Absence of Polar Effect of Frameshift Mutations in the *E* Gene of the *Escherichia coli* *argECBH* Cluster

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SUMMARY

Four arginine auxotrophs have been obtained in *Escherichia coli* K12 following treatment with the acridine half-mustard ICR 191. Their properties show that they carry mutations in the *E* gene of the *argECBH* cluster, and their reversion patterns indicate that these mutations are frameshifts. The mutations exert no polar effect on the expression of the *argC*, *B* and *H* genes. These results accord with earlier suggestions that *E* is not transcribed on to the same messenger RNA molecule as *C*, *B* and *H*.

INTRODUCTION

The nine structural genes and single regulator gene for the enzymes mediating arginine synthesis from glutamate in *Escherichia coli* K12 (Fig. 1) constitute one of the best-known systems of functionally related genes showing incomplete clustering (Maas, 1961; Glansdorff, Sand & Verhoef, 1967; Taylor, 1970). Four of these genes, *argB*, *C*, *E* and *H*, appear to be contiguous; the gene order (Glansdorff, 1965) is *E-C-B-H* (Fig. 1). Genes *argB*, *C*, *E* and *H* code respectively for *N*-acetyl- γ -glutamokinase, *N*-acetylglutamic γ -semialdehyde dehydrogenase, acetylornithinase, and argininosuccinase (Fig. 1); we shall call the enzymes B, C, E and H.

The functional organization of this cluster has still not been completely elucidated. The observation of polar effects of certain nonsense and deletion (presumably frameshift) mutations in *argC* and *B* on the expression of *B* and *H* and of *H* respectively (Cunin, Elseviers, Sand, Freundlich & Glansdorff, 1969) and of a *C* mutation, probably frameshift, on the expression of *B* and *H* (Vogel, Baumberg, Bacon, Jones, Unger & Vogel, 1967; Ashcroft, 1970) suggests that *C*, *B* and *H* are transcribed on to a single polycistronic messenger RNA molecule in the direction $\overrightarrow{CBH}$. Cunin *et al.* (1969) reported that of five amber *argE* mutations isolated, none showed a polar effect on the expression of *C*, *B* and *H*, suggesting that *E* is not part of the *CBH* operon (i.e. is not transcribed on to the same messenger as the latter three genes). The properties of a mutant carrying a deletion probably spanning the *E-C* boundary suggest (Elseviers, Cunin & Glansdorff, 1969) that *argE* is transcribed on to a separate messenger in the same direction as *C*, *B* and *H*, but later work (N. Glansdorff & R. Cunin, personal communication; Ashcroft, 1970) casts doubt on this interpretation. Frameshift mutations in an operon usually exert a polar effect on promoter-distal genes (Martin, Whitfield, Berkowitz & Voll, 1966), probably due to the generation of terminator codons in the phase-shifted region of the messenger (Martin, 1967). Such mutations are readily induced in *Escherichia coli* by the acridine half-mustard ICR-191 (3-chloro-7-methoxy-9-[3-chloroethyl-aminopropyl-amino]acridine) (Berger, Brammar & Yanofsky, 1968).

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Fig. 1. Genes and enzymes of arginine synthesis in *Escherichia coli* K12. The gene *argR* is a regulator gene, mutations in which permit arginine enzyme synthesis at elevated rates unaffected by the presence of arginine.

We report here the isolation of four *argE* mutants following treatment with this mutagen: their reversion patterns indicate that they carry frameshifts. No polar effect on the expression of *argC*, *B* or *H* could be detected, supporting the conclusion of Cunin *et al.* (1969) that *E* is transcribed on to one messenger and *C*, *B* and *H* on to another.

METHODS

Bacterial strains (all derivatives of *Escherichia coli* K12) are listed in Table 1.

Media and growth of cultures. Minimal media and L-broth have been described (Baumberg, 1970). Nutrient broth was Oxoid no. 2. Overnight nutrient broth cultures were grown from single colonies on a nutrient agar plate. Strains for lysis by P1*k*c and recipient cultures in transductions were grown in L-broth.

Preparation of cell extracts and enzyme assays. To grow up cultures for enzyme assay, 0.1 ml. of an overnight broth culture was inoculated into minimal salts-glucose medium (10 ml.) supplemented with amino acids as required and grown overnight at 37° on an orbital incubator. The minimal medium overnight culture was diluted 20- to 100-fold in identical

medium, and incubation was continued under the same conditions. Bacterial growth was followed as O.D. at 660 nm., a correction being applied for the nonlinear relationship between O.D. and bacterial mass for O.D.'s above about 0.3.

Cells were harvested at O.D. (660 nm.) 0.6 to 0.7, and centrifuged at 10,000 *g* for 10 min. at 4°. The cells were resuspended in 0.1 M-potassium phosphate buffer, pH 7, containing reduced glutathione at 10⁻³ M, and broken in an MSE 100 watt ultrasonic disintegrator operating at maximum amplitude for 20 sec. (0°). Extracts were centrifuged at 38,000 *g* for 15 min. at 4°. Enzyme B was assayed immediately; if the other assays were to be performed later, extracts were stored at -20°.

Enzyme B was assayed by the method of Baich & Vogel (1962), with the following modifications to the reaction mixture: α -*N*-acetyl-L-glutamic acid, adjusted to pH 7.4 with NaOH, 30 μ moles; MgCl₂, 15 μ moles; ATP, 6 μ moles; hydroxylammonium chloride, adjusted to pH 7.4 with NaOH, 2 mmoles; and extract, in a total volume of 1.5 ml. Following incubation at 37°, the reaction was stopped by the addition of 1 ml. 3 N-HClO₄. One ml. of Fe(ClO₄)₃ (O. Frederick Smith Chemical Co., Columbus, Ohio) in 0.1 N-HClO₄ was then added, denatured protein removed by centrifugation, and the absorbance due to α -*N*-acetyl- γ -L-glutamyl hydroxamate read at 510 nm. Blank tubes were run in which the substrate was added only after stopping the reaction. A standard curve was constructed for γ -L-glutamyl hydroxamate (Sigma Chemical Co. Ltd, London).

Enzyme C was assayed essentially by the method of Baich & Vogel (1962). The reaction mixture contained: glycine-NaOH buffer, pH 9.5, 50 μ moles; α -*N*-acetylglutamic γ -semi-

Table 1. *Bacterial strains*

Strain	Mating type	Relevant markers	Source and/or reference
BL290*	F ⁻	<i>his</i> , <i>trp</i>	W. J. Brammar
6P‡	F ⁻	<i>his</i> , <i>ile</i> , <i>met</i> , † <i>pro</i>	Baumberg (1970)
P4X*	Hfr P4X	<i>metB</i> †	N. Glansdorff
P4XB2	Hfr P4X	<i>metB</i> , <i>argR</i>	N. Glansdorff
MN42, EC(B), EC-I, Sup102, CB-I	Hfr P4X	<i>metB</i> , carrying deletions of various parts of the <i>argECBH</i> cluster	N. Glansdorff; Cunin <i>et al.</i> (1969)
BL291	F ⁻	<i>his</i> , <i>trp</i> , <i>argE171</i>	See text
BL292	F ⁻	<i>his</i> , <i>trp</i> , <i>argE176</i>	See text
BL293	F ⁻	<i>his</i> , <i>trp</i> , <i>argE179</i>	See text
BL294	Hfr P4X	<i>metB</i> , <i>argE181</i>	See text
BL295	F ⁻	<i>his</i> , <i>ile</i> , <i>met</i> , <i>pro</i> , <i>argE113</i>	See text
BL332	F ⁻	<i>his</i> , <i>ile</i> , <i>met</i> , <i>pro</i> , <i>argE162</i>	See text
BL333	Hfr P4X	<i>argE181</i>	See text
BL334	F ⁻	<i>his</i> , <i>ile</i> , <i>pro</i> , <i>argE113</i>	See text
BL336	F ⁻	<i>his</i> , <i>ile</i> , <i>pro</i> , <i>argE162</i>	See text
BL338	Hfr P4X	<i>argE171</i>	See text
BL350	Hfr P4X	<i>argE176</i>	See text
BL357	Hfr P4X	<i>argE179</i>	See text
BL382	Hfr P4X	<i>argE113</i>	See text
BL397	Hfr P4X	<i>argE162</i>	See text
BL402	Hfr P4X	<i>argE171</i> , <i>argR</i>	See text
BL432	Hfr P4X	<i>argE176</i> , <i>argR</i>	See text
BL451	Hfr P4X	<i>argE179</i> , <i>argR</i>	See text
BL470	Hfr P4X	<i>argE181</i> , <i>argR</i>	See text
BL478	Hfr P4X	<i>argE113</i> , <i>argR</i>	See text
BL483	Hfr P4X	<i>argE162</i> , <i>argR</i>	See text

* These strains are suppressor-free.

† These *met* loci are co-transducible with the *argECBH* cluster by phage P1*kc*.

‡ This strain carries a strong amber suppressor.

aldehyde, 1.3 μ moles; K_2HPO_4 , 40 μ moles; NADP, 0.25 μ moles; bovine serum albumin, 1.5 mg.; and extract, in a total volume of 1.5 ml. The increase in absorbance due to formation of NADPH₂ was followed continuously with a Unicam SP 800 spectrophotometer. Some loss of enzyme activity occurred if the total protein concentration in the reaction mixture fell below 1 mg./ml.; this was prevented by the bovine serum albumin. α -N-Acetylglutamic γ -semialdehyde was prepared according to Vogel & McLellan (1970), except that it was eluted from the Amberlite IR-4B column with a 0.05–0.5 M- K_2SO_4 gradient. Fractions containing no 2-oxoglutarate were pooled, adjusted to pH 7.0 and stored at -20° till use. The presence of K_2SO_4 in the solution had no effect on the enzyme C assay. Material prepared and stored in this way became unusable after a few weeks; when such aged solutions were used, extracts known to be devoid of enzyme C activity gave an appreciable rate of NADPH₂ formation in the assay.

Enzyme E was assayed by the method of Vogel & Bonner (1956).

Enzyme H was assayed according to Theil, Forsyth & Jones (1969), except that after the addition of NaOH to the assay mixture boiled with ninhydrin + citric acid each tube was shaken violently until all trace of blue colour had vanished. A further yellow colour due to argininosuccinate disappeared on standing for 2 h. or was removed by placing the tubes in boiling water for 5 min. Barium argininosuccinate was obtained from Sigma or was prepared by the method of Ratner (1957) using a protein extract of *Escherichia coli* W 160-37 (F_{14}^+), a partial diploid for the *argECBH* cluster.

Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951).

Mutagenesis techniques and mutant isolation. Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) (Ralph N. Emanuel Ltd, London) followed the procedure of Adelberg, Mandel & Chen (1965). Treated cultures underwent two or more cycles of penicillin enrichment for ornithine auxotrophs (Davis, 1948), before spreading on supplemented minimal agar plates + arginine (1 μ g./ml.). After incubation at 37° for 48 h., minute colonies (diameter 0.5 to 1 mm.) were selected for further study and frequently proved to be arginine auxotrophs.

In mutagenesis by the acridine half-mustard ICR 191, an exponentially growing culture in supplemented minimal salts medium was diluted (100 to 300 cells/ml.) into several lots of similar medium + arginine. ICR 191 (kindly donated by Dr H. J. Creech, Institute for Cancer Research, Philadelphia) was added (10 to 25 μ g./ml.). All operations involving ICR 191 were carried out in a dark room under a red safety light, and the cultures, enclosed in aluminium foil, were incubated on an orbital incubator at 37° for 48 h. The culture that had grown at the highest ICR 191 concentration was then diluted and spread on supplemented minimal agar plates + arginine so as to obtain about 200 colonies per plate. These were replicated on to similar plates without and with arginine to identify Arg⁻ colonies for further study. In some experiments, the ICR 191-treated culture was subjected to penicillin enrichment and arginine auxotroph isolation as described above; however, with this technique no *argE* mutants were obtained.

In reversion studies, supplemented minimal agar plates without arginine or a precursor were spread with $5-8 \times 10^7$ cells from a nutrient broth-grown culture washed with and resuspended in 0.1 M-potassium phosphate buffer. A drop of the mutagen (NG, 0.05 ml. of a 2 mg./ml. aqueous solution; ICR-191, 0.1 ml. of a 100 μ g./ml. aqueous solution) was added to the centre of the plate. Plates were incubated at 37° for 2 days.

Transduction

This was carried out according to Siegel & Bryson (1967).

RESULTS AND DISCUSSION

Isolation of mutants, reversion studies, and deletion mapping. We have collected Arg⁻ strains from six mutant isolation experiments in four of which we used ICR 191 to obtain frameshift mutations and in two, NG to provide base-substitution mutations for comparison. Starting strains were BL290 and P4X (for ICR 191 mutagenesis) and 6P (NG mutagenesis). Arginine auxotrophs obtained in these experiments were streaked on supplemented minimal medium containing acetylornithine, ornithine or arginine. The *argE* mutants will grow on arginine but not on acetylornithine, and will also grow on ornithine if enzymes F, G and H are present. It was reasoned that, although *argE* polar mutations would diminish the level of enzyme H, they should still permit at least slow growth on ornithine, since even a strain, carrying a strongly polar *argB* mutation, in which the activity of enzyme H is only 1 % of the wild-type maximum still shows very slow growth on ornithine (Cunin *et al.* 1969).

We therefore intended to examine arginine auxotrophs that failed to respond to acetylornithine but grew normally or slowly on ornithine. In fact, of the 23 mutants categorized (20 following ICR 191 and 3 following NG mutagenesis), all grew normally on ornithine. From each experiment, one such isolate was selected for further study; the isolates following ICR 191 treatment were denoted BL291, BL292, BL293 (derived from BL290) and BL294 (derived from P4X) and those following NG treatment BL295 and BL332.

The mutants were tested for revertibility to arginine independence by ICR 191 and NG (Table 2). Each mutagen strongly reverts only the mutants obtained following its own use. These results provide strong presumptive evidence that BL291, BL292, BL293 and BL294 carry frameshift *arg* mutations, and BL295 and BL332 base-substitution *arg* mutations. This is consistent with the appreciably higher spontaneous reversion rate of BL295 and BL332 (Oeschger & Hartman, 1970). The slight revertibility of BL292 by NG is not contradictory since NG reverts frameshift mutations occasionally (Riyasaty & Atkins, 1968; Yourno & Heath, 1969; Newton, 1970; Oeschger & Hartman, 1970); the opposite event, reversion of a base-substitution mutation by an acridine half-mustard, is much rarer (Whitfield, Martin & Ames, 1966; Berger *et al.* 1968), and was not observed for BL295 and BL332.

Evidence that the six mutations map in the *argE* region was obtained by P1*k*c transduction. The donors were the mutants (BL291, BL292 and BL293) or their *met*⁺ equivalents obtained by transduction (BL333, BL334 and BL336 from BL294, BL295 and BL332 respectively). The recipients were a series of derivatives of P4X carrying various deletions extending into the *argECBH* cluster (Fig. 2).

The results are shown in Table 3. It is seen that the six mutants failed to yield *arg*⁺ recombinants when crossed with MN42, EC(B) or EC-I, but gave such recombinants when crossed with Sup 102 or CB-I. All six mutations therefore map in the region between the

Table 2. *Reversion of ICR 191- and NG-induced mutations by ICR 191 and NG*

Reversion frequencies were obtained as described under Methods.

Mutant	Mutagen		
	ICR 191	NG	None (control)
BL291	> 200 colonies	0	0
BL292	> 250	3	0
BL293	> 200	0	0
BL294	> 250	0	0
BL295	0	> 200	2
BL332	0	> 200	4

Fig. 2. The extent of deletions carried by derivatives of P4X that were used in the approximate location of *argE* mutations isolated in this study. The locus *ppc* controls the enzyme phosphoenolpyruvate carboxylase, closely linked to (and possibly contiguous with) *argE*. The evidence that *sup 102* extends into *argE* is biochemical and physiological (see Elseviers *et al.* 1969), and is not conclusive.

'left' end of *EC-1* (which may lie outside the *argE* structural gene) and the 'left' end of *sup 102*. The mutations in BL291, BL292, BL293, BL294, BL295 and BL332 were designated *E171*, *E176*, *E179*, *E181*, *E113* and *E162*.

Arginine enzyme activities in the *argE* mutants. The *argR*⁺ strains BL338, BL350, BL357, BL382 and BL397, carrying *E171*, *E176*, *E179*, *E113* and *E162*, and the *argR* strains BL402, BL432, BL451, BL470, BL478 and BL483, carrying *E171*, *E176*, *E179*, *E181*, *E113* and *E162* respectively were constructed by *P1kc* transduction of P4X and P4XB2. These strains together with BL294 and their parents P4X and P4XB2 were grown in arginine-supplemented minimal medium and assayed for arginine enzymes as described in Methods. The results are presented in Table 4.

It is clear that none of the *argE* mutations is strongly polar, nor is there any consistent appearance of a weak polar effect (distal gene expression 50% or more of wild-type) if all the data are given equal weight. Since the enzyme C and B assays are less reliable than the enzyme H assay, particularly at low activities, we have calculated the ratio of enzyme H activity for each strain to that for the parent strain; if these values are consistently well below

Table 3. *The ratio of arg⁺ to met⁺ transductants (expressed as a percentage) from crosses between the putative argE mutants and various argECBH deletion mutants*

Donor	Recipient				
	MN42	EC(B)	EC-1	Sup 102	CB-1
BL291	< 0.02*	< 0.03	< 0.03	5.4	5.1
BL292	< 0.04	< 0.03	< 0.03	7.5	14
BL293	< 0.04	< 0.03	< 0.04	7.4	14
BL333	< 0.03	< 0.03	< 0.04	6.3	9.2
BL334	< 0.03	< 0.01	< 0.06	1.1	7.2
BL336	< 0.02	< 0.02	< 0.08	8.0	4.6

* When no *arg*⁺ recombinants were found, the ratio is given as less than that percentage which would have been calculated had one *arg*⁺ recombinant been obtained.

1 for a particular *argE* mutation, the possibility of the mutation being polar must be considered. In fact, on this basis *E176* and *E181* might be claimed to be about 25 and 20% polar respectively (i.e. distal gene expression was lowered by these amounts).

Let us consider the implications of such an assertion. It could imply that *argECBH* is a single unit of transcription, and that of four frameshift mutations obtained, two showed no detectable polar effect while two showed polar effects of 25% or less. The implausibility of this is clear if we consider results obtained with other systems. Martin *et al.* (1966) reported that all of 14 *Salmonella typhimurium* LT2 *hisC* frameshifts were more than 50% polar; Newton (1970) found that of 31 *Escherichia coli* K12 *lacZ* frameshifts, only 2 were less than 50% polar. Weakly polar nonsense mutations are similarly rare (Martin *et al.* 1966; Fink & Martin, 1967; Zipser, Zabell, Rothman, Grodzicker & Wenk, 1970).

It might be argued that our selection technique excludes extremely polar *argE* mutations (enzyme H activity less than 1% of wild-type), implying that *argE* polar mutations affecting the levels of enzymes B, C and H do in fact occur, but are either extremely polar (over 99%) or very weakly polar (less than 50%). While this possibility cannot be ruled out, the *argE* gene would be quite unique in this respect, for not only are such weakly polar mutations relatively uncommon, as pointed out above, but extremely polar mutations have not been encountered (Martin *et al.* 1966; Newton, 1970) except when new genetic material has been inserted into the operon affected (Jordan, Saedler & Starlinger, 1968; Shapiro, 1969; Malamy, quoted in Newton, 1970). This is unlikely to result from ICR 191 treatment.

In view of the infrequency of weakly polar mutations in these systems, we believe the hypothesized isolation of four such frameshifts in *argE* to be extremely improbable. We consider it more likely that the four *argE* frameshift mutations do not exert a polar effect on C, B and H, the observed variations in enzyme levels resulting from scatter in the measured activities or possibly small inter-strain differences; and that these results support the contention of Cunin *et al.* (1969) that *argE* is not part of the same operon as *argC*, B and H.

Table 4. Specific activities (μ moles product formed/mg. protein/h.) of enzymes coded for by genes *argECBH* in arginine-grown cultures of the *argE* mutants and of the parent strains

<i>argR</i> ⁺ Strains	Enzyme				$\frac{\text{H activity in mutant}}{\text{H activity in parent}}$
	E	C	B	H	
P4X	4.3	0.10	0.09	0.22	1
BL338	0	0.19	0.18	0.31	1.4
BL350	0	0.08	0.17	0.16	0.73
BL357	0	0.13	0.20	0.31	1.4
BL294	0	0.12	0.12	0.19	0.86
BL382	0	0.07	0.06	0.27	1.2
BL397	0	0.10	0.16	0.20	0.91
<i>argR</i> Strains					
P4XB2	6.1	2.8	2.9	7.4	1
BL402	0	n.d.*	2.0	4.6	0.62
BL432	0	n.d.	1.7	5.5	0.74
BL451	0	n.d.	2.8	5.5	0.74
BL470	0	n.d.	2.4	5.8	0.78
BL478	0	2.7	2.5	6.8	0.92
BL483	0	3.2	2.3	6.6	0.89

* n.d. = not determined.

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