in *E. coli* Includes a Rho-Independent Attenuator Sequence

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Summary. 1. The two polypeptide chains that comprise aspartate carbamoyltransferase in *Escherichia coli* are encoded by adjacent cistrons expressed in the order, promoter-leader-catalytic cistron-regulatory cistron (p-leader-*pyrBI*). These two cistrons and their single control region have been cloned as a 2,800 base pair (bp) fragment (The minimal coding requirement for the catalytic and regulatory polypeptides is about 1,350 bp plus control regions). The genes contained by this fragment are subject to normal repression controls and thus possess the intact control regions.

2. By deleting an internal fragment with specific restriction endonucleases, it was possible to construct shortened fragments which no longer produced the regulatory polypeptide. In these cases the expression of the catalytic cistron was normal and subject to repression upon growth in the presence of uracil. Since the *pyrB* cistron retained transcriptional control, the regulatory polypeptide was not required for expression or control of the catalytic cistron. As expected, the catalytic trimer (M_r=100,000 daltons) from these deletion mutants had no effector response nor did it exhibit homotropic kinetics for aspartate. The enzyme was identical to the c_3 trimer purified from the native holoenzyme by neohydrin dissociation.

3. Insertion of Mu d1(*lac* Ap^r) into the structural region of *pyrB* had a negative effect on the expression of *pyrI*. This supports the idea that the catalytic and regulatory polypeptide chains of aspartate carbamoyl-transferase are encoded by a single bicistronic operon. Detailed restriction analysis of the cloned *pyrBI* region has produced a genetic map of restriction sites which is colinear with the published amino acid sequences of the two polypeptides. These maps indicate that the 3′-terminus of the catalytic cistron is adjacent to the 5′-terminus of the regulatory cistron and separated by 10–20 bp.

4. DNA sequence analysis of the 5′-proximal regions of *pyrBI* revealed that an extensive leader sequence separated the promoter and first structural gene *pyrB*. This leader of approximately 150 bp contains an attenuator sequence and the translational signals required for the production of a leader polypeptide of 43 amino acids.

In this paper we describe the structural organization of *pyrBI*, and provide a detailed analysis of its regulatory region including its DNA sequence.

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Introduction

De novo pyrimidine (UMP) biosynthesis (see Fig. 1) in both *Escherichia coli* and *Salmonella typhimurium* is regulated allosterically at aspartate carbamoyltransferase (ATCase; Gerhart and Pardee 1962) and carbamoylphosphate synthetase (CPSase; Pierard et al. 1965; Anderson and Meister 1965) or transcriptionally throughout (*carAB* to *pyrF*) in response to fluctuations in endogenous nucleotide pools (Williams and O'Donovan 1973; Kelln et al. 1975; Schwartz and Neuhard 1975). While the evidence for the involvment of various nucleotide pools in the repression/derepression of the *pyr* genes is clear, extensive searches for a suitable aporepressor have not been successful (O'Donovan and Neuhard 1970; Kelln and O'Donovan 1976). O'Donovan and Gerhart (1972) reported a putative *pyrR* which resulted in derepression of the *pyr* genes, but upon subsequent analysis it was found to be a mutation in UMP kinase (*pyrH*) (Justesen and Neuhard 1975). This leaky mutant produced greatly reduced levels of UDP and UTP, thereby causing derepression of the de novo pathway. Kelln has isolated *cis*-acting mutations ("operator-like") which are derepressed for *pyrB* (R.A. Kelln, personal communication) and recently, Jensen et al. (1982) reported the derepression of *pyrB* in a strain of *S. typhimurium* possessing an altered RNA polymerase (mapping in the *rpoBC* gene cluster). While this observation suggests a regulatory role for RNA polymerase in the expression of *pyrB*, the regulatory control of *pyrBI* remains undefined. The present study details the structural organization of the *pyrBI* region of the *E. coli* chromosome and presents evidence consistent with sequential expression of the catalytic and regulatory polypeptides from a bicistronic operon (po *pyrB*, *pyrI*). Similar results describing a bicistronic operon encoding ATCase have been obtained for *S. typhimurium* (G. Michaels and R.A. Kelln submitted to Mol. Gen. Genet).

The aspartate carbamoyltransferase (EC 2.1.3.2) of *E. coli* is a multimeric enzyme possessing allosteric control sites on regulatory polypeptides which are distinct from the catalytic subunits (Gerhart and Schachman, 1965). The ATCase of *E. coli* and other enteric bacteria (Wild et al. 1980) and yeast phosphofructokinase (EC 2.2.1.11; Laurent et al. 1978) are the only reported multimeric enzymes comprised of catalytic subunits and separable regulatory subunits. The native holoenzyme of *E. coli* is a dodecamer composed of six identical catalytic polypeptides (functional as a trimer, c_3) and six identical regulatory polypeptides (functional as

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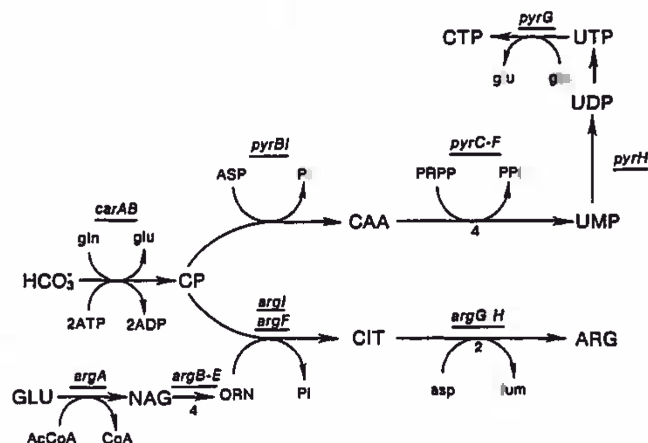

Fig. 1. De novo biosynthetic pathways for pyrimidine and arginine with genetic loci of *E. coli* indicated. GLU, glutamate; NAG, N-acetyl-glutamate; ORN, ornithine; CIT, citrulline; asp, asparate; fum, fumarate; ARG, arginine; HCO_3^-, bicarbonate; gln, glutamine; glu, glutamate; ATP, adenosine-5'-triphosphate; ADP, adenosine-5'diphosphate; CP, carbamoylphosphate; ASP, aspartate; CAA, carbamoylaspartate; UMP, uridine-5'monophosphate; UDP, uridine-5'diphosphate; UTP, uridine-5'-triphosphate; CTP, cytidine-5'-triphosphate; PRPP, 5'-phosphoribosyl-3'-pyrophosphate; Pi, inorganic phosphate; PPi, inorganic pyrophosphate. The genetic loci encode the respective biosynthetic enzymes: *argA*, amino acid acetyltransferase (EC 2.3.1.1.); *argB* acetylglutamate kinase (EC 2.7.2.8); *argC*, N-acetyl-γ-glutamyl-phosphate reductase (EC 1.2.1.38); *argD*, acetylornithine aminotransferase (EC 2.6.1.11); *argE*, acetylornithine deacetylase (EC 3.5.1.16); *argI*, *argF*, duplicate genes for ornithine carbamoyltransferase (EC 2.1.3.3); *argG*, argininosuccinate synthetase (EC 6.3.4.5); *argH*, argininosuccinate lyase (EC 4.3.2.1); *carAB*, glutamine (light) and ammonia (heavy) subunits of carbamoylphosphate synthesase (EC 6.3.5.5 or EC 6.3.4.16); *pyrBI*, catalytic and regulatory subunits of aspartate carbamoyltransferase (EC 2.1.3.2); *pyrC* dihydroorotase (EC 3.5.2.3); *pyrD*, dihydroorotate oxidase (EC 1.3.3.1); *pyrE*, orotate phosphoribosyltransferase (EC 2.4.2.10); *pyrF*, orotidine-5'phosphate decarboxylase (EC 4.1.1.23), *pyrG*, CTP synthetase, (EC 6.3.4.2); *pyrH*, UMP kinase (EC 2.7.4.4). The genetic nomenclature is that of *E. coli* (Bachmann and Low 1980) and the enzyme nomenclature is from Dixon and Webb (1979). Multiple biosynthetic steps are indicated as CAA to UMP in 4 steps catalyzed by enzymes encoded by genes *pyrC* to *pyrF*

a dimer, r_2) (Weber 1968). Only the catalytic trimer is required for the transcarbamoylation reaction: carbamoylphosphate + aspartate → carbamoylaspartate + Pi. Such catalysis is not subject to allosteric modification. The structural architecture of the holoenzyme is described as $2(c_3):3(r_2)$ in which each catalytic site in one trimer is cooperatively associated with a catalytic site in the other trimer through a regulatory dimer (Cohlberg et al. 1972). The interaction of these two types of polypeptides (defined as the r:c produced an elaborate model for this allosterically regulated enzyme, and biochemical studies have detailed the nature of the multiple active sites and the R⇄T transitions of the enzyme (Kantrowitz et al. 1981; Monaco et al. 1978).

Despite the extensive studies with ATCase, virtually nothing is known about the structural organization and regulation (i.e. the genetic control) of the cistrons encoding the two polypeptides of the enzyme. It has been shown (Wild et al. 1981) that the regulatory cistron is closely linked to the catalytic cistron (designated *pyrB*, map position 96 minutes, aspartate carbamoyltransferase, EC 2.1.3.2, catalytic subunit) (Bachmann and Low 1980). The regulatory cistron (designated *pyrI*) encodes the regulatory polypeptide and was first identified by Feller et al. (1981) when they described an altered ATCase defective in its regulatory polypeptide. In this paper we provide a definition of the regulatory and catalytic cistrons of ATCase and initiate an analysis of the control of their genetic expression.

Materials and Methods

Preparation of Bacterial Strains and Plasmids

F'-plasmid DNA was purified from *E. coli* K-12 (KLF17/KL132) carrying F'117 (F'*pyrB*$^+$, *argI*$^+$; ECGSC #4255) according to the procedures of Deonier and Mirels (1977). Recombinant DNA plasmids of various sizes were produced by ligation of restriction endonuclease-produced fragments of F'117 into pBR322 (Bolivar et al. 1977). The chimeric plasmids were transformed into appropriate recipient strains as described previously (Dagert and Ehrlich 1979). Transformed strains were identified by a rapid screening procedure (Davis et al. 1980) and plasmid DNA was prepared according to the cleared lysate method (Katz et al. 1973). *E. coli* TB2 was derived from insertion and subsequent excision of Mu d1(*lac* Ap^r) (Casadaban and Cohen 1979) in wild-type K-12. The resultant strain was lacking OTCase and ATCase. Thus the parental strain is presumed to have been initially *argF*$^-$, *argI*$^+$. Some *E. coli* K-12 strains possess two structural genes for OTCase (Glansdorff et al. 1967). Upon excision of Mu d1(*lac* Ap^r) (Bukhari 1976) the strain became Ap^s and non-revertible for *argI*, *pyrB* and *pyrI* (the simplest explanation is that imprecise excision removed part of the *pyrB*/*argI* region).

Restriction endonucleases were obtained from Bethesda Research Labs (Rockville, Maryland), New England Biolabs (Beverly, Massachusetts) and Boehringer-Mannheim (Indianapolis, Indiana) and they were used according to the suppliers' recommendations. Fragment digests were subjected to electrophoresis on vertical acrylamide gels (6–10%) or horizontal submersible agarose gels (0.7%) prepared in Tris-borate buffer, pH 8.3 (0.089 M Tris base, 0.089 M boric acid, and 2.5 mM Na_2 EDTA). After electrophoresis, the gels were stained with ethidium bromide

in the text. The enzyme structure was deduced from molecular weight determinations on Sephadex G-200 (Wild et al. 1980)

Plasmid*	Restriction site+	Fragment Molecular size (kb)	Genotype	Enzyme ATCase	Structure OTCase
pPB-h101	*Hind*III-*Hind*III	14	$pyrB^+$, $pyrI^+$, $argI^+$	$2(c_3):3(r_2)$	c_3
pPB-h102	*Eco*RI-*Eco*RI	12	$pyrB^+$, $pyrI^+$, $argI^+$	$2(c_3):3(r_2)$	c_3
pPB-h103	*Sal*I-*Sal*I	10	$pyrB^+$, $pyrI^+$, $argI^+$	$2(c_3):3(r_2)$	c_3
pPB-h104	*Pst*I-*Pst*I	6.0	$pyrB^+$, $pyrI^+$,	$2(c_3):3(r_2)$	–
pPB-h105	*Sal*I-*Pst*I	2.8	$pyrB^+$, $pyrI^+$,	$2(c_3):3(r_2)$	–
pPB-c201	*Pst*I-*Pst*IΔ(*Bgl*II-*Bgl*II)	5.3	$pyrB^+$,	c_3	–
pPB-c202	*Pst*I-*Pst*IΔ(*Hpa*I-*Hpa*I)	3.5	$pyrB^+$,	c_3	–
pAI-101	*Pst*I-*Pst*I	5.0	$argI^+$,	–	c_3
pAI-102	*Hpa*I-*Hpa*I	3.0	$argI^+$,	–	c_3

pPB-c201 was produced by the deletion of a 650 bp fragment from pPB-h104 with *Bgl*II. Similarly, pPB-c202 resulted from the deletion of a 2.5 kb fragment with *Hpa*I (see Fig. 2). Enzymatic assay, purification, and molecular weight determination were performed to verify the nature of various cistronic products. Enzyme assays for ATCase in cell-free extracts of *E. coli* cells have been described earlier (Wild et al. 1980). Molecular weights of ATCase were estimated by ascending G-200 chromatography. The enzyme was purified according to the procedures of Gerhart and Holoubek (1967) and was separated into catalytic and regulatory subunits by exposure to the mercurial, neohydrin (Yang et al. 1978).

The phenotypes resulting from the various cloned fragments can best be defined by the resulting enzymatic structures. Thus plasmid pPB-h104, containing *pyrB*, *pyrI*, and *argI*, produces the intact holoenzyme for ATCase [M_r = 300,000, $2(c_3):3(r_2)$] and the typical OTCase trimer [M_r = 100,000; c_3]. Similarly, plasmids such as pPB-c201 ($pyrB^+$) produced only the catalytic trimer (c_3) of ATCase and lacked the regulatory dimer (r_2) and OTCase. The presence of the regulatory subunit in cell-free extracts was verified by specific antibody precipitation or titration against functional catalytic subunit (c_3) according to the technique of Perbal and Hervé (1972).

DNA Sequence Determination

DNA fragments used for sequence determination were isolated from preparative polyacrylamide gels by the methods of Maxam and Gilbert (1980). Fragments were dephosphorylated and then labeled at their 5′ ends by using bacterial alkaline phosphatase and polynucleotide kinase (Bethesda Research Laboratories, Rockville, Maryland) and [γ-^{32}p] ATP (Schwartz-Mann). The labeling reaction was followed by digestion with an appropriate restriction enzyme, and the desired fragments were isolated by preparative polyacrylamide gel electrophoresis. Sequence determination of the isolated fragments was accomplished by using the C, C+T, A>C, A+G, and G specific-cleavages described by Maxam and Gilbert (1980).

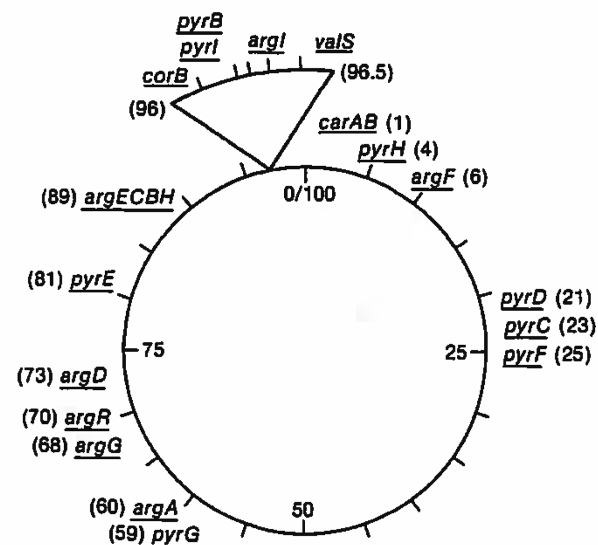

Fig. 2. The genetic map of *E. coli* K-12, highlighting the gene loci involved in the de novo biosynthesis of arginine and pyrimidines. A description of the genetic nomenclature is presented in the legend to Fig. 1. Additional gene loci include *corB*, *argR*, and *valS* which encode respectively cobalt resistance (magnesium transport), the aporepressor for arginine, and the tRNA synthetase for valine (Bachmann and Low 1980)

Results

Cloning and Sublconing of pyrB, pyrI, and argI

The *pyrBI/argI* region of the *E. coli* K-12 chromosome has been subcloned into pBR322 from F′117 (F′$pyrB^+$, $pyrI^+$, $argI^+$) using several restriction endonucleases (see Table 1). The genetic map of *E. coli* K-12 is presented in Fig. 2 and the genetic loci involved in arginine and pyrimidine biosynthesis are highlighted. The biosynthetic enzymes

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Fig. 3. Analysis of the *pyrBI-argI* region of the *E. coli* by restriction endonuclease fragment mapping. Overlapping restriction endonuclease fragments were used to define the location of *pyrB*, *pyrI*, and *argI* loci relative to one another. The various DNA fragments, cloned into pBR322 and described in Table 1, are represented in numbers of base-pairs (0–18,000) (It should be noted that *pyrB* and *argI* encode respective catalytic polypeptides of ATCase and OTCase while *pyrI* codes for the regulatory polypeptide of ATCase. There is no arginine locus analogous to *pyrI*). The gene loci are oriented on the *E. coli* map in such a manner that *argI* is close to 0 minutes and that expression is counterclockwise for both, presumably, therefore from the same strand as suggested by Syvanen & Roth (1973) for *S. typhimurium*

unique to each pathway (Fig. 1) are dispersed throughout the chromosome, yet the appropriate gene loci are subject to cooperative regulatory controls upon repression/derepression (Beckwith et al. 1962; Williams and O'Donovan 1973; Kelln et al. 1975; and Schwartz and Neuhard 1975 for pyrimidines/and Vogel 1961; Elseviers et al. 1972; Vogel et al. 1971; Leisinger and Haas 1975 for arginine). The *pyrBI* and *argI* gene loci are characterized by the dramatic levels of derepression which can be obtained (the derepression/repression ratio may approach 500 for these three loci) while the other enzymes of the pathways vary only 2–4 fold (Vogel et al. 1971; Williams and O'Donovan 1973; Kelln et al. 1975; Schwartz and Neuhard 1975). By using overlapping restriction mapping and secondary digestion of cloned fragments, it has been possible to characterize the genetic organization of the cistrons encoding ATCase (*pyrBI*) and OTCase (*argI*). As summarized in Fig. 3, the following observations can be made:

(i) The *pyrBI* gene region is located approximately 3,000 bp from *argI* gene;

(ii) The *pyrB* and *pyrI* cistrons are immediately adjacent, with *pyrI* distal from *argI*;

(iii) There is a dramatic similarity in the restriction patterns of *pyrBI* and the *argI* gene region. (The *Pst*I – *Bst*EII – *Hpa*I recognition sites are virtually identical in location for both regions). It has been shown already that the *argI* and *pyrB* polypeptides possess some N-terminal sequence homologies indicative of gene duplication (Gigot et al. 1977). Thus, the organization of the *pyrBI*/*argI* region may

the reported amino acids sequences of the ATCase cistrons. The precise limits of the genes can be determined from the sizes of their polypeptide products: 300 amino acids for the *pyrB* product, 152 amino acids for the *pyrI* product and approximately 300 amino acids for the *argI* product. The colinear maps of the restriction sites of *pyrBI* and corresponding amino acid sequences are presented in Fig. 4. Since all of these restriction enzymes are class II endonucleases which recognize specific base sequences, it is possible to determine the corresponding cognate amino acids (see Legend for Fig. 4). These colinear maps reveal several additional structural details of the *pyrBI* gene region:

(i) The reported amino acid sequences approximate the restriction site map;

(ii) The N-terminus of the regulatory polypeptide encoded by *pyrI* lies alongside the C-terminus of the catalytic polypeptide, encoded by pyrB; approximately 10–20 bp separate the two coding regions.

Organization of pyrB and pyrI into an Operon

The structural arrangement of *pyrBI* region suggests that the two cistrons are organized as a bicistronic operon. Further evidence to support this contention was obtained by the concommitant loss of both the catalytic and regulatory polypeptides upon insertion of Mu d1(*lac* Ap^r) into *pyrB* of the chromosome. This Mu lysogen was Ap^r, *pyrB*$^-$, and *pyrI*$^-$, yet *argI*$^+$. Following incubation at 42° C, single colony isolates were screened for *pyrBI*$^-$ and Ap^s. It was

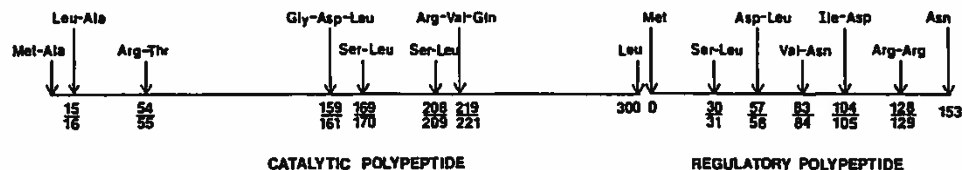

Fig. 4. The colinear map of the *pyrB-pyrI* region and its polypeptides. Since class II restriction endonucleases recognize specific inverted palindromic sequences, it is possible to determine the corresponding di- and tri-peptides encoded by such DNA segments. For example: The *Hpa* I restriction site, 5′-GTTAAC-3′, has the potential to encode N-Val-Asn when transcribed and translated in the first position reading frame; N-Leu-Thr in the second reading frame or a chain-terminating UAA in the third. Only one Val-Asn sequence is observed for the amino acid sequence of the regulatory polypeptide at residues 83/84 and the Leu-Thr combination is not observed. Similar logic applies for the *Bgl* II restriction sites, 5′-AGATCT 3′, which unequivocally designates Asp-Leu at amino acid residues 57/58. Theoretically, the restriction of the DNA with *Bgl* II and *Hpa* I should produce a fragment of 80 bp in length. This is presented in the top schematic figure. Thus the locations of the regulatory and catalytic cistrons are established accurately. It was possible to locate the unique position for all restriction endonucleases recognizing 6 or more base-pairs. Using endonucleases that recognize 5 bp sites it was possible to estimate the location relative to DNA fragment sizes. By comparing the known polypeptide map to the restriction endonuclease map it was possible to determine that between 10–20 base pairs separate the catalytic and regulatory cistrons

lytic trimers and regulatory dimers occurs only in the cytoplasm and not on transcriptionally active polysomes. Furthermore, the same study showed that there is less than five percent production of r in the absence of c. The results of our study support the earlier suggestion by Perval and Herve (1972) that the *pyrB* and *pyrI* cistrons comprise a bicistronic operon in which the catalytic cistron is promoter-proximal.

The Regulatory Polypeptide has no Role in the Expression of the Catalytic Cistron

The plasmid pPB-h104 was subjected to internal deletion by *Hpa*I which removed approximately 2,500 bp including the carboxy-terminal region of *pyrI*. In a separate deletion, 650 bp including the last two-thirds (95 amino acid residues) of *pyrI* were removed. In each case, *pyrB* was responsive to typical repression conditions in the presence/absence of exogenous uracil (50 µg/ml) for both plasmids (Table 2). Relevant plasmids (Table 1) were characterized by acrylamide gel electrophoresis and by auxotrophic-characteristics. After the internal fragments were removed, the resulting plasmids were transformed into competent TB2 cells. The molecular weights of ATCase from the various plasmids were determined by chromatography on Sephadex G-200 as described in earlier reports (Wild et al. 1980). The strains containing the catalytic *pyrB* and partial deletion of the *pyrI* cistron produced only catalytic polypeptides and all enzymatic activity was recovered as catalytic trimers (M_r = 100,000 daltons. Thus, the presence of a functional *pyrI* gene product is not required for normal *pyrB* expression and regulation.

Analysis of the Regulatory Region of pyrBI by DNA Sequence Determination

The DNA sequence of the promoter region of *pyrBI* is presented in Fig. 5. The nucleotide sequence contains three

Table 2. Repression of ATCase formation in various plasmid constructs of *E. coli*. The specific activities are expressed as micromoles of carbamoylaspartate produced per minute reaction time per milligram of protein from a cell-free extract. The strains and plasmids are described in the text and Table 1. Repression index is calculated as the ratio of specific activity without uracil/specific activity with uracil for each strain

Strain	Genotype	Specific activity	Repression index
K12 (min)	$pyrB^+$, $pyrI^+$	12	0
K12 (+U)	$pyrB^+$, $pyrI^+$	4.0	3.0
TB2 (min)	$pyrB^-$, $pyrI^-$	<0.1	–
TB2:pPBh104 (min)	$pyrB^+$, $pyrI^+$	188	0
TB2:pPBh104 (+U)	$pyrB^+$, $pyrI^+$	70	2.7
TB2:pPBc201 (min)	$pyrB^+$, $pyrI^-$	225	0
TB2:pPBc201 (+U)	$pyrB^+$, $pyrI^-$	134	1.7
TB2:pPBc202 (min)	$pyrB^+$, $pyrI^-$	151	0
TB2:pPBc202 (+U)	$pyrB^+$, $pyrI^-$	39	3.8

sites that are organized spatially along the DNA in agreement with the consensus "idealized" promoter sequence (Pribnow 1975; Gilbert 1976; Rosenberg and Court 1979). A classic RNA polymerase recognition site (R_c) is centered ten base pairs (−10) from the presumptive transcriptional initiation site designated I (bp = +1). A sigma "recognition site" (R_σ) is located approximately thirty-five base pairs (−35) preceding the transcriptional initiation site. The R_c sequence, TATAATG, represents the "idealized" Pribnow box which serves as the base specific contact sequence for the RNA polymerase core (Pribnow 1979). Twelve base pairs separate the R_c sequence from R_σ, thus defining an ideal promoter sequence covering approximately 40 bp. This precedes the translation initiation of the catalytic polypeptide of ATCase (+153) which can be identified by fitting

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