Molec. Gen. Genetics 110, 197–210 (1971)

A Genetic and Biochemical Study of Streptomycin- and Spectinomycin-Resistance in *Salmonella typhimurium*

T. YAMADA and J. DAVIES
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Received November 17, 1970

Summary. In *Salmonella typhimurium*, streptomycin resistance can occur by mutation at the *strA* or the *strB* locus. *StrA* mutants have altered ribosomes which are refractory to the drug in cell-free amino acid incorporation systems, and in ^{3}H-dihydrostreptomycin binding studies. *StrB* mutants, unlike the *strA* mutants, are resistant to several aminoglycoside antibiotics and resistance is not due to a mutational change in the cell's protein synthetic machinery. Spectinomycin resistant mutants of *S. typhimurium* also fall into two classes, only one of which is ribosomal in mechanism. The *spcA* and *spcB* loci are closely linked to *strA*, *aroC*, and *argG* on the *S. typhimurium* linkage map.

Introduction

In *Escherichia coli*, high-level streptomycin (str-r)[1] and spectinomycin (spc-r)[1] resistance generally result from low-frequency mutational events which lead to alterations of specific protein components of the 30s ribosome subunit (Ozaki, Mizushima, and Nomura, 1969; Bollen, Davies, Ozaki, and Mizushima, 1969); in all cases so far, it has been possible to demonstrate a clear difference in properties between the 30s subunits of the sensitive and resistant strains. The genetic loci for str-r and spc-r are closely linked on the *E. coli* chromosome, and are considered to be part of a group of genetic markers which define the structural elements for the 30s ribosomal subunit (Flaks, Leboy, Birge, and Kurland, 1966). There is one locus determining str-r (*strA*) and one locus for spc-r (*spcA*) in *E. coli* and no cross-resistance between the two antibiotics has been reported. (The only exceptions apply to resistance to these antibiotics determined by R-factors.) Extensive mapping of str-r (Breckenridge and Gorini, 1970) and str-d mutations (Birge and Kurland, 1970) has further confirmed the map position of the *strA* locus.

In *Bacillus subtilis*, the same situation would seem to apply, since the *str* and *spc* loci are linked to a number of other antibiotic resistance loci, all of which are presumed to code for structural units of the bacterial ribosome (Goldthwaite, Dubnau, and Smith, 1970). However, a recent report by Harford and Sueoka (1970) suggests that there may be two unlinked loci for spc-r in *Bacillus subtilis*; *in vitro* studies have not been performed to establish that these resistance mutations do affect the ribosome in *B. subtilis*.

It has been known for some time that there are two separate genetic loci (*strA* and *strB*) which determine resistance to streptomycin in *Salmonella typhimurium* (Sanderson and Demerec, 1965), and we have examined strains carrying

1 The notation str-s, str-r, and str-d refers to the phenotypes of streptomycin sensitivity, resistance, and dependence; similarly for spectinomycin phenotypes (spc-s and spc-r).

mutations in these loci in an attempt to determine the mechanism of resistance. By analogy between the two linkage maps (Taylor, 1970; Sanderson, 1970) it would seem that the *strA* locus in *S. typhimurium* would correspond to the *strA* locus in *E. coli*, both loci determining the structure of a 30s ribosomal protein; the *strB* locus in *S. typhimurium* has no corresponding locus in *E. coli*. We have been able to confirm the former supposition, but have failed to show how mutations at *strB* confer resistance. The nature of spc-r in *S. typhimurium* has not been studied previously and we wish to describe some experiments concerning such resistant mutants. A number of high-level spc-r mutants have been isolated and found to map close to the *strA* locus on the *S. typhimurium* chromosome, linked to *aroC* and *argG*. Surprisingly, our data suggest that not all of these spc-r mutants affect ribosomes, although they do map in the region coding for the ribosomal protein genes in *Salmonella* species (Sypherd, O'Neil, and Taylor, 1969). There appear to be two closely linked *spc* loci in *S. typhimurium*, one of which determines a ribosomal component (probably a protein), while the other does not. In addition, we have examined an anomalous *S. typhimurium* mutant *argG*10, which was previously isolated as a pleiotropic arginine-requiring, str-r mutant by Demerec and his co-workers (Demerec, Lahr, Balbinder, Miyake, Ishidsu, Mizobuchi, and Mahler, 1960). The ribosomes from this str-r mutant are indistinguishable in property from the ribosomes of the wild-type parent strain, and the *argG*10 mutant behaves like a *strB* mutant in growth and cross-resistance characteristics. This suggests that there may be more than one locus for the *strB* phenotype in *S. typhimurium*.

Materials and Methods

Strains

The bacterial strains and their sources are listed in Table 1. Spontaneous str-r and spc-r mutants were isolated by plating *ca.* 3×10^9 cells on ML plates containing either 200 μg/ml streptomycin sulphate, or 400 μg/ml spectinomycin sulphate. After incubation at 37° for 3–5 days, the colonies were picked and checked for drug-resistance versus dependence, and for parental markers. In most cases only drug-resistant mutants were found.

Wild type phage P22 was obtained from J. S. Gots.

Culture Media and Drugs

Bacteria were grown in ML broth [10 g Bactotryptone, 5 g Bacto yeast extract, and 10 g sodium chloride per 1000 ml] or M9 minimal medium [3 g KH_2PO_4, 6 g Na_2HPO_4, 0.25 g $MgSO_4\cdot7H_2O$, 5 g NaCl, 10 g NH_4Cl, 2.0 mg vitamin B_1, and 10 g glucose per 1000 ml]. This minimal medium was supplemented as necessary for various auxotrophs. Growth in these media was monitored by measuring OD_{550} in a Bausch & Lomb Spectronic 20. Solid media were prepared by adding Bactoagar, 13 g/1000 ml to the above. Streptomycin sulphate was obtained from Nutritional Biochemicals Corporation, and spectinomycin sulphate from the Upjohn Company.

Transduction

Phage stocks were prepared by growth in liquid medium and were stored in T2-buffer (Hershey and Chase, 1952) after chloroform treatment. In transduction experiments the recipient bacteria were mixed with phage at a multiplicity of 5 and incubated at 37° for 6 minutes before plating on selective media. The transductants were picked with a sterile toothpick on to the same selective plates, and then replica plated to score for antibiotic markers. Since *S. typhimurium* has a relatively high basal level of resistance to spectinomycin, replica plating was carried out when colonies were still small, on to plates containing

Table 1. *Description of strains of S. typhimurium*

Strain	Genotype	Source
TY1 (≡LT2)	wild-type	J. S. Gots
TY5	*strB* 57, *nic* 5	K. E. Sanderson
TY6	*strA* 201, *hisF* 1009, *xyl* 1, *metA* 22, *trpE* 22	K. E. Sanderson
TY11	*aroC* 36	K. E. Sanderson
TY12	*aroC* 26	K. E. Sanderson
TY14	*argG* 10 (str-r)	K. E. Sanderson, B. A. D. Stocker
TY15	*cysG* 382	K. E. Sanderson
TY17, TY18, TY19	*strA*	spontaneous mutants from TY1
TY34, TY37, TY44, TY46	*strB*	spontaneous mutants from TY1
TY31, TY32, TY33	*spcA*	spontaneous mutants from TY1
TY52	*spcB*	spontaneous mutants from TY1
TY51	*spcB* (also low level str-r)	spontaneous mutants of TY1
TY58	*argG* 459, *proAB* 47	B. A. D. Stocker
TY59	*argG* 454, *proAB* 47	B. A. D. Stocker
TY66	*strA*, *spcB*	spontaneous spc-r mutant of TY19
TY78	*aroC* 36, *spcA*	spontaneous spc-r mutant of TY11

400 μg/ml of drug. When *argG* was used as selective marker in strains which carry the *argG* mutation as suppressor of *proAB* (Kuo and Stocker, 1969), the selective plates contained proline to support the growth of arg^+ pro^- transductants. When str-r or spc-r were used as selected markers, samples of mixed recipient bacteria and transducing phage were spread on ML plates without drug and incubated for 5 hours at 37°. At this time, an appropriate quantity of antibiotic was placed under the agar and the plate was allowed to stand at 5° for 12 hours to allow absorption and diffusion of the drug. The plates were then incubated at 37° to allow growth of resistant clones. The resistant transductants were purified on media containing drug before replica plating.

Preparation of Cell Free Extracts, Ribosomes and Supernatant Fractions

Cells were harvested from early log phase cultures in ML broth, and washed twice with standard buffer (0.01 M Tris, pH 7.8, 0.01 M magnesium acetate, 0.05 M ammonium chloride, and 0.006 β-mercaptoethanol). The cells were broken by alumina grinding and S-30 extracts prepared essentially as described by Nirenberg (1964). Ribosomes and supernatant fractions were obtained by centrifugation at $100000 \times g$ for three hours. The supernatant was dialysed against 2000 volumes of standard buffer. The ribosome pellet was resuspended in standard buffer and centrifuged through 20 ml of the same buffer containing 34% sucrose, in a Spinco 30 rotor for 10 hours at 30000 r.p.m. The clear pellet of ribosomes was resuspended in standard buffer. All cell fractions were stored in liquid nitrogen.

Cell-Free Amino Acid Incorporation

Polypeptide synthesis was carried out in S-30 extracts as described by Nirenberg (1964) with a few minor modifications.

Antibiotic-Binding Studies

^{3}H-labelled dihydrostreptomycin and ^{3}H-labelled dihydrospectinomycin were prepared by ^{3}H-sodium borohydride (Amersham-Searle) reduction of the drugs in aqueous solution. The excess borohydride was destroyed by the addition of acetic acid and the lyophilized product

was repeatedly evaporated to dryness in the presence of methanol to remove the last traces of borate. Specific activities of the labelled drugs was the order of 10^6 c.p.m. per microgram.

For binding, the reaction mixture (300 μl) containing 30–40 OD_{260} units of ribosomes, 0.01 M $MgCl_2$, 0.01 M Tris, pH 7.6, 0.01 M NH_4Cl and ^3H-labelled antibiotic (as indicated in figure legend) was incubated at 37° for 30 minutes (dihydrostreptomycin) or 60 minutes (dihydrospectinomycin), cooled, and diluted with ice-cold standard buffer. This sample was layered on top of 20 ml of standard buffer containing 34% sucrose and centrifuged at 30000 r.p.m. for 15 hours in a Spinco 30 rotor. The pellet was resuspended in distilled water (200 μl) and small samples were counted in a Packard scintillation counter using a toluene-based fluid containing "Biosolv" (Beckman Instruments). Results are expressed as c.p.m. of drug bound per 10 OD_{260} of ribosomes.

Results

Growth and Cross-Resistance Properties of str-r and spc-r Strains of S. typhimurium

High-level str-r and spc-r mutants of *E. coli*, when grown in the absence of drug, usually show little difference in growth rate from the parent sensitive strain. However certain spc-r or str-r mutants do show markedly reduced growth rates (Anderson, 1969; Couturier, Desmet, and Thomas, 1964; Nashimoto and Nomura, 1970). Wild-type strains of *S. typhimurium* (e.g., TY1) are sensitive to the aminoglycoside antibiotics streptomycin, spectinomycin, neomycin, paromomycin, and kanamycin; the minimal inhibitory concentrations for these drugs are greater for *S. typhimurium* than for *E. coli*. This is especially true in the case of spectinomycin, since the wild type strain TY1 will continue to grow (at a reduced growth rate) when challenged with 50 μg/ml of the drug. The growth curves for several *S. typhimurium* strains in rich medium are shown in Fig. 1. It can be seen that *strA* mutants have a doubling time close to that of the sensitive parent, whereas the *strB* and *argG*10 mutants grow more slowly than the wild-type. When these four strains were grown in medium containing streptomycin (50 μg/ml), the growth of the sensitive parent was completely inhibited, the *strA* mutant was unaffected, while the *strB* and *argG*10 strains continued to grow at a slightly reduced rate (Fig. 2). When the drug concentration was increased to 200 μg/ml, the *strB* and *argG*10 mutants were still capable of slow growth while the *strA* mutant continued to grow unimpeded. When tested with other aminoglycoside antibiotics, the wild-type (str-s), and *strA* mutant were sensitive to neomycin (20 μg/ml), kanamycin (20 μg/ml) and paromomycin (20 μg/ml), whereas the *strB* and *argG*10 mutants were resistant to these drugs, although there was a slight reduction in growth rate when the drug was added. This result clearly differentiates between *strA* and *strB* mutants in *S. typhimurium*.

The *spc* mutants that we have isolated were tested for resistance to other aminoglycoside antibiotics. With the exception of TY5 (which is resistant to spectinomycin and streptomycin), all of these mutants showed normal sensitivity to streptomycin, neomycin, paromomycin, and kanamycin.

Genetic Mapping of str-r and spc-r Strains

Previous experiments on the mapping of the *strB* locus by Demerec and his colleagues (Sanderson and Demerec, 1965) which have been confirmed by Roth, Anton, and Hartman (1966) have shown that the *strB* locus is situated near *hisS* at 80 minutes on the *S. typhimurium* linkage map. The *strA* locus has been

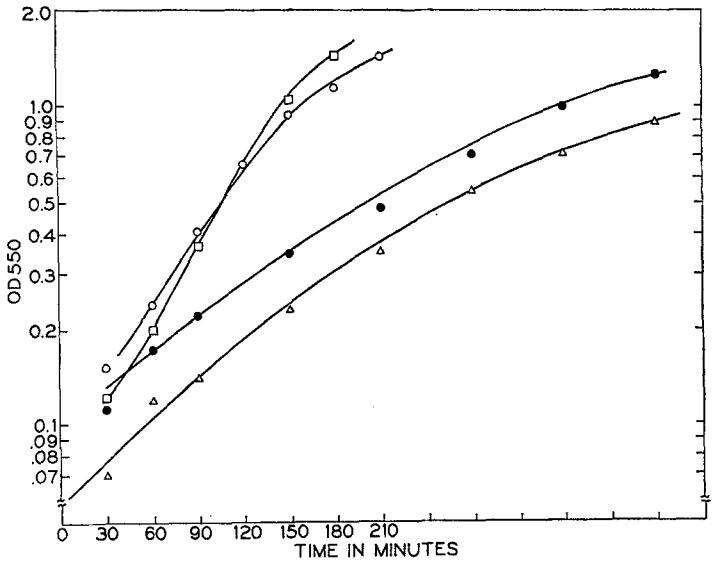

Fig. 1. Growth of *S. typhimurium* strains in rich medium (□——□) TY·1 (wild-type), (●——●)TY·5 (*strB*), (○——○) TY·6 (*strA*), (△——△) TY14 (argG10)

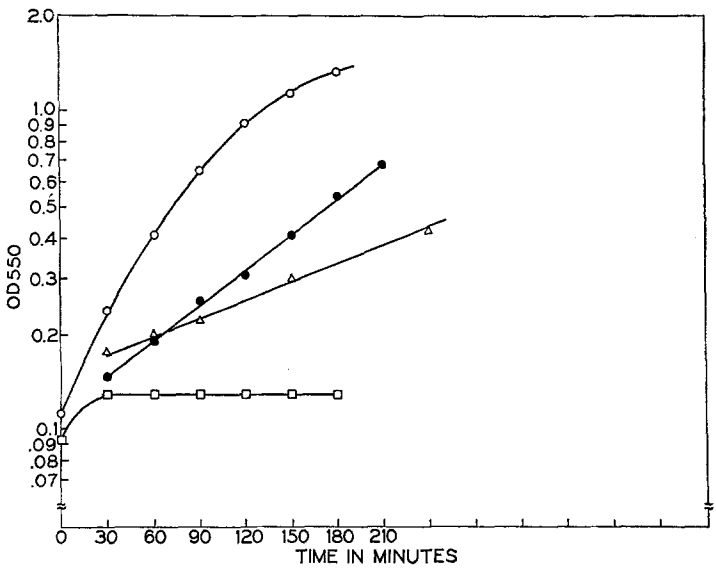

Fig. 2. Growth of *S. typhimurium* strains in rich medium containing 50 μg/ml streptomycin. Symbols the same as for Fig. 1

placed at minute 108, close to *argG* and *aroC* (Kuo and Stocker, 1969; Nishioka and Eisenstark, 1970) and we have confirmed this map position by P22 transduction using *strA* mutants isolated in this laboratory (Table 2). No additional transductional analysis of *strB* mutants has been carried out.

Table 2. *P22 transduction analysis of str-r mutants*

	Donor	Recipient	Transductants		
			arg$^+$	*arg*$^+$ *str-r*	*arg*$^+$ *str-r*/*arg*$^+$ (%)
(i)	TY6 (*strA*)	TY58 (*argG*)	438	162	37
	TY17 (*strA*)	TY58 (*argG*)	102	41	40
	TY18 (*strA*)	TY58 (*argG*)	318	112	35
	TY19 (*strA*)	TY58 (*argG*)	114	39	34
	TY5 (*strB*)	TY58 (*argG*)	106	0	<0.94
	TY37 (*strB*)	TY58 (*argG*)	228	0	<0.44
	TY44 (*strB*)	TY58 (*argG*)	331	0	<0.33
			cys$^+$	*cys*$^+$ *str-r*	*cys*$^+$ *str-r*/*cys*$^+$ (%)
(ii)	TY6 (*strA*)	TY15 (*cysG*)	311	8	2.57
	TY17 (*strA*)	TY15 (*cysG*)	345	11	3.2
	TY18 (*strA*)	TY15 (*cysG*)	443	22	4.96
	TY46 (*strB*)	TY15 (*cysG*)	332	0	<0.31
			aro$^+$	*aro*$^+$ *str-r*	*aro*$^+$ *str-r*/*aro*$^+$ (%)
(iii)	TY6 (*strA*)	TY11 (*aroC*)	343	2	0.58
	TY17 (*strA*)	TY11 (*aroC*)	115	2	1.74
	TY6 (*strA*)	TY12 (*aroC*)	229	1	0.44
	TY17 (*strA*)	TY12 (*aroC*)	230	1	0.43

Table 3. *P22 transduction analysis of spc-r mutants*

	Donor	Recipient	Transductants		
			arg$^+$	*arg*$^+$ *spc-r*	*arg*$^+$ *spc-r*/*arg*$^+$ (%)
(i)	TY51 (*spcB*)	TY59 (*argG*)	111	26	23.4
	TY52 (*spcB*)	TY58 (*argG*)	268	53	19.8
	TY31 (*spcA*)	TY58 (*argG*)	138	7	5.0
	TY32 (*spcA*)	TY58 (*argG*)	355	11	3.1
	TY33 (*spcA*)	TY58 (*argG*)	222	5	2.26
			cys$^+$	*cys*$^+$ *spc-r*	*cys*$^+$ *spc-r*/*cys*$^+$ (%)
(ii)	TY51 (*spcB*)	TY15 (*cysG*)	313	0	<0.32
	TY52 (*spcB*)	TY15 (*cysG*)	336	8	2.38
			aro$^+$	*aro*$^+$ *spc-r*	*aro*$^+$ *spc-r*/*aro*$^+$ (%)
(iii)	TY51 (*spcB*)	TY11 (*aroC*)	242	14	5.8
	Ty52 (*spcB*)	TY11 (*aroC*)	231	5	2.17
	TY51 (*spcB*)	TY12 (*aroC*)	310	19	6.15
	TY52 (*spcB*)	TY12 (*aroC*)	232	9	3.88
	TY31 (*spcA*)	TY11 (*aroC*)	226	29	12.8
	TY32 (*spcA*)	TY11 (*aroC*)	177	23	13.0
	TY33 (*spcA*)	TY11 (*aroC*)	236	29	12.3
	TY31 (*spcA*)	TY12 (*aroC*)	233	39	16.7
	TY32 (*spcA*)	TY12 (*aroC*)	334	65	19.5
	TY33 (*spcA*)	TY12 (*aroC*)	341	48	14.0

Table 4. *P22 transduction analysis of str-r, spc-r double mutant*

	Donor	Recipient	Transductants			
			aro$^+$	*aro*$^+$ *spc-r*	*aro*$^+$ *str-r*	*aro*$^+$ *str-r spc-r*
(i)	TY66 (*strA, spcB*)	TY11 (*aroC*)	333	18	4[a]	3
	TY66 (*strA, SpcB*)	TY12 (*aroC*)	345	25	7[b]	5
			cys$^+$	*cys*$^+$ *spc-r*	*cys*$^+$ *str-r*	
(ii)	TY66 (*strA, SpcB*)	TY15 (*cysG*)	539	0	8	
			arg$^+$	*arg*$^+$ *str-r*	*arg*$^+$ *spc-r*	*arg*$^+$ *spc-r str-r*
(iii)	TY66 (*strA, spcB*)	TY58 (*argG*)	216	28	8[c]	7

[a] 3 spc-r. — [b] 5 spc-r. — [c] 7 str-r.

Table 5. *Cross between spcA and spcB mutants*

Donor	Recipient	*aro*$^+$	*aro*$^+$ *spc-s*	*aro*$^+$ *spc-s*/*aro*$^+$ (%)
TY52 (*spcB*)	TY78 (*aroC, spcA*)	533	33	6.1
TY31 (*spcA*)	TY78 (*aroC, spcA*)	475	0	<0.21

In like manner, we have carried out a transductional analyses of *spc* mutants and have found these mutants to be closely linked to *strA*, *aroC*, and *argG* (Table 3). The *argG* mutants used in this analysis were arginine-requiring suppressors of proline auxotrophs described by Kuo and Stocker (1969); attempts to map our spc-r mutants relative to the original *argG*10 mutant proved to be difficult, since this strain is somewhat resistant to spectinomycin. In Table 4 we provide mapping data for a *strA spcB* double mutant; in general the linkage data from transduction analysis of such double mutants gives lower frequencies than the corresponding single resistant mutants. The results from all of these crosses indicate that there are two closely linked loci for spc-r in *S. typhimurium*. One *spc* locus (*spcB*) is close to *argG* and *strA*, and the other (*spcA*) is close to *aroC*. Since the *spcA* and *spcB* mutants are separate, it should be possible to demonstrate recombination between them. The results in Table 5 provide further evidence for two separate *spc* loci, since spc-s recombinants are generated in a cross between a *spcA* and a *spcB* mutant. In Fig. 3 we show a preliminary linkage map for the "*str-spc*" region of the *S. typhimurium* chromosome.

Properties of Cell-Free Extracts and Ribosomes of strA, strB, and argG10 Mutants

When cell-free extracts of str-r mutants of *E. coli* are compared with extracts from str-s strains for their ability to synthesize polypeptides, it is found that the str-r extracts are not affected by the addition of the drug, while str-s extracts are inhibited. The same results were obtained using cell-free systems containing purified ribosomes from sensitive- and resistant-strains (Flaks, Cox, and White

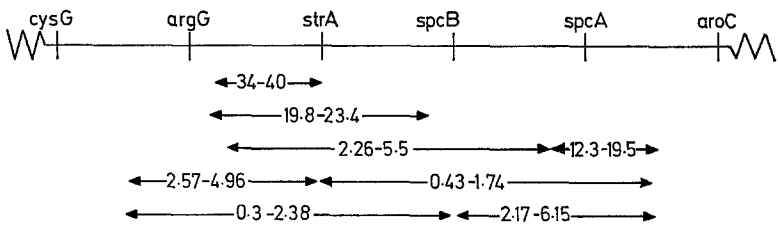

Fig. 3. Gene order map for the *strA* region of the *S. typhimurium* chromosome. This map is derived from the transduction frequencies given in Tables 2, 3, and 4. This order is preferred to the order *aroC-spcA-spcB-argG-strA-cysG*, from consideration of the results of transduction with the double mutant given in Table 4

1962). More recently, it has been shown that ^{3}H-labelled dihydrostreptomycin can bind to ribosomes from a sensitive-, but not a resistant-strain (Kaji and Tanaka, 1968). We have carried out similar incorporation studies with various str-r mutants of *S. typhimurium*; cell-free extracts of wild-type, *strA*, *strB*, and *argG*10 strains were prepared by alumina grinding and tested for their response to poly U and to R17 RNA in the presence and absence of streptomycin. Several assays for streptomycin action *in vitro* were used: a) inhibition of poly U-directed phenylalanine synthesis; b) the production of coding errors during the translation of poly U; or c) inhibition of polypeptide synthesis stimulated by bacteriophage R17 RNA. Inhibition of poly U-directed phenylalanine incorporation is not a good index of sensitivity since somewhat variable results are obtained. The other two assays are more reliable. The results shown in Table 6 indicate that polypeptide synthesis in extracts of the *strA* mutant is clearly resistant to the inhibitory or misreading action of streptomycin, whereas extracts of the wild-type (str-s), *strB* and *argG*10 strains are equally sensitive to the drug. We conclude that streptomycin resistance in the *strB* and *argG*10 mutants does not result from a mutational alteration in the protein synthetic machinery of these strains.

The properties of the ribosomes from these strains was confirmed by binding studies with ^{3}H-dihydrostreptomycin; as shown in figure 4 the ribosomes from the *strA* mutant fail to bind the labelled drug. The ribosomes from the wild type, *strB* and *argG*10 mutants bind labelled drug to the same extent. Therefore *strB* and *argG*10 mutants possess ribosomes and protein synthetic machinery sensitive to streptomycin, and some other mechanism for resistance must be considered.

Properties of Cell-Free Extracts and Ribosomes of spc-r Mutants

The ribosomes from spc-r mutants of *E. coli* are resistant to the drug in cell-free incorporation studies (Davies, Anderson, and Davis, 1965) and fail to bind ^{3}H-dihydrospectinomycin under normal incubation conditions (Bollen, Helser, Yamada, and Davies, 1969). Cell-free extracts and purified ribosomes were prepared from a number of spc-r mutants of *S. typhimurium* (both *spcA* and *spcB*) and compared to the sensitive parent in amino acid incorporation and antibiotic binding studies. R17 RNA was used as messenger RNA in the amino acid incorporation experiments, since poly U-directed polypeptide synthesis is not

Table 6. *Effects of streptomycin on amino acid incorporation by cell-free extracts of S. typhimurium strains*

	Incorporation (c.p.m. per reaction mixture)			
	TY1 (wild-type)	TY5 (*strB*)	TY6 (*strA*)	TY14 (*argG*10)
(i) Poly U-directed phenylalanine incorporation				
Complete system	560	690	1350	2225
— poly U	137	116	47	43
+ str (10 μg/ml)	280	330	1160	2600
+ neomycin (10 μg/ml)	—	—	530	—
(ii) Poly U-directed system: misreading				
Complete system	170	183	53	136
— poly U	65	90	414	37
+ str (10 μg/ml)	1020	1250	72	3800
+ neomycin (10 μg/ml)	—	—	2280	—
(iii) R17 RNA-directed system				
Complete system	270	670	1500	943
— mRNA	28	43	75	98
+ str (10 μg/ml)	68	58	1750	104

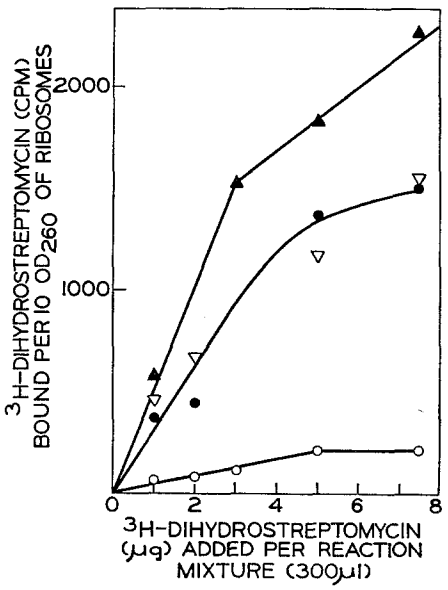

Fig. 4. The binding of ^{3}H-dihydrostreptomycin to purified ribosomes from str-s and str-r strains of *S. typhimurium*. Experimental details are described in Materials and Methods. (▽——▽) TY·1 (wild-type), (●——●) TY·5 (*strB*), (○——○) TY·6 (*strA*), (▲——▲) TY·14 (*argG*10)

Table 7. *Effects of spectinomycin on R17 RNA-directed amino acid incorporation by cell-free extracts of S. typhimurium strains*

	Incorporation (c.p.m. per reaction mixture)			
	TY1 (wild-type)	TY31 (*spcA*)	TY51 (*spcB*)[a]	TY52 (*spcB*)
Complete system	2238	1966	350	515
— messenger RNA	95	178	27	49
+ spc 0.5 μg/ml	1050	2200	151	194
+ spc 1 μg/ml	773	2342	168	129

[a] Amino acid incorporation in extracts of *spcB* mutants is usually low; it is not known if this has any significance.

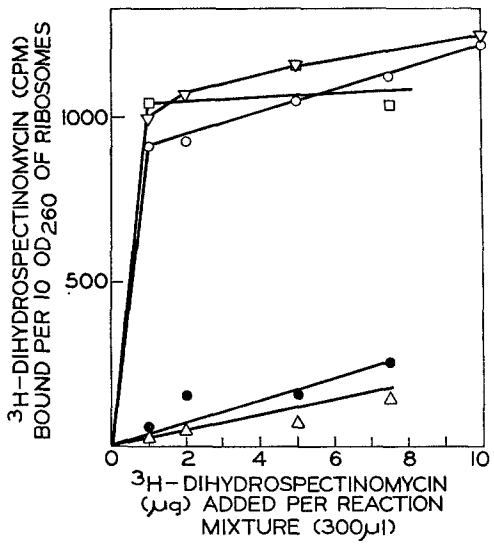

Fig. 5. The binding of ^{3}H-dihydrospectinomycin to purified ribosomes from spc-s and spc-r strains of *S. typhimurium*. Experimental details are described in Materials and Methods. (▽——▽) TY·1 (wild-type), (□——□) TY52 (*spcB*), (○——○) TY66 (*spcB*), (△——△) TY33 (*spcA*), (●——●) TY 32 (*spcA*)

sensitive to spectinomycin. The results of these experiments (Table 7, and Fig. 5) support the notion that there are two classes of spc-r mutants, since cell-free amino acid incorporation by *spcA* mutants is resistant to the inhibitory effect of the drug and *in vitro* incorporation by *spcB* mutants is as sensitive as that of cell-free extracts of the sensitive host. These results were confirmed by ^{3}H-dihydrospectinomycin binding studies (Fig. 5) which show that purified ribosomes from *spcA* strains do not bind appreciable amounts of the drug; within the same concentration range the sensitive parent and *spcB* mutants had equal ability to

bind the drug. It would therefore appear that there are two distinct *spc* loci in *S. typhimurium*, which have a similar phenotype but different mechanisms of resistance.

Discussion

S. typhimurium and *E. coli* are two very closely related gramnegative species with a great deal of similarity (apparent) in their genetic make-up (in some cases polarity of operons is reversed); intergeneric matings (Baron, Gemski, Johnson, and Wohlhieter, 1968) and phage infections (Okada and Watanabe, 1968) are possible. In *Escherichia coli*, considerable effort has been exerted to define the genetic determinants for the ribosome, and *strA* and *spcA* are the two best-defined ribosome markers; these mutations are single-step, low frequency events (10^{-9} to 10^{-10}) which affect genes which determine the synthesis of two separate proteins of the 30 s ribosome subunit (Ozaki *et al.*, 1969; Bollen *et al.*, 1969). These two loci are closely linked and are situated on the linkage map near to other known ribosome markers. No other chromosomal loci for str-r and spc-r have been reported for *E. coli*, although it seems likely that mutations might occur which prevent uptake of the drug by the cell; these would presumably be low-level resistant mutants (for example, tetracycline and chloramphenicol resistance in *E. coli*: Reeve, 1968). It is conceivable that researchers may have overlooked low-level str-r and spc-r mutations in *E. coli*, since the selection often involved immediate exposure to a high concentration of drug. There is an unusual class of antibiotic resistant mutants in *E. coli* which are related to a deficiency in hemin synthesis (Beljanski and Beljanski, 1957).

We have shown that the two known *str* loci in *S. typhimurium* control different functions; one (*strA*) is probably a gene for a 30 s ribosomal protein and is strictly analogous to the *strA* locus in *E. coli*. The frequency of spontaneous appearance of *strA* mutants is approximately 10^{-9} in both organisms. We do not know the mechanism of resistance resulting from a mutation in the *strB* locus in *S. typhimurium*; there is no analogous locus in *E. coli*. These mutants, which are pleiotropic (Demerec *et al.*, 1960), appear with a higher spontaneous frequency (10^{-7}) than *strA* mutants and are usually seen after a 3–5 day incubation compared to the appearance of *strA* mutants after 2–3 days. The fact that *strB* mutants are resistant to other aminoglycoside antibiotics might suggest that resistance in these mutants is due to a general inability of aminoglycosides to penetrate the cell. We have attempted to measure differences in uptake of ^{3}H-dihydrostreptomycin by various strains, but these experiments were not successful in distinguishing sensitive- and resistant-mutants, presumably because of the large amount of drug which attaches nonspecifically to the outer surface of the cells. In addition, we have examined cell-free extracts of *strB* mutants for enzymes which are known to inactivate streptomycin by adenylylation or phosphorylation (Benveniste, Yamada, and Davies, 1970; Ozanne, Benveniste, Tipper, and Davies, 1969), but no evidence for such inactivation has been found.

Is the *argG*10 mutant to be classified as a *strB* mutant? This strain was isolated by Demerec and his coworkers (Demerec *et al.*, 1960) and until recently this was the only *argG* mutant available; *argG*10 is a one-step mutant which

became (simultaneously) arginine-requiring and streptomycin-resistant and was assumed to be *strA* because of its chromosomal location. Kuo and Stocker (1969) have confirmed that the *arg* mutation is in the true *argG* locus, since it failed to recombine with other *argG* mutants in transduction. We can therefore conclude that the *argG*, str*A*, and *strB* loci are not coincident and it is probable that the str-r character of *argG*10 is the pleiotropic result of mutation to arginine auxotrophy, by an unknown mechanism. However, none of the other *argG* auxotrophs described by Kuo and Stocker became simultaneously str-r. It is clear from our results that this mutant does not have the same phenotype as *strA* mutants, and is more closely related to the *strB* phenotype in being pleiotropic, non-ribosomal in nature, and exhibiting cross-resistance to other aminoglycoside antibiotics. The growth characteristics of *strB* and *argG*10 mutants are similar, and quite different from *strA* mutants.

Only one *strB* mutant has been mapped accurately (Roth *et al.*, 1966) and it is possible that there are other sites on the *S. typhimurium* chromosome which determine this phenotype; the *argG*10 mutant may be another member of this class. A genetic and biochemical analysis of *strB* mutants would be worthwhile, since such a study may provide data to explain the formation of these non-ribosomal, pleiotropic str-r mutants. There are well documented examples of multiple alleles for str-r and str-d in other systems, such as *Pneumococcus* (Rotheim and Ravin, 1961) and *Proteus* (Hofemeister and Bohme, 1967). These studies have been mainly genetic and it has not yet been established whether these *str*-mutations affect ribosome function in these strains.

Mutations in spc-r have not previously been studied in *S. typhimurium*, and our data suggest that, as with str-r in this strain, there are at least two loci for spc-r (*spcA*, *spcB*). Mutations at the two loci are phenotypically different; *spcA* mutants are likely to be alterations in a ribosomal protein and probably correspond to *spcA* mutants in *E. coli*. The mechanism of resistance in *spcB* mutants is not known, and we can only present the negative evidence that *spcB* mutants do not possess altered ribosomal components, neither do they inactivate the drug by adenylylation (Benveniste *et al.*, 1970). Since the map location for the *spcA* and *spcB* loci is in the region of the *S. typhimurium* chromosome which contains ribosomal genes (O'Neil, Baron, and Sypherd, 1969), we cannot rule out that *spcB* resistance is due to a mutational alteration in the protein synthetic machinery which cannot be detected in our cell-free amino acid incorporation system. Equally likely is the possibility that *spcB* resistant mutants have a decreased permeability for the drug; this would suggest a specific transport system for spectinomycin, since we do not find resistance to other drugs as a result of a mutation in the *spcB* allele. Although there appears to be only one spc-r determinant in *E. coli* (Bollen *et al.*, 1969; Anderson, 1969), a recent report by Harford and Sueoka (1970) suggests the existence of two separate *spc* loci in *B. subtilis*; the latter mutants have not yet been examined at the ribosome level.

Acknowledgements. This work was supported by a research grant from N.I.H. We wish to thank Drs. K. E. Sanderson, B. A. D. Stocker and J. S. Gots for bacterial strains and bacteriophage. Dr. G. B. Whitfield Jr., of the Upjohn Company, supplied us with generous quantities of spectinomycin sulphate. We thank Drs. C. G. Kurland and M. Nomura for their comments on the manuscript.

References

Anderson, P.: Sensitivity and resistance to spectinomycin in *Escherichia coli*. J. Bact. **100**, 939–947 (1969).

Baron, L. S., Gemski, P., Jr., Johnson, E. M., Wohlhieter, J. A.: Intergeneric bacterial matings. Bact. Rev. **32**, 362–369 (1968).

Beljanski, M., Beljanski, N.: The formation of respiratory enzymes by a streptomycin-resistant and hemin-requiring mutant of *Escherichia coli*. Ann. Inst. Pasteur **92**, 396–412 (1957).

Benveniste, R., Yamada, T., Davies, J.: Enzymatic adenylylation of streptomycin and spectinomycin by R-factor-resistant *Escherichia coli*. Infection and Immunity **1**, 109–119 (1970).

Birge, E. A., Kurland, C. G.: Phenotypic reversion of the streptomycin dependence phenotype. Submitted to Molec. Gen. Genetics, 1970.

Bollen, A., Davies, J., Ozaki, M., Mizushima, S.: Ribosomal protein conferring sensitivity to the antibiotic spectinomycin in *Escherichia coli*. Science **165**, 85–86 (1969).

— Helser, T., Yamada, T., Davies, J.: Altered ribosomes in antibiotic-resistant mutants of *E. coli*. Cold Spr. Harb. Symp. quant. Biol. **34**, 95–100 (1969).

Breckenridge, L., Gorini, L.: Genetic analysis of streptomycin resistance in *Escherichia coli*. Genetics **65**, 9–25 (1970).

Couturier, M., Desmet, L., Thomas, R.: High pleiotrophy of streptomycin mutations in *Escherichia coli*. Biochem. biophys. Res. Commun. **16**, 244–248 (1964).

Davies, J., Anderson, P., Davis, B. D.: Inhibition of protein synthesis by spectinomycin. Science **149**, 1096–1098 (1965).

Demerec, M., Lahr, E. L., Balbinder, E., Miyake, T., Ishidsu, J., Mizobuchi, K., Mahler, B.: Bacterial genetics. Carnegie Inst. Wash. Year B **59**, 426–441 (1960).

Flaks, J. G., Cox, E. C., White, J. R.: Inhibition of polypeptide synthesis by streptomycin. Biochem. biophys. Res. Commun. **7**, 385–389 (1962).

— Leboy, P. S., Birge, E. A., Kurland, C. G.: Mutations and genetics concerned with the ribosome. Cold Spr. Harb. Symp. quant. Biol. **28**, 623–631 (1966).

Goldthwaite, C., Dubnau, D., Smith, I.: Genetic mapping of antibiotic resistance markers in *Bacillus subtilis*. Proc. nat. Acad. Sci. (Wash.) **65**, 96–103 (1970).

Harford, N., Sueoka, N.: Chromosomal location of antibiotic resistance markers in *Bacillus subtilis*. J. molec. Biol. **51**, 267–286 (1970).

Hershey, A. D., Chase, M.: Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. gen. Physiol. **36**, 39–56 (1952).

Hofemeister, J., Bohme, H.: Streptomycin-abhängige Mutanten von *Proteus mirabilis*; ihre Genetik, Suppression und Modifikation. Molec. Gen. Genetics **99**, 219–247 (1967).

Kaji, H., Tanaka, Y.: Binding of dihydrostreptomycin to ribosomal subunits. J. molec. Biol. **32**, 221–230 (1968).

Kuo, T. T., Stocker, B. A. D.: Suppression of proline requirement of *proA* and *proAB* deletion mutants in *Salmonella typhimurium* by mutation to arginine requirement. J. Bact. **98**, 593–598 (1969).

Nashimoto, H., Nomura, M.: Structure and function of bacterial ribosomes. XI. Dependence of 50S ribosomal assembly on simultaneous assembly of 30S subunits. Proc. nat. Acad. Sci. (Wash.) 1970, in press.

Nirenberg, M. W.: In: Methods in enzymology (S. P. Colowick and N. O. Kaplan, eds.), vol. 6, p. 17–23. New York: Academic Press 1964.

Nishioka, Y., Eisenstark, A.: Sequence of genes replicated in *Salmonella typhimurium* as examined by transduction techniques. J. Bact. **102**, 320–333 (1970).

Okada, M., Watanabe, T.: Transduction with phage P1 in *Salmonella typhimurium*. Nature (Lond.) **218**, 185–187 (1968).

O'Neil, D. M., Baron, L. S., Sypherd, P. S.: Chromosomal location of ribosomal protein cistrons determined by intergeneric bacterial mating. J. Bact. **99**, 242–247 (1969).

Ozaki, M., Mizushima, S., Nomura, M.: Identification and functional characterization of the protein controlled by the streptomycin-resistant locus in *E. coli*. Nature (Lond.) **222**, 333–339 (1969).

Ozanne, B., Benveniste, R., Tipper, D., Davies, J.: Aminoglycoside antibiotics: inactivation by phosphorylation in *Escherichia coli* carrying R-factors. J. Bact. **100**, 1144–1146 (1969).

Reeve, F. C. R.: Genetic analysis of some mutations causing resistance to tetracycline in *Escherichia coli*. Biochem. J. **106**, 35P (1968).
Roth, J. R., Anton, D. N., Hartman, P. E.: Histidine regulatory mutants in *Salmonella typhimurium*. I. Isolation and general properties. J. molec. Biol. **22**, 305–323 (1966).
Rotheim, M. B., Ravin, A. W.: The mapping of genetic loci affecting streptomycin resistance in pneumococcus. Genetics **46**, 1619–1634 (1961).
Sanderson, K. E.: Current linkage map of *Salmonella typhimurium*. Bact. Rev. **34**, 176–193 (1970).
— Demerec, M.: The linkage map of *Salmonella typhimurium*. Genetics **51**, 897–913 (1965).
Sypherd, P. S., O'Neill, D. M., Taylor, M. M.: The chemical and genetic structure of bacterial ribosomes. Cold Spr. Harb. Symp. quant. Biol. **34**, 77–84 (1969).
Taylor, A. L.: Current linkage map of *Escherichia coli*. Bact. Rev. **34**, 155–175 (1970).

Communicated by W. Maas

Dr. J. Davies
Department of Biochemistry
University of Wisconsin
Madison, Wisconsin 53706/U.S.A.