Molecular Cloning and Expression in *Escherichia coli* of the *recA* Gene of *Legionella pneumophila*

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Interspecific complementation of an *Escherichia coli* *recA* mutant with a *Legionella pneumophila* genomic library was used to identify a recombinant plasmid encoding the *L. pneumophila* *recA* gene. Recombinant *E. coli* strains harbouring the *L. pneumophila* *recA* gene were isolated by replica-plating bacterial colonies on medium containing methyl methanesulphonate (MMS). MMS-resistant clones were identified as encoding the *L. pneumophila* *recA* analogue by their ability to protect *E. coli* HB101 from UV exposure and promote homologous recombination. Subcloning of selected restriction fragments and Tn5 mutagenesis localized the *recA* gene to a 1.7 kb *Bgl*II–*Eco*RI fragment. Analysis of minicell preparations harbouring a 1.9 kb *Eco*RI fragment containing the *recA* coding segment revealed a single 37.5 kDa protein. Insertional inactivation of the cloned *recA* gene by Tn5 resulted in the disappearance of the 37.5 kDa protein, concomitant with the loss of RecA function. The *L. pneumophila* *recA* gene product did not promote induction of a λ lysogen; instead, the presence of the heterologous *recA* gene caused a significant reduction in spontaneous and mitomycin-C-induced prophage induction in *recA*⁺ and *recA* *E. coli* backgrounds. Despite the lack of significant genetic homology between the *L. pneumophila* *recA* gene and the *E. coli* counterpart, the *L. pneumophila* RecA protein was nearly identical to that of *E. coli* in molecular mass, and the two proteins showed antigenic cross-reactivity. Western blot analysis of UV-treated *L. pneumophila* revealed a significant increase in RecA antigen in irradiated versus control cells, suggesting that the *L. pneumophila* *recA* gene is regulated in a manner similar to that of *E. coli* *recA*.

INTRODUCTION

Legionella pneumophila is a Gram-negative bacterium widely distributed in natural and man-made fresh-water habitats (Fliermans *et al.*, 1981; Stout *et al.*, 1982). To date, a growing list of related *Legionella* species comprising at least 33 serogroups have been classified (Thacker *et al.*, 1985). Results from DNA–DNA hybridization experiments suggest that the *Legionellaceae* are unrelated to other known bacterial families (Brenner *et al.*, 1979, 1981). In contrast to our increasing understanding of the bacteriology and ecology of this group of bacteria, basic information on the genetic organization and gene expression in *L. pneumophila* is currently lacking.

Recombination-deficient (Rec[−]) *L. pneumophila* strains would be a valuable tool in developing methods to genetically characterize metabolic and pathogenic properties of *L. pneumophila*. The availability of Rec[−] *L. pneumophila* strains would permit a number of currently difficult or impossible methodologies including: (1) stable maintenance of R' plasmids resulting from *in vivo* cloning, (2) recovery from *L. pneumophila* recipient strains of cloned genes mobilized from *Escherichia coli*-based gene libraries, and (3) an analysis of the recombinational requirements

Abbreviation: MMS, methyl methanesulphonate.

associated with the reversible phenotypic conversion between virulent and avirulent isolates (Elliot & Johnson, 1982; McDade & Shephard, 1979).

Homologous recombination in *E. coli* is controlled in part by the *recA* gene product (Clark, 1973). The RecA protein possesses a variety of regulatory and catalytic activities involved in homologous recombination, DNA repair mechanisms, and induction of prophage λ (McEntee *et al.*, 1979; Roberts *et al.*, 1978; Walker, 1984). Recently, *recA* gene analogues from a number of bacteria have been identified by heterospecific complementation of *recA* mutations in *E. coli* (Better & Helinski, 1983; Finch *et al.*, 1986; Geoghegan & Houghton, 1987; Goldberg & Mekalanos, 1986; Keener *et al.*, 1984; Koomey & Falkow, 1987; Ohman *et al.*, 1985). In this report I describe the cloning, characterization and expression in *E. coli* of the *recA* analogue of *L. pneumophila*, and demonstrate that the *L. pneumophila recA* gene product possesses considerable functional and antigenic similarity to its *E. coli* counterpart. In addition, the *L. pneumophila recA* gene appears to be induced by UV treatment.

METHODS

Bacterial strains, bacteriophage and plasmids. These are listed in Table 1.

Media. *L. pneumophila* was grown in liquid and on solid buffered charcoal yeast extract medium (BCYE) (Edelstein, 1981). *E. coli* strains were grown in L-medium or M9 or M63 minimal media (Maniatis *et al.*, 1982). Antibiotics used for *E. coli* selection were at the following concentrations in $\mu\text{g ml}^{-1}$: carbenicillin (Cb), 100;

Table 1. *Bacterial strains, bacteriophage and plasmids*

Strain	Genotype	Source or reference
<i>Legionella pneumophila</i>		
Philadelphia 1	Wild-type (serogroup 1)	J. Hammell (Hahnemann University School of Medicine, Philadelphia)
Togus 1	Wild-type (serogroup 2)	
Bloomington 2	Wild-type (serogroup 3)	
Los Angeles 1	Wild-type (serogroup 4)	
Other <i>Legionella</i> spp.		
<i>L. bozemanii</i>	Wild-type	
<i>L. gormanii</i>	Wild-type	
<i>L. micdadei</i>	Wild-type	
<i>Escherichia coli</i>		
HB101	F ⁻ <i>hsdS20 recA13 supE44 rpsL20 ara-14 proA2 leu lacY1 galK2 xyl-5 mtl-1</i>	Boyer & Roulland-Dussoix (1969)
LE392	F ⁻ <i>supF58 supE44 hsdR514 galK2 trpR55 metB1 lacY1 tonA53 mtl-1 xyl-5</i>	Silhavy <i>et al.</i> (1984)
EMG24	HfrH, prototrophic	ATCC 23740
P678-54	<i>minA</i>	Adler <i>et al.</i> (1967)
JC10240	HfrPO45 <i>srl-300::Tn10 recA56 thr-300 ilv-318 rpsE300</i>	Csonka & Clark (1980)
N99cI ⁺	K12 λ lysogen	Pharmacia
LD99	N99cI ⁺ <i>srl-300::Tn10 recA56</i>	This study
<i>Bacteriophage</i>		
$\lambda::\text{Tn5}$	<i>b221 cI857 cIII::Tn5 Oam Pam Sam</i>	Berg (1977)
<i>Plasmids</i>		
pBR322	ColE1 <i>bla</i> Tc ^R	Bolivar <i>et al.</i> (1977)
pUC19	ColE1 <i>bla</i>	Yanisch-Perron <i>et al.</i> (1985)
pACYC184	p15A Cm ^R Tc ^R	Chang & Cohen (1978)
pLAD110	pBR322 <i>rec</i> ⁺ 6.5 kb insert in <i>Bam</i> HI site	This study
pLAD111	pUC19 with 1.9 kb <i>Eco</i> RI fragment of pLAD110 insert; <i>rec</i> ⁺	This study
pLAD112	1.7 kb <i>Bgl</i> II- <i>Eco</i> RI fragment of pLAD110 inserted in <i>Bam</i> HI <i>Eco</i> RI sites of pUC19; <i>rec</i> ⁺	This study
pLAD113	800 bp <i>Bgl</i> II- <i>Bam</i> HI fragment of pLAD110 inserted in <i>Bam</i> HI site of pUC19	This study

kanamycin (Km), 50; and tetracycline (Tc), 10. Medium containing methyl methanesulphonate (MMS) was prepared by spreading 0.1 ml of 2% (v/v) solution on the surface of L-agar plates.

General recombinant DNA methods. Large-scale plasmid DNA was prepared by a cleared lysate method (Clewell & Helinski, 1969) and purified by CsCl density-gradient centrifugation in the presence of ethidium bromide (Stougaard & Molin, 1981). Small-scale plasmid preparations were performed by the method of Holmes & Quigley (1981). Restriction endonuclease digests of plasmid DNA were performed according to the manufacturer's specifications. DNA fragments were analysed by electrophoresis through agarose or acrylamide gels containing TBE buffer (0.089 M-Tris pH 8.0, 0.089 M-boric acid, 0.002 M-EDTA) and ethidium bromide (0.5 µg ml⁻¹). Physical mapping of recombinant plasmids was performed by single and multiple restriction enzyme digestion followed by electrophoresis in agarose or acrylamide gels. The positions of Tn5 insertions in recombinant plasmids were located by examining the resulting alteration in the restriction digestion pattern based on a published map of Tn5 (Jorgensen *et al.*, 1979). Chromosomal DNA was purified as described by Goldberg & Ohman (1984).

Construction of an *L. pneumophila* genomic library. High-molecular-mass *L. pneumophila* Philadelphia 1 DNA (50 µg) was partially digested with *Sau*3A to generate fragments predominantly 4–10 kb in length. The resulting genomic DNA fragments and molecular mass standards (*Hind*III digested λ DNA) were separated by electrophoresis through a 0.6% (w/v) agarose gel (20 × 20 cm) for 13 h at 2 V cm⁻¹. Following electrophoresis, the outer gel lanes containing the molecular mass standards were sliced from the gel, stained in ethidium bromide (0.5 µg ml⁻¹), rinsed free of excess dye, and repositioned beside the gel slab on a UV transilluminator. The gel slab was protected from direct UV exposure by aluminium foil. Exposure of stained size markers to UV light located the region of the gel slab containing fragments ranging in size from 4 to 8 kb. The appropriate portion of the gel was excised with a clean razor and placed in as many 17 × 100 mm polypropylene snap-cap tubes as required. The agarose was mashed with a sterile glass rod, mixed with an equal volume of phenol (Aldrich) previously redistilled and equilibrated with TE buffer (0.01 M-Tris/HCl pH 8.0, 0.001 M-EDTA), and frozen at -70 °C for 1 h. The gel/phenol mixture was centrifuged at 15000 g in a Sorvall SM-24 rotor for 30 min to separate the phases. The aqueous phase was removed and re-extracted with phenol as just described followed by two consecutive extractions with chloroform/isoamyl alcohol (24:1, v/v). The partially digested chromosomal DNA was then precipitated with ethanol in the presence of 2 M-ammonium acetate. The precipitated DNA was collected by centrifugation, washed with 70% (v/v) ethanol, and desiccated until dry. The dried DNA pellet was dissolved in 0.1 ml TE and stored at 4 °C for no longer than two weeks.

Vector DNA (pBR322) was prepared as described above. Following digestion with *Bam*HI, linearized pBR322 was dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals). Phosphatase was removed from the reaction mixture by heating at 68 °C in the presence of 0.01 M-EDTA for 15 min, followed by two extractions with TE-saturated phenol, then chloroform/isoamyl alcohol (24:1, v/v). Dephosphorylated pBR322 and partially digested chromosomal DNA were mixed at a molar ratio of 1:2 (vector:insert) with T4 DNA ligase (Pharmacia), and incubated for 4 h at room temperature, followed by 18 h at 4 °C. Portions of the ligation mixture were used to transform CaCl₂-treated *E. coli* HB101. Transformants were selected on L-agar plates containing Cb. The entire library was spread in 0.1 ml volumes on L-agar containing Cb, and following overnight incubation the resulting bacterial colonies (about 200 per plate) were washed from 100 plates in L-broth, pelleted by centrifugation and resuspended to one-half the original volume in L-medium containing 50% (v/v) glycerol. The bacterial suspension was then stored in 1 ml portions at -70 °C.

Transposon mutagenesis of cloned DNA. Tn5 was transferred from λ::Tn5 to a recombinant-plasmid-containing derivative of *E. coli* HB101, as described for λNK561 (Silhavy *et al.*, 1984). The resulting transductants, selected by plating on Km-containing L-agar, were spread at a density of 300–500 c.f.u. per plate. Following overnight growth, colonies washed from 20 plates were pooled and a portion then used as inoculum for 1 litre of L-medium (with Km) contained in a 2.8 litre Fernbach flask. The culture was grown overnight at 37 °C with vigorous aeration then used as a source of plasmid DNA. Plasmid molecules which contained Tn5 insertions were identified by transformation of competent *E. coli* HB101 followed by selection for resistance to Km. Km^r colonies were then screened for the loss of *recA* function by sensitivity to MMS and UV light.

UV irradiation. Sensitivity to UV exposure was tested by plating appropriate dilutions of mid-exponential-phase cultures on agar plates and exposing them to UV light at the indicated doses. UV dose was determined with a UV dosimeter. Alternatively, RecA activity was tested by shielding from the UV one half of an agar plate cross-streaked with single colonies from strains to be tested. Following UV exposure (10 J m⁻²), plates were wrapped to prevent photoreactivation and incubated at 37 °C.

Minicell analysis. Minicells were prepared from plasmid-containing derivatives of *E. coli* P678-54 and labelled with [³⁵S]methionine (ICN Radiochemicals) as described by Goldberg & Mekalanos (1986). SDS extracts of labelled minicells were separated by electrophoresis through 12.5% (w/v) acrylamide gels (Laemmli, 1970), stained to localize size markers, then dried under a vacuum at 80 °C. Dried gels were exposed to X-OMAT AR film (Eastman Kodak) for 24–36 h to visualize radiolabelled protein bands.

Bacterial conjugation. Cultures of *E. coli* EMG24 and plasmid-containing derivatives of *E. coli* HB101 were grown to mid-exponential phase in L-medium (or L-medium with added Cb) from overnight starter cultures, then

washed twice in L-medium to remove antibiotic from the HB101 strains. Samples (1 ml, 5×10^7 c.f.u.) of EMG24 (donor) and HB101 (recipient) strains were mixed, incubated at 37 °C for 1 h, then washed three times in sterile saline to remove L-medium components. The washed mating mixture was then plated on M9 minimal medium containing either leucine or proline, each at 100 µg ml⁻¹, and Cb to counterselect donor strains. The frequency of recombination in plasmid-containing HB101 strains was determined.

Induction of prophage λ . Plasmid-containing derivatives of two *E. coli* λ lysogens, N99cI⁺ (Rec⁺) and LD99 (*recA56*), were grown at 37 °C to mid-exponential phase in L-medium and induced by the addition of mitomycin C (10 µg ml⁻¹). After 1 h incubation in the presence of mitomycin C, 1 ml samples of each culture were removed and lysed by the addition of chloroform (0.02 ml). The lysed culture samples were centrifuged for 1 min in a microcentrifuge to remove cell debris, and the resulting supernatant fraction was titrated for λ on a lawn of *E. coli* LE392. Strain LD99 was constructed by mating N99cI⁺ with JC10240, selecting for the inheritance of tetracycline resistance, and then screening for the loss of sorbitol utilization (Csonka & Clark, 1980).

Preparation of anti-RecA antibody. Two female New Zealand White rabbits were immunized with 500 µg each of purified *E. coli* RecA protein (US Biochemical Corp.) emulsified in Freund's complete adjuvant (1.0 ml) in a total volume of 2.0 ml. Several (8–10) subcutaneous injections were made along the back of each rabbit, each delivering approximately 0.1 ml of immunogen. The remainder of the immunogen (200–250 µg) was administered intramuscularly in a hind quarter. At monthly intervals, two successive booster immunizations were given to the rabbits as just described except that the RecA protein was emulsified in Freund's incomplete adjuvant. One week after the second booster immunization, blood was drawn from the rabbits and sera prepared. Anti-RecA antibody was assessed by Western blot analysis (Towbin *et al.*, 1979). The anti-RecA antibody titre of the pooled sera exceeded 10000.

Induction of the *Legionella recA* gene. Two 10 ml samples of a mid-exponential-phase culture of *L. pneumophila* in BCYE broth (about 1×10^8 ml⁻¹) were harvested by centrifugation at 5000 g for 10 min, washed in normal saline and resuspended in 1 ml saline. The bacterial suspensions were each placed in the bottom of 30 mm sterile Petri plates and one was irradiated with UV (10 J m⁻²) from a germicidal lamp. The control suspension received no UV treatment. The suspensions were transferred to 250 ml shake flasks containing 50 ml pre-warmed BCYE broth and incubated with shaking (200 r.p.m.) for 1 h at 37 °C. Following incubation, the bacterial cells were pelleted by centrifugation as above, resuspended in 0.1 ml sterile water, and prepared for SDS-PAGE by the addition of 2 × sample buffer containing 10% (v/v) 2-mercaptoethanol followed by boiling for 10 min. Samples (10 µl) of each preparation were fractionated by SDS-PAGE on a 12.5% acrylamide gel as described above, transferred to nitrocellulose by electroblotting and probed with the anti-RecA antibody preparation. Alkaline-phosphatase-conjugated goat anti-rabbit IgG (Promega) served as the second antibody. The relative quantity of RecA protein in control and experimental gel lanes was determined by scanning the Western blot with a model GS 300 transmittance/reflectance scanning densitometer (Hoeffer Scientific Instruments).

Southern blot analysis. This was done as described by Silhavy *et al.* (1984). Genomic digests were separated on a 0.7% agarose gel, transferred to nitrocellulose and probed with a ³²P-labelled, nick-translated DNA probe. Hybridization was performed at 42 °C in 50% (w/v) formamide, 5 × SSC (pH 7.0), 5 × Denhardt's, 0.1% SDS, and 0.1 mg denatured salmon sperm DNA ml⁻¹. SSC (1 ×) is 0.15 M-NaCl, 0.015 M-sodium citrate (pH 7.0). Denhardt's reagent (1 ×) is 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone. Post-hybridization washes were as follow: 2 × 5 min in 1 × SSC, 0.1% SDS at 25 °C; 2 × 5 min in 0.1 × SSC, 0.1% SDS at 42 °C; and 1 × 5 min in 0.1 × SSC, 0.1% SDS at 55 °C. Radioactive fragments were detected by exposure to X-OMAT AR film.

RESULTS

*Isolation of the *L. pneumophila recA* gene*

Because of its *recA* genotype, *E. coli* HB101 is highly sensitive to UV light and chemical mutagens. This fact was exploited as a means to isolate the *L. pneumophila recA* analogue by interspecific complementation. Recombinant *E. coli* HB101 isolates which harboured the *L. pneumophila recA* gene were easily identified as those clones in which the RecA⁻ phenotype was suppressed.

Approximately 10000 *L. pneumophila* gene library members were spread on L-agar plates at a density of about 250 per plate. The bacterial colonies were replica-plated onto L-agar medium containing MMS. Four colonies which showed MMS-resistant (MMS^R) growth were picked from the appropriate master plates, streaked for isolation, and retested for their RecA phenotype. All four isolates conferred resistance to MMS and to an exposure of UV light which killed a pBR322-containing derivative of *E. coli* HB101.

Plasmid DNA isolated from the four putative *recA* clones was used to transform competent *E. coli* HB101. The resulting transformants were tested for MMS resistance to ensure that the

Fig. 1. Restriction map of the *L. pneumophila* DNA segment containing the *recA* gene. *L. pneumophila* DNA is designated by the boxed area; the pBR322 sequences of pLAD110 are defined by the thin lines. The position of Tn5 insertions which abolished the RecA⁺ phenotype conferred by pLAD110 are designated by filled triangles. The open triangle shows the position of a Tn5 insertion which did not affect RecA activity. Plasmids pLAD111, pLAD112 and pLAD113 are pUC19 containing selected fragments of pLAD110 as shown. RecA activity of strains harbouring subcloned plasmids was assessed by UV sensitivity as described in Methods. The hatched area in the pLAD110 map denotes the approximate location of the *recA* gene. The direction of *recA* transcription is shown by the arrow above the gene map. Restriction enzyme abbreviations are: E, *Eco*RI; Bg, *Bgl*II; P, *Pst*I; B, *Bam*HI; S, *Sal*I.

observed RecA phenotype was linked to the recombinant plasmid and not due to spontaneous reversion of the *E. coli* *recA* mutation. Transformants containing the putative *L. pneumophila* *recA* gene sequences from all four original clones conferred resistance to MMS.

Mapping, subcloning, and transposon mutagenesis of the *L. pneumophila* *recA* gene

Restriction analysis of plasmid DNA from the MMS^R isolates revealed that the four recombinant plasmids contained identical 6.5 kb inserts of *L. pneumophila* DNA. A restriction map of one of these plasmids, called pLAD110, is shown in Fig. 1. Transposon mutagenesis and subcloning of selected restriction fragments were used to determine the approximate location of the *recA* gene on the 6.5 kb insert of pLAD110. Sixteen Tn5 inserts which abolished the RecA⁺ phenotype were shown to map in a 700 bp region of pLAD110. The locations of representative inserts, including the two which mapped furthest apart (3 and 6) yet still within the putative *recA* coding region, are shown in Fig. 1. Of ten Tn5 inserts which did not affect the RecA phenotype of pLAD110, the one which mapped closest to the cluster of inserts within the *recA* gene mapped approximately 270 bp to the right of insert number 6 (Fig. 1). Since RecA activity was expressed by a 1.7 kb *Bgl*II-*Eco*RI subclone (pLAD112), the left end of the *recA* gene resides between the Tn5 insert number 3 and the *Bgl*II site defined by the subclones pLAD112 and pLAD113.

Expression of the *L. pneumophila* *recA* gene in *E. coli*

Having isolated and mapped the *L. pneumophila* *recA* gene, experiments were done to further characterize the RecA phenotype conferred by pLAD110 and derivative recombinant plasmids.

The degree of UV sensitivity of the *recA* strain *E. coli* HB101 harbouring various plasmids is illustrated in Fig. 2. HB101(pLAD110) showed approximately 10⁵-fold higher resistance than HB101(pBR322) to a comparable UV exposure (Fig. 2). Inactivation of MMS resistance by Tn5 insertion in pLAD110 also resulted in sensitivity to UV exposure. Strain HB101(pLAD110.5-3), containing a Tn5 insertion mapping within the *recA* gene of pLAD110 (Tn5 insert number 3 in Fig. 1), displayed UV sensitivity approximately equivalent to that of HB101(pBR322) (Fig. 2). In contrast, a Tn5 insertion which mapped outside the *recA* coding region, as in pLAD110.5-20, had no effect on the UV resistance expressed by HB101(pLAD110) (Fig. 2).

In addition to induction of DNA repair mechanisms, the *recA* gene of *E. coli* plays a mandatory role in homologous recombination. To further characterize functional characteristics of the *L. pneumophila* *recA* gene product, its ability to promote recombination in *E. coli* HB101 was tested. The results of recombination between a prototrophic *E. coli* HfrH strain (EMG24) and plasmid-containing derivatives of HB101 are shown in Table 2. Recombination

Fig. 2. Survival of various plasmid-containing isolates of *E. coli* HB101. Bacterial strains were irradiated with various doses of UV light, then survival was measured and expressed as a percentage of the value for the appropriate non-irradiated control strain. Symbols represent HB101 harbouring the following plasmids: ○, pLAD110; ●, pLAD110.5-20; △, pLAD110.5-3; ▲, pBR322.

Table 2. *Recombination in E. coli strains*

Recipient strains were incubated with HfrH strain EMG24 for 1 h prior to plating on the appropriate minimal medium. Frequency is expressed as the number of recombinants per donor.

Recipient	Frequency of recombinants	
	Pro ⁺	Leu ⁺
HB101(pBR322)	2×10^{-6}	9×10^{-6}
HB101(pLAD110)	7.1×10^{-3}	1.1×10^{-2}
HB101(pLAD110.5-3)	1.1×10^{-6}	3×10^{-7}
HB101(pLAD110.5-20)	1.2×10^{-3}	2.3×10^{-2}

between EMG24 and HB101(pLAD110) or HB101(pLAD110.5-20) was approximately 10^3 -fold greater than that observed when HB101(pBR322) or HB101(pLAD110.5-3) were used as recipient strains. Low-level recombination between EMG24 and HB101 in the absence of a functional *recA* gene was attributed to F' formation.

Cleavage of LexA and λ repressor proteins in *E. coli* requires a normally quiescent proteolytic activity of the RecA protein. Expression of this activity is mediated by a number of chemical and physical treatments and represents activation of the SOS response in *E. coli*. Cleavage of the λ repressor by the *L. pneumophila* RecA protein was tested by assessing the induction of prophage λ in plasmid-containing *recA* and *recA*⁺ *E. coli* backgrounds. The addition of mitomycin C ($10 \mu\text{g ml}^{-1}$) to strain N99cI⁺ (*recA*⁺) harbouring pUC19 resulted in a nearly 100-fold increase in λ plaque production over the observed level of spontaneous induction (Table 3). The presence of the cloned *L. pneumophila recA* gene reduced the level of both spontaneous and mitomycin-C-induced prophage induction by nearly 100-fold. In addition, the *L. pneumophila recA* gene product failed to promote λ induction in a *recA* *E. coli* background.

Identification of the L. pneumophila recA gene product and UV-induced synthesis of the RecA protein

Minicell analysis was used to investigate the product of the *L. pneumophila recA* gene. In an attempt to eliminate other *L. pneumophila* protein products which may be encoded on pLAD110,

Table 3. Spontaneous and mitomycin-C-mediated induction of λ prophage in plasmid-containing strains

Strain	Prophage induction (p.f.u. ml ⁻¹) following treatment with:	
	No mitomycin C	Mitomycin C at 10 μ g ml ⁻¹
N99cI ⁺ (pUC19)	7.8×10^5	4.3×10^7
N99cI ⁺ (pLAD111)	2.3×10^3	4.0×10^5
LD99(pUC19)	100	100
LD99(pLAD111)	0	0

a 1.9 kb *Eco*RI fragment containing the entire *L. pneumophila* *recA* gene was subcloned into the single *Eco*RI site of pACYC184. The resulting plasmid pLAD211 and the pACYC184 vector were used to transform the minicell-producing *E. coli* strain P678-54. Minicells were isolated from both plasmid-containing derivatives of P678-54 and labelled with [³⁵S]methionine. Following SDS-PAGE of labelled cell extracts and autoradiography, a single 21 kDa protein band representing the monomeric form of the pACYC184-encoded type I chloramphenicol acetyltransferase was observed in minicells containing only vector DNA (Fig. 3, lane A). In contrast, the pLAD211-containing minicell preparation contained a major 37.5 kDa protein and a minor 35 kDa protein band (Fig. 3, lane B). The 37.5 kDa protein is nearly identical in molecular mass to the *E. coli* RecA protein (Sancar *et al.*, 1980) and presumably is the *L. pneumophila* counterpart. The 35 kDa protein is probably a product of proteolytic cleavage of the native RecA protein. A similar finding was reported for the *recA* gene product of *Vibrio cholerae* (Goldberg & Mekalanos, 1986).

To determine whether the 37.5 kDa protein was in fact the product of the *recA* gene, proteins encoded by pLAD110 and two Tn5-mutated, RecA⁻ derivatives were examined by minicell analysis (Fig. 4). The 37.5 kDa protein appeared only in ³⁵S-labelled extracts of the pLAD110-containing strain (Fig. 4, lane A). Mutation of pLAD110 to the RecA⁻ phenotype coincided with the disappearance of the 37.5 kDa protein. This finding, although not indisputable, strongly suggested that the 37.5 kDa protein was the product of the *L. pneumophila* *recA* gene. In place of the 37.5 kDa protein, the RecA⁻ mutants encoded the Tn5-derived 28 000 kDa aminoglycoside 3'-phosphotransferase, the IS50R-encoded 54 kDa and 58 kDa P2 inhibitor and transposase, respectively (Johnson *et al.*, 1982), and truncated versions of the RecA protein (Fig. 4, lanes B and C). The two Tn5-containing pLAD110 derivatives examined were those spaced farthest apart yet still within the *recA* coding region (Fig. 1). The sizes of the truncated RecA proteins encoded by pLAD110.5-3 and pLAD110.5-6 were consistent with *recA* transcription starting to the right of the *Bam*HI site and proceeding towards the *Bgl*III site, as shown by the arrow in Fig. 1. These data are also consistent with the cloned *recA* gene being under control of an endogenous promoter, since the direction of *recA* transcription in pLAD110 is opposite that of the interrupted tetracycline-resistance gene of pBR322.

Induction of the *L. pneumophila* *recA* gene was examined by Western blot analysis of extracts of UV-treated and control *L. pneumophila* cells, using antibody against *E. coli* RecA protein as probe. Irradiation of *L. pneumophila* with UV resulted in a significant increase in the level of the RecA protein present in cell extracts (Fig. 5). A scan of the blot indicated a four- to fivefold increase in detectable RecA in UV-irradiated cells. In addition to the 37.5 kDa form of RecA, the 35 kDa breakdown was also observed.

Southern blot analysis

Genomic digests of various strains of *L. pneumophila* and other *Legionella* species were analysed by Southern blotting to determine sequence relatedness of the cloned *recA* gene to the analogous DNA segment in other strains. When probed with a ³²P-labelled 1.9 kb *Eco*RI fragment containing the *recA* gene of the *L. pneumophila* Philadelphia 1 strain (serogroup 1),

Fig. 3

Fig. 4

Fig. 3. Expression of the *L. pneumophila recA* gene in minicells. Minicells were isolated and labelled with [35 S]methionine as described in Methods. SDS-soluble extracts of labelled minicell preparations were separated by electrophoresis through a 12.5% polyacrylamide gel. The gel was dried and exposed to X-ray film to produce the autoradiogram shown. Lane A, pACYC184; lane B, pLAD211. The position of molecular mass markers was determined by staining the gel prior to drying. Markers were: myosin (H-chain), 200 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; α -chymotrypsinogen, 25.7 kDa; β -lactoglobulin, 18.4 kDa; lysozyme, 14.3 kDa. The type I chloramphenicol acetyltransferase encoded by pACYC184 is designated by CAT.

Fig. 4. Analysis of proteins encoded by pLAD110 and Tn5-interrupted derivatives. Minicell preparations, molecular mass standards, and conditions for preparing the autoradiogram from the dried polyacrylamide gel, were as described for Fig. 3. Lane A, pLAD110; lane B, pLAD110.5-3; lane C, pLAD110.5-6. Molecular mass values shown to the right are for the native RecA protein (37.5 kDa) and truncated derivatives (35 kDa and 19 kDa). The pBR322-encoded β -lactamase and the Tn5-encoded aminoglycoside phosphotransferase are designated by BLA and APT, respectively.

EcoRI genomic digests of strains representing serogroups 1, 2, 3 and 4 yielded a single 1.9 kb band (Fig. 6, lanes 1–4). In contrast, a faint 6.0 kb fragment was seen in the lanes containing the non-*pneumophila* DNA digests (Fig. 6, lanes 5–7). The weak hybridization signal obtained with the non-*pneumophila* species suggests limited sequence relatedness between the *recA* gene of *L. pneumophila* and the three other *Legionella* species tested here. An *EcoRI* digest of *E. coli* genomic DNA did not hybridize with the *L. pneumophila recA* probe (Fig. 6, lane 8).

DISCUSSION

The *recA* gene participates in several important functions in *E. coli*, including homologous recombination, DNA repair and induction of the SOS response and prophage λ . In recent years,

Fig. 5

Fig. 6

Fig. 5. UV induction of *L. pneumophila* *recA*. Extracts of control (lane 1) and UV-treated (lane 2) *L. pneumophila* were separated by SDS-PAGE, electrotransferred to nitrocellulose and probed with antibody raised against *E. coli* RecA protein. Pre-stained molecular mass markers (lane 3) were: phosphorylase *b*, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; α -chymotrypsinogen, 25.7 kDa; β -lactoglobulin, 18.4 kDa and lysozyme, 14.3 kDa.

Fig. 6. Southern blot analysis. *EcoRI* genomic digests of bacterial strains were separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with a ^{32}P -labelled 1.9 kb DNA segment containing the entire *L. pneumophila* *recA* gene. Lanes 1-4 contained 1-3 μg of DNA from *L. pneumophila* serogroups 1-4, respectively. The remaining lanes contained identical preparations of DNA from *L. bozemanii* (lane 5), *L. gormanii* (lane 6), *L. micdadei* (lane 7), and *E. coli* (lane 8).

recA gene analogues from several widely divergent bacterial species have been cloned by complementation of *E. coli* *recA* mutations (see Introduction). A similar approach was used in this report to clone the *L. pneumophila* *recA* gene and partially characterize the encoded RecA protein.

Heterologous *recA* genes described thus far promote homologous recombination and UV resistance in *E. coli* *recA* host strains. DNA repair and recombination promoting activities of the RecA proteins are thus highly conserved among widely divergent species. The ability to cleave λ *ci* and induce SOS repair mechanisms, however, are properties thus far not reported with

heterologous *recA* products apart from RecA proteins of other members of the *Enterobacteriaceae* (Keener *et al.*, 1984). With regard to *cI* cleavage, the *L. pneumophila recA* gene product is no exception to this observation. In fact, the presence of the *L. pneumophila recA* gene in an *E. coli* strain lysogenic for λ resulted in an inhibition of induction by mitomycin C. These data probably indicate that the *L. pneumophila* RecA protein is capable of binding but not cleaving or inactivating *cI*. UV induction of the *L. pneumophila recA* gene and the resulting increase in RecA protein indicates that the *L. pneumophila recA* gene is regulated by UV damage. Whether regulation of *L. pneumophila recA* involves cleavage of a LexA analogue by an activated RecA protein, however, is not known. The inability to demonstrate proteolytic inactivation of λ *cI* by the *L. pneumophila recA* product is not necessarily indicative of a unique SOS-like response. Rather, these data are probably a reflection of the high degree of specificity required by *E. coli* RecA to bind and cleave *cI*.

Kokjohn & Miller (1987) reported that the product of the *Pseudomonas aeruginosa recA* gene cross-reacted with antibody raised against purified *E. coli* RecA protein. Based on this observation it was anticipated that the *L. pneumophila* RecA protein might also share immunological cross-reactivity with that of *E. coli*. Though expected (and observed), immunological cross-reactivity between the *recA* gene products of *L. pneumophila* and *E. coli* is of significant interest. As shown here and by others there is little or no detectable gene sequence homology (by Southern hybridization analyses) or restriction endonuclease cleavage patterns among the various cloned *recA* genes, yet functionally they are highly conserved. Immunological cross-reactivity of *recA* gene products is no doubt a reflection of the observed functional conservation. A comparison of *recA* gene sequences is certainly warranted.

This work was initiated as a first step towards the construction of *recA* strains of *L. pneumophila* by a gene replacement technique. Such strains will be invaluable in the long-term goals of characterizing the genetic organization of *L. pneumophila*. They will be most useful as recipients for plasmid-mediated chromosomal transfer experiments. Although marker transfer has been demonstrated, R' structures were not isolated (Dreyfus & Iglewski, 1985). Stabilization of R' plasmids in a *recA* background will allow *in vivo* cloning of *L. pneumophila* genes. Additionally, the role of recombination in reversible avirulent conversion can be assessed. Finally, the *recA* gene product of *E. coli* participates in a number of regulatory and catalytic processes. Conservation of heterologous *recA* gene function in *E. coli* indicates that the RecA protein plays similar roles in other bacterial species. The *recA* gene is therefore a potentially good candidate for analysis of gene function and regulation in *L. pneumophila*.

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