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 2.5 x 10⁻³ M CaCl₂, 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 W66.

(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 Ampicillin to str | 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−  | +                         | 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                         | +                 |                               |
| KLF4/AB2463       | Low (1968)                            | F’  | r                         | +                 | 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 facilitate 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) | RM (µg/ml) | ActD (µg/ml) | GV (µg/ml) | Azide (µg/ml) |
|----------|------------------|--------------|-----------|-------------|----------|--------------|
|          |                  | (a)          | (b)       | (c)         | (d)      | (e)          |
| D21      | +                | 20           | 1         | >10         | 3        | 20           |
| D22      | envA             | 0.55         | 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 cotransduction 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                    |
|       |         |           |                    |                            | 22 leu+ - envA - azi*            |
|       |         |           | Thr+               | 900                        | 0 thr+ - envA                    |
| 4     | D22     | E64-11    | Leu+               | 286                        | 23 leu+ - envA                    |
|       |         |           |                    |                            | 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...
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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

| Transductant | PEA (%) | D-Amp (µg/ml) | RM (µg/ml) | GV (µg/ml) | ActD (µg/ml) | Azide (µg/ml) | Suggested genotype |
|--------------|---------|---------------|------------|------------|--------------|---------------|-------------------|
| 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 µm³ (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 µm³ 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 t 113, which was grouped as having normal resistance (see Table 4) showed a normal size distribution. The two transductants t 116 and t 117, 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).
(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 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.

Fig. 1. Growing cells with a cell volume larger than 5·0 μm³ 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 μm³. Growth was followed by optical density readings with a Klett photometer (upper part).
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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 such clone as donor it was possible to introduce its F' KLF4envA 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.

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 μm³, while a lower threshold setting of 10 corresponded to 5·0 μm³.
Table 5. F-duction crosses with the F' KLF4

| No. | Donor                  | Recipient | Selected phenotype | No. of colonies tested | % of colonies tested that score as: |
|-----|------------------------|-----------|--------------------|------------------------|------------------------------------|
| 1   | KLF4/AB2463            | D22       | Amp-10             | 49                     | Donor: 61, Amp-10: 100, Pro+: 84   |
| 2   | KLF4/AB2463            | D22       | Amp-10             | 95                     | Donor: 100, Amp-10: 100, Pro+: 100 |
| 3   | KLF4/AB2463            | D22       | Pro+               | 62                     | Donor: 100, Amp-10: 100, Pro+: 100 |
| 4   | KLF4/envA/AB2463       | D22       | Amp-10             | No resistant colonies  |                                                   |
| 5   | KLF4/envA/AB2463       | D22       | Pro+               | 23                     | Donor: 100, Amp-10: 0, Pro+: 100    |
| 6   | KLF4/envA/AB2463       | D22       | Amp-10             | No resistant colonies  |                                                   |
| 7   | KLF4/envA/AB2463       | D22       | Pro+               | 22                     | Donor: 100, Amp-10: 0, Pro+: 100    |
| 8   | KLF4/envA/AB2463       | D22       | Pro+               | 100                    | Donor: 80, Amp-10: 97, Pro+: 100    |
| 9   | KLF4/envA/AB2463       | D22       | Pro+               | 56                     | Donor: 100, Amp-10: 100, Pro+: 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 x 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...
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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 $5 \times 10^{-6}$/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 re-suspended 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 β-ampicillin (10, 20 and 30 µg/ml respectively) and incubated at 28 °C. The frequency of ampicillin-resistant clones were $4 \times 10^{-5}$ at an ampicillin concentration of 10 µg/ml, and $2 \times 10^{-6}$ 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 rif\(_A\), 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 env\(_A\) locus.

Table 8. Transduction experiments with ampicillin-resistant revertants of the env\(_A\) mutant D22

| Cross | Donor    | Recipient | Selected phenotype | No. tested | D-Amp (1 \(\mu\)g/ml) | RM (1 \(\mu\)g/ml) | Azide (100 \(\mu\)g/ml) |
|-------|----------|-----------|--------------------|------------|----------------------|-------------------|----------------------|
| 1     | D22S1    | E64-11    | Leu\(^+\)          | 150        | 149                  | 149               | 88                   |
|       |          |           | Thr\(^+\)          |            | 99                   | 99                | 99                   |
| 2     | D22M1    | E64-11    | Leu\(^+\)          | 95         | 47                   | 47                | 43                   |
| 3     | D22M2    | E64-11    | Leu\(^+\)          | 98         | 65                   | 65                | 62                   |

The maximal concentration (\(\mu\)g/ml) permitting growth of D220 (env\(_A\), amp\(_A^+\)) 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 env\(_A\) was cotransducible with the leu marker (Table 3) was used for testing whether ampicillin resistance was due to a reversion in the env\(_A\) 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 \(\mu\)g/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 \(\mu\)g/ml, while normal env\(_A\), amp\(_A^+\) strains did not grow at this concentration. All of the transductants 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 env\(_A\) containing strains. Cross 3 also gave Leu\(^+\) transductants with an ampicillin resistance of about 1 \(\mu\)g/ml at a frequency of 28%. 

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(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 KLF4envA/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 KLF4envA. Compared to the haploid strains KLF4 did not show any effect on the resistance pattern. However, with KLF4envA 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          |
| KLF4envA/D22S14       | envA, sup-200+/envA, sup-200 | 5            | 1          |
| KLF4/D22M14           | envA+, sup-201+/envA, sup-201 | 15           | 1          |
| KLF4envA/D22M14       | envA, sup-201+/envA, sup-201 | 5            | 1          |
| KLF4/D22M24           | envA+, sup-202+/envA, sup-202 | 2            | 0-1        |
| KLF4envA/D22M24       | envA, sup-202+/envA, sup-202 | 5            | 0-5        |

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 Holling (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, ft5A, has been found to map close to leu (Van de Putte, Van Dillewijn & Rösch, 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.
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 \textit{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 \textit{envA} gene was a transposition, reversion or other aberration involving a recombination step, seems unlikely since strain D22 and its \textit{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 \textit{envA} region of the chromosome (see Table 8). If \textit{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 E84-11 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\textit{envA} were introduced into strains D22S14, D22M14 and D22M24 (containing \textit{recA}) it was found that KLF4\textit{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 \textit{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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