Orchestrated delivery of *Legionella* effectors by the Icm/Dot secretion system

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Abstract

Legionella pneumophila uses the Icm/Dot Type IV secretion system (T4SS) to translocate a record number (300) of bacterial effectors into the host cell. Despite recent breakthrough progress in determining the structure and the localization of the secretion machinery, it is still a challenge to understand how the delivery of so many effectors is organized to avoid bottleneck effect and to allow effective manipulation of the host cell by *L. pneumophila.* Here, we demonstrate that secretion of effectors is ordered and so precisely set up that it lines-up with the delivery timing required for the function of the effectors in the cell. We observe notably that the secretion order of 4 effectors targeting Rab1 is fully consistent with the sequence of their actions on Rab1. Importantly, we show that the timed delivery of an effector is not dependent on its concentration, nor on its picking-up by chaperone proteins. Conversely, this control involves c-di-GMP signaling, as a c-di-GMP synthesizing enzyme, namely the diguanylate cyclase Lpl0780/Lpp0809, significantly contributes to accurate triggering of effector secretion via a post-translational control of the T4SS machinery at the bacterial pole.

Significance

Type 3, 4 and 6 secretion systems are multiprotein complex known to be crucial for infectious cycle of many bacterial pathogens. Despite considerable progress on several fronts in structure-function analysis of these systems, one of the blackest boxes in our understanding is the signal that triggers the activation of effectors transfer. This is particularly true for the Icm/Dot T4SS in *L. pneumophila* that deals with the translocation of a record number of 300 effectors. We demonstrate that Icm/Dot secretion is timely fine-tuned and most importantly, that the complex orchestration of so many effector actions relies at least in part on the defined timing of their translocation into the host cell. Also, we highlight for the first time a post-translational control of a T4SS by c-diGMP signaling.

Introduction

Legionella pneumophila is the causative agent of the severe pneumonia known as Legionnaires' disease or legionellosis. Pathogenic strains of *Legionella* emerge in the environment after intracellular multiplication in amoeba. Bacteria are disseminated by water aerosols and when inhaled into lungs, engulfed by alveolar macrophages. Within amoeba and human macrophages, *L. pneumophila* evades endocytic degradation and triggers the biogenesis of a Legionella-containing vacuole (LCV), a rough endoplasmic reticulum-like compartment permissive for its intracellular multiplication (1).

Crucial for hijacking host-cell vesicles trafficking necessary for LCV biogenesis, and subsequently for intracellular multiplication of *L. pneumophila,* is the Type 4 Secretion System (T4SS) Icm/Dot (for Intracellular multiplication and Defect in organelle trafficking) (2, 3). The Icm/Dot system is a complex machinery located at the bacterial pole (4-6) and composed of 27 proteins involved in (i) a multiprotein apparatus for secretion (7), (ii) a coupling protein complex (DotL/IcmO; DotM/IcmP; DotN/IcmJ) (8, 9) and (iii) chaperone proteins that associate with the coupling protein complex and are involved in some specific substrate recognition for presentation to the translocon (IcmS; IcmW; LvgA) (10-12). This machinery translocates an exceptionally large repertoire of effectors, over 300 proteins, into the host-cell cytosol (13).

Despite major progress on the Icm/Dot structure thanks to electron cryotomography technology (5, 14, 15), much still needs to understand the functioning of the machinery, in particular regarding the secretion control of so many effectors. The triggering of effector translocation is dependent upon phagocytosis and requires the active participation of the host cells (16). However, it could be assumed that all the 300 effectors are not simultaneously translocated into the host cytosol. Despite some subtle differential expression patterns of the Icm/Dot encoding genes, expression of most of them increases continuously during intracellular growth of *L. pneumophila*, thus resulting in an available Icm/Dot apparatus when the bacteria are released in the extracellular medium for a second round of infection. Consistently, most of the Icm/Dot substrates encoding genes are mainly upregulated during the transmissive phase (17). Besides, inhibitors of protein synthesis are not effective at inhibiting Icm/Dot-dependent effector translocation (16). Thus, activity of the Icm/Dot system does not rely on *de novo* synthesis of structural Icm/Dot components or effector proteins.

A critical component of the translocated substrates that allow their Icm/Dot recognition is a carboxyl-terminal signal of less than 30 amino acids with both short polar amino acids and a glutamate-rich region (E-box) (18, 19). However, *Legionella* likely employs multiple sophisticated molecular mechanisms to regulate the export of some effectors, as reported for SidJ effector (20). The secretion of this Icm/Dot substrate is mediated by dual signal sequences that include a conventional C-terminal domain needed for the secretion at early points of infection, and an internal motif efficient at later time points (20). Moreover, some (such as SidJ) but not all Icm/Dot effectors seem to be picked-up by the IcmS/W chaperone proteins (12, 21).

Here, we sought to gain information on the delivery timing of Icm/Dot effectors during infection. In this purpose, we developed a kinetic translocation assay, which enables a fine monitoring of the Icm/Dot substrates translocation during a large time frame. We demonstrate that the Icm/Dot substrates translocation is fine-tuned during infection, independently of effectors synthesis, and in addition to the phagocytosis-dependent triggering of the overall translocation. In particular, we highlight various translocation patterns depending on effector, some of them accumulating into the host cells while others being only transiently detected. We also observe that translocation of some effectors occurs right after the contact with the cell while others are secreted as late as 2 hours post-infection. Finally, we underline that the sequential action of effectors most likely relies on a fine-tuned control of their ordered translocation and that c-di-GMP signaling contributes to this orchestrated secretion.

Results

Kinetic assay of effector translocation reveals distinctive and effector-specific profiles. We sought to find out the temporal activity of the Icm/Dot system in the initial phase of monocyte infection during which the translocation of effectors determines the fate of the bacterium. In order to directly compare the translocation dynamics of multiple effectors, we resorted to use the β-lactamase translocation reporter system (22) that was been successfully used to monitor T3SS and T4SS effectors translocation of various bacteria. In addition, the reporter system proved effective in the identification of about 300 Icm/Dot substrates (13). Typically, an effector protein fused on its N-terminus to the TEM-1 β-lactamase is detected in host cells by the cleavage of the β-lactam ring of CCF4, a fluorescent substrate of TEM-1 that accumulates in eukaryotic cells. In typical translocation assay CCF4 is added 1h post-infection to quantify the concentration of the cleaved/intact β-lactamase substrate (emission ratio 460/530 nm). This method provides a quantitative measure of the effector fusion translocated into the host cell, but it is limited to a single time point. A live kinetic assay using CCF4-preloaded cells provided a more dynamic image of effector translocation, but the observable timeframe was limited to less than 90 minutes as a consequence of CCF4 leakage (16). Here, we developed a multiple end-point assay to follow the level of translocated effector along an extended time frame. We took advantage that protonophores, such as carbonyl cyanide m-chlorophenyl hydrasone (CCCP), completely inhibits the Icm/Dot T4SS activity (16), allowing us to freeze translocation at different time points before addition of CCF4. To test this method, a TEM1-LepA protein expressing fusion was introduced in *L. pneumophila* Lens and used to infect U937-derived phagocytes at a MOI of 20. CCCP (10 μM) was then added at different time points from 0 to 180 min post-infection (Fig. 1A).

Consistent with previous observations, we found that the level of translocated LepA steadily increases during the first hour to reach a plateau (16). However, and unexpectedly, a sudden burst of LepA levels is observed at a later time point (~100 min.) before returning to lower and steady levels (Fig. 1A). No translocation was detected when cells were infected by the isogenic mutant strain deleted of the *dotA* gene or when TEM-1 was fused to the housekeeping protein Enoyl-acyl CoA reductase FabI (Fig. 1A). We then used this method to analyse the profiles of translocated effectors for LegK4, SidJ, SidM and SidC. Despite the fact that these effectors are constitutively expressed at similar levels (Fig. S1), none showed profiles similar to that of LepA and each effector showed distinct, and even opposing, profiles (Fig. 1B). For instance, LegK4 levels are maximal at the earliest time point, confirming that Icm/Dot translocation could occur immediately after the host-cell contact, then rapidly decrease to background levels 1h post-infection. SidJ levels slowly but steadily increase over time while SidM levels sharply increase at 90 minutes to then reach a plateau that likely represents a CCF4 substrate-limiting step. Reminiscent of the burst observed for LepA levels at ~100 minutes, SidC levels also showed a burst at 35 minutes before returning to background levels (Fig. 1B).

Altogether, translocated effector levels can be accessed over the course of several hours and with a temporal resolution that was not previously available. Importantly, the new data draw an unexpected complex picture of translocation profiles characterized by specific timing of increasing levels, which can be either transient or stable.

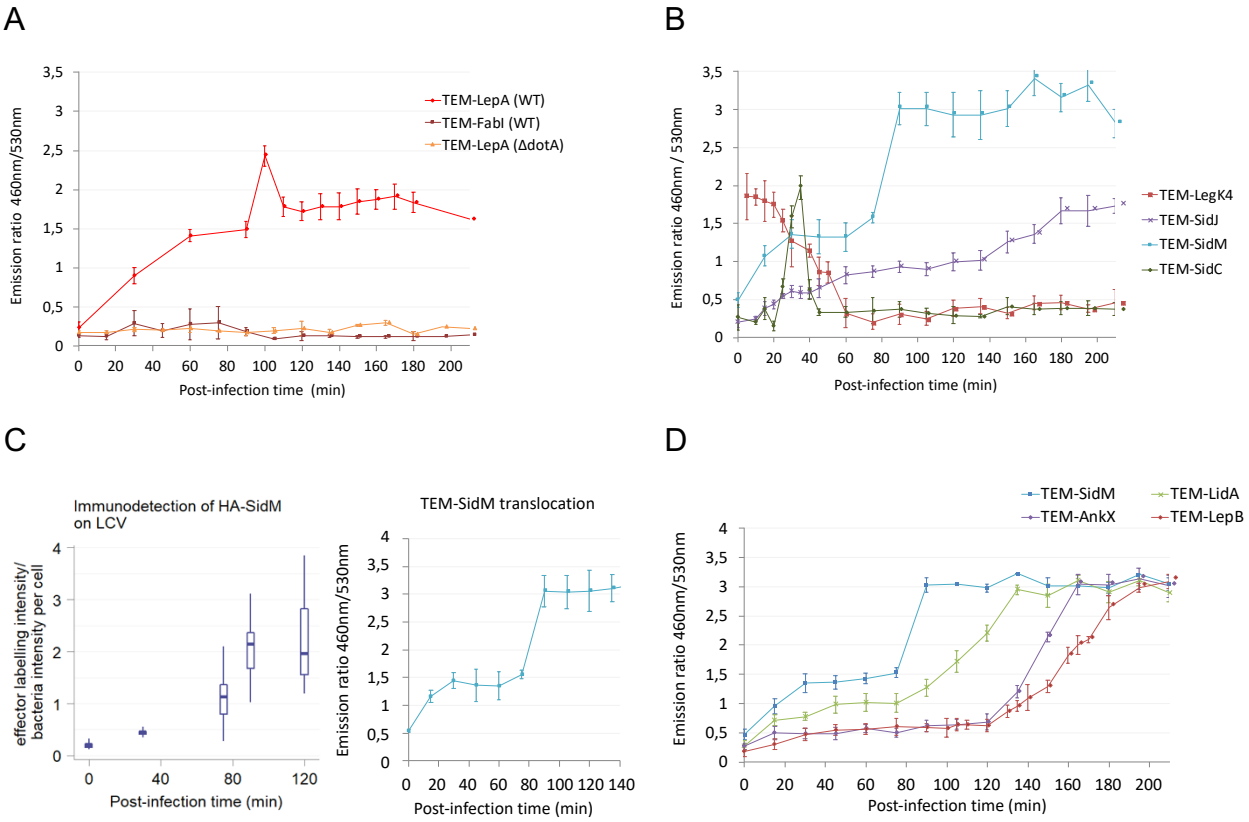

Fig. 1. Secretion kinetics can adopt different profiles depending on tested TEM-effector fusions and are consistent with their biological functions. *(A)* Secretion kinetics assays of TEM-LepA fusion protein. U937 cells were infected with wild-type (WT) and *ΔdotA* Lens strains harboring a TEM-LepA or a TEM-FabI expression plasmid (MOI 20). *(B)* Secretion kinetic of TEM-SidC, TEM-SidM, TEM-SidJ, TEM-LeB and TEM-LegK4 in the WT Lens strain. *(C)* Correlation between secretion kinetic of TEM-SidM and the immunodetection of HA-SidM on LCV. *Left panel*, Quantitative analysis of HA-SidM presence on LCV at 0, 30, 75, 90, and 120 min post-infection of U937 cells infected with HA-SidM and mCherry producing *L. pneumophila* (MOI 50). The average intensity of HA labeling was reported to the average intensity of bacteria fluorescence for each U937 cell on at less 20 cells per condition (Fig. S2). Central box represents the values from the lower to upper quartile (25 th to 75 th percentile) for each condition. The middle line represents the median. *Right panel,* Secretion kinetic of TEM-SidM fusion in the WT Lens strain. *(D)* Secretion kinetics of TEM-SidM, TEM-LidA, TEM-AnkX and TEM-LepB in the WT Lens strain. Results are obtained from 3 independent experiments made in triplicates and are presented as means ± SD.

Kinetic translocation profiles are consistent with functional consequences. The specific profiles of effector translocation levels suggest that a specific mix of different effectors at a given time could determine the succession of events that follows *L. pneumophila* phagocytosis. One of the best characterized series of events is the biogenesis of the LCV which involves the seemingly sequential action of Icm/Dot effectors targeting the host cell small GTPase Rab1. The GEF activity of SidM/DrrA is known to activate Rab1 on the LCV surface (23, 24), and its AMPylase activity maintains Rab1 in a GTP-linked active form (25, 26). Consistently, SidM/DrrA was detected on the early LCV during the first 3 hours post-infection and with maximal association at 1 hour post-infection (27). Using a similar microscopy-based immunofluorescence method and *L. pneumophila* expressing a HA-tagged SidM, we measured the level of SidM associated to the LCV relative to mCherry-expressing bacteria (Fig. S2) (Fig. 1C). We confirmed that HA-SidM is found on the LCVs as early as 30 min post-infection. At 75 minutes, HA-SidM was increasingly associated with LCVs to reach a maximum at 90 minutes post-infection (Fig. 1C). The kinetic of translocated levels of TEM-SidM is highly consistent with the dynamics of localisation of HA-SidM on the LCV, attesting to the relevance of the profiles gathered from the translocation kinetics (Fig. 1C). In addition to SidM, AnkX and LidA also contribute to the activation of Rab1 on the LCV, and to the subsequent ER recruitment to the vacuole (28, 29). In contrast, the GAP activity of LepB catalyzes the Rab1 GTP-hydrolysis and results in the removal of Rab1 from the LCV surface (27). The kinetic of LepB levels was previously determined but at the low time resolution of 1 time point per hour (27, 30).

A better time-resolved analysis of the translocated levels of the LepB, SidM, AnkX and LidA effectors was performed as described above. Similar to SidM, the three other translocated proteins display a pattern with relatively low levels followed by a steady increase to reach a CCF4-limiting plateau (Fig. 1D). However, each effector is characterized by specific timing of increasing levels. As described above, translocated TEM-SidM is detected as early as 30 minutes and its levels accumulated quickly, reaching a plateau at 90 min post-infection. TEM-LidA follows a similar pattern but significantly delayed. AnkX levels begin to significantly increase later than those of LidA but rise more. LepB levels rise nearly 2 hours after those of SidM to slowly reach a plateau at 3 hours post-infection (Fig. 1D). Strikingly, the accumulation kinetics of these effectors are in agreement with the function of the corresponding effectors during infection: SidM would be translocated first to activate the Rab1 GTPase on the LCV; it would be followed by AnkX and LidA that would promote this Rab1 activation; finally, LepB would be the last translocated effector to remove Rab1 from the LCV, thus terminating Rab1-dependent ER recruitment on the LCV. This experiment highlights that the complex orchestration of effector actions relies at least in part on the defined timing of translocated levels of some of the numerous Icm/Dot effectors into the host cells.

Robust timing of effector translocation, independently of chaperone activity and effector concentration. The finding that various effectors begin to accumulate in the host cell with a specific timing suggests that the translocation apparatus exerts a control on the translocation process. Such control has been previously documented for type III secretion systems (T3SS). The main factors contributing to the timing of effector translocation are the relative concentration of effectors in the bacterial cytoplasm and the involvement of chaperones (31). The timing at which various *L. pneumophila* effectors begin to accumulate in the cell is strikingly different despite the fact that they are all ectopically and constitutively expressed at similar levels (Fig. S1). Yet, we could not rule out that small difference in effector concentration dictates the timing of translocation. To directly test this hypothesis, we monitored the translocation profiles of our model effector, LepA, expressed at increasing levels. Macrophages were infected with TEM-LepA expressing bacteria grown in medium containing increasing IPTG concentrations from 0.5 µM to 500 µM, and β-lactamase activities were measured as described above. From 0.5 µM to 50 µM IPTG, increased expression levels of TEM-LepA result in increasing levels of translocated LepA (Fig. 2A).

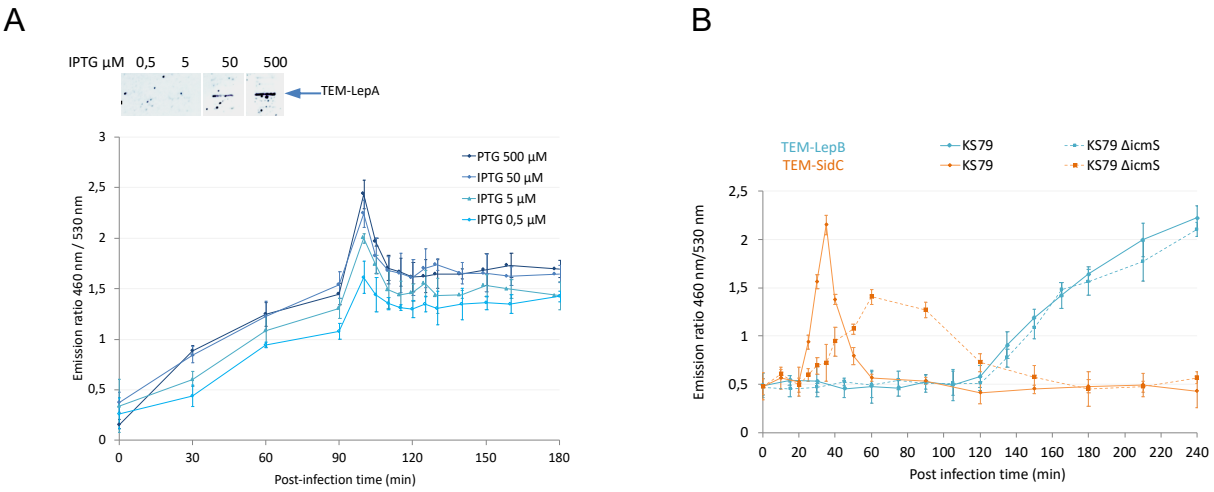

Fig. 2. Timing of effector translocation is independent of effector concentration and chaperone activity. *(A)* Secretion kinetics of TEM-LepA translocation after expression induction by a range of IPTG concentrations corresponding to different levels of synthesis assessed by Western blot with anti-TEM (top panel). *(B)* Secretion kinetics of the IcmS-dependent TEM-SidC and the IcmS-independent TEM-LepB fusions in KS79 *L. pneumophila* Philadelphia and in the *ΔicmS* mutant strain. The results are representative of 2 independent experiments and are presented as means ± SD.

Increasing further LepA expression levels with 500 µM IPTG did not significantly increase the translocated LepA levels, indicating that Icm/Dot-dependent translocation is a rate-limited step (data not shown). Most importantly, the kinetic profile of LepA translocation was largely unaffected by the expression levels of LepA, disproving the hypothesis that the timing of effector translocation is controlled by effector expression levels.

Some effectors, such as SidC and SidJ (12, 32) have been described to be translocated in a chaperone IcmS/IcmW/LvgA-dependent manner, whereas others such as LepB are not (33). Interestingly, the two effectors requiring the IcmS/IcmW chaperone-like adaptor complex for translocation still show distinct translocation profile. For instance, SidC levels abruptly increase at an early time point to rapidly become undetectable, while SidJ levels rise slowly and steadily all along the 3 hours of the time course (Fig. 1B). Yet, recognition by the IcmS-IcmW may determine the timing and efficiency of effector translocation. We tested this hypothesis by following SidC and LepB levels translocated by the Philadelphia-1 derived strain KS79 and its isogenic mutant Δ*icmS*. Remarkably, the kinetics of LepB and SidC translocation by the Lens and Philadelphia strains are nearly identical, indicative of the robustness of the timing of effector translocation (Fig. S3). Expectedly, the absence of IcmS had no impact on the kinetic of LepB translocation (Fig 2B). However, the absence of IcmS had a major impact of SidC levels which rose less rapidly, were generally lower but lasted for an extended time (Fig. 2C). Rather than delivering a short burst of SidC, the Δ*icmS* seemed to translocate the same amount of SidC but over a larger time frame (Fig. 2C). However, and importantly, the lack of IcmS had no incidence on the timing at which translocated levels begin to rise (at ~30 minutes). Thus, the Icm/Dot system translocates effectors with a defined timing that does not depend on effector concentration or chaperone activity. The mechanism that controls the timing of translocation of each effector is unknown but may be related to the properties of the effector protein (E-box, folding rate, stability) and how each effector interacts with the Icm/Dot system (affinity). Nevertheless, timing of effector translocation may also result from intrinsic and/or external modulations of the activity of the Icm/Dot system.

Timing of Icm/Dot effector translocation depends on c-di-GMP signaling. We previously reported that three c-di-GMP-metabolizing enzymes directly contributed to the ability of *L. pneumophila* Lens strain to infect both protozoan and mammalian cells. Mutants with deletions of the corresponding genes (*lpl0780*, *lpl0922* and *lpl1118*) were partially defective for Icm/Dot-dependent processes such as escape of the LCV from the host degradative endocytic pathway and efficient endoplasmic reticulum recruitment to the LCV (34). A snapshot of effector translocation, 1 hour post-infection, revealed effector-specific alterations in translocation efficiencies. While some effectors appeared unaffected, others appeared translocated less efficiently or over-translocated (34). Interestingly, these phenotypes were robustly conserved in Paris strain but only for the *lpp0809/lpl0780* deletion mutant (Fig. S4). The sequences of Lpl0780 and Lpp0809 are strongly conserved (97,87 % identity, 99,61 % similarity) and as expected, the *lpl0780* gene can rescue the phenotype of the Δ*lpp0809* strain (Fig. S4A). Then, we sought to address the potential role of diguanylate cyclase Lpp0809/Lpl0780 in the control of effector translocation, reasoning that difference in effector translocation efficiencies in the previous snapshot actually resulted from alterations in the timing of effector translocation. Hence, we analysed the translocation kinetics of our model TEM-LepA effector in the strain deleted of the gene *lpl0780* (Fig. 3A). Interestingly, the burst of LepA levels appeared delayed by nearly 1 hour in *lpl0780* mutant. The introduction of the *lpl0780* gene on a plasmid restores quite totally the optimal delivery of the LepA effector in the Δ*lpl0780* strain (Fig. 3A), suggesting a role of Lpl0780/Lpp0809, and subsequently of c-diGMP signaling, in the control of the right timing of effector secretion.

The diguanylate cyclase Lpl0780/Lpp0809 acts at a post-transcriptional level and locally at the bacterial poles. To go further on the Icm/Dot secretion control by c-di-GMP, we adressed the question whether the diguanylate cyclase Lpl0780/Lpp0809 could impact the expression of genes encoding components of the secretory apparatus and /or effectors, which could interfere with secretion of the model effector LepA in the Δ*lpl0780* mutant. RNA sequencing showed that only 12 genes (among 2966) were significantly differentially expressed ($P < 0.01$ and $\log_2$ fold change of >1 or <−1) between Δ*lpl0780* strain and the WT Lens strain (Table S4). Noteworthy, the fold changes remain quite weak and none of these genes can be connected to T4SS machinery or effectors expression, thus suggesting a post-transcriptional control. Among the described modes of action of c-di-GMP (35), we then privileged the hypothesis of a post-translational control close to the Icm/Dot secretion machinery by the diguanylate cyclase Lpl0780/lpp0809. Given that the secretion apparatus was recently described to be located at the bacterial poles (4, 6, 8), we tested this hypothesis by determining the cell localization of Lpp0809-sfGFP fusion protein expressed from a plasmid or from the chromosome (Fig. 3B). Importantly, we checked that in both cases the Lpp0809-sfGFP fusion proteins are functional, as demonstrated by their ability to restore intracellular replication of the Δ*lpp0809* strain (Fig. S5A-B). The plasmid version of Lpp0809-sfGFP localizes at both poles in 30% of the cells, and sometimes in the form of foci along the bacterium (Fig. 3C, 3E). Moreover, internal foci are observed for larger cells (Fig. 3D), as described for the machinery component DotF proposed to be targeted to the pole at the midcell (6). To check that the Lpp0809 polar localization is not due to overexpression of the fusion protein or to the sfGFP tag, localization of Lpp0809-sfGFP and 4xHA-Lpp0809 fusion proteins synthesized from chromosome was established. In half of the bacteria, the chromosomal version of Lpp0809-sfGFP is also detectable, despite weak fluorescence, at the poles (Fig. S5CDE) with a polarity score similar to that of T4SS components outside the complex core, such as DotB or DotL (14). Bipolar localization is also observed by immunodetection of 4xHA-Lpp0809 (Fig. S5F) and often close to the coupling complex protein DotN

(Fig. 3F). Together, these data suggest that the diguanylate cyclase Lpl0780/Lpp0809 could modulate the local pool of c-di-GMP near the Icm/Dot machinery, thus controlling directly or indirectly the efficiency of the sequential picking-up of effectors.

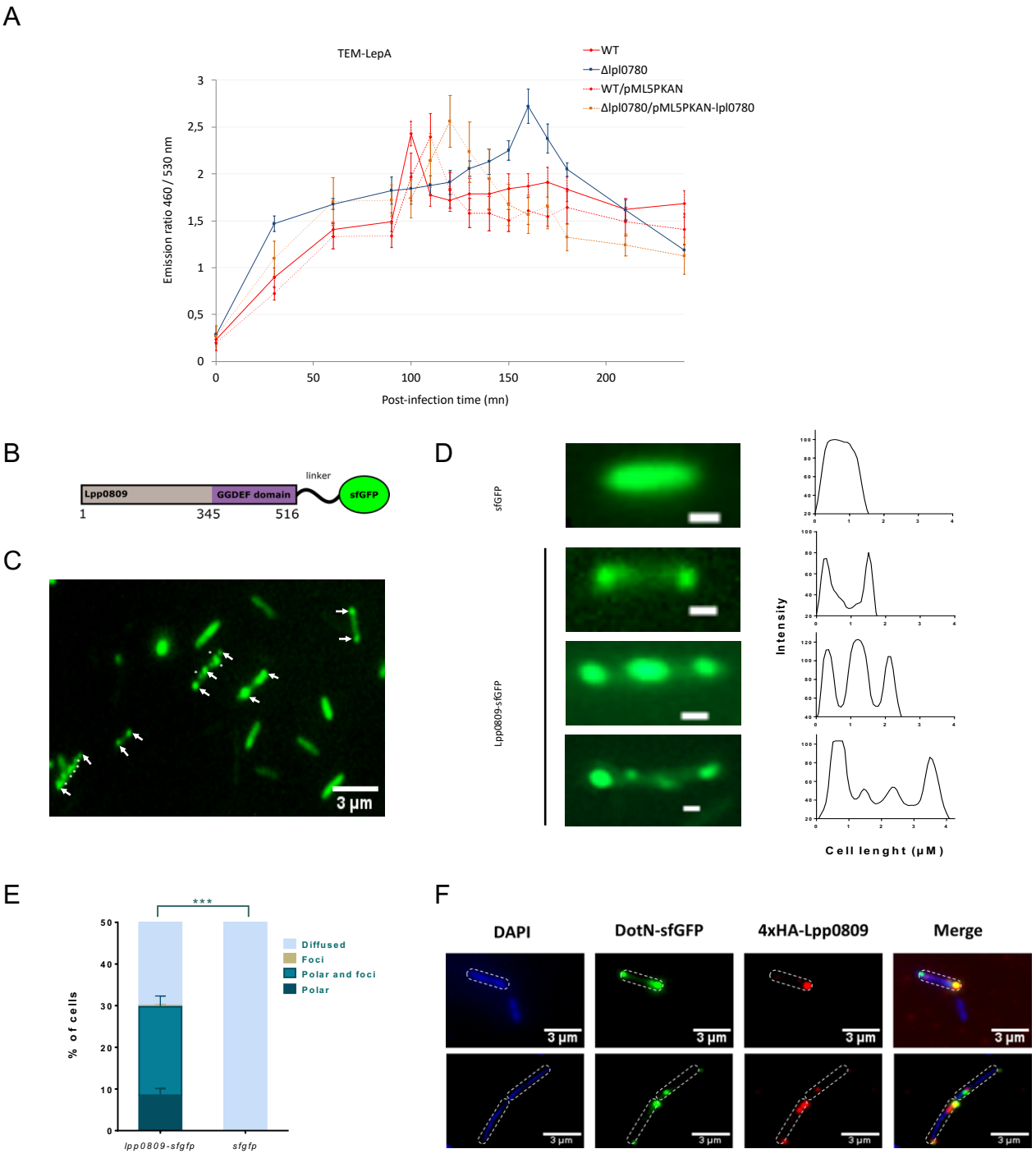

Fig. 3. Lpl0780/Lpp0809 is required for the optimal timing of effector delivery by the Icm/Dot system and is localized at the bacterial poles *(A)* The introduction of the *lpl0780* gene restores quite totally the optimal delivery of the LepA effector in the Δ*lpl0780* strain. *(B)* Schematic representation of Lpp0809-sfGFP fusion. *(C)* Localization of recombinant Lpp0809-sfGFP in Paris strain expressing the pML5PKAN-*lpp0809-sfgfp* fusion, collected in stationary phase. Cells with polar localization (arrowheads) and foci localization (asterisk) of Lpp0809-sfGFP are indicated (scale bar = 3µm). *(D)* ImageJ analysis of fluorescence intensity along the axis of representative cells (scale bar = 0,5µm). *(E)* Percentage of cells displaying cytoplasmic, foci or polar localization of Lpp0809-sfGFP (n ≥ 200 bacteria in stationary phase). *(F)* Localization in Paris strain of recombinant DotN-sfGFP (green), 4xHA tag labelled Lpp0809 (red) and DAPI staining (blue) (scale bar = 3µm). Results of Fig. 3A, E are representative of 3 independent experiments and are presented as means ± SD.

Discussion

Delivery of effector proteins that hijack host cell processes to the benefit of the bacteria is a mechanism widely used by bacterial pathogens. Effector delivery is achieved by complex effector injection devices such as the Type III, the Type IV and the Type VI secretion systems (T3SS, T4SS, T6SS). Although considerable progresses have been made on several fronts in structure-function analysis of these secretion systems, one of the blackest boxes in our understanding is the signal that triggers the activation of effectors transfer. Type III and Type VI secretions are known to respond to environmental stimuli and can be triggered *in vitro* by using various chemicals (36, 37) while effectors delivery by most of the T4SS occurs only in response to establishment of productive contact with targets cells (16, 38, 39). Important insights about activation of DNA transfer by the paradigm model of the *Agrobacterium* T4SS have been proposed (40-44). However, understanding how the Icm/Dot T4SS of *L. pneumophila* manage effective secretion of over 300 protein effectors, both avoiding a bottleneck effect and promoting optimal secretion for bacteria intracellular replication, is a real challenge.

Here, we developed a kinetic translocation assay on *L. pneumophila* T4SS Icm/Dot that allows a fine monitoring of substrates secretion into the host cells, and thus could be a useful tool to decipher the signals that control the T4SS translocation triggering. It is based on the β-lactamase translocation reporter system combined with the effect of the protonophore CCCP added at various time slots. CCCP is a protonophore that collapses the proton gradient and could interfere with the energy requirement of the Icm/Dot system and thus stop the secretion (16). The CCCP-combined β-lactamase assay is more sensitive and allows a monitoring on larger time frame than the real time β-lactamase, based on the real time detection of CCF4 substrate hydrolysis as the TEM-effector fusion is being translocated by the infecting population (16). The physiological relevance of the translocation kinetics obtained with the CCCP-combined β-lactamase assay was validated by demonstrating that the time-dependent SidM translocation into the host cell was consistent with the kinetics of SidM retrieving on the LCV. Also, we observed with the β-lactamase translocation assay that SidM accumulated into the host cells from 75 min to 200 min, consistently with the western-blot detection of SidM in the host cell between 1h to 3h post-infection (30). Finally, we found that SidC is early translocated, between 20 min to 45 min post-infection, which is rather similar with the presence of SidC detected by immunofluorescence on the LCV at 1h post-infection (45).

Thanks to the CCCP-combined β-lactamase assay we clearly established that the delivery into the host cells of each Icm/Dot effector is finely tuned. We observed that translocation of some effectors (e.g LegK4) is very early, immediately after the contact between the bacteria and its host cell, while that of others (e.g LepB) starts only 2h post-infection. The very early translocation is consistent with the Icm/Dot secretion apparatus already assembled before the contact with the host cell (5, 6). However, late translocation of some effectors strongly supports the occurrence of other signaling mechanisms to control their picking-up and translocation than the availability of the secretion device and the phagocytosis-dependent triggering of the overall translocation. Significantly, we demonstrated that the early translocation is not due to the effector picking-up by the chaperone protein IcmS. Otherwise, the IcmS protein is essential to an efficient translocation rate. Importantly, we also showed that the timed delivery of an effector is not dependent on its concentration and synthesis. This result is consistent with transcriptomic studies previously showing that transcription of most of the effectors is synchronously upregulated in the transmissive phase, and it also excludes an impact of a potential post-transcriptional control of effector synthesis on the start of the secretion. Above all, we showed that translocation is so precisely set up that it lines-up with the functionality of each effector as demonstrated with the sequential action of Icm/Dot effectors which target the host cell small GTPase Rab1.

Besides, we highlighted that in addition to the control of translocation triggering, the arrest of translocation was also tightly tuned. Translocation of some effectors is stopped quickly after the beginning of the translocation (e.g SidC), while that of others is maintained for a long time period, until 4h post-infection (e.g SidJ). Consistently, it has emerged that *L. pneumophila* is able to achieve temporal regulation of an effector using the ubiquitin-proteasome system (46). Indeed, after establishing its replicative niche, the *L. pneumophila* effector SidH is degraded by the host proteasome. Most remarkably another effector protein LubX is able to mimic the function of an eukaryotic E3 ubiquitin ligase and polyubiquitinates SidH, targeting it for degradation (47, 48). Together, these data clearly demonstrate that *L. pneumophila* temporally controls the function of Icm/Dot effectors inside host cells by (1) fine-tuning the trigerring of secretion, (2) controlling the arrest of secretion, and (3) modulating the half-time of life of some effectors after their delivery into the cells. Additionally, it is clear that the function of these effectors is also controlled spatially by their addressing to the appropriate host cell compartment (49, 50).

Returning to the onset of secretion, we propose that it would be controlled by c-di-GMP signaling. Thus, we demonstrated that a c-di-GMP synthesizing enzyme, namely the diguanylate cyclase Lpl0780/Lpp0809, significantly contributes to accurate triggering of effector secretion. We observed that this enzyme most likely exerts a post-translational control of the T4SS machinery rather than a control of its synthesis. Given its localization at the bacterial pole with the T4SS machinery and by comparison with previously described post-translational controls of other bacterial secretion systems (except the T4SS) by binding of c-di-GMP (51-56), it is tempting to hypothesize that the c-di-GMP could modify the interactions between the coupling protein complex, the chaperones and others unknown partners to finely orchestrate loading of the 300 effectors on the Icm/Dot in coherence with their role during the infection. Our results reveal an unpublished c-di-GMP dependent control of the type 4 secretion and pave the way to decipher the molecular mechanism involved.

Materials and Methods

Bacterial strains, plasmids and primers. See *SI Materials and Methods* and Tables S1, S2 & S3

Kinetic assay of TEM translocation. Kinetic assays of TEM-effector translocation were made by improving previous end-point measurement translocation assay described by (22) and adapted to the T4SS effector translocation of *L. pneumophila* (34). It relies on multiples end-point measurements allowed by the adding of the CCCP protonophore. See more details in *SI Materials and Methods.*

Fluorescence and immunofluorescence microscopy. *L. pneumophila* proteins were immunodetected by the procedure previously described for *E. coli* and adapted to *L. pneumophila (6)*. Details protocols in *SI Materials and Methods.*

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