THORNLEY, M. J. & YUDKIN, J. (1959). *J. gen. Microbiol.* **20**, 365–372

The Origin of Bacterial Resistance to Proflavine

2. Spontaneous Mutation to Proflavine Resistance in *Escherichia coli*

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SUMMARY: Experiments were performed to demonstrate the production in *Escherichia coli* of spontaneously occurring mutants resistant to proflavine. The fluctuation test of Luria & Delbrück (1943) suggested that such mutants arise. This was confirmed by a modification of the technique of Lederberg & Lederberg (1952). By this method of indirect selection by replica plating, two separate first-step mutants were isolated; from one of these was isolated a second-step mutant, and from this a third-step mutant. The resistance of the third-step mutant was about 100 times greater than that of the original sensitive strain. By the same technique, two first-step mutants to chloramphenicol resistance were isolated. None of the 6 mutants had come into contact with either drug until the time of testing. Cross-resistance between proflavine and chloramphenicol was shown by 5 of the 6 mutants; the sixth showed an increase in proflavine resistance though not in resistance to chloramphenicol.

We have used two methods to demonstrate that mutants to proflavine resistance may arise spontaneously in cultures of *Escherichia coli*. The first was the fluctuation method of Luria & Delbrück (1943). This test, however, has been the subject of justifiable criticism (Eriksen, 1949; Dean & Hinshelwood, 1952). In particular, it has been pointed out that minor differences in environment can account for apparent fluctuations in numbers of resistant organisms. Our own results showed rather small fluctuations which might possibly have been accounted for in this way. We have therefore also used the more conclusive indirect selection method of Lederberg & Lederberg (1952).

METHODS

Organism. In all the experiments described in this paper we used strain 36 derived by us from *Escherichia coli* NCTC 8196. It was originally grown by us from a single colony, and had been transferred through a large number of broth cultures before the experiments began.

Media. The media and cultural conditions were the same as those described in the previous paper (Thornley & Yudkin, 1959).

Indirect selection by replica plating. Our method was a slight modification of that of Lederberg & Lederberg (1952). A nutrient agar plate was inoculated with 10^6 – 10^7 organisms of a sensitive strain and incubated at 37°. This formed the master plate for the first set of replicates. The incubation period was of importance; in early experiments 24 hr. was used, but later a 5 hr.

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incubation period was found to give better results (see Discussion). A square of velveteen, sterilized by autoclaving, was mounted with the pile upwards on a rubber bung 7 cm. in diameter, by means of a rubber band. The velvet had a triangle as a marker stitched in the centre. The master plate was pressed on the velvet pad, so that some of the bacteria were transferred to corresponding positions on the velvet, the position of the marker being traced on the back of the plate with grease pencil. A series of plates with increasing concentrations of proflavine was pressed in turn on the velvet pad, and the position of the marker traced on each. These drug plates were incubated for 24 hr. and, in the meantime, the master plate was kept at 2°. Colonies growing on the drug plates could be traced back to the corresponding area of the master plate by the system of markers. Where colonies grew at the same position on two or more proflavine plates of different concentrations, the area on the master plate was presumed to contain a microcolony of resistant organisms. An inoculum was taken from this point with the tip of a straight 'Nichrome' wire inoculating needle, and transferred to a new master plate for the second set of replicas. This inoculum was spread over this master plate by a bent glass rod. In the earlier experiments, an intermediate subculture was made from the inoculum into broth, and the new master plate was inoculated from this after overnight incubation.

The second set of replicas was carried out in exactly the same way as the first, including (in later experiments) the 5 hr. incubation period for the master plate. By means of these 2 cycles of replication, the proportion of resistant organisms was considerably increased, and therefore the master plate for the third set of replicas was usually inoculated in a different way. This was by streaking with the wire needle in a criss-cross pattern, giving fine lines of growth after 5 hr. of incubation. This modification, suggested by Dr Jehudith Sinai, enabled resistant microcolonies to be located more accurately. Under a low-power binocular microscope, a very small inoculum was picked from the appropriate position on the streak on the third master plate with a sharpened steel needle, and placed on the next master plate where it was spread over the surface by a bent glass rod. It was essential that the amount transferred should be sufficiently small to give not more than about 100 widely spaced colonies. This master plate (4th set) was incubated for 5 hr., the replicas on drug agar were made, and the master plate was re-incubated overnight. The final step in the process was to pick off a resistant colony from this master plate. As the colonies were well separated, the incubation could be prolonged to 24 hr. before picking off, without any loss of resolution, and with the obvious advantage that colonies were clearly visible, whereas 5 hr. microcolonies were very difficult to locate when widely scattered. It was still desirable, however, to use only 5 hr. of incubation before making the replicas (see Discussion).

Cultures of the resistant strain finally obtained were plated out and picked from discrete colonies twice, in order to make reasonably sure that they were in fact pure strains. From the final discrete colony, inoculation was made on to several slopes, and these formed the culture of each strain.

Assessment of resistance. The same three methods were used as in the pre-

ceding paper. These were dilution counts, counting on spread agar plates, and measuring the length of the streak on gradient plates. Dilution counts could not be used for the more resistant strains, since concentrations of proflavine greater than 100 $\mu\text{g./ml.}$ reacted with the peptone solution, which became turbid. This reaction did not occur in the solid medium at concentrations of proflavine up to 1000 $\mu\text{g./ml.}$ We have therefore confined the results we present to those derived either from spread plates or from gradient plates.

RESULTS

Fluctuation tests

Two separate experiments were carried out, exactly according to the method of Luria & Delbrück (1948). Calculations from the results showed in each instance a variance in numbers of resistant organisms in the separate cultures which was significantly greater than that expected by random distribution ($P < 0.1$). Nevertheless, the variance was not large and we felt it might possibly have been due to environmental factors.

Indirect selection by replica plating

Isolation of proflavine-resistant strains. The proflavine-resistant mutants obtained by replica plating were named M1 to M5 in the order of their isolation. The method of isolation is summarized in Table 1.

Table 1. *Summary of replica-plating experiments for isolation of proflavine-resistant mutants of Escherichia coli*

Selection	No. of cycles of replication and type of inoculum*	Incubation period of master-plate (hr.)	Subcultures from master-plate	Proflavine conc. used ($\mu\text{g./ml.}$)
S36 $\longrightarrow$ M1	4 (C, C, S, S)	24	Broth or slopes	5-50
S36 $\longrightarrow$ M2, M3	7 (C, C, X, S, X, X, S)	24 (5 for last)	Direct†	10, 20, 50
M2 $\longrightarrow$ M4	4 (C, C, X, S)	5	Direct (broth for last)	100, 150
M4 $\longrightarrow$ M5	4 (C, C, X, S)	5	Direct	400, 500

* C = crowded plate, 10^6 - 10^7 organisms; X = criss-cross streaks; S = single colony.

† Direct on to next master plate for subsequent replica plating.

M1. This was derived from sensitive strain 36 by a series of 4 replica plating operations, with plates containing 5-50 $\mu\text{g.}$ proflavine/ml., and with an intermediate subculture in broth between sets of replica plating. It was regarded as a first-step mutant, and repeated attempts were made to isolate a second-step mutant from it. They were all unsuccessful, although more resistant strains could be easily obtained by direct selection from proflavine plates.

M2. Another first-step mutant was obtained from strain 36, 7 sets of replicas being necessary on plates containing proflavine 10, 20 and 50 $\mu\text{g./ml.}$ From the final set, two colonies M2 and M3 were picked off, which originally had different colony forms. They were found to have exactly the same resistance, and the difference in colony form disappeared after a few subcultures. We therefore retained only M2 for further work.

M4. A master plate was made from M2. Concentrations of proflavine 100–150 $\mu\text{g./ml.}$ were used for making the replicas. Four replications yielded a pure culture of strain M4.

M5. Here M4 was used to make the master plate, and the drug plates contained proflavine 400 and 500 $\mu\text{g./ml.}$ Four sets of replicas were needed to give a pure culture of strain M5. Two attempts to obtain a yet more resistant strain from M5 were unsuccessful.

Isolation of chloramphenicol-resistant mutants. From the sensitive strain 36, 2 first-step mutants were isolated, L1 and L2. L2 was slightly more resistant than L1. The concentrations of chloramphenicol used for the replica plates were 2, 3 and 4 $\mu\text{g./ml.}$

The resistance of first-step mutants

Both M1 and M2 were included in this category, since the chance of picking up a double mutant during replica plating is remote. Strain M2 corresponded in resistance to most of the strains previously obtained from strain 36 plated on proflavine-containing agar (see Thornley & Yudkin, 1959). The pattern observed here differed from that described by Cavalli & Maccacaro (1952) for chloramphenicol. These authors found first-step mutants which had many different degrees of resistance. The resistance shown by M1, on the other hand, was only once encountered amongst a large number of strains picked from colonies on proflavine agar. Thus, the concentration of proflavine which decreased the count to 1 in 1000 was 55 $\mu\text{g./ml.}$ for strain M1, and 82 $\mu\text{g./ml.}$ for strain M2. It was thought probable that this unusual strain was isolated in our earlier attempts at replica plating because of its exceptionally good growth in competition with other strains. When mixtures of two strains were subcultured 14 times in broth, M1 held its own with its parent strain 36 and overgrew a more resistant strain obtained by 'training'. At this stage, we had not isolated other first-step mutants by replica plating, so that we did not test the relative growth of M1 against these. If, however, as seems likely from these earlier results, M1 was unusually vigorous, the intermediate broth culture which we were then using between sets of replicas could well have resulted in overgrowth of second-step mutants by this strain. This would account for our failure to isolate second-step mutants from M1.

Step-wise mutation to proflavine resistance

The strains M2, M4 and M5 were regarded as a series representing first-, second- and third-step mutants to proflavine resistance. Resistance to different drug concentrations was determined by spread plate counts for the sensitive strain and for each of the mutant strains (Figs. 1 and 2; Table 2).

Fig. 1. Resistance of sensitive *Escherichia coli* 36 and of mutants. The curves represent counts on spread plates containing varying amounts of proflavine. Each curve represents one determination of resistance; the determination was repeated on different occasions. 36 = sensitive organisms; M2 = first-step mutant, from 36; M4 = second-step mutant, from M2; M5 = third-step mutant, from M4.

Fig. 2. Resistance of first-step mutant M1, from sensitive *Escherichia coli* 36.

Table 2. Resistance of sensitive and mutant strains of *Escherichia coli* 36
(Proflavine concentration giving a survival of 10^{-2} and 10^{-3} of the original organisms.
Calculated by interpolation from Fig. 1.)

Survival	Strain 36	Strain M2	Strain M4	Strain M5
	Proflavine concentration, log $\mu\text{g./ml.}$			
10^{-2}	0.602 ± 0.031	1.873 ± 0.040	2.330 ± 0.028	2.532 ± 0.028
10^{-3}	0.665 ± 0.023	1.917 ± 0.032	2.416 ± 0.025	2.655 ± 0.028

The exact resistance of each strain and the exact differences between strains varied slightly according to the method of measurement. If we take the criterion of resistance as the concentration of drug which decreases the number of colonies to 1 in 1000 on spread plates, then the relative resistance of the strains may be expressed thus:

$$36 \xrightarrow{\times 18} M2 \xrightarrow{\times 3.2} M4 \xrightarrow{\times 1.8} M5.$$

The strain of highest resistance, M5, is approximately 100 times more resistant than sensitive strain 36. If we use the criterion of the diminution in colony count to 1 in 100, the increase between strains 36 and M5 is 85 times.

First-step mutants to chloramphenicol resistance

The resistance of the two chloramphenicol-resistant mutant strains L1 and L2 was compared with that of the sensitive strain 36 by growth on plates with increasing concentrations of chloramphenicol, and by gradient plates. The factor of increased chloramphenicol resistance was 7 for strain L1 and 10 for strain L2. Spread plate counts with strains 36 and L1 also gave a factor of about 7 for the ratio of chloramphenicol concentrations needed to reduce the colony count by 99%.

Cross resistance of mutants to proflavine and chloramphenicol

The 4 mutants isolated for resistance to proflavine, and the 2 for resistance to chloramphenicol, were each tested on gradient plates containing the other drug. Table 3 shows the increase in resistance between successive mutants, both to proflavine and to chloramphenicol, the number indicating the factor by which the resistance increases.

Table 3. *Cross-resistance of mutants to proflavine and chloramphenicol*

36 = original sensitive strain *Escherichia coli* 36.

Increase in resistance from one step to the next shown thus: P $\times$ 8, i.e. proflavine resistance increased eightfold; C $\times$ 10, i.e. chloramphenicol resistance increased tenfold.

DISCUSSION

The final procedure which we adopted for indirect selection by replica plating was found to be most effective and enabled a series of 4 or 5 replica platings to be carried out in 1 week. The omission of intermediate culture on broth was intended to eliminate the period of competitive growth between sensitive and mutant organisms. It is well known that mutants often tend to grow more slowly than the wild type in the absence of drug. Strain M1 was unusual in being able to persist through many subcultures in the presence of the sensitive strain 36. It is noteworthy that this strain M1 was the only one isolated in the early stages of this work, when broth subcultures were being used. A period of incubation of the master plate, shorter than the usual 18 or 24 hr., was suggested by Lederberg as one of the possible variations of the technique, and has been used by several other workers (e.g. Sneath, 1955). It has the obvious advantage of better resolution on the replica plates, since the very small

colonies will not spread sideways. Also, in the early stages of the process, where a large inoculum is used, there is less chance of resistant colonies being covered over by surrounding sensitive organisms during the growth period. There is another advantage in the final stages of the enrichment process, when a master plate with isolated colonies is used. If such a colony is developing from a sensitive organism, and a mutation to resistance occurs during its growth, the final colony will consist of a mixture of sensitive and resistant organisms. It will therefore give colonies on the replica plates. Nevertheless, on subcultures from the master plate, predominantly sensitive organisms will be found if they have a growth advantage over the mutants. The use of a period of 5 hr. for incubation of the master plate, with a maximum colony size of about 1000 organisms, very much decreases the chance of mutations occurring during colony growth. That is, the colony which gives replicas on the drug plate is more likely to have arisen from a single mutant organism.

In practice, results which could be explained by this process were encountered during the isolation of M2 from 36. In one of the later sets of replicas, 3 master plates were inoculated, each with about 200 organisms, incubated for 24 hr. and replicated. Seven colonies on the master plates were thought to be resistant judging by the colonies on the drug plates, and inocula were picked from these seven positions, in some cases in duplicate, giving a total of 12 subcultures. All proved to consist mainly of sensitive organisms, some with a small proportion of resistant ones. Since using the 5 hr.-incubation period, we have always found that colonies corresponding in position to those on drug plates consisted only of resistant organisms. Lam & Sevag (1955) do not seem to have considered this possibility. They picked streptomycin-resistant colonies from the drug plates of a replica series, and the colonies in corresponding positions on the master plate, and they found a much higher resistance in the colonies obtained from the drug plates. This is exactly as would be expected if a mutation had occurred during colony growth on the master plate. These authors, however, interpreted their results as evidence that contact with the drug was necessary to develop resistance.

It seems certain from our results that proflavine resistance in *Escherichia coli* may be acquired by a series of mutational steps, since increased resistance acquired in the complete absence of drug may be demonstrated by replica-plating. The same is true of the acquisition of at least a low degree of chloramphenicol resistance.

Of the 6 sets of mutants studied, 5 had increased resistance both to chloramphenicol and to proflavine. The sixth (from M2 to M4) showed an increase in proflavine resistance only. It would thus seem that the property of proflavine resistance may result from at least two different mechanisms of resistance. One would appear to affect resistance to both drugs, and the other resistance to proflavine only. This situation is similar to that observed by Cavalli (1952) for cross-resistance to chloramphenicol and terramycin.

We are grateful to the Medical Research Council for a personal grant to one of us (M.J.T.).

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(Received 21 October 1958)