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Promoter Mapping and Selection of Operator Mutants by Using Insertion of Bacteriophage Mu in the *argECBH* Divergent Operon of *Escherichia coli* K-12

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The analysis of a large number of Arg mutants obtained by inserting phage Mu in the *argECBH* cluster of genes confirmed the "facing" arrangement proposed earlier for the promoters of *argE* (*argEp*) and *argCBH* (*argCBHp*) and clarified remaining ambiguities regarding the localization of *argEp*. Casadaban and Cohen's Mu d *lac* phages (M. Casadaban and S. N. Cohen, Proc. Natl. Acad. Sci. U.S.A. **76**:4530–4533, 1979) were used to construct strains where either an intact or a truncated *lacZ* gene was fused to *argC* or *argB*. Several operator-constitutive mutations could be selected for in such strains; the mutations affected both arms of the cluster, thereby defining one common operator region for both directions of transcription.

The occurrence of divergent operons transcribed from promoters facing each other over an internal operator region was disclosed some years ago by the study of deletions affecting the *argECBH* divergent gene cluster in *Escherichia coli* (12). Other mutations specifically identified an operator region (2, 3, 7, 12, 16) or displayed a dual effect; certain nucleotide substitutions in the sequence which codes both for the leader of the *argE* message and for the promoter of *argCBH* (*argCBHp*) were found to increase the rate of translation of the *argE* message and to adversely affect *argCBHp* (2, 9; J. Piette, A. Boyen, R. Cunin, D. Charlier, M. Crabul, F. Van Vliet, N. Glansdorff, C. Squires, and C. L. Squires, manuscript in preparation). However, no point mutation specifically altering the promoter for *argE* (*argEp*) could be isolated up to now. The location of *argEp* was left undefined in a 0.3-kilobase-long segment containing the proximal part of *argC*, between the ends of two deletions: *sup-400* (Fig. 1.; 12), which does not affect *argE*, and *sup-102*, which abolishes expression of that gene without hampering transcription of *argH*. A more refined mapping was needed to interprete DNA sequence data.

The lack of specific techniques for isolating *argEp* "up" or "down" mutants prompted us to investigate the effects of insertion of bacteriophage Mu in the proximal part of the *argCBH* operon in order to localize the *argE* promoter. In addition to the thermoinducible Mu cts62 strain, we used the defective Mu d *lac* ampicillin-resistant (Ap^r) derivatives constructed by Casadaban and Cohen (reference 6 and unpublished data); this approach enabled us to obtain fusion between *argC*- or *argB*-encoded proteins and β-galactosidase with a view to sequencing the amino-proximal ends of those enzymes. Moreover, fusion strains were used to investigate whether operator mutations obtained by selecting for constitutive, *argC*-dependent β-galactosidase synthesis would provide evidence for a specific *argCBH* repressor-binding site.

MATERIALS AND METHODS

Media. Minimal medium 132 and complex media 869, 856, and 855 have been described (14). When needed, supplements to minimal medium were as follows: ampicillin, 25 μg/ml; L-arginine hydrochloride, 100 μg/ml; *N*-α-L-acetylarginine, 25 μg/ml; L-methionine, 50 μg/ml; thiamine hydrochloride, 1 μg/ml; lactose and glucose, 0.2% (wt/vol).

Strains. Table 1 lists the bacterial and phage strains used.

Phage manipulations. (i) Lambda derivatives. The standard transduction technique has been described (17). For the plate recombination and complementation tests, recipient cells were first streaked in patches of about 1.5 cm^2 on minimal medium with the necessary supplement. After 24 h of growth at 32°C, they were replicated onto minimal plates (without arginine) seeded with equivalent quantities (about 2.10^9) of the transducing phages and of the parental helper phage λ 199 in soft agar. The lysates were obtained by thermoinduction of the appropriate MN42 λ^- lysogen as previously described (17). The plates were incubated for 48 h at 32°C.

(ii) Mu cts62. To prepare Mu cts lysogens, a fresh lysate of phage Mu was spotted onto 10^8 cells of strain

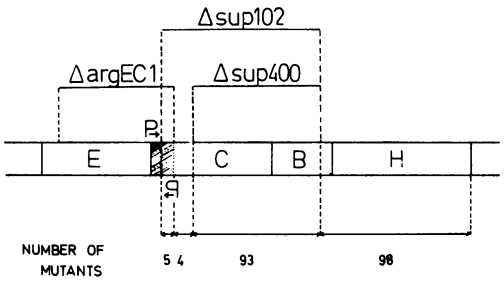

FIG. 1. Deletion mapping of thermosensitive mutants of the *argCBH* locus recovered after lysogenization of strain P4X with thermoinducible bacteriophage Mu *cts*62.

P4X poured in soft agar (855) on a plate of medium 856 supplemented with $CaCl_2$ (5 mM) and $MgSO_4$ (10 mM). Independent cultures were inoculated from different places within the area of the spots after 15 h of incubation at 32°C. Lysogens containing a Mu phage inserted at an *arg* locus were identified by replica plating after two successive penicillin enrichments (15).

(iii) Mu *cts* d (Ap^r *lac*) and Mu *cts* d II301 (Ap^r *lac*). Lysates of these phages (and of the Mu *cts* helper) were prepared from strains Mal103 and Mal315, respectively, and used to prepare ampicillin-resistant lysogens of strain MC4100 as previously described (6). Lysogenic cells were then either submitted to two successive D-cycloserine enrichments (using 50 μg of D-cycloserine per ml for 90 min) to obtain Arg mutants or plated directly on minimal plates supplemented with lactose, thiamine, acetylarginine, and ampicillin (see text).

Reversion tests of Mu *cts* insertions. The reversion test was adapted from Bukhari (4) as follows. From a particular Mu lysogen, 50 temperature-resistent derivatives were isolated after incubating 2×10^8 cells on plates of medium 856 for 2 h at 43°C. The 50 colonies were purified once and pooled in groups of 10. The pools were grown up to stationary phase in supplemented minimal medium, and samples of about 10^{10} cells of each culture were screened for the presence of revertants on minimal medium.

Enzyme assays. *N*-α-L-acetylornithinase (EC 3.5.1.16), arginosuccinase (EC 4.3.2.1), and ornithine carbamoyltransferase (EC 2.1.3.2) were assayed as described previously (12). β-Galactosidase was assayed according to Miller (18).

RESULTS

Rationale. Any *argEp* down mutant would be recognized as a slow utilizer of *N*-α-acetylarginine, a relatively low-affinity substrate for acetylornithinase, the *argE*-encoded enzyme (1). The deacylation of acetylarginine rather than acetylornithine offers the advantage of bypassing the whole pathway. The polar effect that a Mu phage exerts on the distally located genes would be recognized by slow growth on ornithine (12).

Screening for insertions at the *argECBH* locus was readily achieved by replica plating (see Materials and Methods) on lawns of λ d *arg* transducing phages carrying either the whole *argECBH* cluster or a particular deletion (*argEC1*, *sup-102*, *sup-400*, or *argBH100*; Fig. 1). Mu insertions in *argH* would be complemented by λ *sup-102* or *sup-400*; insertions in *argE* would be complemented by λ *sup-400* and *argBH100*; insertions in *argB*, in *argC*, or in the control region would not be complemented by any of those phages but would recombine with them to various extents. This plate test proved to be a good screening tool; for example, recombination between mutant *argEC1* and *sup-400* (carrying two deletions ending about 0.2 kilobases apart) gave about 50 colonies on a 1.5-cm^2 patch.

TABLE 1. Strains used

Strain	Genotype	Source or reference
Bacteria		
MC4100	F$^-$ *araD139* Δ(*lacIPOZYA*)*U169 rpsL thi*	5
Mal103	F$^-$ Mu *cts* d1 (Ap^r *lac*) Mu *cts* Δ(*proB lacIPOZYA*)*XIII rpsL*	6
Mal315	F$^-$ Mu *cts* d II301 (Ap^r *lac*) *mal*(λ^r)::Mu *cts* *araD139* Δ(*araAIOC leu*)*7697* (*proAB*, *lacIPOZYA*)*XIII rpsL*	M. Casadaban
P4X λ$^-$	Hfr *metB relA*, cured of λ	17
P4XB2 λ$^-$	As P4XB2 λ, *argR*	12
MN42 λ$^-$	Hfr *metB* Δ(*ppc-argECBH*) *relA*, cured of λ	17
Phage		
Mu *cts*62	Thermoinducible Mu-1 phage	A. Toussaint
λ13	d *ppc argECBH bfe*	17
λ13 *sup-102*	d *ppc sup-102 bfe*	17
λ13 *sup-400*	d *ppc sup-400 bfe*	17
λ13 *argEC1*	d *ppc argEC1 bfe*	17
λ13 *argBH100*	d *ppc argBH100 bfe*	2
363	P1-like transducing phage	14

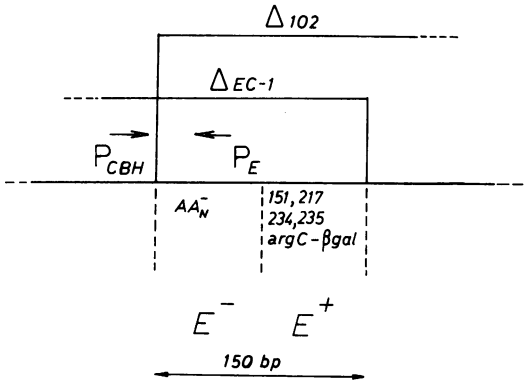

FIG. 2. Detail of Fig. 1: mutations mapping in the overlap between the *argEC1* and *sup-102* deletions. Only mutant AA^-_N shows impaired utilization of acetylarginine, hence the assignment of *argEp* to the left-hand side of the overlap. bp, Base pairs.

Promoter mapping using Mu cts62. From a total of 60 independent cultures of Hfr strain P4X exposed to phage Mu *c*ts62, about 300 Arg mutants were recovered which lysed on exposure to 43°C. They could be (i) genuine insertions at an *arg* locus, (ii) rearrangements (such as deletions; 13) due to phage Mu interaction with an *arg* locus, or (iii) spontaneous Arg mutants lysogenized by phage Mu. The latter should be rare since, in our hands, two successive applications of the penicillin enrichment procedure did not bring the proportion of spontaneous Arg mutants in a wild-type population to more than 1%. Categories (i) and (ii) should constitute the majority, insertions being expected to outnumber other rearrangements (4).

A group of 200 mutants found to be affected at the *argCBH* locus by their ability to be complemented by transducing phage λ 13 and by their slow growth in the presence of ornithine were mapped more precisely by the plate test described above. The data (Fig. 1) suggested no conspicuous clustering of target sites when considering the respective lengths of the regions involved. The mutants mapping in the segment defined by the left-hand extremities of deletions *sup-102* and *sup-400* (AA^-_N, 151, 23, 216, 217, 234, 235, 274, and 278) were analyzed further with the *argC* enzyme–β-galactosidase protein fusion strain described in the next section. All except strains 23 and 216, which were probably short deletions, appeared to be genuine Mu insertions as indicated by the occurrence of Arg^+ revertants in cultures or thermoresistant derivatives (4) and by concomitant loss of thermosensitivity and acquisition of the Arg^+ phenotype during transduction by phage 363. All but one (AA^-_N) grew normally on acetylarginine as the source of arginine. The mutation of strain AA^-_N was localized with four other mutations (including the *argC* enzyme–β-galactosidase protein fusion strain) in the overlap between the *argEC1* and the *sup-102* deletions (Fig. 2). The results of the plate test were confirmed by standard transduction techniques (17), in conditions where 1,000 recombinants were obtained in the 0.2-kilobase-long *argEC1–sup-400* interval.

Enzyme assays performed on mutants AA^-_N and 151 and on their *argR* derivatives (Table 2) confirmed that the slow utilization of ornithine was due to the polar effect that the Mu prophage exerts on *argH*. The data also confirmed that mutant AA^-_N had a very low specific activity for acetylornithinase. However, this was not the case for mutant 151, which also harbored a Mu prophage in the *argEC1–sup-102* overlap. *argE* expression was less severely affected by mutation AA^-_N than by the *sup-102* deletion, suggesting that the insertion had left *argEp* partially intact. In addition, strain AA^-_N showed the characteristic, unique among the 165 mutants investigated, of reverting spontaneously, albeit at a low frequency (about 10^{-8}), to slow-growing Arg^+ revertants. This feature suggested that the insertion could be confined to a control region distinct from the structural *argC* gene, so that most of the revertants would consist of imprecise excision of Mu or of rearrangements allowing some expression of the adjacent *argE* and *argC* genes. By contrast, one would expect the reversion pattern of a Mu insertion within a structural gene to be much more restricted.

TABLE 2. Enzyme specific activities in mutants harboring Mu insertions in the *argEC1-sup102* overlap

Strain[a]	Sp act (μmol/h per mg of protein)		
	Acetylornithinase (*argE*)	Arginosuccinase (*argH*)	Ornithine carbamoyltransferase (*argF argI*)
AA^-_N	0.4	0.05	
AA^-_N *argR*[b]	1.6	0.2	1,144
151	2.2	0.05	
151 *argR*	30	0.1	1,615
P4XB2 *argR*	40	3.1	1,170
P4X *sup-102 argR*	0.2	5.0	

[a] All cultures were grown in minimal medium supplemented with arginine.

[b] *argR* derivatives were obtained by transducing (14) the insertion into a P4XB2 (*argR*) derivative carrying the *ppc argECBH* deletion no. 42 (12), selecting for Ppc^+ recombinants, and checking for the presence of Mu as in the original mutant (see text).

TABLE 3. Specific activities of acetylornithinase and arginosuccinase in thermoresistant derivatives of strain N325 (Mu insertion in *argC*)

Strain[a]	Sp act (μmol/h per mg of protein)	
	Acetylornithinase	Arginosuccinase
T5	0.04	0.1
T5 *argR*[b]	0.47	0.55
T7	0.03	0.12
T7 *argR*	0.24	0.70
N325[c]	4.0	0.02

[a] Cultures were grown in minimal medium supplemented with arginine.

[b] *argR* (derepressed) derivatives; see Table 2.

[c] A culture of N325 derepressed by using acetylarginine (25 μg/ml) as the source of arginine gave 59 and 0.02 U for acetylornithinase and arginosuccinase, respectively.

Many thermoresistant derivatives of a Mu *c*ts lysogen are deletions eliminating all or part of the phage DNA as well as adjacent regions (13). Strain N325, with a Mu *c*ts inserted in *argC*, was used to generate deletions entering the control region. Derivatives resisting exposure to 43°C were selected on complex medium 856. Among about 2,000 of these, replica plating disclosed many colonies growing better than the parental strain in the presence of ornithine, an indication that the polar effect of Mu was at least partially released and thus that *argCBHp* was left intact. Among these, two grew very slowly in the presence of acetylarginine, suggesting that *argEp* was affected.

As expected from the above analysis of insertion mutants, the recombination test proved these two strains (T5 and T7) to harbor deletions reaching into the *argEC1–sup-102* overlap. This was confirmed physically by heteroduplex analysis after transfer of one of these mutations (T5) on a λ 13 transducing phage (data not shown). Enzyme assays (Table 3) showed that *argE* expression was low, albeit still repressible in both mutants, whereas the polar effect on *argH* was considerably reduced. Therefore, the deletions would appear to have damaged *argEp* but left *argCBHp* largely intact. The residual polar effect observed on *argH* could have been due to remnants of an imprecisely excised prophage, to a translational frameshift or to an alteration of the *argC* ribosome-binding site.

Inserting the lactose operon into *arg* genes: selection of regulatory mutants. The defective Mu *c*ts d (Ap^r *lac*) phage constructed by Casadaban and Cohen (6; Fig. 3, line 1) makes it possible to insert the gene for β-galactosidase, devoid of a functional promoter, into any gene (*x* in Fig. 3), the malfunctioning of which can be alleviated by some growth condition. If the phage is properly oriented (Fig. 3), transcription proceeds unimpeded from the promoter of gene *x* (P_x) into *lac*. Such an insertion mutant in the *argC* or *argB* gene of a Lac$^-$ strain would become Lac$^+$ if the arginine regulon was derepressed, for example, if acetylarginine was used as the source of arginine at a low concentration (25 μg/ml) (8). Regulatory mutants could then be selected as Lac$^+$ colonies in the presence of arginine. Phage Mu *c*ts d II301 (M. Casadaban, unpublished data; Fig. 3, line 2) contains a truncated *lacZ* gene that may become functional downstream from the translational start of an actively transcribed gene; a "fused" protein is obtained, composed of a fragment of the NH_2-proximal sequence of gene *x* linked by Mu-encoded amino acids to the truncated β-galactosidase. The phenotype of such fusion strains and their potentialities as sources of regulatory mutants would be the same as in the foregoing case. In addition, they might considerably facilitate the determination of the NH_2-proximal amino acid sequence of the gene product becoming fused to β-galactosidase.

(i) Operon fusion strains. Ampicillin-resistant Arg mutants were obtained after two successive D-cycloserine enrichments performed at 29°C with a population of Lac$^-$ 4100 cells; they were screened for their Lac phenotype in the presence of arginine and acetylarginine, respectively. Among a sample of 40 strains found to be Lac$^+$ in the presence of acetylarginine and Lac$^-$ in the presence of arginine, almost all possible types of insertion into *arg* genes were represented (except in *argE*), including several ones in *argC* or *argB*, as indicated by growth and plate recombination tests. One of the latter (no. 20) was assayed and found to synthesize 20 times as much β-galactosidase when acetylarginine (25 μg/ml) was the source of arginine rather than this amino acid itself (383 versus 20 U/h per mg of protein).

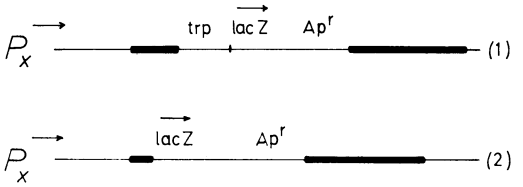

FIG. 3. Simplified scheme for the structure of fusion strains. (1) Operon fusion: a *lacZ* gene devoid of promoter becomes expressed from P_x over a Mu fragment devoid of transcriptional stop signals (6). (2) Protein fusion: a truncated *lacZ* gene (the first seven codons are lacking) becomes expressed from P_x over a 116-base-pair Mu fragment without translational stop signals (Casadaban, unpublished data). Ap, ampicillin resistance gene; Trp, part of the *trp* operon. Filled boxes indicate Mu DNA.

Spontaneous mutants of strain 20 able to use lactose in minimal medium supplemented with arginine (100 μg/ml) were obtained at a frequency of about 5×10^{-7}. In a first batch of nine mutants, all displaying high specific activities of β-galactosidase in the presence of arginine, two were affected in the arginine repressor as judged from their elevated level of ornithine carbamoyltransferase (encoded by the unlinked *argF* and *argI* genes), and one was an operator-constitutive mutant (see below). The rest probably harbored transpositions of the Mu d *lac* prophage, since the genetic linkage between the Lac^+ phenotype and the *arg* region was disrupted (Arg^+ transductants remained Lac^+).

The putative operator-constitutive mutation was recovered by transducing to Arg^+ the P4X *argEC1* strain with a 363 phage lysate grown on the operator-constitutive mutant. The resulting transductant displayed a marked reduction of repressibility for both *argE* and *argH* (Table 4), thus for both arms of the divergent cluster. A second batch of mutants was screened out by the same procedure. Nine strains were found to harbor *argR* mutations, and nine others contained Mu d *lac* transpositions. Transduction into P4X *argEC1* and enzyme assays (see above) proved the seven remaining strains to consist of four operator mutants very similar to the one above (data not shown) and three strains in which constitutive β-galactosidase synthesis was due to a mutation on the Mu d *lac* prophage itself.

(ii) Protein fusion strains. Strains which synthesize a β-galactosidase fused to the NH_2-proximal part of the *argC* or *argB* products were isolated directly by plating a population of cells of strain 4100 lysogenized with phage Mu *cts* d II301 on minimal medium supplemented with lactose, acetylarginine, and ampicillin at 29°C. About 1 cell in 10^2 grew on this medium, and among 3,000 cells, 2 Arg mutants were identified by replica plating on Lac plates devoid of arginine. By the above-mentioned plate recombination test, one strain was found to be an *argC-lacZ* fusion very early in the *argC* gene, within the *argEC1–sup-102* overlap (see Fig. 1 and previous section). The other did not recombine with the *sup-400* deletion; therefore, it was probably an *argB-lacZ* fusion. Like the above *arg-lacZ* operon fusion (no. 20), both strains synthesized 20 times more β-galactosidase when acetylarginine (25 μg/ml) was used instead of arginine.

TABLE 4. Acetylornithinase and arginosuccinase in an operator-constitutive mutant derived from operon fusion strain 20

Strain	Supplement to minimal medium	Sp act (μmol/h per mg of protein)	
		Acetylornithinase	Arginosuccinase
P4XB2 *argR*	None	84	8.5
Operator-constitutive mutant	None	82	5.7
	Arginine	38	3.3
P4X (wild type)	None	23	1.3
	Arginine	8	0.2

DISCUSSION

The properties of mutants harboring Mu prophages inserted near the *argE-argC* junction were found to be in full agreement with our previous conclusions (2, 12) that the promoter of *argE* (*argEp*) faces the promoter of *argCBH*. Moreover, the data localized *argEp* in the overlap between the *argEC1* and *sup-102* deletions, thus a relatively small segment of 100 to 150 nucleotides (17). The properties of two deletion mutants (T5 and T7) obtained as thermoresistant derivatives of a Mu insertion in *argC* and which extend into this region support these conclusions. Since no other local alteration of *argEp* could be obtained up to now, these results will be very useful for the interpretation of the recently determined DNA sequence of the *argE-argC* region (9, 19; Piette et al., manuscript in preparation). The fact that the *sup-102* deletion affects the expression of *argE* more severely than the AA^-_N, T5, and T7 mutations suggests that *argEp* might be contained entirely in the *argEC1–sup-102* overlap.

Inserting Casadaban and Cohen's (6) Mu d *lac amp* phages into *argECBH* to recover *arg-lacZ* fusions or strains producing a hybrid β-galactosidase protein under arginine control proved considerably easier and more straightforward than the older, multistep Casadaban procedure for constructing fusions (5) already applied to *argA* (11) and *argE* by T. Eckhardt (unpublished data). When used to isolate regulatory mutants, the one-step fusion strains gave rise to a large proportion of Mu d *lac* transpositions. This was no surprise, since Mu d *lac amp* can still transpose. Spontaneous regulatory mutants of both the *argR* and *argECBH* operator-constitutive type could, however, be recovered even from a limited sample; at least one of the operator-constitutive mutations proved to be a single-base-pair deletion and not a rearrangement (Piette et al., manuscript in preparation). It is conceivable that the relative frequencies of regulatory mutants and transpositions could be altered by mutagenesis or by making the inserted Mu unable to transpose. The method is presently being applied to fusions between *lac* and *car* or *pyr* genes (unpublished data from our laboratory) in order to unravel the still elusive mechanism which regulates pyrimidine biosynthesis

and, in concert with arginine repression, controls the synthesis of carbamoylphosphate synthetase.

The above-described operator mutants were all found to be derepressed for *argE*, even though selection had been focused on *argC*-dependent β-galactosidase synthesis. Taking previous data into account, it now appears that this dual effect on both arms of the *argECBH* operon is typical of most operator mutants affecting this cluster of genes, whether they have been obtained by selection for *argE* (2, 3, 7) or for *argCBH* (16; this paper). We had, however, reported *cis*-dominant, *argE*-specific derepressed mutants as well (2, 7). On the basis of sequence data (Piette et al., manuscript in preparation), it appears that the relevant mutations might open up new promoters in the vicinity of the control region, though their affecting a specific *argE* operator has not been excluded. Therefore, at present only one operator region has been defined in the *argECBH* cluster; the actual number of repressor binding sites could be larger, however (Piette et al., manuscript in preparation).

Regarding the mechanism responsible for control of enzyme synthesis in the arginine pathway, it is of interest that in the very proximal *argC-lac* protein fusion strain mentioned above, β-galactosidase synthesis is extensively modulated by the intracellular arginine level. This shows that the *argC* product is not an essential regulatory element for its own production, as was already suggested by in vitro evidence (10).

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