

## Repression of *Escherichia coli* Carbamoylphosphate Synthase: Relationships with Enzyme Synthesis in the Arginine and Pyrimidine Pathways

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Cumulative repression of *Escherichia coli* carbamoylphosphate synthase (CPSase; EC 2.7.2.9) by arginine and pyrimidine was analyzed in relation to control of enzyme synthesis in the arginine and pyrimidine pathways. The expression of *carA* and *carB*, the adjacent genes that specify the two subunits of the enzyme, was estimated by means of an in vitro complementation assay. The synthesis of each gene product was found to be under repression control. Coordinate expression of the two genes was observed under most conditions investigated. They might thus form an operon. The preparation of strains blocked in the degradation of cytidine and harboring leaky mutations affecting several steps of pyrimidine nucleotide synthesis made it possible to distinguish between the effects of cytidine and uridine compounds in the repression of the pyrimidine pathway enzymes. The data obtained suggest that derivatives of both cytidine and uridine participate in the repression of CPSase. In addition, repression of CPSase by arginine did not appear to occur unless pyrimidines were present at a significant intracellular concentration. This observation, together with our previous report that *argR* mutations impair the cumulative repression of CPSase, suggests that this control is mediated through the concerted effects of regulatory elements specific for the arginine and pyrimidine pathways.

The production of carbamoylphosphate (CP) for the arginine and pyrimidine biosynthetic pathways in *Escherichia coli* is achieved by a single carbamoylphosphate synthase (glutamine) (CPSase; EC 2.7.2.9) using glutamine as nitrogen donor (36, 38). This enzyme is governed by control mechanisms that take its dual function into consideration. Its activity is modulated by the antagonistic effects of an inhibitor, uridine 5'-monophosphate (UMP), and activators, ornithine and inosine 5'-monophosphate, whereas its synthesis is subject to cumulative repression by arginine and pyrimidines (4, 35, 36, 38).

Both the arginine and pyrimidine pathways are controlled by enzyme repression (7, 28, 44) and feedback inhibition (11, 45). Repression of the eight arginine pathway enzymes, in spite of the scattering of their structural genes at several loci of the *E. coli* genome (40), is mediated by a single regulatory gene, *argR* (17, 28). The variations in the expression of the *arg* genes with the conditions of repression by arginine are not strictly coordinate but qualitatively coordinate or "correlative" (9, 15, 44). Mutations

at the *argR* gene lead to constitutive synthesis of all eight enzymes of the pathway. Control of enzyme synthesis in the pyrimidine biosynthetic pathway, although it was demonstrated very early (47), has since remained poorly understood. Five enzymes coded by unlinked genes (*pyrB* to *pyrF*; Fig. 1) are involved in the synthesis of UMP from aspartate and CP; all five are repressed by exogenous pyrimidines (8, 47). Attempts at selecting *pyrR* mutations rendering the synthesis of all five enzymes constitutive have failed (8, 37). The participation in the regulation of the *E. coli* pyrimidine pathway of a control by sequential induction similar to the one demonstrated in *Saccharomyces cerevisiae* by Lacroute (24) cannot be completely ruled out.

Cumulative repression of CPSase consists of a partial repression of the enzyme after growth in the presence of either arginine or a pyrimidine base or nucleoside, and in an almost total repression in the presence of both end products (38). The significant finding that *argR* mutations reduce the repressibility of CPSase strongly suggests a direct participation of the

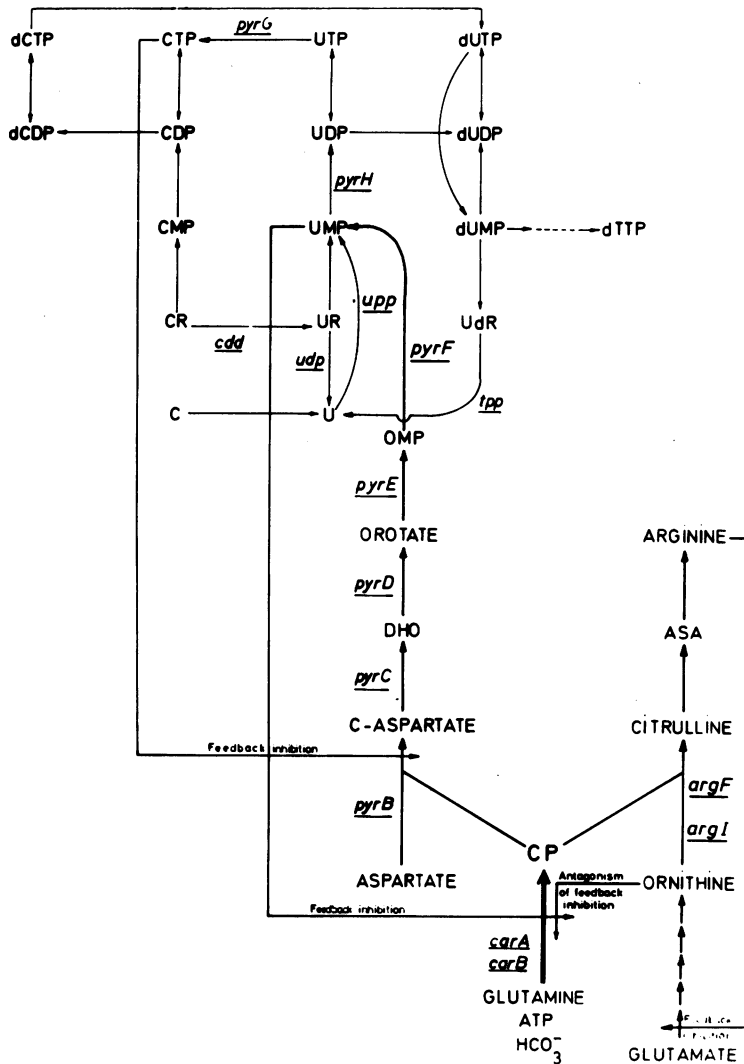


FIG. 1. Pathways of CP, arginine, and pyrimidine biosyntheses in *Escherichia coli* and their main feedback controls. The following gene designations are used: *carA*, light subunit of CPSase (glutamine) (EC 2.7.2.9); *carB*, heavy subunit of CPSase (glutamine) (EC 2.7.2.9); *argF* and *argI*, for isomeric OTCase (EC 2.1.3.3); *pyrB*, for ATCase (EC 2.1.3.2); *pyrC*, for dihydroorotase (EC 3.5.2.3); *pyrD*, for DHODEHase (EC 1.3.3.1); *pyrF*, for orotidine 5'-monophosphate pyrophosphorylase (EC 2.4.2.10); *pyrH*, for orotidine 5'-monophosphate decarboxylase (EC 4.1.1.23); *pyrG*, for CTP synthetase (EC 6.3.4.2); *pyrH*, for UMP kinase (EC 2.7.4.-); *udp*, for uridine phosphorylase (EC 2.4.2.3); *upp*, for UMP pyrophosphorylase (EC 2.4.2.9); *tpp*, for thymidine phosphorylase (EC 2.4.2.4); and *cdd*, for cytidine deaminase (EC 3.5.4.5). ASA, Argininosuccinic acid; C-Aspartate, carbamoylaspartate; DHO, dihydroorotate; OMP, orotidine 5'-monophosphate; C, cytosine; U, uracil; UR, uridine; CR, cytidine; UdR, deoxyuridine; UDP and UTP, uridine 5'-di- and triphosphate; CMP and CDP, cytidine 5'-mono- and diphosphate; dCMP, dCDP, and dCTP, deoxycytidine 5'-mono-, di-, and triphosphate; dTTP, thymidine 5'-triphosphate.

arginine repressor in cumulative repression (37). A similar involvement of a pyrimidine repressor is conceivable but could not be verified for lack of *pyrR* mutants.

The subunit composition and the organization of the genetic determinants of CPSase

have been established recently (29, 43). Adjacent genes, *carA* and *carB*, code for the two subunits of the enzyme. A large subunit of molecular weight 130,000, a product of *carB*, catalyzes the synthesis of CP from ammonia but not from glutamine. It also carries the bind-

ing sites of the effectors. The ability to use glutamine as the amino group donor is provided by the product of *carA*, a light subunit (molecular weight, 42,000) that bears the glutamine-binding site.

Important similarities exist between the metabolism of pyrimidines and its control in *E. coli* and *Salmonella typhimurium* (33). Most observations made concerning *E. coli* CPSase, including cumulative repression, have now been repeated in *S. typhimurium* (1, 2). The selection of *pyrR* mutations has similarly failed in this bacterium (19, 21, 32). Extensive studies of the metabolism of pyrimidines and pyrimidine nucleosides have been performed in *S. typhimurium* (6, 7, 30, 31). They have provided the means of specifically altering the cellular pools of uridine or cytidine compounds by using specially prepared strains. This approach has led to the proposal that different pyrimidine compounds are involved in the repression of the various pyrimidine enzymes. For instance, it was suggested that the expression of *pyrB* gene for aspartate carbamoyltransferase (ATCase; EC 2.1.3.2) is predominantly regulated by a uridine compound, whereas the synthesis of CPSase would be controlled by a cytidine derivative (1, 23, 31, 41, 46).

Our aim in the present work was to analyze further the mechanism of cumulative repression in *E. coli*. In a first set of experiments we have studied the expression of *carA* and *carB* under a variety of conditions. In a second approach we have investigated the relationship between cumulative repression and repression of the arginine and pyrimidine pathway enzymes. This approach included the application to *E. coli* of some of the methodology employed for identifying the co-repressors of the pyrimidine pathway in *S. typhimurium*, and the study of the role of arginine in the repression of CPSase and ATCase.

## MATERIALS AND METHODS

**Abbreviations.** A list of abbreviations and gene designations used in this work is given in the legend of Fig. 1. Cdx indicates the phenotype of complete inability to utilize cytidine as a total pyrimidine source in a *cdd* background.

**Chemicals.** Nucleosides, nucleotides, CP, and most other biochemicals were from Sigma Chemical Co., St. Louis, Mo., or Boehringer, Mannheim, Germany. [ $^{14}\text{C}$ ]NaHCO<sub>3</sub> and [ $^{14}\text{C}$ ]UMP were from the Radiochemical Centre, Amersham, England. [ $^{14}\text{C}$ ]aspartic acid was purchased from IRE, Belgium. 5-Fluorouracil, 5-fluorouridine, and 5-fluorodeoxycytidine were generous gifts from Hoffmann-La Roche Co., Basel, Switzerland, through the courtesy of G. Verly (Hoffmann-La Roche, Brussels, Belgium).

**Bacterial strains, growth media, and genetic**

**techniques.** All strains used in this work (Table 1), except a few derivatives of strain KL16 (Hfr, *thi*) used in strain preparations, are derived from strain P4X (Hfr, *metB*) (5). Strain JC411ER11, harboring the amber mutation *argR11*, was kindly provided by L. Gorini. Strain FPR41, bearing a leaky *pyrF* mutation, was given by B. Perbal.

All media, culture conditions, and procedures used in matings and transductions have been described previously, as have mutagenesis and mutant selections (13, 29).

Cells used for enzyme assays were grown on a rotary shaker at 37°C in minimal medium 132 supplemented with 0.5% glucose (wt/vol) and, when specified, with L-arginine (100 µg/ml), uracil (50 µg/ml), or cytidine (100 µg/ml). Other metabolite requirements were satisfied as described previously (29). Unless otherwise mentioned, cells were harvested by centrifugation at mid-log phase.

**Isolation of strains.** The procedures used to obtain a set of *E. coli* strains affected in pyrimidine metabolism are similar to those described for *E. coli* by other authors, or are adapted from procedures developed for *S. typhimurium*. The details given here are limited to a few relevant points. The presence of selected mutations has been verified by enzyme assays.

Selection of a *cdd* mutation (affecting cytidine deaminase; EC 3.5.4.5) in strain KL16AU (Hfr, *thi argG carB8*) was performed by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis followed by penicillin counterselection, with deoxyuridine (40 µg/ml) as a pyrimidine source, combined with screening for resistance to 5-fluorodeoxycytidine (0.5 µg/ml) plus uracil (50 µg/ml) (22). The strain obtained was devoid of measurable residual cytidine deaminase activity.

Strain P4XLTF1507 (Hfr, *metB tpp udp pyrH cdd*) was prepared from strain P4X171D1 Azu<sup>r</sup> (Hfr, *metB argA upp*) through the following steps: (i) A *tpp* mutant was isolated by selecting for resistance to 5-fluorouracil (2.5 µg/ml) plus deoxyadenosine (100 µg/ml) and screening for inability to use thymidine (1,000 µg/ml) as a carbon source (3). (ii) A *udp* mutant was isolated by selecting for resistance to 5-fluorouracil (10 µg/ml) plus adenosine (50 µg/ml) and screening for inability to use uridine (1,000 µg/ml) as a carbon source (39). (iii) Transfer of a *upp*<sup>+</sup> allele to the resulting strain was performed by mating its phenocopy with KL16 *argG* (Hfr, *thi argG*) and selection of an Arg<sup>+</sup> Azu<sup>r</sup> recombinant, P4XLTD1 (Hfr, *metB tpp udp*). (iv) Selection of a bradytrophic *pyrH* mutation in the latter *udp* derivative was accomplished by a procedure described for *S. typhimurium* (32). This procedure, originally designed for the selection of *pyrR* mutations, has been shown to yield *pyrH* mutations (19, 21). It is based on selection for resistance to a mixture of 5-fluorouracil and 5-fluorouridine (1 µg/ml) in a *udp* strain. The resistant mutants (1 spontaneous mutant in 2 × 10<sup>7</sup> cells) were screened for pyrimidine overproduction. Two percent of these resistant mutants were able to feed mutant P4XSB22 (Hfr, *metB pyrE*) and proved to be efficient uracil or uridine overproducers. They were identified as *pyrH* mutants through enzyme assay. Mutants LTF15 and

TABLE 1. Designations and derivations of strains

Strain	Sex	Genotype	Origin, method of construction or reference
P4X	Hfr P4X	<i>metB</i>	(5)
P4XB2	Hfr P4X	<i>metB argR</i>	From P4X, this laboratory
P4XSB22	Hfr P4X	<i>metB pyrE</i>	From P4X, after ultraviolet induction, this laboratory
Jef8	Hfr P4X	<i>metB thrA carB8</i>	From a <i>thrA</i> derivative after ICR191 mutagenesis (29)
Mi178	Hfr P4X	<i>metB thrA carA178</i>	From a <i>thrA</i> derivative of P4X spontaneously (29)
P4XSB4	Hfr P4X	<i>metB pan</i>	From P4X after ultraviolet induction, this laboratory
JC411ER11	F <sup>-</sup>	<i>thi his metB aroE24 argR11 strA</i>	(20)
FPR41	Hfr H	<i>pyrF</i>	Revertant obtained by B. Perbal from strain YA287 (8)
KL16	Hfr KL16	<i>thi</i>	(5)
KL16 <i>argG</i>	Hfr KL16	<i>thi argG</i>	From KL16, this laboratory
KL16AU	Hfr KL16	<i>thi argG carB8</i>	From KL16 <i>argG</i> , this laboratory
KL16AU <i>cdd</i>	Hfr KL16	<i>thi argG carB8 cdd</i>	From KL16AU, this work
P4XSB171D1	Hfr P4X	<i>metB argA</i>	From P4X, this laboratory
P4XSB171D1 Azu <sup>R</sup>	Hfr P4X	<i>metB argA upp</i>	From P4XSB171D1, this laboratory
P4XLTD1	Hfr P4X	<i>metB tpp udp</i>	From P4X171D1 Azu <sup>R</sup> , this work
P4XLTF15	Hfr P4X	<i>metB tpp udp pyrH</i>	From P4XLTD1, this work
P4XLTE3	Hfr P4X	<i>metB tpp udp pyrH</i>	From P4XLTD1, this work
P4XLTF1507	Hfr P4X	<i>metB tpp udp pyrH</i>	From P4XLTF15, this work
P4XG3	Hfr P4X	<i>metB tpp udp cdd pyrG</i>	From P4XLTD1, this work
P4XG51	Hfr P4X	<i>metB tpp udp cdd pyrG carA50</i>	From P4XG3, this work
P4XG81	Hfr P4X	<i>metB tpp udp cdd Cdx pyrG</i>	From P4XG51, this work
P4XG81 PyrG <sup>±</sup>	Hfr P4X	<i>metB tpp udp cdd Cdx pyrG</i>	From P4XG81, this work
P4XG91	Hfr P4X	<i>metB tpp udp cdd Cdx</i>	From P4XG81, this work
P4XG91 PyrF <sup>±</sup>	Hfr P4X	<i>metB tpp udp cdd Cdx pyrF</i>	From P4XG91, this work

LTE3, for example, displayed considerably decreased levels of this enzyme (1 and 0.7 units/mg of protein, in contrast to 22 units/mg of protein in the wild-type strain). A recipient strain harboring a *pan* mutation was transduced by the *pyrH* mutants LTF15 and LTE3; 2 and 2.5 percent, respectively, of the Pan<sup>+</sup> recombinants were found to be *pyrH* as tested by pyrimidine excretion, indicating a slightly different location from that of the homologous gene in *S. typhimurium* (19, 32). (v) Transfer of the *cdd* marker of KL16AU (see above) was accomplished by a mating with a phenocopy of P4XLTF15, and Arg<sup>+</sup>His<sup>+</sup> recombinants were selected. Strain P4XLTF1507 (Hfr, *metB tpp udp pyrH cdd*) was obtained in this way.

Strain P4XG81 (Hfr, *metB tpp udp cdd Cdx pyrG*) was prepared from P4XLTD1 (Hfr, *metB tpp udp*) in several steps: (i) Transfer of the *cdd* mutation of KL16AU *cdd* was performed by a mating with the phenocopy of a *his* derivative of P4XLTD1, and Arg<sup>+</sup>His<sup>+</sup> recombinants were selected. The *cdd* mutation was required to reduce cytidine deamination and allow the selection of *pyrG* mutations. (ii) By penicillin counterselection, a mutant was isolated that required cytidine for growth, P4XG3 (Hfr, *metB tpp udp cdd pyrG*). This *pyrG* mutation was co-transducible with *argA* and *cysC* (Table 2); this suggests a location similar to that of the homologous gene in *Salmonella* (6). (iii) Strain P4XG51 (Hfr, *metB tpp udp cdd pyrG carA50*) was prepared

TABLE 2. Localization of the *pyrG* marker by transduction: recipient *argA cysC*, donor *pyrG*

Classes of recombinants	Cross-over <sup>a</sup> regions	Genetic constitution of recombinants	
		Arg <sup>+</sup>	Cys <sup>+</sup>
Arg <sup>+</sup> Pyr <sup>+</sup> Cys <sup>+</sup>	1-2-3-4	2	2
Arg <sup>+</sup> Pyr <sup>-</sup> Cys <sup>+</sup>	1-4	13	5
Arg <sup>-</sup> Pyr <sup>-</sup> Cys <sup>+</sup>	2-4		40
Arg <sup>-</sup> Pyr <sup>+</sup> Cys <sup>+</sup>	3-4		278
Arg <sup>+</sup> Pyr <sup>+</sup> Cys <sup>-</sup>	1-2	106	
Arg <sup>+</sup> Pyr <sup>-</sup> Cys <sup>-</sup>	1-3	8	

<sup>a</sup> Cross-over regions as shown in parentheses if order is (1)-*argA*-(2)-*pyrG*-(3)-*cysC*-(4).

by transfer of mutation *carA50* in a *thr*<sup>-</sup> derivative of P4XG3. (iv) By penicillin counterselection, a Cdx strain was isolated which carried an additional mutation (see Results) preventing the use of cytidine as a total pyrimidine source. The resulting strain, which displays an absolute requirement for uracil in the presence of cytidine, was transduced to Car<sup>+</sup>, yielding strain P4XG81 (Hfr, *metB tpp udp cdd Cdx pyrG*).

Strain P4XG91 (Hfr, *metB tpp udp cdd Cdx*) was obtained by transducing strain P4XG81 to prototrophy for cytidine. Strain P4XG81 PyrG<sup>±</sup> (Hfr, *metB*

*tpu udp cdd Cdx pyrG*) is a spontaneous revertant of P4XG81 able to grow at a very low rate in the absence of cytidine. Strain P4XG91 *PyrF<sup>+</sup>* (Hfr, *metB tpu udp cdd Cdx pyrF*) was prepared by transducing the leaky *pyrF* mutation of strain FPR41 into a *trp<sup>-</sup>* derivative of P4XG91 and selecting for *Trp<sup>+</sup> Pyr<sup>+</sup>* recombinants.

**Enzyme Assay.** The preparation of cell extracts and assays for CPSase, ornithine carbamoyltransferase (OTCase; EC 2.1.3.3), UMP pyrophosphorylase (EC 2.4.2.9), and dihydroorotate dehydrogenase (DHODEHase; EC 1.3.3.1) have been described previously (29, 37). ATCase was assayed by the method of Perbal and Hervé (34), UMP kinase was assayed by the method of Ingraham and Neuhaud (19), thymidine phosphorylase (EC 2.4.2.4) was assayed by the method of Razzle and Khorana (40), uridine phosphorylase (EC 2.7.4.-) was measured by the method of Pritchard and Ahmad (39), cytidine deaminase was assayed by the method of Karlström (22), and cytidine 5'-triphosphate (CTP) synthetase (EC 6.3.4.2) was determined by the method of Liberman (26). Protein content was estimated by the method of Lowry et al. (27). Specific activities are expressed as units (nanomoles of product formed per minute) per milligram of protein.

**In vitro complementation assay of *car* products.** Extracts of mutants Jef8 (Hfr, *metB thrA carB8*) and Mi178 (Hfr, *metB thrA carA178*) carrying *car* mutations that complement efficiently in vitro (29) were used for assaying, respectively, the products of *carA* and *carB*. The complementation test was performed as described before (29); the extracts tested were supplemented with increasing amounts of each of the two complementing mutants. Recorded values (expressed as glutamine-dependent activities) are the activities reached at saturation by the complementing extracts.

## RESULTS

**Repression of *carA* and *carB* expression by arginine and pyrimidines.** The levels of the glutamine- and ammonia-dependent activities of CPSase have been estimated after growth under conditions achieving various levels of repression by arginine and pyrimidines. As shown in Table 3, cumulative repression of CPSase in the wild-type strain P4X was observed with either nitrogen donor. Repression was partial in the presence of arginine and

TABLE 3. Effect of growth conditions on expression of *carA* and *carB* genes

Strain	Relevant genotype <sup>a</sup>	Addition to minimal medium (μg/ml)	CPSase activity <sup>b</sup>		Estimation of total <i>car</i> gene products by in vitro complementation <sup>c</sup>	
			Glutamine dependent	Ammonia dependent	<i>carA</i> product	<i>carB</i> product
P4X		None	20.0	2.3	19.7	18.5
		Arginine, 100	14.7	1.5	15.5	14.8
		Uracil, 50	8.7	1.0	8.8	8.7
		Arginine, 100 } + uracil, 50 }	1.2	0.2	1.3	1.2
P4XB2	<i>argR</i>	None	41.8	4.2	41.0	40.7
		Arginine, 100 } + uracil, 50 }	21.5	2.0	22.2	21.0
P4XSB22 <sup>d</sup>	<i>pyrE</i>	Uracil, 7.5 <sup>e</sup>	70.8 (67.2) <sup>f</sup>	4.2 (4.8) <sup>f</sup>	86.3	70.7
P4XSB22 <sup>d</sup>	<i>pyrE</i>	Arginine, 100 } + uracil, 7.5 <sup>e</sup> }	54.3 (66.1) <sup>f</sup>	5.2 (6.5) <sup>f</sup>	87.0	56.8
Jef8	<i>carB8</i>	Arginine, 100 } + uracil, 7.5 <sup>e</sup> }	ND <sup>g</sup>	ND <sup>g</sup>	100.0	ND <sup>g</sup>
Jef8	<i>carB8</i>	Arginine, 100 } + uracil, 50 }	ND <sup>g</sup>	ND <sup>g</sup>	0.8	ND <sup>g</sup>

<sup>a</sup> Only the relevant genotypes for these experiments are given here; further information is given in Materials and Methods.

<sup>b</sup> Activity was estimated with either glutamine (0.01 M) or ammonia (0.1 M) as the nitrogen donor. Specific activities are given as nanomoles of CP formed per minute per milligram protein.

<sup>c</sup> The products of *carA* and *carB* were estimated by in vitro complementation with extracts of mutant Mi178 (*carA178*) and Jef8 (*carB8*), respectively. Both gene products are expressed as glutamine-dependent specific activities.

<sup>d</sup> The CPSase level of strain P4XSB22 grown in minimal medium with excess arginine and uracil is identical to that of strain P4X grown under the same conditions.

<sup>e</sup> Cells grown on a limiting amount of uracil (7.5 μg/ml) were harvested 3 h after the plateau corresponding to exhaustion of uracil has been reached, to achieve maximal derepression of CPSase.

<sup>f</sup> Some variation was observed in the CPSase levels of this mutant after such growth conditions. For this reason, we have indicated in parentheses the data of another experiment.

<sup>g</sup> ND, Not detectable.

uracil alone; it was almost total in the presence of both arginine and uracil. Both activities were enhanced when derepression was achieved by starving the pyrimidine auxotrophic mutant P4XSB22 for uracil or by using the *argR* mutant P4XB2. Yet, an excess synthesis of one of the subunits of the enzyme would not be easily detected by such measurements. Indeed, the glutaminase activity of the isolated *carA* gene product can be estimated only with purified preparations. In addition, the ammonia activity of the isolated heavy subunit (*carB* product) cannot be easily distinguished from that of the aggregate of the two products. A complementation assay (see Materials and Methods) was developed in vitro for determining, after growth under various conditions, the level of each of the *car* products. The results of an analysis based on this assay are presented in the last two columns of Table 3. Coordinate expression of *carA* and *carB* was observed in P4X and P4XB2 under all conditions tested, since no significant excess of *carA* or *carB* could be detected. Only after starving strain P4XSB22 for pyrimidines could a 20 to 50% excess of the light subunit be detected. Excess synthesis of the heavy subunit was never observed.

The same experimental approach has been used for studying the repression of the synthesis of the light subunit in strain Jεf8, which bears a large deletion covering the entire *carB* gene (29). The repressibility and derepressibility of the light subunit are not affected in this strain. Large deletions affecting the *carA* gene have not been selected so far. Nevertheless, all the *carA* mutants we have examined display a normal repression of *carB* expression (data not shown).

**Preparation of a strain blocked in the degradation of cytidine.** With the aim of investigating the relationships between cumulative repression and control mechanisms of the synthesis of the other pyrimidine enzymes, we have, as was done previously in *S. typhimurium*, prepared strains that are blocked in the interconversion of cytidine and uridine compounds. Such strains allow the independent manipulation of the pools of these two groups of metabolites.

In *S. typhimurium*, exogenous cytidine does not accumulate as such in the cell but is readily deaminated to uridine. A *cdd* mutation (gene for cytidine deaminase) is required to build up a sufficient intracellular pool of cytidine compounds and permit the recovery of *pyrG* mutations, which affect CTP synthetase and are accompanied by a specific requirement for cytidine (6, 31). We have observed a similar situa-

tion in *E. coli*: strain P4XLTD1 (*tpp udp*), previously constructed for the selection of *pyrH* mutations, had to be made *cdd* before it could be used successfully for the recovery of a *pyrG* mutation. Yet the resulting strain, when made *car* (thus harboring the constellation of mutations *tpp udp cdd pyrG car*), was still capable of growth at a significant rate (generation time, 200 min) in the presence of cytidine as a total pyrimidine source. An additional mutation, the Cdx phenotype, had to be selected (see Materials and Methods) to abolish completely this ability to use cytidine. Whether it affects a residual cytidine deaminase activity or another activity could not be determined so far. Thus, strain P4XG91 (*tpp udp cdd Cdx*), which was eventually prepared, is totally blocked in the conversion of cytidine to uridine nucleotides. It was used both for further strain preparations and as such for testing cytidine compounds as possible co-repressors in repression of CPSase.

A comparison of repression of CPSase, ATCase, and DHODEHase in strains P4X (wild type) and P4XG91 is presented in Table 4. As expected from the rapid conversion of cytidine to uridine, repression by cytidine and uracil was comparable in the wild-type strain, except for CPSase, which was slightly less repressible by cytidine than by uracil. Repression by cytidine of CPSase and DHODEHase was little affected in strain P4XG91, which is blocked in the degradation of cytidine. In contrast, repression of ATCase by cytidine was considerably, but not totally, suppressed. These observations were confirmed by comparing the enzyme levels obtained, for both strains, after growth in the presence of arginine plus cytidine or arginine plus uracil. These various findings suggest that a cytidine compound is involved in the repression of CPSase and DHODEHase, whereas a uridine derivative is mainly implicated in the repression of ATCase. Nevertheless, a limited repression of the latter enzyme by cytidine was observed, suggesting that a cytidine compound might also play a role in the repression of ATCase. The fact that in both strains CPSase was more efficiently repressed by uracil than by cytidine seems to indicate that uridine as well as cytidine compounds are involved in the repression of CPSase.

**Repression of CPSase and other pyrimidine enzymes in bradytrophic pyrimidine mutants.** Confirmation of the proposal made above was sought by studying strains which are similar to P4XG91 but which harbor additional leaky mutations, making pyrimidine limitations possible (see Table 5).

The first strain studied, a bradytrophic *pyrF* derivative, grew slowly on minimal medium.

TABLE 4. Levels of CPSase, ATCase, and DHodehase of wild-type strain P4X and mutants P4XG91 and P4XB2 after growth under various conditions

Strain (relevant genotype <sup>a</sup> )	Enzyme	Enzyme specific activities <sup>b</sup> as function of additions to minimal medium <sup>c</sup>					
		None	Arginine	Cytidine	Uracil	Arginine + cytidine	Arginine + uracil
P4X (wild type)	CPSase <sup>d</sup>	17.3	8.7	8.3	6.3	0.8	0.8
	ATCase	208	115	15	15	23	23
	DHodehase	3.0	3.3	1.2	2.2	2.0	2.2
P4XG91 ( <i>tpp udp cdd Cdx</i> )	CPSase <sup>d</sup>	15.7	10.5	10.0	5.8	4.2	0.7
	ATCase	162	105	125	20	82	25
	DHodehase	3.8	2.8	1.7	1.3	1.8	1.8
P4XB2 ( <i>argR</i> )	CPSase <sup>d</sup>	34.2	29.7	29.5	32.0	21.7	23.0
	ATCase	200	120	22	25	20	28
	DHodehase	3.0	3.2	2.2	2.0	1.7	2.2

<sup>a</sup> Only the relevant genotype for this experiment is given here. Further information is given in Materials and Methods.

<sup>b</sup> Specific activities are given as nanomoles of product formed per minute per milligram of protein.

<sup>c</sup> Additions are made at the following concentrations (final) in  $\mu\text{g/ml}$ : arginine, 100; cytidine, 100; and uracil, 50.

<sup>d</sup> Only the glutamine-dependent activity of CPSase has been estimated.

TABLE 5. Levels of CPSase, ATCase, and DHodehase in different bradytrophic pyrimidine mutants after growth under various conditions

Strain (relevant genotype) <sup>a</sup>	Enzyme	Growth behavior and enzyme specific activities <sup>b</sup> as function of addition to minimal medium <sup>c</sup>					
		None	Arginine	Cytidine	Uracil	Arginine + cytidine	Arginine + uracil
P4XG91 <i>PyrF</i> <sup>±</sup> ( <i>tpp udp cdd Cdx pyrF</i> )	CPSase <sup>d</sup>	43.3	41.3	26.2	5.5	24.7	0.5
	ATCase	7,750	8,500	8,580	33	8,330	38
	DHodehase	19.2	15.0	40	1.7	4.3	2.0
	Generation time (min) <sup>e</sup>	300	305	255	50	260	50
P4XG81 <i>PyrG</i> <sup>±</sup> ( <i>tpp udp cdd Cdx pyrG</i> )	CPSase <sup>d</sup>	50.2	35.5	9.3	7.0	3.8	0.5
	ATCase	16,660	16,660	227	57	152	52
	DHodehase	24.2	25.3	3.3	2.2	2.3	2.3
	Generation time (min) <sup>e</sup>	375	345	55	50	50	50
P4XLTF1507 ( <i>tpp udp cdd pyrH</i> )	CPSase <sup>d</sup>	65.0	61.7	89.8	60.8	90.0	56.7
	ATCase	1,160	8,660	6,920	830	5,000	660
	DHodehase	7.2	22.2	4.2	4.7	5.5	4.8
	Generation time (min) <sup>e</sup>	65	270	140	65	NM <sup>f</sup>	60

<sup>a</sup> Only the relevant genotype for this experiment is given here; further information is given in Materials and Methods.

<sup>b</sup> Specific activities are given as nanomoles of product formed per minute per milligram of protein.

<sup>c</sup> Additions are the following concentrations (in  $\mu\text{g/ml}$ ) (final): arginine, 100; cytidine, 100; uracil, 50.

<sup>d</sup> Only the glutamine-dependent activity of the enzyme has been estimated.

<sup>e</sup> Generation time is 50 min for a wild-type strain on minimal medium.

<sup>f</sup> NM, Not measurable.

CPSase, ATCase, and DHodehase were derepressed under these conditions, which should cause a general limitation of pyrimidine compounds. Neither the growth rate nor the levels of CPSase and the other pyrimidine enzymes were significantly affected by the presence of

arginine in the growth medium. Addition of exogenous cytidine to increase the intracellular concentration of cytidine compounds while maintaining the limitation in the supply of uridine compounds resulted in a slight improvement of growth and in partial repression of

CPSase; DHodehase was more efficiently, although not totally, repressed, whereas the ATCase level was not significantly affected. The simultaneous addition of cytidine and arginine did not result in a more efficient repression of CPSase than cytidine alone. Thus, exogenous cytidine, in the condition of limited synthesis of uridine compounds, cannot exert a maximal repression of CPSase and DHodehase. Two explanations could be proposed for this finding: (i) uridine compounds also participate in the repression of these enzymes; and (ii) the pool of cytidine compounds that can be formed from exogenous cytidine is smaller than the pool that is formed from uracil and is insufficient to exert maximal repression.

Bradytrophic *pyrG* mutant P4XG81 *PyrG*<sup>+</sup> provided the means of distinguishing between these two possibilities (Table 5). When grown on minimal medium, this strain was expected to synthesize limiting amounts of cytidine compounds. In contrast, uridine compounds should accumulate in considerably larger amounts than in the wild-type strain. Under such conditions, the levels of all three enzymes tested were considerably increased: that of ATCase was particularly elevated. Addition of cytidine to the growth medium restored a wild-type growth rate and notably reduced the level of all three enzymes. Repression of CPSase was considerably greater than it was in the *pyrF* bradytrophic mutant grown in the same medium. In both strains, the cytidine compounds are predominantly formed from exogenous cytidine: their intracellular concentrations are likely to be similar. The data thus support the first of the above explanations: the participation of uridine compounds, either alone or in a concerted manner with cytidine compounds, in the repression of CPSase. Similarly, the high ATCase level that could be reached in this strain supports the idea of the involvement of cytidine compounds in the repression of this enzyme. In this respect, it should be mentioned that, under conditions of cytidine starvation, total repression of ATCase by uracil could not be reached in strain P4XG81 (*tpp udp cdd Cdx pyrG*). Specific ATCase activity was 420 units per mg of protein in contrast with an activity of 33 units in the presence of cytidine plus uracil.

The bradytrophic *pyrH* mutant P4XLTF1507, although it displays a considerably reduced level of UMP kinase, grows relatively fast on minimal medium. Partial derepression of ATCase and DHodehase occurred under such conditions (Table 5). In addition, this partial *pyrH* block prevented repression by uracil. Similar behavior has been observed in some *pyrH* mutants of *S. typhimurium*; this was misleading

for a while, since it was a characteristic expected of the as yet undescribed *pyrR* mutants (19, 21, 32). In the case of *E. coli*, we have extended this observation to CPSase, which is markedly derepressed (Table 5). Growth on minimal medium of this *pyrH* bradytrophic strain was expected to result in the following intracellular conditions: increased concentration of UMP (a significant pyrimidine overproduction is indeed observed), and decreased concentration of cytidine and uridine compounds (except UMP). It can be assumed that, for such a *pyrH* strain, it may be difficult to establish a balance between the unusually high UMP pool that must be maintained to allow a sufficient synthesis of the other pyrimidine nucleotides, and the unfavorable effects of such an elevated UMP concentration on the activity of CPSase. Perturbation of this balance, for example, by lowering the intracellular concentration of ornithine (the positive effector of CPSase) or by raising the CTP pool (the feedback inhibitor of ATCase), might be expected to result in still more precarious conditions. This is probably what happened when arginine or cytidine was added to minimal medium. These additions provoke severe reductions of growth rate and prevent pyrimidine excretion. An important derepression of ATCase was observed (Table 5). CPSase was not repressed by arginine, and was even more derepressed in the presence of cytidine. DHodehase was derepressed in the presence of arginine and repressed in the presence of cytidine. The behavior of this mutant again suggests a participation of both cytidine and uridine compounds (other than UMP) in repression of CPSase.

**Interactions of cumulative repression with control of enzyme synthesis in the arginine and pyrimidine pathways.** In several experiments described in the preceding sections, it was observed that repression of CPSase by arginine was limited or even negligible under conditions of severe pyrimidine starvation. Such was the case, for instance, with bradytrophic *pyrF* or *pyrH* mutants (Table 5).

This point was investigated by submitting mutant P4XSB22 (*pyrE*) to conditions of pyrimidine starvation, with or without arginine added to the medium (Table 3). In most cases (figures in parentheses), identical CPSase levels were attained whether arginine was present or not. Occasional differences were observed (figures without parentheses) but were found to result from a slight de-coordination in the expression of *carA* and *carB*; in both cases, the in vitro complementation assay revealed equivalent production of the preponderant gene product, that of *carA*. Yet, repression of OTCase by



arginine is normally effective under such conditions (3,000 units of OTCase per mg of protein in the absence of arginine, versus 25 units in its presence). Consequently, repression of CPSase by arginine does not seem to occur unless pyrimidine derivatives are present at a significant intracellular concentration. Such observations suggest a concerted role in the repression of CPSase by arginine and pyrimidine.

In a previous report (7) we mentioned that significant repression of ATCase by arginine occurs in *argR*<sup>-</sup> as well as in *argR*<sup>+</sup> strains, thus rendering unlikely a direct effect of the product of *argR* on the synthesis of ATCase. This experiment has been repeated and extended to DHODEHase. No indication of a repression of this enzyme by arginine was obtained (Table 4). However, the repression of ATCase by arginine is a reproducible feature and is unaffected even by an *argR* nonsense mutation (*argR11*). Such findings exclude a direct participation of the *argR* product in the repression of ATCase. They also eliminate the possibility that a significant part of the expression of *pyrB* (the ATCase structural gene) would be due to read-through transcription from the adjacent *argI* gene, one of the OTCase structural determinants (25). Thus, repression by arginine of ATCase is likely to result from the diversion of excess CP toward the pyrimidine pathway under conditions where ornithine synthesis is blocked through feedback inhibition of the first enzyme of the arginine pathway.

## DISCUSSION

The contiguous genes *carA* and *carB* show coordinate expression under most conditions investigated. They might thus constitute an operon. Yet, extreme derepression, as achieved by maintaining a pyrimidine auxotroph under conditions of pyrimidine starvation, results in a deviation from coordination, in which a slight excess of the *carA* subunit is produced. This cannot be accounted for by a preferential degradation of the *carB* product, since the relative levels of the two *car* products remain unchanged after the cells have been maintained in a state of pyrimidine starvation for as long as 8 h (not shown). It is possible to reconcile these data with the existence of a *car* operon if we assume that they are an expression of natural polarity, transcription proceeding from *carA* into *carB*; this assumption receives some support from the fact that the control of *carA* expression is not affected by deletion *carB8*, which covers all known *carB* mutations. A definitive answer concerning the direction of

transcription in the *car* locus has to await a more detailed study of polar effects in the expression of *carA* and *carB*, a study that is presently in progress.

Several examples of autogenous regulation of gene expression, a mechanism by which a protein controls the expression of its own structural gene, have been described and discussed recently (16). However, our finding that repression of the light subunit of CPSase is not affected by deletion *carB8* demonstrates that the product of this gene is not required for cumulative repression of CPSase. Likewise, no evidence for a need of the *carA* product in repression of CPSase could be obtained.

The second part of this work was devoted to a comparison of the control of the synthesis of CPSase with that of two other pyrimidine pathway enzymes, ATCase and DHODEHase. The expression of the *pyr* genes has been previously studied by Beckwith et al. (8), who reported coordinate expression of the supposedly linked *pyrC*, *pyrD*, *pyrE*, and *pyrF* genes but non-coordinate variations of ATCase, the product of *pyrB*. A reevaluation of these data has shown that all five *pyr* genes are separated (33, 42). Furthermore, non-coordinate expression of *pyrD* and *pyrE* has since been observed (10). We have observed the levels of CPSase, ATCase, and DHODEHase in a series of strains that harbor partial blocks in genes *pyrF*, *pyrG*, and *pyrH*, in addition to a set of mutations that totally separate the metabolism of cytidine compounds from that of uridine compounds. These enzymes were found to obey similar controls, since all three were repressed by excess pyrimidine and derepressed under condition of pyrimidine starvation. Nevertheless, their levels vary in a markedly non-coordinate fashion. The maximal derepression ratios (from fully derepressed to fully repressed levels) are 650-fold for ATCase, 15-fold for DHODEHase, and 20-fold for CPSase (125-fold when the level obtained in the presence of both arginine and uracil is taken as the repressed level).

Conclusions regarding the nature of the corepressors involved in the repression of each of these enzymes have been drawn from such experiments. Cytidine compounds are without any doubt dominant over uridine compounds in the repression of DHODEHase. The ATCase level is regulated mainly by the effects of uridine compounds. In contrast, several indications of a participation of both cytidine and uridine compounds in repression of CPSase have been obtained. For instance, cytidine represses CPSase efficiently in strains blocked in the degradation of this nucleoside. Yet, repression is only partial if uridine compounds are

limiting (*pyrF* background). The value of conclusions drawn from the study of cultures growing slowly under conditions of pyrimidine starvation has been questioned, inasmuch as such conditions might lead to general derepression (23, 41). However, evidence for such a phenomenon is lacking. Furthermore, the fact that each enzyme considered here responds in a different way to variations of the two groups of pyrimidine derivatives shows that we are indeed dealing with specific effects. The data obtained here concerning the repression of ATCase and DHODEHase in *E. coli* are in agreement with the major implications of the work on *S. typhimurium* (23, 31, 41, 46). In contrast, our conclusions regarding the pyrimidine co-repressors of CPSase are at variance with those reached in this organism (1) where a cytosine compound alone was concluded to be responsible for the repression of CPSase. Yet, the latter conclusion was based solely on the observation that cytidine but not uracil represses CPSase synthesis in strain DL38 (*cdd*, *cod*) (1). The interpretation of such behavior is difficult unless it is supposed that this strain harbors an additional defect such as a *upp* mutation (affecting UMP pyrophosphorylase) that prevents the uptake of uracil and its conversion into UMP (37).

Other groups, working on *S. typhimurium*, have attempted to obtain further information from the measurement of pyrimidine pools. Such measurements have provided useful data, some of which have been extrapolated to *E. coli* for the interpretation of the results obtained here, but they did not permit the precise identification of the pyrimidine co-repressors (12, 23, 30, 41).

Therefore, we are developing a completely different approach, which consists in the characterization of the regulatory elements by means of a deoxyribonucleic acid-directed, cell-free system for CPSase synthesis. A transducing phage carrying the *car* region ( $\lambda$ *dcarAB*) has been isolated, and its deoxyribonucleic acid has been used for the in vitro synthesis of CPSase (18). This system is being used for the study of cumulative repression of CPSase. Similar steps are being taken for performing in vitro synthesis of ATCase and OTCase (P. Halleux, N. Glansdorff, M. Crabeel, and A. Piérard, Abstr. 10th Fed. Eur. Biochem. Soc. Meet., Paris, July 1975, abstr. no. 849).

The in vitro approach should also be suitable for studying the relationships between repression of CPSase and repression of enzymes of the arginine and pyrimidine pathways. This topic was dealt with in the final part of this work and has led to the idea that the effects of arginine and the pyrimidine co-repressors in the repres-

sion of CPSase are concerted. This conclusion, together with the involvement of the product of *argR* in repression of CPSase (37), leads almost inescapably to the proposal that arginine and pyrimidine repressors do in some way interact in achieving CPSase repression. Some substantiation of this hypothesis is reported in an accompanying paper (14), which describes the transducing  $\lambda$ *dcar* phage. This phage multiplies as a plasmid when introduced in a *car* strain in the absence of a helper phage. The resulting strain, when grown on minimal medium, displays from 20- to 30-fold the CPSase level of a wild-type strain on the same medium, suggesting that a large number of copies of the *car* gene exists in the cell. In addition, simultaneous derepression of the levels of ATCase (coded by *pyrB*) and OTCase (coded by *argF* and *argI*) was observed. We conclude that in such a strain, the larger number of copies of the *car* operator region available creates a shortage of two regulatory elements: one involved in the repression of the arginine pathway enzymes, including the product of *argR*, the other participating in the repression of the pyrimidine enzymes. The two elements would act either side by side or in the form of an aggregate on the *car* operator.

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