Molec. gen. Genet. 127, 1—18 (1973)

Electrophoretic and Immunological Studies on Ribosomal Proteins of 100 *Escherichia coli* Revertants from Streptomycin Dependence*

R. Hasenbank, C. Guthrie, G. Stöffler, H. G. Wittmann, L. Rosen and D. Apirion

Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, Germany
Department of Microbiology, Washington University Medical School St. Louis, U.S.A.

Received August 3, 1973

Summary. Revertants from streptomycin dependence to independence were isolated as single step mutants from six different streptomycin dependent strains. The ribosomal proteins from 100 such mutants were analyzed by two-dimensional polyacrylamide gel electrophoresis and some of them were also examined by immunological techniques. Altered proteins were found in 40 mutants, 24 in protein S4 and 16 in protein S5. No change in any other protein was detected.

Altered S5 proteins migrated into five different positions on the polyacrylamide plate and it can be concluded that the mutant proteins differ from the wild type probably by single amino acid replacements. The altered S4 proteins migrated into 17 different positions on the plate. Extensive changes of length, both shorter and longer than wild type S4 protein, are postulated for many of the mutant S4 proteins.

Analysis of the ribosomal proteins of four *ram* mutants revealed altered S4 proteins in two of them. The alterations in these mutant proteins are probably very similar to those found in streptomycin independent mutants.

Among the revertants there was no apparent correlation between the protein alteration and the particular response to streptomycin.

These studies suggest a strong interaction between protein S12, which confers streptomycin dependence, and protein S4 or S5, which can suppress this dependence.

Introduction

Mutation from streptomycin sensitivity to streptomycin dependence in *E. coli* results in an alteration in 30S ribosomal protein S12 (in the nomenclature of Wittmann *et al.*, 1971). This has been shown by demonstrating that 30S subunits reconstituted using protein S12 from a streptomycin dependent mutant and all other proteins from streptomycin sensitive ribosomes, are streptomycin dependent in a cell-free system (Birge and Kurland, 1969).

Reversion from streptomycin dependence to independence is, in most cases, due to a second mutation which maps close to the first site (Hashimoto, 1960), and this non-allelic mutation leads to an alteration in the ribosomal particle (Brownstein and Lewandowski, 1967; Apirion and Schlessinger, 1967; Apirion *et al.*, 1969). By electrophoretic and immunological methods, protein S4 was found to be altered in eight of fourteen revertants studied (Deusser *et al.*, 1970). Similar

* Paper No. 60 on "Ribosomal Proteins". Preceding paper is by B. Wittmann-Liebold, Hoppe-Seyler's Z. physiol. Chemie, in press.

findings were described by Kreider and Brownstein (1971). These are in good agreement with results of Birge and Kurland (1970) who used reconstitution experiments to demonstrate that mutant protein S4 is responsible for the suppression of the streptomycin dependent phenotype *in vitro*. It was subsequently shown that the phenotypic change from dependence to independence *in vivo* can also be associated with an alteration in protein S5 (Stöffler *et al.*, 1971; Kreider and Brownstein, 1972). The finding that mutations affecting either protein S4 or S5 can lead to suppression of the streptomycin dependent phenotype, which results from mutations in protein S12, suggests that these three proteins function cooperatively with each other in the ribosome.

Since the revertants investigated in our previous studies (Deusser *et al.*, 1970; Stöffler *et al.*, 1971) all originated from a single streptomycin dependent strain (N522), we decided to analyze revertants derived from several streptomycin dependent strains of independent origin. This was done to test the possibility that suppression of different *str*^{d} mutations in S12 might require mutations in proteins other than S4 or S5. Electrophoretic and immunological comparison of 100 independently isolated mutants from streptomycin dependence to independence derived from several different streptomycin dependent strains are described in this paper.

Materials and Methods

Mutant Isolation

Streptomycin dependent (Str-D) mutants N522, N523, N530 and N543 were isolated from *E. coli* K12 strain AB774 (see Apirion *et al.*, 1969 and Fig. 1). Spontaneous revertants from the four streptomycin dependent mutants were obtained as described by Deusser *et al.* (1970).

Str-D mutants N522 and N523 were isolated after treatment with the acridine half mustard ICR 191 (Silengo *et al.*, 1967). N530 was a spontaneous mutant and N543 was isolated after treatment of strain AB774 with N-methyl-N-nitro-N-nitrosoguanidine (Apirion, 1966). Another set of spontaneous revertants (d101–d1048) were isolated from a spontaneous streptomycin dependent mutant (d10) of *E. coli* K12 strain A19 (Fig. 1) in the following way: Single colonies of d10 were grown in broth plus streptomycin (200 μg/ml) at 37° C. Aliquots were centrifuged, washed and spread on tryptone plates after a ten-fold dilution. After incubation for several days at 37° C, five discrete colonies were picked from each plate and streaked on tryptone plates. Single purified colonies were grown in tryptone broth and spot-tested on tryptone plates containing 0, 10, 20, 50, 200 μg/ml streptomycin and 10, 20, 30, 50, 100 μg/ml spectinomycin. Only one mutant per isolation with a given phenotype was retained for further analysis. Mutant EB 30-su6 (Birge and Kurland, 1970) and *ram* mutants (Rosset and Gorini, 1969) T776-39:3, T776-40:4, T777-4:2 and T779-25:3 were kindly provided by Dr. C. G. Kurland, Uppsala and Dr. L. Gorini, Boston, respectively. Transduction experiments were done with bacteriophage P1.

Two-dimensional Polyacrylamide Gel Electrophoresis

Ribosomes were isolated by differential centrifugation. Ribosomal proteins were extracted with acetic acid, lyophilized and separated by two-dimensional polyacrylamide gel electrophoresis as described by Kaltschmidt and Wittmann (1970).

Immunochemical Investigations

a) Antisera: Five different antisera against pure ribosomal protein S4 as well as five different antisera against protein S5 were used. Both proteins S4 and S5 were purified from

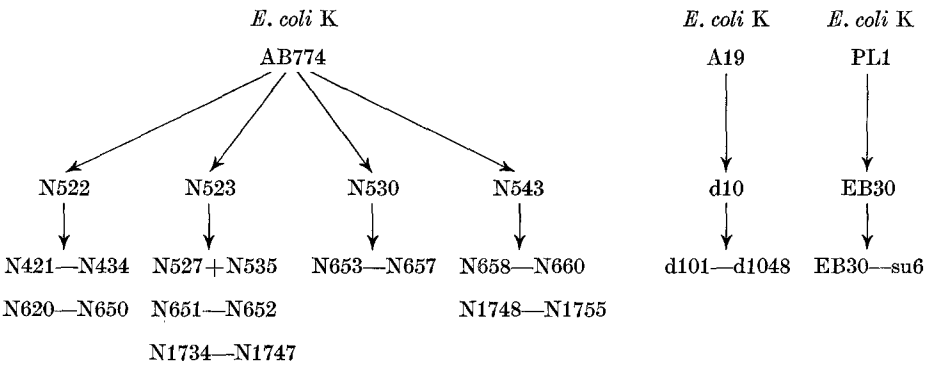

Fig. 1. Pedigree of the mutants described in this paper

E. coli A19 ribosomes as described by Hindennach *et al.* (1971). One anti S4-serum as well as one anti S5-serum was raised in sheep; the others were raised in rabbits. Titers were estimated and recorded as mg specific antibody per ml serum as previously described by Stöffler and Wittmann (1971).

b) Immunological methods, including double-diffusion tests, quantitative immunoprecipitation, cross-absorption tests and intra gel cross-absorption were performed as described previously (Stöffler and Wittmann, 1971).

Immunoelectrophoresis was performed in 1.5% agarose (Behring-Werke AG, Marburg/Lahn, Germany) in a sodium barbital buffer, pH 8.6, containing 1 M urea at 150 V (constant), 115 mA/cm; running time: 4 hours. After the electrophoretic separation of ribosomal proteins, the agarose plates were briefly equilibrated with sodium barbital buffer, pH 8.6, containing 0.75 M LiCl, to remove urea. Slots were cut out and filled with anti-S4 and anti-S5 sera, respectively. The plates were developed for 2–3 days at room temperature. Photographic documentations were done with both unstained and stained plates. The staining solution contained 0.5% azocarmin in 5% acetic acid.

Nomenclature

The following nomenclature was used for phenotypes: Str-S, Str-R, Str-D and Str-I, respectively, indicate sensitivity to, resistance to, dependence on and independence from streptomycin. Genotypes *str*^{s}, *str*^{r}, *str*^{d} and *str*^{i} designate alleles causing sensitivity to, resistance to, dependence on and independence from streptomycin.

Results and Discussion

Types of Mutants. Our previous studies (Deusser *et al.*, 1970; Stöffler *et al.*, 1971) were performed with revertants from a single streptomycin dependent mutant, N522. For the reason mentioned above (see "Introduction") we decided to add to this analysis revertants that were isolated from a number of independently isolated streptomycin dependent mutants. Four sets of mutants were studied (Fig. 1):

a) 79 mutants derived from four different streptomycin dependent mutants (N522, N523, N530 and N543) which were isolated from strain AB774. b) 20 mutants derived from the streptomycin dependent mutant d10 isolated from strain A19 (Fig. 1). c) Mutant EB 30-su6 which is a derivative of EB 30, a streptomycin

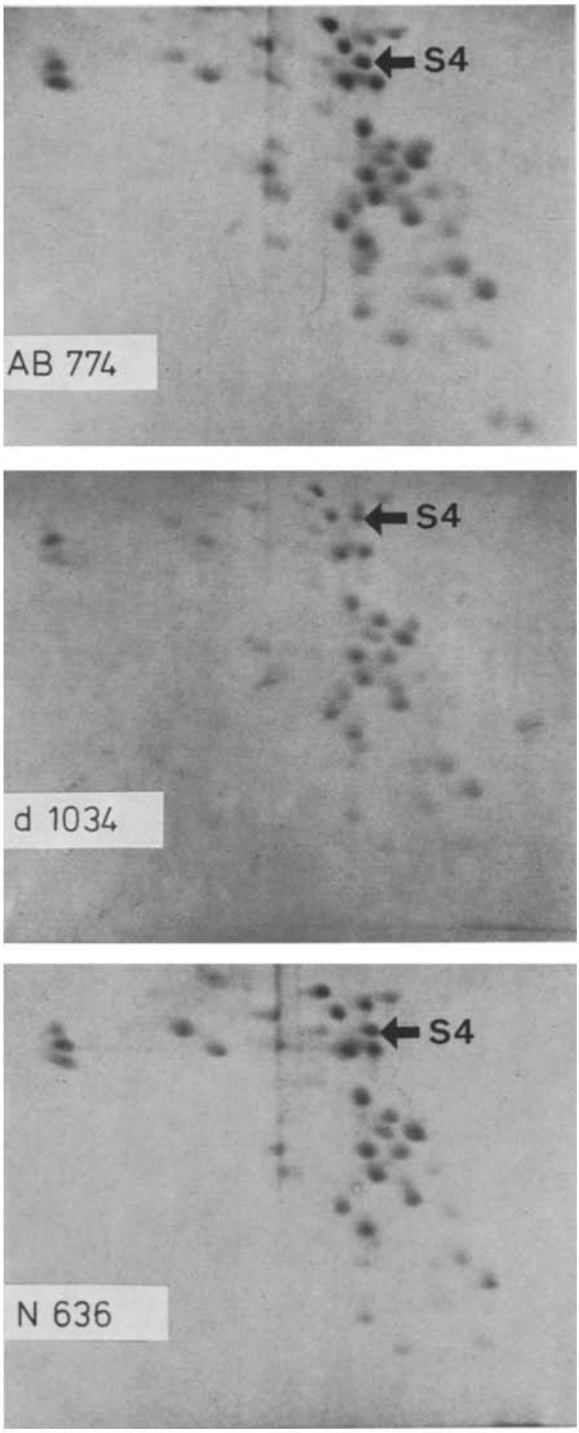

Fig. 2a

Fig. 2a and b. Two-dimensional gel electropherograms of wild type AB774 and five revertants from streptomycin dependence

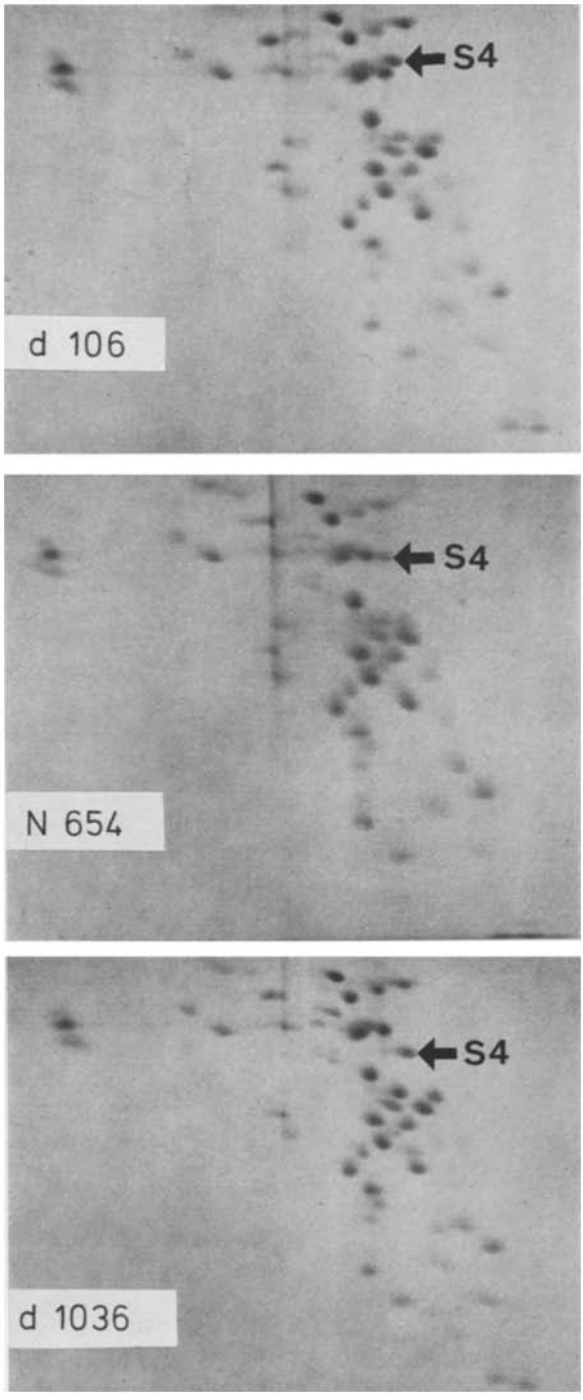

Fig. 2b

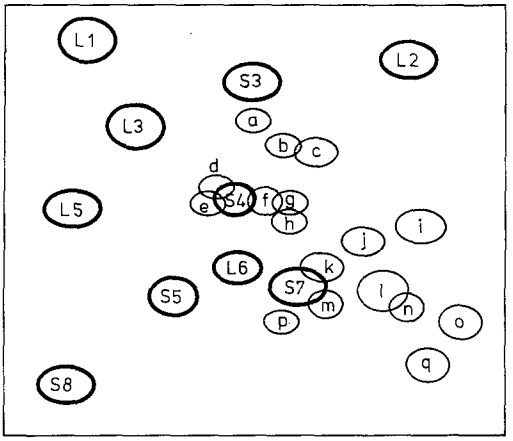

Fig. 3. Location of mutant S4 proteins on the two-dimensional gel electropherogram (for mutants see Table 1)

dependent mutant of *E. coli* K12 (Birge and Kurland, 1970). d) Four strains (T776-39:3, T776-40:4, T777-4:2 and T779-25:3) with *ram* mutations (Rosset and Gorini, 1969) which, when introduced into streptomycin dependent mutants, allow growth without streptomycin (Bjare and Gorini, 1971).

Number of Mutants with Altered Proteins. After isolation of ribosomes from the mutants, the ribosomal proteins were extracted and separated by two-dimensional electrophoresis. The protein patterns of the mutants were photographed and compared to each other and to *E. coli* strains AB774 and A19. The results are given in Figs. 2–4 and in Table 1, in which mutants with altered proteins are listed. Mutants with unchanged patterns are omitted from the Table. 40 of the 100 mutants had electrophoretically altered proteins. 24 of these 40 mutants had altered S4 proteins whereas 16 were altered in S5. In addition, two of the four investigated *ram*-mutants also showed an electrophoretically altered S4. No protein other than S4 or S5 was unambiguously shown to be altered in any of these mutants. This result suggests that, regardless of the streptomycin dependent allele present, reversion from streptomycin dependence is caused exclusively by alteration in proteins S4 or S5. More exhaustive investigation of the mutants which did not reveal electrophoretically altered S4 or S5 proteins would be necessary in order to rigorously prove this conclusion.

In our previous studies (Deusser *et al.*, 1970), five of 14 mutants analyzed were found by electrophoretic methods to have altered proteins; immunological techniques revealed changes in another three mutants. By extrapolation from these results, one should expect to find among the 100 mutants approximately 20–25 mutants with proteins altered in a similar manner (i.e. by exchange of neutral amino acids), such that the alteration can be detected by immunological but not by electrophoretic methods. This would then bring the number of mutants with electrophoretically or immunologically detectable altered S4 or S5 proteins to a total of about 60–65.

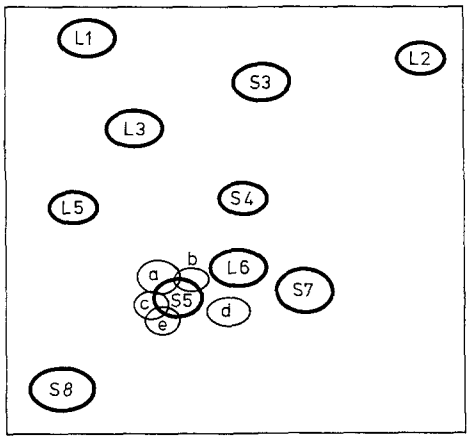

Fig. 4. Location of mutant S5 proteins on the two-dimensional gel electropherogram (for mutants see Table 1)

Table 1. Mutants altered in protein S4 or S5

Mutant	Parental strain	Altered protein	Protein position[a]	Streptomycin phenotype[b]	Mutant	Parental strain	Altered protein	Protein position[a]	Streptomycin phenotype[b]
N421	N522	S5	5a	R	N660	N543	S5	5d	S
N422	N522	S4	4c	R	N1733	N523	S4	4k	R
N425	N522	S4	4o	R	N1734	N523	S4	4n	L
N432	N522	S4	4p	R	N1737	N523	S5	5a	L
N433	N522	S4	4q	R	N1739	N523	S5	5d	R
N527	N523	S5	5a	L	N1741	N523	S5	5a	L
N535	N523	S5	5b	R	N1743	N523	S4	4b	L
N627	N522	S4	4d	R	N1745	N523	S5	5a	L
N628	N522	S4	4g	R	N1746	N523	S4	4i	S
N634	N522	S4	4e	R	N1749	N543	S4	4e	R
N635	N522	S5	5c	R	N1753	N543	S5	5a	L
N636	N522	S4	4h	R	N1755	N543	S5	5a	L
N637	N522	S5	5d	R	d106	d10	S4	4j	L
N639	N522	S4	4h	R	d1016	d10	S4	4e	R
N643	N522	S5	5a	R	d1023	d10	S5	5a	L
N647	N522	S4	4h	R	d1034	d10	S4	4a	L
N650	N522	S5	5e	R	d1036	d10	S4	4q	R
N651	N523	S4	4q	L	d1043	d10	S4	4o	S
N652	N523	S4	4b	L	su6	EB-30	S4	4m	
N653	N530	S5	5a	R	T776/39:3		S4	4f	
N654	N530	S4	4l	R	T776/40:4		S4	4h	

[a] Designate position of electrophoresis on a two-dimensional gel plate (see Fig. 3 and 4).
[b] Classified according to the response to streptomycin. S = sensitive similar to the original AB774 strain; R = resistant, to 200 μg per ml; L = low level resistance, withstands lower level of streptomycin than 200 μg/ml.

Altered S5 Proteins. Based on the electrophoretic mobilities of the mutant S5 proteins in two-dimensional gel electrophoresis, only a few types of alterations are observed (Table 1). Of the 100 revertants investigated, 16 revealed an alteration in protein S5. The altered S5 proteins migrated in two-dimensional electrophoresis to five different positions only (Fig. 4). S5 from 10 different revertants (class a) migrated to the same position, i.e. further towards the anode than wild type S5. A mutant of this class was previously characterized (Stöffler *et al.*, 1971). Three other mutant S5 proteins migrated to unique positions (b, c, e; see Fig. 4). Only three S5 proteins (position d) are more basic (or less acidic) than the wild type S5; these three proteins also moved to an identical position. Since mutants with electrophoretically identical S5 proteins (e.g. position 5a; Fig. 4, Table 1) exhibited differences in their phenotypes (see Table 1) one could conclude that their mutational alterations might not be identical. However, all S5a mutants from any single str^d parent (e.g. N523 or N543) exhibited the same streptomycin phenotype, although the streptomycin phenotype does vary across the different parents (Table 1 and below).

The distance by which mutant S5 proteins are separated from wild type S5 protein on polyacrylamide plates corresponds to the distance (about 3 mm) by which the S5 proteins from *E. coli* strains B and K are separated (Kaltschmidt *et al.*, 1970). Since proteins S5B and S5K differ in the exchange of one amino acid, namely alanine versus glutamic acid (as found by Wittmann-Liebold and Wittmann, 1971), it is likely that the various S5 proteins of the mutants described in this paper also differ by only one amino acid exchange. Mutant proteins which differ by a larger number of amino acids are separated by greater distances on polyacrylamide plates (Funatsu *et al.*, 1972) than the mutant S5 proteins shown in Fig. 4.

The amino acid difference in protein S5 between *E. coli* strains K and B is located in tryptic peptide T1 (Wittmann-Liebold and Wittmann, 1971). The amino acid exchanges in S5 proteins mutated to spectionmycin resistance are located in tryptic peptide T10 (Funatsu *et al.*, 1971). Neither of these amino acid exchanges can be detected by immunochemical methods (Kaltschmidt *et al.*, 1970; Stöffler, unpublished results). We therefore conclude that none of the animals used for immunization recognized a determinant in the altered regions, i.e. in either tryptic peptide T1 or T10. However, six revertants from streptomycin dependence to independence with an altered electrophoretic mobility of protein S5 gave partial cross-reactions when compared with the wild type S5 protein in double diffusion tests. Therefore we conclude that the alterations of protein S5 in revertants from streptomycin dependence to independence are located in a region of protein S5 different from that altered in spectinomycin resistant mutants or that responsible for the K and B strain-specific amino acid difference. That the mutations in the S5 protein which cause resistance to spectinomycin and reversion from streptomycin dependence, respectively, are located in two separate parts of the S5 protein, is also indicated by genetic analysis.

Two independently isolated spectinomycin resistant mutants, N223 and N225, were isolated from strain N421 which contains a mutation to streptomycin dependence in S12 and to streptomycin independence in S5 (see Stöffler *et al.*, 1971 and Table 1). A series of transduction experiments were carried out with N223

and N225. When N223 (*spc*^{r}, *str*^{i}, *str*^{d}) was the donor and N141 (*spc*^{s}, *str*$^{i+}$, *str*^{s}) was the recipient, from 62 Str-R transductants isolated, 21 were sensitive to spectinomycin. All the transductants were resistant to and not dependent on streptomycin, which means that in about 30% of the transductants, a crossover took place between the *str*^{i} site and the *spc* site.

Similar results were obtained in a transduction experiment using N225 and N141. In this case, 17 out of 47 Str-R transductants were sensitive to spectinomycin. Again, none of the transductants were dependent on streptomycin. When the selection was carried out for spectinomycin resistance, most of the transductants were sensitive to streptomycin: 104 from 154 when N223 was used, and 136 from 159 when N225 was used.

Assuming that both, mutation to spectinomycin resistance and to reversion from dependence, are in the same gene, then the order of the markers can be either *spc str*^{i} *str*^{d} or *str*^{i} *spc str*^{d}. The data presented here, suggest that the first order is more likely. If the second order were the correct one, then, when Str-R transductants were selected for, most of them should have been resistant to spectinomycin. (Since none of them was dependent on streptomycin, they should have included both the *str*^{d} and the *str*^{i} allele). However, in experiments with both strains about one third of the transductants were sensitive to spectinomycin. Both sets of data (selection of Spc-R or Str-R transductants) are compatible with the first order. These data also suggest that *str*^{i} by itself does not confer any resistance to streptomycin. The finding that most of the Spc-R transductants were sensitive to streptomycin indicates that any crossover between *spc* and *str*^{d}, regardless of whether or not the *str*^{i} allele is included, leads to sensitivity to streptomycin.

Two unexpected results were obtained in these experiments: First, no Str-D transductants were isolated; and second, a very high frequency of crossing over took place between the *str*^{i} site and the *spc* site (assuming that they are in the same gene). The first observation most likely results from the fact that strain N522 is a very poor grower; thus any transductant that carries the unsuppressed str_{522} allele is at a very severe disadvantage and is selected against. The second phenomenon could be a consequence of the fact that to get Str-R transductants, both alleles str^d_{522} and str^i_{421} have to be included in the transducting fragments. If these two alleles are quite far apart (and they are likely to be since they are in two different genes) then the chance of the third allele spc_{223} or spc_{225} being included in the same transducing fragment is reduced and the apparent distance between the two sites (*str*^{i} and *spc*) becomes exaggerated.

In oder to test whether mutations to streptomycin independence and to altered S5 proteins are closely linked, the ribosomal proteins from twenty-five transductants resistant to streptomycin were analyzed by two-dimensional gel electrophoresis. In all cases the normal S5 protein of the recipient N141 was replaced in the transductants by the altered S5 protein. This result is a strong indication that the altered S5 protein is involved in the reversion from streptomycin dependence to independence and that a single mutational event leads to an altered S5 protein which in turn suppresses streptomycin dependence.

The observation that mutations in protein S5 could suppress streptomycin dependence (Stöffler *et al.*, 1971) encouraged the attempt to screen selectively for such suppressors in the presence of spectinomycin. Although no cross-resistance

between streptomycin and spectinomycin is known (Weisblum and Davies, 1968), it was thought that a subclass might exist having an alteration in protein S5 which simultaneously suppresses streptomycin dependence and causes resistance to spectinomycin. Such a search was unsuccessful. Under conditions where many thousands of streptomycin independent mutants could be isolated in the absence of spectinomycin, none were found in its presence, even at spectinomycin concentrations as low as 30 μg/ml. The simplest explanation for this finding is that two different and non-overlapping regions of the protein are involved in the two functions (suppression of streptomycin dependence and resistance to spectinomycin). This conclusion is in agreement with the immunochemical and genetic data presented above.

On the other hand, the possibility was also considered that such a mutant (*spc*^{r}, *str*^{i}) would be non-viable. (It should perhaps be noted that the action of spectinomycin on sensitive cells is bacteriostatic rather than bacteriocidal, so that, at least theoretically, cells of the desired type should not be prevented from arising on the spectinomycin-containing plates). To investigate this possibility, *str*^{i} mutants were screened for their ability to grow on plates containing 10, 20, 30 and 50 μg/ml spectinomycin. The parental strain A19 grows normally on 10–20 μg/ml and gives a variable response at 30 μg/ml spectinomycin. It was found (Table 2) that a significant proportion (10 out of 20) of the streptomycin independent mutants showed an increased sensitivity to spectinomycin compared to wild type cells. Although the extent of this hypersensitivity is somewhat variable, it is not simply an artifact of the slower growth of some of the revertants on plates, as can be concluded from studies in liquid media (data not shown). There does not appear to be a direct correlation between the phenotypic response to spectinomycin and the specific protein alteration (Table 2 cf. Table 1).

Altered S4 Proteins. Electrophoretic alterations in S4 proteins were observed in 26 of the mutants studied. In contrast to mutations in S5 proteins, the altered S4 proteins showed a great variety in their electrophoretic mobility. S4 proteins from the 26 mutants moved to 17 different positions on the two-dimensional electrophoresis plate (Table 1, Fig. 3); only the remaining nine migrated towards positions overlapping with one of the other 17 different S4 protein positions. Five electrophoretic positions were occupied by more than one mutant S4 protein, namely positions b and o by two, positions e and q by three and position h by four proteins (Table 1). However, it is not excluded that proteins migrating to the same position on the two-dimensional electrophoresis plate are also different from each other and that their movement to the same position is only coincidental. Differences should be expected at least for the S4 proteins migrating into electrophoretic positions o and q, since the phenotypes of these mutants belonging to the same electrophoretic group are not the same (Table 1). However, as already mentioned for protein S5a, such phenotypic variations were only found across different parents whereas within a given streptomycin dependent parent all mutants of the same electrophoretic type have the *same* streptomycin phenotype (Table 1). This supports the idea that the phenotype of a given revertant reflects the different interactions between S12 and S4 rather than the alteration in protein S4 alone.

Table 2. Phenotype of streptomycin-dependent revertants of d10

Mutant	Streptomycin[a]	Spectinomycin[b]	Altered protein[c]
d101	20	30–50	—
d105	200	30–50	—
d106	50	30 (HS)	S4
d108	200	30 (HS)	—
d1010	20	30–50	—
d1014	200	20 (HS)	—
d1016	200	30 (HS)	S4
d1017	20	30–50	—
d1020	50	30 (HS)	—
d1021	200	20 (HS)	—
d1023	50	30–50	S5
d1029	50	30–50	—
d1032	200	30 (HS)	—
d1034	50	30–50	S4
d1035	200	20 (HS)	—
d1036	200	20 (HS)	S4
d1042	200	20 (HS)	—
d1043	20	30–50	S4
d1047	200	30–50	—
d1048	50	30–50	—
d10(str^d)	200(50[d])	n.t.	
A19(str^s)	5–10	30–50	

Mutants were isolated as described in Materials and Methods and spottested on antibiotic containing tryptone plates. Plates were scored after overnight incubation at 37°C. The results shown were obtained in a minimum of two tests.

[a] Highest conc. (μg/ml) tested to which cells are resistant on plates.
[b] Lowest conc. (μg/ml) tested which inhibits cell growth on plates; (HS) = hypersensitive.
[c] See Table 1; dash indicates no electrophoretic alteration found;
[d] Minimum conc. required for growth.
n.t. = not tested.

All 26 mutants with an electrophoretically altered S4 protein showed only partial immunological cross-reaction with the antisera raised against wild type S4. Ribosomal proteins from ten randomly selected mutants with electrophoretically unaltered S4 or S5 proteins were examined by immunochemical methods. Three of them showed a reduced cross-reaction with the anti-S4 wild type sera.

It is remarkable that the altered S4 proteins in all but two cases (positions d and e) migrate faster in the first dimension towards the cathode than S4 from the wild strain, revealing that these mutations lead to more basic S4 proteins (Fig. 3). These results were confirmed by immuno-electrophoresis. Furthermore, it was observed that many S4 proteins move significantly faster or slower in the second dimension of the electrophoresis than the wild type S4 protein. Since the mobility in the second dimension is mainly according to size of the protein, this finding suggests a difference in molecular weight between S4 proteins from wild type and several mutants. Determination of molecular weights by SDS gel electrophoresis (Funatsu *et al.*, 1972) confirmed this expectation.

Comparison of the electrophoretic migration positions of the S4 proteins described in this paper with the analysis of molecular weights carried out for some of these mutants by Funatsu *et al.* (1972) allows a prediction of the molecular weights: S4 proteins which migrate into positions a, b and c (Fig. 3) should be longer than S4 of wild type. The S4 proteins in electrophoretic positions k to q should have lower molecular weights than the wild type protein. It seems very likely that S4 proteins which migrate into electrophoretic positions d, e and f have about the same length as S4 of the parental strain. It is difficult to predict unambiguously from the migration in the two-dimensinal electrophoresis to which class (longer, shorter or the same length) the mutant proteins corresponding to positions g, h, i and j belong. Studies with isolated mutant proteins are necessary to clarify this point.

Since more data about molecular weights, amino acid composition, tryptic peptides and sequences of the C-terminal regions became available (Funatsu *et al.*, 1972), a better interpretation of the immunochemical results is possible. Protein S4 of mutant N422 is longer by about 20 amino acids than the wild type S4, which contains about 200 amino acids (E. Schiltz and J. Reinbolt, unpublished results). S4 from N422 showed only a partial cross-reaction with anti wild type S4 sera. This result is unexpected if one assumes that S4 protein from N422 has an unchanged sequence until amino acid position 200, the normal C-terminal end of parental S4 protein. It could be explained if one assumes that the tail of 20 additional amino acids leads to a conformational change in the sequence 1–200. Since it is known that most of the specific antibodies against ribosomal proteins are directed against sequence specific determinants rather than against conformational specific determinants (Stöffler and Wittmann, 1971), this interpretation seems rather unlikely. On the other hand, a frame-shift mutation as well as a deletion or insertion within the S4 cistron would easily account for the reduced cross-reactivity of S4 derived from mutant N422, because in these cases the antigenic determinants in the C-terminal region should be altered and therefore lead to a reduced cross-reaction.

S4 proteins from mutants N425 and N433 also showed a significant decrease of immunological cross-reactivity when developed against anti wild type S4 sera. At least one determinant should be located within the last twenty amino acids of the wild type S4 protein, since proteins S4 from N425 and N433 are shorter than wild type S4 protein by 20 and 30 amino acids, respectively (Funatsu *et al.*, 1972). In addition, when one anti S4 serum (rabbit R128) was developed against S4 of N425 and N433 the precipitation line against N425 led to spur formation over N433. From the results it can be concluded that the length of the protein chain (corresponding to wild type) is longer in N425 than in N433. Amino acid sequence studies on these mutant proteins, now in progress, are expected to give more detailed information.

ram mutants. Three findings show that *ram* mutants (Rosset and Gorini, 1969) although isolated by a different selection method, are equivalent to the S4 mutants studied in this paper. Firstly, the introduction of the *ram* allele into streptomycin dependent mutants results in streptomycin independence (Bjare and Gorini, 1971). Secondly, protein S4 is altered in *ram* mutants as demonstrated by Zimmermann *et al.* (1971). Thirdly two-dimensional electrophoresis show that

Table 3. Streptomycin phenotypes of the examined revertants

Strain	Streptomycin phenotype									Total tested	Total altered
	S			L			H				
	S4	S5	none	S4	S5	none	S4	S5	none		
d10	1		3	2	1	4	2		7	20	6
N522							10	5	30	45	15
N523	1		1	4	4	1	1	2	4	18	12
N530							1	1	3	5	2
N543		1			2	1	1		6	11	4
	2	1	4	6	7	6	15	8	50		
		7			19			73		99*	39*

* Not including ram-mutants and EB30-su6.

altered S4 proteins from *ram* mutants are located on the electrophoresis plate among the altered S4 proteins of revertants from streptomycin dependence (Fig 3 and Table 1). Of the four mutants analysed, two showed electrophoretically altered proteins. One of them fell into group h, together with three mutants derived from the streptomycin dependent strain N522.

Therefore, it can be expected that the proteinchemical alterations in the S4 proteins of the *ram* mutants are of a similar type to those of the other mutants described in this paper. Proteinchemical studies on the difference between S4 proteins from wild type and from *ram* mutants are in progress (v. Acken, unpublished results).

Single Mutations that Can Cause Multiple Alterations in a Protein. The results presented here on the altered S4 proteins as well as the analysis of some of these mutant proteins (Funatsu *et al.*, 1972; Donner and Kurland, 1972) leave little doubt that multiple amino acid changes can occur in protein S4 in various revertants from streptomycin dependence to independence. While the complete sequence of these altered proteins is not yet known, it is quite clear that some of the proteins are shorter while others are longer than the wild type S4 proteins. The results also show that these multiple changes are near the carboxyl end of the protein. Since all the revertants were isolated without mutagenesis and their frequency is much higher than the frequency of mutation from streptomycin sensitivity to resistance, we conclude that they most probably result from single step mutations.

There are a number of ways to explain these results; three possible interpretations will be discussed here:

1) Mutant S4 proteins shorter than S4 of the parental type could be caused by mutation of a sense codon to a termination codon, and longer protein chains by a change from the termination codon into a sense codon. Although there is no difficulty in explaining the observed variety of *shorter* mutant proteins by this mechanism, one should expect only one protein chain *longer* than that of wild type, namely that which is translated until the next termination codon. We have

however observed longer proteins of different lengths and therefore the possibility mentioned in this paragraph is not likely.

2) The changes in protein length could be due to incorrect processing of a hypothetical precursor protein, "pro-S4". The incorrect cleavage might be due either to a defective processing enzyme or to amino acid replacements in the S4 protein leading to alterations in the tertiary protein structure. By this mechanism a great variety of shorter or longer chains can be explained.

3) Deletion or insertion of nucleic acid pieces corresponding to one or more codons would explain shorter or longer protein chains but the proteins should have the same C-terminus as wild type S4 protein. However proteinchemical studies on the mutant proteins have revealed different C-terminal regions (Funatsu *et al.*, 1972). Therefore another possibility must be considered. Deletion (or insertion) of a nucleic acid region not corresponding to one or more codons, caused e.g. by deletion of a number of nucleotides which is not a multiple of three, would lead to an altered frame shift and therefore to a different amino acid sequence in the C-terminal region. In this way protein chains shorter than wild type can be explained depending on the length and the position of the deletions or insertions. This seems indeed to be the mechanism by which the different lengths of the mutant S4 proteins originate (E. Schiltz, J. Reinbolt, W. Puls and H. G. Wittmann, unpublished results).

Comparison of Revertant Phenotype with Protein Alteration. Mutants from Str-D to Str-I show a variety of responses to streptomycin addition to growth media. While they do not require this antibiotic for growth, some revertants become sensitive, some resistant and some hypersensitive to streptomycin (Hashimoto, 1960; Kreider and Brownstein, 1972). Since all the revertants were analyzed both for their response to streptomycin and for alterations in S4 or S5 proteins (Table 1), possible correlations were looked for. No particular pattern could be found. It seems that most str^d alleles can give rise to streptomycin independent strains which fall into a variety of phenotypic classes with respect to the response to streptomycin. On the other hand in a given class one can find mutants with either S4 or S5 alterations in different positions.

It is worth noting that all the revertants from strain N522 could withstand 200 μg of streptomycin (see Table 1), which could classify them as Str-R strains. This seems to be a specific attribute of this particular allele (str^d_{522}). Of the three major phenotypic classes of revertants with respect to streptomycin, the L-class (low level resistance) seems to have significantly more members with electrophoretically altered S4 or S5 proteins than do the other two classes. As previously mentioned, only one obvious "hot spot" was observed, position 5a, where 10 out of the 16 observable S5 mutants were gathered.

The streptomycin independent mutants from the streptomycin dependent parent d10 were screened for their response to streptomycin and spectinomycin and several classes were found (see Table 2). Again there do not appear to be any obvious correlations between phenotypic response to streptomycin and the protein alteration. For example, both d106 and d1023 are resistant to 50 μg/ml streptomycin and sensitive to 200 μg/ml, yet the former is altered in S4 and the latter in S5.

General Remarks. There are a number of interesting points that the experiments described here raise:

a) Regardless of the streptomycin dependent allele, reversion to streptomycin independence occured by changes in proteins S4 or S5 only. This is true although streptomycin dependent mutants with different alleles were used. The S12 proteins from the streptomycin dependent mutants used were found by sequence analysis to be different (Funatsu and Wittmann, 1972; Itoh and Wittmann, 1973).

b) It was unexpected that the ribosome would remain functional with such extensive changes in one of its structural proteins, namely S4, since it is envisaged as a rather conservative organelle which is strict in the kind of altertation that it can withstand. Most of the revertants studied here have growth rates comparable to those of the streptomycin sensitive strains from which the streptomycin dependent mutants have been isolated. This suggests: 1) the extensive changes in the C-terminal region of the S4 protein do not interfere with the assembly of the ribosome even though protein S4 interacts directly with the 16S rRNA; and 2) the C-terminal region of protein S4 is not critical for the accurate functioning of the ribosome (at least not in revertants from streptomycin dependence).

c) The fact that mutant S4 proteins longer than wild type were observed suggest that stretches of untranslated nucleotides exist between neighbouring cistrons coding for ribosomal proteins. Reading into the next ribosomal protein cistron would affect this protein and would be revealed in two-dimensional gel electrophoresis. However no modification of any other ribosomal protein has been found.

d) Prior to these experiments, analysis of ribosome mutations with a similar phenotype, e.g. mutants resistant to streptomycin or to spectinomycin, revealed that only very few amino acid positions in the mutant ribosomal proteins are altered. We might thus expect that reversion from streptomycin dependence to independence could result from amino acid replacements in a few different positions. While the alterations in protein S5 seem to be of this nature, it was rather surprising that a large variety of extensive changes in protein S4 could lead to reversion from streptomycin dependence. This raises the possibility that there is a sequence in the C-terminal region of protein S4 which is required for the expression of streptomycin dependence. Removal or extensive alteration of this region would then be enough to abolish the dependency of the ribosome on streptomycin. The absence of this sequence in protein S4 can be achieved in a number of ways, as shown in this paper, since the sequence of the protein can apparently be varied without greatly affecting the biosynthesis and functioning of the ribosome.

e) It is clear from these studies that proteins S4, S5 and S12 interact intimately in the functioning of the ribosome. Whether or not these interactions involve physical contact among the involved proteins remains to be seen. The fact that no other ribosomal protein was altered in any of the revertants suggests that reversion from streptomycin dependence can occur only by altering proteins S4 or S5. Of course if alteration in other proteins did not change their charge, they could not have been detected by the electrophoretic techniques used. If alterations in proteins other than S4 or S5 did occur, then based on our experience at least some of them should have been detected by the techniques used.

f) At present we know three types of alterations in protein S5: those causing resistance to spectinomycin, those reverting streptomycin dependence and the alteration which is connected with the difference between *E. coli* strain K and B. From the genetic, immunological and sequence analyses discussed above it can be concluded that each of these three types of alterations is restricted to a unique part of the S5 protein.

g) While the changes in protein S5 are very likely due to single site point mutations causing the replacement of only one amino acid, the altered S4 proteins probably arise via one step mutations (as discussed above) yet involve multiple alterations in the amino acid sequences. Among the various possibilities available to explain one step mutations that cause multiple changes in proteins, we prefer the suggestion that insertions and deletions are responsible for the observed alterations. This hypothesis is amenable to direct test by immunological and sequence analyses, some of which are currently in progress.

h) Protein S4 was shown to be a key-protein in the assembly of the 30S subunit (Mizushima and Nomura, 1970) and to bind specifically and independently to 16S RNA (Schaup *et al.*, 1970, 1971; Garrett *et al.*, 1971; Zimmermann *et al.*, 1972). A decreased binding of protein S4-su6 to 16S RNA was demonstrated by Green and Kurland (1971) and the possibility of a correlation of a reduced binding constant of S4/16S RNA with phenotypic suppression of streptomycin dependence was discussed. However, investigations of the binding of S4 proteins isolated from six mutants (N422, N424, N425, N428, N430 and N433) revealed that such a correlation between the protein S4/16S RNA binding constant and the phenotypic suppression of streptomycin dependence does not exist (Daya *et al.*, 1972).

i) Revertants fall into a number of phenotypic classes with respect to response to streptomycin but there is no detectable correlation between the phenotype and the nature of the protein alteration that led to reversion (see Table 1). It should be noted that all mutants of the same electrophoretic type derived from any single streptomycin dependent parent exhibited the same streptomycin phenotype. However the phenotype varies across parents (Table 1). Therefore the phenotype of any given revertant might reflect the interactions of the altered proteins S12 as well as S4 or S5 and should not be dependent upon the alteration in protein S4 or S5 only.

References

Apirion, D.: Altered ribosomes in a suppressor strain of *Escherichia coli*. J. molec. Biol. **16**, 285–301 (1966)

Apirion, D., Schlessinger, D.: Reversion from streptomycin dependence in *Escherichia coli* by a further change in the ribosome. J. Bact. **94**, 1275–1276 (1967)

Apirion, D., Schlessinger, D. Phillips, S., Sypherd, P.: *Escherichia coli:* Reversion from streptomycin dependence, a mutation in a specific 30S ribosomal protein. J. molec. Biol. **43**, 327–329 (1969)

Birge, E. A., Kurland, C. G.: Altered ribosomal protein in streptomycin dependent *Escherichia coli*. Science **166**, 1282–1284 (1969)

Birge, E., Kurland, C. G.: Reversion of a streptomycin dependent strain of *Escherichia coli*. Molec. gen. Genet. **109**, 356–369 (1970)

Bjare, U., Gorini, L.: Drug dependence reversed by a ribosomal ambiguity mutation ram. J. molec. Biol. **57**, 423–435 (1971)

Brownstein, B. L., Lewandowski, L. J.: A mutation suppressing streptomycin dependence. I. An effect of ribosome function. J. molec. Biol. **25**, 99–109 (1967)

Daya-Grosjean, L., Garrett, R. A., Pongs, O., Stöffler, G., Wittmann, H. G.: Properties of the interaction of ribosomal protein S4 and 16S RNA in *Escherichia coli* revertants from streptomycin dependence to independence. Molec. gen. Genet. **119**, 277–286 (1972)

Deusser, E., Stöffler, G., Wittmann, H. G., Apirion, D.: Ribosomal proteins XVI. Altered S4 proteins in *Escherichia coli* revertants from streptomycin dependence to independence. Molec. gen. Genet. **109**, 298–302 (1970)

Donner, D., Kurland, C. G.: Changes in the primary structure of a mutational altered ribosomal protein S4 of *Escherichia coli*. Molec. gen. Genet. **115**, 49–53 (1972)

Funatsu, G., Puls, W., Schiltz, E., Reinbolt, J., Wittmann, H. G.: Ribosomal proteins, XXXI. Comparative studies on S4 proteins of six *Escherichia coli* revertants from streptomycin dependence to independence. Molec. gen. Genet. **115**, 131–139 (1972)

Funatsu, G., Schiltz, E., Wittmann, H. G.: Ribosomal proteins, XXVII. Localisation of the amino acid exchanges in protein S5 from *Escherichia coli* mutants resistant to spectinomycin. Molec. gen. Genet. **114**, 106–111 (1971)

Funatsu, G., Wittmann, H. G.: Ribosomal proteins XXXIII. Location of amino acid replacements in protein S12 isolated from *Escherichia coli* mutants resistant to streptomycin. J. molec. Biol. **68**, 547–550 (1972)

Garrett, R. A., Rak., K. H., Daya, L., Stöffler, G.: Ribosomal proteins, XXIX. Specific protein binding sites on 16S rRNA of *Escherichia coli*. Molec. gen. Genet. **114**, 112–124 (1971)

Green, M., Kurland, C. G.: A mutationally altered ribosomal protein with a defective RNA binding site. Nature (Lond.) New Biol. **234**, 273–274 (1971)

Hashimoto, K.: Streptomycin resistance in *Escherichia coli* analyzed by transductions. Genetics **45**, 49–62 (1960)

Hindennach, I., Stöffler, G., Wittmann, H. G.: Ribosomal proteins. Isolation of the proteins from 30S ribosomal subunits of *Escherichia coli*. Europ. J. Biochem. **23**, 7–11 (1971)

Itoh, T., Wittmann, H. G.: Amino acid replacements in protein S5 and S12 of two *Escherichia coli* revertants from streptomycin dependence to independence. Molec. gen. Genet. **127**, 19—32 (1973)

Kaltschmidt, E., Stöffler, G., Dzionara, M., Wittmann, H. G.: Ribosomal proteins XVII. Comparative studies on ribosomal proteins of four strains of *Escherichia coli*. Molec. gen. Genet. **109**, 303–308 (1970)

Kaltschmidt, E., Wittmann, H. G.: Ribosomal proteins, VII. Two-dimensional polyacrylamide gel electrophoresis for fingerprinting of ribosomal proteins. Analyt. Biochem. **36**, 401–412 (1970)

Kreider, G., Brownstein, B. L.: A mutation suppressing streptomycin dependence, II. An altered protein in the 30S ribosomal subunit. J. molec. Biol. **61**, 135–142 (1971)

Kreider, G., Brownstein, B. L.: Ribosomal proteins involved in the suppression of streptomycin dependence in *Escherichia coli*. J. Bact. **109**, 780–785 (1972)

Mizushima, S., Nomura, M.: Assembly mapping of 30S ribosomal proteins from *Escherichia coli*. Nature (Lond.) **226**, 1214–1218 (1970)

Rosset, R., Gorini, L.: Ribosomal ambiguity mutation. J. molec. Biol. **39**, 95–112 (1969)

Schaup, H. W., Green, M., Kurland, C. G.: Molecular interactions of ribosomal components I. Identification of RNA binding sites for individual 30S ribosomal proteins. Molec. gen. Genet. **109**, 193–205 (1970)

Schaup, H. W., Green, M., Kurland, C. G.: Molecular interaction of ribosomal components. II. Site-specific complex formation between 30S proteins and ribosomal RNA. Molec. gen. Genet. **112**, 1–8 (1971)

Silengo, L., Schlessinger, D., Mangiarotti, G., Apirion, D.: Induction of mutations to streptomycin and spectinomycin in *Escherichia coli* by N-methyl-N′-nitro-N-nitrosoguanidine and acridine half mustard ICR 191. Mutation Res. **4**, 701–703 (1967)

Stöffler, G., Deusser, E., Wittmann, H. G., Apirion, D.: Ribosomal proteins XIX. Altered S5 ribosomal proteins in an *Escherichia coli* revertant from streptomycin dependence to independence. Molec. gen. Genet. **111**, 334–341 (1971)

Stöffler, G., Wittmann, H. G.: Sequence differences of *Escherichia coli* 30S ribosomal proteins as determined by immunochemical methods. Proc. nat. Acad. Sci. (Wash.) **68**, 2283–2287 (1971)

Weisblum, B., Davies, J.: Antibiotic inhibitors of the bacterial ribosome. Bact. Rev. **32**, 493–528 (1968)

Wittmann, H. G., Stöffler, G., Hindennach, I., Kurland, C. G., Randall-Hazelbauer, L., Birge, E. A., Nomura, M., Kaltschmidt, E., Mizushima, S., Traut, R. P., Bickle, T. A.: Correlation of 30S ribosomal proteins of *Escherichia coli* isolated in different laboratories. Molec. gen. Genet. **111**, 327–333 (1971)

Wittmann-Liebold, B., Wittmann, H. G.: Ribosomal proteins, XX. Isolation and analysis of the tryptic peptides of proteins S5 from strains K and B of *Escherichia coli*. Biochim. biophys. Acta (Amst.) **251**, 44–53 (1971)

Zimmermann, R. A., Carvin, R. D., Gorini, L.: Alterations of a 30S ribosomal protein accompanying ram mutation in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S. **68**, 2263–2267 (1971)

Zimmermann, R. A., Muto, A., Fellner, P., Ehresmann, C., Branlant, C.: Localization of ribosomal protein binding sites on 16S ribosomal RNA. Proc. Nat. Acad. Sci. U.S.A. **69**, 1282–1286 (1972)

Communicated by E. Bautz

R. Hasenbank
G. Stöffler
H. G. Wittmann
Max-Planck-Institut
D-1000 Berlin 33
Ihnestr. 63–73

L. Rosen
D. Apirion
Dept. of Microbiology
Washington University
Box 8093
St. Louis, Miss. 63110
U.S.A.

C. Guthrie
Department of Biochemistry
and Biophysics
University of California
San Francisco Medical Center
San Francisco, California 94122
U.S.A.