

The *Legionella pneumophila* Two-Component Regulatory Systems that Participate in the Regulation of Icm/Dot Effectors

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Abstract *Legionella pneumophila*, the causative agent of Legionnaires' disease, actively manipulates intracellular processes to establish a replication niche inside their host cells. The establishment of its replication niche requires a functional Icm/Dot type IV secretion system which translocates about 300 effector proteins into the host cells during infection. This enormous number of effectors should be coordinated at the level of gene expression, in order to be expressed and translocated at the correct time and appropriate amounts. One of the predominant ways in bacteria to regulate virulence gene expression is by the use of two-component systems (TCSs). To date, four TCSs have been shown to be involved in the regulation of Icm/Dot effector-encoding genes: The PmrAB and CpxRA TCSs that directly control, and the LetAS and LqsRS TCSs that indirectly control the level of expression of effector-encoding genes. According to our current knowledge, these four TCSs control the expression of about 70 effector-encoding genes. The regulation by different TCSs divides the effectors into groups of co-regulated effector-encoding genes that are probably co-expressed at a similar time during infection and might perform related functions. In addition, examples of interplay between these TCSs were already reported indicating that they form part of a regulatory network that orchestrates the expression of *L. pneumophila* effector-encoding genes during infection.

Abbreviations

GAP	GTPas activating protein
GEF	Guanine nucleotide exchange factor
Icm/Dot	Intracellular multiplication/Defect organelle trafficking
LCV	<i>Legionella</i> containing vacuole
TCS	Two-component system

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1 Introduction

Legionella pneumophila is an opportunistic human pathogen that multiplies within alveolar macrophages and causes a severe pneumonia known as Legionnaires' disease. In order to establish a replicative niche inside eukaryotic cells, *L. pneumophila* modulates host cell functions by the delivery of about 300 effector proteins through the Icm/Dot Type-IV secretion system (reviewed in Ensminger and Isberg 2009; Franco et al. 2009; Gomez-Valero et al. 2011; Shin and Roy 2008). The numerous effectors that take part in the establishment of the *L. pneumophila* containing vacuole (LCV); the many host cell pathways manipulated by *L. pneumophila* effectors (Dorer et al. 2006; O'Connor et al. 2012) and the stepwise process that occurs during the establishment of the LCV inside host cells (Horwitz 1983a); suggest that the effectors translocated by the Icm/Dot secretion system will most likely be regulated at the level of gene expression, in order to coordinate a successful infection (other levels of regulation of effectors translocation such as the recognition by components of the secretion complex and chaperons probably contribute to this stepwise process as well).

Two-component systems (TCSs) are widespread signal transduction devices in bacteria that enable them to respond to environmental stimuli mainly via changes in gene expression. These systems are used by many pathogenic bacteria that utilize multiple TCSs in order to control the expression of their virulence genes (Fass and Groisman 2009; Gooderham and Hancock 2009; Gotoh et al. 2010; Vogt and Raivio 2012). The TCSs are classically composed of a membrane-integrated sensor histidine kinase and a cytoplasmic transcriptional regulator containing an N-terminal receiver domain and a C-terminal DNA binding domain (helix-turn-helix domain). Generally, stimuli detected by the sensor histidine kinase lead to its autophosphorylation. Then, the phosphoryl group from the histidine residue is

transferred to an aspartic acid residue in the receiver domain of the response regulator, thus leading to its activation (Jung et al. 2012; Laub and Goulian 2007).

To date, four TCSs have been found to regulate the expression of *L. pneumophila* effector-encoding genes (Table 1 and Fig. 1): (i) the CpxRA TCS was shown to directly activate or repress the expression of 12 effector-encoding genes and several *icm/dot* genes (Altman and Segal 2008; Gal-Mor and Segal 2003a); (ii) the PmrAB TCS was shown to directly activate the expression of 43 effector-encoding genes (Al-Khodori et al. 2009; Zusman et al. 2007); (iii) the LetAS TCS was shown to indirectly regulate the expression of four effector-encoding genes (Rasis and Segal 2009; Shi et al. 2006) and (iv) the LqsRS TCS that was shown to indirectly regulate the expression of 12 effector-encoding genes (Tiaden et al. 2010; Tiaden et al. 2007).

These four TCSs, the different functions mediated by the effectors they regulate and the interplay between them are the focus of this chapter.

2 The CpxR-CpxA Two-Component System

The *L. pneumophila* CpxR-CpxA TCS consists of the CpxR response regulator and the CpxA sensor histidine kinase (Gal-Mor and Segal 2003a). The CpxRA TCS has been studied in many bacteria, where CpxA was shown to sense misfolded proteins in the bacterial envelope and to activate (phosphorylate) CpxR. Phosphorylated CpxR was shown to regulate pilus assembly, adherence, and biofilm formation (Hunke et al. 2012; Vogt and Raivio 2012). Moreover, CpxR was shown to be required for host cell invasion in several species, including pathogenic *Escherichia coli* and *Salmonella enterica* (Humphreys et al. 2004; Nevesinjac and Raivio 2005).

The involvement of the CpxRA TCS in *L. pneumophila* virulence was first identified in a genetic screen looking for a direct regulator of the *icmR* gene (Gal-Mor and Segal 2003a). Later, by using bioinformatic approaches aimed at identifying additional genes that harbor the CpxR regulatory element (GTAAAnnnnnnGWAAA, W indicates T or A) this system was shown to participate in the regulation of two additional *icm/dot* genes (*icmV* and *icmW*), the *lvgA* gene and 11 effector-encoding genes (Altman and Segal 2008). The *L. pneumophila* CpxRA TCS was found to activate the expression of all the *icm/dot* genes it regulates, as well as five effector-encoding genes, and to repress the expression of six other effector-encoding genes (Altman and Segal 2008). However, deletion mutants in the genes coding for CpxR as well as CpxA were found to have no intracellular growth phenotype when examined in different host cells (Gal-Mor and Segal 2003a). The environmental stimuli that activate the *L. pneumophila* CpxA sensor kinase have not been discovered yet, but since CpxR-activated effectors were shown to translocate into host cells early during infection (see below), the CpxA activation might be related to *L. pneumophila* attachment to host cells like in the case of *E. coli* (Nevesinjac and Raivio 2005).

Table 1 *L. pneumophila* two-component systems involved in effectors regulation

Response regulator	Sensor kinase	Type of regulation	Additional components	Effectors regulated	Type of HTH ^a	References
CpxR-lpg1438	CpxA-lpg1437	Direct	–	CegC1, LegA10, CegC2, Ceg7, LegA11, Ceg18, CegC3, CegC4, SidH, SidM/DrrA, SidD, Ceg33	WHTH	Altman and Segal (2008); Gal-Mor and Segal (2003a)
PmrA-lpg1292	PmrB-lpg1291	Direct	–	Ceg2-7, SidE, Ceg8-10, SdhA, Ceg11, LegA9, Ceg14/SidL, Ceg15, Ceg17-21, SidG, Ceg22, LegC5, Ceg23, SidB, Ceg24-25, LegLC8, LegAU13, SdeC, SdeB, SdeA, Ceg28-30, LegA14, SdbB, LepB, Ceg32/SidL, SidF, Ceg33, VipE, Ceg34	WHTH	Al-Khodor et al. (2009); Zusman et al. (2007)
LetA-lpg2646	LetS-lpg1912	Indirect	RsmY, RsmZ, CsrA, LetE	VipA, YlfB/LegC2, RalF, YlfA/LegC7	HTH_LUXR	Bachman and Swanson, (2004); Edwards et al. (2010); Forsbach-Birk et al. (2004); Gal-Mor and Segal (2003b); Hammer et al. (2002); Lynch et al. (2003); Molofsky and Swanson (2003); Rasis and Segal (2009); Sahr et al. (2009)
LqsR-lpg2732	LqsS-lpg2734	Indirect	LqsA, LqsT	Lpg0081, lpg0294, lpg0634 lpg0967, SidK, RavN, lpg1453, MavB, CegC4, SdbB, lpg2844, lpg3000	No HTH	Kessler et al. (2013); Tiaden et al. (2008, 2007, 2010)

^a WHTH winged helix-turn-helix, HTH_LUXR A LuxR type helix-turn-helix

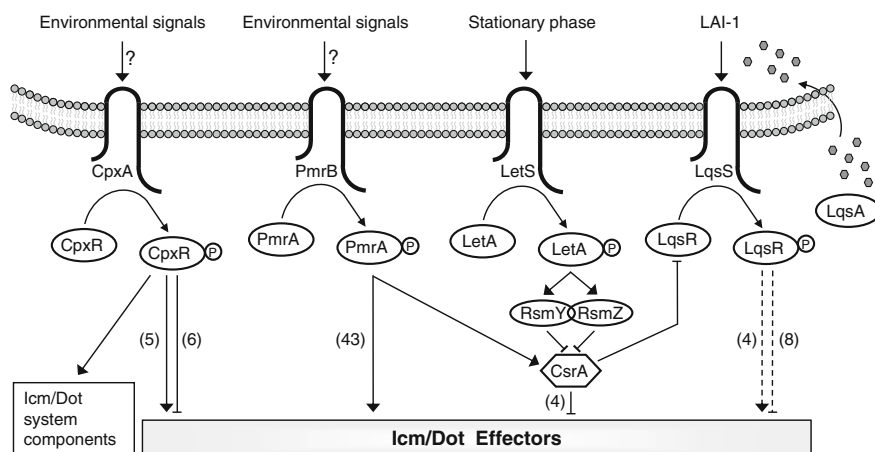


Fig. 1 Model of the TCSs that control the expression of the *L. pneumophila* Icm/Dot effector-encoding genes. The four TCSs (CpxRA, PmrAB, LetAS and LqsRS) and the components of the LetAS-RsmYZ-CsrA regulatory cascade as well as LqsA which is part of the Lqs system are schematically illustrated. The environmental signals sensed by CpxA and PmrB are currently not known, and the phosphorylation of these components is expected to be activated by transfer of the phosphate group to their cognate response regulators CpxR and PmrA respectively, which then directly activate or repress the transcription of their target effector-encoding genes. During stationary phase, the LetAS TCS activates the expression of the sRNAs RsmY and RsmZ that thus sequester CsrA from its target mRNAs and relieve the CsrA post-transcriptional repression. The *csrA* gene was also shown to be under the regulation of the PmrA transcriptional regulator and LqsR was shown to be repressed by CsrA. The quorum sensing system Lqs is being activated by binding of LAI-1 (*Legionella* auto inducer-1, generated by LqsA) to LqsS that thus activates the LqsR response regulator by phosphorylation. LqsR does not contain any DNA binding domain and it affects gene expression in an unknown way. The number of effector-encoding genes which were shown to be regulated by each of these TCSs is indicated in brackets. Solid lines and dashed lines indicate direct and indirect regulation, respectively. Solid arrows and T-shaped symbols indicate activation and repression, respectively

The function of most of the effectors regulated by the CpxRA TCS is currently not known. Two of the effectors that were found to be repressed by CpxR (LegA10 and LegA11) (Altman and Segal 2008) contain an Ankyrin domain (Table 2), which probably indicates that they interact with yet unknown host proteins. One effector (SidM/DrrA) that was shown to be strongly activated by CpxR (Altman and Segal 2008) was studied intensively. SidM/DrrA was found to recruit the host cell factor Rab1 to the LCV, it was shown to function as a guanine nucleotide exchange factor (GEF) for Rab1 as well as a GDP dissociation inhibitor (GDI) displacement factor (GDF) for Rab1 (Machner and Isberg 2006; Machner and Isberg 2007; Neunuebel et al. 2011). Besides these activities the amino-terminal domain of SidM/DrrA was shown to AMPylate Rab1, leading to its covalent modification. The AMPylation by SidM/DrrA limits the access of GTPase activating proteins (GAPs), thereby keeping Rab1 constitutively active (Muller et al. 2010; Murata et al. 2006). Interestingly, it was found that another effector-

Table 2 Effector proteins with known function which are regulated by TCSs

Lpg#	Gene product	Regulator	Motif ^a	Brief functional description ^b	References
Ipg0038	LegA10/AnkQ	CpxR	AR		de Felipe et al. (2005)
Ipg0436	LegA11/AnkJ	CpxR	AR		de Felipe et al. (2005); Habyarimana et al. (2008)
Ipg2464	SidM/DrrA	CpxR		GEF and GDF for Rab1, AMPylates Rab1	Machner and Isberg (2006); (2007) Muller et al. (2006, 2010); Neunuebel et al. (2011)
Ipg2465	SidD	CpxR		deAMPylates Rab1	Neunuebel et al. (2011); Tan and Luo (2011)
Ipg0246	Ceg9	PmrA		Trafficking (yeast)	Heidman et al. (2009)
Ipg0376	SdhA	PmrA	GRIP	Vacuole integrity, anti-apoptotic	Creasey and Isberg (2012); Ge et al. (2012); Laguna et al. (2006)
Ipg0402	LegA9	PmrA	AR	Preventing autophagy	Khweek et al. (2013)
Ipg0437	Ceg14/SidL	PmrA		Inhibits eEF1A	Fontana et al. (2011)
Ipg1121	Ceg19	PmrA		Trafficking (yeast)	Heidman et al. (2009)
Ipg1488	LegC5/l-gt3	PmrA		Glucosyltransferase of eEF1A	Belyi et al. (2008); Hurtado-Guerrero et al. (2010)
Ipg2144	LegAU13/AnkB	PmrA	AR/F-box	Generates polyubiquitinated proteins on the LCV, supply amino acids for bacterial growth	Price et al. (2009, 2010, 2011)
Ipg2452	LegA14	PmrA	AR	Trafficking (yeast)	Heidman et al. (2009)
Ipg2490	LepB	PmrA		GAP for Rab1	Ingmundson et al. (2007); Neunuebel et al. (2011)
Ipg2504	Ceg32/SidI	PmrA		Inhibits eEF1A	Shen et al. (2009)
Ipg2584	SidF	PmrA		Anti-apoptotic, phosphoinositide phosphatase	Banga et al. (2007); Hsu et al. (2012)
Ipg0390	VipA	LetA		Trafficking, binds actin	Franco et al. (2012); Shohdy et al. (2005)
Ipg1884	Y1fB/LegC2	LetA		Trafficking	(de Felipe et al. (2008)
Ipg1950	RaIF	LetA	Sec7	GEF for ARF1	Nagai et al. (2002)
Ipg2298	Y1fA/LegC7	LetA		Trafficking	Campodonico et al. (2005); de Felipe et al. (2008)
Ipg0634		LqsR		Trafficking (yeast)	Heidman et al. (2009)
Ipg0695	LegA8/AnkX	LqsA	AR	Phosphocholates Rab1	Goody et al. (2012); Mukherjee et al. (2011); Pan et al. (2008); Tan et al. (2011)
Ipg0968	SidK	LqsR		Inhibition of vacuolar ATPase	Xu et al. (2010)

^a AR Ankyrin repeat, *F-box* F-box domain that mediates ubiquitination of proteins, *GRIP* A domain sufficient for targeting to the Golgi

^b *Trafficking (yeast)* The involvement in trafficking was shown only in yeast using the CPY system

encoding gene *sidD*, which is located adjacent to *sidM/drrA*, harbors the opposite enzymatic activity and it functions as a deAMPyator for Rab1 (Neunuebel et al. 2011; Tan and Luo 2011). Examination of the effect of CpxR on the expression of *sidD* revealed that CpxR represses the level of expression of *sidD* as opposed to its activation of the level of expression of *sidM/drrA* (Zusman and Segal, unpublished results). The connection between the regulation and function of these two effectors will be discussed in Sect. 6.1.

3 The PmrA-PmrB Two-Component System

The *L. pneumophila* PmrA-PmrB TCS consists of the PmrA response regulator and the PmrB sensor histidine kinase (Zusman et al. 2007). The PmrAB TCS was studied extensively in *Salmonella enterica* serovar Typhimurium where it functions as the major regulator of lipopolysaccharide modification genes (Gunn 2008). The PmrA regulator was shown to be activated when its cognate sensor PmrB detects mildly acidic pH (Perez and Groisman 2007) or the presence of Fe³⁺ (Wosten et al. 2000). The *S. enterica* PmrAB TCS was found to be active when the bacteria are inside macrophages and during infection of mice (Merighi et al. 2005).

The involvement of the PmrAB TCS in *L. pneumophila* virulence was first identified in a bioinformatic analysis of several *L. pneumophila* effector-encoding genes that were found to contain a conserved regulatory element at their upstream regulatory region. This regulatory element consists of a tandem repeat sequence (cTTAATatT, lower case letters indicate less conserved nucleotides) with a spacer of two nucleotides (Zusman et al. 2007). Bioinformatic and literature searches of bacterial regulatory elements and regulatory factors revealed that a very similar sequence to the one described above has been previously identified in *S. enterica*, and it was shown to be recognized by the PmrA response regulator (Marchal et al. 2004; Wosten and Groisman 1999). Later, the *L. pneumophila* PmrAB TCS was found to activate the expression of many *L. pneumophila* effector-encoding genes (Al-Khodori et al. 2009; Zusman et al. 2007) and the identification of the PmrA regulatory element in the upstream regulatory region of many hypothetical proteins led to their validation as novel effector-encoding genes (Burststein et al. 2009; Zusman et al. 2007). In line with these results, the gene encoding for PmrA was shown to be required for intracellular growth of *L. pneumophila* in amoeba (Zusman et al. 2007). The environmental stimuli that activate the *L. pneumophila* PmrB sensor kinase are not known, but since *L. pneumophila* was shown to inhibit phagosome-lysosome fusion early during infection (Horwitz 1983b), PmrB activation might be related to the pH levels of the LCV, like in the case of *S. enterica* (Perez and Groisman 2007). Currently, the *L. pneumophila* PmrAB regulon consists of 43 effector-encoding genes, it is the largest effectors regulon, and it includes about 15 % of the known *L. pneumophila* effectors.

Of the large number of effectors that were shown to be regulated by the PmrAB TCS, the function of 11 effectors was uncovered (Table 2), and related functions

among these effectors might indicate for the time during infection when the PmrAB TCS activates the expression of its target effectors. Two groups of PmrAB regulated effectors with related functions arise: (i) Three effectors (SidI, SidL, and Lgt3) were found to interact with components of the eukaryotic translation elongation machinery (eEF1A and eEF1B), interactions which lead to inhibition of host protein synthesis (Belyi et al. 2008; Fontana et al. 2011; Hurtado-Guerrero et al. 2010; Shen et al. 2009). Lgt3 was shown to function as a glucosyltransferase of eEF1A and the way by which the two other effectors (SidI and SidL) inhibit translation elongation is currently not known. Two additional effectors (Lgt1—Lpg1368 and Lgt2/LegC8—Lpg2862) were shown to function as glucosyltransferases of eEF1A (Belyi et al. 2008; Hurtado-Guerrero et al. 2010), but there is no information regarding their regulation. (ii) Three effectors (SdhA, SidF, and LegAU13/AnkB) seem to be involved in maintenance of the LCV in the host cell. Two of these effectors (SdhA and SidF) were shown to have anti-apoptotic activities (Banga et al. 2007; Laguna et al. 2006). SdhA was shown to actively stabilize the integrity of the LCV during intracellular replication (Creasey and Isberg 2012; Ge et al. 2012) and SidF was shown to contribute to apoptosis resistance of *L. pneumophila*-infected cells by specifically interacting with two proapoptotic members of the Bcl2 protein family (Banga et al. 2007). Beside these two effectors, the effector LegAU13/AnkB that harbors an ankyrin domain and an F-box motif, was shown to generate polyubiquitinated proteins on the LCV, and degradation of these proteins supply amino acids required for bacterial growth (Price et al. 2011). It is important to note that these three effectors (SdhA, SidF, and LegAU13/AnkB) are expected to perform their function after the establishment of the LCV (about 6 h post infection), when the bacteria grow exponentially inside the LCV (Horwitz 1983a). This result was indeed found with the effector SidI (described above) which was found to be expressed during exponential phase when examined in vitro (Shen et al. 2009). An additional PmrAB regulated effector with a known function is LepB which was shown to function as a GAP for Rab1, and was found to translocate into host cells and to perform its function several hours post-infection (Neunuebel et al. 2011) (see Sec. 6.1).

4 The LetA-LetS Two-Component System

The *L. pneumophila* LetA-LetS TCS consists of the LetA response regulator and the LetS sensor histidine kinase (Hammer et al. 2002). The LetAS TCS was found to be present in several γ -proteobacteria, in many of which it bears different names such as the *Pseudomonas aeruginosa*—GacS-GacA, *E. coli*—BarA-UvrY, *S. enterica*—BarA-SirA, and *Vibrio cholerae*—VarS-VarA. In most pathogenic bacteria that harbor this TCS was shown to be involved in virulence gene expression (Lapouge et al. 2008).

The involvement of the LetAS TCS in *L. pneumophila* virulence was first identified in a genetic screen looking for mutants that express the flagellin gene

poorly (Hammer et al. 2002). Later, the signal transduction pathway from LetS to individual effector-encoding genes was found to comprise a regulatory cascade (Hovel-Miner et al. 2009; Rasis and Segal 2009; Sahr et al. 2009). During stationary phase LetS activates LetA by a four-step phosphorelay (Edwards et al. 2010). Activated (phosphorylated) LetA positively regulates the transcription of two small regulatory RNAs, RsmY, and RsmZ, which act in a redundant fashion to jointly antagonize CsrA, a small RNA-binding protein that negatively regulates the expression of effectors-encoding genes (Hovel-Miner et al. 2009; Rasis and Segal 2009; Sahr et al. 2009). Sequestration of CsrA by RsmY and RsmZ leads to de-repression of CsrA-repressed mRNAs (Rasis and Segal 2009). A deletion mutant of LetA was found to be defective for intracellular growth of *L. pneumophila* in amoeba (Gal-Mor and Segal 2003b; Hammer et al. 2002; Lynch et al. 2003), and the gene encoding for CsrA was found to be essential for *L. pneumophila*, however, mutants containing a reduced level of this regulator were shown to be attenuated for intracellular multiplication in amoeba (Forsbach-Birk et al. 2004; Molofsky and Swanson 2003). In addition, the expression of the gene encoding for CsrA was shown to be activated by the PmrA response regulator described above (Rasis and Segal 2009). The current number of effector-encoding genes regulated by the LetAS-RsmYZ-CsrA regulatory cascade is rather small and includes only four effector-encoding genes (Rasis and Segal 2009; Shi et al. 2006), however, it is highly likely that additional *L. pneumophila* effectors will be found to be regulated by the LetAS-RsmYZ-CsrA regulatory cascade. The level of expression of additional effector-encoding genes was found to be affected by a deletion of *letA* or *letS* (Edwards et al. 2010; Shi et al. 2006), but it is not known if these effectors are regulated by the regulatory cascade described above.

Functional analyses of the four effectors regulated by the LetAS-RsmYZ-CsrA regulatory cascade were already performed and all these effectors were found to be involved in vesicular trafficking (Table 2). RalF, which was the first effector identified in *L. pneumophila*, functions as a GEF for Arf1 (ADP ribosylation factor) and it was shown to localize on the LCV early during infection (Nagai et al. 2002). The VipA effector was found to bind actin in vitro and directly polymerize actin microfilaments. During macrophage infection, VipA was found to be associated with actin patches and early endosomes indicating for its role in modulating organelle trafficking (Franco et al. 2012). The paralogous effectors YlfA and YlfB were also shown to be involved in vesicular trafficking and they were found within large structures that colocalized with anti-KDEL antibodies in mammalian cells (Campodonico et al. 2005; de Felipe et al. 2008). All the effectors known to be regulated by the LetAS-RsmYZ-CsrA regulatory cascade were found to be involved in vesicular trafficking that takes place during the establishment of the LCV. This result might indicate that effectors which are expressed at the end of an infection cycle (the equivalent of stationary phase) are translocated into host cells and perform their function early during the next infection cycle, when the bacteria actively modulate organelle trafficking.

5 The LqsR-LqsS Two-Component System

The *L. pneumophila* LqsR-LqsS TCS consists of the LqsR response regulator and the LqsS sensor histidine kinase (Spirig et al. 2008). The prototype of this system is the *V. cholerae* CqsAS (*Cholerae* quorum sensing) quorum sensing system which includes an autoinducer synthase (CqsA) and its cognate sensor (CqsS) (Miller et al. 2002). The CqsA and CqsS quorum sensing system in *Vibrio* promotes cell density-dependent regulation of virulence and biofilm formation (Miller et al. 2002; Henke and Bassler 2004).

The Lqs system was first identified in *L. pneumophila* by a bioinformatic analysis of the *L. pneumophila* genome with the aim of identifying a homologous system to the CqsAS system from *V. cholerae* and the corresponding *L. pneumophila* proteins were termed LqsA and LqsS (*Legionella* quorum sensing) (Tiaden et al. 2007). In *L. pneumophila*, a gene encoding for a putative response regulator (that lacks a DNA binding motif) is located between *lqsA* and *lqsS*, and this gene was termed *lqsR*. The autoinducer synthase LqsA was shown to catalyze the production of the diffusible signaling molecule 3-hydroxypentadecan-4-one (*Legionella* auto inducer-1—LAI-1, Fig. 1) that is presumably recognized by the sensor kinase LqsS, which in turn probably activates LqsR (Spirig et al. 2008; Tiaden et al. 2010). Recently an ‘orphan’ homologue of LqsS termed LqsT was identified which probably also respond to LAI-1 (Kessler et al. 2013). DNA microarray experiments revealed that LqsR affects the expression of genes involved in virulence including 12 effector-encoding genes (Tiaden et al. 2007). In addition, a transcriptome analysis of the $\Delta lqsA$, $\Delta lqsS$, and $\Delta lqsT$ mutants indicated that the level of expression of several other effector-encoding genes was changed in these mutants (Kessler et al. 2013; Tiaden et al. 2010). The expression of LqsR itself was found to require the RpoS sigma factor, and it was also found to be dependent to a smaller extent on the response regulator LetA (Tiaden et al. 2007). Moreover, the production of LqsR was found to be regulated at a post-transcriptional level by the sRNAs RsmY and RsmZ and by CsrA (Sahr et al. 2009). These results indicate that the Lqs system is involved in the regulation of gene expression during stationary phase, similarly to the LetAS TCS. However, since the LqsR response regulator lacks any known DNA binding motif it is currently not known how it affects gene expression.

The function of two effectors whose level of expression was changed by the Lqs system was determined (Table 2). The effector AnkX was found to catalyze the transfer of phosphocholine to Rab1, which like SidM/DrrA contributes to the activation of Rab1 on the LCV (Goody et al. 2012; Mukherjee et al. 2011; Pan et al. 2008; Tan et al. 2011). The function of the effector SidK was also uncovered and it was found to specifically target the host v-ATPase, a multisubunit complex responsible for organelle acidification in eukaryotic cells. SidK was found to specifically interact with VatA, a key component of the proton pump and this binding was shown to result with inhibition of ATP hydrolysis and proton translocation (Xu et al. 2010). The functions mediated by these two effectors are

expected to be required early during infection which correlates well with their activation during stationary phase, similarly to effectors regulated by the LetAS TCS.

6 Interplay Between Different TCSs in The Regulation of *L. pneumophila* Effectors

The pioneering work performed during the early 1980 s by Marcus Horwitz demonstrated that the establishment of the LCV in host cells is a sequential process that includes several steps, a process which was more carefully described later on (Horwitz 1983a, b; Kagan and Roy 2002; Robinson and Roy 2006; Tilney et al. 2001). It is clear today that this stepwise process, which occurs similarly in both human macrophages and amoeba (Abu Kwaik 1996), is mediated by the numerous effectors translocated via the Icm/Dot secretion system (Gomez-Valero et al. 2011; Isberg et al. 2009; Segal et al. 2005). One way to accomplish these stepwise events that occur on the LCV is to translocate different sets of effectors in a timely fashion. The different sets of effectors might be regulated at the level of gene expression which should result with sequential expression and translocation of different sets of effectors which are likely to perform their functions group after group. Since the current knowledge about the regulatory factors that control the expression of effector-encoding genes is rather limited (the regulatory factors that control the expression of more than 200 effectors are not known) it is impossible to build a complete picture about *L. pneumophila* effectors regulation in relation to their function during infection. However, the interplay between different TCSs in relation to the function of different effectors during infection starts to uncover.

6.1 Effectors Manipulating Rab1 are Regulated by Both the CpxRA and the PmrAB TCSs

One of the best studied host factors manipulated by *L. pneumophila* is Rab1. Six effectors (SidM/DrrA, SidD, AnkX, Lem3, LidA, and LepB) were shown to target this host factor (reviewed in Neunuebel and Machner 2012), and the regulation of the genes encoding for some of these effectors is already known. The effector SidM/DrrA was found to activate Rab1 by functioning as a Rab1-GEF (Machner and Isberg 2006; Murata et al. 2006) and as an AMPylator for Rab1 early during infection (Muller et al. 2010; Neunuebel et al. 2011), and it was shown before to be activated by CpxR at the level of transcription (Altman and Segal 2008). SidD (which is located adjacent to *sidM/drrA*) was shown to deAMPylate Rab1, thus counteracting the activity of SidM/DrrA (Neunuebel et al. 2011; Tan and Luo 2011), and it was found to be repressed by CpxR at the level of transcription

(Zusman and Segal, unpublished results). In addition, LepB that functions as a Rab1-GAP (Ingmundson et al. 2007) was shown to be activated by PmrA (Zusman et al. 2007). Examination of the localization of these three effectors on the LCV during the first hours post-infection indicated that SidM/DrrA was localized first to the LCV, during the time when the LCV is being established, while SidD and LepB were found on the LCV several hours post-infection (Neunuebel et al. 2011), at the end of the LCV establishment. Thus, effectors activated by CpxR (SidM/DrrA) seem to be translocated very early during infection and to contribute to the LCV establishment, and effectors repressed by CpxR (SidD) as well as effectors activated by PmrA (LepB) are probably translocated into host cells several hours post-infection. This observation also fits the function of several other PmrA activated effectors (SdhA, SidF, and LegAU13/AnkB) which are likely to perform their function when the bacteria grow exponentially in the LCV.

6.2 The Regulatory Switch Between the PmrAB and the LetAS Regulated Effectors

Another type of interplay between regulators is the direct regulation of one regulator by another regulator. This type of regulation was found between the PmrAB TCS and the CsrA translational repressor, which is a part of the LetAS-RsmYZ-CsrA regulatory cascade (Rasis and Segal 2009). The direct activation of the CsrA encoding gene by PmrA suggests a regulatory switch between two groups of effectors. At the same time when the expression of the effector-encoding genes which are under the regulation of the PmrAB TCS will be activated, the expression of the CsrA-encoding gene will be activated as well, consequently this activation should lead to an increase in the amount of the CsrA post-transcriptional repressor in the bacterial cell that in turn will lead to repression of the group of effector-encoding genes that are under the repression of CsrA. Moreover, the function of the post-transcriptional repressor CsrA was found to be dependent on the LetAS TCS which is activated during stationary phase (Rasis and Segal 2009; Shi et al. 2006) and the PmrA regulated effectors seem to be expressed during exponential phase (Shen et al. 2009). Thus, the likely scenario is that PmrA activates the expression of its target effector-encoding genes during exponential phase at the same time when the level of the sRNAs RsmY and RsmZ anti-repressors in the bacterial cell is low, and then the increase in the amount of the CsrA repressor by PmrA will most likely result with a strong reduction in the level of expression of the effectors repressed by CsrA (Fig. 2). Thus, this interplay between two regulators of effector-encoding genes should result with two apparent groups of effectors: One group of effectors which are activated by the PmrAB TCS and are expressed during exponential phase and the second group of effectors which are de-repressed by the LetAS TCS and are expressed during stationary phase.

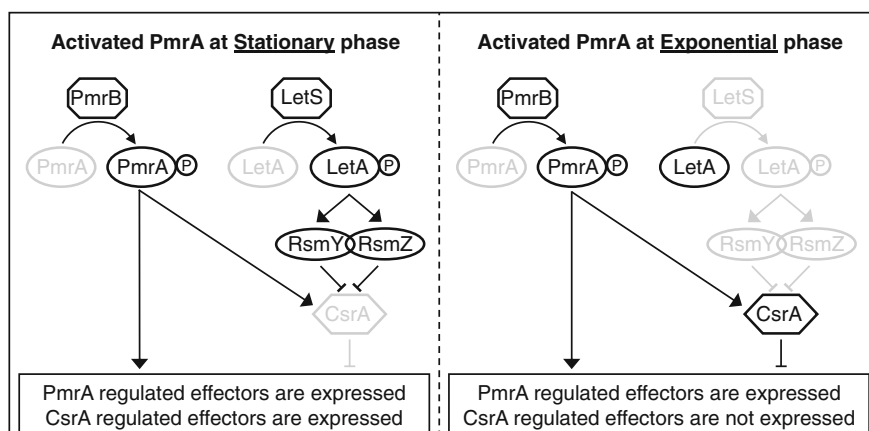


Fig. 2 The interplay between effectors regulated by PmrAB TCS and the LetAS-RsmYZ-CsrA regulatory cascade in relation to growth phase. The activation by the PmrA response regulator might occur during exponential or stationary phase of growth, when the LetAS-RsmYZ-CsrA regulatory cascade is inactivated or activated, respectively. If the PmrAB TCS activation occurs at stationary phase when the level of expression of the small sRNAs RsmY and RsmZ is very high, the activation of the level of expression of the gene encoding for CsrA by PmrA will be sequestered by the two sRNAs (*left side*). If the PmrAB TCS activation occurs at exponential phase (which is more likely, see text) when the level of expression of the small sRNAs RsmY and RsmZ is very low, the activation of the level of expression of the gene encoding for CsrA by PmrA will result with a reduction in the level of expression of effectors regulated by the LetAS-RsmYZ-CsrA regulatory cascade at the same time when effectors directly regulated by PmrA will be activated (*right side*). This interplay between these two regulatory systems will lead to a regulatory switch between the effectors regulated by the two systems. The regulatory components which are present and active at each growth phase are marked in black, the regulatory components which are absent or inactive are marked in grey

6.3 Effectors Directly Regulated by Both the CpxRA and PmrAB TCSs

An additional type of interplay between regulators is the direct regulation of a single effector by multiple TCSs. This situation was described thus far for three effectors (Ceg7, Ceg18, and Ceg33) which were shown to be directly regulated by both the CpxRA and the PmrAB TCSs (Altman and Segal 2008). The joint regulation by these TCSs includes two possible scenarios since CpxR was shown to function as a repressor or as an activator of effector-encoding genes and PmrA was shown to function only as an activator. Ceg07 and Ceg18 were found to be repressed by CpxR and activated by PmrA, and Ceg33 was found to be activated by both of these TCSs. Taking into account that these TCSs probably respond to different environmental stimuli (currently there is no information regarding the environmental stimuli that activate the CpxA or PmrB sensor histidine kinases) this type of regulation will result with effectors that will be expressed under conditions that activate both TCSs as well as with effectors that will be repressed

under a certain condition and activated under another condition. Considering all the results described above showing that SidM/DrrA (activated by CpxR) was found early on the LCV and LepB and SidD (activated by PmrA and repressed by CpxR, respectively) were found later on the LCV, the joint regulation by these TCSs should result with effectors that will be translocated both early and late into the host cells (effectors activated by both TCSs), and with effectors that will be translocated only late into the host cells (effectors repressed by the CpxRA TCS and activated by the PmrAB TCS).

7 Conclusions

The study of the regulation of the numerous effectors translocated into host cells by *L. pneumophila* already uncovered four TCSs that participate in this process. However, the ways by which the majority of the effector-encoding genes are regulated is largely unknown. Further study of these TCSs and the environmental stimuli that activate them as well as identification of additional TCSs and other regulators of gene expression that coordinate the regulation of the *L. pneumophila* effector-encoding genes will deepen our understanding on the ways by which such a multicomponent pathogenesis system is controlled at the level of gene expression in order to result with a successful infection.

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