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Interaction of the Cytoplasmic Membrane and Ribosomes in *Escherichia coli*; Altered Ribosomal Proteins in Sucrose-Dependent Spectinomycin-Resistant Mutants

Munehiko Dombou, Takeshi Mizuno*, and Shoji Mizushima
Department of Agricultural Chemistry, Nagoya University, Chikusa-ku, Nagoya 464 Japan

Summary. Alterations in the ribosomes of sucrose-dependent spectinomycin-resistant (Suc^d-Spc^r) mutants of *Escherichia coli* were studied. Subunit exchange experiments showed that 30S subunits were responsible for the resistance of ribosomes to spectinomycin in all Suc^d-Spc^r mutants tested.

Proteins of 30S ribosomes were analyzed by carboxymethyl cellulose column chromatography based on their elution positions. Mutants YM22 and YM93 had an altered 30S ribosomal protein component, S5, and mutant YM50 had an altered protein, S4. Although a shift of elution position was not detected for all the 30S ribosomal proteins from mutant YM101, the amount of protein S3 was appreciably lowered in the isolated 30S subunits. A partial reconstitution experiment with protein S3 prepared from both the wild-type strain and YM101 revealed that the mutant had altered protein S3 which is responsible for the spectinomycin resistance.

These alterations in 30S subunits are discussed in relation to the interaction between ribosomes and the cytoplasmic membrane.

Introduction

Starting with the identification of the ribosomal protein responsible for the streptomycin resistance (Ozaki, Mizushima and Nomura, 1969), extensive analyses on the alteration of ribosomal proteins in the antibiotic resistant strains have been carried out. Spectinomycin resistance in *Escherichia coli* has also been extensively studied and specific amino acid residues in ribosomal protein S5 were found to be responsible for the resistance (Bollen et al., 1969; Funatsu et al., 1971). However, all of these alterations in ribosomal proteins have been discussed only in relation to the ribosomal function, that is protein synthesis.

Recently, another type of spectinomycin resistant mutant, sucrose-dependent spectinomycin-resistant (Suc^d-Spc^r) mutants, were isolated by Miyoshi and Yamagata (1976). These mutants can grow in the presence of spectinomycin only when the medium is supplemented with 20% sucrose. In a previous paper (Mizuno et al., 1976), we showed that a single mutation was responsible for alterations in both the cytoplasmic membrane and the ribosomes in the mutants. In these mutants, a specific cytoplasmic membrane protein, I-19, was missing and the polypeptide synthetic activity of the isolated ribosomes was resistant to spectinomycin. Since the Suc^d-Spc^r mutation has been mapped at the *aroE-strA* region (Miyoshi and Yamagata, 1976) where a gene cluster for ribosomal proteins has been demonstrated to exist (Jaskunas et al., 1974), the primary change of the mutation seemingly occurs in one of the ribosomal proteins.

In this work we analyzed ribosomal proteins of some of these mutants and found an alteration in specific proteins of the 30S subunits. The results are discussed in relation to the interaction between ribosomes and the cytoplasmic membrane.

Materials and Methods

Bacterial Strains. All strains used in this study are listed in Table 1. All Suc^d-Spc^r mutants were isolated from W4626phe$^-$ (wild-type strain) by Miyoshi and Yamagata (1976) on nutrient agar plates containing 20% (w/v) sucrose and 100 μg of spectinomycin per ml.

Media and Buffers. The following media and buffers were used. M3su medium; Difco antibiotic medium no. 3 supplemented with

* *Present address*: Mitsubishi-Kasei Institute of Life Science, Machida, Tokyo 194, Japan

For offprints contact: M. Dombou

Table 1. Strains of *E. coli* used

Strain	Genotype and relevant characteristics	Reference
W4626phe$^-$	F$^-$ *purE trp phe* lac_{85} gal_2 xyl_2 *mal mtl ara Str* (λ); spectinomycin-sensitive wild-type strain	(Miyoshi and Yamagata, 1976)
YM22, YM50, YM93, YM101	Suc^d-Spc^r mutants from W4626phe$^-$; spectinomycin resistant in the presence of 20% sucrose	(Miyoshi and Yamagata, 1976)

10 μg of adenine per ml, 40 μg of tryptophan per ml, and 20% (w/v) sucrose. Modified M9 medium; 42 mM NH_4Cl, 0.5% glucose, 1 μg of thiamine chloride per ml, 50 μg each of 19 amino acids (all L-amino acids except leucine) per ml, 10 μg of adenine per ml, and 20% (w/v) sucrose.

Preparation of 70S Ribosomes and Ribosomal Subunits. Cells were grown at 37° C on a reciprocal shaker and harvested at the late exponential phase of growth. The preparation of 70S ribosomes and ribosomal subunits was carried out as described previously (Mizuno and Mizushima, 1974).

Assay of R17 Phage RNA Directed in vitro Polypeptide Synthesis. In vitro polypeptide synthesis was carried out as described by Wallace et al. (1974). The "S-100" extracts, R17 phage ribonucleic acid and a mixture of initiation factors were prepared as described previously (Mizuno and Mizushima, 1974; Mizuno et al., 1976). Radioactivity measurements were carried out in 10 ml of toluene-base scintillation fluid with a Packard 3320 Tri-Carb scintillation spectrometer.

Analysis of 30S Ribosomal Proteins by CM-cellulose Column Chromatography. Cells were grown in modified M9 medium. When the culture reached the early exponential phase of growth (A_{660}; 0.15), 5 to 10 μCi of [^{14}C]leucine (330 mCi/m mole) or 50 to 100 μCi of [^{3}H]leucine (32 Ci/m mole) were added to the medium and cultivation continued to the late exponential phase of growth (A_{660}; 0.65). The cells were then harvested and the 30S ribosomal subunits prepared as described above. Usually, ^{14}C-labelled wild-type 30S subunits (about 10^5 cts/min in 15 A_{260} units) and ^{3}H-labelled mutant 30S subunits (about 10^6 cts/min in 15 A_{260} units) were combined and mixed with 200 A_{260} units of nonradioactive wild-type 30S subunits and the mixture used for protein analysis. Procedures for the analysis of 30S subunit proteins on a CM-cellulose column were the same as those described by Otaka et al. (1968); for the nomenclature, see Muto et al. 1975), except the total volume of the elution buffer was 500 ml. Proteins in individual peak fractions after the chromatography were analyzed by one-dimendional polyacrylamide gel electrophoresis can identify all 30S Traub et al. (1971). The combination of CM-cellulose chromatography and polyacrylamide gel electrophoresis can identify all 30S subunit proteins.

Purification of Protein S3. The 30S subunits were treated with 1 M LiCl according to the method of Homann and Nierhaus (1971) and protein S3 was isolated from the extracts on a CM-cellulose column according to the method of Otaka et al. (1968).

Chemicals. Spectinomycin sulfate was a generous gift from Upjohn Co. L-[U-^{14}C] valine with a specific activity of 280 mCi/m mole and L-[U-^{14}C] leucine with a specific activity of 330 mCi/m mole were obtained from the Radiochemical Centre, Amersham. L-[4,5-$^3H_2$] leucine with a specific activity of 32 Ci/m mole was obtained from Daiichi Pure Chem. Co. Carboxymethyl cellulose (CM52) was obtained from Whatman Ltd. Nonion NS210 (polyoxyethylene nonylphenolether; n=10) was a product of Nippon Oils and Fats Co. Ltd.

Table 2. Effect of spectinomycin on R17 phage RNA directed polypeptide synthesis with hybrid ribosomes

	Source of subunits		Spectinomycin	[^{14}C]-valine incorporation (cts/min)	Inhibition by spectinomycin (%)
	30S subunit	50S subunit			
a)	Wild	Wild	+	1384	74
			−	5336	
	YM22	Wild	+	2417	53
			−	5189	
	Wild	YM22	+	1772	73
			−	6632	
	YM22	YM22	+	3446	28
			−	4786	
	Wild 70S		−	13106	−
	YM22 70S		−	13375	−
b)	Wild	Wild	+	1029	80
			−	3773	
	YM50	Wild	+	782	44
			−	1134	
	Wild	YM50	+	933	83
			−	3727	
	YM50	YM50	+	884	49
			−	1407	
	Wild 70S		−	4443	−
	YM50 70S		−	2701	−
c)	Wild	Wild	+	805	69
			−	1974	
	YM93	Wild	+	1039	38
			−	1505	
	Wild	YM93	+	963	66
			−	2279	
	YM93	YM93	+	1225	41
			−	1904	
	Wild 70S		−	7118	−
	YM93 70S		−	6054	−
d)	Wild	Wild	+	992	66
			−	2959	
	YM101	Wild	+	276	45
			−	502	
	Wild	YM101	+	1602	72
			−	5880	
	YM101	YM101	+	628	27
			−	865	
	Wild 70S		−	6546	−
	YM101 70S		−	8199	−

Ribosomal subunits were prepared from W4626phe$^-$ (wild) and Suc^d-Spc^r mutants (YM22, YM93, YM50 and YM101). The R17 phage RNA directed polypeptide synthesis was assayed with 0.7 A_{260} units of 30S subunits and 1.4 A_{260} units of 50S subunits Incubation was carried out for 30 min at 34° C. Spectinomycin (0.4 μM) was added when indicated. All values were subtracted from the radioactivity incorporated in the absence of ribosomes

Results

Subunit Exchange Experiments. In order to determine which ribosomal subunit is responsible for the spectinomycin resistance in Suc^d-Spc^r mutants, subunit exchange was carried out between ribosomes from the wild-type strain and Suc^d-Spc^r mutants, and the polypeptide synthetic activity of these ribosomes was measured in the presence and absence of spectinomycin (Table 2). Ribosomes were more resistant to spectinomycin when the 30S subunits from the mutants were used regardless of the source of the 50S subunits, indicating that the 30S subunits are responsible for the spectinomycin resistance in all of the Suc^d-Spc^r mutants tested.

When the 30S subunits from YM101 were used, the polypeptide synthetic activity was remarkably reduced regardless of the source of the 50S subunits, while the 70S ribosomes from YM101 were almost as active as those from the wild-type strain (Table 2d). This indicates that the 30S subunits from YM101 had suffered some damage in the process of subunit separation. This was confirmed in an experiment which will be described later.

Alterations in 30S Ribosomal Proteins. The Suc^d-Spc^r mutation has been mapped at the *aroE-strA* region by Miyoshi and Yamagata (1976), where a cluster of many ribosomal protein genes has been demonstrated to exist. The evidence indicates the possibility that some proteins in the 30S subunits are responsible for the mutation. Therefore, 30S ribosomal proteins prepared from Suc^d-Spc^r mutants were compared with those from the wild-type strain using column chromatography on CM-cellulose. The 30S ribosomal proteins from a Suc^d-Spc^r mutant labelled with [^{14}C]leucine and those from the wild-type strain labelled with [^{3}H]leucine were co-chromatographed on a CM-cellulose column. The results using YM22, YM93, YM50 and YM101 are presented in Figure 1.

As shown in Figure 1a, the difference in protein peaks between the wild-type strain and YM22 was restricted to fractions 50 to 58. A similar anomaly was also noticed for 30S proteins from YM93 (Fig. 1b). Polyacrylamide gel electrophoresis showed that these fractions contained proteins S10, S5 and S2 in that order of elution. In order to identify the altered ribosomal protein in YM22, fractions 50 to 62 were analyzed individually by polyacrylamide gel, and radioactivity in individual proteins counted. The ^{3}H radioactivity of protein S5 (wild-type strain) showed a maximum at fraction 56, whereas the ^{14}C radioactivity of the same protein (YM22) showed a maximum at fraction 52 (Fig. 2a). On the other hand, no difference was found between YM22 and the wild-type strain in elution profiles from the CM-cellulose chromatography of proteins S10 and S2 (Fig. 2b and c). Therefore, it is concluded that protein S5 of YM22 is different from that of the wild-type strain. A similar shift of elution position of protein S5 on the CM-cellulose column chromatogram was also demonstrated for YM93 (data not shown).

Figure 1c shows that the difference in protein peaks between the wild-type strain and YM50 was restricted to fractions 108 to 116. Since these fractions contained protein S4 alone and the examination on the polyacrylamide gel revealed that the radioactivity profiles in Figure 1c were exclusively due to protein S4, it is concluded that protein S4 of YM50 is different from that of the wild-type strain.

Although no difference was observed in peak positions between the wild-type strain and YM101, some anomalies were found in fractions 102 to 110 (Fig. 1d). These fractions contained proteins S3 and S7. Individual fractions belonging to this region were analyzed by polyacrylamide gel electrophoresis and the radioactivity in protein bands was counted separately. Although no difference was observed between the wild-type strain and YM101 in the peak positions of radioactive S3 and S7, the ^{14}C(YM101)/^{3}H(wild-type strain) ratio in protein S3 was appreciably lower than that in protein S7. Table 3 shows these ratios together with those of some other proteins and the total protein mixture of 30S subunits from which they were prepared. Although radioactivity values especially those for ^{14}C were rather low, we have obtained essentially the same result with the other independent experiment. From the results in Figure 1d and Table 3, it is evident that the amount of protein S3 is appreciably lowered in the isolated 30S subunits

Table 3. Reduced amount of protein S3 in YM101

30S subunit protein	^{3}H cts/min (wild-type strain)	^{14}C cts/min (YM101)	(^{14}C/^{3}H) $\times 100$
S1	663	65	9.8
S10	844	66	7.8
S7	3350	189	5.6
S3	1382	32	2.3
S4	2903	186	6.4
TP30	6.6×10^5	5.0×10^4	7.6

One and half ml of each of the following fractions seen in Figure 1d was analyzed by polyacrylamide gel electrophoresis and the radioactivity in individual bands measured as described in Figure 2; fraction 30 (for protein S1), fraction 64 (for protein S10), fraction 106 (for protein S7), fraction 108 (for protein S3) and fraction 113 (for protein S4). TP30; total 30S subunit proteins applied to the CM-cellulose column for Figure 1d. The efficiencies of radioactivity measurements were 37% for ^{14}C and 55% for ^{3}H

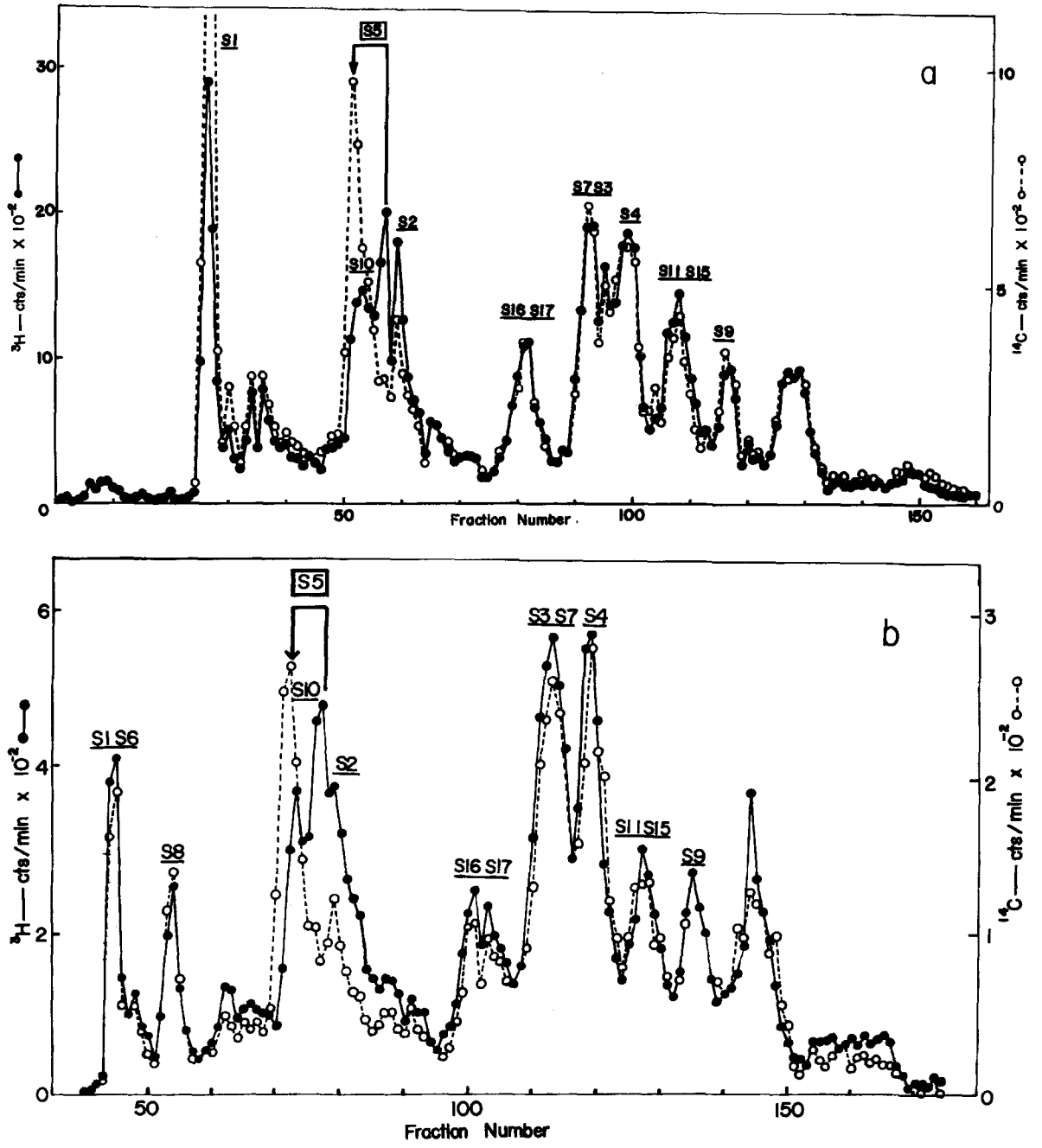


Fig. 1a–d. CM-cellulose column chromatography of the 30S ribosomal proteins from W4626phe$^-$ (wild-type strain) and Suc-^{d}-Spc^r mutants. ●——● [^{3}H]Leucine-labelled W4626phe$^-$ protein (**a–d**). ○---○ [^{14}C]Leucine-labelled Suc^d-Spc^r mutant protein (**a**; YM22, **b**; YM93, **c**; YM50 and **d**; YM101). The volume of each fraction was 2.5 ml. One ml of each fraction was used for the radioactivity measurement. The efficiencies of radioactivity measurements were 59% for ^{14}C and 32% for ^{3}H

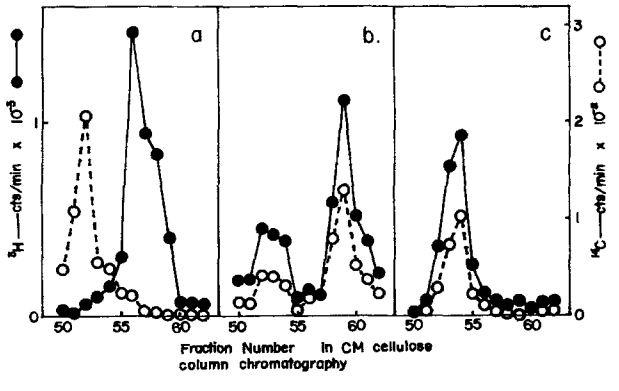
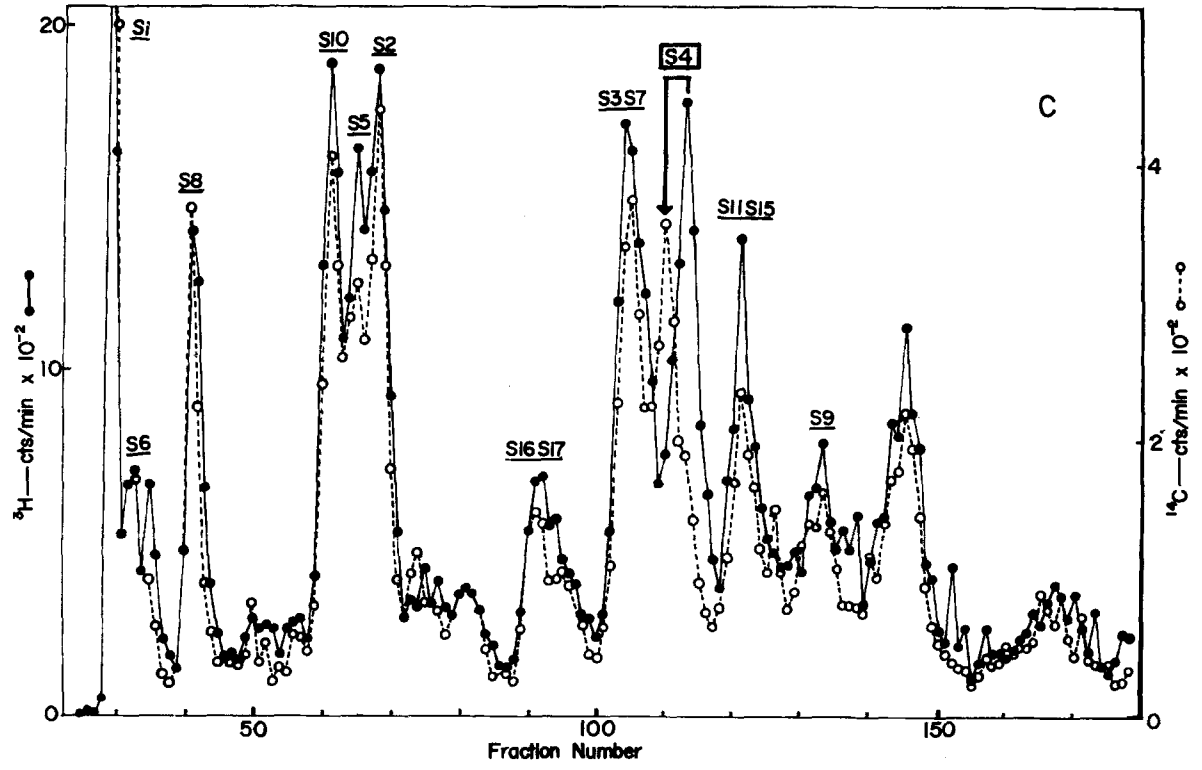


Fig. 2a–c. Elution profiles of proteins S5, S2 and S10 of YM22 from the CM-cellulose. Fraction numbers correspond to those in the CM-cellulose column chromatography shown in Figure 1a. One and half ml of each of fractions 50 to 62 was concentrated to 0.1 ml by means of dry Sephadex G-100 and analyzed by polyacrylamide gel electrophoresis. After staining with Amido-black, protein bands containing S2, S5 and S10 were cut out individually, digested with 0.2 ml of 30% hydrogen peroxide-0.3% ammonia water, dissolved in 10 ml of nonion-toluene (1:2) scintillation fluid and used for radioactivity measurement. ●——● [^{3}H]Leucine-labelled W4626phe$^-$ protein (**a–c**); ○---○ [^{14}C]Leucine-labelled YM22 protein (**a–c**). (**a**) S5, (**b**) S2 and (**c**) S10


c
S1
S10
S2
S4
S3 S7
S5
S8
S11 S15
S6
S16 S17
S9
^{3}H—cts/min x 10^{-2}
^{14}C—cts/min x 10^{-2}
Fraction Number

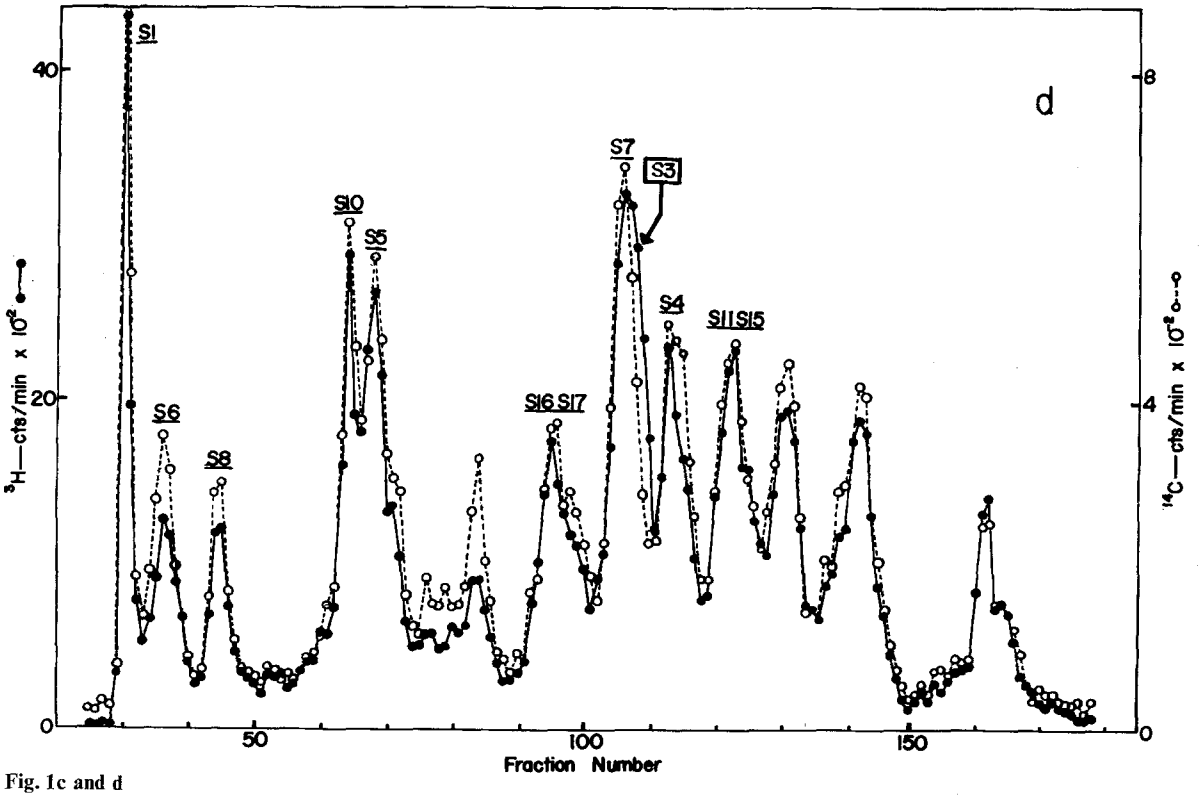

d
S1
S7
S3
S10
S5
S4
S11 S15
S6
S16 S17
S8
^{3}H—cts/min x 10^{-2}
^{14}C—cts/min x 10^{-2}
Fraction Number

Fig. 1c and d

Table 4. Effect of protein S3 from the wild-type strain and YM101 on R17 phage RNA directed polypeptide synthetic activity of ribosomes

	Source of subunits		Source of protein S3	Spectinomycin	^{14}C-Valine incorporation (cts/min)	Inhibition by spectinomycin (%)
	30S subunit	50S subunit				
a)	YM101	YM101	–	+	602	34
				–	916	
	YM101	YM101	YM101	+	901	23
				–	1173	
	YM101	YM101	Wild	+	942	60
				–	2373	
b)	Wild	Wild	–	+	665	80
				–	3269	
	Wild	Wild	YM101	+	650	81
				–	3420	
	Wild	Wild	Wild	+	1019	76
				–	4255	

Ribosomes (10.5 A_{260} units of 30S subunits and 21 A_{260} units of 50S subunits) and protein S3 (23 µg) were incubated under the reconstitution conditions (total volume 1.6 ml) (Traub and Nomura, 1969). Ribosomes were recovered by centrifugation and analyzed for polypeptide synthetic activity as described in Materials and Methods. Incubation for polypeptide synthesis was for 30 min at 34° C. Spectinomycin (0.4 µM) was added when indicated

of YM101. The possibility that protein S3 of YM101 migrated to a different position on the gel has been excluded, since the purified S3 of YM101 and that of the wild-type strain migrated to the same position. The lower content of protein S3 in YM101 may account for the appreciably weaker activity of the isolated 30S subunits of YM101 in polypeptide synthesis (Table 2).

Alteration of Protein S3 in YM101. In order to determine whether the lower content of protein S3 is due to an alteration in the protein of YM101 and that this alteration is responsible for the spectinomycin resistance, the following experiment was carried out: The 30S subunits and the 50S subunits from the YM101 were incubated under the reconstitution conditions (Traub and Nomura, 1969) with protein S3 prepared either from the wild-type strain or YM101, and the polypeptide synthetic activity examined in the presence and absence of spectinomycin (Table 4a). Incubation with protein S3 from the wild-type strain resulted in an increase in the polypeptide synthetic activity with a concomitant increase in the sensitivity to spectinomycin. On the other hand, incubation with protein S3 from YM101 did not result in any changes.

As a control, the same experiment was performed with the 30S subunits from the wild-type strain. Neither protein S3 from the wild-type strain nor that from YM101 caused any changes in either the polypeptide synthetic activity or the sensitivity to spectinomycin (Table 4b). From these results, it is concluded that protein S3 had been altered in YM101, and the alteration is responsible for both the spectinomycin resistance and the unstable situation of the protein in the ribosomes. Essentially the same result was obtained with protein S3 prepared through a phosphocellulose column according to the method of Held et al. (1973).

Discussion

The results presented in this paper show that an alteration in either protein S3, S4 or S5 of the 30S ribosomal subunits is responsible for the resistance to spectinomycin in Suc^d-Spc^r mutants. Since Suc^d-Spc^r mutants had been isolated through nitrosoguanidine treatment, alterations in other ribosomal proteins which could not be detected on the CM-cellulose column may occur, and such alterations may in fact be responsible for the Suc^d-Spc^r mutations. However, the fact that protein S5 is altered in the same manner in two independently isolated mutants, YM22 and YM93, strongly supports the view that protein S5 is responsible for the spectinomycin resistance in these mutants. The partial reconstitution experiment with protein S3 also confirmed that altered protein S3 is responsible for the spectinomycin resistance in YM101. On the other hand, we found only one such mutant that showed an alteration in protein S4.

The Suc^d-Spc^r mutation is pleiotropic, giving rise to hypersensitivity to antibiotics, dyes and detergents, and abnormality in cell morphology (Miyoshi and Yamagata, 1976), and the absence of the specific cytoplasmic membrane protein, I-19 (Mizuno et al., 1976) as well as alterations in ribosomal proteins. From the following evidence, it has been suggested that the primary step of the mutation is the alteration of a ribosomal protein and other alterations take place as a result of the ribosomal alteration; (i) the Suc^d-Spc^r mutation was mapped at the *aroE-strA* region where a gene cluster of ribosomal proteins has been demonstrated to exist (Jaskunas et al., 1974) and (ii) the absence of cytoplasmic membrane protein I-19 was also demonstrated by treatment of the wild-type cells with spectinomycin, whereas such a phenomenon was not observed with a *spcA* mutant in which the ribosomes are resistant to spectinomycin (Mizuno et al., 1977). The fact that the ribosomal proteins were altered upon mutation further supports this view. It has been demonstrated that protein S5 is responsible for spectinomycin resistance in *spcA* mutants (Bollen,

Table 5. Pleiotropy in Suc^d-Spc^r mutants

Strain	Mutant type[a] (Miyoshi and Yamagata, 1976)	Proteins in cytoplasmic membrane[b] (Mizuno et al., 1976)			Altered ribosomal protein
		I-13	I-19	I-24	
W4626phe$^-$ (wild-type)	−	+	+	+	−
YM22	I	+	−	−	S5
YM50	I	+	−	−	S4
YM93	I	+	−	−	S5
YM101	II	−	−	+	S3

[a] I = those unable to grow on sucrose-free medium in the presence of spectinomycin. II = those unable to grow on sucrose-free medium irrespective of the presence or absence of spectinomycin

[b] + = present; − = absent

et al., 1969; Dekio and Takata, 1969) and either valine or serine in peptide T10 of the wild-type strain is changed to glutamic acid or proline, respectively (Funatsu et al., 1971). Since YM22 and YM93 are clearly different from the *spcA* mutants in terms of sucrose dependence, the alteration in protein S5 in the former must be different from that in the latter.

Although all Suc^d-Spc^r mutants show spectinomycin resistance in the presence of sucrose and lack protein I-19 in the cytoplasmic membrane, there are some differences in their phenotypes as summarized in Table 5. Mutant YM22 and YM93 that have altered protein S5 show the same characteristics, while YM101 is different from these mutants in sucrose dependence and membrane proein profile as well as in alteration of ribosomal protein. Although another type of ribosomal alteration (protein S4 in YM50) caused alterations similar to those in YM22 and YM93, they are different in hypersensitivity to some antibiotics. dyes and detergents (Miyoshi and Yamagata, 1976). Thus depending on the site of alteration in the ribosomes, phenotypic expressions in other parts of the cells were different to some extent.

The fact that the alteration in either protein S3, S4 or S5 resulted in similar alterations in other parts (most probably in the membrane) of the cells suggests that those ribosomal proteins are topologically related in the 30S subunits. The idea was supported by the following evidence: (i) Alterations in protein S4 or S5 suppress the streptomycin dependence controlled by protein S12 (Kreider and Brownstein, 1972; Deusser et al., 1970), (ii) protein S5 is essential for the in vitro assembly of protein S3 (Mizushima and Nomura, 1970), (iii) the cold-sensitivity of Spc^r mutants can be relieved by a secondary alteration in either protein S2, S3 or S5 (Nashimoto and Uchida, 1975), and (iv) cross-linked pairs of S3–S4, S3–S5 and S4–S5 are formed by the treatment of the 30S subunits with cross-linking reagents (Sommer and Traut, 1976; Lutter et al., 1975; Peretz et al., 1976). Furthermore, by means of immune electron microscopy, one of the antibody reactive sites of each of proteins S3, S4 and S5 has been mapped on a specific locus of the 30S subunit surface close to each other. Adopting the ribosome model of Tischendorf et al. (1975), it is extremely interesting that this locus could closely face on the cytoplasmic membrane, even if the major interaction site of the ribosomes with the cytoplasmic membrane is the 50S subunits as believed in eukaryotic cells (Sabatini et al., 1966). Thus it is probable that an alteration in the specific site of the 30S subunits directly affects the cytoplasmic membrane as suggested in previous papers (Mizuno et al., 1976; Mizuno et al., 1977).

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