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pyrB Mutations as Suppressors of Arginine Auxotrophy in *Salmonella typhimurium*

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Salmonella typhimurium strains which produce high constitutive levels of aspartate transcarbamoylase due to the *pyrH700* mutation were found to grow more slowly in minimal medium than *pyrH*$^+$ controls. The addition of arginine or citrulline but not ornithine restored normal growth rates. This requirement for arginine was completely suppressed by *pyrB* mutations and partially suppressed by *pyrC* and *pyrD* mutations. No suppression was observed with mutants at the *pyrF* locus. Introduction of leaky mutation *argI2002* resulted in a more extreme arginine requirement and accentuated suppression by *pyrB* mutations. Suppression by the *pyrC* and *pyrD* mutations was reduced as a result of the incorporation of the leaky *argI2002* allele. These results indicate that in *pyrH700* strains carbamoyl phosphate is preferentially directed toward the formation of intermediates in the pyrimidine biosynthetic pathway. Arginine auxotrophy results from the reduced availability of carbamoyl phosphate for the biosynthesis of arginine. Suppression of this arginine dependence for growth is used as a convenient positive selection technique for *pyrB* mutations.

Although extensive studies (13, 18, 32) have been conducted on the structure, conformational changes, and ligand interactions of aspartate transcarbamoylase (ATCase) from *Escherichia coli*, there have been relatively few investigations aimed at the systematic isolation and biochemical characterization of mutant forms of this regulatory enzyme. Mutant strains of *Salmonella typhimurium* and *E. coli* lacking ATCase activity have been isolated (7, 40), and pyrimidine-overproducing mutants have been described (26). However, no general positive selection technique is available for mutants deficient in ATCase activity. This paper describes such a technique, which is based on novel features of pyrimidine and arginine metabolism whereby the flow of carbamoyl phosphate, a common intermediate in pyrimidine and arginine biosyntheses, is altered in certain strains so as to cause arginine auxotrophy.

In *S. typhimurium* and *E. coli* the carbamoyl phosphate formed as a result of the catalytic activity of carbamoyl phosphate synthetase (CPSase) is utilized both by ATCase as a substrate for pyrimidine biosynthesis and by ornithine transcarbamoylase (OTCase) as a substrate for the biosynthesis of arginine. Because of the dual requirement of carbamoyl phosphate for the formation of both pyrimidines and arginine, CPSase has apparently acquired unusual properties; its activity and synthesis are modulated by intermediates of both pathways (Fig. 1). The structural genes for the two subunits of CPSase are designated *pyrA* in *S. typhimurium* and *carAB* in *E. coli*. In both genera expression of the structural genes for CPSase is repressed cumulatively by arginine and pyrimidine nucleotides (1, 29); in addition, CPSase is subject to feedback inhibition by UMP and to activation by ornithine (3, 5, 28). The structural gene for OTCase, *argI*, is also subject to repression by arginine (21), but the enzyme is apparently not sensitive to feedback inhibition (4, 22). Expression of the structural gene for ATCase, *pyrB*, is repressed by UDP or UTP (33, 38), and the enzyme is inhibited by CTP, the endproduct in the pyrimidine biosynthetic pathway (14, 27).

High levels of ATCase synthesis have been found in *S. typhimurium* (17, 19) and *E. coli* (29) strains carrying certain alleles of the UMP kinase structural gene, *pyrH*. Because of the partially defective kinase in these strains, the levels of UDP and UTP are reduced, with a consequent derepression of *pyrB*. Moreover, since exogenous uracil enters the pyrimidine pathway before the reaction catalyzed by UMP kinase, ATCase synthesis is not repressed when uracil is added. In these strains allosteric effects may also be operative. At elevated levels of UMP, there may be inhibition of CPSase, and at lowered CTP levels ATCase would not be inhibited. As a consequence of all of these factors, the bulk of the carbamoyl phosphate in *pyrH700* strains may be utilized by ATCase. Hence, as shown

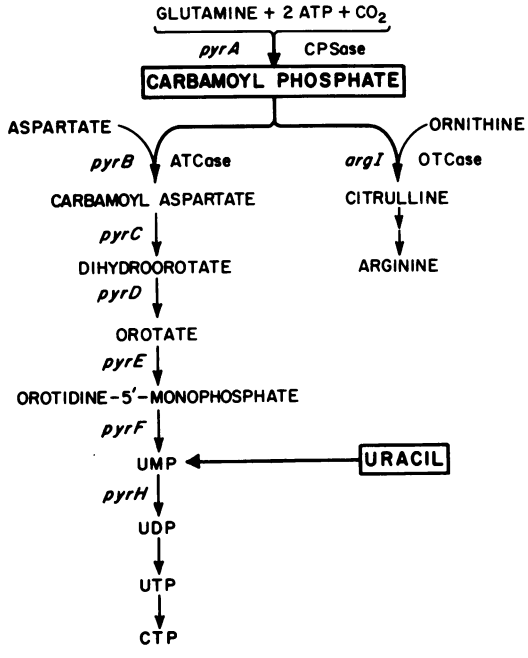

FIG. 1. *Biosynthetic pathways of arginine and pyrimidines. Genetic symbols for the enzymes are shown in italics. The genes and enzymes relevant to the discussion are shown. Only the pertinent reactants and products of individual reactions are presented.*

below, the amount of carbamoyl phosphate for the OTCase reaction is decreased, leading to arginine auxotrophy. In addition, mutations in *pyrB* can efficiently suppress this requirement for arginine, thereby providing a powerful positive selection technique for mutants deficient in ATCase activity.

(The results presented in this paper were taken from a Ph.D. thesis presented by D.D.J. to the University of California, Berkeley.)

MATERIALS AND METHODS

Strains. Strains used in this investigation are derivatives of *S. typhimurium* LT2, with the exception of strain TR3200, which is a derivative of LT7 (Table 1). The *pyrH700* mutation (formerly HD58) was isolated by O'Donovan and Gerhart as a pyrimidine excreter (26). Mutation *pyrB655* is a large deletion isolated by Syvanen and Roth (36). Strains carrying Tn*10* insertions were kindly provided by J. Roth and C. Beck.

The F393 *argF lac proAB* (P22 *pyrB*) episome was derived by specialized transduction into F128; the donor was the cointegration product of *E. coli* K-12 F128 *argF lac proAB* and F117 *argI pyrB*, as described by Kaye et al. (20). F393 strains do not produce progeny phage, are sensitive to P22 infection, and are unable to complement 15 P22 amber mutations (20). Both the specialized transducing phage (20) and the recipient F128 (16) complement *argI* mutations; however, no complementation was observed using F393 which had been transduced to tetracycline resistance by phage grown on an *argF282*::Tn*10* donor (C. Beck, personal communication). Thus, F393 does not appear to carry the entire P22 *pyrB arg* genome.

TABLE 1. *S. typhimurium strains used in this investigation*

Strain	Genotype
TR3200[a]	*amtA1 proAB47 pyrB655 trp-130*
TR3505[a]	Wild-type strain LT2
TR4574[a]	*fol-101 leuD798 pyrB655 pyrH700*
TR4639[a]	*fol-101 leuD798 pyrH700*
TR4640[a]	*fol-101 leuD798 pyrB655 pyrH700*/F393 *lac*$^+$ (P22 *pyrB*$^+$)
TR4701[a]	*fol-101 leuD798 pyrH700*/F393 *lac*$^+$ (P22 *pyrB*$^+$)
HS2002	*argI2001 fol-101 leuD798 pyrB655 pyrH700*
HS2044	*argI2002 fol-101 leuD798 pyrB655 pyrH700*
HS2045	*argI2002 fol-101 leuD798 pyrB655 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)
HS2060	*argI2002 fol-101 pyrB655 pyrD2266*::Tn*10 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)
HS2109	*argI2002 fol-101 pro-1646 pyrB655 pyrH700*/F393 *argF280 lac*$^+$ *pro*$^+$ (P22 *pyrB*$^+$)
HS2200	*argI2002 fol-101 leuD798 pyrB655 pyrC691*::Tn*10 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)
HS2201	*argI2002 fol-101 leuD798 pyrB655 pyrF696*::Tn*10 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)
HS2202	*fol-101 leuD798 pyrB655 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)
HS2203	*fol-101 leuD798 pyrB655*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)
HS2204	*fol-101 leuD798 pyrB655 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB707*)
HS2205	*argI2002 fol-101 leuD798 pyrB655 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB707*)
HS2217	*fol-101 leuD798 pyrB655 pyrC691*::Tn*10 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)
HS2218	*fol-101 leuD798 pyrB655 pyrD2266*::Tn*10 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)
HS2219	*fol-101 leuD798 pyrB655 pyrF696*::Tn*10 pyrH700*/F393 *argF280 lac*$^+$ (P22 *pyrB*$^+$)

[a] Kindly provided by J. Roth.

Media. The minimal glucose medium used was the E medium of Vogel and Bonner (37) containing 0.25% (wt/vol) glucose for the liquid medium and 2% (wt/vol) glucose for the solid medium (2% agar). The citrate-free E medium of Berkowitz et al. (8) containing 0.20% (wt/vol) lactose was used for the minimal lactose medium. Unless otherwise indicated, minimal media were supplemented with auxotropic require-

ments at 100 μg/ml for amino acids and 20 μg/ml for uracil. Difco nutrient broth (NB) containing 0.5% (wt/vol) NaCl was used as a rich medium.

Transductional crosses. The non-lysogenizing transducing phage P22 HT105/int-201 constructed by J. Roth was used for all transductional crosses. Phage lysates containing approximately 10^{10} plaque-forming units per ml were prepared from bacterial NB cultures. Crosses were preformed by spreading 0.1 ml of this lysate with 0.1 ml of a recipient NB culture (1×10^8 to 2×10^8 cells) on selective plates. Transductants were picked after 24 to 48 h at 37°C. Nonlysogenic phage-free isolates were purified by streaking for single colony isolates on green indicator medium (10) and testing for P22 sensitivity. Tetracycline-resistant transductants carrying a Tn*10* insertion were selected on NB plates containing 15 μg of tetracycline per ml.

Episome transfer. F′ episomes were transferred by spot tests on selective plates.

Mutagenesis. Bacterial cultures were mutagenized with diethyl sulfate and ICR-191, as described by Roth (31). The isolation of independent mutations was made highly probable by spreading HS2109 on selective plates immediately after diethyl sulfate treatment. A penicillin selection (31) was used for the isolation of mutation *argI2001* and the ICR-101-induced mutation *pro-1646*. Mutation *argF280* was selected as a spontaneous tetracycline-sensitive excision product of *argF282*::Tn*10*. Mutation *pyrB707* was produced spontaneously in strain HS2045 as described below.

Reversion test. Mutants were grown overnight in NB, and 0.1 ml of culture was spread on minimal glucose plates supplemented with only the amino acid requirements and 1% (vol/vol) NB. Pyr$^+$ revertants were scored after 72 h at 37°C.

Cell extracts. Cultures in late exponential phase (absorbance at 650 nm, 0.7) were harvested by centrifugation and rinsed with 40 mM potassium phosphate buffer, pH 7.0. Cells were resuspended in buffer and disrupted by sonic oscillation. Cell debris was removed by centrifugation for 30 min at 40,000 × *g*.

RESULTS

Expression of *pyrB* alleles in *pyrH700* strains. As Table 2 shows, *pyrH700* strains carrying an *S. typhimurium* or *E. coli pyrB*$^+$ allele produced considerably higher levels of ATCase activity than did the wild-type strain, LT2. Synthesis of ATCase in strain TR4639 was elevated 27-fold over the wild-type level, which is in agreement with the observation of O'Donovan and Gerhart (26). In strain TR4640 the *S. typhimurium pyrB*$^+$ allele was removed by the *pyrB655* deletion and complemented by an *E. coli* episomal *pyrB*$^+$ allele. Activity in this strain was 65-fold greater than the wild-type level. The 2.4-fold enhancement of activity in strain TR4640 over that of TR4639 was presumably due to the higher gene dosage commonly found for episomal genes (34). The ATCase activity in the merodiploid strain TR4701 was approximately the sum of the activities exhibited by strains TR4639 and TR4640.

TABLE 2. *ATCase activity in pyrH700 strains*

Strain	Relevant genotype[a]	Sp act[b]
TR3505	Wild type	7.28
TR4639	*pyrH700*	199.0
TR4640	*pyrH700 pyrB655*/F (P22 *pyrB*$^+$)	471.0
TR4701	*pyrH700*/F (P22 *pyrB*$^+$)	783.0

[a] TR4639, TR4640, and TR4701 also carry *fol-101 leuD798*.

[b] Specific activities are expressed as micromoles of carbamoyl aspartate produced per hour per milligram of total crude protein at 30°C in 40 mM potassium phosphate, pH 7.0. Measurements were made by the method of Davies et al. (11), using 4 mM [^{14}C]carbamoyl phosphate and 20 mM aspartate. Protein concentration was determined by the method of Lowry et al. (25), using bovine serum albumin as a standard. Cultures were grown in minimal glucose medium supplemented with 100 μg of leucine per ml (no uracil).

In all subsequent experiments *pyrB* alleles were carried on F393 *argF280 lac proAB* (P22 *pyrB*). This took advantage of the enhanced expression of the episomal *pyrB*$^+$ allele and provided a Lac$^+$ selection for transfer of pyrB independent of Pyr and Arg phenotypes.

Arginine-dependent growth of *pyrH700* strains. All *pyrH700* Pyr$^+$ strains used in this study grow significantly faster on minimal plates when the medium is supplemented with arginine. Similar results were observed in liquid minimal medium. The culture of strain HS2202 containing arginine grew with a generation time of 67 min (comparable to that of the wild type), whereas the unsupplemented culture grew with a significantly longer generation time (164 min) (Fig. 2A). The *pyrH*$^+$ control, strain HS2203, grew at a wild-type rate (generation time, 53 min) and showed no arginine dependence.

When exogenous citrulline was substituted for arginine (Fig. 2B), the growth rate of strain HS2202 remained high (generation time, 69 min). In contrast, an ornithine-supplemented culture grew at a slower rate (generation time, 171 min). A nutritional requirement for citrulline which is not satisfied by ornithine would be expected in strains which are unable to utilize carbamoyl phosphate for arginine biosynthesis, as observed in mutants which lack CPSase or OTCase activity (2, 35).

Suppression of arginine-dependent growth by *pyr* mutations. If the requirement for arginine in the *pyrH700* mutants is attributable to the preferential flow of carbamoyl phosphate into the pyrimidine pathway, then any block in the pyrimidine pathway that interferes with this flow should suppress the arginine requirement.

Strains isogenic to HS2202 containing an additional mutation in *pyrB* (HS2204), *pyrC*

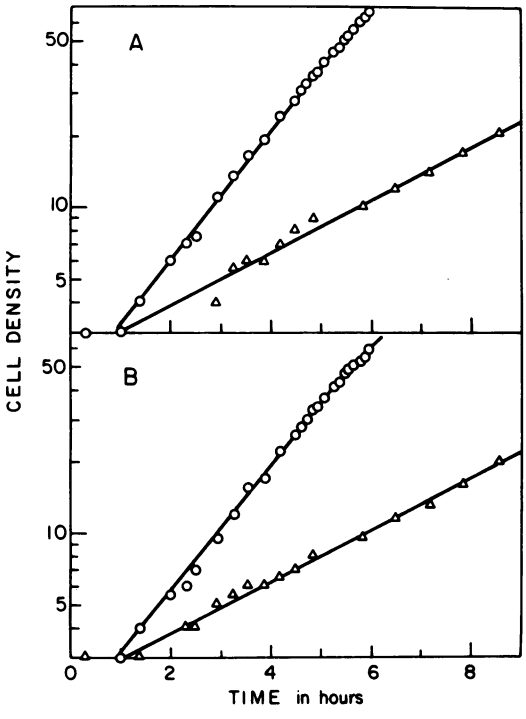

FIG. 2. *Growth of strain HS2202 [fol-101 leuD798 pyrB655 pyrH700/F393 argF280 lac⁺ (P22 pyrB⁺)] on lactose minimal medium supplemented with 20 μg of uracil per ml and 100 μg of leucine per ml. All cultures were simultaneously inoculated 1:100 with an overnight culture grown in lactose minimal medium containing 20 μg of uracil per ml, 100 μg of leucine per ml, and limiting arginine (10 μg/ml). Cell growth was measured in a Klett-Summerson photoelectric colorimeter with a no. 66 filter, and the reading on the instrument was taken as an indication of cell density. (A) Effect of arginine. Symbols: ○, culture supplemented with 100 μg of arginine per ml; △, control containing no arginine. (B) Effects of citrulline and ornithine. Symbols: ○, culture supplemented with 100 μg of citrulline per ml; △, culture supplemented with 100 μg of ornithine per ml.*

(HS2217), *pyrD* (HS2218), or *pyrF* (HS2219) were constructed and tested for suppression of the arginine requirement in the presence of uracil. Growth kinetics in the presence and absence of exogenous arginine are summarized in Table 3. The *pyrB707* strain grew at a high rate and showed no arginine dependence. The *pyrC691*::Tn*10* and *pyrD2266*::Tn*10* strains also grew at high rates in the presence of arginine and exhibited slightly reduced growth rates (after an initial lag period of about 30 min) in the absence of arginine. In contrast, a striking arginine requirement was seen for the *pyrF696*::Tn*10* strain. Thus, mutations in *pyrB*, *pyrC*, and *pyrD* but not in *pyrF* appear to reduce the flow of carbamoyl phosphate into the pyrimidine pathway.

TABLE 3. *Suppression of the pyrH700 arginine requirement by other pyr mutations*

Strain	*pyr* mutation	Generation time (min)[a]		Ratio[b]
		+Arginine	−Arginine	
HS2202	None	67	164	2.45
HS2204	*pyrB707*	57	57	1.00
HS2217	*pyrC691*::Tn*10*	62	72	1.16
HS2218	*pyrD2266*::Tn*10*	57	63	1.11
HS2219	*pyrF696*::Tn*10*	57	160	2.80

[a] Liquid minimal lactose cultures were grown as described in the legend to Fig. 2 in the presence and absence of arginine. Uracil was present at 20 μg/ml.

[b] The arginine requirement is expressed as the ratio of the generation time in the absence of arginine to the generation time in the presence of arginine.

Effect of a leaky *argI* mutation on the utilization of carbamoyl phosphate. Since overproduction of ATCase apparently caused a decreased availability of carbamoyl phosphate for the reaction catalyzed by OTCase, perhaps an *argI* mutation which is leaky in a *pyrB* background would also lead to a reduced ability of OTCase to compete for carbamoyl phosphate in a *pyr*⁺ background.

A completely blocked Arg⁻ isolate (HS2002) was derived from strain TR4574 by using diethylsulfate mutagenesis. This isolate grew in the presence of citrulline but not ornithine and was complemented by F393 *argF*⁺ but not by F393 *argF282*::Tn*10*. This mutation *argI2001* gave a leaky revertant *argI2002* (HS2044), whose leaky phenotype cotransduced with tetracycline resistance in mutation *argI1833*::Tn*10*. The F393 *argF280* (P22 *pyrB*⁺) episome was then introduced to yield strain HS2045, which carries the *argI2002* allele but is otherwise isogenic to HS2202.

When strain HS2045 was plated on minimal plates without arginine, pinpoint colonies were observed after 48 h. Insertion of mutation *pyrB707* (strain HS2205) resulted in a strain which formed large colonies. In contrast, the introduction of *pyrC691*::Tn*10* (HS2200), *pyrD2266*::Tn*10* (HS2060), and *pyrF696*::Tn*10* (HS2201) yielded strains which still produced pinpoint colonies. Clearly, the arginine requirement for growth of strains carrying mutation *argI2002* is greater in a *pyrH700 pyrB*⁺ background (as seen by the comparison between strains HS2045 and HS2202) than in a *pyrH700 pyrB707* background (HS2205 versus HS2204). Thus, the suppression of the arginine phenotype of mutation *pyrH700* by the *pyrB707* mutation

is more pronounced in the presence of the *argI2002* allele. The failure of mutations *pyrC691*::Tn*10* and *pyrD2266*::Tn*10* to suppress the requirement for arginine indicates that in strains containing the *argI2002* allele the preferential flow of carbamoyl phosphate into the pyrimidine pathway is not as dependent on the reactions catalyzed by the enzymes coded by *pyrC* and *pyrD*.

Selection of *pyrB* mutations as suppressors of the *pyrH700* arginine phenotype. Arg^+ revertants can be readily selected from *argI2002 pyrH700*/F393 *lac*$^+$ (P22 *pyrB*$^+$) strains. They appear spontaneously at a frequency of 10^{-5} (in terms of the number of cells plated [Table 4]), and this frequency is significantly increased by ICR-191 and diethyl sulfate mutagenesis (Table 4).

These revertants were screened for the presence of *pyrB* mutations by first testing for a uracil requirement. If a requirement was detected, the episome was transferred to a *pyrB655* strain (TR3200) (selecting for Lac^+) and tested again for a uracil requirement. Isolates which failed to complement mutation *pyrB655* in this test were assigned to the *pyrB* locus. Many of the Arg^+ isolates had only a slight requirement for uracil, indicating that the suppression phenotype of *pyrB* mutations is more sensitive than the pyrimidine auxotrophy phenotype. The frequency at which suppressors appear in *pyrB* is summarized in Table 4, which shows that many of the tested Arg^+ revertants were found to be Pyr^-. Virtually all of those revertants carried a *pyrB* mutation. As Table 4 shows, elimination of the requirement of arginine for growth provided a sensitive selection technique for detecting mutations at the *pyrB* locus.

The *pyrB* mutations identified in this study are not in general a consequence of prophage excision since all of the 15 chemically induced *pyrB* mutations tested were found to revert spontaneously and an appreciable fraction (4 of 45) of the spontaneous mutations also reverted spontaneously.

DISCUSSION

The arginine auxotrophy of the strains containing the *pyrH700* mutation can be readily interpreted in terms of a competition between ATCase and OTCase for a single carbamoyl phosphate pool. Alterations in the organism which lead either to increased consumption of carbamoyl phosphate for the reaction catalyzed by ATCase or to decreased production of carbamoyl phosphate tend to decrease its availability for arginine biosynthesis.

Mutations in the UMP kinase structural gene (*pyrH*) which lead to a partially defective kinase are correlated with high levels of ATCase activity and excretion of uracil (17, 19, 26). The low level of UMP kinase activity resulting from the *pyrH700* mutation apparently reduces the pools of endogenous pyrimidine nucleoside di- and triphosphates, which in turn derepress the structural genes for the enzymes implicated in pyrimidine biosynthesis. In addition, feedback inhibition of ATCase would be precluded because of the decreased concentration of CTP. Thus, in strains carrying the *pyrH700* allele high levels of uninhibited ATCase would be produced, and the carbamoyl phosphate would be utilized preferentially for the formation of pyrimidine intermediates, which are subsequently excreted.

In principle, the availability of carbamoyl phosphate for arginine biosynthesis may also be reduced by a decrease in the synthesis of carbamoyl phosphate. Because of the partially defective UMP kinase in *pyrH700* mutants, the

TABLE 4. *Selection of pyrB mutations*

Mutagenesis	Strain	Reversion frequency[a]	No. of colonies			% Yield of *pyrB*
			Arg^+	Pyr^-[b]	*pyrB*[c]	
Spontaneous	HS2045	1×10^{-5}	90	51	45	50
ICR-191	HS2045	4×10^{-4}	64	19	19	30
Diethyl sulfate	HS2109[d]	1×10^{-3}	54	19	17	31

[a] Cultures were mutagenized as described in the text, diluted, and spread on NB plates and on minimal lactose plates supplemented with 100 μg of leucine per ml and 20 μg of uracil per ml. Plates were scored for Arg^+ revertants after 24 to 36 h at 37°C. The spontaneous appearance of Arg^+ colonies on the selection plates was probably more frequent than the actual occurrence of reversions in the original unselected population because the cells may have divided several times after plating. However, for cells which were treated with mutagen before plating, the increased appearance of Arg^+ colonies does reflect the number of revertants in the unselected population.

[b] Number of Arg^+ colonies which were detectably Pyr^-.

[c] Number of Arg^+ Pyr^- colonies which did not complement *pyrB655*.

[d] Strain HS2109 carries the additional mutation *pro-1646*, which improves maintenance of F393 *lac proAB* (P22 *pyrB*).

concentration of UMP would be expected to increase in these mutants. Since UMP is an inhibitor of CPSase, the synthesis of carbamoyl phosphate would be reduced. Therefore, the increased utilization of carbamoyl phosphate for pyrimidine biosynthesis and the decreased formation of carbamoyl phosphate in strain HS2202 containing the *pyrH700* allele would lead to the nutritional requirement for arginine or citrulline which is not satisfied by ornithine.

A mutational block at the ATCase step would restore the availability of carbamoyl phosphate for arginine synthesis by preventing the flow of carbamoyl phosphate into the pyrimidine pathway and by reducing the production of the inhibitor UMP. An analogous mechanism based on altering the flow of carbamoyl phosphate has been proposed by Bolivar et al. (9), which accounts for the unusual nutritional requirements of an *E. coli* strain which was partially defective in CPSase. They suggested that the reduced rate of carbamoyl phosphate synthesis caused a more pronounced growth dependence for arginine than for pyrimidines because of the level of ATCase activity. This arginine requirement was suppressed by the introduction of a *pyrB* mutation. Although it is tempting to attribute the inadequate supply of carbamoyl phosphate in strain HS2202 to the inhibition of its synthesis by UMP, it seems unlikely that this inhibition of CPSase would contribute significantly to the *pyrB*-suppressible requirement for arginine. Activation of CPSase by ornithine is thought to overcome the inhibition by UMP during arginine starvation (5, 28). In addition, the failure of mutation *pyrF696*::Tn*10* to suppress arginine auxotrophy would require that orotidine 5'-monophosphate function as an inhibitor of CPSase in the same way as UMP. As yet there have been no reports of the inhibition of the CPSase of *E. coli* or *S. Typhimurium* by orotidine 5'-monophosphate. (In experiments with CPSase from *E. coli*, S.G. Powers [unpublished data] has found that orotidine 5'-monophosphate is a very poor inhibitor compared with UMP.)

Mutations which interfere with the flow of carbamoyl phosphate into the pyrimidine pathway apparently suppress the partial requirement for arginine exhibited by the strains carrying the *pyrH700* allele. Among the pyrimidine mutants which were tested, two types of suppressors of arginine auxotrophy were found: (i) mutations at the *pyrB* locus, which lead to defective ATCase and thereby block directly the entry of carbamoyl phosphate into the pyrimidine pathway; and (ii) mutations at the *pyrC* and *pyrD* loci, which interfere with successive biosynthetic reactions in the pyrimidine pathway and thereby block entry of carbamoyl phosphate indirectly. Presumably the second type of suppressor prevents the formation of intermediates like orotate which can be excreted readily (39, 41), with the result that there is an accumulation of carbamoyl aspartate, the product of the ATCase-catalyzed reaction. A *pyrD* mutation may cause accumulation of carbamoyl aspartate since the reverse direction of the *pyrC*-controlled reaction (dihydroorotate to carbamoyl aspartate) is thermodynamically favored over the forward direction (24). If high endogenous levels of carbamoyl aspartate are produced, the net flow of carbamoyl phosphate into the pyrimidine pathway may be reduced because carbamoyl aspartate either acts as a noncompetitive inhibitor of carbamoyl phosphate binding (30) or contributes toward increasing the reverse reaction catalyzed by ATCase (23).

The inability of mutation *pyrF696*::Tn*10* to suppress the arginine requirement of strain HS2202 indicates that the intermediates preceding the formation of UMP may be excreted efficiently and do not lead to an accumulation of carbamoyl aspartate. Some *E. coli* derivatives excrete large quantities of orotic acid due to a nonauxotrophic lesion at the *pyrF* locus (39). The ability of strain HS2202 to excrete this intermediate efficiently would account for the suppression of the arginine requirement by mutation *pyrD2266*::Tn*10* and the lack of suppression by mutation *pyrF696*::Tn*10*.

An increase in the sensitivity of the selection technique was achieved by increasing the relative advantage of ATCase over OTCase in the competition for carbamoyl phosphate. This was accomplished by the introduction of leaky mutation *argI2002* into strain HS2202, which caused a reduction in the utilization of carbamoyl phosphate for the OTCase-catalyzed reaction. As a consequence of this leaky mutation in the gene coding for OTCase, the suppression of arginine auxotrophy by mutation *pyrB707* was accentuated. Moreover, mutations *pyrC691*::Tn*10* and *pyrD2266*::Tn*10* failed to suppress arginine auxotrophy in this background. Apparently in these strains carbamoyl aspartate can be excreted, and its accumulation does not sufficiently reduce the ability of ATCase to utilize carbamoyl phosphate and thereby to suppress the arginine requirement significantly. An *E. coli* strain blocked at *pyrC* has been reported to excrete carbamoyl aspartate (15).

It could be argued that the slow growth of strains containing the *pyrH700* allele is caused by an accumulation of a toxic pyrimidine intermediate rather than by competition for carba-

moyl phosphate. Indeed, the accumulation of carbamoyl aspartate in yeasts (6) has been shown to be toxic. However, to account for the suppression of this phenotype by exogenous arginine in the present studies, one must postulate that (i) toxicity of the pyrimidine intermediate(s) is directed specifically at the arginine biosynthetic pathway, or (ii) the levels of the toxic intermediate(s) are modulated by the arginine level, presumably through repression of CPSase synthesis. Since the arginine requirement in strain HS2202 was suppressed by mutations *pyrC691*::Tn*10* and *pyrD2266*::Tn*10*, it appears that an accumulation of either carbamoyl aspartate or dihydroorotate does not contribute to the toxicity. Failure of mutation *pyrF696*::Tn*10* to suppress requires that the first toxic intermediate must precede the *pyrF*-controlled step. However, since orotic acid is excreted efficiently (39) and since the *pyrE*-controlled step is reversible (12), these intermediates would not be expected to accumulate to high levels. A mechanism involving the toxic accumulation of orotic acid or orotidine 5'-monophosphate cannot be eliminated rigorously; however, it appears to be unlikely.

Suppression of arginine auxotrophy by *pyrB* mutations in *pyrH700 argI2002* strains provides a convenient selection technique for mutations at this locus. Pyrimidine auxotrophs appear in high yield (in excess of 30%) and are specific for the *pyrB* locus. Since suppression requires only a modest reduction in ATCase activity, the technique is sensitive even to very leaky mutations. Large numbers of independent *pyrB* isolates can be obtained from a single preparation when mutations are induced in the absence of growth. These isolates can then be screened for a variety of interesting phenotypes. Detailed characterizations of such isolates in terms of genetic fine structure and biochemical properties of the mutant ATCase are now in progress.

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LITERATURE CITED

1. **Abd-El-Al, A., and J. L. Ingraham.** 1969. Control of carbamyl phosphate synthesis in *Salmonella typhimurium*. J. Biol. Chem. **244**:4033–4038.
2. **Abd-El-Al, A., and J. L. Ingraham.** 1969. Cold sensitivity and other phenotypes resulting from mutation in *pyrA* gene. J. Biol. Chem. **244**:4039–4045.
3. **Abdelal, A. T. H., and J. L. Ingraham.** 1975. Carbamylphosphate synthetase from *Salmonella typhimurium*. Regulation, subunit composition, and function of the subunits. J. Biol. Chem. **250**:4410–4417.
4. **Abdelal, A. T. H., E. H. Kennedy, and O. Nainan.** 1977. Ornithine transcarbamylase from *Salmonella typhimurium*: purification, subunit composition, kinetic analysis, and immunological cross-reactivity. J. Bacteriol. **129**:1387–1396.
5. **Anderson, P. M., and A. Meister.** 1966. Control of *Escherichia coli* carbamyl phosphate synthetase by purine and pyrimidine nucleotides. Biochemistry **5**: 3164–3169.
6. **Bach, M.-L., and F. Lacroute.** 1972. Direct selective techniques for the isolation of pyrimidine auxotrophs in yeast. Mol. Gen. Genet. **115**:126–130.
7. **Beckwith, J. R., A. B. Pardee, R. Austrian, and F. Jacob.** 1962. Coordination of the synthesis of the enzymes in the pyrimidine pathway of *E. coli*. J. Mol. Biol. **5**:618–634.
8. **Berkowitz, D., J. M. Hushon, H. J. Whitfield, Jr., J. Roth, and B. N. Ames.** 1968. Procedure for identifying nonsense mutations. J. Bacteriol. **96**:215–220.
9. **Bolivar, F., M. Galván, and J. Martuscelli.** 1976. Biochemical and genetic characterization of a carbamyl phosphate synthetase mutant of *Escherichia coli* K12. J. Gen. Microbiol. **94**:142–148.
10. **Bresch, C.** 1953. Genetical studies on bacteriophage T_1. Ann. Inst. Pasteur Paris **84**:157–163.
11. **Davies, G. E., T. C. Vanaman, and G. R. Stark.** 1970. Aspartate transcarbamylase. Stereospecific restrictions on the binding site for L-aspartate. J. Biol. Chem. **245**: 1175–1179.
12. **Flaks, J. G.** 1963. Nucleotide synthesis from 5-phosphoribosylpyrophosphate. III. Orotidine 5'-phosphate pyrophosphorylase. Methods Enzymol. **6**:148–152.
13. **Gerhart, J. C.** 1970. A discussion of the regulatory properties of aspartate transcarbamylase from *Escherichia coli*. Curr. Top. Cell. Regul. **2**:275–325.
14. **Gerhart, J. C., and A. B. Pardee.** 1962. The enzymology of control by feedback inhibition. J. Biol. Chem. **237**: 891–896.
15. **Gerhart, J. C., and A. B. Pardee.** 1964. Aspartate transcarbamylase, an enzyme designed for feedback inhibition. Fed. Proc. **23**:727–735.
16. **Hoppe, I., and J. Roth.** 1974. Specialized transducing phages derived from *Salmonella* phage P22. Genetics **76**:633–654.
17. **Ingraham, J. L., and J. Neuhard.** 1972. Cold-sensitive mutants of *Salmonella typhimurium* defective in uridine monophosphate kinase (*pyrH*). J. Biol. Chem. **247**:6259–6265.
18. **Jacobson, G. R., and G. R. Stark.** 1973. Aspartate transcarbamylases, p. 225–308. *In* P. D. Boyer (ed.), The enzymes, vol. 9, 3rd ed. Academic Press Inc., New York.
19. **Justesen, J., and J. Neuhard.** 1975. *pyrR* identical to *pyrH* in *Salmonella typhimurium*: control of expression of the *pyr* genes. J. Bacteriol. **123**:851–854.
20. **Kaye, R., J. Barravecchio, and J. Roth.** 1974. Isolation of P22 specialized transducing phage following F'-episome fusion. Genetics **76**:655–667.
21. **Kelln, R. A., and G. A. O'Donovan.** 1976. Isolation and partial characterization of an *argR* mutant of *Salmonella typhimurium*. J. Bacteriol. **128**:528–535.
22. **Legrain, C., and V. Stalon.** 1976. Ornithine carbamoyltransferase from *Escherichia coli* W. Purification, structure and steady-state kinetic analysis. Eur. J. Biochem. **63**:289–301.
23. **Legrain, C., V. Stalon, N. Glansdorff, D. Gigot, A. Piérard, and M. Crabeel.** 1976. Structural and regulatory mutations allowing utilization of citrulline or carbamoylaspartate as a source of carbamoylphosphate in *Escherichia coli* K-12. J. Bacteriol. **128**:39–48.

24. **Lieberman, I., and A. Kornberg.** 1954. Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. II. Dihydroorotic acid, ureidosuccinic acid, and 5-carboxymethylhydantoin. J. Biol. Chem. **207**:911-924.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
26. **O'Donovan, G. A., and J. C. Gerhart.** 1972. Isolation and partial characterization of regulatory mutants of the pyrimidine pathway in *Salmonella typhimurium*. J. Bacteriol. **109**:1085-1096.
27. **O'Donovan, G. A., H. Holoubek, and J. C. Gerhart.** 1972. Regulatory properties of intergeneric hybrids of aspartate transcarbamylase. Nature (London) New Biol. **238**:264-266.
28. **Piérard, A.** 1966. Control of the activity of *Escherichia coli* carbamoyl phosphate synthetase by antagonistic allosteric effectors. Science **154**:1572-1573.
29. **Piérard, A., N. Glandsdorff, D. Gigot, M. Crabeel, P. Halleux, and L. Thiry.** 1976. Repression with *Escherichia coli* carbamoyl phosphate synthase: relationships with enzyme synthesis in the arginine and pyrimidine pathways. J. Bacteriol. **127**:291-301.
30. **Porter, R. W., M. O. Modebe, and G. R. Stark.** 1969. Aspartate transcarbamylase. Kinetic studies of the catalytic subunit. J. Biol. Chem. **244**:1846-1859.
31. **Roth, J. R.** 1970. Genetic techniques in studies of bacterial metabolism. Methods Enzymol. **17A**:3-35.
32. **Schachman, H. K.** 1974. Anatomy and physiology of a regulatory enzyme—aspartate transcarbamylase. Harvey Lect. **68**:67-113.
33. **Schwartz, M., and J. Neuhard.** 1975. Control of expression of the *pyr* genes in *Salmonella typhimurium*: effects of variations in uridine and cytidine nucleotide pools. J. Bacteriol. **121**:814-822.
34. **Stetson, H., and R. L. Somerville.** 1971. Expression of the tryptophan operon in merodiploids of *Escherichia coli*. I. Gene dosage, gene position and marker effects. Mol. Gen. Genet. **111**:342-351.
35. **Syvanen, J. M., and J. R. Roth.** 1972. Structural genes for ornithine transcarbamylase in *Salmonella typhimurium* and *Escherichia coli* K-12. J. Bacteriol. **110**: 66-70.
36. **Syvanen, J. M., and J. R. Roth.** 1973. Structural genes for catalytic and regulatory subunits of aspartate transcarbamylase. J. Mol. Biol. **76**:363-378.
37. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97-106.
38. **Williams, J. C., and G. A. O'Donovan.** 1973. Repression of enzyme synthesis of the pyrimidine pathway in *Salmonella typhimurium*. J. Bacteriol. **115**:1071-1076.
39. **Womack, J. E., and G. A. O'Donovan.** 1978. Orotic acid excretion in some wild-type strains of *Escherichia coli* K-12. J. Bacteriol. **136**:825-827.
40. **Yan, Y., and M. Demerec.** 1965. Genetic analysis of pyrimidine mutants of *Salmonella typhimurium*. Genetics **52**:643-651.
41. **Yates, R. A., and A. B. Pardee.** 1956. Pyrimidine biosynthesis in *Escherichia coli*. J. Biol. Chem. **221**:743-756.