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Conditional mutations affecting the cell envelope of *Escherichia coli* K-12

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SUMMARY

Amongst a collection of temperature sensitive (TS) mutants of *Escherichia coli* K-12, some have been found which can grow at the restrictive temperature (42 °C) if the osmotic pressure of the medium is raised by the addition of sodium chloride (1 %) or sucrose (12·5 %). These mutants are described as temperature sensitive osmotic remedial (TSOR) mutants. At the restrictive temperature they are not osmotically fragile, but do display decreased resistance to inhibitory agents such as deoxycholate, actinomycin D and acridine orange; they also show release of the periplasmic enzyme ribonuclease. These results indicate a change in the cell's outer permeability barrier. The genes affected in six of the mutants have been located on the *E. coli* linkage map. The mutations, which occur at loci not previously described, have been named *envM–envT* to indicate their effect on the cell *env*elope.

1. INTRODUCTION

There is now considerable knowledge about the structure of the cell envelope of Gram-negative bacteria, but little is yet known about the control of envelope synthesis or the genetics of the processes involved. The rigidity of the envelope is principally due to a layer composed of peptidoglycan (also known as mucopeptide or murein). Loss of integrity of this layer results in the formation of osmotically fragile cells which, in hypotonic conditions, form spheroplasts and then lyse. Mangiarrotti, Apirion & Schlessinger (1966) used broth containing 20 % sucrose to isolate wall-defective mutants which were dependent for survival on high osmotic strength medium. Mutants with defective cell walls could also be obtained by using conditionally expressed mutations, such as in temperature sensitive mutants in which the mutant phenotype is expressed only at a restrictive (elevated) temperature. Mutants of this type would be expected to be dependent upon sucrose under the restrictive conditions.

In this laboratory we commenced a programme of study of cell envelope genetics in *Escherichia coli* K-12 by isolating temperature sensitive mutants in which the effect of the mutation was overcome by the addition of salt or sucrose to the medium. Our collection of mutants includes a number of strains which lyse and resemble the mutants recently described by Matsuzawa *et al.* (1969), Lugtenberg, de Haas-Menger & Ruyters (1972) and Taketo, Hayashi & Kuno (1972). Lysis of these wall-

defective mutants can be prevented by elevating the osmotic pressure of the growth medium at the restrictive temperature (42 °C). We have isolated another class of temperature sensitive mutants which grow at 42 °C on medium of increased osmotic pressure. These mutants, in contrast to mutants with defective peptidoglycan, are not osmotically fragile at 42 °C in any media. Such mutants, in which the role of the high osmotic pressure does not appear to be that of physically supporting spheroplasts, are referred to as temperature sensitive osmotic remedial (TSOR). In this paper we describe the properties of several mutants of this type.

2. MATERIALS AND METHODS

(i) *Bacterial strains*

All organisms used in this work are derivatives of *Escherichia coli* K-12, and their genotypes are given in Table 1. The temperature sensitive mutants were originally isolated after nitrosoguanidine mutagenesis of strain KA56 as described elsewhere

Table 1. *Organisms used*

Strain	Sex	Genotype*
AB3282	F⁻	*thi-1, leu-351, proA2, trp-356, his-4, ilvC7, argE3, galK2, lacY1, mal-358, str-704, tsx358*
JC5088	HfrPO45	*recA56, ilv⁻, thr⁻, spc^r*
JP500	HfrH	*thi⁻, galEPL5, tyrR356*
JP534	F⁻	*trp-26, his-10, pyrF40*
JP1537	F⁻	*thi-1, thr-1, pan-1, aroP⁻, tonA351, azi-351, str^r*
JP5059	F⁻	*argH1, metB1, rif-352*
KA6	F′	*met⁻*, gal$^{+/+}$ (carries the F8 episome)
KA56	HfrH	*thi⁻, galEPL5*

Strain	Sex	ts mutation†	Other markers
JP1154	HfrH	*env-395*	As in KA56
JP1174	HfrH	*envN394*	As in KA56
JP1378	F⁻	*envT406*	As in KA56
JP5001	F⁻	*envP396*	Arg⁺ derivative of AB3282
JP5005	F⁻	*envN394*	Pro⁺ derivative of AB3282
JP5018	F⁻	*env-405*	Arg⁺ derivative of AB3282
JP5020	F⁻	*env-404*	Arg⁺ derivative of AB3282
JP5023	F⁻	*envM392*	*trp-26 his-10*
JP5046	F⁻	*envQ397*	*argA21 lysB10 str-9*

* Symbols used are those of Taylor (1970). Numbers refer to allele numbers allotted to mutations in this laboratory, in accord with the system used by E. A. Adelberg and A. J. Pittard.

† Mutations of TS mutants were originally given allele numbers with the prefix *ts*. When the effected genes were identified, the mutations were given a name more indicative of the phenotype they cause. Thus, *env* mutations affect the cell *env*elope.

(Russell & Pittard, 1971). Temperature sensitive (TS) mutants are capable of growth on nutrient agar at 32 °C, but not at 42 °C. Because the mutants which are described below have altered cell envelopes, we have adopted the nomenclature *env* for the genes affected. Fig. 1 shows the positions of relevant genetic markers on the *E. coli* linkage map.

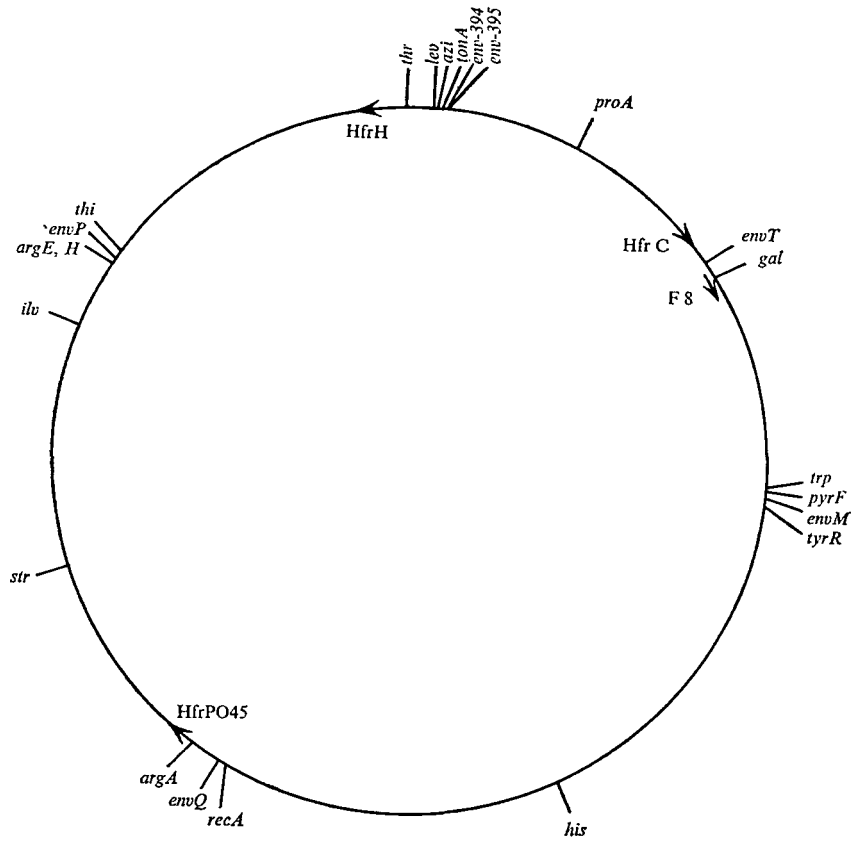

Fig. 1. Genetic linkage map of *E. coli* K-12, based on that of Taylor (1970) and showing the positions of markers referred to in this paper and the origins of transfer of the male strains used.

(ii) *Materials*

The minimal medium used was half-strength medium 56 described by Monod, Cohen-Bazire & Cohn (1951) supplemented with 0·2 % carbon source, together with thiamine and amino acids in the concentrations used by Adelberg & Burns (1960). Nutrient broth consisted of Oxoid Nutrient Broth no. 2, plus 0·3 % Oxoid yeast extract, solidified for plates with 1 % Oxoid Agar no. 1. 'Salt' and 'sucrose' plates consisted of nutrient agar with sodium chloride (1 %, w/v) or sucrose (12·5%, w/v) added. Dyes and other inhibitors were sterilized separately before adding to molten nutrient agar.

(iii) *Genetic methods*

Conjugation and transduction methods have been described recently (Russell & Pittard, 1971). All genetic transfer experiments were carried out at 32 °C, at which temperature all the TS donor strains are capable of genetic transfer.

(iv) *Growth studies*

For the assessment of growth on solid media a suspension of the organism was prepared in a drop of buffer to give a density of about 10^8 cells/ml. A loop of this suspension was then applied to the plates in a streak about 1 in. long.

For liquid medium studies, logarithmic phase cultures of the strains to be tested were inoculated into 125 ml Erlenmeyer flasks equipped with side arms and containing 10 ml of appropriate medium. The optical density of the cultures was followed using a Klett–Summerson photoelectric colorimeter fitted with a no. 66 filter. The initial density in each flask was about 3×10^7 cells/ml (10 Klett units). Aliquots for viability determinations were taken at various times and other samples were examined by phase-contrast microscopy. Each strain was incubated at 32° and 42 °C with the addition of sodium chloride (1 %) or sucrose (12·5 %) as required.

(v) *Release of ribonuclease*

Release of ribonuclease was determined by a modification of the method used by Lopes, Gottfried & Rothfield (1972). Nutrient agar and nutrient agar salt plates were prepared containing 1·5 % RNA. Logarithmic phase cultures of the strains to be tested were spotted on to pairs of plates. One plate of each pair was then incubated for 4 h at 32 °C and the other the same time at 42 °C. The plates were then flooded with 0·1 N hydrochloric acid. The surface of the plates became opaque as a result of the precipitation of the RNA. Strains which had released ribonuclease were surrounded by a zone of clearing.

3. RESULTS

(i) *Growth of the mutant strains*

Amongst our collection of TS mutants, which were selected solely on the basis of the temperature sensitivity of their growth, many were found which were enabled to grow at the restrictive temperature (42 °C) if extra salt (1 %) or sucrose (12·5 %) was included in the solid medium (Table 2). Such mutants are described as temperature sensitive osmotic remedial (TSOR), and the eight *env* mutants described in this report were the first eight stable, non-lysing TSOR mutants which we came across in our screening procedures. It was subsequently found that the inclusion of one of a number of other salts or sugars to an equivalent osmotic pressure could also restore growth. This suggested that there was a general correction rather than an affect specific to a particular solute. Growth was not restored by the addition of the peptidoglycan precursors glucosamine, D-alanine, D-glutamate or meso-diaminopimelate. Addition of oleate was also without effect.

The growth of the mutants was examined further in liquid medium (nutrient broth). All strains grew well at 32 °C at rates approximating that of the parent strain. At 42 °C three general types of growth were observed (Fig. 2, Table 2); with some strains mass increase ceased with 60 min of the temperature shift (type A), with others growth continued for the duration of the experiment (5 h) but at a decreasing rate (type B) and a final group (type C) grew almost as well at 42 °C as at

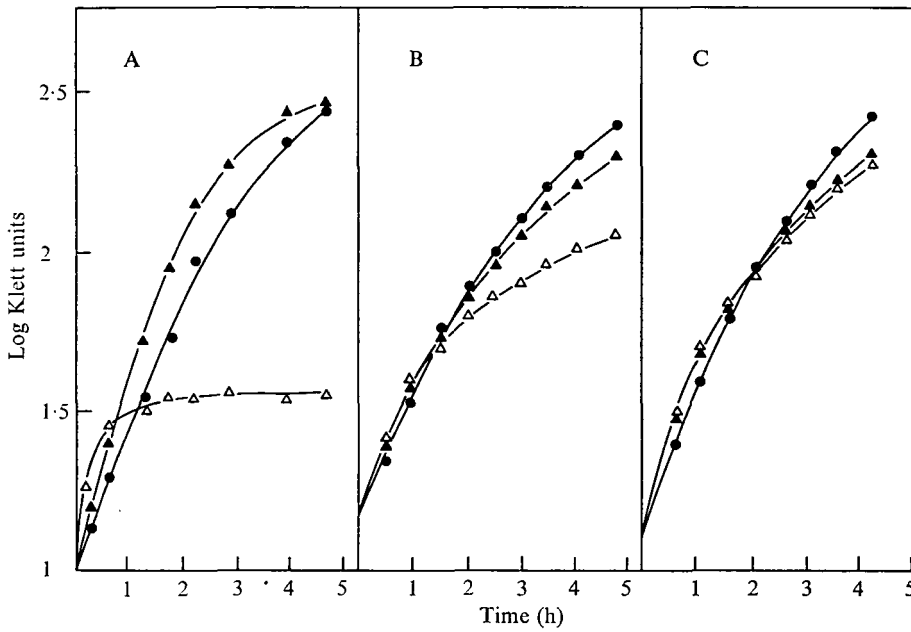

Fig. 2. Types of growth observed in liquid medium (nutrient broth). Details of the experiments are described in Materials and Methods. Examples shown: type A – *envM392*; type B – *env-395*; type C – *envT406*. ●, 32 °C; △, 42 °C; ▲, 42 °C with 1 % NaCl added.

Table 2. *Growth of TS mutants*

Strain carrying mutation	Growth of streak on plates after 24 h at 42 °C* NB	NB + 1 % NaCl	NB + 12·5 % sucrose	Type of growth† curve in liquid medium
Wild-type	+ + +	+ + +	+ + +	Wild-type
envM392	−	+ + +	+ + +	A
envN394	−	+ + +	+ + +	B
env-395	+	+ +	+ + +	B
envP396	−	+ +	+ +	A
envQ397	−	+ + +	+ + +	B
env-404	−	+ +	+ + +	C
env-405	−	+ +	+ + +	A
envT406	−	+ +	+ +	C

* + + +, wild-type amount of growth; −, no visible growth; + and + +, intermediate levels.

† See Fig. 2.

32 °C even though they failed to give visible growth on solid media at 42 °C. Type C mutants continued to grow for at least ten generations after a shift at 42 °C in liquid medium, whereas they grow for less than three generations on solid media. The reason for this difference in growth under the two conditions is not known. No strains lysed in broth at 42 °C. After 5 h incubation at 42 °C aliquots of the cultures

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were diluted into four volumes of distilled water. No lysis was observed suggesting the cells were not osmotically fragile.

With all mutants whose growth rate was reduced at 42 °C, the addition of sodium chloride (1 %) to the medium resulted in stimulation of growth. In some cases growth was restored to the rate found at 32 °C (Fig. 2 A). This is in agreement with the results obtained using solid media.

(ii) *Viability and morphology*

The viability of each strain was determined after incubation in liquid medium at 42 °C for various periods of time. No marked decrease in the viable count was observed for any of the mutants; all were capable of forming colonies on nutrient plates at 32 °C or on nutrient plates supplemented with sodium chloride (1 %) at 42 °C, even when growth in the liquid medium had been stopped for a period of several hours. When examined under phase-contrast microscopy after incubation in nutrient broth at 42 °C for 4–5 h, all strains were seen to have retained their rod-shaped morphology although *env-394* strains tended to form chains.

(iii) *Envelope changes*

During studies of the osmotic fragility of the mutants growing at 42 °C, we examined their susceptibility to the surface active agent sodium deoxycholate (DOC), and found that they were much more sensitive to it than was the wild-type.

Table 3. *Sensitivity of TSOR mutants to inhibitors, when grown on nutrient agar plates + 1 % NaCl or 12·5 % sucrose at 42 °C*

Strain-carrying mutation	Inhibitors							
	DOC	MBl	AO	TPTC	ActD	Nov	Rif	Amp
Wild-type	.	.	.	.	.	.	.	.
envM392	S	.	.	.	.	.	S	.
envN394	S	.	S	S	S	S	.	.
env-395	S	.	.	.	S	S	.	.
envP396	S	S	S	S	S	S	S	.
envQ397	S	S	.	.	S	.	.	S
env-404	S	.	S	.	S	.	.	.
env-405	S	.	.	S	S	S	.	S
envT406	S	.	.	.	S	S	.	.

S = sensitive (no visible growth after 24 h).

Abbreviations of inhibitors and concentrations used: DOC, sodium deoxycholate (10 mg/ml); MBl, methylene blue (10 μg/ml); AO, acridine orange (50 μg/ml); TPTC, triphenyl tetrazolium chloride (500 μg/ml); ActD, actinomycin D (Calbiochem) (5 μg/ml); Nov, novobiocin (Upjohn) (40 μg/ml); Rif, rifampicin (Lepetit) (1 μg/ml); Amp, ampicillin (Glaxo) (2·6 μg/ml).

DOC-sensitivity is known in some situations to be associated with more general permeability changes (see Discussion) so, in order to extend observations of this abnormal DOC-sensitivity of the mutants, streak tests were carried out at 32 °C and 42 °C on plates containing added salt or sucrose and a wide range of dyes and anti-

biotics at concentrations which do not inhibit the parent organism. Although all the mutants grew well on salt or sucrose plates at 42 °C, on such plates they were inhibited by at least two of the following: acridine orange, triphenyl tetrazolium chloride, actinomycin D, novobiocin, rifampicin or ampicillin at the concentrations employed (Table 3).

(iv) *Release of ribonuclease*

Mutant strains were tested to see if ribonuclease (a periplasmic enzyme) was released from the cells at 42 °C. All mutant strains released ribonuclease when cells were incubated at 42 °C on nutrient agar plates containing 1 % sodium chloride. No release occurred at 32 °C or with two control strains (AB3282, KA56) at either temperature.

(v) *Genetic mapping*

The mutant phenotypes described above were all consequences of single mutations, as demonstrated by the facts that simultaneous reversion to wild-type growth (TS^+) and wild-type envelope properties could be found in strains carrying each of the *env* mutations, and that the temperature sensitivity of growth and of envelope properties were not separated in any genetic transfer experiments.

The first step in the mapping of the *env* mutations in strains which were HfrH donors was to cross them with the recipient strain AB3282. Such experiments served three purposes: (i) helped to separate the *env* mutation from any other auxotrophic mutations induced by the mutagen; (ii) allowed a preliminary conclusion to be reached as to the map position of the *env* marker by studying the pattern of its inheritance as compared with that of the other known genetic markers in AB3282; (iii) yielded F^- strains carrying known mutations in addition to the *env* alleles for use in further experiments as required.

env-392

Conjugation experiments showed that *env-392* is closely linked to the *trp* locus, so attempts were made to co-transduce it with *trp*. When Trp^+ transductants were

Table 4. *A transduction experiment determining the order of* trp, env-392 *and* tyrR

Donor JP500 *trp*$^+$ *env*$^+$ *tyrR356*
Recipient JP5023 *trp*$^-$ *env-392 tyrR*$^+$

A (selected)	B (unselected)	B/A
Trp^+ Env^+	$TyrR^-$	15 %
Trp^+ $TyrR^-$*	Env^+	96 %

* The *tyrR356* allele confers resistance to 0·2 mM 3-fluorotyrosine.

selected using strain JP534 (*trp*$^-$ *pyrF*$^-$) as recipient and a Trp^+ Pyr^+ *env-392* strain as donor, 53/322 (16·5 %) were found to be TS. When Pyr^+ was selected, 119/145 (82 %) were found to be TS. *trp* and *pyrF* were 23 % co-transducible. 93 % of

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Trp+ TS transductants were also Pyr+, whereas only half of the Trp+ Pyr+ transductants had also received TS. The order of these three markers is therefore *trp pyrF env-392*. The tyrosine biosynthesis regulator gene *tyrR* also maps in this region (Im, Davidson & Pittard, 1971) and, as the experiment described in Table 4 demonstrates, *env-392* lies between *trp* and *tyrR*. The gene affected by *env-392* has been named *envM*. A final gene order of *trp pyrF envM tyrR* can be deduced.

env-394

The cross between JP1174 and AB3282 revealed a high degree of linkage between *env-394* and *leu*, so a *leu*− *env-394* strain was crossed with KA56 (HfrH) in an interrupted mating at 32 °C. The result showed that the wild-type allele of *env-394* was transferred just $4\frac{1}{2}$ min after *leu*+. In order to locate *env-394* in greater detail,

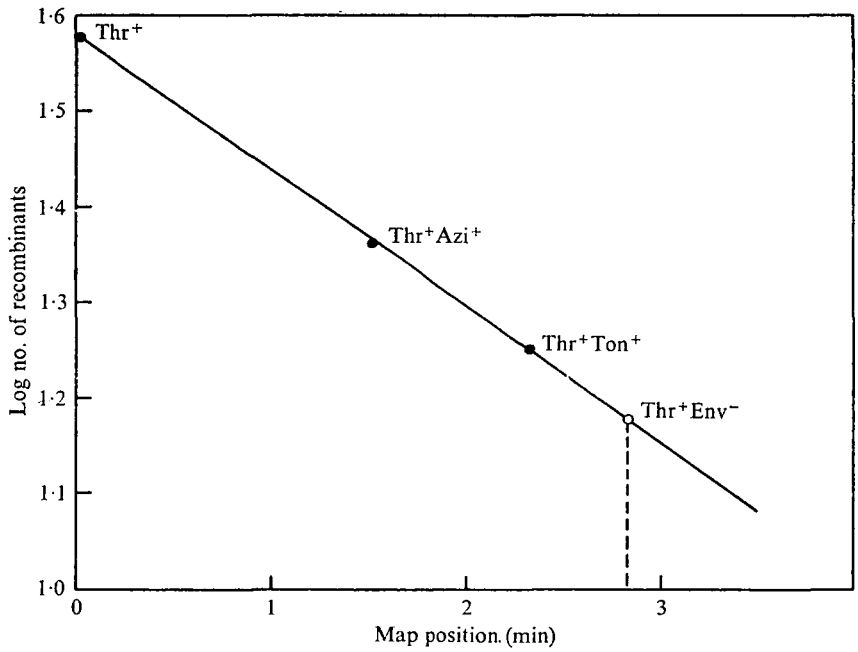

Fig. 3. Frequency of cotransfer with *thr* of *azi*, *tonA* and *env-394* markers from the donor strain JP1174 (HfrH *thr*+ *azi*+ *tonA*+ *env-394*) when crossed with JP1537 (F− *thr*− *azi*− *tonA*− *env*+). Thr+ recombinants were selected, and inheritance of the other characters examined as unselected markers. The reference markers *thr*, *azi* and *tonA* are located according to Taylor (1970).

JP1174 was crossed with strain JP1537 for 30 min, and Thr+ recombinants selected. These recombinants were tested for their temperature sensitivity and resistance to azide and bacteriopage T5. The results are plotted in Fig. 3, and locate *env-394* close to minute 3 on the linkage map. Although this result would predict linkage of *env-394* to the *dapD* locus described by Bukhari & Taylor (1971), we have not been able to obtain TS transductants when P1 propagated on JP1174 was used to

introduce ability to grow without DAP into a *dapD*12 strain. The mutation *env-394* is in a gene designated *envN*.

env-395

Mapping of the mutation carried by JP1154 gave results almost identical to those described JP1174 above, so *env-395* can also be located at minute 3 on the linkage map. It is not yet known whether *envN394* and *env-395* are allelic.

env-396

The cross between an HfrH *env-396* strain and AB3282 indicated linkage of *env-396* to the *argE* gene, so attempts were made to co-transduce *env-396* with various genes in the 70–80 min region of the chromosome, using phage P1 grown on

Table 5. *A three-point transduction determining the order* arg env-396 thi

Class of recombinant			
arg	*env*	*thi*	No.
1	0	0	69
1	1	1	28
1	1	0	3
1	0	1	0

P1 grown on JP5001 (*arg*$^+$ *env-396 thi*$^-$) was used to introduce Arg$^+$ into JP5059 (*argH1 env*$^+$ *thi*$^+$).

1 = marker from donor; 0 = marker from recipient.

a *env-396* donor. Co-transduction with *argH* at a frequency of 31 % and with *thi* at a frequency of 96 % was found and, in order to map *env-396* further, the three point transductional cross described in Table 5 was performed. The result allowed *env-396* to be located just before minute 78 on the linkage map, the gene order being *argH envP thi*. The gene affected by *env-396* has been named *envP*.

env-397

The preliminary mating of the HfrH strain carrying *env-397* with AB3282 indicated that the mutation lay distal to the *his* locus, so an interrupted conjugation was performed with a *his*-4 *env-397* strain as recipient and a donor with origin of transfer that of HfrH. The first TS$^+$ recombinants appeared 22 min after the first His$^+$ ones. As the experiment was carried out at 32 °C, at which temperature the rate of chromosome transfer is about half that at 37 °C, this result allowed the conclusion that the *env-397* mutation lay approximately 11 min from *his* on the linkage map, i.e. close to minute 49. Bacteriophage P1 growth on a *ts*-397 strain was used to transduce *glyA*$^+$, *tyrA*$^+$, *pheA*$^+$ and *cysC*$^+$ into strains with mutations in these genes, and co-transduction of *env-397* was found with *pheA* at the low frequency of 2/300, and with *cysC* at a frequency of 7/300. These linkages are very similar to those obtained for the *recA* gene (Willetts, Clark & Low, 1969) so, in order to determine the relative

orders of *env-397* and *recA*, the experiment described in Table 6 was performed. The results revealed a gene order of arg*A* *env-397* *recA*. The mutation *env-397* affects *envQ*.

Table 6. *The order of* arg A env-397 recA

Class of recombinant			
arg	*env*	*rec*	No.
1	1	1	53
1	0	0	39
1	1	0	8
1	0	1	0

The segregation pattern of unselected markers from the cross JP5046 (F^- *argA21 env-397 rec*$^+$) × JC5088 (Hfr PO45 *arg*$^+$ *env*$^+$ *recA56*) is shown. Arg$^+$ was selected.

The *recA* character was detected by testing for UV-sensitivity as described by Willetts *et al.* (1969).

1 = marker from donor; 0 = marker from recipient.

env-406

Strain JP1378, although obtained from the Hfr strain KA56, lost its donor capability at some stage during its isolation. In order to map *env-406* therefore, a spontaneous streptomycin-resistant derivative of JP1378 was prepared, and this used as recipient in matings with a range of donor strains having different points of origin of chromosome transfer. It was found that after a 15 min conjugation with the donor strain KA6, which carries the F8 episome, Gal$^+$ and Gal$^+$TS$^+$ recombinants were obtained in a ratio of 500 : 1. Such a ratio is expected between the high frequency of transfer of *gal* (on the episome) and the lower frequency of transfer of a chromosomal marker. The result shows that the wild-type allele of *env-406* is on the chromosome (rather than the F8 episome) quite close to *gal* and on the anticlockwise side of it. As a donor strain with the origin of transfer of HfrC does not yield TS$^+$ recombinants early, *env-406* must lie between the origin of transfer of HfrC at minute 13 and the *gal* locus at minute 17. The affected gene has been named *envT*.

env-404 and *env-405*

These two mutations have not yet been mapped in detail, but preliminary experiments indicate that *env-404* lies between minute 17 and minute 30 on the genetic map, and that *env-405* lies between minutes 74 and 78. *env-404* is not co-transducible with *trp*, so is not allelic with *envM392*. Similarly, *env-405* is not co-transducible with *argH*, and so cannot be an allele of *envP*.

4. DISCUSSION

The results presented in this paper raise a number of questions about the class of mutants examined: what is the nature of the cell surface change which results in increased sensitivity to a wide range of inhibitory agents, how is this change related to the temperature sensitivity of growth of the mutants, and what is the mechanism

by which temperature sensitivity can be overcome by changing the osmotic strength of the growth medium?

The 'natural resistance' of Gram-negative bacteria to the inhibitory agents listed in Table 3 is believed to be due to the outer membrane, composed largely of lipopolysaccharide (LPS) phospholipid and protein. Loss of components of the outer membrane either by mutation (Eriksson-Grennberg, Nordström & Englund, 1971; Makëlä & Stocker, 1969; Tamaki, Sato & Matsuhashi, 1971), by chemical removal (Leive, 1968; Muschel & Gustafson, 1968) or by physical treatments such as freezing or drying (Hambleton, 1971; Ray, Jezeski & Busta, 1971) results in a greatly increased sensitivity, presumably because the inhibitors can now penetrate to their target sites. Alterations to the outer membrane can also result in release of periplasmic enzymes, such as ribonuclease (Lopes *et al.* 1972). Thus, on the basis of altered permeability properties and their leakage of ribonuclease, our *env* mutants appear to have alterations to their outer membrane at the restrictive temperature.

Evidence is accumulating that LPS is the component responsible for many of the permeability properties of the outer membrane. Mutants are known in Salmonella which have lost almost all of the core part of the LPS. Such mutants show altered permeability characteristics like the mutants described in this paper but, with a single unexplained exception (Wilkinson, Gemski & Stocker, 1972), are otherwise normal with regards to morphology and growth. The presumed loss of LPS in our mutants might, however, be a consequence of an alteration in the 'deep' part of the LPS macromolecule, a region for which no mutants have yet been reported, or be a secondary consequence of an alteration to some other (vital) component of the cell.

The fact that mapping of our eight *env* mutations revealed the existence of seven or eight distinct loci indicates that a large number of genes is concerned with maintaining the integrity of the cell envelope. A number of mutants which show altered envelopes, manifested by increased sensitivity to one or more of the inhibitors we have used, has previously been reported. In no case has the precise metabolic defect been identified but in several cases the responsible mutation has been mapped (Ennis, 1971; Higa & Mandel, 1970; Imae, 1968; Otsuji & Higashi, 1970; Nakamura, 1968; Nozawa & Mizuno, 1968; Normark, 1969, 1970; Schlief, 1969; Sekiguchi & Iida, 1967; Sugino, 1966). Only in the case of the *envA* and *envB* mutants of Normark (1969, 1970) has the mutation responsible for sensitivity changes been shown to have a marked effect on cell growth. Apparently loss of the outer permeability barrier's function does not necessarily impair growth. None of our *env* mutations which have been accurately located appear to be closely linked to other mutations already known to result in altered permeability.

Several types of mutation are known to alter indirectly the outer membrane's permeability to dyes and the cell's sensitivity to deoxycholate. One group consists of *tol* mutations, which confer tolerance to infection by certain colicines (Holland *et al.* 1970; Nagel de Zwaig & Luria, 1967; Whitney, 1971). Another group of mutants believed, on a number of criteria, to have mutations affecting their cytoplasmic membranes have been shown to have increased permeability, and sensitivity

to deoxycholate (Hirota *et al.* 1969; Ricard, Hirota & Jacob, 1970). Finally, there is evidence from a number of sources that the permeability properties of the outer membrane can be substantially altered by events not having any obvious immediate connexion with the cell envelope. The infection of the cell with a derepressed sex factor can alter its permeability by some unknown mechanism (Dowman & Meynell, 1970; Romero *et al.* 1971) as can inhibition of protein synthesis (Matzura & Broda, 1968; Russell, 1972).

The nature of the phenomenon of osmotic remediability is obscure (Russell, 1972). Many TS mutants, including ones in which the affected cell component is not associated with the cell envelope, are osmotic remedial. As recently shown by one of us (Russell, 1972), TSOR mutations affecting aminoacyl-tRNA synthetases exhibit phenotypes very similar to that of the *env* mutants described in this paper, displaying altered outer membrane properties when growing on media of high osmotic strength at the restrictive temperature. The changed envelope properties are apparently a consequence of unbalanced growth due to the defect in protein synthesis being only partially suppressed by the medium. A similar explanation may be applicable to the *env* mutants, i.e. the phenotype on high-osmotic strength medium may be due to the *env* mutation being only leakily expressed, while total blockage of the *env* function on normal media stops growth completely.

The genetic map locations of the *env* mutations tell us nothing of their possible functions, but do suggest that they are in previously unreported genes. None of them have been found to map at a locus occupied by a gene already known to affect LPS or any other component of the outer cell membrane. The results of Russell (1972) suggest that temperature sensitivity of protein synthesis could result in a phenotype similar to that seen with the *env* mutants, but none of the *env* loci identified coincide with known protein synthesis genes. The only description of a cytoplasmic membrane mutation which maps close to an *env* gene is that of Wijsman (1972), who reported a mutation mapping very close to our *envM392* mutation. Wijsman did not report whether he tested his mutant (which is TSOR like the *env* mutants) for changed permeability characteristics at the restrictive temperature.

Experiments are continuing in an attempt to identify the precise metabolic defects in the *env* mutants. As a first step towards this aim, experiments designed to determine whether the mutations affect cell components which are directly concerned with the synthesis or stability of the outer membrane, or whether the TS lesions affect cell structures or activities which could indirectly result in outer membrane changes (e.g. cytoplasmic membrane, protein synthesis) are being performed.

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