Restriction Alleviation by Bacteriophages Lambda and Lambda Reverse

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Deletion analysis indicated that the phage λ restriction alleviation gene(s) *ral* resides between the *cIII* and *N* genes. The Ral^+ phenotype was expressed only when λral^+ carried a modification such that it was resistant to restriction by the host specificity system. Under these conditions, *Ral* function protected superinfecting unmodified phages from restriction by *EcoK* or *EcoB* but not from restriction by *EcoP1*. *Ral*-protected phage DNA was not concomitantly *K* and *B* modified, but rather received only the modification specified by the system of the restricting host. Possible mechanisms for *Ral* action are discussed. Of the other lambdoid phages tested, the hybrid phage λrev had *Ral* activity, whereas $\phi 80vir$ and one λ -P22 hybrid did not. The restriction alleviation activity of λrev called *Lar*, may be the same as the activity expressed in *sbca*⁻ strains of *Escherichia coli*, but it was functionally separable from exonuclease VIII activity (the product of the *recE* gene), which is also expressed in *sbca*⁻ strains.

Host modification restriction systems were first described by Arber and Dussoix (2, 11) and have been identified in a number of species and strains of bacteria (41). Presumably, modification and restriction have evolved to protect bacteria from invading foreign DNA, although additional roles have not been ruled out. The modification restriction loci of *Escherichia coli* K-12 and *E. coli* B and the SB system of *Salmonella typhimurium* are allelic and include three genes (*hsdS*, *hsdM*, and *hsdR*) (5, 15, 24, 53). The specificity of the system is determined by the *hsdS* gene (5). Modification and restriction in these bacterial species have been designated type I and are characterized by an enzyme complex which requires the cofactors S-adenosylmethionine, ATP, and Mg^{2+} to restrict unmodified DNA but only the S-adenosylmethionine cofactor to modify DNA (28, 32, 37, 55). Methylation occurs within the recognition sequence (45, 52), whereas endonucleolytic cleavage occurs outside this sequence and shows no apparent sequence preference for the actual DNA cleavage reaction (1, 23, 38).

Bacteriophages have evolved several ways to disrupt the complex process of modification and restriction and thus escape lethal cleavage. The temperate phage Mu is resistant to restriction by a variety of restriction enzymes because of methylation by a rather nonspecific modification function (*mom*) after induction from the pro-

phage state (51). The T-even phages escape restriction because DNA is glucosylated (12). T3 and T7 express *ocr* functions immediately upon infection, which prevent not only restriction but also modification by *EcoB* or *EcoK* (27, 47, 48). Although T3*ocr*⁻, T7*ocr*⁻, and T-even glucosylation-defective phages are sensitive to restriction, the rare phages that do escape restriction are modified. Another virulent phage, T5, must have some mechanism(s) to escape restriction by *EcoRI*, as T5 DNA has six sites sensitive to *EcoRI* in vitro (54), yet no *EcoRI* restriction or modification occurs in vivo (8).

The λ restriction alleviation function, *Ral*, was first characterized by Zabeau (M. Zabeau, Ph.D. thesis, Rijkuniversiteit Gent, Ghent, Belgium, 1974), whose observations have been published recently (9, 10, 58). Here I show that λ and λrev (a recombinant between the *Rac* prophage of *E. coli* K-12 and λ [25]) have functions (*Ral* and *Lar*) which can prevent restriction by *EcoB* and *EcoK*. In addition, I suggest that the *Lar* activity of λrev is the same activity that is expressed in *sbca*⁻ mutants of *E. coli* (44).

MATERIALS AND METHODS

Strains. Phage and bacterial strains are described in Table 1. M8521 is strain M5221 of H. Greer (18) selected to grow on eosin methylene blue (EMB) plates.

Media. λ broth contained 1% tryptone, 0.25% NaCl, and 0.1% yeast extract; λ plate agar and top agar contained, in addition, 1.1 and 0.65% agar, respectively, but no yeast extract. LB plate agar contained 1%

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TABLE 1. *List of bacterial and phage strains*

Strain	Restriction-modification phenotype	Other markers	Source or reference
Bacteria			
M72	K	su ⁻ lacamtrpamstr ⁻	E. Signer
M8002	K	M72(λ cIind831)	This work
M8521*	K	M72(bio255cI857H1)	18
M5222	K	M72(bio247cI857H1)	18
M8521(P1)	K, P1		This work
M5222(P1)	K, P1		This work
Q1	K	B1 ⁺ revertant of C600, ϕ 80 ^R	43
Q8002	K	Q1(λ cIind831)	I. Herskowitz
Q8003	K	Q1(λ imm ⁴³⁴ T)	I. Herskowitz
Q8004	K	Q1(λ imm ²¹)	I. Herskowitz
Q8100	O	C600 (r ⁻ m ⁻)	M. Meselson via F. W. Stahl
Q8803	O	r ⁻ m ⁻ gal ⁻ met ⁻ [803]	W. B. Wood via J. Engel
Q8209	B	r _B ⁺ m _B ⁺ F ⁺ [2027]	W. B. Wood via J. Engel
Q8302	B	Q8209(λ ind831)	This work
Q8303	B	Q8209(λ imm ⁴³⁴ T)	This work
Q8304	B	Q8209(λ imm ²¹)	This work
QD5003	K	Ymel, suIII ⁺	16
QA8002	K	CR63(h^{80} att ⁸⁰ cI857) λ^R	This work
QA8004	K	CR63(h^{80} att ⁸⁰ imm ²¹) λ^R	This work
QW11	K	recA56 recB21 [JC7507]	J. Clark via E. Signer
MI742	K	recA ⁻ str ⁻ su ⁻ (gal- λ) Δ	21
MI8000	K	MI742 (λ b2cI857Oam501Sam7)	A. Strathern
MI8000 λ^R	K	MI8000 made λ^R , mal ⁺	This work
AB1157	K	AB1157 suII	E. Signer
JC5519	K	recB21 recC22 str31	J. Gillen
JC8679	K	recB21 recC22 sbcA23	J. Gillen
JC8689	K	Rec ⁻ , UV ^s derivatives	J. Gillen
JC8690	K	JC8679 lacking	J. Gillen
JC8691	K	ExoVIII activity	J. Gillen
Phage			
λ bio10cI857			42
λ bio10imm ²¹ cI ⁻			
λ bio252cI857			
λ bio252imm ²¹ cI ⁻			
λ imm ⁴³⁴ cI6T			W. F. Dove
λh^{80} bio243imm ⁴³⁴ cI6T			E. Signer
λh^{80} att ⁸⁰ cI857			E. Signer
λ Sp ⁱ -imm ^{P22} c25(OP) ^{P22}			S. Hilliker
ϕ 80vir			E. Signer
λ rev exbio10cI857p4			This work
λ b2int102red3cI857p4			I. Herskowitz
P1c ⁺ CM			L. Rosner

tryptone, 0.5% NaCl, 0.5% yeast extract, and 1.5% agar. Low-salt tryptone top agar contained 1% tryptone, 0.6% agar, and 10⁻³ M MgCl₂. SM for phage dilutions has been described previously by Weigle et al. (56) and was supplemented with 0.01 M MgSO₄ and 0.01% gelatin. EMB agar was as described by Campbell (7), but with 0.05% maltose as a sugar supplement. OM medium for scoring λ deletion mutations contained sodium citrate and was as described by Manly et al. (36).

Phage stocks. Phage stocks were made from single plaques by the confluent lysis method. The single-plaque isolations were done in the same bacterial

strains used to make the stocks. Modified phage stocks were made in the following hosts: Q8100 for λ .O, Q8803 for λh^{80} .O, Q1 for λ .K, QD5003 for λh^{80} .K, Q8209 for λ .B, Q8100(P1) for λ .P, and Q1(P1) for λ .K,P.

Plating conditions. Plating bacteria were prepared daily by diluting a freshly saturated overnight culture 100-fold into λ broth containing 0.2% maltose and growing to a density of 5 $\times$ 10⁸ cells per ml (if λh^{80} phages were to be used, low-salt tryptone medium was used). A diluted phage sample (0.1 ml) was mixed with 0.2 ml of plating bacteria, adsorbed at room temperature for 20 min, mixed with 2.5 ml of top agar, and poured onto 1-day-old λ medium plates. λ top agar

and λ plate agar were used for λ . Low-salt tryptone top agar and λ plate agar were used for h^{80} and $\phi 80$. Plates were incubated at 37°C unless otherwise indicated.

Construction of lysogens. λ lysogens were formed by spotting phage at approximately 10^6 phage per ml onto bacteria in a λ agar or LB agar plate. After incubation at 34°C, the surviving bacteria were purified and tested for immunity and resistance.

To make P1 lysogens of M5222, M8521, Q1, and Q8100, 0.2-ml portions of plating bacteria were poured onto LB agar plates containing 2.5 mM CaCl_2 . P1c^+ CM was spotted onto these bacterial lawns, and single colonies were purified from the lysis areas on λ agar plates containing 12.5 μg of chloramphenicol per ml. Colonies of purified isolates were tested on EMB plates for the ability to restrict $\lambda\text{imm}^{434}\text{cI.K}$ and the failure to restrict $\lambda\text{imm}^{434}\text{cI.K.P}$.

Coinfections. A fresh overnight culture was diluted 100-fold into λ broth containing 0.2% maltose and grown to a density of 1×10^8 to 2×10^8 cells per ml at 37°C on a reciprocating shaker. Bacteria were centrifuged and then suspended at 10^8 cells per ml in 0.01 M MgSO_4 when the helper was $h\lambda$ and in 0.001 M MgSO_4 when the helper was h^{80} ; 0.2-ml portions of bacteria and 0.2-ml portions of helper phage were mixed in conical 12-ml glass centrifuge tubes at a final multiplicity of infection (MOI) of 5 (unless otherwise indicated) and adsorbed at room temperature for 15 min. LB broth (3 ml) was added to each tube and the infected cells were aerated by shaking at 37°C for 10 min. A sample was diluted into SM containing chloroform and titrated for unadsorbed phage. The infected cells were centrifuged, 0.2 ml of test phage (MOI, 0.25, unless otherwise indicated) was added, and the pellet was blended with a Vortex mixer to suspend the bacteria. After further phage adsorption for 15 min at room temperature, the cells were (i) either diluted 100-fold into prewarmed LB broth and grown for a single cycle of growth to measure phage yield or plated directly with the appropriate indicator bacteria for infective center formation or both and (ii) diluted into SM containing CHCl_3 to measure unadsorbed phage. When several indicator strains were used to determine the yields of individual phage types, λvir was titrated on all indicator strains to be sure that the phage plating efficiencies were comparable and within the experimental sampling error.

Control infections were done in parallel; in these infections both helper and test phages were modified to determine (i) whether the Ral helper effect was specific for stimulating unmodified phage growth and (ii) whether phage yields under nonrestricting conditions were normal. Each coinfection described below was done at least twice in the host indicated and, in several instances, in additional bacterial strains.

Single cycle of growth. M8521 and M5222 (lysogens carrying defective Ral^+ and Ral^- prophages, respectively) were grown under both noninducing (33°C) and inducing (40°C) conditions and used subsequently at the corresponding temperatures to test their abilities to support the growth of both modified and unmodified superinfecting phages; 100-fold dilutions of saturated cultures were grown to a density of 2×10^8 cells per ml and then suspended in 0.01 M MgSO_4 . A

0.2-ml portion of bacteria and 0.2 ml of phage were mixed at a final MOI of 0.25 and adsorbed for 20 min at room temperature. Infected cells were diluted 100-fold into prewarmed LB broth at the corresponding temperatures and 100-fold into SM containing CHCl_3 to measure unadsorbed phage. At 10-min intervals samples were withdrawn, treated with CHCl_3 , and titrated. The endpoint and phage yields of the single cycles of growth were determined when the phage titers reached a plateau.

Ral effect on the modification of nonreplicating phage. Coinfections were performed as described above, except that both the helper and test phages were added at MOIs of 10, and after adsorption of the test phage λ antibody was added at a final K of 45 before plating for infective centers.

Isolation of $\lambda\text{rev exbio10cI857}$. $\lambda\text{red}^- \text{gam}^-$ phages, such as $\lambda\text{bio10cI857}$, cannot grow in recA^- hosts and are said to exhibit the Fec^- phenotype (59). To enrich for λrev in a stock of $\lambda\text{bio10cI857}$, phage were grown for a single lytic cycle in the recA^- strain MI742 at an MOI of 50. λrev was isolated at a frequency of 5×10^{-9} from this stock as large plaque formers on the recA^- strain MI742. (The frequency of isolation of λrev from a stock of $\lambda\text{bio252cI857}$ treated in parallel was 9.6×10^{-9} .) Besides the Fec^+ phenotype, $\lambda\text{rev exbio10cI857}$ shared the following properties with previously isolated λrev phage: it grew in P2 lysogens (60), it grew on OM medium plates, and it grew much more poorly than its parent ($\lambda\text{bio10cI857}$) at 43°C.

RESULTS

Ral function of λ prevents restriction of superinfecting bacteriophage. The data in Table 2 are consistent with the hypothesis that λ has a function, Ral, which can prevent restriction of unmodified phage by the host specificity system. Ral appeared to be present after infections with hybrid phages carrying the bio252 substitution but not after infections with hybrid phages carrying bio10 (Table 2, coinfections 1 through 4). Preinfection with $\lambda\text{bio252cI857}^{21}\text{.K}$ and $\lambda\text{bio252cI857.K}$ partially blocked restriction of superinfecting $\lambda\text{imm}^{434}\text{.0}$, increasing the yield of λimm^{434} by factors of 143 and 17, respectively. In other coinfections (Table 2, coinfections 5 through 8), both $\text{Ral}^+ \lambda\text{bio252.0}$ and $\text{Ral}^- \lambda\text{bio10.0}$ helper phage yields were approximately 10^{-3} , indicating Ral^+ and Ral^- phages were restricted to the same extent. In addition, the Ral^+ helper phages (coinfections 5 and 7) did not block restriction of superinfecting $\lambda\text{imm}^{434}\text{.0}$. Thus, restricted phages could not direct the synthesis of Ral, which may be the reason why $\lambda\text{ral}^+\text{.0}$ phages fail to prevent their own restriction.

The magnitude of the Ral effect varied with the growth phase, the culture conditions, the bacterial strain, and the genotype of the infecting phage. Even the relatively small Ral effect shown in Table 2 would have a sizeable selective

TABLE 2. Expression of *Ral* during coinfection

Coinfection ^a		Phage yields during coinfection		Helper effect
No.	Helper phage	Yield of helper	Yield of λ imm ⁴³⁴ .K	
1	λ bio252imm ²¹ .K	162 (88) ^b	4.3×10^{-1} (25)	143
2	λ bio10imm ²¹ .K	122	6.0×10^{-3}	2.0
3	λ bio252imm ¹ .K	88	5.0×10^{-2}	17
4	λ bio10imm ¹ .K	135	9.0×10^{-3}	3.0
5	λ bio252imm ²¹ .O	2×10^{-3} (9.4×10^{-4})	7.0×10^{-3} (170)	2.3
6	λ bio10imm ²¹ .O	4×10^{-3}	1.0×10^{-2}	3.3
7	λ bio252imm ¹ .O	2×10^{-4}	5.0×10^{-3}	1.7
8	λ bio10imm ¹ .O	1×10^{-4}	1.0×10^{-2}	3.3
9	None		3.0×10^{-3} (160)	

^a The superinfecting phage was λ imm⁴³⁴.O. Coinfections were in bacterial strain Q1, as described in the text. *imm* λ helper phages carried cI857, *imm*²¹ phages were cI⁻, and the test phage was λ imm⁴³⁴.cI6T. Yields of helper phages were determined by plating on Q8003 (r_K^+ m_K^+ , λ), and yields of test phages were determined by plating on Q8002 (r_K^+ m_K^+ , λ) or Q8004 (r_K^+ m_K^+ , λ imm²¹).

^b Yields in parentheses are from coinfections done in parallel in which the superinfecting test phage (λ imm⁴³⁴.K) was modified.

advantage after many generations. λ ral.0 would not have to be able to protect itself in order for the *ral* gene to show a selective advantage. In coinfections such as those described in Table 2, λ ral⁺ in the wild could prevent restriction of a superinfecting λ ral⁺ phage carrying an inappropriate modification; in such a case, perpetuating your kind is the same as perpetuating yourself.

Ral activity does not require host Rec or phage Red recombination functions. In the experiments shown in Table 2, *Ral* activity was assayed by the ability to rescue phage carrying the immunity region of 434. Since this region does not carry a known K restriction site, I felt that *Ral* might act by stimulating recombination of the helper with restricted fragments of DNA rather than by rescuing entire genomes. Table 3 shows that *Ral* did not require host recombination function RecA or RecB. Preinfection with λ ral⁺ (in this case, λ bio252imm²¹.cI⁻.K) increased the number of infective centers formed by superinfecting λ bio10cI857.0 131-fold. Likewise, *Ral* did not act by stimulating Red rescue of restricted fragments of DNA since both the *Ral*⁺ helper phage λ bio252imm²¹ and the test phage λ bio10cI857.0 were Red⁻.

Ral expression in the prophage state is under immunity control. To determine which λ genes were essential for *Ral* function and whether *Ral* was expressed in the prophage state, the λ cI857 lysogens carrying the H1 deletion and the *bio* substitutions shown in Fig. 1 were infected with λ imm⁴³⁴.0 before and after thermal induction. λ imm⁴³⁴.0 was able to grow in M8521 (*bio*255cI857 H1) at 40 but not at 34°C (Table 4), implying that the *Ral* activity of the prophage was under immunity control. *Ral* activity was not expressed in M5222 (*bio*247cI857) at either 34 or 40°C. These results showed that

TABLE 3. *Ral* does not require host RecAB or phage Red recombination functions for activity^a

Helper phage	No. of infective centers produced upon coinfection of QW11 (<i>recA</i> ⁻ <i>recB</i> ⁻)		
	Helper phage	Test phage	Helper effect
λ bio252imm ²¹ .K	0.21 (0.17) ^b	6.8×10^{-4} (0.01)	131
λ bio10imm ²¹ .K	0.14	8.0×10^{-5}	1.5
None		5.2×10^{-5}	1

^a The superinfecting test phage was λ bio10.O. Coinfections were performed in strain QW11 as described in the text. Helper phages carried *imm*²¹.cI⁻; the superinfecting phage was λ bio10cI857. Infective center formation of λ bio10cI857 was assayed on strain Q8004 (r_K^+ m_K^+ , λ imm²¹), whereas helper phage infective centers were determined on strain Q8002 (r_K^+ m_K^+ , λ).

^b The numbers in parentheses are from coinfections done in parallel in which the superinfecting test phage (λ bio10.K) was modified.

FIG. 1. Prophage map of λ indicating relevant substitutions and deletions. The regions of DNA deleted by the various deletions and substitutions are indicated by the black lines. The figure is not drawn to genetic or physical scale. The *bio* and *att80* substitutions each carry a K restriction site, and the morphogenesis region probably carries three K sites. K restriction recognition sites *sk2* and *sk1* are indicated on the map (39).

the *Ral* function(s) is located to the right of the *bio*255 substitution and is at least partially deleted by the *bio*247 deletion. *ral* may be located to the left of the immunity 2S1 substitution, as

TABLE 4. *Ral* expression by a defective λ cI857 prophage after thermal induction^a

Bacterial strain	Defective prophage	Temp (°C)	Yields of the following superinfecting phages before and after thermal induction of prophage:	
			λ bio10imm ²¹ .O	λ bio10imm ²¹ .K
M8521	bio255cI857H1	33	2.0×10^{-4}	7.5
		40	0.11 (275)	33 (4.4)
M5222	bio247cI857H1	33	6.4×10^{-4}	7.5
		40	9.0×10^{-4} (1.4)	60 (8.0)

^a Single cycles of growth were accomplished as described in the text. The numbers in parentheses are the increases in plating efficiency at 40 compared with the plating efficiency at 33°C. Phage yields were determined by plating on strain Q1 ($r_K^+ m_K^+$). The phage yield determined on strain Q1 was 0.72 that determined on strain Q8100 ($r^- m^-$).

λ bio252imm²¹ hybrids had *Ral* activity (Tables 2 and 3). Alternatively, the λ imm²¹ phage may have a hybrid *Ral* function partially encoded by the immunity region of phage 21 and partially encoded by the *ral* gene of λ .

In the experiments shown in Table 4, the progeny phage produced in the presence of *Ral* had an efficiency of plating of 0.72 ± 0.06 on a restricting host (Q1) compared with the isogenic nonrestricting host (Q8100), whereas the control for efficiency of plating (λ vir.K) plated with an efficiency of 0.90 ± 0.04 . This result is consistent with the hypothesis that at least 80% of the *Ral*-protected progeny are fully modified or that all of the *Ral*-protected phage lack modification at one site.

***Ral* alleviates restriction by *EcoK* and *EcoB* but not by *EcoP1*.** To determine whether *Ral* could act on other restriction systems, the coinfections shown in Tables 5 and 6 were performed. Preinfection of the $r_B^+ m_B^+$ strain Q8209 with the *Ral*⁺ helper λ bio252cI857.B increased the yield of superinfecting unmodified λ imm⁴³⁴.cI.B by a factor of 46 (Table 5). Clearly, *Ral* could alleviate *EcoB* restriction as effectively as it could alleviate *EcoK* restriction.

However, in the experiments shown in Table 6, under conditions in which *Ral* alleviated *EcoK* restriction substantially, no specific effect of *Ral* on *P1* restriction was observed. At a high temperature, where *Ral* was expressed in the M8521(*P1*) lysogen, there was a 2,750-fold increase in the plating efficiency of λ imm⁴³⁴.P, but only a 7.7-fold increase in the plating efficiency of λ imm⁴³².K, which was not significantly different from the 2.9-fold effect on the totally modified control (λ imm⁴³⁴.K,P).

***Ral* is not a methylase with both K specificity and B specificity.** From the results of the experiments shown in Table 4, I concluded that the progeny resulting from a *Ral*-protected infection were K modified. Because *Ral* protects DNA from *EcoB*, it was of interest to determine whether *Ral* was a methylase which acted independently of the host modification and restric-

TABLE 5. *Ral* alleviates *E. coli* B restriction^a

Helper phage	Phage yields of λ imm ⁴³⁴ during coinfection of an $r_B^+ m_B^+$ host		
	Helper phage	Test phage	Helper effect
λ bio252cI857.B	29 (11) ^b	0.46 (5)	46
λ bio10cI857.B	23 (25)	0.004 (10)	0.4
λ cI857.B	43 (45)	4.9 (16)	490 ^c
λ rev exbio10cI857.B	9 (45)	1.9 (9)	190 ^d
None		0.01 (30)	

^a The superinfecting test phage was λ imm⁴³⁴.O. Coinfections were performed in strain Q8209 ($r_B^+ m_B^+$) as described in the text. λ imm⁴³⁴ carried cI6T. λ imm⁴³⁴ phage yields were calculated on strain Q8302($r_B^+ m_B^+$, λ), and helper phage yields were determined on strain Q8803 ($r_B^+ m_B^+$, λ imm⁴³⁴).

^b The yields in parentheses are for the corresponding coinfections in which the superinfecting phage (λ imm⁴³⁴.cI.B) was modified.

^c The helper effect was due to *Ral* and *Red*.

^d The helper effect was due to *Lar* and *Der*.

tion system to modify DNA at both K and B sites. Preinfection of the $r_K^- m_K^-$ host Q8100 with the *Ral*⁺ phage λ bio252cI857.0 did not result in B and K modifications of the superinfecting λ imm⁴³⁴.0 (data not shown). However, it is conceivable that *Ral* methylated only the parental phage DNA and that this parental modification was subsequently lost through rounds of replications under the $r_K^- m_K^-$ conditions. To observe the effect of *Ral* on parental phage, the infections shown in Table 7 were performed. In these experiments, I tried to determine whether superinfecting parental DNA became B modified by *Ral* without enlisting the help of *EcoB*. To score the modification acquired by parental phage DNA, infections were performed in lysogens homoimmune to the superinfecting phage. In this way, DNA replication was blocked by the repressor of the resident prophage, whereas DNA maturation and morphogenesis functions were supplied by the heteroimmune helper phage. In conclusion, when the $r_K^+ m_K^+$ lysogen M8002 was superinfected with λ b2int⁻.red⁻cI⁻p4.K after preinfection with either *Ral*⁺ or *Ral*⁻ helper phage, the progeny phages were not B modified, as they failed to form plaques

TABLE 6. *Ral* does not alleviate P1 restriction

P1 lysogen	Temp (°C)	Efficiency of plating of: ^a		
		$\lambda imm^{434}.K$	$\lambda imm^{434}.P$	$\lambda imm^{434}.K.P$
M8521 (P1)	34	6.0×10^{-6}	4.0×10^{-5}	0.38
	40	4.6×10^{-5} (7.7) ^b	0.11 (2,750)	1.1 (2.9)
M5222 (P1)	34	3.7×10^{-5}	6.9×10^{-5}	0.65
	40	7.7×10^{-5} (2.1)	4.9×10^{-4} (7.1)	1.1 (1.7)

^a Efficiency of plating of λimm^{434} on P1 lysogens in the presence (40°C) and absence (34°C) of *Ral*. λimm^{434} was $\lambda imm^{434}.cI6T$. Phage were titrated as described in the text. Indicator cultures were grown and petri plates were incubated at the temperatures indicated. Efficiency of plating was measured with respect to the titer on strain Q8100 ($r^- m^-$).

^b The numbers in parentheses are the increases in plating efficiency for each phage/host combination at the higher temperature.

TABLE 7. *Ral* is not a methylase which modifies parental phage DNA upon injection^a

Coinfection	Superinfecting phage	Helper phage	Phage yields from coinfections of $r_K^+ m_K^+ \lambda$ lysogen			
			Yield of superinfecting phage in the following hosts:			Yield of helper phage
			$r^- m^-$	$r_K^+ m_K^+$	$r_B^+ m_B^+$	
1	$\lambda b2cI^- p4.K$	$\lambda bio252imm^{21}.K$	0.25	0.32	3.1×10^{-4}	13
2	$\lambda b2cI^- p4.K$	$\lambda bio10imm^{21}.K$	0.32	0.38	$<1 \times 10^{-3}$	20
3	$\lambda b2cI^- p4.B$	$\lambda bio252imm^{21}.K$	0.09	0.09	0.02	18
4	$\lambda b2cI^- p4.B$	$\lambda bio10imm^{21}.K$	9.5×10^{-4}	6.4×10^{-4}	6.4×10^{-4}	22

^a The procedure for the *Ral* rescue of nonreplicating phage is described in the text. The MOIs of both helper phage and test phage were each 10. Helper phages carried $imm^{21}.cI^-$; $\lambda b2cI^- p4$ was $\lambda b2int102red3cI857p4$. Coinfections were in strain M8002, a λ lysogen of bacterial strain M72. (M72 does not exhibit any MOI effect on the ability to restrict unmodified phage over a MOI range of 0.25 to 15 [data not shown].) $\lambda cI^- p4$ were scored as small plaques on the following λimm^{21} lysogens: strain Q8104 ($r^- m^-$), strain Q8004 ($r_K^+ m_K^+$), and strain Q8304 ($r_B^+ m_B^+$). The yields of helper phages were determined by plating on the λ lysogen Q8002 ($r_K^+ m_K^+$). In addition, $\lambda cI^- p4$ were scored on strain M1742, a *RecA*⁻ host (data not shown). Yields were equivalent to the yields found with strain Q8004; thus, $\lambda cI^- p4$ did not rescue the *bio* region of the helper phage.

on an $r_B^+ m_B^+$ host. As maturation of unreplated phage requires recombination to produce DNA of maturable length (46), there was a question as to whether the putative *EcoB* modification of the parental superinfecting DNA could have been lost by recombination with the replicating helper DNA. That this was unlikely was shown by additional experiments (Table 7, coinfections 3 and 4), in which the test phage already carried a B modification. In coinfection 3, $\lambda b2int^- red^- cI^- p4.B$ phage matured under restricting conditions but, in the presence of *Ral*, retained parental modification (or at least a good fraction did, as the yields calculated on the isogenic $r_B^+ m_B^+$ and $r_K^+ m_K^+$ strains were 0.02 and 0.09, respectively). Since parental modification was retained on the *Ral*-protected phage during the necessary recombination and maturation steps, it is likely that any *de novo* *Ral* modification of *EcoB* sites on $\lambda b2int^- red^- cI^- p4.K$ (Table 7, coinfection 1) would also have survived subsequent recombination and maturation if it had actually occurred. Thus, it is unlikely that *Ral* is a methylase which can recognize *EcoB* and *EcoK* sites independently of the host modification restriction system.

λ -P22 hybrid $\lambda Spi^- imm^{P22} c25(OP)^{P22}$ and $\phi 80$ do not express *Ral* functions. *S. typhimurium* has a modification and restriction system (*hdsSB*) which is allelic to the systems of *E. coli* K-12 and *E. coli* B (53). Because the arrangement of genes in *Salmonella* phage P22 is like the arrangement in λ (4), it was of interest to determine whether P22 carried a *Ral* function in a position homologous to the position of *Ral* in λ . The λ -P22 hybrid $\lambda Spi^- imm^{P22} c25(OP)^{P22}$ carries a substitution of P22 DNA extending from *red* through the *ren* genes of λ (Fig. 1) (22, 50). Since this hybrid carries the P22 *erf* gene, it was necessary to test *Ral* activity on a phage whose restricted fragments could not be rescued by *Erf*-promoted recombination (Table 8). The unmodified test phage chosen was $\lambda h^{80} att^{80}.cI857.0$, which has at least two K restriction sites in DNA segments nonhomologous to the λ -P22 hybrid; one of these sites (*sk* $\phi 80$) segregates with *h80*, and the other (*sk*2) segregates with immunity of λ (39). Hence, progeny phage were scored as being measured by *Ral* if they had both the immunity of λ and the host range of $\phi 80$. Under conditions in which preinfection by $\lambda bio-252imm^{21}.cI^- .K$ stimulated the formation of in-

TABLE 8. $\lambda\text{Spi}^- \text{imm}^{\text{P22}}(\text{OP})^{\text{P22}}$ is phenotypically Ral^- ^a

Helper phage	Infective centers made by the following superinfecting phages during coinfections:	
	$\lambda\text{H}^{\text{80}} \text{att}^{\text{80}}.0$	$\lambda\text{imm}^{\text{434}}.0$
$\lambda\text{Spi}^- \text{imm}^{\text{P22}}(\text{OP})^{\text{P22}}.K$	1.3×10^{-3} (0.21)	0.23 ^b (0.60)
$\lambda\text{bio252imm}^{\text{21}}.K$	0.23 (0.40)	NT ^c (NT)
$\lambda\text{bio10imm}^{\text{21}}.K$	7.3×10^{-4} (NT)	NT (NT)
None	1.1×10^{-3} (0.67)	4.0×10^{-4} (0.90)

^a Coinfections were performed in bacterial strain QD5003 as described in the text. Infective centers of helper phages were determined on strain Q8003 (data not shown, but all were approximately 1). The numbers in parentheses are the yields in parallel coinfections of the corresponding modified superinfecting phages. Infective centers of superinfecting $\lambda\text{imm}^{\text{434}}\text{cI6T}$ were assayed in strain Q8004, and infective centers of superinfecting phage $\lambda\text{H}^{\text{80}} \text{att}^{\text{80}}\text{cI857}$ were assayed in strain QA8002.

^b Erf-promoted rescue of imm^{434} .

^c NT, Not tested.

fective centers by superinfecting $\lambda\text{H}^{\text{80}} \text{att}^{\text{80}}\text{cI857}.0$ 200-fold, $\lambda\text{Spi}^- \text{imm}^{\text{P22}}\text{c25}(\text{OP})^{\text{P22}}$ had no effect. The rescue of $\lambda\text{imm}^{\text{434}}.0$ (a phage with more extensive homology with the λ -P22 hybrid) was probably due to Erf rescue of the immunity 434 region of restricted DNA. The lack of restriction alleviation by the λ -P22 hybrid was not due to expression of an inhibitor in the P22 immunity region or to insufficient expression of a Ral from the P22 leftward promoter, because $\lambda\text{bio252imm}^{\text{P22}}(\text{OP})^{\text{P22}}$, which contains the P22 promoter, could supply the Ral function of λ during coinfection (data not shown).

Similarly, another lambdoid phage ($\phi 80$) was tested for restriction alleviation. In the experiments shown in Table 9, $\phi 80$ did not express a Ral function. Preinfection with $\phi 80\text{vir}$ did not prevent restriction of $\lambda\text{imm}^{\text{434}}.0$ by EcoK , as the yield of $\lambda\text{imm}^{\text{434}}$ was not increased by the presence of the $\phi 80\text{vir}$ helper.

λrev has a restriction alleviation function, Lar . λrev (isolated as a revertant of $\lambda\text{red}^- \text{gam}^-$, which formed plaques on RecA^- hosts) carries a substitution of foreign DNA between $b2$ and $c\text{III}$ which codes for a new attachment site, as well as an integrase and recombination function (Fig. 1) (17, 59). λrev has rescued the recE gene from *E. coli* and expresses its product, exonuclease VIII (ExoVIII), upon infection (13), which gives rise to the Der^+ phenotype (Red in reverse) and the ability of this " Red^- " gam^- phage to plate under RecA^- conditions. The recE gene is expressed in sbca^- mutants of *E. coli* (49). sbca^- mutants were selected as mitomycin C-resistant, Rec^+ revertants of $\text{recB}^- \text{recC}^-$ strains (3) and have the additional phenotype that they exhibit a substantially increased plating efficiency of $\lambda.0$ (44).

These data suggested that λrev was probably created by a recombination event with *E. coli* DNA which picked up the recE gene and a nearby " int " gene, indicating that the sbca locus may be part of a cryptic prophage (17). The

TABLE 9. $\phi 80$ is phenotypically Ral^- ^a

Helper phage	Phage yields on coinfection	
	Yield of helper	Yield of $\lambda\text{imm}^{\text{434}}$
$\phi 80$	105 (135)	0.02 (12)
None		0.04 (55)

^a The superinfecting phage was $\lambda\text{imm}^{\text{434}}.0$. The coinfections were performed in bacterial strain QD5003 as described in the text. Yields in parentheses are for parallel coinfections where the test phage was modified. $\lambda\text{imm}^{\text{434}}$ was $\lambda\text{imm}^{\text{434}}\text{cI6T}$, and $\phi 80$ was $\phi 80\text{vir}$. The yields of $\lambda\text{imm}^{\text{434}}$ were determined on the $\phi 80^{\text{R}}$ host strain Q1, and $\phi 80\text{vir}$ were assayed on strain QD5003.

increased plating efficiency of $\lambda.0$ on sbca^- strains indicates that this cryptic prophage might also express a Lar function analogous to the Ral function of λ . To test this hypothesis, plaque-forming phage were selected on a recA^- host from a stock of the Ral^- phage $\lambda\text{bio10cI857}$ (see above). This $\lambda\text{rev exbio10}$ had the same properties as previously isolated λrev phages (17, 59): (i) it grew in recA^- and polA^- bacterial strains and P2 lysogens (60), (ii) it plated on OM medium plates containing citrate, indicating that it had a net deletion of phage DNA. (iii) and it grew poorly at a high temperature (43°C).

$\lambda\text{rev exbio10cI857}$ appeared to have a Lar function which prevented restriction of the superinfecting $\lambda\text{H}^{\text{80}} \text{bio243imm}^{\text{434}}.0$ phage (Table 10). The number of $\text{r}_\text{K}^+ \text{m}_\text{K}^+$ cells infected with $\lambda\text{H}^{\text{80}} \text{bio243imm}^{\text{434}}.0$ which formed infective centers increased 50-fold in the presence of the λrev helper phage, when $\lambda\text{H}^{\text{80}} \text{imm}^{\text{434}}$ infective centers were selected for (Table 10). This was probably a minimum Lar effect since coinfection with the λrev helper phage reduced the probability of infective center formation approximately five-fold when the superinfecting phage was modified (Table 10). These $\lambda\text{H}^{\text{80}} \text{imm}^{\text{434}}$ phages probably retained the bio substitution, as no $\lambda\text{H}^{\text{80}} \text{revimm}^{\text{434}}$ recombinants were scored as infective

TABLE 10. *λrev* is phenotypically *Ral*^a

Helper phage	Infective centers and yields produced by the following phages during coinfection			
	<i>λh</i> ⁸⁰ <i>bio243imm</i> ^{434b}		<i>λrevimm</i> ^{434c}	
	Infective centers	Yield	Infective centers	Yield
<i>λrev exbio10cI857.K</i>	0.05 (0.08)	35 (27)	0.02 (0.02)	0.7 (0.7)
None	0.001 (0.38)	208 (116)	<5.3 × 10 ⁻⁵ (<3.1 × 10 ⁻⁴)	

^a The superinfecting phage was *λh*⁸⁰*bio243imm*⁴³⁴.O. Coinfections were performed as described in the text. *λh*⁸⁰*bio243imm*⁴³⁴ carried the *cI6T* mutation. The numbers in parentheses are from parallel coinfections where the superinfecting phage was modified. Infective centers were determined on *rK*⁺ *mK*⁺ hosts.

^b Infective centers were determined on strain QA8002 (*λ*^R, *λh*⁸⁰*cI857*). Plaques were very tiny due to the heteroimmune prophage exclusion exhibited by certain *λh*⁸⁰ prophages against certain *λh*⁸⁰ superinfecting phages (P. Toothman and D. Forbes, unpublished data). No infective centers were detected on strain MI8000 *λ*^R (*recA*⁻, *λ* lysogen), indicating that the *λh*⁸⁰*imm*⁴³⁴ infective centers on strain QA8002 carried *bio243* and not *rev*. The helper effect was due to *Lar*.

^c Infective centers and yields of *rev imm*⁴³⁴ recombinants were assayed on strain MI8000 (*Rec*⁻, *λ*). The helper effect was due to *Der*.

centers on the appropriate selection host (data not shown). It is unlikely that the *λh*⁸⁰*bio243imm*⁴³⁴ progeny were rescued by the *Der* recombination function, unless for some other reason *EcoK* failed to restrict both the site in the *bio* substitution and the site (*sk2*) between *bio243* and *imm*⁴³⁴. However, if such were the case, *λh*⁸⁰*bio243imm*⁴³⁴ should have behaved as if it had only one restriction site (*sk1*), and it would not have been restricted so efficiently (i.e., only 50-fold instead of 1,000-fold) (39).

Lar and *Der* have very different effects on the average burst size of the phages which they rescue, where average burst is defined as yield per infective center. The average burst of *λh*⁸⁰*imm*⁴³⁴, *Lar*-protected phage was large (equivalent to the burst of the modified controls) (Table 10). The average burst of the *λrevimm*⁴³⁴ recombinants rescued by *Der* was small, approximately 1 (Table 10). This difference may reflect the times that *Lar* and *Der* act during infection (early and late, respectively). However, from these data it is impossible to tell whether *Der* acted before or after *Lar* or even whether *Der* acted in the same cells as *Lar*.

Table 11 presents further evidence that *Lar* and *Der* are separate functions. Gillen et al. (14) isolated *Rec*⁻ UV-sensitive revertants of *recB*⁻ *recC*⁻ *sbca*⁻ mutants in an attempt to isolate mutations in the *recE* structural gene. Three such revertants which contained no detectable ExoVIII protein are listed in Table 12. Two of these (JC8689 and JC8690) simultaneously reverted to the stringent restriction phenotype. These mutants may be *sbca*⁻ revertants or deletions of *recE* and *lar*. However, JC8691 retained its restriction alleviation activity despite its loss of ExoVIII. Thus, *Lar* activity expressed in *sbca*⁻ strains is separable from, or at least does not require, ExoVIII activity.

TABLE 11. Restriction alleviation activity of *sbca*⁻ strains of *E. coli* is not due to ExoVIII activity^a

Bacterial strain	Efficiency of plating of <i>λimm</i> ⁴³⁴ .O	Restriction alleviation
JC5519 (<i>recB</i> ⁻ <i>recC</i> ⁻)	2.0 × 10 ⁻⁵ (0.50)	1
JC8679 (<i>recB</i> ⁻ <i>recC</i> ⁻ <i>sbca</i> ⁻)	1.3 × 10 ⁻¹ (0.56)	6.5 × 10 ³
JC8689 (<i>Rec</i> ⁻ revertant)	6.0 × 10 ⁻⁵ (0.34)	3
JC8690 (<i>Rec</i> ⁻ revertant)	6.0 × 10 ⁻⁵ (0.38)	3
JC8691 (<i>Rec</i> ⁻ revertant)	1.3 × 10 ⁻² (0.38)	6.5 × 10 ²

^a Phage were titrated as described in the text. *λimm*⁴³⁴ was *λimm*⁴³⁴*cI6T*. The numbers in parentheses are the plating efficiencies of *λimm*⁴³⁴.K. Restriction alleviation was measured with respect to the plating efficiency on strain JC5519. Bacterial strains JC8689, JC8690, and JC8691 were *Rec*⁻ revertants of strain JC8679 and contained no enzymatically active ExoVIII protein (Jane Gillen, personal communication).

TABLE 12. *Ral* is expressed in AB1157^a

Helper phage	Phage yields during coinfections		Helper effect
	Yield of helper	Yield of <i>λimm</i> ⁴³⁴	
<i>λbio252cI857.K</i>	17 (16)	0.02 (9)	20
<i>λbio10cI857.K</i>	15 (20)	0.001 (6)	1
<i>λrev exbio10cI857.K</i> ^b	32 (32)	1.1 (7)	1,100
None		0.001 (55)	

^a The superinfecting phage was *λimm*⁴³⁴.O. Coinfections were performed in strain AB1157 as described in the text. Yields of helper phages were determined on strain Q8003, and yields of *λimm*⁴³⁴ were determined on strain Q8002. *λimm*⁴³⁴ carried *cI6T*. Yields in parenthesis are from parallel coinfections where the superinfecting phage was modified.

^b The coinfecting phage supplied *Der* function as well as *Lar*.

The *λrev* isolated by Zissler et al. (59) and the *λrev* isolated by Gottesman et al. (17) also supplied *Lar* during coinfections and alleviated *EcoB* restriction.

***Ral* activity does not require transactivation of host *Lar* activity.** To test whether

Ral activity required host Lar function, the coinfection experiments described in Table 12 were done. The coinfection host was AB1157. The observations that *sbcA*⁻ mutants cannot be isolated in AB1157 (49), that *λrev* cannot lysogenize AB1157 efficiently (17), and that AB1157 is *Rac*⁻ (35) all suggested that AB1157 was deleted for the cryptic prophage containing *recE* and *lar*. Recently, Kaiser and Murray (25) have shown that *λrev* is formed by recombination with the *Rac* prophage, and these workers have confirmed that the *Rac* prophage is deleted in AB1157. As Table 12 shows, both *λbio252c1857* and *λrev exbio10c1857* express restriction alleviation activity in coinfection experiments in an AB1157 genetic background. Therefore, the restriction alleviation activity expressed in *E. coli* K-12 strains after infection by *λbio252c1857* or *λrev exbio10c1857* does not result from the depression of the host Lar function under *sbcA* control.

DISCUSSION

Ral expression is under control of the *cI* gene in *λ* and is expressed early during infection by *λral*⁺-modified phage. Unmodified phage which superinfect a *λral*⁻-infected cell are much less likely to be restricted, and the progeny of those phage which do escape restriction are fully or almost fully modified.

The Ral function of *λ* can prevent restriction of unmodified phage DNA by the *EcoK* and *EcoB* enzymes. Ral does not seem to be a methylase which can recognize and methylate the appropriate bases within the A(N)⁸TGC recognition sequence found in both *EcoK* and *EcoB* sites (26, 31, 40). If this were so, the nonreplicating phage which mature in the presence of Ral should have both *EcoK* and *EcoB* modifications (Table 7). Thus, the specificity of modification which occurs in the presence of Ral is the specificity defined by the host modification and restriction system.

The observation that Ral can save phage from restriction in the absence of recombination functions is consistent with the observation of Zabeau et al. (58) that Ral acts to rescue entire genomes, since it protects nonhomologous phage P2 from restriction at low MOIs.

Under *Ral*⁻ conditions, the Rec and Red recombination systems seem to differ in the ability to recombine helper phage with restricted fragments. The simplest interpretation of the data in Table 8 is that the *λ*-P22 hybrid does not have a Ral function analogous to the Ral function of *λ*, which can prevent restriction of nonhomologous DNA, at least under the conditions in which the *λRal* function is active. The Red function of this hybrid (Erf of P22) can effi-

ciently rescue the immunity region of phage *λimm*⁴³⁴, indicating that Red can recombine restricted fragments and helper phages. There is no evidence that Rec can perform similarly, since infective centers (which should be the most sensitive indicator of such a phenomenon, as opposed to phage yield) are not increased significantly by a *λred*⁻*ral*⁻ helper, compared with no helper at all. This may be due to expression of inhibitors of *recBC* nuclease, such as *gam*, by the unmodified test phage used in these experiments, or, more likely, it is an intrinsic property of the Rec recombination system (perhaps, simply, restricted fragments are better substrates for nuclease digestion than for recombination).

In vitro studies on the kinetics of modification and restriction of unmodified DNA by *EcoB* and *EcoK* have shown that it is modified slowly and restricted rapidly (55). Several ways Ral might stimulate modification at the expense of restriction are as follows. Four stages have been recognized in the restriction process. First, S-adenosylmethionine is bound to the enzyme which undergoes activation; this form of the enzyme binds nonspecifically to DNA. Next, ATP is bound, and the enzyme undergoes a conformation change, binds specifically and tightly to the unmodified DNA, and, in the continued presence of ATP, restricts the DNA (20, 57). Ral might interact directly with the restriction enzyme complex to produce an *r*⁻*m*⁺ phenocopy, resembling the *hsdR* mutant *EcoK*-18, which forms short-lived recognition complexes that cannot be stabilized by ATP and cannot restrict unmodified DNA (DNA.0), but still can methylate it (6, 20). Debrouwere et al. (9) have suggested that the Ral effect may be mediated by a direct interaction with rho. Evidence for such an interaction is all indirect, but it is an interesting model.

In vitro, *EcoK* and *EcoB* restrict unmodified DNA in the presence of S-adenosylmethionine, ATP, and Mg²⁺, but *EcoK* and *EcoB* can modify DNA.0 if ATP and Mg²⁺ are left out of the reaction mixture (19, 37). Thus, potentially, Ral might act to stimulate modification by lowering ATP concentrations or Mg²⁺ concentrations or both in the cells. Another possibility is that Ral may be a DNA-binding protein that interacts with DNA at the *EcoB* and *EcoK* recognition sites to change the local structure of the DNA and make the DNA a more favorable substrate for modification or a less favorable substrate for restriction.

The Lar function expressed by *λrev* and *sbcA*⁻ strains may prevent restriction in a fashion analogous to the Ral function of *λ*. The observation that *λ* can rescue the reverse functions (an integrase, a general recombination function

[Der], and a restriction alleviation function [Lar]) from many strains of *E. coli* suggested that *rev* is part of a lambdoid prophage. Subsequently, Kaiser and Murray (25) demonstrated that *rev* is homologous to the *Rac* prophage (33, 35), which is very close to the *sbca* locus (34). Thus, the *recE* gene is involved in (i) recombination during transfer of *rac*⁺ by Hfr mating with *rac*⁻ *recB*⁻ or *rac*⁻ *recC*⁻ recipients (35), (ii) recombination proficiency in *recB*⁻ *sbca*⁻ and *recC*⁻ *sbca*⁻ strains (30, 49), and (iii) recombination proficiency by *λrev* (13). It is not known whether the Lar expressed in *sbca*⁻ strains and by *λrev* is coded for by a gene distinct from *recE*. However, it is clear that ExoVIII, which is coded for by the *recE* gene (29), is not required for Lar activity. The *recE* gene product with a molecular weight of 1.4×10^5 may contain separate domains for the exoVIII and Lar activities (13). It remains to be determined whether *recE* and *lar* are separate genes.

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