

## Coordinated Alterations in Ribosomes and Cytoplasmic Membrane in Sucrose-Dependent, Spectinomycin-Resistant Mutants of *Escherichia coli*

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Alterations in cytoplasmic membrane and ribosomes from sucrose-dependent spectinomycin-resistant ( $Suc^d-Spc^r$ ) mutants of *Escherichia coli*, mutants that are resistant to spectinomycin in the presence of 20% sucrose but sensitive in the absence of sucrose, were studied. The protein composition of cytoplasmic membrane was analyzed by gel electrophoresis on polyacrylamide gel containing 8 M urea and 0.5% sodium dodecyl sulfate, which assured the reproducible separation of 28 protein bands. A major protein band, I-19, was missing in all cytoplasmic membrane preparations from 10  $Suc^d-Spc^r$  mutants. Besides protein I-19, proteins I-13 and I-24 were missing in some mutants. On the other hand, the protein composition of cytoplasmic membrane from a sucrose-independent spectinomycin-resistant mutant was indistinguishable from that from the wild-type strain. The polypeptide synthetic activity of ribosomes from  $Suc^d-Spc^r$  mutants was resistant to spectinomycin. Studies on a revertant obtained from one of these mutants without any selection for sensitivity to spectinomycin revealed that a single mutation was responsible for both the ribosomal alteration, i.e., spectinomycin resistance, and the lack of protein I-19 in the cytoplasmic membrane. Studies on a transductant obtained with a  $Suc^d-Spc^r$  mutant as the donor also confirmed the single-mutation concept. It was concluded that in  $Suc^d-Spc^r$  mutants an alteration in the ribosomes caused the deletion of protein I-19 from cytoplasmic membrane.

Cytoplasmic membrane and ribosomes are major organelles in bacterial cells. These organelles have been studied independently to determine their separate functions. But evidence has also accumulated in support of the view that these separate functions are interrelated. For example, the structural interaction between cytoplasmic membrane and ribosomes (4, 18, 20, 21) and a specific function of the ribosomes bound to cytoplasmic membrane (3) have been reported. In higher organisms, the existence of ribosomes bound to endoplasmic reticulum and their specific role in the formation of particular proteins have been well established (7, 16, 17).

Spectinomycin-resistant mutants of *Escherichia coli* are interesting from this point of view. Some spectinomycin-resistant mutations affect the stability of sex factors (23), and others make the assembly of the 50S subunit as well as the 30S subunit of ribosomes sensitive to cold (13). These phenomena could be explained by

assuming some interaction between ribosomes and cytoplasmic membrane. Recently, another type of spectinomycin-resistant mutant, a sucrose-dependent, spectinomycin-resistant ( $Suc^d-Spc^r$ ) mutant, was isolated by Miyoshi and Yamagata (10). These mutants can grow in the presence of spectinomycin when the medium is supplemented with 20% sucrose. The mutants seemed to be very useful for the elucidation of the structural and functional interaction between ribosomes and cytoplasmic membrane for the following reasons: (i) they were resistant to spectinomycin, which inhibits protein synthesis on ribosomes, and the mutation was mapped between the *aroE* and *strA* loci where a gene cluster for ribosomal proteins has been reported (9, 19), suggesting an alteration in the ribosomes of these mutants; (ii) mutant cells were hypersensitive to antibiotics, dyes, and detergents, indicating alterations in the cell envelope; and (iii) on the basis of genetic

analysis, it was considered that a single mutation might be responsible for the two alterations.

By the investigation to be described, we revealed an alteration in the ribosomes and the deletion of one major protein from cytoplasmic membrane prepared from *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants. Such alterations were coordinated, suggesting some functional or structural interaction between ribosomes and cytoplasmic membrane.

#### MATERIALS AND METHODS

**Bacteria and growth conditions.** All *Suc<sup>d</sup>-Spc* mutants of *E. coli* and strains derived from the mutants were isolated by Miyoshi and Yamagata on nutrient agar plates containing 20% sucrose and 100  $\mu$ g of spectinomycin per ml (10). A sucrose-independent spectinomycin-resistant (*Spc<sup>r</sup>*) mutant was isolated from *E. coli* W4626 *phe<sup>-</sup>* spontaneously. All strains discussed in this communication are listed in Table 1.

Cells were grown in a reciprocating shaker at 37 C in medium M<sub>50</sub> (Difco antibiotic medium no. 3 supplemented with 10  $\mu$ g of adenine per ml, 40  $\mu$ g of tryptophan per ml, and 20% [wt/vol] sucrose). The cells were harvested at the late exponential phase of growth, washed once with 20% sucrose solution, and used for the preparation of membranes and ribosomes.

**Preparation and analysis of membranes.** Outer and cytoplasmic membranes were prepared according to the method described previously (12), with one modification. Cells from 1 liter of culture were suspended in 18 ml of 20% sucrose instead of water and were used for the preparation of spheroplasts.

For preparation of the total-membrane fraction, spheroplasts prepared by the method of Mizushima and Yamada (12) were recovered by centrifugation at 93,000  $\times$  *g* for 1 h. By centrifugation the outer membranes that had been released from the cells were also recovered in the pellet. Spheroplasts thus recovered were homogenized in 5 mM MgCl<sub>2</sub> containing 1  $\mu$ g of deoxyribonuclease per ml and centrifuged under the same conditions. The supernatant was used as the cytoplasmic protein fractions. The pellet was suspended in water, dialyzed against 1% ethylenediaminetetraacetic acid (pH 7.0) overnight at 4 C, recovered by centrifugation under the same conditions, and used as the total-membrane fraction.

Polyacrylamide gel electrophoresis of membrane proteins was carried out as described previously (12).

**Preparation of ribosomes and assay of in vitro polypeptide synthesis.** Ribosomes were prepared as previously described (11) and salt-washed in a mixture of 1 M NH<sub>4</sub>Cl, 30 mM MgCl<sub>2</sub>, 6 mM  $\beta$ -mercaptoethanol, and 10 mM tris(hydroxymethyl)amino-methane-hydrochloride (pH 7.8) overnight at 4 C. "S 100" extracts were obtained from S 30 extracts (11) of the wild-type strain by centrifugation at 93,000  $\times$  *g* for 6 h. A crude mixture of initiation factors was obtained from the first wash of wild-type ribosomes with 1 M NH<sub>4</sub>Cl (8). In vitro polypeptide synthesis was carried out according to the method of Wallace et al. (22) with some modifications: 9.9 mM magnesium acetate, 24  $\mu$ g of R17 phage ribonucleic acid, and 30  $\mu$ g of a crude mixture of initiation factors were used. The polypeptide synthetic activity was stimulated 10-fold with the crude mixture of initiation factors. Radioactivity measurements were carried out in 10 ml of toluene scintillation fluid with a Packard 3320 Tri-Carb scintillation spectrometer.

TABLE 1. Strains of *E. coli* used

Strain	Genotype and relevant characteristics	Reference
W4626 <i>phe<sup>-</sup></i>	<i>F<sup>-</sup>purE<sup>-</sup> trp<sup>-</sup> phe<sup>-</sup> lac<sup>-88</sup> gal<sup>-2</sup> xyl<sup>-2</sup> mal<sup>-</sup> ara<sup>-</sup> str<sup>r</sup>(<math>\lambda</math>); spectinomycin-sensitive wild-type strain</i>	24
YM22	} <i>Suc<sup>d</sup>-Spc<sup>r</sup></i> mutants from W4626 <i>phe<sup>-</sup></i> ; spectinomycin resistant in the presence of 20% sucrose	} 10
YM26		
YM37		
YM50		
YM54		
YM69		
YM84		
YM85		
YM93	} Spontaneous revertant from YM50	} 10
YM101		
YM50R3		
W4626 <i>phe<sup>-</sup> Spc<sup>r</sup> 2</i>	Sucrose-independent, spectinomycin-resistant ( <i>Spc<sup>r</sup></i> ) mutant from W4626 <i>phe<sup>-</sup></i>	This article
AB2834	<i>F<sup>-</sup>thi<sup>-</sup> aroE<sup>-</sup> mala<sup>-</sup> T6<sup>r</sup></i>	15
AB2834 <i>Suc<sup>d</sup>-Spc<sup>r</sup> 50</i>	Transductant of AB2834 from YM50 selected for spectinomycin resistance	10

YM69

**Chemicals.** Spectinomycin sulfate was a generous gift of Upjohn Co. [ $^{14}\text{C}$ ]valine with a specific activity of 225 mCi/mmol was obtained from Daiichi Pure Chemicals Co.

## RESULTS

**Analysis of cytoplasmic membrane proteins by gel electrophoresis.** The protein composition of cytoplasmic membranes of *E. coli* W4626 *phe*<sup>-</sup> (wild-type strain) and *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutants was analyzed by electrophoresis in polyacrylamide gel containing 8 M urea and 0.5% sodium dodecyl sulfate. A typical profile of cytoplasmic membrane proteins from the wild-type strain is shown in Fig. 1A. Twenty-eight protein bands were reproducibly observed in all preparations examined. Individual bands were tentatively numbered from I-1 to I-28. Cytoplasmic membranes were prepared from three typical *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutants, YM50, YM69, and YM101, and protein composition was compared with that from the wild-type strain (Fig. 1B, C, and D). It is immediately clear that one major

band, I-19, was missing in the cytoplasmic membrane from these mutants. In addition to protein I-19, proteins I-13 and I-24 were missing in YM101 and YM50, respectively. Many other *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutants were similarly examined. As summarized in Table 2, protein I-19 was missing in cytoplasmic membranes from all the *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutants examined. Therefore, it was concluded that the lack of I-19 in cytoplasmic membrane was related to the *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutation.

Protein I-19 was unique in its color on a gel. This protein band was stained in reddish purple, whereas others around the protein appeared more bluish. This made it much easier to identify the protein band among many others.

The lack of protein I-19 in the membrane of *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutants was confirmed with membrane preparations prepared by different procedures. The total-membrane fraction was prepared from both the wild-type strain and mutants, and the protein composition was analyzed by gel electrophoresis (Fig. 2). A band

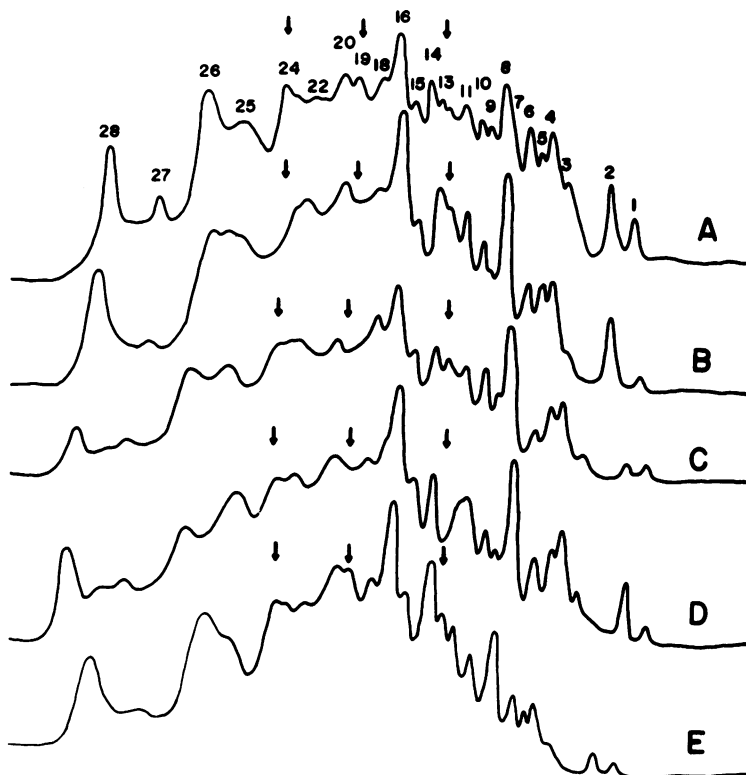


FIG. 1. Scans of polyacrylamide gels of cytoplasmic membrane proteins prepared from *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutants. Cytoplasmic membranes prepared from (A) W4626 *phe*<sup>-</sup> (wild-type strain), (B) YM50, (C) YM69, (D) YM101, and (E) W4626 *phe*<sup>-</sup> *Spc*<sup>2</sup> were heated at 100 C for 5 min in the presence of sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis. Arrows indicate band position of proteins I-13, I-19, and I-24. Tops of gels are to the right.

TABLE 2. Lack of cytoplasmic membrane proteins in *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants

Strain	Protein band of cytoplasmic membrane <sup>a</sup>		
	I-13	I-19	I-24
W4626 <i>phe<sup>-</sup></i>	+	+	+
YM22	+	-	-
YM26	+	-	+
YM37	+	-	+
YM50	+	-	-
YM54	+	-	+
YM69	+	-	+
YM84	+	-	+
YM85	+	-	+
YM93	+	-	-
YM101	-	-	+

<sup>a</sup>Cytoplasmic membranes were prepared from *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants, and protein composition was analyzed by polyacrylamide gel electrophoresis after heating at 100 C for 5 min in the presence of sodium dodecyl sulfate. +, Present; -, absent.

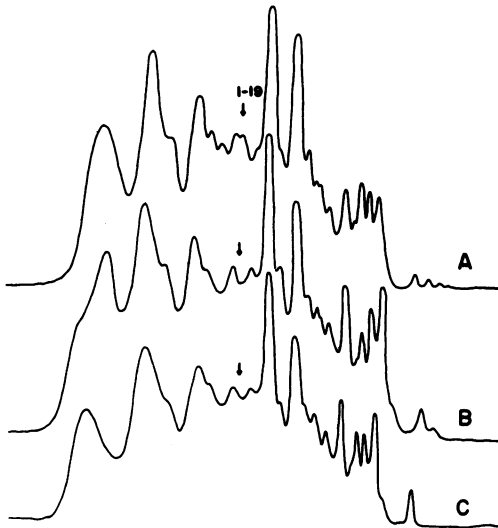


FIG. 2. Scans of polyacrylamide gels of total-membrane fractions. Total-membrane fractions prepared from (A) W4626 *phe<sup>-</sup>* (wild-type strain), (B) YM50, and (C) YM93 were heated at 100 C for 5 min in the presence of sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis. Arrows indicate band position of protein I-19. Tops of gels are to the right.

corresponding to protein I-19 was missing in the preparation from the mutants, but present in that from the wild-type strain. The crude envelope fraction prepared by grinding with alumina gave the same result (data not shown). Protein composition of outer membrane and cytoplasmic protein fractions was also examined on

polyacrylamide gel under the same conditions. No difference was observed between the wild-type strain and *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants (data not shown). Thus, changes in protein composition resulting from *Suc<sup>d</sup>-Spc<sup>r</sup>* mutation were detected only in cytoplasmic membrane preparations, and protein I-19 was the only protein that was missing in all mutants of this kind. A protein band that corresponded to protein I-19 was not detected in outer membrane or ribosome-containing cytoplasmic protein fractions. Examination with polyacrylamide gel also revealed that protein I-19 did not correspond to any ribosomal proteins (data not shown).

**Cytoplasmic membrane proteins of *Spc<sup>r</sup>* mutant.** The *Suc<sup>d</sup>-Spc<sup>r</sup>* mutant is different from the *Spc<sup>r</sup>* mutant (10). To examine whether the lack of protein I-19 in the cytoplasmic membrane is a characteristic feature of the *Suc<sup>d</sup>-Spc<sup>r</sup>* mutation or not, the cytoplasmic membrane from the *Spc<sup>r</sup>* mutant (W4626 *phe<sup>-</sup>-Spc<sup>r</sup>2*) was compared with that from the wild-type strain (Fig. 1E). No difference between the two strains was found in protein composition; i.e., protein I-19 was present in the cytoplasmic membrane of the *Spc<sup>r</sup>* mutant. This result indicates that the *Suc<sup>d</sup>-Spc<sup>r</sup>* mutation is a novel type of spectinomycin-resistant mutation and that the lack of protein I-19 in the cytoplasmic membrane is a characteristic feature of *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants.

**Characterization of ribosomes from *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants.** Some alteration in the ribosomes of *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants was suggested, because the mutation was mapped between the *aroE* and *strA* loci, where a gene cluster for ribosomal proteins has been reported (9, 19). To confirm this hypothesis, we examined salt-washed ribosomes from *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants for sensitivity to spectinomycin by an in vitro assay of polypeptide synthesis. Figure 3 shows that ribosomes from *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants, as well as *Spc<sup>r</sup>* mutants, were resistant to spectinomycin, indicating that the *Suc<sup>d</sup>-Spc<sup>r</sup>* mutation has caused some alteration in ribosomes.

**Coordinated alteration in ribosomes and cytoplasmic membrane.** To examine whether the lack of protein I-19 in the cytoplasmic membrane from *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants is related to an alteration in ribosomes, two critical experiments were undertaken. Since *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants are hypersensitive to various chemicals such as rifampin, actinomycin D, methylene blue, acriflavine, dodecyl sulfate and deoxycholate, it was possible to isolate revertants without any selection for spectinomycin. Some of these revertants were spectinomycin sensitive. The salt-washed ribosomes from one such revertant

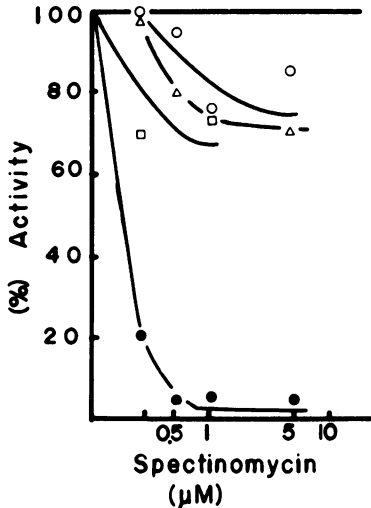


FIG. 3. Sensitivity to spectinomycin of salt-washed ribosomes prepared from *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants. *In vitro* polypeptide synthetic activity was assayed with salt-washed ribosomes prepared from W4626 *phe<sup>-</sup>* (wild-type strain) (4,218 counts/min) (●), YM50 (6,682 counts/min) (○), YM93 (7,116 counts/min) (□), and W4626 *Spc<sup>2</sup>* (3,009 counts/min) (Δ). Numbers in parentheses show radioactivity incorporated in the absence of spectinomycin. Radioactivity incorporated without ribosomes has been extracted.

(YM50R3) were also sensitive to spectinomycin (Fig. 4), and protein I-19 was present in the cytoplasmic membrane (Fig. 5A and B).

The *Suc<sup>d</sup>-Spc<sup>r</sup>* mutation was transducible by phage P1, and the sucrose dependence was not separable from the spectinomycin resistance in the process of transduction (10). One such transductant (AB2834 *Suc<sup>d</sup>-Spc<sup>r</sup> 50*), obtained with YM50 as the donor and AB2834 as the recipient, was characterized in terms of sensitivity of ribosomes to spectinomycin and protein composition of cytoplasmic membrane. Figures 4 and 5C and D show that the alteration in ribosomes and the lack of protein I-19 in cytoplasmic membrane were co-transduced with the *Suc<sup>d</sup>-Spc<sup>r</sup>* mutation. Therefore, it was concluded that these alterations are coordinated.

## DISCUSSION

In the present article, we have described the alteration of ribosomal sensitivity to spectinomycin and lack of protein I-19 in the cytoplasmic membrane in all *Suc<sup>d</sup>-Spc<sup>r</sup>* mutants, with no exceptions. Furthermore, experiments with revertants and transductants indicated that the alterations in both ribosomes and cytoplasmic membrane are coordinated; i.e., a single muta-

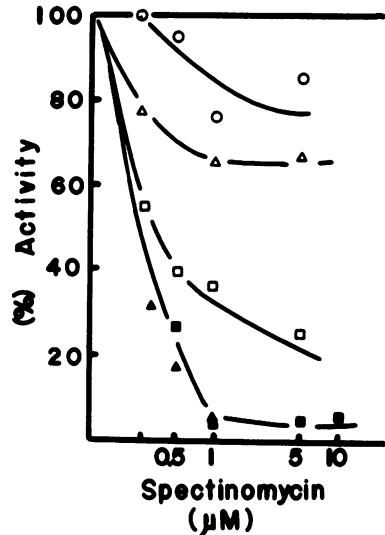


FIG. 4. Sensitivity to spectinomycin of salt-washed ribosomes prepared from revertant YM50R3 and transductant AB2834 *Suc<sup>d</sup>-Spc<sup>50</sup>*. *In vitro* polypeptide synthetic activity was assayed with salt-washed ribosomes prepared from W4626 *phe<sup>-</sup>* (wild-type strain) (4,680 counts/min) (■), YM50R3 (12,154 counts/min) (□), AB2834 *Suc<sup>d</sup>-Spc<sup>50</sup>* (2,485 counts/min) (Δ), and AB2834 (6,829 counts/min) (▲). YM50 (○) was cited from Fig. 3 for comparison. Numbers in parentheses show radioactivity incorporated in absence of spectinomycin. Radioactivity incorporated without ribosomes has been subtracted.

tion, the *Suc<sup>d</sup>-Spc<sup>r</sup>*, is responsible for both alterations.

There are two ways of explaining these phenomena. One possibility is that the *Suc<sup>d</sup>-Spc<sup>r</sup>* mutation primarily causes an alteration in one of the ribosomal constituents, which in turn results in the lack of protein I-19 in cytoplasmic membrane; another possibility is that a primary alteration in cytoplasmic membrane causes an alteration in the ribosomes. We favored the first possibility, because the following evidence suggests that the primary alteration is related to ribosomes: (i) the mutation was mapped between the *aroE* and *strA* loci where a gene cluster for ribosomal proteins has been demonstrated, and (ii) the only known target of spectinomycin action is the ribosome (14). Assuming that the alteration in the ribosomes is a primary event caused by the mutation, one can possibly explain the lack of protein I-19 in the cytoplasmic membrane from the mutants as follows. The assembly of protein I-19 in the cytoplasmic membrane is stabilized by ribosomes, and an alteration in the ribosomes causes the deletion of protein I-19 from the

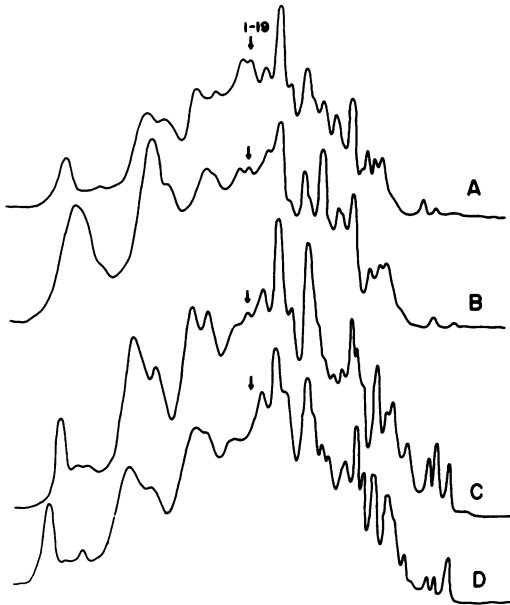


FIG. 5. Scans of polyacrylamide gels of cytoplasmic membrane proteins prepared from revertant YM50R3 and transductant AB2834 *Suc*<sup>d</sup>-*Spc*<sup>r</sup>50. Cytoplasmic membranes prepared from (A) W4626 *phe*<sup>-</sup> (wild-type strain), (B) YM50R3, (C) AB2834, and (D) AB2834 *Suc*<sup>d</sup>-*Spc*<sup>r</sup>50 were heated at 100 C for 5 min in the presence of sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis. Arrows indicate band position of protein I-19. Tops of gels are to the right.

cytoplasmic membrane. This possibility was supported by another finding: the treatment of the wild-type strain with spectinomycin caused the deletion of protein I-19 from the cytoplasmic membrane (T. Mizuno, H. Yamagata, and S. Mizushima, unpublished data). This deletion could be interpreted as a result of the spectinomycin-induced conformational change in the ribosomes, because such a deletion was not observed on a sucrose-independent *Spc*<sup>r</sup> mutant whose ribosomes were resistant to the antibiotic.

Assuming that the *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutation primarily alters ribosomes and that the alteration affects the assembly of protein I-19 in cytoplasmic membrane, protein I-19 might be detectable in some cell fractions from the mutants. So far, we have failed to find the protein in fractions of outer membrane, cytoplasmic protein, and ribosomes. However, this does not necessarily mean that those fractions are free of the protein. For example, since the total amount of proteins in the cytoplasmic protein fraction was about eight times as much as that in the cytoplasmic membrane fraction, it would

be difficult to detect the protein in the fraction by gel electrophoresis under the conditions used in the present study. Although protein I-19 was not found in the isolated cytoplasmic membrane, we are not sure whether the protein is also missing in the cytoplasmic membrane in living cells. If the possible interaction of ribosomes with cytoplasmic membrane via protein I-19 is essential for the bacterial growth, the protein has to be localized close to the cytoplasmic membrane even in the mutants. The relationship between the biochemical alterations described in the present article and the sucrose dependence of the mutants has not yet been studied. Also, we have not yet studied other proteins lacking in some of the mutants.

It has been demonstrated that the ribosomal protein conferring spectinomycin sensitivity controlled by the *spcA* locus is ribosomal protein S5 (1, 2, 5), and either valine or serine in S5-peptide T10 of the wild-type strain is changed to glutamic acid or proline, respectively, in *SpcA* mutants (6). Since the *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutant is clearly different from the *Spc*<sup>r</sup> mutant, the altered site of ribosomes in the *Suc*<sup>d</sup>-*Spc*<sup>r</sup> mutant must also be different from that in the *Spc*<sup>r</sup> mutant. The identification of a ribosomal component responsible for the mutation is in progress in our laboratory.

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