SEARCH FOR REGULATORY LOGIC IN DE NOVO PYRIMIDINE BIOSYNTHESIS IN BACTERIA

by

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ABSTRACT

The de novo pyrimidine biosynthetic pathway produces the pyrimidine end products uridine-5'-triphosphate (UTP) and cytidine-5'-triphosphate (CTP), which lead to the synthesis of new DNA and RNA for cell growth. Aspartate transcarbamoylase (ATCase, E.C. 2.1.3.2.) catalyzes the first committed step of de novo metabolic flux into pyrimidine biosynthesis and is controlled through feedback inhibition of existing enzyme by CTP and repression of enzyme formation by UTP in E. coli. ATCase is encoded by a single bicistronic operon pyrBI in members of the Enterobacteriaceae; however, allosteric regulation of ATCase varies among classes of enteric bacteria. Some of these variations include activation by the end product CTP. In order to gain insight into this regulatory logic, repression of pyrBI expression has been studied in heterologous gene systems of S. marcescens and P. vulgaris transformed into an E. coli host.

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INTRODUCTION

Cellular metabolism is characterized by a complex system of interactive checks and balances which work continually to maintain biochemical balance. Several different types of regulatory mechanisms are utilized to control the activities of various biosynthetic and catabolic pathways, such as that of pyrimidine *de novo* biosynthesis and degradation. The *de novo* pyrimidine biosynthetic pathway provides the pyrimidine nucleotides uridine-5'-triphosphate (UTP) and cytidine-5'-triphosphate (CTP), which are used to synthesize RNA and lead to the synthesis of DNA precursors, as shown in Figure 1. The end products UTP and CTP (and metabolic intermediates such as UMP) regulate their own production at two levels: transcriptionally (1) and allosterically (2). Transcriptional regulation involves the direct control of gene expression; repression of *pyrBI*, *pyrE*, and *pyrF* by UTP is an example of this type of regulation (3,4). In the other type of regulation, allosteric interactions affect the activity of the first enzyme of the pathway, aspartate transcarbamoylase (ATCase, E.C. 2.1.3.2.) (2). These two

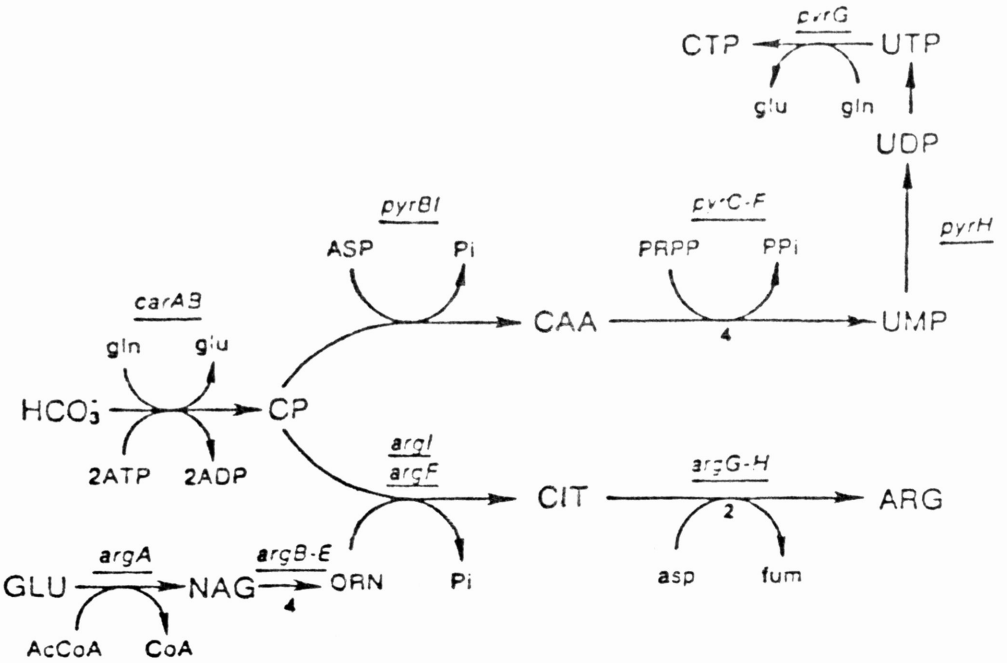

Fig. 1. De novo biosynthetic pathways for pyrimidine and arginine with genetic loci of *E. coli* indicated. GLU, glutamate; NAG, N-acetyl-glutamate; ORN, ornithine; CIT, citrulline; asp, asparate; fum, fumarate; ARG, arginine; HCO_3-, bicarbonate; gln, glutamine; glu, glutamate; ATP, adenosine-5′-triphosphate; 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*

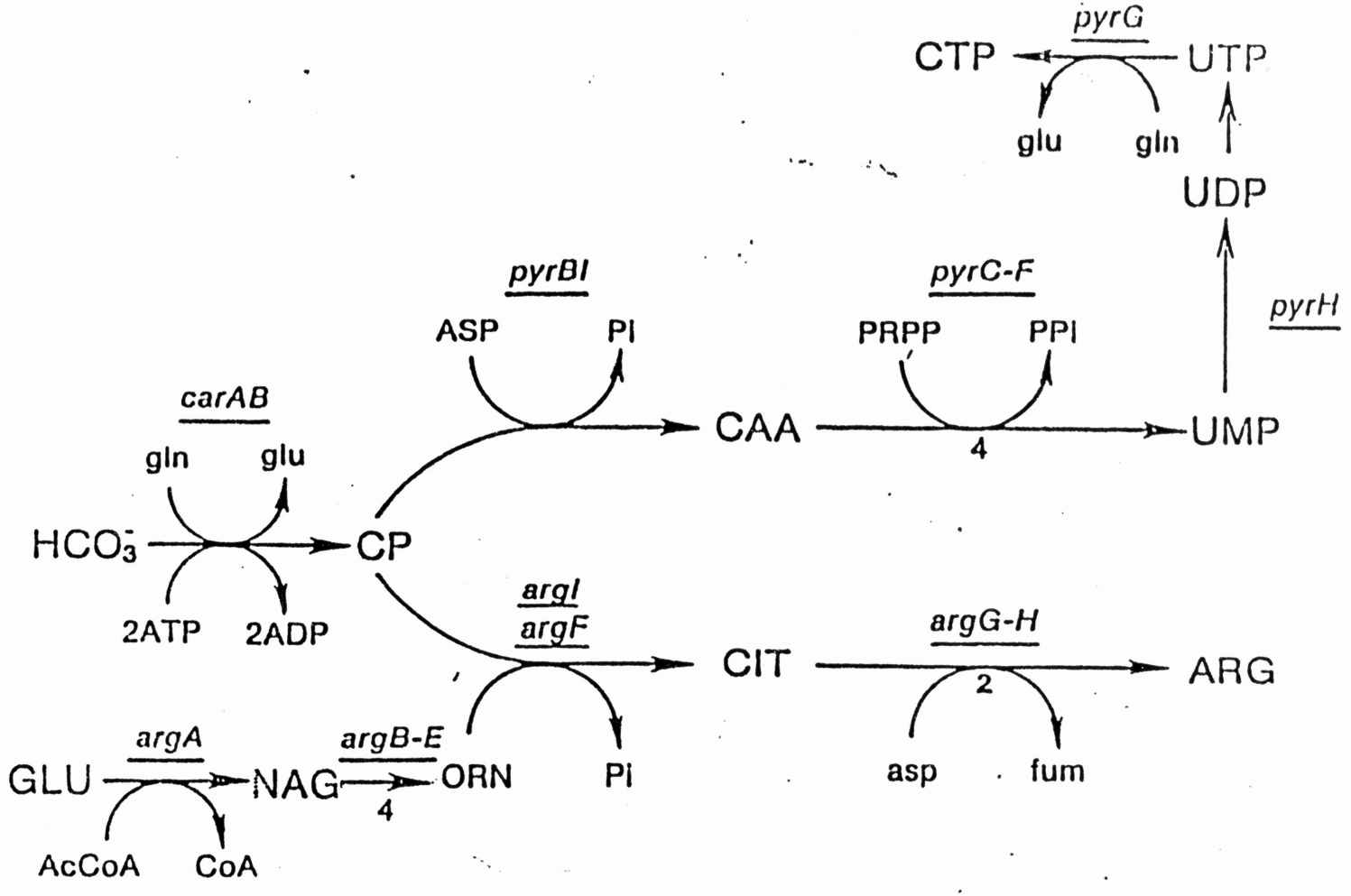

Figure 1. Pyrimidine Biosynthetic Pathway

types of regulation cooperate to coordinate the control of UTP and CTP levels in the cell.

ATCase catalyzes the first committed step of pyrimidine biosynthesis and involves the condensation of carbamoyl phosphate with aspartate to form carbamoyl aspartate and release inorganic phosphate. As a logical consequence of its position in the pathway, regulation of gene expression leading to formation of ATCase primarily determines the amount of UTP and CTP synthesized. Expression of the genes coding for ATCase is repressed in various wild type bacteria grown in media supplemented with uracil, as reported by J. L. Cooper-Johnson in her M.S. Thesis, Texas A&M University. Exogenous uracil may be converted to UTP and CTP by one of several interactive salvage pathways in the cell. When UTP, one of the end products, is present in large amounts, de novo biosynthesis of pyrimidines is de-emphasized; less ATCase is made; and the genes coding for ATCase are repressed.

The ATCase holoenzyme of Escherichia coli is composed of six catalytic polypeptides (functional as a trimer) and six regulatory polypeptides (functional as a dimer) (5). These subunits associate to form a dodecamer, represented as $2(c_3):3(r_2)$ (6). As shown in Figure 2, the catalytic and regulatory polypeptide chains are encoded in a single bicistronic operon (designated pyrBI) (7,8,9).

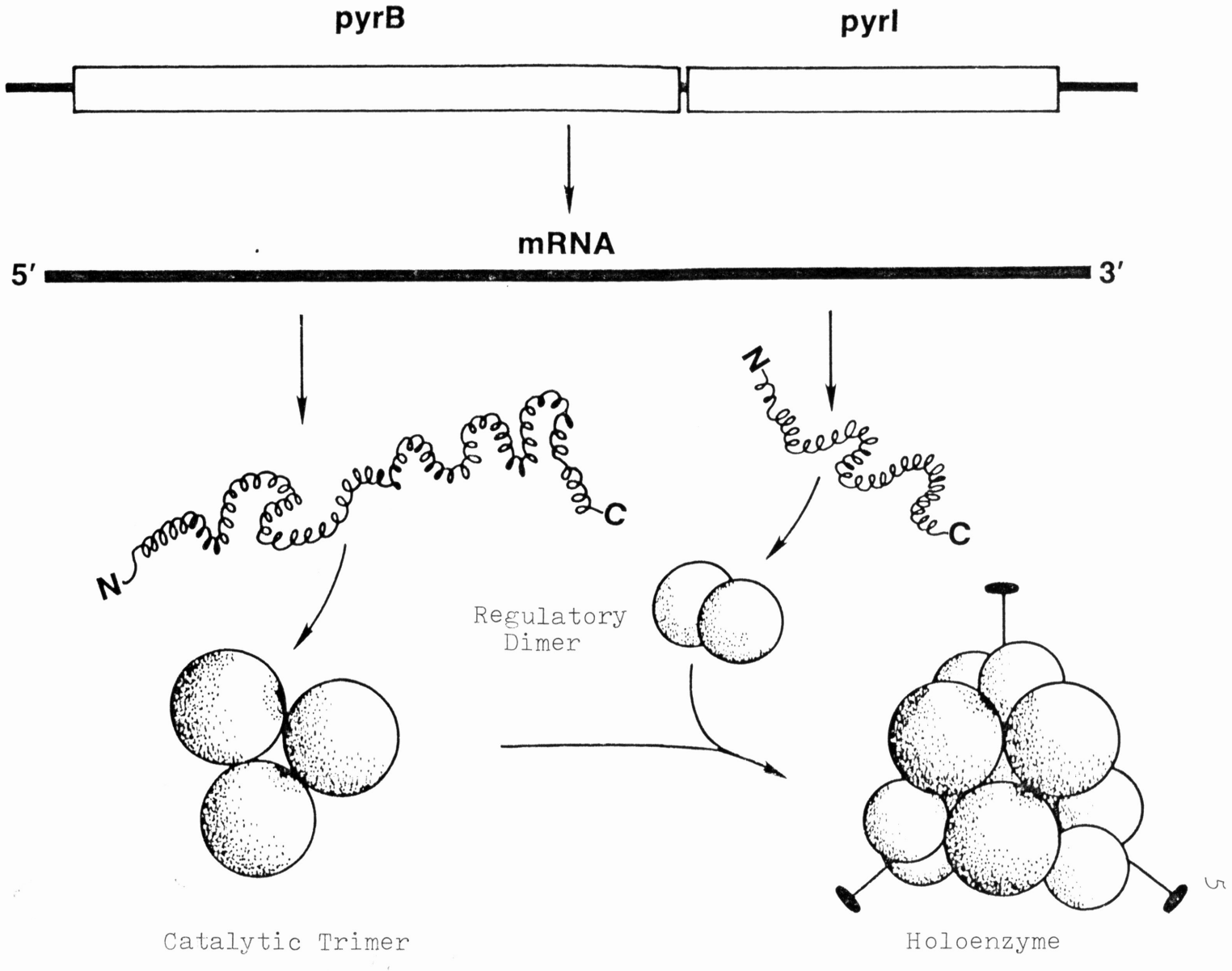

Figure 2. Organization of pyrBI Operon

Repression of gene expression has been shown to be accomplished by at least two quite different methods. One mechanism would involve a specific repressor molecule, or "apoprotein", which would bind to a control region within the promoter region of the targeted operon and block transcription. Such a repressor has not been identified for the pyrimidine pathway (10,11). Another common system which could accomplish repression or derepression is attenuation. Attenuation involves interrupted transcription caused by formation of a secondary structure within the mRNA of a transcribed leader polypeptide (12). Certain secondary structures, designated rho-independent terminators, are capable of terminating transcription by arresting the progression of RNA polymerase. In amino acid biosynthetic operons, the ability of this structure to terminate transcription depends upon the level of amino acyl-tRNAs required for translation of the leader peptide. In the case of pyrimidine biosynthesis, this ability appears to depend upon the availability of UTP (9,13). In the attenuator model for E. coli pyrBI, coupling of transcription and translation is necessary for expression of the gene. If uracil levels are low, coupled transcription / translation will result in expression of the gene. If enough uracil is present, termination will occur. A model for the leader region of E. coli pyrBI is presented in Figure 3.

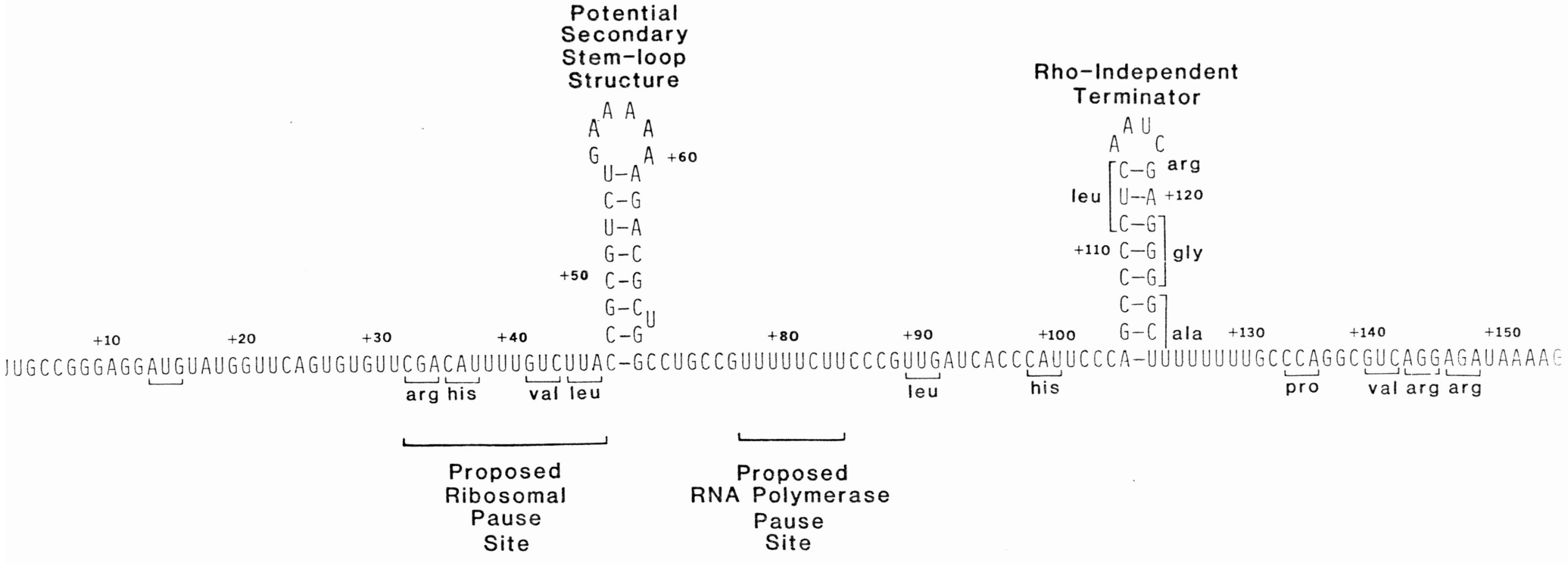

Figure 3. E. coli pyrBI Leader Region

In a second type of regulation, (i.e. at the level of enzyme catalysis), ATCase is subject to allosteric inhibition by CTP and activation by ATP (2). CTP binds to an allosteric site on the regulatory dimer of ATCase, inhibiting the catalytic activity of the enzyme. In this manner, the presence of an adequate amount of CTP decreases synthesis of carbamoyl aspartic acid, which results in a reduction of pyrimidine biosynthesis via the de novo pathway.

Allosteric regulation varies among enteric bacteria, and the regulatory subunit appears to dictate the nature of allosteric response (14-16). In E. coli, CTP inhibits ATCase activity by decreasing the affinity for substrate binding. Thus, the accumulation of metabolic end products decreases continued synthesis. However, ATCases of other enteric bacteria, such as Proteus vulgaris and Serratia marcescens, are activated by CTP (14-15). Surprisingly, the presence of an end product of the pathway serves to activate the pathway even further. This type of allosteric regulation suggests the presence of a regulatory logic that differs dramatically from that of E. coli.

There are a number of variables which could influence the effect of CTP on different ATCases. The amount of ATCase, UTP/CTP pool levels, and the levels of substrates carbamoyl phosphate and aspartate all play an important role

in regulation of the pathway. In addition, the efficiency of ATCase at different aspartate concentrations varies among different bacteria (14). These factors, when combined with allosteric control by CTP, are thought to regulate pyrimidine biosynthesis in a "logical" manner in E. coli, although the logic is not always readily apparent in some other enteric bacteria.

To gain some insight into this diverse regulatory logic, regulation at the level of gene expression has been examined for the pyrBI operons of P. vulgaris , S. marcescens , and E. coli to determine if repression occurs in the presence of uracil. The pyrBI genes from different bacteria were transformed in the same host E. coli strain, which has a deletion in pyrBI and must rely upon the cloned genes for growth in minimal media (9). Thus, regulation of the newly introduced ATCase genes can be observed.

TABLE 1 BACTERIAL STRAINS

Strain	Genotype	Reference
E. coli K12 (E63)	wild type	9
E. coli HB101	pro^- leu^- thi^- $hsdR^-$ $endA^-$ $recA^-$ $rpsL20^-$ $ara14^-$ $galK2^-$ $xyl5^-$ $mtl1^-$ $supE44^-$	24
E. coli HB101-4442	HB101 + $pyrB^-$	25
E. coli TB2	$pyrB^-$ $pyrI^-$ arg^-	9

TABLE 2 PLASMIDS

Plasmid	Antibiotic Resistance	Genotype	Size (kilobases)	Reference
pPBhl04-Ec[a]	$Tc^{R}Ap^{S}$	$pyrB^{+}$ $pyrI^{+}$	6	9
pPBhl01-Pv[b]	$Tc^{S}Ap^{R}$	$pyrB^{+}$ $pyrI^{+}$	>20	22
pPBhl01-Sm[c]	$Tc^{S}Ap^{R}$	$pyrB^{+}$ $pyrI^{+}$	>20	22

[a] *E. coli*
[b] *P. vulgaris*
[c] *S. marcescens*

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Bacterial strains were stored in 40% glycerol/TYE at -20°C. Transformations were performed as outlined by Dagert and Ehrich (17). Selective platings were performed on tryptone-yeast extract (TYE) plates containing the appropriate antibiotics in the following concentrations: ampicillin, 40ug/ml and tetracycline, 25ug/ml. TYE liquid media consists of 10 grams Bactotryptone, 5 grams yeast extract and 5 grams sodium chloride per liter of distilled deionized water. Fifteen grams of Bactoagar per liter of TYE were added to make plates.

Growth conditions

Cultures started from single colony isolates of strains listed in Table 1 were grown at 37°C, with shaking, to late exponential phase in 500 mls minimal media (M56) (18) in the presence or absence of supplemental uracil (50ug/ml). Additional supplements to M56 were added to a final concentration of 0.1% case amino acids, 0.2% glucose, and 10ug/ml B_1.

Preparation of cell-free extracts

Cells were harvested at 4°C by centrifugation for ten minutes at 6000 rpm. Cell pellets were washed with 100 mls of 40mM potassium phosphate, pH 7.0, per 500 mls of liquid culture harvested. The washed cells were centrifuged again in the same way and then stored as frozen pellets overnight at -20°C.

After thawing, each pellet was resuspended in 3 mls of 40mM potassium phosphate, pH 7.0, containing 0.20mM zinc acetate and 1mM dithiothreitol. The resuspended cells were disrupted by treatment with a Heat Systems Model SW 185 Sonic Oscillator (Plainview, N. Y.) at a setting of 5 for a total of 150-180 seconds (5 to 6 thirty second pulses). Each sample was cooled in an ice-salt bath between pulses to maintain the temperature below 25°C.

The sonicated extract was centrifuged at 15,000 rpm for ten minutes. The supernatant was collected and centrifuged at 18,000 rpm for 30 to 60 minutes at 4°C. The extract was subsequently dialyzed for 2 hours against 40mM potassium phosphate buffer, 0.02mM zinc acetate, pH 7.0 at 4°C.

Enzyme assay

ATCase activity was assayed by measuring the amount of carbamoyl aspartate (CAA) formed at 28°C according to the method of Gerhart and Pardee (19), as modified by Prescott

and Jones (20). Each reaction tube contained 0.16% carbamoyl phosphate, 40mM potassium phosphate, pH 7.0, cell-free extract, and 5mM, 20mM, or 100mM K-aspartate in a final volume of 2.0 mls.

All reactions were performed in duplicate. Except for carbamoyl phosphate, all components of the reaction mix were preincubated for 2 to 3 minutes at 28°C. In each case the reaction was started by the addition of carbamoyl phosphate which had been prepared immediately before use and kept on ice. At 10-, 20-, and 30-minute intervals, 0.5 ml reaction samples were withdrawn from the reaction mix and stopped with an acid color solution. The tubes were then incubated at 60°C for one hour and 50 minutes to allow color reaction to occur.

When high protein concentrations caused precipitation in reaction tubes, samples were centrifuged at slow speed (1100 g) in a desk-top centrifuge before absorbance was measured. CAA production was quantitated at 466 nm. in a Gilman 300-N spectrophotometer zeroed against water. Appropriate controls were performed to evaluate colorimetric background due to substrate and extract constituents.

Specific activity is expressed as nanomoles CAA formed per minute per milligram protein. Total protein concentration in each cell-free extract was estimated using

the method of Lowry (21). Recrystallized bovine serum albumin was used as the standard.

Materials

Antipyrine, bovine serum albumin, CAA, diacetyl monoxime, dilithium carbamoyl phosphate, and D,L-dithiothreitol were purchased from Sigma Chemical Company. Sulfuric acid and glacial acetic acid were reagent grade.

RESULTS

ATCase assays were performed on cell-free extracts from cells grown in the presence or the absence of uracil. From these ATCase assays and subsequent Lowry protein estimation, specific activities were calculated. Tables 3 through 6 show results obtained from ATCase assays. Values for specific activities of ATCase in E63 and pPBh104 in TB2 are similar to results reported earlier (9).

Care was taken to ensure that frozen samples were thoroughly mixed after thawing. In order to compare assay data with protein concentrations, the cell-free extract was mixed consistently. The substrate aspartate was also mixed after thawing to ensure that a consistent amount of aspartate was used in each assay.

The cloned ATCase genes from *S. marcescens* and *P. vulgaris* were expressed well in *E. coli*. For this to occur, the *E. coli* RNA polymerase must be able to recognize the promoter regions of *S. marcescens* and *P. vulgaris*. In fact, ongoing studies in our laboratory have produced comparable promoter sequences for *S. marcescens* and *P. vulgaris* (22). The specific activity of ATCase from transformed bacteria was about ten times higher than that of wild type bacteria since the ATCase genes were present on a multi-copy number plasmid, as

TABLE 3 SPECIFIC ACTIVITY - 5mM ASPARTATE

Strain	Origin of ATCase	Specific Activity[a] -Uracil	Specific Activity[a] +Uracil	Repression Index
E63	*E. coli*	7.6	1.3	5.8
HB101	*E. coli*	64.6	4.0	16.2
HB101-4442:pPBh104-Ec	*E. coli*	132.3	57.6	2.3
TB2:pPBh104-Ec	*E. coli*	77.9	61.6	1.3
HB101-4442:pPBh101-Pv	*P. vulgaris*	ND[b]	ND	ND
HB101-4442:pPBh101-Sm	*S. marcescens*	ND	ND	ND

[a]nanomoles carbamoylaspartic acid/minute/mg. protein
[b]ND: not detectable

Calculation: [(O.D./min.)(.196lumoles CAA/O.D.)(20)(dilution factor)]/(mg. protein/ml.)

Repression Index = Specific Activity(-U)/Specific Activity(+U)

TABLE 4 SPECIFIC ACTIVITY - 20 mM ASPARTATE

Strain	Origin of ATCase	Specific Activity* -Uracil	Specific Activity* +Uracil	Repression Index
E63	*E. coli*	32.9	10.2	3.2
HB101	*E. coli*	126.2	7.1	17.8
HB101-4442:pPBh104-Ec	*E. coli*	301.7	101.8	3.0
TB2:pPBh104-Ec	*E. coli*	130.2	92.8	1.4
HB101-4442:pPBh101-Pv	*P. vulgaris*	281.8	108.9	2.6
HB101-4442:pPBh101-Sm	*S. marcescens*	405.4	82.8	4.9

*Units as in Table 3

TABLE 5 SPECIFIC ACTIVITY - 100 mM ASPARTATE

Strain	Origin of ATCase	Specific Activity* -Uracil	Specific Activity* +Uracil	Repression Index
E63	*E. coli*	50.2	18.6	2.7
HB101	*E. coli*	122.6	7.2	17.0
HB101-4442:pPBh104-Ec	*E. coli*	241.3	96.0	2.5
TB2:pPBh104-Ec	*E. coli*	201.6	110.0	1.8
HB101-4442:pPBh101-Pv	*P. vulgaris*	647.4	244.9	2.6
HB101-4442:pPBh101-Sm	*S. marcescens*	692.4	106.3	6.5

*Units as in Table 3

TABLE 6 REPRESSION INDEX SUMMARY

Strain	Origin of ATCase	Average Repression Index
E63	*E. coli*	3.9[a]
HB101	*E. coli*	16.9[a]
HB101-4442:pPBh104-Ec	*E. coli*	2.6[a]
TB2:pPBh104-Ec	*E. coli*	1.5[a]
HB101-44442:pPBh101-Pv	*P. vulgaris*	2.6[b]
HB101-4442:pPBh101-Sm	*S. marcescens*	5.7[b]

[a] 5mM, 20mM, and 100mM values averaged.
[b] 20mM and 100mM values averaged.

compared to wild type bacteria, which have only one copy of the genes coding for ATCase on a chromosome.

HB101 had an extremely high repression index, possibly due to existant stressed nucleotide metabolism. HB101 is an E. coli strain which has been genetically manipulated so that it is unable to perform certain types of DNA repair and recombination. Its genotype is listed in Table 1.

Repression occurred in all cells studied. Janet L. Cooper-Johnson reported that wild type E. coli, P. vulgaris and S. marcescens show repression by uracil. However, the mechanism by which repression is accomplished is unknown.

DISCUSSION

ATCase assays were performed in order to determine if pyrBI genes from enteric bacteria cloned on a plasmid and transformed into an E. coli host strain could be expressed and regulated in the presence of supplemental uracil. Controls carried out in this experiment included the assay of both wild type strains and of E. coli pyrBI genes on a plasmid, transformed into the same E. coli host strain.

Assays were performed using different concentrations of aspartate because of different enzymatic characteristics. $[S]_{0.5}$ values for aspartate were calculated by determining the substrate concentration at which the reaction velocity is half of the maximal velocity in a velocity versus substrate plot. $[S]_{0.5}$ values for different bacteria are listed in Table 7 (23). $[S]_{0.5}$ for P. vulgaris and S. marcescens are higher than 5mM aspartate, and assays using 5mM often yield no slopes at all. Table 6 shows average repression indices for bacteria studied. Values at 20mM and 100mM aspartate were averaged for S. marcescens and P. vulgaris. Values at three aspartate concentrations were averaged for E. coli.

The extent of repression was quite similar for each cloned pyrBI, as indicated by repression indices in Tables 3-6. S. marcescens transformants had an average

TABLE 7 $[S]_{0.5}$ FOR ENTERIC BACTERIA

Native Holo ATCase	$[S]_{0.5}$ aspartate	*Nucleotide Effectors	
		CTP	ATP
E. coli	5mM	-	+
S. marcescens	20mM	+	+
P. vulgaris	29mM	+	+

* See reference 23.

repression index of 5.7, as compared to 2.6 for *P. vulgaris* transformants. The repression indices for cloned *E. coli pyrBI* in two different *E. coli* host strains were slightly different. Since HB101 had an extremely high repression index, the extent of repression found in HB101-4442 may have been affected by stressed nucleotide metabolism.

Cloned *pyrBI* from *S. marcescens* and *P. vulgaris* in an *E. coli* host have shown repression in the presence of uracil. The repression mechanism appears to be functionally similar among these three ATCases in that all are repressed in an *E. coli* host, in spite of the fact that the components required for gene expression are provided by a different kind of bacteria. It seems unlikely that the specific repressor model would function in transformed cells studied because recognition of known different specific sequences within *S. marcescens* and *P. vulgaris* by a repressor molecule would be necessary. Following this argument, it is proposed that repression is accomplished by some form of attenuation.

Differences in the extent of repression could be explained by differences in the secondary structures involved in attenuation. ATCase genes from *S. marcescens* and *P. vulgaris* are currently being sequenced . The leader regions of *S. marcescens* and *P. vulgaris* seem

to be similar to that of E. coli, so attenuation is a possible mechanism for repression, although all of the details have not been clarified (22). Poly-uridylate regions and "pause sites" in mRNA appear to contribute to attenuation, and differences in these regions would have an effect on the extent of repression.

It would be valuable to study these plasmids in other E. coli hosts, such as TB2, which is a wild type K12 strain with a pyrBI deletion (9). Since HB101 appears to have stressed nucleotide metabolism, results from this study may not completely represent what would be observed in another E. coli background. Another future study should be done using host bacteria which overproduce ATCase due to a mutation in the gene for a second enzyme in the pyrimidine biosynthetic pathway. Whereas repression is the result of a high concentration of uracil, derepression occurs as the result of an inability to satisfy cellular requirements for uracil. A leaky mutation in pyrH or pyrF gene results in bacteria which cannot efficiently produce UTP using this de novo pathway. Derepression results in thousand fold increases in ATCase production, as shown in Figure 4.

Although the end product activation of ATCase by allosteric control seems illogical for P. vulgaris and S. marcescens, the ATCase genes are regulated by repression in native bacteria and in E. coli. Recent

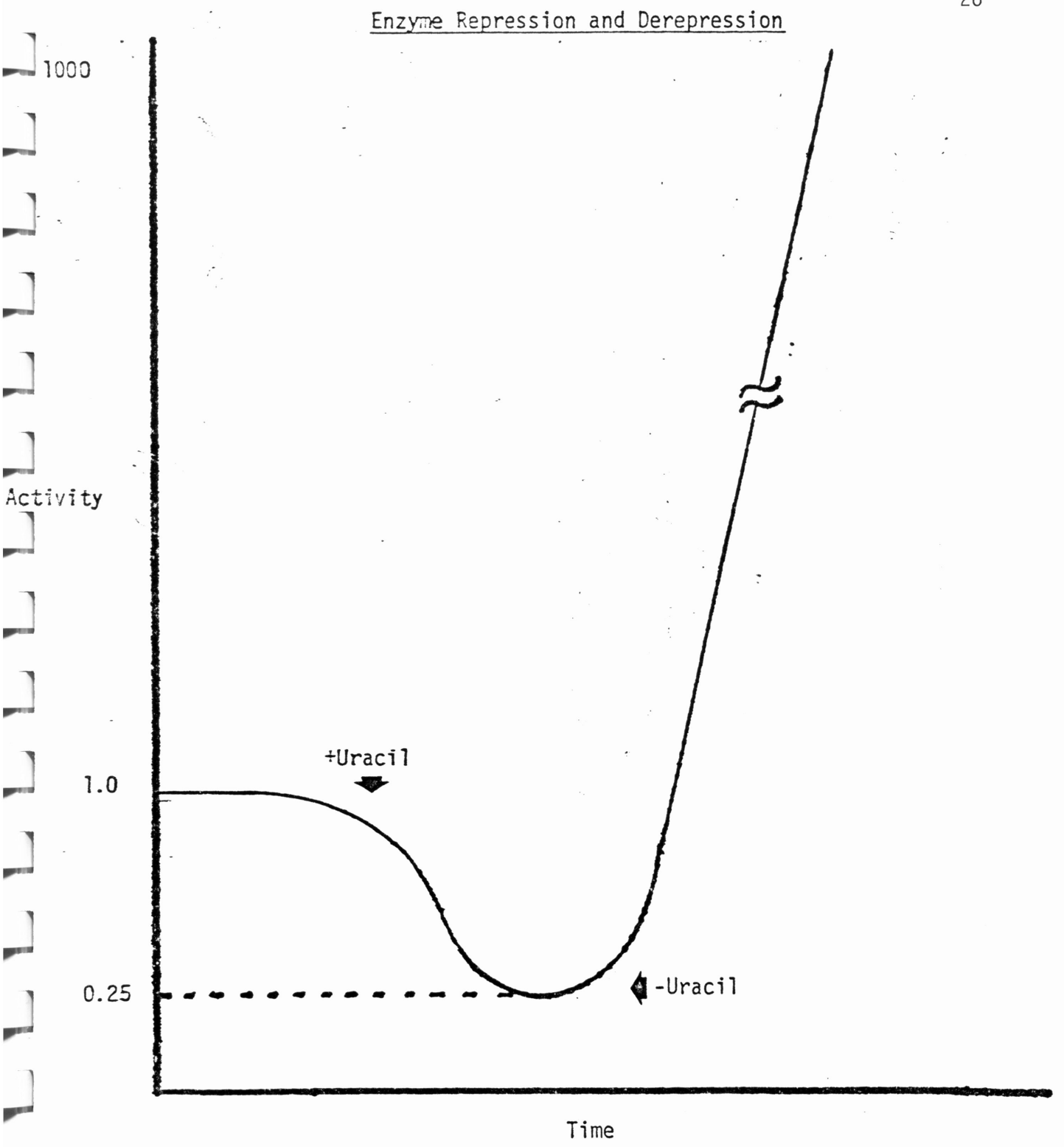

Figure 4. Repression and Derepression of ATCase

studies in our laboratory have demonstrated that *S. marcescens* and *P. vulgaris* maintain endogenous nucleotide pools which are 300 to 400 percent higher than *E. coli*, as reported by J. L. Cooper-Johnson in her M.S. Thesis. Perhaps regulation at the level of gene expression is more important than allosteric control.

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