Molec. gen. Genet. 158, 129–139 (1977)

MGG

Improved Electrophoretic and Immunochemical Techniques for the Identification and Characterization of Mutant Proteins, Applied to Ribosomal Protein S8 in *Escherichia coli* Mutants

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Summary. The ribosomal proteins of 11 mutants which are sensitive to starvation at elevated temperature and of 36 transductants derived from them were studied with several electrophoretic, immunochemical and proteinchemical methods. The following results were obtained: (1) Ribosomal protein S8 is altered in three of these mutants. (2) The amino acid exchange in protein S8 of mutant N4128 is Glu→Lys in position 59 of the protein chain. (3) Temperature sensitivity and inability to recover from starvation at elevated temperatures are caused by the same mutational event which is, however, unrelated to the alteration in protein S8.

Several electrophoretic and immunological procedures were applied during the characterization of these mutants. A modified immunoelectrophoresis on cellulose acetate gels was developed, and proved to be the most applicable procedure for the detection of mutationally altered ribosomal proteins. This procedure may gain general importance for detecting mutational alterations in other proteins.

Introduction

The ribosome of Escherichia coli is a complex organelle built up from 54 ribosomal proteins and three different RNA molecules (cf. Brimacombe et al., 1976; Stöffler and Wittmann, 1977, for recent reviews). Information leading to a clearer understanding of the structural and functional properties of this organelle can be derived from the analysis of mutants. One source for ribosomal mutations are strains which show different responses to ribosomal antibiotics as compared to the parental strains, i.e. they become more resistant or mor sensitive to an antibiotic. Such ribosomal mutants have been useful for the identification of ribosomal proteins altered in strains resistant to streptomycin, spectinomycin or erythromycin (see Wittmann and Wittmann-Liebold, 1974; Stöffler and Wittmann, 1977, for reviews).

In this paper we report the analysis of ribosomal proteins of mutants which are sensitive to starvation at elevated temperature (Phillips et al., 1969) and of transductants derived from them. Protein S8 was found to be altered in some of these mutants and the amino acid replacement was localized in a mutant S8 protein chain.

In addition, we describe several new electrophoretic and immunological procedures that were developed during the characterization of these mutants. They are of general importance for detecting mutational alterations in ribosomal and other proteins.

Material and Methods

Isolation of Starvation Temperature (sts) Mutants was done according to Phillips et al. (1969). The following mutants were studied: N4003, N4050, N4106, N4128, N4306, N4308, N4310, N4312, N4334, N4335 and N4435.

Preparation of Cells and Ribosomes. Cells were grown in rich medium (yeast extract and peptone enriched with glucose), harvested in the late log-phase and broken by grinding with aluminium powder (ALCOA A-305). Ribosomes were isolated by differential centrifugation and washed with 0.5 M ammonium chloride (Hindennach et al., 1971).

Biochemical Procedures. Ribosomal proteins were extracted from 70S ribosomes with acetic acid (Hardy et al., 1969). Two-dimensional gel electrophoresis was according to Kaltschmidt and Wittmann (1970), cellulose acetate gel electrophoresis according to Stöffler (1967) with some modifications (G. Stöffler and R. Ehrlich, unpublished results). Cellogel was from Chemetron, Milano-Italy. Isolation of protein S8 from the wild type and the mutants was done as previously described (Hindennach et al., 1971). SDS-gel electrophoresis was according to Weber and Osborne (1968); two-dimensional gel electrophoresis using SDS in the second dimension has previously been described (Geisser, Tischendorf, Stöffler and Wittmann, 1973).

Immunological Techniques

a) Specific antisera against individual ribosomal proteins were raised in rabbits and characterized according to Stöffler and Wittmann (1971).

b) Ouchterlony double diffusion was performed as described (Stöffler and Wittmann, 1971; Stöffler et al., 1971).

c) Immunoelectrophoresis: Three different methods have been compared. (1) Immunoelectrophoresis on agarose plates was used as described previously (Hasenbank et al., 1973, and in more detail by Zubke, 1977). (2) Immunoelectrophoresis on cellulose acetate gel was done by embossing wells for the application of antisera with the Beckman Embosser R-103. (3) Following electrophoresis, the cellulose acetate strips were soaked with antisera specific to an individual ribosomal protein (G. Stöffler and R. Ehrlich, unpublished results).

d) Preparation of S8-antibody complexes from the total mixture of 70S ribosomal proteins (TP70) was similar as described for quantitative immuno-precipitation (Stöffler and Wittmann, 1971). 20–40 mg of total 70S ribosomal proteins were dissolved in 1 ml of 4 M urea. The solution was diluted with 0.05 M Tris/HCl pH 8.2, 0.5 M LiCl to a final concentration of 1.0 M urea. 120 µl antiserum (anti-S8) was added per 1 mg of TP70. The incubation mixture was allowed to stand for 2 h at 37° C and then for 48 h at 4° C. After centrifugation (10 min × 8.000 rpm) the pellet was washed three times in 0.05 M Tris/HCl pH 8.2, 0.5 M LiCl, dissolved in formic acid, dialysed against 1% acetic acid and lyophilized (Zubke, 1977). This precipitate was applied onto SDS-polyacrylamide gels (Weber and Osborne, 1968) or on one-dimensional polyacrylamide gels (Reisfeld et al., 1962; Kaltschmidt and Wittmann, 1969; see also Zubke, 1977).

Protein-Chemical Methods

a) Isolation and purification of protein S8 from 30S ribosomal subunits of E. coli N4128 and N4003 was done by CM-cellulose chromatography and gel filtration on Sephadex G-100 as previously described (Hindennach et al., 1971). The purity of the isolated proteins was tested by two-dimensional gel electrophoresis (Kaltschmidt and Wittmann, 1970) and by electrophoresis on cellulose acetate gels (Stöffler, 1967).

b) Cleavage by trypsin: 5 mg protein S8 from the wild type and from mutant N4128 were dissolved in 1 ml double distilled water, and the pH was brought to 7.8 with 0.05% ammonia. 50 µg TPCK-treated trypsin were added, and after 4 h at 37° C the solution was lyophilized.

c) Separation of the tryptic peptides: About 0.2 mg protein hydrolysate, dissolved in 10 µl 5% formic acid, were spotted on a cellulose thin layer plate (Macherey and Nagel; Düren, Germany) and separated by the fingerprint method. First dimension: High voltage electrophoresis at pH 4.4 in pyridine, acetic acid, acetone, water (2:4:15:73,v/v) for 2 h at 400 V. Second dimension: Ascending chromatography in pyridine, n-butanol, acetic acid, water (50:75:15:60,v/v). Ninhydrin stained spots were cut out and eluted with 6 N hydrochloric acid. Besides the isolation of peptides by thin layer chromatography 4 mg tryptic peptides were separated on Sephadex G15 (200 × 1 cm) in 20% acetic acid.

d) Amino acid analysis: Peptides were hydrolysed in 5.7 N hydrochloric acid (0.02% β-mercaptoethanol) under nitrogen for 20 h or for 40 h. The analyses were carried out in a Durrum D-500 analyzer at a 1–5 nanomol range.

e) Carboxypeptidase treatment: About 100 nanomoles of peptide were dissolved in 50 µl sodium borate buffer, pH 8.0, at 37° C. 5 µg of the carboxypeptidases A and B (Worthington, U.S.A.) were added, aliquots were taken after 20, 60 and 200 min for amino acid analysis.

f) Cleavage of proteins with CNBr was done as described by Funatsu et al. (1972).

Results and Discussion

Analysis of sts (starvation temperature sensitive) Mutants

For the isolation of mutants, cells were starved to deplete their ribosome content; those that failed to recover from starvation were selected (Phillips et al., 1969). About one quarter of the isolated mutants tested in vitro have functionally altered ribosomes (Apirion et al., 1969). Characterization of *sts*-mutants revealed a variety of different phenotypes (Apirion et al., 1969; Phillips et al., 1969). Thus all mutants grew slower than the parental strain even at 30° C. Some of the mutants showed an increased sensitivity to ribosome antibiotics, while some of the *sts*-mutants had an increased ability to suppress nonsense codons.

We therefore anticipated that the observed pleiotropic response in *sts*-mutants should be reflected by alterations of several ribosomal protein genes. In order to detect as many alterations as possible the ribosomal proteins from the *sts*-mutants were analyzed by four different methods: two-dimensional polyacrylamide gel electrophoresis, cellulose acetate gel electrophoresis, immunoelectrophoresis and double-immunodiffusion (Table 1). One reason for using several methods is the experience that alterations in a few ribosomal proteins are difficult to detect by two-dimensional polyacrylamide gel electrophoresis. This is true for proteins which migrate very slowly during the first dimension. On the other hand some of these proteins give sharp bands in cellulose acetate gel electrophoresis (Stöffler, 1967; G. Stöffler and R. Ehrlich, unpublished results).

Electrophoretic Procedures. When 70S proteins were tested by two-dimensional gel electrophoresis, a 30S protein, namely S8, was found to be unequivocally altered in two of the mutants (N4003, N4128). The alteration was also detected by each of the three other methods applied. Figure 1 shows the positions of altered S8 proteins in mutants N4003 and N4128 drawn into the two-dimensional polyacrylamide gel electropherogram of the wild type strain D10.

Cellulose acetate electropherograms of 70S proteins from four mutants as well as 30S and 70S proteins from the wild type strain D10 are shown in Figure 2 (top). They revealed differences in the migration of the protein band containing S8 in mutants N4003 and N4128.

Table 1. Summary of results of 11 *sts* mutant-strains

	Electrophoretic techniques		Molecular weights[a,b]	Immuno-electrophoretic techniques Methods[c]	Immuno-diffusion[d] experiments with anti-$S8_{WT}$	Analysis of CNBr-Fragments	
	2D	CAG		A B C		CAG[e]	Ippt[f]
N4003*	S8 more acidic than in WT	S8 more acidic than in WT**	ident.	S8 more acidic than in WT	reduced reaction	altered	no alteration detected
N4050	ident.	ident	ident.	ident.	ident.	n.d.	n.d.
N4106	ident.	ident.	ident.	ident.	ident.	n.d.	n.d.
N4128	S8 more basic	S8 more basic**	ident.	S8 more basic	reduced reaction	altered	altered
N4306*	ident.	ident.	ident.	ident.	ident.	n.d.	n.d.
N4308*	ident.	ident.	ident.	ident.	ident.	n.d.	n.d.
N4310*	ident.	ident.	ident.	ident.	ident.	n.d.	n.d.
N4312*	ident.	ident.	ident.	ident.	ident.	n.d.	n.d.
N4334*	ident.	ident.	ident.	ident.	reduced reaction	n.d.	no alteration detected
N4335	ident.	ident.	ident.	ident.	ident.	n.d.	n.d.
N4435	ident.	ident.	ident.	ident.	ident.	n.d.	n.d.

Ident. means: identical as wild type protein; n.d. means: not determined; * = Ribosomes thermosensitive in vitro (Apirion et al., 1969)
** = Also performed with purified proteins (Fig. 2)

[a] Method of Geisser et al. (1973)
[b] SDS-electrophoresis of immunoprecipitates
[c] For description of methods A, B, C see Materials and Methods and Figure 3
[d] See Materials and Methods and Figure 4
[e] Purified mutant S8 proteins were analyzed (see Fig. 8)
[f] Immunoprecipitates were analyzed (see Fig. 9)

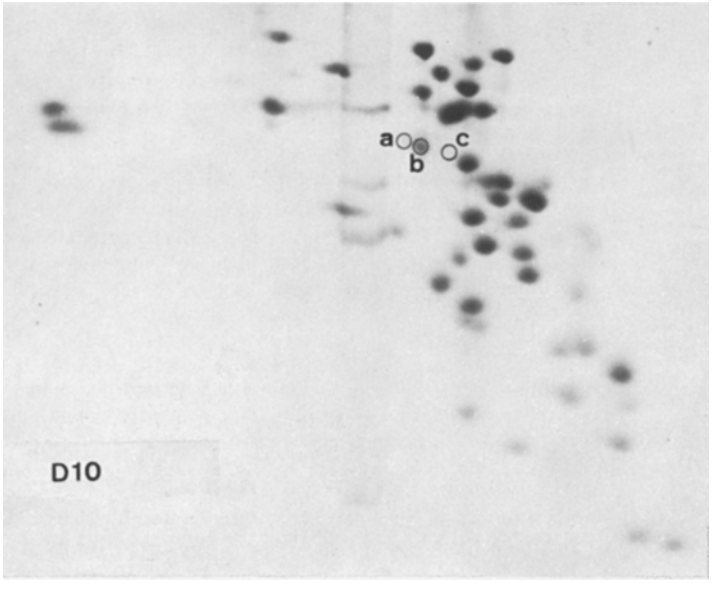

Fig. 1. Two-dimensional electropherogram of 70S ribosomal proteins. The positions of proteins S8 are encircled. a: S8 from N4003; b: S8 from D10; S8 from N4128

The differences between wild type and mutant proteins became more apparent when the densitometer tracings of the electropherograms were compared (Fig. 2, middle). The densitometer patterns were compared with the profile of 30S proteins in which the S8 band contains (in contrast to 70S proteins) only a single protein. Comparison of the protein profiles shows that in the wild type the peak containing protein S8 (small arrow) becomes somewhat smaller in the mutants. It does not disappear completely because it contains protein L5 besides protein S8 in TP70. The altered mutant protein S8 migrates to a

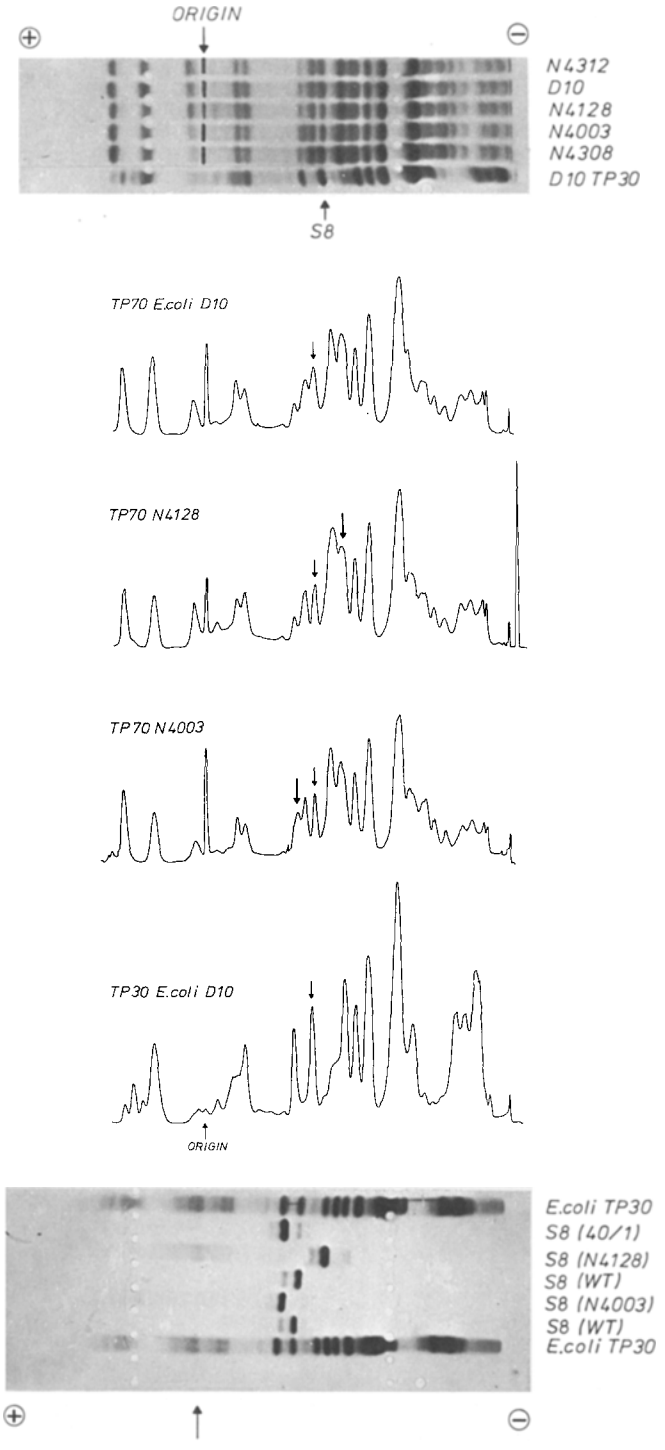

Fig. 2. *Top:* Cellulose acetate gel electrophoresis of 45 µg TP30 and 100 µg TP70 from wild type (D10) ribosomes, and of 100 µg TP70 from each of the mutants N4003, N4128, N4308, N4312. Electrophoresis was performed in a buffer containing 0.1 M Tris, 0.1 M Bicine, 0.05 M β-mercaptoethanol and 8 M urea, pH 8.8, at 350 Volt (4.2 mA) for 115 min. Staining was with Coomassie brilliant blue R250. In TP70, protein S8 comigrates with protein L5. Therefore, the band containing protein S8 does not disappear in the electropherograms of TP70 from mutants N4003 and N4128. The staining intensity of this band is, however, reduced. This can also be seen in the densitometer tracings shown below. *Middle:* Densitometer tracings of the separation profiles of TP30 and TP70 of D10 ribosomes and of TP70 from mutants N4003 and N4128. The positions of the wild type protein S8 and of the altered mutant proteins S8 are indicated by small and large arrows, respectively. *Bottom:* Cellulose acetate gel electrophoresis of TP30 (60 µg) and of the pure S8 proteins from wild type and mutants N4003, N4128, and the unrelated mutant 40/1 (3 µg each). Experimental conditions were as described above except that the running time was shorter (85 min)

new position (indicated by large arrows): in mutant N4128 at the cathodic side, in mutant N4003 at the anodic side of wild type S8 (Fig. 2, middle).

The alterations are more clearly seen, when 30S proteins or purified S8 proteins are electrophoresed (Fig. 2, bottom). It is interesting that the altered S8 protein purified from mutant V40/1 which was isolated by a different method (Wittmann et al., 1974) migrates to the same position as that of S8 from mutant N4003. It remains to be seen whether this is caused by identical amino acid replacements in proteins S8 of both mutants.

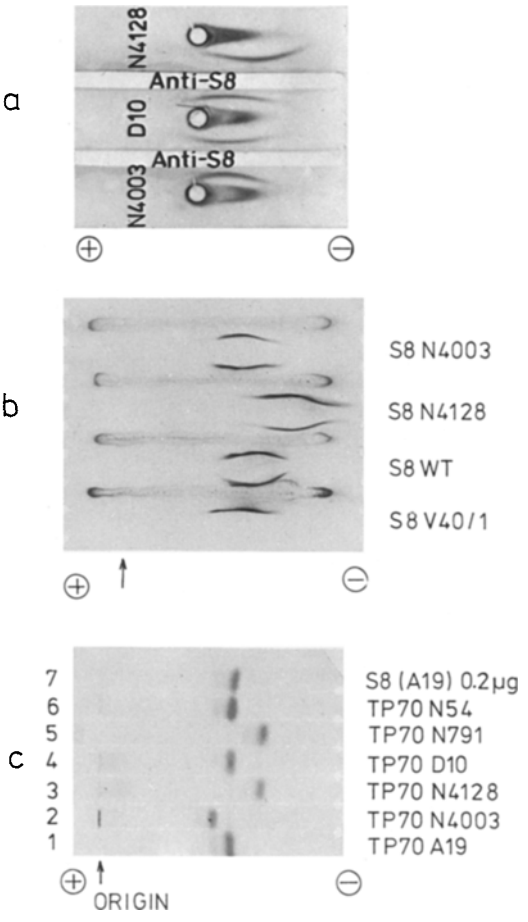

Fig. 3a–c. Immunoelectrophoresis. **a** Immunoelectrophoresis in 1.5% agarose in a sodium barbital buffer, pH 8,6, containing 4 M urea. 300 µg of TP70 were applied as indicated in the Figure. Electrophoresis was at 10 V/cm (constant), 115 mA; running time: 4 h. Following electrophoresis the agarose plates were briefly equilibrated with sodium barbital buffer, pH 8.6, containing 0.75 M LiCl to remove urea. 250 µl anti-S8 serum (rabbit 362) was filled into each trough; diffusion was for 48 h. **b** Immunoelectrophoresis on cellulose acetate gels. Electrophoretic separation was done under the conditions described in Legend to Figure 2 (running time: 85 min). 5 µg of each purified S8 protein was applied as indicated. Following electrophoresis the urea was removed (see Materials and Methods). The anti-S8 used has been enriched by affinity chromatography on CNBr activated Sepharose 4B to which pure wild type S8 protein was coupled. The procedure was similar to the one described by Tischendorf and Stöffler (1975). Each of the embossed troughs was filled with 1 mg specific antibody, dissolved in 40 µl 0.02 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS-buffer). Diffusion was overnight in a moist chamber and unreacted protein was removed by several washes with PBS-buffer. Staining was with Coomassie brilliant blue R250. **c** Modified immuno-electrophoresis on cellulose acetate gels. 25 µg of TP70 extracted from wild type, mutant and transductant ribosomes were applied as indicated in positions 1–6. 0.2 µg of pure protein S8 from E. coli A19 was applied in position 7. Electrophoresis was as in Figure 2 (running time: 95 min). The strips were not directly stained but soaked overnight in an antiserum to protein S8 (rabbit 362); the excess antiserum was removed by several washes with PBS-buffer and the antigen-antibody complexes were stained with Coomassie brilliant blue R250. The stain reacted with the antibodies which are predominant in the antigen-antibody complexes. Without the amplifier effect, contributed by the soaking in antiserum, the small amount of proteins would hardly be visible by direct staining (cf. amounts of TP70 applied in Fig. 2)

Immunoelectrophoresis. The altered mobilities of the S8 proteins in mutants N4003 and N4128 could also be demonstrated by three immuno-electrophoretic techniques. A major problem had to be overcome in order to accomplish a reasonable separation of ribosomal proteins. Ribosomal proteins are poorly soluble in standard-buffers and hence badly separated. They separate well, however, in urea or SDS-containing buffers.

Therefore, attempts were made to separate ribosomal proteins in agarose gels in the presence of urea: after electrophoresis the urea was removed by a brief dialysis of the gels and immunodiffusion was started (Hasenbank et al., 1973; Zubke, 1977). A photograph of this electrophoresis is shown in Figure 3a, and it confirms the electrophoretic results concerning the altered mutant proteins (see above and Fig. 2).

Although agarose is probably the best material for immunodiffusion (Ouchterlony, 1958; Crowle, 1973), the electrophoretic separation of ribosomal proteins is by far better on cellulose acetate gels than on agarose gels (Stöffler and Ehrlich, unpublished observations). We separated ribosomal proteins on cellulose acetate gels (as in Fig. 2), removed the urea by dialysis against phosphate buffered saline and filled specific antibody to protein S8 (enriched by affinity chromatography) in the embossed wells (Fig. 3b). The resolution of this procedure is better than that of agarose gels (compare Fig. 3a and b).

By far the best and most sensitive procedure was a modified immunoelectrophoresis technique (G. Stöffler and R. Ehrlich, unpublished). Small amounts of 70S proteins are first separated by cellulose acetate gel electrophoresis; urea is removed, and the strips are soaked in the antiserum (anti-S8). Only the S8 ~ anti-S8 complex remains bound to the gel after washing it with phosphate buffered saline. The antigen-antibody complex and the bound antibody molecules are stained with Coomassie brilliant blue (Fig. 3c). Instead of precipitin arcs, as formed in standard-immunoelectrophoretic systems (see Fig. 3a, b), distinct precipitin-bands are formed by the use of this procedure. The results confirm the conclusions about the migration of mutant S8 proteins (Fig. 2), and the electropherogram looks as if pure S8 proteins were electrophoresed. It should, however, be kept in mind that a mixture of more than 50 ribosomal proteins is electrophoresed and only that protein

against which the specific antibody is directed is stained with this method. This a very useful method for the investigation of altered ribosomal proteins.

The alterations in protein S8 differ in the two mutants (Figs. 1–3): In N4003 protein S8 migrates further to the anode (and is therefore more acidic), while protein S8 in mutant N4128 migrates more towards the cathode (and is therefore more basic) than protein S8 in the parental strain D10 or in any other standard E. coli strain. The distances of the two mutant S8 proteins from the S8 wild type protein are not identical. The distance between S8 from wild type and S8 from N4128 is larger than that between wild type S8 and S8 from N4003 (see Figs. 1–3). Therefore, the alteration in protein S8 from N4128 should be due to a difference of more than one charge.

The possibility exists that the mutant protein is altered in size as has been found with many of the S4 mutants (Funatsu et al., 1972; Hasenbank et al., 1973). Following electrophoresis in the first dimension we therefore performed a second dimension electrophoresis in SDS-polyacrylamide gels (Geisser et al., 1973). In this second dimension all three S8 proteins migrated identically, indicating that the proteins are not significantly altered in size.

Double-Immunodiffusion. Since protein S8 was found to be electrophoretically altered in two of the *sts*-mutants analysed, we tested the antigenic capacity of the ribosomal proteins from all mutants towards antibodies prepared against a purified S8 protein from the wild type by double diffusion in Ouchterlony plates. These experiments were performed since previous studies on ribosomal mutants have revealed that more mutational alterations can be detected by double-immunodiffusion than by electrophoretic procedures (Stöffler et al., 1971; Hasenbank et al., 1973; see also Stöffler, 1974).

In fact, an additional mutant (N4334) with a reduced cross-reactivity with anti-wild type S8 sera was found (Fig. 4a). In addition, semiquantitative immunodiffusion experiments, carried out according to the procedure of Wright (1959), indicated that the amount of protein S8 in this particular mutant is reduced. The electrophoretically altered S8 proteins from mutants N4003 and N4128 also gave only a partial identity reaction when compared with the wild type S8 protein (Fig. 4b).

Thus three of the strains (N4003, N4128 and N4334) revealed antigenic differences as compared to the parental strain D10. The antigenic properties of protein S8 from strain N4003 were different from that of S8 from N4128. This was concluded from the occurrence of a "double spur" which formed when anti-S8 was developed against the two mutants, filled into two adjacent wells (Fig. 4b).

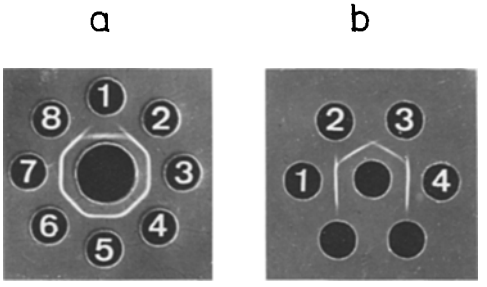

Fig. 4a and b. Immunodiffusion. The antibody preparation has been enriched by affinity chromatography on CNBr activated agarose columns to which wild type protein S8 was coupled. Two sheep sera and three rabbit sera (each animal immunized with wild type protein S8) were pooled. **a** Centerwell: 0.5 mg enriched anti-S8 antibody; the peripheral wells contained each 150 µg of TP70 in (1) N4334; in (2) D10; in (3) N4308; in (4) N4312; in (5) and (8) E. coli K A19; in (6) N4435; in (7) N4310. **b** Centerwell: 0.35 mg anti-S8 (the same preparation as in Figure 4a); in (1) and (4) 150 µg TP70 E. coli K A19; in (2) 150 µg N4128; in (3) 150 µg N4003

Do Mutational Alterations Occur in Other Ribosomal Proteins?

a) Extra-Protein Near L7. Two-dimensional polyacrylamide gel electrophoresis of ribosomes from some *sts*-mutants and their transductants (see below) revealed an extra-protein spot which migrated slightly faster in the first dimension and slightly slower in the second dimension than protein L7. An extra band corresponding to this spot was also observed by cellulose acetate gel electrophoresis (Fig. 5).

In order to test whether this new protein is related to protein L7, the 70S proteins of the strains containing the new protein were separated by cellulose acetate electrophoresis and the electropherogram was treated with antiserum against L7 as described in Materials and Methods. Cross-reaction was only found with proteins L7 and L12 but not with the new protein showing that it was not related to proteins L7/L12.

Since protein S6 migrates on the two-dimensional electropherogram very close to proteins L7/L12 and to the new protein, the possibility existed that the latter was related to protein S6. Therefore, identical experiments were done with anti-S6 as just described for anti-L7: there was also no cross-reaction between anti-S6 and the new protein.

The results demonstrate that the protein under consideration was neither related to proteins L7/L12 nor to S6. It cannot be a modification product or a precursor of these proteins but is apparently a non-ribosomal protein which becomes more strongly associated with the ribosomes of some *sts*-mutants than with wild type ribosomes.

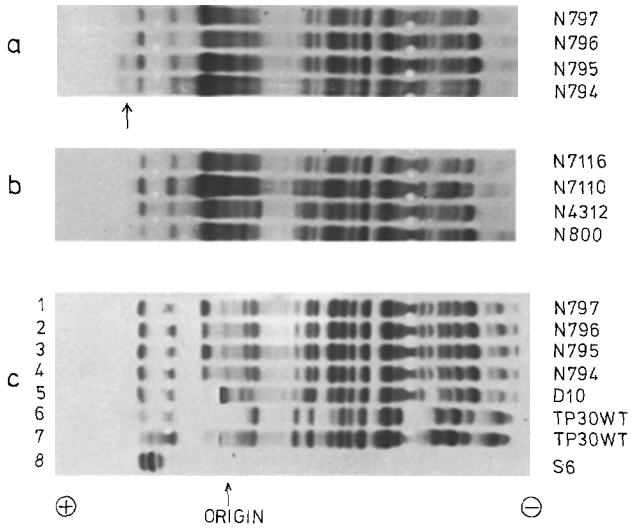

Fig. 5a–c. Cellulose acetate gel electrophoresis. **a and b** Electropherograms of *sts*-mutants and tranductants. TP70 was prepared from non-salt-washed ribosomes. An additional protein band migrating faster towards the anode is indicated by an arrow. **c** Positions 1–5: Electropherogram of TP70 from four transductants and the wild type strain D10. Comparison of TP30 from E. coli MRE 600 from ribosomes prepared according to Lührmann (1975) in position 6 and from ribosomes prepared according to Hindennach et al. (1971) in position 7. Different forms of protein S6 (Hitz et al., 1976) are shown in position 8

b) Modified Protein S3. The two-dimensional gel electropherograms of 70S proteins from several *sts*-mutants and transductants gave some indication of alterations in protein S3. It is, however, known that protein S3 is easily degraded (Craven et al., 1969) and several different modification products have recently been isolated and characterized (Wittmann and Stöffler, unpublished results). They elute at other positions from CM-cellulose columns than the true protein S3. The different elution behaviour is paralleled by different mobilities in the two-dimensional polyacrylamide gel electrophoresis and in the cellulose acetate gel electrophoresis (Fig. 6).

The proteins with somewhat different electrophoretic mobilities were studied by immunodiffusion and immunoelectrophoresis using antisera to protein S3. Indeed, each of the bands, including the faintly stained ones, reacted with anti-S3. The occurrence of the modified S3 bands depends on the procedure used for the purification of ribosomes and also on the conditions of storage of the extracted ribosomal proteins. For instance storage of proteins in urea containing solutions at pH 7.5–8.6, even at −20° C, lead to the formation of several bands for proteins S3. The best procedure for preventing modification of protein S3 is to prepare ribosomes according to Lührmann (1975) and to store the extracted proteins lyophilized at −20° C. These data show that the modification of protein S3 is unrelated to the Sts-phenotype.

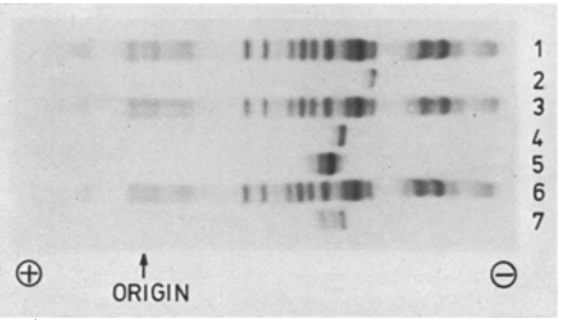

Fig. 6. Electropherogram of a cellulose acetate gel run with modified proteins S3. Positions 1, 3 and 6: E. coli TP30 (40 µg each); position 2: native protein S3 (4 µg); position 4: modified product A (4 µg); position 5: modified product B with satellite bands (4 µg); position 7: modified products A, B and C (4 µg). Electrophoretic conditions were as described in the Legend to Figure 2 (running time 80 min)

Proteinchemical Studies

In order to exactly characterize the mutational alteration in protein S8 the elucidation of the amino acid exchange of the mutant protein is required. Therefore, protein S8 was isolated from ribosomes of mutant N4128 and analyzed by protein-chemical methods.

The Identification of the Amino Acid Exchange in Protein S8 of Mutant N4128

The primary structure of protein S8 from *E. coli* wild type has been established (Stadler and Wittmann-Liebold, 1976). The determination of the sequence of protein S8 from mutant N4128 was based on a comparison of the fingerprint pattern and on the amino acid analysis of the tryptic peptides from the wild type and the mutated protein.

Fingerprints of the tryptic peptides of the S8 proteins from wild type and from mutant N4128 were made and compared. The peptide map of N4128 contains one spot more than the wild type S8 protein. All other spots are in the same relative positions. The composition of this additional peptide was Thr_1, Leu_2, Lys_1 which corresponds to the C-terminal region of peptide T16 from wild type (see Stadler and Wittmann-Liebold, 1976). The replacement of the glutamic acid residue in position 59 of the wild type protein S8 by a basic lysine residue in the mutant protein provides an additional site for trypsin cleavage. The occurrence of a new tetrapeptide with the same amino acid composition as found for the addi-

tional peptide eluted from the mutant protein is explained in this way.

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                    57          60          63
Wild type:      ...Glu-Leu-Glu-Leu-Thr-Leu-Lys...
                           ↓
Mutant N4128:   ...Glu-Leu-Lys-Leu-Thr-Leu-Lys...
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Accordingly, peptide T16 from mutant N4128 should be shorter by four residues than the wild type peptide T16 and should have the sequence ...Glu-Leu-Lys at its C-terminus. In order to test this, the mutant protein S8 from N4128 was cleaved with trypsin and peptide T16 was isolated by gel filtration on Sephadex G15. It was then treated with carboxypeptidases A and B to establish the sequence of the C-terminal amino acids. The sequence was found to be ...Glu-Leu-Lys, exactly as expected.

The replacement of glutamic acid by lysine in position 59 results in a mutant protein with one negative charge less and one positive charge more than in the wild type protein. This agrees very well with the relatively drastic electrophoretic difference between the wild type and mutant protein as observed with each of the electrophoretic techniques (Fig. 1–3).

Comparison of CNBr-Fragments

Protein S8 contains four methionine residues at positions 2, 9, 26 and 95 (Stadler and Wittmann-Liebold, 1976) and hence at least five fragments should be obtained after cleavage of this protein with CNBr (Fig. 7).

The N-terminal dipeptide serine-homoserine is not precipitable by TCA because of its small size; therefore it is not seen on staining the cellulose acetate gels after electrophoresis of the CNBr fragments. Assuming complete cleavage, only four fragments are therefore expected. The CNBr-cleavage products of wild type protein S8, separated by cellulose acetate gel electrophoresis showed, however, eight bands (Fig. 8). Six of them were heavily stained, viz. 1–6. The two others (bands 7 and 8) were, although faintly stained, reproducibly observed. The occurrence of eight bands showed that protein S8 is incompletely cleaved by CNBr, and hence some of the bands represent overlapping fragments. Incomplete cleavage by CNBr is a general experience in protein-chemical studies.

Comparison of the CNBr-fragments of wild type S8 with those from mutant protein $S8_{N4128}$ and mutant protein $S8_{N4003}$ revealed obvious differences in their electrophoretic patterns (Fig. 8). In mutant N4128 only a single band, namely band 1, migrates identically to the wild type S8 protein. All other bands

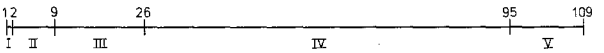

Fig. 7. Distribution of methionine residues in protein S8 (for amino acid sequence see Stadler and Wittmann-Liebold, 1976). Protein fragments expected after CNBr treatment are designated I-V

migrate faster towards the cathode. The amino acid exchange in protein S8 of mutant N4128 was localized in position 59 (see above) and hence in the CNBr-fragment IV (Fig. 7). All those faster migrating bands should be overlapping fragments that contain fragment IV.

Comparison of the CNBr fragments of proteins S8 from the wild type and mutant N4003 shows that band 1 migrates significantly slower as compared to both the wild type and mutant N4128 (Fig. 8). In addition, a new very faint band occurs which migrates between band 1 and 2 of the wild type fragments. Finally, band 3 disappeared and co-migrates with band 2 of the wild type. The latter result becomes more pronounced by comparing the densitometer patterns (not shown).

The application of cellulose acetate gel electrophoresis will be of great help for the protein-chemical analysis of wild type and mutant proteins. It is an excellent method for the separation of peptides of large and medium size and therefore supplements the fingerprint technique on thin layer plates. Although the latter method is very useful for the separation of peptides up to a size of approximately 20–30 amino acids, it has severe limitations in resolving a mixture of large peptides.

Analysis of S8-Antibody Precipitates

We attempted the analysis of S8 proteins purified by precipitation with anti-S8. The purpose of this experiment was to find out whether one can analyse protein S8 prepared by immunoprecipitation directly from the total mixture of ribosomal proteins. If successful, such a technique could eliminate the necessity to purify the mutant protein in question by the more laborious column chromatographic techniques.

Total ribosomal proteins were prepared from strains D10, N4003 and N4128. They were reacted with anti-S8, and the precipitates were washed repeatedly, freeze-dried and analyzed by four electrophoretic procedures: disc-electrophoresis, the split-gel modification of the first method, one-dimensional SDS polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis. The precipitates contained only S8 and S8-specific antibody. Analysis by SDS polyacrylamide gel electro-

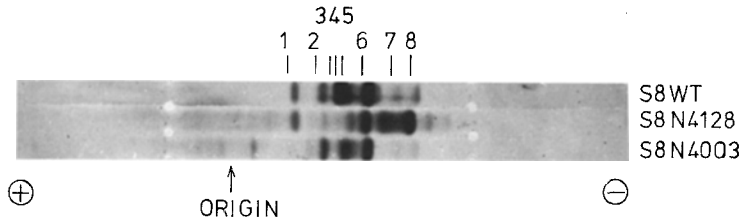

Fig. 8. Electropherograms of CNBr fragments derived from purified S8 proteins. The fragments from the wild type protein (15 µg), from mutant N4128 (50 µg) and mutant N4003 (60 µg) are compared. The numbers 1–8 correspond to the bands obtained from wild type protein S8 and not to the fragments designated with numbers I-V (Fig. 7). Electrophoretic conditions as in Figure 2 (running time: 80 min)

phoresis showed that the molecular weights of all three proteins, i.e. of the S8 proteins of strain D10 and of the mutants N4003 and N4128 are identical. The three other methods confirmed the data described above that each of the three proteins differs in their electrophoretic mobility.

An example of a disc-electrophoresis with an immunoprecipitate is shown in Figure 9a. Under the selected experimental conditions, IgG-molecules do not migrate into the gel but remain at the origin. One strong band (number 2) is seen which migrates identically with protein S8. This result was confirmed by the split-gel-technique and by one-dimensional electrophoresis in SDS (Zubke, 1977). Using the latter method, the L- and H-chains migrate into the gel and can be used as simultaneous markers for molecular weight determinations. In fact, during the characterization of several mutants altered in protein S4, differences in the molecular weights of S4 proteins from various mutants were detected and their molecular weights could be estimated by this procedure (Zubke, 1977).

Cleavage of S8~Anti-S8 Complexes with CNBr. Three immunoprecipitates were cleaved with CNBr: $TP70_{A19}$~anti-S8, $TP70_{N4003}$~anti-S8 and $TP70_{N4128}$~anti-S8. As controls a purified anti-S8-IgG preparation and pure protein S8 were treated with CNBr under identical conditions (Fig. 9b, c). Analysis of the cleavage products was performed by three methods: disc-electrophoresis, split-gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis. An example of comparative analysis of the products obtained from the wild type and mutant N4128 by the split-gel technique is given in Figure 9d. This shows that one fragment from the mutant N4128 migrates faster than its corresponding fragment of the wild type (Fig. 9d).

The main problem of this method lies in the fact that certain fragments of the ribosomal protein migrate to identical positions as the CNBr-fragments derived from the antibody-molecule (compare Figs. 9b, c). A better resolution and thus an easier

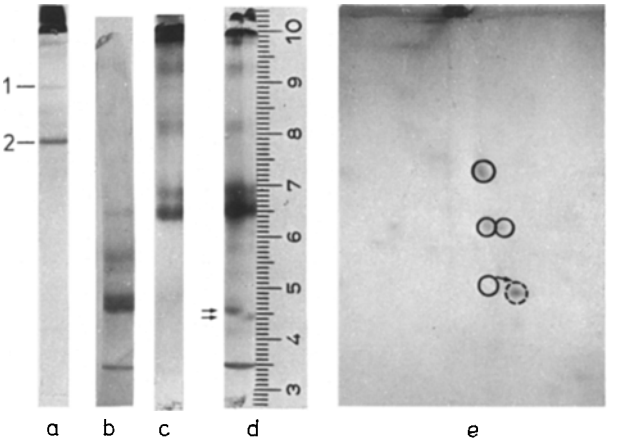

Fig. 9a–e. Disc gel electrophoresis **a** Load: 100 µg of the immunoprecipitate [TP70 wild type plus anti-S8]. **b** Load: 50 µg CNBr cleavage products from pure S8 wild type protein. **c** Load: 50 µg CNBr cleavage products from purified IgG. **d** Disc electrophoresis (split-gel modification), left side: 250µg of the immunoprecipitate [$TP70_{wild\ type}$~anti-S8]; right side: 250µg immunoprecipitate [$TP70_{N4128}$~anti-S8]. Both immunoprecipitates were applied after cleavage with CNBr. Electrophoretic conditions: 90 V, 3 mA (per tube), running time: 4 h; staining: amido black. **e** Two-dimensional electropherogram of the peptides obtained after treatment of the immunoprecipitate with CNBr. The CNBr-peptides of the wild type S8 protein are encircled by a continuous line and the peptide altered in mutant N4128 is encircled by a broken line

identification of the CNBr-fragment can be obtained by their analysis on two-dimensional polyacrylamide gels. With this method an alteration in the digest of mutant N4128 has been detected (Fig. 9e). Due to the polydispersity of the different antibody-species and also due to the better resolution on two-dimensional gels, far more antibody-fragments are seen. The intensity of the fragments of the antigen-protein S8 is, however, stronger than those of the antibody-fragments (Fig. 9e).

The results indicate that analysis of specific immunoprecipitates is a practicable method. Discrimination of antibody-fragments from antigen-derived fragments is not as much a problem as the high amounts of antiserum required for this method. Both difficulties can, however, easily be overcome by the use

of radioactively labelled antigens, e.g. ribosomal proteins.

Genetic Studies

To determine whether or not the mutation in the altered S8 protein also causes temperature sensitivity, two types of transduction experiments were carried out. In the first series strain D10 was the donor and strain N4128 the recipient and selection was made for the ability to grow at 43° C. 152 transductants were analyzed; all were *sts*$^+$, i.e. they were able to survive starvation for a carbon source for three days at 43° C (see Phillips et al., 1969). The ribosomal proteins of 20 of the 152 transductants, randomly picked, were analyzed by two-dimensional gel electrophoresis, cellulose acetate gel electrophoresis and immunoelectrophoresis. In all the transductants, the altered S8 was still present (for examples see Fig. 5).

A second series of transductants were isolated in which one of the transductants, viz. N791, from the first experiment was the recipient and strain N54 (a derivative of strain D10) was the donor. We selected for the ability to grow at 45° C.

The ribosomal proteins of sixteen such transductants were analyzed by two-dimensional polyacrylamide gel electrophoresis, cellulose acetate gel electrophoresis and immunoelectrophoresis and in all of them the original pattern seen in the recipient strain N791, namely the appearance of the altered S8, was observed.

The genetic studies indicate strongly that the same mutational event caused temperature sensitivity and inability to recover from starvation at elevated temperatures. This is indicated by the fact that all Ts$^+$-transductants are also Sts$^+$, i.e. these two characteristics are inseparable. The experiments reported here also show that mutant N4128 carries more than one mutation. In the second transduction experiment in which selection was made for the ability to grow at 45° C, the pattern of the altered S8 proteins of the transductants remain the same as that of the recipient strain. In other words, while the ability to grow at 45° C was regained it did not involve genes that affect the ribosomal protein S8. Therefore, we can conclude that the temperature sensitive phenotype of the mutants is not caused by the altered S8 protein.

General Conclusions

Alterations in almost all of the proteins from the small and the large subunit of E. coli ribosomes have recently been observed in temperature-sensitive mutants and in revertants from a strain with a novel type of streptomycin dependence (Isono et al., 1976, 1977; Dabbs and Wittmann, 1976; E.R. Dabbs, manuscript in preparation). Some of the methods described in this paper should be very useful to further characterize these and other mutants. In particular, the methods can be used for the detection of altered acidic ribosomal proteins like S1, S6, L7, L9 and L12. Alterations in these proteins are rather difficult to detect by two-dimensional polyacrylamide gel electrophoresis since only a few acidic proteins which can be used as markers migrate into this region of the gel plate. Similarly, as has been shown for protein S8 in this paper, alterations in those proteins (e.g. L4, L10, S10, L21) which barely migrate out of the sample gel during the first dimension of the two-dimensional gel electrophoresis should also be easier detectable by cellulose acetate electrophoresis or by the various immunological procedures described. Furthermore, as shown for protein S3, products arising by degradation or by modification are discernible from mutated proteins.

The modified immunoelectrophoresis (Fig. 3c) is obviously the optimal procedure to decide whether a protein is actually lacking in ribosomes or only hidden by migration into the position of another ribosomal protein. The latter problem has frequently impeded the identification of mutated ribosomal proteins (Deusser et al., 1970; Stöffler et al., 1971; Hasenbank et al., 1973; Wittmann et al., 1974). Finally, the methodology should also be applicable for the detection of mutationally altered components of complex structures other than ribosomes.

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Communicated by E. Bautz

Received October 1, 1977