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# Mutant of *Escherichia coli* with Anomalous Cell Division and Ability to Decrease Episomally and Chromosomally Mediated Resistance to Ampicillin and Several Other Antibiotics

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In a mutation experiment with a rough, ampicillin-resistant strain, we isolated two smooth mutants which were both sensitive to ampicillin and carried defects in the cell envelope. One of the strains (with the *envA* gene) is hindered in its completion of septa and forms chains of cells. The *envA* gene has been mapped to a position between *leu* and *proB*, at 2 to 4 min. The *envA* gene decreased the resistance mediated by both episomal and chromosomal genes for resistance to several antibiotics. During growth the *envA* mutant was characterized by abnormal ratios between viable count or cell count and optical density. The ratio between viable count and optical density was affected during shift-up and shift-down experiments. When compared to the parent strain, the *envA* mutant was found to be more resistant to ultraviolet irradiation on plates. Prestarvation for tryptophan had a protective effect against irradiation both on the parent strain and the *envA* mutant.

During our recent work with an ampicillin-resistant strain of *Escherichia coli*, it was observed that some classes of penicillin-resistant recombinants always produced smooth colonies (4). To investigate a possible relationship between capsule formation and ampicillin resistance, a number of smooth mutants were isolated from a rough parent strain that contains the *ampA* gene. Two such mutants were studied further. During normal growth, one produces chains of cells that can be transformed into filaments. Since the cell envelope was affected, the mutation was designated *envA*. When tested for ampicillin resistance on plates, *envA* reduced the resistance mediated by *ampA* by a factor of 40. *EnvA* also decreased both chromosomally and episomally mediated resistance to several other antibiotics. The *envA* mutation, which mapped between *leu* and *proB*, was found to increase tolerance to ultraviolet (UV) irradiation.

The other smooth mutant isolated was also more UV-resistant than the parent strain. It produces sphere-like cells and the mutation (*envB*) maps near streptomycin resistance (*unpublished results*). Both of the mutants have been studied by electron microscopy (*unpublished results*).

## MATERIALS AND METHODS

**Strains.** All strains used were *E. coli* K-12. Their properties are shown in Table 1. Strains D11 and D21 are derived from strain RC711 of Meynell and Datta (14) as described by Boman et al. (4). The mutant D22 was obtained from the rough, moderately ampicillin-resistant, *ampA*-containing strain D21 in the following way. About $10^8$ cells per ml of D21 were grown in 10 ml of LB medium for 2.5 hr in the presence of ethylmethanesulfonate (EMS) at a concentration of 10 $\mu$g/ml. After removing EMS by centrifugation, the cells were grown in LB medium to a concentration of $8 \times 10^8$ cells per ml. An appropriate dilution was spread on 100 plates containing minimal medium. After 40 hr of incubation at 37 C, the plates were examined for smooth colonies. Among an estimated 30,000 colonies, 133 were more or less mucoid. Replica plating and single-cell tests revealed that two mucoid clones were significantly more sensitive to ampicillin than the parent strain D21. The most sensitive was the *envA* mutant D22. The other smooth mutant, designated D23, is described separately by Normark (*unpublished results*).

The R factor R1 (14) carrying resistance to ampicillin, chloramphenicol, sulfonamides, and kanamycin, was obtained from Datta.

**Media and growth conditions.** The compositions of the minimal medium and the rich LB medium are described elsewhere (16). In tryptophan-starvation ex-

TABLE 1. *Strains of E. coli K-12 and their relevant characters*[a]

| Strain | Reference | Sex | Streptomycin genotype | Ampicillin genotype | Envelope genotype | Morphological characteristics | Other relevant markers |
|---|---|---|---|---|---|---|---|
| D11 | Boman et al. (4) | $F^-$ | *strA* | + | + | Rough | *proB, trp, his* |
| D21 | Boman et al. (4) | $F^-$ | *strA* | *ampA* | + | Rough | *proB, trp, his* |
| D22 | This paper | $F^-$ | *strA* | *ampA* | *envA* | Smooth, chain-forming | *proB, trp, his* |
| HfrH | *See* (21) | Hfr | + | + | + | Rough | |
| P4x | *See* (21) | Hfr | + | + | + | Rough | *metB* |

[a] Hfr strain transfers the chromosome as follows: HfrH, *O-thr-proB, trp;* P4x, *O-proB-thr-metB.* Abbreviations for genes according to Taylor and Trotter (21).

periments, the basal medium was supplemented with 0.2% glucose and 0.2% casein hydrolysate (Oxoid L41). Experiments were performed at 37 C unless otherwise stated. Growth was recorded by optical density readings made with a Klett Summerson colorimeter with a W66 filter.

**Materials.** D-Ampicillin (α-aminobenzylpenicillin) and penicillin G (benzylpenicillin) were kindly provided by AB Astra, Södertälje, Sweden. D-Cycloserine and streptomycin were from AB Kabi, Stockholm, Sweden; novobiocin was from Merck, Sharp & Dohme, Rahway, N.J.; kanamycin was from AB Ferrosan, Malmö, Sweden; chloramphenicol was from Erco, Stockholm, Sweden; and nalidixic acid was from Winthrop Ltd., Surbiton on Thames, England.

**Viable counts and cell counts.** Viable counts of a culture were made by spreading 0.1 ml of appropriate dilutions in 0.9% NaCl. For the mutant D22, colonies were counted after about 20 hr of incubation at 37 C. Cells were counted under a microscope with a Petroff-Hausser bacteria counter from C. A. Hausser & Sons, Philadelphia. Chains as well as filaments were always counted as a single cell.

**Phase microscopy.** Cultures were examined microscopically with a Leitz Ortholux microscope equipped with dark-phase optics and a Polaroid camera. Cultures were generally treated with 5% formaldehyde before examination. Plates were photographed with a Cordis immunodiffusion camera.

**Determination of resistance.** Resistance was tested by replica plating or by single-cell colony formation on plates with different concentrations of antibiotic [for a recent discussion of these methods see Nordström et al. (16)].

**Determination of penicillinase activity.** Novick's iodometric method (17) was used, adapted by Burman et al. (5) to a Technicon AutoAnalyzer.

**Standard mating procedure.** Strains to be mated were pregrown at 37 C in LB to an optical density of 100 Klett units (about $4 \times 10^8$ cells per ml). Of these cultures, 9 ml of $F^-$ cells were mixed with 1 ml of Hfr cells in a prewarmed 300- or 500-ml flask at 37 C without rotation. Samples (0.1 ml) were withdrawn and diluted with 5 or 10 ml of 0.9% NaCl. They were blended with a Vortex Super Mixer for 40 to 60 sec, and 0.1 ml was spread on plates.

**Irradiation with UV light.** Plates with bacteria were irradiated by a UV lamp mounted at a distance of 27.5 cm in a box lined with aluminum foil. The bulb was of type TUV 6W from Philips, Holland. Photoreactivation was prevented by keeping the plates wrapped in aluminum foil until after incubation.

## RESULTS

**Morphological characterization of strain D22.** Strain D22 was isolated as a mutation from rough to smooth colony morphology. This difference is illustrated in Fig. 1, which shows streaks of the parent strain D21 and the *envA* mutant D22 on the same plate photographed after 1, 2, and 3 days of incubation at 30 C. Besides the difference in colony morphology, the figure also demonstrates that D22 grows significantly more slowly than D21 both in minimal medium (Fig.1) and in rich medium, in which the difference is greater. Microscopic examination showed that cultures of D22 at different stages of growth produced linear aggregates of cells of varying lengths. This is shown in Fig. 2b, which includes the parent strain D21 as a control (Fig. 2a). Figure 2b shows rather clear constrictions in actively growing D22, which indicates that this strain differs from the filament-producing *lon* mutants first described by Howard-Flanders et al. (8). We have therefore decided to refer to the anomaly in D22 as "chain formation," and we will in later sections provide evidence indicating that "chain formation" differs from filament formation.

**Resistance of D22 to ampicillin and some other antibiotics.** We compared the ampicillin resistance of D22 with that of some related strains. Figure 3 shows single-cell colony formation on different concentrations of ampicillin. In agreement with previous results, the *ampA* gene in D21 was found to give a 10-fold increase in resistance (16). However, the *envA* locus in D22 decreased the resistance mediated by *ampA* to such an extent that D22 was considerably less resistant than the wild-type strain D11.

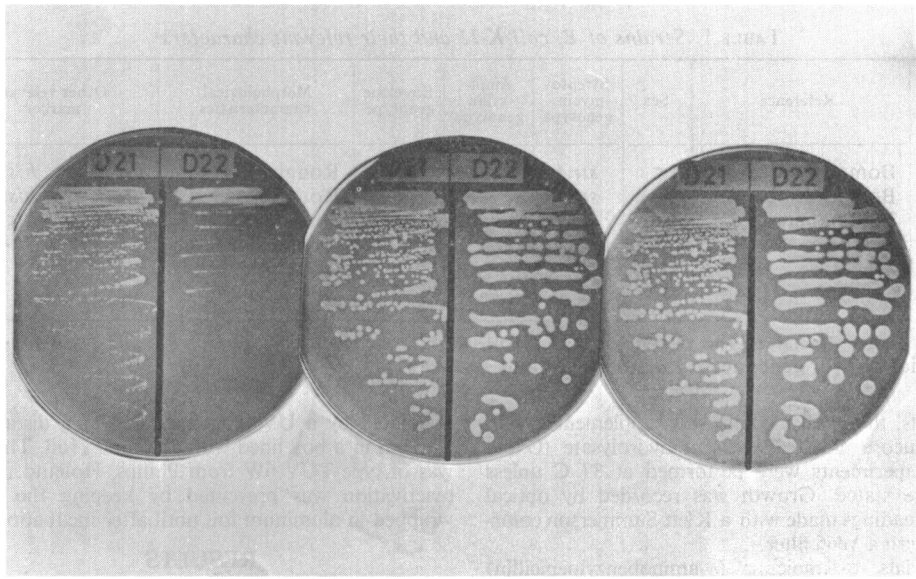


FIG. 1. *Streaks of D21 and D22 (with envA) on a plate with minimal medium. Photographs were taken after 1 day (left), 2 days (middle), and 3 days (right) of incubation at 30 C.*

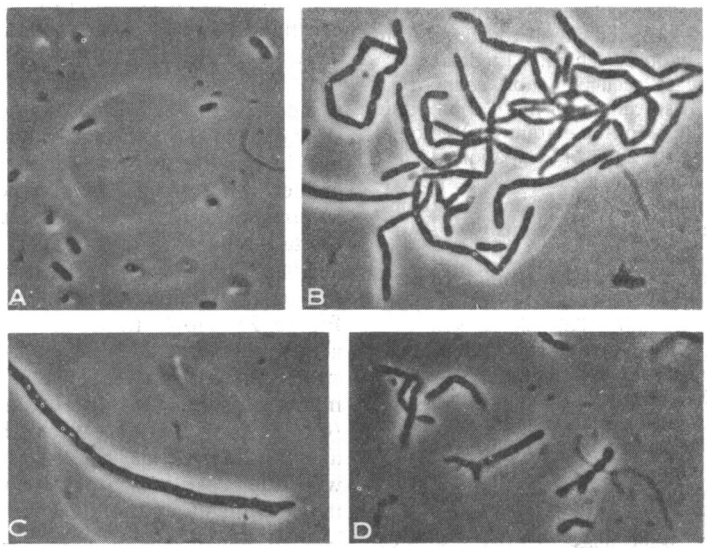


FIG. 2. *Upper part, cells of the parent strain D21 (a) and the envA mutant D22 (b). Both strains were grown in the rich LB medium, and samples were taken toward the end of the exponential growth phase. The lower part shows a filament (c) and irregular cells (d) of D22 obtained after prolonged exponential growth. Phase-contrast microscopy.* × *1,100.*

We also compared the effect of ampicillin in liquid cultures of the same three strains. Under these conditions, D11 was the most sensitive (Fig. 4). At ampicillin concentrations of 10 μg/ml, D11 started to lyse after 45 min; however, with the *ampA*-carrying strain D21, no effect was recorded. The growth curve for D22 (*ampA*, *envA*) showed first a decrease and then an increase in cell mass. This type of curve is often obtained with penicillinase-producing organisms and indicates arrest of the initial course of lysis when enough ampicillin has been hydrolyzed.

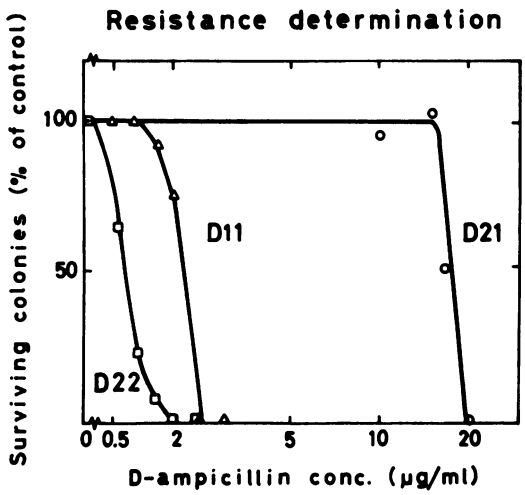


FIG. 3. *Resistance determination by single-cell colony formation on plates with different concentrations of D-ampicillin.*

We therefore compared the penicillinase activity of buffer-suspended cells of D21 and D22 pregrown in different media. No significant difference between the two strains was found, and we conclude that strain D22 still contains the penicillinase known to be associated with the *ampA* gene (11). Significant differences between resistance in liquid cultures and on plates have previously been observed (5). In this case, we believe that the difference is related to abnormalities in the viable count.

The lower part of Fig. 4 shows the relative increase or decrease in viable count obtained with an ampicillin concentration of 10 μg/ml. Significant killing was obtained only with D11, whereas the two other strains survived this antibiotic level.

It was recently demonstrated that the *ampB* mutation, which on solid medium enhances both chromosomal and episomal penicillin resistance, also changed the resistance to chloramphenicol, kanamycin, novobiocin, and cycloserine (16). We studied the resistance pattern of D22 (*ampA*, *envA*) and its parent strain D21 (*ampA*) with and without the presence of an R factor. The antibiotics used were selected on the basis of their known interference with either cell wall formation or protein or deoxyribonucleic acid (DNA) synthesis. The *envA* mutation decreased the resistance to all antibiotics tested except cycloserine (Table 2), resistance to which was unaffected or slightly increased, as was the case for strain D22-R1. Table 2 shows that *envA* affected both the resistance mediated by two chromosomal genes for resistance to ampicillin

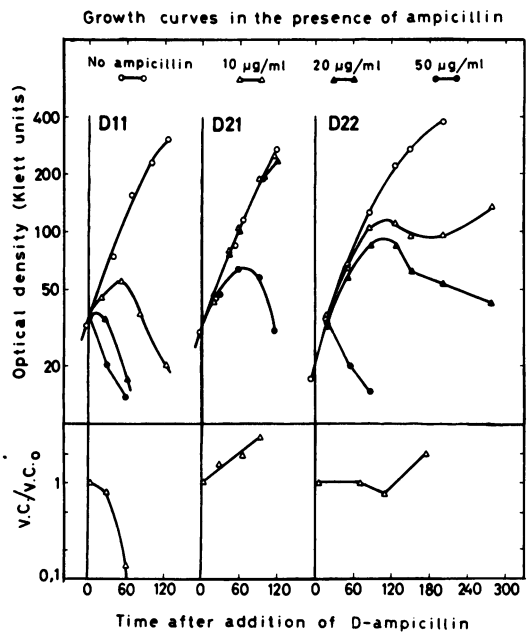


FIG. 4. *Effect of ampicillin on liquid cultures of D11 (wild type), D21 (ampA), and D22 (ampA, envA). The cells were growing exponentially in a rich medium when ampicillin was added at zero-time. Cell mass was followed by optical density readings with a Klett photometer (upper part); viability is given as viable counts at a given time (V.C.) over viable counts at zero-time ($V.C._0$).*

TABLE 2. *Effect of envA on resistance to different antibiotics*

| Antibiotic tested | Resistance[a] on plates (μg/ml) | | | |
|---|---|---|---|---|
| | D21 (+)[b] | D22 (*envA*)[b] | D21-R1 (+)[b] | D22-R1 (*envA*) |
| D-Ampicillin | 18 | 0.5 | 100 | 6 |
| Penicillin G | 200 | 5 | 200 | 40 |
| D-Cycloserine | 20 | 20 | 15 | 25 |
| Novobiocin | 60 | 10 | | |
| Chloramphenicol | 2.0 | 0.2 | 300 | 50 |
| Kanamycin | 1 | 0.6 | 1,000 | 200 |
| Streptomycin | 1,500 | 500 | | |
| Nalidixic acid | 3 | 0.5 | 3 | 0.5 |

[a] Resistance was determined by single-cell-colony formation, and the results are given as the concentration at which 100% growth was obtained. The resistance factor R1 (14) mediates resistance to ampicillin, chloramphenicol, kanamycin, and sulfonamide. All four strains carry the chromosomal *ampA* and *strA* genes for ampicillin and streptomycin resistance, respectively.

[b] Symbols + and *envA* indicate genotypes.

(*ampA*) and streptomycin (*strA*) and the R factor-mediated resistance to ampicillin, chloramphenicol, and kanamycin.

**Genetic mapping of the envA locus.** We performed a number of interrupted-mating experiments with HfrH and P4x, two donor strains with reverse orders of injecting the chromosome. From the known history of the strains and the available data, D22 could be expected to contain the *ampA* gene at 82 min (4, 7) and in addition a new gene, *envA*, which would suppress the resistance mediated by *ampA*. In a cross between a wild-type, sensitive donor and D22 (*ampA*, *envA*), selection for the Amp-10 phenotype (mediated by *ampA*) should therefore reflect the entrance of the *envA*$^+$ gene. If the changes in colony morphology and the growh rate (*see* Fig. 1) were linked to ampicillin sensitivity, then for the same reasons it should also be possible to map *envA* by scoring only for rough colonies and fast growth rate on plates with rich medium. That both of these assumptions were correct is indicated from the results obtained with HfrH (Fig. 5) and with P4x (Fig. 6). In all four experiments the entrance of *proB*$^+$ was used as a time reference.

In the experiment shown at the right of Fig. 5, 100 rough and fast-growing colonies were picked and tested for resistance. All were found to have the *ampA* type of resistance. In the experiments shown at the right of Fig. 6, the number of rough colonies on the *proB*$^+$ plates were counted. Their

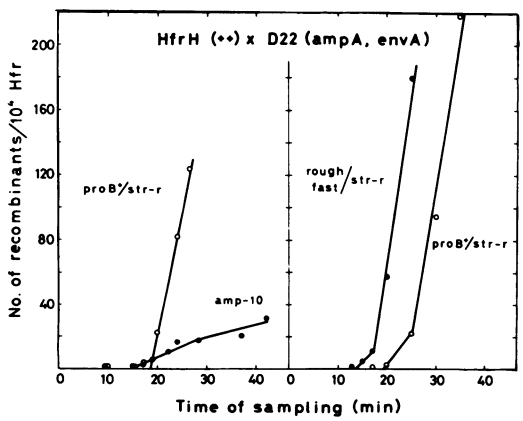


FIG. 5. *Two separate interrupted mating experiments in which Hfr Hayes was crossed to D22. Since envA decreases the penicillin resistance mediated by ampA, it was possible to select for envA*$^+$ *only by ampicillin resistance without any counter-selection (left). Since envA gives slow growth and smooth colonies, it was also possible to select for rough and fast-growing colonies by using streptomycin resistance as counter-selection (right).*

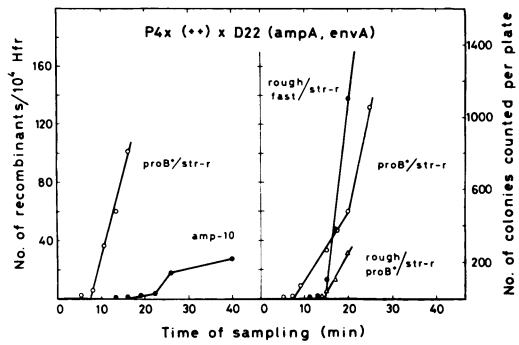


FIG. 6. *Two separate interrupted mating experiments using P4x as donor and D22 as recipient. Selection conditions as in Fig. 5, except that, in addition, rough and proB*$^+$ *streptomycin-resistant recombinants were scored for.*

appearance is plotted in Fig. 6 (triangles) and extrapolates to the same entrance time as found for the fast-growing colonies. In the same experiment, 50 smooth and 50 rough *proB*$^+$ recombinants were picked and tested for ampicillin resistance. All of the smooth recombinants were found to be sensitive, whereas all of the rough type showed the resistance phenotype of *ampA*.

The differences between the entrance of *envA* and *proB* varied from 3 to 7 min. However, since selection pressure with ampicillin clearly decreased the number of recombinants, it is reasonable to assume that scoring for rough colonies and fast growth gave better data. These experiments indicate that the *envA* gene is located at 6 to 7 min from *proB*, that is, at 2 to 4 min, using the time scale of Taylor and Trotter (21). This position, the relevant genes, and the injection orders of the Hfr strains are shown in Fig. 7.

**Chain formation in D22 during normal growth and in shift experiments.** A disturbance in cell division can be expected to give rise to decreased viable counts. In particular, the relation between cell mass, followed by optical density and viable count, should be drastically affected. We therefore compared strains D21 and D22 during a normal growth experiment in a rich medium. The inoculum was 1% of the volume of a full-grown culture, and optical density, microscopic cell counts, and viable counts were followed for 218 min of growth. During the entire experiment, the number of colony formers was considerably lower in the *envA* mutants D22 than in the parent strain D21 (Fig. 8). A quantitative interpretation of the viable counts indicates that the length of the chains in the beginning was close to 100 cells, while at the end of the experiment it had decreased to less than 10. However, the cell

count showed that the initial difference between D21 and D22 was only about 10-fold; in the end, it was reduced to 2-fold. This discrepancy would indicate that 80 to 90% of the D22 cells were killed when they were diluted and transferred from the liquid to the solid medium.

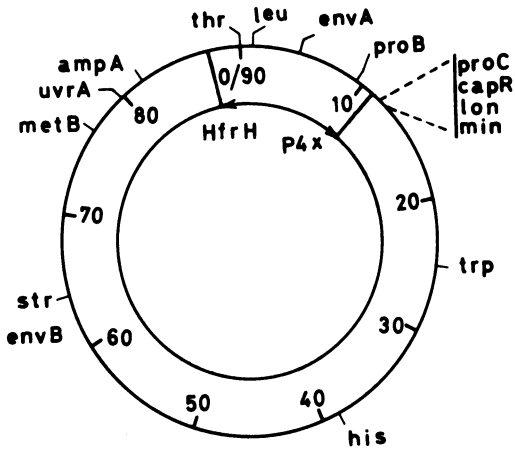


FIG. 7. *Circular chromosome of E. coli K-12 with envA, envB, and selected markers relevant for cell envelope effects. Arrows indicate order of gene transfer of the Hfr strains used.*

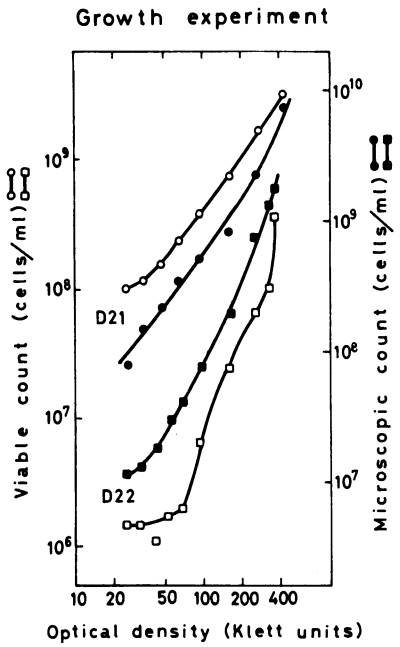


FIG. 8. *Comparable growth experiments in rich LB medium with strain D21 (parent strain) and D22 (envA mutant). Growth was followed by optical density, viable cell count, and microscopic cell count.*

Transitions between different steady states of growth are known to induce changes in the proportions of the fundamental components of the bacterial cell (12, 19). A change from a minimal medium to a rich medium (shift up) produces an immediate rise in the slope of the optical density curve due to increasing cell size. On the other hand, for viable counts, there is a transition period before the new steady-state rate of growth is achieved. A change from a rich medium to a minimal medium (shift down) causes adjustment to a smaller cell size (12). When these types of experiments were applied to D21 and D22, there was a clear difference between the strains (Fig. 9 and 10). Whereas D21 behaved normally in both types of shift, a shift up with D22 first produced a 10-fold decrease in viable count, followed after 1 hr by a very rapid increase in the number of viable cells.

In shift-down experiments with D22 (*see* Fig.

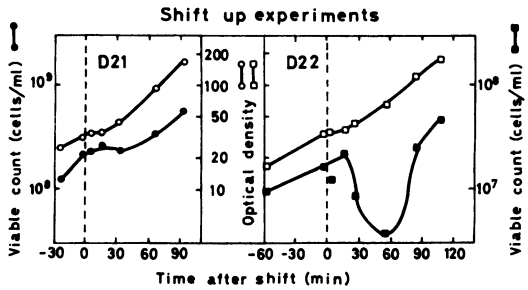


FIG. 9. *Shift-up experiments with D21 and D22 (envA). Before zero-time, the cells were grown in minimal medium with glucose as carbon source. Shift was performed by filtration and washing on the filter. The filter was then transferred into prewarmed rich LB medium.*

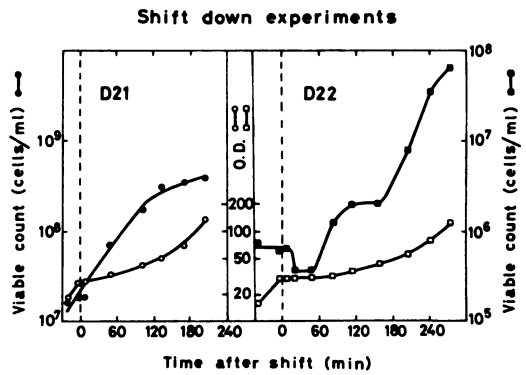


FIG. 10. *Shift-down experiments with strains D21 and D22 (envA). The media and the shift technique were the same as in Fig. 9. Before zero-time, the rich medium was used; after zero-time, minimal medium with glucose as carbon source was used.*

10), a viable count curve was obtained which indicates that synchronous division was induced. The data in Fig. 9 and 10 can be interpreted as due to differences in cell size between D21 and D22. A shift up tends to increase this difference, that is, to increase chain length in D22, while a shift down clearly reduces the difference between the two strains. When a full-grown culture of D22 was used as inoculum, the first growth phase was characterized by an increase in optical density and constant viable count (Fig. 8 and 10). This result was interpreted as being due to chain elongation, which was confirmed by microscopic examination.

**UV irradiation experiments.** UV irradiation of *E. coli* B and some mutants of K-12 will induce a lethal type of filament formation (3). Moreover, the experiment described in the previous section (*see* Fig. 8) indicated that most cells of D22 were killed when they were plated. It was therefore expected that UV irradiation on plates would enhance the killing of D22 (*envA*) and make the strain more UV-sensitive than the parent strain D21. However, the effect of UV irradiation was the reverse (Fig. 11). Up to 40-sec irradiation of D22 did not cause any significant decrease in viable counts, whereas the same treatment killed nearly 80% of the parent strain D21.

Tryptophan starvation in an otherwise rich medium stops DNA synthesis after allowing the cell to complete one round of the circular chromosome (12). Figure 11 also shows two experiments of this type. In the *envA* mutant, D22, prestarvation for tryptophan produced an almost threefold increase in viable counts, and UV irradiation enhanced this increase to a factor of 10. Both in D22 and in the parent strain, D21, prestarvation for tryptophan protected against UV irradiation.

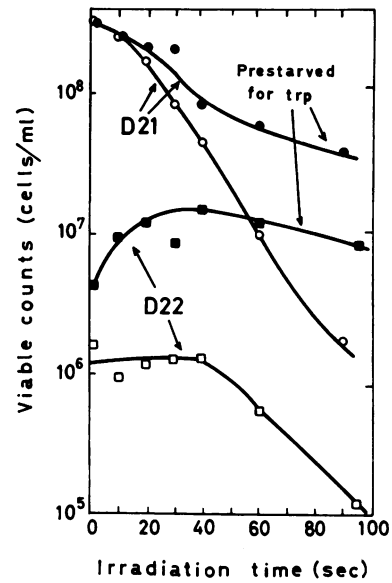


FIG. 11. *Survival curves for D21 (parent strain) and D22 (envA mutant) after exposure to UV irradiation on plates. Both strains require tryptophan. Before irradiation, the cells were either pregrown in a rich LB medium (open symbols) or 90-min prestarved for tryptophan (trp) in an otherwise rich medium containing 0.2% of casein hydrolysate (filled symbols).*

## DISCUSSION

**Nature of the deficiency in strain D22.** It is known that for *E. coli* strain B and certain mutants of strain K-12, mild exposure to agents which disturb DNA synthesis renders the cell unable to divide. Instead, filaments are formed that die after reaching a certain critical length. The interpretation generally given to this phenomenon is that the strains have an imbalance between DNA synthesis and either membrane or cell wall biosynthesis (3, 22). Despite the fact that D22 can form filaments easily, it is clear that the strain is different from the mutants previously described. Strain D22 forms chains during ordinary growth, whereas the *fil*, *lon*, and *uvs* mutants normally have to be induced to form filaments (3). D22 is morphologically different from the two mutants described by Adler et al. (1, 2), one of which seems to resemble our *envB* mutant (*unpublished results*). The *envA* and *envB* mutants have recently been compared to the parent strain by using electron microscopy (*unpublished results*). The morphology suggests that the *envA* gene may in some way be concerned with the association between the inner and outer layers of the wall.

The physiological data presented in this paper seem to be consistent with the hypothesis that strain D22 has normal DNA synthesis but carries a defect in cell envelope formation, which creates an imbalance affecting cell division. Under conditions that are known to decrease or stop DNA synthesis in normal cells, we found an increase in the number of viable cells of D22, as would be expected if the imbalance had been counteracted. Such conditions were used in the UV irradiation and tryptophan starvation experiments shown in Fig. 11. However, our killing curves with UV irradiation of D22 differ in shape from those recently obtained by Kantor and Deering (10) and Donch and Greenberg (6) with *uvs*-carrying strains.

DNA synthesis is known to decrease in relation to septum formation in a shift-down experiment (*see* Fig. 10) and when the cells leave the exponential growth phase toward the end of a

growth experiment (*see* Fig. 8). Chain formation presumably involves a situation in which a number of septa have been blocked at nearly the same stage in their formation. If in a shift-down experiment the imbalance were relieved temporarily, these septa would be completed within a short time, giving the synchronous growth observed in Fig. 10.

We also tried some conditions which, according to our hypothesis, would make the imbalance more severe. Shift-up experiments are known to produce an increase in DNA synthesis which, after only 40 to 50 min, is followed by an increased rate of cell division (12). Figure 9 shows that during this period there is a considerable decrease in the viable count of D22. Microscopic examination has shown that during prolonged exponential growth in liquid cultures the chains of D22 are transformed into filaments and elongated irregular structures (Fig. 2c and d). It seems reasonable that the long exponential-growth phase which follows the dilution and plating of a D22 chain on a rich medium creates the same type of imbalance observed in liquid cultures and therefore gives a high percentage of killing. Suit et al. (20) observed similar types of misformed cells during thymine starvation and with mitomycin C treatment of *E. coli* C.

Plating on penicillin, which inhibits a late step in cell wall formation (9, 23), would be expected to increase the imbalance created by ordinary plating. This explains why the *envA* mutant D22 is highly sensitive to ampicillin when tested on plates. Since the cell wall of gram-negative bacteria contains many proteins and since chloramphenicol inhibits protein formation, it is possible to explain the increased sensitivity to chloramphenicol as being due to an additional imbalance between DNA and cell wall formation. This interpretation is consistent with the morphological observations of Morgan et al. (15).

**Mapping of the envA locus.** An anomaly in viable count could be expected to constitute a serious hindrance to genetic mapping. However, the fact that we have selected for the entrance of the wild-type gene rather than the mutated *envA* locus would seem to eliminate this as a source of error. However, among the $proB^+$ recombinants, there will be both *envA* and $envA^+$. The low survival of the first group can explain why, in the experiment in which $proB^+$ was injected before $envA^+$ (Fig. 6), the slope for the $proB^+$ recombinants was low in the beginning and then rose after the entrance of $envA^+$. Since Hfr strains with the reverse order of injection of the chromosome (Fig. 7) gave results in good agreement, we conclude that the *envA* locus maps at 2 to 4 min, using the time scale of Taylor and Trotter (21).

The *envA*-containing strain D22 was isolated as a smooth colony from a rough parent strain (Fig. 1). Reeve (18) recently observed a correlation between smooth colony morphology and resistance to chloramphenicol and tetracycline. The mapping position of *envA* clearly shows that the mutation must be different from the *lon* mutants (8) as well as the *capR* mutants analyzed by Markovitz et al. (13). *CapR*, *lon*, and *min* are all linked to *proC* at 10 min (*see* Fig. 7 and references 6, 13, and 21). Whether the capsule produced by D22 is similar to or different from that of *capR* mutants is being investigated.

**Episomal and chromosomal resistance.** We previously described another gene, *ampB*, which has the property to enhance both chromosomal and episomal ampicillin resistance (16). In addition to the effect on ampicillin resistance, it also affects chloramphenicol resistance and several properties related to the bacterial surface (Nordström and Burman, *in preparation*). It is interesting that *envA* in several cases decreases the antibiotic resistances which are enhanced by *ampB*. Like *ampB*, *envA* acts on both chromosomal and episomal genes for antibiotic resistance. The reason for these effects may be related to the function of the original wild-type alleles which, after mutations, have become resistance genes.

ACKNOWLEDGMENTS

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