JOURNAL OF BACTERIOLOGY, Dec. 1970, p. 1294-1298

Vol. 104, No. 3
Printed in U.S.A.

Physiological Streptomycin Resistance in a Multiauxotroph of *Escherichia coli* Strain 15 T⁻

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Received for publication 31 August 1970

Escherichia coli strain WWU was found to be moderately resistant to streptomycin when grown in a minimal medium, although the strain was sensitive if grown in nutrient broth. Transfer experiments showed that cells grown in minimal medium retain the resistant state for a period of time after dilution into nutrient broth; and conversely, sensitive cells grown in nutrient broth were sensitive after dilution into minimal medium for a period of time. The kinetics of transition from resistant to sensitive and from sensitive to resistant were observed, and kinetics of ^{3}H-dihydrostreptomycin accumulation by resistant and sensitive cells were compared. The data suggested that cells grown in minimal medium were physiologically resistant because they accumulated streptomycin poorly. Inactivation per incorporated antibiotic molecule was the same in resistant and sensitive cells.

Streptomycin is an aminoglycoside antibiotic with a wide range of effects on *Escherichia coli* (2). One result of the cell-streptomycin interaction is loss of viability, which has been attributed to an interaction with the ribosomes (9). More specifically, the sensitivity has been associated with a particular protein subunit of the core proteins of the 30*S* ribosomal particle (6, 11).

Mutational and genetical data have generally supported the initial observation by Hashimoto (5) that a single-step genetic alteration could confer resistance to high levels of streptomycin. Genetically stable *E. coli* strains have also been observed which have a low-level resistance to streptomycin (resistant to concentrations of 5 to 10 μg/ml). Permeability barriers (3) and enzymatic inactivation of the antibiotic (4, 10, 13) are mechanisms of low-level resistance.

In addition to resistance resulting from mutation in the cell, several instances of relative resistance to streptomycin have been reported and ascribed to antagonistic or competitive effects of various ions in the media (7, 12). In this report we describe the moderate resistance of *E. coli* strain WWU grown in a minimal medium. The same strain is sensitive to streptomycin if grown in nutrient broth. However, in this instance we find evidence that the differential resistance results from the physiology of the cell. This conclusion is chiefly supported by the *gradual* alteration of the cell's resistant condition upon transfer from one medium to another.

By measuring simultaneously the incorporation of antibiotic and the inactivation of cell viability, with cells grown either in nutrient broth or in minimal medium, it was apparent that both types of cells were equally sensitive to the antibiotic once incorporated. The physiological resistance resulted from a reduced permeability to streptomycin.

MATERIALS AND METHODS

Bacterial strains. A multiauxotroph of *E. coli* strain 15 T⁻, known as WWU, was the principal organism used in this study (1). A single-step, high-level, streptomycin-resistant mutant of strain WWU was also used.

Media. The minimal-salts, defined medium has previously been described (1). It contained thymidine, uridine, tryptophan, arginine, methionine, and proline to satisfy the auxotrophic requirements of WWU. The nutrient broth was 8 g of Nutrient Broth (Difco) preparation per liter of distilled water, sterilized at 121 C for 20 min. For solid media, 15 g of agar (Difco) per liter was incorporated in the appropriate liquid media.

Streptomycin. Streptomycin sulfate was obtained from Pfizer Laboratories (New York, N.Y.). A concentrated stock solution of 100 mg/ml was made in distilled water and stored at −20 C in 1-ml portions.

^{3}H-dihydrostreptomycin (^{3}HSm) was obtained from Mallinckrodt Chemical Works (St. Louis, Mo.)

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as a dry powder with a specific activity of 262 Ci/mole. ^{3}HSm was dissolved in distilled water (5.6 mg/2.8 ml) and stored at −20 C. A further dilution of 1:4 was made as required (500 μg/ml, final concentration).

Growth and inactivation. Cultures inoculated from stock strains were grown overnight in minimal or nutrient medium, with shaking at 37 C. Experimental cultures (10 ml) were inoculated (0.1 ml) from the overnight cultures and grown at 37 C with aeration to mid-exponential phase.

Inactivation by streptomycin was tested by adding streptomycin to these cultures and assaying for viability on nutrient agar. Buffer solution (minimal medium without glucose and the supplements) was used for dilutions. Colonies were counted after 12 hr of incubation at 37 C.

Transfer experiments. Cells were grown in the first growth medium to a viable titer of approximately 2×10^8 organisms per ml. Samples (0.1 ml) were transferred to 10-ml amounts of prewarmed second growth medium in a set of culture tubes. At intervals of time after transfer, streptomycin was added to individual cultures in the second growth medium. The effect of streptomycin on cells was determined by viability assays and measurements of accumulated, radiolabeled antibiotic.

Accumulation of ^{3}H-dihydrostreptomycin. Samples (0.1 ml) of cultures growing in the presence of ^{3}HSm were individually added to 10-ml amounts of buffer solution held in an ice bath. Each diluted sample was filtered onto a membrane filter (Millipore Corp., HAWP 02500); the filter was rinsed once with 10 ml of buffer solution, dried with hot air, and placed in 15 ml of scintillation fluid for counting.

The toluene - ethanol - 2,5 - diphenyloxazole - dimethyl 1,4-bis-2-(5-phenyloxazolyl)-benzene scintillation fluid (779 ml, 230 ml, 4 g, and 0.25 g, respectively) had a 15% counting efficiency by counting samples of a known activity.

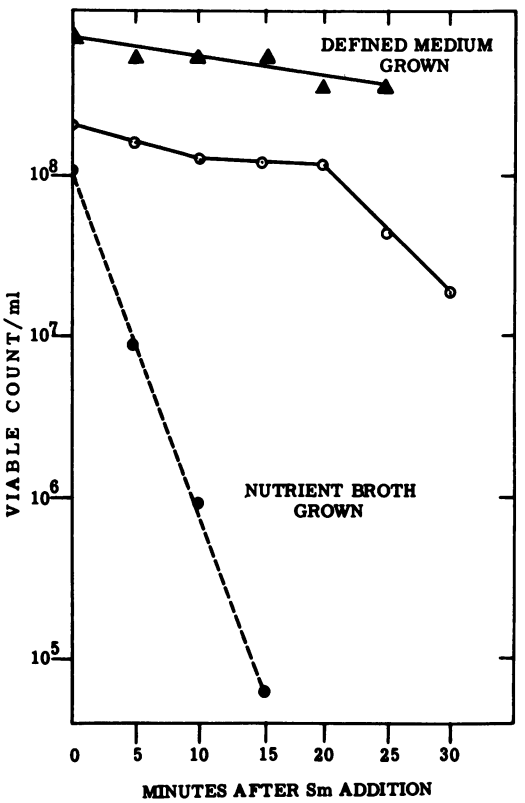

FIG. 1. *Inactivation of E. coli by streptomycin in different media. Streptomycin was added to exponentially growing cultures of E. coli WWU in nutrient broth (broken line) or minimal medium (solid line). Final concentrations of streptomycin were 10 μg/ml (●), 50 μg/ml (▲), and 100 μg/ml (○). Viable counts were determined by plating diluted samples on nutrient agar.*

RESULTS

Inactivation by streptomycin. *E. coli* WWU showed different sensitivities to streptomycin in the two media. Cells grown in minimal medium were more resistant to streptomycin than were cells grown in nutrient medium. Streptomycin at 10 μg/ml in nutrient broth reduced viability by a factor of 10^{-3} in 15 min. In minimal medium a concentration of 50 μg/ml caused only a slight decline in viable titer, and a concentration of 100 μg/ml caused a slight loss of viability in the first 20 min followed by a faster rate of killing (Fig. 1).

Transfer experiments. Cells grown in minimal medium were transferred into nutrient broth and challenged with streptomycin (10 μg/ml) after various periods of incubation in nutrient broth. By this means the kinetics of change from resistance to sensitivity could be estimated.

Figure 2 shows a gradual change in the sensitivity of the cells. At least a 40-min incubation in nutrient broth is required for cells to lose their resistance and become sensitive.

A similar gradual change in sensitivity was observed in the converse experiment. Cells grown in nutrient broth were transferred into minimal medium and challenged with streptomycin (100 μg/ml) after periods of incubation in minimal medium. At 90 min after transfer into minimal medium, these cells had become resistant to streptomycin (Fig. 3).

The gradual change in sensitivity as seen in the transfer experiments suggested that a physiological change took place in the cells when they became either sensitive or resistant. However, some of the observed resistance might have been the result of an antagonism between streptomycin and phosphate. This was a small effect at most. Strain WWU grown in defined medium with 0.2 mM phosphate instead of the normal 64 mM

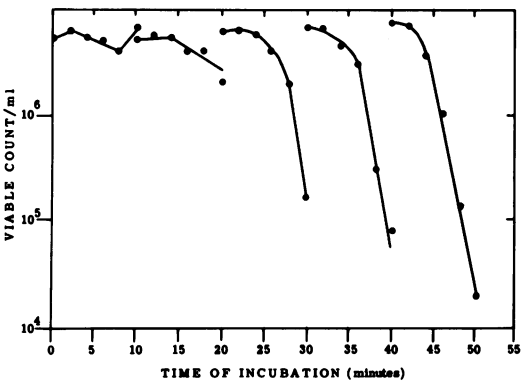

FIG. 2. *Increasing sensitivity of cells transferred to nutrient broth. Cells growing in minimal medium were identically diluted into five equal portions of nutrient broth. Streptomycin was added to a concentration of 10 μg/ml in each culture after incubation periods of 0, 10, 20, 30, and 40 minutes, respectively. A viable count was determined in each culture just before adding streptomycin and every 2 min after adding streptomycin.*

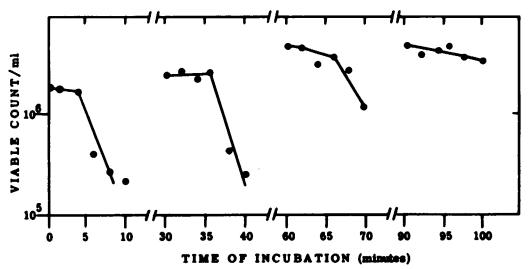

FIG. 3. *Decreasing sensitivity of cells transferred to minimal medium. Cells growing in nutrient medium were identically diluted into four equal portions of minimal medium. Streptomycin was added to a concentration of 100 μg/ml in each culture after incubation periods of 0, 30, 60, and 90 min, respectively. A viable count was determined in each culture just before adding streptomycin and every 2 min after adding streptomycin.*

phosphate showed the same gradual change in resistance when transferred into nutrient medium. Cells growing in nutrient medium with phosphates added to the concentration present in defined medium were moderately resistant, but they were immediately sensitive upon transfer to normal nutrient medium.

Accumulation of ^{3}H-dihydrostreptomycin. Radioactive dihydrostreptomycin was used to determine the correlation between accumulation of antibiotic within the cells and inactivation of the cells. Control experiments showed ^{3}HSm to be equivalent to streptomycin (*data not given*).

Cells grown in minimal medium and exposed to ^{3}HSm at 10 μg/ml showed no significant accumulation of radioactivity in 30 min. With cells grown in nutrient broth, a steady increase in accumulated radioactivity occurred during exposure to ^{3}HSm (Fig. 4).

The uptake kinetics of physiologically changing cells were examined by using ^{3}HSm. Cells were transferred from minimal medium to nutrient broth as described above. The rates of accumulation and inactivation were observed after various periods of incubation in the second medium. The general increase in sensitivity was accompanied by a gradual increase in the rate of ^{3}HSm accumulation (Fig. 5).

Inactivation as a function of accumulated antibiotic. The accumulation results showed sensitivity to parallel permeability. More specifically, the results suggested that the cytoplasm and presumably ribosomes were equally sensitive to streptomycin in cells growing in minimal medium or nutrient medium. This point could be made more explicit by replotting the data of Fig. 5 to show viability as a function of accumulated antibiotic (Fig. 6). Although the kinetics of inactivation (sensitivity) of the cells in three different physiological conditions differed markedly, in-

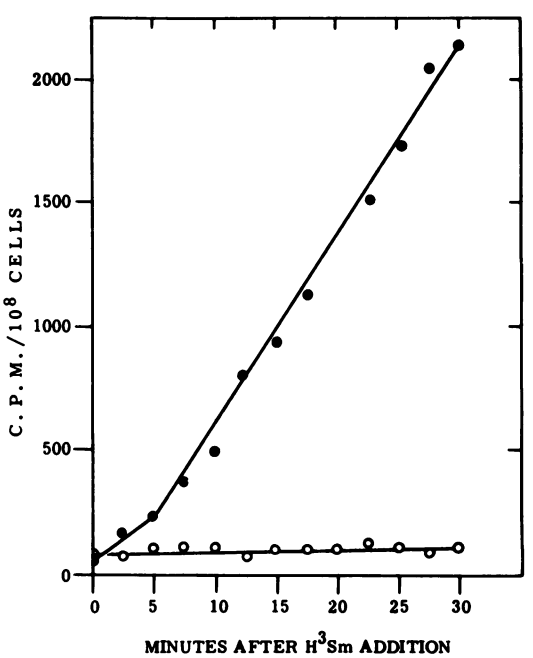

FIG. 4. *Uptake of ^{3}HSm by cells in minimal or nutrient medium. ^{3}H-Sm was added to a concentration of 10 μg/ml to exponential cultures of E. coli WWU in nutrient (●) or minimal (○) medium. Samples were taken during incubation to determine uptake of the tritium labeled antibiotic. The cells were washed with a salts buffer before liquid scintillation counting. The measured radioactivity was normalized to a standard cell number determined from the viable titer just before antibiotic addition.*

activation as a function of ^{3}HSm accumulation could be represented by a single straight line.

Test of a streptomycin-resistant mutant. To demonstrate that poor accumulation of ^{3}HSm was not in fact a function of resistant ribosomes, a single-step, high-level, streptomycin-resistant mutant was examined. This mutant was resistant to streptomycin at 1000 μg/ml in nutrient broth. Accumulation of ^{3}HSm was observed with this mutant, and survival after the accumulation of 1.2×10^5 ^{3}HSm molecules per cell was greater than 98%. Accumulation could occur in the absence of the specific complexing which caused cell inactivation.

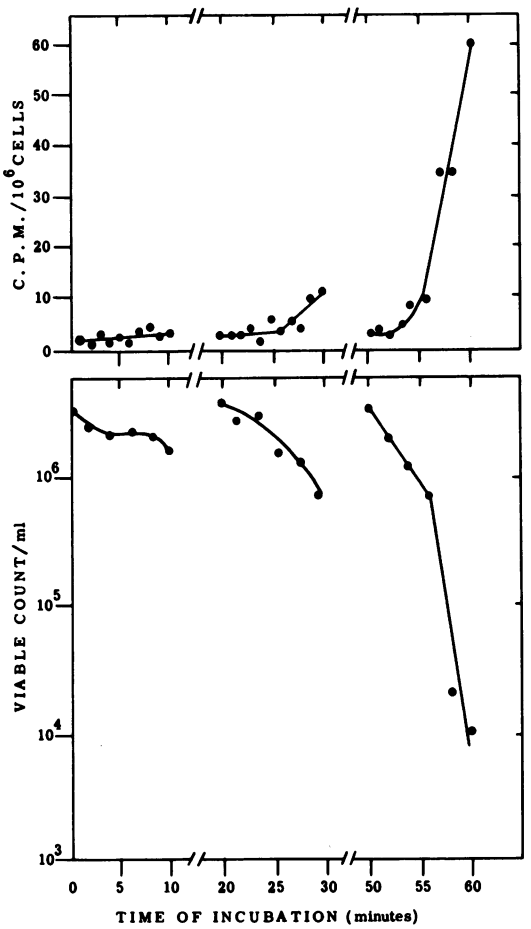

FIG. 5. *Increasing sensitivity and ^{3}H-Sm accumulation after transfer to nutrient broth. Cells growing in minimal medium were identically diluted into three portions of nutrient broth. ^{3}H-Sm was added to a concentration of 10 μg/ml in each culture after incubation periods of 0, 20, and 50 min, respectively. A viable count was determined in each culture just before and every 2 min after adding ^{3}H-Sm. Concomitantly, samples were removed and assayed for ^{3}H-Sm uptake.*

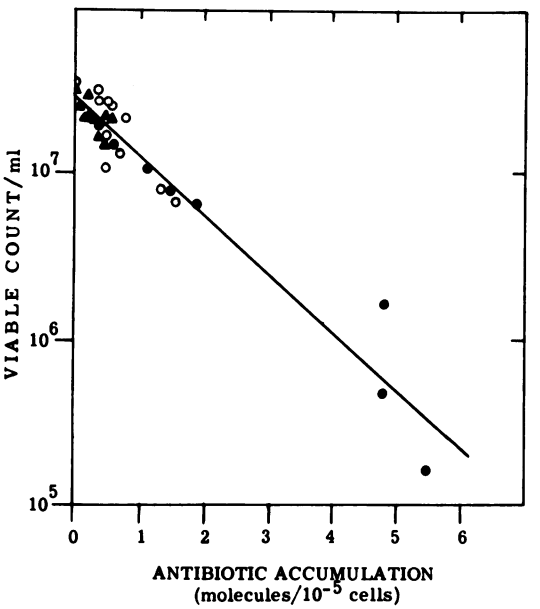

FIG. 6. *Cell survival as a function of ^{3}H-Sm uptake. Cells were challenged with ^{3}H-Sm at three stages of the transition from resistance to sensitivity in nutrient broth (see Fig. 5). The kinetics of cell survival and ^{3}H-Sm uptake were determined in each stage: after 0 min of incubation in nutrient broth (▲), after 20 min of incubation in nutrient broth (○), and after 50 min of incubation in nutrient broth (●). The straight line drawn through the data points has a slope indicating a probability of one inactivation event per 1.2×10^5 accumulated antibiotic molecules.*

Effect of media on a permeability mechanism. It was noted that filtering cells grown in nutrient broth and suspending them in minimal medium before further dilution into minimal medium made the cells immediately resistant to streptomycin upon transfer to minimal medium. Sensitivity could be restored if fresh nutrient broth was added in addition to the filtered cells. Filtered cells transferred to defined medium showed no significant uptake of ^{3}HSm, whereas, when a one-hundredth dilution of nutrient broth was also added (80 μg/ml), the cells did accumulate ^{3}HSm.

It should be emphasized that this dependence of sensitivity on a small supplement of nutrient material was a characteristic of nutrient broth-grown cells only. Cells grown in minimal medium, which showed a reduced permeability to the antibiotic and hence resistance, were not immediately sensitive upon transfer to a total nutrient medium (Fig. 2). Moreover, the addition of a one-hundredth dilution of nutrient broth to cells which had made the normal transition from sensitivity to resistance (as seen in Fig. 3) did not alter the resistant response of these cells. Nutrient broth-grown cells (sensitive

cells) diluted into minimal medium were still sensitive only when a small amount of the nutrient medium was transferred to the defined medium in addition to the cells.

DISCUSSION

The differential sensitivity of *E. coli* strain WWU to streptomycin was very pronounced. Although high concentrations of streptomycin cause considerable killing both in nutrient broth and in minimal medium, moderate concentrations (10 to 50 μg/ml) caused little killing in minimal medium.

It does not seem possible to explain this insensitivity simply in terms of streptomycin antagonism by constituents of the medium. When cells were switched from one medium to another they retained the state of sensitivity (or resistance) observed in the first medium.

Cells growing or grown in minimal medium incorporated radio-labeled dihydrostreptomycin at less than 1/40 of the rate of cells growing in nutrient broth. These cells incubated in nutrient broth did not become sensitive until they become permeable. Moreover, as they gradually became more permeable they gradually became more sensitive. Loss of viability was, at all times, an unvarying function of incorporated antibiotic.

Although inactivation of bacteria by streptomycin is generally plotted against incubation time, it is apparent in Fig. 6 that a possibly fundamental aspect of inactivation by streptomycin can be observed if survival is plotted against accumulated antibiotic molecules. Inactivation is exponential.

Hence the target theory of drug action, as first proposed many years ago by Paul Ehrlich, can be examined in the analytical form of contemporary target theory for radiation action (8). Both concepts attribute survival to "total escape" of a critical target within the cell. Accordingly, the data of Fig. 6 suggest a D_{37} dose of 120,000 ^{3}HSm molecules, or that for every 120,000 possible adsorption sites in *E. coli* for ^{3}HSm molecules there is one critical "target" site. Adsorption of streptomycin to this "target" destroys the colony forming ability of the cell.

The studies reported here do not allow a decision as to whether the physiological resistance of cells grown in minimal medium results from a novel barrier to streptomycin or a deficient permeation system. It is interesting to note, however, that the sensitivity of cells from nutrient broth, in minimal medium, was dependent on small supplements of nutrients, that the high-level resistant mutant still incorporated ^{3}HSm, and that the concentration of incorporated ^{3}HSm exceeded the concentration of exogenous ^{3}HSm after a short period of uptake (10^5 ^{3}HSm molecules per cell is approximately a concentration of 100 μg/ml).

It is not unreasonable that cells grown in nutrient broth contain a permeation system for streptomycin, which concentrates streptomycin inside the cell. This system would be of little consequence when high concentrations of antibiotic are in the medium, since a large concentration gradient would assure rapid accumulation. However, when exogenous antibiotic is at a low concentration, this system would cause the accumulation of antibiotic within the cell. Cells growing in minimal medium would lack this hypothetical permeation system, would have a very low endogenous streptomycin concentration, and would have a small probability of a lethal interaction with streptomycin.

ACKNOWLEDGMENTS

We thank Eli Lilly and Co., Indianapolis, for a Grant-in-Aid to one of us (R.C.B.) which made this work possible.

LITERATURE CITED

1. Bockrath, R. C., M. Osborn, and S. Person. 1968. Nonsense suppression in a multiauxotrophic derivative of *Escherichia coli* 15 T$^-$: identification and consequences of an amber triplet in the deoxyribomutase gene. J. Bacteriol. **96**:146–153.
2. Brock, T. D. 1966. Streptomycin. Symp. Soc. Gen. Microbiol. **16**:131–168.
3. Gunderson, W. B. 1965. Reduced streptomycin killing in *E. coli* carrying the Mu-factor in its extrachromosomal state. Acta Pathol. Microbiol. Scand. **65**:627–635.
4. Harwood, J. H., and D. H. Smith. 1969. Resistance factor-mediated streptomycin resistance. J. Bacteriol. **97**:1262–1271.
5. Hashimoto, K. 1960. Streptomycin resistance in *Escherichia coli* analyzed by transduction. Genetics **45**:49–62.
6. Ozaki, M., S. Mazushima, and M. Nomura. 1969. Identification and functional characterization of the protein controlled by the streptomycin-resistant locus in *E. coli*. Nature (London) **222**:333–339.
7. Plotz, P. H., D. T. Dubin, and B. D. Davis. 1961. Influence of salts on the uptake of streptomycin by *Escherichia coli*. Nature (London) **191**:1324–1325.
8. Setlow, R. B., and E. C. Pollard. 1962. Molecular Biophysics. Addison-Wesler Publ. Co., Mass.
9. Spotts, C. R., and R. Y. Stanier. 1961. Mechanism of streptomycin action on bacteria: a unitary hypothesis. Nature (London) **192**:633–637.
10. Takasawa, S., R. Uthara, M. Okanishi, M. Maeda, and H. Umezawa. 1968. Studies on adenylstreptomycin, a product of streptomycin inactivation by *E. coli* carrying the R-factor. J. Antibiot. (Tokyo) Ser. A **21**:477–484.
11. Traub, P., and M. Nomura. 1968. Streptomycin resistance mutation in *Escherichia coli*: altered ribosomal protein. Science **160**:198–199.
12. Tzagaloff, H., and W. W. Umbreit. 1963. Influence of streptomycin on nucleotide excretion in *Escherichia coli*. J. Bacteriol. **85**:49–52.
13. Umezawa, H., S. Takasawa, M. Okanishi, and R. Utahara. 1968. Adenylstreptomycin, a produce of streptomycin inactivated by *E. coli* carrying R factor. J. Antibiot. (Tokyo) Ser. A. **21**:81–82.