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Identification of Closely Linked Loci Controlling Ultraviolet Sensitivity and Refractivity to Colicin E2 in *Escherichia coli*

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Mutants (phenotypic symbol Ref-II) refractory to colicin E2 have been isolated in several strains of *Escherichia coli* K-12, and a *ref*II locus has been mapped 1 to 2 min counter clockwise to *thr*. A small number of Ref-II mutants are also ultraviolet (UV)-sensitive and the uv^s locus in one such strain has been mapped close to the *ref*II locus near *thr*. The Ref-II mutation alone does not affect recombinant formation in F^- strains, but the Ref-II, UV^s strains behave in many respects like Rec^- mutants, giving reduced recombination frequencies in crosses with male strains. It is suggested that the *ref*II and uv^s loci correspond to closely linked if not identical genes, concerned in some way in the activity of one or more deoxyribonucleases, and that the Ref-II, UV^s mutants arise as the pleiotropic expression of a single gene or of a deletion or polar mutation affecting linked genes.

The adsorption of colicin to specific receptors in the cell surface of sensitive bacteria is followed by characteristic intracellular changes and ultimately by cell death (18). The colicin molecules which apparently fail to act in cell-free systems (11, 17) seemingly promote their specific effects in vivo from the extracellular attachment site. Although colicin receptors probably reside in the bacterial cell wall (3, 4), the attached colicin, in order to act, must presumably interact in some specific way with the cytoplasmic membrane of the affected cell. Nevertheless, gross physical changes in the properties of the cell membrane do not accompany colicin action (14, 16). The presence of colicin at the cell surface therefore introduces some subtle change in the cell membrane, triggering a series of events eventually leading to a specific and lethal intracellular change. This series of steps may be envisaged as the "colicin pathway" connecting the extracellular colicin to its intracellular target, fixation to the colicin receptor being the first step in this process. In the case of colicins E2 and E3, this and possibly some subsequent steps in the pathway are shared (6), although ultimately two quite distinct targets are affected. Thus, colicin E2 induces deoxyribonucleic acid (DNA) degradation, whereas E3 induces a specific inhibition of protein synthesis (16).

Resistant mutants lacking the colicin E receptor have been isolated, and a genetic locus controlling in some way receptor formation has been mapped between *met* and *thi* (10). Mutants resistant to colicin E, which nevertheless still retain the colicin E receptor, have also been isolated. Such mutants which are presumably blocked at some post-fixation point in the "colicin pathway" are designated refractory to colicin E (6). Mutants refractive (or tolerant) to colicin E have been isolated by several workers (2, 6, 15, 19, 20) and fall into two main groups. The major group includes multirefractory mutants, refractory to colicin E2 and E3 or to colicin E1, E2, and E3. Several classes of this type of mutant, which are often also refractory to other unrelated colicins, have been mapped at one of at least three distinct sites closely linked to *gal*. The second group of refractory mutants includes strains refractory to only a single colicin. Two such classes are known: E1-refractory mutants, first isolated by Clowes and Moody (2) and designated by us Ref-I mutants, and E2 refractory mutants (Ref-II) (6). All Ref-I strains appear to map at a single locus linked to *thy* (6), whereas (as described in this paper) refractivity of the Ref-II type is probably controlled by a single locus closely linked to *thr*.

Ref-II mutants are conditional mutants refractory to colicin E2 at low temperature but largely sensitive at high temperature. Detailed properties of these mutants, in which DNA breakdown, following E2 adsorption, takes place

at 40 C but not at 30 C, have been described elsewhere (8). A small number of Ref-II mutants, in addition to E2 refractivity, also show an increased sensitivity to ultraviolet (UV)-irradiation, although this effect is not temperature-dependent (7). The genetic properties and the location of the UV sensitivity locus of one such strain are also described in this paper.

MATERIALS AND METHODS

Organisms. The following E2-sensitive strains were used in this study: *Escherichia coli* HfrH (T1^r *str*^{s} λ$^-$); HfrR4 (Col-I^r *str*^{s} *met*$^-$) and F$^-$, strain 203 (*thi*$^-$ *met*$^-$ *arg*$^-$ *thr*$^-$ *leu*$^-$ *gal*$^-$ *trp*$^-$ *his*$^-$ *str*^{r}) (6); ASH10 (F$^-$, *thi*$^-$ *met*$^-$ *thy*$^-$ *lac*$^-$ *leu*$^-$ *str*^{r}) and ASH1 (Hfr, *thi*$^-$ *met*$^-$ *thy*$^-$ *leu*$^-$ *str*^{r}) (7). The source of colicin E2 in these studies was *Salmonella typhimurium* LT2, 906 carrying the E2 (P9) factor, and the culture conditions, production, and assay of colicin were all as reported previously (6). The origins of the Hfr strains used are shown in Fig. 1.

Mating conditions. Linkage analysis and selection for different recombinant classes was carried out as described previously (6). In interrupted mating experiments, male and female strains were first grown in nutrient broth (NB) to 5 × 10^7 cells/ml and 5 × 10^8 cells/ml, respectively; equal volumes were mixed and incubated at 37 C with gentle shaking for 5 min. The culture was then diluted 10-fold in NB to reduce further pairing; 0.5-ml samples were removed at intervals, diluted 10-fold in ice-cold buffer, and blended for 1 min with a flask shaker (B.T.L. Laboratory Centre, Birmingham, England) before plating for recombinants. For the direct selection of E2-refractive recombinants, samples from the mating mixture were blended in ice-cold NB plus streptomycin; these samples were then incubated at 37 C for 90 min without shaking, to allow expression of E2 refractivity before plating on NB plates plus 10^4 units of colicin E2. Plates were incubated at 25 C, and the E2-refractive colonies were then replica-plated to plates containing colicin E2 and colicin E3 at 25 and 40 C to confirm the Ref-II phenotype. For the direct selection of UV-resistant recombinants, blended samples were also allowed 90 min of expression time before plating, in this case at 37 C, on PEP plates [1% peptone (Oxoid), 0.1% Beef Extract (Difco), 1% sodium chloride], plus streptomycin. The plates were then irradiated with a UV dose of 260 ergs with a low-pressure mercury lamp (Hanovia Ltd) set at 46 cm. The presence of streptomycin in samples during incubation for expression of the UV^r and E2-refractivity markers prevented further growth and initiation of transfer by the *str*^{s} male strain. For the selection of T1^r recombinants, samples were plated (after blending) directly onto NB plates previously spread with 10^7 T1 phage particles.

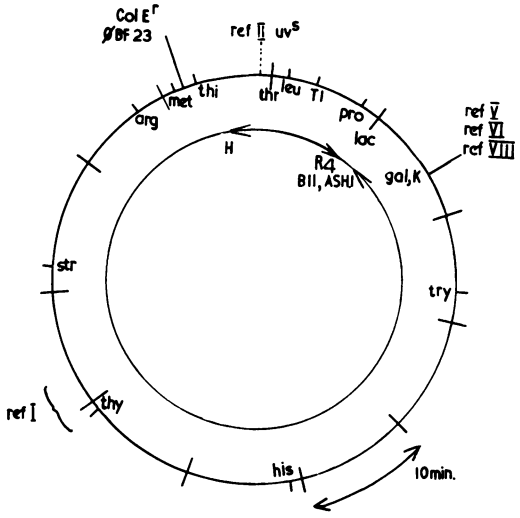

FIG. 1. *Linkage map of E. coli K-12 showing the origin of the Hfr strains used and the location of colicin E-refractory loci.*

RESULTS

Isolation of mutants refractory to colicin E2. Ref-II mutants refractory to E2 at 30 C but sensitive at 40 C were readily isolated from strain ASH10 (6). However, Ref-II mutants were not previously obtainable from other strains. In the present work, further attempts were made to isolate Ref-II mutants in additional strains, and it was observed that, if selection for, and sensitivity tests with, E2 refractory mutants were carried out at 25 C rather than 30 C, such mutants did appear in several strains (Table 1). The frequency of Ref-II mutants was, however, usually lower than with ASH10, and some strains still failed to produce any such mutants.

Linkage analysis of the Ref-II mutation. Several independently isolated Ref-II mutants of strain ASH10 were crossed with an HfrH male strain. Selection was made for *leu*, T1^r, and *lac* recombinants and, in each case, the recombinants were replica-plated to colicin E2 and E3 lawns at 25 and 40 C to determine the proportion that were still resistant to E2 at 25 C. The distribution of the E2-sensitivity marker among selected markers in a typical cross is shown in Table 2. The results indicated that the *ref*II locus was linked to *leu*; this was confirmed in crosses where selection was made for recombinants for the distal marker, *lac*. In all, 26 Ref-II, F$^-$ strains were crossed with the HfrH strain, and the proportion of the unselected markers, *leu* T1^r and E2-sensitivity, among *lac* recombinants was determined by replica plating. The results (Table 2) clearly indicated the presence of a single *ref*II locus closely linked with and to the left of the *leu* locus (Fig. 1).

Mapping of the refII locus by interrupted mating. Confirmation of the position of the *ref*II locus was also sought from interrupted

mating experiments. Mutants refractory to colicin E2 (Ref-II) were isolated in an HfrH strain (ASH54) and in HfrR4 (ASH55). The E2-refractory mutants were crossed with the F^-, colicin E-sensitive strain 203, and the time of entry of the *ref*II locus was determined as described above. As anticipated, colicin E2 refractivity enters close to the *thr* and *leu* loci (Fig. 2). The results with both male strains indicated a map position 1 to 2 min counterclockwise to the *thr* locus (*see* Fig. 1). The different scales used in Fig. 2 should be noted; in several experiments using ASH54, reduced numbers of Ref-II recombinants relative to other classes were obtained. Low (13) reported such an effect previously for markers very near the origin of the transferred chromosome.

Mapping studies with a UV-sensitive Ref-II mutant. It was reported previously (7) that, out of 20 Ref-II mutants examined, three strains were found to be UV-sensitive in addition to being refractive to colicin E2. Attempts were

TABLE 1. *Characteristics of some E. coli K-12 Ref-II mutants*

Strain no.	Origin	Sex	Response to UV	Other markers												
				met	*thi*	*arg*	*thy*	*his*	*try*	*gal*	*lac*	*leu*	*thr*	T1	*str*	λ
ASH50	AP[a] mutation from ASH10	F^-	R	−	−	+	−	+	+	+	−	−	+	S	R	+
ASH51	AP mutation from ASH10	F^-	R	−	−	+	−	+	+	+	−	−	+	S	R	+
ASH52	AP mutation from ASH10	F^-	R	−	−	+	−	+	+	+	−	−	+	S	R	+
ASH53	AP mutation from ASH10	F^-	R	−	−	+	−	+	+	+	−	−	+	S	R	+
ASH54	AP mutation from HfrH	Hfr	R	+	+	+	+	+	+	+	+	+	+	R	S	−
ASH55	AP mutation from HfrR4	Hfr	R	−	+	+	+	+	+	+	+	+	+	S	S	+
ASH57	AP mutation from 203	F^-	R	−	−	−	+	−	−	−	−	−	−	S	R	+
ASH101	AP mutation from ASH1	Hfr	R	−	−	+	−	+	+	+	+	−	+	S	R	−
ASH102	AP mutation from ASH1	Hfr	R	−	−	+	−	+	+	+	+	−	+	S	R	−
ASH112	AP mutation from ASH10	F^-	S	−	−	+	−	+	−	+	−	−	+	S	R	+
ASH113	AP mutation from ASH10	F^-	S	−	−	+	−	+	+	+	−	−	+	S	R	+

[a] 2-Amino purine.

TABLE 2. *Linkage analysis of Ref-II mutants*[a]

Ref-II mutants tested (21)	Recombinants			E2[s]	Type strains tested (24)	Selected marker	No. tested	Proportion of unselected markers		
	Selected marker	Input male	No. tested					T1[r] *str*[r]	*leu str*[r]	E2[s]
		%		%				%	%	%
(e.g., ASH50)	*leu str*[r]	2.7	199	72	(e.g., ASH52)	*lac str*[r]	333	86	79	54
	T1[r] *str*[r]	1.2	96	56						
	lac str[r]	0.8	228	34						
(e.g., ASH51)	*leu str*[r]	2.0	200	74	(e.g., ASH53)	*lac str*[r]	224	83	72	56
	T1[r] *str*[r]	1.2	124	49						
	lac str[r]	0.9	98	33						

[a] An HfrH strain (E2[s] T1[r] λ[−] *str*[s]) was crossed with several Ref-II derivatives of the F^- strain ASH10 (*met*[−] *thi*[−] *leu*[−] *lac*[−] *thy*[−] λ[+] Col-I[r] *str*[r]). Exponential cultures (10^8 cells/ml) of each strain were mixed; after 90 min at 37 C, selection was made for various recombinant classes; subsequently, the proportion of the unselected markers, including E2 sensitivity, was determined by replica-plating.

therefore made to map the UV-sensitivity locus in one such Ref-II mutant, ASH112. Interrupted mating experiments were carried out with this strain in crosses with two UV-resistant E2-sensitive male strains, HfrH and HfrR4. The time of entry of UV resistance was determined in each case, and the results showed (Fig. 3) that this UV-sensitivity locus is also located close to the *thr* marker. When the UV-resistant recombinants were replica-plated to NB plates containing colicin E2, all colonies appeared to be E2-sensitive. In strain ASH112, therefore, the UV-sensitivity locus and the *ref*II locus are extremely closely linked.

The rec⁻ character of Ref-II, UV^s mutants. Previous studies (7) indicated that the UV sensitivity of the Ref-II, UV^s mutants was of the Rec type. The recombination frequencies obtained with ASH112 and another UV^s Ref-II mutant, ASH113, in crosses with an HfrH strain were therefore determined (Table 3). Although the UV marker was transferred early in these crosses, markedly reduced recombination frequencies were obtained. Significantly different recombination-deficiency indices were obtained with the two mutants; the possible significance of this will be discussed.

Effect of the Ref-II mutation upon genetic recombination. Since the majority of Ref-II mutants, unlike ASH112, are not UV-sensitive, it was not anticipated that mutations at the *ref*II locus would affect recombination frequencies. As indicated in Table 2, this expectation was fulfilled; F⁻ strains carrying the Ref-II mutation all gave normal recombination frequencies. Moreover, when crosses were carried out at 25 C (where E2 refractivity is maximally expressed), similar results were obtained.

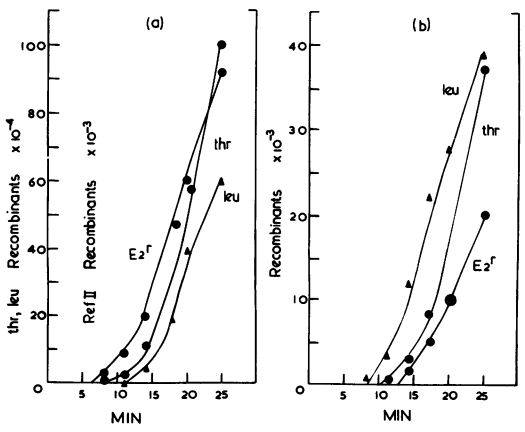

FIG. 2. *Time of entry of the colicin E2-refractory locus. (a) ASH54 (HfrH) crossed with E2-sensitive F⁻ strain 203; (b) ASH55 (HfrR4) crossed with E2-sensitive 203.*

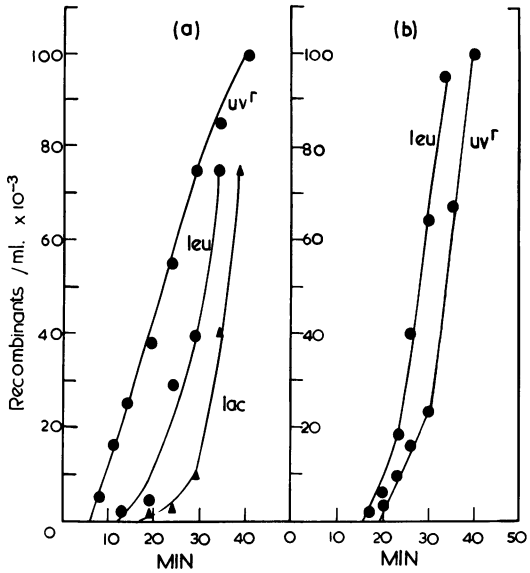

FIG. 3. *Time of entry of the UV^s marker in ASH112. (a) HfrH crossed with ASH112; (b) HfrR4 crossed with ASH112.*

Aberrant recombination frequencies were, however, sometimes obtained with Ref-II derivatives of ASH1, a male strain transferring the *ref*II locus as a tail marker (see Fig. 1). Table 4 shows a comparison of the results of crosses of a Ref-II derivative of HfrH (ASH54) and two Ref-II male strains (ASH101 and ASH102) derived from ASH1. The number of recombinants obtained in each case is presented as a percentage of that obtained in crosses with the corresponding E2-sensitive (*ref*+) males. Somewhat variable results were obtained with ASH101, the frequency of recombinants sometimes approaching that of the wild type, but ASH102 always gave the same low yield. The possibility was tested that the reduction in recombination frequency was due to, e.g., restriction of "Ref-II DNA" in the *ref*+ recipient. ASH102 was therefore crossed with a Ref-II derivative (ASH57) of the F⁻ strain 203. The results (Table 4) showed that, although both male and female strains carried a Ref-II mutation, the same marked reduction in recombinant formation occurred.

DISCUSSION

Mutation to E2 refractivity may be envisaged as a change in one of two main types of system. The Ref-II mutants at low temperature may fail to (i) transmit the effect of the adsorbed colicin through the cytoplasmic membrane, (ii) initiate

TABLE 3. *Reduced recombination frequencies with F⁻ strains carrying Ref-II UV^s mutations*[a]

Donor	Recipient	Recombinants as percentage of input male			Recombination-deficiency index (UV^r/UV^s)
		leu str^r	T1^r *str*^{r}	*lac str*^{r}	
HfrH	ASH10 $(UV)^r$	2.2	1.9	0.7	
HfrH	ASH113 (UV^s)	0.005		0.002	4.5×10^2
HfrH	ASH112 (UV^s)	0.33	0.17	0.09	7

[a] Mating conditions were as described in Table 2. The recombination deficiency index (1) was determined from the frequency of *leu str* recombinants in each cross.

TABLE 4. *Recombination frequencies obtained with male (Ref-II) strains*[a]

Donor	Recipient	Recombinants as percentage of that obtained with wild-type (*ref*$^+$) males				
		leu str^r	*lac str*^{r}	*gal str*^{r}	*try str*^{r}	*his str*^{r}
HfrH (Ref-II)	203 (*ref*$^+$)	100	100		100	
ASH101 (Ref-II)	203 (*ref*$^+$)			5	8	13
ASH102 (Ref-II)	203 (*ref*$^+$)			4	4	2
ASH102 (Ref-II)	ASH57 (Ref-II)			4	4	2

[a] Crosses carried out at 37 C as described in Table 2.

nuclease attack. Some specific change in the cell membrane could account for the former possibility, whereas, lack of a deoxyribonuclease, inhibition of its activity, or possibly even modification of its substrate DNA could account for the latter. Previous reports (6, 19) indicated that some early steps in the colicin E pathway were in fact common to both E2 and E3. Moreover, it has also been shown (5a) that the two colicins probably contain regions of similar structure. The specific refractivity of Ref-II mutants to E2, therefore, suggests that these mutants are blocked in some distal step in the pathway, e.g., perhaps some step affecting a deoxyribonuclease. This hypothesis gained some support from the finding (7) that several Ref-II mutants also showed an increased sensitivity to UV irradiation, a situation that has been presumed to arise after the loss of, or a change in, the activity of deoxyribonucleases or other repair enzymes (9). Genetic analysis of Ref-II mutants has now provided further indications that the *ref*II gene product may in some way be concerned with the activity of a deoxyribonuclease.

The mapping studies in this work were greatly facilitated by the proximal position of the *ref*II locus on the HfrH chromosome. This has allowed a fairly precise position, 1 to 2 min counterclockwise to *thr*, to be assigned to it. The position of the UV-sensitivity locus in the Ref-II mutant ASH112 was found to be extremely closely linked to the *ref*II locus. This finding, together with the high incidence of mutants like ASH112 among Ref-II strains, makes it improbable that E2 refractivity and UV sensitivity arose in these strains by two independent mutations. It appears more likely that the concommitant UV sensitivity of certain Ref-II mutants may be explained as the pleiotropic effects of a single gene or a polar mutation or deletion affecting closely linked genes. Fine-structure genetic analysis with bacteriophage P1 and reversion studies with the UV-sensitive Ref-II mutants should provide the answer to these alternatives.

Previous physiological studies (7) showed that the UV-sensitive Ref-II mutants resembled in several respects Rec^- rather than UVR^- mutants. Furthermore, it was observed in the present experiments that reduced recombination frequencies were obtained with ASH112 and ASH113 as recipients in crosses with male strains. Moreover, experiments (*unpublished*) have shown that the entry of the β-galactosidase gene, whether present on the chromosome or on a sex factor, from male donors is normal in these mutants. Such strains appear, therefore, to fulfill all the requirements of Rec mutants, and selection for E2 refractivity could be a convient method of isolating this particular group of *rec*$^-$ mutants. The two strains ASH112 and ASH113 were significantly different in their capacity for recombinant formation. Clark (1) has observed that strains with low-deficiency indices show "cautious" UV-induced DNA breakdown, whereas those mutants with high-deficiency indices show "reckless" breakdown.

Preliminary results (*unpublished*) indicate that both ASH112 and ASH113 are rather cautious in this respect, and alternative reasons for their markedly different deficiency indices must be sought.

The Ref-II mutation itself was found to have no effect upon recombination frequencies when present in the F^- and when crosses were carried out at 37 C or at 25 C, the latter temperature being that at which E2 refractivity is maximally expressed. In contrast, markedly reduced recombination frequencies were sometimes obtained in crosses in which the *ref*II locus was present in the male strain. This effect has been observed only with Ref-II derivatives of ASH1, a male strain carrying the *ref*II gene as a tail marker. The phenomenon is not due to an incompatibility between male and female DNA, comparable for example to that observed in interspecies restriction, since, if both male and female strains carry the *ref*II gene, reduced recombination frequencies are still observed. Experiments are now in progress to determine whether this effect is found with other male strains, especially those carrying a distal *ref*II locus, and also to identify the defective step in the conjugation process in these mutants.

The function of the *ref*II gene product in E2 action is still unclear, although it seems probable that it is associated with some late step in the pathway and possibly with the activity of a deoxyribonuclease. It seems unlikely, however, that the *ref*II gene determines the synthesis of a specific deoxyribonuclease. An alternative hypothesis that is now being considered is that the *ref*II gene determines the formation of a component of the cell membrane which may bind, and hence regulate, the activity of one or more deoxyribonucleases. In this connection, the map position of the *ref*II locus and the closely linked uv^s locus is particularly intriguing. Previous workers reported the location of rec^- (21) and r^-m^- (restriction-modification; 12, 22), genes in this relatively small region of the chromosome delineated by the origin of HfrH and *thr*. It is tempting to think that all these genes may constitute one or more operons concerned with the formation and regulation of various deoxyribonucleases and repair enzymes. Further studies will be directed toward determining the relationship of all these genes to each other.

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