

## Restriction alleviation and modification enhancement by the Rac prophage of *Escherichia coli* K-12

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### Summary

Bacteriophage  $\lambda$  encodes an antirestriction function, Ral, which is able to modulate the activity of the *Escherichia coli* K-12 restriction and modification system, EcoKI. Here we report the characterization of an analogous function, Lar, expressed by *E. coli sbcA* mutants and the hybrid phage  $\lambda$ .reverse. *E. coli sbcA* mutants and  $\lambda$ .reverse both express genes of the Rac prophage, and we have located the *lar* gene immediately downstream of *recT* in this element. The *lar* gene has been cloned in an expression plasmid, and a combination of site-directed mutagenesis and labelling of plasmid-encoded proteins has enabled us to identify a number of translational products of *lar*, the smallest of which is sufficient for restriction alleviation. Lar, like Ral, is able both to alleviate restriction and to enhance modification by EcoKI. Lar, therefore, is functionally similar to Ral and the nucleotide sequences of their genes share 47% identity, indicating a common origin. A comparison of the predicted amino acid sequences of Lar and Ral shows only a 25% identity, but a few short regions do align and may indicate residues important for structure and/or function.

### Introduction

Bacteriophages use numerous mechanisms to escape host-encoded restriction and modification (R-M) systems (see Bickle and Krüger, 1993, and references therein). The activity of the *Escherichia coli* K-12 R-M enzyme, EcoKI, is modulated by a number of anti-restriction systems, including the Ocr activity of phages T3 and T7 (Studier, 1975), and the Ral activity of phage  $\lambda$  (Zabeau *et al.*, 1980). Ocr has been shown to competitively bind EcoKI, and block both restriction and modification of the phage (Bandyopadhyay *et al.*, 1985). However, Ral is

different; it alters the activity of the restriction complex so that unmodified DNA that escapes restriction is efficiently methylated (G. King and N. Murray, unpublished). EcoKI is a member of the Type IA R-M enzymes, which are the only prokaryotic DNA methyltransferases (MTases) shown to have a strong preference for hemimethylated DNA *in vivo*, and are also the only systems in which restriction is known to be alleviated by Ral (Loenen and Murray, 1986). One *ral* homologue, that of phage P22, has been sequenced; the predicted amino acid (aa) sequences of the two gene products are almost identical (Semerjian *et al.*, 1989). *E. coli sbcA* hosts (Templin *et al.*, 1972) and a derivative of phage  $\lambda$ , designated  $\lambda$ .reverse ( $\lambda$ .rev; Zissler *et al.*, 1971), express a Ral-like activity (Simmon and Lederberg, 1972; Toothman, 1981). In *sbcA* mutants, the *recE* and *recT* genes, the general recombination functions of a defective lambdoid prophage, Rac, have been activated (Kushner *et al.*, 1974; Clark *et al.*, 1993; Kolodner *et al.*, 1994).  $\lambda$ .rev also expresses the *recE* and *recT* genes (Gottesman *et al.*, 1974) acquired by a recombination event between  $\lambda$  and the excised Rac prophage (Kaiser and Murray, 1979). The gene encoding the Ral-like activity of the Rac prophage has been termed *lar* (Toothman, 1981).

The Type IA R-M systems are encoded by three genes: *hsdR*, *hsdM*, and *hsdS*. The HsdM and HsdS subunits together form a MTase that methylates two adenine residues in complementary strands of a bipartite DNA recognition sequence. In the presence of the HsdR subunit the complex can also act as an endonuclease, binding to the same target sequence but cutting the DNA some distance from this site. Whether the DNA is cut or modified depends on the methylation state of the target sequence. When the target site is unmodified, the DNA is cut. When the target site is hemimethylated, that is when only one adenine residue in the target is methylated, the complex acts as a maintenance MTase modifying the DNA so that both strands become methylated. This maintenance MTase activity ensures that the pattern of DNA methylation is preserved after replication. The enzyme recognizes a completely modified target sequence, but dissociates from the DNA (Suri *et al.*, 1984; Dryden *et al.*, 1993; Powell *et al.*, 1993).

Experiments dependent on expression of *ral* from a multicopy plasmid suggest that Ral alleviates restriction by changing the activity of EcoKI from a maintenance MTase to a *de novo* MTase, which efficiently methylates

unmodified DNA (Loenen and Murray, 1986). The efficient methylation of unmodified DNA is also observed with Ral and the *EcoKI* MTase alone, and mutations have been isolated in *hdsM* which mimic the activity of Ral (Kelleher *et*

*al.*, 1991). We are interested in the molecular mechanisms of these activities, and in defining important domains or residues within Ral and *EcoKI*.

Here we report the identification of *lar*, the gene

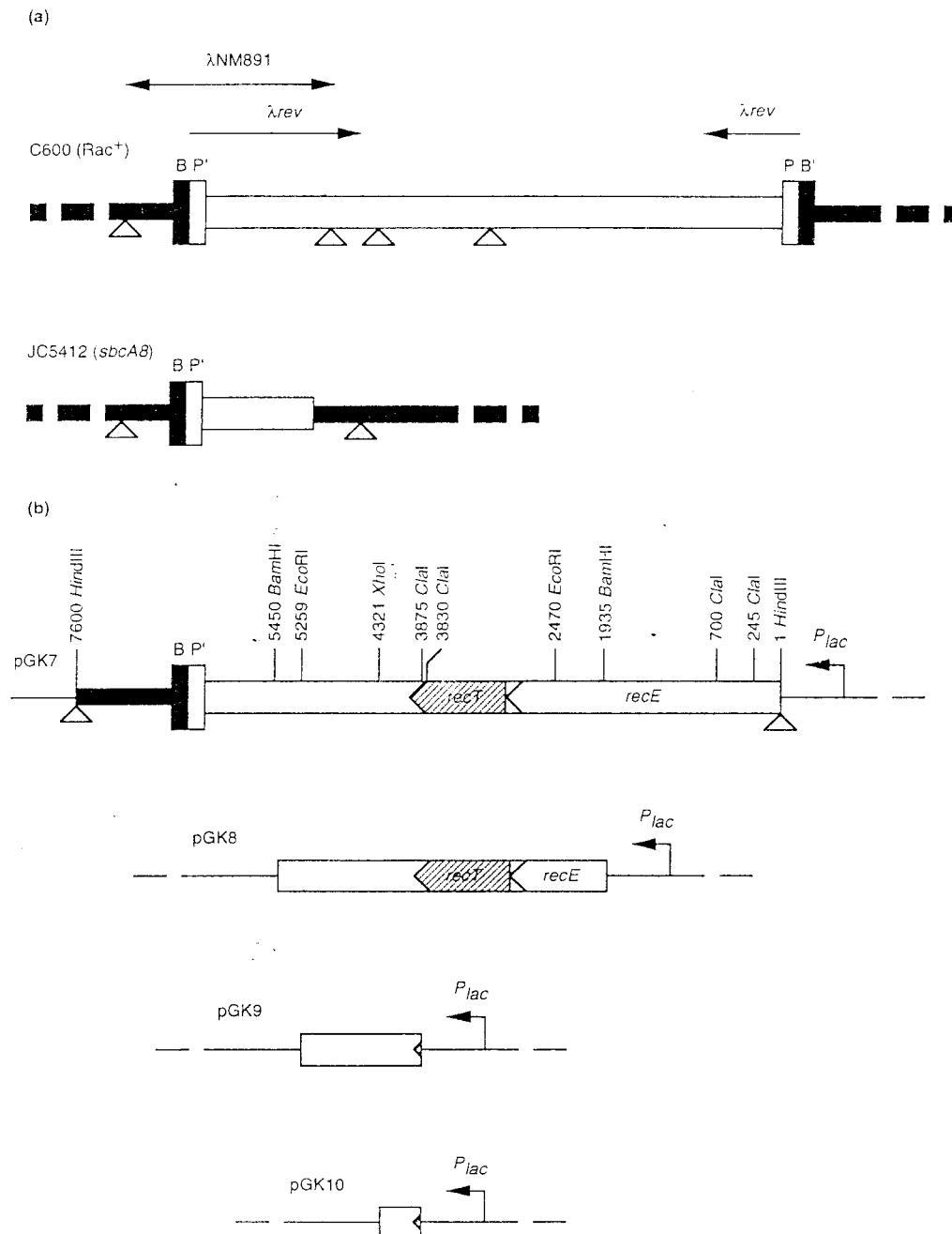


Fig. 1. Localization of the region encoding *lar*.

a. Origin of  $\lambda$ .rev,  $\lambda$ .NM891 and the Rac prophage integrated in the chromosome of the *E. coli* strain C600 and an *sbcA* mutant (*sbcA8*; JC5412). The DNA common to  $\lambda$ .rev and the *sbcA8* mutant is contained in  $\lambda$ .NM891.

b. The 7.6 kb *HindIII* fragment from  $\lambda$ .NM891 was cloned in pUC18 (Yanisch-Perron *et al.*, 1985) to generate pGK7. Segments of this 7.6 kb *HindIII* fragment were cloned and screened for restriction-alleviation activity. The *BamHI* fragment was cloned in pUC18 to generate pGK8. The *Clal-EcoRI* fragment was excised from pGK8, converted to a blunt-ended fragment using Klenow polymerase, and ligated to pUC18 cut with *SmaI*, to generate pGK9. pGK10 was similarly made with the 500 bp *XhoI-EcoRI* fragment from pGK9. Thick line, *E. coli* chromosome; thin line, pUC18 DNA; open box, Rac DNA; PB', hybrid Rac attachment site; open triangles, *HindIII* target sites; *P<sub>lac</sub>*, *lacUV5* promoter in pUC18; DNA sequence numbering as Clark *et al.* (1993).

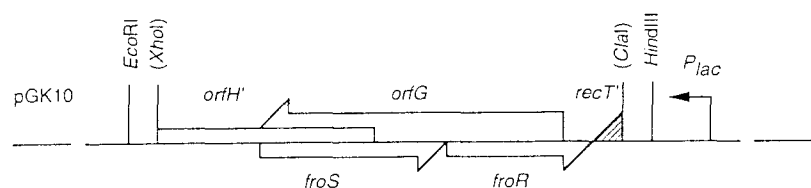


Fig. 2. Schematic showing the open reading frames within the 500 bp *ClaI*–*XhoI* DNA fragment cloned in pGK10. Predicted length of gene products; *OrfG*, 77 aa; *OrfH'*, 66 aa (truncated); *FroR*, 39 aa; *FroS*, 40 aa. Brackets indicate restriction sites in the *Rac* prophage lost during cloning.

responsible for *Ral*-like activity in *E. coli sbcA* hosts and *λ.rev* (Toothman, 1981). We have cloned *lar* and used an expression plasmid to identify its gene product. A comparison of the predicted aa sequences of *Ral* and *Lar* has highlighted a number of common motifs.

## Results

### Identification of the region encoding *lar*

To identify the region of the *Rac* prophage that encodes *lar*, a 7.6 kb *HindIII* fragment from *λ.NM891* (Kaiser and Murray, 1979) that includes DNA common to the *E. coli sbcA8* mutant and *λ.rev* was cloned in pUC18 to generate pGK7 (Fig. 1). An *EcoKI* restriction-proficient strain (NM710) was transformed with pGK7 and the restriction-alleviation activity of the plasmid was examined by comparing the titre of *λ.v.0* on this strain versus a non-restricting strain (NM792). In this screen, a similar titre indicates restriction alleviation. pGK7 alleviated restriction, and the *lar* gene was further localized by cloning segments of the 7.6 kb *HindIII* fragment in pUC18 (Fig. 1; see legend for experimental details). The smallest DNA fragment that conferred restriction-alleviation activity was a 500 bp insert in pGK10.

The region of the *Rac* prophage in pGK10 has been sequenced (Clark *et al.*, 1993; GenBank Accession Number L23927) and contains four open reading frames (ORFs), *orfG* and *orfH'* (truncated) in one orientation, and *froR* and *froS* in the other (Fig. 2). All of the clones that had *Lar* activity contained a plasmid insert oriented such that the *lac* promoter produces a transcript of *orfG*. In order to examine the orientation dependence of the restriction-alleviation phenotype, the 500 bp *EcoRI*–*HindIII* fragment from pGK10 was cloned in pUC19, to generate

pGK11, then removed and recloned in pUC18, to generate pGK12. pGK11 and pGK12, therefore, differ only in the orientation of the 500 bp insert relative to the *lac* promoter. Restriction-alleviation activity was unaffected by the orientation of the insert (Table 1), suggesting that there is sufficient *lar* expression to alleviate restriction even when the gene is in the opposite orientation to the *lac* promoter. In these experiments, transcription was neither enhanced nor opposed by activation of the *lac* promoter.

Table 2. Modification-enhancement activity of *lar*.

Host	Eop of progeny phages
NM526(DE3) pT7-6	$4.6 \times 10^{-3}$
NM526(DE3) pGK15	1.0

A restriction-deficient, modification-proficient strain (NM526) was transformed with the plasmid of interest and infected with unmodified *λ* phages (*λ.NM325.0*). After a single round of infection, the degree of phage modification was estimated by measuring the efficiency of plating (eop) of progeny phages on a restricting strain (Ymel) relative to that on a non-restricting derivative (NM526) (see the *Experimental procedures*).

### Identification of the gene encoding *lar*

A 290 bp fragment including *orfG* (nucleotides 3883–4172. Clark *et al.*, 1993) was amplified by the polymerase chain reaction (PCR) and cloned in pT7-6 to generate pGK15. This plasmid was found both to alleviate restriction and to enhance modification by *EcoKI* (Tables 2 and 3). *orfG* is the only complete ORF transcribed from the T7 promoter in pGK15, although *froR* and *froS* are read in the opposite orientation. To test whether *orfG* encoded *lar*, an amber codon was generated within *orfG* which would have no effect on the predicted aa sequence of *froS*. Codon 42 of *orfG* was changed from AAG to TAG. The resulting plasmid (pGK16; K42Am) was examined for restriction-alleviation activity in both a *sup* (NM777) and *supF* (Ymel) host (Table 3). Restriction was not alleviated in a *sup* host, but was alleviated in a *supF* host. The open reading frame *orfG*, therefore, encodes a *Ral*-like anti-restriction activity that is almost certainly the *Lar* activity identified by Toothman (1981).

### Localization of the *Lar* start codon

Examination of the DNA sequence at the start of *lar*

Table 1. Orientation dependence of *lar* activity.

Host	Titre <i>λ.v.0</i>
NM794	$4.5 \times 10^{10}$
NM777 pUC18	$2.4 \times 10^7$
NM777 pGK11	$2.1 \times 10^{10}$
NM777 pGK12	$3.8 \times 10^{10}$

Restriction-alleviation activity was determined for each host by measuring the titre of unmodified *λ*. phages (*λ.NM325.0*) normalized against the titre of modified *λ*. This corrects for variation resulting from factors other than restriction (see the *Experimental procedures*).

**Table 3.** Restriction-alleviation activity of *lar* mutants.

Host	Lar	Titre $\lambda$ .0
NM777(DE3) pT7-6		$3.3 \times 10^7$
NM777(DE3) pGK15	Wild type	$4.0 \times 10^{10}$
NM777(DE3) pGK16	K42Am	$2.5 \times 10^7$
NM777(DE3) pGK17	M1I	$3.1 \times 10^{10}$
NM777(DE3) pGK18	M10I	$2.8 \times 10^{10}$
NM777(DE3) pGK19	M14I	$1.5 \times 10^{10}$
NM777(DE3) pGK20	M1Am	$3.6 \times 10^{10}$
NM777(DE3) pGK21	M10Am	$2.8 \times 10^{10}$
NM777(DE3) pGK22	M14Am	$1.1 \times 10^7$
NM777(DE3) pGK23	M10Am M14I	$2.6 \times 10^7$
Ymel(DE3) pT7-6		$1.7 \times 10^7$
Ymel(DE3) pGK15	Wild type	$2.7 \times 10^{10}$
Ymel(DE3) pGK16	K42Am	$2.2 \times 10^{10}$
Ymel(DE3) pGK22	M14Am	$2.1 \times 10^7$
Ymel(DE3) pGK23	M10Am M14I	$8.9 \times 10^6$

Restriction-alleviation activity was determined for each host by measuring the titre of unmodified  $\lambda$  phages ( $\lambda$ .NM325.0) normalized against the titre of modified  $\lambda$ . (see the *Experimental procedures*).

identified a number of possible translational start codons. The DNA codon ATG encodes the most common translational initiation codon (see McCarthy and Gualerzi, 1990, and references therein), and there are three of these close to the start of *orfG* at positions 1, 10, and 14 (see Fig. 3). To identify which ATG was the translational initiation codon, we mutated each ATG codon individually to one for isoleucine; the first ATG codon was changed to ATA (pGK17) and codons 10 and 14 were changed to ATC (pGK18 and pGK19, respectively). We anticipated that a mutation to the ATG codon required for translational initiation would abolish Lar activity. However, all three mutants retained restriction-alleviation activity (Table 3), indicating that a single ATG codon was unlikely to be wholly responsible for translational initiation.

To identify the minimum coding sequence required for restriction-alleviation activity, each of the three ATG codons was mutated to an amber codon. An amber codon is recognized as a translational 'stop' codon in a *sup<sup>-</sup>* host, whereas, in a *sup<sup>F</sup>* host, a tyrosine residue is substituted at this position. An amber mutation in either the first (M1; pGK20) or second (M10; pGK21) ATG codons did not affect activity, but a change to the third ATG codon (M14; pGK22) abolished activity, both in a *sup<sup>-</sup>* and *sup<sup>F</sup>* host. This showed that translation of the

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AAGCTTCAGAGGAATAATTCAGCCTGGCGGTGTAATGCACCGC 3915
                                     M H R Lar
CAACTTGAAATATTTTATGAGAAAAATTATGAGATATGAC 3960
Q L E I F F M R K I M R Y D Lar

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**Fig. 3.** The DNA and predicted aa sequence of the 5' region of *lar*. The first 6 bp in this sequence comprise the *Hind*III recognition site, introduced by the PCR and used to clone *lar*. DNA sequence numbering is as in Clark *et al.* (1993).

DNA sequence upstream of the second ATG codon was not required for restriction alleviation. To ascertain whether the third ATG codon could be mutated to one for isoleucine (ATC) when no translation occurred upstream of the second methionine codon, a double mutant was made (M10Am M14I; pGK23). This double mutant is unable to alleviate restriction in either a *sup<sup>-</sup>* or a *sup<sup>F</sup>* host. To further the interpretation of these results, the polypeptides produced by each of the *lar* clones were examined.

#### Identification of the *lar* gene product

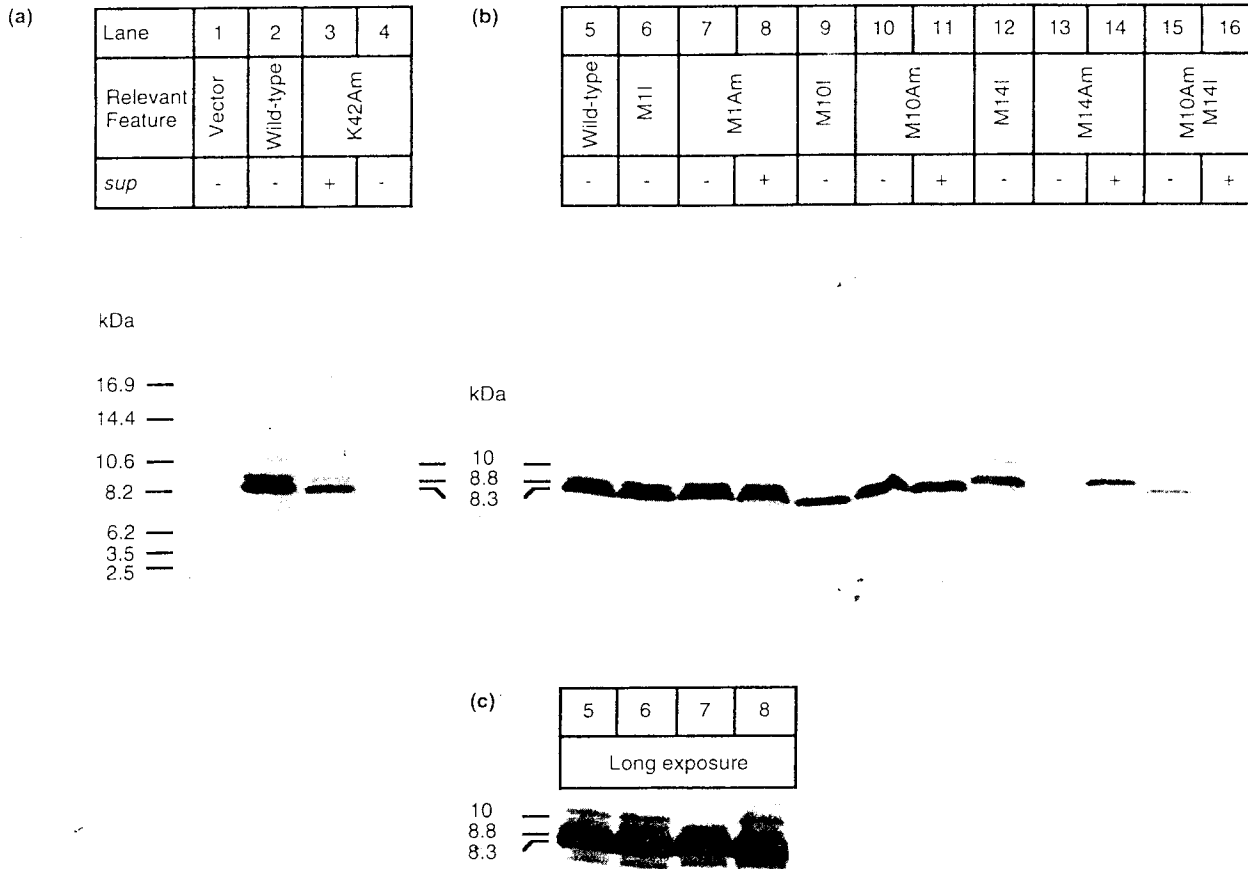
The products of the *lar* clones were visualized by the selective labelling of proteins encoded by genes under the control of the T7 promoter (Tabor, 1990). Differently sized polypeptides were detected depending on both the plasmid and the suppressor genotype of the host (Fig. 4, a and b). The host strains alone, BL21(DE3)*sup<sup>-</sup>* or BL21(DE3)*sup<sup>F</sup>*, produced no labelled protein (data not shown), nor did pT7-6 in either the *sup<sup>-</sup>* (lane 1) or *sup<sup>F</sup>* host (data not shown). pGK15 (wild-type *lar*; lanes 2 and 5) produced two main products, with estimated molecular masses of 8.8 kDa, and 8.3 kDa; the smaller of these is the more abundant.

The proteins produced by the *lar* mutants were similarly examined. In a *sup<sup>-</sup>* host, pGK16 (K42Am) produced no polypeptides of an appropriate size for Lar (lane 4), although a number of smaller products were seen, consistent with truncated forms of Lar. In contrast, in a *sup<sup>F</sup>* host (lane 3) both the 8.8 kDa and 8.3 kDa proteins were produced by pGK16. This correlates well with the observed phenotype; pGK16 does not alleviate restriction in a *sup<sup>-</sup>* host, but does in a *sup<sup>F</sup>* host.

Both pGK18 (M10I; lane 9) and pGK21 (M10Am), in a *sup<sup>-</sup>* (lane 10) or *sup<sup>F</sup>* host (lane 11), produced only the 8.3 kDa polypeptide, from which we concluded that the second ATG (M10) is the initiation codon for the 8.8 kDa protein.

The third ATG codon (M14) is the major translational start of *lar*, encoding the 8.3 kDa protein, as this polypeptide was not detected in either a *sup<sup>-</sup>* or a *sup<sup>F</sup>* host when the third ATG codon was mutated to an amber codon (M14Am; pGK22; lanes 13 and 14, respectively). Nevertheless, pGK19 (M14I; lane 12) and pGK23 (M10Am M14I; lanes 15 and 16) both produced small quantities of the 8.3 kDa protein. The ATC codon in pGK19 and pGK23, therefore, appears to be used as a poor translational start in these mutants. It seemed unlikely that the codons between the second and third ATG codons could encode the translational start of the 8.3 kDa protein as this polypeptide was not visualized in a *sup<sup>F</sup>* host transformed with pGK22 (M14Am), and yet the 8.8 kDa polypeptide (starting at M10) was detected (lane 14).

A 10 kDa polypeptide, specific to the *lar* clones, can be



**Fig. 4.** Visualization of the *lar* gene products. a. Lane 1, BL21(DE3) pT7-6 (vector); lane 2, BL21(DE3) pGK15 (wild-type); lane 3, NM793(DE3) pGK16 (K42Am); lane 4, BL21(DE3) pGK16. b. Lane 5, BL21(DE3) pGK15 (wild-type); lane 6, BL21(DE3) pGK17 (M11); lane 7, BL21(DE3) pGK20 (M1Am); lane 8, NM793(DE3) pGK20; lane 9, BL21(DE3) pGK18 (M10I); lane 10, BL21(DE3) pGK21 (M10Am); lane 11, NM793(DE3) pGK21; lane 12, BL21(DE3) pGK19 (M14I); lane 13, BL21(DE3) pGK22 (M14Am); lane 14, NM793(DE3) pGK22; lane 15, BL21(DE3) pGK23 (M10Am M14I); lane 16, NM793(DE3) pGK24. c. A longer exposure of the autoradiograph showing the 10 kDa Lar polypeptide in lanes 5–8.

visualized after longer exposures of the autoradiograph (Fig. 4c). The initiation codon for this low-abundance polypeptide must precede the first ATG codon since, in a *supF* host, pGK20 (M1Am) produced the 10 kDa, 8.8 kDa and

8.3 kDa proteins (lane 8) but, in a *sup* host, this clone only produced the two smaller polypeptides (lane 7). pGK17 (M11) did not affect the polypeptides produced (lane 6).

*Comparisons between the predicted amino acid sequences of Ral and Lar*

The minimum aa sequence required for Lar activity starts at the methionine encoded by the third ATG codon (M14). The predicted aa sequence of this polypeptide was compared with that predicted for Ral using the GAP Program of the University of Wisconsin Genetics Computer Group (GCG 7.3, 1991; see Fig. 5). The two proteins are much more dissimilar than  $\lambda$  Ral and P22 Ral (Semerjian *et al.*, 1989). A number of regions do align, and these may indicate important structural and/or functional roles for residues within the two proteins.

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Lar MRYDNVKKPCPFCGCPSPVTVKAISGYRAKNCNGCESRTGYGG 54
      ..... : | : : |||. ....
Ral .....MTTIDKNQWCGQFK.RCNGCKLQSECMV 37

SEKEAL.....ERWNRKRTG.....NNGGVHV 77
... : : : | : : | : :
KPEEMFPVMEDGKYVDKWAIRTTAMIARELGKQNNKAA... 66
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**Fig. 5.** Comparison of the predicted amino acid sequence of Ral and Lar (starting at M14) (see text). Gap weight 3.0; length weight 0.1; lines indicate identity; dots indicate similarity; % similarity, 52%; % identity, 25%.

## Discussion

We have identified *lar*, the gene responsible for restriction-alleviation and modification-enhancement activity of the *E. coli* Rac prophage. This gene has been sequenced previously, although its function was unknown and it was termed *orfG* (Clark *et al.*, 1993). The *lar* gene, cloned in pT7-6, can be translated from three initiation codons to produce polypeptides with estimated molecular masses of 10 kDa, 8.8 kDa and 8.3 kDa. pGK21 (M10Am) alleviates restriction in a *sup* host and yet only produces the 8.3 kDa protein, demonstrating that this is the only form of Lar that is required for activity. We conclude that the translational initiation codon for this protein is codon 14 (ATG). However, when codon 14 is changed to an ATC (pGK19 and pGK23) the 8.3 kDa polypeptide is still produced, although, as is apparent from the labelling experiments with pGK19 (M14I; Fig. 4B, lane 12), the amount of the 8.3 kDa polypeptide is much reduced relative to the 8.8 kDa protein. It has been shown that ATC can be used to initiate protein synthesis (Romero and Garcia, 1991) and our results appear to indicate that, in pGK19 and pGK23, this codon can be used as a poor translational initiation codon. Restriction was not alleviated by pGK23 (M10Am M14I), although there may not have been sufficient Lar produced in these phenotypic assays to alleviate restriction as transcription of the gene encoding T7 polymerase was not induced. The restriction-alleviation and modification-enhancement assays provided clear phenotypes with uninduced, wild-type *lar* (pGK15). It seemed preferable to use these conditions to examine Lar activity rather than those in which the cell physiology is perturbed by the gross overproduction of Lar, dependent upon T7 RNA polymerase. The initiation codon for the 10 kDa form of Lar appears to be before the first ATG codon (M1), and that for the 8.8 kDa polypeptide is the second ATG codon (M10). The 8.3 kDa protein is the most abundant, although it is not possible to predict what forms would be produced from the Rac prophage and whether the different forms have any biological significance.

The Rac prophage includes genes that can serve the same functions as the early genes of phage  $\lambda$ , including integration, recombination, immunity and replication (Low, 1973; Gottesman *et al.*, 1974; Diaz *et al.*, 1979). The *recE* and *recT* genes from Rac can substitute for the general recombination genes of  $\lambda$ , *exo* and *bet*, respectively (Kushner *et al.*, 1974; Clark *et al.*, 1993). In  $\lambda$ , *ral* is upstream of the recombination genes, whereas in the Rac prophage, *lar* is immediately downstream of *recT*. The *ral*, *exo* and *bet* genes are all transcribed from  $p_L$  in  $\lambda$  and it is logical to expect that they are also cotranscribed in Rac. Other genes in this transcriptional unit include those required by  $\lambda$  for excision (*xis*) and integration (*int*); analogous functions in the Rac prophage may be

encoded in the DNA sequence downstream of *lar*. Despite the rearrangement of gene order in Rac relative to  $\lambda$ , the DNA sequences of *ral* and *lar* show 47% identity (data not shown), suggesting that the two genes have a common origin.

Ral and Lar are both able to alleviate restriction and enhance modification by the Type IA R-M systems and an examination of their predicted aa sequences may, therefore, help identify key residues involved in structure and/or function. Cysteine residues are commonly used to stabilize the structure of proteins, either forming disulphide bridges or associating with divalent metal ions (Branden and Tooze, 1991). Lar contains a sequence of cysteine residues, Cys-X<sub>2</sub>-Cys-X<sub>17</sub>-Cys-X<sub>2</sub>-Cys, that resembles a zinc-binding motif. Ral does not contain the same motif but it does contain a symmetrical sequence, Cys-X<sub>5</sub>-Cys-X<sub>2</sub>-Cys-X<sub>5</sub>-Cys, which may serve a similar role. When the aa sequences of Ral and Lar are compared, a number of regions align. The identities CNGC and W--RTT may be relevant. The cysteines in the sequence CNGC may be of structural relevance, whereas the W--RTT residues may indicate a region of functional importance. The best conservation at the nucleotide level is seen in the region encoding W--RTT (data not shown). Ral is amenable to structural analysis by nuclear magnetic resonance, and polypeptide produced by solid-phase synthesis (Brown *et al.*, 1993) and *in vivo* from the cloned gene (G. King and N. Murray, unpublished) will be used for this purpose. Given the structure of Ral, the motifs identified here should help us to further distinguish important residues and define targets for site-directed mutagenesis.

## Experimental procedures

### Strains, phages, and microbiological methods

Bacterial strains and phages are described in Table 4. Media and general methods have been described previously (Murray *et al.*, 1977). NM710 was used as the host for the preparation of K-modified lysates ( $\lambda$ .K), and NM679 was used as the host for unmodified lysates ( $\lambda$ .0). Bacteria were made lysogenic for  $\lambda$ .DE3 by a co-infection with  $\lambda$ .NM75, to provide *Int in trans*, and lysogens with the immunity of phage 21 were selected by plating on L-agar seeded with  $\lambda$ .*imm*<sup>21</sup>*cl* ( $\lambda$ .NM507) and a derivative with the host range of  $\phi$ 80 ( $\lambda$ .NM508).

NM793(DE3) was made by a series of P1 transductions. An efficient donor of *supF*, tagged with a closely linked transposon, was generated by the P1-mediated transfer of *trpC::Tn10* from a *sup* strain (W3110*trpC::Tn10*) to the *supF* strain, Ymel. Tetracycline-resistant transductants of Ymel were selected on L-agar supplemented with 10  $\mu$ g ml<sup>-1</sup> tetracycline, and screened for the retention of *supF* by their ability to suppress  $\lambda$ .*Sam7* ( $\lambda$ .NM123) but not  $\lambda$ .*Pam3* ( $\lambda$ .NM435). Phage P1 grown on this host (NM787) was used to transduce BL21(DE3). Tetracycline-resistant transductants

Table 4. Strains and phages.

Strain/Phage <sup>a</sup>	Relevant features	Source
<i>E. coli</i> K-12 derivative		
NW2	$\Delta(mrr-hsd-mcrB)$	Woodcock <i>et al.</i> (1989)
CAG12081	<i>zj3061::Tn10fnr501</i>	Singer <i>et al.</i> (1989)
W3110	<i>sup<sup>+</sup></i>	C. Yanofsky
W3110 <i>trpC::Tn10</i>	<i>trpC::Tn10</i>	C. Yanofsky
NM679	W3110 $\Delta(mrr-hsd-mcr)$	See text
NM777 <sup>b</sup>	W3110 <i>lar</i>	See text
NM794	NM777 <i>hsdR</i> $\Delta$ 4	See text
LE451	C600 <i>recA</i> <i>srl::Tn10</i> $\Delta(rac)$	Diaz <i>et al.</i> (1979)
NM710	C600 <i>srl::Tn10</i> $\Delta(rac)$	See text
NM792	NM710 <i>hsdR</i> $\Delta$ 4	See text
Ymel <sup>b</sup>	<i>supF</i>	Lab. collection
NM526 <sup>b</sup>	Ymel <i>hsdR</i> $\Delta$ 4	See text
NM787	Ymel <i>trpC::Tn10</i>	See text
<i>E. coli</i> B derivative		
BL21 <sup>b</sup>	<i>sup<sup>+</sup>hsdS</i>	Studier and Moffat (1986)
NM793 <sup>b</sup>	BL21 <i>supF</i>	See text
Phage		
P1 <i>kc</i>		Lennox (1955)
DE3	$\lambda$ <i>int::lacI lacUV5-T7gene1imm<sup>21</sup></i>	Studier and Moffat (1986)
NM22	$\lambda$ <i>Nam7Nam53c126</i>	Lab. collection
NM75	$\lambda$ <i>b2red3imm<sup>+</sup></i>	Lab. collection
NM123	$\lambda$ <i>cl857Sam7</i>	Lab. collection
NM243	$\lambda$ <i>vir</i>	Lab. collection
NM325	$\lambda$ <i>bio232</i> $\Delta(int-ral)imm434c1$	Lab. collection
NM435	$\lambda$ <i>Pam3c126</i>	Lab. collection
NM507	$\lambda$ <i>b2imm<sup>21</sup>c1</i>	Lab. collection
NM508	$\lambda$ <i>h<sup>80</sup>imm<sup>21</sup>c1</i>	Lab. collection
NM891	$\lambda$ <i>recE</i>	Kaiser and Murray (1979)

a. NM numbers refer to stock numbers in this laboratory.

b. Lysogenic for  $\lambda$ DE3, where appropriate.

were selected for as before and screened for the presence of *supF* by their ability to suppress  $\lambda$ *Nam7Nam53* ( $\lambda$ NM22), a phage chosen to avoid complications of complementation by the resident *imm<sup>21</sup>* prophage. Phage P1 was grown on the *trpC<sup>+</sup>* host and used to regenerate a prototrophic strain (NM793). These were selected on M9-minimal agar and screened for the presence of the suppressor mutation as described above.

The *lar* and *hsd* deletion derivatives of W3110 were also made by P1 transduction. The former (NM777) using CAG12081 (Singer *et al.*, 1989) as the donor of *fnr501* ( $\Delta$ *fnr-lar*), and the latter (NM679) by first introducing the temperature-sensitive lethal mutation *dnaC325* by cotransduction with *zj3::Tn10*. *dnaC<sup>+</sup>* recombinants were selected following transduction with a P1 lysate made on the *hsd* deletion strain, NW2 (Woodcock *et al.*, 1989). Transductants selected at 42 °C were screened for Hsd<sup>-</sup>, McrB<sup>-</sup> and tetracycline sensitivity.

The *hsdR* $\Delta$ 4 derivatives of Ymel (NM526), NM710 (NM792) and NM777 (NM794) were made by transferring *hsdR* $\Delta$ 4 from a  $\lambda$ *hsd* phage to the bacterial chromosome (Gough and

Murray, 1983). NM710 is a *recA<sup>+</sup>* derivative of the *rac* deletion strain LE451 (Diaz *et al.*, 1979), made by transferring the *recA<sup>+</sup>* allele from a  $\lambda$ *recA<sup>+</sup>* phage to the host chromosome.

#### Restriction-alleviation tests

Bacteria transformed with plasmids were grown at 37 °C until mid-log phase, in L-broth supplemented with 10 mM MgSO<sub>4</sub> and 100  $\mu$ g ml<sup>-1</sup> ampicillin. Samples (0.2 ml) were infected with appropriate dilutions of  $\lambda$ NM325.0 or  $\lambda$ NM325.K for 15 min, and plated in top layer on BBL-agar to assay infective centres. Plaques were counted after overnight incubation and the titre of  $\lambda$ NM325.0 was normalized against the titre of  $\lambda$ NM325.K.

#### Modification-enhancement tests

Bacteria transformed with plasmids were grown as outlined above. Samples (0.2 ml) were infected with  $\lambda$ NM325.0 at a multiplicity of infection of 0.1, and incubated for 15 min to allow adsorption of phages. The infected cells were harvested and resuspended in 10 ml of L-broth supplemented with 100  $\mu$ g ml<sup>-1</sup> ampicillin. Cultures were incubated with aeration at 37 °C for 70 min to allow a single round of infection, and the proportion of modified progeny phage was estimated from the titre on NM777 relative to that on a restriction-deficient derivative (NM794).  $\lambda$ NM325.K was used as a control to ensure that the plating efficiencies of the indicator bacteria were comparable.

#### Plasmids, DNA manipulation and sequencing

Preparation, cloning and analysis of DNA was carried out essentially as described by Midgley and Murray (1985). Competent bacteria were prepared by electroporation (Heery and Dunican, 1989) and small-scale DNA isolation performed by the method of He *et al.* (1990). Details of the construction of plasmids used to localize restriction-alleviation activity are given in the legend to Fig. 1. The PCR, and site-directed mutagenesis using the PCR, were performed as described by Higuchi (1990), with a concentration of 50  $\mu$ M dNTPs, and 0.2  $\mu$ M primers. DNA fragments generated by the PCR were cloned in pT7-6 (Tabor, 1990), which does not have a ribosome-binding site upstream of the polylinker and therefore relies on sequences cloned downstream of the T7 promoter to initiate translation. Template DNA used in the PCR was  $\lambda$ NM891 unless otherwise stated. The oligonucleotide primers used to generate the insert in pGK15, and flanking primers used for site-directed mutagenesis, are 5'-GGAAGCTTCAGGGAATAATTCAG and 5'-GGGAATCTTCTTTTCAA-TAGTGG. Complementary pairs of primers were used to generate mutations and only one primer of each pair is described. The primers used to generate the mutations are: pGK16 5'-TACCGAGCGTAGTGTAAC, pGK17 5'-GTGTAATACACCGCCAAC, pGK18 5'-TTTATCAGAAAAATTATGAG, pGK19 5'-GAAAAATTATCAGATATGAC, pGK20 5'-GTGTATAGCACCGCCAAC, pGK21 5'-TTTTAGAGAAAAATTATGAG, pGK22 5'-GAAAAATTTAGAGATATGAC. The -40 universal primer, 5'-GTTTTCCAGTCACGAC, was used as a flanking primer with pGK11 DNA template in the PCR to generate the fragment cloned in pGK17 and pGK20; the

usual flanking primer annealed too close to the mutagenic primer to generate a manageable product. pGK21 was used as the template in the PCR to generate the insert in pGK23 with the mutagenic primers used for pGK19, in order to generate a double mutant. All plasmid inserts generated by the PCR were analysed by double-strand DNA sequencing, using Sequenase 2.0 (Amersham Life Science). pGK17 and pGK20 both have an additional mutation (G to A) outside any ORF, immediately 5' to the PCR primer. We were unable to isolate mutants using these primers without a mutation at this position. All other plasmids contain the expected sequence (Clark *et al.*, 1993).

#### Analysis of polypeptides

Gene products were visualized by the selective labelling of polypeptides encoded by genes under the control of the T7 promoter, essentially as described by Tabor (1990). BL21(DE3) and NM793(DE3) were used as the hosts for these experiments and expression of the T7 polymerase gene was induced by the addition of 0.6 mM IPTG (Studier and Moffat, 1986). Samples were denatured and reduced in 'cracking' buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 10% 2-mercaptoethanol, 100 mM DTT), the polypeptides separated by Tricine-SDS-PAGE (Shägger and von Jagow, 1987), and visualized by autoradiography.

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