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A genetic map of several mutations affecting the mucopeptide layer of *Escherichia coli*

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SUMMARY

Several temperature-sensitive mutants of *Escherichia coli* were isolated which lyse at the restrictive temperature. Some of these possess a biochemically defined lesion in cell-wall mucopeptide synthesis. Three genes, termed *murC*, *E* and *F*, have been localized between the *azi* and *leu* markers. From transductional data a fine structure map was constructed of the *mur* mutations, establishing the order of the genes. The genetic relationship between these cell wall genes and neighbouring genes involved in cell division is discussed.

1. INTRODUCTION

Among temperature-sensitive mutants of *Escherichia coli* K-12, several showed lysis when grown at the restrictive temperature; they were denoted by TKL (A. Rörsch, unpublished). The TKL strains were analysed biochemically by Lugtenberg, who found that many of the mutations involve genes for the 'adding enzymes' (Ito & Strominger, 1962*a*, *b*; Comb, 1962) which synthesize the precursor of the mucopeptide layer of the cell wall. In this process, UDP-*N*-acetylglucosamine* is converted in two steps to UDP-*N*-acetyl-muramic acid, to which are added, sequentially, L-alanine, D-glutamic acid, *m*-diaminopimelic acid and D-alanyl-D-alanine.

Preliminary evidence indicated that many *lts* mutations in TKL strains which lyse at the restrictive temperature are closely linked and are also linked to the *fts* marker, which gives rise to filament formation (van de Putte, van Dillewijn & Rörsch, 1964). It seemed of interest to investigate the relationship between the mutations in this complex of genes concerned with cell division and cell-wall synthesis, in view of the frequent occurrence in bacteria of clustering of genes with related functions for regulational purposes. In this paper some of the genes specifying the biochemical steps mentioned are characterized, their order is reported and their genetic relation with the *fts* mutations is discussed.

* Abbreviation: UDP = uridine diphosphate. *lts* = mutation giving rise to lysis at 42° C.

Table 1. *Strains of* Escherichia coli *K-12*

Strain	Sex	Genetic characters
KMBL 49	F⁻	*thr leu thi pyrF thyA lac tonA*
KMBL 146	F⁻	KMBL 49 *his ilvA arg tsx*
KMBL 158	F⁻	KMBL 146 *trp*
TKL 11, 15, 19, 22, 24	F⁻	KMBL 158 *lts*
TKL 7, 39 and 46	F⁻	KMBL 146 *lts*
TKF 2, TKF 10	F⁻	KMBL 49 *fts*
TKF 12, TKF 15	F⁻	KMBL 146 *fts*
H 1119	F⁻	*purE lts*
ST 222, ST 640	F⁻	*thr leu trp his thy thi ara lac gal xyl mtl str lts*
KMBL 171	HfrH	*met azi*

All strains were provided by Dr A. Rörsch, except H 1119 (from Dr P. G. de Haan) and ST 222 and ST 640 (from Dr M. Matsuhashi).

2. MATERIAL AND METHODS

(i) *Bacterial and phage strains*

The genotypes of the strains can be found in Table 1. The TKL and TKF strains were obtained after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine of strains derived from CR 34 (Okada, Yanagisawa & Ryan, 1960). Transducing phage was ϕ363 from Dr A. Rörsch.

(ii) *Media*

Minimal agar contained per litre 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0·1 g $MgSO_4$, with Difco Bacto agar at 1·5 %. Where required additions were the following: amino acids and nucleotides at 40 μg/ml, thiamine at 4 μg/ml. For the selection of thermo-resistant recombinants a medium was used of lower ionic strength: 1·5 g Na_2HPO_4, 0·75 g KH_2PO_4, 0·5 NH_4Cl, 0·05 g $MgSO_4$/litre. In addition, in some cases media very poor in ions were used: 2E medium containing per litre 300 mg Na_2HPO_4, 150 mg KH_2PO_4, 100 mg NH_4Cl and 10 mg $MgSO_4$; or 1 E medium containing half these concentrations. Nutrient broth contained 8 g Difco Nutrient broth and 5 g NaCl/l. Peptone agar contained 8 g Difco nutrient broth, 5 g Difco peptone and 15 g Difco Bacto agar per litre. Sodium azide was added to a final concentration of 2·3 mM.

(*iii*) *Conjugation and transduction methods*

For conjugation log-phase cells growing in nutrient broth were mixed at 28 °C, so as to give approximately 2×10^7 Hfr cells and 2×10^8 F⁻ cells/ml. After 3 h the mixture was plated on appropriate media to select recombinants, using the auxotrophy of the Hfr parent for counter-selection. Heat-tolerant recombinants were scored on minimal medium of low ionic strength, transferred to 42 °C after a period of 4 h at 28 °C to allow complete expression. The relative frequencies of different recombinants in such an uninterrupted mating at 28 °C do not deviate essentially obtained from those at 37 °C.

Transduction with phage 363 was performed mainly according to the method of Lennox. The lysates used for transduction were normally obtained at 28 °C, following the method of Signer (1966). Phage titres ranged from 1 to 5×10^9. The number of transductants at 28 °C is not significantly lower than at 37 °C.

Recombinant colonies were tested for the presence of unselected markers by suspending them in saline and streaking on the appropriate media, in which, to prevent contamination with parental types, the original selective procedure was repeated.

3. RESULTS

(i) *Characterization of the strains*

When TKL strains are grown at the restrictive temperature (42 °C), lysis occurs (Wijsman, 1972). Since a concentration of 10% sucrose in the medium is able to stabilize the spheroplasts formed, it is concluded that the lesion affects the cell wall rather than the cytoplasmic membrane. The growth of several of the strains is restored by the addition of 20% sucrose to the plate; in others this is not the case, but a correlation of this phenomenon with the enzymic function affected (as described below) was not found.

(ii) *Location of the mutations*

The TKL strains were mated to strain KMBL 171, and from the number of *lts*$^+$ recombinants in relation to the gradient of transmission of the other markers it was concluded that the *lts* mutations here studied are located near *leu*, to which they are at least 80% linked. Close linkage of *lts* mutations to *leu* and *azi* was confirmed by transduction with phage 363 (Table 2). Since some *fts* mutations, in strains forming filaments at the restrictive temperature, had likewise been located near *leu* by van de Putte *et al.* (1964), they were compared with the *lts* mutations. Three-point crosses presented in Table 3 provide evidence that both *lts* and *fts* mutations are located between *leu* and *azi*. In this connexion it is relevant that *fts–12* is the marker closest to *azi*, as will appear below. On account of its high linkage to *azi* (92%) in conjugation with KMBL 171, it was anticipated that the *lts* mutation in strain H 1119, too, might be located among the other *lts* and *fts* mutations.

(iii) *The sequence of the mutational sites*

It seemed of interest to find the exact order of the temperature-sensitive mutations by intercrossing strains carrying different alleles of *leu*. Originally all the strains considered, except H 1119, were *leu*$^-$. The *leu*$^+$ allele was introduced into all of them and the resulting strains were used as a host for phage 363. With the lysates obtained, three-factor transductions could be performed with the *leu*$^-$ strains as recipient. When no more than two crossovers are necessary to produce a *leu*$^+$ *lts*$^+$ recombinant (Fig. 1, left), the percentage of the *leu*$^+$ allele among heat-tolerant recombinations is high, indicating that, in the example, *lts-1* is located to the left of *lts-2*. On the other hand, when at least four crossovers are necessary to produce *leu*$^+$*lts*$^+$ recombinants, the ratio *leu*$^+$*lts*$^+$/*lts*$^+$ will be low (Fig. 1, right).

Table 2. *Cotransduction of* lts *or* fts *with* leu *and* azi

(Donor strain: KMBL 171 *leu*$^+$ ts$^+$ *azi*$^-$. Recipient strain: *leu*$^-$ ts$^-$ *azi*$^+$.)

Selected marker	Unselected marker	Recipient strains: TKL 15	TKL 19	TKL 39	TKL 11	TKL 46	H 1119	TKL 22	TKF 15	TKF 12
lts$^+$ or *fts*$^+$	*leu*$^+$									
No. of recombinants examined		40	40	220	39	100	—	60	98	115
No. having inherited the unselected marker		36	35	163	26	63	—	41	65	64
Cotransduction		90%	88%	79%	67%	63%	—	69%	66%	56%
leu$^+$	*lts*$^+$ or *fts*$^+$									
No. of recombinants examined		100	144	90	100	50	50	163	120	170
No. having inherited the unselected marker		94	118	50	48	31	35	97	62	76
Cotransduction		94%	81%	55%	48%	62%	70%	60%	52%	45%
lts$^+$ or *fts*$^+$	*azi*$^-$									
No. of recombinants examined		40	—	42	39	100	—	60	98	75
No. having inherited the unselected marker		37	—	41	35	86	—	57	80	67
Cotransduction		92%	—	98%	90%	86%	—	95%	81%	90%

Table 3. *Frequency of unselected markers among* leu$^+$ *transductants of some TKL or TKF strains*

(Donor strain: KMBL 171 *leu*$^+$ ts$^+$ *azl*$^-$. Recipient strain: *leu*$^-$ ts$^-$ *azi*$^+$.)

		Recipient strains					
No of *leu*$^+$ recombinants examined:		20	11	100	14	50	80
Unselected markers							
lts of *fts*	*azi*						
+	−	12 (60)	5 (45)	35 (35)	4 (29)	24 (48)	24 (30)
−	+	6 (30)	4 (36)	52 (52)	9 (64)	26 (52)	52 (65)
+	+	2 (10)	1 (9)	13 (13)	1 (7)	0 (0)	4 (5)
−	−	0 (0)	1 (9)	0 (0)	0 (0)	0 (0)	0 (0)

Figures in parentheses are the percentage frequency of the various genotypes among the *leu*$^+$ recombinants.

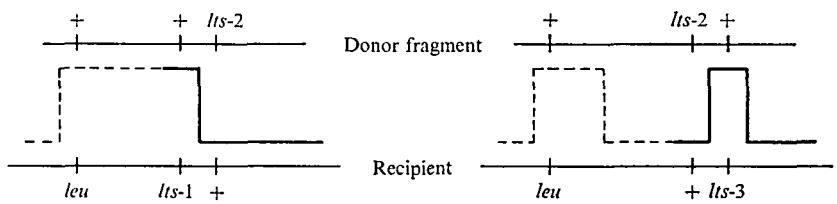

Fig. 1. A comparison between two hypothetical crosses with a high (left) and a low (right) fraction of *leu*$^+$ among *lts*$^+$ recombinants, respectively. Full line: crossover essential for *lts*$^+$ recombinant formation. Dashed: crossover pattern resulting in *lts*$^+$ *leu*$^+$ recombinants.

In this way the order of different mutations can be deduced (Gross & Englesberg, 1959).

In Table 4 there is a convincing discontinuity between high values for the ratio mentioned, with a minimum of 50 %, and low values with a maximum of 26 %. For instance, in the reversed 39 × 46 crosses, the ratio is 65 % when TKL 46 *leu*$^+$ is donor, but 15 % when TKL 39 *leu*$^+$ is donor. From this pattern it may be concluded that the order of the mutations is as follows:

leu...(lts-19, lts-15...lts-39)...lts-46...(lts-1119...lts-7, fts-10, fts-15)...fts-12...azi.

In one case *azi* was introduced in the donor strain, to test the crossing-over pattern independently. Since *azi* and *leu* are situated on different sides of the thermosensitive mutations, their frequency among heat-resistant recombinants is expected to be inversely related. From the cross TKF 12 × phage 363 (TKL 15 *leu*$^+$ *azi*$^-$), 30 heat-tolerant recombinants were analysed; only four of these (13 %) carried the *leu*$^+$ allele, a 'low' value, indicating that at least four crossovers are involved. Of these 30 recombinants, 21 (69%) carried the *azi*$^-$ allele, pointing to the requirement for only two crossovers for the formation of *lts*$^+$ *azi*$^-$ recombinants. The outcome of this cross confirms the order *leu...lts-15...fts-12...azi.*

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Table 4. *Ratio of the numbers of transductants per* 0·1 *ml plated when thermosensitive* lts *and* fts *strains carrying different alleles of* leu *are crossed by transduction*

		leu$^+$ *lts*$^+$/*lts*$^+$		*leu*$^+$ *lts*$^+$/*leu*$^+$	
Recipient	Donor (*leu*$^+$)	No.	%	No.	%
TKL 39	TKL 15	0/14	< 8	0/360	< 1
		0/8	< 15	0/176	< 1
	TKL 46	17/26	65	17/317	6
	TKL 7	8/12	66	—	
	TKF 10	50/70	74	50/3210	1·5
	TKF 12	21/28	75	21/650	3
TKL 46	TKL 15	31/139	22	31/2840	1
	TKL 19	49/188	26	49/3390	1·5
	TKL 39	33/220	15	33/2540	1
	H 1119	50/100*	50	13/97	13
	TKL 7	79/87	90	79/390	20
	TKL 22	219/309	69	219/1400	15
	TKF 10	54/99	55	54/500	11
	TKF 15	186/260	71	186/940	19
H 1119 *str leu*	TKL 46	6/74	9	6/1010	1
	TKL 7	25/40	62	—	—
TKF 15	TKF 46	0/72	2	0/198	1
TKF 12	TKL 15	4/30*	13	4/69*	6
	TKL 39	14/160	8	14/1000	1·5
	TKF 10	0/200	0·5	0/330	1
	TKF 15	32/216	15	32/8000	0·5

All numbers were directly scored on the selection plates, except those marked with *, resulting from the analysis of isolated colonies.

A number of thermosensitive mutants are very leaky, producing 'lawns' when whole cultures are plated at the restrictive temperature, so that heat-tolerant recombinants cannot be selected. In fact, virtually only TKL 46 behaved as a good recipient, the viability of recombinants in strains such as TKL 39 being low at 42 °C. The ratio *leu*$^+$*lts*$^+$/*leu*$^+$, in which selection is made for *leu*$^+$ instead of for *lts*$^+$ transductants, was now introduced for comparison with the *leu*$^+$*lts*$^+$/*lts*$^+$ ratio. As Table 4 shows, the two were found to give the same information regarding the ordering of sites, even though in crosses between temperature-sensitive mutants in general the ratio of *leu*$^+$ transductants at 42°/28° is much lower than in the case of wild-type strains (115/89 for KMBL 146), so that discrimination between 'high' and 'low' ratios becomes less easy. Accordingly, the *leu*$^+$ *lts*$^+$/*leu*$^+$ ratio alone can be used to order those mutations from which no *lts*$^+$ recombinants could be directly selected. The results are given in Table 5 and show that the order of the mutations is the following:

leu...(lts-19, lts-15...lts-39)...lts-46...lts-119...lts-7...lts-22...fts-10...
fts-15...fts-12...azi.

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Table 5. *Ratio of the numbers of transductants per* 0·1 *ml plated when 'leaky' thermosensitive* lts *and* fts *strains carrying different alleles of* leu *are crossed*

		leu+ *lts*+/*leu*+	
Recipient	Donor (*leu*+)	No.	%
TKL 7	TKL 46	0/86	1
TKL 22	TKL 46	27/1950	1·5
	H 1119	9/100*	9
	TKL 7	10/2320	0·5
	TKF 10	272/720	37
TKF 10	H 1119	2/105*	2
	TKL 22	37/356	11
TKF 15	TKF 10	15/452	3

All numbers were directly scored on the selection plates, except those marked with *, resulting from the analysis of isolated colonies.

Table 6. *Genetic symbols for some of the enzymes concerned with the synthesis of the mucopeptide layer*

Enzymes	Genetic symbol	Mutants showing defective activity*
L-Alanine-adding enzyme	*murC*	H 1119, ST 622
D-Glutamic acid-adding enzyme	*murD*	
m-Diaminopimelic acid-adding enzyme	*murE*	TKL 11, 15, 19, 24, 39
D-Alanyl-D-alanine-adding enzyme	*murF*	TKL 46
D-Alanine: D-alanine ligase	*ddl*	ST 640

* Data of Dr E. J. J. Lugtenberg.

4. DISCUSSION

(*i*) *Correlation of the biochemical and the genetic data*

The genetic map gains its interest from the findings of Lugtenberg, de Haas-Menger & Ruyters (1972), who have tested the enzymic activity in the mutants, in comparison with the wild type, of the enzymes listed in Table 6.

Of the five mutants in which the diaminopimelic acid-adding enzyme activity is affected, *lts-24* has not been mapped precisely because of its leaky character, but it can be cotransduced with *leu* (90% cotransduction). When diaminopimelic acid (20 μg/ml) is added, growth at 42° is restored. This observation may be compared with reports on some mutants acyl-tRNA synthetases for which, too, the addition of even a small surplus of their amino acid substrate restores sufficient *in vivo* activity (Neidhardt, 1966; several references in Folk & Berg, 1970).

In H 1119 the activity of the L-alanine-adding enzyme is strongly affected; the same is found for ST 622. The temperature-sensitive mutation of ST 622 was reported to be located near *leu* (Matsuzawa *et al.* 1969). The same is true for ST 640 (Matsuzawa *et al.* 1969), but here the activity of the D-alanine: D-alanine ligase is affected. All the enzymes mentioned are fully active in strains with an *fts* mutation.

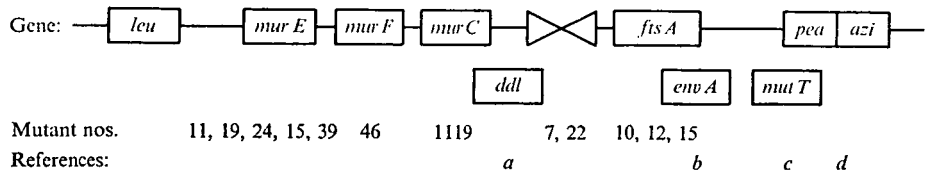

Fig. 2. Genetic sequence of cell envelope loci between *leu* and *azi*. Rectangles indicate genes, but distances are shown only approximately, the *mur* genes, for example, being possibly contiguous. The numbers of the mutants refer to the TKL number, except for *murC* (H number) and for *fts* (TKF number). The aberrant symbol used for the site where mutations 7 and 22 are found is meant to indicate that they are likely not to specify a separate gene, but possibly a DNA fragment with a regulatory rôle. References: *a* = Matsuzawa *et al.* (1969); *b* = Normark *et al.* (1969); *c* = Cox & Yanofsky (1969); *d* = Yura & Wada (1968).

In view of the biochemical data it seems warranted to give the genetic symbol *mur* to genes specifically concerned with the synthesis of the pentapeptide precursor of the 'murein' (Weidel & Pelzer, 1964). Two enzymes concerned with the synthesis of UDP-*N*-acetyl-muramic acid, a pyruvate transferase and an enolpyruvate reductase, have been described for *Enterobacter* (Gunetileke & Anwar, 1966, 1968), but may be assumed to be present in *E. coli* as well. The symbols *murA* and *murB* are reserved for these two enzymes; a mutant in *murB* was described and mapped by Matsuzawa *et al.* (1969). It is located at about 78 min on the current map, far away from the present cluster.

Symbols for the adding enzymes and for the D-alanine : D-alanine ligase are found in Table 6. They have been used in the genetic map (Fig. 2), which shows that the genes *murE*, *murF* and *murC* are very close to each other, apparently forming a genetic unit, to which the as yet unidentified gene *murD* as well as *ddl*, located in this region by Matsuzawa *et al.* (1969), may also belong.

The mutant TKL 7 does not appear to be affected in one particular enzyme of those tested, although its behaviour was abnormal (E. J. J. Lugtenberg, personal communication). TKL 22 is interesting in that its growth at 42 °C can be restored by the addition of 5 mg/ml D-alanine in synthetic medium. It remains to be seen whether *lts–7* and *lts-22* affect regulatory functions or other cell wall enzymes.

(ii) *On the nature of the* fts *mutations*

Whether the *fts* mutations affect one gene or more than one cannot yet be said. Their concentration between the *mur* region and *azi* points to a specific role of the *fts* gene(s) in the process of cell division. Filament formation as such could also be a result of an aspecific weakening of the cell wall (Bazill, 1967). In this respect it is interesting to find that both TKL 7 and TKL 22, whose mutations are located between the *mur* genes and the *fts* complex, form short filaments shortly before or during lysis. Their phenotype, intermediary between lysis and filament formation, is in remarkable agreement with their position between the *lts* and *fts* mutations.

Taylor (1970) has claimed that *azi* is an older synonym of *fts*. It must be emphasized, however, that the *fts* mutations do not confer any increased resistance to

sodium azide at 28 °C, unlike the mutants of type 7, described by Yura & Wada (1968), which also form filaments at 42 °C. Furthermore, the *fts* mutations mapped all reside to one side of the classical *azi* mutation in strain HfrH. However, in this respect these *fts* mutations may represent a special case, because they were selected by filtration at 42 °C, followed by recovery at 28 °C (van de Putte *et al.* 1964). For some of the TKF mutants isolated at random the process of filtration at the restrictive temperature would already be lethal; this phenomenon might have a genetic basis, even though van de Putte (1967) has found that these random mutations, too, are located near *leu*. It is concluded that the *fts* mutations mapped are not located in the *azi* gene, while for other *fts* mutations fine structure data are needed.

(iii) *The relations with neighbouring gene complexes*

Whatever their relationship may mean causally, a close correlation is found between the phenotypes and the loci of the mutations. Taylor (1960) gives 0·5 min as the distance between *leu* and *azi*. Of the 10–15 genes that can be accommodated on such a segment of the genophore, possibly the greater part is known at present. Of these genes several are concerned with mucopeptide synthesis, and these possibly form an operon. The mutations in *fts*, *envA*, giving rise to chain formation (Normark, Boman & Matsson, 1969), *pea* (Yura & Wada, 1968) and *azi* affect the process of cell division; of these, *pea* and *azi* have a special relation with the function of the membrane (Yura & Wada, 1968). The filament-forming *azi* mutant of type 7 is reported by Yura & Wada to degrade its DNA when shifted to the restrictive temperature (another difference with *fts* in view of the data of van de Putte *et al.* 1964).

A relation with DNA replication of this complex of cell-division genes seems to be provided by the adjacent *mutT1* allele, which is supposed by Cox & Yanofsky (1969) to induce changes in the normal base sequence of the DNA by coding for a protein that is a component of an error-detecting system associated with DNA replication. The mutation has been located between *azi* and *leu*, very close to *azi*; the authors mentioned the possibility that *mutT* and *azi* are synonymous.

When their sequence becomes known, a complementation analysis involving all the mutations mentioned would be worth while in revealing the number of genes. It seems to be unlikely that the close topographic relationship of these cell-envelope and cell-division genes is fortuitous, although the selective advantage of this clustering, perhaps concerned with regulation, remains to be studied.

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