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Operator-Constitutive Mutants in the Threonine Operon of *Escherichia coli* K-12

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Three *Escherichia coli* K-12 mutant strains resistant to DL-α-amino-β-hydroxyvaleric acid were isolated in which the expression of the *thr* operon is constitutive. The localization and dominance properties of the mutations involved, called *thrO*, are those of operator mutations. The gene sequence is *OABC* as suggested by earlier studies.

Three of the structural genes coding for the threonine biosynthetic enzymes belong to the same operon (16) located at 0 min on the genetic map of *Escherichia coli* (15). These genes, *thrA*, *thrB*, and *thrC*, code for aspartokinase I-homoserine dehydrogenase I (adenosine 5'-triphosphate:L-aspartate 4-phosphotransferase [EC 2.7.2.4] and L-homoserine:nicotinamide adenine dinucleotide oxidoreductase [EC 1.1.1.3]), homoserine kinase (adenosine 5'-triphosphate:L-homoserine *O*-phosphotransferase [EC 2.7.1.39]), and threonine synthase (*O*-phosphohomoserine phospholyase [EC 4.2.99.2]), respectively.

The biosynthesis of these enzymes is subject to multivalent repression by threonine and isoleucine (3). The complex regulatory mutants isolated previously suggest the involvement of charged isoleucyl-transfer ribonucleic acid (2, 17) and charged threonyl-transfer ribonucleic acid (8, 9) in the regulation of the threonine operon. This work represents our first attempt to obtain simple, derepressed mutants that may yield more information on the mechanisms involved in this regulation.

The selection procedure utilizes the properties of a structural analogue of threonine, DL-α-amino-β-hydroxyvaleric acid (AVA), which prevents the growth of wild-type *E. coli* K-12 by inhibiting aspartokinase I and homoserine dehydrogenase I (1). Mutants resistant to this analogue have already been isolated (1); the mutations were localized in the *thrA* gene and affect feedback inhibition by threonine. Since other kinds of regulatory mutants able to overcome growth inhibition by AVA could be expected, a new systematic analysis of resistant mutants was performed. To facilitate their characterization, the mutants were isolated from a strain lacking aspartokinase II-homoserine dehydrogenase II (*metLM*) and aspartokinase III (*lysC*).

MATERIALS AND METHODS

Media and chemicals. The media used in this work were described previously (16). L-Amino acids required for the growth of auxotrophs were used at 1 mM, except for *meso*-diaminopimelic acid (0.1 mM) and L-homoserine (0.5 mM). For the repression studies, L-threonine was used at 5 mM.

Crystalline sodium penicillin G was from Specia. Ethyl methane sulfonate was obtained from Eastman Kodak Co. All of the amino acids were purchased from Merck; AVA was from Sigma Chemical Co. Homoserine phosphate was prepared by the method of Wormser and Pardee (18), using *E. coli* K-12 homoserine kinase kindly supplied by Ben Burr.

Bacterial and phage strains. The strains of *E. coli* employed are described in Table 1. Allele numbers were allocated to the Service de Biochimie Cellulaire —Institut Pasteur by the *E. coli* Genetic Stock Center of Yale University.

Transductions with P1*vir* or P1*kc* phages were performed by the method of Lennox (6). Transductions with λ*dthr*$_c$ (11, 14) and its derivatives were described previously (16).

Phage λ*imm21 b2* was provided by P. Brachet.

Enzyme assays. Cells grown in minimal medium and harvested while still in logarithmic phase were suspended in buffer (10 mM, potassium phosphate [pH 7.2] containing 2 mM threonine and 0.1 mM isoleucine; 0.1 mM pyridoxal phosphate) and sonically disrupted.

A 2-h centrifugation at 105,000 × *g* was performed whenever homoserine kinase and aspartokinase were assayed (16). Aspartokinase I, homoserine dehydrogenase I, and homoserine kinase were measured as described previously (10, 16). The assay for threonine synthase takes advantage of an accessory threonine deaminase activity of this enzyme (12) (B. Burr, personal communication) and measures the rate

TABLE 1. *List of bacterial strains*

Strain	Genotype								Sex	Origin/reference
	thrO	*thrA*$_1$	*thrA*$_2$	*thrB*	*thrC*	*metLM*	*lysC*	Other loci		
ML52[a]	+	+	+			+	+			
MI158	+	+	+	+	+	+	+	*pyrA53, thi-1*	HfrH	M. Iaccarino
RCB169	+	+	+	+	+	+	+	*metA, argH, rifR6*	HfrP 4×6	Molecular Biology Department, Institut Pasteur Collection
GT1	+	+	+	+	+	*1005*	*1004*		F$^+$	16
GT3	+	*1016*	+	+	+	*1005*	*1004*		F$^+$	16
GT10	+	+	+	+	+	*1005*	*1004*	*serB22, pyrA53 pro-1001*	F$^+$	16
GT13	+	+	+	+	*1001*	*1005*	*1004*	*serB22, pro-1001*	F$^+$	16
GT14	+	*1101*	+	+	+	*1005*	*1004*	*serB22, pro-1001*	F$^+$	16
GT18	+	*1101*	+	+	+	*1005*	*1004*	*pro-1001*	F$^+$	Ser$^+$ transductant of GT14 P1*kc* grown in MI158
GT190	+	+	+	+	+	*1005*	*1004*		HfrP 4×6	See text
GT191	*1025*	+	+	+	+	*1005*	*1004*		HfrP 4×6	Spontaneous mutant resistant to AVA of GT190
GT193	*1026*	+	+	+	+	*1005*	*1004*		HfrP 4×6	Spontaneous mutant resistant to AVA of GT190
GT196	*1027*	+	+	+	+	*1005*	*1004*		HfrP 4×6	Spontaneous mutant resistant to AVA of GT190
GT280	*1025*	+	+	*1020*	+	*1005*	*1004*		HfrP 4×6	Thr$^-$ derivative of GT191 after mutagenesis
GT282	*1027*	+	+	*1021*	+	*1005*	*1004*		HfrP 4×6	Thr$^-$ derivative of GT196 after mutagenesis
GT288	*1026*	*1024*	+	+	+	*1005*	*1004*		HfrP 4×6	Thr$^-$ derivative of GT193 after mutagenesis

[a] For strain ML52, the *thr* mutation is not identified.

of formation of α-ketobutyrate (4). Final concentrations in 0.2 ml of the reaction mixture were: 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonate adjusted to pH 8 with KOH; 100 mM ammonium sulfate; 0.1 mM pyridoxal phosphate; 50 μg of bovine serum albumin; and 10 mM homoserine phosphate. The reaction was carried out at 46 C for 10 min and was stopped by the addition of 0.2 ml of 2,4-dinitrophenylhydrazine (0.1%) in 2 N hydrochloric acid. After the addition of 0.5 ml of 96% ethanol and 0.5 ml of 5 N sodium hydroxide, the absorption was measured at 440 nm. The blank value, obtained in the absence of homoserine phosphate, was subtracted from the measured absorbance.

Threonine deaminase was assayed by measuring the rate of formation of α-ketobutyrate (4) according to Szentirmai and Umbarger (13).

The protein concentration was estimated by the biuret method (5).

Selection of mutants resistant to AVA. The mutants were isolated from Hfr strain GT190. This strain was obtained by two sequential P1 transductions from the *metLM lysC* F$^+$ strain GT1 into the *argH metA* strain RCB169.

About 5 × 10^8 cells of strain GT190 were plated on a minimal medium containing DL-α-amino β-hydroxyvaleric acid (20 mM). After 2 days at 37 C, colonies were purified and tested for threonine excretion by streaking on a lawn of ML52 (10^7 cells per plate). The three independent mutants studied in this paper were selected from three separate clones of GT190.

Construction of merodiploids for a cis-trans test. Threonine auxotrophs were selected from constitutive mutants by ethyl methane sulfonate mutagenesis followed by penicillin enrichment (7). The *thr* mutations were identified through complementation analysis with λ*dthr* phage strains carrying mutations in one of the four cistrons (16) and were also checked by enzyme assay. Thr$^+$ transductants were then selected by using a λ*dthr* phage carrying a mutation in a gene different from the one present on the bacterial chromosome.

The genetic characteristics of the merodiploids were verified as follows. The strains were induced at 41 C, and the lysate obtained was shown to carry the *thr* mutation of the original lysate. Curing of these merodiploids was performed with λ*imm21 b2* (14), and the cured strains were shown to carry the original *thr* mutation present on the nonlysogenized parent.

RESULTS

Isolation of mutants. Spontaneous mutants resistant to AVA were isolated from the *metLM lysC* strain GT190. Three classes of mutants could be recognized according to their phenotype regarding threonine excretion. Mutants of the first class do not excrete threonine and show no alteration in the level of homoserine dehydrogenase I that is normally inhibited by threonine; they were not studied further.

Mutants of the second class, excreting large amounts of threonine, were found to be *thrA*

mutants that produce an aspartokinase I-homoserine dehydrogenase I resistant to threonine inhibition; as such, they do not differ from those described previously (1). Only three independent mutants of the third class (called *thrO* mutants) will be described in this paper. They excrete smaller amounts of threonine than class II mutants, and their homoserine dehydrogenase activity is normally sensitive to feedback inhibition by threonine.

Enzyme levels in AVA-resistant mutants. The AVA-resistant mutants were grown in a minimal medium and in a minimal medium containing threonine (repressing conditions). Cell extracts were prepared and assayed for aspartokinase I, homoserine dehydrogenase I, homoserine kinase, threonine synthase, and threonine deaminase. As seen in Table 2, the four activities involved in threonine biosynthesis are coordinately derepressed in the mutants. In the absence of added threonine, the activity is four- to fivefold higher in the mutants than in the wild type, and only a slight repression is obtained in the presence of threonine. Threonine deaminase, on the other hand, which is not involved in threonine biosynthesis, is not derepressed in these mutants.

TABLE 2. *Specific activities of enzymes in parent and mutant strains grown in a minimal medium (MM) and in a minimal medium supplemented with threonine (T)*

Strain	Growth medium	Sp act[a]				
		AKI	HDHI	HSK	TS	TD
GT190	MM	30	200	50	0,7	50
	T[b]	18	90	25	ND	ND
GT191	MM	175	920	310	4,5	45
	T[b]	155	850	250	ND	ND
GT193	MM	160	800	300	4,7	35
	T[b]	170	620	178	ND	ND
GT196	MM	200	820	350	6	37
	T[b]	180	860	280	ND	ND

[a] Specific activities are given in nanomoles of product formed per minute per milligram of protein. AKI, Aspartokinase I; HDHI, homoserine dehydrogenase I; HSK, homoserine kinase; TS, threonine synthase; TD, threonine deaminase; ND, not done.

[b] Threonine was used at 5 mM for repression studies.

Localization of the mutations. Stocks of phage P1 grown on the different constitutive mutants were used to transduce strain GT18 (*thrA*$_1$*1101*). Threonine excretion was used as a test for the presence of the constitutive mutation in the *thrA*$_1$$^+$ recombinants. In two transductants that excreted threonine, the level of homoserine dehydrogenase activity was verified to be as high as that in the constitutive donor. The constitutive mutations are at least 90% cotransducible with *thrA*$_1$ and are therefore located either inside or very close to the *thr* operon.

The localization of the mutation relative to the operon was done as follows. Thr auxotrophs were selected for each of the constitutive mutants and characterized as *thrB* in two instances and *thrA*$_1$ in the third case. Stocks of phage P1 grown on these double mutants (*thrO thrB* or *thrO thrA*$_1$) were used to transduce strain GT14 (*serB thrA*$_1$*1101*) or GT13 (*serB thrC*). Analysis of Thr$^+$ recombinants for serine auxotrophy and threonine excretion demonstrated that *thrO* segregates, like *serB*, as a marker located to the left of the *thrA*$_1$*1101* (Table 3), the order being *serB thrO thrA*$_1$ *thrA*$_2$ *thrB thrC*.

Cis-trans test. Merodiploids containing two copies of the threonine operon were constructed (see above). The copy located on the chromosome carries the constitutive mutations as well as a mutation in one of the cistrons of the threonine operon. The other copy located on phage λ*dthr* carries a mutation on a different cistron of the operon. The results (Table 4) indicate clearly that the *thrO* mutations exert an effect only in the *cis* position: the activity

TABLE 3. *Ordering of serB, thrO, thrA*$_1$*, and thrC*

Donor	Recipient	No. of Thr$^+$ transductants analyzed	Unselected markers (%)		
			Threonine excretors	Ser$^+$	Threonine excretors and Ser$^+$
GT280 (*thrO1025, thrB1020*)	GT14 (*thrA*$_1$*1101, serB22*)	124	83	51	49
	GT13 (*thrC1001, serB22*)	135	4	12	1
GT282 (*thrO1027, thrB1021*)	GT14	170	95	90	90
	GT13	72	7	53	7
GT288 (*thrO1026, thrA*$_1$*1024*)	GT14	52	42[a]	82	41
	GT13	320	2	23	2

[a] The fact that the percentage of threonine excretors is lower than that of Ser$^+$ recombinants in this cross is probably due to negative interference.

TABLE 4. *Specific activities of enzymes in partial diploids and in parental strains grown in a minimal medium (MM) and in a minimal medium supplemented with threonine (T)*

Strain	Growth medium	Sp act[a]		
		AKI (*thrA*$_1$ product)	HDHI (*thrA*$_2$ product)	HSK (*thrB* product)
GT6 (*thrO*$^+$, *thrA*$_1^+$, *thrA*$_2^+$, *thrB*$^+$, *thrC*$^+$) (λd*thrO*$^+$, *thrA*$_1^+$, *thrA*$_2^+$, *thrB*$^+$, *thrC*$^+$)	MM	62	370	105
GT280 (*thrO*^{c}, *thrA*$_1^+$, *thrA*$_2^+$, *thrB*$^-$, *thrC*$^+$)	MM[b]	135	820	<5
GT280 (*thrO*^{c}, *thrA*$_1^+$, *thrA*$_2^+$, *thrB*$^-$, *thrC*$^+$) (λd*thrO*$^+$, *thrA*$_1^+$, *thrA*$_2^-$, *thrB*$^+$, *thrC*$^+$)	MM	170	1,140	60
	T[c]	180	1,250	49
GT282 (*thrO*^{c}, *thrA*$_1^+$, *thrA*$_2^+$, *thrB*$^-$, *thrC*$^+$)	MM[b]	109	430	<5
GT282 (*thrO*^{c}, *thrA*$_1^+$, *thrA*$_2^+$, *thrB*$^-$, *thrC*$^+$) (λd*thrO*$^+$, *thrA*$_1^+$, *thrA*$_2^-$, *thrB*$^+$, *thrC*$^+$)	MM	180	580	90
	T[c]	180	720	46
GT288 (*thrO*^{c}, *thrA*$_1^-$, *thrA*$_2^+$, *thrB*$^+$, *thrC*$^+$)	MM[d]	5	560	ND
GT288 (*thrO*^{c}, *thrA*$_1^-$, *thrA*$_2^+$, *thrB*$^+$, *thrC*$^+$) (λd*thrO*$^+$, *thrA*$_1^+$, *thrA*$_2^+$, *thrB*$^+$, thrC$^+$)	MM	20	700	ND
	T[c]	13	750	ND

[a] See footnote *a* of Table 2.
[b] MM + 1 mM threonine.
[c] Threonine was used at 5 mM for repression studies.
[d] MM containing 0.5 mM homoserine, 0.1 mM diaminopimelic acid, and 1 mM lysine.

that is coded only by the phage (*trans* to *thrO*^{c}) is at the wild-type level and is still repressible by threonine, whereas the activity that is coded only by the bacterial chromosome (*cis* to *thrO*^{c}) is elevated and not repressible.

DISCUSSION

This work demonstrates that, by selecting for resistance to AVA, it is possible to obtain strains that have all of the characteristics of operator-constitutive mutants in the *thr* operon. The corresponding mutations are closely linked to the *thrA* side of the *thrA thrB thrC* cluster and lead to derepression of the *thr* operon only when it is located in *cis* position. The properties of such mutants provide further evidence for the existence and polarity of the *thr* operon. Knowledge of the exact nature and target of the mutations described here will require further information on the mechanism involved in the regulation of the *thr* operon. Functionally, the strains behave as operator-constitutive mutants and may be used as such for studies on the in vitro expression of the operon.

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