# A STUDY OF THE PYRIMIDINE BIOSYNTHESIS PATHWAY AND ITS REGULATION IN TWO DISTINCT ORGANISMS: *Methanococcus jannaschii* AND *Pseudomonas aeruginosa*

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*Methanococcus jannaschii* is a thermophilic methane producing archaebacterium. In this organism genes encoding the aspartate transcarbamoylase (ATCase) catalytic (PyrB) and regulatory (PyrI) polypeptides were found. Unlike *Escherichia coli* where the above genes are expressed from a biscistronic operon the two genes in *M. jannaschii* are separated by 200-kb stretch of genome. Previous researchers have not been able to show regulation of the *M. jannaschii* enzyme by the nucleotide effectors ATP, CTP and UTP. In this research project we have genetically manipulated the *M. jannaschii pyrI* gene and have been able to assemble a 310 kDa *E. coli* like enzyme. By using the second methionine in the sequence we have shown that the enzyme from this organism can assemble into a 310 kDa enzyme and that this enzyme is activated by ATP, CTP and inhibited by UTP. Thus strongly suggesting that the second methionine is the real start of the gene.

The regulation of the biosynthetic pathway in *Pseudomoans aeruginosa* has previously been impossible to study due to the lack of CTP synthase (*pyrG*) mutants. By incorporating a functional uridine (cytidine) kinase gene from *E. coli* it has been possible to isolate a *pyrG* mutant. In this novel mutant we have been able to independently manipulate the nucleotide pools and study its effects on the enzymes in the biosynthetic

pathway. The enzyme asapartate transcarbamoylase was repressed 5-fold when exogenous uridine was high and cytidine was low. The enzyme dihydroorotate was repressed 9-fold when uridine was high. These results suggest that a uridine compound may be the primary repressing metabolite for the enzymes encoded by *pyrB* and *pyrC*. This is the first study to be done with the proper necessary mutants in the biosynthetic pathway of *P aeruginosa*. In the past it has been impossible to vary the internal UTP and CTP pools in this organism.

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The support of my family has been invaluable. I thank my parents for believing in me and being here in spirit rather than person while I have been in the United States.

Finally Chris Trahan, has been a constant pillar to lean on while I have been doing this program. I thank you for all your support and thank you for being my inspiration.

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# CHAPTER I

# INTRODUCTION

In 1996 Bult *et al*. reported the complete genome sequence of *Methanococcus jannaschii*. The size of the entire genome was reported to be 1.66 mega-bases, which included two plasmids of 58 kb and 16 kb respectively. A total of 1738 protein coding genes were identified, though only 38% of them were assigned putative cellular roles. The complete genome sequence of this thermophilic autotrophic archaeon allowed for the first time the ability to compare genetic components and biochemical pathways among the three domains of life.

In 1977 Fox and Woese proposed the placement of Archaebacterium into a distinct kingdom of its own. Although Archaea are cytoplasmically prokaryotic in nature, they are related at the molecular level more specifically to Eukaryota. Many of the genes related to transcription, translation, and replication are more similar to those found in Eukaryota, although one does find genes that are required for cell division and central metabolism to bear resemblance to Prokaryota genes.

There are three orders that comprise the methanogens, the *Methanobacteriales*, the *Methanococcales* and the *Methanomicrobiales*. The *Methanococcales* have one family *Methanococcaceae*, and one genus *Methanococcus*, which is composed of six species of themophilic and mesophilic organisms. Members of this genus are Gram-negative or Gram-positive irregular cocci, 1-2 μm in diameter. *M. jannaschii* is the most extensively studied of the six

species in the genus *Methanococcus* (Fig 1). Leigh (1983) first isolated *M. jannaschii* from a sediment sample collected from the sea floor surface at the base of a 2600 m deep hydrothermal vent in the East Pacific Rise and the Guaymus Basin (Jones *et al*, 1983). *M. jannaschii* can grow at pressures of up to 200 atm and has an optimal temperature for growth of 85°C, a pH growth optimum of 6.0 – 7.0 and grows optimally at a salinity of 0.4 – 0.7 M NaCl. *M. jannaschii* utilizes hydrogen ($H_2$) and carbon dioxide ($CO_2$) as substrates for methanogenesis. When growing on $H_2$ and $CO_2$, the methanogens are autotrophic with $CO_2$ serving as both a carbon source and as the electron acceptor. Formate can act as an electron acceptor to be reduced to methane ($CH_4$) for one of the three strains of *M. jannaschii*.

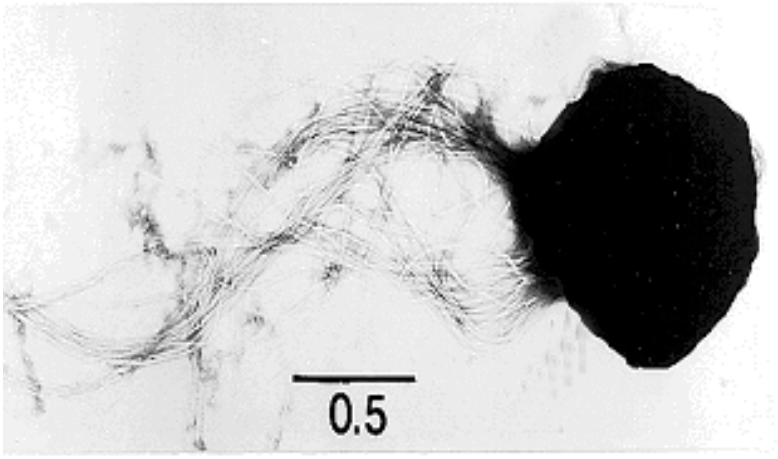



Fig 1. Photomicrograph of *M. jannaschii* seen as irregular cocci. Adapted from The *Methanococcus jannaschii* functions database.

Web address http://geta.life.uiuc.edu/~nikos/Methanococcus_jannaschii.html

**Synthesis of pyrimidine nucleotides *de novo***

The pyrimidine biosynthetic pathway has been studied in detail in bacteria (Yates & Pardee, 1956a, 1956b, 1957; Beckwith *et al*., 1962; Hayward & Belser, 1965; Yan & Demerec, 1965; Hutson & Downing, 1968; Isaac & Holloway, 1968; Condon *et al*., 1976, Foltermann *et al*., 1981; Grogan & Gunsalus, 1993), fungi (Lacroute, 1968; Caroline, 1969), plants (Kafer & Thornburg, 1999) and mammals (Hager & Jones, 1967; Nakinishi *et al*., 1968; Jones, 1980).

There are nine enzymatic steps, which ultimately result in the formation of the pyrimidine ribonucleotides UTP and CTP (Fig 2). The pyrimidine ring is assembled first and then linked to ribose phosphate to form the initial pyrimidine nucleotide OMP. The precursors of the pyrimidine ring are carbamoylphosphate and aspartate. The initial step in the pathway is the formation of carbamoylphosphate, which is produced in the reaction catalyzed by carbamoylphosphate synthetase (CPSase; carbamoylphosphate: L-aspartate carbamoyltransferase, EC 6.3.55). Carbamoylphosphate plays a dual role, being required not only for pyrimidine but also for arginine biosynthesis. The *carAB* genes encode CPSase in *Escherichia coli*. Figure 6 shows the fate of carbamoylphosphate in overall microbial metabolism.

The next step in the pathway involves the carbamoylation of aspartate by aspartate transcarbamoylase (ATCase; EC 2.1.3.2) to form N-carbamoylaspartate and inorganic phosphate. This reaction is the first committed step in the synthesis of pyrimidine nucleotides *de novo*. The genes encoding ATCase in *E. coli* are *pyrBI* and in *Pseudomonas* are *pyrBC′* .

Carbamoylaspartate cyclizes with the loss of water to yield dihydroorotate. At this stage the pyrimidine ring has formed. The reaction is catalyzed by

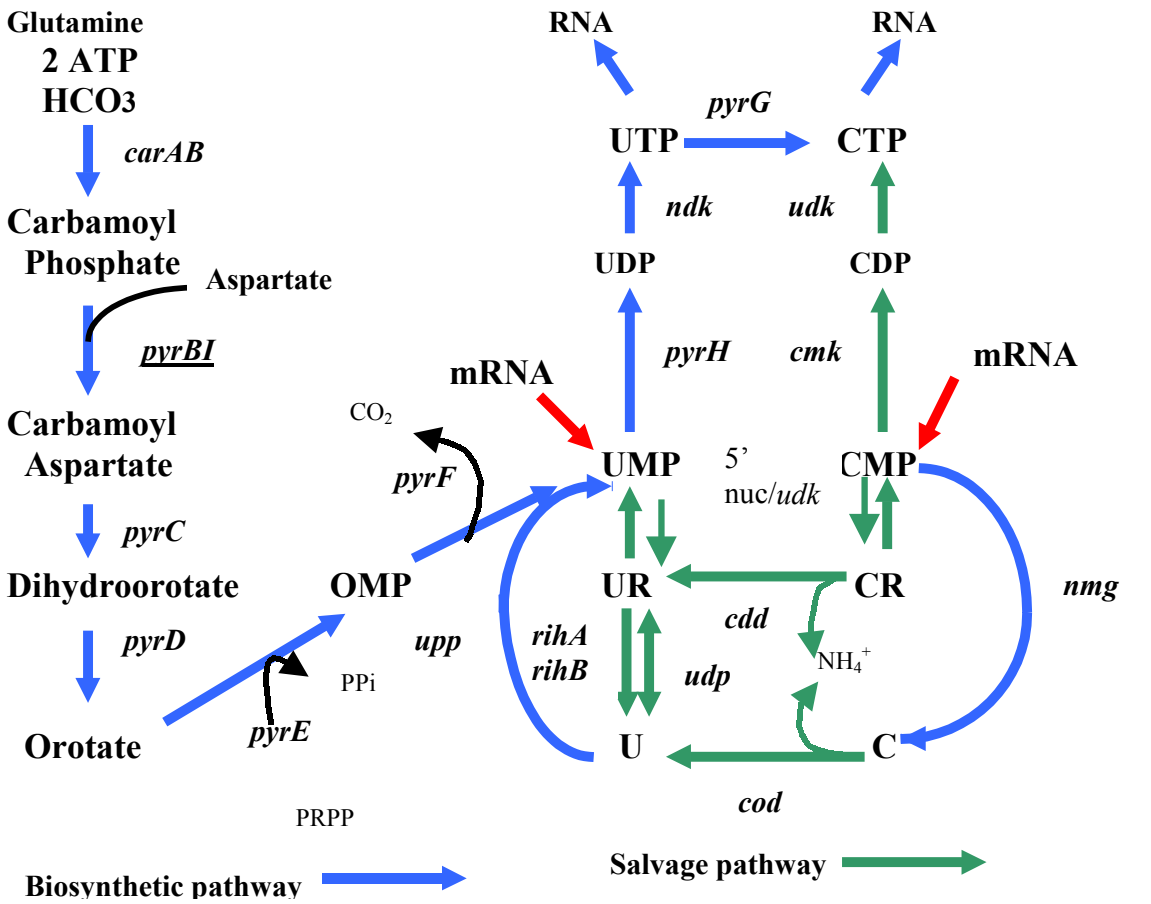
dihydroorotase (DHOase; EC 3.5.2.3) encoded by the *pyrC* gene. The dihydroorotate so formed is then oxidized to orotate by dihydrooorotate dehydrogenase (DHOdehase; EC 1.3.3.1) encoded by *pyrD*. DHOdehase is a flavoprotein which is membrane associated.

The next step in the pathway involves the acquisition of a ribose phosphate group. Orotate phosphoribosyltransferase (OPRTase; EC 2.4.2.10) catalyzes the reaction where orotate a free pyrimidine, reacts with PRPP to yield orotidine-5'-monophosphate (OMP) and pyrophosphate. This reaction is driven forward by the hydrolysis of pyrophosphate. The *pyrE* gene encodes the enzyme is responsible for this conversion.

Decarboxylation of OMP is carried out by OMP decarboxylase (OMPdecase; EC 4.1.1.23), which yields uridine-5'- monophosphate (UMP). The *pyrF* gene encodes OMP decarboxylase. UMP is then phosphorylated by a specific UMP kinase to form uridine-5'-diphosphate (UDP). UMP kinase (UMPK; EC 2.7.4.4) utilizing the *gamma* phosphate of ATP. UTP is produced by the phosphorylation of UDP by nucleoside diphosphokinase. (NDK; EC 2.7.4.6)

The final step in the pyrimidine pathway is the amination of UTP to cytidine-5'-triphosphate (CTP) by the enzyme CTP synthetase. (CTPsase; EC 6.3.42). For this enzyme, encoded by *pyrG*, glutamine serves as the amino donor for the amination.

ATCase is an especially interesting regulatory enzyme. ATCase from *E. coli* is one of the most highly characterized enzymes. The nature of this enzyme in *M. jannaschii*, *E. coli* and in *Pseudomonas* species will be discussed later.




**Biosynthetic pathway genes & enzymes:**
*carAB*: Carbamoylphosphate synthetase
*pyrB*: Aspartate transcarbamoylase
*pyrC*: Dihydroorotase
*pyrD*: Dihydroorotate dehydrogenase
*pyrE*: Orotate phosphoribosyltransferase
*pyrG*: Cytidine 5'-triphosphate synthetase
*pyrH*: Uridine 5'-monophosphate kinase
*ndk*: Nucleotide diphosphokinase

**Salvage genes & enzymes:**
*cdd*: Cytidine deaminase
*cmk*: Cytidine 5'-monophosphate kinase
*cod*: Cytosine deaminase
*nmg*: Nucleoside monophosphate glycosylase
*upp*: Uracil phosphoribosyltransferase
*udk*: Uridine kinase
*udp*: Uridine phosphorylase
*rihA*: Ribonucleoside hydrolase A
*rihB*: Ribonucleoside hydrolase B

Fig 2. The pyrimidine biosynthetic and salvage pathways in *E. coli*.

**Recycling of pyrimidine and purine nucleotides**

Pyrimidine nucleotide biosynthesis can be divided into three phases (O'Donovan *et al*, 1989). Phase 1 involves the production of the pyrimidine ribonucleotides UTP and CTP. Phase 2 yields the deoxyribonucleotides dCTP and the ultimate pyrimidine nucleotide dTTP. The final phase, phase 3 involves the salvage pathways. The salvage phase can be divided into two parts, the ribonucleotide salvage pathway and the second one being the deoxyribonucleotide salvage pathway (Fig 3). The nucleotide salvage pathway functions to supply the pentose portions of nucleotides and nucleosides as carbon and energy source and the amino group of cytosine compounds as nitrogen source. Pyrimidine bases and nucleosides are returned to the pool as triphosphates and thus are recycled and not excreted into the medium.

The salvage pathway in prototrophs provides a balance between RNA synthesis and the biosynthetic pathway, while it supplies all the necessary requirements for pyrimidines in auxotrophs (O'Donovan & Shanley, 1995). Figure 3 shows the salvage enzyme pathway in the archetype organism *E. coli*. The latter half of this dissertation deals specifically with the salvage pathway, which is discussed in greater detail in Chapter II.

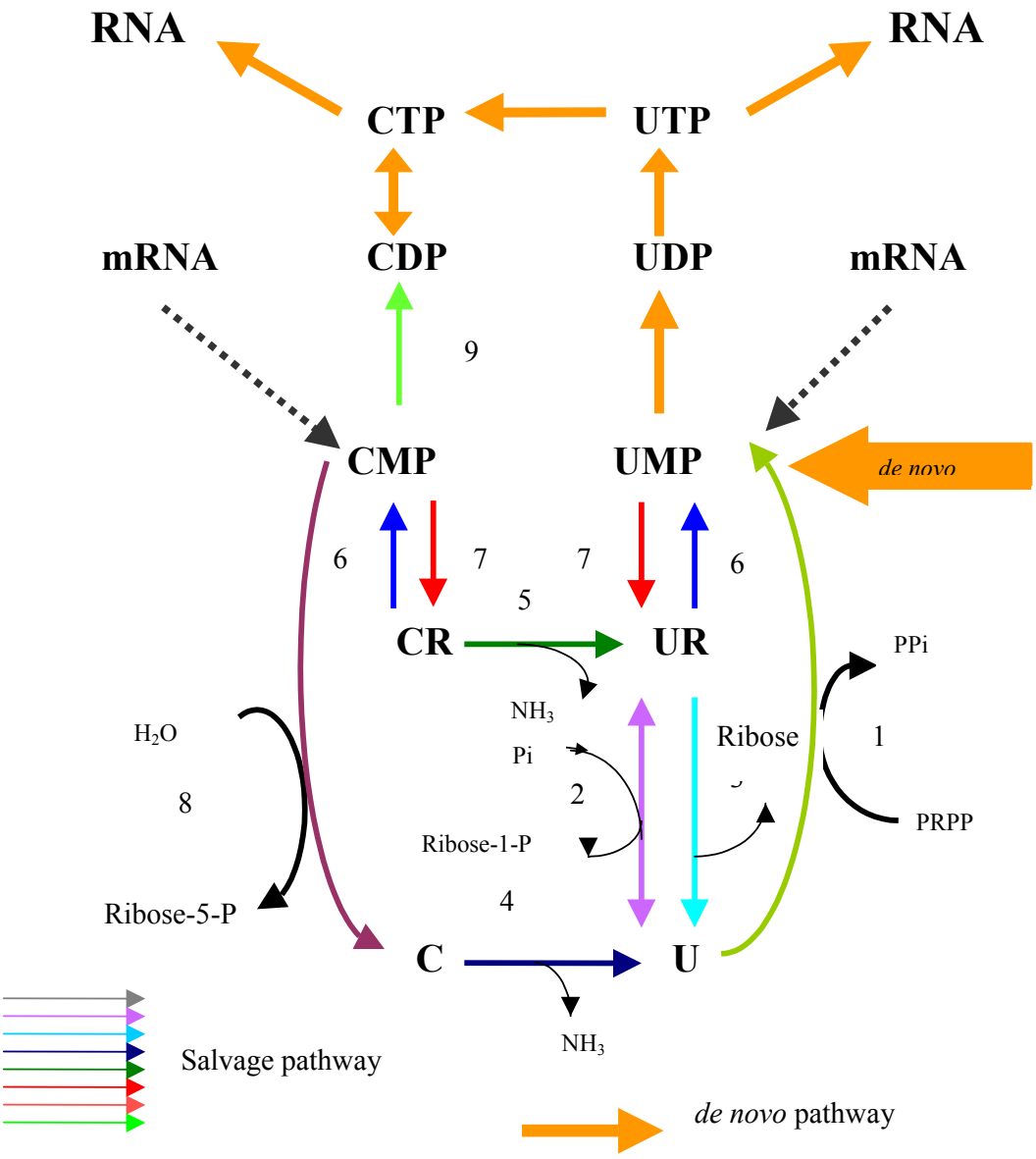



Fig 3. Pyrimidine salvage pathway in *Escherichia coli*. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. ribonucleoside hydrolase A & B (Rih), 4. cytosine deaminase (Cod), 5. cytidine deaminase (Cdd), 6. uridine kinase (Udk), 7. 5'-nucleotidase, 8. CMP glycosylase, 9. CMP kinase (Cmk)

**Classification of bacterial aspartate transcarbamoylase**

Bacterial ATCases are divided into three classes. Bethell & Jones (1969) first proposed these classes, namely Class A, Class B and Class C. The classification is based on the molecular weight of the ATCases and their response to the nucleotide effectors (ATP, CTP and UTP).

Class B ATCase, in which the enzymes of *E. coli* and other *Enterobacteriaceae* have been placed has a molecular mass of approximately 300 kDa and shows sigmoid saturation curves with both substrates, aspartate and carbamoylphosphate. The subunit structure of Class B ATCase is $2(c_3)$: $3(r_2)$.

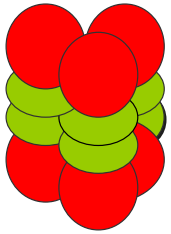
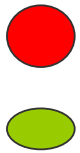
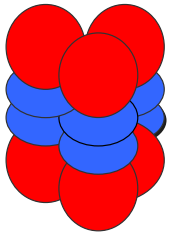
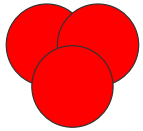
Class C ATCases are the smallest in size and have a molecular mass of 100-140 kDa. Class C enzymes are not regulated and are simple catalytic trimers ($c_3$). A representative within this class of ATCases is the *Bacillus subtilis* enzyme, which has a structural organization of ($c_3$).

The largest ATCases are those that belong to Class A and have been studied in the genus *Pseudomonas*. The ATCase of *P. fluorescens* (Neumann & Jones 1964; Adair & Jones 1972), *P. putida* (Condon, *et al.*, 1976; Shurr *et al.*, 1995), *P. aeruginosa* (Isaac & Holloway, 1968) and *Burkholderia cepacia* (Linscott, Ph.D. dissertation, 1996) have all been extensively studied. Purified ATCase from *P. fluorescens* was thought to be a dimer (Neumann & Jones, 1964; Adair & Jones, 1972) until it was shown to consist of a 1:1 ratio of 34-kDa and 45-kDa polypeptides, which are arranged in a dodecamer (Bergh & Evans 1993). The $K_M$ for aspartate in this study was reported to be 2.75 mM and that for carbamoylphosphate to be 14 μm. ATCase of *P. aeruginosa* (Isaac & Holloway 1968, Schurr *et al.*, 1995) was reported

to have a molecular mass of 480 kDa, whilst the $K_M$ of aspartate in this species was 1.3 mM. ATP and UTP inhibited the enzyme. CTP was also an inhibitor but not to the same extent as ATP and UTP. The ATCase of *P. putida* (Condon *et al.*, 1976) was reported to be inhibited by pyrophosphate, ATP, UTP and CTP. When carbamoylphosphate was limiting, CTP was the most influential inhibitor of the enzyme. The structural organization of the Class A ATCase encoded by *pyrBC'* in *Pseudomonas* species is dodecameric and may be represented as follows $2B_3$: $3C'_2$. Schurr *et al*, (1995) showed that the ATCase of *P. putida* required an inactive DHOase to be functional.

Hughes *et al*, (1999) conducted an extensive study of the ATCase of *Streptomyces griseus*. In this organism, hyperbolic curves were obtained for ATCase activity when velocity-substrate plots for aspartate and carbamoylphosphate were made. The ATCase enzyme was inhibited by ATP, CTP, UTP and GTP. This is typical of Class A ATCase found in other organisms. Purification studies of ATCase and DHOase showed that the two activities were present as a single enzyme complex. Two other organisms, namely *Deinococcus radiophilus* and *Thermus aquaticus* also show this ATCase/DHOase activity in their holoenzyme structure. Many more organisms may also possess this unique complex. This led Hughes *et al,* (1999) to suggest a subtyping of the Class A ATCases. Class $A_1$ ATCases would be those that have both ATCase and DHOase activity and Class $A_2$ would be those that have degenerate DHOase subunits as one finds in *Pseudomonas*. Figure 4 shows all the classes of ATCases.

Fig 4.  The Classes of Bacterial ATCase

| ATCase Class | Holoenzyme | Subunit Structure | Representative organisms |
|---|---|---|---|
| **Class A (480 kDa)** |  |  *pyrB* catalytic 34 kDa<br>*pyrC'* active or inactive. 45 kDa | **Inactive DHOase**<br>*Pseudomonas putida*<br>*Pseudomonas aeruginosa*<br>**Active DHOase**<br>*Deinococcus radiophilus*<br>*Thermus aquaticus* |
| **Class B (300 kDa)** |  |  *pyrB* catalytic 34 kDa<br>*pyrI* regulatory 17 kDa | *Escherichia coli*<br>*Salmonella typhimurium*<br>*Serratia marcescens*<br>*Neisseria meningitidis*<br>*Proteus vulgaris*<br>*Pyrococcus abyssi* |
| **Class C (100 kDa)** |  |  *pyrB* catalytic 34 kDa | *Bacillus subtilis*<br>*Bacillus caldolyticus*<br>*Streptococcus pyogenes* |

**Regulation by transcriptional attenuation of *E. coli pyrBI* operon**

The *pyr* genes can be regulated by mechanisms of transcriptional and translational attenuation. In *E. coli* the catalytic and the regulatory polypeptides of ATCase are expressed from adjacent genes, *pyrB* and *pyrI* respectively, which are present in a bicistronic operon. The pathway for pyrimidine biosynthesis is regulated at the level of enzyme synthesis by attenuation (Roof *et al.*, 1982; Turnbough *et al.*, 1983; Schachman, 1983) and enzyme activity is regulated by allosteric inhibition by nucleotide effectors (Gerhart & Pardee, 1962, 1964).

The *pyrBI* genes encoding ATCase and the *pyrE* gene encoding OPRTase all have upstream leader sequences that allow for attenuational control. Turnbough *et al*, (1983) reported the presence of two promoter regions located at –350 bp and –160 bp upstream of the translational start of the *pyrB* gene. The promoter region located at –160 bp was observed to be more active *in vivo* than the region located at –350 bp (Navre & Schachman, 1983). Transcripts initiated at both promoters are terminated at a region of dyad symmetry, 40 bp from the start of the *pyrB* gene. The *rho*-independent terminator is high in GC residues, which allows for the formation of a stem loop secondary structure. The stem loop structure is followed by a string of uridine residues, which typifies other attenuators found in some amino acid biosynthetic operons. A second stem loop formation can also be observed at –100 bp upstream of the *pyrB* start (Fig 5a).

Based on the information a model has been proposed for the mechanism of attenuational control of pyrimidine biosynthesis in *E. coli*.

At high intracellular levels of UTP, RNA polymerase transcribes the message through the region of dyad symmetry. This allows for the formation of the stem loop secondary structure corresponding to the termination loop and transcription is terminated. Therefore the structural genes are not transcribed (Fig 5b).

At low UTP concentrations, the RNA polymerase pauses at a site located –80 bp from the start of the *pyrB* gene. This pausing allows the translating ribosome to catch up, and this linking does not allow the formation of the stem loop structure. The coupling of the ribosome and RNA polymerase allows for the transcription of the *pyrBI* operon and the subsequent expression of ATCase (Fig 5c).

**Fig 5a Promoter-Regulatory Region**

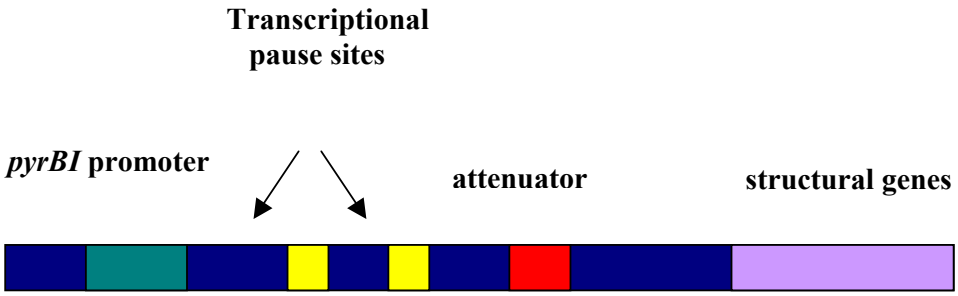



**Fig 5b High UTP-No or Weak Transcriptional Pausing**

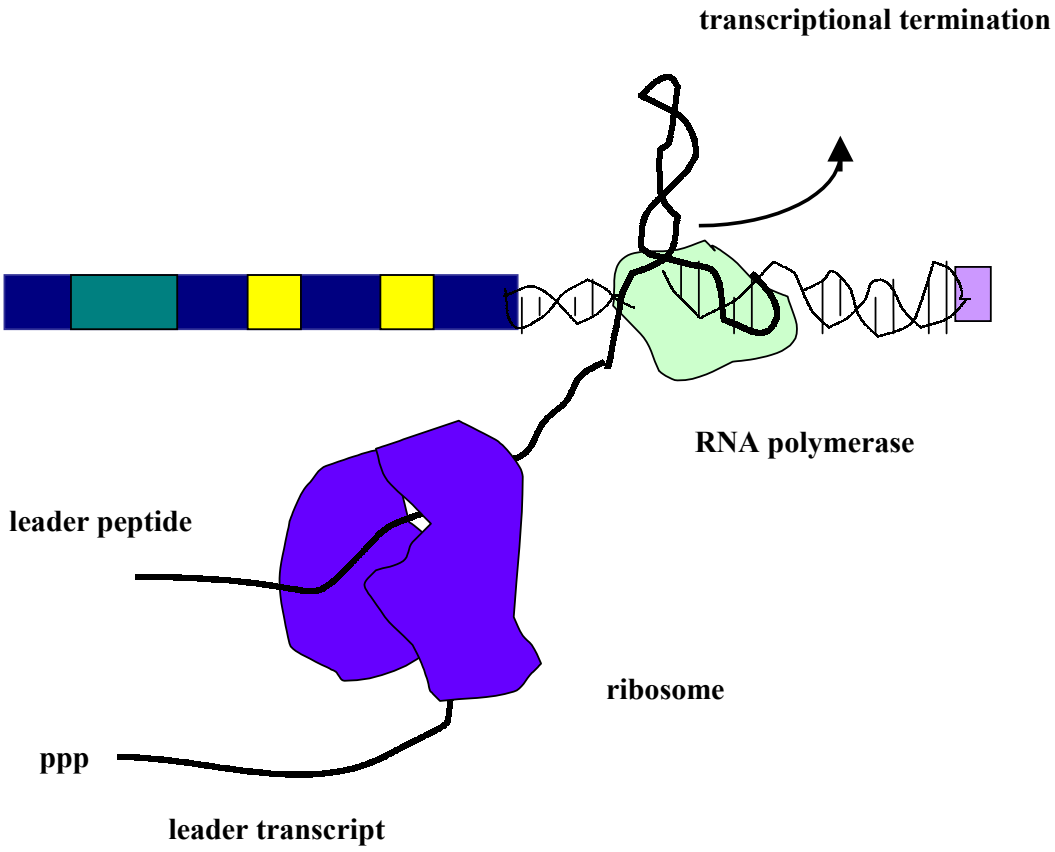
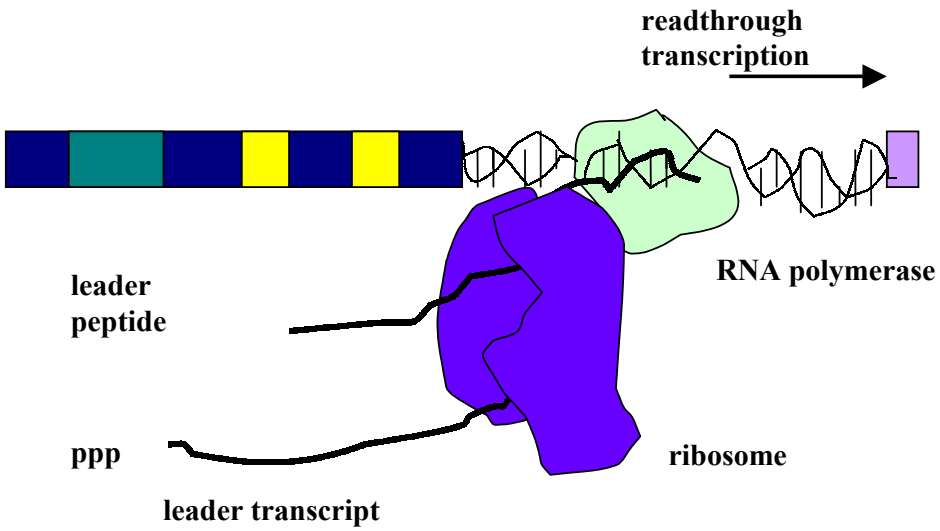


**Fig 5c Low UTP-Strong Transcriptional Pausing**



**Regulation of pyrimidine biosynthesis at the level of enzyme activity in *E. coli* and *Pseudomonas*.**

Enzyme activity is controlled at three steps in the *de novo* synthesis of pyrimidines in *E. coli*. CPSase, is inhibited by UMP and activated by ornithine and IMP. ATCase the first committed step in the pathway is inhibited by CTP and UTP, and activated by ATP (Gerhart & Pardee, 1962). CTP synthetase encoded by my favorite gene, *pyrG*, is inhibited by CTP and activated by UTP (Long and Pardee, 1967; Long and Koshland, 1978).

In the genus *Pseudomonas* regulation at the level of enzyme activity is similar to that found in *E. coli*. CPSase activity is inhibited by UMP and activated by ornithine and N-acetylornithine (Abdelal *et al*., 1983). ATCase activity which is encoded by *pyrBC*' is inhibited by ATP, CTP and UTP (Issac and Holloway, 1968; Condon *et al*, 1976; Vickrey, 1993; Schurr *et al*, 1995).

**Regulation by attenuation of the *Bacillus subtilis* pyrimidine biosynthetic (*pyr*) gene cluster**

In *Bacillus subtilis* the *pyr* map as a single gene cluster on the chromosome (Lerner & Switzer, 1986). All of the genes on the cluster are expressed as a single polycistronic transcriptional message. Transcription is controlled by the availability of UTP. Switzer *et al* (1999) have shown that it is controlled by an attenuation mechanism.

Transcriptional attenuation is controlled at three sites within the 5' sequence of the transcript. The first gene in the cluster is a UMP- dependent RNA binding protein encoded by *pyrR*. When pyrimidine nucleotide levels are high (UTP) in the *B. subtilis* cell, UMP binds to the PyrR protein. This in turn binds to the *pyr* mRNA in a sequence specific manner. This binding promotes the formation of a *rho*-independent terminator, which inhibits the expression of the downstream *pyr* genes (Lu *et a.l*, 1995; Lu and Switzer, 1996; Turner *et al.*, 1998; Switzer *et al.*, 1999).

**Regulation of pyrimidine biosynthesis in *Pseudomonas*.**

Various researchers (Isaac & Holloway, 1968; Condon *et al.*, 1976) have tried to elucidate the regulation of pyrimidine biosynthesis in the pseudomonads. Regulation of pyrimidines in *Pseudomonas* has been elusive and researchers have yet to ascertain the control of the pathway. It has not been possible to study the mechanism of control at the level of gene expression. However research in our lab (Patel & Kumar, unpublished data) have studied the presence of a regulatory protein, PyrR, in *P. aeruginosa* upstream of the *pyrBC*' genes. The *pyrR* gene product is thought to regulate the expression of the later pyrimidine genes, *pyrD*, *pyrE* and *pyrF* by binding to a specific sequence. PyrR is also thought to autoregulate its own expression.

**Aspartate transcarbamoylase of thermophiles and hyperthermophiles**

Microorganisms have the ability to inhabit almost any enviroment. The discovery of life in extreme conditions opened up a whole new perspective on the fundamentals of applied biology. "Extremophiles" are organisms that can thrive in extreme conditions. These include those organisms that can withstand very low temperatures (between 0°C and 10°C) as in the case of the psychrophiles. Thermophiles and hyperthermophiles are organisms that can withstand very high temperatures (60°C to 113°C). Organisms that can survive at pressure (40 atm) are known as barotolerant organisms or barophiles. Alkalophiles are those organisms that can survive at pH>10 and acidophiles are those that can survive at pH 3.0 or lower. All these organisms are prokaryotes belonging to the eubacterial or archaea domains of life (Cunin, 1997). The ATCases of many of these organisms provide insights into the ancestral forms of the enzyme and therefore it is important to study the enzymes in these extremophiles. As in all bacteria, carbamoylphosphate (CP) is required in the arginine and pyrimidine biosynthetic pathways. Fig. 6 shows the many pathways in which CP is involved. CP is a highly thermolabile metabolite and it may even be toxic at high temperatures by being converted to cyanide. Therefore, thermophilic bacteria must have evolved strategies to protect against degradation of CP and its possible toxic effects. Thus, it is important to study these two intertwined pathways. ATCase appears to be ubiquitous and therefore its study in extremophilic archaea and eubacteria is useful when one considers it for comparative phylogenetic studies.

The genes for ATCase from all of the following bacteria have been studied. *Thermus aquaticus* ZO5 (Van de Casteele *et al.*, 1997), *Thermus thermophilus, Thermatoga maritima, Pyrococcus abyssi* and *Sulfolobus solfataricus* ATCases have all been cloned and subsequently characterized by complementing a *pyrB* deficient *E. coli* strain. All of the ATCases from *T. aquaticus, P. furiosus, P. abyssi, T. maritima* and *S. solfataricus* are regulated (Van de Casteele, 1994 and Purcarea *et al.*, 1994). All show cooperativity for the use of aspartate in that velocity-substrate plots are sigmoidal for both aspartate and carbamoylphosphate.

*P. abyssi* ATCase shows cooperativity for the use of aspartate and carbamoylphosphate. (Van de Casteele, 1994 and Purcarea, 1995). The ATCase of *T. maritima* and *P. furiosus* is inhibited by UTP and CTP, whilst the enzyme from *T. aquaticus* is inhibited by UTP (Van de Casteele, 1994; Purcarea *et al.*, 1994; Van de Casteele, 1997). Enzyme assays conducted at 37°C showed that the *P. abyssi* ATCase was inhibited by CTP, UTP and activated by ATP. When the same assay was performed at 90°C it was observed that the enzyme was inhibited by CTP only. UTP, CTP and ATP activate the *S. solfataricus* enzyme.

When the catalytic polypeptides of ATCase (*pyrB*) and the regulatory (*pyrI*) polypeptides are aligned from the extremophiles and *E. coli* it can be seen that there are strong similarities between them. Fig. 7 shows the *pyrB* alignments from various organisms and Fig. 8 shows the *pyrI* alignments from various organisms. In *S. solfataricus* the *pyrBI* genes are organized as part of a *pyrB I- pyrE* divergent operon.

This type of arrangement for pyrimidine genes is novel when one compares it to other closely related organisms.

As pointed out earlier the entire genome of *M. jannaschii* has been sequenced (Bult *et al*., 1996) and it has been shown that two ORFs show similarity to the *P. abyssi* and *S. solfataricus pyrB* and *pyrI* genes. In *E. coli*, the two genes are present as part of a *pyrBI* bicistronic operon. In *M. jannaschii* the two coding regions are separated by a 200 kb stretch of genome. Genes that are unrelated to the metabolism of CP are present within this 200 kb space. When one aligns the pyrB gene product of *E. coli* and *M. jannaschii* they exhibit 47% amino acid identity and 67% amino acid similarity (Fig 9) are exhibited between them. Amino acid comparison of the *M. jannaschii* PyrI protein shows 35% identity and 52% similarity to the *E. coli* PyrI protein (Fig 10). Residues comprising the zinc binding site and nucleotide effector binding sites are all conserved in the *M. jannaschii* PyrI. *M. jannaschii* differs from other recently characterized ATCases from other archaeabacteria (*P. abyssi* and *S. acidocaldarius*), which contain an enterobacteria-like *pyrBI* operon (Purcarea *et al.*, 1997 and Durbecq *et al.*, 1999).

It seemed important to ascertain if the *M. jannaschii* PyrB and the PyrI polypeptides were capable of assembly into an active 310 kDa *E. coli* like ATCase and if the putative complex could be regulated.

In this chapter we present data to show that the *M. jannaschii* ATCase has the capacity to assemble into an *E. coli* like enzyme and that the nucleotide effectors ATP, CTP and UTP regulate the complex.

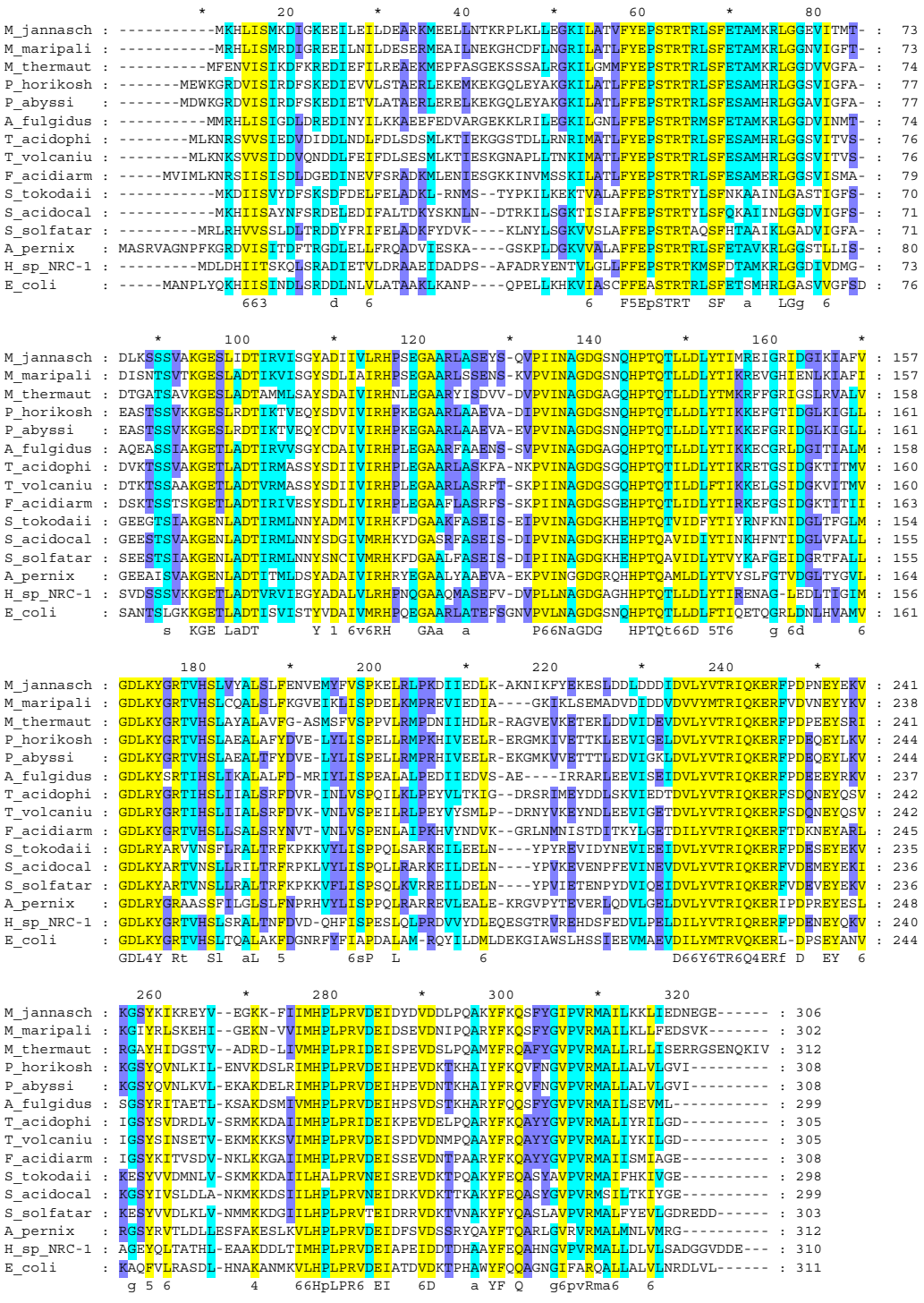



Fig 7. Amino acid alignment of *M. jannaschii pyrB* gene with other known organisms *pyrB* genes.

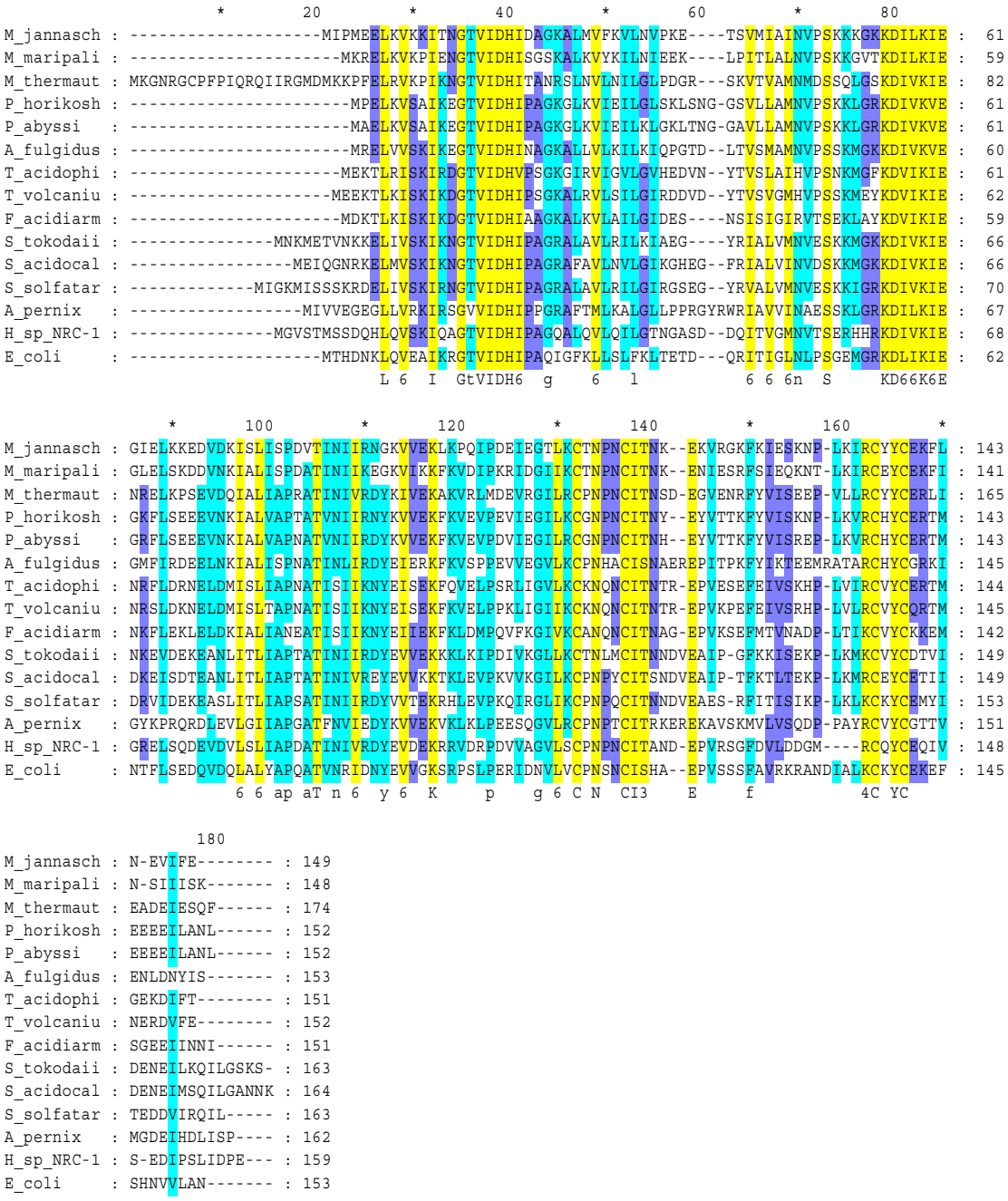



Fig. 8 Amino acid alignment of *M. jannaschii pyrI* gene with other known organisms *pyrB* gene.

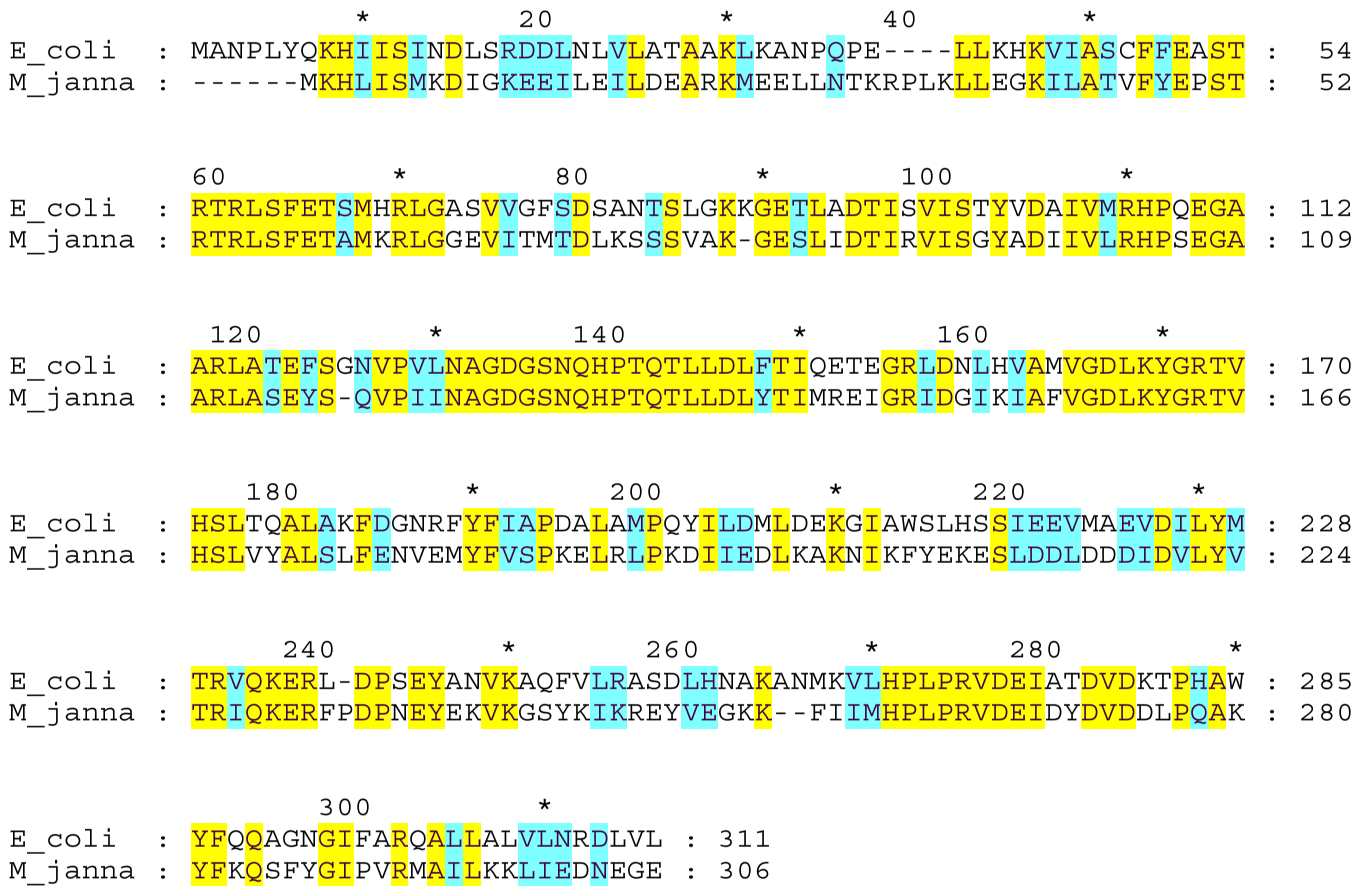



Fig 9. Amino acid alignment of *E. coli pyrB* gene and *M. jannaschii pyrB* gene.

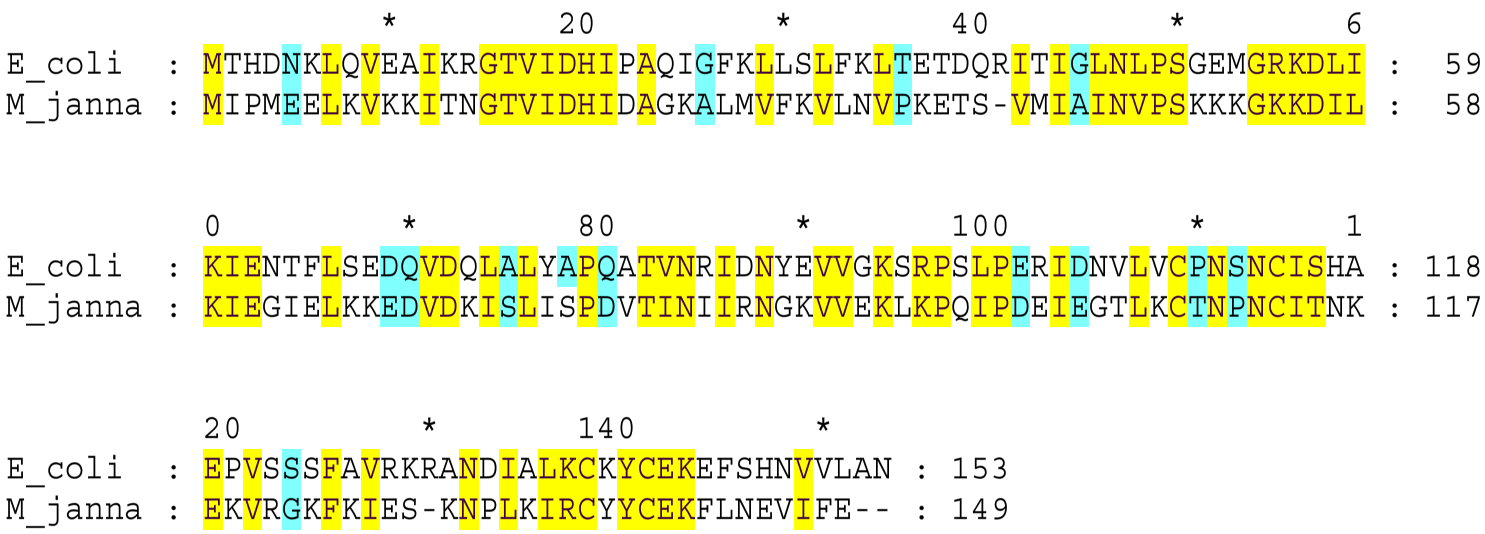



Fig 10. Amino acid alignment of *E. coli pyrI* gene and *M. jannaschii pyrI* gene.

Table 1. Known pyrimidine genes in thermophilic organisms.

| Organism | pyrimidine genes | organization of genes |
|---|---|---|
| *M. jannaschii* | *pyrB* and *pyrI* | genes separated by 200-kb |
| *P. abyssi* | *pyrB* and *pyrI* | genes are fused, one transcript |
| *S. solfataricus* | *pyrB* and *pyrI* | genes next to each other, not fused |
| *S. acidocaldarius* | *pyrB* and *pyrI* | genes separated by 1 bp |
| *Ta. Maritime* | *pyrB* and *pyrI* | genes are fused, one transcript |
| *Thermus ZO5* | *pyrR-pyrB-bbc-pyrC* | genes in an operon |
| *B. caldolyticus* | *pyrR-pyrP-pyrC-pyrD* | *de novo* genes associated with 2 |
| | *pyrA-pyrF-pyrE* | salvage genes |

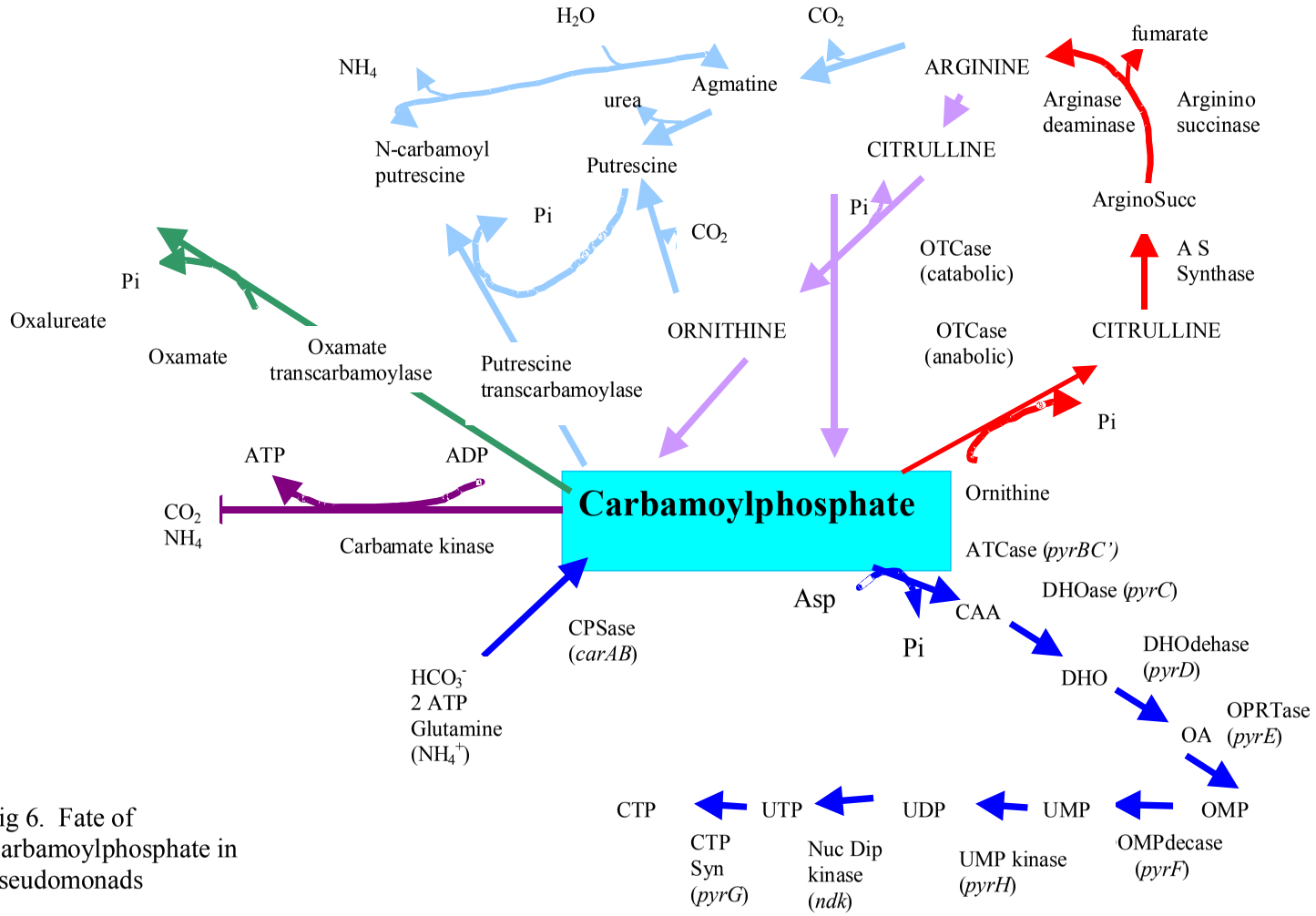



Fig 6. Fate of Carbamoylphosphate in Pseudomonads

# CHAPTER I

# MATERIALS AND METHODS

**Bacterial strains, plasmids, media and growth conditions.**

The bacterial strains and plasmids used in this study are listed in Table 2. The genotype of *Escherichia coli* strain TB2 (*argI* $^{-}$, *argF* $^{-}$ and *pyrBI* $^{-}$) was confirmed by checking for growth in *E. coli* minimal medium (Miller, 1992) with the addition of arginine, uracil or both at a concentration of 50 μg $ml^{-1}$. For the transformation experiments, the recombinant plasmids were selected on the appropriately supplemented *E. coli* minimal medium. Antibiotics were added at the following concentrations: ampicillin 100 μg $ml^{-1}$ and kanamycin 50 μg $ml^{-1}$. The carbon source used was glycerol at 0.2%. All *E. coli* TB2 strains harbouring the selected plasmids were grown at 37°C with shaking at 250 rpm.

**Preparation of competent cells.**

All *E. coli* TB2 competent cells for transformation experiments were prepared using the calcium chloride method of Dagert & Ehrich (1979) with slight modifications. The cultures were grown in 50 ml of *E. coli* minimal medium to an optical density at 600 nm ($OD_{600}$) of 0.2 to 0.4 and chilled on ice at 0 to 4°C for 10 min. The cells were harvested by centrifugation at 1875 xg at 4°C for 15 min, the pellet was resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 20 to 25 min. The cells were centrifuged at 833 xg at 4°C for 15 min, the pellet was

resuspended in 0.7 ml of ice cold $CaCl_2$ and incubated overnight on ice. The following day glycerol was added to a final concentration of 15% and the cells were separated into 200 µl aliquots. Cells were then frozen at –80°C and could used for up to three months.

Table 2. List of Strains and Plasmids

| Strain or Plasmid | Genotype or relevant property |
|---|---|
| **Strain** | |
| *E. coli* TB2 | Δ*pyrBI*, *arg I*, *argF* |
| **Plasmids** | |
| pUC18 | pMB1 origin, high copy |
| pBJR28 | *E. coli pyrB* |
| pEK2 | *E. coli pyrBI* |
| pK184 | Kanamycin resistant compatible plasmid |
| AMJAE67 | 1.26 kb insert containing the *M. jannaschii pyrI* gene in pUC18 |
| AMJPK84 | 1.2 kb insert containing the *M. jannaschii pyrB* gene in pUC18 |
| pCRII vector | Invitrogen linearized plasmid |
| pSPMI | Unmodified *pyrI* cloned into the *Eco*RI and *Bam*HI sites of pK184 |
| pSPMI'2 | Shine Dalgarno and deletion of the first 3 amino acids cloned into pCRII vector |
| pSPMI3 | Shine Dalgarno and change of the native GTG start codon to an ATG start cloned into pCRII vector |
| pSPMI'4 | Shine Dalgarno and deletion of the first 3 amino acids cloned into pK184 vector |
| pSPMI5 | Shine Dalgarno and change of the native GTG start codon to an ATG start cloned into pK184 vector |

**Transformation of *E. coli* TB2**.

Transformation of *E. coli* TB2 with the plasmids *E. coli pyrB* (pBJR28) (Fig 11), *E. coli pyrBI* (pEK2) (Fig 12) and *M. jannaschii pyrB* (AMJPK84) (Fig 13) were conducted according the method described by Huff *et al*, (1990). Approximately 40 ng of plasmid DNA were mixed with 200 µl of the $CaCl_2$ treated cells and the mixture was incubated on ice for 15 min. The cells were then heat shocked at 42°C for 2 min, and then placed back into the ice bucket for a further 15 min. One ml of LB broth was added to the cells, which were then incubated at 37°C for 1 h. After 1 h the cells were plated on LB medium, plasmid insertion was selected for by ampicillin resistance at a concentration of 100 µg $ml^{-1}$.

Further analysis was conducted to show that expression of the plasmid genes satisfied the pyrimidine requirement of *E. coli* TB2. The colonies that grew on the LB ampicillin plates were replica plated onto three addition plates, of *E. coli* minimal medium, of *E. coli* minimal medium supplemented with arginine and of *E. coli* minimal medium plus arginine and uracil. Untransformed *E. coli* TB2 cells were used as a control.

**Preparation of cell extracts for aspartate transcarbamoylase activity gels.**

Starter cultures of cells containing the above plasmids pBJR28, pEK2, AMJPK84 and *E. coli* TB2 with no plasmid were grown in 5 ml of *E. coli* minimal medium overnight with the addition of glucose as the carbon source, thiamine

(vitamin $B_1$), arginine, and ampicillin. *E. coli* minimal medium (50 ml) with the above additions was used and the starter cultures were used as the inoculum. The flasks were shaken at 37°C at 250 rpm and grown to an $OD_{600}$ of 0.5 to 0.7. The cells were harvested by centrifugation at 1875 xg at 4°C for 15 min and resuspended in 1 ml of ATCase buffer (5 ml Tris-HCl pH 8.0, 200 µl 1M β-mercaptoethanol, 2 µl 1M $ZnSO_4$, 20 ml 100% glycerol and 75 ml $ddH_2O$). The cells were disrupted by sonication for 3 min in an ethanol ice bath. The sonicated extract was centrifuged at 12,000 xg at 4°C for 30 min. The supernatant (clarified extract) was transferred to a sterile microcentrifuge tube and used to assay ATCase on an activity gel.

**Heat treatment of clarified cell extract.**

Thirty microliters (30 µl) of clarified cell extracts were heated at 65°C and 80°C for 0, 10, and 20, 30 min. This part of the experiment was conducted in order to test the thermostability and subunit structure of *E. coli pyrB* (pBJR28), *E.coli pyrBI* (pEK2) and *M. jannaschii pyrB* (AMJPK84). After the period of heat treatment, the extracts were centrifuged at 10,000 xg for 1 min. The clear supernatant was then used for further analysis.

**Non-denaturing polyacrylamide aspartate transcarbamoylase activity gels.**

A nondenaturing polyacrylamide gel with 5% stacking gel and an 8% separating gel was used. The gel was prepared by first pouring the separation gel, which contained 2.67 ml of the stock solution of acrylamide (30% w/v acrylamide and

1 % w/v bis-acrylamide in dd$H_2O$), 2.5 ml of Buffer B (1.5 M Tris-HCL, pH 8.8), and 4.83 ml of dd$H_2O$. Ammonium persulfate (0.02 g) was added to the mixture. The gel was polymerized with the addition of 5 µl of N, N, N' N' - tetramethylenediamine (TEMED). The mixture was gently inverted and poured into the assembled Bio-Rad mini protean II apparatus. A space of 2 cm was left at the top to pour the stacking gel. The gel was overlaid with *N*- butanol to prevent drying and was allowed to polymerize for approximately 30 to 40 min after which the stacking gel was prepared. The stacking gel contained 0.67 ml of acrylamide, 1 ml of buffer C (0.5 M Tris, pH 6.8) and 2.3 ml dd$H_2O$, ammonium persulfate (0.01g) and 5µl of TEMED. A 10 well comb was inserted and the gel was allowed to polymerize. 16 µl of sample was mixed with 5µl of 5X loading buffer (312.5 mM Tris, 50% v/v glycerol, and 0.05% w/v bromophenol blue in dd$H_2O$). The samples were loaded onto the gel. The chamber was filled with gel running buffer (25 mM Tris, 192 mM glycine in dd$H_2O$) and electrophoresis was carried out 150 V for 1 h at room temperature.

**Aspartate transcarbamoylase activity stain of the nondenaturing polyacrylamide gels.**

The gels were stained specifically for ATCase activity by the method described by Bothwell (1975) as modified by Kedzie (1987). The principle behind method is that when ATCase catalyses the reaction between carbamoylphosphate and aspartate to produce carbamoylaspartate, an inorganic phosphate is generated. This phosphate group reacts with lead nitrate to form a white precipitate. A modified version of the above method by Kedzie (1987) was performed. The gels were placed in 250 ml of 50 mM, histidine buffer pH 7.0 for 5 to 10 min that had been warmed at

65°C in a waterbath. 5 ml of 1 M aspartate and 10 ml of 0.1 M carbamoylphosphate was added and the gels were incubated at room temperature on a rocking shaker. The reactants were removed by rinsing the gel in 100-200 ml of warm dd$H_2O$ 3 times. Lead nitrate at a concentration of 3mM was added to another 250 ml of histidine buffer, pH 7.0, which was then poured onto the gel. After 10 min, the lead nitrate was removed by washing three times in warm dd$H_2O$. ATCase activity was observed at the site of lead phosphate precipitation. The gel was left overnight at room temperature to increase the visibility of the bands. The gel was stained with 1% sodium sulfate for 3 min and washed three times with dd$H_2O$ to convert the white lead nitrate to black lead sulfide. The gels were soaked in 10% glycerol and dried to preserve them.

**Cloning of the *M. jannaschii pyrI* gene into the compatible vector pK184.**

The *M. jannaschii pyrI* (AMJE67) plasmid (Fig 14) was obtained from the American Type Culture Collection (ATCC 623859). In order to co-express this plasmid with the *M. jannaschii pyrB* and with the *E. coli pyrB* gene (pBJR28) it was necessary to clone it into the compatible plasmid pK184 (Fig 15) (GenBank accession number U00800). Plasmid pK184 DNA was digested with the restriction enzymes *Eco*RI and *Bam* HI to linearize the plasmid and allow for the ligation of the *M. jannaschii pyrI* gene fragment, which had been previously isolated from AMJE67 using the same enzymes. The 1.26 kb *pyrI* gene fragment was purified from the agarose gel using the Sephaglas Band Prep Kit® (Amersham-Pharmacia) and ligated into pK184 to produce pSPM1 (Fig 16).

**Cotransformational studies using *E. coli pyrB* and *M. jannaschii pyrI*, and *M. jannaschii pyrB* and *M. jannaschii pyrI*.**

*E. coli* TB2 cells were transformed using the method described above. DNA from plasmids expressing the *E. coli pyrB* (pBJR28) and *M. jannaschii pyrI* (pSPMI), *M. jannaschii pyrB* (AMJPK84) and pSPMI was used. The insertion of both plasmids was confirmed by extraction of plasmid using the alkaline lysis method. The cell extracts were run on 8% nondenaturing gels. This experiment failed to produce a 310 kDa holoenzyme.

**Addition of an *E. coli* like Shine Dalgarno sequence into the native *M. jannaschii pyrI by* polymerase chain reaction.**

Sequence analysis of the upstream DNA of the *M. jannaschii pyrI* did not reveal an *E. coli* consensus Shine Dalgarno sequence. We also observed that 6 bases in from the designated start there was another methionine residue which was coded for by ATG. Thus, 2 synthetic oligonucleotide primers were designed and synthesized by of Biosynthesis, Inc. The first primer was used to incorporate an *E. coli* Shine Dalgarno (SD) sequence, as well to change the original GTG start codon to an ATG start codon. The second was used to incorporate an *E. coli* (SD) sequence and delete the first three amino acids present in the native clone. PCR using *Taq* polymerase (Saiki *et al*., 1998) was used to amplify the products (Table 3). The following conditions were used. This enzyme generates 3'T overhangs and this allows for the direct cloning into the pCR$^{TM}$II Vector (Fig 17) from The Original TA

Cloning ® Kit (Invitrogen). After ligation and transformation clones, which contained the modification were selected on LB plus kanamycin 50 μg $ml^{-1}$, with 5 bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) at 0.003% and isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.03%. White colonies appearing on the medium were selected and grown in 5 ml of LB medium with 50 μg $ml^{-1}$ of kanamycin. Insertion of the modified *pyrI*'s were confirmed by restriction digest and by DNA sequencing. The clones produced were termed pSPMI'2 (addition of SD sequence and deletion of the 1st 3 amino acids) and pSPMI3 (addition of an SD sequence and change of the original GTG to and ATG start codon) (Fig 18 and 19).

Table 3 Oligonucleotides used for PCR of modified *M. jannaschii pyrI*

| **Primer** | **Sequence (5'- 3')** | **Use** |
|---|---|---|
| MetIF | GGAGGAGAAATGTAACTATGATTCCTATGGAGGAGTTAAAA | SD sequence & GTG → ATG |
| MetIF | GGAGGAGAAATTAACTATGGAGGAGTTAAAAGTTAAAAAA | SD sequence & Deletion of First two amino acids |
| MetIR | TTTGAAATACTTCTTCCTTTAATTCCATAATACCA | Reverse primer |

**DNA sequencing of pSPMI'2 and pSPMI3**

Sequencing of double-stranded DNA was performed with the Sequenase® kit (U.S. Biochemicals Corp.) based on the dideoxy method of Sanger *et al*., (1977 & 1980). All reactions were carried out according to manufacturer's specifications. M13 –20 forward primer and M13 reverse primer were used to sequence the recombinant plasmids and confirm the orientation of the inserts.

**Cloning of pSPMI'2 and pSPMI3 into the compatible vector pK184**

It was necessary to clone the modified *pyrI* genes, first into pUC19, and then into pK184, due to the lack of enzymes that were available for the purpose of cloning into pK184. Therefore, the insert from pSPMI2 and pSPMI3 was excised with the restriction enzymes *Kpn*I *and Xho*I. The vector pUC19 was also digested with the same enzymes and then a ligation reaction was set up. *E. coli* DH5α was transformed with the DNA. Cells with the recombinant plasmids were selected on LB agar plates with ampicillin at a concentration of 100 μg $ml^{-1}$. The plates also contained 5 bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) at 0.003% and isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.03%. Presumptive transformants containing the insert appeared as white colonies and were picked. Plasmid DNA was extracted using the alkaline lysis method.

Upon confirmation of the insert of the appropriate size, by agarose gel electrophoresis, the plasmid DNA was digested with the enzymes *Kpn*I and *Sal*I. This was also true of pK184. Ligation of the modified *pyrI* genes with the compatible

vector pK184 was set up. *E. coli* DH5α cells were transformed and plated out on LB agar with kanamycin at a concentration 50 μg $ml^{-1}$. The plasmids were named pSPM4 (Fig 20) and pSPM5 (Fig 21). The plasmids containing the inserts were selected for cotransformational studies along with *M. jannaschii pyrB* (AMPK84).

**Cotranformational studies with the various modified *M. jannaschii pyrI* plasmids and the *M. jannaschii pyrB* plasmid.**

*E. coli* TB2 cells were transformed with the above plasmids. Table 4 lists the plasmids. The insertion of both plasmids was confirmed by isolation of plasmid DNA. Cell extracts were prepared for ATCase activity gels as described earlier. Clarified cell extracts were heated at 80°C for 20 min before loading onto nondenaturing ATCase activity gels.

Table 4

| Plasmids | Description |
|---|---|
| AMPK84 + pSPMI2 vector | *M. jannaschii pyrB* and modified *pyrI'* in pCRII |
| AMPK84 + pSPMI3 vector | *M. jannaschii pyrB* and modified *pyrI* in pCRII |
| AMPK84 + pSPMI4 vector | *M. jannaschii pyrB* and modified *pyrI* in pK184 |
| AMPK84 + pSPMI5 vector | *M. jannaschii* pyrB and modified *pyrI'* in pK184 |

**Effector response of 310 kDa Aspartate Transcarbamoylase of *M.* jannaschii.**

Cell extract was assayed for ATCase activity by measuring the amount of carbamoylaspartate (CAA) produced at 20 min at 37°C. The method used was the color reaction described by Prescott & Jones, 1969. ATCase assays were performed to determine the Vmax, $K_M$ and nucleotide effector response. A tribuffer system (0.05 M MES, 0.1 M diethanolamine, and 0.051 M N-ethymorpholine) was used. The assay was conducted at pH 9.5 in a microtiter plate. Aspartate was varied from a final concentration of 5 mM to 60 mM and carbamoylphosphate was kept at a concentration of 5 mM. The total volume of the assay was 200 µl. Effector response could be measured by adding UTP, CTP, or ATP at a final concentration of 1 mM into the microtiter plate wells. A blank control containing all ingredients minus the enzyme was used. This reading was then subtracted from the final reading for the experimental reaction. An aliquot of the enzyme was incubated at 80ºC and then 1 µl of it was used in the reaction. The assay reaction plate was preincubated at 37ºC for 2 to 3 min. The reaction was initiated with the addition of cabamoylphosphate. At 20 min the reaction was stopped with the addition of 100 µl of stop mix (2 parts antipyrine (5 mg/ml) in 50% sulfuric acid and 1 part monoxime (8 mg/ml) in 5 % acetic acid). After the addition of the stop mix clear tape was applied to the top of the wells to prevent the evaporation of the reaction mixture. The color was developed at 60ºC in a waterbath. The assay was read at 450 nm in a kinetic microplate reader by molecular devices. Velocity substrate curves were generated by plotting the specific activity of the enzyme (µmol CAA/min/µg of protein). The protein concentration was measured by the method described by Bradford (1976) with Bovine Serum Albumin

as the standard. The µmol produced was calculated by generating a CAA standard curve.

***In vitro* expression of modified PyrI from *M. jannaschii*.**

The *M. jannaschii* PyrI protein was expressed using the *E. coli* S30 extract for Circular DNA (Promega). This procedure allows one to observe the transcription and translation of DNA sequences that have been cloned into plasmid vectors. The technique is based on the method described by Zubay (1973) and allows for the characterization of polypeptides from cloned genes.

One µg of pSPMI4 and pSPMI5 DNA (individually) was used as the template. The DNA was mixed with 5 µl of amino acid mixture, which was devoid of methionine, 20 µl of s30 Premix without amino acids, 15 µCi of [$^{35}$ S] methionine (NEN Dupont) and 15 µl of s30 Circular extract. Nuclease-free water was added to a final volume of 50 µl. The components were mixed by vortexing and then centrifuged at 12,000 xg for 5 s to collect the mixture at the bottom of the tube. The reaction was incubated at 37$^{\circ}$C for 2 h. The reaction was terminated after 2 h by placing the tubes on ice for 5 min. A 5 µl aliquot was transferred to a fresh tube and was precipitated by adding 20 µl of acetone. This step removes the polyethylene glycol (PEG) present in the reaction mixture. The supernantant was aspirated and the pellet was dried for 15 min in a vacuum desiccator. The expressed proteins were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were then visualized by autoradiography.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).**

SDS is an anionic detergent, which denatures proteins by binding or wrapping around the protein backbone. The method denatures the protein into its individual subunits and thus separates them based on their molecular weight. SDS binds specifically to proteins and confers a negative charge to them. It is necessary to reduce the disulfide bridges in proteins so that they would adopt a random coil configuration. This allows for the proteins to be separated based on its molecular weight. Therefore separation of proteins using SDS-PAGE is determined by molecular weight rather than its intrinsic electrical charge. A 15% SDS polyacrylamide separating gel with a 5% stacking gel was used to analyze the samples. The apparatus used to conduct the electrophoresis was the Mini-Protean II™ chamber (Bio-Rad).

The gel was prepared by first pouring the 15% separating gel, which contained 5 ml of the stock solution of acrylamide (30% w/v acrylamide and 0.8 % w/v bis-acrylamide in $ddH_2O$), 2.5 ml of Buffer B (1.5 M Tris-HCl, pH 8.8, 0.4% w/v SDS in $ddH_20$), and 2.5 ml of $ddH_2O$. Ammonium persulfate (0.02 g) was added to the mixture and mixed by vortexing gently to dissolve the ammonium persulfate. The gel was polymerized with the addition of 5 µl of N, N, N' N' - tetramethylenediamine (TEMED). The mixture was gently inverted and poured into the assembled apparatus. A space of 2 cm was left at the top to pour the stacking gel. The gel was overlaid with *n* - butanol to prevent drying and was allowed to polymerize for approximately 30 to 40 min after which the stacking gel was prepared. The stacking gel contained 0.67 ml of Solution A, 1 ml of buffer C (0.5 M Tris, pH 6.8, 0.4% w/v SDS) and 2.3

ml $ddH_2O$, ammonium persulfate (0.01 g) and 5 µl of TEMED. A 10 well comb was inserted and the gel was allowed to polymerise for 1 h. The gel was placed into the apparatus and the tank filled up with denaturing gel running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS w/v, pH 8.3). The sample were mixed at a ratio of 4:1 with the gel loading dye (60 mM Tris-HCI, pH 6.8, 25% glycerol v/v, 2% SDS w/v, 14.4 mM β-mercaptoethanol, 0.1% Bromophenol blue) in a sterile microfuge tube. The samples and the standards were boiled for 2 min and cooled on ice. The samples and the standards were loaded onto the gel and electrophoresed for 90 min at 150 V. The proteins were stained with Coomasse Blue staining solution (45% methanol v/v, 10% acetic acid v/v, 0.1% Coomasse Brilliant Blue R-250 w/v in $ddH_2O$) for 10 min with gentle rocking. The gel was destained with a solution of 10% methanol v/v and 10% glacial acetic acid v/v in $ddH_2O$ for 3 h. The gel was dried on a Whatmann™ filter paper and an X-ray film was placed on top of the dried gel in a Kodak™ X-ray exposure holder.

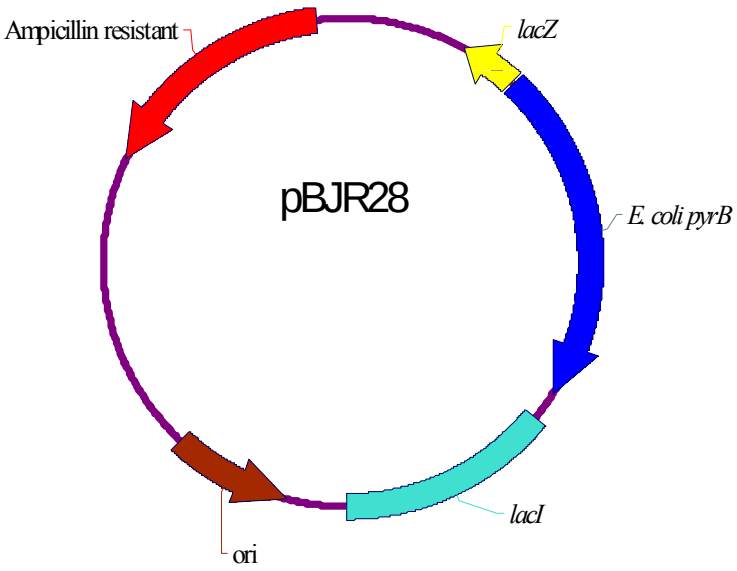



Fig 11. Plasmid pBJR28 constructed by Jill Ruley. The plasmid contains the entire 1.1 kb *pyrB* gene fragment from the plasmid pEK2.

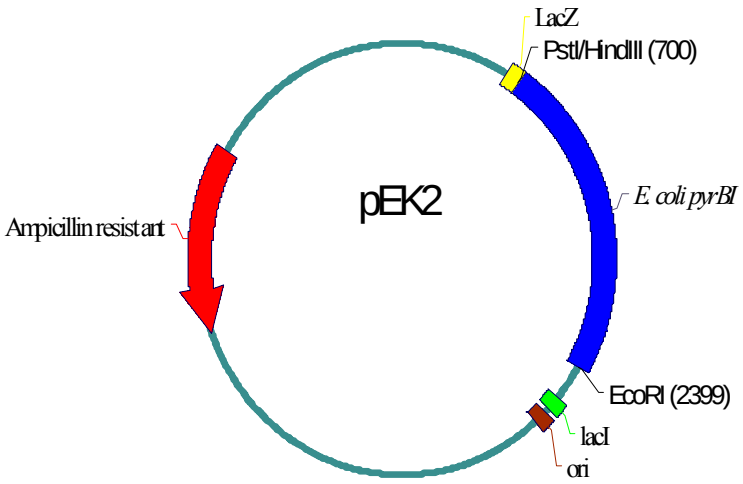



Fig 12. Schemmatic representation of plasmid pEK2. The plasmid contains the entire *pyrBI* operon of *E. coli*.

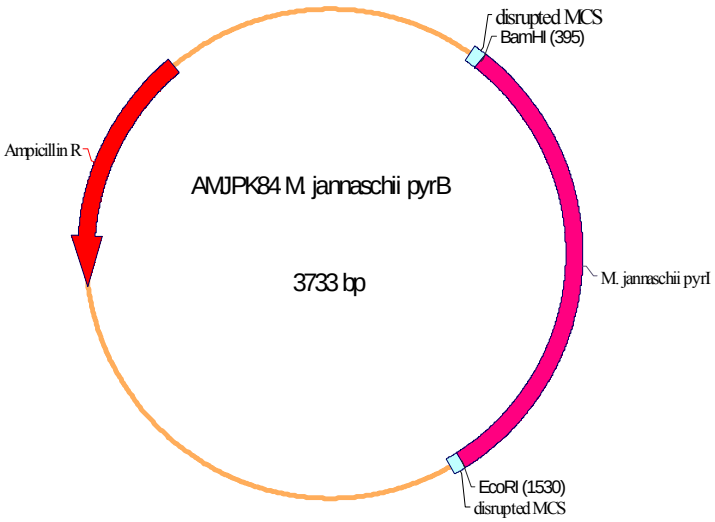



Fig 13. Plasmid AMJPK84 containing the intact 1.2 kb *M. jannaschii pyrB* gene in pUC18. Plasmid was obtained from the American Type Culure collection.

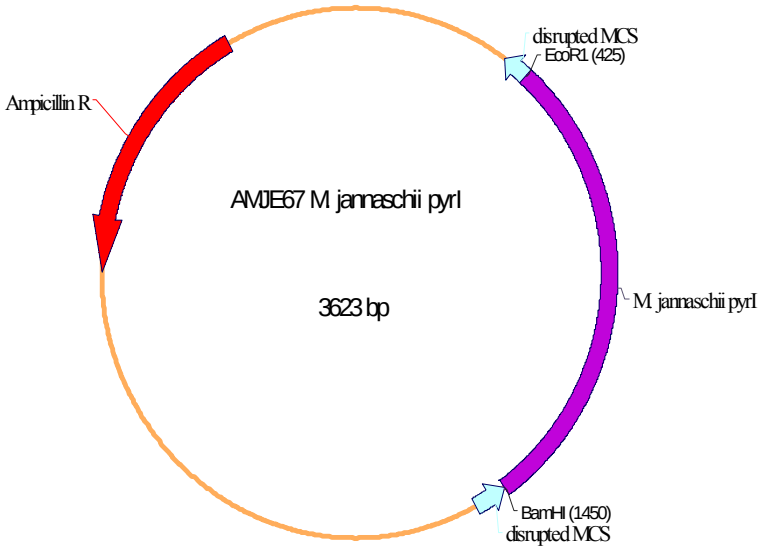



Fig 14. Plasmid AMJ67 containing the entire 1.26 kb *M. jannaschii pyrI* gene in pUC18. Plasmid was obtained from the American Type Culture collection (ATCC 623859).

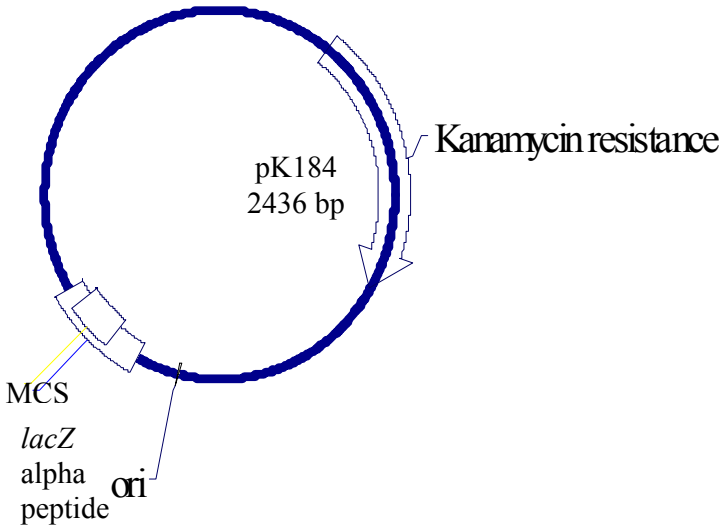



Fig 15. Schematic representation of plasmid pK184.

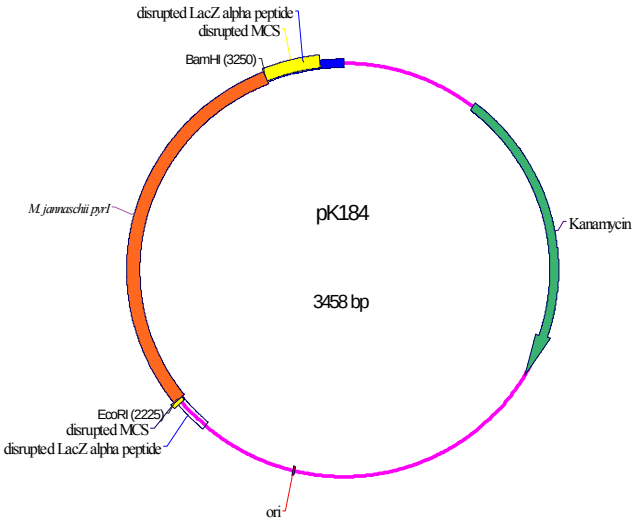



Fig 16. The *M. jannaschii* 1.26 kb *pyrI* gene fragment from AMJE67 cloned into the pK184 plasmid at the *Eco*RI and *Bam*HI restriction sites

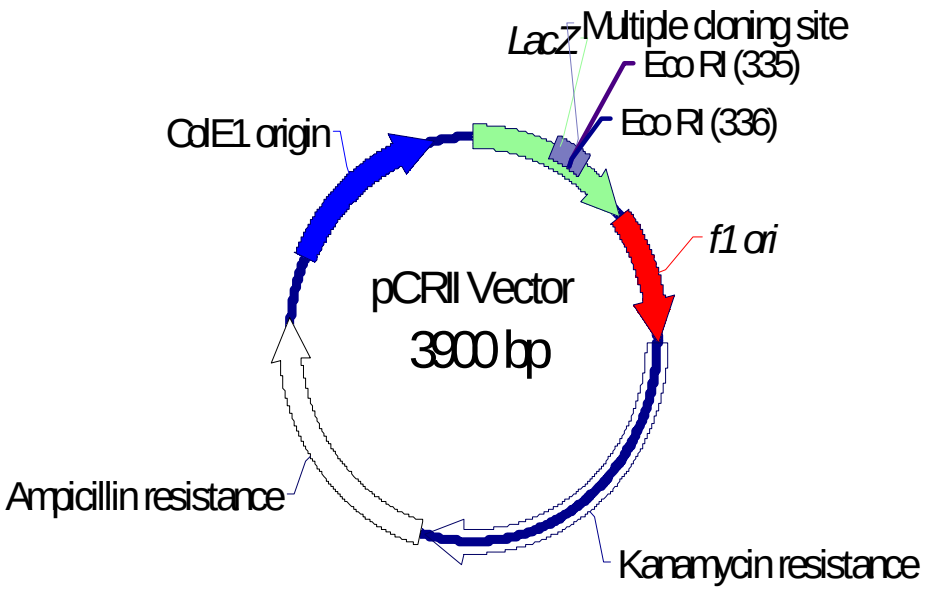



Fig 17. Schematic representation of pCRII™ vector (Invitrogen®).

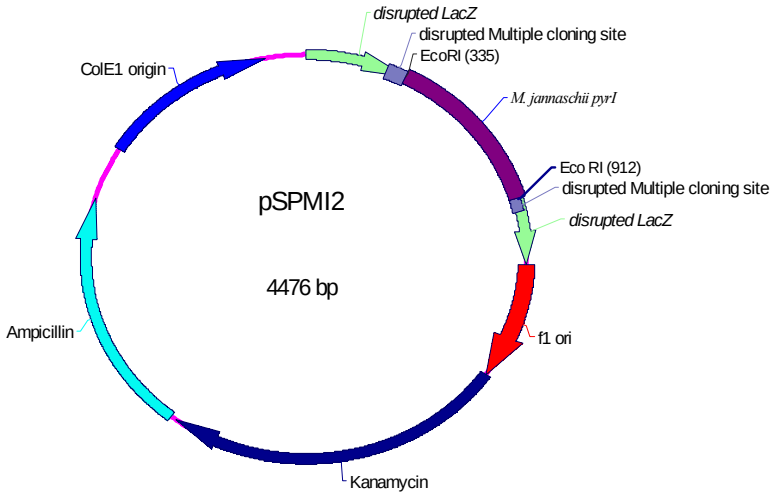



Fig.18 Schematic diagram of the pCRII vector harbouring the modified *M. jannaschii pyrI*. Addition of an *E. coli* like Shine Dalgarno change of the native GTG start to an ATG start. Insert size is 576 bp.

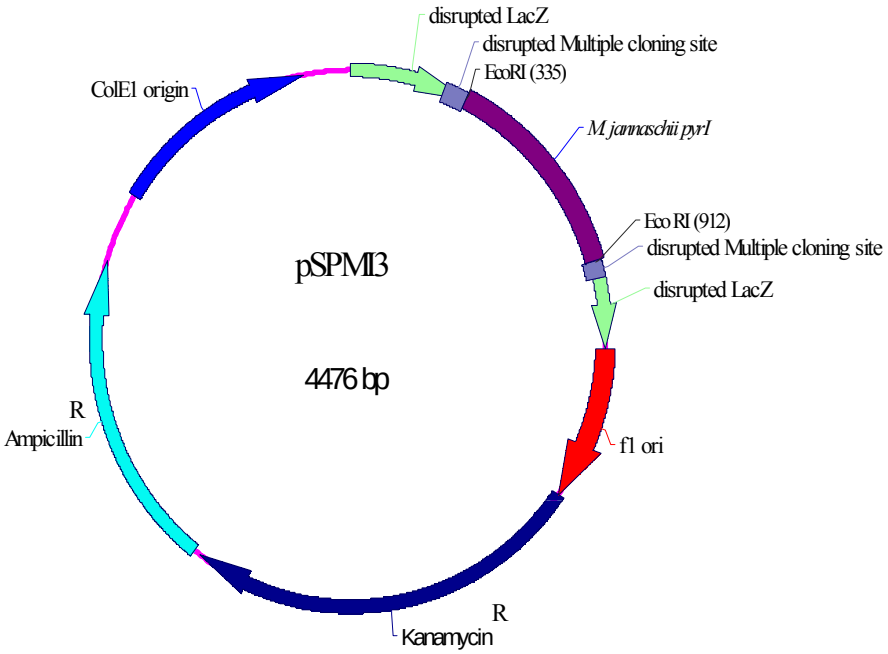



Fig.19 Schematic diagram of the pCRII vector harbouring the modified *M. jannaschii pyrI*. Addition of an *E. coli* like Shine Dalgarno and deletion of the 1st 3 amino acids 568 bp.

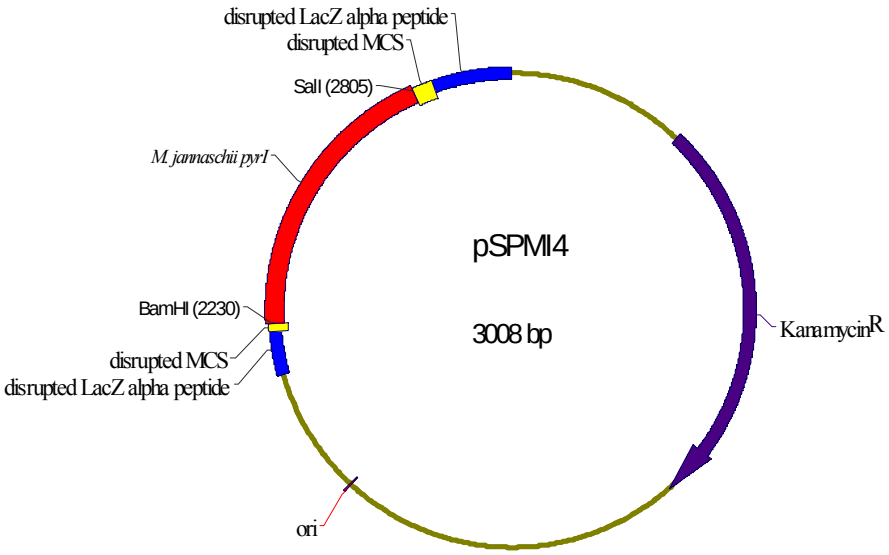



Fig. 20 Schematic diagram of recombinant plasmid containing the modified *M. jannaschii pyrI* subcloned from pUC19 into pK184. The insert size is 576-bp and contains the modified *pyrI* native sequence. Shine-Dalgarno sequence has been added and the native GTG start codon changed to ATG.

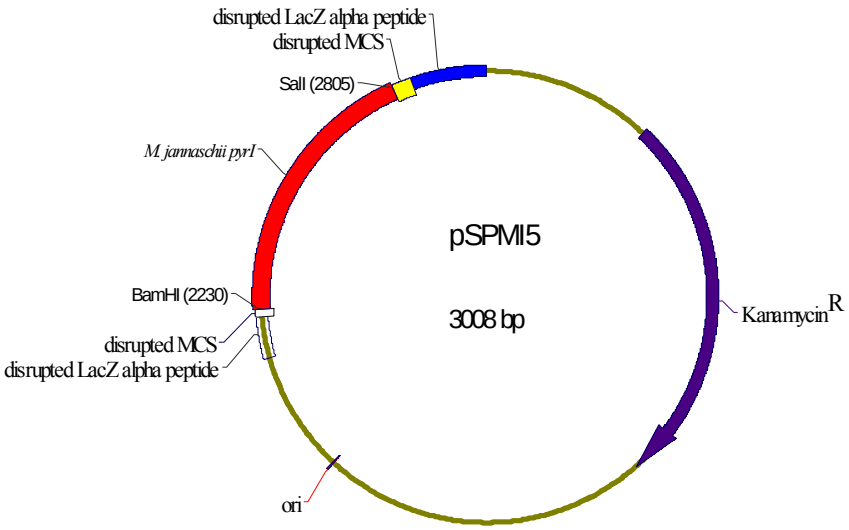



Fig. 21 Schematic diagram of recombinant plasmid containing the modified M. jannaschii pyrI subcloned from pUC19 into pK184 plasmid. The insert size is 568-bp and contains the modified pyrI native sequence. Shine Dalgarno added and 1st three amino acids deleted as to use the second methionine ATG start.

# CHAPTER I

# RESULTS AND DISCUSSION

## Active ATCase from various plasmids

Cell extracts from *E. coli* TB2 cells containing various plasmids were run on ATCase activity gels (Fig 21). *E. coli* TB2 cells containing the *E. coli pyrBI* from expressed from the plasmid pEK2 showed aggregated forms of ATCase and bands corresponding to 310 kDa and 270 kDa. Cells containing the *E. coli pyrB* only (pBJR28) showed a 100 kDa ATCase band corresponding to the catalytic *pyrB* trimer. Cells containing the *M. jannaschii pyrB* gene (AMJPK84) similarly showed the 100 kDa ATCase band corresponding to the catalytic trimer.

## Thermostability of active ATCase encoded by *E. coli pyrB* (pBJR28), *E. coli pyrBI* (pEK2) and *M. jannaschii pyrB* (AMJPK84) at 65°C.

The thermostability of the *E. coli* ATCase was compared to that of *M. jannaschii*. *E. coli* TB2 cells containing the plasmids pBJR28 (*E. coli pyrB*), pEK2 (*E. coli pyrBI*) and AMJPK84 (*M. jannaschii pyrB*) were grown at 37°C. 30 µl of clarified cell extracts were heated at 65°C for 0, 10, 20 and 30 min. ATCase activity gels showed that the *E.*

*coli* enzyme remained fully active after the heat treatment. *E. coli* TB2 cells containing the pBJR28 plasmid showed 100 kDa ATCase bands up to 30 min of incubation at 65°C. At time 0 the *E. coli TB2* cells containing the pEK2 plasmid showed a holoenzyme at 310 kDa. The 310 kDa holoenzyme form disappeared after heating presumably degrading into the catalytic trimer form as the incubation time increased. In contrast the *E. coli* TB2 sample containing the *M. jannaschii pyrB* gene showed very little or no activity at time 0 (no heat treatment). As the sample was incubated at 65°C the ATCase seemed to become active and its activity increased as the incubation time increased (Fig 22). This result suggests that the *E. coli* enzyme is sensitive to temperature and the enzyme is less active at higher temperatures. The *M. jannaschii* catalytic subunit responds to high temperatures and therefore is more thermostabile.

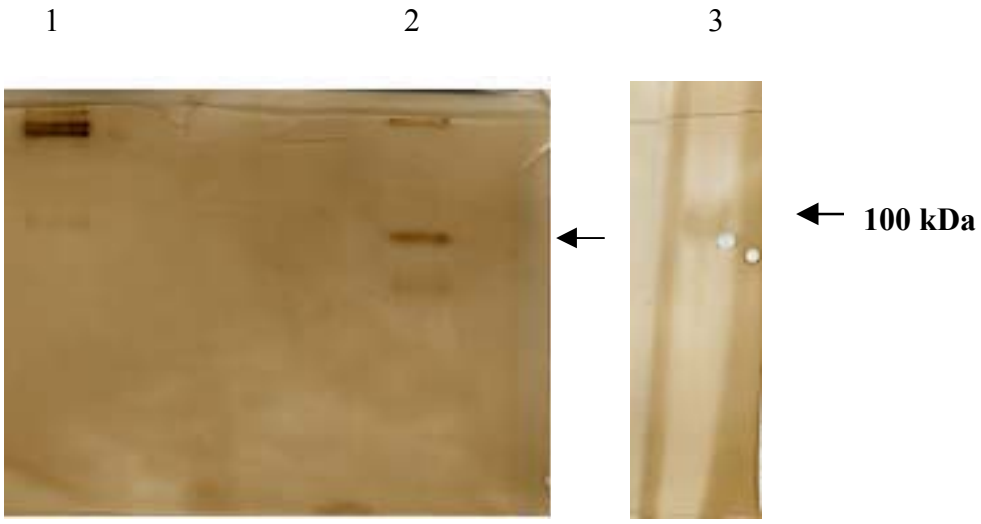



Fig 21. ATCase expressed from various plasmids. Lane 1. The *E. coli pyrBI* gene products showing the aggregated form of ATCase along with the 310 kDa ($2C_3$:$3R_2$), the 270 kDa ($2C_3$:$2R_2$), and the 100 kDa ($C_3$) forms. Lane 2 shows the *E. coli pyrB* gene product (C3). Lane 3 shows the *M. jannaschii pyrB product*.

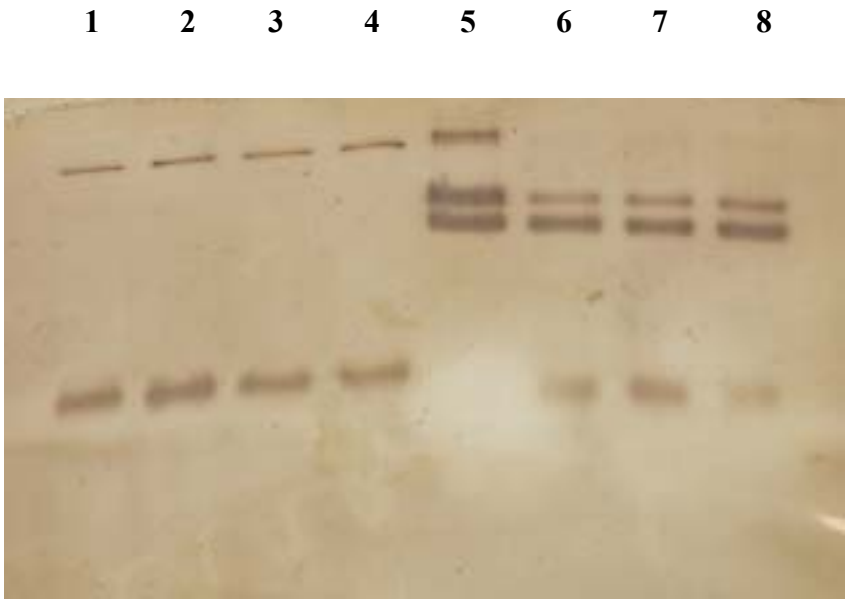



Fig 22a. Thermosensitivity of ATCase expressed from plasmids pEK2 and pBJR28 in *E. coli* TB2. Cell extracts were heated to 65°C and loaded onto the gel. Lanes 1-4. *E. coli pyrB* (pBJR28) incubated at 65°C for 0, 10, 20, and 30 min, respectively. Lanes 5-8. *E. coli pyrBI* (pEK2) incubated at 65°C for 0, 10, 20, and 30 min.

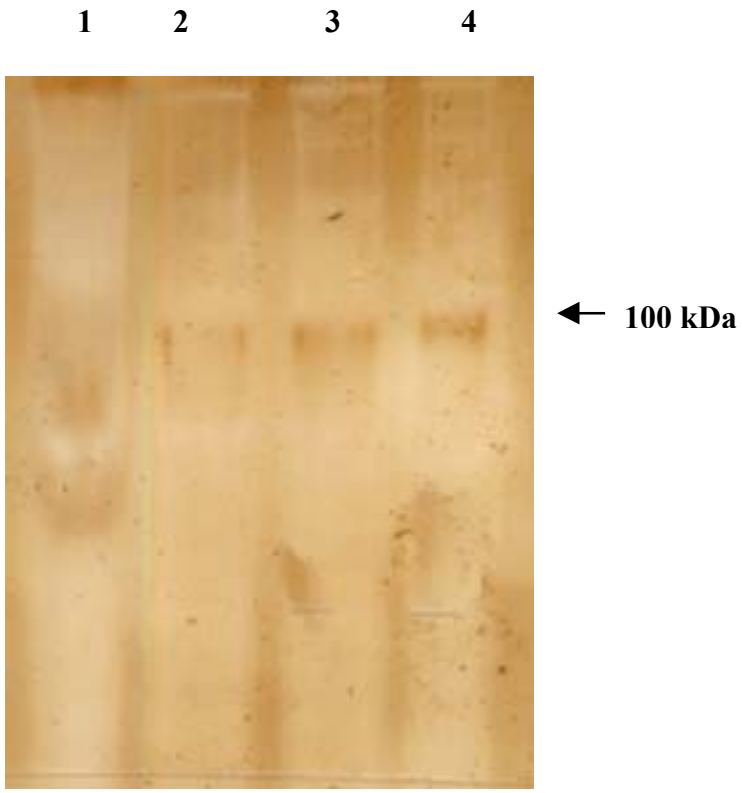



Fig 22b. Thermostability of *E. coli* TB2 cells harboring the *M. jannaschii pyrB* (AMPK84) plasmid. Cell extracts were heated at 65°C. Lane 1 heat treatment for 0 min. Lane 2 heat treatment for 10 min. Lane 3 heat treatment for 20 min. Lane 4 heat treatment for 30 min.

**Thermostability of active ATCase from *E. coli pyrB* (pBJR28), *E. coli pyrBI* (pEK2) and *M. jannaschii pyrB* (AMJPK84) at 80°C.**

The various protein products expressed from plasmid DNAs expressed in *E. coli* TB2 were incubated at 80°C for 0, 10, 20 and 30 min. Results from this experiment showed that the *E. coli pyrB* and *E. coli pyrBI* expressed form the plasmids containing cell extract are denatured with heat
(Fig 23). The lanes on the activity gel containing the *E. coli pyrB* expressed product and *E. coli pyrBI* gene product samples showed no ATCase activity and this suggests that the *E. coli* enzyme is very sensitive to high heat. In contrast the *M. jannaschii pyrB* gene product retained activity after heating at 80°C for 30 min.

At high temperatures, pyrimidine biosynthesis is confronted with the thermostability of not only the enzymes of the pathway but also with the stability of the substrates. One of the ATCase substrates, carbamoylphosphate, is thermolabile with a half-life of 30 min at 37°C. At higher temperatures such as 80°C the half-life is reduced dramatically. Thus microorganisms grow optimally at high temperatures must have developed a strategy to utilize the CP very quickly. One of the mechanisms of protecting CP at 100°C is involves its rapid conversion to a heat stable compound such as carbamoylasaprtate, dihydroorotate or orootate. This could then satisfy pyrimidine and arginine biosynthesis (Purcarea., 1996).

**1 2 3 4 5 6 7 8 9**

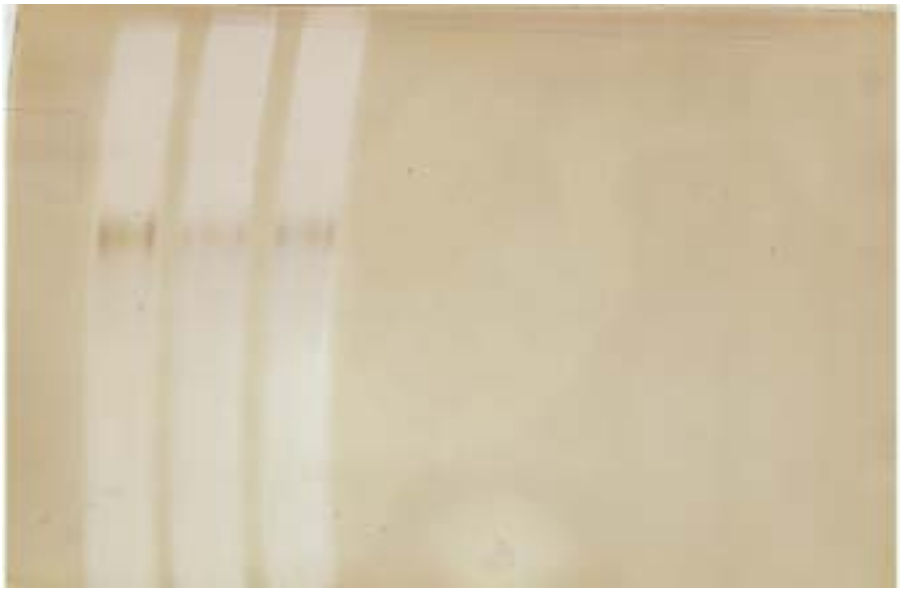


Fig 23. Thermostability of ATCase encoded by *E. coli pyrB* (pBJR28), *E. coli pyrBI* (pEK2) and *M. jannaschii pyrB* (AMPK84). Lane 1. *E. coli* TB2 cell extract harboring plasmid AMPK84, 10 min of incubation at 80˚C. Lane 2. *E. coli* TB2 harboring plasmid AMPK84, 20 min of incubation at 80˚C. Lane 3. *E. coli* TB2 harboring plasmid AMPK84, 30 min. Lane 4. *E. coli* TB2 harboring plasmid pBJR28, 30 min. Lane 5. *E. coli* TB2 harboring plasmid pBJR28, 20 min. Lane 6. *E. coli* TB2 harboring plasmid pBJR28, 10 min. Lane 7. *E. coli* TB2 harboring plasmid pEK2, 30 min. Lane 8. *E. coli* TB2 harboring plasmid pBJR28, 20 min. Lane 9. *E. coli* TB2 harboring plasmid pBJR28, 10 min.

**Formation of a 310 kDa ATCase enzyme from *M. jannaschii*.**

*E. coli* TB2 cells were transformed with the previously described plasmids. Pairs of plasmids were cotransformed (Table 4). The recombinants were able to satisfy the pyrimidine requirement of *E. coli* TB2. The insertion of both plasmids was confirmed by isolation of plasmid DNA. Cell-free extract was prepared as described in the materials and method section. Clarified cell extract was heated for 20 min at 80°C and loaded onto a non-denaturing ATCase activity gel. The results from this experiment show that the ATCase from *M. jannaschii* is capable of assembling into a 310 kDa *E. coli*-like holoenzyme
(Fig 24). The plasmid that was constructed by altering the original GTG start codon to an ATG start was able to produce a 310 kDa enzyme. A similar result was observed with the recombinant plasmid where the second internal methionine (ATG) was used instead of the designated methionine (GTG).

Kantrowitz and coworkers (1999) showed that only the *M. jannaschii* pyrB gene product exhibited catalytic activity as trimer and that the *M. jannaschii* pyrI gene product existed as a dimer. Preliminary characterization of the enzyme conducted by the above researchers suggested that the enzyme was similar to the *E. coli* ATCase with regard to the molecular mass. However in this paper no data upholding the suggestion was presented. Here I present data that shows the *M. jannaschii* ATCase is capable of assembling into a 310 kDa ATCase.

1 2 3 4 5 6 7 8 9

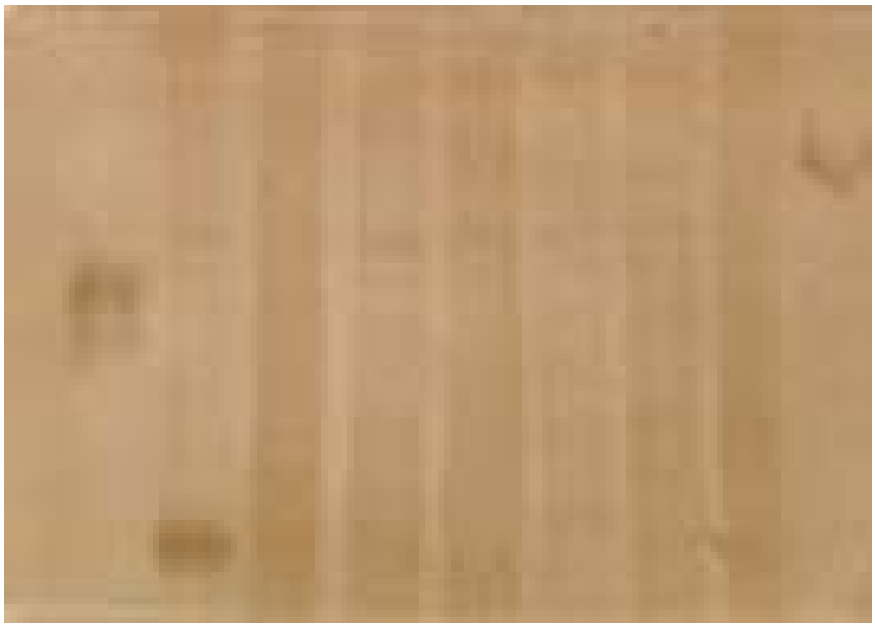


Fig 24. Nondenaturing polyacrylamide gel stained for ATCase activity. Lane 1, Purified *E. coli* holoenzyme. Lane 2, *E.coli pyrB* gene product. Lane 3, *M. jannaschii pyrB* gene product. Lane 4, *M. jannaschii pyrB* + *M. jannaschii pyrI* gene products. Lane 5 *M. jannaschii pyrB* + *M. jannaschii pyrI'* gene products. Lane 6, *M. jannaschii pyrB* + *M. jannaschii pyrI* gene products. Lane 7, *M. jannaschii pyrB* + *M. jannaschii pyrI'* gene products , Lane 8 *E. coli* TB2 cell extract and Lane 9 *Pseudomonas aeruginosa* purified ATCase.

**ATCase assay data and effector response of the modified *pyrI* genes of *M. jannaschii***

ATCase assays were performed to determine the $V_{max}$, $K_M$ and nucleotide effector response for the *M. jannaschii pyrB* and the two genetically modified *pyrI*'s. Partially purified cell sonicates were assayed for ATCase activity by measuring the amount of CAA produced at 37°C for 20 min using the colorimetric method described by Prescott & Jones 1969. The assay was conducted in a microtiter plate and the absorbance was read at 450 nm in a kinetic microplate reader.

The KM for aspartate was calculated to be 20 mM in both of the cases (Figs 26 & 29). It was also observed that the $K_M$ for carbamoylphosphate was in accordance with findings in 1999 at 88 μM of CAA $min^{-1}$ (data not shown).

In contrast to their data we observed regulation of the ATCase by nucleotide effectors. For the *M. jannaschii pyrB* and the *pyrI* where the GTG start codon was changed to an ATG start codon along with the addition of an *E. coli* like Shine Dalgarno no regulation by the nucleotides UTP, ATP and CTP was observed (Fig 28). However we did assemble a 310 kDa holoenzyme.

When an *E. coli* Shine Dalgarno was added along with the removal of the $1^{st}$ 3 amino acids we observed regulation of the ATCase enzyme by the nucleotide effectors UTP, CTP and ATP. At low aspartate concentration (0–5 mM) and saturating carbamoylphosphate (5 mM) CTP, ATP and UTP inhibited the activity of ATCase. At

saturating levels of aspartate (40-60 mM) enzyme activity was activated by CTP and ATP and slightly inhibited by UTP (Fig 30).

The native *pyrI* start codon was designated by GenBank to be a GTG start because of the alignment with the amino acids sequence of *E. coli*. However if one looks at the amino acid alignment (Fig 10) it can be seen that a second methionine is preceded by the amino acids methionine, isoleucine and proline in the order of MIPM. It is our belief that the internal methionine is in-fact the correct start for the *M. jannaschii pyrI* gene which would make perfect genetic sense when one considers the G + C content of 31% for *M. jannaschii*. Other researchers have used the 1$^{st}$ methionine as the proposed start and appear to have failed in their endeavors to see regulation of the *M. jannaschii* ATCase holoenzyme by the nucleotide effectors CTP, ATP and UTP respectively.

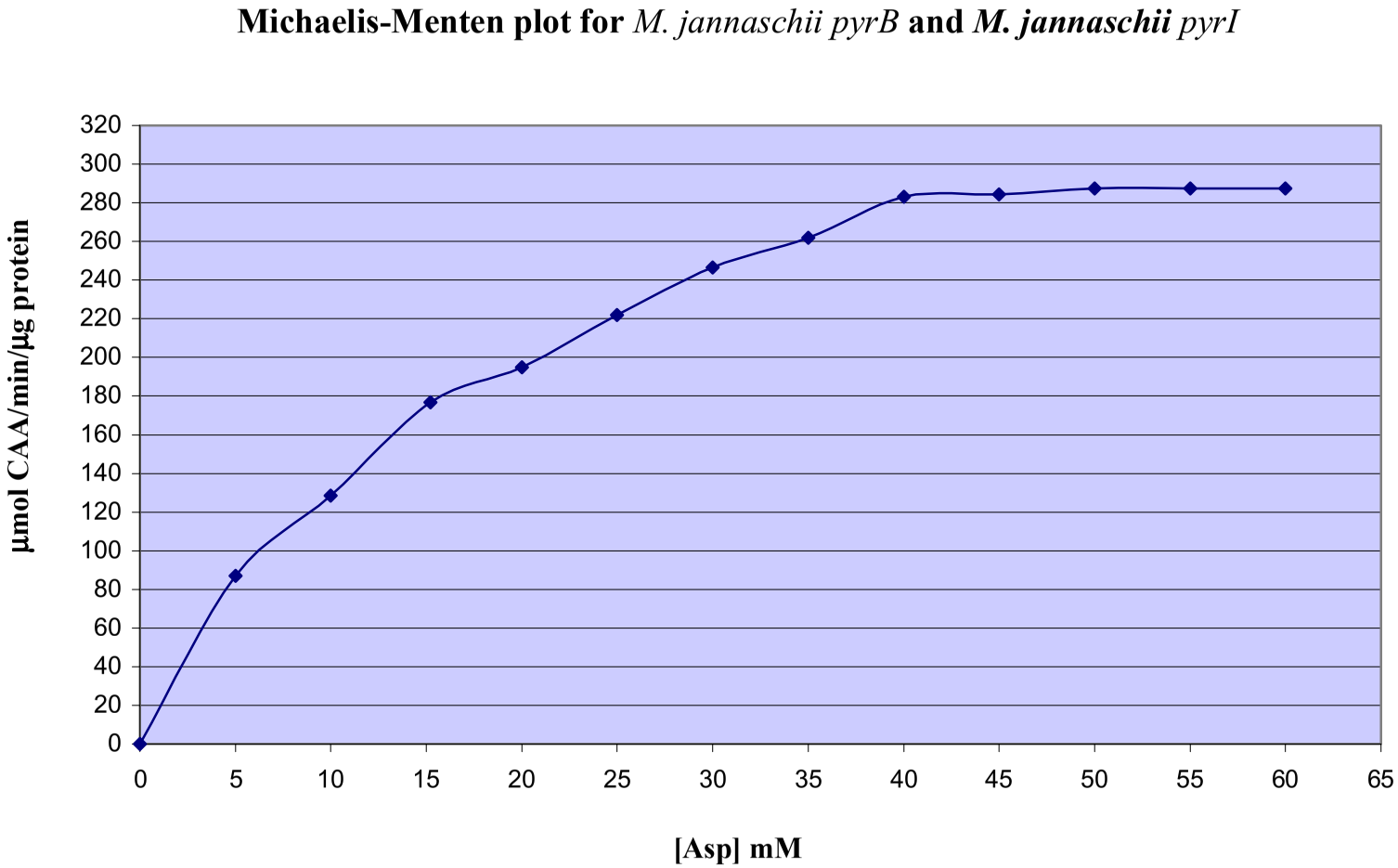



Fig 25 Michaelis-Menten plot for *M. jannaschii pyrB* and *M. jannaschii pyrI* (plasmid pSPMI4)

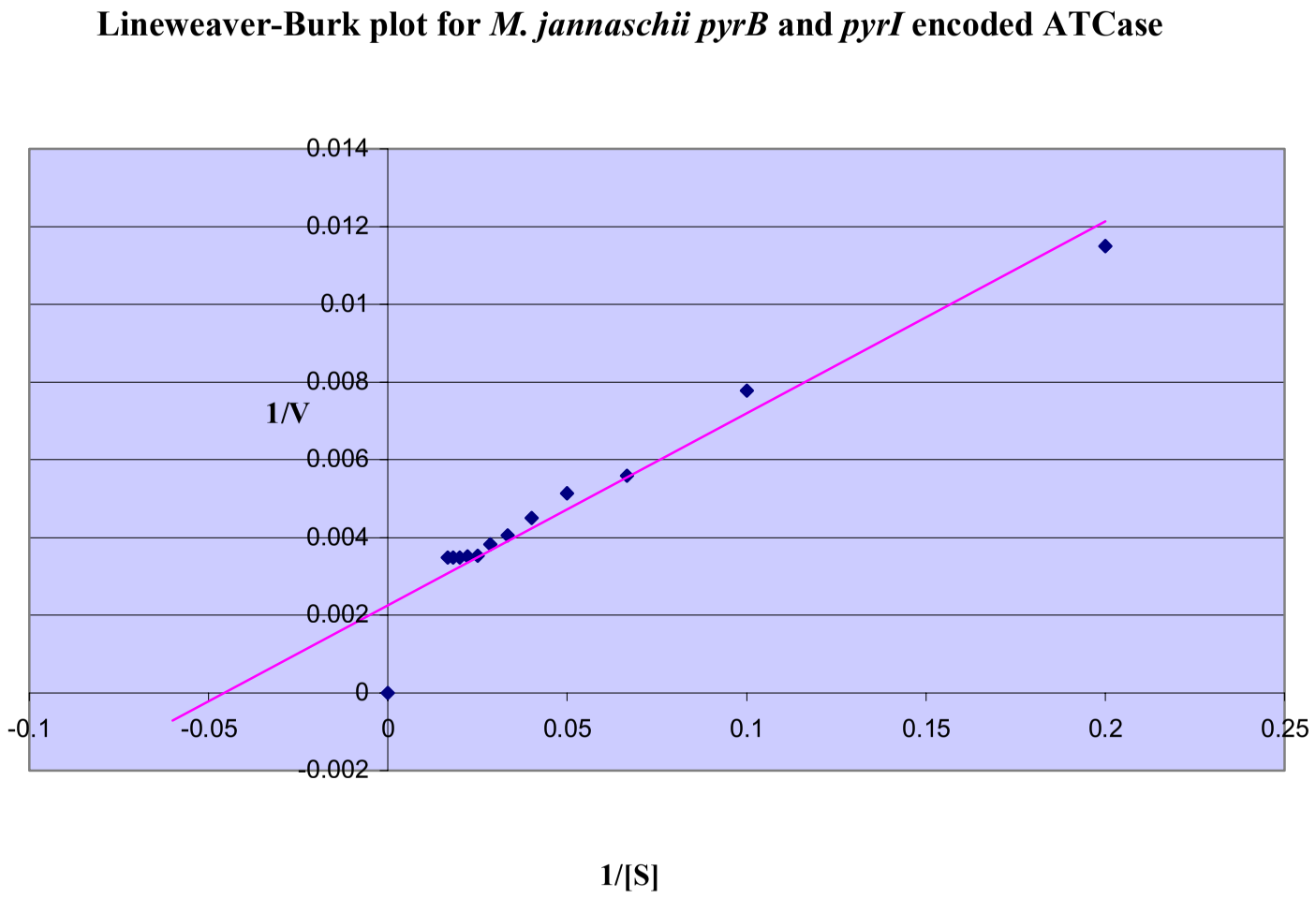



Fig 26. Lineweaver-Burk plot for *M. jannaschii pyrB* and *M. jannaschii pyrI* gene products.

**Effector response for *M. jannaschii* ATCase expressed from *M. jannaschii pyrB* and *M. jannaschii pyrI***

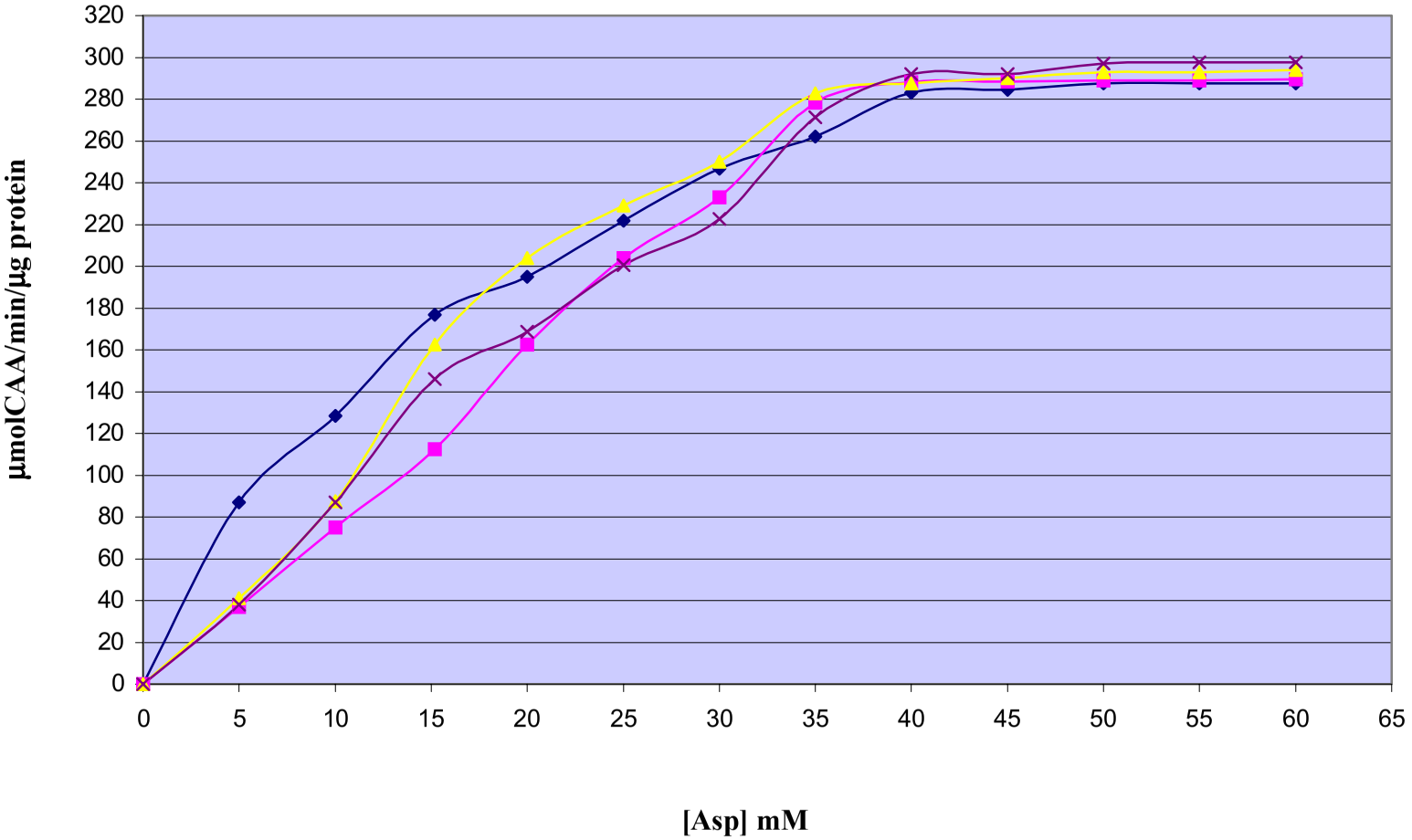



Fig 27 Nucleotide effector response curves.


Fig 28 Michaelis-Menten plot for *M. jannaschii* ATCase expressed from *M. jannaschii pyrB* and *pyrI'*.

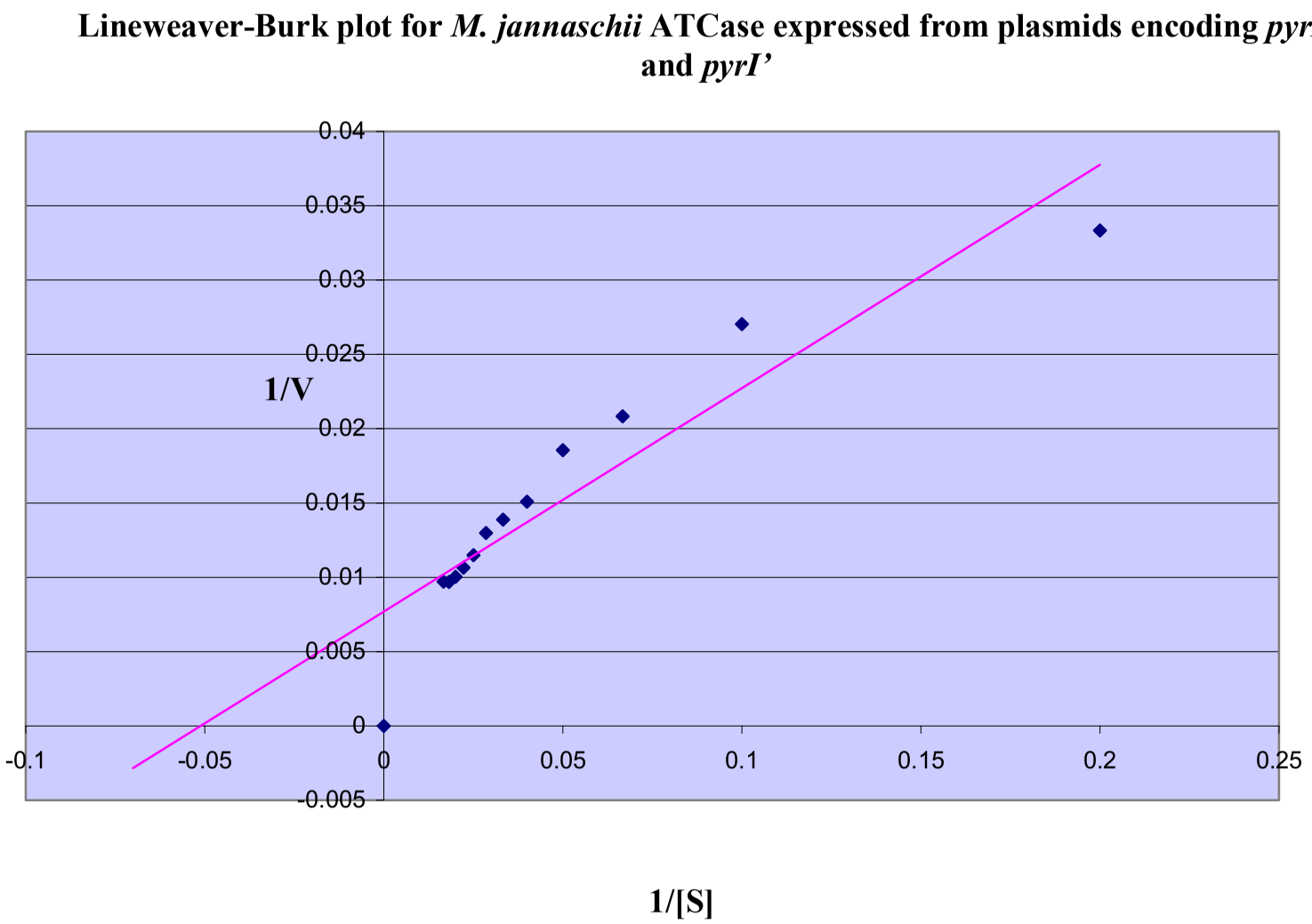



Fig 29 Lineweaver-Burk plot for ATCase expressed from *M. jannaschii pyrB* and *pyrI'*.

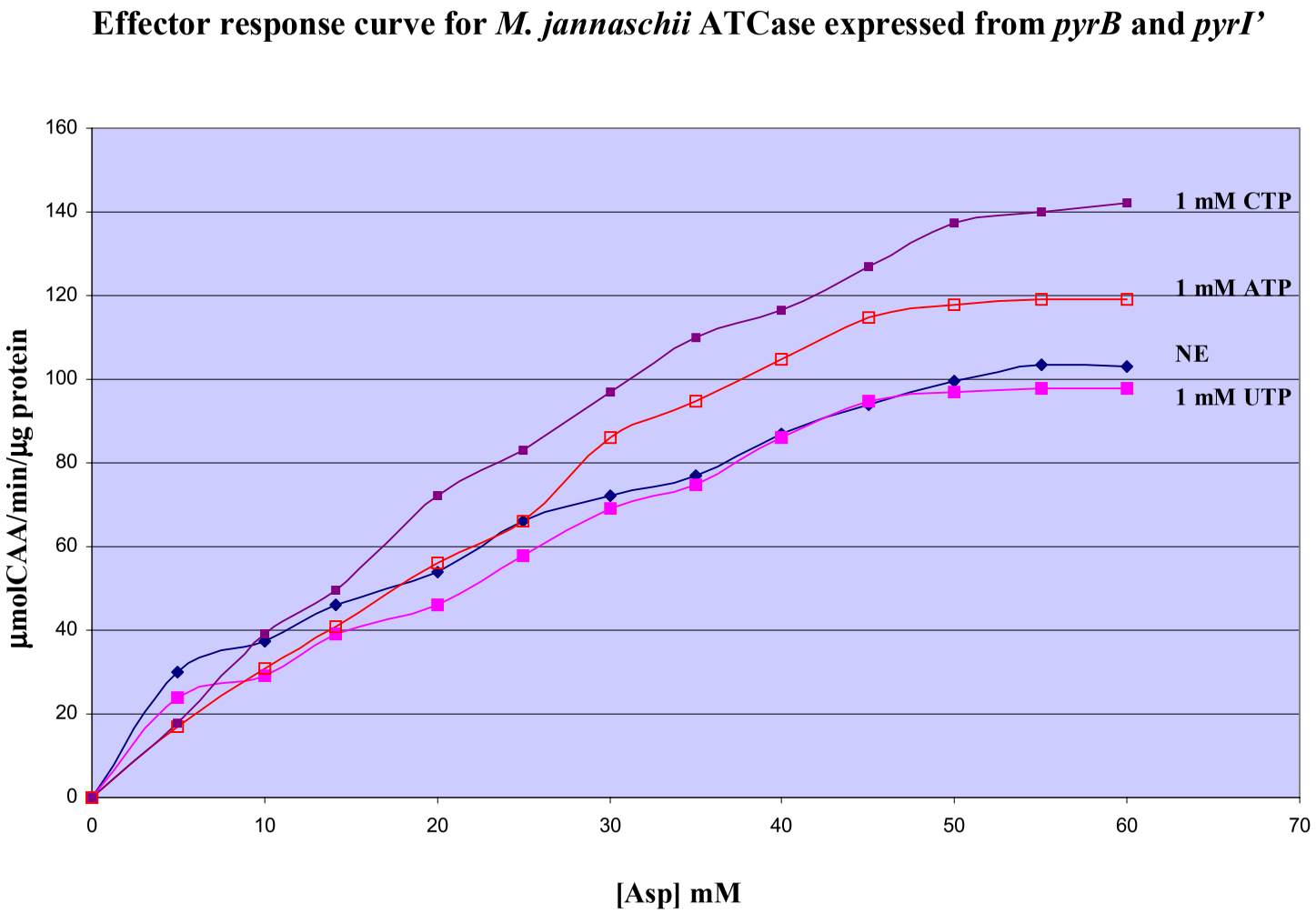



Fig. 30 Effector response curve for ATCase expressed from *M. jannaschii pyrB* and *pyrI'* .

**In vitro transcription translation analysis of pSPMI4 and pSPMI5.**

The *M. jannaschii* PyrI protein was expressed using the *E. coli* S30 extract. This procedure follows the basic method described by Zubay in 1973. The procedure allows one to directly observe the transcription and translation of specific DNA sequences and thus allows for the characterization of polypeptides from specific genes. In this case the two clones described earlier as pSPMI4 and pSPMI5 were used with one μg of plasmid DNA in each case. A 15% SDS gel was used to determine the molecular weight of the *M. jannaschii* PyrI protein. Results from this experiment showed the formation of a 17 kDa polypeptide from each of the plasmids (Fig 31a). The native size of the polypeptide is 34 kDa as observed in figure 31b from the same plasmids.

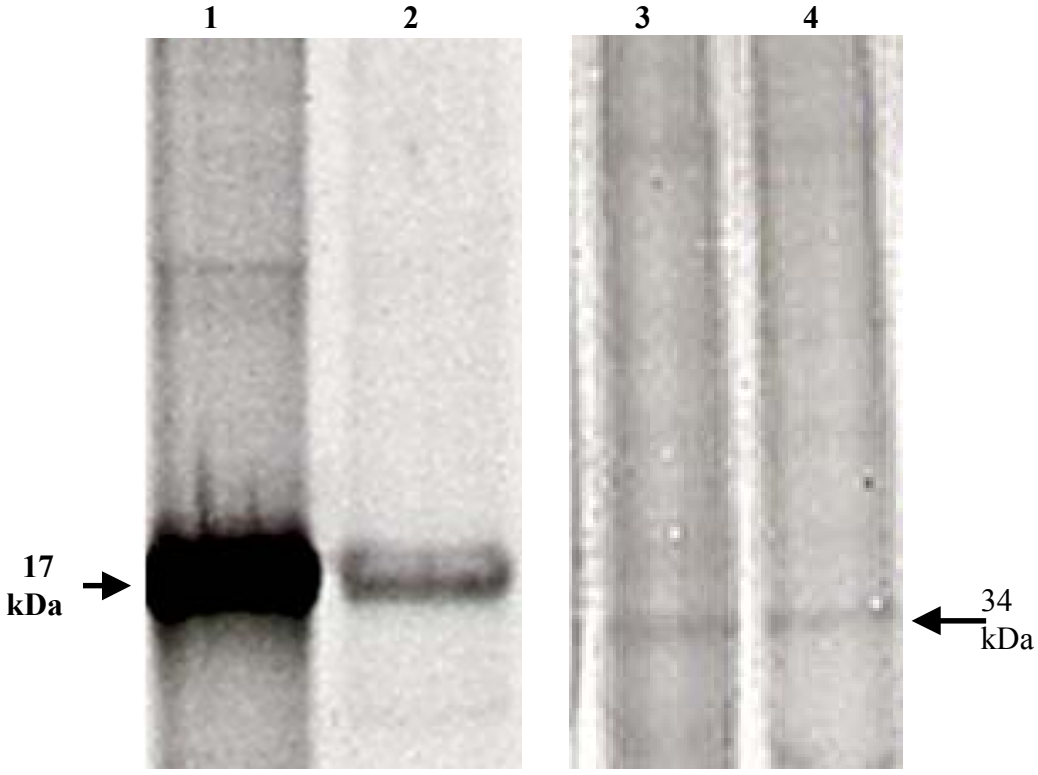



Fig 31a & 31b. Zubay analysis of *M. jannaschii* modified *pyrI* clones. Figure 31a shows the results of a 15% SDS PAGE gel. Lane 1 shows the product of the plasmid pSPMI5 and lane 2 shows the product of the plasmid pSPMI4. Both recombinant plasmids showed the formation of a 17kDa protein product. Figure 31b shows the results of a 15% native gel. Lane 1 shows the 34 kDa subunit size from the plasmid pSPMI4 *M. jannaschii pyrI* and lane 2 shows the product of pSPMI5 *M. jannaschii pyrI'*.

# CHAPTER I

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# CHAPTER II

## INTRODUCTION

The genus *Pseudomonas* is defined as having unicellular straight Gram negative rods. With the exception *Pseudomonas mallei*, all species are motile with one or more polar flagella. The G + C content of the pseudomonads is 58% to 69% (Mandel, 1966).

Pseudomonads have an oxidative metabolism and all pseudomonads lack the enzyme phosphofructokinase. Therefore they cannot utilize the Embden-Meyerhof glycolytic pathway (Entner & Doudoroff, 1952; Conway, 1992). Instead pseudomonads use the Entner-Doudoroff pathway to form pyruvate from carbohydrates. Glucose is converted to glucose-6-phosphate by glucokinase encoded by *glk*. Glucose-6-phosphate is further metabolized to 6-phosphogluconate by the product of the *zwf* gene, which encodes glucose-6-phosphate dehydrogenase. Two enzymes specific to ED convert 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate. Aldolase encoded by *eda* then catalyzes the formation of pyruvate and glyceraldehydes-3-phosphate. The two products can then be further metabolized via the EMP glycolytic pathway and the tricarboxylic acid cycle (TCA).

The genus *Pseudomonas* includes the opportunistic human pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* is a ubiquitous environmental bacterium first named by Migula in the early 1900. *P. aeruginosa* is versatile in that it can grow at

temperatures of 4˚C to 43˚C and the bacterium can grow in many different environments such as soil, marshes, marine environments, plants and animal tissue. Pigment production by *P. aeruginosa* is a key identification factor for this organism. Pyocyanin is a blue-green pigment, which is unique to this speciesmicroorganism. Other pigments such as pyoverdin (yellow), pyorubin (red) and pyomelanin (brown) are also produced by this organism. The pigment production is an iron scavenging mechanism from the surrounding medium and these pigments also act as virulence factors. Scientific evidence is also emerging that the production of pigment is linked to the pyrimidine biosynthetic pathway.

*P. aeruginosa* is a human pathogen because of its resistance to certain antibiotics and disinfectants, which would eliminate other bacterial species. *P. aeruginosa* is a common source of bacteraemia in burn victims, urinary tract infections in patients who are catheterized, cancer patients and AIDS patients (Stover *et al.*, 2000). It does not usually cause disease in healthy individuals but is very persistent in immunosuppressed persons.

Cystic fibrosis (CF) is an autosomal recessive disorder, which is prevalent in approximately 1 in 2,500 live births. The prevalence of this disease amongst Caucasian populations is very high and is characterized by the excessive mucus production in the lungs of affected individuals. The excess mucus build up in (CF) patients is a perfect environment for *P. aeruginosa* to colonize. Thus *P. aeruginosa* is a major cause of morbidity and mortality in CF patients, usually due to the lack of effective antibiotics available to treat the infection. Death of the patient occurs due to pulmonary failure.

**Pyrimidine biosynthesis.**

Pyrimidine and purines are essential for the building materials ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). These molecules are required for the passage of genetic information to succeeding generations in bacteria, fungi, plants and mammals. Therefore the study of the synthesis of these macromolecules is imperative if one is to understand evolutionary relationship between the three domains of life. Two pathways form pyrimidine ribonucleotides, the *de novo* and the salvage pathway.

The pyrimidine biosynthetic pathway has been studied in detail in bacteria (Yates & Pardee, 1956a, 1956b, 1957; Beckwith *et al.*, 1962; Hayward & Besler, 1965; Yan & Demerec, 1965; Hutson & Downing, 1968; Isaac & Holloway, 1968; Condon *et al.*, 1976, Foltermann *et al.*, 1981; Grogan & Gunsalus, 1993), fungi (Lacroute, 1968; Caroline, 1969), plants (Kafer & Thornburg, 1999) and mammals (Hager & Jones, 1967; Nakinishi *et al.*, 1968; Jones, 1980).

There are nine enzymatic steps, which ultimately result in the formation of the pyrimidine ribonucleotides UTP and CTP (Fig 2). The pyrimidine ring is assembled first and then linked to ribose phosphate to form the initial pyrimidine nucleotide OMP. The precursors of the pyrimidine ring are carbamoylphosphate and aspartate. The initial step in the pathway is the formation of carbamoylphosphate, which is produced in the reaction catalyzed by carbamoylphosphate synthetase (CPSase; carbamoylphosphate: L-aspartate carbamoyltransferase, EC 6.3.55). Carbamoylphosphate plays a dual role, being required

not only for pyrimidine but also for arginine biosynthesis. The *carAB* genes encode CPSase in *Escherichai. coli*.

The next step in the pathway involves the carbamoylation of aspartate by aspartate transcarbamoylase (ATCase; EC 2.1.3.2) to form N-carbamoylaspartate and inorganic phosphate. This reaction is the first committed step in the synthesis of pyrimidine nucleotides *de novo*. The genes encoding ATCase in *E. coli* are *pyrBI* and in *Pseudomonas* are *pyrBC'* .

Carbamoylaspartate cyclizes with the loss of water to yield dihydroorotate. At this stage the pyrimidine ring has formed. The reaction is catalyzed by dihydroorotase (DHOase; EC 3.5.2.3), encoded by the *pyrC* gene. The dihydroorotate so formed is then oxidized to orotate by dihydrooorotate dehydrogenase (DHOdehase; EC 1.3.3.1), encoded by *pyrD*. DHOdehase is a flavoprotein which is membrane associated.

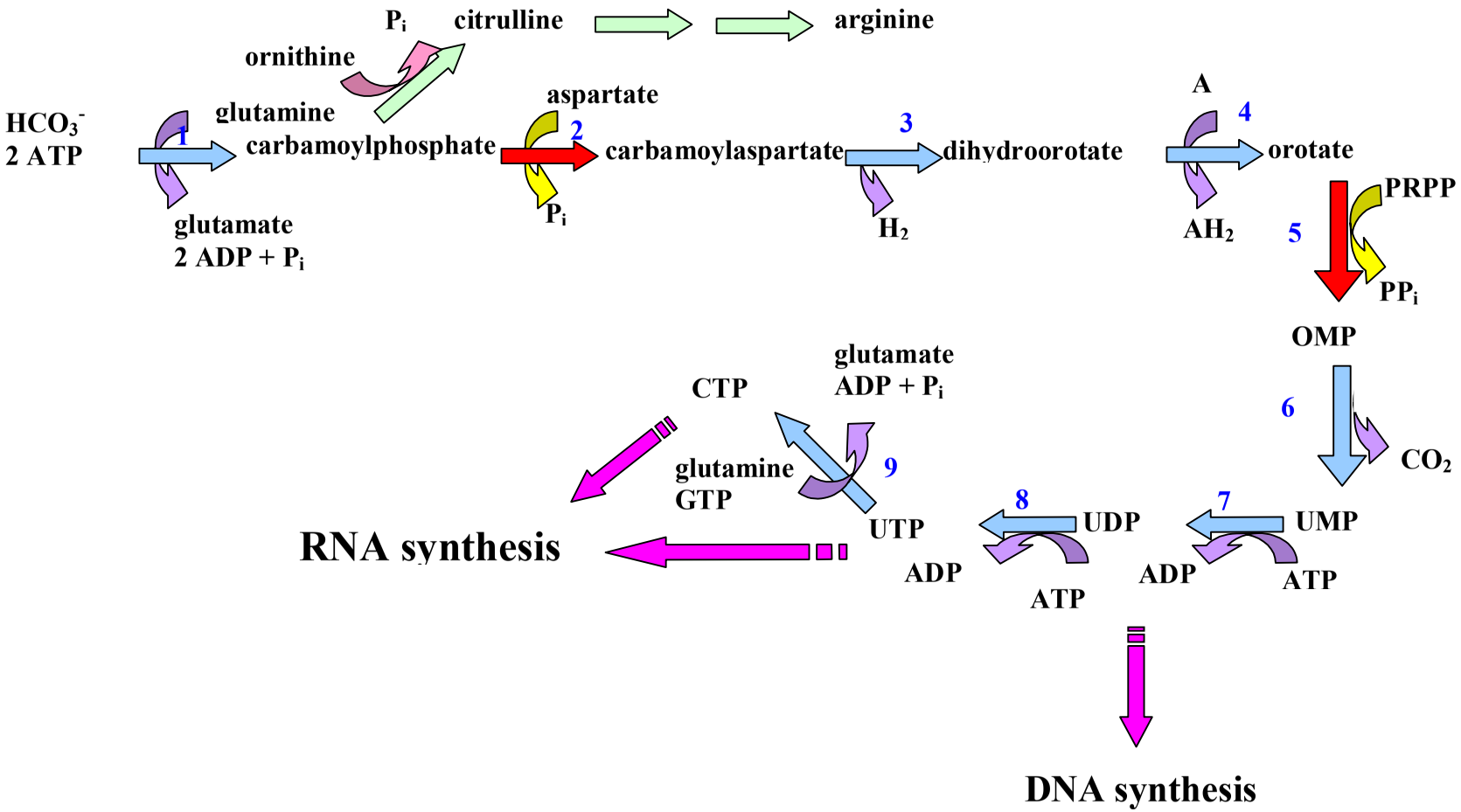
The next step in the pathway involves the acquisition of a ribose phosphate group. Orotate phosphoribosyltransferase (OPRTase; EC 2.4.2.10) catalyzes the reaction where orotate a free pyrimidine, reacts with PRPP to yield orotidine-5'-monophosphate (OMP) and pyrophosphate. This reaction is driven forward by the hydrolysis of pyrophosphate. The *pyrE* gene encodes the enzyme is responsible for this conversion.

Decarboxylation of OMP is carried out by OMP decarboxylase (OMPdecase; EC 4.1.1.23), which yields uridine-5'- monophosphate (UMP). The *pyrF* gene encodes OMP decarboxylase. UMP is then phosphorylated by a specific UMP kinase to form uridine-5'-diphosphate (UDP). UMP kinase (UMPk; EC 2.7.4.4) utilizing the *gamma* phosphate

of ATP. UTP is produced by the phosphorylation of UDP by nucleoside diphosphokinase. (NDK; EC 2.7.4.6)

The final step in the pyrimidine pathway is the amination of UTP to cytidine-5'-triphosphate (CTP) by the enzyme CTP synthetase. (CTPase; EC 6.3.42). For this enzyme, encoded by *pyrG*, glutamine serves as the amino donor for the amination. CTP synthetase is feedback inhibited by CTP and activated by UTP (Neuhard & Nygaard, 1987).

**Pyrimidine pathway**



**Salvaging of pyrimidine nucleotides.**

The salvage pathway is responsible for the scavenging of pyrimidine nucleotides, nucleosides and nucleobases that are formed during mRNA and rRNA degradation and hence recycling them to replenish the lost store and using them as a source of carbon and energy. The pyrimidine biosynthetic pathway may be missing in some obligate parasites, but no organism is devoid of the salvage pathway in one form or another.

D A Beck (1995) has studied the pyrimidine salvage pathway in almost 44 prototrophic bacteria and 11 auxotrophs, which were selected because of specific mutations that they contained in biosynthetic and salvage pathways O' Donovan & Shanley, 1995). This study was conducted using High Performance Liquid Chromatography and offered much insight into the salvage pathways of a wide range of microorganisms. The prototype organism used was *E. coli* and the researcher discovered 8 more variations of the salvage pathways in other organisms. Through this study, many groups emerged which differed in the enzymes that were responsible for salvaging pyrimidines. Figure 2 and 3 show the enzymes involved in the salvaging of pyrimidine nucleotides in *E. coli* and *P. aeruginosa* respectively.

The following is a general list of all the compounds in the pyrimidine salvage pathway and how they are utilized in an organism. Some organisms have two pathways by which to produce UMP from uracil (U). The first pathway is the catalysis of uracil by the enzyme uracil phosphoribosyltransferase (Upp). One molecule of PRPP is utilized in this reaction (Anderson *et al.*, 1992). The second pathway for the formation of UMP can

be conducted by the enzyme uridine phosphorylase (Udp) when a high amount of ribose-1-phosphate is present. Uridine can then be converted to UMP by the enzyme uridine kinase (Udk). GTP donates its γ-phosphate for this reaction (Neuhard & Nygaard, 1987).

Cytosine deaminase (CodA) catalyzes the deamination of cytosine to uracil in an irreversible reaction (West *et al.*, 1982)

Uridine is a substrate for at least 4 different salvage enzymes. Uridine phosphorylase (Udp), uridine kinase (Udk), ribonucleoside hydrolase A and B (Rih) and uridine hydrolase. In *E. coli* only the first three of the enzymes have been found (Beck, 1995). Cytidine in enteric bacteria is phosphorylated to CMP by uridine (cytidine) kinase. When cytidine is available to *E. coli* much of it is converted to uridine by cytidine deaminase (CodA) by a hydrolytic cleavage (Ashley & Bartlett, 1984). In *Pseudomonas* cytidine has also been shown to be a substrate for ribonucleoside hydrolase (Terada *et al*., 1967; Beck, 1995).

The salvaging of pyrimidines is important when one considers that some organisms do not have a pyrimidine biosynthetic pathway. If a mutation was to occur in the biosynthetic pathway (making a pyrimidine auxotroph) the salvage pathway would in effect take over and continue to make the cell survive.

Breakdown products of mRNA (the 5' mononucleotides UMP and CMP) can be toxic to the cell and therefore are removed very quickly (O' Donovan, 1978). If they are allowed to accumulate they will undoubtly cause bacterial cell death. A mutation that affected the process of mRNA degradation in a cell would have the result of self

destruction. The cell would die because of the buildup of the toxic 5' monophosphates (O' Donovan & Shanley 1995).

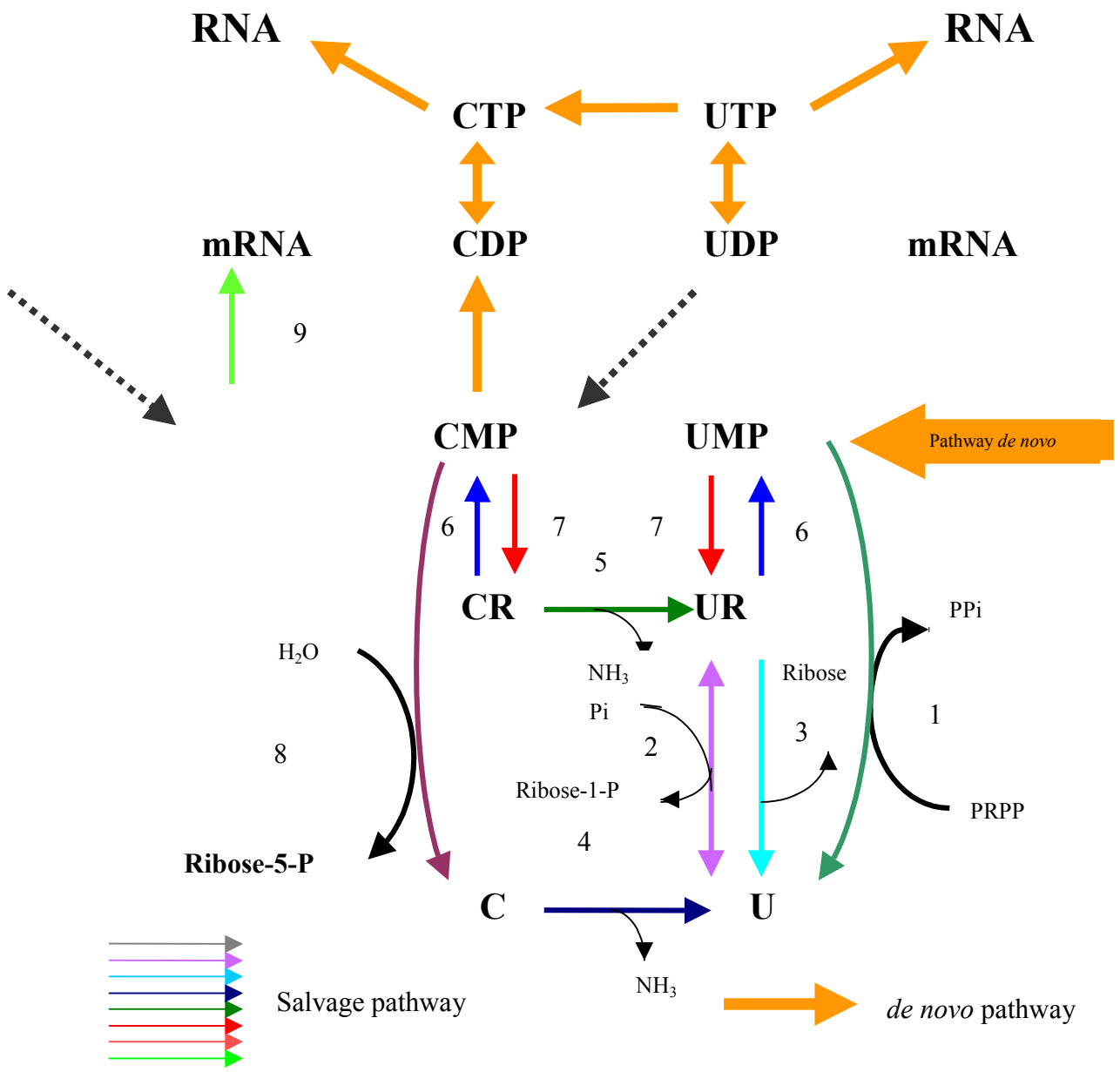



Fig 2. Pyrimidine salvage pathway in *Escherichia coli*. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. ribonucleoside hydrolase A & B (Rih), 4. cytosine deaminase (CodA), 5. cytidine deaminase (Cdd), 6. uridine kinase (Udk), 7. 5'-nucleotidase, 8. CMP glycosylase (Cmg), 9. CMP kinase (Cmk)

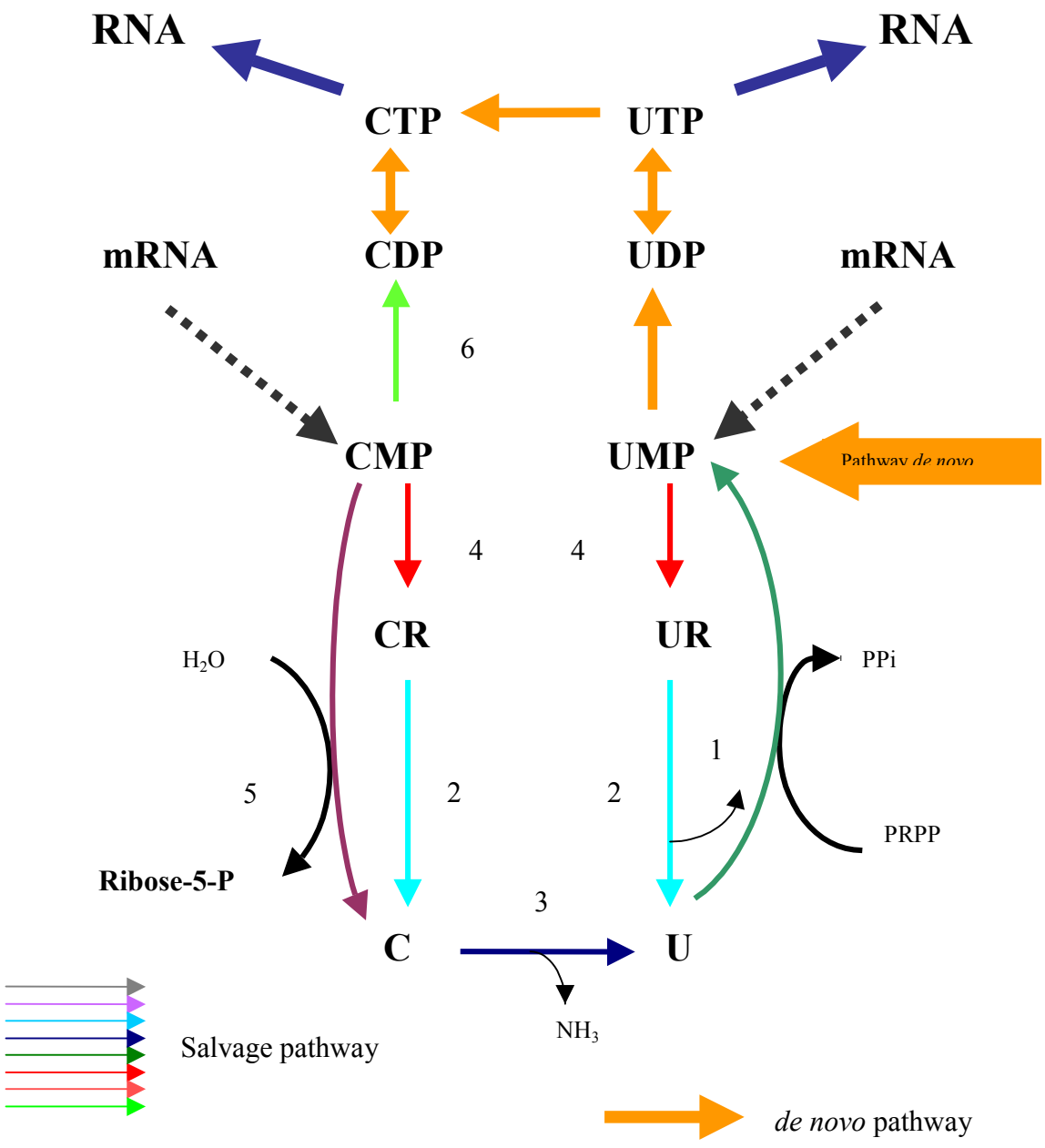



Fig 3. Pyrimidine salvage pathway in *P. aeruginosa*. Enzymes are 1. uracil phosphoribosyltransferase (Upp), 2. ribonucleoside hydrolase A & B (Rih), 3. cytosine deaminase, 4. 5'-nucleotidase, 5. CMP glycosylase (Cmg), 6. CMP kinase (Cmk)

**CTP Synthase.**

CTP synthase (EC 6.3.4.2) encoded by *pyrG* catalyzes the following reaction:

$$\text{UTP + ATP + L-glutamine} \xrightarrow[Mg^{2+}]{GTP} \text{CTP + L–glutamate + ADP + Pi}$$

Ammonia can be substituted for both GTP and glutamine in which case the reaction is

$$\text{UTP + } NH_3 \text{ + ATP} \xrightarrow{Mg^{2+}} \text{CTP + ADP + Pi}$$

The nucleotide CTP plays essential roles in the bacterial cell. It is required for growth and metabolism for all organisms and therefore must be available. CTP is used for the synthesis of nucleic acids (Traut, 1988) and membrane phospholipids (Kennedy, 1986). In *E. coli* the enzyme exists as a dimer ($M_r$ =105,000) of two identical subunits when the substrates UTP and ATP are not present. In the presence of UTP, ATP and $Mg^{2+}$ the enzyme exists as a tetramer of $M_r$ = 210,000. (Koshland & Levitzki, 1974; Koshland, 1978). The gene encoding CTP synthase has been cloned from *Bacillus subtilis* (Trach *et al.*, 1988), *Saccharomyces cerevisiae* (Ozier-Kalogeropoulos *et al.*, 1991), human beings (Yamauchi *et al.*, 1990), *Azospirillum brasilense* (Zimmer & Hundeshagen, 1994), *Cricetulus griseus* (Chinese hamster cells, Zhai *et al.*, 1995), *Mycobacterium leprae* (Smith & Robison, 1994), *Chlamydia trachomatis* (Tipples & McClarty, 1995), *Lactococcus lactis* (Steen *et al.*, 2001) and *Nitosomonas europaea* (Mahony & Miller, 1998). The enzyme contains a conserved glutamine amide transfer domain, which is characteristic of glutamine amidotranferases.

The enzyme has been purified to homogeneity from *E. coli* and it has been characterized with respect to its kinetic, physical, and enzymological properties. The enzyme exhibits complex kinetic properties including negative cooperativity for the effector GTP and the substrate glutamine. Positive cooperativity is seen with the substrates ATP and UTP. This is only if one of the nucleotides varied whilst the other is held at a constant level. This is under non-saturating conditions. If one of the nucleotides is saturating then normal Michaelis-Menten curves are observed for the other substrate. When the substrates bind a conformational change is observed which facilitates the binding of subsequent molecules. The second binding would therefore cause polymerization and hence facilitate the formation of the tetramer from the dimer (Yang *et al.*, 1994). CTP synthetase is a key enzyme in the synthesis of pyrimidine nucleotides.

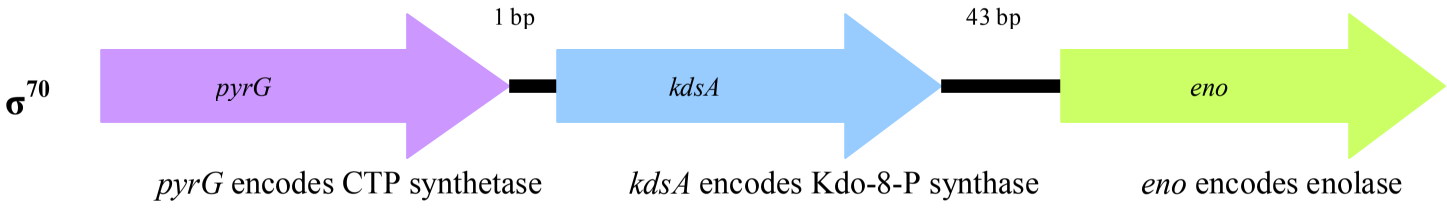
Walsh *et al*, (1999) discovered that in *P. aeruginosa* CTP synthetase encoded by *pyrG* was the first gene is a tricistronic operon. The three genes are located at 28.2 to 29.9 min on the *P. aeruginosa* 75 min map. The start codon of *kdsA* is 1 nucleotide downstream of the stop codon of *pyrG* and there are 43 nucleotides between the stop codon of *kdsA* and the start of *eno*. The gene order is therefore *pyrG*, *kdsA* and *eno* (Fig 4).

A sigma 70 ($\sigma^{70}$)-like promoter was identified upstream of *pyrG* but no promoter-like sequences was detected upstream of *kdsA* and *eno* thus suggesting that the three genes are co-transcribed. *P. aeruginosa* produces two types of cell surface lipolysaccharides (LPS), an A band and a B band which differ in the nature of the O antigen attached to the lipid A-core (Lam *et a.l*, 1996). The lipid A-core is required for

the stability of the outer membrane and also is shown to contribute to the intrinsic drug resistant of the organism (Hancock, 1998). Biosynthesis of the lipid A-core occurs at the cytoplasmic face of the inner membrane. The first sugar in the inner core region is 3-deoxy-D-*manno*-octulosonic acid (Kdo). The synthesis of this sugar involves the condensation of phosphoenolpyruvate and arabinose-5-phosphate by the enzyme Kdo-8-P synthase encoded by *kdsA*. Enolase catalyzes the formation of phosphoenolpyruvate (PEP) from phosphoglycerate. PEP is one of the substrates for Kdo-8-P synthase. CTP is required to activate Kdo before it is transferred to the lipid-A core (Walsh *et al.*, 1999) and high amounts of CTP is required during LPS synthesis. Thus, the presence of these genes together in an operon in *P. aeruginosa* is not an accident. In *Chlamydia trachomatis pyrG* is in part of an operon that has another Kdo biosynthetic *kdsB* gene immediately upstream (Tipples & McClarty, 1993; Wylie *et al.*, 1996). In *E. coli pyrG* and *eno* are part of an operon (Weng & Zalkin, 1986) whilst in *Nitosomonas europea pyrG* and *eno* are linked but are separated by 400 bp and most likely transcribed by separate promoters (Mahony & Miller, 1998).

Analysis of the PAO1 genome did not reveal any other copies of the *pyrG* and therefore a mutant that requires cytidine for growth would be a disaster if it were not an in-frame deletion due to the fact that a mutation in *pyrG* would be deleterious to the LPS synthetic operon.

***P. aeruginosa kdsA* operon.**






**Involvement of *eno*, *kdsA* and *pyrG* gene products in the biosynthesis of Kdo.**

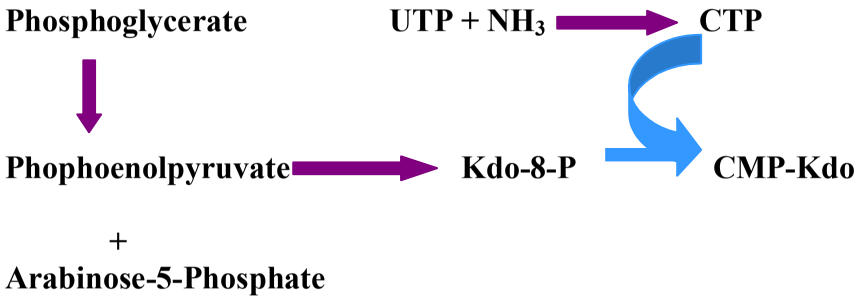



Fig 4a & 4b. The *P. aeruginosa* operon including the *pyrG* , *kdsA* and *eno* genes. Involvement of *eno*, *kdsA* and *pyrG* in the biosyntheis of Kdo.

**Regulation of pyrimidine enzymes in *P. aerguginosa*.**

As in *Escherichia coli* the pyrimidine biosynthetic pathway shares in the production of UTP and CTP for RNA with the pyrimidine salvage pathway. While salvage pathways are essential in providing a balance of RNA synthesis in prototrophs, they are obligately required to satisfy pyrimidine requirements in auxotrophs. Salvage pathways also return mRNA degraded monomers, the 5' monophosphates, to their triphosphate levels for RNA resynthesis. A portion of the 5' monophosphates (CMP) is further degraded to cytosine, which is subsequently deaminated to uracil for the conversion to UMP.

*Pseudomonas aeruginosa* lacks the salvage enzyme uridine (cytidine) kinase and therefore cannot convert uridine to UMP and cytidine to CMP. Thus, a pyrimidine auxotroph (Pyr $^{-}$) in a *upp* $^{-}$ background cannot grow when fed exogenous uracil. In the absence of uridine (cytidine) kinase it is impossible to study the regulation of the pyrimidine enzymes in *P. aeruginosa* (Fig 3).

Various authors have shown that in *E. coli* and *S. typhimurium* the expression of aspartate transcarbamoylase (*pyrBI*), orotate phosphoribosyltransferase (*pyrE*) and orotidine-5'-monophosphate decarboxylase (*pyrF*) is under negative control by a uridine nucleotide.

A cytidine nucleotide has been shown to exert control on carbamoylphosphate synthetase (*carAB*), dihydroorotase (*pyrC*) and dihydroorotate dehydrogenase (*pyrD*) (O' Donovan *et al.*, 1989; Smith *et al.*, 1980; Schwartz & Neuhard, 1975). In the above

organisms with ~50% G + C, it has been observed that a uridine compound was the primary repressing metabolite for the expression of *pyrB* gene. It is thought that a cytidine compound may be the primary repressing metabolite in organisms, which have a higher G + C composition such as *P. aeruginosa*.

To test this hypothesis it was necessary to isolate a *pyrG* mutant in *Pseudomonas,* in a strain with a functional *udk* gene. Such a strain could be fed exogenous cytidine because it would be a requirement for growth. Metabolic blocks were created systematically in *P. aeruginosa* starting with the isolation of a *upp*$^-$ strain and then electroporating the *E. coli udk* gene on the plasmid pDEB1 into this strain. Expression of the *udk* gene was confirmed by 5-fluroanalog plate assays. By way of an in-frame deletion and subsequent gene replacement method the CTP synthase (*pyrG*) was inactivated on the chromosome.

This hitherto unavailable mutant allowed the independent manipulation of the intracellular UTP and CTP pools by exogenous feeding of or starving for uridine and cytidine nucleosides.

Assays for the pyrimidine enzymes encoded by *pyrB-F* were conducted with the cells grown in all possible combinations of high and low cytidine and or uridine nucleoside levels.

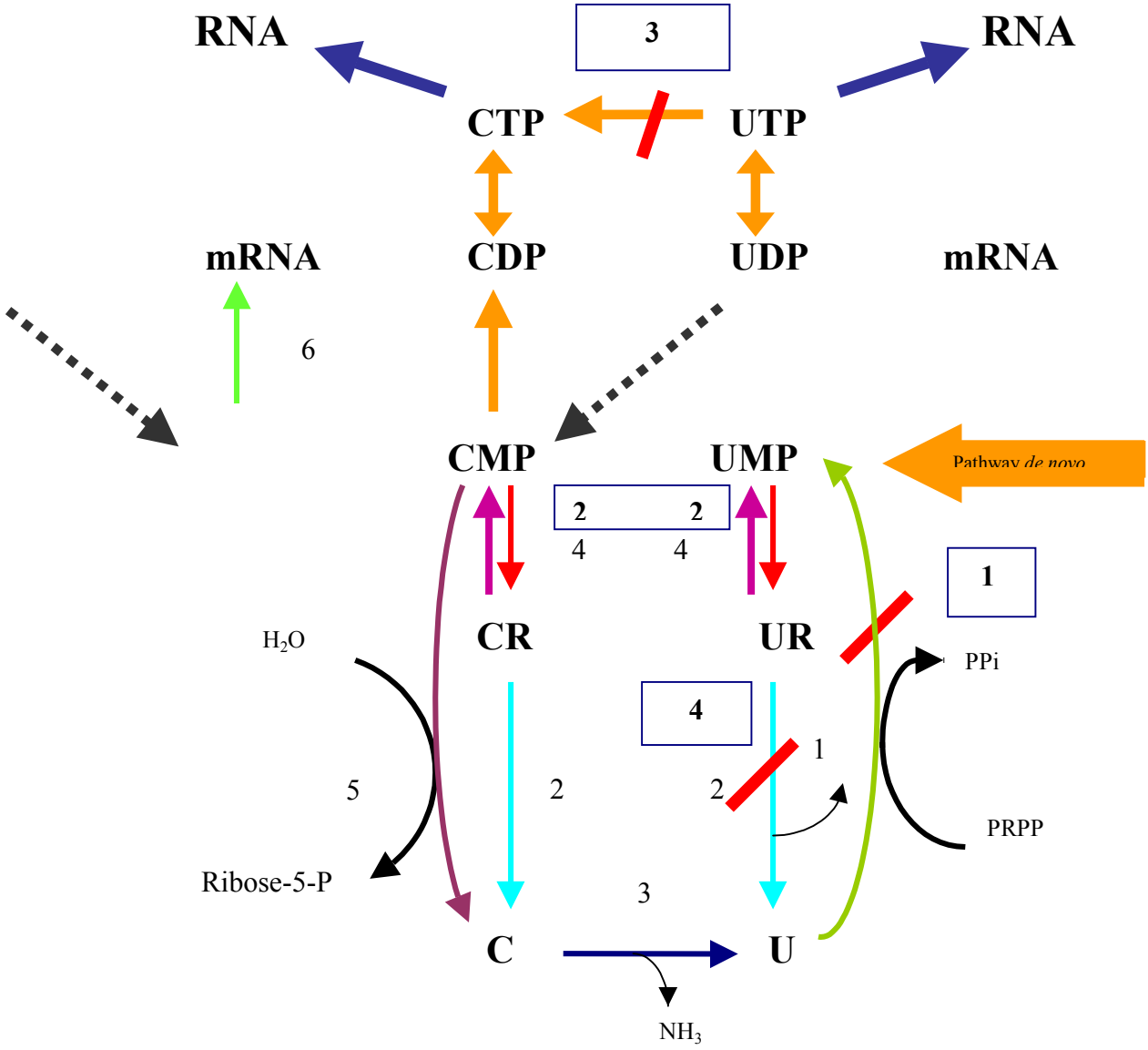



Fig 5. Red lines show the metabolic blocks that were created in *P. aeruginosa* so that uridine and cytidine could be fed to increase the nucleotide pools. The uridine (cytidine) kinase gene from *E. coli* was expressed in *P. aeruginosa*. Numbers in boxes represent the order in which the blocks were created.

# CHAPTER II

# MATERIALS AND METHOD

**Bacterial strains, plasmids, media and growth conditions.**

The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) enriched medium (Miller, 1992), *Pseudomonas* minimal medium (Ornston & Stanier, 1966) supplemented with Hutners's Metals 44 (Murray *et al*, 1981). For biparental mating experiments transconjugates were selected on *Pseudomonas* isolation agar (PIA). Antibiotics were added at the following concentrations: carbenicillin, 600 μg $ml^{-1}$, ampicillin, 100 μg $ml^{-1}$ and 50 μg $ml^{-1}$; gentamicin, 20 μg $ml^{-1}$ and 100 μg $ml^{-1}$. The carbon source used was glucose at a concentration of 0.2 %. All *E. coli* and *P. aeruginosa* strains were grown at 37˚C. *P. aeruginosa* used for the biparental mating experiment was grown at 42˚C in an attempt to inactivate its restriction modification system.

**Isolation of *P. aeruginosa* (PAO1) chromosomal DNA.**

Chromosomal DNA from *P. aeruginosa* (PAO1) was isolated using the method described by Berns & Thomas Jr., (1965). A starter culture of the above organism was grown in 5 ml of *Pseudomonas* minimal medium supplemented with 0.2 % glucose. A 1% inoculum was used to initiate the growth in a larger volume of 50 ml. Cells were grown to an $OD_{600}$ of 0.5 to 0.7. The cells were harvested at 1875 xg at 4˚C and placed

on ice for 20 min. The cooled cells were washed in 30 ml of a 50 mM Tris pH 8.0 and 20 mM ethylenediamine tetra acetic acid (EDTA) buffer for pelleting at 1875 xg for 20 min. The pellet was resuspended in 50 ml of the above buffer. Pronase at 5 mg/ml final concentration and 0.1 mls 10 % sodium dodecyl sulfate (SDS) were added. The solution containing the cells and the buffer were transferred to silconized corex tubes and incubated at 37˚C for 3 to 8 hrs. The solution was extracted 4X with an equal volume of phenol equilibrated with 50 mM Tris-HCl pH 8.0 and 1 mM EDTA. Phenol contamination was removed by extracting with 15 ml of diethyl ether. The DNA was dialyzed against 3 changes of Tris-EDTA pH 8.0. The purity and quantity of DNA was read at 280 nm and 260 nm respectively in as Shimadzu 500 UV spectrophotometer. A DNA concentration of .265 μg/μl was recovered using the above preparation.

Table 2. List of Strains and plasmids.

| Strain or Plasmid | Genotype or relevant property |
|---|---|
| **Strains** | |
| *P. aeruginosa* PAO1 | Wild type |
| *E. coli* | |
| SM10 | thi-1 thr leu *ton*A *lacY supE recA*::RP4-2-Tc::Mu ($kn^R$) |
| HPS1 | e14- (*mcrA*) *recA*1 *end*A1 *gyr*A96 thi-1 *hsd*R17 *supE*44 *relA*1 Δ(*lac-proAB*) rif zxx::miniTn5lac4 ($lacI^{q+}$ *lacZ* ΔM15) $rifampicin^R$ $chlorophenicol^R$ |
| **Plasmids** | |
| pEUK20 | *E. coli udk* gene |
| pUCP19 | *E. coli-Ps* shuttle vector |
| pDEB1 | 780 bp *E. coli udk* gene subcloned from pEUK20 |
| pCRII | $Amp^R$ $Kn^R$ PCR cloning vector |
| pSPG1 | 2.1 kb PCR product cloned into PCR II vector |
| pEXGm | $Gm^R$: $oriT^+$ $SacB^+$, gene replacement vector with MCS of pUC18 |
| pEX18Δ*pyrG* | $Gm^R$: $oriT^+$ $SacB^+$, gene replacement vector with MCS of pUC18. *pyrG* (-195 bp) cloned into the *Eco*RI and *Kpn*I restriction sites. |
| pGmΩ1 | $Amp^R$ aacC1; gentamicin cassette with omega loops |
| pGmCJF5 | *nuh*::$Gm^R$ in pCJF5; Gm cassette cloned into the *Sca*I site |

**Polymerase chain reaction and cloning of CTP Synthetase (*pyrG*) from *P. aeruginosa*.**

Primers for the amplification of *pyrG* from *P. aeruginosa* were designed using Prophet 5.0™ gene analysis software.

The upstream primer (5'GGTGGCCAACATTCCTGG-3') and the downstream primer (5'-CACGGAACGAGTGGATCG-3') were synthesized by BioSyntheis, Inc. The 2.1 kb DNA fragment incorporating the entire *pyrG* gene from *P. aeruginosa* was amplified using *Taq* polymerase using the method of PCR (Saiki *et al.*, 1988). The fragment generated was cloned into the pCRII™ vector from The Original TA Cloning ® Kit (Invitrogen) (Fig. 8). After ligation and transformation into INVαF' competent cells, clones which contained the *pyrG* gene were selected on LB plus ampicillin 50 μg $ml^{-1}$, with 5 bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) at 0.003% and isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.03%. White colonies appearing on the medium were selected and grown in 5 ml of LB medium with 50 μg $ml^{-1}$ of ampicillin. Insertion of the *pyrG* gene was confirmed by restriction digestion and by DNA sequencing. Five positive clones were isolated and characterized (Fig 9).

**Complementation of *E. coli* JF646.**

*E. coli* JF646, which is auxotrophic for cytidine, was transformed with the plasmids and the vector controls (pCRII vector). The transformants were grown on selective medium which, lacked cytidine. Complementation was confirmed by the growth of *E. coli* JF646

without cytidine and recovering the plasmids from colonies growing on the selective medium.

**Isolation of *P. aeruginosa* (PAO1) *upp*⁻ mutants.**

Wild type strains of *P. aeruginosa* were grown at 37˚C in 5 ml of *Pseudomonas* minimal medium with glucose at a concentration of 0.2%. The overnight cultures (100 µl) was spread onto *Pseudomonas* minimal agar with 0.2% glucose. Crystals of 5-fluorouracil were placed in the center of the plate with a sterile spatula. The plates were incubated at 37˚C for two to three days upon which a zone of killing was observed. Colonies appearing within the zone of killing were identified as *upp*⁻ and were picked and grown in 5 ml of *Pseudomonas* minimal medium to confirm the genotype. The overnight culture was spread onto a new *Pseudomonas* minimal medium plate. Crystals of 5-fluorouracil were placed in the center of the plate. The plates were incubated for two days. The *upp*⁻ mutant was confirmed by the growth of the cells up to the 5-fluorouracil crystals in the center of the plate.

**Preparation of Electro competent *upp*⁻ *P. aeruginosa* (PAO1).**

A single colony of *P. aeruginosa upp*⁻ cells were inoculated into 5 ml of *Pseudomonas* minimal medium. The cells were grown at 37˚C at 250 rpm. 100 µl of the culture were used as the inoculum for 100 ml of *Pseudomonas* minimal medium. The cells were grown at 37˚C to an $OD_{600}$ of 0.5 to 0.7 was reached. The flask was chilled on ice for 20 min and transferred to two chilled 50 ml centrifuge tubes. The sample was centrifuged at

5000 xg for 20 min at 4˚C. The pellet was gently resuspened by swirling in 10 ml of ice cold $H_2O$. The above step was repeated 5 times. The cells were resuspended in 5 ml of ice-cold $H_2O$ and centrifuged at 5000 xg for 10 min. The pellet was resuspended in 2 ml of 10% glycerol (v/v) and transferred to pre-chilled microcentrifuge tubes in 50 µl aliquots. The cells were frozen on dry ice and stored at -80˚C until use.

**Confirmation of the expression of uridine (cytidine) kinase (*udk*) in *P. aeruginosa* by flouroanalog plate assays.**

*P. aeruginosa upp*$^-$ mutants transformed with the plasmid pDEB1 were grown overnight in 5 ml of *Pseudomonas* minimal medium with 0.2% glucose and 600 μg ml$^{-1}$ of carbencillin. One hundred μl of the overnight culture was spread plated onto *Pseudomonas* minimal agar which was then divided into two halves. On one side of the plate, crystals of fluorocytidine were placed in the center whilst on the other side crystals of fluorouridine was placed. A zone of killing would indicate the expression of the *E. coli* uridine (cytidine) kinase gene in *P. aeruginosa*.

**Cloning of the *P. aeruginosa* CTP synthase gene (*pyrG*) into pUCP19.**

A 2.1 kb DNA fragment containing the entire *pyrG* gene was excised from the plasmid pSPG1 using the restriction enzymes *Kpn*I and *EcoR*I. The DNA fragment was agarose gel purified and ligated into the pUCP19 plasmid, which had also been digested with the same enzymes. After ligation and transformation into DH5α competent cells, clones, which contained the *pyrG* gene were selected on LB plus ampicillin 100 μg ml$^{-1}$, with X-gal at 0.003% and IPTG at 0.03%. White colonies appearing on the medium were selected and grown in 5 ml of LB medium with 100 μg ml$^{-1}$ of ampicillin. Upon confirmation of the cloned gene of the appropriate size the plasmid was digested with the restriction enzyme *Bss*HII. This enzyme was chosen because it would remove a portion (~195 bp) from the internal region of *pyrG*, however it would not cut the plasmid DNA. The linearized plasmid with the 195 bp deletion was agarose gel purified using Gelase

and circularized using $T_4$ DNA ligase. This method created an in-frame deletion of the *pyrG* gene (Fig 10).

**Cloning of Δ*pyrG* into the suicide vector pEX18Gm.**

pEX18Gm (Genbank Accession number AF047518) is a gene replacement vector which was developed by Hoang *et al*, (1998) (Fig 11). This vector is useful for gene replacement studies in *P. aeruginosa*. The vector incorporates (i) A counter-selectable *sacB* marker, (ii) a *lacZ*α-allele for blue white screening, (iii) the multiple cloning site from pUC18 and, (iv) gentamicin resistance gene. The plasmid can be used to replace wild type gene sequences with cloned genes because it allows for one to track the gene replacement process. Thus this plasmid was used for the replacement of the wild type *pyrG* sequence with the Δ*pyrG*. The Δ*pyrG* gene was excised from pUCP19 using the restriction enzymes *Kpn* I and *Eco* RI. The 1.756 bp *pyrG* gene was agarose gel purified and cloned into plasmid pEX18Gm, which had been digested with the same enzymes. After ligation and transformation into DH5α competent cells, clones, which contained the Δ*pyrG* gene were selected on LB plus gentamicin 20 μg $ml^{-1}$, with X-gal at 0.003% and IPTG at 0.03%. White colonies appearing on the medium were selected and grown in 5 ml of LB medium with 20 μg $ml^{-1}$ of gentamicin. Plasmid DNA was isolated using the alkaline lysis method. Insertion of the Δ*pyrG* gene was confirmed by restriction digest and agarose gel electrophoresis.

**Preparation of competent cells.**

All *E. coli* SM10 and *E. coli* HPS1 competent cells for transformation experiments were prepared using the calcium chloride method of Dagert & Ehrich (1979) with slight modifications. The cultures were grown in 50 ml of *E. coli* minimal medium with the appropriate antibiotics to an $OD_{600}$ of 0.2 to 0.4 and chilled on ice at 0 to 4°C for 10 min. The cells were harvested by centrifugation at 1875 xg at 4°C for 15 min, the pellet was resuspended in 20 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 20 to 25 min. The cells were centrifuged at 833 xg at 4°C for 15 min, the pellet was resuspended in 0.7 ml of ice cold $CaCl_2$ and incubated overnight on ice. The following day glycerol was added to a final concentration of 15% and the cells were separated into 200 µl aliquots. Cells were then frozen at –80°C and could used for up to three months.

**Transformation of *E. coli* HPS1 and E. coli SM10 with pEX18Gm Δ*pyrG*.**

Transformation of *E. coli* HPS1 with the plasmid pEX18 Δ*pyrG* (Fig 12) ), was conducted according the method described by Huff *et al*, (1990). Approximately 40 ng of plasmid DNA were mixed with 200 µl of the $CaCl_2$ treated cells and the mixture was incubated on ice for 15 min. The cells were then heat shocked at 42°C for 2 min, and then placed back into the ice bucket for a further 15 min. LB broth at a volume of 1 ml was added to the cells, which were then incubated at 37°C for 1 h. After 1 h the cells were plated on LB medium, plasmid insertion was selected for by gentamicin resistance at a concentration of 20 µg $ml^{-1}$.

Transformation of *E. coli* SM10 with the plasmid pEX18Δ*pyrG* (Fig 12), was conducted according the method described by Huff *et al*, (1990). *E. coli* SM10 cells were used for the biparental mating experiment because it allows for the mobilization of plasmid sequences into the chromosome of wild-type cells. Approximately 40 ng of plasmid DNA were mixed with 200 µl of the $CaCl_2$ treated cells and the mixture was incubated on ice for 15 min. The cells were then heat shocked at 42°C for 2 min, and then placed back into the ice bucket for a further 15 min. LB broth at a volume of 1 ml was added to the cells, which were then incubated at 37°C for 1 h. After 1 h the cells were plated on LB medium, plasmid insertion was selected for by gentamicin resistance at a concentration of 20 µg $ml^{-1}$.

**Isolation of *pyrG*$^{-}$ (cytidine requiring mutants) by biparental mating.**

The gene replacement plasmid, pEX18 Δ*pyrG*, was mobilized into *P. aeruginosa* PAO1 *upp*$^{-}$, *udk*$^{+}$, by biparental mating as described by De Lorenzo & Timms (1994). Five ml cultures of *E.coli* SM10 harboring the pEX18 Δ*pyrG* plasmid (donor strain) were grown at 37°C overnight in LB medium. Five ml cultures of *P. aeruginosa* (PAO1, *upp*$^{-}$, *udk*$^{+}$) were grown in *Pseudomonas* minimal medium with µg $ml^{-1}$ carbenicillin 600 at 42°C overnight in an attempt to inactivate its restriction modification system (recipient strain). 40 µl of the recipient strain (*P. aeruginosa*, PAO1) were placed into a sterile microfuge tube and pelleted at 12,000 xg in a bench top centrifuge. The cells were washed 3 times with sterile phosphate buffered saline (pH 7.3). 40 µl of the donor strain was added to the same tube and the above procedure was again performed. The donor

and recipient cells were transferred to a 15 ml tube, which contained 5 ml of 10 mM $MgSO_4$. The solution containing the donor and recipient strain were transferred to a 5 ml sterile syringe, which had a Nalgene filter unit containing a 0.45 μm Filter. The solution was pushed through the filter unit. The apparatus was disassembled and the filter was removed with sterile forceps and placed on an LB agar plate bacteria side up. The plate was incubated at 37°C for 16 to 18 h. Sterile forceps were used to remove the filter off the LB plate and it was placed into a tube containing 10 ml of 10 mM $MgSO_4$. The bacteria were resuspened in the solution and serial dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ were made. The serial dilution of $10^{-3}$ was plated on PIA plates supplemented with 50 μg $ml^{-1}$ of cytidine and incubated at 37°C overnight. PIA plates select against *E. coli* strains. Colonies appearing on the plates were replica-plated on 3 plates. Plate 1 was *Pseudomonas* minimal medium supplemented with 50 μg $ml^{-1}$ cytidine, 100 μg $ml^{-1}$ gentamicin and glucose at a concentration of 0.2% as the carbon source. Plate 2 was *Pseudomonas* minimal medium supplemented with 50 μg $ml^{-1}$ cytidine, carbenicillin at 600 μg $ml^{-1}$ and glucose at a concentration of 0.2 %. Plate 3 was *Pseudomonas* minimal medium supplemented with 50 μg $ml^{-1}$ cytidine, 10 % sucrose, carbenicillin 600 μg $ml^{-1}$ and glucose at 0.2 %. Colonies that were sucrose sensitive, carbenicillin resistant and gentamicin resistant were picked and inoculated into 5 ml LB broth supplemented with cytidine at a concentration of 50 μg $ml^{-}$. The tube was incubated overnight at 37°C to increase the number of double crossovers. Serial dilutions $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ were made and 100 μl of the $10^{-3}$ dilution was plated onto *Pseudomonas* minimal agar plates supplemented with cytidine at 50 μg $ml^{-1}$ and

carbenicillin at 600 μg $ml^{-1}$. Transconjugates were replica-plated onto 3 plates. Plate 1 was *Pseudomonas* minimal medium supplemented with 50 μg $ml^{-1}$ cytidine, 100 μg $ml^{-1}$ gentamicin and glucose at a concentration of 0.2% as the carbon source. Plate 2 was *Pseudomonas* minimal medium supplemented with 50 μg $ml^{-1}$ cytidine, 600 μg $ml^{-1}$ carbenicillin and glucose at a concentration of 0.2 %. Plate 3 was *Pseudomonas* minimal medium supplemented with 50 μg $ml^{-1}$ cytidine, 10 % sucrose, 600 μg $ml^{-1}$ carbenicillin and glucose at 0.2 %. Colonies of ,phenotype carbenicillin resistant, sucrose resistant, gentamicin sensitive and cytidine-requiring were isolated. These colonies were designated PAO1 *upp*$^{-}$, *udk*$^{+}$, *pyrG*$^{-}$. Strains were confirmed for the requirement of cytidine by plating on *Pseudomonas* minimal agar with and without cytidine.

**Small scale chromosomal isolation of wild-type and mutant *pyrG*$^{-}$ strains.**

Small scale genomic DNA preparation from wild type *P. aeruginosa* (PAO1) and 5 cytidine-requiring mutants was conducted. Five ml of each of the samples were grown independently to saturation overnight. 1.5 ml of the overnight culture was transferred to a microfuge tube and the cells were pelleted for 2 min at 10,000 xg. The cells were resuspended in 575 μl of Tris-EDTA solution and resuspeneded by repeated pipetting. 30 μl of 10 % (w/v) SDS and 3 μl of 20 mg proteinase K $ml^{-1}$ was added. The samples were mixed and incubated at 37°C for 1 h. 100 μl of 5 M NaCl was added and mixed thoroughly. 80 μl of CTAB/NaCl was added and the mixture was incubated at 65°C for 10 min. An equal volume of 24:1 chloroform/isoamyl alcohol was added, the mixture was mixed thoroughly and centrifuged for 5 min at 12,000 xg. The supernatant was

removed to a fresh microfuge tube and the sample was extracted with 25:24:1 phenol/chloroform/isoamyl alcohol and placed into a fresh tube. DNA was precipitated by the addition of 600 µl of isopropanol centrifuged and washed once with 70 % ethanol and dried briefly in a vacuum dessicator. The quantity and the protein contamination of the DNA samples were measured by UV spectrophotometer at $Abs_{260}$ and $Abs_{280}$ respectively.

**Verification of *pyrG* deletion in mutant strain by PCR.**

The deletion of the *pyrG* gene and its subsequent integration into *P. aeruginosa* (PAO1*, upp*$^{-}$, *udk*$^{+}$) chromosome was verified by PCR (Saiki, 1988) on chromosomal DNA extracted from the strain. The DNA from five mutant strains and wild-type DNA was extracted using the above method. The PCR primers initially used to isolate the *pyrG* gene were used to verify the deletion using *Taq* DNA polymerase. The following conditions for the PCR reaction were used: Step 1: denaturation at 95$^{\circ}$C for 5 min, Step 2: denaturation at 95$^{\circ}$C for 2 min, Step 3: Annealing at 56$^{\circ}$C for 1 min, Step 4: polymerisation at 72$^{\circ}$C for 3 min, Step 5: repeat step 2-4 29 times, Step 6: 72$^{\circ}$C for 5 min and Step 7: 4$^{\circ}$C for 99 h. Products formed by the PCR reaction were observed using agarose gel electrophoresis.

**Inactivation of the uridine hydrolase gene (*nuh*) by gentamicin cassette mutagenesis in specially constructed cytidine requiring strains of *P. aeruginosa*.**

The *P. aeruginosa* uridine hydrolase gene was cloned into the pZero vector to produce the plasmid pCJF5 by Christopher Fields in our lab. A 1.6 kb *Sma*I fragment containing the gentamicin-resistant (Gm) cassette was isolated from plasmid pGMΩ1 (Fig 13) (Schweizer, 1993). A *Sca*I digest of pCJF5 allowed for the direct insertion of the Gm cassette into the internal region of uridine hydrolase gene (Fig 14). *P. aeruginosa upp*$^-$, *udk*$^+$, *pyrG*$^-$ was made electrocompetent and the plasmid was electroporated into the cells as described above. The cells were plated on *Pseudomonas* minimal agar supplemented with 50 μg ml$^{-1}$ cytidine and 20 μg ml$^{-1}$uridine. The antibiotics 600 μg ml$^{-1}$ carbenicillin, 200 μg ml$^{-1}$ gentamicin were added at the mentioned concentrations.

**Confirmation of a uridine hydrolase (nuh) mutant in *P. aeruginosa*.**

*Pseudomonas* minimal agar plates supplemented with uridine and cytidine at a concentration of 50 μg ml$^{-1}$ were streaked with *P. aeruginosa upp*$^-$, *udk*$^+$, *pyrG*$^-$ *nuh*$^+$. Another plate with the same supplements was streaked with the isogenic *P. aeruginosa upp*$^-$, *udk*$^+$, *pyrG*$^-$ *nuh*$^-$. If the *P. aeruginosa upp*$^-$, *udk*$^+$, *pyrG*$^-$ *nuh*$^-$ grew on the plate then this would prove that they are not uridine hydrolase mutants because of the uridine hydrolase's ability to make ribose, which could satisfy the carbon source requirement.

**Growth conditions of *pyrG*$^-$, *upp*$^-$, *udk*$^+$ *nuh*$^-$ strains for *pyr* assays.**

*P. aeruginosa upp*$^-$, *udk*$^+$, *pyrG*$^-$ *nuh*$^-$ and its isogenic *P. aeruginosa upp*$^-$, *udk*$^+$, *pyrG*$^-$, *nuh*$^-$ strains were grown in *Pseudomonas* minimal medium under var conditions of high and low uridine and cytidine. At least 50 μg ml$^{-1}$ of cytidine was required for the cells to grow successfully. All mutant strains were grown with glucose as the carbon source, 600 μg ml$^{-1}$ carbenicillin and 200 μg ml$^{-1}$ gentamicin. Table 2 describes the various conditions concentrations of uridine and cytidine used.

Table 2

| Strain | Condition for growth |
|---|---|
| *P. aeruginosa* | $UR_{20}$ and $CR_{50}$, $UR_{200}$ and $CR_{200}$, $UR_{20}$ and $CR_{200}$, and $UR_{200}$ and $CR_{50}$ |
| *P. aeruginosa upp*$^-$, *udk*$^+$, *pyrG*$^-$ | $UR_{20}$ and $CR_{50}$, $UR_{200}$ and $CR_{200}$, $UR_{20}$ and $CR_{200}$, and $UR_{200}$ and $CR_{50}$ |
| *P. aeruginosa upp*$^-$, *udk*$^+$, *pyrG*$^-$, *nuh*$^-$ | $UR_{20}$ and $CR_{50}$, $UR_{200}$ and $CR_{200}$, $UR_{20}$ and $CR_{200}$, and $UR_{200}$ and $CR_{50}$ |

**Preparation of cell extract for *pyr* enzyme assays.**

All strains were grown in 100 ml of *Pseudomonas* minimal medium with the necessary additions. Bacteria were grown to 100 Klett units and harvested by centrifugation at 6,000 xg for 20 min. The cells were resuspended in 1 ml of 40 mM phosphate buffer pH 8.0. Cells were disrupted by sonication and transferred to a microfuge tube. The clarified extract was centrifuged at 10, 000 xg for 4 min at which time a 200 µl sample was removed and placed into a sterile tube for dihydroorotate dehydrogenase (*pyrD*) encoded enzyme assays. The remainder was centrifuged at 12, 000 xg for a further 20 min. The supernantant was removed for enzymes encoded *pyrB-pyrF*.

**Enzyme assays.**

All assays were carried out at 37˚C. Changes in absorbance were monitored spectrophotometrically using a Shimadzu UV-mini spectrophotometer 1240. Specific activites of the enzymes assayed were determined under conditions in which product formation (or substrate utilization) was proportional to extract and time and expressed as µmol $min^{-1}$ $mg^{-1}$ protein. All values represent the mean of three independent determinations.

**Aspartate transcarbamoylase (ATCase) assay.**

ATCase enzyme assays were performed using the method described by Gerhart & Pardee (1962). The specific activity (μmol $min^{-1}$ $mg^{-1}$) was determined by monitoring the enzymatic production of carbamoylaspartate (CAA) in 10 min at 37°C using the colorimetric method of Prescott & Jones (1969). A 1 ml reaction volume contained the following components: 40 μl of Tri-buffer (Ellis & Morrison, 1982), pH 9.5, (51 mM diethanolamine, 51 mM *N*-ethylmorpholine and 100 mM MES), 50 μl of 100 mM potassium aspartate (pH 9.5), 100 μl of 50 mM carbamoylphosphate (dilithium salt), 10 μl of clarified extract, 800 μl $ddH_2O$. In addition to the reaction tubes, a control was also prepared by substituting $ddH_2O$ for larified extract. The ATCase assay tubes were prepared in advance, without the addition of carbamoylphosphate, and preincubated at 37°C for 2 min. The reaction was initiated by the addition of carbamoylphosphate and the tubes were incubated at 37° for 10 min. After 10 min, the reaction was terminated by the addition of 1 ml of color mix (2 parts of 5 mg $ml^{-1}$ of antipyrine in 50% sulfuric acid (v/v) with 1 part of 8 mg $ml^{-1}$ of 2, 3-butanedione monoxime in 5% acetic acid (v/v)). The reaction tubes were mixed by vigorous vortexing, and incubated at 65°C for 2 h in the light to allow development of the color. The tubes were capped with marbles to minimize evaporation. After 2 h, the absorbance units at 466 nm ($A_{466}$) was measured, using the control tube to blank the spectrophotometer. The μmoles of CAA produced were determined using a CAAstandard curve, prepared with known concentration of

CAA ranging from 25 to 300 μM in the standard assay reaction mix and under the same color development conditions .

**Dihydroorotase (DHOase) assay.**

The DHOase assay tubes contained the following components in a 1 ml reaction volume: 100 μl of 1 M Tris (pH 8.6), 100 μl of 10 mM EDTA, 100 μl of 20 mM dihydroorotate (in 0.1 M phosphate buffer, pH 7.5), 10 μl clarified extract and 690 μl of $ddH_2O$ (Beckwith *et al*., 1962). The DHOase assay tubes were prepared in advance, without the dihydroorotate, and pre-incubated at 37°C for 2 min. The reaction was initiated by the addition of dihydroorotate and the tubes were incubated at 37°C for 10 min. After 10 min, the reaction was terminated by the addition of 1 ml of color mix, the tubes were vortexed and incubated at 65°C for 2 h in the light. The $A_{466}$ units were measured using a control tube, containing all reaction components except dihydroorotate, to blank the spectrophotometer.

**Dihydroorotate dehydrogenase assay.**

Dihydroorotate dehydrogenase activity was measured spectrophotometrically by monitoring the conversion of dihydroorotate to orotate at 290 nm. The dihydroorotate dehydrogenase reaction mix was prepared in a quartz cuvette containing the following components in a 1 ml reaction volume: 0.1 M Tris (pH 8.6), 6 mM $MgCl_2$, 1 mM dihydroorotate (in 0.1 M phosphate buffer, pH 7.5), 50 μl clarified extract and $ddH_2O$ to a 1 ml total volume. The reaction cuvette was prepared in advance, without the

dihydroorotate, and incubated at 37°C for 2 min. The reaction was initiated with the addition of dihydroorotate, and the $A_{290}$ units were noted immediately, this was the blank reading. The cuvette was then incubated at 37°C for 10 min, after which the $A_{290}$ units were measured. The blank reading was subtracted from the 10 min reading. An increase in absorbancy of 1.93 is equivalent to a change in substrate concentration of 1 mM (relative to the blank reading).

**Orotate phosphoribosyltransferase (OPRTase) assay.**

OPRTase activity was measured spectrophotometrically by the method of Smith *et al*., (1980). The conversion of orotate to OMP was monitored at 295 nm. The OPRTase reaction mix was prepared in a quartz cuvette containing the following components in a 1 ml reaction volume: 0.1 M Tris (pH 8.6), 6 mM $MgCl_2$, 0.25 mM orotate, 0.6 mM 5-phosphoribosyl-1-pyrophosphate (PRPP), 50 µl clarified extract and $ddH_2O$ to a 1 ml total volume. The reaction cuvette was prepared in advance, without the orotate, and incubated at 37°C for 2 min. The reaction was initiated with the addition of orotate, and the $A_{295}$ units were recorded immediately, this was the blank reading. The cuvette was then incubated at 37°C for 10 min, after which the $A_{295}$ units were measured. The 10 min reading was subtracted from the blank reading. A decrease in absorbancy of 3.67 is equivalent to an increase in orotidine 5'-monophosphate (OMP) concentration of 1 mM (relative to the blank reading).

**OMP decarboxylase assay.**

OMP decarboxylase activity was measured spectrophotometrically by monitoring the conversion of OMP to uridine 5'-monophosphate (UMP) at 285 nm. The OMP decarboxylase reaction mix was prepared in a quartz cuvette containing the following components in a 1 ml reaction volume: 0.1 M Tris (pH 8.6), 6 mM $MgCl_2$, 0.2 mM OMP, 50 µl clarified extract and $ddH_2O$ to a 1 ml total volume. The reaction cuvette was prepared in advance, without the OMP, and incubated at 37°C for 2 min. The reaction initiated with the addition of OMP, and the $A_{285}$ units were noted immediately, this was the blank reading. The cuvette was then incubated at 37°C for 10 min, after which the $A_{285}$ units were measured. The 10 min reading was subtracted from the blank reading. A decrease in absorbancy of 1.38 is equivalent to a decrease in OMP concentration of 1 mM (relative to the blank reading).

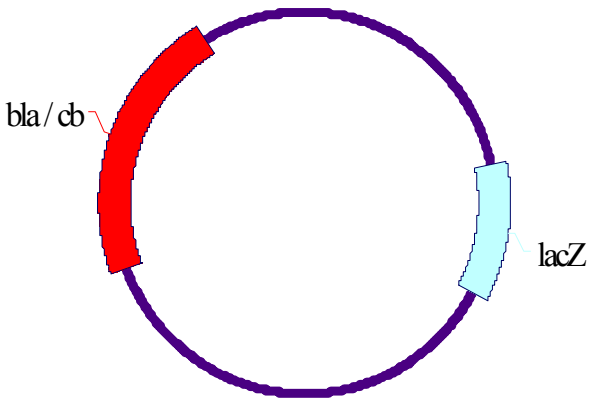



Fig 6. Plasmid pUCP19. The plasmid contains the ampicillin resistant gene which confers carbenicillin resistance in Pseudomonads.

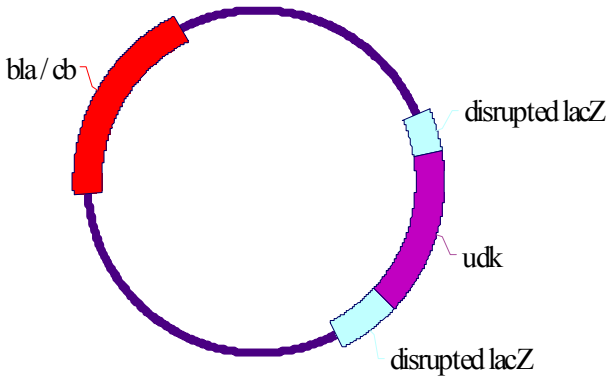



Fig 7. Construction of pDEB (Beck, 1995). The plasmid contains the *E. coli* 780 bp uridine (cytidine) kinase gene from pEUK20 (Kelln, 1988). The host plasmid is pUCP19.

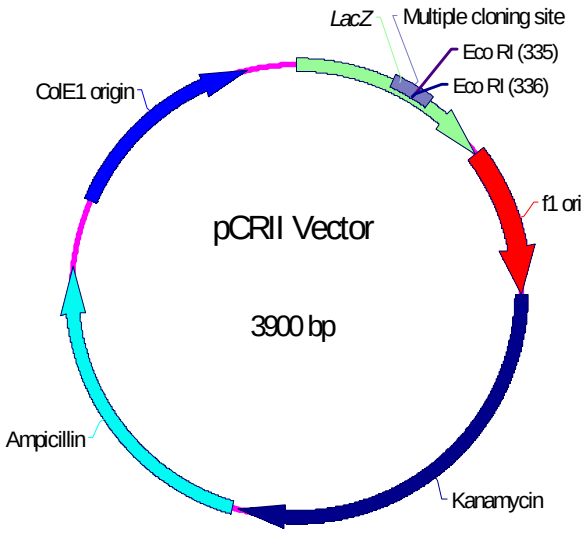



Fig 8 Schematic diagram of pCR™ II vector (Invitrogen). The multiple cloning site is located within the *lac*Zα gene which allows for blue, white selection. The plasmid contains a kanamycin and ampicillin resistant gene.

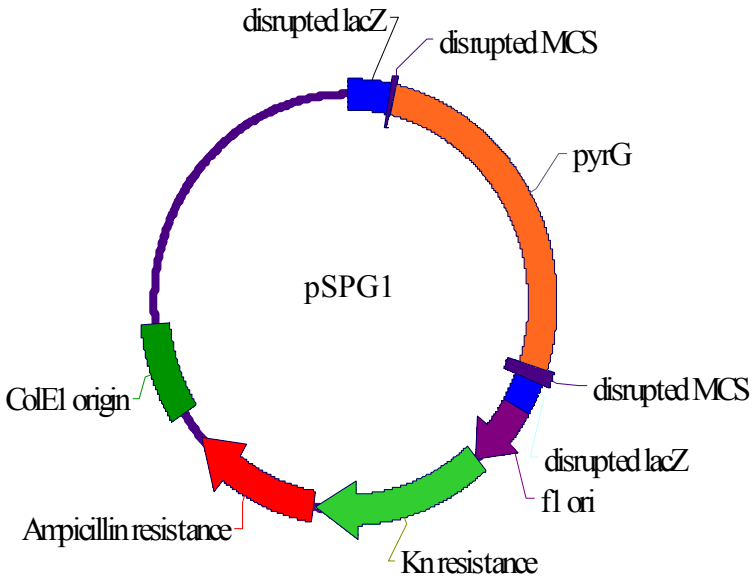



Fig 9. Construction of pSPG1, entire *pyrG* gene cloned into the multiple cloning site of the plasmid.

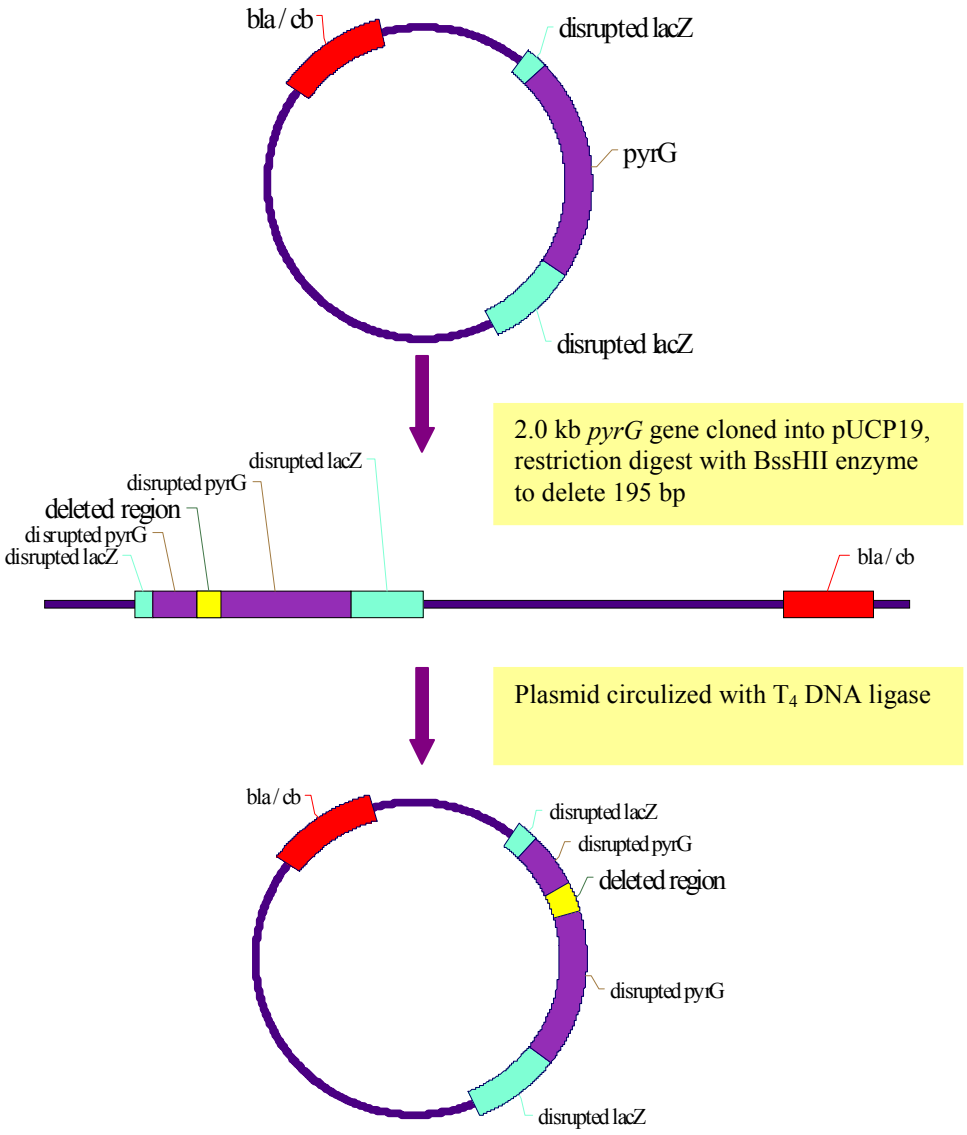



Fig 10. Construction of the *pyrG* deletion in pUCP19. The 2.0 kb pyrG gene was cloned into pUCP19 and the geletion was conducted in the plasmid by digesting with the *BssH*II enzyme.

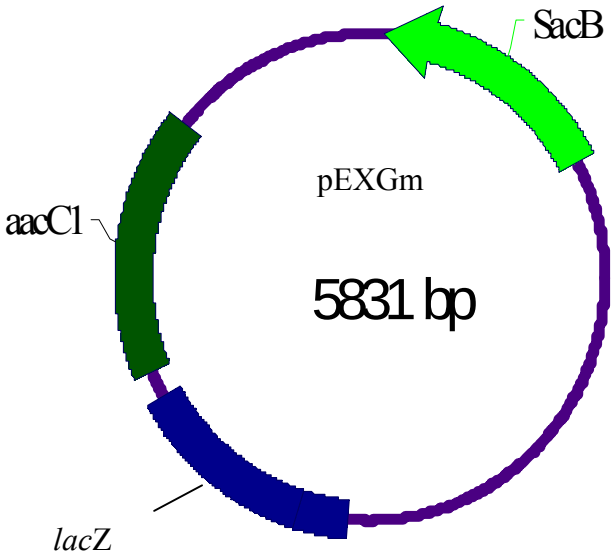



Fig 11. Gene replacement plasmid pEX18Gm. Plasmid size is 5.831 kb and has the convienient multiple cloning site of pUC18. This vector has the gentimicin resistant marker and the *Bacillus subtilis SacB* gene, which confers sucrose sensitivity and thus allows for the identification of single and double crossovers.

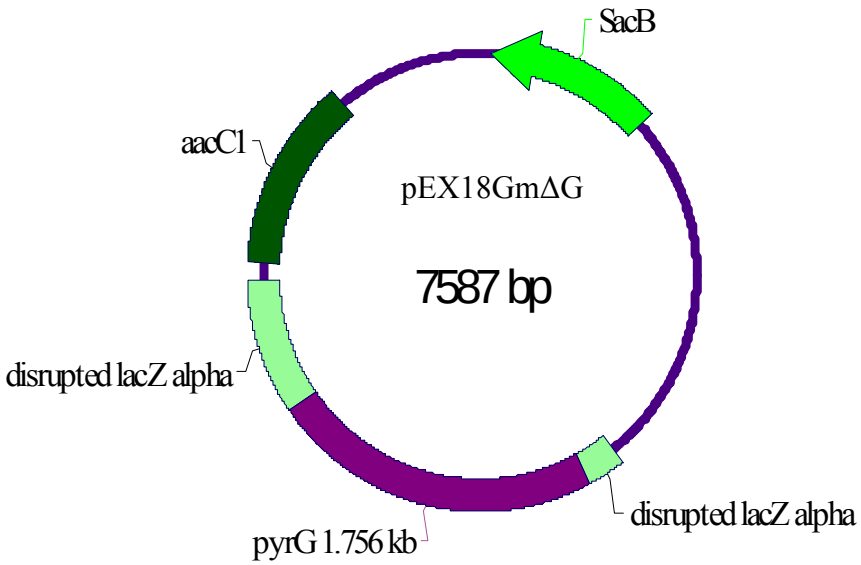



Fig 12. Gene replacement plasmid containing the deleted 1.756 kb *pyrG* gene subcloned from pUCP*ΔpyrG*. This plasmid was used to replace the wild type *pyrG* gene in *P. aeruginosa upp*$^{-}$, *udk*$^{+}$.

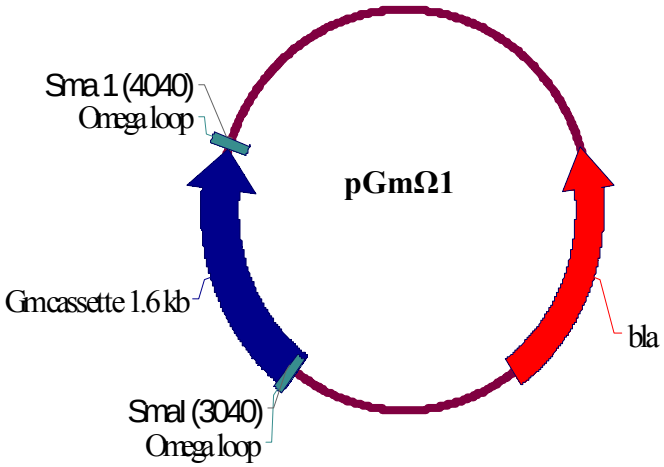



Fig 13. Small broad host range plasmid pGmΩ1 plasmid (Schweizer, 1993). The plasmid contains the 1.6 kb gentamicin resistant gene cassette. The gentamicin resistant cassette can be used for site specific insertion and deletion mutagenesis.

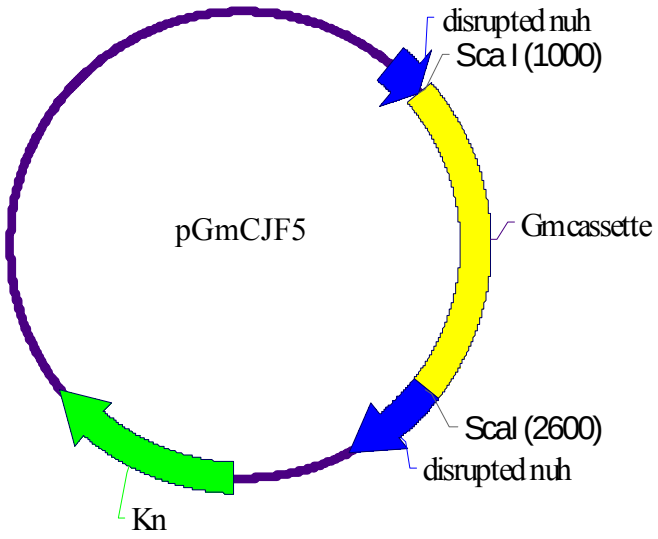



Fig 14. Schematic diagram of pCJF5. The resulting 7.3 kb plasmid contains the 1.6 kb gentamicin cassette fragment, digested from pGMΩ1 (Schweizer, 1993). The Gm cassette was cloned into the Sca1 restriction site of plasmid pCJF5. Expression of uridine hydrolase was inactivated by the insertion of the Gm cassette.

# CHAPTER II

# RESULTS AND DICUSSION

**Cloning of the CTP synthase (*pyrG*) of *P. aeruginosa*.**

Chromosomal DNA from *P. aeruginosa* was prepared and used to conduct PCR using the method described by Saiki *et al* (1988). A product of the size 2.0 kb was produced and was subsequently cloned into the pCRII™ vector (Invitrogen) to produce the construct pSPG1 (Fig 8). The isolation of the *pyrG* gene was confirmed by complementing the *E. coli* pyrimidine auxotroph JF646.

**Isolation of *P. aeruginosa upp*$^-$ strain.**

The pyrimidine analog 5'- fluorouracil was used to isolate a *P. aeruginosa upp*$^-$ mutant. A pyrimidine analog is a pyrimidine base which has an additional functional group attached to one of the members of the pyrimidine ring. The analog 5'fluorouracil has a fluoro group attached to the number 5 carbon on the pyrimidine ring. In a bacterial cell the analog is recognized as a uracil compound and is quickly taken up by the enzyme uracil permease (*uraA*). Next uracil phosphoribosyltransferase encoded by the *upp* gene converts it to FUMP. The ultimate toxic compund molecule is 5FdUMP, which inhibits thymidylate synthase.

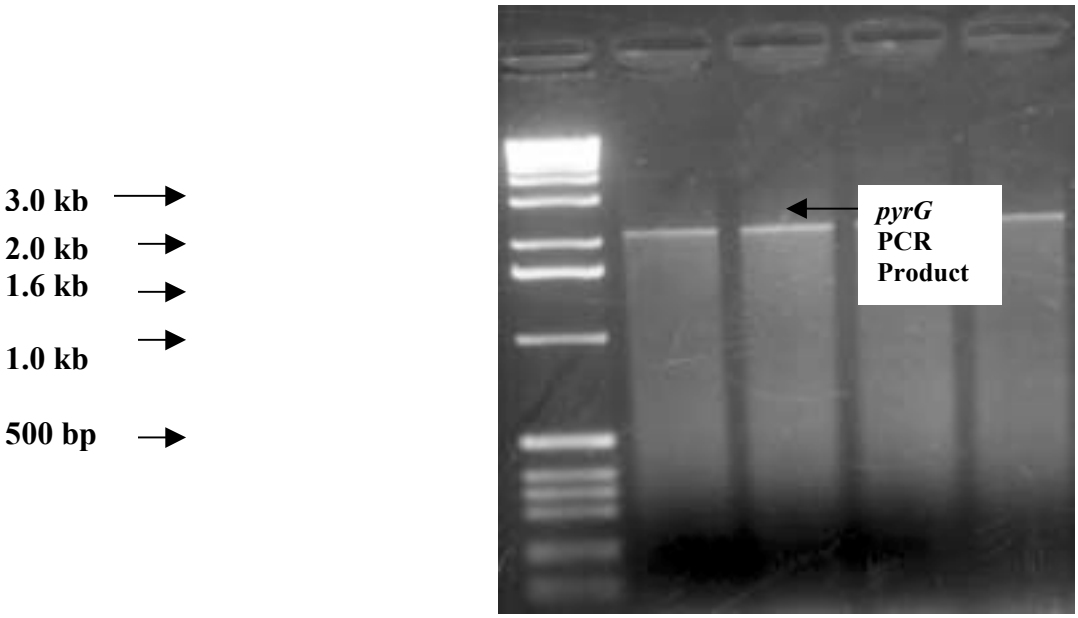



Fig 15. A photograph of the *pyrG* gene isolated from PAO1 chromosomal DNA. The size of the fragment is 2.1 kb. The product generated was cloned into the pCRII™ vector (Invitrogen®).

In any given population of bacteria some cells are naturally resistant to 5'- fluorouracil, *upp*$^-$. These *upp* mutants have no uridine phosphoribosyltransferase and thus are unable to catalyze the reaction of fluorouracil to fluorouridine 5' monophosphate (FUMP). In this study I have isolated a *P. aeruginosa upp*$^-$ strain, which is resistant to 5' fluorouracil (Fig 16). Figure 16 shows the plate assay performed in order to isolate such a mutant. Crystals of 5'-fluorouracil were placed in the middle of the plate. *P. aeruginosa upp*$^-$ mutants were able to grow right up to the crystals. This was the first step in reaching that pot of gold at the end of the rainbow, which in my case the pot of gold is excluded and all that I am left with is the wish of finding *pyrG* mutants in *Pseudomonas*.

**Expression of the *E. coli* uridine (cytidine) kinase (*udk*) gene in *P. aeruginosa*.**

Organisms with a functional uridine (cytidine) kinase gene are sensitive to the pyrimidine analog 5'-fluorouridine because the enzyme can catalyze the reaction of FUR to FUMP, which is toxic to the cell. Such organisms are also sensitive to 5'fluorocytidine because uridine (cytidine) kinase can also catalyze the reaction of cytidine to CMP and ultimately CTP. 5'-fluorocytidine can be converted to the toxic nucleotide levels.

Beck (1995) showed that *P. aeruginosa* does not possess a functional uridine (cytidine) kinase and does not have a cytidine deaminase gene. She cloned the *E. coli* udk gene into pUCP19. This construct was named pDEB1 (Fig 7). By finding that *P. aeruginosa* became sensitive to FUR and FCR only after she introduced the *E. coli*

uridine (cytidine) kinase gene. Beck showed that the introduced *udk* was expressed in *P. aeruginosa*.

I used this plasmid to electroporate it into a *P. aeruginosa upp*$^-$ strain.

I plated the transformed cells onto *Pseudomonas* minimal medium and then placed crystals of 5-fluorouridine and 5-fluorocytidine in the middle of the plate. The results of this experiment showed a zone of killing around the FUR and FCR crystals. The zone of killing indicates that the *udk* gene can be expressed in *P. aeruginosa upp*$^-$ (Fig 17).

Until a functional uridine (cytidine) kinase was incorporated into *P. aeruginosa* it was not possible to convert UR (FUR) or CR (FCR) to their nucleotide derivatives. Once *udk* was expressed, it became possible to feed exogenous nucleosides to pyrimidine auxotrophs as well as sensitizing *P. aeruginosa* to FUR and FCR. Thus, the isolation of a *pyrG* mutant became feasible. This in turn allowed the independent manipulation of the UTP and CTP pools.

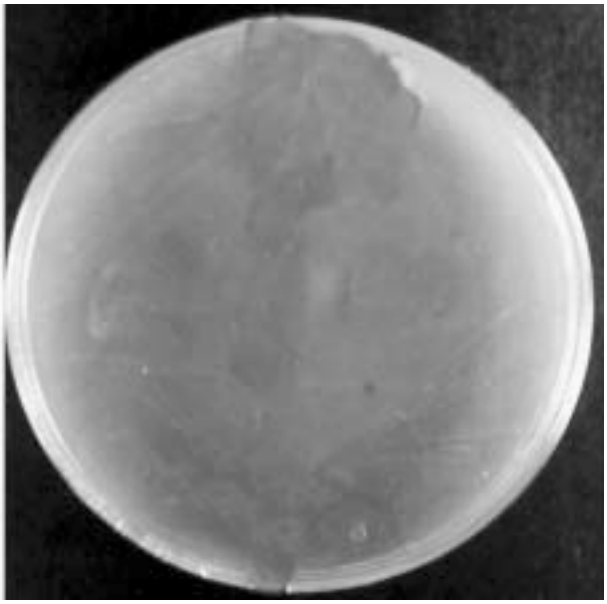


Fig 16. *P. aeruginosa* PAO1 *upp*$^{-}$ strain isolation. Crystals of 5' fluorouracil were placed in the center of the plate. No zone of killing was observed.

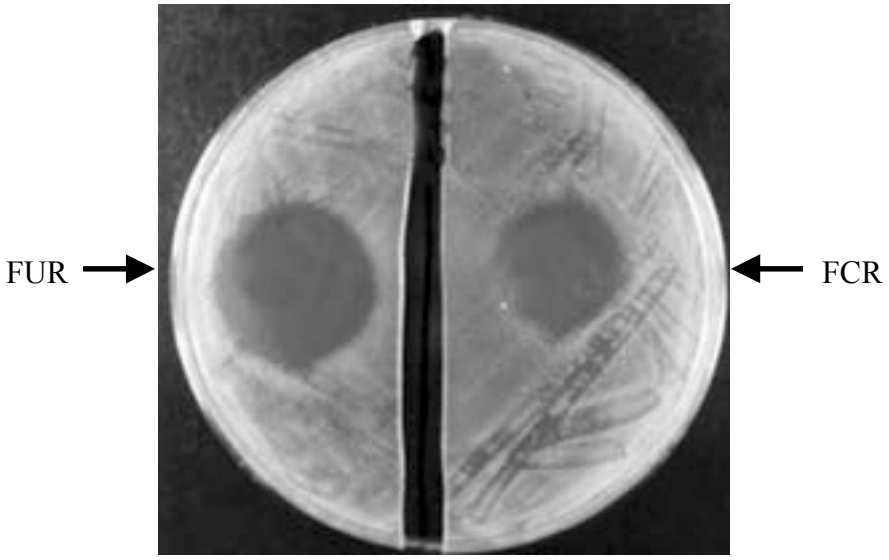



Fig 17. Expression of the *E. coli* uridine (cytidine) kinase gene is *P. aeruginosa* PAO1 *upp*$^{-}$ strain. On the left half of the plate crystals of fluorouridine were placed in the and on the right half of the plate crystals of fluorocytidine were placed. Zones of killing indicate the expression of the *udk* gene.

**Isolation of cytidine requiring mutants in *P. aeruginosa*.**

CTP synthase mutants were isolated using the in-frame deletion technique described by Schweizer and Houng (1993). The *pyrG* gene was sub-cloned from pSPG1 into the pUCP19 vector. An in-frame deletion was created in this vector by restriction digest using the enzyme *Bss*HII. This restriction enzyme created a 195 bp deletion in the internal region of the *pyrG* gene. The vector was circularized and the truncated fragment was cloned into the gene replacement vector pEX18Gm (Fig 10, 11 & 12). The pEX18 *pyrG* plasmid was incorporated into the *P. aeruginosa upp*$^{-}$ *udk*$^{+}$ strain by the method of biparental mating described by De Larenzo & Timms (1993).

Like its *E. coli* counterpart the *pyrG* gene of *P. aeruginosa* encodes CTP synthase. This was confirmed by genome analysis and only one copy of the gene was found. The disruption (deletion) in *pyrG* resulted in the cells requirement for 50 μg ml$^{-1}$cytidine, *pyrG* mutants are cytidine auxotrophs.

In *E. coli* and *S. typhimurium* cytidine is rapidly deaminated to uridine by cytidine deaminase. Accordingly, in *E. coli pyrG* mutants must be isolated in a *cdd*$^{-}$ background. *P. aeruginosa* does not have this enzyme but instead has a very active nucleoside hydrolase, which must be mutated along with *pyrG* to study regulation. This is discussed below. Chromosomal DNA from *P. aeruginosa* wild type cells and the mutants isolated was prepared. PCR was conducted to confirm the presence of the deleted *pyrG* gene (Fig 18).

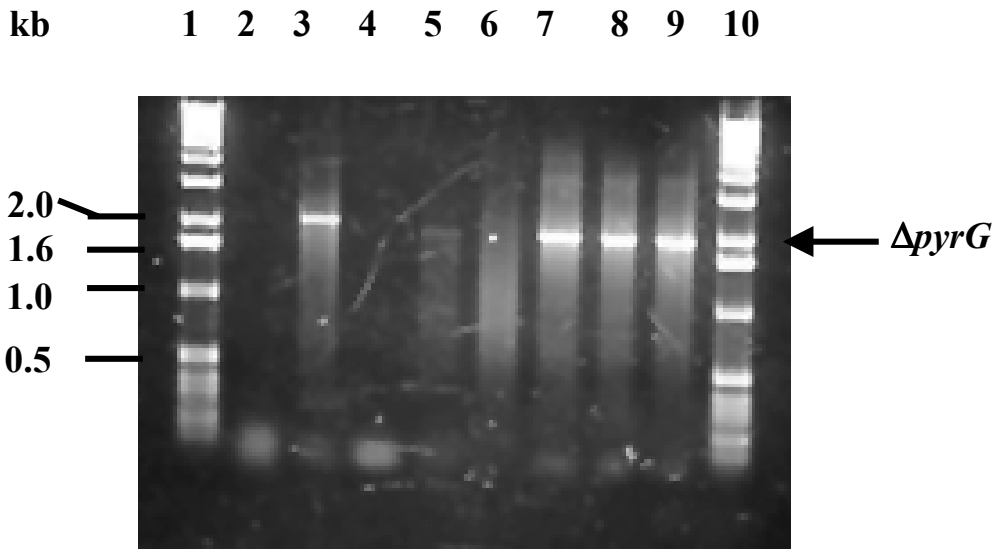



Fig 18. Verification of *pyrG* inframe deletion in *P. aeruginosa* PAO1 *upp*$^{-}$ *udk*$^{+}$. Lane1 1 kb ladder, Lane 2 Negative control, Lane 3 Wild type, Lane 4 Negative control, Lane 5-9 Δ*pyrG*, Lane 10 1 kb Ladder

**Cassette mutagenesis of uridine hydrolase in *P. aeruginosa upp$^-$ udk$^+$ pyrG$^-$*.**

The uridine hydrolase gene was shown to be very active in *P. aeruginosa* by Christopher Fields (unpublished data, 2001). This caused a problem because uridine was added to the medium to feed the cells and to elevate the UTP pools in *P. aeruginosa*. Initial *pyr* enzyme assays did not show much effect on the *de novo* enzymes. This led to the conclusion that the uridine added was rapidly broken down to uracil by uridine hydrolase. Indeed, Fields showed that the hydrolase was induced by growth on uridine. The uracil was excreted into the surrounding medium. Therefore it was imperative to construct a uridine hydrolase mutant in the *P. aeruginosa upp$^-$ udk$^+$ pyrG$^-$*. A 1.6 kb gentamicin cassette was inserted into the middle of the uridine hydrolase gene. The resultant construct was electroporated into the above strain. The mutation was confirmed by plating the cells onto *Pseudomonas* minimal medium, which had uridine or cytidine only as the carbon source. A uridine hydrolase plus mutant would be able to break these compounds down and use the ribose liberated as a carbon source. A uridine hydrolase minus mutant would not be able to do this. Inability of the cells to use uridine or cytidine as a carbon source confirmed the mutation in the hydrolase gene.

**Enzyme assays for *pyrB-pyrF* in the specially constructed cytidine requiring (*pyrG*) mutants of *P. aeruginosa*.**

Enzyme assays for ATCase (*pyrB*), DHOase (*pyrC*), DHOdecase(*pyrD*), OPRTase (*pyrE*) and OMPdecase (*pyrF*) were carried out on the specially constructed cytidine requiring mutants. The cells were grown in 100 ml of *Pseudomonas* minimal medium with the appropriate antibiotics. This experiment allowed us to ascertain the effects on the *pyr* enzymes when the cells were grown in different levels of uridine and or cytidine. In a *pyrG*$^-$ *upp*$^-$ *udk*$^+$ the specific activity for the enzyme ATCase was 342 μmol min$^{-1}$ mg$^{-1}$ protein. In comparison the specific activity of the *pyrG*$^-$ *upp*$^-$ *udk*$^+$ *nuh*$^-$ was 71 μmol min$^{-1}$ mg$^{-1}$ protein. This showed that when the level of uridine was high (200 μg ml$^{-1}$) and cytidine low (50 μg ml$^{-1}$) there is a 5-fold repression of the enzyme ATCase, suggesting that a uridine compound (UMP, UDP or UTP) is the primary repressing metabolite for ATCase (Table 3, Fig 19).

A more dramatic change was observed in the enzyme dihydroorotase. When uridine and cytidine levels were at 20 μg ml$^{-1}$ and 50 μg ml$^{-1}$ there was a 5-fold repression of the enzyme. When both uridine and cytidine were at 200 μg ml$^{-1}$ there was a 4.6-fold repression in the enzyme. The most change observed in the enzyme was when uridine was 200 μg ml$^{-1}$ and cytidine was 50 μg ml$^{-1}$. Then there was a 9-fold repression in the enzyme. This again suggests that a uridine compound or high UTP has a repressive effect on the DHOase enzyme (Table 4, Fig 20).

For the enzyme encoded by *pyrD*, a 2-fold repression was observed when the cells were grown in high uridine and low cytidine. This result is not as significant as those

observed for the earlier enzymes in the pathway (Table 5, Fig 21). When the cells were grown in high uridine and low cytidine the enzyme OMP decarboxylase was derepressed 1.4-fold in the *pyrG*$^{-}$ *upp*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ when compared to *pyrG*$^{-}$ *upp*$^{-}$ *udk*$^{+}$ (Table 6, Fig 22). Finally the enzyme encoded by *pyrF* was repressed 3-fold when grown in high uridine and low cytidine (Table 7, Fig 23).

This is the first study to be done with the proper necessary mutants in the biosynthetic pathway of *P. aeruginosa*. In the past it has been impossible to vary the internal pools of UTP and CTP for the lack of a *pyrG* mutation. Currently work is underway in our laboratory to quantify these pools by way of High Performance Liqiud Chromatography. This will provide ultimate data to discover if a uridine or a cytidine nucleotide is the primary repressing metabolite.

None of this would be possible without the presence of a functional uridine (cytidine) kinase in *P. aeruginosa*. This presence allowed the isolation of a *pyrG* mutant, which in turn required a nucleoside mutant for full expression.

Table 3. Specific activities (ATCase) in *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ strain, and its isogenic *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ strain.

| **Strain** | **Growth conditions** | | | |
|---|---|---|---|---|
| | **$Ur_{20}/Cr_{50}$** | **$Ur_{200}/Cr_{200}$** | **$Ur_{200}/Cr_{50}$** | **$Ur_{20}/Cr_{200}$** |
| | **Specific activity in µmol min$^{-1}$ mg protein** | | | |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ | 75 | 258 | 342 | 123 |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ | 106 | 98 | 71 | 78 |

Table 4. Specific activities (DHOase) for *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ strain, and its isogenic *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ strain.

| **Strain** | **Growth conditions** | | | |
|---|---|---|---|---|
| | **$Ur_{20}/Cr_{50}$** | **$Ur_{200}/Cr_{200}$** | **$Ur_{200}/Cr_{50}$** | **$Ur_{20}/Cr_{200}$** |
| | **Specific activity in µmol min$^{-1}$ mg protein** | | | |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ | 49 | 57 | 43 | 26 |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ | 9.3 | 12.4 | 4.9 | 10 |

Table 5. Specific activities (DHOdecase) for *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ strain, and its isogenic *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ strain.

| **Strain** | **Growth conditions** | | | |
|---|---|---|---|---|
| | **$Ur_{20}/Cr_{50}$** | **$Ur_{200}/Cr_{200}$** | **$Ur_{200}/Cr_{50}$** | **$Ur_{20}/Cr_{200}$** |
| | **Specific activity in µmol min$^{-1}$ mg protein** | | | |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ | 42 | 61 | 49 | 26 |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ | 36 | 32 | 27 | 31 |

Table 6. Specific activities (OPRTase) for *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ strain, and its isogenic *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ strain.

| **Strain** | **Growth conditions** | | | |
|---|---|---|---|---|
| | **$Ur_{20}/Cr_{50}$** | **$Ur_{200}/Cr_{200}$** | **$Ur_{200}/Cr_{50}$** | **$Ur_{20}/Cr_{200}$** |
| | **Specific activity in µmol min$^{-1}$ mg protein** | | | |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ | 81 | 44 | 36 | 37 |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ | 115 | 25 | 52 | 69 |

Table 7. Specific activities (OMPdecase) for *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ strain, and its isogenic *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ strain.

| **Strain** | **Growth conditions** | | | |
|---|---|---|---|---|
| | **$Ur_{20}/Cr_{50}$** | **$Ur_{200}/Cr_{200}$** | **$Ur_{200}/Cr_{50}$** | **$Ur_{20}/Cr_{200}$** |
| | **Specific activity in µmol min$^{-1}$ mg protein** | | | |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ | 23 | 22 | 72 | 11 |
| *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$ | 27 | 32 | 27 | 14 |

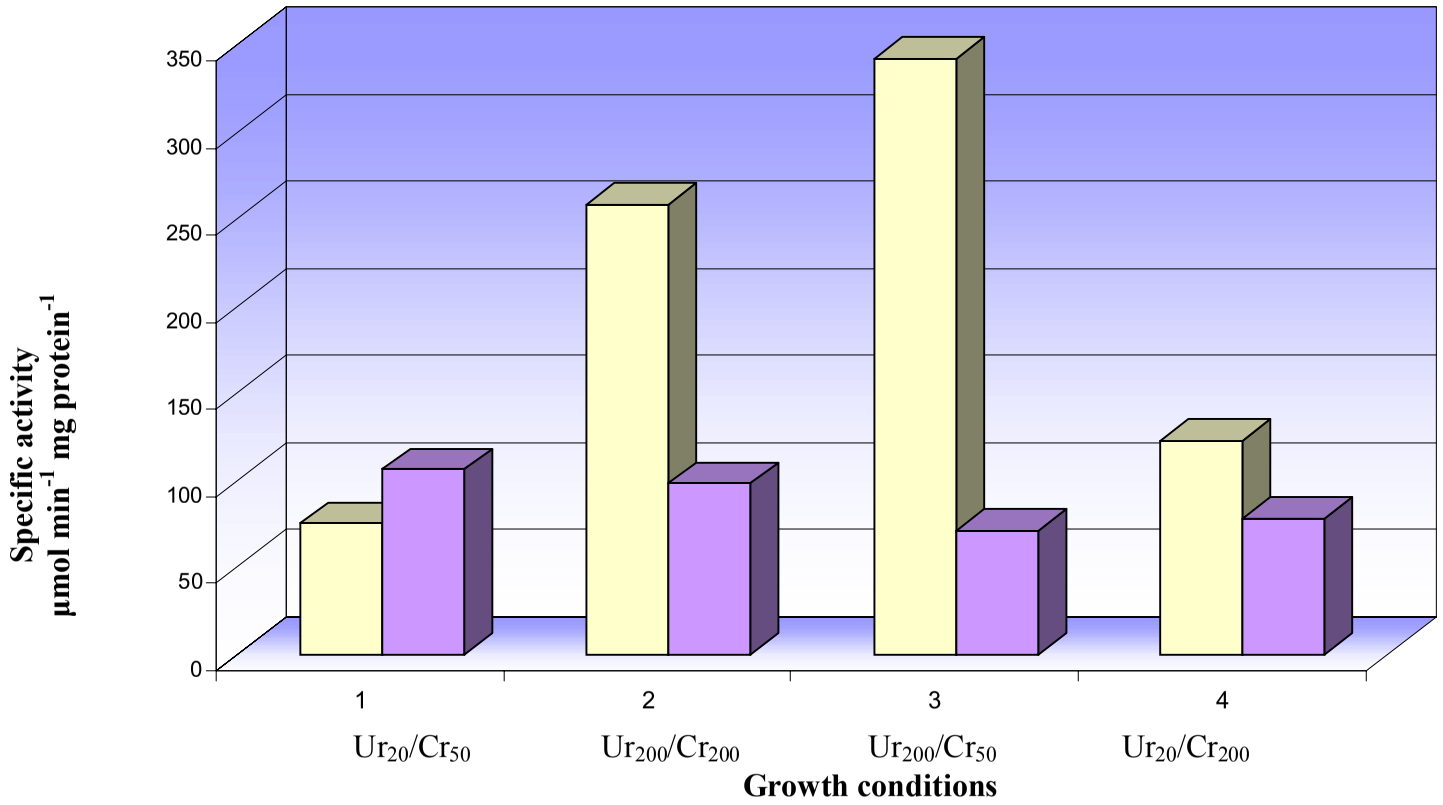



Fig 19. ATCase activity in *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ versus its isogenic strain *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$

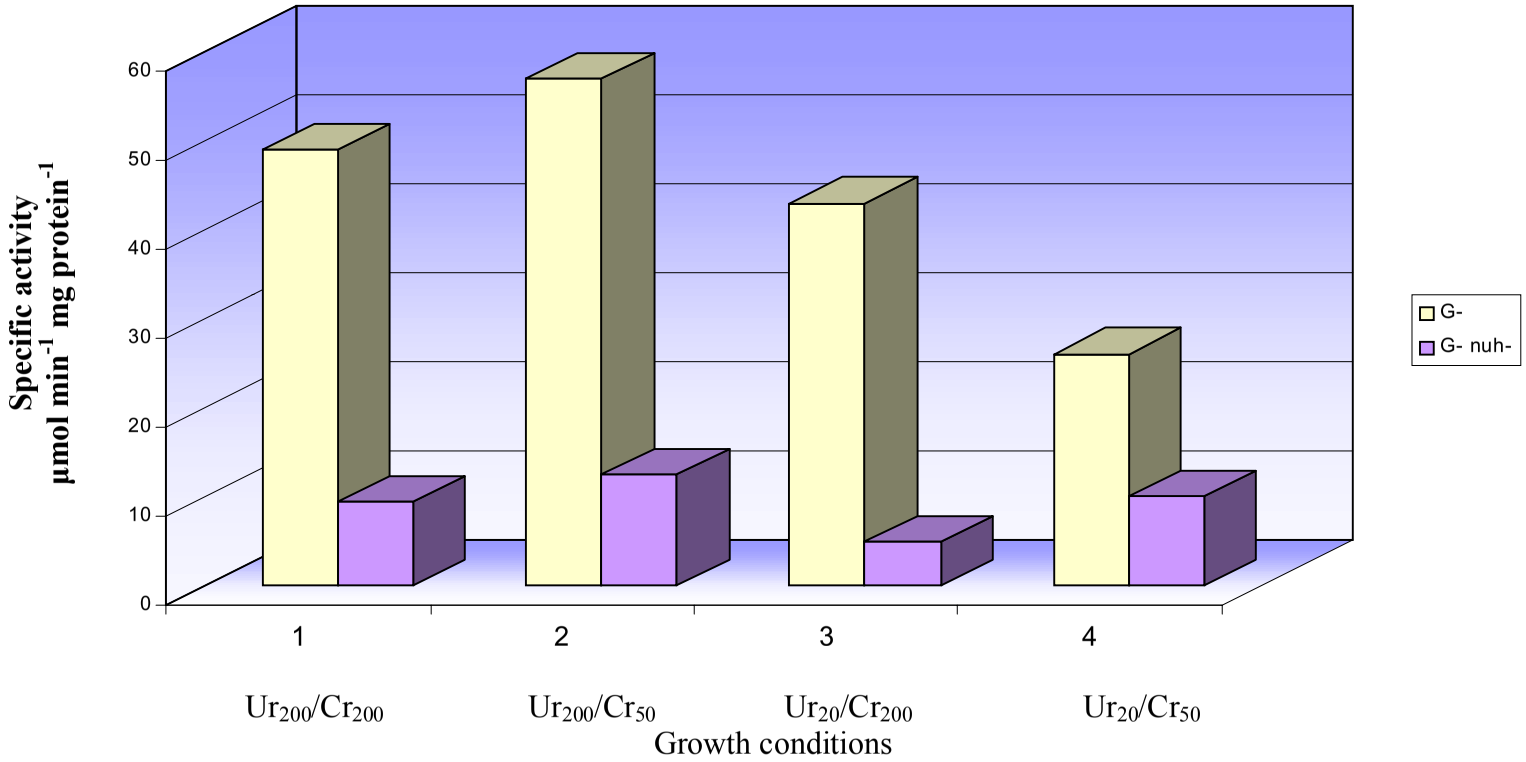



Fig 20. DHOase activity in *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ versus its isogenic strain *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$.

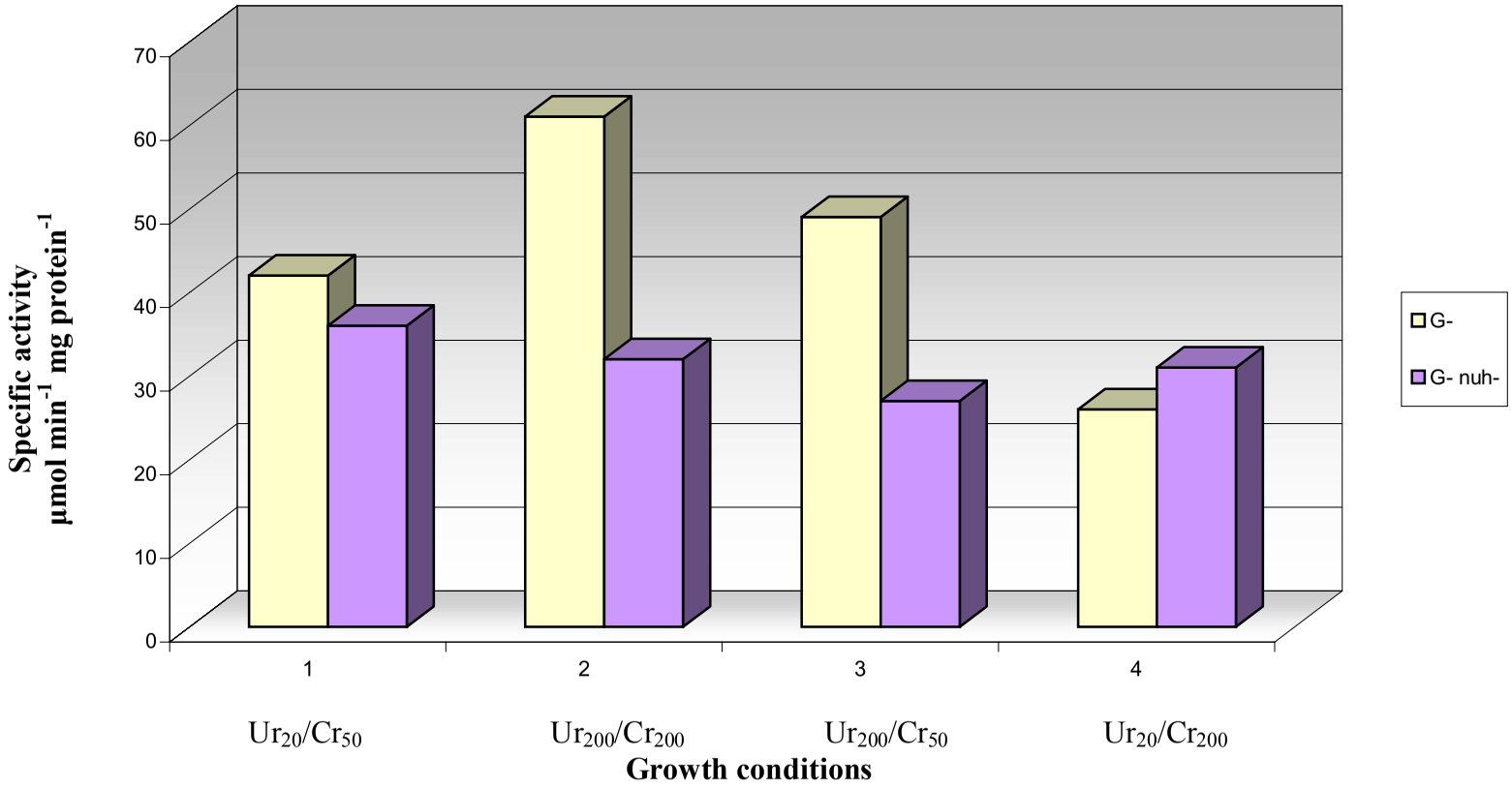



Fig 21. DHOdecase activity in a *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ strain versus its isogenic strain *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$

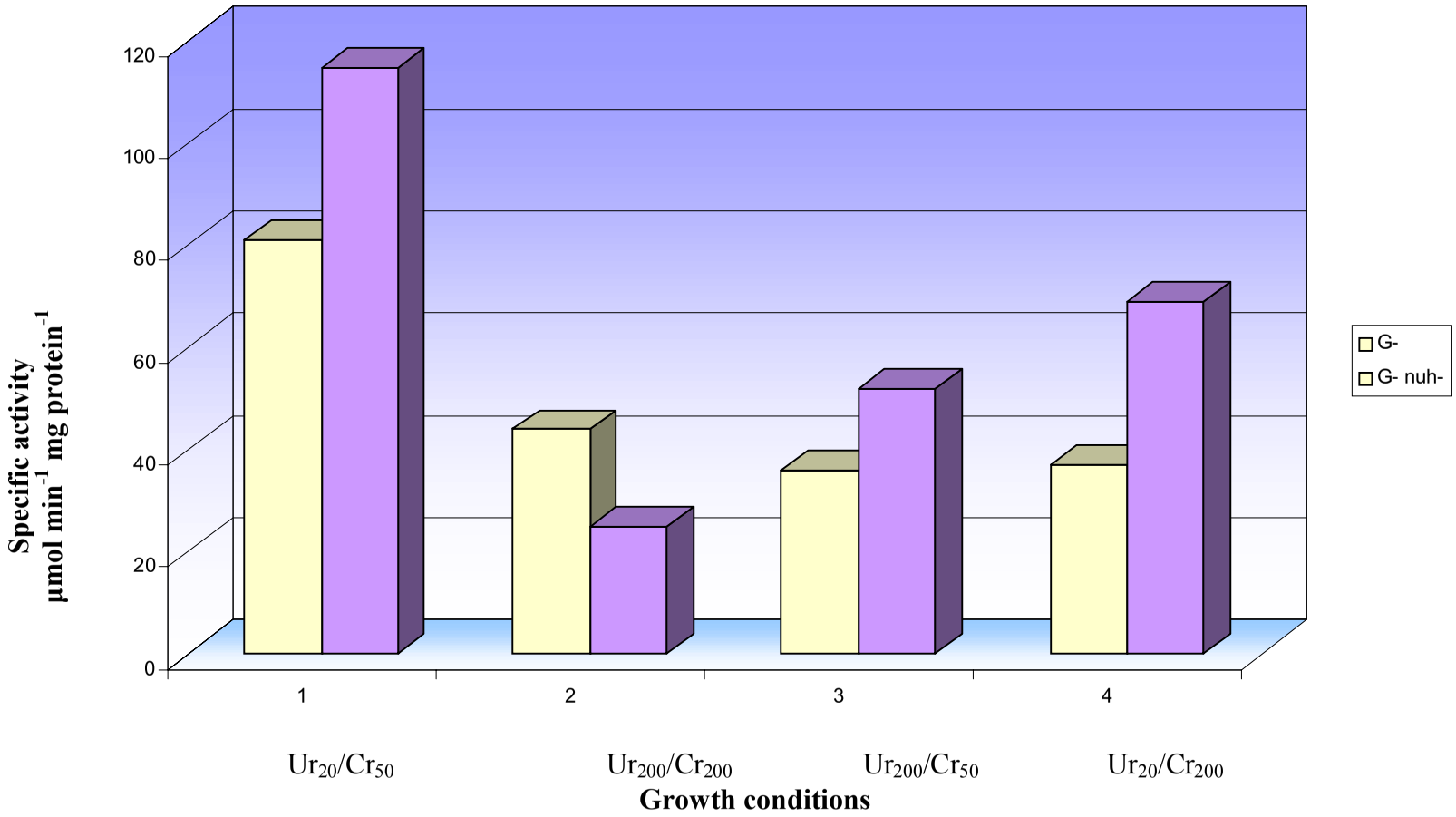



Fig 22. OPRTase activity in a *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ strain versus its isogenic strain *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$

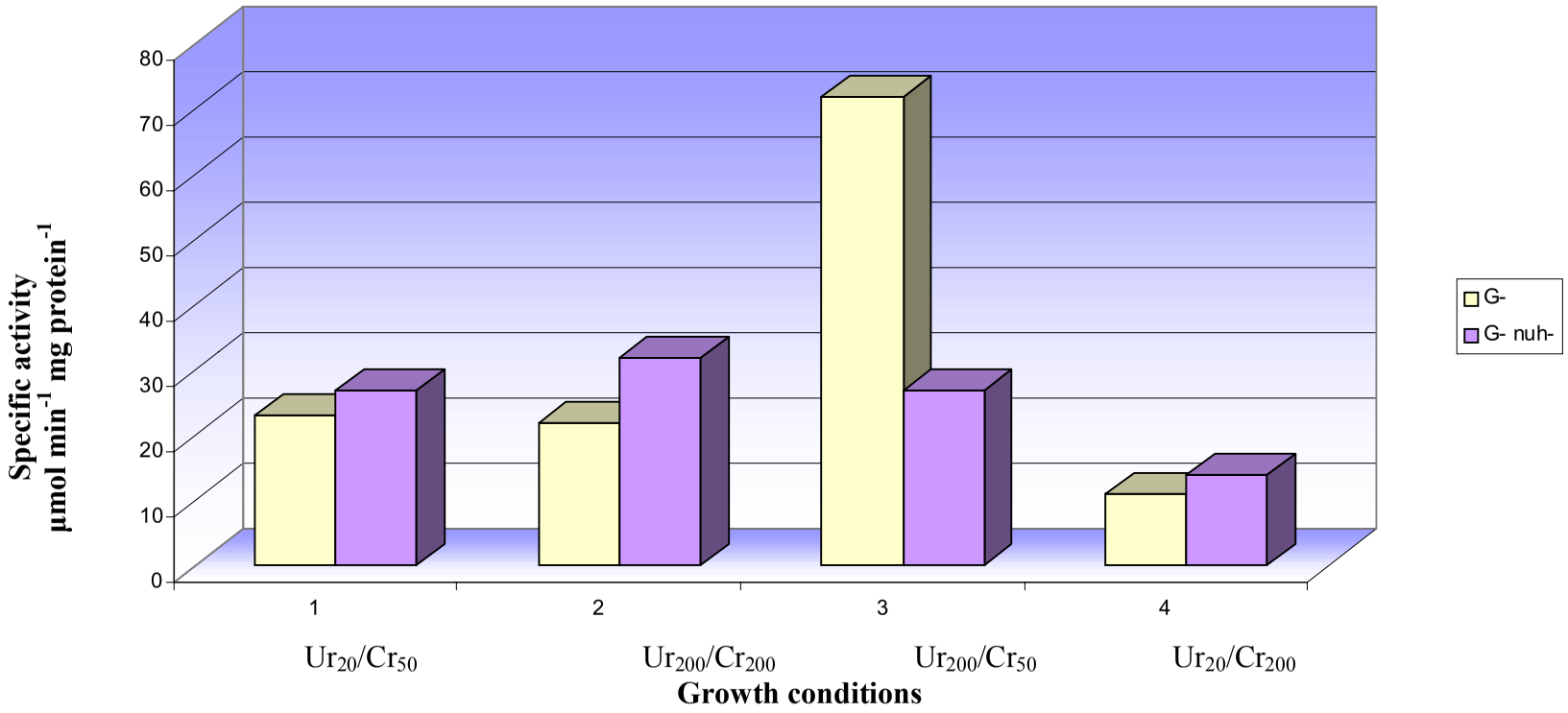



Fig 23. Assay data for OMPdecase in *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ versus its isogenic strain *upp*$^{-}$ *pyrG*$^{-}$ *udk*$^{+}$ *nuh*$^{-}$

CHAPTER II

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