THE DUAL GENETIC CONTROL OF ORNITHINE TRANSCARBAMYLASE SYNTHESIS IN *ESCHERICHIA COLI* K12

N. GLANSDORFF^a, G. SAND^a AND C. VERHOEF^b

^a Laboratory of Microbiology, University of Brussels, Brussels (Belgium) and

^b Laboratory of Microbiology, Utrecht (The Netherlands)

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SUMMARY

Mutations at 2 distant genes influence ornithine transcarbamylase synthesis in *Escherichia coli* K12. The wild type alleles of either of them restores to an OTC-less strain the ability to synthesize this enzyme under feedback control by arginine.

INTRODUCTION

Among bacterial mutants, examples in which a specific metabolic block may find its origin in mutations at more than one genetic locus are scarce or non-existent, if certain defects in regulation or permeability are barred. Indeed, mutations that affect the structure of a given bacterial enzyme are usually clustered at a single locus. This prevalent one-locus-one-enzyme relationship contrasts with the intricate polygenic control of certain proteins in higher organisms, as, for example, the phosphatases of *Aspergillus nidulans*¹.

The present results, however, show that *E. coli* K12 contains 2 loci, quite distant from each other, at which mutations may specifically affect the cell capacity to produce ornithine transcarbamylase (OTC), the enzyme that performs the sixth step of arginine biosynthesis. Peculiarly, the wild type allele of either of these loci restores to an OTC-less strain the ability to synthesize this enzyme; in both cases the restored synthetic ability is still repressible by arginine. The first known of these loci, already mapped by MAAS AND MAAS¹² in an *E. coli* K12-*E. coli* B hybrid, has been called *argF* in the first terminology of *E. coli* arg determinants based on DEMEREC's nomenclature (refs. 4, 13). The symbol *argI* will be used here to designate the new locus.

Abbreviations: *Ade*, adenine; *arg*, arginine; *can*, canavanine; *cit*, citrulline; *his*, histidine; *lac*, lactose; *leu*, leucine; *met*, methionine; *pro*, proline; *rha*, rhamnose; T5, phage T5; *thi*, thiamine; *thr*, threonine; *str*, streptomycin; Hfr, male, donor; F⁻, female, recipient; s, sensitive; r, resistant; OTC, ornithine transcarbamylase.

TABLE I

THE STRAINS USED

Strain	<i>ade</i>	<i>arg</i>	<i>his</i>	<i>leu</i>	<i>met</i>	<i>pro</i>	<i>thi</i>	<i>thr</i>
Hfr B-7	+	+	+	+	—	+	+	+
Hfr R ₄	+	+	+	+	+	+	+	+
Hfr R ₄	+	+	+	—	+	<i>proA</i>	+	—
Hfr P ₄ X	+	+	+	+	<i>metB</i>	+	+	+
Hfr P ₈₀₁	+	+	+	+	+	+	—	—
Hfr P ₁₀	+	+	+	—	+	+	—	—
Hfr P ₇₂	+	+	+	+	<i>metB</i>	+	—	+
Hfr HfrH	+	+	+	+	+	+	—	+
Hfr 30SO	+	+	+	+	+	+	—	+
F [−] K ₁₂	<i>adeK</i> *	+	+	—	+	<i>proA</i>	+	—
F [−] K ₁₂	<i>adeK</i>	+	+	—	+	+	+	—

* *AdeK* (ref. 16) is probably identical with *pur B* (ref. 17). Hfrs B-7, P₈₀₁ and P₇₂ were obtained through the courtesy of R. Lavallé. All Hfr's were streptomycin-sensitive and the F[−] strains resistant.

MATERIALS AND METHODS

Strains. Derivatives of *E. coli* K₁₂, listed in Table I, were used.

Interrupted mating experiments. Exponentially-growing broth cultures of Hfr and F[−] strains were mixed in the proportion 1:10 respectively. Samples were taken at intervals, diluted in broth and shaken vigorously for 30 sec. Aliquots were plated on selective media. All manipulations were carried out in a hot room at 37°.

Transduction experiments. Phage 363 was used¹⁰; techniques as in GLANSDORFF⁸.

Selection of recombinants. Samples of 0.1 ml were plated on minimal medium supplemented with glucose (0.2%) and, whenever required, with streptomycin (200 µg/ml) and growth factors in the following final concentrations (µg/ml): adenine (20), L-arginine (100), L-leucine (40), L-methionine (40), L-proline (30), thiamine (1), L-threonine (100).

Enzyme measurements. Bacteria were grown aerobically at 37° in minimal medium supplemented with nutritional requirements (see above). Enzyme activity was measured in cells permeabilized by toluol or in ultrasonic extracts, and is respectively expressed in µmoles of citrulline formed/h per mg dry weight or per mg protein.

OTC assay: see ref. 13. Argininosuccinase was assayed according to ref. 15; however the concentration of argininosuccinate in the incubation mixture was raised to 10 µmoles/ml.

RESULTS

Isolation of an ornithine transcarbamylase-less mutant (OTC[−]) and construction of various derivatives

An OTC-less mutant is expected to grow on citrulline but not on ornithine (Fig. 1). Such an organism has been isolated from the *thr leu proA* derivative of Hfr R₄. After exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine¹ this strain was submitted to penicillin treatment⁹, growth in the presence of citrulline being carried out in alternation with exposure to penicillin in the presence of ornithine.

A mutant exhibiting the expected phenotype was crossed with F[−] K₁₂ *adeK*

Fig. 1. Map of the *arg* loci of *E. coli* K12, with an outline of the corresponding enzymic steps (gene-enzyme correspondence as in ref. 13). The map is not precisely to scale; as explained in the text, *argF* and *I* are cotransducible with *proA* and *adeK* respectively. See ref. 16 and refs. 3, 18, for precise localization of *adeK* and *proA*. Whereas Hfr 3050 and Hfr H have the same origin of transfer, this is not known for Hfr P4X and R4. Order of proximal markers transferred by Hfr P72: see ref. 7.

thr leu, and a $F^- ade^+ thr leu cit str^R$ recombinant was isolated, in order to obtain a *cit* recipient for mating experiments. The following derivatives were then prepared: an $F^- thr^+ leu adeK proA cit str^R$ recombinant from a cross between K12 *adeK thr leu proA*, and a *thr^+* revertant of Hfr R4 *thr leu proA cit*; and an $F^- T5^R thr leu proA cit str^R$ recombinant from a cross between $F^- thr leu cit str^R$ and a $T5^R$ derivative of Hfr R4 *thr leu proA cit*.

Ornithine transcarbamylase in the mutant

Extracts were prepared from cultures of Hfr R4 *thr leu proA cit* and $F^- thr leu cit$ (see above) growing exponentially in minimal medium supplemented with arginine and other requirements. Under the latter conditions, *arg^+* *E. coli* K12 strains exhibit an OTC specific activity of about 1.5 units per mg protein. In both organisms the activity was equal to or less than 0.2 units. More precise measurements were hindered by interference of an unknown substance giving a faint red colour with the reagents utilized. A similar observation has been made with OTC-less mutants of *Saccharomyces cerevisiae* (A. PIERARD, unpublished).

Mapping the OTC⁻ mutation by interrupted mating experiments

A set of 8 Hfr *str^S* strains, at least 6 of which exhibit distinct origins of transfer (Fig. 1), were crossed either with F⁻ *thr leu cit* or F⁻ *thr leu proA cit str^R*. Aliquots were shaken and plated at intervals for the selection of *cit⁺ str^R*, *thr⁺ leu⁺ str^R* or *pro⁺ str^R* recombinants. The results are grouped in Table II. In crosses No. 2, 3, 4, 6 and 9, the order deduced from entrance curves has been confirmed by recombinant analysis.

TABLE II

TRANSFER OF *thr*, *leu*, *pro* AND *cit* LOCI BY VARIOUS Hfr STRAINS

Recipient	Donor	Cross No.	Order of transfer*
<i>thr⁻, leu⁻, cit⁻</i>	P10	1	no early transfer of <i>cit⁺</i>
	P72	2	o ---- <i>cit</i> ---- <i>thr-leu</i>
	30SO	3	o ---- <i>thr-leu</i> ---- <i>cit</i>
	Hfr H	4	o ---- <i>thr-leu</i> ---- <i>cit</i>
	P801	5	<i>cit</i> ---- <i>thr-leu</i> ---- o
	P4X	6	<i>thr-leu</i> ---- <i>cit</i> ---- o
	R4	7	<i>cit</i> ---- <i>thr-leu</i> ---- o
	B7	8	<i>thr-leu</i> ---- <i>cit</i> ---- o
<i>thr⁻, leu⁻, proA⁻, cit⁻</i>	P4X	9	<i>thr-leu</i> ---- <i>proA</i> ---- <i>cit</i> ---- o

* Distances: 7-10 min transfer time between *cit* and *thr-leu* in crosses 3, 4, 6, 8; 2-3 min in crosses 2, 5, 7; 0.5 min between *cit* and *proA* in cross 9.

Hfr P10 transferred no *cit⁺* allele during the period investigated (30 min). The 7 other Hfr strains fall into 2 categories (see Table II and Fig. 1).

(1) Hfr P72, P801 and R4, which transfer a *cit⁺* allele situated very close to *thr-leu* and to the left of it (as the chromosome is usually drawn).

(2) Hfr 30SO, H, P4X and B-7, which transfer a *cit* allele located on the right of *thr-leu*, at a distance corresponding to 7-10 min of transfer time. To this category belong both Hfrs (P4X and 30SO) used by MAAS AND MAAS¹² to map an OTC⁻ mutation found in *E. coli* B (GORINI, quoted by the latter authors) and mapped in a B-K12 F⁻ hybrid.

Rather than assume the existence of 2 classes of Hfr strains as regards the location of a unique *cit* locus, we considered that each of them might contain 2 *cit* loci, one on the left of *thr-leu* (*argI*), one on the right (*argF*), the wild type allele of either of these loci being able to compensate the deficiency of the F⁻ strain. Indeed, in a mating between such an Hfr and F⁻ *thr leu cit* only the entrance of the first transferred *cit⁺* allele would be observed; on the selective plates, the few *cit⁺* recombinants due to the integration of the distal allele—entering F⁻ cells about 12 min later—would be interspersed among recombinants carrying the proximal allele.

We can see from Fig. 1 that the results of Table II do not contradict this hypothesis; however, since Hfr R4 does not transfer a *cit* marker before *thr-leu*, we are led to assume either that the origin of the R4 chromosome lies between *proA* and *argF*, or, if this origin is identical with that of P4X, that *argF* is absent from, or non-functional in, R4. A corollary of this hypothesis is that it should be possible to manipulate *argI* and *F* independently of each other by cotransduction with neighbouring genes. Moreover this type of experiment could show whether R4 does or does not contain an *argF* allele to the right of *thr-leu*. The following section provides an answer to this question.

Mapping the cit loci by transduction experiments

Among the markers which a priori could be cotransducible with one of the *cit* alleles, we considered *proA* (0.5 min of transfer from *argF*) and *adeK* (between the transfer origins of Hfr Pro and 30SO, Fig. 1).

The F⁻ strain *adeK leu proA cit* was used as recipient in a set of transductions which involved as donors several of the Hfrs tested in the mating experiments. *Ade*⁺, *pro*⁺, *cit*⁺, *ade*⁺ *cit*⁺, *pro*⁺ *cit*⁺, *thr*⁺ *cit*⁺ and *leu*⁺ *cit*⁺ transductants were selected.

TABLE III

TRANSDUCTION ANALYSIS OF LINKAGE BETWEEN *cit*, *adeK*, *proA*, *thr* AND *leu*
Transductants/0.1 ml of phage-infected bacterial suspension (multiplicity: 0.2 to 0.1)

Donor	Recipient	<i>cit</i> ⁺	<i>ade</i> ⁺	<i>pro</i> ⁺	<i>thr</i> ⁺ <i>cit</i> ⁺	<i>leu</i> ⁺ <i>cit</i> ⁺	<i>ade</i> ⁺ <i>cit</i> ⁺	<i>pro</i> ⁺ <i>cit</i> ⁺
P72	<i>adeK</i> , <i>cit</i>	880	970	770	—	—	7	183
30SO	<i>thr</i> ⁺ , <i>leu</i> ,	300	1210	870	—	—	6	300
P4X	<i>proA</i>	2860	3340	1900	—	0	20	221
P801		4240	5280	2940	—	2	40	710
R4		2540	4660	2360	—	0	34	2
P72	<i>adeK</i> , <i>cit</i> ,	6600	5480	—	0	0	60	—
30SO	<i>thr</i> , <i>leu</i>	2310	2890	—	0	0	28	—
R4	<i>cit</i> , <i>thr</i> , <i>leu</i> , <i>proA</i>	—	—	5240	—	—	—	2

Table III gives the results of these experiments. It is clear that *adeK* is always weakly but definitely cotransduced with a *cit* marker (*argI*); the cotransduction index is 0.5% to 1%. Neither *leu* nor *thr* are cotransducible with a *cit* allele. A strong cotransduction of *proA* with a *cit* marker (*argF*) always occurs (about 25%) *except* when Hfr R4 is used as the donor. This strain thus seems to carry only *argI* in a functional state. It is noteworthy that R4 is precisely the strain that was used as starting material to isolate the OTC⁻ mutant; moreover, to our knowledge, R4 is up to now the only K12 strain to have permitted the isolation of such a mutant.

The cotransduction of *proA* and *argF* enabled us to prepare a Hfr *pro*⁺ *argF*⁺, from the *proA cit* derivative of Hfr R4. As expected, the new *pro*⁺ *argF*⁺ Hfr, when crossed with an F⁻ *proA argF*, transfers *argF*⁺ before *proA*⁺, in the first 2 min of mating.

The possibility of handling *argI* and *F* separately has prompted us to determine their respective contributions to the synthesis of OTC.

Enzymic constitution of strains carrying argI, argF or both

To disclose the role of each *cit* allele in OTC synthesis we determined the OTC specific activity in an (*ade*⁺ *argI*⁺) *argF proA* and a (*pro*⁺ *argF*⁺) *argI adeK* transductant, both issuing from transduction No. 1 (Table III). Added to the results (Table IV) are those obtained with an *ade*⁺ revertant of the latter transductant, in order to detect any non-specific effect of adenine, which is known to be slightly growth inhibitory. These organisms were cultured with and without arginine, in order to test the repressibility of OTC synthesis. As reference, we measured the specific activity of argininosuccinase, the last enzyme of the biosynthesis.

In Table IV the enzymic constitutions of the above transductants are compared with those of Hfr R4 (*argI*⁺ *argF*) and Hfr P4X (*argI*⁺ *argF*⁺). We completed this set

of results by determining the maximal capacity of OTC production in strains carrying the genotypes (*argI*⁺ *argF*⁺), (*argI* *argF*⁺) and (*argI*⁺ *argF*), respectively; this was achieved by assaying OTC in canavanine-resistant mutants of Hfr P4X (*argI*⁺ *argF*⁺),

TABLE IV

ORNITHINE TRANSCARBAMYLASE AND ARGININOSUCCINASE-SPECIFIC ACTIVITIES IN *argF argI*⁺, *argF*⁺ *argI* AND *argF*⁺ *argI*⁺ STRAINS (μmoles/h/mg dry weight)

Strain	Ornithine transcarbamylase		Argininosuccinase	
	- <i>arg</i>	+ <i>arg</i>	- <i>arg</i>	+ <i>arg</i>
(<i>pro</i> ⁺ <i>argF</i> ⁺) <i>argI adeK</i>	7.8	1.3	0.67	0.1
(<i>pro</i> ⁺ <i>argF</i> ⁺) <i>argI ade</i> ⁺	9.4	2.0	0.55	0.1
idem, <i>can</i> ^r	268	—	3.9	—
(<i>ade</i> ⁺ <i>argI</i> ⁺) <i>argF proA</i>	22.0	1.8	0.46	0.1
R4 (<i>argI</i> ⁺ <i>argF</i>)	29.2	5.4	0.30	0.1
idem, <i>can</i> ^r	685	—	3.7	—
P4X (<i>argF</i> ⁺ <i>argI</i> ⁺)	35.0	1.0	0.7	0.1
idem, <i>can</i> ^r	600*	—	3.5	—

* Values up to 800 are not uncommon among *can*^r derivatives of K12 strains.

Hfr R4 (*argI*⁺ *argF*) and the *ade*⁺ revertant of transductant (*pro*⁺ *argF*⁺) *argI adeK*; indeed, canavanine is a powerful selective agent of *R*^{-arg} mutants, in which the enzymes of arginine biosynthesis are constitutively synthesized at the highest rate attainable by wild type strains¹¹. We selected such mutants in AF canavanine medium¹¹ and again used the argininosuccinase assay as a check of the regulatory properties of the 3 strains isolated; argininosuccinase-specific activities actually measured (3.5 to 4.0 units/mg dry weight) are typical of *R*^{-arg} mutants.

From Table IV we gather the following information: (1) *argF*⁺ *argI* and *argF* *argI*⁺ organisms both have appreciable, although different, OTC specific activities; (2) OTC synthesis is repressible in both types of strain; (3) OTC-specific activity is 2 or 2.5 times lower in *argF*⁺ *argI* than in *argF* *argI*⁺ organisms whether it is determined in *R*⁺_{arg} or *R*^{-arg} derivatives of these strains. Yet the internal supply of arginine is not restricted in *argF*⁺ *argI* *R*⁺_{arg} cells, since argininosuccinase synthesis is not substantially derepressed in the latter (lines 1 and 2) and since OTC synthesis in an *argF*⁺ *argI* organism can be further derepressed by the introduction of a *can*^R allele (line 3). There could be a slight, genuine overproduction of OTC in K12 strains, as suggested independently by the observation that, in Hfr P4X, low doses of canavanine reduce OTC synthesis by about 20% without influencing the growth rate (GLANSDORFF, unpublished); in addition, there could exist, between the carbamylases synthesized by *argF*⁺ *argI* and *argF* *argI*⁺ cells, such a difference that their relative activities *in vitro* would not truly parallel their relative efficiencies *in vivo*.

DISCUSSION

We have shown that 2 unlinked loci, *argF* and *I*, must be taken into account in order to understand the genetic control of OTC synthesis in *E. coli* K12. Either of the wild type alleles of these loci confers to an OTC-less strain the ability to synthesize this enzyme under feedback control by arginine. This could be explained in at least two ways: (1) *argF* and *argI* code for different subunits of a heteropolymeric OTC; (2)

argF and *argI* each codes for a protein able to carbamylate ornithine. The 2 loci could be the products, more or less differentiated by mutation, of the duplication of an ancestral OTC gene.

The ability of each locus alone to compensate the deficiency of an OTC-less strain obviously favours the second hypothesis, but is by no means incompatible with the first: indeed the *argF* and *argI* mutations considered here might determine an *arg*⁻ phenotype only when they are combined in the same genome. Hypothesis 2 is also favoured by the fact that attempts to isolate OTC-less mutants from various K12 strains failed until Hfr R₄, genuinely *argI*⁺ *argF*⁻, was used. A critical test of the aforementioned possibilities (and of any other) would be to examine the properties of mutants harbouring a deletion of *argF* or *argI*. In this respect, some preliminary evidence again favours hypothesis 2: the XIII *lac-pro* deletion mutant² is reported to lack the loci *lac* and *proB* (the latter situated between *lac* and *proA*); the strains harbouring this mutation are *arg*⁺. We carried out transductions between different *F*⁻ *leu*⁻, *proA*⁻, *cit*⁻ recipients and a XIII donor, kindly provided by H. NIJKAMP. In a typical experiment 62640 *leu*⁺ recombinants were recovered, 19040 *cit*⁺ and no *pro*⁺. The latter result could mean that the deletion extends itself beyond *proB*, over *proA* and consequently over *argF*, which is situated between *lac* and *proA*; the *cit*⁺ recombinants would then only reflect the integration of the *argI*⁺ allele present in the donor. However, the experiment is not demonstrative since failure to recover *proA*⁺ transductants does not prove that the *proA* locus is absent in XIII strains. If further experiments confirmed that a strain harbouring a deletion of *argF* (or *argI*) is nevertheless *arg*⁺, hypothesis 2 would appear very likely.

More elaborate interpretations are not excluded: one of the *arg* loci, say *argF*, could act as a suppressor of *argI* deficiencies. However, at present, it seems difficult to visualize such a situation in more precise terms. Indeed, such a suppressor (in the broader sense of this term) not only ought to be present in several independently isolated Hfrs, each of them provided with a functional *argI* locus, but should also suppress the phenotypes of two independent OTC-less mutants: the present one and the *E. coli* K12-*E. coli* B hybrid isolated by MAAS AND MAAS¹² from an OTC-less mutant of *E. coli* B. Considering the situation the other way around (*argI* as suppressor of *argF* defects) raises similar difficulties. Along this line it may be mentioned that aspartate transcarbamylase, the genetic locus of which is close to *adeK* and *argI*, and which may modify the amount of enzymes in the arginine pathway by provoking interferences of regulation between arginine and pyrimidine biosynthesis^{8,20} is detectable in the OTC-less mutant; neither is its synthesis derepressed nor is this enzyme modified in its response to its allosteric inhibitor (M. MERGEAY, personal communication).

A comparison between ornithine transcarbamylases extracted from the wild type, *argF*⁺ *argI*⁻ and *argF*⁻ *argI*⁺ strains, combined with further genetic studies, will be required before definite conclusions can be drawn.

In Hfr R₄, *argF* is not functional, or at least is unable to compensate the defect of an OTC-less strain. As an *argF*⁺ R₄ Hfr may be constructed by transduction, it is reasonable to think that a recent ancestor of this strain carried the 2 loci in a functional state. If the *argF*⁺ *argI*⁺ condition was only weakly favoured by selection with respect to the *argF*⁻ *argI*⁺ condition, a strain of the latter genotype could have arisen and been maintained in the artificial surroundings of the laboratory.

It is not yet known whether the B and W strains of *E. coli* are similar to the K12 strains as regards the genetic control of ornithine transcarbamylase. For *E. coli* B, no mapping involving B donors has yet been reported; the most recent map of the chromosome of the W strain¹⁹ shows a *cit* marker in the region where *argF* is found in K12; the relevant experiments have not been published. *ArgI* is situated near *pyrB* (ref. 17) which directs aspartate transcarbamylase synthesis; a similar situation occurs in *Bacillus subtilis*¹⁴, where, however, only one *cit* locus has been reported.

As regards the topology of the *E. coli* chromosome, the sequence *proA-argF-lac* is to be substituted for the order *argF-proA-lac* (ref. 17).

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