JOURNAL OF BACTERIOLOGY, Aug. 1985, p. 500–505
0021-9193/85/080500-06$02.00/0
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Vol. 163, No. 2

Toxicity of the Pyrimidine Biosynthetic Pathway Intermediate Carbamyl Aspartate in *Salmonella typhimurium*

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Received 11 December 1984/Accepted 6 May 1985

Growth of *Salmonella typhimurium pyrC* or *pyrD* auxotrophs was severely inhibited in media that caused derepressed *pyr* gene expression. No such inhibition was observed with derepressed *pyrA* or *pyrB* auxotrophs. Growth inhibition was not due to the depletion of essential pyrimidine biosynthetic pathway intermediates or substrates. This result and the pattern of inhibition indicated that the accumulation of the pyrimidine biosynthetic pathway intermediate carbamyl aspartate was toxic. This intermediate is synthesized by the sequential action of the first two enzymes of the pathway encoded by *pyrA* and *pyrB* and is a substrate for the *pyrC* gene product. It should accumulate to high levels in *pyrC* or *pyrD* mutants when expression of the *pyrA* and *pyrB* genes is elevated. The introduction of either a *pyrA* or *pyrB* mutation into a *pyrC* strain eliminated the observed growth inhibition. Additionally, a direct correlation was shown between the severity of growth inhibition of a *pyrC* auxotroph and the levels of the enzymes that synthesize carbamyl aspartate. The mechanism of carbamyl aspartate toxicity was not identified, but many potential sites of growth inhibition were excluded. Carbamyl aspartate toxicity was shown to be useful as a phenotypic trait for classifying pyrimidine auxotrophs and may also be useful for positive selection of *pyrA* or *pyrB* mutants. Finally, we discuss ways of overcoming growth inhibition of *pyrC* and *pyrD* mutants under derepressing conditions.

In *Salmonella typhimurium* and *Escherichia coli*, de novo synthesis of UMP (Fig. 1), the precursor of all pyrimidine nucleotides, is catalyzed by six enzymes encoded by six unlinked genes and operons (21). This pathway is regulated at the level of both enzyme activity and gene expression. The first enzyme of the pathway, carbamyl phosphate synthetase (encoded by *pyrA*), is essential for both pyrimidine and arginine biosynthesis (Fig. 1). Its activity is subject to feedback inhibition by UMP and activation by ornithine, IMP, and phosphoribosylpyrophosphate (2, 4, 23). Aspartate transcarbamylase (encoded by *pyrB*), the first enzyme uniquely committed to pyrimidine nucleotide biosynthesis, is sensitive to feedback inhibition by CTP and to activation by ATP (8, 20). Regulation at the level of gene expression is noncoordinate and complex. The expression of *pyrA* (designated *carAB* in *E. coli*) is regulated by cumulative repression requiring a pyrimidine nucleotide and arginine (1, 24, 26). Repression by arginine is mediated by the arginine repressor protein encoded by *argR* (12, 14, 25). The expression of *pyrB* (designated *pyrBI* in *E. coli*), *pyrE*, and *pyrF* appears to be repressed by a uridine nucleotide, whereas *pyrC* and *pyrD* expression appears to be repressed primarily by a cytidine nucleotide (24, 30). Recent studies in *E. coli* indicate that *pyrBI* (16, 29, 33, 34) and *pyrE* (27, 28) expression is regulated by an attenuation control mechanism that is sensitive to the relative rates of UTP-dependent transcription within a leader region preceding the *pyr* structural gene(s) and coupled translation of the leader transcript. Expression of *pyrE* and *pyrF*, but not that of other *pyr* genes, apparently requires a *trans*-acting regulatory factor encoded by a putative regulatory gene designated *pyrS* in *E. coli* (19). The mechanism by which this putative regulatory factor affects gene expression is not known. At present, there is nothing known about the regulatory mechanisms controlling *pyrC* and *pyrD* expression.

In our continuing studies of the mechanisms controlling *pyr* gene expression in *S. typhimurium*, we discovered that the growth of certain pyrimidine auxotrophs, namely *pyrC* and *pyrD* mutants, was inhibited in media that caused pyrimidine limitation and derepressed *pyr* gene expression. In this report we present experiments that show that this growth inhibition is due to the accumulation of high intracellular levels of the pyrimidine biosynthetic pathway intermediate carbamyl aspartate (also called ureidosuccinate) (Fig. 1). Although the site of growth inhibition was not identified, potential uses and effects of carbamyl aspartate toxicity in the study of *pyr* gene expression are described.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are derivatives of *S. typhimurium* LT2. They are listed with their genotypes in Table 1. Construction of strains by phage P22-mediated transduction was as previously described (6).

Media and culture methods. The MOPS (morpholinepropanesulfonic acid) minimal medium described by Neidhardt et al. (17) was used as the liquid medium. To grow cells, 5 or 10 ml of MOPS medium supplemented with 0.04% glucose, 5 μg of uracil per ml, and 0.5 mM arginine (*pyrA* strains only) was inoculated with the desired strain and incubated overnight at 37°C with shaking (250 rpm). A portion of this overnight culture was diluted 1:50 into MOPS medium containing 0.4% glucose with a pyrimidine source and other supplements as indicated in the text. This culture was incubated at 37°C with shaking. Growth was followed by measuring the increase in optical density at 650 nm. The medium used for plates contained phosphate-buffered N^-C^- medium (3), 10 mM NH_4Cl, 1.5% Difco agar, and other supplements as indicated.

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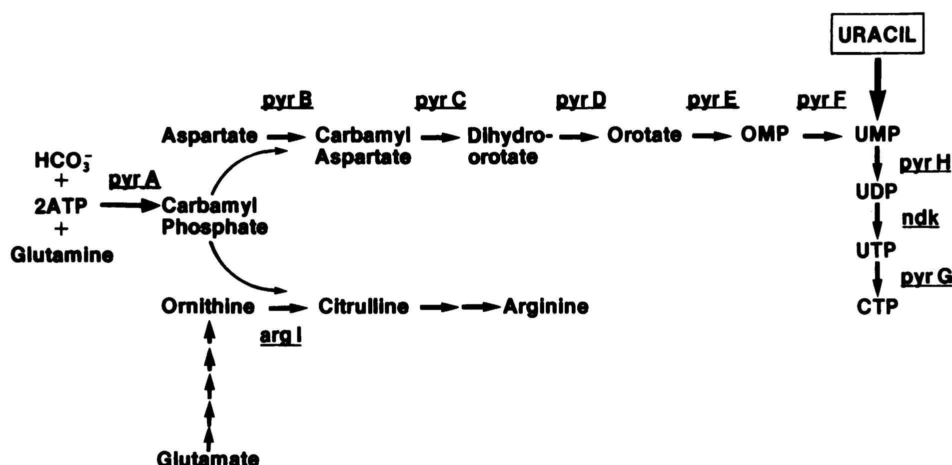

FIG. 1. Pyrimidine and arginine biosynthetic pathways of *S. typhimurium*. Gene designations and encoded enzymes are: *pyrA*, carbamyl phosphate synthetase (EC 6.3.5.5); *pyrB*, aspartate transcarbamylase (EC 2.1.3.2); *pyrC*, dihydro-orotase (EC 3.5.2.3); *pyrD*, dihydroorotate oxidase (EC 1.3.3.1); *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); *ndk*, nucleosidediphosphate kinase (EC 2.7.4.6); and *argI*, ornithine transcarbamylase (EC 2.1.3.3).

Aspartate transcarbamylase assay. Samples were withdrawn from cultures at indicated times, and cell extracts were prepared as previously described (6), except that 50 mM potassium phosphate (pH 7.0) was used as extraction buffer. Enzymatic activity was measured as previously described (6).

Protein determination. Protein samples were assayed by the method of Lowry et al. (15) with crystalline bovine serum albumin as the standard.

RESULTS

Selective growth inhibition of *pyrC* and *pyrD* auxotrophs under pyrimidine-limiting conditions. The pyrimidine auxotrophic strains pyrAΔ81, TT460 (*pyrB*), pyrCΔ73, and pyrDΔ121 grew similarly in MOPS minimal medium containing 0.2 mM uracil (and 0.5 mM arginine for pyrAΔ81), with doubling times of 44 ± 1 min (data not shown). This growth rate is the same as that of wild-type *S. typhimurium* because of the rapid transport of uracil into cells and conversion to UMP (35). Cells grown on uracil contain elevated levels of pyrimidine nucleotides, which cause repressed *pyr* gene expression (6, 30). The same four pyrimidine auxotrophs were grown in minimal medium with 0.5 mM orotate, which is a pyrimidine pathway intermediate (Fig. 1). Growth on 0.5 mM orotate causes pyrimidine limitation because of the inefficient transport of this intermediate into cells (7). It previously has been shown that growth of *pyrA* and *pyrB* mutants on 0.5 mM orotate causes derepressed *pyr* gene expression (6). The growth patterns of the four auxotrophs were found to be strikingly different (Fig. 2). Strains pyrAΔ81 and TT460 (*pyrB*) grew exponentially, with doubling times of 65 and 52 min, respectively, until required nutrients were depleted. Growth of strains pyrCΔ73 and pyrDΔ121 was, in contrast, dramatically inhibited after the first 2 h of incubation. The doubling times for these two strains increased to more than 600 min. Growth of strain pyrCΔ73 was slightly more inhibited than that of pyrDΔ121.

The first 2 h of growth shown in Fig. 2 apparently represented a transition from growth on uracil, the pyrimidine source in the cultures used as inocula, to growth on orotate. The amount of growth supported by uracil carried over with each inoculum was measured by growing strain pyrEΔ125, which cannot use orotate as a pyrimidine source (Fig. 1), under the same conditions used to grow the other four auxotrophs. The amount of uracil-supported growth was small (Fig. 2).

The results (Fig. 2) were not dependent on using MOPS minimal medium. Similar growth patterns were obtained when MOPS medium was replaced by the phosphate-buffered N^-C^- medium containing 10 mM NH_4Cl (data not shown). The only significant difference in using the two media was that orotate-grown cells tended to form clumps in N^-C^- medium but not in MOPS medium.

Evidence for carbamyl aspartate toxicity. The results (Fig. 2) indicate that either the synthesis or accumulation of high levels of carbamyl aspartate is responsible for inhibiting the growth of *pyrC* and *pyrD* strains. This was confirmed by constructing *pyrC* mutants that contain an additional mutation in either *pyrA* or *pyrB*, which will prevent carbamyl aspartate synthesis. Strains JL3507 (*pyrA pyrC*) and JL3508 (*pyrB pyrC*) grew without inhibition on 0.5 mM orotate; each culture grew with a doubling time of 52 min (Fig. 3). The presence of a Tn*10* element in these two strains did not contribute to the suppression of growth inhibition because

TABLE 1. Bacterial strains

Strain	Genotype	Source and comments
LT2-Z	Wild type	B. Ames
pyrAΔ81	Δ*pyrA81*	B. Ames
TT460	*pyrB692*::Tn*10*	J. Roth
pyrCΔ73	Δ*pyrC73*	B. Ames
pyrDΔ121	Δ*pyrD121*	B. Ames
pyrEΔ125	Δ*pyrE125*	B. Ames
JL3403	*zab-403*::Tn*10*	J. Ingraham; Tn*10* is 80% cotransducible with *pyrA* by P22
JL3488	*zab-403*::Tn*10*	P22(JL3403) × pyrAΔ81
JL3489	Δ*pyrA81 zab-403*::Tn*10*	P22(JL3403) × pyrAΔ81
JL3506	Δ*pyrC73 zab-403*::Tn*10*	P22(JL3488) × pyrCΔ73
JL3507	Δ*pyrA81* Δ*pyrC73 zab-403*::Tn*10*	P22(JL3489) × pyrCΔ73
JL3508	*pyrB692*::Tn*10* Δ*pyrC73*	P22(TT460) × pyrCΔ73

strain JL3506 (*pyrC*), which also contains this transposon, was inhibited exactly as strain pyrCΔ73 (Fig. 3).

The synthesis of high levels of carbamyl aspartate could inhibit growth by depleting essential pyrimidine pathway intermediates. A possibility consistent with the pattern of growth inhibition was that excessive synthesis of carbamyl aspartate depleted the carbamyl phosphate pool, which would cause arginine starvation (Fig. 1). This possibility was tested by growing strains pyrCΔ73 and pyrDΔ121 as described in the legend to Fig. 2, except that 0.5 mM arginine was included in the growth medium. Growth inhibition was identical to that shown in Fig. 2, indicating that arginine limitation was not the cause of growth inhibition. Unless carbamyl phosphate synthetase is highly sensitive to product inhibition, the pattern of growth shown in Fig. 2 indicates that energy deprivation caused by ATP depletion is not the cause of inhibition. However, we excluded this possibility unambiguously by examining the nucleotide pools in strain pyrCΔ73 grown on orotate essentially as described in Fig. 2. Nucleotide pool sizes were measured as previously described (5) in two samples collected before and 165 min after the onset of growth inhibition (data not shown). The ATP pool size was the same in both samples and was similar to that in uracil-grown cells. The only nucleoside triphosphate pool size that was substantially different in the two samples was that of UTP. As expected, the UTP pool was much (approximately 10-fold) smaller in the inhibited cells. The levels of guanosine tetraphosphate (ppGpp) also were measured in this experiment and were found to be lower after inhibition. This result indicates that cells were not starved for glutamine or aspartate, which are required for carbamyl aspartate synthesis, or for any other amino acid. These results indicate that it is not the depletion of essential metabolites by excessive carbamyl aspartate synthesis but the accumulation of toxic levels of this intermediate that causes growth inhibition.

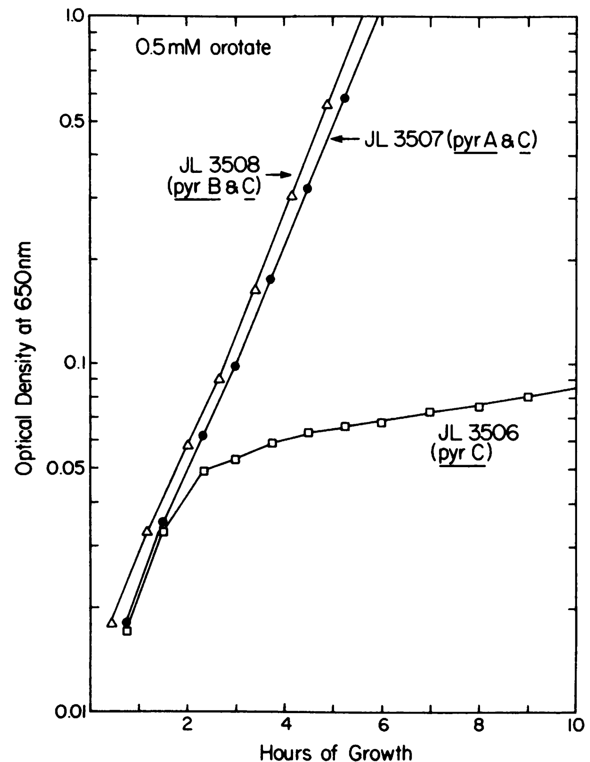

FIG. 3. Growth of strains JL3506, JL3507, and JL3508 under pyrimidine-limiting conditions. Cultures (20 ml) were grown in MOPS minimal medium supplemented with 0.4% glucose, 0.5 mM arginine (JL3507 only), and 0.5 mM orotate.

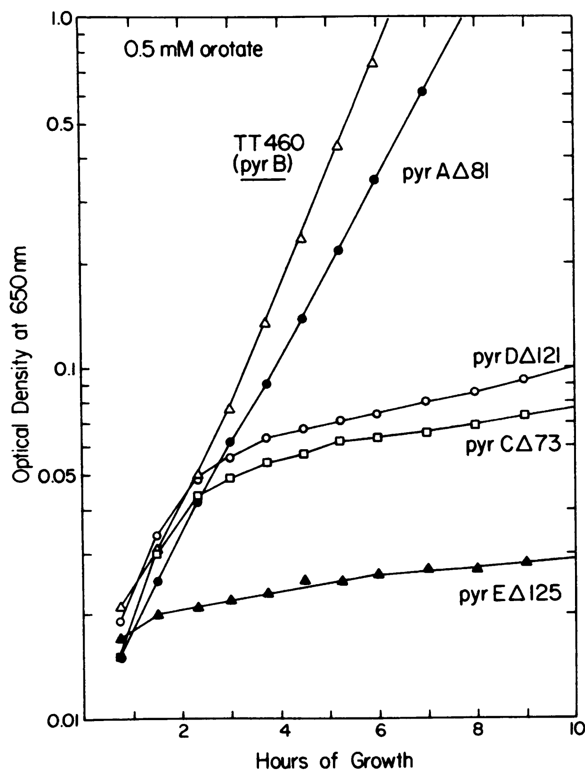

FIG. 2. Growth of *pyr* auxotrophs under pyrimidine-limiting conditions. Cultures (20 ml) of the indicated strains were grown in MOPS minimal medium supplemented with 0.4% glucose, 0.5 mM arginine (pyrAΔ81 only), and 0.5 mM orotate.

To obtain additional evidence for carbamyl aspartate toxicity, we attempted to demonstrate a correlation between growth inhibition and levels of the enzymes that synthesize carbamyl aspartate in a *pyrC* auxotroph. Strain pyrCΔ73 was grown in minimal medium supplemented with 0.5, 1.0, 2.5, or 5.0 mM orotate. Increasing the orotate concentration results in less severe pyrimidine limitation in *pyr* auxotrophs, apparently due to increased orotate transport (7). The growth inhibition observed at 0.5 mM orotate was increasingly suppressed as the orotate concentration increased (Fig. 4). At 5.0 mM orotate, growth of pyrCΔ73 was similar to that of uracil-grown cells, except for a short period of diauxie within the first hours of incubation. This break in the growth curve was presumably due to the shift from using uracil to using orotate as the sole pyrimidine source. Samples were taken from each culture in Fig. 4, and the levels of the *pyrB* gene product aspartate transcarbamylase were measured. Increasing the orotate concentration resulted in decreased levels of this enzyme (Table 2). Regulation of the synthesis of carbamyl phosphate synthetase, the *pyrA* gene product, on the orotate-supplemented medium was previously shown to be similar to that of aspartate transcarbamylase (6). These experiments establish a direct correlation between the severity of growth inhibition of a *pyrC* auxotroph and the level of the pyrimidine enzymes which catalyze the synthesis of carbamyl aspartate. In pyrimidine-limited cultures of a *pyrC* auxotroph, in which feedback

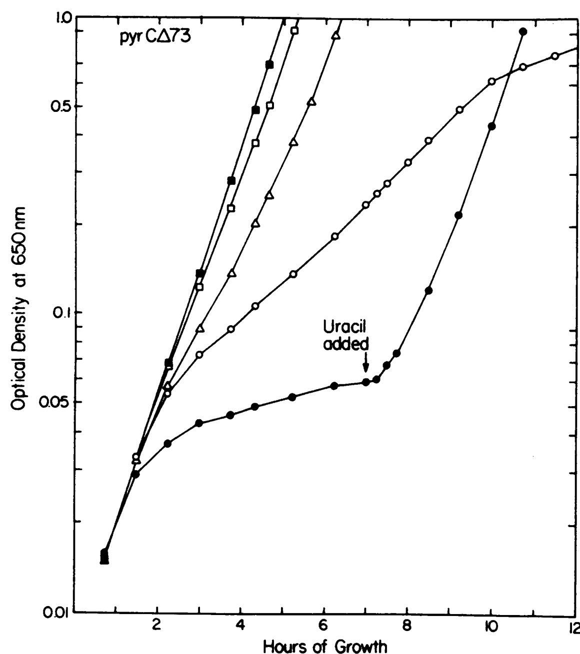

FIG. 4. Growth of strain pyrCΔ73 on uracil or various concentrations of orotate. Cultures (60 ml) were grown in MOPS minimal medium supplemented with 0.4% glucose and either 0.5 (●), 1.0 (○), 2.5 (△), or 5.0 mM (□) orotate or 0.2 mM uracil (■). At 7 h of growth, uracil (final concentration, 0.2 mM) was added to the culture containing 0.5 mM orotate.

inhibition of pyrimidine enzyme activities would be minimal, the aspartate transcarbamylase level should reflect the intracellular concentration of carbamyl aspartate.

In the experiment shown in Fig. 4, uracil was added to the 0.5 mM orotate culture of pyrCΔ73 to determine how rapidly the growth inhibition could be reversed. As can be seen, within 1 h after uracil addition the growth rate returned to that of uracil-grown cells. This result may indicate that once elevated rates of carbamyl aspartate synthesis cease, accumulated pools of this intermediate can be rapidly eliminated by the cell. We have found that the addition of dimethyl sulfoxide (1%) to the medium stimulates growth of strain pyrCΔ73 on orotate. This can be attributed to the ability of dimethyl sulfoxide to permeabilize the cell membrane and promote leakage of carbamyl aspartate from the cell.

Attempts to identify the site of carbamyl aspartate-mediated growth inhibition. Exogenously supplied carbamyl aspartate is toxic to the yeast *Saccharomyces cerevisiae*. Toxicity is alleviated in large part by purines, and it has been proposed that carbamyl aspartate may inhibit one of the first five enzymes of the purine nucleotide biosynthetic pathway (13). However, we found that none of several purines tested (50 μg of inosine, guanosine, or adenosine per ml) reverses the growth inhibition of strain pyrCΔ73 on 0.5 mM orotate. The addition of thiamine (5 μg/ml), which requires an early purine biosynthetic pathway intermediate for its synthesis (18), also had no effect, even when added in conjunction with purines. We thought that carbamyl aspartate might interfere with folate interconversions, because folate cofactors are involved in the early steps of purine biosynthesis and because carbamyl aspartate resembles the *p*-aminobenzoate/L-glutamate portion of tetrahydrofolate. However, this explanation appears incorrect because a combined supplement of

TABLE 2. Specific activities of aspartate transcarbamylase in strain pyrCΔ73 grown on uracil or various concentrations of orotate[a]

Pyrimidine source (mM)	ATCase (nmol/min per mg of protein)
Uracil (0.2)	17.7 (1.0)[b]
Orotate (5.0)	43.5 (2.5)
Orotate (2.5)	131 (7.4)
Orotate (1.0)	764 (43.2)
Orotate (0.5)	1,418 (80.1)

[a] Aspartate transcarbamylase (ATCase) activity was measured in one sample (40 ml) from each of the cultures in Fig. 4. A sample was harvested at 7 h of growth from the 0.5 mM orotate culture, at 8 h of growth from the 1.0 mM orotate culture, and at an optical density at 650 nm of 0.5 from the other cultures.

[b] Numbers within parentheses represent relative specific activities.

inosine (50 μg/ml), thiamine (5 μg/ml), and thymidine (20 μg/ml), which should spare the major folate requirements of the cell (9, 22), failed to stimulate growth of strain pyrCΔ73 on orotate. Carbamyl aspartate also resembles *N*-carbamyl-β-alanine (also called ureidopropionate), an intermediate in the conversion of uracil to pantothenate (31). However, pantothenate and β-alanine also failed to counteract carbamyl aspartate toxicity. Other vitamins tested, including biotin, pyridoxine, and nicotinamide, were similarly inactive.

Classification of pyrimidine auxotrophs by using carbamyl aspartate toxicity. Although the growth inhibition of *pyrC* and *pyrD* mutants caused by carbamyl aspartate accumulation creates some difficulty in working with these strains (see below), this property can also be useful. For example, it permits the rapid classification of uncharacterized pyrimidine auxotrophs. This has been particularly useful in screening large numbers of auxotrophs generated by Tn*10* hops and Mu *d*1 (Ap^r *lac*) insertions (6). When the protocol summarized in Table 3 is used, unknown *pyr* auxotrophs can be classified within 1 day into four groups: (i) *pyrA*, (ii) *pyrB*, (iii) *pyrC* or *pyrD*, and (iv) *pyrE* or *pyrF*. Exact identification within groups (iii) and (iv) is done by cotransductional mapping or by assaying pyrimidine enzyme activities. The use of the glycerol plate in Table 3 is not required for classification, but it is useful in confirming the identification of *pyrC* and *pyrD* mutants. Growth of these two auxotrophs on 1 mM orotate is not limited for pyrimidines when glycerol is the carbon source and no growth inhibition is observed. Apparently, transport of orotate is much more efficient in cells grown on glycerol than when glucose is the carbon source (37).

TABLE 3. Classification of pyrimidine auxotrophs by nutrient requirements and growth characteristics[a]

Strain	Glucose and uracil	Glucose, orotate, and arginine	Glycerol and orotate
pyrA	−	+	−
pyrB	+	+	+
pyrC or *pyrD*	+	±[b]	+
pyrE or *pyrF*	+	−	−

[a] The indicated strain was streaked on N^-C^- plates supplemented with 10 mM NH_4Cl, 0.4% of the indicated carbon source, 0.2 mM uracil or 1 mM orotate as a pyrimidine source, and 0.5 mM arginine where indicated. The plates were incubated at 37°C for 1 day.

[b] Light and mottled growth.

DISCUSSION

This investigation was initiated to determine the cause of the selective growth inhibition of *pyrC* and *pyrD* auxotrophs when grown under pyrimidine-limiting conditions. The results presented indicate that this inhibition is due to the accumulation of toxic levels of the pyrimidine pathway intermediate carbamyl aspartate. Growth conditions that cause derepressed *pyr* gene expression appear to be required to maintain high levels of this intermediate. Carbamyl aspartate toxicity is not observed with *pyrC* and *pyrD* auxotrophs grown under conditions of pyrimidine excess. The absence of growth inhibition apparently reflects low carbamyl aspartate levels caused by the repression of *pyrA* and *pyrB* expression and by feedback inhibition of residual *pyrA* and *pyrB* gene product activity by the high levels of pyrimidine nucleotides in these cells.

The growth inhibition of strain pyrCΔ73 was slightly greater than that of strain pyrDΔ121 (Fig. 2). This difference presumably reflects the utilization of carbamyl aspartate in the synthesis of dihydroorotate, the next pyrimidine pathway intermediate, in the *pyrD* strain. Because the synthesis of dihydroorotate is readily reversible (21), the carbamyl aspartate level should still remain relatively high in this strain. It has not been determined whether the accumulation of dihydroorotate contributes to growth inhibition.

The possible explanation that growth inhibition was caused by the preferential utilization of carbamyl phosphate for the synthesis of pyrimidine pathway intermediates resulting in arginine auxotrophy was clearly excluded. This result was of particular interest because it had been shown that arginine limitation was responsible for the growth inhibition of a leaky *pyrH* strain of *S. typhimurium* (Fig. 1), which contains a partially defective UMP kinase (11). In this strain, which also contains derepressed levels of the pyrimidine biosynthetic enzymes (11), the carbamyl phosphate pool is apparently insufficient to support the synthesis of required amounts of arginine in addition to the synthesis of pyrimidine pathway intermediates. A likely reason for the inadequate supply of carbamyl phosphate is feedback inhibition of carbamyl phosphate synthetase by UMP (1), which accumulates to very high levels in the *pyrH* strain (30). This feedback inhibition would apparently not be substantially overcome by the accumulation of enzyme activators (1, 2). No such feedback inhibition would occur in orotate-grown *pyrC* and *pyrD* auxotrophs, which should result in much higher levels of carbamyl phosphate. There may be other factors contributing to a larger carbamyl phosphate pool in the *pyrC* and *pyrD* auxotrophs, including less flow of carbamyl phosphate into the pyrimidine pathway because of the absence of readily excretable intermediates (11, 36).

When added exogenously, carbamyl aspartate is not toxic to *S. typhimurium*. Unlike *Saccharomyces cerevisiae* (10), *S. typhimurium* does not possess a transport system for this intermediate. Mutant strains (*usp*, ureidosuccinate permease) have been isolated that can use carbamyl aspartate as a pyrimidine source because of increased permeability (32, 38). The addition of up to 0.25 mM carbamyl aspartate to a culture of a *usp* mutant does not cause growth inhibition, although this concentration is sufficient to cause greater repression of *pyrB* expression than that found in uracil-grown cells (38). It appears that the accumulation of toxic levels of carbamyl aspartate requires the block in the pyrimidine biosynthetic pathway that is provided by a *pyrC* or *pyrD* mutation.

The mechanism by which high levels of carbamyl aspartate inhibit growth in *S. typhimurium* is presently unknown. It does not appear to involve the inhibition of synthesis of purine nucleotides, amino acids, folates, thiamine, pantothenate, or several other vitamins. In the experiment shown in Fig. 4, the growth inhibition of strain pyrCΔ73 was rapidly reversed by the addition of uracil to the culture medium. Although this result may simply reflect the cessation of high levels of carbamyl aspartate synthesis caused by increased levels of pyrimidine nucleotides, it raises the possibility that the site of inhibition is the synthesis of pyrimidine nucleotides. This possibility was not explored further in this study.

There are both positive and negative consequences of carbamyl aspartate toxicity for the study of *pyr* gene expression. As described, this property is useful in the rapid classification of pyrimidine auxotrophs. Another potential use of carbamyl aspartate toxicity is for the positive selection of *pyrA* and *pyrB* mutations. Mutations in either gene permit the growth of a *pyrC* auxotroph on minimal-glucose plates supplemented with 0.5 mM orotate and arginine. A similar selection employing suppression of arginine auxotrophy caused by a leaky *pyrH* mutation has been used to isolate *pyrB* mutations (11). This selection, however, is not useful for *pyrA* mutations. The major disadvantage associated with carbamyl aspartate toxicity is the inability to grow *pyrC* and *pyrD* auxotrophs under pyrimidine-limiting conditions. Such growth conditions are required to study derepressed *pyrC* and *pyrD* expression. To circumvent this problem, a *pyrA* or *pyrB* mutation can be introduced into the strain of interest. This can be easily accomplished by transduction with Tn*10* insertions in or near *pyrA* and *pyrB*.

ACKNOWLEDGMENTS

We thank John Ingraham and Bruce Ames, in whose laboratories a portion of this work was done, for their support and encouragement.

This work was supported by Public Health Service grants GM 29466 (C.L.T), AI 05526 (J. Ingraham), and GM 19993 (B. Ames) from the National Institutes of Health. B.R.B. was supported by a postdoctoral fellowship from the Damon Runyon-Walter Winchell Cancer Fund (DRG-113-F) and presently by Genentech, Inc.

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