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Selection of Penicillin-sensitive Mutants of *Escherichia coli* following Ultraviolet Irradiation

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SUMMARY: By taking advantage of the long lag period before minimal therapeutic concentrations of penicillin kill bacteria, five mutants of the K12 strain of *Escherichia coli* (*Bacterium coli*) have been isolated following ultraviolet irradiation, which were 10-50 times more sensitive to penicillin than K12. This supports the theory that development of antibiotic-resistant forms is by mutation and selection.

It appears from the work of Demerec (1945, 1948) that in any given population of penicillin-sensitive organisms there are a few more resistant ones and these are believed to arise by spontaneous mutation. There would seem to be no strong reason why the same population of bacteria should not contain a few organisms more sensitive than the rest; the main difficulty in demonstrating this is one of selecting a minute proportion of sensitive ones from a mass of more resistant bacteria.

Many people have shown that strains of bacteria with an induced resistance to penicillin will sometimes become more sensitive simply by repeated subculture in the absence of the drug (Todd, Turner & Drew, 1945; Chain & Duthie, 1945). Very little work has been done on these lines with organisms which are naturally resistant to penicillin, even before contact with the drug.

One case where a naturally resistant organism has become somewhat more sensitive is that reported by Plough & Grimm (1949); in the course of testing auxotrophic mutants derived from *Salmonella typhimurium*, by ultraviolet irradiation they isolated a cysteine-requiring mutant which was 4-16 times more sensitive to penicillin than the original, depending on the medium used for testing. Recently Voureka (1951) has reported the production of variants from *Escherichia coli* (*Bacterium coli*) by growth in presence of antiserum and chloramphenicol; one of these, variant β , was almost 10 times more sensitive to penicillin than the original.

A consideration of the early compiled tables of penicillin sensitivities of various organisms is striking for the very wide variations in sensitivity within many species. Thus, strains of *Esch. coli* vary in sensitivity from 15 to 5000 units per ml. (u./ml.) (Florey *et al.* 1949). It seems likely that these differences have not been induced by previous contact with the drug (as is the case with most resistant staphylococci), at any rate, these strains do not change in penicillin sensitivity merely on repeated subculture in drug-free media. It seems quite probable that these different strains have arisen in nature by

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mutations, and perhaps similar changes can be brought about in the laboratory by methods designed to increase the number of mutants, e.g., irradiation with ultraviolet light.

The peculiar bacteriostatic and bactericidal effects of penicillin are such that selection of any more sensitive forms appeared possible. Parker & Marsh (1946) have shown that the viability of staphylococci after exposure to penicillin begins to decrease only after a lag period, which is longer for lower penicillin concentrations; if the penicillin is removed before the end of this lag period the organisms will begin to grow after a few hours' delay. This suggested the possibility of selecting sensitive variants by plating out on a medium containing as high a concentration of penicillin as will allow the wild type to grow freely; if the penicillin is then removed by addition of penicillinase, any more sensitive variants could begin to grow provided that the concentration of the drug to which they had been exposed was near to the minimum effective dose for those variants, and that the time of exposure had not been too long. By this means the wild type would have a start of several hours over any sensitive variants which would then be likely to appear as very small colonies.

The K12 strain of *Esch. coli* was chosen for the present work for the following reasons:

(a) Most of the work on bacterial genetics has been done with K12 and it has been shown to recombine (Lederberg, 1947).

(b) It will grow readily on inorganic salts plus glucose.

(c) Because of its minimal growth requirements there is the possibility that any metabolic differences in sensitive organisms would be more readily detected (Davis, 1950).

EXPERIMENTAL

The K12 strain of *Esch. coli* was used; this will grow on 60 u. penicillin/ml., but not on 90 u./ml. The size of inoculum has very little effect on the apparent sensitivity so that active penicillinase production can be ruled out. Cultures were grown on nutrient broth overnight; 3 ml. of such a culture were irradiated for 1 min. in an open Petri dish by ultraviolet light (this leaves approx. 10^{-4} of the organisms viable); dilutions of the irradiated culture were made in broth; usually 1/50 and 1/500. These dilutions were then incubated for 1 hr. to allow for delayed phenotypic expression (Davis, 1950), then, 0.2 ml. of each dilution was spread on to each of ten 6 in. nutrient agar plates containing 500 u. penicillin/ml. After 8 hr. the plates were removed from the incubator and sprayed with a solution containing 1000 u. penicillinase (Schenley Labs. Inc.)/ml.; after another 4 hr. or so, contact photographic prints were made of all the plates; by this time the K12 colonies had grown to a reasonable size. Incubation was then continued for a further 24 hr. making a total period of incubation of about 40 hr. At this time the plates were examined for secondary colonies by placing them in position on the photographic print and shining blue light through them from beneath; a yellow light shining from above enabled one to pick off any secondary colonies, which appeared yellowish and were usually

minute, in contrast to the others which were bluish. Usually plates with a total of 400–500 colonies yielded one or two secondary colonies. These secondary colonies were picked off, inoculated into small broth tubes and incubated for 6–8 hr. at which time they were streaked out on penicillin ditch plates containing 300 u. penicillin/ml. of agar in the ditch; a streak of the original K12 (8 hr. culture) was included for comparison.

Penicillin sensitivities of the isolated variants were further tested by tube assay in nutrient broth using bulk dilutions of penicillin of 50, 25, 12, 6, 3 and 1 u./ml., in a total of 2 ml. The tubes were read after 16 hr. Inocula of approximately 1000 organisms were used; with much larger inocula all strains appeared to have sensitivities approaching that of K12, presumably due to the early appearance of reverse mutants.

Minimal medium was that of Davies & Mangioli (1950).

RESULTS

From the first 140 secondary colonies only one (A) was significantly more sensitive than K12. This was then used as the stock strain for further irradiation instead of K12, the penicillin content of the plates being decreased to 10 u./ml., to allow normal growth of this organism. From 190 secondary colonies using A as stock, four more sensitive variants were isolated (2A, 3A,

Table 1. *Titration of variants in nutrient broth with inoculum of c. 1000 organisms/tube*

Organism	Penicillin concentrations in units/ml.						
	50	25	12	6	3	1	0
K12	++	+++	+++	+++	+++	+++	+++
K12	+++	+++	+++	+++	+++	+++	+++
A	—	—	+	+++	+++	+++	+++
A	—	—	—	++	+++	+++	+++
2A	—	—	—	+	+++	+++	+++
2A	—	—	—	+	+++	+++	+++
3A	—	—	—	+	+++	+++	+++
3A	—	—	—	+	+++	+++	+++
4A	—	—	—	—	+++	+++	+++
4A	—	—	—	—	+	+++	+++
5A	—	—	—	—	—	+	+++
5A	—	—	—	—	—	++	+++

+++ = good growth to — = no growth.

4A and 5A). The strain 4A was then used for stock and the penicillin concentration of the plates decreased to 2 u./ml. No more sensitive strains appeared from 105 secondary colonies, so the penicillin concentration was decreased to 0.5 u./ml., again without success; finally a concentration of 0.1 u./ml. was tried without producing any more sensitive strains than 4A. Altogether, 200 secondary colonies from 4A were tested.

Table 1 shows the sensitivity tests for these five variants by tube assay with minimal inocula.

The variants were tested against coli phages T1, T2, T3 and T4; like K12 they were all sensitive to T1, T2 and T4 and were not affected by T3.

All the variants have been subcultured at least 10 times and 4A has been subcultured over 20 times in broth without loss of sensitivity; at the end of the 20 subcultures 4A showed a fair proportion of more resistant colonies and had to be purified.

As a control to the experiments a K12 culture was diluted and plated out on penicillin agar without ultraviolet irradiation; secondary colonies did appear in about the same numbers as with the irradiated strain, but out of 150 secondary colonies tested none had any greater sensitivity than K12.

DISCUSSION

The great majority of the secondary colonies had the same sensitivity as the parent stock, and one must assume that they were derived from organisms which were in an unfavourable state for growth and required a long lag before commencing active growth. They may be analogous to the persisters of Bigger (1944). However, the minority of the secondary colonies were more sensitive variants and the numbers were roughly one from 2×10^4 surviving bacteria (or possibly 2×10^8 original bacteria). It will be recalled that Demerec (1945) arrived at a figure of 1 in 10^7 for spontaneous penicillin-resistant mutants in staphylococcus. Insufficient numbers of bacteria were examined to form a really good non-irradiated control, so that there is no clear proof that these sensitive strains are produced as a result of the ultraviolet irradiation. Nevertheless, in so far as it is extremely unlikely that any adaptation will go against the pressure selection gradient, these more sensitive strains would seem to be true mutants of K12.

It is interesting that in spite of the sensitivity having been increased by 10–15 times from K12, all of the mutants except 5A could grow on minimal medium. In the case of penicillin there is a general tendency for resistant bacteria to be more nutritionally independent than sensitive ones. Gale & Rodwell (1949) have trained sensitive staphylococci to grow with fewer amino-acids in the medium and in the course of this training the organisms became concurrently penicillin resistant, in spite of having had no contact with the drug. This seems analogous to the experiments of McIlwain (1943) in training *Corynebacterium diphtheriae* to grow without pantothenic acid when it became concurrently insensitive to pantooyltaurine, and one might argue by analogy that penicillin antagonizes or in some way interferes with the absorption of one or more amino-acids (Gale & Rodwell, 1949). It has, however, been pointed out that there are bacteria, notably *Bacillus subtilis*, which are extremely sensitive to penicillin even though they grow on completely inorganic media with glucose (Hunter & Baker, 1949).

During the course of this work I had the opportunity to test 161 temperature-sensitive primary mutants of K12 for penicillin sensitivity; all of the mutants would grow on minimal medium at 25° but required added nutrilites for growth at 37°. Eight of these primary mutants were more penicillin sensitive

than K12; Table 2 shows the growth requirements of these organisms (Leupold, U., personal communication).

Since these are primary mutants the increased sensitivity must be associated with the increased growth needs; it is, however, clear that there is no common requirement for all these mutants.

Table 2. *Growth requirements and penicillin sensitivities of K12 mutants*

No.	Requirements at 37°	Penicillin sensitivity (μ ./ml.)
2520	Methionine + lysine	3
2463	Methionine or valine or α -aminobutyric acid	1
580	Lysine or isoleucine + valine or α -aminobutyric acid	3
1253	Threonine or yeast nucleic acid	6
1424	Methionine	6
2430	Unknown	3
998	Unknown	3
1779	Unknown	3

We have in all, then, thirteen mutants of *Esch. coli*, all considerably more sensitive to penicillin than K12; nine of these mutants associate their sensitivity with increased nutritional requirements of various sorts, four do not. Hunter & Baker (1949) have shown that Gale's original hypothesis that 'The sensitivity of a cell to penicillin is determined by the degree to which its growth processes are dependent upon assimilation of preformed amino-acids rather than their synthesis', is no longer tenable when applied to different bacterial species. The present results show that even within one species the hypothesis does not always hold true.

It is, nevertheless, difficult to avoid the belief that there is a relationship between penicillin sensitivity and amino-acid assimilation or metabolism; any derangement of amino-acid metabolism in the mutants A, 2A, 3A and 4A, however, must be an internal one as it is not reflected externally in the form of increased nutritional requirements.

It appears that there are many different alterations in bacterial metabolism which can affect penicillin sensitivity, and since many of these (Davis, 1950) enzymic reactions of metabolism have been shown to be genetically controlled, it is clear that, indirectly at least, variations in the degree of penicillin sensitivity are under the same genetic control.

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