CASSETTE SYSTEMS FOR CREATING INTERGENERIC HYBRID ATCASES

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Cassette systems for creating intergeneric hybrid ATCases were constructed. An *Mlu*I restriction enzyme site was introduced at the carbamoylphosphate binding site within the *pyrB* genes of both *Pseudomonas putida* and *Escherichia coli*. Two hybrids, *E. coli pyrB* polar domain fused with *P. putida pyrB* equatorial domain and *P. putida pyrB* polar domain fused with *E. coli pyrB* equatorial domain, are possible. The intergeneric *E. coli-P. putida* hybrid *pyrB* gene was constructed and found to encode an active ATCase which complemented an *E. coli* Pyr^- strain. These hybrids are useful for kinetic and expression studies of ATCase in *E. coli*.

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INTRODUCTION

The study of pyrimidine biosynthesis is important due to the role that pyrimidines play in forming the genetic material that is necessary for cellular growth and for the passing of genetic information to subsequent generations. Pyrimidines serve as building blocks in the informational macromolecules ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). They are also found in all cell membranes as UDP-glucose or dTDP-rhamnose for example. Pyrimidines are found in all organisms and are six-membered, aromatic heterocyclic ring compounds. Pyrimidine nucleosides consist of nitrogenous bases, cytosine and thymine in DNA and cytosine and uracil in RNA, linked to a pentose sugar. In DNA the sugar is 2'-deoxyribose, whereas in RNA it is ribose. Nucleotides are nucleosides with phosphate groups added.

Uridine-5'-monophosphate (UMP; Fig. 1) serves as a precursor for all the pyrimidine nucleotides. The biosynthesis of UMP, referred to as the pyrimidine biosynthetic pathway, is accomplished in six enzymatic steps. This pathway appears to be universal and follows

the same sequence in all organisms thus far studied including bacteria, fungi, plants, and animals (O'Donovan & Neuhard, 1970; Neuhard & Kelln, 1996).

Pyrimidine biosynthetic pathway.

The enzyme carbamoylphosphate synthetase (CPSase, EC 6.3.5.5) catalyzes the first step in pyrimidine synthesis. The reaction utilizes bicarbonate, ammonium ions or glutamine, and consumes two molecules of adenosine-5'-triphosphate (ATP) leading to the formation of one molecule of carbamoylphosphate and adenosine-5'-diphosphate (ADP) (Anderson & Meister, 1965; Kalman *et al*., 1966). Carbamoylphosphate is required for both arginine and pyrimidine synthesis (Abdelal *et al*., 1969).

The first committed step in pyrimidine biosynthesis is the production of carbamoylaspartate (CAA) by aspartate transcarbamoylase (ATCase, EC 2.1.3.2). Aspartate is carbamoylated at the amino group, producing CAA and releasing inorganic phosphate. The enzyme dihydroorotase (DHOase, EC 3.5.2.3) then catalyzes the cyclization of CAA, resulting in the release of a molecule of water and production of dihydroorotate (DHO).

Following this, DHO is oxidized to orotate (OA) in a reaction catalyzed by dihydroorotate dehydrogenase (DHOdehase, EC 1.3.3.1). The first pyrimidine nucleotide is then produced by the transfer of ribose-5'-phosphate from 5'-phosphoribosyl-1'-pyrophosphate (PRPP) to OA to form orotidine-5'-monophosphate (OMP), a reaction catalyzed by orotate phosphoribosyltransferase (OPRTase, EC 2.2.4.10). OMP is decarboxylated by the enzyme OMP decarboxylase (OMPdecase, EC 4.1.1.23) in the final step in the production of UMP.

UMP serves as a precursor for the production of the pyrimidine nucleoside triphosphates, uridine-5'-triphosphate (UTP) and cytidine-5'-triphosphate (CTP). In sequential steps, UMP is further phosphorylated to uridine-5'-diphosphate (UDP) by the highly specific UMP kinase (EC 2.7.4.4). A non-specific enzyme, nucleoside diphosphate kinase (NDK, EC 2.7.4.6), further phosphorylates UDP to form UTP. CTP synthetase (EC 6.3.4.2) is responsible for conversion of UTP to CTP by transferring an amino group from glutamine.

Aspartate Transcarbamoylase.

Aspartate transcarbamoylase (ATCase) catalyzes the first committed step in pyrimidine biosynthesis (Fig. 1). Aspartate transcarbamoylase of *Escherichia coli* is the epitome of a regulatory enzyme, exhibiting all the hallmarks of allosteric kinetic behavior. Thus, ATCase has emerged as one of the best characterized bacterial enzymes to date. Three classes of bacterial ATCase (A-C) have been described, based on varying molecular weights of the holoenzyme, quaternary structure (Fig. 2), and enzyme kinetics (Bethell & Jones, 1969; Wild *et al*., 1980).

Representative of Class C ATCase: *Bacillus subtilis.*

A representative of the class C ATCase is found in *Bacillus subtilis* (Brabson and Switzer, 1975). Class C ATCases are 100 kDa catalytic trimers that are not associated with any other polypeptides. This form of ATCase is not regulated by nucleotide effectors in *Bacillus* or *Staphylococcus* and thus displays Michaelis-Menten saturation kinetics when velocity-substrate plots are made. However, the ATCase of wheat (Yon, 1984), also found as a catalytic trimer, exhibits a sigmoid curve when velocity-

substrate plots are made, indicating that it is also regulated.

Representative of Class B ATCase: *Escherichia coli.*

The *E. coli* ATCase is a representative of the class B ATCases. Class B ATCase holoenzymes have a molecular mass of ~310 kDa, are composed of two catalytic homotrimers coupled to three regulatory homodimers, are allosterically regulated and bind substrates cooperatively which results in sigmoidal saturation kinetics when velocity-substrate plots are made. The ATCase of *E. coli* is activated by ATP and inhibited by CTP and UTP in the presence of CTP (O'Donovan *et al.*, 1989). The genes *pyrBI* of *E. coli* (Pauza *et al.*, 1982; Roof *et al.*, 1982) encode the dodecameric enzyme form of aspartate transcarbamoylase (Fig. 3). Six identical catalytic polypeptides, organized as two enzymatically functional catalytic trimers (c_3) and six identical regulatory polypeptides organized as three regulatory dimers (r_2) define the holoenzyme. The $2c_3{:}3r_2$ dodecameric structure is conserved among the enteric ATCases. *E. coli pyrB* encodes the catalytic polypeptide of 34 kDa, while *pyrI* encodes the regulatory polypeptide of 17 kDa. In *E. coli* the nucleotide effectors bind to the

dimeric regulatory subunits encoded by the *pyrI* gene (red arrow in Fig. 3).

Representative of Class A ATCase: *Pseudomonas putida.*

The *P. putida* ATCase is a representative of the class A ATCase (Schurr *et al.*, 1995). Class A ATCase holoenzymes have molecular masses of about 480 kDa, contain a dodecameric structure, and exhibit hyperbolic curves when velocity-substrate plots are made. Pyrophosphate, ATP, UTP, and CTP inhibited the ATCase of *P. putida*, examined by Condon *et al*. (Condon *et al.*, 1976). The genes *pyrBC'* of *P. putida* (Schurr *et al.*, 1995) encode the dodecameric holoenzyme form of aspartate transcarbamoylase (Fig. 3). The *pyrB* gene encodes a 36 kDa polypeptide that does not produce an active trimer as does *E. coli* and *Bacillus* (Fig. 4 and 5). To be active, the *pyrB* gene requires the participation of the overlapping *pyrC'* gene which, encodes a DHO-ase like 46 kDa PyrC' polypeptide to produce a 480 kDa dodecameric holoenzyme. The enzyme is regulated by ATP and to a lesser extent by UTP and CTP (Schurr *et al.*, 1995). The 46 kDa chain is not a homolog of the class B 17 kDa regulatory polypeptide, since the binding site for

nucleotide effectors has been located on the 36 kDa chain (Shepherdson & McPhail, 1993).

Background information and research.

Computer comparisons of *P. putida* ATCase with other known ATCases using the Pileup program of the University of Wisconsin GCG Package revealed an 11 amino acid extension at the amino terminus. This 11 amino acid overhang did not align with any other known bacterial or eukaryotic ATCase amino acid sequences. A comparison of the amino acids of the overhang with those of the regulatory polypeptide of *E. coli* suggested that the N-terminal extension contained a possible nucleotide binding site for effectors (Kumar *et al.*, 1994).

From this information the following hypothesis was derived: namely that the effector binding site (regulatory region) for the *P. putida* ATCase holoenzyme is located at the N-terminus region of the *pyrB* encoded 36 kDa polypeptide. Three lines of evidence suggest that this binding site appears at the N-terminus. (1) Bergh & Evans, 1993, in *P. fluorescens* used labeled ATP to show that ATP bound to the 36 kDa polypeptide encoded by the *pyrB* gene, (2) GCG motif computer analysis showed that a putative ATP

binding site was located at the N-terminus region of the *pyrB* gene of *P. putida* (Schurr *et al*., 1995), and (3) Alan Kumar showed by constructing various deletion mutants of the *P. putida* ATCase, that ATP binding was indeed localized at the N-terminus region of the *pyrB* gene (Kumar *et al*., 1994).

A second hypothesis postulates that the 11 amino acid overhang of *P. putida* PyrB does not allow for proper trimer formation thereby precluding catalytic trimer activity (Fig. 5) (Kumar *et al*. 1999).

METHODS

Bacterial strains and plasmids used.

The bacterial stains and plasmids to be used are listed in Table 1.

Table 1: Strains and Plasmids used.

Strain or plasmid	Genotype or Description	Source
Strains		
E. coli TB2	*pyrBI, argF*	W. D. Roof
E. coli DH5α	F^-, ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*) U169, *deoR, recA1, endA1, hsdR17,* ($r_k^- m_k^-$, *supE44* λ^-, *thil-1, gyrA96, relA1*	GIBCO-BRL
Plasmids		
PALTER®-*Ex*1	Tet, Amp, selectable markers, SP6, T7, *tac* promoters, *lacZ*	PROMEGA
pAK10	*P. putida pyrB*; 1-kbp insert in pUC19 with orientation identical to that of the ***lac* p**romoter	A. P. Kumar
pBJR28	*E. coli pyrB;* 1-kbp insert in pUC18	J. R. Ruley
pDBPC′	*P. putida pyrC′;* 2.3-kbp insert in pK184	D. Brichta

DH5α allows for the prevention of undesirable restriction of cloned DNA and recombination with host chromosomal DNA because DH5α is *recA*- and lacks the *E. coli* K restriction system. DH5α is also *lacZ- (Δ α-lac)* which allows for blue-white colony selection when complemented with the α-polypeptide encoded by various vectors. TB2 is a strain of *E. coli* with an extensive deletion within the *argI-pyrBI* region that eliminates ATCase activity.

Media and growth conditions.

E. coli strains DH5 and TB2 were grown and maintained in Luria-Bertani (LB) broth (10 g/l Difco Bacto-Tryptone, 5 g/l Difco Yeast Extract, and 10 g/l sodium chloride) or LB agar (15 g/l Difco Agar). *E. coli* TB2 was also grown in defined medium, *E. coli* minimal medium (Ecmm) broth (10.5 g/l dibasic K_2HPO_4, 4.5 g/l monobasic KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, 0.5 g/l Na_3citrate, 1 ml 1 M $MgSO_4$, 1 ml 1000x B1 (thiamine), 10 ml 20% dextrose or .2% carbon source) or Ecmm agar (15 g/l Difco Agar). These cultures were grown at 37°C, and liquid cultures shaken at 250 rpm in a New Brunswick Scientific Co. Series 25 Incubator Shaker. Antibiotics were added to the medium depending upon which

plasmids the cells contained, as follows: cells containing pAK10, pBJR28, pBpals, pBEaAS, pNELLS, pNPS, pCELLS, pCPS, pBEP, and pUC19 received 100 μg of ampicillin per ml, cells which contained pALTER®-Ex1, pBpals, and pBEaAS received 12.5 mg tetracycline in 80% ethanol per ml, and cells which contained pDBPC' received 60 g kanamycin per ml.

Preparation of cell extracts.

Cell extract was prepared by breaking the cells using sonication. Cell pellets from freshly harvested cells were resuspended in the appropriate breaking buffer for the assays to be performed. Typically, 1 ml of ATCase breaking buffer (2 mM β-mercaptoethandol, 20 μM $ZnSO_4$, and 50 mM Tris-HCl, pH 8.0) was added per 1 g wet weight of pellet. The cell suspension was sonicated using a Branson Cell Disruptor 22 for 5 min sonication per sample. The power setting were set at 3 for output control and pulse for a 50% duty cycle. The tip contained a diameter of 5 mm capable of vibrating at 20 kiloHertz. The sonicated suspension was transferred to a 50 ml oak ridge and centrifuged at 4°C for 30 min at 33,000 x g in a Sorvall RC5 (SA600 rotor). The resulting supernatant was transferred

to a sterile 15 ml, disposable conical tube and stored at 4°C until the cell extract was analysed by ATCase activity gel.

Preparation of competent cells for transformation.

Escherichia coli DH5α and TB2 competent cells were produced according to a slight modification of the procedure of Dagert & Ehrich (1979). A single colony of *E. coli* DH5α or TB2 was picked from a LB agar plate with a sterile inoculating loop and used to inoculate 5 ml of sterile LB broth. The bacterial culture was grown overnight at 37°C with shaking at 250 rpm. A sterile flask containing 50 ml of LB Broth was then inoculated with 500 µl of the overnight culture and grown, typically 2-3 hours (h) at 37°C with shaking at 250 rpm until an absorbency reading at 650 nm reached between 0.2 and 0.5. A loopful of the culture was then streaked onto an LB plate, which was incubated overnight at 37°C to check purity. The remainder of the cell culture was then transferred aseptically to a 50 ml disposable, conical tube and chilled on ice for 20 min.

The cells were harvested by centrifugation in a Sorvall RT6000B Refrigerated Centrifuge for 10 min at 4°C in a Sorvall H1000B rotor at 1,800 x g. The supernatant was poured off, and the pellet was resuspended in 20 ml of cold (0-4°C) 0.1 M $CaCl_2$. Then, the cells were harvested by centrifugation at 833 x g for 15 min. After the supernatant was poured off, cells were resuspended by gentle vortexing in 2 ml of 0.1 M $CaCl_2$ and then stored overnight on ice in a 4°C refrigerator. Next, 150 µl of sterile 100% glycerol was added and mixed to achieve a final concentration of 15% glycerol. Aliquots of 400 µl were distributed to 0.5 ml microcentrifuge tubes and either kept on ice for use within a few hours or stored at -80°C for up to 1 month.

Transformation of DNA into *E. coli* DH5α and TB2 strains were carried out using the heat shock method of Mercer and Loutit, 1979.

Isolation of plasmid DNA by alkaline lysis.

Plasmid DNA (pDNA) was isolated according to the alkaline lysis method (Zhou *et al.*, 1990). After incubation of transformed cells overnight at 37°C with

shaking at 250 rpm, 1.5 ml of the 5 ml culture was transferred to a sterile 1.5 ml microcentrifuge tube. The 1.5 ml culture was centrifuged for 1 min. at 10,000 x g in a Savant Speed Fuge HSC10K microcentrifuge while the remainder of the overnight culture was stored at 4°C. The supernatant was poured off and the bacterial pellet resuspended by vortexing in 300 µl TENS solution (77.5% Tris-EDTA (TE) Buffer, pH 8, 0.1 M NaOH, and 0.5% SDS). This tube was then placed on ice and 150 µl of 3 M sodium acetate, pH 5.2, was added. Then the contents of the tube were mixed by gentle vortex and placed on ice. In order to pellet the cell debris and most of the chromosomal DNA, the tube was centrifuged for 2 min at 10,000 x g at 4°C, and the supernatant, approximately 450 µl, was transferred to a fresh, 1.5 ml microcentrifuge tube. Next, two volumes, 900 µl, of 100% cold ethanol (EtOH) was added and the tube was placed on dry ice (-78.5°C solid carbon dioxide (CO_2)) for at least 10 min. To pellet plasmid DNA, the tube was centrifuged at 10,000 x g for 10 min at 4°C and the supernatant was removed by pouring off and pulling remaining excess off with a drawn-out Pasteur pipette. To

remove residual salts, 1 ml of -20°C (v/v) 70% EtOH was added, tube inverted, and then centrifuged for 5 min at 10,000 x g at 4°C. The supernatant was removed by pouring off and pulling remaining excess off with a drawn-out Pasteur pipette, and the pellet was dried for approximately 2-3 min in a Savant Speed Vac™ Concentrator. The plasmid DNA pellet was resuspended by vortexing in 30-40 µl of sterile ddH_2O. To degrade any RNA present in the sample, 1 µl of RNase A, pre-heated for 5 min in a 65°C water bath, was added and mixed briefly on a vortex mixer. After a brief centrifugation to collect the tube contents at the bottom of the tube, the tube was placed in a 37°C water bath for 30 min. All plasmid DNA was analyzed by agarose minigel electrophoresis by loading 5 µl of plasmid DNA combined with 1 µl of 5X Loading Buffer on a 1% agarose gel. The agarose gel was placed in 1X TAE and electrophoresed a 55 V for approximately 1 h 15 min. The remaining plasmid DNA was stored at 4°C.

Genetic manipulations.

Fill-in reactions, 3' overhang removal to form blunt ends or 5' overhang fill-in to form blunt ends, were carried out at 12°C for 20 min using T4 DNA polymerase and a 0.2 mM final concentration of dNTPs (New England Biolabs, Beverly, MA). Phenol extraction, ether extraction, and ethanol precipitation followed in order to remove the polymerase. Restriction digestions and ligations were performed according to the specifications of the manufacturer (New England Biolabs, Beverly, MA).

Agarose minigel electrophoresis.

All DNA fragments, including those from PCR, rapid plasmid preparations, and restriction enzyme digestions, were analyzed by agarose gel electrophoresis. The gel was prepared by addition of agarose to 1% (w/v) in TAE buffer (40 mM Tris-Acetate and 1 mM EDTA, pH 8.0). The components were swirled to mix, weighed, and then brought to a boil on a hot stir plate. Once all the agarose was in solution, the mixture was allowed to cool briefly and then weighed again. Distilled H_2O was added to replace the fluid lost by boiling. Then 1 µl of 10 mg/ml ethidium bromide (EtBr) was

added to the gel and allowed to mix for a 1% final gel concentration. The gel mix was poured into a gel tray containing a comb and allowed to cool until solidified. The comb was then removed.

The gel tray containing the gel was placed in an electrophoresis chamber containing 1X TAE buffer. DNA samples were prepared for loading on the gel by mixing predetermined amounts of DNA and 5X sample loading buffer (25% v/v glycerol, 0.5% w/v SDS, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol, and 50.0 mM EDTA) and then adding the mixture to the desired well. Gels were electrophoresed for approximately 1 h 15 min at 55 V. When DNA bands were hard to visualize, the DNA was stained by placing the gel in an ethidium bromide solution (0.5 μg ml^{-1}) for 30 min followed by rinsing and destaining in ddH_2O to remove residual ethidium bromide from the gel. The gel was then observed and photographed over UV radiation using a Fotodyne transilluminator and Polaroid MP-4 camera. Polaroid type 55 film was used in order to generate positives as well as negatives, and exposure and development times were typically 35 s and 25 s,

respectively. All negatives were rinsed for 10 min under tap water before being air-dried.

Purification of DNA fragments.

Immediately following separation of DNA fragments by agarose gel electrophoresis, DNA bands of interests were purified by electroelution. DNA recovery is achieved by electrophoresis of the DNA from a small slice of electrophoresis gel into a sealed piece of buffer filled dialysis tubing.

A razor blade was used in order to excise the slice of agarose containing the DNA band of interest. Care was taken to cut as close to the DNA band as possible. A 10 mm dialysis tubing of approximately 6 inches in length was thoroughly rinsed with distilled H_2O both inside and outside of the tubing and handled with gloves. A dialysis bag clip was placed at one end of the tubing and the excised agarose was placed in the dialysis tubing using a narrow spatula. Approximately 150 µl of 0.25X TAE buffer was placed inside the dialysis tubing and the other end of the dialysis tubing was closed with another dialysis bag clip making sure no air bubbles were present within the closed dialysis

tubing. The dialysis bag was then placed into a horizontal agarose gel electrophoresis unit. The unit was filled with 0.25X TAE buffer until it just covered the dialysis tubing. A rubber stopper was placed on top of the clips in order to weigh down the dialysis tubing. Electroelution then proceeded for 2 h at 163 V. Upon completion of electroelution, the current was reversed for 10 s by switching the red and black leads. Using a Fisher brand micropipette tip, the buffer containing desired DNA was carefully removed from the bag and placed in a 1.5 ml microcentrifuge tube. The gel within the dialysis tubing was rinsed with 100 µl of 0.25X TAE buffer and transferred to the microcentrifuge tube.

In order to remove any contaminating proteins, a phenol extraction was employed. One volume of water saturated phenol, pH 8, approximately 250 µl, was added to the tube under the hood while wearing protective gloves. The tube was vortexed and then centrifuged at 10,000 x g for 1 min. The bottom layer of phenol was then removed with a drawn-out Pasteur pipette. This phenol extraction was repeated once more to insure the extraction of proteins.

The following ether extraction was performed twice to remove the residual phenol. One volume of ether, approximately 250 µl, was added to the microcentrifuge tube under the hood, vortexed briefly, and then centrifuged at 10,000 x g for 10-15 s. The ether, or top layer, was removed under the hood with a drawn-out Pasteur pipette.

Next, the tube was centrifuged at 10,000 x g for 10 min to pellet any residual agarose debris. The supernatant was transferred to a new microcentrifuge tube with a drawn-out Pasteur pipette. The new microcentrifuge tube was centrifuged at 10,000 x g for 5 min and the supernatant was again transferred to a new microcentrifuge tube with a drawn-out Pasteur pipette to ensure agarose debris removal.

The final step was to ethanol precipitate the DNA. One tenth the volume, about 25 µl, of 3 M sodium acetate and three volumes, about 750 µl, of 100% cold EtOH were added to the microcentrifuge tube. The tube was vortexed and placed in –80 C freezer or on dry ice for 4-10 min, followed by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant was removed with a drawn-out Pasteur pipette and discarded. To remove residual salts, 1 ml of -20°C (v/v) 70% EtOH was added, tube inverted, and then

centrifuged for 5 min at 10,000 x g at 4°C. The supernatant was removed with a drawn-out Pasteur pipette, and the pellet was dried for approximately 2-3 min in a Savant Speed Vac™ Concentrator. The DNA pellet was resuspended by vortexing in 7-30 µl of ddH_2O. For verification of DNA purity and yield, and analysis of a small quantity of the eluted fragment by agarose gel electrophoresis was generally carried out.

Design of mutagenic oligonucleotides.

Oligonucleotide primers for polymerase chain reaction (PCR) were designed for PCR cloning and PCR site-directed mutagenesis. Two primers were designed for each gene terminus of interest. A forward primer matched the coding strand, and a reverse primer was prepared which matched the complementary strand. Primers were designed with restriction enzyme sites to facilitate plasmid cloning. For the single base mutations, four 24mer oligonucleotides were designed with the mismatch located at the center (Fig. 6). The mutagenic oligonucleotides produce a silent mutation, which does not change the amino acid sequence, creating an *Mlu* I restriction enzyme site within the

conserved carbamoylphosphate domain of the *pyrB* gene (Fig. 6). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and Biosynthesis (Lewisville, TX). The primer sequences and locations are detailed in Table 2.

Table 2. Oligonucleotide primers for PCR.

Primer Name	Description	Sequence (5'-3')
EcBALTF	pBEaAS forward primer	GAG GCC TCT AGA CTG CAG AAT CAC
Ecmut-40	*E. coli pyrB* -40 bp upstream from STRT conserved site	CCA GAG CTG TTG AAG CAC
EcmutM1R	*E. coli pyrB* reverse mutagenic primer	GAG GCG GGT ACG CGT AGA GGC TTC
EcmutF	*E. coli pyrB* forward mutagenic primer	GAA GCC TCT ACG CGT ACC CGC CTC
EcBALTR	pBEaAS reverse primer	CAG GAA ACA GGA TCC
PpBAF	*P. putida pyrB* forward primer	GCC CTC GAG CGC CAG G
Ppmut-40F	*P. putida pyrB* -40 bp upstream from STRT conserved site	GTC CCG TTG CTG CGC GGC
Ppmut3	*P. putida pyrB* reverse mutagenic primer	GGT GCG GGT ACG CGT CGA GTT CTC
Ppmut	*P. putida pyrB* forward mutagenic primer	GAG AAC TCG ACG CGT ACC CGC ACC
PpBBR	pBpals reverse primer	GGC GAA TTC GAG CTC

Polymerase chain reaction.

PCR was performed on DNA fragments ranging from 260 bp to 1.3 kpb in size. *Taq* DNA polymerase was used with elongation times of 30 s to 1 min, 50 s at 72 C. The *Taq* DNA polymerase was contained in the premixed, double-concentrated PCR master solution (Boehringer Mannheim, Indianapolis, IN). During sample preparation, all reagents

and reaction mixes were stored on ice. For a 50 µl total reaction volume placed in a thin-walled, 0.6 ml PCR tube, 25 µl of PCR master mix was added. This gives the final reaction mix 1.25 units of *Taq* DNA polymerase in 0.005% (w/v) Brij 35, 0.2 mM each of dATP, dCTP, dGTP, and dDTP, 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. An additional 0.6 µl of 25 mM $MgCl_2$ was added resulting in an overall concentration of 1.8 mM $MgCl_2$. Appropriate volumes of forward and reverse oligonucleotide primers were added to give 60 ng of each. Between 100-300 ng of template DNA were included in the reaction mix. The final reaction volume was achieved by addition of water to give a total of 50 l. The sample was layered with an equal volume of sterile mineral oil to minimize evaporation.

The PCR was performed on an MJ Research PTC-100 Thermal Programmable Controller (MJ Research, Inc., Watertown, MA). Initial denaturation was for 5 min at 94 C, followed by primer annealing for 2 min at a specific annealing temperature depending on the primers used. Elongation proceeded for 40 s to 1 min 15 s at 72 C depending on desired fragment size. The DNA was again denatured at 94 C for 2 min. The sample was cycled through

the annealing, elongation, and denaturation phases for a total of 30 times. After completion of these steps, an additional elongation period of 5 min was performed. The samples were subsequently stored at 4 C.

All products were analyzed by agarose gel electrophoresis. A 1.0% (w/v) agarose gel was used for analysis of PCR products of all fragment sizes. The products were typically prepared for loading on the gel by mixing 10 l with 2.5 l of 5X sample loading buffer.

Cloning of PCR products.

Cloning of purified PCR products was achieved using the TA Cloning Kit dual promoter (Invitogen, Carlsbad, CA). In order to avoid degradation of the single 3' A-overhangs on the PCR products and resulting reduction in ligation efficiency, ligations were always set up on the same day that PCR products were generated. Typically 3-5 µl (approximately 50-100 ng) of the PCR product was added to a 0.6 ml, thin walled microcentrifuge tube on ice. Next, 1µl of 10X Ligation buffer (60 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 50 mM NaCl, 1 mg ml^{-1} bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol, 10 mM

spermidine), 2 μl of pCR II vector (25 ng μl^{-1}), and sterile water to a total volume of 9 μl were included in the PCR tube. The addition of 1 μl of T4 DNA Ligase (4.0 Weiss units) brought the total ligation reaction volume to 10 μl. The ligation reaction was incubated at 14°C for 16 h in an MJ Research PTC-100 Thermal Programmable Controller.

Transformation was achieved by adding the entire 10 μl ligation reaction into 200 μl of competent *E. coli* DH5α in a 1.5 ml microcentrifuge tube. The mixture was incubated on ice for 20 min and then heat shocked in a 42°C water bath for 2 min. The mixture was then diluted with 1 ml of LB broth and incubated for 1 h with shaking at 250 rpm at 37°C. During this incubation period, 50 μl of 2% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and 10 μl if 100 mM isopropylthiogalactoside (IPTG) were added to three LB agar + Ampicillin (Amp, 100 μg ml^{-1}) plates per ligation with a sterile spreader. These LB plates were then placed in a 37°C incubator for 30 min to allow the added reagents to dry.

Following the 1 h incubation, 100 μl and 20 μl of the transformed cells were plated onto two different LB + Amp +

X-gal + IPTG plates using a sterile spreader. The remaining transformed cells were pelleted by centrifugation at 10,000 x g for 4-5 s. The supernatant was poured off and the pellet was resuspended in the remaining drop by brief mixing with a vortex mixer. The resuspended cells were then plated onto the remaining LB + Amp + X-gal + IPTG plate. These plates were then incubated at 37°C for 16 h to 24 h.

The pCR II vector contained an ampicillin resistance gene, thus allowing transformed cells to grow in ampicillin. Fifteen white colonies (containing *E. coli* DH5α with pCR II vector + insert) and one blue colony (containing *E. coli* DH5α with pCR II vector only), selected to serve as a control, were selected from the transformation plates and inoculated into 5 ml LB broth containing Amp (100 μg ml^{-1}) by using sterile toothpicks. The transformed cells were incubated 12 h to 24 h at 37°C with shaking at 250 rpm.

Analysis of plasmid DNA by restriction enzyme digestion.

To determine which plasmids had inserts of the desired size, a restriction enzyme digestion was employed in order

to remove the insert from the pCR II vector. The restriction enzyme, *Eco*RI (New England Biolabs, Beverly, MA), was chosen based on the fact that it has only two cut sites in the pCR II vector, which reside on either side of the insert. Therefore, *Eco*RI digestions of plasmid DNA should produce only two bands, 3.9 kbp for the vector and the desired length for the insert, when separated by agarose gel electrophoresis.

To set up a restriction enzyme digest, the following contents were added to a 0.6 ml microcentrifuge tube: 4-5 µl of plasmid DNA isolated by alkaline lysis, 1 µl of *Eco*RI restriction enzyme, 2 µl of 10X *Eco*RI buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.025% Triton X-100, pH 7.5 @ 25°C), and ddH_2O up to a total volume of 20 µl. The contents were then incubated in a 37°C water bath for 2 h, followed by heat inactivation of the *Eco*RI in a 65°C water bath for 20 min. The *Eco*RI digest was then analyzed by loading a combination of 20 µl of the *Eco*RI digest and 5 µl of 5X Loading Buffer on a 1% agarose gel placed in 1X TAE. The sample was electrophoresed at 55 V for 1 h 15 min.

Preparation of plasmid DNA for sequencing.

After plasmid analysis by restriction digestion, plasmids believed to be a desired clone were further purified for double stranded (ds) DNA sequencing. Of the remaining 3.5 ml of the desired overnight culture previously used for isolation of plasmid DNA by alkaline lysis, 10 µl was removed and used to inoculate 10 ml of LB broth containing freshly added antibiotic of the appropriate kind and amount. This culture was incubated for 16-24 h at 37°C with shaking at 250 rpm.

Plasmid DNA was purified from this culture using the RPM Spin Midi Kit and protocol (BIO 101, Inc., La Jolla, CA). Specific reagents included in each solution were not available from BIO 101. The 10 ml LB broth culture was transferred to a 15 ml sterile centrifuge tube and harvested by centrifugation in a RT6000B Refrigerated Centrifuge for 10 min at 4°C in a Sorvall H1000B rotor at 1800 x g. The supernatant was poured off, and the pellet was resuspended in 500 l of sterile ddH_2O by vortex. Cells were transferred to a 2 ml microcentrifuge tube and centrifuged at 10,000 x g for 20 s. Again the supernatant was poured off. The supernatant was poured off and the

remaining excess pulled off with a drawn-out Pasteur pipette. At this stage, the cell pellet was either stored at -20°C until ready to process or used immediately. Those pellets that were stored at -20°C were first thawed for 8 min at 37°C before continuing with the following plasmid purification.

Plasmid DNA isolation and purification began with the addition of 200 µl of Pre-Lysis Solution. The bacterial pellet was completely resuspended in this solution by mixing on a vortex mixer. Next, 400 µl of Alkaline Lysis Solution was added, and the tube was inverted gently 15 times. Following this, 300 µl of ice-cold Neutralizing Solution was added, and the tube was shaken vigorously 3-5 times until a uniform white precipitate formed. The tube was then centrifuged for 5 min at 10,000 x g, and the supernatant was carefully transferred to a new 2 ml microcentrifuge tube.

GLASSMILK Spin Buffer containing a silica matrix was incubated for 10 min in a 65°C water bath, and then 900 µl of this Spin Buffer was added. The tube was inverted gently for 5 min at room temperature and then centrifuged for 5 s at 10,000 x g at room temperature. The supernatant

was decanted, leaving the plasmid DNA bound to the GLASSMILK, and 500 µl of Wash Solution containing EtOH was added. The GLASSMILK/plasmid DNA complex was resuspended by gentle pipetting up and down, and the solution was then transferred to a kit-supplied SPIN Filter. This filter was centrifuged for 20 s at 10,000 x g to separate the GLASSMILK/plasmid DNA complex from the Wash Solution. The Catch Tube containing the Wash Solution was emptied and the Spin Filter reassembled. A second wash was performed by adding an additional 500 µl of Wash solution and recentrifuging for 5 min at 10,000 x g to dry the GLASSMILK/plasmid DNA complex trapped by the Spin Filter. The Spin Filter was then transferred to a new kit-supplied Catch Tube, and 100 µl of Elution Solution containing Tris-HCl and EDTA was added to remove the plasmid DNA from the GLASSMILK. The plasmid DNA was eluted by gently stirring and pipetting the solution up and down with a 200 µl pipette until the GLASSMILK/plasmid DNA complex was completely resuspended. The Catch Tube was then centrifuged at 10,000 x g for 5 min to collect the eluted DNA in the Catch Tube. The Spin Filter containing the GLASSMILK was discarded. The eluted plasmid DNA was analyzed by agarose minigel

electrophoresis by loading a combination of 5 µl of the eluted plasmid DNA with 1 µl of 5X Loading Buffer on a 1% agarose gel in 1X TAE.

Double stranded DNA sequencing.

In order to verify the genetic makeup of the clones created, the plasmids were analyzed by double stranded DNA sequencing using the dideoxyribonucleotide method of chain termination (Sanger *et al*., 1977). Sequencing reactions were set up according to a slight modification of the protocol provided for the T7 Sequenase™ version 2.0 DNA sequencing kit (Amersham Life Science, Inc., Cleveland, OH). For each plasmid to be sequenced, both M13 Forward (-40) and M13 Reverse primers were utilized, thus allowing sequence to be read from opposite ends of the insert. Approximately 5 µg of plasmid DNA was placed into a 1.5 ml microcentrifuge tube, and ddH_2O was added to achieve a total volume of 30 µl. To denature the DNA, 3 µl of freshly prepared 2 N NaOH was added, and the mixture was incubated at room temperature for 5 min. Next, 120 µl of –20°C 100% EtOH was added along with 5 µl of 3 M sodium acetate, pH 5.0. This was mixed and incubated at –78.5°C in solid CO_2

for 5 min. This was then centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was removed without disturbing the pellet using a siliconized, drawn-out Pasteur pipette. To remove residual salts, 200 µl of –20°C 70% EtOH was added, and the tube was centrifuged for 5 min at 10,000 x g at 4°C. The supernatant was again removed with a siliconized, drawn-out Pasteur pipette. This 70% EtOH wash was repeated, and the pellet was dried for 3-5 min in a Savant Speed Vac™ Concentrator. The pellet was resuspended in 7 µl ddH_2O with two freeze-thaw cycles consisting of incubation of the tube at –78.5°C in solid CO_2 for 10 min followed by a 5 min incubation in a 65°C water bath. Following each 65°C incubation, the plasmid DNA and ddH_2O were mixed with a vortex mixer and centrifuged at 10,000 x g for 4 s to collect the mixture at the bottom of the tube.

Following this initial denaturation and cleanup, 2 µl of T7 Sequenase Reaction Buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, and 250 mM NaCl) from the T7 Sequenase Kit was added along with 1 µl of either M13 Forward (-40) or M13 Reverse primers (0.5 pmol μl^{-1}). Incubation at 37°C for 45 min allowed the primer to anneal to the template. After

placing this tube on ice, four 0.5 ml tubes were labeled and filled with 2.5 µl of the appropriate termination mix (80 µM dGTP, 80 µM dCTP, 80 µM dTTP, 50 mM NaCl, and 8 µM of the appropriate ddNTP) from the red-capped tubes from the T7 Sequenase Kit. The termination tubes were stored on ice until use. In another 0.5 ml tube, a 1:5 diluted labeling mix was prepared, usually by adding 2 µl of the T7 Sequenase dGTP Labeling Mix (7.5 µM dGTP, 7.5 µM dCTP, and 7.5 µM dTTP) from the green-capped tube to 8 µl of ddH_2O. The 1:5 Labeling Mix was stored on ice. Diluted T7 Sequenase DNA polymerase was prepared in a separate 0.5 ml tube by adding the following components on ice, all from the T7 Sequenase Kit: 1.2 µl of inorganic pyrophosphatase (5 U ml^{-1} in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 50% v/v glycerol), 7.2 µl of glycerol enzyme dilution buffer (20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA, and 50% v/v glycerol), and 1.2 µl of T7 Sequenase™ version 2.0 DNA polymerase (13 U $µl^{-1}$ in 20 mM KPO_4, pH 7.4, 1 mM DTT, 0.1 mM EDTA, and 50% v/v glycerol).

The four termination tubes were then pre-warmed in a 39°C heating block. The following components were added to the ice-cold annealed DNA mixture: 1 µl of 0.1 M

Dithiothreitol (DTT from the T7 Sequenase Kit), 2 μl of the 1:5 Labeling Mix, 1 μl of Dimethylsulfoxide (DMSO), 0.5 μl of Redivue [α-^{35}S] dATP (12.5 μCi μl^{-1}), and 2 μl of diluted T7 Sequenase DNA Polymerase. This mixture was centrifuged at 10,000 x g for 3 s. To allow chain termination to occur, 3.5 μl of the labeling reaction mixture was added to each of the four termination tubes and incubated for 20 min at 39 °C. Next, 4 μl of Stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) from the T7 Sequenase Kit was added to each termination tube and mixed by pipetting up and down. The tubes were then centrifuged at 10,000 x g for 3 s and frozen at –20 °C.

Some DNA sequence reactions were performed and provided by *ACGT*, Inc. (Northbrook, IL).

Preparation of polyacrylamide sequencing gels.

A gel cassette was assembled to provide a reservoir for the sequencing gel. The sequencing plates measured 50.8 cm x 40.6 cm and were cleaned twice with glass cleaner followed by 100% EtOH. The inside of the notched plate was siliconized with chlorinated organopolysiloxane in heptane to prevent the gel from adhering to this plate. The

outside of the non-notched plate had a thermometer so that the temperature of the gel could be regulated. After adding spacers to the long dimensions of one plate, the other gel plate was positioned on top of the plate with the spacers, and all of the sides except for the top were taped together.

All samples were analyzed on a 6% polyacrylamide sequencing gel prepared by mixing the following components in a 250 ml beaker: 42.4 g of urea, 20 ml of a 29% acrylamide/1% bis-acrylamide mixture stored at 4°C, and 10 ml of 10X TBE sequencing buffer (890 mM Tris base, 890 mM boric acid, and 20 mM EDTA). These first three ingredients were stirred and heated until they dissolved, and then ddH_2O was added up to 100 ml. The mixture was again stirred until it dissolved completely. Undissolved particles were removed from the solution by filtering it through a Buchner funnel containing a piece of Whatman No. 50 filter paper, and the filtrate was collected in a 250 ml Erlenmeyer vacuum filtration flask. Next, 0.12 g of solid ammonium persulfate was added and the solution swirled to mix. After covering the flask with a rubber stopper, the solution was degassed *in vacuo* until all gasses were

removed (about 3-5 min), and the contents were then carefully poured into a 250 ml beaker.

Immediately before pouring the solution into the gel cassette, 20 µl of N, N, N', N' -Tetramethylethylenediamine (TEMED) was added, and the solution was briefly stirred with a stir bar and magnetic stirrer. The gel was then poured by pulling up 60 ml of the solution into a 60 cc syringe and then slowly and steadily discharging the solution into one corner of the gel plates, which had previously been set at a 45° angle. The gel cassette was then placed on rubber stoppers in a horizontal position, and the comb was inserted with its teeth flush with the outside edge of the plate. Clamps were placed over the two plates at the level of the comb in order to prevent movement of the comb, and plastic wrap was used to cover the open end of the plate to prevent the gel from drying out. The gel was allowed to polymerize for a minimum of 3 h (preferably overnight).

Loading and electrophoresing the sequencing gel.

After allowing the gel to polymerize, the shark's tooth well-forming comb and the tape holding the two plates together were removed. The plates holding the gel were

then placed into the gel chamber apparatus, and 1X TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) was added to the upper and lower chambers. Air bubbles were removed from the bottom of the gel cassette, and the well-forming comb was inserted with the shark's teeth facing the top of the gel. A 60 cc syringe was used to eject 1X TBE at the top surface of the gel in order to remove any debris that was present. In order to warm up the plates to prevent renaturation of DNA, the gel was pre-electrophoresed for 1 h at 72 W (approximately 1600 V) after adding 2 µl of Stop solution to one of the outer wells on each side of the gel.

Once the plates had reached a temperature of 50-55°C, the power supply was turned off, and a needle and syringe was used to wash out each of the wells to be used with 1X TBE. Samples to be loaded were first denatured at 90°C for 2 min followed by a 5 min quick cool on ice. Next, 3 µl of sample was loaded in each well with the order from left to right always being GATC. Samples were electrophoresed at 72 W (approximately 1600 V) until the xylene cyanol dye had migrated a distance of 30 cm. The same samples were then reloaded, and the xylene cyanol was allowed to migrate 60

cm. The 60 cm run allowed sequence to be read further into the insert. Longer runs were employed when necessary.

Following completion of electrophoresis, the power supply was turned off, the plates were removed from the sequencing chamber and placed on the bench top, and the siliconized, notched plate was separated from the gel using a spatula. A 43 cm x 35 cm piece of Whatman 3 MM paper was placed on top of the gel, and a pipette was rolled on top of the Whatman paper to allow the gel to adhere to the Whatman paper. The Whatman paper, with gel adhered, was placed gel side up on the bench top, and the gel was covered with plastic wrap. The gel was dried for 3 h at 76°C in a Drygel Sr. Slab Gel Dryer Model SE 1160 supplied by Hoefer Scientific Instruments (San Francisco, CA) attached to a Savant refrigerated solvent trap.

Autoradiography of sequencing gels.

After the sequencing gel was dried completely, the Saran wrap was removed, and the gel adhered to Whatman paper was placed in a Fisher Biotech metal cassette with the gel side facing up. The number of counts min^{-1} was measured using a W. B. Johnson GSM-5 Survey Meter (Mountain Lakes, NJ) in order to determine the exposure time. In a

dark room, Kodak XAR-5 film was placed in a dark drawer to expose the film for approximately 72-120 h. The film was developed using an All Pro 100 developer.

Nondenaturing polyacrylamide activity gels.

Cell extract was prepared as described previously for the ATCase activity assay. Samples of typically 24 µl of cell extract were mixed with 6 µl of 5X loading buffer (312.5 mM Tris, pH 6.8, 50% v/v glycerol, and 0.05% w/v bromophenol blue in ddH_2O). The total volume of 30 µl was loaded onto a nondenaturing polyacrylamide gel (PAGE) with a 5% stacking gel and an 8% separating gel. Partially purified ATCase from *E. coli* K12 (holoenzyme and catalytic trimer) and *P. aeruginosa* PAO1 with known molecular mass were used as standards. The gel was run in a Bio-Rad Mini-Protean II cell at 100 V for 2.5 h at room temperature. The gel was prepared by first pouring the separating gel, which contained 2.67 ml of the stock solution of acrylamide (29% w/v acrylamide and 1% w/v bis-acrylamide in ddH2O), 2.5 ml of Buffer B (1.5 M Tris-HCl, pH 8.8), and 4.83 ml of ddH_2O. Ammonium persulfate (0.02 g) was added to the mixture to remove dissolved oxygen. Just prior to pouring the gel, 5 µl of N, N, N', N' - tetramethylethylenediamine

(TEMED) was added and mixed in by gentle inversion. The solution was poured into the gel plates leaving a 2 cm gap at the top. The separating gel was covered with a layer of N-butanol to prevent drying and allowed to stand at room temperature for 1 h to polymerize. The butanol layer was poured off, and the stacking gel poured. The stacking gel contained 0.67 ml of the stock solution of 30% acrylamide, 1 ml of Buffer C (0.5 M Tris, pH 6.8), and 2.3 ml of ddH_2O. Ammonium persulfate (0.01 g) and TEMED (5 µl) were added. Once the stacking gel was poured, a comb was inserted between the plates to form the wells. The stacking gel was allowed to polymerize for 30 min. The running buffer contained 25 mM Tris and 192 mM glycine in ddH_2O at a pH of 8.8.

The gels were stained specifically for ATCase activity by a procedure developed by Bothwell (Bothwell 1975) and further modified by K. Kedzie (1987), who used histidine instead of imidazole. In this study, one slight modification to the procedure were made as indicated. When the electrophoresis was complete, the plates were separated, and the gel was placed in 250 ml 50 mM histidine, pH 7.0, at 4°C, for 5 min while gently shaking.

After this equilibration, 5 ml of 1 M aspartate and 10 ml of 0.1 M carbamoylphosphate were added to the gel and allowed to react for 20 min (modified to extend time from 10 min to allow resolution of faint bands). The gel was rinsed 3 times with ice-cold ddH_2O to remove the reactants. Orthophosphate released enzymatically and trapped in the gel was precipitated 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 ddH_2O. ATCase activity was visible as white bands at the site of lead phosphate precipitation. Intensity of the bands generally increased with storage overnight at 4°C in ddH2O. For better visualization of the bands, the gels were sometimes stained with 1% sodium sulfide for 3 min. Brown lead sulfide then replaced the white lead phosphate bands. The gel was destained in ddH_2O to remove background.

To retain the gels in a permanent form, they were preserved by drying between two sheets of Promega gel drying film while suspended between a Promega gel drying frame. After placing in the apparatus, they were allowed to dry at room temperature overnight. The dried gel was removed from the frame and excess drying film removed from

the edges with scissors. The dried gel could be stored indefinitely.

RESULTS AND DISCUSSION

Clones pBEaAS and pBpals.

In preparation for site directed mutagenesis, two clones were constructed, *E. coli pyrB* and *P. putida pyrB* cloned into the pALTER®-*Ex*1 vector. The plasmid naming scheme for pBEaAS is as follows: **p**lasmid, *pyr**B***, ***E***. *coli*, p**A**LTER®-*Ex*1, Jesus **A**rroyo (researcher), and Luci **S**impson. The plasmid naming scheme for pBpals is as follows: **p**lasmid, *pyr**B***, ***P***. *putida*, p**A**LTER®-*Ex*1, **L**uci, and **S**impson. Plasmid pBEaAS (Fig. 7), which carries the *pyrB* of *E. coli* in pALTER®-*Ex*1, was constructed by restriction endonuclease digestion of pBJR28 and pALTER®-*Ex*1 with *Bam*HI and *Pst*I followed by ligation (Fig. 8). Plasmid pBpals (Fig. 9), which carries the *pyrB* of *P. putida* in pALTER®-*Ex*1, was constructed by restriction endonuclease digestion of pAK10 and pALTER®-*Ex*1 with *Eco*R1 and *Hin*dIII followed by ligation using T4 DNA ligase (Fig. 10). These ligated mixtures were then transformed into competent *E. coli* DH5 cells followed by selection on $LBamp_{100}$ plates. Restriction enzyme digestion and DNA sequencing (Sanger *et al.*, 1977) across

the vector-insert junctions (Figs. 11 & 12) have verified these constructs.

Restriction endonuclease digestion of pBEaAS with *Bam*HI and *Pst*I produced two DNA fragments, one of approximately 5.8 kbp in size, which represents the linear pDNA of the vector, pALTER®-*Ex*1, and the other of approximately 1.4 kbp in size, representing the insert DNA of *E. coli pyrB* (Fig. 11). Restriction endonuclease digestion of pBpals with *EcoRI* and *Hin*dIII produced two DNA fragments, one of approximately 5.8 kbp in size, which represents the linear pDNA of the vector, pALTER®-*Ex*1, and the other of approximately 1 kbp in size, representing the insert DNA of *P. putida pyrB* (Fig. 11).

Sequencing across the insert-vector junction of pBEaAS using both M13 Forward and M13 Reverse sequencing primers verified the presence of *E. coli pyrB* from pBJR28 within the pALTER®-*Ex*1 vector in orientation identical to that of the T7 promoter. Sequencing across the insert-vector junction of pBpals using both M13 Forward and M13 Reverse sequencing primers verified the presence of *P. putida pyrB* from pAK10 within the pALTER®-*Ex*1 vector in orientation identical to that of the *tac* promoter. These nucleotide

sequences were compared to known sequence using the NCBI BLAST algorithm tool (Altschul *et al.*, 1997) and are shown in Figure 12. Clones pBEaAS and pBpals were originally created in order to perform site-directed mutagenesis utilizing the pALTER -*Ex*1 system. Attempts made using the pALTER -*Ex*1 system produced no results. A change in strategy was developed and these clones were used to perform PCR site-directed mutagenesis instead.

Clone pBJR28.

Sequencing across the insert-vector junction of pBJR28 using both M13 Forward and Reverse sequencing primers clarified the exact identity and orientation of the insert DNA. This sequence was compared to known sequence using the NCBI BLAST algorithm tool and is shown in Figure 18. The sequence comparison found a partial section of *pyrI* to be inverted. From this sequence a detailed plasmid map of pBJR28 was constructed (Fig. 19).

Clone pAK10.

Sequencing across the insert-vector junction of pAK10 using both M13 Forward and Reverse sequencing primers

clarified the exact identity and orientation of the insert DNA. This sequence was compared to known sequence using the NCBI BLAST algorithm tool and is shown in Figure 20. From this sequence a detailed plasmid map of pAK10 was constructed (Fig. 21).

Clones pNELLS, pCELLS, pNPS, and pCPS.

In order to construct hybrid genes, an *Mlu*I restriction enzyme site was introduced at the carbamoylphosphate binding site within the *pyrB* genes of both *E. coli* and *P. putida*. This silent mutation was created by site-directed mutagenesis using PCR, which was also used to amplify all four domains individually (polar and equatorial domains of both *E. coli* and *P. putida*) and in so doing created a *Mlu* I restriction enzyme site in all four products. The original clones used for site-directed mutagenesis were pBEaAS and pBpals. The PCR product was then cloned into the PCR vector pCR™II. These four clones were named pNELLS (**p**lasmid, **N**-terminus, ***E****. coli*, **L**ayman (researcher), **L**uci **S**impson), pCELLS (**p**lasmid, **C**-terminus, ***E****. coli*, **L**ayman (researcher), **L**uci **S**impson), pNPS (**p**lasmid,

N-terminus, ***P****. putida*, **S**impson), and pCPS (**p**lasmid, **C**-terminus, ***P****. putida*, **S**impson).

Clones pNELLS and pCELLS (Fig.13) were created by PCR amplification of the desired *E. coli pyrB* terminus using pBEaAS as the template DNA. Amplification of the N-terminus for the construction of pNELLS was performed using oligonucleotides EcBALTF and ECBmutM1R, an annealing temperature of 44°C, and an elongation time of 1 min. Amplification of the C-terminus for the construction of pCELLS was performed using oligonucleotides EcBmutF and EcBALTR, an annealing temperature of 39°C, and an elongation time of 1 min. 15 s.

Clones pNPS and pCPS (Fig. 14) were created by PCR amplification of the desired *P. putida pyrB* terminus using pBpals as the template DNA. Amplification of the N-terminus for the construction of pNPS was performed using oligonucleotides PpBAF and Ppmut3, an annealing temperature of 60°C, and an elongation time of 40 s. Amplification of the C-terminus for the construction of pCPS was performed using oligonucleotides PpBBR and Ppmut, an annealing temperature of 45°C, and an elongation time of 1 min 15 s.

These PCR products were then ligated into the pCR™II cloning vector and analyzed by *EcoRI* restriction enzyme digestion. Restriction endonuclease digestion of pNELLS, pCELLS, pNPS, and pCPS with *EcoRI* produced two DNA fragments, one of approximately 3.9 kbp in size, representing the linear pDNA of the vector, pCR™II, and the other ranging from 260 bp to 1.06 kbp, representing the insert DNA of each clone (Fig. 15).

Confirmation of the mutagenic site was shown by *Mlu*I digests (Fig. 15) and DNA sequencing (Figs. 16 and 17).

Restriction endonuclease digestion of pNELLS, pCELLS, pNPS, and pCPS with *Mlu*I produced one DNA fragment for each clone ranging from 4.16 to 4.95 kbp in size representing linearized pDNA of the vector plus insert. The sizes of the DNA fragments differed depending on the insert size of each clone (Fig. 15).

Sequencing across the insert-vector junction of pNELLS, pCELLS, pNPS, and pCPS using both M13 Forward and M13 Reverse sequencing primers verified the presence of the insert DNA and the *Mlu*I restriction enzyme site in each of the four clones (Fig. 16). Sequence identified the insert DNA of pNELLS and pCELLS, N-terminus and C-terminus of *E.*

coli pyrB, to be in orientation identical to that of the *Lac* promoter. Sequence identified the insert DNA of pNPS and pCPS, N-terminus and C-terminus of *P. putida pyrB,* to be in orientation opposite to that of the *lac* promoter. These nucleotide sequences were compared to the known sequence using the NCBI BLAST algorithm tool and are shown in Fig. 17.

Clones pNELLS, pCELLS, pNPS, and pCPS are cassette systems, which can be utilized to create intergeneric hybrid ATCases. Such hybrid ATCases are useful in the study of the relationship of function and structure with genetic regions and domains from different genera or species. Clones pNPS and pCELLS could be used to create a *P. putida-E. coli* hybrid *pyrB.* This hybrid gene could then be used to test the hypotheses described earlier. Typically, the hybrid gene would be transformed into *E. coli* strain TB2, with no ATCase activity, to test for complementation. Failure to complement would suggest that the N-terminal region of *P. putida pyrB* prohibits formation of a trimer as it does in *P. putida* wild type. Complementation would indicate that an active ATCase can be produced.

The size of the hybrid ATCase could then be determined on a native non-denaturing PAGE and stained for activity. The hybrid ATCase could be purified from *E. coli* cells containing the plasmid insert, and kinetics performed on the enzyme. An intact *E. coli* trimer is active but not regulated. Based on work in our laboratory, the N-terminal region of the *P. putida* PyrB polypeptide contains the effector binding site. The active hybrid ATCase (presumably a trimer) could be tested for regulation with effector molecules, ATP, CTP, and UTP. If regulation were observed, this would further confirm our finding on the effector binding site.

The same hybrid *pyrB* could also be cotransformed with an *E. coli pyrI* on a compatible plasmid to determine if an *E. coli*-like dodecamer is formed. If dodecamer formation is possible, then this enzyme could be tested for inhibition by UTP, CTP, and activation by ATP. The native *E. coli* holoenzyme is not inhibited by UTP alone. Wild type *E. coli* ATCase holoenzyme displays sigmoidal kinetics when velocity-substrate plots are made. ATP activation and CTP inhibition are also seen. It would be interesting to learn if the hybrid holoenzyme maintained similar kinetics.

Clones pNELLS and pCPS were used to create an *E. coli*-*P. putida* hybrid *pyrB* described below.

Clone pBEP.

In order to test the hypotheses, an intergeneric hybrid *pyrB* gene was constructed between *E. coli* and *P. putida*. The plasmid naming scheme for pBEP is as follows: **p**lasmid, *pyr**B***, ***E****. coli*, and ***P****. putida*. Plasmid pBEP (Fig. 22), which carries the *E. coli* polar domain of *pyrB* and the *P. putida* equatorial domain of *pyrB* was constructed by manipulation of pNELLS and pCPS. First, pNELLS was cut with restriction enzyme *Mlu*I and *Eco*RV, which opens up or linearizes the vector DNA. Second, pCPS was similarly cut with restriction enzyme *Kpn*I followed by a fill-in reaction to create a blunt end for compatability with *Eco*RV. Third, pCPS was double digested with *Mlu*I and *Not*I. This double restriction enzyme digestion performed two different functions. The *Mlu*I digest cut out the insert DNA of pCPS and allowed for compatability with the vector DNA, while the *Not*I digest helped to prevent the vector DNA of pCPS from ligating into the linear pNELLS vector by creating incompatible ends. Finally, the two digested clones were ethanol precipitated followed by a 20:1 insert to vector

ligation using T4 DNA ligase (Fig. 23). The ligated mixture was then transformed into competent *E. coli* TB2 cells followed by selection on $LBamp_{100}$ plates. *EcoRI* and *Mlu*I restriction enzyme digestion has verified this construct (Fig. 24).

Restriction endonuclease digestion of pBEP with *EcoRI* produced two DNA fragments, one of approximately 3.9 kbp in size, which represents the linear pDNA of the vector, pCR II, and the other of approximately 1.6 kbp, representing the insert DNA of *E. coli-P. putida pyrB* (Fig. 24). Restriction endonuclease digestion of pBEP with *EcoRI* and *Mlu*I produced three DNA fragments, one of approximately 3.9 kbp in size, representing the linear vector DNA, one of approximately 820 bp in size, which represents the *P. putida* equatorial domain DNA fragment of *pyrB*, and one of approximately 440 bp in size, which represents the *E. coli* polar domain DNA fragment of *pyrB* (Fig. 24).

The creation of the *E. coli-P. putida* hybrid *pyrB* enables the study of the N-terminal extension of *P. putida pyrB*. This clone allows for the determination of whether the N-terminus of the *P. putida* PyrB polypeptide precludes assembly of an ATCase trimer and thus inhibits ATCase function.

The pBEP clone was transformed into the same *E. coli* strain TB2 and analyzed for complementation. Growth of the transformants in Ecmm medium at 30°C indicates that an active trimer was assembled. Cell extracts were run on a non-denaturing PAGE gel stained for ATCase activity (Foltermann, 1986)(Fig. 25). The pBEP clone produced a 100 kDa catalytic trimer. This result strongly suggests that the N-terminal region of *P. putida* PyrB prevents the formation of an active trimer, as seen in *P. putida* wild type PyrB. Our lab has shown that the *Pseudomonas pyrB* gene encodes a polypeptide that exists only as monomers (Kumar *et al*. 1999).

The pBEP clone can also be used to determine if the N-terminus region of *P. putida* PyrB participates in binding nucleotide effectors for the purpose of regulation of ATCase activity. This could be achieved by cotransformation of pBEP with pDBPC' (a *P. putida pyrC'* which lacks the *P. putida pyrB* clone). If regulation is not found for the holoenzyme formed from the cotransformation of pBEP with pDBPC', then this would further confirm the regulatory function of the N-terminus region of *P. putida* PyrB. To date the only information we

have on the *Pseudomonas* PyrC' polypeptide is that it is required for assembly into a dodecamer and to provide structural stability (Kumar *et al.*, 1999). If regulation is observed for the hybrid holoenzyme, then a function for the *Pseudomonas* PyrC' polypeptide would be discovered.

Since the *E. coli-P. putida pyrB* gene contains the *E. coli* upstream promoter-leader sequence, it could be tested in TB2 for repression of this active hybrid ATCase even though the gene is mainly *Pseudomonas*. To date, only two-fold repression is observed in *Pseudomonas*. Another advantage of this repressible *pyrB* gene would be for cotransformation with the *Pseudomonas pyrC'* gene, pDBPC', to see if a 480 kDa holoenzyme is formed. The proportion of PyrB: PyrC' could also be tested. Knowing this ratio is important in studies of assembly into a dodecamer.

REFERENCES

Abdelal, A., Kessler, D. P. & Ingraham, J. L. (1969). Arginine-auxotrophic phenotype resulting from a mutation in the *pyrA* gene of *Escherichia coli* B/r. *Journal of Bacteriology* **97**, 466-468.

Anderson, P. M. & Meister, A. (1965). Evidence of an activated form of carbon dioxide in the reaction catalyzed by *Escherichia coli* carbamyl phosphate synthetase. *Biochemistry* **4**, 2803-2809.

Bergh, S. T. & Evans, D. R. (1993). Subunit structure of class A aspartate transcarbamoylase from *Pseudomonas fluorescens*. *Proc. Natl. Acad. Sci. USA.* ***90***, 1918-9822.

Bethell, M. R. & Jones, **M. E. (1969).** Molecular size and feedback-regulation characteristics of bacterial aspartate transcarbamylases. *Archives of Biochemistry and Biophysics* ***134***, 352-365.

Bothwell, M. (1975). Ph.D. thesis. University of California, Berkeley.

Brabson, J. S. & Switzer, R. L. (1975). Purification and properties of *Bacillus subtilis* aspartate transcarbamolase. *J. Biol. Chem.* ***250(22)***, 8664-8669.

Condon, S., Collins, J. K. & O'Donovan, G. A. (1976). Regulation of arginine and pyrimidine biosynthesis in *Pseudomonas putida. J. Gen. Micro.* ***92***, 375-383.

Dagert, M. & Ehrich, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli*. *Gene* ***6***, 23-28.

Foltermann, K. F., Beck, D. A., & Wild, J. R. (1986). In vivo formation of hybrid aspartate transcarbamoylases from native subunits of divergent members of the family *Enterobacteriaceae. J. Bacteriol*. **167**, 285-290.

Kalman, S. M., Duffield, P. H. & Brozowski, T. (1966). Purification and properties of a bacterial carbamylphosphate synthetase. *Journal of Biological Chemistry* **241**, 1871-1877.

Kedzie, K. M. (1987). Characterization of the *pyrB* gene of *Serratia marcescens* and hybrid gene formation with the *pyrB* gene of *Escherichia coli*, leading to the production of chimeric ATCases. Ph.D. dissertation. Texas A&M University : College Station.

Kenny, M. J., McPhail, D. & Shepherdson, M. (1996). A reappraisal of the diversity and class distribution of aspartate transcarbamoylase in Gram-negative bacteria. *Microbiology* ***142***, 1873-1879.

Kumar, A. P., Brichta, D. M., Schurr, M. J. & O'Donovan, G. A. (1994). Deletion of a 34 amino acid N-terminal region of the aspartate transcarbamoylase in *Pseudomonas putida* abolishes regulatory nucleotide effector responses. abstr. K-36, p282. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology. ASM, Washington, D. C.

Kumar, A. P., Brichta, D. M., Segars, D., & O'Donovan, G. A. (1999). Assembly of aspartate transcarbamoylase in *Pseudomonas putida*. abstr. *In* Abstracts of the 99th General Meeting of the American Society for Microbiology, ASM, Washington, D. C.

Kumar, A. P., Schurr, M. J., Campbell, A. L., & O'Donovan, G. A. (1993). Removal of a short amino terminal peptide changes effector responses of *Pseudomonas putida* aspartate transcarbamoylase. abstr. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology. ASM, Washington, D. C.

Mercer, A. A., & Loutit, J. S. (1979). Transformation and transfection of *Pseudomonas aeruginosa*: effects of metal ions. *J. Bacteriol* **140,** 37-42.

Neuhard, J. & Kelln, R. A. (1996). Biosynthesis and conversions of pyrimidines. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn., pp. 580-599. Edited by F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger. Washington, DC: American Society for Microbiology.

Neuhard, J. & Nygaard, P. (1987). Purines and Pyrimidines. In *Escherichia coli* and *Salmonella typhimurium*: *Cellular and Molecular Biology*, pp. 445-473. Edited by F. C. Neidhardt. Washington, DC: American Society for Microbiology.

O'Donovan, G. A., Herlick, S., Beck, D. E. & Dutta, P. K. (1989). UTP/CTP ratio, an important regulatory parameter for ATCase expression. *Arch. Microbiol.* ***153***, 19-25.

O'Donovan, G. A. & Neuhard, J. (1970). Pyrimidine metabolism in microorganisms. *Bacteriological Reviews* ***34***, 278-343.

O'Donovan, G. A. & Shanley, M. S. (1995). Pyrimidine Metabolism in *Pseudomonas*. *Paths to Pyrimidines: An International Newsletter* ***3***, 49-59.

Pauza, C. D., Karels, M. J., Navre, M. & Schachman, H. K. (1982). Genes encoding *Escherichia coli* aspartate transcarbamoylase: the *pyrB-pyrI* operon. *Proc. Natl. Acad. Sci. USA* ***79***, 4020-4024.

Ruley, J. (1992). Creation and characterization of an *Escherichia coli* and *Pseudomonas putida* hybrid aspartate transcarbamoylase. M.S. thesis. University of North Texas: Denton.

Roof, W. D., Folterman, K. F. & Wild, J. R. (1982). The organization and regulation of the *pyrBI* operon in *E. coli* includes a rho-independent attenuator sequence. *Mol. Gen. Genet.* ***187***, 391-400.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* ***74***, 5463-5467.

Shepherdson, M. & McPhail, D. (1993). Purification of aspartate transcarbamoylase from *Pseudomonas syringae*. *FEMS Microbiol. Lett.* ***114***, 201-206.

Schurr, M. J., Vickrey, J. F., Kumar, A. P., Campbell, A. L., Cunin, R., Benjamin, R. C., Shanley, M. S. & O'Donovan, G. A. (1995). Aspartate transcarbamolyase genes of *Pseudomonas putida*: Requirement for an inactive dihydroorotase for assembly into the dodecameric hololenzyme. *Journal of Bacteriology* ***177***, 1751-1759.

Wild, J. R., Foltermann, K. F. & O'Donovan, G. A. (1980). Regulatory divergence of aspartate transcarbamoylases within the *Enterobacteriaceae. Archives of Biochemistry and Biophysics* ***201***, 506-517.

Yon, R. J. (1984). Regulatory kinetics of wheat-germ aspartate transcarbamoylase. Adaptation of the concerted model to account for complex kinetic effects of uridine 5'-monophosphate. *Biochem J* ***221,*** 281-287.

Zhou, C., Yang, Y. & Jong, A. Y. (1990). Mini-prip in ten minutes. *BioTechniques* ***8***, 172-173.

Figure 1. Pyrimidine biosynthetic pathway in *Escherichia coli* and *Salmonella typhimurium*. Gene symbols and the enzymes they encode are: *ndk* – nucleoside diphosphate kinase; *pyrA* (*carAB*) – carbamoylphosphate synthetase; *pyrBI* – aspartate transcarbamoylase; *pyrC* – dihydroorotase; *pyrD* – dihydroorotate dehydrogenase; *pyrE* – orotate phosphoribosyltransferase; *pyrF* - OMP decarboxylase; *pyrG* – CTP synthetase; *pyrH* – UMP kinase. Adapted from Neuhard & Nygaard (1987).

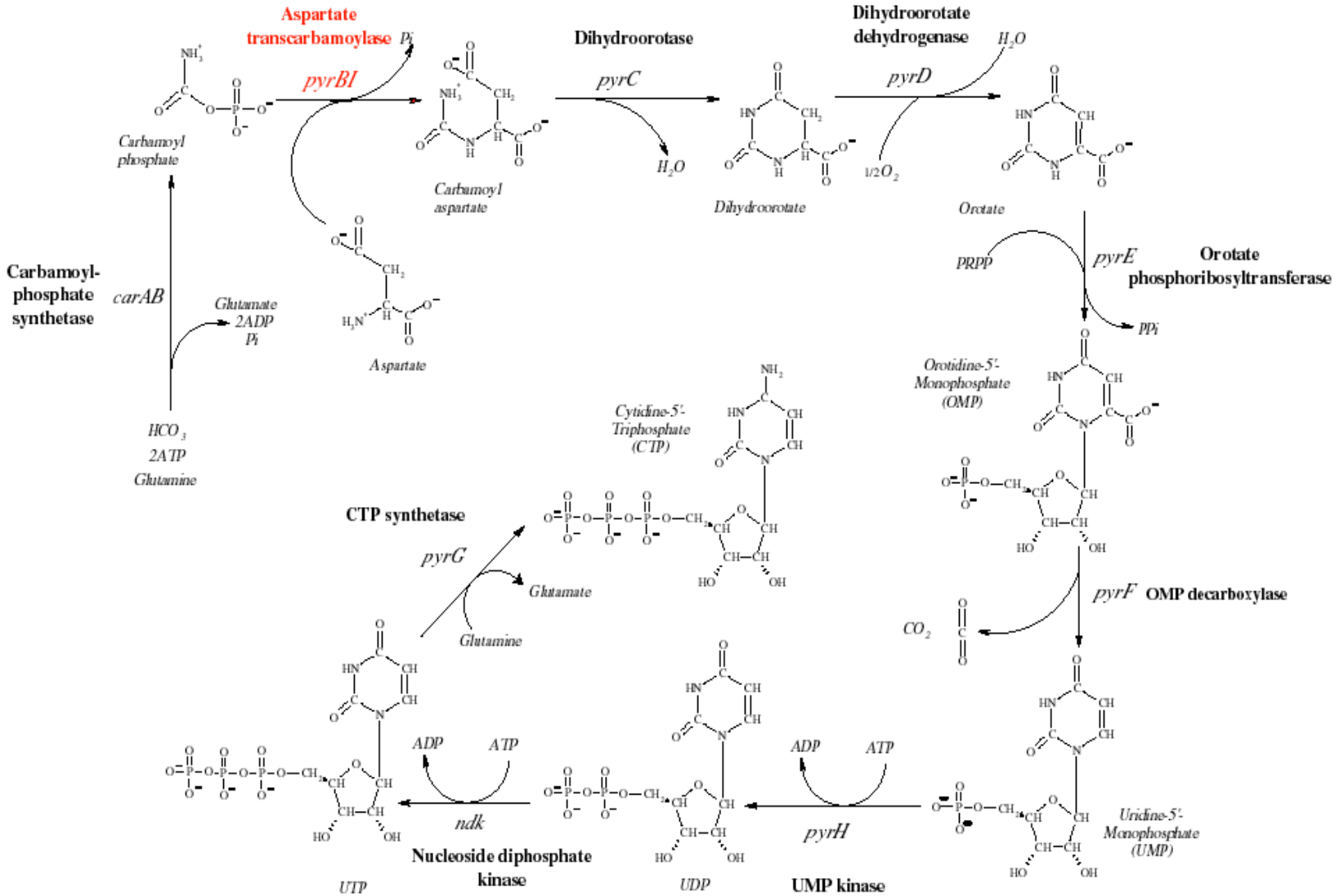

Aspartate transcarbamoylase
Pi
pyrBI
Carbamoyl phosphate
Carbamoyl aspartate
Dihydroorotase
pyrC
H2O
Dihydroorotate dehydrogenase
pyrD
H2O
1/2O2
Dihydroorotate
Orotate
PRPP
pyrE
Orotate phosphoribosyltransferase
ppi
Orotidine-5'-Monophosphate (OMP)
pyrF
OMP decarboxylase
CO2
Uridine-5'-Monophosphate (UMP)
Carbamoyl-phosphate synthetase
carAB
Glutamate
2ADP
Pi
Aspartate
HCO3
2ATP
Glutamine
Cytidine-5'-Triphosphate (CTP)
CTP synthetase
pyrG
Glutamate
Glutamine
ADP
ATP
pyrH
UMP kinase
UDP
ADP
ATP
ndk
Nucleoside diphosphate kinase
UTP

Figure 2. Classes of bacterial ATCase (not drawn to scale). Adapted from Bergh & Evans (1993).

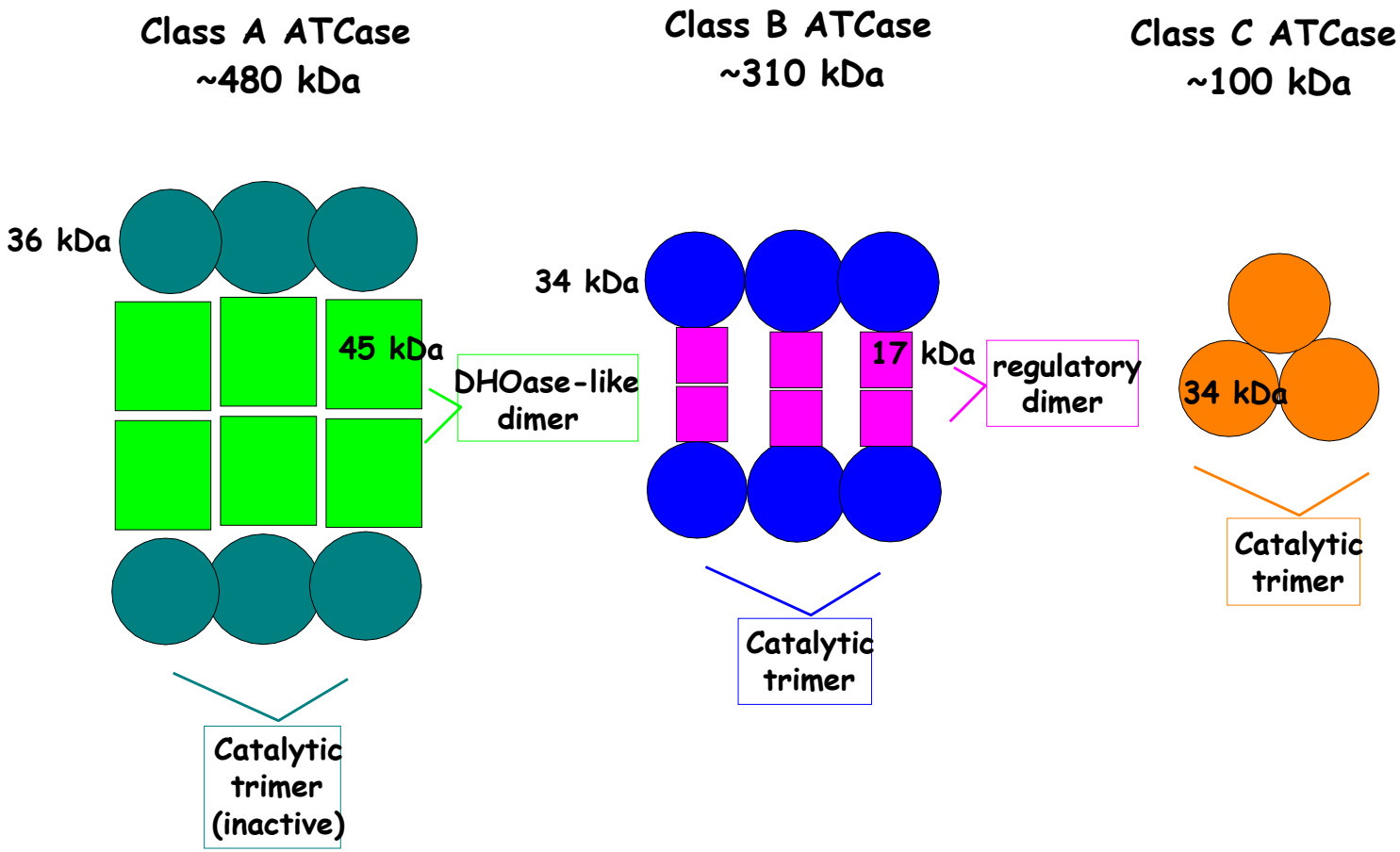


Figure 3. Assembly of ATCase holoenzyme from *E. coli* and *P. putida* (not drawn to scale).

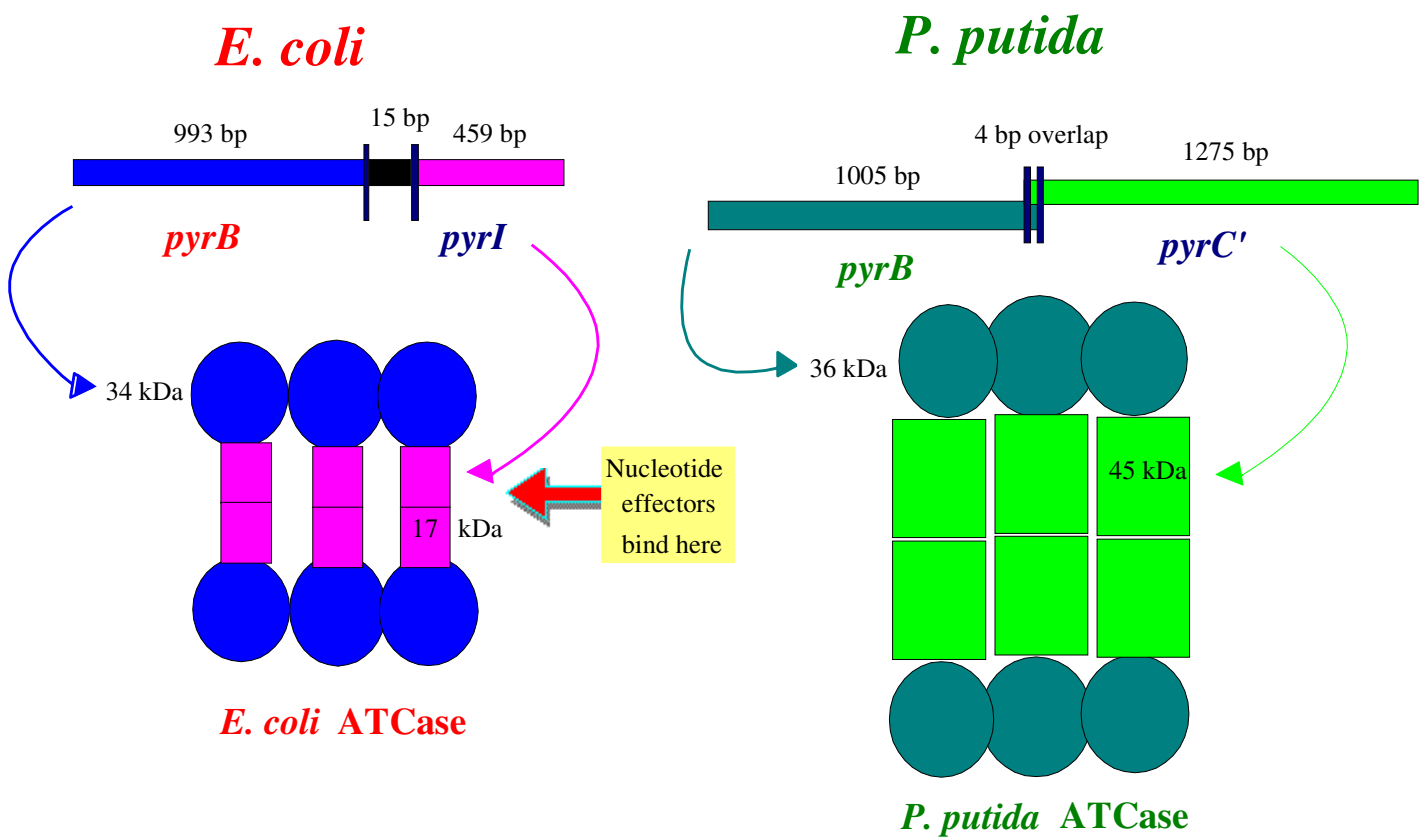


Figure 4. Assembly of PyrB from *E. coli* and *P. putida*. Rectangular bars represent DNA genes and circles represent the polypeptides encode by the genes.

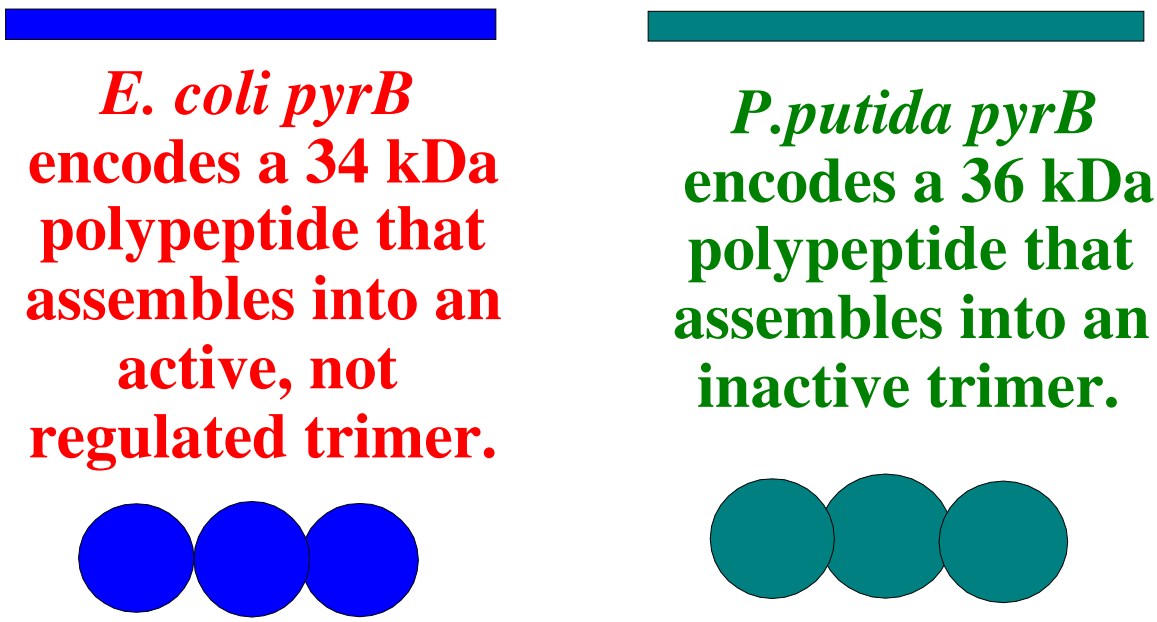


Figure 5. Assembly of PyrB from *E. coli* and *P. putida*.

Blue lines represent PyrB polypeptides encoded by *pyrB*.

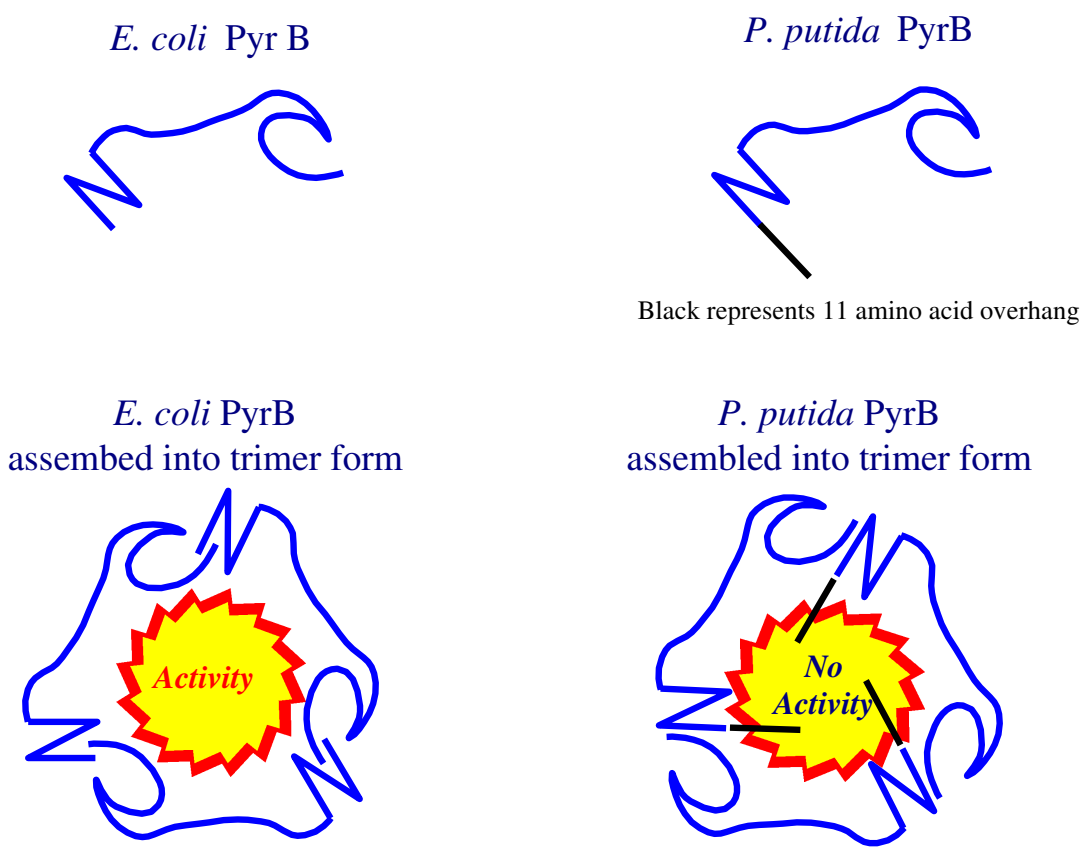


Figure 6. Mutagenic Oligo Design. *Mlu*I restriction enzyme sequence is indicated in purple. Standard single letter codes for bases are adenosine (A), cytidine (C), guanosine (G), and thymidine (T). Single letter codes for the amino acids are serine (S), threonine (T), and arginine (R).

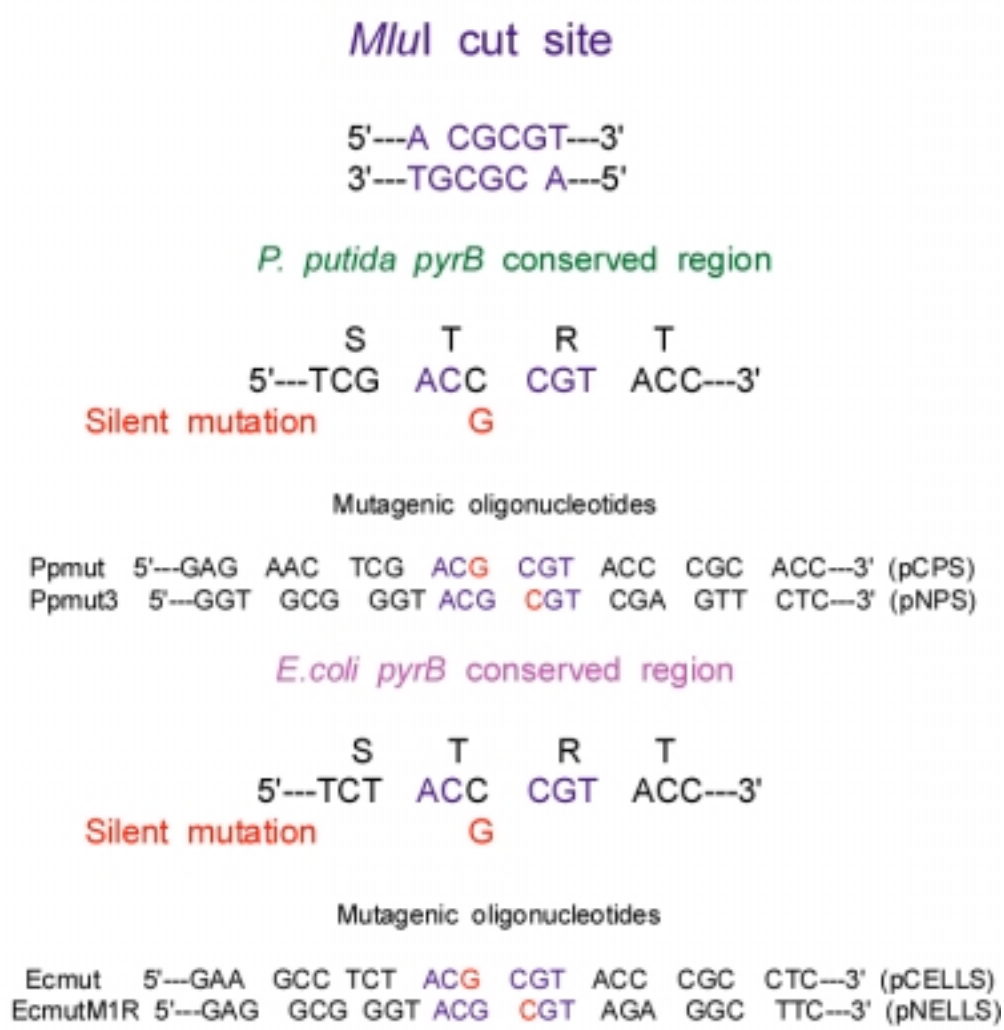


Figure 7. pBEaAS plasmid map.

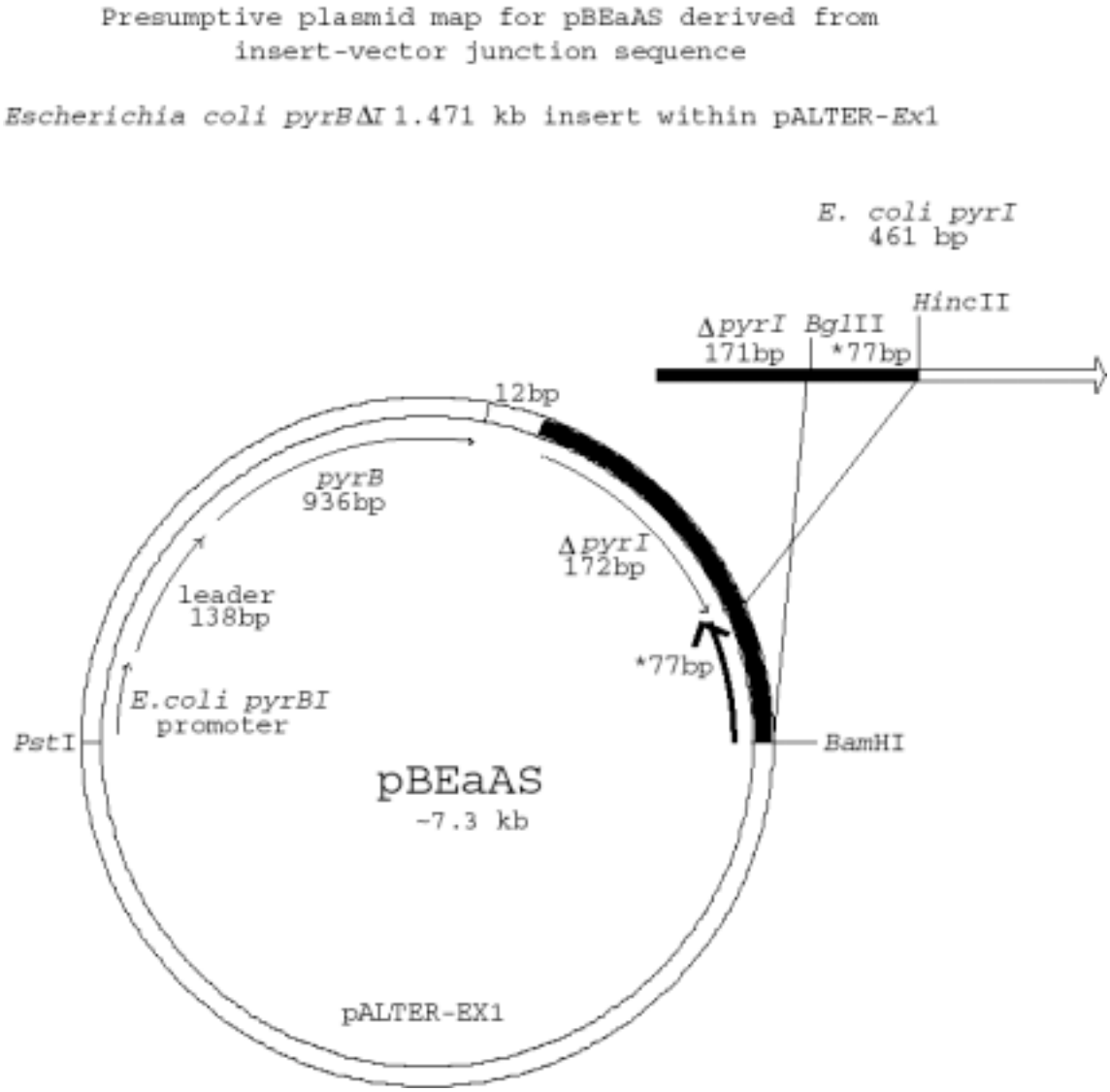


Figure 8. Schematic diagram demonstrating the genealogy of pBEaAS.

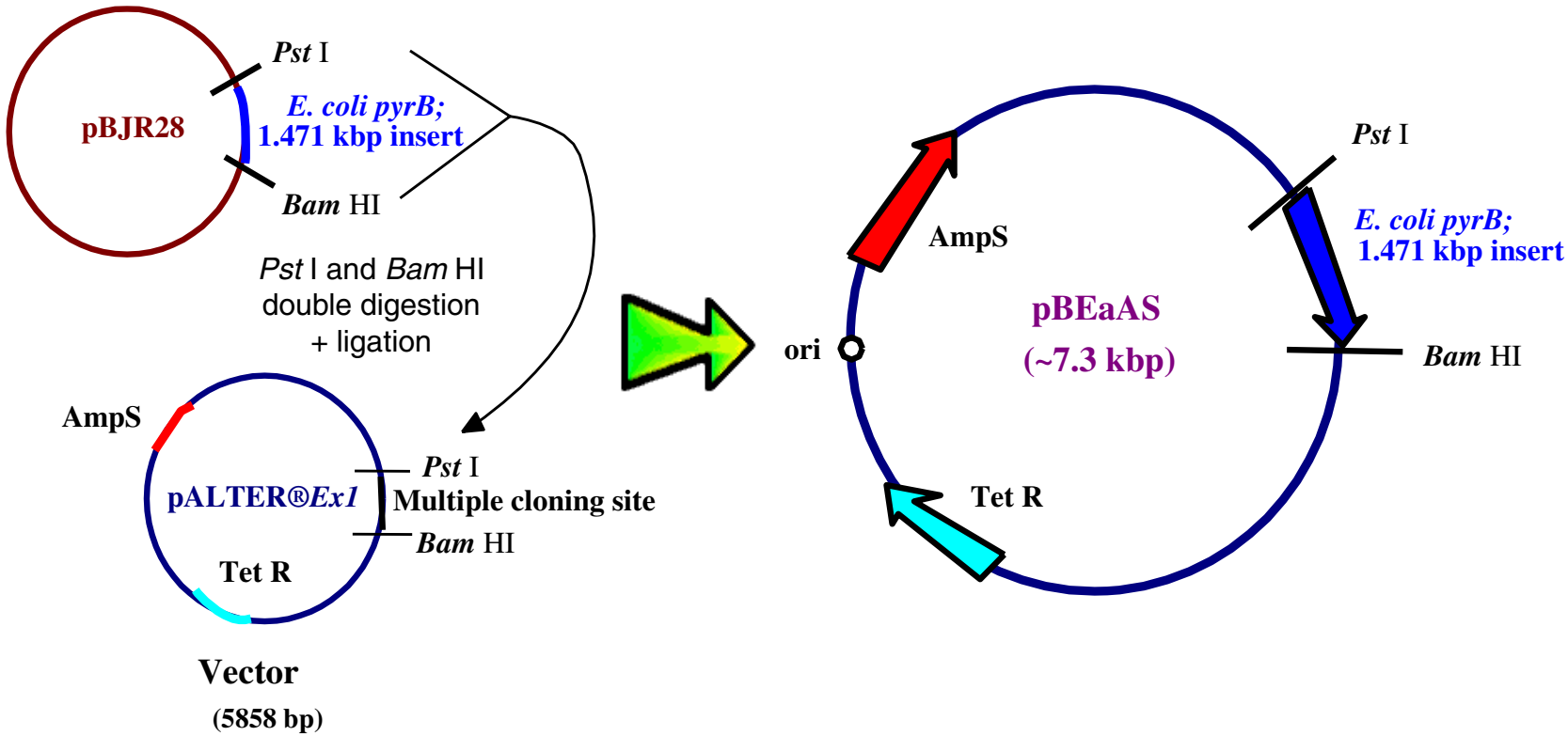


Figure 9. pBpals plasmid map.

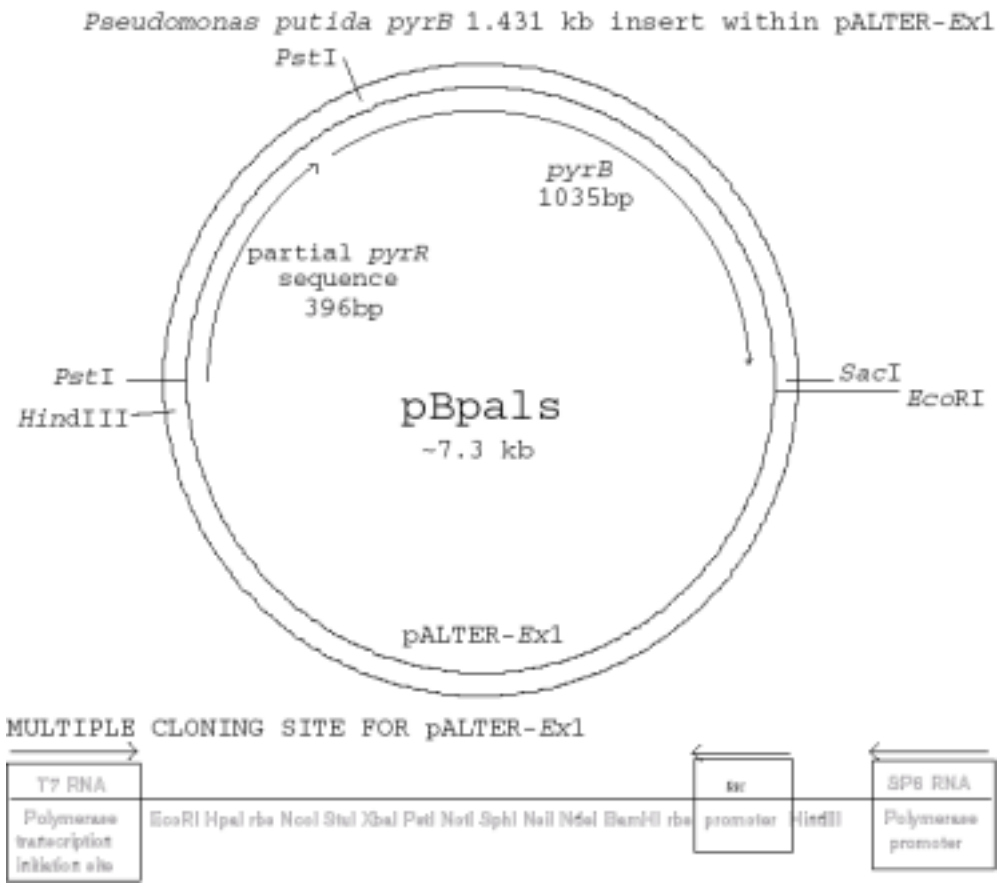


Figure 10. Schematic diagram demonstrating the genealogy of pBpals.

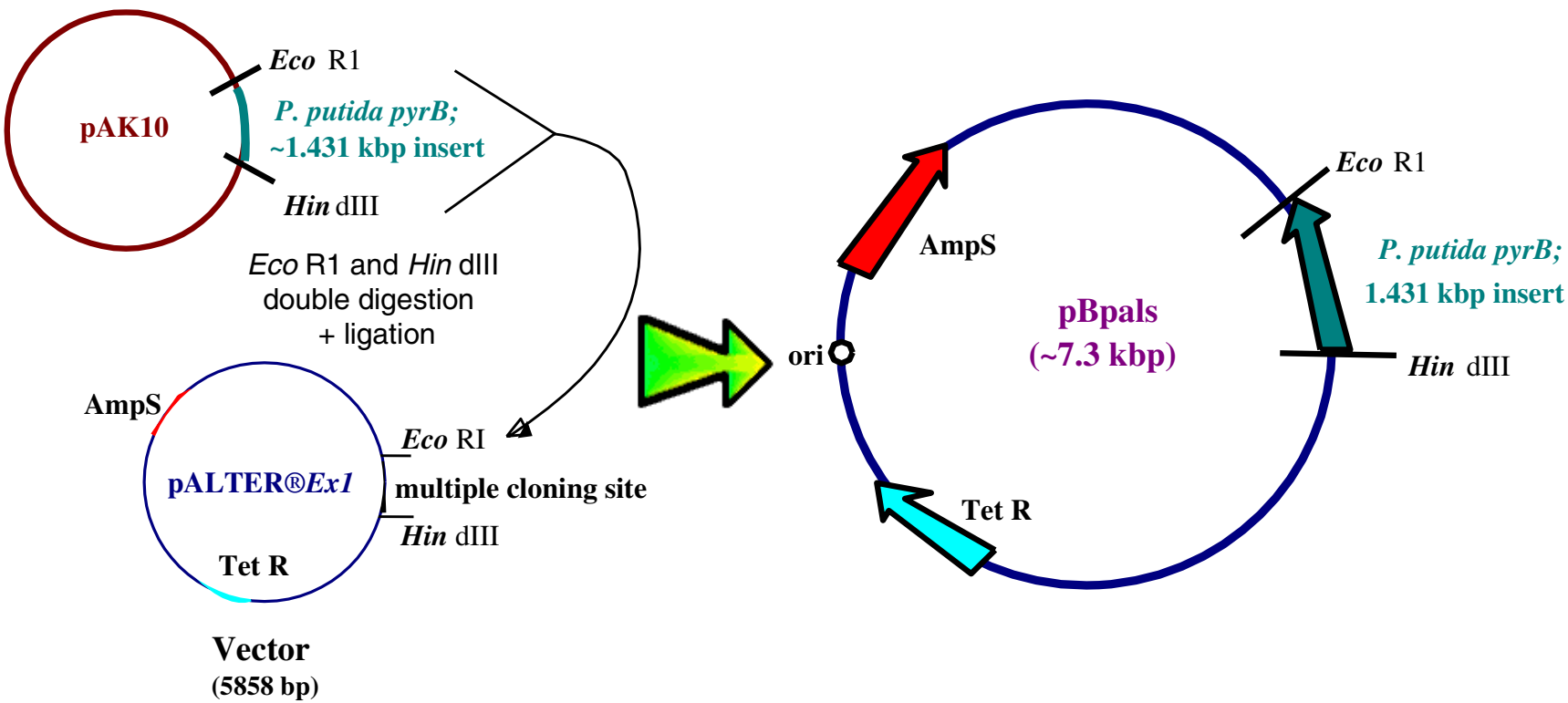
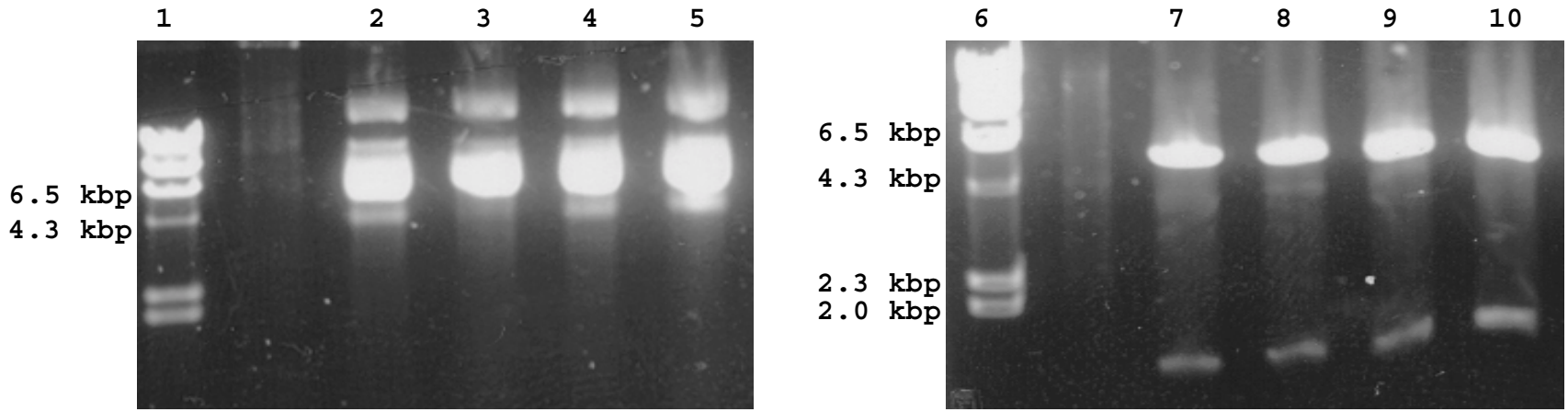


Figure 11. An agarose gel depicting restriction enzyme digestion of pBEaAS and pBpals.



Lane 1 and 6. Lambda DNA - *Hin*dIII Digest DNA size marker.

Lane 2 and 3. Undigested pBEaAS.

Lane 4 and 5. Undigested pBpals.

Lane 7 and 8. BamHI and PstI digestion of pBEaAS - ~**5.9 kbp** linear vector, ~**1.47 kbp** insert DNA.

Lane 9 and 10. *Eco*RI and *Hin*dIII digestion of pBpals - ~**5.9 kbp** linear vector, ~**1.43 kbp** insert DNA.

Figure 12. Insert-vector junction DNA sequence comparison of pBEaAS and pBpals with known *E. coli pyrBI*, *P. putida pyrB*, and pALTER-*Ex*1 cloning vector sequences. The *E. coli*, *P. putida*, and pALTER-*Ex*1 sequences were obtained from GenBank and are available on the Internet at http://www.ncbi.nlm.nih.gov. Query A is the M13 Forward primer pBEaAS sequence read from a DNA sequencing polyacrylamide gel. Subject A is the *E. coli pyrBI* operon encoding for ATCase with a length of 1593 bp. Query B is the M13 Reverse primer pBEaAS sequence read from a DNA sequencing polyacrylamide gel. Subject B is the cloning vector pALTER-*Ex*1 complete vector sequence of 5858 bp. Subject C is the *E. coli pyrBI* operon encoding ATCase with a length of 1410 bp. Query C is the M13 Forward pBpals sequence read from a DNA sequencing polyacrylamide gel. Subject D is the *P. putida pyrB* gene and *pyrC'* gene with a length of 1403 bp. Query D is the M13 Reverse primer sequence of pBpals read from a DNA sequencing polyacrylamide gel. Subject E is the *P. putida pyrR* gene and *pyrB* gene with a length of 778 bp.

```
Identities = 86/90 (95%), Gaps = 4/90 (4%)

Query A:25 ctgcagaatcacatcaaataaaaatgcatataccttgacttttaattcaaataaaccgtt
84         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct A:340ctgcagaatcacatcaaataaaaatgcatataccttgacttttaattcaaataaaccgtt
399

Query A:85 t----tgacaaaatattgcatcaaatgctt 110
           |    |||||||||||||||||||||||||
Sbjct A:400tgcgctgacaaaatattgcatcaaatgctt 429

Identities = 70/73 (95%), Gaps = 3/73 (4%)

Query B:1  gacaattaa-catcggctcgtata-tgt-tggaattgtgagcggataacaatttcacaca
57         ||||||||| ||||||||||||| ||| ||||||||||||||||||||||||||||||
Sbjct B:145gacaattaatcatcggctcgtataatgtgtggaattgtgagcggataacaatttcacaca
86

Query B:58 ggaaacaggatcc 70
           |||||||||||||
Sbjct B:85 ggaaacaggatcc 73

Identities = 39/39 (100%)

Query B:27 tggaattgtgagcggataacaatttcacacaggaaacag 65
           |||||||||||||||||||||||||||||||||||||||
Sbjct B:257tggaattgtgagcggataacaatttcacacaggaaacag 219

Identities = 62/64 (96%), Gaps = 2/64 (3%)

Query B:74 gatctgatcaaaatcgaaaataccttttgagtgaagatc-agtagatc-actggcattg
131        |||||||||||||||||||||||||||||||||||||| |||||||| ||||||||||
Sbjt C:1117gatctgatcaaaatcgaaaataccttttgagtgaagatcaagtagatcaactggcattg
1176

Query B:132 tatg 135
            ||||
Sbjct C:1177tatg 1180

Identities = 78/80 (97%), Gaps = 2/80 (2%)

Query C:30 tcactgggcgttctcctggtcgaattgaccgttgccgcgttctgcccgctcatggccatg
89         |||||||||||||||||||||||||||| ||||| ||||||||||||||||||||||||
Sbjt D:1403tcactgggcgttctcctggtcgaattgac-gttgc-gcgttctgcccgctcatggccatg
1346

Query C:90  gacagcacggccatgcgcac 109
            ||||||||||||||||||||
Sbjct D:1345gacagcacggccatgcgcac 1326
```

```
Identities = 27/28 (96%), Gaps = 1/28 (3%)

Query D:1 ctaccgcgacgactt-agccagaacggc 27
          ||||||||||||||| ||||||||||||
Sbjct D:55ctaccgcgacgacttcagccagaacggc 82

Identities = 27/28 (96%), Gaps = 1/28 (3%)

Query D:1  ctaccgcgacgactt-agccagaacggc 27
           ||||||||||||||| ||||||||||||
Sbjct E:409ctaccgcgacgacttcagccagaacggc 436
```

Figure 13. Plasmid maps of pNELLS and pCELLS. Green arrows represent sequencing primers. Navy blue arrows represent primers associated with

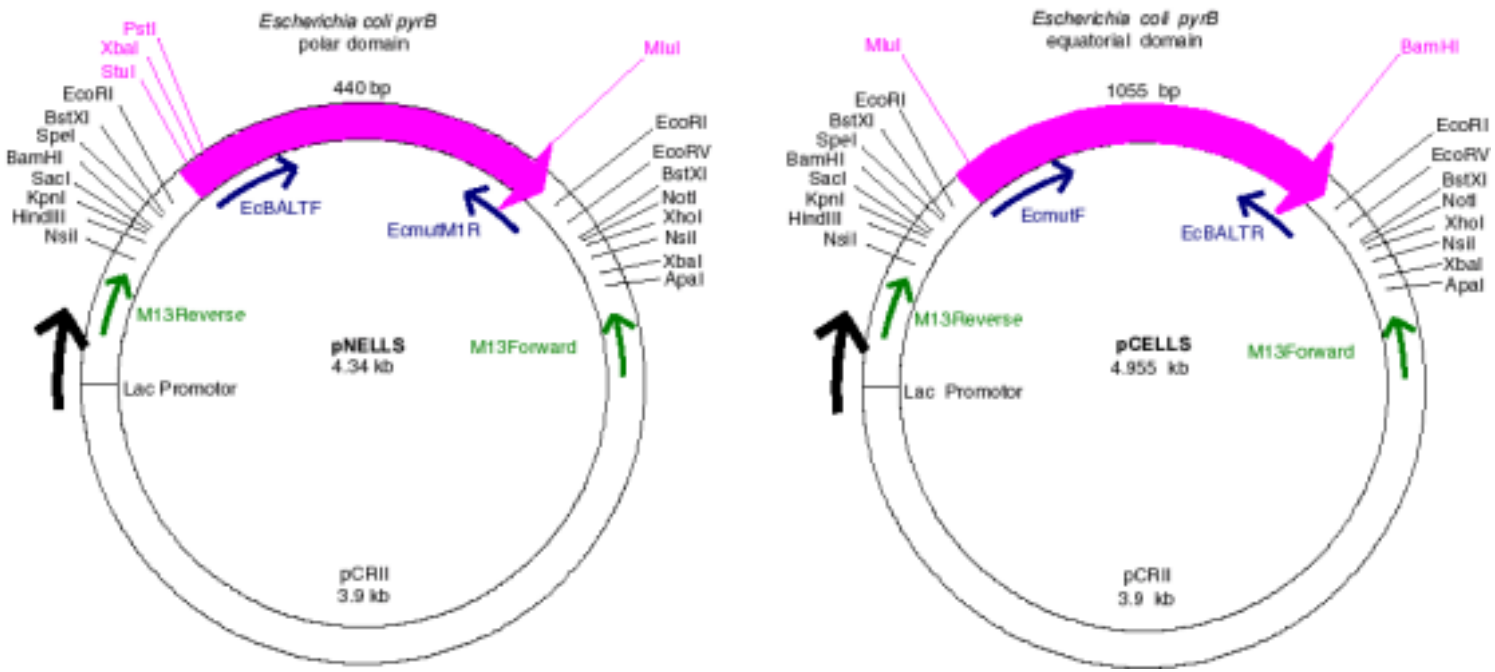


Figure 14. Plasmid maps of pNPS and pCPS. Green represents sequencing primers. Navy blue arrows represent primers associated with insert DNA.

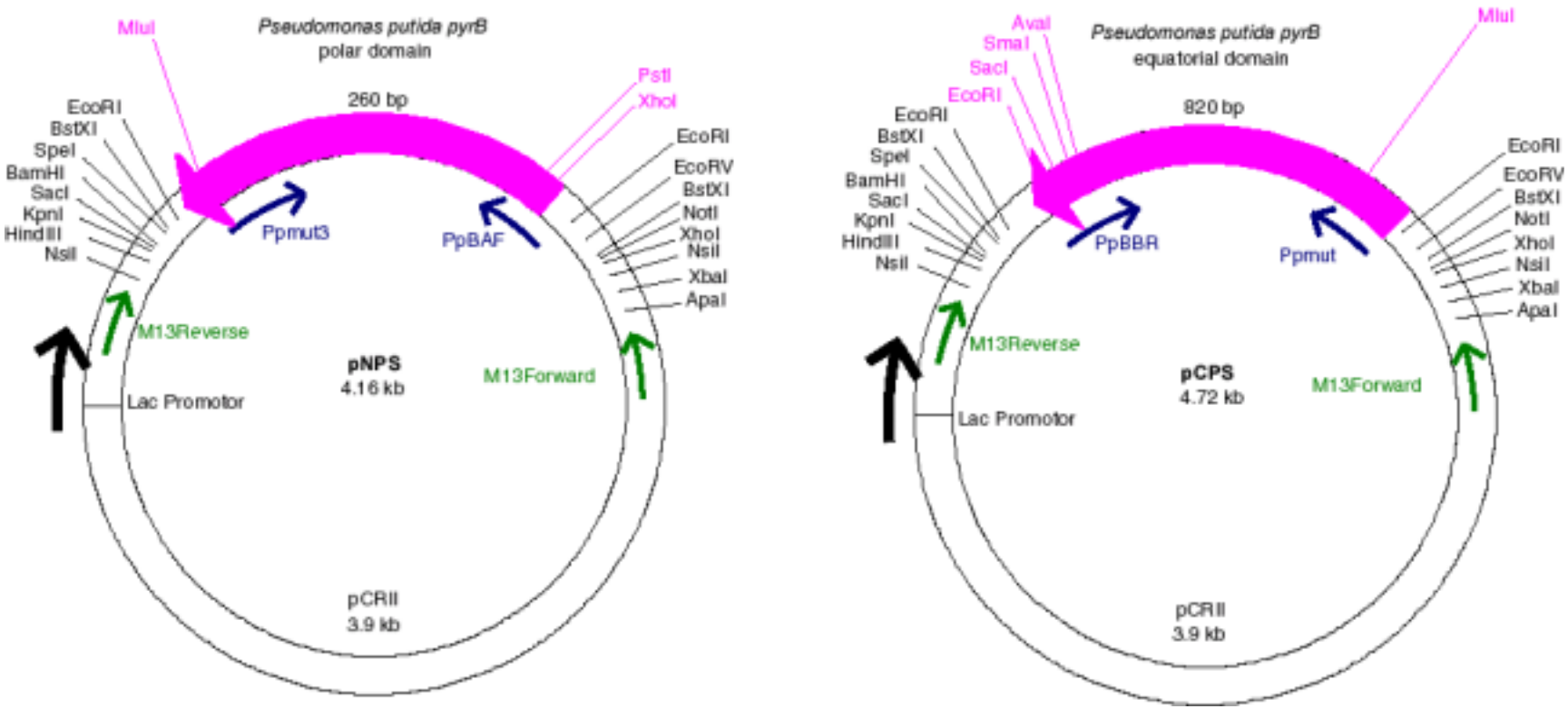
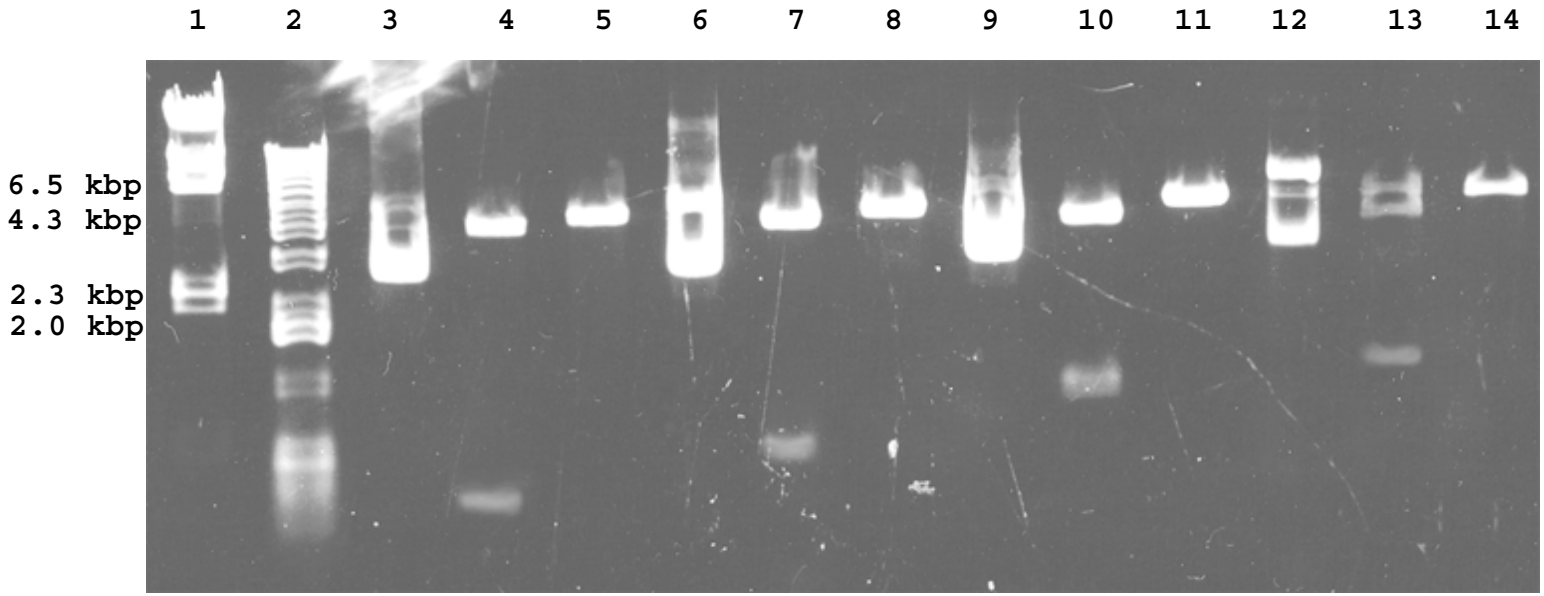


Figure 15. An agarose gel depicting the *Eco*RI and *Mlu*I restriction enzyme digestion of pNELLS, pCELLS, pNPS, and pCPS.



Lane 1. Lambda DNA-*Hin*dIII Digest size marker.
Lane 2. pGEM DNA size marker.
Lane 3. Undigested pNPS.
Lane 4. *Eco*RI digest of pNPS - **3.9 kbp** linear vector, **260 bp** insert DNA.
Lane 5. *Mlu*I digest of pNPS - **4.16 kbp** linear vector + insert DNA.
Lane 6. Undigested pNELLS.
Lane 7. *Eco*RI digest of pNELLS - **3.9 kbp** linear vector, **440 bp** insert DNA.
Lane 8. *Mlu*I digest of pNELLS - **4.34 kbp** linear vector + insert DNA.
Lane 9. Undigested pCPS.
Lane 10. *Eco*RI digest of pCPS - **3.9 kbp** linear vector, **820 bp** insert DNA.
Lane 11. *Mlu*I digest of pCPS - **4.72 kbp** linear vector + insert DNA.
Lane 12. Undigested pCELLS.
Lane 13. *Eco*RI digest of pCELLS - **3.9 kbp** linear vector, **1055 bp** insert DNA.
Lane 14. *Mlu*I digest of pCELLS - **4.955 kbp** linear vector + insert DNA.

Figure 16. DNA sequence of the *Mlu*I site in pNELLS, pCELLS, pNPS, and pCPS. An autoradiograph of a chain-terminator sequencing gel, showing the sequence of single stranded M13 recombinant DNA. Purple represents the *Mlu*I cut site DNA sequence and red represents the single base mutation.

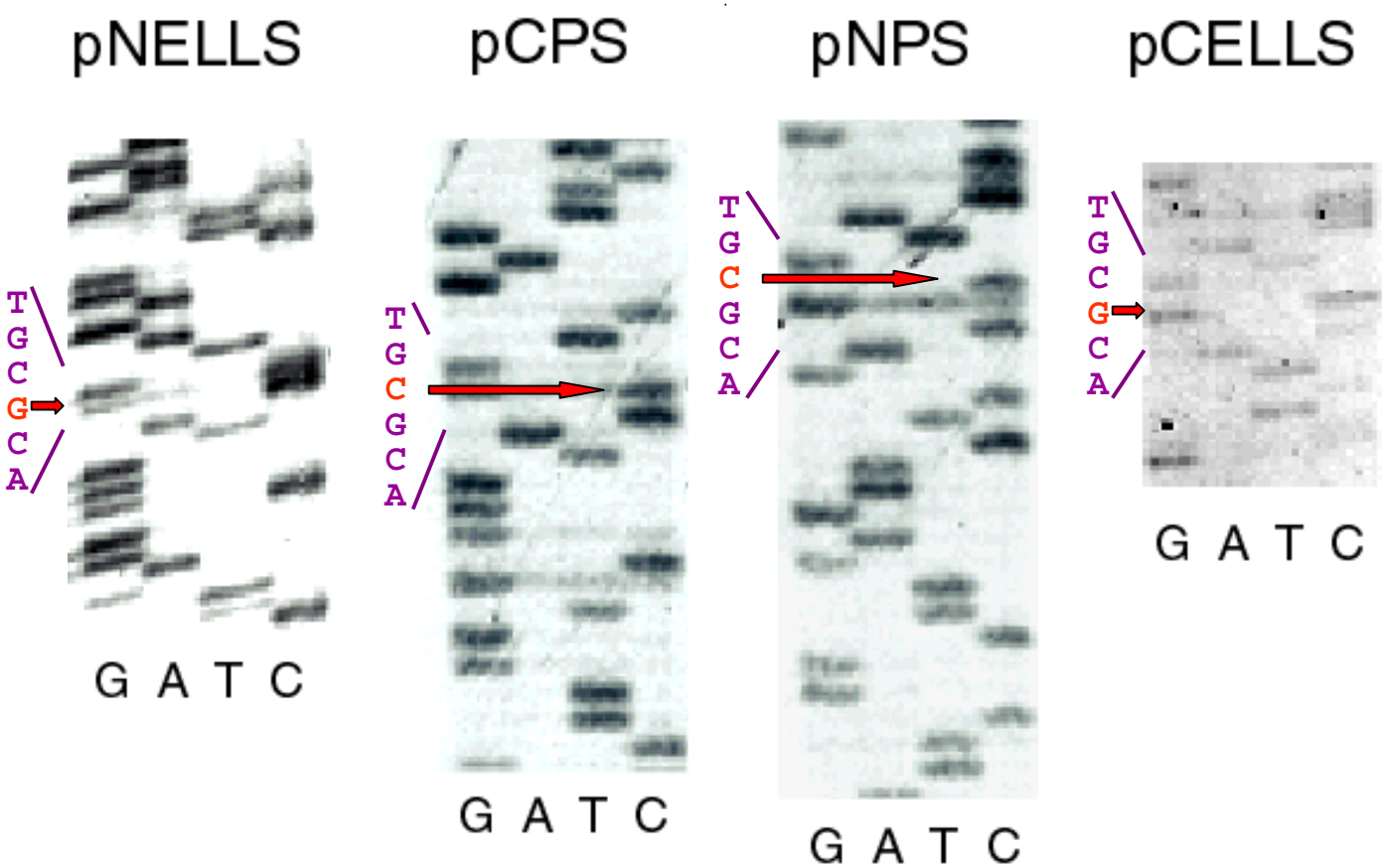


Figure 17. Insert-vector junction DNA sequence comparison of pNELLS, pCELLS, pNPS, and pCPS with known *E. coli*, *P. putida,* and pALTER-*Ex*1 cloning vector sequences. The *E. coli*, *P. putida,* and pALTER-*Ex*1 sequences were obtained from GenBank and are available on the Internet at http://www.ncbi.nlm.nih.gov. Query A is the M13 Forward primer sequence of pNELLS provided by AGCT, Inc. Subject A is the *E. coli pyrBI* operon encoding for ATCase with a length of 1593 bp. Subject B is the cloning vector, pALTER-*Ex*1, complete vector sequence with a length of 5858 bp. Query B is the M13 Forward primer sequence of pCELLS provided by AGCT, Inc. Subject C is the *E.coli pyrBI* operon encoding ATCase with a length of 1410 bp. Query C is the M13 Reverse primer sequence of pCELLS provided by AGCT, Inc. Query D is the M13 Forward primer sequence of pNPS provided by AGCT, Inc. Subject D is *P. putida pyrB* gene and *pyrC'* gene with a length of 1403 bp. Query E is the M13 Forward primer sequence of pCPS provided by AGCT, Inc. Query F is the M13 Reverse primer sequence of pCPS provided by AGCT, Inc. Red represents the single base mutation, purple represents the *Mlu*I restriction enzyme site, and blue represents inverted partial *pyrI* sequence.

```
Identities = 436/453 (96%), Gaps = 7/453 (1%)

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733

Query A:111ctctggttgcgggtttgctttcagtttcgccgctgtcgccagcaccagattaaggtcatc
170        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct A:732ctctggttgcgggtttgctttcagtttcgccgctgtcgccagcaccagattaaggtcatc
673

Query A:171gcgactaaggtcgtttatggaaatgatatgtttctgatatagcggattagccatctttta
226        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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613

Query A:227tctcctgacgcctgggcaaaaaaaagcccctcgattgaggggctgggaatgggtgatcaa
283        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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553

Query A:284cgggaagaaaaacggcaggccagcgtctttttttcagacgcggtaagacaaaatgtcgaac
343        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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493

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403        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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433

Query A:404cccaagcatttgatgcaatattttgtcagcgcaaacggtttatttgaattaaaagtcaag
463        | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct A:432cgcaagcatttgatgcaatattttgtcagcgcaaacggtttatttgaattaaaagtcaag
373

Query A:464gtatatgcatttttatttgatgtgattctgcag 496
           |||||||||||||||||||||||||||||||
Sbjct A:372gtatatgcatttttatttgatgtgattctgcag 340

Identities = 224/231 (96%), Gaps = 4/231 (1%)

Query A:576agcttgagtattctatagtgtcacctaaatagcttggcgtaatcatggtcatagctgttt
635        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct B:165agcttgagtattctatagtgtcacctaaatagcttggcgtaatcatggtcatagctgttt
224

Query A:636cctgtgtgaaattggtatccgctcacaattccacacaacatacgagccggaagcataaag
695        ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct B:225cctgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagcataaag
284
```

```
Query A:696tgtaaagcctgggggtgcctaatgagtgagctaactcacattaa-ttgcgttgcgctcac
754        |||||||||| ||||||||||||||||||||||| |||||||| ||||||||||||||||
Sbjct B:285tgtaaagcct-ggggtgcctaatgagtgagctaac-cacattaatttgcgttgcgctcac
342

Query A:755tggccgcttttcagtcgggaaacctgtcg-gccagctgcattaatgaatcg 804
           || |||||||| |||||||||||||||||| |||||||||||||||||||
Sbjct B:343tgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcg 393

Identities = 662/672 (98%)

Query B:151gatctttgcggcccatctcgccagaaggcaggttcagaccaatggtgatgcgctgatccg
210        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt C:1120gatctttgcggcccatctcgccagaaggcaggttcagaccaatggtgatgcgctgatccg
1061

Query B:211tttcggtcagcttgaacagactcaacagcttaaaaccgatctgggcggggatatggtcaa
270        | |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjt C:1060tctcggtcagcttgaacagactcaacagcttaaacccgatctgggcggggatatggtcaa
1001

Query B:271ttaccgtgccgcgtttaatagcttcaacctgcaatttattatcgtgtgtcatctctattt
330        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt C:1000ttaccgtgccgcgtttaatagcttcaacctgcaatttattatcgtgtgtcatctctattt
941

Query B:331ccccttacagtaccagatcgcgattcagaaccagtgccagtaacgcctggcgagcgaaaa
390        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:940ccccttacagtaccagatcgcgattcagaaccagtgccagtaacgcctggcgagcgaaaa
881

Query B:391tcccgttgcctgcctgctggaagtaccaggcgtgtggcgttttatcaacatccgtcgcaa
450        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:880tcccgttgcctgcctgctggaagtaccaggcgtgtggcgttttatcaacatccgtcgcaa
821

Query B:451tctcatcaacacgcggcagcggatgcagcactttcatattggctttggcgttgtggagat
510        |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:820tctcatcaacgcgcggcagcggatgcagcactttcatattggctttggcgttgtggagat
761

Query B:511cgctggcgcgaagaacaaactgcgctttcacgttggcgtactcggacgggtccagacgct
570        ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:760cgctggcacgaagaacaaactgcgctttcacgttggcgtactcggacgggtccagacgct
701



Query B:571ctttttgcacgcgggtcatgtacaggatgtctacttccgccatcacttcttcaatagagc
630        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:700ctttttgcacgcgggtcatgtacaggatgtctacttccgccatcacttcttcaatagagc
641
```

```
Query B:631tgtgcagactccatgcgatccctttttcatcgagcatatccagaatgtattgcggcattg
690        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:640tgtgcagactccatgcgatccctttttcatcgagcatatccagaatgtattgcggcattg
581

Query B:691ccagcgcgtccggcgcgatgaagtaaaaacggttgccgtcgaacttcgctaacgcctgag
750        |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct C:580ccagcgcgtccggcgcaatgaagtaaaaacggttgccgtcgaacttcgctaacgcctgag
521

Query B:751tcagggagtgaacggtgcggccatatttcaggtcaccaaccattgcgacgtggagaatgt
810        |||||||||||||| |||||||||||||||||||||||||||||||||||||||| |||
Sbjct C:520tcagggagtgaaccgtgcggccatatttcaggtcaccaaccattgcgacgtggagattgt
461

Query B:811ccagacgcccct 822
           ||||||||||||
Sbjct C:460ccagacgcccct 449

Identities = 81/81 (100%)

Query B:70 gatctgatcaaaatcgaaaatacctttttgagtgaagatcaagtagatcaactggcattg
129        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:1117gatctgatcaaaatcgaaaatacctttttgagtgaagatcaagtagatcaactggcattg
1176

Query B:130tatgcgccgcaagccacggtt 150
           |||||||||||||||||||||
Sbjct C:1177tatgcgccgcaagccacggtt 1197

Identities = 230/234 (98%)

Query C:52 gcctctacgcgtacccgcctctctttcgaaacatctatgcaccgcctgggggccagcgtg
111        |||||||| |||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct C:154gcctctacccgtacccgcctctctttcgaaacatctatgcaccgcctaggggccagcgtg
213

Query C:112gtgggcttctccgacagcgccaatacatcactgggtaaaaagggcgaaacgctggccgat
171        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:214gtgggcttctccgacagcgccaatacatcactgggtaaaaagggcgaaacgctggccgat
273

Query C:172accatttcggttatcagcacttacgtcgatgcgatagtgatgcgtcatccgcaggaaggt
231        |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct C:274accatttcggttatcagcacttacgttgatgcgatagtgatgcgtcatccgcaggaaggt
333



Query C:232gcggcgcgcctggccaccgagttttccggcaatgtaccggtactgaatgccggt 285
           |||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct C:334gcggcgcgccttgccaccgagttttccggcaatgtaccggtactgaatgccggt 387

Identities = 449/465 (96%), Gaps = 9/465 (1%)
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```
Query C:281ccggtactgaatgccggtgatggctccaaccaacatccgacgcaaaccttgctggactta
340        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:370ccggtactgaatgccggtgatggctccaaccaacatccgacgcaaaccttgctggactta
429

Query C:341ttcactattcaggaaacccaggggcgtctggacaatctccacgtcgcaatggttggtgac
400        |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:430ttcactattcaggaaaccgaggggcgtctggacaatctccacgtcgcaatggttggtgac
489

Query C:401ctgaaatatggccgcaccgttcactccctgactcaggcgttagcgaagttcgacggcaac
460        |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:490ctgaaatatggccgcacggttcactccctgactcaggcgttagcgaagttcgacggcaac
549

Query C:461cgtttttacttcatcgcgccggacgcgctggcaatgccgcaatacattctggatatgctc
520        ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:550cgtttttacttcattgcgccggacgcgctggcaatgccgcaatacattctggatatgctc
609

Query C:521gatgaaaaagggatcgcatggagtctgcacagctctattgaagaagtgatggcggaagta
579        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct C:610gatgaaaaagggatcgcatggagtctgcacagctctattgaagaagtgatggcggaagta
669

Query C:580gacatcctgtacatgacccgcgtgcaaaaagagcgtctggacccgtcccagtacgcccaa
639        |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||| ||||
Sbjct C:670gacatcctgtacatgacccgcgtgcaaaaagagcgtctggacccgtccgagtacg-ccaa
728

Query C:640cgtgaaagcgcagttttggtcttcgcgccagcgatctccacaaacgccaaaagccaatat
699        |||||||||||||| |||| |||||| ||||||||||||||| |||||| ||||||||||
Sbjct C:729cgtgaaagcgcag-tttgttcttcgtgccagcgatctccac-aacgcc-aaagccaatat
785

Query C:700gaaaagtgcttgcatccgctgcccgcgtggttgatgagattgcga 744
          | ||||||| |||||||||| |||||  ||||||||||||||||
Sbjct C:786g-aaagtgc-tgcatccgctg-ccgcg-cgttgatgagattgcga 826

Identities = 273/277 (98%), Gaps = 1/277 (0%)

Query D:52 gccctcgagcgccaggacctcgcctccgcttccgccctttaagagtcccccgcgatgacg
111        |||||||||||||||||||| |||||  ||||||||||||||||||||||||||||||||
Sbjct D:346gccctcgagcgccaggacct-gcctcgccttccgccctttaagagtcccccgcgatgacg
404



Query D:112ccaatcgacgccaagcgcccgctgcagctcaatgatcagggccagctgcgccacttcctc
171        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:405ccaatcgacgccaagcgcccgctgcagctcaatgatcagggccagctgcgccacttcctc
464
```

```
Query D:172tcgctcgacggtttgccccgcgaactgctcaccgagatcctcgacaccgccgactccttc
231        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:465tcgctcgacggtttgccccgcgaactgctcaccgagatcctcgacaccgccgactccttc
524

Query D:232ctggaagtcggtgcccgggccgtgaagaaagtcccgttgctgcgcggcaagaccgtgtgc
291        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:525ctggaagtcggtgcccgggccgtgaagaaagtcccgttgctgcgcggcaagaccgtgtgc
584

Query D:292aacgtgttcttcgagaactcgacgcgtacccgcacca 328
           |||||||||||||||||||||||| ||||||||||||||
Sbjct D:585aacgtgttcttcgagaactcgacccgtacccgcacca 621

Identities = 743/749 (99%), Gaps = 4/749 (0%)

Query E:28 gagaactcgacgcgtacccgcaccaccttcgaactggcggcccagcgcctgtcggccgac
87         |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:597gagaactcgacccgtacccgcaccaccttcgaactggcggcccagcgcctgtcggccgac
656

Query E:88 gtgatcagcctgaacgtgtcgacctcctcgaccagcaagggcgagaccctgttcgacacc
147        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:657gtgatcagcctgaacgtgtcgacctcctcgaccagcaagggcgagaccctgttcgacacc
716

Query E:148ctgcgcaacctcgaagccatggcggccgacatgtttgtggtacgccattccgactccggc
207        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:717ctgcgcaacctcgaagccatggcggccgacatgtttgtggtacgccattccgactccggc
776

Query E:208gcggcgcacttcatcgccgagcacgtgtgcccggacgtcgccgtgatcaacggtggtgac
267        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:777gcggcgcacttcatcgccgagcacgtgtgcccggacgtcgccgtgatcaacggtggtgac
836

Query E:268ggccgccacgcgcacccgacccagggcatgctcgacatgctcaccattcgccgccacaag
327        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:837ggccgccacgcgcacccgacccagggcatgctcgacatgctcaccattcgccgccacaag
896

Query E:328ggcagcttcgagaacctctcggtagccatcgtcggcgacatcctgcactcgcgcgtggcc
387        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:897ggcagcttcgagaacctctcggtagccatcgtcggcgacatcctgcactcgcgcgtggcc
956

Query E:388cgctccgacatgctggcgctcaaagcgctgggctgcccggacatccgggtgatcggcccg
447        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct D:957cgctccgacatgctggcgctcaaagcgctgggctgcccggacatccgggtgatcggcccg
1016
```

```
Query E:448aaaaccctgatcccgatcggcatcgagcagtacggcgtaaaggtctacaccgacctcgcc
506        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt D:1017aaaaccctgatcccgatcggcatcgagcagtacggcgtaaaggtctacaccgacctcgcc
1076

Query E:507gaaggcctgaaggatgtcgacgtggtgatcatgctgcgcctgcaacgcgagcgcatggcc
566        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt D:1077gaaggcctgaaggatgtcgacgtggtgatcatgctgcgcctgcaacgcgagcgcatggcc
1136

Query E:567ggcggcctgctgcccagcgagggcgagttctaccg-ctgttcgggctgaccaccgcgcgc
625        |||||||||||||||||||||||||||||||| |||||||| ||||||||||||||||
Sbjt D:1137ggcggcctgctgcccagcgagggcgagttctaccgcctgttcggcctgaccaccgcgcgc
1196

Query E:626ctgg-ctgcgccaagcctgacgccatcgtcatgcacccgggcccgatcaaccgtggtgtg
684        |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt D:1197ctggcctgcgccaagcctgacgccatcgtcatgcacccgggcccgatcaaccgtggtgtg
1256

Query E:685gaaatcgagtcggcggtggccgacggc-agcactcggtgatcctcaaccaggtcacctac
743        |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjt D:1257gaaatcgagtcggcggtggccgacggcaagcactcggtgatcctcaaccaggtcacctac
1316

Query E:744 ggcatcgccgtgcgcatggccgtgctgtc 772
             |||||||||||||||||||||||||||||
Sbjct D:1317ggcatcgccgtgcgcatggccgtgctgtc 1345

Identities = 578/586 (98%), Gaps = 7/586 (1%)

Query F:93 tcactgggcgttctcctggtcgaattgacgttgcgcgttctgcccgctcatggccatgga
152        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt D:1403tcactgggcgttctcctggtcgaattgacgttgcgcgttctgcccgctcatggccatgga
1344

Query F:153cagcacggccatgcgcacggcgatgccgtaggtgacctggttgaggatcaccgagtgctt
212        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt D:1343cagcacggccatgcgcacggcgatgccgtaggtgacctggttgaggatcaccgagtgctt
1284

Query F:213gccgtcggccaccgccgactcgatttccacaccacggttgatcgggcccgggtgcatgac
272        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt D:1283gccgtcggccaccgccgactcgatttccacaccacggttgatcgggcccgggtgcatgac
1224


Query F:273gatggcgtcaggcttggcgcaggccaggcgcgcggtggtcaggccgaacaggcggtagaa
332        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt D:1223gatggcgtcaggcttggcgcaggccaggcgcgcggtggtcaggccgaacaggcggtagaa
1164
```

```
Query F:333ctcgccctcgctgggcagcaggccgccggccatgcgctcgcgttgcaggcgcagcatgat
392        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt D:1163ctcgccctcgctgggcagcaggccgccggccatgcgctcgcgttgcaggcgcagcatgat
1104

Query F:393caccacgtcgacatccttca-gccttcggcgaggtcggtgtagacctttacgccgtactg
451        ||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjt D:1103caccacgtcgacatccttcaggccttcggcgaggtcggtgtagacctttacgccgtactg
1044

Query F:452ctcgatgccgatcgggatcagggttttcgggcccgatcacccggatgtccgggcagccca
511        |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjt D:1043ctcgatgccgatcgggatcagggttttcggg-ccgatcacccggatgtccgggcagccca
985

Query F:512gcgctttgagcgccagcatgtcggagcgggccacgcgcgaatgcaggatgtcgcccgacg
571        |||||||||||||||||||||||||||||||||||||||| |||||||||||| ||||||
Sbjct D:984gcgctttgagcgccagcatgtcggagcgggccacgcgcgagtgcaggatgtcg-ccgacg
926

Query F:572atggctaccgagagggttctcgaagctgccctttgtggcggcgaatggtgagcatgtcga
631        |||||||||||||| |||||||||||||||| |||||||||||||||||||||||||||||
Sbjct D:925atggctaccgaga-ggttctcgaagctgccc-ttgtggcggcgaatggtgagcatgtcga
868

Query F:632 gcatg-cctgggtcgggtgcgcgt-gcggccgtcaccaccgttgat 675
            |||||| |||||||||||||||| ||||||||||||||||||||
Sbjct D:867 gcatgccctgggtcgggtgcgcgtggcggccgtcaccaccgttgat 822
```

Figure 18. Insert-vector junction DNA sequence comparison of pBJR28 with known *E. coli pyrBI* sequence. The *E. coli* sequence was obtained from GenBank and is available on the Internet at http://www.ncbi.nlm.nih.gov. Query A is the M13 Forward primer sequence of pBJR28 read from a DNA sequencing polyacrylamide gel. Subject A is the *E. coli pyrBI* operon start with leader peptide sequence with a length of 1581 bp. Query B is the M13 Reverse primer sequence of pBJR28 read from a DNA sequencing polyacrylamide gel. Subject B is the *E.coli pyrBI* operon encoding ATCase with a length of 1410 bp.

```
Identities = 253/268 (94%), Gaps = 10/268 (3%)

Query A: 1 attcaaataaaccgtttgcgctgacaaaatattgcatcaaatgcttgcgccgcttctgac
68         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct A:384attcaaataaaccgtttgcgctgacaaaatattgcatcaaatgcttgcgccgcttctgac
443

Query A:69 gatgagtata-tgccggacaatttgccgggaggatgtatggttcagtgtgt-cgacattt
126        |||||||||| |||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct A:444gatgagtataatgccggacaatttgccgggaggatgtatggttcagtgtgttcgacattt
503

Query A:127tgtcttaccgcgtctgaaaaaagacgctggcctgccgtttttcttcccgttgatcaccca
186        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct A:504tgtcttaccgcgtctgaaaaaagacgctggcctgccgtttttcttcccgttgatcaccca
563

Query A:187ttcccag--cctcaatcgaggggc---ttcccccgcaggcgt-aggagataaaagatggc
240        |||||||  ||||||||||||||   ||    | ||||||| ||||||||||||||||
Sbjct A:564ttcccagcccctcaatcgaggggctttttttgcccaggcgtcaggagataaaagatggc
623

Query A:241taat-cgctatatcag-aacatatcatt 266
           |||| |||||||||| ||||||||||
Sbjct A:624taatccgctatatcagaaacatatcatt 651

Identities = 174/178 (97%), Gaps = 1/178 (0%)

Query B:37 gatctttgcggcccatcacgccagaaggcaggttcagaccaatggtgatgcgctgatccg
96         |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjt B:1120gatctttgcggcccatctcgccagaaggcaggttcagaccaatggtgatgcgctgatccg
1061

Query B:97 tttcggtcagcttgaacagactcaacagcttaaaaccgatctgggcggggatatggtcaa
156        | |||||||||||||||||||||||||||||| |||||||||||||||||||||||||
SbjctB:1060tctcggtcagcttgaacagactcaacagcttaaacccgatctgggcggggatatggtcaa
1001

Query B:157 ttaccgt-ccgcgtttaatagcttcaacctgcaatttattatcgtgtgtcatctctat
213         ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct B:1000ttaccgtgccgcgtttaatagcttcaacctgcaatttattatcgtgtgtcatctctat
943

Identities = 34/36 (94%)

Query B: 1  gatcaactggcattgtatcccccgcaagccacggtt 36
            |||||||||||||||||| | ||||||||||||||
Sbjct B:1162gatcaactggcattgtatgcgccgcaagccacggtt 1197
```

Figure 19. pBJR28 plasmid map.

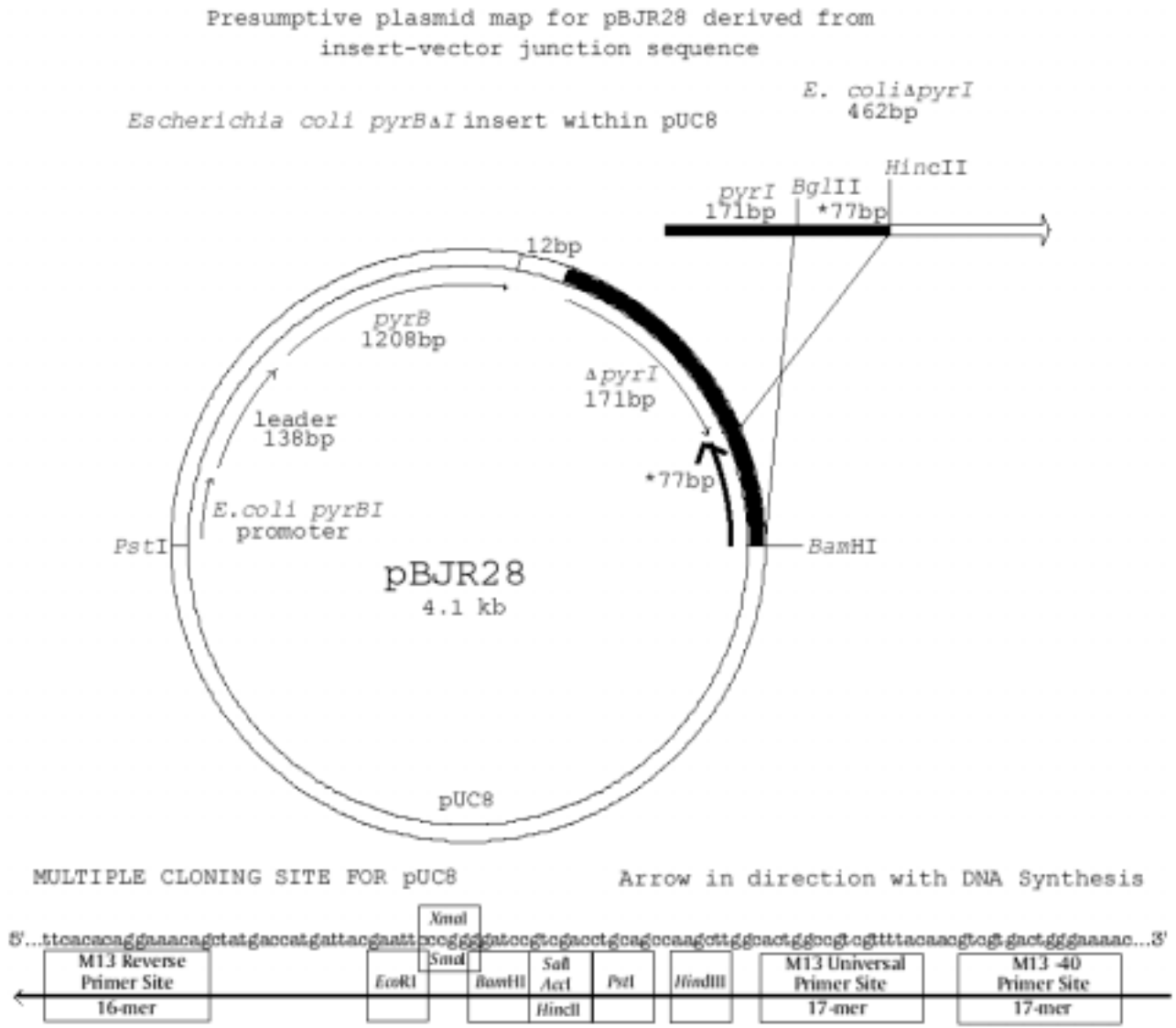


Figure 20. Insert-vector junction DNA sequence comparison of pAK10 with known *P.putida pyrBC'* sequence. The *P. putida* sequence was obtained from GenBank and is available on the Internet at http://www.ncbi.nlm.nih.gov. Query A is the M13 Forward primer sequence of pAK10 read from a DNA sequencing polyacrylamide gel. Subject A is the *P. putida pyrB* and *pyrC'* gene with a length of 1403 bp. Query B is the M13 Reverse primer sequence of pAK10 read from a DNA sequencing polyacrylamide gel. Subject B is the *P. putida pyrR* and *pyrB* gene with a length of 778 bp.

```
Identities = 246/261 (94%), Gaps = 14/261 (5%)

Query A: 6 tcactgggcgt-ctcctggtcgaattgacgttgcgcgt-ctgcccgctcatggccatgga
63         |||||||||||| |||||||||||||||||||||||| |||||||||||||||||||||
Sbjt A:1403tcactgggcgttctcctggtcgaattgacgttgcgcgttctgcccgctcatggccatgga
1344

Query A: 64cagcacggccatgcgcacggcgatgccgtagc-gac-tggtt-aggatcaccgagt-ct-
118        |||||||||||||||||||||||||||||||  ||| ||||| |||||||||||| ||
Sbjt A:1343cagcacggccatgcgcacggcgatgccgtaggtgacctggttgaggatcaccgagtgctt
1284

Query A:119-ccgtcggccaccgccgactcgatttccacaccacggttgatcgggcccgggtgcatgac
177         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjt A:1283gccgtcggccaccgccgactcgatttccacaccacggttgatcgggcccgggtgcatgac
1224

Query A:178gatggcgt-aggctt-gcgcagg-caggcgcgcggtggtcaggccgaacaggcggtagaa
233        |||||||| ||||||| |||||||| ||||||||||||||||||||||||||||||||||
Sbjt A:1223gatggcgtcaggcttggcgcaggccaggcgcgcggtggtcaggccgaacaggcggtagaa
1164

Query A:234 ctcg--ctcgctgggcagcag 252
             ||||  ||||||||||||||
Sbjt A:1163 ctcgccctcgctgggcagcag 1143

Identities = 216/232 (93%), Gaps = 9/232 (3%)

Query B: 4 tctcgttctaccgcacgacgacttccagccagaaca-cct-cacccgcaacttcgcccgt
61         |||||||||||||  ||||||||| |||||||||  ||| ||||||||| |||||||||
Sbjct B:402tctcgttctaccgc--gacgacttc-agccagaacggcctgcacccgcaagttcgcccgt
458

Query B: 62acgagctgcctttcgaggtcgagggaccagcacctggtgctggtggatgacgtactgatg
121        |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct B:459ccgagctgcctttcgaggtcgaggg-ccagcacctggtgctggtggatgacgtactgatg
517

Query B:122agcggtcgcacacgcggcgcggcgctcaacgaactgttcgattacggccgcccgg-cagc
180        ||||||||||   | ||||||||||||||||||||||||||||||||||||| ||||
Sbjct B:518agcggtcgcaccatccgcgcggcgctcaacgaactgttcgattacggccgcccggccagc
577

Query B:181gt-accctggtctgcctgctggacctggat-ccggcgaattgccgatccgtc 230
           || |||||||||||||||||||||||| ||||||||||||||||||||
Sbjct B:578gtcaccctggtctgcctgctggacctggatgccggcgaattgccgatccgtc 629

Identities = 21/21 (100%)

Query B:235ccggcgaattgccgatccgtc 255
           |||||||||||||||||||||
Sbjct B:609ccggcgaattgccgatccgtc 629
```

Figure 21. pAK10 plasmid map.

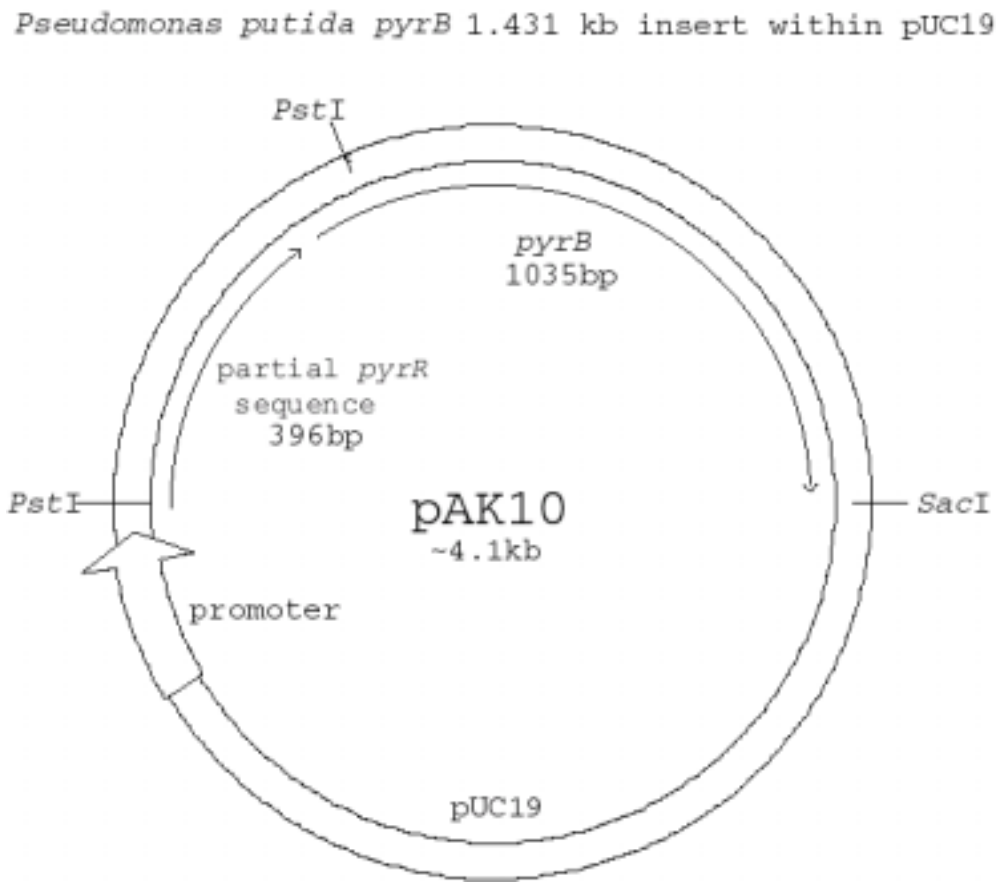


Figure 22. pBEP plasmid map. Green arrows represent sequencing primers. Navy blue arrows represent primers associated with insert DNA.

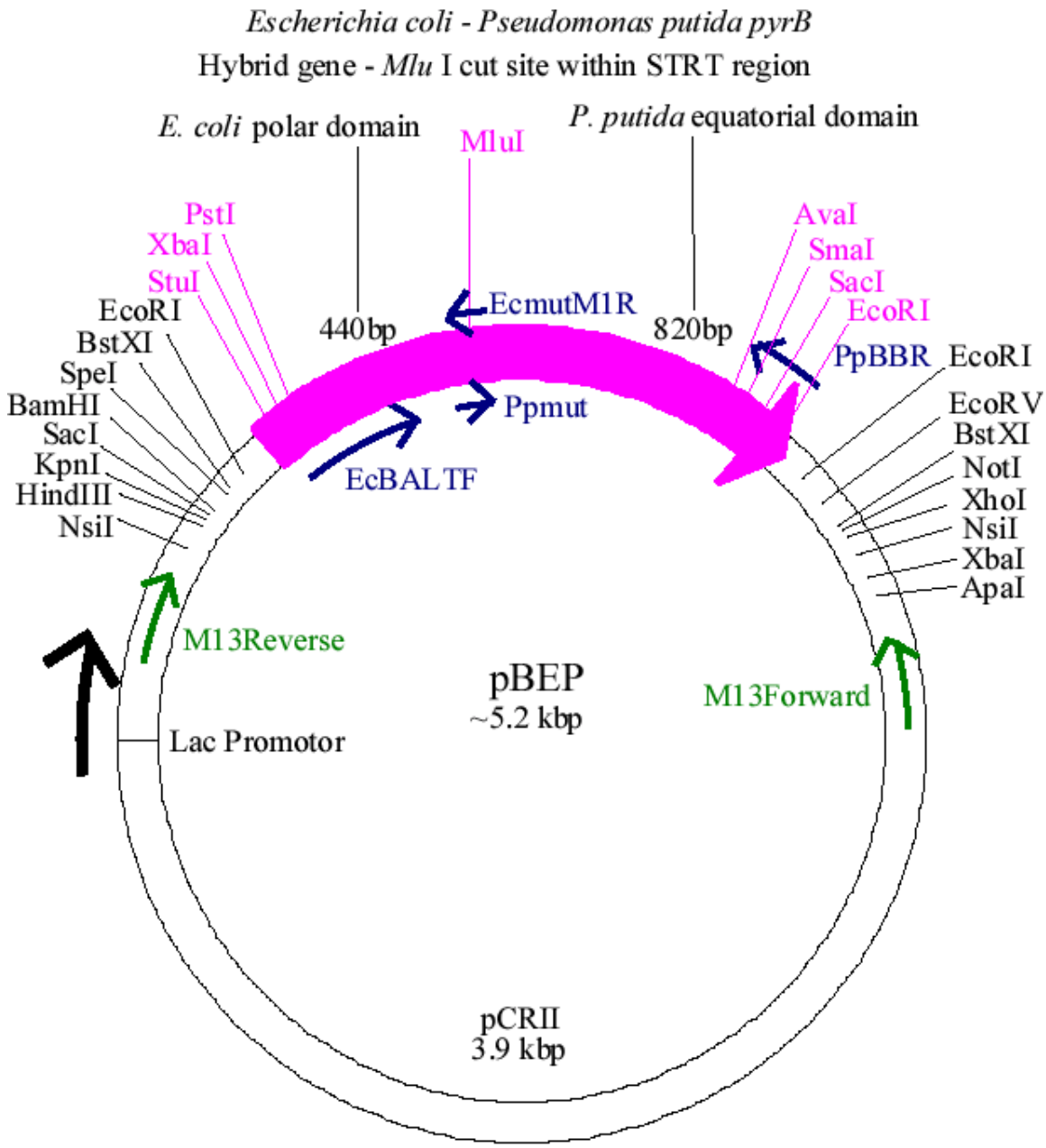


Figure 23. Schematic diagram demonstrating the genealogy of pBEP.

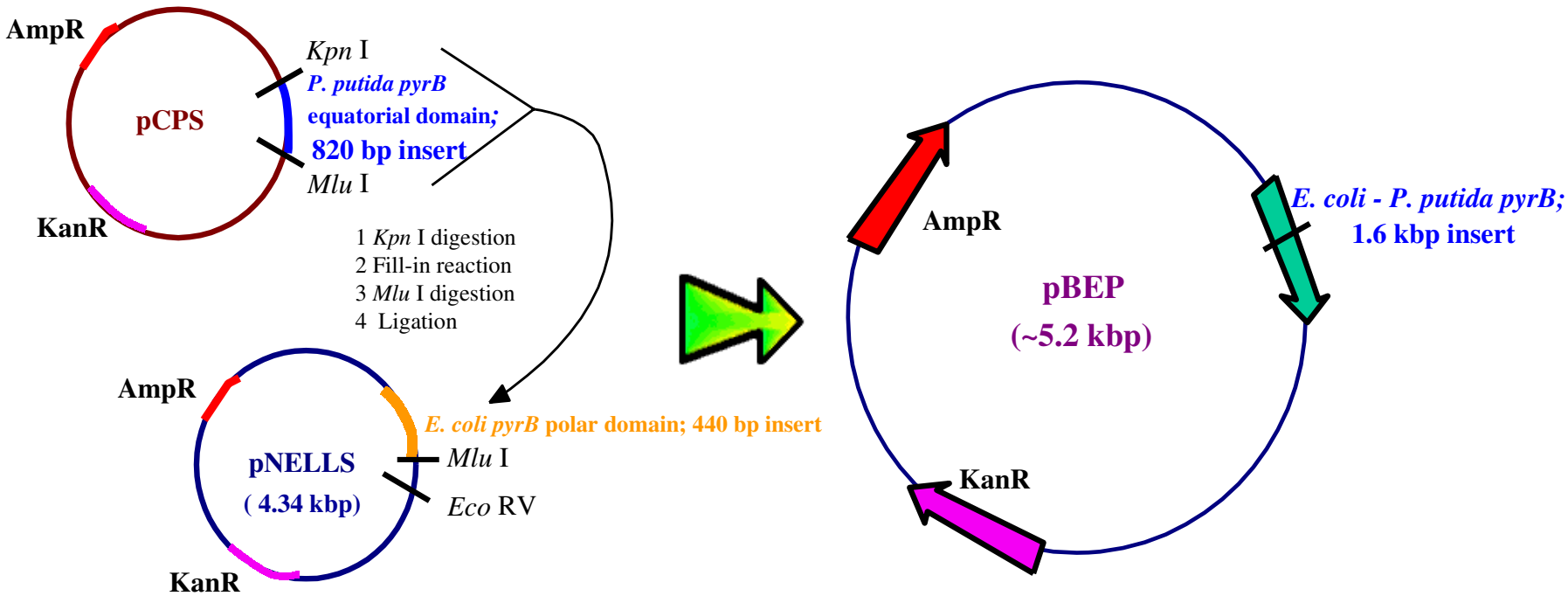
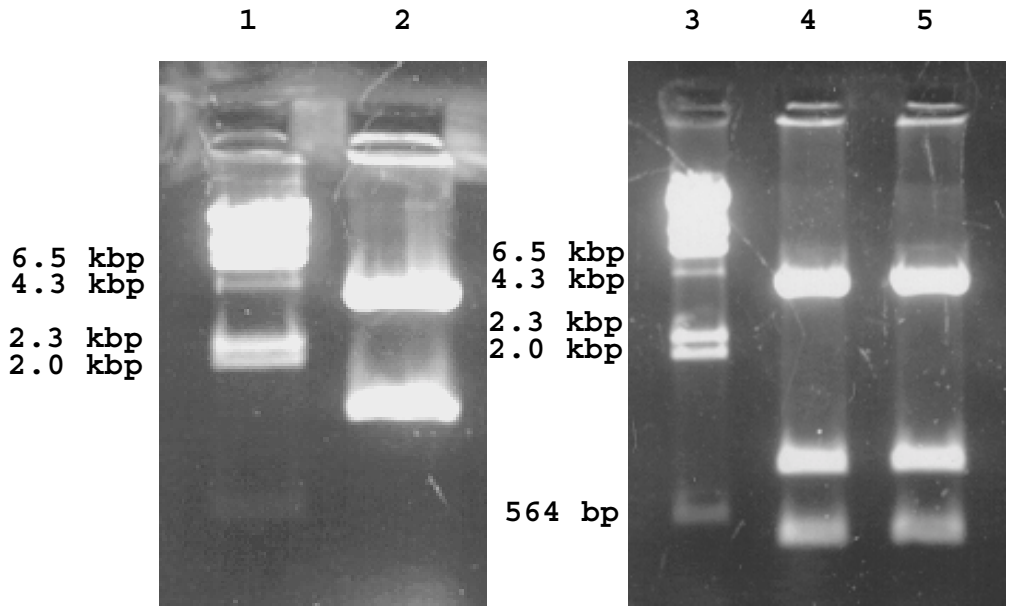


Figure 24. An agarose gel depicting the *Eco*RI and *Mlu*I restriction enzyme digestion of pBEP.

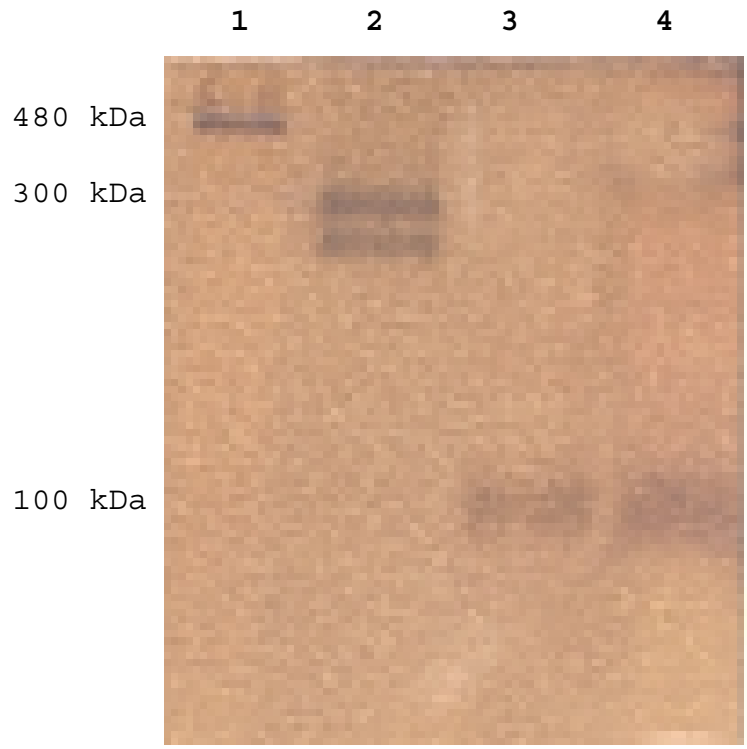


Lane 1 and 3. Lambda DNA- *Hin*dIII digest size marker.

Lane 2. *Eco*RI digest of pBEP - **3.9** kbp linear vector, **1.6 kbp** insert DNA.

Lane 4 and 5. *Eco*RI and *Mlu*I digestion of pBEP - **3.9 kbp linear** vector, **820 bp** *E. coli pyrB* polar domain insert DNA, **440 bp** *P. putida pyrB* equatorial domain insert DNA.

Figure 25. A nondenaturing polyacrylamide activity gel of the ATCase from pBEP.



Lane 1. *P. putida* ATCase holoenzyme.

Lane 2. *E. coli* ATCase holoenzyme.

Lane 3. pBJR28 *E. coli* ATCase trimer within *E. coli* TB2.

Lane 4. pBEP hybrid ATCase trimer within *E. coli* TB2.