Biochemical and Genetic Characterization of a Carbamyl Phosphate Synthetase Mutant of *Escherichia coli* K12

By F. BOLIVAR, MATILDE GALVÁN AND J. MARTUSCELLI

Departamento de Biología Molecular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México 20, D.F., México

(Received 31 July 1975, revised 4 November 1975)

SUMMARY

An unusual *Escherichia coli* K12 mutant for carbamyl phosphate synthetase is described. The mutation was generated by bacteriophage MuI insertion and left a 5 % residual activity of the enzyme using either ammonia or glutamine as donors. The mutation is recessive to the wild-type allele and maps at or near the *pyrA* gene, but the mutant requires only arginine and not uracil for growth. By a second block in the *pyrB* gene it was possible to shift the accumulated carbamyl phosphate to arginine biosynthesis. The K_m values and the levels of ornithine activation and inhibition by UMP were normal in the mutant enzyme.

INTRODUCTION

In *Escherichia coli* the only enzyme responsible for the biosynthesis of carbamyl phosphate is carbamyl phosphate synthetase (CPS). This metabolite has a dual role in bacterial physiology: it is utilized for the synthesis of both arginine and pyrimidines (Pierard *et al.*, 1965). In *E. coli*, the CPS is coded by the *pyrA* (or *capA*) gene that is located in the first minute in the standard genetic map between the *leu* and *thr* markers (Taylor & Trotter, 1972). Several phenotypes resulting from mutations in this gene have been reported: auxotrophs for arginine and uracil (Pierard *et al.*, 1965), arginine-sensitive (Eisenstark, 1967), uracil-sensitive (Pierard *et al.*, 1965) and arginine auxotrophs (Abd-El-Al, Kessler & Ingraham, 1969). However, all the mutants giving such diverse phenotypes map in the *pyrA* gene which apparently codes for the CPS heavy subunit.

During the isolation of arginine auxotrophs in *E. coli* K12 a mutant was identified which was phenotypically similar to the one isolated by Abd-El-Al *et al.* (1969). The mutation was obtained after infection by bacteriophage MuI, a mutagenic agent known to cause polar mutations (Nomura & Engbaek, 1972; Buxton, 1975). The biochemical and genetic analyses are presented in this communication.

METHODS

Bacterial strains. The strains utilized in this study are described in Table 1.

Phage strains. Bacteriophages P1_{kt} and P1_{vir}, and the mutator phage MuI were obtained from Dr A. L. Taylor.

Media. The minimal medium was M56 which contains (per litre deionized water): Na₂HPO₄, 8.67 g; KH₂PO₄, 5.29 g; (NH₄)₂SO₄, 2.0 g; 10 % MgSO₄·7H₂O, 2.0 ml; 1 % Ca(NO₃)₂, 1.0 ml; 0.05 % FeSO₄·7H₂O, 1.0 ml; 0.1 % thiamine, 0.2 ml; and 20 % glucose, 10 ml; the last two reagents were added separately. The required supplements were

Table 1. *Bacterial strains*

Strain designation	Mating type	Genotype*	Source or derivation
JM1	Hfr H	<i>thi</i>	A. L. Taylor
JM3	Hfr	<i>leu thr lac thi strA</i>	A. L. Taylor
JM18	F ⁻	<i>leu thr lac (λ) strA</i>	A. L. Taylor
JM38	Hfr	<i>pyrA53 thi</i>	JM1, nitrosoguanidine-induced
JM43	F ⁺	<i>pyrA50† (Mu1) (λ)</i>	A. L. Taylor
JM44	F ⁻	<i>his ade argE pyrB thi strA</i>	A. L. Taylor
JM46	Hfr	<i>(λ)</i>	A. L. Taylor
JM89	F ⁺	<i>pyrA13† (Mu1) (λ)</i>	Mu1 induced mutagenesis of JM46. A. L. Taylor
JM139	Hfr	<i>thr leu ilv recA56</i>	F. Bastarrachea
JM405	F ⁻	<i>thr his pyrB thi strA</i>	JM3 crossed with JM44, ArgE ⁺ Ade ⁺ selection
JM406	F ⁻	<i>pyrA13† (Mu1) his pyrB thi strA</i>	JM405 transduced to Thr ⁺ with P1 grown on JM89
JM407	F ⁻	<i>pyrA13† (Mu1) his strA</i>	JM406 transduced to PyrB ⁺ with P1 grown on JM1
JM409	F'	<i>F' thr⁺ leu⁺/leu thr</i>	F. Bastarrachea
JM411	F ⁻	<i>pyrA13† (Mu1) leu lac (λ) strA</i>	JM18 transduced to Thr ⁺ with P1 grown on JM89
JM412	F ⁻	<i>pyrA13† (Mu1) leu lac (λ) strA thyA</i>	JM411 aminopterin selection
JM413	F ⁻	<i>pyrA13† (Mu1) leu lac (λ) strA recA56</i>	JM139 crossed with JM412, Thy ⁺ selection
JM414	F'	<i>F' pyrA⁺ leu⁺/pyrA13† (Mu1) lac leu recA56 (λ) strA</i>	JM409 crossed with JM413, Leu ⁺ selection

* According to Taylor & Trotter (1972). The Mu1 prophage is inserted into the bacterial gene preceding the symbol †.

added at 40 µg ml⁻¹ and were also sterilized separately. Complete medium was L-broth (Difco yeast extract, 5 g l⁻¹; Difco tryptone, 10 g l⁻¹; NaCl 5 g l⁻¹; pH 7.4). Bacterial growth was measured in a Klett-Summerson colorimeter equipped with a red filter.

Genetic procedures. Mating conditions, conjugation, transduction, preparation of phage stocks and interrupted mating experiments were performed according to Taylor & Trotter (1967) and Adelberg & Burns (1960). The linkage test was used to demonstrate that a mutation was Mu1 induced (Bukhari & Taylor, 1971).

Mutagenesis. Nitrosoguanidine mutagenesis was performed under the conditions described by Adelberg, Mandel & Chen (1965), followed by ampicillin enrichment. Bacteriophage Mu1 mutagenesis was by the method of Taylor (1963).

Enzymic assays. Bacterial cultures (500 ml) were grown in supplemented minimal medium to exponential-growth phase. After two washings the concentrated cells were sonicated with a Branson cell disruptor at full power during 4 min, with 30 s pulses. The temperature during sonication never exceeded 7 °C. The extract was centrifuged in the cold at 10000 g for 15 min to remove cell debris and the supernatant was chromatographed through a G-10 Sephadex column to remove low molecular weight compounds. The CPS activity was measured using Na₂CO₃ labelled with ¹⁴C (New England Nuclear) as one of the substrates (Anderson & Meister, 1965). The ornithine transcarbamylase (OTC) was assayed by measuring citrulline by a colorimetric procedure (Hunninghake & Grisolia, 1966). The protein concentration was estimated by the Folin phenol method (Lowry *et al.*, 1951) using bovine serum albumin (Sigma) as standard.

Chemicals. The enzymic substrates were obtained from Calbiochem and Sigma. Other chemicals were of the best quality available.

Fig. 1. Growth of strain JM89 in minimal medium and supplemented with different metabolites. ○, Growth in minimal medium with or without uracil; ●, growth in minimal medium plus citrulline or arginine; □, growth in minimal medium plus arginine and uracil.

RESULTS

Mutant JM89 was isolated after phage Mu1 mutagenesis of strain JM46. In supplemented minimal plates it grows in citrulline or arginine but not in ornithine or uracil. This is the classic phenotype for OTC-deficient mutants. However, since it is now known that *E. coli* K12 is diploid for the OTC gene, *argF* and *argI* (Glansdorff, Sand & Verhoef, 1967), and since double Mu1-induced auxotrophs have not been reported, it was of interest to study this mutant isolated in one step. As shown in Fig. 1, the mutant grows in citrulline or arginine in liquid medium, but more important, this growth is stimulated by uracil, which does not stimulate OTC mutants. A similar phenotype was described in an *E. coli* B/r mutant (Abd-El-Al *et al.*, 1969). To further exclude the possibility that this could be an OTC mutant, we assayed this enzyme in cell-free extracts. The level of OTC activity in the mutant was similar to that of the parental strain.

Since JM89 is able to grow in arginine alone, it must produce sufficient carbamyl phosphate for pyrimidine biosynthesis but not sufficient for arginine synthesis. So the mutation seems to be in the *pyrA* gene which codes for the CPS activity. By a colorimetric method which measures citrulline, we did not detect any CPS activity in the presence of excess OTC. However, when the activity was assayed using ^{14}C -labelled carbonate as substrate, we detected a residual activity of 5 % CPS in the mutant (Table 2).

We mapped the mutation and the results are presented in Table 3. When the transductional donors are mutants in which Mu1 is the mutagenic agent, the cotransduction frequency between *thr* and *leu* is drastically reduced in comparison with a point mutation donor. This means that with the Mu1 genome (molecular weight 2.5×10^7 daltons) linearly inserted in a gene the possibility of a P1 transducing particle carrying adjacent markers is reduced (Martuscelli *et al.*, 1971).

Table 2. *Determination of CPS activity in cell-free extracts using glutamine or ammonia as nitrogen donor*

The cells were grown in minimal medium plus arginine and uracil.

Strain	<i>pyrA</i> allele	Specific activity $\pm$ S.E.M.*	
		Glutamine (10 μ M)	Ammonia (100 μ M)
JM46	Wild type	1.575 $\pm$ 0.005	1.523 $\pm$ 0.02
JM89	<i>pyrA13</i> [†] (MuI)	0.082 $\pm$ 0.0006	0.08 $\pm$ 0.0005

* As μ mol carbamyl phosphate produced/h per mg protein. The differences between the mean enzymic activities of the two strains are highly significant ($P \ll 0.001$).

[†] See footnote to Table 1.

Table 3. *Mapping of different pyrA mutations by P1 transduction*

Recipient	Donor	Mutagenic agent for the <i>pyrA</i> mutation in donor	Percentage of Thr ⁺ transductants that scored as	
			Pyr ⁻	Leu ⁺
JM18	JM38	NTG	25-40	10
JM18	JM43	MuI	0.5	ND
JM18	JM89	MuI	0.4	ND

NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ND, not detectable.

Eighty arginine auxotrophic independent transductants resulting from the cross between JM89 as donor and JM18 as recipient were purified and all of them were MuI lysogens. This indicates that in strain JM89, MuI is inserted in the *pyrA* gene or nearby.

To demonstrate that JM89 produces a limiting amount of carbamyl phosphate that for some reason is not available or insufficient for arginine biosynthesis, we constructed a double mutant *pyrA13, pyrB*. The gene *pyrB* codes for the enzyme aspartate transcarbamylase (ATC) that catalyses the second step in the pyrimidine pathway so that *pyrB* mutants are only uracil auxotrophs. To obtain the double mutant, JM405 (a *thr pyrB* strain) was transduced with P1 grown on JM89, and selected for Thr⁺ in the presence of arginine and uracil. Several arginine auxotrophs were obtained and one of them, JM406, was saved for further characterization after two isolations. This putative *pyrA13, pyrB* mutant, in spite of harbouring the *pyrA13* allele, does not require arginine for growth in contrast to the parental strain JM89. This would be possible if one assumes that the carbamyl phosphate accumulated by the *pyrB* mutation can now be utilized for arginine biosynthesis. That JM406 is indeed a double mutant was shown by transducing the strain with P1 grown on the prototroph JM1 and selecting for Ura⁺ in the presence of arginine, generating in this way strain JM407. The growth pattern of JM407 is similar to JM89, which implies the presence of the *pyrA13* allele.

The next step was to perform dominance tests in JM89. For this we crossed JM409 (an F' carrying the first minute of the *E. coli* chromosome and hence the *pyrA* gene in the wild state) with JM413 (a *recA56* JM89 derivative), and selected in minimal medium with streptomycin, and in minimal medium with arginine, uracil and streptomycin. The total number of recombinants in both selections was the same. One exconjugant from the former selection was purified and labelled JM414. This strain can grow in minimal medium without supplements. Its growth pattern and the fact that the number of recombinants was the same in both selections, indicates that the *pyrA13* allele is recessive to the wild-type *pyrA* allele present in strain JM409.

Table 4. *Effect of allosteric modulators on the CPS activity in cell-free extracts*

Strain	Specific activity ($\mu\text{mol carbamyl phosphate produced/h per mg protein}$)						
	Without additions	Plus UMP (10^{-4} M)	Activity (%)	Plus Orn (10^{-2} M)	Activity (%)	UMP+Orn	Activity (%)
JM46	1.56	0.338	21.6	2.832	175	1.236	79
JM89	0.079	0.014	17.7	0.135	169	0.054	68

We attempted to purify the CPS from the parent and the mutant strains according to Anderson & Meister (1965). We found that under conditions in which the wild-type enzyme could be purified the mutant-type enzyme was very unstable, and so far more difficult to purify.

To rule out the presence of a CPS inhibitor in the mutant strain, mixtures of wild-type and mutant cell-free extracts were made using equal amounts of protein from the wild-type and mutant raw extracts. The resulting specific activity in the mixture was the same as the sum of the individual values. This experiment excludes the presence of inhibitors in the mutant.

The next step was to characterize both enzymes by kinetic parameters. Anderson & Meister (1965) and Kalman, Duffield & Brzozowski (1966) have demonstrated that NH_4^+ can be utilized instead of glutamine by the CPS from *E. coli*. As can be seen in Table 2 the enzyme from the wild-type strain uses glutamine ($10 \mu\text{M}$) and ammonia ($100 \mu\text{M}$) as nitrogen donor. In spite of the fact that the mutant strain has only 5 % CPS residual activity, it uses both nitrogen substrates efficiently. The effect of allosteric effectors on enzymic activity is well known (Anderson & Marvin, 1968; Pierard, 1966; Pierard *et al.*, 1965). The effect of UMP and ornithine on the enzyme activity from wild-type and mutant cells is shown in Table 4 and it is clear that these modulators have a similar effect on both enzymes. For the mutant enzyme K_m values of 1.44×10^{-3} , 3.8×10^{-4} and 3.5×10^{-3} M were found for ATP, glutamine and bicarbonate respectively. These values agree with those obtained for the parental strain JM46 and are similar to those reported by Anderson & Meister (1966).

DISCUSSION

We are dealing with an *E. coli* mutant generated by the insertion of the Mu1 genome into the bacterial chromosome. It has normal OTC activity, cotransduces with *thr*, is recessive to the wild-type *pyrA* allele, and has low CPS activity. All the *pyrA* mutants so far isolated by Mu1 mutagenesis have a classical phenotype requiring arginine and uracil (A. L. Taylor, personal communication). This is reasonable if one assumes that Mu1 phage integrates linearly in the host chromosome and induces polar effects on distal genes (Nomura & Engbaek, 1972; Buxton, 1975). In the mutant we report here, the phage did not completely abolish the CPS function, since the strain did not require uracil although its growth was stimulated by it. Evidently the mutant was synthesizing a limited amount of carbamyl phosphate that it is preferentially utilized by the pyrimidine pathway. One would expect this, since the aspartate transcarbamylase Michaelis constant for carbamyl phosphate, the next enzyme in the pyrimidine path, is about 20 times lower than that for OTC, the next enzyme in the arginine pathway (1×10^{-5} M versus 1.9×10^{-4} M, respectively; Porter, Modebe & Stark, 1969; Rogers & Novelli, 1962).

There are several alternative explanations of how a Mu1-induced mutation can still have residual CPS activity. The simplest is that the Mu1 genome is integrated at the end of

the *pyrA* gene, leaving most of the gene intact, so that an incomplete polypeptide with greatly reduced catalytic activity is generated. This would be equivalent to the six frame shift mutants with non-auxotrophic phenotypes reported by Pierard *et al.* (1973) that map at the operator distal end of the gene for the heavy subunit.

We know from Trotta *et al.* (1973) that the CPS from *E. coli* is composed of two non-identical subunits. They found that after purification the enzyme is dissociated into a heavy and a light polypeptide. The former catalyses the ammonia-dependent carbamyl phosphate synthesis and harbours the allosteric sites for NH_4^+ , ornithine, IMP and UMP. The light chain catalyses the glutamine hydrolysis activity. It is hard to imagine in which gene MuI is integrated in our mutant, since none of the kinetic parameters are altered and only the V_{max} is affected.

Another possibility is that MuI is inserted in an unknown regulatory gene for the activity or biosynthesis of CPS adjacent to the *pyrA* gene. It would be interesting to measure the other catalytic activities of CPS in the mutant strain, for example bicarbonate-dependent ATP cleavage, and ATP- and bicarbonate-dependent hydrolysis of γ -glutamyl hydroxamate, to see if these activities are affected by the mutation.

We thank Dr A. L. Taylor for bacterial strains and Dr K. Willms for reviewing the manuscript.

REFERENCES

- ABD-EL-AL, A., KESSLER, D. P. & INGRAM, J. L. (1969). Arginine auxotrophic phenotype resulting from a mutation in the *pyrA* gene of *Escherichia coli* B/r. *Journal of Bacteriology* **97**, 466–468.
- ADELBERG, E. A. & BURNS, S. N. (1960). Genetic variation in the sex factor of *Escherichia coli*. *Journal of Bacteriology* **79**, 321–330.
- ADELBERG, E. A., MANDEL, M. & CHEN, G. C. C. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K-12. *Biochemical and Biophysical Research Communications* **18**, 788–795.
- ANDERSON, P. M. & MARVIN, S. V. (1968). Effect of ornithine, IMP, and UMP on carbamyl phosphate synthetase from *Escherichia coli*. *Biochemical and Biophysical Research Communications* **32**, 928–934.
- ANDERSON, P. M. & MEISTER, A. (1965). Evidence for an activated form of carbon dioxide in the reaction catalyzed by *Escherichia coli* carbamyl phosphate synthetase. *Biochemistry* **4**, 2803–2809.
- ANDERSON, P. M. & MEISTER, A. (1966). Bicarbonate-dependent cleavage of adenosine triphosphate and other reactions catalyzed by *Escherichia coli* carbamyl phosphate synthetase. *Biochemistry* **5**, 3157–3163.
- BUKHARI, A. I. & TAYLOR, A. L. (1971). Genetic analysis of diaminopimelic acid- and lysine-requiring mutants of *Escherichia coli*. *Journal of Bacteriology* **105**, 844–854.
- BUXTON, R. J. (1975). Genetic analysis of thymidine-resistant and low-thymine requiring mutants of *Escherichia coli* K-12 induced by bacteriophage Mu-1. *Journal of Bacteriology* **121**, 475–484.
- EISENSTARK, A. (1967). Linkage of arginine sensitivity (*ars*) and uracil arginine requiring (*pyrA*) loci of *Salmonella typhimurium*. *Nature, London* **213**, 1263–1264.
- GLANSBORFF, N., SAND, G. & VERHOEF, C. (1967). The dual genetic control of ornithine transcarbamylase synthesis in *Escherichia coli* K-12. *Mutation Research* **4**, 743–751.
- HUNNINGHAKE, D. & GRISOLIA, S. (1966). A sensitive and convenient micromethod for estimation of urea, citrulline and carbamyl derivatives. *Analytical Biochemistry* **16**, 200–205.
- KALMAN, S. M., DUFFIELD, P. H. & BRZOWSKI, T. (1966). Purification and properties of a bacterial carbamyl phosphate synthetase. *Journal of Biological Chemistry* **241**, 1871–1877.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MARTUSCELLI, J., TAYLOR, A. L., CUMMINGS, D. J., CHAPMAN, V. A., DELONG, S. S. & CAÑEDO, L. (1971). Electron microscopic evidence for linear insertion of bacteriophage Mu-1 in lysogenic bacteria. *Journal of Virology* **8**, 551–563.
- NOMURA, M. & ENGBAER, E. (1972). Expression of ribosomal protein genes as analyzed by bacteriophage Mu-induced mutations. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 1526–1530.
- PIERARD, A. (1966). Control of the activity of *Escherichia coli* carbamyl phosphate synthetase by antagonistic allosteric effectors. *Science, New York* **154**, 1572–1573.

- PIERARD, A., GLANSORFF, N., MERGEAY, M. & WIAME, J. M. (1965). Control of the biosynthesis of carbamyl phosphate in *Escherichia coli*. *Journal of Molecular Biology* **14**, 23-36.
- PIERARD, A., GRENSON, M., GLANSORFF, N. & WIAME, J. M. (1973). A comparison of the organization of carbamyl phosphate synthesis in *Saccharomyces cerevisiae* and *Escherichia coli*, based on genetical and biochemical evidence. In *The Enzymes of Glutamine Metabolism*, pp. 483-503. Edited by S. Prusiner and E. R. Stadtman. New York: Academic Press.
- PORTER, R. W., MODEBE, M. O. & STARK, G. R. (1969). Aspartate transcarbamylase. Kinetic studies of the catalytic subunit. *Journal of Biological Chemistry* **244**, 1846-1859.
- ROGERS, P. & NOVELLI, G. D. (1962). Purification of ornithine transcarbamylase from derepressed cells of *Escherichia coli* w. *Archives of Biochemistry and Biophysics* **96**, 398-407.
- TAYLOR, A. L. (1963). Bacteriophage-induced mutation in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **50**, 1043-1051.
- TAYLOR, A. L. & TROTTER, C. D. (1967). Revised linkage map of *Escherichia coli*. *Bacteriological Reviews* **31**, 332-353.
- TAYLOR, A. L. & TROTTER, C. D. (1972). Linkage map of *Escherichia coli* strain K-12. *Bacteriological Reviews* **36**, 504-524.
- TROTTA, P. P., PINKUS, L. M., WELLNER, V. P., ESTIS, L., HASCHEMEYER, R. H. & MEISTER, A. (1973). Structure function relationships in glutamine-dependent carbamyl phosphate synthetase. In *The Enzymes of Glutamine Metabolism*, pp. 431-482. Edited by S. Prusiner and E. R. Stadtman. New York: Academic Press.