Regulation of Arginine and Pyrimidine Biosynthesis in *Pseudomonas putida*

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SUMMARY

Repression of biosynthetic enzyme synthesis in *Pseudomonas putida* is incomplete even when the bacteria are growing in a nutritionally complex environment. The synthesis of four of the enzymes of the arginine biosynthetic pathway (*N*-acetyl- α -glutamokinase/*N*-acetylglutamate- γ -semialdehyde dehydrogenase, ornithine carbamoyltransferase and acetylornithine- δ -transaminase) could be repressed and derepressed, but the maximum difference observed between repressed and derepressed levels for any enzyme of the pathway was only 5-fold (for ornithine carbamoyltransferase). No repression of five enzymes of the pyrimidine biosynthetic pathway (aspartate carbamoyltransferase, dihydro-orotase, dihydro-orotate dehydrogenase, orotidine-5'-phosphate pyrophosphorylase and orotidine-5'-phosphate decarboxylase) could be detected on addition of pyrimidines to minimal asparagine cultures of *P. putida* A90, but a 1.5- to 2-fold degree of derepression was found following pyrimidine starvation of pyrimidine auxotrophic mutants of *P. putida* A90. Aspartate carbamoyltransferase in crude extracts of *P. putida* A90 was inhibited *in vitro* by (in order of efficiency) pyrophosphate, CTP, UTP and ATP, at limiting but not at saturating concentrations of carbamoyl phosphate.

INTRODUCTION

Regulation of the synthesis of biosynthetic monomers such as amino acids, purines and pyrimidines in the enteric bacteria is clearly understood in terms of allosteric inhibition of key enzymes and repression of synthesis of biosynthetic enzymes. The control is so effective that little or no wasteful formation of either the small molecules or the macromolecules needed for their biosynthesis occurs, irrespective of the nutritional composition of the bacterial environment. Although information is still limited, it appears that the pseudomonads may not be so efficient in regulating enzyme biosynthesis, especially under conditions of an abundance of biosynthetic monomers in their external environment.

The synthesis of biosynthetic monomers in pseudomonads appears to be efficiently regulated through feed-back inhibition of strategically-placed allosteric proteins. Addition of a mixture of the 20 amino acids contained in proteins to the culture medium caused a 75% reduction in endogenous synthesis of amino acids in *Pseudomonas putida* (Collins, 1974). Several workers (Udaka, 1966; Jensen, Nasser & Nester, 1967; Cohen, Stanier & Le Bras, 1969; Robert-Gero, Poiret & Cohen, 1970; Chou & Gunsalus, 1971; Isaac & Holloway, 1972; Haas, Kurer & Leisinger, 1972) have demonstrated the existence of allosteric enzymes, regulated by end-product inhibition, in key positions in amino acid biosynthetic pathways in pseudomonads. Likewise pyrimidine biosynthesis is probably

regulated through allosteric inhibition of aspartate carbamoyltransferase (Neumann & Jones, 1964; Isaac & Holloway, 1968).

However, evidence for efficient regulation of the biosynthesis of anabolic enzymes in pseudomonads is not convincing. There is no report of significant repression or derepression of a complete biosynthetic pathway. Constitutive synthesis of enzymes and minor variations between repressed and derepressed enzyme levels appear to be the rule rather than the exception (Udaka, 1966; Ramos *et al.*, 1967; Waltho, 1968; Isaac & Holloway, 1968, 1972; Robert-Gero *et al.*, 1970; Chou & Gunsalus, 1971; Maurer & Crawford, 1971; Voellmy & Leisinger, 1972).

Unlike enteric bacteria, strains of *P. putida* do not respond to complex supplementation of a minimal medium (containing a readily metabolizable carbon source) by growing appreciably faster (Collins & Condon, 1972). In a search for biosynthetic systems which do not respond to an abundance of biosynthetic monomers in the external environment, the regulation of both the arginine and pyrimidine pathways was examined in this bacterium.

METHODS

Bacterial strains. Strains A90 and A775 of *Pseudomonas putida* were originally obtained from the Department of Bacteriology and Immunology, University of California, Berkeley, California, U.S.A., and were classified by Stanier, Palleroni & Doudoroff (1966).

Growth media and growth measurements. The basal medium used contained, per litre: KH_2PO_4 , 6.8 g; Na_2HPO_4 , 7.1 g; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; MgSO_4 , 0.6 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 79 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.2 mg; nitrilotriacetic acid, 0.2 g; Hutner's metals '44' (Cohen-Bazire, Sistrom & Stanier, 1957), 1 ml. The carbon source, asparagine, and supplementary amino acids and pyrimidines were added after separate sterilization. For growth experiments, liquid cultures (100 ml or less) were grown in 250 ml Erlenmeyer flasks, and aerated by shaking in a Gallenkamp metabolic shaking incubator. The growth temperature was maintained at $30 \pm 0.2^\circ\text{C}$. Growth was monitored by measurements of E_{660} using a Pye Unicam SP500 spectrophotometer.

Shift-down experiments were performed with cultures growing exponentially in Difco Casamino acid-supplemented minimal asparagine medium. When exponential growth was established, the bacteria were centrifuged, washed and divided into two portions. One portion was suspended in minimal asparagine medium while the other was resuspended in the original Casamino acid-supplemented minimal asparagine medium as a control.

Preparation of cell-free extracts. Extracts were prepared from sonically-disrupted exponentially growing bacteria in either 0.1 M-sodium-potassium phosphate buffer, pH 7.0 (for arginine enzyme assays) or 0.02 M-tris-HCl buffer, pH 8.0 (for pyrimidine pathway enzymes) containing 1 mM-2-mercaptoethanol. Extracts were dialysed for 12 to 18 h against the same buffer at 2°C . The protein concentration of the extracts was determined according to Lowry *et al.* (1951).

Enzyme assays. With the exception of *N*-acetyl- α -glutamokinase and *N*-acetylglutamate- γ -semialdehyde dehydrogenase which were assayed at 20°C , the enzymes were assayed at 30°C . A unit of enzyme activity is expressed in μmol of substrate reacting/min.

N-acetyl- α -glutamokinase, EC. 2.7.2.8 and *N*-acetylglutamate- γ -semialdehyde dehydrogenase, EC. 1.2.1.38. These two enzyme activities were assayed simultaneously according to the procedure of Baich & Vogel (1962).

Acetylornithine- δ -transaminase, EC. 2.6.1.11. The procedure of Albrecht & Vogel (1964) was used.

Ornithine carbamoyltransferase, EC. 2.1.3.3. This biosynthetic enzyme was assayed by a modification (Sellers, 1974) of the original (Archibald, 1944) method. In this modification citrulline was measured by the colorimetric method of Prescott & Jones (1969) for the assay of carbamoyl aspartate and other ureido compounds. The assay mixture contained, per ml: 100 μ mol tris-HCl buffer, pH 8.5; 5 μ mol L-ornithine, pH 8.5; 400 μ g carbamoyl phosphate (dilithium salt); and cell-free extract. At pH 8.5 an inducible catabolic ornithine carbamoyltransferase also present in this strain is inactive (Stalon *et al.* 1967).

Aspartate carbamoyltransferase, EC. 2.1.3.2. This enzyme was assayed according to the optimal conditions described by Neumann & Jones (1964) for *Pseudomonas fluorescens*. The carbamoyl aspartate produced was determined colorimetrically (Prescott & Jones, 1969). In the experiment on allosteric inhibition of aspartate carbamoyltransferase, the substrates carbamoyl phosphate and L-aspartate were varied as described in the text.

Dihydro-orotate dehydrogenase, EC. 1.3.3.1. The method of O'Donovan & Gerhart (1972) was used.

Dihydro-ototase, EC. 3.5.2.3. This was assayed by the method of Beckwith *et al.* (1962). The carbamoyl aspartate formed from dihydro-orotate was determined colorimetrically (Prescott & Jones, 1969).

Orotidine-5'-phosphate pyrophosphorylase, EC. 2.4.2.10. The method of Beckwith *et al.* (1962) as modified by Wild (1971) was used. The assay mixture contained, per ml: 0.6 μ mol 5-phospho- α -D-ribose 1-diphosphate; and cell-free extract. After 5 min pre-incubation at 30 °C, the reaction was started by the addition of L-orotate (10 μ mol), and followed by observing the decrease in E_{295} .

Orotidine-5'-phosphate decarboxylase, EC. 4.1.1.23. A modification of the method of Beckwith *et al.* (1962) was used. The assay mixture contained, per ml: 10 μ mol tris-HCl buffer, pH 8.7; 2 μ mol $MgCl_2$; and cell-free extract. After 10 min pre-incubation at 30 °C, the reaction was started by the addition of orotidine 5'-phosphate (0.2 μ mol), and its disappearance was followed spectrophotometrically at 285 nm.

Isolation of mutants. Auxotrophic mutants which required either a pyrimidine or a pyrimidine plus arginine for growth were isolated by penicillin counterselection of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated cultures of *P. putida* $\Delta 90$. The mutants were characterized by assaying for pyrimidine and arginine pathway enzymes. Three of these mutants were used in the present study: *P. putida* $\Delta 90$ *pyrA*, defective in the carbamoyl phosphate synthetase reaction; *P. putida* $\Delta 90$ *pyrB*, defective in the aspartate carbamoyltransferase reaction; *P. putida* $\Delta 90$ *pyrF*, defective in the orotidine-5'-phosphate decarboxylase reaction.

RESULTS

Withdrawal of an amino acid supplement from P. putida cultures

When either of the two *P. putida* strains growing exponentially for many generations in a medium containing asparagine (as the primary carbon source) plus Casamino acids was harvested, washed and transferred to a minimal asparagine medium, complete cessation of growth did not occur (Fig. 1). Growth continued at a reduced rate until the full minimal asparagine rate was achieved after 70 to 90 min. Thus, even in the presence of a full complement of amino acids, a repression of the synthesis of any of the amino acid biosynthetic enzymes sufficient to prevent growth is not a characteristic of this bacterium.

Fig. 1. Shift-down of *P. putida* strains A90 (a) and A775 (b) from Casamino acid-supplemented asparagine minimal medium (○) to minimal asparagine medium (●) at 30 °C. Exponentially-growing cells from the Casamino acid media were harvested at zero time by centrifugation, washed and resuspended in the minimal asparagine medium. Growth was followed as E_{660} .

Table 1. Derepression of arginine biosynthetic enzymes in *P. putida* A90 *pyrA* growing in minimal asparagine medium containing uracil (20 µg/ml) and arginine (100 µg/ml) on deprivation of arginine

	Duration of starvation (h)	...	Specific activity*		
			0	1	2
<i>N</i> -acetyl- α -glutamokinase + <i>N</i> -acetylglutamate- γ -semialdehyde dehydrogenase†	100		100	186	126
Acetylornithine- δ -transaminase	100		100	83	63
Ornithine carbamoyltransferase	100		100	528	468

* The specific activity of arginine enzymes, expressed as a percentage of the value obtained for cells grown in the presence of arginine. The specific activities (units/mg protein) corresponding to 100% activity for *N*-acetyl- α -glutamokinase + *N*-acetylglutamate- γ -semialdehyde dehydrogenase, acetylornithine- δ -transaminase, and ornithine carbamoyltransferase were 1.83, 0.83 and 0.13, respectively.

† Assayed simultaneously.

Regulation of synthesis of arginine biosynthetic enzymes in *P. putida* A90

Enzymes of the arginine biosynthetic pathway were assayed in cell-free extracts of *P. putida* A90 grown from inoculation in minimal asparagine media both in the presence and absence of arginine (100 µg/ml). The level of ornithine carbamoyltransferase in the presence of arginine was 39 % of that in its absence, indicating a 2.5-fold repression of synthesis by arginine. *N*-acetyl- α -glutamokinase and *N*-acetylglutamate- γ -semialdehyde dehydrogenase (assayed together) were repressed by only 30%. The specific activity of acetylornithine- δ -transaminase was not reduced by growth in the presence of arginine – in fact it increased by 25%. These results were confirmed in a derepression experiment with the auxotrophic mutant *P. putida* A90 *pyrA*. Bacteria were grown for many generations in a minimal asparagine medium containing 100 µg arginine plus 20 µg uracil/ml, harvested, and resuspended in a similar medium but without the arginine. Extracts made 1 and 2 h after removal of the arginine were assayed for arginine biosynthetic enzymes and compared with an extract of similar bacteria grown in the presence of arginine (Table 1). Fivefold derepression of ornithine carbamoyltransferase and approximately 2-fold derepression of *N*-acetyl- α -

Table 2. *Derepression of pyrimidine biosynthetic enzymes in P. putida* A90 *pyrF* growing in minimal asparagine medium containing uracil (20 µg/ml) on deprivation of uracil

Duration of starvation (h) ...	Specific activity*			
	0	0.5	1.0	1.5
Aspartate carbamoyltransferase	100	129	140	162
Dihydro-orotase	100	115	120	173
Dihydro-orotate dehydrogenase	100	77	120	175
Orotidine-5'-phosphate pyrophosphorylase	100	134	133	172
Orotidine-5'-phosphate decarboxylase	0	0	0	0

* The specific activity of pyrimidine pathway enzymes following uracil starvation, expressed as a percentage of the value obtained for cells grown in the presence of uracil. The specific activities (units/mg protein) corresponding to 100% activity for aspartate carbamoyltransferase, dihydro-orotase, dihydro-orotate dehydrogenase and orotidine-5'-phosphate pyrophosphorylase were 0.34, 0.25, 0.0026 and 0.017, respectively.

glutamokinase and/or *N*-acetylglutamate- γ -semialdehyde dehydrogenase occurred after 1 h, while the specific activity of acetylornithine- δ -transaminase dropped by approximately 11% during the same period. The levels of all arginine pathway enzymes assayed decreased during the second hour of arginine starvation.

Regulation of synthesis of pyrimidine pathway enzymes in P. putida A90

The presence of uracil in the growth medium did not repress synthesis of any pyrimidine pathway enzyme measured. The levels of five enzymes of the pyrimidine biosynthetic pathway (aspartate carbamoyltransferase, dihydro-orotase, dihydro-orotate dehydrogenase, orotidine-5'-phosphate pyrophosphorylase and orotidine-5'-phosphate decarboxylase) in extracts of *P. putida* A90 grown in minimal asparagine medium with 20 µg uracil/ml, were similar to those in minimal asparagine medium without uracil. As uracil fully satisfied the pyrimidine requirements of pyrimidine auxotrophic mutants, it is unlikely that the lack of repression was due to transport difficulties or to failure to convert uracil to the various phosphorylated pyrimidine derivatives required for growth. It is also unlikely that the lack of repression was due to catabolism of uracil, since *P. putida* cannot use uracil as carbon or nitrogen source. The level of pyrimidine pathway enzymes in *P. putida* A90 is regulated, however, as starvation of pyrimidine auxotrophs does result in a modest increase in the specific activity of all the enzymes of the pathway. When *P. putida* A90 *pyrF*, growing exponentially in asparagine minimal medium containing 20 µg uracil/ml, was transferred to a similar medium but without uracil, the concentration of pyrimidine pathway enzymes gradually increased by about 62 to 75% over a 90 min period (Table 2). A similar result was obtained with another mutant, *P. putida* A90 *pyrB*, which was switched from uracil to cytosine as a pyrimidine source. Cytosine fulfilled the pyrimidine requirement for auxotrophic mutants of *P. putida* only with difficulty, as the growth rate was reduced 7- to 8-fold compared with uracil supplementation. Growth of a pyrimidine auxotrophic mutant of *P. putida* A90 with cytosine, therefore, is growth under conditions of pyrimidine limitation.

When *P. putida* A90 *pyrB* growing exponentially in minimal asparagine medium containing 50 µg each of both uracil and cytosine/ml was harvested and resuspended in the same medium containing cytosine alone, the concentration of pyrimidine pathway enzymes increased approximately 2-fold after 3 h.

Table 3. *Allosteric inhibition of aspartate carbamoyltransferase in extracts of P. putida* A90

Inhibitor	Inhibitor concn (mM)	Activity* (%)	
		L-Aspartate† limiting	Carbamoyl phosphate‡ limiting
None		100	100
Pyrophosphate	0.05	94	104
	0.5	85	24
	5.0	47	7
CTP	0.05	100	116
	0.5	95	96
	5.0	83	16
UTP	0.05	91	80
	0.5	93	76
	5.0	91	50
ATP	0.05	100	111
	0.5	87	105
	5.0	81	68
UMP	0.05	103	116
	0.5	97	115
	5.0	91	125

* The specific activity corresponding to 100% activity was 0.31 units/mg protein.

† L-Aspartate, 1 mM; carbamoyl phosphate, 10 mM.

‡ L-Aspartate, 10 mM; carbamoyl phosphate, 0.5 mM.

Fig. 2. Allosteric inhibition of aspartate carbamoyltransferase activity (extinction at 466 nm/10 min) in dialysed extracts of *P. putida* A90. The enzyme was assayed alone (○) and in the presence of 5 mM-CTP (●) or 5 mM-UTP (□). The concentration of L-aspartate was saturating at 10 mM while the concentration of carbamoyl phosphate was varied as indicated.

Allosteric inhibition of aspartate carbamoyltransferase

The pyrimidine pathway in *P. putida* A90 might be regulated *in vivo* through allosteric inhibition of aspartate carbamoyltransferase under conditions of carbamoyl phosphate limitation. With carbamoyl phosphate saturation (and even with L-aspartate limitation) only sodium pyrophosphate of the possible effectors tested had any great inhibitory effect

on the *in vitro* assay of aspartate carbamoyltransferase in dialysed extracts of *P. putida* A90. With carbamoyl phosphate limitation, however, several pyrophosphorylated compounds tested inhibited the activity to varying degrees depending on their concentration (Table 3). The greatest degree of inhibition was noted with sodium pyrophosphate. Of the three nucleoside triphosphates, CTP, UTP and ATP, CTP proved the most effective inhibitor. UMP, the only non-pyrophosphorylated derivative tested, proved slightly stimulatory. The superiority of CTP over UTP at 5 mM can be seen from Fig. 2, in which is shown activity-substrate concentration plots of aspartate carbamoyltransferase (in dialysed extracts of *P. putida* A90) alone and in the presence of either CTP or UTP. In this experiment L-aspartate was kept at saturation levels while the carbamoyl phosphate concentration was varied. At 5 mM, CTP was a much more effective allosteric inhibitor than UTP. At low carbamoyl phosphate concentrations, CTP caused 90 to 100% inhibition whereas UTP only barely (5 to 10%) inhibited the enzyme.

DISCUSSION

This study shows that end-product repression of synthesis of the pathway enzymes makes a contribution to the regulation of the arginine biosynthetic pathway in *P. putida* A90. The repressive effect is not spectacular. The greatest difference between repressed and derepressed levels was only 5-fold (for ornithine carbamoyltransferase), in marked contrast to the 80-fold repression of the same enzyme in *Escherichia coli* K12 (Maas, 1965). This result agrees with those of other investigators of the regulation of arginine biosynthesis in pseudomonads. Repression of synthesis of ornithine carbamoyltransferase by arginine has been reported by several groups (Udaka, 1966; Ramos *et al.*, 1967; Chou & Gunsalus, 1971; Voellmy & Leisinger, 1972; Isaac & Holloway, 1972); the extent was about 2- to 6-fold, except for experiments in which arginine was the sole carbon source, where 50- to 100-fold repression was observed (Voellmy & Leisinger, 1972; Isaac & Holloway, 1972).

Information on the regulation of synthesis of the other enzymes of the arginine biosynthetic pathway in pseudomonads is meagre. In *P. aeruginosa*, synthesis of only one (*N*-acetylglutamate- γ -semialdehyde dehydrogenase) of the seven other enzymes was repressed by arginine (Voellmy & Leisinger, 1972; Isaac & Holloway, 1972) and the repression reported was only 2-fold. Chou & Gunsalus (1971) observed that the synthesis of *N*-acetyl- α -glutamokinase in *P. putida* ppG1 was repressed by acetylglutamate and acetylornithine, while synthesis of ornithine acetyltransferase (and ornithine carbamoyltransferase) was repressed by arginine. Our work indicates that the synthesis of at least one of the two enzymes *N*-acetyl- α -glutamokinase and *N*-acetylglutamate- γ -semialdehyde dehydrogenase (which were assayed together) is slightly repressed by arginine and derepressed by arginine limitation in *P. putida* A90. Chou & Gunsalus (1971) and Voellmy & Leisinger (1972) reported that acetylornithine- δ -transaminase was inducible by arginine in *P. putida* ppG1 and *P. aeruginosa* respectively, a result which we confirm here for *P. putida* A90.

Similar observations have been made with other biosynthetic pathways in pseudomonads. Waltho (1968) could find no evidence of repression control of the phenylalanine pathway in *P. aeruginosa*. Robert-Gero *et al.* (1970) working on repression of aspartokinase synthesis and Maurer & Crawford (1971) on the regulation of the tryptophan pathway in strains of *P. putida* found that the presence of the amino acid in the bacterial environment caused only minor repression of enzymes of the specific biosynthetic pathway. Maurer & Crawford (1971) did, however, isolate analogue-resistant mutants which had 15- to 20-fold derepressed levels of three of the tryptophan biosynthetic enzymes. The mutations mapped

outside the tryptophan structural genes, suggesting a similarity with the *trpR* mutations in *E. coli*. It is possible, therefore, that in pseudomonads the wild-type aporepressor element of the tryptophan biosynthetic pathway does not respond dramatically to changes in the concentration of tryptophan but that a much larger response can be produced by genetic changes in the aporepressor.

The synthesis of the pyrimidine pathway enzymes in *P. putida* A90 was not significantly altered by the presence of exogenous uracil, but the specific activity of the enzymes increased 1.5- to 2-fold on pyrimidine starvation of auxotrophic mutants, indicating some regulation over synthesis of pyrimidine biosynthetic enzymes. This result differs from that of Isaac & Holloway (1968), who failed to detect repression or derepression of pyrimidine enzyme synthesis in *P. aeruginosa*.

Our observations on inhibition of aspartate carbamoyltransferase suggest that, as in other bacteria, it probably plays an important role in the regulation of pyrimidine biosynthesis. The enzyme appears to resemble that in *P. fluorescens* (Neumann & Jones, 1964; Adair & Jones, 1972) more closely than that in *P. aeruginosa* (Isaac & Holloway, 1968). In both *P. fluorescens* and *P. putida* pyrophosphate and the nucleotides CTP, UTP and ATP were inhibitory at limiting carbamoyl phosphate concentrations and pyrophosphate was the most effective inhibitor. Aspartate carbamoyltransferase in dialysed extracts of *P. putida* A90 differs, however, from that of *P. fluorescens* in that little allosteric inhibition was detected under conditions of carbamoyl phosphate saturation and also in that CTP was the most powerful trinucleotide effector tested.

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