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Bactericidal Action of Streptomycin and Comparison with Spectinomycin in Heterozygotes of *Escherichia coli*

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Str^s/*str*^{r} heterozygotes of *Escherichia coli* K-12 are shown to be sensitive to the lethal as well as the inhibitory action of streptomycin. The rate of killing was lower in heterozygotes than in sensitive homozygotes, and among heterozygotes it was lower in those with a higher proportion of resistant ribosomes. These strains also differed, in a parallel manner, in the kinetics of inhibition of growth and protein synthesis by streptomycin. Similar results were obtained with spectinomycin and corresponding merodiploid strains. Since spectinomycin is purely bacteriostatic and stabilizes polysomes, it must block resistant ribosomes behind inhibited sensitive ribosomes; hence, these results are consistent with an initial similar polysomal blockade by streptomycin. However, since streptomycin causes gradual polysome breakdown, its dominant lethal action must involve some mechanism other than a permanent polysomal blockade.

Though several actions of streptomycin (Str) on the ribosome have been established, the mechanism of its irreversible, lethal effect on the cell remains unclear. The presence of both fully sensitive and resistant ribosomes in *str*^{r}/*str*^{s} heterozygotes (14) provides an opportunity to throw light on this mechanism by determining whether the interaction of Str with the sensitive ribosomes has consequences that prevent the resistant ribosomes from subsequently resuming protein synthesis. This paper will show that Str does have such consequences: in heterozygotes of *Escherichia coli* K-12, not only does Str inhibit growth (8) but it is also rapidly lethal. However, the rate of killing is lower in heterozygotes than in a sensitive homozygote, and among heterozygotes it is lower in those strains with the resistant allele on the episome rather than the chromosome, which thus has a higher proportion of resistant ribosomes.

The different strains also showed parallel differences in the kinetics of inhibition of growth and protein synthesis by Str. Very similar variations in the kinetics of inhibition were obtained with spectinomycin (Spc) in corresponding merodiploid strains. The significance of these kinetics for the lethal action of Str, and for the proposal (7) that in heterozygotes Str causes polysomal blockade of resistant ribosomes, behind inhibited sensitive ribosomes, will be discussed.

MATERIALS AND METHODS

Construction of merodiploids. A previous paper (14) described the culture media, the construction of the isogenic merodiploids, and the genetic terminology used in this work. A genetic map of a typical merodiploid, FS9D, is shown in Fig. 1; this strain is a *trans* double heterozygote, with *spc*^{r} and *str*^{s} on the episome, and *spc*^{s} and *str*^{r} on the chromosome. The *str*^{r} allele used in these studies (str^r-1) is "restrictive" in its effect on the phenotypic suppression of certain auxotrophic mutations by Str (5): when transduced into *E. coli* B strain T19-32 (kindly supplied by L. Gorini), which is *str*^{s} *arg*$^\pm$ (Str-suppressible) *leu*$^-$ (not Str-suppressible), it converted the phenotype of Str^R Arg$^-$ Leu$^-$ (neither Str-suppressible).

All merodiploids were stored on methionine-maltose-minimal slants to select against loss of the episome, and they were periodically repurified and checked for merodiploidy as previously described (14).

Inhibition of cell growth and protein synthesis. For studies of the kinetics with which Str or Spc inhibits cell growth and macromolecule synthesis, cells grown overnight in methionine-maltose-minimal medium were diluted into methionine-glucose-minimal medium to allow more rapid growth, and the antibiotic was added after one or two mass doublings. Cell mass was estimated by measurement with a Lumetron colorimeter of the optical density (490 nm) of cultures in sidearm flasks. To measure protein synthesis, ^{14}C-leucine was added, and samples of 1.0 ml were transferred at

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intervals into 1.0 ml of cold 10% trichloroacetic acid. The precipitates were collected on membrane filters (HA; Millipore Corp.) and were counted with a liquid scintillation counter.

Viability. For studies of viability, Str was added to cells growing in methionine-glucose-minimal medium, and viable counts were determined in duplicate on arginine-methionine-glucose-minimal medium. The survivors were scored for frequency of Str^R segregants by replica-plating onto arginine-methionine-glucose minimal-Str (200 μg/ml) plates, and for frequency of Mal^- segregants by replica-plating onto MacConkey-maltose plates. Experiments were discarded if more than 2% Mal^- or Str^R segregants were found; this value was rarely exceeded up to 3 hr after addition of Str to the growing culture.

MIC. The minimal inhibitory concentration (MIC), the least concentration that prevented visible growth after 40 hr of incubation at 37 C, was determined by spotting with a replica inoculator approximately 10^4 cells onto methionine-maltose-minimal plates containing approximately twofold increments of Str or Spc, from 1.0 to 5,000 μg/ml.

Chemicals. Spc sulfate was a gift of The Upjohn Co. Radiochemicals were from New England Nuclear Corp. Str and other chemicals were from commercial sources.

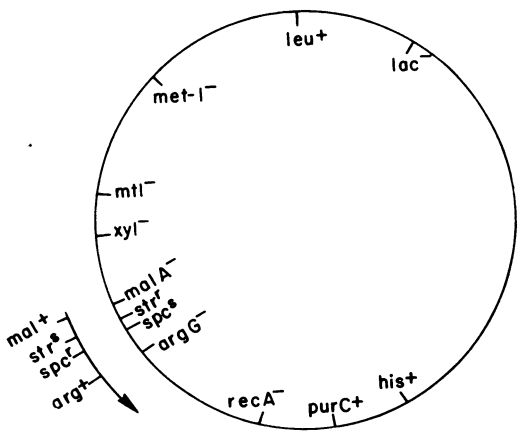

FIG. 1. *Genetic map of the heterozygous merodiploid FS9D, adapted from the Taylor and Trotter (15) map of E. coli.*

TABLE 1. *Minimal inhibitory concentrations of spectinomycin (Spc) and streptomycin (Str) for merodiploids*

Strain	MIC (μg/ml)	
	Spc	Str
FS5D *spc*^{r} *str*^{s}/F*spc*^{s} *str*^{r}	80	5
FS7D *spc*^{s} *str*^{r}/F*spc*^{s} *str*^{r}	40	2,500
FS9D *spc*^{s} *str*^{r}/F*spc*^{r} *str*^{s}	80	5
FS10D *spc*^{r} *str*^{s}/F*spc*^{r} *str*^{s}	3,000	5
FS12D *spc*^{s} *str*^{r}/F*spc*^{s} *str*^{s}	80	5
FS13D *spc*^{r} *str*^{s}/F*spc*^{s} *str*^{s}	80	5
FS14D *spc*^{s} *str*^{r}/F*spc*^{r} *str*^{r}	80	5,000
FS15D *spc*^{r} *str*^{s}/F*spc*^{r} *str*^{r}	3,000	5

[a] The lowest concentration of antibiotic at which no visible growth occurred on methionine maltose-minimal plates containing Spc or Str onto which were spotted approximately 10^4 colony-forming units of the indicated merodiploids. Plates were read after incubation for 40 hr at 37 C.

RESULTS

Inhibition of growth of merodiploids by Str and Spc. The MIC of Str or of Spc was determined for isogenic merodiploid strains carrying different combinations of sensitivity and resistance at the *str-A* and *spc* loci; the same four alleles were used throughout. Not only were the heterozygotes for *str* and *spc* phenotypically sensitive, as previously observed, but the MIC for the heterozygotes was found to be identical to that for the homozygotes (Table 1). Similarly, the kinetics of inhibition of growth in liquid medium by moderate concentrations of Str was essentially identical for the two

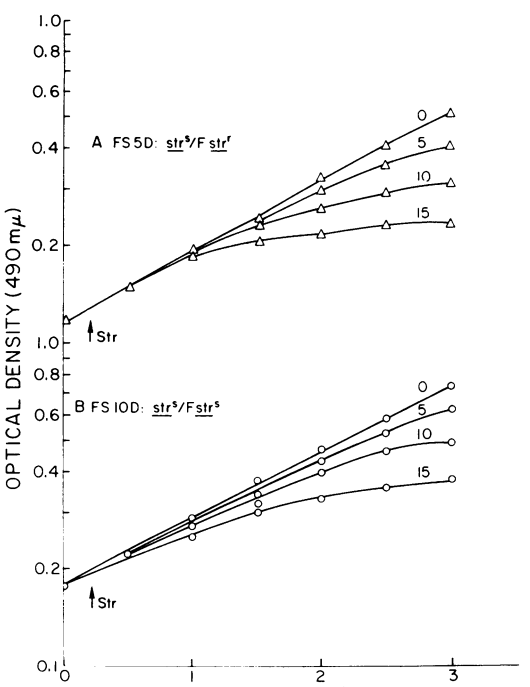

FIG. 2. *Effect of low concentrations of streptomycin (Str) on growth of a heterozygote (FS5D) and a sensitive homozygote (FS10D). Str was added to a series of identical cultures growing in methionine-glucose-minimal medium in side-arm flasks, with shaking. The concentrations (μg/ml), indicated by the numbers adjacent to the curves, are near the MIC for both strains (Table 1). Optical densities were measured at intervals.*

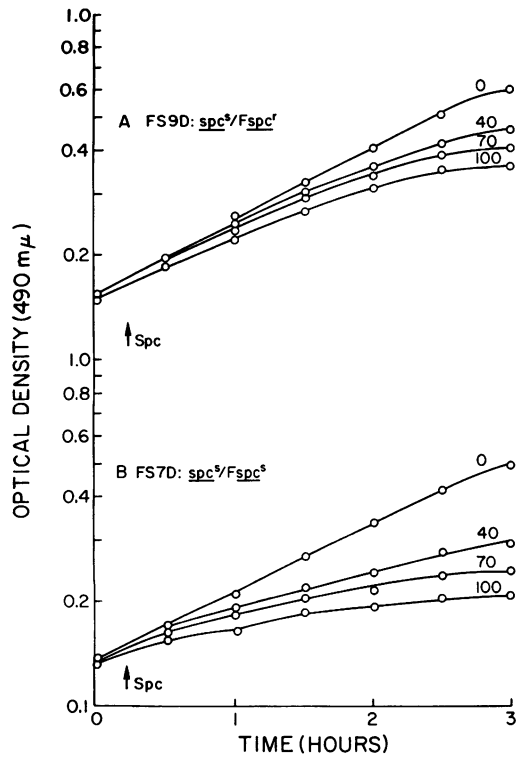

FIG. 3. *Effect of low concentrations of spectinomycin (Spc) on growth of a heterozygote and a sensitive homozygote. Conditions as in Fig. 2, except for use of Spc instead of Str.*

strains (Fig. 2). With Spc, the sensitive homozygote showed a slightly faster and more marked response than the heterozygote (Fig. 3).

Though the heterozygotes and the sensitive homozygotes had a similar MIC, there was some difference in the kinetics of the response to higher concentrations of either antibiotic (50 μg of Str/ml, 150 μg of Spc/ml). With Str (Fig. 4), the time of onset of inhibition was similar in the various strains, but secondary lysis, seen with the sensitive homozygote, was slower with the heterozygotes (FS9D, FS12D) carrying *str*^{r} on the chromosome and was not seen at all (in 5.5 hr) with the heterozygotes (FS5D, FS15D) carrying *str*^{r} on the episome. With Spc (Fig. 5), the homozygote ceased growing by 1 hr, but all heterozygotes continued to grow slowly for the observed 5.5 hr; again, resistance was greater when the resistant allele was on the episome (FS9D, FS14D) rather than on the chromosome (FS5D, FS13D).

Inhibition of protein synthesis. The kinetics of inhibition of protein synthesis was studied under the same conditions. (Since the label was added to growing cultures only shortly before the Str or Spc, the limited incorporation in the presence of the inhibitor appears in exaggerated dimensions on the exponential plot used.)

As Fig. 6 shows, after the addition of Str (50 μg/ml), the sensitive homozygote (FS10D) was completely inhibited within 20 to 60 min, whereas the episome-resistant heterozygotes (FS5D and FS15D) continued to incorporate ^{14}C-leucine slowly for at least 4 hr; the chromosome-resistant

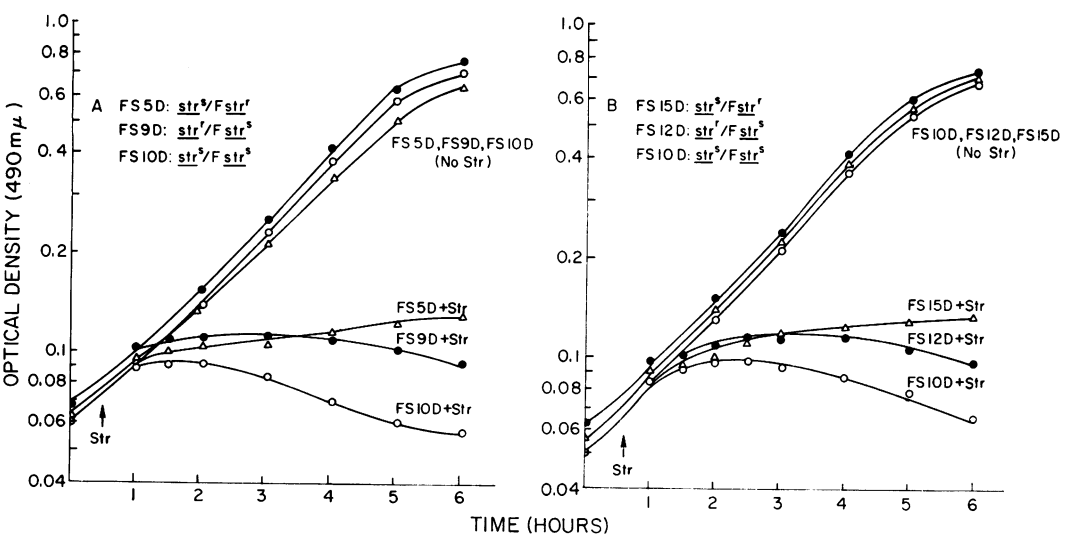

FIG. 4. *Effect of streptomycin (Str) on growth of heterozygotes and a sensitive homozygote. With each strain, 50 μg of Str/ml (10 times the MIC) was added to one half of a culture growing as in Fig. 2.*

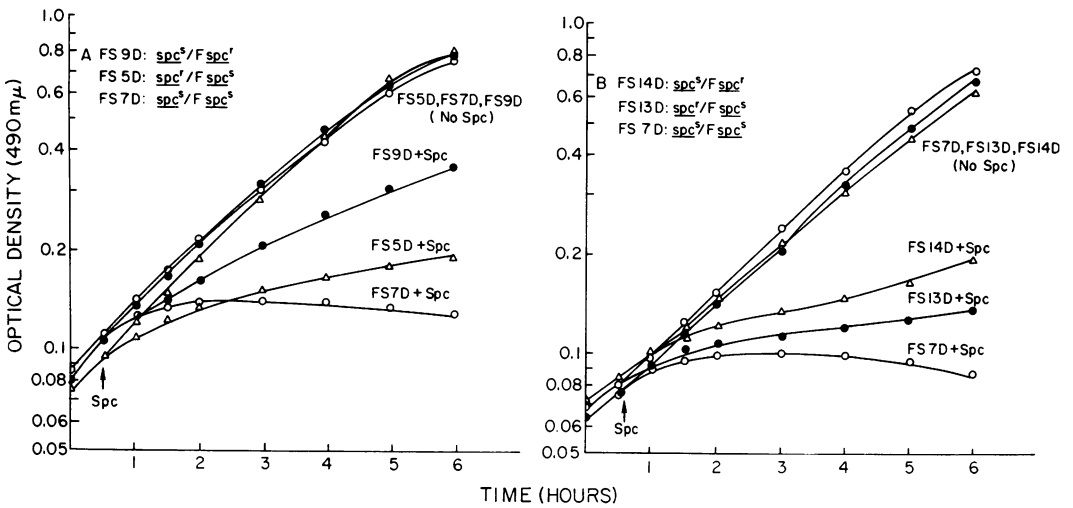

FIG. 5. *Effects of spectinomycin (Spc) on growth of heterozygotes and a sensitive homoyzygote. Experiment as in Fig. 4, except for the strains and the addition of Spc (150 μg/ml) instead of Str. This concentration is two to five times the MIC for the various strains (Table 1).*

heterozygotes (FS9D and FS12D) were intermediate in their response. Thus, during 4 hr in the presence of Str, the sensitive homozygote incorporated only 6%, and the inhibited heterozygotes 8 to 17%, of the amount incorporated by the parallel uninhibited control.

With Spc (Fig. 7), the results were roughly similar, except that the inhibition did not have the lag characteristic of Str, and it was less complete, even for the sensitive homozygote. In 4 hr after the addition of Spc, the *spc*^{s} homozygote (FS7D) incorporated only 3%, and the heterozygotes 7 to 22%, of the amount incorporated by the uninhibited controls.

Killing of heterozygotes by Str. As Fig. 8 shows, the *str*^{s}/*str*^{r} heterozygotes exhibited somewhat less killing by 50 μg of Str/ml in 3 hr (10^{-3} to $10^{-4.2}$ survivors) than that observed with the sensitive homozygote ($10^{-4.4}$ to $10^{-4.7}$ survivors), but the response was nevertheless large. As with the kinetics of protein synthesis, strains with *str*^{r} on the episome (FS5D, FS15D) respond more slowly than those with *str*^{r} on the chromosome (FS9D, FS12D).

Dissociation of protein synthesis and killing. Comparison of the effect of Str on protein synthesis (Fig. 6) and viability (Fig. 8), in the same experiment, shows that in those heterozygotes with the more fully expressed (episomal) resistance substantial protein synthesis continued (to a degree somewhat obscured by the exponential plot) for some time after 99% of the cells had been killed. Indeed, with strain FS5D the synthesis between 1.1 hr (the time of 99% killing) and 4.0 hr nearly equaled that during the initial 1.1 hr, and the curve of incorporation had not completely leveled off at 4.0 hr.

DISCUSSION

It has been suggested (7) that the dominance of sensitivity to Str in a heterozygote is due to blockade of resistant ribosomes behind inhibited sensitive ribosomes in polysomes. However, Str was recently shown to cause gradual breakdown of polysomes, in the cell (10) as well as in extracts (12); hence, it seems unlikely to produce a permanent blockade. Nevertheless, an initial blockade might be important in its inhibitory action. To throw light on this possibility, we have compared the kinetics of inhibition by Str and by Spc, which also acts on the small ribosomal subunit, selects for highly resistant mutants (4), and exhibits dominance of sensitivity over resistance in heterozygotes (2). Spc undoubtedly causes polysomal blockade, since it is purely bacteriostatic and inhibits chain extension; moreover, it has been shown to stabilize polysomes, in heterozygotes (B. J. Wallace, *personal communication*) as well as in sensitive cells (6). Both Str and Spc are known to inhibit growth and protein synthesis in sensitive cells only gradually; the lag presumably reflects the kinetics of entry, since in vitro an adequate concentration of Str (11) or of Spc (1) immediately and quite completely inhibits polysomes formed on natural messenger.

Either antibiotic inhibited both protein synthesis and growth somewhat more slowly in heterozygotes than in fully sensitive homozygotes.

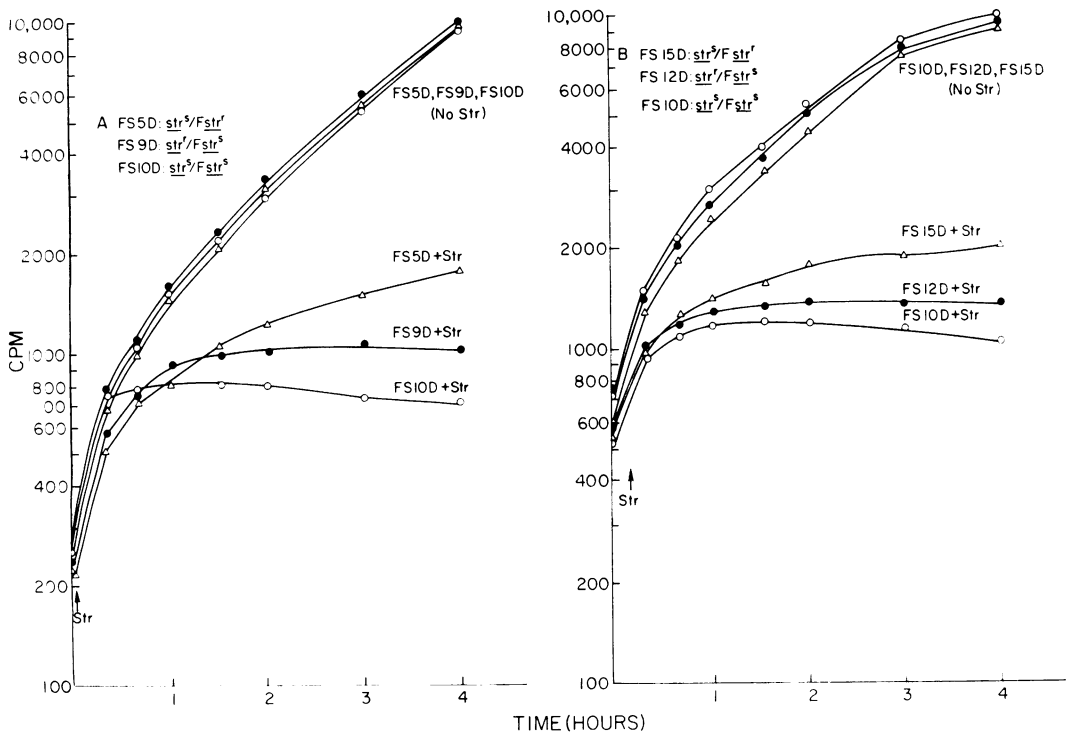

FIG. 6. *Effect of streptomycin (Str) on protein synthesis in heterozygotes and a sensitive homozygote. To cultures growing as in Fig. 2, ^{14}C-leucine was added (0.01 μCi per 10 μg per ml) when the optical density was approximately 0.06; Str (50 μg/ml) was added to half of each culture 60 min later, when the optical density was approximately 0.10. Samples were analyzed for incorporated ^{14}C as described in Materials and Methods.*

Moreover, the slowing was more pronounced (Fig. 6 and 7) in strains with the resistant allele on the episome rather than on the chromosome, paralleling the somewhat higher proportion of resistant ribosomes in the former strains (ca. 40% versus 30%; 14). The model of polysomal blockade fits these differences, since the cumulative blockade of resistant behind sensitive ribosomes is necessarily a gradual process, and it is not surprising that this slowing is more pronounced the higher the proportion of resistant units. The similarity in the kinetics of inhibition by Str and by Spc, both in sensitive and in heterozygous strains, suggests that Str may initially cause polysomal blockade, even though the Str-inhibited ribosome does not remain permanently fixed on the messenger.

The K-12 heterozygotes used in this study are shown to be rapidly killed by Str. Any proposed mechanism for the lethal action of Str must be able to account for this dominance of sensitivity to killing, as well as for the contrasting observations of Breckenridge and Gorini (3) in a heterozygote of *E. coli* B. This strain exhibited only 0 to 90% killing in 1 to 5 hr with 100 μg of Str/ml, which led to the conclusion that Str has a primarily bacteriostatic action in heterozygotes. A possible explanation for the discrepancy is provided by our observations on the effects of varying the ratio of resistant to sensitive ribosomes: killing, like inhibition, was slower in heterozygotes than in an isogenic sensitive homozygote, and the slowing was greater in those K-12 heterozygotes with 40% instead of 30% resistant ribosomes (Fig. 8). It would be interesting to know whether the B-strain heterozygote that exhibited much less killing has a higher proportion of resistant ribosomes.

Since inhibition of ribosomes by Str is followed by gradual release, both from polypeptide-bearing polysomes (10, 12) and from initiation complexes (9, 13), the lethal action of Str in heterozygotes could not be accounted for by blockade if the released ribosomes were inert. Moreover, it is clear that the misreading effect of Str, though invaluable in localizing its action and in probing ribosomal function, also does not account for killing. In further study of the lethal action of Str, Wallace et al. (16) have recently obtained evidence that the Str-ribosomes released from in-

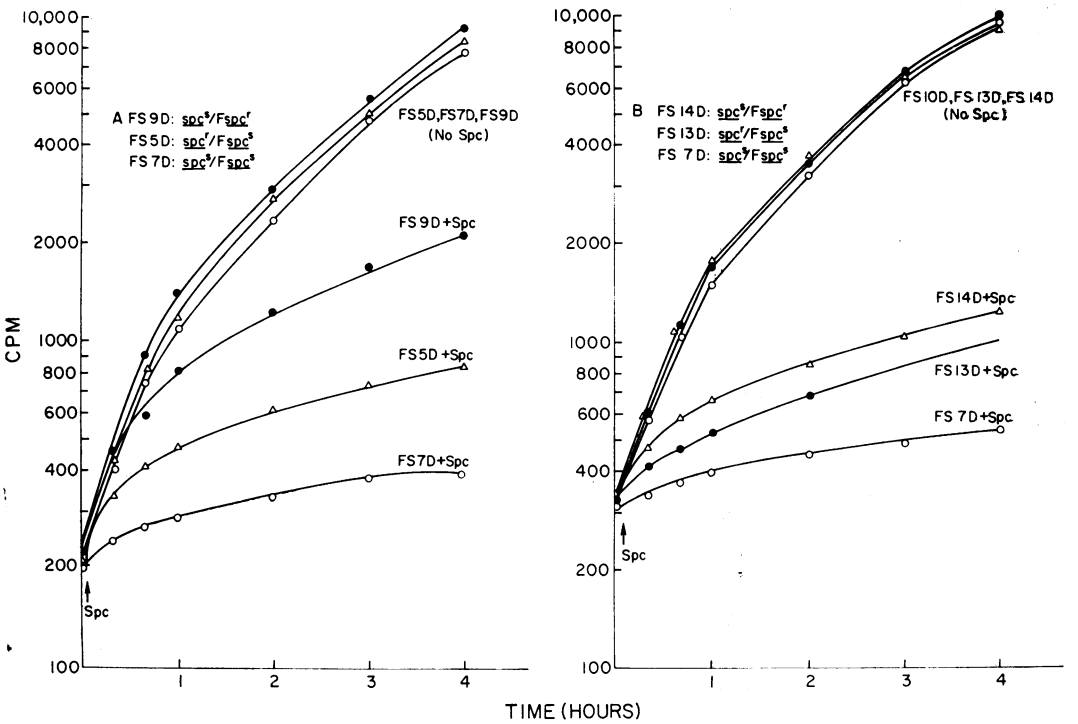

FIG. 7. *Effect of spectinomycin (Spc) on protein synthesis in heterozygotes and a sensitive homozygote. Experiment as in Fig. 6, except for use of different strains and addition of Spc (150 μg/ml) instead of Str.*

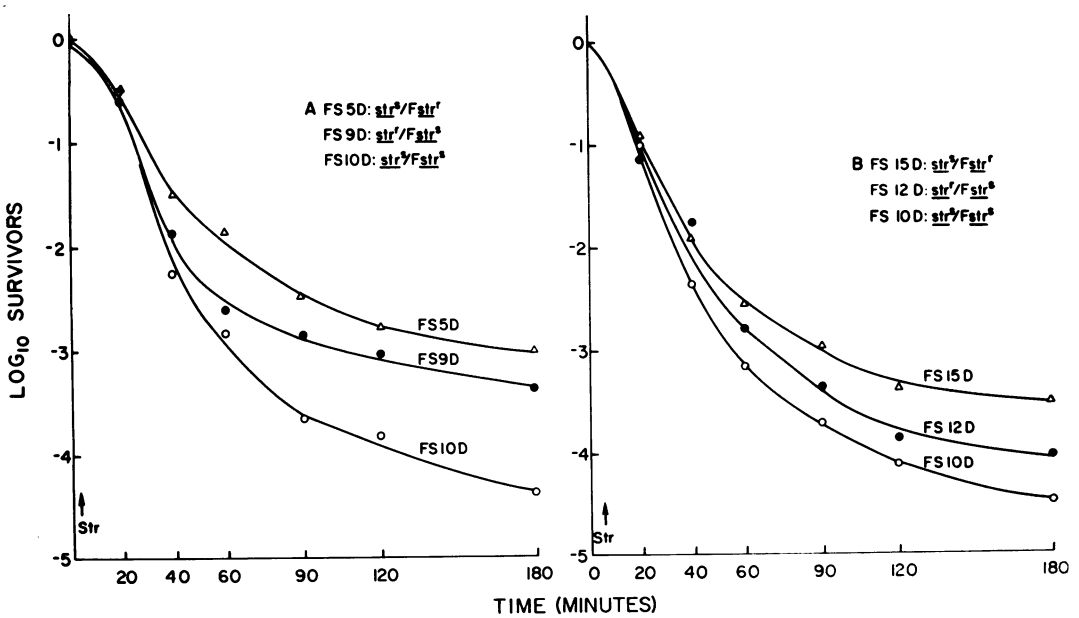

FIG. 8. *Killing by streptomycin (Str) of heterozygotes and a sensitive homozygote. In the experiment of Fig. 6, with 50 μg of Str/ml added to an exponentially growing culture, viability was quantitated at intervals. Survivors were checked for resistant segregants (see Materials and Methods).*

hibited polysomes are not inert: in Str-killed sensitive cells the messenger ribonucleic acid in polysomes turns over rapidly even after protein synthesis has ceased, and in extracts of Str-killed heterozygotes the resistant ribosomes resume activity when provided with fresh messenger ribonucleic acid. It could be concluded that the Str-released ribosomes reinitiate but do not translate. The resulting dynamic, constantly renewed polysomal blockade can account for the present observation that Str can permanently block the activity of the resistant ribosomes in heterozygotes.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Anderson, P., J. Davies, and B. D. Davis. 1967. Effect of spectinomycin on polypeptide synthesis in extracts of *Escherichia coli*. J. Mol. Biol. **29**:203–215.
2. Apirion, D. 1967. Three genes that affect *Escherichia coli* ribosomes. J. Mol. Biol. **30**:255–275.
3. Breckenridge, L., and L. Gorini. 1969. The dominance of streptomycin sensitivity re-examined. Proc. Nat. Acad. Sci. U.S.A. **62**:979–985.
4. Davies, J., P. Anderson, and B. D. Davis. 1965. Inhibition of protein synthesis by spectinomycin. Science **149**:1096–1098.
5. Gorini, L., G. A. Jacoby, and L. Breckenridge. 1966. Ribosomal ambiguity. Cold Spring Harbor Symp. Quant. Biol. **31**: 657–670.
6. Gurgo, C., D. Apirion, and D. Schlessinger. 1969. Polyribosome metabolism in *Escherichia coli* treated with chloramphenicol, neomycin, spectinomycin or tetracycline. J. Mol. Biol. **45**:205–220.
7. Lederberg, E. M., L. Cavalli-Sforza, and J. Lederberg. 1964. Interaction of streptomycin and a suppressor for galactose fermentation in *E. coli* K-12. Proc. Nat. Acad. Sci. U.S.A. **51**:678–682.
8. Lederberg, J. 1951. Streptomycin resistance: a genetically recessive mutation. J. Bacteriol. **61**:549–550.
9. Lelong, J. C., M. A. Cousin, D. Gros, M. Grunberg-Manago, and F. Gros. 1971. Streptomycin induced release of fMet-tRNA from the ribosomal initiation complex. Biochem. Biophys. Res. Commun. **42**:530–537.
10. Luzzatto, L., D. Apirion, and D. Schlessinger. 1969. Polyribosome depletion and blockage of the ribosome cycle by streptomycin in *Escherichia coli*. J. Mol. Biol. **42**:315–335.
11. Modolell, J., and B. D. Davis. 1968. Rapid inhibition of polypeptide chain extension by streptomycin. Proc. Nat. Acad. Sci. U.S.A. **61**:1279–1286.
12. Modolell, J., and B. D. Davis. 1969. Mechanism of inhibition of ribosomes by streptomycin. Nature (London) **224**:345–348.
13. Modolell, J., and B. D. Davis. 1970. Breakdown by streptomycin of initiation complexes formed on ribosomes of *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **67**:1148–1155.
14. Sparling, P. F., J. Modolell, Y. Takeda, and B. D. Davis. 1968. Ribosomes from *Escherichia coli* merodiploids heterozygous for resistance to streptomycin and to spectinomycin. J. Mol. Biol. **37**:407–421.
15. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of *Escherichia coli*, Bacteriol. Rev. **31**:332–353.
16. Wallace, B. J., P. C. Tai, E. L. Herzog, and B. D. Davis. 1971. Ribosomes complexed with streptomycin: dynamic polysomal blockade and impaired response to dissociation factor. Symposium on Molecular Mechanisms of Antibiotic Action, *in press*. Elsevier, Amsterdam.