

Interaction of Cytoplasmic Membrane and Ribosomes in *Escherichia coli*: Spectinomycin-Induced Disappearance of Membrane Protein I-19

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Incubation of *Escherichia coli* with spectinomycin caused the disappearance of a major protein from the cytoplasmic membrane. This protein, called "I-19," was not a ribosomal protein. Its disappearance was not a result of the direct action of spectinomycin on the cytoplasmic membrane, but a result of its action on ribosomes. The disappearance was specifically induced by spectinomycin, and other antibiotics such as neomycin, erythromycin, and chloramphenicol had no effect. Although growth was not required for spectinomycin-induced disappearance of protein I-19 from the cytoplasmic membrane, the disappearance was not observed under conditions where protein synthesis was inhibited completely either by the addition of chloramphenicol or by cooling in ice. It is suggested that at least some ribosomes interact with the cytoplasmic membrane and that a modification of the mode of interaction through the action of spectinomycin on ribosomes caused the deletion of membrane protein I-19.

In a previous paper (10), we showed the following; (i) ribosomes from sucrose-dependent, spectinomycin-resistant ($Suc^d Spc^r$) mutants (8) are resistant to spectinomycin; (ii) a major cytoplasmic membrane protein, I-19, was missing from the membrane in all these mutants; and (iii) such alterations in ribosomes and membrane were coordinated. Since the mutation responsible for the $Suc^d Spc^r$ phenotype has been mapped between the *aroE* and *strA* loci, where a gene cluster for ribosomal proteins has been reported (7, 14), we postulate that the deletion of protein I-19 from the cytoplasmic membrane is mediated by an alteration in the ribosome. If the assumption is correct, one can imagine that an alteration of ribosomal structure induced by spectinomycin in a spectinomycin-sensitive bacterium would also result in the loss of protein I-19 from the cytoplasmic membrane, since spectinomycin is known to interact with ribosomes and to inhibit protein synthesis (1-3, 6, 17).

In this paper, we present evidence supporting the idea that protein I-19 is released from the cytoplasmic membrane as a result of a conformational changes in the ribosomes induced by spectinomycin. The result is discussed in connection with the interaction between ribosomes and cytoplasmic membrane.

MATERIALS AND METHODS

Bacteria and growth conditions. *Escherichia coli* W4626Phe⁻ (*pureE trp phe lac-85 gal-2 mtl xyl-2 mal*

str [λ]), a spectinomycin-sensitive strain (19), and W4626Phe⁻Spc^r10-2 (20) and W4626Phe⁻Spc^r2 (10), spectinomycin-resistant mutants derived from W4626Phe⁻, were used. Bacteria were grown in M3su medium supplemented with 20% (wt/vol) sucrose as described previously (10).

Preparation of membranes and analysis of membrane proteins. Cytoplasmic membranes were prepared according to the method described previously (11) with one modification (10). Briefly, cells from 1 liter of culture were suspended in 18 ml of 20% sucrose instead of water and treated with lysozyme (226 μ g/ml) and ethylenediaminetetraacetate (EDTA) (1.2 mM) for preparing spheroplasts. Spheroplast membranes were obtained by suspending the spheroplasts in 5 mM MgCl₂, and the preparation was dialyzed overnight against 1% EDTA (pH 7.0). The cytoplasmic membrane was separated from the outer membrane by isopycnic sucrose gradient centrifugation.

Polyacrylamide gel electrophoresis of membrane proteins was carried out in 8 M urea-0.5% sodium dodecyl sulfate as described previously (11). All samples were heated at 100°C for 5 min in 1% sodium dodecyl sulfate-1% 2-mercaptoethanol before electrophoresis.

Preparation of ribosomes and assay of in vitro protein synthesis. Ribosomes, S-100 extracts, R17 phage ribonucleic acid, and a mixture of initiation factors were prepared as described previously (9, 10). In vitro polypeptide synthesis with salt-washed ribosomes was carried out according to the method of Wallace et al. (17) with some modifications (10). Radioactivity measurements were carried out in 10 ml of toluene-based scintillation fluid with a Packard 3320 Tri-Carb scintillation spectrometer. Ribo-

somal subunits for gel electrophoretic analysis were prepared from the spheroplast lysate as described previously (10).

Chemicals. Spectinomycin sulfate (specific activity, 611 mg/g) was a generous gift of The Upjohn Co. [^{14}C]valine (specific activity, 225 mCi/mmol) was obtained from Daiichi Pure Chemicals Co. Chloramphenicol and neomycin sulfate were from Sigma Chemical Co., and erythromycin was from Dainihon Chemicals Co.

RESULTS

Effect of spectinomycin on protein composition of the cytoplasmic membrane. Spectinomycin is a specific inhibitor of protein synthesis. At the mid-log phase of growth, spectinomycin was added to the culture of *E. coli* W4626Phe⁻ to a final concentration of 100 $\mu\text{g}/\text{ml}$. Bacterial growth measured turbidimetrically continued at a reduced rate during the first 2 h, followed by an almost complete cessation of growth; the cell mass doubled during the 2-h growth period (Fig. 1). Cells obtained by growth in spectinomycin-containing medium were harvested at intervals, and purified cytoplasmic membrane fractions were prepared. The composition of the cytoplasmic membrane protein of these preparations is shown in Fig.

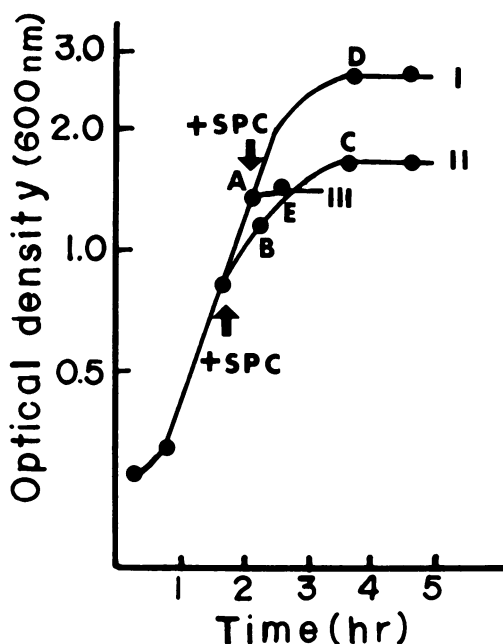


FIG. 1. Inhibition of growth of *E. coli* W4626Phe⁻ by spectinomycin. Spectinomycin was added to cultures at the time indicated by arrows. The concentrations of spectinomycin in curves I, II, and III were 0, 100, and 200 $\mu\text{g}/\text{ml}$, respectively. Cells were harvested at the indicated points (A-E) and used for the preparation of cytoplasmic membrane.

2A-C. It is immediately clear that one major band, I-19, was greatly reduced in the cytoplasmic membrane from spectinomycin-treated cells. Significant disappearance of protein I-19 from the cytoplasmic membrane was seen after a 30-min incubation, and the disappearance was almost complete after 2 h. On the other hand, the profile of other cytoplasmic membrane proteins was essentially not altered even after the 2-h incubation with spectinomycin. Although differences were sometimes observed in protein bands other than I-19, they were not reproducible. From our previous work (10), protein I-19 does not correspond to any ribosomal protein by urea-sodium dodecyl sulfate-gel electrophoresis (Fig. 3). Furthermore, the protein could not be extracted from the cytoplasmic membrane by treatment with either 8 M urea or 1 M KCl or by overnight dialysis against 27 mM disodium EDTA (pH 7.0). These results strongly suggest that protein I-19 is a membrane protein.

A possible explanation for the disappearance is that the phenomenon is not a result of a specific effect of spectinomycin, but a result of the cessation of bacterial growth. To test this possibility, we examined the protein composition of cytoplasmic membrane prepared from cells grown in the absence of spectinomycin and harvested in the stationary phase of growth (Fig. 1D). As shown in Fig. 2D, the amount of protein I-19 in the cytoplasmic membrane was essentially the same as that from the exponentially growing cells (Fig. 2A). Furthermore, the cessation of cell growth caused by the addition of chloramphenicol or by cooling in ice did not result in the disappearance of protein I-19 from the cytoplasmic membrane (see Fig. 7A and 8A). Alternatively, the reduction of protein I-19 content in the membrane could be due to specific inhibition of protein I-19 biosynthesis, since 100 μg of spectinomycin per ml was not sufficient to inhibit completely the net increase in bacterial cell mass, as shown in Fig. 1. However, this possibility was also excluded, because treatment with 200 μg of spectinomycin per ml for 30 min resulted in a significant decrease in the amount of protein I-19 without any significant increase in the cell mass (Fig. 1E and 2E). Thus, we conclude that spectinomycin induced the release of protein I-19 from the cytoplasmic membrane.

The disappearance was specific in that it was induced by spectinomycin but not by neomycin, erythromycin, or chloramphenicol. None of these latter antibiotics induced the disappearance of protein I-19 at concentrations that almost completely inhibited cell growth (data not shown).

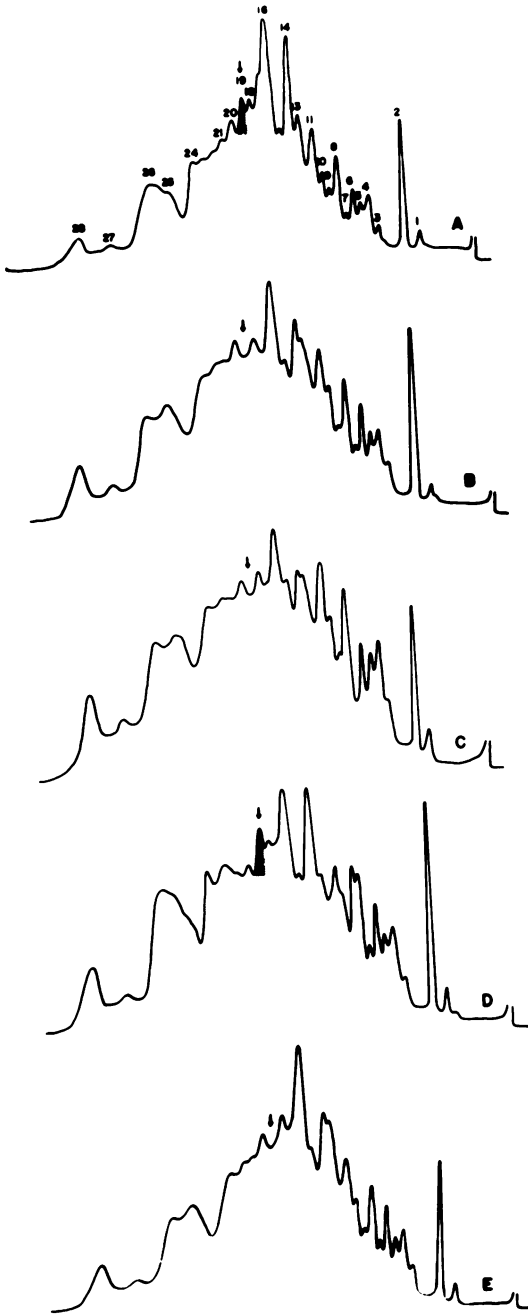


FIG. 2. Scans of polyacrylamide gels of cytoplasmic membrane proteins prepared spectinomycin-treated cells. Cells were harvested at the points A through E indicated in Fig. 1, and cytoplasmic membranes were prepared from the cells. (A) Cells from log-phase growth; (B and C) cells grown in the presence of 100 μg of spectinomycin per ml for 30 and 120 min, respectively; (D) cells from stationary-phase growth; (E) cells grown in the presence of 200 μg of spectinomycin per ml for 30 min. Arrows indicate

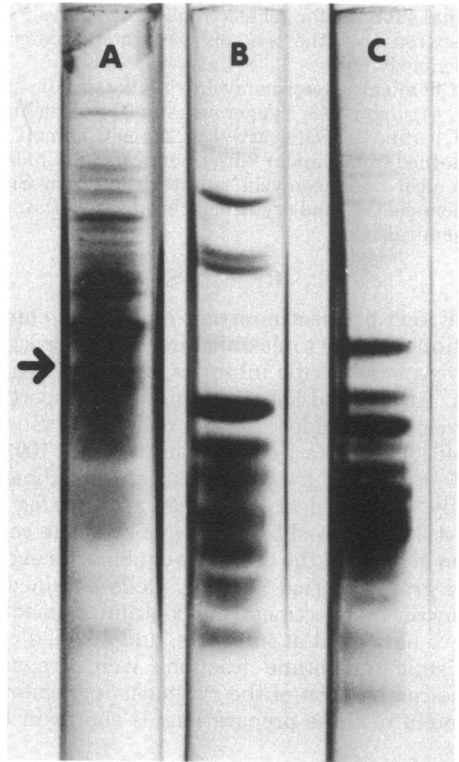


FIG. 3. Urea-sodium dodecyl sulfate-gel electrophoretic patterns of cytoplasmic membrane and ribosomal subunits. (A) Cytoplasmic membrane; (B) 30S ribosomal subunit; (C) 50S ribosomal subunit. Arrow indicates band position of protein I-19.

Effect of spectinomycin on protein composition of the cytoplasmic membrane fraction from spectinomycin-resistant (Spc^r) mutants. Although the only known target of spectinomycin action is the ribosomes, it has not yet been excluded that spectinomycin may also act directly on the cytoplasmic membrane. To test this possibility, the effect of spectinomycin on the protein composition of the cytoplasmic membrane of Spc^r mutants was analyzed. Ribosomes from the Spc^r mutant (W4626Phe⁻ Spc^r 10-2) were resistant to spectinomycin, indicating that the ribosomes were free from spectinomycin action (Fig. 4). Then the effect of spectinomycin on protein I-19 in the cytoplasmic membrane of the Spc^r mutant was examined. We showed in a previous paper (10) that a Spc^r mutant has protein I-19 in the cytoplasmic membrane. Figure 5 shows that protein I-19 in the cytoplasmic membrane from a Spc^r mutant was not affected by the 2-h incubation with

band position of protein I-19. Gel origin is to the right.

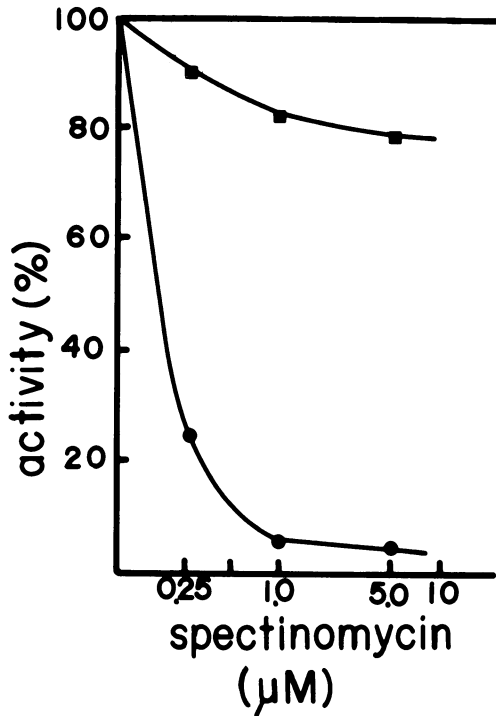


FIG. 4. Sensitivity to spectinomycin of salt-washed ribosomes prepared from a *Spc^r* mutant. *In vitro* polypeptide synthetic activity was assayed with salt-washed ribosomes prepared from W4626Phe⁻ (●) (4,572 cpm) and W4626Phe⁻Spc^r10-2 (■) (3,151 cpm). S-100 fraction and a mixture of initiation factors were prepared from the wild-type strain (W4626Phe⁻). Numbers in parentheses show radioactivity incorporated in the absence of spectinomycin. Radioactivity incorporated without ribosomes has been subtracted.

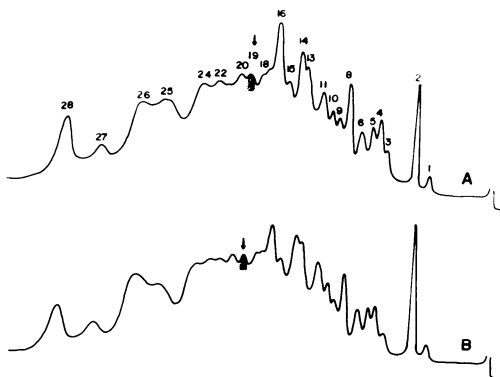


FIG. 5. Effect of spectinomycin on composition of cytoplasmic membrane protein of a *Spc^r* mutant. Cytoplasmic membrane prepared from W4626Phe⁻Spc^r10-2, which had been cultured in the presence (B) or absence (A) of 100 µg of spectinomycin per ml, was analyzed by polyacrylamide gel electrophoresis. Arrows indicate the band position of protein I-19. Gel origin is to the right.

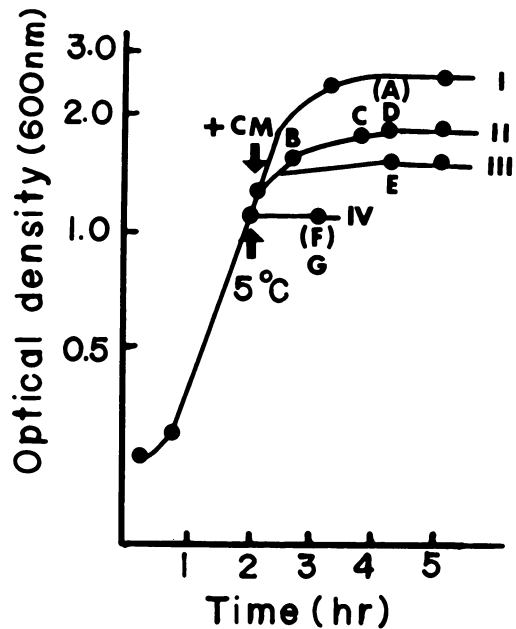


FIG. 6. Effect of chloramphenicol or cold treatment on bacterial growth in the presence of spectinomycin. 100 µg (curve II) or 300 µg (curve III) of chloramphenicol per ml was added to cultures at the time indicated by an arrow. After 10 min of cultivation, 100 µg of spectinomycin per ml was added and the cultivation was continued. The growth curves with 100 and 300 µg of chloramphenicol per ml alone (without spectinomycin) were essentially the same as curves II and III, respectively. Another culture was cooled to 5°C within 10 min in ice water at the mid-log phase of growth followed by treatment with 200 µg of spectinomycin per ml, and the culture was kept in ice for 60 min (curve IV). The growth curve in the cold in the absence of spectinomycin was the same as curve IV. Curve I shows a control experiment without spectinomycin. Points A through G indicate the position where cells were harvested for the preparation of cytoplasmic membrane.

spectinomycin. The same result was obtained with another independently isolated spontaneous *Spc^r* mutant (W4626Phe⁻Spc^r2) (10). These results strongly suggest that the deletion of protein I-19 from the cytoplasmic membrane is a result of the action of spectinomycin on ribosomes.

Effect of inhibition of protein synthesis on spectinomycin-induced disappearance of protein I-19 from the cytoplasmic membrane. To examine the relationship between the disappearance of protein I-19 and ribosome function, the effect of chloramphenicol or cold treatment of cells on spectinomycin-induced disappearance of protein I-19 was studied. In one experiment, chloramphenicol was added to the culture 10 min before the addition of spectinomy-

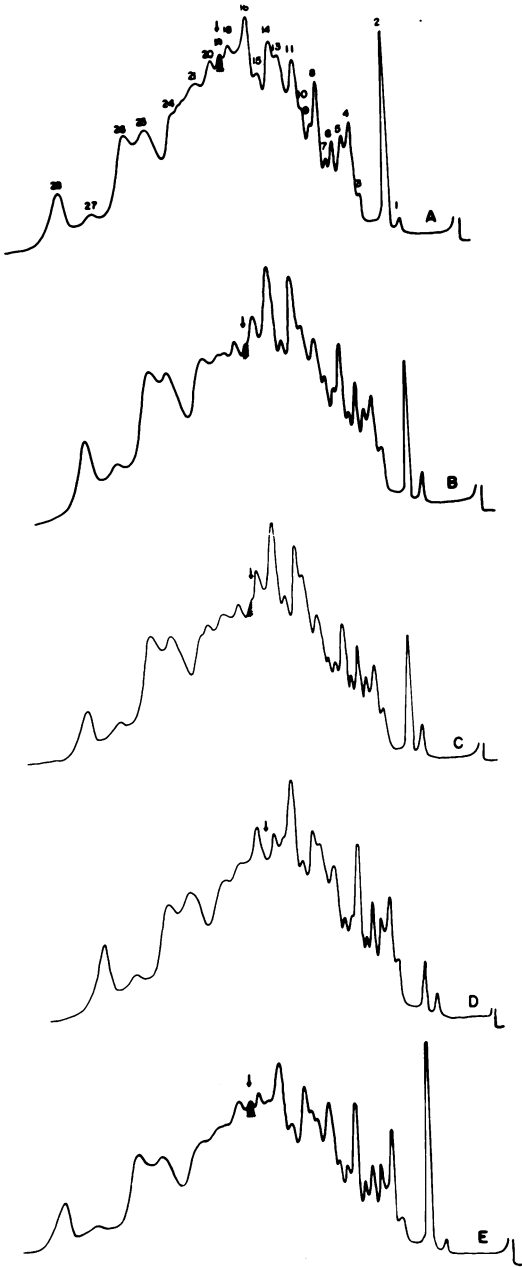


FIG. 7. Effect of chloramphenicol on the spectinomycin-induced disappearance of protein I-19. Cytoplasmic membranes were prepared from cells harvested at positions A through E indicated in Fig. 6. (A) Cells grown in the presence of 100 μg of chloramphenicol per ml for 120 min; (B to D) cells grown in the presence of both 100 μg of chloramphenicol per ml and 100 μg of spectinomycin per ml for 30, 90, and 120 min, respectively; (E) cells grown in the presence of both 300 μg of chloramphenicol and 100 μg of spectinomycin per ml for 120 min. Membrane preparations were analyzed by polyacrylamide gel electro-

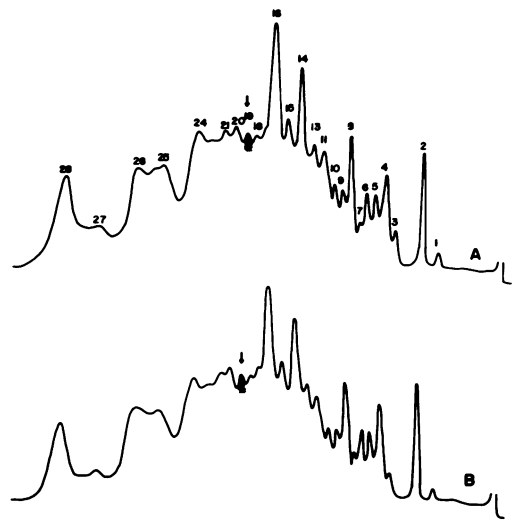


FIG. 8. Effect of cold treatment on spectinomycin-induced disappearance of protein I-19. Cells were cooled to 5°C, treated with (B) and without (A) spectinomycin, and harvested at positions G and F, respectively, indicated in Fig. 6. Cytoplasmic membranes were prepared and analyzed by polyacrylamide gel electrophoresis. Arrows indicated band position of protein I-19. Gel origin is to the right.

cin, and cultivation was continued. Although 100 μg of chloramphenicol per ml strongly inhibited the growth of cells, the inhibition was incomplete, suggesting that the ribosomes were still functioning slowly under these conditions (Fig. 6, curve II). Treatment with chloramphenicol resulted in a delay of the disappearance of protein I-19 from the cytoplasmic membrane (Fig. 7A-D). When the concentration of chloramphenicol was increased to 300 $\mu\text{g}/\text{ml}$, bacterial growth stopped almost completely and the spectinomycin-induced disappearance of protein I-19 was not observed (Fig. 6, curve III; Fig. 7E). In another experiment, 100 μg of spectinomycin per ml was added first, and chloramphenicol was added after 1.5 min. Irrespective of the order of addition of antibiotic, chloramphenicol showed the same effect on the disappearance of protein I-19; i.e., the disappearance of protein I-19 was inhibited partially with 100 μg of chloramphenicol and completely with 300 μg of chloramphenicol per ml. Essentially the same result was obtained when the culture was cooled to 5°C at the mid-log phase of growth to stop cell growth completely (Fig. 6, curve IV) and treated with 200 μg of spectinomycin per ml at 5°C for 60 min (Fig. 8).

phoresis. Arrows indicate band position of protein I-19. Gel origin is to the right.

DISCUSSION

In the present paper, we show that spectinomycin caused the deletion of a protein, I-19, from the cytoplasmic membrane. The protein does not correspond to any of the ribosomal proteins characterized by gel electrophoresis. Furthermore, the effect of spectinomycin on the cytoplasmic membrane protein was not a result of the direct action of spectinomycin on the cytoplasmic membrane, but a result of its action on ribosomes, since the disappearance of protein I-19 was not observed in the *Spc^r* mutants, in which the ribosome was shown to be resistant to antibiotic action. Moreover, the spectinomycin-induced disappearance of protein I-19 from the cytoplasmic membrane was not a result of the specific inhibition of protein I-19 synthesis by the antibiotic.

We have examined the action of spectinomycin, neomycin, erythromycin, and chloramphenicol, which are known to act on ribosomes. Each antibiotic has a specific target on ribosomes. Chloramphenicol and erythromycin interact with 50S subunits (18); spectinomycin and neomycin also interact with 30S subunits, but their modes of action differ, indicating that they probably interact with different sites on the 30S subunit. The disappearance of protein I-19 occurred only when bacteria were treated with spectinomycin. These results suggest that the disappearance of protein I-19 could not be ascribed to inhibition of protein synthesis, but that a specific structural change of ribosomes induced by spectinomycin was involved. The action of streptomycin could not be examined, since the strain was resistant to the antibiotic. Binding of spectinomycin to ribosomes is insufficient to cause the disappearance of protein I-19, since the complete inhibition of ribosomal function by chloramphenicol or by cold treatment whether preceding or following spectinomycin treatment suppressed the spectinomycin effect. Although spectinomycin itself strongly inhibits ribosomal function as well as bacterial growth, even 300 μg of spectinomycin per ml was insufficient to inhibit these processes completely.

In a previous paper (10) we showed that the alteration in ribosomes and the lack of protein I-19 in the cytoplasmic membrane of *Suc^d Spc^r* mutants are coordinated; i.e., a single mutation is responsible for these alterations. We also suggested that the *Suc^d Spc^r* mutation primarily caused a structural alteration in a ribosomal constituent, which, in turn, resulted in the lack of protein I-19 in the cytoplasmic membrane. The altered ribosomal constituent is now tentatively identified with two *Suc^d Spc^r* mutants.

They are S-5 in strain YM93 and S-4 in strain YM50 (M. Dombou, T. Mizuno, and S. Mizushima, manuscript in preparation). It should be emphasized that the alteration in the ribosomes caused by the *Suc^d Spc^r* mutation and that caused by treatment of the wild-type strain with spectinomycin produced the same effect in the composition of cytoplasmic membrane protein. It is highly probable, therefore, that the *Suc^d Spc^r* mutation and spectinomycin cause some alteration in a certain part of ribosomes that is important for interaction with the cytoplasmic membrane. Although several workers have studied the role of ribosomes in the membrane fraction of bacteria (4, 5, 13, 15, 16), structural and functional relationships between ribosomes and the cytoplasmic membrane have not been well established. Two independent results discussed here (10; this paper) can be best interpreted by the existence of structural interaction between ribosomes and the cytoplasmic membrane. Cytoplasmic membrane protein I-19 may play an important role in the interaction.

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