Identification of novel loci involved in entry by *Legionella pneumophila*

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***Legionella pneumophila* is primarily an intracellular pathogen during infection; thus, the mechanisms of entry into host cells are likely to be important for pathogenesis. Several *L. pneumophila* mutants that display an enhanced-entry (Enh) phenotype were isolated by selecting for bacteria that enter host cells at a higher frequency than wild-type. In the course of characterizing the genetic basis of one of these mutants, C3, a strategy was developed for the isolation of laboratory-media-repressed virulence determinants from *L. pneumophila*. Screens for dominant mutations using a genomic DNA library from C3 resulted in the isolation of three cosmids that confer an Enh phenotype to wild-type *L. pneumophila*. Transposon mutagenesis of these cosmids allowed identification of three loci that affect entry. Analysis of the putative proteins encoded by these loci, designated *rtxA* and *enhC*, demonstrated similarity to repeats in the structural toxin protein and the secreted Sel-1 protein from *Caenorhabditis elegans*, respectively. *L. pneumophila* *rtxA* and *enhC* mutants display significantly reduced entry into host cells, compared to wild-type bacteria. The phenotype that the cosmids containing these loci confer is most likely due to elevated expression resulting from their presence on multicopy vectors. The use of increased gene copy number to overexpress genes that are normally repressed under laboratory growth conditions is generally applicable to the isolation of virulence determinants from *L. pneumophila* and other bacterial pathogens.**

Keywords: phagocytosis, cytotoxicity, Sel-1, invasion, RTX

INTRODUCTION

Legionella pneumophila is the causative agent of the potentially lethal pneumonia Legionnaires' disease. *L. pneumophila* is a Gram-negative bacterium that, although it can persist for up to a year in tap water (Skaliy & McEachern, 1979), replicates almost exclusively intracellularly during infection (Glavin *et al.*, 1979) and in its natural freshwater habitat (Fields *et al.*, 1989; Rowbotham, 1986; Wadowsky *et al.*, 1988). Since *L. pneumophila* must enter host cells in order to cause disease (Davis *et al.*, 1983), entry is considered a primary mechanism for the pathogenesis of these bacteria. However, exactly how entry occurs is not well under-

stood. Macrophages are the primary cell type that *L. pneumophila* replicates within during infection (Davis *et al.*, 1983; Winn & Myerowitz, 1981), but it has been suggested that epithelial cells may also play a role (Mody *et al.*, 1993). In addition to the conventional phagocytic mechanism observed in most organisms, entry of *L. pneumophila* into monocytes has been shown to occur by an unusual mechanism termed 'coiling phagocytosis' (Cirillo *et al.*, 1994; Horwitz, 1984; Rechnitzer & Blom, 1989). Although this asymmetrical pseudopod coiling mechanism appears to be consistently observed in *L. pneumophila* (Cirillo *et al.*, 1994, 1999; Rechnitzer & Blom, 1989) and spirochaetes (Cheng *et al.*, 1999; Rittig *et al.*, 1992, 1998; Szczepanski & Fleit, 1988), it is almost never (Horwitz, 1984) or only randomly (Rittig *et al.*, 1994) observed in other bacterial species. The possibility that the entry mechanism used by *L. pneumophila* plays an important role in subsequent intracellular events is supported by recent observations that differences in intracellular trafficking

Abbreviations: Enh, enhanced entry (phenotype); RTX, structural toxin. The GenBank accession numbers for the *enh1* and *enh2* loci reported in this paper are AF057703 and AF057704, respectively.

occur within five (Roy *et al.*, 1998) to fifteen (Wiater *et al.*, 1998) min after entry.

Several mechanisms of *L. pneumophila* adherence to host cells have been demonstrated, including opsonization with complement (Husmann & Johnson, 1992; Marra *et al.*, 1990; Payne & Horwitz, 1987) and specific antibodies (Horwitz & Silverstein, 1981; Husmann & Johnson, 1992; Nash *et al.*, 1984). In support of mechanisms involving bacterial opsonization, both Fc (Husmann & Johnson, 1992) and complement receptors (Marra *et al.*, 1990; Payne & Horwitz, 1987) have been shown to mediate adherence. Complement-mediated adherence has been shown to result in phagocytosis by monocytes (Payne & Horwitz, 1987) and is thought to involve the *L. pneumophila* major outer-membrane protein (Bellinger-Kawahara & Horwitz, 1990; Krinos *et al.*, 1999). However, no specific *L. pneumophila* mutants have been constructed in this gene, which prevents elucidation of its exact role in entry. It has been suggested that non-opsonic adherence mechanisms may also exist (Gibson *et al.*, 1994; Rodgers & Gibson, 1993). Potential host-cell receptors for non-opsonic adherence have been suggested (Harb *et al.*, 1998; Venkataraman *et al.*, 1997) and a type IV pilus that can mediate this type of adherence has been described (Stone & Abu Kwaik, 1998). However, in the absence of this pilus *L. pneumophila* adheres at 53 % of the wild-type levels, suggesting the presence of multiple adherence mechanisms.

In the current study, we designed a strategy to identify additional bacterial factors involved in entry of *L. pneumophila* into host cells. This strategy is based on the observation that entry by *L. pneumophila* is enhanced after growth in the environmental host *Acanthamoeba castellanii*, compared to growth on laboratory media (Cirillo *et al.*, 1994). This observation suggests that the genes involved in entry into host cells by *L. pneumophila* are down-regulated when grown on laboratory media. Using chemical mutagenesis and a positive-selection strategy, we isolated mutants that display enhanced entry into host cells when grown on standard laboratory media (BCYE agar). Screens for the presence of a dominant mutation in one of these mutants allowed the identification, sequencing and characterization of three loci that have the ability to confer an enhanced entry (Enh) phenotype. Identification of these genes may help to clarify the unusual mechanism of entry utilized by *L. pneumophila*. In addition, the strategy used to isolate these genes should be generally applicable to the identification of other regulated virulence determinants from *L. pneumophila* and a wide range of bacterial pathogens.

METHODS

Bacterial strains and growth conditions. The strains and plasmids used for these experiments are described in Table 1. The *L. pneumophila* strain AA100 has been shown to be virulent in both *in vitro* and *in vivo* models of infection (Moffat *et al.*, 1994) and was passaged no more than twice in

the laboratory before use in these studies, to prevent loss of virulence. AA100 was grown on BCYE agar (Edelstein, 1981) for 3 d at 37 °C in 5 % CO₂. The *Escherichia coli* strains were grown in Lennox broth (LB, Difco) at 37 °C. When necessary, kanamycin and/or chloramphenicol (Sigma) were added at 25 µg ml⁻¹ to bacterial growth media. The strain *yec47* was constructed by infecting XL-1 Blue with λpir and screening for the presence of a phage lysogen as described previously (Kolter *et al.*, 1978). This strain was used to propagate plasmids that carry an R6K origin of replication.

DNA manipulations. DNA manipulations were carried out essentially as described by Sambrook *et al.* (1989). Direct electroporation proved to be a rapid and efficient method for transferring plasmids from *L. pneumophila* to *E. coli*. For this technique, colonies containing the plasmid of interest were suspended in 1 ml sterile distilled water at 4 °C in an Eppendorf tube, washed twice with sterile distilled water and mixed 1:1 with freshly thawed XL-1 Blue electroporation-competent cells, prepared and frozen as described by Dower *et al.* (1988). Electroporation was then carried out in the same manner as for *E. coli* (Dower *et al.*, 1988) and dilutions plated on LB agar with kanamycin.

The vector pJDC15 was constructed on the backbone of pTLP4 (Moffat *et al.*, 1994; Stibitz *et al.*, 1986). The streptomycin sensitivity gene and *cos* site were removed by digestion with *Sph*I and replaced with the *Sph*I fragment containing the kanamycin-resistance gene from pYUB8 (McAdam *et al.*, 1995). The resulting plasmid, designated pJDC1, was then cut with *Pvu*II to remove the chloramphenicol-resistance gene and ColE1 origin of replication and replace them with the R6K origin of replication (Kolter *et al.*, 1978) carried on a *Sma*I fragment from pBDJ121 (Jones & Falkow, 1994). The promoterless streptomycin-sensitive *rpsL* allele was inserted into the *Bam*HI and *Xba*I sites of the resulting plasmid, pJDC6, by PCR from pTLP4 with the addition of flanking *Bgl*II and *Xba*I sites creating plasmid pJDC7. The promoterless chloramphenicol-resistance gene was inserted into the *Xba*I and *Sac*I sites of pJDC7 by PCR from pTLP4 with the addition of *Sac*I and *Bam*HI sites on the 5' end and a *Xba*I site on the 3' end. The resulting vector, pJDC8, was digested with *Sac*I/*Bam*HI and ligated to the L5 mycobacteriophage promoter produced by PCR from pYUB215 (Cirillo *et al.*, 1991) with the addition of flanking 5' *Sac*I and 3' *Bam*HI sites. The *sacB* gene (Blomfield *et al.*, 1991) was cloned into the resulting plasmid, pJDC13, by ligation of the *sacB*-containing *Eco*RV fragment of pCVD442 (Mobley *et al.*, 1993) into the *Eco*RV site, producing pJDC15. pJDC15 is a suicide plasmid in any bacterial strain that does not contain the Pir protein *in trans* and carries kanamycin and chloramphenicol resistance, and streptomycin and sucrose sensitivity as well as an origin of transfer for conjugation. These characteristics make pJDC15 an ideal vector for allelic exchange in a number of bacterial species.

Cell culture. HEP-2 cells (ATCC CCL23), established from a human epidermoid carcinoma, were grown in RPMI 1640 plus 5 % heat-inactivated foetal calf serum (Gibco). THP-1 cells (ATCC TIB202), a human monocytic cell line, were grown in RPMI 1640 plus 10 % heat-inactivated foetal calf serum.

Phenotypic characterization of strains. Assays to determine the intracellular growth rate of each strain were carried out essentially as described previously (Cirillo *et al.*, 1999). The THP-1 cells used for viability assays were seeded into 24-well tissue culture dishes at 1.5 × 10⁶ cells per well in RPMI 1640 plus 10 % serum, 5 µg LPS ml⁻¹ (Difco; *E. coli* 0127:B8) and

Table 1. Bacterial strains and plasmids

Strain/plasmid	Characteristic	Source/reference
Strains		
<i>E. coli</i>		
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qΔM15 Tn10 (Tet^r)</i>]	Stratagene
χ2819	F- <i>lacY1 glnV44 galK2 galT22 λ(cI857 b2 redβ3 S7)</i> <i>recA56 ΔthyA57 metB1 hsdR2</i>	R. Curtiss, III*
ψec47	XL-1 Blue <i>λpir</i>	Current study
<i>L. pneumophila</i>		
AA100	Serogroup 1, clinical isolate no. 130b, naturally arising Sm ^r strain	Engleberg <i>et al.</i> (1984)
C7	AA100 mutant displaying enhanced entry (Enh1)	Current study
C8	AA100 Enh1	Current study
C1	AA100 Enh2	Current study
C2	AA100 Enh2	Current study
C9	AA100 Enh2	Current study
C10	AA100 Enh2	Current study
C3	AA100 Enh3	Current study
C11	AA100 Enh3	Current study
ψlp24	AA100 <i>ΔrtxA</i>	Current study
ψlp30	AA100 <i>ΔenhC</i>	Current study
Plasmids/cosmids		
pYUB289	Km ^r Ap ^r p15Aori <i>cos</i>	V. Balasubramanian†
1A3	Enh1 Km ^r Ap ^r p15Aori <i>cos</i>	Current study
2A4	Enh2 Km ^r Ap ^r p15Aori <i>cos</i>	Current study
2A6	Enh2 Km ^r Ap ^r p15Aori <i>cos</i>	Current study
pKV32	Cm ^r Ap ^r	Visick & Ruby (1997)
pJDC15	Km ^r <i>sacB</i> R6Kori	Current study
pJDC19	<i>enh2</i> (C3) Km ^r Ap ^r p15Aori <i>cos</i>	Current study
pJDC20	<i>enh1</i> (AA100) Km ^r Ap ^r p15Aori <i>cos</i>	Current study
pJDC23	<i>enh1</i> (C3) Km ^r Ap ^r p15Aori <i>cos</i>	Current study
pJDC24	<i>enh2</i> (AA100) Km ^r Ap ^r p15Aori <i>cos</i>	Current study

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40 U human γ -interferon ml⁻¹ (Boehringer Mannheim). The cells were incubated for 2 d with this medium and fresh medium added for an additional 24 h to allow activation of the monocytes prior to entry. Bacteria were added to the cells at a m.o.i. of 10–100 and incubated at 37 °C for 5 min, washed three times with warm PBS, and suspended in fresh medium with human γ -interferon and LPS before lysis of different sample wells with water every 2 h for 24 h. Dilutions of the resulting lysates were plated to determine c.f.u. at each time point.

Growth rate in laboratory media was assessed essentially as described by Byrne & Swanson (1998). Bacteria were inoculated from BCYE agar into 25 ml BYE broth (Edelstein, 1981) at a concentration of 10⁵ c.f.u. ml⁻¹ and grown at 37 °C with agitation. Optical density readings were taken at 600 nm and dilutions plated on BCYE agar every 2 h for 24 h.

The presence of pili and flagella was assessed by transmission electron microscopy of negatively stained specimens essentially as described by Chandler *et al.* (1980) and Rodgers *et al.* (1980). The bacteria were suspended at 10⁶ c.f.u. ml⁻¹ in PBS and a drop of this suspension applied to Formvar carbon

coated copper grids. The samples were then stained with 1% uranyl acetate and examined by transmission electron microscopy.

The ultrastructural morphology of the bacteria was examined as described previously (Cirillo *et al.*, 1994). Samples were fixed in 2% glutaraldehyde and 1% OsO₄ for 2 h and stained with 0.5% uranyl acetate overnight at 4 °C. Samples were then embedded and thin sections examined by transmission electron microscopy.

Motility was assessed using microscopy, essentially as described by Wei & Bauer (1998). Bacteria were grown in BYE to stationary phase and diluted to 10⁴ c.f.u. ml⁻¹ in sterile deionized water prior to examination with a Nikon TE300 inverted microscope with differential-interference-contrast optics. Digital video captures were obtained from at least three independent fields from three independent suspensions and the number of bacteria quantitated using NIH Image 1.61 on selected frames. Repeated examination of the captured video allowed quantitation of the number of motile bacteria in each field. The total number of bacteria per field used was always greater than 50, resulting in quantitation of the

percentage of motile bacteria from greater than 450 bacteria per strain.

Entry and adherence assays. Entry assays were carried out essentially as described previously (Cirillo *et al.*, 1994). HEp-2 cells were seeded in 24-well tissue culture dishes (Falcon) at a concentration of 2.5×10^5 cells per well and allowed to adhere overnight at 37 °C. The bacteria to be assayed were suspended and diluted in the same medium as the cells that were to be infected. After adding the bacteria at an m.o.i. of 100 they were allowed to interact with cells for various times, though all data shown are for 90 min. The cells were then washed with PBS and incubated in the appropriate culture medium plus 100 µg gentamicin ml⁻¹ for 2 h. After antibiotic treatment, the cells were washed with PBS, then with water and lysed by incubation for 10 min in 1 ml water followed by vigorous pipetting. In the case of THP-1 cells, the bacteria were allowed to interact with cells for various times, though all data shown are for 30 min and the assays were carried out in suspension. This requires that the cells be pelleted by centrifugation at 100 *g* for 1 min before each change of solution. After lysis, the number of intracellular bacteria was determined by plating for c.f.u. on BCYE (*L. pneumophila*) or LB (*E. coli*) agar. Entry levels were determined by calculating the percentage of the inoculum that became gentamicin-resistant over the course of the assay [i.e. % entry = $100 \times (\text{c.f.u. gentamicin-resistant} / \text{c.f.u. inoculum})$]. To correct for variation in levels of uptake between experiments, entry is reported relative to AA100 (i.e. relative entry = % entry test strain / % entry AA100). Adhesion was tested in a similar manner to that for entry, except that bacteria were added to the cells, mixed and immediately washed three times to remove non-adherent bacteria prior to lysis.

Chemical and transposon mutagenesis. AA100 was mutagenized at a concentration of 1×10^8 c.f.u. ml⁻¹ with 30 µg EMS ml⁻¹ for 1 h at 37 °C in minimal A buffer (Miller, 1972). This method of EMS treatment resulted in a level of 1.25 % survival of *L. pneumophila*. The pool of mutagenized bacteria was then washed twice in minimal A buffer and grown overnight on BCYE agar. The pool was suspended in RPMI 1640 and aliquots (10⁶ c.f.u.) were used to isolate enhanced entry mutants.

Cosmids carrying *L. pneumophila* genomic fragments were mutagenized with a chloramphenicol-resistant mini-Tn10 transposon (Alexeyev & Shokolenko, 1995) carried on pKV32 (Visick & Ruby, 1997). The plasmid pKV32 has an R6K γ -origin of replication, which prohibits its replication in strains that do not carry the π protein *in trans* (Miller & Mekalanos, 1988). Mutagenesis of the cosmids 1A3, 2A4 and 2A6 with this transposon was carried out by transformation of the XL-1 Blue strain containing each of these cosmids with pKV32. The resulting transformants were then allowed to recover in SOC medium (Dower *et al.*, 1988) with 2 mM IPTG for 1 h at 37 °C to induce transposition (Alexeyev & Shokolenko, 1995). The culture was then incubated for an additional 4 h in the presence of kanamycin and IPTG. Aliquots were plated on LB agar plates with kanamycin and chloramphenicol. The 100–200 colonies that arose from transposition events were pooled, plasmid DNA isolated by alkaline lysis (Sambrook *et al.*, 1989) and transformed into XL-1 Blue. This procedure allows isolation specifically of those transposition events into the cosmid. Individual colonies were then screened for the presence of unique insertions into the cosmid by restriction analysis. Unique transposon insertions were then electroporated into AA100 using the same method described for *E. coli* (Dower *et al.*, 1988) and tested for the ability to confer enhanced entry.

Selection for enhanced-entry mutants. Mutants that displayed enhanced entry were isolated through the use of a modified HEp-2 cell entry assay. This selective entry assay was accomplished by growing EMS-mutagenized *L. pneumophila* strain AA100 on BCYE agar at 37 °C for 5 d and then pooling the resulting colonies in RPMI 1640. A standard entry assay into HEp-2 cells was carried out with this suspension as described above, except that the bacteria were only allowed to interact with the HEp-2 cells for 5 min. The bacteria that entered during this assay were grown on BCYE agar for 5 d and individual colonies suspended in PBS with 50% (v/v) glycerol and kept frozen at –70 °C until use. To test the entry phenotype of individual clones, they were grown on BCYE agar for 3 d and compared to AA100 in a standard entry assay. Those clones that displayed greater than a twofold increase in entry over AA100 were considered to have an Enh phenotype.

Library construction and screening for dominant mutations.

Contiguous and non-contiguous *L. pneumophila* strain AA100 and C3 genomic DNA libraries were constructed in the cosmid vector pYUB289. pYUB289 was constructed by first producing a *PacI* cassette in SuperCos 1 (Stratagene) as described previously for pYUB328 (Balasubramanian *et al.*, 1996). The resulting cassette and adjacent *cos* site were moved to pACYC177 by digestion with *NheI/AatII*, isolation of the appropriate DNA fragments and ligation. The resulting cosmid carries a single *cos* site, the *PacI* cassette, kanamycin resistance, ampicillin resistance and the low copy-number origin of replication p15A. To construct the *Legionella* genomic libraries, total genomic DNA was isolated as described for *E. coli* (Silhavy *et al.*, 1984), digested partially with *Sau3AI* to produce fragments of approximately 20 kbp in length for non-contiguous libraries or 50 kbp for contiguous libraries. For non-contiguous libraries, these fragments were ligated to *BamHI*-cut and dephosphorylated pYUB289. For contiguous libraries, these fragments were dephosphorylated and ligated to *BamHI*-cut pYUB289. The resulting ligations were *in vitro* packaged with Gigapack II Gold (Stratagene) packaging mix and used to infect χ 2819 for *in vivo* packaging (Jacobs *et al.*, 1986). Over 10000 kanamycin-resistant χ 2819 colonies were pooled for *in vivo* packaging, producing a lysate that had a titre of greater than 10⁹ cosmid-containing phages ml⁻¹. This lysate was used to infect XL-1 Blue and plated on LB agar plates containing kanamycin such that approximately 20000 colonies were produced on ten plates. The resulting colonies were pooled in two separate pools of approximately 10000 colonies each and plasmid prepared from them.

To screen for dominant mutations, the two resulting plasmid preparations from the non-contiguous C3 library were electroporated independently into AA100 and dilutions plated on BCYE agar with kanamycin. Approximately 10000 kanamycin-resistant AA100 colonies from each transformation were pooled and clones that conferred the enhanced-entry phenotype isolated in the same manner as the original isolation of EMS-mutagenized enhanced-entry mutants. Individual colonies were isolated following the selective entry assay and their ability to enter HEp-2 cells compared to wild-type AA100. Those clones that entered HEp-2 cells at two-fold higher frequencies than AA100 were considered to confer the Enh phenotype. For each of the transposon insertions, the ability to confer the Enh phenotype was tested in a similar manner except that the purified plasmids carrying insertions were directly transformed into AA100 and tested for the Enh phenotype.

To ensure that the cosmid carried by each clone was responsible for the enhanced entry rather than the acquisition of a spontaneous mutation in the host bacteria, each cosmid

that conferred the Enh phenotype was transferred from AA100 into XL-1 Blue, purified, retransformed into AA100 and retested in the entry assay. Cosmids were transferred into *E. coli* by the technique of direct electroporation.

Isolation and subcloning of contiguous *enh* loci. To demonstrate that the Enh phenotype was due to the loci indicated by transposon mutagenesis and not the result of scrambled genes, we identified and subcloned each of the loci involved from the contiguous genomic libraries of C3 and AA100. A 609 bp region beginning 33 bp upstream of the putative translational start for *rtxA* was used as a probe in colony hybridizations to isolate the contiguous cosmid clones that contain the *enh1* locus. A 438 bp region beginning 590 bp upstream of the putative translational stop for *enhC* was used as a probe in colony hybridizations to isolate the contiguous cosmid clones that contain the *enh2* locus. Restriction and Southern analyses confirmed that the *enh1* and *enh2* loci on these cosmids were on 5265 bp *EcoRI* and 5263 bp *SwaI* fragments, respectively, that were the same size in C3 and AA100 chromosomal DNA digests (data not shown). One of each of the cosmids isolated in this manner containing *enh1* or *enh2* was digested with either *EcoRI* or *SwaI*, respectively, and the appropriate size fragment was purified and ligated into *EcoRI* or *ScaI* digested pYUB289. The resulting cosmids, pJDC19, pJDC20, pJDC23 and pJDC24, are listed in Table 1. The presence of the complete contiguous loci on each of these subclones was confirmed by restriction and sequence analysis (data not shown).

Construction and complementation of Δ *rtxA* and Δ *enhC* mutations. In-frame deletions were constructed in the *rtxA* and *enhC* genes by overlapping PCR in a similar manner to that described previously (Fang *et al.*, 1999; Horton *et al.*, 1989; Sandhu *et al.*, 1992). An in-frame deletion was constructed in the *rtxA* gene, producing the amino-terminal 6 aa and the carboxy-terminal 124 aa of RtxA. This construct does not contain domains thought to be required for RTX (structural toxin) activity (Boehm *et al.*, 1990; Welch, 1991). An in-frame deletion was constructed in the *enhC* gene, producing the amino-terminal 12 aa and the carboxy-terminal 158 aa of EnhC. The resulting mutations were then cloned into the *NotI* site of pJDC15, transformed into AA100 and plated on kanamycin to select for single-crossover events resulting from integration of the plasmids by homologous recombination. These recombinants were then plated on BCYE plus 5% sucrose plates to select for the presence of the desired mutations in the chromosome and loss of pJDC15. The presence of the appropriate Δ *rtxA* and Δ *enhC* mutations in the *L. pneumophila* chromosome were confirmed by Southern analysis and extended PCR (data not shown). The resulting Δ *rtxA* and Δ *enhC* mutants were designated ψ lp24 and ψ lp30, respectively. Complementation of these mutations was performed by transformation with the appropriate plasmids pJDC20 (Δ *rtxA*) and pJDC24 (Δ *enhC*) that contain only the complete wild-type *enh* locus and promoter region. Each of the resulting mutants and complementing strains were then compared to wild-type in entry assays.

Southern analysis and colony hybridization. Probes were labelled by nick translation or PCR with digoxigenin using the methods suggested by the manufacturer of the Genius System (Boehringer Mannheim). Membranes were prepared by the suggested methods for both colony hybridization and Southern analysis. Hybridization and washes were carried out at high stringency.

DNA sequence analysis. DNA sequence analysis was carried out initially using a forward primer TrnF (5'-CCACTAGT-

TCTAGAGCGGCC-3') from the mini-Tn10 transposon (Alexeyev & Shokolenko, 1995). The sequence was continued by primer walking directly on the cosmid of interest. All regions were sequenced completely in both directions using Big Dye Terminator (Applied Biosystems) cycle sequencing and subsequent analysis on an ABI 310 automated sequencing apparatus (Applied Biosystems). Sequence analysis and assembly was carried out using Gene Construction Kit 2 (Textco) and comparison with known sequences using BLAST (Altschul *et al.*, 1997). Analysis of putative signal sequences was done using SignalP (Nielsen *et al.*, 1997), potential protein profiles using ProfileScan (Lüthy *et al.*, 1994; Thompson *et al.*, 1994), and secondary structure and multiple sequence alignments using Lasergene (DNASTAR) software.

Statistical analyses. All *in vitro* experiments were carried out in triplicate and repeated at least twice. Significance of the results was analysed using ANOVA. Values of $P < 0.05$ were considered significant.

RESULTS

Isolation of *L. pneumophila* enhanced-entry mutants

EMS mutagenesis and selective-entry assays were used to produce a pool of mutagenized *L. pneumophila* that is enriched for mutants that have acquired an enhanced ability to enter cells when grown on BCYE agar. Enh mutants were isolated using selective-entry assays from two independent EMS-mutagenized AA100 pools of approximately 10^6 c.f.u. Twenty clones isolated from each selection were screened individually for their ability to enter HEP-2 cells relative to wild-type AA100. Results from a representative assay are shown in Fig. 1a. From the 40 clones screened in HEP-2 cells, 22 clones, or approximately 55%, displayed the Enh phenotype. Thus, the selection procedure was relatively successful for the isolation of Enh mutants. Eleven of these clones were also assayed for their ability to enter THP-1 cells (Fig. 1b). These clones were classed into three phenotypic groups that display significantly enhanced entry ($P < 0.005$): Enh1 (C7 and C8), that enter both HEP-2 and THP-1 cells at greater than twofold higher levels than wild-type; Enh2 (C1, C2, C9 and C10), that enter HEP-2 cells at higher levels than wild-type but not THP-1 cells; and Enh3 (C3 and C11), that enter both HEP-2 and THP-1 cells at greater than fivefold higher levels than wild-type. We observed significantly different levels of entry in these clones compared to wild-type in entry assays for various times from 5 min to either 30 min (THP-1 cells) or 90 min (HEP-2 cells). The data shown (Fig. 1) are for the 30 and 90 min entry assays in THP-1 and HEP-2 cells, respectively.

Isolation of cosmids that confer the Enh phenotype to wild-type *L. pneumophila*

The Enh mutant C3 was chosen for further analysis due to its high level of enhanced entry into both HEP-2 and THP-1 cells. We first compared the phenotypic characteristics of the C3 mutant to wild-type *L. pneumophila*. We characterized the C3 Enh mutant in terms of intracellular replication rate, growth rate in laboratory media, the presence of pili, the presence of flagella,

Fig. 1. Entry of individual EMS-mutagenized *L. pneumophila* clones into epithelial cells (a) and monocytes (b) after a selective-entry assay. Data points and error bars represent the mean of triplicate samples from a representative experiment and their standard deviations. All experiments were performed at least three times.

motility, colony morphology and ultrastructural morphology. None of these phenotypic characteristics were significantly different from wild-type *L. pneumophila* (data not shown). The only apparent difference between the C3 mutant and wild-type *L. pneumophila* was its entry phenotype.

A cosmid library of C3 total genomic DNA was constructed and transferred into wild-type AA100 to allow isolation of the genes responsible for the C3 Enh phenotype. Due to the possibility that the C3 Enh phenotype was due to multiple mutations, we utilized a non-contiguous genomic library for these experiments. A low copy-number cosmid vector was chosen for these studies to reduce the possibility of copy-number effects, such as toxicity of *L. pneumophila* gene products in *E. coli*, on the comprehensiveness of the library. Recombinant cosmids that conferred the Enh phenotype were isolated using selective-entry assays. A total of 19 cosmids were isolated from two independent pools of 20000 AA100 transformants with the C3 genomic library. The ability of each of these recombinant clones

Fig. 2. Entry into epithelial cells (a) and monocytes (b) of wild-type (AA100) *L. pneumophila* and AA100 transformed with the parent cosmid vector (pYUB289) or cosmids from the C3 mutant total genomic DNA library. Data points and error bars represent the mean of triplicate samples from a representative experiment and their standard deviations. All experiments were performed at least three times. The * symbols in panel (a) indicate those cosmid clones that were selected for further analysis.

to enter HEp-2 cells was compared to wild-type in standard entry assays (Fig. 2a). Seven cosmid-containing clones consistently displayed the Enh phenotype, two from the first pool (containing cosmids 1A3 and 1A7) and five from the second pool (containing cosmids 2A3, 2A4, 2A6, 2A7 and 2A9). A restriction map of each of these cosmids was constructed using *Bam*HI (data not shown). All of these cosmids had unique restriction maps excluding 2A4 and 2A6, which were identical. Cosmid 1A3 consistently displayed the highest relative entry of those tested. On the basis of these data, cosmids 1A3, 2A4 and 2A6 were chosen for further analysis.

Fig. 3. The ability of parent cosmid 1A3 (a) and 2A6 (b) and cosmid carrying different Tn10 insertions to confer enhanced entry. If the mutagenized cosmid conferred significantly lower relative entry than the parent cosmid it was considered to be within a locus involved in the Enh phenotype. Mutagenized cosmid are shown in the same order as shown in Fig. 4. Designations for the loci identified are shown below each graph. Data points and error bars represent the mean of triplicate samples from a representative experiment and their standard deviations. All experiments were performed at least three times.

Cosmids 1A3, 2A4 and 2A6 were purified in *E. coli* and then retransformed into AA100 to determine whether the Enh phenotype conferred by these cosmid was due

to the plasmid or a spontaneous mutation in the host bacterium. Transformations from AA100 to *E. coli* were carried out by direct electroporation. The ability of

Fig. 4. Determination of loci responsible for enhanced entry phenotype by transposon mutagenesis of cosmids 1A3 and 2A6. The position of each unique transposon insertion is indicated. The symbols indicate transposon insertions that result in cosmids that confer levels of entry to AA100 that are (–) or are not (+) significantly different from that of the parent cosmid (1A3 or 2A6). All transposon insertions were tested at least three times.

AA100 carrying the purified cosmids relative to wild-type *L. pneumophila* to enter HEP-2 cells was then determined. Entry of these transformants was comparable to that obtained with the original clones carrying the cosmids (data not shown). These results indicate that the observed phenotype can be attributed to activities present on the cosmid rather than in the host bacterium. The entry phenotypes of 2A4 and 2A6 into monocytes were the same as wild-type *L. pneumophila* (Fig. 2b). Only cosmid 1A3 conferred the Enh phenotype into both monocytes and epithelial cells, though at four- to eightfold lower levels than the original C3 Enh mutant. We assayed the adherence of these clones to HEP-2 cells and THP-1 cells to determine whether adherence was affected as well as entry. We found a direct correlation between the entry and adherence phenotype of all of the clones isolated as well as the original C3 mutant (data not shown). In addition, the level of adherence conferred by each clone relative to each other was comparable to their level of entry. These data suggest that adherence and entry are intimately linked in *L. pneumophila* and that differences in adherence may significantly contribute to the observed Enh phenotype. The only cosmid that conferred a similar phenotype to the original C3 mutant was cosmid 1A3. For this reason 1A3 was the only cosmid characterized further, with the exception of mapping the loci involved and the construction of physical maps for cosmids 2A4 and 2A6.

Identification of enhanced-entry (*enh*) loci

Detailed physical maps of cosmids 1A3, 2A4 and 2A6 were constructed using *Bam*HI, *Nhe*I and *Xho*I (data not shown). Cosmids 2A4 and 2A6 have identical physical maps with these three enzymes. However, cosmid 1A3 has no apparent regions of overlap with cosmids 2A4 and 2A6. Southern analysis was carried out with AA100 chromosomal DNA and purified 1A3, 2A4

and 2A6 cosmid DNA digested with *Bam*HI, using 1A3 and 2A6 separately as probes (data not shown). No overlapping regions were identified by Southern analysis for 1A3 with the 2A4 and 2A6 cosmids. However, 2A6 hybridized with all restriction fragments of 2A4, indicating that these cosmids are identical. Thus, only cosmids 1A3 and 2A6 were analysed further.

Transposon mutagenesis of cosmids 1A3 and 2A6 was carried out in XL-1 Blue using mini-Tn10. Greater than 100 transposon insertions in each cosmid were isolated and partially mapped with restriction endonucleases to determine the location of each transposon insertion (data not shown). At least 26 unique transposon insertions per cosmid were tested for their ability to confer the Enh phenotype of the original cosmid in HEP-2 cells (Fig. 3). The location and phenotype of each transposon insertion is shown in Fig. 4. Two different loci, designated *enh1* (~4 kbp) and *enh2* (~5 kbp), on cosmid 1A3 were involved in the ability of this cosmid to confer the Enh phenotype. In the case of cosmid 2A6 one locus, designated *enh3* (~4 kbp), was required. Since only cosmid 1A3 confers a phenotype similar to the C3 mutant strain, all further studies were carried out on the *enh1* and *enh2* loci from this cosmid. To confirm that each locus, separate from adjoining fragments, was sufficient to confer the Enh phenotype and this phenotype was due to the presence of contiguous chromosomal fragments rather than scrambled genes, we identified and subcloned these loci from our contiguous C3 and AA100 genomic libraries.

Colony hybridization was used to identify cosmids in these contiguous libraries that carried *enh1* and *enh2*. Contiguous fragments of approximately 5 kbp that contained each of these loci were subcloned into pYUB289 and the resulting cosmids pJDC19, pJDC20, pJDC23 and pJDC24 (Table 1) were examined for their ability to confer the Enh phenotype individually (Fig. 5). The contiguous fragments from both C3 and AA100

Fig. 5. Entry of wild-type (AA100) *L. pneumophila* and AA100 transformed with 1A3, pJDC19, pJDC20, pJDC23 or pJDC24 into epithelial cells (a) and monocytes (b). Data points and error bars represent the mean of triplicate samples from a representative experiment and their standard deviations. All experiments were performed at least three times.

conferred the Enh phenotype. However, the levels of enhanced entry conferred by *enh1* and *enh2* separately were not quite as high as the original cosmid 1A3 (threefold as compared to fivefold) that contains both loci. These data suggest that the presence of both loci has a synergistic effect on entry. In addition, the fact that both loci from the mutant C3 and wild-type strain AA100 confer similar levels of enhanced entry suggests that the Enh phenotype conferred by *enh1* and *enh2* is due to gene copy-number effects. This conclusion is further supported by sequence analysis of these loci from C3 and AA100.

Sequence analysis of the *enh1* locus

Initial sequence analysis was carried out from the mini-Tn10 insertions that resulted in an Enh-negative phenotype on the cosmid 1A3. This strategy allowed rapid identification of the genes required to confer this phenotype. The two Enh-negative transposon insertions in the *enh1* locus were within a gene that encodes a protein with significant amino acid sequence identity to several leukotoxins or cytotoxins that are members of the repeats in the RTX family including *frpC* from *Neisseria meningitidis* (accession no. L06299; 23% identity over 1106 aa), *hlaA* from *Pasteurella haemolytica* (accession no. M20730; 23% identity over 634 aa) and *cyaA* from *Bordetella bronchiseptica* (accession no. Z37112) and *Bordetella pertussis* (accession no. X14199; 22% identity over 795 aa). This ORF was named *rtxA* based on these data. The complete sequence of the 5265 bp *EcoRI* fragment containing the *enh1* locus was determined for both C3 and AA100, and the organization of the genes present is shown in Fig. 6. There were no differences between the nucleotide sequence of C3 and AA100 in this region. The region upstream of *rtxA* gene contains a second ORF (216 aa) that is transcribed in the same direction as the *rtxA* gene and overlaps it by 10 aa. This ORF was found to have amino acid similarity to the asparagine-rich antigen from *Plasmodium falciparum* (accession no. 102334; 25% identity over 163 aa) and the outer membrane usher protein (UseH) for CS6 fimbriae from *E. coli* (accession no. 1706159; 31% identity over 106 aa). This similarity is primarily in asparagine-rich regions of the protein. This ORF was named *arpB* to indicate that it is an asparagine-rich protein (Fig. 6). Upstream of this ORF there is a region of approximately 300 bp that does not appear to contain any large ORFs. A potential -10 sequence, AATATTG, was found 146 bp upstream and a potential -35 sequence, GTATTGTAAG, was found 178 bp upstream of the ATG for *arpB*. No other putative -10 and -35 sequences were found upstream of *rtxA*.

Sequence analysis of the *enh2* locus

Sequence analysis of the *enh2* locus was begun in the same manner as for *enh1*. Three mini-Tn10 insertions in the *enh2* locus gave an Enh-negative phenotype. Sequence analysis from these transposon insertions outward resulted in the identification of an ORF with significant amino acid similarity to the *sel-1* gene product from *Caenorhabditis elegans* (accession no. U50828; 25% identity over 550 aa), the chitin synthase 4 gene product from *Candida albicans* (accession no. AB003310; 24% identity over 275 aa) and the *SKT5* gene product from *Saccharomyces cerevisiae* (accession no. S65415; 25% identity over 361 aa). Sequence analysis was carried out on the complete 5263 bp *enh2* locus for both C3 and AA100 encompassing this ORF and two other ORFs that appear to be transcribed in the same direction (Fig. 6). There were no differences between the nucleotide sequence of C3 and AA100 in this region. The two upstream ORFs transcribed in the same direction

Fig. 6. Sequence analysis of the *enh1* and *enh2* loci. Gene names and the direction of transcription of each of the ORFs found within these loci are shown directly below the construct. The size of each locus is shown in parentheses.

Fig. 7. Characteristics of the *L. pneumophila* *rtxA* gene (a) and alignment of the 64 aa highly conserved region including the three carboxy-terminal RTX repeat sequences (b). Locations of putative translational start, stop and ribosome-binding sequences are indicated. White boxes indicate the positions of individual 9 aa RTX repeat sequences within the coding region of *rtxA*. Bold indicates residues that are conserved in the majority of the aligned sequences. Numbers adjacent to the alignment indicate the position of the aligned amino acids within the coding sequence of the RTX proteins. Abbreviations for the aligned RTX repeat regions are: LpRtxA.f1, *L. pneumophila* amino-terminal region; LpRtxA.f2, *L. pneumophila* carboxy-terminal region; BbClyA, *B. bronchiseptica* CyaA repeat region; BpCyaA, *B. pertussis* CyaA; EcHlyA, *E. coli* HlyA; AcClyIIA, *Actinobacillus pleuropneumoniae* ClyIIA; AnHlyA, *Anabaena* sp. strain PCC 7120 HlyA; NeFrpA, *N. meningitidis* FrpA; and PaHla1, *Pas. haemolytica* Hla1.

Fig. 8. Characteristics of the *L. pneumophila* *enhA*, *enhB* and *enhC* genes (a) and alignment of the 154 aa conserved region with similar regions of other proteins (b). Locations of putative translational start, stop and ribosome-binding sequences are indicated. White boxes indicate the positions of putative secretory signal sequences, the box filled with a grid indicates the TPR domain and boxes filled with diagonals indicate the two highly conserved regions. Bold indicates residues that are conserved in the majority of the aligned sequences. Numbers adjacent to the alignment indicate the position of the aligned amino acids within the coding sequence of the proteins. Abbreviations for the aligned conserved regions are: LpEnhC.d1, *L. pneumophila* amino-terminal conserved region; LpEnhC.d2, *L. pneumophila* carboxy-terminal conserved region; CeSel1, *Cae. elegans* Sel-1; CaCsr, *Can. albicans* chitin synthase regulatory factor; ScSkt5, *S. cerevisiae* Skt5p.

Fig. 9. Entry into epithelial cells (a) and monocytes (b) by wild-type (AA100) *L. pneumophila*, AA100 containing in-frame deletions in *rtxA* ($\Delta rtxA$) and *enhC* ($\Delta enhC$), $\Delta rtxA$ expressing the *enh1* locus from a plasmid ($\Delta rtxA/pJDC20$) and $\Delta enhC$ expressing the *enh2* locus from a plasmid ($\Delta enhC/pJDC24$). Data points and error bars represent the mean of triplicate samples from a representative experiment and their standard deviations. All experiments were performed at least three times.

had no significant similarity to any sequence in the available protein and DNA sequence databases. Based upon the Enh phenotype of this locus, these ORFs were given the gene names *enhA*, *enhB* and *enhC*. All three *enh* genes have an upstream region that closely resembles the *E. coli* consensus promoter sequence (Rosenberg & Court, 1979; Youderian *et al.*, 1982).

Characterization of the *rtxA* and *enhABC* genes

Analysis of the sequence of the *rtxA* gene revealed the presence of several distinctive characteristics (Fig. 7). Standard translational start (ATG) and stop (TAA) sequences were present, allowing the translation of a protein of 1208 aa. However, a somewhat unusual ribosome-binding site (AAGTAG) was found upstream of the translational start. Eight repeats of the RTX consensus sequence in two separate regions of the protein (at aa 519 and 1060) were found within the *L. pneumophila rtxA* gene (Fig. 7a). The first of these regions has five 9 aa RTX repeats, whereas the second region has only three. The 9 aa repeats were nearly identical for all copies present in the *L. pneumophila rtxA* gene. When the carboxy-terminal regions of the RTX repeats were aligned, additional sequence similarity was observed in the 40 aa carboxy-terminal of the repeats. An alignment of the two *L. pneumophila* RTX repeat regions with similar RTX regions from other species is shown in Fig. 7b. The positions of glycine, aspartate and phenylalanine residues continue to be highly conserved in this region.

The *enhA*, *enhB* and *enhC* genes each have standard translational start, translational stop and ribosome-binding sites (Fig. 8a). The *enhA* gene would encode a protein of 240 aa, *enhB* a protein of 142 aa and *enhC* a protein of 1200 aa. In addition, all three genes encode proteins with a hydrophobic alpha-helical region in their amino terminus that has an amino acid sequence characteristic of standard secretory signal sequences in Gram-negative bacteria. The potential signal sequence cleavage sites for *enhA*, *enhB* and *enhC* are between amino acids 19 and 20, 27 and 28, and 21 and 22, respectively.

The putative *enhC* gene product has several characteristics that are reminiscent of eukaryotic proteins. A region was found between amino acids 223 and 257 (Fig. 8a) that fits the profile for eukaryotic tetratricopeptide (TPR) regulatory proteins (n value = 8.4). However, only one repeat region was found within *enhC*, whereas at least seven are usually found in other TPR proteins (Goebel & Yanagida, 1991; Sikorski *et al.*, 1990). The strongest amino acid sequence similarity between the protein encoded by *enhC* and known protein sequences was with the *sel-1* gene product from *Cae. elegans* and the *SKT5* gene product from *S. cerevisiae*. There is a conserved domain of Sel-1 that has previously been observed to be similar to a region of Skt5p (Grant & Greenwald, 1997). This region of Sel-1 is the region of highest sequence similarity to EnhC. However, instead of a single conserved region, EnhC has two regions that have similarity to each other as well as to the conserved region of Sel-1 (Fig. 8). Alignment of the conserved regions of EnhC with all of the proteins that display high similarity to EnhC reveals the presence of this conserved region in the chitin synthase 4 gene from *Can. albicans* as well (Fig. 8b). A characteristic of this region is conserved alanine repeats every 7–8 aa with highly conserved leucine, tyrosine and glycine residues. Pre-

diction of the secondary structure of this conserved domain suggests a beta sheet, alpha helix, turn (proline at amino acid 62–63 in alignment), alpha helix, beta sheet structure. The alpha-helical domains are hydrophobic and represent potential transmembrane regions. The phenotypic effect of *enhC* on *L. pneumophila* entry and similarity to eukaryotic proteins, particularly Sel-1, suggest that *enhC* is a secreted protein that is involved in the interaction of *L. pneumophila* with host cells.

Mutations in the *rtxA* and *enhC* genes affect entry

To clearly demonstrate that the genes identified are involved in entry of *L. pneumophila*, we constructed in-frame deletions in *rtxA* and *enhC* and attempted to complement them with a plasmid containing the appropriate *enh* locus. The ability of the resulting strains to enter epithelial cells and monocytes was then compared to the wild-type *L. pneumophila* strain AA100 (Fig. 9). Each of the mutations significantly ($P < 0.006$) affects the ability of *L. pneumophila* to enter both cell types. This reduced-entry phenotype can be complemented by expression of the appropriate *enh* locus from a low-copy-number plasmid. However, no complementation was observed in the mutants containing the plasmid vector without the appropriate *enh* locus (data not shown). These results indicate that the *enh1* and *enh2* loci are important for entry of *L. pneumophila* into monocytes.

DISCUSSION

We have isolated two loci, *enh1* and *enh2*, that when present on multicopy vectors enhance entry of wild-type *L. pneumophila* into epithelial cells and monocytes. These loci were identified as the result of attempts to isolate the genes responsible for the Enh phenotype of the *L. pneumophila* entry mutant C3. This strategy assumes that the Enh phenotype is due to a dominant mutation. There are a number of potential dominant mutations that could be responsible for the Enh phenotype, including mutations within bacterial receptor(s), regulatory genes or other proteins involved in the synthesis and/or secretion of bacterial receptor(s). Despite the fact that a dominant mutation was not identified, the possibility that the C3 phenotype is due to a dominant mutation still exists. This possibility can not be ruled out until all cosmids that have the ability to confer an Enh phenotype to wild-type *L. pneumophila* have been fully characterized. However, it is equally likely that the mutation in C3 is recessive. If a recessive mutation is responsible for the C3 Enh phenotype, the genes involved could be isolated by transformation of a genomic library of wild-type *L. pneumophila* into C3 and screening for the loss of enhanced entry. This approach is complicated by the fact that there is no positive selection for the loss of the ability to enter host cells in *L. pneumophila*. Thus, the library must be screened in individual entry assays, requiring at least 300 entry assays to screen it in entirety. We are currently in the process of investigating this possibility as well as re-

examining other cosmids (1A7, 2A3, 2A4 and 2A6) that confer the Enh phenotype.

The *enh1* locus contains two genes, *rtxA* and *arpB*. The *rtxA* gene product has eight RTX repeats, which are thought to be involved in the Ca^{2+} -binding activity that is required for toxin activity and host-cell attachment (Boehm *et al.*, 1990; Welch, 1991). The involvement of this domain in binding to host cells has been observed with RTX toxins including the *Actinobacillus actinomycesemcomitans* leukotoxin (Lally *et al.*, 1997), *E. coli* haemolysin (Lally *et al.*, 1997) and *B. pertussis* cytotoxin (Glaser *et al.*, 1989). An observation that supports a role for the *rtxA* gene in adherence and entry is that RTX toxins use $\beta 2$ integrins as receptors on target cells (Lally *et al.*, 1997). Previous studies in *L. pneumophila* have demonstrated a role for complement receptors, which are $\beta 2$ integrins, in both adherence and entry by *L. pneumophila* (Marra *et al.*, 1990; Payne & Horwitz, 1987). These data combined with the presence of an RTX in *L. pneumophila* suggest that further investigation of direct interactions of *L. pneumophila* with complement receptors is warranted.

The *enh2* locus contains three genes, *enhA*, *enhB* and *enhC*, that have putative Gram-negative signal sequences. The observation that *enhC* affects the ability of *L. pneumophila* to enter host cells suggests the presence of a novel mechanism of entry that has not been observed in other bacterial systems. The presence of unusual mechanisms of entry in *L. pneumophila* is not surprising considering that entry has been shown to occur by coiling phagocytosis (Horwitz, 1984), which has not been observed at significant levels in other bacterial species with the exception of spirochaetes (Rittig *et al.*, 1992, 1998). The role of *enhC* in entry may be through a direct interaction with a target-cell receptor or through regulation of the available receptors on the host cell, similar to Sel-1 (Grant & Greenwald, 1996). Unfortunately, the receptor for Sel-1 on target cells is not yet known, which would facilitate analysis of its interaction with these cells. Studies are ongoing to differentiate between the possible role of *enhC* in entry as well as the roles of the two other genes present at this locus.

The current study has laid a foundation for further examination of the entry mechanisms utilized by *L. pneumophila*. The strategy used to isolate the *enh1* and *enh2* loci should be broadly applicable to the isolation of virulence determinants from bacterial pathogens. This is not the first time that gene dosage effects have been used to control the regulation of genes of interest (Aiba *et al.*, 1982; Blanc-Potard *et al.*, 1999; O'Sullivan *et al.*, 1996). However, this is the first time that these effects have been utilized to directly select for genes of interest, particularly virulence determinants. One caveat of this strategy may be that the operator regions present on the fragments present in multiple copies could saturate global regulators, resulting in down- or up-regulation of unlinked loci in the same regulon. This potential problem was not observed in the current study, since only transposon insertions in coding regions affected the

Enh phenotype conferred by these plasmids. One might expect that overexpression of genes under growth conditions where they are not normally expressed would be detrimental to the bacteria. Thus, the use of the low copy-number p15A origin of replication is likely to have contributed to the success of the current study by producing only moderately enhanced levels of expression. Application of this strategy to the analysis of the molecular determinants of *L. pneumophila* entry has provided insight into the potential mechanisms involved and should lead to a better understanding of the pathogenesis of Legionnaires' disease.

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