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A genetical study of thymineless mutants of *E. coli* K12

By S. I. ALIKHANIAN, TAMILLA S. ILJINA, EZA S. KALIAEVA, SVETLANA V. KAMENEVA and V. V. SUKHODOLEC

Kurchatov's Institute of Atomic Energy, Moscow, USSR

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1. INTRODUCTION

Mutations of a gene controlling biosynthesis of thymidylate synthetase cause thymine-requiring mutants (*thy*) to occur (Cohen & Barner, 1954). The mutant cells, in contrast to the prototrophs, are capable of using external thymine for growth (Crawford, 1958). It is interesting that although a *thy*$^+$ strain does not utilize external thymine, nevertheless all the enzymes necessary for incorporation of external thymine into DNA are present in extracts of this strain (Mantsavinos & Zamenhof, 1961). It is possible to suppose that there is a mechanism in the cell for switching over the thymidilate biosynthesis from one pathway to another.

Unlike other auxotrophs, which stop dividing in the absence of the necessary growth factor, thymineless mutants die when deprived of thymine. The mechanism of thymineless death is not yet clear. However, numerous investigations (Maaløe & Hanawalt, 1961; Menningmann & Szybalski, 1962; Maaløe, 1963) have shown that death in the absence of thymine is due to disturbances in normal DNA replication, while biosynthesis of other cell components appears to be normal.

The phenomenon of thymineless death in *E. coli* K12 prevents the isolation of *thy* auxotrophs by the usual technique. However, these mutants can readily be selected by the method of Okada, Yanagisawa & Ryan (1960, 1961) in which wild-type bacteria are exposed simultaneously to thymine and aminopterin; only *thy* mutants can utilize the exogenous thymine and so are selected. Thymine mutants of *E. coli* K12 isolated by this technique have already been used for genetical studies. Kitsuji (1964) located the thymine locus within the chromosomal segment between the *str* and *his* loci. He also demonstrated that *thy*$^+$ recombinants could be obtained in crosses of different thymine mutants. Ishibashi, Sugino & Hirota (1964) have shown that the *thy* and *arg*B loci are adjacent to each other. Alikhanian, with colleagues, carried out a detailed genetical investigation of 150 *thy* mutants related to thymidylate biosynthesis (1965 and Appendix). In the present paper the data obtained in the investigation of these 150 *thy* mutants are summarized and more recent results presented.

2. MATERIALS AND METHODS

(i) *Strains of bacteria and phage*

We used *E. coli* K12 strain Hfr3.OSO *thi*$^-$*lac*$^-$ for isolating thymine mutants. The original strain, when crossed, transferred markers in the sequence: O-*thr-leu-lac-his* (Fig. 1). 150 thymine-requiring mutants were numbered from

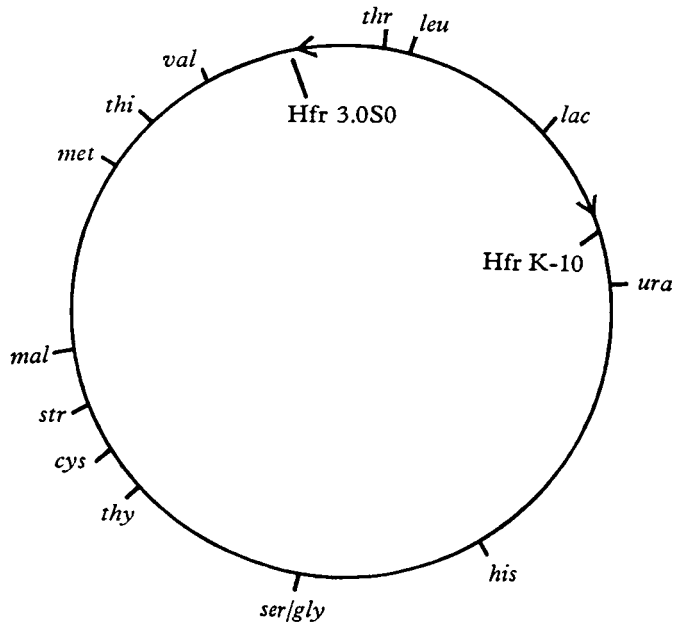

Fig. 1. Genetical map of *E. coli* K12.

1 to 151 (there was no *thy*72) according to their sequence of isolation. We used also the following *E. coli* K12 strains:

(1) P-678 F⁻ *thr⁻leu⁻gal⁻str-r*;
(2) S21 F⁻ *mal⁻cys⁻his⁻str-r*; this strain was isolated as a *ura⁺str-r* recombinant from a cross PA330.JA287 *mal⁻his⁻ura⁻str-r* × Hfr K10 *cys⁻*; this *cys* mutation was obtained by us, but not identified in terms of *cys*A, *cys*B and so on.
(3) PA260 F⁺ *ser/gly, str-r*; this was obtained from PA260 F⁻ *ser/gly, str-r*.

Bacteriophage P1*kc* (Lennox, 1955) was also employed, and the *Sh*16 strain of *Shigella dysenteriae* was used as an indicator culture.

Strains P-678, Hfr 3.OSO, PA330.JA287, PA260, and 58-161 and also P1*kc* phage were obtained from Dr F. Jacob; Hfr K10 from Dr C. Levinthal; and *Sh*16 strain of *Sh. dysenteriae* from Dr S. E. Luria, for whose courtesy we are very grateful.

(ii) *Isolation of* thy *mutants*

A modification of the method of Okada *et al.* (1960, 1961) was used. The proportion of *thy⁻* mutants isolated appears to depend on two opposing factors. One is the number of mutants produced, which should increase with the initial population density; the other is the period available for their accumulation, during growth of the population to about 10^9 cells/ml., which decreases with increase of the initial population density. In our experiments we have found the optimal initial density to be 10^5 bacteria/ml. From those tubes which failed to yield mutants, inocula of

about 10^5 bacteria were transferred to fresh media containing aminopterin and thymine: a mutagen ratio of 1:10 was used instead of 1:1 (Okada *et al.*, 1961) to increase the efficiency of incorporation of thymine analogues. By these means, and using the following mutagens, 150 *thy* mutants have been isolated:

5-iododeoxyuridine: 99 mutants (*thy*1 to *thy*100)
Ultra-violet light: 10 mutants (*thy*111 to *thy*119)
5-bromouracil: 18 mutants (*thy*120 to *thy*137)
5-bromodeoxyuridine: 1 mutant (*thy*151)
Spontaneous: 13 mutants (*thy*138 to *thy*150)

(iii) *Method of crossing*

To produce recombinants, broth cultures of Hfr or F^+ and F^- bacteria were grown overnight at 37°C. in a shaker and then transferred to fresh broth. After 1·5–2 hours' growth, the F^- strain was centrifuged and resuspended to a population density 5- to 10-fold greater than that of the Hfr or F^+ culture. Equal volumes of the two suspensions were then mixed and gently shaken at 37°C. for 2·5 hours. The mixture was finally centrifuged and the bacteria resuspended and plated on an appropriate selective medium. Recombinants were scored for non-selective markers by streaking on further selective media.

(iv) *Genetic analysis of* thy *mutants*

To accomplish reciprocal crosses 23 *thy* mutations (Nos. 2, 3, 4, 7, 9, 10, 13, 14, 15, 33, 64, 78, 83, 86, 87, 89, 108, 111, 120, 127, 128, 130, 151) were transferred from Hfr 3.OSO into S21 F^- and F^- *cys*, *thy*, *his*, *str-r* recombinants were selected among *mal*$^+$*str-r* progeny. Mutations 2, 3, 4, 7 and 9 were also transferred into S21 *val-r*, so that streptomycin-sensitive strains S21 F^- *cys*, *thy*, *his*, *str-s*, *val-r* were selected.

In order to map *thy* mutations, three-factor reciprocal crosses were made between mutants as follows: S21 *cys*$^-$*thy*2*str-r* × Hfr 3.OSO *cys*$^+$*thy*3 *str-s* and S21 *cys*$^-$*thy*3*str-r* × Hfr 3.OSO *cys*$^+$*thy*2*str-s*. In each cross the number of *cys*$^+$*thy*$^+$*str-r* recombinants was evaluated. The frequency of *thy*$^+$ recombinations obtained was expressed as the ratio of *cys*$^+$*thy*$^+$*str-r* recombinants to a *cys*$^+$*str-r* standard recombinant class.

The arrangement of *thy* mutations relatively to the *cys* marker was established by comparing the frequencies of wild-type recombinants in two reciprocal crosses. In one arrangement a quadruple crossover was necessary to obtain a *cys*$^+$*thy*$^+$*str-r* recombinant, while in the other a double crossover was sufficient.

The recombinant ratio $\frac{cys^+thy^+str\text{-}r}{thy^+str\text{-}r}$ has been used as an additional criterion for establishing the order of *thy* mutants. The ratio will clearly be greater in whichever of the two reciprocal crosses requires only two crossovers to yield *cys*$^+$*thy*$^+$*str-r* recombinants.

(v) *Burst size of phage P*1kc

To investigate the burst size from thymineless mutants, the bacterial cells were prepared as follows: a sample of overnight culture grown in thymine-containing minimal medium was transferred into fresh minimal medium with thymine, grown to about 6–8 $\times 10^8$ organisms/ml., centrifuged twice and resuspended in $CaCl_2$ buffer (0·25 μg./ml.; a cofactor for phage adsorption). Then the cells were aerated at 37°C. for 60 min. to reduce the internal thymine content. For evaluation of average burst size we used the technique of Ellis & Delbrück (1939). In the P1*kc* experiments we used the following medium (grams per litre of distilled water): NH_4Cl, 5·0; NH_4NO_3, 1·0; K_2HPO_4, 3·0; KH_2PO_4, 1·0; Na_2SO_4, 2·0; $MgSO_4.7H_2O$, 0·1; glucose, 2·0; pH = 7. Additional ingredients were used in the following concentrations (in μg./ml.): thiamine, 10·0; thymine, 25·0; L-cysteine, 25; DL-threonine, 150; L-leucine, 70; streptomycin, 200 μg./ml.; thymidine (as a selective agent), 150; and valine (as a selective agent), 100.

(vi) *Thymineless death*

To investigate mutants for 'thymineless death', log-phase bacteria were transferred into minimal thymineless medium at about 10^4 organisms/ml. A count of viable cells was made after 4 hours' incubation at 37°C. with aeration, by plating on a complete medium.

(vii) *Thymine incorporation*

Log-phase bacteria were grown to about 5×10^8 cells/ml. in liquid minimal medium (see section V), supplemented with 20 μg./ml. thymine for *thy*$^-$*tlr*$^+$ and 1 μg./ml. for *thy*$^-$*tlr* mutants. After washing, the cells were resuspended in minimal medium without thymine and aerated for an hour at 37°C. to exhaust the intracellular thymine. (Omitting this procedure of thymine starvation did not change the results of incorporation.) ^{14}C-thymine or ^{14}C-thymidine was then added; and at various times thereafter samples were transferred to chilled buffer containing unlabelled thymine and washed three times by centrifugation to remove the label. The pellets were finally dissolved with formic acid, 0·2 ml. samples were dried on aluminium planchettes and their radioactivity determined. In some cases washed cells were treated with 0·2 N $HClO_4$ to measure radioactivity that had been incorporated in the acid-insoluble fraction.

3. RESULTS

(i) *Thymineless death*

A characteristic feature of thymineless mutants is that they die because of unbalanced growth in the absence of thymine (Cohen & Barner, 1954). The phenomenon has been found in two mutants, B-3 and 15T$^-$. It was of interest to investigate whether thymineless death is characteristic of all our mutants. Of our

150 mutants, 134 were investigated; the remaining 16 were unstable *thy* mutants. All died in the absence of thymine. In some, the lag preceding death was prolonged so that death of all the cells occurred after 6 hours' thymine starvation instead of the usual 3 hours.

(ii) *Loss of a sex-factor from thymineless mutant cells*

A test of the mutants for Hfr character has shown that most of them have lost this character and behave as F^+, although all of them originate from the same Hfr 3.OSO strain. F^+ 3·OSO *thy* mutants in mating with F^- P-678 form *thr*$^+$*leu*$^+$*str-r* recombinants with a frequency of about 0·04 to 0·05% donor cells, that is, about 100-fold lower than the frequency when Hfr 3.OSO *thy* mutants are used.

The F^+ mutants, just like typical F^+ strains, transfer their sex factor to recombinants; in matings with F^- strains all the progeny appear to be F^+. The Hfr to F^+ conversion seems to result from aminopterin treatment because the untreated Hfr 3.OSO strain itself is reasonably stable: a test of 50 single-colony Hfr 3.OSO isolates has shown all of them to be Hfr.

Some of the *thy* mutants appear to be sterile in matings with P-678 and S21 F^- strains. These mutants seem, therefore, to have lost their F-factors, though the mechanism of this phenomenon is not clear.

In total, only 45 of the mutants retained the Hfr character; 103 mutants were found to be F^+, and 2 to be F^- strains (*thy*113 and *thy*144).

(iii) *Location of the* thy *locus on the chromosome*

In crosses of F^- S21 (*cys*$^-$*thy*$^-$*str-r*) with Hfr 3.OSO (*cys*$^+$, *thy*$^+$, *str-r*) the *thy* marker was found to be linked to the *cys* marker; when selection of *cys*$^+$*str-r* recombinants was made nearly 70% of the offspring carried the Hfr *thy*$^+$ marker. Linkage of *thy* and *cys* was also shown by linked transduction of these markers by P1*kc* bacteriophage.

The order of *cys* and *thy* was determined by crosses where these markers were unselected (Table 1). The results favour the order of genes: *mal-cys-thy*. According to this sequence (1) the probability of *cys*$^+$ integration is greater than that of *thy*, and (2) the *cys*$^+$*thy*$^+$ recombinant class appears to be more probable (two crossovers) than *cys thy* (four crossovers).

In order to localize the *thy* locus in relation to the *ser/gly* marker the genetic constitutions of *str-r val-r* and *cys*$^+$*val-r* recombinants were determined in a cross S21 F^-*cys*$^-$*thy*$^-$*str-s val-r* × F^+ *ser/gly*$^-$*str-r val-s*. It was found that among *str-r val-r* recombinants 74% were *cys*$^+$, 54% were *thy*$^+$, and only 6% were *ser/gly* recombinants. This result led to the suggestion that *thy* and *ser/gly* are not linked or, at least, not tightly linked. 126 *cys*$^+$*val-r* were tested for *thy* and *ser/gly* markers. Among them were found 19 *thy*$^+$*ser/gly*, 24 *thy*$^-$*ser/gly*$^+$, 11 *thy*$^-$*ser/gly*$^-$, and 72 *thy*$^+$*ser/gly*$^+$. These data suggested the following gene order: *cys-thy-ser/gly*, since (1) the frequency of *thy* integration (91/126) was higher than that of *ser/gly*

(30/126) and (2) the *thy*$^+$*ser*/*gly*$^+$ recombinant class was more frequent (two crossovers) than *thy ser*/*gly* (four crossovers).

Table 1. *The genetical constitution of* mal$^+$, val-r *recombinants with respect to genes* cys *and* thy *in the cross:*
Hfr 3.*OSO*, mal$^+$cys$^+$thy$^-$val-s × *F*$^-$*S*21, mal$^-$cys$^-$thy$^+$val-r.

Male strain	Absolute number of recombinants					Integration of markers of Hfr strain (%)	
	cys$^-$*thy*$^+$	*cys*$^+$*thy*$^-$	*cys*$^-$*thy*$^-$	*cys*$^+$*thy*$^+$	Total	*cys*$^+$	*thy*$^-$
Hfr 3.OSO *thy*2	100	21	4	44	169	38·4	14·8
Hfr 3.OSO *thy*3	135	21	15	34	205	26·8	17·6
Total	235	42	19	78	374	32·1	16·3

(Each line represents the results of one conjugation experiment.)

(iv) *The fine genetic mapping of the* thy *locus*

In crosses between Hfr 3.OSO and PA330-JA287, or its S21 derivative, transfer of a thymine marker occurs with a frequency of about 1% of the donor cell input. A sufficient number of recombinants can thus be obtained for high resolution genetic analysis of the *thy* locus. In matings between Hfr and F$^-$ *thy* mutants, we have generally found at least 100,000 recombinants of the standard class *cys*$^+$*str-r* per millilitre of conjugation mixture, except in the case of F$^-$ S21 strains *thy*33, *thy*89, *thy*120 and *thy*130 where this number was reduced to not more than 20,000. Thus if wild-type (*thy*$^+$) recombinants were absent from 1 ml. of conjugation mixture, the frequency of recombination should be less than 0·0005% of the number of standard class recombinants (confidence limit = 0·5).

This resolving power should be adequate to detect the lowest recombination frequency likely to be found. Experimentally, if *thy*$^+$ recombinants were not found in the sample containing 100,000 *cys*$^+$*str-r* cells, they were also absent from 10-fold and even 50-fold greater volumes of conjugation mixture.

On the other hand, mapping of the F$^+$ mutants has been made with less precision. In matings with F$^+$ mutants not more than 5000 *cys*$^+$*str-r* recombinants are generally formed per millilitre of conjugation mixture. When *thy*$^+$ recombinants are absent from such crosses the recombination frequency is only 0·01% of 5000 *cys*$^+$*str-r* recombinants (confidence limit = 0·5).

As a result of matings between 45 Hfr *thy* mutants, 14 sites were found within the *thy* locus. A mutant from each site was chosen for reciprocal crosses. More than 50 combinations of reciprocal crosses allow us to arrange the sites in the following order: *cys*-3-130-2-120-128-33-7-89-87-86-111-78-83-108. In the establishment of this order the crosses between adjacent mutants are 'critical' (Table 2).

As shown in Table 2, the location of a pair of mutations with respect to the *cys* gene is suggested by comparing the ratios *cys*$^+$*thy*$^+$*str-r*/*thy*$^+$*str-r* and *cys*$^+$*thy*$^+$*str-r*/

Table 2. *Reciprocal three-factor crosses between* thy *mutants**

No. of cross	Designation of *thy* mutants crossed		Total number of recombinants			Recombinant ratios (%)		No. of independent crosses performed
	F⁻	Hfr	*cys*⁺*thy*⁺*str-r*	*thy*⁺*str-r*	*cys*⁺*str-r* (× 1000)	*cys*⁺*thy*⁺*str-r* / *thy*⁺*str-r*	*cys*⁺*thy*⁺*str-r* / *cys*⁺*str-r*	
1	3	130	2430	—	98	—	2·480	1
	130	3	387	861	44	45·0	0·884	1
2	130	2	88	138	409	68·8	0·021	2
	2	130	115	402	973	28·6	0·012	3
3	2	120	14	66	265	21·2	0·005	4
	120	2	8	82	342	9·8	0·002	2
4	120	128	101	188	118	53·8	0·086	3
	128	120	496	1193	640	41·6	0·077	3
5	128	33	158	489	822	32·3	0·019	4
	33	128	0	2	6	< 25·0	< 0·008	3
6	33	7	2	6	4	33·3	0·050	3
	7	33	294	1059	5125	27·8	0·006	5
7	7	89	110	170	280	64·0	0·039	1
	89	7	0	0	2·5	—	< 0·020	1
8	89	87	5	9	11	55·6	0·045	2
	87	89	25	53	331	47·2	0·008	2
9	87	86	308	762	440	40·4	0·070	5
	86	87	425	1961	778	21·6	0·055	4
10	86	111	1261	2535	813	49·8	0·155	1
	111	86	281	617	195	45·5	0·144	1
11	111	78	19	45	270	42·2	0·007	2
	78	111	4	32	211	12·5	0·002	2
12	78	83	210	775	151	27·8	0·139	2
	83	78	14	161	53	8·7	0·026	1
13	83	108	170 (107)**	253	282	42·3	0·060	3
	108	83	304 (227)**	638	1539	35·6	0·020	4

* In each experiment 0·5 ml. conjugation mixture was plated on:
minimal medium (MM) + streptomycin (S) for selection of *cys*⁺*thy*⁺*str-r* recombinants;
MM + S + cysteine for selection of *thy*⁺*str-r* recombinants;
MM + S + thymine for selection of *cys*⁺*str-r* recombinants.

** In these crosses the *thy*⁺*str-r* recombinants were not counted in all the experiments; the *cys*⁺*thy*⁺*str-r* recombinant number used for evaluating *cys*⁺*thy*⁺*str-r*/*thy*⁺*str-r* is therefore less and is given in brackets.

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cys$^+$ *str-r* in both combinations of a reciprocal cross. The significance of the difference between these ratios was assessed by entering the pooled data in a 2×2 table, computing and evaluating χ^2 with one degree of freedom.

In two crosses only (Nos. 4 and 10 of Table 2) has the χ^2 test shown the difference between the *cys*$^+$*thy*$^+$*str-r*/*cys*$^+$*str-r* ratios not to be reliably significant (less than 90% probability). In addition, the *cys*$^+$*thy*$^+$*str-r*/*thy*$^+$*str-r* ratios do not differ appreciably (less than 90% of probability) in crosses Nos. 6 and 8, and are not

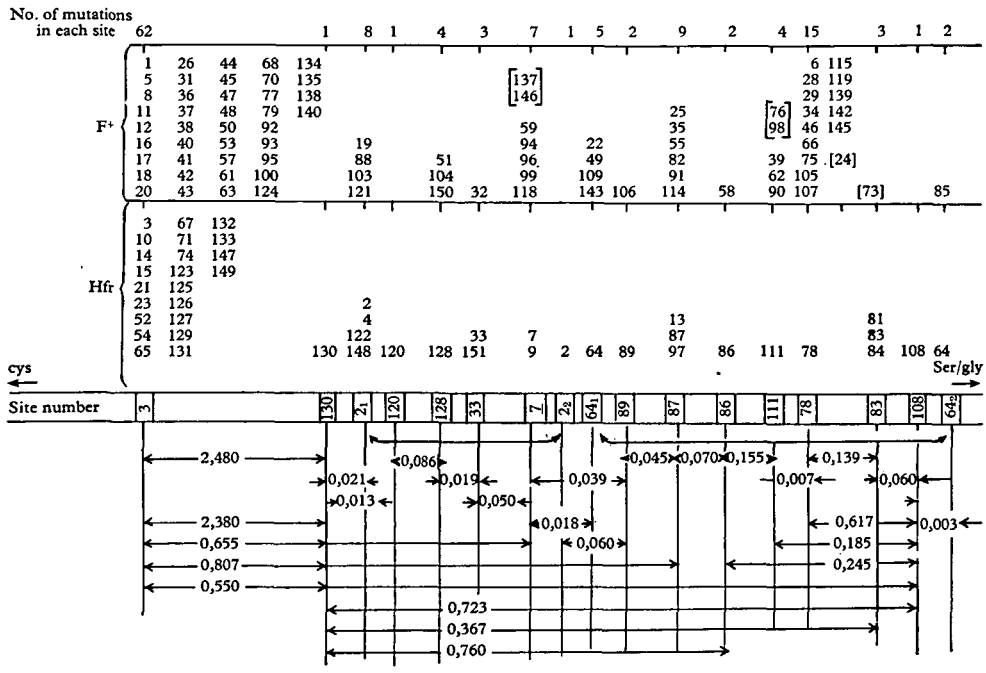

Fig. 2. Genetical map of the *thy* locus in *E. coli*. Two of 150 *thy* mutants originating from Hfr 3·OSO, *thy*13 and *thy*144 (both F$^-$), have not been mapped. 134 mutants are given in the map; the remaining 14 *thy* mutants (27, 30, 56, 60, 69, 80, 101, 102, 110, 112, 116, 117, 136, 141—all F$^+$) are located within the *thy* locus, but their precise location has not been established because of their strong reversibility. The precise orientation of sites 2_2 and 64_1 relative to each other has not been determined.

significant enough (more than 90% but less than 95% of probability) in crosses Nos. 4 and 13. No data contradictory for the outlined site order have been obtaining in other crosses.

To estimate the distances between mutations, the greater of the two values of *cys*$^+$*thy*$^+$*str-r*/*cys*$^+$*str-r* from the reciprocal crosses has been taken as reflecting the probability of a single crossover between sites. Some of these distances are shown in Fig. 2. Some of the estimates of distances are seen to be mutually inconsistent, but the cause of these inconsistencies is not yet clear. It will be seen that most of the F$^+$ mutants have been distributed between separate sites with the exception of Nos. 24, 73, 76, 98, 137, and 146, which give recombinants in matings with the representatives

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of all sites. These mutants are conventionally attributed to those sites with which they have the lowest frequency of recombination. A common feature of all the mutants attributed to one site is their inability to form *thy*$^+$ recombinants in crosses with the mutant whose number is used to indicate the site. However, every mutant attributed to a particular site has been tested by mating with at least one of the neighbouring site mutants. Thus, all the mutants of site 78 form *thy*$^+$ recombinants in mating with *thy*111 and vice versa. Mutants of the most numerous site, No. 3, all recombine with *thy*2 and *thy*7.

An attempt was made to carry out reciprocal crosses between the different mutants within one site. For this purpose reciprocal Hfr crosses were made between *thy*3, *thy*10, *thy*14, *thy*15 and *thy*127. In none of these combinations have *thy*$^+$ recombinants been obtained. In view of the high resolving power of the method used, it seems clear that all these mutants really belong to the same site. The same test cannot be used for the F$^+$ 3.OSO mutants, where the lower resolving power may be insufficient to distinguish closely linked sites.

In order to investigate whether *thy* mutants result from mutations in one or more cistrons, experiments with abortive transduction were carried out using the temperate phage P1*kc*. When *thy* mutants were infected with phage from the wild-type strain and plated on enriched minimal medium, minute colonies were detected in addition to the large colonies normally expected as a result of transduction. The frequency of occurrence of such minute colonies was, however, lower than that of the large ones. When *thy* bacteria were infected with phage from any of the *thy* mutants, including those from other sites, no minute colonies appeared. These experiments suggest that the *thy*$^+$ character is due to a single cistron.

One of the most interesting findings of this study was the discovery that all the mutations mapping at site No. 3 are temperature-sensitive, i.e. are auxotrophic only at 37°C. and grow normally on minimal medium at 28°C. In addition, this site is clearly a 'hot spot' as Fig. 2 shows, since nearly half the total number of mutations isolated arise there.

(v) *A gene controlling the quantitative thymine requirement of* thy *mutants*

Thymineless mutants fell into two groups according to their quantitative thymine requirements. Mutants of the first type require 10–20 μg./ml. thymine for growth, while the other type requires 0·5–1·0 μg./ml. only. In populations of the first type, variants arise which can grow at the lower concentration of thymine. However, both types of mutation are identical in the efficiency with which thymidine satisfies their thymine need and require not more than 0·5–1·0 μg./ml. thymidine for normal growth. A genetical study of these latter mutants has shown that the low requirement is due to mutation at another locus unlinked to *thy*, and designated as *tlr* (thymine low requirement). When mutants *thy* 64*tlr*$^-$ and *thy*83*tlr*$^-$ were crossed with various F$^-$ *thy* strains (requiring 20 μg./ml. thymine), this locus was found to map at the proximal end of the Hfr 3.OSO chromosome and to be linked to the *thr* locus (see Fig. 1).

(vi) *Burst size of P1*kc *phage from the* thy *mutants*

When the burst sizes of P1*kc* phage from *thy⁻tlr⁻* and *thy⁻tlr⁺* cells were compared, that from *thy⁻tlr⁻* was found to reach a maximum at a concentration of thymine at least 10-fold lower than was required for maximum burst size from *thy⁻tlr⁺* (Fig. 3). The burst size in the latter case was always lower than that from the *thy⁻tlr⁻* strain. When thymidine was used as a growth factor it was shown that *thy⁻tlr⁺* and, *thy⁻tlr⁻* strains did not differ in their thymidine requirements. The high burst size

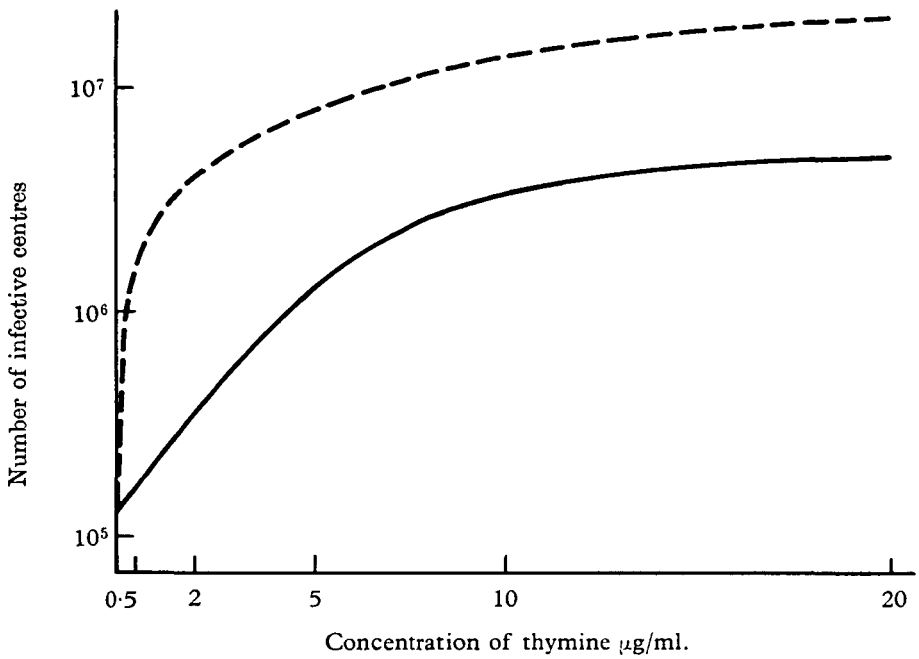

Fig. 3. The burst size of phage P1*kc* from *E. coli* mutants *thy⁻tlr⁺* (solid line) and *thy⁻tlr⁻* (broken line) as a function of thymine concentration in the medium.

is demonstrated for mutants of both types at low concentration of thymidine in the medium (0·1 μg./ml.). All this implies that there is a specificity for thymine requirement by these mutants. It is likely that a mutation in the *tlr* gene results in the activation of some enzyme promoting the thymine–thymidine conversion.

(vii) *The influence of* thy *and* tlr *mutations on* ^{14}C*-thymine incorporation by* E. coli *K*12 *cells*

In an investigation of the kinetics of ^{14}C-thymine incorporation into *thy⁻tlr⁺* and *thy⁻tlr⁻* strains, data were obtained indicating that at low thymine concentration (0·5 μg./ml.), sufficient for growth of the *thy⁻tlr⁻* mutant but not for that of the mutant with high thymine requirement (*thy⁻tlr⁺*), there is a difference in exogenous thymine incorporation. The results are shown in Table 3.

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Table 3. *Kinetics of incorporation of* ^{14}C*-thymine* by* thy⁻tlr⁺ *and* thy⁻tlr⁻ *strains at 0·5 μg./ml. concentration of thymine*

	thy⁻tlr⁺		*thy⁻tlr⁻*	
Minutes	Counts/min.	Cells/ml.	Counts/min.	Cells/ml.
15	372	$6{\cdot}8 \times 10^8$	10,428	$6{\cdot}0 \times 10^8$
30	552	$5{\cdot}5 \times 10^8$	24,498	$6{\cdot}4 \times 10^8$
60	708	$6{\cdot}2 \times 10^8$	33,000	$6{\cdot}9 \times 10^8$
90	1,008	$5{\cdot}7 \times 10^8$	31,572	$5{\cdot}7 \times 10^8$
120	1,056	$6{\cdot}4 \times 10^8$	40,800	$5{\cdot}0 \times 10^8$
150	1,269	$5{\cdot}2 \times 10^8$	40,668	$2{\cdot}1 \times 10^8$

* Specific activity 10,000 counts/min. per μg.

Table 4. *Kinetics of incorporation of* ^{14}C*-thymine* by* thy⁻tlr⁺ *and* thy⁻tlr⁻ *strains at 20 μg./ml. concentration of thymine (*^{14}C*-thymine constituted one-fifth of total thymine)*

	thy⁻tlr⁺		*thy⁻tlr⁻*	
Minutes	Counts/min.	Cells/ml.	Counts/min.	Cells/ml.
10	2,365	5×10^8	3,545	5×10^8
20	4,869	5×10^8	5,973	5×10^8
40	9,865	5×10^8	8,990	5×10^8
60	16,154	5×10^8	13,060	5×10^8

* Specific activity 10,000 counts/min. per μg.

At higher concentrations of thymine, sufficient for growth of *thy⁻tlr⁺* mutants, the kinetics of ^{14}C-thymine incorporation by *thy⁻tlr⁺* and *thy⁻tlr⁻* mutants has been shown to be the same (Table 4). These results show that the *thy⁻tlr⁺* mutant cannot grow at low thymine concentration because of an insufficient supply with exogenous thymine. A high concentration of thymine in the medium is necessary for a normal input of external thymine into the *thy⁻tlr⁺* cells. Mutants carrying the additional mutation *thy⁻tlr⁻* are able to utilize exogenous thymine more efficiently.

In experiments with ^{14}C-labelled uracil and cytosine it was shown that equal amounts of these compounds penetrate into both *thy⁻tlr⁺* and *thy⁻tlr* cells. These results suggest that the *tlr* gene specifically controls thymine incorporation into cells.

It was known from the literature (Crawford, 1958) that wild-type *E. coli* cells (*thy⁺*) do not utilize exogenous thymine for DNA synthesis. At the same time it was shown (Mantsavinos & Zamenhof, 1961) that a reversion of the 15T⁻ *E. coli* strain to prototrophy did not result in loss of ability to utilize external thymine.

We investigated ^{14}C-thymine and ^{14}C-thymidine incorporation into wild-type cells of *E. coli* Hfr 3.OSO. As one can see from Table 5, thymine penetrates the cells

Table 5. *Kinetics of incorporation of* ^{14}C*-thymine* and* ^{14}C*-thymidine* by the cells of* E. coli 3.*OSO* thy$^+$

	Thymine			Thymidine			
	0·01 μM	0·2 μM		0·01 μM		0·2 μM	
Minutes	Total uptake (counts/min.)	Total uptake (counts/min.)	% acid insoluble	Total uptake (counts/min.)	% acid insoluble	Total uptake (counts/min.)	% acid insoluble
10	10	112	47	325	42	1720	47
30	27	112	47	346	59	2200	52
50	27	165	69	388	62	2240	56
70	35	204	59	405	74	2600	65
90		230	65	442	62	4000	55

* Specific activity 3,500,000 counts/min. per μmole.

less readily than thymidine both at low and high concentrations. However, that part of the label incorporated into the acid-insoluble fraction is approximately the same for both when high concentrations were used.

Experiments with spontaneous *thy*$^+$ revertants of *thy*$^-$*tlr*$^+$ and *thy*$^-$*tlr*$^-$ mutants, as well as with *thy*$^+$ transductants obtained from the same strains, were also performed (Table 6). The prototrophic revertants and transductants isolated from the

Table 6. *The incorporation of* ^{14}*C-thymine* by wild cells of* E. coli *K*12 *Hfr* 3.*OSO* thy$^+$*-revertants* (*R*), *and* thy$^+$*-transductants* (*T*)

Designation of strains assayed	Counts/min. per 10^9 cells	Designation of strains assayed	Counts/min. per 10^9 cells
thy$^-$ 104	270	*thy*$^-$ 104 *tlr*	12,460
thy$^-$ 95	160	*thy*$^-$ 95 *tlr*	12,000
T 1	8	T *tlr* 1	70
T 2	8	T *tlr* 2	122
T 3	16	T *tlr* 3	132
T 4	10	T *tlr* 4	194
T 5	20	T *tlr* 5	86
R 1	12	R *tlr* 1	136
R 2	10	R *tlr* 2	182
R 3	20	R *tlr* 3	118
R 4	8	R *tlr* 4	164
R 5	10	R *tlr* 5	156
E. coli K12 Hfr 3.OSO	16		

Thymine concentration was 1 μg./ml.; labelled thymine constituted half of the total thymine; time of labelling was 30 min.

* Specific activity 26,000 counts/min. per μg.

thy$^-$*tlr*$^+$ mutants did not differ much from the wild-strain *E. coli* 3.OSO in their incorporation of external thymine. The efficiency of incorporation of external thymine by the *thy*$^+$*tlr*$^-$ revertants and transductants is higher than by *thy*$^+$*tlr*$^+$ ones, but greatly reduced in comparison with that of the original *thy*$^-$*tlr*$^-$ mutant. Thus, when the synthesis of thymine is disturbed, as a result of a *thy*$^-$ mutation, the cells start to utilize external thymine; and vice versa, when the capacity to synthetize thymine is restored, the incorporation of external thymine stops. We obtained a similar effect in our experiments with aminopterin treatment of a wild strain. Aminopterin is known to inhibit thymidilate synthesis. An estimation of ^{14}C-thymine incorporation into cells, untreated and treated with aminopterin, has shown that an inhibition of thymidilate synthesis and subsequent exhaustion of its intermediates in the cells makes them capable of incorporating external thymine.

(viii) *A gene for thymidine sensitivity*

The growth of some *thy*$^-$*tlr*$^-$ and *thy*$^-$*tlr*$^+$ strains has been shown to be completely inhibited when thymidine is added to the medium at concentrations of 10 μg./ml. and higher (Fig. 4). But in a great number of *thy*$^-$*tlr*$^-$ mutants thymidine does not

inhibit growth; only 3 of 13 independently isolated *thy*$^-$*tlr*$^-$ mutants were found to be thymidine-sensitive (*td-s*), the remainder being thymidine-resistant (*td-r*).

A test for viability has shown that *td-s* bacteria cannot divide in thymidine medium; however, they remain viable. After prolonged incubation (up to 24 hours) of *td-s* cells in thymidine medium they resume their growth, possibly by the digestion of thymidine in the medium by thymidine phosphorylase action (Rachmeller, Gerhardt & Rosner, 1961). A *td-s* mutation is phenotypically expressed when it is

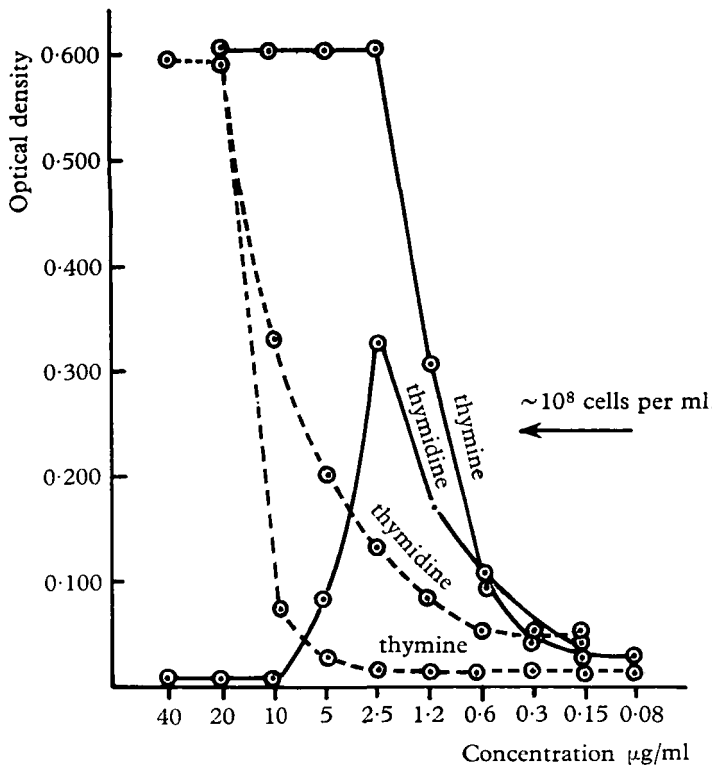

Fig. 4. The rate of bacterial growth of *E. coli* Hfr 3.OSO *thy*8 (broken line) and Hfr 3.OSO *thy*8 *td-s* (solid line) as a function of thymine and thymidine concentration. Density assessed after 15 hours' incubation on a shaker at 37°C. The mutation symbol *td-s* denotes thymidine sensitivity.

transferred into a prototrophic (*thy*$^+$) strain so that the *td* locus can rather easily be located on the chromosome. It has been found by a series of crosses that *td* is located at the proximal end of the Hfr 3.OSO chromosome, close to *thr* and *leu*. In Table 7 the results of a cross are shown where the localization of *td* was determined in regard to *thr* and *leu* (three-point test). As the data show, the most probable gene order is: *td-thr-leu*.

We have retained the *tlr* symbol for the locus controlling the quantitative thymine requirement only, without influencing thymidine sensitivity. As discussed earlier, *tlr* is also located at the proximal segment of Hfr 3.OSO. The *tlr* and *td* loci

are linked with the *thr* locus and with each other. The most probable gene order is: *tlr-td-thr*.

Table 7. *The genetical constitution of* gal$^+$str-r *recombinants for genes* td, thr, *and* leu *in a cross: Hfr* 3.*OSO* thy87 td-s (td-s, thr$^+$, leu$^+$) × *P*-678 *F*$^-$ (td-r thr$^-$ leu$^-$)

Recombinant classes	Number of recombinants	Number of crossovers sufficient to form recombinants when gene order is:		
		td-thr-leu	*thr-td-leu*	*thr-leu-td*
leu$^+$*thr*$^+$*td-s*	81	2	2	2
leu$^+$*thr*$^+$*td-r*	12	2	4	4
leu thr td-s	4	4	4	2
leu thr td-r	20	2	2	2
leu$^+$*thr td-s*	1	4	2	2
leu$^+$*thr td-r*	10	2	2	4
leu thr$^+$*td-s*	3	4	4	4
leu thr$^+$*td-r*	2	4	4	4
	Total: 133			

It is curious that when the P1*kc* phage propagates on *td-s* mutants, addition of thymidine to the medium inhibits not only the division of the *td-s* cells themselves, but also multiplication of the phage inside them (Table 8). It should be noted that the thymidine inhibitory effect could be reversed by addition of any riboside, but not of deoxyribosides, to the growth medium.

Table 8. *Phage P1*kc *burst sizes on the thymidine-resistant* (thy8 td-r tlr$^+$) *and thymidine-sensitive* (thy8 td-s tlr) *strains in the presence of thymidine*

Thymidine in medium	*thy8 td-r tlr*$^+$			*thy8 td-s tlr*$^-$		
	Infectious centres × 10^{-4}		Average burst size	Infectious centres × 10^{-4}		Average burst size
μg./ml.	20 min.	120 min.	per cell	20 min.	120 min.	per cell
0·0	5·0	10	2	10·0	13	1
0·1	3·6	190	22	7·8	350	45
0·25	6·0	240	40	7·3	350	50
0·5	6·6	260	40	7·3	220	31
1·0	6·5	220	40	5·4	80	15
2·0	8·4	230	34	6·6	38	6
5·0	8·6	230	26	7·0	36	5
10·0	8·2	220	26	6·3	33	5

4. DISCUSSION

The data presented show that all the *thy* mutants isolated by the aminopterin technique result from a mutation in one chromosomal region between the *cys* and *ser/gly* genes. The *thy* locus represents a complex linearly ordered structure. It is interesting that, in spite of the large number of mutations studied, a comparatively

small number of sites has been found. Thus, about half of all the *thy* mutations are located at one site (No. 3). The irregularity in the distribution of the mutations among sites reflects either a different intragenic frequency for spontaneous and induced mutagenesis, or an unequal probability of the phenotypic expression of mutations at different sites of the *thy* locus.

The site No. 3 is of interest not only because it comprises a 'hot spot' located rather far from other sites but because all mutations there are temperature-dependent. The functional importance of the site No. 3 might be clarified by study of the thymidylate synthetase protein in mutants. It seems very probable that the temperature sensitivity of site No. 3 mutants is due to production of a temperature-sensitive thymidylate synthetase protein. But we have no evidence as to whether the accumulation of mutants at this site is due to a unique conformation of DNA molecule there, or to some selective bias in the aminopterin technique.

Distances between sites on the genetical map appeared sometimes to contradict the sequence of sites established by a comparison of recombination frequencies in the two combinations of a reciprocal cross. This paradox could be due not only to structural peculiarities of the mutations themselves, but also to some kind of negative interference exaggerating only the distance between very closely linked sites.

Our experiments show that the cells of the *thy*$^+$ Hfr 3.OSO strain of *E. coli* K12 take up thymine much less effectively than thymidine. However, when the internal pathway for thymidylate biosynthesis is disturbed by a *thy*$^-$ mutation, or by addition of aminopterin, the incorporation of exogenous thymine improves. This suggests that some product of the thymidylate pathway may specifically inhibit thymine uptake. Nevertheless *thy*$^-$ mutants retain the differential response of the wild-type strain, requiring *c.* 20 μg./ml. thymine for growth as against 1·0 μg./ml. thymidine.

Mutation of the *tlr* gene seems to provide better thymine penetration into the cells. There are some data (Breitman & Bradford, 1964; Pritchard, 1965, and our data for the burst sizes of P1*kc* from the *thy*$^-$*tlr*$^+$ and *thy*$^-$*tlr*$^-$ bacteria) which suggest that the reason for 'low' thymine-requirement is the capacity of *thy*$^-$*tlr*$^-$ mutants to produce thymidine more effectively. Pritchard explained this capacity by accumulation in these double mutants of deoxy-adenosine which can participate in the exchange reaction:

$$\text{d-adenosine} + \text{thymine} \rightleftarrows \text{thymidine} + \text{adenine}.$$

Whether this reaction is the only reason for 'low' thymine requirement and serves as a pump for exogenous thymine, or whether it accompanies some different alteration of the controlling mechanism, is not yet clear. In any case the comparison of our data and data obtained by the study of various thymineless bacteria (Harrison, 1965) shows that there are some metabolic differences between different bacterial strains that require more careful investigation.

The results of genetical mapping of the *tlr* mutants show the *tlr* gene to be closely linked to a gene controlling sensitivity for thymidine. The *td* gene function in thymine metabolism is not yet clear.

SUMMARY

The 150 independently isolated *thy*$^-$ mutants of *E. coli* K12 Hfr 3.OSO were studied genetically and phenotypically. Variants were found among the mutants in respect to the lag period of thymineless death, and temperature sensitivity. The latter correlates with mutations located at a specific site on the genetical map.

The *thy* locus is located between the *cys* and *ser/gly* genes, and is a linear structure where 134 *thy* mutants are distributed over more than 17 sites. The site distribution of the mutants is not regular: about a half of them (62) are localized within one site and all these are temperature-sensitive.

Two further genes involving utilization of thymine—*tlr* and *td*—were found. Mutations of *tlr* lead to a reduced thymine requirement (0·5 μg./ml. instead of 20 μg./ml.). A mutation of *td* results in thymidine sensitivity.

This latter character is expressed when the *td-s* allele is transferred into *E. coli* K12, prototrophic for thymine, by conjugation. Thymidine inhibition can be reversed by the addition of any riboside to the growth medium. Both genes map at the proximal end of the Hfr 3.OSO chromosome and are linked with the *thr* gene. The most probable gene order is: *tlr-td-thr*.

The following results have been obtained from ^{14}C-thymine incorporation experiments with wild-type cells, as well as with *thy*$^-$*tlr*$^+$ and *thy*$^-$*tlr*$^-$ cells: (1) Wild-type cells incorporate exogenous thymine extremely poorly, but incorporate thymidine better. (2) The *thy*$^-$*tlr*$^+$ mutants are able to incorporate thymine only when high concentration are used, but can utilize a low concentration of thymidine. (3) The *thy*$^-$*tlr*$^-$ mutants are able to incorporate exogenous thymine as well as thymidine at low concentration. (4) The *tlr* mutation is a thymine-specific one.

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APPENDIX

Further details of the experiments described can be found in the following publications in Russian (summary in English).

ALIKHANIAN, S. I., ILJINA, T. S., KALIAEVA, E. S., KAMENEVA, S. V. & SUKHODOLEC, V. V. (1965). The production of and study on *Escherichia coli* K12 mutants with impaired thymidilate synthetizing system. *Microbiologia* **XXXIV**, 4.

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