sporolactobacillus inulinus*. Morphologically similar organisms that do not form endospores, such as *Lactobacillus fermentum,* have been described in some detail (Chassy and Murphy, 1993) and serve as excellent controls for the study of endospore-forming organisms. While *Sporosarcina ureae* and *Lactobacillus fermentum* have been morphologically and metabolically characterized, *Sporolactobacillus inulinus* has not. Taxonomically, cocci that differentiate into true endospores are placed in the genus *Sporosarcina*. Currently, two species of this genus are recognized: *S. halophila* and *S. ureae*. These bacteria share some characteristics with the

micrococci as their cell wall lacks diaminopimelic acid and contains lysine (Priest, 1992).

The DNA base composition (G + C ratio) is also used to differentiate *Sporosarcina* from the micrococci. *Sporosarcina* has a low G + C ratio (43%) compared to the micrococci which have a high G + C ratio (78%) (Priest, 1992). Bacteria isolated initially from chicken feeds and subsequently from the rhizospheres of plants have been placed in the genus *Sporolactobacillus* because they share characteristics with both *Bacillus* and *Lactobacillus* (Norris, 1981). In particular, *Sporolactobacillus* differentiates into true endospores, but like the lactobacilli, lack catalase, grow optimally under microaerophilic (or anaerobic) conditions, and produce lactic acid in fermentation. Physiologically, the genus resembles *Bacillus coagulans* (although the latter is catalase positive with homolactic fermentation) and rRNA analyses show significant sequence homology between *Sporolactobacillus* and *B. coagulans* (Norris, 1981). Any aerobic endospore-forming bacteria excluded from the genera *Sporolactobacillus* and *Sporosarcina* have been placed in the genus *Bacillus*. As mentioned previously, the genus *Lactobacillus* is catalase negative, carries out lactic acid

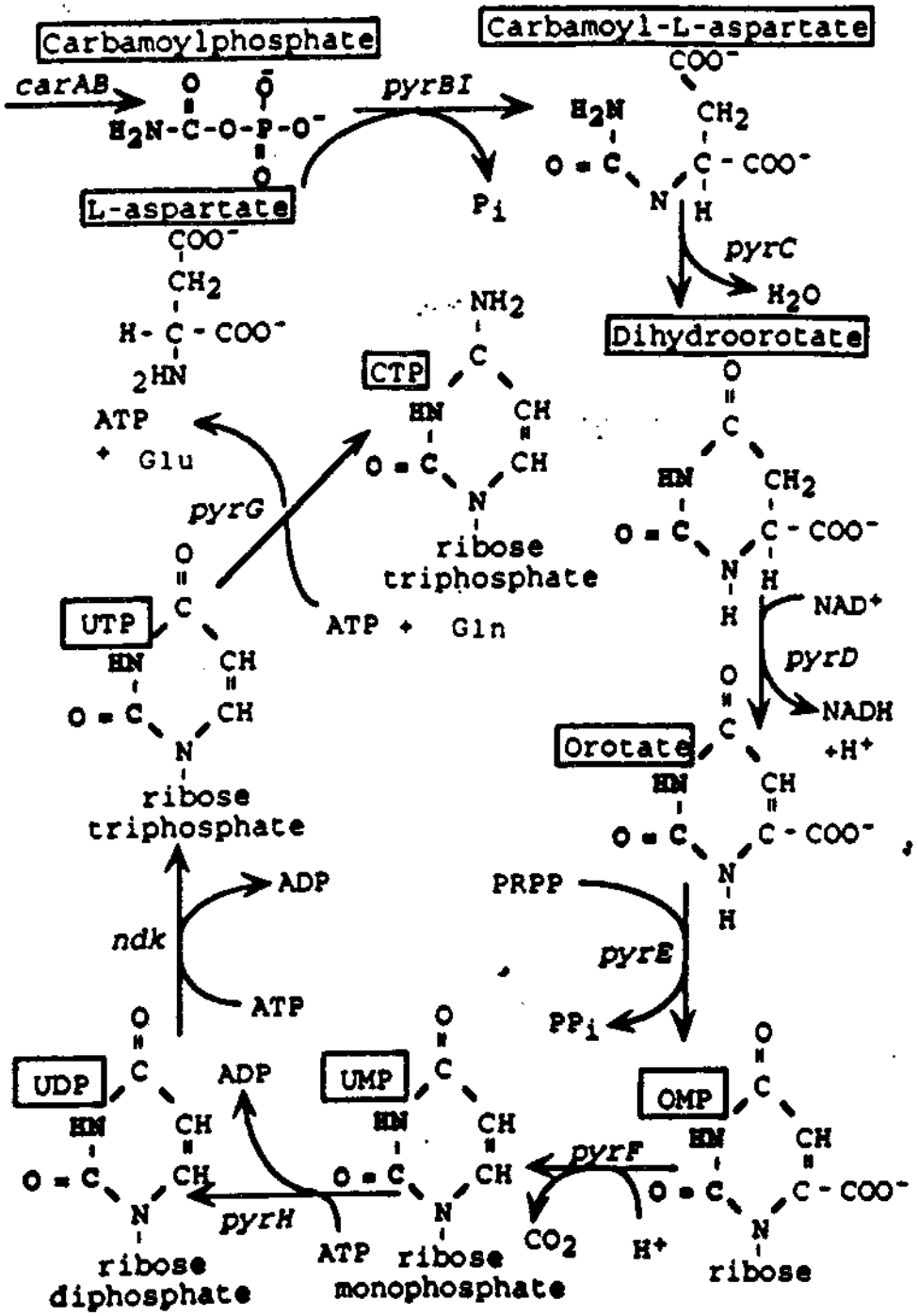
fermentation, and grows optimally in microaerophilic conditions, with growth occurring up to 42°C, making *Lactobacillus* slightly thermophilic. This genus is somewhat similar to *Bacillus* in morphological terms (Gram-positive rods) and in rRNA homology (Norris, 1981). Lactobacilli are also classified on the basis of the enzymes present in sugar metabolism in their respective heterofermentative pathways. My interest in these organisms stems from their peculiar characteristics with respect to pyrimidine biosynthesis and pyrimidine salvage. Thus, it is important to study the nature of the most significant enzyme in pyrimidine biosynthesis, aspartate transcarbamoylase (ATCase; EC 2.1.3.2), and to identify the enzymes present in pyrimidine salvage pathways. Questions to be addressed include: (1) Since *Sporosarcina ureae* is a true endospore-former, does it have a pyrimidine pathway or an ATCase like those of *Bacillus,* or (2) since *S. ureae* is a coccus, does it behave like *Micrococcus*? These questions will be explored for the enzyme ATCase and for the pyrimidine salvage pathways both of which characteristics have been used previously as taxonomic markers (Neuhard, 1987). Analogous questions will be applied to *Lactobacillus fermentum* and *Sporolactobacillus inulinus* and answers sought as for (1) and (2).

The Pyrimidine *de novo* Pathway. The *de novo* pathway of pyrimidine biosynthesis is universal and almost identical in all organisms. While the assimilation of pyrimidine nucleotides is similar, the regulation of these compounds is quite distinct in different organisms. The *Escherichia coli* pathway has gained the most attention over a number of years and it is this pathway that will be described in detail. Pyrimidine biosynthesis is generally regulated at the level of enzyme activity (feedback inhibition and activation) and enzyme synthesis (attenuation or repression). Since ATCase is the committed step in pyrimidine biosynthesis in *E. coli*, it is regulated. ATCase has been extensively studied and is one of the best characterized bacterial enzymes, exhibiting all of the characteristics of an allosteric enzyme.

In *E. coli*, the biosynthesis of pyrimidines is accomplished through six unlinked genes, two of which constitute small operons. These are designated as *carAB*, *pyrBI*, *pyrC*, *pyrD*, *pyrE* and *pyrF*. These genes encode the enzymes for synthesis of UMP, the precursor of all pyrimidine ribonucleotides (Neuhard, 1987). The pathway also provides precursors for deoxyribonucleotide synthesis, namely dCTP and dTTP (O'Donovan and Neuhard, 1970).

The first enzyme of the pathway, encoded by the operon *carAB*, is carbamoylphosphate synthetase (CPSase;EC 2.7.2.5). The reaction it catalyzes is the condensation of HCO_3^- or CO_2 and NH_3 (from glutamine) with the consumption of two moles of ATP per mole carbamoylphosphate formed (Abdelal and Ingraham, 1975). This enzyme is present in microorganisms and in the cytosol of all eukaryotic cells capable of forming pyrimidine nucleotides. Eukaryotes also have a CPSase that uses ammonia as a substrate instead of glutamine. It is associated with citrulline formation in the pathway for arginine biosynthesis. ATCase in eukaryotes is strikingly different from the *E. coli* enzyme. This came to light through analysis of ATCase inhibition by N-phosphonoacetyl-L-aspartate (PALA). This compound, synthesized as an analog of the putative transition state complex formed between the two substrates, inhibits pyrimidine synthesis in mammalian cells. However, cells eventually develop resistance to it, because levels of ATCase rise in these cells beyond the capacity of PALA to inhibit all activity. These resistant cells were found to contain similarly elevated levels of carbamoylphosphate synthetase and dihydroorotase. The explanation for this observation came with the discovery of a single protein

Figure 1. The pyrimidine *de novo* pathway from *E. coli*. *carAB*, carbamoylphosphate synthetase; *pyrBI*, aspartate transcarbamoylase (catalytic and regulatory subunits, respectively); *pyrC*, dihydroorotase; *pyrD*, dihydroorotate dehydrogenase; *pyrE*, orotate phosphoribosyl transferase; *pyrF*, orotidylate decarboxylase; *pyrH*, uridylate kinase; *ndk*, nucleoside diphosphate kinase; *pyrG*, cytidine triphosphate synthetase; PRPP, 5'-phosphoribosyl-1'-pyrophosphate.


Carbamoylphosphate
carAB
L-aspartate
pyrBI
Pi
Carbamoyl-L-aspartate
pyrC
H2O
Dihydroorotate
NAD+
pyrD
NADH
+H+
Orotate
PRPP
pyrE
PPi
OMP
pyrF
CO2
H+
ribose
UMP
ribose
monophosphate
pyrH
ATP
UDP
ADP
ndk
ATP
ribose
diphosphate
UTP
ATP + Glu
pyrG
ATP + Gln
CTP
ribose
triphosphate
ribose
triphosphate

containing three identical polypeptide chains, each of a molecular weight of 230 kDa that catalyzes all three reactions. George Stark has given this trifunctional enzyme the acronym CAD, for the respective enzymatic activities involved with this single polypeptide (Stark, 1973).

The first committed step of pyrimidine nucleotide biosynthesis is encoded by the second operon in the enteric bacteria, *pyrBI*. This enzyme, ATCase, is involved in the carbamoylation of the amino group of carbamoylphosphate to produce carbamoylaspartate. The enzyme is activated by adenosine triphosphate (ATP) and feedback inhibited by cytidine triphosphate (CTP) (Gerhart and Pardee, 1962; Gerhart and Schachman, 1965). On the level of enzyme synthesis, ATCase is regulated by attenuation in which the concentration of uridine triphosphate (UTP) determines whether or not *pyrBI* is transcribed and translated into the active enzyme. These two mechanisms of regulation will be addressed in more detail later in this paper since ATCase is one of the focal points of this study.

In the third reaction of the pathway, encoded by *pyrC*, the pyrimidine ring is closed by dihydroorotase (DHOase; EC 3.5.2.3) with the formation of L-dihydroorotate. The equilibrium in this reaction is pH-dependent, becoming more

favorable at low pH values. In most organisms studied, DHOase is a very unstable enzyme (Neuhard, 1987). L-dihydroorotate is then oxidized to orotate, catalyzed by the enzyme dihydroorotate dehydrogenase (DHOdehase; EC 1.3.3.1) which is encoded by *pyrD*. Orotate then combines with the phosphoribosyl group of 5-phosphoribosyl-1-pyrophosphate (PRPP) to form orotidine-5-monophosphate (OMP), the first pyrimidine mononucleotide to be synthesized. The reaction is catalyzed by orotate phosphoribosyltransferase (OMPppase; EC 2.4.2.10) and is encoded by *pyrE*. In the ensuing reaction, OMP is decarboxylated to uridine-5-monophosphate (UMP) by the *pyrF* encoded OMP decarboxylase (OMPdecase; EC 4.1.1.23). A highly specific UMP kinase, encoded by *pyrH*, catalyzes the phosphorylation of UMP to uridine-5-diphosphate (UDP), with ATP as the external source of the phosphate. UDP is subsequently phosphorylated to uridine-5-triphosphate (UTP) by nucleoside diphosphokinase, *ndk*, which is non-specific for the diphosphate base of the last substrate. The ultimate step in the biosynthesis of pyrimidine nucleotides is catalyzed by cytidine-5-triphosphate synthase, which is encoded by *pyrG*. In this reaction, cytidine-5-triphosphate (CTP) is formed by the reaction of UTP with glutamine serving as the amino donor.

The reaction is driven by the concomitant hydrolysis of ATP to ADP and an inorganic phosphate.

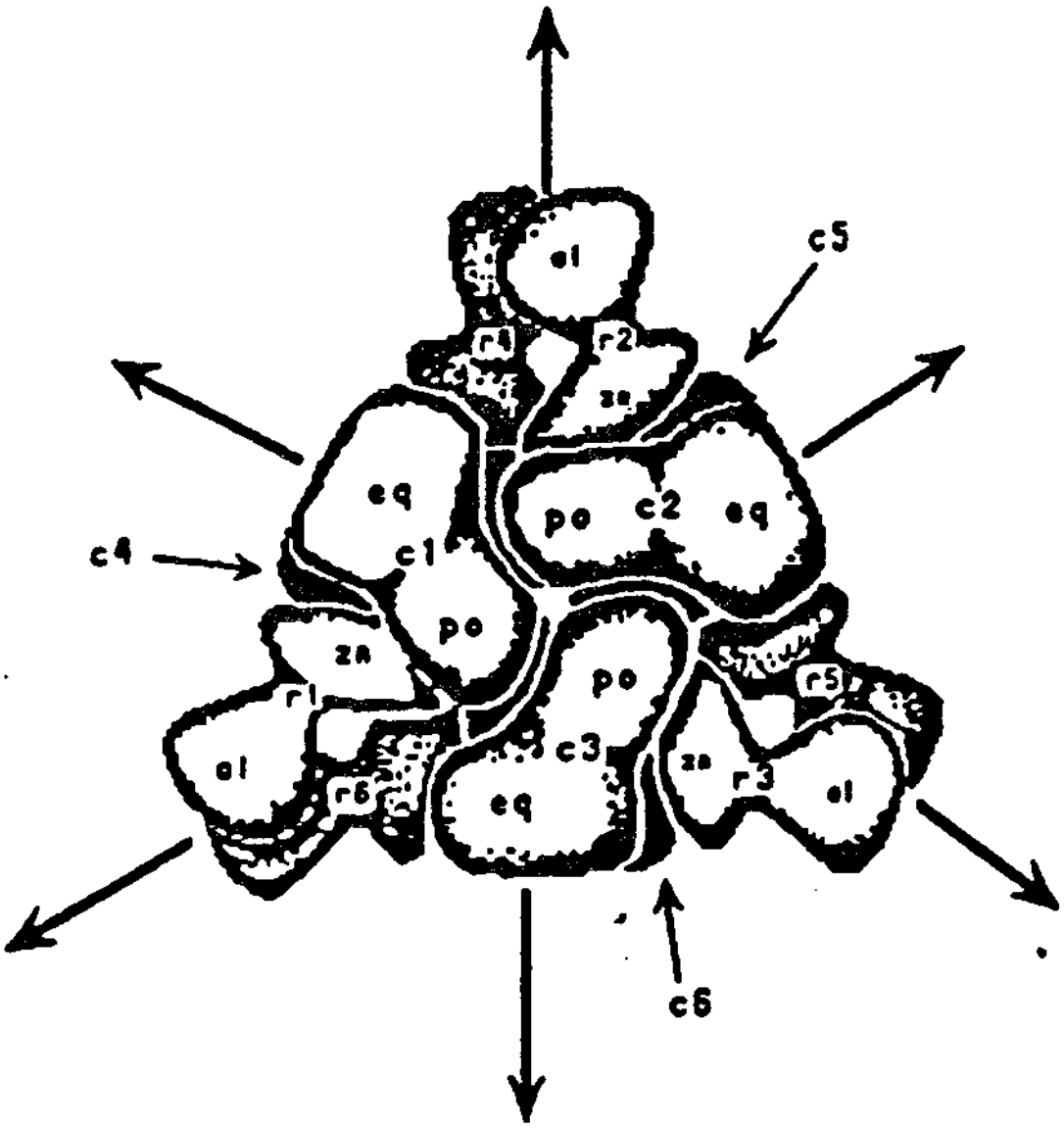
***E. coli* ATCase.** The ATCase from *E. coli* remains one of the best studied regulatory enzymes. The catalytic activity of ATCase is dependent on the formation of an active site from half-sites on separate subunits (Honzatko *et al.*, 1982; Kantrowitz *et al.*, 1980; Rosenbusch and Weber, 1971). The enzyme consists of two trimeric proteins ($2c_3$) while three dimeric polypeptides ($3r_2$) bind nucleotide effectors (ATP and CTP) to give regulatory effects. ATP enhances activity while CTP inhibits activity.

The *pyrBI* genes lie contiguous to one another and are expressed coordinately. The *pyrB* gene encodes the catalytic polypeptide chain (c) while the *pyrI* gene encodes the regulatory polypeptide chain (r). These c and r chains have molecular weights of 33 and 17 kDa, respectively (Allewell, 1989). Together, these polypeptide chains form a dodecamer (Figure 2) denoted in shorthand as $2c_3:3r_2$. The structural derivation of ATCase from *E. coli* stems greatly from the work of Lipscomb. The three-dimensional structure is now known with a resolution of 2.5Å (Honzatko *et al.*, 1982; Ke *et al.*, 1984). The amino acid sequence of ATCase is known for 11 different enzymes. In *E. coli*, two tryptophan

residues and a cysteine residue are found near the catalytic site of the enzyme. The catalytic chain containing these residues is characterized by a complexity of alpha helices, accompanied by two domains, denoted polar (amino-terminus) and equatorial (carboxy-terminus) (Honzatko *et al.*, 1982). The actual catalytic site is found concealed between the interfaces of two adjacent catalytic chains of the same trimer with the amino acid sequence of the active site being histidine, proline, threonine, and glutamine (HPTQ). The r chain also two domains, the allosteric domain, and the zinc domain, which correspond to the amino-terminal (allosteric) and carboxy-terminal (zinc) regions (Gerhart and Schachman, 1965). The principle nucleotide binding site is found in the amino-terminal regulatory chain with a group of four cysteine residues near the carboxy-terminal region. This "coalition" of zinc ions to these cysteine residues is required for the interactions between the catalytic and regulatory subunits.

The interaction of the regulatory subunits of *E. coli* ATCase plays an important role in the kinetics of the enzyme. The binding of ATP and CTP to the regulatory sites was shown to be anticoorperative, by which these compete for a single site on the regulatory subunit (Buckman, 1970;

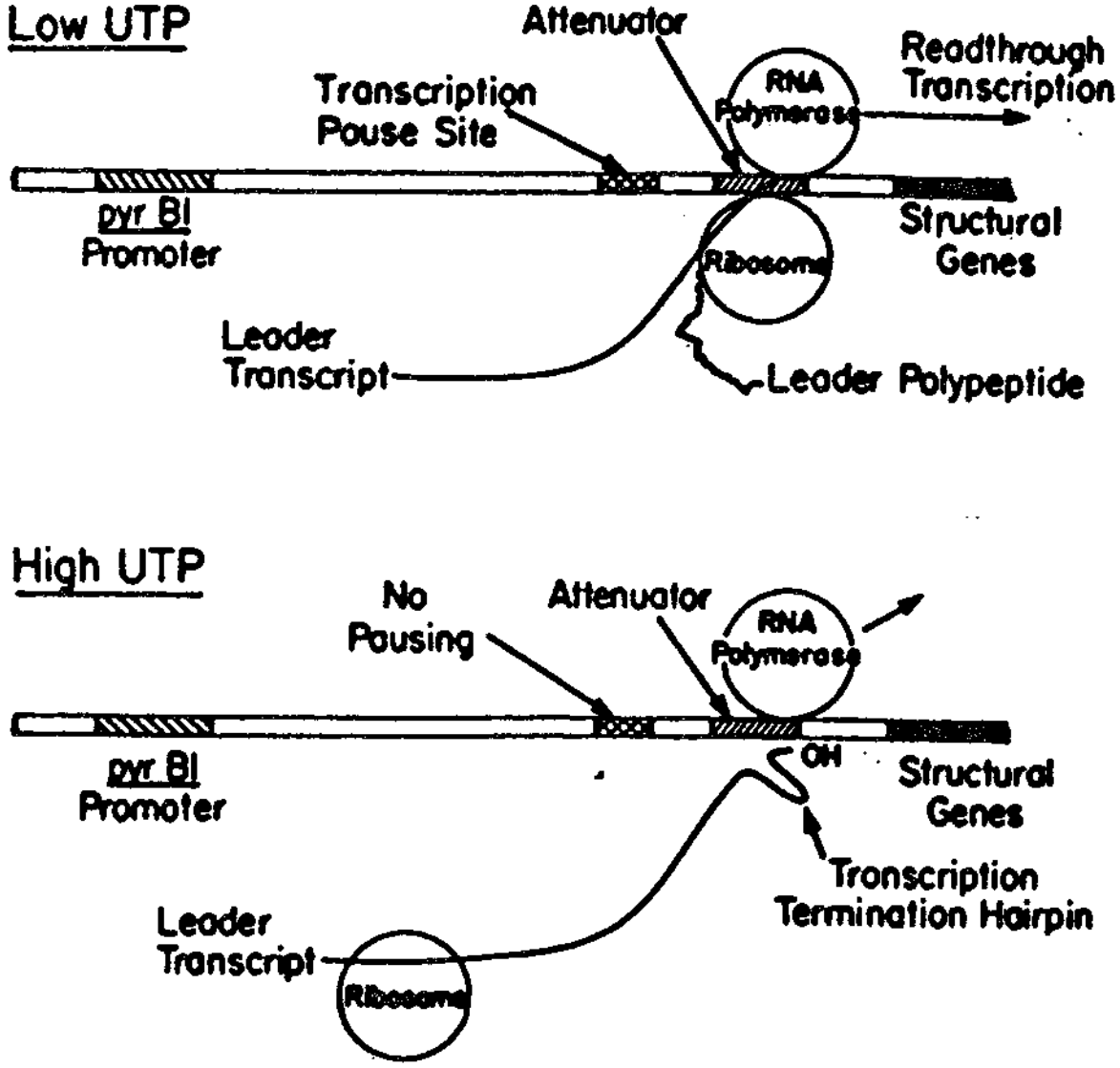
Figure 2. The quaternary structure of *E. coli* ATCase. Taken from Krause *et al.*, 1987.


c5
c4
c6
eq
c1
po
c2
eq
zn
r1
po
po
c3
eq
zn
r3
el
el
r6
r5
r4
r2
zn

Winland-Gray *et al.*, 1973). The catalytic subunit itself exhibits Michaelis-Menten kinetics when velocity vs. substrate plots are made. It does not show any inhibition by CTP or activation by ATP (Gerhart and Schachman, 1965). It has also been shown that UTP works in union with CTP to magnify the inhibition of the enzyme (Corder and Wild, 1989). By these mechanisms of activation and inhibition of ATCase, purine and pyrimidine nucleotides are maintained at a relatively balanced level in the cell.

Attenuation of *E. coli* ATCase. Regulation of ATCase from *E. coli* is not only at the level of enzyme activity, but also at the level of translation. As first proposed by Roof (Roof, 1982) and later by Turnbough (Turnbough, 1983), a unique model for regulation of operon expression exists in which low UTP levels cause close coupling of transcription and translation of a *pyrBI* leader region (Figure 3). This close coupling suppresses transcriptional termination at an attenuator proceeding the structural genes. When the intracellular level of UTP is low as a consequence of pyrimidine limitation, RNA polymerase stalls at the UTP-sensitive pause site proceeding the attenuator. This pause permits a ribosome to initiate translation of a 44-amino acid leader polypeptide, while continuing to translate up to

Figure 3. Model for the regulation of *pyrBI* operon expression as taken from Turnbough (Turnbough, 1983). The model shows the relative positions of RNA polymerase and the translating ribosome when UTP levels are either low or high.


Low UTP
Attenuator
Readthrough Transcription
RNA Polymerase
Transcription Pouse Site
pyr BI Promoter
Structural Genes
Ribosome
Leader Transcript
Leader Polypeptide
High UTP
No Pausing
Attenuator
RNA Polymerase
pyr BI Promoter
OH
Structural Genes
Tronscription Termination Hairpin
Leader Transcript
Ribosome

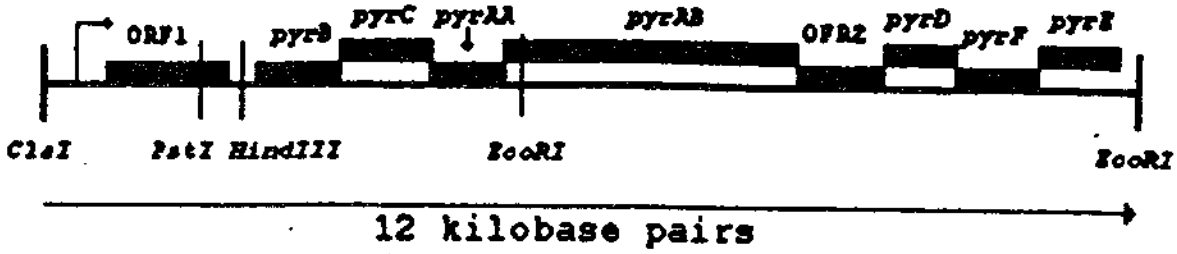
the stalled RNA polymerase. When the polymerase eventually passes this pause site and transcribes the attenuator, the formation of the attenuator encoded RNA hairpin necessary for termination is precluded or immediately disrupted by the adjacent translating ribosome. The disruption of termination of the leader transcript allows RNA polymerase to continue transcription into the structural genes. Translation of the leader polypeptide is then terminated within the ribosome binding site preceding the ATCase catalytic subunit. When the intracellular level of UTP is high, RNA polymerase does not pause during the transcription of the leader region. This rapid transcription does not permit a ribosome to bind to the transcript and catch up to RNA polymerase before the formation of the attenuator-encoded hairpin. The result is termination of transcription before the structural genes can be transcribed (Turnbough, 1983).

***Bacillus subtilis* ATCase.** The ATCase of *B. subtilis* presents interesting comparative biochemical contrasts to the *E. coli* enzyme. Native *B. subtilis* ATCase is a trimer of catalytic polypeptides and, unlike the *E. coli* enzyme, neither has or binds regulatory subunits. The enzyme activity is not affected by nucleotides, and substrate

saturation obeys Michaelis-Menten kinetics. Although *B. subtilis* ATCase does not exhibit any inhibition by nucleotides, strong evidence has accumulated suggesting the *B. subtilis* enzyme is regulated by transcription termination-antitermination (Quinn and Switzer, 1993). The *de novo* genes for pyrimidine biosynthesis are grouped in one operon (Quinn and Switzer, 1993). The *B. subtilis pyr* operon contains 10 genes and covers 12 kilobases (kb) of DNA (Figure 4). The genes are encoded in order of open reading frame 1A, ORF1B, *pyrB, pyrC, pyrAA, pyrAB,* ORF2, *pyrD, pyrF,* and *pyrE* (Quinn and Switzer, 1993); and are seemingly transcribed as a single mRNA from one promoter. Extensive overlapping of adjacent ORFs in coordinately regulated gene clusters are seen in Figure 4 with *B. subtilis* and are thought to reflect a mechanism of translational coupling that ensures formation of equivalent numbers of polypeptides from the various cistrons (Zalkin and Ebbole, 1988).

Classification of ATCases. In 1969, Bethell and Jones described three classes of bacterial ATCases as seen in Table 1. The characterization of these ATCases corresponded to three major classes of bacteria. Gram-positive bacteria, such as *Bacillus,* typically have Class C ATCase enzymes with a molecular weight of 100 kDa and hyperbolic kinetics

Figure 4. Schematic diagram of the *B. subtilis pyr* operon as adapted from Switzer (Quinn and Switzer, 1993). A dark box represents the reading frame for each gene, with the overlap between boxes reflecting the overlap between reading frames in an exaggerated form. Selected restriction sites important in cloning the DNA are indicated. The arrow represents the start of transcription.


ORF1
pyrB
pyrC
pyrAA
pyrAB
ORF2
pyrD
pyrF
pyrE
ClaI
PstI
HindIII
EcoRI
EcoRI
12 kilobase pairs

Bethell and Jones, 1969; Brabson and Switzer, 1975; Neumann and Jones, 1964). The enzymes consist of catalytic trimers, resembling the unregulated catalytic trimers of *E. coli*, and are not inhibited by any pyrimidine nucleotides. Class B ATCases are found in Gram negative facultative anaerobic bacteria, such as *E. coli* and *Salmonella typhimurium*.

Table 1. Characteristics of Bacterial ATCase Enzymes.

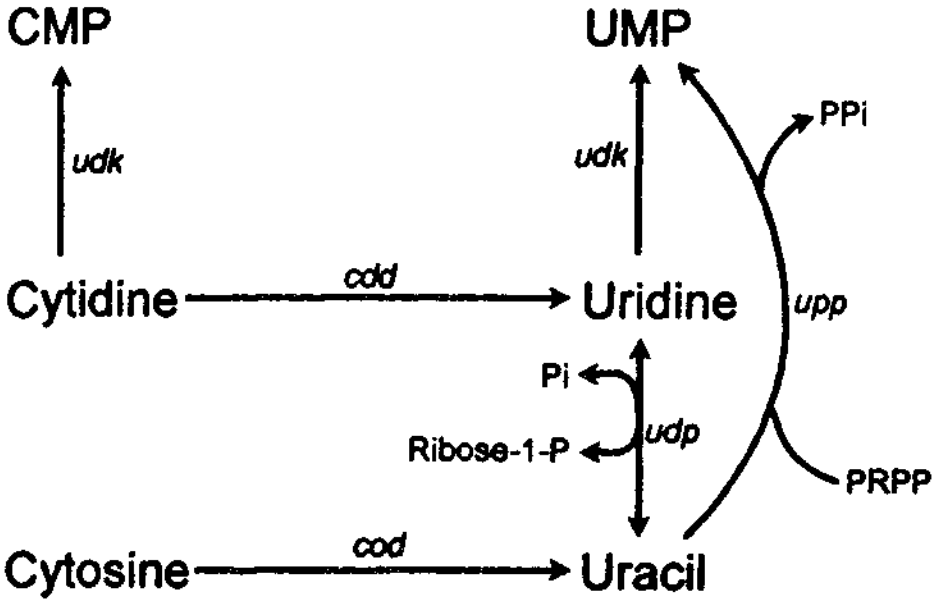
Distinction	**Class A**	**Class B**	**Class C**
Molecular Weight	360-480 kDa	300-320 kDa	100-140 kDa
Substrate saturation curve in absence of nucleotide	Hyperbolic - No cooperativity	Sigmoidal - Cooperativity	Hyperbolic - No cooperativity
Response to exogenous pyrimidines	UTP;CTP inhibit	CTP inhibits, ATP activates	No response to ATP, CTP or UTP
Heat and organomercural treatment	No dissociation	Dissociation	Not applicable

These enzymes are dodecameric ($2c_3:3r_2$) in form with a molecular weight of approximately 300 kDa and exhibit sigmoidal substrate kinetics, indicating cooperativity. Class A enzymes are found in *Pseudomonas putida, Pseudomonas fluorescens,* and *Pseudomonas aeruginosa* with a much larger molecular weight of approximately 480 kDa (Schurr, Ph.D.

Dissertation, University of North Texas, 1993; Vickrey, Ph.D. Dissertation, University of North Texas, 1993). In general, the *Pseudomonas* holoenzyme is inhibited by all three nucleotide triphosphates, CDP, pyrophosphates, and orthophosphate at limiting concentrations of carbamoylphosphate (Vickrey, Ph.D. Dissertation, University of North Texas, 1993).

Pyrimidine salvage. In addition to the enzymes for synthesis of nucleotides *de novo* described in the preceding sections of this paper, enzymes are widely distributed in both mammalian tissues and microorganisms that catalyze the synthesis of pyrimidine mononucleotides from pyrimidine bases. Many bacteria take up pyrimidine bases efficiently for nucleotide synthesis, and a phosphoribosyltransferase has been identified in all studied species of bacteria (O'Donovan and Neuhard, 1970). By contrast, exogenous pyrimidines are utilized poorly by mammalian cells. The most widely known salvage pathways of the enteric bacteria include *E. coli* and *Salmonella typhimurium* (Figure 5). Pyrimidine analogs have helped immensely in the elucidation of the enzymes involved in the salvage pathway (O'Donovan and Neuhard, 1970) and are included here for completeness. Cytosine deaminase, encoded by the *cod* gene, catalyzes the

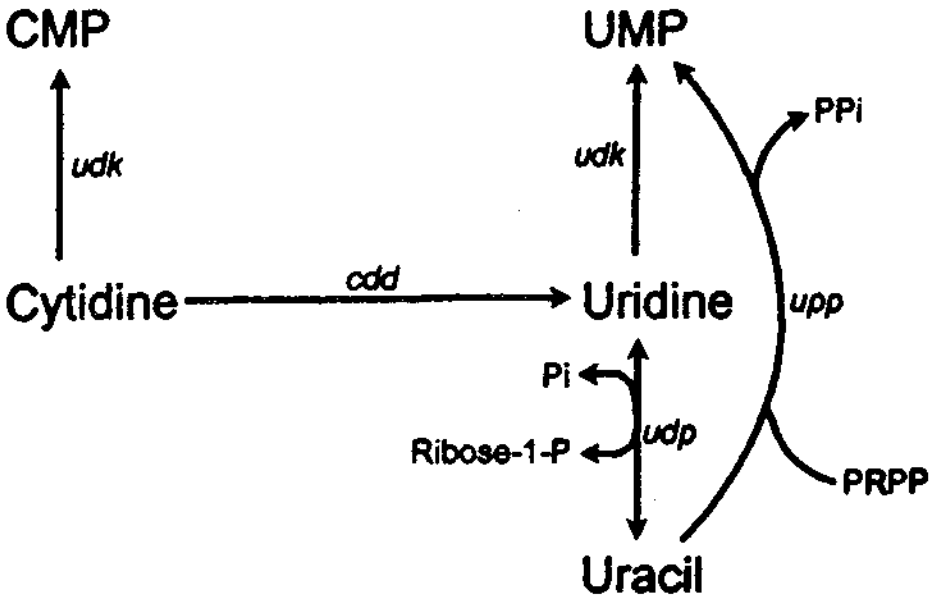
Figure 5. Metabolism of pyrimidine bases and nucleosides in *Escherichia coli* and *Salmonella typhimurium*. Individual reactions are identified by gene symbols. *cod*, cytosine deaminase; *cdd*, cytidine deaminase; PRPP, 5'-phosphoribosyl-1'-pyrophosphate; *udp*, uridine phosphorylase; *upp*, uracil phosphoribosyltransferase; *udk*, uridine kinase. Adapted from O'Donovan and Neuhard, 1970.


CMP
UMP
udk
udk
PPi
Cytidine
cdd
Uridine
upp
Pi
Ribose-1-P
udp
PRPP
Cytosine
cod
Uracil

deamination of cytosine to uracil which is then converted to UMP directly by uracil phosphoribosyltransferase (*upp*). To a lesser extent in the wild type or in a *upp* mutant, uracil may be converted directly to uridine by uridine phosphorylase (*udp*) acting in reverse. Cytosine deaminase seems to be a non-specific enzyme since several analogs, such as 6-azacytosine, isocytosine, 5-fluorocytosine, and 5-azauracil, can act as substrates (O'Donovan and Neuhard, 1970). Cytosine deaminase-deficient mutants are easily characterized since they are 5-fluorocytosine resistant and 5-fluorouracil sensitive (Neuhard, 1968; Neuhard and Ingraham, 1968). In a similarly irreversible reaction, cytidine is deaminated to uridine by cytidine deaminase (*cdd*). Uridine kinase catalyzes a non-specific reaction to which either uridine or cytidine can serve as substrates and are converted to their respective mononucleotides. Uridine kinase has been reported to be feedback inhibited by CTP and UTP in *E. coli* and *Salmonella typhimurium* (Anderson and Brockman, 1964; Neuhard, 1968).

Pyrimidine salvage in *B. subtilis*. The pyrimidine salvage pathway of *B. subtilis* and related organisms is shown in Figure 6. The pathways by which pyrimidine bases and nucleosides are converted to the nucleotide level vary

Figure 6. Pyrimidine salvage in *B. subtilis*. Individual reactions are identified by gene symbols. *cdd*, cytidine deaminase; PRPP, 5'-phosphoribosyl-1'-pyrophosphate; *udp*, uridine phosphorylase; *udk*, uridine kinase; *upp*, uracil phosphoribosyltransferase.


CMP
UMP
udk
udk
PPi
Cytidine
cdd
Uridine
upp
Pi
Ribose-1-P
udp
PRPP
Uracil

among the different bacteria studied. A key enzyme is uracil phosphoribosyltransferase, which is present in all Gram-positive bacteria (Nygaard, 1993). Each function has been ascertained by studies of mutant strains with single or multiple blocks in the pathway (Rima, 1977; Neuhard, 1983). As in *E. coli*, mutants that are deficient in particular salvage enzymes can be isolated by selecting to resistance to pyrimidine analogs. Mutants defective in these enzymes require exogenous pyrimidines for growth. In *B. subtilis*, uracil is converted directly to UMP by uracil phosphoribosyltransferase (*upp*) or converted to UMP in two steps by the combination of uridine phosphorylase (*udp*) and uridine kinase (*udk*). Uridine is also taken up directly and phosphorylated to UMP by the non-specific nucleoside kinase. Thus, mutants defective in the *upp* gene can still take up uracil and convert it to UMP by the intermediate formation of uridine catalyzed by pyrimidine nucleoside phosphorylase and uridine kinase. *B. subtilis* and several *Lactobacillus* species lack the ability to deaminate cytosine (Neuhard, 1983). So far, cytosine deaminase activity has not been reported in any Gram-positive bacteria (Neuhard, 1983). Enterics and similar bacteria are the only bacteria that

possess cytosine deaminase activity and can meet pyrimidine requirements with cytosine.

Several peculiarities have been noted in the lactobacilli. For example, reduction of the ribonucleotides to deoxyribonucleotides occurs at the triphosphate level, not at the diphosphate level as seen in most other organisms. Lactobacilli also exhibit the ability to synthesize all four deoxyribonucleotides if given any one of them exogenously. A number of the lactobacilli contain cytidine deaminase activity (Nygaard, 1993) as do most *Bacillus* species. Many lactobacilli also require vitamin B_{12} for growth because B_{12} is involved in the reduction of ribonucleoside triphosphates. This requirement can be met by any one of the naturally occurring deoxynucleosides when supplied exogenously with the other free bases (Goulian and Beck, 1966).

MATERIALS AND METHODS

Bacterial strains used. The following bacterial strains were used (Table 2) in this research.

Table 2. Bacterial Strains and Various Characteristics.

Bacterium	Optimal pH	O_2 Conditions	Optimal Temp.
Lactobacillus fermentum	5.5 - 7.0	Tolerant	37°C
Sporolacto-bacillus inulinus	5.5 - 7.0	Tolerant	37°C
Sporosarcina ureae	8.0 - 8.5	Aerobic	25°C
Micrococcus luteus	6.5 - 7.5	Aerobic	25°C

Media and growth conditions. All bacterial strains were grown in 100 ml of complex media as micro-cultures for inoculation to a one liter Fernbach flask. Cultures of *Sporosarcina ureae* and *M. luteus*, being obligate aerobes and slightly psychrophilic, were grown at 25°C with aeration. Cultures of *L. fermentum* and *Sporolactobacillus inulinus*, which are microaerophilic, were grown at 37°C with no aeration. The complex medium for "micro" cultures for *Sporosarcina ureae* was prepared by dissolving 0.8 grams of nutrient broth in 100 ml of distilled-deionized water (ddH_2O) in a 250 ml Pyrex beaker. The contents are then poured into an 250 ml flask and autoclaved at 121°C, at 15 lb. psi for 20 minutes. Since *Sporosarcina ureae* is

considered to be a basophile (Yamada, 1976), urea beads (SIGMA Chemical) must be filter sterilized and aseptically added to the medium to obtain a pH of 8.5. "Micro" cultures of *Sporosarcina ureae* typically were inoculated at 25°C for 48 hours before the entire 100 ml were added to a one liter preparation of *Sporosarcina ureae* minimal medium. The minimal medium for this organism was prepared according to the method of Yamada *et al.* (Yamada, 1976). To 760 ml of ddH_2O, the following chemicals from stock solutions were added to give the final concentration of each (in parentheses): $MnCl_2$ (2×10^{-3} mM), $FeSO_4$ (9×10^{-3} mM), $MgSO_4$ (0.415 mM), KCl (4.6 mM), K_2HPO_4 (1 mM), and NaCl (5 mM). This solution was autoclaved at 121°C, 15 lb. psi for 20 minutes and allowed to cool to room temperature. The carbon and energy source was glutamine or asparagine (final concentration 20 mM). Solutions of filter sterilized biotin (1 µg/ml), cysteine (5 µg/ml), and $(NH_4)_2SO_4$ (0.2 mg/ml) were also aseptically added. If minimal medium agar plates were needed, 15 grams of Difco Bacto-agar were added before the solution was autoclaved. The remaining requirements were added to the solution before the plates could be poured.

For the cultivation of *L. fermentum* and *Sporolactobacillus inulinus*, Lactobacilli MRS medium was utilized

(Rogosa *et al.*, 1960). This medium was prepared by adding 55 grams of MRS to one liter of ddH_2O and autoclaving to sterilize. Agar plates contained 15 grams of Difco Bacto-Agar in addition to the above.

In growing *B. subtilis* as a control organism, minimal medium was prepared after the method of Cox and Hanson (Cox and Hanson, 1968). To 808 ml of ddH_2O, the following stock solutions were added to give final concentrations which are given in parentheses: K_2HPO_4 (80 mM), KH_2PO_4 (60 mM), sodium citrate (3 mM), $(NH_4)_2SO_4$ (20 mM), $MnSO_4$ (2×10^{-2} mM), $FeSO_4$ (2×10^{-2} mM), and $MgCl_2$ (0.5 mM). A separately sterilized solution of glucose was aseptically added to give a final concentration of 20 mM. Agar plates were made using the above mentioned method and adding 15 grams per liter of Difco Bacto-Agar.

Harvesting of bacterial cells. All bacterial strains were harvested in log phase at approximately 100 Klett Units ($\approx 10^8$ cells/ml) by evenly dispensing the contents of a one liter flask into four (4) Sorvall GS-3 rotor bottles (500 ml capacity). The cells were then centrifuged at 4°C in a Sorvall RC5C centrifuge using a GS-3 rotor for 10 minutes at 16,000 x g. The supernatant was decanted without disturbing the bacterial cell pellets. Depending on the experiment for

which the bacterial cells were to be used, the cells were washed once in either 20 ml of high performance liquid chromatography (HPLC) breaking buffer (10 mM Tris; 0.3 grams reagent grade Tris[hydroxymethyl]aminomethane in 250 ml ddH_2O pH with HCl to 7.0; 1 ml of 1 M $MgCl_2$) or 20 ml of an ATCase breaking buffer (50 mM Tris pH 8.0; 0.02 mM $ZnSO_4 \cdot 7H_2O$ and 2 mM β-mercaptoethanol). The suspended solution was then transferred to a Sorvall Oakridge centrifuge tube (50 ml capacity) and centrifuged for 15 minutes at 4°C and 16,000 x g in a SA600 Sorvall rotor. The liquid medium was decanted without disturbing the bacterial cell pellet. The pellet was then weighed to quantitate the amount of cells obtained and to determine the amount of buffer in which to resuspend the pellet before breaking the cells. This final cell suspension was broken or stored at -20°C until needed.

Preparation of cell-extracts. Harvested 5-10 ml suspended pellets were broken by passing once or twice through a 0.148 cm outlet orifice French pressure cell at 10,000 lb. psi. This mixture became increasingly viscous when a satisfactory rupture of the bacterial cells occured. The cell-extract was spun in a capped Oakridge centrifuge tube using the SA600 rotor at 4°C and 39,000 x g for one hour. It was necessary to keep the cell-extract ice-cold at

this point to ensure that no protein denaturation will occur. Another method that was used to break bacterial cells was the Branson Sonifier Cell Disrupter Model 200. This sonicator was set up with the output control adjusted to 4.5, timer position on hold, with the duty cycle adjusted to 50% and pulsed. The time of sonication depended on the bacterium being disrupted. Obtainment of suitable breakage of *Sporosarcina ureae* usually occurred in two, 15 minute intervals with cooling of the horn tip with an ice-water and ethanol bath between sonications. While *L. fermentum* acquired good breakage in three 15 minute intervals with the addition of 0.5 mm glass beads, *Sporolactobacillus inulinus* could be broken only by using the French press. Overall, preference was given to the French press for cell disruption due to the fact that Gram-positive cells are more difficult to break than are Gram-negative cells.

Determination of protein concentration. In determining the protein concentration of cell-extracts, the Lowry method (1951) of protein concentration measurement was used with bovine serum albumin (BSA) as the protein standard. The BSA standard curve was created by assembling a series of 10 tubes with a total volume of 200 µl ranging from 0-90 µg BSA in 10 µg increments using 0.1% BSA stock solution obtained

from Pierce Chemical (Rockford, IL.). For determining the protein concentrations in crude extracts, 5 µl of three dilutions (1:5, 1:10, 1:20) were added to a total volume of 200 µl (i.e. 5 µl sample in 195 µl of ddH_2O; 10 µl sample in 190 µl of ddH_2O; 20 µl sample in 180 µl of ddH_2O). To each tube starting with the blank of the standard curve, 0.8 ml of freshly prepared alkaline copper reagent (0.5 ml 2% Na/K tartrate; 0.5 ml 1% $CuSO_4 \cdot 5H_2O$; 49 ml of Na_2CO_3 in 0.1 N NaOH) was added. These tubes were then allowed to stand at room temperature for 10 minutes, noting the time each addition was made. After the 10 minute period, 0.1 ml 1 N Folin reagent (1 M Folin stock per 1 ml ddH_2O) was added while vortexing the tube. The mixing of each tube is extremely important for the correct color development, thus determining protein concentration. The tubes were allowed to stand at room temperature for 30 minutes to one hour. All tubes were read at A_{660} in a Beckman model D325 spectrophotometer. Protein concentration measurements can be calculated by plotting the cell-extract absorbance reading on the standard graph of A_{660} vs. µg BSA constructed with varying concentrations of BSA.

Non-denaturing ATCase activity gels. The size of ATCase can be observed in crude extracts using a non-

denaturing polyacrylamide gel by exploiting the ortho-phosphate released during the reaction (Bothwell, 1975). Cell-extracts for polyacrylamide electrophoresis were prepared as above. A 50 ml sample from a 100 ml culture of each bacterium was collected in a 50 ml Oakridge centrifuge tube by centrifugation in a Sorvall RC5C centrifuge using a SA600 rotor at 4°C for 10 minutes at 16,000 x g. The supernatant was decanted and the pellet was resuspended in approximately 2 ml of ATCase breaking buffer and disrupted accordingly.

A 6% acrylamide gel was prepared from a 40% bis-acrylamide; acrylamide (1:39) stock solution. The normal procedure for construction of the activity gel was to add 9.64 ml of 40% bisacrylamide, 7.5 ml separating buffer (36.3 grams Tris per 100 ml ddH_2O; adjust pH to 8.9 with concentrated HCl), and 4.48 ml of 40% sucrose to a 100 ml graduated cylinder and volumetrically add ddH_2O up to 60 ml total volume. The acrylamide solution was then placed in a filter flask with 0.15 grams of ammonium persulfate to catalyze polyermization. To ensure the removal of oxygen from the solution, it was degassed for 10 minutes. Once degassed, 25 µl of TEMED (Tetramethylethylenediamine) was added to the bottom of a clean 250 ml beaker and the gel

solution slowly poured into the beaker to allow the contents to blend. The solution was gently swirled before pouring into a 20.5 cm x 20.5 cm gel cast assembly. With this concentration of TEMED, complete polymerization occurred in approximately 1 1/2 hours. If the gel was to run on a different day then it was made, then the gel cast assembly would be covered in Saran wrap and placed at 4°C until the samples were ready to be electrophoresed. The gel was stored no longer than 48 hours.

When ready for electrophoresis, the gel was loaded with approximately 25 µl of cell-extract and mixed with 5 µl of 5X loading dye for activity. This loading dye consisted of 1 ml glycerol (for weight), 1 ml running buffer (1.2 grams Tris; 5.76 grams glycine per two liters of ddH_2O), and 0.25 mg bromophenol blue. Running buffer was placed at both positive and negative ends of the gel cast apparatus as to supply sufficient conductivity from the top portion of the gel to the bottom. The power supply (BioRad Xi3000) was set at 5 Watts and timed for 8-10 hours, or until the bromo-phenol blue from the loading dye reached the bottom of the gel.

Non-denaturing gradient gels. In order to approximate the molecular weight of ATCase, 2%-20% gradient gels (Dr.

Mark Shanley, personal communication) were utilized. In preparing the gradient gel, two flasks were needed, one for the 2% acrylamide and one for the 20% acrylamide solutions. To prepare the 2% acrylamide solution, 2 ml of 30:0.8 ratio of acrylamide to bis, 3.75 ml resolving buffer (3 M Tris HCl, pH 8.8), 0.7 ml 1.5% ammonium persulate (in H_2O) were added to 20.55 ml ddH_2O. For the 20% acrylamide solution, 20 ml of the same ratio bisacrylamide, 3.75 ml resolving buffer, 0.7 ml 1.5% ammonium persulfate, and 4.5 grams of sucrose were added to 2.75 ml ddH_2O. Each solution was degassed for 10 minutes. To each solution, 10 µl of TEMED were added and swirled to mix. The contents of each flask were poured into the respective reservoirs of a gradient maker with gentle swirling from a stir bar. A peristalic pump was used to "pour" the gel at a flow rate of 3-5 ml/minute, giving the higher concentrations of acrylamide at the bottom of the gel and the lower concentrations at the top. Once the gel polymerized, samples could be loaded as they were with the 6% activity gel. The running buffer used for the gradient gels was the same used for the activity gels. Given the higher gel concentrations, duplicate samples with molecular weight standards (SIGMA chemical) were electrophoresed for 24-26 hours at 4°C and a power

setting of 5 Watts. Once the electrophoresis process was complete, the gel was sliced between the duplicate samples. One set to be stained for activity, one set to be stained for protein with coomassie blue. The coomassie blue stain was used to visualize the molecular weight standards so that they could be compared with the ATCase bands to estimate their molecular weight.

ATCase activity stain. After electrophoresis for ATCase activity, the gel must be stained. This was achieved by placing the gel in a glass dish and equilibrating for five minutes in 250 ml of ice cold 50 mM histidine (2.62 grams of histidine-free base per 250 ml ddH_2O) at pH 7.0 Histidine hydrochloride cannot be used as a primary buffer because this will change the pH of the system and affect results. After equilibration, 5 ml of 1 M aspartate (0.78 grams of aspartate per 5 ml ddH_2O) and 10 ml of 0.1 M carbamoylphosphate (0.153 grams of carbamoylphosphate per 10 ml ddH_2O) were added to the glass dish containing the gel and histidine base. The gel was then placed on a floor shaker with gentle shaking for 10 minutes to allow adequate time for a reaction to occur. After the 10 minute reaction time, the gel was rinsed three times with ice cold ddH_2O to wash away the reactants. The enzymatically released

orthophosphate trapped in the gel was precipitated upon addition of 250 ml of ice cold 3 mM lead nitrate (0.25 grams of lead nitrate per 250 ml ddH_2O) in ice cold 50 mM histidine. After 10 minutes of gentle swirling, the lead nitrate mixture was removed with three washes of ice cold ddH_2O. The gel was stored overnight at 4°C as this aided in visualizing the ATCase bands. The resolution of the water-insoluble lead phosphate precipitate was attained by converting it to a dark lead sulfide precipitate through submersion of the gel in 300 ml of 1% sodium sulfide for five minutes. If background levels of sulfide remained high after this rinse, the gel was further destained with 0.7% nitric acid.

Coomassie blue stain. To visualize the molecular weight standards used in the gradient gels, it was necessary to perform a coomassie blue stain. To the section of the gradient gel to be stained, 200 ml of "pre-stain" (900 ml methanol in 100 ml ddH_2O) was poured over the gel in a Pyrex dish and equilibrated for two hours. After the two hour equilibration, the "pre-stain" was decanted and 250 ml of coomassie blue stain (900 ml methanol, 100 ml ddH_2O and one gram of coomassie blue powder) was poured onto the gel and gently swirled overnight. After overnight staining, the

coomassie blue was decanted and 250 ml of "destain" (800 ml methanol in 200 ml ddH_2O) was poured onto the gel and gently swirled for 2 hours, or until a visual contrast depicting the bands could be seen.

ATCase spot assay. A modification of the complete ATCase assay, referred to as a spot assay (or range finding), was performed to estimate the proper dilution (if any) of a cell-extract to use in a complete assay. The spot assay also allows the following of ATCase activity in fractions from molecular weight or ion exchange chromatography. A 20 µl sample from cell-extracts was transferred into 430 µl of pre-mix (40 mM Tris, pH 9.5, 10 mM aspartate, and 5 ml ddH_2O). The 0.45 ml mixture was blended on a vortex and then incubated for three to five minutes in a 30°C water bath. The reaction was initiated by the addition of 50 µl 0.1 M carbamoylphosphate. After 20 minutes of incubation at 30°C, 1.5 ml of acid color mix (stop mix) was added and immediately mixed. Color development was achieved by placing these tubes at 60°C for 20 minutes to two hours. The tubes were then read at A_{466} in a Beckman spectrophotometer.

Acid color mix preparation. The acid color mix solution consisted of two solutions, antipyrine in 50% H_2SO_4

and monoxime in 10% acetic acid. Each of these were made and kept separately for long periods of time at 4°C in light protected bottles (Gerhart and Pardee, 1962; Prescott and Jones, 1969). Acid color mix used in the assay consisted of a mixture of two parts antipyrine and one part monoxime. The antipyrine solution was prepared by mixing a one liter of 50% H_2SO_4 from reagent grade H_2SO_4 and ddH_2O. A one liter glass graduated cylinder was filled with 500 ml of ddH_2O and placed in a tray of ice water. The cylinder with the ddH_2O was allowed to cool for approximately 30 minutes. 500 ml of reagent grade H_2SO_4 was added in 100 ml increments with a cooling period between additions of 10 to 15 minutes. The mixture of this acid solution generated extreme heat and was always performed with extreme caution. Once the solution was cool to the touch, five grams of antipyrine were added and allowed to dissolve. Monoxime in 10% acetic acid was(diacetyl monoxime) in one liter of 5% acetic acid. made by dissolving eight grams of 2,3-butanedione-monoxime

ATCase enzyme assay. For kinetic determinations of ATCase, one liter of bacterial cells were grown in either *Sporosarcina ureae* minimal medium, Lactobacilli MRS, *B. subtilis* minimal medium, or nutrient broth (*Micrococcus luteus*). After growth, the one liter culture was harvested

at 4°C by centrifugation at 16,000 x g for 10 minutes. The concentrated pellets were washed in 20 ml of ATCase breaking buffer. The suspension of cells was then transferred into a 50 ml Oakridge centrifuge tube and spun at 16,000 x g for 10 minutes. After the 10 minute spin, the supernatant was decanted and the pellet weighed. These cells were resuspended in twice the weight per volume in ATCase buffer and either disrupted or stored at -20°C until needed.

The cell-extract was centrifuged in a RC5C centrifuge (SA600 rotor) at 39,000 x g for one hour. ATCase activity was assayed by measuring the amount of carbamoylaspartate produced in 20 minutes at 30°C according to the method of Gerhart and Pardee (Gerhart and Pardee, 1962), with modifications using the color development procedure of Prescott and Jones (Prescott and Jones, 1969). Assays were performed in order to determine whether or not the ATCases from the organisms in question exhibit hyperbolic kinetics similar to the *Bacillus* enzyme.

Assay tubes contained the following reagents in a final volume of 2 ml: 1.48 ml ddH_2O, 80 µl Tris buffer (pH 9.5), 200 µl of varying concentrations of aspartate, 40 µl of cell-extract (enzyme), and 200 µl of carbamoylphosphate. All assay tubes were prepared in advance (without carbamoyl-

phosphate) and preincubated at 30°C for five minutes. The reaction was initiated by the addition of 200 µl carbamoyl-phosphate to each assay tube at 20 second intervals. At 10 minute intervals for 30 minutes, 500 µl of the sample were added to a stop mix solution at 20 second intervals (1 ml stop mix, 0.5 ml ddH_2O), which was on ice. After addition of the sample to the stop mix tubes, marbles acting as "caps" were placed on the top of each tube (to prevent evaporation) and placed in a 60°C water bath for 110 minutes for color development. Proceeding the color development period, the tubes were placed on ice and in the dark for one minute for cooling. The contents of the tubes were then read at A_{466} in a Beckman spectrophotometer to determine the amount of carbamoyl aspartate formed. Positive and negative controls (pre-mix blanks with either aspartate or carbamoyl-phosphate, buffer, substrates, and cell-extracts) were also assayed to determine background color.

High performance liquid chromatography assays. Cultures of 100 ml (grown to a density of 10^9 cells/ml) were harvested at 10,000 x g for 15 minutes at 4°C. The cell pellet was resuspended in 2 ml of 10 mM Tris HCl buffer (pH 7) and broken with either the Branson sonifier or the French press. The mixture was again centrifuged at 10,000 x g at

4°C for 10 minutes. The pellet was discarded while the supernatant was used for enzyme assays. Pyrimidine salvage enzyme assays were performed in 1.5 ml microcentrifuge tubes at physiological growth temperatures. The assay mixture contained 200 µl of 1 mM substrate (cytosine, cytidine, or uridine, respectively) and 20 µl of cell-extract which starts the reaction. Incubation times varied depending on the organism. After incubation, 20 µl of the assay mix were injected onto a reverse phase column (Beckman Ultrasphere ODS 5 µm, 259 cm x 4.6 mm I.D., particle size 5 µm, pore size 100 Å). Utilizing a Waters Model 510 pump, a U6K injector, and a variable wave-length SpectroMonitor 5000 photodiode array detector (LCD Analytical), peak heights were quantified on a Waters Model 740 Data Module and the LCTalk software package (LCD Analytical) in conjunction with an IBM personal computer. Compounds were detected by monitoring the column effluent at 254 nm with a sensitivity set at 0.06 absorbance units full scale (a.u.f.s.). Individual nucleosides or bases were identified using retention times established by monitoring known standards and their retention times on the reversed phase column.

RESULTS

Enzyme assays. The primary goal of this study was to determine if other endospore-forming organisms, such as *Sporosarcina ureae* and *Sporolactobacillus inulinus*, have a similar ATCase and pyrimidine recycling scheme as *B. subtilis*. It is known that the ATCase of *B. subtilis* exhibits hyperbolic kinetics (Switzer, 1982; Figure 7) is active as a catabolic trimer, and has a molecular weight of 100 kDa. Kinetic studies with the *Sporosarcina ureae* ATCase suggest that the enzyme from this endospore-forming organism also exhibits hyperbolic kinetics (Figure 8), although the Km_{asp} of 2.5 mM for the enzyme is greater than the Km_{asp} of 0.5 mM for the *Bacillus* enzyme.

The ATCase from *L. fermentum* has been shown to exhibit hyperbolic kinetics but appears to be saturated at lower concentrations of aspartate than is the ATCase from *B. subtilis*, which had a Km_{asp} of 0.5 mM. The *L. fermentum* ATCase also has been shown to display repression (Marouf, Ph.D. Dissertation, Texas A&M University, 1973) when grown in 100 µg of uracil per ml. The ATCase from *Sporolactobacillus inulinus* was expected to give the same kinetic results as *L. fermentum*.

Figure 7. Velocity substrate plot for the aspartate transcarbamoylase from *B. subtilis* at various concentrations of aspartate.

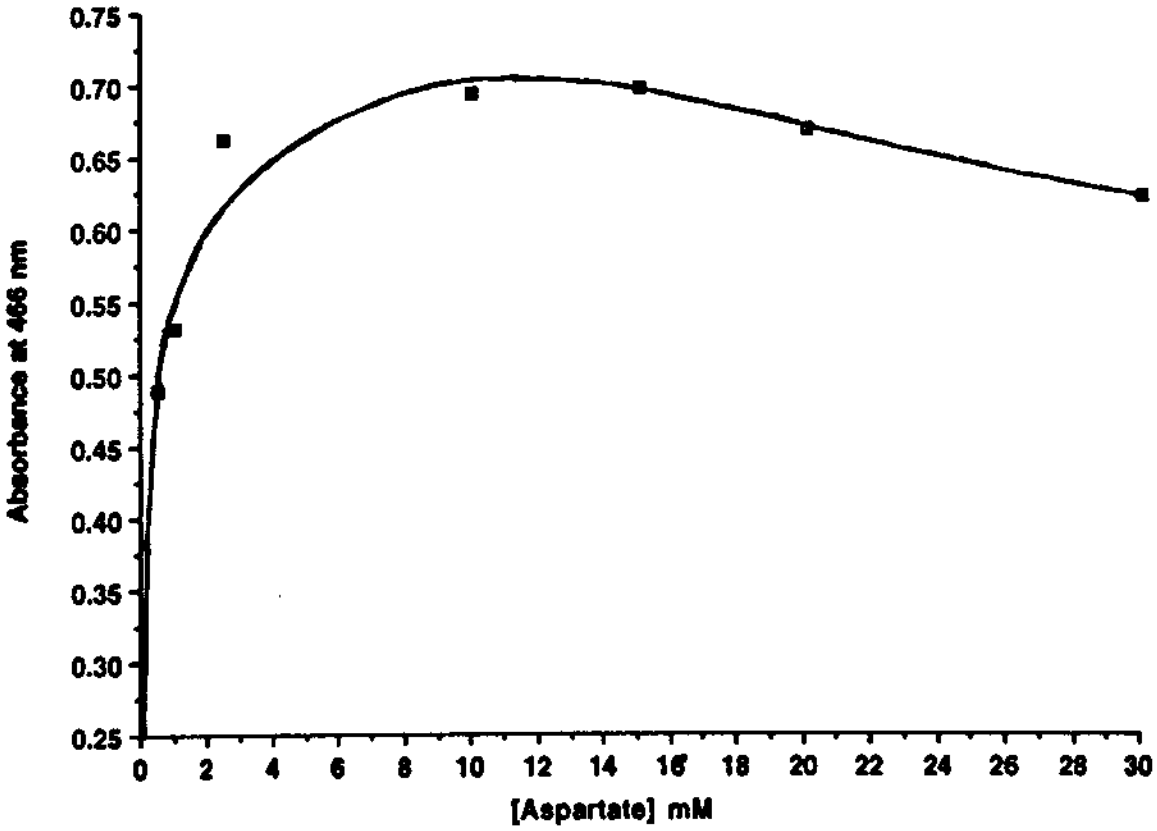
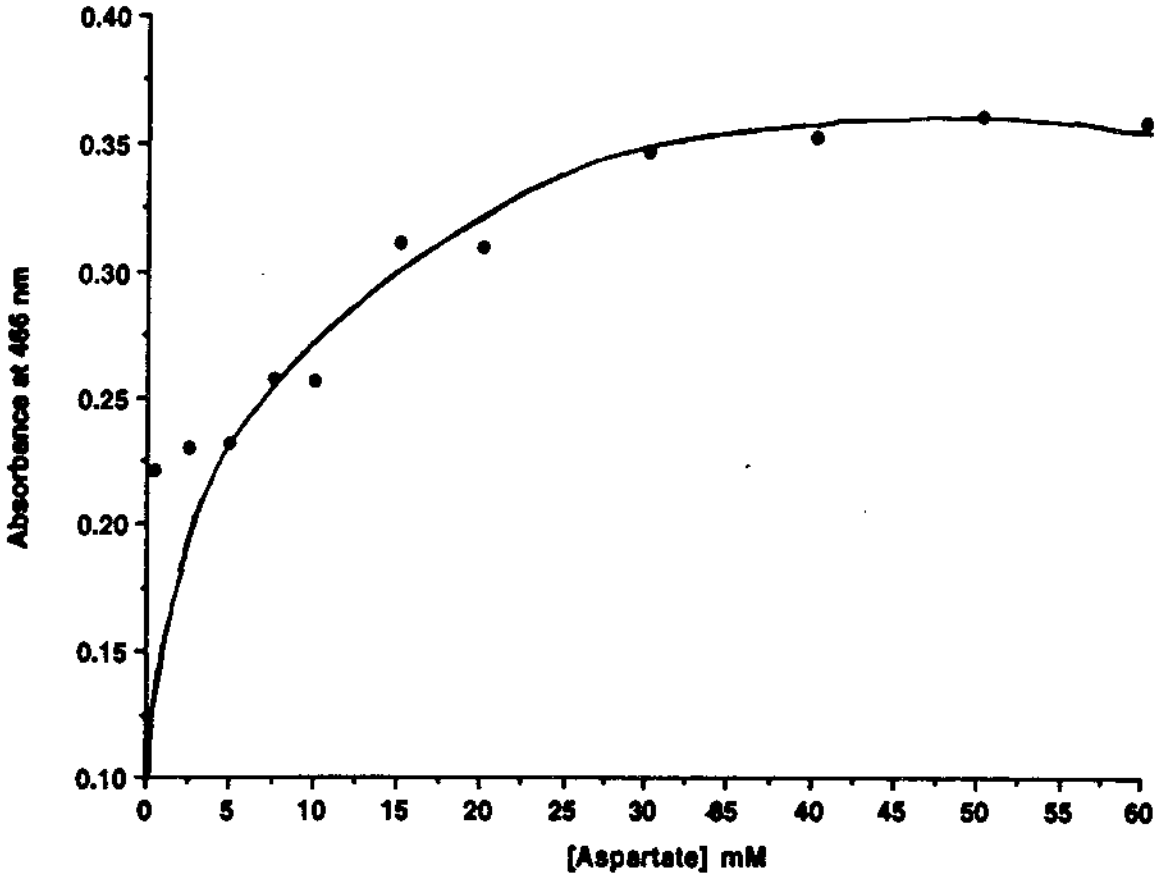

0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
Absorbance at 466 nm
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
[Aspartate] mM

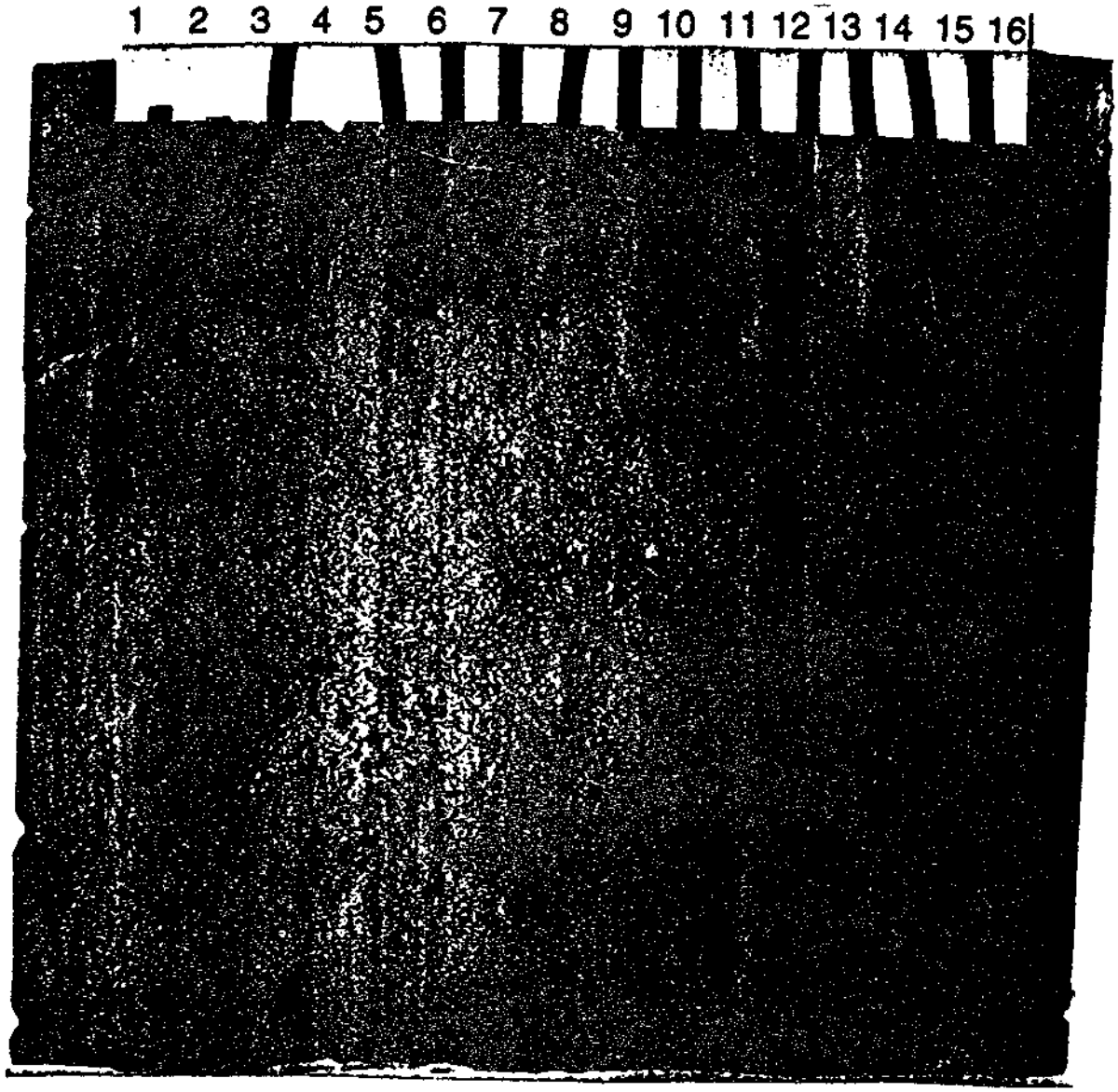
Figure 8. Velocity substrate plot for the aspartate transcarbamoylase from *Sporosarcina ureae* at various concentrations of aspartate.


0.40
0.35
0.30
0.25
0.20
0.15
0.10
Absorbance at 486 nm
0
5
10
15
20
25
30
35
40
45
50
55
60
[Aspartate] mM

ATCase activity stains. Although activity stains are not an accurate approximation of molecular weight (M_r), a suitable estimation of the size of the enzyme can be obtained. ATCase activity stains of *Sporosarcina ureae* (lane 3, Figure 9) revealed a band that migrated (to the bottom of the gel) an identical distance to that of the *B. subtilis* ATCase. A lighter band from *L. fermentum* (lane 5) migrated the same distance as *Sporosarcina* and *B. subtilis*, indicating that the *L. fermentum* enzyme was a similar size to that of *B. subtilis* and *Sporosarcina*. As can be seen in Figure 10, a faint band of *Sporolactobacillus* (lane 9) migrated about the same distance as the other three organisms suggesting a similar size for the ATCase for this organism. The *M. luteus* enzyme (land 10) produced a band of similar size to that of the *Pseudomonas aeruginosa* standard (lane 12). This was repeated twice with the same result found each time. The band corresponding to the *M. luteus* ATCase always migrated the same distance as did the *P. aeruginosa* ATCase. This unusual finding will be treated further in the Discussion.

ATCase gradient gels. ATCase gradient gels were used as a means of estimating the molecular weight of ATCases from the organisms of this study. In Figure 10, it can be

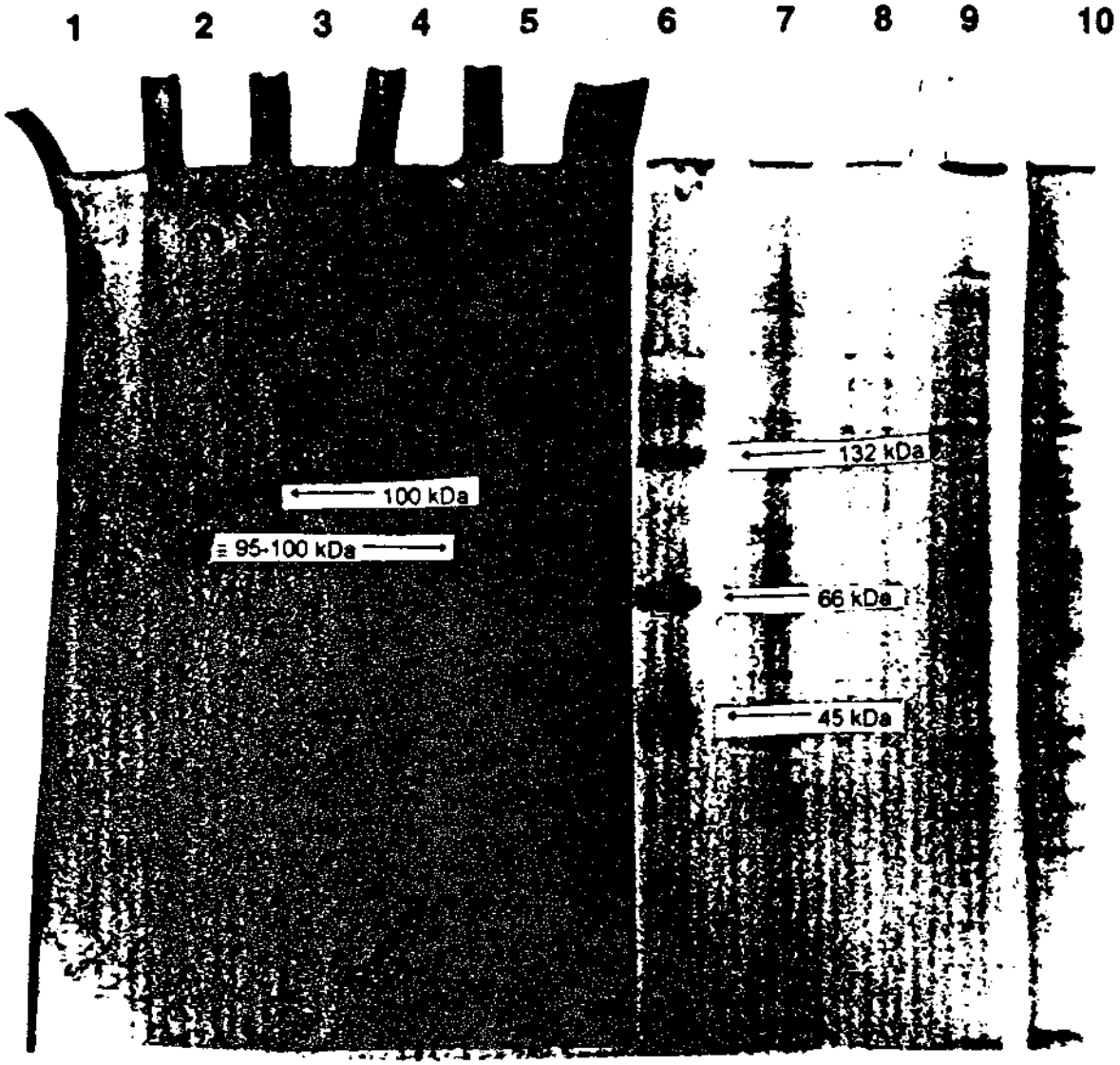
Figure 9. ATCase activity stain of the organisms involved in this study. Lanes are numbered at the top of each well. Lane assignments are: (1), *E. coli* ($2c_3$:$2r_2$); (2)-(3) *Sporosarcina ureae*; (4)-(5), *L. fermentum*; (6)-(8), *B. subtilis*; (7)-(9), *Sporolactobacillus inulinus*; (10)-(11), *M. luteus*; (12), *Pseudomonas aeruginosa*; (13), *Sporosarcina ureae*; (14), *Methanococcus jannaschii*; (15)-(16), no sample loaded.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

seen that *Sporosarcina ureae* (lane 5) gave a smeared band that migrated a distance nearly equal to the of the ATCase from B. subtilis (lanes 1 and 2). Since the molecular weight of the ATCase from *B. subtilis* is known to be 100 kDa (in agreement with the published results of Swizter (Switzer, 1982) and molecular weight standards), the result here suggests that the ATCase from *Sporosarcina ureae* can be roughly estimated between 95-100 kDa. The *M. luteus* band (lane 4) either did not migrate of the well or did not stain. If the former is the case, then this would suggest a very high molecular weight for the *Micrococcus* enzyme. Since the *L. fermentum* and *Sporolactobacillus inulinus* bands migrated identically as did the *B. subtilis* and *Sporosarcina ureae* enzymes on previous activity gels, it was inferred that these enzymes would give similar results as *Bacillus* and *Sporosarcina ureae* on a gradient gel.

HPLC assays. Pyrimidine salvage schemes can be used to taxonomically classify various organisms in order to differentiate between different species or identify like organisms. They are used in this study to determine if the organisms used are similar to *B. subtilis* with respect to pyrimidine salvage. Enzymes of pyrimidine salvage were determined using known retention times of substrates

Figure 10. ATCase gradient gel to estimate molecular weights of ATCase. Lanes are numbered at the top of each well. Lane assignments are as follows: (1)-(2), *B. subtilis*; (3), *Methanococcus jannaschii*; (4), *M. luteus*; (5) *Sporosarcina ureae*; (6), Molecular weight standards; (7), *M. luteus*; (8), *Methanococcus jannaschii*; (9)-(10), *B. subtilis*.


1
2
3
4
5
6
7
8
9
10
100 kDa
95-100 kDa
132 kDa
66 kDa
45 kDa

(cytosine, uracil, cytidine, and uridine, respectively. Figure 11). As can be seen from Figure 11, retention times of cytosine, uracil, cytidine, and uridine were measured to detect product formation from the respective enzymatic reactions of the salvage enzymes involved in the recycling of nucleotides.

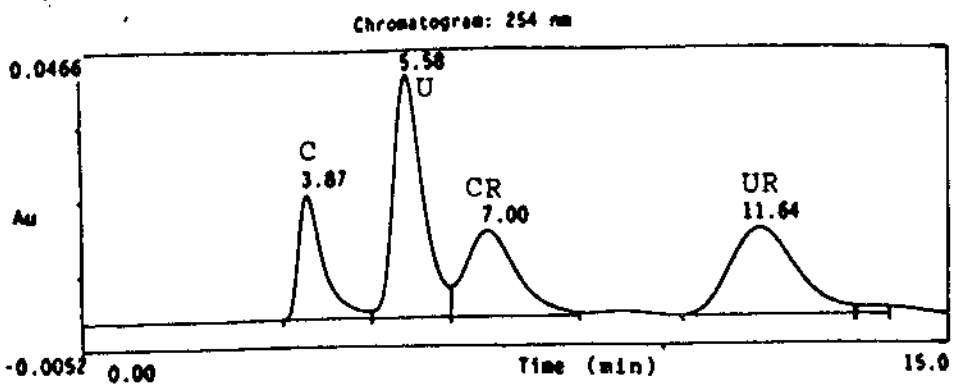
For example, to test for the presence of cytosine deaminase in cell-extracts of *Sporosarcina ureae* (Figure 12 top), the cell-extract was incubated for in cytosine for one hour at physiological growth conditions. If cytosine deaminase activity is present, then increasing amounts of uracil will accumulate in the mixture due to the enzymatic deamination of cytosine to uracil. When assayed, the accumulation of uracil would be detected by the HPLC at the corresponding retention time (and peak) for this compound. However as Figure 12 shows, no uracil peak is present with incubations of cell-extract in cytosine. Therefore, since uracil is not accumulating in the assay mixture, cytosine deaminase activity is absent. Next, cell-extracts were incubated in cytidine (Figure 12, middle) to determine the presence of cytidine deaminase. Cytidine deaminase catalyzes the enzymatic deamination of cytidine to uridine. The accumulation of uridine is detected in the same manner

as stated above. By the result indicated by the incubation of cell-extracts with cytidine, there was accretion of uridine showing the presence of cytidine deaminase activity. If either nucleoside hydrolase or uridine phosphorylase was present, the uridine formed would be enzymatically converted to uracil, since both nucleoside hydrolase (non-specific) and uridine phosphorylase (specific for uridine) catalyze this reaction. The presence of a very small peak which corresponded to uracil was seen. However, in this case, nucleoside hydrolase activity was be ruled out due to the fact that no cytosine peak was observed (Figure 12, middle). Nucleoside hydrolase can use either uridine or cytidine as substrates and cleaves off the ribose sugar to give either uracil or cytosine as products, respectively. Finally, cell-extracts of *Sporosarcina ureae* were incubated in uridine to verify the presence of uridine phosphorylase. As seen in the bottom of Figure 14, a small uracil peak was observed, indicating the presence of uridine phosphorylase.

Next, the morphologically similar *M. luteus* was assayed. Cell-extracts with no incubations revealed a unusually high cytosine background, but did not affect results with other assays of *M. luteus*. Cell-extracts of *M. luteus* incubated with cytosine (Figure 13, top) were assayed

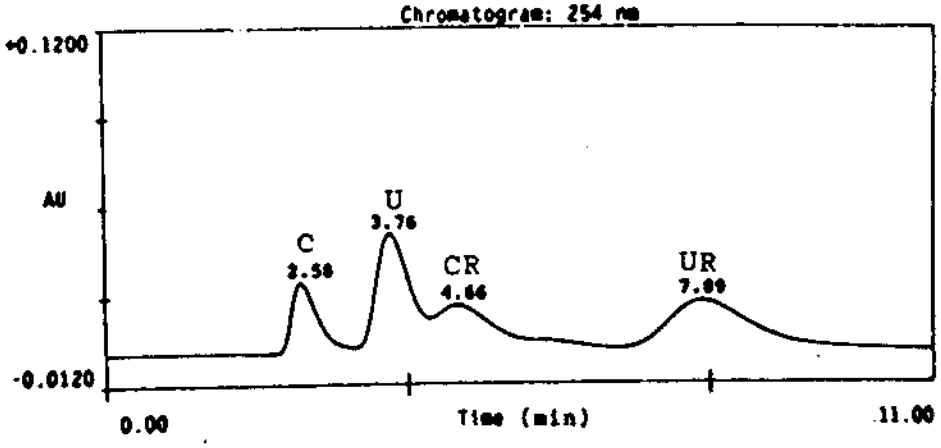
Figure 11. Determination of retention times and peaks from cytosine, uracil, cytidine, and uridine for 11 and 15 minute HPLC assays of cell-extracts of the organisms involved with this study. Substrates are detected in order of cytosine, uracil, cytidine, and uridine.

15 min run



Substrate	Retention Time (in min)
Cytosine	3.87
Uracil	5.58
Cytidine	7.00
Uridine	11.64

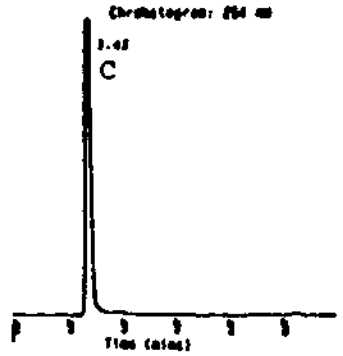
11 min run



Substrate	Retention Time (in min)
Cytosine	2.58
Uracil	3.76
Cytidine	4.66
Uridine	7.89

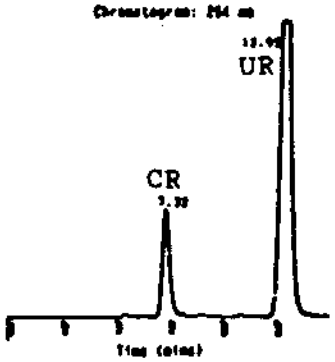
Figure 12. 15 minute HPLC assays of cell-extracts of *Sporosarcina ureae* incubated in cytosine (top), cytidine (middle), and uridine (bottom). The corresponding retention times and peaks from each incubation determined salvage enzymes present.

Incubated with Cytosine - 15 min run



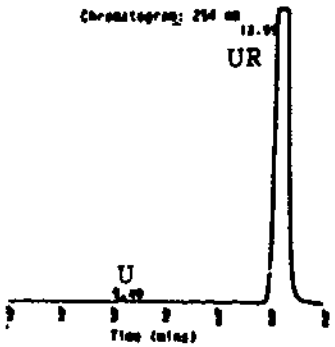
Substrate	Retention Time (in min)
Cytosine	3.43
Uracil	—
Cytidine	—
Uridine	—

Incubated with Cytidine - 15 min run



Substrate	Retention Time (in min)
Cytosine	—
Uracil	—
Cytidine	7.32
Uridine	12.96

Incubated with Uridine - 15 min run



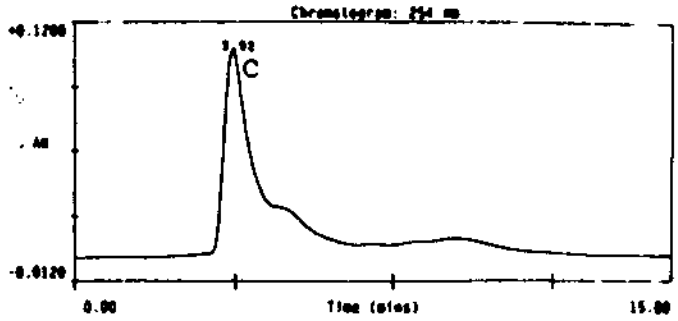
Substrate	Retention Time (in min)
Cytosine	—
Uracil	5.40
Cytidine	—
Uridine	12.96

revealed the presence of only a cytosine peak. Since a uracil peak was not observed, cytosine deaminase activity was not present. Incubation in cytidine (Figure 13, middle) revealed two peaks aside from the cytosine background and cytidine itself. A large peak of uridine (retention time of 11.41 min) was observed which indicated the presence of cytidine deaminase. A uracil peak (retention time of 5.50 min) was also seen and suggested the presence of uridine phosphorylase. Cell-extracts incubated in uridine (Figure 13, bottom) revealed a uracil peak with a retention time of 5.59 minutes, verifying uridine phosphorylase activity.

11 minute assays were employed with cell-extracts of *L. fermentum* (Figure 14). Incubations of cell-extracts with cytosine revealed a uracil peak (retention time of 3.63 min.) which indicated the presence of cytosine deaminase. This was an unusual observation for a Gram-positive organism and will be treated further in the Discussion. Incubations of cell-extracts of cytidine gave a uracil peak (retention time: 3.63 min.) and a cytosine peak (retention time: 2.53) min., Figure 14). The cytosine peak is due to the fact that cytidine is being converted to cytosine via nucleoside hydrolase, while cytosine is being converted to uracil via cytosine deaminase. No uridine peak was observed, therefore

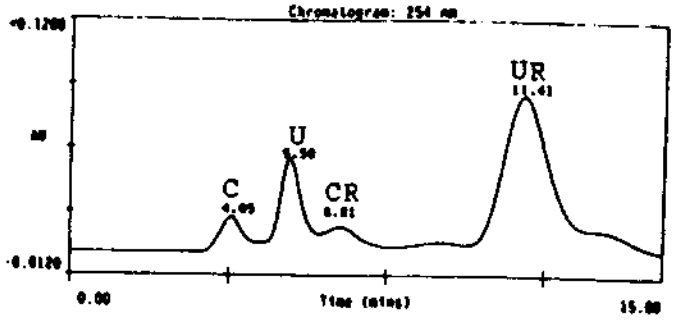
Figure 13. 15 minute HPLC assays of cell-extracts of *Micrococcus luteus* incubated in cytosine (top), cytidine (middle), and uridine (bottom). The corresponding retention times and peaks from each incubation determined salvage enzymes present.

Incubated with Cytosine - 15 min run



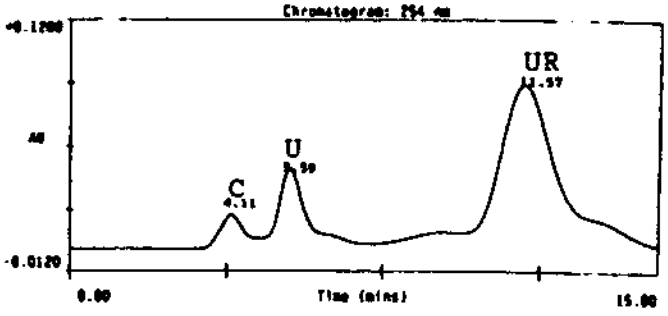
Substrate	Retention Time (in min)
Cytosine	3.92
Uracil	——
Cytidine	-----
Uridine	——

Incubated with Cytidine - 15 min run



Substrate	Retention Time (in min)
Cytosine	4.05 (background)
Uracil	——
Cytidine	6.81
Uridine	11.41

Incubated with Uridine - 15 min run



Substrate	Retention Time (in min)
Cytosine	4.11 (background)
Uracil	5.59
Cytidine	——
Uridine	11.57

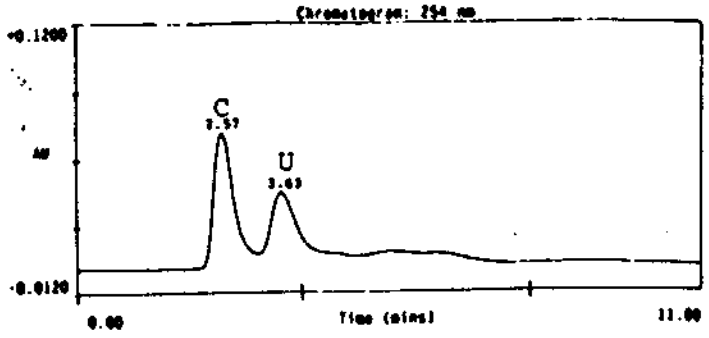
cytidine deaminase is not present. Nucleoside hydrolase activity was further verified by incubations of cell-extracts with uridine, as this assay gave rise to a uracil peak with a retention time of 3.62 minutes.

15 minute assays with cell-extracts of *Sporolactobacillus inulinus* (Figure 15) incubated as above gave the same results as did the salvage scheme of *L. fermentum*. In incubations of cytosine (Figure 15, top), a uracil peak (retention time of 6.23 min) was observed verifying the presence of cytosine deaminase. *Like L. fermentum*, cytidine deaminase activity was not observed. (Figure 15, middle) although nucleoside hydrolase activity was present from the appearance of the cytosine (retention time of 4.24 min) from the cytidine substrate. From the bottom of Figure 15, cell-extracts incubated with uridine gave rise to a uracil peak (retention time of 5.96 min) to further verify the activity of nucleoside hydrolase.

Following Figures 14 and 15, Table 3 summarizes the enzymes present of pyrimidine salvage for each organism with respect to results obtained by HPLC assays.

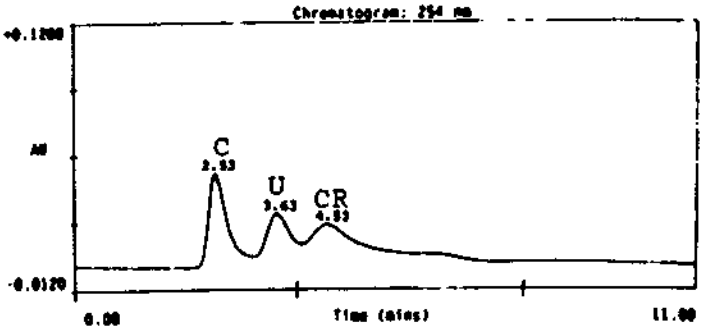
Figure 14. 11 minute HPLC assays of cell-extracts of *L. fermentum* incubated in cytosine (top), cytidine (middle), and uridine (bottom). The corresponding retention times and peaks from each incubation determined salvage enzymes present.

Incubated with Cytosine - 11 min run



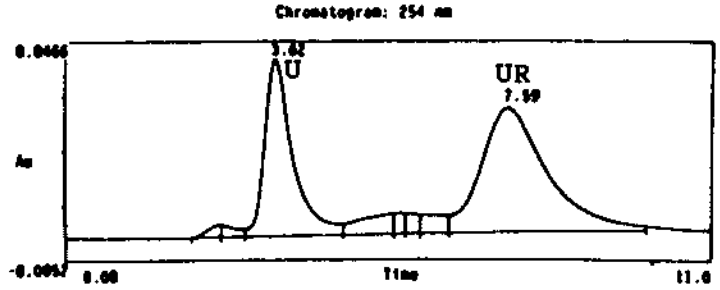
Substrate	Retention Time (in min)
Cytosine	2.57
Uracil	3.63
Cytidine	——
Uridine	——

Incubated with Cytidine - 11 min run



Substrate	Retention Time (in min)
Cytosine	2.53
Uracil	3.63
Cytidine	4.53
Uridine	——

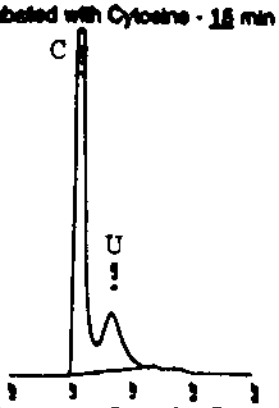
Incubated with Uridine - 11 min run



Substrate	Retention Time (in min)
Cytosine	——
Uracil	3.62
Cytidine	——
Uridine	7.59

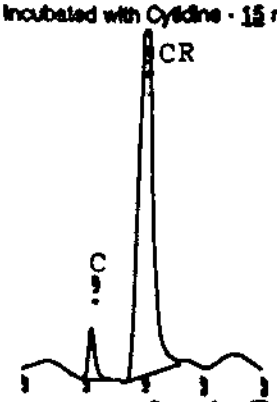
Figure 15. 15 minute HPLC assays of cell-extracts of *Sporolactobacillus inulinus* incubated in cytosine (top), cytidine (middle), and uridine (bottom). The corresponding retention times and peaks from each incubation determined salvage enzymes present.

Incubated with Cytosine - 15 min run



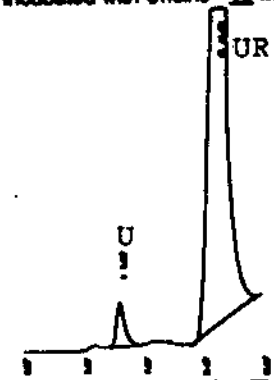
Substrate	Retention Time (in min)
Cytosine	4.16
Uracil	6.23
Cytidine	—
Uridine	—

Incubated with Cytidine - 15 min run



Substrate	Retention Time (in min)
Cytosine	4.24
Uracil	—
Cytidine	7.49
Uridine	—

Incubated with Uridine - 15 min run



Substrate	Retention Time (in min)
Cytosine	—
Uracil	5.98
Cytidine	—
Uridine	12.25

Table 3. Summary of Salvage Enzymes.

Organism	*cdd*	*udp*	*cod*	*nuh*
Sporosarcina ureae	+	+	-	-
M. luteus	+	+	-	-
L. fermentum	-	-	+	+
Sporolactobacillus inulinus	-	-	+	+

DISCUSSION

In recent years, many morphological similarities have been cited between *Bacillus* species and the genus *Sporosarcina* (Pechman *et al.*, 1976). While there are similarities between these two genera, the taxonomic position of the genus *Sporosarcina* is still a matter of controversy. At present, any Gram-positive aerobic endospore-forming coccus is placed in the genus *Sporosarcina*. Beijerinck first described the endospore-forming, motile organism which occurred in packets of eight cocci as *Planosarcina ureae* (Beijerinck, 1901), but this was later rejected and the genus name *Sporosarcina* was substituted. The genetic relationship between *Sporosarcina ureae* and certain members of the genera *Bacillus* and *Micrococcus* has been studied by Herndon and Bott (Herndon and Bott, 1969). This study revealed that DNA homology

was higher between *Sporosarcina ureae* and *B. subtilis* (74%) than between *Sporosarcina ureae* and *Micrococcus luteus* (19%). With regard to ATCase and pyrimidine salvage, the results of this study support the proposal that *Sporosarcina ureae* is closely related to *Bacillus* species. As stated earlier, no known organism is devoid of pyrimidine salvage. Thus, determining which pyrimidine recycling enzymes are present can be a powerful tool to aid in characterizing and differentiating between similar though not identical genera. However, from the results of these or earlier experiments, it should not be rationalized that all endospore-forming organisms are genetically or morphologically similar.

The question of whether the ATCase of *Sporosarcina ureae* behaved like that of the morphologically similar *M. luteus* was next explored. Preliminary findings (data not shown) suggest that the ATCase from *M. luteus* follows hyperbolic kinetics. It is not known if the enzyme is inhibited by nucleotide effector molecules mainly because of low specific activity of the native enzyme. One substantial difference found between the ATCase of *Sporosarcina ureae* and that of *M. luteus* was the size and molecular weight of the respective ATCase (Figure 10). The ATCase from *M. luteus* is much larger than that of *Sporosarcina*, comparing

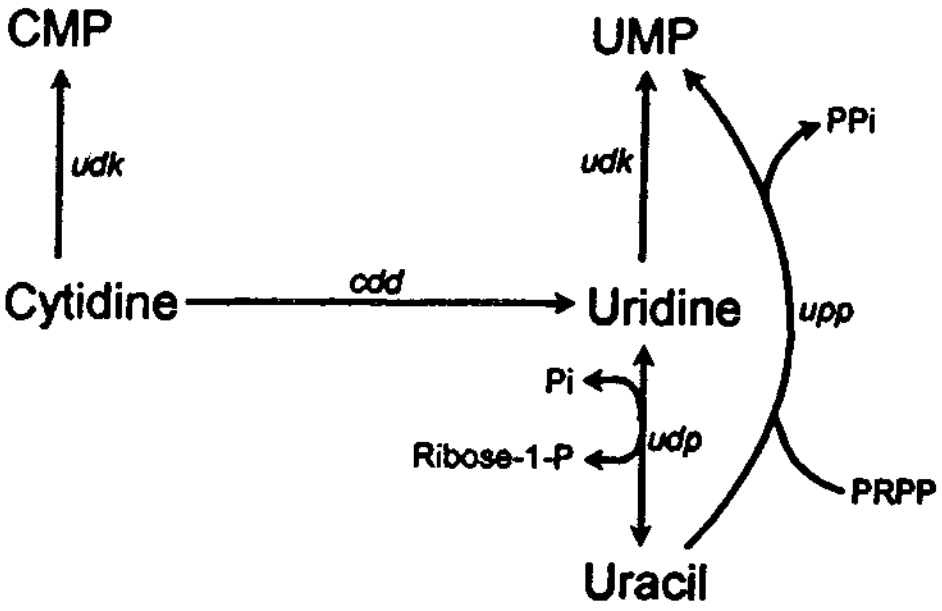
to the size of the *P. aeruginosa* enzyme. *Sporosarcina ureae* and *M. luteus* do share similarities with respect to pyrimidine salvage, both are identical to that of *B. subtilis* (Figure 16). This suggests that there are more similarities between *Sporosarcina* and *Micrococcus* than just morphology, however, it is clear that *Sporosarcina* is more closely related to *Bacillus* than to *Micrococcus*, as Herndon and Bott first suggested with DNA homology. It seems that only one of the major differences between the two organisms is morphology. Apparently, this difference has justified *Sporosarcina* to be grouped into a separate genus than *Bacillus* in *Bergey's Manual*.

The lactobacilli are a truly unique group. They flourish in acidic environments and therefore inhibit the growth of competing bacteria. They have immense importance in food microbiology as they are used for the production of many food and dairy products. Although the lactobacilli have been extensively characterized, a morphological and genetically similar organism, *Sporolactobacillus*, has not gained much attention. *Sporolactobacillus* differentiates into true endospores while the lactobacilli do not. Growth environments of these two genera are very similar, both thrive in acidic and aerotolerant conditions. This leads to

the question of how similar are the ATCases and pyrimidine salvage of these bacteria. Both appear to have ATCase of approximately 100 kDa (see Figure 9), suggesting that the ATCase from these organisms is active as a catalytic trimer, as found in *Bacillus* or *Sporosarcina*. *L. fermentum* follows hyperbolic kinetics and is not affected by nucleotide effectors. Pyrimidine salvage in *Sporolactobacillus inulinus* seems is similar to that of *L. fermentum* (Figure 17), indicating that these organisms are very similar, if not identical, with respect to pyrimidine metabolism. Both organisms contain cytosine deaminase. This is key finding because as of 1983, no Gram-positive organism was reported to have cytosine deaminase activity (Neuhard, 1983).

While these organisms are similar in their pyrimidine metabolism pathways, it is still apparent that the lactobacilli warrant a respective taxonomic position distinct from the genus *Bacillus*. *Sporolactobacillus* exhibits many characteristics of *Bacillus* as well as *Lactobacillus*. Given these similarities, *Sporolactobacillus* could be placed in the genus *Bacillus* or *Lactobacillus*. Interestingly, *Bergey's Manual* of systematic bacterial nomenclature has *Sporolactobacillus* as a separate genus. From the data derived from this study, it is suggested that

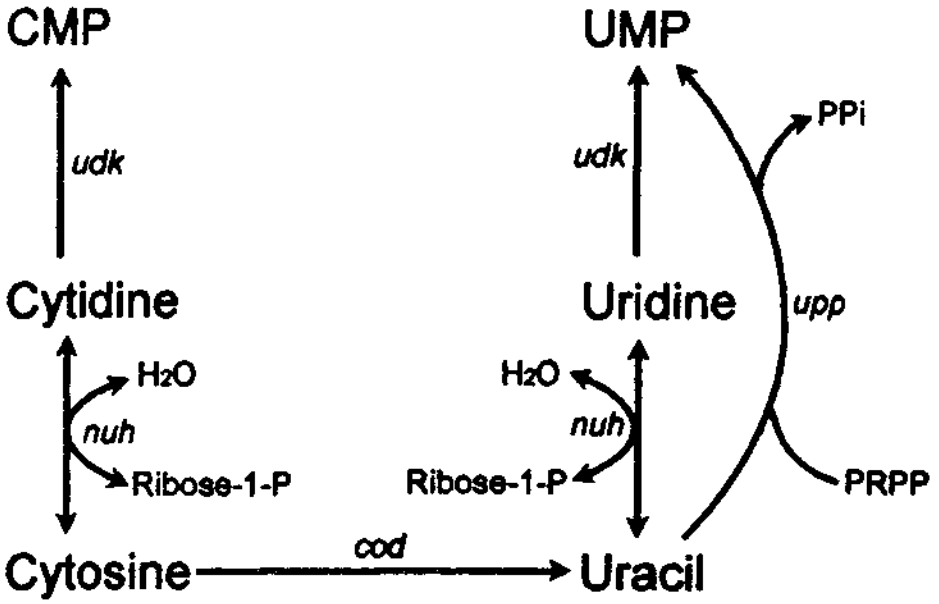
Figure 16. Proposed pyrimidine salvage pathway for *Sporosarcina ureae* and *M. luteus*. Individual reactions are identified by gene symbols. *cdd*, cytidine deaminase; PRPP, 5'-phosphoribosyl-1'-pyrophosphate; *udp*, uridine phosphorylase; *udk*, uridine kinase; *upp*, uracil phosphoribosyltransferase.


CMP
UMP
udk
udk
PPi
Cytidine
cdd
Uridine
upp
Pi
Ribose-1-P
udp
PRPP
Uracil

Sporolactobacillus be placed with the lactobacilli due to its almost identical biochemistry (i.e. sugar fermentation, growth conditions, and pyrimidine salvage). Since the pyrimidine salvage pathway of this organism differs vastly from *Bacillus*, *Sporolactobacillus inulinus* should not be placed within that genus.

In summary, studies of the ATCase from *Sporosarcina ureae* revealed Michaelis-Menten kinetics with a Km_{asp} of 2.5 mM (Figure 8), while the molecular weight of enzyme was estimated to be approximately 95-100 kDa (Figure 10). These findings indicate that the ATCase from *Sporosarcina* was identical to the size and molecular weight of the ATCasefrom *B. subtilis* (Switzer, 1982). The ATCase from *L. fermentum* and *Sporolactobacillus inulinus* were identical in size and molecular weight (Figure 9), while the *L. fermentum* enzyme exhibited hyperbolic kinetics similar to *Sporosarcina* and *B. subtilis* (Marouf, Texas A&M University Dissertation, 1973). While the former organisms were classical representatives of Class C ATCases, *M. luteus* was not. Referring to Figure 9, the ATCase band from *M. luteus* migrated a distance identical to that of ATCase band from *P. aeruginosa*. The molecular weight of the *P. aeruginosa* enzyme has been deduced to be approximately 480 kDa (Vickrey, Ph.D. Dissertation,

Figure 17. Proposed pyrimidine salvage pathway for *L. fermentum* and *Sporolactobacillus inulinus*. Individual reactions are identified by gene symbols. *cod*, cytosine deaminase; *nuh*, nucleoside hydrolase; PRPP, 5'-phosphoribosyl-1'-pyrophosphate; *udk*, uridine kinase; *upp*, uracil phosphoribosyltransferase.


CMP
udk
Cytidine
H2O
nuh
Ribose-1-P
Cytosine
cod
UMP
udk
PPi
Uridine
upp
H2O
nuh
Ribose-1-P
PRPP
Uracil

University of North Texas, 1993). This suggests a similar size and molecular weight for *M. luteus* ATCase.

The results of the HPLC assays have revealed distinct similarities and differences between these related group of bacteria. HPLC assays of cell-extracts from *Sporosarcina ureae* verified the suspicion that salvage enzymes are the same as the salvage enzymes from *B. subtilis* (Figure 12); cytidine deaminase and uridine phosphorylase were present while cytosine deaminase and nucleoside hydrolase were not. Given the results of these data, *Sporosarcina* belongs with the *Bacilliceae*. *M. luteus*, being morphologically similar to *Sporosarcina*, was shown to consist of the same salvage enzymes as *Sporosarcina* and *B. subtilis* (Figure 13), containing both cytidine deaminase and uridine phosphorylase and devoid of cytosine deaminase and nucleoside hydrolase. The HPLC assays of cell-extracts from *L. fermentum* (Figure 14) and *Sporolactobacillus inulinus* (Figure 15) revealed that these organisms have identical salvage pathways similar to *Pseudomonas* species and opposite of *B. subtilis*. Both contain cytosine deaminase and nucleoside hydrolase while cytidine deaminase and uridine phosphorylase are absent. The only apparent difference that exists *between L. fermentum and Sporolactobacillus inulinus* is that fact that

the latter differentiates into true endospores. Therefore, it is suggested that *Sporolactobacillus* should be grouped with the lactobacilli.

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