CREATION AND CHARACTERIZATION OF AN *ESCHERICHIA COLI* AND *PSEUDOMONAS PUTIDA* HYBRID ASPARTATE TRANSCARBAMOYLASE

THESIS

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By

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Aspartate transcarbamoylase (ATCase) is encoded by the *pyrBI* genes in *E. coli*. Expression of these genes is reduced four-fold by attenuation when grown on uracil. Using plasmid, pRO1727, the *pyrB* and the *pyrBI* genes from *E. coli* were cloned into a *P. putida pyrB* auxotroph. A recombinant *pyrB* gene was recovered that encoded a functional hybrid ATCase with a molecular weight of 470 kDa. This is similar in size to the *P. putida* enzyme. The hybrid ATCase was inhibited by ATP, CTP and UTP, something never seen for the *Pseudomonas* enzyme, whereas *E. coli* ATCase is inhibited by CTP and activated by ATP. Expression of the hybrid ATCase was reduced four-fold by growth in uracil. This suggests attenuation since the recombinant *pyrB* gene contains the promoter and part of the *pyrB* from *E. coli* and the rest from *Pseudomonas pyrB*.

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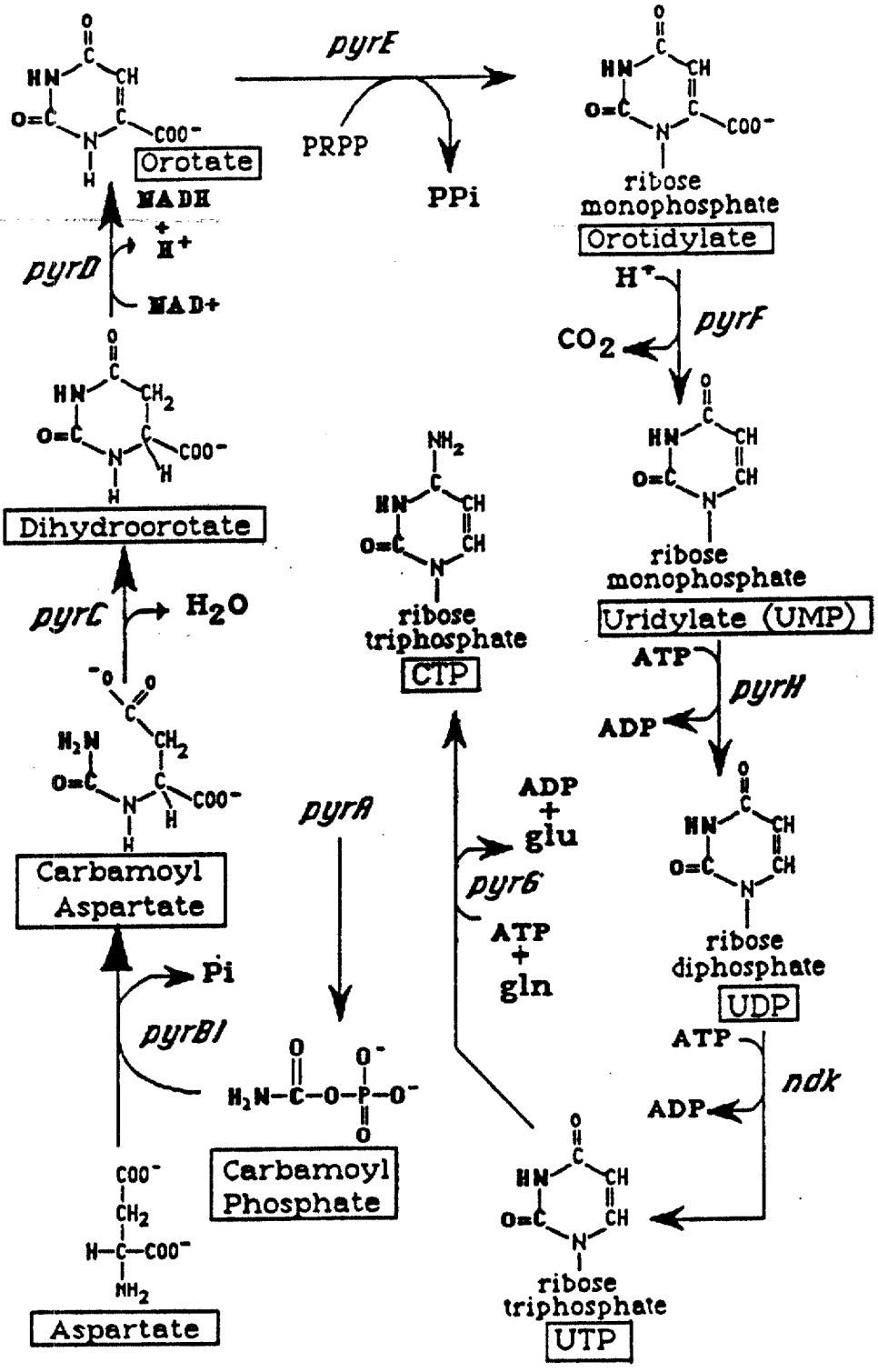
INTRODUCTION

The *de novo* pathway for pyrimidine biosynthesis is universal and virtually identical in all organisms. It has been dutifully studied in bacteria (6, 7, 15, 22, 68, 72, 73, 74), in fungi (11, 36), and in mammals (21, 43). Although the sequence of events is similar, the regulation of the pathway differs between organisms. The pathway, in general, is controlled at the level of enzyme activity by feedback inhibition and activation, and at the level of enzyme synthesis by repression and attenuation. Aspartate transcarbamoylase of *Escherichia coli* is the epitome of a regulatory enzyme, exhibiting all the hallmarks of allosteric kinetic behavior. It is for this reason that the enzyme in *E. coli* has captured the spotlight of many studies for a number of years. Thus, ATCase has emerged as one of the best characterized bacterial enzymes to date. ATCase from *E. coli* is discussed in full, so that it can be further compared and contrasted to the enzyme from *Pseudomonas putida*.

Pyrimidine Pathway. In *E. coli* K-12, six consecutive, unlinked genes and small operons designated as *carAB*, *pyrBI*, *pyrC*, *pyrD*, *pyrE*, and *pyrF* encode the six enzymes of the pyrimidine pathway (Figure 1). This pathway is responsible for the synthesis of UMP, which is a precursor of all

Figure 1. The pyrimidine *de novo* pathway. *pyrA* = carbamoylphosphate synthetase, *pyrB* = aspartate transcarbamoylase, *pyrC* = dihydroorotase, *pyrD* = dihydroorotate dehydrogenase, *pyrF* = orotidylate decarboxylase, *pyrG* = cytidine triphosphate synthetase, *pyrH* = uridylate kinase, *ndk* = nucleoside diphosphate kinase, PRPP = 5-phosphoribosyl-1-pyrophosphate.

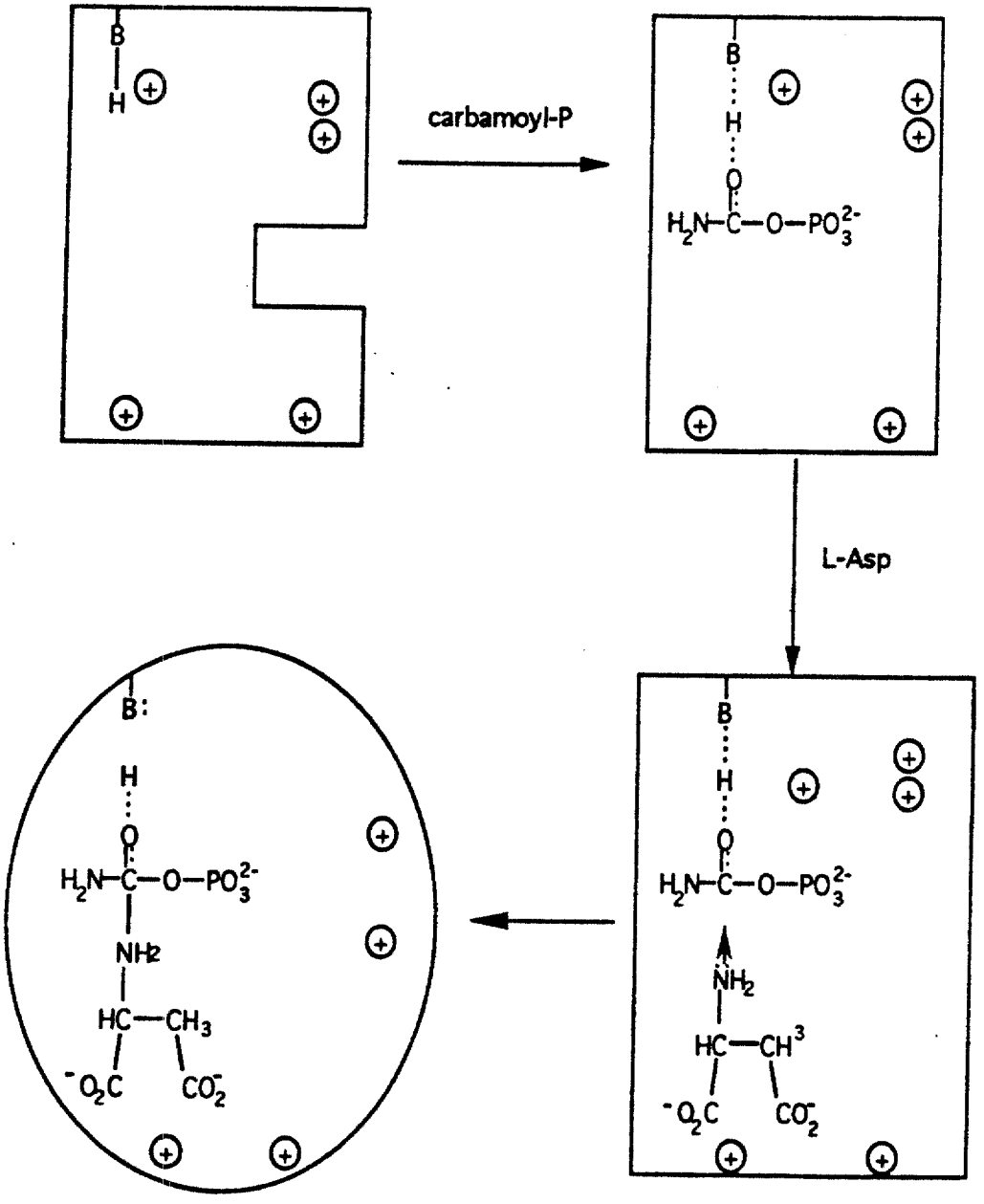

pyrE
PRPP
PPi
Orotate
NADH
H+
pyrD
NAD+
Dihydroorotate
pyrC
H2O
Carbamoyl Aspartate
Pi
pyrBI
Aspartate
pyrA
Carbamoyl Phosphate
ribose monophosphate
Orotidylate
H+
CO2
pyrF
ribose monophosphate
Uridylate (UMP)
ATP
ADP
pyrH
ribose diphosphate
UDP
ATP
ADP
ndk
ribose triphosphate
UTP
ADP + glu
pyrG
ATP + gln
ribose triphosphate
CTP

pyrimidine ribonucleotides (46). The pathway also provides the precursors for deoxyribonucleotide biosynthesis, leading to the formation of dCTP and ultimately, dTTP (49).

The first enzyme of the pathway, encoded by the operon *car*AB, is carbamoylphosphate synthetase (CPSase). In the enteric bacteria, it functions in two biosynthetic pathways--pyrimidine and arginine. CPSase couples the cleavage of two molecules of ATP to the formation of one molecule of carbamoylphosphate from bicarbonate and ammonium or glutamine (1) Synthesis of this enzyme is controlled by cumulative repression by both arginine and a cytidine nucleotide, both of which are end products of their respective pathways (40, 51).

The enzyme catalyzing the first reaction unique to pyrimidine biosynthesis is encoded by the second operon, *pyrBI* (13, 66). This enzyme, aspartate transcarbamoylase, is involved in the carbamoylation of the amino group of carbamoylphosphate to produce carbamoylaspartate. Early experiments and NMR studies (57) allowed Jacobson and Stark (27) to predict that the reaction involved the interaction of certain residues on the enzyme with the carbonyl group of carbamoylphosphate, enhancing the polarization of the latter, thus favoring its nucleophilic attack by the amino group of aspartate (Figure 2). The synthesis of ATCase is controlled

Figure 2. Diagramatic representation of the nucleophilic attack of the amino group of aspartate upon the carbonyl group of carbamoylphosphate, all of which is facilitated by ATCase.


carbamoyl-P
L-Asp

by attenuation, and possibly repression, while its activity is controlled by allosteric inhibition and activation. Because ATCase is the focus of this paper, these two mechanisms are covered later in greater detail.

Carbamoylaspartate is next cyclized with the elimination of water to form dihydroorotate. This reaction is catalyzed by the enzyme dihydroorotase (DHOase), which is coded by the *pyrC* gene. The product of this reaction is then oxidized to orotate, with oxygen serving as the external oxidant. This reaction is catalyzed by dihydroorotate dehydrogenase, encoded by the *pyrD* gene. Orotate then combines with the phosphoribosyl group of 5-phophoribosyl-1-pyrophosphate (PRPP) to form orotidine-5-monophosphate (OMP), the first pyrimidine nucleotide produced. This reaction is catalyzed by orotate phosphoribosyltransferase, which is encoded by the *pyrE* gene. Next, OMP is decarboxylated to uridine-5-monophosphate (UMP) by the enzyme OMP decarboxylase, encoded by *pyrF*. Highly specific UMP kinase, encoded by *pyrH*, catalyzes the phosphorylation of UMP to UDP, using ATP as the source of phosphate. Uridine-5-diphosphate is further phosphorylated to UTP by the enzyme nucleoside diphosphokinase, a non-specific diphosphate kinase encoded by *ndk*. The final step, the production of CTP from UTP, is catalyzed by the enzyme CTP synthetase, encoded by *pyrG*. In this reaction, glutamine serves as the amino donor.

Expression of the *pyrBI* operon, together with the *pyrE* and *pyrF* genes, is repressed by a uridine nucleotide, whereas, the expression of *carAB*, *pyrC* and *pyrD* is repressed by a cytidine nucleotide. Thus, nucleotide repression serves to regulate the flow of the pyrimidine pathway at the level of enzyme synthesis. Also, the *pyrE* gene possesses an attenuator region very similar in structure and regulation to that of *pyrBI* (45) which serves as another control mechanism. It is clear that *pyrBI* occupies an important position in the regulation of the pyrimidine pathway, and it is for this reason that ATCase has been chosen for this study.

***E. coli* ATCase.** The catalytic activity of ATCase is dependent on the formation of an active site from half-sites and separate subunits (30, 56, 23). In essence, each active enzyme is a trimeric protein containing three active sites. Secondly, *E. coli* and the enteric bacteria contain a second polypeptide, with regulatory properties only ,which efficiently binds nucleotide effectors (ATP and CTP) when regulating enzymatic activity.

The *pyrBI* genes lie adjacent to one another and are expressed coordinately. The *pyrB* gene encodes the catalytic (c) polypeptides, while the *pyrI* gene encodes for regulatory (r) polypeptides. These c and r chains have molecular weights of 33 and 17 Kd, respectively (4). They are expressed in the order: promoter, leader, catalytic gene,

and regulatory gene. Together, these polypeptide chains form a dodecamer, containing two trimeric (c_3) catalytic subunits and three dimeric (r_2) regulatory subunits (Figure 3). The dodecameric form is written in shorthand, as follows: $2c_3:3r_2$. For illustrative purposes however, the enzyme may be schematically drawn by relating the three c chains of each C subunit on a three-fold axis, while relating pairs of c chains in the two C subunits and pairs of r chains within the three R dimers on three two-fold molecular axes (Figure 4).

The structural elucidation of ATCase from *E. coli* stems largely from the contributions of Lipscomb's laboratory. The three-dimensional structure of the enzyme is now known with a resolution of 2.5 Å (23, 31). The enzyme is known to extend 50 Å from its center, along a three-fold axis and approximately 75 Å along the two-fold molecular axes, in the direction of the regulatory chains.

The unliganded native ATCase was once considered to have only three types of subunit contacts, c:c, r:r, and c:r. Crystallographic studies, however, indicate that the contacts between enzyme subunits are much more extensive than were previously thought. For instance, not only are there intrasubunit c:c contacts (c1-c3), but there also lies intersubunit c:c contacts (c1-c4) between opposite catalytic subunits. In addition, the c:r contacts are found to be of two types. The first of these (c1-r1) is found between the

Figure 3. Schematic diagram showing the dodecameric structure of *E. coli* ATCase.

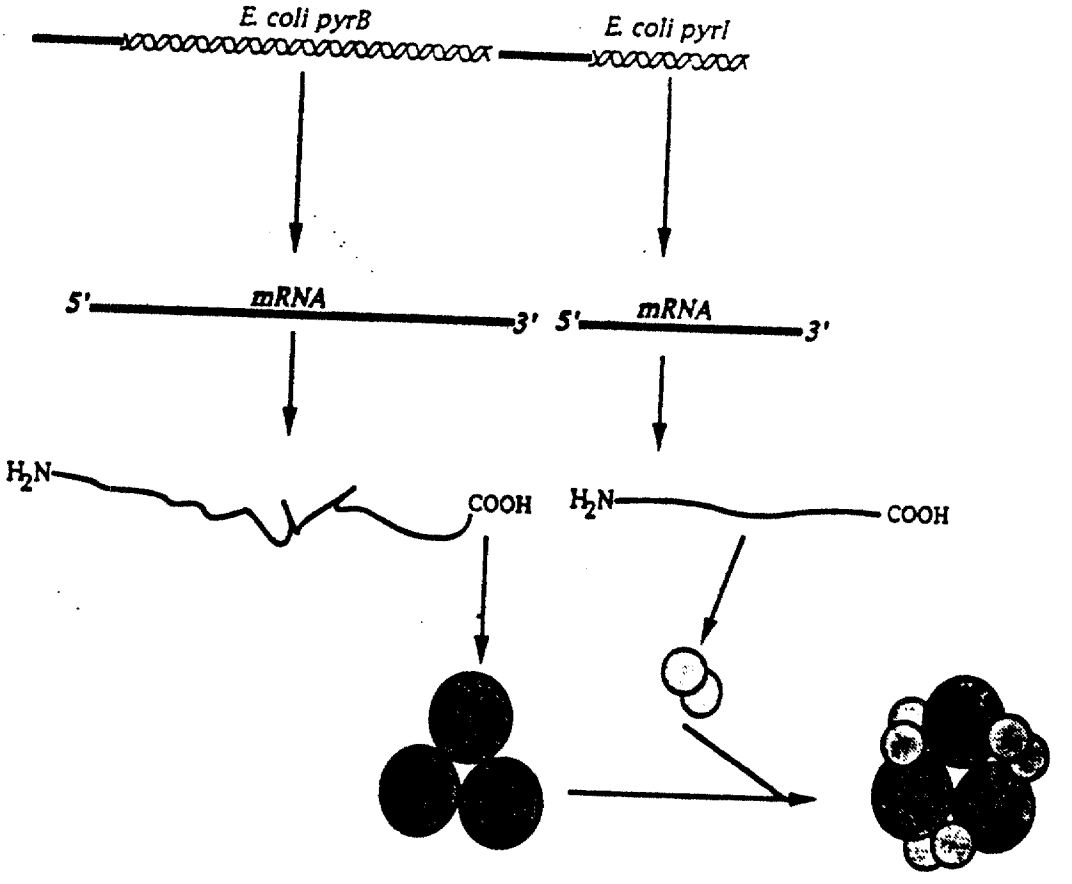
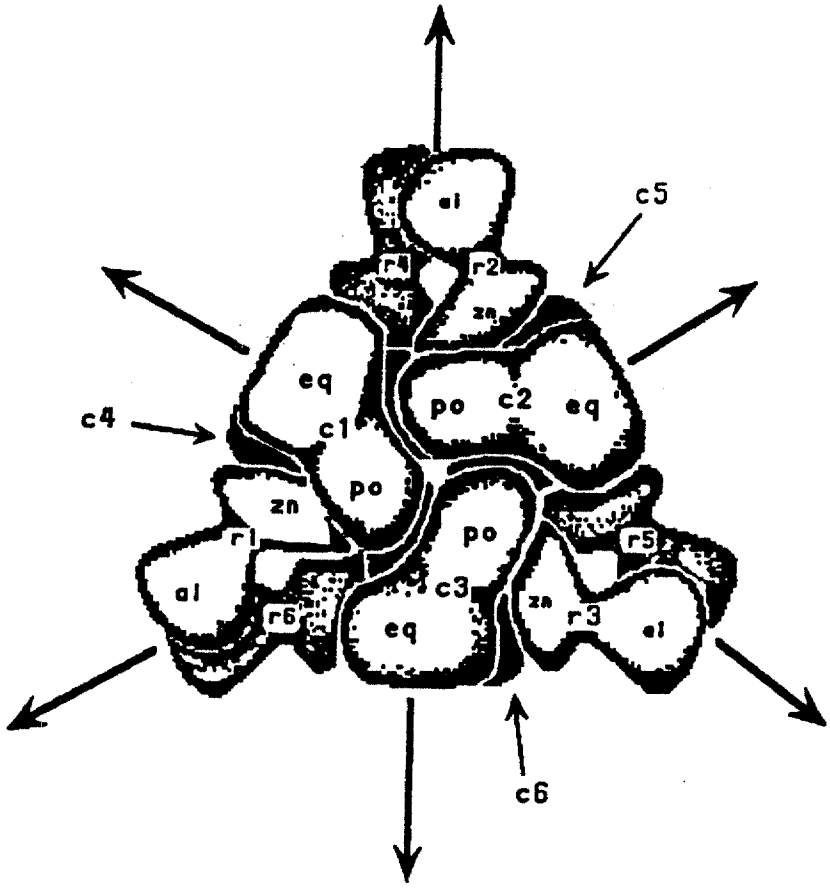

E. coli pyrB
E. coli pyrI
5'
mRNA
3'
5'
mRNA
3'
H2N
COOH
H2N
COOH

Figure 4. Quaternary structure of the *E. coli* ATCase. Adapted from Krause *et al* (34).

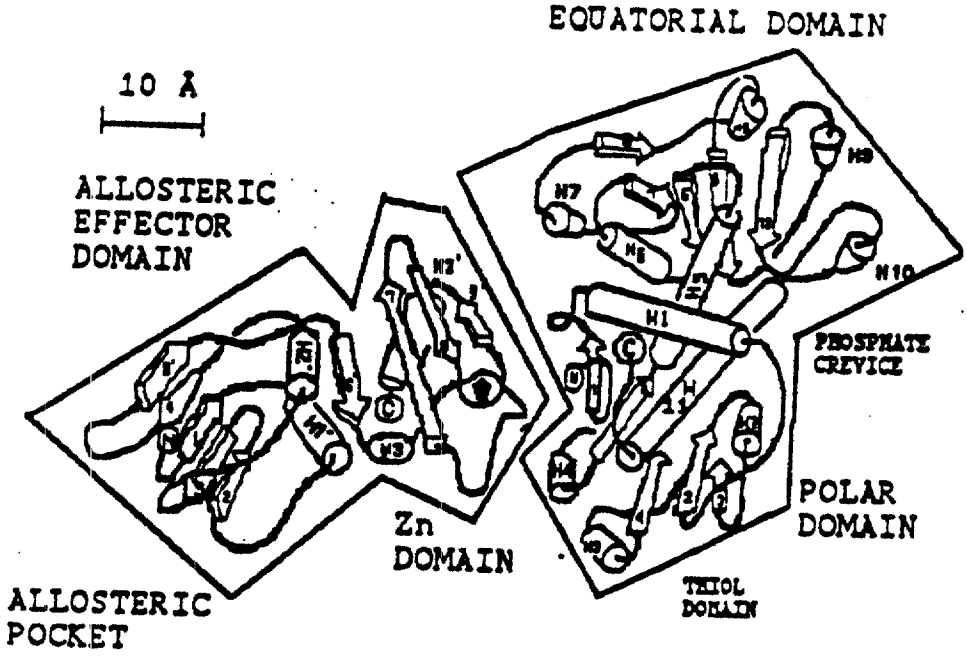

al
r4
r2
zn
c5
eq
po
c2
eq
c4
c1
po
zn
r1
po
r5
al
r6
c3
zn
r3
eq
al
c6

polar domain of a catalytic chain and the zinc domain of an adjacent regulatory chain, while the second (c1-r4) lies between the equatorial domain of the same catalytic chain and the zinc domain of a second regulatory chain (3).

The amino acid sequence of the two polypeptide chains is known in *E. coli*. The catalytic chain contains two tryptophan residues, as well as a cysteine near the catalytic site of the enzyme. This particular chain is characterized by an entanglement of alpha helices, accompanied by two domains, termed polar and equatorial, which correspond to the Amino (N) and carboxy (C) -terminal regions of the c chain (Figure 5) (23). Carbamoylphosphate binds between two c chains in such a way as to interact with the polar domains of both (35, 34, 54, 65). L-Aspartate, on the other hand, interacts with groups in the equatorial domain of a single chain, with the exception of Lys 84. The actual catalytic site is found sequestered between the interfaces of two neighboring catalytic chains of the same trimer, in what is said to be the "phosphate crevice."

The corresponding domains of the r chains are the allosteric and zinc domains, which again correspond to the N- and carboxy (C)-terminal regions, respectively (18). The primary nucleotide binding site is found in the N-terminal region of consistent with the amino acid sequence of the regulatory chain, which reveals a cluster of four cysteine

Figure 5. Tertiary structure of the *E. coli* ATCase showing one catalytic subunit and one regulatory subunit. Adapted from Krause *et al* (34).


EQUATORIAL DOMAIN
10 Å
ALLOSTERIC
EFFECTOR
DOMAIN
Zn
DOMAIN
PHOSPHATE
CREVICE
POLAR
DOMAIN
THIOL
DOMAIN
ALLOSTERIC
POCKET
REGULATORY
SUBUNIT
CATALYTIC
SUBUNIT

residues in the C-terminal region. It is this association of the metal ion to these residues that is an absolute requirement for the attachment of and virtual communication between the catalytic and regulatory subunits

Hybrids. The native holoenzyme can be dissociated by treatment with heat (56) or organomercurials, such as neohydrin (69) or parachloromercuribenzoate (16). These chemicals displace the zinc ions between the regulatory and catalytic subunits, causing the enzyme to fall apart into its constituent R and C subunits. Because it is possible to obtain individual active subunits, and then to reconstitute the enzyme, with properties indistinguishable from those of the original enzyme, it is also possible and quite advantageous to create several kinds of hybrid ATCase molecules.

One such hybrid enzyme was composed of catalytic subunits made of both active and inactive catalytic chains (19). These molecules were reconstituted to contain two (out of a possible six) active chains, with these two chains located either in the same or different catalytic subunits. The results of this very elegant experiment suggested that the two catalytic trimers do not inhibit interactions or cooperativity between all six catalytic sites (19).

Further, interspecific hybrids were formed from members of the family *Enterobacteriaceae*, by simply dissociating

their constituent catalytic and regulatory subunits and then reforming them into hybrid enzymes (48, 14). Of particular interest and importance, is the construction of two hybrid enzymes, formed from the purified catalytic chains derived from either *E. coli* or *Serratia marcescens*, and added to the purified regulatory chains derived from the other species. It should be noted that ATCase in *E. coli* is inhibited by CTP and activated by ATP, while the enzyme in *S. marcescens* is activated by both effectors. If the resultant ATCase enzyme contained the regulatory subunit from *S. marcescens*, the enzyme was found to be activated by both CTP and ATP, as displayed by the *Serratia* native enzyme. Moreover, if the regulatory subunit in the hybrid was derived from *E. coli*, the new enzyme was found to be inhibited by CTP and activated by ATP. Again, this effector response matches that of *E. coli*'s native ATCase enzyme. It was therefore concluded that heterotropic responses of the enzyme are dependent upon the origin of the regulatory subunits (58)(5).

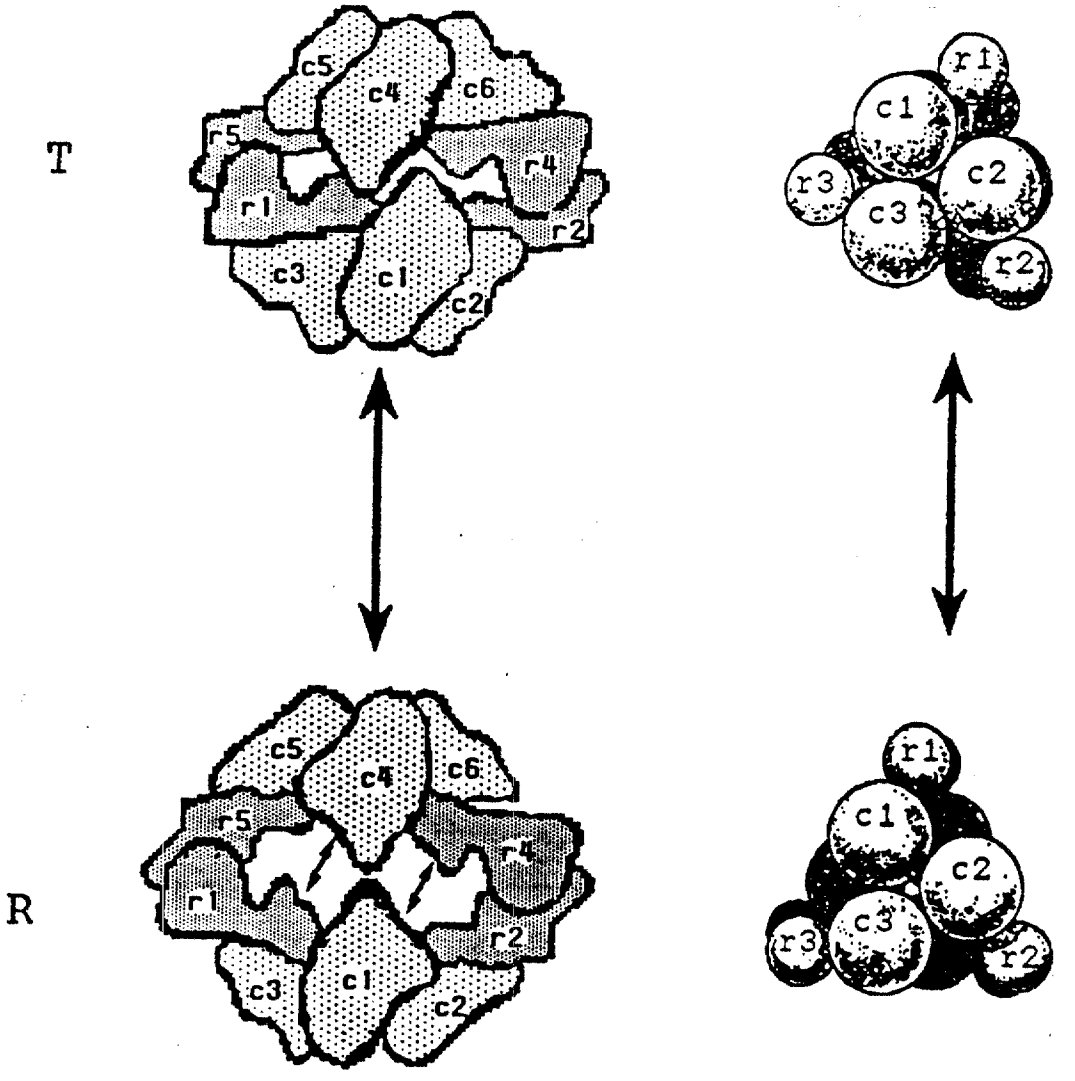
More recently, Houghton *et al* created a chimeric enzyme genetically, in which the carbamoylphosphate binding site of ornithine transcarbamoylase (OTCase) was fused to the aspartate binding site of *E. coli* ATCase. OTCase, encoded by the sixth enzyme of the arginine pathway, catalyzes the carbamoylation of the amino group of ornithine, in a reaction very similar to that of ATCase. The carbamoylphosphate

binding domains of OTCase and ATCase also show highly similar amino acid sequence homology. This enzyme was created by the gene fusion of *argI* and *pyrB in vitro* at a common *Bst*EII cleavage site. Complementation of uracil-requiring *E. coli pyrB* mutants using plasmids containing this created enzyme was successful.

Analysis of the amino acid sequence homology of the ATCases from bacteria to mammals, reveals that there is a homology range of 30 to 60 percent (42). Using this degree of sequence homology, Wild *et al*, fused genic regions to form a chimeric enzyme, composed of *E. coli* (aspartate binding domain) and hamster (carbamoylphosphate binding domain) (24, 64). Again, complementation of *E.coli* pyrimidine auxotrophs by this chimeric enzyme was observed (25).

R and T states. Aspartate transcarbamoylase is found to exist in two forms: a taut conformation (T-state), which has a low affinity for aspartate, and the relaxed conformation (R-state), which has a high affinity for aspartate (Figure 6)(3). Transitions from the taut to the relaxed enzymatic structure involve the simultaneous breaking of interface localized bonds, and the formation of others at different interface sites (29, 32, 38, 37). That is, the binding of carbamoylphosphate to the enzyme triggers a change in the tertiary structure, mainly at the interfaces between the zinc and allosteric domains within the r chain and at the

Figure 6. Schematic representation of the T to R transition of the *E. coli* ATCase. c = catalytic chain, r = regulatory chain. Adapted from Gouaux *et al* (20).


T
R
c5
c4
c6
r5
r4
r1
r2
c3
c1
c2
r1
c1
r3
c2
c3
r2

subunit interfaces at c1-c4 and c1-r4. Next, L-aspartate binds with an almost simultaneous formation of new bonds between both the polar and equatorial domains in the c chain, and the c1-r1 and c1-c2 interfaces between subunits. Accompanied by this sequence of events, is the movement of the c1-c4, c1-r4, and zinc-allosteric interfaces.

Regulation. The regulation of ATCase in *E. coli*, as mentioned previously, is through allosteric inhibition by CTP and activation by ATP. The binding of ATP and CTP to the regulatory sites was shown to be anticooperative, in that they compete for a single site on the regulatory subunit (59, 10, 67, 61). In addition, UTP has recently been shown to work in synergy with CTP, amplifying the inhibitory effects of CTP (12). A primary-secondary effects model has been used to interpret the heterotropic interactions involved in the binding of these effectors to the enzyme. Here, the nucleotides act by altering the affinity of the catalytic site for aspartate via local conformational changes. This model accounts for the fact that ATP reverses the effect of CTP and vice-versa (62). For instance, when ATP binds to the regulatory site, it promotes a local conformational change that increases the affinity for aspartate to the catalytic site. This is said to be the primary effect. The secondary effect occurs when in the presence of aspartate. Here, the conformational changes made by ATP increase the level of

saturation of the catalytic sites by aspartate. This increases the level of saturation of the catalytic site by aspartate and shifts the T and R equilibrium towards the R form. The opposite occurs when CTP binds to a regulatory site. Once again, CTP promotes a local conformational change that decreases the affinity of aspartate for the catalytic site. In the presence of aspartate, these conformational changes decrease the saturation of the catalytic sites for this substrate and shift the T and R equilibrium torward the T form. This interplay serves to balance the production of purine and pyrimidine ribonucleoside triphosphates within the cytoplasm of a bacterial cell.

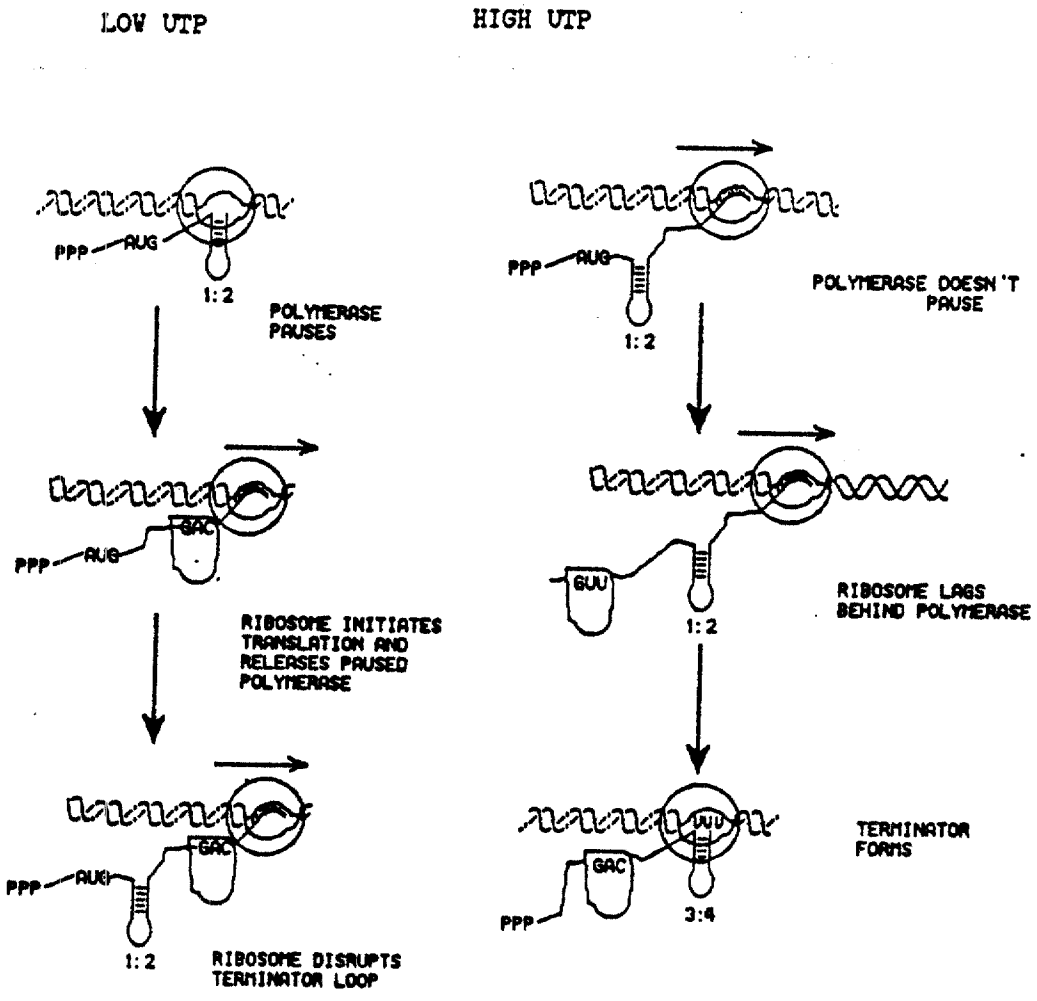
Attenuation. Upon sequencing the upstream region, including the promoter region of the *pyrBI* operon (44, 55, 63), several putative regulatory elements were identified. Included in these was a *rho*-independent termination or attenuator region located only 23 base pairs before the structural gene of *pyrB*. Transcription initiated at the *pyrBI* promoters is efficiently (98%) terminated at this site. If termination does not occur at the attenuator, the leader transcript encodes a 44-amino acid polypeptide which terminates three nucleotides before the start of the ATCase sequence (55, 44, 63).

A strong transcriptional pause site was also found approximately 20 base pairs upstream from the attenuator.

This region encodes a sequence in the transcript which is rich in uridine. Thus, when levels of UTP are low (conditions of high gene expression), the transcribing RNA polymerase pauses at the attenuator region which is a GC-rich region of dyad symmetry, followed by a poly A sequence, requiring UTP for the extension of the mRNA. A translating ribosome then overtakes the pausing RNA polymerase, thereby precluding the formation of the terminator stem and loop structure necessary for transcriptional termination. However, when the level of UTP is high (conditions of low gene expression), the RNA polymerase does not pause and the terminator stem and loop structure is formed before the ribosome can overtake the polymerase (Figure 7). Thus, it is the pausing of the RNA polymerase in the pyrimidine genes that is important in attenuation (regulation at the level of transcription), rather than the stalling of the ribosome, as found in the amino acid genes (regulation of the level of translation)(28).

Enzyme Kinetics. In *E. coli*, ATCase displays cooperative sigmoidal kinetics for substrates, aspartate and carbamoylphosphate, when the initial velocity is plotted against the substrate concentration. (17, 8). The K_m for aspartate is found to be 5.5 mM, whereas the K_m for carbamoylphosphate is 0.2 mM. Cytidine triphosphate (CTP) is found to enhance this sigmoidicity for both the aspartate and

Figure 7. Diagram of the attenuation mechanism in *E. coli*. Adapted from Yanofsky (71).


LOW UTP
HIGH UTP
PPP
AUG
1:2
POLYMERASE PAUSES
GAC
RIBOSOME INITIATES TRANSLATION AND RELEASES PAUSED POLYMERASE
AUU
RIBOSOME DISRUPTS TERMINATOR LOOP
POLYMERASE DOESN'T PAUSE
GUU
RIBOSOME LAGS BEHIND POLYMERASE
UUU
3:4
TERMINATOR FORMS

carbamoylphosphate curves. The catalytic subunit alone displays simple Michaelis-Menten kinetics at low concentrations, when the carbamoylphosphate concentration is varied with limiting aspartate (8, 52). However, no cooperativity is observed when there are very low concentrations of aspartate in half or fully saturating carbamoylphosphate. Also, in low concentrations of aspartate, the catalytic subunit was four times as active as the holoenzyme. This four-fold activity was lost when the regulatory subunit was added in reconstitution studies.

Enzyme Classification. In 1969, Bethell and Jones identified three broad classes of bacterial ATCases, which are listed in the following table.

Table 1. Classification of Bacterial ATCase Enzymes

	Class A	Class B	Class C
Molecular weight (MW)	360 kDa	300 kDa	100-140 kDa
Substrate saturation curves in absence of nucleotide	Hyperbolic	Sigmoidal	Hyperbolic
pyrimidine effector response	Response UTP^-. CTP^-	Response CTP^-	No response
Heat and organomecural treatment	No dissociation	Dissociation	---------

The characteristics of these ATCases corresponded to three different major classes of bacteria. The Gram negative facultative anaerobic bacteria, such as *E. coli*, possess a class B ATCase, which are dodecameric in form, with a molecular weight of approximately 300 kDa, and sigmoidal substrate kinetics. Gram positive bacteria, such as *Bacillus*, have typical class C enzymes, with a molecular weight of 100 kDa and hyperbolic kinetics (7, 9, 47). These enzymes consist of catalytic trimers, corresponding to the unregulated hyperactive form of *E. coli*, with no regulation by nucleotides.

The Class A ATCases were found in *Pseudomonas*, with a much larger molecular weight at 360 kDa and Michaelis-Menten kinetics (2, 7, 47). The *Pseudomonas* holoenzyme was inhibited by all triphophates, CDP, pyrophosphate, and orthophosphate at limiting concentrations of carbamoylphosphate. In 1972, Adair and Jones showed that the ATCase from *P. fluorescens* was composed of two identical subunits, with a molecular weight of 180 kDa. They were not, however, successful in uncoupling catalytic activity from regulatory functions. Based on this information, the ATCase from *Pseudomonas* has long been thought to be encoded by one gene, and therefore assumed to be composed of fused catalytic and regulatory chains. Recent studies on *P. putida* and *P.*

aeruginosa, however, show there is no separate regulatory gene, but that the first in the sequence (*pyrB*) solely encodes ATCase, with shared catalytic and regulatory activities. The second gene (*pyrC*, rather than *pyrI*), encodes the third enzyme in the pyrimidine pathway, namely dihydroorotase (M.J. Schurr Ph.D. Dissertation, UNT, 1992).

Not only are the ATCases of *E. coli* and *P. putida* very different in their enzyme kinetics, molecular weight, and gene arrangement, but, as mentioned before, the enzyme in *E. coli* is regulated by an attenuator mechanism, not seen previously in *Pseudomonas*. This can be tested by expressing the *E. coli* enzyme in *Pseudomonas*. One of the aims of this research was therefore to see if attenuation works in *P. putida*.

In attempts to answer this question, the leader, promoter, and part of the *E. coli pyrB* were recombined into a *pyrB* of *P. putida* (detailed below). This region is responsible for the attenuator mechanism found in *E. coli*. Further, because the catalyic and regulatory functions in *P. putida* appear to be encoded by the same gene, efforts to uncouple catalytic and regulatory activities, as in the other hybrid research, would prove to be futile. Therefore, the near 100 percent sequence homology of the carbamoylphosphate binding site between that of the enzyme of *E. coli* and *P.*

putida, served as a most prominent site for targeted homologous recombination.

MATERIALS AND METHODS

Chemicals Tris (hydroxymethyl)-aminomethane (trizma base), kanamycin sulfate, tetracycline hydrochloride, uracil, nitrilotriacetic acid, dithiothreitol, spermidine, the disodium salts of ATP, CTP, UTP, L-dihydroorotic acid, lysozyme chloride from chicken egg white, salmon sperm DNA, blue dextran, 2,3-butanedione monoxime, antipyrine, polyethylene glycol, L-aspartic acid, L-histidine, succinic acid and carbamoylphosphate were supplied by the Sigma Chemical Co. Boric acid (H_3BO_3), EDTA, magnesium chloride ($MgCl_2*6H_2O$), calcium chloride ($CaCl_2$), cobalt nitrate ($Co(NO_3)_2*6H_2O$), ferric sulfate ($FeSO_4*7H_2O$), cupric sulfate ($CuSO_4*5H_2O$), dipotassium phosphate (K_2HPO_4), potassium phosphate (KH_2PO_4), dextrose (glucose), ammonium acetate (CH_3COONH_4), magnesium acetate (CH_3COOMg), potassium hydroxide (KOH), sodium borate ($Na_2B_4O_7 * 10\ H_2O$), ammonium molybdate ($(NH_4)_6Mo_7O_{24} * 4\ H_2O$), magnesium sulfate ($MgSO_4 * 7\ H_2O$), dibasic sodium phosphate (Na_2HPO_4), ammonium sulfate ($(NH_4)_2SO_4$), agar and sodium chloride (NaCl) were provided by Fisher Scientific Co. Ampicillin monosodium salt, 5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X-gal), acrylamide,

agarose, bis-acrylamide, ammonium persulfate, bromophenol blue and cesium chloride were molecular biology grade reagents supplied by IBI. Anhydrous glycerol, glycine, sulfuric acid (H_2SO_4) and zinc sulfate ($ZnSO_4*7\ H_20$) were obtained from J.T. Baker Co. Bacto tryptone, Bacto-yeast extract and MacConkey were purchased from Difco. Xylene cyanol, sodium dodecyl sulfate (SDS) and NNN',N'Tetramethylethylenediamine (TEMED) were electrophoresis purified reagents supplied by BioRad. Isopropyl-B-thio-galactopyranoside (IPTG) was obtained from Bethesda Research Laboratories. Radioactive (γ-^{32}P) ATP, (α-^{35}S) ATP, and [^{35}S] methionine were supplied by DuPont NEN. Manganese sulfate ($MnSO_4$) was purchased from Mallinkrodt. Mineral oil was supplied by Sargent. Ethanol (200 proof), used for DNA precipitations, was obtained from Midwest Solvent Company of Illinois. The sodium salt of dextran sulfate and Sephacryl S-300 high resolution gel matrix were both supplied by Pharmacia. XAR5 X-ray film was bought from Kodak. Restriction enzymes were obtained from all of the following sources: Boehringer Mannheim, BRL, IBI, New England Biolabs and U.S. Biochemicals. The deionized water was distilled through a Corning Megapure System MP-1 and "used as ddH_20" in making solutions for this research.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in the following table.

TABLE 2. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or Reference
Strains		
P. putida		
PpN1137 (pyrB1137)	*pyrB*	B.W. Holloway
2825	*E.coli pyrB* on plasmid pRO-1727 in PpN1137	This study
HES-6	*E.coli pyrBI* on pRO-1727 in PpN1137	This study
E.coli		
DH5α	F⁻ f80d *lacZ* DM15 D(*lacZYA-argF*) U169 *endA1 hsdR17* (r_k^- m_k^+)	BRL

Table 2 continued.

Strain or plasmid	Genotype or description	Source or Reference
	recA1 supE44	
	1- *thi-1 gyrA relA1*	
TB-2	Δ*pyrBI argF*	W. Roof
Plasmids		
pUC19	Ap^r *lacZ* DM15 (70)	
pEK-2	*E.coli pyrBI* in pUC7	E.Kantrowitz
pRO-1727	tet^r, Ap^r, *Pseudomonas* origin of replication	R. Olson
pBJR-28	*E.coli pyrB* in pUC19	This study

The plasmid pEK-2 was obtained from professor Evan Kantrowitz of Boston college. This plasmid contains the *pyrBI* genes of *E. coli*, as well as a multiple cloning site of the ATCase enzyme. The plasmid pBJR-28 contains the *E. coli pyrB* on pUC-19 in DH5α for screening purposes. TB-2, a Δ*pyrBI E. coli* strain, was used to check the activity of the ATCase

enzyme encoded by the *pyrB* gene on pBJR-28. Strain 2825 contains the *E. coli pyrB* in a *Pseudomonas* compatible plasmid, pRO-1727, and transformed into a *P. putida pyrB*$^-$ strain, Ppn1137. pRO-1727 was kindly provided by Ron Olson. HES-6, on the other hand, contains the *E. coli pyrBI* genes on pRO-1727 in PpN1137.

Media and growth conditions. *E. coli* and *P. putida* were grown in Luria-Bertani (LB) broth at 37°C and 30°C respectively with aeration (41). Appropriate antibiotics were added to the medium as follows: cells containing the pUC series plasmids, ampicillin (100 µg/ml), and cells containing pRO-1727, tetracycline (35 µg/ml).

Hutner's "Metals 44 " is incorporated into the preparation of *Pseudomonas* minimal medium. The following ingredients were dissolved, in sequence, in a total of 800 ml ddH_2O in the making of "Metals 44": 2.5 g EDTA (free acid), 10.95 g $ZnSO_4*7\ H_2O$, $FeSO_4*7\ H_2O$, 1.54 g $MnSO_4*H_2O$, 392.0 mg $CuSO_4*5\ H2O$, 251.0 mg $CuSO_4$ (anhydrous), 250.0 mg $Co(NO_3)_2*6H_2O$ or 177.0 mg $Na_2B_4O_7*10\ H_2O$. In order to retard precipitation, a few drops of H_2SO_4 were added to the above solution, which was then brought up to a liter with ddH_2O. The final solution of"Metals 44" appears lime-green in color and can be stored at room temperature for an indefinite period of time. Again, this solution was incorporated into the preparation of the concentrated base used in the

Pseudomonas minimal medium. This solution was prepared in the following manner: nitrilotriacetic acid (20.0 g) was dissolved, separately in 600 ml of water and neutralized with approximately 14.6 g of KOH The other components were dissolved in the following order: 28.9 g $MgSO_4$ (anhydrous), 6.67 g $CaCl_2*7H_2O$, 18.5 mg $(NH_4)_6Mo_7O_{24}*7$ H_2O, 198 mg $FeSO_4*7$ H_2O and 100 ml "Metals 44". The pH was adjusted to 6.8, and the volume was then brought up to one liter. When adjusting the pH of this solution, a precipitate was momentarily formed, but with constant stirring, this problem was overcome. The color of the solution also changed from a deep yellow to a nice straw color when a pH of 6.8 was approached. The final solution can be stored for at least one year over toluene or in the cold. One liter of *Pseudomonas* minimal medium (M9) contained: 25 ml of 0.5 M Na_2HPO_4, 25 ml of 0.5 M KH_2PO_4, 10 ml of 10 percent $(NH_4)_2SO_4$, 10 ml concentrated base and 930 ml of ddH_2O. The basal medium was supplemented with 10 mM succinate as a carbon source (50).

The *E. coli* minimal A medium contained: 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g sodium citrate in one liter. These components were sterilized in 988 ml ddH2O and supplemented with sterile 0.2 percent glucose, 10 µg/ml thiamine, and 1 mM $MgSO_4$ (41).

When growing a *E. coli* or *Pseudomonas* Pyr auxotroph, their respective minimal media were supplemented with uracil

(50 µg/ml). Both media had 1.5 percent agar added to them. If plates were desired, however, the agar, resuspended in 500 ml ddH2O, was autoclaved, while the basal medium was also brought up to 500 ml. The solutions were then mixed after being autoclaved and cooled to 55°C and subsequently poured into plates.

Sterile YT medium, "predispensed" into 5 ml tubes, was used for screening rapid plasmid preparations. YT contained 8 g Bacto tryptone, 5 g Bacto yeast extract and 5 g NaCl per liter.

A "modified" TB medium was found to be of optimal composition for bulk isolation of plasmid DNA. The original TB medium contained 12 g Bactotryptone, 24 g Bacto-yeast extract, 0.04 percent glycerol and 2.3 g KH_2PO_4 and 12.5 g K_2HPO_4 per liter (33). The modified medium contained increased amounts of Bactotryptone and Bacto-yeast extract, identical to the amounts called for in the making of LB medium. In extact quantification, this medium contained 10 g Bactotryptone, 5 g Bacto-yeast extract, 10 g NaCl, 4 ml 100 percent glycerol, 2.3 g KH_2PO_4 and 12.5 g K_2HPO_4 per liter. In preparing this medium, it was necessary to separately autoclave and dissolve KH_2PO_4 and K_2HPO_4 in 100 ml ddH_2O, while the rest of the components were likewise separately dissolved in 900 ml ddH_2O. The ingredients were thereafter mixed after having been cooled to 25°C. With this modified

medium there was a 10-fold increase in plasmid yield, which was approximately 10 mg plasmid DNA per liter. TB, on the other hand, gave only a 5 to 7-fold increase in plasmid yield per liter (60).

The cells were then grown at the appropriate temperature in a New Brunswick model G25 incubator shaker.

Genetic Manipulations. Restriction digests were performed according to the manufacturer's specifications (BRL, USB, New England Biolabs). The digests were incubated in a Precision GCA water bath at each enzyme's appropriate temperature. Completion and/or success in cutting the DNA was further verified by loading 1.5 µl of the DNA sample on an 0.8 percent agarose gel (see detailed protocol)

Restriction enzymes were removed by phenol extraction and followed by an ethanol precipitation. Ligations were set up in accordance with the parameters calculated and set by MacLigate, a Macintosh free-ware computer program. After phenol extracting and ethanol precipitating the vector and target DNA, the ligation was set up using a sterile 0.5 ml microfuge tube and by adding the following contents: 1) the vector DNA and target DNA fragments in a ratio of 3 moles to one, respectively, with a total concentration of no more than 0.5 µg total DNA. The optimal concentration was found to be 1 µg total DNA/ml; *e.g.* 20 µl total volume of ligation is equal to 20 ng total DNA in ligation, 2) 1 µl of T4 DNA

Ligase (approximately 1 Weiss unit of enzyme), 3) 4 µl of 5X ligation buffer, consisting of 250 mM Tris-HCl (pH 7.6), 25.0 percent (w/v)PEG 8000, 50.0 mM magnesium chloride, 5.0 mM ATP, and 5.0 mM DTT, 4) sterile ddH_2O up to a total of 20 µl. The actual stock 5X DNA ligation buffer was made with all of the components listed above, with the exception of ATP and DTT. The ATP (20 mM) and DTT (20 mM) were prepared and stored separately at -20°C, and were added to the ligation prior to incubation. The stock ligation buffer was stored at -20°C for an extended period of time. The ligation mixture was incubated at room temperture (23-26°C) overnight, and then diluted with an equal volume of TE buffer (10 mM tris, 1 mM EDTA, pH 8.0) before being added to competent cells the following day. This ligation mix can be stored at 4°C for short periods of time or at -20°C for longer time periods.

Transformations were performed according to the method of Huff *et al* (26). *E. coli* cells were grown to an absorbance of 0.5-0.7 at a wavelength of 550nm. Next, the cells were and transferred to a sterile 50 ml conical tube and put on ice for 20 minutes. They were centrifuged for 5 min at 4°C at 3000 rpm in a table-top Sorvall model RT6000B. The supernatant was then decanted, while the pellet finally resuspended in 25 ml of cold sterile, 0.1 M $MgCl_2$. The cells were centrifuged once again, and the supernatant decanted, while the pellet was resuspended in 5 ml of iced, sterile 0.1

M $CaCl_2$. The cells were then placed on ice for a minimum of 2 hours. Next, 200 µl of cells were removed and added to 10 µl of diluted ligation mix in a microfuge tube. This mixture was placed on ice for 40 min and subsequently heat shocked for 2 min at 42°C. After this incubation, 800 µl of YT broth were added to the samples which were then grown for 40 min on a shaker incubator at 37°C to allow expression of the antibiotic resistance gene. Finally, 100 µl of these cells were plated onto selective medium. For instance, cells that were transformed with pUC plasmid derivatives were plated onto YT medium supplemented with 100 µg/ml ampicillin, IPTG, and X-gal. Occasionally cells were plated onto MacConkey medium supplemented with 100 µg/ml ampicillin and IPTG instead of using X-gal. The cells that were not transformed were kept at 4°C for up to one month. After 3 days of storage, an outgrowth step was no longer necessary.

Rapid Plasmid Preparation The screening of recombinant plasmids was done by extracting plasmids using a plasmid preparation method ("rapid prep"). An aliquot of 1.5 ml of overnight culture was centrifuged for 1 min in a microfuge at 10,000 x g to pellet the cells. The supernatant was decanted leaving 50-100 µl of medium with the cell pellet, and then vortexed at high speed to resuspend the cells. The disruption of the cells was done by the addition of 300 µl of TENS (10 mM Tris, 1 mM EDTA pH 8.0, 0.1 N NaOH

and 0.5 percent SDS made fresh each time), vortexed 2-5 s until the mixture became sticky. The chromosomal DNA and cellular debris were precipitated by the addition of 150 µl of 3.0 M sodium acetate pH 5.2 and vortexed for 2-5 seconds. The chromosomal DNA and cellular debris was pelleted for 2 min at 10,000 x g in a microfuge. The supernatant was transferred to fresh tubes and mixed well with 0.9 ml of 100 percent ethanol that has been cooled to -20°C.The tubes were spun for 2 min to pellet plasmid DNA and RNA. The supernatant was discarded and the pellet rinsed twice with 1 ml of 70 percent ethanol. The pellet was dried in a Savant speedvac for 2-3 min and resuspended in 20-40 µl of TE buffer or distilled water for further use. Restriction digestion of the plasmids required elimination of the RNA and this was accomplished by the addition of 1 µl of RNase (10 mg/ml) to the DNA and incubation for 1/2 hour at 37°C. At this point the DNA was clean enough to be electrophoresed through a 0.8 percent agarose gel or cut with a restriction enzyme.

Agarose Minigel Electrophoresis Agarose minigel electrophoresis was used to check the completion of restriction digests, as well as the presence of plasmids in cloning experiments. The ends of an acrylic gel tray were closed by using masking tape as a barrier. A well former or comb was placed at one end of tray, in order to form wells

for the containment of samples. An agarose solution was poured into the tray until it just covered the area of the tray (approximately 75 percent of the depth of the tray). A 1 percent w/v agarose solution was made as follows: 0.8 g electrophoresis grade agarose, 20.0 ml 5X TBE Agarose Gel Electrophoresis Running Buffer and 80.0 ml distilled water. The solution was then placed into an Erhlenmeyer flask that had been previously tare weighted. The flask was then placed in a microwave oven for approximately 2 min, or until the agar had completely melted. The solution in the flask was swirled occasionally. The flask was weighed and distilled water was added to compensate for evaporation of liquid accounted for in the original mass. The solution was allowed to cool (gel) for approximately 45 min, or until it was completly solidified. The well former was removed carefully, so as not to tear the gel. The gel tray, with solidified agarose, was placed in the electrophoresis tank. The tank was filled with single strength TBE agarose gel running buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) until the level was 1-2 mm higher than the top of the gel. The DNA is mixed with 5X agarose gel electrophoresis loading buffer (25.0 glycerol, 0.5 percent SDS, 0.1 percent bromophenol blue, 0.1 percent xylene cyanol and 50.0 mM EDTA), and the digest was loaded into a gel slot. A molecular weight marker of *Hin*DIII cut-Lambda DNA was usually

loaded into the first lane. Electrophoresis was usually done at 60 volts for about 1 hour. The gel was then stained in ethidium bromide at 0.5 µg/ml and then destained by submerging in water for 5 minutes. Destaining removed the residual ethidium bromide from the gel. This particular step was repeated for longer periods of time if background levels were too high. The gel was examined under a UV lamp.on a Fotodyne transilluminator. Successful gels were photographed using a Polaroid MP-4 camera, with the f-stop set on 4.5, and a 1 min exposure. Polaroid type 55 film was used because a negative is generated with this film. This was convenient for generating additional pictures for the use in figures.

Recovery of DNA from Agarose Gels by Electroelution. When a specific restriction fragment from a plasmid was subcloned, it was often necessary to separate that piece from other DNA fragments. This was accomplished by digesting the plasmid with the necessary restriction enzyme(s), running the fragments out on a agarose gel (0.8 percent), staining the gel to visualize the fragment and then cutting the DNA out of the gel. There are many methods to remove DNA from agarose, but electroelution was found to be the most efficient. Using a narrow spatula, the small piece of agarose, containing the DNA of interest, was pushed into an approximately 6 inch length of 10 mm dialysis tubing. A dialysis bag clip was placed at one end of the bag.

Approximately 500 µl of 0.25X TAE buffer was pipetted inside the dialysis tubing. The open end of the dialysis tubing was secured with another clip. The dialysis bag was placed into a horizontal agarose gel electrophoresis unit which had been partially filled with 0.25X TAE buffer. The level of buffer was adjusted until it just covered the dialysis tubing and electroelution was carried out for 3 hours at 80 volts. Upon completion of electroelution, the current was reversed for 15 s by switching the red and black leads. Before the dialysis bag was opened,the excised gel slice was moved around against the sides of the dialysis bag to further aid in dislodging the DNA. Using a siliconized Pasteur pipette, the TAE buffer was removed from the dialysis tubing and transferred to a microfuge tube. The gel slice was then rinsed in the dialysis tubing with 100 µl of 0.25X TAE buffer. The rinsed contents were transferred to microfuge tube with a Pasteur pipette. The eluate in the tube was phenol-extracted twice to help remove contaminating agarose debris. This was done by adding 1 volume (typically about 500 µl) of phenol to the microfuge tube and vortexing well and centrifuging at 10,000 rpm for 1 minute. The phenol (the bottom layer) was removed with a drawn out pipette. Then 1 volume (500 µl) of ether was added to the microfuge tube which was vortexed briefly and centrifuged at 10,000 x g for approximately 10-15 seconds. The ether (upper layer) was

removed under the hood using a drawn out Pasteur pipette. The residual ether was allowed to evaporate with the tube open at room temperature for a few min. The supernatant was then transferred to another microfuge tube with a drawn out Pasteur pipette, and the tube was centrifuged at 10,000 x g for 5 minutes. The supernatant was transferred to a third microfuge tube and the DNA was concentrated in a Speed Vac Concentrator to reduce the total volume to about 200 µl. The DNA was then ethanol-precipitated by the addition of 0.1 volume of 3 M sodium acetate and 3 volumes of 100 percent ethanol. The tube containing the DNA was vortexed and placed on dry ice for approximately 10 min and was centrifuged for 15 min at 4°C in a microfuge. The supernatant was removed with a drawn out Pasteur pipette, 1 ml of 70 percent ethanol was added and centrifuged for 5 min at 10,000 x g. The supernatant removed as before and the pellet dried in a Savant Speed Vac for 5 minutes. The pellet was dissolved in 25 µl TE buffer. Repeated vortexing, freezing at -70°C and thawing at 65°C helped to redissolve the pellet. For verification of DNA purity and yield, analysis of a small quantity of the eluted fragment was generally carried out by running 5 µl of the DNA on an 0.8 percent agarose gel. This was electrophoresed at 80 V until the bromophenol blue reached the end of the gel. The gel was stained with ethidium bromide at 0.5 µg/ml for 5 min, destained in

deionized water for approximately 2 minutes and viewed under ultraviolet lamp.

Isolation of Plasmid DNA by Alkaline Lysis. The cells from 1 liter of modified TB medium were collected using a Sorvall GSA rotor at 8,000 x g for 10 minutes in a Sorvall RC5C centrifuge. The bacterial pellet was resuspended in 20 ml with 0.15 M NaCl. This was repelleted in a Sorvall SA600 rotor at 8,000 xg for 10 min, and the pellet resuspended in 10 ml of a solution containing the following ingredients: 50.0 mM Tris, 25.0 percent sucrose (pH 8.0), and 5.0 mg lysozyme per ml. This solution was autoclaved, and prior to use, lysozyme at 5 mg/ml was added. It was transferred to a SA600 40 ml rotor tube and allowed to stand at room temperature for 5 minutes. Then 4 ml of 0.25 M Na2 EDTA (pH 8.0) were added, and the tube incubated on ice for 5 min, prior to the addition of 5 ml of 5 M NaCl. Next, 2 ml of 10 percent SDS were added to the above, mixed throughly and placed on ice for 2 h at 4°C. This was followed by the spinning of the tube in a Sorvall SA600 rotor at 16,350 x g for 60 min. The cellular DNA and bacterial debris, at this point, should form a very "tight" pellet at the bottom of the tube. The supernatant was poured off into a graduated cylinder and one volume of isopropanol was added. The contents were then frozen at -20°C overnight. The next day, the contents were melted in a tray of tap water and

centrifuged at 8,000 x g, 4°C in a Sorvall GSA rotor. The pellet was resuspended in 8 ml 10 mM Tris, 1 mM EDTA pH 8.0. Pure RNAse, at a concentration of 20 µg/ml, was added in order to destroy the RNA present in the sample. At this point, a sample of the DNA product was checked on an agarose gel for the presence of plasmid. Meanwhile, the rest of the suspension was kept at 4°C stirring. The undissolved material was removed by centrifugation at 10,000 x g for 10 min in a SA600 rotor. Cesium chloride (5.3 g/5 ml TE) was added to the supernatant and 600 µl of ethidium bromide at 10 mg/ml was added to the Sorvall T-1270 ultracentrifuge tube. These tubes were balanced to 0.01 g and sealed. Mineral oil or a 1.06 g CsCl/ml TE solution was used to balance the tubes. Tubes were sealed with Ultracrimp™ caps and crimping device. The tubes were centrifuged for approximately 40 h at 36,000 x g in a Sorvall OTD75B ultracentrifuge, using a T-1270 rotor. The plasmid DNA in the tube could be seen by using a handheld UV lamp. Using a 3 ml disposable syringe with a 20G hypodermic needle, the lower band was removed by first inserting the needle through the side of the tube, directly below the band of interest, and slowly drawing the liquid out. The bevel of the needle was kept pointing down to reduce the chance of accidentally drawing in the upper (chromosomal). A second 16G needle was inserted into the top of the tube to allow air to enter the tube as the lower band

is removed. Otherwise, air bubbles may enter the tube at the point of needle insertion and disrupt the band separation. The ethidium bromide was extracted using butanol, saturated with TE buffer (or water). Up to 5-6 extractions were required. This was continued until the upper butanol layer was free of color (pink). Fewer extractions were required if care were taken each time to remove as much of the butanol layer as possible. Cesium chloride was removed by dialysis against 2 liters of T.E. buffer, with 3 changes. The concentration of DNA was determined by reading the absorbance at 260 nm (1.0 O.D.= 50 µg DNA).

Nondenaturing Activity Polyacrylamide Gels

Bacteria were grown overnight at 37°C for *E.coli* and 30°C for *P. putida* to an approximate absorbance of 1.0 at 590 nm in 5 ml of YT. The bacterial cells were centrifuged, resuspended in 300 µl of ATCase breaking buffer (50 mM Tris, 2.0 mM mercaptoethanol, and 20 mM $ZnCl_2$) and sonicated for 30 s using a Branson Cell Disruptor 200. The cell-free extract was centrifuged for 10 min at 10,000 x g. Samples of 20 µl of cell-free extract were mixed with 20 µl of loading buffer which contained 1 ml running buffer, 1 ml glycerol and 0.25 mg bromophenol blue. This total volume of 40 µl was loaded onto a 6 percent nondenaturing polyacrylamide gel and run at 5 watts for 10 hours. The stock solution of acrylamide contained: 40 percent w/v acrylamide and 1 percent w/v N,N'-

bis-methylene acrylamide in ddH_2O. The separating gel buffer contained: 36.3 g Tris (pH 8.9) in 100 ml ddH_2O. A 6 percent acrylamide gel contained: 9.64 ml of 40 percent acrylamide, 7.5 ml separating gel buffer, 4.48 ml 40 percent sucrose and 38.38 ml ddH_2O. Ammonium persulfate, in the amount of 150 mg, was added to this mixture and then degassed for 10 minutes. Prior to pouring the gel, 35 µl of TEMED was added to the gel mixture. The polyacrylamide gel was poured between 20.5 cm X 20.5 cm siliconized glass plates. The running buffer contained 10 mM Tris and 40 mM glycine, at a pH of 8.3. The gel was stained specifically for ATCase activity by a procedure developed by Bothwell (Bothwell, 1975, Dissertation, University of California, Berkeley) and further modified by K. Kedzie, (1987 Ph.D. dissertation, Texas A&M University, College Station), who replaced histidine for imidazole. After the bromophenol blue had reached the bottom of the gel, the plates were separated, and the gel was placed in 250 ml ice-cold 50 mM histidine pH 7.0 for 5 minutes. Next, 5 ml of 1.0 M aspartate and 10 ml of 0.1 M carbamoylphosphate were added to the gel and allowed to react for 20 min. The gel was rinsed 3X with ice-cold distilled water to remove reactants. The enzymatic release of orthophosphate trapped in the gel was allowed to precipitate by the addition of 3 mM lead nitrate in ice-cold 50 mM histidine, pH 7.0. Lead nitrate was removed after 10

min with 3 changes of ice-cold water. The gel was further stained with 300 ml 5 percent ammonium sulfide, which turned the white lead phosphate precipitate into dark lead sulfide bands (Bothwell, 1975, Dissertation, University of California, Berkeley). This last step is optional, with bands becoming visible with the addition of just lead nitrate, as descibed in the second-to-last step.

Aspartate transcarbamoylase Assays. In assaying ATCase, 1 liter of cells was grown in M9 medium in the presence and absence of uracil at 50 µg/ml. These cells were harvested at 4°C by centrifugation at 16,000 x g for 10 minutes. The pellets were washed in approximately 1 ml of ATCase breaking buffer per gram of cells. The washed cells were centrifuged and then either stored by freezing at -20°C or immediately disrupted twice at 1500 pounds psi, using a 4 ml French Press. The cell-free extract was centrifuged at 12,000 x g for 1 h at 4°C in a Sorvall RC5C centrifuge, using a SA400 rotor.

ATCase activity was assayed by measuring the amount of carbamoyl aspartate produced in 20 min at 30°C according to the method of Gerhart and Pardee (17), with modifications using the color development procedure of Prescott and Jones (53). Assays were performed to determine either the optimal Km_{asp} or Km_{cp} at pH 8.0. In the determination of K_{masp}, the assay tubes contained the following reagents in a final

volume of 2.0 ml: 40 mM Tris (pH 8.0), 4.5 mM carbamoylphosphate, 0.2 ml diluted enzyme preparation, 0.5 mM to 50 mM potassium aspartate. Further, the effector response was examined by the addition of 5.0 mM of ATP, CTP or UTP. The assay tubes, used in determining the Km_{cp}, contained the following reagents, also in a final volume of 2.0 ml: 40 mM Tris (pH 8.0), 0.078 mM to 6.0 mM carbamoylphosphate, 0.2 ml diluted enzyme preparation, 20 mM potassium aspartate. All assay tubes were prepared in advance (without carbamoylphosphate) and preincubated at 30°C for 5 minutes. The reaction was initiated by the addition of 200 µl carbamoylphosphate (8 mg/ml) for the aspartate curve. The carbamoylphosphate curve was initiated with 200 µl of carbamoylphosphate of varying concentrations (0.078 mM - 6.0 mM). At 10 min intervals up to 30 min, 0.5 ml of the reaction mix was pipetted into "stop tubes" that were maintained on ice and contained 0.5 ml ddH_2O and 1 ml color mix (0.5 mg/ml antipyrine in 50 percent sulfuric acid and 0.8 mg/ml monoxime in 5 percent acetic acid). These tubes remained on ice until all samples were added. Color was developed by the incubation of the tubes in a 60°C bath, capped with marbles to limit evaporation,and exposed to the lighting of the room. After 110 min of incubation, the assay tubes were cooled to room temperature and read in a Beckman DU-40 spectrophotometer set at 466 nm. Positive and negative

controls were performed to determine background color development of buffer, substrates, and cell extracts. The kinetic curves were generated, plotting specific activity of the enzyme (nmol carbamoylaspartate/min/mg protein) versus the concentration of aspartate or carbamoylphosphate. The Km_{asp} and Km_{cp} were determined from the intercepts of the x-axis on 1/S *versus* 1/V plots.

Spot Assay. A slightly modified and somewhat shortened ATCase assay, appropriately named the "spot assay," was used to predict the amount of enzyme to be used in a complete assay. It was also employed to detect the presence of the enzyme in fractions obtained in its partial purification. In this assay, a 20 µl sample from a column fraction or a cell free extract was mixed with 430 µl of a "pre-mix." The pre-mix contained 40 mM Tris (pH 8.0) and 20 mM aspartate (pH 8.0). These tubes were incubated at 30°C for 5 min, and the reaction was initiated with the addition of 50 µl of 8 mg carbamoylphosphate per ml. After 20 min, 1.5 ml of stop color mix was added to the reaction tube, and this was incubated at 60°C for 110 min. The tubes were read, using the optical density of 466 nm.

Protein Concentration Determination. Protein was quantified by the method of Lowry et al.(39). Bovine serum albumin (BSA) at 0-100 µg was used to produce a standard curve. The BSA was divided into 11 tubes at 10 µg increments

as follows: the 0 µg tube solely contained 200 µl ddH_2O, while the 10 µg tube contained 190 µl ddH_2O + 10 µl 0.1 percent BSA (100 µl dessicated BSA and 900 µl ddH_2O yields a 1 µg BSA/ml solution). The rest of the tubes used in the standard curve were set up in an according manner. Three different volumes (5 µl, 10 µl and 20 µl) were taken from the unknown samples and brought up to 200 µl total volume. Eight hundred µl alkaline copper reagent (0.5 ml 2 percent sodium-potassium tartrate, 0.5 ml 1 percent copper sulfide, mixed before adding 49 ml of 2 percent sodium carbonate in 0.1 N sodium hydroxide) was added to all tubes, and they were allowed to stand at room temperature for 10 minutes. Folin reagent (100 µl, 1 N commercial preparation diluted 1:1 with ddH_2O) was added to all the tubes, which were then incubated at room temperature for 30 minutes. The absorbance was read at 660 nm. Hence, the absorbance was plotted against the concentration of BSA to establish a standard curve.

Radioactive labelling of probe. The probe used to determine the extent of crossover between *P. putida* and *E. coli* was pMJS-29. This plasmid carries the *pyrBC* from *P. Putida*. Hence, 10 µg of this plasmid was cut with *Hin*dIII and *Xba*I simultaneously, using BRL React 2 Buffer, overnight and at 37°C. This digest was electrophoresed through a 0.8 percent agarose gel for 2 h, and the 3.1 Kb band was excised. "Gene Clean" was used to separate the DNA from the agarose:

the agarose band was weighed in a microfuge tube (0.34 g), and 3 volumes of 6M NaI (1.02 ml) was added to the tube. This was incubated at 55°C until the agarose melted. Then, 5 ul of Geneclean glass milk was added, vortexed, and incubated on ice for 10 min to allow DNA to bind to Geneclean's silica matrix. The tube was microfuged for 1 min to pellet the glass beads. The glass beads/DNA pellet was subsequently washed 3 times in Geneclean's New Solution, which contained an undisclosed amount of NaCl, Tris, EDTA and ethanol. This was followed by the addition of 20 µl of TE to the pellet, which was then resuspended by vortexing, and finally incubated at 55°C for 10 min. The glass beads were pelleted for 1 min and the supernatant was drawn off.

The DNA fragment (*pyrBC* from *P. putida*) was then random prime labelled using Boehringer Mannheim Random labelling kit. First, 5 µl of this DNA was diluted in 15 µl TE, which was then boiled for 3 min and placed quickly on ice. In this step, the DNA is denatured. Next, 10 µl of this DNA was taken and dNTPs were added(dATP, dGTP, dTTP, 1 µl of 10 mM solution), 2 µl of polymerase buffer, 1 µl of Klenow fragment and 5 µl of 3000 Ci/mmol α^{32}P dCTP. This reaction was incubated at 37°C for 30 min.

Southern Blots Chromosomal DNA from *P. putida* strains HES-6 and 2825 (10 µg) was digested with 16 units *Eco*RI, *Pst*I and *Bam*HI individually for 2 hours at 37°C. The

digested fragments were electrophoresed overnight at 20 volts through 0.8 percent (w/v) agarose, and the DNA was transferred to a GeneScreen-Plus membrane for hybridization. First, the gel was placed in 0.25 M HCl for approximately 15-20 min to destroy purine residues, a procedure commonly referred to as "acid nicking". This improved the efficiency of the transfer of larger molecular weight DNA bands to the membrane. The DNA in the gel was then denatured by placing the gel in 1.5 M NaCl and 0.5 M NaOH for 30 min. The gel was further neutralized by soaking it in 0.5 M Tris (pH 8.0) and 1.5 M NaCl for 30 min, followed by its transfer to a dish containing 10X SSC for 15-30 minutes. A stock solution of 20X SSC diluted to the proper concentration contained 175.3 g of NaCl and 88.2 g of sodium citrate (pH 7.0) in 1 liter. In order to transfer the DNA to the GeneScreen Plus nylon membrane, a sandwich was made (described below). First, a single sheet of plastic food wrap was placed on the benchtop. Next, three or four sheets of Whatman 3MM filter paper, cut to the size of the gel and saturated with 2X SSC, were placed on top of the plastic wrap. The gel was then placed on top of the saturated Whatman paper and overlayed with a piece of presoaked (in 2X SSC) GeneScreen Plus, cut to the exact size of the gel. When the GeneScreen membrane was placed on the gel, care was taken not to trap air bubbles between the membrane and the gel. This was usually accomplished by

gently rolling a sterile pippette on the membrane surface, so that all air pockets were essentially released. This was followed by the placement of a 2X SSC saturated sheet of 3MM paper on top of the membrane, which was then overlayed with a stack of blotting paper (paper towels trimmed to the correct size worked fine). A large weight such as a catalog or lead brick was placed on top of this stack, in order to allow maximum absorbance and transfer. This was left undisturbed overnight. The following morning, the membrane was ready for the hybridization procedure. First, the membrane was prehybridized by soaking it in 10 ml of Prehybridization Solution which consisted of: 1.0 percent SDS, 50 percent formamide and 10.0 percent dextran sulfate. The solution was added to a plastic hybridizaton bag containing the membrane. The plastic bag was heat-sealed at the top and incubated with constant agitation for at least 24 h at 42 °C. The probe and 200 µl of 5 mg/ml salmon sperm DNA was boiled for 3 min and placed on ice quickly. This again was done in order to denature the strands of DNA. The sealed top edge of the bag was cut off and 0.5 to 1.0 ml of the probe solution was carefully added by using a 1 ml syringe fitted with a 20G needle. The probe solution consisted of: salmon sperm DNA (150 µg/ml) and 1.0 to 4.0 x 10^5 cpm of probe typically in 1 ml of solution. Again, care was taken to remove any air that was introduced during the injection of the probe solution.

The plastic bag was resealed and incubated with constant slow agitation for 24 h overnight in a New Brunswick Gyrotory water bath shaker Model G76. The membrane was removed from the hybridization bag and washed once with 100 ml of 5X SSC and 0.1 percent SDS at room temperature for 5 min with constant agitation. 5X SSC is made by diluting a stock of sterile 20x SSC, which contains 175.3 g NaCl and 88.2 g Sodium citrate at pH 7. It was again washed twice with 200 ml of a solution containing 5X SSC and 0.1 percent SDS at 65°C for 1 h with constant agitation. The membrane, with the DNA side face up, was placed on a sheet of Whatman #1 filter paper and allowed to dry at room temperature. This filter was then placed in a Kodak X-ray cassette (25.4 cm X 20.3 cm). The membrane was autoradiographed on Kodak XAR5 film for an appropriate period of time (generally 1-14 days, depending on the level of radioactivity) in the presence of an intensifying screen.

RESULTS

Subcloning of *E.coli pyrB* and *pyrBI*. In testing the attenuation hypothesis in *Pseudomonas*, two different *E. coli pyrB* subclones were made. The first construct in the series, contained the gene encoding the catalytic subunit (*pyrB*) only. The gene was obtained from a 5.5 kb plasmid,

pEK-2, by performing a *Hind*III/*Bgl*II cut on 20 µg of the plasmid. This generated a 1.1 kb fragment, which was detected on 0.8 percent agarose gel, and then electroeluted and precipitated. A fill-in reaction was performed, and the fragment blunt-end ligated into the *Hinc*II site of pUC-18. This construct, which was called pBJR28, was further transfromed into *E. coli* DH5α in order to provide a quick screening method (Figure 8). Plasmids of transformants were further obtained by rapid preparation ("rapid prep"), followed by digestion of the plasmid DNA with *Eco*RI, so that the approximate size of the insert could be calculated (Figure 9). An insert of the appropriate size of 1.4 kb was found in several of the screened transformants. Their rapid prep DNA was further transformed into an *E. coli pyrB* auxotroph, TB-2. This strain was employed with the prospects of measuring the activity of the plasmid-encoded ATCase alone and without the conflicting presence of a parental enzyme. Transformants were plated on LB medium containing 100 µg per ml ampicillin. Ampicillin resistant colonies were assumed to contain pUC genes conferring resistance and were further transferred to *E. coli* minimal medium, in the absence of uracil. In order to grow in the absence of uracil, these cells must contain a functional *pyrB* gene. With this principle in mind, these colonies were transferred and grown in 5 ml of LB ampicillin (100 µg/ml) tubes at 37°C shaking.

Figure 8. Subcloning the *E. coli pyrB*.

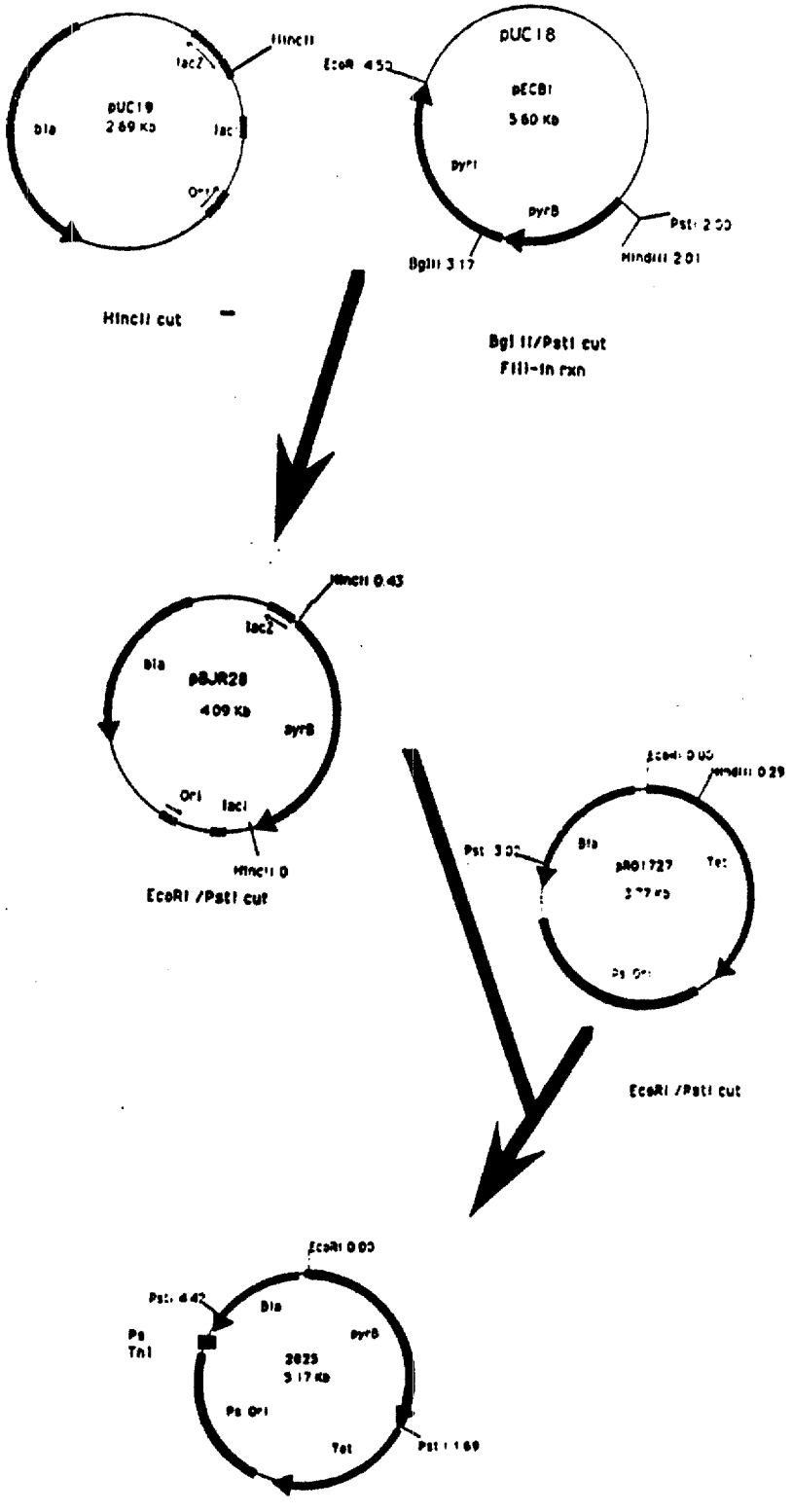
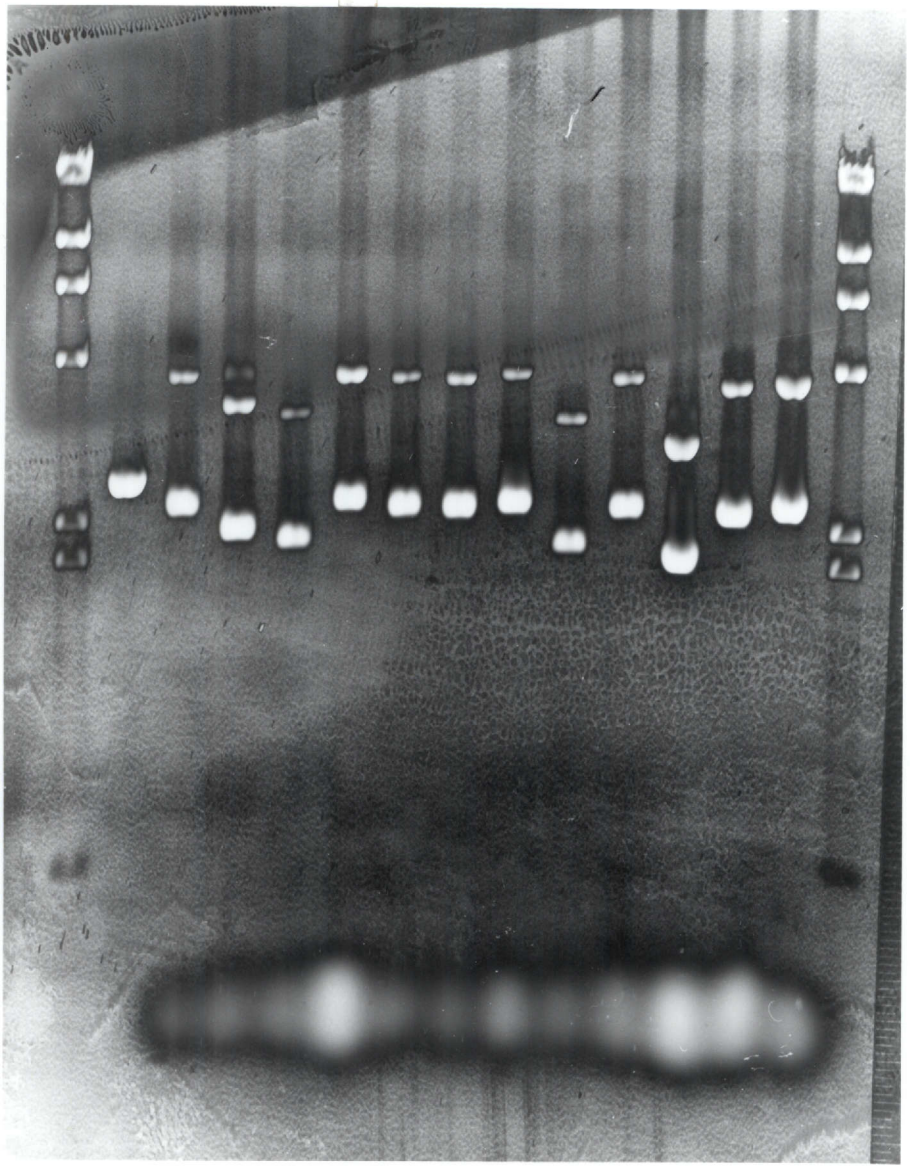

pUC19
2.69 Kb
HincII
lacZ'
Bla
lacI
Ori
pUC18
pECB1
5.60 Kb
EcoR 4.52
pyrF
pyrB
PstI 2.00
HindIII 2.01
BglII 3.17
HincII cut
BglII/PstI cut
Fill-in rxn
HincII 0.43
lacZ'
Bla
pBJR28
4.09 Kb
pyrB
Ori
lacI
HincII 0
EcoRI /PstI cut
Bla
Tet
Ps Ori
EcoRI /PstI cut
EcoRI 0.00
PstI 4.42
Bla
pyrB
Ps
Tnl
2825
3.17 Kb
Ps Ori
Tet
PstI 1.69

Figure 9. 0.8 percent Agarose gel showing pBJR28 cut with *Eco*RI. * indicates lane containing pBJR28 DNA. The first and last lanes contain *Hind*III cut Lambda.

*



An activity gel was performed on colony number 28, to determine if the ATCase. catalytic subunit was indeed present. The activity gel (Figure 10) shows a band of 100 kDa, identical to that of the *pyrB* of the wild-type *E. coli*. This recombinant was prepared in bulk as a precautionary step in case the plasmid proved to be important in later phases of the research.

The second construct contained the genes encoding both the catalytic and regulatory subunits (*pyrBI*) of the *E. coli* ATCase (Figure 11). The two genes for the *E. coli* holoenzyme were cut again from the plasmid pEK-2, with the restriction enzymes *Hin*dIII and *Eco*RI. This yielded a fragment that was 2.7 kb, a size somewhat similar to that of pUC-8. Therefore, it was necessary to separate the fragment containing the two genes from the pUC plasmid by utilization of a *Sca*I site centrally located in the pUC region. These fragments were run on a 0.8 percent gel and the upper fragment of 2.7 kb was digest generated two fragments of pUC8, which were virtuallyidentical in size (1.3 Kb). These fragments were discarded, as their sizes only served to highlight the larger fragment containing the genes in question. In order to subclone and ultimately express the *E. coli pyrBI* genes in *Pseudomonas*, a suitable vector was required. Therefore pRO1727, a plasmid containing the *Pseudomonas* origin of replication as well as a gene encoding tetracycline

Figure 10. Nondenaturing ATCase activity polyacrylamide gel. Lane 1: *P. putida* PRS2000, Lane 2: Standard Bovine Serum Albumin (MW = 132kD), Lane 3: Standard urease (MW = 545kD, 272kD), Lane 4: *P. putida pyrB1137*, plus uracil, Lane 5: *P. putida* (PRS2000) minus uracil, Lane 6: *P. putida* HES-6, containing the *E. coli pyrBI*, Lane 7: *P. putida* 2825, containing the *E. coli pyrB*, Lane 8: *E. coli* DH5α containing plasmid (pEK-2)

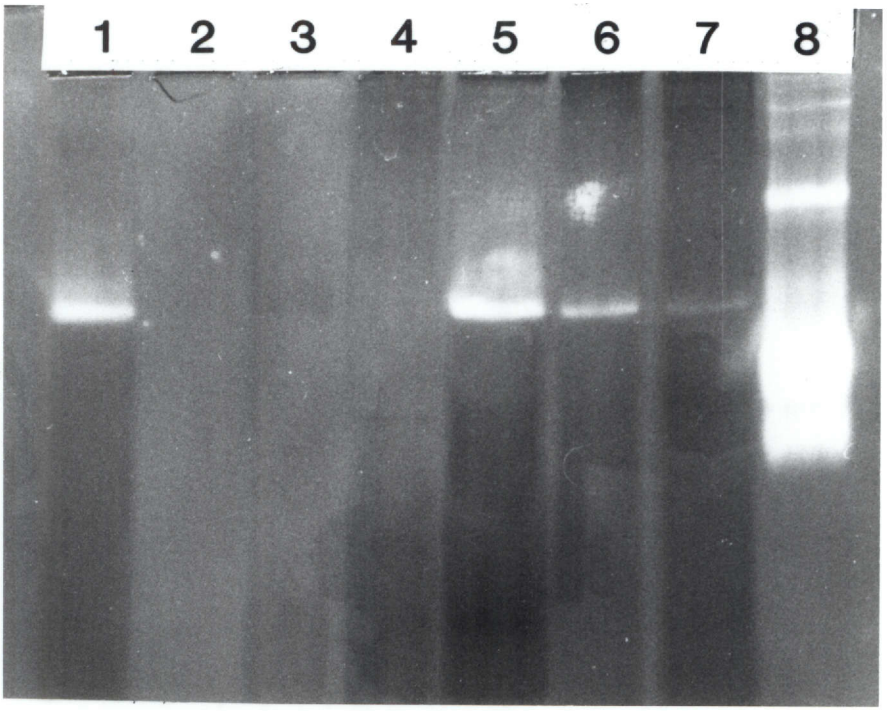
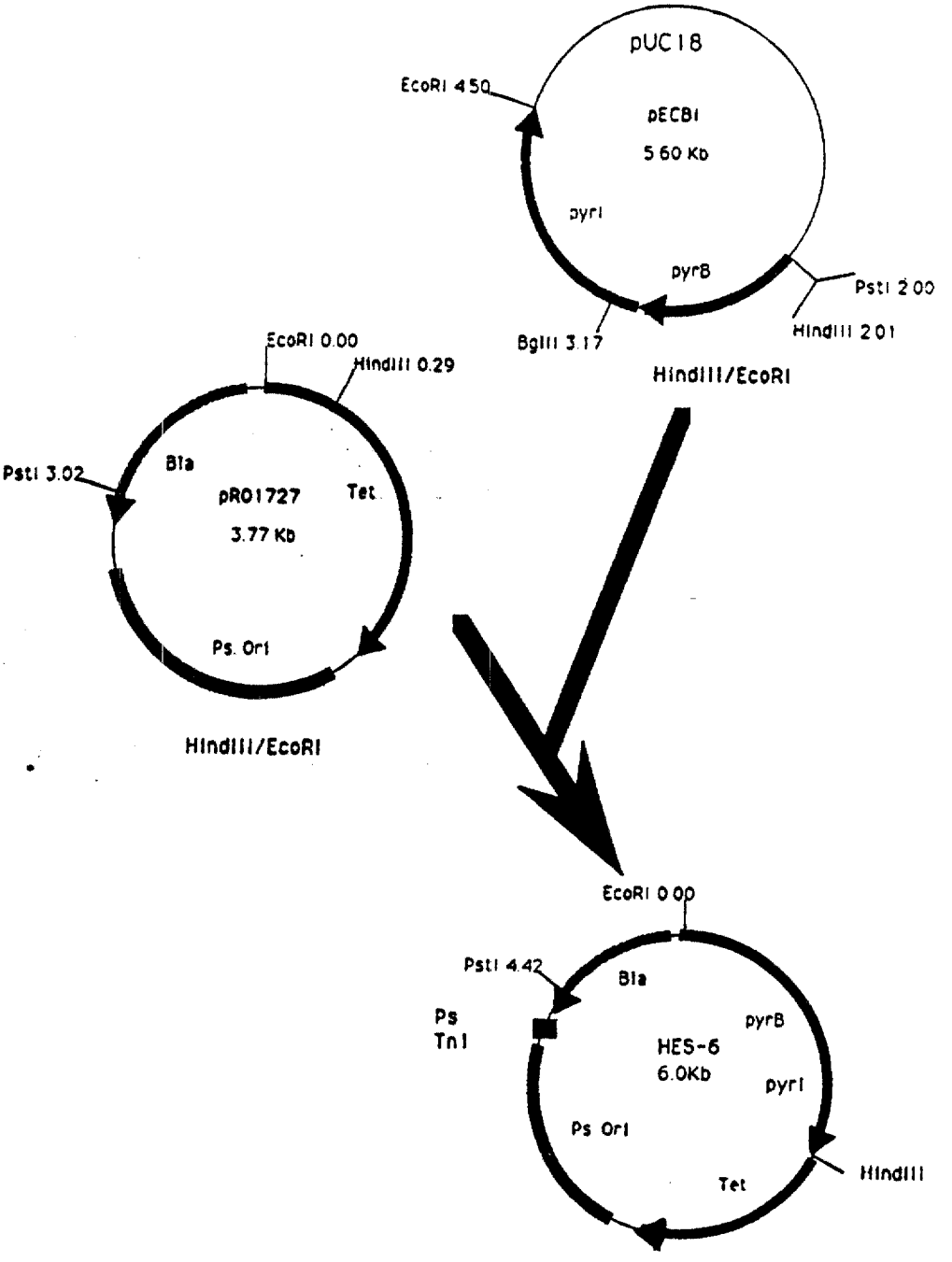

1
2
3
4
5
6
7
8

Figure 11. Subcloning the *E. coli pyrBI*.


pUC18
EcoRI 4.50
pECB1
5.60 Kb
pyrI
pyrB
PstI 2.00
BglII 3.17
HindIII 2.01
HindIII/EcoRI
EcoRI 0.00
HindIII 0.29
PstI 3.02
Bla
pRO1727
Tet
3.77 Kb
Ps. Ori
HindIII/EcoRI
EcoRI 0.00
PstI 4.42
Bla
Ps
Tn1
pyrB
HES-6
6.0Kb
pyrI
Ps Ori
Tet
HindIII

resistance, was obtained from Dr. Ron Olsen. The *E. coli* catalytic subunit of ATCase was retrieved first by performing an *Eco*RI/*Pst*I digest on pBJR-28. This 1.1 kb fragment containing the *pyrB* gene was electroeluted from a 0.8 percent agarose gel, ligated into plasmid pRO1727, cut with *Eco*RI/*Pst*I before being transformed into a *P. putida pyrB1137*. The nature of this mutation is proposed to be a single base deletion. Next, the *pyrBI* genes from *E. coli* were subcloned (detailed above), and ligated into pRO1727, cut with *Hin*dIII/*Eco*RI. Transformants containing the *pyrB* and *pyrBI* genes were screened on YT/tetracycline (35 µg/ml). Approximately 100 colonies arose on these plates, and rapid preps were performed on all of these. Because no plasmid was recovered from any of these colonies, the rapid prep procedure as a whole was assumed to have failed. It is for this reason that rapid preps were performed repeatedly, each time still producing the same results. Thus, ligations and transformations were repeated, using the same plasmid constructs, as used previously. Efforts were also made to make fresh competent cells and to reconstitute all of the solutions and buffers involved in the transformation and rapid plasmid preparation procedures. It was clear that plasmid DNA is not present and that prototrophy must be conferred on the chromosome of the mutant strain.

Activity gel. Although there was no plasmid present

in these transformants, they still displayed *pyrB* prototrophy, as evidenced by their growth in the absence of uracil. Hence, an activity gel on these transformants was performed in order to ultimately check for the presence of ATCase activity (refer back to Figure 10). The transformed *P. putida* cells, containing functional ATCase catalytic subunit from *E. coli*, were expected to have a MW of 100 kDa, identical to that of the wild-type *E. coli* ATCase catalytic subunit. The transformed *P. putida* cells containing the *E. coli pyrBI* genes, on the other hand, were expected to have a MW identical to the *E. coli* holoenzyme (310 kDa). This result was not obtained. Instead, transformed strains both showed individual bands that were identical in MW (470 kDa) to the wild-type *P. putida* ATCase/DHOase complex,regardless of whether it contained the *pyrB* or *pyrBI* plasmid constructs.

Enzyme kinetics. In an attempt to characterize the enzymes, ATCase assays were performed on HES-6 and 2825. First, the Km_{Asp} of the mutant ATCases from strains HES-6 and 2825 were measured at a value of 11.6 mM (Figure 12 and 13). Because 2825 contained only the *pyrB* of the *E. coli* ATCase, is was expected to have a Km_{ASP} of 5.5 mM, identical to that of the wild-type *E. coli*. Further, the aspartate curves of both strains were hyperbolic in nature, which is what was truly expected for only the strain containing the *E. coli*

Figure 12. ATCase activity of PRS2000, 2825 and HES-6 as a function of aspartate concentration. The carbamoylphosphate concentration was held constant at 4.5 mM.

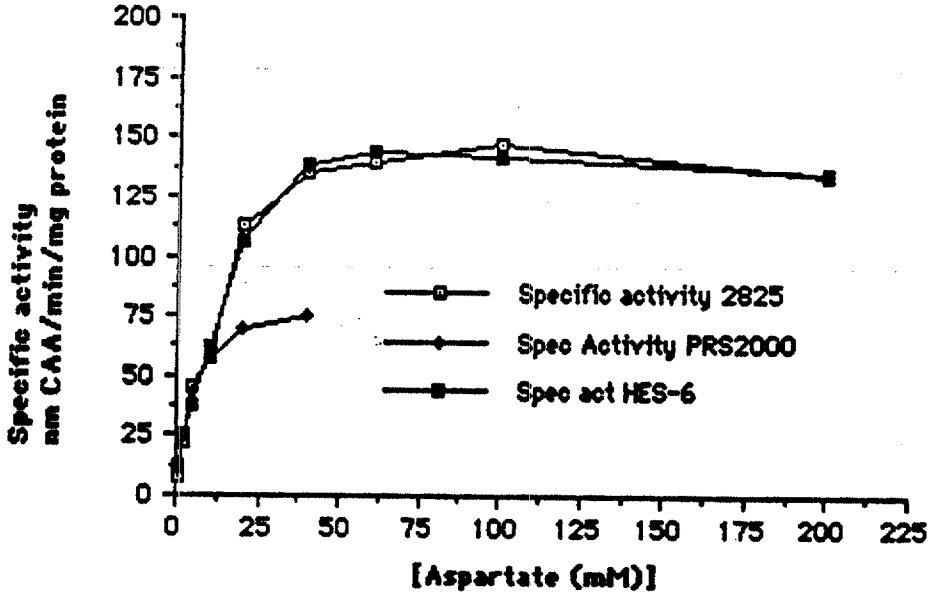
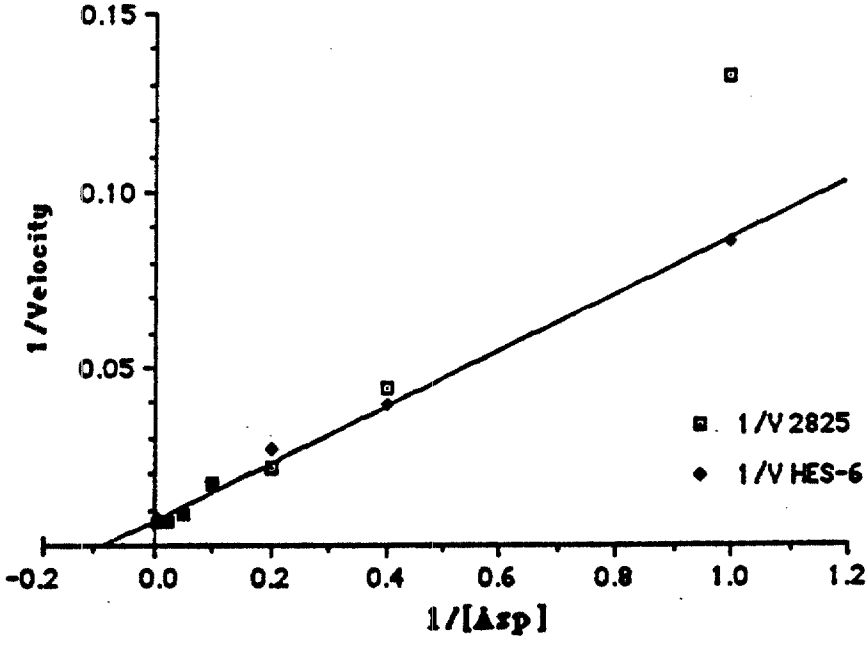

Specific activity
nm CAA/min/mg protein
200
175
150
125
100
75
50
25
0
0
25
50
75
100
125
150
175
200
225
[Aspartate (mM)]
Specific activity 2825
Spec Activity PRS2000
Spec act HES-6

Figure 13. Lineweaver-Burke plot of enzyme activity of 2825 and HES-6 as a function of aspartate concentration.


0.15
0.10
0.05
1/Velocity
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1/[Asp]
1/V 2825
1/V HES-6

pyrB gene. Ironically, this result was also simultated in the strain containing the *E. coli pyrBI* genes, in which case the curves were expected not to be hyperbolic, but rather sigmoidal like the *E. coli* holoenzyme. The sigmoidicity of the holoenzyme's kinetic curves serves as a indirect illustration of the enzyme's cooperativity. Hence, cooperativity was naturally expected in the strain containing the *E. coli pyrBI*. The hyperbolic kinetic curves witnessed in this case are a classic behavior hallmark of the *Pseudomonas* enzyme, where there is a lack of cooperativity. Next, the Km_{cp} for 2825 and HES-6 was measured at 3.2 mM (Figure 14 and 15). This value is approximately 5 times higher than the wild-type *P. putida* ATCase, which is 0.6 mM. Again, the curves were hyperbolic in nature. HES-6 and 2825 also had a $Vmax_{Asp}$ of approximately 30 mM and a $Vmax_{cp}$ of 7 mM. The wild-type *P. putida*, on the other hand, has a Vmax of 10 mM, and a $Vmax_{cp}$ of 9.12 mM. In the presence of effectors (ATP, CTP, and UTP), the ATCases from strains 2825 and HES-6 showed maximal (100 percent) inhibition, which again is very different from the wild-type E. coli enzyme,. which is activated by ATP and inhibited by CTP (Table 3). Wild-type *P. putida* ATCase, on the other hand, shows only minimal (15 percent) inhibition to these effectors. Thus, the enzymes may show identical MW's to the wild-type *P.*

Table 3. Effector response of ATCase

	No effector	ATP*	CTP*
E. coli holoenzyme (*pyrBI*)	100	180	37
E.coli catalytic trimer (*pyrB*)	100	100	100
P. putida holoenzyme	100	34	51
Cloned *pyrBI* in *P. putida*	100	0**	0**
Cloned *pyrB* in *P. putida*	100	0**	0**

* 2.0 mM Effectors

** Concentrations above 125 μM show complete inhibition.

putida holoenzyme, but they exhibit distinctly different kinetics.

Growth curves. In the course of the research, it was observed that the mutants grew considerably slower than the *P. putida* wild-type strain. To this end, a growth curve was generated in minimal medium in the presence and absence of uracil. A generation time of 2.5 h was observed for strains HES-6 and 2825 (Table 4).The isogenic wild-type *P. putida* strain had a generation time of 1.0 h, as did strain *pyrB1137* when grown in uracil.

Testing for attenuation. Detection of the attenuator mechanism was performed in the mutant strains by growth in the presence and absence of uracil. A four-fold

difference in specific activity was seen in the wild-type *E. coli* ATCase, with the km_{ASP} of 5 in the presence of uracil and

Table 4. Growth rates for PRS2000, PpN1137, HES-6 and 2825.

	Generation Time (30°C, hours)
P. putida PRS2000	1.0
P. putida pyrB1137	1.0
**P. putida pyrB1137* with *E. coli pyrB*	2.5
**P. putida pyrB1137* with *E. coli pyrBI*	2.5

* The cells were grown in *Pseudomonas* minimal medium supplemented with 40 mM Succinate. The *P. putida pyrB1137* was also supplemented with 50 µg/ml uracil.

20 in its absence. The km_{Asp} of 2825 was 25 in the presence of uracil, while in its absence it was 75 (Table 5).

Table 5. Effect of growth on uracil on ATCase specific activity.

	+ URACIL	- URACIL
Wild-type *P. putida*	20	30
Wild-type *E. coli*	5	20
E. coli cloned *pyrB* in *P.putida*	47	140
E. coli pyrBI in *P. putida*	35	140

Figure 14. ATCase activity of PRS2000, 2825 and HES-6 as a function of carbamoylphosphate concentration.

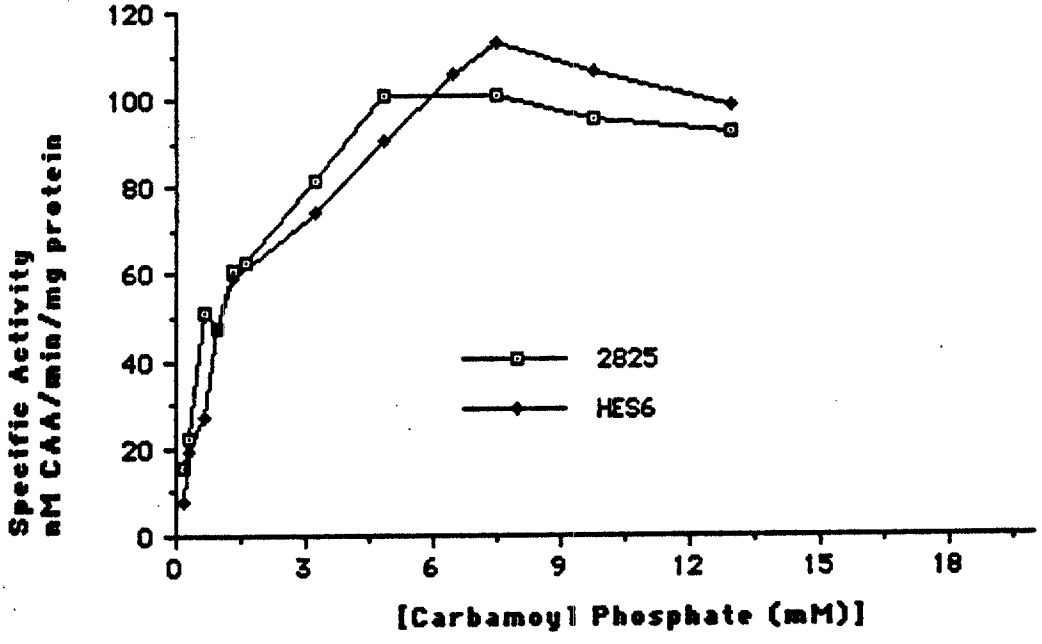
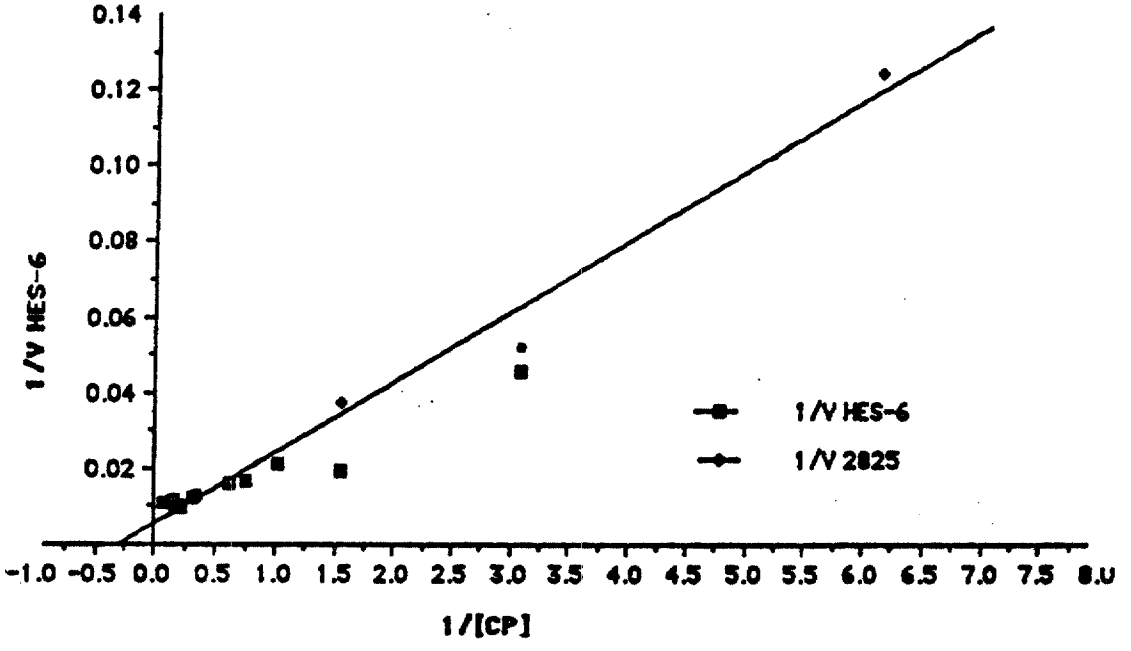

Specific Activity
nM CAA/min/mg protein
120
100
80
60
40
20
0
0
3
6
9
12
15
18
[Carbamoyl Phosphate (mM)]
2825
HES6

Figure 15. Lineweaver-Burke plot of the enzyme activity of 2825 and HES-6 as a function of carbamoylphosphate concentration.


0.14
0.12
0.10
0.08
0.06
0.04
0.02
1/V HES-6
1/V HES-6
1/V 2825
-1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0
1/[CP]

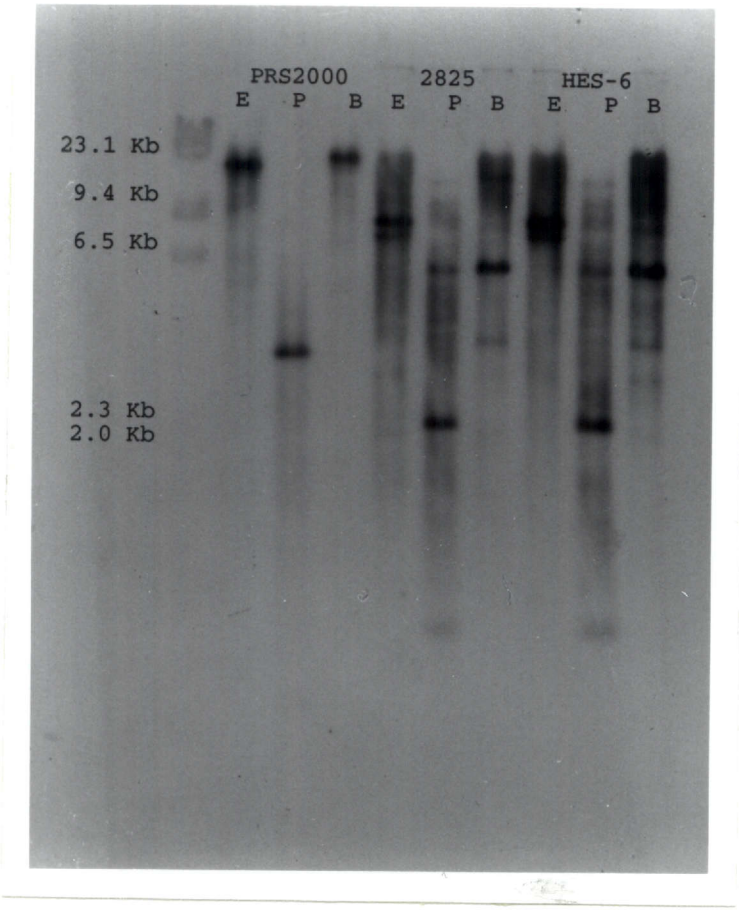
These results closely mimic those of the wild-type *E. coli* strain, and thus it is believed that attenuation in *Pseudomonas* is responsible for these results. Almost identical results were found in HES-6, indicating a possible similarity in composition between the two strains.

Southern blot analysis. Southern blot analysis is shown in Figure 16. The wild-type *P. putida pyrBC* and strains 2825 and HES-6 were individually cut with *Eco*RI, *Pst*I, and *Bam*HI. The *Eco*RI cuts for the wild-type *P.putida* chromosome generated a single fragment with an approximate size of 20 kb. The chromosomal DNA of 2825 and HES-6, on the other hand, generated an additional fragment when cut with *Eco*RI. These two fragments of 8 and 9 kb, are generated from a seemingly smaller chromosome. Similar results were obtained when 2825 and HES-6 were cut with *Pst*I and *Bam*HI, in which one and two additional fragments were generated, respectively. Hence, this overt addition of new restriction sites is clearly indicative of a cross-over and insertion event in the *PyrB1137* strain.

DISCUSSION

The *pyrB* gene, which encodes the catalytic trimer of ATCase in *E. coli*, was cloned into a Pyr$^-$ auxotroph of *P. putida* (PpN1137) using the broad host range plasmid pRO1727

Figure 16. Southern blot analysis of PRS2000, 2825, and HES-6. E = *Eco*RI, P = *Pst*I, B = *Bam*HI.


PRS2000
2825
HES-6
E P B E P B E P B
23.1 Kb
9.4 Kb
6.5 Kb
2.3 Kb
2.0 Kb

as the vector. Selection was made for Pyr$^+$ prototrophy and tetracycline resistance (Tc^r; 35 μg/ml). Isolated colonies were picked, purified and tested for pyrimidine prototrophy. A single colony was selected and grown in succinate-supplemented *Pseudomonas* minimal medium. The cells were harvested, disrupted in a French pressure cell and assayed for ATCase, using 5 mM carbamoyl phosphate and varying concentrations of aspartate (500 nM - 400 mM). An active ATCase was observed on a 6 percent polyacrylamide activity gel.

When the *P. putida* PpN1137 strain was tested for the presence of the *pyrB*-carrying plasmid, no plasmid was found. Since *P. putida* stably inherited the *pyrB*$^+$ gene of *E. coli* and remained resistant to tetracycline, the plasmid is believed to have been inserted into the *P. putida* chromosome. Southern blot analysis revealed a different chromosomal band pattern for 2825 and HES-6, as compared to that of the wild-type *P. putida*. Thus, additional restriction sites could only be spawned by the insertion of "foreign" DNA into the chromosome.

The molecular weight of the ATCase from the transformed Pyr$^+$ *P. putida* was next investigated on a 6 percent activity gel. We expected to find a molecular weight of 100 KDa, that of the catalytic trimer of *E. coli.*, because *P. putida* PpN1137 had no ATCase. Instead, we found an active ATCase

with a MW of 484 KDa, which is nearly identical to the *P. putida* complex of Schurr *et al*, the *P. aeruginosa* of Vickrey *et al* and the *P. fluorescens* of Bergh and Evans. These *Pseudomonas* enzymes are thought to exist in dodecameric complexes with the *pyrC* encoded DHOase, replacing the *pyrI* encoded *E. coli* regulatory polypeptide. The *pyrB* encoded ATCase polypeptide (A) has a MW of 33 KDa. Thus, the putative dodecamer is suggested at $2A_3:3D_2$, which is analogous to the *E. coli* ATCase dodecameric structure.[2(99) + 3(88.4)]

Effector response was measured in the presence of UTP, CTP and ATP. These nucleotides are known to inhibit the *Pseudomonas* enzyme only minimally (~15 percent) when assayed at the approximate physiological concentration of aspartate and carbamoylphosphate at pH 8.0. All three nucleotides completely inhibited the enzyme from the transformed *P. putida* strain 2825. The *E. coli* enzyme, on the other hand is inhibited by CTP only and activated by ATP. Thus, this strain is distinct from either the *E. coli* or *P. putida* wild-type holoenzyme.

Since the PpN1137 of *P. putida* had no catalytic activity and the transformed *P. putida* contained an active enzyme, it was important to ascertain the velocity-substrate response of the enzyme to aspartate. We expected to find Michaelis-Menten hyperbolic kinetics because of the assumed presence of

the *E. coli pyrB* encoded ATCase, with a K_m equivalent to that of the *E. coli* catalytic trimers (~10 mM), and with no response to nucleotide effectors ATP or CTP because of the absence of the *E. coli pyrI* encoded regulatory polypeptide. Indeed, we found Michaelis-Menten kinetics, but the K_m differed from that known for the *E. coli* catalytic trimer (refer to Table 3). Once again, the result simulated that found for the *Pseudomonas* enzyme. Coupled with a MW of 470 KDa this result suggested that an active *Pseudomonas* enzyme was present in the transformed *P. putida*.

Since the *E. coli pyrB* gene that we used carried an active promoter (including the leader sequence), we next determined the level of expression of ATCase in the *P. putida* transformed strain. To this end, strain 2825 was grown in minimal succinate medium in the presence and absence of uracil. As controls, wild-type *P. putida* and wild-type *E. coli* were similarly treated. Table 6 gives the results of these experiments. As can be seen here, the *P. putida* wild-type enzyme showed little or no response to growth in uracil, while the expression of the wild-type *E. coli* enzyme was decreased 4-fold in growth in uracil. The ATCase from the transformed *P.putida* behaved just like its *E. coli* counterpart, with a 3 to 4-fold decrease in expression when grown in uracil. This suggests that the *E. coli* promoter

(and its attenuated leader sequence as well) functions in *P. putida* background.

It now appears that the *pyrB*$^{+}$ gene on plasmid 2825 had recombined into the *P. putida pyrB* gene. Again, a Southern blot suggests the incorporation of *E. coli* DNA as evidenced by the addition of multiple restriction sites. In additon, the evidence of an attenuator mechanism in these strains leads us to believe that the *E. coli* attenuator region is inserted into the *P. putida* chromosome. This recombinational event must have also occurred at a locus promoter-distal to the mutant *pyrB1137* allele because the strain was restored to prototrophy. We repeated the original experiment, this time using a cloned *pyrBI* gene (encoding the ATCase holoenzyme, MW 300 KDa). The same *P. putida pyrB1137* auxotroph was transformed with the recombinant plasmid HES-6 (refer back to Table 1). Pyr^{+} prototrophy and tetracycline resistance were selected for and ATCase was examined as before from the transformed *P. putida*. Results obtained were similar to those found earlier for the cloned *E. coli pyrB* gene.

The exact crossover junctions have not yet been established. However, this point must be in the region of maximal sequence homology encoding the carbamoylphosphate binding site or polar domain of the ATCase polypeptide. Since the two hybrid enzymes produced from two different constructs of the *E. coli pyrBI* were nearly identical in both

sets, the crossover points are believed to be the same in both sets. A double cross-over, as opposed to a single cross-over, is believed to have occurred because of lack of DNA probed, as evidenced by the summation of fragments generated in a single digest. These numbers, compared to those generated in the digests on the wild-type chromosome, suggests that the chromosomes of 2825 and HES-6 are somewhat smaller in size. Of course, this is pure speculation based on the number values generated on a Southern blot. Whether or not there has been an ommision in the *P. putida* chromosome has yet to be answered by a sequence analysis of 2825 and HES-6. What this does suggest is that the strains are not revertants, but true hybrids.

In conclusion, the results of this experiment have global relevance because any mutant allele of *pyrB* can be used in this way (i.e. with a plasmid borne *pyrB* or *pyrBI*) to create a *pyr*$^+$ prototroph that possesses a hybrid ATCase.

In addition, because Lipscomb *et al* (personal communication to Gerard A. O'Donovan) are constantly seeking new diverse sources of hybrids for the purposes of assessing their three-dimensional structures, this research would prove to be very fruitful. Such enzymes appear to be stably inherited and can be carefully purified to homogeniety. This can be accomplished easily by utilizing *P. putida* probes for the 5' end of the *pyrB* gene and the 3' end of the *pyrC* gene,

and perform a polymerase chain reaction on the two genes (*pyrBC*). This, in turn, can be subcloned into wild-type *E. coli* cells, which can then be grown up to gram quantities, and their crude extract used in column purification (G200 or HPLC).

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