Genetic studies on the streptomycin resistance of bacteria were conducted by Demerec (1948), Hsie and Bryson (1950), Hotchkiss (1952, 1955), Ushiba et al. (1954, 1955), Hashimoto (1957), Hsu (1957), Tsukamura et al. (1958), etc. It was reported by these authors that the genetic system controlling streptomycin resistance in *Escherichia coli*, *Pneumococci* and *Salmonella* consists of many factors that differ in their potency, and phenotypes of high resistance levels are produced by mutation of a single gene as well as by mutations of polygenes. Genetic information concerning the drug resistance-system of genus *Mycobacterium* is yet little. Hsie and Bryson (1950) described that streptomycin resistance in *Mycobacterium ranae* is a "facultative multi-step pattern" and Ushiba et al. (1957) did that gradual increase of streptomycin resistance in *Mycobacterium 607* occurs spontaneously, that is, in a manner excluding the induced mutation by streptomycin. Tsukamura et al. (1958) reported that the genetic system controlling streptomycin resistance of *Mycobacterium tuberculosis* also consists of multiple factors and phenotypes of high resistance levels are produced by a single gene mutation or by mutations of multiple factors. The present study has been designed to conduct systematic observations on the streptomycin resistance of mycobacteria.

**Principle of the study**

It appears to be a general rule in the classical genetics to suppose the existence of one genotype corresponding to one character. In the field of the bacterial genetics, the character is not observed in a cell but in a bacterial population. Characters of a clone, *i.e.*, those of a bacterial population derived from a single cell would correspond to those of an individual of higher organisms. The character of clones in relation to drug resistance is the population structure of clones in relation to drug resistance. The present study will be conducted by assuming a hypothesis that one type of population structure of clones corresponds to one genotype.

In order to determine numbers of genes responsible for different levels of resistance, it appears most important to determine numbers of types of the population
structure in clones isolated by the first-step selection of the parent sensitive clone previously not exposed to any drug. It would be probable that clones obtained by the first-step selection, that is, first-step clones are produced by a single gene mutation, for a chance that they could be mutants produced by double or more mutations occurring in the same cell appears to be extremely rare.

**Methods**

*Strain.* Mycobacterium avium, strain Jucho, was used throughout the study. The parent clone was derived from a single colony of the stock culture.

*Medium.* Sauton medium and Sauton agar medium were used. Sauton medium consisted of 40 ml. of glycerin, 4.0 gm. of sodium glutamate, 0.5 gm. of dibasic potassium phosphate, 0.5 gm. of magnesium sulfate, 2.0 gm. of sodium citrate, 0.05 gm. of ferrous sulfate, and 960 ml. of distilled water. The pH was adjusted to 7.2, and the resulting medium was dispensed in 50 ml. amounts into 200 ml. Erlenmeyer flasks and autoclaved at 115°C for thirty minutes. Sauton agar medium consisted of Sauton medium containing 3 per cent agar. The Sauton agar medium was dispensed in 20 ml. amounts to Petri dishes, 9 cm. in diameter. Dihydrostreptomycin sulfate (Meiji Co.) was dissolved aseptically in distilled water and added to the medium immediately before use.

*Calculation of viable bacterial numbers.* Seven-day-old cultures in Sauton medium were homogenized by shaking with glass beads and suspended in physiological saline. The suspension* was tenfold diluted. Each 0.1 ml. of different dilutions were inoculated to a series of Sauton agar medium plates containing graded concentrations of streptomycin including control plates without drug. The plates inoculated were incubated at 37°C for five days and counts of viable colonies were made.

*Determination of population structure of clones.* A single whole colony was picked from colonies grown on medium containing any drug concentration and the isolate was subcultured in a drug-free Sauton medium to form a large population. The resulting clone, a bacterial population derived from a single colony, was tested for its population structure. The population structure was expressed as a survival curve, i.e., a series of survival ratios for the test clone on various concentrations of streptomycin.

It may be said that the method adopted in the present study is a developmental form of the method described by Demerec (1948) and Hsie and Bryson (1950). In the present study, population structures were determined of various first-step clones (clones derived from single colonies obtained by the first-step selection of the parent clone with various concentrations of streptomycin). The analysis of the population structure was repeated on second-step to multi-step clones until a highly resistant clone was

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* The cell suspension consisted mostly of single cells and clumps of several cells. It was demonstrated that, under the conditions tested, only one cell is viable among about five cells, because the cell suspension used here gave about one fifth of viable counts given by a cell suspension prepared from a one-day-old culture, which exhibited the same optical density. It has been thus considered that each colony may be derived from a single viable cell.
Results and discussion

1. Survival curve of the parent sensitive clone.
   Survival curve of the parent clone, i.e., survival ratios of it on various concentrations of streptomycin are shown in Fig. 1. The survival ratio slowly decreased until it reached 0.3 µg/ml of streptomycin and decreased very rapidly at the concentration of 0.5 µg/ml of the drug. Thereafter, the ratio again decreased slowly. Survivors on media containing extremely high concentrations (more than 100 µg/ml.) of streptomycin were found at rates of about $10^{-8}$.

   First-step clones were analysed for their population structure, which were derived from single colonies grown on various concentrations of streptomycin by inoculating the parent clone. The results are shown also in Fig. 1.

   Population structures of first-step clones derived from colonies grown on media containing less than 0.3 µg/ml of streptomycin were similar to the population structure of the parent clone. As described in the principle of the study, the results suggest that these clones have the same genotype as that of the parent clone, i.e., the wild genotype. They suggest also that, under the conditions tested, a significant increase of streptomycin-resistant cells in number caused by mutagenic effect of streptomycin (Akiba et al., 1957) cannot be observed.

   Population structures of clones derived from single colonies grown on media containing more than 0.5 µg/ml of streptomycin were quite different from the population structure of the parent clone. As described in the principle of the study, the results suggest that these clones have the same genotype as that of the parent clone, i.e., the wild genotype. They suggest also that, under the conditions tested, a significant increase of streptomycin-resistant cells in number caused by mutagenic effect of streptomycin (Akiba et al., 1957) cannot be observed.

Fig. 1. Population structures (survival curves) of the parent sensitive clone, first-step resistant clones and second-step resistant clones of Mycobacterium avium (strain Jucho), showing the step-wise variability in degree of streptomycin resistance (population structure). Each of the first-step clones were isolated from a single colony of the parent clone growing on the concentration of streptomycin indicated by the arrows. For example, S5R indicates population structure (survival curve) of a first-step clone isolated from a single colony growing on the concentration of 5 µg/ml of streptomycin. S5-20R indicates the population structure of a second-step clone derived from a single colony of the clone S5R growing on the concentration of 20 µg/ml of streptomycin.

Fig. 2. Population structures (survival curves) of a first-step clone S0.7R and second-step and third-step clones derived from the clone S0.7R, showing the step-wise variability in population structure (degree of resistance). S0.7R indicates the population structure of a first-step clone isolated from a single colony of the parent clone growing on the concentration of 0.7 µg/ml of streptomycin. S0.7-1R, S0.7-3R, S0.7-5R, S0.7-10R, and S0.7-100R represent population structures of second-step clones isolated from a single colony of the clone S0.7R growing on the concentrations of 1 µg/ml, 3 µg/ml, 5 µg/ml, 10 µg/ml, of streptomycin, respectively (indicated by the arrows on the curve of S0.7R). S0.7-10-100R represents the population structure of a third-step clone isolated from a single colony of the clone S0.7-10R growing on the concentration of 100 µg/ml of streptomycin.
Second-step clones were obtained by planting the first-step clones onto media containing various concentrations of streptomycin and isolating single colonies, and third-step clones were done by planting the second-step clones onto media containing various concentrations of the drug and isolating colonies on the media.

The second-step and third-step clones represented various population structures according to the concentration used for the second- or third-step selections (Fig. 1 and 2). Survivors on media containing more than 100 μg/ml of streptomycin always produced fully resistant population.

The existence of various population structures of clones produced by further selections of the first-step clones suggests that there are various genotypes that may be produced by double or triple or more mutations of genes. It appears thus that clones representing fully resistant population structures can also be produced by double or multiple mutations.

In view of the results obtained, it is suggested that there are multiple genes that differ in their potency and highly or fully resistant clones are produced by a single gene mutation and by double or multiple mutations.

**Summary**

Genetic studies on the streptomycin resistance of *Mycobacterium avium* (strain Jucho) were conducted by the population analysis method assuming a hypothesis that one type of the population structure of first-step clones corresponds to one genotype. The resulting conclusions are as follows:

There are multiple genotypes producing different population structures of clones. Both intermediate and fully resistant population structures of clones are produced by a single gene mutation or by double or multiple mutations.

**References**


