MUTATION OF STREPTOMYCIN-DEPENDENT MYCOBACTERIUM RANAE SELECTED FROM A SULFATHIAZOLE-RESISTANT VARIANT

DIRAN YEGIAN AND VERA BUDD

Ray Brook State Tuberculosis Hospital, Ray Brook, New York

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The recent extensive use of antibacterial agents has been instrumental in developing new procedures in the study of bacterial inheritance. Much has been written on the genetic aspects of bacterial resistance to penicillin, streptomycin, and other chemotherapeutic agents, and it is now conceded that these resistant variants arise by spontaneous mutation. In the case of streptomycin there has also been described a variant that requires this agent for growth (Miller and Bohnhoff, 1947; Paine and Finland, 1948; Yegian and Budd, 1948). These streptomycin-dependent strains provide a practical method of studying reverse mutation, for by plating large populations on medium free from streptomycin it is possible to select nondependent variants for analysis.

This report describes the variants obtained in this manner from a streptomycin-dependent strain of *Mycobacterium ranae* isolated directly from the parent streptomycin-sensitive culture and those obtained from a streptomycin-dependent strain selected from a sulfathiazole-resistant variant of this microorganism. In the latter case two "factors" are involved, and this is reflected in the variety of morphological and biological variants recovered.

**EXPERIMENTAL PROCEDURES AND RESULTS**

The test organism, *M. ranae*, is a rapidly growing acid-fast bacillus that is nonpathogenic for mammals. It grows readily on nutrient glycerol agar at 37 C and produces in 3 days colonies that are dry and slightly wrinkled (figure 1). Microscopically they reveal a fine network with a slightly heaped-up center and a lobulated edge. This parent culture is sensitive to sulfonamides and streptomycin.

The development of a strain of *M. ranae* highly resistant to sulfathiazole was accomplished by serial transfer through increasing concentrations of the drug in liquid sorbitan monoooleate albumin medium until good growth was obtained in the presence of 100 mg per cent of sulfathiazole. This resistant strain grows much more slowly than the parent strain even in the absence of sulfathiazole (Yegian et al., 1946). The young colonies (figure 2) are composed of coarser strands and the edges are more irregular than those of the parent *M. ranae*.

The same procedure was used to isolate streptomycin-dependent strains from the parent and sulfathiazole-resistant cultures (Yegian et al., 1949). Large populations of each were plated on series of nutrient glycerol agar plates containing 100 μg of streptomycin per ml. Colony counts were made at intervals and individual colonies were transferred to medium with and without streptomycin to determine their status in relation to this drug.
In the case of the parent culture it was found that the colonies that appeared within 7 days were all resistant to streptomycin. These colonies are morphologically similar to the sulfathiazole-resistant types but show only a slight tendency to grow more slowly than those of the parent type. After 14 days both streptomycin-resistant and streptomycin-dependent colonies were obtained, and at 21 days the majority were streptomycin-dependent. These dependent organisms grow even more slowly than the sulfathiazole-resistant strain and are uniformly dry and irregularly angulated. Microscopically they resemble a tangle of cut threads with loose, irregular single strands protruding.

The plates inoculated with sulfathiazole-resistant M. ranae were not examined until the 14th day of incubation because of the slower rate of growth. At this time only streptomycin-resistant colonies were obtained, streptomycin-dependent colonies appearing in the period between the 21st and 28th days. These dependent colonies are similar to those obtained directly from the parent culture but tend to be round and more regular in outline (figure 3). They develop very slowly.

The isolation of nondependent variants from these dependent strains was accomplished in the following manner: The turbid growths obtained in sorbitan monooleate albumin medium containing 100 μg of streptomycin per ml were washed thoroughly to remove the streptomycin. The bacilli were resuspended in physiological salt solution to make heavy suspensions of approximately equal turbidity and 1-ml quantities of these suspensions were plated on nutrient glycerol agar without streptomycin. The plates were incubated for 21 days, at the end of which time colony counts were made.

The streptomycin-dependent strain isolated directly from the streptomycin-sensitive parent gave rise to only 2 colonies or fewer per plate in the absence of the drug. These colonies resembled the parent M. ranae morphologically and were uniformly sensitive to streptomycin.

The streptomycin-dependent strain isolated from the sulfathiazole-resistant culture, however, yielded 100 times more colonies on streptomycin-free medium. Three morphologically distinct colony types were observed. The majority, which appeared first and comprised 85 per cent of the total number of colonies, were large rough colonies that presented microscopically a wavy, heaped-up appearance and an irregular edge (figure 4). Sixty of these colonies, chosen at random, were tested; 37, or 62 per cent, were found to be resistant to both sulfathiazole and streptomycin, and 23, or 38 per cent, were resistant to sulfathiazole only.

Second in order of appearance came a small, flat, rough type of colony (figure 5) that made up 5 per cent of the total number of colonies. Analysis of 67 of these revealed that 45, or 67 per cent, were resistant to both streptomycin and sulfathiazole; 19, or 28 per cent, were resistant to sulfathiazole only; and 3, or 5 per cent, were streptomycin-resistant.

Last to appear, after 15 to 20 days of incubation, were the small smooth colonies that made up the remaining 10 per cent of the total. These were shiny and slightly elevated with a regular contour (figure 6). Fifty-four of these colonies were analyzed and yielded four types with regard to drug sensitivity: 82, or 60 per cent, were resistant to both sulfathiazole and streptomycin; 7, or 13 per cent, were resistant to sulfathiazole only; 9, or 16 per cent, were resistant to strepto-
Figure 1. Colonies of parent strain of *M. ranae*. Incubated 3 days.

Figure 2. Colony of sulfathiazole-resistant *M. ranae*. Incubated 6 days.

Figure 3. Colonies of sulfathiazole-resistant, streptomycin-dependent *M. ranae*. Incubated 10 days.

Figures 4, 5, and 6. Mutants derived from sulfathiazole-resistant, streptomycin-dependent *M. ranae* in the absence of streptomycin.

Figure 4. Large, rough colony. Incubated 6 days.

Figure 5. Small, flat colony. Incubated 8 days.

Figure 6. Small, smooth colony. Incubated 10 days.

All illustrations were taken at a magnification of 60.
mycin only; and 6, or 11 per cent, were sensitive to both drugs. This last type was like the parent _M. ranae_ in drug sensitivity but differed in growth rate and colony morphology. These figures were compiled from several experiments.

Cultures of all three morphological types were plated out, and each gave rise to the same type of progeny. Further studies revealed that all three grew in the presence of _para_-aminosalicylic acid and penicillin, as does the original parent strain of _M. ranae_. Films stained by the gram and Ziehl-Neelsen techniques revealed no differences in staining properties or cellular morphology.

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**SUMMARY**

The back mutations derived from streptomycin-dependent _Mycobacterium ranae_ offer a new and promising approach in the study of bacterial genetics. Streptomycin-dependent variants isolated directly from the sensitive parent strains of certain microorganisms mutate back either to the parent sensitive type (Miller and Bohnhoff, 1947; Yegian and Vanderlinde, 1949) or to streptomycin-resistant variants (Paine and Finland, 1948; Yegian _et al._, 1949) with no discernible change in colony morphology.

The problem becomes more complex, however, if a sulfathiazole-resistant variant is selected from the parent sensitive culture and then from this a streptomycin-dependent culture is obtained for study, as reported here. In this instance we are dealing with perhaps two genetic "factors": resistance to sulfathiazole and dependence upon streptomycin for growth. Colonies obtained from this dependent variant in the absence of streptomycin present a complicated genetic behavior and yield three distinct morphological types, which include four variants that differ with regard to drug sensitivity.

At this time it is futile to postulate a classical genetic theory, but the possibility of an apparent segregation of hereditary variants without fusion might be considered.

Finally, the use of streptomycin-dependent variants lends itself well to further analysis of the mechanism of bacterial inheritance.

**REFERENCES**


