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PhoA gene fusions in *Legionella pneumophila* generated *in vivo* using a new transposon, Mud*phoA*

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Summary

To enable effective use of *phoA* gene fusions in *Legionella pneumophila*, we constructed Mud*phoA*, a derivative of the mini-Mu phage Mu dII4041, which is capable of generating gene fusions to the *Escherichia coli* alkaline phosphatase gene (EC 3.1.3.1). Although an existing fusion-generating transposon, Tn*phoA*, has been a useful tool for studying secreted proteins in other bacteria, this transposon and other Tn*5* derivatives transpose inefficiently in *Legionella pneumophila*, necessitating the construction of a more effective vector for use in this pathogen. Using Mud*phoA* we generated fusions to an *E. coli* gene encoding a periplasmic protein and to an *L. pneumophila* gene encoding an outer membrane protein; both sets of fusions resulted in alkaline phosphatase activity. We have begun to use Mud*phoA* to mutate secreted proteins of *L. pneumophila* specifically, since this subset of bacterial proteins is most likely to be involved in host–bacterial interactions. This modified transposon may be useful for studies of other bacteria that support transposition of Mu, but not Tn*5*, derivatives.

Introduction

Bacterial proteins that mediate the virulence of pathogens are most likely to be expressed at the bacterial surface or exported from the bacterial cell. In the case of *Legionella pneumophila*, several secreted (or exported) proteins with potential roles in pathogenesis have been identified. These include Mip, a 24 kDa surface protein (Cianciotto *et al.*, 1989), MSP, a 38 kDa protease (Dreyfus and Iglewski, 1986), and legiolysin, a 39 kDa protein that confers haemolysis, pigment production and fluorescence activities (Williams *et al.*, 1991). Several genetic methods for mutational analysis in *L. pneumophila* have been reported. Among these methods are allelic exchange of mutant for wild-type genes mediated by tri-parental conjugational matings, introduction of Tn*5* on the broad-host-range plasmid pRK340, and introduction of bacteriophage Mu on the broad-host-range plasmid pRK2 (Cianciotto *et al.*, 1988; Dreyfus and Iglewski, 1985; Keen *et al.*, 1985; Mintz and Shuman, 1987).

One way of identifying genes encoding proteins that are localized specifically to the bacterial envelope or periplasm is by using transposons that generate fusions between host proteins and enzymes that are active only when secreted. For example, Tn*phoA* generates fusions to alkaline phosphatase and Tn*blaM* to β-lactamase, both normally periplasmic proteins in *Escherichia coli* (Manoil and Beckwith, 1985; Broome-Smith *et al.*, 1990). We found that Tn*phoA* functions poorly as an insertional mutagen in *L. pneumophila*. Alternatively, Mu transposons are known to be functional in this bacterium (Mintz and Shuman, 1987). We therefore decided to modify Mu dII4041 by ligating a *phoA* gene fragment near its right end, thereby constructing Mu dII*phoA* (herein called Mud*phoA*). Mu dII4041 was chosen because it contains a *Bam*HI site 116 base pairs (bp) from the Mu right end and ligations into this site are capable of maintaining an open reading frame from the Mu right end through the ligated fragment (Castilho *et al.*, 1984; Kahmann and Kamp, 1979). Similar mini-Mu (Ap,*lac*) transposons have been constructed for *in vivo* generation of transcriptional and translational gene fusions by ligating an *E. coli lac* fragment into the *Bam*HI site (Casadaban and Chou, 1984).

Results

*Construction of Mud*phoA

We inserted the *E. coli* alkaline phosphatase gene (*phoA*) near the right end of mini-Mu transposon Mu dII4041(Castilho *et al.*, 1984) (Fig 1). To facilitate this construction, Mu dII4041 was first transposed into plasmid pUC18Δ, using strain BAC101 (Table 1). A DNA fragment encoding the *phoA* gene, missing amino-terminal sequences, was ligated into the *Bam*HI and *Xho*I sites on pUC18Δ::Mu dII4041. The Tn*5* kanamycin resistance gene (*neo*) (Beck *et al.*, 1982) was inserted adjacent to the *phoA* fragment. The resulting transposon, Mu dII*phoA*

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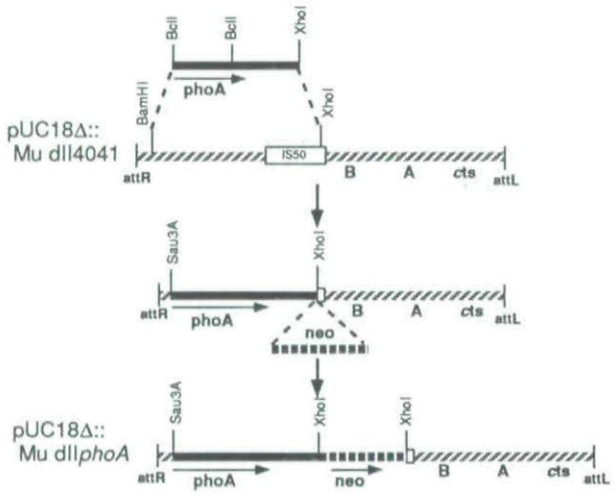

Fig. 1. Construction of Mud*phoA*. Mu dII4041 was transposed onto pUC18Δ, a derivative of pUC18 that lacks the small *Pvu*II fragment containing the multiple cloning sequence (Yanisch-Perron *et al.*, 1985). The *Bcl*I–*Xho*I fragment containing the *E. coli* alkaline phosphatase (*phoA*) gene was isolated from pSMJ11.4::Tn*phoA* (see Table 1) and ligated to pUC18Δ::Mu dII4041, replacing the *Bam*HI–*Xho*I fragment in Mu dII4041, which contained the *neo* gene (Km^R) and most of the IS50 sequences (Manoil and Beckwith, 1985). An *Xho*I fragment from pSMJ11.4::Tn*phoA* containing the Km^R gene (*neo*) was ligated to the unique *Xho*I site on pUC18Δ::Mud*phoA*. All further work was done using the clone with the Km^R gene transcribed in the same direction as the *phoA* gene. Mu right end and Mu left end refer to the right and left Mu terminal repeat ('attachment') sequences, respectively. A and B are the essential Mu transposition-replication genes and *c*ts is the temperature-sensitive Mu repressor gene, *c*ts62 (Casadaban and Chou, 1984).

(Mud*phoA*), possesses only those Mu sequences necessary for transposition, it is defective for making phage particles, and it has a temperature-sensitive repressor gene (*c*ts62).

Characterization of the junction between the inside Mu right end and phoA

For Mud*phoA* to be effective in generating protein fusions, its *phoA* fragment must be capable of expressing alkaline phosphatase activity, and the junction between the right end of Mu and *phoA* must maintain a translational reading frame through the Mu right end. The *Bcl*I–*Xho*I fragment from Tn*phoA* lacks the *phoA* promoter, ribosome-binding site, and signal peptide sequences, as well as the coding sequence for 28 amino acids beyond the signal sequence cleavage point, to Asp-29 (Chang *et al.*, 1986). Since Tn*5*–*phoA* junctions up to the 40th amino acid beyond the signal cleavage point are still capable of generating normal levels of phosphatase activity (Manoil and Beckwith, 1985), we expected that the *phoA* fragment in Mud*phoA* would also express the activity.

Ligation of the 5'-end of the *phoA* fragment into the right end of Mu dII4041 maintains not only the reading frame of the *phoA* gene but also the *Sau*3A restriction site at the Mu right end–*phoA* junction (Fig. 1). To confirm that this site was preserved, we analysed a deletion derivative of pUC18Δ::Mud*phoA*, since it was easier to map the *Sau*3A site on this smaller plasmid (Fig. 2). The presence of this junctional *Sau*3A site was taken as evidence that the ligation was precise and that the junction maintained the correct translational reading frame.

*Transposition of Mud*phoA *and construction of a double Mu lysogen, EA17*

To test whether Mud*phoA* would transpose as expected, strain M8820Mucts was transformed with pUC18Δ::Mud*phoA* and one of the transformants was purified. Plasmid

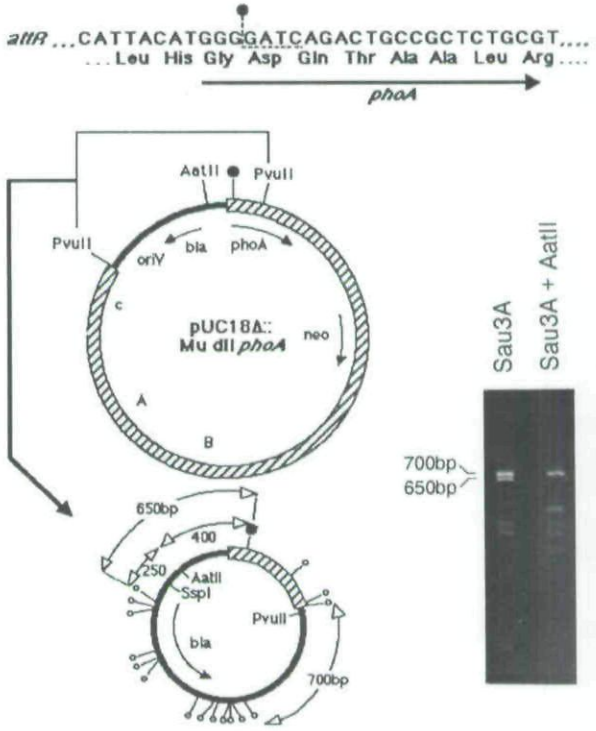

Fig. 2. Analysis of the Mu dII4041-*phoA* ligation junction. The inferred sequence of the junction is shown at the top of the figure with the junctional *Sau*3A site marked by a solid circle. pUC18Δ::Mud*phoA* was digested with *Pvu*II and religation of the smaller fragment resulted in recovery of a deletion plasmid that contained the junctional *Sau*3A site (●) as well as several other *Sau*3A sites (○). The 700 bp and 650 bp fragments indicated on the circular map are shown in the restriction enzyme analysis of the pUC18Δ::Mud*phoA* deletion plasmid (lower right). As indicated on the map, digestion of the 650 bp *Sau*3A fragment with *Aat*II results in two smaller fragments of 400 bp and 250 bp. Note the disappearance of the 650 bp fragment on the gel in the double digest. Double digestion with *Ssp*I and *Sau*3A also eliminated the 650 bp fragment and produced fragments of the expected size (data not shown).

Table 1. Bacterial strains and plasmids.

Strain/Plasmid	Description or Genotype	Source or Reference
E. coli		
M8820	F⁻, *araD139*, Δ(*ara–leu*)*7697*, Δ(*proAB-argF-lacIPOZYA*)XIII, *rpsL*	Casadaban (1975)
M8820Mu	F⁻, *araD139*, Δ(*ara–leu*)*7697*, Δ(*proAB-argF-lacIPOZYA*)XIII, *rpsL*, Mu *c*⁺	Casadaban and Chou (1984)
M8820Mucts	M8820 with Mu cts62	Casadaban and Chou (1984)
MG1655	Prototroph, F⁻, λ⁻	Guyer *et al.* (1981)
JC10240	Hfr, *thr300, recA56, srl300*::Tn*10*(Tc^R), *relA1, rlv318, spoT, thi1,rpsE2300*, λ⁻	Csonka and Clark (1979)
BAC101	M8820 *recA56 srl*::Tn*10*(Tc^R) Mu *c*ts62 with Mu dII4041	Casadaban and Chou (1984)
EA4	M8820Mucts, *recA56, srl300*::Tn*10* (Tc^R)	This work
EA17	EA4 with Mud*phoA* prophage	This work
χ2981	*E. coli* K-12, F⁻ Δ41[*proB-lacYZ*],λ⁻ T3r, Δ*asdA4, zhf-2*::Tn*10, cycA1*	R. Curtiss
χ2981 (pTLP7::Mud*phoA*)	χ2981 with pTLP7::Mud*phoA*	This work
χ2981(pRK212.1)	χ2981 with pRK212.1	J. E. Rogers
CC118	*araD139*, Δ(*ara, leu*)*7696*, Δ *lacX74, phoA*Δ20, *galE, galK, thi, rpsE, rpoB, argE*$_{am}$ *recA1*	Manoil and Beckwith (1985)
L. pneumophila		
AA100	*L. pneumophila* SG1 130b	Engleberg *et al.* (1984a)
AA103	*L. pneumophila* SG1 130b high-frequency conjugation recipient	Cianciotto *et al.* (1988)
AA110	AA103 with Mud*phoA* fusion	This work
AA111	AA103 with Mud*phoA* fusion	This work
AA112	AA103 with Mud*phoA* fusion	This work
AA113	AA103 with Mud*phoA* fusion	This work
AA114	AA100 with pNC31.5::Mud*phoA*	This work
AA118	AA103 with Mud*phoA* fusion	This work
Plasmids		
pUC18Δ	pUC18, *Pvu*II deletion removing the polylinker region, Ap^R	This work
pUC18Δ::Mu dII4041	pUC18Δ with Mu dII4041 insertion, Ap^R, Km^R	This work
pUC18Δ::Mud*phoA*	pUC18Δ with Mud*phoA*, Ap^R Km^R	This work
pSMJ11.4::Tn*phoA*	Ap^R, *pal*::Tn*phoA*	Engleberg *et al.* (1991)
pNC31.5	Ap^R, Cm^R, *oriT, mip*	Cianciotto *et al.* (1989)
pTLP7	Cm^R, *sacB, oriT*	This work
pTLP7::Mud*phoA*	Cm^R, Km^R, *sacB, oriT*	This work
pRK212.1	Ap^R, Tc^R, Tra⁺	Figurski *et al.* (1976)

DNA isolated from this strain was compared to pUC18Δ::Mud*phoA* by restriction enzyme digestions to ensure that pUC18Δ::Mud*phoA* had been stably introduced and had not undergone any rearrangements or deletions. The restriction mapping indicated that the two plasmids were identical. M8820Mucts harbours a complete Mu prophage that is capable of making phage particles. Since it also possesses the temperature-sensitive repressor gene (*c*ts62), phage growth and transposition are inducible at the permissive temperature of 42°C (Castilho *et al.*, 1984).

A culture of M8820Mucts (pUC18Δ::Mud*phoA*) was grown at 30°C and then shifted to 42°C to initiate transposition and lysis. A lysate prepared from this culture was used to transduce *E. coli* strain MG1655 to kanamycin resistance (Km^R). The M8820Mucts (pUC18Δ::Mud*phoA*) strain produced lysate titres of 10^8–10^9 plaque-forming units (pfu) per ml when titred on MG1655, and the frequency of Km^R transduction was 10^{-3}–10^{-4} per recipient cell at a multiplicity of infection of 1. Among the Km^R transductants, 97% were ampicillin sensitive (Ap^S), indicating that most Mu particles in the lysate containing Mud*phoA* did not contain pUC18Δ. We concluded that the separation of these antibiotic markers was probably caused by the transposition of Mud*phoA* at the permissive temperature.

We adapted the scheme of Castilho *et al.* (1984) to test the ability of Mud*phoA* to generate active *phoA* fusion proteins. In this scheme a donor strain is constructed carrying one copy each of Mud*phoA* and the helper prophage Mucts. The target plasmid is then introduced into the donor strain, transposition is derepressed by raising the temperature, and the lysate generated by this process is used to transduce a Mu-immune recipient. In

some transductants, homologous recombination between Mu sequences results in recovery of Mu insertions in the target plasmid (Castilho *et al.*, 1984).

To prevent the formation of target plasmid multimers, which would be recombinationally unstable following Mu insertion, it was necessary to construct this donor strain in a recombination-deficient background. Accordingly, M8820Mu*cts* was transduced with a P1 lysate made from strain JC10240 (*recA*56 *srl*::Tn*10* (Tc^R)) (Csonka and Clark, 1979), selecting for tetracycline resistance. The resulting strain, EA4, was purified and tested for the *recA* mutation by u.v. light sensitivity (Maniatis *et al.*, 1982). Strain EA4 was then transduced by a M8820Mu*cts* (pUCΔ::Mud*phoA*) lysate, followed by selection for Km^R lysogens in which Mud*phoA* had inserted despite Mu immunity. While the frequency of obtaining these double Mu lysogens was low, one was selected, i.e. strain EA17. Chromosomal DNA from strains EA17 and EA4 and pUC18Δ::Mud*phoA* plasmid DNA was cut with *Hin*dIII and probed with radiolabelled pUC18Δ::Mud*phoA*. Comparison of the hybridizing fragments among these DNAs showed that EA17 contained the same Mu fragments as EA4 as well as fragments identical to pUC18Δ::Mud*phoA* that were internal to Mud*phoA*, but no fragments specific to the vector pUC18Δ. In addition, alkaline lysis preparations of EA17 produced no detectable plasmid DNA. We concluded from this evidence (data not shown) that EA17 contained chromosomal copies of the two Mu genomes.

*Isolation and expression of Mud*phoA *fusions in β-lactamase and the* L. pneumophila mip *gene*

The β-lactamase gene on pBR322 encodes a periplasmic protein (Bolivar *et al.*, 1977). Tn*phoA* insertions into this gene generate active *phoA* fusions (Manoil and Beckwith, 1985). To test for Mud*phoA* fusions to this gene, pBR322 was introduced into EA17 by transformation. One transformant was induced for phage growth and transposition at 42°C. The lysate from this strain was used to transduce the Mu-immune, *recA*$^+$ strain M8820Mu*c*$^+$, and to recover potential Mud*phoA* insertions. The Mu-immune strain M8820Mu*c*$^+$ was used as a recipient to reduce the likelihood of additional transpositions by the incoming Mud*phoA*.

Table 2 shows the frequencies of the various phenotypes generated by pBR322::Mud*phoA* insertion. The frequency of insertions into the Ap^R and Tc^R genes was lower than expected given the size of these regions on pBR322 (Sutcliffe, 1979). About 14% of the Mud*phoA* insertions in the Ap^R gene were PhoA$^+$, which is close to the expected frequency (16.7%) of Mud*phoA* in-frame translational fusions (two orientations, three reading frames)

We next isolated fusions to an *L. pneumophila* gene, *mip*, whose product is an outer membrane protein. When *mip* is cloned on an *E. coli* replicon, its protein product localizes to the *E. coli* surface (Engleberg *et al.*, 1984a,b). A plasmid carrying a cloned copy of *mip*, pNC31.5 (Cianciotto *et al.*, 1989), was introduced into EA17 by transformation. The resulting strain was induced for phage growth and two *mip*::Mud*phoA* insertions having alkaline phosphatase activity were isolated following transduction of M8820Mu*c*$^+$. We introduced the *mip*::Mud*phoA* fusion plasmids into *L. pneumophila* strain AA100 by electroporation. Transformants expressed PhoA activity on XP-containing agarose plates (see the *Experimental procedures*), confirming that Mud*phoA* fusions can be expressed in *L. pneumophila* as well as in *E. coli*.

Table 2. Phenotype of Km^R transductants with a Mu lysate from EA17(pBR322).

Phenotype	Number counted	%
Ap^R, Tc^R, PhoA$^-$	995	94.5
Ap^R, Tc^S, PhoA$^-$	20	2.0
Ap^S, Tc^R, PhoA$^-$	30	3.0
Ap^S, Tc^R, PhoA$^+$	5	0.5

To confirm that the alkaline phosphatase activity of these strains resulted from fusions to Mud*phoA* and not from other events (such as constitutive phosphatase mutations), strain CC118, an *E. coli* strain that has a *phoA* deletion (*phoA* Δ20) (Manoil and Beckwith, 1985) was transformed with each of the presumed PhoA$^+$ plasmids. All of the resulting CC118 strains acquired alkaline phosphatase activity, demonstrating that the PhoA$^+$ phenotype was of plasmid origin.

The insertion points of Mud*phoA* in both the β-lactamase gene and the *mip* gene were mapped and the sizes of the expected fusion proteins were estimated. Several *bla*::Mud*phoA* insertions mapped in a region approximately 300 bp from the beginning of the *bla* gene while other *bla*::Mud*phoA* insertions mapped approximately 400, 500, 600 and 800 bp into the *bla* gene. Of the two *mip*::Mud*phoA* insertions, one mapped approximately 200 bp upstream and the other 100 bp downstream from a unique *Hin*dIII site located near the *C*-terminal end of *mip* (Engleberg *et al.*, 1989).

We also analysed whole cell lysates of the CC118 strains containing plasmids with Mud*phoA* fusions by immunoblotting with anti-alkaline phosphatase antibody and anti-Mip monoclonal antibody (12F4) (Cianciotto *et al.*, 1989). Anti-alkaline phosphatase antibody produced reactive bands in each of the *bla*::Mud*phoA* samples and the molecular weights of these protein species closely approximated the predicted sizes based on restriction mapping. In the case of the *mip*::Mud*phoA* samples, both anti-alkaline phosphatase and anti-*mip* antibody reacted

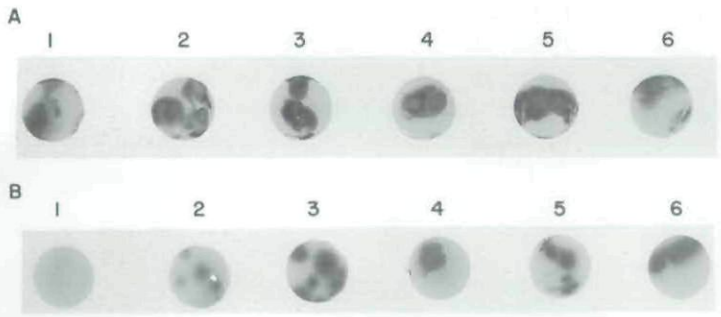

Fig. 3. Detection of alkaline phosphatase activity on XP-agarose plates. In panel A, the XP-agarose has been adjusted to a pH of 7.0 and in panel B to a pH of 11.0. In both panels, disc 1 is AA103, discs 2–5 are PhoA$^+$, Mud*phoA* insertion mutants AA110, AA111 AA112, and AA113 and disc 6 is AA114, a PhoA$^+$ *L. pneumophila* carrying pNC31.5::Mud*phoA* (*mip*::Mud*phoA*). At pH 7, all of the strains, including AA103 which lacks a Mud*phoA* insertion, produce a blue colour due to the native *L. pneumophila* phosphatase (Muller, 1981). At pH 11.0, only the *L. pneumophila* strains with Mud*phoA* insertions that resulted in *E. coli* alkaline phosphatase activity produce a blue colour.

with the same bands, which also closely approximated the predicted molecular weights (data not shown). We concluded from these observations that Mud*phoA* insertions into the *bla* and *mip* genes resulted in the formation of stable fusion proteins.

*Construction of a delivery vector, pTLP7::Mud*phoA

For delivery of Mud*phoA* into *Legionella*, we constructed a vector, pTLP7, that has both selectable and counterselectable genetic markers. We included the broad-host-range plasmid pRK2 origin of transfer sequence, *oriT* (Guiney and Yakobson, 1983), to mobilize the vector into *L. pneumophila* using transfer functions provided *in trans* by a helper conjugational plasmid, pRK212.1 (Figurski *et al.*, 1976). Counterselection of the delivery vector facilitates the isolation of chromosomal Mud*phoA* insertions since any kanamycin-resistant transconjugants remaining after counterselection must be due to non-vector, or chromosomal, copies of Mud*phoA*. We chose the *sacB* allele of *Bacillus subtilis* as the counterselectable marker (Gay *et al.*, 1985). The *sacB* gene encodes a sucrose-inducible levansucrase that synthesizes levans, branched polymers of fructose. Synthesis of these polymers within the periplasm of *L. pneumophila* is lethal. Thus, *L. pneumophila* carrying this gene will not grow on buffered charcoal yeast extract (BCYE) supplemented with sucrose, and only cells that have lost the allele are able to survive (Cianciotto *et al.*, 1988).

Strain EA17 was transformed with pTLP7 and Mud*phoA* insertions into the plasmid were isolated. One of these pTLP7::Mud*phoA* plasmids was purified, and the insertion was mapped to ensure that Mud*phoA* had not inserted into a strategically important sequence, such as the *sacB* gene. pRK212.1 and pTLP7::Mud*phoA* were then introduced by transformation into strain χ2981, a diaminopimelic acid (dap) auxotroph, since exclusion of diaminopimelic acid from selective BCYE medium counterselects the *E. coli* donor strains in conjugal matings.

*Isolation of Mud*phoA *insertions with alkaline phosphatase activity in* L. pneumophila

We used tri-parental conjugative matings to introduce pTLP7::Mud*phoA* into *L. pneumophila* (Engleberg *et al.*, 1988), and screened the resulting Km^R exconjugants for *phoA* activity. Strain AA103 was used as the recipient strain because it generates higher frequencies of transconjugants in such matings (Cianciotto *et al.*, 1988). We did not subject the mating mixtures to sucrose counterselection, since simultaneous selection for Km^R and sucrose counterselection reduced the total number of transconjugants by about 10-fold. Plasmid counterselection could be more efficiently applied after isolation of PhoA$^+$ transductants. In a typical experiment, we screened between 1000–2000 Km^R transductants. The frequency of PhoA$^+$ isolates was about 0.1%. Some positive colonies developed colour more rapidly than others, probably indicating different levels of phosphatase expression.

Figure 3 demonstrates the PhoA screening assay using XP-agarose indicator plates. At pH 7, both PhoA$^+$ and PhoA$^-$ strains exhibited phosphatase activity, seen as an intense blue colour on the XP-agarose indicator medium (Fig. 3A). By raising the pH of the medium, the background activity produced by the native *L. pneumophila* phosphatase was minimized (Fig. 3B, disc 1), whereas strains with PhoA$^+$ gene fusions continued to produce a blue colour (Fig. 3B, discs 2–7). Strain AA114 (*mip*::Mud*phoA*) was included to show the expression of a Mud*phoA* gene fusion to a known *L. pneumophila* surface protein. PhoA$^+$ strains AA110 and AA112 consistently produce a blue colour that is less intense than the colour produced by AA111, AA113 or AA114.

The PhoA$^+$ *L. pneumophila* isolates were also analysed by Southern hybridization (Fig. 4). Figure 4, panel A shows genomic DNA from four PhoA$^+$ strains, digested with *Hin*dIII and probed with pTLP7:: Mud*phoA*. The Mud*phoA* insertions are different in each strain, since the

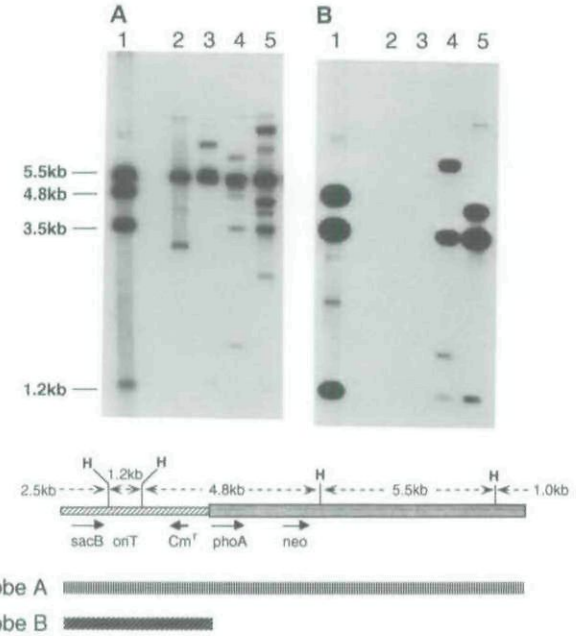

Fig. 4. Southern hybridization of *L. pneumophila* having Mud*phoA* insertions. Lane 1 (control), pTLP7::Mud*phoA*; lane 2, AA110; lane 3, AA111; lane 4, AA112; lane 5, AA113. The *Hin*dIII restriction map of pTLP7::Mud*phoA* is shown below the photographs. The thinner, diagonally striped segment corresponds to pTLP7 vector sequences and the thicker, darkly shaded segment is Mud*phoA*, inclusive of the Mu right and left ends. The corresponding sequences used as probes are indicated below the map. In panel A, the filter was probed with pTLP7::Mud*phoA* (probe A). In panel B , the filter was washed free of probe A and reprobed with pTLP7 (probe B) to identify vector-specific fragments. Note that all of the samples in panel A have a 5.5 kilobase (kb) internal Mud*phoA* fragment. Note that AA110 and AA111 have only one Mud*phoA* insertion each, and they are free of vector sequences. AA112 and AA113 have retained vector sequences as well as transpositions. The high molecular-weight band in the AA110, AA111, AA112, and AA113 samples (lanes 2–5) is presumably pRK212.1 which has a unique *Hin*dIII site and shares the *oriT* sequence with the probe (Figurski *et al.*, 1976).

sizes of the junctional bands, determined by the positions of flanking genomic *Hin*dIII sites, are different. Each Mud*phoA* insertion would therefore produce three bands, one corresponding to the 5.5-kb internal fragment, and two variable junction fragments. AA111 (Fig. 4, lane 3) has three fragments, indicating a single insertion. AA110 (Fig. 4, lane 2) has two fragments that react strongly; one corresponds to the 5.5-kb internal fragment. The third fragment is probably the weaker band at approximately 4.0 kb. AA112 and AA113 (Fig. 4, lanes 4 and5) have multiple insertions. By comparing lane 1 (pTLP7::Mud*phoA*) with AA112 and AA113 (Fig. 4, lanes 4 and 5) it appears that these last two strains contain vector-specific fragments. Since we did not subject the matings to counterselection to eliminate the vector, the presence of these bands is not surprising. The spontaneous loss of the vector in AA110 and AA111 is consistent with the behaviour of ColE1 replicons in *L. pneumophila* (Engleberg *et al.*, 1988). To confirm this interpretation, the same filter was probed with pTLP7 (Fig. 4B). Vector sequences were present in strains AA112 and AA113 (lanes 4,5) but not in AA110 and AA111. Moreover, the vector fragments in AA112 and AA113 were of unexpected sizes, indicating some rearrangement of the vector sequences.

*Detection of Mud*phoA *fusion proteins*

To detect the presence of fusion proteins in the PhoA$^+$ *L. pneumophila* strains, we performed immunoblotting of whole cell lysates with anti-alkaline phosphatase antibody (kind gift of Dr David A. Lowe)(Fig. 5). In each case, the antibody detected fusion proteins with apparent molecular weights greater than that of native alkaline phosphatase (Fig. 5, lane 1). The fusion proteins in strains AA111, AA113 and AA114 (lanes 4, 6, 7) reacted more intensely than those in strains AA110 and AA112 (Fig. 5, lanes 3 and 5). This pattern of reactivity correlated with the differences in intensity of blue colour detected on XP-agarose indicator plates for these strains (Fig. 3). The smaller bands seen with most of the samples presumably correspond to alkaline phosphatase degradation products. These products also appear to be of different sizes and intensities, except for one fragment common to all the samples that is slightly smaller than the alkaline phosphatase monomer (Fig. 5, lane 1). Presumably this smaller, common band represents the product of the truncated *phoA* fragment used in Mud*phoA*.

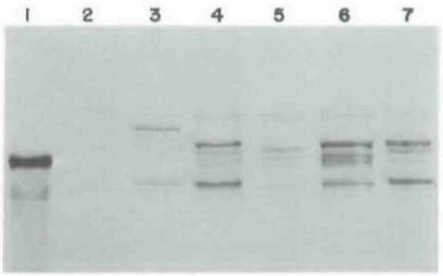

Fig. 5. Immunoblot of Mud*phoA* fusion proteins with anti-PhoA serum. Whole cell lysates were prepared from *L. pneumophila* strains as described by Pearlman *et al.* (1985), electrophoresed in a SDS–polyacrylamide gel (8%), transferred to nitrocellulose and reacted with anti-alkaline phosphatase antibody. Lane 1, denatured alkaline phosphatase; lane 2, AA103; lane 3, AA110; lane 4, AA111; lane 5, AA112; lane 6, AA113; lane 7, AA114. Note the absence of reactive protein in AA103 and the presence of strongly reacting bands in the other lanes which contain samples from PhoA$^+$ *L. pneumophila* strains. In lane 7, the *mip::phoA* fusion protein had a predicted size of 56 kDa based on restriction enzyme analysis of the insertion site on pNC31.5. The other strains show variably sized bands at least one of which is larger than alkaline phosphatase monomer in each case.

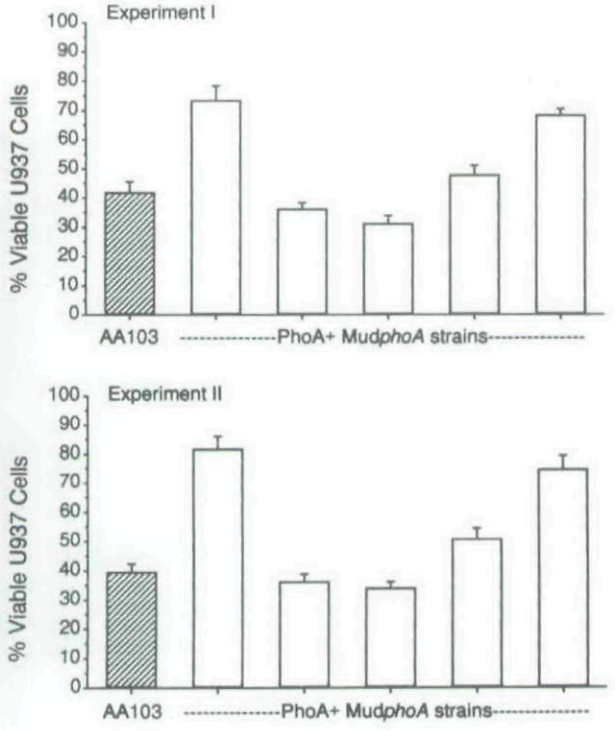

Fig. 6. Reproducible cytopathic effect of PhoA⁺ Mud*phoA* mutants of *L. pneumophila* in a U937 cell assay. The two experiments shown were performed on separate days with separate cultures of bacteria and monocytic cells with inocula of 10^6 bacteria. After 72 h at 37°C, the U937 cell viability was determined by staining with MTT (% viable U937 cells = mean OD_{550} for each strain ÷ mean OD_{550} of uninoculated, control U937 cell wells × 100). AA103 is the virulent, parent strain and contains no Mud*phoA* insertions. PhoA⁺ mutants studied were (from left to right): AA110, AA111, AA112, AA113, and AA118.

*Relative infectivities of PhoA⁺ Mud*phoA L. pneumophila *mutants in U937 cell assays*

Mud*phoA* contains the temperature-sensitive repressor allele *cts*(62) that regulates transposition in a temperature-dependent manner. The utility of Mud*phoA* in identifying virulence factors in *L. pneumophila* (or any other pathogen) depends upon the effect, if any, on bacterial cell viability of growth at 37°C. That is, if Mu transposition is sufficiently derepressed at 37°C to kill Mud*phoA*-containing bacteria, all strains having functional transposons would appear to be attenuated in virulence assays conducted at this temperature. Since we intend to screen PhoA⁺ Mud*phoA L. pneumophila* mutants for an *in vitro* U937 cell cytopathicity effect (CPE) at 37°C (Pearlman *et al.*, 1988), it is important to insure that any observed reduction in bacterial virulence is caused by insertional inactivation of a virulence-related gene rather than by bacterial death resulting from derepressed Mu transposition.

To address this concern, we tested the plating efficiencies of PhoA⁺ Mud*phoA L. pneumophila* mutant strains at 30°C and 37°C. In all strains examined, there was no significant difference in the cfu numbers of individual Mud*phoA L. pneumophila* mutant strains grown at either temperature (data not shown). Next, we conducted several independent experiments to compare PhoA⁺ Mud*phoA L. pneumophila* mutants and the parent strain, AA103, in the cytopathicity assay using U937 cells. Figure 6 shows the results of two of these assays. Clearly, this screening assay detects reproducible differences between the PhoA⁺ Mud*phoA* mutants; some are as virulent as the parent strain, whereas two mutants were significantly attenuated. These experiments suggest that any secondary transposition induced by growth at 37°C is not sufficient to affect the infectivity of these strains, as measured by the U937 cell assay.

Discussion

We described construction of Mud*phoA*, a derivative of Mu dII4041 (Castilho *et al.*, 1984) that can be used to generate alkaline phosphatase gene fusions *in vivo*. To generate a PhoA⁺ fusion, Mud*phoA* must insert into a gene with an amino-terminus encoding a signal peptide, since alkaline phosphatase is normally active only when it is exported from the cytoplasm (Michaelis *et al.*, 1983; Hoffman and Wright, 1985; Manoil and Beckwith, 1985). Insertions of Mud*phoA* in several different positions in the gene for the periplasmic β-lactamase protein gave fusion proteins with alkaline phosphatase activity. Based on restriction mapping, many of these insertions were in the same region of the β-lactamase gene, possibly indicating non-random Mu-insertion activity or lethality of certain insertions (Castilho *et al.*, 1984; Hall and Silhavy, 1981). Two different insertions in the gene for the membrane-localized Mip protein of *L. pneumophila* similarly also resulted in enzyme activity, both in *E. coli* and in *L. pneumophila*.

It is reasonable to assume that detection of PhoA⁺ Mud*phoA* fusions in *L. pneumophila* will identify genes encoding signal peptides, a hallmark of proteins that are secreted from the cytoplasm. Secreted proteins are those most likely to be involved in mediating *L. pneumophila* pathogenesis; therefore we can use Mud*phoA* to identify and to mutate the genes encoding these proteins. An advantage of using Mud*phoA* is that chromosomal insertions can be selected *in vivo* under different environmental conditions, thereby allowing identification of selectively expressed genes that may be important for virulence, but are otherwise difficult to identify using *in vitro* methods. For example, one can select for PhoA⁺ Mud*phoA* insertions at lower temperatures, under iron limitation, oxygen stress or low/high osmolarity — conditions known to affect expression of virulence determinants in other pathogenic bacteria.

Mintz and Shuman reported the ability of Mu to transpose to many sites within the *Legionella* genome, by generating insertion mutations and *lac* gene fusions (Mintz and Shuman, 1987). We have obtained similar results with Mud*phoA*. All of the fusion strains isolated to date stably maintain the PhoA$^+$ phenotype after several passages on BCYE/Km media.

Fusions generated by Mud*phoA* insertion contain 38 extra amino acids resulting from translation of the Mu right end sequence (Fig. 2). The presence of this extra sequence had no effect on *lac* fusion protein expression (Castilho *et al.*, 1984); however, its effect on the secretion and dimerization of PhoA$^+$ fusion proteins was unpredictable. Our results indicate that this linking peptide does not prevent expression of the enzyme, although proteolytic cleavage in this region seems to occur frequently, judging by the products seen in Fig. 5.

To screen *L. pneumophila* strains for the presence of *E. coli* alkaline phosphatase activity, we developed an *in situ* assay for PhoA activity. This assay helped us overcome several technical difficulties associated with screening for PhoA activity in *L. pneumophila*. For example, the black colour of BCYE medium interferes with blue colour detection of PhoA activity, and incorporation of the colour indicator substrate, XP, into BCYE is toxic to *L. pneumophila*. In addition, the native phosphatase activity of *L. pneumophila* masks the *E. coli* phosphatase activity at pH levels that permit bacterial growth; however, this background activity becomes insignificant at a pH of about 11.0.

The broad-host-range plasmid pRK212.1, which was used in this study to provide transfer functions, is itself transferred at a high frequency (Fig. 4). It is possible to eliminate this plasmid either by passage on non-selective media or by 'curing'. Its use may be avoided by introducing Mud*phoA* from an *E. coli* strain carrying the Tra functions on the chromosome or by placing Mud*phoA* on a conjugative 'suicide' vector (Engleberg *et al.*, 1988; Miller, 1972; Keen *et al.*, 1985). In any case, the presence of pRK212.1 in *L. pneumophila* confers no detectable change on intracellular infectivity (J. E. Rogers, unpublished observations). In some cases (Fig. 4B) the delivery vector, or derivatives of it, is present in the fusion strains. The presence of the apparently modified vector fragments suggests possible Mu-induced inversions or deletions (Pato, 1989). Sucrose counterselection effectively eliminates *sacB*-containing plasmids from *L. pneumophila*, but it is not known how this counterselection will effect these unexplained derivatives (Cianciotto *et al.*, 1988).

We have isolated several PhoA$^+$ *L. pneumophila* transconjugants, and we have found two of these strains (AA110, AA118) to be significantly attenuated in a U937 cell-infectivity assay. However, certain biological features of the transposon impose limitations on the use of Mud*phoA* for identification of virulence genes. For example, since derepression of Mu *c*ts(62) phage transposition can be reliably induced at 42°C, it is unlikely that animal challenge experiments could be performed using Mud*phoA* mutants. The febrile temperatures would probably induce enough secondary transposition to limit the growth and survival of *L. pneumophila* strains that carry Mud*phoA*. A more immediate concern is that some leakiness of the Mu *c*ts(62) might occur at the lower, semi-permissive temperature of 37°C and result in a similar bacterial attrition in the U937 cell-infectivity assay. At the very least, passage of the bacteria at 37°C might be expected to result in a heterogeneous population of bacteria containing multiple copies of the transposon in diverse chromosomal locations.

To limit secondary transpositions, we routinely maintain and cultivate all *L. pneumophila* (Mud*phoA*) strains at 30°C or store them at –70°C. All bacterial inocula for the U937 cell assay are grown at 30°C, and any bacteria isolated from cell cultures (at 37°C) are discarded. By exercising this precaution, we have shown that most PhoA$^+$ *L. pneumophila* strains are as cytopathic as the parental strain in the U937 cell assay. This normal level of cytopathicity has been demonstrated in repeated experiments on different days, using different cultures of the *L. pneumophila* test strains and different batches of differentiated U937 cells. In the same experiments, the two PhoA$^+$ mutants that were attenuated in our initial screening assay were comparably attenuated in all subsequent assays. We conclude from this experience that *L. pneumophila* does not become attenuated in the U937 cell assay simply because of the presence of either Mud*phoA* or a PhoA$^+$ gene fusion. Furthermore, the attenuated PhoA$^+$ mutants are likely to acquire this phenotype as a result of transposon-induced mutations in virulence-related genes.

The multiplicity of Mud*phoA* transpositions in *L. pneumophila* presents a second potential problem in characterizing the phenotype of attenuated mutants, since most transconjugants contain more than one copy of the transposon (typically 1–4 copies). Although it is likely that only one of these copies is responsible for the PhoA$^+$ activity, it is by no means certain that the fused gene is also responsible for the attenuated phenotype. For this reason (and because of the additional concern about possible secondary transpositions, outlined above), all mutations of interest will eventually have to be isolated by molecular cloning and then reintroduced into a wild-type strain by homologous recombination. This manoeuvre will determine whether the PhoA$^+$ gene fusion or some other Mud*phoA* insertion is responsible for the attenuated phenotype. In addition, a reconstructed strain will not have a functional transposon and could be used for animal challenge studies and extended phenotypic characterization.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The *E. coli* and *L. pneumophila* bacterial strains used in this study are listed in Table 1. Strain BAC101, obtained from M. J. Casadaban, was our source for Mu dII4041 (Casadaban and Chou, 1984). Strain CC118 is an *E. coli phoA* deletion strain obtained from C. Manoil (Manoil and Beckwith, 1985). *L. pneumophila* strain AA100 is a serogroup 1 clinical isolate, 103b (Los Angeles), and AA103 is a derivative of AA100 that is a high-frequency conjugal recipient (Engleberg *et al.*, 1988; Cianciotto *et al.*, 1988).

Plasmids used include pUC18Δ, which was derived in this lab. from pUC18 by deletion of the small *Pvu*II fragment containing multiple cloning sequences (Yanisch-Perron *et al.*, 1985). pRK212.1 is a deletion derivative of pRK2 that is Km^S (Figurski *et al.*, 1976).

E. coli strains were routinely cultured from frozen stocks onto Luria–Bertani (LB) medium or in LB broth containing antibiotics as required. Antibiotics were used at the following concentrations; kanamycin (Km) 25 μg ml^{-1}, tetracycline (Tc) 15 μg ml^{-1}, chloramphenicol (Cm) 20 μg ml^{-1} and ampicillin (Ap) 50 μg ml^{-1}. *L. pneumophila* strains were also cultured from frozen stocks onto BCYE medium, containing 25 μg ml^{-1} kanamycin as required.

Genetic and DNA manipulations

Chromosomal DNA was prepared from *E. coli* and *L. pneumophila* as described by Ausubel *et al.* (1987). Plasmid DNA preparation, DNA cloning and Southern hybridizations were performed using standard methods (Maniatis *et al.*, 1982). DNA restriction enzymes were used as suggested by the manufacturers. ^{32}P-labelled DNA probes were made with the multi-prime kit (Amersham).

Cloning and isolation of recombinant plasmids were done in *E. coli* strain HB101. Transformation of temperature-sensitive Mud*phoA*-containing plasmids was performed by electroporation, using a Bio-Rad Gene Pulser apparatus (Bio-Rad) or by the method of Chung *et al.* (1989). Other transformations were by standard procedures (Maniatis *et al.*, 1982). *E. coli* and *L. pneumophila* cells were prepared for electroporation by the method of Dower *et al.* (1988). Methods for handling bacteriophage Mu and P1-mediated transduction of *E. coli* were as described by Bukhari and Ljungquist (1977) and Silhavy *et al.* (1984).

Tri-parental bacterial conjugations for introduction of Mud*phoA* into *L. pneumophila* were performed as described by Engleberg *et al.* (1988), with minor modifications. Mating mixtures of AA103, χ2981(pRK212.1) and χ2981 (pTLP7::Mud*phoA*) were incubated on nitrocellulose filters on the surface of BCYE medium for 6 h at 30°C. The mating mixtures were then resuspended and plated onto BCYE/Km at appropriate dilutions to obtain 100–200 colonies per plate. The plates were incubated for an average of 6 days at 30°C, until the colonies had reached optimum size for transfer to XP-agarose plates.

Plate assays for detection of PhoA fusions

For detection of alkaline phosphatase activity in *E. coli* strains resulting from Mud*phoA* insertions, we incorporated 5-bromo-4-chloro-3-indolyl phosphate (XP) at a concentration of 40 μg ml^{-1} into the selective LB media. The high phosphate concentration in LB medium effectively suppressed expression of the native *E. coli* alkaline phosphatase, allowing easy detection of PhoA activity resulting from Mud*phoA* fusion proteins.

This strategy fails to detect *E. coli* alkaline phosphatase activity in *L. pneumophila* because the black background colour of BCYE prevents visualization, XP inhibits *L. pneumophila* growth, and *L. pneumophila* produces a broad pH range (pH 4–8) phosphatase activity that is not inhibited by high phosphate concentration (Muller, 1981). To circumvent these technical problems, we developed XP-containing agarose plates for detection of $PhoA^+$ *L. pneumophila* strains.The XP-agarose plates were made as follows: 1.5 g of electrophoresis grade agarose was mixed with 100 ml of a 0.1 molar solution of 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) buffer (Sigma). The suspension was then brought to pH 11.0 with 10 M NaOH and heated to dissolve the agarose completely. After the solution cooled to approximately 40°C, XP (40 mg ml^{-1} in dimethylformamide) was added at a concentration of 40 μg ml^{-1} and 10 ml of the XP-agarose solution was allowed to solidify in Petri plates.

L. pneumophila Km^R transconjugant colonies were lifted onto nitrocellulose filters, and the filters were applied colony-side-down onto the surface of XP-agarose plates. $PhoA^+$ colonies were generally apparent after 1–2 h incubation at room temperature. In each experiment $PhoA^+$ and $PhoA^-$ control strains were included on the filters to assess the development of blue colour because of *E. coli* alkaline phosphatase activity. When $PhoA^+$ colonies were detected, master plates were re-incubated at 30°C until good colony growth was achieved (about 3 days). The $PhoA^+$ colonies were identified, picked, and streaked for purity on BCYE/Km media. After re-isolation, strains were always re-tested on fresh XP-agarose plates. The XP-agarose plates were efficient at detecting alkaline phosphatase activity as long as the plates were used when freshly made. After about 48 h the plates began to lose specificity, probably owing to a spontaneous reduction in pH.

Antibodies and immunoassays

To detect alkaline phosphatase fusion proteins, whole cell lysates of strains were prepared and reacted with anti-alkaline phosphatase antibody. *L. pneumophila* strains were grown on plates for 48–72 h at the appropriate temperature. Cells were suspended in water to an optical density at 550 nm (OD_{550}) of 1.0 and samples were boiled for 5 min in the presence of SDS and 2-mercaptoethanol (Pearlman *et al.*, 1985). The lysates were centrifuged briefly to pellet debris, and equal amounts were electrophoresed in an 8% discontinuous SDS–polyacrylamide gel. Alkaline phosphatase (Sigma) was treated as above and included in the immunoassays. The separated proteins were transferred to nitrocellulose, and immunoblots were prepared as previously described (Cianciotto *et al.*, 1989). Horseradish peroxidase-conjugated goat anti-rabbit antibody (Cappel Industries) was used as the secondary antibody and diaminobenzidine (Sigma) was used as the colour indicator substrate.

U937 cell cytopathicity

Determination of the cytopathic effects of *L. pneumophila*

strains was performed as described by Pearlman *et al.* (1988) with slight modifications. Bacteria grown on either BCYE or BCYE/Km at 30°C were added to adherent U937 cells in 96-well plates (10^5 cells per well), and the infection was allowed to proceed at 37°C for 72 h. The infected monolayers were neither washed nor treated with antibiotics during this incubation. At the end of the incubation, the U937 cell-cytopathic effect was determined as previously described. The vital stain tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was used to distinguish live from dead U937 cells. In all experiments, a set of eight replicate infections for each bacterial strain was examined. The lowest and highest OD_{550} readings were eliminated from the set of values used to calculate an average and standard deviation.

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