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Segregation of the Mutator Property of Plasmid R46 from Its Ultraviolet-Protecting Property

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Summary. Plasmid R46 (an R factor conferring resistance to ampicillin, sulfonamides, streptomycin and tetracycline) reduces the bactericidal effect of UV irradiation but increases its mutagenic effect (reversion of *hisG46*), and raises the frequency of spontaneous reversion (mutator effect). Putative deletion mutants of R46 were obtained by transduction of the plasmid, then two successive conjugal transfers. Plasmids of five of six deletion classes, each with a different combination of drug resistance traits, retained conjugative ability and the UV-protecting, mutagenesis-enhancing and mutator effects of R46. (pKM101, used in the Ames system to enhance responsiveness to chemical mutagens, is one such mutant of R46.) Plasmids of a sixth class, represented by pKM115, conferred resistance only to streptomycin and were non-conjugative. All of several such plasmids (of independent origin) had a much stronger mutator effect than did R46, but lacked UV-protecting ability and did not enhance the mutagenic effect of UV irradiation. We infer that R46 possesses: (i) a gene, *uvp*, which increases capacity for error-prone repair of UV-damaged DNA, and thus causes both UV protection and enhancement of UV mutagenesis; (ii) gene(s) whose action in the absence of gene *uvp* greatly increases the frequency of spontaneous reversion of *hisG46*. A plasmid of another incompatibility group, pLS51, has UV-protecting and mutagenesis-enhancing effect but lacks the mutator property; introduction of pLS51 into a clone of *hisG46* carrying a pKM115-type plasmid greatly reduced its spontaneous reversion rate, as expected if pLS51 also has a *uvp* gene able to modulate the mutator effect of R46-derived gene(s) in the pKM115-type plasmid.

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Introduction

Some conjugative plasmids, recognizable as R or Col factors, reduce the susceptibility of their *Salmonella typhimurium* or *Escherichia coli* hosts to the lethal effect of ultraviolet (UV) irradiation but increase its mutagenic effect, in respect of reversion of certain mutations causing auxotrophic character (Howarth, 1965, 1966; MacPhee, 1972, 1973a, b; Mortelmans and Stocker, 1976). An R-factor plasmid, R46, produced both these effects and, in addition, increased the frequency of spontaneous reversion of some point-mutant histidine-requiring (*his* mutant) derivatives of *S. typhimurium* LT2 (Mortelmans and Stocker, 1976). (We call such ability to increase the frequency of spontaneous mutation the "mutator" property of the plasmid, by analogy with the term used for chromosomally located genes with this effect, to differentiate the effect from the ability of the plasmid to increase the mutagenic effect of UV irradiation.) Mutator effect, UV protection and enhancement of UV mutagenesis were absent in *recA* mutant hosts but undiminished in a *uvrB* mutant host (defective in the excision-repair of UV-damaged DNA) and in a *polA* host lacking in vitro DNA polymerase I activity. This led us to surmise that the plasmid produced all three effects by enhancing the *recA*-dependent error-prone (therefore mutagenic) process for repair of damaged DNA (Witkin, 1976). However,

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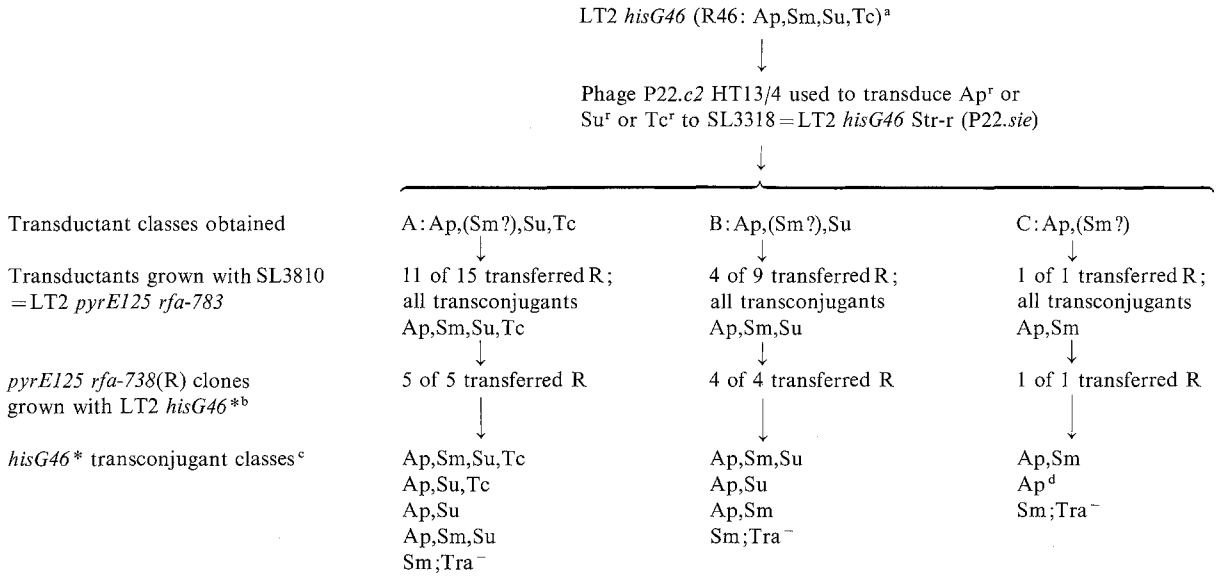

Fig. 1. Origin of deletion mutants of R46

[a] R46 is Tra$^+$, confers UV-protection, enhances UV mutagenesis and increases frequency of spontaneous reversion of *hisG46*

[b] *hisG46** is a single-colony re-isolate of LT2 *hisG46* suspected to differ genetically from it

[c] The plasmids in transconjugants of all classes except those resistant only to streptomycin were Tra$^+$, conferred UV protection, enhanced UV mutagenesis and increased spontaneous reversion frequency. Transconjugants resistant only to streptomycin did not transfer streptomycin-resistance by conjugation, were as UV-sensitive as *hisG46* and no more susceptible than it to UV mutagenesis and reverted to His$^+$ at high frequency (ca. 100 colonies per plate of enriched defined medium)

[d] Plasmid pKM101 came from one such *hisG46** transconjugant resistant only to ampicillin

our tests of *his* mutants of known codon character showed that though R46 increased the frequency of spontaneous reversion of all tested nonsense and of some missense mutants, it had no such effect on some other missense mutants or on frameshift mutants, at least when tested in *uvr*$^+$ hosts. By contrast the plasmid increased the frequency of UV-induced reversion (calculated per 10^8 survivors) for all the non-deletion *his* mutants tested, nonsense, missense or frameshift. The difference in range of mutations susceptible to the mutator effect or to the enhancement of mutagenic effect of UV irradiation might indicate that the plasmid produces its effect on spontaneous mutation frequency by some mechanism other than that involved in enhancement of mutagenic effect of UV irradiation and in UV protection. If so at least two plasmid genes are presumably involved and one might hope to find mutant forms of R46 lacking one gene function or the other, as a result of point or deletion mutation. This paper describes the isolation of several classes of R46 mutants, some at least of them probably arising by deletion since they lack two or more resistance traits of the original plasmid. One such mutant plasmid, which we named pKM101, was found (McCann et al., 1975) to enhance the mutagenic effect of a chemical mutagen to a somewhat greater extent than did R46 itself; for this reason plasmid pKM101 was introduced into several of the strains used in the Ames system for detection of mutagenic activity—the resulting strains, TA100 and TA98, are now extensively used. We also encountered clones of LT2 *hisG46* with properties which we attribute to the presence in them of a class of deletion mutant of R46 lacking the UV-protecting ability of R46 but with increased mutator activity; we named one such postulated mutant plasmid pKM115. The first part of the Results section of this paper describes the steps (transduction of the plasmid, followed by two conjugal transfers) which yielded pKM101, pKM115 and other mutant plasmids. This information (Fig. 1) is relevant to the question of how these plasmids arose from plasmid R46 but less so to the interpretation of their properties, described in the next section of the Results. We then describe the modification of the properties of a clone inferred to harbor pKM115 by introduction of an unrelated UV-protecting plasmid. Reports by others on the behavior of R46 or pKM101 and relevant to our observations are considered in the Discussion section.

In the present paper all tests on frequency of mutation, spontaneous or induced by irradiation, involved *S. typhimurium* strain LT2 *hisG46*; the *his* mutant allele in this strain is of missense type, has a fairly low frequency of spontaneous reversion and responds

Table 1. Bacterial strains

Strain	Description	Reference
Salmonella typhimurium strains		
hisG46	LT2 *hisG46*	Hartman et al., 1971
trpD1	LT2 *trpD1*	Howarth, 1965
trpB68	LT7 (ColI-LT7) *trpB68*	Ozeki et al., 1962
SL1127	LT2 *proA46 purC7* Str-r (R46)	Drabble and Stocker, 1968
SL1145	LT2 *proA46 purC7* Str-r (R6)	Drabble and Stocker, 1968
SL1148	LT2 *proA46 purC7* Str-r (pLS51)	Drabble and Stocker, 1968
SL3318	LT2 *hisG46* Str-r (P22.*sie*)	
SL3810	LT2 *pyrE125 rfa-738*	Kuo and Stocker, 1972

Strain *hisG46* was provided by B. Ames. All other strains are from Stocker collection. Genotype symbols accord with standard nomenclature, except that by *Salmonella* genetics usage *S. typhimurium* strains differing from wild-type by a single mutation are indicated by parent strain symbol and their (unique) mutation number, instead of by strain number. Phenotype symbol: Str-r, streptomycin-resistant. Plasmid R6 is the same as R-Munich, of Drabble and Stocker (1968); plasmid pLS51, formerly called R6-Tc and derived from R6 by Drabble and Stocker (1968), confers resistance only to tetracycline. Strain LT7 *trpB68* resembles LT7 by the indicated colicinogeny

to the mutagenic effect of many chemical mutagens and for this reason is included in the Ames system for detection of mutagenic activity (Ames et al., 1975).

Materials and Methods

Bacterial Strains, Phages and Plasmids. Table 1 lists the *Salmonella typhimurium* strains used. R46 is an R factor of incompatability group N, conferring resistance to ampicillin, streptomycin, sulfonamides and tetracycline. It was previously known as R-Brighton or R1818 and is probably identical to TP120 (Anderson and Datta, 1965; Datta and Kontomichalou, 1965; Drabble and Stocker, 1968; Grindley et al., 1973; Mortelmans and Stocker, 1976); it appears as entry No. 79 in the Novick (1974) catalog. For transduction we used phage P22.*c2* HT13/4, a "high-transducing" mutant of phage P22.*c2* (Schmieger, 1972); strains to be used as transductional recipients were first made lysogenic for P22.*sie* (superinfection-exclusion negative) (Walsh and Meynell, 1967; Levine, 1972).

Media. The nutrient broth and nutrient agar used were "Oxoid" nutrient extract broth number two (CM67) and "Oxoid" blood agar base (CM55), prepared as directed by manufacturer. The defined medium used was Davis minimal medium, with glycerol, 5 ml/l, as energy source, solidified with New Zealand agar [Davis Gelatin (N.Z.) Ltd., Woolston, New Zealand], 15 g/l, and supplemented when appropriate with required amino acids or other growth factors. His^+ revertants were selected on defined medium supplemented with nutrient broth, 10 ml/l, hereinafter called enriched defined medium; this medium supports growth of *his* auxotrophs to a final population of about 3×10^9 colony-forming units per plate (Mortelmans and Stocker, 1976).

Genetic Methods. For conjugal transfer of R plasmids we inoculated tubes of broth with donor and recipient bacteria in the ratio 1:10 and incubated the mixture, unshaken, at 37° C for about 18 h, then streaked out on defined medium selective for the recipient strain, with an antibiotic: ampicillin, 25 µg/ml; streptomycin, 100 µg/ml; sulfathiazole, 50 µg/ml; or tetracycline, 25 µg/ml. Colonies picked from selection plates were purified by single-colony re-isolation on the selective medium, then purified clones were tested for each of the resistances of the donor.

For transduction, phage P22.*c2* HT13/4 was propagated on a broth culture of a strain carrying R46; after 6 h incubation at 37° C with aeration the phage-infected culture was centrifuged and the supernatant was held at 60° C for 60 min; sterility was checked by culture. The lysate was mixed with recipient culture, to give a phage: bacteria ratio of about 5:1. The transduction mixture, after standing 25 min at 37° C, was centrifuged and the cells washed twice in defined medium; samples of the washed-cell suspension, and of dilutions, were spread on plates of defined medium with histidine and a single drug, ampicillin, 25 µg/ml, or sulfathiazole, 50 µg/ml, or tetracycline, 25 µg/ml. The strain used as recipient, SL3318, is LT2 *hisG46* Str-r made lysogenic for P22.*sie*. Such lysogeny protects against killing by P22.*c2* without reducing the frequency of transduction (Stocker, unpublished). Drug-resistant colonies were counted after two days' incubation. After single-colony re-isolation on selective medium clones were tested for resistance to all relevant drugs.

Rates of spontaneous and UV-induced reversion to His^+ were measured as previously described (Mortelmans and Stocker, 1976). In brief, plates of enriched minimal medium were spread with about 10^8 washed cells from an overnight unshaken broth culture. The plates were incubated, either unirradiated or after exposure to UV from a bactericidal lamp, dose 6 J/m^2. His^+ colonies in the thin film of "background" growth were counted after two days' incubation. Their number on control plates is directly proportional to the frequency of spontaneous reversion per bacterium per generation. The number of colonies per irradiated plate, less the number per non-irradiated plate, shows the number of induced mutations per plate. Viable counts, on nutrient agar plates incubated without irradiation or after exposure to UV, 6 J/m^2 (see below), then allow calculation of the number of induced mutations per 10^8 survivors. Survival after UV exposure was determined as previously described (Mortelmans and Stocker, 1976). In brief, colonies were counted on nutrient agar plates inoculated with drops, volume 0.01 ml, of decimal dilutions of washed-cell suspensions from overnight broth cultures, incubated without irradiation or after exposure to UV fluences in the range 5-60 J/m^2. (The conditions used for testing UV survival, that is irradiation of dilute bacterial suspensions on the surface of nutrient plates, differed from those used for UV mutagenesis, in which relatively dense bacterial suspensions spread on the surface of enriched defined medium were irradiated. However, in our experience the survival of UV-irradiated *S. typhimurium* LT2 does not depend on the medium on which tested, simple defined or nutrient broth agar.) For screening tests of UV sensitivity broth cultures, including *hisG46* R^- and *hisG46*(R46) as controls, were streaked across nutrient agar plates which were incubated after exposure to UV, 0, 20, 40, 60, 80 or 100 J/m^2. A strain carrying a UV-protecting plasmid such as R46 gave confluent growth even after exposure to 80 J/m^2, whereas an R^- strain or one carrying a non-protecting plasmid gave confluent growth only for doses up to 40 J/m^2.

Results

1. Isolation of Deletion-Mutant Forms of Plasmid R46

Transduction of R46 Resistances into SL3318. The first stage in the procedures which led to the isolation

of pKM101, pKM115 and other deletion mutant derivatives of R46 was the transduction of resistance traits of R46 by phage P22, previously observed (Drabble and Stocker, 1968) to result in isolation of incomplete (deletion) forms of the plasmid. We used phage P22.*c2* HT13/4 grown on LT2 *hisG46*(R46) and as recipient SL3318 (=*hisG46* Str-r lysogenic for P22.*sie*) (see Methods). The number of transductant colonies per plate inoculated with ca. 8×10^7 colony-forming units of SL3318 was about 500 for the ampicillin and sulfathiazole selection plates, and about 6 for the tetracycline plates. There were no colonies on control plates, without phage. Testing of (non-purified) transductants, 100 from each selection, showed that 272 (class A) transductants had all the testable resistances of R46, 27 (class B) transductants were resistant to ampicillin and sulfathiazole but sensitive to tetracycline, and one (class C) transductant was ampicillin-resistant but sensitive to tetracycline and sulfathiazole. (Plasmid-determined streptomycin-resistance could not be tested, because of the chromosomally determined resistance of the recipient strain.) Fifteen class A transductants (resistant to ampicillin, sulfathiazole and tetracycline), numbered T1 through T15, nine class B transductants (resistant to ampicillin and sulfathiazole), numbered T16 through T20 and T22 through T25, and the single class C transductant (resistant to ampicillin), numbered T21, were further tested. The plasmids in transductants T1 through T25 were assigned corresponding plasmid symbols, pKM1 through pKM25.

Conjugal Transmission of Transduced Plasmids to Strain SL3810. Transductants T1 through T25 were tested for ability to transfer any one of their resistance traits by conjugation. A "rough" strain, SL3810 (= LT2 *pyrE125 rfa-738*), unable to adsorb P22, was used as conjugal recipient, to prevent possible transduction by phage released by the lysogenic transductants to be tested as conjugal donors; strain SL3810 is streptomycin-sensitive, and so allowed recognition of plasmid-determined streptomycin-resistance. Twenty-five mixed cultures, each comprising one transductant together with SL3810 as recipient, after overnight incubation were streaked on defined medium with uracil and one antibacterial agent, to select for SL3810 clones which had acquired a resistance trait. Eleven of the fifteen crosses of class A transductants, four of the nine crosses of class B transductants and the cross of the only class C transductant gave colonies on each applicable selection medium. The other nine crosses gave no colonies on any selection; the four class A and five class B transductants concerned presumably housed plasmids which had lost *tra* genes during transduction. All tested transconjugants from the sixteen fertile crosses were found to have acquired all the observed plasmid-determined resistances of their donor parent (i.e., to ampicillin, sulfonamides and tetracycline; or to ampicillin and sulfonamides; or to ampicillin), and also streptomycin-resistance, masked by chromosomally determined resistance in the donors. Thus all the 16 transduced plasmids which retained conjugative ability also retained the streptomycin-resistance trait of R46, and none lost any resistance trait during conjugal transfer from transductant to rough recipient. The plasmids in the rough transconjugants, one from each of the sixteen fertile crosses, were therefore assumed identical to those (pKM1, pKM2, etc.) in the donors concerned.

Second Conjugal Transfer of Transduced Plasmids, from SL3810 to hisG46.* For tests on their ability to confer UV-protection, etc., we decided to transfer some of the sixteen previously transduced plasmids now shown to be transmissible by conjugation, from the *pyrE125 rfa-738* transconjugants to our "standard" host, LT2 *hisG46*. In accordance with our custom we first made a single-colony re-isolate of strain LT2 *hisG46*, so as to get rid of any His^+ revertants which might have accumulated in the stock culture. As described below we later suspected that the LT2 *hisG46* re-isolate (which was not preserved after the crosses were completed) differed genetically from our stock culture of strain LT2 *hisG46*; we shall indicate this clone as *hisG46**, the asterisk indicating the hypothetical mutation. The ten clones chosen for testing as conjugal donors [themselves transconjugants derived from the crosses of SL3318 transductants as donors to SL3810 (=LT2 *pyrE125 rfa-738*) as recipient] comprised the following: five transconjugants with all four resistances of R46, obtained by crosses of SL3810 as recipient with class A transductants; four transconjugants resistant to ampicillin, sulfathiazole and streptomycin, from crosses of SL3810 with class B transductants; and one transconjugant, resistant to ampicillin and streptomycin, from the cross of SL3810 with the class C transductant. (See Table 3 for the numbers assigned to the strains tested and for their origin.) Each of these ten clones was grown in mixed culture with LT2 *hisG46**. Samples from each mixture were streaked on defined medium with histidine and ampicillin or sulfathiazole or streptomycin or tetracycline, but without uracil, required by the donor strains. All applicable selections yielded growth, i.e., *hisG46** (R) transconjugants, from each of the ten matings. Testing of single colonies picked from the selection plates showed that many of them had fewer resistance traits than their donor parent. Tests on 374 colonies (eleven from each selection plate) showed that transconjugants with all the resis-

Table 2. Number of transconjugant clones of various resistance patterns obtained in ten crosses, of donors carrying previously transduced plasmids to *hisG46** as recipient

Resistance pattern of *hisG46** transconjugant class	Donors used, resistances and donor label		
	Ap,Sm,Su,Tc (1R,2R,3R,4R and 5R)	Ap,Sm,Su (6R,7R,9R and 10R)	Ap,Sm (8R)
	Number of transconjugant clones of indicated type and donors in crosses from which obtained		
Ap,Sm,Su,Tc	147 (1R,2R,3R,4R,5R)	N.A.	N.A.
−,Sm,−,−	26 (1R,2R,3R,4R,5R)	8 (10R)	4 (8R)
Ap,−,Su,Tc	21 (2R,3R,5R)	N.A.	N.A.
Ap,−,Su,−	20 (3R,5R)	4 (6R,9R)	N.A.
Ap,Sm,Su,−	6 (1R,2R)	117 (6R,7R,9R,10R)	N.A.
Ap,Sm,−,−	0	3 (9R)	12 (8R)
Ap,−,−,−	0	0	6 (8R)
Total tested	220	132	22

Resistances: Ap, ampicillin; Sm, streptomycin; Su, sulfonamides, Tc, tetracycline. N.A., not applicable. Eleven colonies were tested from each applicable selection (for a single resistance trait) for each of the ten crosses. The recipient in each cross was *hisG46**, a re-isolate of *hisG46*. The donors were ten clones of constitution SL3810(R), numbered 1R through 10R. Donors 1R, 2R, 3R, 4R and 5R had plasmids derived from five class A transductants, respectively, T2, T3, T7, T8 and T14, housing plasmids pKM2, pKM3, pKM7, pKM8 and pKM14. Donors 6R, 7R, 9R and 10R had plasmids derived from class B transductants, respectively, T18, T20, T22 and T23, housing plasmids pKM18, pKM20, pKM22 and pKM23. The plasmid in donor 8R came from the class C transductant, T21, housing plasmid pKM21

tances of their donor parent were obtained in all ten crosses; in nine of the crosses they made up the majority of the tested clones. Transconjugants with fewer resistance traits than their donor parent fell into six classes (Table 2). Transconjugants of each of four of these "incomplete" or "deletion" classes were obtained in two or more of the ten crosses. All the six transconjugants resistant only to ampicillin came from cross #8, in which the plasmid of the donor, pKM21 (derived from the class C transductant), conferred only ampicillin and streptomycin resistances. [The plasmid in one such transconjugant resistant only to ampicillin, assigned symbol pKM101 (Table 3), is that used in the Ames mutagenicity test system.] The three transconjugant clones resistant only to ampicillin and streptomycin all came from only one of the four crosses in which the donor was resistant to ampicillin, streptomycin and sulfonamides (Table 2). Transconjugants resistant only to streptomycin, inferred to have acquired mutant plasmids of the pKM115 type (see below), were obtained from eight of the ten crosses. This makes it likely that such plasmids are of uniform composition and result from a characteristic event which occurred with some frequency during the crosses concerned.

Possible Explanations for Production of Many Mutant Plasmids in the Ten Crosses. We considered three hypotheses to explain the frequency of production of deletion-mutant plasmids during the ten conjugal crosses described above, despite their rarity in other conjugal crosses. (i) R46 might be inherently unstable when carried by, or transmitted by conjugation from, SL3810 (=LT2 *pyrE125 rfa-738*); (ii) frequent incomplete conjugal transmission might be a consequence of the previous transduction of the plasmid (e.g., if the only plasmids successfully transduced were those which had lost segments of the plasmid genome, facilitating their fitting into a P22 phage head but diminishing their stability during conjugal transfer); (iii) the deletions might have resulted from the use, as conjugal recipient, of an exceptional clone of LT2 *hisG46*, prone to loss of segments of the plasmid during its acquisition by conjugation. In additional experiments (K.E. Mortelmans, Ph.D. thesis, Stanford, 1975) strain SL3810 carrying R46, either previously transduced or transferred only by conjugation, was crossed with *hisG46* as recipient; no or very few mutants of R46 were obtained, a result tending to disprove hypotheses (i) and (ii). Hypothesis (iii) could in theory have been directly tested by repetition of the crosses, using as recipient the single-colony re-isolate of LT2 *hisG46* which had been used in the ten crosses; however, this clone (referred to above as *hisG46**) had not been preserved. To obtain a culture of the hypothetical *hisG46** type we therefore examined a transconjugant, SL3379, obtained in one of the ten crosses and resistant only to ampicillin,

Table 3. Mutants of R46 obtained by conjugal transfer of previously transduced plasmids: strain and plasmid number assignments of transconjugants taken as "type" examples, summary of properties and origin

Strain number	Plasmid number	Resistances	Transfer ability	UV		Mutator activity	Ancestry	
				Protection	Mutagenesis enhancement		Conjugational donor	Transductant ancestor
SL3379	pKM101	Ap,−,−,−	+	+	+	+	8R (Ap,Sm)	T21 (pKM21)
SL3363	pKM102	Ap,−,Su,Tc	+	+	+	+	3R (Ap,Sm,Su,Tc)	T7 (pKM7)
SL3375	pKM103	Ap,−,Su,−	+	+	+	+	6R (Ap,Sm,Su)	T18 (pKM18)
SL3371	pKM104	Ap,Sm,Su,−	+	+	+	+	7R (Ap,Sm,Su)	T20 (pKM20)
SL3385	pKM110	Ap,Sm,−,−	+	+	+	+	9R (Ap,Sm,Su)	T22 (pKM22)
SL3368	pKM115	−,Sm,−,−	−	−	−	++	1R (Ap,Sm,Su,Tc)	T1 (pKM1)

All strains are derivatives of LT2 *hisG46**. Transfer ability: tested by conjugation with LT2 *trpD1*. UV mutagenesis enhancement: + indicates number of UV-induced revertants per plate inoculated with about 10^8 bacteria and irradiated, 6 J/m^2, was about 200, compared with 10–27 for *hisG46* R$^-$; − indicates no enhancement. Mutator effect: + indicates about 30 spontaneous revertants per plate of enriched defined medium, compared to about 3 for *hisG46* R$^-$; ++ indicates about 100 revertants per plate. Conjugational donors are clones of SL3810 (=LT2 *pyrE125 rfa-738*) given plasmids by conjugation with indicated transductants; their plasmids had all the resistance traits detected in the transductants (and also streptomycin-resistance, masked in the transductants) and are assumed identical with the plasmids in the transductants, whose plasmid numbers are shown

therefore inferred to have acquired a mutant plasmid of the pKM101 type. Two of fifty colonies obtained by streaking out a broth culture of SL3379 were ampicillin-sensitive. The two segregants, designated SL3439 and SL3440, had also lost both the UV-resistance of their pKM101-carrying parent and its high susceptibility to mutagenic effect of UV (6J/m^2) irradiation. They were inferred to have lost pKM101, and therefore to represent the hypothetical *hisG46** genotype. Both were tested, in parallel with our stock culture of *hisG46,* as recipients in conjugational crosses with SL1127 (=LT2 *purC7 proA46* Str-r carrying R46) as donor. Selection was made on defined medium with histidine and either ampicillin or tetracycline. Thirty transconjugant clones each of the two crosses (15 from each selection) were scored for all resistance traits. All thirty from the cross in which the recipient was *hisG46* from our stock culture had all the four resistance traits of R46. By contrast the thirty SL3939 and SL3940 transconjugants selected as ampicillin-resistant included eleven which were sensitive to sulfathiazole, streptomycin and tetracycline; and the thirty selected as tetracycline-resistant included three which were resistant to ampicillin and sulfathiazole but sensitive to streptomycin. This result supports hypothesis (iii).

2. *Properties of Mutant Derivatives of Plasmid R46*

As noted above the presumed deletion mutant forms of plasmid R46 obtained by the ten conjugal crosses (Table 2) fell into six classes in respect of drug-resistance traits. One transconjugant of each class was taken as the "type" strain and given an SL stock number (Table 3); the plasmid (or presumed plasmid) of each type strain was similarly allotted a number in the pKM series (Table 3). Transconjugant LT2 *hisG46** clones inferred to carry mutant forms of R46, three clones for each of the six classes defined by drug-resistance traits, after purification by single-colony isolation were tested for ability to transmit their resistance trait(s) to LT2 *trpD1* by conjugation. All the tested clones of five of the six classes transmitted all their resistances. Clones of the sixth class, resistant only to streptomycin did not transmit this trait (see below). Representatives, at least three for each class, of the five classes able to transmit were tested qualitatively for UV sensitivity and for frequency of spontaneous and UV-induced reversion to His$^+$ (Table 4). All the tested clones were less sensitive to UV irradiation than *hisG46* R$^-$ and about as resistant as *hisG46*(R46); and all of them gave about as many spontaneous and UV-induced His$^+$ revertants as did *hisG46*(R46). Thus the plasmids present in these five classes, though deficient of one or more resistance trait, retained the UV-protecting effect of R46 and its ability to increase frequency of spontaneous reversion and to enhance mutagenic effect of UV irradiation.

Properties of Plasmids Conferring Only Streptomycin-Resistance. The sixth class of *hisG46** transconjugant comprised 38 clones resistant only to streptomycin, obtained from eight of the ten crosses (Table 2). In the initial tests all of three such clones failed to transfer streptomycin-resistance to LT2 *trpD1* by conjugation; another 32 clones of the same class were similarly tested, also with negative results. All of 15 transconjugants of this class in the screening test appeared

Table 4. Resistance patterns and effect on UV-sensitivity and on frequency of spontaneous and UV-induced reversion of plasmids of six mutant classes, obtained by conjugal transfer of transduced R46

Strain and plasmid number	Resistance pattern	UV protection	His$^+$ colonies/plate	
			Non-irradiated	Irradiated 6 J/m^2
SL3379:pKM101	Ap, −, −, −	+	34	204
2 others	Ap,, −, −, −	+	32, 33	207, 217
SL3363:pKM102	Ap, −,Su,Tc	+	26	208
12 others	Ap, −,Su,Tc	+	24-42	186-282
SL3376:pKM107	Ap, −,Su, −	+	38	256
3 others	Ap, −,Su, −	+	32-39	196-226
SL3371:pKM104	Ap,Sm,Su, −	+	27	214
17 others	Ap,Sm,Su, −	+	20-40	185-271
SL3385:pKM110	Ap,Sm, −, −	+	25	205
2 others	Ap,Sm, −, −	+	29, 31	215, 226
SL3368:pKM115	−,Sm, −, −	−	105	120
14 others	−,Sm, −, −	−	86-131	84-135
Transconjugants with complete R46	Ap,Sm,Su,Tc	+	19-37	180-235
hisG46 R$^-$	N.A.	N.A.	2-4	10-27

Strains are LT2 *hisG46** given mutant forms of R46 by conjugation with LT2 *pyrE125 rfa-738* clones which had obtained their plasmids by conjugation with transductants. Strains were not all tested on same day. Figures for control strains, *hisG46* R$^-$ and *hisG46* (R46), are ranges, from several experiments. UV protection determined by qualitative test. Number of His$^+$ colonies/plate are mean of two plates, of enriched defined medium, or range of means for set of three or more strains. N.A. = not applicable

about as UV-sensitive as *hisG46* R$^-$: the UV-dose/log-survival curves for six such clones were indistinguishable from those obtained with *hisG46* R$^-$. Each of these 15 clones gave about 100 spontaneous His$^+$ revertant colonies per plate of enriched defined medium (Table 4), compared with an average of about 24 for *hisG46*(R46) and of about 3 for *hisG46* R$^-$: thus the spontaneous reversion frequency in these clones was about thirtyfold higher than in the R$^-$ strain, compared with about tenfold higher for strains carrying R46 itself. The mean number of revertant colonies per plate of enriched defined medium exposed to UV, 6 J/m^2, after inoculation with about 10^8 cells, was not significantly greater than the number on non-irradiated control plates (Table 4): thus exposure to this dose of irradiation, calculated to permit about 40% survival, had no detectable mutagenic effect on any of these 15 strains.

The level of streptomycin resistance of two transconjugants resistant only to streptomycin was determined by spreading inocula of ca. 800 bacteria on nutrient agar plates with graded concentrations of streptomycin. Both clones appeared about as resistant as *hisG46*(R46), showing ca. 100% efficiency of colony formation at 25 μ/ml (compared with less than 1% for *hisG46* R$^-$) and ca. 2% at 50 μg/ml, as expected if their resistance resulted from presence in them of a mutant form of R46 which retained its streptomycin-resistance gene. (Several of these transconjugants when tested after several months storage at −70° instead showed only a very slight degree of resistance of streptomycin (L. Valdivia and Stocker, unpublished data); however, all the experiments described in the present paper concern the transconjugants as originally isolated.)

We surmised that the high frequency of reversion to His$^+$ of this class of transconjugant resulted from presence of a mutant plasmid. An alternative explanation was considered: the LT2 *hisG46** culture used as recipient in the ten crosses from which the clones were isolated might have contained a clone of *mut* (mutator) mutants, causing increased frequency of mutation both to (low-level) streptomycin resistance and to His$^+$; on this hypothesis the clones resistant only to streptomycin isolated from eight of the conjugation mixtures would be not transconjugants, of genotype *hisG46** (R), but two-step mutants, of genotype *hisG46* mut*$^-$ *str*$^-$ (where *mut* and *str* are sets of mutant alleles at unspecified chromosomal loci governing, respectively, spontaneous mutability and streptomycin sensitivity). If this were so the two properties, high reversion frequency and low-level streptomycin resistance, would probably be due to chromosomal mutations only distantly linked, and so they would not be transmitted together at high frequency, by conjugation or transduction, and would not be liable to simultaneous loss by loss of a plasmid. As noted above clones of this sort did not transmit streptomycin-resistance to LT2 *trpD1* by conjugation. We attempted to transduce streptomycin-resistance from one of these clones to SL3428 (= LT2 *hisG46* lysogenic for P22.*sie*). Two high-transducing variants of phage P22, P22.*c2* HT13/4 and P22.*c*$^+$ HT105/1 (Schmieger, 1972), were propagated on SL3367 (= LT2 *hisG46** carrying a plasmid of the pKM115 type). The lysates of the clone resistant only to streptomycin failed to evoke any streptomycin-resistant transductants, though control lysates, i.e., the same two phages grown on *hisG46*(R46), evoked many such.

As we failed to transfer the streptomycin-resistance of the exceptional clones either by conjugaton or by transduction, the possibility remained that their streptomycin-resistance and high frequency of spontaneous reversion to His$^+$ resulted from chromosomal mutations, respectively at *str* and *mut* loci. We there-

fore looked for spontaneous loss of these two properties. An overnight broth culture of strain SL3370 (=*hisG46** carrying pKM117, a pKM115-like plasmid) was streaked out on nutrient agar; of 100 colonies tested two proved to be sensitive to streptomycin, 25 μ/ml, and were inferred to be "cured" of their plasmids. These two clones showed a spontaneous reversion frequency similar to that of *hisG46* R^- (mean number of His^+ revertants per plate 3.0, compared to 3.3 for the R^- control). After exposure to UV irradiation, 6 J/m², the number of UV-induced His^+ revertants per plate for the two "cured" clones was about the same as for the control *hisG46* R^- (means for the "cured" clones 20.1 and 18.3, compared to 19.3 for the R^- control strain). The UV sensitivity of these two clones was similar to that of the control *hisG46* R^-. The simultaneous loss of resistance to streptomycin (25 μ/ml), high spontaneous reversion frequency and insusceptibility to UV-induced reversion makes it likely that these properties were determined by mutant plasmid pKM117, of the pKM115 type, derived from R46.

3. Effect of Co-Presence of a pKM115-Type Plasmid and a UV-Protecting Plasmid Lacking Mutator Effect

We thought that the absence in pKM115 and similar plasmids of an R46 gene determining UV-protecting ability (and enhancement of UV mutagenesis) might account for their greater mutator effect, compared to that of R46, and therefore that introduction into a *hisG46**(pKM115)-type strain of a UV-protecting plasmid lacking mutator effect might reduce the mutator effect of the pKM115-type plasmid and give a phenotype like that of *hisG46*(R46). We had available a conjugative plasmid unrelated to R46 which conferred resistance to tetracycline, gave UV protection and enhanced UV mutagenesis (to about the same extent as R46) but had no obvious effect on frequency of spontaneous reversion of *hisG46* (Table 5). This plasmid, previously indicated R6-Tc (Drabble and Stocker, 1968; Stocker, unpublished observations), we now name pLS51. It was originally encountered in the course of a conjugal transmission of R6 [=R-Munich of Drabble and Stocker, 1968: =No. 39 in the Novick (1974) catalog]. Plasmid pLS51 was transferred from SL1148 (=LT2 *proA46 purC7* Str-r carrying pLS51) by conjugation, with selection for tetracycline-resistance. to SL3369 (=LT2 *hisG46** carrying pKM116, a pKM115-type plasmid). Three clones of independent origin, inferred from their drug resistances to carry both pKM116 and pLS51, were tested for UV sensitivity, UV mutagenic effect and spontaneous reversion frequency (Table 5).

Table 5. UV resistance and yield of spontaneous and UV-induced His^+ revertants of LT2 *hisG46* carrying R46, its derivative pKM116, R6, its derivative pLS51 or pKM116 and pLS51

Strain number	Plasmid(s) carried	Survival, 6 J/m² UV	Number of His^+ colonies per plate	
			Non-irradiated	Irradiated 6 J/m²
hisG46	none	40%	3.6	27
SL3405	R6	87%	16	157
SL3341	pLS51	94%	3.6	165
SL3302	R46	85%	30	183
SL3369*	pKM116	37%	115	113
SL3409*	pKM116+pLS51	89%	23	203
SL3410*	pKM116+pLS51	91%	25	169
SL3411*	pKM116+pLS51	87%	18	196

All strains are descendants of LT2 *hisG46*, those marked * *via* LT2 *hisG46**. Plasmid pLS51, formerly called R6-Tc and derived from R6, confers resistance only to tetracycline. Number of His^+ colonies per plate: mean of three plates

All three resembled *hisG46*(R46), and differed from their parent, *hisG46** carrying a pKM115-like plasmid, in UV resistance, frequency of UV-induced His^+ revertants, and, as surmised above, in frequency of spontaneous reversion.

Discussion

The first step in the procedure which yielded many mutant plasmids (including pKM101 and pKM115) was transduction of R46 by phage P22.*c2* HT13/4, with selection for resistance to ampicillin or sulfathiazole or tetracycline (the transductional recipient used being streptomycin-resistant by chromosomal mutation plasmid-determined streptomycin-resistance could not be selected for or tested). Many transductants had lost tetracycline-resistance and/or conjugative ability. We think it likely that the others may have suffered deletion of other plasmid genes; the plasmids in the 25 transductants chosen for further study were therefore allotted symbols, pKM1 through pKM25. The second step was the conjugal transfer of the plasmids of 16 Tra^+ transductants to a "rough" recipient, SL3810. The 16 plasmids thus transferred were all found to have the streptomycin-resistance gene of R46, and there was no loss of any other resistance trait during their transfer to the "rough" recipient; the SL3810(R) clones obtained were therefore presumed to house plasmids identical to those in the transductants serving as conjugal donors. In the third step ten of the "rough" transconjugants were used as conjugal donors, in ten parallel crosses in each of which the recipient was the same re-isolated clone of LT2 *hisG46* (termed *hisG46**).

Each of the crosses yielded, amongst others, transconjugants lacking one or more of the resistance traits of the donor concerned. Thus many of the mutant plasmids finally obtained, of six different classes (Table 2), resulted from two successive mutations, the first during transduction and the second during the second conjugal transfer of a transduced plasmid. For instance pKM101, conferring only ampicillin-resistance and now used in the Ames system (and all of several mutant plasmids of the same type) resulted from loss of tetracycline and sulfathiazole resistance during transduction (to give pKM21) and loss of streptomycin resistance during subsequent conjugation. Plasmids of the pKM115 type conferring low-level streptomycin resistance, high reversion frequency and (apparent) reduction of mutagenic effect of UV were obtained in eight of the ten crosses, and most of them came from transductants housing plasmids not known to be different from R46 itself. If as we believe the clones in question owe these phenotypic traits to presence of deletion derivatives of R46 a particular deletion must have occurred on at least eight occasions.

The isolation of mutant plasmids from all of ten parallel crosses on one occasion, compared with their absence or rarity in other crosses, indicated some peculiarity in the ten crosses. Repeat crosses using the same donors showed that the peculiarity was not in the genotype of the donors; and no peculiarity in the conditions of the mating experiment were known. The third possible explanation, that the *hisG46* culture used as recipient in the ten crosses (referred to above as *hisG46**) differed from standard *hisG46* (for instance as maintained in our stock culture collection), could not be directly tested, because the reisolate had not been preserved after completion of the crosses. However, mutants of R46 were also encountered in later crosses in which we used as recipient either of two R^- segregants from a clone, of inferred constitution *hisG46**(pKM101), obtained in one of the ten crosses. This result favors the hypothesis of inadvertent use of a mutant clone of *hisG46* in the ten crosses. Plasmids which behave as stable units in one species may readily dissociate into separate units, or lose parts of their genome, when transferred into bacteria of other species (Nisioka et al., 1969); it would therefore not be surprising if the ability of *S. typhimurium* to accept R46 by conjugation and maintain it intact were subject to alteration by mutation.

Many ColI plasmids and many R factors of certain groups both reduce the lethal and increase the mutagenic effect of UV irradiation (Howarth, 1965, 1966; Drabble and Stocker, 1968; Siccardi, 1969; MacPhee, 1972, 1973a, b; Mortelmans and Stocker, 1976; Tweats et al., 1976; Monti-Bragadin et al., 1976; Walker, 1977; Babudri and Monti-Bragadin, 1977); the extent of these effects was about equal for all of several UV-protecting plasmids tested (K.E. Mortelmans, Ph.D. thesis, Stanford University, 1975). It is surmised (Mortelmans and Stocker, 1976) that UV-protection and enhancement of UV mutagenesis both reflect a plasmid-determined increase in ability to effect repair of UV-damaged DNA by some error-prone mechanism. We propose that the UV-protecting and mutagenesis-enhancing action of R46 (and of most of its derivatives, including pKM101) result from the action of a *uvp* (*UV-p*rotection) gene or genes causing increased repair capacity, and that other UV-protecting plasmids possess similar *uvp* genes.

Some but not all UV-protecting plasmids increase the frequency of spontaneous reversion of mutation *hisG46* (K.E. Mortelmans, Ph.D. thesis, Stanford University, 1975), the degree of this mutator effect varying according to plasmid. Our motive for seeking mutant forms of R46 was to test whether the mutator property of the plasmid could be separated from its UV-protecting ability. One of the two predicted products of such separation, a plasmid with mutator property but not UV-protecting, was, we believe, discovered in the transconjugants resistant only to streptomycin, isolated from eight of ten crosses. These clones were all as UV-sensitive as *hisG46* R^- but reverted to His^+ at about X30 higher frequency than did *hisG46* R^-, and so at about X3 higher frequency than *hisG46* carrying R46 or any other class of deletion mutant of it (Table 4). Exposure of 15 such clones to UV irradiation, 6 J/m^2 (allowing survival of ca. 40%), caused no increase in the number of revertant colonies per plate (means and s.d. 104.9 ± 14.4 for the non-irradiated sets, 103.2 ± 15.1 for the 15 irradiated sets). The large number of spontaneous revertant colonies per plate might have masked a weak mutagenic effect, such as 10 UV-induced revertant colonies per plate. However, the data at least suggest that UV irradiation (6 J/m^2) of clones inferred to carry pKM115-type plasmids is less effective in inducing reversion than similar irradiation of *hisG46* R^-. We surmised that the high spontaneous reversion frequency of these clones resulted from R46-derived gene(s) acting in the absence of a modifying effect of the *uvp* gene of R46, and that introduction of an unrelated UV-protecting plasmid by supplying *uvp* function might reduce the high spontaneous mutation frequency. Plasmid pLS51, a UV-protecting plasmid derived from R6 and thus unrelated to R46, was introduced by conjugation into a *hisG46** clone carrying a pKM115-type plasmid; three transconjugants inferred from their drug-resistances to carry both

pLS51 and pKM116 as predicted produced spontaneous His^+ revertants at only the same rate as did *hisG46*(R46) (Table 5). The introduction of the UV-protecting plasmid also resulted in reappearance of UV mutagenesis, to about the same extent as in *hisG46*(R46); if, as we suspect, the mutagenic effect of UV is reduced in clones carrying pKM115-type plasmids, we surmise that this effect, like high spontaneous reversion rate, results from action of R46-derived genes acting in the absence of *uvp* function. Our data do not show whether the relatively weak mutator effect of the parent plasmid, R46 (and its other deletion derivatives, including pKM101), results from the action of the same plasmid genes, modulated by that of gene *uvp;* the kinds of mutation whose spontaneous frequency is increased by R46 have been determined (Mortelmans and Stocker, 1976) but the effect of pKM115-type plasmids has been observed only in respect of mutation *hisG46,* because of the difficulty of transferring such plasmids to other hosts, as discussed below.

Walker (1978) has isolated mutants of pKM101 which have simultaneously lost UV-protecting effect, ability to enhance UV mutagenesis and mutator effect, an observation which seems to argue against mutator effect resulting from action of genes other than those responsible for the other two effects. However, a mutation affecting expression of a plasmid operon containing both the *uvp* gene and gene(s) responsible for mutator effect might account for the occurrence of such mutants.

We have above assumed that the special properties (streptomycin-resistance, high spontaneous reversion frequency, apparent absence of UV mutagenesis) in certain clones reflects the presence in them of a particular type of deletion mutant of plasmid R46, typified by plasmid pKM115. However, we did not succeed in proving the presence of such plasmids by showing their transfer to other hosts, by conjugation or transduction. Our best evidence that the three properties of these clones are plasmid-determined is the observation that two out of 100 single-colony isolates from one such clone had lost all three properties, being streptomycin-sensitive and showing the same frequency of spontaneous and UV-induced reversion as strain *hisG46* R^-. An alternative hypothesis, of selection, from the recipient *hisG46** culture used in the ten crosses, of mutants resistant to low concentrations of streptomycin, arising in a *mut* (mutator) mutant clone, might explain both the streptomycin resistance and the high spontaneous mutation frequency but would not account for diminished UV mutagenesis, nor perhaps for the reduction of high spontaneous mutation frequency by introduction of an unrelated UV-protecting plasmid (Table 5). Later experiments in this laboratory have produced some additional evidence favoring the existence of pKM115-type plasmids. Dr. Esther Lederberg (personal communication) examined DNA from one clone and found suggestive evidence for the presence of a small plasmid; such experiments are complicated by the presence in LT2 lines of a large cryptic plasmid (Smith et al., 1973; Spratt et al., 1973). L. Valdivia and B.A.D. Stocker (unpublished observations) found that clones inferred to carry pKM115-type plasmids were slightly resistant to spectinomycin, as expected since the R46 gene causing streptomycin-resistance also determines resistance to spectinomycin. However, the evidence for existence of pKM115-type plasmids remains incomplete.

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