DOMINANCE OF STREPTOMYCIN SENSITIVITY OVER DEPENDENCE IN *ESCHERICHIA COLI* K12 MERODIPLOIDS[1]

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TWO kinds of mutations are known which affect bacterial sensitivity to streptomycin: those conferring resistance to and others which confer dependence upon the antibiotic. Genetic analysis of such mutants from *Escherichia coli* has been confined to recombinational analysis by conjugation (NEWCOMBE and NYHOLM 1950) and by transduction (HASHIMOTO 1960). By means of both types of tests, the existence of multiple alleles at a single *str A* locus has been inferred, but remains unconfirmed by complementation tests.

Studies of the dominance-recessiveness relationships among *str A* alleles as well as complementation tests have been hampered due to difficulties found in constructing stable merodiploids carrying pairs of *str A* alleles. Stable merodiploids remain the best system amenable for this kind of study.

Several workers have shown dominance of the wild-type, streptomycin-sensitive allele (*str* A^+), over resistant ones (LEDERBERG 1951; APIRION and SCHLESSINGER 1968b; SPARLING *et al.* 1968). Recent work of this type has been performed with the aid of stable F-merogenotes carrying the *str A* gene (APIRION and SCHLESSINGER 1968b; SPARLING *et al.* 1968).

We have continued the genetic analysis of the *str A* mutations of *E. coli*. With the aid of KLF41, an F-merogenote constructed by Low (1968), and of mutant episomes derived from it, we have been able to construct merodiploids homozygous and heterozygous for *str A* alleles. Here we describe results which show that alleles conferring streptomycin sensitivity and dependence can coexist inside the same cell and that sensitivity is dominant over dependence.

MATERIALS AND METHODS

Bacterial strains: All strains are derivatives of *E. coli* K12 (Table 1). Strain KLF41/JC1553 was generously provided by DR. BROOKS LOW and is termed here MX2. KLF41 spans approximately the chromosomal region from min 60 to 66 of the standard *E. coli* map (TAYLOR and TROTTER 1967), a segment at least encompassing *arg G* on one side and *mal A* on the other (Figure 1). It has been proposed that part of this region determines the structure of the 30S ribosomal subunit (LEBOY, COX and FLAKS 1964).

To prepare stable merodiploids for *str A* genes it was necessary that the F^- recipients carry one or more recessive mutations which can be complemented by dominant, wild-type genes present in KLF41. Since this F-merogenote carries the *arg* $G+$ gene it seemed expedient to start with an F^- strain carrying the *arg* G^- *str* $A+$ loci. Strain W3350 was subjected to two successive

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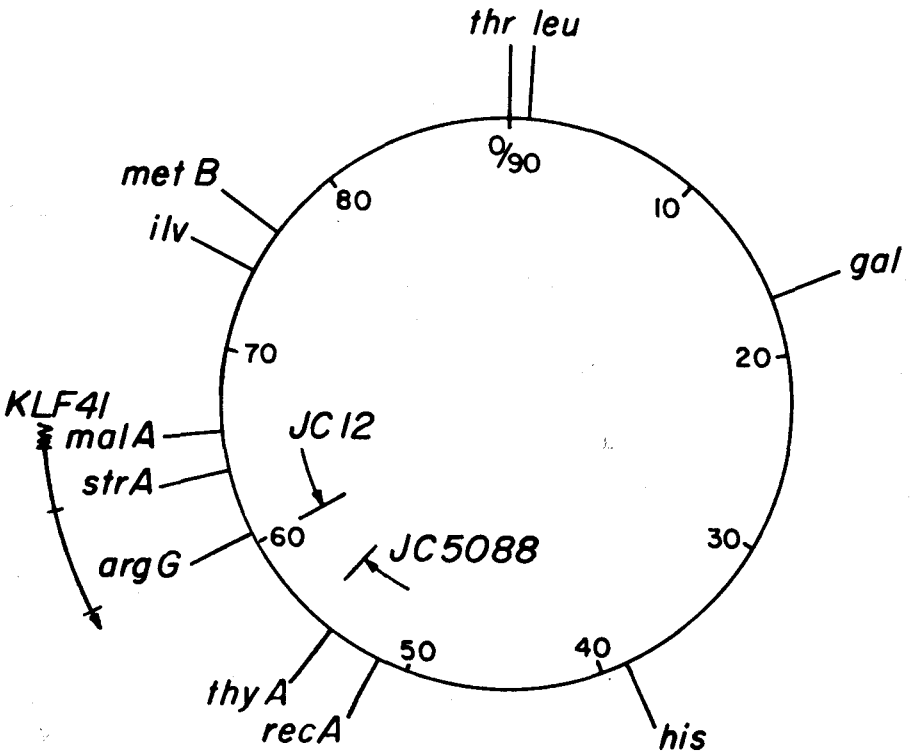

FIGURE 1.—Genetic map of *E. coli* showing location of relevant genes, points of origin of Hfr strains used as indicated by the inner arrows, and the approximate length of the F′ factor, KLF41 (from TAYLOR and TROTTER 1967 and Low 1968).

treatments with the mutagen ethyl methanesulfonate and strain MX30 (*leu-26 arg G31*) isolated. The *arg G* mutant was selected among all *arg* mutants isolated by its ability to mother merodiploid progeny when mated with a KLF41 male in plates selective for Arg+ offspring. That the mutation we were dealing with was probably in *arg G* was also supported by the inability of MX30 to grow in minimal medium supplemented with ornithine or citrulline in place of arginine. Furthermore, we made sure we were not dealing with an *arg G* mutation phenotypically suppressible by streptomycin (GORINI and KATAJA 1964, 1965), since if it were, it would render impossible recognition and handling of Arg+ merodiploid progeny in streptomycin-containing media after crosses involving MX30 or its *str A* derivatives. The *arg G31* mutation was found not to be suppressed by streptomycin in the *str A*+ strain MX30 nor in a number of Sm^R and Sm^D mutants derived from it.

The Sm^D mutants used as recipients in the crosses with KLF41 males to be described were isolated after independent treatments of strain MX30 with the mutagen N-methyl-N′-nitro-N-nitrosoguanidine (ADELBERG, MANDEL and CHEN 1965).

Construction of MX55. For the Rec+ strain MX32, a nearly isogenic Rec− derivative was required. The procedure employed to obtain it involved isolation of a Thy− derivative using the trimethoprim method (STACEY and SIMSON 1965). This was followed by mating of the Thy− Rec+ mutant obtained with Hfr JC5088 which transfers *thy*+ and its *rec A56* mutation early in conjugation; consequently these were inherited with a high degree of linkage. One Thy+ Rec− (UV-sensitive) derivative termed MX55 was isolated, purified, and tested for inheritance of female markers such as Gal− Leu− Arg− and Sm^D.

Nomenclature: Symbols for genotypes and phenotypes follow the recommendations of DEMEREC *et al.* (1966). The only deviation from conventional nomenclature is the use here of the phenotypic symbols Sm^R and Sm^D inserted as (*r*) and (*d*), respectively, into the genotypic designations of *str A* mutations. This helps to identify immediately the phenotype conferred by these alleles in the haploid cells in which they were originally isolated. All F-merogenotes described here are termed after the original KLF41 regardless of any genotypic changes in its linked markers. Allele numbers for new mutations were assigned from a block kindly allotted to us by DR. BARBARA BACHMANN of Yale University.

Media and mating conditions: The media and culture methods used were those described by ADELBERG and BURNS (1960). For broth crosses MX2 cells were grown overnight in liquid minimal medium devoid of arginine. This was diluted 1:50 in L broth, incubated at 37°C to mid-log phase and 1 ml mixed with 9 ml of the recipient cells in complex media. The proportion of the populations which retained KLF41 at the time of mating was always checked. Matings in L broth were performed using a male to female ratio of 1:10 at a total cell concentration of about 2×10^8 cells/ml. Interruption of matings were carried out with the aid of a vibratory device of the type described by Low and WOOD (1965). All other conditions for mating in broth or in plates have been described previously (BASTARRACHEA and CLARK 1968).

Purification of strains. Strains were purified by at least two cycles of single colony isolations on the same minimal medium in which they arose. Haploid strains were maintained on L slants with or without streptomycin, as required. All KLF41 merodiploids were maintained on slants of the appropriate minimal medium devoid of arginine.

Scoring of the streptomycin phenotype: Progeny colonies from conjugation experiments were picked with sterile toothpicks and patched on master plates of the same medium on which they arose. After they grew, they were replica-plated on the same minimal medium with and without streptomycin.

Streptomycin-sensitive colonies were identified by their ability to grow only on plates devoid of streptomycin. For scoring the phenotype as resistant or dependent, the master plates were replicated as above. Dependent clones showed heavier growth on the streptomycin plates than on plates without it, while resistant clones grew equally well on both plates. Plates devoid of streptomycin showing normal growth of resistant clones and less confluent residual growth of the dependents were used to replicate new plates with and without streptomycin. Upon incubation, resistant clones grew equally well on both plates while dependent ones grew only sporadically on streptomycin plates and not at all on plates lacking streptomycin.

A more sensitive alternative test consisted of inoculating 10^4 to 10^5 cells into 6–7 ml of L broth with and without 100 μg/ml of streptomycin. Even after prolonged incubation, sensitive clones only grew to apparent turbidity ($\geqq 10^7$ cells/ml) in media without streptomycin. Resistant clones grew in both media and dependent ones only grew in media with streptomycin.

Acridine orange curing: This was done as previously described (HIROTA 1960). To prepare inocula for those experiments, KLF41 merodiploid strains were grown overnight in the appropriate liquid minimal medium devoid of arginine, to reduce the number of cells which had lost KLF41 spontaneously. Unless otherwise stated, acridine orange (obtained from Eastman Organic Chemicals) was used at a final concentration of 25 μg/ml for curing Rec+ merodiploids and at 10 μg/ml for curing Rec⁻ merodiploids (BASTARRACHEA and WILLETTS 1968). At the end of the incubation period the cultures were diluted and spread onto appropriate plates. These were L plates with or without streptomycin, and minimal medium plates either with or without arginine and streptomycin singly and in combination. Colonies were patched on master plates of the same medium on which they were isolated and replicated, when applicable, on differential plates for scoring their responses to the presence or absence of arginine or streptomycin and for their ability to sexduce F⁻ *arg G*⁻ cells.

MS2 phage sensitivity: The male- specific bacteriophage MS2 was propagated and assayed on the double male strain JC182 (CLARK 1963) using the media and conditions described by DAVIS and SINSHEIMER (1963). Sm^S and Sm^R strains were tested for maleness by mixing in top agar 0.1 ml of an MS2 suspension (1000 plaque-forming units) with 0.1 ml of an overnight

bacterial culture. After pouring the suspensions on MS plates, these were incubated overnight to allow development of plaques.

Concentrations of streptomycin > 50 μg/ml strongly inhibited MS2 infection of Sm^D strains (BROCK 1962). Therefore, after growth overnight on streptomycin medium, Sm^D cells were mixed with phage, poured on plates without streptomycin, and incubated under conditions of streptomycin depletion.

Transduction tests: These were made using bacteriophage P1 *vir a* (WILLETTS *et al.* 1969). Plate lysates were made according to WILLETTS *et al.* (1969) except that for streptomycin-dependent strains the plates contained 100 μg/ml of streptomycin and the $CaCl_2$ concentration was raised to 10^{-2} M. Transductions were carried out at multiplicities of infection of 0.05. Details for growth of the recipients, phage adsorption, and the method for selecting transductants were those described by HASHIMOTO (1960).

Other methods: Rec⁻ strains were identified by both their inability to mother conjugational Arg+ recombinants when mated with Hfr strain JC12 and by their UV-sensitivity. These tests were performed by the replica-plating techniques described by CLARK and MARGULIES (1965).

RESULTS

Most of the data presented in this paper were collected from two main crosses: one between the KLF41-harboring strain MX2, and the F⁻ Sm^D Rec^+ strain MX32 and the other between MX2 and MX55, a *rec A56* mutant derivative of MX32 (Table 1).

One advantage of using MX2 as donor lies in its Rec⁻ phenotype. MX2, being a KLF41 *str* A^+/*str A(r)309* diploid, transfers its autonomous F-merogenote but does not undergo donor recombination (CLOWES and MOODY 1966) necessary for mobilization of chromosomal markers (SCAIFE and GROSS 1963). Therefore, in

TABLE 1

Bacterial strains used

Strain	Relevant genotypic characteristics										Sm phenotype	Mating type
	KLF41		Chromosome									
	argG	strA	argG	strA	metB	ilv	thr	leu	gal	his	recA		
MX2	+	+	6	309	1	+	+	2	6	1	1	Sm^S	F-prime
MX17			6	309	1	+	+	+	+	1	1	Sm^R	F⁻
W3350			+	+	+	+	+	+	1,2	+	+	Sm^S	F⁻
MX30			31	+	+	+	+	26	1,2	+	+	Sm^S	F⁻
MX32			31	900	+	+	+	26	1,2	+	+	Sm^D	F⁻
MX50	+	+	31	900	+	+	+	26	1,2	+	+	Sm^S	F-prime
MX52	+	del	31	900	+	+	+	26	1,2	+	+	Sm^D	F-prime
MX55			31	900	+	+	+	26	1,2	+	56	Sm^D	F⁻
MX56	+	+	31	900	+	+	+	26	1,2	+	56	Sm^S	F-prime
MX57	+	del	31	900	+	+	+	26	1,2	+	56	Sm^D	F-prime
JC12			+	+	1	+	+	+	6	+	+	Sm^S	Hfr
JC5088			+	+	+	318	300	+	+	+	56	Sm^S	Hfr

Genotypic symbols are those recommended by TAYLOR and TROTTER (1967).

The *strA309* allele confers to haploid cells the ability to resist $\geqq 1000$ μg/ml of streptomycin. Sm^D strains carrying the *strA900* allele grow similarly well in the presence of 50–1000 μg/ml of streptomycin; 10 μg/ml not being sufficient to support full growth.

crosses involving MX2 as a donor, only the *str* A^+ allele on the F-merogenote is transferred.

Cross MX2 × MX32. KLF41 was transferred from MX2 to the F^- Sm^D Rec^+ recipient MX32 in a standard conjugation experiment. After a 2 hr mating, the conjugation was interrupted and the suspension diluted and plated. Selection was applied for Arg^+ [His^+ Met^+] progeny using minimal agar plates with and without streptomycin. Arg^+ colonies which arose on the plates were patched on the same minimal medium in which they were isolated. After 48 hrs of incubation, when growth was well developed, they were replica-plated on minimal medium plates with arginine or streptomycin, with both and with neither, and on minimal plates containing lawns of the F^- Sm^R Rec^- strain MX17, selective for Arg^+ [Gal^+ Leu^+] and for Arg^+ [Sm^R Gal^+ Leu^+] merodiploid progeny. This allowed us to confirm their phenotype towards arginine and streptomycin and to know whether or not they harbored F-merogenotes carrying *arg* G^+ and *str* A^+. Since we did not know whether both alleles conferring Sm^S and Sm^D could coexist inside the same cell, and if so, which was dominant over the other, we reasoned that heterozygous sensitive/dependent merodiploids having the genotype of the primary zygotes should be the most frequent class isolated. As shown below, this reasoning proved to be incorrect.

As seen in Table 2, the number of colonies isolated in streptomycin-containing plates (and eventually identified as Sm^D) was 103-fold higher than that obtained in media devoid of streptomycin. It was also found that of all Sm^D clones tested, 89.4% were not donors and probably were Arg^+ Sm^D haploid recombinants. The remaining 10.6% were *arg* G^+ donors to MX17. All these donors, one of which was purified and termed MX52, transferred their F-merogenotes to MX17 recipients in media selective for Arg^+ [Gal^+ Leu^+] progeny both with and without streptomycin. It was further found that all of these merodiploid progeny were

TABLE 2

Genetic analysis of the crosses MX2 × MX32 and MX2 × MX55

Cross	Selection	Per cent frequency of conjugants	Number of colonies tested for unselected markers	Per cent frequency of unselected markers			
				Sm^S	Sm^D	Donors of $argG^+$ to MX17 -Sm	Donors of $argG^+$ to MX17 +Sm
MX2 X MX32	Arg^+ [His^+ Met^+]	1.7×10^{-1}	850	100		23	0
MX2 X MX32	Arg^+ [His^+ Met^+ Sm^R]	17.5	1697		100	10.6	10.6
MX2 X MX55	Arg^+ [His^+ Met^+]	1.2×10^{-1}	400	100		100	0
MX2 X MX55	Arg^+ [His^+ Met^+ Sm^R]	2.4×10^{-2}	400		100	100	100

MX2 was grown in L broth for about 10 generations before mating. The frequency of spontaneous haploid segregants in these cultures was always around 0.02%. MX32 and MX55 were grown in L broth plus streptomycin. When these cultures reached densities of about 2×10^8 cells/ml, they were centrifuged, washed once and resuspended in the same initial volume of prewarmed L broth. They were immediately mixed with male cultures in adequate proportions and mated for 2 hrs.

In plate matings to ascertain ability to transfer *argG*+ to MX17, counterselection for Gal^+ Leu^+ was always applied.

Sm^R and donors to MX30 in plates selective for Arg^+ [His^+ Met^+] progeny. If F-merogenotes of the MX52 type had carried the *str* A^+ gene, then *arg* G^+ transfer to MX17 should have taken place only in media devoid of streptomycin. Moreover, it would have been expected that all progeny from such crosses would be Sm^S due to the dominance of *str* A^+ in heterozygous *str* A^+/*str A(r)309* diploids. Therefore, genotypically Arg^+ Sm^D clones from the cross MX2 × MX32 which are *arg* G^+ donors to MX17 must be either: (a) KLF41 *arg* G^+ *str A del/ arg G31* (or *arg* G^+) *str A(d)900* hemizygous for the *str A* gene, or (b) homozygous KLF41 *arg* G^+ *str A(d)900/arg G31* (or *arg* G^+) *str A(d)900*, in which case streptomycin dependence would be recessive to resistance as shown by the Sm^R phenotype of all the progeny obtained after crossing these donors with MX17.

To decide between these alternatives, two Arg^+ Sm^R clones from a cross between MX52 × MX17 were purified (MX60 and MX61) and used to transduce the *arg G31 str* A^+ strain MX30. If MX52 were diploid for *str A(d)900*, then MX60 and MX61 would be heterozygous *str A(d)900/str A(r)309* and consequently their P1 lysates should transduce both Sm^R and Sm^D to MX30. Alternatively, if MX52 were hemizygous for *str A(d)900* and the *str A* region of its F-merogenote were deleted, then MX60 and MX61 should carry only the *str A(r) 309* chromosomal gene. Consequently, P1 should transduce only the Sm^R phenotype. The results showed that P1 lysates from MX60 and MX61 only transduced Sm^R to MX30. Moreover, Arg^+ (the F-merogenote marker *arg* G^+ of MX60 and MX61) was also transduced to MX30, but as expected, unlinked to Sm^R. Under similar conditions, control P1 lysates made on MX52 transduced Sm^D but not Sm^R to MX30. This and additional evidence supports the genotype KLF41 *arg* G^+ *str A del/arg G31 str (d)900* for MX52.

str A *genotype of F-merogenote in sensitive/dependent heterodiploids:* Direct evidence for dominance of *str* A^+ over *str A(d)900* came from the analysis of the Arg^+ Sm^S offspring obtained from the original cross MX2 × MX32. All Arg^+ colonies arising on selective plates without streptomycin were Sm^S, and of these 23% were found to transfer *arg* G^+ to the *arg G6* recipient MX17. This transfer, however, took place only in media devoid of streptomycin indicating that the *str* A^+ allele was present in their F-merogenotes since the KLF41 *str* A^+/*str A (r)309* zygotes which should have been formed were killed in the presence of streptomycin due to dominance of the *str* A^+ allele. Confirmation for this conclusion came when it was shown that the progeny obtained on selective media without streptomycin after mating the Arg^+ Sm^S donors with MX17 were all Sm^S. On the other hand, all Arg^+ Sm^S, not donors to MX17, were considered haploid recombinants. This, however, is probably an oversimplification since F-merodiploids arising by recombination carrying the recessive *arg G31* allele in the F-merogenote could have been formed, but were overlooked by our screening procedures.

str A *genotype of chromosome in sensitive/dependent heterodiploids:* To demonstrate existence of the dependent allele in the chromosome of the heterodiploids, 26 Arg^+ Sm^S colonies which were donors of *arg* G^+ to MX17 were tested for their ability to segregate cells able to grow in the presence of strepto-

mycin. Twenty-one of the clones readily segregated such progeny at frequencies from 10^{-4} to 10^{-5}, well above the frequency of 10^{-9} for spontaneous mutation of *str* A^+ to *str* A^-. Upon further testing all segregant clones selected in the presence of streptomycin were found to be Sm^D. Therefore, these 21 clones which were Sm^S and did segregate Sm^D cells most probably represent heterozygous KLF41 *str* A^+/*str A(d)900* merodiploids. Consequently, streptomycin sensitivity is dominant over dependence. One of these was purified and termed MX50. The remaining 5 *arg* G^+ donor clones did not segregate Sm^D cells and were regarded as homozygous sensitive merodiploids.

Other observations relevant to the cross MX2 × MX32 have also been made. Thus, the low frequency of Sm^S offspring obtained may be related to the lack of proportionality noticed in the number of Arg^+ colonies recovered in minimal plates without streptomycin. It was consistently found that as the dilution went 10-fold higher—i.e., from 10^{-1} to 10^{-2}, the number of colonies recovered from the 10^{-2} dilution were not one tenth of those obtained at 10^{-1}, but were about one-thousandth as many. This indicates that the KLF41 *str* A^+/*str A(d)900* zygotes which should have been formed have difficulty in outgrowing into colonies. After primary growth most of these colonies could be serially transferred without serious difficulty. This lack of proportionality also indicates that the frequency number of Sm^S offspring appearing in Table 2, which is calculated from the number of colonies scored at the lowest dilution plated (10^{-1}), may be small by virtue of this property.

A priori it might appear as if the amount of streptomycin bound to the recipient cells prior to mating would affect the viability of the Sm^S zygotes formed. Instead of this, a beneficial cell-crowding effect was noticed. A number of tests such as (a) streptomycin starvation of the Sm^D recipients prior to mating, (b) incubation of the selection plates to allow growth of the merozygotes under anaerobic conditions or (c) plating of samples of the mating mixture at intervals between 0 and 4 hrs of incubation, did not help to achieve proportionality in the number of colonies recovered with increasing dilution of the mating mixture, but rather (a) and (b) treatments decreased their overall frequency.

While the reason for this effect remains unknown, it is noteworthy that the residual streptomycin bound to the recipients is not bactericidal, otherwise no stable heterozygotes could be isolated. One possible explanation could be that the dominant Sm^S phenotype is not manifested early enough after zygote formation but only after cell division proceeds and the streptomycin molecules are diluted out in the sister cells. In pneumococcus transformants the Sm^R phenotype is completely manifested only after 180 min of DNA fixation (EPHRUSSI-TAYLOR 1962).

Heterozygous KLF41 *arg* G^+ *str* A^+/*arg G31 str A(d)900* merodiploids such as MX50 continually segregate haploid cells of the parental type MX32. In addition, recombinational events between homologous regions of the F-merogenote and the chromosome can give rise to: (a) homozygous segregants containing a recombinant F-merogenote or a recombinant chromosome, (b) gene transposition, (c) recombinant segregants arising by recombination followed by segrega-

tion, or (d) insertion of the circular F-merogenote into the chromosome resulting in the formation of Hfr merodiploids. Recombinational events involving homologous crossing over do not occur in merodiploids carrying *rec A* mutations (Low, 1968). On the other hand, deletion of genes in the F-merogenote or in the chromosome, possibly mediated by gene products other than that of *rec A* (FRANKLIN 1967; INSELBURG 1967), result in merodiploids hemizygous for previously diploid markers. Most of the aforementioned classes of segregants have been found to occur in cultures of MX50. A detailed study on the nature of these genetic interactions, however, is beyond the scope of this paper.

Cross MX2 × MX55: In order to circumvent the difficulties created by the genetic events mediated by products of the *rec A* gene that occur in the zygotes from the cross MX2 × MX32, a cross between MX2 and MX55, a *rec A56* mutant derivative of MX32 was performed. The results of this cross are also given in Table 2 to facilitate comparison with those obtained in the cross MX2 × MX32. It was predicted that in plates selective for Arg$^+$ [His$^+$ Met$^+$] merodiploid progeny, one class should be preferentially isolated; namely, heterozygous merodiploids carrying *arg G*$^+$ and *str A*$^+$ in the F-merogenote and *arg G31 str A(d)900* in the chromosome. Consequently, most if not all progeny should be donors of *arg G*$^+$ to MX17 only in media without streptomycin, selective for Arg$^+$ [Gal$^+$ Leu$^+$] offspring. As shown in the lower part of Table 2, this is the result which was obtained. One of these merodiploids was purified and termed MX56. These results, together with those showing: (a) that the progeny obtained after crossing merodiploids of the MX56 type with MX17 were Sm^S, and (b) that MX56 segregates haploid cells with the recessive Sm^D phenotype (see below), confirmed unambigously the dominance of streptomycin sensitivity over dependence. A lack of proportionality similar to that observed during selection of Arg$^+$ [Gal$^+$ Leu$^+$] in the cross MX2 × MX32 was observed in the cross MX2 × MX55.

For the merodiploid progeny expected in plates selective for Arg$^+$ [His$^+$ Met$^+$ Sm^D] offspring, we predicted that its frequency should be much lower than that obtained from the cross MX2 × MX32 since most recombinational events occurring in the zygotes from the latter cross would not have occurred in those from the cross MX2 × MX55. Therefore, most if not all Arg$^+$ Sm^D progeny should result from deletions in the *str A* region of the F-merogenote and 100 percent of such progeny should be *arg G*$^+$ donors to MX17 in plates selective for either Arg$^+$ [Gal$^+$ Leu$^+$] or Arg$^+$ [Gal$^+$ Leu$^+$ Sm^R] offspring. As summarized in Table 2, the findings fitted nicely with the predictions. One of the isolated merodiploids was purified and termed MX57. This evidence, along with the facts that (a) all Arg$^+$ progeny obtained by mating merodiploids of the MX57 type with MX17 were identified by replica-plating as Sm^R, and (b) that MX57 segregates haploid cells of the MX55 parental type are in favor of a genetic constitution of the type KLF41 *arg G*$^+$ *str A del/arg G31 str A(d)900* for the Arg$^+$ Sm^D merodiploids arising from the cross MX2 × MX55. These findings also add support to the current notion of deletion events occurring independently of a functional *rec A* gene.

TABLE 3

Growth response of merodiploid strains to L broth of pH 7.8 with and without acridine orange

Strain	Derivation	Genotype F-merogenote/chromosome	Supplements to L broth None	AO	Sm	Sm+AO
MX2	JC12 x JC1553	$argG^+$ $strA^+$/argG6 strA(r)309	20	48	22	48
MX50	MX2 x MX32	$argG^+$ $strA^+$/argG31 strA(d)900	70	40	40	70
MX52	MX2 x MX32	$argG^+$ strA del/argG31 strA(d)900	--	--	20	20
MX56	MX2 x MX55	$argG^+$ $strA^+$/argG31 strA(d)900	96	--	48	72
MX57	MX2 x MX55	$argG^+$ strA del/argG31 strA(d)900	--	--	48	48

Acridine orange (AO) was added at final concentrations of 25 μg/ml for the Rec^+ strains and at 10 μg/ml for Rec^- strains. Streptomycin (Sm) was added at a final concentration of 100 μg/ml. The inocula used were about 10^4 cells/ml from overnight cultures in minimal medium. The numbers indicate approximately the hours at which cell densities of 10^9/ml were reached. Absence of growth was recorded after 90 hrs of incubation.

Acridine orange elimination of KLF41 merogenotes: Merodiploid strains representing most of the typical classes isolated and characterized from the crosses previously described were analyzed for their response to acridine orange. For comparison, the behavior of the parental strain MX2 was also determined. The results obtained are given in Tables 3 and 4.

The sensitive-resistant heterozygote MX2, developed growth on all four media employed; acridine orange efficiently eliminated KLF41 in L broth. L plus streptomycin medium was selective for cells that lost their F-merogenotes and for those carrying F-merogenotes that lacked part or all of the *str A* region. In the presence of both streptomycin and acridine orange 100% of the survivors had lost KLF41.

The two sensitive/dependent heterodiploids MX50 and MX56 behaved similarly in that they grew slower in L broth than in minimal broth and grew under stationary conditions but not under shaking. One possible explanation, among others, would be that in these strains spontaneous loss of KLF41 occurs more rapidly either in complex medium or under strongly aerobic conditions, the Sm^D segregants being unable to survive and multiply in the absence of streptomycin.

For MX50 (*rec* A^+) cells, the addition of acridine orange to L broth caused selection of haploid segregants carrying a recombinant *str* A^+ gene. Since no selection pressure was exerted, no Arg^+ recombinants were found. Under the same conditions, MX56 (*rec A56*) cells did not yield recombinants and consequently no survivors were found.

In L plus streptomycin medium, MX50 heterodiploids yielded mostly haploid segregants but these were the only permissive survivors in MX56 cultures. A possible curing effect of acridine orange on MX50 and MX56 in L broth containing streptomycin was obscured since the very presence of streptomycin was sufficient to select haploid segregants. In the *rec* A^+ strain MX50, acridine orange apparently stimulated recombination between chromosome and F-merogenote,

TABLE 4

Characterization of populations obtained after treatment of merodiploids with acridine orange

Strain	Populations from experiment in Table 3		Percentages of most probable genotypes	Per cent curing deduced
MX2	L	100	KLF41 argG$^+$ strA$^+$/argG6 strA(r)309	
	L+AO	4	KLF41 argG$^+$ strA$^+$/argG6 strA(r)309	
		96	haploid argG6 strA(r)309	96
	L+Sm	42	KLF41 argG$^+$ strA del/argG6 strA(r)309	
		58	haploid argG6 strA(r)309	
	L+Sm+AO	100	haploid argG6 strA(r)309	a
MX50	L	76	KLF41 argG$^+$ strA$^+$/argG31 strA(d)900	
		24	haploid argG$^+$ strA$^+$	
	L+AO	100	haploid argG31 strA$^+$	b
	L+Sm	12	KLF41 argG$^+$ strA(d)900 (or strA del)/argG31 strA(d)900	
		88	haploid argG31 strA(d)900	
	L+Sm+AO	28	KLF41 argG$^+$ strA(d)900 (or strA del)/argG31 strA(d)900	
		54	haploid argG31 strA(d)900	
		18	haploid argG$^+$ strA(d)900	c
MX52	L+Sm	97	KLF41 argG$^+$ strA del/argG31 strA(d)900	
		3	haploid argG31 strA(d)900	
	L+Sm+AO	91	KLF41 argG$^+$ strA del/argG31 strA(d)900	
		9	haploid argG31 strA(d)900	6
MX56	L	100	KLF41 argG$^+$ strA$^+$/argG31 strA(d)900	
	L+Sm	100	haploid argG31 strA(d)900	a
	L+Sm+AO	100	haploid argG31 strA(d)900	a
MX57	L+Sm	98	KLF41 argG$^+$ strA del/argG31 strA(d)900	
		2	haploid argG31 strA(d)900	
	L+Sm+AO	99	KLF41 argG$^+$ strA del/argG31 strA(d)900	
		1	haploid argG31 strA(d)900	0

Cultures from experiments in Table 3 were diluted and spread over L plates with or without streptomycin, as required. In every case, at least 200 colonies were patched on plates of the same medium and after they grew, were replica-plated on minimal medium plates to score their response to the presence and absence of arginine or streptomycin. They were also mated with MX17 cells on plates selective for Arg+ [Gal+ Leu+] or Arg+ [Gal+ Leu+ Sm^R] merodiploid progeny.

[a] Selection of haploid segregants, not curing.

[b] Curing and/or selection preceded by recombination.

[c] Segregation preceded by recombination (see text).

an effect that has been previously noticed (BASTARRACHEA and WILLETTS 1968). In fact, it cannot be stated that *strA*+ recombinants isolated after treating MX50 cells with acridine orange in L broth are not due to the same effect.

Strains MX52 and MX57 only grew in L broth plus streptomycin. Under these conditions the level of spontaneous haploid segregants was low, of the order of 2 to 9%. The addition of acridine orange to this medium (25 μg/ml for the *rec A*+ strain MX52 and 10 μg/ml for the *rec A56* strain MX57) did not cure these cells of their KLF41 merogenotes (Table 4). Experiments not shown in the tables revealed that streptomycin (100 μg/ml) did not allow curing of KLF41 even when the acridine orange concentration was raised up to 100 μg/ml for MX52 and 25 μg/ml for MX57.

The fact that concentrations of acridine orange of 200 μg/ml for the Rec^+ merodiploid MX52 and of 50 μg/ml for the Rec^- merodiploid MX57 did not allow growth of these strains in the presence of streptomycin (100 μg/ml) indicates that the antibiotic interferes with acridine orange curing but does not alter the original sensitivity of Rec^+ and Rec^- cells to the dye (BASTARRACHEA and WILLETTS 1968). These observations support the proposal that the two well-known effects of acridine orange (elimination of F-merogenotes and inhibition of cell growth) are exerted at different topological levels or at structurally distinct targets of the bacterial cell.

The results obtained in experiments with F-merodiploid Sm^R strains (BASTARRACHEA and TAM, unpublished) indicate that streptomycin completely inhibits acridine orange curing of F-merogenotes when added to cells pregrown on streptomycin, but only partially inhibits the curing of cells pregrown without streptomycin. These results suggest that the inhibition of curing by acridine orange is an effect due to the streptomycin molecules on the cells exposed to it, independent of the *str A* genotype.

MS2 sensitivity of KLF41 merodiploids: Characteristic maleness of the merodiploid strains MX50, MX52, MX56 and MX57 was also confirmed by demonstrating their sensitivity to the male-specific bacteriophage, MS2. This is in contrast to the resistance to MS2 exhibited by F^- strains of *E. coli* K12 such as MX30 or MX17 (Table 1).

Other crosses: MX33 and MX37, two additional Sm^D strains isolated by independent treatments of MX30 with nitrosoguanidine were also used as recipients in crosses with MX2. The results from such crosses as to frequencies of Arg^+ transfer and the quality of the progenies obtained were very similar to those described for the cross MX2 × MX32.

DISCUSSION

We have described results which show that after matings between appropriate male and female strains of *E. coli* K12, persistent heterozygous merodiploids carrying the streptomycin-sensitive allele in the F-merogenote and the streptomycin dependent allele in the chromosome can be isolated in streptomycin-free selective media.

Dominance of streptomycin sensitivity over dependence in these heterodiploids was inferred from their ability to grow and maintain their diploid state in media devoid of streptomycin. Culturing of the heterodiploids in streptomycin media resulted in selection of haploid Sm^D segregants. This was taken as proof for the existence of the streptomycin-dependent allele in the chromosome of the heterodiploids.

Proof for the existence of the streptomycin-sensitive allele in the F-merogenotes was obtained after these were transferred by conjugation to an Sm^R recipient. The resultant progeny were Sm^S, presumably as a result of dominance of the sensitive allele over the resistant one.

Attempts to cure with acridine orange the sensitive/dependent merodiploids

failed (Tables 3 and 4). This was due to the fact that in medium selective for haploid cells with the recessive Sm^D phenotype, the very presence of streptomycin was sufficient to select this kind of cell.

The main conclusion derived from these experiments, i.e., that streptomycin sensitivity is dominant over dependence has also been supported in a recent paper (BRECKENRIDGE and GORINI 1969).

The criterion used throughout this paper to establish the dominance of streptomycin sensitivity over dependence was based upon the inability of the heterodiploids to grow in media containing 100 μg/ml of streptomycin. BRECKENRIDGE and GORINI (1969) have recently shown that the dominance of streptomycin sensitivity over resistance in *E. coli* merodiploids is manifested as a sudden but reversible inhibition of growth. This is in contrast to the rapid cell-killing effect exerted by streptomycin in either *str* A^+ haploid cells or in homozygous *str* A^+/*str* A^+ cells. Experiments to elucidate the nature of the primary effect of *str* A^+ on cell viability in the sensitive/dependent merodiploids are hampered by the slow growth of these cells in liquid medium (Table 3; also BRECKENRIDGE and GORINI 1969).

Thus far, we have relied on the assumption that the Sm^D mutation we have studied most intensively (*str A(d)900*) occurs readily in the *str A* gene. Genetic studies by NEWCOMBE and NYHOLM (1950) and by HASHIMOTO (1960) indicated that in conjugational and transductional crosses between high level resistant and dependent *E. coli*, few, if any, streptomycin-sensitive recombinants were formed. This was interpreted as indicating that the two mutations had occurred in the same functional locus.

It is known, however, that Sm^D mutants can revert to the Sm^S or Sm^R phenotypes and that most, if not all of such revertants are due to suppressor mutations which map close to the *str A* chromosomal region (HASHIMOTO 1960; BROWNSTEIN and LEWANDOWSKI 1967; APIRION and SCHLESSINGER 1967a). Consequently, revertants of this type carry, in addition to the original *str A* mutation, a suppressor mutation which affects the same or perhaps another ribosomal protein.

If the Sm^D suppressors map outside of the *str A* gene, it is conceivable that, in our diploids, dominance of streptomycin sensitivity over dependence can be due to the expression of a gratuitous suppressor present in KLF41. We believe that this is not the case because: a) we ignore the possibility of the suppressor mutations changing the cell response to streptomycin when present simultaneously with *str* A^+ and b) the emergence of a suppressor mutation in an Sm^D strain is a rare event, of the order of 10^{-8}, and consequently the chances of its having arisen under nonselective conditions in strain JC12 from which KLF41 was isolated (Low 1968) are rather slim.

Recently, fractionation and reconstruction experiments of the bacterial ribosomal subunits carried out by OZAKI *et al.* (1969) and by BIRGE and KURLAND (personal communication), have indicated that a single structural protein of the 30S ribosomal subunit (the P10 protein of OZAKI *et al.* equivalent to the protein 15 of BIRGE and KURLAND) is responsible for either the Sm^S, Sm^R or the Sm^D phenotype and consequently that these three are allelic.

Several important questions remain to be answered before we understand the molecular basis underlying the observed dominance of streptomycin sensitivity over dependence. Among these are: (1) How many copies of the F-merogenote are made per cell in relation to those of the chromosome? (2) Have both *str A* alleles equal probabilities of being transcribed and translated into proteins? (3) Are both protein products assembled into ribosomes? If so, (4) are both kinds of ribosomes engaged in protein synthesis? Experiments are now in progress in our laboratory attempting to answer some of these questions.

In vitro studies with ribosomes isolated from streptomycin sensitive/resistant heterozygotes have led SPARLING *et al.* (1968) to conclude that ribosomes are assembled from a common precursor pool of proteins to which both *str A* alleles contribute, and also to note an excess of sensitivity over resistance. These authors, however, were cautious in inferring that such an excess could contribute to, but was not in itself sufficient to explain the dominance of sensitivity over resistance.

Mutational alterations in a number of different loci that specify proteins of the 30S subunit, including the *str A* loci (DAVIES *et al.* 1964; PESTKA *et al.* 1965), the *nek* loci (APIRION and SCHLESSINGER 1968a) and the *ram* loci (ROSSET and GORINI 1969) strongly influence the fidelity of translation of the genetic code. This is exemplified by the phenotypic suppression exerted by streptomycin on a number of mutations *in vivo* which depends upon the permissiveness of a particular *str* A^+ allele (review–GORINI and DAVIES 1968), by the restrictive effects of Sm^R mutations on the activity of nonsense suppressors (LEDERBERG *et al.* 1964; COUTURIER *et al.* 1964; GARTNER and ORIAS 1966; KUWANO *et al.* 1968; OTSUJI and AONO 1968), and by the restrictive effects of both Sm^R and Sm^D mutations on streptomycin phenotypic suppression (APIRION and SCHLESSINGER 1967b).

All these phenomena are being pursued *in vivo*, and *in vitro* with ribosomes of the different heterozygotes in order to arrive at a more coherent picture of the mechanism of action of streptomycin and of the role of the *str A* protein in the modulation of ribosome structure and function (OZAKI *et al.* 1969). An approach of this sort has recently been reported (BRECKENRIDGE and GORINI 1969).

Finally, we should mention that our present genetic analysis was not limited only to the sensitive/dependent merodiploids. We have also characterized other types of progeny obtained from the two main crosses described in RESULTS (MX2 × MX32 and MX2 × MX55). Among these progenies we observed a large number of merodiploids which apparently carried deletions at the *str A* locus of their merogenotes. In fact, all Sm^D progeny obtained after crossing MX2 with the *rec A56* recipient MX55 (Table 2, line 4) have been attributed to deletions. The frequency of occurrence of these events was of the order of $2.4 \times 10^{-2}\%$. Occurrence of such a high frequency of deletions (which is independent of *rec A* function) at the *str A* locus of the F-merogenotes, however, does not necessarily imply that similar events are normal occurrences at the chromosomal *str A* locus. Similar frequencies (<0.01%) for other possible deletions have been observed for the loss of drug-resistant traits from R factors harbored by *E. coli* (FALKOW *et al.* 1966), though in this case the cells were haploid for the R factor genes and their relationship to *rec A* functions was not investigated. This is in contrast to

the rarity (frequencies 10^{-7} to 10^{-8}) of appearance of deletions independent of the *rec A* function which are found in certain well-studied chromosomal regions such as the *trp-tonB* region (FRANKLIN 1967).

Deletions at the *str A* locus of the F-merogenotes can be sought as a result of extraordinary recombinational events other than those requiring the *rec A* products. These events can either occur as errors during replication or as single breakage-and-reunion events of DNA segments after the formation of loops.

If deletions at the chromosomal *str A* locus do not occur as frequently as those at the *str A* locus of the F-merogenotes, then we might hypothesize that perhaps they occur as errors of replication. This is an especially interesting possibility since we still know of no evidence indicating that F-mediated and chromosomal vegetative replication occur via similar mechanisms.

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SUMMARY

Persistent heterozygous Arg^+ merodiploids which carry the streptomycin-sensitive *str* A^+ allele on the F-merogenote and the streptomycin-dependent *str(d)900* allele on the chromosome were isolated from crosses between F-prime strain MX2 of the following genetic constitution: KLF41 *arg* G^+ *str* A^+/*arg G6, str A(r)309 rec A1*, and the streptomycin-dependent F^- recipient MX32, *arg G31 str A(d)900 rec* A^+. In these merodiploids, streptomycin sensitivity is dominant over dependence. Segregation of cells with the recessive Sm^D phenotype, curing with acridine orange, sensitivity to the male-specific bacteriophage MS2, and ability to transfer their F-merogenotes in conjugation experiments were means used to characterize them. These merodiploids continually segregate recombinant and parental types but can be maintained indefinitely by continuous selection. By crossing MX2 with MX55, a *rec A56* derivative of MX32, similar KLF41 *arg* G^+ *str* A^+/*arg G31 str A(d)900* but Rec^- merodiploids were isolated which obligately maintained their heterozygous state in media devoid of streptomycin. Culturing in streptomycin media was selective for Sm^D segregants of the MX55 parental type.

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