GENETIC ANALYSES OF MUTATIONS FROM STREPTOMYCIN DEPENDENCE TO INDEPENDENCE IN SALMONELLA TYPHIMURIUM[1]

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Received May 29, 1962

STREPTOMYCIN-DEPENDENT bacterial mutants require the antibiotic for sustained growth. Mutations occur in dependent cells that eliminate the requirement for streptomycin. The ease with which these independent mutants may be detected has led to the use of this system in many studies of mutagenesis (DEMEREC 1951; BERTANI 1951; DEMEREC, BERTANI and FLINT 1951; DEMEREC and HANSON 1951; GLOVER 1956; IYER and SZYBALSKI 1958). NEWCOMBE and NYHOLM (1950), NEWCOMBE (1952), and BERTANI (1951) observed that different independent mutants varied in their level of resistance from as sensitive as wild type to fully resistant. HASHIMOTO (1960) conclusively demonstrated that the mutation to the sensitive independent type was not due to a genetic reversion to wild type, but was due to a secondary genic alteration that suppressed the original dependent phenotype.

The experiments reported here are concerned with the genetic analysis of mutations from high-level streptomycin dependence to independence in *S. typhimurium*, strains LT-2 and LT-7, by means of the transducing bacteriophage P22. The data obtained in this system provide answers to the following questions: (1) Is the primary mutation to high-level streptomycin dependence pleiotropic? (2) Is there a genetic basis for the different levels of resistance displayed by streptomycin-independent mutant types arising from dependent populations? (3) Do true genic reversions from dependence to wild type ever occur and, if so, under what conditions?

MATERIALS AND METHODS

Bacterial strains: Salmonella typhimurium strains LT-7 and LT-2 were obtained from DR. PHILIP E. HARTMAN, The Johns Hopkins University, Department of Biology, Baltimore, Md. Strain LT-7 (MIYAKE 1960) was a mixture of stable and mutable cells. The stable type was isolated by streaking a dozen colonies onto nutrient agar with and without 1 mg/ml of dihydrostreptomycin sulfate. Hundreds of streptomycin-resistant colonies developed from the mutable

[1] This work was presented at the Annual Meetings of the Society of American Bacteriologists, May 1960 (MATNEY, GOLDSCHMIDT and BAUSUM 1960).

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isolates, but comparatively few arose from stable isolates. A stable LT-7 stock was used to obtain the streptomycin-dependent isolates, since mutations to high-level resistance are induced by ultraviolet light in LT-7 and not in LT-2. Once obtained in LT-7, the mutations were transduced into the more stable LT-2 strain.

Phage: PLT-22 transducing phage was obtained from DR. PHILIP E. HARTMAN. Phage stocks for transduction were prepared essentially as described by HARTMAN (1956) except that the donor cell cultures were grown in Difco Brain Heart Infusion broth. Streptomycin was added to yield a final concentration of 1 mg/ml when the donor strain was streptomycin dependent. When phage-sensitive nonlysogenic transductants were needed for further genetic analyses by transduction, these were obtained by using ultraviolet-irradiated virulent mutants of PLT-22 phage as described by GOLDSCHMIDT and LANDMAN (1962).

Media: Complete media: either nutrient broth (8.0 g dehydrated Difco Nutrient Broth plus 5.0 gm NaCl per liter) or brain heart infusion broth (BHI = 37 g dehydrated Difco BHI per liter).

Minimal medium: minimal salts of VOGEL and BONNER (1956) plus 0.2 percent glucose.

Agar media: 15 g Difco Bacto-Agar per liter of broth medium.

Diluting fluid: 8.0 g NaCl per liter of H_2O.

Streptomycin: Dihydrostreptomycin sulfate; unless stated otherwise, added to yield a final concentration of 1 mg per ml.

Nomenclature of genetic symbols: The primary mutations considered in this work involve the high level, single step, streptomycin resistance locus, which will be designated *str* as in the nomenclature of HASHIMOTO (1960). The wild-type allele will be referred to as *str-s*, since it renders the cell sensitive to the antibiotic. Mutants that have an absolute growth requirement for streptomycin are designated *str-d*. Separate isolates are identified with a number, e.g., *str-d-72*, *str-d-73*. Other high-level resistant types characterized by their growth on nutrient agar with and without 1 mg/ml dihydrostreptomycin sulfate are: *str-r*, indifferently resistant; *str-pr*, a partially resistant type that grows better in the absence of streptomycin; *str-pd*, a partially dependent type that grows better in the presence of the antibiotic; *str-min*, a heterogeneous group that forms very minute colonies in the presence of streptomycin and varies in its growth response without streptomycin from as well as wild type to no growth at all (dependent). Approximately three percent of the spontaneous *str-min* mutants are pink in color and are suspected to have some aberration in their cytochromes.

Detailed studies were confined to the *str-d* mutants and strains derived from them. Secondary modifier (*mod*) mutations alter the dependent phenotype by abolishing the requirement for methionine and reducing the requirement for streptomycin. Mutations at some locus other than *str*, which eliminate the requirement for both streptomycin and methionine, have been designated as *su* (suppressor). Secondary mutations, modifier (*mod*) and suppressor (*su*), retain the numerical identity of the primary mutation. Thus *str-d-73/mod*$^+$*/su-73-1* refers to a suppressed isolate of an unmodified (+ = wild type) *str-d-73* strain.

Membrane filter technique for the isolation of UV-induced streptomycin-dependent mutants: The membrane filter techniques for the isolation of UV-induced high-level streptomycin-resistant or streptomycin-dependent mutants in *Escherichia coli* B/r (MATNEY, SHANKEL and WYSS 1958) were applied to *S. typhimurium* strain LT-7. Log phase cells growing in BHI broth were impinged onto membrane filters (ca. 2×10^8 cells per filter). The membranes were placed on BHI agar plates (soft agar, as used for *E. coli*, results in confluence of colonies of Salmonella) and exposed to sufficient ultraviolet radiation to effect a 90 percent reduction in viability. The plates were incubated for four hours at 37°C and the membranes transferred to BHI-streptomycin agar. After two days' incubation at 37°C, a variety of resistant colony sizes was noted. When picked and streaked on nutrient agar with and without streptomycin, the following responses were noted. The largest colonies were indifferent to streptomycin (*str-r*). The smallest colonies consisted of the *str-min* phenotypes. Colonies of intermediate size fell into three groups: *str-pr*, *str-pd*, and *str-d*. Less than five percent of the induced mutations were of the desired *str-d* type.

Isolation of modified dependent strains: Modified dependent strains were isolated from dependent strains by selection on minimal agar plus streptomycin.

Isolation of streptomycin-independent mutants: Nondependent mutants were isolated from dependent strains by selection on nutrient agar lacking streptomycin.

Membrane filter techniques for measuring the delayed development of streptomycin resistance in transduced cells: When wild-type recipient cells are infected by transducing phage from streptomycin-dependent donor cells, there is a delayed expression of several hours before the infected cells become resistant to streptomycin. This delayed expression is conveniently measured by impinging a suitable number of phage-infected cells onto each of several membrane filters that are placed on warm BHI agar plates. After varying periods of incubation at 37°C, the membranes are transferred to BHI agar containing streptomycin. The minimum incubation time permitting the subsequent appearance of the largest number of streptomycin-dependent colonies is the delayed expression time.

The results illustrated in Figure 1 show a typical delayed expression pattern for the transduction of *str-d* into *str-s* recipients. Approximately four hours of incubation were required for maximum expression of the *str-d* phenotype.

Similar experiments have been performed with transduction of *str-r* into *str-s* recipients. The frequency of transduction of *str-r* was approximately 5×10^{-7} to 1×10^{-6} per phage with multiplicities of infection between 0.06 and 17.1 phage per bacteria.

str-s was transduced into *str-d* recipients by plating the phage-infected cells on nutrient agar lacking streptomycin. The frequencies of transduction were approximately the same as for the reciprocal transductions with the two *str-d* strains used routinely in this study (i.e., *str-d-73* and *str-d-72*). In contrast to the *E. coli* P1 system (HASHIMOTO 1960), all *str-d* recipients tested were transduced to *str-s*.

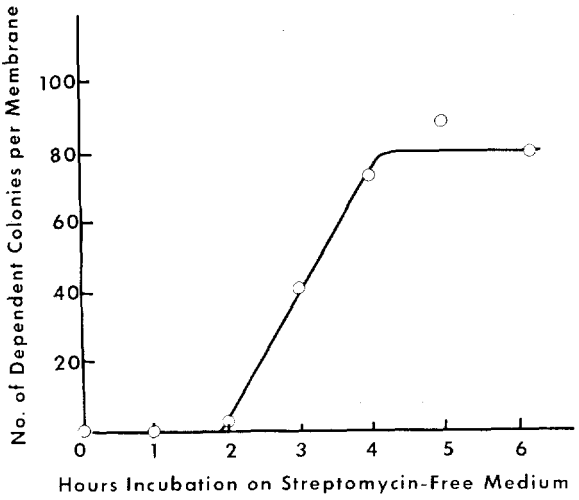

FIGURE 1.—Delayed phenotypic expression of streptomycin-dependence in transduced cells.

EXPERIMENTAL RESULTS

Pleiotropic nature of the primary mutation to streptomycin dependence: All primary isolates of *str-d* mutants were unable to grow on minimal agar plus streptomycin. Auxanographic techniques were used to determine which compounds would satisfy the growth factor requirements. As WATANABE and WATANABE (1959a) previously pointed out, wild-type *S. typhimurium* displays a higher resistance (ca. 100-fold) to streptomycin on minimal agar than on nutrient agar. This observation suggested that dependent mutants might require much higher concentrations of antibiotic to grow on the basal minimal agar than on nutrient agar. When the streptomycin concentration was increased to 5 mg/ml in the minimal agar clear-cut results were obtained that indicated that L-methionine (20 μg/ml) would satisfy the growth requirements of all the freshly isolated *str-d* mutants checked.

Modifier mutation of str-d: When 10^8 washed *str-d* cells from overnight cultures were plated on minimal agar plus streptomycin, several hundred colonies were observed. Some of these colonies were picked and purified by streaking on minimal agar plus streptomycin. These isolates were found to differ from the original *str-d* culture in several ways: they plated at essentially 100 percent efficiency on minimal agar plus streptomycin (compared with nutrient agar plus streptomycin) instead of 10^{-5}–10^{-6}; they required much less streptomycin for optimum growth (see Figure 2); and their rate of growth was much more rapid (division rate in log phase = 1.6 × that of original *str-d* in nutrient broth plus streptomycin).

The mutation in these cultures was shown to be due to a modifier at some other locus than the *str* locus by the following experiment: The prototrophic (modified) *str-d* strains were used as donors in transduction experiments with a wild-type *str-s* recipient. All of the resulting dependent transductants were found to be

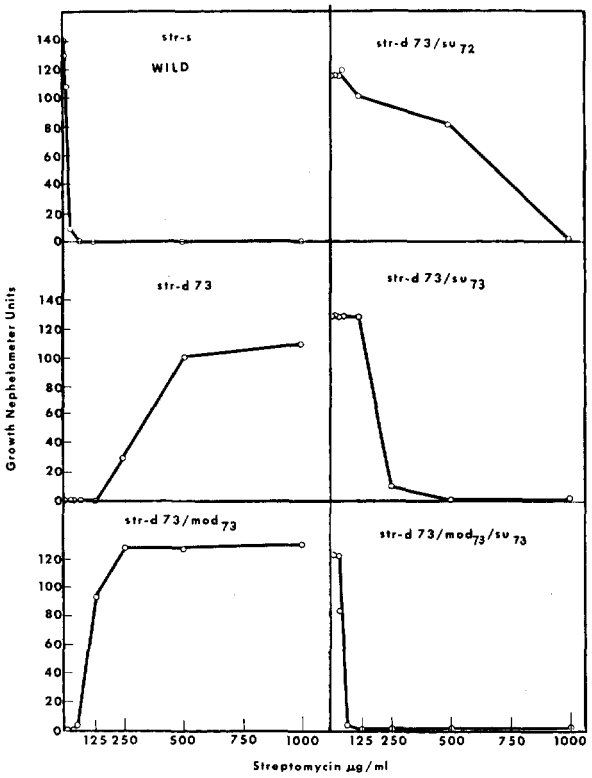

FIGURE 2.—Effect of various concentrations of dihydrostreptomycin on the growth response of wild type, *str-d-73* and its modified and suppressed derivatives.

auxotrophic. Thus loss of the methionine requirement with a concomitant reduction in the requirement for streptomycin must be due to a second mutational event in the genome, which is too distant from the *str* locus to be jointly transduced.

The high spontaneous mutation rate of the modifier precluded the possibility of transducing it into an unmodified dependent recipient. It was difficult to maintain unmodified auxotrophic dependent stocks. Cultures of the auxotrophic dependent stocks were tested prior to use by streaking on minimal plus streptomycin agar to make certain they were not modified to the prototrophic *str-d/mod* type. When necessary, the unmodified dependent type was reclaimed by transducing the primary dependent mutation into wild-type strains.

Suppressor mutations of str-d: When sufficient numbers of dependent cells (either modified or unmodified strains) were spread onto the surface of agar media without streptomycin, scattered colonies developed that could be shown to no longer require either streptomycin or methionine. The level of streptomycin resistance of these independent mutants, as judged by growth on nutrient agar plates (or broth, see Figure 2) containing graded concentrations of antibiotic, remained rather uniform for a given dependent strain, i.e., independent mutants

arising from *str-d-72* were capable of good growth on nutrient agar containing 1 mg streptomycin per ml whereas none of the mutants arising from *str-d-73* grew on this medium. High-titer phage stocks were prepared on representative independent mutant stocks of both *str-d-72* and *str-d-73*. When wild-type LT-2 was used as the recipient, only streptomycin-dependent methionineless transductants were obtained. Therefore, independent mutants, regardless of their level of resistance, were due to separate suppressor mutations.

The effect of specific mutations on the resistance level displayed by streptomycin-independent types: The origin of modified and suppressed derivatives of streptomycin-dependent strains is outlined in Figure 3. The effect of specific mutations of each of the three mutant types on the final level of resistance displayed by the suppressed dependent form was determined. Strains *str-d-72* and *str-d-73*, together with their respective modified and suppressed derivatives, were selected for intensive study, since streptomycin-independent mutants arising from *str-d-72* grew on nutrient agar containing 1 mg/ml of dihydrostreptomycin sulfate and those arising from *str-d-73* did not.

High-titer P22 phage stocks were prepared on suppressed mutants of both strains. A suppressor mutation from one strain could then be transduced into either a modified or unmodified dependent version of the other. The resistance level of the resulting streptomycin-independent transductant was determined by picking a representative number of colonies and streaking them onto a series of nutrient agar plates containing successively greater amounts of streptomycin

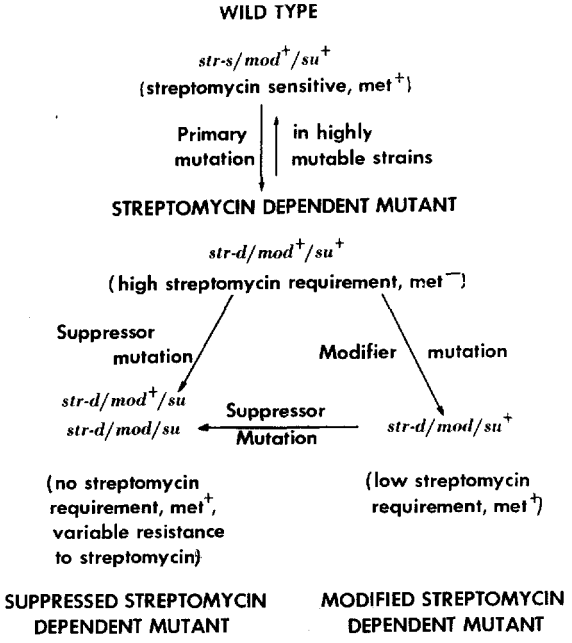

FIGURE 3.—The origin of streptomycin-dependent strains and their modified and/or suppressed derivatives.

(0, 5, 10, 20, 50, 100, 250, 500, and 1000 μg/ml). The highest streptomycin concentration permitting visible growth after overnight incubation was arbitrarily chosen as the level of resistance. The results presented in Table 1 are arranged in genotypic pairs, which differ in but one of the three types of mutation. The first pair contains no modifier mutations (+ = wild-type allele), the same suppressor mutation (*su-72*), but different dependent mutations (*str-d-72* and *str-d-73*); the strain containing the *str-d-72* mutation is more than twice as resistant to streptomycin as the one containing *str-d-73* (500 μg/ml *vs.* > 1000 μg/ml). The second genotypic pair also differs only in the specific *str-d* mutation present. In this case both suppressed strains displayed the same resistance to streptomycin. It is clear that the primary dependent mutation can exert an influence on the resistance level of the *su-72* suppressed mutant but this effect is nullified in the presence of *su-73*. The role of the specific suppressor mutation on streptomycin resistance is better illustrated by rearranging the same genotypes. *su-72* confers a greater level of resistance than does *su-73* and this effect is more marked with its native *str-d-72* than with *str-d-73*. The modifier mutations could not be transduced because of their high spontaneous mutation rates. Their effect on the resistance level of the suppressed types was determined by their presence or absence. The remaining four pairs of genotypes indicate that the presence of *mod-73* invariably rendered the cell quite sensitive to streptomycin (< 50 μg/ml) whereas *mod-72* enhanced the resistance.

As shown in Table 2, appropriate combinations of the six mutations that occurred in the two dependent strains resulted in streptomycin-independent types, which ranged in their level of resistance from as sensitive as wild type to

TABLE 1

The effect of specific mutations on the level of streptomycin resistance of suppressed dependent (independent) types

Mutational type tested	Mutations present in recipient cell		Suppressor mutation contributed by phage	Level of resistance of independent transductant (μg/ml in nutrient agar)
	str-d	Modifier		
Dependence	73	+	72	500
	72	+	72	>1000
	73	+	73	250
	72	+	73	250
Suppressor	73	+	73	250
	73	+	72	500
	72	+	73	250
	72	+	72	>1000
Modifier	73	+	73	250
	73	73	73	< 50
	73	+	72	500
	73	73	72	< 50
	72	+	73	250
	72	72	73	1000
	72	+	72	>1000
	72	72	72	>1000

TABLE 2

The genotypic basis for the wide range of resistances displayed by suppressed mutants of streptomycin-dependent strains of Salmonella typhimurium

Genotype			Level of resistance
str-d	*mod*	*su*	μg/ml in nutrient agar
73	73	73	< 50
73	+	73	250
72	+	73	250
73	+	72	500
72	72	73	1000
72	+	72	>1000
72	72	72	>1000

+ = wild-type allele.

fully resistant. Such an analysis establishes a genetic basis for the early observations (NEWCOMBE and NYHOLM 1950; BERTANI 1951) that all levels of streptomycin resistance were manifested by independent mutants that arose from streptomycin-dependent cultures. It was noted that in general the *mod* and *su* mutations that occurred in *str-d-73* strain lowered the level of resistance (*mod-73* was more effective) and that conversely the auxiliary mutations occurring in *str-d-72* enhanced the level of resistance. Such a finding suggests that the primary mutation, *str-d*, has a directional effect on the type of subsequent mutational changes that occur.

Test for allelism of suppressor mutations: HASHIMOTO (1960) reported that all suppressor mutations of streptomycin dependence that were tested were either allelic or closely linked in *E. coli* B/r. Experiments were performed to determine if the mutations were linked in *S. typhimurium*. The genotypes of the parental and possible recombinant types are schematically drawn in Figure 4. Only streptomycin-sensitive recipients could be used. Resistant (or dependent) transductants were selected on BHI plus streptomycin after four hours' preincubation on BHI to allow for phenotypic expression. The colonies were tested for dependence by velveteen replication to BHI agar plates with and without streptomycin. The results are presented in Table 3. The cross between *str-d-120/*

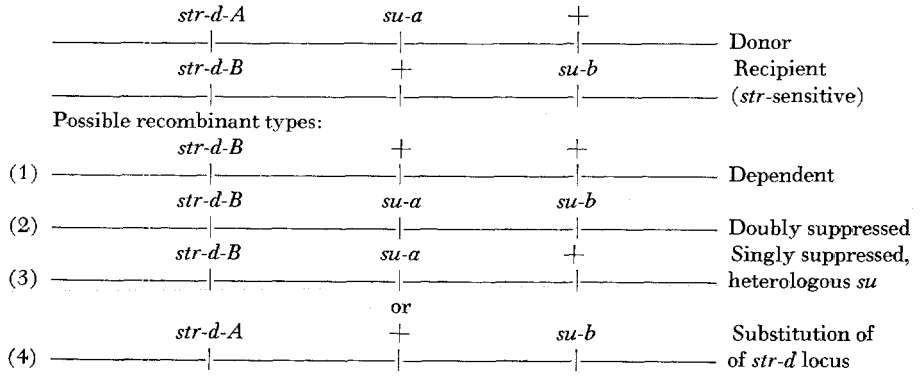

FIGURE 4.—Transductional analysis of suppressor mutations for allelism.

TABLE 3

Genetic analysis of suppressor mutations occurring in streptomycin-dependent strains of Salmonella typhimurium

Recipient		Donor		Colonies appearing on BHI + 1 mg/ml stm		Frequency of transduction
Genotype	Resistance level*	Genotype	Resistance level	Number	Phenotype	
str-d 120/ su-120-3	> 10 < 100	Homologous control		0	.....	.....
str-d 120/ su-120-3	> 10 < 100	*str-d 121/ su-121-1*	> 100 <1000	97	dependent	1×10^{-7}
str-d 120/ su-120-3	> 10 < 100	*str-d 73/ su-73-1*	> 100 <1000	12.5	dependent	3.5×10^{-9}
str-d 120/ su-120-1	> 100 <1000	Homologous control		12	resistant	.....
str-d 120/ su-120-1	> 100 <1000	*str-d 121/ su-121-1*	> 100 <1000	372	resistant	3.7×10^{-7}

* Level of stm in μg/ml that permitted growth on nutrient agar.

su-120–3 and *str-d-121/su-121–1* yielded only dependent transductants and the frequency was within the range expected if a wild-type donor had been used; this cross must be between nonallelic suppressed strains in which there was essentially no linkage between the two suppressor loci. The cross of this same recipient with *str-d-73/su-73–1* yielded only dependent colonies, but the frequency is so low that either these are spontaneous reversions of the suppressor or there is close linkage of the two suppressor mutations. The third cross between *str-d-120/su-120–1* and *str-d-121/su-121–1* yielded only resistant colonies. Since the dependent recombinant was not observed it would indicate that there were no crossovers between the two suppressor mutations. The resistant phenotype might be due to substitution of a heterologous suppressor (Figure 4(3)) or substitution of the *str-d* locus (Figure 4(4)) since the results in Table 1 indicated that the specific suppressor may alter the resistance level of the suppressed strain. In either case the data suggest that *su-120–1* and *su-121–1* are within the same locus and that it is distinct from the closely linked *su-120–3* and *su-73–1* mutations.

The occurrence of true reversions to wild type: A strain of LT-2 bearing the *str-d-72* mutation was employed as a recipient in several transduction experiments that involved the transfer of *su* mutations. The cell control plates routinely contained five or six streptomycin-independent colonies. During the course of one transduction experiment it was noted that this strain had become highly unstable as evidenced by the cell control plates, which contained hundreds of independent colonies as shown in Figure 5. Several of the many small independent colonies were tested and found to be fully resistant to streptomycin (typical for *su* mutants of *str-d-72*). The large colonies, on the other hand, constituted a new type because they were as sensitive to streptomycin as wild type. They were suspected of being true revertants and were purified by streaking and selecting isolated clones with typical morphology. When these mutable

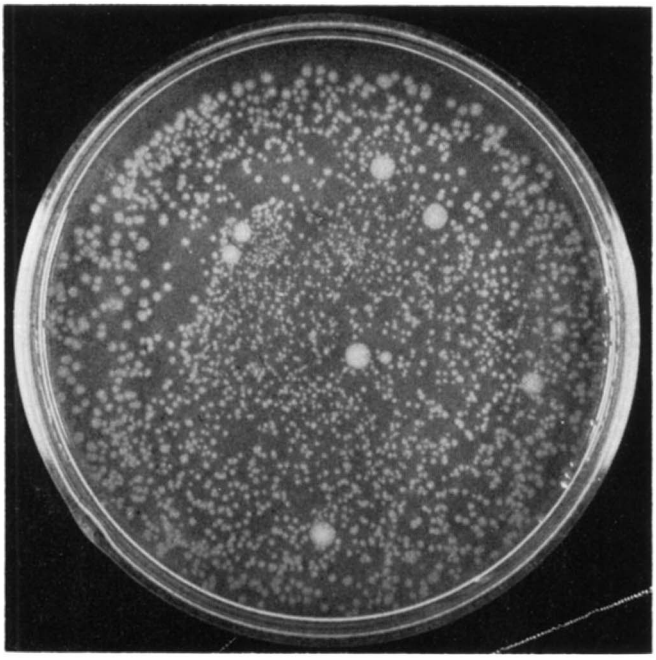

FIGURE 5.—The appearance of large revertant colonies and small suppressed colonies arising from a population of genetically unstable dependent cells on streptomycin-free medium.

sensitive cells were spread onto nutrient agar plates containing 1 mg/ml of dihydrostreptomycin sulfate many resistant colonies developed. Several of the largest colonies were found to be indifferent to streptomycin (*str-r*), i.e., grew very well in its presence or absence. This wild-type behavior was in sharp contrast to sensitive suppressed dependent strains, which gave rise exclusively to dependent mutants when plated onto streptomycin nutrient agar.

A mutable revertant strain was used in several transduction experiments. The results presented in Table 4 show that the revertant behaved exactly like wild

TABLE 4

Transduction analysis of revertants

Donor	Recipient	Transductant
Wild type	Wild type	None
'Revertant'	Wild type	None
str-d/su	Wild type	*str-d*
Wild type	*str-d*$_{72}$	Wild type*
'Revertant'	*str-d*$_{72}$	Wild type*
str-d/su	*str-d*$_{72}$	Resistant (*str-d*$_{72}$/*su*)
str-d$_{72}$	Wild type	*str-d*$_{72}$†
str-d$_{72}$	'Revertant'	*str-d*$_{72}$†

* Sensitive streptomycin-independent transductant.
† Transduction frequency of 10^{-6} per phage particle.

type in each cross. Phages produced by the revertant strain were not capable of transducing high-level streptomycin resistance or dependence but rather were capable of transducing dependent cells to wild type. Revertant cells were transduced to dependence with the same frequency as wild-type recipient cells.

Dependent mutations *str-d-100*, *str-d-107*, and *str-d-111* were isolated in the mutable version of LT-7. Populations of each dependent isolate gave rise to large streptomycin-independent mutant colonies when sufficient numbers of cells were plated on nutrient agar devoid of the antibiotic. These colonies were purified and judged to be revertants on the basis that, like wild type, they gave rise to the complete spectrum of high-level streptomycin-resistant mutants.

Large revertant-type mutants have not been observed to arise from stable dependent populations, even following treatment with either ultraviolet light or $MnCl_2$ under conditions that permitted the development of induced *su* mutants.

Apparently true reversions from streptomycin dependence to wild type do occur but they seem to be confined to unstable dependent strains that carry an active mutator gene.

DISCUSSION

The single-step mutation in *S. typhimurium* to high-level streptomycin dependence was found to confer a requirement for methionine as well as streptomycin. The pleiotropic nature of this mutation was first suggested by NEWCOMBE and NYHOLM (1950). They reported that streptomycin-dependent mutants of *E. coli* K-12 seemed to have growth factor requirements other than the antibiotic and that this might be influenced by a modifier gene. Modifier mutations in the present study eliminated the requirement for methionine and reduced, by tenfold, the amount of streptomycin needed for growth. The pleiotropic nature of mutations to an intermediate level of streptomycin resistance in *S. typhimurium*. No recombination between dependent alleles was observed. In this case concomitant deficiencies for thiamine and nicotinic acid were noted. The discovery of a methionine locus near the high-level streptomycin-resistant/dependent region in *E. coli* K-12 (MATNEY, MCDONALD and GOLDSCHMIDT 1962), tempts one to consider the primary mutation to streptomycin dependence as being a large overlapping alteration.

Further evidence of the multisite nature of the primary dependent mutation stems from the inability to obtain wild-type recombinants in crosses involving dependent mutations of discrete origin. HASHIMOTO (1960) cross-transduced six dependent strains of *E. coli*, and we have performed similar experiments with *S. typhimurium*. No recombination between dependent alleles was observed. In addition, the fact that no appreciable number of wild-type recombinants have been obtained in crosses between *str-r* and *str-d* (NEWCOMBE and NYHOLM 1950; HASHIMOTO 1960) attests both to the allelic nature of these mutational types and to their multisite nature.

Neither modifier nor suppressor mutations appeared to be linked to the *str* locus in *S. typhimurium*. Two separable suppressor loci were found. These results are quite different from the genetic analyses performed by HASHIMOTO

(1960) with *E. coli* B/r and the P1 transducing phage. He found one suppressor locus that was linked to *str*. Whether this discrepancy is due to different suppressor loci in the two bacterial systems or to the size of the genetic information transduced by the two phages (GLANVILLE and DEMEREC 1960) is not certain.

Finally, the question has been raised as to whether the *str-d* mutation may be a deletion, since all streptomycin sensitive independent mutants of genetically stable dependent strains were found to be due to suppressor mutations, both in the present study and in *E. coli* B/r (HASHIMOTO 1960). However, the appearance of true reversions to wild type in genetically unstable dependent strains of *S. typhimurium* tends to eliminate the possibility of the *str-d* mutation being a deletion.

SUMMARY

The mutation to streptomycin dependence in *Salmonella typhimurium* has a pleiotropic effect resulting in a requirement for methionine as well as streptomycin. Modifier mutations at another locus abolish the requirement for methionine and reduce the requirement for streptomycin. Suppressor mutations eliminate the requirement for both methionine and streptomycin. The level of streptomycin resistance displayed by a suppressed independent strain is determined by the interactions among the specific dependence mutation, modifier mutation, and suppressor mutation. No reversions to true wild type were observed with any genetically stable dependent strain. Dependent strains that were unstable, presumably because of a mutator gene, were observed to yield apparently true reversions to wild type in four different strains. Neither the modifier nor the suppressor mutations were closely linked to the streptomycin dependence locus. At least two different unlinked suppressor loci were detected.

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