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MOLECULAR PATHOGENESIS

Study of *Legionella* Effector Domains Revealed Novel and Prevalent Phosphatidylinositol 3-Phosphate Binding Domains

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ABSTRACT *Legionella pneumophila* and other *Legionella* species replicate intracellularly using the Icm/Dot type IV secretion system. In *L. pneumophila* this system translocates >300 effectors into host cells and in the *Legionella* genus thousands of effectors were identified, the function of most of which is unknown. Fourteen *L. pneumophila* effectors were previously shown to specifically bind phosphoinositides (PIs) using dedicated domains. We found that PI-binding domains of effectors are usually not homologous to one another; they are relatively small and located at the effectors' C termini. We used the previously identified *Legionella* effector domains (LEDs) with unknown function and the above characteristics of effector PI-binding domains to discover novel PI-binding LEDs. We identified three predicted PI-binding LEDs that are present in 14 *L. pneumophila* effectors and in >200 effectors in the *Legionella* genus. Using an *in vitro* protein-lipid overlay assay, we found that 11 of these *L. pneumophila* effectors specifically bind phosphatidylinositol 3-phosphate (PI3P), almost doubling the number of *L. pneumophila* effectors known to bind PIs. Further, we identified in each of these newly discovered PI3P-binding LEDs conserved, mainly positively charged, amino acids that are essential for PI3P binding. Our results indicate that *Legionella* effectors harbor unique domains, shared by many effectors, which directly mediate PI3P binding.

KEYWORDS Icm/Dot, *Legionella*, PI3P-binding domains, effectors

Legionella pneumophila is a Gram-negative bacterial species found in aquatic environments (1). In nature, *L. pneumophila* multiplies inside a variety of protozoan cells that serve as its environmental reservoir (2, 3). When people inhale aerosols containing *L. pneumophila*, the bacteria infect alveolar macrophages and can cause a life threatening pneumonia called Legionnaires' disease (4, 5). Instead of being degraded by defense mechanisms of protozoa and human macrophages, *L. pneumophila* subverts host cell processes and grows within a specialized *Legionella*-containing vacuole (LCV) (6, 7). To establish the LCV inside eukaryotic cells, *L. pneumophila* modulates host cell functions using the Icm/Dot type IV secretion system. The Icm/Dot system is essential for *L. pneumophila* pathogenesis, since it delivers effector proteins into the host cells, and these effectors directly modulate various host cell processes, resulting in the biogenesis of the LCV (reviewed in references 8 to 12). The genome of *L. pneumophila* encodes more than 300 different effectors and in most cases the deletion of individual effector-encoding genes, or even groups of several effector-encoding genes in a single mutant, has a modest effect on the ability of the bacteria to survive and multiply in both amoeba and mammalian host cells (13–15). The function of most of the *L. pneumophila* effectors remains unknown, but several effectors were shown to modulate important cellular processes, including vesicular trafficking, apoptosis, ubiquitination, autophagy, signal transduction, lipid metabolism, host gene expression, and others (reviewed in references 12, 16, and 17).

Citation Nachmias N, Zusman T, Segal G. 2019. Study of *Legionella* effector domains revealed novel and prevalent phosphatidylinositol 3-phosphate binding domains. Infect Immun 87:e00153-19. https://doi.org/10.1128/IAI.00153-19.

Editor Craig R. Roy, Yale University School of Medicine

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Received 22 February 2019
Returned for modification 19 March 2019
Accepted 27 March 2019
Accepted manuscript posted online 8 April 2019
Published 21 May 2019

TABLE 1 PI4P- and PI3P-binding *L. pneumophila* effectors

Locus tag[a]	Name	Size (aa)	Position of PI-BD (aa)[b]	PI binding[c]	Localization[d]	Effector function	Reference(s)
lpg0160	RavD	325	190–325	PI3P	LCV	Helps to prevent encounters of the LCV with lysosomes	59
lpg0695	AnkX	949	491–949	PI3P, PI4P	LCV	Phosphocholinates Rab1 and Rab35	60–64
lpg0940	LidA	729	60–180	PI3P, PI4P	LCV	Modulates Rab1 to render it persistently active	33, 65–69
lpg1101	Lem4	322	201–322	PI4P	LCV	Phosphotyrosine phosphatase	70, 71
lpg1683	RavZ	502	329–423	PI3P	AP	Irreversible deconjugation of Atg8/LC3	40, 42, 72
lpg1978	SetA	644	401–644	PI3P	LCV	Glucosyltransferase, subverts vesicle trafficking	73, 74
lpg2222	LpnE	375	ND	PI3P	LCV	Binds polyphosphate 5-phosphatase OCRL1	75–77
lpg2311	RidL	1,167	867–1167	PI3P	LCV	Binds the retromer subunit Vps29 and inhibits retrograde trafficking	78–81
lpg2464	SidM/DrrA	647	544–647	PI4P	LCV	AMPylates Rab1 and Rab35, activates Rab1	33, 82–85
lpg2510	SdcA	908	605–771	PI4P	LCV	E3 ubiquitin ligase, recruits ER to the LCV	32
lpg2511	SidC	917	609–776	PI4P	LCV	E3 ubiquitin ligase, recruits ER to the LCV	32, 43, 86–90
lpg2603	Lem28	434	326–434	PI4P	LCV	ND	70
lpw03701	LtpD	679	472–626	PI3P	LCV	Binds a phosphatase involved in PI production	91
lpp0356	LtpM	639	469–639	PI3P	LCV	Glycosyltransferase	92

[a]lpp0356 is found in *L. pneumophila* strain Paris, and lpw03701 is found in *L. pneumophila* strain 130b and not in *L. pneumophila* strain Philadelphia-1.
[b]PI-BD, phosphoinositide-binding domain; ND, not determined.
[c]PI3P, phosphatidylinositol 3-phosphate; PI4P, phosphatidylinositol 4-phosphate.
[d]LCV, *Legionella*-containing vacuole; AP, autophagosomes.

Phosphoinositides (PIs) are low-abundance phospholipids, composed of diacylglycerol (DAG) and phosphatidylinositol. The carbohydrate moiety of PI compounds can be phosphorylated at the positions 3′, 4′, and/or 5′, thus forming seven different PI lipids. Distinct PIs, together with other regulatory factors, define organelle identity, such as phosphatidylinositol 3-phosphate (PI3P), which is present mainly on early endosomes, and phosphatidylinositol 4-phosphate (PI4P), which is enriched at the Golgi complex (18, 19). In addition, PIs play a major role in the trafficking processes between organelles and are crucial regulators of eukaryotic signal transduction (18, 19). PIs mediate their functions in part through the binding of their head groups to cytosolic proteins or cytosolic domains of membrane proteins. Thus, they can regulate and/or recruit proteins to specific organelles. Typically, the binding of proteins to PIs involves electrostatic interactions with the negative charges of the phosphate(s) on the inositol ring. Specific PI-binding domains have been described in eukaryotic proteins (20–22), some of which specifically bind PI3P or PI4P. The FYVE domain (named after the four proteins harboring the domain: Fab1p, YOTB, Vac1p, and EEA1) is between 60 and 80 residues long, and its PI3P-binding site is formed by the positively charged amino acid residues in the conserved WxxD, (R/K)(R/K)HHCR, and RVC sequence (23–25). The Phox homology (PX) domain is generally between 110 and 140 residues long, and its consensus binding motif for PI3P binding is $R(Y/F)x_{23-30}Kx_{13-23}R$ (26, 27). The Pleckstrin homology (PH) domain is a widespread domain family harbored by a variety of proteins which bind different PIs, including PI4P; it is generally between 100 and 120 residues long and is highly homologous in terms of three-dimensional structure and secondary structure, and the interaction with PIs is mediated by lysine and arginine residues (28, 29).

Beside their important role in cellular organelle identity and signal transduction, PIs also play a major role during bacterial infection (30). Several *L. pneumophila* effectors, most of which contribute to the establishment of the LCV, were shown to bind specific PIs, usually PI3P or PI4P (31). The first effector which was reported to bind PI4P was SidC (32), which was followed by SidM, and other effectors that all bind specifically to PI4P (33). In addition, several effectors, most of which are localized to the LCV, were shown to bind PI3P, and two effectors were shown to bind both PI3P and PI4P (see Table 1 and references therein). Altogether, 14 *L. pneumophila* effectors were shown to bind specific PIs, but in most cases the domains required for PI binding were found not homologous to one another. Only three *L. pneumophila* effectors that bind PI4P (SidM, Lem4, and Lem28) have a C-terminal homologous domain (here named PI4P-M). The two homologous effectors SidC and SdcA bind PI4P via the same domain (here named PI4P-C),

which is not homologous to the PI4P-M domain. Other effectors that bind PI3P or both PI3P and PI4P are not homologous to one another, not even at their PI-binding domain. It should be noted that PI3P and PI4P are present on the LCV, and their temporal and spatial dynamics are involved in LCV formation. The *L. pneumophila* wild-type strain was found to acquire PI3P within a minute after uptake by *Dictyostelium discoideum* and to gradually lose this PI within 2 h. During these 2 h, PI4P steadily accumulated on the LCV of wild-type *L. pneumophila* and was maintained on it for at least 8 h (34). The temporal localization of these two PIs is likely to dictate the time postinfection in which specific effectors are localized to the LCV.

The *Legionella* genus includes more than 50 species, most of which are pathogenic, and they all contain the conserved Icm/Dot type IV secretion system (35, 36). In a previous study, we predicted thousands of effectors in 41 *Legionella* species, and this high number of effectors made it possible to identify conserved effector domains in these *Legionella* species (35). *Legionella* effector domains (LEDs) are conserved across effector orthologous groups, but they are not present in other known proteins. LEDs represent the building blocks of numerous *Legionella* effectors, yet their function is unknown. We were interested to determine whether some of these LEDs function as PI-binding domains. To this end, we looked for LEDs which have properties similar to known PI-binding domains in effectors. We found three such LEDs, which are present in a dozen *L. pneumophila* effectors and in more than 200 predicted effectors in the 41 *Legionella* species examined. Examination of these *L. pneumophila* effectors for PI binding revealed that they specifically bind PI3P, thus almost doubling the number of *L. pneumophila* effectors that bind PIs and establishing the importance of LEDs for discovering effector function.

RESULTS

Identification of common features of *L. pneumophila* effector PI-binding domains. Currently there are 14 *L. pneumophila* effectors which were shown to specifically bind PI3P and/or PI4P (Table 1 and references therein). In most cases the domains which bind the same PI (or different PIs) are not homologous, suggesting that this function was developed multiple times during evolution. Analysis of 12 *L. pneumophila* effectors for which the PI-binding domain was characterized to some extent (Table 1 and Fig. 1) revealed several common features of these nonhomologous PI3P or PI4P effector-binding domains: (i) most of these domains are located at the C-terminal ends of the effectors (all except in LidA), (ii) these domains are usually small and comprise about 100 amino acids (similar to the eukaryotic PI-binding domains), and (iii) most of the effectors harboring these PI-binding domains also harbor either catalytic or protein binding domains at their N-terminal regions (Fig. 1). We used these three features to discover novel PI-binding LEDs in *Legionella* effectors, as described below.

Identification of LEDs whose features resemble the features of known PI-binding domains. In a previous study, we identified a large number of effectors in the *Legionella* genus, and this information made it possible to identify and analyze conserved LEDs (35). This analysis revealed 99 LEDs, consisting of 53 well-characterized mostly eukaryotic domains which were previously described (9, 37) and 46 newly discovered conserved domains which share no homology with any known domains (35). We used the three features described above to examine the 46 novel LEDs in order to identify LEDs harboring the same properties. Our analysis revealed three such LEDs: LED006, LED027, and LED035. These three LEDs have the same characteristics: they are always located at the C-terminal ends of the effectors, they are small (about 100 amino acids [aa]), and in some effectors they are found together with catalytic or protein binding domains (Fig. 2). In addition, we compared the protein architectures (different domain combinations) of *Legionella* effectors which harbor these three novel LEDs to the characterized PI4P-binding domain present in SidM/DrrA and two other effectors (see the introduction). This comparison indicated that in some effectors the PI4P-M-binding domain and these three LEDs are found together with the same catalytic/protein binding domains (Fig. 2 and see Fig. S1 in the supplemental material). Impor-

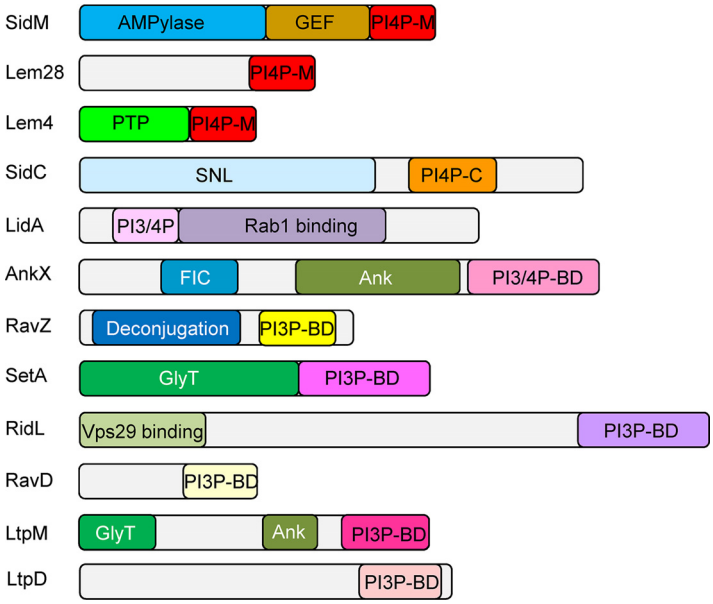

FIG 1 Domain architecture of *L. pneumophila* effectors that bind PIs. The known domains of each effector that contains a PI-binding domain are shown. The domains presented are as follows: PI4P-M, PI4P-binding domain of SidM; PI4P-C, PI4P-binding domain of SidC; PI3P-BD, PI3P-binding domain; PI3/4P-BD, PI3P and PI4P-binding domain; deconjugation, a domain related to cysteine proteases, required for Atg8 deconjugation reaction; AMPylase, AMPylation of Rab1; GEF, GTP-GDP exchange factor for Rab1; GlyT, glycosyltransferase; SNL, N-terminal ubiquitin ligase domain; FIC, filamentation induced by cAMP, used by AnkX for phosphocholination of Rab1; Ank, ankyrin repeat; PTP, protein tyrosine phosphatase; Vps29 binding, domain that binds the Vps29 retromer subunit. Domains with the same name and different colors perform the same function but are not homologous.

tantly, these three LEDs are highly prevalent in the 41 *Legionella* species examined (found in 230 effectors; see Table 2), and the architectures presented in Fig. 2 are only of representative effectors that contain additional known domains or LEDs. Most of the effectors containing these three predicted PI-binding LEDs do not contain additional LEDs or any known domains. This architecture is found in several *L. pneumophila* effectors and in numerous effectors in the *Legionella* genus (this architecture is presented by the uppermost effector shown for each LED in Fig. 2). The three LEDs identified have average size similar to the PI4P-M-binding domain and have the same distance from the end of the effector, as well as a similar degree of homology among the sequences of individual LEDs (Table 2). The similar characteristics found between the three predicted PI-binding LEDs and the validated PI4P-M-binding domain suggest that the newly identified LEDs might also bind PIs.

The three novel LEDs are found in 14 *L. pneumophila* effectors. We decided to focus our study on all of the *L. pneumophila* effectors in which these three novel LEDs were identified (Table 3). (i) LED006 was found in eight *L. pneumophila* effectors (CegC2, LegK1, Ceg22, LegC5, Lem9, LegC6, LepB, and lpg2327), one of which (LepB) was previously shown to be localized to the LCV (38), and its N-terminal domain specifically converts PI3P into PI3,4P (39), but there was no indication that it binds PIs at its C-terminal end. Two of the other effectors containing LED006 (LegK1 and LegC5) also contain catalytic domains, i.e., a serine/threonine kinase domain and a glycosyltransferase domain, respectively, but there was no information about their ability to bind PIs. (ii) LED027 was found in three *L. pneumophila* effectors (RavZ, Ceg19, and lpg1961), one of which (RavZ) was previously shown to bind PI3P and to be localized to autophagosomes (40). The PI3P-binding region of RavZ and the amino acids of LED027 overlap. The other two effectors harboring this LED were not studied before. (iii) LED035 was also found in three *L. pneumophila* effectors (RavB, Lem21, and MavH), none of which was studied prior to our study. During the course of this study, Kubori et al. characterized Lem21 (also named LotA) and found that it binds PI3P at the region which

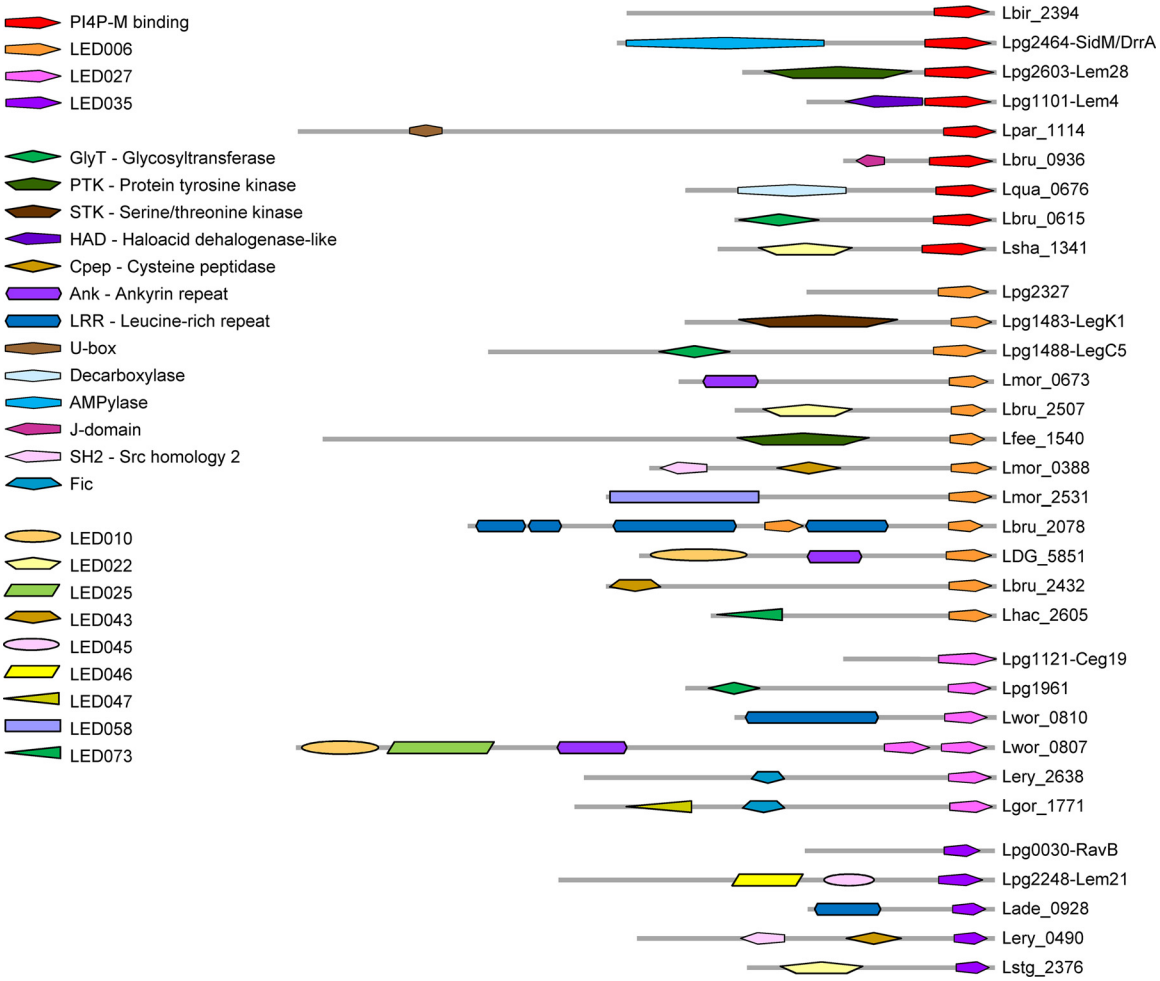

FIG 2 Architectures of effectors containing the PI4P-M-binding domain and the three predicted PI-binding LEDs. Each protein architecture containing the PI4P-M-binding domain, LED006, LED027, or LED035 is represented by a single effector. Several known domains, as well as unknown LEDs, were found in the same architecture with two or three of the domains known or predicted to bind PIs (see summary in Fig. S1). The novel LEDs were originally described elsewhere (35). For each of the analyzed C-terminal domains, the upper effector represents a large group of effectors containing the domain with no other known domain or LED. The effectors presented are indicated on the right by their locus tag or gene name.

covers the amino acids of LED035 (41). The 13 effectors that were not examined for PI binding prior to our study and contain one of these three novel LEDs were examined as described below.

LEDs from eleven *L. pneumophila* effectors bind PI3P *in vitro*. To determine whether the LEDs of these 13 effectors bind PIs *in vitro*, we generated N-terminal glutathione *S*-transferase (GST) fusions from all of these 13 effectors that included only the LED sequence region (Table 3). We were able to express and purify 12 of these fusions (all except CegC2) and assayed their binding to PIs and other lipids immobilized

TABLE 2 Properties of the PI4P-M binding domain and the predicted PI-binding LEDs

Domain	Avg size (aa)	Avg distance from C terminus (aa)	No. of LEOGs in the genus[a]	No. of effectors in *L. pneumophila*	No. of effectors in the genus[b]	Avg E value to the LED consensus
PI4P-M	99	10	12	3	36	4.8×10^{-8}
LED006	69	20	32	8	138	6.9×10^{-8}
LED027	82	48	11	3	51	3.7×10^{-8}
LED035	66	44	7	3	41	2.5×10^{-21}

[a]LEOGs, *Legionella* effector orthologous groups.
[b]The LEDs identified in the *L. pneumophila* validated effectors and the predicted effectors in the 41 *Legionella* species examined are described in supplementary Table 9 in reference 35.

TABLE 3 *L. pneumophila* effectors harboring the predicted PI-binding LEDs

LED	Locus tag	Name	Effector size (aa)	Position of LED (aa)	Other domain[a]	Known function(s)[b]	Reference(s)
LED006	lpg0126	CegC2	1,102	1027–1084			
	lpg1483	LegK1	529	450–522	STK	Activates NF-κB	93, 94
	lpg1484	Ceg22	269	189–260			
	lpg1488	LegC5	865	750–838	GlyT	Inhibits eEF1A	95, 96
	lpg1491	Lem9	413	298–386			
	lpg1588	LegC6	672	556–644			
	lpg2327		297	181–269			
	lpg2490	LepB	1,294	1210–1283		GAP for Rab1 on the LCV	38, 39, 97–99
LED027	lpg1121	Ceg19	256	146–250		Vesicle trafficking	73
	lpg1683	RavZ	502	329–423		Deconjugation of Atg8/LC3 on autophagosomes	40, 42, 72
	lpg1961		510	432–499	GlyT		
LED035	lpg0030	RavB	304	201–263			
	lpg2248	Lem21	744	622–702	LED45, 46	Deubiquitinase	41
	lpg2425	MavH	268	158–217			

[a]STK, serine/threonine kinase; GlyT, glycosyltransferase.
[b]GAP, GTase activating protein; LCV, *Legionella*-containing vacuole.

onto nitrocellulose membranes (Fig. 3A). Under these conditions, all seven *L. pneumophila* effectors harboring LED006 were found to specifically bind PI3P (Fig. 3B). One of the two *L. pneumophila* effectors harboring LED027 (Ceg19) also bound PI3P, and the other effector (lpg1961) did not bind any lipid (Fig. 3C) (RavZ, which harbors LED027, was previously reported to bind PI3P [42] and was therefore not examined). In addition, all the *L. pneumophila* effectors harboring LED035 were found to bind PI3P, and one of them (RavB) also bound PI3,5P and, to a much weaker extent, PI5P (Fig. 3D). These results validate that LED006, LED027, and LED035 are indeed PI-binding domains and showed that they all specifically bind PI3P. These LEDs are found in 14 *L. pneumophila*

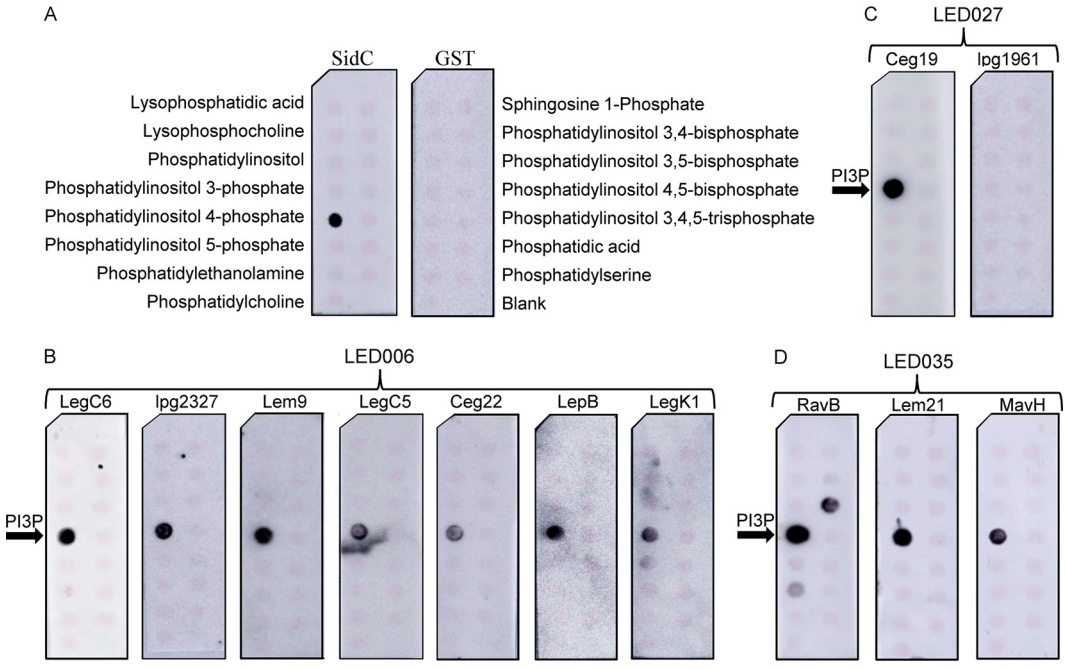

FIG 3 The three novel LEDs bind specifically to PI3P *in vitro*. Affinity-purified GST fusion proteins (150 pmol) of SidC and GST controls (A), LED006 from LegC6, lpg2327, Lem9, LegC5, Ceg22, LepB, or LegK1 (B), LED027 from Ceg19 or lpg1961 (C), and LED035 from RavB, Lem21, or MavH (D) were assayed for binding to different synthetic lipids (100 pmol) immobilized to nitrocellulose membranes by a protein-lipid overlay assay using an anti-GST antibody. The different lipids immobilized to the strip are indicated in panel A. PI3P, phosphatidylinositol 3-phosphate.

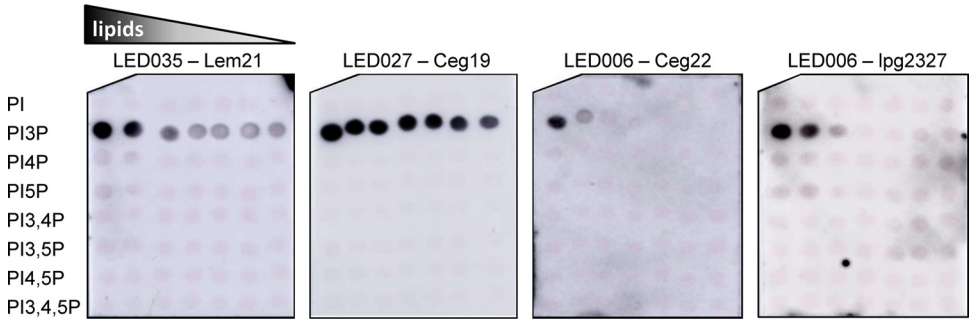

FIG 4 Three PI3P-binding LEDs bind PI3P *in vitro* with different affinities. The binding of the affinity-purified GST fusion proteins of several different LEDs—LED027 from Ceg19, LED035 from Lem21, and LED006 from Ceg22 or from lpg2327 (300 pmol)—to 2-fold serial dilutions of PIs (100 to 1.56 pmol) immobilized on nitrocellulose membranes was analyzed by a protein-lipid overlay assay using an anti-GST antibody.

effectors and in 230 effectors in the 41 *Legionella* species examined, all of which are thus predicted to bind PIs.

LED027 and LED035 bind PI3P more strongly than LED006. In order to compare whether the binding affinities of the three LEDs to PI3P are similar, we examined the binding of representative effectors containing LED006, LED027, and LED035 to PIs, using PI arrays onto which the lipids were spotted in 2-fold dilution series. Using these PI arrays, we found that LED027 and LED035 (from the effectors Ceg19 and Lem21, respectively) bind exclusively to PI3P with similarly high affinities (Fig. 4). In contrast, under the same conditions, LED006 from the two effectors Ceg22 and lpg2327 (see below for the difference of LED006 between these two effectors) were found to specifically bind PI3P, but with considerably lower affinity than LED027 and LED035 (Fig. 4). These results indicate that even though the examined LEDs have similar properties and bind the same PI, their PI3P-binding affinities are different.

Conserved tyrosine and lysine residues are essential for PI3P binding by LED027. LED027 is one of the two LEDs which were found to bind PI3P with high affinity. In order to identify critical amino acids in LED027 that are required for PI3P binding, we generated a protein multiple sequence alignment of LED027 from effectors and predicted effectors in the *Legionella* genus. Figure 5A depicts an alignment of LED027 from ten effectors from different orthologous groups (35), including the two *L. pneumophila* effectors containing LED027 that were found to bind PI3P (Fig. 3 and 4) (42). (lpg1961, which harbors LED027 but did not bind PIs, was excluded from the alignment.) Since PI3P is negatively charged and previous analyses indicated that positively charged amino acids, as well as polar and aromatic amino acids, are involved in PI binding (23, 26), we paid special attention to conserved positively charged amino acids, as well as to conserved polar and aromatic amino acids, found in the LED alignment. This analysis revealed two highly conserved amino acids: tyrosine and lysine (marked by arrows in Fig. 5A). To determine the importance of these amino acids for PI3P binding by Ceg19 (representing LED027), they were individually changed to alanines from Ceg19. GST fusions of these two mutated versions of Ceg19 were purified and examined for PI binding using the PI arrays (Fig. 5B). Both of these mutants completely lost their ability to bind PI3P (with the same amounts of GST-tagged proteins used in both analyses; Fig. 5B and Fig. S2). These results validate the importance of the conserved tyrosine and lysine of Ceg19 for PI3P binding. The same amino acids were also found to be important for PI binding by eukaryotic PI-binding domains (see the introduction).

Conserved adjacent arginine-histidine and histidine-serine are required for LED035 PI3P binding. Like LED027, LED035 was also found to bind PI3P with high affinity (Fig. 4). Multiple sequence alignment was performed using LED035 from effectors from different ortholog groups, as described above. This sequence analysis revealed five conserved amino acids: a single tyrosine, an adjacent arginine-histidine,

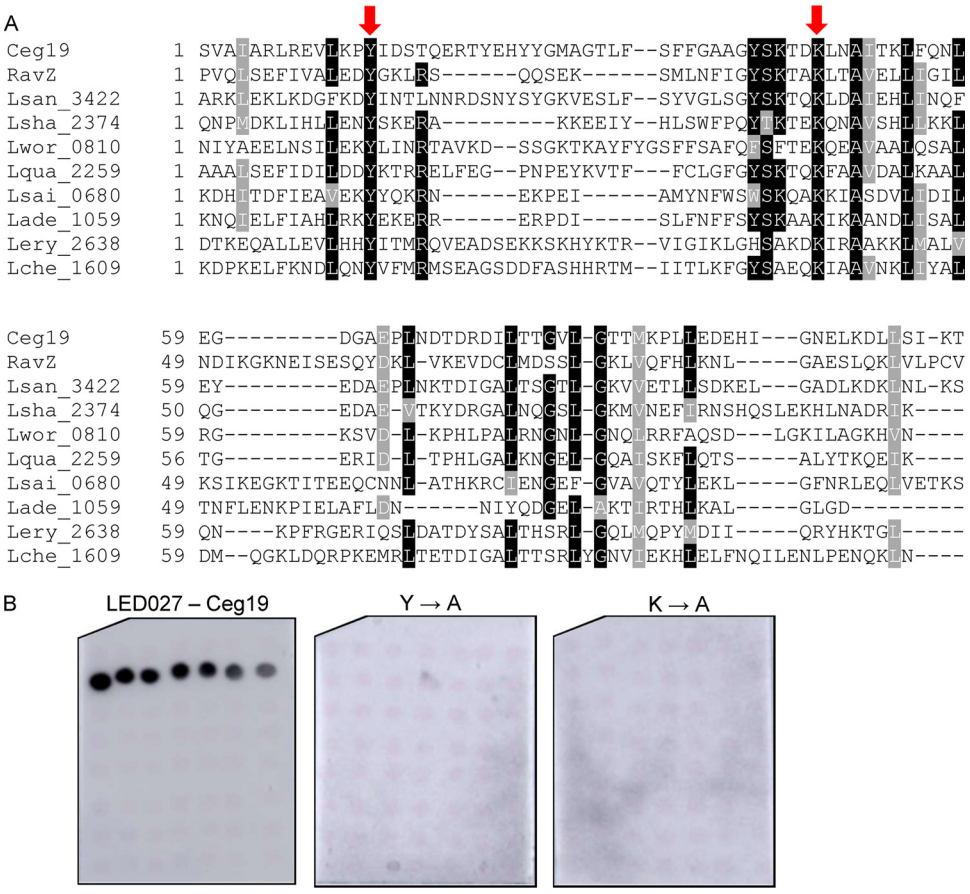

FIG 5 Identification of amino acids critical for PI3P binding by Ceg19. (A) Multiple sequence alignment of ten effectors or predicted effectors harboring LED027. The conserved amino acids tyrosine and lysine that were mutated are marked with red arrows. (B) The binding of the affinity-purified GST fusion proteins of the wild-type effector Ceg19 or the two mutants with mutations in the tyrosine and lysine residues (Y160A and K193A) of the same effector (300 pmol) to 2-fold serial dilutions of PIs (100 to 1.56 pmol) immobilized on nitrocellulose membranes was analyzed by a protein-lipid overlay assay using an anti-GST antibody.

and an adjacent histidine-serine (marked by arrows in Fig. 6A). Comparing the sequence alignments of LED027 and LED035 (Fig. 5A and 6A) revealed a conserved tyrosine in both LEDs and, at a similar distance, like the essential lysine in LED027, conserved adjacent arginine-histidine residues were identified in LED035. To determine the importance of these conserved amino acids for PI3P binding of LED035, they were mutated in RavB. Three mutants were constructed in which the single tyrosine or each of the adjacent amino acid pairs were changed to alanines. GST-fusions containing the three mutated RavB were purified and examined for PI binding. This analysis indicated that the mutants with mutations in the adjacent arginine-histidine or the adjacent histidine-serine completely lost the ability to bind PI3P (with the same amounts of GST fusion proteins used in both analyses; Fig. 6B and Fig. S2). However, surprisingly, the mutation in the conserved tyrosine had no effect on PI3P binding (Fig. 6B), even though in Ceg19, which also binds PI3P with high affinity, a conserved tyrosine residue was found to be important for PI3P binding (Fig. 5). These results demonstrate the importance of the two adjacent and conserved amino acids (arginine-histidine and histidine-serine) for the PI3P binding of RavB and show that different LEDs which bind PI3P with high affinity use different amino acids to achieve the same function.

A single lysine or adjacent histidine-arginine residues located at a similar position are required for LED006 PI3P binding. Of the LEDs we analyzed in this study, LED006 is the most prevalent, both in *Legionella* predicted effectors and specifically in *L. pneumophila* validated effectors. However, the binding of LED006 to PI3P is

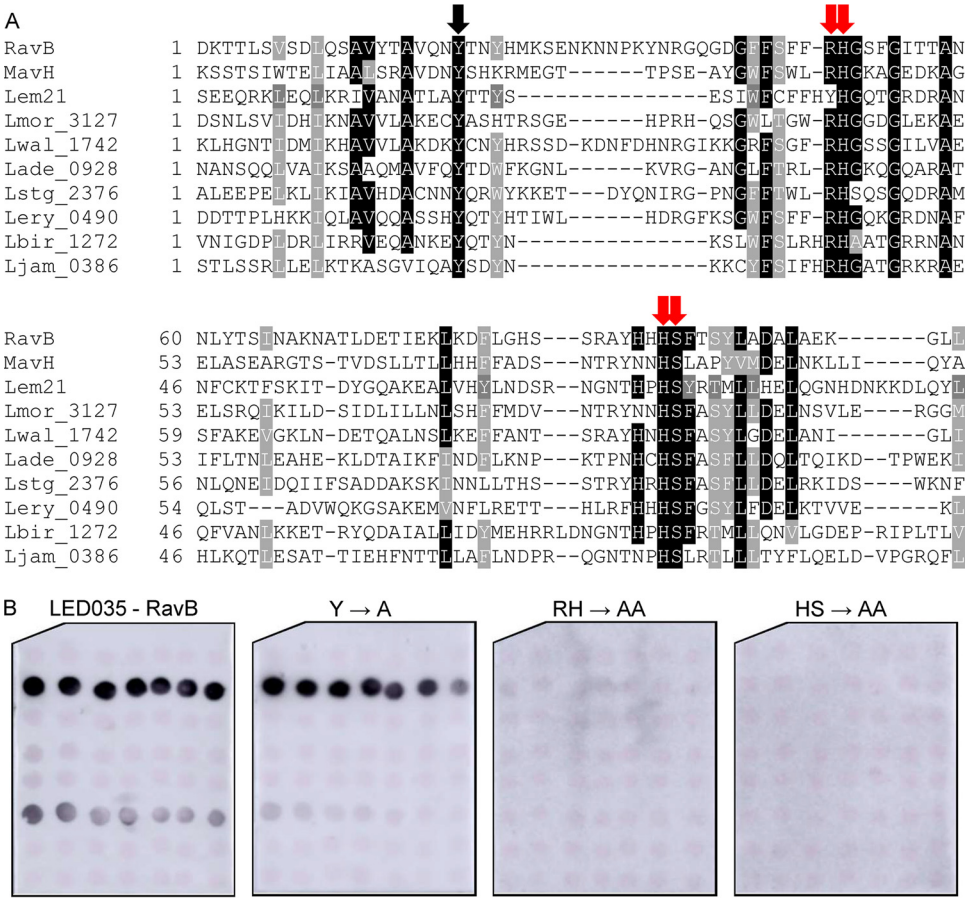

FIG 6 Identification of amino acids critical for PI3P binding by RavB. (A) Multiple sequence alignment of ten effectors or predicted effectors harboring LED035. The conserved amino acid tyrosine and the adjacent arginine-histidine or histidine-serine that were mutated are marked with red arrows if the mutation affected the PI3P binding or with black arrows if the mutation did not affect PI3P binding (see panel B). (B) Binding of affinity-purified GST fusion proteins of the wild-type effector RavB or the three mutants with mutations in the single tyrosine, the adjacent arginine-histidine, and the adjacent histidine-serine (Y185A, RH213-214AA, and HS257-258AA) of the same effector (300 pmol) to 2-fold serial dilutions of PIs (100 to 1.56 pmol) immobilized on nitrocellulose membranes was analyzed by a protein-lipid overlay assay using an anti-GST antibody.

weaker than that of the other PI3P-binding LEDs we identified. To test whether LED006 harbors amino acids essential for PI3P binding similar to LED027 and LED035, a multiple sequence alignment was constructed for the LED region of all the effectors harboring LED006 (Fig. 7A). This analysis revealed several conserved amino acids. Following the importance of positively charged amino acids and aromatic amino acids that we demonstrated for PI3P binding, we decided to examine three pairs of amino acids: phenylalanine-lysine, histidine-arginine, and two adjacent phenylalanine residues (marked by arrows in Fig. 7A). To determine the importance of these amino acids for PI3P binding, these amino acids were changed to alanines in Ceg22. GST fusions of the three mutants were purified, and their examination revealed that only the adjacent histidine-arginine are critical for PI3P binding and that the other mutations did not affect PI3P binding (Fig. 7B and Fig. S2).

When analyzing the LED006 sequence alignment generated for the seven *L. pneumophila* effectors that were found to bind PI3P (Fig. S3), we realized that four of them (LegC6, Lem9, LegC5, and lpg2327) lack the histidine-arginine pair that was found to be critical for PI3P binding by Ceg22. These adjacent histidine-arginine residues were absent in 27 of the 138 effectors and predicted effectors harboring LED006 in the 41 *Legionella* species examined. A multiple sequence alignment of *Legionella* validated and predicted effectors lacking the histidine-arginine residues revealed that either a

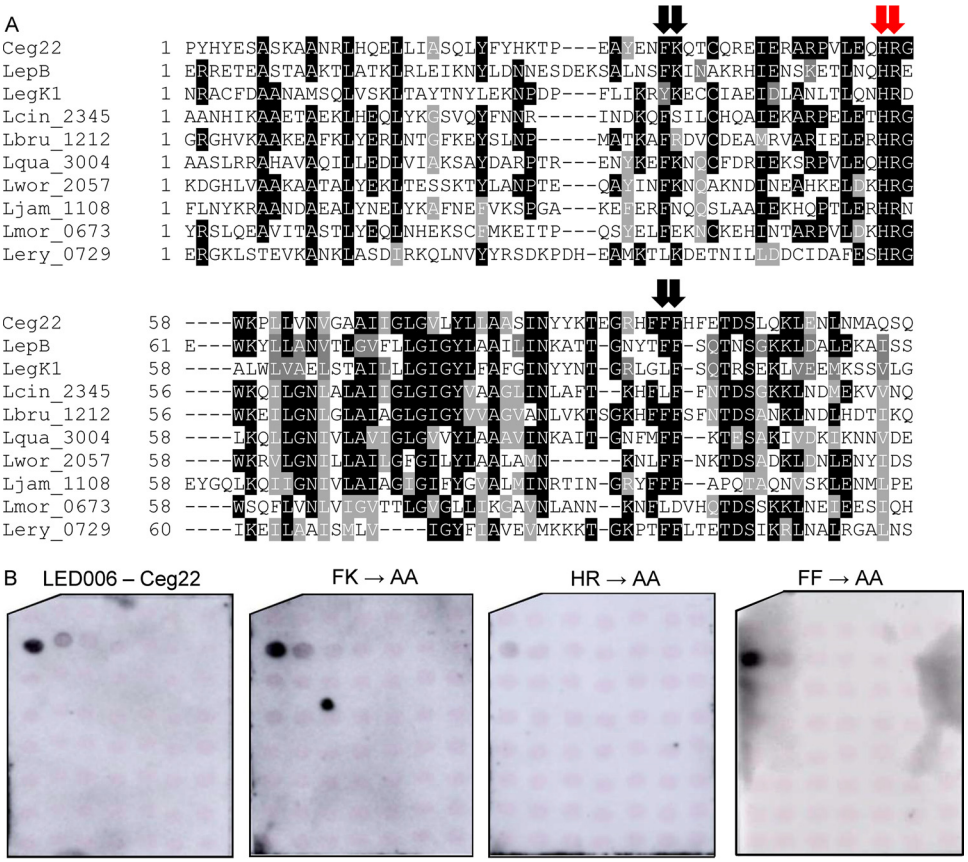

FIG 7 Identification of amino acids critical for PI3P binding by Ceg22. (A) Multiple sequence alignment of ten effectors or predicted effectors harboring LED006. The conserved adjacent phenylalanine-lysine, histidine-arginine, and the two phenylalanine residues that were mutated are marked with red arrows if the mutation affected PI3P binding or with black arrows if the mutation did not affect PI3P binding (see panel B). (B) Binding of affinity-purified GST fusion proteins of the wild-type effector Ceg22 or the three mutants with mutations in the adjacent phenylalanine-lysine, the adjacent histidine-arginine, and the adjacent phenylalanine residues (FK189-190AA, HR207-208AA, and FF245-246AA) of the same effector (300 pmol) to 2-fold serial dilutions of PIs (100 to 1.56 pmol) immobilized on nitrocellulose membranes was analyzed by a protein-lipid overlay assay using an anti-GST antibody.

lysine or an arginine residue is present in a position similar to the missing histidine-arginine residues (marked by a red arrow in Fig. 8A and boxed in blue in Fig. S3). In addition, 12 amino acids downstream from this position, another conserved lysine residue was identified (marked by single black arrow in Fig. 8A). To determine the importance of these amino acids in the PI3P binding of LED006, these two single lysine residues, the adjacent phenylalanine-lysine residues, and the two adjacent phenylalanine residues (the latter two were also mutated in Ceg22) were changed to alanines in lpg2327. Examination of the PI binding of these mutants revealed that only the lysine residue located at a position similar to the conserved histidine-arginine found in the majority of the effectors harboring LED006 was critical for PI3P binding, and the other mutants constructed had no effect (or a very mild effect in the mutant with a mutation in the two adjacent phenylalanine residues) on PI3P binding (Fig. 8B and Fig. S2). Combined, these results indicate the importance of either adjacent histidine-arginine residues or a lysine residue at a specific position for LED006 PI3P binding (see below).

Comparison of the *Legionella* PI3P-binding domains identified. The analysis of the three newly discovered PI3P-binding LEDs presented here makes it possible to compare the domains and amino acids required for PI binding. A comparison that included the three novel LEDs, as well as the PI4P-M-binding domain (Fig. 9), led us to two main findings. The first is that the amino acids essential for PI binding by *Legionella*

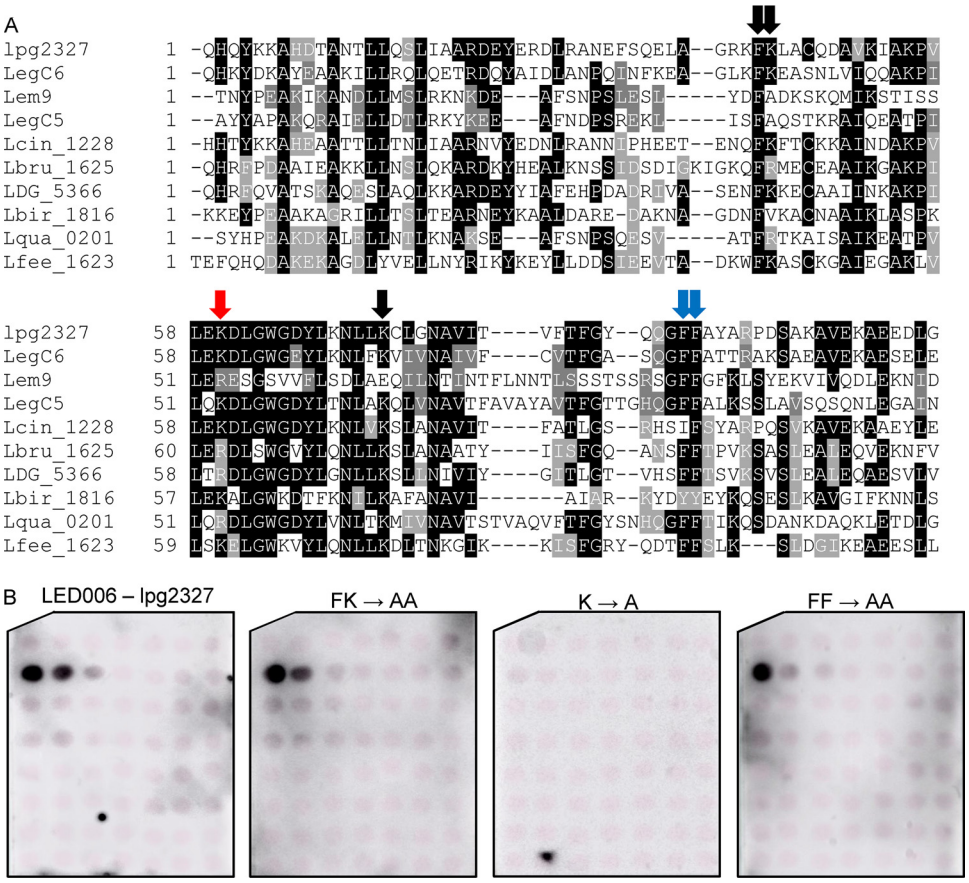

FIG 8 Identification of an amino acid critical for PI3P binding present in a subset of effectors containing LED006. (A) Multiple sequence alignment of ten effectors or predicted effectors harboring LED006. The conserved adjacent phenylalanine-lysine and the two adjacent phenylalanine residues, as well as the single lysine or lysine/arginine that were mutated, are marked with red arrows if the mutation affected PI3P binding, with a blue arrow if the mutation had a weak effect on PI3P binding, or with black arrows if the mutation did not affect PI3P binding (see panel B). (B) Binding of affinity-purified GST fusion proteins of the wild-type effector lpg2327 or the three mutants with mutations in the adjacent phenylalanine-lysine, the single lysine, or the two adjacent phenylalanine residues (FK220-221AA, K237A, and FF268-269AA) of the same effector (300 pmol) to 2-fold serial dilutions of PIs (100 to 1.56 pmol) immobilized on nitrocellulose membranes was analyzed by a protein-lipid overlay assay using an anti-GST antibody.

effectors are usually positive or aromatic amino acids. This observation also fits the known information about the eukaryotic PX domain, which binds PI3P and harbors the consensus $R(Y/F)x_{23-30}Kx_{13-23}R$ sequence (26, 27); the eukaryotic FYVE domain, which binds PI3P and harbors the consensus WxxD, (R/K)(R/K)HHCR, and RVC sequence (23–25); and the PI4P-C domain, present in the *Legionella* homologous effectors SidC and SdcA, in which arginine, tryptophan, and phenylalanine residues are required for PI4P binding (43). Our second finding is that the residues essential for PI binding in the newly identified PI3P-binding LEDs and the PI4P-M-binding domain are located at or close to the end of an alpha-helix or a beta-strand structure (Fig. 9). The same was found in the PI4P-C domain (43) and in eukaryotic PI-binding domains (22). There was not even a single case of an essential PI-binding residue located in the middle of an alpha-helix or a beta-strand. There were three cases where we mutated amino acids located in the middle of a secondary structure (phenylalanine and lysine in Ceg22 and lpg2327 and a lysine residue in lpg2327, marked in black in Fig. 9), but their mutagenesis did not affect the PI3P binding of LED006.

The analysis performed with the three newly discovered PI3P-binding LEDs uncovered similarities and differences in their PI-binding properties and demonstrated that the study of LEDs can reveal new information regarding the function mediated by groups of effectors containing the same LEDs.

FIG 9 Comparison of the regions of the LEDs that were found to contain amino acids critical for PI binding. The domains of effectors that were found to bind PI3P or PI4P are presented. For each of the three newly identified PI3P-binding LEDs, as well as for the PI4P-M-binding domain, the amino acid sequence of two effectors is presented. The sequence of the upper effector representing each LED mutagenized and the amino acids required for PI3P binding identified in it are presented. The predicted secondary structure (red cylinder, alpha-helix; green arrow, beta-strand [according to JPred]) for each LED is presented between the pair of sequences. In the upper sequences, amino acids in red and yellow were found critical for PI binding, amino acids marked in boldface were mutagenized and found not to be required for PI binding, and amino acids marked in blue and yellow had a small effect on PI3P binding. In the lower sequences, amino acids located at the same position as the ones mutated in the upper sequences are marked in boldface.

DISCUSSION

Previous studies have shown that the *Legionella* genus contains thousands of effectors (35, 36). These effectors are expected to function inside host cells during infection and to modulate host cell processes to the benefit of the bacteria. One of the biggest challenges in the *Legionella* pathogenesis field is to uncover the functions of these effectors in order to discover all the pathways and processes modulated by these human pathogens during infection. Since the number of effectors is so large, novel approaches are required to uncover effector functions; one such approach was presented here and it involves the study of LEDs (35). About half of the LEDs are uncharacterized protein domains found in various orthologous groups of *Legionella* effectors. Deciphering the function of these basic effector building blocks will allow us to better understand the effector domain architecture and the function mediated by multiple effectors. Using several properties of known *Legionella* effector PI-binding domains, we identified here three LEDs that specifically bind PI3P. Our results are a proof of concept that effectors can be studied by characterizing their LEDs. The finding that 12 of the 13 *L. pneumophila* effectors harboring LED006, LED027, or LED035 that were examined here were found to bind PI3P suggests that the majority of the 230 *Legionella* effectors (Table 2) harboring these LEDs likely bind PI3P.

It was previously demonstrated, using several *L. pneumophila* effectors, that PI3P- and PI4P-binding domains are involved in the localization of effectors in the host cell, in most cases to the LCV (Table 1 and references therein). The finding that 11 *L. pneumophila* effectors containing LED006, LED027, or LED035 bind PI3P considerably expands the repertoire of catalytic domains (such as serine/threonine kinase and cysteine peptidase) and protein binding domains (such as leucine-rich repeat) that might be recruited to the LCV, since they are found together with these three LEDs as part of the same effector (Fig. 2). In addition, our findings can also direct the study of the function of additional LEDs with unknown function. According to our analysis, there is only a single PI-binding LED in each effector. Therefore, LEDs of unknown function that are found on the same effector, together with a PI-binding LED, are most likely

catalytic or protein-protein binding LEDs (see the list in Fig. 2). For example, LED022 (present in 58 effectors in the 41 *Legionella* species examined) is found on the same effectors together with LED006, LED035, or the PI4P-M-binding domain and always at the N terminus (Fig. 2 and Fig. S1). Analysis of LED022, which is on average 150 amino acids long, revealed several highly conserved amino acids, including four adjacent residues (data not shown). These amino acids might serve as part of a catalytic core of this LED, and additional study of this LED might lead to the identification of its function in connection to the PI-binding LEDs associated with it in several effectors.

Several eukaryotic domains were reported to specifically bind PIs in numerous eukaryotic proteins (20, 21). Comparison of these domains to the *Legionella* PI-binding domains revealed one clear difference. The *Legionella* PI-binding domains are almost exclusively located at the effector's C-terminal end (in all the effectors examined beside LidA). However, the eukaryotic PX domain which binds PI3P and the PH domain which binds PI4P were shown to be located at either the N-terminal or the C-terminal end of the different proteins in which they were found (26, 27). This difference might be related to the fact that the translocation signal of the Icm/Dot effectors is located at the ~25 C-terminal amino acids of the effectors (44–47). The C-terminal part of the effectors probably enters the host cells before its N-terminal part; thus, the location of the effectors' PI-binding domains at the C-terminal end (right next to the translocation signal) might facilitate the localization of these effectors to the LCV even before the entire effector has entered the host cell.

It is well established that *Legionella* effectors harbor domains typically found in eukaryotic proteins, such as ankyrin, F-box, and U-box domains and more (36, 37, 48, 49). The analysis performed here indicates that the three novel PI3P-binding domains identified, even though not homologous to eukaryotic PI-binding domains, share the same amino acids critical for PI binding (22). This observation suggests either convergent evolution, i.e., bacteria and eukaryotes independently evolved similar mechanisms for PI binding, or that these domains represent an ancient acquisition from eukaryotes that was followed by many changes, leading to lack of homology, except in residues critical for PI binding.

The LEDs, three of which were studied here, are present in numerous effectors and share relatively low degree of homology to each other. However, as we showed here, LEDs present in different effectors can perform the same function. Thus, the study of LEDs, which are the building blocks of effectors, has the potential to uncover functions mediated by numerous effectors, as well as to uncover the way by which a combination of LEDs present in the same effector contribute to the overall functions mediated by effectors during infection.

MATERIALS AND METHODS

Bacterial strains and media. The *L. pneumophila* wild-type strain used in this work was JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* Philadelphia-1, which is a wild-type strain in terms of intracellular growth (50). The *Escherichia coli* strains used in this work were MC1022 (51) and BL21(DE3) (52). Bacterial media, plates, and antibiotics were previously described (53).

Plasmid construction. N-terminal GST fusions were constructed for LED006 from eight effectors (CegC2, LegK1, Ceg22, LegC5, Lem9, LegC6, LepB, and lpg2327), for LED027 from two effectors (Ceg19 and lpg1961), and for LED035 from three effectors (RavB, Lem21, and MavH) by amplification of their coding regions and cloning into the pGEX-5x3 vector (Amersham). The resulting plasmids are listed in Data Set S1 in the supplemental material, and the primers used for the cloning are listed in Data Set S2 in the supplemental material.

To construct GST fusions containing site-directed mutations in amino acids predicted to be involved in PI3P binding of the effectors examined, site-directed mutagenesis was performed by the PCR overlap extension approach (54), in a way similar to that described previously (55). The following amino acids were mutated: Ceg22 (FK-189-190, HR-207-208, and FF-245-246), lpg2327 (FK-220-221, K-237, K-250, and FF-268-269), Ceg19 (Y-160 and K-193), and RavB (Y-185, RH-213-214, and HS-257-258). All of the single and double mutants constructed contained changes to a single or double alanine residues, respectively. The primers used for the mutagenesis are listed in Data Set S2 in the supplemental material, and the plasmids resulting from the site-directed mutagenesis are listed in Data Set S1.

Protein purification. BL21(DE3) strains containing the GST fusion protein encoding plasmids were lysed using B-PER (Thermo Fisher) or sonication. For sonication, the pellet was resuspended in 5 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.1% Triton X-100, and 1 mg/ml lysozyme, complete

mini-protease inhibitor [Roche]; pH 8), and the cells were then disrupted by sonication in 10-s on/off intervals. For lysis using the B-PER reagent, pellets were resuspended in 5 ml of B-PER reagent supplemented with lysozyme, DNase I, and complete mini-EDTA-free protease inhibitor (Roche) in the concentrations suggested by the manufacturer, and the lysate was then gently mixed for 15 min. For both lysis methods, the soluble fraction was collected by centrifugation at 10,000 × *g* for 10 min, 0.5 ml of reduced glutathione resin (GE Healthcare) was added, and the mixture was gently shaken at room temperature for 60 min. After incubation, the resin was washed three times with 10 ml of phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2HPO_4 [pH 7.3]). The proteins were eluted using 0.5 mM Tris buffer (pH 8.0) containing 10 mM reduced glutathione.

Lipid overlay assay. A lipid overlay assay was performed as previously described (56), with few modifications. In brief, the lipid strips or PI arrays (Echelon Biosciences) were blocked for 1 h in TBST (50 mM Tris, 3 mM KCl, 137 mM NaCl [pH 7.5], 0.1% Tween 20) containing 5% skim milk at room temperature and then incubated overnight with the GST fusion protein of choice (150 pmol for the lipid strips and 300 pmol for the PI arrays) at 4°C. On the following day, the membrane was incubated for 2 h at room temperature in TBST containing 5% skim milk and a goat anti-GST antibody (Amersham) diluted 1:2,000. The membrane was then incubated with for 1 h at room temperature in TBST containing 5% skim milk and a horseradish peroxidase-conjugated donkey anti-goat antibody (Jackson Immunoresearch Laboratories, Inc.) diluted 1:10,000. Signal was detected using an Imager 600 (GE Healthcare). Western blot analysis was performed in a similar way.

Bioinformatic analyses. Protein multiple sequence alignments were performed using the MAFFT program (57) and visualized using BoxShade. Protein secondary structure predictions were performed using JPred (58).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00153-19.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.01 MB.

ACKNOWLEDGMENTS

We thank Marika Linsky and David Burstein for reading the manuscript.

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