JOURNAL OF BACTERIOLOGY, June 1974, p. 990-998

Vol. 118, No. 3
Printed in U.S.A.

Threonine Locus of *Escherichia coli* K-12: Genetic Structure and Evidence for an Operon

J. THÈZE AND I. SAINT-GIRONS

Service de Biochimie Cellulaire, Institut Pasteur, 75015 Paris, France

Received for publication 17 December 1973

Three genes, *thrA*, *thrB*, and *thrC*, were previously defined and localized in the threonine locus of *Escherichia coli* K-12. *thrA*, *thrB*, and *thrC* specify the enzymes aspartokinase I-homoserine dehydrogenase I, homoserine kinase, and threonine synthetase, respectively. A complementation analysis of the threonine cluster using derivatives of a lambda phage carrying the threonine genes ($\lambda dthr_c$) demonstrates that: (i) *thrB* and *thrC* each consist of a single cistron; and (ii) *thrA* is composed of two cistrons, $thrA_1$ and $thrA_2$, although it specifies a single polypeptide chain. $thrA_1$ and $thrA_2$ correspond to aspartokinase I and homoserine dehydrogenase I, respectively. Their relative order is established. The demonstration of polar effects of mutations (nonsense or induced by phage Mu) in *thrA* and *thrB* is taken as evidence for the existence of a *thrA thrB thrC* operon, transcribed in this order.

The three structural genes coding for threonine biosynthetic enzymes are clustered at 0 min on the genetic map of *Escherichia coli* (29). These genes, *thrA*, *thrB*, and *thrC*, respectively, code for aspartokinase I-homoserine dehydrogenase I (adenosine triphosphate [ATP]:L-aspartate 4-phosphotransferase [EC 2.7.2.4]; and L-homoserine:nicotinamide adenine dinucleotide oxidoreductase [EC 1.1.1.3]), homoserine kinase (ATP:L-homoserine *O*-phosphotransferase [EC 2.7.1.39]) and threonine synthetase (*O*-phosphohomoserine phospholyase [EC 4.2.99.2]). Their order is shown in Fig. 1. Aspartokinase I and homoserine dehydrogenase I are carried by a single polypeptide chain (7). This bifunctional enzyme is therefore specified by a single gene, *thrA*. We use the following nomenclature for the different types of *thrA* mutants: $thrA_1$, mutants that lack aspartokinase I and retain homoserine dehydrogenase I; $thrA_2$, mutants that lack homoserine dehydrogenase I and retain aspartokinase I; $thrA_1A_2$, mutants that lack both activities.

This paper presents a complementation analysis of the threonine cluster. This analysis was greatly facilitated by the availability of a strain of phage lambda ($\lambda dthr_c$) transducing the threonine genes, recently isolated by W. J. Schrenck and R. Weisberg (manuscript in preparation). The existence of polar effects (8, 12) of mutations in the *thr* cluster demonstrates that *thrA*, *thrB*, and *thrC* belong to the same operon. Partial polar effects caused by nonsense mutations were identified by enzyme assay. Total polar effects produced by insertion of prophage Mu were analyzed by complementation.

(This paper is part of a thesis presented by J. T. in partial fulfillment of the requirements for the Dr.Sc. degree from the University of Paris.)

MATERIALS AND METHODS

Media. Broth consisted of 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl.

Minimal medium (5) was supplemented with thiamine hydrochloride (1 μg/ml) and 4% glucose (or lactose when specified) as a carbon source. The concentration of L-amino acids was 10^{-3} M except when otherwise indicated; L-homoserine and *meso*-diaminopimelic acid (Dpm) were respectively used at 2×10^{-4} and 10^{-4} M.

The corresponding solid media were prepared by incorporating 1.5% agar (Difco) into the liquid media.

Chemicals. Crystalline sodium penicillin G was from Specia. Penicillinase was purchased from Mann Research Laboratories. Ethyl methane sulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were obtained from Eastman Kodak Co. and Aldrich Chemical Co., respectively. All of the amino acids and inorganic compounds were purchased from Merck & Co. Trimethoprim was a generous gift of the Wellcome Research Laboratories. Thiaisoleucine was synthesized and kindly given by G. Kenyon.

Bacteria. The strains of *E. coli* K-12 used were described in Table 1. The *E. coli* Genetic Stock Center (Yale University) has allocated allele numbers 1000 to 1200 for the use of the Service de Biochimie Cellulaire.

Phages. $\lambda dthr_c$, isolated by W. J. Schrenk and R. Weisberg (manuscript in preparation), is a phage

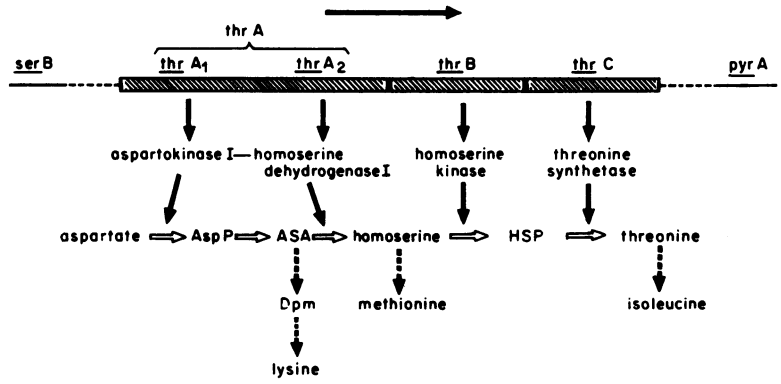

FIG. 1. *Threonine system of E. coli K-12. The genetic map of this locus was previously established (29). The genetic structure and the direction of transcription of the threonine operon (given by the upper arrow) are discussed in the text. The isofunctional enzymes aspartokinase II-homoserine dehydrogenase II and aspartokinase III are not shown since most of the strains used in this work lacked these three activities. The second enzyme of this pathway, aspartic semialdehyde dehydrogenase, is coded by the gene asd localized at 66 min on the map (26). Abbreviations: aspP, aspartyl phosphate; ASA, aspartic semialdehyde acid; Dpm, diaminopimelic acid; HSP, homoserine phosphate.*

transducing all of the threonine genes. The genotype of the parental phage is λ*CI857,S7*.

λ*dthr*$_c$ has the threonine region integrated in the region coding for the late functions of λ (28). Lysates of this phage were prepared by thermoinduction at 42 C of strain J.S.12.120 (see Table 1) and treatment with chloroform.

φ80p.*suIII*, given by M. Hofnung, is a plaque-forming phage carrying the amber suppressor *suIII*.

The following phage strains carrying nonsense mutations were used to identify the nature of bacterial suppressors: λ*susN53* (amber), given by P. Brachet; λ*CI,susN251* (ochre) and λ*CII2002 susO261* (UGA), given by R. Thomas; and T4WA1 (UGA), given by G. A. Jacoby. The corresponding *su*$^+$ bacterial strains allowing growth of these phages are listed in Table 1. Mu-1 phage was provided by G. Lindahl, λ*imm 21, b2* phage was provided by P. Brachet, and P1*vir* was provided by M. Yarmolinky.

Transduction of threonine mutations induced by Mu integration. Transductions with phage P1*vir* were performed according to the method of Lennox (16). To prevent the induction of the Mu prophage during transduction, the acceptor strain GT64 was previously lysogenized by Mu-1. Strain GT64 is a Lac$^-$ derivative of strain GT10, obtained after phage Mu mutagenesis (3).

Conjugation. Matings were carried out according to the method of Jacob and Wollman (13).

Strain GT100 was obtained by conjugation between strain GT99 as a donor and strain GT2 as a recipient. The mating was interrupted after 20 min of conjugation in order to avoid the reintroduction of the wild-type alleles of *metLM* and *lysC*. Among the Pro$^+$ recombinants, we selected a clone that had received the *lacZu239* character.

Enzyme assays. Aspartokinase I and homoserine dehydrogenase I activities were measured as previously described (20, 32). Homoserine kinase activity was measured by coupling homoserine-dependent adenosine diphosphate production to pyruvate kinase (ATP:pyruvate phosphotransferase [EC 2.7.1.40]) and lactate dehydrogenase (D-lactate:nicotinamide adenine dinucleotide oxidoreductase [EC 1.1.1.27]) (27).

Estimation of protein concentration. The concentration of protein was determined by the biuret method (10).

Enzymological identification of threonine auxotrophs. Strains requiring threonine only for growth must lack homoserine kinase or threonine synthetase. Mutants lacking homoserine kinase are classified as *thrB*. Mutants possessing homoserine kinase are assumed to lack threonine synthetase and are classified as *thrC*. The extracts of threonine auxotrophs were obtained from cells grown with a limited concentration of threonine (1.5×10^{-4} M) in order to avoid a complete repression of the homoserine kinase activity.

Selection of threonine mutants. The threonine mutants reported in Table 2 were derived from strains HfrH, GT100, and GT200. They were obtained after mutagenesis by ethyl methane sulfonate, at a concentration that permits 5% survival, and penicillin enrichment. Strains GT121 and GT132 were obtained by ultraviolet mutagenesis and penicillin selection in solid medium (1).

Threonine was added to minimal medium to select mutants derived from strain HfrH, but threonine, methionine, lysine, and Dpm were added to select mutants derived from strains GT100 and GT200. Lys$^-$, Met$^-$, and Lys$^-$Met$^-$ mutants were first eliminated. Among the remaining auxotrophs, we selected those which grew on homoserine, homoserine plus lysine plus Dpm, threonine, or threonine plus methionine plus lysine plus Dpm.

Identification of nonsense mutations. Two methods were used to identify nonsense mutations. Drops of φ80p.*suIII* were applied to petri dishes spread with bacteria. They were then incubated for 24 to 48 h at 37 C. The appearance of a large number of colonies indicated an amber mutation. The threonine

TABLE 1. *Characteristics of strains used*

Strain	Genotype[a]	Origin
HfrH	*str*$^+$	F. Jacob
HfrCU383	*str*$^+$, *thrC* (amber)	F. Jacob
HfrH3300u239	*str*$^+$, *lacZu239* (amber)	F. Jacob
Gif 102	*thrA*$_2$*1015*, *metLM1005*, *lysC1004*	(14)
GT2	*metLM1005*, *lysC1004*, *pro1001*	(29)
GT10	*pro1001*, *pyrA53*, *serB22*	(29)
GT12	*thrB1000*, *metLM1005*, *lysC1004*, *pro1001*, *serB22*	(29)
GT13	*thrC1001*, *metLM1005*, *lysC1004*, *pro1001*, *serB22*	(29)
GT14	*thrA*$_1$*1101*, *metLM1005*, *lysC1004*, *pro1001*, *serB22*	(29)
GT25	HfrH, *thrB1007*	This paper
GT28	HfrH, *thrC1010*	This paper
GT64	*pro1001*, *pyrA53*, *serB22*, *lac-1000* (Mu-1)[b]	This paper[c]
GT65	*thrA*$_2$*1015*, *lysC1004*, *metLM1005* (λd*thrA*$_2$*1015*)	This paper
GT99	HfrH, *str*$^+$, *lacZu239* (amber), *thy-1000*	Trimethoprim-resistant strain of HfrH3300u239 (22)
GT100	*metLM1005*, *lysC1004*, *lacZu239* (amber)	This paper[c]
GT200	*metLM1005*, *lysC1004*, *lacZu239* (amber), thiaileuR	Thiaisoleucine-resistant strain of GT100 (24)
CA275	HfrC, *thi*, *lacZu125* (amber), *trp* (amber), *suIII*$^+$	J. D. Smith
CAJ64	*str*$^+$, *thi*, *lac*, *su*$^+$ (UGA), (λ)	J. D. Smith
CAJ70	*su*$^+$ (UGA)	J. D. Smith
CA13B	*su*$^+$ (UAA)	R. Thomas
AT905	HfrKL16, *thr-9* (Mu-1), *thi-1*	A. Taylor
AT2338	*thr-7* (Mu-1), *thi-1*	A. Taylor
KMBL366	HfrH, *thi-8*, *met-102*, *thr-101* (Mu-1)	P. van de Putte
KMBL397	HfrH, *thi-8*, *met-102*, *thr-102* (Mu-1)	P. van de Putte
KMBL1114	HfrH, *thi-8*, *cysB125*, *galK1381*, *thr-103* (Mu-1)	P. van de Putte
KMBL1273	F$^-$, *thr-104* (Mu-1cts4)	P. van de Putte
KMBL1288	F$^-$, *thr-105* (Mu-1cts4)	P. van de Putte
KMBL1294	F$^-$, *thr-106* (Mu-1cts4)	P. van de Putte
KMBL1299	F$^-$, *thr-107* (Mu-1cts4)	P. van de Putte
KMBL1554	F$^-$, *thr-110* (Mu-1cts4)	P. van de Putte
KMBL1207	*thi-209*, Δ*pro-lac-111*, *thr-109* (Mu-1), suII/Fts114*lac*	P. van de Putte
RH2103	*str*, *gal*, *thr* (Mu-1)	M. Faelen
RH2107	*str*, *gal*, *thr* (Mu-1)	M. Faelen
MX223	*leu*, *thy*, *thr* (Mu-1),(λind$^-$)	L. Caro
J.S.12.120	*strA*, *trpR2*, *thr*, *recA*$_1$ (λ*CI857*, *S7*) (λ*dthr*$_c$)	W. J. Schrenk and R. Weisberg

[a] Genetic symbols used are explained in references 26 and 29.
[b] Mu, Mutations induced by phage Mu integration.
[c] Constructions of strains GT64 and GT100 are described in Materials and Methods.

mutants derived from strain HfrH were spread on minimal medium. The other mutants derived from strains GT100 and GT200 carry a *lac* amber mutation and were tested in minimal medium containing lactose as a source of carbon. The suppression of the *lac* mutation serves as an internal control in these experiments.

Thr$^+$ revertants were obtained from presumptive nonsense mutations not suppressed by φ80p.*suIII*. We spread bacteria on a solid medium containing 5×10^{-3} M L-methionine to avoid the growth of revertants owing to the recovery of aspartokinase II-homoserine dehydrogenase II (21). A crystal of nitrosoguanidine was placed in the center of the petri dishes. After 3 to 4 days of incubation at 37 C, 20 or 30 of the colonies surrounding the crystal were purified. We screened these revertants for the presence of a suppressor with a set of phage strains carrying nonsense mutations.

Isolation of λdthr phages carrying a threonine mutation. Six λ*dthr*$_c$ derivatives (λ*dthr*$^-$) each carrying a different threonine mutation were isolated from the following strains, lysogenized by λ*dthr*$_c$ and λ*CI857*, *S7*: Gif 102, GT12, GT13, GT14, GT25, and GT28. (The threonine mutations of these strains were identified enzymologically.) Each of these strains could be cured of λ*dthr*$_c$ by superinfection by λ*imm21*, *b2*. From each merodiploid, auxotrophs (either for threonine or for threonine plus methionine plus lysine plus Dpm) were obtained by penicillin enrichment. The majority (about 99%) had lost λ*dthr*$_c$. In order to recognize strains carrying a recombinant λ*dthr*$^-$, we screened for the ability of the auxotrophic clones to produce *thr* transducing phages.

Two hundred survivors of the penicillin treatment were analyzed by the replica plating technique. The original petri dish was replicated on minimal medium

(plate 1) and two plates (plates 2 and 3) containing threonine (or threonine plus methionine plus lysine plus Dpm). Plates 1 and 2, incubated at 37 C, indicated the auxotrophic clones. Plate 3 was incubated for 3 h at 30 C and 4 h at 42 C and then sprayed with chloroform. To detect the production of $\lambda dthr_c$, plate 3 was replicated onto minimal medium spread with a threonine auxotroph (plate 4). The threonine mutation of this last strain was localized in a different gene from that of the merodiploid analyzed. We selected the clones, known to be auxotrophs from the results of plates 1 and 2, that were able to produce transductants on plate 4. Of the 200 clones analyzed for each merodiploid, only one or two auxotrophic clones produced *thr* transducing phages. Each transducing phage was shown to carry the *thr* mutation of the merodiploid from which it was isolated. The names of the phages produced by this method are: $\lambda dthrA_1 1101$, $\lambda dthrA_2 1015$, $\lambda dthrB1000$, $\lambda dthrB1007$, $\lambda dthrC1001$, and $\lambda dthrC1010$.

Test for complementation. A threonine mutant was grown overnight in broth. The culture was then resuspended in 10^{-2} M $MgSO_4$ and shaken 30 min at 37 C, and 5×10^7 bacteria were spread on a petri dish containing the necessary supplements. Drops of 10^{-1} and 10^{-2} dilutions of the various $\lambda dthr^-$ lysates were then applied to a small area on the plate, which was incubated at 30 C. Appearance of confluent growth in the area within 36 h of incubation indicated complementation. Phage $\lambda dthr_c$, which complemented all the threonine mutants tested, was always used as a control.

RESULTS

Complementation analysis of mutants with a simple threonine requirement. A mutation leading to a simple threonine requirement must be localized in *thrB* or *thrC*, the genes respectively coding for homoserine kinase and threonine synthetase (Fig. 1). When such mutations were carried by strains derived from strains GT100 or GT200, which possess only aspartokinase I-homoserine dehydrogenase I, the mutants grew slowly in the presence of L-threonine and better in the presence of L-threonine plus L-methionine plus L-lysine plus Dpm. This can be explained by the fact that L-threonine inhibits the activity (23) and represses the synthesis (9) of aspartokinase I-homoserine dehydrogenase I and thus leads in these strains to a phenotypic limitation in homoserine and Dpm.

To determine the number of cistrons in *thrB* and *thrC*, 53 threonine auxotrophs derived from strains HfrH, GT100, and GT200 were analyzed by complementation with the six $\lambda dthr_c$ derivatives. All of the mutants complemented with $\lambda dthrA_1 1101$ and $\lambda dthrA_2 1015$. Twenty-eight mutants did not complement with $\lambda dthrB1000$ and $\lambda dthrB1007$ but did complement with the two phages carrying *thrC* mutations. Conversely, 25 mutants complemented with the two phages carrying *thrB* mutations but did not complement with $\lambda dthrC1001$ and $\lambda dthrC1010$. These two classes contained, respectively, seven and five mutants whose mutations were suppressible by $\phi 80p.suIII$. The missense mutants as well as the nonsense ones behaved unambiguously in the complementation test. Mutations leading to a requirement for threonine alone could then be localized either in the *thrB* cistron or in the *thrC* cistron (Table 2). It was further verified that five mutants identified as *thrB* by complementation, strains GT134, GT142, GT144, GT147, and GT148, lacked homoserine kinase activity. On the other hand, this enzymatic activity was present in the crude extracts of six mutants classified as *thrC* (GT121, GT140, GT143, GT146, GT155, and GT157).

Complementation analysis of mutants requiring homoserine or homoserine plus Dpm. Among the mutants isolated from strains GT100 and GT200 (two strains lacking aspartokinase II-homoserine dehydrogenase II and aspartokinase III), the clones able to grow on L-homoserine or L-homoserine plus L-lysine plus Dpm were expected to be *thrA* mutants. These mutants were analyzed by complementation with the six $\lambda dthr^-$ phages. The eight strains found to grow on homoserine alone complemented with $\lambda dthrB1000$, $\lambda dthrB1007$, $\lambda thrC1001$, $\lambda dthrC1010$, and $\lambda dthrA_1 1101$ but not with $\lambda dthrA_2 1015$, and were thus placed in the $thrA_2$ category. All of the 13 strains that grew on homoserine plus Dpm plus lysine complemented with $\lambda dthrB1000$, $\lambda dthrB1007$, $\lambda dthrC1001$, $\lambda dthrC1010$, and $\lambda dthrA_2 1015$ but not with $\lambda dthrA_1 1101$, and therefore belonged to the $thrA_1$ category. Among the auxotrophs growing on homoserine plus Dpm plus lysine, we found none of the $thrA_1A_2$ type. A list of the $thrA_1$ and $thrA_2$ mutants isolated is given in Table 2.

Partial polar effects of nonsense mutants of thrA. A final category of auxotrophs consists of four strains requiring threonine plus methionine plus lysine plus Dpm: GT132, GT153, GT201, and GT206. The complementation test showed that the corresponding mutations were localized in the *thrA* gene: strains GT132 and GT153 were $thrA_2$, and strains GT201 and GT206 were $thrA_1A_2$ (see also Table 2). The *thrA* mutations of strains GT132 and GT201 were suppressed by $\phi 80p.suIII$. The nature of the mutations in strains GT153 and GT206 was investigated by testing the capacity of revertants of these strains to allow the growth of phages carrying different types of nonsense mutations. Strains GT153 and GT206 both

carried UGA mutations. In the same manner, we found that strain Gif 102, which produces a fragment of aspartokinase I-homoserine dehydrogenase I having only aspartokinase I activity (14, 31), carried an ochre mutation.

Strains GT132, GT153, GT201, GT206, and Gif 102 were assayed for aspartokinase I, homoserine dehydrogenase I, and homoserine kinase activities (Table 3). The enzymatic assays corroborate the genotypes determined by complementation. Strains GT132 and GT153 possessed aspartokinase I activity and lacked homoserine dehydrogenase I, as did strain Gif 102, whereas strains GT201 and GT206 lacked both activities. Homoserine kinase activity was not detectable in any of these strains under the

TABLE 2. *Threonine mutants*[a]

Class of threonine mutants	Original strain		
	HfrH	GT100	GT200
thrA$_1$*A*$_2$			201, 206
thrA$_1$		138, 139, 152	209, 211, 220, 227, 230, 233, 237, 241, 243, 251
thrA$_2$		132, 136, 153	203, 208, 214, 217, 223, 232, 238
thrB	20, 21, 22, 23, 24, 25, 26, 27, 30, 31	134, 142, 144, 147, 158	204, 205, 207, 213, 215, 218, 222, 228, 236, 239, 245, 248, 252
thrC	28, 29	121, 140, 143, 146, 155, 157	210, 212, 216, 219, 221, 224, 225, 226, 229, 234, 235, 240, 242, 244, 246, 249, 253

[a] Complete name of a mutant is GT followed by a number. The mutants are classified according to parental strain and nature of threonine mutation. An underlined number indicates that the mutant carried an identified nonsense mutation.

TABLE 3. *Specific activities of aspartokinase I, homoserine dehydrogenase I, and homoserine kinase in some Thr$^-$ strains and their parental strains*[a]

Strain	Sp act[a]			Threonine mutation	Nature of the threonine mutation	Supplements added to minimal medium[b] for growth
	Aspartokinase	Homoserine dehydrogenase	Homoserine kinase			
GT100	28	206	53	0	0	None
GT121[c]	27	85	25	*thrC*	Amber	met, lys, Dpm, and thr[d]
GT136[c]	33	<2	83	*thrA*$_2$	Not known	HS
GT132[c]	34	<2	<5	*thrA*$_2$	Amber	met, lys, Dpm, and thr[d]
GT153[c]	20	<2	<5	*thrA*$_2$	UGA	met, lys, Dpm, and thr[d]
GT200	180	800	320	0	0	None
GT208[e]	53	<2	107	*thrA*$_2$	Not known	HS
GT212[e]	31	280	86	*thrC*	Amber	met, lys, Dpm, and thr[d]
GT201[e]	5	<2	<5	*thrA*$_1$*A*$_2$	Amber	met, lys, Dpm, and thr[d]
GT206[e]	5	<2	<5	*thrA*$_1$*A*$_2$	UGA	met, lys, Dpm, and thr[d]
Gif102	20	<2	<5	*thrA*$_2$	Ochre	HS
GT65[f]	60	<2	10	*thrA*$_2$/*thrA*$_2$[f]	Ochre	HS

[a] Expressed as nanomoles of product per minute per milligram of protein.
[b] Abbreviations: met, methionine; lys, lysine; Dpm, diaminopimelic acid; HS, homoserine; thr, threonine.
[c] Strains derived from GT100.
[d] Threonine concentration was 1.5×10^{-4} M.
[e] Strains derived from GT200.
[f] GT65 is a merodiploid constructed by lysogenization of Gif 102 with λ*dthrA*$_2$*1015* (see Table 1).

conditions used. Under the same growth conditions, the *thrC* amber mutants GT121 and GT212 exhibited homoserine kinase activity. Strains GT136 and GT208, $thrA_2$ mutants which grow on homoserine, also had this enzymatic activity. The absence of detectable homoserine kinase was particularly striking in the case of strains GT201 and GT206, which were derived from strain GT200, a strain derepressed for aspartokinase I-homoserine dehydrogenase I and homoserine kinase (see Table 1).

These results suggest that nonsense mutations in *thrA* exert polar effects on the expression of *thrB* (19). The complementation test indicates, however, that strains GT132, GT153, GT201, GT206, and Gif 102 are $ThrB^+$ and $ThrC^+$. This phenomenon may be explained by assuming that a partial polar effect of these mutations reduces but does not abolish the expression of *thrB*. This situation is well illustrated by strain Gif 102, which does not exhibit homoserine kinase activity but is able to grow on homoserine and thus must synthesize threonine from homoserine. Further, a small amount of homoserine kinase is measurable in strain GT65 diploid for the $thrA_2$*1015* mutation of strain Gif 102.

The fact that the other four strains did not grow on homoserine could be explained if the *thrA* mutation they carry exerts a stronger polar effect (17, 19, 33, 34) on the expression of *thrB* than does $thrA_2$*1015*. Nevertheless, when homoserine was only used for threonine biosynthesis, as in minimal medium containing 5×10^{-4} M L-isoleucine plus 10^{-3} M L-methionine plus 2×10^{-4} M L-homoserine, the strains GT132, GT153, GT201, and GT206 were able to grow. On the contrary, total polar mutants of *thrA* induced by phage Mu integration (GT70 and GT78) could not grow in this medium. This result indicates that strains GT132, GT153, GT201, and GT206 retain a residual homoserine kinase activity in agreement with the results of the complementation tests.

Total polar effects induced by phage Mu integration. Phage Mu is known to induce total polar effects in operons (15, 30). Threonine auxotrophs selected after phage Mu mutagenesis were thus analyzed by complementation. The requirement of these strains for threonine alone does not exclude the possibility that phage Mu is integrated in *thrA*: homoserine and aspartate semialdehyde could be synthesized by aspartokinase II-homoserine dehydrogenase II and aspartokinase III. The presence of these isofunctional enzymes would preclude the complementation analysis of *thrA*. We thus transduced all of the threonine mutations due to phage Mu insertion into strain GT64, a phage Mu lysogenic strain that lacks aspartokinase II-homoserine dehydrogenase II and aspartokinase III activities (see Materials and Methods).

In these transductions, Ser^+ transductants were selected and then screened for the Thr^- character (Table 4). The frequency of cotransduction is generally low in comparison with the frequency (60%) previously found between *serB22* and threonine point mutations (25). The cotransduction frequency is known to decrease in such a cross where one of the mutations results from phage Mu integration (4). These results demonstrate that the threonine mutations occurring after phage Mu insertion are localized at the usual threonine locus.

From each cross, one Ser^+ Thr^- transductant was purified and analyzed by complementation. The results obtained with 14 strains are given in Table 4. Four different phenotypes were observed: either *thrC* alone was inactivated, or both *thrB* and *thrC*, or $thrA_2$, *thrB*, and *thrC*, or $thrA_1$, $thrA_2$, *thrB*, and *thrC* were inactivated. It was verified that all of those strains still carried *thrC:* they gave Thr^+ recombinants when crossed with three Hfr strains with different *thrC* mutations (HfrH GT28, HfrH GT29, and HfrCU383).

DISCUSSION

The mutants of the threonine locus obtained in strains lacking the isofunctional enzymes aspartokinase II-homoserine dehydrogenase II and aspartokinase III fall into four classes according to their auxotrophic requirements: threonine, homoserine, homoserine plus lysine plus Dpm, and threonine plus methionine plus lysine plus Dpm.

It appears that auxotrophic mutations leading to a simple threonine requirement are localized in genes *thrB* and *thrC*. Complementation analysis shows that these two genes are each composed of a single cistron. The first is the structural gene for homoserine kinase, and the second is the structural gene for threonine synthetase.

Among the 21 non-polar *thrA* mutants we identified by complementation, 13 had lost aspartokinase I but retained homoserine dehydrogenase I activity ($thrA_1$ type), and eight had lost homoserine dehydrogenase I but retained aspartokinase I activity ($thrA_2$ type). Thus, most of *thrA* mutants lose only one activity. The results imply that the *thrA* gene is organized into two cistrons: $thrA_1$ and $thrA_2$, specifying aspartokinase I and homoserine dehydro-

TABLE 4. *Analysis by complementation of total polar effects induced by phage Mu integration into different strains*[a]

Name of strain	Name of original strain	Cotransduction with *serB22* (%)	Complementation with λ*dthr*$_c$ and derivatives[b]							Gene(s) not expressed[c]
			A$_1$*1101*	*A*$_2$*1015*	*B1000*	*B1007*	*C1001*	*C1010*	λ*dthr*$_c$	
GT66	AT2338	2.5	+	+	+	+	−	−	+	*thrC*
GT69	KMBL1207	4	+	+	+	+	−	−	+	*thrC*
GT74	RH2107	45[d]	+	+	+	+	−	−	+	*thrC*
GT75	KMBL1299	1	+	+	+	+	−	−	+	*thrC*
GT79	RH2103	13	+	+	+	+	−	−	+	*thrC*
GT68	KMBL1288	1.5	+	+	−	−	−	−	+	*thrB, thrC*
GT77	MX223	6	+	−	−	−	−	−	+	*thr*$_2$*A*$_2$, *thrB, thrC*
GT70	KMBL1273	0.5	−	−	−	−	−	−	+	*thrA*$_1$*A*$_2$, *thrB, thrC*
GT71	KMBL1114	14	−	−	−	−	−	−	+	*thrA*$_1$*A*$_2$, *thrB,thrC*
GT72	KMBL1294	48[d]	−	−	−	−	−	−	+	*thrA*$_1$*A*$_2$, *thrB, thrC*
GT73	KMBL397	6.2	−	−	−	−	−	−	+	*thrA*$_1$*A*$_2$, *thrB, thrC*
GT76	KMBL366	5	−	−	−	−	−	−	+	*thrA*$_1$*A*$_2$, *thrB, thrC*
GT67	AT905	4	−	−	−	−	−	−	+	*thrA*$_1$*A*$_2$, *thrB, thrC*
GT78	KMBL1554	10	−	−	−	−	−	−	+	*thrA*$_1$*A*$_2$, *thrB, thrC*

[a] Threonine mutations, obtained by phage Mu mutagenesis, were transduced from the original strains listed in the second column (see also Table 1) into GT64, a strain carrying the *serB22* allele.

[b] Complete name of a λ*dthr*$_c$ derivative is λ*dthr*$_c$ followed by the name of the threonine allele it carries. The symbol "+" indicates that the complementation was effective with the corresponding λ*dthr*$_c$ derivative. λ*dthr*$_c$ was used as a control.

[c] Gene(s) "inactivated" by polar effect in the corresponding strain analyzed.

[d] Differed widely from the other results for unexplained reasons.

genase I, respectively. In light of the fact that most *thrA* mutants were either of the *thrA*$_1$ or *thrA*$_2$ types, it is important to point out that the initial selection was made on threonine plus methionine plus lysine plus Dpm, which did not counterselect the *thrA*$_1$*A*$_2$ type. This result is consistent with the fact that aspartokinase I and homoserine dehydrogenase I are carried by independent regions of a single polypeptide chain (31).

The different types of *thrA* nonsense mutations and those induced by phage Mu insertion in *thrA* confirm the existence of and permit us to localize the two subregions of this gene. Among 13 *thrA* mutants, composed of five nonsense and eight phage Mu-induced mutants, nine *thrA*$_1$*A*$_2$ and four *thrA*$_2$ and no *thrA*$_1$ mutants were found. Thus, *thrA*$_1$ appears to be proximal to the promoter end of the gene, whereas *thrA*$_2$ is distal.

The proposed fine genetic structure of *thrA* is in agreement with previous biochemical investigation on aspartokinase I-homoserine dehydrogenase I (31). A carboxy-terminal fragment of 55,000-dalton mass has been isolated from the native aspartokinase I-homoserine dehydrogenase I subunit after mild proteolysis. This fragment carries the homoserine dehydrogenase I activity and should in part correspond to the polypeptide coded by the *thrA*$_2$ subregion. Further, a polypeptide of 45,000 daltons possessing only aspartokinase I activity has been purified from Gif 108, a derivative of Gif 102. This second fragment corresponds to the amino-terminal portion of the aspartokinase I-homoserine dehydrogenase I subunit and is likely to be in part coded by the *thrA*$_1$ subregion. The mass of the native subunit is 85,000 daltons.

The hypothesis of gene fusion has been suggested to explain the origin of the bifunctional enzyme aspartokinase I-homoserine dehydrogenase I (31). A similar situation has been artificially produced by the fusion of two adjacent genes of the histidine operon (35). In the case of *thrA*, it would be interesting, on the other hand, to produce an artificial separation leading to *thrA*$_1$ and *thrA*$_2$ genes, which would then produce independent polypeptides.

The analysis by complementation of the total polar effects induced by phase Mu integration allows us to conclude that the three threonine genes belong to the same operon transcribed in the following order: $\overrightarrow{thrA_1A_2BC}$. This is the only arrangement compatible with the existence and the pattern of the total polar effects observed. In agreement with this organization, nonsense mutations in *thrA* exert partial polar effects on the expression of *thrB*.

The threonine operon was first thought to be under the control of a multivalent repression

system dependent on the concentration of free threonine and isoleucine (9). It now appears that the corresponding transfer ribonucleic acids (tRNA's) play the major role. In strains treated with borrelidin, an antibiotic known to inhibit threonyl tRNA synthetase (11), the synthesis of aspartokinase I-homoserine dehydrogenase I (18) and homoserine kinase is derepressed. The same derepression has been found in a strain (Tir 8) carrying a mutation in an isoleucyl tRNA synthetase (6, 24). However, in this latter strain there are two other mutations which could play a role in the regulation of the threonine operon (2; M. Coker and H. E. Umbarger, Bacteriol. Proc., p. 135-136, 1970).

ACKNOWLEDGMENTS

We wish to thank P. Van de Putte, A. Taylor, A. Bukhari, M. Faelen, L. Caro, G. A. Jacoby, J. D. Smith, R. Thomas, M. Hofnung, P. Brachet, G. Lindhal, and M. Yarmolinsky for providing strains and phages. We are deeply indebted to: W. J. Schrenk and R. Weisberg for providing $\lambda dthr_c$ phage; M. Schwartz for numerous suggestions, criticisms, and discussions; and D. Margarita for her excellent technical assistance. In particular, we wish to thank G. N. Cohen for his encouragement and his constant interest in this work. We thank B. Burr and G. Kaplan for their help during the preparation of the manuscript.

This investigation was supported by grants from the Delegation Générale à la Recherche Scientifique et Technique, the Centre National de la Recherche Scientifique (E.R. no. 85), and the National Institutes of Health. I.S.G. is indebted to the Philippe Foundation for a fellowship during the course of this work.

LITERATURE CITED

1. Adelberg, E. A., and J. W. Myers. 1953. Modification of the penicillin technique for the selection of auxotrophic bacteria. J. Bacteriol. **65**:348-353.
2. Blatt, J. M., and H. E. Umbarger. 1972. On the role of isoleucyl t-RNA synthetase in multivalent repression. Biochem. Genet. **6**:99-118.
3. Boram, W., and J. Abelson. 1971. Bacteriophage Mu integration: on the mechanism of Mu-induced mutations. J. Mol. Biol. **62**:171-178.
4. Bukhari, A., and A. L. Taylor. 1971. Genetic analysis of diaminopimelic acid- and lysine-requiring mutants of *Escherichia coli*. J. Bacteriol. **105**:844-854.
5. Cohen, G. N., and H. V. Rickenberg. Concentration spécifique réversible des amino acides chez *Escherichia coli*. Ann. Inst. Pasteur (Paris) **91**:693-720.
6. Dwyer, S. B., and H. E. Umbarger. 1968. Isoleucine and valine metabolism of *Escherichia coli*. XVI. Pattern of multivalent repression in strain K-12. J. Bacteriol. **95**:1680-1684.
7. Falcoz-Kelly, F., J. Janin, M. Véron, P. Truffa-Bachi, and G. N. Cohen. 1972. Revised structure of aspartokinase I-homoserine dehydrogenase I of *Escherichia coli* K12. Evidence for four identical subunits. Eur. J. Biochem. **28**:507-519.
8. Franklin, N., and S. E. Luria. 1961. Transduction by bacteriophage P1 and the properties of the lac genetic region in *E. coli* and *S. dysenteriae*. Virology **15**:299-311.
9. Freundlich, M. 1963. Multivalent repression in the biosynthesis of threonine in *Salmonella typhimurium* and *Escherichia coli*. Biochem. Biophys. Res. Commun. **10**:277-282.
10. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum protein by means of the biuret reaction. J. Biol. Chem. **177**:751-756.
11. Hütter, R., K. Poralla, H. G. Zachau, and H. Zähner. 1966. Uber die Wirkungsweise von Borrelidin. Hemmung des Threonineinbaus in SRNA. Biochem. Z. **344**:190-196.
12. Jacob, F., and J. Monod. 1961. On the regulation of gene activity. Cold Spring Harbor Symp. Quant. Biol. **26**:193-211.
13. Jacob, F., and E. L. Wollman. 1961. Sexuality and the genetics of bacteria. Academic Press Inc., New York.
14. Janin, J., P. Truffa-Bachi, and G. N. Cohen. 1967. Subunits of the complex protein carrying the threonine sensitive aspartokinase activity in a mutant of *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **26**:429-434.
15. Jordan, E., H. Saedler, and P. Starlinger. 1968. 0° and strong polar mutations in the *gal* operon are insertions. Mol. Gen. Genet. **102**:353-363.
16. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190-206.
17. Martin, R. G., D. F. Silbert, D. W. E. Smith, and H. J. Whitfield. 1966. Polarity in the histidine operon. J. Mol. Biol. **21**:357-369.
18. Nass, G., K. Poralla, and H. Zähner. 1969. Effect of the antibiotic borrelidin on the regulation of threonine biosynthetic enzymes in *E. coli*. Biochem. Biophys. Res. Commun. **34**:84-91.
19. Newton, W. A., J. R. Beckwith, D. Zipser, and S. Brenner. 1965. Nonsense mutants and polarity in the lac operon of *Escherichia coli*. J. Mol. Biol. **14**:290-296.
20. Patte, J. C., G. Le Bras, T. Loviny, and G. N. Cohen. 1963. Rétro-inhibition et répression de l'homosérine déshydrogénase d'*Escherichia coli*. Biochim. Biophys. Acta **67**:16-30.
21. Patte, J. C., G. Le Bras, and G. N. Cohen. 1967. Regulation by methionine of the synthesis of a third aspartokinase and a second homoserine dehydrogenase in *Escherichia coli* K12. Biochim. Biophys. Acta **136**:245-257.
22. Stacey, K. A., and E. Simson. 1965. Improved method for the isolation of thymine-requiring mutants of *Escherichia coli*. J. Bacteriol. **90**:554-555.
23. Stadtman, E. R., G. N. Cohen, G. Le Bras, and H. de Robichon-Szulmajster. 1961. Feedback inhibition and repression of aspartokinase activity in *Escherichia coli* and *Saccharomyces cerevisiae*. J. Biol. Chem. **236**:2033-2038.
24. Szentirmai, A., M. Szentirmai, and H. E. Umbarger. 1968. Isoleucine and valine metabolism of *Escherichia coli*. XV. Biochemical properties of mutants resistant to thiaisoleucine. J. Bacteriol. **95**:1672-1679.
25. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of *Escherichia coli*. Bacteriol. Rev. **31**:332-353.
26. Taylor, A. L., and C. D. Trotter. 1972. Linkage map of *Escherichia coli* strain K-12. Bacteriol. Rev. **36**:504-524.
27. Thèze, J., L. Kleidman, and I. Saint Girons. 1974. Homoserine kinase from *Escherichia coli* K-12: properties, inhibition by L-threonine, and regulation of biosynthesis. J. Bacteriol. **118**:577-581.
28. Thèze, J., and D. Margarita. 1974. Characterization of a bacteriophage λ transducing the threonine operon of *Escherichia coli* K-12. Mol. Gen. Genet., in press.
29. Thèze, J., D. Margarita, G. N. Cohen, F. Borne, and J. C. Patte. 1974. Mapping of the structural genes of the three aspartokinases and of the two homoserine dehydrogenases of *Escherichia coli* K-12. J. Bacteriol. **117**: 133-143.
30. Toussaint, A. 1969. Insertion of Mu-1 within prophage λ: a new approach for studying the control of the late

functions in bacteriophage λ. Mol. Gen. Genet. **106**:89–92.
31. Véron M., F. Falcoz-Kelly, and G. N. Cohen. 1972. The threonine sensitive dehydrogenase and aspartokinase activities of *Escherichia coli* K-12. The two catalytic activities are carried by two independent regions of the polypeptide chain. Eur. J. Biochem. **28**:520–527.
32. Wampler, D. E., and E. W. Westhead. 1968. Two aspartokinases from *Escherichia coli*. Nature of the inhibition and molecular changes accompanying reversible inactivation. Biochemistry **7**:1661–1671.
33. Whitfield, H. J., R. G. Martin, and B. N. Ames. 1966. Classification of amino transferase (C gene) mutants in the histidine operon. J. Mol. Biol. **21**:335–355.
34. Yanofsky, C., and J. Ito. 1966. Nonsense codons and polarity in the tryptophan operon of *Escherichia coli*. J. Mol. Biol. **21**:313–334.
35. Yourno, J., T. Kohno, and J. R. Roth. 1970. Enzyme evolution: generation of a bifunctional enzyme by fusion of adjacent genes. Nature (London) **228**:820–824.