GENETIC MANIPULATION OF ASPARTATE TRANSCARBAMYLASES FROM *ESCHERICHIA COLI* AND *SERRATIA MARCESCENS*

by

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ABSTRACT

Aspartate transcarbamylase (ATCase, EC 2.1.3.2) allosterically modulates de novo pyrimidine biosynthesis in the enteric bacteria. This modulation differs in Escherichia coli and Serratia marcescens, with CTP allosterically inhibiting ATCase from E. coli and activating the enzyme in S. marcescens. The enzyme is composed of two catalytic trimers and three regulatory dimmers encoded by the pyrB and pyrI genes respectively. These adjacent cistrons are organized into a single control region to achieve coordinated biosynthesis of the catalytic and regulatory polypeptide chains necessary to form the holoenzyme. The genes for ATCase from the two organisms may be manipulated by in vitro recombinant DNA techniques. After the structure of the pyrBI genes encoding ATCase are characterized, they may be genetically altered and then transformed back into appropriate strains. The in vivo effects may then be studied. Eventually, DNA sequencing may be done for evaluation of evolutionary trends.

ACKNOWLEDGMENT

My sincerest thanks go to Mark S. Shanley, whose excellence as a teacher is only surpassed by his work as a scientist. I would also like to express appreciation to the members of the Wild-O'Donovan lab, whose support and friendship have meant a great deal to me. And finally, Dr. Wild, thank you for giving me this opportunity.

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INTRODUCTION

The relationships among bacterial species have been widely studied in an effort to elucidate species divergence and organization within various families. The family Enterobacteriaceae is no exception, and numerous comparative evaluations have been made (1-3). According to Canovas et al., examination of regulatory control systems will yield insight into the significance of the conservation of a given biochemical pathway within taxonomically similar bacteria (4). The enzyme aspartate transcarbamylase is ideally suited for studies of regulation because it possesses a complex regulatory-catalytic interaction mechanism that differs characteristically among divergent members of Enterobacteriaceae (5).

Aspartate transcarbamylase (ATCase, EC2.1.3.2) catalyzes the first unique step in the de novo biosynthesis of pyrimidine nucleotides in Escherichia coli and other enteric bacteria. Carbamyl phosphate is condensed with aspartic acid in the presence of ATCase to yield carbamyl aspartic acid and inorganic phosphate (Figure 1). Regulation of this enzyme is carried out at all three levels within the cell: metabolic, enzymatic, and genetic.

Yates and Pardee proposed feedback inhibition to be a metabolic control mechanism of pyrimidine biosynthesis

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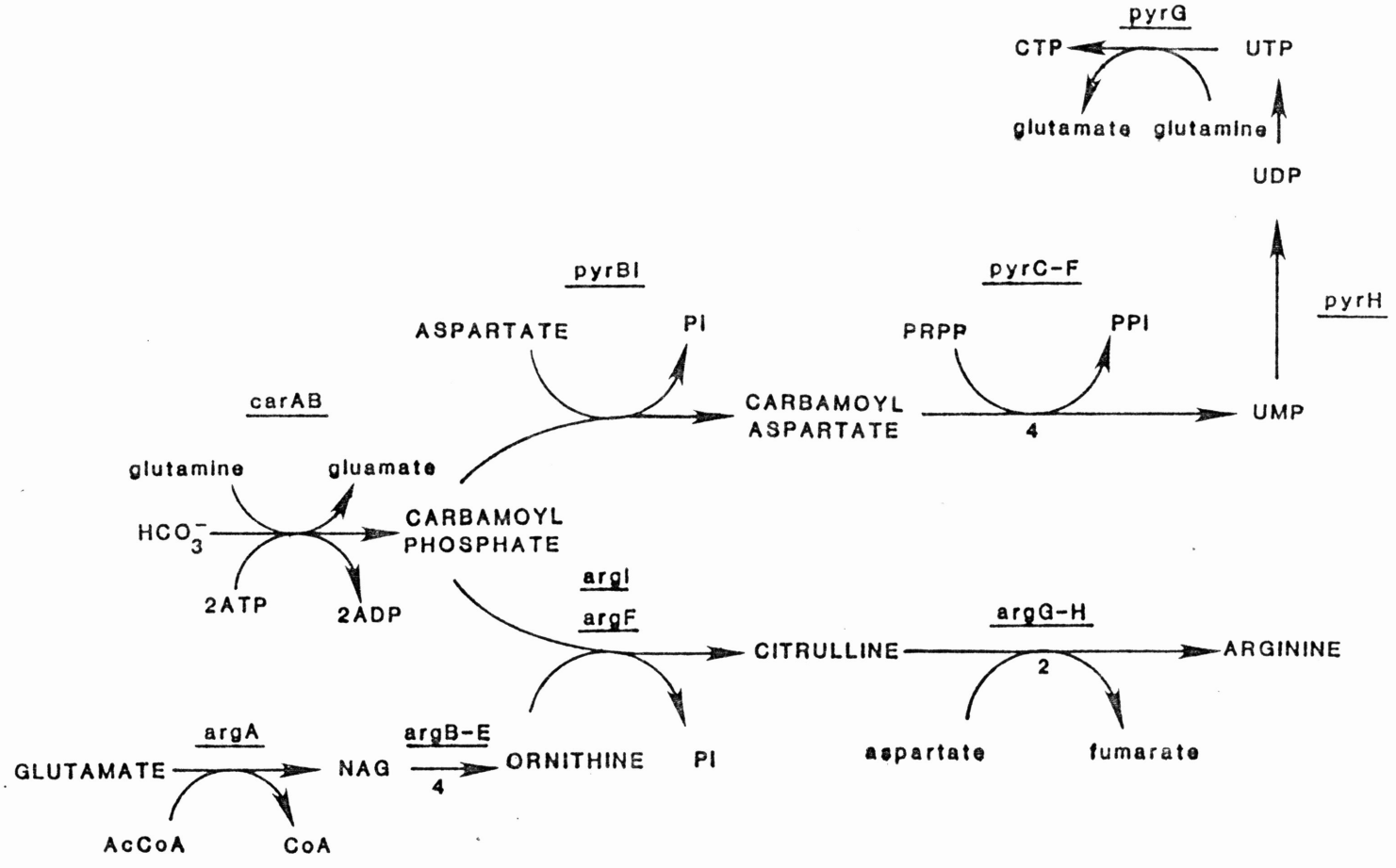

Figure 1. Pyrimidine biosynthetics pathway in E. coli

in E. coli (6). They found that the ultimate endproduct of the pathway, CTP, inhibits ATCase and thus prevents the accumulation of most intermediates in the pathway both in vivo and in vitro. Gerhart and Pardee also demonstrated that inhibition was specific for cytosine derivatives among the pyrimidine compounds they examined (7).

Enzymatically, cytosine derivatives behave as negative allosteric effectors in E. coli by binding a site on the enzyme other than the active site. It has been shown that ATP serves as a positive allosteric effector in E. coli, facilitating production of a balanced pool of purines and pyrimidines to carry out nucleic acid biosynthesis (7).

In Serratia marcescens, a more divergent member of the enteric bacteria whose own ATCase is structurally similar to the ATCase of E. coli, ATP allosterically activates the enzyme; however, CTP has been shown to activate ATCase as well (8).

On a genetic level, there is evidence that the amount of ATCase synthesized in E. coli is controlled by a repressor mechanism (9). At this time, though , very little is known about this repressor or its operator. The regulatory region of the genes encoding the enzyme in E. coli, including the DNA sequence, has been examined by Roof et. al. who confirm a rho-independent attenuator sequence within the control region of the genes (10).

The characteristics of ATCase in the enteric bacteria have been widely studied (review 11). The enzyme has a molecular weight of approximately 310,000, but is dissociated upon treatment with mercurials to yield two identical subunits (C_3) that are trimers of catalytic polypeptide chains and three identical regulatory subunits (r_2) composed of dimeric proteins to yield a composition of $2(C_3):3(r_2)$ (Figures 2 and 3).

While the catalytic subunits are active catalytically and pyrimidine biosynthesis may be achieved without the regulatory subunits, only the holoenzyme is subject to feedback inhibition or activation by CTP (12). The dissociated subunits can be recombined to yield a functional holoenzyme with the r:c domains of interaction re-established (13).

ATCase from E. coli is encoded by the pyrBI genes, which map at 96.0 minutes and are linked, producing a bicistronic message that codes for the catalytic and regulatory polypeptide chains (14) (Figure 4).

Comparatively little is known about ATCase production in Serratia marcescens on a molecular level. Shanley (personal communication) has verified the presence of the pyrBI genes encoding ATCase catalytic and regulatory subunits; however, the genes have yet to be characterized.

The goal of this research is to further elucidate the expression of ATCase in S. marcescens. Moreover, attempts

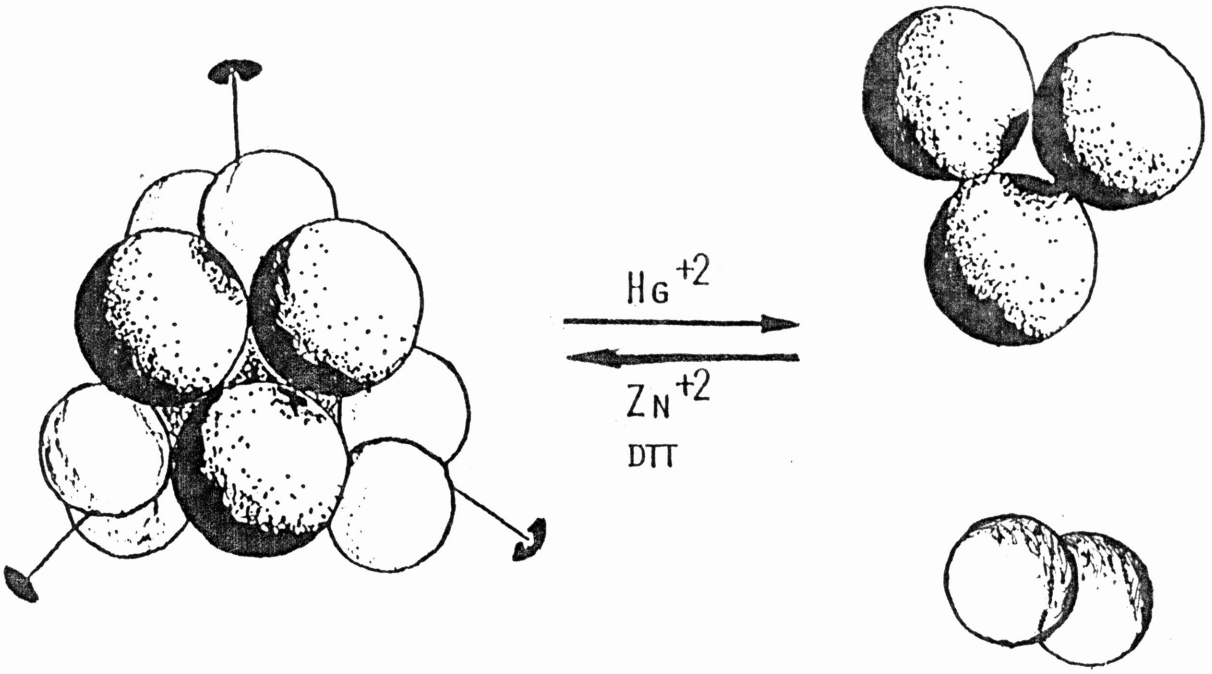

Figure 2. Dissociation of ATCase with mercurials

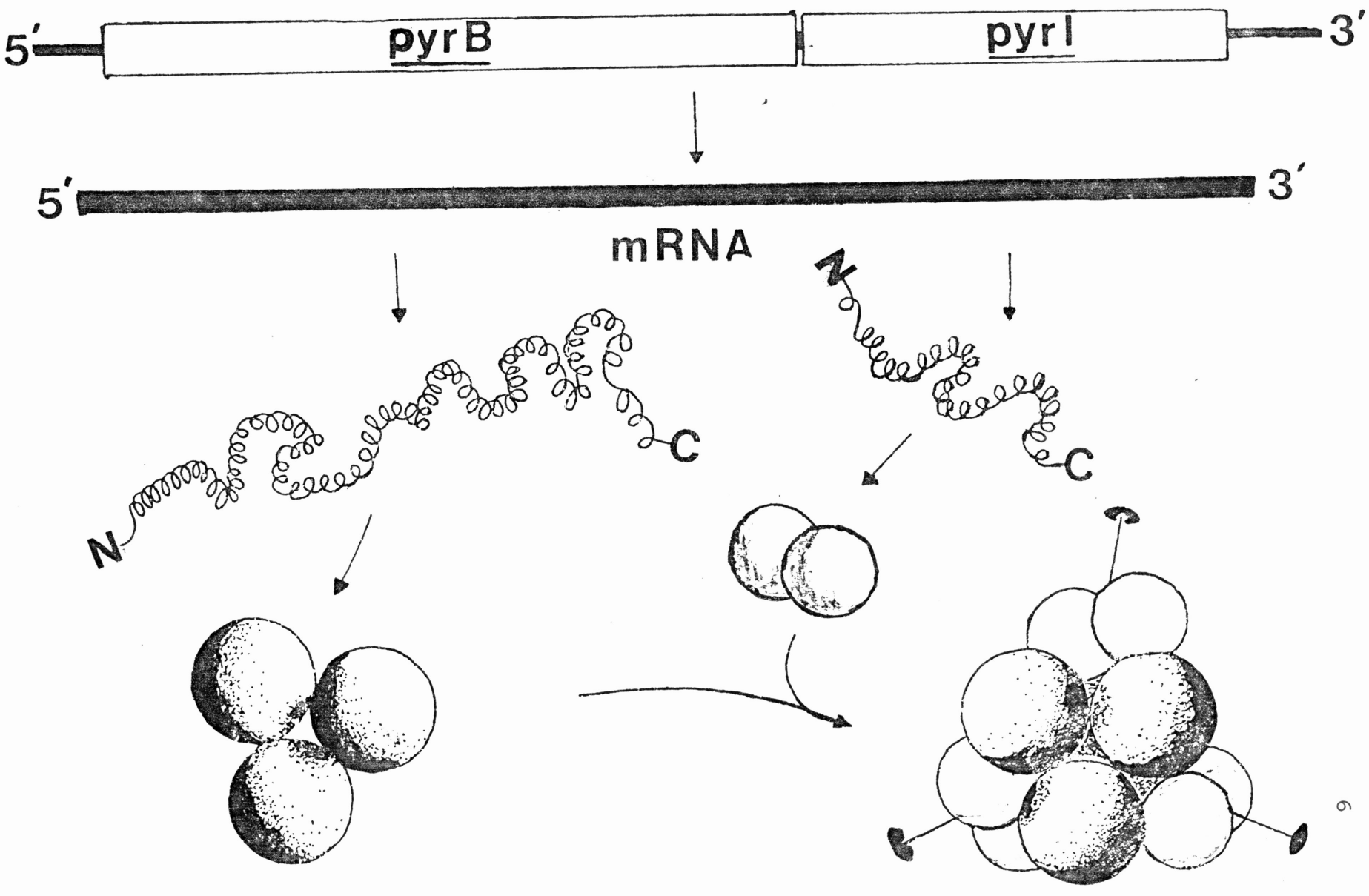

Figure 3. Holoenzyme construction

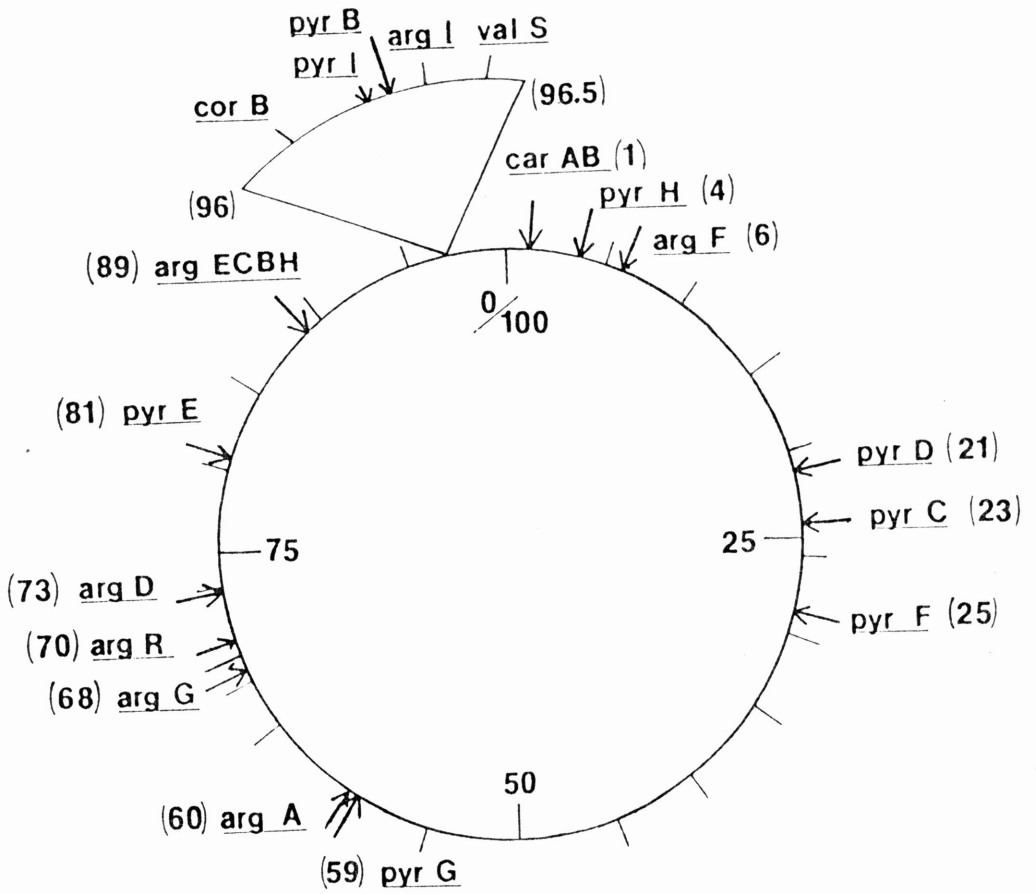

Figure 4. The pyrBI genes map at 96.0 minutes in E. coli

have been made to achieve expression of ATCase from S. marcescens in E. coli and to produce active hybrid enzymes.

MATERIALS AND METHODS

Preparation of Bacterial Strains and Plasmids

The pyrimidine-requiring auxotrophic mutant of *E. coli* K-12 utilized in this research was constructed by William D. Roof using Mu dl(*lac* Ap^r) to insert and subsequently delete genomic DNA (15). The resulting strain, HB101 4442, was *pyrB*$^-$ and was sensitive to both ampicillan and tetracycline.

The vector used for transformation and plasmid construction, pBR322 (16), was purified through a 48 hour ultracentrifugation in cesium chloride immediately following triton lysis (17). Purified plasmid was extracted from the gradient, dialyzed against 2 changes of DNA buffer (20mM Tris-HCl, 1mM Na_2.EDTA, pH7.4), and stored at 4^oC.

The recombinant plasmids were constructed according to Figures 5 and 6. Genomic *S. marcescens* HY wild type DNA was isolated by Mark Shanley, and restriction endonucleses obtained from Bethseda Research Labs in Rockville, Maryland were used to cleave the DNA in a series of partial digestions (17). The DNA fragments were ligated with T_4 DNA ligase (also obtained from BRL) into appropriately restricted pBR322, generating plasmids containing various-sized inserts. Plasmid pPB-r101 was constructed by Karen F. Foltermann, shown in Figure 6.

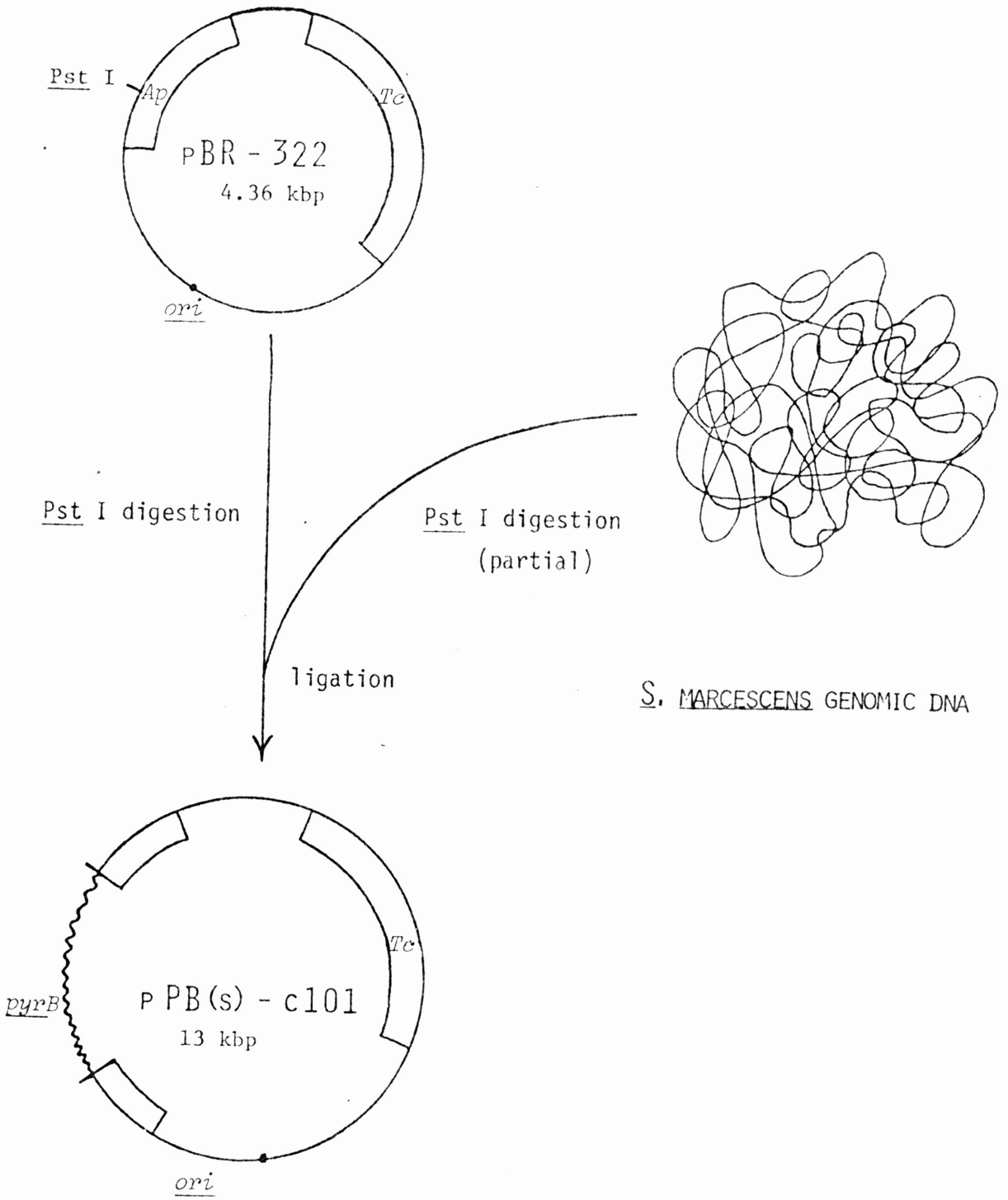

Figure 5. Construction of pPB(s)-c101. Plasmid pPB(s)-c402 is a PstI subclone of pPB(s)-c101

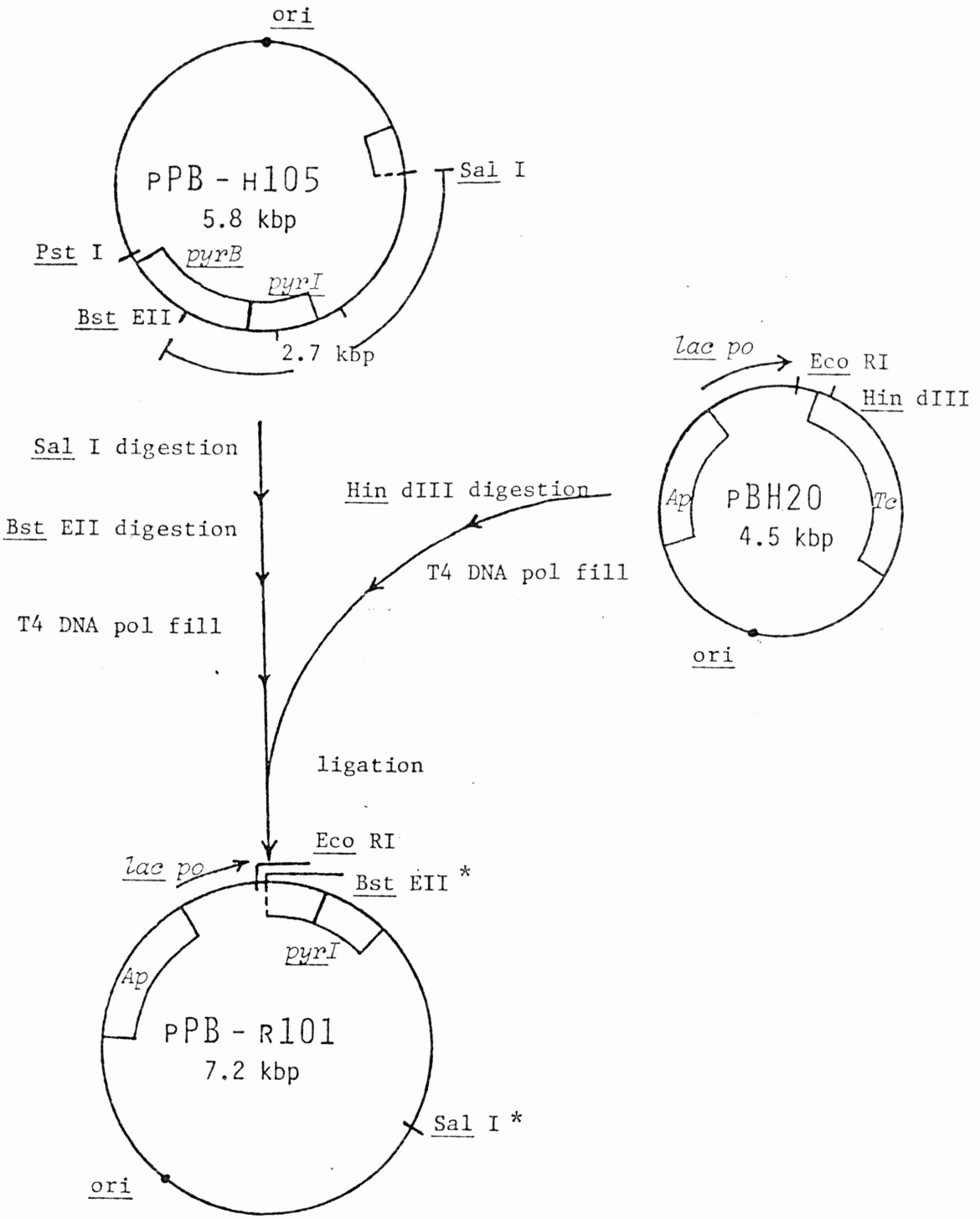

Figure 6. Construction of pPB-r101 by Karen F. Foltermann

Growth Conditions

Bacteria were grown at 37^{o} in TF medium (100 mMTris, pH7.8, 10% Tris Salts, 0.05mM $MgCl_2.6H_2O$, 20% NH_4Cl) supplemented with 2 ug/ml thiamin, 0.1% case amino acids, and 0.2% glucose per liter of distilled deionized water. Auxotrophs were also grown at 37^{o}C in TF + dextrose + B_1 + uracil (25 ug/ml) + case amino acids (TFDCB+U).

Overnight growth, resulting in stationary phase cutures, provided maximum yield in screening procedures.

Transformation and Phenotypic Selection

Constructed plasmids were transformed into HB101 4442 competent cells according to the method of Maniatis et al. (17). Cells were then streaked onto selective media plates: TYE (10g bactotryptone, 5g yeast extract, 5g NaCl per liter distilled deionized water, pH 7.0), TYE + Ampicillan (40 ug/ml), TYE + Tetracycline (25 ug/ml), TFDCB and TFDCB+U.

Plasmid Screening

Phenotypically selected cells were screened for the presence of plasmids using a quick plasmid isolation procedure (17) and subsequently subjected to gel electrophoresis on horizontal submersible agarose gels (0.7%) prepared in Tris-borate buffer (0089M Tris, 0.089 M boric acid, 2.5mM Na_2EDTA, pH 8.3). After electrophoresis, the gels were stained with ethidium bromide

(0.5 ug/ml) and destained in Tris-borate buffer. The DNA could then be observed and photographed under UV light. Plasmid pPB(s)-c402, a subcloned PstI digest, and plasmid pPB(s)-502, a HindIII digest, were thus screened for further analysis and purified as detailed previously.

Restriction Endonuclease Characterization

Isolated plasmids were digested with restriction endonucleases according to the supplier's recommendations. The restricted fragments were simultaneously electrophoresed with known standard digestions using horizontal agarose (0.7%) gels as previously described. Estimations of molecular size were then made using plots of log Base Pairs vs. distance migrated (mm), and a map was constructed as in Figure 7.

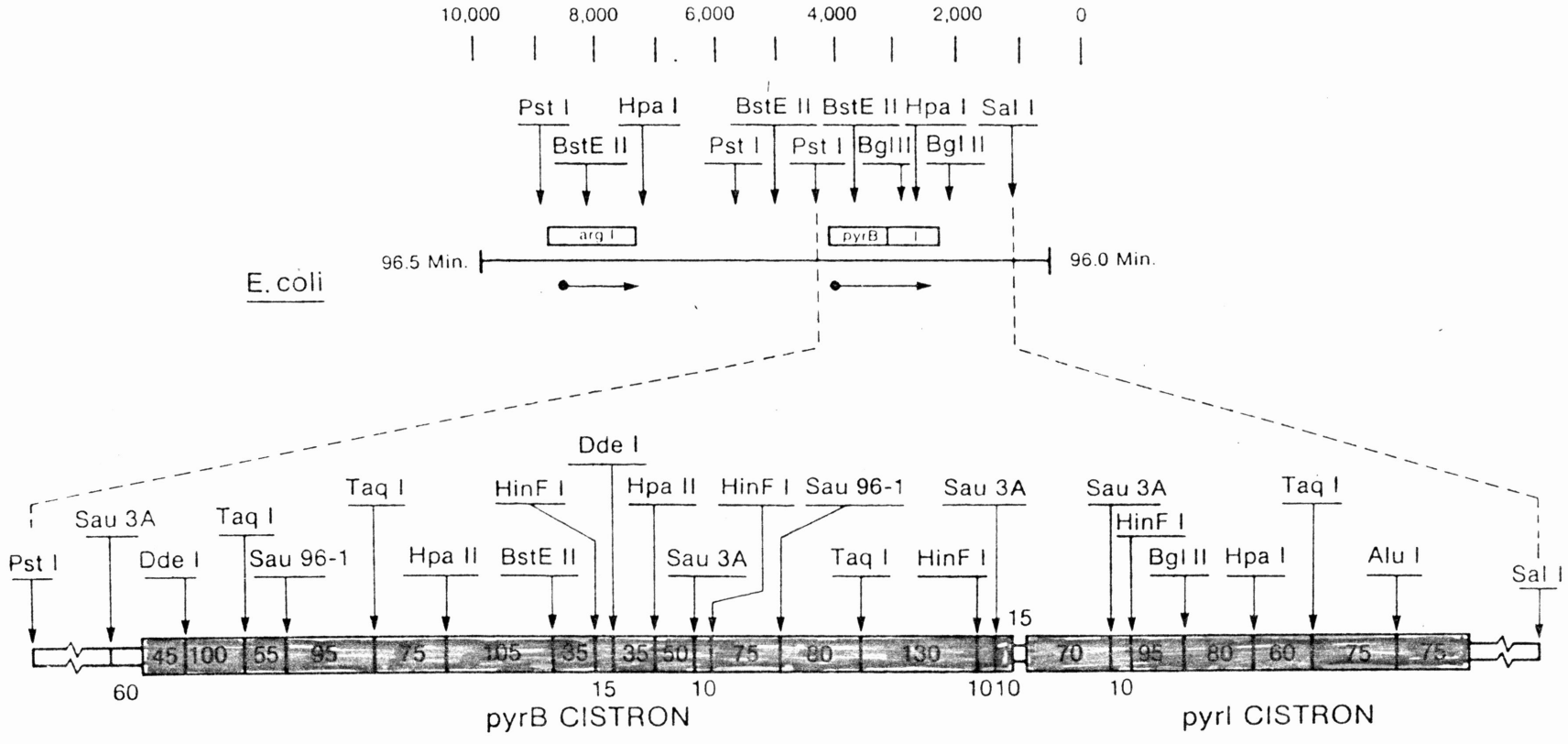

Figure 7. Restriction endonuclease map of E. coli pyrBI gene region

RESULTS

Expression of pPB(s)-c402 and pPB(s)-501 in *E. coli*

After transformation of HB101 4442 with pPB(s)-c402 and pPB(s)-501, colonies were streaked on selective media and the following results were obtained.

Table 1. Transformation Results

Strain	Media				
	TYE	TYE+Ap	TYE+Tc	TFDCB	TFDCB+U
pPB(s)-c402	+	+	-	+	+
pPB(s)-501	+	-	+	+	+
pBR322(control)	+	-	-	-	-

These results demonstrate that both plasmids transform HB101 4442 *pyrB*$^-$ cells to prototrophy, and that DNA derived from *S. marcescens* can be successfully cloned into *E. coli*.

Plasmid purification and electrophoresis indicated that pPB(s)-c402 has a molecular of approximately 6 kilobases, thus containing an insert of approximately 1.5-1.6 kibobases. Plasmid pPB(s)-501 has a molecular size of over 22 kilobases, with an insert size of over 18 kilobases. More precise size approximations could not be made due to inaccuracy of standard comparisons within that size range.

Restriction Endonuclease Characterization

Plasmid pPB(s)-c402 was most easily characterized due to the small size of inserted S. marcescens DNA. Only a crude restriction map was constructed because future sequencing of the insert would yield more information at a greater efficiency. The workable map is illustrated in Figure 8.

Plasmid pPB(s)501 was much more difficult to characterize due to initial purification problems and its large size. However, restriction sites are outlined in Figure 9. These sites have not been ordered, nor is their orientation necessarily correct.

Hybrid ATCase Formation

Plasmid pPB(s)-c101, containing only pyrB from S. marcesens (Mark Shanley, personal communication), was transformed with Karen F. Foltermann's pPB-r101, which contains E. coli pyrI under lac control, into E. coli HB101 4442. The results in Table 2 were obtained, with expected results outlined in Table 3.

Table 2. Hybrid Transformation Results

	Media			
	TYE	TYE+Ap	TYE+Tc	TYE+AptTc
hybrid cells	TMTC	+	+	+
	TFDCB+Ap	TFDCB	TFDCB+U	
hybrid cells	+	TMTC	TMTC	

TMTC=too many count

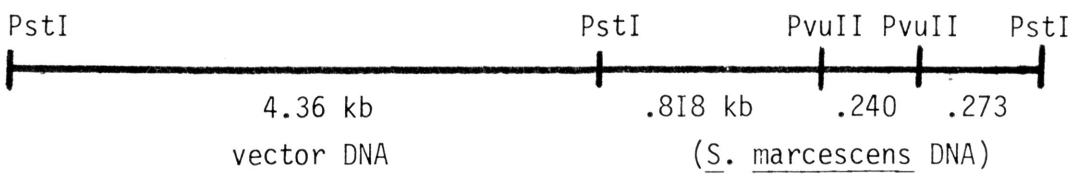

Figure 8. Restriction sites of pPB(s)-c402

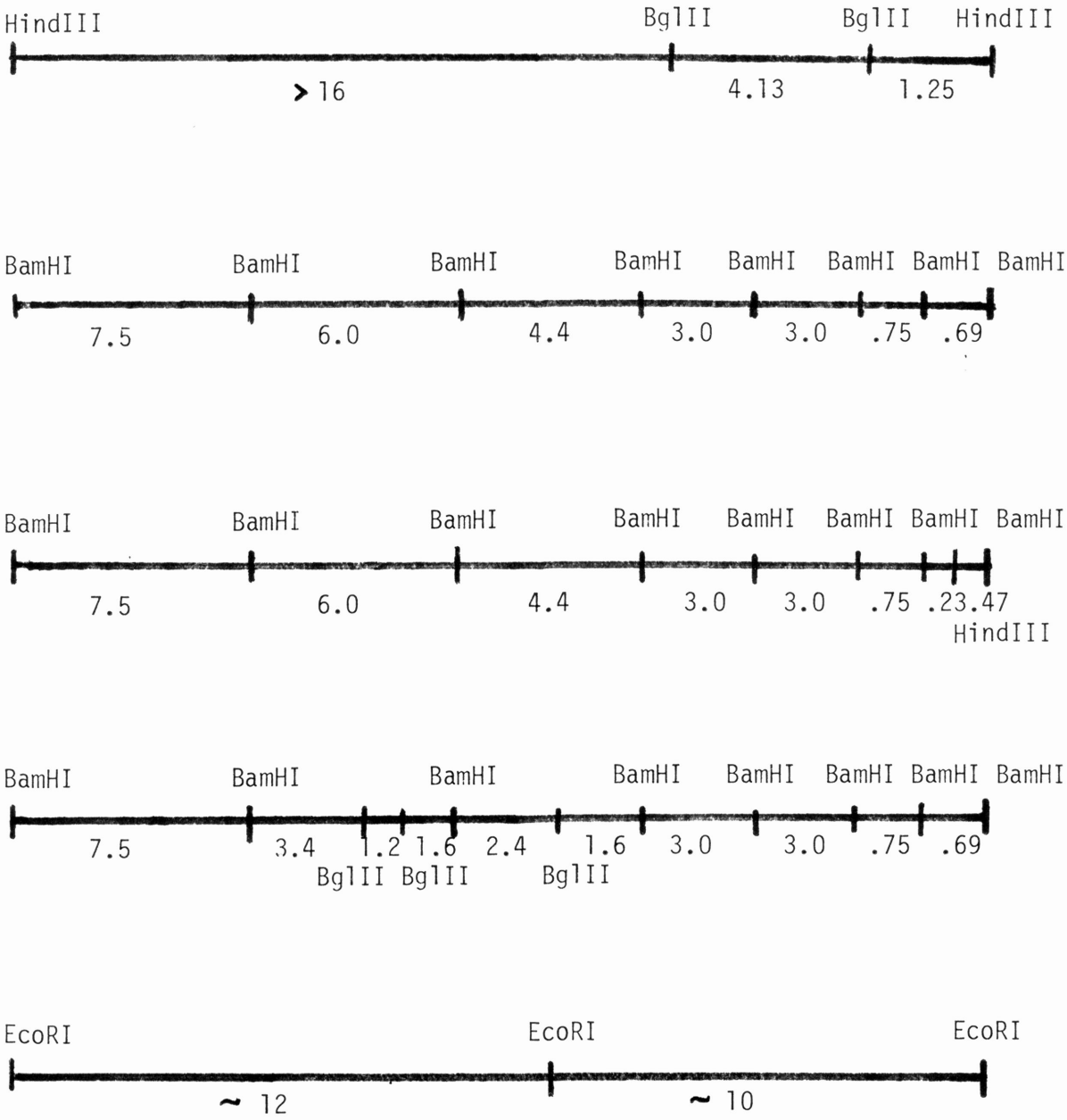

Figure 9. Restriction sites of pPB(s)-501

Table 3. Hybrid Transformation Predicted Results

	pPB-r101	pPB(s)-c101	Hybrid
ATCase Activity	-	+	+
Ampicillan response	Ap^r	Ap^s	Ap^r
Tetracycline response	Tc^s	Tc^r	Tc^r

Cells were re-picked to verify phenotypes, and colonies from TYE+Ap+Tc, TFDCB+Ap, and TYE+Ap plates were disrupted to test for inserts. Figure 10 verifies the presence of the two plasmids pPB(s)-c101 and pPB(s)-501, which are coding for the catalytic and regulator subunits of ATCase, respectively, to form a hybrid enzyme.

Mark Shanley has performed kinetic analyses on the hybrid ATCase, and his results indicate that effector response is present, with ATP activating the enzyme and CTP inhibiting the enzyme. He also notes an increase substrate requirement for the enzyme.

Discussion

Although E. coli and S. marcescens are both members of the family Enterobacteriaceae, the regulation of aspartate transcarbamylase in both organisms is divergent. ATP yields a positive effector response in both, yet CTP negatively effects E. coli and positively effects S. marcescens (8). One may thus question the regulatory logic of S. marcescens and ask how the genes encoding ATCase in the two organisms differ.

Top Well		Bottom Well	
Lane(s)		Lane(s)	
1	pBR322	1	pBR322
2	pPB(s)-c101	2	pPB(s)-c101
3-10	from TYE+Ap+Tc	3-9	from TYE+AptTc
11-20	from TFDCB+Ap	10-18	fromTFDCB+Ap
		18-20	from TYE+Ap

0.7% agarose gel in 1X TBE

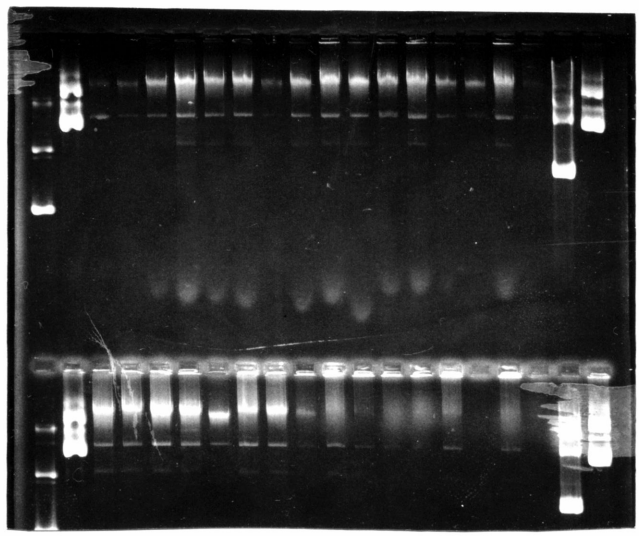

Figure 10. Gel electrophoresis of isolated plasmids

In an effort to understand the differences between the two cistrons, comparisons have been made at the molecular level. It is apparent that enough similarities exist between the two organisms to allow expression of S. marcescens ATCase in E. coli. Mark Shanley (unpublished data) has verified that the ATCase produced in E. coli encoded by S. marcescens DNA maintains the effector response present in S. marcescens; i.e., ATP activation and CTP inhibition. This evidence supports the claim that ATCase expression is regulated at the molecular level as well.

The fact that S. marcescens catalytic subunits can bind substrate in E. coli also implies that these subunits should be similar to those normally present in E. coli, and thus the genes may be homologous. Karen M. Kedzie (unpublished data) has begun sequencing the insert in pPSc402 containing S. marcescens pyrB, and initial sequence analysis has indicated conservation of the first twenty amino acids of the catalytic polypeptide when compared to E. coli.

However, restriction analysis of pPP(s)-c402 and pPB(s)-501 has not supported complete DNA sequence homology between E. coli and S. marcescens. Although amino acid sequence has been conserved, single base changes within the DNA sequence have altered restriction endonuclease cleavage sites, and the restriction maps generated do not completely correlate. At this time it is hard to predict where the greatest regions of homology will occur, but one may

hypothesize that the pyrB sequence will be quite similar in both organisms and that the pyrI regulatory sequence may be very different to elicit the CTP positive effector response in S. marcescens.

Formation of the hybrid ATCase supports this hypothesis in that the r:c domains of interaction are conserved when the regulatory subunits of E. coli bind the catalytic subunits of S. marcescens to form the hybrid enzyme. Because the effector response is characteristic of E. coli; the conclusion may be drawn that the regulatory subunit alone is responsible for regulation of expression.

Further research is being done in an effort to form a hybrid enzyme assembled with regulatory subunits from S. marcescens pyrI and catalytic subunits from E. coli pyr B. Plasmid pPB(s)-501 contains both pyrB and pyrI from S. marcescens. When the insert is further characterized, it will be possible to subclone a fragment containing only pyrI into M13mp8 (17). Because the regulatory gene is promoter distal, the promoter will be lost when pyrI is isolated, and the gene must be placed under control of the lac promoter-operator for sufficient expression. This new plasmid will be transformed into JM103 simultaneously with a plamid containing only pyrB from E. coli. Selective pressure will be applied, and phenotypically desired cells, if any, will be analyzed for effector response. A molecular weight determination will be made, and eventually pyrI of

S. marcescens will be sequenced.

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