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Genetics of a chain-forming mutant of *Escherichia coli*

Transduction and dominance of the *envA* gene mediating increased penetration to some antibacterial agents

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SUMMARY

We have recently described a chain-forming mutant of *Escherichia coli* which showed a decreased resistance to ampicillin and several other antibiotics (Normark, Boman & Mattson, 1969). The gene mediating drug sensitivity was denoted *envA* and by conjugation mapped at 2–4 min. Transduction experiments have now shown that *envA* is located between *leu* and *azi* at 1·5 min. The mapping was facilitated by the finding that *envA* mediated sensitivity to actinomycin D, rifampicin and gentian violet. The *envA* locus could be genetically differentiated from the *pea* locus mediating resistance to phenethyl alcohol (Yura & Wada, 1968). Studies using partial diploids revealed that *envA* was recessive to its wild-type allele both when located on an episome and on the chromosome.

Assuming that revertants from *envA* to the wild-type allele could be selected as ampicillin-resistant derivatives, such mutants were isolated and their phenotype characterized. Reversion to ampicillin resistance was accompanied by reversion to insensitivity to actinomycin D. However, not all revertants exhibited wild-type tolerance to rifampicin. Three different ampicillin-resistant revertants were studied genetically. The results indicate that these strains contain suppressor mutations in the *envA* region of the chromosome. It is suggested that the *envA* gene, directly or indirectly, affects the EDTA sensitive 'permeability barrier' of the surface layer of *Escherichia coli*.

1. INTRODUCTION

It is known that *Escherichia coli* is generally impermeable to charged drugs such as actinomycin D. Leive (1965) has suggested that the outer EDTA-sensitive surface layer of *E. coli* is responsible for this permeability control. This additional 'permeability barrier' is presumably due to the lipopolysaccharide-containing structure which has been shown to be released in part by EDTA (Leive, Shovlin & Mergenhagen, 1968). In view of this finding and the fact that our chain-forming mutant containing the *envA* gene had a decreased resistance to many antibiotics, additional information on the integrity of this 'barrier' and hence of passage of certain antibacterial agents may be gained by a detailed study of this strain.

The gene *envA* mediating drug sensitivity was previously mapped by conjugation at 2–4 min (Normark *et al.* 1969). Here I describe a system for cotransduction

between *leu*, *envA* and *azi*. The cotransduction analysis was facilitated by the finding that *envA* mediated sensitivity to actinomycin D, gentian violet and rifampicin in addition to other drugs previously reported (Normark *et al.* 1969). Ampicillin-resistant revertants of an *envA*-containing strain were studied genetically and it could be concluded that they contained suppressors in the *envA* region of the chromosome. In partial diploids these suppressors were only partially recessive to their wild-type alleles while the *envA* gene was completely recessive in heterozygotes.

2. MATERIALS AND METHODS

(i) *Strains*

All strains used were *E. coli* K12 and are listed in Table 1. Strain D21 is derived from strain RC711 of Meynell & Datta (1966) as described by Boman *et al.* (1968). RC 711 seems to be the same as J62 but cured for the F-factor (see pedigree given by Clowes & Hayes, 1968). Strain D22 is an EMS-induced mucoid mutant derived from the rough strain D21 (Normark *et al.* 1969). *recA* derivatives of D21 and D22 were isolated as *his*$^+$ recombinants obtained in crosses with the *recA*-containing Hfr strain KL16-99 of Low (1968). Such *recA* strains were given numbers ending with 4. The *recA* gene was scored by the ultraviolet sensitivity method of Clark & Margulies (1965). A Philips TUV 6 W lamp was used at a distance of 27·5 cm.

(ii) *Media and growth conditions*

The minimal medium used was medium E of Vogel & Bonner (1956). It was supplemented with 0·2 % glucose, 1 μg/ml of thiamine and 25 μg/ml of the L-epimer of the required amino acid. The complete medium used was LB of Bertani (1951) supplemented with medium E and 0·2 % glucose. LA plates contained LB, 0·2 % glucose and $2{\cdot}5 \times 10^{-3}$ M $CaCl_2$, solidified with 1·5 % agar.

Unless otherwise stated the experiments were performed at 37 °C. The bacteria were cultivated on a rotary shaker and growth was recorded by optical density readings using a Klett–Summerson colorimeter with filter W 66.

(iii) *Materials*

D-Ampicillin (pure D-epimer of α-aminobenzylpenicillin) was kindly provided by AB Astra, Södertälje, Sweden. Actinomycin D (ActD) was obtained from Merck, Sharp and Dohme, Rahway, N.J., U.S.A.; gentian violet (GV) and sodium azide from Merck, Darmstadt, Germany; *N*-methyl-*N*-nitroso-*N*-nitroguanidine (NG) from K and K Laboratories, Inc., Hollywood, Calif., U.S.A.; phenethyl alcohol (PEA) and streptomycin from AB Kabi, Stockholm, Sweden. Rifampicin (RM) was kindly obtained as a gift from I. Oeschger.

(iv) *Determination of resistance*

The bacteria to be tested by single cell test were grown in LB at 37 °C on a rotary shaker and harvested in the logarithmic growth phase. About 100–400 cells were spread on LA plates containing different concentrations of the antibacterial agent.

Table 1. *Strains of* Escherichia coli *K12 and their relevant characters*

Strain	Source and/or derivation	Sex	Response to str	Ampicillin genotype	Envelope genotype	Other relevant markers
D21	Boman *et al.* (1968)	F⁻	r	*ampA1*	+	*proA*, *trp*, *his*
D214	Recombinant from KL16-99 × D21	F⁻	r	*ampA1*	+	*proA*, *trp*, *recA*
D22	Normark *et al.* (1969)	F⁻	r	*ampA1*	*envA*	*proA*, *trp*, *his*
D224	Recombinant from KL16-99 × D22	F⁻	r	*ampA1*	*envA*	*proA*, *trp*, *recA*
D220	Recombinant from KL25 × D22	F⁻	r	+	*envA*	*proA*, *his*
SY111	Summers & Siegel (1969)	F⁻	r	*ampA1*	*envA*	*proA*, *trp*, *his*, *rifA*
La51	AmpA transductant of PA2004	F⁻	r	*ampA1*	+	*pyrB*, *thr*, *leu*, *his*
La514	Recombinant from KL16-99 × La51	F⁻	r	*ampA1*	+	*pyrB*, *thr*, *leu*, *recA*
E64-11	*azi* and *met* derivative of C600	F⁻	s	+	+	*thr*, *leu*, *met*, *azi*, *pea*
AB1157	Low (1968)	F⁻	r	+	+	*thr*, *leu*, *proA*, *argE*, *his*
AB2463	Low (1968)	F⁻	r	+	+	*thr*, *leu*, *proA*, *argE*, *his*, *recA*
HfrH (Hayes)	Hayes (1968)	Hfr	s	+	+	.
KL25	Low (1968)	Hfr	s	+	+	.
KL16-99	Low (1968)	Hfr	s	+	+	*recA*
KLF4/AB2463	Low (1968)	F′	r	+	+	*thr*⁺*leu*⁺*proA*⁺/*thr*, *leu* *proA*, *argE*, *his*, *recA*

Injection order for Hfr strains: KL25 injects O-*ilv-ampA-proA*, KL16-99 injects O-*lysA-his-proA*, HfrH injects O-*thr-leu-proA*. Strain RC711, of which strain D21 and D22 are derivatives, has been shown to contain *proA* instead of *proB* (E. C. R. Reeve, personal communication).

(v) *Determination of cell size*

Cell size was determined in a Coulter counter model B, equipped with a 70 μm diameter aperture (Coulter Electronics Ltd., Dunstable, England). Calibration material with known particle diameter was obtained from Coulter Electronics Ltd., Dunstable, England. Cells were grown in LB or minimal media. Samples of 10 or 100 μl were taken at intervals and diluted in 0·15 M-NaCl which had been filtered through a 0·45 μm membrane to eliminate particle background.

(vi) *Mating and transduction procedure*

F′ KLF4 transfer to F^- recipients was performed as follows. About 0·1 ml from an overnight LB culture of the F^- recipient was inoculated into 10 ml of LB in a 250 ml flask and incubated with rapid shaking at 37 °C to an optical density of 50 Klett units (about 2×10^8 cells/ml). The donor strain was grown overnight in minimal medium to avoid loss of the F′ KLF4. About 0·2 ml was inoculated into 10 ml LB and grown at 37 °C to 50 Klett units. The donors and recipient were mixed in a ratio of 1:1 and the mating mixture was incubated for 30–45 min with slow shaking.

The standard mating procedure using Hfr donors was the same as described earlier by Normark *et al.* (1969). The transduction procedure with phage P1bt was according to the modification described by Eriksson-Grennberg (1968).

3. RESULTS

(i) *Transduction analyses of the* envA *locus*

It was previously shown by Normark *et al.* (1969) that the *envA* marker present in the chain-forming mutant D22 mediated a drastic decrease in episomal and chromosomal resistance to ampicillin and many other drugs. Although interrupted mating experiments showed the *envA* gene was at 2–4 min on the map of *E. coli* (Normark *et al.* 1969), a more detailed mapping of the *envA* gene was considered necessary, especially in order to judge whether the highly pleiotropic characters of strain D22 were due only to the *envA* gene or whether other loci were involved. To faciliate transduction mapping, a resistance characterization of the potential donors and recipients was needed. These are given in Table 2 and show that the *envA* mutant D22 was sensitive to actinomycin D (ActD), rifampicin (RM) and gentian violet (GV). Resistance to sodium azide was also investigated, since preliminary experiments showed the *envA* marker to be closely linked to *azi*. Strain D22 contains the *ampA* allele at 82 min (Eriksson-Grennberg, 1968), while strain AB1157 and strain E64-11, used as recipients in transduction crosses, are both $ampA^+$. It was therefore important to compare the donor strains with an $ampA^+$ derivative of the mutant D22. Such an $ampA^+$, $envA^+$-containing strain, denoted D220 was isolated as a recombinant more ampicillin-sensitive than strain D22 (Table 2) in a cross with Hfr KL25, which injects the *ampA* region of the chromosome early during conjugation. The assumption that strain D220 had lost the

ampA gene was confirmed by a cross HfrH × D220 in which no ampicillin-resistant recombinants could be obtained. In previous mapping of the *envA* gene in D22 such crosses were found to give ampicillin-resistant clones as soon as *envA* was replaced by its wild-type allele (Normark *et al.* 1969).

Table 2. *Resistance of strains used in transduction experiments*

Strains	Envelope genotype	D-amp (μg/ml) (*a*)	RM (μg/ml) (*b*)	ActD (μg/ml) (*c*)	GV (μg/ml) (*d*)	Azide (μg/ml) (*e*)
D21	+	20	1	> 10	3	20
D22	*envA*	0·5	0·01	0·1	0·1	20
D220	*envA*	0·25	0·01	0·1	0·1	20
E64–11	+	2	1	> 10	2	150
La51	+	15	1	> 10	3	.
AB1157	+	1	1	> 10	3	.

Resistance was estimated as the ability to form single cell colonies on plates with different concentrations of antibacterial agent tested at the following concentrations in μg/ml. (*a*) D-Ampicillin: 0·1, 0·25, 0·5, 1, 2, 3, 5, 10, 15, 20, 30; (*b*) rifampicin: 0·01, 0·05, 0·1, 0·5, 1, 2, 5, 10; (*c*) actinomycin D: 0·1, 0·25, 0·5, 1, 2, 5, 10; (*d*) gentian violet: 0·1, 0·25, 0·5, 1, 2, 3, 5, 10, (*e*) sodium azide: 5, 10, 20, 50, 100, 150, 200. Strains D21, D22 and La51 have the *ampA* allele which normally mediates a tenfold increase in ampicillin resistance. E64–11 contains the *azi* marker mediating resistance to azide.

Since strain D22 contains the *ampA* gene, it should be possible to obtain *envA*$^+$ transductants by selecting for ampicillin resistance. When phage P1bt, grown on the *envA*$^+$, *ampA*$^+$ and *azi* strain E64-11, was used for transduction with strain D22 as recipient no transductants were obtained when cells were spread on ampicillin plates immediately after the phage treatment. However, if the cells were incubated in LB for 2–4 h before plating, to allow for phenotypic expression, rough ampicillin-resistant transductants were obtained with a frequency of 3×10^{-6}/viable clone of D22. These ampicillin-resistant transductants were tested for unselected markers. As shown in cross 1, Table 3, 76 % of the *envA*$^+$ transductants were *leu* and 96 % were *azi*. Among 199 *envA*$^+$ transductants 149 had received both *leu* and *azi*. No cotransduction was found with the *thr* marker.

Using cotransduction with *leu*, the *envA* gene could be transferred to a number of strains. With selection for the Leu$^+$ phenotype the contransduction between *leu*$^+$ and *envA* was 23–26 % using three different recipient strains (Table 3). In cross 2 the recipient strain La51 contained the *ampA* gene, and drug-sensitive transductants were scored on LA plates containing ampicillin (no growth on 10 μg/ml) and on LA plates containing gentian violet (no growth on 2 μg/ml). The recipient in cross 3, strain AB1157, forms rough colonies while the *envA* mutant D22 forms slightly mucoid colonies. In this cross mucoid colony formers were scored for on minimal plates. Of 600 *leu*$^+$ clones, 148 formed mucoid colonies, while among 900 *thr*$^+$ clones no mucoid colony formers were found. Of 87 such mucoid and *leu*$^+$ transductants 84 were found to be sensitive to ampicillin (no growth on 1 μg/ml) and to actinomycin D (no growth on 10 μg/ml). Only three clones had a resistance

close to that of the recipient. Of 55 *leu*$^+$ rough transductants all carried the recipient's resistance to the antibacterial agents tested. Of 100 rough *thr*$^+$ transductants none showed the *envA* phenotype for drug sensitivity while eight had an intermediate resistance. The results from cross 3 (Table 3) therefore suggest that the drug sensitivity and mucoid colony formation observed in strain D22 were caused by the same genetical event.

Table 3. *Transduction experiments with the* envA *gene using phage P1bt*

Cross	Donor	Recipient	Selected phenotype	No. of transductants tested	Cotransduction between genes (%)
1	E64-11	D22	Amp-10*	199	76 *envA*$^+$-*leu* 96 *envA*$^+$-*azi* 0 *envA*$^+$-*thr*
2	D22	La51	Leu$^+$	89	26 *leu*$^+$-*envA* 2·3 *leu*$^+$-*thr*
			Thr$^+$	96	0 *thr*$^+$-*envA* 1 *thr*$^+$-*leu*$^+$
3	D22	AB1157	Leu$^+$	600	25 *leu*$^+$-*envA*
			Thr$^+$	900	0 *thr*$^+$-*envA*
4	D22	E64-11	Leu$^+$	286	23 *leu*$^+$-*envA* 22 *leu*$^+$-*envA*-*azi*$^+$ 0·4 *leu*$^+$-*thr*$^+$
			Thr$^+$	261	0 *thr*$^+$-*envA* 0 *thr*$^+$-*envA*-*azi*$^+$ 2·3 *thr*$^+$-*leu*$^+$
			Thr$^+$, Leu$^+$	24	0 *thr*$^+$, *leu*$^+$-*envA* 0 *thr*$^+$, *leu*$^+$-*envA*-*azi*$^+$

* Selection was on LA plates containing ampicillin at 10 μg/ml (ampicillin phenotype defined by Nordström *et al.* 1968).

All transductants in cross 4 (Table 3) were examined for the presence of *envA* by scoring for sensitivity to ampicillin (no growth on 1 μg/ml) and to gentian violet (no growth on 2 μg/ml), respectively. The cotransduction between *leu*$^+$ and *azi*$^+$ was found to be 22 %, while *leu*$^+$ and *envA* were cotransduced at a frequency of 23 %. Of the *leu*$^+$, *envA* transductants 96 % also carried the *azi*$^+$ marker, while all *leu*$^+$, *azi*$^+$ transductants carried *envA*. No *thr*$^+$ transductants carried the *envA* gene. These results indicate that *envA* is located to the right of *leu* between *leu* and *azi*, probably very close to *azi*.

Since resistance to phenethyl alcohol (PEA) has been shown by Yura & Wada (1968) to be controlled by the *pea* gene located very close to *azi*, it was considered important to differentiate between the *envA* and the *pea* markers. Strain C600 of which E64-11 is a derivative is more PEA-resistant than other *E. coli* K12 strains (Yura & Wada, 1968). Eight purified *leu*$^+$ transductants from cross 4 (Table 3) were studied in detail for resistance to PEA, ActD, RM, GV, azide and ampicillin (Table 4). It was found that six transductants carried the same resistance to PEA as

the recipient strain E64-11, while two were more PEA-sensitive. Of five *leu*$^+$, *envA* transductants only one was PEA-sensitive, which means that the *envA* and *pea*$^+$ alleles of strain D22 could be separated genetically.

Table 4. *Resistance pattern of eight purified* leu$^+$ *transductants from cross 4 in Table 3*

Strains	PEA (%)	D-Amp (μg/ml)	RM (μg/ml)	GV (μg/ml)	ActD (μg/ml)	Azide (μg/ml)	Suggested genotype
Transductant							
112	0·05	2	1	2	> 10	150	*envA*$^+$, *azi*, *pea*$^+$
113	0·14	3	1	2	> 10	150	*envA*$^+$, *azi*, *pea*
114	0·14	2	1	2	> 10	150	*envA*$^+$, *azi*, *pea*
116	0·14	0·25	0·01	0·1	0·1	5	*envA*, *azi*$^+$, *pea*
117	0·10	0·25	0·01	0·1	0·1	5	*envA*, *azi*$^+$, *pea*$^+$
119	0·14	2	1	2	> 10	150	*envA*$^+$, *azi*, *pea*
120	0·14	0·25	0·01	0·1	0·1	50	*envA*, *azi*, *pea*
121	0·14	0·25	0·01	0·1	0·1	5	*envA*, *azi*$^+$, *pea*
D22 (donor)	0·05	0·5	0·01	0·1	0·1	20	*envA*, *azi*$^+$, *pea*$^+$
E64-11 (recipient)	0·14	2	1	2	> 10	150	*envA*$^+$, *azi*, *pea*
D21	0·12	20	1	3	> 10	20	*envA*$^+$, *azi*$^+$, *pea*$^+$
D220	0·05	0·25	0·01	0·1	0·1	20	*envA*, *azi*$^+$, *pea*$^+$

Resistance was estimated as the ability to form single cell colonies on plates with different concentrations of antibacterial agents tested. The concentrations of PEA used was the following (% v/v): 0·05, 0·1, 0·12, 0·14, 0·16, 0·18 and 0·20. Concentration of the other antibacterial agents used were the same as in Table 2.

One of the main characteristics of the *envA* mutant D22 is its tendency to form chains during exponential growth in the rich LB medium (Normark *et al.* 1969). Using an electronic Coulter counter the size distribution of D22 cells was compared to cells of the parent strain D21. Fig. 1 shows that stationary phase cells of D21 and D22 grown in LB medium had approximately the same cell volume. After 2 h of exponential growth in rich medium 75% of the counted particles of the *envA* mutant D22 exceeded a volume of 5·0 μm^3 (Fig. 1). Using the parent strain D21 only 4% of the particles reached this size. With D22 the size distribution was the same at different temperatures (30, 37 and 42 °C). However, in minimal medium the fraction of particles larger than 5·0 μm^3 was reduced to 25%. The results are in agreement with the previous microscopic observations which indicated that with D22 chain-formation was predominant during exponential growth in rich medium (Normark *et al.* 1969).

The size distributions of three *leu*$^+$ transductants from cross 4 (Table 3) were examined after 2 h of exponential growth in rich medium. Fig. 2 (left part) shows that transductant t113, which was grouped as having normal resistance (see Table 4) showed a normal size distribution. The two transductants t116 and t117, which scored as extremely sensitive to ampicillin and gentian violet, both showed the same pattern as the *envA*-containing reference strain D220 (Fig. 2, middle and right parts).

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(ii) *Merodiploids for different alleles of the* envA *locus*

The dominance relationships of the *envA* and *envA*$^+$ alleles were studied in strains D21, D22 and in their respective *recA* derivatives. The latter strains, D214 and D224, were isolated as u.v.-sensitive recombinants in crosses with the *recA*-containing Hfr strain KL16-99 (Low, 1968). The F′ KLF4, which covers the region 88–10 min (Low, 1968), was used for preparing partial diploids of the *envA* gene at 1·5 min. Using strains D22 or D224 as recipients it was possible to isolate

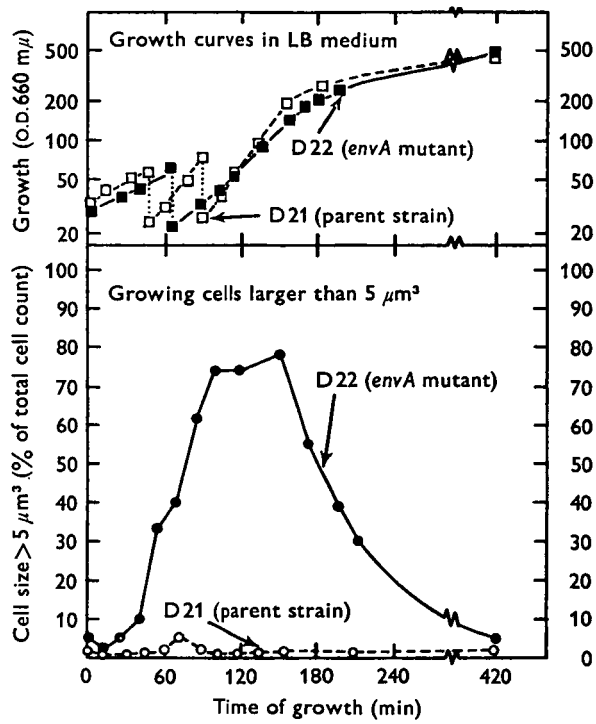

Fig. 1. Growing cells with a cell volume larger than 5·0 μm^3 during growth in LB medium. Strains D21 and D22 were grown overnight in LB medium, diluted 10^{-1} with prewarmed LB at zero time and incubated at 37 °C on a rotary shaker. Prewarmed LB was added during the experiment to prolong the exponential growth phase. At intervals a sample of 10 μl was diluted in 10 ml 0·15 M-NaCl and the cell count was read at different threshold values in a Coulter counter model B. The settings were the following: aperture current 1/374, amplification 1/4, upper threshold 100. The total cell count was estimated at a lower threshold level of 2. Using spherical particles with known diameter a lower threshold level of 10 was found to correspond to a particle volume of 5·0 μm^3. Growth was followed by optical density readings with a Klett photometer (upper part).

clones containing the episome KLF4 by selecting for Pro$^+$ clones. It was also possible to obtain F′-containing clones by selection for ampicillin resistance, which was the first evidence that *envA* is recessive to its wild-type allele. The presence of the episome KLF4 was tested by mating on plates using as recipient strain AB2463 (*recA*) and scoring for clones with Thr$^+$, Leu$^+$ and Pro$^+$ phenotypes.

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From cross 1 in Table 5, one partial diploid was incubated in LB for several generations. After streaking on LA plates, 50 clones were picked and tested for the presence of KLF4 and for ampicillin resistance. Of these clones 47 were still ampicillin-resistant and could transfer KLF4, two were ampicillin-sensitive and could not transfer the episome, while one ampicillin-sensitive clone still contained KLF4. This F-ductant, which was believed to contain *envA* on its episome, was crossed to strain AB2463 with selection for Thr$^+$, Leu$^+$ and Pro$^+$ clones. Using one

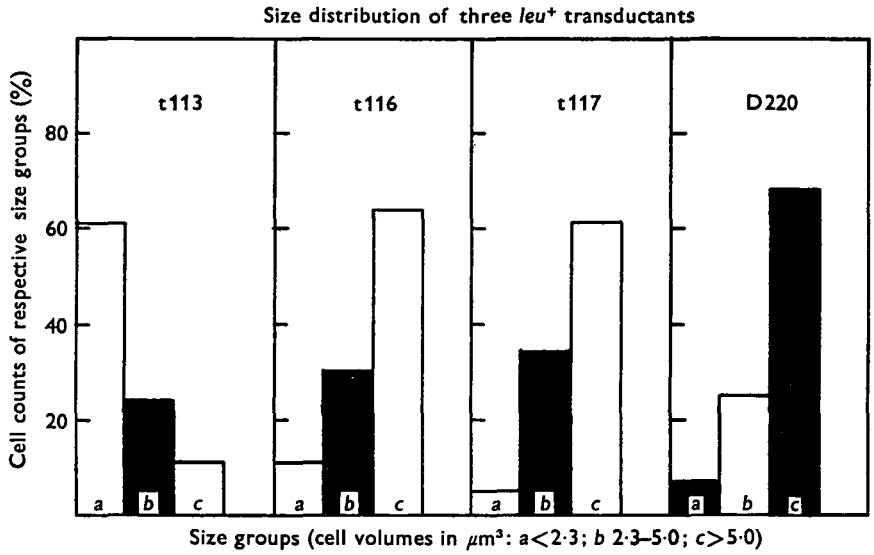

Fig. 2. Cell size distribution of three *leu*$^+$ transductants from cross 4, Table 3. Experimental conditions as in Fig. 1. A cell size distribution was made after 2 h exponential growth in LB. A lower threshold setting of 5 corresponded to a particle volume of 2·3 μm^3, while a lower threshold setting of 10 corresponded to 5·0 μm^3.

such clone as donor it was possible to introduce its F′ KLF4*envA* into strains D21, D22, D214 and D224. The results of these crosses are summarized in Table 5. When introducing this F′ into strains D22 and D224 the Pro$^+$ clones isolated were still ampicillin-sensitive (crosses 5 and 7). No F-ductants were obtained when selecting for ampicillin resistance (crosses 4 and 6). In cross 8 using D21 as recipient strain most F′ clones were ampicillin-resistant. However, a segregation to ampicillin sensitive clones was observed at a low frequency. With the *recA*-containing strain D214 as recipient no ampicillin-sensitive clones were obtained. This indicates that the ampicillin-sensitive clones obtained in cross 8 were caused by a recombination event between the episome and the chromosome. These recombinants were also sensitive to actinomycin D.

To obtain a more complete characterization of the merodiploids prepared Table 6 gives their single-cell resistance to some of the antibacterial agents used previously.

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Table 5. *F-duction crosses with the F' KLF4*

No.	Donor	Recipient	Selected phenotype	No. of colonies tested	% of colonies tested that score as: Donor	Amp-10	Pro+
1	KLF4/AB2463	D22	Amp-10	49	61	100	84
2	KLF4/AB2463	D224	Amp-10	95	100	100	100
3	KLF4/AB2463	D224	Pro+	62	100	100	100
4	KLF4*envA*/AB2463	D22	Amp-10	No resistant colonies			
5	KLF*envA*/AB2463	D22	Pro+	23	100	0	100
6	KLF*envA*/AB2463	D224	Amp-10	No resistant colonies			
7	KLF4*envA*/AB2463	D224	Pro+	22	100	0	100
8	KLF4*envA*/AB2463	D21	Pro+	100	80	97	100
9	KLF4*envA*/AB2463	D214	Pro+	56	100	100	100

Amp-10 phenotype was scored for on plates containing ampicillin at 10 μg/ml. Ampicillin phenotype was defined by Nordström *et al.* (1968). Donor capacity (the presence of KLF4 and KLF4*envA*) was tested by replica plating on a lawn of the recipient strain AB2463 selecting for Thr+, Leu+ and Pro+ clones.

Table 6. *Resistance of merodiploids containing different alleles of the* envA *locus*

Strain	Genotype	Morphological characteristics	D-Amp (μg/ml)	RM (μg/ml)	ActD (μg/ml)
D214	*envA*+	Rough	20	1	> 10
D224	*envA*	Mucoid, chain-forming	0·5	< 0·1	0·1
KLF4/D214	*envA*+/*envA*+	Rough	10	1	> 10
KLF4*envA*/D214	*envA*/*envA*+	Rough	20	1	> 10
KLF4/D224	*envA*+/*envA*	Rough	7	0·5	10
KLF4*envA*/D224	*envA*/*envA*	Mucoid, chain-forming	0·5	< 0·1	1

Resistance was tested by single-cell colony formation at the following concentrations (μg./ml). D-Ampicillin: 0·5, 1, 2, 5, 7, 10, 15, 20 and 30. Rifampicin: 0·1, 1, 2, 5 and 10. Actinomycin D: 0·1, 0·5, 1, 2 and 10. Strain KLF4/D224 grew poorly at an actinomycin D concentration of 10 μg/ml, while this concentration had no visible effect on the growth of D214, KLF4/D214 and KLF4*envA*/D214.

(iii) *Drug-resistant revertants of the* envA *mutant D22*

Since the *envA* gene mediates sensitivity to ampicillin, it was assumed that revertants from *envA* to the wild-type allele could be selected as ampicillin-resistant mutants of strain D22. Such revertants could be isolated with a frequency of 5×10^{-8}/viable D22 clone on plates containing ampicillin at 10 μg/ml. Among 20 such ampicillin-resistant revertants tested, none was as ampicillin-resistant as D21, the *ampA*-containing parent strain of D22. However, all revertants showed a change from mucoid to rough colony formation. The resistance pattern of one ampicillin-resistant revertant D22S1 is shown in Table 7. The partial reversion to ampicillin resistance in D22S1 was accompanied by an apparently complete reversion to actinomycin D insensitivity, as well as a wild-type level of tolerance to

gentian violet and rifampicin. The size distribution of the cells was found to be the same for fast exponentially growing cells of D21 and D22S1, which indicates that a complete reversion of the chain formation trait also occurred. However, strain D22S1 was considerably more sensitive to PEA than strain D21, as was also true for its parent strain D22. Spontaneous revertants of strain D22 were also selected on a rifampicin concentration of 1 μg/ml. The frequency was 5×10^{-8}/viable D22 clone. Of 25 such revertants all were found to be ampicillin-resistant.

To test whether reversion to ampicillin resistance was always followed by an increased tolerance to other antibacterial agents, the following mutation experiment was performed: stationary phase cells of strain D22 were washed and resuspended in 0·1 M potassium phosphate (pH 6·0) and treated for 30 min with *N*-methyl-*N*-nitroso-*N*-nitroguanidine at 200 μg/ml. Afterwards the cells were washed twice, spread on plates with different concentrations of D-ampicillin (10, 20 and 30 μg/ml respectively) and incubated at 28 °C. The frequency of ampicillin-resistant clones were 4×10^{-5} at an ampicillin concentration of 10 μg/ml, and 2×10^{-6} at 20 μg/ml, respectively. No mutants were obtained on plates with ampicillin at 30 μg/ml. Since the *ampA*-containing parent strain D21 cannot form single-cell colonies on ampicillin concentrations higher than 20 μg/ml, none of the mutants showed a resistance higher than the Amp-10 phenotype. All revertants formed rough colonies, and all grew well on plates containing actinomycin D at 2 μg/ml. Of 31 isolated revertants, 26 grew on plates containing gentian violet (2 μg/ml) and 18 tolerated a rifampicin concentration of 2 μg/ml. Two of these ampicillin-resistant revertants, strains D22M1 and D22M2, were further characterized. Their resistance at 37 °C to ampicillin, ActD, RM, GV and PEA was included in Table 7.

Table 7. *Resistance pattern of drug-resistant revertants of strain D22*

Strains	D-amp (μg/ml)	RM (μg/ml)	ActD (μg/ml)	GV (μg/ml)	Azide (μg/ml)	PEA (%)
D22S1	15	1	> 10	3	20	< 0·05
D22M1	15	0·5	> 10	3	20	< 0·05
D22M2	5	0·1	2	2	20	< 0·05
SY111	0·5	> 50	0·1	0·1	.	.

All strains are believed to contain the *envA* mutation as well as the *ampA* marker, which alone mediates a tenfold increase in ampicillin resistance. Comparative data showing the effects of these genes are given in Table 2. Resistance was tested by single cell colony formation. Concentrations of the antibacterial agents were the same as in Table 4, except for strain SY111, where the highest concentration of rifampicin tested was 50 μg/ml.

(iv) *Resistance mutation not affecting the envelope of the* envA *mutant D22*

Summers & Siegel (1969) have recently described a rifampicin-resistant mutant of strain D22. Except for its rifampicin resistance this mutant strain, SY111, in all respects resembles its parent strain D22 (see Table 7). The gene mediating rifampicin resistance was mapped by crossing strain SY111 with the Hfr strains KL25 and

HfrH, and selecting for Pro$^+$ recombinants. When strain KL25 was used as donor, 52 % of the Pro$^+$ recombinants were rifampicin-sensitive. A cross with HfrH and SY111 gave rise only to rifampicin-resistant Pro$^+$ clones. These results show that the gene mediating rifampicin resistance maps between the origin of KL25 and HfrH; that is, between 73 and 87 min. This locus, tentatively designated *rifA*, may be allelic to the rifampicin resistance genes recently described (Tocchini-Valentini, Marino & Colvill, 1968; Ezekiel & Hutchins, 1968). Thus both mapping and resistance pattern show the existence of rifampicin resistance without any direct relationship to the *envA* locus.

Table 8. *Transduction experiments with ampicillin-resistant revertants of the* envA *mutant D22*

Cross	Donor	Recipient	Selected phenotype	No. tested	No. of transductants growing on: D-Amp (1 μg/ml)	RM (1 μg/ml)	Azide (100 μg/ml)
1	D22S1	E64-11	Leu$^+$	150	149	149	88
			Thr$^+$	99	99	99	99
2	D22M1	E64-11	Leu$^+$	95	47	47	43
3	D22M2	E64-11	Leu$^+$	98	65	65	62

The maximal concentration (μg/ml) permitting growth of D220 (*envA*, *ampA*$^+$) would be: for amp, 0·25; RM, 0·01; and for azide, 20. Resistance was tested by replica plating.

(v) *Genetic analysis of ampicillin-resistant revertants of strain D22*

The fact that *envA* was cotransducible with the *leu* marker (Table 3) was used for testing whether ampicillin resistance was due to a reversion in the *envA* gene or to a mutation located elsewhere on the chromosome. To discriminate between these alternatives, phage stocks of P1bt, grown on the ampicillin-resistant revertants D22S1, D22M1 and D22M2 were used as donors in transduction experiments with strain E64-11 as recipient. The results of these crosses are shown in Table 8. In cross 1, using strain D22S1 as donor, only one transductant obtained was sensitive to ampicillin (no growth on 1 μg/ml) when selecting for Leu$^+$ clones. However, about 40 % of the Leu$^+$ transductants had a slightly decreased resistance to ampicillin and rifampicin. None of the transductants tested formed mucoid colonies or gave rise to chain formation.

In cross 2 (Table 8) with the ampicillin-resistant revertant D22M1 as donor, ampicillin-sensitive and rifampicin-sensitive Leu$^+$ transductants were obtained at a frequency of 50 %. These transductants could form colonies at an ampicillin concentration of 0·5 μg/ml, while normal *envA*, *ampA*$^+$ strains did not grow at this concentration. All of the transdutants in cross 2 showed rough colony morphology and a normal size distribution.

In cross 3 (Table 8) mucoid, ampicillin-sensitive and rifampicin-sensitive transductants segregated at a frequency of 34 %. These transductants showed the same size distribution as *envA* containing strains. Cross 3 also gave Leu$^+$ transductants with an ampicillin resistance of about 1 μg/ml at a frequency of 28 %.

(vi) *Effects of an F' factor on the ampicillin-resistant revertants of D22*

In order to get a better understanding of the reversion phenotypes exhibited by D22S1, D22M1 and D22M2, merodiploids were prepared in the following way. By mating KL16-99 with the revertants, *his*$^+$ and *recA* recombinants were isolated and designated D22S14, D22M14 and D22M24 respectively. These strains were then crossed with KLF4/AB2463 and KLF4*envA*/AB2463, selecting for Pro$^+$ clones. These were purified and tested for ability to transfer the Thr$^+$ and Leu$^+$ phenotype to the recipient strain La514. Merodiploids were tested for resistance to ampicillin and rifampicin as shown in Table 9. The results show that there was a considerable difference in resistance between strains containing KLF4 and KLF4*envA*. Compared to the haploid strains KLF4 did not show any effect on the resistance pattern. However, with KLF4*envA* present, there was a considerable decrease in ampicillin resistance while resistance to rifampicin was less affected.

Table 9. *Resistance of partial diploids of ampicillin-resistant revertants of D22*

Strain	Suggested envelope genotype	D-Amp (μg/ml)	RM (μg/ml)
D22S14	*envA*, *sup*-200	15	1
D22M14	*envA*, *sup*-201	15	1
D22M24	*envA*, *sup*-202	5	0·5
KLF4/D22S14	*envA*$^+$, *sup*-200$^+$/*envA*, *sup*-200	15	1
KLF4*envA*/D22S14	*envA*, *sup*-200$^+$/*envA*, *sup*-200	5	1
KLF4/D22M14	*envA*$^+$, *sup*-201$^+$/*envA*, *sup*-201	15	1
KLF4*envA*/D22M14	*envA*, *sup*-201$^+$/*envA*, *sup*-201	2	0·1
KLF4/D22M24	*envA*$^+$, *sup*-202$^+$/*envA*, *sup*-202	5	0·5
KLF4*envA*/D22M24	*envA*, *sup*-202$^+$/*envA*, *sup*-202	2	0·1

Symbols *sup*-200, *sup*-201 and *sup*-202 stand for the respective suppressor gene in strains D22S1, D22M1 and D22M2 and their *recA* derivatives D22S14, D22M14 and D22M24. *envA*$^+$, *sup*-200$^+$, *sup*-201$^+$, *sup*-202$^+$ indicate wild-type alleles. Resistance was estimated by single-cell colony formation.

4. DISCUSSION

(i) *Mapping and dominance on the* envA *locus*

Many of the results presented depend on the method of estimating resistance as the ability of a cell to form a colony at different concentrations of the agent tested. Nordström *et al.* (1968) have shown that for ampicillin this method normally gives rise to sharp drops in colony counts, and accuracy was estimated to be within a factor of 2. However, for chloramphenicol the accuracy of the method was less satisfactory. In the present study it was observed that the determinations of resistance to rifampicin, azide and gentian violet were less accurate than those for ampicillin. For this reason, care has been exercised in the interpretation of minor variations observed in the resistance pattern.

The conjugation mapping performed earlier had shown *envA* to be located at 2–4 min (using the time scale of Taylor & Trotter, 1967). When selecting for

envA$^+$ transductants (cross 1, Table 3) the cotransduction with the *leu* marker was 76%, while only 23–26% of *leu*$^+$ transductants inherited the *envA* gene (crosses 2–4 in Table 3). Such inequalities of reciprocal cotransduction frequencies have been noted by other workers (Taylor & Trotter, 1967). In this case, however, the lower viability of *envA*-containing strains is the most likely explanation for this discrepancy. It is thus reasonable to assume that the selection of *envA*$^+$ transductants gives the best linkage information. The cotransduction frequencies given in Table 3 therefore suggest that the most likely gene order would be *thr–leu–envA–azi*. Since the location of both *leu* and *azi* are determined with relatively high precision (Taylor & Trotter, 1967), the present data suggest that *envA* is located at 1·5 min.

Yura & Wada (1968) have shown that resistance to PEA is determined by the *pea* marker located very close to *azi*. The fact that four out of five tested *leu*$^+$, *envA* transductants carried the same resistance to PEA as the PEA-resistant recipient strain E64-11 shows that *envA* and *pea* can be genetically differentiated. Recently Helling (1968) has reported on some mutations mediating high mutation rates, *mutT1 mut-2*, which are located between *leu* and *azi*. The genetic locus for potassium transport has been mapped in the *leu* region (Lubin & Kessel, 1960) and a gene affecting cell division, *ftsA*, has been found to map close to *leu* (Van de Putte, Van Dillewijn & Rörsch, 1964). Despite their different physiological effects these four genes, which should be closely linked, may all be concerned with the envelope or the cell membrane, indicating that many membrane functions are controlled by genes in the *envA* region of the chromosome. Leive (1965) has shown that in *E. coli* sensitivity to actinomycin can be produced by treatment with ethylenediamine tetraacetate (EDTA). The fact that *envA* mediates sensitivity to actinomycin as well as to ampicillin, an antibiotic acting outside the cytoplasmic membrane, suggests that the EDTA-sensitive surface layer is affected by the *envA* gene. Mutants of *E. coli* sensitive to actinomycin D have been described by Sekugushi & Iida (1967), but no genetic data are yet available. An important conclusion from the resistance analyses of *envA* and *envA*$^+$ strains is that the normal resistance found in *E. coli* to many antibiotics is caused by a permeability barrier. The *envA* mutation causes for example a 100-fold decrease in the resistance to rifampicin, revealing that the RNA polymerase even *in vivo* is very sensitive to this drug.

The *envA* mutation was found to be recessive in partial diploids both with an episomal and chromosomal location (Table 6), in contrast to the *capR* gene described by Markowitz & Rosenbaum (1965). However, most merodiploid recombinants tested did not reach the same resistance level as the control strain D214. This may be explained at least in part by the lower growth-rate observed for the partial diploids. In addition, the gene product of *envA* may possibly be able to compete with that produced by the wild-type allele. Diploids for the *envA* gene showed a slightly higher tolerance to actinomycin D than the control strain D224. This observation is consistent with the hypothesis that *envA* makes a gene product with an impaired function.

(ii) *The properties of the back mutants with increased ampicillin resistance*

The fact that revertants to ampicillin resistance could be obtained spontaneously at a normal mutation frequency makes it unlikely that the *envA* gene contains a large deletion. Reversion to ampicillin resistance was followed by reversion to rough colony formation, normal cell division and a wild-type response to other antibacterial agents. It was possible to isolate revertants showing only a partial reconstruction of the resistance pattern found in the parental strain D21. However, in no case was a revertant found which in all tested respects was indistinguishable from the parent strain D21. The possibility that in strain D22 the original mutation in the *envA* gene was a transposition, reversion or other aberration involving a recombination step, seems unlikely since strain D22 and its *recA* derivative D224 showed the same mutation frequency to ampicillin and rifampicin resistance.

The genetic studies of three ampicillin-resistant revertants can be interpreted to mean that they all contain suppressor mutations in the *envA* region of the chromosome (see Table 8). If *envA* is a point mutation in a gene, one would expect that intragenic suppressor mutations would give rise to clones with resistance pattern in between that observed for strains D22 and D21.

It is known that strain E64-11 as well as D21 and its derivatives contains amber suppressors (Signer, Beckwith & Brenner, 1965; L. Isaksson & O. Sköld, personal communication). It is therefore excluded that the set of suppressors here described are of a similar nature.

When the episomes KLF4 and KLF4*envA* were introduced into strains D22S14, D22M14 and D22M24 (containing *recA*) it was found that KLF4*envA* gave a considerable decrease in the resistance to ampicillin. Resistance to rifampicin was also affected but to a lesser extent (Table 9). F′ strains containing KLF4 did not show a significant difference in resistance when compared to the haploid strains. These results would be expected if the suppressors present in the ampicillin-resistant revertants were only partially recessive to their wild-type alleles.

Some of the transduction experiments have given results which are difficult to interpret (e.g. crosses 2 and 3 in Table 8). It should therefore be emphasized that conclusions concerning the nature of the gene products of the *envA* gene and its suppressors should await chemical and physiological studies on the cell envelopes of the respective mutants, which currently have been initiated.

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