1 **Mobile element integration reveals an atypical chromosome dimer resolution system in**
2 ***Legionella pneumophila***

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4 Beth Nicholson¹, Shayna R. Deecker¹ and Alexander W. Ensminger^{1,2#}

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6 ¹ Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada.

7 ² Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada.

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11 #Address correspondence to Alexander W. Ensminger, alex.ensminger@utoronto.ca

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ABSTRACT

In bacteria, the mechanisms used to repair DNA lesions during genome replication include homologous recombination between sister chromosomes. This can lead to the formation of chromosome dimers if an odd number of crossover events occurs. The concatenated DNA must be resolved before cell separation to ensure genomic stability and cell viability. The broadly conserved *dif*/Xer system counteracts the formation of dimers by catalyzing one additional crossover event immediately prior to cell separation. While *dif*/Xer systems have been characterized or predicted in the vast majority of proteobacteria, no homologs to *dif* or *xer* have been identified in the order *Legionellales*. Here we report the discovery of a distinct *dif*/Xer system in the intracellular pathogen *Legionella pneumophila*. The *dif* site was uncovered by our analysis of *Legionella* mobile element-1 (LME-1), which harbors a *dif* site mimic and integrates into the *L. pneumophila* genome via site-specific recombination. We demonstrate that *lpg1867* (herein named *xerL*) encodes a tyrosine recombinase that is necessary and sufficient for catalyzing recombination at the *dif* site, and that deletion of *dif* or *xerL* causes filamentation and slow growth phenotypes. In addition, our data show that LME-1 can be classified as an integrative mobile element exploiting Xer (IMEX). The identification of LME-1 as an IMEX of *Legionella*'s atypical *dif*/Xer system further highlights the extent to which diverse mobile elements have independently evolved to exploit this critical cellular machinery.

34 INTRODUCTION

35 The circular nature of bacterial chromosomes brings about a problem for the partitioning of
36 genetic information to daughter cells: the formation of chromosome dimers during replication.
37 Dimers are generated when homologous recombination-mediated repair of DNA lesions results in an
38 odd number of crossover events between sister chromosomes. The concatenated DNA must be
39 resolved into monomers for proper cell division to occur. The essential and broadly conserved
40 bacterial *dif*/Xer system overcomes this topological problem by catalyzing one additional crossover
41 event immediately prior to cell separation (Barre et al., 2001; Steiner & Kuempel, 1998). The *dif*
42 (deletion-induced filamentation) site is a conserved ~30-bp DNA element on the chromosome that
43 contains binding sites for either two copies of a single tyrosine recombinase or one copy of two
44 different ones (Blakely et al., 1991, 1993; Castillo et al., 2017; Le Bourgeois et al., 2007; Leroux et
45 al., 2013; Sciochetti et al., 2001). In a highly regulated process, a nucleoprotein complex containing
46 the Xer tetramer and two aligned *dif* sites forms a recombination synapse, and site-specific
47 recombination between them deconcatenates the chromosome dimer into monomers (Thanbichler,
48 2010).

49 Dimer resolution via site-specific recombination by tyrosine recombinases was first
50 discovered in plasmids (Austin et al., 1981; Summers & Sherratt, 1984) including ColE1, in which a
51 DNA sequence named *cer* was found to be essential for plasmid monomerization and stability
52 (Summers & Sherratt, 1984). Recombination between *cer* sites was later found to require catalysis
53 by XerC (Colloms et al., 1990) and XerD (Blakely et al., 1993) encoded on the *E. coli* genome.
54 These findings, along with homology between the chromosomal *dif* site and *cer*, led to the discovery

of the *dif*/XerCD chromosome dimer resolution (CDR) pathway in *E. coli* (Blakely et al., 1991, 1993; Clerget, 1991; Kuempel et al., 1991) and later in several other species of bacteria (Debowski et al., 2012; Jensen, 2006; Le Bourgeois et al., 2007; Leroux et al., 2013; Sciochetti et al., 2001; Val et al., 2008) and archaea (Cortez et al., 2010; Duggin et al., 2011). Subsequent dissection of the *dif*/Xer recombination machinery has been aided by another group of mobile genetic elements, named integrative mobile elements exploiting Xer (IMEX) (Das et al., 2011, 2013; Fournes et al., 2016; Hassan et al., 2010; Huber & Waldor, 2002; Midonet et al., 2014; Midonet & Barre, 2014; Val et al., 2005). IMEXs, which include lysogenic phages, genomic islands, and plasmids, contain a *dif*-mimic sequence which allows them to integrate into the chromosome at the *dif* site using the host's Xer recombination machinery (Das et al., 2013; Midonet & Barre, 2014).

Homologs of *xerC* and *xerD* have been predicted to be present in the vast majority of proteobacterial species (Carnoy & Roten, 2009). Exceptions to this include two groups of bacteria that utilize a single-recombinase system. The *Streptococci*/*Lactococci* use a single recombinase XerS and an atypical *dif* site (Le Bourgeois et al., 2007) and a group of epsilon-proteobacteria, including *Helicobacter* and *Campylobacter* species, uses a single-recombinase XerH system (Carnoy & Roten, 2009). While *dif*/Xer homologs have been detected in almost 90% of proteobacteria, no *dif* or Xer homologs could be detected in the order *Legionellales*, despite encoding RecA, RecBCD, and RecF, which are thought to be responsible to dimer formation during replication (Carnoy & Roten, 2009). How then do the members of the order *Legionellales* overcome the threat to genome stability that chromosome dimers impose? It is possible that they use an entirely different method for CDR, or that its *dif*/Xer system is sufficiently divergent from others to avoid detection by sequence-based homology searches.

Several strains of *Legionella pneumophila* harbor a phage-like integrative mobile genetic element, named *Legionella* mobile element-1 (LME-1) (Deecker et al., 2021; Lüneberg et al., 2001; Rao et al., 2016). We previously found that integration into the genome requires a 22-bp attachment site (*att*) in LME-1 that is identical to a sequence on the *L. pneumophila* genome (Rao et al., 2016). Even though LME-1 is only integrated in a small proportion of sequenced *L. pneumophila* isolates, the chromosomal *att* site is present in all *L. pneumophila* isolates and *Legionella* species described to date (Table S1), a level of conservation that suggests an important function. We report here that the LME-1 *att* site on the chromosome meets several criteria of the missing *dif* site in *Legionella*: size ~30 bp, low GC-content, some degree of palindromicity, positioned close to the replication terminus, and in a non-coding region with varying flanking genes. We identify a single recombinase that is both necessary and sufficient for site-specific recombination at this site. Together, our results suggest that *L. pneumophila* uses a distinct single-recombinase system for CDR and that LME-1 is an IMEX of that system.

RESULTS

The LME-1 attachment site is invariably close to the *Legionella* replication terminus.

We previously determined that LME-1 integrates into the *L. pneumophila* genome via a 22-bp attachment (*att*) site (Rao et al., 2016). The corresponding *att* site on the chromosome is contained within a broader 29-bp sequence that is conserved across all sequenced *L. pneumophila* strains and *Legionella* species (**Fig. 1A, Table S1**), suggesting that it performs an essential function. While the *att* site itself is conserved, its genomic neighbourhood varies (Rao et al., 2016) (**Table S1**), indicating that the intrinsic function of this sequence may be unrelated to its flanking genes. In a search for attributes of the *att* site that may hint at its function, we analyzed its chromosomal location in all *L. pneumophila* strains with available genome sequences (**Table S1**). We found that

Figure 1 | The DNA sequence hijacked by LME-1 is invariably proximal to the site of GC-skew inflection and resembles a *dif* site. **A)** Sequence alignment of the LME-1 *att* site region in different *L. pneumophila* strains (left panel) and other *Legionella* species (right panel). The dashed-line box denotes the 22-nt sequence that corresponds to the *att* site present on LME-1. The grey arrows indicate the inverted repeat portion of the sequence. **B)** Circos plots showing GC-skew across the chromosome of several *L. pneumophila* strains (left panel) and other *Legionella* species (right panel). Each ring corresponds to one genome, with inside-to-outside rings corresponding to the genomes listed top-to-bottom in the alignments in panel A. The black line indicates the position of the *att* site in each genome. **C)** Alignment of the LME-1 *att* site with *dif* sites from bacterial species with established *dif*/Xer systems. The binding sites for XerC and XerD homologs (named CodV and RipX, respectively in *B. subtilis*) (Blakely et al., 1991, 1993; Sciochetti et al., 2001), XerS (Nolivos et al., 2010), and XerH (Bebel et al., 2016; Leroux et al., 2013) are underlined.

despite the disparate gene neighbourhoods, the location of the *att* site was roughly opposite the origin of replication in all cases. In fact, GC-skew analysis of each genome revealed that the *att* site was invariably close to the cumulative GC-skew maximum, which occurs at the site of replication termination (Grigoriev, 1998) (**Fig. 1B, Table S1**). The *att* site is present within a small ~8-kb window between 35 kb and 43 kb from the GC-skew inflection point (**Table S1**). This specificity of positioning extended to the *att* sites of other *Legionella* species, which are located between 89 bp and 79 kb from the point of GC-skew inflection (**Fig. 1B, Table S1**).

The proximity of the LME-1 *att* site to the terminus region is reminiscent of bacterial *dif* sites, so we next compared the *att* site to the *dif* sites of several bacteria with established *dif*Xer systems. In addition to its proximity to the terminus, the LME-1 *att* site shows several similarities to known *dif* sites, including (i) intergenic location (**Table S1**), (ii) size of ~30 bp, (iii) low G+C content, and (iv) a short central region flanked by left and right arms with some degree of dyad symmetry (**Fig. 1C**). However, the LME-1 *att* site is distinguished from known *dif* sites by the perfect dyad symmetry of its 10-bp arms and the short 4-bp central region separating them. The arms do not show homology to any known Xer-binding motifs (**Fig. 1C**) (Carnoy & Roten, 2009).

The *L. pneumophila* *dif* site supports highly efficient RecA-independent DNA recombination.

The similarities between the chromosomal LME-1 *att* site (hereafter referred to as the *Legionella* *dif* site) and established *dif* sites suggest that it likely contains two binding sites for one or more tyrosine-type recombinases that catalyze site-specific recombination (Castillo et al., 2017). To assess the ability of the *L. pneumophila* *dif* site to undergo site-specific recombination, we used an intermolecular recombination assay (**Fig. 2A**). In this assay, a non-replicative plasmid (pJB4648) containing a gentamicin-resistance marker and a 22-bp portion of the *dif* site (corresponding to the LME-1 *att* site) is transformed into *L. pneumophila* strain Lp02. If site-specific recombination occurs between the *att* site on the plasmid and the chromosomal *dif* site, the entire plasmid will be

126 integrated into the genome and confer gentamicin resistance to the cell. We compared the number of
 127 transformants resulting from the *att*-containing plasmid to one with a control 22-bp sequence derived
 128 from the *L. pneumophila* genome near the *dif* site. To evaluate the efficiency of recombination
 129 supported by the *dif* site, we also included a plasmid containing 2200 bp of unrelated intergenic
 130 sequence from *L. pneumophila*, which can integrate into the genome via a single-crossover
 131 homologous recombination event. Considering that chromosomal *dif* site-specific recombination is
 132 known to be RecA-independent (Domínguez et al., 2011; Kuempel et al., 1991; Le Bourgeois et al.,
 133 2007), we also assessed the integration of each of these plasmids in a *recA* deletion strain (**Fig. 2B**).
 134 To control for any strain-to-strain differences in overall transformation efficiency, we normalized the
 135 number of transformants for each plasmid to the number resulting from transformation with a
 136 replicative plasmid (pJB1806_GmR). The *att* site-containing plasmid integrated into the genome at a

high rate, with an efficiency that was 50-fold higher than that of the 2200-bp control. In contrast, the 22-bp control sequence did not generate any transformants, indicating that the number of integration events was lower than the detection limit of 1 CFU per $\sim 2 \times 10^9$ cells. In the $\Delta recA$ strain, no transformants were detected for the 2200-bp control which relies on homologous recombination for integration, while the *att* site-containing plasmid integrated at a level similar to that in the wildtype strain (**Fig. 2B**). In both the wildtype and $\Delta recA$ strains, integration of the *att*-containing plasmid at the chromosomal *dif* site was confirmed by PCR-amplification of the *dif* region and Sanger sequencing (data not shown). These data indicate that, similar to other *dif* sites, site-specific recombination at the *L. pneumophila dif* site is efficient and recA-independent.

***Legionella* Xer/*dif* is likely a single-recombinase system.**

We next aimed to identify the recombinases that catalyzes recombination at the *Legionella dif* site. No orthologs of XerC, XerD, XerS, or XerH have been identified in *Legionella* using sequence homology (Carnoy & Roten, 2009). However, HHPred analysis revealed that six *L. pneumophila* genes present in strain Lp02 (*lpg0980*, *lpg0981*, *lpg1070*, *lpg1085*, *lpg1867*, and *lpg2057*) show structural similarity to tyrosine-type recombinases (probability >99%). We therefore repeated our intermolecular recombination assay in strains containing individual deletions of these six genes, along with a strain lacking the *dif* site. As expected, deletion of the *dif* site resulted in no detectable integration of the *att* site-containing plasmid. Deletion of *lpg1867* also specifically abolished integration, while deletion of each of the other five recombinases had no significant effect (**Fig. 3A**). We also performed rescue experiments using expression plasmids to confirm that the loss of recombination at the *dif* site in the $\Delta lpg1867$ strain was due to loss of Lpg1867 protein function and not any secondary mutations acquired during strain generation. Expression of Lpg1867 in the $\Delta lpg1867$ strain resulted in near-wildtype levels of recombination (**Fig. 3B**). In contrast, no recombination was detected with an empty vector control or when a point mutant of Lpg1867

Figure 3 | The *L. pneumophila* Xer is encoded by *lpg1867* and does not require a partner recombinase. Shown are dot plots for assays detecting intermolecular recombination between a plasmid containing the 22-bp *att* site and the chromosomal *dif* site, as described for Figure 2. **A**) Intermolecular recombination in wildtype Lp02 and strains containing deletions of the *dif* site or one of six tyrosine-type recombinases. Shown in pink are the *dif*-deletion strain and *lpg1867*-deletion strain, which both displayed levels of recombination below the limit of detection. **B**) The wildtype and $\Delta lpg1867$ strains were transformed with plasmids expressing wildtype Lpg1867 or a predicted catalytic point mutant, Y387F. Wildtype Lpg1867, but not the catalytic mutant, rescued the recombination defect in the $\Delta lpg1867$ strain. **C**) Recombination in the wildtype strain was compared to that in a 5-deletion strain (*lpg0980*, *lpg0981*, *lpg1070*, *lpg1085*, and *lpg2057* deleted) and a pan-deletion strain, with all six recombinases deleted (i.e, 5-deletion plus *lpg1867* deletion). **D**) The wildtype strain and pan-deletion strain were transformed with expression plasmids as in panel B. Wildtype Lpg1867, but not the catalytic mutant, fully rescued the recombination defect in the pan-deletion strain.

(Y387F) was expressed. The Y387F mutant is predicted to be catalytically inactive based on the equivalent catalytic tyrosine mutation in other tyrosine recombinases (Blakely et al., 1993; Gibb et al., 2010; Pan et al., 1993).

We next asked whether one of the other five putative tyrosine recombinases might be involved along with Lpg1867 in recombination at the *dif* site, despite having no effect upon individual deletion. To address this, we generated two multiple-deletion strains: one with five putative recombinases deleted (all except *lpg1867*), and a pan-deletion strain with all six deleted. In our intermolecular recombination assay, integration of the *att*-containing plasmid was similar to wildtype levels in the 5-deletion strain and nonexistent in the pan-deletion strain (**Fig. 3C**). Overexpression of Lpg1867, but not Lpg1867-Y387F, again rescued the recombination defect (**Fig. 3D**). Collectively, these data indicate that Lpg1867 is both necessary and sufficient to catalyze site-specific recombination at the *L. pneumophila dif* site.

Deletion of *dif* or *lpg1867* induces filamentation and inhibits growth.

Loss of a functional chromosome dimer resolution system causes defects in cell division, which can result in a variety of phenotypes including slow growth and filamentation (the *dif* site was named for its deletion-induced filamentation phenotype). Consistent with reported findings for *dif* or *xer* deletions in other species (Blakely et al., 1991; Debowski et al., 2012; Farrokhi et al., 2019; Hendricks et al., 2000; Kuempel et al., 1991; Sciochetti et al., 2001), we observed that loss of *dif* or Lpg1867 in *L. pneumophila* led to filamentation in a subpopulation of cells (**Fig. 4A**). Notably, and consistent with a reduction in chromosome dimer formation in the absence of RecA (Pérals et al., 2000; Steiner & Kuempel, 1998) we observed a decrease in filament formation upon deletion of *recA* in the Δ *lpg1867* background (**Fig. 4A**).

Deletion of *dif* or *lpg1867* also results in a visible reduction in colony growth on solid media (**Fig. 4B**). Again, this defect is alleviated upon additional deletion of *recA* in the Δ *lpg1867*

Figure 4 | *dif*- and *lpg1867*-deletion strains exhibit filamentation and altered growth on solid media. **A)** DAPI-stained cells of the indicated *L. pneumophila* strains, showing filamentation upon deletion of the *dif* site or *lpg1867* and rescue when *recA* is deleted in the $\Delta lpg1867$ background. The scale bars represent 10 μm . **B)** CYET spread plates after 4 days of growth at 37°C with visible differences in colony size (left panel). Each colony radius is plotted as a separate data point, with the geometric mean shown by the purple line (right panel). The reduction in colony size in the $\Delta lpg1867$ strain is rescued by the additional deletion of *recA*. **C)** Distribution of colony sizes in the 5-deletion strain and pan-deletion strain (left panel) and in the pan-deletion strain expressing wildtype or mutant Lpg1867 (right panel). Data are plotted as in panel B. Wildtype Lpg1867, but not the catalytic mutant Lpg1867-Y387F, rescues the small colony phenotype observed in the pan-deletion.

187 background (Fig. 4B), consistent with the growth inhibition being caused by an inability to resolve
 188 chromosome dimers. The slow growth phenotype is also present in the recombinase pan-deletion
 189 strain, but not the 5-deletion strain, and the defect is rescued by expression of wildtype Lpg1867 but
 190 not the catalytic mutant (Fig. 4C), mirroring the effects of these deletions on recombination (Fig.
 191 3C,D). Taken together, these data support a model in which a single site-specific recombinase,
 192 Lpg1867 (which we designate here as XerL), catalyzes recombination between two *dif* sites to
 193 resolve chromosome dimers in *Legionella*.

194

LME-1 stability is modulated by Lpg1867 (XerL) and the formation of a modified downstream *dif* site.

We have previously shown that LME-1 integrates into the *L. pneumophila* chromosome at what we now know to be the *dif* site (Rao et al., 2016). Other invasive elements known to integrate at the *dif* site of their host (IMEXs) do not encode their own recombinase and instead utilize the host Xer for integration/excision (Das et al., 2013; Midonet & Barre, 2014). Similarly, LME-1 does not appear to encode its own recombinase or integrase (Deecker et al., 2021). Our intermolecular recombination assay indicates that XerL can facilitate integration of circular DNA containing the LME-1 22-bp *att* site (**Fig. 2, Fig. 3**). To investigate whether XerL is also involved in LME-1 excision, we overexpressed XerL in *L. pneumophila* strain Murcia-4983, which naturally contains LME-1 stably integrated at its *dif* site (Rao et al., 2016). Using an established qPCR-based assay (Rao et al., 2016), we quantified LME-1 excision in this strain upon XerL overexpression and found that excision was increased >25 fold compared to a control strain transformed with empty vector (**Fig. 5A**). Interestingly, the catalytic mutant Lpg1867-Y387F did not enhance excision, and in fact inhibited excision by >5 fold (**Fig. 5A**). A possible explanation for this is that the mutant Lpg1867 is acting as a dominant-negative by binding the *dif/att* sites but not catalyzing recombination.

LME-1 is stably integrated into the Murcia-4983 genome (only excised in ~1% of the population), despite being flanked by two *att* sites (Rao et al., 2016). The sequence of the LME-1 *att* site is identical to the *L. pneumophila dif* site, but only encompasses 22-bp of it. Recombination between this 22-bp sequence and the chromosomal *dif* site results in *att*-site duplication, which generates an intact *dif* site upstream and a modified *dif* site downstream of LME-1 that contains 3 substitutions (**Fig. 5B**). We next asked whether these modifications to the downstream *dif* site contribute to the stability of the integrated form of LME-1. To address this, we developed an excision assay in which a kanamycin-resistance cassette was used as a proxy for LME-1 by inserting it into the Lp02 Δ *lpg1867*

Figure 5 | The sequence of the LME-1 *att* site contributes to its low level of excision, which increases upon Lpg1867 (XerL) overexpression. **A)** Shown is the effect of overexpression of wildtype Lpg1867 or the catalytic mutant Lpg1867-Y387F on LME-1 excision compared to an empty vector control in *L. pneumophila* strain Murcia-4983. The change in LME-1 excision was determined using an established qPCR-based assay. **B)** Comparison of the wildtype *dif* site in strain Murcia-4983 and the sequences flanking LME-1 resulting from *att* site duplication upon integration into the chromosome. The upstream copy of the *dif* site remains intact, but the downstream copy has three nucleotide differences (pink letters). These differences are within the inverted repeat regions of the *dif* site (denoted by the grey arrows), which presumably contain binding sites for Lpg1867 (XerL). **C)** Schematic representation of the intramolecular recombination assay used to assess the effect of flanking sequencing on LME-1 excision. Wildtype and mutant Lpg1867 expression plasmids were transformed into two strains made in an Δ *lpg1867* background: a *dif*-KanR-*att* (DKA) strain in which the KanR gene is flanked by the sites typically flanking LME-1, and a *dif*-KanR-*dif* (DKD) strain in which the same reporter is flanked by two wildtype *dif* sites. Recombination between the two sites leads to excision of the KanR reporter, which is quantified by plating on selective and nonselective media. **D)** The percent of cells with kanamycin resistance prior to induction of Lpg1867 (wildtype or mutant) and after 24 and 48 hours are plotted. A loss in kanamycin resistance corresponds to recombination between the two sites flanking the KanR reporter. The only strain with significant loss of kanamycin resistance was the DKD strain with wildtype Lpg1867 overexpressed, in which only ~2% of cells maintained the reporter even prior to induction (likely due to leaky expression of Lpg1867 from the plasmid).

strain at the *dif* site (**Fig. 5C**). We made two strains: a *dif-KanR-att* (DKA) strain in which the KanR gene is flanked by the sites typically flanking LME-1, and a *dif-KanR-dif* (DKD) strain in which the same reporter is flanked by two wildtype *dif* sites. We then overexpressed wildtype or mutant Lpg1867 in both of these strains. To quantify excision of the reporter, we determined the proportion of kanamycin-resistant cells in the population prior to induction of expression and at 24-h and 48-h post induction by colony counting on selective and nonselective plates. We found that the LME-1-like reporter in the DKA strain was remarkably stable, even when wildtype Lpg1867 was overexpressed (**Fig. 5D**). In contrast, kanamycin resistance was lost in the vast majority (98%) of cells in the DKD strain containing the wildtype Lpg1867 expression plasmid, even prior to induction (likely due to leaky expression), and no kanamycin-resistant cells remained after 24 h. However, no excision was detected in the DKD strain containing the catalytic mutant Lpg1867 expression plasmid (**Fig. 5D**). The stability of the reporter in the DKA strain suggests that the short 22-bp *att* site of LME-1 likely contributes to the stability of its integrated form by preventing it from being flanked by two intact *dif* sites.

DISCUSSION

The discovery of the *L. pneumophila* CDR system has solved two mysteries of the pathogen's biology: the function of the conserved DNA sequence that is hijacked by LME-1, and how *Legionella* species are able to resolve chromosome dimers despite having no homologs to known *dif* sites and Xer recombinases. Similarly, no *dif*/Xer components have been identified in members of the other *Legionellales* family, *Coxiellaceae* (Carnoy & Roten, 2009), which includes the human pathogen *Coxiella burnetii*. A priority going forward will be to characterize the CDR pathway(s) in *Coxiellaceae* using *L. pneumophila* as a genetically tractable surrogate.

The *Legionella dif*/Xer system is distinct in several ways from those characterized in other species. The *dif* site arms are a 10-bp inverted repeat with no interruptions, whereas other *dif* sites show only partial dyad symmetry between the two arms, even for single-recombinase systems. Additionally, *dif* and *xerL* are separated by >400 kb in the *L. pneumophila* genome, whereas in the other bacterial single-recombinase systems (*i.e.* *difSL*/XerS of *Streptococci* and *Lactococci* and *difH*/XerH of *Campylobacter* and *Helicobacter*), the *dif* site is always near the *xer* gene, indicating they may have been acquired as a single module (Carnoy & Roten, 2009; Castillo et al., 2017; Le Bourgeois et al., 2007; Leroux et al., 2013).

With no apparent *dif* or Xer homologs in *Legionella*, the CDR system was instead unearthed through our investigation of LME-1 and its attachment site. In turn, the discovery of the *Legionella dif* site and XerL has allowed us to explore new LME-1 biology. We found that the 22-bp LME-1 *att* site is sufficient for integration of circular DNA at the *L. pneumophila dif* site, and that overexpression of XerL boosts LME-1 excision 25-fold. These findings are consistent with LME-1 belonging to the IMEX class of integrative mobile elements, which exploit the host CDR machinery for integration and in some cases excision (Das et al., 2013; Midonet & Barre, 2014). The mechanistic details of LME-1 integration and excision, and how they relate to the strategies of other IMEXs, are interesting topics of future study. One intriguing aspect of LME-1 is the stability of its integrated form. Our reporter excision assay showed that the modified *dif* site and wildtype *dif* site that flank the integrated LME-1 do not recombine with each other, or at least recombine at a very low level that is not detectable in our assay. Another stably integrated IMEX, the gonococcal genomic island (GGI) of *Neisseria gonorrhoeae*, generates a similarly modified *dif* site (*dif*_{GGI}) at one end of its integrated form, while the wildtype *dif* (*dif*_{Ng}) remains intact at the other (Fournes et al., 2016). The *Neisseria* CDR system uses XerC and XerD homologs, and recombination takes place

when the FtsK DNA translocase pauses at the *dif*/Xer complex and activates XerD. Interestingly, FtsK does not stop at the *dif*_{GGI}-XerC/D complex, but rather translocates through it, which likely leads to disassembly of the complex and precludes recombination between the *dif*_{NG} and *dif*_{GGI} (Fournes et al., 2016). It is possible that this same FtsK-dependent process contributes to the stability of the integrated form of LME-1, although we have yet to determine whether XerL requires FtsK for activation. Addressing both of these questions is a priority for our future investigation of the *L. pneumophila* CDR system and its associated IMEX, LME-1.

The importance of resolving chromosome dimers for the maintenance of genomic stability means that the *dif* site and Xer recombinases are highly conserved (Carnoy & Roten, 2009; Kono et al., 2011), making them an attractive target for integration by IMEXs. The diversity of IMEX elements suggests that *dif*/Xer is a pathway that has been hijacked independently across a broad range of bacteria (Das et al., 2013; Midonet & Barre, 2014), even in a system as distinct as the one in *Legionella*. The discovery of the *Legionella dif*/Xer pathway and its associated IMEX, which had been missed in homology-based searches due to divergence from known CDR components, warrants a closer look at other bacteria in which no *dif*/Xer system has been identified to date.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *L. pneumophila* Lp02 strain was used as the background for all *L. pneumophila* strains generated in this study. The *L. pneumophila* Murcia-4983 strain, which harbors LME-1, is an environmental isolate collected during the 2001 outbreak in Murcia, Spain (García-Fulgueiras et al., 2003). All *L. pneumophila* strains were grown at 37°C in N-(2-acetamido)-2-aminoethanesulfonic

acid (ACES) buffered yeast extract supplemented with 100 ug/mL thymidine (AYET) and on charcoal AYET (CYET) agar plates.

Deletion strains were made using a scar-free suicide-cassette method described recently (Bailo et al., 2019) with some minor modifications. Briefly, linear DNA containing a *mazF*-KanR cassette flanked by ~1.5-kb homology arms was introduced into the parental strain via natural transformation. Cells in which the gene of interest had been replaced with the *mazF*-KanR cassette via homologous recombination were selected by plating on media containing kanamycin. As *mazF* gene expression is under an IPTG-inducible promoter, colonies can be screened on kanamycin- and IPTG-containing plates to identify cells that are IPTG-sensitive and kanamycin-resistant. In a second natural transformation, linear DNA containing only the homology arms (resulting in gene deletion) was introduced, and cells in which the cassette was excised were selected on plates containing IPTG. Colonies were then screened to identify those that were IPTG-resistant and kanamycin-sensitive, and the deletion was verified by PCR screening and Sanger sequencing.

The reporter strains used for the intramolecular recombination (reporter excision) assays were generated by introducing linear DNA containing the *dif*-KanR-*dif* or *dif*-KanR-*att* cassette (flanked by ~1.5 kb homology arms around the chromosomal *dif* site) into the Lp02 Δ *lpg1867* strain. Strains containing the cassette were selected on plates containing kanamycin, and the correct integration of the cassettes was verified by Sanger sequencing.

The pJB4648-based plasmids used for the intermolecular recombination assays were constructed by cloning the 22-bp *att* site sequence, a control 22-bp sequence, or 2200 bp of intergenic Lp02 sequence into pJB4648 digested with XhoI and ApaI. The gentamicin-resistant plasmid pJB1806-GmR was generated by first amplifying the gentamicin resistance gene and promoter from pJB4648 and cloning it in place of the chloramphenicol resistance gene in plasmid pJB1806. Protein expression plasmids used for the intermolecular recombination and colony size assays were made by cloning the *lpg1867* gene (wildtype or modified to encode the Y387F

mutation) into the multiple cloning site of pNT562 (Nishida et al., 2017). The tet-on-pJB1806-GmR-based expression plasmids used for the reporter excision assays were generated by replacing the *lacIq* gene and tac promoter in pJB1806-GmR with the *tetR* gene and tetracycline-responsive promoter from pLJR965 (Rock et al., 2017) and then cloning in the wildtype or mutant Lpg1867 ORF immediately after the promoter, as described by Rock and colleagues (Rock et al., 2017).

GC-skew analysis

The cumulative GC-skew maximum value for each *Legionella* genome (shown in Table S1) was determined using the GenSkew java app (<https://genskew.csb.univie.ac.at>) with default settings. The whole-genome GC-skew profiles and *att* site locations for seven *L. pneumophila* isolates and seven *Legionella* species, selected to show a range of cGC-skew maxima locations relative to the origin, were plotted using Circos (Krzywinski et al., 2009). The GC-skew profiles of these genomes were normalized for genome length. The selected *L. pneumophila* isolates and *Legionella* species are: *L. pneumophila*: Philadelphia_2 (GenBank accession number CP015929.1), Philadelphia_1 (AE017354.1), E7_O (CP015954.1), Paris (CR628336.1), NY23 D-7705 (CP021261.1), NCTC11985 (LT906452.1), and subsp. *fraseri* strain D-4058 (CP021277); *Legionella* species: *L. israelensis* L18-01051 (CP041668.1), *L. micdadei* (LN614830.1), *L. spiritensis* NCTC11990 (LT906457.1), *L. sainthelensi* NCTC12450 (LR134178.1), *L. longbeachae* NSW150 (FN650140.1), *L. oakridgensis* NCTC11531 (LR134286.1), and *L. fallonii* LLAP-10 (LN614827.1).

Recombination assays

For the intermolecular recombination (plasmid integration) assays, each strain was transformed with a pJB4648-based plasmid containing the 22-bp *att* site sequence, a control 22-bp sequence, or 2200 bp sequence as indicated. The strains were also transformed with the replicative plasmid pJB1806-GmR to control for strain-to-strain differences in general transformation

efficiency. Plasmid concentrations were determined using a Quant-iT PicoGreen dsDNA kit (Invitrogen), and 200 ng of each plasmid was electroporated into 2 ODU of *L. pneumophila* Lp02 cells as described previously (Rao et al., 2013). After an 8-h recovery in liquid AYET media shaking at 37°C, dilutions of each sample were plated onto CYET plates supplemented with 15 µg/mL gentamicin and incubated at 37°C for 4 days. Colonies were counted using GeneTools analysis software (Syngene), and the results were plotted using GraphPad Prism version 9.2.0.

For the intramolecular recombination assay, the DKA and DKD strains containing the kanamycin-resistance cassette were transformed with tet-on-pJB1806-GmR containing wildtype Lpg1867, Lpg1867-Y387F, or empty vector by electroporation as described above. Transformants were maintained on CYET plates containing both gentamicin and kanamycin until induction of protein overexpression. Two-day old patches were resuspended in AYET supplemented with gentamicin only, and an aliquot was taken for 0-h timepoint (no induction) CFU counting on selective (CYET+kan) and nonselective (CYET) plates. Liquid cultures were made from the resuspended patches in 2 mL of AYET supplemented with gentamicin and anhydrotetracycline (100 ng/mL) at a starting OD₆₀₀ of 0.2, and incubated with shaking at 37°C. After 24 h, each sample was plated for CFU counting and also back-diluted to OD 0.2 for further incubation. At the 48-h timepoint, a final CFU plating was performed. The number of colonies that grew on CYET+kan were compared to the number that grew on CYET to determine the proportion of cells that had lost kanamycin resistance. The results were plotted using GraphPad Prism version 9.2.0.

Fluorescent microscopy

Overnight cultures of Lp02, Lp02Δdif, Lp02Δlpg1867, and Lp02Δlpg1867ΔrecA were grown from a 2-day old patch in AYET medium. Bacteria were collected at post-exponential phase (OD₆₀₀ 4.0 to 4.5). Approximately ~1x10⁶ cells (1 ODU) from each strain were washed once with 1 mL of 1X PBS before a final resuspension in 500 µL of 1X PBS. The bacteria were stained with 15 µg/mL

of DAPI (Roche) for 30 minutes at room temperature and imaged using a 63x oil immersion lens on a Zeiss Axio Imager.M2 microscope.

Colony size assay

The distribution of colony size was determined by resuspending 2-day-old patches of the various strains in AYET. The OD₆₀₀ of each cell suspension was determined, and the appropriate dilutions made so that ~200-300 cells were added to each plate. Spread plates were incubated at 37°C for 4 days before imaging using a SynGene system with GeneSnap software (Syngene). Colony diameter was determined using ColTapp automated image analysis software (Bär et al., 2020), and plotted using GraphPad Prism version 9.2.0.

Quantitative PCR analysis of LME-1 excision

The quantitative PCR (qPCR) analysis of LME-1 excision was performed as described by Rao and colleagues with some slight modifications (Rao et al., 2016). Briefly, overnight cultures of *L. pneumophila* str. Murcia-4983 carrying either a pNT562 plasmid, a pNT562 plasmid expressing Lpg1867 or a pNT562 plasmid expressing Lpg1867-Y387F were grown from a 2-day-old patch in AYE medium supplemented with 20 µg/mL of kanamycin and 500 µM of IPTG. Bacteria were collected at post-exponential phase (OD₆₀₀ 4.0 to 4.5) and genomic DNA was extracted using a NucleoSpin Tissue Kit (Machery-Nagel) and quantified using a Quant-iT PicoGreen dsDNA kit (Invitrogen). LME-1 excision was quantified by qPCR using SsoAdvanced Universal SYBR Green Supermix (BioRad) and a CFX96 Real-Time System (BioRad). Plasmids containing the amplification products were used as standards for the standard curve. All excision quantifications were done for three biological replicates of each isolate. The excision frequency for each isolate was calculated as described by Rao and colleagues using the following formula (Rao et al., 2016):

$$\text{Excision Frequency} = \frac{\text{Amplicon (chrF\&chrR)}}{\text{Amplicon (chrF\&chrR)} + [\text{Amplicon (chrF\&lmeR)} + \text{Amplicon(lmeF\&chrR)}] / 2}$$

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SUPPLEMENTARY MATERIAL:

Table S1 | The att site is close to the cumulative GC-skew maximum in all sequenced *L.*

***pneumophila* strains and *Legionella* species.** The nucleotide positions of the att site and cumulative GC-skew maximum (cGC skew max) are shown for each *L. pneumophila* strain and *Legionella* species with a completed genome sequence available in Genbank. The cGC skew maximum corresponds to the site of replication termination (Grigoriev, 1998). The genes immediately upstream and downstream of each att site are also indicated.