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Sucrose-Dependent Spectinomycin-Resistant Mutants of *Escherichia coli*

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Spectinomycin-resistant (Spc^r) mutants of *Escherichia coli* were isolated from nutrient agar plates containing 20% sucrose and 100 μg of spectinomycin per ml. About one-third of the Spc^r mutants thus obtained were sucrose dependent (Suc^d) and were classified into two types: I, those unable to grow on sucrose-free medium in the presence of spectinomycin; and II, those unable to grow on sucrose-free medium irrespective of the presence of spectinomycin. Most of these mutants were hypersensitive to antibiotics, dyes, and detergents and were abnormal in cell morphology, suggesting changes in cell envelopes. Reversion experiments indicated that the sucrose-dependent spectinomycin resistance and hypersensitivity to various chemicals were not independently induced properties. The Suc^d-Spc^r mutations of type I mutants were transducible by phage P1 and were mapped at the *strA-aroE* region.

Spectinomycin is an antibiotic that inhibits protein synthesis (2, 3, 9). Spectinomycin-resistant (Spc^r) mutants of *Escherichia coli* have an altered S5 ribosomal protein (6, 10). The Spc^r mutations are known to be pleiotropic; they affect the growth rate of the cell (3), especially at low temperature (20), alleviate the restriction of suppression by the Str^r mutation (14), and make ribosomal assembly cold sensitive (20). In addition, some Spc^r mutations affect the stability of sex factors in *E. coli*, suggesting ribosomal involvement in the control of episome replication (Eps property, references 29 and 30).

One of possibilities we proposed to explain the Eps property of the Spc^r mutation was that the altered ribosomes or ribosomal proteins affected some function of the bacterial membrane that controls the episome replication (29). Mutants altered in spectinomycin resistance and cell membrane organization were sought to support the above assumption. In the hope of finding such mutants, we started to isolate sucrose-dependent, spectinomycin-resistant mutants from a strain of *E. coli*. Mutants of *E. coli* isolated as being sucrose dependent (Suc^d) were reported to be defective in the cell envelope (16). Temperature-sensitive mutants that lysed at 42 C but were protected by 20% sucrose appeared to be defective in cell wall synthesis (17). Temperature-sensitive deoxyribonucleic acid synthesis mutants of *E. coli* were able to grow at nonpermissive temperature if salts or sugars were added to the medium. These mutants appeared to be altered in the membrane components (24). These results suggest that sensitivity to an osmotic environment is a good criterion for the identification of membrane or cell wall mutants.

This paper describes the isolation of Suc^d-Spc^r mutants of *E. coli* and some of their properties that suggest an alteration of the cell envelope and ribosomes.

MATERIALS AND METHODS

Bacterial and phage strains. *E. coli* W4626Phe$^-$, F$^-$*purE*$^-$Trp$^-$Phe$^-$*lac*$_{\Delta 5}$$^-$*gal*$_2$$^-$*xyl*$_2$$^-Mal^-Mtl^-Ara^-$Str^r (λ), was a derivative of strain W4626 (29). Strain AB2834, F$^-$Thi$^-$*aroE*$^-$*malA*$^-$T6^r (22), was obtained through H. Nashimoto. P1*vir* was described previously (28).

Media. Penassay broth medium no. 3 (M3, Difco) supplemented with 10 μg of adenine and 40 μg of tryptophan per ml was used as a basal nutrient medium. Modified M3 media were as follows. M3susp was made by adding spectinomycin and 20% sucrose. M3su was made by adding 20% sucrose. M3sp was made by adding spectinomycin. The concentration of spectinomycin was 100 μg/ml except where otherwise indicated. Solid media contained 1.35% agar. TY medium was described previously (28). M9 medium contained (per liter): Na_2HPO_4, 6 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.5 g; NH_4Cl, 1 g; thiamine, 1 mg; and glucose, 2 g.

Chemicals. Chemicals were obtained from the following sources: spectinomycin sulfate (specific activity, 641 μg/mg), a gift from The Upjohn Co.; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, Aldrich

Chemical Co.; methylene blue, Chroma-Gesellschaft; acriflavine, Tokyo Kasei Kogyo Ltd.; sodium deoxycholate (DOC), Difco Laboratories; sodium lauryl sulfate (SDS), Wako Pure Chemical Industries; rifampin, Mann Research Laboratories; and actinomycin D, Merck & Co., Inc.

Test for the Suc^d-Spc^r phenotype. Approximately 10^5 cells were spotted on M3, M3susp, M3su, and M3sp agar plates. Those which were able to grow on M3susp but failed to grow on M3sp plates were regarded as being Suc^d-Spc^r.

Test for antibiotic and chemical sensitivity. Approximately 10^5 cells were spotted on M3susp, M3su, and M3 agar plates containing the appropriate test chemical. Antibiotics, dyes, and detergents were added after the agar had cooled to 60 to 70 C. The concentrations of added chemicals were as follows: rifampin, 1 μg/ml; actinomycin D, 3 μg/ml; methylene blue, 100 μg/ml; acriflavine, 10 μg/ml; DOC, 0.1%; and SDS, 0.02%. These chemicals did not affect the growth of wild-type strain W4626Phe$^-$ at the above-mentioned concentrations. Strains that were inhibited at these concentrations were classified as being hypersensitive.

Transduction procedures. P1 lysates were prepared as described by Lennox (15). Phage P1*vir*, grown on donor cells, were added to recipient cells at a multiplicity of infection of 0.5 in TY medium. After 30 min of incubation at 37 C, anti-P1 serum was added and the cells were spread on suitable selecting plates. To select Str^r or Spc^r transductants, the cells were distributed into tubes and grown for five generations before being spread.

RESULTS

Isolation of Suc^d-Spc^r mutants. W4626Phe$^-$ cells were treated with 30 to 50 μg of nitrosoguanidine per ml at 37 C for 30 min by the procedure of Adelberg et al. (1). The mutagenized cells were washed, suspended in M3su broth (5×10^8 cells/ml), and diluted 20-fold with M3su broth, and 5-ml portions were distributed into tubes (each tube assures one independent selection). The tubes were incubated at 37 C for five bacterial generations, 5×10^8 cells from each tube were spread on M3susp and M3sp agar plates, and the plates were incubated for 2 days at 37 C. Colonies appearing on M3susp plates were isolated and tested for the Suc^d-Spc^r phenotype (Table 1). Through six experiments, the frequency of appearance of Spc^r mutants on M3susp plates was higher than that on M3sp plates. About one-third of Spc^r mutants isolated from M3susp plates failed to grow on M3sp plates, indicating their Suc^d-Spc^r phenotype. As a result of six independent experiments of 25 independent selections, 178 Suc^d-Spc^r mutants were obtained. Most of these Suc^d-Spc^r mutants were able to grow on M3sp plates if 2% NaCl was added to the medium, suggesting that their Suc^d-Spc^r property was related to an osmotic environment. Some of these mutants were able to grow on M3 agar plates, and others failed to grow on M3 plates as well as on M3sp plates. The former were classified tentatively as type I and the latter were classified as type II, although a considerable number of mutants of intermediate property existed (Table 2).

Growth of Suc^d-Spc^r mutants in liquid media. Exponential-phase cultures of the various Suc^d-Spc^r mutants grown in M3su broth were diluted 10^4-fold into M3, M3susp, M3su, and M3sp broth, and subsequent growth was observed. The results were consistent with those of the spot tests; viable cells did not increase in M3sp (type I) or in M3 as well as M3sp (type II). Typical growth curves of YM50 (type I), YM69 (type II), and W4626Phe$^-$ (wild type) are shown in Fig. 1. The initial fall in the number of viable

TABLE 1. *Frequency of appearance of Spc^r and Suc^d-Spc^r mutants*

Expt	Frequency of appearance of Spc^r mutants		No. of Spc^r mutants isolated from M3susp plates	No. of Suc^d-Spc^r mutants
	M3sp plate	M3susp plate		
1	1.5×10^{-7}	7.6×10^{-7}		
2	5.4×10^{-7}	6.4×10^{-7}		
3	1.0×10^{-7}	6.9×10^{-7}	25	10
4	4.7×10^{-7}	7.1×10^{-7}	19	10
5	3.8×10^{-6}	1.4×10^{-5}	19	10
6	1.1×10^{-6}	4.3×10^{-6}	47	20
7			194	33
8			195	95

TABLE 2. *Classification of Suc^d-Spc^r mutants*

Bacteria	Growth on plates				No. of mutants classified[a]
	M3-susp	M3-su	M3-sp	M3	
W4626Phe$^-$ (wild type)	−	+	−	+	
W4626Phe$^-$Spc^{r}10-2[b]	+	+	+	+	
Mutant type I	+	+	−	+	54
II	+	+	−	−	20
I/II[c]	+	+	−	±	37

[a] Of the 178 mutants obtained from six independent experiments with 25 independent selections, a total of 111 was classified.

[b] Sucrose-independent Spc^r mutant of W4626Phe$^-$.

[c] Intermediate type.

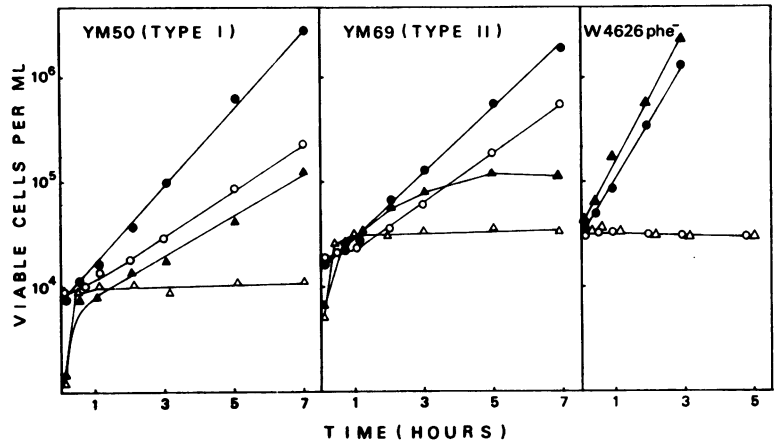

FIG. 1. *Growth of Suc^d-Spc^r mutants in M3 and modified M3 broth. Exponential-phase cultures of YM50, YM69, and W4626Phe$^-$ in M3su broth were diluted 10^4-fold into M3susp, M3su, M3sp, and M3 broth and incubated at 37 C. At various times, portions were diluted and plated on M3su agar plates. Symbols:* ○, *M3susp broth;* ●, *M3su broth;* △, *M3sp broth;* ▲, *M3 broth.*

YM50 and YM60 cells in M3 and M3sp was likely due to osmotic shock at the time they were diluted into the media.

Hypersensitivity of Suc^d-Spc^r mutants to antibiotics, dyes, and detergents. One of the possible characteristics of envelope mutants is an alteration of the permeability barrier to various chemicals. Since these Suc^d-Spc^r mutants were expected to be defective in the cell envelope, they were tested for sensitivity to rifampin, actinomycin D, methylene blue, acriflavine, DOC, and SDS by spot testing. The sensitivity pattern of 13 mutants is shown in Table 3. Most mutants were hypersensitive to one or more of these chemicals. For example, YM10 was sensitive to rifampin on M3susp and M3su plates, to actinomycin D, acriflavine, DOC, and SDS on the M3susp plate, and to methylene blue on the M3 plate. The results of such experiments for 111 mutants are summarized in Table 4, in which mutants are regarded as being hypersensitive if they were sensitive to the chemicals on any one of M3susp, M3su, and M3 plates.

Analysis of revertants from Suc^d-Spc^r mutants. To determine whether the properties of hypersensitivity to various chemicals and sucrose-dependent spectinomycin resistance resulted from a single mutation, reversion experiments were carried out. Suc^d-Spc^r cells were spread on M3 agar plates containing the above-mentioned concentrations of rifampin, actinomycin D, methylene blue, acriflavine, DOC, and SDS together. After 3 to 4 days of incubation at 37 C, colonies appeared at the frequency 10^{-8} to 10^{-9}. Revertants thus obtained spontaneously were tested for spectinomycin resistance. They fell into three groups depending on their phenotype of spectinomycin resistance: (1) Spc^r, irrespective of the presence of sucrose; (2) Suc^d-Spc^r of type I; and (3) Spc^s, irrespective of the presence of sucrose (Table 5). Groups 1 and 2 represent partial revertants, and group 3 represents full revertants. Since the process obtaining these revertants did not include any

TABLE 3. *Hypersensitivity of Suc^d-Spc^r mutants to antibiotics, dyes, and detergents*[a]

Mutant	Type	Hypersensitivity to: Rif, 1 μg/ml	Act D, 3 μg/ml	MB, 100 μg/ml	AF, 10 μg/ml	DOC, 0.1%	SDS, 0.02%
YM10	I	AB	A	C	A	A	A
YM20	II	*	A	AB	*	A	AB
YM22	I	A	A	ABC	AB	A	AB
YM26	II	AB	AB	AB	*	*	*
YM29	I	ABC	A	ABC	*	AC	*
YM35	II	AB	AB	AB	*	*	*
YM50	I	C	A	*	AC	C	*
YM61	II	AB	AB	AB	*	AB	*
YM69	II	AB	A	B	*	*	*
YM97	I	ABC	ABC	*	*	ABC	A
YM101	II	*	A	*	*	*	*
YM105	I	A	ABC	C	AC	ABC	A
YM107	II	A	A	AB	*	*	*

[a] Symbols: A, B, and C corresponds to M3susp, M3su, and M3 plates, respectively, on which each mutant showed hypersensitivity to a testing chemical. Since all strains used were not able to grow on an M3sp plate, hypersensitivity on M3sp plates was not tested. The asterisk (*) indicates that the mutant did not show hypersensitivity on any plate. Abbreviations: RIF, rifampin; Act D, actinomycin D; MB, methylene blue; AF, acriflavine.

selective procedure for spectinomycin sensitivity, the frequencies of appearance of revertants of group 3 among the revertants from strains YM50, YM101, and YM105 (2/87, 6/24, and 6/20, respectively) were high enough to indicate that the sucrose-dependent spectinomycin resistance and hypersensitivity to various chemicals of these mutants were induced by a single mutation. The appearance of partial revertants could be explained by a possible mutation that suppresses or modifies the Suc^d-Spc^r mutation.

TABLE 4. *Summary of hypersensitivity of Suc^d-Spc^r mutants to the tested chemicals*[a]

Chemicals	No. of sensitive mutants
1 μg of rifampin per ml	102
3 μg of actinomycin D per ml	31
100 μg of methylene blue per ml	62
10 μg of acriflavine per ml	31
0.1% DOC	78
0.02% SDS	36

[a] The number of mutants tested was 111.

TABLE 5. *Analysis of revertants of Suc^d-Spc^r mutants*

Mutant	No. of revertants tested	No. of revertants showing the phenotype: Spc^r [a]	Suc^d-Spc^r	Spc^s [a]
YM22	12	0	12	0
YM26	41	41	0	0
YM37	23	0	23	0
YM50	87	12	73	2
YM54	8	5	3	0
YM69	31	26	5	0
YM72	17	7	10	0
YM84	7	0	7	0
YM85	11	11	0	0
YM101	24	8	10	6
YM105	20	7	7	6

[a] Spc^r or Spc^s irrespective of the presence of sucrose.

Mapping of Suc^d-Spc^r mutations. Spectinomycin is known to affect the function of the ribosome (7, 26), and Spc^r mutations are responsible for the alteration of ribosome structure (6, 10). All known Spc^r mutations in *E. coli* were mapped at a region between the *aroE* and *strA* loci (2, 7, 25). Many ribosomal genes have been reported to cluster at this region (8). Therefore, P1 transduction experiments were carried out to examine whether Suc^d-Spc^r mutations located at this region. Type I mutants, YM50 and YM93, were used as the donor. Since these strains were Str^{r}Suc^d-Spc^r*aroE*$^+$, AB2834 (Str^{s}Spc^s*aroE*$^-$) was used as the recipient. Type II mutants were not used as the donor because of difficulty in preparing a high-titer P1 lysate from them. Transductants of Str^r, Spc^r, and *aroE*$^+$ markers were selected on M3su agar plates containing 100 μg of streptomycin per ml, M3susp agar plates, and M9 agar plates, respectively. Transductants were then tested for the configuration of nonselected markers. With strain YM93 as the donor, the sucrose dependence of Spc^r transductants was tested on M3susp and M3sp agar plates, each containing 200 μg of spectinomycin per ml (Table 6). All Spc^r transductants showed the Suc^d-Spc^r property; that is, sucrose dependence of type I mutants did not separate from Spc^r mutation throughout these experiments, indicating that Suc^d-Spc^r property was caused by a single mutation or closely linked mutations. Suc^d-Spc^r mutations were cotransducible with both Str^r and *aroE*$^+$ markers. The frequency of cotransduction between Str^r and *aroE*$^+$ markers was

TABLE 6. *Transduction of the Suc^d-Spc^r property by phage P1*[a]

Donor	Selected marker	No. of transductants tested	Segregation of nonselected markers
YM50	Spc^r	49	Suc^d-Spc^r/Str^{r}Aro$^+$ Str^{r}Aro$^-$ Str^{s}Aro$^+$ Str^{s}Aro$^-$ 49 / 14 23 10 2
YM50	Str^r	50	Spc^{s}Aro$^+$ Spc^{s}Aro$^-$ Suc^d-Spc^{r}Aro$^+$ Suc^d-Spc^{r}Aro$^-$ 0 14 14 22
YM50	Aro$^+$	49	Spc^{s}Str^s Spc^{s}Str^r Suc^d-Spc^{r}Str^r Suc^d-Spc^{r}Str^s 13 3 26 7
YM93	Spc^r	47	Suc^d-Spc^r/Str^{r}Aro$^+$ Str^{r}Aro$^-$ Str^{s}Aro$^+$ Str^{s}Aro$^-$ 47 / 24 13 9 1

[a] The recipient was AB2834, Str^{s}Spc^s*aroE*$^-$, throughout the above experiments.

lower than that between Suc^d-Spc^r and other markers. The numbers of transductants of Str^{r}Spc^s*aroE*$^+$ or Str^{s}Suc^d-Spc^r*aroE*$^-$ markers was much smaller than those of the others. These results indicated that the Suc^d-Spc^r mutation(s) is located between *strA* and *aroE* loci.

Morphological changes of Suc^d-Spc^r mutants. Another characteristic of Suc^d-Spc^r mutants was changes in cell morphology. Twelve mutants selected rather arbitrarily were observed with a microscope, after incubation in M3, M3susp, M3su, and M3sp broth. All mutants observed showed a filamentous or an amorphous form in at least one medium. Filament formation at nonpermissive conditions (M3sp for type I mutants; M3 and M3sp for type II mutants) was remarkable. Figures 2A through 2D are phase-contrast micrographs of filamentous cells of strains YM93 and YM101 and amorphous cells of strain YM50 compared with the wild-type strain W4626Phe$^-$. Forked cells were found among the filamentous cells, although the number was less than 1% of the total (Fig. 2E).

DISCUSSION

About one-third of the Spc^r mutants isolated from M3 agar plates containing 20% sucrose and 100 μg of spectinomycin per ml were shown to be sucrose dependent. Thus, the frequency of appearance of Suc^d-Spc^r mutants was markedly high under our experimental conditions. One trivial explanation for the appearance of Suc^d-Spc^r mutants is that the action of spectinomycin is counteracted by sucrose and, consequently, some low-level resistant mutants are obtained from the sucrose-containing medium. However, this is not the case because most of the Suc^d-Spc^r mutants obtained showed resistance to 200 to 400 μg of spectinomycin per ml

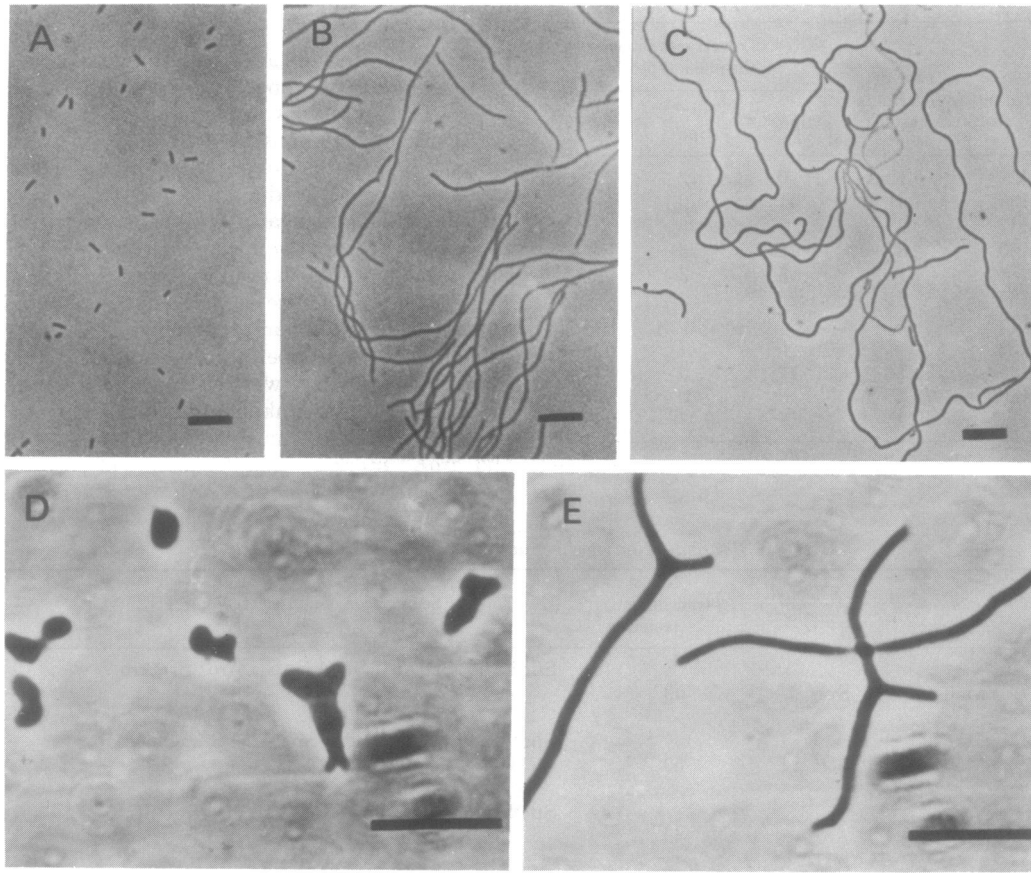

FIG. 2. *Phase-contrast micrographs of Suc^d-Spc^r mutants. (A) Wild-type strain W4626Phe$^-$ in M3 broth; (B and C) filamentous cells of YM 93 and YM101 in M3sp broth; (D) amorphous cells of YM50 in M3su broth; (E) forked cells among filamentous cells of YM93 in M3sp broth. Bar indicates 10 μm.*

and the sensitivity of the wild-type strain to spectinomycin is only slightly affected by the presence of 20% sucrose.

Hypersensitivity of Suc^d-Spc^r mutants to antibiotics, dyes, and detergents is reminiscent of various colicin-tolerant mutants with altered membranes (13, 18, 27), as well as other cell envelope mutants (19, 21, 23), suggesting that these Suc^d-Spc^r mutants are also defective in the cell wall or membrane. These results are similar to those observed with sucrose-dependent mutants (4, 5, 16).

Isolation of revertants that were wild type with regard to spectinomycin resistance, sucrose dependence, and sensitivity to various chemicals was successful in three mutants, including both type I (YM50 and YM105) and type II (YM101), without any selection for spectinomycin sensitivity. The sucrose dependence of type I mutants did not separate from spectinomycin resistance in the process of transduction. The mutations were mapped at a locus between *strA* and *aroE*, suggesting that they are mutations of a ribosomal gene. From these results, it is concluded that the Suc^d-Spc^r mutation is a single mutation that most likely affects either the sensitivity of the ribosome to spectinomycin or the organization of the cell envelope. A biochemical analysis of the ribosomes and cell envelope of these mutants that verifies this inference will be reported in a separate paper (T. Mizuno, H. Yamada, H. Yamagata, and S. Mizushima, submitted for publication).

Assuming that the above inference is correct, how does the mutation of the ribosomal gene affect the organization of the cell envelope? A clue to this question may be found in the possible interaction of ribosomes and membranes. There have been a number of reports on membrane-bound ribosomes in bacteria (11, 12), and the idea that bacterial ribonucleic acid, deoxyribonucleic acid, and protein synthesis occurs in a membrane-associated process has become popular with analogy to eukaryotes. Although it is still unclear why significant amounts of ribosomes are bound to bacterial membranes, the functional or structural interaction of the ribosomes and membranes is presumably possible. There are two possibilities that account for the properties of Suc^d-Spc^r mutants. (i) The conformational change of membrane-bound ribosomes produced by the Suc^d-Spc^r mutation directly affects membrane organization. (ii) The ribosomal alteration is responsible for some abnormality in the protein synthesis mechanism, so that some protein necessary for membrane organization is not synthesized in an active form.

Most of Suc^d-Spc^r mutants stopped division and became filamentous under nonpermissive conditions, suggesting that bulk protein synthesis was not inhibited. Analysis of this process will give more information about the property of Suc^d-Spc^r mutants. An examination of the stability of sex factors in these mutants is now being planned.

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